Optimization of a two-phase fermentation process for the production of polyhydroxybutyrate (PHB) from organic and inorganic (industrial waste) substrate

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Optimization of a two-phase fermentation process for the production of polyhydroxybutyrate (PHB) from organic and inorganic (industrial waste) substrate

Thesis submitted in fulfilment of the requirements of the degree of Doctor (Ph.D.) in Applied Biological Sciences: Environmental Technology

Optimalisatie van een tweefasig fermentatieproces voor de productie van polyhydroxybutyraat (PHB) uit organisch en anorganisch (industrieel afval) substraat

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List of abbreviations

CDM	Cell dry mass (total biomass)
DO	Dissolved oxygen
DSC	Differential scanning calorimetry
GC	Gas chromatograph
GHG	Greenhouse gases
GPC	Gel permeation chromatography
HA	Hydroxyalkanoate
HB	Hydroxybutyrate
HD	Hydroxydecanoate
HHx	Hydroxyhexanoate
HO	Hydroxyoctanoate
HTD	Hydroxytetradecanoate
HV	Hydroxyvalerate
LB	Lenox broth
LEL	Lower explosion limit
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PA	Polyamide
PB	Polybutadiene
PBS	Polybutyrate succinate
PCL	Polycaprolactone
PDI	Polidispersity index
PE	Polyethylene
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PLA	Polylactic acid
PP	Polypropylene
PVC	Polyvinyl chloride
RCC	Residual cell (biomass) concentration
SBR	Styrene butadiene rubber
TGA	Thermo-gravimetric analysis

List of symbols

$\mu_{ps_{het}}$	Specific PHB production rate at heterotrophic phase	g PHB/g cell/h
$\mu_{ps_{aut}}$	Specific PHB production rate at autotrophic phase	g PHB/g cell/h
CO_2^*	Carbon dioxide saturation concentration	g/L
C_b	Molar concentration of base solution	mol/L
D_{lCO2}	Diffusion coefficient of carbon dioxide	cm^2/s
D_{lO2}	Diffusion coefficient of oxygen	cm^2/s
F_1	Substrate feeding at phase 1	L
F_2	Substrate feeding at phase 2	L
$F_N(t)$	Feeding rate of nitrogen	L/h
$F_{Na}(t)$	Feeding rate of NaOH	L/h
$F_S(t)$	Feeding rate of substrate	L/h
H_2^*	Hydrogen saturation concentration	g/L
K_{IN}	Nitrogen inhibition constant for growth	g/L
K_{IS}	Substrate inhibition constant for growth	g/L
K_N	Nitrogen saturation constant for growth	g/L
K_{PHB}	Intracellular PHB content half-saturation constant	g/L
K_{PIN}	Nitrogen inhibition constant for PHB production	g/L
K_{PIS}	Substrate inhibition constant for PHB production	g/L
K_{PS}	Substrate saturation constant for PHB production	g/L
K_S	Substrate saturation constant for growth	g/L
K_{pCO2}	Carbon-di-oxide saturation constant for PHB production	g/L
K_{pH2}	Hydrogen saturation constant for PHB production	g/L
K_{pIO2}	Oxygen inhibition constant for PHB production	g/L
K_{pO2}	Oxygen saturation constant for PHB production	g/L
K_{xCO2}	Carbon-di-oxide saturation constant for growth	g/L
K_{xH2}	Hydrogen saturation constant for growth	g/L
K_{xO2}	Oxygen saturation constant for growth	g/L
M_S	Molecular weight of the substrate	g/mol
N_F	Nitrogen concentration on the feeding solution	g/L
Na_F	Na^+ concentration on the feeding solution	g/L
Na_{pm}	Additional Na^+ concentration at which PHB production is zero	g/L
Na_{xm}	Additional Na^+ concentration at which growth rate is zero	g/L
O_2^*	Oxygen saturation concentration	g/L
P_{het}	PHB produced by heterotrophic process	g/L
P_{aut}	PHB produced by autotrophic process	g/L
P_g	Partial pressure	atm
Q_b	Base flow rate	L/h
S_F	Substrate concentration in the feeding solution	g/L
S_{Rf}	Relative sensitivity function	

V_1	Working volume at the end of phase 1	L
Y_{NS}	Molar ration of ammonium and substrate consumption	
Y_{pCO2}	PHB yield over carbon dioxide	g PHB/g CO_2
Y_{pH2}	PHB yield over hydrogen	g PHB/g H_2
Y_{pO2}	PHB yield over oxygen	g PHB/g O_2
Y_{pS}	PHB yield over substrate	gPHB/g substrate
Y_{xCO2}	Biomass yield over carbon dioxide	g PHB/g CO_2
Y_{xH2}	Biomass yield over hydrogen	g PHB/g H_2
Y_{xN}	Biomass yield over nitrogen	g cell/g NH_4^+ - N
Y_{xO2}	Biomass yield over oxygen	g PHB/g O_2
Y_{xS}	Biomass yield over substrate	g cell/g substrate
Y_{xp}	Biomass yield over PHB	g cell/ g PHB
$f_{PHB(max)}$	Maximum PHB to biomass ratio	
$f^0_{PHB(max)}$	Maximum PHB to biomass ratio with no additional Na^+	
f_{PHB}	PHB to biomass ratio, P/X	
k_H	Henry's constant	L.atm/mol
n_p	Na^+ inhibition coefficient on PHB production kinetics	
n_{pf}	Na^+ inhibition coefficient on maximum PHB to biomass fraction	
n_x	Na^+ inhibition coefficient on growth kinetics	
μ_N	Specific nitrogen consumption rate	g nitrogen/g cell/h
$\mu^{max}_{ps_{het}}$	Maximum specific PHB production rate at heterotrophic phase	g PHB/g cell/h
$\mu^{max}_{ps_{aut}}$	Maximum specific PHB production rate at autotrophic phase	g PHB/g cell/h
μ_p	Specific PHB production rate	g PHB/g cell/h
μ_{ps}	Specific PHB production rate on substrate	g PHB/g cell/h
μ^{max}_{ps}	Maximum specific PHB production rate on substrate	g PHB/g cell/h
μ_s	Specific substrate consumption rate	g substrate/g cell/h
μ_x	Specific biomass growth rate	g cell/g cell/ h
μ_{xp}	Specific growth rate on PHB	g cell/ g cell/h
μ^0_{xp}	Specific growth rate on PHB with no additional Na^+	g cell/g cell/h
μ^{max}_{xp}	Maximum specific growth rate on PHB	g cell/g cell/h
μ_{xs}	Specific growth rate on substrate	g cell/g cell/h
μ^0_{xs}	Specific growth rate on substrate with no additional Na^+	g cell/g cell/h
μ^{max}_{xs}	Maximum specific growth rate on substrate	g cell/g cell/h
ρ_{FN}	Density of the nitrogen feeding solution	g/L
ρ_{FNa}	Density of the NaOH feeding solution	g/L
ρ_{FS}	Density of the substrate feeding solution	g/L
ρ_{ps}	PHB production rate on substrate	g PHB/L/h
ρ_w	Water density	g/L
ρ_{xp}	Biomass growth rate on PHB	g cell/L/h
ρ_{xs}	Biomass growth rate on substrate	g cell/L/h
C_x	Degree of crystallinity	%

H_m	Melting enthalpy	J/g
$k_L a$	Volumetric mass transfer coefficient	h^{-1}
M_n	Number average molecular weight	kDa
M_w	Average molecular weight	kDa
N	Nitrogen concentration	g/L
P	PHB production	g/L
S	Substrate concentration	g/L
T_d	Degradation temperature	°C
T_g	Glass transition temperature	°C
T_m	Melting temperature	°C
$V(t)$	Bioreactor working volume	L
X	Residual biomass concentration	g/L
X_0	Initial biomass concentration (at time=0)	g/L
X_m	Residual biomass concentration at which specific growth rate is zero	g/L
$D(t)$	Dilution rate	h^{-1}
E	Nash-Sutcliffe model efficiency coefficient	
$F(t)$	Overall feed flow rate	L/h
j	Objective function	
p	Perturbation factor	
y	Variable	
α	Biomass density inhibition coefficient	
β	PHB production saturation power coefficient	
δ	Combined relative sensitivity function	
η	Correction factor for alkali addition	
θ	Parameter	

Summary

Polyhydroxybutyrate (PHB) is an intracellular energy and carbon storage material synthesized by a variety of microorganisms, which has become of considerable industrial interest and of environmental importance as a biodegradable and biobased plastic. Even though PHBs are regarded as an effective substitute for petroleum-based plastics, the high production cost has hampered their commercial application. A lot of effort has been devoted to reduce the production cost by developing more efficient processes for the production of PHB, and by using inexpensive renewable resources or secondary raw materials as substrate. This PhD research focused on the development and optimization of a new and sustainable two-phase fermentation process for pure culture PHB production, by using either pure or (industrial) waste organic substrate and carbon dioxide (CO_2) as carbon source. To this end, lab-scale experiments were combined with modelling and simulation work. Overall, the work contributes to attain commercial viability and to increas the sustainability of PHB production.

The most frequently applied pure culture PHB production method concerns a two-phase fed-batch fermentation process that consists of a cell growth phase under favorable growth conditions to yield a high cell density (phase 1), followed by a PHB production phase under imbalanced growth conditions by limiting a nutritional element, such as nitrogen, phosphate, or oxygen (O_2), to trigger PHB synthesis (phase 2). Most often heterotrophic conditions are applied during both phases; the resulting process is termed heterotrophic-heterotrophic PHB production. A wide variety of organic substrates can be used, either pure substrates such as glucose, sucrose, starch, or cellulose, or waste substrates such as molasses, whey and waste glycerol. However, also autotrophic-autotrophic PHB production is possible, by applying bacteria which use CO_2 as a carbon source, hydrogen (H_2) as an energy source and O_2 as electron acceptor. Even though autotrophic PHB production is an interesting process option to reduce the concentration of the greenhouse gas CO_2, its application is limited by the fact that the O_2 concentration in the gas phase needs to be kept below the lower level of explosion. This limitation can be overcome through heterotrophic-autotrophic PHB production, consisting of a heterotrophic growth phase on organic substrate, followed by autotrophic PHB production on CO_2, H_2, and O_2, which constitutes the ultimate goal of this thesis. Fermentation through a pure culture of *Cupriavidus necator* was considered throughout the work. This bacterial species is a metabolically versatile organism capable of shifting between heterotrophic and autotrophic growth.

The **first part** of the work (chapters 2, 3 and 4) concerns the study of **heterotrophic-heterotrophic PHB production.** The main challenge in fed-batch fermentation is to control the substrate concentration within an optimal range, thereby avoiding limiting and inhibiting

concentration levels. Previously developed strategies to control the substrate concentration during fed-batch fermentation of PHB exhibit drawbacks such as the absence of feedback control, lack of sensitivity, being expensive and/or limited to a particular substrate. To overcome these limitations, a three-stage control strategy independent of the organic substrate was developed for automated substrate feeding in a two-phase fed-batch process for PHB production (**Chapter 2)**. The optimal feeding strategy was determined by using glucose as the substrate and for a culture of *C. necator*. The combined substrate feeding strategy consisting of exponential feeding, followed by a novel method based on alkali-addition monitoring, which resulted in a maximal cell concentration in the growth phase (phase 1). In the PHB accumulation phase (phase 2), a constant amount of substrate was dosed, based on the estimated amount of biomass produced in the phase 1 and on the specific PHB accumulation rate. Through this control strategy, the glucose concentration was maintained within its optimal range of 10-20 g/L. Maximal cell concentration and PHB production of 164 and 125 g/L, respectively, were obtained when nitrogen feeding was stopped at 56 g/L of residual biomass. The three-stage feeding strategy was validated using waste glycerol as the sole carbon source for PHB production, resulting in a PHB production of 65.6 g/L and PHB content of 62.7% while keeping the glycerol concentration within its optimal range.

A mechanistic model describing pure culture heterotrophic-heterotrophic PHB production was developed in **Chapter 3**. The model was calibrated and validated for two different organic substrates, glucose and waste glycerol. In both cases, PHB production was triggered by applying nitrogen limitation. The simulation results matched the experimental observations very well. Biomass growth on PHB during non-limiting (growth) conditions was found non-negligible, even in the presence of substrate. Biomass growth was clearly inhibited by the biomass density. Even though the presence of nitrogen inhibits PHB production, some PHB production during the growth phase (growth-associated PHB production) was detected. Other phenomena described by the model included non-linear product inhibition of PHB production. The accumulated impurities from the waste substrate negatively affected the obtained maximum PHB content. Overall, the developed mathematical model provided an accurate prediction of the dynamic behavior of heterotrophic biomass growth and PHB production in a two-phase pure culture system.

Chapter 4 evaluated the effect of sodium (Na^+) concentration on the growth and PHB production by *C. necator*. Both biomass growth and PHB production were inhibited by Na^+. Biomass growth became zero at 8.9 g/L Na^+ concentration while PHB production was completely stopped at 10.5 g/L Na^+. A mathematical model for pure culture heterotrophic PHB

production was set up to evaluate the Na^+ inhibition effect. The parameters related to Na^+ inhibition were estimated based on shake flask experiments. The model was subsequently validated based on fed-batch experiments. The accumulated Na^+ showed non-linear inhibition effect on biomass growth and PHB content but linear inhibition effect on PHB production kinetics. Fed-batch experiments revealed that a high accumulation of Na^+ due to a prolonged growth phase, using NaOH for pH control, decreased the subsequent PHB production.

The **second part** of the thesis deals with **autotrophic-autotrophic PHB production**. In **Chapter 5**, a mathematical model based on mass balances was set up to describe autotrophic PHB production. The model takes into account the stoichiometry and kinetics of biomass growth and PHB formation as well as the physical transfer from the gas phase to the liquid fermentation broth. The developed model was calibrated and validated based on independent experimental datasets from literature obtained for *C. necator*. The obtained simulation results accurately described the dynamics of autotrophic biomass growth and PHB production. The effect of O_2 and/or nitrogen stress conditions, and the gas mixture composition in terms of O_2 and H_2 was investigated through scenario analysis. As a major outcome, a higher maximum PHB production was obtained under O_2 stress conditions compared to nitrogen stress conditions. At high O_2 fractions in the gas mixture, which would result in H_2 limitation before O_2 limitation, PHB production could be increased by applying nitrogen stress. The effect of the reactor type was assessed through comparing a continuous stirred tank reactor (CSTR) with an air-lift fermentor. The developed model forms the basis for future design with minimum experimentation of suitable control strategy aiming at a high PHB production.

The **third part** of the thesis is devoted to **heterotrophic-autotrophic PHB production**. The technical feasibility of *C. necator* for sustainable autotrophic PHB production from CO_2 following heterotrophic cell growth was evaluated in **Chapter 6**. In this cultivation method, cell growth occurred under heterotrophic conditions using two different organic substrates: glucose and waste glycerol. In both cases, PHB biosynthesis was triggered by applying nitrogen and O_2 limitation at three different cell mass concentrations under autotrophic conditions using a gas mixture of CO_2, O_2 and H_2. To ensure the test conditions relevant for industrial application, O_2 concentration was kept below the safety value during autotrophic PHB production phase. PHB production from CO_2 on waste glycerol grown cell mass resulted in a PHB production of 28 g/L, which makes up the highest value ever reported in literature for PHB synthesis from CO_2 at an O_2 concentration below the lower explosion limit of 5 vol%. The fermentation performance decreased when delaying the phase shift, i.e. when nutrient limitation was applied at higher cell mass concentrations. It was further shown that PHB

production from CO_2 at high cell mass concentration is metabolically feasible, but under the tested conditions the O_2 mass transfer was limiting PHB accumulation. Characterization of the produced PHB revealed that the organic carbon source affected the properties of PHB. Overall, it can be concluded the cultivation method developed in this thesis research work led to the production of PHB with properties similar range to commercial PHB and PHB typically found in literature. In order to compete with current heterotrophic-heterotrophic cultivation systems, the O_2 transfer rate should be enhanced to achieve a higher PHB productivity.

In **Chapter 7**, a model for heterotrophic-autotrophic PHB production was setup based on previously established models for heterotrophic-heterotrophic (Chapter 3) and autotrophic-autotrophic (Chapter 5) PHB production processes. The model was validated on the experimental datasets obtained with different organic substrates at different switching points (Chapter 6). The developed mathematical model provided an accurate prediction of the dynamic behavior of heterotrophic biomass growth and autotrophic PHB production. The effect of O_2 and ammonium-nitrogen (NH_4^+-N) on biomass growth and PHB production were investigated. The optimal O_2 concentration for PHB production was determined as 0.224 mg/L. The optimal nitrogen concentration for biomass growth was 0.60-0.70 g NH_4^+-N/L, while PHB production was maximal under nitrogen free condition. Heterotrophic-autotrophic PHB production is currently economically less attractive than heterotrophic-heterotrophic PHB production. Further process optimization and possible carbon taxes may stimulate its application in future.

Chapter 8 offers some general conclusions and perspectives drawn from this work. It includes some critical remarks on the implications of this thesis for PHB production in terms of the used substrate as well as concerning process optimization. This chapter further contains suggestions for future research, with the final aim to improve the economical and practical (industrial) feasibility of the process.

Samenvatting

Polyhydroxybutyraat (PHB) is een intracellulair reservemateriaal voor energie en koolstof dat gesynthetiseerd wordt door een verscheidenheid aan micro-organismen. PHB geniet industrieel belang als een biologisch afbreekbaar en bio-gebaseerde plastic. Hoewel PHBs worden beschouwd als een effectief alternatief voor kunststoffen gebaseerd op aardolie, belemmeren de hoge productiekosten hun commerciële toepassing. Veel inspanningen zijn reeds geleverd om de productiekosten van PHB te verminderen door de ontwikkeling van efficiëntere processen en door het gebruik van goedkope hernieuwbare of secundaire grondstoffen als substraat. Dit doctoraatsonderzoek richtte zich op de ontwikkeling en optimalisatie van een nieuw en duurzaam tweefasig fermentatieproces voor PHB productie met een reincultuur uit een zuiver organisch substraat, industriële organische reststromen of CO_2 als koolstofbron. Daartoe werden experimenten op labo schaal gecombineerd met modellering- en simulatiewerk. Het werk draagt bij tot een verhoogde commerciële levensvatbaarheid en duurzaamheid van PHB productie.

De meest toegepaste productiemethode voor PHB met een reincultuur betreft een tweefasig *fed-batch* fermentatieproces. De eerste fase bestaat uit celgroei onder gunstige groeiomstandigheden om tot een hoge celdichtheid te komen. Om PHB synthese te activeren dienen de micro-organismen op een bepaald punt in de cultivatie onder stress te worden gebracht door het reduceren van een nutriëntenstroom, zoals stikstof, fosfaat of zuurstof. Meestal worden beide fasen onder heterotrofe condities uitgevoerd. Het resulterend proces wordt dan heterotrofe-heterotrofe PHB productie genoemd. Een grote verscheidenheid van organische substraten kan worden gebruikt, hetzij zuivere substraten zoals glucose, sucrose, zetmeel of cellulose, of reststromen zoals melasse, wei en afval glycerol. Ook autotrofe PHB productie is mogelijk door het gebruik van bacteriën die koolstofdioxide (CO_2) als koolstofbron kunnen aanwenden met waterstof (H_2) als energiebron en zuurstof (O_2) als elektronacceptor. Hoewel autotrofe PHB productie een interessante technologie is om de concentratie van het broeikasgas CO_2 te verminderen, is de toepassing beperkt omdat de zuurstofconcentratie in de gasfase onder de onderste explosiegrens dient te blijven. Deze beperking kan overwonnen worden door heterotrofe-autotrofe PHB productie, bestaande uit een heterotrofe groeifase uit organisch substraat, gevolgd door autotrofe PHB productie uit CO_2, H_2 en O_2, wat het uiteindelijke doel van dit proefschrift vormt. Als reincultuur werd *Cupriavidus necator* gekozen, een metabolisch veelzijdige bacterie die in staat is zowel heterotroof als autotroof te groeien.

Een **eerste deel** van het werk (hoofdstukken 2, 3 en 4) betreft de studie van **heterotrofe-heterotrofe PHB productie**. De belangrijkste uitdaging in *fed-batch* fermentatie is om de

substraatconcentratie binnen een optimaal bereik te controleren zodat limiterende en inhiberende concentratieniveaus vermeden worden. Eerder ontwikkelde strategieën om de substraatconcentratie in *fed-batch* fermentatie van PHB te controleren vertonen nadelen, zoals de afwezigheid van een *feedback* controle strategie, een gebrek aan gevoeligheid, de kost en/of de toepasbaarheid voor slechts één bepaald substraat. Om deze beperkingen te overwinnen werd een driefasige regelstrategie ontwikkeld onafhankelijk van het organische substraat. Daarin wordt substraatvoeding geautomatiseerd in een tweefasig *fed-batch* proces voor PHB productie (**Hoofdstuk 2**). De optimale voedingsstrategie werd ontwikkeld met behulp van glucose als substraat en *C. necator* als reincultuur. De gecombineerde substraatvoedingsstrategie bestaat uit exponentieel voeden, gevolgd door een nieuwe methode op basis van basedosering. Dit resulteerde in een maximale celconcentratie in de groeifase (fase 1). In de PHB accumulatiefase (fase 2) werd een constante hoeveelheid substraat gedoseerd, gebaseerd op de geschatte hoeveelheid biomassa die in de fase 1 gevormd werd en de specifieke PHB accumulatiesnelheid. Door deze regelstrategie kon de concentratie aan glucose binnen het optimale bereik van 10-20 g/L gehouden worden. Maximale cel- en PHB-concentraties van 164 en 125 g/L respectievelijk, werden verkregen wanneer de stikstofdosering werd gestopt bij 56 g/L residuele biomassa. De voedingsstrategie werd gevalideerd met afval glycerol als de enige koolstofbron voor PHB productie. Een PHB concentratie van 65.6 g/L en PHB gehalte van 62.7% werd hierbij bereikt terwijl glycerol in zijn optimale concentratierange werd gecontroleerd.

Een mechanistisch model dat heterotrofe-heterotrofe PHB productie door een reincultuur beschrijft, werd ontwikkeld in **Hoofdstuk 3**. Het model werd gecalibreerd en gevalideerd voor twee verschillende organische substraten, glucose en afval glycerol. In beide gevallen werd PHB productie getriggerd door een limitatie aan stikstof. De simulatieresultaten kwamen zeer goed overeen met de experimentele waarnemingen. Biomassagroei uit PHB tijdens niet-limiterende (groei)condities bleek niet verwaarloosbaar te zijn, zelfs in aanwezigheid van substraat. Biomassagroei werd duidelijk geïnhibeerd door de biomassadensiteit. Hoewel de aanwezigheid van stikstof PHB productie remt, werd enige PHB productie gedurende de groeifase waargenomen. Andere verschijnselen beschreven door het model waren niet-lineaire PHB inhibitie. De geaccumuleerde onzuiverheden uit het afvalsubstraat hadden een negatieve invloed op de verkregen maximale PHB inhoud. In het algemeen kan gesteld worden dat het ontwikkelde wiskundige model nauwkeurig het dynamische gedrag van heterotrofe biomassagroei en PHB productie in een tweefasige reincultuur kan voorspellen.

Hoofdstuk 4 evalueerde het effect van natrium (Na^+) concentratie op de groei en PHB productie van *C. necator*. Zowel de biomassagroei als PHB productie werden geïnhibeerd door Na^+. De groei van biomassa werd nul bij 8,9 g/L Na^+ terwijl PHB productie geheel werd gestopt bij 10,5 g/L Na^+. Een wiskundig model voor heterotrofe PHB productie door een reincultuur werd ontwikkeld om het Na^+ inhibitie effect te evalueren. De parameters gerelateerd aan Na^+ remming werden geschat op basis van schudflesexperimenten. Het model werd vervolgens gevalideerd met *fed-batch* experimenten. De geaccumuleerde Na^+ vertoonde een niet-lineair inhibitie effect op de biomassagroei en PHB inhoud, maar een lineair inhibitie effect op de PHB productiekinetiek. *Fed-batch* experimenten onthulden dat een hoge accumulatie van Na^+ die te wijten was aan een langere groeifase met NaOH als base voor pH controle, de PHB productie verlaagde.

Een **tweede deel** van het proefschrift behandelt **autotrofe-autotrofe PHB productie**. In **Hoofdstuk 5** werd een wiskundig model op basis van massabalansen ontwikkeld om autotrofe PHB productie te beschrijven. Het model houdt rekening met de stoichiometrie en kinetieken van biomassagroei en PHB vorming evenals de fysische overdracht van de gasfase naar de vloeibare fermentatievloeistof. Het ontwikkelde model werd gecalibreerd en gevalideerd op basis van onafhankelijke experimentele datasets uit de literatuur verkregen voor *C. necator*. De verkregen simulatieresultaten beschreven nauwkeurig de dynamieken van autotrofe biomassagroei en PHB productie. Het effect van O_2 en/of stikstof stresscondities, en de gassamenstelling wat O_2 en H_2 betreft, werd onderzocht door scenario analyse. Een belangrijk resultaat was dat een hogere PHB concentratie bekomen werd onder O_2 stressomstandigheden dan onder stikstof stress. Bij hoge O_2 fracties in het gasmengsel, hetgeen zou resulteren in H_2 limitatie voordat O_2 limitatie plaatsvindt, kon de PHB productie verhoogd worden bij stikstof limitatie. Het effect van het type reactor werd beoordeeld door het vergelijken van een continu geroerde tank reactor met een *air-lift* fermentor. Het ontwikkelde model vormt de basis voor toekomstig werk om met een minimum aan experimenten een geschikte controlestrategie te bepalen voor een hoge PHB productie.

Het **derde deel** van het proefschrift is gewijd aan **heterotrofe-autotrofe PHB productie**. De technische haalbaarheid van duurzame autotrofe PHB productie uit CO_2 volgend op heterotrofe celgroei werd geëvalueerd in **Hoofdstuk 6**. In deze cultivatiemethode vond celgroei plaats onder heterotrofe omstandigheden met twee verschillende organische substraten: glucose en afval glycerol. In beide gevallen werd PHB biosynthese getriggerd door stikstof en O_2 te limiteren bij drie verschillende celmassaconcentraties onder autotrofe condities bestaande uit een gasmengsel van CO_2, O_2 en H_2.

Om te verzekeren dat de gekozen testcondities industrieel relevant zijn, werd de O_2 concentratie tijdens autotrofe PHB productiefase onder de veiligheidswaarde gehouden. PHB productie uit CO_2 door op afval glycerol gegroeide celmassa resulteerde in een PHB concentratie van 28 g/L, de hoogste waarde gerapporteerd in de literatuur bij een O_2 concentratie onder de onderste explosiegrens van 5 vol%. De fermentatieprestatie nam af bij het vertragen van de faseverschuiving, *i.e.* wanneer nutriëntlimitatie werd toegepast bij hogere celmassaconcentraties. Verder werd aangetoond dat PHB productie uit CO_2 bij een hoge celmassaconcentratie metabolisch mogelijk is, maar de massa overdracht van O_2 belemmerde PHB accumulatie onder de geteste condities. Karakterisatie van het geproduceerde PHB toonde aan dat de organische koolstofbron de eigenschappen van PHB beïnvloedde. In het algemeen kan echter gesteld worden dat de eigenschappen van PHB geproduceerd door de cultivatiemethode ontwikkeld in dit proefschift vergelijkbaar waren met die van commercieel PHB en PHB typisch gerapporteerd in de literatuur. Om te kunnen concurreren met de huidige heterotrofe-heterotrofe cultivatiesystemen, moet de transfersnelheid van zuurstof echter verbeterd worden zodat een hogere productiviteit van PHB bekomen kan worden.

In **Hoofdstuk 7** werd een model voor heterotrofe-autotrofe PHB productie ontwikkeld op basis van de eerder ontwikkelde modellen voor heterotrofe-heterotrofe (Hoofdstuk 3) en autotrofe-autotrofe (Hoofdstuk 5) PHB productie. Het model werd gevalideerd met de experimentele datasets verkregen voor de verschillende organische substraten bij verschillende faseverschuivingen (Hoofdstuk 6). Het ontwikkelde wiskundige model is in staat het dynamische gedrag van heterotrofe biomassagroei en autotrofe PHB productie nauwkeurig te voorspellen. Het effect van O_2 en ammonium-stikstof (NH_4^+-N) op de biomassa groei en PHB productie werd tevens onderzocht. De optimale O_2-concentratie voor PHB productie was 0.224 mg/L. De optimale stikstofconcentratie voor biomassagroei was 0.60-0.70 g NH_4^+-N/L, terwijl PHB productie maximaal was onder stikstofvrije condities. Heterotrofe-autotrofe PHB productie is momenteel economisch minder aantrekkelijk dan heterotrofe-heterotrofe PHB productie. Verdere procesoptimalisatie en mogelijke koolstoftaksen kan dit proces stimuleren in de toekomst.

Hoofdstuk 8 biedt een aantal algemene conclusies en perspectieven getrokken uit deze doctoraatstudie. Het omvat een aantal kritische opmerkingen over de implicaties van dit proefschrift voor PHB productie in termen van het gebruikte substraat evenals de betreffende procesoptimalisatie. Dit hoofdstuk bevat suggesties voor toekomstig onderzoek, met het uiteindelijke doel om de economische en praktische (industriële) haalbaarheid van het proces te verbeteren.

Chapter 1:

General introduction

1.1. Introduction

Plastics have become an indispensable part of our daily life. Since the 1950s, the production of plastics has shown a steady increase with about 9% per year. Global production reached 299 million tons in 2013. The production of plastics is so far mainly based on fossil fuels and takes place through energy intensive petrochemical processes (Shen et al., 2009). The environmental impact of these conventional fossil-based plastics is not associated only to their production process, but also to their non-biodegradability, making them persistent to the environment. They are therefore becoming a serious pollution issue (Castilho et al., 2009; Marshall et al., 2013). The world's growing environmental awareness and limited fossil fuels reserves have directed research and industrial attention towards the production of bioplastics as alternatives for petrochemical based synthetics. Bioplastics encompass the materials which are either biobased or biodegradable or both. They can be produced fully or partially from biomass and can be tailored to be fully or partially biodegradable.

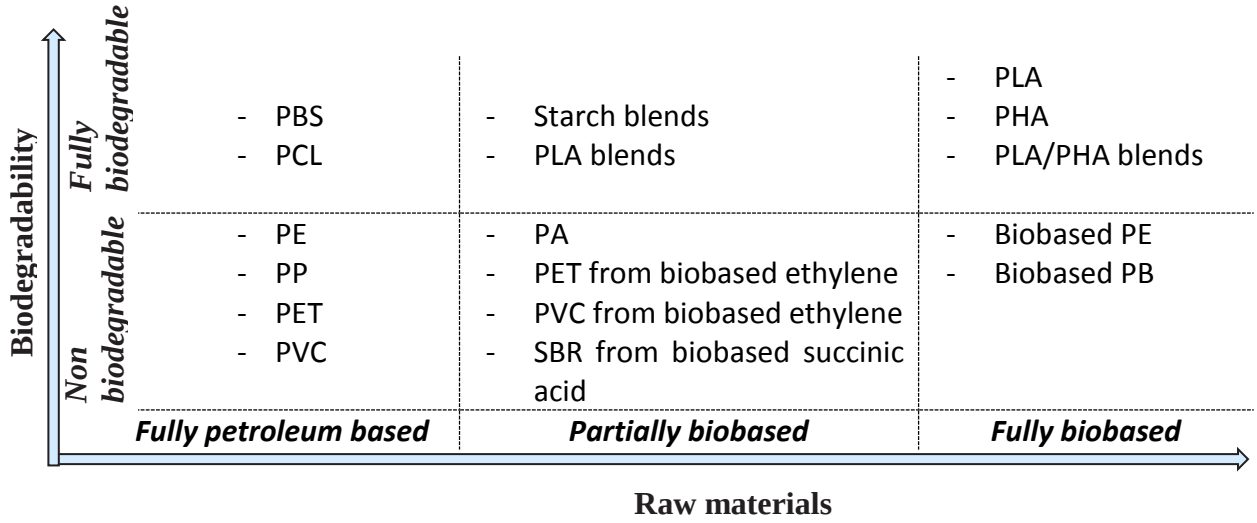


Figure 1.1. Classification of plastics based on source and biodegradability (Shen et al., 2009). (PA=Polyamide, PB=Polybutadiene, PBS=Polybutyrate succinate, PCL=Polycaprolactone, PE=Polyethylene, PET=Polyethylene terephthalate, PHA=Polyhydroxyalkanoate, PLA=Polylactic acid, PP=Polypropylene, PVC=Polyvinyl chloride, SBR=Styrene butadiene rubber).

Among the alternatives, an interesting bioplastic are the fully biobased and biodegradable polyhydroxyalkanoates (PHAs) (Figure 1.1). In this chapter the structure of PHAs as well as of

polyhydroxybutyrate (PHB) (Section 1.2) and available production methods (Section 1.3) are discussed in detail. Section 1.4 presents the properties and Section 1.5 explores the applications of PHB. Finally, the objectives and outline of this PhD thesis are summarized in Section 1.6.

1.2. Polyhydroxyalkanoates (PHAs) and polyhydroxybutyrate (PHB)

Among the different types of bioplastics, a lot of attention has been devoted to PHAs, which are synthesized by a number of microorganisms as intracellular storage material. PHAs constitute an attractive alternative to petrochemically synthetized plastics due to their comparable physical and chemical properties, and because of their biodegradability and biocompatibility as additional advantages.

PHAs are polyesters of hydroxyalkanoic acids, composed of hydroxy fatty acids with the most common structure shown in Figure 1.2. The side group (R in Figure 1.2) varies from methyl (C_1) to tridecyl (C_{13}) (Madison and Huisman, 1999). PHA are termed polyhydroxybutyrate (PHB) if R is a methyl ($-CH_3$) group, polyhydroxyvalerate (PHV) if R is an ethyl ($-CH_2CH_3$) group, and polyhydroxyhexanoate (PHHx) if R represents a propyl ($-CH_2CH_2CH_3$) group.

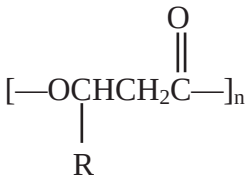


Figure 1.2. General structure of a polyhydroxyalkanoate (PHA)

Polyhydroxybutyrate (PHB) is the most common and well-studied PHA. It was first isolated and characterized by Lemoigne in 1925 (Doi, 1990). Since then, a number of studies have been performed with various bacterial strains such as Gram-positive bacteria (Findlay and White, 1983; Williamson and Wilkinson, 1958), Gram-negative bacteria (Forsyth et al., 1958), photosynthetic bacteria (Hassan et al., 1996; Hassan et al., 1997; Hassan et al., 1998) including cyanobacteria (Jau et al., 2005; Jensen and Sicko, 1971) to identify which one has the highest PHB production capacity. Macrae and Wilkinson (1958) found that PHB production by *Bacillus megaterium* was stimulated when the ratio of glucose to nitrogen in the culture medium was high, while intracellular degradation of PHB occurred in the absence of carbon and energy sources. By the year 1973 it was well recognized that the storage material PHB fulfilled a similar role for bacteria as starch and glycogen for higher organisms *i.e.* eukaryotic (Dawes and Senior, 1973). First it was thought that the produced PHB was a homopolymer,

comprising hydroxybutyrate (HB) as the sole monomer. Later, it was discovered that PHB also contained other types of monomers besides the already present HB such as hydroxyvalerate (HV), hydroxyhexanoate (HHx), hydroxyheptanoate (HHp), hydroxyoctanoate (HO) (Wallen and Rohwedder, 1974; Findlay and White,1983; De Smet et al., 1983). Besides, a large variety of monomers with straight, branched, saturated, unsaturated and also aromatic structures were also found as a constituent in copolymers of PHB (Steinbüchel and Valentin, 1995; Witholt and Kessler, 1999). The presence of various monomers in the copolymer appeared to be dependent on the substrate used as carbon and energy source.

Intensive research on PHB production by pure cultures of bacteria has been conducted, mostly for seeking an inexpensive carbon source to reduce the production cost and applying genetic engineering to improve the productivity. Another method to reduce the production cost is to use a microbial mixed culture which allows saving energy (no sterilization is required) and reduces fermentation equipment costs (less expensive materials for reactor construction) (Serafim et al., 2008). Despite these advantages, the yield (<65%) and volumetric productivities still remain low, and other metabolites and extracellular polymeric substances are being produced (Serafim et al., 2008).

Since the current industrial processes are based on the pure culture fermentation, this PhD research only focused on pure culture process.

1.3. Pure-culture PHB production processes

PHB production through microbial fermentation takes place under heterotrophic and/or autotrophic conditions. While most works were carried out using organic substrates as carbon source, *i.e.* heterotrophic PHB production, (Choi et al., 1997; Ryu et al., 1997; Steinbuchel, 2001; Wang and Lee, 1997) attempts to produce PHB from carbon dioxide (CO_2), *i.e.* autotrophic or heterotrophic-autotrophic PHB production, using hydrogen (H_2) as an energy source, have also been undertaken (Tanaka et al., 1994; Tanaka and Ishizaki, 1994; Volova and Kalacheva, 2005).

1.3.1. Heterotrophic process

Heterotrophic PHB production is a bioprocess that uses an organic substrate as carbon and energy source. The provided substrate is able to support bacterial growth, maintenance functions and reserve polymer synthesis (Wang et al., 2012).

Most research and industrial production of PHB has focused on usage of pure cultures. Under optimal process conditions, the microorganisms have the ability to accumulate PHB until 80% of the cell dry mass (CDM) (Posada et al., 2011).

Heterotrophic biomass growth on an organic substrate proceeds mostly according to the following equation (Doran, 1995):

$$C_wH_xO_y + (4w - \frac{y}{2} + \frac{x}{4} - 1.91Y_{XS})\ O_2 + 0.19\ Y_X\ NH_4^+ \rightarrow Y_X\ CH_{1.74}O_{0.46}N_{0.19} + (w - Y_X)\ CO_2 + \frac{x - 1.17Y_X}{2}\ H_2O + 0.19\ Y_X\ H^+ \quad (1.1)$$

In Eq. 1.1, $C_wH_xO_y$ denotes the organic substrate, and $CH_{1.74}O_{0.46}N_{0.19}$ is the chemical composition of *Cupriavidus necator* biomass (without PHB) (Ishizaki and Tanaka, 1990), a model organism for PHB production (Asenjo and Suk, 1985). Y_X is the yield of biomass over organic substrate. During cultivation, oxygen (O_2) and NH_4^+ are mandatory for bacterial growth as an electron acceptor and as a nitrogen source respectively and CO_2 is produced as a side-product. PHB production is suppressed due to excess of NH_4^+ (Kim et al., 1994).

Heterotrophic PHB production from an organic carbon source is represented according to the Eq. 1.2 (Akiyama et al., 2003).

$$C_wH_xO_y + (w - \frac{y}{2} + \frac{x}{4} - \frac{9}{2}Y_P)\ O_2 \rightarrow Y_P\ C_4H_6O_2 + (w - 4Y_P)\ CO_2 + (\frac{x}{2} - 3Y_X)\ H_2O \quad (1.2)$$

Here $C_4H_6O_2$ is the chemical composition of PHB monomer. Y_P is the yield of PHB over organic substrate.

Most lab-scale studies and established industrial processes for PHB production work with pure sugars such as glucose, fructose, sucrose etc. as carbon source, ensuring high productivity and proper metabolic functioning of the strain. Nevertheless, these substances are expensive and several efforts have been developed to identify low cost carbon sources, namely industrial and agricultural waste substrate such as waste glycerol, molasses, dairy whey, corn syrup, starch residues etc. There are still some concerns about the final PHB content as well as a high productivity.

Two prevalent cultivation methods are employed for pure culture PHB production, depending on the microorganism used; growth associated and non-growth associated mode.

1.3.1.1 Growth associated PHB production

Growth associated PHB production is an one step process, in which PHB production is conducted in parallel with bacterial growth. In this case there is low nutrient inhibition effect on the PHB production process. Organisms like *Bacillus mycoides*, *Azohydromonas lata* etc. are able to produce PHB in growth associated mode with high productivity in a nutrient rich medium. It is possible to further increase the PHB productivity however applying a stress condition (*i.e.* nitrogen limitation) (Koller and Muhr, 2014).

1.3.1.2 Non-growth associated PHB production

The non-growth associated manner consists of two phases. First, biomass is grown under favourable conditions especially at optimal level of carbon, nitrogen and oxygen (O_2) concentration. The second phase is PHB accumulation under nutrient limiting conditions namely nitrogen (Arifin et al., 2011; Kulpreecha et al., 2009; Pradella et al., 2012; Ramachandrana and Amirul, 2013), phosphorus (Haas et al., 2008; Ryu et al., 1997; Shang et al., 2007) or O_2 limitation (Ishizaki and Tanaka, 1991). Due to the higher productivity reached, most researchers select the pure culture non-growth associated process to produce PHB.

Various bacteria have the ability to synthesize PHB such as *Cupriavidus necator*, *Alcaligenes latus*, *Azotobacter vinelandii*, certain *Pseudomonas* as well as genetically modified *Escherichia coli* strains etc. Among them *C. necator* is the most extensively studied micro-organism due to its high productivity (Ashby et al., 2002). It can produce significant amounts of PHB from different carbon substrate such as glucose, fructose, glycerol, oil and even waste substrate (Table 1.1).

Table 1.1: Comparison of PHB production and productivity using *Cupriavidus necator* (formerly known as *Ralstonia eutropha* and *Alcaligenes eutrophus*) as an organism in a fed-batch fermentation process in order of increased PHB concentration.

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

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

1.3.2. Autotrophic process

A gas mixture of CO_2, H_2 and O_2 is used for cell growth and PHB production. Hydrogen oxidizing bacteria such as *C. necator* have the ability to grow and produce PHB through autotrophic metabolism, using CO_2 as carbon source and H_2 as energy source (Table 1.1). CO_2 is a greenhouse gas of which the concentration in the atmosphere is related with the global warming phenomenon. In attempts to counteract climate change, emitted CO_2 can be a valuable source of carbon which can be utilized in the production of commercially valuable products such as PHB. Autotrophic biomass growth (Eq. 1.3) (Ishizaki and Tanaka, 1990) and PHB production (Eq. 1.4) (Ishizaki and Tanaka, 1991) are described by equations 1.3 and 1.4 respectively.

$$21.36\ H_2 + 6.21\ O_2 + 4.09\ CO_2 + 0.76\ NH_4^+ \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.7\ H_2O + H^+ \quad (1.3)$$

$$33\ H_2 + 12\ O_2 + 4\ CO_2 \rightarrow C_4H_6O_2 + 30\ H_2O \quad (1.4)$$

Until now autotrophic PHB production has only been conducted in pure culture non-growth associated manner using *C. necator* as organism. The gas composition which attains sufficient cell growth has a ratio of H_2:O_2:CO_2 = 7:1:1. Such a gas composition lies completely within the gas-explosion range and is therefore too dangerous to work with. Considering safety issues, the O_2 concentration in the gas phase should be kept below the lower level of explosion (LEL) for O_2, between 6 and 6.9 vol% (Takeshita and Ishizaki, 1996). However, under those low O_2 concentrations, limited growth as well as low PHB production were achieved (Tanaka et al., 1995).

1.3.3. Heterotrophic-autotrophic process

The heterotrophic-autotrophic process consists of a heterotrophic phase for exponential growth using an organic substrate promoting a high cell density culture followed by an autotrophic phase for PHB production using a gas mixture of CO_2, O_2 and H_2 at an O_2 concentration under the LEL. The advantage of this cultivation system is that a high cell concentration can be obtained as O_2 can be supplied under non-limiting conditions during the cell mass growth phase, while in the autotrophic phase PHB biosynthesis will be triggered when the O_2 concentration is below its critical value of LEL.

1.4. Properties of PHB

PHB has attracted a lot of industrial attention due to its wide range of properties. Its properties are comparable with conventional fossil fuel based plastics *i.e.* PE, PP etc. PHB is a non-toxic and biodegradable thermoplastic of which the properties are influenced by the rate of polymerization and the molecular weight distribution (Doi, 1990). Co-polymers of PHB differ in their properties depending on the composition of the monomers, the length of the side chain and the functional groups in the polymer. Homo- and co-polymers of PHB have a wide range in degree of polymerization that reflects in wide range of physical properties like melting point, glass transition temperature, crystallinity, mechanical properties etc. (Table 1.2).

Some PHBs are similar in their material properties to polypropylene (PP) and offer a good resistance to moisture and good aroma barrier properties. Pure PHB is relatively brittle and stiff. Addition of plasticizers with PHB improves the flexibility and elongation properties while reducing crystallinity (Fabra et al., 2014). The properties of PHB can be improved by either blending with an other polymer (Bartczak et al., 2013; Modi et al., 2013) or using a mixed substrates during the culture period (Chia et al., 2010; Iqbal and Amirul, 2014). These improved properties of PHB can lead to a broader range of industrial applications.

Table 1.2: Range of typical properties of PHB and co-polymer of PHB (Akaraonye et al., 2010; Chia et al., 2010; Iqbal and Amirul, 2014; Reddy et al., 2009; Volova et al., 2013; Xie and Chen, 2008; Zhao and Chen, 2007).

Properties	Unit	PHB	P(HB-co-HV[1])	P(HB-co-HHx[2])
Weight average molecular weight (M_w)	kDa	105 – 613	922–1111	440 – 635
Polydispersity index	–	1.75 – 5.87	2.51 – 3.49	2.03 – 3.6
Melting point (T_m)	°C	120 – 180	145 – 179	94 – 129
Glass transition temperature (T_g)	°C	-35 – 8.2	-1.73 – 8.5	-1.75 – 0.6
Degradation temperature (T_d)	°C	226 – 290	224 – 295	239 – 285
Crystallinity	%	8 – 47	48 – 76	–
Tensile strength	MPa	18 – 40	2.4 – 42	4.5 – 36
Young modulus	MPa	3.5 – 19	1.2 – 63	135 – 117
Elongation	%	4 – 19	42 – 123	12 – 40

[1] 0.9-65 % of hydroxyvalerate (HV)
[2] 5-12 % of hydroxyhexanoate (HHx)

PHB is biodegradable under both aerobic and anaerobic conditions. A number of organisms in nature are able to degrade PHB through depolymerisation and enzymatic hydrolysis to oligomers and monomers and then metabolic degradation to water and CO_2 (Jendrossek et al., 2002; Lim et al., 2005; Sridewi et al., 2006). The melting point is also considered an indicator of biodegradability which decreases with increasing melting point. In addition, high order structures have a high crystallinity which results in an increased melting point and decreased biodegradability (Nishida and Tokiwa, 1992). Abe and Doi (1999) reported that higher crystallinity reduces the PHB degradability because it results in less amorphous regions on which organisms can attack.

1.5. Application of PHB

The wide range of properties of PHB and its copolymers make it an attractive biopolymer for various applications involving packaging, medical and coating materials. The main difficulty is to produce a specific copolymer with desired properties.

1.5.1. Industrial applications

PHB is used to make small disposable articles such as shampoo bottles and packaging materials including food packages (Hocking and Marchessault, 1994). It is also used for bags, paper, disposable utensils, cups etc. Foils, films and diaphragms can also possible be made with PHB. PHB latex can be used as water-resistance surface of cover paper or cardboard (Lauzier et al., 1993). Hard articles such as combs, pens etc. are made of PHB because of its high crystallinity (Chen, 2005). The copolymer P(HB-HHx) is used to make flushable, nonwovens, binders, flexible packing, thermoformed articles, synthetic paper and medical devices (Chen et al., 2001). P(HB-HV) has gas barrier properties and is useful for food packaging, plastic beverage bottles, coated paper milk cartons etc. (Hocking and Marchessault, 1994). PHB blend with PHO is also known as an elastomer for the production of food additives (Clarinval and Halleux, 2005) .

1.5.2. Medical applications

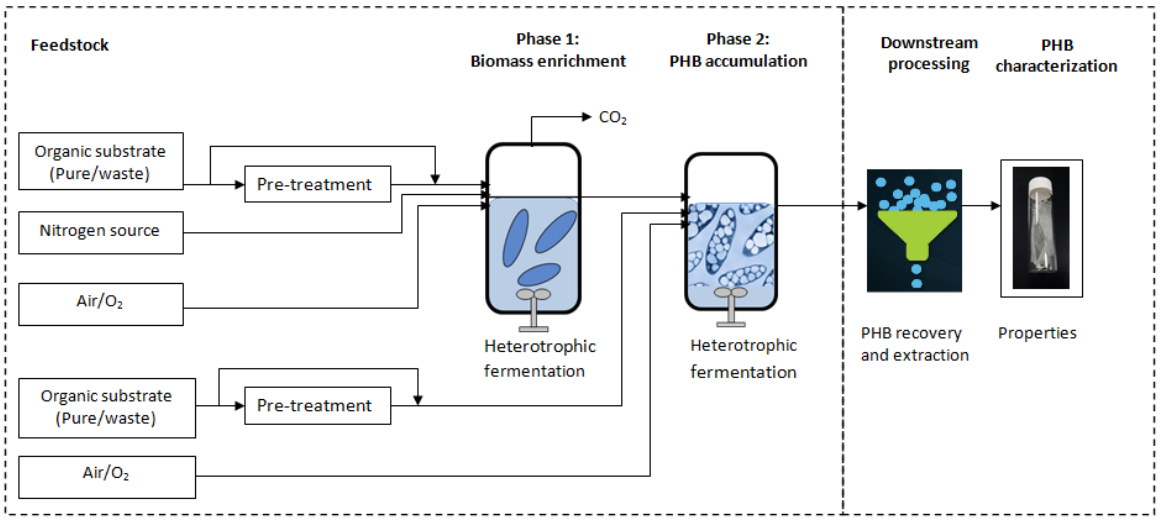
PHB and its copolymers are extensively used as pharmaceutical products in surgery, transplantology, tissue engineering, pharmacology etc. In tissue engineering, the cells are grown *in vitro* on PHB to construct “tissue” for implantation purposes (Shinoka et al., 1998).

PHB and P(HB-co-HHx) are the most extensively studied biopolymers for tissue engineering and controlled internal drug delivery system. PHB has also been found to be a suitable scaffold for preparing autologous cardiovascular tissue (Qu et al., 2006; Shangguan et al., 2006).
PHB is frequently used as bone plates, osteosynthetic materials and surgical sutures (Steinbuchel and Fuchtenbusch, 1998). Hydroxyapatite ($Ca_5(PO_4)_3OH$) incorporated into PHB can be used in hard tissue regeneration (Doyle et al., 1991). The combination of hydroxyapatite with PHB and P(HB-HV) leads to similar mechanical strength as that of human bones which is beneficial for bone tissue engineering (Galego et al., 2000). The graft copolymer of methyl methacrylate and PHB blocks can be used as bone cement in orthopedic applications (Nguyen and Marchessault, 2006). Moreover the biocompatible property of PHB makes it well-suited for skincare products (Chen and Wu , 2005).

1.6. Objectives of the research – thesis outline

The overall goal of this doctoral research was to develop and optimize a new and sustainable two-stage heterotrophic-autotrophic fermentation process for the production of PHB. In contrast to using pure carbon sources, as typically applied in industry and mostly reported in literature so far, the final goal was to use industrial wastes as substrate in both phases instead of pure carbon sources. By using cheap raw materials and optimizing the process, it was aimed to increase the productivity and to reduce the overall production cost. To this end, a step-wise research approach was applied.
Part I of this thesis focuses on the optimization of the **heterotrophic-heterotrophic PHB production** process (i.e., reference process) by a combination of experimental and modeling work using either glucose (pure) or waste glycerol as substrate (Figure 1.3a). A substrate control strategy independent of the organic carbon source was developed to obtain a high cell density culture with high PHB productivity and content for a fed-batch fermentation process (**Chapter 2**). To determine the optimal feeding strategy, glucose was first used as a substrate and *Cupriavidus necator* DSM 545 as the model organism. To induce PHB biosynthesis and accumulation, imbalanced growth conditions were enforced through nitrogen limitation. The developed feeding strategy was then validated using waste glycerol as the sole carbon source.

(a)



(b)

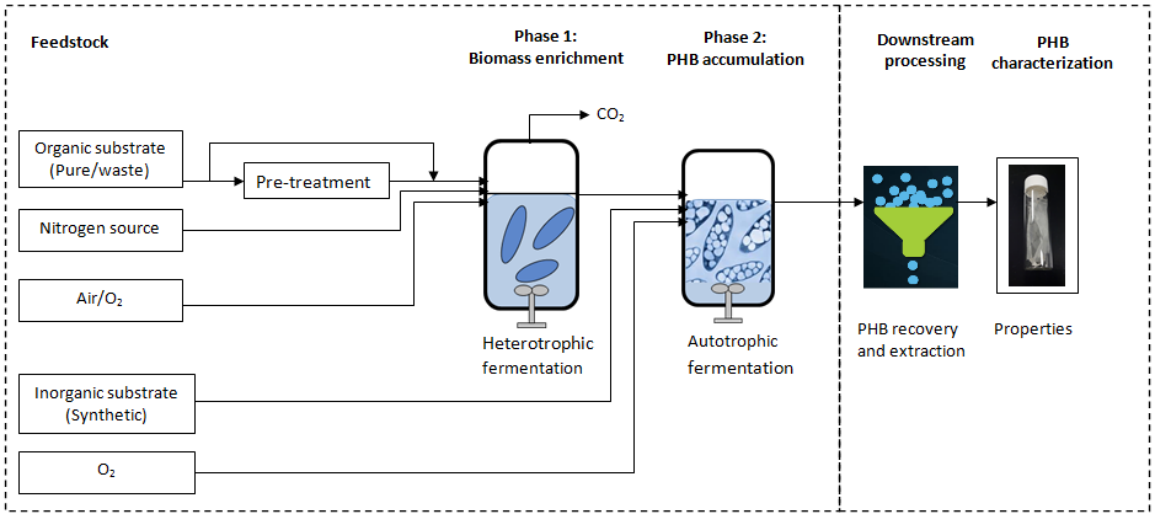


Figure 1.3. Overview of the PHA production process, (a) heterotrophic-heterotrophic, (b) heterotrophic- autotrophic.

A mathematical model for heterotrophic-heterotrophic PHB production was developed to describe the biomass growth and the PHB production phases (**Chapter 3**). A parameter sensitivity analysis was carried out, followed by model calibration to estimate the most sensitive parameter values. Various model structures were evaluated, assessing the importance of aspects such as cell density inhibition and biomass growth on PHB, which were not considered previously in pure culture PHB production processes. Product inhibition of PHB production was evaluated as well. The simulation results were validated for two different substrates, being (pure) glucose and waste glycerol, based on independent experimental datasets.

The impurities mainly sodium (Na^+) contained in waste glycerol adversely affected the microbial behaviour. Therefore, in **Chapter 4**, shake flask experiments were carried out to fully characterize the effect of Na^+ on biomass growth and PHB production. A mathematical model was set up based on the model developed in Chapter 3, incorporating the findings, to describe the effect of sodium on heterotrophic biomass growth and PHB production.

Part II describes the **autotrophic-autotrophic PHB production** process using inorganic substrates (mixture of gases H_2, O_2, CO_2) as carbon and energy source. Since no model was available for autotrophic PHB production, a mathematical model for autotrophic PHB production was developed in **Chapter 5** to describe the process dynamics, including the evolution and influence of substrate and nutrients. The ultimate target was to extend this model to a heterotrophic-autotrophic model by combining with the heterotrophic model (Chapter 3). **Chapter 5** comprises the optimization of autotrophic PHB production through modeling and simulation. An autotrophic PHB production model was set up to describe the process dynamics, including the evolution and influence of substrate and nutrients. The model was subsequently calibrated and validated based on literature data. The effect of oxygen and/or nitrogen limitation on the PHB production was assessed. The optimal composition of the gas mixture to ensure maximum PHB production was evaluated. Finally, the influence of the reactor configuration was elaborated on.After optimizing the heterotrophic-heterotrophic and autotrophic-autotrophic PHB production, the research activities were directed towards the optimization of **heterotrophic-autotrophic PHB production** (**Part III)**. The technical feasibility of using *C. necator* DSM 545 for autotrophic PHB production from a gas mixture (CO_2, H_2, O_2), following heterotrophic cell growth from an organic substrate was evaluated in **Chapter 6**. To ensure that test conditions were relevant for later industrial application, a safety margin of 2.0 vol% below the LEL of 5 vol% O_2 was taken into account during autotrophic cultivation. The influence of the organic carbon source on autotrophic PHB accumulation was evaluated using two different organic substrates, glucose and waste glycerol, as carbon source for cell mass growth. PHB biosynthesis was induced under imbalanced growth conditions by limiting nitrogen and O_2 at different cell mass concentrations. Furthermore, the biopolymers were characterized with different techniques and compared with polymers synthesized on solely organic carbon sources (using glucose and waste glycerol in Part I) and a commercial polymer to evaluate the influence of the fermentation mode and substrates on the properties of the biopolymers.

In **Chapter 7**, heterotrophic-autotrophic PHB production was modelled based on previously established models for heterotrophic-heterotrophic (Chapter 3) and autotrophic-autotrophic PHB production (Chapter 5) processes. The model was validated on the experimental datasets of Chapter 6. Subsequently, the model was used in view of process optimization in terms of maximizing PHB production, to examine the influence of operating parameters, O_2 and ammonium-nitrogen.

In **Part IV**, **Chapter 8** offers some final considerations and conclusions, reaching out to the broader research field of PHB production and productivity from organic or inorganic carbon source. The chapter discusses the need for process optimization and the implications of the present work for model development for PHB production. Some recommendations for future research are presented, as well as an outlook to the future evolution of PHB production and the implications in industrial scale.

PART I:

Heterotrophic-heterotrophic PHB production

Chapter 2:

A robust fed-batch feeding strategy independent of the carbon source for optimal polyhydroxybutyrate production

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Abstract

A three-stage control strategy independent of the organic substrate was developed for automated substrate feeding in a two-phase fed-batch culture of *Cupriavidus necator* DSM 545 for the production of the biopolymer polyhydroxybutyrate (PHB). The optimal feeding strategy was determined using glucose as the substrate. A combined substrate feeding strategy consisting of exponential feeding and a novel method based on alkali-addition monitoring resulted in a maximal cell concentration in the biomass growth phase. In the PHB accumulation phase, a constant substrate feeding strategy based on the estimated amount of biomass produced in the first phase and a specific PHB accumulation rate was implemented to induce PHB under limiting nitrogen at different biomass concentrations. Maximal cell (CDM) concentration and PHB production of 164 and 125 g/L were obtained when nitrogen feeding was stopped at 56 g/L of residual biomass; the glucose concentration was maintained within its optimal range. The developed feeding strategy was validated using waste glycerol as the sole carbon source for PHB production, and the three-stage control strategy resulted in a PHB production of 65.6 g/L and PHB content of 62.7% while keeping the glycerol concentration constant. It can thus be concluded that the developed feeding strategy is sensitive (feeding based on small change in pH), robust (i.e., independent of PHB producing organism), inexpensive, and applicable to fed-batch culture for PHB production independent of the carbon sour ce.

Keywords: Automatic substrate feeding; polyhydroxybutyrate (PHB); fed-batch fermentation; biomass growth; PHB accumulation; waste glycerol.

2.1. Introduction

Polyhydroxybutyrate (PHB) is an intracellular storage material that is synthesized by a number of microorganisms and has become of considerable industrial interest and of environmental importance as a biodegradable and biobased polyester. Although PHB is regarded as an effective substitute for conventional plastics for such applications as medical and agricultural uses (Reddy et al., 2003) and food packaging (Bucci et al., 2005), the full-scale commercialization of this biopolymer is hampered by its high production cost compared to other (bio)polymers (Chanprateep, 2010). The factors affecting the economics of PHB include the raw materials, process design, and downstream processing (Atlic et al., 2011; Kosior et al., 2006).

According to Shen et al. (2009), 50% of the total production costs can be attributed to the raw materials of which the carbon source for growth and polymer accumulation accounts for 70-80%. Thus, to attain bulk commercial viability and to further improve the sustainability profile of PHB production by fermentation, it is desirable to use waste carbon sources instead of pure substrates. A wide spectrum of industrial by-products, such as whey, molasses, starch, and waste glycerol, have already been studied with regard to PHB production (Akaraonye et al., 2010).

The production of biodiesel by the transesterification of oil with a short chain alcohol generates approximately 10% (w/w) glycerol as a co-product stream. Although pure glycerol is an important feedstock with applications found in the food, drug, and pharmaceutical industries, glycerol from biodiesel cannot be used in these applications due to the presence of impurities and requires further refinement prior to its use. As refining waste glycerol is expensive, it is important to search for alternative applications in which crude glycerol can be used as is with no refinement needed. Within this context, the biological conversion of crude glycerol to higher value chemicals, such as PHB, is an attractive alternative (Ashby et al., 2011; Posada et al., 2011). Indeed, utilizing crude glycerol as a cheap feedstock to produce PHB could increase the economic performance of both the biodiesel and biopolymer industries, though it should be noted that the presence of impurities adversely affects the quality of the polymer by reducing its molecular mass (Cavalheiro et al., 2009; Koller et al., 2006; Madden et al., 1999). Two prevalent cultivation methods are employed for PHB production, depending on the microorganism used. The more frequently applied method is a two-phase fermentation process that consists of a cell-growth phase under favorable growth conditions to yield a high cell density, followed by a PHB production phase under imbalanced growth conditions by limiting a nutritional element, such as nitrogen, phosphate, or oxygen, to trigger PHB synthesis and accumulation (Atlic et al., 2011; Kim et al., 1994; Pradella et al., 2012). The model organism for this cultivation process is *Cupriavidus necator* (formerly known as *Ralstonia eutropha, Alcaligenes eutrophus*, and *Wautersia eutropha*) (Cavalheiro et al., 2009; Pohlmann et al., 2006; Reinecke et al., 2009). For two-phase fermentation processes, the time at which nitrogen limitation is initiated, the choice of limiting nutrient, and the fermentation strategy are of utmost importance for maximizing PHB yield and productivity (Atlic et al., 2011; Wang et al., 2012). The second cultivation mode consists of a single-phase process with PHB accumulating in a growth-associated manner. Although PHB synthesis occurs under nutrient-sufficient conditions, it has been reported that applying nitrogen limitation enhances the final PHB

content, making the recovery more economic. A well-known growth-associated PHB producer is *Alcaligenes latus* (Wang and Lee, 1997; Wang et al., 2012).

Fed-batch operation is the most widely applied method in order to achieve high cell densities, productivity, and yields of PHB compare to batch culture (Lee et al., 1999). In comparison with continuous fermentation, the fed-batch mode ensures the same retention time for all bacteria, resulting in uniform distribution PHB content in all the cells. The main challenge in fed-batch fermentation is to control the substrate concentration within an optimal range, thereby avoiding limiting and inhibiting concentration levels. As a result, the substrate feeding strategy is crucial for successfully obtaining high cell density cultures. Several feeding strategies have been proposed to improve PHB productivity and yield, such as continuous feeding (Hafuka et al., 2011), pH stat (Arifin et al., 2011; Kulpreecha et al., 2009), and dissolved oxygen (DO) stat (Lee et al., 2000a, Lee et al., 2000b; Park et al., 2001), in addition to control strategies based on the carbon dioxide (CO_2) evolution rate or using a carbon source analyzer (Kim et al., 1994). However, all the feeding strategies developed to date carry important drawbacks. Continuous feeding is a simple method without feedback mechanism, such that over- or underfeeding is likely to occur, thus affecting the metabolism of the microorganisms. Substrate feeding strategies with indirect feedback control, such as pH or DO stat, are based on the finding that DO or pH increases sharply upon the depletion of a carbon source. When the pH or DO becomes higher than its set point, the nutrient is added at a predetermined rate to the fermentor (Lee et al., 1999). Due to the nature of this feeding method, the substrate concentration cannot be kept at the desired level and will oscillate from the set point value to zero. During the periods of carbon depletion, the biomass growth rate and thus the final productivity can be adversely affected. Furthermore, as no biomass growth occurs during imbalanced growth conditions, no sharp DO or pH increase is expected upon carbon depletion, resulting in cell starvation due to substrate exhaustion resulting from the improper control of the substrate (Kim et al., 1994; Wang and Lee, 1997). On-line monitoring systems are more efficient. The CO_2 evolution rate can be obtained from mass spectrometry measurements, allowing an estimation of the substrate requirement based on the conversion efficiency. The use of a carbon source analyzer allows the direct measurement of the substrate concentration in the reactor. However, such systems are expensive; moreover, an online substrate analyzer is limited to a specific type of (pure) substrate. As a consequence, it cannot be used when applying a waste stream as the carbon source.

The aim of this research work was to optimize the overall fermentation process for the production of PHB independent of the carbon source used. A sensitive, robust and inexpensive

substrate control strategy independent of the carbon source used for a fed-batch fermentation process was developed to obtain a high cell density culture with high PHB productivity and content. To determine the optimal feeding strategy, glucose was used as a substrate and *Cupriavidus necator* DSM 545 as the model organism. To induce PHB biosynthesis and accumulation, imbalanced growth conditions were enforced through nitrogen limitation. The developed feeding strategy was then validated using waste glycerol as the sole carbon source.

2.2. Materials and methods

2.2.1. Organism

Cupriavidus necator (formerly known as *Ralstonia eutropha* and *Alcaligenes eutrophus*) DSM 545 was used as the microorganism. According to the DSMZ website (www.dsmz.de), this strain, a mutant of *C. necator* DSM 529, constitutively expresses glucose-6-phosphate dehydrogenase.

2.2.2. Carbon sources

The experiments were performed using either glucose (Merck, Germany, 650 g/L) or waste glycerol; the latter was kindly provided by a local biodiesel industry (Oleon, Belgium) and contained 85% (w/w) glycerol (see Section 2.8). The sodium and potassium content were 1.5% (w/w) and less than 0.1% (w/w) respectively. The conductivity and density of the waste glycerol was 78.6 mS/cm and 1260 g/L respectively.

2.2.3. Culture media

Lennox broth (LB) medium (Invitrogen, Life Technologies Europe B.V., Belgium) was used as the seed medium for preculture 1 and was autoclaved at 121°C for 20 minutes. The seed medium for preculture 2 contained 10 g/L carbon source, 3 g/L $(NH_4)_2SO_4$, 1.5 g/L KH_2PO_4, 4.47 g/L $Na_2HPO_4.2H_2O$, 0.2 g/L $MgSO_4.7H_2O$, and 1 mL/L trace element solution. For the fermentation culture, the initial medium consisted of 12 g/L glucose or 17 g/L waste glycerol, 4 g/L $(NH_4)_2SO_4$, 13.3 g/L KH_2PO_4, 1.2 g/L $MgSO_4.7H_2O$, 1.87 g/L citric acid, and 10 mL/L trace element solution. The trace element solution of the mineral salt medium for preculture 2 and the fed-batch experiments had the following composition: 10 g/L $FeSO_4.7H_2O$, 2.25 g/L $ZnSO_4.7H_2O$, 1 g/L $CuSO_4.5H_2O$, 0.5 g $MnSO_4.5H_2O$, 2 g/L $CaC1_2.2H_2O$, 0.23 g/L $Na_2B_4O_7.10H_2O$, 0.1 g/L $(NH_4)_6Mo_7O_{24}$, and 35% HC1 10 mL/L. The solution was filter sterilized through a 0.2-µm polyethersulfone (PES) filter (Whatman, UK). The carbon source

and $MgSO_4.7H_2O$ were separately autoclaved at 121°C for 20 minutes. All three solutions were aseptically added to the medium after cooling; the pH of the medium was adjusted to 6.80 with 5 M NaOH.

2.2.4. Inoculum preparation

Stock cultures of *C. necator* DSM 545 were stored at -20°C in 2-mL cryovials containing 0.5 mL of 80% glycerol (Merck, Germany) and 1 mL of a late exponential-phase liquid culture in LB medium. These stock cultures were used to inoculate preculture 1 by transferring 200 µL to 5 mL of LB medium in 15-mL test tubes. The preculture was cultivated in an orbital shaker (Innova 42, Eppendorf, USA) for 24 hours at 30°C and 200 rpm. Subsequently, 2 mL of the strain was sub-cultured for 24 hours at 30°C and 180 rpm in 100 mL of preculture 2 seeding medium in 500-mL baffled flasks. When using waste glycerol as the carbon source, successive sub-culturing was performed five times to ensure a good adaptation of the microorganisms to the glycerol substrate. Lastly, the seed culture was used to inoculate baffled flasks (4 vol% inoculum, Section 2.5) or the bioreactor (12.5 vol% inoculum, Section 2.6).

2.2.5. Shake flask experiment

The effect of the glucose concentration on the growth of *C. necator* DSM 545 was investigated by monitoring the initial growth rate as previously described in literature (Cavalheiro et al., 2009). Preculture 2 (4 mL) was inoculated into 100 mL of fermentation medium supplemented with glucose ranging from 5 to 60 g/L in 500-mL baffled flasks. The flasks were incubated at 180 rpm and 30°C for 10 hours to ensure favourable conditions for biomass growth. Samples were then collected for analysis, as described in Section 2.2.8. All the shake flask experiments were conducted in duplicate to confirm the precision of the results.

2.2.6. Fed-batch experiments

Fed-batch experiments were performed in a 3-L bioreactor (Applikon Biotechnology, the Netherlands). The setup was equipped with on-line monitoring and an EZ-control system (Applikon Biotechnology, the Netherlands) used to control the stirring speed, DO, foam formation, pH and temperature. The DO concentration level was regulated at 55% of air saturation for phase 1 (biomass growth) and 30% of air saturation for phase 2 (PHB production) using a cascade control strategy consisting of the agitation speed (850 up to 1000 rpm) and air and/or oxygen flow. Foaming was controlled using 30% antifoam C emulsion (Sigma-Aldrich Chemie, GmbH, Germany), and the pH was maintained at 6.80 by adding acid

(2 M H_2SO_4) or base (5 M NaOH or 20% NH_4OH). The process temperature was fixed at 30°C. A computer-based software program, BioXpert, was used to implement the developed feeding strategy for controlling the carbon source concentration in the fermentor at the desired level. Samples were collected at regular time intervals and analyzed according to Section 2.2.8.

2.2.7. Development of feeding strategy using glucose as the carbon source

Separate feeding strategies were developed for each phase of the two-phase fermentation process using glucose as the carbon source. Each feeding strategy was evaluated at least twice per substrate to confirm its applicability.

2.2.7.1. Phase 1: Biomass growth

Two types of substrate feeding strategies, exponential feeding and two-stage feeding consisting of exponential feeding, followed by feeding based on alkali-addition monitoring, were developed to control the substrate concentration in phase 1 within an optimal range. In parallel, nitrogen was added using NH_4OH as a base to control the pH.

Exponential feeding

To maintain a maximal cell growth within the exponential growth phase, the substrate should be added according to its consumption. Thus, the concept of exponential feeding is based on the exponential growth of residual biomass (RCC: defined as the difference between the cell dry mass (CDM) and PHB production) (with concentration X, in g/L):

$$\frac{dX}{dt} = \mu_x X$$

$\Leftrightarrow$

$$X = X_0 e^{\mu_x (t - t_0)} = X_0 e^{\mu_x \Delta t} \quad (2.1)$$

$\Leftrightarrow$

$$\Delta X = X_0 (e^{\mu_x \Delta t} - 1) \quad (2.2)$$

where t denotes time (h), μ_x is the specific biomass growth rate (1/h), and X_0 represents the initial (at $t=t_0$) residual biomass concentration (g/L). During exponential feeding, the amount of feed solution needed to keep the substrate concentration constant is the amount of substrate consumed ($\frac{\mathbf{1}}{\boldsymbol{Y_{XS}}} \Delta X.V$) (g substrate) divided by the substrate concentration in the feed solution S_F (g/L) and is expressed as:

$$\frac{\Delta F_1}{\Delta t} = \frac{1}{Y_{xS}}\frac{1}{S_F}\frac{\Delta X}{\Delta t}.V = \frac{1}{Y_{xS}}\frac{1}{S_F}\frac{1}{\Delta t}X_0(e^{\mu_x \Delta t} - 1).V \quad (2.3)$$

where ΔF_1 is the volume (L) of feed solution fed to the fermentor for a period (Δt) at phase 1, V is the working volume of the fermentor medium (L), Y_{XS} is the biomass yield (g biomass/g substrate), and S_F is the substrate concentration in the feeding solution (g/L). Given Eq. 2.3, it is clear that the accuracy of dosing depends on accurate knowledge of the microbial growth parameters μ and Y_{XS} and the initial biomass concentration X_0, in addition to the reactor volume and the feed concentration, which are known.

Alkali-addition monitoring

An indirect feedback-control feeding strategy based on alkali-addition monitoring was developed from the mass balance of biomass growth. The stoichiometry of residual biomass (RCC) growth (Eq. 2.4) using glucose as the sole carbon source was determined given the residual biomass yield with glucose (Y_{XS}) and the elementary biomass composition and by subsequently applying (elemental) balances for C, N, charge, H, and O. The composition of *C. necator* cells was taken from Ishizaki and Tanaka, (1990), and Y_{XS} was measured in a batch experiment.

$$C_6H_{12}O_6 + 1.97\ O_2 + 0.72\ NH_4^+ \Rightarrow$$
$$3.79\ CH_{1.74}O_{0.46}N_{0.19} + 2.21\ CO_2 + 0.72\ H^+ + 3.78\ H_2O \quad (2.4)$$

Protons (H^+) are produced during biomass growth, decreasing the pH of the mineral medium solution; thus, the medium needs to be neutralized using alkali to maintain the pH at the optimum level for the growth of *C. necator*. Based on the mass balance equation (Eq. 2.4), the substrate feeding rate was estimated from the amount of alkali supplied to keep the pH constant. The substrate feeding rate based on the supplied alkali needed to keep the substrate concentration constant is the amount of substrate consumed per hour ($\frac{\mathbf{1}}{\boldsymbol{Y_{NS}}} C_b\ Q_b M_S$) (g substrate/h) divided by the substrate concentration in the feed solution S_F (g/L) and is expressed as:

$$\frac{dF_1}{dt} = \frac{1}{Y_{NS}}\frac{1}{S_F} C_b\ Q_b M_S \quad (2.5)$$

where C_b is the molar concentration of the base solution (mole/L), Q_b is the base flow rate (L/h), M_S is the molecular weight of the substrate, and Y_{NS} denotes the molar ratio between the

ammonium and substrate consumption and is equivalent to the molar ratio between proton production and substrate consumption ($Y_{NS(glucose)}$=0.72).

Combined substrate feeding

A two-stage feeding strategy for biomass growth (phase 1) was developed, consisting of exponential feeding for the first 10 hours, followed by feeding based on alkali-addition monitoring. This strategy was termed 'combined substrate feeding'.

2.2.7.2. Phase 2: PHB accumulation

As imbalanced growth conditions by limiting a nutritional element (in this case nitrogen) triggers PHB synthesis, the feed should contain only the substrate and no nitrogen. The substrate feeding rate and substrate consumption rate are directly proportional to the PHB accumulation rate, which is in turn related to the residual biomass concentration (X) and specific PHB accumulation rate (μ_p, g PHB/g biomass/h). Therefore, the feeding rate of the feed solution in phase 2 needed to keep the substrate concentration constant is the amount of substrate consumed per hour ($\frac{1}{Y_{PS}} X\mu_p V$) (g substrate/h) divided by the substrate concentration in the feed solution S_F (g/L) and is expressed as

$$\frac{dF_2}{dt} = \frac{1}{Y_{PS}}\frac{1}{S_F}\frac{dP}{dt} = \frac{1}{Y_{PS}}\frac{1}{S_F} X\mu_p V \tag{2.6}$$

where P is the amount of PHB (g), F_2 is the volume of feed solution fed to the fermentor during phase 2 (L), μ_p is the specific PHB accumulation rate (g PHB/g biomass/h), and Y_{PS} is the yield of PHB (g PHB/g substrate). Although the total amount of residual biomass remains the same during phase 2, the biomass concentration decreases due to dilution through the added feed volume and can be calculated as

$$X = \frac{X_1 V_1}{V_1 + \int_0^{F_2} dF_2} \tag{2.7}$$

where X_1 denotes the residual biomass concentration (g/L) and V_1 is the working volume at the end of phase 1 (L).

2.2.8. Analytical procedures

The glucose concentration in the medium was determined off-line by the phenol-sulfuric acid method using glucose as a standard, as originally described by Dubois et al. (1956). The glycerol concentration was determined off-line through HPLC using MilliQ water as the mobile phase. The concentrations of ammonium (NH_4^+-N) were evaluated off-line colorimetrically with standard Hach Lange cuvette tests (Hach Lange Gmbh, Germany). The growth of *C. necator* was roughly monitored by measuring the optical density (OD) using a spectrophotometer (UV-1800, Shimadzu, Japan) at 600 nm, with the sufficient dilution of the culture broth. Moreover, a gravimetrical method was used to determine the exact cell concentration, which was expressed as cell dry mass (CDM). Hereto, culture broth (15-20 mL) was centrifuged (SORVALL RC6+ centrifuge, Thermo Scientific, Clintonpark Keppekouter, Belgium) at 7000 x g in pre-weighted screw-cap tubes for 30 min at 4°C. The cell pellets were washed with distilled water, re-centrifuged, frozen at -20°C, and lyophilized until a constant weight. CDM was determined as the weight difference between tubes containing the cell pellets and empty tubes. For the PHB analysis, dried samples and external standards (PHB, Biomer) were subjected to methanolysis in the presence of 50 vol% methanol and 50 vol% NaOH. The resulting 3-hydroxybutyric acids were analyzed by HPLC using 0.05% H_3PO_4 as the mobile phase, Prevail Organic Acid column (particle size 5µm) at 40°C and UV detector (Agilent Technologies, 1200 Series). Residual cell concentration (RCC) was defined as the difference between biomass (CDM) and PHB production. Waste glycerol concentration was determined off-line through HPLC using ultrapure water (resistivity, 18.2 MΩ.cm at 25°C) as a mobile phase with Monochrom 5 Diol column at 25°C and ELSD detector (Alltech 3300 ELSD). The ultrapure water was produced using a Milli-Q device (Merck Millipore, Germany) and 0.22 µm membrane filter. The sodium quantification in waste glycerol was performed at 589 nm in a flame atomic absorption spectrophotometer (PerkinElmer AAnalyst 300) after dilution with water. All the analytical measurements were verified with an external standard; the measurement error was found always lower than 5%.

2.2.9. PHB extraction

After lyophilization, 1 g of dried cells was resuspended in 100 mL chloroform for 24 h with vigorous agitation at room temperature. After extraction, the cellular debris was separated by filtration (Whatman, Schleicher and Schuell, 75 mm radius). The chloroform fraction containing the solubilized polymer was poured into cold ethanol to precipitate the polymer.

After filtration, PHB was resuspended into chloroform, and the precipitation procedure was repeated twice to further purify the polymer. The precipitated polymer was filtered and dried.

2.2.10. PHB characterization

The average molecular weight (M_w) was determined by gel permeation chromatography (GPC) using a Waters Breeze™ System with a combination of three column series (PSS SDV analytical 1000 Å, 5 µm, 300 x 8.00; PSS SDV analytical 100000 Å, 5 µm, 300 x 8.00; PSS SDV analytical 1000000 Å, 5 µm, 300 x 8.00) and equipped with a 2414 differential refractive index detector. Chloroform was used as the eluent at 35°C, and the applied flow rate was 1.0 ml/min. A calibration curve was obtained using narrow polystyrene standards (Polymer Laboratories) in the M_w range of 580-1,930,000 g/mol.

2.3. Results and discussion

2.3.1. Development of a feeding strategy using glucose as the carbon source

2.3.1.1. Phase 1: Biomass growth

The development of a feeding strategy in a fed-batch culture for biomass growth to control the substrate concentration at its optimal level is essential to attain a maximal cell concentration and high biomass productivity. In addition, this approach affects the overall PHB productivity by preventing premature shifting to phase 2. In this study, two types of glucose feeding strategies, exponential feeding and combined substrate feeding, were evaluated to maintain the glucose concentration within an optimal range. First, a series of shake flask experiments were performed to determine the optimal glucose concentration (Figure 2.1). The results indicated that the initial concentration of glucose significantly affected the specific growth rate, which was found to be at a maximum at an initial glucose concentration ranging between 10 and 20 g/L, corresponding to previously reported values (Kim et al., 1994; Lee and Yao, 1991). A decrease in growth rate was observed at higher glucose concentrations.

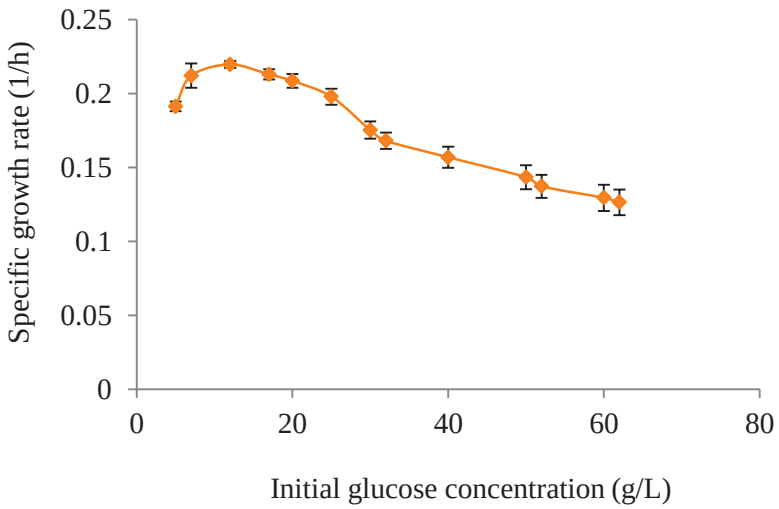


Figure 2.1. The effect of initial glucose concentration on the specific growth rate of *C. necator* DSM 545 in fermentor medium at shake-flask scale.

Exponential feeding of glucose

A series of batch experiments at the bioreactor level were performed with *C. necator* DSM 545 to determine the initial residual biomass concentration (X_0), specific biomass growth rate (μ_x, using Eq. 2.1), and biomass yield (Y_{XS}) (data not shown), as X_0=0.4 (±0.03) g/L, μ_x=0.149 (±0.012) 1/h, and Y_{XS}=0.5 (±0.015) g biomass/g glucose, respectively. These values were applied in an initial fed-batch fermentor experiment to evaluate the exponential feeding strategy (Figure 2.2a). The results showed that a biomass concentration of 24 g/L (CDM) was attained after 24 h and contained 8% PHB. The glucose concentration could be maintained within its optimal range (10 to 20 g/L) during the first 16 h of fermentation but then decreased from 15 to 2 g/L. The experiment was stopped after 24 h because glucose became limiting for biomass growth, and this decrease in glucose concentration indicated that X_0 and/or μ_x were higher than the estimated values: X_0=0.438 g/L and μ_x=0.157 1/h. Therefore, a second fed-batch experiment using these parameter values was set up (Figure 2.2b). The glucose concentration was again controlled within its optimal range during 17 h of fermentation but then gradually increased and reached 35.5 g/L after 32 h; at that point, growth ceased, and a maximal biomass concentration (CDM) of 42 g/L was obtained. The average specific growth rate μ_x was calculated as 0.131 1/h, which was lower than the applied value, causing the overfeeding of glucose. Thus, the shake flask experiments confirmed the effect of glucose inhibition on the growth rate when the concentration was higher than 20 g/L (Figure 2.1).

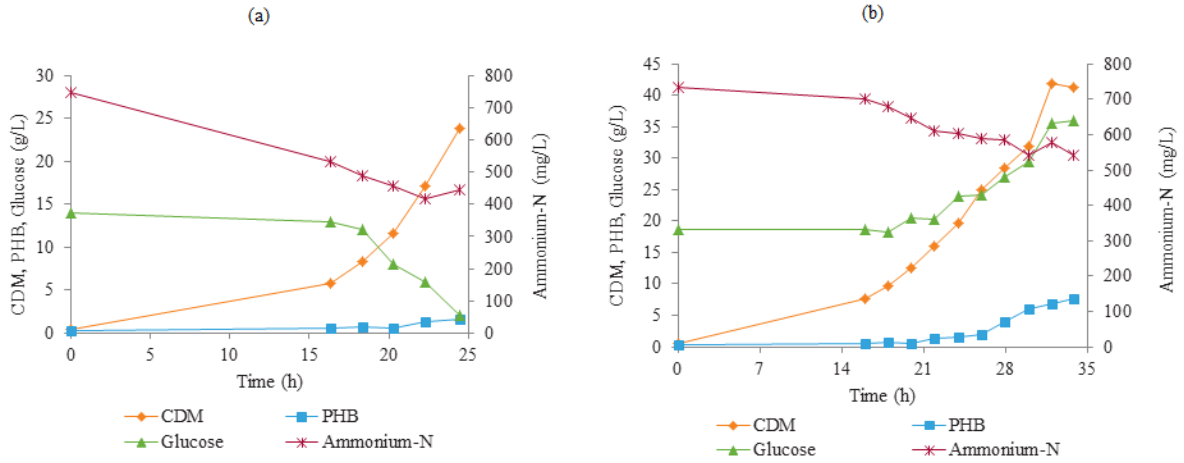


Figure 2.2. Cell biomass production of *C. necator* DSM 545 in fed-batch cultivation using exponential glucose feeding with (a) X_0 = 0.4 g/L and μ_x = 0.143 1/h, and (b) X_0 = 0.438 g/L and μ_x = 0.157 1/h.

The exponential feeding strategy has been developed to allow cells to grow at constant specific growth rates (Lee et al., 1999). Nonetheless, several authors have reported the necessity of feedback or feed-forward control to compensate for fluctuations due to process perturbations and parameter inaccuracies (Nor et al., 2001; Qiu et al., 2009). Our study confirmed that this simple feeding technique was ineffective to maintain the substrate concentration at the optimal level for *C. necator* DSM 545. Exponential feeding resulted in long-term over- or underfeeding due to deviations in the parameter values from the initially estimated values, resulting in growth repression or cell starvation. Moreover, the results indicated the need for a feeding strategy with a feedback control mechanism to compensate for fluctuations due to parameter inaccuracies.

Combined feeding of glucose

A feedback-control glucose feeding strategy based on alkali-addition monitoring was developed (Eq. 2.5) and used in fed-batch culture in an effort to reach a high-density culture. As NH_4OH is volatile, part of the NH_4^+ will be stripped as NH_3, which results in a higher need for the addition of base than is stoichiometrically needed according to Eq. 2.4. Consequently, by using 20% (NH_3-basis) NH_4OH as an alkali, glucose feeding based on Eq. 2.5 may result in overfeeding, causing the premature termination of biomass growth. Therefore, the proposed feeding strategy (Eq. 2.5) was extended with a correction factor, η, resulting in the following control law (Eq. 2.8).

$$\frac{dF_1}{dt} = \eta \frac{1}{Y_{NS}} \frac{1}{S_F} C_b \, Q_b M_S \qquad (2.8)$$

There are a number of operating and process parameters, including temperature, air flow rate, flow pattern, stirring speed, method of NH_4OH dosing, that affect the NH_3 loss, and thus the correction factor value. As these parameters may vary with the experimental setup, it is very important to determine η for every experimental setup.

Experiments were performed to determine η from the correlation between the stoichiometrically needed and actually pumped amount of 20% (NH_3-basis) NH_4OH (Figure 2.3). The results showed that an average of 25% more alkali was pumped; hence, the value of η was set to 0.75. Due to the buffering capacity of the mineral medium and low biomass production, it was observed that no alkali was added during the first 10 to 12 h of fermentation (Figure 2.3). Although only a low amount of biomass was produced during that period, the substrate was consumed for biomass growth. As the objective of this study was to develop a feeding strategy independent of the carbon source used for controlling the carbon source at its optimal level, we decided to include an additional feeding strategy for the period that alkali was not added. As a result, combined feeding was applied during phase 1, which consisted of exponential feeding during the first 10 hours using Eq. 2.3 with X_0=0.4 g/L and μ_x=0.149 1/h, as determined from the batch experiments at the bioreactor level, followed by alkali-addition monitoring using Eq. 2.8. It should be noted that as an alternative for exponential feeding, higher initial substrate concentrations can be used. In this case, the feeding is only based on alkali-addition monitoring. The results showed that the cell concentration (CDM) reached a maximal level of 73.5 g/L after 33 h, with μ_x 0.141 1/h (Figure 2.4). PHB accumulation began after 25 h and increased to 6 g/L, corresponding to a final PHB content of 8%. Despite a small decrease in the glucose concentration at the end of the fermentation due to simultaneous biomass growth and PHB formation, glucose was overall properly controlled at its optimal level. Indeed, the feeding of glucose was based on the stoichiometry of residual biomass growth and thus the PHB production at the end of the biomass growth phase was not taken into account. By incorporating the PHB production in Eq. 2.4, the decrease of substrate concentration can be avoided.

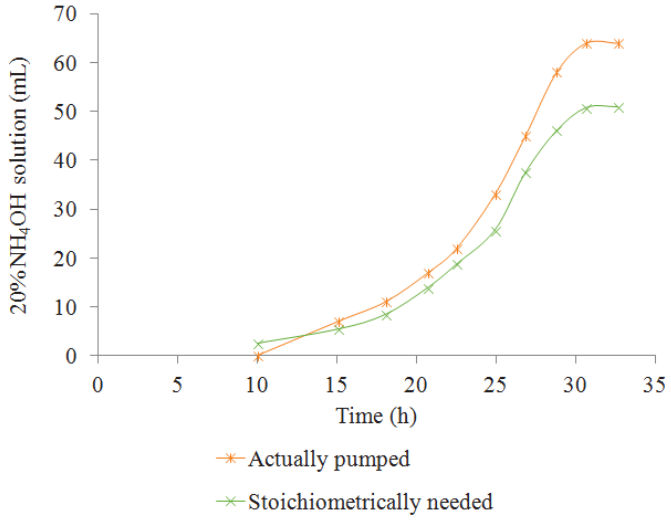


Figure 2.3. Theoretical and actual NH_4OH (20% NH_3 basis) feeding profile during cell biomass production of *C. necator* DSM 545 in fed-batch cultivation.

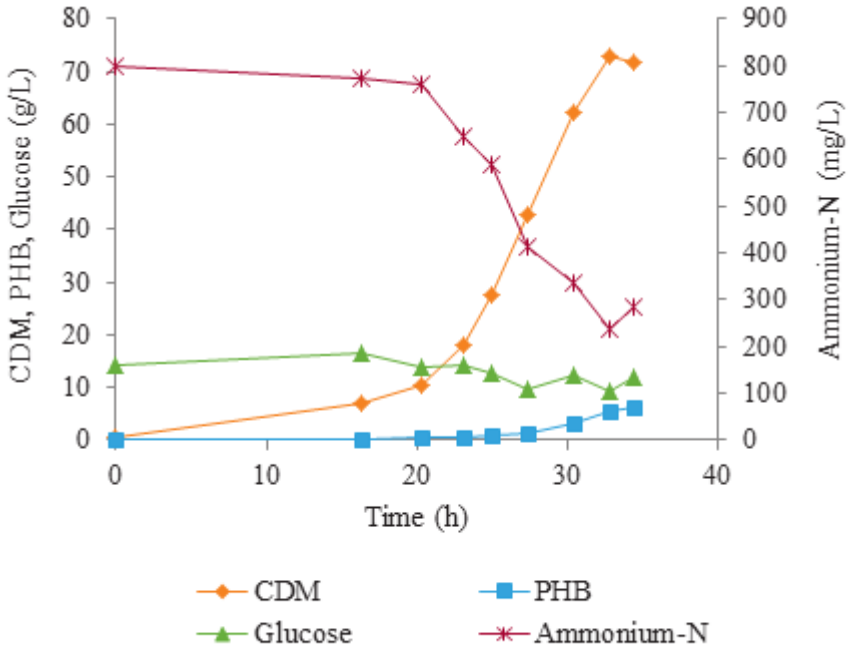


Figure 2.4. Cell biomass production of *C. necator* DSM 545 in fed-batch cultivation using combined (exponential feeding for first 10 hours and then alkali addition monitoring) glucose feeding.

In fed-batch cultivation, several directly or indirectly measured variables are used for control purposes. The directly measured variables include the pH, DO concentration, OD, substrate concentration, pressure, and gas outflow composition. The indirectly determined variables include the specific growth rate, cell concentration, oxygen uptake rate, CO_2 evolution rate, and respiratory quotient are estimated or calculated from one or more of the directly measured variables (Lee et al., 1999). Except for pH and DO, the determination of the variables requires dedicated sensors or analytical equipment that are not commonly installed in bioreactors. Furthermore, a substrate feeding strategy coupled with the measurement of pH (pH-stat) or DO (DO-stat) is also far from optimal, as the cells will be exposed to oscillations in substrate concentration. In this study, a feedback control strategy was developed for automated substrate feeding in fed-batch *C. necator* DSM 545 culture with the aim of maintaining the substrate concentration within its optimal range. The control strategy uses the online estimation of the cell concentration as a performance indicator of the fed-batch culture, an estimation that is based on the base consumption rate and the ratio between substrate conversion and acid production, as determined from the mass balance. A direct comparison of the results obtained using this organism with literature data is difficult, as the biomass growth phase is not represented as a separate phase in literature but as an integrated phase for PHB production. However, it can be concluded that the feeding strategy was efficient because glucose was controlled at its optimal level throughout the culture period, even at the highest cell density (CDM) of 73.5 g/L. It should be noted that the cells had already accumulated a small amount of PHB during the growth phase (phase 1), consistent with previous reports (Berezina et al., 2013; Kim et al., 1994). This shows that imbalanced growth conditions are a sufficient, but in no case a necessary condition for PHB accumulation.

The great advantage of this feeding strategy is that only online monitoring of the amount of base added during fermentation is required. As a consequence, the method is applicable to bioreactors equipped with standard pH probes, representing a low investment cost. In addition, the method can maintain the substrate concentration at its optimal value, thus allowing the cells to grow at a maximum specific growth rate. It should be noted however that the success of this feeding strategy mainly depends on the accuracy of the base dosing. Indeed, possible losses of base during dosing should be taken into account and need to be evaluated for every system, as this affects the feeding strategy and thus the substrate concentration.

2.3.1.2. Phase 2: PHB accumulation

After optimizing the feeding strategy for biomass growth, PHB synthesis was triggered by applying nitrogen limitation in the presence of glucose, which was achieved by replacing NH_4OH feeding with NaOH for pH control. The glucose concentration was maintained at the optimal level in phase 2 (PHB production phase) using the feeding strategy according to Eqs. 2.6 and 2.7. The value of μ_p was determined to be 0.09 g PHB/g biomass/h, and Y_{PS} was taken from the literature as 0.30 g PHB/g glucose (Nonato et al., 2001; Rossell et al., 2006). To determine the maximal PHB production and productivity, nitrogen limitation was applied when the residual biomass concentration reached 49, 56, and 62 g/L. The residual biomass was estimated based on the amount of total glucose fed or and consumption of ammonium-nitrogen in phase 1, as described in Eq. 2.9.

$$X = \frac{F_1 * Y_{XS}}{S_F} = (N_0 + \eta C_b \, Q_b M_S) Y_{XN} \tag{2.9}$$

where N_0 was the initial nitrogen concentration (g/L) and Y_{XN} was the biomass yield over nitrogen (g biomass/g nitrogen).

The correctness of the estimated value was later confirmed by analytical results with less than 2% variation. The results are summarized in Table 2.1, and the overall results of PHB accumulation applying nitrogen limitation at 49 and 56 g/L residual biomass concentration (RCC) are shown in Figure 2.5.

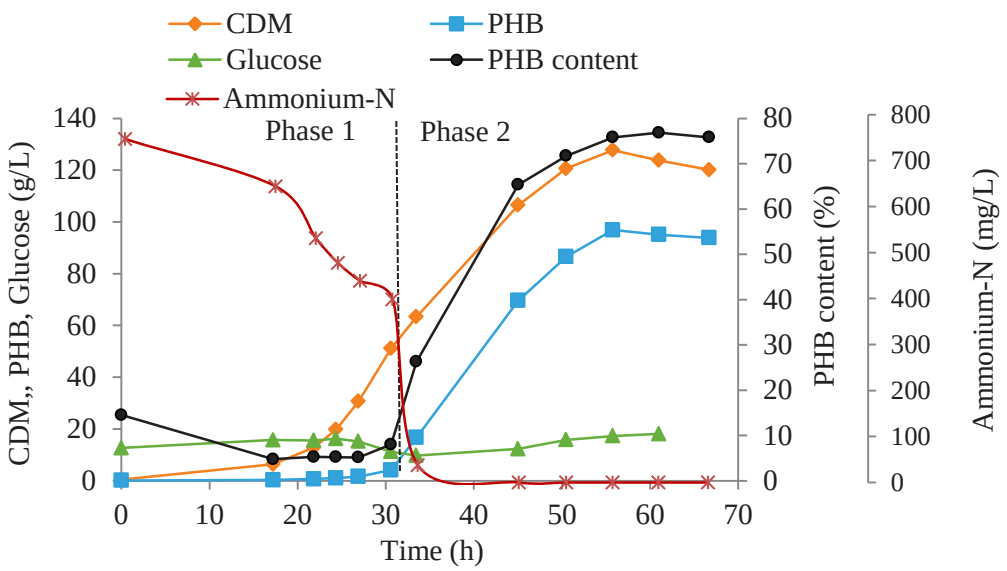
When nitrogen was limited at the residual biomass concentration of 49 g/L, the nitrogen in the culture broth became depleted after a short time, thereby triggering PHB synthesis (Figure 2.5a). The maximal biomass (CDM) and PHB production were 127.7 g/L and 97 g/L, respectively, after 56 h, resulting in a PHB content of 75.8% and PHB productivity of 1.74 g PHB/L/h. The glucose concentration in the medium fluctuated between 10 and 20 g/L; however, as this is considered to be the optimal range, it did not affect the growth and PHB production. In total, 438 g (673 mL of 65% glucose solution) glucose was fed during fermentation, resulting in Y_{PS}=0.22 g PHB/g glucose. When applying nitrogen limitation at a 56 g/L residual biomass concentration, a maximal biomass (CDM) concentration of 164 g/L and PHB production of 125 g/L were achieved after 62 h (Figure 2.5b). The PHB content and productivity increased to 76.2% and 2.03 g PHB/L/h, and the glucose concentration (13-16 g/L) was properly maintained within its optimal range. In this experiment, 523 g of glucose (806 mL of 65% glucose solution) was added, which resulted in Y_{PS}=0.24 g PHB/g glucose. In both

experiments, the PHB production reached its maximum concentration 26 h after shifting to phase 2. In order to totally consume the residual glucose, we advise to stop the glucose feeding 25 h after shifting to phase 2. When delaying nitrogen limitation until the residual biomass concentration reached 62 g/L, the process became unstable due to excessive foaming, which could not be controlled by the addition of antifoam. For all experiments, the DO concentration was regulated at 55% air saturation for phase 1 and 30% air saturation for phase 2 using a cascade control strategy; during the experiments, DO varied from 49 to 61% in phase 1 and 25 to 32% in phase 2.

A number of studies have focused on efficient process design for PHB production, and an overview of the final cell concentration (CDM), PHB production, PHB content, and productivity obtained from various cultures applying different feeding strategies, substrates, and microorganisms is given in Table 2.1. In the present study, the glucose concentration was maintained at its optimal level using a three-stage feeding strategy consisting of combined feeding at phase 1, followed by constant feeding at phase 2.

Our experimental results show that although this strain accumulated a low amount of PHB in the first phase (maximum 16%), imbalanced growth conditions by limiting a nutritional element such as nitrogen enhanced the PHB content (maximum 75.8%) and productivity. This is consistent with recently (and older) published studies where ammonium limitation was imposed to promote PHB synthesis by the same strain (Cavalheiro et al., 2012; Spoljaric et al., 2013a). The latter study also confirms that an insufficient supply of nitrogen or phosphorus can be regarded as the main regulating factor for redirection of carbon flux from biomass to PHB synthesis for *C. necator*. Furthermore, it can be observed that applying nitrogen limitation at a higher cell concentration (RCC) increased the final cell concentration (CDM), PHB production, and PHB productivity, though the PHB content stayed constant. The highest values were obtained when NH_4OH feeding was stopped at the residual biomass concentration of 56 g/L. Furthermore, the process became unstable in an attempt to further enhance fermentation performance by delaying nitrogen limitation at a residual biomass concentration of 62 g/L. The importance of the timing of nitrogen limitation, the optimal residual biomass concentration for shifting to phase 2, and the instability of the culture at higher cell concentrations correspond to the findings of Kim et al. (1994) (Table 2.1).

(a)



(b)

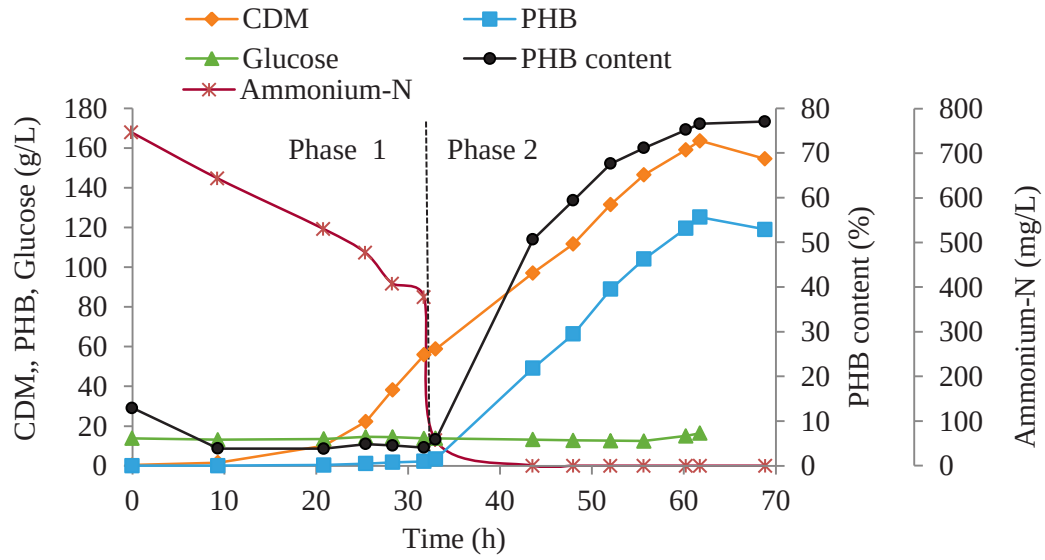


Figure 2.5. Cell biomass and PHB production of *C. necator* DSM 545 in fed-batch cultivation using three-stage glucose feeding. Nitrogen limitation was applied at (a) 49 and (b) 56 g/L residual biomass concentration (RCC).

Table 2.1: Comparison of different substrate feeding strategy in a fed-batch process for PHB production using nitrogen limitation.

Feeding strategy	Strain	Substrate	Residual cell concentration (RCC) at onset of N limitation (g/L)	CDM concentration (g/L)	PHB production (g/L)	PHB content (%)	PHB productivity (g/L/h)	Reference
CO_2 evolution rate	*Alcaligenes eutrophus* NCIMB 11599	Glucose	44	124	92	74	1.87	Kim et al., 1994
Online glucose analyzer	*A. eutrophus* NCIMB 11599	Glucose	56	164	121	74	2.42	
pH stat	*A. eutrophus* NCIMB 11599	Glucose	/	55	10	18	0.25	
	Bacillus megaterium BA-019	Molasses	/	72.6	30.5	42	1.27	Kulpreecha et al., 2009
	Escherichia coli	Glucose	30	89.8	36	40	1.87	Arifin et al., 2011
DO stat and continuous	*Alcaligenes latus* DSM 1123	Sucrose	35	111.7	98.7	88	4.94	Wang and Lee, 1997
pH-DO-stat	Recombinant *E. coli* strain HMS174/pTZ18u-PHB	Molasses	/	39.5	31.6	80	1.00	Liu et al., 1998
Continuous	*A. eutrophus*	Glucose	/	40	18	45	0.45	Du et al., 2000
Fixed rate in multistage process	*Cupriavidus necator* DSM 545	Glucose	25	81	63	78	1.85	Atlic et al., 2011
Pulsed feeding	*C. necator* DSM 545	Soybean oil	20	83	67	80	2.5	Pradella et al., 2012
Exponential + alkali addition monitoring + constant	*C. necator* DSM 545	Glucose	49	127.7	97	75.8	1.74	This study
			56	164	125	76.2	2.03	This study
			62	Process became unstable				This study

Based on the comparison of PHB production was triggered by nitrogen limitation using various microorganisms, substrates, and feeding strategies (Table 2.1), the highest values reported to date were obtained by Kim et al. (1994); however, an online glucose analyzer was used to control the glucose concentration in that study. Although Kim et al. (1994) were able to efficiently produce PHB, the use of an online glucose analyzer is limited to the substrate used and is expensive. Moreover, fluctuations in glucose concentration can occur due to delays in measurement time (Lee et al., 1999). In the present study, comparable results were obtained using an inexpensive and robust feeding strategy that, importantly, can be applied for the production of PHB independent of the carbon source used.

2.3.2. Validation of feeding strategy using waste glycerol as a carbon source

To demonstrate that the developed three-stage feeding strategy is independent from the carbon source used, the feeding strategy was validated using waste glycerol derived from a biodiesel production plant as the carbon source for PHB production. First, a series of shake flask experiments were performed to determine the optimal waste glycerol concentration, which was found to be 10 to 30 g/L (data not shown). For fed-batch culture, combined substrate feeding consisting of an initial 10 hours of exponential feeding (Eq. 2.3) followed by alkali addition monitoring (Eq. 2.8) was used for the biomass growth phase using the parameter values (previously determined by a number of batch experiments at the bioreactor level) X_0=0.4 g/L, μ=0.161 1/h, Y_{XS}=0.48 g biomass/g glycerol, and Y_{NS}=0.37 mole of NH_4^+/mole of glycerol. After 30 h, nitrogen limitation was initiated to trigger PHB biosynthesis. In the PHB accumulation phase, waste glycerol was added using the feeding strategy described by Eqs. 2.6 and 2.7 with parameter values (previously determined by a number of batch experiments at the bioreactor level) of μ_p=0.11 g PHB/g biomass/h and Y_{PS}=0.52 g PHB/g glycerol. The higher Y_{PS} from glycerol compared to glucose (Y_{PS}=0.3 g PHB/g glucose) can be explained by the more reduced intracellular state of glycerol-grown cells than cells grown on glucose under similar conditions of oxygen availability. This has a significant effect on the intracellular redox state, which causes the cells to direct carbon flow toward the synthesis of more-reduced products such as PHB when glycerol was used than when glucose was used in order to achieve redox balance (San et al., 2002).

As shown in Figure 2.6, the biomass (CDM) and PHB productions obtained after 48 h were 104.7 g/L and 65.6 g/L, respectively, resulting in a PHB content of 62.7%. The maximum PHB productivity was as high as 1.36 g PHB/L/h, and the glycerol concentration was maintained at its optimal level using this three-stage feeding strategy. To ensure maximum PHB production

and utilization of residual glycerol, we advise to stop the glycerol feeding 16 h after shifting to phase 2. The main limitation of the feeding strategy and the current existing methods is the lack of a feedback control parameter to determine the end point of substrate feeding. To maximize the product yield on substrate, substrate feeding should be stopped upon the observed pH decrease at the end of the fermentation process. This will result in a 50% reduction of the residual carbon source. The reason for this pH decrease is still unclear and urges for further investigation.

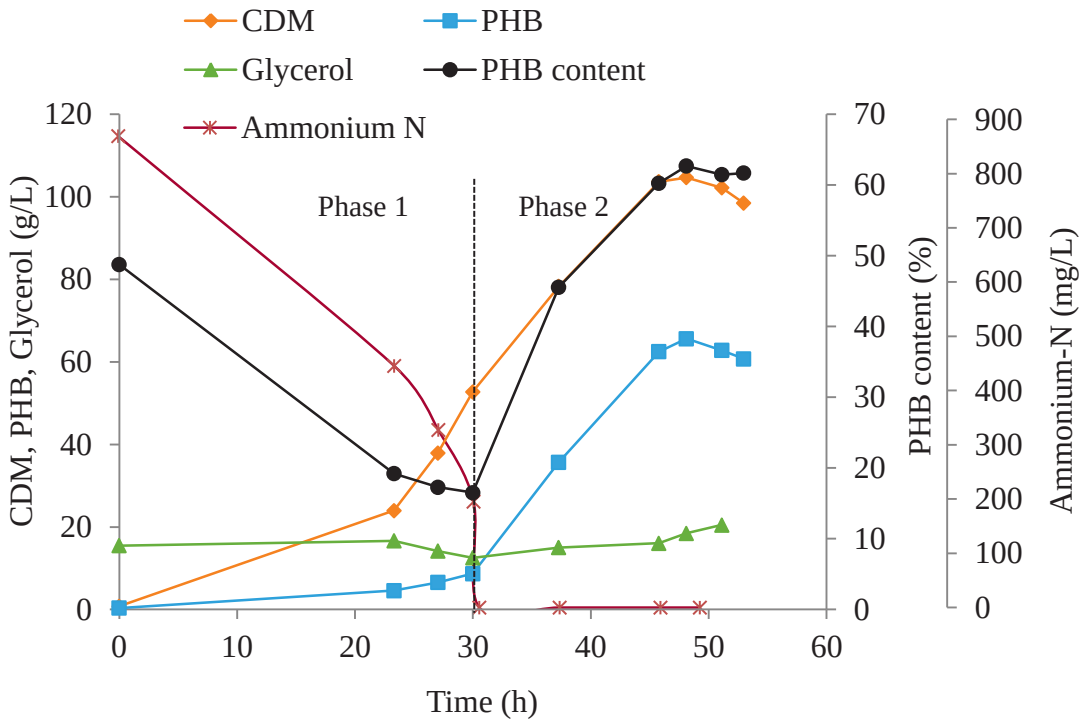


Figure 2.6. Cell biomass and PHB production of *C. necator* DSM 545 in fed-batch cultivation using three-stage feeding of waste glycerol. Nitrogen limitation was applied at 44 g/L residual biomass concentration.

Table 2.2 compares the results from a number of studies on the production of PHB from pure and waste glycerol using various production strains. However, the substrate feeding strategy was not clearly described in these studies. Only Cavalheiro et al. (2012) reported the pulse addition of waste glycerol for phase 1 and constant feeding of diluted waste glycerol for phase 2. From this overview, it can be concluded that the results obtained in the present study resulted in the highest reported values for PHB production from waste glycerol and even for pure glycerol as the carbon source. Only Koller et al. (2006) and Ibrahim and Steinbuchel (2009) reported a higher PHB content from waste glycerol and pure glycerol, respectively, whereas

Cavalheiro et al. (2009) achieved a higher PHB productivity using pure glycerol. Nevertheless, the CDM and PHB productions in these cases were much lower compared to the present study.

Table 2.2: Comparison of PHB production and productivity using glycerol and waste glycerol as a carbon source in a fed-batch fermentation process.

Substrate	Strain	CDM concentration (g/L)	PHB production (g/L)	PHB content (%)	PHB Productivity (g/L/h)	Reference
Pure glycerol	*Cupriavidus necator* DSM 545	82.6	51.2	62	1.52	Cavalheiro et al., 2009
Pure glycerol	*C. necator* JMP 134	102	57.1	56	1.31	Posada et al., 2011
Pure glycerol	*Zobellella denitrificans* MW 1	81.2	54.32	66.9	1.09	Ibrahim and Steinbuchel, 2009
Waste glycerol	*C. necator* DSM 545	104.7	65.6	62.7	1.36	This study
Waste glycerol	*C. necator* DSM 545	76.2	38.1	50	1.1	Cavalheiro et al., 2009
Waste glycerol	*C. necator* DSM 545	30.19	10.9	36.1	0.17	Cavalheiro et al., 2012
Waste glycerol	*Osmophilic organism* (unidentified)	21.3	16.2	76	0.09	Koller et al., 2006
Waste glycerol	*C. necator* JMP 134	-	-	58-60	-	Mothes et al., 2007
Waste glycerol	*Burkholderia cepacia* ATCC 17759	23.6	7.4	31	-	Zhu et al., 2010

2.3.3. Product characterization

Based on the PHB characterization results, the average molecular weight of PHB decreased from $1.23x10^6$ Da to $6.24x10^5$ Da when waste glycerol was used as the carbon source instead of glucose, whereas the polydispersity index increased from 1.18 to 1.59. The molecular mass values in this study have the same order of magnitude as those obtained by other authors for PHB from glycerol (Cavalheiro et al., 2009) and from glucose (Madden et al., 1999). Furthermore, the lower molecular weight in the presence of glycerol corresponds to previous reports (Cavalheiro et al., 2009; Koller et al., 2006; Madden et al., 1999). It has been demonstrated by Madden et al. (1999) that carbon sources present in the medium during the PHB accumulation phase by *C. necator* act as chain-transfer agents in the chain termination step of the polymerization process.

2.4. Conclusions

- A new three-stage substrate feeding strategy for PHB production independent from the organic substrate was developed. The proposed feeding strategy consists of exponential feeding and feeding based on alkali-addition monitoring for biomass growth, followed by constant feeding for PHB production.
- The substrate concentration was controlled within its optimal range during the fed-batch culture.
- Using this feeding strategy and initiating nitrogen limitation at the optimal time resulted in maximal cell and PHB production.
- The developed substrate control strategy has the additional advantages of being sensitive (i.e., feeding based on small change in pH), robust (i.e., independent of PHB producing organism) and inexpensive.
- The developed feeding strategy can be used for other types of fermentation processes that require pH control to achieve high cell density cultures.

Chapter 3:
Modeling pure culture heterotrophic production of polyhydroxybutyrate (PHB)

This chapter was published as:
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Abstract

In this contribution a mechanistic model describing the production of polyhydroxybutyrate (PHB) through pure-culture fermentation was developed, calibrated and validated for two different substrates, namely glucose and waste glycerol. In both cases, non-growth-associated PHB production was triggered by applying nitrogen limitation. The occurrence of some growth-associated PHB production besides non-growth-associated PHB production was demonstrated, although it is inhibited in the presence of nitrogen. Other phenomena observed experimentally and described by the model included biomass growth on PHB and non-linear product inhibition of PHB production. The accumulated impurities from the waste substrate negatively affected the obtained maximum PHB content. Overall, the developed mathematical model provided an accurate prediction of the dynamic behavior of heterotrophic biomass growth and PHB production in a two-phase pure culture system.

Keywords: fermentation, polyhydroxybutyrate (PHB), cell density inhibition, product inhibition, simulation.

3.1. Introduction

Polyhydroxybutyrate (PHB) is a biodegradable and bio-based plastic, which is synthesized by a wide variety of organisms as an intracellular storage material from renewable resources and has the potential to substitute conventional fossil fuel based plastics for a wide range of applications. Despite its advantages, PHB is still commercially behind the petroleum based synthetic plastics. Although rising oil price and environmental consciousness generated interest on commercial production of PHB, the major drawback is the high production cost. The factors affecting the economics of PHB include the costs for raw materials and downstream processing as well as the lack of an optimal control strategy (Atlic et al., 2011; Patnaik, 2005). To overcome these limitations, a number of studies aimed at better understanding and optimization of the fermentation process (Dias et al., 2005; Dias et al., 2008; Khanna and Srivastava, 2008; Spoljaric et al., 2013a).

Fed-batch operation is typically applied to achieve a high cell density culture, which is a prerequisite for a high productivity and yield, particularly in cases of intracellular products. Two prevalent cultivation methods are employed for PHB production depending on the microorganisms used. The most frequently applied method is a two-phase fermentation process

consisting of a biomass growth phase under favorable growth conditions to yield a high cell density, followed by a PHB production phase under imbalanced growth conditions by limiting a nutritional element such as nitrogen, phosphate or oxygen to trigger PHB synthesis and accumulation (Grousseau et al., 2013; Patwardhan and Srivastava, 2008; Ryu et al., 1997). During the two-phase fermentation processes, cell growth and PHB production need to be balanced to obtain a higher productivity, avoiding incomplete production because of late shifting to stress conditions, at a too high biomass concentration or because of premature shifting at a too low biomass concentration. The second cultivation mode consists of a single-phase process during which PHB is accumulated in a growth-associated manner (Yaname et al., 1996; Ackermann and Babel, 1997).

Cupriavidus necator is a model organism which has a strong ability to produce PHB in a non-growth-associated manner. Heterotrophic biomass growth of *C. necator* on an organic substrate (typically) takes place according to Eq. 3.1 (Doran, 1995).

$$\mathrm{C_wH_xO_y} + (4\mathrm{w} - \frac{y}{2} + \frac{x}{4} - 1.91\mathrm{Y_X})\ \mathrm{O_2} + 0.19\ \mathrm{Y_X}\ \mathrm{NH_4^+} \rightarrow \mathrm{Y_X}\ \mathrm{CH_{1.74}O_{0.46}N_{0.19}} + (\mathrm{w} - \mathrm{Y_X})\ \mathrm{CO_2} + \frac{x - 1.17\mathrm{Y_X}}{2}\ \mathrm{H_2O} + 0.19\ \mathrm{Y_X}\ \mathrm{H^+} \quad (3.1)$$

In Eq. 3.1, $C_wH_xO_y$ denotes the organic substrate, and $CH_{1.74}O_{0.46}N_{0.19}$ is the chemical composition for *C. necator* (Ishizaki and Tanaka, 1990). Y_X is the yield of biomass over organic substrate. During bacterial growth, O_2 is consumed as well as NH_4^+ as a nitrogen source, while CO_2 is produced as a side-product. At this stage, PHB production is suppressed by excess NH_4^+ supply (Kim et al., 1994).

Under stress conditions, i.e. under nutrient limitation, the organic carbon source is used for PHB production according to Eq. 3.2 (Akiyama et al., 2003).

$$\mathrm{C_wH_xO_y} + (\mathrm{w} - \frac{y}{2} + \frac{x}{4} - \frac{9}{2}\mathrm{Y_P})\ \mathrm{O_2} \rightarrow \mathrm{Y_P}\ \mathrm{C_4H_6O_2} + (\mathrm{w} - 4\mathrm{Y_P})\ \mathrm{CO_2} + (\frac{x}{2} - 3\mathrm{Y_X})\ \mathrm{H_2O} \quad (3.2)$$

in which $C_4H_6O_2$ represents the chemical composition of PHB monomer. Y_P is the yield of PHB over organic substrate. With respect to the limiting nutrient, most researchers applied nitrogen limitation (Cavalheiro et al., 2012; Kim et al., 1994; Pradella et al., 2012), some others used phosphate limiting conditions (Grousseau et al., 2013; Ryu et al., 1997; Shang et al., 2007) to stimulate the PHB production process.

The microbial production of PHB comprises a number of complex process steps including biomass growth, intracellular polymer accumulation, biomass decay, maintenance etc. in which

a number of process operation variables are involved (Penloglou et al., 2012). Various mathematical models have been constructed to analyze the underlying mechanisms for the PHB production through heterotrophic cultures (Dias et al., 2005; Khanna and Srivastava 2008; Marang et al., 2013). Most of them were used as a powerful tool to determine the kinetics and process parameters of microbial growth and PHB synthesis (Lee et al., 1997; Penloglou et al., 2012; Shang et al., 2007), as well as to develop adequate feeding strategies (Khanna and Srivastava, 2008; Patwardhan, 2004) aiming at maximum PHB production.

In this study, a mathematical model for pure culture heterotrophic PHB production has been developed to describe the biomass growth and the PHB production phases. A parameter sensitivity analysis has been carried out, followed by a model calibration to estimate the most sensitive parameter values. Various model structures have been evaluated, assessing the importance of aspects such as cell density inhibition and residual biomass growth on PHB, which were not been considered previously in pure culture PHB production processes. Product inhibition of PHB production was evaluated as well. The simulation results have been validated for two different substrates, being (pure) glucose and waste glycerol, based on independent experimental datasets.

3.2. Materials and methods

3.2.1. Organism, carbon source, culture media and inoculum

Cupriavidus necator (formerly known as *Ralstonia eutropha* and *Alcaligenes eutrophus*) DSM 545 was used as microorganism. The carbon sources were used same as mentioned in Chapter 2. Culture media and inoculum were prepared as previously described in chapter 2.

3.2.2. Fed-batch experiments

A lab-scale fermentation unit as described in Chapter 2 was used in this study. A three-stage substrate feeding strategy was applied to control the substrate concentration at the desired level, consisting of an initial 10 hours of exponential feeding and then feeding based on alkali-addition (coupled with NH_4OH feeding for pH control) during biomass growth (phase 1), followed by constant feeding during PHB production (phase 2) (Chapter 2). Samples were taken at regular time intervals for analysis.

3.2.3. Analytical procedures

The concentrations of glucose, glycerol, ammonium-nitrogen (NH_4^+-N), sodium (Na^+), biomass (expressed as cell dry mass, CDM) and PHB were determined as described in Chapter 2.

The PHB yield on substrate (Y_{ps}) was determined from the slope of the PHB versus substrate concentration profile in the absence of nitrogen. The residual biomass yield over substrate (Y_{xs}) was determined from the slope of the residual cell versus substrate concentration profile while correcting for substrate consumption for PHB production.

3.2.4. Model stoichiometry and kinetics

The model for heterotrophic PHB production takes into account four main processes: (1) residual biomass growth on carbon substrate; (2) residual biomass growth on PHB; (3) PHB production and (4) maintenance. The model stoichiometry and kinetics are listed in Table 3.1 and Table 3.2, respectively. The stoichiometric and kinetic parameter values are listed in Table S3.1, while Table S3.2 summarizes the operating parameter values (in Appendix S.3).

Table 3.1: Stoichiometry of the heterotrophic PHB production model.

Component → Process ↓	Substrate (*S*) (g substrate/L)	Nutrient (*N*) (g ammonium-N/L)	Residual biomass (RCC) (*X*) (g cell/L)	PHB (*P*) (g PHB/L)
1. Biomass growth on S	$-1/Y_{xs}$	$-1/Y_{xN}$	1	
2. Biomass growth on P		$-1/Y_{xN}$	1	$-1/Y_{xp}$
3. PHB production	$-1/Y_{ps}$			1
4. Maintenance	-1			

There is a metabolic interaction between growth and PHB production based on substrate, as substrate (carbon) overflow gives the advantage to PHB production. This phenomenon is implicitly incorporated in the model kinetics through the parameter values. By using higher saturation and inhibition constant for substrate in PHB production than in biomass growth (K_{PS} (=4.1)> K_S (=1.2) and K_{PIS} (=80)> K_{IS} (=16.73)), advantage is given to PHB production at high substrate concentrations. The respiration rate may become limiting at low substrate concentration, however such conditions did not prevail in our reactor, in which the substrate concentration was kept constant.

The biomass (CDM) was assumed to be composed of two components: residual biomass (RCC) (*X*) and PHB (*P*), which were taken up as separate state variables. The oxygen concentration in the process was reasonably assumed not to vary beyond a (relatively wide) non-limiting and non-inhibiting range, so oxygen did not need to be taken up as a state variable. Carbon dioxide was not taken up as an explicit state variable either, as its production does not affect the conversion processes and its concentration as such was not considered of interest in this study. Cell death was not modelled as a separate process, but could be considered lumped together with cell growth through the estimation of maximum specific growth rate (μ_{xs}^{max}).

The first process, residual biomass growth is limited by too low and inhibited by too high organic substrate (*S*) and nutrient concentrations (*N*), which was modelled through Haldane kinetics for both substrate and nutrient (Eq. 3.3), as in Lee et al. (1997).

Luong et al. (1988), reported that residual biomass growth may take place on the produced PHB besides substrate. This phenomenon was taken up as a second process. The proposed reaction rate expression (Eq. 3.5) reflects that the specific growth rate on PHB is limited by the intracellular PHB fraction (f_{PHB}) and that nitrogen again constitutes the limiting nutrient, which becomes inhibiting at high concentrations.

High residual biomass densities may negatively affect biomass growth rate, which can be described by the logistic growth model for self-limited population growth (Verhulst, 1838). Mulchandani et al. (1988) used this type of expression to describe microbial polysaccharide synthesis and clearly mentioned its applicability for PHB producing organisms with a very large 'cell density inhibition coefficient (α)' value. In this study, the logistic growth model was applied for residual biomass growth on substrate as well as on PHB (Eqs. 3.4 and 3.6, respectively).

Overall, three different models were distinguished for further model calibration and model selection. *Model A* considers residual biomass growth on substrate only, while *Model B* takes into account residual biomass growth on PHB. *Model C* constitutes a further extension of *Model B* including cell density inhibition for residual biomass growth on both substrate and PHB.

The actual PHB production (process 3) was described to take place under nitrogen limitation. In fact, the availability of nitrogen source in the medium determines which process takes place: residual biomass growth (process 1 and 2, for $N > K_N$) or PHB production (process 3, for $N < K_{PIN,}$ note that K_{PIN} was assumed equal to K_N in this study, see Table S3.1 in Appendix). At extremely high nitrogen concentrations ($N > K_{IN} >> K_{PIN}$), biomass growth (process 1 and 2) is inhibited.

Table 3.2: Kinetic expressions of the heterotrophic PHB production model.

Process	Reaction rate		Model
1. Biomass growth on S	$\rho_{xs} = \mu_{xs} X$ With		
	$\mu_{xs} = \mu_{xs}^{max} \left(\frac{S}{K_S + S + S^2/K_{IS}}\right)\left(\frac{N}{K_N + N + N^2/K_{IN}}\right)$	(3.3)	A, B
	$\mu_{xs} = \mu_{xs}^{max} \left(\frac{S}{K_S + S + S^2/K_{IS}}\right)\left(\frac{N}{K_N + N + N^2/K_{IN}}\right)\left[1 - \left(\frac{X}{X_m}\right)^{\alpha}\right]$	(3.4)	C
2. Biomass growth on P	$\rho_{xp} = \mu_{xp} X$ With		
	$\mu_{xp} = \mu_{xp}^{max} \frac{f_{PHB}}{K_{PHB} + f_{PHB}}\left(\frac{N}{K_N + N + N^2/K_{IN}}\right)$	(3.5)	B
	$\mu_{xp} = \mu_{xp}^{max} \frac{f_{PHB}}{K_{PHB} + f_{PHB}}\left(\frac{N}{K_N + N + N^2/K_{IN}}\right)\left[1 - \left(\frac{X}{X_m}\right)^{\alpha}\right]$	(3.6)	C
3. PHB production	$\rho_{ps} = \mu_{ps} X$ With		
	$\mu_{ps} = \mu_{ps}^{max} \left(\frac{S}{K_{PS} + S + S^2/K_{PIS}}\right)\left[1 - \left(\frac{f_{PHB}}{f_{PHB(max)}}\right)^{\beta}\right]\frac{K_{PIN}}{N + K_{PIN}}$	(3.7)	A, B, C
4. Maintenance	$\rho_m = m_s X$	(3.8)	A, B, C

3.2.5. Mass balances

The mass balances of the different components in the bioreactor comprise two main contributions: macroscopic transport and biochemical conversion. The macroscopic transport in and out of the fermentor was described by the dilution rate *D(t)* (1/h), i.e. the ratio between the overall flow rate *F(t)* (L/h) of the feed medium into the fermentor and the volume *V(t)* (L) of the bioreactor (Eq. 3.9).

$$D(t) = \frac{F(t)}{V(t)} \qquad (3.9)$$

A fed-batch process was considered (no outgoing flow), in which organic substrate and nutrient (ammonium) were fed to the fermentor in such a way that their concentration in the fermentor was kept constant at the levels corresponding with maximum production, being *S* and *N*, respectively. The overall feed flow rate *F(t)* (L/h) was determined by the flow rates of the

organic substrate and nutrient solutions (F_S (L/h) and F_N (L/h), respectively) as well as by the substrate and nitrogen concentrations in these feed solutions (S_F (g/L) and N_F (g/L), respectively) and their densities (ρ_{FS} (g/L) and ρ_{FN} (g/L), respectively), through the Eq. 3.10.

$$F(t) = \frac{dV(t)}{dt} = F_S(t)\frac{\rho_{FS}-S_F}{\rho_w} + F_N(t)\frac{\rho_{FN}-N_F}{\rho_w} \quad (3.10)$$

Eq. 3.10 only accounts for the water flow. This was justified because of the substrate added was consumed, which made that the volume change during the fed-batch fermentation was lower than the amount of feeding solution added. The substrate from the feeding solution was consumed, leaving only the water to cause a volume change. The volume change by PHB accumulation was not significant and thus neglected.

Mass balances were set up for the substrate and nitrogen concentrations in the fermentor (see Appendix S3.1), from which the feed flow rates of the organic substrate and nutrient solutions were subsequently determined, given that the substrate and nutrient concentrations were maintained at a constant (optimal) level:

$$\frac{dS(t)}{dt} = \frac{F_S(t)S_F}{V(t)} - D(t)S - \mu_S X(t) = 0 \quad (3.11)$$

$$\Leftrightarrow \quad F_S(t) = \frac{1}{S_F}\left(S\frac{dV(t)}{dt} + \mu_S X(t)V(t)\right) \quad (3.12)$$

$$\frac{dN(t)}{dt} = \frac{F_N(t)N_F}{V(t)} - D(t)N - \mu_N X(t) = 0 \quad (3.13)$$

$$\Leftrightarrow \quad F_N(t) = \frac{1}{N_F}\left(N\frac{dV(t)}{dt} + \mu_N X(t)V(t)\right) \quad (3.14)$$

μ_S (g substrate/g cell/h) and μ_N (g nitrogen/g cell/h) denote the specific substrate consumption rate and the specific nitrogen consumption rate, respectively:

$$\mu_S = \frac{\mu_{xs}}{Y_{xs}} + \frac{\mu_{ps}}{Y_{ps}} + m_S \quad (3.15)$$

$$\mu_N = \frac{\mu_{xs}+\mu_{xp}}{Y_{xN}} \quad (3.16)$$

Assuming that neither biomass nor PHB were present in the feed solutions, the residual biomass and PHB production profiles during fed batch culture were obtained from their respective mass balances:

$$\frac{dX(t)}{dt} = \big(\mu_x - D(t)\big)X(t) \quad (3.17)$$

$$\frac{dP(t)}{dt} = \mu_p X - D(t)P(t) \quad (3.18)$$

in which μ_x (g cell/g cell/h) and μ_p (g PHB/g cell/h) denote the specific residual biomass growth rate and the specific PHB production rate, respectively:

$$\mu_x = \mu_{xs} + \mu_{xp} \quad (3.19)$$

$$\mu_p = \mu_{ps} - \frac{\mu_{xp}}{Y_{xp}} \quad (3.20)$$

3.2.6. Sensitivity analysis

A local sensitivity analysis of different kinetic parameters around their default values (Table 3.3) was performed in order to identify which parameters were the most sensitive. Only the parameters that have a considerable influence on the model output, were subsequently considered in the parameter estimation.

The sensitivity function was defined as the partial derivative of the variable (y) to the parameter (θ), which was approximated numerically through the finite difference method assuming local linearity:

$$\frac{\partial y(t)}{\partial \theta} = lim_{\Delta\theta \to 0} \frac{y(t,\theta+\Delta\theta) - y(t,\theta)}{\Delta\theta} \quad (3.21)$$

The perturbation $\Delta\theta$ was very low and defined by

$$\Delta\theta = p.\theta \quad (3.22)$$

in which p denotes the perturbation factor. The perturbation needs to be taken small enough for the linear approximation to be valid and large enough to avoid numerical instabilities. A typical value for the perturbation factor (p) of 10^{-4} was therefore selected.

The sensitivity function described by Eq. 3.21 is an absolute sensitivity function, of which the value depends on the value of the variable and the parameter considered. Besides, its unit of sensitivity function is different for different parameters, which further hampers its interpretation and the comparison of the sensitivity of different parameters. To overcome these

difficulties, the relative sensitivity function $S_{Rf}(t)$ of variable y towards parameter θ was defined as Eq. 3.23.

$$S_{Rf}(t) = \left|\frac{\partial y(t)}{\partial \theta} . \frac{\theta}{y(t)}\right| \quad (3.23)$$

The relative sensitivity function is dimensionless and allows comparing the sensitivity of different parameters at a time instant *t*. To compare the overall sensitivity of different parameters over the considered time period, the combined relative sensitivity function (δ) is obtained from the average of $S_{Rf}(t)$ for a given parameter θ as Eq. 3.24.

$$\delta = \left|\frac{\sum_{i=1}^{n}\left(\frac{\partial y_i(t)}{\partial \theta} . \frac{\theta}{y_i(t)}\right)}{n}\right| \quad (3.24)$$

in which *n* denotes a freely chosen number of virtual measurements.
In this study, δ >2 was used as a selection criteria for the most sensitive parameters, of which the values were estimated for model calibration. This threshold was selected as a compromise between a significant effect on model outcome and keeping a minimum number of parameters.

3.2.7. Model calibration

In view of parameter estimation, an objective function ($J(\theta)$) was defined to obtain the best possible fit between the model predictions and experimental data, as obtained by minimizing the sum of squared errors (Eq. 3.25):

$$J(\theta) = \sum_{i=1}^{n}\left(y_i(t) - y_i^m(t,\theta)\right)^2 \quad (3.25)$$

$y_i(t)$ represents the experimental data observations of the model outputs, in this case the residual cell (RCC) and the PHB production, while $y_t^m(t,\theta)$ denotes the model predictions corresponding with the given parameter set θ at time *t*. During the model calibration, the 'Nelder-Mead simplex direct search' estimation algorithm (Nelder and Mead, 1965), an constrained nonlinear optimization method, was used.

3.2.8. Model validation

During model validation, the model predictions were compared with an independent experimental dataset. For this purpose, the Nash-Sutcliffe model efficiency coefficient (Nash and Sutcliffe, 1970) was used to quantitatively describe the accuracy of model outputs and in this way assess the predictive power of the model:

$$E = 1 - \frac{\sum_{i=1}^{n}\left(y_i(t) - y_i^m(t)\right)^2}{\sum_{i=1}^{n}(y_i(t) - \bar{y})^2} \quad (3.26)$$

Nash–Sutcliffe efficiencies range from -∞ to 1. An efficiency of 1 ($E = 1$) corresponds to a perfect match of modelled outcome to the observed data. An efficiency of 0 ($E = 0$) indicates that the model predictions are as accurate as the mean of the observed data, whereas an efficiency less than zero ($E < 0$) indicates that the observed mean is a better predictor than the model. Essentially, the closer the model efficiency is to 1, the more accurate the model is.
The Nash-Sutcliff criterion was complemented with visual inspection to evaluate the model fit.

3.3. Results and discussion

The model behaviour was evaluated for two different substrates; pure glucose and waste glycerol. Model calibration and validation were performed based on independent experimental datasets. The model calibration was based on the growth phase (phase 1) only, while both phases in the PHB production process (phase 1 and 2) were accounted for during validation.

3.3.1. Model calibration for biomass growth (phase 1) on glucose substrate

To describe biomass growth on glucose, three different models (A, B and C) were considered and calibrated on experimental data. The simulation outcomes from the calibrated models were compared to identify the most suitable model to describe the process.

3.3.1.1.Experimental data for the biomass growth phase

The experimental results for biomass growth on glucose (Figure 3.1, discrete markers), show that after 33 h the total biomass concentration reached a maximal level of 73.5 g/L, while growth-associated PHB production amounted to 6 g/L, corresponding to a PHB content of 8%. Throughout the experiment the glucose concentration was maintained within its optimal range (10-20 g/L), in which the specific growth rate remained almost constant (Chapter 2). Although

the ammonium nitrogen concentration decreased from 0.78 to 0.45 g/L, nitrogen was sufficient for biomass growth (Repaske, 1961).

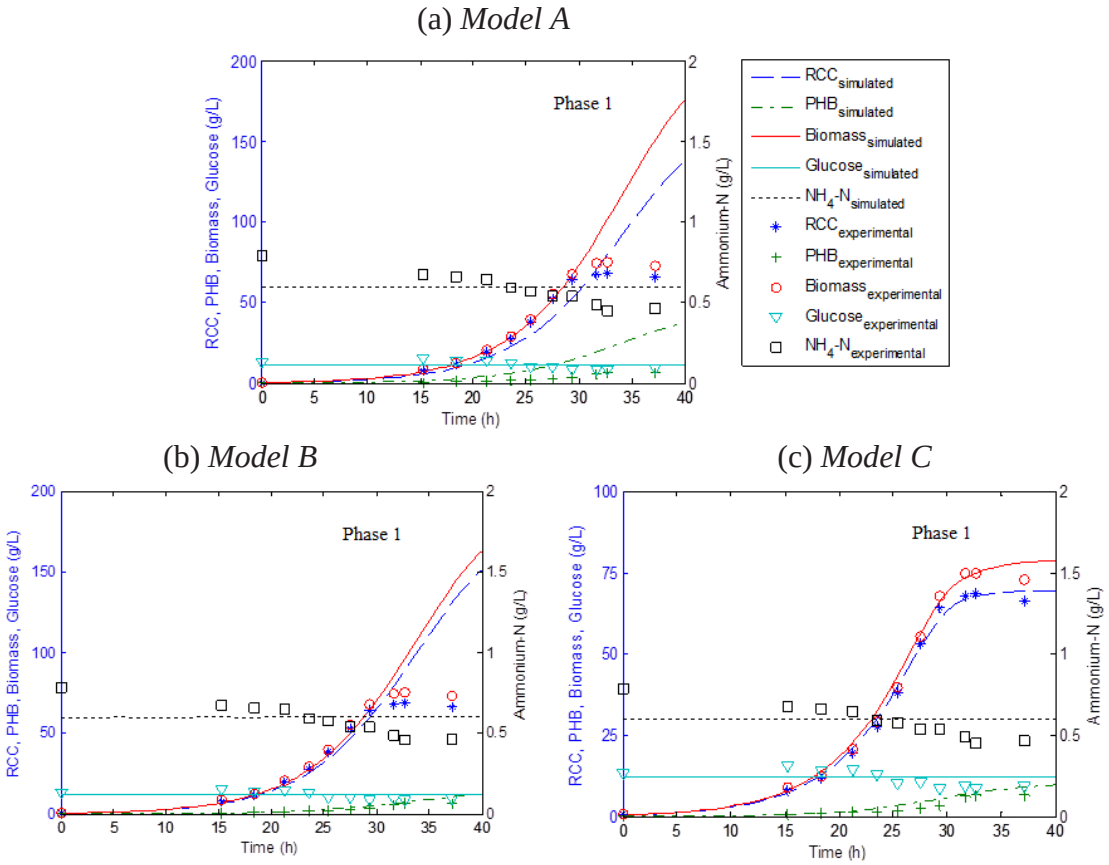

Figure 3.1. Model calibration results for glucose substrate. Comparison between the simulation outcome and experimental observations for (a) *Model A*, (b) *Model B* and (c) *Model C*.

3.3.1.2.Model A

For this model considering residual biomass growth on substrate only and neglecting biomass self-inhibition, the parameters K_{IS}, K_{IN}, $\mu_{xs}^{\max}$ and μ_{ps}^{max} were found most sensitive with respect to the model output variables RCC and PHB (δ > 2, Table S3.3 in Appendix). The estimated parameter values are listed in Table 3.3 and were in agreement with literature values (Table S3.1 in Appendix S3). Figure 3.1a compares the calibrated model output with the experimental observations. The experimental RCC concentrations are underestimated up to the moment that the maximal RCC is reached, while the PHB production is overestimated by the model with corresponding Nash-Sutcliffe model efficiency coefficients (E) for RCC and PHB amounting to 0.66 and -0.23, respectively (Table S3.4). This can be explained by the fact that the conversion of PHB into biomass is not considered in *Model A*.

Table 3.3: Initial and estimated values of most sensitive kinetic parameters used in the model calibration.

Parameter	Initial value	Unit	Estimated parameter value			
			Using glucose			Using waste glycerol
			Model A	*Model B*	*Model C*	*Model C*
K_{IS}	17.430	g substrate/L	20.092	17.323	16.728	37.136
K_{IN}	1.500	g N/L	1.567	-	-	-
$\mu_{XS}^{\max}$	0.410	g cell/g cell/h	0.491	0.446	0.46	0.328
μ_{ps}^{max}	0.180	g PHB/g cell/h	0.239	0.222	0.217	0.232
K_{PIN}	0.254	g N/L	-	0.265	0.262	0.679
μ_{xp}^{max}	0.180	g cell/g cell/h	-	0.149	0.126	0.136
K_{PHB}	0.250	g PHB/g cell	-	-	0.148	0.138

3.3.1.3.Model B

Model B accounts for residual biomass growth on the produced PHB, besides growth on organic substrate, in presence of nitrogen. K_{IS}, $\mu_{XS}^{\max}$, μ_{ps}^{max}, K_{PIN} and μ_{xp}^{max} were found as the most sensitive parameters and their values were subsequently estimated (Table 3.3). Figure 3.1b shows that the PHB production is predicted better by *Model B* than by *Model A*, while the RCC is still not so well predicted (E=0.72 for RCC and E=0.91 for PHB, see Table S3.4). In particular, the simulated biomass concentration keeps rising while the experimentally determined biomass concentration reaches a constant level at the end of the experiment (around 73 g/L).

The experimental data shows PHB accumulation under non-limiting conditions, i.e. during the biomass growth phase (Figure 3.1) that incorporated in the model (Eq. 3.7). Microorganisms have been reported to use this intracellular PHB, besides organic substrate, for residual biomass growth in presence of nitrogen (Luong et al., 1988). By comparing the simulation results for the model only considering biomass growth on organic substrate (*Model A*) and also including residual biomass growth on PHB (*Model B*) with the experimental results (Figure 3.1a and b), it is clear that biomass growth on PHB during non-limiting conditions indeed cannot be

neglected, in this respect demonstrating the better predictive capacity of *Model B* over *Model A*. The proposed reaction rate expression (Eq. 3.5) reflects that the specific growth rate on PHB is limited by the intracellular PHB fraction (f_{PHB}) and that nitrogen again constitutes the limiting nutrient, which becomes inhibiting at high concentrations.

3.3.1.4.Model C

Residual biomass self-inhibition on growth was considered in *model C*. The parameters K_{IS}, $\mu_{XS}^{\max}$, μ_{ps}^{max}, K_{PIN}, μ_{xp}^{max} and K_{PHB} were found most sensitive; estimated in the model calibration. This results in a good accordance between the experimental data and the simulation results (Figure 3.1c), which is also reflected by values of the Nash-Sutcliffe model efficiency coefficients close to 1 (E= 0.99 and 0.94 for RCC and PHB, respectively, see Table S3.4). Comparing the simulated and experimental biomass concentrations profiles (Figure 3.1c), it is clear that cells cannot grow in an unlimited way as was assumed in *Model B*. It is quite unlikely that oxygen limitation was the reason for the growth stop, given the relatively high DO levels applied in the process. It was also verified that the biomass concentration could not be enhanced by adding essential nutrients (phosphate, $MgSO_4$, trace elements) at the middle and at the end of the cultivation period. Instead, the negative effect of high cell density on the biomass growth rate, described by Mulchandani et al. (1988) was put responsible for limiting biomass growth on substrate and on PHB (Eqs. 3.4 and 3.6, respectively). This maximum residual biomass concentration, for which the specific growth rate becomes zero, was determined experimentally as X_m =68 g cell/L. The same value (68±1 g cell/L) was found for both substrates, glucose and waste glycerol, and was confirmed by repeated experiments, which strengthens the hypothesis of biomass density inhibition and justifies its description through the logistic expression.

The relationship between the biomass growth and cell density depends on cell density inhibition coefficient (α), of which the value depends on the microbial species, the physicochemical properties of the culture medium and the operation conditions (Luong et al., 1988). α =1 denotes a linear relation between the specific growth rate and the biomass concentration, corresponding with logistic growth kinetics. However, Strehaiano et al. (1983) experimentally demonstrated that the specific growth rate decreased nonlinearly with increasing biomass concentration. When α >1, the growth lies in between exponential and logistic patterns. This is also the case in this study, in which the α-value of 5.8 given by Mulchandani et al. (1988) but not previously evaluated on experimental data, was shown to adequately simulate the experimental behaviour.

3.3.2. Model validation for biomass growth and PHB production (phase 1 and 2) on glucose substrate

Model C was subjected to validation on two independent experimental datasets, both considering the two phases of the PHB production process.

3.3.2.1.Experimental data for PHB production

Two distinct experimental datasets were considered, which differed in the time instant at which nitrogen (N) limitation was imposed (switching point from phase 1 to phase 2) to enhance the PHB productivity. When stopping the N feed at a RCC of 49 g /L, the maximal biomass (CDM) and corresponding PHB productions 128 g/L and 97 g/L, respectively, were obtained after 56 h (Figure 3.2a), corresponding with a PHB content of 75.8%. When applying nitrogen limitation at 56 g/L residual biomass concentration, a maximal biomass (CDM) concentration of 164 g/L and corresponding PHB production of 125 g/L are achieved after 62 h (Figure 3.2b), corresponding with 76.2% PHB. In both cases, the glucose concentration was at its optimal level with a very small fluctuation and this fluctuation almost did not affect the biomass growth and PHB production.

(a) (b)

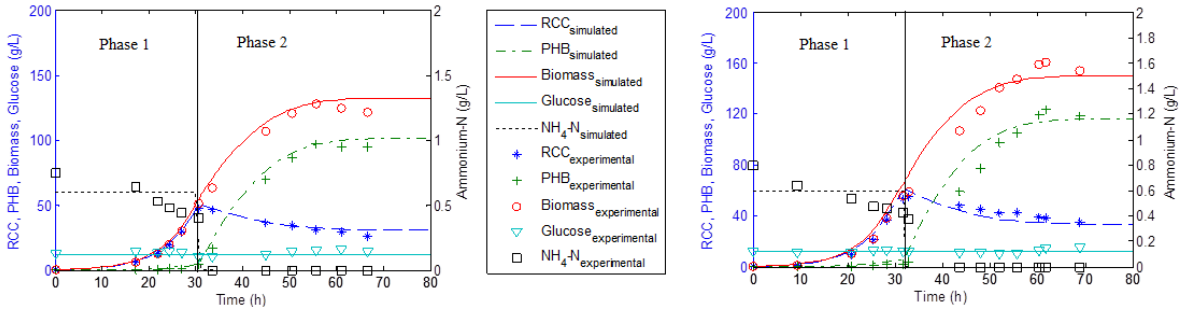

Figure 3.2. Model validation results for glucose substrate. Comparison between simulation outcome for *Model C* and experimental observations for two independent datasets, corresponding with a stop in the nitrogen feed at (a) 49 g/L and (b) 56 g/L residual cell concentration (RCC).

3.3.2.2.Model validation

The behaviour of *Model C* was simulated for two different switching points, stopping nitrogen feeding at 49 and 56 g/L residual cell concentration (RCC). The validation consists of a visual comparison between the model prediction and the experimental observations (Figure 3.2) besides the calculation of Nash-Sutcliffe model efficiency coefficient (E) for RCC and PHB

(Table S3.4). In both cases the models predict the experimental observations quite well. The E-values corresponding with an operation switch at 49 g/L RCC amount to 0.97 for RCC and 0.98 for PHB, while these for an operation switch at 56 g/L RCC are 0.93 for RCC and 0.96 for PHB, all of which are close to 1, thus indicating a very good model fit.

The availability of nitrogen determines whether biomass growth or PHB production will take place. Both non-growth-associated and growth-association PHB production are described by Eq. 3.7 in this study. During the biomass growth phase (phase 1), no nitrogen limitation is imposed, resulting in a high biomass growth and a low growth-associated PHB accumulation. Non-growth-associated PHB production is triggered during the PHB production phase (phase 2) by stopping the nitrogen feeding. Growth and non-growth-associated PHB production have been reported as two separate processes by several research groups (Mulchandani et al., 1989; Patwardhan and Srivastava, 2004; 2008). However, this phenomenon can be explained only by the inhibition effect of nutrient concentration (Spoljaric et al., 2013a).

Fermentation end products are known to negatively affect microbial activities. The accumulation of PHB results in a decreasing PHB formation, described by Eq. 3.7. implying that the cells are not capable to produce PHB in an unlimited way but that the specific PHB production rate approaches to zero as f_{PHB} approaches $f_{PHB(max)}$. The relation between the specific PHB production rate and product concentration depends on the empirical PHB saturation power coefficient (β). Lee et al. (1997) indicated a linear relation (β =1) whereas other author (Patwardhan and Srivastava 2004; 2008) used a proportional relation, but in reality it is not universally applicable. The value β =3.85 determined by Dias et al. (2005; 2006) for PHB production through mixed cultures was confirmed in this study to be applicable as well for pure culture PHB production.

3.3.3. Model calibration for biomass growth (phase 1) on waste glycerol substrate

Model C was calibrated using waste glycerol considering only biomass growth (phase 1). Figure 3.3 illustrates a typical time course of the fed batch cultivation of *C. necator* in the bioreactor. A maximum biomass concentration of 84.4 g/L and corresponding PHB production of 18.3 g/L were obtained after 37 hours of cultivation period, corresponding with a PHB content of 21.7%. The most sensitive kinetic parameters K_{IN}, K_{PHB}, K_{PIN}, μ_{XS}^{max}, μ_{PS}^{max} and μ_{XP}^{max} were estimated (Table 3.3). The resulting model predicted the process performance well (Figure 3.3), Nash-Sutcliffe model efficiency coefficient for RCC and PHB were calculated as 0.96 and 0.83, respectively.

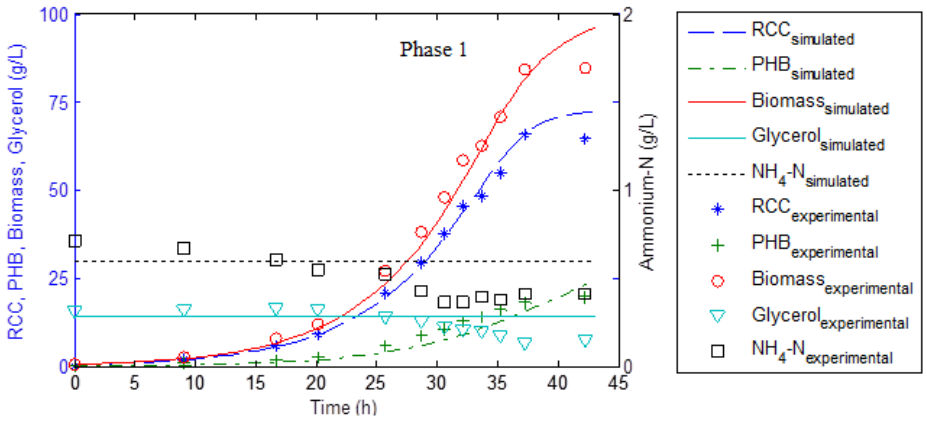

Figure 3.3. Model calibration results for waste glycerol substrate. Comparison between the simulation outcome from *Model C* with experimental observations using waste glycerol as a substrate only the growth phase.

A high growth-associated PHB content was obtained using waste glycerol (21.7% PHB, Figure 3.3) as a substrate compare to using glucose (8% PHB content, Figure 3.1), while the same nitrogen concentration level was maintained in both cases. The higher degree of growth-associated PHB production using waste glycerol compared to glucose is reflected by a higher nitrogen inhibition constant (lower nitrogen inhibition) in the model, namely K_{PIN} =0.68 and 0.26 for waste glycerol and glucose, respectively (Table 3.3).

3.3.4. Model validation for biomass growth and PHB production (phase 1 and 2) on waste glycerol substrate

Two experimental datasets concerning biomass growth and PHB production on waste glycerol were available for validation of the proposed model (*Model C*). These datasets differed in the time instant at which nitrogen limitation was applied to stimulate the PHB production, being 7 and 44 g/L residual cell concentration (RCC), in order to examine the effect of the time instant at which the shift was applied. When nitrogen was limited at a residual cell concentration of 7 g/L, a maximal biomass (CDM) concentration of 30.7 g/L and corresponding PHB production of 21.6 g/L were obtained after 40 h (Figure 3.4a), corresponding with 70.4% PHB. When applying nitrogen limitation at 44 g/L residual biomass concentration, a maximal biomass (CDM) concentration of 104.7 g/L and corresponding PHB production of 65.6 g/L were achieved after 48 h (Figure 3.4b), corresponding with a PHB content of 62.7%. For both datasets, the model predictions agree well with the experimental results in terms of residual cell

concentration (RCC), PHB and total biomass concentration (CDM). The E-values corresponding with an operation switch at 7 g/L RCC amount to 0.95 for RCC and 0.94 for PHB, while these for an operation switch at 44 g/L RCC were 0.95 for RCC and 0.93 for PHB, all of which were close to 1, thus indicating a very good model fit.

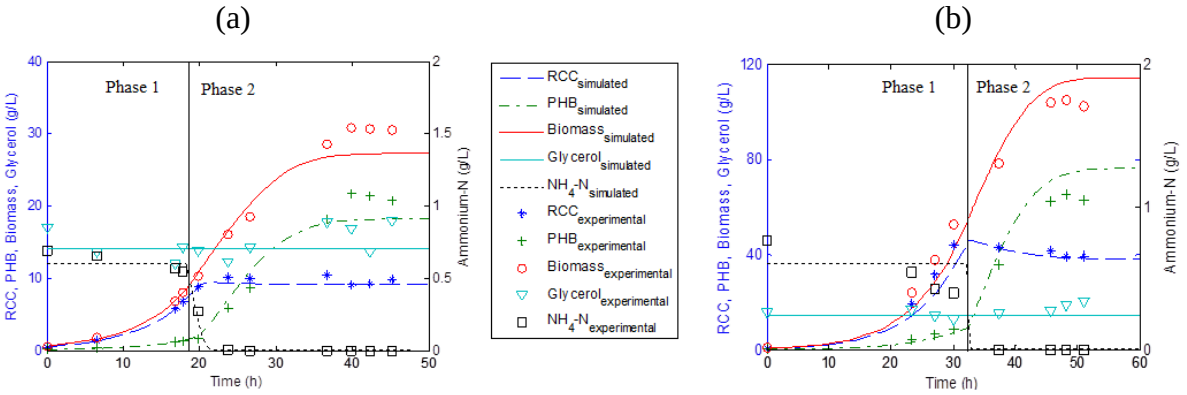

Figure 3.4. Model validation results for waste glycerol substrate. Comparison of between simulation outcome for *Model C* and experimental observations, stopping the nitrogen feed at (a) 7 g/L and (b) 44 g/L residual cell concentration (RCC).

The time instant at which the nitrogen limitation is imposed, hardly affects the PHB content in case of glucose substrate: 76% PHB content is obtained for both switching points considered. However, using waste glycerol as substrate, PHB contents of 70.4% and 62.7% were obtained when applying nitrogen limitation at 7g/L and 44 g/L RCC, respectively. It is hypothesized that the lower PHB content obtained in the case of waste glycerol for a later application of nitrogen limitation, i.e. at an increased cell density, is due to the accumulation of impurities in the culture medium , which negatively affect the PHB accumulation metabolism of *C. necator* (Posada et al., 2011), and thus result in a decreased PHB content.

The accumulation of impurities most likely sodium (Na^+) can also be seen as the reason for the lower PHB content obtained from waste glycerol compared to glucose, being maximum 70.4% and 76.2%, respectively, in this study. The lower PHB content obtained on waste glycerol as a carbon source instead of glucose corresponds to literature. A high PHB content (74%) was obtained by Kim et al. (1994) among others using glucose as substrate, whereas for glycerol maximum 56 to 67% PHB content was obtained (Cavalheiro et al., 2009; Ibrahim and Steinbuchel, 2009; Posada et al., 2011).

Concerning the model simulation results for waste glycerol, the PHB content was underestimated for the switching point 7 g/L RCC (Figure 3.4a) and overestimated for the

switching point 44 g/L RCC (Figure 3.4b). This is mathematically translated as a deviation of $f_{PHB(max)}$ from the used value ($f_{PHB(max)}$=2), dependent on the switching point. The maximum PHB to active biomass ratio ($f_{PHB(max)}$) was experimentally determined at 2.4 for the switching point 7 g/L RCC and at 1.65 for switching point 44 g/L RCC. Using this experimentally determined $f_{PHB(max)}$ value for each dataset results in a better model prediction, corresponding with higher values for the Nash-Sutcliffe model efficiency coefficient (E) value, being 0.98 for PHB both at switching point 7 g/L RCC and at switching point 44 g/L RCC. In view of further model applications, further understanding of the relation between the metabolic activities and accumulated impurities is required. For instance, relationships between the maximum PHB content ($f_{PHB(max)}$) and the accumulation of impurities from the waste substrate could be established.

3.4. Conclusions

- A mechanistic model for fed-batch pure culture two-phase PHB production was developed, calibrated and validated for two different substrates, namely glucose and waste glycerol.
- Biomass growth on PHB during non-limiting (growth) conditions was found non-negligible, even in the presence of substrate.
- Biomass growth was clearly inhibited by the biomass density.
- Growth-associated PHB production took place besides non-growth-associated PHB production.
- Product (PHB) inhibition during pure culture PHB production under nitrogen limitation followed a nonlinear relationship.
- The improved description of the above regulating factors led to an improved model structure, validated by a better fit to the experimental data.

Chapter 4:

Effect of sodium accumulation on heterotrophic growth and polyhydroxybutyrate (PHB) production by *Cupriavidus necator*

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Abstract

This study evaluates the effect of sodium (Na^+) concentration on the growth and PHB production by *Cupriavidus necator*. Both biomass growth and PHB production were inhibited by Na^+ as a result of osmotic stress: biomass growth became zero at 8.9 g/L Na^+ concentration while PHB production was completely stopped at 10.5 g/L Na^+. A mathematical model for pure culture heterotrophic PHB production was set up to describe the Na^+ inhibition effect. The parameters related to Na^+ inhibition were estimated based on shake flask experiments. The accumulated Na^+ showed non-linear inhibition effect on biomass growth but linear inhibition effect on PHB production kinetics. Fed-batch experiments revealed that a high accumulation of Na^+ due to a prolonged growth phase, using NaOH for pH control, decreased the subsequent PHB production. The model was validated based on independent experimental datasets, showing a good agreement between experimental data and simulation results.

Keywords: fermentation, polyhydroxybutyrate (PHB), Na^+ inhibition, biomass growth, PHB production, mathematical modeling, simulation.

4.1. Introduction

The application of polyhydroxybutyrate (PHB), a biodegradable plastic, produced from renewable resources, is promising (Akaraonye et al., 2010). Given its physical properties, PHB has the potential to substitute conventional fossil fuel-based plastics; however it is still commercially behind petroleum-based plastics. The major drawback is the high production cost, which is dominated for approximately 50% by the raw material costs (Choi and Lee, 1999). To attain bulk commercial viability and to further improve the sustainability of PHB production, it is desirable to use waste and surplus materials for PHB biosynthesis. The latter is also advantageous in terms of waste management. A number of recent studies were conducted on the potential of waste glycerol, produced in the biodiesel industry, for pure culture PHB production (Cavalheiro et al., 2012). The PHB production was significantly lower than that obtained on pure substrate. This was attributed to the presence of impurities in the waste glycerol from biodiesel industry namely sodium (Na^+) (Cavalheiro et al., 2009; Mothes et al., 2007).

A two-phase fed-batch process is typically applied for PHB production by pure culture fermentation. The first phase is characterized by favourable conditions for biomass growth, while in the second phase PHB production is realized under stress conditions

Cupriavidus necator is the most widely used organism for pure culture PHB production. Heterotrophic biomass growth of *C. necator* (phase 1) on an organic substrate using NaOH for pH control is described by Eq. 4.1 (Doran, 1995).

$$\begin{aligned} C_wH_xO_y + (4w - \frac{y}{2} + \frac{x}{4} - 1.91Y_X)\ O_2 + 0.19\ Y_X\ NH_4^+ \ +\ 0.19\ Y_X\ NaOH \\ \rightarrow Y_X\ CH_{1.74}O_{0.46}N_{0.19} + (w - Y_X)\ CO_2 + \frac{x - 1.55Y_X}{2}\ H_2O + 0.19\ Y_X\ Na^+ \end{aligned} \quad (4.1)$$

In Eq. 4.1, $C_wH_xO_y$ denotes the organic substrate, and $CH_{1.74}O_{0.46}N_{0.19}$ is the chemical composition for *C. necator* (Ishizaki and Tanaka, 1990). Y_X is the yield of biomass over organic substrate. During bacterial growth, NH_4^+ is consumed as a nitrogen source, while CO_2 is produced a side-product. At this stage, PHB production is suppressed by excess NH_4^+ concentration (Kim et al., 1994).

Under stress conditions, i.e. under nutrient limitation, the organic carbon source is used for PHB production according to Eq. 4.2 (Akiyama et al., 2003).

$$C_wH_xO_y + (w - \frac{y}{2} + \frac{x}{4} - \frac{9}{2}Y_P)\ O_2 \rightarrow Y_P\ C_4H_6O_2 + (w - 4Y_P)\ CO_2 + (\frac{x}{2} - 3Y_X)\ H_2O \quad (4.2)$$

in which $C_4H_6O_2$ represents the chemical composition of PHB monomer. Y_P is the yield of PHB over organic substrate. With respect to the limiting nutrient, most researchers applied nitrogen limitation (Cavalheiro et al., 2012; Kim et al., 1994; Pradella et al., 2012), while some others used phosphate limiting conditions (Grousseau et al., 2013; Ryu et al., 1997; Shang et al., 2007) to stimulate the PHB production process.

A lower PHB production and PHB content were observed when NaOH was used for pH control instead of NH_4OH (Passanha et al., 2013; Yeo et al., 2008). Passanha et al. (2014) reported a 30% higher PHB content using 6.5 g/L NaCl in nutrient medium by *C. necator* strain and then PHB content decreased with increasing NaCl concentration. Ibrahim and Steinbuchel (2009) achieved maximum PHB accumulation using 20 g/L NaCl by *Zobellella denitrificans* MW1.

While there are clear indications that Na^+ originating from the substrate or added for pH control negatively affects PHB production, the effect of Na^+ on the individual growth phase and PHB production phase is not yet completely clear. In this contribution, shake flask

experiments were carried out to fully characterize the effect of Na^+ on biomass growth and PHB production. A mathematical model was set up, incorporating these findings, to describe the effect of Na^+ on pure culture heterotrophic PHB production. The model was validated through fed-batch experiments.

4.2. Materials and methods

4.2.1. Organism, media and inoculum preparation

C. necator DSM 545 was used as microorganism. Culture media and inoculum (preculture 1 and 2) were prepared as previously described in Chapter 2.

4.2.2. Shake flask experiment

The effect of the Na^+ concentration on the growth of *C. necator* DSM 545 was investigated by monitoring the growth rate on glucose in presence of different concentrations of Na^+. Four mL of preculture 2 medium was inoculated into 100 mL of fermentation medium in 500-mL baffled flasks, to which Na^+ was added ranging from 0 to 8.90 g/L. The flasks were incubated for 10 hours at 180 rpm and 30°C to ensure favourable conditions for biomass growth. Samples were then collected for analysis.

After 24 hours of biomass growth without additional Na^+, 25 mL of the culture was transferred to 75 mL of ammonium nitrogen free fermentation medium that contained added Na^+ ranging from 0 to 10.5 g/L to evaluate its effect on PHB production. The flasks were incubated at 180 rpm and 30°C and sample was taken after 10, 26 and 38 hours for analysis.

4.2.3. Fed-batch experiments

Fed-batch experiments were performed in a 3-L bioreactor (Applikon Biotechnology, the Netherlands) (see Chapter 2). The pH was maintained at 6.80±0.05 by adding 5M NaOH or 2M H_2SO_4. The feed solution for phase 1 and 2 contained 650 g/L glucose with 164 g/L $(NH_4)_2SO_4$ and 650 g/L glucose, respectively. The software program, BioXpert, was used to control the glucose concentration as well as nitrogen in the fermentor at the desired level (glucose 10-20 g/L, NH_4^+-N 0.5-0.8 g/L for phase 1 and NH_4^+-N free in phase 2) through implementing the feeding strategy developed in Chapter 2. Samples were collected at regular time intervals and analysed.

4.2.4. Analytical procedures

The concentrations of glucose, ammonium-nitrogen (NH_4^+-N), sodium (Na^+), biomass (expressed as cell dry mass, CDM) and PHB were determined as described in Chapter 2.

4.3. Model development

4.3.1. Process stoichiometry and kinetics

A model for heterotrophic PHB production was set up based on the model developed in Chapter 3 (Model C) to evaluate the effect of Na^+ in the culture medium. The model stoichiometry and kinetics are displayed in Table 4.1 and Table 4.2, respectively. The stoichiometric and kinetic parameter values are listed in Table S4.1, while Table S4.2 summarizes the operating parameter values (in Appendix S4). The biomass was assumed to be composed of two components: residual biomass concentration (RCC) (X) and PHB (P), which were taken up as separate state variables.

Table 4.1: Stoichiometry of the heterotrophic PHB production model.

Component → Process ↓	Substrate (S) (g substrate/L)	Nutrient (N) (g ammonium-N/L)	Residual Biomass (X) (g /L)	PHB (P) (g PHB/L)
1. Biomass growth on substrate	$-1/Y_{xs}$	$-1/Y_{xN}$	1	
2. Biomass growth on PHB		$-1/Y_{xN}$	1	$-1/Y_{xp}$
3. PHB production	$-1/Y_{ps}$			1
4. Maintenance	-1			

The model considers four main processes: (1) biomass growth on carbon substrate (Eq. 4.3 and 4.4); (2) biomass growth on PHB (Eq. 4.5 and 4.6); (3) PHB production (Eq. 4.7 and 4.8) and (4) maintenance (Eq. 4.9). Both the yield and maintenance are assumed to be unaffected due to additional Na^+. The yield was verified through shake flask experiments and found almost same as without Na^+ (with maximum 7% variation).

The negative effect of Na^+ concentration on biomass growth and PHB production was described by modified logistic kinetics. It was assumed that biomass growth on substrate and on PHB was reduced by Na^+ in the same way, described by the same parameters Na_{xm} and n_x. The accumulation of Na^+ during biomass growth when using NaOH for pH control is quantified by $Y_{Na,x}$ (g Na^+/g residual biomass). As for PHB production, Na^+ negatively affects the specific PHB production rate (parameters Na_{pm} and n_{pf}) as well as the PHB content in terms of the maximum PHB to active biomass ratio ($f_{PHB(max)}$, affected through parameters Na_{pm} and n_{pf} , Eq. 4.8).

Table 4.2: Kinetic expressions of the heterotrophic PHB production model.

Process	Reaction rate
1. Biomass growth on substrate	$\rho_{xs} = \mu_{xs} X$ With $\mu_{xs} = \mu_{xs}^0 \left[1 - \left(\frac{Na}{Na_{xm}}\right)^{n_x}\right]$ (4.3) $\mu_{xs}^0 = \mu_{xs}^{max} \left(\frac{S}{K_S + S + S^2/K_{IS}}\right)\left(\frac{N}{K_N + N + N^2/K_{IN}}\right)\left[1 - \left(\frac{X}{X_m}\right)^{\alpha}\right]$ (4.4)
2. Biomass growth on PHB	$\rho_{xp} = \mu_{xp} X$ With $\mu_{xp} = \mu_{xp}^0 \left[1 - \left(\frac{Na}{Na_{xm}}\right)^{n_x}\right]$ (4.5) $\mu_{xp}^0 = \mu_{xp}^{max} \frac{f_{PHB}}{K_{PHB} + f_{PHB}} \left(\frac{N}{K_N + N + N^2/K_{IN}}\right)\left[1 - \left(\frac{X}{X_m}\right)^{\alpha}\right]$ (4.6)
3. PHB production	$\rho_{ps} = \mu_{ps} X$ With $\mu_{ps} = \mu_{ps}^{max} \left(\frac{S}{K_{PS} + S + S^2/K_{PIS}}\right)\left[1 - \left(\frac{f_{PHB}}{f_{PHB(max)}}\right)^{\beta}\right]\frac{K_{PIN}}{N + K_{PIN}}\left[1 - \left(\frac{Na}{Na_{pm}}\right)^{n_p}\right]$ (4.7) $f_{PHB(max)} = f_{PHB(max)}^0 \left[1 - \left(\frac{Na}{Na_{pm}}\right)^{n_{pf}}\right]$ (4.8)
4. Maintenance	$\rho_m = m_s X$ (4.9)

4.3.2. Mass balances

The volume of the fed-batch bioreactor (*V(t)*, in L) changed with time due to the addition of the feed solution (with flow rate $F_S(t)$, in L/h and density ρ_{FS}, in g/L) containing the glucose and nitrogen, (with concentrations (S_F and S_N, in g/L) and due to the addition of sodium hydroxide solution (with flow rate $F_{Na}(t)$, in L/h, density ρ_{FNa} and concentration Na_F in g/L) (Eq.4.10):

$$\frac{dV(t)}{dt} = F_S(t)\frac{\rho_{FS} - S_F - S_N}{\rho_w} + F_{Na}(t)\frac{\rho_{FNa} - Na_F}{\rho_w} = F(t) \quad (4.10)$$

The overall feed flow rate $F(t)$ (L/h) determines the dilution rate D(t) (1/h).:

$$D(t) = \frac{F(t)}{V(t)} \quad (4.11)$$

Given that the substrate concentrations in the fermentor ($S(t)$, in g/L) were controlled to a constant level throughout the experiment, the individual mass balance for substrate (Eq. 4.12) was used to determine the substrate feed flow rate (Eq. 4.13). The nitrogen concentration (*N(t)*, in g/L) was calculated from the mass balance for nitrogen (Eq. 4.14):

$$\frac{dS(t)}{dt} = \frac{F_S(t)S_F}{V(t)} - D(t)S(t) - \mu_s X(t) = 0 \quad (4.12)$$

$$\leftrightarrow F_s(t) = \frac{1}{S_F}(S\,F(t) + \mu_S\,X(t)\,V(t)) \quad (4.13)$$

$$\frac{dN(t)}{dt} = \frac{F_S(t)N_F}{V(t)} - D(t)N - \mu_N X(t) \quad (4.14)$$

The terms μ_S and μ_N denote the specific organic substrate and nitrogen consumption rate (g/g/h) respectively. (Eqs. S4.1 and S4.2 in Appendix S4).

Sodium accumulated in the medium of fed-batch fermentation because of base addition for the neutralization of protons (H^+) produced during active biomass growth. The Na^+ concentration $Na(t)$ (g/L) and flow rate of base solution $F_{Na}(t)$ (L/h) were determined by Eqs. 4.15 and 4.16 respectively.

$$\frac{dNa(t)}{dt} = \frac{F_{Na}(t)Na_F}{V(t)} - D(t)Na \quad (4.15)$$

$$F_{Na}(t) = \frac{1}{Na_F}(\mu_x Y_{Na,x} X(t)V(t)) \quad (4.16)$$

in which terms μ_X denotes specific biomass growth rate (g/g/h) (Eqs. S4.1 in Appendix S4). The residual biomass (Eq. S4.3) and PHB production (Eq. S4.4) profiles resulted from their respective mass balances (detailed in Appendix S3.1).

4.3.3. Model calibration and validation

All model parameters concerning Na^+ inhibition (Na_{xm}, Na_{pm}, n_x, n_p, n_{pf}) were estimated (model calibration) based on the best possible fit between the model predictions and experimental data observations, in this case residual biomass (RCC) and PHB production, as obtained by minimizing the sum of squared errors.

As for model validation, the model predictions were compared with three independent experimental datasets. Besides visual comparison, the Nash-Sutcliffe model efficiency coefficient (E) (Nash and Sutcliffe, 1970) was used to quantify the accuracy of model outputs and in this way assess the predictive power of the model. Nash–Sutcliffe efficiency coefficients range from -∞ to 1. Essentially, the closer the model efficiency is to 1, the more accurate the model is.

4.4. Results and discussions

The developed model was calibrated and validated based on distinct experimental datasets. Shake flask experiments were conducted for model calibration, to determine the parameter values related to Na^+ inhibition. Three datasets obtained from lab-scale fed-batch fermentation were used for model validation; the first one concerned the growth phase only (phase 1), the second and third one concerned both biomass growth and PHB production (phase 1 and 2), but differed in starting point of phase 2 in the PHB production process. In all three cases, NaOH was used to control pH throughout the experiments.

4.4.1. *Model calibration for biomass growth (phase 1) using shake flask fermentation*

To determine the parameter values related to Na^+ inhibition on biomass growth of *C. necator*, a series of shake flask experiments were conducted which differed in the amount of Na^+ added (Figure 4.1, discrete markers). The decrease of the specific growth rate with increasing Na^+ concentration was first slowly and then more rapidly, from 0.192 g/g/h when no Na^+ was added, to 0 g/g/L for a Na^+ concentration of 8.9 g Na^+/L.

The parameter values related to Na^+ inhibition on biomass growth (Eqs. 4.3 and 4.5), Na_{xm} and n_x, were estimated as 8.9 g/L and 1.91 respectively. A good accordance between the experimental data and the simulation results was obtained (Figure 4.1), which is also reflected by the value of the Nash-Sutcliffe model efficiency coefficient (E-value= 0.95).

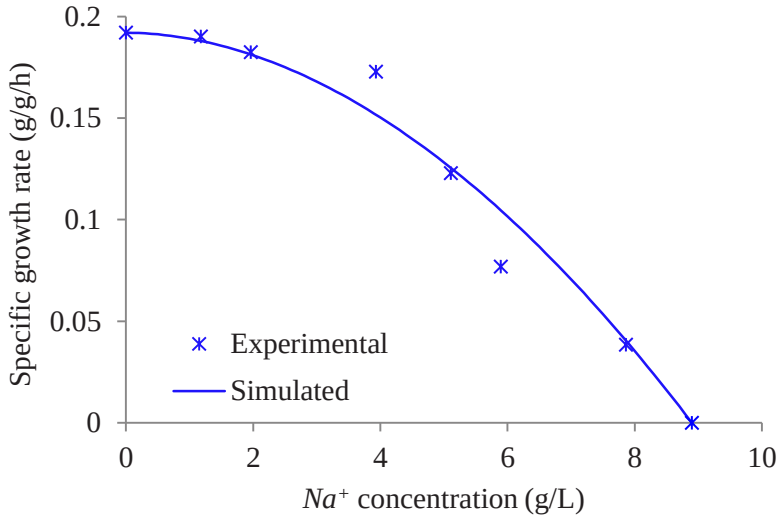


Figure 4.1. Effect of sodium concentration in mineral medium on specific biomass growth rate (μ_x).

The value of the inhibition coefficient n_x depends on the microbial species, the physicochemical properties of the culture medium and the operating conditions (Luong et al., 1988). A value n_x =1 denotes a linear relation, corresponding with logistic growth kinetics. For n_x >1, the growth lies in between exponential and logistic patterns and is termed as 'modified logistic'. The n_x-value of 1.91, estimated in this study, confirms the experimental findings of Mothes et al. (2007), who also demonstrated that the specific growth rate decreased nonlinearly with increasing Na^+ concentration.

4.4.2. *Model calibration for PHB production (phase 2) using shake flask fermentation*

The specific PHB production rate (μ_p, g/g/h) and the PHB accumulation capacity in terms of maximum PHB to residual biomass ratio ($f_{PHB(max)}$) was determined through shake flask experiments for different Na^+ concentration (Figure 4.2, discrete markers). The $f_{PHB(max)}$ decreased from 3.5 without Na^+ addition to zero at Na_{pm} = 10.5 g Na^+/L. At the latter point, the specific PHB production rate was also zero. The value of n_{pf} was estimated at 1.23, indicating a slight nonlinearity in the relation between the $f_{PHB(max)}$ and the added Na^+ concentration, while the specific PHB production rate linearly decreased with increasing Na^+ concentration (n_p = 1). Figure 4.2 compares the model output of specific PHB production rate and $f_{PHB(max)}$ with the experimental observations. The resulting model simulations fit very well with the experimental observations (Figure 4.2); the Nash-Sutcliffe model efficiency coefficients were 0.98 in both cases.

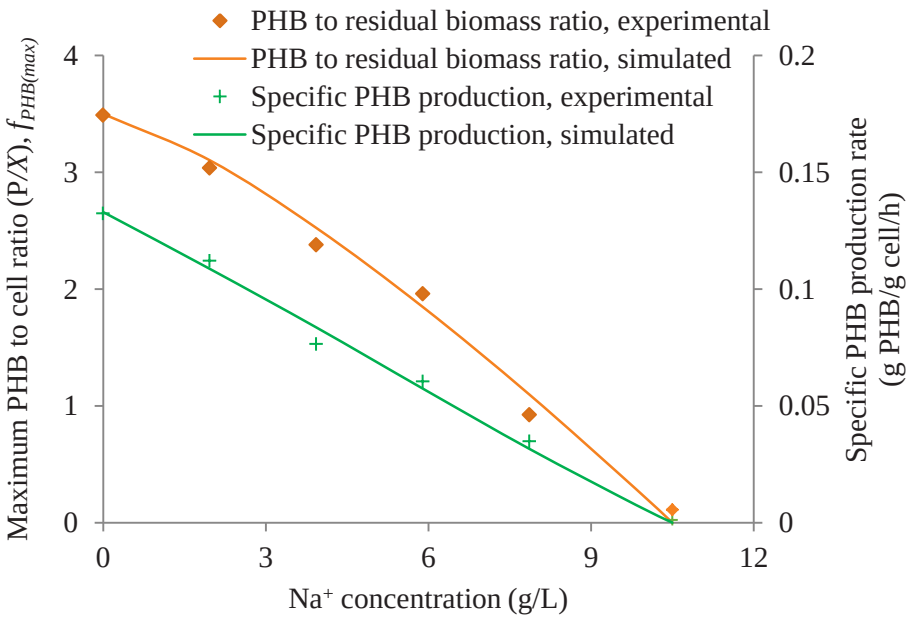


Figure 4.2. Effect of sodium concentration in mineral medium on specific PHB production rate (μ_p) and maximum PHB production capacity ($f_{PHB(max)}$).

4.4.3. *Model validation for cell growth (phase 1) using fed-batch fermentation*

A fed-batch experiment to grow *C. necator* was conducted using glucose as substrate and NaOH for pH control. Ammonium-nitrogen was controlled through feeding of $(NH_4)_2SO_4$

combined with glucose feeding. NaOH was fed to neutralize the produced protons (H^+) during biomass growth, resulting in the accumulation of Na^+ (Eq. 4.1). A biomass (CDM) concentration of 47.7 g/L and corresponding PHB production of 4.8 g/L, i.e. 10%, were achieved after 45 hours of cultivation(Figure 4.3, discrete markers).

The calibrated model described the experimental observations quite well (Figure 4.3); with corresponding E-values of 0.97 for RCC and of 0.87 for PHB.

The maximum biomass concentration (CDM) achieved at the end of the growth phase (47.7 g/L after 45 hours) was 37% lower than the one obtained under exactly the same conditions but applying NH_4OH instead of NaOH for pH control (73.5 g/L after 33 hours, Chapter 3), which can probably be attributed to the osmotic stress due to accumulated Na^+. The experimentally observed biomass growth stopped after 45 hours. At that time, the accumulated Na^+ concentration amounted to 9.58 g/L, which is close to the value 8.9 g/L determined from batch experiments, while all other conditions were favourable for growth.

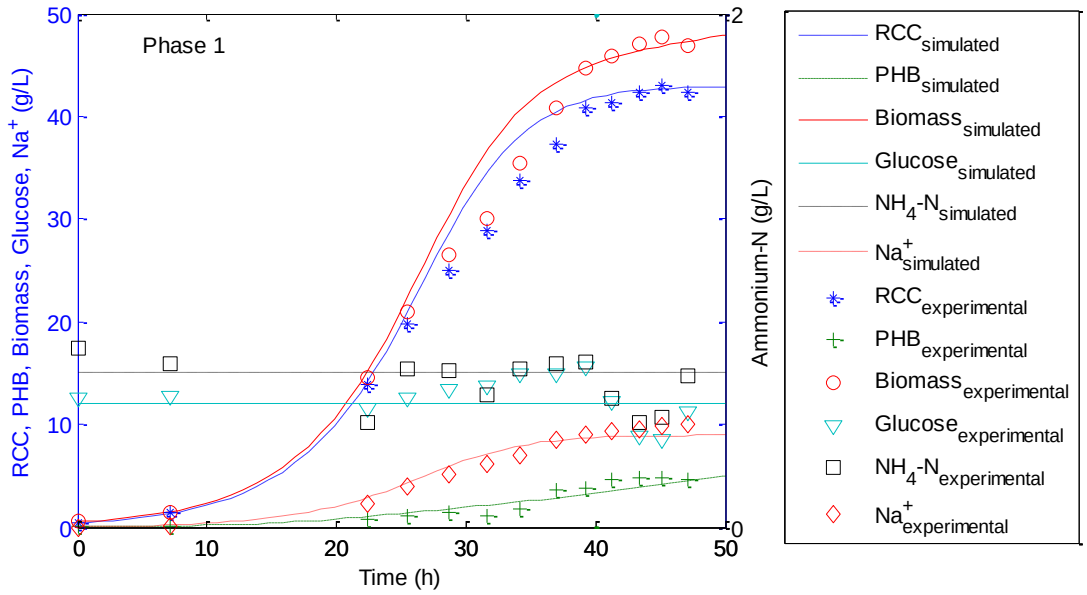


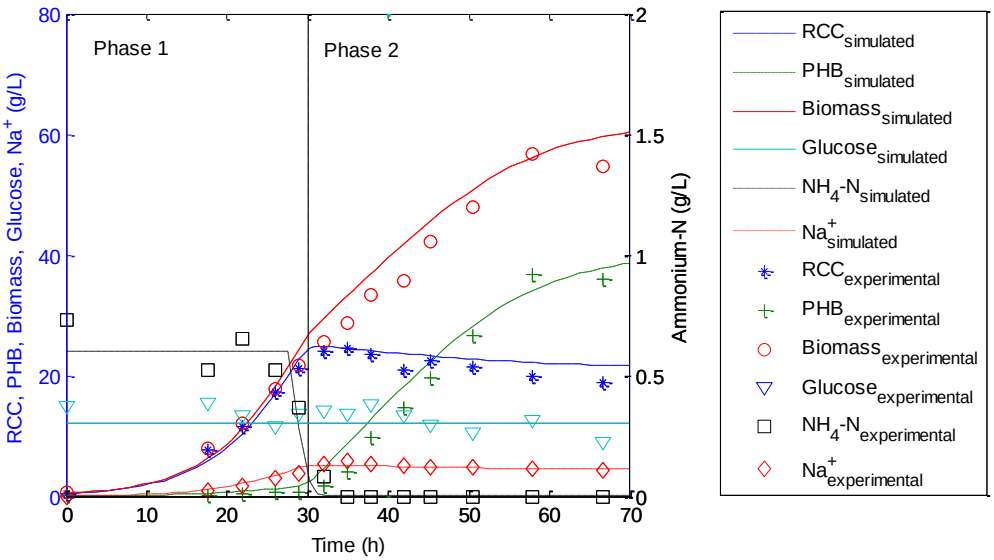
Figure 4.3. Model validation results for growth phase (phase 1). Comparison between simulation outcome (full lines) and experimental observations (discrete markers).

4.4.4. Model validation for cell growth and PHB production (phase 1 and 2) using fed-batch fermentation

Two distinct experiments were conducted involving both biomass growth and PHB production by *C. necator* using NaOH for pH control in the first phase. These experiments differed in the time instant at which nitrogen (N) limitation was imposed to enhance the PHB productivity. The switching point from phase 1 to phase 2 was set at 19 and 36 g/L residual cell concentration (RCC) for the first and second experiment, respectively (Figure 4.4). When stopping the nitrogen feed at RCC of 19 g/L, a maximal biomass (CDM) of 56.7 g/L and PHB productions of 36.8 g/L, corresponding with a PHB content of 64.8%, were obtained after 58 hours (Figure 4.4a). When applying nitrogen limitation at 36 g/L RCC, a maximal biomass concentration of 63.8 g/L and PHB production of 27.3 g/L, corresponding to 42.9% PHB content, were achieved after 77 hours (Figure 4.4b). For both datasets, the model predictions agreed very well with the experimental results in terms of residual biomass concentration (RCC; E-values 0.97 and 0.98 for switching point at 19 g/L and 36 g/L, respectively), PHB (E-values 0.95 and 0.92) and total biomass concentration.

Comparing the two datasets, the PHB content clearly decreased (from 64.8% to 42.9%) when delaying the shift to phase 2 (at RCC = 36g/L instead of 19 g/L, respectively), despite the higher residual biomass concentration produced (CDM = 63.8 g/L instead of 56.7 g/L, respectively). This was attributed to inhibition by the Na^+ which was added (as NaOH) for pH control during the growth phase and thus accumulated in the medium, inhibiting the subsequent PHB production step both in terms of specific PHB production rate (μ_{ps}) and PHB content ($f_{PHB(max)}$). This finding was earlier observed by Breedveld et al. (1993). These authors reported that Na^+ inhibition on PHB biosynthesis pathway, causing PHB degradation and increasing the cellular trehalose content, resulted in a decreased PHB content. When using NH_4OH instead of NaOH for pH control, the time instant at which the nitrogen limitation was imposed, hardly affects the PHB content: a 76.2% PHB content was obtained using glucose as substrate (Chapter 2).

(a)



(b)

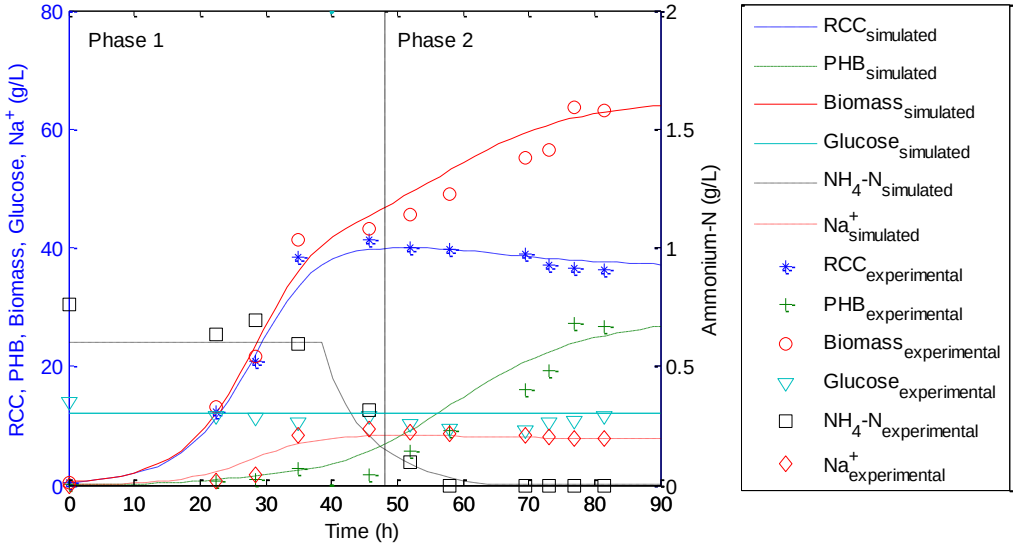


Figure 4.4. Model validation results for a complete fed-batch process of PHB production (phase 1 and 2). Comparison between simulation outcomes (full lines) and experimental observations (discrete markers) for two independent datasets, corresponding with a stop in the nitrogen feed at (a) 19 g/L and (b) 36 g/L residual biomass concentration (RCC).

Besides the addition of NaOH for pH control, also the substrate may provoke Na^+ inhibition. According to literature, a lower PHB content (56 to 67%) is obtained on waste glycerol (Cavalheiro et al., 2009; Ibrahim and Steinbuchel, 2009; Posada et al., 2011) than on glucose (74 to 76% PHB content) (Kim et al.,1994; Chapter 2). Mothes et al. (2007) found a decrease

of the PHB content from 70% (pure glycerol) to 48% by *C. necator* using 5.5% NaCl contained waste glycerol. This was attributed to the accumulation of Na^+ in the culture medium, which negatively affects the PHB accumulation metabolism of *C. necator* (Posada et al., 2011), and thus results in a decreased PHB content. Moreover, using waste glycerol as substrate, the PHB content decreased by delaying the time instant at which nitrogen (N) limitation was imposed because of increasing accumulation of Na^+ (Chapter 3). Seemingly contradictory, a recent study (Passanha et al., 2014) reported the improvement of PHB production using *C. necator* by the addition of NaCl medium. They obtained a PHB content of 61% on VFA as carbon source; the PHB content was increased to 80% in the presence of 2.56 g/L Na^+ but decreased to 20% upon further addition of Na^+ to 5.90 g/L. However, they used a nutrient medium, not containing any Na^+ while in this study mineral medium was used, already containing 1.69 g/L Na^+. The maximum PHB content of 78% obtained on the same mineral medium in this study is very close to the maximum PHB content of 80% obtained by Passanha et al. (2014) using a nutrient medium to which Na^+ was added to 2.56 g/L. However, since mineral medium is used more often than the more expensive nutrient medium, most authors reported a decreasing PHB production with addition of Na^+ (Cavalheiro et al., 2009, Mothes et al., 2007).

To overcome the Na^+ inhibition problem, Ibrahim and Steinbuchel (2009) suggested to use halophilic or moderate halophilic bacteria such as *Zobellella denitrificans* MW1 that can work under high Na-salt containing condition. They obtained a PHB content of 71% PHB in the presence of 20 g/L NaCl. However, the application of *Zobellella denitrificans* is hampered by its relatively high optimal temperature (41°C) and lower PHB productivity compared to *C. necator*.

4.5. Conclusion

- A mathematical model for heterotrophic PHB production was set up to evaluate the effect of sodium on biomass growth and PHB production. Shake flask experiments confirmed that both phases were inhibited by sodium due to osmotic stress. These experimental data were used for model calibration, yielding the inhibition parameters for both biomass growth and PHB production by *C. necator*. The model was subsequently validated based on fed-batch experiments.

- The inhibition of both biomass growth and PHB content by the addition of sodium to a mineral medium was characterized as non-linear, while the inhibition of the specific PHB production rate was found linear.
- As a result of inhibition through sodium added for pH control in the growth phase, a lower maximum PHB content and concentration were obtained by applying the nitrogen stress to stimulate PHB production at higher residual biomass concentrations, i.e. when delaying the shift from the growth phase to the PHB production phase.
- Inhibition of PHB production by sodium concentration may also be due to its presence in waste carbon sources. It is further influenced by the culture medium and differs among bacteria.

PART II:

Autotrophic-autotrophic PHB production

Chapter 5:

Polyhydroxybutyrate (PHB) production from CO_2: model development and process optimization

This chapter was published as:
Mozumder, M.S.I., Garcia-Gonzalez, L., De Wever, H., Volcke, E.I.P., 2015. Poly(3-hydroxybutyrate) (PHB) production from CO_2: model development and process optimization. Biochemical Engineering Journal 98, 107-116.

Abstract

The biosynthesis of poly(3-hydroxybutyrate) (PHB) directly from carbon dioxide (CO_2), is a sustainable alternative for non-renewable, petroleum-based polymer production. The conversion of CO_2 implies a reduction of greenhouse gas emissions. Hydrogen oxidizing bacteria such as *Cupriavidus necator* have the ability to store PHB using CO_2 as a carbon source, i.e. through an autotrophic conversion. In this study, a mathematical model based on mass balances was set up to describe autotrophic PHB production. The model takes into account the stoichiometry and kinetics of residual biomass growth and PHB formation as well as physical transfer from the gas phase to the liquid fermentation broth. The developed model was calibrated and validated based on independent experimental datasets from literature, obtained for *C. necator*. The obtained simulation results accurately described the dynamics of autotrophic biomass growth and PHB production. The effect of oxygen (O_2) and/or nitrogen stress conditions, as well as of the gas mixture composition in terms of O_2 and hydrogen (H_2) was investigated through scenario analysis. As major outcome, a higher maximum PHB production was obtained under oxygen stress conditions compared to nitrogen stress conditions. At high O_2 fractions in the gas mixture, which would result in H_2 limitation before O_2 limitation, PHB production can be increased by applying nitrogen stress. The effect of the reactor type was assessed through comparing a continuous stirred tank reactor (CSTR) with an air-lift fermentor. The developed model forms the basis for future design with minimum experimentation of suitable control strategy aiming at a high PHB production.

Keywords: CO_2, autotrophic cultivation, modeling, dynamic simulation, poly(3-hydroxybutyrate) (PHB), nutrient limitation, bioreactor configuration.

5.1. Introduction

CO_2 is the primary greenhouse gas (GHG) emitted through human activities, of which the combustion of fossil fuels for energy and transportation dominate about 90% of the total anthropogenic CO_2 emissions (Aresta and Dibenedetto, 2004; Oliver et al., 2013). The production of value-added chemicals from CO_2 feedstock could help to reduce the GHG emissions and close the carbon cycle. PHB production from CO_2 would be an example of sustainable future technologies aiming at saving natural resources and energy (Zakrzewska-Trznadel, 2011). To ensure PHB production from CO_2 is sustainable, H_2 should be produced from renewable energy sources.

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Poly(3-hydroxybutyrate) (PHB) is a biodegradable and bio-based plastic, synthesized by a variety of organisms as an intracellular storage material from renewable resources. Although it has the potential to substitute conventional plastics based on fossil fuels for a wide range of applications, PHB is still commercially behind petroleum-based synthetic plastics due to its high production cost. The factors affecting the economics of PHB include the costs for raw material and downstream processing as well as the lack of an optimal control strategy for the production process. To attain bulk commercial viability and to further improve the sustainability profile of PHB production, CO_2 could be used as a feedstock for PHB production by *Cupriavidus necator* (formerly known as *Alcaligenes eutrophus* (Davis et al., 1969), *Ralstonia eutropha* (Yabuuchi et al., 1995) and *Wautersia eutropha* (Vaneechoutte et al., 2004)). This model organism has a strong ability to accumulate PHB in either a heterotrophic or an autotrophic way, i.e. using organic substrate or CO_2 as a carbon source, respectively. In the latter case, C. *necator* is capable of producing PHB up to 80% of the dry cell weight, in a non-growth-associated manner, as demonstrated by Tanaka and Ishizaki (1994) and Tanaka et al. (1995). Two processes are distinguished, namely biomass growth and subsequent PHB production, which are realized in two distinct phases to achieve a high PHB production and PHB content. The stoichiometry for biomass growth from CO_2 (phase 1) was determined by Ishizaki and Tanaka (1990) as:

$$21.36\ H_2 + 6.21\ O_2 + 4.09\ CO_2 + 0.76\ NH_4^+ \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.7\ H_2O + 0.76\ H^+ \quad (5.1)$$

in which $C_{4.09}H_{7.13}O_{1.89}N_{0.76}$ represents the elemental composition of *C. necator* (without PHB). Ammonium (NH_4^+) is used as a nitrogen source for biomass growth. Under stress conditions, i.e. under nutrient (nitrogen (Tanaka et al., 2011; Volova and Kalacheva, 2005; Volova et al., 2013b)) or oxygen (Tanaka et al., 1995; Ishizaki et al., 1993) limitation, PHB production is stimulated. The stoichiometric equation for autotrophic PHB production (phase 2) was found as (Ishizaki and Tanaka, 1991):

$$33\ H_2 + 12\ O_2 + 4\ CO_2 \rightarrow C_4H_6O_2 + 30\ H_2O \quad (5.2)$$

in which $C_4H_6O_2$ represents the chemical composition of PHB monomer.

Most studies regarding PHB biosynthesis from CO_2 used a conventional fermentation set-up with continuous feeding of a gas mixture consisting of H_2, O_2 and CO_2, while the exhaust gas was either discharged or recycled (Ishizaki and Tanaka, 1991; Takeshita and Ishizaki, 1996; Volova and Voinov, 2003). Achieving a high density of PHB producing bacteria through autotrophic cultivation is not easy due to the low solubility of gases, which causes the gas transfer to the liquid phase to be the limiting factor for biomass growth as well as for PHB production. Increasing the mass transfer coefficient of gases (CO_2, H_2 and O_2) leads to both a higher biomass production (expressed in g/L), a higher PHB production (in g/L) and productivity (in g/L/h) (Takeshita and Ishizaki, 1996). Process optimization in terms of PHB production as well as productivity indeed required to make the production of PHB economically attractive in comparison with petrochemical plastics.

So far, most experimental work has been conducted in view of optimizing PHB production through autotrophic fermentation. The focus of these experimental studies was to assess the influence of O_2 and nitrogen stress conditions on the PHB productivity, to determine the process stoichiometry (Ishizaki and Tanaka, 1991), to identify physical and kinetic parameters affecting the process, such as the kinematic viscosity, density, surface tension, heat and mass transfer coefficient (Volova and Voinov, 2003), to evaluate the effect of mass transfer on biomass and PHB production (Takeshita and Ishizaki, 1996), to assess the potential of producing poly(hydroxyalkanoate) (PHA) copolymers from CO_2 (as the main carbon source) combined with organic substrates (Volova et al., 2013b; Volova et al., 2004) and to increase the autotrophic PHB production (g/L) and productivity (g/L/h) using a basket type agitation system (Tanaka et al., 1995) or air-lift fermentor (Taga et al., 1997). Overall, it is clear that a better process understanding and optimization are required to make autotrophic PHB production successful in the future.

Modeling and simulation are useful tools in view of optimizing PHB production processes. Concerning heterotrophic biomass growth and PHB production by *C. necator*, a number of models are available in literature. Some are based on a simplified metabolic reaction for a single substrate (Spoljaric et al., 2013b) or mixed substrates (Spoljaric et al., 2013a), aiming to determine kinetic parameters and optimize the feeding strategy for fed-batch cultivation. A similar, single-substrate model was applied by Horvat et al. (2013) for the optimization of a continuous five-stage process. A complex metabolic network was considered by Lopar et al. (2013) to analyze the metabolic status in PHB producing cells within all steps of the latter process. Besides metabolic models, several macroscopic models for heterotrophic PHB production were proposed, to develop the substrate and nutrient feeding strategy (Khanna and

Srivastava, 2006; Patwardhan and Srivastava, 2008; Shahhosseini, 2004), to evaluate the effect of pH on biomass growth and PHB production (Faccin et al., 2012), or to study the growth and PHB production mechanism (Chapter 3).

An earlier model concerning autotrophic growth and PHB production was set up and validated to experimental data by Heinzle and Lafferty (1980). However, in their model, only nitrogen was considered, limiting biomass growth and inhibiting PHB production, while CO_2, H_2 and O_2 were not taken up as state variables and the influence of gas transfer was not considered. The latter features were included in the present study, allowing to describe the dynamics of and interaction between NH_4^+, CO_2, H_2 and O_2. The model was subsequently calibrated and validated based on literature data. It was proven a useful tool to gain insight in the process mechanisms and in view of process optimization. The effect of O_2 and/or nitrogen limitation on the PHB production was assessed. The composition of the gas mixture was optimized to ensure maximum PHB production. Finally, the influence of the reactor configuration was elaborated on.

5.2. Materials and methods

5.2.1. Stoichiometry and kinetics

The model for autotrophic PHB production took into account two main processes: (1) biomass growth and (2) PHB production. The model stoichiometry and kinetics are summarized in Table 5.1 and Table 5.2, respectively. The stoichiometric and kinetic parameter values are listed in the Appendix (Table S5.1). The stoichiometric coefficients were determined by the stoichiometric equations for growth (Eq. 5.1) and for PHB production (Eq. 5.2), which were based on the *overall* consumption of gaseous substrate and on the *overall* biomass and PHB production (Ishizaki and Tanaka, 1990; 1991). In this way, maintenance was lumped in the stoichiometry and therefore not considered as a separate process. The biomass was assumed to be composed of two components: residual biomass (residual cell concentration (RCC), denoted by *X*) and PHB (*P*), which were taken up as separate state variables.

The first process concerned residual biomass growth in the presence of gaseous substrates H_2, O_2 and CO_2, and ammonium-nitrogen (*N*) in the liquid phase. Residual biomass growth limitation by the substrates H_2, O_2 and CO_2 was described as Monod kinetics. Due to the low solubility of gases, these substrate concentrations were reasonably assumed not to be in the inhibiting range. Residual biomass growth was described to be limited by low ammonium-

nitrogen concentrations, while very high concentrations were assumed to have an inhibition effect, as is the case for heterotrophic growth (Belfares et al., 1995). The combined limitation and inhibition effect was modelled through Haldane kinetics for ammonium nitrogen concentration (Eq. 5.3 in Table 5.2), as in Lee et al. (1997). The second process, PHB production, was limited by low substrate (H_2, O_2 and CO_2) concentrations and inhibited by high O_2 concentration (O_2>K_{PIO2}) (Takeshita et al., 1993).

Table 5.1: Stoichiometry of the autotrophic PHB production model.

Component → Process ↓	H_2 (g/L)	O_2 (g/L)	CO_2 (g/L)	NH_4-N (g/L)	Residual biomass RCC) (*X*) (g/L)	PHB (*P*) (g/L)
1. Biomass growth	$-1/Y_{xH2}$	$-1/Y_{xO2}$	$-1/Y_{xCO2}$	$-1/Y_{xN}$	1	
2. PHB production	$-1/Y_{pH2}$	$-1/Y_{pO2}$	$-1/Y_{pCO2}$			1

Table 5.2: Kinetic expressions of the autotrophic PHB production model.

Process	Reaction rate
1. Biomass growth	$\rho_{xs} = \mu_{xs} X$ With $\mu_{xs} = \mu_{xs}^{max} \left(\frac{H_2}{K_{xH2}+H_2}\right)\left(\frac{O_2}{K_{xO2}+O_2}\right)\left(\frac{CO_2}{K_{xCO2}+CO_2}\right)\left(\frac{N}{K_N+N+N^2/K_{IN}}\right)$ (5.3)
2. PHB production	$\rho_{ps} = \mu_{ps} X$ With $\mu_{ps} = \mu_{ps}^{max} \left(\frac{H_2}{K_{pH2}+H_2}\right)\left(\frac{O_2}{K_{pO2}+O_2+\frac{O_2^2}{K_{pIO2}}}\right)\left(\frac{CO_2}{K_{pCO2}+CO_2}\right)\left[1-\left(\frac{f_{PHB}}{f_{PHB(max)}}\right)^{\beta}\right]\frac{K_{PIN}}{N+K_{PIN}}$ (5.4)

The effect of nitrogen and O_2 concentration on residual biomass growth and/or PHB production is schematically represented in Figure 5.1. High O_2 concentrations ($O_2 > K_{xO2}$) stimulate the residual biomass growth but inhibit PHB production. At an intermediate O_2 concentration ($K_{xO2} < O_2 < K_{PIO2}$) both residual biomass growth and PHB production take place (Figure 5.1a). Low O_2 concentration ($K_{pO2} < O_2 < K_{xO2}$) stimulates PHB production and limits the growth but very low O_2 concentration ($O_2 < K_{pO2}$) limits PHB production. The availability of nitrogen

source in the medium also determines which process takes place: high nitrogen concentration ($N>K_{IN}$) inhibits both residual biomass growth and PHB production, intermediate nitrogen concentration ($K_{IN}>N>K_N$) stimulates biomass growth (process 1) and low concentration ($N<K_{PIN}$) stimulates PHB production (process 2, note that K_{PIN} was assumed equal to K_N in this study, see Table S5.1 in Appendix). The PHB production process under nitrogen limitation was described as in Spoljaric et al. (2013a). It is clear that both nitrogen and oxygen concentration were control handles for PHB production. Note that it was assumed that the double limitation, by both nitrogen and O_2, could be described by combining the descriptions for single nitrogen or single O_2 limitation.

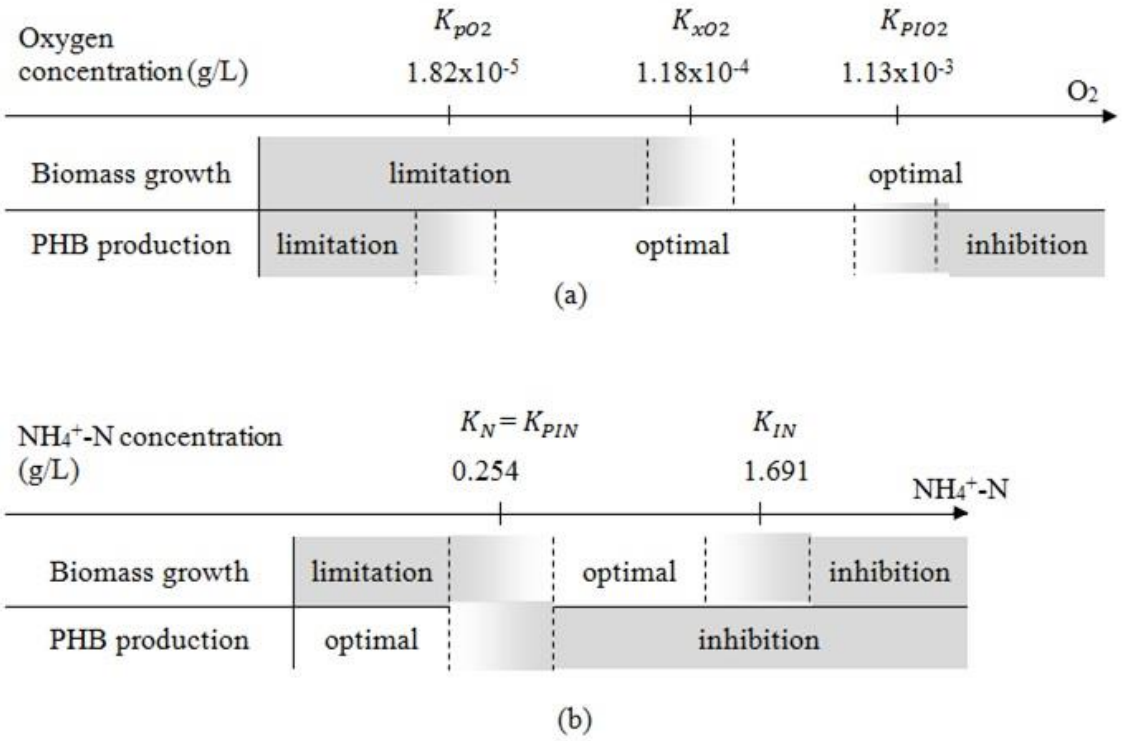

Figure 5.1. Influence of oxygen (a) and nitrogen (b) concentration on biomass growth and PHB production.

Fermentation end products are known to negatively affect microbial activities, which was described in the model through modified logistic kinetics (Mulchandani and Luong, 1989). The saturation of PHB resulted in a decreasing PHB formation (described by Eq. 5.4 in Table 5.2), implying that the cells were not capable to produce PHB in an unlimited way but that the specific PHB production rate approached zero as PHB to active biomass ratio (f_{PHB}) approached its maximum, $f_{PHB(max)}$. The value β=3.85 determined by Dias et al. (2005; 2006) for PHB production through mixed cultures and used in Chapter 3 for pure culture PHB production was also applied in this study.

5.2.2. Mass balances

Mass balances were set up for the gaseous substrates (H_2, O_2, CO_2) and nitrogen concentrations in the fermentor, from which substrate and nitrogen concentrations were subsequently determined (Eqs. 5.5-5.8), given that the nitrogen concentration was maintained at a constant (optimal) level during the growth phase. In the overall process, only a very small amount of N containing solution was needed for biomass growth (during phase 1), while there was no outgoing stream. Therefore the liquid volume in the process was assumed constant.

$$\frac{dH_2(t)}{dt} = k_L a_{H2}(H_2^* - H_2) - \left(\frac{\mu_{xs}}{Y_{xH2}} + \frac{\mu_{ps}}{Y_{pH2}}\right)X \tag{5.5}$$

$$\frac{dO_2(t)}{dt} = k_L a_{O2}(O_2^* - O_2) - \left(\frac{\mu_{xs}}{Y_{xO2}} + \frac{\mu_{ps}}{Y_{pO2}}\right)X \tag{5.6}$$

$$\frac{dCO_2(t)}{dt} = k_L a_{CO2}(CO_2^* - CO_2) - \left(\frac{\mu_{xs}}{Y_{xCO2}} + \frac{\mu_{ps}}{Y_{pCO2}}\right)X \tag{5.7}$$

$$\frac{dN(t)}{dt} = \frac{F_N N_F}{V} - \frac{\mu_{xs}}{Y_{xN}}X = 0 \;\Leftrightarrow\; F_N = \left(\frac{1}{N_F}\right)\left(\frac{\mu_{xs}}{Y_{xN}}\right)XV \tag{5.8}$$

$H_2^*,\ O_2^*,\ CO_2^*$ represent the equilibrium liquid phase concentrations corresponding with the gas phase composition of H_2, O_2, CO_2 respectively as expressed by Henry's law (Eq. 5.9). The solubility of a gas (C^* ($H_2^*,\ O_2^*,\ CO_2^*$), g/L) is the inverse of Henry's constant (k_H, atm/g/L), multiplied by the partial pressure of the gas (P_g, atm).

$$C^* = P_g / k_H \tag{5.9}$$

The Henry's constant (k_H) of each gas was calculated from the gas solubility at standard conditions (pure gases at 30°C and 1 atm pressure (Dean, 1985), see Appendix – Table S5.2).
The overall volumetric mass transfer coefficients for H_2 ($k_L a_{H2}$) and for CO_2 ($k_L a_{CO2}$) were calculated from that of O_2 ($k_L a_{O2}$) according to Eq (5.10) (Ishizaki et al., 2001) and Eq (5.11) (de Heyder et al., 1997) respectively.

$$k_L a_{H2} = 0.280(k_L a_{O2})^{1.29} \tag{5.10}$$

$$k_L a_{CO2} = \sqrt{\frac{D_{lCO2}}{D_{lO2}}}\, k_L a_{O2} \quad (5.11)$$

in which D_{lCO2} is the diffusion coefficient for CO_2 (1.77×10^{-5} cm^2/s (Treybal, 1955)) and D_{lO2} is the diffusion coefficient for O_2 (2.50×10^{-5} cm^2/s (de Heyder et al., 1997)).

The residual biomass (RCC) (X) and PHB (P) concentration profiles were obtained from their respective mass balances.

$$\frac{dX}{dt} = \mu_{xs} X \quad (5.12)$$

$$\frac{dP}{dt} = \mu_{ps} X \quad (5.13)$$

5.2.3. Model calibration and validation

In view of parameter estimation, an objective function ($J(\theta)$) was defined to obtain the best possible fit between the model predictions and experimental data taken from literature, as obtained by minimizing the sum of squared errors (Eq. 5.14):

$$J(\theta) = \sum_{i=1}^{n} \left(y_i(t) - y_i^m(t,\theta)\right)^2 \quad (5.14)$$

$y_i(t)$ represents the experimental data observations, in this case residual cell (RCC) and PHB production, while $y_i^m(t,\theta)$ denotes the model predictions corresponding with the given parameter set θ at time t. During the model calibration, the 'Nelder-Mead simplex direct search' estimation algorithm (Nelder and Mead, 1965), an unconstrained nonlinear optimization method, was used.

During model validation, the model predictions were compared with two independent experimental datasets taken from literature. For this purpose, the Nash-Sutcliffe model efficiency coefficient (E) (Nash and Sutcliffe, 1970) was used to quantitatively describe the accuracy of model outputs and in this way assess the predictive power of the model.

$$E = 1 - \frac{\sum_{i=1}^{n} \left(y_i(t) - y_i^m(t)\right)^2}{\sum_{i=1}^{n} (y_i(t) - \bar{y})^2} \quad (5.15)$$

Nash–Sutcliffe efficiency coefficients range from -∞ to 1. A value $E = 1$ corresponds to a perfect match of the model outcome to the observed data. An efficiency of 0 ($E = 0$) indicates that the model predictions are as accurate as the mean of the observed data, whereas an efficiency less than zero ($E < 0$) indicates that the observed mean is a better predictor than the model. Essentially, the closer the model efficiency is to 1, the more accurate the model is.

5.3. Results and discussion

The developed model was calibrated and validated based on three distinct experimental datasets, differing in operating conditions and taken from literature (Ishizaki and Tanaka, 1991). In the experiment used for model calibration, PHB production was triggered applying O_2 limitation. Two other datasets were used for model validation; the first one concerned PHB production under nitrogen limitation and the second one used a gas mixture containing a relatively high O_2 fraction. Different scenarios were analysed to find out the optimal balance between O_2 and nitrogen stress conditions in view of maximal PHB production, i.e. the final PHB production (in g/L), and maximal PHB productivity, i.e. the PHB production rate over the whole period of the experiment (in g/L/h). The effect of the gas composition (O_2 fraction) on PHB production was evaluated. Finally the effect of reactor configuration was elaborated on, thus completing the overview of experimental data on autotrophic PHB production available in literature.

5.3.1. Model calibration

To describe autotrophic biomass growth and PHB production, the developed model was first calibrated on experimental data (Figure 5.2, discrete markers, (Ishizaki and Tanaka, 1991)) in which O_2 limitation was applied to stimulate the PHB production. A gas mixture of O_2:H_2:CO_2 = 15:75:10 was used as substrate. The initial nitrogen concentration in the medium was set to 1.06 g/L NH_4^+-N (using 5 g/L $(NH_4)_2SO_4$). Nitrogen sufficient condition was maintained using 4% ammonium water that was used to control the pH. The microorganism grew at a high specific growth rate from an initial biomass concentration of 0.30 g/L until the dissolved O_2 concentration in the culture broth became insufficient for residual biomass growth. This condition of O_2 limitation was established after 24 hours, leading to enhanced PHB accumulation, which implies the start of phase 2. Note that residual biomass growth continued

even in the PHB production phase (see RCC in Figure 5.2a). At the end of the experiment approximately 50 g/L biomass (CDM) with 53% PHB content was produced.

Most model parameter values were taken from literature (Table S5.1 in Appendix). The maximum PHB to active biomass ratio, $f_{PHB(max)}$, was calculated based on literature concerning autotrophic growth in a CSTR type bioreactor (Ishizaki and Tanaka, 1991; Tanaka et al., 1995). No literature values were available for the maximum specific autotrophic PHB production rate and the saturation constant of O_2 for PHB production, which were estimated through model calibration as μ_{ps}^{max} = 0.26 g PHB/g cell/h and K_{pO2} =1.82x10^{-5} g O_2/L, respectively. The saturation constants for CO_2 in both growth (K_{xCO2}) and PHB production (K_{pCO2}) were both set equal to the saturation constant for O_2 during growth, K_{xO2} = 1.18x10^{-4} g CO_2/L. Note however that the values of K_{xCO2} and K_{pCO2} were not sensitive to the model for sufficiently high CO_2 concentration, which prevail in all available literature reports on autotrophic PHB production (typical CO_2 concentration in liquid medium >0.1 g/L>> K_{xO2}, for about 10% CO_2 the in gas mixture). The volumetric mass transfer coefficient for O_2 ($k_L a_{O2}$) was assumed as 340 h^{-1}, determined by Tanaka and Ishizaki (1994) using a similar fermentor (typical CSTR) and operating conditions as Ishizaki and Tanaka (1991). Given the insignificant change of viscosity with a high cell concentration and intracellular PHB production (Mulchandani and Luong, 1989; Volova and Voinov, 2003), the change of $k_L a_{O2}$ with biomass growth was neglected. The total pressure inside the fermentor was assumed to be 1.5 atm, considered as maximum allowable level for a glass jacket fermentor (Tanaka et al., 1995). The initial biomass and nitrogen concentrations were set to their experimental values. The initial concentrations of O_2, H_2 and CO_2 in the culture broth were determined as their saturation concentration corresponding with their partial pressure.

Figure 5.2 compares the calibrated model output with the experimental observations. In this model all the parameter values associated with biomass growth (Eq. 5.3) were taken from available literature (see Table S5.1). Nevertheless, the simulation results agreed well with the experimental data (Figure 5.2), which is also reflected by values of the Nash-Sutcliffe model efficiency coefficients € of 0.92 and 0.96 for RCC and PHB, respectively. At the end of the experiment, the biomass growth rate decreased for an unclear reason, an observation which was not described by the model.

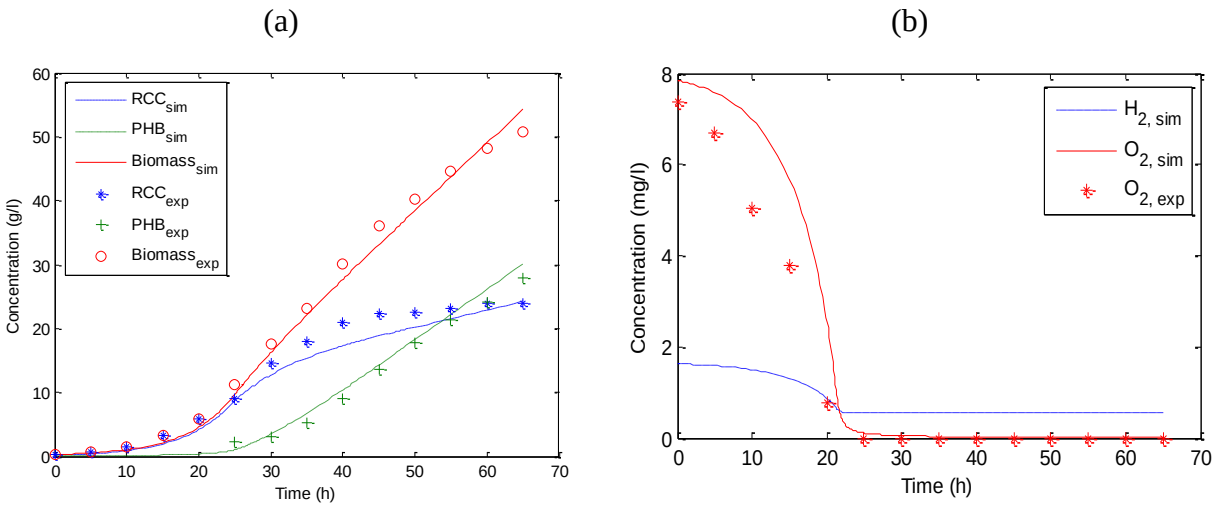

Figure 5.2. Model calibration results for autotrophic biomass growth, PHB production (a) and oxygen and hydrogen concentration profile in medium (b). Comparison between the simulation outcome and experimental observations from Ishizaki and Tanaka (1991) under the gas composition O_2:H_2:CO_2=15:75:10 and oxygen stress conditions to stimulate the PHB production.

5.3.2. Model validation

Two distinct experimental datasets were used for model validation. In the first one, nitrogen stress was applied to stimulate the PHB production while O_2 concentration was kept around 2.9 mg/L during the PHB production phase (Figure 5.3a, (Ishizaki and Tanaka, 1991)). The exact composition of the gas mixture and the procedure for maintaining a sufficiently high O_2 concentration in phase 2 were not given for this experiment. However, as the data originated from the same source as the one used for model calibration, the same gas composition (O_2:H_2:CO_2=15:75:10) was applied also for this simulation run. Once the O_2 concentration in the liquid phase reached 2.9 mg/L, it was set constant in the simulation, in accordance with the available experimental data. The initial ammonium-nitrogen concentration in the medium was 1.06 g/L NH_4^+-N (≈5 g/L $(NH_4)_2SO_4$); no additional ammonium was supplied. After a while the available ammonium was consumed due to biomass growth, resulting in a N-limiting condition, which suppressed residual biomass growth and stimulated PHB production. The biomass concentration (CDM) increased from an initial concentration of 0.45 g/L to 27 g/L after 80 hours of cultivation, with a PHB production of 16 g/L (Figure 5.3a).

The developed model predicts the experimental observations quite well, with Nash–Sutcliffe model efficiency coefficients (E) of 0.98 for RCC and 0.91 for PHB, which were close to 1 and thus indicated a very good model fit. So, although the parameters related to nitrogen limitation and inhibition on biomass growth and PHB production were taken from Chapter 3 describing a heterotrophic process, they also appeared to be very well applicable for autotrophic conditions. The second dataset for model validation concerned an experiment conducted with a gas mixture containing a higher O_2 concentration (O_2:H_2:CO_2=25:65:10) than the first one, while maintaining nitrogen sufficient conditions (Ishizaki and Tanaka, 1991). The main aim of the increased O_2 concentration was to increase the O_2 transfer rate as well as the PHB productivity. But the experiment resulted in poor PHB production (Figure 5.3b), which was attributed to limitation of H_2 before O_2. The latter hypothesis of Ishizaki and Tanaka (1991) was confirmed here by the model simulation, which matched the experimental observations very well with E-values of 0.93 and 0.97 for RCC and PHB respectively.

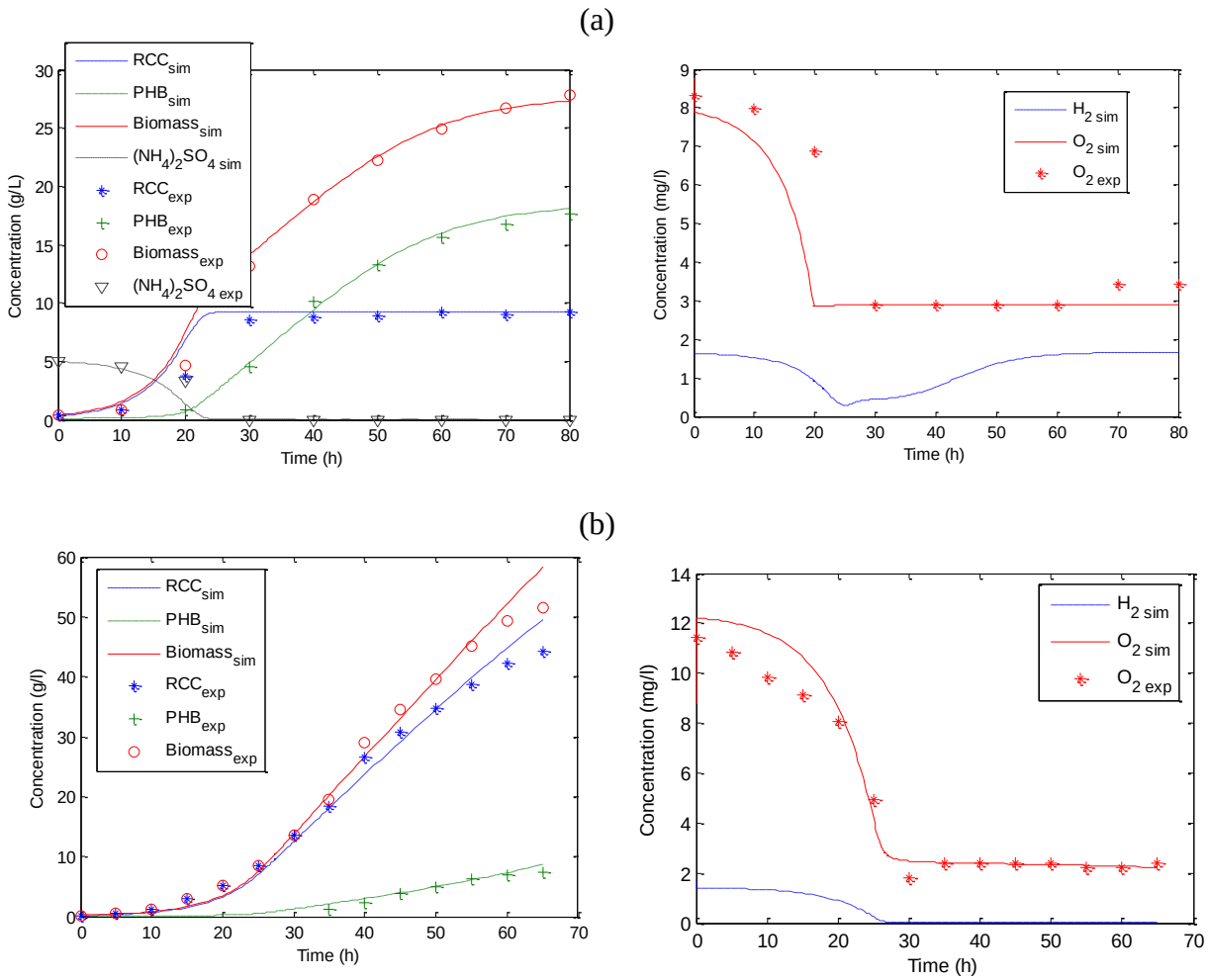

Figure 5.3. Model validation results for autotrophic biomass growth, PHB production and bulk oxygen and hydrogen concentration. Comparison between the simulation outcome (full lines) and experimental observations from Ishizaki and Tanaka (1991) (discrete markers) for two distinct cases: either applying nitrogen limitation and oxygen sufficient condition to stimulate the PHB production (a) or applying a gas composition of O_2:H_2:CO_2=25:65:10 (b).

5.3.3. Effect of oxygen and/or nitrogen stress conditions

The validated model was applied for scenario analysis, to determine the optimal operating conditions for maximum PHB production. The effect of both O_2 stress conditions at different nitrogen concentrations and nitrogen stress conditions at various O_2 levels were simulated. In all cases, a O_2:H_2:CO_2 gas mixture of 15:75:10 was used, while the initial NH_4^+-N concentration was set at 1.06 g/L and the initial RCC concentration was 0.30 g/L. The volumetric O_2 mass transfer coefficient ($k_L a_{O2}$) was set at 340 h^{-1}. For the scenarios concerning

O_2 stress, the time instant at which O_2-limitation occurred was determined by the O_2 content of the gases, which at a given moment became insufficient for growth of the increasing biomass concentration (Figure 5.4, left). As for the nitrogen limiting conditions, biomass growth was suppressed and PHB production was stimulated as soon as the initial NH_4^+-N concentration was consumed due to biomass growth (Figure 5.4, right).

Under O_2 stress, PHB production and productivity increased with decreasing nitrogen concentrations in the medium from 1.06 g/L to 0.5 g/L (Figure 5.4, left; Table S5.3), which demonstrates that nitrogen limitation is an additional control handle to trigger PHB accumulation. However, when further decreasing the nitrogen concentration to 0.01 g/L, both PHB production and productivity decreased (Figure 5.4, left) since very low nitrogen concentrations also limit biomass growth. The PHB production kept increasing in time under O_2 stress conditions due to continued growth of active biomass, also in the PHB production phase (Figure 5.4).

Applying nitrogen stress conditions, biomass growth was suppressed in the PHB production phase, resulting in a fixed maximum PHB production (17 g/L) at various O_2 concentrations, although the production rate increased with decreasing O_2 concentration in the medium (Figure 5.4, right). The overall PHB yield was higher under nitrogen stress conditions than under O_2 stress conditions (Table S5.3 in Appendix), because nitrogen limitation causes biomass growth to stop and substrates to be used for PHB production only.

The continued growth of resudial biomass in the PHB production phase under oxygen stress conditions resulted in higher cell density compared to nitrogen stress condition, leading to a higher maximum PHB production. This confirms the experimental findings of Ishizaki and Tanaka (1991), who found 27 g/L PHB production under oxygen stress conditions after 60h of cultivation whereas under nitrogen stress conditions the maximum PHB production was 16 g/L after 80h of cultivation. The maximum PHB productivity was found at 0.5 g/L nitrogen concentration, at which an intermediate growth rate (slope of RCC) was maintained. This is in agreement with the observations concerning heterotrophic PHB production of Grousseau et al. (2013), who found the maximum PHB production to correspond with intermediate values of the specific biomass growth rate.

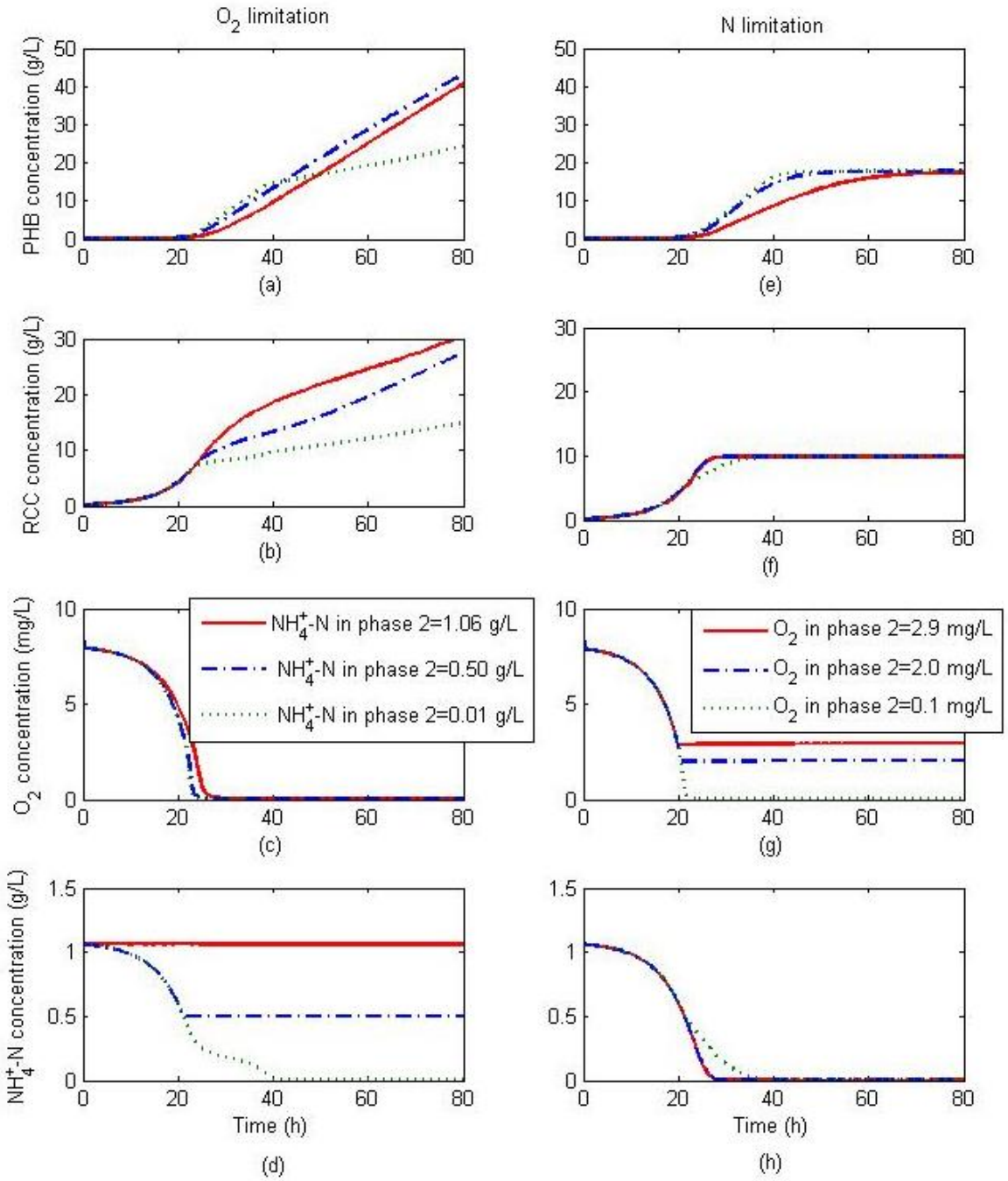

Figure 5.4. Scenario analysis concerning the effect of oxygen stress (left) and nitrogen stress (right) on PHB production.

5.3.4. Effect of gas mixture composition (oxygen fraction) on PHB production

Achieving a high cell density (RCC) is a prerequisite for maximum PHB productivity (Ienczak et al., 2013) and can be achieved by increasing the limiting substrate namely O_2. Given that all gaseous substrates need to be fed into the reactor and that the gas composition influences the

mass transfer from gas to liquid phase, it is relevant to study the effect of the gas mixture composition on PHB production and productivity.

To determine the optimal gas mixture composition leading to maximum PHB production under oxygen stress conditions, simulations were conducted for various O_2 and H_2 concentrations, while keeping the nitrogen concentration fixed (at 1.06 g/L NH_4^+-N) (Figure 5.5, left). The RCC concentration increased with increasing O_2 fraction while the PHB production and productivity increased with increasing O_2 fraction from 15% to 20% and decreased when further increasing the O_2 fraction to 25%. The low PHB production at low O_2 fraction was due to a low O_2 transfer rate resulting in O_2 limitation on PHB production ($O_2 < K_{pO2}$). By increasing the O_2:H_2 ratio to 2:7 (20% O_2 and 70% H_2 in Figure 5.5), the O_2 transfer rate as well as RCC concentration increased to obtain the maximum PHB production and productivity. The corresponding O_2 concentration in the culture medium during the PHB production phase was around 3.17×10^{-5} g/L, which fulfilled K_{pO2} (1.182×10^{-5} g/L)<O_2 < K_{xO2} (1.18×10^{-4} g/L)< K_{pIO2} and thus stimulated the PHB production (see Figure 5.1). Note that, for heterotrophic PHB production with the same organism, Lefebvre et al. (1997) found that the optimal O_2 concentration in the culture medium, corresponding to maximum PHB production, was between 7.60×10^{-5} to 3.04×10^{-4} g/L. Further increasing the O_2 fraction at the expense of H_2 decreased the PHB production because H_2 limitation was obtained before O_2 limitation. Even though keeping relatively high O_2 concentration was advantageous for active biomass growth, a too high O_2 concentration should be avoided in view of optimal PHB production. This phenomenon was experimentally confirmed by Ishizaki and Tanaka (1991) using O_2:H_2=25:65. In this study it was found that the maximum PHB production under O_2 stress conditions was obtained at O_2:H_2=2:7. Note that this gas composition O_2:H_2:CO_2=2:7:1 was also applied by Takeshita and Ishizaki (1996) to ensure sufficient O_2 and H_2 for biomass growth.

The effect of the oxygen and hydrogen fractions in the gas mixture was also evaluated while applying nitrogen stress to stimulate the PHB production (Figure 5.5, right). The NH_4^+-N concentration was kept constant at the initial level of 1.06 g/L until a concentration of 25 g/L residual biomass was reached, after which the NH_4^+-N feeding was stopped. The maximum PHB production and productivity under N-stress were obtained for a gas mixture composition of O_2:H_2=22:68. At O_2:H_2=25:65, the PHB production and productivity decreased due to a relatively lower H_2 transfer rate. An O_2 fraction below 20% resulted in a lower PHB production because of a lower O_2 transfer rate. The overall PHB yields were higher under nitrogen stress conditions than under O_2 stress conditions (Table S5.4 in Appendix), as was also found when applying different O_2 and N_2 concentration levels (Table S5.3).

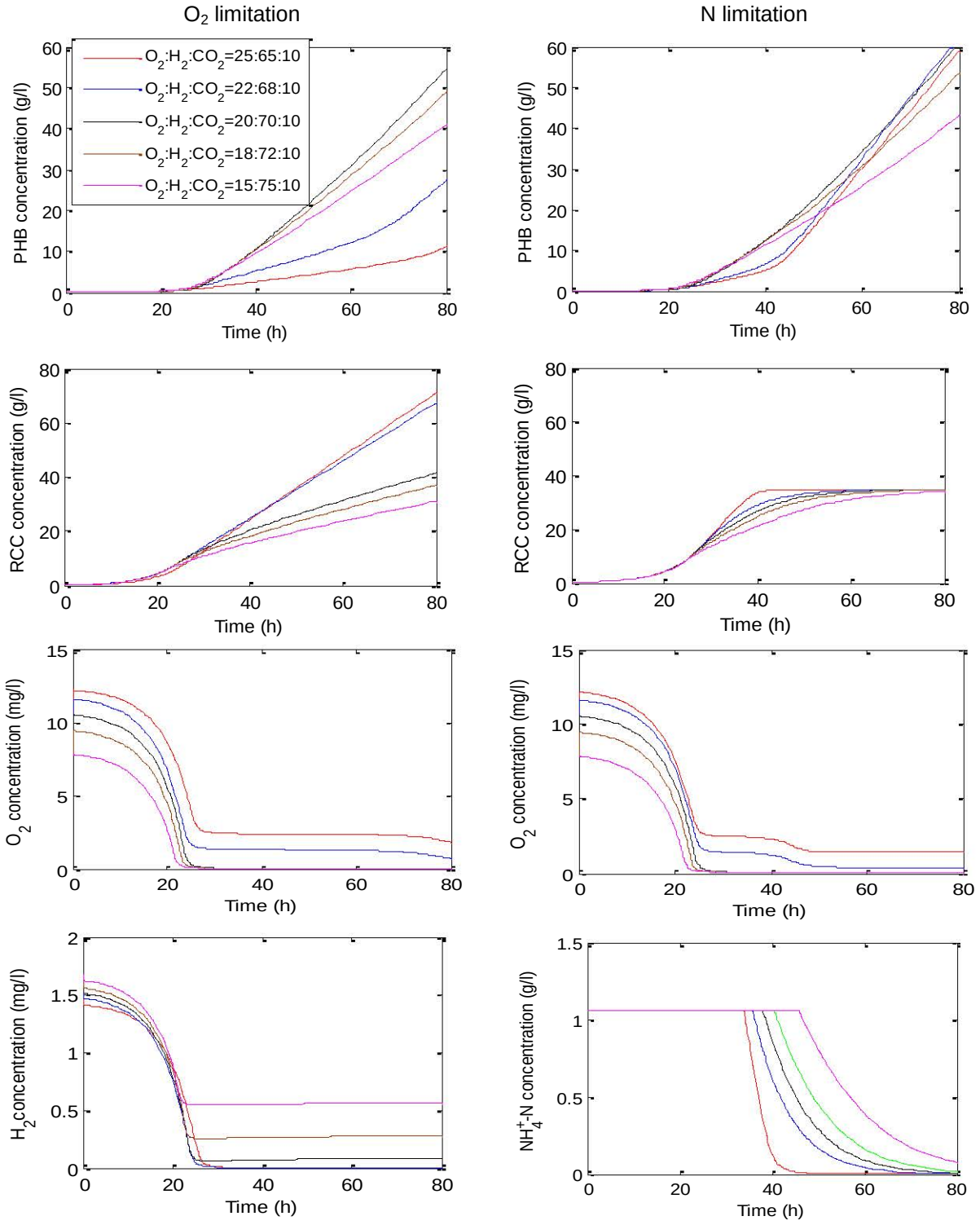

Figure 5.5. Scenario analysis concerning the influence of gas composition on PHB production, under oxygen stress (left) and nitrogen stress (right).

Comparing the results obtained for a high O_2 fraction (O_2:H_2=25:65) under nitrogen stress and O_2 stress, it is clear that the application of nitrogen stress increases the PHB production even at

high O_2 fractions causing H_2 limitation when applying only O_2 stress to trigger PHB production. This was confirmed experimentally by Volova and Kalacheva (2005) and Volova et al. (2003), who conducted autotrophic cultivations with a high O_2 fraction (O_2:H_2=2:6) and produced 63% PHB content applying nitrogen stress.

5.3.5. Effect of reactor configuration

Most literature references dealing with autotrophic PHB production considered a continuous stirred tank reactor configuration, except Taga et al. (1997), who used an air-lift fermentor to get high PHB production. In their experiment a gas mixture with composition O_2:H_2:CO_2=5:85:10 was supplied to the fermentor under 30 kPa overpressure at a $k_L a_{O2}$ of 250 h^{-1}. The pre-culture was grown on fructose as carbon source and then used for autotrophic PHB production, leading to a lag phase of around 10 hours. The growth of active biomass was stopped at 10 g/L cell concentration (RCC), while PHB production was stimulated. Even though the exact stress condition was not mentioned by the authors, it was assumed to be N-limitation since no residual biomass growth was observed in the PHB production phase.

After 120h, PHB production reached 49.2 g/L corresponding to 82% PHB content. A simulation was conducted considering the 10h lag phase and 82% PHB content and the outcome was compared with experimental results of Taga et al. (1997). Figure 5.6 shows that the developed model was able to describe the process kinetics very well with E value very close to 1 (0.93 for RCC and 0.98 for PHB), confirming the applicability of the model for different fermentor types. Although the reason behind the high PHB content in the air-lift fermentor was not elaborated on by the authors, the model is able to predict the results by only adapting the parameter value of maximum PHB to active biomass ratio ($f_{PHB(max)}$). In view of process optimization for other reactor types, further understanding of the relation between the metabolic activities and the reactor design is required. Relationships between the maximum PHB content $f_{PHB(max)}$ and the reactor type could be established.

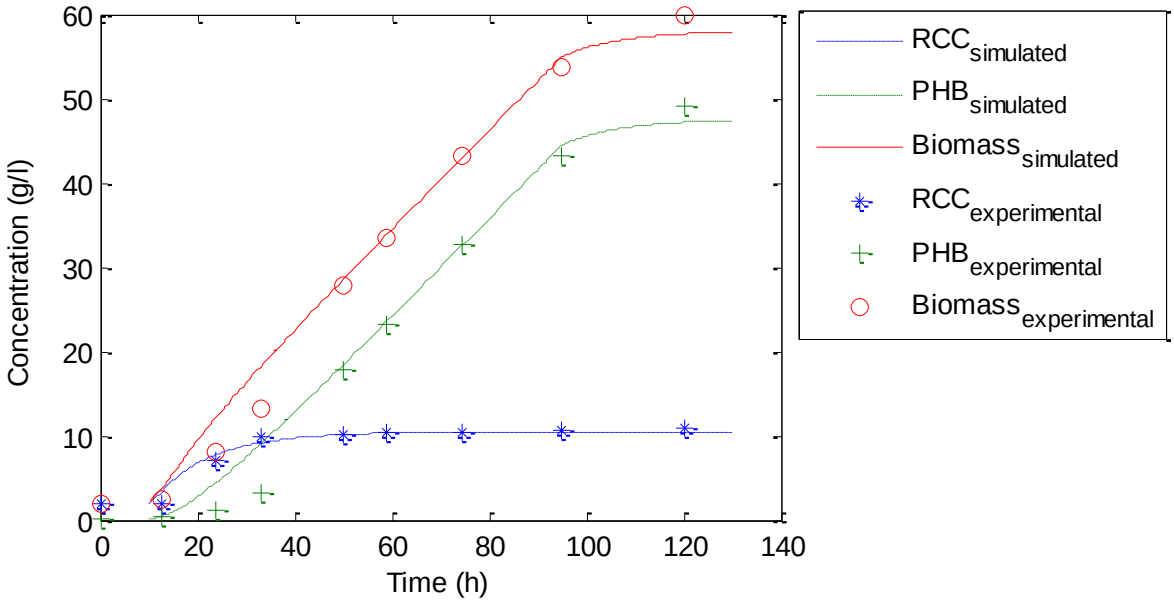

Figure 5.6. Model adaptation for air-lift fermentor with comparing the simulation outcome and experimental observations from Taga et al. (1997) using nitrogen limiting condition.

5.3.6. Potential of autotrophic versus heterotrophic PHB production

The potential of autotrophic versus heterotrophic PHB production was assessed comparing the maximum specific biomass growth rate (μ_{xs}^{max}), maximum specific PHB production rate (μ_{ps}^{max}) and PHB content ($f_{PHB(max)}$) for autotrophic and heterotrophic PHB production (Table 5.3). The maximum specific biomass growth rate was higher under heterotrophic condition while the maximum specific PHB production rate was slightly higher under autotrophic condition. Using a CSTR type reactor, the obtained PHB content ($f_{PHB(max)}$) was higher for heterotrophic compared to autotrophic culture, while an exceptionally high PHB content was reported by Tage et al. (1997) during autotrophic PHB production in an air-lift fermentor. Moreover, autotrophic PHB production implies a CO_2 reduction, which constitutes an additional advantage compared to heterotrophic PHB production. Given the high specific heterotrophic biomass growth rate, the high specific autotrophic PHB production rate and the advantages of using CO_2 as a feedstock, an alternative cultivation method consisting of heterotrophic biomass growth (phase 1) and autotrophic PHB production (phase 2), seems to warrant further investigation.

Table 5.3: Comparison between autotrophic and heterotrophic PHB production processes.

Parameter → / Process ↓	Maximum specific biomass growth rate, μ_{xs}^{max} (g cell/g cell/h)	Maximum specific PHB production rate, μ_{ps}^{max} (g PHB/g cell/h)	Maximum PHB to active biomass ratio $f_{PHB(max)}$
Autotrophic	0.29 (Siegel and Ollis, 1984)	0.26 (This study)	For CSTR: 1.78±0.32 (Average value, from Ishizaki and Tanaka, (1991); Tanaka et al. (1995)) For air-lift fermentor: 4.5 (Taga et al. 1997)
Heterotrophic	0.41- 0.82 (Horvat et al., 2013; Shahhosseini, 2004; Chapter 3)	0.21- 0.25 (Horvat et al., 2013; Chapter 3)	For CSTR reactor: 3.3 (Chapter 3)

5.4. Conclusions

- A mathematical model for autotrophic PHB production was developed and was calibrated and validated using literature data.
- Both biomass growth and PHB production needed O_2 as substrate, but low O_2 concentrations stimulated PHB production while high concentrations inhibited it.
- The continued growth of residual biomass in the PHB production phase under O_2 stress conditions resulted in higher cell density compared to nitrogen stress conditions, leading to a higher PHB production.
- The optimal O_2:H_2 ratio for maximum PHB production under O_2 limiting conditions was 2:7.
- When applying O_2 stress to trigger PHB production, the addition of nitrogen stress increased the PHB production even at high O_2 fractions causing H_2 limitation.
- The model can be used for the development with minimum experimentation of control strategies aiming high autotrophic PHB production.

PART III:

Heterotrophic-autotrophic PHB production

Chapter 6:

Sustainable autotrophic production of polyhydroxybutyrate (PHB) from CO_2 using a two-stage cultivation system

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Abstract

The technical feasibility of *Cupriavidus necator* DSM 545 for sustainable autotrophic polyhydroxybutyrate (PHB) production from CO_2 using a two-stage cultivation system was evaluated. In this cultivation method, cell mass growth occurred under heterotrophic conditions using two different organic substrates, namely glucose and waste glycerol. In both cases, PHB biosynthesis was triggered by applying nitrogen and oxygen limitation at three different cell mass concentrations under autotrophic conditions using a gas mixture of H_2, O_2 and CO_2. To ensure that the test conditions were relevant for later industrial application, O_2 concentration was kept below the safety value during autotrophic PHB production. PHB production from CO_2 on waste-glycerol grown cell mass resulted in a PHB production of 28 g/L, which is the highest reported value in literature for PHB synthesized from CO_2 at an O_2 concentration below the lower explosion limit of 5 vol%. The fermentation performance decreased when nutrient limitation was delayed at higher cell mass concentrations. Furthermore, it was shown that PHB production from CO_2 at high cell mass concentration is metabolically feasible, but under the tested conditions the mass transfer of O_2 was limiting PHB accumulation. Characterization of the produced polymers showed that the organic carbon source affected the properties of PHB. Overall, the cultivation method developed in this study provided PHB with properties similar to a commercial PHB and PHB typically found in literature. It can be concluded that heterotrophic-autotrophic production of PHB by *C. necator* is a promising cultivation method to reduce the overall production cost of PHB. In order to compete with the current heterotrophic cultivation system, the oxygen transfer rate must however be enhanced to achieve a higher PHB productivity.

Keywords: polyhydroxybutyrate, *Cupriavidus necator*, heterotrophic-autotrophic cultivation, waste glycerol, CO_2, characterization.

6.1. Introduction

Polyhydroxybutyrate (PHB) is a biodegradable and bio-based plastic, synthesized by a variety of organisms as an intracellular storage material from renewable resources. Although it has the potential to substitute conventional fossil fuel based plastics for a wide range of applications, PHB is still commercially behind the petroleum based synthetic plastics. The major drawback is the high production cost which is dominated for approximately 50% by the raw material costs (Choi and Lee, 1999). To attain bulk commercial viability and to further improve the

sustainability profile of PHB production, it is desirable to use waste and surplus materials for PHB biosynthesis. In addition, the conversion of waste materials to PHB is advantageous for waste management. Both liquid (such as crude glycerol) and gaseous substrates (using CO_2 as feedstock) have been studied for PHB production (Akaraonye et al., 2010; Castilho et al., 2009; Koller et al., 2010).

Cupriavidus necator is a metabolically versatile organism capable of shifting between heterotrophic growth (utilizing organic compounds as carbon and energy source) and autotrophic growth (utilizing CO_2 as carbon source and H_2 or formate as energy source). In addition, the bacterium can accumulate PHB up to 80% of the dry cell weight in a non-growth-associated manner (Pohlmann et al., 2006). Two cultivation methods exist to utilize CO_2 for PHB production by *C. necator*. The most frequently applied cultivation method uses a gas mixture of CO_2, H_2 and O_2 for both cell mass growth (phase 1) and PHB accumulation (phase 2) according to Eq. 6.1 (Ishizaki and Tanaka, 1991) and Eq. 6.2 respectively (Ishizaki et al., 2001).

$$21.36\ H_2 + 6.21\ O_2 + 4.09\ CO_2 + 0.76\ NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.7\ H_2O \quad (6.1)$$

$$33\ H_2 + 12\ O_2 + 4\ CO_2 \rightarrow C_4H_6O_2 + 30\ H_2O \quad (6.2)$$

A gas composition ratio of H_2:O_2:CO_2=7:2:1 is needed to attain sufficient biomass growth by avoiding gas-limited conditions (Takeshita and Ishizaki, 1996), but lies within the gas-explosion range. Several solutions have been proposed to solve the explosion risk problem. The cultivation could be carried out by applying other terminal electron acceptors such as nitrate than O_2. This results however in an extreme reduction in the cell yield and growth (Tiemeyer et al., 2007). A better strategy is to reduce the O_2 content in the gas phase below the explosion limit. Depending on the method used (EN 1839, 2012), the lower explosion limit (LEL) of O_2 in H_2 has been estimated to range from 4.0 vol% (Schroder et al., 2004), 5 vol% (Coward and Jones, 1952) to 6.9 vol% (Ishizaki et al., 1993). By reducing the O_2 concentration below its LEL, the driving force for mass transfer of O_2 decreases, increasing the risk for mass transfer limitation. As a result, a lower cell mass concentration is attained at the end of cell mass growth phase, yielding a lower final PHB production and productivity.

In the second cultivation method for PHB production from CO_2, the formation of cell mass occurs under heterotrophic conditions (phase 1) (Chapter 2), followed by PHB accumulation (phase 2) using a gas mixture of CO_2, H_2 and O_2 (Eq. 6.2). Similar to the first cultivation method, the O_2 concentration in the mixture of substrate gases needs to be maintained below

LEL to avoid gas detonation. The advantage of this cultivation system is that a high cell mass concentration and thus productivity can be obtained during the cell mass growth phase as O_2 can be supplied under non-limiting conditions, while in the second autotrophic phase PHB biosynthesis will be triggered when the O_2 concentration is below its critical value which is reported to be 3% (Tanaka and Ishizaki, 1995).

The objective of this study was to evaluate the technical feasibility of *C. necator* DSM 545 for sustainable autotrophic PHB production (phase 2) from a gas mixture (CO_2, H_2, O_2) that followed heterotrophic cell mass growth (phase 1) from an organic substrate. To ensure that test conditions were relevant for later industrial application, a safety marge of 2.0 vol% below the LEL of 5 vol% O_2 was taken into account during autotrophic cultivation (NFPA 69, 2014). The influence of the organic carbon source on the formation of key enzymes of autotrophic metabolism was evaluated in terms of PHB accumulation by using two different organic substrates, glucose and waste glycerol, as carbon source for cell mass growth. PHB biosynthesis was induced under imbalanced growth conditions by limiting nitrogen and O_2 at different cell mass concentrations. Furthermore, the biopolymers were characterized with different techniques and compared with polymers synthesized on solely organic carbon sources and a commercial polymer to evaluate the influence of the fermentation mode and substrates on the properties of the biopolymers.

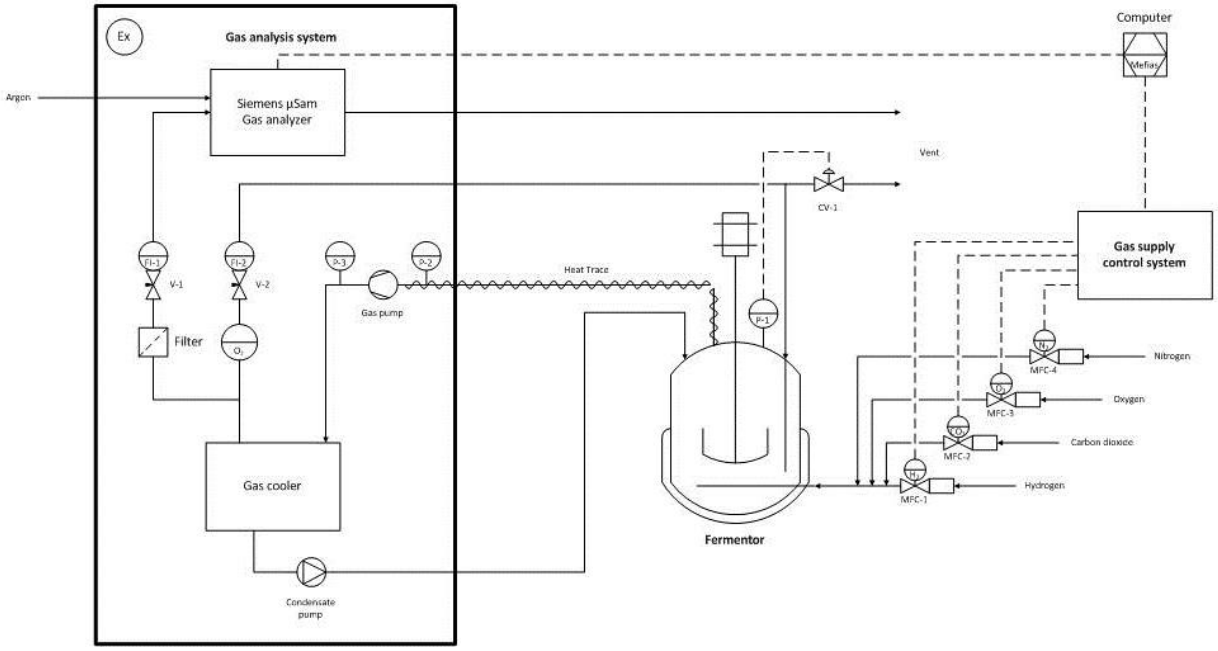


Figure 6.1. Scheme of the set-up for heterotrophic-autotrophic cultivation of polyhydroxybutyrate (PHB); MFC: mass flow controller; CV: control valve; P: pressure transmitter ; V needle valve; FI: flow indicator.

6.2. Materials and methods

6.2.1. Organism, media and inoculum preparation

The microorganism, culture media and inoculum preparation used in this study were the same as previously reported (Chapter 2).

6.2.2. Set-up

The set-up for heterotrophic-autotrophic production of PHB, consisted of a bioreactor, online gas analysis system and gas control system. A schematic representation is shown in Figure 6.1.

6.2.2.1. Bioreactor

A 7-L, double jacketed, lab-scale fermentor unit with an EZ-Control system (Applikon Biotechnology, the Netherlands) for on-line monitoring and controlling of the stirring speed, dissolved oxygen (DO), foam formation, pH and temperature was used. The DO concentration was maintained around 55% of air saturation for cell mass growth phase (phase 1) using a cascade control strategy consisting of the agitation speed (850 up to 950 rpm), air and/or oxygen flow. These relatively high DO levels were chosen to ensure that the process was not limited by the O_2 concentration. In PHB production phase (phase 2), the stirring speed was kept constant at 1400 rpm and O_2 concentration was measured by a gas analysis system (Section 6.2.2.2.) and kept constant below the safety level of 3 vol% by the gas control system (Section 6.2.2.3.). The pH was controlled at 6.80 by adding acid (2 M H_2SO_4) or base (20% NH_4OH in cell mass growth (phase 1) and 5 M NaOH in PHB production (phase 2)). Foam formation was measured through a level contact (conductivity) sensor and was controlled by the addition of 30% antifoam C emulsion (Sigma-Aldrich Chemie, GmbH, Germany). The process temperature was measured by a Platinum resistance thermometer sensor (PT 100) and kept constant at 30 °C through the double jacket using the EZ-Control thermocirculator. The head space of the bioreactor was kept at atmospheric pressure during cell mass growth phase and at an overpressure of 40 mbar during PHB production phase. The pressure was controlled by a pressure transmitter (Keller, PR-35XHT) and a pneumatic control valve (Badger Meter, ATC type 755) as back pressure control valve.

6.2.2.2. Gas analysis system

Gas from the bioreactor outlet was continuously withdrawn via a heated traced tubing using a gas sample pump (Bühler, PS2 Eexd) at a minimal flow rate of 164 L/min and dried by a gas cooler (Bühler, EGK2 Ex). The condensate was returned to the bioreactor using a build in

peristaltic pump. The gas was splitted in two streams. One stream was pumped through a gas filter (Swagelok in-line filter, F-Series, 0.5 µm) and a variable area flowmeter (Krohne, DK 800/R/k1) to an on-line gas chromatograph (GC) (MicroSAM, Siemens) to determine the gas composition (H_2, O_2, CO_2 and N_2) of the head space of the bioreactor. The GC was equipped with three micro thermal conductivity detectors and Argon was used as the carrier gas. A second stream was resupplied to the bioreactor through a gas return line and a variable area flowmeter (Krohne, DK 800/R/k1). Dependent on the (over)pressure (setpoint) of the head space in the bioreactor, a part of this stream was discharged to the atmosphere. This vent was connected with the a gas counter (Schlumberger, Gallus 2000) to monitor the gas exit.

6.2.2.3. Gas control system

An in-house developed software program (MeFiAS) interfaced with the gas analysis system and controlled the supply of gases and safety during fermentation. Gases, supplied from compressed gas cylinders (Air Products, grade BIP, purity 5.7), were fed separately in the culture liquid medium to obtain a constant head space gas composition of H_2:O_2:CO_2=84:2.8:13.2 (vol%). During autotrophic fermentation, the O_2 concentration was kept below the safety level of 3 vol%.

6.2.3. Fermentation

The seed culture was inoculated at 12.5 vol% into 2.5 L fermentation medium containing either 10 g/L glucose or 17 g/L waste glycerol (Chapter 2). Separate feeding strategies were used for each phase of the two-phase fermentation process. A computer-based software program, BioXpert, was used to control the organic carbon source concentration in the bioreactor at the desired level using a two-stage substrate feeding strategy. This feeding strategy consisted of an initial 10 h of exponential feeding followed by feeding based on alkali-addition which was coupled with NH_4OH feeding for pH control. During the growth phase, the cell mass concentration was estimated based on the amount of total substrate added (Chapter 2). When the cell mass concentration deviated 5 g/L from the desired cell mass concentration for switching to PHB production phase (phase 2), feeding of the organic substrate was stopped to consume the residual organic substrate and ammonium feeding was replaced with NaOH for pH control. It was ensured that C:N ratio was in balance for cell demand to obtain maximal PHB accumulation from CO_2. When the nitrogen concentration was approximately below 100 mg/L, gases were continuously sparged in the bioreactor to keep the gas composition in the head space constant at a ratio of H_2:O_2:CO_2=84:2.8:13.2 vol% as mentioned in Section 6.2.2.3.

Under these conditions, nitrogen and oxygen became limited, triggering PHB synthesis. Samples were taken at regular time intervals for analysis during cultivation.

6.2.4. Analytical procedures

The concentrations of glucose, glycerol, ammonium (NH_4^+-N), biomass (expressed as cell dry mass, CDM) and PHB was determined as previously described (Chapter 2).

6.2.5. Calculations

The residual cell concentration (RCC) is defined as

$$RCC = CDM - PHB \quad (6.3)$$

The PHB content is expressed as

$$PHB\ content\ (\%) = PHB \times 100/CDM \quad (6.4)$$

The PHB fraction produced from gaseous CO_2 is defined as

$$PHB\ fraction\ (\%) = \left(PHB_{phase\ 2} - PHB_{phase\ 1}\right) \times 100/PHB_{phase\ 2} \quad (6.5)$$

Gas uptake for each gas is calculated as

$$Gas\ uptake = Gas\ inlet - Gas\ outlet \quad (6.6)$$

The gas conversion efficiency is calculated as

$$Gas\ conversion\ efficiency\ (\%) = (Gas\ inlet - Gas\ outlet) \times 100/Gas\ inlet \quad (6.7)$$

The PHB yield from the different gases is calculated as

$$Y_{PHB/gas}(g\ PHB\ /g\ gas) = PHB\ /Gas\ uptake \times Liquid\ volume \quad (6.8)$$

6.2.6. PHB extraction and characterization

The biopolymers produced in this study were extracted as previously described (Chapter 2). PHB produced from glucose and waste glycerol (Chapter 2) and PHB purchased from a commercial manufacturer (Biomer, Germany) were characterized by means of gel permeation chromatography (GPC), differential scanning calorimetry (DSC), thermo-gravimetry (TGA) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR). GPC analysis was performed using a Waters Breeze™ System with a combination of three column series (PSS SDV analytical 1000 Å, 5 µm, 300 × 8.00; PSS SDV analytical 100000 Å , 5 µm, 300 × 8.00; PSS SDV analytical 1000000 Å, 5 µm, 300 × 8.00) and equipped with a Waters 2414 differential refractive index detector. Chloroform was used as the eluent at 35°C, and the applied flow rate was 1.0 mL/min. A calibration curve was obtained using narrow polystyrene standards (Polymer Laboratories) in the range of 580-1930000 g/mol. ^{1}H NMR spectra were recorded at 25°C on a Bruker Avance 300 MHz Ultrashield spectrometer. The polymer samples were

dissolved in deuterated chloroform. Proton spectra were recorded at 300 MHz. DSC measurements were carried out on a Mettler-Toledo DSC1/700 instrument. The apparatus was calibrated using indium of high purity. Samples were sealed in aluminum pans and analysed. Measurements were performed under an 100 mL/min N_2 flow rate according to the following protocol: the sample was placed for 5 min at -100°C, followed by a first heating from -100°C to 230°C at 10°C/min. The sample was allowed to stabilize for 2 min at 230°C prior to performing a second cooling from 230°C to -100°C at 10°C/min followed by a 2 min isotherm at -100°C. This allows to create a similar thermal history for all samples. The second heating was then performed from -100°C to 230°C at 10°C/min. Glass transition temperature (T_g), melting temperature (T_m) and total melting enthalpy (ΔH_m) were determined from the second heating run of the DSC endothermic peaks. The degree of crystallinity (X_c) of PHB was calculated assuming that the ΔH_m value of 100% crystalline PHB is 146 J/g (Barham et al., 1984). TGA analysis was performed on a Mettler-Toledo TGA/SDTA851e instrument. The sample was heated from 25°C to 800°C at a rate of 10°C/min under a nitrogen flow of 100 mL/min. Degradation temperature (T_d) was set, for comparison, as the temperature at which 10 wt.% loss occurs.

6.3. Results and discussion

This study aimed to evaluate autotrophic PHB production from a gas mixture (CO_2, H_2, O_2) following heterotrophic cell mass growth on an organic substrate. The choice of organic carbon source is essential from technological and economical point of view as the substrate influences the growth rate, the degree of synthesis of key enzymes for chemolithoautotrophic metabolism (Bowien and Schlegel, 1981; Friedrich et al., 1981) and raw material cost. This concept has already been applied using either fructose or acetic acid as organic carbon source for cell mass growth followed by autotrophic PHB production at 6.7 vol% O_2 (Sugimoto et al., 1999; Tanaka and Ishizaki, 1994). The best results were obtained when the cells were cultivated with fructose in the heterotrophic growth phase (Tanaka and Ishizaki, 1994). However, this carbon source is expensive and should be replaced by lower cost substrates. In this study, glucose was chosen since this feedstock is generally utilized for PHB production and is indeed less expensive compared to fructose. Waste glycerol was selected as second carbon source as this is an industrial by-product from the biodiesel production for which the biological conversion of crude glycerol to higher value chemicals, such as PHB, is an interesting application (Posada et al., 2011). The enzymes necessary for autotrophic metabolism can be influenced by the organic

substrate used for growth. It was reported that key enzymes of autotrophic energy generation and CO_2 fixation were not effected during the growth on glycerol (Friedrich et al., 1981). This gives an additional advantage for using of waste glycerol in growth phase.

6.3.1. Effect of heterotrophic phase on autotrophic PHB production

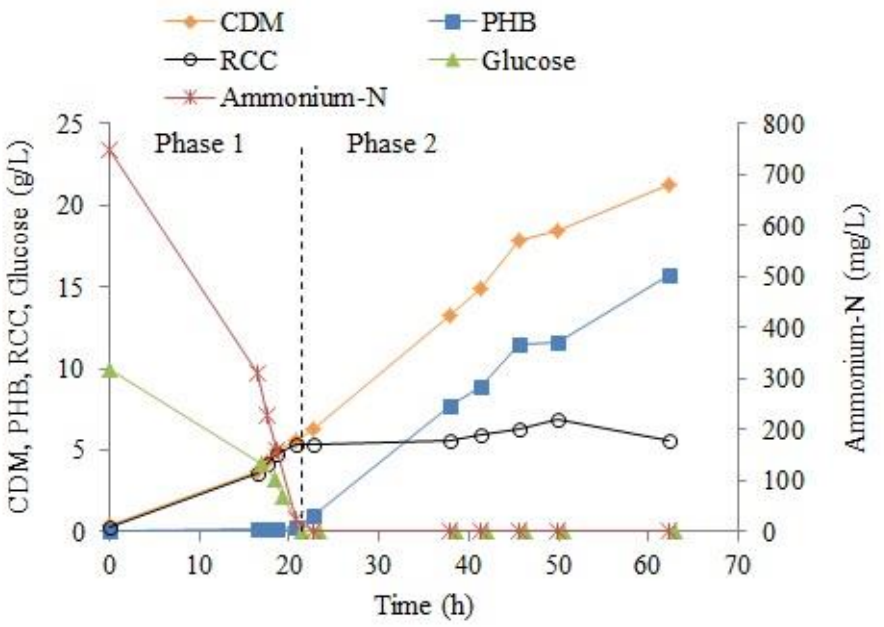
Autotrophic PHB production at O_2 concentration below the safety value, that followed heterotrophic cell mass growth on either glucose or waste glycerol was evaluated at three different cell mass concentrations. Shifting to PHB production phase was done at low, intermediate and high residual cell concentration (RCC) (5, 15 and 40 g/L). The overall results are shown in Figure 6.2 and 6.3 for glucose and waste glycerol, respectively, and are summarized in Table 6.1.

6.3.1.1. Autotrophic PHB production on glucose-grown cells

For autotrophic PHB production at low cell mass concentrations (5 g/L RCC), heterotrophic growth was carried out in batch mode using 10 g/L glucose and 750 mg/L nitrogen (Figure 6.2a), according to the mass balance of cell mass growth (Chapter 2). When nitrogen concentration was around 160 mg/L, a gas mixture of H_2:O_2:CO_2 was supplied to the bioreactor to keep the head space's gas composition constant at a ratio of 84.0:2.8:13.2. Under these conditions, O_2 became limited. As nitrogen was still present, the cells started to accumulate PHB in addition to cell mass growth using the residual glucose (2 g/L). Once glucose and nitrogen became depleted, carbon flux was redirected towards PHB production and PHB was accumulated using CO_2 as feedstock. The overall results are summarized in Table 6.1. The yield of PHB over different gases was calculated as the ratio of the amount of PHB formed in the PHB production phase to the gas uptake. During the process, a part of the gas stream was discharged to the atmosphere. For the first experiment performed (i.e., glucose with 5 g/L RCC), this discharged gas was not counted and therefore the yield cannot be calculated. Figure 6.2b shows the time course for autotrophic PHB production of cells grown over glucose when applying nutrient limitation at intermediate cell mass concentration (15 g/L RCC). During the growth phase (phase 1), cell mass concentration increased exponentially to 15 g/L. Glucose and ammonia feeding was stopped at 10 g/L CDM to consume the residual glucose (10 g/L) and nitrogen (0.687 g/L) in the culture medium (Y_{XS}=0.5 g cell mass/g glucose (Chapter 2)), ensuring maximal PHB accumulation over CO_2. When nitrogen concentration was 5 mg/L, autotrophic cultivation was initiated. No lag phase was observed as the cells immediately

started to accumulate PHB due to O_2 and nitrogen limitation. The overall results are given in Table 6.1.

(a)



(b)

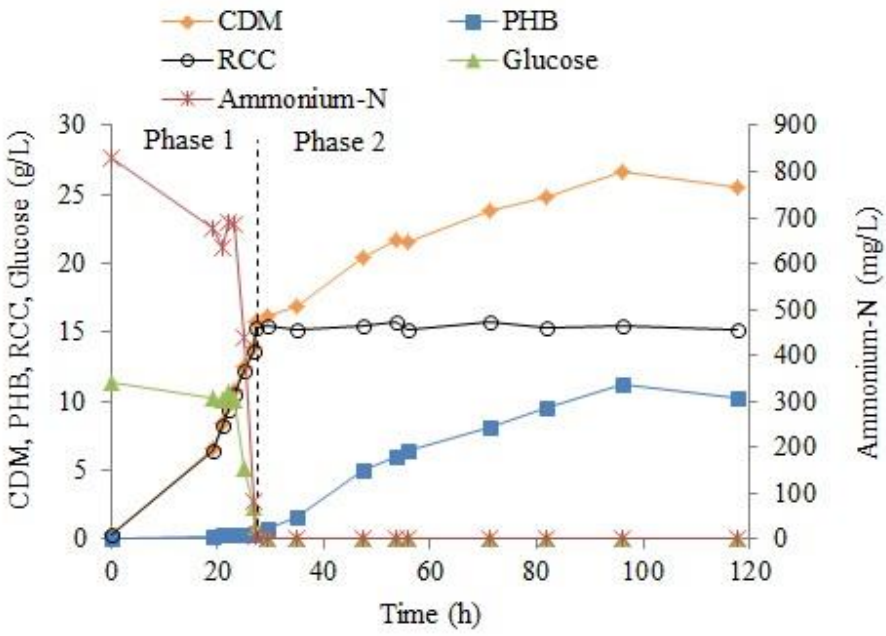


Figure 6.2. Time course of two-stage cultivation of *C. necator* DSM 545 using glucose in the heterotrophic growth phase (phase 1) and a gas mixture composed of H_2:O_2:CO_2 = 84.0:2.8:13.2 in the autotrophic PHB production phase (phase 2). Nutrient limitation was applied at (a) 5 and (b) 15 g/L biomass concentration. CDM, cell dry mass; PHB: polyhydroxybutyrate; RCC: residual cell concentration, defined as the difference between CDM and PHB production.

When delaying nitrogen and oxygen limitation at high cell mass concentration (40 g/L RCC), the cells were not able to accumulate PHB under autotrophic conditions and hence the PHB productivity and yields were not possible to evaluate. After 150h of autotrophic cultivation the cell mass concentration decreased to 30 g/L due to starvation (Table 6.1).

6.3.1.2. Autotrophic PHB production on waste glycerol-grown cells

As PHB production from CO_2 by cells grown on waste glycerol at low and intermediate cell mass concentrations was performed in a similar way as detailed in Section 6.3.1.1, the presentation of the results is limited to Table 6.1 and Figure 6.3.

With respect to PHB production at high cell-density (35 g/L RCC), no PHB accumulation was observed even after 92h of autotrophic cultivation. However, upon dilution of the cell mass concentration to 9 g/L with mineral salts medium, the cells started to accumulate PHB under autotrophic conditions. After 186h, cell mass concentration increased to 20 g/L with a PHB content of 48%.

Table 6.1. Summary of the results obtained in this study for the production of PHB from CO_2 using a heterotrophic-autotrophic cultivation system.

Heterotrophic phase				Autotrophic phase								
Substrate	CDM[1] (g/L)	PHB[2] (g/L)	RCC[3] (g/L)	CDM (g/L)	PHB (g/L)	RCC (g/L)	PHB content[4] (%)	PHB fraction[5] (%)	Overall PHB productivity[6] (g/L/h)	$Y_{PHB/CO2}$[7] (g PHB/g CO_2)	$Y_{PHB/H2}$[7] (g PHB/g H_2)	$Y_{PHB/O2}$[7] (g PHB/g O_2)
Glucose	5	0.2	4.8	21	16	5	74	99	0.252	-	-	-
Glucose	16	0.4	15.6	27	11	16	41	96	0.116	0.47	0.79	0.25
Glucose	42	2	40	29	0.1	28.9	0.3	-	-	-	-	-
Waste glycerol	10	3	7	18	13	5	72	76	0.187	0.35	0.79	0.27
Waste glycerol	19	6	13	44	28	16	61	80	0.168	-	0.79	0.27
Waste glycerol	47	11	36	31	0.9	30.1	3	-	-	-	-	-

[1]CDM, cell dry mass; [2]PHB, polyhydroxybutyrate; [3]RCC, residual cell concentration, calculated as the difference between CDM and PHB production; [4]PHB content, calculated as the percentage of the ratio of the PHB production to the CDM concentration; [5]PHB fraction produced from gaseous CO_2, calculated as the percentage of the ratio of the PHB production formed in the PHB production phase to the total PHB production; [6]PHB productivity, calculated as the final PHB production divided by the duration of the fermentation; [7]$Y_{PHB/gas}$, PHB yield from the different gases, calculated as the ratio of the amount of PHB formed in the PHB production phase to the gas uptake.

Table 6.2. Overview of the literature results for the production of PHB from CO_2 using a *chemolithoautotrophic* cultivation system in increasing order of final PHB production per cultivation system. In addition, the highest reported values obtained in literature for heterotrophic cultivation from glucose and waste glycerol are given.

Organic substrate	H_2:O_2:CO_2 (vol %)	Strain	CDM[1] at onset of phase 2 (g/L)	CDM concentration (g/L)	PHB[2] concentration (g/L)	PHB content[3] (%)	Overall PHB productivity[4] (g/L/h)	Limitation	K_La[5] (1/h)	Bioreactor	Reference
-	70:10:10	*Ideonella* sp. strain O-1	1	7	5	78	0.208	N	-	CSTR	Tanaka et al., 2011
-	60:20:10	*C. necator* B-5786	10	12	8	63	0.105	N	-	CSTR	Volova et al., 2004
-	70:20:10	*C. necator* ACM 1296	10	16	6	38	0.150	O_2	-	CSTR	Darani et al., 2006
-	60:20:10	*C. necator* ATCC 17699	5	18	14	78	0.200	N	-	CSTR	Sonnleitner et al., 1979
-	Not given	*C. necator* ATCC 17697	9	27	16	59	0.225	N	-	CSTR	Ishizaki and Tanaka, 1991
-	60:20:10	*C. necator* B-5786	10	30	22	75	0.314	N	310-420	CSTR	Volova and Voinov, 2003
-	75:15:10	*C. necator* ATCC 17697	9	60	36	60	0.550	O_2	-	CSTR	Ishizaki and Tanaka, 1991
-	85:5:10	*C. necator* ATCC 17697	10	59	46	79	0.548	O_2	300	Air lift[6]	Taga et al., 1997
-	85:5:10	*C. necator* ATCC 17697	10	60	49	82	0.408	O_2	250	Air lift	Taga et al., 1997
-	85:5:10	*C. necator* ATCC 17697	10	69	56	81	0.609	O_2	340	Air lift[7]	Taga et al., 1997
-	85.2:6.3:8.3	*C. necator* ATCC 17697	29	91	62	68	1.55	O_2	2970	CSTR	Tanaka et al.,, 1995
Waste glycerol	-	*C. necator* DSM 545	53	105	66	63	1.360	N	-	CSTR	Chapter 2
Glucose	-	*C. necator* NCIMB 11599	70	164	121	74	2.420	N	-	CSTR	Kim et al., 1994

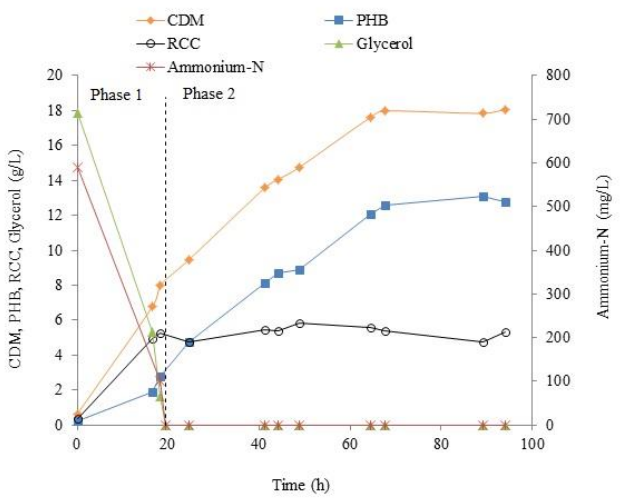
[1]CDM, cell dry mass; [2]PHB, polyhydroxybutyrate; [3]PHB content, calculated as the percentage of the ratio of the PHB production to the CDM concentration; [4]PHB productivity, calculated as the final PHB production divided by the duration of the fermentation; [5]K_La, mass transfer coefficient; [6]Addition of 0.1% carboxymethylcellulose to the fermentation medium; [7]Addition of 0.05% carboxymethylcellulose to the fermentation medium.

Table 6.3. Overview of the literature results for the production of PHB from CO_2 using a *heterotrophic-chemolithoautotrophic* cultivation system in increasing order of final PHB production per cultivation system. The gas composition ratio which lies within the gas-explosion range (using lower explosion limit of 5.0 vol% O_2) is indicated in bold. In addition, the highest reported values obtained in literature for heterotrophic cultivation from glucose and waste glycerol are given.

Organic substrate	H_2:O_2:CO_2 (vol %)	Strain	CDM[1] at onset of phase 2 (g/L)	CDM concentration (g/L)	PHB[2] concentration (g/L)	PHB content[3] (%)	Overall PHB productivity[4] (g/L/h)	Limitation	K_La[5] (1/h)	Bioreactor	Reference
Waste glycerol	84.0:2.8:13.2	*C. necator* DSM 545	47	31	0.9	3	-	N + O_2	340	CSTR	This study
Glucose	84.0:2.8:13.2	*C. necator* DSM 545	42	29	0.1	0.3	-	N + O_2	340	CSTR	This study
Glucose	84.0:2.8:13.2	*C. necator* DSM 545	16	27	11	41	0.116	N + O_2	340	CSTR	This study
Acetic acid	**86.5:6.5:10**	***C. necator* ATCC 17697**	**5**	**23**	**13**	**55**	**0.152**	**O_2**	**-**	**CSTR**	**Sugimoto et al., 1999**
Waste glycerol	84.0:2.8:13.2	*C. necator* DSM 545	10	18	13	72	0.187	N + O_2	340	CSTR	This study
Fructose	**83.0:5.3:10.6**	***C. necator* ATCC 17697**	**10**	**27**	**15**	**56**	**0.237**	**O_2**	**340**	**CSTR**	**Tanaka and Ishizaki, 1994**
Glucose	84.0:2.8:13.2	*C. necator* DSM 545	5	21	16	74	0.252	N + O_2	340	CSTR	This study
Fructose	86.5:4.9:9.8	*C. necator* ATCC 17697	4	26	22	85	0.309	O_2	340	CSTR	Tanaka and Ishizaki, 1994
Fructose	**84.1:6.7:10.3**	***C. necator* ATCC 17697**	**15**	**43**	**24**	**56**	**0.632**	**O_2**	**340**	**CSTR**	**Tanaka and Ishizaki, 1994**
Waste glycerol	84.0:2.8:13.2	*C. necator* DSM 545	19	46	28	61	0.168	N + O_2	340	CSTR	This study
Waste glycerol	-	*C. necator* DSM 545	53	105	66	63	1.360	N	-	CSTR	Chapter 2
Glucose	-	*C. necator* NCIMB 11599	70	164	121	74	2.420	N	-	CSTR	Kim et al., 1994

[1]CDM, cell dry mass; [2]PHB, polyhydroxybutyrate; [3]PHB content, calculated as the percentage of the ratio of the PHB production to the CDM concentration; [4]PHB productivity, calculated as the final PHB production divided by the duration of the fermentation; [5]K_La, mass transfer coefficient.

(a)



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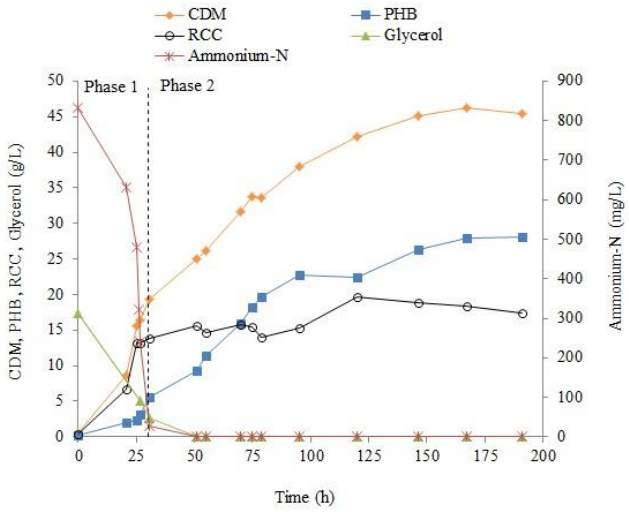

(c)

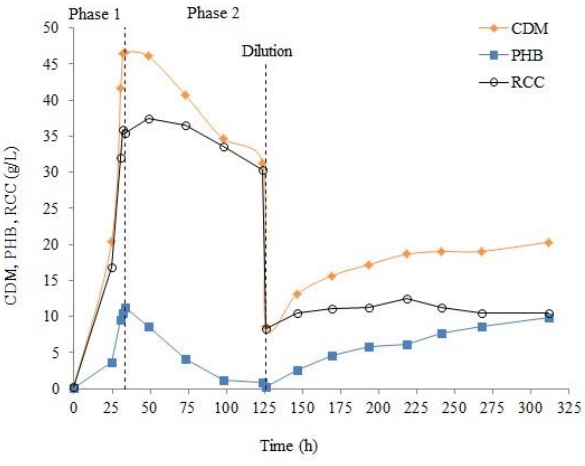

Figure 6.3. Time course of two-stage cultivation of *C. necator* DSM 545 using waste glycerol in the heterotrophic phase and a gas mixture composed of H_2:O_2:CO_2 = 84.0:2.8:13.2 in the autotrophic phase. Nutrient limitation was applied at (a) 10, (b) 19 and (c) 46 g/L biomass concentration. CDM: cell dry mass; PHB: polyhydroxybutyrate; RCC: residual cell concentration, defined as the difference between CDM and PHB production.

6.3.1.3. Evaluation of the heterotrophic-autotrophic process

A number of studies have been focusing on the production of PHB from CO_2 using either a chemolithoautotrophic or heterotrophic-chemolithoautotrophic cultivation system. An overview of these results is given in Table 6.2 and 6.3, respectively. For industrial scale application, a high cell-density culture with high PHB content and PHB productivity is preferable keeping the O_2 concentration in the gas phase below the LEL. Higher cell mass concentration and lower gas concentration inherently implicates an increased risk of mass transfer limitation, causing premature shifting to PHB production phase in the chemolithoautotrophic cultivation system or incomplete PHB accumulation in the heterotrophic-chemolithoautotrophic process. The latter system seems the most promising approach for the conversion of CO_2 to PHB as higher cell mass concentration and growth rate can be obtained in the growth phase under heterotrophic conditions compared to autotrophic conditions. From Table 6.3, it can indeed be concluded that the highest reported PHB production from CO_2 at a O_2 concentration below the LEL in literature was obtained in the present study using waste glycerol-grown cell mass.

The prerequisite for heterotrophic-autotrophic cultivation is the activation of the autotrophic metabolism of *C. necator*, which is shown to be affected by the organic source (Friedrich et al., 1981). The results in this study showed that independent from the organic carbon source used, maximal PHB content, PHB production and PHB productivity was obtained when nutrient limitation was imposed at low cell mass concentration (5 g/L RCC). Delaying nutrient limitation at intermediate cell mass concentration (15 g/L RCC) reduced the fermentation performance. When shifting to PHB production phase at too high cell mass concentrations, PHB accumulation did no longer occur (Table 6.1). A decrease of PHB content and overall productivity with increasing cell mass concentration from 4 to 10 g/L at the time point of shifting to PHB production phase was also observed by Tanaka and Ishizaki (1994). When these authors imposed nutrient limitation at a higher cell mass concentration (at 15 g/L), PHB productivity was enhanced, but the final PHB content did not increase. It was postulated that the lower PHB content at higher cell-density could be attributed to damage of the autotrophic growth ability of the microorganisms as a result of prolonged heterotrophic cultivation (Tanaka and Ishizaki, 1994).

In this study, autotrophic PHB production resumed upon dilution of waste glycerol-grown cells from high to low cell mass concentration (Figure 6.3c). This confirms that key enzymes for autotrophic metabolism were formed during heterotrophic growth but that PHB accumulation

was restricted by mass transfer limitation of O_2. The transferred oxygen at higher cell densities probably goes to maintainance. This is the reason for the decrease in biomass at phase 2 (Figure 6.3c). It should be noted that the higher PHB productivities obtained by Tanaka and Ishizaki (1994) must have resulted from the higher O_2 concentration (4.9 – 6.7 vol%) used in the gas phase during autotrophic cultivation as a similar volumetric mass transfer coefficient (k_La) value was determined. Furthermore, in contrast to the findings of Tanaka and Ishizaki (1994) and Sugimoto et al. (1999), no adaptation of the bacteria to assimilate CO_2 was required when switching from heterotrophic to autotrophic metabolism (Figure 6.2 and 6.3). The lag phase observed by these authors might be related to the organic substrates used as the key enzymes for autotrophic metabolism were found at intermediate activities in fructose-grown cells, while the formation of the enzymes was completely repressed during biomass growth on acetate (Friedrich et al., 1981). However, despite the inhibitory effect of acetate on the enzymes, the cells were still able to fixate CO_2.

Accumulation of PHB during the heterotrophic cell mass growth phase was consistent with previous reports (Berezina, 2013; Chapter 2). It is however not clear why cell mass grown on waste glycerol accumulated more PHB (30% PHB content) compared to cells grown on glucose (2 to 5% PHB content). This difference probably explains why the additional PHB produced from CO_2 in the autotrophic phase was higher for the glucose-grown cells compared to waste glycerol-grown cells. However, the PHB fraction produced from CO_2 was still higher than 75%. The gas consumption efficiency for glucose-grown and glycerol-grown cell mass was 85% and 55%, respectively.

It can be concluded that heterotrophic-autotrophic production of PHB by *C. necator* is a promising cultivation method to reduce the overall production cost of PHB. PHB production from CO_2 on waste-glycerol grown cell mass under conditions relevant for industrial application resulted in the highest reported PHB production synthesized from CO_2 so far. In addition, it was shown that PHB production from CO_2 at higher cell mass concentration is metabolically feasible, but under the tested conditions the mass transfer of O_2 was limiting PHB accumulation. In order to compete with the current heterotrophic cultivation system, the oxygen transfer rate must be enhanced to achieve a higher PHB productivity. Using a continuous stirred tank bioreactor (CSTR), the k_La can be increased by reducing the gas bubble diameter, increasing the gas hold-up time, operation at elevated pressure, the addition of chemicals or catalysts to the fermentation medium, etc. (Garcia-Ochoa and Gomez, 2009; Mohammadi et al., 2011). Increase of gas velocity can also improve the k_La, however the high gas flow rate may adversely affect the substrate gas conversion. Although CSTR is the most widely used reactor

for gas fermentation (Ishizaki et al., 2001; Mohammadi et al., 2011), other bioreactor configurations such as airlift reactors and bubble columns could also be of interest.

6.3.2. Biopolymer characterization

The biopolymers produced by autotrophic fermentation at low cell mass concentration when grown on glucose ($PHB_{GLUCOSE\text{-}CO2}$) and waste glycerol ($PHB_{GLYCEROL\text{-}CO2}$) were characterized by different techniques. In addition, PHB produced by *C. necator* DSM 545 on solely glucose ($PHB_{GLUCOSE}$) and waste glycerol ($PHB_{GLYCEROL}$) from our previous work (Chapter 2) and commercial PHB (PHB_{BIOMER}) were analyzed to evaluate if the fermentation mode and substrate affected the properties of the biopolymer. To the best of our knowledge, this is the first time reported in literature that PHB produced from CO_2 was characterized in this extent.

6.3.2.1. Chemical structure

For all samples, the peaks observed in the ^1H NMR spectra coincided and the spectrum of $PHB_{GLUCOSE\text{-}CO2}$ is shown in Figure 6.4. Three peak groups related to the polymer were observed: a multiplet at 5.2 ppm which is characteristic for the methine group (signal 1), a doublet of quadruplet at 2.5 ppm which corresponds to a methylene group adjacent to an asymmetric carbon atom bearing a single proton (signal 2), and a doublet at 1.2 ppm which corresponds to the methyl group coupled to one proton (signal 3). These chemical shifts correspond to the characteristics of PHB homopolymer (Oliveira et al., 2007; Rodriguez-Contreras et al., 2013), which is consistent with the previous finding for PHB produced from CO_2 as the sole feedstock (Ishizaki et al., 2001). In addition, the spectrum of $PHB_{GLYCEROL\text{-}CO2}$ showed resonance at 3.7 ppm which corresponds to the terminal esterification of glycerol to PHB accumulated during the cell mass growth phase through the primary hydroxyls (C1 or C3 positions of glycerol) (Ashby et al., 2011). In all spectra, two other signals at 1.6 ppm and 7.2 ppm were observed which are due to water and chloroform, respectively.

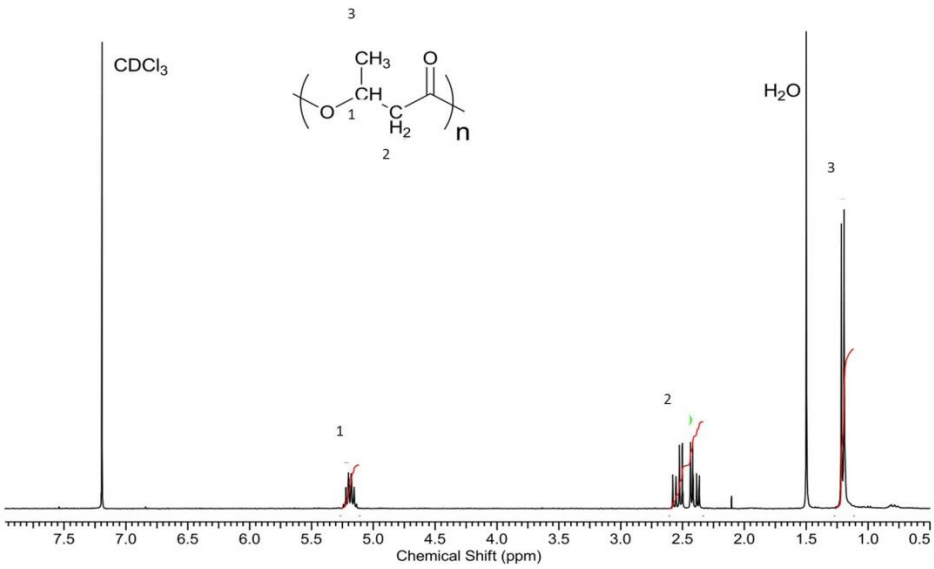

Figure 6.4. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) spectrum of polyhydroxybutyrate (PHB) extracted from a two-stage cultivation of *C. necator* DSM 545 using glucose in the heterotrophic phase and a gas mixture composed of H_2:O_2:CO_2 = 84.0:2.8:13.2 in the autotrophic phase ($PHB_{GLUCOSE\text{-}CO2}$). 1: multiplet at 5.2 ppm, characteristic for the methine group; 2: doublet of quadruplet at 2.5 ppm, characteristic for the methylene group adjacent to an asymmetric carbon atom bearing a single proton; 3: doublet at 1.2 ppm, characteristic for the methyl group coupled to one proton; $CDCl_3$, deuterated chloroform; H_2O, water.

6.3.2.2. Molar mass distribution

The results summarized in Table 6.4 show that PHB produced in this study ($PHB_{GLYCEROL\text{-}CO2}$ and $PHB_{GLUCOSE\text{-}CO2}$) had a lower molecular weight compared to the polymers produced from the organic substrates ($PHB_{GLYCEROL}$ and $PHB_{GLUCOSE}$), but a higher molar mass compared to PHB produced using solely CO_2 as feedstock (PHB_{CO2}) (Volova et al., 2013a). Moreover, although CO_2 was used as carbon source in the PHB accumulation phase, lower molar mass was obtained when cell mass was grown on glycerol ($PHB_{GLYCEROL\text{-}CO2}$) instead of glucose ($PHB_{GLUCOSE\text{-}CO2}$) which is probably related to PHB accumulation during the cell mass growth phase. Molar mass of PHB has indeed been reported to be affected by carbon source, microorganism and conditions of cultivation (Sudesh et al., 2000). The lower molecular weight in the presence of glycerol corresponds to previous reports (Chapter 2) and can be explained by esterification of glycerol with PHB resulting in chain termination (end-capping) as

demonstrated (Madden et al., 1999) and confirmed by our ^{1}H NMR results for $PHB_{GLYCEROL-CO2}$. In addition, the cultivation time did not affect the molecular weight of PHB.

The molecular mass values in this study are in the same range as commercial PHB (PHB_{BIOMER}) and PHB reported in literature. A typical average molecular weight (M_w) of PHB produced from wild-type bacteria is usually in the range of 10-3000 kDa with a polydispersity index (PDI) of around two (Khanna and Srivastava, 2005; Sudesh et al., 2000).

Table 6.4. Molar mass distribution of PHB synthesized from different substrates.

Sample	M_n[1] (kDa)	M_w[2] (kDa)	PDI[3]	Reference
PHB_{CO2}[4]	308	625	2.03	Volova et al., 2013a
$PHB_{GLYCEROL-CO2}$[5]	549	749	1.36	This study
PHB_{BIOMER}[6]	564	844	1.50	This study
$PHB_{GLYCEROL}$[7]	624	992	1.59	Chapter 2
$PHB_{GLUCOSE-CO2}$[8]	744	1222	1.64	This study
$PHB_{GLUCOSE}$[9]	1235	1457	1.18	Chapter 2

[1]M_n, number average molecular weight; [2]M_w, weight average molecular weight; [3]PDI, polydispersity index; [4]PHB_{CO2}, PHB produced using solely CO_2 as feedstock; [5]$PHB_{GLYCEROL-CO2}$, PHB produced using CO_2 as feedstock on biomass grown on waste glycerol; [6]PHB_{BIOMER}, commercial PHB produced by Biomer; [7]$PHB_{GLYCEROL}$, PHB produced using solely waste glycerol as feedstock; [8]$PHB_{GLUCOSE-CO2}$, PHB produced using CO_2 as feedstock on biomass grown on glucose; [9]$PHB_{GLUCOSE}$, PHB produced using solely glucose as feedstock.

6.3.2.3. Thermal properties

DSC and TGA results are summarized in Table 6.5 and the thermogram of $PHB_{GLUCOSE-CO2}$ is shown in Figure 6.5. Thermo-analysis by DSC of the polymers resulted in multiple melting peaks, which can be interpreted as the presence of different inter-lamellae dimensions in the crystallites, and differences in the microstructure of the polymers. Respectively two and three melting peaks were observed with $PHB_{GLUCOSE-CO2}$ and $PHB_{GLYCEROL-CO2}$ of which the maxima of the first two melting peaks of $PHB_{GLYCEROL-CO2}$ were about 20°C lower. Autotrophic production of PHB in cells grown on glucose ($PHB_{GLUCOSE-CO2}$) leads to higher melting temperatures (T_m) compared to $PHB_{GLUCOSE}$, which can be an asset.

Table 6.5. Thermal properties of PHB synthesized from different substrates.

Sample	T_m[1] (°C)			T_g[2] (°C)	X_c[3] (%)	T_d[4] (°C)	Reference
	1	2	3				
$PHB_{GLYCEROL\text{-}CO2}$[5]	123.83	141.33	148.17	-4.46	62	280	This study
$PHB_{GLUCOSE\text{-}CO2}$[6]	146.33	158.83	-	-1.21	58	255	This study
$PHB_{GLYCEROL}$[7]	161.14	167.8	-	-	55	250	This study, Chapter 2
$PHB_{GLUCOSE}$[8]	124.67	142.17	148.67	-6.64	52	270	This study, Chapter 2
PHB_{BIOMER}[9]	149.83	161.83	-	-	60	255	This study

[1]T_m, melting temperature from the second heating run of the differential scanning calorimetry (DSC) endothermic peaks; [2]T_g, glass transition temperature from the second heating run of the DSC endothermic peaks; [3]X_c, degree of crystallinity; [4]T_d, degradation temperature at which 10 wt.% loss occurs; [5]$PHB_{GLYCEROL\text{-}CO2}$, PHB produced using CO_2 as feedstock on biomass grown on waste glycerol; [6]$PHB_{GLUCOSE\text{-}CO2}$, PHB produced using CO_2 as feedstock on biomass grown on glucose; [7]$PHB_{GLYCEROL}$, PHB produced using solely waste glycerol as feedstock; [8]$PHB_{GLUCOSE}$, PHB produced using solely glucose as feedstock; [9]PHB_{BIOMER}, commercial PHB produced by Biomer.

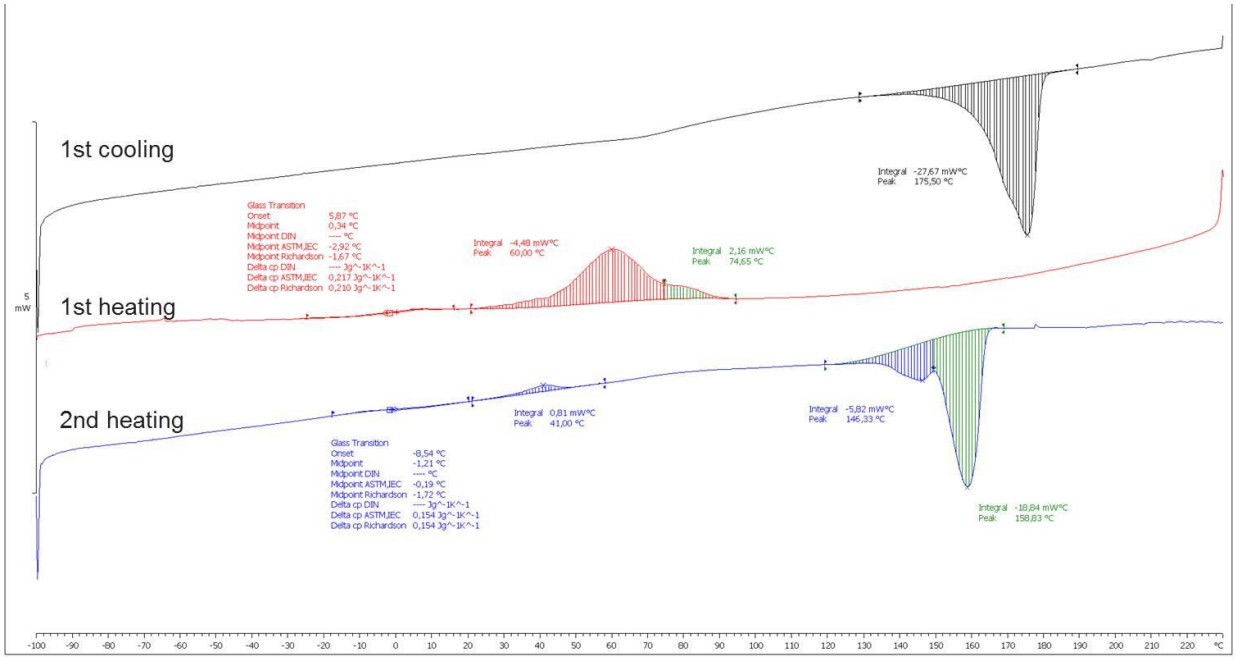

Figure 6.5. Differential scanning calorimetry (DSC) analysis of polyhydroxybutyrate (PHB) extracted from a two-stage cultivation of *C. necator* DSM 545 using glucose in the heterotrophic phase and a gas mixture composed of H_2:O_2:CO_2 = 84.0:2.8:13.2 in the autotrophic phase ($PHB_{GLUCOSE\text{-}CO2}$). Glass transition temperature (T_g), melting temperature (T_m) and total melting enthalpy (ΔH_m) were determined from the second heating run of the DSC endothermic peaks.

In addition, the thermal properties of this polymer were similar to those of commercial PHB (PHB_{BIOMER}). It should be noted that recrystallization was observed for $PHB_{GLYCEROL\text{-}CO2}$ during the second heating. The degradation temperature (T_d) was on the other hand the highest for $PHB_{GLYCEROL\text{-}CO2}$, however all polymers present degradation temperatures above 250 °C. The degree of crystallinity (X_c) was similar for all polymers.

Our results are in accordance with the general thermal properties for PHB as reported in the literature (Sudesh et al., 2000; Volova et al., 2013a), where a crystallinity degree comprised between 50-80%, a glass transition temperature (T_g) between -4°C and 1°C and T_m about 160 to 180°C have been reported.

6.3.2.4. Evaluation of the biopolymer's characteristics

Although CO_2 was used as carbon source for PHB production, the organic substrate of the heterotrophic phase affected the properties of the polymer. The biopolymer produced by autotrophic production on glucose-grown cell mass had a higher molar mass and T_m compared to PHB produced from CO_2 that followed heterotrophic growth on waste glycerol, indicating less impurities present in the crystallites and higher degree of perfection of the crystallites. The degradation temperature was on the other hand the highest for the latter polymer. Despite these differences, it can be concluded that *C. necator* DSM 545 produced the homopolymer PHB from CO_2 in the heterotrophic-autotrophic cultivation system with comparable characteristics to those of a commercial biopolymer and PHB typically reported in literature.

6.4. Conclusions

- PHB was produced from CO_2 using a heterotrophic-autotrophic cultivation system at an oxygen concentration below the LEL for two organic substrates, namely glucose and waste glycerol, by limiting nitrogen and oxygen at three cell mass concentrations.
- PHB production from CO_2 on waste-glycerol grown cell mass under conditions relevant for industrial application resulted in the highest reported PHB production synthesized from CO_2 so far.
- Independent of the organic carbon source, the fermentation performance decreased when delaying nutrient limitation at a higher cell mass concentration.
- The low mass transfer rate of oxygen was the main bottleneck for obtaining high PHB productivity and PHB production from CO_2.

- Characterization of the produced polymers showed that the organic carbon source affected the properties of PHB. Overall, the proposed cultivation method however provides PHB with similar properties to a commercial PHB and PHB typically found in literature.
- In order to compete with the current heterotrophic cultivation systems, research work should focus on increasing the oxygen mass transfer rate.

Chapter 7:

Modeling and optimization of heterotrophic-autotrophic polyhydroxybutyrate (PHB) production

Abstract

In this study, heterotrophic-autotrophic polyhydroxybutyrate (PHB) production was modelled based on previously established models for heterotrophic-heterotrophic and autotrophic-autotrophic PHB production processes. The model was validated on experimental datasets obtained with different organic substrates and applying different switching points from growth phase to PHB production phase. The developed mathematical model provided an accurate prediction of the dynamic behavior of heterotrophic biomass growth and autotrophic PHB production. The effect of oxygen (O_2) and ammonium-N (NH_4^+-N) on biomass growth and PHB production were investigated. Moreover economic evaluation based on the cost of feedstock for PHB production was explored.

Keywords: polyhydroxybutyrate (PHB), heterotrophic-autotrophic cultivation, CO_2, mathematical modelling, simulation.

7.1. Introduction

Polyhydroxybutyrate (PHB) is an intracellular storage material that is synthesized by a number of microorganisms and has become of considerable industrial interest and of environmental importance as a biodegradable and bio-based plastic. Although PHB is regarded as an effective substitute for conventional plastics, commercialization of this biopolymer is hampered by its high production cost compared to other (bio)polymers (Chanprateep, 2010).

PHB is mostly produced through pure-culture fermentation, in which a cell growth phase under nutrient-sufficient conditions is followed by a PHB production phase triggered by applying nutrient (typically nitrogen) limitation, making up a two-phase process. Most often heterotrophic conditions are applied during both phases, employing a wide variety of organic substrates, either pure substrates such as glucose, sucrose, starch, or cellulose, or waste substrates such as molasses, whey and glycerol. However, also autotrophic PHB production is possible by applying bacteria which use carbon dioxide (CO_2) as a carbon source and hydrogen (H_2) as an energy source (Ishizaki and Tanaka, 1991; Sugimoto et al., 1999; Volova et al., 2013b). Autotrophic PHB production is an interesting process option to reduce the concentration of the greenhouse gas CO_2 and in this way contribute to climate change mitigation. However, autotrophic PHB production is limited by the fact that the oxygen (O_2) concentration in the gas phase needs to be kept below the lower level of explosion, i.e. between 6 and 6.9% O_2 by volume in the gases mixture of CO_2, H_2, and O_2 (Takeshita and Ishizaki,

1996). The O_2 transfer rate becomes very small under this condition, which seriously hampers the cell growth because of O_2 limitation (Tanaka and Ishizaki, 1994). As a result, autotrophic-autotrophic PHB production, i.e. combining autotrophic growth and autotrophic PHB production cannot be realized in an economically feasible way.

To overcome this limitation, a new cultivation method, namely heterotrophic-autotrophic PHB production, was proposed by Tanaka and Ishizaki, (1994). This method consists of a heterotrophic growth (phase 1) on organic substrate, during which nutrient-sufficient conditions are maintained, followed by autotrophic PHB production (phase 2) on CO_2, H_2, and O_2 while applying nitrogen limitation.

Cupriavidus necator (formerly known as *Ralstonia eutropha*, *Alcaligenes eutrophus*, and *Wautersia eutropha*) is a well-known and well-studied hydrogen oxidizing bacterium that has ability to grow under both heterotrophic condition using organic carbon and autotrophic condition using gas mixture (CO_2, O_2 and H_2). In the presence of sufficient O_2 and nitrogen, *C. necator* ($CH_{1.74}O_{0.46}N_{0.19}$) grows on organic substrate ($C_xH_yO_z$) according to Eq 7.1.

$$\begin{aligned} \mathrm{C}_w\mathrm{H}_\mathrm{x}\mathrm{O}_y + (4\mathrm{w} - \frac{y}{2} + \frac{x}{4} - 1.91\mathrm{Y}_X)\ \mathrm{O}_2 + 0.19\ \mathrm{Y}_X\ \mathrm{NH}_4^+ + 0.19\ \mathrm{Y}_X\ \mathrm{NaOH} \\ \rightarrow \mathrm{Y}_X\ \mathrm{CH}_{1.74}\mathrm{O}_{0.46}\mathrm{N}_{0.19} + (\mathrm{w} - \mathrm{Y}_X)\ \mathrm{CO}_2 + \frac{x - 1.55\mathrm{Y}_X}{2}\ \mathrm{H}_2\mathrm{O} + 0.19\ \mathrm{Y}_X\ \mathrm{Na}^+ \end{aligned} \quad (7.1)$$

Under stress condition such as under O_2 and/or nitrogen limitation, *C. necator* has the capability to produce PHB using organic carbon as Eq. 7.2 or using CO_2 as carbon source and H_2 as energy source as Eq. 7.3 (Tanaka and Ishizaki, 1994).

$$\mathrm{C}_w\mathrm{H}_\mathrm{x}\mathrm{O}_y + (\mathrm{w} - \frac{y}{2} + \frac{x}{4} - \frac{9}{2}\mathrm{Y}_P)\ \mathrm{O}_2 \rightarrow \mathrm{Y}_P\ \mathrm{C}_4\mathrm{H}_6\mathrm{O}_2 + (\mathrm{w} - 4\mathrm{Y}_P)\ \mathrm{CO}_2 + (\frac{x}{2} - 3\mathrm{Y}_X)\ \mathrm{H}_2\mathrm{O} \quad (7.2)$$

$$33H_2 + 12O_2 + 4CO_2 \rightarrow C_4H_6O_2 + 30\,H_2O \quad (7.3)$$

Although the heterotrophic-autotrophic process has a high potential to produce PHB from CO_2, till now only a limited number of studies were conducted. Tanaka and Ishizaki (1994) proved the feasibility of the concept using fructose as the carbon source during the heterotrophic growth phase and a gas mixture of CO_2:H_2:O_2=10.3:84.1:6.7 during the autotrophic PHB production phase. Sugimoto and his collaborators (1999) used acetic acid for heterotrophic growth but found a low PHB productivity in the subsequent phase due to the inhibitory effect of acetic acid on enzymes related to autotrophic metabolism. In this thesis (Chapter 6) the feasibility of heterotrophic-autotrophic PHB production was demonstrated using waste glycerol

for the heterotrophic growth phase and maintaining an O_2 fraction lower than 3% in the gas mixture during the autotrophic phase, to ensure safe operational conditions.

In this chapter, heterotrophic-autotrophic PHB production was modelled based on previously established models for heterotrophic-heterotrophic and autotrophic-autotrophic PHB production processes. The model was validated on the experimental datasets of Chapter 6. Subsequently, the model was used in view of process optimization in terms of maximizing PHB production, to examine the influence of operating parameters; O_2 and NH_4^+-*N*.

7.2. Modeling heterotrophic-autotrophic PHB production

7.2.1. Process stoichiometry and kinetics

The model for heterotrophic-autotrophic PHB production was based on previously established models, for heterotrophic-heterotrophic PHB production (Chapter 3) and for autotrophic-autotrophic PHB production (Chapter 5). Four processes were considered: heterotrophic biomass growth on organic substrate and on PHB, PHB production on organic substrate and autotrophic PHB production using CO_2 as carbon source. Autotrophic biomass growth was not taken into account given that the autotrophic phase was only started when the nitrogen concentration had become very low, thus preventing biomass growth. The model stoichiometry and the corresponding kinetics are given in Tables 7.1 and 7.2 respectively. The stoichiometric and kinetic parameter values are listed in the Appendix in Chapter 3 and 5 (Table S3.1 and S5.1).

Cell growth, both on organic substrate and on PHB, is limited by too low concentrations of nitrogen and inhibited when these concentrations are too high, which is modeled through Haldane kinetics. The effect of organic substrate on cell growth is also described by the Haldane kinetics, while growth on PHB is limited (not inhibited) by the intracellular PHB fraction. Monod kinetics was included to describe the limitation effect of O_2 on both biomass growth processes; it was assumed that the O_2 concentration was never sufficiently high to be inhibitory. Cell density inhibition of biomass growth on substrate and PHB (see Chapter 3) was taken into account by a modified logistic growth expression.

PHB production, both heterotrophic and autotrophic, is inhibited by nitrogen, as expressed by a non-competitive inhibition equation; product (PHB) inhibition was modeled through a modified logistic expression.

Table 7.1: Stoichiometry of the heterotrophic-autotrophic PHB production system.

Component → Process ↓	Substrate (S)	Nutrient (N)	Oxygen (O_2)	Residual biomass (X)	PHB (P_{hat})	Process rate
1. Biomass growth on substrate	$-1/Y_{xs}$	$-1/Y_{xN}$	$-1/Y_{xO}$	1		$\mu_{xs}\ X$
2. Biomass growth on PHB		$-1/Y_{xN}$	$-1/Y_{xO}$	1	$-1/Y_{xp}$	$\mu_{xp}\ X$
3. Heterotrophic PHB production	$-1/Y_{ps}$		$-1/Y_{pO}$		1	$\mu_{ps_{het}}X$
Component →	H_2	O_2	CO_2		PHB (P_{aut})	Process rate
4. Autotrophic PHB production	$-1/Y_{pH2}$	$-1/Y_{pO2}$	$-1/Y_{pCO2}$		1	$\mu_{ps_{aut}}X$

Table 7.2: Process kinetics of the heterotrophic-autotrophic PHB production system.

Process	Kinetic expression
1. Biomass growth on substrate	$\mu_{xs} = \mu_{xs}^{max}\left(\frac{S}{K_s+S+\frac{S^2}{K_{IS}}}\right)\left(\frac{N}{K_N+N+\frac{N^2}{K_{IN}}}\right)\left(\frac{O_2}{K_{xO2}+O_2}\right)\left[1-\left(\frac{X}{X_m}\right)^{\alpha}\right]$
2. Biomass growth on PHB	$\mu_{xp} = \mu_{xp}^{max}\frac{f_{PHB}}{K_{PHB}+f_{PHB}}\left(\frac{N}{K_N+N+\frac{N^2}{K_{IN}}}\right)\left(\frac{O_2}{K_{xO2}+O_2}\right)\left[1-\left(\frac{X}{X_m}\right)^{\alpha}\right]$
3. PHB production on organic substrate	$\mu_{ps_{het}} = \mu_{ps_{het}}^{max}\left(\frac{S}{K_{ps}+S+\frac{S^2}{K_{PIS}}}\right)\left(\frac{O_2}{K_{pO2}+O_2+\frac{S_2^2}{K_{PIO2}}}\right)\left[1-\left(\frac{f_{PHB}}{f_{PHB(\max)}}\right)^{\beta}\right]\frac{K_{PIN}}{N+K_{PIN}}$
4. PHB production on gaseous substrate	$\mu_{ps_{aut}} = \mu_{ps_{aut}}^{max}\left(\frac{H_2}{K_{pH_2}+H_2}\right)\left(\frac{O_2}{K_{pO_2}+O_2+\frac{S_2^2}{K_{PIO_2}}}\right)\left(\frac{CO_2}{K_{pCO_2}+CO_2}\right)\left[1-\left(\frac{f_{PHB}}{f_{PHB(\max)}}\right)^{\beta}\right]\frac{K_{PIN}}{N+K_{PIN}}$

Oxygen limitation and inhibition of PHB production, was modeled through Haldane kinetics, and so was the effect of organic substrate on heterotrophic PHB production. Due to their low

solubility, the concentrations of H_2 and CO_2 during autotrophic PHB production were reasonably assumed not to be in the inhibiting range; their limitation effect was modeled through Monod kinetics.

7.2.2. Mass balance

The mass balances over the fermentor for heterotrophic-autotrophic PHB production comprised two main contributions: macroscopic transport (inflows and outflows) and biochemical conversion (transformation of substrate and nutrients into biomass; residual biomass and PHB). The corresponding terms for the heterotrophic growth phase and for the autotrophic PHB production phase were described in Chapter 3 and in Chapter 5, respectively.

The total PHB production in the heterotrophic-autotrophic system was calculated as the sum of the amount of PHB produced during the heterotrophic growth condition (P_{het}) and the produced PHB under autotrophic condition (P_{aut}).

7.2.3. Model validation

The model was applied to simulate the four experimental datasets described in Chapter 6. The Nash-Sutcliffe model efficiency coefficient (E) (Nash and Sutcliffe, 1970) was used to quantitatively describe the accuracy of model outputs and in this way assess the predictive power of the model. Essentially, the closer the model efficiency is to 1, the more accurate the model is.

7.3. Results and discussion

7.3.1. Model validation

The model for heterotrophic-autotrophic PHB production was validated on the experimental datasets presented in Chapter 6. These datasets differed in the substrates applied during the heterotrophic growth phase, being pure glucose or waste glycerol, and in the RCC for which the autotrophic PHB production phase was initiated, namely around 5 and 16 g/L RCC.

Figure 7.1 displays the model validation results for heterotrophic-autotrophic PHB production using glucose or waste glycerol for growth. During the heterotrophic growth phase, sufficient substrate (initially 10 g/L glucose), O_2 (55% of air saturation) and nitrogen (initially 0.75 g/L NH_4^+-N) concentrations were maintained to favor growth instead of PHB production. When the desired RCC was achieved, nitrogen limitation was imposed to stimulate PHB production, by stopping the NH_4^+-N feeding. The organic substrate feeding was stopped and a gas mixture of

H_2:O_2:CO_2=84:2.8:13.2 vol% was supplied, thus realizing autotrophic conditions. The model describes the experimental observations obtained with glucose quite well, with Nash-Sutcliffe model efficiency coefficients E=0.91 (0.92) for RCC and E=0.87 (0.81) for PHB when the autotrophic phase at 5 (respectively 16) g/L RCC (Figure 7.1a-b). Also for the experimental data using waste glycerol for growth, the model predictions agreed well with the experimental results in terms of RCC, PHB and total biomass concentration (Figure 7.1c-d). The E-values corresponding with an operation of autotrophic phase at 5 (16) g/L RCC are 0.95 (0.82) for RCC and 0.86 (0.97) for PHB, all of which are close to 1, thus indicating a good model fit.

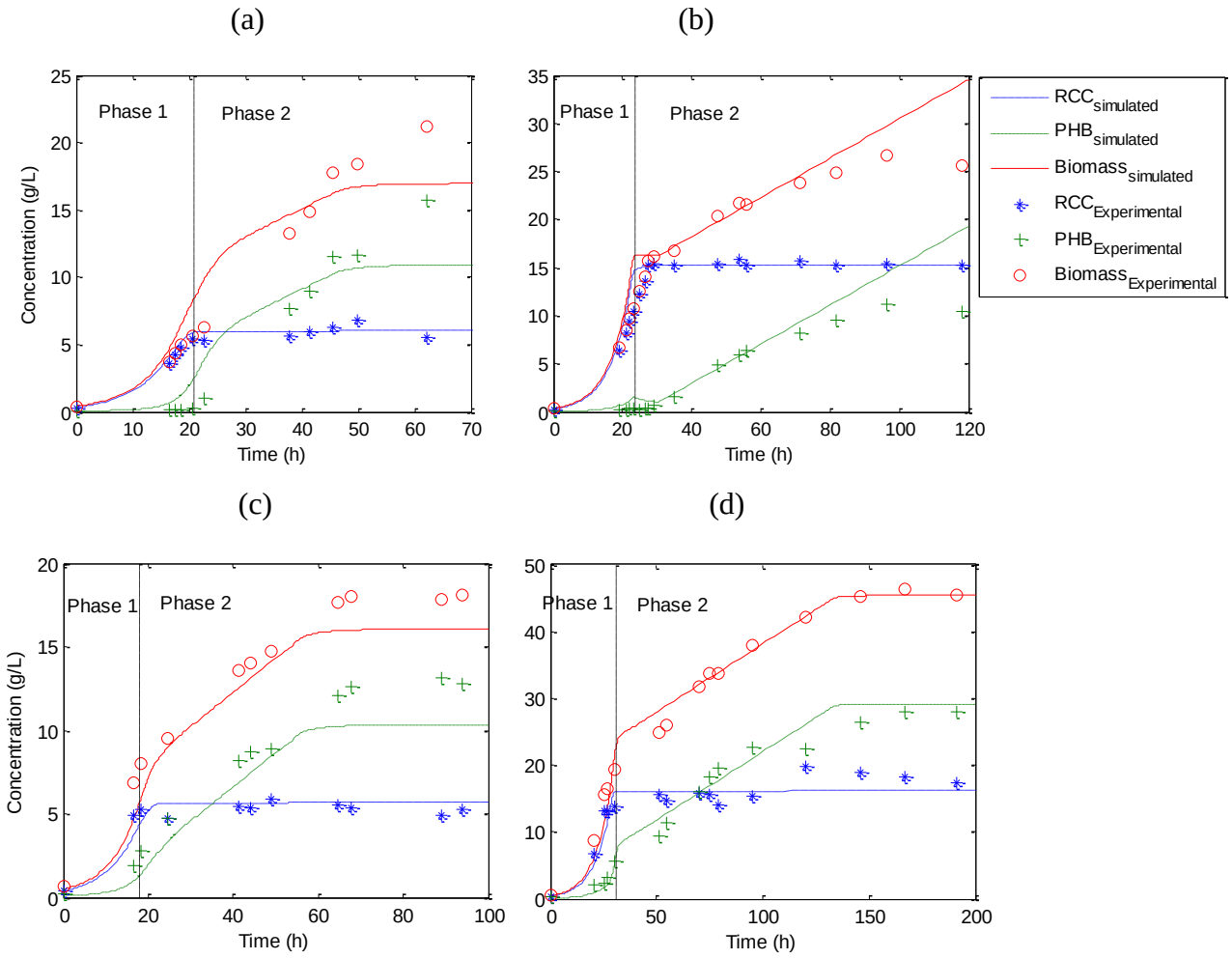

Figure 7.1. Validation of the model, for heterotrophic-autotrophic PHB production, with $f_{PHB(\max)}$=1.78, using glucose (a, b) or waste glycerol (c, d) as a substrate in the heterotrophic phase (phase 1) and starting the autotrophic phase at 5 g/L RCC (a, c) and 16 g/L RCC (b, d).

Even though the simulation results match the experimental data quite well, it can be noted that the final biomass and PHB production are not so well predicted in the cases of Figure 7.1a-b-c, while better results are obtained in the case of Figure 7.1d. This could be remedied (see Figure 7.2) by adjusting the value of the maximum PHB to RCC ratio, $f_{PHB(\max)}$, according to the experimental data obtained in Chapter 6, instead of using the default value $f_{PHB(\max)}$=1.78 (Chapter 5).

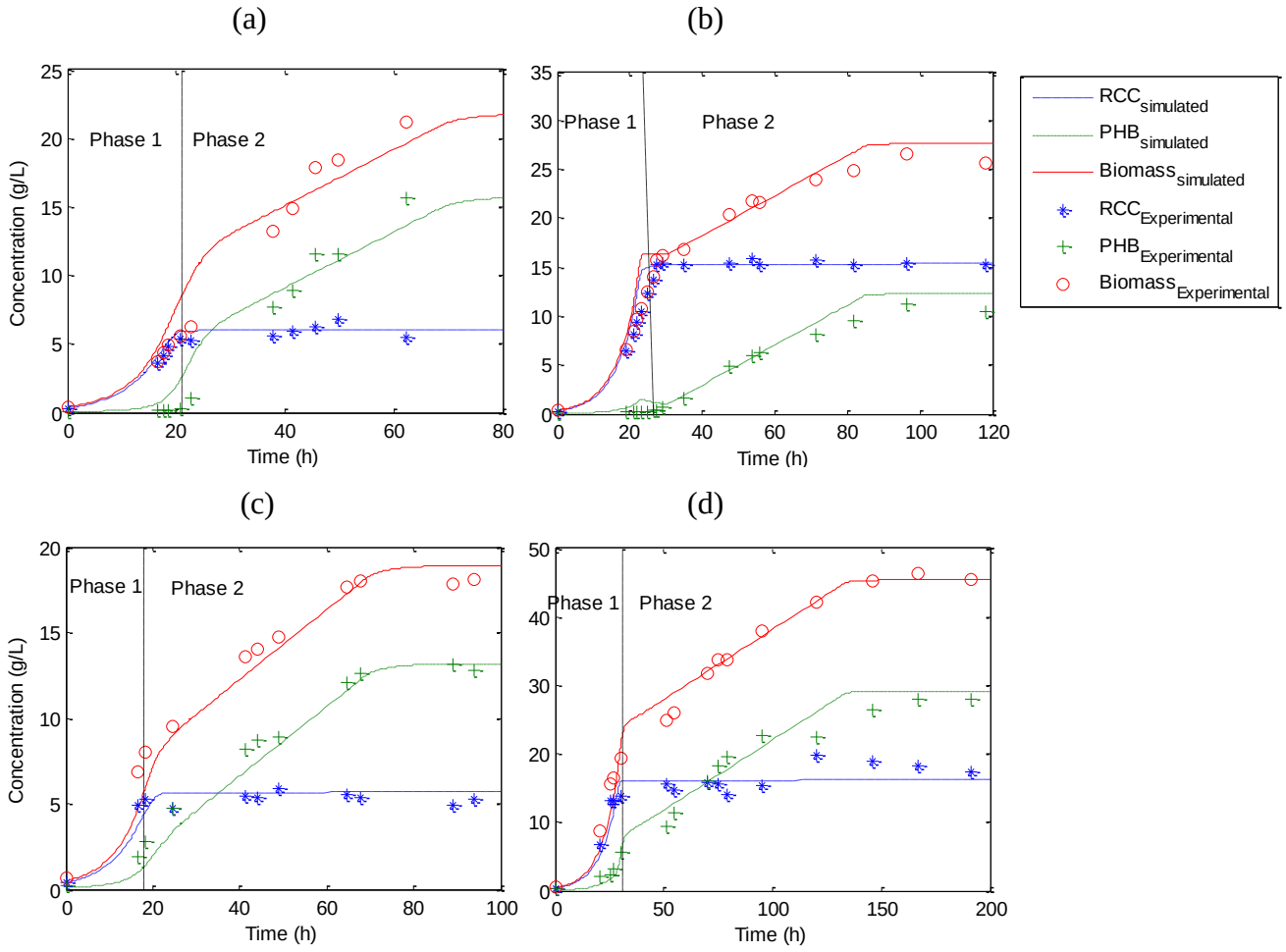

Figure 7.2. Validation of the model validation for heterotrophic-autotrophic PHB production, with adjustment of $f_{PHB(\max)}$ according to Table 7.3, using glucose (a, b) and waste glycerol (c, d) as a substrate in the heterotrophic phase (phase 1) and starting the autotrophic phase at 5 g/L RCC (a, c) and 16 g/L RCC (b, d). Comparison between the simulation outcome (full lines) and experimental observations (discrete markers).

Apparently the maximum PHB to RCC ratio obtained during autotrophic PHB production depends on the organic substrate used in and on the duration of heterotrophic growth phase. The $f_{PHB(\max)}$ decreased when delaying the start of the autotrophic PHB production phase

(Table 7.3). This is in line with the findings of Friedrich et al. (1981), who stated that the specific activity of the enzymes linked to autotrophic metabolism but synthetized during heterotrophic growth was considerably affected by a prolonged heterotrophic growth phase. Besides, $f_{PHB(\max)}$ also depends on the organic substrate used in heterotrophic phase (Table 7.3). Further research is recommended to elucidate the mechanisms behind both phenomena, leading to the establishment of correlations for their description, which could be integrated into the model.

Table 7.3: Dependency of maximum PHB to RCC ratio, $f_{PHB(max)}$ on organic substrate type and switching conditions.

Organic substrate in heterotrophic phase	RCC at the autotrophic phase	$f_{PHB(\max)}$	Reference
Fructose	4	5.6	Tanaka and Ishizaki, 1994
Fructose	10	1.3	Tanaka and Ishizaki, 1994
Fructose	15	1.3	Tanaka and Ishizaki, 1994
Acetic acid	5	1.2	Sugimoto et al., 1999
Glucose	5	(16/5)=3.2	Calculated from Chapter 6
Glucose	16	(11/16)=0.7	Calculated from Chapter 6
Waste glycerol	5	(13/5)=2.6	Calculated from Chapter 6
Waste glycerol	16	(28/16)=1.8	Calculated from Chapter 6

During the heterotrophic growth phase, organic substrate and nitrogen were fed in stoichiometric amounts such that they were both consumed when switching to the autotrophic PHB production phase. While an ideal stoichiometric ratio can easily be applied and maintained during simulation, in practice dosing of nitrogen or glucose had to be adjusted just before switching to the second phase, due to ammonia stripping and/or inaccuracies in the feeding equipment. Besides, transition between the two phases took around 30-40 minutes during which, sometimes a small amount of substrate had to be provided to guarantee the survival of the bacteria. During simulation, additional substrate or nitrogen dosages associated with the switching phase were not accounted for as such, but lumped into the initial dosages of substrate and nitrogen concentration. Nevertheless, the simulation results still matched the experimental data very well.

The good agreement between the experimental data and simulation results is remarkable, given that no model calibration was made for heterotrophic-autotrophic PHB production as such, but the model structure and all model parameters - except $f_{PHB(\max)}$ - were obtained for heterotrophic-heterotrophic PHB production (Chapter 3) and autotrophic-autotrophic PHB production (Chapter 5). This underlines the general applicability of the developed model.

7.3.2. Optimization of the heterotrophic-autotrophic process

The validated model was applied for scenario analysis, to determine the optimal operation conditions, in terms of O_2 and nitrogen concentrations in the fermentation medium, resulting in maximum specific biomass growth rate and maximum specific PHB production rate. In all cases glucose was used as carbon source during heterotrophic growth (phase 1) and nitrogen limitation was imposed at 5 g/L RCC to shift to the PHB production phase ($f_{PHB(max)}$=2.6). A gas mixture of H_2:O_2:CO_2 was supplied for autotrophic PHB production (phase 2).

7.3.2.1. Effect of dissolved oxygen concentration on growth and PHB production

The role of the dissolved oxygen (DO) concentration on specific biomass growth and specific PHB production rate was examined through model simulation. During the growth phase (phase 1) nitrogen was maintained at 0.7 g/L NH_4^+-*N*, while the PHB production phase (phase 2) was nitrogen free. Figure 7.3 displays the (total) specific growth rate, on substrate and on PHB, and the (total) specific PHB production rate, both autotrophic and heterotrophic, in terms of the DO concentration. The biomass growth rate remained almost the same for DO concentrations above 1.5 mg/L (equivalent to 20% air saturation concentration) – note that the model implicitly assumed that the O_2 concentration was never sufficiently high to be inhibitory for growth. Below 1.5 mg O_2/L, the growth rate decreased due to O_2 limitation.

As for PHB production, the optimal oxygen concentration was found at 0.224 mg/L (equivalent to 3% air saturation concentration). Below and above this concentration the specific PHB production rate decreases due to limitation and inhibition, respectively.

The optimal O_2 concentration for maximum biomass growth is higher than that for maximum PHB production. This is not only true for heterotrophic biomass growth, but also for autotrophic biomass growth (see Chapter 5, Figure 5.1). During autotrophic growth on CO_2 and H_2, the high optimal O_2 levels to reach maximum biomass growth cannot be maintained for safety reasons (explosion risk). For this reason, the heterotrophic-autotrophic process is

preferred over the autotrophic-autotrophic PHB production process (Tanaka and Ishizaki, 1994).

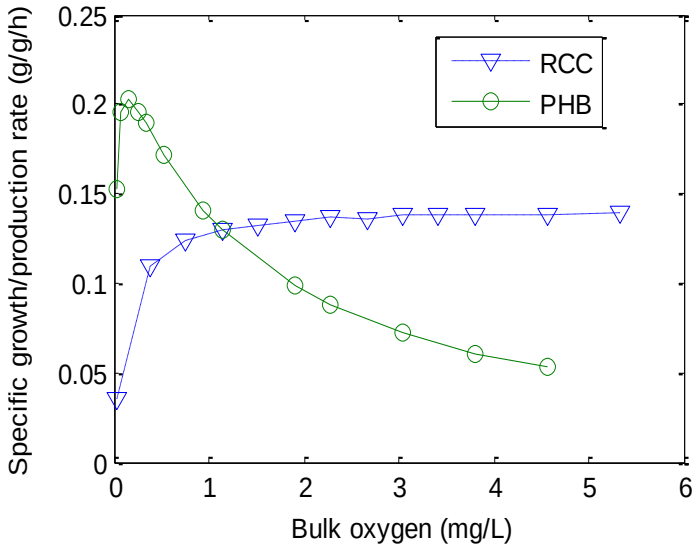

Figure 7.3. Effect of O_2 concentration on specific biomass (RCC) growth rate and specific PHB production rate. Biomass growth was conducted at 0.70 g/L NH_4^+-*N*; PHB production was under nitrogen-free conditions.

7.3.2.2. Effect of ammonium-N (NH_4^+-*N*) on growth and PHB production

The effect of NH_4^+-*N* concentration on biomass growth and PHB production is summarized in Figure 7.4. The O_2 concentration was maintained at 4.18 mg/L in the growth phase (55% air saturation, as in Chapter 2) and at 2.28 mg/L (30% air saturation) in the PHB production phase. NH_4^+-*N* has both a limitation and inhibition effect on growth; the maximum specific growth rate was observed at a nitrogen concentration of 0.6-0.7 g/L. Below this concentration the growth rate decreased due to limitation; a linear decrease of the maximum specific growth rate with increasing nitrogen concentrations above this value was observed due to inhibition. This linear decrease corresponds with the findings of Belfares et al. (1995) for the same organism (*C. necator*) who reported a linear inhibition effect at NH_4^+-*N* concentration above 2 g/L. Gahlawat and Srivastava (2013) found the same optimal nitrogen concentration of 0.7 g/L for *Azohydromonas australica*, of which the growth rate increased with increasing NH_4^+-N concentrations below this value and then decreased, first slowly and then rapidly once beyond 11 g/L NH_4^+-*N*. It should be noted however that there are no merits to work with such high nitrogen concentrations. In most fed-batch experiments, NH_4^+-*N* feeding is regulated by the pH

control, using NH_4OH as a base. The ratio of NH_4^+-N consumption and H^+ production during the growth (phase 1) is 1:1 (according to Eq. 7.1), therefore there is no possibility of overfeeding or accumulation of NH_4^+-*N*, such that nitrogen inhibition will not be observed in practice. On the other hand during growth, the fermentation medium is rich in nitrogen, magnesium and phosphate, which could lead to the production of struvite ($NH_4MgPO_4 \cdot 6H_2O$) crystals (Scudder 1928), resulting in a decreased NH_4^+-*N* concentration, as observed in Chapter 2 during growth. So from a technical point of view, the most challenging part in realizing the optimal nitrogen concentration corresponding with the maximum growth rate, is to overcome the NH_4^+-*N* limitation effect. The specific PHB production rate was the highest when NH_4^+-*N* became zero (Figure 7.4), which is in agreement with the fact that nitrogen stress is applied to stimulate PHB production (Cavalheiro et al., 2012; Tan et al., 2011).

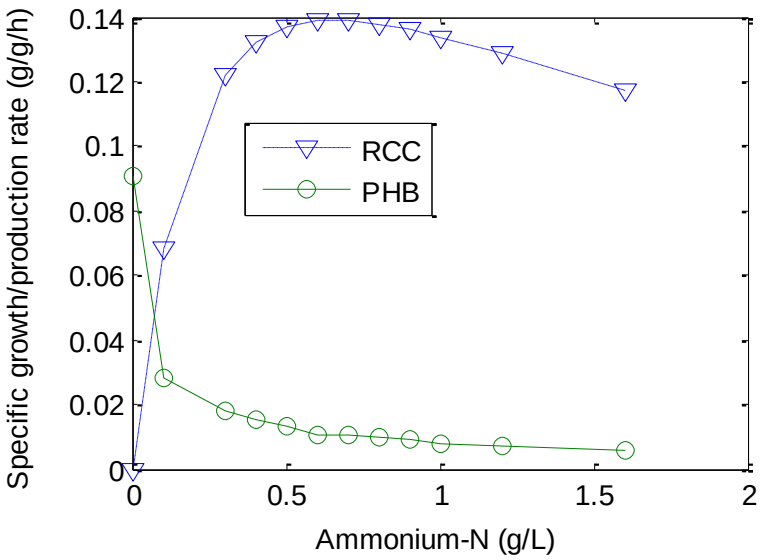

Figure 7.4. Effect of NH_4^+-*N* concentration on specific biomass (RCC) growth rate and specific PHB production rate. The biomass growth was conducted at 4.18 mg/L and PHB production was at 2.28 mg/L O_2 concentration.

7.4. Economic evaluation of PHB production

In this study, two biotechnological processes, heterotrophic-heterotrophic (Chapter 2) and heterotrophic-autotrophic (Chapter 6), have been investigated for the production of PHB. The PHB productivity, PHB content, PHB yield and the cost of substrates considerably affect the overall production cost of PHB (Choi and Lee 1997). On the basis of the theoretical mass

balances of PHB production over glucose (Eq. 7.1) and taking into account an approximate glucose price of 450 euro/ton,, the carbon substrate cost is 1.89 euro/kg PHB while for waste glycerol it is 1.2 euro/kg PHB. Today, the price of PHB is 4.5 euro/kg of which the raw material costs account for 40 to 50% of the production costs (Shen et al., 2009). The production cost of PHB is still much higher compared to conventional petroleum-based plastics such as polyethylene (PE) or polyethylene terephthalate (PET) which ranges between 1.10-1.50 euro/kg, and biobased polymers such as polylactic acid (PLA, 1.9 euro/kg) (Shen 2009). Industry expects however that the PHB price will drop along with their capacity expansions. In the heterotrophic-autotrophic PHB production process, the total substrate cost (glucose, H_2 and CO_2) is 2.65 euro/kg PHB, of which 80% is taken up by H_2. Although based on the substrate cost and obtained experimental results, heterotrophic PHB production seems more attractive, 3.18 kg and 1.08 kg CO_2 are emitted to produce 1 kg of PHB from glycose and glycerol respectively, while 1.53 kg CO_2 is converted to produce 1 kg of PHB under heterotrophic-autotrophic process. A dedicated attributional life-cycle assessment of the whole process is essential to measure and map the environmental benefits of the autotrophic fermentation process. The possible future application of carbon taxes could make heterotrophic-autotrophic PHB production more attractive. Europe and USA imposed carbon taxes for the use of fossil fuel either in energy production or transportation (OECD, 2013). Still now no country applied the carbon tax for the CO_2 emission from non-energy industrial processes. According to the report of Carbon Tax Center (CTC), USA, CO_2 releases for converting fossil fuels to plastics or petrochemicals in non-combustion processes will be taxed very soon at the same level as CO_2 emitted in the production of energy or for transportation purposes.

Besides the imposement of the carbon tax, the heterotrophic-autotrophic PHB production process could become more efficient by increasing the PHB productivity and the PHB content, e.g. by maintaining the O_2 and NH_4^+-N concentrations at their optimal level during both the growth phase and the PHB production phase, as demonstrated in this chapter.

Table 7.4: Substrate cost for heterotrophic-heterotrophic and heterotrophic-autotrophic process.

Process	Substrate cost			CO_2 emission (kg CO_2/kg PHB)
	Phase I (euro/kg PHB)	Phase II (euro/kg PHB)	Total (euro/kg PHB)	
Heterotrophic-heterotrophic (glucose[1])	0.39	1.50	1.89	3.18
Heterotrophic-heterotrophic (waste glycerol[2])	0.39	0.82	1.20	1.08
Heterotrophic-autotrophic (glucose – H_2[3]/CO_2[4])	0.39	2.27	2.65	-1.53
	Total production cost (euro/kg)			
Commercial PHB	1.12-1.60[5]			-
Polyethylene (PE)	1.50[5]			-
Polyethylene terephthalate (PET)	1.10-1.50[5]			-
PLA	1.9[5]			

[1]Price of glucose: 450 euro/ton (market price)
[2]Price of waste glycerol: 450 euro/ton (price given by Oleon, Belgium)
[3]Price of H_2: 2800 euro/ton (market price)
[4]Price of CO_2: 65 euro/ton (market price)
[5]Shen 2009

7.5. Conclusions

- A mathematical model for heterotrophic-autotrophic PHB production process was developed based on existing models for heterotrophic-heterotrophic PHB production and autotrophic-autotrophic PHB production. The simulation results matched experimental data very well, without the need for calibration.
- While it is generally known that oxygen stress conditions stimulate PHB production, too low O_2 concentrations may result in oxygen limitation. The optimal O_2 concentration for PHB production was determined as 0.224 mg/L.

- The optimal nitrogen concentration for biomass growth was 0.60-0.70 g /L NH_4^+-*N*, while PHB production was maximal under nitrogen free condition.
- Heterotrophic-autotrophic PHB production is currently economically less attractive than heterotrophic-heterotrophic PHB production. Further process optimization and possible carbon taxes may stimulate its application in future.

PART IV:

Epilogue

Chapter 8:

Conclusions and future perspectives

The objective of this thesis was to optimize the pure culture polyhydroxybutyrate (PHB) production process in terms of productivity, using either pure or waste organic substrate or CO_2 as inorganic substrate.

The global demand for PHB is increasing: while in 2013 the production capacity amounted to 34,046 tonnes, market data forecast the global production capacities will increase to approximately 40,656 tonnes by 2015 and to 141,351 tonnes by 2018 (European Bioplastics, 2014). Industrial PHB production mainly relies on pure culture fermentation using organic substrates such as glucose and sucrose. Typically a two-phase process is applied, involving a cell growth phase under nutrient-sufficient conditions (phase 1), followed by a PHB production phase triggered by applying nutrient (typically nitrogen) limitation (phase 2). In order to meet the increasing demand for PHB and at the same time reduce the dependency on food substrates, alternative substrates, alternative process configurations and alternative process operation strategies were evaluated in this doctoral research.

8.1. Effect of substrate on PHB production

Three different substrates were evaluated for PHB production: glucose as a pure organic substrate, waste glycerol as a waste organic substrate and a gas mixture of CO_2, H_2 and O_2. In the latter case, when the gas mixture was used for PHB production, biomass growth was preceded by heterotrophic growth on either glucose or waste glycerol.

Table 8.1: Maximum PHB production and productivity obtained in this doctoral research work through different substrates and processes.

Process	Substrate	Final PHB production (g/L)	PHB productivity (g/L/h)	PHB content (%)
Heterotrophic-Heterotrophic	Glucose-glucose	125	2.02	76.5
	Waste glycerol-waste glycerol	65.6	1.36	62.7
Heterotrophic-Autotrophic	Glucose-pure gas mixture	15.7	0.25	76
	Waste glycerol-pure gas mixture	28	0.168	61

The most efficient cultivation method in terms of final PHB production and productivity was achieved when applying glucose as the sole carbon source during fermentation (Table 8.1). Implementation of this process at industrial scale can however result in a lower PHB productivity due to oxygen mass transfer limitation. An optimal balance between PHB productivity and final PHB production should be found. The PHB production obtained from waste glycerol during this doctoral research work (Chapter 3) was the highest reported value for PHB production from waste glycerol and was even higher than reported for pure glycerol. To achieve a higher process efficiency, waste glycerol could be refined to remove the inhibitory impurities. Since such pretreatment step would increase the production costs, the improvement in process efficiency should be balanced against the total economics. Another option would be to use genetically modified organisms that can better cope with the stress of impurities. Although PHB production from CO_2 on waste-glycerol grown cell mass also resulted in the highest reported PHB production synthesized from CO_2 so far under non-explosive conditions (Chapter 6), its process performance is still far from the heterotrophic processes. Future research should focus on increasing the mass transfer rate of O_2.

8.2. Biopolymer characterization

Characterization of produced PHB (from all of three substrates) confirmed that all of them consisted of hydroxybutyrate (HB). However, the organic carbon source affected the PHB properties: PHB produced from pure glucose has a higher molecular weight and a lower polydispersity index compared to PHB which was produced from waste glycerol and from an inorganic gas mixture. PHB produced from the latter two sources showed very similar physical and thermal properties, which were also similar to the characteristics of commercial PHB and of PHB reported in literature (Chapter 6). In addition, it was observed that the cultivation time did not affect the molecular weight of PHB.

Until now, autotrophic biopolymer production targeted PHB, while little research efforts have focused on the PHA copolymers (such as PHBV) from CO_2 (Park et al., 2014). These copolymers have a greater ductility and toughness than PHB. Such characteristics could extend the applications of PHA. Therefore, further research should be oriented towards the production of PHA copolymers from CO_2 by adding co-substrates during phase 2.

8.2. Process control

8.2.1. Substrate control strategy

For heterotrophic-heterotrophic PHB production, the optimal glucose and glycerol concentrations, leading to the maximum growth rate of *C. necator* DSM 545, were determined as 10-20 g/L and 10-30 g/L respectively (Chapter 2). A combined (organic) substrate feeding strategy consisting of exponential feeding and feeding based on alkali-addition monitoring resulted in a maximum cell concentration during the biomass growth phase (phase 1). In the PHB production phase (phase 2), a constant substrate feeding strategy based on the estimated amount of biomass produced in the phase 1 and a specific PHB accumulation rate was implemented to control the substrate concentration. The PHB production was triggered using nitrogen limitation. Overall, the developed three-stage substrate feeding strategy was well able to control the substrate within its optimal range and was independent from the type of organic substrate (Chapter 2). The developed substrate control strategy has the additional advantages of being sensitive (i.e., feeding based on small change in pH), robust (i.e., independent of PHB producing organism) and inexpensive. In addition, this control strategy could be used for other types of fermentation processes that require pH control to achieve high cell densities. The main limitation of the developed feeding strategy and the current existing methods is the lack of a feedback control parameter to determine the end point of substrate feeding. To maximize the the product yield on substrate, substrate feeding could be stopped upon the observed pH decrease at the end of the fermentation process. This would result in a 50% reduction of the residual carbon source. However, the actual reason for the observed pH decrease is still unclear and urges for further investigation.

During heterotrophic-autotrophic PHB fermentation (Chapter 6), biomass was grown under heterotrophic conditions maintaining the substrate concentration in its optimal range using the same combined feeding strategy as in Chapter 2. In the following autotrophic PHB production phase, the O_2 concentration was maintained below the lower explosion limit (LEL) maintaining a gas composition of H_2:O_2:CO_2 = 84:2.8:13.2 (vol%) (Chapter 6). The gas composition was analysed through an online gas-chromatograph (GC) and adjusted by an in-house developed software program and by supplying the individual gases.

8.2.2. Effect of time instant for switching to PHB production

For heterotrophic PHB production on pure glucose, the time instant at which stress conditions were imposed, to switch to the PHB production phase, hardly affected the PHB content. A

higher PHB production was obtained by delaying the application of stress condition, i.e. when applying nitrogen limitation at higher cell densities. However, at very high cell densities, problems arose in controlling the produced foam during the process (Chapter 2).

When using waste glycerol as a substrate, the obtained final PHB content decreased by delaying the time instant at which nitrogen stress was applied, i.e. with increasing cell concentrations. This was attributed to the accumulated impurities, sodium in particular, which probably imposed an osmotic stress on the cells (Chapter 3). Also when using NaOH for pH control during the growth phase (phase 1), a lower final PHB content and a lower PHB production was obtained when applying the nitrogen stress at a higher cell concentration, when delaying the time instant of the phase shift (Chapter 4). This contrasts with the observations without sodium inhibition (Chapter 2).

During autotrophic-autotrophic PHB production, O_2 limitation occurs at a certain cell mass concentration, resulting in an automatic shift to the PHB production phase (Chapter 5). Below this critical concentration, increasing the cell concentration in phase 1 does not affect the PHB content but results in a higher PHB production.

As for heterotrophic-autotrophic PHB production, the final PHB content decreased when prolonging the heterotrophic growth phase, i.e. by delaying the application of stress condition. This was explained through damage on the autotrophic growth ability of the microorganisms resulting from the longer heterotrophic growth period, however further research is necessary to elucidate the underlying mechanism (Chapter 6).

8.2.3. Effect of mass transfer of gaseous substrate

The mass transfer of the gaseous substrates from the gas phase to the liquid phase is determined by a number of factors, including the gas mixture composition, the applied pressure and the mass transfer coefficient. There is an optimal gas composition of H_2, O_2 and CO_2 which should be applied in both phases leading to maximal PHB production (Chapter 5). However, this composition cannot be maintained in practice because of explosion risks at high O_2 fractions. When maintaining the O_2 concentration below the lower explosion limit (LEL), the mass transfer limitation of O_2 became a barrier in achieving a high PHB production (Chapter 6). Further improvement of the heterotrophic-autotrophic process to increase the O_2 mass transfer by high-pressure fermentations or the addition of external devices such as hollow fibre membrane and/or rotor-stator spinning disc could be tested. Possible adverse effects of high pressure on the PHB producing organism needs to be accounted for.

Since biomass growth allows higher O_2 concentration compared to the PHB production phase, the heterotrophic-autotrophic process is more favourable over the autotrophic-autotrophic PHB production process to keep a high productivity while minimizing the risk of explosion (Chapter 7).

8.3. Role of modeling and simulation

During this doctoral research, mathematical models for pure culture PHB production were developed based on mass balances, process stoichiometry and kinetics. The aim was to describe biomass growth and PHB production and for subsequent use in view of process optimization. All the kinetics were considered as independent process although there are metabolic interactions between growth and PHB production based on substrate. These metabolic interactions on substrate did not affect the heterotrophic-heterotrophic model (Chapter 3 and 4) as the substrate concentration was assumed to be constant.

Dedicated models for heterotrophic-heterotrophic PHB production were set up to evaluate important aspects such as cell density inhibition on growth, biomass growth on PHB, PHB inhibition (Chapter 3) and sodium inhibition on both biomass growth and PHB production (Chapter 4), which had not been considered previously for pure culture PHB production processes. From the evaluation of these models it was found that PHB production took place during the growth phase and the produced PHB was simultaneously used for cell growth (Chapter 3). Sodium was found to have a non-linear inhibition effect on both the specific biomass growth rate and the final PHB content, and a linear inhibition effect on the specific PHB production rate (Chapter 4). Given that the extent of PHB production inhibition by sodium depends on type of bacteria and on the culture medium, the parameters related to sodium inhibition need to be adjusted accordingly.

The uncertainty of estimated parameters were determined through the Fisher Information Matrix (FIM) but unrealistically high errors (standard deviations) were obtained (results not shown). This was attributed to the highly nonlinear model structure, reflected by a strong effect of the parameter perturbation range on the results. Besides, a strong correlation between parameters was observed. These issues could be remedied by performing a more detailed sensitivity analysis, resulting in optimal experimental designs determining the operating conditions leading to improved identifiability and more reliable estimation of parameters.

Based on the developed model, a maximum PHB production and concentration of 134 g/L and 2.33 g/L/h could be theoretically achieved when triggering PHB biosynthesis at 63 g/L RCC

applying glucose as carbon source and the process conditions (T, pH, DO etc) as stated in Chapter 2. However, under these conditions, excessive foaming was observed during cultivation (Chapter 2). Furthermore, at industrial scale, meeting the high oxygen demand - wich is paired with high PHB productivity - will be the main challenge.

The models developed for autotrophic-autotrophic PHB production (Chapter 5) and for heterotrophic-autotrophic PHB production (Chapter 7) were the very first efforts in describing these processes in view of optimizing PHB production. Both autotrophic-autotrophic and heterotrophic-autotrophic models were validated with experimental data sets and used for subsequent process optimization. O_2 played a vital role on the overall process; both cell growth and PHB production needed O_2 as substrate, but low O_2 concentrations stimulated PHB production while high concentrations inhibited it (Chapter 7). The optimal dissolved oxygen (DO) concentration for autotrophic biomass growth and PHB production was predicted as 2.25 mg/L (20% air saturation) and 0.25 mg/L (3% air saturation), respectively. If these DO concentrations would have been used in a heterotrophic-autotrophic set-up, the PHB productivity could be improved by a factor of 4 (i.e., 0.67 g/L/h) when triggering PHB biosynthesis at 15 g/L RCC with waste glycerol as carbon source for cell growth under the process conditions (T, pH, NH_4^+-N, pressure, k_La etc) as stated in Chapter 6.

The continuous growth of residual biomass in the PHB production phase under O_2 stress conditions resulted in higher cell density compared to nitrogen stress condition, leading to a high PHB production (Chapter 5). The PHB productivity in heterotrophic-autotrophic culture could be further improved to 0.77 g/L/h by applying oxygen stress condition with DO concentration 0.25 mg/L and maintaining 0.50 g/L NH_4^+-N in PHB production phase. The high oxygen demand to obtain this high PHB productivity in a heterotrophic-autotrophic process will however be the main challenge.

8.4. Sustainability of PHB production

A full cradle-to-gate Life Cycle Assessment (LCA) study for PHB production by fermentation using renewable organic feedstocks (Harding et al., 2007), showed that PHB production is superior to polyolefins production in all life cycle categories, removing any doubts raised on the merits of PHB production. Including the effects of polymer disposal (i.e. cradle-to-grave) will further strengthen the environmental advantages of PHB as incineration of polyolefins adds additional negative environmental impacts to their life cycles. As different raw materials are used in the heterotrophic-autotrophic process, a dedicated LCA assessment of this process

needs to be performed to assess the sustainability of PHB production. The LCA will allow to calculate the different greenhouse gases and Natural Capital impacts. Pioneering the valuation of external impacts and benefits such as the cost of climate change, biodiversity, water quality, air quality etc. for this process will bring a new but crucial dimension of environmental arguments.

To ensure the sutainability of PHB production from CO_2, H_2 should be produced from excess renewable energy – which is currently curtailed – coming from wind and solar power (by water electrolysis or splitting). When no excess energy is available, PHB could be produced under heterotrophic conditions, whereas autotrophic PHB production will take place in periods of excess energy. Future research should therefore investigate how flexible the fermentation system is under conditions of discontinuous (renewable) energy supply.

Experimental work in this doctoral research encompassed only the use of pure synthetic gases. In future the challenging switch to the use of real (industrial) CO_2 off-gases should be made to study the effect of impurities of the CO_2-gas source on the biocatalysts activity and process efficiency. Indeed, gas feedstock characteristics (flows, CO_2 level, impurities etc.) differ depending on industry, location, etc. The impact of impurities on bioprocesses is still largely unknown and urges for investigation.

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Appendix

Appendix S3: Supplementary material with Chapter 3

S3.1. Derivation of mass balances describing biomass growth and PHB production in a fed-batch reactor

All mass balances correspond to the general principle of mass conservation:

Accumulation = Transport (In – Out) + Conversion (Production - Consumption)

The overall mass balance expresses that the accumulation of mass in the reactor is caused by the incoming mass from the substrate and nutrient feed streams, while there is no outgoing stream:

$$\frac{d(\rho_w V(t))}{dt} = F_S(t)(\rho_{FS} - S_F) + F_N(t)(\rho_{FN} - N_F) \quad [\text{g/h}] \qquad \text{(S3.1)}$$

The density of the culture broth ρ_w can be assumed contant, such that Eq. S3.1 can be written as

$$\frac{dV(t)}{dt} = F_S(t)\frac{\rho_{FS} - S_F}{\rho_w} + F_N(t)\frac{\rho_{FN} - N_F}{\rho_w} = F(t) \qquad \text{(3.10)}$$

in which F(t) is defined as the overall feed flow rate (L/h).

The individual mass balance for substrate in the fed-batch fermentor (no outgoing flow) is derived as:

$$\frac{d(V(t)S(t))}{dt} = F_S(t)S_F - \mu_S X(t)V(t) \qquad \text{(S3.2)}$$

Given that the reactor volume in thefed-batch fermentor is not constant, the term on the left hand side is expanded as,

$$\frac{d(V(t)S(t))}{dt} = V(t)\frac{d(S(t))}{dt} + S(t)\frac{d(V(t))}{dt} \qquad \text{(S3.3)}$$

Substitution of Eq. S3.3 in Eq. S3.2, followed by subsitution of Eq. 3.10 and rearrangement results in

$$V(t)\frac{d(S(t))}{dt} + S(t)\frac{d(V(t))}{dt} = F_S(t)S_F - \mu_S X(t)V(t) \quad \text{(S3.4)}$$

$$\Leftrightarrow \quad V(t)\frac{d(S(t))}{dt} + S(t)F(t) = F_S(t)S_F - \mu_S X(t)V(t) \quad \text{(S3.5)}$$

$$\Leftrightarrow \quad \frac{dS(t)}{dt} = \frac{F_S(t)S_F}{V(t)} - \frac{F(t)}{V(t)}S(t) - \mu_S X(t) \quad \text{(S3.6)}$$

Defining the dilution rate as the ratio between the overall feed flow rate and the reactor volume

$$D(t) = \frac{F(t)}{V(t)} \quad \text{(3.9)}$$

Eq. (S.6) becomes

$$\frac{dS(t)}{dt} = \frac{F_S(t)S_F}{V(t)} - D(t)S(t) - \mu_S X(t) \quad \text{(3.11)}$$

Analogously, the mass balances for nitrogen, residual biomass (RCC) and PHB for in the fed-batch reactor volume are derived as (note that residual biomass and PHB are assumed not to be present in the feed streams):

$$\frac{dN(t)}{dt} = \frac{F_N(t)N_F}{V(t)} - D(t)N - \mu_N X(t) \quad \text{(3.13)}$$

$$\frac{dX(t)}{dt} = \mu_x X(t) - D(t)X(t) \quad \text{(3.17)}$$

$$\frac{dP(t)}{dt} = \mu_p X - D(t)P(t) \quad \text{(3.18)}$$

S3.2. Model parameter values

The stoichiometric and kinetic parameter values applied in this study are summarized in Table S3.1.; the values for the operating parameters are given in Table S3.2.

Table S3.1. Stoichiometric and kinetic parameter values of the heterotrophic PHB production model.

Parameter		Value	Unit	References
Stoichiometric parameters				
Y_{ps}	PHB yield over substrate	0.30	g PHB/g glucose	Experimentally determined in this study
		0.52	g PHB/g glycerol	Experimentally determined in this study
Y_{xN}	biomass yield over nitrogen	8.9	g Biomass/g N	Theoretically calculated
Y_{xp}	biomass yield over PHB	0.88	g Biomass /g PHB	Dias et al., 2006
Y_{xs}	biomass yield over substrate	0.48	g Biomass /g glucose	Tanadchangsaeng and Yu, 2012
		0.48	g Biomass /g glycerol	Experimentally determined in this study
Kinetic parameters				
μ_{XS}^{max}	maximum specific biomass growth rate over substrate	0.41	g cell/g Biomass /h	Du et al., 2001
μ_{PS}^{max}	maximum specific PHB production rate	0.09	g PHB/g Biomass /h	Du et al., 2001
μ_{XP}^{max}	maximum specific biomass growth rate over PHB	0.18	g Biomass /g PHB/h	Dias et al., 2005
K_S	saturation constant for substrate in growth	1.2	g substrate/L	Cougnon et al., 2011
K_{IS}	substrate inhibition constant for growth	17.43	g substrate/L	Lee et al., 1997

K_N	saturation constant for nitrogen in growth	0.254	g N/L	Patnaik, 2006
K_{IN}	nitrogen inhibition constant for growth	1.5	g N/L	Lee et al., 1997
K_{PS}	saturation constant for substrate in PHB production	4.1	g substrate/L	Lee et al., 1997
K_{PIS}	substrate inhibition constant for PHB production	80	g substrate/L	Lee et al., 1997
K_{PHB}	saturation constant for PHB in growth	0.14	g PHB/g Biomass	Dias et al., 2005
K_{PIN}	nitrogen inhibition constant for PHB production	0.254	g N/L	Assumed equal to K_N
X_m	maximum residual cell concentration at which specific growth rate is zero	68	g cell/L	Experimentally determined in this study
$f_{\mathrm{PHB(max)}}$	PHB to active biomass ratio, P/X	3.3 (for glucose)		Experimentally determined in this study
		2 (for waste glycerol)		Assumed in this study
α	cell density inhibition coefficient	5.8		Mulchandani and Luong, 1989
β	production (PHB) saturation power coefficient	3.85		Dias et al., 2006
m_S	specific glucose consumption for maintenance	0.02	g substrate/g Biomass /h	Frigon et al., 2006

Table S3.2. Operating parameter values.

Operating parameters		Value	Unit
S (glucose)	substrate concentration in culture broth	12	g/L
S (waste glycerol)		14	g/L
N	ammonium nitrogen concentration in culture broth	0.60	g/L
S_F (glucose)	substrate concentration in feeding solution	650	g/L
S_F (waste glycerol)		850	g/L
N_F	ammonium nitrogen concentration in feeding solution	164	g/L
ρ_{FS} (glucose)	density of substrate feeding solution	1230	g/L
ρ_{FS} (waste glycerol)		1260	g/L
ρ_{FN}	density of ammonium nitrogen feeding solution	1040	g/L
ρ_w	density of culture broth	1000	g/L

S3.3. Model calibration and validation results

The results of the model sensitivity analysis have been summarized in Table S3.3. Table S3.4 gives an overview of the model efficiency coefficients obtained during model calibration (phase 1) and validation (phase 1 and 2) for both substrates (glucose and waste glycerol).

Table S3.3: Combined relative sensitivity functions (δ) of various model parameters with respect to the output variables RCC and PHB.

	Model A		Model B		Model C			
	Without N-limitation		Without N-limitation		Without N-limitation		With N-limitation	
Variable (y) → / Parameter (θ) ↓	RCC	PHB	RCC	PHB	RCC	PHB	RCC	PHB
K_S	0.1110	0.0551	0.0687	0.0574	0.0477	0.0456	0.0146	0.2527
K_{IS}	5.2633	2.6114	3.2574	2.7197	2.2619	2.1623	0.6691	2.6027
K_N	1.3970	0.6932	1.0682	0.2885	0.7479	0.2326	0.2392	1.8212
K_{IN}	2.3108	1.1465	1.7668	0.4771	1.2370	0.3847	0.3716	1.8166
μ_{XS}^{max}	35.5287	17.6271	21.9865	18.3570	15.2668	14.5946	4.5160	15.5298
K_{pS}	0.0006	0.1846	0.0529	0.8068	0.0376	0.7891	0.0128	0.8643
K_{pIS}	0.0001	0.0356	0.0102	0.1555	0.0073	0.1521	0.0024	0.2240
$f_{PHB(max)}$	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0310	1.1074
β	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0006	0.0264
μ_{ps}^{max}	0.0120	3.5192	1.0074	15.3818	0.7174	15.0442	0.2408	22.1562
$K_{PIN} = K_N$	0.0064	1.8946	0.5423	8.2808	0.3862	8.0990	0.1183	8.7426
μ_{xp}^{max}	-	-	0.2827	4.8574	0.2142	15.6119	0.0756	2.7475
K_{PHB}	-	-	0.0907	1.8291	0.0667	2.0529	0.0230	2.8234
α	-	-	-	-	0.0045	0.0037	0.0000	0.0000
X_m	-	-	-	-	0.7150	0.3400	0.0000	0.0000

Table S3.4: Nash-Sutcliffe model efficiency coefficient (E) for RCC and PHB productions for the proposed models using estimated parameter value.

Substrate	Phase	Model	E value for RCC	E value for PHB
Glucose	Phase 1	*Growth model A*	0.66	-0.23
		Growth model B	0.72	0.91
		Growth model C	0.99	0.94
	Phase 1 and 2	Model validation with switching point 49 g/L	0.97	0.98
		Model validation with switching point 56 g/L	0.93	0.96
Waste glycerol	Phase 1	Model validation with using *Growth model C*	0.96	0.83
	Phase 1 and 2	Model validation with switching point 7 g/L	0.95	0.94
		Model validation with switching point 44 g/L	0.95	0.93

Appendix S4: Supplementary materials with Chapter 4

S4.1. Mass balances related to cell growth and PHB production

The specific organic substrate consumption rate μ_S (g substrate/g cell/h) reflects that organic substrate is used for growth, PHB production and maintenance (Eq. S4.1) whereas the nitrogen consumption rate μ_N (g N/g cell/h) described the use of nitrogen for the biomass growth on substrate and on PHB (Eq. S4.2)

$$\mu_s = \frac{\mu_{xs}}{Y_{xS}} + \frac{\mu_{ps}}{Y_{pS}} + m_s \quad \text{(S4.1)}$$

$$\mu_N = \frac{\mu_{xs} + \mu_{xp}}{Y_{xN}} \quad \text{(S4.2)}$$

The active cell and PHB production profiles could be calculated from Eqs. S4.3 and S4.4 respectively, taking into account that both are not present in feeding solution.

$$\frac{dX(t)}{dt} = [\mu_x - D(t)]\, X(t) \quad \text{(S4.3)}$$

$$\frac{dP(t)}{dt} = \mu_p X(t) - D(t)P(t) \quad \text{(S4.4)}$$

μ_x (g cell/g cell/h) denotes the specific cell growth on organic substrate and PHB (Eq. S4.5), while μ_p (g PHB/g cell/h) represents the specific PHA production rate (Eq. S4.6):

$$\mu_x = \mu_{xs} + \mu_{xp} \quad \text{(S4.5)}$$

$$\mu_p = \mu_{ps} - \frac{\mu_{xp}}{Y_{xp}} \quad \text{(S4.6)}$$

S4.2. Model parameter values

The stoichiometric and kinetic parameter values applied in this study are summarized in Table S4.1.; the values for the operating parameters are given in Table S4.2.

Table S4.1. Stoichiometric and kinetic parameter values of the heterotrophic PHB production model.

Parameter		Value	Unit	References
Stoichiometric parameters				
Y_{ps}	PHB yield over substrate	0.30	g PHB/g glucose	Chapter 3
Y_{xN}	biomass yield over nitrogen	8.90	g cell/g N	Chapter 3
Y_{xp}	biomass yield over PHB	0.88	g cell/g PHB	Dias et al., 2006
Y_{xs}	biomass yield over substrate	0.48	g cell/g glucose	Tanadchangsaeng and Yu, 2012
$Y_{Na,x}$	addition of sodium for pH control due to unit residual biomass production	0.184	g Na /g cell	Theoretically calculated based on Eq. 4.1
Kinetic parameters				
μ_{XS}^{max}	maximum specific biomass growth rate over substrate	0.46	g cell/g cell/h	Chapter 3
μ_{PS}^{max}	maximum specific PHB production rate	0.217	g PHB/g cell/h	Chapter 3
μ_{XP}^{max}	maximum specific biomass growth rate over PHB	0.126	g cell/g PHB/h	Chapter 3
K_S	saturation constant for substrate in growth	1.2	g substrate/L	Cougnon et al., 2011

K_{IS}	substrate inhibition constant for growth	16.728	g substrate/L	Chapter 3
K_N	saturation constant for nitrogen in growth	0.254	g N/L	Patnaik, 2006
K_{IN}	nitrogen inhibition constant for growth	1.5	g N/L	Lee et al., 1997
K_{PS}	saturation constant for substrate in PHB production	4.1	g substrate/L	Lee et al., 1997
K_{PIS}	substrate inhibition constant for PHB production	80	g substrate/L	Lee et al., 1997
K_{PHB}	saturation constant for PHB in growth	0.148	g PHB/g cell	Chapter 3
K_{PIN}	nitrogen inhibition constant for PHB production	0.262	g N/L	Chapter 3
X_m	maximum residual biomass concentration at which specific growth rate is zero	68	g cell/L	Chapter 3
$f^0_{PHB(max)}$	maximum PHB to active biomass ratio, *P*/*X*	3.5		Experimentally determined in this study
α	cell density inhibition coefficient	5.8		Mulchandani and Luong, 1989
β	production (PHB) saturation power coefficient	3.85		Dias et al., 2006
m_S	specific glucose consumption for maintenance	0.02	g substrate/g cell/h	Frigon et al., 2006
Na_{xm}	maximum *Na* concentration at which specific growth rate is zero	8.9	g Na^+/L	Estimated in this study
Na_{pm}	maximum *Na* concentration at which specific PHB production rate is zero	10.5	g Na^+/L	Estimated in this study
n_x	*Na* inhibition coefficient on growth kinetics	1.91		Estimated in this study
n_p	*Na* inhibition coefficient on PHB production kinetics	1.0		Estimated in this study

n_{pf}	*Na* inhibition coefficient on maximum PHB fraction	1.2		Estimated in this study

Table S4.2. Operating parameter values.

Operating parameters		Value	Unit
S	substrate concentration in culture broth	12	g/L
N	ammonium nitrogen concentration in culture broth	0.60	g/L
S_F	substrate concentration in feeding solution	650	g/L
N_F	ammonium nitrogen concentration in feeding solution	164	g/L
ρ_{FS}	density of substrate feeding solution	1230	g/L
ρ_{FNa}	density of 5M NaOH solution	1090	g/L
ρ_w	density of culture broth	1000	g/L

Appendix S5: Supplementary materials with Chapter 5

Table S5.1. Stoichiometric and kinetic parameter values of the autotrophic PHB production model.

Parameter		Value	Unit	References
Stoichiometric parameters				
Y_{xH2}	biomass yield over hydrogen	2.27	g cell/g H_2	Ishizaki and Tanaka, 1990, after unit conversion
Y_{xO2}	biomass yield over oxygen	0.49	g cell/g O_2	
Y_{xCO2}	biomass yield over carbon dioxide	1.53	g cell/g CO_2	
Y_{xN}	biomass yield over nitrogen	9.10	g cell/g NH_4^+-N	
Y_{pH2}	PHB yield over hydrogen	1.30	g PHB/g H_2	Ishizaki and Tanaka, 1991, after unit conversion
Y_{pO2}	PHB yield over oxygen	0.22	g PHB/g O_2	
Y_{pCO2}	PHB yield over carbon di oxide	0.49	g PHB/g CO_2	
Kinetic parameters				
μ_{XS}^{max}	maximum specific biomass growth rate	0.29	g cell/g cell/h	Siegel and Ollis, 1984
μ_{PS}^{max}	maximum specific PHB production rate	0.26	g PHB/g cell/h	Estimated in this study
K_{xH2}	saturation constant for H_2 in growth	9.20×10^{-6}	g H_2/L	Siegel and Ollis, 1984
K_{xO2}	saturation constant for O_2 in growth	1.18×10^{-4}	g O_2/L	Siegel and Ollis, 1984
K_{xCO2}	saturation constant for CO_2 in growth	1.18×10^{-4}	g CO_2/L	Assumed
K_{pH2}	saturation constant for H_2 in PHB production	9.20×10^{-6}	g H_2/L	Assumed as K_{xH2}
K_{pO2}	saturation constant for O_2 in PHB production	1.82×10^{-5}	g O_2/L	Estimated in this study

K_{pCO2}	saturation constant for CO_2 in PHB production	$1.18x10^{-4}$	g CO_2/L	Assumed
K_{pIO2}	oxygen inhibition constant for PHB production	0.00113	g O_2/L	Tanaka et al., 1995
K_N	saturation constant for nitrogen in growth	0.254	g N/L	Patnaik, 2006
K_{IN}	nitrogen inhibition constant for growth	1.691	g N/L	Chapter 3
K_{PIN}	nitrogen inhibition constant for PHB production	0.254	g N/L	Assumed as K_N
$f_{PHB(max)}$	maximum PHB to active biomass ratio, P/X (for CSTR type bioreactor)	1.78±0.32		Average value, (Ishizaki and Tanaka, 1991 and Tanaka et al., 1995)
β	production (PHB) saturation power coefficient	3.85		Dias et al., 2006
$k_L a_{(O2)}$	mass transfer coefficient for oxygen	340	h^{-1}	Tanaka and Ishizaki, 1994
P	total pressure	1.50	atm	Tanaka et al., 1995

Table S5.2. Calculated Henry's constant (k_H) for gases calculated at standard condition (Dean, 1985).

Gases	k_H	
	atm/mol/L	atm/g/L
H_2	1350	675
O_2	887	27.72
CO_2	35	0.79

Table S5.3: Effect of oxygen and nitrogen stress on PHB productivity and yield using a gas mixture O_2:H_2:CO_2=15:75:10 (numerical values corresponding with Figure 5.4).

O_2 (mg/L) (phase 2)	NH_4^+-N (g/L) (phase 2)	PHB productivity (g/L/h)	Overall PHB yield		
			gPHB/gO_2	gPHB/gH_2	gPHB/gCO_2
Limitation	1.06	0.51	0.16	0.91	0.39
	0.5	0.54	0.17	0.95	0.41
	0.01	0.30	0.17	0.96	0.41
2.9	Limitation	0.25	0.18	0.98	0.42
2.0		0.31	0.18	0.98	0.42
0.1		0.42	0.18	0.98	0.42

Table S5.4: Influence of gas composition on PHB productivity and yield (numerical values corresponding with Figure 5.5).

			Overall PHB yield		
O_2:H_2:CO_2	Stress condition	PHB productivity (g/L/h)	gPHB/gO_2	gPHB/gH_2	gPHB/gCO_2
25:65:10	O_2 limitation	0.14	0.10	0.50	0.26
22:68:10		0.34	0.14	0.73	0.34
20:70:10		0.68	0.16	0.91	0.39
18:72:10		0.61	0.16	0.91	0.39
15:75:10		0.51	0.16	0.91	0.39
25:65:10	N limitation	0.74	0.17	0.97	0.41
22:68:10		0.78	0.18	0.98	0.42
20:70:10		0.76	0.17	0.98	0.41
18:72:10		0.67	0,17	0.95	0.41
15:75:10		0.54	0.16	0.89	0.39

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List of publications:

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