



LUND UNIVERSITY

Microbial production of 3-hydroxypropionic acid and poly(3-hydroxypropionate): Investigation of *Lactobacillus reuteri* propanediol utilization pathway enzymes

Sabet Azad, Ramin

2015

[Link to publication](#)

Citation for published version (APA):

Sabet Azad, R. (2015). *Microbial production of 3-hydroxypropionic acid and poly(3-hydroxypropionate): Investigation of Lactobacillus reuteri propanediol utilization pathway enzymes*. [Doctoral Thesis (compilation), Biotechnology]. Division of Biotechnology, Lund University.

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Microbial production of 3-hydroxypropionic acid and poly(3-hydroxypropionate)

Investigation of *Lactobacillus reuteri* propanediol
utilization pathway enzymes

Ramin Sabet-Azad



LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Engineering, Lund University, Sweden.
To be defended at Center for Chemistry and Chemical Engineering. Date 30th of
April 2015 and time 10.00.

Faculty opponent

Prof. An-Ping Zeng, Hamburg University of Technology, Germany

Organization LUND UNIVERSITY Department of Biotechnology P.O Box 124 SE 221 00 Lund, Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue 2015-04-30	
Author(s) Ramin Sabet-Azad		Sponsoring organization VINNOVA	
Title and subtitle Microbial production of 3-hydroxypropionic acid and poly(3-hydroxypropionate) : Investigation of <i>Lactobacillus reuteri</i> propanediol utilization pathway enzymes			
Abstract <p>Concerns regarding environmental issues such as greenhouse gas emissions and climate change have led to a shift within the research community and chemical and energy industry sectors for finding sustainable routes for producing fuels and chemicals from renewable resources, thereby minimizing our dependence on petroleum. The C3-chemical 3-hydroxypropionic acid has been identified as a top candidate for the biobased chemical industry. This platform chemical is a β-hydroxy acid containing two functional groups (hydroxyl and carboxyl) enabling its conversion into value-added chemicals such as 1,3-propanediol, acrolein, malonic acid, acrylamide and acrylic acid, which can be used in resins, coatings, paints, adhesives, lubricants, and in the textile industry as anti-static agent. Polymerized 3-HP, poly(3-hydroxypropionate) (poly(3-HP)), is a biodegradable and stable polymer which, besides its potential role as a biomaterial, can be degraded to 3-HP monomer. In recent years, a dramatic increase in the interest for microbial production of 3-HP and poly(3-HP) has been observed. Metabolic engineering and recombinant expression of various enzymatic pathways in a number of bacterial strains have been suggested and implemented, with mainly renewable glucose and glycerol as substrates.</p> <p>This thesis presents a novel pathway called the propanediol utilization pathway present in <i>Lactobacillus reuteri</i> that catalyzes dehydration of glycerol to 3-hydroxypropionaldehyde (3-HPA) and further to 3-HP by a series of reactions catalyzed by propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW). Through structural modeling and kinetic characterization of PduP, its 3-HPA consuming ability was confirmed and catalytic mechanism proposed. PduP, PduL and PduW-mediated conversion of 3-HPA to 3-HP was confirmed through their recombinant expression in <i>Escherichia coli</i>. 3-HPA produced from glycerol by <i>L. reuteri</i> was used as a substrate for conversion to 3-HP by the recombinant <i>E. coli</i>. A yield of 1 mol/mol was reached with a titer of 12 mM 3-HP. Depletion of the cofactor NAD⁺ required for the catalysis of 3-HP to 3-HP-CoA, was deemed responsible for the low titer. Regeneration of NAD⁺, used up in PduP catalyzed reaction, was achieved by recombinant expression of NADH oxidase (Nox) from <i>L. reuteri</i> in <i>E. coli</i> expressing PduP, PduL and PduW. The final 3-HP titer by this recombinant strain was at least twice that of <i>E. coli</i> carrying solely PduP, PduL and PduW.</p> <p>For the production of poly(3-HP), PduL and PduW in the recombinant strain were replaced by polyhydroxyalkanoate synthase of <i>Chromobacterium</i> sp. that converts 3-HP-CoA to poly(3-HP). A poly(3-HP) content of up to 40% (w/w) cell dry weight was reached in an efficient and cheap process requiring no additives or expensive cofactors.</p>			
Key words Platform chemicals; biopolymers; 3-hydroxypropionic acid; 3-hydroxypropionaldehyde; glycerol; poly(3-hydroxypropionate); polyhydroxyalkanoates; <i>Escherichia coli</i> ; <i>Lactobacillus reuteri</i> ; propanediol utilization pathway; propionaldehyde dehydrogenase; biotransformation; cofactor regeneration; NADH oxidase			
Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language English	
ISSN and key title		ISBN 978-91-7634-273-6	
Recipient's notes		Number of pages 132	
		Security classification	

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature



Date

2015-03-23

Microbial production of 3-hydroxypropionic acid and poly(3-hydroxypropionate)

Investigation of *Lactobacillus reuteri* propanediol
utilization pathway enzymes

Ramin Sabet-Azad



LUND
UNIVERSITY

Front page photograph: Mikael Risedal, Lund Universitets Bild- och Mediabank.

Doctoral thesis

© Ramin Sabet-Azad

Faculty of Engineering, Biotechnology

ISBN 978-91-7623-273-6 (printed)

ISBN 978-91-7623-274-3 (pdf)

ISRN LUTKDH/TKBT – 15 / 1163 - SE

Printed in Sweden by Media-Tryck, Lund University

Lund 2015



KLIMATKOMPENSERAT
PAPPER



Till min Mor och Far

تقديم به پدر و مادرم

Abstract

Concerns regarding environmental issues such as greenhouse gas emissions and climate change have led to a shift within the research community and chemical and energy industry sectors for finding sustainable routes for producing fuels and chemicals from renewable resources, thereby minimizing our dependence on petroleum. The C3-chemical 3-hydroxypropionic acid has been identified as a top candidate for the biobased chemical industry. This platform chemical is a β -hydroxy acid containing two functional groups (hydroxyl and carboxyl) enabling its conversion into value-added chemicals such as 1,3-propanediol, acrolein, malonic acid, acrylamide and acrylic acid, which can be used in resins, coatings, paints, adhesives, lubricants, and in the textile industry as anti-static agent. Polymerized 3-HP, poly(3-hydroxypropionate) (poly(3-HP)), is a biodegradable and stable polymer which, besides its potential role as a biomaterial, can be degraded to 3-HP monomer. In recent years, a dramatic increase in the interest for microbial production of 3-HP and poly(3-HP) has been observed. Metabolic engineering and recombinant expression of various enzymatic pathways in a number of bacterial strains have been suggested and implemented, with mainly renewable glucose and glycerol as substrates.

This thesis presents a novel pathway called the propanediol utilization pathway present in *Lactobacillus reuteri* that catalyzes dehydration of glycerol to 3-hydroxypropionaldehyde (3-HPA) and further to 3-HP by a series of reactions catalyzed by propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW). Through structural modeling and kinetic characterization of PduP, its 3-HPA consuming ability was confirmed and catalytic mechanism proposed. PduP, PduL and PduW-mediated conversion of 3-HPA to 3-HP was confirmed through their recombinant expression in *Escherichia coli*. 3-HPA produced from glycerol by *L. reuteri* was used as a substrate for conversion to 3-HP by the recombinant *E. coli*. A yield of 1 mol/mol was reached with a titer of 12 mM 3-HP. Depletion of the cofactor NAD^+ required for the catalysis of 3-HP to 3-HP-CoA, was deemed responsible for the low titer. Regeneration of NAD^+ , used up in PduP catalyzed reaction, was achieved by recombinant expression of NADH oxidase (Nox) from *L. reuteri* in *E. coli* expressing PduP, PduL and PduW. The final 3-HP titer by this recombinant strain was at least twice that of *E. coli* carrying solely PduP, PduL and PduW.

For the production of poly(3-HP), PduL and PduW in the recombinant strain were replaced by polyhydroxyalkanoate synthase of *Chromobacterium* sp. that converts 3-HP-CoA to poly(3-HP). A poly(3-HP) content of up to 40% (w/w) cell dry weight was reached in an efficient and cheap process requiring no additives or expensive cofactors.

Popular summary

Since the onset of the 20th century, human society has been using non-renewable resources, mainly oil, for the production of fuels and chemicals that are now an integral part of our everyday life. In the last few decades however, concerns regarding environmental effects, geopolitical issues and the eventual depletion of oil have led to us re-evaluating our dependency on this resource. Current production of a majority of everyday chemicals is based on refining petroleum to a small number of other molecules, also known as platform chemicals, which can then be converted to a much larger number of chemicals through various processes.

In our efforts to move from a fossil to biobased economy in which renewable resources like sugars or glycerol, obtained through plants, trees, grasses, and/or as residues/wastes of agro-/forestry based industries, will constitute the feedstock for industry, sustainable technologies for processing of the biomass and its components in an environmentally-friendly manner need to be developed. Biotechnological production of platform chemicals from the biomass feedstocks is mainly based on replacing traditional chemical reactors with microorganisms. Microorganisms can be considered small reactors as they contain mechanisms for the conversion of a large variety of natural as well as synthetic molecules to others. These mechanisms are driven by enzymes which are in turn encoded in their genomes. As microorganisms are present in pretty much every type of environment imaginable on the planet, the number of naturally occurring reactions is very high. Some microorganisms are known to produce biodegradable plastics as a protection mechanism in harsh conditions. Microbial processes for the production of chemicals and materials are based in water and require no organic solvents. They can be performed in lower temperatures and are very specific. As petroleum is cheap however, there is no economic incentive to shift to these greener processes. Thus, it is important to develop microbial methods for the production of these chemicals from cheap resources and in high concentrations and yields.

This thesis deals mainly with the production of the platform chemical 3-hydroxypropionic acid (3-HP) and its polymer poly(3-hydroxypropionate). These products are not available commercially. 3-HP is of great promise as it can be further converted into wide array of chemicals, e.g. resins, coatings, lubricants and in the textile industry as anti-static agent. Poly(3-HP) is a biodegradable polymer that can replace certain fossil-based polymers in different applications.

3-HP is produced in smaller amounts by certain microorganisms that grow slowly and/or are expensive to cultivate. Therefore, a copy and paste-strategy has been implemented for moving some of the reactions to well-known organisms that are easier and cheaper to cultivate. *Lactobacillus reuteri*, a probiotic bacteria, contains a

mechanism for the production of 3-hydroxypropionic acid from glycerol, which is currently produced in large amounts as a byproduct from the production of bio-diesel from several plant oils. By copying relevant genes and transferring them to the cheaply cultivated bacterium *Escherichia coli*, it was proven that 3-HP could be produced with a high yield from glycerol. Such a strategy does require the understanding of the metabolic system in the bacteria in order to avoid any interference and to incorporate strategies for improving the formation of the product in a selective and clean manner. Such strategies were implemented in the present work, thereby increasing the final concentration of 3-HP. Some key enzymes in these processes were studied further in order to gain a better understanding of their function and structure.

In the same manner, a relevant gene from *Lactobacillus reuteri* and a gene from another bacteria, *Chromobacterium* sp. known to produce a bioplastic, were copied and pasted in *Escherichia coli*, resulting in a strain with the capacity to produce poly(3-hydroxypropionate) in a cheap and efficient manner.

List of papers

This thesis is based on the following papers, which are provided towards the end of the text. The papers will be referred to in the text by their Roman numerals

- I Sabet-Azad R., Linares-Pastén J.A., Torkelson L., Sardari R.R.R., Hatti-Kaul R. Coenzyme A-acylating propionaldehyde dehydrogenase (PduP) from *Lactobacillus reuteri*: Kinetic characterization and molecular modeling. *Enzyme & Microbial Biotechnology* 2013. 53:235-242.
- II Sabet-Azad R., Sardari R.R.R., Linares-Pastén J.A., Hatti-Kaul R. Production of 3-hydroxypropionic acid from 3-hydroxypropionaldehyde by recombinant *Escherichia coli* co-expressing *Lactobacillus reuteri* propanediol utilization enzymes. *Bioresource Technology* 2015. 180:214-221.
- III Sabet-Azad R., Dishisha T., Arieta V., Hatti-Kaul R. Cofactor regeneration enhances the production of 3-hydroxypropionic acid from 3-hydroxypropionaldehyde: Integrating *Lactobacillus reuteri* NADH oxidase and propanediol utilization pathway enzymes in *Escherichia coli*. 2015. (Manuscript)
- IV Linares-Pasten J.A., Sabet-Azad R., Pessina L., Sardari R.R.R., Ibrahim M.H.A., Hatti-Kaul R. Efficient poly(3-hydroxypropionate) production from glycerol using *Lactobacillus reuteri* and recombinant *Escherichia coli* harboring *L. reuteri* propionaldehyde dehydrogenase and *Chromobacterium* sp. PHA synthase genes. *Bioresource Technology* 2015. 180:172-176.

Paper I, II and IV and are reproduced by the permission of Elsevier.

My contribution to the papers

The overall idea for this work was provided by Professor Rajni Hatti-Kaul

- I I planned this study in cooperation with Javier Linares-Pastén and performed the experiments on cloning and expression of PduP. I and Lisa Torkelson contributed equally to the kinetic characterization of PduP. I performed the structural characterization of PduP. Roya Sardari performed the production of the substrate 3-HPA. I performed data analysis and wrote the first draft of the manuscript under the supervision of Javier Linares-Pastén and Rajni-Hatti-Kaul.
- II I planned this work in cooperation with Javier Linares-Pastén. I performed most experimental parts. Roya Sardari performed the experiments concerning production and in situ removal of 3-HPA. I performed data analysis and wrote the first draft under the supervision of Rajni Hatti-Kaul.
- III I planned this work in cooperation with Tarek Dishisha. Victor Arieta performed the cloning and expression of NADH oxidase under my supervision. I and Victor Arieta contributed equally to the kinetic characterization of NADH oxidase. I performed shake flask bioconversion of 3-HPA to 3-HP. I and Tarek Dishisha contributed equally for bioreactor conversion of 3-HPA to 3-HP. I performed data analysis and in cooperation with Tarek Dishisha wrote the first draft of the manuscript under the supervision of Rajni Hatti-Kaul.
- IV I was involved in the experimental parts regarding production and analysis of poly(3-HP) together with Javier Linares-Pastén and Mohammed Ibrahim, as well as revising the first draft written by Javier Linares-Pastén. Javier Linares-Pastén, Laura Pessina and Mohammad Ibrahim performed the cloning and expression of PduP and PhaC. Roya Sardari produced 3-HPA. The work was performed under the supervision of Rajni Hatti-Kaul.

Contents

1 Introduction	1
1.1 Scope of the Thesis	2
2 Industrial Biotechnology for the Production of Platform Chemicals and Polymers	3
2.1 The chemical industry	3
2.2 Petroleum-based production of platform chemicals and materials	3
2.3 Industrial biotechnology	4
2.3.1 Platform chemicals from the bio-based industry	5
2.3.2 Bio-based polymers	6
2.3.3 Renewable resources for the production of platform chemicals	6
3 3-Hydroxypropionic Acid and Poly (3-Hydroxypropionate)	9
3.1 3-Hydroxypropionic acid as a C3 platform chemical	9
3.1.1 Chemical synthesis of 3-hydroxypropionic acid	10
3.1.2 Native microbial production of 3-HP	10
3.1.3 Recombinant microbial production of 3-HP	12
3.2 Poly(3-hydroxypropionate): a biodegradable polymer	19
3.2.1 Chemical and enzymatic methods for poly(3-HP) production	19
3.2.2 Recombinant microbial production of poly(3-HP)	19
4 <i>Lactobacillus reuteri</i> and the Propanediol Utilization Pathway	23
4.1 Lactic acid bacteria and <i>L. reuteri</i>	23
4.2 Metabolism of glucose and glycerol in <i>L. reuteri</i>	23
4.2.1 Propanediol utilization pathway	25
4.2.2 Propionaldehyde dehydrogenase (PduP) (Paper I)	27
5 Production of 3-Hydroxypropionic Acid and Poly(3-Hydroxypropionate) Using Engineered <i>Escherichia coli</i>	29
5.1 <i>Escherichia coli</i> as a microbial factory	29
5.1.1 <i>Escherichia coli</i>	29
5.1.2 <i>E. coli</i> metabolism	29
5.1.3 Engineering of <i>E. coli</i>	31
5.2 Production of 3-HP using Pdu pathway engineered in <i>E. coli</i> (Paper II)	32
5.3 Integration of cofactor regeneration for continuous production of 3-HP by <i>E. coli</i>	35

5.3.1 NADH/NAD ⁺ regeneration systems	35
5.3.2 NADH-oxidase and 3-HP production (Paper III)	37
5.4 Production of poly(3-hydroxypropionate) in <i>E. coli</i> (Paper IV)	41
Conclusions and Future Perspectives	43
Acknowledgments	45
References	47

1 Introduction

Human society has, for the larger part of the 20th and in to the 21st centuries, mainly relied on petrochemistry for the production of fuels, chemicals and materials [1]. Fractional distillation of crude oil as part of petroleum refining processes result in the production of liquid hydrocarbon mixtures (naphta), jet fuel (e.g. kerosene), diesel and liquified petroleum gases. Naphta and petroleum gases can be further processed for obtaining commodity petrochemicals such as benzene, xylene, toluene, ethene and propene. These chemicals are regarded as platforms, meaning that they can be utilized as building blocks for the production of a wide array of chemicals and materials used within most areas of everyday life [2, 3]. The usage of petroleum for the production of these essential products however has major shortcomings that need to be adressed. Petroleum is a non-renewable resource, which is in conflict with the ever-increasing global demand for goods. Extreme crude oil price fluctuations related to geopolitical issues and its increased or decreased output are destabilizing factors affecting the chemical industry as well as the global economy. Furthermore, environmental issues such as the emission of greenhouse gases, acid rains and the release of toxic and non-degradable compounds in air, soil and water are partly affiliated with the processing of petroleum and utilization of petroleum derived chemicals and fuels.

An alternative for the chemical industry is to shift to renewable resources as raw materials. Residual biomass streams from agriculture, forestry or agro-industrial processes constitute the most important resources being investigated. However, with the change in raw materials, a technology shift is required. Biotechnology, based on microorganisms and their enzymes as catalysts, is one of the key enabling technologies for the bio-based production of fuels, chemicals and materials in an environmentally sustainable fashion [4]. This is based on the natural ability of the microorganisms to utilize organic carbons present in biomass for growth and survival through a multitude of metabolic pathways involving enormous numbers of metabolites of interest [2]. Furthermore, modern biotechnology enables recombinant expression of enzymes in, and engineering of, standard microbial hosts to produce specific products and avoiding the production of byproducts. Certain enzymes are capable of accepting synthetic substrates that are structurally similar to their native substrates.

Earlier adapatations of the chemical industry to sustainable biotechnology involved the microbial production of biofuels such as ethanol from wood and food source biomass [5]. Modern biotechnology however strives for minimizing the utilization of food sources for the production of fuels, chemicals and materials by instead using byproducts, generated through the processing of biomass, for their valorization by their microbial conversion to a number of platform chemicals. These byproducts' ready availability and low prices make them potent alternatives to crude oil.

In order to reach high titers, productivities and yields of the products, thus gaining leverage towards petroleum-based production systems which have been optimized for decades, microbial production strains with higher tolerance towards the product, cheaper cultivation methods and faster growth rates than those of the native strains [6] are necessary. Additionally, synthetic enzymatic pathways can be constructed by the recombinant expression of a number of enzymes from different microorganisms in one host strain.

The work presented in this thesis was performed within the framework of a research project entitled “Industrial Biotechnology for the Production of Platform Chemicals” (BioVinn) involving an academic-industrial collaboration financed by the Swedish Governmental Agency for Innovation Systems (Vinnova).

1.1 Scope of the Thesis

This thesis aims for the study and utilization of a microbial pathway consisting of propanediol utilization pathway (pdu) enzymes of *Lactobacillus reuteri* for the production of the platform chemical 3-hydroxypropionic acid (3-HP) as well as its polymer poly(3-hydroxypropionate) in *Escherichia coli*.

Paper I deals with the structural modelling and kinetic characterization of NAD⁺-dependent propionaldehyde dehydrogenase (PduP), the first of three enzymes catalyzing 3-HP formation from the substrate 3-hydroxypropionaldehyde (3-HPA), itself obtained by dehydration of glycerol by *Lactobacillus reuteri*.

Paper II presents the recombinant expression of three enzymes of the propanediol utilization pathway, PduP, phosphotransacylase (PduL) and propionate kinase (PduW), in *Escherichia coli* BL21(DE3) for the production of 3-HP from 3-HPA. A verification of the pathway was performed, as well as an evaluation of titers, productivities and yields of 3-HP obtained from 3-HPA by growing or resting *E. coli* recombinantly expressing PduP, PduL and PduW.

Paper III deals with the kinetic characterization and structural modeling of water-forming NADH oxidase and its co-expression in *E. coli* BL21(DE3) with PduP, PduL and PduW for increasing final titers of 3-HP in resting conditions by the regeneration of cofactor NAD⁺ from NADH.

Paper IV presents the recombinant expression of PduP and a highly active polyhydroxyalkanoate synthase (PhaC) from *Chromobacterium* sp. strain USM2 in *E. coli* BL21(DE3) for the production of poly(3-HP).

2 Industrial Biotechnology for the Production of Platform Chemicals and Polymers

2.1 The chemical industry

The chemical industry encompasses the vast number of companies related to the production of commodity-, life science-, specialty-, and consumer product chemicals. Commodity chemicals are e.g. platform petrochemicals, polymers and inorganics, while specialty chemicals are those utilized in more specific applications in e.g. wood-, textile- or engineering industries. (www.essentialchemicalindustry.org).

The production of chemicals up until the 20th century was based on utilizing coal or renewable resources such as wood for the production of alcoholic fuels, organic acids and similar basic chemicals, and industrial processing was mainly through bacterial or fungi fermentations. With the discovery of petroleum and natural gas, processes were developed and optimized for the conversion of these non-renewable resources to a few platform chemicals that act as building blocks for the production of most chemicals and materials essential for the modern society, thereby largely replacing the sustainable processes of the pre-petroleum era [1].

2.2 Petroleum-based production of platform chemicals and materials

The two most common petrochemical groups are alkenes such as propene and ethene and aromatics such as benzene, toluene and xylene. These few chemicals serve as platforms for the enormous number of chemicals and materials utilized by the textile-, food-, transportation-, housing-, recreation-, communication- and health/hygiene industries (Figure 2.1) [7, 8]. The heavy hydrocarbons of crude oil are cracked to the smaller petrochemicals, fuels and gases by fluid catalytic cracking at high temperatures using heat tolerant acidic catalysts, followed by their separation through distillation. The alkenes are obtained as off-gases while the aromatics are present in the naphtha [9]. These commodity chemicals are priced at up to 1\$ per kg [1].

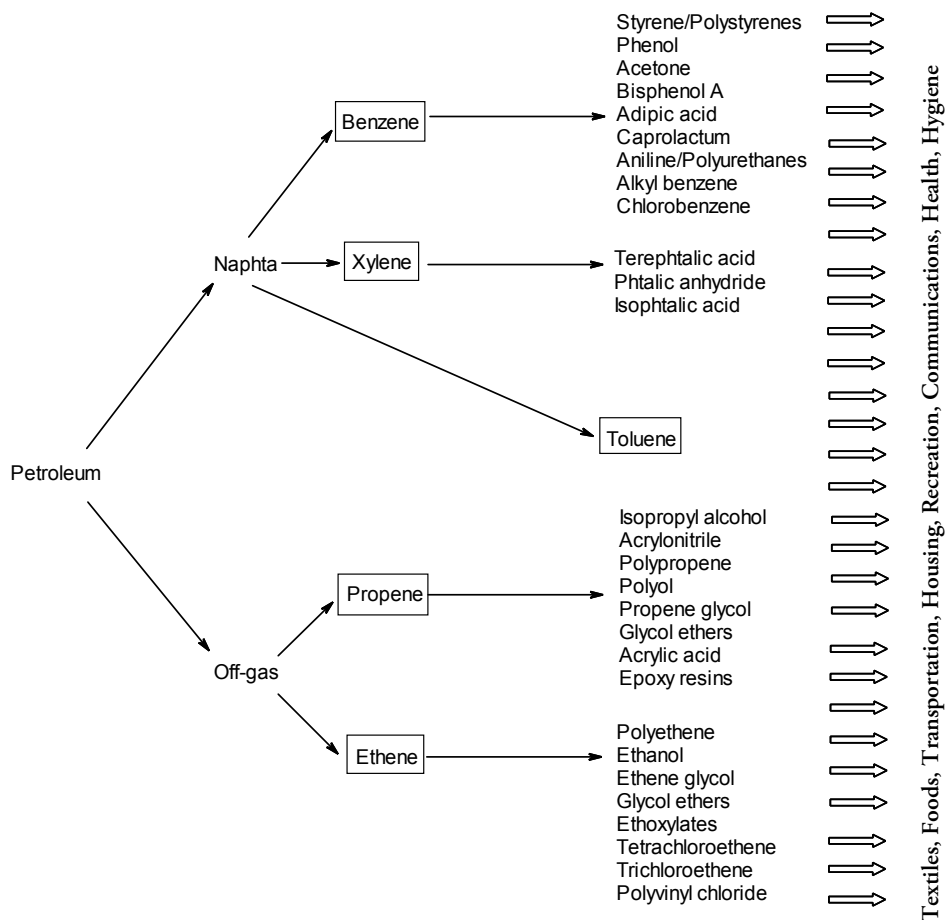


Figure 2.1
Platform chemicals derived from petroleum and their valorization for the production of various chemicals [7, 8].

2.3 Industrial biotechnology

The unsustainability in relying on non-renewable petroleum and its processing, resulting in negative environmental effects, has led to a shift of focus towards the utilization of the mechanisms of life for the production of chemicals, fuels and materials. Enzymes and microorganisms have been studied and implemented in a large number of processes as a means to reduce the role of petroleum and instead utilize organic matter, biomass, as precursor. The main advantages of biotechnological processes are the stereospecificity and selectivity of enzymatic reactions, lower temperatures of the reactions and the reintroduction of formed CO₂ for the production of biomass,. Shifting from a petroleum-based industry to a bio-based one

however requires the implementation of novel processes and development of efficient microbial factories for obtaining high titers, yields and productivities of the compounds of interest.

2.3.1 Platform chemicals from the bio-based industry

An alternative to petrochemicals are bio-based platform chemicals with straight chains or as branched compounds of two to six carbons [2, 7]. These platform chemicals can be produced either *in vitro* through enzyme catalysis or *in vivo* utilizing whole cells expressing the enzymes of a certain pathway.

Platform chemicals require certain characteristics which enable their valorization. They need to contain at least one functional group such as a carboxyl or hydroxyl which can be utilized for their conversion or interlinkage [2]. Additionally, microbial production of these chemicals require the presence of some native metabolic pathway where enzymes catalyze their formation. Alternatively several pathways can be combined, which separately form or utilize intermediates, for the production of platform chemicals [10].

A number of compounds have been identified as potential platform chemicals for C2-C6 chemistry and also for aromatic products (Figure 2.2) [2]. Lactic acid, succinic acid and 1,3-propanediol (1,3-PD) are today produced microbially in an industrial setting [2].

Organic acids in particular have generated much interest, due to their presence as intermediates or end-products in nearly all metabolic pathways as well as their functional carboxyl-group [11]. **Paper II** and **III** deal with the microbial production of the platform carboxylic acid chemical 3-hydroxypropionic acid (3-HP).

C2	C3	C4	C5	C6
Ethanol	Lactic acid	Butyric acid	Glutaconic acid	Adipic acid
Acetic acid	3-HP	Succinic acid	Itaconic acid	Glucaric acid
Glycine	Malonic acid	Malic acid	3-Hydroxyvaleric acid	Caprolactone
Ethene	Propene	Fumaric acid	Xylitol	6-Aminocaproic acid
Ethene glycol	Acrylonitrile	Succinonitrile	Glutaric acid	
	1,2-Propanediol	Aspartic acid	1-Pentanol	
	1,3-Propanediol	GABA	2-Methyl-1-butanol	
	Propionic acid	1-Butanol	3-Methyl-1-butanol	
	Acrylic acid	2-Butanol	Cadavarine	
	Acrylamide	1,4-Butanediol	N-Methyl-2-pyrrolidone	
		Putrescine	1,5-Pentadiol	
		Tetrahydrofuran		

Figure 2.2

Various C2-C6 chemicals proposed as building blocks for the bio-based chemical industry [2].

2.3.2 Bio-based polymers

Petroleum-derived polymers such as polyethylene, polypropene and polyvinylchloride stand for over 96% percent of the plastics market [10]. However, focus is shifting towards the replacement of these polymers with degradable polyhydroxyalkanoates (PHAs), produced naturally by certain microorganisms due to stress mechanisms for the storage of carbon and energy reserves [12]. The global production capacities for bio-based polymers in 2014 was 1.67 million tonnes and is expected to increase to 6.7 million tonnes by 2018 (en.european-bioplastics.org).

There are a number of naturally occurring PHAs, however most abundant is poly(3-hydroxybutyrate) (poly(3-HB)), produced by microorganisms such as *Ralstonia eutropa* and *Bacillus megaterium* by the reduction of acetoacetyl-CoA, itself derived from the condensation of two acetyl-CoA [13]. Other naturally occurring polyhydroxyalkonates are co-polymers of 3-HB and 3-hydroxyvalerate, 4-hydroxybutyrate or 5-hydroxyvalerate [12] and co-polymers of 3-hydroxyoctanoate and 3-hydroxyhexanoate or 3-hydroxydodecanoate [14].

With a melting temperature of 177 °C and glass transition temperature of 3 °C, similar to those of polypropene (174 °C and -13 °C) and polyethylene (130 °C and -36 °C), poly(3-HB) can be utilized in a broad temperature range and has gained interest as an alternative to the petroleum-derived polymers. However poly(3-HB) is a brittle polymer with an elongation at break of 6%, in comparison with polypropene and polyethylene with elongations at break of 400% and 620%, respectively [15]. In **Paper IV**, microbial synthesis of the non-naturally occurring poly(3-hydroxypropionate) (poly(3-HP)) with a temperature range of 77 °C-to -22°C and elongation at break of 634% is described.

2.3.3 Renewable resources for the production of platform chemicals

Sustainable production of platform chemicals through the means of biotechnology require carbohydrate substrates which are renewable. Renewable biogenic resources (biomass) such as oil plants, starch plants, sugar beets and canes can be treated for the production of hexoses such as glucose and galactose and pentoses such as xylitol, fructose and arabinose. These monosaccharides can be metabolized by various microorganisms as carbon sources and as substrates for the formation of platform chemicals [2].

Two pressing issues need to be addressed in order to eliminate the drawbacks associated with bioindustrial production of chemicals, fuels and materials. The cost of petroleum is inhibiting the growth of the biobased industry. In order to achieve an economically viable transition, substrates need to be of low value. As petrochemicals are valued at 1\$ per kg, the starting material for bio-based platform chemicals and polymers, their production and processes related to their downstream processing need

to be at or below this cost. Furthermore, various biomasses are utilized as food sources, which is in direct competition for their utilization as materials for the bioindustry.

A solution to these two issues is the utilization of side-streams or byproducts from agriculture, forestry and related industries, which possess low or no value for the production of platform chemicals and materials. One approach is that of synthetic gas (syngas) fermentation, where gasified residual side-streams containing CO, CO₂ and H₂ are used by certain carbon-fixing microorganisms for the production of value-added chemicals. However, drawback with syngas technology are low productivities, a low gas-liquid mass transfer coefficient and product inhibition [16]. Another approach is that when byproducts can be utilized as substrates directly.

2.3.3.1 Glycerol as a renewable resource

Biodiesel has been gaining momentum as a biofuel. It is composed of mono-alkyl esters with low toxicity and biodegradability. It is also compatible with conventional diesel engines [17] and its production is expected to grow rapidly; in two years (2010-2012) U.S. biodiesel production grew 2.8 times, up to 3.7 billion liters. Production of biodiesel, through the transesterification of vegetable oils, leads to the formation of the byproduct glycerol. For every 10 parts of biodiesel produced, 1 part crude glycerol is produced as a waste stream with its price reaching as low as a few cents per kg in the mid '00's [18]. Crude glycerol contains between 38% and 96% glycerol, with methanol and ash contents up to 14% and 29%, respectively [19].

Glycerol is a polyol and is utilized in a wide array of applications. The highest usage of glycerol is in the cosmetics, soap and pharmaceutical industries, however it is utilized in a number of other applications in the food and tobacco industries, etc. [20].

Glycerol is a naturally occurring compound and many microorganisms have the ability to either utilize it as carbon source or as an electron acceptor. Its low price, high abundance and its production as a waste product thus makes it a top candidate for the production of various biochemicals. **Papers I, II, III and IV** utilize 3-hydroxypropionaldehyde (3-HPA), obtained from the bioconversion of glycerol by *L. reuteri*, for the production of 3-HP or poly(3-HP).

3 3-Hydroxypropionic Acid and Poly(3-Hydroxypropionate)

3.1 3-Hydroxypropionic acid as a C3 platform chemical

3-HP has been identified as one of the top bio-based platform chemicals [3, 7, 10]. The molecule is a β -hydroxyacid and a structural isomer of lactic acid (2-hydroxypropionic acid). Its two functional groups (hydroxyl and carboxyl) enable its chemical or biochemical valorization to a larger number of industrially significant derivatives (Figure 3.1) [7, 21], among which 1,3-propanediol (1,3-PD), with a projected market volume of 0.2 million tonnes per year, and acrylic acid, with a market volume of 0.9 million tonnes per year [3], are themselves building blocks for the production of solvents, fibers, polymers, coatings, paints and adhesives [22]. The projected market volume of 3-HP is 3.6 million tonnes per year [11]. Furthermore, the self polymerization of 3-HP, resulting in poly(3-HP) is of great interest as the polymer is biodegradable with low melting temperature and high flexibility [15].

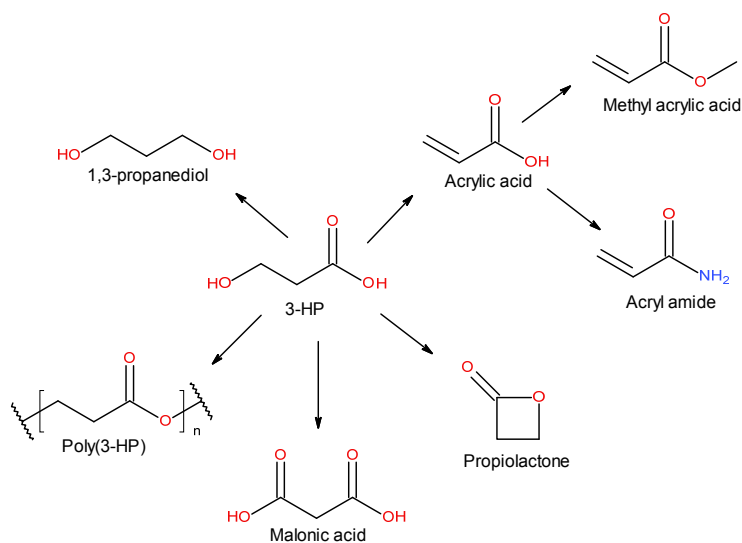


Figure 3.1
3-HP and its derivatives

3.1.1 Chemical synthesis of 3-hydroxypropionic acid

While chemical synthesis of 3-HP is possible, it has not yet been implemented on large scale. Unfavorable economics due to expensive substrates and/or toxicity of substrates and intermediates involved in these processes are the main reasons for the chemical synthesis not being a viable method for industrial scale production of 3-HP [23, 24].

3-HP can be produced through alkali- or acid hydrolysis of β -propiolactone, a carcinogenic compound which in turn is formed by reacting ketone with formaldehyde [25]. Ethene chlorohydrin, derived from ethene and chlorine, can be converted to 3-HP via the intermediate β -hydroxypropionitrile and its reaction with sodium cyanide, a toxic compound [23].

The usage of acrylic acid for the chemical synthesis of 3-HP in presence of alkali and through acid catalysis has been reported. However, high costs associated with the substrate, with increases of 60-63% during one year (2011) [23] have made large scale process based on the hydration of acrylic acid to 3-HP economically unfeasible. Furthermore, the current main chemical method for the production of acrylic acid, based on two-stage oxidation of petroleum-based propene [26] makes the process questionable from an environmental point of view.

Catalytic conversion of allyl alcohol to 3-HP based on utilizing carbon-supported gold catalyst has been reported [27] and resulted in a selective yield of maximum 98% 3-HP when doping the gold catalyst with copper. The reusability of the catalyst was however a major bottleneck, with yields decreasing after each cycle.

While liquid phase oxidation of 3-HPA and 1,3-PD can lead to 3-HP formation, the costs as well as difficulties in obtaining these substrates constitute a major drawback [23].

3.1.2 Native microbial production of 3-HP

3.1.2.1 The 3-HP and 3-HP/4-hydroxybutyrate cycles

3-HP is found as an intermediate in a process for the conversion of inorganic carbon (CO_2) into metabolizable carbon sources. The pathway of utilizing acetyl CoA carboxylase and propionyl CoA carboxylase for the fixation of CO_2 for obtaining glyoxylic acid, the 3-HP cycle (Figure 3.2) [28], is predominantly found in thermophilic and acidophilic microorganisms of the domain archaea [29] such as *Acidianus brierleyi* [30], *Metallosphaera sedula* [31], *Acidinaus ambivalens* [29] and *Sulfolobus metallicus* [32]. Its presence has even been reported in the thermophilic and photosynthetic bacterium *Chloroflexus aurantiacus* [32]. A similar pathway to the 3-HP cycle, 3-HP/4-hydroxybutyrate (4-HB) cycle, has been reported from *Metallosphaera sedula* and key genes of this pathway are found in *Sulfobacillus*,

Archaeoglobus and *Cenarchaeum* sp. Its difference from the 3-HP cycle is the formation of 4-HB from succinyl-CoA and further conversion of 4-HB through 4-HB-CoA and acetoacetyl-CoA to two acetyl-CoA [33].

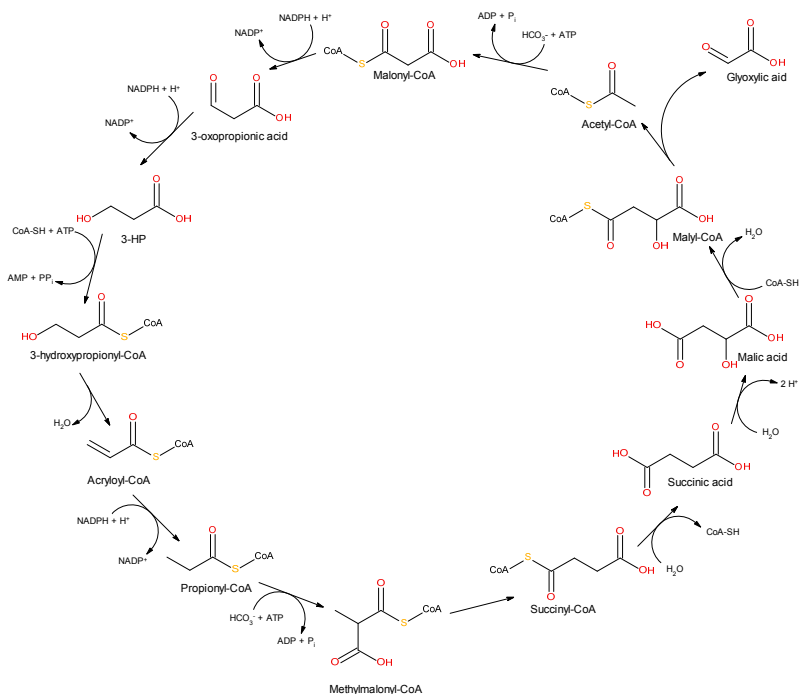


Figure 3.2
The 3-hydroxypropionate cycle.

3.1.2.2 Production of 3-HP from acrylic acid

A number of microorganisms have been shown to contain pathways for the conversion of acrylic acid to 3-HP, either as an intermediate or end-product. Cleavage of gaseous dimethylsulfoniopropionate (DMSP), abundant in marine environments, by *Alcaligenes faecalis* M3A results in extracellular production of dimethylsulfide (DMS) and acrylic acid by DMSP lyase. Further conversion of acrylic acid by the microorganism through as of yet uncharacterized mechanisms resulted in the extracellular production of 3-HP possibly as an intermediate towards the production of a yet unknown compound [34]. A strain of *Byssoschlamys*, isolated from air dust, has been shown to produce 3-HP from acrylic acid, however with a high demand for strict pH control and buffer type, reaching a yield of 70% (v/v) in the presence of an additional energy source (glucose) [35]. Two fungal strains of the species *Geotrichum* and *Trichoderma*, isolated from petrochemical activated sludge, have also been shown to produce 3-HP and acetic acid from acrylic acid after 4 days

of cultivation in the presence of 2 g/L acrylic acid for a number of cycles in buffered conditions [36]. A more industrial approach was applied and reported for native production of 3-HP from acrylic acid by *Rhodococcus erythropolis* LG12, isolated from soil. The proposed pathway, based on a cascade reaction of acrylyl-CoA synthase, enoyl-CoA hydratase and 3-hydroxyisobutyryl-CoA hydrolase, was able to produce up to 17.5 g/L 3-HP with a yield of 0.44 mol/mol and productivity of 0.22 g L⁻¹ h⁻¹ [37].

The high costs associated with acrylic acid as presented earlier, as well as the low productivities of these 3-HP producing strains are major drawbacks in using these native processes for large scale 3-HP production.

3.1.2.3 Production of 3-HP as an antimicrobial agent

The endophytic fungi *Diaphorthe phaseolorum* [38], *Meloidogyne incognita* and four strains of *Melanconium betulinum* [39] have been shown to produce 3-HP as an antimicrobial agent. However no metabolic pathways are presented, and therefore clarification is necessary in the mechanism by which these strains produce 3-HP.

3.1.2.4 Production of 3-HP from glycerol

Lactobacillus sp. [40-42], *Klebsiella pneumoniae* [43], *Citrobacter freundii* [44,45], *Clostridium* sp. [46,47] and *Pantoea agglomerans* [48] are able to natively convert glycerol to 3-HPA in aerobic or microaerobic conditions [49]. The dehydration of glycerol to 3-HPA is catalyzed by cofactor B₁₂ or S-adenosylmethionine dependent glycerol dehydratase and followed in *L. reuteri* by an NAD⁺-dependent oxidative branch resulting in 3-HP and ATP formation and an NADH-dependent reductive branch resulting in 1,3-PD formation [50]. *K. pneumoniae* has been extensively studied for native 1,3-PD production from glycerol. The presence of a propanediol utilization pathway in the strain shows its 3-HP producing ability [51]. The mechanisms of 3-HP production from glycerol through the propanediol utilization pathway is detailed in chapter 4 and forms the basis for this thesis.

3.1.3 Recombinant microbial production of 3-HP

An approach widely investigated to industrialize 3-HP production is the heterologous expression of various 3-HP producing pathways in microbial hosts. A large number of research- and industrial groups have been proposing and implementing various pathways in *E. coli*, *K. pneumoniae*, *S. cerevisiae* and *Lactobacillus* sp. using mainly glucose and glycerol as substrates in order to reach significant titers, yields and productivities of the end product. Proposed pathways for 3-HP production are shown in Figure 3.3.

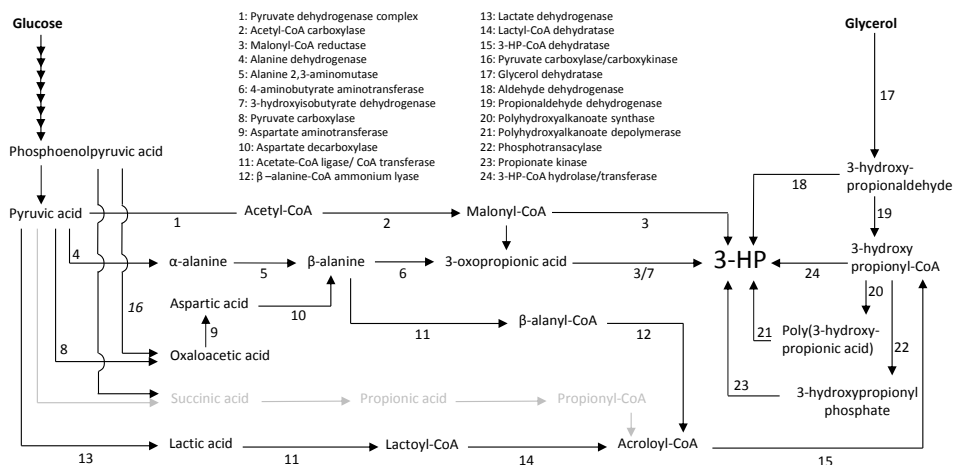


Figure 3.3
Proposed pathways for recombinant 3-HP production from glucose and glycerol.

3.1.3.1 Microbial production of 3-HP from glucose

The U.S. based company Cargill has patented 7 pathways for the production of 3-HP from glucose [21, 22, 52, 53]. Since none of these pathways have been detected in native systems, recombinant expression of several enzymes is required. While glucose is the starting material, these pathways are presented with the intermediates pyruvic acid or phosphoenolpyruvic acid (PEP) as substrates, obtained through glycolysis. Cofactor dependency, ATP production and favourable thermodynamics are of key importance in evaluating these different pathways. The net yield from the glycolysis is 2 ATP and 2 NADH per two molecules of pyruvic acid formed.

Pathway 1 (Figure 3.4) (Enzymes 1, 2, 3 and 3/7) involves the conversion of pyruvic acid to 3-HP via acetyl-CoA [21]. No net gain of ATP per glucose is obtained via this pathway and the strict NADPH dependency of malonyl-CoA reductase [54] for the conversion of malonyl-CoA to 3-oxopropionic acid is an issue leading to cofactor imbalance. Recombinant *E. coli* utilizing pathway 1 for 3-HP production from glucose has been developed and in combination with a cofactor regeneration system resulted in a final titer of 2.14 mM with a yield of 0.02 mol/mol glucose [55].

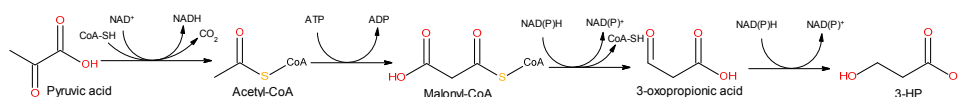


Figure 3.4
3-HP production from pyruvic acid via acetyl-CoA.

Pathway 2 (Figure 3.5) (Enzymes 4, 5, 6 and 3/7) proposes the production of 3-HP from pyruvic acid through α -alanine to β -alanine, followed by its conversion to 3-HP via 3-oxopropionic acid [53]. A net yield of 2 ATP per glucose is obtained. Alanine dehydrogenase catalyzes the conversion of pyruvic acid and glutamic acid (Glu) to α -alanine and α -ketoglutaric acid (AKG). Regeneration of Glu requires NADH. NAD(P)H is required for the conversion of 3-oxopropionic acid to 3-HP, thus the reaction is not redox balanced. Furthermore, no naturally occurring alanine 2,3-aminomutase, for the conversion of α -alanine to β -alanine, has been reported to date. Instead, B₁₂-dependent lysine 2,3-aminomutase has been engineered for the transfer of the amino group from the α - to β -position from the carboxyl group of the amino acid [53].

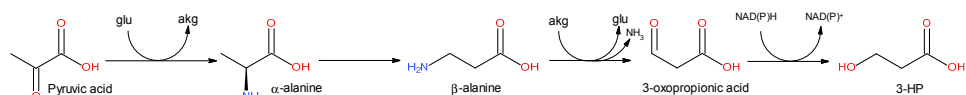


Figure 3.5

3-HP production from pyruvic acid via α -alanine, β -alanine and 3-oxopropionic acid.

Pathway 3 (Figure 3.6) (Enzymes 4, 5, 11, 12, 15 and 24) involves the conversion of pyruvic acid to 3-HP via α -alanine, β -alanine, β -alanyl-CoA, acryloyl-CoA and 3-HP-CoA [21, 53]. As ATP is required for the conversion of β -alanine to β -alanyl-CoA, no net yield of ATP per glucose is achieved. Redox neutrality is reached starting from glucose. As stated for pathway 2, the conversion of α -alanine to β -alanine requires alanine 2,3-aminomutase activity which has not been detected to date in natural systems.

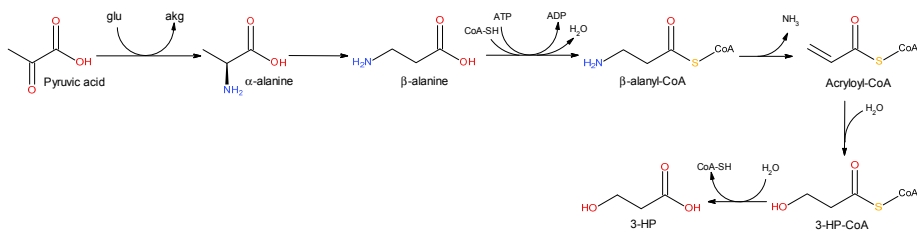


Figure 3.6

3-HP production from pyruvic acid via α -alanine, β -alanine and 3-HP-CoA.

Pathways 4 (Figure 3.7) (Enzymes 8/16, 9, 10, 6 and 3/7) and 5 (Figure 3.8) (Enzymes 8/16, 9, 10, 11, 12, 15 and 24) are similar to pathways 2 and 3, however starting from pyruvic acid or phosphoenolpyruvic acid to obtain intermediates oxaloacetic acid and aspartic acid [21, 52]. Conversion of phosphoenolpyruvic acid to pyruvic acid and oxaloacetic acid leads to the production of 1 ATP. Direct conversion of phosphoenolpyruvic acid to oxaloacetic acid and its further conversion to 3-HP

however leads to a net gain of 2 ATP per glucose for pathway 4 and no gain of ATP per glucose for pathway 5. In contrast, pyruvic acid to 3-HP leads to a net loss of 2 ATP per glucose for pathway 4 and no net gain of ATP per glucose for pathway 5. Metabolic engineering of *S. cerevisiae* with enzymes for pathway 4 resulted in a final titer of 13.7 g/L 3-HP and a yield of 0.13 mol/mol glucose [56].

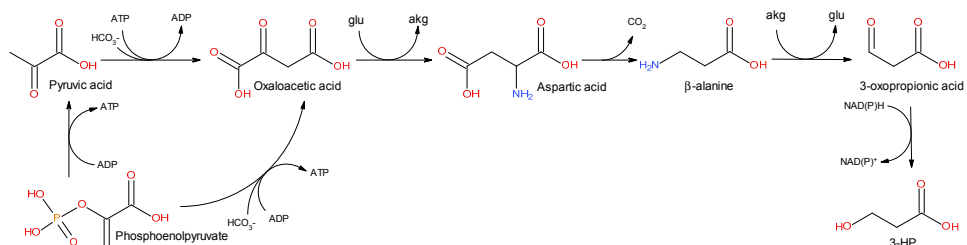


Figure 3.7

3-HP production from pyruvic acid or phosphoenolpyruvic acid via oxaloacetic acid and 3-oxopropionic acid.

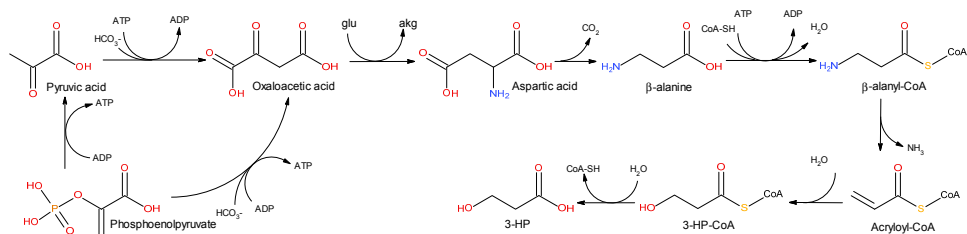


Figure 3.8

3-HP production from pyruvic acid or phosphoenolpyruvate via oxaloacetic acid and 3-HP-CoA.

Pathway 6 (Figure 3.9) (Enzymes 13, 11, 14, 15) involves conversion of pyruvic acid to 3-HP via lactic acid [21]. The ATP-dependency of CoA-transferase, for the conversion of lactic acid to lactoyl-CoA, leads to no net gain of ATP from glucose. Pyruvic acid conversion to lactic acid requires NADH, hence starting from glucose the reaction is redox neutral.

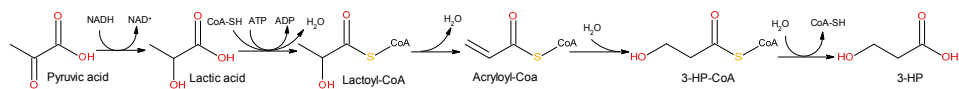


Figure 3.9

3-HP production from pyruvic acid via lactic acid.

3-HP production via propionic acid has been proposed, with starting materials being phosphoenolpyruvic acid or pyruvic acid [21]. The reaction is based on succinic acid fermentation by *Actinobacillus* sp [57]. A net loss of 0.33 ATP per 3-HP formed as

well as the possible irreversibility of 3-HP to propionyl-CoA [22] are drawbacks in the further study of this pathway.

3.1.3.2 Microbial production of 3-HP from glycerol

3-HP formation from glycerol is based on two metabolic pathways. Both pathways utilize cofactor B₁₂-dependent or B₁₂-independent glycerol dehydratase for the conversion of glycerol to 3-HPA. While a CoA-independent route utilizes NAD⁺-dependent aldehyde dehydrogenase, found in various microorganisms, for the direct oxidation of 3-HPA to 3-HP (Pathway A), a CoA-dependent pathway utilizes propanediol utilization pathway enzymes involved in native 3-HP production from glycerol by *Klebsiella pneumoniae* or *Lactobacillus* sp. (Pathway B). Studies involving 3-HP production by pathway A or pathway B are presented in Table 3.1. **Paper II** and **III** cover 3-HP production through pathway B.

3.1.3.2.1 Glycerol dehydratase

Glycerol dehydratase is a rather complex system consisting of up to five interacting subunits for activation and catalysis of glycerol to 3-HPA. Coenzyme B₁₂-dependent glycerol dehydratase have been found in *Salmonella*- [58], *Citrobacter* [59], *Klebsiella*- [60, 61] and *Lactobacillus* [62-64] sp. The latter two, specifically those of *K. pneumoniae* (DhaB123 and reactivase GdrAB) [60, 65] and *Lactobacillus brevis* (DhaB123 and reactivase DhaR12) [66, 67] have been utilized for native and recombinant production of 3-HP from glycerol. Homolysis of a cobalt to carbon (Co-C) bond of cofactor B₁₂ occurs in the presence of the substrate, thus generating an adenosyl radical. This radical then serves as an intermediate hydrogen acceptor from the C1 of the substrate, thus generating a radical substrate. This is followed by enzyme mediated transfer of the hydroxyl group of C2 to C1 of the substrate, return of hydrogen from the adenosyl-group and release of H₂O, thus obtaining the aldehyde. However, inactivation of the enzyme occurs due to its irreversible bond to the cobalamin-group. A reactivase is activated by ADP, binds to the enzyme, and releases the cobalamin, after which an ATP-dependent release of the reactivase from the enzyme occurs [65, 68].

B₁₂-independent glycerol dehydratase has been reported from *Clostridium butyricum*, instead utilizing S-adenosyl methionine for its activation. The catalysis is proposed to be initiated by the radical cleavage of S-adenosylmethionine by an [4Fe-4S]⁺-cluster catalyzed by a glycerol dehydratase activating enzyme, followed by a radical propagation to Cys433 of glycerol dehydratase, which transfers the radical to the ligand, causing the release of a C2 hydroxyl and water formation. A proton is transferred from C1 to Glu435 and the radical is transferred back to Cys433 [69, 70].

The high oxygen sensitivity of glycerol dehydratases is a limiting factor for utilizing the enzyme in combination with oxidative enzymatic catalysis of 3-HPA to 3-HP [69].

Table 3.1
Summary of studies for the production of 3-HP from glycerol

Production strain	Pathway	Heterologous enzyme(s) 1	Heterologous enzyme(s) 2	Metabolic engineering	Mode of production	Production method	Titer (g/L)	Yield (mol/mol)	Productivity (g/L/h)	Reference
<i>Lactobacillus</i> sp.	A	-	-	-	Batch	Culture tubes	2.8	0.55	0.07	Garai-Ibbabe et al., 2008 [42]
<i>E. coli</i>	A	DhaB ¹	AldH ⁵	-	Batch	250 mL shake flask	0.6	0.48	0.02	Raj et al., 2008 [71]
<i>E. coli</i>	A	DhaB ¹	AldH ⁵	-	Fed-batch	5 L bioreactor	31.0	0.35	0.43	Raj et al., 2009 [72]
<i>K. pneumoniae</i>	A	DhaB ¹ + GdrAB ³	AldH ⁵	-	Batch	3 L bioreactor	2.9	0.10	0.12	Zhu et al., 2009 [73]
<i>E. coli</i>	A	DhaB ¹ + GdrAB ³	AldH ⁵ /KGSADH ⁶	-	Fed-batch	5 L bioreactor	38.7	0.35	0.54	Rathnasingh et al., 2009 [74]
<i>K. pneumoniae</i>	A	DhaB ¹ + GdrAB ³	PuuC ⁷	dDhaT ¹⁰	Fed-batch	5 L bioreactor	16.0	0.23	0.67	Ashok et al., 2011 [75]
<i>K. pneumoniae</i>	B	-	PduP ⁸	-	Fed-batch	5 L bioreactor	6.8	-	0.23	Luo et al., 2011a [76]
<i>K. pneumoniae</i>	A	-	dDhaK ⁹ + DhaT ¹⁰	-	Batch	5 L bioreactor	1.4	0.07	0.06	Luo et al., 2011b [77]
<i>K. pneumoniae</i>	B	-	PduP ⁸	dDhaP ⁹	Batch	5 L bioreactor	1.9	0.10	0.08	Luo et al., 2012 [78]
<i>K. pneumoniae</i>	A	-	AldH ⁵	-	Fed-batch	5 L bioreactor	24.4	0.18	1.02	Huang et al., 2012 [79]
<i>K. pneumoniae</i>	A	-	AldH ⁵ /KGSADH ⁶ /PuuC ⁷	dDhaT ¹⁰ + dYqjD ¹²	Fed-batch	1.5 L bioreactor	16.3	0.40	0.30	Ko et al., 2013 [80]
<i>K. pneumoniae</i>	A	-	KGSADH ⁶	-	Fed-batch	1.5 L bioreactor	22.7	0.35	0.38	Kumar et al., 2012 [81]
<i>E. coli</i>	A	DhaB ¹ + DhaR ⁴	AldH ⁵	-	Fed-batch	2.5 L bioreactor	14.3	0.15	0.26	Kwak et al., 2013 [66]
<i>K. pneumoniae</i>	A	-	KGSADH ⁶	dDhaA ¹⁰	Fed-batch	1.5 L bioreactor	11.3	0.27	0.94	Kumar et al., 2013 [82]
<i>K. pneumoniae</i>	A	-	Various aldehyde dehydrogenases	-	Fed-batch	5 L bioreactor	48.9	0.41	1.75	Huang et al., 2013 [83]
<i>K. pneumoniae</i>	A	DhaB ¹ + GdrAB ³	PuuC ⁷	dDhaT ¹⁰ + dYqjD ¹²	Fed-batch	5 L bioreactor	28.1	0.40	0.58	Ashok et al., 2013 [84]
<i>K. pneumoniae</i>	A	-	PuuC ⁷	dGlpK ¹¹ + dDhaT ¹⁰	Fed-batch	5 L bioreactor	22.0	0.30	0.46	Ashok et al., 2013 [85]
<i>Pseudomonas denitrificans</i>	A	DhaB ¹ + GdrAB ³	PuuC ⁷	-	Batch	250 mL shake flask	4.9	0.68	0.13	Zhou et al., 2013 [86]
<i>K. pneumoniae</i>	A	-	AldH ⁵ + AOX ¹¹	dDhaT ¹⁰	Batch	250 mL shake flask	0.9	0.03	0.04	Li et al., 2014 [87]
<i>K. pneumoniae</i>	A	-	AldH ⁵ + AOX ¹¹	dDhaT ¹⁰	Fed-batch	5 L bioreactor	3.0	0.03	0.14	Li et al., 2014 [87]
<i>E. coli</i>	A	DhaB ¹ + GdrAB ³	KGSADH ⁶	-	Batch	250 mL shake flask	2.7	0.31	-	Senkararayanan et al., 2014 [88]
<i>E. coli</i>	A	DhaB ¹ + GdrAB ³	KGSADH ⁶	-	Batch/ Fed-batch	1.5 L bioreactor	42.3	0.31	0.86	Senkararayanan et al., 2014 [88]
<i>E. coli</i>	A	DhaB ¹ + GdrAB ³	AldH ⁵	dAckA ¹³ + dYqjD ¹²	Batch	250 mL shake flask	16.3	0.28	0.34	Jung et al., 2014 [89]
<i>E. coli</i>	A	DhaB ¹ + GdrAB ³	AldH ⁵	dAckA ¹³ + dYqjD ¹²	Fed-batch	5 L bioreactor	40.5	0.26	1.26	Jung et al., 2014 [89]
<i>L. reuteri</i>	B	-	-	Pdu ¹³	Fed-batch	3 L bioreactor	10.6	0.5	1.08	Dishisha et al., 2014 [50]
<i>E. coli</i>	A	DhaB ¹ + DhaR ⁴	PSALDH ¹²	dGlpK ¹¹ + dYqjD ¹²	Fed-batch	2.5 L bioreactor	57.3	0.86	1.59	Kim et al., 2014 [67]
<i>L. reuteri</i> + <i>E. coli</i>	B	-	PduP ⁸ + PduL ¹³ + PduW ¹⁴	-	Fed-batch + Fed-batch	3 L bioreactor/ 1 L shake flask	1.1	0.68	0.09	Paper II
<i>L. reuteri</i> + <i>E. coli</i>	B	-	PduP ⁸ + PduL ¹³ + PduW ¹⁴ + Nox ¹⁵	-	Fed-batch + Fed-batch	3 L bioreactor/ 1 L shake flask	(2.0)	0.53	0.15	Paper III
<i>L. reuteri</i> + <i>E. coli</i>	B	-	PduP ⁸ + PduL ¹³ + PduW ¹⁴ + Nox ¹⁵	-	Fed-batch + Fed-batch	3 L bioreactor/ 1 L bioreactor	(6.1)	0.41	0.30	Paper III

¹Glycerol dehydratase of *K. pneumoniae*; ²Glycerol dehydratase of *L. brevis*; ³Glycerol dehydratase of *E. coli* K12; ⁴α-ketoglutaric semialdehyde dehydrogenase of *Acetivibrio bromilense*; ⁵Aldehyde dehydrogenase 1 of *K. pneumoniae*; ⁶Propionaldehyde dehydrogenase of *L. reuteri*; ⁷Aldehyde dehydrogenase 2 of *K. pneumoniae*; ⁸1,3-PD oxidoreductase of *Pseudomonas* sp.; ⁹Semialdehyde dehydrogenase of *Pseudomonas aeruginosa*; ¹⁰Propionaldehyde dehydrogenase of *L. reuteri*; ¹¹Propionate kinase of *L. reuteri*; ¹²NADH oxidase of *L. reuteri*; ¹³Glycerol dehydrogenase of *K. pneumoniae*; ¹⁴1,3-PD oxidoreductase 2 of *K. pneumoniae*; ¹⁵D-lactate dehydrogenase of *K. pneumoniae*; ¹⁶Glycerol kinase of *E. coli*; ¹⁷1,3-PD oxidoreductase of *E. coli*; ¹⁸Overexpression of the *pdu* operon; ¹⁹Glycerol Kinase of *E. coli*.

3.1.3.2.2 CoA-independent pathway (Pathway A)

Nearly all studies reported on the production of 3-HP from glycerol are based on pathway A, which is initiated by dehydration of glycerol to 3-HPA by glycerol dehydratase followed by oxidation of 3-HPA to 3-HP by NAD⁺-dependent aldehyde dehydrogenase (Figure 3.10). Aldehyde dehydrogenases from *E. coli* K12 (AldH), *K. pneumoniae* (PuuC) and *Azospirillum brasiliense* (KGSADH) have been recombinantly expressed or overexpressed in *E. coli*, *K. pneumoniae* or *Pseudomonas nitroficans* in combination with glycerol dehydratase from *K. pneumoniae* or *L. brevis*.

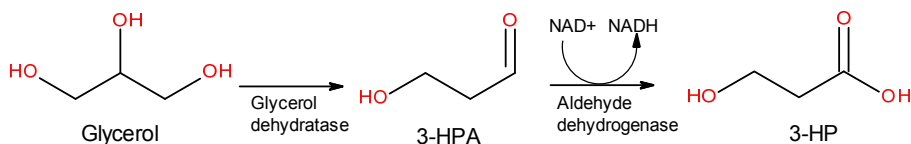


Figure 3.10
CoA-independent production of 3-HP from glycerol (Pathway A).

3.1.3.2.3 CoA-dependent pathway (Pathway B)

The native abilities of *Lactobacillus* species [41, 42, 50] and *K. pneumoniae* [77] to produce 3-HP and 1,3-PD from glycerol form the basis for pathway B. Dehydration of glycerol to 3-HPA by glycerol dehydratase is followed by a cascade reaction of NAD⁺- and CoA-dependent propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and ATP-producing propionate kinase (PduW) (Figure 3.11). It has been shown that deletion of PduP in *K. pneumoniae* leads to a decrease in final titers of 3-HP from glycerol [78]. In **Paper I**, the conversion of 3-HPA to the intermediate 3-HP-CoA is presented. **Paper II** is the first report on the recombinant expression of PduP, PduL and PduW for the production of 3-HP, where 12 mM 3-HP was produced before NAD⁺ depletion occurs. An NAD⁺ regeneration mechanism is present natively in *Lactobacillus reuteri* [50] and *K. pneumoniae* [41, 60] through the production of 1,3-PD by NADH-dependent 1,3-PD oxidoreductase. In order to increase the final yield of 3-HP from glycerol by pathway B, an alternative NAD⁺ regeneration system has to be applied for avoiding 1,3-PD production. **Paper III** presents a novel method for regeneration of NAD⁺ by the co-expression of NADH oxidase of *L. reuteri* that liberates water as the co-product, resulting in a 3-HP concentration at the very least two times higher that obtained in **Paper II**.

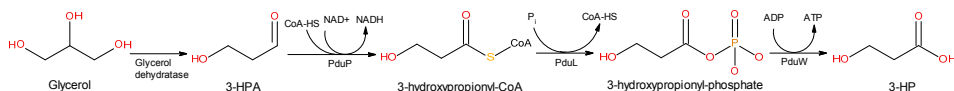


Figure 3.11
CoA-dependent production of 3-HP from glycerol (Pathway B).

3.2 Poly(3-hydroxypropionate): a biodegradable polymer

Poly(3-HP) is a short chain length (scl) PHA which has generated interest due to its potential usage as a flexible and stable biomaterial. A low melting temperature of about 77 °C, glass transition temperature of -22 °C, a highest ϵ_b (elongation at break) value of 634 % as well as its biodegradability makes the polymer a candidate for the replacement of petroleum-derived thermoplastics and is an alternative to more brittle biopolymers such as poly(3-HB) [15].

3.2.1 Chemical and enzymatic methods for poly(3-HP) production

Various non-microbial methods exist for the production of poly(3-HP). In the presence of effective catalysts such as FeCl_3 , SnCl_4 and NaOH and even less effective catalysts such as NaCl , Ca(OH)_2 , HCl and acetic acid, polymerization of β -propiolactone to poly(3-HP) with a highest molecular weight of 1 kg/mol occurs when the substrate is added slowly to a 150 °C solution of the acid, base or salt catalysts in an inert solvent [90]. The carcinogenic properties of β -propiolactone is however a major drawback [25, 91]. In another approach, 3-HP can be used as a substrate for the construction of 3-HP macrocyclic ester trimers to a yield of 30 wt.% via acid-catalyzed self-condensation, which in turn can be used for the production of poly(3-HP) by ring opening polymerization in the presence of a Zn-alkoxide catalyst in CH_2Cl_2 at room temperature. 90% of the trimer was converted to poly(3-HP) in 30 min with a highest molecular weight of 67 kg/mol [92] However, the method requires 3-HP which is not available commercially.

Methyl 3-HP can be utilized as a substrate in a solvent-free enzymatic system for the production of poly(3-HP). Lipase (Novozymes 435) successfully catalyzed the polymerization of 3-HP to poly(3-HP) at 55 °C with an 85 % molar yield after 30h and a largest molecular weight of ca 2.2 kg/mol. Methyl formed in the process is however inhibitory for the generation of polymers of larger molecular weight [93]. Furthermore, the high costs associated with the substrate is a major drawback.

3.2.2 Recombinant microbial production of poly(3-HP)

In contrast to other PHAs, no microorganisms are known to produce poly(3-HP) natively [15]. There are however mechanisms and pathways that have been utilized for the recombinant production of poly(3-HP), with starting materials being acetyl-CoA, glycerol or 1,3-PD. All pathways are via 3-HP and 3-HP-CoA and require a polyhydroxyalkanoate synthase (PhaC) for the polymerization of 3-HP-CoA. Studies involving poly(3-HP) production from glucose, glycerol and 3-HPA by the expression of relevant enzymes in various host strains can be seen in Table 3.2.

Table 3.2

Summary of studies on the recombinant production of poly(3-HP).

Production strain	Substrate	Heterologous enzymes	Mode of production	Production method	Additives	Content of cells wt.% (Wt _{PHB} /Wt _{CoA})	Titer (g L ⁻¹)	Volumetric productivity (g L ⁻¹ h ⁻¹)	Reference
<i>E. coli</i>	Glycerol	DhaB ¹ + PduP ² + PhaC ³	Fed-batch	14 L bioreactor	di-Na ⁺ and K ⁻ -Na ⁻ -tartrate	11.98	1.42	0.03	Andreessen et al., 2010 [94]
<i>E. coli</i>	Glucose	Mcr ⁴ + PrpE ⁵ + PhaC ³	Fed-batch	Shake flask	Biotin and NaHCO ₂	0.98	0.01	1.39 * 10 ⁻⁴	Wang et al., 2012 [95]
<i>E. coli</i>	Glycerol	DhaB ⁶ + GdrAB ⁷ + PduP ² + PhaC ³	Fed-batch	5 L bioreactor	Vitamin B ₁₂	46.4	10.1	0.12	Wang et al., 2013 [96]
<i>Shimwellia blattae</i>	Glycerol	DhaT ⁸ + AldD ⁹ + Pct ¹⁰ + PhaC ³	Fed-batch	2 L bioreactor	-	9.8	0.40	0.01	Heinrich et al., 2013 [97]
<i>S. blattae</i>	Glycerol	DhaT ⁸ + AldD ⁹ + Pct ¹⁰ + PhaC ³	Fed-batch	2 L bioreactor	-	14.5	0.74	0.015	Andreessen et al., 2014 [98]
<i>K. pneumoniae</i>	Glycerol	DhaB ⁶ + GdrAB ⁷ + AldH ¹¹ + PrpE ⁵ + PhaC ³	Batch	250 mL shake flask	-	12.7	0.24	0.005	Feng et al., 2015 [99]
<i>E. coli</i>	3-HPA	PduP ¹² + PhaC ¹³	Fed-batch	2 L baffled shake flask	-	40.0	0.26	0.065	Paper IV

¹Glycerol dehydratase of *C. butyricum*; ²Propionaldehyde dehydrogenase of *S. enterica*; ³Polyhydroxyalkanoate synthase of *R. eutropha*; ⁴Malonyl-CoA reductase of *Chloroflexus aurantiacus*; ⁵Propionyl-CoA synthase of *E. coli*; ⁶Glycerol dehydratase of *K. pneumoniae*; ⁷Glycerol dehydratase reactivase of *K. pneumoniae*; ⁸Polyhydroxyalkanoate synthase of *Cupriavidus necator*; ⁹1,3-Propanediol oxidoreductase of *Pseudomonas putida*; ¹⁰Aldehyde dehydrogenase of *P. putida*; ¹¹Propionate CoA-transferase of *Clostridium propionicum*; ¹²Propionaldehyde dehydrogenase of *L. reuteri*; ¹³Polyhydroxyalkanoate synthase of *Chromobacterium* sp.

3.2.2.1 Poly(3-HP) production from acetyl-CoA

Acetyl-CoA is formed through glycolysis and can be utilized for the formation of poly(3-HP) via malonyl-CoA, oxaloacetic acid, 3-HP and 3-HP-CoA (Figure 3.12). In aerobic conditions, lower yields of poly(3-HP) can be expected when utilizing this pathway as the tricarboxylic acid (TCA) cycle utilizes acetyl-CoA for ATP and NADH-production.

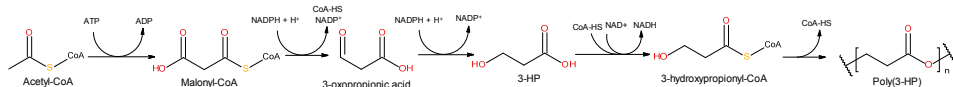


Figure 3.12

Poly(3-HP) production from acetyl-CoA.

3.2.2.2 Poly(3-HP) production from 1,3-PD or glycerol

Conversion of 1,3-PD and glycerol to poly(3-HP) occurs through intermediate 3-HPA (Figure 3.13). While 1,3-PD conversion to 3-HPA requires the reversible action of 1,3-PD oxidoreductase, glycerol conversion to 3-HPA requires the action of glycerol dehydratase. Oxidation of 3-HPA to 3-HP is followed by CoA-acylation of 3-HP to 3-HP-CoA by NAD⁺-dependent PduP. Polymerization of 3-HP-CoA is achieved by PhaC, thus obtaining poly(3-HP). Kinetic and structural characterization of PduP is performed in **Paper I**. PduP is further detailed in chapter 4.2.2. Conversion of 3-HPA to poly(3-HP) via 3-HP and 3-HP-CoA by PduP of *L. reuteri* and PhaC of *Chromobacterium* sp. is presented in **Paper IV**.

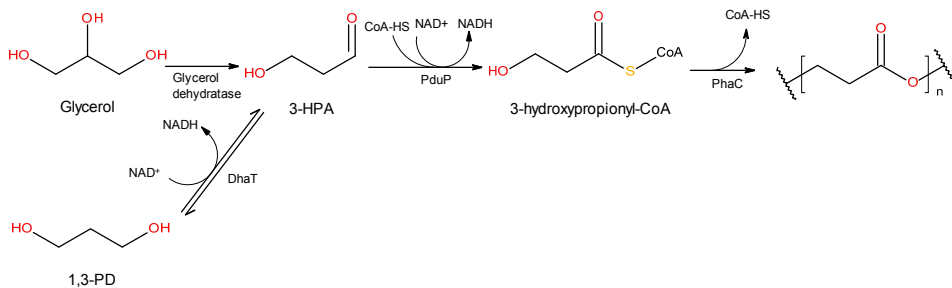


Figure 3.13
Poly(3-HP) production from glycerol or 1,3-PD

3.2.2.3 Polyhydroxyalkanoate synthase (*PhaC*)

The polymerization of CoA thioesters, such as 3-HP-CoA, are performed by polyhydroxyalkanoates synthases (Pha). Over 60 Pha genes of eubacteria have been characterized and even more from prokaryotes have been identified through genomic sequencing [14]. Pha is found in over 45 microorganisms [100] and is utilized for the storage of carbon and energy reserves without any significant effect on the osmotic pressure of the cell [12]. Pha can be divided into four classes based on their primary structures, substrate specificity and subunit composition [101]:

- Class I Phas contain only one subunit, PhaC (61-72 kDa), and utilize CoA thioesters of 3-hydroxy fatty acids of 3-5 carbon atoms.
- Class II Phas are similar to Class I Phas, instead utilizing CoA thioesters of 3-hydroxy fatty acids of 6-14 carbon atoms.
- Class III Phas contain two subunits, PhaC (ca 40 kDa) and PhaE (ca 40 kDa) and prefer 3-hydroxy fatty acids of 3-5 carbons.
- Class IV Phas contain two subunits, PhaC (ca 40 kDa) and PhaR (ca 20 kDa).

The various studies for the recombinant production of poly(3-HP) in *E. coli* utilize mainly Class I *Ralstonia eutropha* PhaC (PhaC_{Re}) for the polymerization of 3-HP-CoA. A conserved triad of amino acids consisting of cysteine, histidine and aspartic acid are proposed to be responsible for the catalytic activity of the enzyme [102]. The aspartic acid activates the hydroxyl group of the 3-hydroxy thioester, while the histidine generates the cysteine thiolate which in turn is responsible for covalent catalysis [103].

Characterization of PhaC from *Chromobacterium* sp strain USM2 (PhaC_{Cs}) revealed a shorter lag phase and an activity higher than that reported for any isolated and purified PhaC for the production of poly(3-HB) where an accumulation of 78 wt.% (wt_{poly(3-HB)}/wt_{CDW}) was achieved in *E. coli*. Furthermore, it was found to utilize a number of 3-hydroxy thioesters as substrates [104]. Recombinant co-expression of codon-optimized PhaC_{Cs} with PduP of *L. reuteri* was achieved in *E. coli* BL21(DE3) for the production of poly(3-HP) from 3-HPA (**Paper IV**).

4 *Lactobacillus reuteri* and the Propanediol Utilization Pathway

4.1 Lactic acid bacteria and *L. reuteri*

L. reuteri is a species of the lactic acid bacteria (*Lactobacillales*) order. The order contains a number of gram-positive bacteria that are identified through their morphology, mode of glucose metabolism and physiological characteristics. Lactic acid bacteria are non-spore forming, non-respiring and aerotolerant cocci or rods which produce lactic acid as their main product of metabolism. Lactic acid bacteria obtain energy by phosphorylation of carbohydrates as either homofermentative (utilizing the glycolysis/Embden-Meyerhof-Parnas (EMB) pathway with production of lactic acid), facultative heterofermentative (utilizing the phosphoketolase pathway with co-production of lactic acid, ethanol and CO₂) or obligatory heterofermentative species (utilizing both pathways). Besides *Lactobacillus*, the order encompasses species of *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Tetrageonococcus*, *Vagococcus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Wessella*, *Lactococcus* and *Streptococcus* [105, 106].

L. reuteri is an obligately heterofermentative bacteria. The strain is found naturally in the gastrointestinal tract of humans and other animals [63]. The strain is considered to be probiotic, meaning it has health enhancing effects on humans. Its probiotic effects are partly attributed to the microorganism's ability to produce the antimicrobial substance reuterin [41, 107], which is a system composing of 3-HPA, HPA-hydrate and HPA-dimer in equilibrium [108]. The strain is considered GRAS (Generally Regarded as Safe).

4.2 Metabolism of glucose and glycerol in *L. reuteri*

As a heterofermentative lactic acid bacteria, glucose metabolism of *L. reuteri* is based on one hand the EMB pathway (Figure 4.1a) and the other the phosphoketolase pathway (PKP) (Figure 4.1b) [106]. End-products are lactic acid, ethanol and CO₂ [105]. While the EMB pathway results in the production of 2 ATP molecules, the PKP pathway results in the formation of 1 ATP. In the presence of external electron acceptors however, ATP-formation and the end-product distribution is different. The presence of fructose with glucose has been seen to enhance the growth of the bacteria, as fructose can be used as an electron acceptor [109] for the production of mannitol

[110], pushing the reaction towards the formation of acetic acid by contributing to an alternative ATP-producing pathway for NAD^+ -regeneration than through the formation of ethanol from acetyl-CoA. Oxygen can be utilized as an electron acceptor by NADH oxidase (**Paper III**), resulting in NAD^+ -regeneration and liberation of H_2O . Other examples of external electron acceptors are citric acid and 1,2-ethanediol [105].

L. reuteri can not utilize glycerol for growth. The conversion of glycerol to dihydroxyacetone-phosphate and its further metabolism via the EMB-pathway requires dihydroxyacetone kinase (DHA) and *L. reuteri* lacks the necessary gene for DHA expression [63]. *L. reuteri* can however utilize glycerol to form 3-HP and 1,3-PD by the same mechanism as that of 1,2-PD conversion to propionic acid and propanol via its propanediol utilization (pdu) pathway [111].

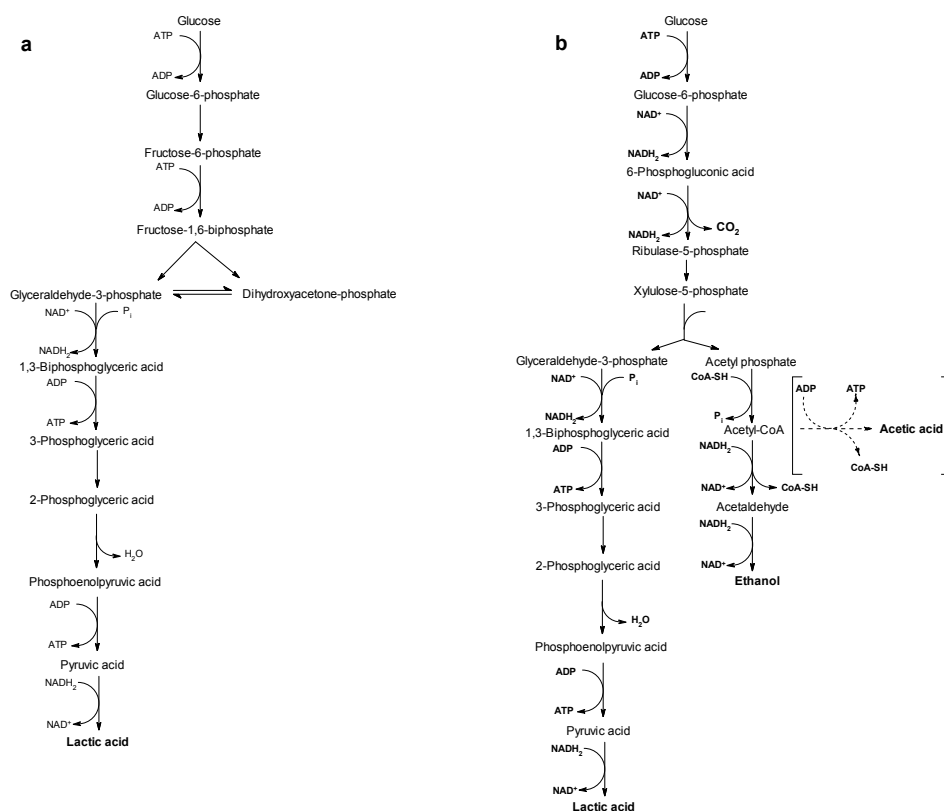


Figure 4.1

Metabolism of glucose in *Lactobacillus reuteri* by a) EMB-pathway resulting in lactic acid production, and b) PKP-pathway resulting in lactic acid, ethanol and CO_2 -production.

4.2.1 Propanediol utilization pathway

The pdu pathway is responsible for the process of utilizing 1,2-propanediol and glycerol, respectively, as electron acceptors in a number of microorganisms. The operon and its mechanism from *Salmonella enterica* [112], *K. pneumoniae* [51] and *Lactobacillus* sp. [62, 63] has been reported. Pdu-enzymes are encoded by the pdu-operon, containing several genes encoding not only catalyzing enzymes but also those responsible for the function and structure necessary for achieving efficient conversion of the substrate to its products. Genetic characterization of *L. reuteri* show a co-regulation of pdu-, cbi-, hem- and cob-operons, with the three last being responsible for cobalamin (B₁₂) biosynthesis, necessary for the activation of glycerol/diol dehydratase [63]. High genomic similarities to the pdu-cob-cluster of *S. enterica* suggests its presence in *L. reuteri* due to horizontal gene transfer [113]. In *S. enterica*, regulatory control of the cluster is based on its induction by 1,2-propanediol or glycerol [114]. In a similar manner, 1,2-propanediol or glycerol induce expression of the pdu-cbi-hem-cob cluster in *L. reuteri* [50].

Cofactor B₁₂-dependent diol/glycerol dehydratase, dehydrating 1,2-propanediol or glycerol to propionaldehyde or 3-HPA, respectively, is encoded by PduCDE. Furthermore, the pdu-operon encodes a microcompartment consisting of structural proteins [115] in which dehydration of 1,2-propanediol or glycerol results in the production of the toxic aldehydes, propionaldehyde or 3-HPA. CoA-acylation by PduP [116] of the aldehydes in the microcompartment is followed by the transport of the intermediates to the cytoplasm of the cell, where PduL [117] and PduW [118] convert the intermediates to propionic acid or 3-hydroxypropionic acid. NADH-dependent 1,3-propanediol oxidoreductase (PduQ), present in the cytoplasm, converts the aldehydes, transported outside the microcompartment, to their respective alcohols propanol or 1,3-PD [119, 120], reaching a redox balance and equimolar amounts of the carboxylic acid and alcohol (Figure 4.3).

4.2.1.1 3-HPA and reuterin

3-HPA is part of a system encompassing the monomer, its dimer (HPA-dimer) and hydrate (HPA-hydrate), called reuterin. An equilibrium exists between the different states and shifts depending on reuterin concentration; While HPA-hydrate is in excess at reuterin concentrations below 1 mM, HPA-dimer increases with increased reuterin concentrations. The monomer concentration is at 0.3 mol fractions at concentrations of 50 mM and decreases to 0.1 mol fractions at around 1 M (Figure 4.2) [108].

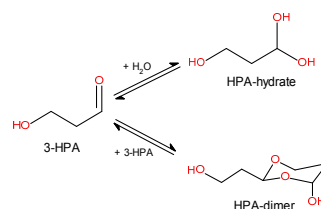


Figure 4.2
The equilibrium of the reuterin system between 3-HPA, HPA-hydrate and HPA-dimer

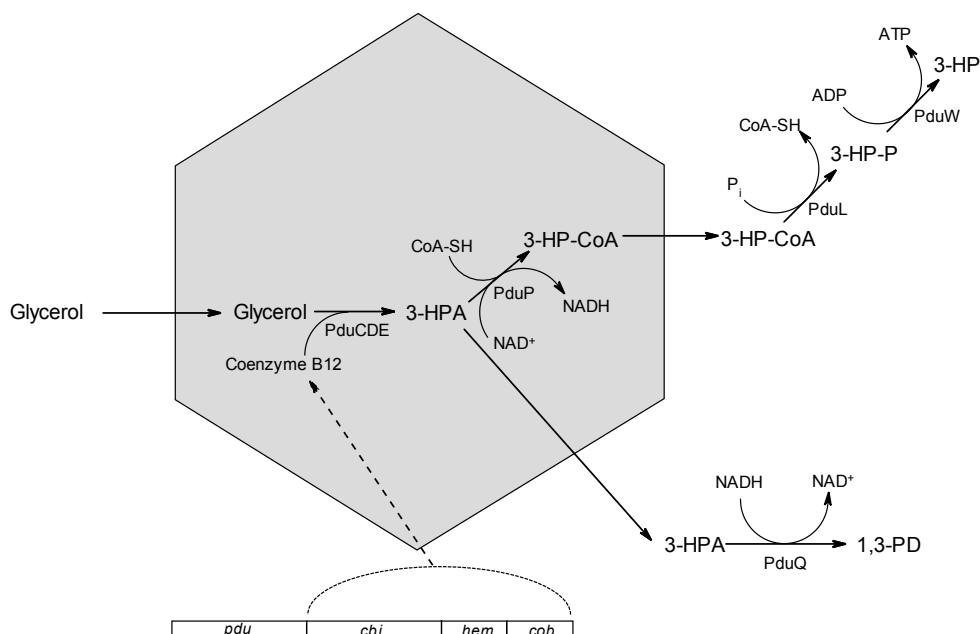


Figure 4.3

Native *pdu*-mediated 3-HP and 1,3-PD production from glycerol and the role of the *cbi-hem-cob* cluster for cofactor B₁₂ synthesis. Microcompartment seen as grey area.

Production of 3-HPA in anaerobic conditions has been observed from a number of microorganisms such as *Lactobacillus* sp. [40, 121], *Klebsiella pneumoniae* [43, 61], *Citrobacter freundii* [44, 45], *Clostridium* sp. [46, 47] and *Pantoea (Enterobacter) agglomerans* [48]. 3-HPA was first identified as an intermediate in the formation of acrolein causing the spoilage of wine [122].

4.2.1.2 *L. reuteri* 3-HPA production and purification

3-HPA production from glycerol by *L. reuteri* was first proposed by Sobolov & Smiley [40] and characterized by Talarico et al. [41, 123]. Probiotic effects of *L. reuteri* are partly attributed to its production of reuterin [107]. Reuterin induces oxidative stress mechanisms in *E. coli*, leading to the expression of a high number of genes related to oxidative stress. Its interaction with thiol groups of proteins and small molecules leads to lower growth rates [124]. Minimal inhibitory concentration of 3-HPA for *E. coli* is 7.5-15 mM while the minimal bactericidal concentration (MBC) is 15-30 mM [125].

L. reuteri in comparison with other 3-HPA producing microorganisms produces the compound extracellularly [126], with a MIC of 30-50 mM and MBC of 60-120 mM [125]. However, up to 140 mM 3-HPA has been produced by *L. reuteri*, after which production ceases due to cell inactivation [127].

3-HPA production can be performed under growing conditions of the microorganism utilizing a mixture of glucose and glycerol, with its further conversion to 1,3-PD [128]. NAD⁺ formed in the process is thus utilized in the metabolism of glucose. The compound can also be produced in resting conditions with the sole addition of glycerol, resulting in 3-HPA formation which is further converted to 3-HP and 1,3-PD by the enzymes of the pdu pathway [50]. By utilizing a scavenger binding to the formed 3-HPA, higher titers can be reached by reducing the inhibition as well as reducing the conversion to 1,3-PD and 3-HP. Examples of scavengers are semicarbazide [49, 129], carbohydrazide [129] and bisulfite [127,130,131].

Fed-batch biotransformation of glycerol in an aqueous system utilizing *L. reuteri* results in the production of up to 40 mM 3-HPA [127] which was utilized in **Paper I** for the kinetic characterization of PduP, in **Paper II** and **III** for the production of 3-HP by PduP, PduL and PduW, and in **Paper IV** for the production of poly(3-HP) by PduP and PhaC_S.

A mixture of free and bisulfite-complexed 3-HPA, obtained through *in situ* complexation of *L. reuteri* produced 3-HPA with bisulfite, binding of the complexed product to Amberlite IRA-400 resin and elution using sodium chloride [131], was utilized in order to verify its usage as a substrate for the production of 3-HP in **Paper II**.

4.2.2 Propionaldehyde dehydrogenase (PduP) (Paper I)

CoA-acylating propionaldehyde dehydrogenase utilizes NAD⁺ and HS-CoA for the CoA-acylation of a number of aldehydes. Its role in the pdu pathway has been established in *S. enterica* [116] and *K. pneumoniae* [78]. Deletion of *pduP* in *K. pneumoniae* led to a dramatic decrease in native 3-HP formation, which verified its role in 3-HP formation in the strain [78]. Kinetic characterization of recombinant *L. reuteri* PduP further indicates that the *pdu*-operon encodes genes for 1,2-propanediol and glycerol utilization. A V_{\max} of 28.9 U/mg and K_m of 28 mM when using propionaldehyde as substrate, in comparison to a highest activity of 18 U/mg before substrate inhibition occurs with 3-HPA concentrations over 7 mM, shows its preference of propionaldehyde over 3-HPA as well as an inhibitory effect on the functions of PduP by 3-HPA, proposed to be due to its interaction with the thiol-group of the catalytic cysteine residue of PduP. Optimal pH and buffer conditions were investigated for the further study and utilization of the pdu-pathway for 3-HP formation, and were found to be at pH 7 in potassium phosphate buffer.

Structural modeling of PduP, based on *Listeria monocytogenes* probable aldehyde dehydrogenase (Egd-E), reveal that PduP consists of two connected domains, with each domain containing a Rossmann-fold (Figure 4.4). Docking of NAD⁺ (Figure 4.5a) and HS-CoA (Figure 4.5b) to the enzyme reveal a shared binding site, where amino

acids Ser417, Ile275, Cys277 and Thr145 play key roles in the binding of the cofactors and the catalytic mechanism of the enzyme. A catalytic mechanism is proposed based on that of CoA-acylating methylmalonate semi-aldehyde dehydrogenase from *Bacillus subtilis* [132]. The mechanism is initiated by the binding of NAD⁺ to the catalytic site, followed by a nucleophilic attack by the sulfur of Cys277, hydride transfer, release of NADH, binding of ⁻S-CoA to the carbonyl carbon of the substrate intermediate and finally release of the product-CoA-complex.



Figure 4.4
Proposal for PduP structure based on *Listeria monocytogenes* Egd-E bound to HS-CoA (red).

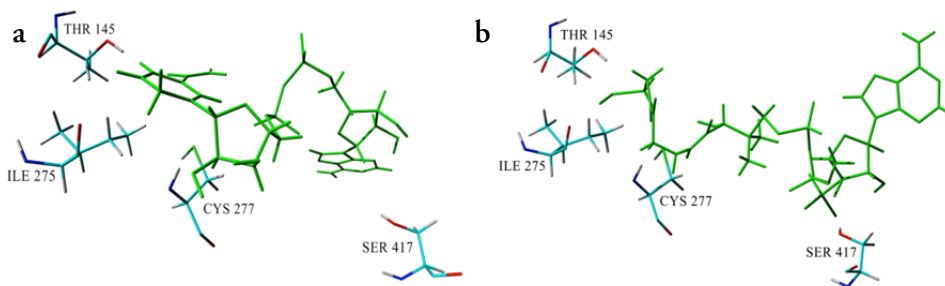


Figure 4.5
Proposed binding site of a) NAD⁺ and b) HS-CoA to PduP and amino acids involved in the catalysis as well as stabilization of the cofactors. Both cofactors (in green) bind to the same site in the enzyme.

5 Production of 3-Hydroxypropionic Acid and Poly(3-Hydroxypropionate) Using Engineered *Escherichia coli*

5.1 *Escherichia coli* as a microbial factory

5.1.1 *Escherichia coli*

E. coli is a species of the Enterobacteriales order and mainly found in the lower intestinal tract of humans and other animals. *E. coli* is gram-negative, non-spore forming, rod-shaped and a facultative anaerobe; In the presence of oxygen, ATP formation is due to aerobic respiration. In the absence of oxygen however, the microorganism switches to anaerobic respiration. The species can be divided in six phylogenetic groups, A, B1, B2, C, D and E [133]. *E. coli* O157:H7 (group E) [133] and *E. coli* O104:H4 (Group B2) [134] are pathogens, producing and releasing verotoxin (shiga-like toxin) that cause internal bleeding and kidney failure in humans [135]. Most *E. coli* strains are however harmless, even beneficial as part of the microflora of mammals [136]. Strains *E. coli* K12 (Group A) and *E. coli* B (Group B1) are non-pathogenic and due to their early isolation and identification [137, 138], the level of research on *E. coli* genomics and metabolomics has been higher than that on any other microorganisms. *E. coli* has been used to study universal cellular mechanisms such as gene regulation, replication, transcription, restriction enzymes and horizontal gene transfer, and enabled systems to be developed for the expression of recombinant proteins and metabolic engineering of *E. coli* and other microorganisms. Various mutants have been developed and are commercially available with regulated or optimized behaviour concerning metabolism and protein production.

5.1.2 *E. coli* metabolism

Glucose and glycerol metabolism in *E. coli* is based on the production of pyruvate through the glycolysis, followed by its conversion to acetyl-CoA entering the TCA cycle or production of acids in anaerobic conditions (Figure 5.1). If the conversion rate of the carbon source to acetyl-CoA surpasses the capacity of the TCA-cycle, acetic acid formation occurs [139]. End products in aerobic conditions are mainly

acetic acid and CO₂. Anaerobic conditions, alternatively called mixed acid fermentation, result in the production of succinic acid, ethanol, acetic acid, formic acid and lactic acid. In the absence or depletion of glucose as well as high accumulation of acetic acid, a glyoxylic acid pathway is activated and enables the utilization of carbon compounds like acetic acid as carbon source [140].

Glycerol can be used as a carbon source by *E. coli*, as it enters the glycolytic pathway through formation of dihydroxyacetone phosphate via glycerol-3-phosphate, utilizing one ATP [141]. The utilization of glycerol as a carbon source can be disrupted through the disruption or knock-out of glycerol kinase (GlpK) [67]. 1,3-PD production is observed in the presence of 3-HPA, through the actions of native 1,3-PD oxidoreductase/alcohol dehydrogenase (YqhD) [Paper II, 142]. Induction of YqhD expression occurs by 3-HPA when cells are in growing conditions in the presence of a carbon source. However, in resting conditions, meaning the lack of any carbon source utilized for growth, YqhD-expression does not occur (Paper II).

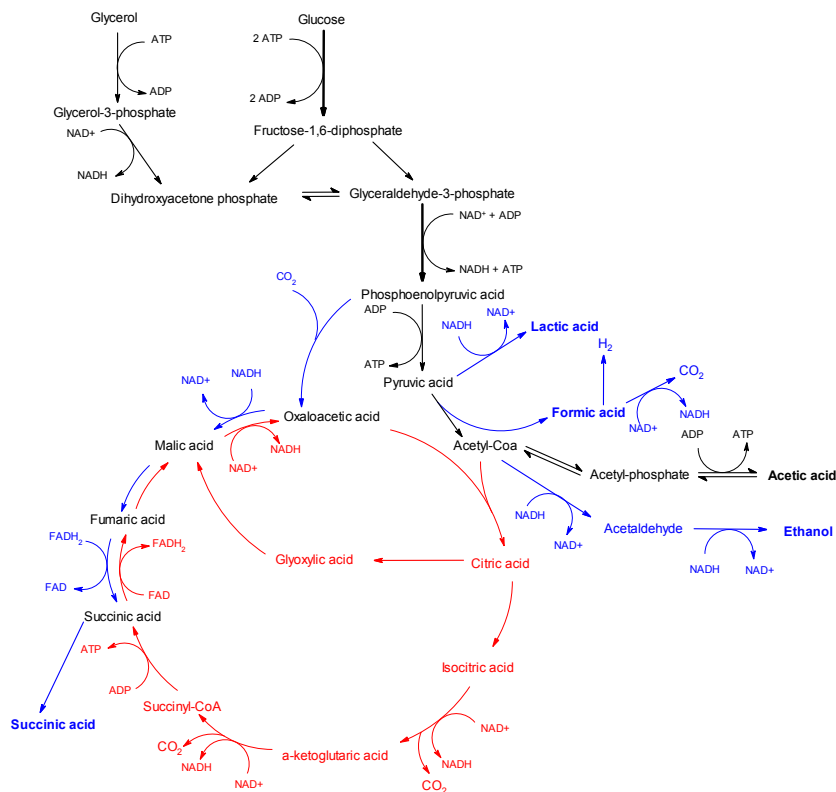


Figure 5.1
Metabolism of glucose and glycerol by *E. coli* in aerobic (red) and anaerobic (blue) conditions.

E. coli BL21 (F- *dcm ompT hsdS*(_{FB} m_B) gal [malB⁺]_{K-12}(λ^S)) is a strain derived from *E. coli* B and widely used as a laboratory strain [138]. In comparison to the other widely used laboratory strain *E. coli* K12 which produces up to 10 g/L acetic acid from glucose when grown in batch conditions, acetic acid formation by *E. coli* BL21 does not surpass 2 g/L, due to higher expression levels of genes related to acetic acid uptake and the glyoxylic acid pathway [143, 144]. High concentrations of acetic acid can be a limiting factor for achieving high cell densities and thus high amounts of recombinant protein [144]. Hence, BL21 cell dry weight yields and growth rates are higher than those of K12 [143].

5.1.3 Engineering of *E. coli*

While early methods for *E. coli* improvement were based on random mutagenesis of the strains, tools are presently available for specific insertion of relevant functions. The availability and high variety of these tools and systems for the recombinant production of proteins facilitates its usage as a host for the production of e.g. biochemicals and biofuels [145]. A number of studies have been focusing on enhancing the production of native compounds, succinic acid, lactic acid, acetic acid and ethanol [146], while other studies focus on the production of biofuels, organic acids, amino acids, sugar alcohols and diols or polymers not naturally produced by the microorganism [145]. Production of native compounds is mainly through the improvement of substrate transportation mechanisms, enhancement of present pathways or removal of pathways leading to byproduct formation. Non-native biochemicals however demand the expression of heterologous pathways. Glycerol can be utilized as a source for the production of biochemicals, biopolymers and biofuels through native or heterologous pathways [147]. Examples of biofuels are H₂ and ethanol, biochemicals are lactic acid, 3-HP, 1,3-PD, 1,2-propanediol, L-phenylalanine, succinic acid, etc. while biopolymers are poly(3-HB) and poly(3-HP) [147].

In an industrial setting, genomic level modification or metabolic engineering, meaning insertion or knock-out of affiliated genes in the genome of the production strain, is preferable. However, for indication of the feasibility or verification of a heterologous pathway, recombinant expression of proteins through the utilization of vectors (plasmids) is a suitable strategy. Introduction of genes through plasmids was the strategy employed for the study of propanediol utilization enzymes for the production of 3-HP and poly(3-HP) in **Papers II, III and IV**. A widely used system for the expression of recombinant genes through plasmids in *E. coli* is the utilization of a bacteriophage T7 promoter system. *E. coli* BL21(DE3) is an engineered strain derived from BL21 where a T7 RNA polymerase is under the control of lactose or its analogue isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible *lacUV5* promoter. A repressor encoded by genomic *lacI* is bound to the *lac* operator of a *lac* promoter and suppressing the expression of T7 RNA polymerase. IPTG displaces the repressor

and transcription of T7 RNA polymerase is activated. In the same manner, T7 compatible plasmids such as those of the PET-system or plasmids constructed for the insertion of two genes such as pCOLADuet-1 have their own set of *lacI* causing repression of inserted genes. The genes placed downstream from T7-promoters carrying a *lac* operator are thus transcribed in the presence of IPTG by T7 RNA polymerase [148, 149]. The plasmids carry a replicon with an origin of replication, a *cis* regulatory element which control the copy number of the plasmid, one or two multiple cloning sites for the insertion of genes as well as a selection marker encoding a specific antibiotic resistance [150]. Drawbacks in the utilization of *E. coli* for the recombinant expression of proteins are the lack of post-translational modification mechanisms as well as its codon usage requiring the synthesis of genes with compatible codons in cases where the codon usage differs between the host strain and native strain [151].

E. coli is able to tolerate acidic conditions down to pH of 3 when in exponential and stationary phase [152, 153]. This tolerance is due to three separate acidic resistance systems induced by acidic conditions [153]. System 1, the glucose-repressed system, is regulated by RNA polymerase and cyclic AMP receptor protein. System 2 requires extracellular glucose and glutamic acid. System 3 is activated under anaerobic conditions and requires extracellular arginine and a complex media with glucose. System 2 and 3 are based on raising internal pH. This tolerance towards acidic conditions make *E. coli* a candidate for the production of carboxylic acids such as 3-HP, as long as acid production is commenced during stationary phase as was the strategy employed for the PduP, PduL and PduW mediated production of 3-HP from 3-HPA (Paper II).

5.2 Production of 3-HP using Pdu pathway engineered in *E. coli* (Paper II)

In order to confirm PduP, PduL and PduW-mediated 3-HP production, *pduP*, *pduL* and *pduW* were introduced in *E. coli* BL21(DE3) by utilizing vector pCOLADuet-1 carrying the genes, each gene under its distinct promoter. 3-HPA produced by *L. reuteri* from glycerol in resting conditions through a fed-batch process (Chapter 4.2.1.2) was utilized as substrate.

The cell lysate of the strain expressing the three enzymes successfully produced 3-HP in equimolar quantities as that of consumed 3-HPA. Mutant strains with disrupted PduP, PduL or PduW were in a similar fashion evaluated for 3-HP formation and 3-HPA consumption. In cell lysate containing PduL and PduW, no 3-HP formation or 3-HPA consumption occur. When utilizing cell lysate containing PduL and PduW, 3-HPA consumption occurs due to the utilization of 3-HPA by PduP. However, no

3-HP formation occurs (Figure 5.2a). When lacking PduW in the cell lysate, 3-HP is formed in equimolar amounts of consumed 3-HPA, however in a lower amount than that in the system with all three enzymes present. This lower amount of 3-HP is formed due to native acetate kinase in *E. coli* which converts 3-HP-P to 3-HP thereby replacing the role of PduW; cell lysate of *E. coli* Δ *ackA* containing PduP and PduL do not produce 3-HP from 3-HPA (Figure 5.2b).

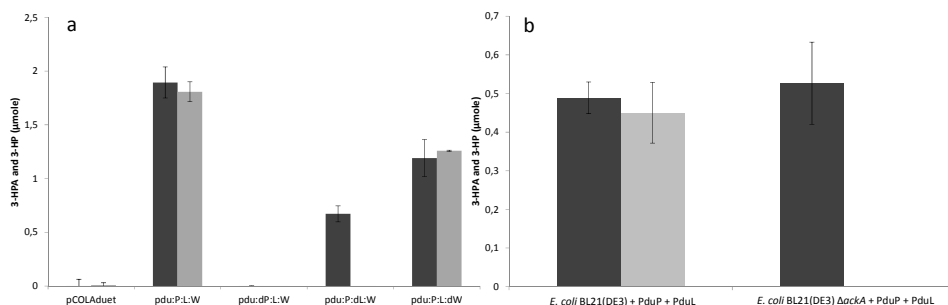


Figure 5.2

End point results in the consumption of 3-HPA (■) and production of 3-HP (▒) by a) clarified cell lysates of *E. coli* BL21(DE3) control strain, PduP, PduL and PduW-expressing strain and dP, dL and dW-mutants, with disrupted PduP, PduL or PduW, respectively and b) clarified cell lysates of *E. coli* BL21(DE3) with or without functional acetate kinase containing PduP and PduL.

1,3-PD is a major byproduct during biotransformation of 3-HPA to 3-HP by *E. coli* expressing PduP, PduL and PduW in growing conditions. 1,3-PD formation requires the induction of *yqhD*, which occurs in growing conditions by the addition of reactive aldehydes such as 3-HPA [Paper II, 142]. In resting conditions however, induction of *yqhD* does not occur and a maximum theoretical yield of 1 mol 3-HP/mol 3-HPA is achieved.

Final titers of 3-HP vary between biotransformation in growing and resting conditions; Growing conditions results in a final titer of 4 mM (0.36 g/L) 3-HP (Figure 5.3a) while resting conditions results in 12 mM (1.08 g/L) (Figure 5.3b). By removing any carbon source utilized for cell growth in the resting conditions, cofactors can be utilized for the specific purpose of 3-HP formation mediated by the recombinant *pdu* enzymes, as seen by the higher titer obtained in resting conditions. The low titers are due to cofactor depletion, as NAD^+ is not regenerated after its utilization by PduP, and thus can be improved through cofactor regeneration [Paper III].

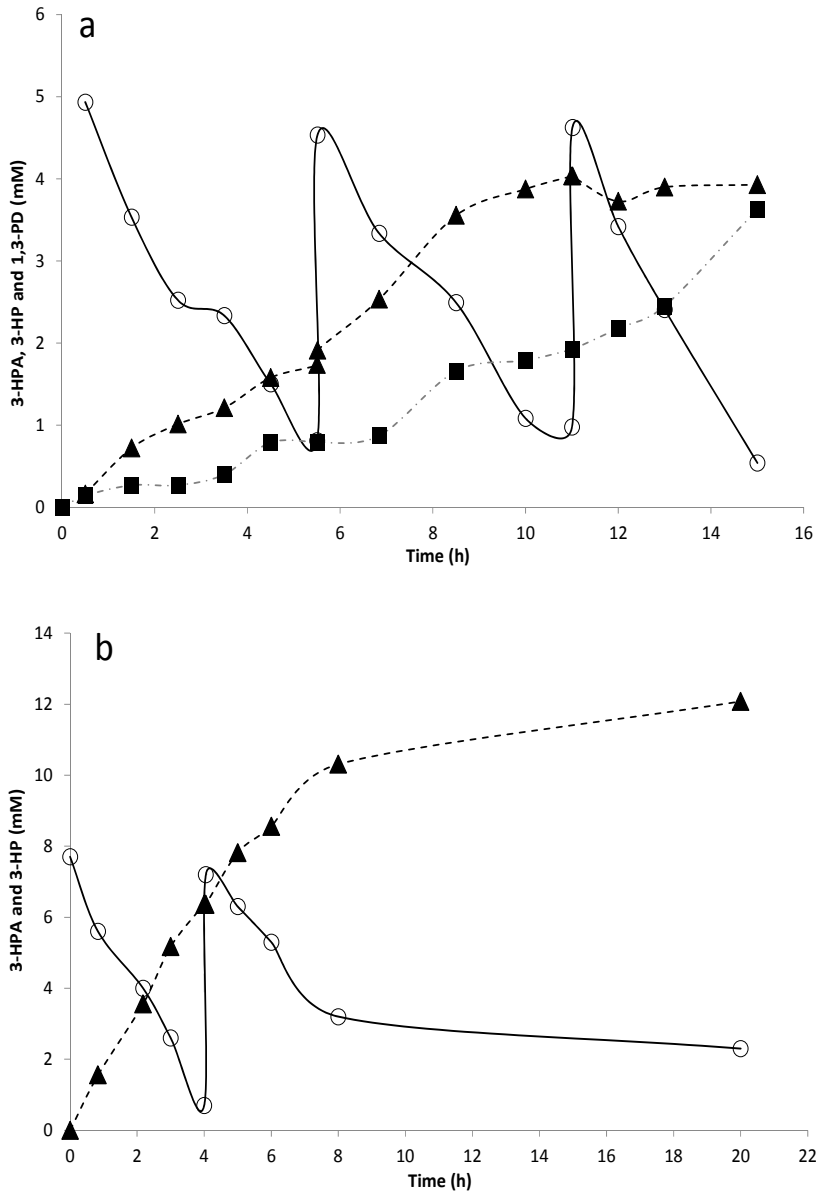


Figure 5.3
 Conversion of 3-HPA (○) to 3-HP (▲) and 1,3-PD (■) by *E. coli* BL21(DE3) expressing PduP, PduL and PduW in a) growing and b) resting conditions. Repeated additions of 3-HPA was performed at its depletion in order to avoid toxic and inhibitory effects associated with higher 3-HPA concentrations.

In order to evaluate the conversion of purified 3-HPA to 3-HP, the 3-HPA recovered from biotransformation reaction by complexation with bisulfite (Chapter 4.2.1.2) was added to a suspension of resting *E. coli* expressing PduP, PduL and PduW. Complete consumption of 14 mM 3-HPA and production of 3-HP up to 12 mM was observed, verifying the maximum titer obtained in resting conditions by *E. coli* expressing PduP, PduL and PduW.

5.3 Integration of cofactor regeneration for continuous production of 3-HP by *E. coli*

Recombinant production of proteins can result in a burden on the microbial hosts, as cellular mechanisms and energy are spent for their expression, thereby affecting other factors such as cell growth [154]. Another challenge is the requirement by certain enzymes or pathways for additional non-protein compounds, cofactors, to be functional. Cofactors are either organic or non-organic and their functions in enzyme catalysis vary considerably [155]. Native metabolic systems strive for a cofactor balance, by means of their regeneration. NADH and NADPH or their oxidized products are necessary for a number of enzymatic redox reactions. NAD⁺ utilized in the TCA cycle is regenerated through oxidative phosphorylation in the presence of oxygen, resulting in the generation of energy in the form of ATP [155]. By inserting a recombinant pathway requiring cofactors, this balance is upset, leading to either a negative impact on vital mechanisms of the host or the pathway not functioning as intended since the cofactors are utilized elsewhere. External addition of cofactors is possible, however resulting in high costs [156]. In the case of 3-HP production through recombinant expression of the NAD⁺-dependent pdu-pathway, an *in vivo* regeneration system is necessary and was implemented in **Paper III**.

5.3.1 NADH/NAD⁺ regeneration systems

NADH is a dinucleotide with an adenosine moiety connected to a nicotinamide moiety via two phosphate groups. The functional adenosine group accepts or donates an electron, thus catalyzing redox reactions. While NADH is the reduced form of the cofactor, NAD⁺ is the oxidized (Figure 5.4).

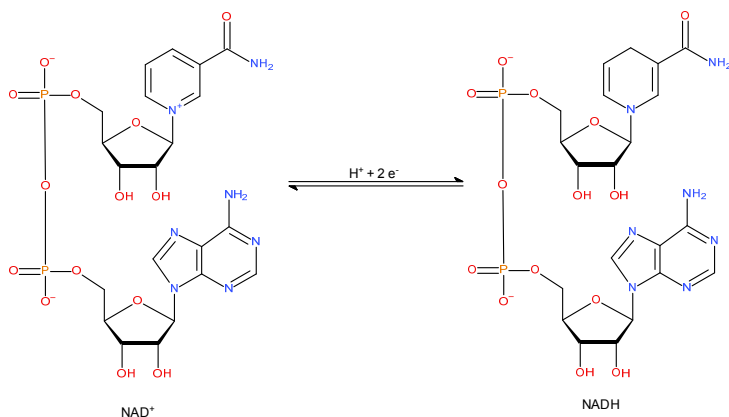


Figure 5.4
Structures and redox reactions of NAD⁺ and NADH.

When introducing a cofactor regeneration system, its compatibility with the process at hand is of utmost importance. Factors to take into account are turnover number of regeneration system, its applicability in various conditions such as acidity and temperature, minimizing its interference with native mechanisms of the host and avoiding the necessity to add additional compounds. Turnover number, meaning moles of formed product per mole cofactor, needs to be equal or higher than that of the cofactor dependent enzyme of the recombinant pathway. If the environmental conditions during biotransformation such as pH, temperature, oxygen levels or presence of inhibiting compounds affect the regeneration system negatively, the system is not feasible. Furthermore, a regeneration system ideally does not require the addition of external compounds or the utilization of native compounds which could affect vital mechanisms of the host cell negatively [157].

Strategies for NAD⁺ or NADH regeneration are either chemical/photochemical, electrochemical or enzyme based [157]. Chemical methods are based on the utilization of H₂ as an electron donor for the regeneration of NADH, carried by one or more proton producing organometallic mediators to the cofactor directly or via an enzyme [158]. Electrochemical methods are based on electrodes providing or accepting electrons either directly or indirectly. Direct electrochemical methods are based on the direct transfer of electrons from electrodes to cofactors under high potentials, while indirect electron transfer incorporates an organic or organometallic mediator carrying electrons to the cofactors directly or coupling the transfer through a second enzyme [159].

These methods can however solely be implemented *in vitro*. Enzyme-based *in vivo* regeneration of cofactors is based on recombinant expression of additional cofactor-dependent enzymes requiring their own set of substrates. NADH and its oxidized product are mainly found in dehydrogenase-catalyzed reactions, and most methods for the regeneration of the cofactors are based on the expression of dehydrogenases or

oxidoreductases catalyzing the reduction or oxidation of ideally cheap substrates. *In vivo* cofactor regeneration systems can also be integrated with native functions of the host strain. Replacement of native *E. coli* formate dehydrogenase with NAD⁺-dependent formate dehydrogenase increases intracellular NADH in *E. coli*, which can be utilized in a recombinant NADH-dependent pathway [160]. This method was implemented for the production of 1,3-PD by *K. pneumoniae*; recombinant expression of NAD⁺-dependent formate dehydrogenase of *Candida boidinii* was achieved in the host strain and resulted in a higher titer of 1,3-PD as well as an up-regulation of NADH-dependent production of lactic acid and ethanol [161].

5.3.2 NADH-oxidase and 3-HP production (Paper III)

NADH oxidase has been detected in several systems as a flavin-dependent, NAD⁺-regenerating and H₂O₂-forming enzyme, utilizing O₂ as an electron acceptor. Its cofactor flavin adenine nucleotide (FAD) acts as a mediator, carrying two electrons from NADH to O₂. [162-166]. Integrating the enzyme for cofactor regeneration *in vivo* would require the degradation of toxic H₂O₂ [167] to H₂O and O₂ by a catalase-mediated reaction.

A different group of FAD-dependent NADH oxidases, detected in a number of microorganisms [168-171] and prevalent in *Lactobacillus* sp. [165, 170, 172-174], circumvent the production of H₂O₂ by direct production of H₂O. In anaerobic mesophiles, H₂O-producing NADH oxidases protect the cells against oxidative stress by the reduction of O₂. The catalytic site of the enzyme contains a conserved cysteine residue responsible for the direct four electron reduction of O₂ to H₂O [171].

Sequence alignment of a number of water-forming NADH oxidases identified an NADH oxidase (Nox) in *L. reuteri*. Structural modeling of *L. reuteri* NADH oxidase was based on the crystal structure of NAD(P)H oxidase of *L. sanfranciscensis* (Figure 5.5). The enzyme consists of a FAD-binding domain, an ADP-binding domain and dimerization domain, with all three domains containing Rossmann folds.

Kinetic characterization of *L. reuteri* Nox revealed its preference for NADH over NADPH. With NADH, A V_{max} of 25.1 U/mg was reached. The higher activity of Nox in relation to PduP indicated that the enzyme would not act as a bottleneck for the regeneration of NAD⁺ required for PduP-mediated conversion of 3-HPA to 3-HP-CoA, which reaches 18 U/mg before substrate inhibition occurs at concentrations above 7 mM 3-HPA.

Co-expression of Nox with PduP, PduL and PduW was achieved in *E. coli* BL21(DE3) (Figure 5.6) through its transformation with pET-28b carrying *nox* and pCOLADuet-1 carrying *pduP*, *pduL* and *pduW*. Shake flask cultivation in resting conditions results in the final formation of at the very least 22 mM 3-HP, with a yield of 0.78 mol/mol 3-HP and productivity of 0.6 mmole g⁻¹ CDW h⁻¹ (Figure 5.7).

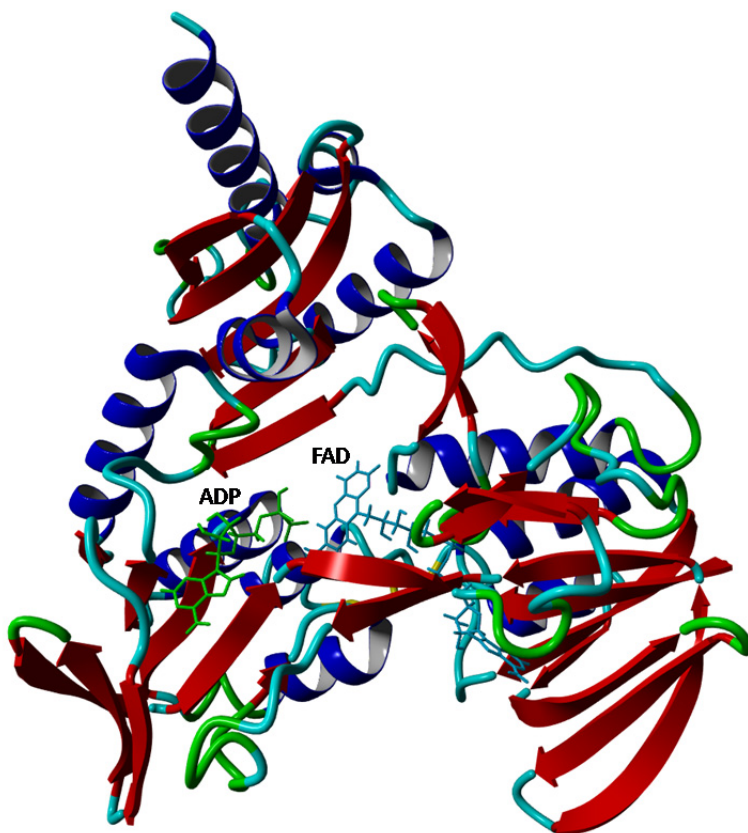


Figure 5.5
Proposed structural model of Nox from *L. reuteri*. The enzyme contains three domains; one ADP-binding, one FAD-binding and one dimerization domain, with each domain containing a Rossman-fold. The ADP residue can be seen in green and FAD in light blue.

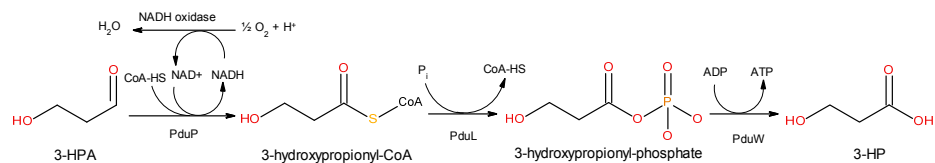


Figure 5.6
PduP, PduL and PduW mediated conversion of 3-HPA to 3-HP, with simultaneous regeneration of NAD^+ by NADH oxidase.

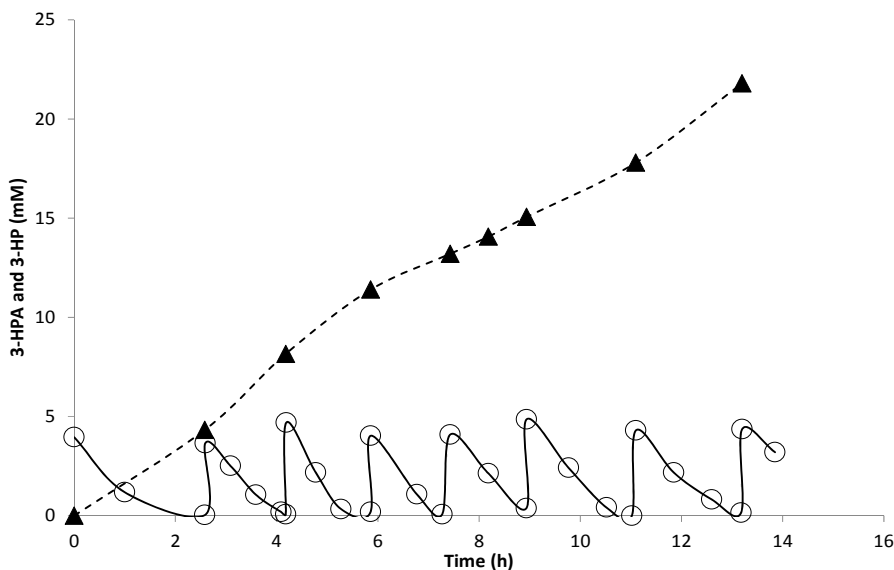


Figure 5.7
Shake flask conversion of 3-HPA (○) to 3-HP (▲) in resting conditions by *E. coli* BL21(DE3) expressing PduP, PduL, PduW and Nox, with repeated additions of 3-HPA.

A major drawback preventing further biotransformation in the setup is however the substrate 3-HPA, obtained from the biotransformation of glycerol by *L. reuteri* (Chapter 4.2.1.2) [127]. 3-HPA was added in pulses of 4 mM, in order to avoid toxic effects on the host strain and inhibition of PduP. Production of 3-HPA through fed-batch biotransformation of glycerol by *L. reuteri* results in up to 40 mM 3-HPA. Each pulse of 3-HPA added to the *E. coli* cultivations thus dilutes the cultivation up to 10 times. Thus, scaling up of the system requires a highly concentrated substrate, which is achieved through the lyophilization of 3-HPA reaching a concentration of 1 M.

The recombinant *E. coli* host was produced, harvested and resuspended in buffered conditions to a cell concentration of OD 11, thereby obtaining a resting cell system. The scaled up system required addition of 3-HPA in a flow rate matching that of PduP, PduL and PduW mediated conversion of it to 3-HP, in order to avoid 3-HPA accumulation and thus toxic effects. However, 0.48 M glycerol present in the lyophilized substrate solution accumulated in the bioreactor, resulting in the induction and expression of YqhD and a final concentration of 65 mM 3-HP and 54 mM 1,3-PD, with a final yield of 0.60 mol/mol 3-HP and productivity of 0.52 mmole g⁻¹ CDW h⁻¹ (Figure 5.8).

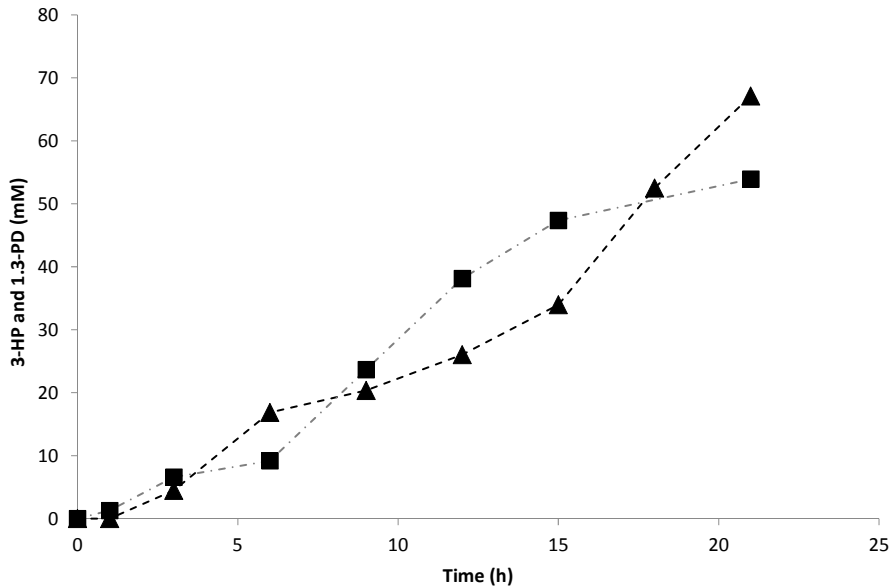


Figure 5.8
Fed-batch bioreactor conversion of 3-HPA to 3-HP (▲) and 1,3-PD (■) by resting cells of *E. coli* BL21(DE3) expressing PduP, PduL, PduW and Nox and utilizing concentrated (1 M) 3-HPA as substrate.

While the study verifies the role of Nox in gaining higher concentrations of 3-HP through the regeneration of NAD^+ utilized by PduP, an alternative method for substrate preparation is necessary. Bisulfite-complexed 3-HPA can be utilized as substrate for the production of 3-HP through the pdu pathway [Paper II], Co-expression of glycerol dehydratase is an option for circumventing problems associated with 3-HPA addition, instead utilizing glycerol as a direct substrate. However, cofactor B_{12} -requirements, or in the case of the cofactor B_{12} -independent glycerol dehydratase of *C. butyricum* S-adenosyl methionine requirements, are drawbacks. Furthermore, the aerobic conditions necessary for cofactor regeneration by NADH oxidase would affect glycerol dehydratase negatively, as the enzymatic system independent of source is inhibited in the presence of oxygen. Glycerol uptake by *E. coli* would result in lower 3-HP yields, as the glycerol would be used as a carbon source by the host. Thus knock out of glycerol kinase (GlpK) as well as YqhD would be required to avoid 1,3-PD and acetic acid formation.

5.4 Production of poly(3-hydroxypropionate) in *E. coli* (Paper IV)

Recombinant expression of PduP and codon-optimized PhaC-gene of *Chromobacterium* sp. (PhaC_{Cs}) was achieved by their insertion in plasmid pCOLADuet-1, followed by transformation of *E. coli* BL21(DE3). Substrate 3-HPA was produced by *L. reuteri*, as described in chapter 4.2.1.2 [127]. Due to toxic effects of 3-HPA on *E. coli* as well as inhibitory effects of the substrate on PduP, 3-HPA was added in a fed-batch mode in pulses of 3 mM to growing cells of the recombinant *E. coli* grown in either glucose and glycerol. While the choice of carbon source does not impact PduP and PhaC_{Cs}-mediated poly(3-HP)-production, 100% utilization of the substrate is achieved when added between 3 and 15 h of induction of PduP and PhaC_{Cs} genes, as opposed to the simultaneous addition of inducer and 3-HPA where solely 50% of the substrate is utilized in the recombinant pathway. Consumption of 330 mg 3-HPA was observed with a continuous growth of cells (Figure 5.9) and production of 260 mg poly(3-HP). A poly(3-HP) content of 40 wt.% ($w_{\text{poly(3-HP)}}/w_{\text{CDW}}$) and molar yield of 0.78 mol poly(3-HP) per mol 3-HPA with an average molecular weight of 0.19 kg/mol was achieved (Figure 5.10).

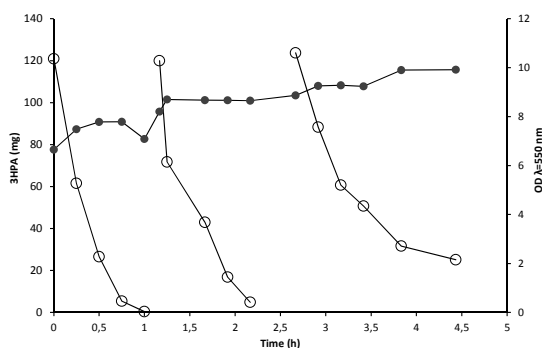


Figure 5.9 Consumption of 3-HPA (○) and growth of *E. coli* BL21(DE3) expressing PduP and PhaC_{Cs} (OD_{550nm}) (●) during repeated additions of 3-HPA for its transformation to poly(3-HP).

The accumulated poly(3-HP) granules of the recombinant strain (<100 nm) are smaller than the average granules produced by native PHA accumulating strains (100-500 nm) (Figure 5.11) [175]. Highly active Phas or alternatively high concentrations of the synthases have been proposed to catalyze the formation of smaller granules and polymers of shorter chain length, as opposed to larger granules and polymer chain length utilizing lower concentrations of Phas [176].

This study confirms an efficient and fast recombinant process utilizing PduP of *L. reuteri* in conjunction with the highly active PhaC of *Chromobacterium* sp. recombinantly produced in *E. coli* for the production of poly(3-HP). The process suffers from the same drawbacks as that of 3-HP-production [Paper II, III]. Direct conversion of glycerol to poly(3-HP) by the recombinant polymer producer requires the expression of cofactor B₁₂-dependent or independent glycerol dehydratase. Deletion of GlpK would be required in order to increase yields of poly(3-HP) from

the substrate. Furthermore, cofactor regeneration by NADH oxidase, as was implemented in **Paper III** for the PduP, PduL and PduW mediated production of 3-HP from 3- HPA, could result in a higher final poly(3-HP) content.



Figure 5.10
Poly(3-HP) produced from 3-HPA by recombinant *E. coli* BL21(DE3) expressing PduP and PhaC_{cs}.

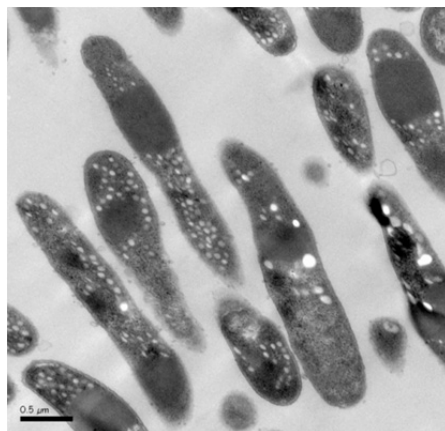


Figure 5.11
Transmission electron micrographs of *E. coli* BL21(DE3) expressing Pdup and PhaC_{cs} accumulating P(3HP) granules (white spots) from 3-HPA.

Conclusions and Future Perspectives

This thesis explains a novel biological route for the production of 3-hydroxypropionic, a chemical considered to be among the top 20 platform chemicals for the bio-based chemical industry. Initially, studies were performed to verify the propanediol utilization pathway present in the probiotic microorganism *Lactobacillus reuteri* as a means for producing 3-HP from glycerol. Once this was demonstrated, improvement of 3-HP production by cofactor regeneration by another enzyme of the same organism was shown. Part of the pdu pathway was also utilized for the production of the stable polymer poly(3-HP).

As the role of glycerol dehydratase for converting glycerol to 3-HPA was known from earlier studies, focus was on the subsequent three enzymes, PduP, PduL and PduW, leading to the formation of 3-HP from 3-HPA. The pathway also produces 1 mole of ATP that is important for maintenance of the cells. Recombinant expression of the enzymes in *E. coli* BL21(DE3) and using the resting cells for transformation of 3-HPA resulted in the highest possible yield of 1 mol 3-HP/mol 3-HPA. The studies also showed that some of the enzymes present natively in *E. coli* need to be disrupted for avoiding the formation of byproducts when using growing cells for biotransformation. The final 3-HP titer could be significantly improved by the co-expression of an O₂-dependent and water-forming NADH oxidase (Nox) for cofactor regeneration, hence providing a very clean system for the production of the platform chemical. The activity of Nox was found to be slightly higher than that of PduP, indicating that the productivity of the PduP, PduL and PduW-mediated production of 3-HP would not be affected by the co-expression of Nox.

In the case of poly(3-HP) production, co-expression of the highly active polyhydroxyalkanoate synthase (PhaC) of *Chromobacterium* sp. USM2 with PduP resulted in high productivity but low titer of small intracellular small granules, up to 40 wt.% (wt_{poly(3-HP)}/wt_{CDW}). The final titer can indeed be improved by the co-expression of Nox for regeneration of NAD⁺ utilized by PduP.}

Although recombinant *E. coli* provides a relatively inexpensive production system due to its fast growth and the possibility to use an inexpensive minimal medium for cultivation, in the current state of development for both 3-HP and poly(3-HP) production it is limited by the toxicity of the 3-HPA substrate that does not allow higher substrate concentrations to be used and in turn limits the product concentration reached. Toxicity of 3-HPA to *E. coli* demands a fed-batch process wherein 3-HPA is added in pulses of lower amounts in order to avoid accumulation of the aldehyde. Further work should focus on tailoring and optimizing the system further for direct conversion of glycerol to 3-HP. Co-expression of glycerol dehydratase is an option for circumventing problems associated with 3-HPA as well as

gaining a pathway for the direct conversion of glycerol to 3-HP in one microbial system. This requires the addition of cofactor B₁₂ or S-adenosylmethionine making it a costly process, however insertion of genes related to the biosynthesis of the cofactors can be performed. The oxygen sensitivity of these glycerol dehydratases due to their mechanism of radical propagations could however be in discourse with O₂-dependent Nox. Furthermore, glycerol kinase related to the utilization of glycerol as a carbon source and 1,3-propanediol oxidoreductase involved in the reduction of 3-HPA to 1,3-PD would need to be knocked out to reach higher yields of 3-HP.

Not much information on the Pdu enzymes is available in the literature. Although recombinant *L. reuteri* PduP was produced and characterized in this work, there is much work needed for the characterization of the other enzymes and determination of their structure. This will enable rational protein engineering of the enzymes for improving the activities of the enzymes with 3-HP and the subsequent metabolites. An alternative pathway for conversion of glycerol to 3-HP involving glycerol dehydratase and NAD⁺-dependent aldehyde dehydrogenase has shown itself to be an effective method in various studies. Although it lacks the ATP-formation, it should also be considered as an alternative after comparison with the Pdu pathway. This pathway could be improved by the co-expression of Nox as done in the present work.

An alternative possibility that may be considered is the use of *L. reuteri* as the production host, since it already contains the mechanisms for the production of 3-HP from glycerol. From the other studies in our laboratory on flux analysis of glycerol conversion to 3-HPA and further to 1,3-PD and 3-HP by *L. reuteri*, it was obvious that glycerol dehydratase has a higher turnover number than the subsequent enzymes. Overexpression of PduP, PduL, PduW and Nox, coupled to the knock out of 1,3-PD oxidoreductase could potentially result in a higher productivity and titer of 3-HP. Even the biopolymer production could potentially be achieved by disruption of *pduL* and *pduW* and introduction of *phaC_{cs}*.

In conclusion it can be stated that while this thesis presents a method for the production of a chemical and a biomaterial which can be utilized as greener alternatives to their petroleum-derived analogues, it also shows that there is much to do before these processes can be considered financially sound alternatives to the cheaper and optimized methods of crude oil valorization. Higher titers and productivities of these products need to be reached, which require additional studies and modifications to the processes and production strains. Furthermore, challenges related to the up-scaling of the processes and downstream processing for their purification need to be addressed.

Many steps have been and are being taken for reaching a way of maintaining our way of life and expanding it to the far reaches of the world in an environmentally sustainable fashion, and many more are needed. This thesis represents one small step towards that goal.

Acknowledgments

I sincerely want to thank my main supervisor Prof. Rajni Hatti-Kaul for your guidance during my PhD-studies. This thesis would not have been possible without your knowledge, enthusiasm, kindness and patience. Thank you for accepting me as a PhD-student and thank you for trusting in me.

To my co-supervisor Javier Linares-Pastén. Without your enthusiasm, friendliness and keen interest in reaching new scientific heights, much of this work would not have existed. Thanks to Shukun Yu, who gave advice and guided me throughout my PhD-studies,

To my main research partners, Roya and Tarek. You two have always been an inspiration to me. Roya, You are truly a great friend and a great colleague, and have always been there to help, in research or in life. I am confident that you will excel in whatever you do. Tarek, your knowledge and interest in biotechnology is astounding. You have contributed to so many ideas, implemented for this thesis and otherwise. I truly look forward to seeing you grow as a researcher and motivate others to follow in your footsteps.

To my student Victor Arieta. Your optimism is contagious, and it showed in the things you have achieved both as my student and in life. I truly wish you a bright future in life and in your career. Lisa Torkelson, thank you for your friendship and for all the things we accomplished while we were working together.

To Jakob Hallquist, Mia Ramgren and Olle Holst, who rekindled my interest in research, which led me to pursue a PhD. Without you, this would not have been possible.

To Emma Poaches and Siv Holmqvist for your help in the administrative matters surrounding my PhD-studies, and for your unwaning kindness. For Frans Peder Nilson, for your guidance in computer-related issues.

To my friends and former colleagues Solmaz and Amin, who brought out the Iranian side of me. As an expat since my baby years, I have always been in between two worlds, but you two made me find my way home. Your kindness, acceptance and friendship will be cherished forever.

To my past and present colleagues at DSP. It is truly a great group of people from every corner of the world. Thank you for making our group enjoyable and friendly and I wish you all the best in life and in research. I look forward to seeing each and everyone of you excel in work and beyond.

And to all others at the Department of Biotechnology. Thank you for making the Department a great place to be at and thank you for making my time as a PhD-student filled with happy memories.

Till alla mina vänner.

Till de i Stockholm; Piotr, Björn, Peter, Shirly, Calle, Kalle och alla de andra. Till mina vänner i Malmö. Till mina vänner i Köpenhamn. Ni vet vilka ni är. Till Michel. Utan ditt stöd och din tålmod och allt det du ger mig hade detta inte varit möjligt.

To my Family, spread all over the world. I truly wish I had you closer.

To my brother Samin. Thank you for being an inspiration in my life. I have always looked up to you and I am so proud of all that you have achieved.

To my parents. Words can not express my admiration for you. You have given me so much and have asked for so little. I will do all that I can to repay all your sacrifice, your kindness, your inspirative spirit, your patience and your love.

References

1. Willke, T., and Vorlop, K. D. (2004) Industrial bioconversion of renewable resources as an alternative to conventional chemistry. *Applied Microbiology and Biotechnology* 66, 131-142.
2. Jang, Y. S., Kim, B., Shin, J. H., Choi, Y. J., Choi, S., Song, C. W., Lee, J., Park, H. G., and Lee, S. Y. (2012) Bio-based production of C2-C6 platform chemicals. *Biotechnology and Bioengineering* 109, 2437-2459.
3. Paster, M., Pellegrino, J. L., and Carole, T. M. (2004) Industrial bioproducts: today and tomorrow. U.S. DOE Report.
4. Gavrilescu, M., and Chisti, Y. (2005) Biotechnology - a sustainable alternative for chemical industry. *Biotechnology Advances* 23, 471-499.
5. Claassen, P. A. M., van Lier, J. B., Lopez Contreras, A. M., van Niel, E. W. J., Sijtsma, L., Stams, A. J. M., de Vries, S. S., and Weusthuis, R. A. (1999) Utilisation of biomass for the supply of energy carriers. *Applied Microbiology and Biotechnology* 52, 741-755.
6. Sauer, M., and Mattanovich, D. (2012) Construction of microbial cell factories for industrial bioprocesses. *Journal of Chemical Technology & Biotechnology* 87, 445-450.
7. Werpy, T., and Peterson, G. (2004) Top value added chemicals from Biomass. Volume I: Results of screening for potential candidates from sugars and synthesis gas. U.S. DOE Report.
8. Matar, S., and Hatch, L. F. (2001) *Chemistry of petrochemical processes*, 2nd ed., Gulf Professional Publishing, Woburn.
9. Fahim, M. A., Alsahhaf, T. A., and Elkilani, A. (2010) Fluidised catalytic cracking. In *Fundamentals of Petroleum Refining* (Fahim, M. A., Alsahhaf, T. A., and Elkilani, A.), Elsevier, Amsterdam, 199-235.
10. Carole, T. M., Pellegrino, J. L., and Paster, M. (2004) Opportunities in the industrial biobased products industry. *Applied Biochemistry and Biotechnology* 15, 113-116.
11. Sauer, M., Porro, D., Mattanovich, D., and Branduardi, P. (2008) Microbial production of organic acids: expanding the markets. *Trends in Biotechnology* 26, 100-108.
12. Anderson, A. J., and Dawes, E. A. (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews* 54, 450-472.
13. Bugnicourt, E., Cinelli, P., Lazzeri, A., and Alvarez, V. (2014) Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. *eXPRESS Polymer Letters* 8, 791-808.
14. Steinbüchel, A., and Lütke-Eversloh, T. (2003) Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochemical Engineering Journal* 16, 81-96.

15. Andreessen, B., Taylor, N., and Steinbuechel, A. (2014) Poly(3-Hydroxypropionate): a promising alternative to fossil fuel-based materials. *Applied and Environmental Microbiology* 80, 6574-6582.
16. Latif, H., Zeidan, A. A., Nielsen, A. T., and Zengler, K. (2014) Trash to treasure: production of biofuels and commodity chemicals via syngas fermenting microorganisms. *Current Opinion in Biotechnology* 27, 79-87.
17. Li, C. L., KK., and Liu, H. (2013) Microbial conversion of waste glycerol from biodiesel production into value-added products. *Energies* 6, 30.
18. Yazdani, S. S., and Gonzalez, R. (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Current Opinion in Biotechnology* 18, 213-219.
19. Yang, F., Hanna, M. A., and Sun, R. (2012) Value-added uses for crude glycerol--a byproduct of biodiesel production. *Biotechnology for Biofuels* 5, 13.
20. Johnson, D. T., and Taconi, K. A. (2007) The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environmental Progress* 26, 338-348.
21. Gokarn, R., Selifonova, O., Jessen, H. J., Gort, S. J., Selmer, T., and Buckel, W. (2007) 3-hydroxypropionic acid and other organic compounds. U.S. patent US7186541B2.
22. Jiang, X., Meng, X., and Xian, M. (2009) Biosynthetic pathways for 3-hydroxypropionic acid production. *Applied Microbiology and Biotechnology* 82, 995-1003.
23. Della Pina, C., Falletta, E., and Rossi, M. (2011) A green approach to chemical building blocks. The case of 3-hydroxypropanoic acid. *Green Chemistry* 13, 1624-1632.
24. Kumar, V., Ashok, S., and Park, S. (2013) Recent advances in biological production of 3-hydroxypropionic acid. *Biotechnology Advances* 31, 945-961.
25. Long, F. A., and Purchase, M. (1950) The kinetics of hydrolysis of b-propiolactone in acid, neutral and basic solutions. *Journal of American Chemical Society* 72, 7.
26. Nexant. (2014) Bio-based acrylic acid: Considerations for commercial viability and success. (<http://www.nexant.com/resources/bio-based-acrylic-acid-considerations-commercial-viability-and-success>)
27. Falletta, E., Della Pina, C., Rossi, M., He, Q., Kiely, C. J., and Hutchings, G. J. (2011) Enhanced performance of the catalytic conversion of allyl alcohol to 3-hydroxypropionic acid using bimetallic gold catalysts. *Faraday Discussions* 152, 367-379.
28. Ishii, M., Chuakrut, S., Arai, H., and Igarashi, Y. (2004) Occurrence, biochemistry and possible biotechnological application of the 3-hydroxypropionate cycle. *Applied Microbiology and Biotechnology* 64, 605-610
29. Hugler, M., Huber, H., Stetter, K. O., and Fuchs, G. (2003) Autotrophic CO₂ fixation pathways in archaea (Crenarchaeota). *Archives of Microbiology* 179, 160-173.
30. Ishii, M., Miyake, T., Satoh, T., Sugiyama, H., Oshima, Y., Kodama, T., and Igarashi, Y. (1996) Autotrophic carbon dioxide fixation in *Acidianus brierleyi*. *Archives of Microbiology* 166, 368-371.
31. Hugler, M., Krieger, R. S., Jahn, M., and Fuchs, G. (2003) Characterization of acetyl-CoA/propionyl-CoA carboxylase in *Metallosphaera sedula*. Carboxylating

- enzyme in the 3-hydroxypropionate cycle for autotrophic carbon fixation. *European Journal of Biochemistry / FEBS* 270, 736-744.
32. Menendez, C., Bauer, Z., Huber, H., Gad'on, N., Stetter, K. O., and Fuchs, G. (1999) Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic Crenarchaeota and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. *Journal of Bacteriology* 181, 1088-1098.
 33. Berg, I. A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007) A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. *Science* 318, 1782-1786.
 34. Ansele, J. H., Pellechia, P. J., and Yoch, D. C. (1999) Metabolism of acrylate to beta-hydroxypropionate and its role in dimethylsulfoniopropionate lyase induction by a salt marsh sediment bacterium, *Alcaligenes faecalis* M3A. *Applied and Environmental Microbiology* 65, 5075-5081.
 35. Takamizawa, K., Horitsu, H., Ichikawa, T., Kawai, K., and Suzuki, T. (1993) Beta-Hydroxypropionic Acid Production by *Byssochlamys* Sp Grown on Acrylic-Acid. *Applied Microbiology and Biotechnology* 40, 196-200.
 36. Dave, H., Ramakrishna, C., and Desai, J. D. (1996) Degradation of acrylic acid by fungi from petrochemical activated sludge. *Biotechnology Letters* 18, 963-964.
 37. Lee, S. H., Park, S. J., Park, O. J., Cho, J., and Rhee, J. W. (2009) Production of 3-hydroxypropionic acid from acrylic acid by newly isolated *rhodococcus erythropolis* LG12. *Journal of Microbiology and Biotechnology* 19, 474-481.
 38. Sebastianes, F. S., Cabedo, N., Aouad, N., Valente, A. M. P., Lacava, P., Azevedo, J., Pizzirani-Kleiner, A., and Cortes, D. (2012) 3-Hydroxypropionic acid as an antibacterial agent from endophytic fungi *Diaporthe phaseolorum*. *Current Microbiology* 65, 622-632.
 39. Schwarz, M., Köpcke, B., Weber, R. W. S., Sterner, O., and Anke, H. (2004) 3-Hydroxypropionic acid as a nematicidal principle in endophytic fungi. *Phytochemistry* 65, 2239-2245.
 40. Sobolov, M., and Smiley, K. L. (1960) Metabolism of glycerol by an acrolein-forming lactobacillus. *Journal of Bacteriology* 79, 261-266.
 41. Talarico, T. L., Casas, I. A., Chung, T. C., and Dobrogosz, W. J. (1988) Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. *Antimicrobial Agents and Chemotherapy* 32, 1854-1858.
 42. Garai-Ibabe, G., Ibarburu, I., Berregi, I., Claisse, O., Lonvaud-Funel, A., Irastorza, A., and Duenas, M. T. (2008) Glycerol metabolism and bitterness producing lactic acid bacteria in cidermaking. *International Journal of Food Microbiology* 121, 253-261.
 43. Abeles, R. H., Brownstein, A. M., and Randles, C. H. (1960) beta-Hydroxypropionaldehyde, an intermediate in the formation of 1,3-propanediol by *Aerobacter aerogenes*. *Biochimica et Biophysica Acta* 41, 530-531.
 44. Mickelson, M. N., and Werkman, C. H. (1940) The dissimilation of glycerol by *Coli-Aerogenes* Intermediates. *Journal of Bacteriology* 39, 709-715.
 45. Homann, T., Tag, C., Biebl, H., Deckwer, W.-D., and Schink, B. (1990) Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. *Applied microbiology and Biotechnology* 33, 121-126.

46. Saint-Amans, S., Girbal, L., Andrade, J., Ahrens, K., and Soucaille, P. (2001) Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose-glycerol mixtures. *Journal of Bacteriology* 183, 1748-1754.
47. Forsberg, C. W. (1987) Production of 1,3-propanediol from glycerol by *Clostridium acetobutylicum* and other *Clostridium* species. *Applied and Environmental Microbiology* 53, 639-643.
48. Barbirato, F., Soucaille, P., and Bories, A. (1996) Physiologic Mechanisms Involved in Accumulation of 3-Hydroxypropionaldehyde during Fermentation of Glycerol by *Enterobacter agglomerans*. *Applied and Environmental Microbiology* 62, 4405-4409.
49. Ulmer, C., and Zeng, A. P. (2007) Microbial production of 3-hydroxypropionaldehyde from glycerol bioconversion. *Chemical and Biochemical Engineering Quarterly* 21, 321-326.
50. Dishisha, T., Pereyra, L. P., Pyo, S. H., Britton, R. A., and Hatti-Kaul, R. (2014) Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol. *Microbial Cell Factories* 13, 76.
51. Toraya, T., Honda, S., and Fukui, S. (1979) Fermentation of 1,2-propanediol with 1,2-ethanediol by some genera of Enterobacteriaceae, involving coenzyme B₁₂-dependent diol dehydratase. *Journal of Bacteriology* 139, 39-47.
52. Marx, A., Wendisch, V. F., Rittmann, D., and Buchholz, S. (2007) Microbiological production of 3-hydroxypropionic acid. Patent WO/2007/042494.
53. Liao, H. H., Gokarn, R., Gort, S. J., Jessen, H. J., and Selifonova, O. (2005) Alanine 2,3-aminomutase. Patent EP1575881.
54. Hugler, M., Menendez, C., Schagger, H., and Fuchs, G. (2002) Malonyl-coenzyme A reductase from *Chloroflexus aurantiacus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO₂ fixation. *Journal of Bacteriology* 184, 2404-2410.
55. Rathnasingh, C., Raj, S. M., Lee, Y., Catherine, C., Ashok, S., and Park, S. (2012) Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *Escherichia coli* strains. *Journal of Biotechnology* 157, 633-640.
56. Borodina, I., Kildegaard, K. R., Jensen, N. B., Blicher, T. H., Maury, J., Sherstyuk, S., Schneider, K., Lamosa, P., Herrgard, M. J., Rosenstand, I., Oberg, F., Forster, J., and Nielsen, J. (2015) Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in *Saccharomyces cerevisiae* via beta-alanine. *Metabolic Engineering* 27, 57-64.
57. Zeikus, J. G., Jain, M. K., and Elankovan, P. (1999) Biotechnology of succinic acid production and markets for derived industrial products. *Applied Microbiology and Biotechnology* 51, 545-552.
58. Bobik, T. A., Havemann, G. D., Busch, R. J., Williams, D. S., and Aldrich, H. C. (1999) The propanediol utilization (pdu) operon of *Salmonella enterica* serovar Typhimurium LT2 includes genes necessary for formation of polyhedral organelles involved in coenzyme B₁₂-dependent 1, 2-propanediol degradation. *Journal of Bacteriology* 181, 5967-5975.
59. Qi, X., Deng, W., Wang, F., Guo, Q., Chen, H., Wang, L., He, X., and Huang, R. (2013) Molecular cloning, co-expression, and characterization of glycerol dehydratase and 1,3-propanediol dehydrogenase from *Citrobacter freundii*. *Molecular Biotechnology* 54, 469-474.

60. Skraly, F. A., Lytle, B. L., and Cameron, D. C. (1998) Construction and characterization of a 1,3-propanediol operon. *Applied and Environmental Microbiology* 64, 98-105.
61. Forage, R. G., and Foster, M. A. (1982) Glycerol fermentation in *Klebsiella pneumoniae*: functions of the coenzyme B12-dependent glycerol and diol dehydratases. *Journal of Bacteriology* 149, 413-419.
62. Sauvageot, N., Muller, C., Hartke, A., Auffray, Y., and Laplace, J. M. (2002) Characterisation of the diol dehydratase pdu operon of *Lactobacillus collinoides*. *FEMS Microbiology Letters* 209, 69-74.
63. Morita, H., Toh, H., Fukuda, S., Horikawa, H., Oshima, K., Suzuki, T., Murakami, M., Hisamatsu, S., Kato, Y., Takizawa, T., Fukuoka, H., Yoshimura, T., Itoh, K., O'Sullivan, D. J., McKay, L. L., Ohno, H., Kikuchi, J., Masaoka, T., and Hattori, M. (2008) Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. *DNA Research* 15, 151-161.
64. Talarico, T. L., and Dobrogosz, W. J. (1990) Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. *Applied and Environmental Microbiology* 56, 1195-1197.
65. Kajiura, H., Mori, K., Tobimatsu, T., and Toraya, T. (2001) Characterization and mechanism of action of a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. *The Journal of Biological Chemistry* 276, 36514-36519.
66. Kwak, S., Park, Y. C., and Seo, J. H. (2013) Biosynthesis of 3-hydroxypropionic acid from glycerol in recombinant *Escherichia coli* expressing *Lactobacillus brevis* dhaB and dhaR gene clusters and *E. coli* K-12 aldH. *Bioresource Technology* 135, 432-439.
67. Kim, K., Kim, S.-K., Park, Y.-C., and Seo, J.-H. (2014) Enhanced production of 3-hydroxypropionic acid from glycerol by modulation of glycerol metabolism in recombinant *Escherichia coli*. *Bioresource Technology* 156, 170-175.
68. Toraya, T. (2000) Radical catalysis of B12 enzymes: structure, mechanism, inactivation, and reactivation of diol and glycerol dehydratases. *Cellular and Molecular Life Sciences* 57, 106-127.
69. O'Brien, J. R., Raynaud, C., Croux, C., Girbal, L., Soucaille, P., and Lanzilotta, W. N. (2004) Insight into the mechanism of the B12-independent glycerol dehydratase from *Clostridium butyricum*: preliminary biochemical and structural characterization. *Biochemistry* 43, 4635-4645.
70. Feliks, M., and Ullmann, G. M. (2012) Glycerol dehydration by the B12-independent enzyme may not involve the migration of a hydroxyl group - a computational study. *The Journal of Physical Chemistry B* 116, 7076-7087.
71. Raj, S. M., Rathnasingh, C., Jo, J. E., and Park, S. (2008) Production of 3-hydroxypropionic acid from glycerol by a novel recombinant *Escherichia coli* BL21 strain. *Process Biochemistry* 43, 1440-1446.
72. Mohan Raj, S., Rathnasingh, C., Jung, W. C., and Park, S. (2009) Effect of process parameters on 3-hydroxypropionic acid production from glycerol using a recombinant *Escherichia coli*. *Applied Microbiology and Biotechnology* 84, 649-657.
73. Zhu, J. G., Ji, X. J., Huang, H., Du, J., Li, S., and Ding, Y. Y. (2009) Production of 3-hydroxypropionic acid by recombinant *Klebsiella pneumoniae* based on aeration and ORP controlled strategy. *Korean Journal of Chemical Engineering* 26, 1679-1685.

74. Rathnasingh, C., Raj, S. M., Jo, J. E., and Park, S. (2009) Development and evaluation of efficient recombinant *Escherichia coli* strains for the production of 3-hydroxypropionic acid from glycerol. *Biotechnology and Bioengineering* 104, 729-739.
75. Ashok, S., Raj, S., Rathnasingh, C., and Park, S. (2011) Development of recombinant *Klebsiella pneumoniae* Δ *dhaT* strain for the co-production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol. *Applied Microbiology and Biotechnology* 90, 1253-1265.
76. Luo, L. H., Seo, J. W., Baek, J. O., Oh, B. R., Heo, S. Y., Hong, W. K., Kim, D. H., and Kim, C. H. (2011) Identification and characterization of the propanediol utilization protein PduP of *Lactobacillus reuteri* for 3-hydroxypropionic acid production from glycerol. *Applied Microbiology and Biotechnology* 89, 697-703.
77. Luo, L. H., Seo, J. W., Oh, B. R., Seo, P. S., Heo, S. Y., Hong, W. K., Kim, D. H., and Kim, C. H. (2011) Stimulation of reductive glycerol metabolism by overexpression of an aldehyde dehydrogenase in a recombinant *Klebsiella pneumoniae* strain defective in the oxidative pathway. *Journal of Industrial Microbiology & Biotechnology* 38, 991-999.
78. Luo, L. H., Kim, C. H., Heo, S. Y., Oh, B. R., Hong, W. K., Kim, S., Kim, D. H., and Seo, J. W. (2012) Production of 3-hydroxypropionic acid through propionaldehyde dehydrogenase PduP mediated biosynthetic pathway in *Klebsiella pneumoniae*. *Bioresource Technology* 103, 1-6.
79. Huang, Y., Li, Z., Shimizu, K., and Ye, Q. (2012) Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol by a recombinant strain of *Klebsiella pneumoniae*. *Bioresource Technology* 103, 351-359.
80. Ko, Y., Ashok, S., Zhou, S., Kumar, V., and Park, S. (2012) Aldehyde dehydrogenase activity is important to the production of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumoniae*. *Process Biochemistry* 47, 1135-1143.
81. Kumar, V., Sankaranarayanan, M., Jae, K. E., Durgapal, M., Ashok, S., Ko, Y., Sarkar, R., and Park, S. (2012) Co-production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cells of recombinant *Klebsiella pneumoniae* J2B strain overexpressing aldehyde dehydrogenase. *Applied Microbiology and Biotechnology* 96, 373-383.
82. Kumar, V., Sankaranarayanan, M., Durgapal, M., Zhou, S., Ko, Y., Ashok, S., Sarkar, R., and Park, S. (2013) Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cells of the lactate dehydrogenase-deficient recombinant *Klebsiella pneumoniae* overexpressing an aldehyde dehydrogenase. *Bioresource Technology* 135, 555-563.
83. Huang, Y., Li, Z., Shimizu, K., and Ye, Q. (2013) Co-production of 3-hydroxypropionic acid and 1,3-propanediol by *Klebsiella pneumoniae* expressing *aldH* under microaerobic conditions. *Bioresource Technology* 128, 505-512.
84. Ashok, S., Sankaranarayanan, M., Ko, Y., Jae, K.-E., Ainala, S. K., Kumar, V., and Park, S. (2013) Production of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumoniae* Δ *dhaT* Δ *yqhD* which can produce vitamin B12 naturally. *Biotechnology and Bioengineering* 110, 511-524.
85. Ashok, S., Mohan Raj, S., Ko, Y., Sankaranarayanan, M., Zhou, S., Kumar, V., and Park, S. (2013) Effect of *puuC* overexpression and nitrate addition on glycerol

- metabolism and anaerobic 3-hydroxypropionic acid production in recombinant *Klebsiella pneumoniae* Δ glpK Δ dhaT. *Metabolic Engineering* 15, 10-24.
86. Zhou, S., Catherine, C., Rathnasingh, C., Somasundar, A., and Park, S. (2013) Production of 3-hydroxypropionic acid from glycerol by recombinant *Pseudomonas denitrificans*. *Biotechnology and Bioengineering* 110, 3177-3187.
 87. Li, Y., Liu, L., and Tian, P. (2014) NAD⁺-independent aldehyde oxidase catalyzes cofactor balanced 3-hydroxypropionic acid production in *Klebsiella pneumoniae*. *Biotechnology Letters* 36, 2215-2221.
 88. Sankaranarayanan, M., Ashok, S., and Park, S. (2014) Production of 3-hydroxypropionic acid from glycerol by acid tolerant *Escherichia coli*. *Journal of Industrial Microbiology & Biotechnology* 41, 1039-1050.
 89. Jung, W. S., Kang, J. H., Chu, H. S., Choi, I. S., and Cho, K. M. (2014) Elevated production of 3-hydroxypropionic acid by metabolic engineering of the glycerol metabolism in *Escherichia coli*. *Metabolic Engineering* 23, 116-122.
 90. Gresham, T. L., Jansen, J. E., Shaver, F. W., and Gregory, J. T. (1948) β -Propiolactone. II. Reactions with salts of inorganic acids. *Journal of the American Chemical Society* 70, 999-1001
 91. Andreessen, B., and Steinbuechel, A. (2010) Biosynthesis and biodegradation of 3-hydroxypropionate-containing polyesters. *Applied and Environmental Microbiology* 76, 4919-4925.
 92. Zhang, D. H. M. T., WB. (2004) A new synthetic route to poly[3-hydroxypropionic acid] (P[3-HP]): Ring-opening polymerization of 3-HP macrocyclic esters. *Macromolecules* 37, 8198-8200.
 93. Song, H., Zhao, Y., Zhang, Y., Kong, W., and Xia, C. (2012) Synthesis of polyhydroxyalkanoates by polymerization of methyl 3-hydroxypropionate in the catalysis of lipase. *Chinese Journal of Catalysis* 33, 432-438.
 94. Andreessen, B., Lange, A. B., Robenek, H., and Steinbuechel, A. (2010) Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. *Applied and Environmental Microbiology* 76, 622-626.
 95. Wang, Q., Liu, C., Xian, M., Zhang, Y., and Zhao, G. (2012) Biosynthetic pathway for poly(3-hydroxypropionate) in recombinant *Escherichia coli*. *Journal of Microbiology* 50, 693-697.
 96. Wang, Q., Yang, P., Liu, C., Xue, Y., Xian, M., and Zhao, G. (2013) Biosynthesis of poly(3-hydroxypropionate) from glycerol by recombinant *Escherichia coli*. *Bioresource Technology* 131, 548-551.
 97. Heinrich, D., Andreessen, B., Madkour, M. H., Al-Ghamdi, M. A., Shabbaj, II, and Steinbuechel, A. (2013) From waste to plastic: synthesis of poly(3-hydroxypropionate) in *Shimwellia blattae*. *Applied and Environmental Microbiology* 79, 3582-3589.
 98. Andreessen, B., Johannngmeier, B., Burbank, J., and Steinbuechel, A. (2014) Influence of the operon structure on poly(3-hydroxypropionate) synthesis in *Shimwellia blattae*. *Applied Microbiology and Biotechnology* 98, 7409-7422.
 99. Feng, X., Xian, M., Liu, W., Xu, C., Zhang, H., and Zhao, G. (2015) Biosynthesis of poly(3-hydroxypropionate) from glycerol using engineered *Klebsiella pneumoniae* strain without vitamin B. *Bioengineered*, doi: 10.1080/21655979.2015.1011027.
 100. Rehm, B. H. (2003) Polyester synthases: natural catalysts for plastics. *The Biochemical Journal* 376, 15-33.

101. Rehm, B. H. (2007) Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. *Current Issues in Molecular Biology* 9, 41-62
102. Rehm, B. H., Antonio, R. V., Spiekermann, P., Amara, A. A., and Steinbuchel, A. (2002) Molecular characterization of the poly(3-hydroxybutyrate) (PHB) synthase from *Ralstonia eutropha*: in vitro evolution, site-specific mutagenesis and development of a PHB synthase protein model. *Biochimica et Biophysica Acta* 1594, 178-190.
103. Jia, Y., Yuan, W., Wodzinska, J., Park, C., Sinskey, A. J., and Stubbe, J. (2001) Mechanistic studies on class I polyhydroxybutyrate (PHB) synthase from *Ralstonia eutropha*: class I and III synthases share a similar catalytic mechanism. *Biochemistry* 40, 1011-1019
104. Bhubalan, K., Chuah, J. A., Shozui, F., Brigham, C. J., Taguchi, S., Sinskey, A. J., Rha, C., and Sudesh, K. (2011) Characterization of the highly active polyhydroxyalkanoate synthase of *Chromobacterium* sp. strain USM2. *Applied and Environmental Microbiology* 77, 2926-2933.
105. Axelsson, L., and Von Wright, A.S. (2012) Lactic acid bacteria: an introduction. In *Lactic acid bacteria: microbiological and functional aspects* (Salminen, S., and Von Wright, A.S.), 4th Ed., Taylor & Francis, Boca Raton.
106. Barrangou, R., Lahtinen, S. J., Ibrahim, F., and Ouweland, A. C. (2012) Genus *Lactobacillus*. In *Lactic Acid Bacteria: Microbiological and Functional Aspects* (Salminen, S., and Von Wright, A. S.), 4th Ed., Taylor & Francis, Boca Raton.
107. Casas, I. A., and Dobrogosz, W. J. (2011) Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. *Microbial Ecology in Health and Disease* 12, 247-285.
108. Vollenweider, S., Grassi, G., Konig, I., and Puhani, Z. (2003) Purification and structural characterization of 3-hydroxypropionaldehyde and its derivatives. *Journal of Agricultural and Food Chemistry* 51, 3287-3293.
109. Arskold, E., Lohmeier-Vogel, E., Cao, R., Roos, S., Radstrom, P., and van Niel, E. W. (2008) Phosphoketolase pathway dominates in *Lactobacillus reuteri* ATCC 55730 containing dual pathways for glycolysis. *Journal of Bacteriology* 190, 206-212.
110. Zaunmuller, T., Eichert, M., Richter, H., and Unden, G. (2006) Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. *Applied Microbiology and Biotechnology* 72, 421-429.
111. Sriramulu, D. D., Liang, M., Hernandez-Romero, D., Raux-Deery, E., Lunsdorf, H., Parsons, J. B., Warren, M. J., and Prentice, M. B. (2008) *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation. *Journal of Bacteriology* 190, 4559-4567
112. Walter, D., Ailion, M., and Roth, J. (1997) Genetic characterization of the pdu operon: use of 1,2-propanediol in *Salmonella typhimurium*. *Journal of Bacteriology* 179, 1013-1022.
113. Santos, F., Vera, J. L., van der Heijden, R., Valdez, G., de Vos, W. M., Sesma, F., and Hugenholtz, J. (2008) The complete coenzyme B12 biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098. *Microbiology* 154, 81-93.

114. Bobik, T. A., Ailion, M., and Roth, J. R. (1992) A single regulatory gene integrates control of vitamin B12 synthesis and propanediol degradation. *Journal of Bacteriology* 174, 2253-2266
115. Havemann, G. D., and Bobik, T. A. (2003) Protein content of polyhedral organelles involved in coenzyme B12-dependent degradation of 1,2-propanediol in *Salmonella enterica* serovar Typhimurium LT2. *Journal of Bacteriology* 185, 5086-5095.
116. Leal, N. A., Havemann, G. D., and Bobik, T. A. (2003) PduP is a coenzyme-a-acylating propionaldehyde dehydrogenase associated with the polyhedral bodies involved in B12-dependent 1,2-propanediol degradation by *Salmonella enterica* serovar Typhimurium LT2. *Archives of Microbiology* 180, 353-361.
117. Liu, Y., Leal, N. A., Sampson, E. M., Johnson, C. L., Havemann, G. D., and Bobik, T. A. (2007) PduL is an evolutionarily distinct phosphotransacylase involved in B12-dependent 1,2-propanediol degradation by *Salmonella enterica* serovar typhimurium LT2. *Journal of Bacteriology* 189, 1589-1596.
118. Palacios, S., Starai, V. J., and Escalante-Semerena, J. C. (2003) Propionyl coenzyme A is a common intermediate in the 1,2-propanediol and propionate catabolic pathways needed for expression of the prpBCDE operon during growth of *Salmonella enterica* on 1,2-propanediol. *Journal of Bacteriology* 185, 2802-2810.
119. Sampson, E. M., and Bobik, T. A. (2008) Microcompartments for B12-dependent 1,2-propanediol degradation provide protection from DNA and cellular damage by a reactive metabolic intermediate. *Journal of Bacteriology* 190, 2966-2971.
120. Kerfeld, C. A., Heinhorst, S., and Cannon, G. C. (2010) Bacterial microcompartments. *Annual Review of Microbiology* 64, 391-408.
121. Sauvageot, N., Gouffi, K., Laplace, J. M., and Auffray, Y. (2000) Glycerol metabolism in *Lactobacillus collinoides*: production of 3-hydroxypropionaldehyde, a precursor of acrolein. *International Journal of Food Microbiology* 55, 167-170.
122. Voisenet, M. E. (1910) Formation d'acroléine dans la maladie de l'amertume de vins. *Comptes Rendus de l'Academie des Sciences* 150, 1614-1616.
123. Talarico, T. L., and Dobrogosz, W. J. (1989) Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. *Antimicrobial Agents and Chemotherapy* 33, 674-679.
124. Schaefer, L., Auchtung, T. A., Hermans, K. E., Whitehead, D., Borhan, B., and Britton, R. A. (2010) The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. *Microbiology* 156, 1589-1599.
125. Cleusix, V., Lacroix, C., Vollenweider, S., Duboux, M., and Le Blay, G. (2007) Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. *BMC Microbiology* 7, 101.
126. Luthi-Peng, Q., Scharer, S., and Puhán, Z. (2002) Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*. *Applied Microbiology and Biotechnology* 60, 73-80.
127. Sardari, R. R., Dishisha, T., Pyo, S. H., and Hatti-Kaul, R. (2013) Improved production of 3-hydroxypropionaldehyde by complex formation with bisulfite during biotransformation of glycerol. *Biotechnology and Bioengineering* 110, 1243-1248.

128. Luthi-Peng, Q., Dileme, F. B., and Puhan, Z. (2002) Effect of glucose on glycerol bioconversion by *Lactobacillus reuteri*. *Applied Microbiology and Biotechnology* 59, 289-296.
129. Krauter, H., Willke, T., and Vorlop, K. D. (2012) Production of high amounts of 3-hydroxypropionaldehyde from glycerol by *Lactobacillus reuteri* with strongly increased biocatalyst lifetime and productivity. *New Biotechnology* 29, 211-217.
130. Rutti, D. P., Lacroix, C., Jeremic, T., Mathis, M., Die, A., and Vollenweider, S. (2011) Development of a reversible binding process for in situ removal of 3-hydroxypropionaldehyde during biotechnological conversion of glycerol. *Biochemical Engineering Journal* 55, 176-184.
131. Sardari, R. R., Dishisha, T., Pyo, S. H., and Hatti-Kaul, R. (2013) Biotransformation of glycerol to 3-hydroxypropionaldehyde: improved production by in situ complexation with bisulfite in a fed-batch mode and separation on anion exchanger. *Journal of Biotechnology* 168, 534-542.
132. Talfournier, F., Stines-Chaumeil, C., and Branlant, G. (2011) Methylmalonate-semialdehyde dehydrogenase from *Bacillus subtilis*: substrate specificity and coenzyme A binding. *The Journal of Biological Chemistry* 286, 21971-21981.
133. Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., Bingen, E., Bonacorsi, S., Bouchier, C., Bouvet, O., Calteau, A., Chiapello, H., Clermont, O., Cruveiller, S., Danchin, A., Diard, M., Dossat, C., Karoui, M. E., Frapy, E., Garry, L., Ghigo, J. M., Gilles, A. M., Johnson, J., Le Bouguenec, C., Lescat, M., Mangenot, S., Martinez-Jehanne, V., Matic, I., Nassif, X., Oztas, S., Petit, M. A., Pichon, C., Rouy, Z., Ruf, C. S., Schneider, D., Turret, J., Vacherie, B., Vallenet, D., Medigue, C., Rocha, E. P., and Denamur, E. (2009) Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genetics* 5, e1000344.
134. Mora, A., Herrera, A., López, C., Dahbi, G., Mamani, R., Pita, J. M., Alonso, M. P., Llovo, J., Bernárdez, M. I., Blanco, J. E., Blanco, M., and Blanco, J. (2011) Characteristics of the shiga-toxin-producing enteroaggregative *Escherichia coli* O104:H4 German outbreak strain and of STEC strains isolated in Spain. *International Microbiology* 14, 121-141.
135. Gyles, C. L. (2014) Shiga toxin-producing *Escherichia coli*: an overview. *Journal of Animal Science* 85, 18.
136. Hudault, S., Guignot, J., and Servin, A. L. (2001) *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. *Gut* 49, 47-55.
137. Bachmann, B. J. (2004) Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology (Neidhardt, F. C.), American Society for Microbiology, Washington D.C.
138. Daegelen, P., Studier, F. W., Lenski, R. E., Cure, S., and Kim, J. F. (2009) Tracing ancestors and relatives of *Escherichia coli* B, and the derivation of B strains REL606 and BL21(DE3). *Journal of Molecular Biology* 394, 634-643.
139. el-Mansi, E. M., and Holms, W. H. (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. *Journal of General Microbiology* 135, 2875-2883.

140. Kornberg, H. L. (1966) The role and control of the glyoxylate cycle in *Escherichia coli*. The Biochemical Journal 99, 1-11
141. Murarka, A., Dharmadi, Y., Yazdani, S. S., and Gonzalez, R. (2008) Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Applied and Environmental Microbiology 74, 1124-1135.
142. Perez, J. M., Arenas, F. A., Pradenas, G. A., Sandoval, J. M., and Vasquez, C. C. (2008) *Escherichia coli* YqhD exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. The Journal of Biological Chemistry 283, 7346-7353.
143. Marisch, K., Bayer, K., Scharl, T., Mairhofer, J., Krempl, P. M., Hummel, K., Razzazi-Fazeli, E., and Striedner, G. (2013) A comparative analysis of industrial *Escherichia coli* K-12 and B strains in high-glucose batch cultivations on process-, transcriptome- and proteome level. PloS One 8, e70516.
144. Shiloach, J., Kaufman, J., Guillard, A. S., and Fass, R. (1996) Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by *Escherichia coli* BL21 (λ DE3) and *Escherichia coli* JM109. Biotechnology and Bioengineering 49, 421-428.
145. Chen, X., Zhou, L., Tian, K., Kumar, A., Singh, S., Prior, B. A., and Wang, Z. (2013) Metabolic engineering of *Escherichia coli*: A sustainable industrial platform for bio-based chemical production. Biotechnology Advances 31, 1200-1223.
146. Forster, A. H., and Gescher, J. (2014) Metabolic engineering of *Escherichia coli* for production of mixed-acid fermentation end products. Frontiers in Bioengineering and Biotechnology 2, 16.
147. Ganesh, I., Ravikumar, S., and Hong, S. (2012) Metabolically engineered *Escherichia coli* as a tool for the production of bioenergy and biochemicals from glycerol. Biotechnology and Bioprocess Engineering 17, 671-678.
148. Sambrook, J., and Russel, D. W. (2001) Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
149. Studier, F. W., and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Journal of Molecular Biology 189, 113-130
150. Rosano, G. L., and Ceccarelli, E. A. (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. Frontiers in Microbiology 5.
151. Ikemura, T. (1985) Codon usage and tRNA content in unicellular and multicellular organisms. Molecular Biology and Evolution 2, 13-34.
152. Warnecke, T., and Gill, R. T. (2005) Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. Microbial Cell Factories 4, 25.
153. Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F., and Foster, J. W. (1999) Control of acid resistance in *Escherichia coli*. Journal of Bacteriology 181, 3525-3535.
154. Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H., and Kompala, D. S. (1990) Plasmid-encoded protein: the principal factor in the "metabolic burden" associated with recombinant bacteria. Biotechnology & Bioengineering 35, 668-681.
155. Berg, J. M., Tymoczko, J. L., and Stryer, L. (2002) Biochemistry, 5th ed., W.H. Freeman and Company, New York

156. Wandrey, C. (2004) Biochemical reaction engineering for redox reactions. *The Chemical Record* 4, 254-265
157. Uppada, V., Bhaduri, S., and Noronha, S. B. (2014) Cofactor regeneration - an important aspect of biocatalysis. *Current Science* 106, 946-957.
158. Abril, O., and Whitesides, G. M. (1982) Hybrid organometallic/enzymic catalyst systems: regeneration of NADH using dihydrogen. *Journal of the American Chemical Society* 104, 1552-1554.
159. Kohlmann, C., Märkle, W., and Lütz, S. (2008) Electroenzymatic synthesis. *Journal of Molecular Catalysis B: Enzymatic* 51, 57-72.
160. Berrios-Rivera, S. J., Bennett, G. N., and San, K. Y. (2002) Metabolic engineering of *Escherichia coli*: increase of NADH availability by overexpressing an NAD⁺-dependent formate dehydrogenase. *Metabolic Engineering* 4, 217-229.
161. Ma, Z., Shentu, X., Bian, Y., and Yu, X. (2013) Effects of NADH availability on the *Klebsiella pneumoniae* strain with 1,3-propanediol operon over-expression. *Journal of Basic Microbiology* 53, 348-354.
162. Kengen, S. W., van der Oost, J., and de Vos, W. M. (2003) Molecular characterization of H₂O₂-forming NADH oxidases from *Archaeoglobus fulgidus*. *European Journal of Biochemistry/FEBS* 270, 2885-2894.
163. Toomey, D., and Mayhew, S. G. (1998) Purification and characterisation of NADH oxidase from *Thermus aquaticus* YT-1 and evidence that it functions in a peroxide-reduction system. *European Journal of Biochemistry/FEBS* 251, 935-945.
164. Niimura, Y., Nishiyama, Y., Saito, D., Tsuji, H., Hidaka, M., Miyaji, T., Watanabe, T., and Massey, V. (2000) A hydrogen peroxide-forming NADH oxidase that functions as an alkyl hydroperoxide reductase in *Amphibacillus xylanus*. *Journal of Bacteriology* 182, 5046-5051.
165. Higuchi, M., Shimada, M., Yamamoto, Y., Hayashi, T., Koga, T., and Kamio, Y. (1993) Identification of two distinct NADH oxidases corresponding to H₂O₂-forming oxidase and H₂O-forming oxidase induced in *Streptococcus mutans*. *Journal of General Microbiology* 139, 2343-2351
166. Rocha-Martin, J., Vega, D., Bolivar, J. M., Godoy, C. A., Hidalgo, A., Berenguer, J., Guisan, J. M., and Lopez-Gallego, F. (2011) New biotechnological perspectives of a NADH oxidase variant from *Thermus thermophilus* HB27 as NAD⁺-recycling enzyme. *BMC Biotechnology* 11, 101.
167. Jang, S., and Imlay, J. A. (2010) Hydrogen peroxide inactivates the *Escherichia coli* Isc iron-sulphur assembly system, and OxyR induces the Suf system to compensate. *Molecular Microbiology* 78, 1448-1467.
168. Sudar, M., Findrik, Z., Domanovac, M. V., and Vasić-Rački, Đ. (2014) Coenzyme regeneration catalyzed by NADH oxidase from *Lactococcus lactis*. *Biochemical Engineering Journal* 88, 12-18.
169. Lopez de Felipe, F., and Hugenholtz, J. (2001) Purification and characterisation of the water forming NADH-oxidase from *Lactococcus lactis*. *International Dairy Journal* 11, 37-44.
170. Riebel, B. R., Gibbs, P. R., Wellborn, W. B., and Bommarius, A. S. (2002) Cofactor regeneration of NAD⁺ from NADH: novel water-forming NADH oxidases. *Advanced Synthesis & Catalysis* 344, 1156-1168.
171. Jia, B., Park, S. C., Lee, S., Pham, B. P., Yu, R., Le, T. L., Han, S. W., Yang, J. K., Choi, M. S., Baumeister, W., and Cheong, G. W. (2008) Hexameric ring structure

- of a thermophilic archaeon NADH oxidase that produces predominantly H₂O. The FEBS Journal 275, 5355-5366.
172. Zhang, Y. W., Tiwari, M. K., Gao, H., Dhiman, S. S., Jeya, M., and Lee, J. K. (2012) Cloning and characterization of a thermostable H₂O-forming NADH oxidase from *Lactobacillus rhamnosus*. Enzyme & Microbial Technology 50, 255-262.
 173. Geueke, B., Riebel, B., and Hummel, W. (2003) NADH oxidase from *Lactobacillus brevis*: a new catalyst for the regeneration of NAD⁺. Enzyme & Microbial Technology 32, 205-211.
 174. Riebel, B. R., Gibbs, P. R., Wellborn, W. B., and Bommarius, A. S. (2003) Cofactor regeneration of both NAD⁺ and NADH and NADP⁺ from NADPH:NADH oxidase from *Lactobacillus sanfranciscensis*. Advanced Synthesis & Catalysis 345, 707-712.
 175. Maehara, A., Ueda, S., Nakano, H., and Yamane, T. (1999) Analyses of a polyhydroxyalkanoic Acid granule-associated 16-kilodalton protein and its putative regulator in the *pha* Locus of *Paracoccus denitrificans*. Journal of Bacteriology 181, 2914-2921.
 176. Gerngross, T. U., and Martin, D. P. (1995) Enzyme-catalyzed synthesis of poly[(R)-(-)-3-hydroxybutyrate]: formation of macroscopic granules in vitro. Proceedings of the National Academy of Sciences of the United States of America 92, 6279-6283.

Paper I



Coenzyme A-acylating propionaldehyde dehydrogenase (PduP) from *Lactobacillus reuteri*: Kinetic characterization and molecular modeling



Ramin Sabet-Azad*, Javier A. Linares-Pastén, Lisa Torkelson,
Roya R.R. Sardari, Rajni Hatti-Kaul

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

ARTICLE INFO

Article history:

Received 22 January 2013
Received in revised form 22 May 2013
Accepted 23 May 2013

Keywords:

Propionaldehyde dehydrogenase
Lactobacillus reuteri
3-Hydroxypropionic acid
3-Hydroxypropionaldehyde
Kinetic characterization
Cofactor binding

ABSTRACT

3-Hydroxypropionic acid (3-HP), an important C3 chemical for a bio-based industry, is natively produced by *Lactobacillus reuteri* from glycerol. Conversion of glycerol occurs via the intermediate 3-hydroxypropionaldehyde (3-HPA), followed by an ATP-producing pathway initiated by the CoA-acylating propionaldehyde dehydrogenase (PduP). The *pduP* gene of *L. reuteri* was cloned and expressed in *Escherichia coli* and the recombinant enzyme was purified to homogeneity for characterization of its activity and properties. Kinetic studies with propionaldehyde as substrate showed a maximum specific activity of 28.9 U/mg, which is 80-fold higher than that reported previously. Maximum activity of 18 U/mg was obtained at 3-HPA concentration of 7 mM, above which substrate inhibition was observed. Substrate inhibition was also seen with coenzyme A at a concentration above 0.5 mM and with NADP⁺ above 9 mM. A structure of PduP is proposed based on homology modeling. *In silico* docking of the co-factors coenzyme A and NAD⁺, respectively, showed a common binding site consisting of amino acids Thr145, Ile275, Cys277 and Ser417, which through site-directed mutagenesis to alanine and kinetic studies, were confirmed as essential for the catalytic activity of PduP.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

3-Hydroxypropionic acid (3-HP) is a C3 chemical that has generated a great deal of interest during the past decade as a potential precursor for the production of bio-based acrylic acid [1–3]. A number of pathways have been engineered in *Escherichia coli* for production of the hydroxyacid from glucose and glycerol, respectively [4–7]. The ability of *Lactobacillus reuteri*, a facultative anaerobe, to produce 3-HP natively has been known since a few years [8,9]. However, until now it has only been assumed that a propanediol utilizing (Pdu) pathway in *L. reuteri* plays a role in this process. Three propanediol utilizing proteins in the pathway, propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW) in a cascade reaction convert aldehyde intermediate (propionaldehyde) to acid product (propionate). The propanediol utilizing operon, mainly responsible for the conversion of 1,2-propanediol to propionate and propanol, has been observed in *Salmonella* sp., *Klebsiella pneumonia* and in some strains of *Lactobacillus* [10–12].

The resting cells of *L. reuteri* produce 3-hydroxypropionaldehyde (3-HPA) from glycerol in a reaction catalyzed by glycerol dehydratase. The 3-HPA enters a reductive pathway leading to the production of 1,3-propanediol (1,3-PD) while an oxidative pathway converts it to 3-HP presumably using the ATP-producing pathway involving the *pdu*-genes [13,14] (Fig. 1). Further studies to confirm the utilization of this pathway for conversion of glycerol to 3-HP conversion, analogous to the confirmed conversion of propionaldehyde to propionic acid, would be of great interest as a route for the heterologous or optimized production of 3-HP.

A kinetic characterization of the first enzyme, PduP of *L. reuteri* JCM 1112, expressed in *Escherichia coli* has been reported earlier [15] with activities at micronit levels. The present study reports on the heterologous expression of the gene encoding PduP from *L. reuteri* DSM 20016 with significantly higher activity and characterization of molecular and activity features of the recombinant enzyme. A structural model of the enzyme is proposed based on homology modeling of the PduP-sequence with known CoA-acylating aldehyde dehydrogenases, cofactor binding sites are identified by *in silico* bonding of PduP with the cofactors and mutagenesis of the identified residues. Furthermore, kinetic study of PduP was performed with propionaldehyde and its role in the proposed 3-HP/1,3-PD producing pathway was verified by using 3-HPA as substrate.

* Corresponding author. Tel.: +4646 222 34 26; fax: +4646 222 47 13.
E-mail address: ramin.sabet.azad@biotek.luse.se (R. Sabet-Azad).

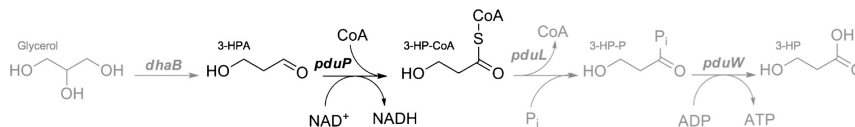


Fig. 1. Proposed pathway of *L. reuteri* 3-HP production from glycerol. Conversion of glycerol is initiated by glycerol dehydratase, resulting in the production of 3-HPA. In a cascade reaction by PduP, PduL and PduW, 3-HP is formed with the production of NADH and ATP. Reactions outside the scope of this study are marked in gray.

2. Materials and methods

2.1. Strains, plasmids and medium

Lactobacillus reuteri DSM 20016 was obtained from the Leibniz Institute DSMZ. The cloning host *Escherichia coli* NovaBlue and expression host *Escherichia coli* BL21(DE3) were purchased from Novagen. De Man, Rogosa and Sharpe (MRS)-medium from Difco (BD) was used for the cultivation of *L. reuteri*. All *E. coli*-strains were cultivated in lysogeny broth (LB)-medium or on LB-agar with supplementation of antibiotics and other selection markers when necessary. LB was obtained from Duchefa Biochemie. Cloning vector pUC19 was purchased from Fermentas and expression vector pET28b was from Novagen.

2.2. Materials

Restriction enzymes BamHI, XhoI, SmaI and DpnI were purchased from Fermentas, while T4 DNA ligase was from New England Biolabs. Propionaldehyde, coenzyme A (HS-CoA), β -nicotinamide adenine dinucleotide (NAD⁺) and β -nicotinamide adenine dinucleotide phosphate (NADP⁺) were purchased from Sigma-Aldrich. Ampicillin and kanamycin were purchased from Sigma-Aldrich and isopropyl- β -D-thiogalactopyranoside (IPTG) and X-Gal were from Fermentas.

2.3. Homology modeling and prediction of co-factor binding sites

Homology modeling of the *L. reuteri* PduP was performed with the aid of CPH-models 3.0, developed by the Center for Biological Sequence Alignment, Technical University of Denmark (DTU). Homology model quality assessment was performed by utilizing the SWISS-MODEL workspace [16] through QMEAN6-scoring [17] and Ramachandran plot. The RMS deviation value was calculated by superimposing alpha carbons of template and model in Swiss PDB-viewer.

The Basic Local Alignment Search Tool (BLASTp, NCBI) was utilized for the detection of enzymes in the Protein Databank (PDB) having sequential resemblance to PduP. Sequence alignments for the identification of conserved amino acids were made by ClustalW sequence alignment (EMBL-EBI).

YASARA (Yet Another Scientific Artificial Reality Application) [18] was used for the study of the molecular model of PduP. Dockings of PduP with NAD⁺, NADP⁺ and HS-CoA, respectively, in a simulated aqueous environment with explicit water molecules, were performed by utilization of AutoDock 4 [19]. Energy minimized structures of the free enzyme and the enzyme docked with NAD⁺, and HS-CoA were obtained in YASARA through the amber99 force field computation, enabling bonds, angles, dihedral angles, Coulomb and Van der Waals forces [20]. Energy computations of free as well as cofactor bound enzyme (PduP-NAD⁺ and PduP-CoA) were done with the GROMOS96 implementation of Swiss-PDB viewer [21].

2.4. Construction of PduP-expressing recombinant *E. coli* strain

L. reuteri DSM 20016 was grown overnight under anaerobic conditions at 37 °C. The cells were harvested and genomic DNA was purified from the cells using GeneJet Genomic DNA kit (Fermentas).

For the amplification of the *pduP* gene by PCR, the primer sequences of Luo et al. [15] were employed with introduction of BamHI and XhoI restriction sites and removal of the native stop codon of PduP. Initial denaturing at 94 °C for 3 min was followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 3 min. A final elongation was performed at 72 °C for 10 min. The PCR product was separated and purified by agarose gel electrophoresis and inserted into pUC19 cloning vector through blunt-end ligation after digesting the vector with SmaI restriction enzyme.

The construct was introduced in *E. coli* NovaBlue by thermal shock transformation. Screening of positive colonies was done by α -complementation of β -galactosidase [22,23]. *pduP* was cloned into pET28b through sequential restriction of the pUC19-PduP construct using BamHI and XhoI, followed by ligation. The pET28b-PduP construct was designated pETPduP-01, encoding PduP followed by a hexahistidine (his₆)-tag on the C-terminus of the protein. The construct was introduced in *E. coli* BL21(DE3) and the recombinant strain was designated *E. coli* BTPduP-01.

The pETPduP-01 construct was verified by nucleotide sequencing. The codon reading frame was corrected by site-directed mutagenesis using the forward primer PduP_SDM_FOR (5'-GAGGAGATATACCATGACAGATTAATG-3') and reverse primer

PduP_SDM_REV (5'-CATTAAATCTGCATGGTATATCTCTCT-3') in a PCR with plasmid pETPduP-01 as template, resulting in the elimination of a supplementary ATGG just upstream from the native start codon. The PCR was conducted with initial denaturation at 98 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 20 s, annealing at 52 °C for 30 s and elongation at 72 °C for 5 min. The reaction was finalized with an elongation step at 72 °C for 7 min. Methylated template DNA was degraded by restriction endonuclease DpnI. The modified vector, designated pETPduP-02 and encoding the corrected PduP-his₆ protein product was introduced in *E. coli* BL21(DE3), resulting in strain *E. coli* BTPduP-02. The same method was used for the construction of PduP containing the point mutations C277A, S417A, T145A, I275A. Forward mutagenic primers used had following sequences (with reverse primers having complementary sequences) (bold text indicates mutagenic sites):

PduP_C277A_FOR (5'-GATATTTT**AGCG**ACTGCTGAAAAGG-3');
 PduP_S417A_FOR (5'-CGGATGCAATG**TGGG**ATCTTGTGTG-3');
 PduP_T145A_FOR (5'-GCCCAAG**TGGG**AACCCCTCAG-3');
 PduP_I275A_FOR (5'-CTTCATTGTAATGATG**GGT**TATGACTGC-3');

The strains were designated BTPduP-C277A, BTPduP-S417A, BTPduP-T145A and BTPduP-I275A, respectively. Transformation of *E. coli* BL21(DE3)-strain with pET28b was performed for the construction of a control strain (BTPET28b-01) lacking the *pduP* gene but identical in all other aspects. Nucleotide sequencing was performed for verifying all the constructs.

2.5. Expression and purification of recombinant PduP

Recombinant *E. coli* strains were grown in LB-medium containing 10 μ g/mL kanamycin under aerobic conditions at 30 °C and shaking speed of 200 rpm. Induction of the enzymes was performed by the addition of 1 mM IPTG when the cultures had reached mid-exponential phase (OD₆₀₀ 0.4–0.6) with subsequent incubation for 4 h at 30 °C and 200 rpm. The cells were then harvested and washed with 20 mM sodium phosphate buffer pH 7.4 containing 0.5 M NaCl.

Lysis of the cells and extraction of proteins was performed by re-suspending and incubating the cells in Bugbuster Protein Extraction Reagent and Lysosane Bioprocessing Reagent (Novagen) according to manufacturer's instructions. The soluble and insoluble protein fractions were separated by centrifugation for 20 min at 23,300 \times g, 4 °C.

The soluble fraction was filtered through a nitrocellulose membrane and the pH of the filtrate was adjusted to 7.4. PduP was purified from the filtrate by binding to a 1 mL HisTrap HP column (GE Healthcare) equilibrated with a binding buffer of 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4, and elution with the buffer containing 500 mM imidazole. In order to remove imidazole and NaCl from the purified protein, dialysis was performed overnight in a buffer containing 20 mM sodium phosphate buffer, pH 7.4 at 4 °C using a cellulose-based dialysis membrane with molecular weight cut off of 10 kDa (SpectrumLabs). The sample was then concentrated by centrifugation in a Centricon tube (molecular weight cut off 30 kDa) at 1600 \times g, room temperature for 15 min. The concentration of the purified protein was estimated by absorbance at 280 nm.

Expression analysis was conducted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 12% acrylamide. The protein bands were stained with Coomassie Brilliant Blue R-250.

2.6. Measurement of enzymatic activity

The activity of PduP was determined by a method developed by Leal et al. [24] with some modifications. The method is based on the oxidation of the substrate aldehyde in the presence of NAD(P⁺) and measuring the production of NAD(P)H spectrophotometrically at 340 nm. The reaction mixture containing 0.43 mM HS-CoA and 5 mM NAD⁺, if not mentioned otherwise, in 50 mM potassium phosphate buffer pH 7.0 supplemented with 1 mM DTT and 0.1 g/L BSA was equilibrated for 5 min at 30 °C before initiating the reaction by addition of 100 mM propionaldehyde. One Unit (U) is defined as the amount of enzyme catalyzing the formation of 1 μ mol NADH formed per minute under the assay conditions described. The crude extract of *E. coli* BTPET28b was used as a negative control in all assays.

Studies on the effects of the buffer (potassium phosphate buffer pH 7.0, HEPES pH 7.0, Tris-HCl pH 7.0), temperature (30–70 °C) and pH (2–12) on the enzyme activity were performed. The pH-profile was determined with Britton-Robinson buffer

containing 40 mM H₂BO₃, 40 mM H₃PO₄ and 40 mM CH₃COOH, and adjusting the pH with NaOH.

The kinetic properties of PduP were determined by varying the concentration of each co-factor (HS-CoA 0.011–6.86 mM, NAD⁺ 0.29–11.43 mM, and NADP⁺ 0.86–21.43 mM, respectively) at propionaldehyde concentration of 200 mM. Kinetic assays were then performed with varied concentrations of the substrates (propionaldehyde 7.1–285.7 mM, 3-HPA 1.1–57.1 mM) with 5 mM NAD⁺ and 0.43 mM HS-CoA. Kinetic properties of the PduP mutants C277A, S417A, I275A and T145A were studied by varying the concentrations of NAD⁺ (0.2–15 mM) and HS-CoA (0.02–2.5 mM) with 100 mM propionaldehyde and all other conditions as above.

2.7. Production of 3-HPA

3-HPA used in this study was produced from glycerol using *L. reuteri* cells according to the method first developed by Lüthi-Peng et al. [25] with modifications as presented by Sardari et al. [26]. *L. reuteri* cells were grown in MRS medium (55 g/L MRS, 20% (v/v) glycerol) at 37 °C for 16 h. The cells were collected and resuspended to a concentration of 21.4 g/L in an aqueous solution containing 200 mM glycerol, bubbled with nitrogen gas and incubated for the production of 3-HPA at 37 °C for 2 h on a rocking table. The cells were then separated by centrifugation and the supernatant was analyzed and used as a source of 3-HPA after filter sterilizing the sample through a 0.2 μm polyethersulfone membrane (VWR).

A negative control in which 3-HPA was removed from the supernatant was also included. 3-HPA removal was done by binding to bisulfite functionalized Amberlite IRA-400 (Cl) resin according to Rütli et al. [27].

2.8. Analysis of glycerol, 3-HPA, 1,3-PD and 3-HP

3-HPA was quantified by a colorimetric method based on dehydration of 3-HPA to acrolein and complexation with di-tryptophan, thus forming a purple complex with a light absorbance at 560 nm [28], with modifications. Three milliliters of concentrated HCl and 0.75 mL of 10 mM di-tryptophan (Sigma–Aldrich) in 50 mM HCl were added to 1 mL of diluted supernatant sample. The sample was incubated for 20 min at 37 °C and absorbance was measured at 560 nm in a UV/vis spectrophotometer. Acrolein was used as standard.

Residual glycerol, 3-HP and 1,3-PD were quantified by HPLC (Jasco) utilizing an Aminex HPX-87H column as reported by Sardari et al. [26].

3. Results

3.1. Identification of the pduP gene of *L. reuteri* DSM 20016

The putative ribosomal protein L29P (NCBI: YP_001272311) of *L. reuteri* DSM 20016 encoded by 1434 nucleotides was identified as a probable propanediol utilizing protein by sequence homology with the propanediol utilization protein PduP of *L. reuteri* JCM 1112 (NCBI: BAG26139) as the template in a BLASTn-query. The template had a completely identical nucleotide sequence with PduP of *L. reuteri* DSM 20016, covering 100% of the sequence. The query identified PduP as a member of the CoA-acylating aldehyde dehydrogenase (AldH)-superfamily.

3.2. Homology modeling and prediction of co-factor binding sites

Two enzymes, the probable aldehyde dehydrogenase from *Listeria monocytogenes* Egd.E (30%, PDB: 3K9D.A) and alcohol dehydrogenase from *Vibrio parahaemolyticus* (31%, PDB: 3MY7.A), were found with similarities above 30% to PduP with sequence coverages above 75%. Protein sequence alignments of the PduP with these enzymes as well as with the characterized CoA-acylating aldehyde dehydrogenases of *Clostridium beijerinckii* (PDB: AF157306) and *Salmonella typhimurium* (PDB: AAA80209) [29] can be seen in Fig. 2.

Homology modeling for the prediction of the tertiary structure of PduP resulted in a model based on the crystal structure of chain A of the probable aldehyde dehydrogenase of *L. monocytogenes*, Egd.E. Quality model assessment of the homology model revealed a QMEAN6-score of 0.616 and a Z-score of -1.717. The Ramachandran plot revealed that none of the total of 386 residues were present in the disallowed regions and 4 residues (1.2%) were present in the generously allowed regions. RMS deviation value of superimposed homologue model on Egd.E was 0.73 Å.

Fig. 3 shows the proposed structural model of PduP with bound HS-CoA. The model indicates an enzyme containing two domains, each containing a typical Rossmann-fold. The proposed NAD⁺ and HS-CoA-binding to the predicted model of PduP is presented in Fig. 4a and b. Energy computations through GROMOS96 resulted in a value of -12,245 kJ/mol for free PduP, -13,323 kJ/mol for the PduP-CoA-complex and -13,285 kJ/mol for the PduP-NAD⁺ complex. The co-factors have a common binding site, with the ADP-moieties surrounded by a GXGXXP-motif and a serine (Ser417), while the NMN-moiety of NAD⁺ is in proximity to an isoleucine (Ile275) and a threonine (Thr145). A Van der Waals interaction occurs between Ile275 and NAD⁺. A cysteine (Cys277) is found close to the thiol-group of HS-CoA and the NMN-moiety of NAD⁺.

3.3. Cloning, expression and purification of PduP

The cloning of pduP in to pET28b was verified by nucleotide sequencing and protein expression in *E. coli* BL21(DE3) through SDS-PAGE analysis (Fig. 5). No band corresponding to the ~55 kDa PduP-his₆ is seen in the crude extract of control strain BTPET28-01, while overexpression of this product is seen in BTPduP-02. Purification of the protein from the clarified BTPduP-02 lysate by immobilized metal ion affinity chromatography yielded 69% recovery of the active enzyme, clearly visible through SDS-PAGE with a molecular weight of ~55 kDa, specific activity of 25 U/mg and purification fold of 14.7 (Table 1).

All PduP-mutants (S417A, C277A, I275A and T145A) were expressed as soluble proteins. The chromatography profiles of these mutants were similar to that obtained for the original PduP.

3.4. Characterization of PduP activity

The PduP activity was evaluated in three different buffers at 30 °C with propionaldehyde as substrate; optimal PduP-activity was observed in potassium phosphate buffer and was almost 30% lower in HEPES-buffer, while no activity was detected with Tris-HCl buffer. The pH profile of PduP was studied using Britton-Robinson buffer between pH 2–12. A bell shaped activity curve was observed with highest activity at pH 7, and no activity at pH 4 and pH 10 at two ends of the curve. Determination of PduP activity at varying temperatures showed activity increase of about 10 U/mg with every 10 °C rise in temperature up to 70 °C at which a maximal specific activity of 57 U/mg was reached. Higher temperatures were not tested since propionaldehyde is a volatile compound and the loss of substrate at these higher temperatures makes the assays less reliable.

The kinetics of PduP activity were determined with both propionaldehyde and 3-HPA as substrates (Fig. 6a and b). With the former, a Michaelis–Menten behavior was seen with a K_m of 28 mM and V_{max} of 28.9 U/mg. Specific activity of 18 U/mg was obtained with 3-HPA as substrate before an inhibition of the enzyme was seen at 3-HPA concentrations above 7 mM.

Activity measurements of PduP at varying concentrations of the cofactors revealed that the activity increased with increase in NAD⁺ concentration without any inhibition (Fig. 7a), while inhibition was observed over 9 mM NADP⁺ (Fig. 7b) and 0.5 mM HS-CoA (Fig. 6c), respectively. Maximum specific activity of PduP with NAD⁺ as co-factor (28.9 U/mg) was almost three-fold higher than that obtained with NADP⁺. No activity was observed with the crude extract of *E. coli* BTPET28b.

3.5. Kinetic studies of PduP with modified co-factor binding sites

Some amino acid residues (Cys277, Ser417, Thr145 and Ile275), expected to be crucial for binding the cofactors based on the predicted model (Fig. 4), were mutated by site-directed mutagenesis.

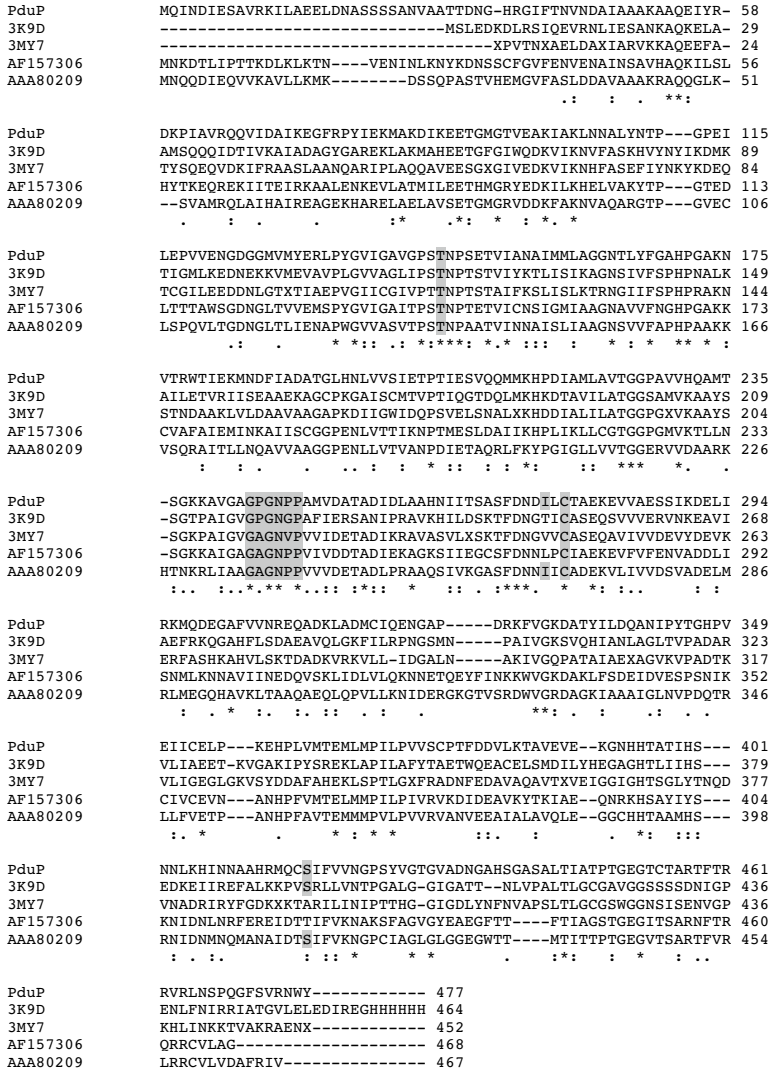


Fig. 2. Alignment of the amino acid sequence of PduP from *L. reuteri* with the sequences of a probable aldehyde dehydrogenase from *Listeria monocytogenes* (3KPD), alcohol dehydrogenase from *Vibrio parahaemolyticus* (3MY7) and the CoA-acylating aldehyde dehydrogenases of *Clostridium beijerinckii* (AF157306) and *Salmonella typhimurium* (AAA80209). Conserved amino acids are marked by an asterisk. Amino acids proposed to play a role in the catalytic mechanism and co-factor binding of the enzymes, based on homology modeling and *in silico* dockings, are marked in gray.

Table 1
Purification scheme of recombinant PduP from *L. reuteri*.

Protein source	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract, <i>E. coli</i> BTPduP-02	10	9.4	31.8 ± 1.0	1.7 ± 0.1	100	1
Purified PduP	2.5	0.9	21.9 ± 0.2	25 ± 0.2	69	14.7

Table 2

Results of kinetic studies of PduP and its mutants S417A, I275A, C277A and T145A, performed using varying concentrations of NAD⁺. Reaction mixtures contained 200 mM propionaldehyde, 0.2–15 mM NAD⁺, 0.43 mM HS-CoA, 1 mM DTT and 0.1 mg/mL BSA. The reactions were performed in 50 mM potassium phosphate buffer, pH 7.0, 30 °C.

	V_{max} (U/mg)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
PduP	36.0 ± 1.0	0.9 ± 0.1	31.2 ± 0.9	32.8
PduP-S417A	18.2 ± 0.9	1.7 ± 0.3	15.6 ± 0.8	9.1
PduP-I275A			No activity detected	
PduP-C277A			No activity detected	
PduP-T417A			No activity detected	

The kinetic properties of the four mutants of PduP in comparison with non-modified PduP with varied concentrations of NAD⁺ can be seen in Table 2. No activity was detected with PduP-C277A, PduP-I275A and PduP-T145A. Only PduP-S417A was found to be active, however with a two-fold increase in K_m and approximately 3.5-fold lower k_{cat}/K_m . With HS-CoA, a V_{max} of 16.8 U/mg was obtained before substrate inhibition was observed (data not shown).

4. Discussion

The structural model of PduP, based on the probable aldehyde dehydrogenase of *L. monocytogenes* (Egd-E), consists of two domains connected by a single strand (Fig. 3). Each domain contains a typical NAD⁺-binding Rossmann-fold. The composite QMEAN6-score (a combination of the six terms C-beta interaction energy, all-atom interaction energy, solvation energy, torsion angle energy, secondary structure agreement and solvent accessibility agreement with an estimated model reliability of 1 at highest and 0 at lowest) of 0.616, Z-score (indicating absolute reliability of the model, in comparison with reference structures, with scores below -4 meaning incorrect modeling in any part of the protein) of -1.77, RMS deviation value (representing the distance between atoms of superimposed proteins with value 1 as an ideal superimposition) of 0.73 Å, and finally the model's Ramachandran plot, reflecting the presence of torsion angles in allowed or disallowed regions thus assessing the three dimensional structure of the protein (no residues in the disallowed region) indicate that the homology model is reliable. Energy computations on the free enzyme and those of the enzyme-cofactor complexes reveal that the free enzyme has a higher potential energy compared to that of

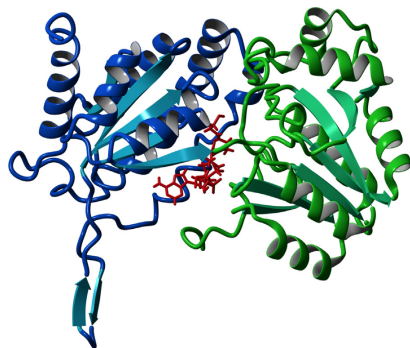


Fig. 3. Proposed structural model of PduP based on homology modeling by CPH models. The structure is based on chain A of the probable aldehyde dehydrogenase of *Listeria monocytogenes* (EgdE). The structure contains two domains (blue and green), each containing a typical Rossmann-fold. The proposed binding site of HS-CoA (molecule in red) in a pocket between the two domains can be seen in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the two enzyme-cofactor-complexes. This indicates the system's preference of complexed enzyme over free enzyme.

Although kinetic parameters of CoA-dependent aldehyde dehydrogenases with both HS-CoA and NAD⁺ have been reported [30,31], there is far less information available regarding the structural interaction between aldehyde dehydrogenases and its two co-factors. Lei et al. [32] were the first to report a shared binding site for HS-CoA and NAD⁺ in the CoA-dependent aldehyde dehydrogenase (acetaldehyde dehydrogenase, DmpG/DmpF) based on hydrogen-deuterium exchange experiments, complementing the structural characterization of the enzyme performed by Manjasetty et al. [33]. A recent study on the crystal structure of an archaeal malonyl-CoA reductase organized in dimers of two dimers in a homotetramer, revealed that the monomers had a binding site shared by its co-factors NADP⁺ and HS-CoA [34]. The shared binding site observed in the two separate CoA-dependent enzymes concur with the proposed mechanism of catalysis of a methylmalonate semialdehyde dehydrogenase, another member of the

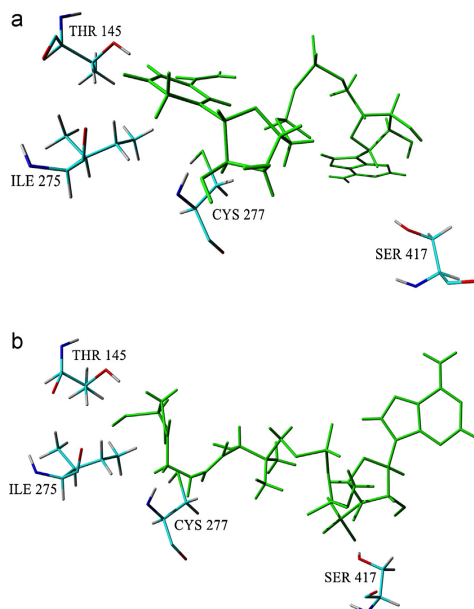


Fig. 4. Proposed binding site of (a) NAD⁺ and (b) HS-CoA, and amino acids involved in the catalysis as well as stabilization of the co-factors. Both co-factors, in green, bind to the same site in the enzyme. The ADP-moiety of both co-factors are in the vicinity of Ser417. Thr145, Ile275 and Cys277 surround the NMN-moiety of NAD⁺ and the thiol-group of HS-CoA. The amino acids in the figure are at a distance of 3 Å or less from the co-factors.

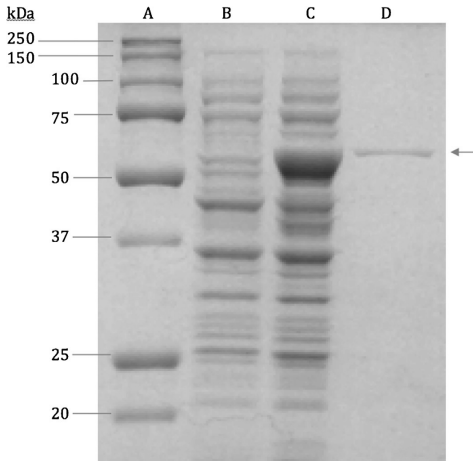


Fig. 5. SDS-PAGE analysis of the crude extracts of strains *E. coli* BTpET28b (B), BTpduP-02 (C), and purified protein product (D). Standard protein ladder is seen in lane A.

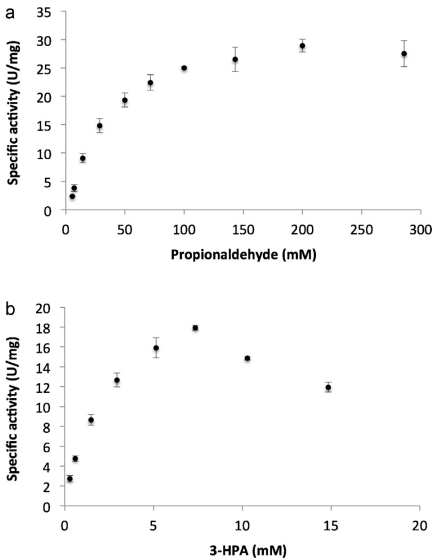


Fig. 6. Specific activity of PduP at 30 °C in 50 mM potassium phosphate buffer pH 7.0 with varying concentrations of (a) propionaldehyde, and (b) 3-HPA. Other components in the reactions were as follows: 5 mM NAD⁺, 0.43 mM HS-CoA, 1 mM DTT and 0.1 mg/mL BSA.

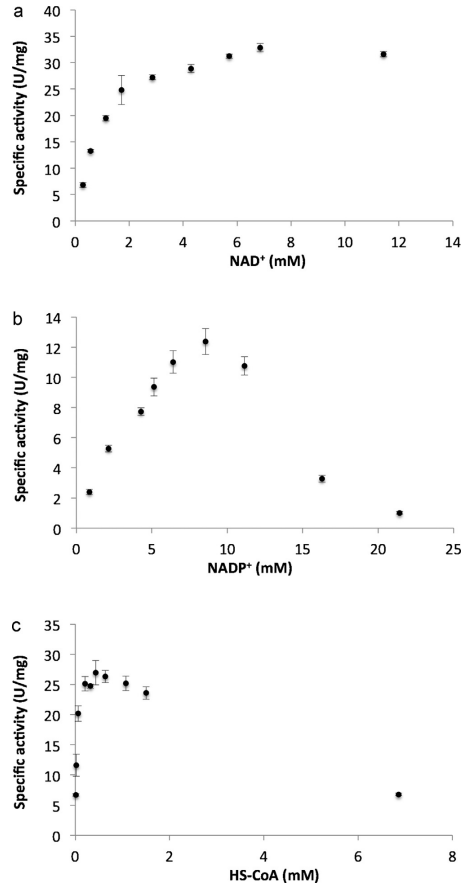


Fig. 7. Specific activity of PduP with varying concentrations of (a) NAD⁺, (b) NADP⁺, and (c) HS-CoA, respectively, at 30 °C in 50 mM potassium phosphate buffer, pH 7.0. Other components in the reactions were as follows: 200 mM propionaldehyde, 0.43 mM HS-CoA (a and b), 5 mM NAD⁺ (c), 1 mM DTT and 0.1 mg/mL BSA.

CoA-dependent aldehyde dehydrogenase subfamily [31]. The catalytic mechanism involves a nucleophilic attack by the sulfur of the catalytic cysteine residue of the enzyme on the carbonyl carbon of the substrate, followed by a hydride transfer, release of NADH, binding of the sulfur of $-S-CoA$ to this same carbonyl carbon of the substrate intermediate, and finally release of the product-CoA complex. While the structures of these CoA-dependent dehydrogenases are not homologous to that of PduP, at the very least this proposed mechanism indicates that the co-factors need to bind to the same domain.

Alignment of the amino acid sequence of PduP with CoA-dependent aldehyde dehydrogenases from *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Clostridium beijerinckii* and *Salmonella typhimurium* shows a number of highly conserved amino acids. The alignment results in combination with the *in silico* docking

and identification of residues at the co-factor binding site of PduP (Fig. 4) gives the possibility to make a structural proposal with a higher degree of confidence.

The highly conserved amino acids Gly242, Ala243 and the Gly244 and Pro245 of a GXGXP-motif, a motif known to interact with the adenosine phosphate (ADP)-moiety of NADH [35], are found to interact with the ADP-moiety of both NAD⁺ and HS-CoA (data not shown). A cysteine residue (Cys277) is equally conserved and in the proposed structure seen in close proximity to the thiol-group of HS-CoA and the NMN-moiety of NAD⁺. Cys277 is proposed as a residue involved in the binding of substrate and heavily involved in the catalytic mechanism of the enzyme, as explained in the mechanism of CoA-dependent aldehyde dehydrogenases above. A serine (Ser417) is adjacent to the C2 hydroxyl oxygen of the ribose in the ADP-moiety of NAD⁺, as reported for Ser12 of acetaldehyde dehydrogenase DmpG/DmpF by Lei et al. [32] where the oxygen of a hydroxyl group of the NAD⁺ ribose forms a hydrogen bond with the hydroxyl group of the serine side chain. An isoleucine (Ile275) is seen to be involved in a van der Waals interaction with the nicotinamide ring of NAD⁺ and is in agreement with the study of Lei et al. [32] where Ile15 stabilized the diphosphate of NAD⁺ and HS-CoA and forms a van der Waals-interaction with the nicotinamide-ring of NAD⁺. The presence of the bulky and hydrophobic Ile275 of PduP at this site indicates a function for properly positioning the nicotinamide ring of NAD⁺ for efficient hydride transfer. The co-factors are oriented such that the sulfur of HS-CoA and the nicotinamide mononucleotide-moiety of NAD⁺ are stabilized between Cys277, Ile275 and Thr145. Pailot et al. [36] showed that the Thr244 in glyceraldehyde-3-phosphate (G3P) dehydrogenase from *Streptococcus mutans* was necessary for an efficient acylation. Thr145 in PduP is proposed to correspond to Thr244 of G3P dehydrogenase based on the configuration and position of the amino acid in the model and its conservation throughout the CoA-dependent aldehyde dehydrogenases.

While the template for homology modeling showed only 30% similarity with PduP and has as of yet not been proven to act as an CoA-acylating aldehyde dehydrogenase, we believe that the proposal for the structure of PduP and mainly its interactions with the two co-factors presented in this study are supported by the limited amount of structural characterization that has been performed for CoA-dependent aldehyde dehydrogenase as well as the high degree of conservation of the proposed catalytic residues throughout a number of enzymes of the same family. However, the crystal structure of PduP should be determined for a complete verification of this model.

Heterologous expression and kinetic characterization of *L. reuteri* PduP in *E. coli*, as reported by Luo et al. [15], indicated that the specific activity of the purified protein with propionaldehyde was as low as 0.294 U/mg. Such low activity could be due to the shift in the codon reading frame due to a faulty forward primer design; an additional ATGC is introduced by the forward primer just upstream from the native start codon of PduP, resulting in an erroneous reading frame commencing with ATG GAT GCA (native start codon in bold). While we did not achieve expression of PduP by the method of Luo et al. [15], by site-directed mutagenesis and correction of the codon reading frame we could express PduP that exhibited significantly higher specific activity, 57 U/mg at 70 °C. With propionaldehyde as substrate, the recombinant enzyme showed Michaelis–Menten behavior with K_m of 28 mM and V_{max} of 28.9 U/mg.

PduP used both NAD⁺ and NADP⁺ as cofactors; the activity with the former was three times higher. Moreover, substrate inhibition was observed with NADP⁺ as well as with HS-CoA (Fig. 7b and c). Inhibition of a CoA-acylating aldehyde dehydrogenase by HS-CoA has been reported earlier and was found to be due to the formation of a dead-end enzyme–CoA complex [29]. An activity of 18 U/mg

with 3-HPA as the substrate was obtained before substrate inhibition was observed, although not as drastic as in the case of NADP⁺ or HS-CoA inhibition.

To further support the proposal of structure and co-factor binding sites, replacement of C277, S417, T145 and I275 with alanine was conducted, thereby obtaining four recombinant variants of the PduP-enzyme. All mutant enzymes were expressed as soluble proteins. During purification, retention times and chromatographic profiles for the four mutants were similar to that of non-mutated PduP. These results indicate with a high probability that the enzymes did not undergo structural misfolding. While the mutants C277A, T145A and I275A-mutants exhibited no detectable activity, S417A did show activity with a two-fold increase in K_m and 3.5-fold decrease in k_{cat}/K_m for NAD⁺ (Table 2). With HS-CoA, inhibition above a concentration of 0.5 mM was still observed, resulting in a maximum specific activity of 16.8 U/mg. Cys277 is essential for catalysis as explained above. With the T145A and I275A-mutations, it seems as though the stabilization of the co-factors is critically disrupted. The higher K_m and lower k_{cat}/K_m values for S417A-PduP with NAD⁺ suggest that Ser417 is involved in the stabilization or the binding of NAD⁺. These results further support the proposal of the critical residues in the co-factor binding site.

In this study, we achieved the highest specific activity reported up to date of *L. reuteri* PduP with 3-HPA as substrate. Furthermore, a structure is proposed for PduP and a shared co-factor binding site based on homology modeling, *in silico docking*, identification of conserved catalytic residues throughout a number of enzymes from the same family and alanine screening of these same residues. PduP is just one of the three enzymes, others being phosphotransacylase (PduL) and propionate kinase (PduW), that in a cascade reaction would convert 3-HPA to 3-HP in an ATP-producing pathway. These enzymes are currently under investigation for their role in 3-HP-production by *L. reuteri*. The verification of the ATP-producing pathway in *L. reuteri* might result in the ability to construct a viable process for the production of 3-HP, either natively in *L. reuteri* or as a heterologous pathway in a host strain such as *Escherichia coli*.

Acknowledgement

The Swedish Governmental Agency for Innovation Systems (Vinnova) is acknowledged for financial support.

References

- [1] Paster M, Pellegrino JL, Carole TM. Industrial bioproducts; Today and Tomorrow. US DOE Report, June; 2003. p. 48–9.
- [2] Willke T, Vorlop KD. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. Appl Microbiol Biotechnol 2004;66:131–42.
- [3] Jang YS, Kim B, Shin JH, Choi YJ, Choi S, Song CW, et al. Bio-based production of C2–C6 platform chemicals. Biotechnol Bioeng 2012;109:2437–59.
- [4] Cie A, Schlarp R, Lantz S, Tzakas M. Renewable acrylic acid. Department of Chemical and Biomolecular Engineering Senior Design Reports. University of Pennsylvania; 2012, available at <http://repository.upenn.edu/cbe.sdr/37> [last accessed 3.12.12].
- [5] Raj SM, Rathnasingh C, Jo JE, Park S. Production of 3-hydroxypropionic acid from glycerol by a novel recombinant *Escherichia coli* BL21 strain. Process Biochem 2008;43:1440–6.
- [6] Rathnasingh C, Raj SM, Lee Y, Catherine C, Ashok S, Park S. Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *Escherichia coli* strains. J Biotechnol 2011;157:633–40.
- [7] Andreesen B, Lange AB, Robenek H, Steinbuechel A. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. Appl Environ Microbiol 2010;76:622–6.
- [8] Morita H, Toh H, Fukuda S, Horikawa H, Oshima K, Suzuki T, et al. Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. DNA Res 2008;15:151–61.
- [9] Garai-Ibabe G, Ibarburu I, Berregi I, Claissse O, Lonvaud-Funel A, Irastorza A, et al. Glycerol metabolism and bitterness producing lactic acid bacteria in cidermaking. Int J Food Microbiol 2008;121:253–61.

- [10] Walter D, Ailion M, Roth J. Genetic characterization of the pdu operon: use of 1,2-propanediol in *Salmonella typhimurium*. J Bacteriol 1997;179:1013–22.
- [11] Luo LH, Kim CH, Heo SY, Oh BR, Hong WK, Kim S, et al. Production of 3-hydroxypropionic acid through propionaldehyde dehydrogenase PduP mediated biosynthetic pathway in *Klebsiella pneumoniae*. Bioresour Technol 2012;103:1–6.
- [12] Sauvageot N, Muller C, Hartke A, Auffray Y, Laplace JM. Characterisation of the diol dehydratase pdu operon of *Lactobacillus collinoides*. FEMS Microbiol Lett 2002;209:69–74.
- [13] Krauter H, Willke T, Vorlop KD. Production of high amounts of 3-hydroxypropionaldehyde from glycerol by *Lactobacillus reuteri* with strongly increased biocatalyst lifetime and productivity. N Biotechnol 2012;29:211–7.
- [14] Yasuda S, Mukoyama M, Horikawa H, Toraya T, Morita H. Process for producing 1,3-propanediol and/or 3-hydroxypropionic acid. U.S. Patent 2007/0148749 A1.
- [15] Luo LH, Seo JW, Baek JO, Oh BR, Heo SY, Hong WK, et al. Identification and characterization of the propanediol utilization protein PduP of *Lactobacillus reuteri* for 3-hydroxypropionic acid production from glycerol. Appl Microbiol Biotechnol 2011;89:697–703.
- [16] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web based environment for protein structure homology modelling. Bioinformatics 2006;22:195–201.
- [17] Benkert P, Biasini M, Schwede T. Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics 2011;27(3):343–50.
- [18] Krieger E, Koraimann G, Vriend G. Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing force field. Proteins 2002;47:393–402.
- [19] Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, et al. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J Comput Chem 1998;19:1639–62.
- [20] Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM, Ferguson DM, et al. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. J Am Chem Soc 1996;117:5179–97.
- [21] van Gunsteren WF, Billeter SR, Eising AA, Hünenberger PH, Krüger P, Mark AE, et al. Biomolecular simulation: the GROMOS96 manual and user guide. Zurich, Switzerland: Vdf Hachschulverlag ETHZ; 1996.
- [22] Horwitz JP, Chua J, Curby RJ, Tomson AJ, Darooq MA, Fisher BE, et al. Substrates for cytochemical demonstration of enzyme activity. I. Some substituted 3-indolyl-beta-D-glycopyranosides. J Med Chem 1964;7:574–5.
- [23] Davies J, Jacob F. Genetic mapping of the regulator and operator genes of the lac operon. J Mol Biol 1968;36:413–7.
- [24] Leal NA, Havemann GD, Bobik TA. PduP is a coenzyme A-acylating propionaldehyde dehydrogenase associated with the polyhedral bodies involved in B12-dependent 1,2-propanediol degradation by *Salmonella enterica* serovar typhimurium LT2. Arch Microbiol 2003;180:353–61.
- [25] Luthi-Peng Q, Scharer S, Puhan Z. Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*. Appl Microbiol Biotechnol 2002;60:73–80.
- [26] Sardari RR, Dishisha T, Pyo SH, Hatti-Kaul R. Improved production of 3-hydroxypropionaldehyde by complex formation with bisulfite during biotransformation of glycerol. Biotechnol Bioeng 2013;110:1243–8.
- [27] Rütli DP, Lacroix C, Jeremic T, Mathis M, Die A, Vollenweider S. Development of a reversible binding process for *in situ* removal of 3-hydroxypropionaldehyde during biotechnological conversion of glycerol. Biochem Eng J 2011;55:176–84.
- [28] Cirlé SJ, Stone L, Boruff CS. Acrolein determination by means of tryptophan: a colorimetric micromethod. Eng Chem Anal 1945;17:259–62.
- [29] Toth J, Ismaiel AA, Chen JS. The ald gene, encoding a coenzyme A-acylating aldehyde dehydrogenase, distinguishes *Clostridium beijerinckii* and two other solvent-producing clostridia from *Clostridium acetobutylicum*. Appl Environ Microbiol 1999;65:4973–80.
- [30] Smith LT, Kaplan NO. Purification, properties, and kinetic mechanism of coenzyme A-linked aldehyde dehydrogenase from *Clostridium kluyveri*. Arch Biochem Biophys 1980;203:663–75.
- [31] Talfournier F, Stines-Chaumeil C, Branlant G. Methylmalonate-semialdehyde dehydrogenase from *Bacillus subtilis*: substrate specificity and coenzyme A binding. J Biol Chem 2011;286:21971–81.
- [32] Lei Y, Pawelek PD, Powlowski J. A shared binding site for NAD⁺ and coenzyme A in an acetaldehyde dehydrogenase involved in bacterial degradation of aromatic compounds. Biochemistry 2008;47:6870–82.
- [33] Manjasetty BA, Powlowski J, Vrielink A. Crystal structure of a bifunctional aldolase-dehydrogenase: sequestering a reactive and volatile intermediate. Proc Natl Acad Sci USA 2003;100:6992–7.
- [34] Demmer U, Warkentin E, Srivastava A, Kockelkorn D, Pötter D, Marx A, et al. Structural basis for a bispecific NAD⁺ and CoA binding site in an archaeal malonyl-coenzyme A reductase. J Biol Chem 2013;288:6363–70.
- [35] Correll CC, Ludwig ML, Bruns CM, Karplus PA. Structural prototypes for an extended family of flavoprotein reductases: comparison of phthalate dioxygenase reductase with ferredoxin reductase and ferredoxin. Protein Sci 1993;2:2112–33.
- [36] Pailot A, D'Ambrosio K, Corbier C, Talfournier F, Branlant G. Invariant Thr244 is essential for the efficient acylation step of the non-phosphorylating glyceroldehyde-3-phosphate dehydrogenase from *Streptococcus mutans*. Biochem J 2006;400:521–30.

Paper II



Production of 3-hydroxypropionic acid from 3-hydroxypropionaldehyde by recombinant *Escherichia coli* co-expressing *Lactobacillus reuteri* propanediol utilization enzymes



Ramin Sabet-Azad*, Roya R.R. Sardari, Javier A. Linares-Pastén, Rajni Hatti-Kaul

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

HIGHLIGHTS

- Co-expression of PduP, PduL and PduW from *Lactobacillus reuteri* in *E. coli*.
- Production of 3-HP from 3-hydroxypropionaldehyde with yield of 1 mol/mol.
- Two-step process using *L. reuteri* and *rE. coli* for 3-HP production from glycerol.

ARTICLE INFO

Article history:

Received 3 September 2014
Received in revised form 30 December 2014
Accepted 31 December 2014
Available online 7 January 2015

Keywords:

Propanediol utilization pathway
Lactobacillus reuteri
3-Hydroxypropionic acid
Two-step biotransformation
Recombinant *Escherichia coli*

ABSTRACT

3-Hydroxypropionic acid (3-HP) is an important platform chemical for the biobased chemical industry. *Lactobacillus reuteri* produces 3-HP from glycerol via 3-hydroxypropionaldehyde (3-HPA) through a CoA-dependent propanediol utilization (Pdu) pathway. This study was performed to verify and evaluate the pathway comprising propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL), and propionate kinase (PduW) for formation of 3-HP from 3-HPA. The pathway was confirmed using recombinant *Escherichia coli* co-expressing PduP, PduL and PduW of *L. reuteri* DSM 20016 and mutants lacking expression of either enzyme. Growing and resting cells of the recombinant strain produced 3-HP with a yield of 0.3 mol/mol and 1 mol/mol, respectively, from 3-HPA. 3-HP was the sole product with resting cells, while growing cells produced 1,3-propanediol as co-product. 3-HP production from glycerol was achieved with a yield of 0.68 mol/mol by feeding recombinant *E. coli* with 3-HPA produced by *L. reuteri* and recovered using bisulfite-functionalized resin.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

3-Hydroxypropionic acid (3-HP), a C3 chemical, has gained tremendous interest in recent years as a building block for several industrially significant chemicals (Paster et al., 2003). The potential to further transform the chemical to obtain value added products such as 1,3-propanediol (1,3-PD), malonic acid, methyl acrylate and foremost bioacrylic acid has galvanized groups around the world with the aim of reaching industrially significant titers and yields of the product from renewable sources such as glucose or glycerol (Andreeßen et al., 2010; Rathnasingh et al., 2009). The abundance of glycerol due to its formation as a by-product from biodiesel (Li et al., 2013) and fatty acid production, makes it an attractive raw material for the chemical industry.

Lactobacillus reuteri, a facultative anaerobe, has been shown to natively produce 3-HP and 1,3-PD in equimolar amounts from glycerol (Dishisha et al., 2014), most likely through the same enzymatic pathway as that for the production of propionic acid and n-propanol from 1,2-propanediol (Sriramulu et al., 2008). The conversion of glycerol is initiated by glycerol dehydratase catalyzed dehydration to 3-hydroxypropionaldehyde (3-HPA) (Morita et al., 2008; Talarico and Dobrogosz, 1990), which is followed on one hand by an ATP-producing oxidative pathway involving CoA-acylating propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW) leading to 3-HP production (Fig. 1) (Yasuda et al., 2007), while a reductive pathway converts 3-HPA to 1,3-PD, simultaneously regenerating NAD⁺ essential for PduP-activity.

The genes encoding PduP, PduL and PduW are present on the *pdu*-operon of a number of microorganisms. PduP, PduL and PduW of *Salmonella enterica* have been characterized using propionaldehyde as substrate (Leal et al., 2003; Liu et al., 2007; Palacios

* Corresponding author. Tel.: +46 46 222 34 26; fax: +46 46 222 47 13.
E-mail address: ramin.sabet_azad@biotek.lu.se (R. Sabet-Azad).

et al., 2003). *S. enterica* PduP was employed in an engineered pathway together with cofactor B12-independent glycerol dehydratase of *Clostridium butyricum* and polyhydroxyalkanoate (PHA) synthase of *Ralstonia eutropha* for the production of poly(3-hydroxypropionic acid) (Poly-3-HP) from glycerol in *Escherichia coli* with a polymer yield of 11.98% per cell dry weight (wt/wt) in a two-step fed-batch fermentation (Andreeßen et al., 2010). In a recent study, an improved yield of poly-3-HP of 46.4% (wt/wt cell dry weight) was achieved using a similar system but with the glycerol dehydratase from *Klebsiella pneumoniae* and an aerobic fed batch fermentation (Wang et al., 2013).

According to a previous study, deletion of native *pduP* in *K. pneumoniae* led to a decrease in final titer of 3-HP from glycerol. The deletion of *pduP* was however possibly compensated by one or more aldehyde dehydrogenases natively present in the organism (Luo et al., 2012). We have earlier reported characterization of recombinant PduP from *L. reuteri*; the enzyme exhibited a specific activity of 18 U/mg at 3-HPA concentration of 7 mM, above which substrate inhibition occurs (Sabet-Azad et al., 2013). The enzyme preferred NAD⁺ as cofactor than NADP⁺. In a separate study, production of 3-HPA from glycerol using resting cells of *L. reuteri* was studied and improved by *in situ* complexation with bisulfite (Sardari et al., 2013a) and subsequent harvesting of the complex on an ion exchange resin (Sardari et al., 2013b). A recent report from our laboratory on flux analysis of the *L. reuteri* Pdu pathway has further shown that the rate of glycerol dehydration is 10 times faster than its subsequent reduction of oxidation of 3-HPA to 1,3-PD and 3-HP, respectively (Dishisha et al., 2014). Thus, for the production of 1,3-PD and 3-HP feeding rate of glycerol has to be kept low in order to prevent the accumulation of 3-HPA.

In the present study, *E. coli* cells engineered with PduP, PduL and PduW from *L. reuteri* DSM 20016, and mutants with only two of the above enzymes in each, were developed to verify and evaluate the pathway for the formation of 3-HP from 3-HPA as substrate. Bioconversions using growing as well as resting cells were performed. The possibility of production of 3-HP from glycerol in a two-step process, involving 3-HPA production by *L. reuteri* promoted by *in situ* complexation with bisulfite (Sardari et al., 2013a), and conversion of 3-HPA to 3-HP by the recombinant *E. coli*, was studied.

2. Methods

2.1. Strains and media

Competent *E. coli* BL21(DE3) was purchased from Novagen. *E. coli* cultivations were made in lysogeny broth (LB)-medium, on LB-agar or in M9 minimal medium (20 g/L glucose, 2 mM MgSO₄, 0.1 mM CaCl₂, 6.8 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl) supplemented with 20 µg/mL kanamycin (Sigma–Aldrich).

2.2. Cloning, expression and purification of recombinant proteins

A construct of pCOLADuet-1 (Novagen) containing the three genes *pduP*, *pduL* and *pduW*, each gene under the control of an

individual T7-promotor, was synthesized by GenScript (Piscataway, USA) and ainoated pCOLADuet:pduP:pduL:pduW.

Individual disruptions of *pduP*, *pduL* and *pduW* were made through the introduction of a stop codon five codons downstream from the start codon of each gene (indicated in bold in primer sequences below) through site directed mutagenesis employing Pfu DNA polymerase. Forward mutagenic primers for the disruption of *pduP* (dPduP-FOR: 5'-ATG GGT ATG CAG ATT **TAA** GAT ATT GAA AGT GC-3'), *pduL* (dPduL-FOR: 5'-ATG GAT GAA GAA CAT **TAA** AGA ACA CTT ATC C-3') and *pduW* (dPduW-FOR 5'-ATG TCA AAA AAA ATA **TAA** GCA ATT AAT TCT GG-3) with template pCOLADuet:pduP:pduL:pduW were employed in a PCR of 1 min initial denaturation at 95 °C followed by 25 cycles of 1 min denaturation at 95 °C, 1 min of annealing at 55 °C and 16 min elongation at 72 °C. A final elongation step was performed at 72 °C for 15 min. Degradation of methylated template DNA was achieved by restriction endonuclease DpnI. Reverse primers were complementary to the forward primers.

Constructs were introduced in the competent *E. coli* BL21(DE3) through thermal shock transformation. *E. coli* BL21(DE3) containing pCOLADuet-1 was developed as a control strain. Construct sequences were confirmed through nucleotide sequencing (GATC Biotech, Germany).

Production of the enzymes was performed in LB-medium under aerobic conditions at 30 °C and 200 rpm. *E. coli* strains were cultivated up to an OD₆₀₀ of 0.6–0.8, after which protein expression was induced by the addition of 1 mM IPTG and subsequent incubation for 4 h. The cells were harvested and lysed with BugBuster Protein Extraction Reagent and Lysogene Bioprocessing Reagent (Novagen) according to product manual. Soluble and insoluble fractions were separated by centrifugation for 20 min at 23,300×g and 4 °C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on soluble and insoluble fractions on gels containing 10% (w/v) acrylamide.

Production and purification of functional recombinant PduP was performed as previously described (Sabet-Azad et al., 2013). Construction of pET28b-PduL, production and purification of PduL was also performed in an identical manner, except for utilizing primers *pduL*-For (5'-CCATGGATGGATGAAGAACATTAAAGAAC-ACTT-3') and *pduL*-Rev (5'-CTCCAGGTTTTCTTCTTGATAA-TAACTTTTCCG-3) for the amplification of *pduL* by PCR.

2.3. Disruption of *ackA* and *yqhD* in *E. coli*

Acetate kinase (*ackA*, NCBI gene ID: 8181271) and alcohol dehydrogenase (*yqhD*, NCBI gene ID: 8180496) of *E. coli* BL21(DE3) were identified through the National Center for Biotechnology Information (NCBI). Disruption of *ackA* and *yqhD* were performed by using targeted group II introns (TargetTron® Gene Knockout System, Sigma–Aldrich). For *ackA*, primers IBS-ac (5'-AAAAAGCT-TATAATTATCCTTACAGTTCCTTCTACGTGCCGCCAGATAGGGTG-3'), EBS1d-ac(5'-CAGATTGTACAATGTGGTATAACAGATAAGTCTTCT-ACCGTAACTTACCCTTCTTGT-3') and EBS2-ac(5'-TGAACGCAAG-TTCTAATTTCCGGTAACTGTCGATAGAGGAAAGTGTCT-3') while for *yqhD*, primers IBS-yq(5'-AAAAAGCTTATAATTATCCTTACAAC-CCTGCTGTGCCGCCAGATAGGGTG-3'), EBS1d-yq(5'-CAGATTGT-

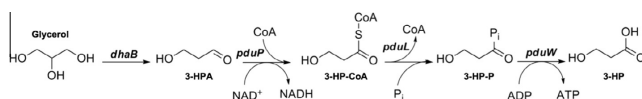


Fig. 1. Proposed pathway for 3-HP production from glycerol by *L. reuteri*. The pathway is initiated by dehydration of glycerol to 3-HPA catalyzed by cofactor B12-dependent glycerol dehydratase. 3-HPA is subsequently metabolized to 3-HP in an oxidative pathway involving three consecutive steps catalyzed by PduP, PduL and PduW. While PduP requires HS-CoA and NAD⁺ as cofactors, the HS-CoA is regenerated in the subsequent reaction catalyzed by PduL in the presence of P_i, while in the last step catalyzed by PduW, the phosphate is transferred to ADP to generate ATP besides 3-HP.

CAATGTGGTGATAACAGATAAGTCTGGCTATTAACCTACCTTTCTT-TGT-3') and EBS2-yq(5'-TGAACGCAAGTTCTCAATTCGGTTGTT-GTCGATAGAGAAAGTGCT-3') were employed for retargeting the introns by PCR with initial denaturation at 94 °C for 30 s followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, with a final elongation at 72 °C for 2 min. Restriction of the PCR-fragments using restriction enzymes Hind III and BsrG I was followed by ligation of the fragments into pACD4K-C and transformation of competent *E. coli* BL21(DE3) with the constructs *dackA*-pACD4K-C and *dyqhD*-pACD4K-C, respectively. Cells were grown to OD₆₀₀ of ~0.2, after which intron expression and genomic insertion was performed by addition of 0.5 mM IPTG, thus obtaining genomically modified Kan^r strains *E. coli* BL21(DE3) *ΔackA* and *E. coli* BL21(DE3) *ΔyqhD*. Knockouts of *ackA* and *yqhD* were confirmed through colony-PCR.

2.4. Production of 3-HPA

3-HPA was prepared by fed-batch biotransformation of glycerol as described elsewhere (Sardari et al., 2013a). Freshly grown cells of *L. reuteri* (5 g cell dry weight) prepared in MRS medium for 8 h at 37 °C and centrifuged, were re-suspended in a 1 L solution containing 2 g glycerol at pH 5 in a 3 L bioreactor (Applikon, The Netherlands), and biotransformation was performed at 37 °C with stirring at 200 rpm and continuous bubbling with N₂ gas. After 1 h of batch biotransformation, an aqueous feeding solution containing 50 g/L glycerol was fed at a rate of 1 mL/min until 5 h. The cells were separated by centrifugation at 15,000 × g and 4 °C for 10 min, and the supernatant was used as a source of 3-HPA after filter sterilization utilizing a 0.2 μm polyethersulfone membrane (VWR), and is henceforth referred to as crude 3-HPA. Product concentrations when produced from crude 3-HPA were calculated by deducting the concentration of 3-HP and 1,3-PD present in the substrate.

2.5. Production of 3-HPA by complexation with bisulfite and recovery of complexed and free 3-HPA

Production of 3-HPA by fed-batch biotransformation of glycerol using *L. reuteri* cells, *in situ* complexation of 3-HPA with bisulfite and purification of a mixture of free and complexed 3-HPA through a column packed with bisulfite-functionalized Amberlite IRA-400 resin (Cl form) was done as described elsewhere (Sardari et al., 2013b). The 3 L Applikon bioreactor was coupled to a tangential flow microfiltration module (Pellicon XL, 0.45 μm, 50 cm² filtration area, Millipore, Bedford, MA, USA) and a column (0.5 × 53 cm) packed with 50.0 g (dry weight) resin.

Cells, grown in MRS-medium as described in Section 2.4, were resuspended in a 1 L solution containing 2 g glycerol and 1.1 g sodium bisulfite. After 1 h of batch biotransformation, a feeding solution containing 74 g/L glycerol and 42 g/L sodium bisulfite was fed to the bioreactor at a rate of 0.92 mL/min and the cell free permeate obtained after passing through the microfiltration unit was pumped upwards through the column packed with bisulfite-functionalized Amberlite IRA-400 and back to the bioreactor. After biotransformation, 3-HPA bound to the resin was eluted using 0.2 M sodium chloride as a mixture of 3-HPA-bisulfite complex and free 3-HPA at a molar ratio of 1:1.

2.6. Production of 3-HP from 3-HPA using clarified lysate of recombinant *E. coli*

The soluble protein fractions of *E. coli* pdu:P:L:W, pdu:dP:L:W, pdu:P:dL:W, pdu:P:L:dW and control pCOLADuet obtained after lysis of the cells (Section 2.2), were used directly for evaluating 3-HP production from crude 3-HPA prepared in Section 2.4.

Reactions of 0.5 mL were run in triplicates in eppendorf tubes containing 10 mM NAD⁺, 0.43 mM HS-CoA, 10 mM ADP, 1 mM DTT, 20 mM KCl, 0.1 mg/mL BSA and 7 mM (3.5 μmol) crude 3-HPA in 50 mM potassium phosphate buffer, pH 7.0. The reactions were initiated by the addition of 30 or 50 μL of the clarified *E. coli* lysate and incubated at 30 °C for 1 h.

3-HP production from 3-HPA was also analyzed for the *ΔackA* mutant under similar conditions as above but by initiating the reaction by addition of purified PduP and PduL to final concentrations of 10 mg/L along with 20 μL of the clarified lysates of *E. coli* BL21(DE3) *ΔackA* and native *E. coli* BL21(DE3) (as control), respectively.

2.7. Production of 3-HP from 3-HPA using whole cells of recombinant *E. coli*

E. coli pdu:P:L:W and control pCOLADuet were grown under aerobic conditions in 1 L baffled shake flasks in 100 mL M9-medium containing 20 g/L glucose. Cells were grown to an OD₆₀₀ of 0.6–0.8, after which protein expression was induced by the addition of 1 mM IPTG and the cultures incubated at 30 °C, 200 rpm for 15 h. One milliliter samples were removed for determining the relationship between cell dry weight and OD₆₀₀.

Bioconversion using growing cells was initiated by the addition of crude 3-HPA (5 mM, produced according to Section 2.4) to the medium. After complete consumption of 3-HPA, an additional 5 mM substrate was added. This procedure was repeated until the 3-HP concentration reached a plateau. The glucose concentration in the medium was continuously monitored.

For bioconversion using resting cells, *E. coli* cells were harvested, re-suspended in filter-sterilized 50 mM potassium phosphate buffer pH 7.0, to an OD₆₀₀ of 3.7 and incubated with 3-HPA at 30 °C, 200 rpm. Two parallel experiments were performed using 8 mM crude 3-HPA obtained by fed-batch cultivation of *L. reuteri* (from Section 2.4) and 14 mM of the preparation containing a mixture of free and bisulfite complexed 3-HPA (prepared in Section 2.5), respectively. An additional 8 mM (crude) or 14 mM (free and bisulfite complexed) 3-HPA was added in cases where the substrate was completely consumed.

For evaluating 1,3-PD production by the resting cells in the above experiment, the native *E. coli* BL21(DE3) and *yqhD*-deficient strain *E. coli* BL21(DE3) *ΔyqhD* were grown in M9-medium containing 20 g/L glucose in aerobic conditions at 30 °C for 15 h, after which crude 3-HPA was added to the culture broth to a final concentration of 4 mM followed by an additional 5 h of incubation. The cells were subsequently harvested and resuspended in filter-sterilized 50 mM potassium phosphate buffer pH 7.0. Crude 3-HPA to a final concentration of 4 mM was added to the resting cell suspensions. 3-HPA consumption and 1,3-PD formation was monitored with time during both growing and resting conditions.

2.8. Analyses of glucose, 3-HPA, 1,3-PD, 3-HP, bisulfite, and cell dry weight

Residual glucose was measured by the ACCU-CHEK Aviva system (Roche, Basel, Switzerland). 3-HPA concentration was determined using a modified colorimetric method based on the dehydration of 3-HPA to acrolein in the presence of HCl (37%), and subsequent complexation with DL-tryptophan, and measuring absorbance at 560 nm (Circle et al., 1945; Sabet-Azad et al., 2013). 1,3-PD, 3-HP and bisulfite were quantified by HPLC (Jasco) using an Aminex HPLX-87H column and detection using RI detector as reported previously (Sardari et al., 2013a).

For determination of cell dry weight, 1 mL of *E. coli* culture samples (Section 2.7), collected in 1.5 mL eppendorf tubes, were centrifuged at 16,000 × g for 10 min, after which the supernatant was

removed. The tubes were placed in a 100 °C oven for 6 h with open lids, after which their weight was determined. A correlation of 0.47 g cell dry weight/OD₆₀₀ was established.

3. Results and discussion

3.1. Identification, cloning and expression of *pduP*, *pduL* and *pduW* of *L. reuteri* DSM 20016 in *E. coli* BL21(DE3)

Formation of 3-HP by native cells of *L. reuteri* in the presence of glycerol is most likely due to the propanediol utilization pathway, encoded by the *pdu*-operon in the organism (Yasuda et al., 2007; Dishisha et al., 2014). This operon is present in a number of strains and its metabolic function has previously been reported (Bobik et al., 1999; Leal et al., 2003; Liu et al., 2007; Palacios et al., 2003) in *S. enterica*. *PduP* of *L. reuteri* DSM 20016 (NCBI: YP_001272311) was identified and characterized in a previous study (Sabet-Azad et al., 2013), where its affinity toward 3-HPA was confirmed. The phosphotransacylase *PduL* (NCBI: YP_001272316) encoded by 645 nucleotides and the putative acetate kinase *PduW* (NCBI: YP_001272309) encoded by 1185 nucleotides were identified through the National Center for Biotechnology Information (NCBI). All plasmid- and *E. coli* constructs developed as part of this study are summarized in Table 1. Protein expression analysis through SDS-PAGE of the cell extracts showed overexpression of *PduP*, *PduL* and *PduW* in *E. coli* *pdu*:P:L:W, seen as bands corresponding to the expected molecular masses of 51 kDa (*PduP*), 24 kDa (*PduL*) and 44 kDa (*PduW*) (Fig. 2). The strains containing disruptions in the sequences of *PduP*, *PduL* and *PduW*, respectively, were found to lack expression of the disrupted genes. No expression of *PduP*, *PduL* or *PduW* was observed in the control pCOLADuet strain.

3.2. Production of 3-HPA from glycerol by *L. reuteri*

A process for the direct conversion of glycerol to 3-HP by *E. coli* would demand the recombinant expression of glycerol dehydratase. This rather complex system, where up to 3 enzymes interact for activation and catalysis of glycerol to 3-HPA, is natively present in *Salmonella*- (Bobik et al., 1999), *Klebsiella*- (Wang et al., 2007) and *Lactobacillus* (Morita et al., 2008; Sauvageot et al., 2002; Talarico and Dobrogosz, 1990) species as cofactor B12-dependent. Its presence as a cofactor B12-independent system in *Clostridium butyricum* has also been reported, instead using S-adenosyl methionine for activation. However, its high oxygen sensitivity limits its usage in combination with enzymes such as *PduP*, *PduL* and *PduW* in *E. coli* (O'Brien et al., 2004). Furthermore, deletion of competing enzymes involved in the consumption of glycerol and natively present in *E. coli* would be necessary for a competitive yield and final titer of 3-HP (Tokuyama et al., 2014).

L. reuteri, on the other hand, produces 3-HPA efficiently and even tolerates relatively high concentrations of the hydroxyaldehyde as compared to other organisms (Cleusix et al., 2012). Fed-batch biotransformation of glycerol using *L. reuteri* cells resulted

in a product containing 3-HPA (50 mM), 3-HP (19.4 mM), 1,3-PD (19.4 mM) and residual glycerol (24 mM). No further conversion of glycerol was possible due to product inhibition.

In order to lower the inhibitory effect of 3-HPA and thus increase its final titer by *L. reuteri*, *in situ* recovery of 3-HPA is required. Although complexation of 3-HPA with semicarbazide or carbohydrazide is very efficient and leads to significant increase in 3-HPA production, the release of 3-HPA from the complex is problematic (Vollenweider and Lacroix, 2004). In comparison, complexation of 3-HPA with bisulfite is an equilibrium process, and the complex is easily dissociated making the free 3-HPA available for the reaction (Sardari et al., 2013b). Fed-batch production of 3-HPA was integrated with a reported method for 3-HPA separation on bisulfite functionalized resin (Sardari et al., 2013b). The product, consisting of a combined concentration of 74 mM free and bisulfite complexed 3-HPA, was obtained that was free of 1,3-PD and 3-HP.

3.3. Production of 3-HP from 3-HPA using clarified cell lysates

Transformation of crude 3-HPA (obtained by fed-batch biotransformation of glycerol as described in Section 2.4) to 3-HP was investigated using clarified cell lysates from the *E. coli* strain expressing functional *PduP*, *PduL* and *PduW* as well as mutants lacking functional expression of *PduP*, *PduL* or *PduW*. The crude 3-HPA solution was added to 0.5 mL of buffered solution of the clarified cell lysates to get the initial 3-HPA concentration of 7 mM. The samples were analyzed after 1 h of incubation. While no consumption of 3-HPA was observed by the strain lacking expression of functional *PduP*, consumption of the hydroxyaldehyde was observed for the mutants lacking *PduL* (0.7 μmol) and *PduW* (1.3 μmol), with production of 3-HP only in the latter case, equimolar to the amount of consumed 3-HPA (Fig. 3a). The highest production of 3-HP was observed by the cell lysate of the strain *pdu*:P:L:W, which was equimolar to the 3-HPA amount consumed (1.9 μmol). This was 1.5-fold higher than that of *pdu*:P:L:dW (Fig. 3a).

Propionate kinase *PduW* is a member of the acetate kinase superfamily and bears 40% similarity with *E. coli* native acetate kinase, *AckA* (NCBI: WP_001362987). It may seem that the lack of *PduW* in *pdu*:P:L:dW is compensated by *AckA*, resulting in 3-HP production by the mutant. To verify the role of *AckA*, the gene encoding the enzyme in the native *E. coli* was disrupted to give a mutant *E. coli* BL21(DE3) *ΔackA*, and 3-HP production from 3-HPA by the clarified cell lysate of the mutant *ΔackA* and the unmodified strain *E. coli* BL21(DE3) in the presence of pure recombinant *PduP* and *PduL* was examined. 3-HP was formed to an equimolar amount of consumed 3-HPA (0.5 μmol) by the unmodified strain, while no 3-HP production was observed by the *ΔackA* strain although 3-HPA was consumed (Fig. 3b). These results confirm the role of *AckA* as a stand-in for *PduW* as well as the role of *PduP*, *PduL* and *PduW* in *L. reuteri* in 3-HP production from 3-HPA, which is formed by dehydration of glycerol by the organism.

Table 1
Plasmid constructs and strains developed for this study.

Strain	Construct	Property
<i>E. coli</i> pCOLADuet	pCOLADuet-1	Control strain, non-modified plasmid in <i>E. coli</i> BL21(DE3)
<i>E. coli</i> <i>pdu</i> :P:L:W	pCOLADuet: <i>pduP</i> : <i>pduL</i> : <i>pduW</i>	Expression of <i>PduP</i> , <i>PduL</i> and <i>PduW</i> , each under a distinct T7-promoter, in <i>E. coli</i> BL21(DE3)
<i>E. coli</i> <i>pdu</i> :dP:L:W	pCOLADuet: <i>dpduP</i> : <i>pduL</i> : <i>pduW</i>	<i>E. coli</i> <i>pdu</i> :P:L:W with disruption of <i>pduP</i>
<i>E. coli</i> <i>pdu</i> :P:dL:W	pCOLADuet: <i>pduP</i> : <i>dpduL</i> : <i>pduW</i>	<i>E. coli</i> <i>pdu</i> :P:L:W with disruption of <i>pduL</i>
<i>E. coli</i> <i>pdu</i> :P:L:dW	pCOLADuet: <i>pduP</i> : <i>pduL</i> : <i>dpduW</i>	<i>E. coli</i> <i>pdu</i> :P:L:W with disruption of <i>pduW</i>
<i>E. coli</i> BL21(DE3) <i>ΔackA</i>	–	<i>E. coli</i> BL21(DE3) with disrupted <i>ackA</i>
<i>E. coli</i> BL21(DE3) <i>ΔyqhD</i>	–	<i>E. coli</i> BL21(DE3) with disrupted <i>yqhD</i>

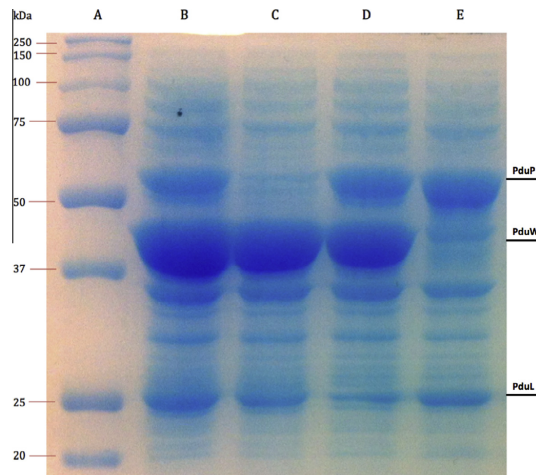


Fig. 2. SDS-PAGE analysis on 10% acrylamide gels of *E. coli* pdu:P:L:W (B), pdu:d:P:L:W (C), pdu:P:dL:W (D) and pdu:P:L:dW (E), with standard protein ladder in lane A.

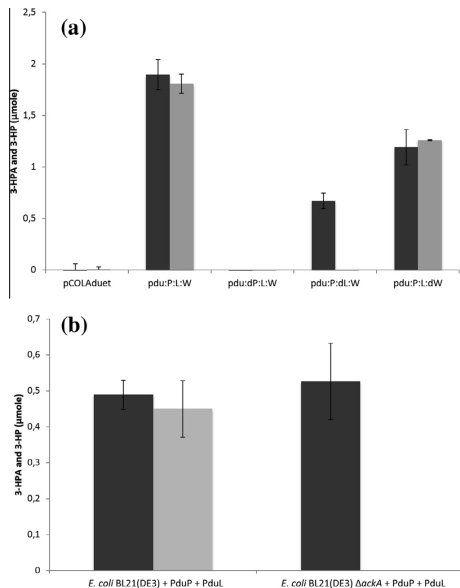


Fig. 3. End point results of the consumption of 3-HPA (■) and production of 3-HP (□) by (a) clarified cell lysates of strains pCOLADuet, pdu:P:L:W, mutant strains lacking functional expression of PduP, PduL or PduW, and (b) *E. coli* BL21(DE3) and *E. coli* BL21(DE3) ΔackA. To 0.5 mL reaction mixture containing 50 mM potassium phosphate buffered system, pH 7.0, 10 mM NAD⁺, 0.43 mM HS-CoA, 10 mM ADP, 1 mM DTT, 20 mM KCl, 0.1 mg/mL BSA and 7 mM crude 3-HPA was added 30 or 50 μl of the clarified cell lysates (and 10 mg/L purified PduP and PduL in case of (b)). The reaction mixture was incubated at 30 °C for 1 h.

3.4. Whole cell production of 3-HP from crude 3-HPA by recombinant *E. coli*

Both growing and resting cells of *E. coli* pdu:P:L:W and control strain pCOLADuet were used for the conversion of crude 3-HPA to 3-HP.

Addition of 5 mM 3-HPA to the growing *E. coli* strains pdu:P:L:W (Fig. 4a) and pCOLADuet (Fig. 4b) resulted in its complete consumption by both the strains in about 5 h. While the pCOLADuet strain did not produce 3-HP, production of the hydroxyacid was observed in the strain overexpressing PduP, PduL and PduW, up to 4 mM after which the production reached a plateau, with a specific production rate of 0.4 mmol g⁻¹ CDW h⁻¹ (36 mg g⁻¹ CDW h⁻¹). Formation of 1,3-PD by both the strains was observed; the rate of production by pCOLADuet was however twice that of pdu:P:L:W. On repeated additions of 3-HPA, total consumption by pdu:P:L:W and pCOLADuet was achieved, until the third addition where consumption of 3-HPA by the control strain leveled off. Combined yields of 3-HP and 1,3-PD were on average 0.5 mol per mol 3-HPA in the early, mid and late phase. The final yield of 3-HP by pdu:P:L:W in these conditions was 0.3 mol/mol 3-HPA. An increasing amount of as yet unknown compound was also detected by HPLC analysis of the product from both pdu:P:L:W and control strains. Work is currently underway for its identification.

The native 1,3-PD producing ability of *E. coli* has been previously reported and ascribed to the presence of an *E. coli* native alcohol dehydrogenase/1,3-PD oxidoreductase (YqhD) (Jarboe, 2011). This was confirmed by disrupting the *yqhD* gene and studying the effect on 1,3-PD formation from 3-HPA by the growing cells of *E. coli* BL21(DE3) ΔyqhD and control *E. coli* BL21(DE3). While 1,3-PD was formed to a yield of 0.5 mol/mol 3-HPA by the native strain, no 1,3-PD formation was observed by the ΔyqhD strain.

For bioconversion using resting cells of *E. coli* pdu:P:L:W, 8 mM crude 3-HPA used was completely consumed in about 4 h in the first batch. 3-HP productivity of 1 mmol g⁻¹ CDW h⁻¹ (90 mg g⁻¹ CDW h⁻¹) was observed, until the production of 3-HP and consumption of 3-HPA leveled off after approximately 8 h, following

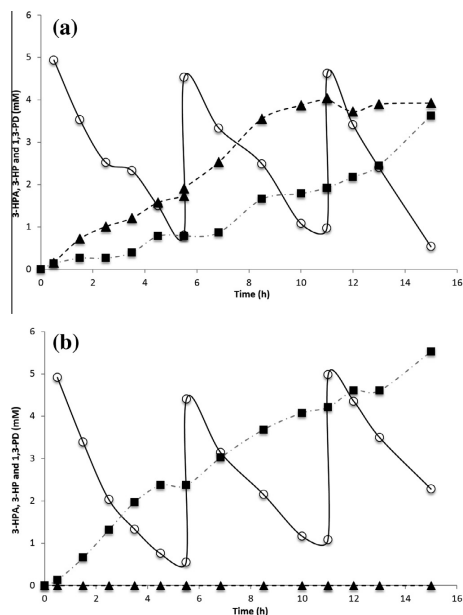


Fig. 4. Profiles of the bioconversion of 3-HPA (○) to 3-HP (▲) and 1,3-PD (■) by growing cells of (a) *E. coli* strain pdu:P:L:W and (b) control pCOLADuet. The bioconversion was conducted at 30 °C in 1 L baffled shake flasks containing 100 mL M9-medium (20 g/L glucose, 2 mM MgSO₄, 0.1 mM CaCl₂, 6.8 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl and 20 µg/mL kanamycin). The experiment was initiated by the addition of 5 mM 3-HPA, which was then repeatedly added to the medium following its consumption by the cells.

repeated addition of crude 3-HPA, giving a final titer of 12 mM 3-HP and yield of 1 mol 3-HP per mol 3-HPA (Fig. 5). No side-product was detected. In contrast, no consumption of 3-HPA or production of 3-HP was observed by the resting cells of the control strain pCOLADuet. The lack of 1,3-PD production by the resting cells, in contrast to that by the growing cells, might be due to addition of 3-HPA under conditions when cells are not stressed and lacking *yqhD* expression. Expression of *YqhD* by *E. coli* would require an activation of *yqhD* through oxidative stress, such as the presence of reactive aldehydes (Pérez et al., 2008). In order to confirm this hypothesis, 3-HPA was added to cultures of *E. coli* BL21(DE3) in growing and resting conditions. As seen in Fig. 6, 1,3-PD production was observed in both growing and resting conditions, thereby explaining the lack of 1,3-PD when adding 3-HPA solely in resting conditions.

While growing cells spend effort in the metabolism of a carbon source for energy and cofactor generation, this behavior is lacking in the resting cells, where a limited pool of cofactors can be applied for specific purposes. Formation of by-products employing resting cells can thus be minimized, leading to higher yields and ideally higher productivities. For the growing-as well resting cell system, the maximum titers were reached after ca 10 h. NAD⁺-regeneration in *L. reuteri* occurs through the production of 1,3-PD by NADH-dependent 1,3-PD oxidoreductase (Dishisha et al., 2014). When utilizing recombinant *E. coli* in resting conditions, the process will

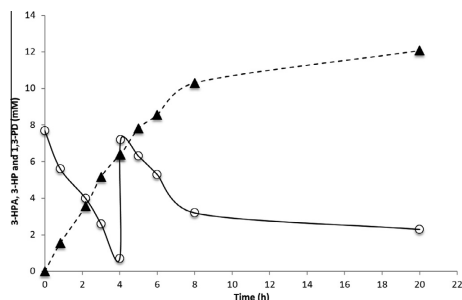


Fig. 5. Profile of the bioconversion of 3-HPA (○) to 3-HP (▲) by resting cells of *E. coli* pdu:P:L:W. The bioconversion was conducted at 30 °C in 1 L baffled shake flasks containing 100 mL 50 mM potassium phosphate buffer, pH 7.0. The reaction was commenced by the addition of 8 mM 3-HPA. The substrate was repeatedly added after its complete consumption was noted in the reaction.

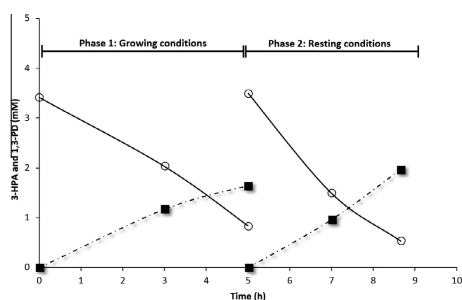


Fig. 6. Profile of the bioconversion of 3-HPA (○) to 1,3-PD (■) by *E. coli* BL21(DE3) in growing and resting conditions. 3-HPA (4 mM) was added to the growing cells (Phase 1) in 1 L baffled shake flasks containing 100 mL M9-medium (20 g/L glucose, 2 mM MgSO₄, 0.1 mM CaCl₂, 6.8 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl and 1 g/L NH₄Cl). Cells were harvested through centrifugation and resuspended in 100 mL 50 mM potassium phosphate buffer, pH 7.0, after which 4 mM 3-HPA was added to the resting cells (Phase 2).

eventually stop with the depletion of NAD⁺ unless a cofactor regeneration system is integrated. The Pdu-pathway leads to the production of ATP, ideally keeping the cells active for a longer period of time even in the resting condition where no carbon-source is present for the generation of energy.

Finally, 3-HPA (14 mM) comprising a 1:1 mixture of free 3-HPA and bisulfite complexed 3-HPA (Sardari et al., 2013b), was used as a substrate for conversion by the resting cells of recombinant *E. coli* pdu:P:L:W, which led to the production of 3-HP with a final titer of 12 mM, yield of 0.85 mol 3-HP/mol 3-HPA but a lowered specific productivity of 0.4 mM g⁻¹ CDW h⁻¹ (36 mg g⁻¹ CDW h⁻¹).

An extensive review on biological production of 3-HP has been performed by Kumar et al. (2013), which presents the results of the various production strategies reported in literature. Table 2 complements these results with strategies published in more recent years. The main focus in the presented strategies is engineering of *E. coli* or *K. pneumoniae*, by direct genomic modulation and/or through expression of native and heterologous enzymes, for the synthesis and over-expression of 3-HP producing pathways. The highest titer (57.3 g/L), yield (0.86 mol per mol glycerol) and

Table 2
Comparison of 3-HP production in this study with recent systems reported in literature.

Strain	Mode of cultivation	Shake flask/ bioreactor	Condition	Titer (g/L)	Average yield (mol 3-HP/mol glycerol)	Productivity (g/L/h)	References
<i>L. reuteri</i> DSM 20016/ <i>E. coli</i> pdu:P:L:W	Two-step process (fed-batch/fed-batch)	3 L bioreactor/ 1 L shake flask	Anaerobic (200 rpm)/aerobic (200 rpm)	1.1	0.68	0.06	This study
<i>E. coli</i> SPC005 (<i>dhaB</i> ¹ <i>gdrAB</i> ² <i>aldH</i> ³ Δ <i>ackA-pta</i> ⁴ Δ <i>yqhD</i> ⁵ Δ <i>glpK</i> ⁶)	Batch	250 mL shake flask	Aerobic (250 rpm)	16.3	0.28	0.34	Jung et al. (2014)
<i>E. coli</i> SPC005 (<i>dhaB</i> ¹ <i>gdrAB</i> ² <i>aldH</i> ³ Δ <i>ackA-pta</i> ⁴ Δ <i>yqhD</i> ⁵ Δ <i>glpK</i> ⁶)	Fed-batch	5 L bioreactor	Aerobic (250 rpm, 1.0 vvm air)	40.5	0.26	1.26	Jung et al. (2014)
<i>E. coli</i> W (<i>dhaB</i> ¹ <i>gdrAB</i> ² <i>KCSADH</i> ⁷)	Batch	250 mL shake flask	Aerobic (250 rpm)	2.7	0.31	–	Sankaranarayanan et al. (2014)
<i>E. coli</i> W (<i>dhaB</i> ¹ <i>gdrAB</i> ² <i>KCSADH</i> ⁷)	Batch/fed-batch	1.5 L bioreactor	Aerobic (650 rpm, 0.5 vvm air)	42.3	0.31	0.86	Sankaranarayanan et al. (2014)
<i>K. pneumoniae</i> Kp(pET-pk- <i>ald</i> ⁸)	Batch	250 mL shake flask	Microaerobic (150 rpm)	0.89	0.03	0.04	Li et al. (2014)
<i>K. pneumoniae</i> Kp(pET-pk- <i>ald</i> ⁸)	Fed-batch	5 L bioreactor	Aerobic (400 rpm, 1.5 vvm air)	3.0	0.03	0.14	Li et al. (2014)
<i>Pseudomonas denitrificans</i> (<i>dhaB</i> ¹ <i>gdrAB</i> ² <i>puuC</i> ⁹)	Batch	250 mL shake flask	Aerobic (200 rpm)	4.9	0.68	0.13	Zhou et al. (2013)
<i>E. coli</i> BL21star(DE3) (<i>dhaB</i> ¹⁰ <i>dhaR</i> ¹¹ <i>PSALDH</i> ¹² Δ <i>glpK</i> ¹³ Δ <i>yqhD</i> ⁵)	Fed-batch	2.5 L bioreactor	Aerobic (1200– 1300 rpm, 1 vvm air)	57.3	0.86	1.59	Kim et al. (2014)

¹ *K. pneumoniae* coenzyme B12-dependent glycerol dehydratase.

² *K. pneumoniae* glycerol dehydratase reactivase.

³ *E. coli* aldehyde dehydrogenase.

⁴ Acetate kinase A-phosphotransacylase.

⁵ Aldehyde reductase.

⁶ Glycerol pathway repressor.

⁷ *E. coli* alpha-ketoglutaric semialdehyde dehydrogenase.

⁸ *Pseudomonas* sp. NAD⁺-independent aldehyde oxidase.

⁹ *K. pneumoniae* aldehyde dehydrogenase.

¹⁰ *L. brevis* glycerol dehydratase.

¹¹ *L. brevis* glycerol dehydratase reactivase.

¹² *Pseudomonas aeruginosa* semialdehyde dehydrogenase.

¹³ Glycerol kinase.

productivity (1.59 g/L/h) achieved to date was through genomic modulation of glycerol metabolism and 1,3-PD production, and recombinant expression of B12-dependent glycerol dehydratase and its reactivating enzyme (DhaB-DhaR) from *Lactobacillus brevis* and semialdehyde dehydrogenase (PSALDH) from *Pseudomonas aeruginosa* in *E. coli* BL21star(DE3) (Kim et al., 2014).

In the present study, while the yield of 3-HPA from glycerol reached 0.68 mol/mol by *L. reuteri*, a yield of 1 mol 3-HP/mol 3-HPA was reached utilizing resting cells of *E. coli*. The yield, titer as well as productivity are comparable to various production systems studied in shake flask conditions, e.g. those involving expression of aldehyde dehydrogenase in *E. coli* (Raj et al., 2008) and NAD⁺-regeneration by aldehyde oxidase in *K. pneumoniae* (Li et al., 2014) for conversion of glycerol to 3-HP, and the construction of a malonyl-CoA pathway in *E. coli* (Rathnasingh et al., 2012) for 3-HP production from glucose. The low productivity in this study, in comparison with a number of widely studied pathways (Kumar et al., 2013) (Table 2), is a consequence of 3-HPA inhibition and slower kinetics of 3-HPA transformation in comparison to its formation (Dishisha et al., 2014), and even in comparison to the alternative pathway involving aldehyde dehydrogenase catalyzed oxidation of 3-HPA to 3-HP. Over-expression of these enzymes for overcoming 3-HPA inhibition, coupled to co-factor regeneration, is expected to increase productivity and final titer of 3-HP. Using a buffered aqueous system without any expensive co-factors such as coenzyme B12 for biotransformation using resting cells can provide an advantage in terms of lower costs including simple downstream processing for obtaining pure 3-HP due to absence of side-products.

4. Conclusions

This study introduces and confirms the propanediol utilization pathway of *L. reuteri*, expressed in *E. coli*, for the production of 3-HP from 3-HPA. Resting cells of the recombinant strain convert 3-HPA quantitatively and at a higher rate to 3-HP in contrast to growing cells, which produce 1,3-PD along with 3-HP. Currently, a cofactor-regeneration system is being engineered in *E. coli* pdu:P:L:W to allow continuous production of 3-HP over a longer period. A further strategy is to develop a production system based on one microbial host with an optimized pathway with *L. reuteri* enzymes for production of 3-HP from glycerol.

Acknowledgement

This work was a part of BioVinn project (Project No. 2008-00849) financially supported by the Swedish Governmental Agency for Innovation Systems (Vinnova).

References

- Andreeßen, B., Lange, A.B., Robenek, H., Steinbüchel, A., 2010. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. *Environ. Microbiol.* 76, 622–626.
- Bobik, T.A., Havemann, G.D., Busch, R.J., Williams, D.S., Aldrich, H.C., 1999. The propanediol utilization (*pdu*) operon of *Salmonella enterica* Serovar Typhimurium LT2 includes genes necessary for formation of polyhedral organelles involved in coenzyme B12-dependent 1,2-propanediol degradation. *J. Bacteriol.* 181, 5967–5975.
- Circle, S.J., Stone, L., Boruff, C.S., 1945. Acrolein determination by means of tryptophan. *Ind. Eng. Chem.* 17, 259–262.

- Cleusix, V., Lacroix, C., Vollenweider, S., Duboux, M., Le Blay, G., 2012. Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. *BMC Microbiol.* 7, 101.
- Dishisha, T., Pereyra, L.P., Pyo, S.H., Britton, R.A., Hatti-Kaul, R., 2014. Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol. *Microb. Cell Fact.* 13, 76.
- Jarboe, L.R., 2011. A broad-substrate range aldehyde reductase with various applications in production of bio-renewable fuels and chemicals. *Appl. Microbiol. Biotechnol.* 89, 249–257.
- Jung, W.S., Kang, J.H., Chu, H.S., Choi, I.S., Cho, K.M., 2014. Elevated production of 3-hydroxypropionic acid by metabolic engineering of the glycerol metabolism in *Escherichia coli*. *Metab. Eng.* 23, 116–122.
- Kim, K., Kim, S.U., Park, Y.-C., Seo, J.H., 2014. Enhanced production of 3-hydroxypropionic acid from glycerol by the modulation of glycerol metabolism in recombinant *Escherichia coli*. *Bioresour. Technol.* 156, 170–175.
- Kumar, V., Ashok, S., Park, S., 2013. Recent advances in biological production of 3-hydroxypropionic acid. *Biotechnol. Adv.* 31, 945–961.
- Leal, N.A., Havemann, G.D., Bobik, T.A., 2003. PduP is a coenzyme-A-acylating propionaldehyde dehydrogenase associated with the polyhedral bodies involved in B12-dependent 1,2-propanediol degradation by *Salmonella enterica* serovar Typhimurium LT2. *Arch. Microbiol.* 180, 353–361.
- Li, C., Keaton, L., Liu, H., 2013. Microbial conversion of waste glycerol from biodiesel production into value-added products. *Energies* 6, 4739–4768.
- Li, Y., Liu, L., Tian, P., 2014. NAD⁺-independent aldehyde oxidase catalyzed cofactor balanced 3-hydroxypropionic acid production in *Klebsiella pneumoniae*. *Biotechnol. Lett.* 36, 2215–2221.
- Liu, Y., Leal, N.A., Sampson, E.M., Johnson, C.L.V., Havemann, G.D., Bobik, T.A., 2007. PduL is an evolutionary distinct phosphotransacylase involved in B12-dependent 1,2-propanediol degradation by *Salmonella enterica* Serovar Typhimurium LT2. *J. Bacteriol.* 189, 1588–1596.
- Luo, L.H., Kim, C.H., Heo, S.Y., Oh, B.R., Wong, W.K., Kim, S., Kim, D.H., Seo, J.W., 2012. Production of 3-hydroxypropionic acid through propionaldehyde dehydrogenase PduP mediated biosynthetic pathway in *Klebsiella pneumoniae*. *Bioresour. Technol.* 103, 1–6.
- Morita, H., Toh, H., Fukuda, S., Horikawa, H., Oshima, K., Suzuki, T., Murukami, M., Hisamatsu, S., Kato, Y., Takizawa, T., Fukuoka, H., Yoshimura, T., Itoh, K., O'Sullivan, D.J., McKay, L.L., Ohno, H., Kikuchi, J., Masaoka, T., Hattori, M., 2008. Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. *DNA Res.* 15, 151–161.
- O'Brien, J.R., Raynaud, C., Croux, C., Girbal, L., Soucaille, P., Lanzilotta, W.N., 2004. Insight into the mechanism of the B12-independent glycerol dehydratase from *Clostridium butyricum*: preliminary biochemical and structural characterization. *Biochemistry-US* 43, 4635–4645.
- Palacios, S., Starai, V.J., Escalante-Semerenca, J.C., 2003. Propionyl coenzyme A is a common intermediate in the 1,2-propanediol and propionate catabolic pathways needed for expression of the *prpBCDE* operon during growth of *Salmonella enterica* on 1,2-propanediol. *J. Bacteriol.* 185, 2802–2810.
- Paster, M., Pellegrino, J.L., Carole, T.M., 2003. Industrial bioproducts: today and tomorrow. US DOE Report. June, 48–49.
- Pérez, J.M., Arenas, F.A., Pradenas, G.A., Sandoval, J.M., Vázquez, C.C., 2008. *Escherichia coli* YqhD exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. *J. Biol. Chem.* 283, 7346–7353.
- Raj, S.M., Rathnasingh, C., Jo, J.E., Park, S., 2008. Production of 3-hydroxypropionic acid from glycerol by a novel recombinant *Escherichia coli* BL21(DE3) strain. *Process Biochem.* 43, 1440–1446.
- Rathnasingh, C., Raj, S.M., Jo, J.E., Park, S., 2009. Development and evaluation of efficient recombinant *Escherichia coli* strains for the production of 3-hydroxypropionic acid from glycerol. *Biotechnol. Bioeng.* 104 (4), 729–739.
- Rathnasingh, C., Raj, S.M., Lee, Y., Catherine, C., Ashok, S., Park, S., 2012. Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *Escherichia coli* strains. *J. Biotechnol.* 157, 633–640.
- Sabet-Azad, R., Linares-Pastén, J.A., Torkelson, L., Sardari, R.R.R., Hatti-Kaul, R., 2013. Coenzyme A-acylating propionaldehyde dehydrogenase (PduP) from *Lactobacillus reuteri*: kinetic characterization and molecular modeling. *Enzyme Microb. Technol.* 53, 235–242.
- Sankaranarayanan, M., Ashok, S., Park, S., 2014. Production of 3-hydroxypropionic acid from glycerol by acid tolerant *Escherichia coli*. *J. Ind. Microbiol. Biotechnol.* 41, 1039–1050.
- Sardari, R.R.R., Dishisha, T., Pyo, S.H., Hatti-Kaul, R., 2013a. Improved production of 3-hydroxypropionaldehyde by complex formation with bisulfite during biotransformation of glycerol. *Biotechnol. Bioeng.* 110, 1243–1248.
- Sardari, R.R.R., Dishisha, T., Pyo, S.H., Hatti-Kaul, R., 2013b. Biotransformation of glycerol to 3-hydroxypropionaldehyde: improved production by *in situ* complexation with bisulfite in a fed-batch mode and separation on anion exchanger. *J. Biotechnol.* 168, 534–542.
- Sauvageot, N., Muller, C., Hartke, A., Auffray, Y., Laplace, J.M., 2002. Characterisation of the diol dehydratase pdu operon of *Lactobacillus collinoides*. *FEMS Microbiol. Lett.* 209, 69–74.
- Sriramulu, D.D., Liang, M., Hernandez-Romero, D., Raux-Deery, E., Lünsdorf, H., Parsons, J.B., Warren, M.J., Prentice, M.B., 2008. *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportion. *J. Bacteriol.* 190, 4559–4567.
- Talarico, T.L., Dobrogosz, W.J., 1990. Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. *Appl. Environ. Microb.* 56, 1195–1197.
- Tokuyama, K., Ohno, S., Yoshikawa, K., Hirasawa, T., Tanaka, S., Furusawa, C., Shimizu, H., 2014. Increased 3-hydroxypropionic acid production from glycerol, by modification of central metabolism in *Escherichia coli*. *Microb. Cell Fact.* 13, 64.
- Vollenweider, S., Lacroix, C., 2004. 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. *Appl. Microbiol. Biotechnol.* 64, 16–27.
- Wang, F., Qu, H., Tian, P., Tai, T., 2007. Heterologous expression and characterization of recombinant glycerol dehydratase from *Klebsiella pneumoniae* in *Escherichia coli*. *Biotechnol. J.* 2, 736–742.
- Wang, Q., Yang, P., Liu, C., Xue, Y., Xian, M., Zhao, G., 2013. Biosynthesis of poly(3-hydroxypropionate) from glycerol by recombinant *Escherichia coli*. *Bioresour. Technol.* 131, 548–551.
- Yasuda, S., Mukoyama, M., Horikawa, H., Toraya, T., 2007. Process for producing 1,3-propanediol and/or 3-hydroxypropionic acid. US Patent US20070148749A1.
- Zhou, S., Catherine, C., Rathnasingh, C., Somasunder, A., Park, S., 2013. Production of 3-hydroxypropionic acid from glycerol by recombinant *Pseudomonas denitrificans*. *Biotechnol. Bioeng.* 110, 3177–3187.

Paper III

Cofactor regeneration enhances the production of 3-hydroxypropionic acid from 3-hydroxypropionaldehyde: Integrating *Lactobacillus reuteri* NADH oxidase and propanediol-utilization pathway enzymes in *Escherichia coli*

Ramin Sabet-Azad^{a,}, Tarek Dishisha^{a,b}, Victor Arieta^a, Rajni Hatti-Kaul^a*

^a *Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*

^b *Department of Microbiology and Immunology, Faculty of Pharmacy, Beni Suef University, Beni Suef, Egypt*

*Corresponding author

Telephone: +46 – 46 222 34 26

Telefax: +46 – 46 222 47 13

E-mail: Ramin.Sabet_Azad@biotek.lu.se

Abstract

Lactobacillus reuteri, a probiotic lactic acid bacterium, has a glycerol metabolic pathway through which it is converted to 3-hydroxypropionaldehyde (3-HPA), 3-hydroxypropionic acid (3-HP) and 1,3-propanediol (1,3-PD). In our earlier studies, we have expressed the genes coding for propionaldehyde dehydrogenase, phosphotransacylase and propionate kinase in *E. coli* for conversion of 3-HPA to 3-HP with accumulation of 1,3-PD as a by-product. In order to enhance the 3-HP yield from 3-HPA, the gene encoding NADH oxidase (Nox), an enzyme that utilizes molecular oxygen as substrate with formation of water as product was isolated from *L. reuteri* and heterologously overexpressed in *Escherichia coli*. As a result, the 3-HP production using recombinant strain was enhanced using intermittent fed-batch mode of operation.

Keywords

NADH oxidase; Cofactor regeneration; 3-Hydroxypropionic acid; 3-Hydroxypropionaldehyde; *Lactobacillus reuteri*; Fed-batch biotransformation

1. Introduction

Significant efforts are continuously being made around the world to move from the current fossil-based economy to a more sustainable economy based on renewable resources. In order to match the efficiency and flexibility of the petrochemical industry, the bio-based industry needs to develop a set of versatile building blocks, or platforms from which a range of products can be derived (Jong et al., 2012). Taking this into consideration, the Department of Energy, USA has identified 30 platform chemicals composed of 1-6 carbon atoms as potential candidates for bio-based production (Werpy et al., 2004). Polyols and organic acids constitute the majority of these chemicals; examples are glycerol, propionic acid and 3-hydroxypropionic acid (3-HP).

3-HP is a structural isomer of lactic acid (2-hydroxypropionic acid). The bi-functionalities, hydroxyl- and carboxyl groups, make it a versatile compound for organic synthesis (Della Pina et al., 2011; Mochizuki & Hirami, 1997; Zhang et al., 2004). 3-HP is an important precursor for acrylic acid (Della Pina et al., 2011) and can be incorporated as a cross-linking agent in coatings, lubricants and antistatic agents for textiles (Kumar et al., 2013). Other applications are expected in food industry, cosmetics and fertilizers (Banner et al., 2011). Currently, there is no commercial process for production of 3-HP, since most of the developed chemical production routes were not environmentally friendly and/or economically unfeasible (Della Pina et al., 2011; Kumar et al., 2013).

Biologically, 3-HP can be obtained in small quantities as an end product of glycerol and acrylic acid metabolism using few microorganisms (Kumar et al., 2013). Jiang et al., 2009 and Henry et al., 2010 have suggested different metabolic pathways for the production of 3-HP from glucose and glycerol (Henry et al., 2010; Jiang et al.,

2009). In order to convert glycerol to 3-HP, two different pathways have been described. The first step in these pathways is the selective dehydration of glycerol yielding 3-hydroxypropionaldehyde (3-HPA) in a reaction catalyzed by glycerol/diol dehydratase. The resulting aldehyde is then oxidized forming 3-HP through aldehyde dehydrogenase, or through the three-step cascade reaction catalyzed by propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL), and kinase (PduW) (Jiang et al., 2009). However, for achieving the redox balance other products are produced simultaneously such as 1,3-PD through 1,3-propanediol oxidoreductase.

The biocatalytic dehydration of glycerol and dismutation of the resulting 3-HPA was achieved yielding equimolar amounts of 1,3PDO and 3HP using resting cells of *Lactobacillus reuteri* (Dishisha et al., 2014). Hence a yield of 50 mol% from glycerol was obtained. In another study we have shown the feasibility for production of 3-HP from 3-HPA using recombinant *E. coli* strain harboring the three enzymes of the propanediol-utilization (pdu) pathway (PduP, L and W) (Sabet-Azad et al., 2015). In this case biotransformation using growing cells led to the accumulation of 1,3-PD in almost equimolar amounts to 3-HP caused by the native 1,3-PD oxidoreductase/alcohol dehydrogenase of *E. coli*, which also provided a means for regeneration of the NADH cofactor formed during the first enzymatic step of 3-HP-coenzyme A formation catalyzed by PduP. On the other hand when the recombinant *E. coli* cells were used in resting conditions, 3-HP was the main product. However, the reaction was stopped as a result of co-factor depletion. Regeneration of NAD^+ from NADH can prolong the biotransformation process and produce 3HP with high yield by resting cells.

Several dehydrogenases/oxidoreductases, such as lactate dehydrogenase and alcohol dehydrogenase, can be used for the generation of NAD^+ from NADH

(Wandrey, 2004). However, the requirement for an additional substrate and the production of an undesired product is a major limitation. On the other hand, the enzyme NADH oxidase (Nox), that utilizes molecular oxygen as substrate, overcomes the need for additional substrate. Several Nox enzymes are present, mostly catalyzing the conversion of O₂ to H₂O₂ with simultaneous NAD⁺ regeneration. Hydrogen peroxide can then be degraded via catalase-mediated reaction into water and oxygen (Higuchi et al., 1993; Kengen et al., 2003; Niimura et al., 2000; Rocha-Martin et al., 2011; Toomey & Mayhew, 1998).

A number of Nox have a unique ability to catalyze the conversion of molecular oxygen directly into water. These enzymes were characterized, purified and cloned from several lactobacilli strains including *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus sanfranciscensis* and others (Geueke et al., 2003; Higuchi et al., 1993; Riebel et al., 2003; Riebel et al., 2002; Zhang et al., 2012).

In the present study, homology of amino acid sequence was used to identify the presence of Nox in *L. reuteri*. The gene encoding the identified protein was amplified, cloned and expressed in *E. coli* BL21(DE3), and the protein purified and characterized. Subsequently, the gene coding for Nox was introduced into the *E. coli* cells bearing the *pduP*, *pduL* and *pduW* genes, to study the effect on production of 3HP from 3HPA (Fig. 1).

2. Materials and methods

2.1. Strains, plasmids and medium

Lactobacillus reuteri DSM 20016 was obtained from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ). Cloning host *E. coli* XL1-Blue and competent *E. coli* BL21(DE3) for enzyme expression were purchased

from Agilent Technologies. The propagation vector pUC19 was purchased from Thermo Scientific, while expression vector pET21a was purchased from Merck Millipore. *L. reuteri* was cultivated in deMan, Rogosa and Sharpe (MRS)-medium (Difco, BD). *Escherichia coli* was cultivated in lysogeny broth (LB)-medium, on LB-agar or in minimal medium M9 (20 g/L glucose, M9 salts, 2 mM MgSO₄, 0.1 mM CaCl₂). Kanamycin (20 µg/mL) and ampicillin (50 µg/mL) (Sigma-Aldrich) were supplemented when necessary for selective pressure. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and X-Gal (Thermo Scientific) were supplemented for blue white screening of transformed *E. coli* XL1-Blue.

2.2. Materials

Pfu DNA polymerase as well as restriction enzymes *Sma*I, *Bam*HI and *Xho*I were purchased from Thermo Scientific. T4 DNA ligase was obtained from New England Biolabs. Coenzyme A (HS-CoA), dithiothreitol (DTT), β-nicotinamide adenine dinucleotide (NAD⁺), β-nicotinamide adenine dinucleotide phosphate (NADP⁺) and adenosine diphosphate (ADP) were purchased from Sigma-Aldrich. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and X-Gal were purchased from Thermo Scientific.

2.3. Cloning and expression of recombinant proteins.

pCOLADuet:pduP:pduL:pduW was constructed as previously described (Sabet-Azad et al., 2015). *L. reuteri* DSM 20016 was grown overnight in aerobic conditions at 37 °C, followed by extraction of genomic DNA utilizing GeneJet Genomic DNA Extraction Kit (Fermentas).

NADH oxidase was amplified by PCR utilizing primers Nox-FOR (5'-ATATAGGATCCATGAAGGTTATTATTGTTGG-3') and Nox-REV (5'-

TATATACTCGAGTTTTTCTAATTCACGTTG-3') introducing *Bam*HI and *Xho*I restriction sites (in bold) and removing the native stop codon of the gene, with an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 3 min. The reaction was finalized with elongation at 72 °C for 10 min. The amplified gene was separated and purified through agarose gel electrophoresis (1% agarose). After digestion of cloning vector pUC19 with *Sma*I, blunt end ligation of linearized pUC19 and the PCR product was performed, thereby obtaining construct pUC19:nox. Thermal shock transformation of *E. coli* XL1-Blue with pUC19 was performed. White colonies, as opposed to blue colonies containing self-circularized or non-restricted pUC19, were isolated and insertion of PCR product in pUC19 verified through agarose gel electrophoresis. Sequential restriction of pUC19:nox and pET21a with *Bam*HI and *Xho*I, respectively, was followed by separation and purification of Nox and ligation of the gene with the linearized pET21a. The final construct, pET21a:nox, encodes Nox integrated with a hexa-histidine tag (his₆) on the C-terminus of the enzyme.

Thermal shock transformation of *E. coli* BL21(DE3) was performed with pET21a:nox, and pET21a:nox and pCOLADuet:pduP:pduL:pduW, respectively. *E. coli* BL21(DE3) strain containing pET21a:nox was designated BTnox, while strain containing pET21a:nox and pCOLADuet:pduP:pduL:pduW was designated BTnoxplw.

2.4. Expression of recombinant proteins and purification of recombinant Nox

E. coli BTnox and BTnoxplw were grown on LB-medium containing appropriate antibiotics in aerobic conditions at 30 °C, 200 rpm. IPTG was added to a final

concentration of 1 mM when cell cultures had reached OD_{620nm} of 0.4-0.6, followed by 4 h (BTnox) or 10 h (BTnoxplw) of additional incubation at 30 °C, 200 rpm.

Cells were harvested by centrifugation for 20 min at 23,300 x g, 4 °C. Bugbuster Protein Extraction Reagent and Lysonase Bioprocessing Reagent (Novagen) were utilized for cell lysis according to manufacturer's instructions. Soluble and insoluble protein fractions were separated by centrifugation as above.

The soluble fraction of BTnox was passed through a 1 mL HisTrap FF Crude column (GE Healthcare). The column was equilibrated using binding buffer (200 mM sodium phosphate, 0.5 mM NaCl, pH 7.4) and Nox-his₆ eluted with elution buffer (binding buffer and 500 mM imidazole), thereby obtaining pure Nox.

The soluble fractions of BTnox and BTnoxplw as well as pure Nox were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 12% (w/v) acrylamide.

2.5. Measurement of enzymatic activity

Kinetic characterization of Nox was based on the oxidation of substrate NAD(P)H which can be measured spectrophotometrically by its absorbance at 340 nm. Reactions were performed in triplicates in 50 mM potassium phosphate buffer pH 7, unless stated otherwise, containing 1 mM DTT with varying NAD(P)H concentrations. Soluble cell crude extracts of strain BTnox as well as purified Nox, respectively, were added for initiation of the reactions after 5 min of incubation of reaction mixtures at 30 °C. Kinetic properties of crude Nox were evaluated using 0-0.15 mM NADH or 0-0.1 mM NADPH. Pure Nox was evaluated using 0-0.15 mM NADH only. One Unit (U) of Nox activity corresponds to the formation of 1 μ mole $NAD(P)^+$ per min.

The effect of Tris-HCl-, HEPES- and potassium phosphate buffers, 50 mM pH 7, as well as that of pH, utilizing citric acid/phosphate buffer 50 mM (pH 4-7) and potassium phosphate buffer 50 mM (pH 6-8) was studied with 0.1 mM NADH as substrate.

2.6. Production of 3-HPA

3-HPA was produced by fed-batch fermentation of *L. reuteri* in MRS-medium, as previously described (Sabet-Azad et al., 2015; Sardari et al., 2013). The supernatant of the fermentation broth, containing a mixture of 50 mM 3-HPA, 19.4 mM 3-HP, 19.4 mM 1,3-PD and 24 mM residual glycerol, was filter sterilized through a 0.2 µm polyethersulfone membrane (VWR), and was used as a “crude” 3-HPA substrate for recombinant *E. coli* for transformation to 3-HP in shake flask experiments.

2.7. Preparation of lyophilized 3-HPA for bioreactor cultivations

L. reuteri cells were produced by inoculating 20 mL of MRS with 1% (v/v) *L. reuteri* stock culture in 20% w/v glycerol. After 17 h at 37 °C, 20 mL of this culture was added to another 200 mL of MRS medium containing 20 mM glycerol and incubated under the same conditions for 8 h. The cells were then harvested by centrifugation at 15,000 xg for 10 min and washed three times in sodium acetate buffer (50 mM, pH 5.0). The cell pellet was resuspended in a total volume of 50 mL of sterile glycerol solution (200 mM), and the biotransformation was allowed to proceed on a rocking table at 37 °C for 2 h. The supernatant was recovered after centrifugation at 8,000 xg for 10 min and filter-sterilized. Fifty milliliters of the solution was frozen to -80 °C and lyophilized. The viscous, light brown liquid was diluted in water to reach a 3-

HPA concentration of 1 M and then used for bioconversion studies. The other impurities were 388 mM 3-HP, 388 mM 1,3-PD and 480 mM glycerol.

2.8. Fed-batch production of 3-HP from 3-HPA by recombinant E. coli expressing PduP, PduL, PduW and Nox via intermittent feeding

Strains BTnoxplw and control strain with empty pET21a and pCOLADuet-1, respectively, were cultivated under aerobic conditions in 1 L baffled shake flasks containing 100 mL M9-medium supplemented with kanamycin and ampicillin. Cells were grown up to an OD₆₀₀ of 0.6, after which induction of protein expression was initiated through the addition of IPTG to a final concentration of 1 mM. The cultures were incubated at 30 °C for 15h, 200 rpm. Cells were harvested by centrifugation at 4 °C, 3900 x g for 20 minutes and resuspended to a final OD₆₀₀ of 3.5 in 100 mL citric acid/potassium phosphate buffer 50 mM at pH 4 or 5, or 100 mL potassium phosphoate buffer 50 mM at pH 6, 7 or 8. Crude 3-HPA was added to a final concentration of 4 mM to each cell suspension. Additional 3-HPA was added to a final concentration of 4 mM to the suspensions in those cases where the substrate was completely consumed. This procedure was repeated for almost 15 h.

2.9. Production of 3-HPA from 3-HP using recombinant E. coli expressing PduP, PduL, PduW and Nox via continuous feeding

E. coli strain BTnoxplw was cultivated in several 1L baffled shake flasks containing 100 mL LB-medium supplemented with kanamycin and ampicillin. Protein expression was induced when cells had reached OD 0.6-0.8, after which they were incubated at 30 °C at 200 rpm for 15h. Cells were harvested by centrifugation at 3,900 ×g for 20 min and resuspended in 100 mM potassium phosphate buffer, pH 7.

The cell suspensions were combined in a 1 L Biostat®-Q bioreactor (B. Braun Biotech International), reaching a final volume of 200 mL and final OD₆₀₀ of 11. Biotransformation was performed at 30 °C with a stirrer speed of 200 rpm and aeration of 0.5 vvm. Concentrated 3-HPA (1 M) was continuously added to the reactor at a flow rate of 0.015 mL/min, based on previously determined parameters for 3-HPA consumption in shake flask conditions (**Section 2.8**).

2.10. Analytical procedures

Analysis of 3-HPA was based on a previously developed method (Circle et al., 1945) with modifications. Two hundred microliter of a suitably diluted sample was mixed with 600 µl HCl for the dehydration of 3-HPA to acrolein. DL-tryptophan (150 µl) was added to the mixture, thereby obtaining an acrolein-chromophore complex which was quantified by absorbance at 560 nm on a spectrophotometer.

Glucose, 3-HP and 1,3-PD were analyzed by HPLC (Jasco) utilizing an Aminex HPX-87H column as described previously (Sardari et al., 2013).

E. coli culture samples (**Section 2.8**) of 1 mL were continuously removed for determining relationship between OD_{620nm} and cell dry weight (g/L); samples were collected in 1.5 mL eppendorf tubes. Centrifugation of samples at 16,000 x g for 10 min was followed by removal of supernatant, drying of cell pellet at 100 °C for 6 h in an oven and finally weighing the pellets.

3. Results and discussion

3.1. Identification of the *nox* gene of *L. reuteri* DSM 20016

The amino acid sequence of the characterized NADH oxidase of *Lactobacillus brevis* (NCBI: AF536177) was used as a template for sequence homology by BlastP (NCBI)

using the non-redundant protein sequence database and limiting search results to *Lactobacillus reuteri* DSM 20016. A FAD-dependent pyridine nucleotide-disulfide oxidoreductase was identified (Lreu_0067, NCBI: 5189747), covering 99% of sequence with an identity of 63%. The identified protein contains 449 amino acids with a predicted molecular weight of 49.6 KDa and a theoretical pI of 5.33 as determined using Protparam tools (<http://www.expasy.ch/tools/protparam.html>).

3.2. Homology model

Molecular modeling is an *in silico* simulation of the behaviour of molecules for evaluation of intra- and inter-molecular interactions. This technique has been widely used for understanding and improving enzymatic activity and selectivity through providing a solid background for choosing the proper protein engineering strategy. The amino acid sequence of *L. reuteri* FAD-dependent pyridine nucleotide-disulfide oxidoreductase was subjected to homology modeling by YASARA Structures Software using the crystal structure of water-forming NAD(P)H oxidase from *L. sanfranciscensis* (PDB: 2CDU, 451 residues with a crystal structure resolution of 1.80 Å) as a template. This enzyme gave the highest quality score of 0.622 (Hooft et al., 1996).

Sequence alignment done by YASARA showed that 442 of 449 target residues (98.4%) were aligned to template residues. Among these aligned residues, the sequence identity was 59.5% and the sequence similarity was 74.7%.

After the side-chains of the model had been built, optimized and fine-tuned, all newly modeled parts were subjected to a combined steepest descent and simulated annealing minimization. Then a full unrestrained simulated annealing minimization was run for the entire model reaching the final model (Fig. 2).

The enzyme consists of a FAD-binding domain, an ADP-binding domain and dimerization domain, with all three domains containing Rossmann folds.

3.3. Expression of recombinant enzymes and purification of Nox

Insertion of Nox in pET21a was verified by nucleotide sequencing. Expression of Nox by *E. coli* BTnox with subsequent enzyme purification as well as Nox, PduP, PduL and PduW by *E. coli* BTnoxplw was verified by SDS-PAGE (Fig. 3). An enzyme corresponding to the expected size of Nox-his₆ (~52 kDa) was produced by strain BTnox. This enzyme was not seen in control strain carrying empty pET21a. Purified Nox, obtained through immobilized metal ion affinity chromatography yielding 35% recovery, purification fold of 3.1 and specific activity of 16.9 U/mg, was seen as a single product of ~52 kDa. Strain BTnoxplw produced enzymes corresponding to the expected sizes of PduP (~55 kDa), PduL (~24 kDa), PduW (~44 kDa) and Nox (~52 kDa).

3.4. Kinetic characterization of Nox

Oxidation of NADH by Nox was evaluated in HEPES-, Tris-HCl- and potassium phosphate buffer (Fig. 4A). The highest activity was found in potassium phosphate buffer, while reactions in Tris-HCl and HEPES resulted in loss of activity by 20% and 32%, respectively.

The pH-profile of Nox was evaluated in citric acid/potassium phosphate buffer (pH 4-6.5) and potassium phosphate buffer (pH 6-8) (Fig. 4B). A pH optimum of 6 was observed, with decrease of activity in a linear manner below and above this condition. This result concurs with previous biochemical characterizations of water-forming NADH oxidases from a number of *Lactobacillus* species (Gueke et al.,

2003; Sudar et al., 2014; Zhang et al., 2012). Further characterization of Nox was however performed at pH 7, for determining the efficiency of co-expressing the enzyme with propanediol-utilization (pdu) enzymes PduP, PduL and PduW. PduP has previously been found to be most active at neutral pH (Sabet-Azad et al., 2013).

Kinetic characterization of Nox with NADH as substrate gave a V_{\max} value of 25.1 U/mg and K_m of 0.055 mM, while oxidation of NADPH showed 3.6 fold lower activity with a V_{\max} of 7.0 U/mg and K_m of 0.073 mM (Fig. 5). The low K_m -value indicates that oxygen might not be a limiting factor when applying Nox in an aerobic process. The specific activity of Nox furthermore indicates that conversion of 3-HPA to 3-HP-CoA by PduP should not be limited due to the depletion of NAD^+ if PduP is produced in equal or lower amounts than Nox, as the activity of PduP is slightly lower (18 U/mg) (Sabet-Azad et al., 2013) than that of Nox.

3.5. Fed-batch production of 3-HP from 3-HPA by recombinant E. coli expressing PduP, PduL, PduW and Nox via intermittent feeding

Shake flask cultivation of *E. coli* BTnoexplw resulted in a continuous production of 3-HP up to 22 mM (Fig. 6A), a titer twice that of recombinant *E. coli* expressing PduP, PduL and PduW (Sabet-Azad et al., 2015), and likely higher still. However, each addition of 3-HPA dilutes the buffered medium. A productivity of 0.6 mmole $\text{L}^{-1} \text{h}^{-1}$ was reached with a yield of 0.78 mol 3-HP per mol 3-HPA. These results confirm that regeneration of NAD^+ by NADH oxidase remedies a cofactor imbalance issue, as seen when utilizing recombinant *E. coli* expressing pdu pathway enzymes; PduP, PduL and PduW, where depletion of NAD^+ resulted in a low final titer of 12 mM 3-HP.

3.6. Production of 3-HPA from 3-HP using recombinant E. coli expressing PduP, PduL, PduW and Nox via continuous feeding

In order to reach high titers of 3-HPA, there is a need to obtain a highly concentrated substrate source. Crude 3-HPA was lyophilized and then resuspended in distilled water thereby obtaining a substrate solution containing 1 M 3-HPA. A fed-batch process was developed, in which 3-HPA was added to the buffered cell suspension of *E. coli* BTnoxp1w at a rate calculated for avoiding accumulation of 3-HPA. Co-production of 3-HP and 1,3-PD was observed in equimolar quantities up to the mid phase where productivity of 1,3-PD was slightly higher. A final titer of 67 mM 3-HP and 54 mM 1,3-PD was achieved (Fig. 6B). Combined yields of 3-HP and 1,3-PD was 1 mol/mol, while that of 3-HP at the end point analysis was 0.60 mol/mole with a specific productivity of 0.52 mmole 3-HP g⁻¹ CDW h⁻¹. Since a higher concentration of the biocatalyst was used, a higher final 3-HP productivity was obtained of 3.1 mmole L⁻¹ h⁻¹.

Two main reasons can account for the accumulation of 1,3-PD when resting cells are used. First, the production of 1,3-PD can be ascribed to the presence of residual glycerol at a concentration of 0.48 M in the crude substrate solution which can be used by the cells as a carbon source thereby perturbing the co-factor balance towards activation of 1,3-propanediol oxidoreductase. No accumulation of glycerol was observed in the cell suspension, indicating its consumption by the recombinant *E. coli* and possibly enabling the induction of YqhD. The second reason is using the continuous feeding strategy that ensures complete utilization and no accumulation of 3-HPA. It was expected that the low doses of 3-HPA would enhance the biocatalytic activity through alleviating the toxicity of 3-HPA on the biocatalyst which is more evident in intermittent feeding. It has previously been shown that 1,3-propanediol

oxidoreductase (YqhD), present natively in *E. coli*, is induced due to oxidative stress in the presence of reactive aldehydes like 3-HPA (Perez et al., 2008; Sabet-Azad et al., 2015). Possibly the better results obtained using intermittent feeding might be a result of accumulation of 3-HPA to relatively high concentration at each feeding pulse which might inhibit the expression or the activity of YqhD. This behavior was solely observed earlier with growing cells of *E. coli* and lacking for resting cells. (Sabet-Azad et al., 2015).

YqhD as well as NADH oxidase are NADH dependent and regenerate NAD^+ for PduP. A lower production of 1,3-PD would have been expected due to direct competition between NADH oxidase and YqhD for the present NADH. This is not evident until a later phase in the process; As 1,3-PD production levels off, the productivity of 3-HP increases, thus leading to a slightly higher yield of 3-HP.

The proposed process is mainly limited by the substrate; a higher final titer of 3-HP could have been expected if the substrate would have been available in higher amounts. Moreover, disruption of native *yqhD* in *E. coli* would be necessary for quantitative conversion of 3-HPA to 3-HP and a better utilization of NADH oxidase activity.

4. Conclusions

The current study shows the possibility of improving the process of 3-HP production by Pdu pathway by integrating cofactor regeneration using Nox. As 3-HPA is not an optimal substrate for the recombinant *E. coli*, a more scalable alternative would be to construct a one-step system by co-expression of glycerol dehydratase in the *E. coli* for the conversion of glycerol to 3-HPA. Cofactor B12-dependent glycerol dehydratase

from *L. reuteri* is presently being investigated for its co-expression with PduP, PduL, PduW and Nox in *E. coli*.

5. Acknowledgement

The authors are grateful to Vinnova (the Swedish Governmental Agency for Innovation Systems) for financial support to the project. The National Council for Research of Brazil (Conselho Nacional de Pesquisa, CNPQ) is acknowledged for supporting Victor Arieta. Dr. Roya R.R. Sardari is acknowledged for producing the 3-HPA utilized in this work.

6. References

- Banner, T., Fosmer, A., Jessen, H., Marasco, E., Rush, B., Veldhouse, J., De Souza, M. 2011. Microbial bioprocesses for industrial-scale chemical production. In: *Biocatalysis for Green Chemistry and Chemical Process Development*, J. Tao, R.J. Kazlauskas, John Wiley & Sons. Hoboken, N.J., pp. 429-467.
- Circle, S.J., Stone, L., Boruff, C.S. 1945. Acrolein determination by means of tryptophan: a colorimetric micromethod. *Industrial and engineering chemistry*, **17**(4), 259-262.
- Della Pina, C., Falletta, E., Rossi, M. 2011. A green approach to chemical building blocks. The case of 3-hydroxypropanoic acid. *Green Chemistry*, **13**(7), 1624-1632.
- Dishisha, T., Pereyra, L.P., Pyo, S.H., Britton, R.A., Hatti-Kaul, R. 2014. Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol. *Microbial Cell Factories*, **13**, 76.
- Geueke, B., Riebel, B., Hummel, W. 2003. NADH oxidase from *Lactobacillus brevis*: a new catalyst for the regeneration of NAD. *Enzyme and Microbial Technology*, **32**(2), 205-211.
- Henry, C.S., Broadbelt, L.J., Hatzimanikatis, V. 2010. Discovery and analysis of novel metabolic pathways for the biosynthesis of industrial chemicals: 3-Hydroxypropanoate. *Biotechnology and Bioengineering*, **106**(3), 462-473.
- Higuchi, M., Shimada, M., Yamamoto, Y., Hayashi, T., Koga, T., Kamio, Y. 1993. Identification of two distinct NADH oxidases corresponding to H₂O₂-forming oxidase and H₂O-forming oxidase induced in *Streptococcus mutans*. *Journal of General Microbiology*, **139**(10), 2343-2351.
- Hoof, R.W., Vriend, G., Sander, C., Abola, E.E. 1996. Errors in protein structures. *Nature*, **381**(6580), 272.
- Jiang, X.L., Meng, X., Xian, M. 2009. Biosynthetic pathways for 3-hydroxypropionic acid production. *Applied Microbiology and Biotechnology*, **82**(6), 995-1003.
- Jong, E.D., Higson, A., Walsh, P., Wellisch, M., Barbosa, M., Blauw, R., Gosselink, R., van Ree, R., Jorgensen, H., Mandl, M., McLaughlin, M., Smith, M.A., Willke, T. 2012. Value added products from biorefineries. IEA Bioenergy.
- Kengen, S.W., van der Oost, J., de Vos, W.M. 2003. Molecular characterization of H₂O₂-forming NADH oxidases from *Archaeoglobus fulgidus*. *European Journal of Biochemistry*, **270**(13), 2885-2894.
- Kumar, V., Ashok, S., Park, S. 2013. Recent advances in biological production of 3-hydroxypropionic acid. *Biotechnology Advances*.
- Mochizuki, M., Hiram, M. 1997. Structural effects on the biodegradation of aliphatic polyesters. *Polymers for Advanced Technologies*, **8**(4), 203-209.
- Niimura, Y., Nishiyama, Y., Saito, D., Tsuji, H., Hidaka, M., Miyaji, T., Watanabe, T., Massey, V. 2000. A hydrogen peroxide-forming NADH oxidase that functions as an alkyl hydroperoxide reductase in *Amphibacillus xylanus*. *Journal of Bacteriology*, **182**(18), 5046-5051.
- Perez, J.M., Arenas, F.A., Pradenas, G.A., Sandoval, J.M., Vasquez, C.C. 2008. *Escherichia coli* YqhD exhibits aldehyde reductase activity and protects

- from the harmful effect of lipid peroxidation-derived aldehydes. *Journal of Biological Chemistry*, **283**(12), 7346-53.
- Riebel, B.R., Gibbs, P.R., Wellborn, W.B., Bommarius, A.S. 2003. Cofactor regeneration of both NAD⁺ and NADH and NADP⁺ from NADPH:NADH oxidase from *Lactobacillus sanfranciscensis*. *Advanced Synthesis & Catalysis*, **345**, 6.
- Riebel, B.R., Gibbs, P.R., Wellborn, W.B., Bommarius, A.S. 2002. Cofactor regeneration of NAD⁺ from NADH: novel water-forming NADH oxidases. *Advanced Synthesis & Catalysis*, **344**, 13.
- Rocha-Martin, J., Vega, D., Bolivar, J.M., Godoy, C.A., Hidalgo, A., Berenguer, J., Guisan, J.M., Lopez-Gallego, F. 2011. New biotechnological perspectives of a NADH oxidase variant from *Thermus thermophilus* HB27 as NAD⁺-recycling enzyme. *BMC Biotechnology*, **11**, 101.
- Sabet-Azad, R., Linares-Pasten, J.A., Torkelson, L., Sardari, R.R., Hatti-Kaul, R. 2013. Coenzyme A-acylating propionaldehyde dehydrogenase (PduP) from *Lactobacillus reuteri*: kinetic characterization and molecular modeling. *Enzyme & Microbial Technology*, **53**(4), 235-42.
- Sabet-Azad, R., Sardari, R.R., Linares-Pastén, J.A., Hatti-Kaul, R. 2015. Production of 3-hydroxypropionic acid from 3-hydroxypropionaldehyde by recombinant *Escherichia coli* co-expressing *Lactobacillus reuteri* propanediol utilization enzymes. *Bioresource Technology*, **180**(0), 214-221.
- Sardari, R.R., Dishisha, T., Pyo, S.H., Hatti-Kaul, R. 2013. Improved production of 3-hydroxypropionaldehyde by complex formation with bisulfite during biotransformation of glycerol. *Biotechnology & Bioengineering*, **110**(4), 1243-8.
- Sudar, M., Findrik, Z., Domanovac, M.V., Vasić-Rački, Đ. 2014. Coenzyme regeneration catalyzed by NADH oxidase from *Lactococcus lactis*. *Biochemical Engineering Journal*, **88**(0), 12-18.
- Toomey, D., Mayhew, S.G. 1998. Purification and characterisation of NADH oxidase from *Thermus aquaticus* YT-1 and evidence that it functions in a peroxide-reduction system. *Eur J Biochem*, **251**(3), 935-45.
- Wandrey, C. 2004. Biochemical reaction engineering for redox reactions. *The Chemical Record*, **4**(4), 254-265.
- Werpy, T., Petersen, G., Aden, A., Bozell, J., Holladay, J., White, J., Manheim, A., Elliot, D., Lasure, L., Jones, S., Gerber, M., Ibsen, K., Lumberg, L., Kelley, S. 2004. Top value added chemicals from biomass, Volume 1—Results of screening for potential candidates from sugars and synthesis gas (U.S. Department of Energy, Oak Ridge, TN, August 2004; available at <http://www.eere.energy.gov/biomass/pdfs/35523.pdf>).
- Zhang, D.H., Hillmyer, M.A., Tolman, W.B. 2004. A new synthetic route to poly[3-hydroxypropionic acid] [P[3-HP]]: Ring-opening polymerization of 3-HP macrocyclic esters. *Macromolecules*, **37**(22), 8198-8200.
- Zhang, Y.W., Tiwari, M.K., Gao, H., Dhiman, S.S., Jeya, M., Lee, J.K. 2012. Cloning and characterization of a thermostable H₂O-forming NADH oxidase from *Lactobacillus rhamnosus*. *Enzyme & Microbial Technology*, **50**(4-5), 255-62.

7. Figure captions

Fig. 1. Proposed metabolic pathway for enhanced conversion of 3-HPA to 3-HP using recombinant *E. coli* expressing propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL), kinase (PduW) and FAD-dependent pyridine nucleotide-disulfide oxidoreductase (Nox) from *L. reuteri*.

Fig. 2. Proposed structural model of *L. reuteri* FAD-dependent pyridine nucleotide-disulfide oxidoreductase (Lreu_0076) built using the water-forming NAD(P)H oxidase of *L. sanfranciscensis* as a template by YASARA Structures software. The structure contains three domains for FAD binding, ADP binding and for dimerization each containing a typical Rosmann fold.

Fig. 3. SDS-PAGE analysis of the crude extracts of strains *E. coli* BTpET28b (control strain) (B), BTPnox (C), BTnoxplw (F) and purified protein product (D) Standard protein ladder is seen in lane A and E.

Fig. 4. Specific activity of *L. reuteri* FAD-dependent pyridine nucleotide-disulfide oxidoreductase with (A) Different buffering system (50 mM of HEPES, Tris-HCl and potassium phosphate) at pH 7, (B) different buffering pH using 50 mM potassium phosphate buffer (■) and 50 mM citrate/phosphate buffer (◆□ in the range between (4 – 8)).

Fig. 5. Specific activity of *L. reuteri* FAD-dependent pyridine nucleotide-disulfide oxidoreductase with varying concentration of NADH and NADPH. (A) crude extract with NADH, (B) Purified enzyme with NADH, and (C) purified enzyme with NADPH.

Fig. 6. Fed-batch biotransformation of 3-HPA (●) to 3-HP (▲) and 1,3-PD (■) using resting cells of recombinant *E. coli* expressing PduP, PduL, PduW and Nox from *L. reuteri*. (A) Intermittent feeding of 3-HPA or (B) continuous feeding of 3-HPA. Experiment details are described in Materials and Methods.

8. Figures

Fig. 1

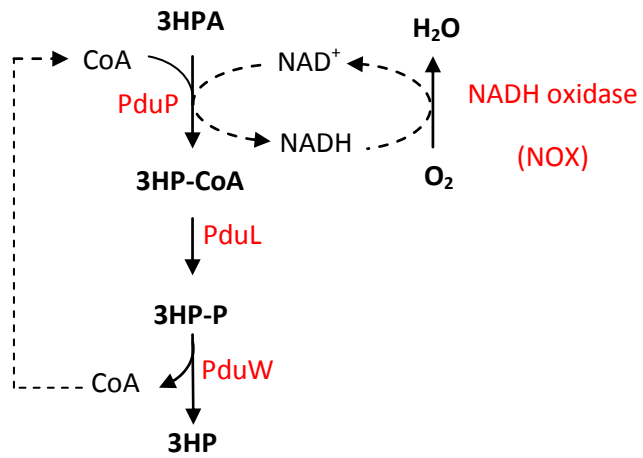


Fig. 2

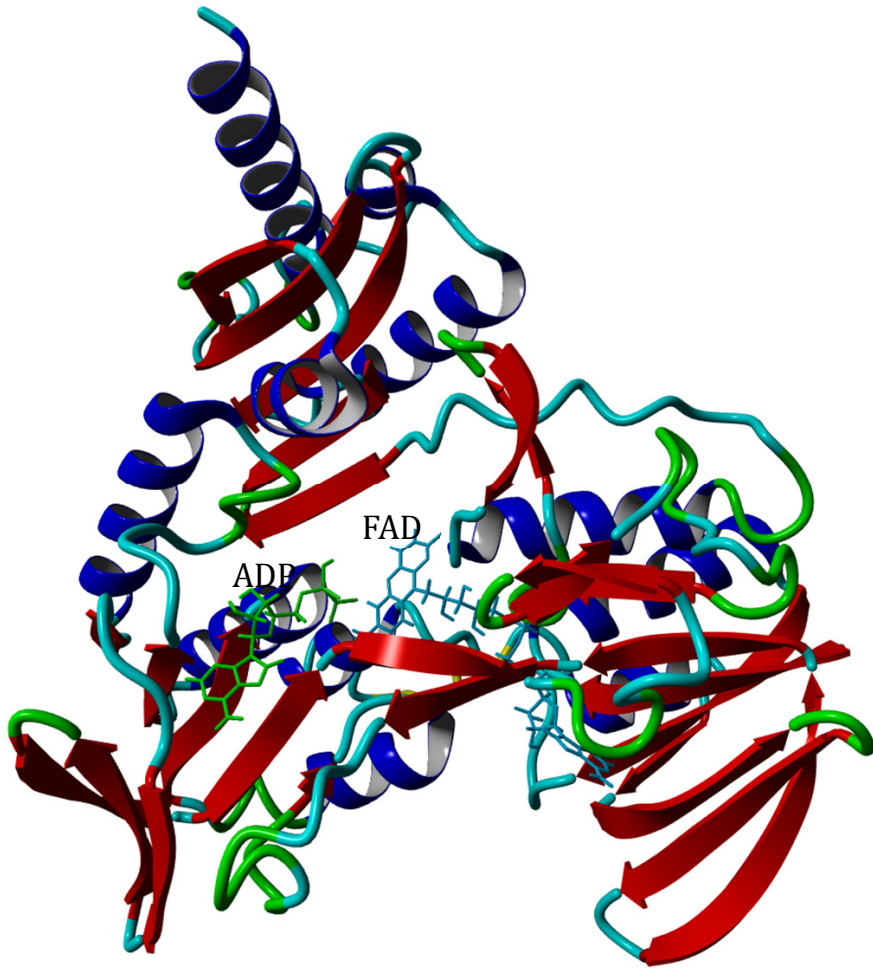


Fig. 3

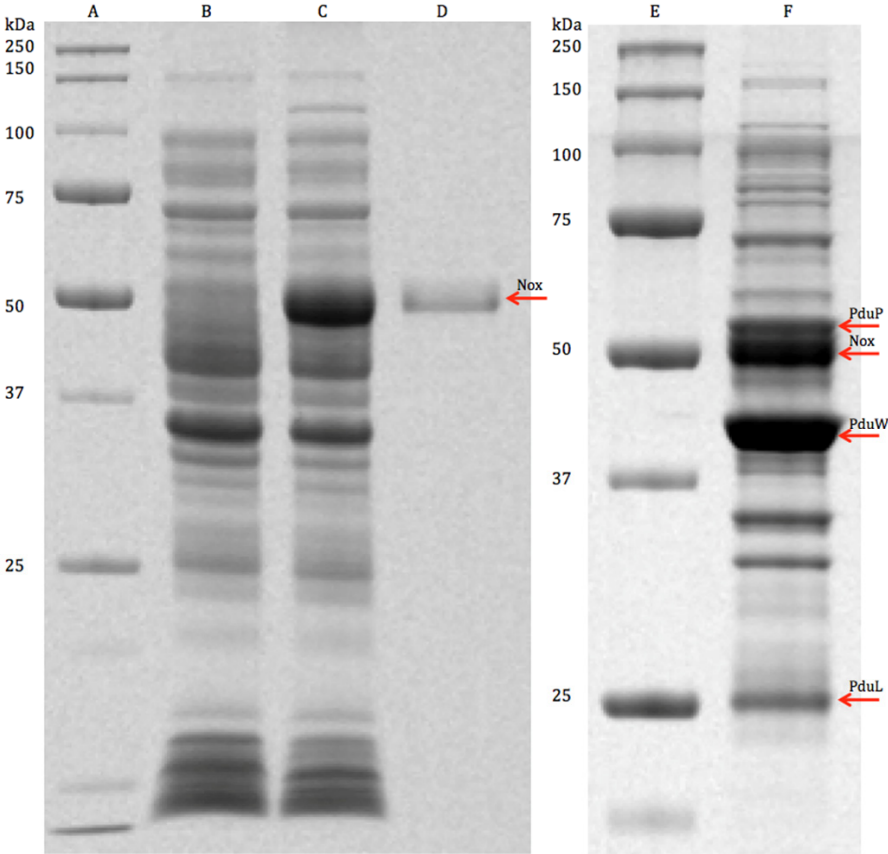


Fig. 4

Fig. 4A

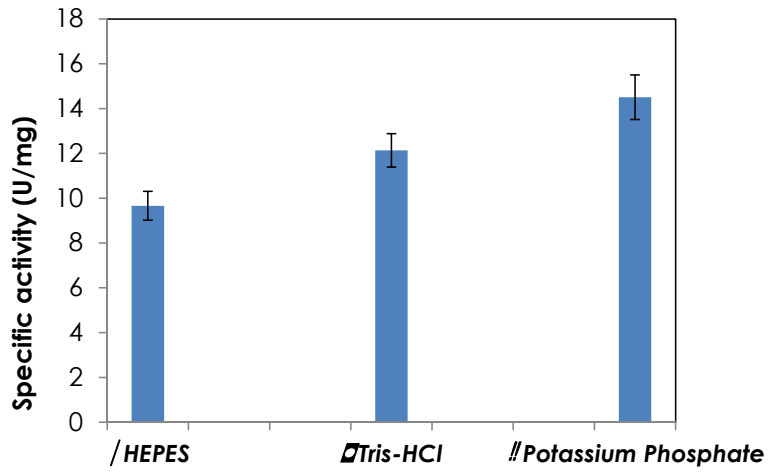


Fig. 4B

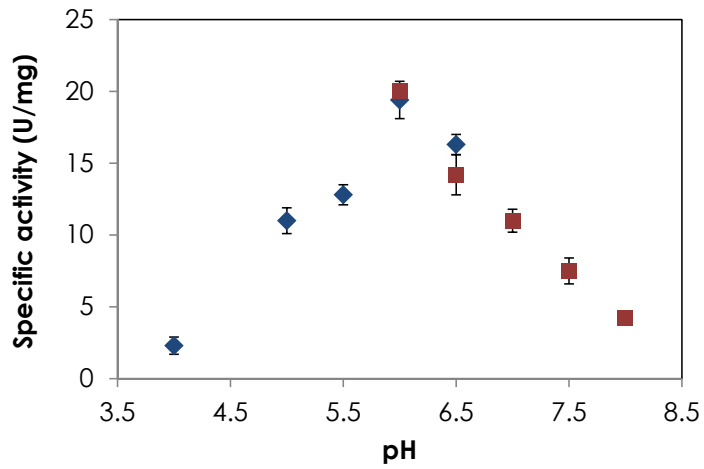


Fig. 5.

Fig. 5A

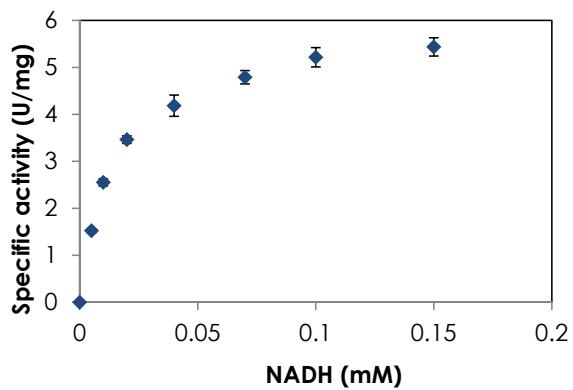


Fig. 5B

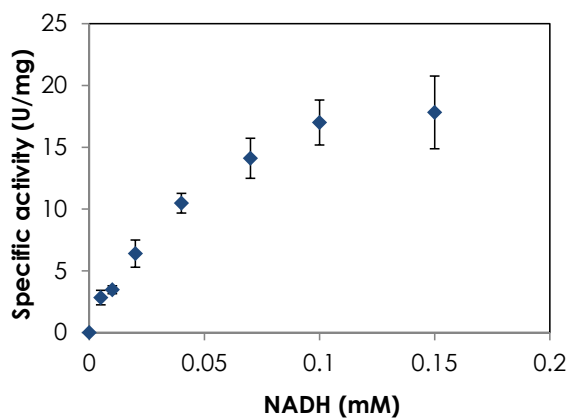


Fig. 5C

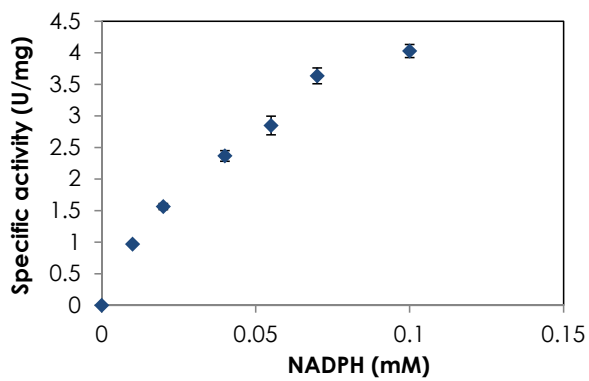


Fig. 6

Fig. 6A

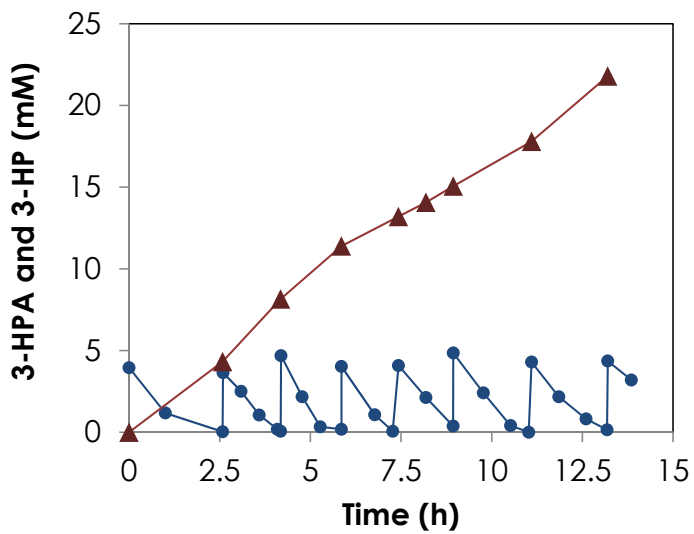
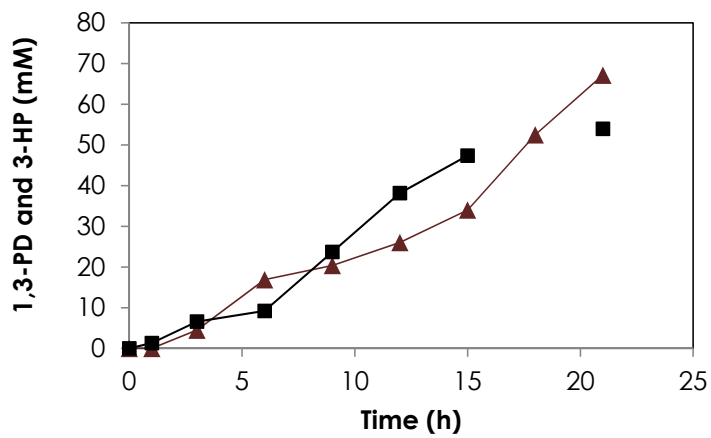


Fig. 6B



Paper IV



Efficient poly(3-hydroxypropionate) production from glycerol using *Lactobacillus reuteri* and recombinant *Escherichia coli* harboring *L. reuteri* propionaldehyde dehydrogenase and *Chromobacterium* sp. PHA synthase genes



Javier A. Linares-Pastén^a, Ramin Sabet-Azad^a, Laura Pessina^a, Roya R.R. Sardari^a,
 Mohammad H.A. Ibrahim^{a,b}, Rajni Hatti-Kaul^{a,*}

^a Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

^b Chemistry of Natural and Microbial Products Department, National Research Centre, Al-Bohoos St., 12622 Cairo, Egypt

HIGHLIGHTS

- P(3HP) production via two-stage process using *L. reuteri* and recombinant *E. coli*.
- Glycerol conversion to reuterin with resting cells of *L. reuteri* in fed-batch mode.
- Co-expression of *L. reuteri* PduP and *Chromobacterium* sp. PHA synthase in *E. coli*.
- Two-stage process gives high P(3HP) content of 40% w/w cell dry weight.

ARTICLE INFO

Article history:

Received 6 November 2014

Received in revised form 27 December 2014

Accepted 29 December 2014

Available online 6 January 2015

Keywords:

Poly(3-hydroxypropionate)

Lactobacillus reuteri

Reuterin

Coenzyme A-acylating propionaldehyde

dehydrogenase

Polyhydroxyalkanoate synthase

ABSTRACT

Poly(3-hydroxypropionate), P(3HP), is a polymer combining good biodegradability with favorable material properties. In the present study, a production system for P(3HP) was designed, comprising conversion of glycerol to 3-hydroxypropionaldehyde (3HPA) as equilibrium mixture with 3HPA-hydrate and -dimer in aqueous system (reuterin) using resting cells of native *Lactobacillus reuteri* in a first stage followed by transformation of the 3HPA to P(3HP) using recombinant *Escherichia coli* strain co-expressing highly active coenzyme A-acylating propionaldehyde dehydrogenase (PduP) from *L. reuteri* and polyhydroxyalkanoate synthase (PhaCcs) from *Chromobacterium* sp. P(3HP) content of up to 40% (w/w) cell dry weight was reached, and the yield with respect to the reuterin consumed by the cells was 78%. Short biotransformation period (4.5 h), lack of additives or expensive cofactors, and use of a cheap medium for cultivation of the recombinant strain, provides a new efficient and potentially economical system for P(3HP) production.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Microbial aliphatic polyesters, polyhydroxyalkanoates (PHAs), have been extensively studied over more than two decades, because of the possibility of using them as renewable, biodegradable plastics instead of the widely used fossil based plastics. Poly(3-hydroxypropionate) (PHP) is an attractive biopolymer due to its useful material properties, biodegradability, biocompatibility, and even for its potential for further valorization to industrially significant chemicals (Andreeßen and Steinbüchel, 2010;

Andreeßen et al., 2014). Unlike other PHAs, no wild type microbial species is known to produce P(3HP) natively. This polymer has been synthesized chemically by ring opening polymerization of 3-hydroxypropionate (3HP) macrocyclic esters (Zhang et al., 2004), and enzymatically by polymerization of methyl 3-hydroxypropionate using immobilized lipase (Novozym[®]435) (Song et al., 2012). However, the high cost of the substrate, 3HP in the former case or methyl-3HP in the latter, is the major drawback in utilizing these methods for large scale production.

In recent years, some studies on production of P(3HP) using recombinant organisms have been reported via three different routes. The first route involves dehydration of glycerol to 3-hydroxypropionaldehyde (3HPA), followed by oxidation and

* Corresponding author. Tel.: +46 46 222 4840; fax: +46 46 222 4713.

E-mail address: Rajni.Hatti-Kaul@biotek.lu.se (R. Hatti-Kaul).

coenzyme A (CoA) ligation to form 3-hydroxypropionyl-CoA (3HP-CoA) and polymerization into P(3HP) (Andreeßen et al., 2010). The second pathway is based on oxidation of 1,3-propanediol (1,3PDO) to 3HPA, followed by its conversion to 3-hydroxypropionic acid (3HP), and ligation to CoA prior to polymerization (Zhou et al., 2011). The third pathway is independent of the carbon source as it uses acetyl-CoA, an intermediate of the central metabolism, as the starting material that is carboxylated to malonyl-CoA, which is reduced to 3HP and the remaining steps as in the second alternative (Wang et al., 2012).

The first route has attracted most interest so far due to the relatively low cost and high abundance of glycerol as raw material than e.g. 1,3PDO, and the higher polymer yields and -productivities obtained as compared to the other routes. The reactions involved are catalyzed by glycerol dehydratase. Coenzyme A-acylating propionaldehyde dehydrogenase (PduP) and polyhydroxyalkanoate synthase (PhaC), respectively. Previously, Andreeßen et al. (2010) have reported P(3HP) content of 11.98% w/w cell dry weight by the expression of glycerol dehydratase from *Clostridium butyricum*, PduP from *Salmonella enterica* serovar *Typhimurium* LT2, and PHA synthase from *Ralstonia eutropha* in *Escherichia coli*. It was necessary to maintain anaerobic conditions during cultivation because of the high sensitivity of glycerol dehydratase to oxygen (Andreeßen et al., 2010). Using the Vitamin-B12 dependent glycerol dehydratase from *Klebsiella pneumoniae* that is less sensitive to oxygen, a higher P(3HP) content of 46.4% (wt/wt [cell dry weight]) was reached (Wang et al., 2013). Recently, this strategy was improved combining chromosomal gene integration (genes involved in the glycerol dehydration) and plasmid addition system (synthase and CoA acylating enzyme genes) based on tyrosine anabolism, with a yield of 67.9% (wt/wt [cell dry weight]) (Gao et al., 2014).

The most critical step in the pathway is that of glycerol dehydration to 3HPA; while the B12-independent glycerol dehydratase from *C. butyricum* is highly sensitive to oxygen (O'Brien et al., 2004; Liu et al., 2010; Felix and Ullmann, 2012) and limits the cultivation conditions and growth of the recombinant *E. coli*, the B12-dependent dehydratase requires reactivation by the expensive B12 cofactor. Some facultative anaerobes like *Lactobacillus reuteri*, *K. pneumoniae*, and many *Enterobacteriaceae* that possess the Vitamin B12 generation enzymatic machinery are able to reactivate the enzyme (Talarico and Dobrogosz, 1990; Santos et al., 2011). 3HPA is produced in high levels by *L. reuteri* when grown in excess of glycerol and is present as an equilibrium mixture with 3HPA-hydrate and -dimer in an aqueous system (Fig. 1), called reuterin and known for its natural antimicrobial activity (Cleusix et al., 2008; Vollenweider et al., 2003). A 3HPA yield up to 85 mol% from glycerol during batch cultivation has been reported (Luthi-Peng et al., 2002). *L. reuteri* has the advantage of being a probiotic microorganism and exhibiting higher resistance to 3HPA than other organisms with a minimum inhibitory concentration value of 30–50 mM for glycerol dehydratase activity and 60–120 mM for growth of the organism. 3HPA production was recently improved in our laboratory by *in situ* recovery using complexation with bisulfite (Sardari et al., 2013a,b) and also by using a mutant over-expressing the glycerol dehydratase (Dishisha et al., 2014).

In the present study, we have designed a production system for improved P(3HP) accumulation comprising conversion of glycerol to 3HPA using native *L. reuteri* in a first stage followed by transformation of 3HPA to P(3HP) using recombinant *E. coli*, co-expressing PduP and PhaC, under aerobic conditions in a minimal medium (Fig. 1). Two recombinant *E. coli* strains were constructed, both containing *pduP* from *L. reuteri* (Sabet-Azad et al., 2013), but differing in the source of *phaC*, which were encoding PhaCre from *R. eutropha* and PhaCcs from *Chromobacterium* sp USM2, respectively. The latter enzyme is known to have a significantly higher activity than that of *R. eutropha* enzyme (Bhubalan et al., 2011).

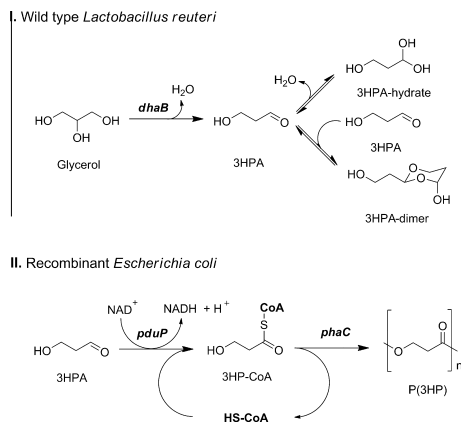


Fig. 1. Reactions involved in the biotransformation of glycerol to poly(3-hydroxypropionate): (I) glycerol dehydration using resting cells of *L. reuteri* to 3HPA, which is in an equilibrium mixture with 3HPA-hydrate and -dimer in aqueous system, and (II) biotransformation of the 3HPA to P(3HP) using recombinant *E. coli* bearing genes encoding PduP and PhaC synthase.

2. Methods

2.1. Bacterial strains, media, and plasmids

L. reuteri DSM 20016 and *R. eutropha* H16 DSM428 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The cloning host *E. coli* NovaBlue and expression strain *E. coli* BL21(DE3) were purchased from Novagen. *L. reuteri* was cultivated at 37 °C in deMan, Rogosa and Sharpe (MRS)-broth (Difco). *R. eutropha* was cultivated in Nutrient broth (5 g peptone and 3 g meat extract in 1 L water). *E. coli* strains were cultivated in LB broth as well as in minimal M9 medium (M9 salts, 0.1 mM CaCl₂, 2 mM MgSO₄) with 20 g/l glucose or glycerol as sole carbon source. All the cultures were maintained on respective media with 1.6% (w/v) agar. Cloning vector pUC19 was purchased from Fermentas, while pCOLADuet vector was from Novagen. Ampicillin (100 µg/mL) was added to the medium to select the *E. coli* strains harboring pUC19 constructs, and 30 µg/mL kanamycin for the selection of the strains harboring pCOLADuet constructs. All antibiotics were purchased from Sigma-Aldrich.

2.2. Production of 3HPA from glycerol

Whole cells of *L. reuteri* DSM 20016 were used as biocatalysts to convert glycerol to 3HPA as described earlier (Sardari et al., 2013a). Freshly grown cells from 2 L MRS broth (5 g cell dry weight), were suspended in a 1 L solution containing 2 g glycerol in a 3 L bioreactor (Applikon, BioBundles, The Netherlands), and biotransformation was performed at pH 5 and 37 °C with agitation at 200 rpm, and continuous bubbling of nitrogen gas. The experiment was started as a batch reaction for 1 h and continued in a fed batch mode by feeding a solution containing 50 g/L glycerol at a rate of 1 mL/min for 6 h. The supernatant was separated from the cells by centrifugation at 15,000g and 4 °C for 10 min and filter sterilized through a 0.2 µm polyethersulfone membrane (VWV, Sweden). The resulting solution was used as a crude source of 3HPA for P(3HP) synthesis.

2.3. Cloning and expression of *pduP* and *phaC* genes

Genomic DNAs were extracted from *L. reuteri* and *R. eutropha* using E.Z.N.A Genomic Isolation Kit (Omega Bio-Tek, USA). The *pduP* gene was amplified from the genomic DNA of *L. reuteri* using the following pair of primers: forward GGATCCGATGCAGA TTAATGATATTGAAAAGTG containing the BamHI restriction site (underlined), and reverse AAGCTTATACCACTGACTGAGAATCC with the HindIII restriction site (underlined). The *phaCre* gene from *R. eutropha* was amplified with the primers: forward GGCCGGCC ACGCGACCGCAAAGCGCC with the FseI restriction site, and reverse TTAATTAAGCCTTGGCTTTCAGGTATCGCC containing the PacI restriction site. A synthetic gene to overexpress the PHA synthase (GenBank: ADL70203.1) from *Chromobacterium* sp. USM2 (PhaCcs) in *E. coli* was designed. The codon optimization was performed using Optimizer Server (<http://genomes.urv.es/OPTIMIZER/>) and analysis of the optimal codons using *E. coli* Codon Usage Analyzer 2.1 (www.faculty.ucr.edu/~mmaduro/codonusage/usage.htm). *PduP* and *PhaCre* amplicons were inserted into pUC19 to propagate the genes and then were subcloned into the multiple cloning sites (MCS) 1 and 2 of pCOLADuet, respectively, giving the final construct pCOLADuet-PduP:PhaCre. On the other hand, *pduP* was subcloned into MCS 1, and the synthetic gene *phaCcs* into MCS 2 of pCOLADuet, giving the final construct pCOLADuet-PduP:PhaCcs. The co-overexpression of PduP/PhaCre and PduP/PhaCcs were performed in LB broth as well as in M9 minimal medium, supplemented with kanamycin and induced with 1 mM IPTG at OD_{550nm} 0.6–1. The level of expression was qualitatively estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on gels using 12% acrylamide.

2.4. Production of P(3HP) using recombinant *E. coli*

The *E. coli* strains BL21(DE3) pCOLADuet-PduP:PhaCcs, BL21(DE3) pCOLADuet-PduP:PhaCre and the negative control BL21(DE3) pCOLADuet (containing the empty plasmid) were grown in M9 minimal medium containing 30 µg/mL kanamycin and 20 g/L glucose or 20 g/L glycerol as the carbon source. Pre-inocula of 4 mL cultures in M9 medium were grown overnight up to OD_{550nm} of 1–1.2, and added to 500 mL M9 medium in 2 L baffled shake flasks. The cultures were incubated at 37 °C and 200 rpm. Recombinant protein co-expression was induced using 1 mM IPTG once the cultures had reached OD_{550nm} of 1.6. The filtered supernatant obtained in Section 2.2 was diluted to contain 120 mg of 3HPA and added to the medium after 15 h of induction. Consumption of 3HPA was monitored and once all the aldehyde was consumed, same amount of 3HPA as above was added. After 3 repetitive additions of 3HPA, the cells were collected for polymer extraction.

2.5. Polymer extraction

The recombinant *E. coli* cells containing the polymer (Section 2.4) were harvested by centrifugation at 800g for 10 min in a Sorvall refrigerated centrifuge (RC5C, USA). The cell pellet was washed twice in deionized water and then re-centrifuged and lyophilized. The polymer was extracted by exhaustive refluxing of the lyophilized cell suspension in 150 mL chloroform for 4 h at ≈60 °C in a Soxhlet apparatus. The extract was concentrated by rotoevaporation and then air dried in a fume hood for polymer film formation.

2.6. Analytical methods

Quantification of 3HPA was done by a modified colorimetric method (Circle et al., 1945): 150 µL of diluted DL-tryptophan

(10 mM in 50 mM HCl) and 600 µL concentrated HCl were added to 200 µL of a suitably diluted sample. The mixture was incubated at 37 °C for 20 min and the absorbance was measured at 560 nm. Concentrations of glucose, glycerol, 1,3-propanediol and 3-hydroxypropionic acid were analyzed by HPLC (Jasco) as described earlier using an Aminex HPLC-87H column connected to a guard column (Biorad, Richmond, USA) with 0.5 mM H₂SO₄ as the mobile phase (Sardari et al., 2013b). Cell density of the recombinant *E. coli* was monitored as optical density at 550 nm after diluting a sample of culture broth 1–10 times in 0.9% NaCl solution. For determining the cell dry weight, the cells were washed twice with water, centrifuged and lyophilized.

The polymer molecular weight was determined by size exclusion HPLC using a Shodex GPC KF 805 column and chloroform as mobile phase. The calibration curve was obtained using polystyrene standard with four molecular sizes: Mn = 6,500,000 g mol⁻¹, from Water Associates; 96,000 and 30,300 g mol⁻¹, from Polymer Laboratories; and 3180 g mol⁻¹, from Agilent Technologies. The molecular structure of the polymer was analyzed by NMR using Bruker DRX 400 MHz. Chemical shifts are given in ppm downfield from signal for Me₄Si with reference to residual CHCl₃. ¹H NMR (400 MHz, CDCl₃) δ 4.36 (t, *J* = 6.3 Hz, 1H, –OCH₂–), 2.66 (t, *J* = 6.3 Hz, 1H, –CH₂COO–), ¹³C NMR (101 MHz, CDCl₃) δ 170.24, 60.02, 33.53.

3. Results and discussion

3.1. Reduction of glycerol into 3HPA by *L. reuteri*

L. reuteri uses glycerol as an electron acceptor and not as a carbon source for growth, hence obviating the formation of several side-products. Glycerol is transformed mainly to 1,3PDO by the growing cells of *L. reuteri* while other by-products are formed as a result of glucose metabolism. The resting cells convert glycerol to 3HPA during a short period of time along with lower amounts of 1,3PDO and 3HP as co-products via the reductive and oxidative branches of the propanediol utilization pathway (Pdu), respectively (Dishisha et al., 2014). The biotransformation is terminated quickly due to the toxic effect of 3HPA.

In the present study, glycerol was dehydrated using resting cells of *L. reuteri* to 3HPA to be used as substrate for the production of P(3HP) by the recombinant *E. coli*. In order to avoid inhibition by the accumulation of 3HPA to toxic levels, glycerol was added to the cell suspension in a fed-batch mode (Sardari et al., 2013a), which provided a product mixture containing 50 mM 3HPA, 19.4 mM 1,3PDO, 19.4 mM 3HP, and 24 mM residual glycerol. The 3HPA yield was 0.45 g/g glycerol with productivity of 13.5 mmol/h (1.06 g/h). Higher productivity of 3HPA and lower accumulation of 1,3PDO and 3HP are possible by *in situ* complexation of 3HPA (Sardari et al., 2013a,b, 2014). Moreover, almost 2-fold increase in 3HPA productivity was achieved when an engineered strain of *L. reuteri* RPRB3007, in which *pdu* operon genes are overexpressed, was used (Dishisha et al., 2014).

3.2. Cloning and expression of *pduP* and *phaC* genes in *E. coli*

Two *E. coli* BL21(DE3) constructs, pCOLADuet-pduP:phaCre and pCOLADuet-pduP:phaCcs expressing *pduP* and *phaC* genes, were developed for the conversion of reuterin to P(3HP) (Fig. 1); *pduP* in both constructs was from *L. reuteri*, but the origin of *phaC* gene differed. *R. eutropha* in the former and a synthetic gene encoding *Chromobacterium* sp. USM2 enzyme in the latter. Both *pduP* and *phaC* genes were under the transcriptional control of T7 promoter. PduP is a coenzyme A-acylating propionaldehyde dehydrogenase able to use 3HPA as a substrate to produce 3HPA-CoA

(Sabet-Azad et al., 2013), which serves as the substrate for polymerization by PhaC (Andreeßen et al., 2010; Wang et al., 2013). PduP from *L. reuteri*, used in this study (28.9 U/mg) (Sabet-Azad et al., 2013), is almost 2-fold more active than the corresponding enzyme from *S. enterica* (15.2 U/mg) (Leal et al., 2003), which was used in the previous works (Andreeßen et al., 2010; Wang et al., 2013). The activity of the PhaCcs from *Chromobacterium* sp. (238 U/mg) (Bhubalan et al., 2011) was 8-fold higher than the synthase from the model strain *R. eutropha* (40 U/mg) (Yuan et al., 2001) used in the earlier reports for P(3HP) production (Andreeßen et al., 2010; Wang et al., 2013).

The synthetic phaCcs gene was designed to encode a 567 amino acid protein product identical to the native PHA synthase from *Chromobacterium* sp. USM2. The optimization of codons for expression in *E. coli* resulted in a nucleotide sequence 83% similar to the native gene. Both constructs, pCOLADuet-pduP:phaCre and pCOLADuet-pduP:phaCcs were verified through nucleotide sequencing, and the protein expression was analyzed by SDS-PAGE. In the first construct, PhaCre was present mainly in the insoluble fraction (not shown) while the PhaCcs from the second construct showed high levels of expression in the soluble phase (Fig. 2). The expression of PduP showed high levels of protein in the soluble phase in both constructs. The construct pCOLADuet-pduP:phaCcs, co-expressing high level of soluble and active recombinant enzymes, is thus an attractive candidate for developing an efficient system for P(3HP) production from 3HPA.

3.3. Production of P(3HP) from reuterin by recombinant *E. coli*

Biotransformation of 3HPA (obtained in Section 3.1) was performed in triplicates in minimal M9 medium with kanamycin and glucose or glycerol as sole carbon source at 37 °C. *E. coli* BL(DE3) pCOLADuet-pduP:phaCcs converted the 3HPA (provided as the product mixture obtained in Section 3.1) to P(3HP) in only 4 h, while no conversion was observed with *E. coli* BL(DE3) pCOLADuet-pduP:phaCre under similar conditions. The lack of activity in the last strain could be attributed to expression of *R. eutropha* PhaCre as insoluble product, and/or short period of biotransformation. In contrast, previous reports on the conversion of glycerol to P(3HP) using *E. coli* harboring the PhaCre (from *R. eutropha*) have shown a lag phase of P(3HP) synthesis of about 5–10 h without clear formation of the polymer, under both anaerobic (Andreeßen et al., 2010) and aerobic conditions (Wang et al., 2013). Studies on the activities of pure PHA synthases from *R. eutropha* and *Chromobacterium* sp. USM2 by measuring the CoA release from butyryl-CoA have shown the former to exhibit a more prevalent lag phase (Bhubalan et al., 2011). In this study we show that *Chromobacterium* sp. USM2 PHA synthase, PhaCcs, was even able to efficiently use 3-hydroxypropyl-CoA as substrate.

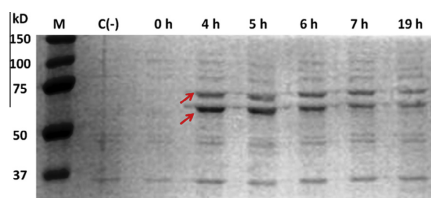


Fig. 2. SDS-PAGE analysis on 12% acrylamide gel of the cell lysate of *E. coli* BL21(DE3) pCOLADuet-pduP:phaCcs. Lane C is analysis of strain harboring empty plasmid pCOLADuet (negative control). The level of expression was monitored from 0 to 19 h after induction. The recombinant proteins, PhaCcs (about 66,000 Da) and PduP (about 53,000 Da), are indicated by arrows. Lane M shows standard protein ladder from BioRad.

The minimal inhibitory concentration (MIC) of 3HPA for *E. coli* has been determined at or below 7.12 mM (Cleusix et al., 2007). Therefore, 3HPA to a final concentration of 3 mM (total amount of 120 mg in 550 mL) was added at a time for biotransformation with growing recombinant *E. coli* strains. When added at the same time as the inducer IPTG, conversion of 3HPA to P(3HP) by *E. coli* BL(DE3) pCOLADuet-pduP:phaCcs reached only 50% during 4 h while 3HPA addition 3 h after the induction time resulted in complete consumption within one hour. This efficient conversion of 3HPA without affecting the bacterial growth was ascribed to the presence of the recombinant enzymes in high amounts. Under these conditions, the average yield of P(3HP) by *E. coli* BL(DE3) pCOLADuet-pduP:phaCcs was 20% and 23% (w/w) cell dry weight using 20 g/L of glycerol and glucose, respectively, as sole carbon source. There was no significant difference in the growth of the recombinant strain with either of the carbon sources, which is consistent with the previous report on *E. coli* with different gluconeogenic carbon sources (Paliy and Gunasekera, 2007). By inducing the heterologous expression at OD_{550nm} of ~1.6, and adding 3HPA 15 h after induction time (average OD_{550nm} of 6.0), the P(3HP) content was improved to 40% (w/w) cell dry weight, i.e. 260 mg polymer was recovered from 651 mg lyophilized bacterial cells.

Polymer accumulation as granules of irregular size in the cytoplasm was observed by transmission electron microscopy (Supplementary information S1). The accumulated P(3HP) granules were small, with an average size of <100 nm and low molecular weight (109,100 Da). The granule size in wild type PHA accumulating cells, often in the range of 100–500 nm, is known to be controlled by different regulatory proteins such as phasins surrounding the granules (Pötter et al., 2005). The absence of phasins in recombinant *E. coli* usually results in large granules (Maehara et al., 1999). The reason underlying the small size of granules in this study could be the high activity and/or expression level of PHA synthase, which prevents the increase in granule size as well as the increase in polymer chain length. Gerngross and Martin (1995) have suggested a general rule that low concentrations of PHA synthase yield large granules and high molecular weight polymers.

Multiple additions of crude reuterin, were successfully converted by *E. coli* BL(DE3) pCOLADuet-pduP:phaCcs to P(3HP); 99.7% of 120 mg added reuterin was consumed within 1 h, 95% of an additional 120 mg reuterin was also consumed within 1 h, and the last addition of 120 mg reuterin was consumed to the extent of 80% after 1.5 h (Fig. 3). During this period (totally 4.4 h), the cell density increased from OD_{550nm} value of 7 to 10. The yield with respect to the reuterin consumed by the cells was 78%. HPLC analysis revealed no production of 3HP and 1,3-propanediol, otherwise formed by oxidation and reduction of 3HPA, respectively. The

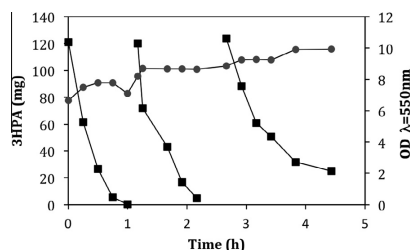


Fig. 3. Profiles of 3HPA consumption (■) and growth of *E. coli* BL21(DE3) pCOLADuet-pduP:phaCcs (OD_{550nm}) (●) during repeated additions of 3HPA for transformation to P(3HP). The zero hour corresponds to the first addition of 3HPA that was done 15 h after induction by IPTG.

relatively high P(3HP) content produced from unprocessed reuterin is promising, considering the short biotransformation period (4.5 h), absence of additives or expensive cofactors, and cheap mineral medium used for biotransformation. The P(3HP) produced was extracted with chloroform (Supplementary information S2), and its molecular structure was confirmed by NMR (Supplementary information S3).

Although the combination of the two new highly active enzymes i.e. PduP from *L. reuteri* and PhaCcs from *Chromobacterium* sp. results in an efficient and robust recombinant strain able to convert 3HPA into P(3HP) in a low cost mineral medium, the production of 3HPA by the native *L. reuteri* requires a rich and expensive culture medium such as MRS. There is a need to develop a low cost medium for *L. reuteri*, e.g. based on agro-industrial by-products such as potato juice, molasses or cheese whey.

4. Conclusion

Glycerol is an attractive raw material for producing value added substances including biopolymers. Co-expression of *L. reuteri* PduP and *Chromobacterium* sp. PhaC in *E. coli*, combined with optimized biotransformation of glycerol to 3HPA by *L. reuteri*, opens a new route to produce P(3HP). Toxicity of 3HPA to *E. coli* requires a substrate concentration below 7 mM for further production of P(3HP). Co-expression of *L. reuteri* glycerol dehydratase would alleviate this limitation, but would require Vitamin B12-supplementation. An alternative strategy being tried is engineering of *L. reuteri* with *Chromobacterium* sp. PhaC and silencing interfering pathways.

Acknowledgements

The work was supported by funding from VINNOVA (The Swedish Agency for Innovation Systems) (Project number: 2008-00849). The authors are grateful to Dr. R. Villagomez for helping with the polymer extraction and NMR analysis, and to Dr. C. Rodriguez-Arza for determination of polymer molecular weight.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2014.12.099>.

References

Andreeßen, B., Steinbüchel, A., 2010. Biosynthesis and biodegradation of 3-hydroxypropionate-containing polyesters. *Appl. Environ. Microb.* 76, 4919–4925.

Andreeßen, B., Lange, A., Robenek, H., Steinbüchel, A., 2010. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. *Appl. Environ. Microb.* 76, 622–626.

Andreeßen, B., Taylor, N., Steinbüchel, A., 2014. Poly(3-hydroxypropionate): a promising alternative to fossil fuel-based materials. *Appl. Environ. Microb.* 80, 6574–6582.

Bhubalan, K., Chuah, J.-A., Shozui, F., Brigham, C., Taguchi, S., Sinskey, A., Rha, C., Sudesh, K., 2011. Characterization of the highly active polyhydroxyalkanoate synthase of *Chromobacterium* sp. strain USM2. *Appl. Environ. Microb.* 77, 2926–2933.

Circle, S.J., Stone, L., Boruff, C.S., 1945. Acrolein determination by means of tryptophane. A colorimetric micro-method. *Ind. Eng. Chem.* 17, 259–262.

Cleusix, V., Lacroix, C., Vollenweider, S., Duboux, M., Le Blay, G., 2007. Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. *BMC Microbiol.* 7, 101.

Cleusix, V., Lacroix, C., Vollenweider, S., Le Blay, G., 2008. Glycerol induces reuterin production and decreases *Escherichia coli* population in an in vitro model of colonic fermentation with immobilized human feces. *FEMS Microbiol. Ecol.* 63, 56–64.

Dishisha, T., Pereyra, L.P., Pyo, S.-H., Britton, R.A., Hatti-Kaul, R., 2014. Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol. *Microb. Cell Fact.* 13, 76.

Feliks, M., Ullmann, G.M., 2012. Glycerol dehydration by the B12-independent enzyme may not involve the migration of a hydroxyl group: a computational study. *J. Phys. Chem. B* 116, 7076–7087.

Gao, Y., Liu, C., Ding, Y., Sun, C., Zhang, R., Xian, M., Zhao, G., 2014. Development of genetically stable *Escherichia coli* strains for poly(3-hydroxypropionate) production. *PLoS ONE* 9, <http://dx.doi.org/10.1371/journal.pone.0097845>.

Gerngross, T.U., Martin, D.P., 1995. Enzyme-catalyzed synthesis of poly[(R)-3-hydroxybutyrate]: formation of macroscopic granules in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6279–6283.

Leal, N.A., Havemann, G.D., Bobik, T.A., 2003. PduP is a coenzyme-a-acylating propionaldehyde dehydrogenase associated with the polyhedral bodies involved in B12-dependent 1,2-propanediol degradation by *Salmonella enterica* serovar Typhimurium LT2. *Arch. Microbiol.* 180, 353–361.

Liu, Y., Gallo, A.A., Florian, J., Liu, Y.S., Mora, S., Xu, W., 2010. QM/MM (ONIOM) study of glycerol binding and hydrogen abstraction by the coenzyme B12-independent dehydratase. *J. Phys. Chem. B* 114, 5497–5502.

Luthi-Peng, Q., Scharer, S., Puhani, Z., 2002. Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*. *Appl. Microbiol. Biotechnol.* 60, 73–80.

Maehara, A., Ueda, S., Nakano, H., Yamane, T., 1999. Analyses of a polyhydroxyalkanoic acid granule-associated 16-kilodalton protein and its putative regulator in the PHA locus of *Paracoccus denitrificans*. *J. Bacteriol.* 181, 2914–2921.

O'Brien, J.R., Raynaud, C., Croux, C., Girbal, L., Soucaille, P., Lanzilotta, W.N., 2004. Insight into the mechanism of the B12-independent glycerol dehydratase from *Clostridium butyricum*: preliminary biochemical and structural characterization. *Biochemistry* 43, 4635–4645.

Paliy, O., Gunasekera, T.S., 2007. Growth of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources and salt contents. *Appl. Microbiol. Biotechnol.* 73, 1169–1172.

Pötter, M., Müller, H., Steinbüchel, A., 2005. Influence of homologous phasins (PhaP) on PHA accumulation and regulation of their expression by the transcriptional repressor PhaR in *Ralstonia eutropha* H16. *Microbiology* 151, 825–833.

Sabet-Azad, R., Linares-Pastén, J.A., Torkelson, L., Sardari, R.R.R., Hatti-Kaul, R., 2013. Coenzyme A-acylating propionaldehyde dehydrogenase (PduP) from *Lactobacillus reuteri*: kinetic characterization and molecular modeling. *Enzyme Microb. Technol.* 53, 235–242.

Santos, F., Spinler, J.K., Sahlner, D.M., Molenaar, D., Teusink, B., De Vos, W.M., Versalovic, J., Hugenoltz, J., 2011. Functional identification in *Lactobacillus reuteri* of a PdcR-like transcription factor regulating glycerol utilization and vitamin B12 synthesis. *Microb. Cell Fact.* 10, 55.

Sardari, R.R.R., Dishisha, T., Pyo, S.-H., Hatti-Kaul, R., 2013a. Improved production of 3-hydroxypropionaldehyde by complex formation with bisulfite during biotransformation of glycerol. *Biotechnol. Bioeng.* 110, 1243–1248.

Sardari, R.R.R., Dishisha, T., Pyo, S.-H., Hatti-Kaul, R., 2013b. Biotransformation of glycerol to 3-hydroxypropionaldehyde: improved production by *in situ* complexation with bisulfite in a fed-batch mode and separation on anion exchanger. *J. Biotechnol.* 168, 534–542.

Sardari, R.R.R., Dishisha, T., Pyo, S.-H., Hatti-Kaul, R., 2014. Semicarbazide-functionalized resin as a new scavenger for *in-situ* recovery of 3-hydroxypropionaldehyde during biotransformation of glycerol by *Lactobacillus reuteri*. *J. Biotechnol.* 192, 223–230.

Song, H., Zhao, Y., Zhang, Y., Kong, W., Xia, C., 2012. Synthesis of polyhydroxyalkanoates by polymerization of methyl 3-hydroxypropionate in the catalysis of lipase. *Chin. J. Catal.* 33, 422–438.

Talarico, T.L., Dobrogosz, W.J., 1990. Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. *Appl. Environ. Microbiol.* 56, 1195–1197.

Vollenweider, S., Grassi, G., König, I., Puhani, Z., 2003. Purification and structural characterization of 3-hydroxypropionaldehyde and its derivatives. *J. Agric. Food Chem.* 51, 3287–3293.

Wang, Q., Liu, C., Xian, M., Zhang, Y., Zhao, G., 2012. Biosynthetic pathway for poly(3-hydroxypropionate) in recombinant *Escherichia coli*. *J. Microbiol.* 50, 693–697.

Wang, Q., Yang, P., Liu, C., Xue, Y., Xian, M., Zhao, G., 2013. Biosynthesis of poly(3-hydroxypropionate) from glycerol by recombinant *Escherichia coli*. *Bioresour. Technol.* 131, 548–551.

Yuan, W., Jia, Y., Tian, J., Snell, K.D., Muh, U., Sinskey, A.J., Lambalot, R.H., Walsh, C.T., Stubbe, J., 2001. Class I and III polyhydroxyalkanoate synthases from *Ralstonia eutropha* and *Allochroatrium vinosum*: characterization and substrate specificity studies. *Arch. Biochem. Biophys.* 394, 87–98.

Zhang, D., Hillmyer, M.A., Tolman, W.B., 2004. A new synthetic route to poly[3-hydroxypropionic acid] (P3-HP): ring-opening polymerization of 3-HP macrocyclic esters. *Macromolecules* 37, 8198–8200.

Zhou, Q., Shi, Z.-Y., Meng, D.-C., Wu, Q., Chen, J.-C., Chen, G.-Q., 2011. Production of 3-hydroxypropionate homopolymer and poly(3-hydroxypropionate-co-4-hydroxybutyrate) copolymer by recombinant *Escherichia coli*. *Metab. Eng.* 13, 777–785.

