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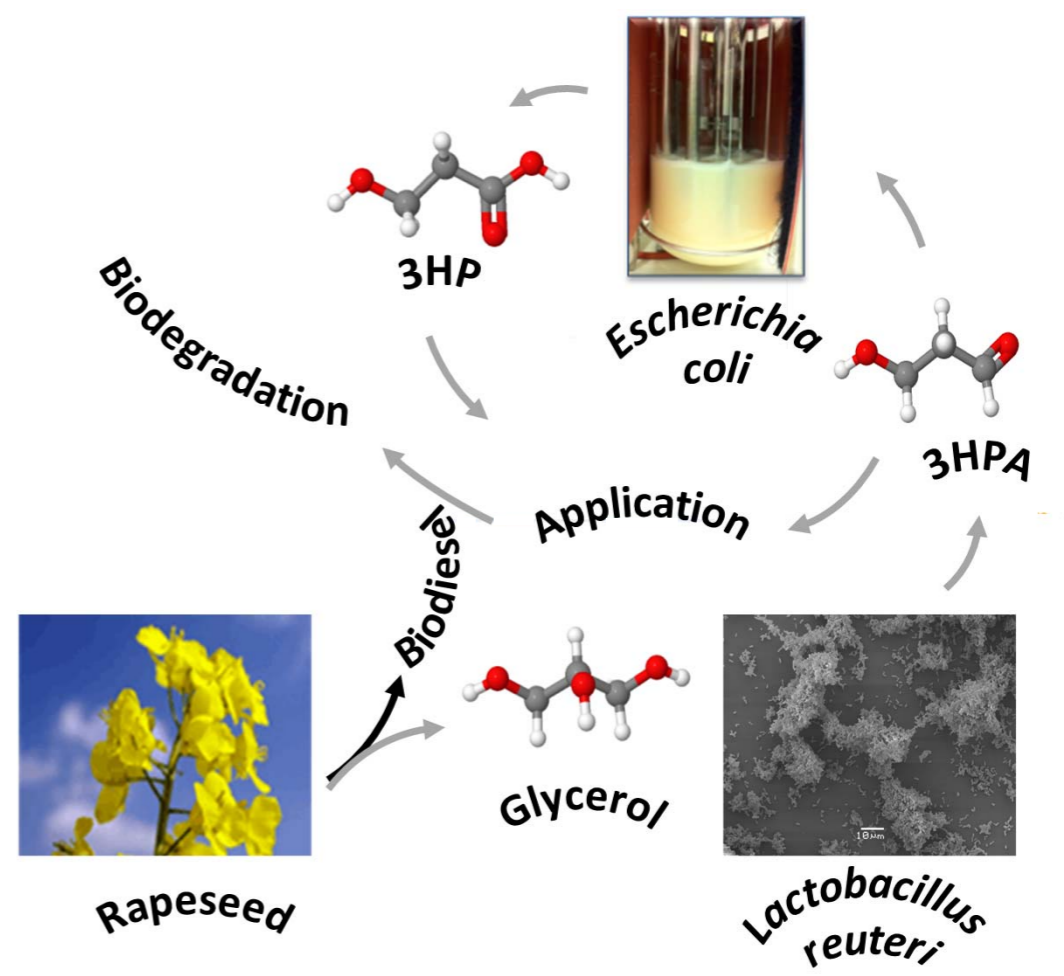


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Roya R.R. Sardari Bio-based C-3 Platform Chemical – Biotechnological Production and -Conversion of 3-Hydroxypropionaldehyde 2013

# Bio-based C-3 Platform Chemical Biotechnological Production and -Conversion of 3-Hydroxypropionaldehyde

Roya. R. R. Sardari



Department of Biotechnology  
Lund University

**Bio-based C-3 Platform Chemical**  
Biotechnological Production and -Conversion of  
3-Hydroxypropionaldehyde

Roya R.R. Sardari



**LUND**  
UNIVERSITY

Doctoral Dissertation  
December 2013

Academic thesis, which by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Wednesday, 11 December 2013 at 10:30 a.m. in Lecture Hall A at the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

The Faculty opponent is Dr. Adrie J. J. Straathof, Delft University of Technology, The Netherlands.

Roya Rezaei Ranjbar Sardari

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تقدیم به پدر و مادر عزیزم

*To my parents and my beloved family*

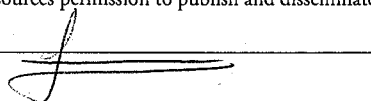


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Title and subtitle <b>Bio-based C-3 Platform Chemical</b> Biotechnological Production and -Conversion of 3-Hydroxypropionaldehyde	
<p>Abstract</p> <p>Demands for efficient, greener, economical and sustainable production of chemicals, materials and energy have led to development of industrial biotechnology as a key technology area to provide such products from bio-based raw materials from agricultural-, forestry- and related industrial residues and by-products. For the bio-based industry, it is essential to develop a number of building blocks or platform chemicals for C2-C6 chemicals and even aromatic chemicals. 3-hydroxypropionaldehyde (3HPA) and 3-hydroxypropionic acid (3HP) are potential platform chemicals for C3 chemistry and even for producing polymers.</p> <p>This thesis presents investigations on the biotechnological routes for the production of a C3 platform chemical, 3HPA from glycerol and its conversion to 3HP. Glycerol, was used as the raw material for production of 3HPA using resting cells of the probiotic bacteria, <i>Lactobacillus reuteri</i>, as the biocatalyst. The antimicrobial effect of the bacteria is attributed to the secretion of "reuterin" that is an equilibrium mixture of 3HPA with its dimer and hydrate forms. Glycerol dehydratase, a Vitamin B12-dependent enzyme, presents in <i>L. reuteri</i>, catalyses the dehydration of glycerol to 3HPA. Production of 3HPA at high concentration results in strong inhibition of the enzyme activity and cell viability, which in turn limits the product yield and -productivity. Different means of <i>in situ</i> capture of 3HPA from the reaction were studied. Complexation of 3HPA with bisulfite in a fed-batch biotransformation of glycerol and subsequent removal through binding to an anion exchange resulted in increase in the production of 3HPA to 5.33 g/g biocatalyst from 0.45 g/g in a batch process. In another approach, <i>in situ</i> removal of 3HPA using semicarbazide-functionalized resin in a batch process, productivity was enhanced 2 fold than that without the resin.</p> <p><i>L. reuteri</i> metabolizes 3HPA further to 1,3-propanediol (1,3PDO) and 3-hydroxypropionic acid (3HP) by reductive and oxidative pathways, respectively. The oxidative pathway, comprises 3 enzymes named propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW). Kinetic characterization and molecular modelling of the first enzyme, PduP, expressed in <i>Escherichia coli</i> was performed. The enzyme had a specific activity of 28.9 U/mg using propionaldehyde as substrate and 18 U/mg with 3HPA as substrate which is the highest specific activity reported up to date. All the Pdu enzymes were then expressed in <i>E. coli</i> in different combinations and used for bioconversion of 3HPA produced by native <i>L. reuteri</i>. Growing cells of the recombinant bacteria with all the three enzymes, <i>E. coli</i> pdu:P:L:W in a fed-batch mode gave 3HP yield of 0.5 mole/mole 3HPA with 1,3PDO as the co-product, while the resting cells gave 3HP yield of 1 mole /mole 3HPA. This showed the possibility of using of Pdu pathway of <i>L. reuteri</i> for production of 3HP.</p>	
Key words Glycerol, 3-hydroxypropionaldehyde, 3-hydroxypropionic acid, biotransformation, <i>Lactobacillus reuteri</i> , <i>In situ</i> complexation.	
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# Abstract

Demands for efficient, greener, economical and sustainable production of chemicals, materials and energy have led to development of industrial biotechnology as a key technology area to provide such products from bio-based raw materials from agricultural-, forestry- and related industrial residues and by-products. For the bio-based industry, it is essential to develop a number of building blocks or platform chemicals for C2-C6 chemicals and even aromatic chemicals. 3-hydroxypropionaldehyde (3HPA) and 3-hydroxypropionic acid (3HP) are potential platform chemicals for C3 chemistry and even for producing polymers.

This thesis presents investigations on the biotechnological routes for the production of a C3 platform chemical, 3HPA from glycerol and its conversion to 3HP. Glycerol, was used as the raw material for production of 3HPA using resting cells of the probiotic bacteria, *Lactobacillus reuteri*, as the biocatalyst. The antimicrobial effect of the bacteria is attributed to the secretion of “reuterin” that is an equilibrium mixture of 3HPA with its dimer and hydrate forms. Glycerol dehydratase, a Vitamin B<sub>12</sub>-dependent enzyme, presents in *L. reuteri*, catalyses the dehydration of glycerol to 3HPA. Production of 3HPA at high concentration results in strong inhibition of the enzyme activity and cell viability, which in turn limits the product yield and -productivity. Different means of *in situ* capture of 3HPA from the reaction were studied. Complexation of 3HPA with bisulfite in a fed-batch biotransformation of glycerol and subsequent removal through binding to an anion exchange resulted in increase in the production of 3HPA to 5.33 g/g biocatalyst from 0.45 g/g in a batch process. In another approach, *in situ* removal of 3HPA using semicarbazide-functionalized resin in a batch process, productivity was enhanced 2 fold than that without the resin.

*L. reuteri* metabolizes 3HPA further to 1,3-propanediol (1,3PDO) and 3HP by reductive and oxidative pathways, respectively. The oxidative pathway, comprises 3 enzymes named propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW). Kinetic characterization and molecular modelling of the first enzyme, PduP, expressed in *Escherichia coli* was performed. The enzyme had a specific activity of 28.9 U/mg using propionaldehyde as substrate and 18 U/mg with 3HPA as substrate which is the highest specific activity reported up to date. All the Pdu enzymes were then expressed in *E. coli* in different combinations and used for bioconversion of 3HPA produced by native *L. reuteri*. Growing cells of the recombinant bacteria with all the three enzymes, *E.*

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# Popular Summary

Microbes are present everywhere in the environment and have quite an intimate relationship with humans. Much of our perception about microbes is as disease causing agents but on the other hand it is also the microbes that present cure for the disease. Besides using microbes for traditional applications, for example for providing antibiotics, production of fermented foods, etc., humans are increasingly taking advantage of the "good" microbes as probiotics for improving health, wastewater treatment, mining of metals, cleaning the environment, and for producing bioenergy, chemicals and materials. The micro-sized organisms contain a complex network of metabolic pathways involving a large number of chemical reactions catalysed by enzymes for utilizing different substances in the environment and converting them to a variety of products.

Lactic acid bacteria comprise an important group of microbes that humans have used for thousands of years to conserve and enhance the nutritional value of sensitive foods. *Lactobacillus* species are a major part of this group. Some *Lactobacillus* species are used for the production of yoghurt, cheese, sauerkraut, pickles, beer, wine, cider several fermented foods, as well as animal feeds, such as silage. *Lactobacillus reuteri* is a major component of the bacteria present in guts of mammals and birds. It has been shown that several different strains of *L. reuteri* have a positive effect on health, including various types of gastrointestinal disorders and oral health. In the late 1980s, it was discovered that *L. reuteri* produced a novel broad-spectrum antibiotic substance by fermentation of glycerol, which was named as "reuterin" after Gerhard Reuter. Reuterin can inhibit the growth of some harmful Gram-negative and Gram-positive bacteria, along with yeasts, fungi and protozoa. Reuterin is a mixture of three components, made of 3-hydroxypropionaldehyde (3HPA) and its derivatives.

This thesis is about 3HPA as a molecule of interest for the chemical industry based on renewable resources. Today, as we become increasingly aware of our dependence on fossil resources to fulfill our needs, and the environmental problems associated with the use of these non-renewable resources, there is a growing interest in the use of renewable resources as raw materials and environment-friendly methods for the production of chemicals, materials and energy. 3HPA is currently not a commercial product. If it could be economically produced from glycerol using the bacteria it can potentially be used as a building block or "platform" for several other chemicals with 3 carbon atoms (C3), e.g. 1,3-propanediol (1,3PDO), 3-hydroxypropionic acid (3HP), acrolein, etc.

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Glycerol, commonly known as glycerine, is produced as a side product of hydrolysis of fats, production of ethanol and biodiesel. Over the past decade or more, biodiesel is being produced from several plant oils such as rapeseed-, soybean- and palm oil, and also from used oils. In this thesis, conversion of glycerol to 3HPA using *L. reuteri* is investigated. When the 3HPA level reaches a certain limit, it starts to affect the cell viability and activity, hence inhibiting its own production. Different strategies to complex 3HPA were studied to improve its production.

*L. reuteri* has also the ability to convert 3HPA to 1,3PDO and 3HP via different pathways. In the thesis, the pathway for 3HP production has been introduced in standard bacteria, *Escherichia coli* by recombinant DNA technology and shown to be active. One of the enzymes of the pathway has further been studied.

The work in this thesis was done in collaboration with Perstorp AB, and was supported by Vinnova, the Swedish Governmental Agency for Innovation Systems.

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# List of Publications

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

- I Sardari, R.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. *Biotechnol. Bioeng.* 110, 1243-1248.
- II Sardari, R.R.R., Dishisha, T., Pyo, S.-H., 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. *J. Biotechnol.* <http://dx.doi.org/10.1016/j.jbiotec.2013.09.009> (Article in press).
- III Sardari, R.R.R., Dishisha, T., Pyo, S.-H., Hatti-Kaul, R., (2013) *In situ* removal of 3-hydroxypropionaldehyde during biotransformation of glycerol using a semicarbazide-functionalized resin (manuscript).
- IV 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. Tech.* 53(4):235-242.
- V Sabet-Azad, R., Sardari, R.R.R., Linares-Pastén, J.A., Hatti-Kaul, R., (2013) Heterologous expression of *Lactobacillus reuteri* propanediol pathway utilizing (Pdu) enzymes in *Escherichia coli* and their evaluation for the production of 3-hydroxypropionic acid from 3-hydroxypropionaldehyde (manuscript).

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# My Contribution to the Papers

The overall idea of the work was provided by Prof. Rajni Hatti-Kaul.

- I I have done the experimental part with 3HPA production and complexation. Tarek Dishisha designed the integration with fed-batch operation for *in situ* complexation. I performed the experiments, data analysis and writing the manuscript with Tarek under supervision of Dr. Sang-Hyun Pyo and Prof. Hatti-Kaul.
  - II I have done the experimental part on 3HPA production, purification and recovery. Tarek designed the integration with fed-batch operation with cell recycling for in situ recovery. I performed the experiments, data analysis and writing the manuscript with Tarek under supervision of Dr. Pyo and Prof. Hatti-Kaul.
  - III I designed and performed all the experiments, data analysis and writing of the first draft of the manuscript. Tarek, Dr. Pyo and Prof. Hatti-Kaul have contributed in formulation of the idea and revision of final manuscript.
  - IV I have contributed with 3HPA production and purification, and writing part of the paper relevant to 3HPA preparation.
  - V I have performed 3HPA production and purification, and also some of the experimental work on 3HP production, and writing part of the paper relevant to 3HPA preparation.
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# Abbreviations

1,3PDO	1,3-Propanediol
3HP	3-Hydroxypropionic acid
3HPA	3-Hydroxypropionaldehyde
CDI	1,1'-carbonyldiimidazole
CDW	Cell dry weight (g)
DCI	Deuterated water and hydrochloric acid
DCM	Dichloromethane
DHA	Dihydroxyacetone
DMF	N,N-dimethylformamide
<i>E. Coli</i>	<i>Escherichia coli</i>
Gly	Glycerol
LAB	Lactic acid bacteria
<i>L. reuteri</i>	<i>Lactobacillus reuteri</i>
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
PduP	Propionaldehyde dehydrogenase
PduL	Phosphotransacylase
PduW	Propionate kinase

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# 1. Introduction

Since the industrial revolution in the mid 19th century, humans have relied on fossil resources – first coal and then mineral oil and -gas - as the base for production of energy, chemicals and materials. The petrochemical industry has made tremendous advances and given us an enormous number of diverse products and totally transformed the modern society. It is difficult to think of any aspect of our lives that is not touched by the chemicals. About 90% of all the chemicals produced have their origin in fossil resources. However, towards the end of the 20th century, increase in the price of the fossil resources and the fear of their depletion due to increasing consumption by the growing global population has reduced the profitability of the chemical industry and forced it to look for alternative resources. Added to this has been the concern over the environmental issues like greenhouse gas emissions, toxic wastes and climate change for which much of the blame has gone to the unsustainable means of production for many chemicals produced from the fossil raw materials [1].

Use of renewable resources is an alternative to fossil resources that has the potential for providing carbon dioxide-neutral clean energy, and environmentally benign chemicals and materials that can be biodegraded once released into the environment after their useful span of life. Biomass from plants, algae, soil, animals, and related industries, constitute the most abundant renewable resource available for industrial production. It is now well understood that the increasing use of biomass for production of fuels, chemicals and materials will inevitably result in competition with food and other industrial sectors, such as forestry. In order to ensure sustainable bio-based production, biomass resources for industrial utilization have been distinguished as any organic by-products with low or no profit, including agricultural food and feed crop residues, wood and wood wastes and residues, grasses, dedicated energy crops and trees, microalgae, animal wastes, municipal wastes, agricultural or industrial waste streams, and other waste materials representing negative profit [2,3]. Industrial waste streams originating from paper making-, food processing-, and even biofuel industry have become important resources for value added production and to increase resource efficiency.

When shifting the raw material base, shift in the processing- and conversion technologies is inevitable. It is also of importance to learn from the structure of the

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petrochemical industry, i.e. to gain diversity from limited number of building blocks, the platform chemicals. These chemicals are transformed into a number of secondary chemicals, which are in turn converted to other downstream products and so on. As the biomass contains a wider range of components than the fossil resources, the potential diversity of products is likely to be large provided the technologies for conversion are available and competitive. The products from the bio-based industry could either possess the same molecular structure as the current fossil based ones or provide an alternative with similar functions [4, 5].

Biotechnology is gaining importance as a key technology area for biomass pre-treatment and further conversion to chemicals, materials and biofuels using microorganisms and enzymes [4]. From the enormous microbial diversity found in nature and the vast metabolic networks and the enzymes it offers, it always seems possible to find microorganisms with the ability to produce desired chemicals from different substrates. The microbial cells have also built up control mechanisms for their metabolic pathways such as feedback inhibition, which limits the formation of products to a level sufficient for their function and survival. Of course this, poses a bottleneck when the microorganisms are used for industrial production where a particular product needs to be produced at high titer and –productivity from the corresponding substrate at high concentration [6]. Different strategies can be used to overcome this limitation, by engineering the microorganism and/or the bio-process. Engineering the microorganism may involve addition or knockout of single or a number of genes or an entire metabolic pathway in the native or heterologous microbial host. At the level of the bioprocess, the fermentation/biotransformation is controlled by substrate addition, product removal, etc [7, 8].

The work presented in the thesis was performed within the framework of two research projects involving academic-industrial collaboration and funded by the Swedish Governmental Agency for Innovation Systems (VINNOVA): “Development of Process Technologies for Immobilized Biocatalysts” and “Industrial Biotechnology for Production of Platform Chemicals” (BioVINN).

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## Scope of the Thesis

This thesis focuses on the aspects of bioprocess development for production of a platform chemical from a renewable raw material. Glycerol, is used as the raw material for production of 3-hydroxypropionaldehyde (3HPA), a chemical produced only as an intermediate in the current chemical processes for 1,3-propanediol production from fossil based raw materials. 3HPA is also produced as a metabolic intermediate by different microorganisms, and exerts an antimicrobial effect. Microbial production of 3HPA as a platform chemical is challenging due to its inhibitory effect on the viability of the producer cells and the enzyme catalyzing its production. The thesis explores different means of *in situ* removal of 3HPA in order to reduce its inhibitory effect and in turn improve its production by probiotic bacteria, *Lactobacillus reuteri*. Furthermore, the thesis also investigates the possibility of utilizing the oxidative pathway in the same organism for further conversion of 3HPA to 3-hydroxypropionic acid (3HP).

The thesis is based on five papers, three of which are published.

**Paper I** describes the conditions for complex formation of 3HPA with sodium bisulfite; the complex forms an equilibrium with the free 3HPA. Addition of bisulfite during fed-batch biotransformation of glycerol reduces the product inhibition.

In **Paper II**, the 3HPA-bisulfite complex is adsorbed onto an ion exchange resin. The binding capacity of the resin for binding the complex was determined and the bound complex was eluted using sodium chloride. The system was integrated with the biotransformation and demonstrated increased productivity of 3HPA and increased biocatalyst operational lifetime.

**Paper III** reports a new method involving a semicarbazide-functionalized resin as an adsorbent for binding 3HPA, and elution using different reagents. *In situ* capture of 3HPA from a reaction mixture also led to improvements in the process.

**Paper IV** involves kinetic and structural characterization of the enzyme, CoA-dependent propionaldehyde dehydrogenase (PduP), which converts 3HPA to 3HP-CoA.

**Paper V** demonstrates the utilization of the three enzymes of the propanediol utilization pathway, PduP, PduL and PduW, engineered in *Escherichia coli* for

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conversion of 3HPA to 3HP. The conversion was studied using crude cell lysate, growing cells and resting cells of *E. coli*.

The chapters ahead provide a background of the area of research and also summarize the results obtained in the different papers. In Chapter 2 the application of biotechnology to the industry based on utilization of renewable resources with focus on bioprocess engineering is discussed. Chapter 3 provides information on lactic acid bacteria (LAB) and more specifically *Lactobacillus reuteri* belonging to the genus *Lactobacillus*. Chapter 4 deals with the platform chemical, 3HPA, its production using chemical and microbial processes, and means of *in situ* removal applied in the thesis work for improving its production. Chapter 5 highlights 3HP, another important platform chemical, and its production from 3HPA using a propanediol utilization pathway of *L. reuteri*. Finally some concluding remarks and suggestions for future studies are presented in Chapter 6.



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## 2. Industrial biotechnology for production of platform chemicals

Already before the petrochemical era, it was known that biotechnology, (although the term was not coined) could be used for the production of industrial chemicals. It has however been considered as an expensive, high tech tool by the chemical industry. With the developments and availability of advanced experimental tools and information in the last few decades, biotechnology has become a powerful technology for industrial production [9].

Also, with increasing global concern on environmental sustainability and resource efficiency, the growing role of biotechnology is being seriously considered [1]. Industrial biotechnology uses biological systems, such as whole cells or enzymes, as reagents or catalysts to make cleaner processes for transformation of renewable resources. Cleaner processes also provide economic and environmental benefits, and possibility to use renewable raw materials reduces our dependence on fossil resources. Industrial biotechnology is considered as a third wave in biotechnology after agriculture- and medical biotechnologies. There are currently some convincing examples showing the great potential of this technology, e.g. production of acrylamide, 1,3-propanediol, lactic acid, Vitamin B<sub>12</sub>, Cephalixin, etc [1,4,11-13].

Since the development of recombinant DNA technology in the 1980s, which enabled the expression of foreign genes in microbial cells, tremendous progress has been made in development of enzymes and microorganisms as industrial biocatalysts [14]. For example, protein engineering allows modification of protein activity, selectivity and stability by making rational- or random (directed evolution) changes in the amino acid composition, while metabolic engineering can be applied for altering the gene expressions in a metabolic pathway in a microorganism to form a product of interest. Using synthetic biology it is now possible to design organisms with desired metabolic pathways [1, 15].

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## 2.1. Biomass as raw material for chemicals

Biomass is a biologically-produced resource which refers to plants or plant-derived materials, human and animal wastes. The biomass potential for heat and power generation as well as chemical production is considerable [16]. In a bio-based chemical industry, industrial biotechnology tools and processes can be used for pre-treatment of biomass to simpler molecules and their further conversion to value added products [17]. Production of bioethanol from cane sugar and corn starch is an example of the largest bio-based product in volume using industrial biotechnology. Besides the issue of competition with food, it has been realized that cost and resource efficiency can be achieved by using biomass wastes and residues with low or no value from agriculture, forestry and related industries, as raw materials and by integrating the production of chemicals or other value added products with that of biofuels. This has led to increasing efforts on developing technologies including biotechnology for converting the various biomass components or by-products to chemicals, etc. For a successful bio-based economy, development of biorefinery on the lines of a petrochemical refinery will be important in order to add value to the raw material as well as achieve resource efficiency. This implies that the raw material is converted to a limited number of building blocks or platforms, which in turn are converted to a range of secondary products, and so on, for different applications [13, 18].

In the context of this thesis, glycerol was used as raw material for C3 platform chemicals.

### 2.1.1. Glycerol, an industrial by-product as raw material

Glycerol (or glycerine) a polyhydric alcohol with three hydroxyl groups, is formed as a by-product in the production of soaps by hydrolysis of fats, production of biodiesel by transesterification of fats, and production of bioethanol. During the World War II, it has also been produced from glucose by yeast, to be used as a component in trinitroglycerine. Glycerol has a wide range of applications in food, pharmaceutical, cosmetics, antifreeze, and many other industries [19].

There has been a considerable increase in the production of biodiesel during the past decade [20]; annual production of biodiesel in 2012 was 969 million gallons [21]. About 10 % of glycerol is formed during biodiesel production. The crude glycerol may contain anywhere between 38 to 96% glycerol and between 14-29% methanol [22]. It can be used in fertilizers and animal feed if the concentration of

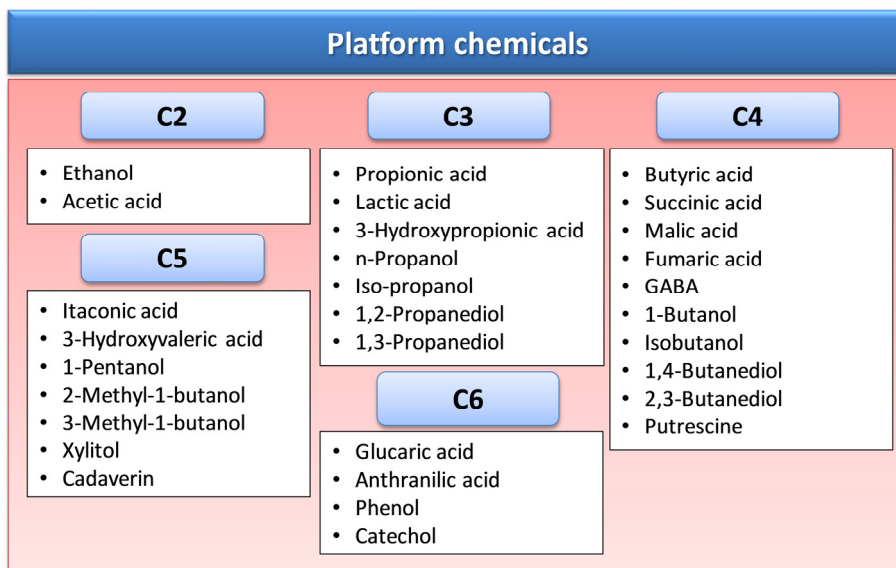
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impurities is controlled [22]. Other applications include as a boiler fuel to produce process steam [23], oxidation or reduction to other three carbon (C3) chemicals [19], and reaction with other substrates to produce higher carbon chemicals [21]. However, these approaches are limited by high viscosity and high autoignition temperature of glycerol in case of combustion, and expensive metal catalysis for synthesis of chemicals [21]. Yet another approach is microbial conversion of glycerol to value added chemicals.

### 2.1.2. Platform chemicals

Platform chemicals are intermediates with functional groups and are used as building blocks for production of a range of chemicals from the raw materials [10]. Production and conversion of the platform chemicals to wide range of marketable products is a very promising approach to make a sustainable market and reduction of biofuel production cost.

In the petrochemical industry, the naphtha fraction from the mineral oil, and gas are fractionated to aromatics (benzene, toluene, xylene) and olefins (ethylene, propylene, etc.) [24], which in turn are processed into other downstream products. Bio-based platform chemicals are biodegradable and the resulting carbon dioxide and water are used for production of plant biomass by photosynthesis [5, 18]. The functional groups on the platform chemicals can be used for different chemical reactions or polymerizations. In this respect, carboxylic acids, alcohols, polyols and aldehydes are the most important platform chemicals for the biobased industry [5]. (Fig. 2.1) shows a list of potential platform chemicals for the future industry.



**Figure. 2.1. Platform chemicals (C2-C6) as potential building blocks for the future with [5]**

Currently, lactic acid and succinic acid are produced industrially by fermentation. 3-Hydroxypropionic acid (3HP) is of special interest due to its application in the synthesis of biodegradable polymers such as poly(3-hydroxypropionic acid) [P(3HP)] with higher strength compared to poly (lactide) [25].

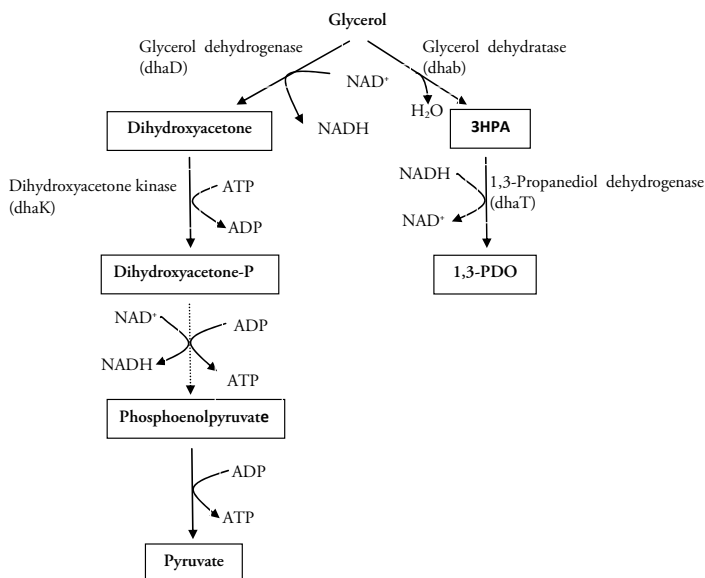
In this thesis, 3-hydroxypropionaldehyde (3HPA) produced from glycerol is considered as a platform chemical (**Papers I-III**) for conversion downstream to 3-hydroxypropionic acid (**Paper V**).

## 2.2. Biotechnological conversion of glycerol

Glycerol can be converted by enzymes as well as whole microbial cells to a range of products. Lipase enzymes can be used to perform esterification reactions between glycerol and different fatty acids [26]. The reactions, e.g. the sites of esterification and chain lengths of the fatty acids are very much dependent on the specificity and selectivity of the enzymes.

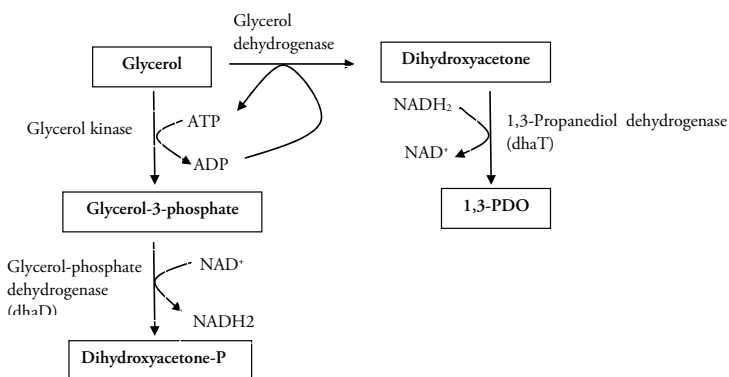
Glycerol is used as carbon source by several bacteria, yeasts and molds for fermentative production of important products such as 1,3-propanediol [9],

propionic acid [27], 3-hydroxypropionaldehyde [28], 3-hydroxypropionic acid, polyhydroxyalkanoates [29], etc. *Citrobacter*, *Klebsiella*, *Clostridium* and *Enterobacter* species grow on glycerol as the sole carbon and energy source under anaerobic conditions. The metabolism of glycerol involves an oxidative and/or reductive pathway. In the former, glycerol is oxidized by a dehydrogenase to dihydroxyacetone (DHA), which is phosphorylated by DHA kinase and enters the glycolysis pathway. In the reductive pathway, glycerol is converted to 1,3-propanediol via 3HPA as an intermediate (Fig. 2.2) [26, 30]. Several *Lactobacillus* strains cannot grow on glycerol, but instead use it as an electron acceptor and convert it to 1,3PDO via 3HPA [31, 32].



**Figure. 2.2. Anaerobic metabolism of glycerol**

*Pediococcus pentosceus* isolated from wine, can utilize glycerol aerobically and does not produce 3HPA. Conversion of glycerol to glycerol-3 phosphate is catalyzed by glycerol kinase before entering glycolysis. Glycerol is also converted to DHA by glycerol dehydrogenase. D-lactate and acetate are the main end products, while some amounts of diacetyl and 2,3-butanediol are also produced (Fig. 2.3) [30, 33].



**Figure. 2.3. Aerobic metabolism of glycerol**

## 2.3. Bioprocesses for production of chemicals: advantages and bottlenecks

In general, a bioprocess is divided into an upstream and a downstream part, the former involving the biocatalyst development and fermentation or bioconversion while the latter includes separation and recovery of the products of interest [4]. Most of the products for chemical industry are low molecular weight and are secreted outside the cells. Some exceptions are e.g. the biopolymers (polyhydroxyalkanoates) and the compatible solutes (such as ectoines) that are accumulated inside the cells. The products could either be associated with the growth of the cells or non-growth related involving single- or multistep bioconversions.

The attractive features of bioprocesses are that they are operated at moderate temperatures and atmospheric pressure and produce less toxic wastes and by-products in contrast to many chemical processes [1]. Production of ethanol from sugars, acetic acid from ethanol (1815) and vitamin C (more than 70 years ago) are three famous examples for the application of industrial biotechnology at industrial scale [34]. Currently, bioprocesses are used for the production of some C2–C6 chemicals [5]. For example, lactic acid is produced by *Sporolactobacillus* with a productivity of 3.8 (g / L .h) [35] and 1,3-propanediol is produced by engineered *E. coli* with a productivity of 3.5 (g / L .h) [36]. Another example is the bio-based production of glucaric acid and adipic acid (C6 chemicals) which are going to be

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scaled up by Rivertop Renewables in United State [5, 37]. No industrial microbial production of aldehydes has been reported [5].

Biotechnological production of chemicals should be efficient enough to be competitive with the chemical industry. The efficiency of a bioprocess is determined in terms of 3 parameters: product yield (g/g or mole/mole substrate), - titer (g/liter), and productivity (g/l.h) [6]. The economy of the process and even the environmental footprint are directly related to the increase in these parameters. High yield results in the need for less raw material, which can normally account for at least 50% of the process costs [38]. The yield of a product is directly related to the amount of raw material used in the process. Higher yield results in the use of less amount of raw material in a process. Moreover, the costly raw materials may be replaced by cheap and waste materials [39]. High product titer reduces the process volume to be handled for downstream processing. High productivity, i.e. “to obtain the highest amount of product in a given volume in the least amount of time” reduces the reactor/process volume [40].

The microbial bioprocesses have invariably low productivity in batch mode owing to the various control mechanisms existing in the cells, such as inhibition by substrate, product or the by-products [9]. Inhibition by the product is also the main cause of low product titre, while the formation of by-products e.g. by the growing cells complicates the downstream processing [4].

Fed-batch mode of fermentation by feeding the substrate at a defined rate is commonly used at industrial scale and is appropriate for lowering the inhibition by substrate [6]. Continuous fermentation in which the substrate feed and product withdrawal occurs continuously can limit product inhibition but also leads to cell washout from the reactor [4, 41]. Use of high cell density reactors in either of the fermentation modes has the possibility to give a robust process with higher productivity as the cells become more resistant to the inhibitory effects [40, 42]. Another attractive approach is to remove the product as it is being formed, i.e. *in situ*, in order to maintain its concentration below the inhibitory level [7].

Downstream processing is an important part of a bioprocess and is also a cost determining step. The target products are separated and purified using one of different separation techniques, based on their physical and chemical properties [4]. It comprises the steps of solid-liquid separation to remove the cells, purification by separation from the by-products and medium components, and formulation [43].

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### 2.3.1. High cell density biotransformations

Immobilization of cells to a solid matrix and recycling of free cells after centrifugation or membrane filtration are the common modes of achieving high density. A number of examples of systems with immobilized cells are available in the literature [40]. Entrapment in a polymer matrix [44] and adsorption to a porous support [27] are two common modes of cell immobilization. A variety of polymers have been used for entrapping cells, including alginate, carrageenan, polyacrylamide, agarose, etc. The porosity of the matrices is important for maintaining unhindered access to the substrates and also for removal of the product, at the same time not compromising the mechanical strength of the gels. In case of growing cells, leakage of cells is observed and leads eventually to rupture of the matrix.

The above problem is avoided by adsorbing the cells on the surface of a support using ionic or other forces. Such supports could be in the form of porous beads or fibrous beds providing large surface area for adsorption [4]. Some microbial strains are able to naturally adsorb to the surface building a biofilm e.g. with the help of polysaccharides produced by the cells. In other cases, biofilm formation can be induced by coating the support by a polycation to which the cells get attached through their negatively charged surface [27, 45]. The adsorbed cells achieve increased resistance to the inhibitory effects existing in the bioreactor [27].

Recycling of free cells after their separation by centrifugation or membrane filtration is an alternative approach used for achieving high cell density [42, 46, 47]. Sequential batch fermentation, by harvesting the cells by centrifugation in between the batches and returning to the fermenter, was recently reported for production of propionic acid from glycerol. The cell density was increased from 4 g cell dry weight per liter after first batch to 21-22 g/liter after 7th batch, which led to increase in propionic acid productivity from 0.2 g/l.h to 1.4 g/l.h [42].

Integration of membrane filtration with the bioreactor is a more common means used for cell recycling. The microbial cells are retained by the microfiltration membrane, while the liquid is removed as permeate. Although the filtration set up is integrated as an external device, even internal cell recycling system have been developed [27].



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### 2.3.2. *In situ* product removal (ISPR)

Most of the products including solvents, organic acids and aldehydes are toxic for the producer cells. In some cases, the product is degraded as soon as it is formed [8]. Both cases make the conditions unsuitable for cells which are used to be under natural physiological conditions [48]. *In situ* product removal or “extractive fermentation or bioconversion” is a technique to remove the product from the vicinity of cells as soon as it is produced [7]. The aim is to minimize the concentration of product in the reaction system allowing increase in cells lifetime, which maximize the yield and productivity of the process. It also reduces the formation of by-products, and as a result the number of downstream processing steps are reduced [7]. Different techniques have been used for *in situ* product removal such as evaporation, extraction, adsorption, crystallization [49], size selective permeation and reverse complex formation [7] and in some cases the combination of two or more can be used for a process. Integration of cell recycling [42] or cell immobilization [27] with a repeated batch or continuous process for product removal is a promising approach to increase the productivity of a bioprocess. An early example of a continuous process with cell recycling is the industrial production of bioethanol by *Saccharomyces cerevisiae* by Chematur in Sweden [41].

**Papers I-III** deal with *in situ* removal of the free 3HPA during its production from glycerol. In **Paper I**, the 3HPA is complexed with bisulfite and the complex remains in equilibrium with the free 3HPA, while in **Paper II** this equilibrium mixture is loaded on to an anion exchange resin that adsorbs the complex and shifts the equilibrium towards more complex formation. In **Paper III**, a semicarbazide functionalized resin is used to bind the 3HPA. In all the cases, increased 3HPA productivity was observed.



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## 3. *Lactobacillus reuteri*: from probiotic to a chemical factory

### 3.1. Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) with the historic name “milk-souring organisms”, are a group of gram-positive, nonsporing, nonrespiring cocci or rods, aerotolerant, acid tolerant, which produce lactic acid as a main end product [50]. The first pure culture of bacteria, “*Bacterium lactis*” was isolated in 1873 by J. Lister. LAB is involved in food and feed fermentations, and also associated with the body of humans and animals. Initially, LAB comprised four genera named *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* and now this group has around 20 genera. However, the principal LAB include *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*, from the food technology perspective [51].

*Lactobacillus* species are a major group of LAB known for its industrial applications. Some *Lactobacillus* species are used for the production of yoghurt, cheese, sauerkraut, pickles, beer, wine, cider several fermented foods, as well as animal feeds, such as silage. The homofermentative strains are also currently used for the production of lactic acid from cane sugar or corn starch. Bio-based L-lactic acid is used for the production of the biodegradable plastic, polylactate (PLA), as an alternative to the traditional route of production from fossil resources [52].

The different members of the LAB group are classified based on morphology (cocci or rods, tetrad forms), the pathway used for glucose fermentation (homo –or –heterofermentative), optimum temperature range for growth (10 - 45 °C), and the variety of sugars utilized [51].

### 3.2. *Lactobacillus reuteri*

*Lactobacillus reuteri* is a distinct species of the genus *Lactobacillus*, which is a wine-related LAB and has very important role in food and feed production and preservation as well as for probiotic properties [50]. The species, culture sources,

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nutritional and physiological characteristics and genetic stability of *L. reuteri* have been well identified [53, 54]. *L. reuteri* inhabits the gastrointestinal tract of humans and animals and birds [55]. Clinical trials have shown *L. reuteri* to be an effective treatment for a variety of ailments such as diarrhea and colic eczema [56, 57]. One reason for the probiotic efficacy of *L. reuteri* is its ability to convert glycerol to 3HPA that is a molecule with antimicrobial activity [58].

### 3.3. Growth and metabolic pathways

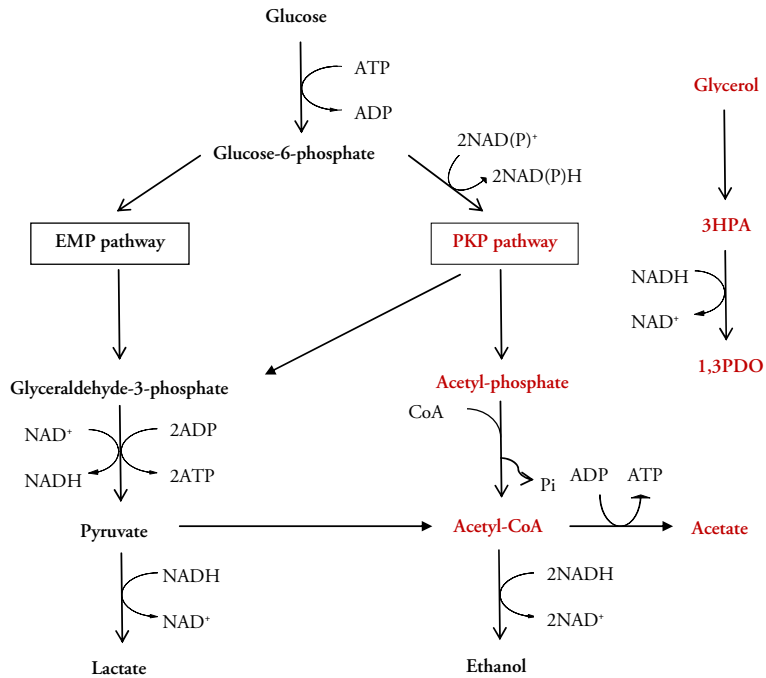
LAB grow in nutritionally rich media by homofermentative or heterofermentative pathways. In general, obligatory homofermentative LAB use the Embden-Meyerhof-Parnaz (EMP) (glycolysis) pathway and produce lactate as a sole end product, whereas heterofermentative LAB use the phosphoketolase (PKP) pathway and produce lactate, ethanol, acetate and carbon dioxide. Facultative heterofermentative LAB can use both pathways depending on the carbon source used in the process (Fig. 3.1) [59].

In homofermentative LAB, pyruvate is used as the main electron acceptor for regeneration of NADH accumulated during sugar metabolism via the glycolytic pathway. Simultaneously, pyruvate is reduced to lactic acid by lactate dehydrogenase. The glycolytic pathway generates more ATP than the PKP pathway [60]. On the other hand, the heterofermentative LAB utilizing glucose by the PKP pathway, use acetyl phosphate besides pyruvate as an electron acceptors yielding ethanol and lactate, respectively. The corresponding reactions are catalysed by bifunctional acetaldehyde-CoA/alcohol dehydrogenase and lactate dehydrogenase. In a parallel reaction, acetyl phosphate is converted to acetic acid with generation of 1 mole ATP per mole acetate formed. The ATP generated from the PKP can be increased to be as efficient as that of glycolysis by addition of an external electron acceptor. In this case, the flux split ratio of acetyl phosphate towards ethanol is decreased and more acetyl phosphate will be available for acetate and make PKP as efficient as EMP [51, 61, 62]. Examples of external electron acceptor are oxygen, glycerol, fructose, citrate, 1,2-propanediol, and 1,2-ethanediol [51].

*L. reuteri* is an obligate heterofermentative microorganism and uses both EMP and PKP pathways for growth on glucose under anaerobic conditions, giving acetate and ethanol as final products [53, 54, 62, 63]. The ethanol formation is due to a redox imbalance. Addition of an electron acceptor is able to balance the

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reduced equivalents produced in PKP pathway [63]. 3HPA produced from glycerol by glycerol-dehydratase can be an external electron acceptor for the PKP pathway (Fig. 3.1).



**Figure. 3.1. The glycolysis and phosphoketolase pathways using glucose as a carbon source and utilization of glycerol as an electron acceptor in *L. reuteri* [63]**



# 4. 3-Hydroxypropionaldehyde: from antimicrobial to platform chemical

## 4.1. 3-Hydroxypropionaldehyde

3-Hydroxypropionaldehyde, also known as 3-hydroxypropanal or 3-oxo-1-propanol, is a C-3 chemical that is a potential building block with high industrial interest for C-3 chemistry in the biobased chemical industry [10]. It serves as a precursor for the synthesis of 1,3-propanediol (1,3PDO), acrolein, 3-hydroxypropionic acid (3HP) and acrylic acid (Fig. 4.1) [28]. Production of 3HPA can be done by both chemical and biochemical transformations.

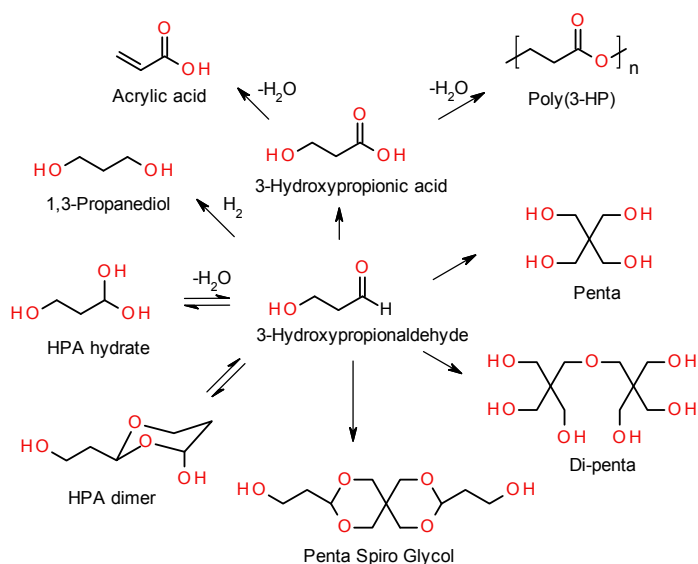
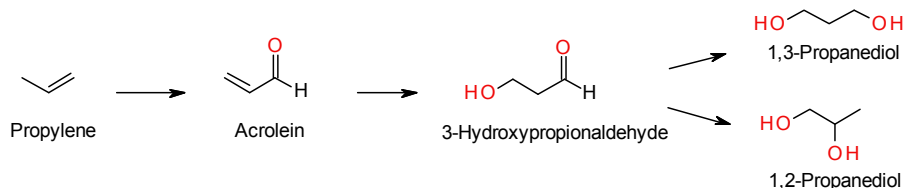


Figure. 4.1. Potential products from 3-hydroxypropionaldehyde

### 4.1.1. 3HPA production from fossil resources

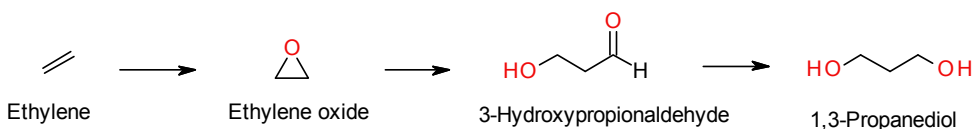
3HPA is currently formed as an intermediate in two different chemical processes for the production of 1,3PDO. The Degussa process uses propylene as substrate

that is converted to acrolein followed by its hydration to 3HPA at mild temperature and pressure [9]. 3HPA is then converted to 1,3-PDO and 1,2-propanediol by catalytic hydrogenation using rubidium catalyst at 90 bars. The yield of 1,3PDO from propylene is 65 % (Fig. 4.2) [9, 64, 65].



**Figure. 4.2. Degussa process for production of 1,3-propanediol from propylene via 3-hydroxypropionaldehyde**

The Shell process uses ethylene as a starting substrate that is oxidized to ethylene oxide. Subsequent hydroformylation of ethylene oxide with carbon monoxide and hydrogen at 150 bars gives 3HPA that is extracted into aqueous phase, and, then hydrogenated to 1,3PDO catalytically using nickel catalyst under high pressure with a final yield of 80 % [9, 66]. In both these processes, separation and purification of 3HPA are not feasible (Fig. 4.3).



**Figure. 4.3. The Shell process for production of 1,3-propanediol from ethylene via 3-hydroxypropionaldehyde**

#### 4.1.2. Microbial production of 3HPA

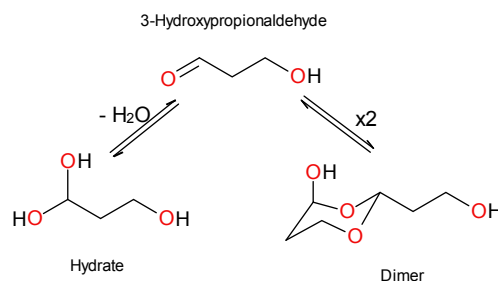
Microbial production of 3HPA was discovered in 1910; The hydroxyl aldehyde is formed as an intermediate during formation of acrolein from glycerol, and was one of the reasons for wine spoilage [67]. In nature, 3HPA is produced from glycerol anaerobically by several microorganisms such as *Bacillus* [68], *Klebsiella (Aerobacter)* [69], *Citrobacter* [70], *Enterobacter* [71], *Clostridium* [72] and *Lactobacillus* [55] and has been suggested to be responsible for their antimicrobial activity and bitterness during wine maturation. It is mainly formed as an intracellular intermediate. The only bacteria which excrete 3HPA as an extracellular



intermediate are those belonging to the genus *Lactobacillus* [30]. Glycerol is dehydrated to 3HPA in a reaction catalyzed by glycerol dehydratase [72, 73]. 3HPA is further reduced to 1,3PDO in a reductive pathway, while in some microorganisms such as *K. pneumonia*, *L. reuteri* and *L. collinoides* it is also oxidised to 3HP in a oxidative pathway [29]. Among all the bacteria producing 3HPA, *K. pneumonia* and *L. reuteri* have shown the production of significant amounts of 3HPA with high tolerance against 3HPA toxicity [58]. 3HPA produced by *K. pneumonia* is intracellular which needs a scavenger to excrete it in the media while the product is secreted out of the cells in *L. reuteri* [74].

## 4.2. HPA system (reuterin)

3HPA together with its dimer form [75] and hydrate form [76] constitute a multicomponent system named HPA system (reuterin). All three components are in an equilibrium in aqueous solution (Fig. 4.4) [77].

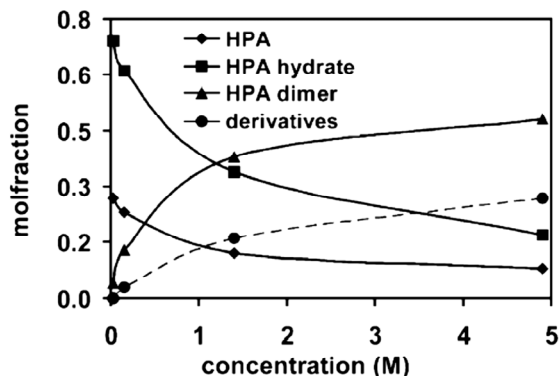


**Figure 4.4. Concentration-dependent equilibrium of 3HPA with its dimer and hydrate**

The molar concentration of 3HPA, dimer and hydrate together as HPA system is determined by tryptophan-HCl (acrolein) assay [77, 78]. This colorimetric method developed by Circle et al [78], and modified Ulmer et al and Vollenweider et al. [77, 79]. 3HPA is dehydrated to acrolein in the presence of concentrated HCl. Acrolein reacts with tryptophan yielding a purple complex [80]. The absorbance of the purple complex is measured spectrophotometrically at 560nm.

The first quantitative determination of the composition of 3HPA, dimer and hydrate has been done using quantitative <sup>13</sup>C NMR in aqueous solution at pH 4.1-7 and temperatures of 4 and 20 °C [77]. The data showed that the composition of HPA system is only dependent on the concentration and not pH. The dimer form of HPA system is dominant at high concentration (4.9 M), is equal with the

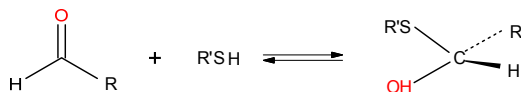
hydrate form at concentration of 1.2 M, is equal with monomer at the concentration of 0.4 M, and is very low at the concentration of 0.03 M when the hydrate form is predominant (69%) and the monomer is 27% of the mixture which means that hydration of HPA system is increased with dilution in aqueous solution(Fig. 4.5) [28].



**Figure. 4.5. Composition of HPA system in aqueous solution at 20 °C [77]**

The stability of HPA system is affected by both pH and temperature. HPA system is unstable under strong acidic conditions (12% DCI) and is destroyed after one day, while showed more stability in a basic environment (pH 8.9) (degraded in 7 days) [77]. Different forms of HPA system in acidic and basic aqueous solutions are aldol dimer and -trimer, acetal tetramer and -trimer, and hemiacetal dimer [81]. HPA system is gradually dehydrated to acrolein at room temperature (20 °C), and is stable at 4 °C for at least 5 months [77].

HPA system (reuterin) is a potent antimicrobial system, which can be used as an agent for sterilization and for fixation of biological tissues with less cytotoxicity instead of glutaraldehyde [82] in the pharmaceutical industry and as a food preservative in food industry [56, 77]. The aldehyde form (3HPA) is the only bioactive component of the HPA system [56]. The interaction of the carbonyl bond of 3HPA, which is highly reactive, with thiol group of small molecules and proteins induces oxidative stress which causes growth inhibition [56]; hemithioacetals are formed by the reversible reactions between thiol groups and 3HPA (Fig. 4.6).



**Figure. 4.6. Formation of hemithioacetals by reversible reaction between an aldehyde and a thiol [56]**

Hence, the concentration of HPA system should be at least 1 mM to inhibit the bacterial growth *in vitro* [56, 58]. The bacteria lose their activity and viability due to toxicity of 3HPA. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for *Escherichia coli* are 7.5-15 mM and 15-30 mM, respectively. *L. reuteri* and *K. pneumonia* cells are more resistant to 3HPA with MIC and MBC of 30-50 mM and 60-120 mM, respectively [58].

### 4.3. Glycerol/diol dehydratase

Two different enzymes called glycerol dehydratase (glycerol hydro-lyase, EC 4.2.1.30 ) and diol dehydratase (D,L-1,2-propanediol hydro-lyase, EC 4.2.1.28) catalyse the production of 3HPA from glycerol and 1,2 propanediol, respectively [83]. Both glycerol and diol dehydratase have similar catalytic properties with slightly different substrate specificity [73]. They are cobalamin (B<sub>12</sub>)- dependent enzymes in *L. reuteri* , *K. pneumonia* [73], and many *Enterobacteriaceae* [84, 85] and B<sub>12</sub>-independent enzymes in *C. butyricum* [72]. The genes encoding the enzymes, are expressed by induction of small quantities of glycerol/1,2 propanediol in the cultivation media in *L. reuteri* [6]. The reaction is catalysed by a free radical mechanism. Coenzyme B<sub>12</sub> contains a covalent carbon-metal bond (C-Co) which is stable in aqueous solution, but easily broken homolytically in the enzymatic reaction, and generates free radicals. Then the enzymes utilize the free radicals to catalyse the reactions. Hence, the coenzyme B<sub>12</sub> is an essential cofactor in this enzymatic reaction. The B<sub>12</sub>-independent enzymes utilize coenzyme S-adenosylmethionine (SAM) instead of B<sub>12</sub> [86].

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## 4.4. Production of 3HPA from glycerol

3HPA is produced from glycerol mostly as an intermediate intracellular and reduced to 1,3PDO by an NAD<sup>+</sup>- dependent oxidoreductase. The production of 3HPA and 1,3PDO can be altered by adding glucose; addition of glucose to the system changes the redox balance in the cells. The NADH produced in glycolysis preferentially oxidized using glycerol as an external electron acceptor [87]. Hence, addition of high amount of glucose results in the production of 1,3PDO as the end product.

Utilization of glycerol in the presence of glucose by growing cells of *Lactobacilli* results in accumulation of 3HPA at glucose : glycerol molar ratio of 0.125 - 0.25 [87, 88].

Production of 3HPA from glycerol can be achieved using either growing cells (i.e. fermentation) or resting cells (bioconversion) of *L. reuteri* [63]. In the process using growing cells, a mixture of glucose and glycerol is used, the former for growth and the latter for conversion to 3HPA [89]. Therefore, the fermentation broth contains 3HPA as well as 1,3PDO, organic acids (lactate and acetate) and ethanol as by-products. The production of 1,3PDO leads to production of acetate in PKP pathway and reduction of ethanol in glycolysis pathway [89]. The accumulation of 3HPA was higher than 1,3PDO at the molar ratio of glucose to glycerol less than 0.33. In contrast, 1,3PDO is the main product at the molar ratio of glucose to glycerol more than 1.6 [90].

Entracellular 3HPA is produced from glycerol by growing cells of *E. agglomerans*, *K. pneumonia*, and *C. freundii*, and convert to 1,3PDO extracellular. 3HPA is secreted to the medium at high concentration, but the maximum concentration is 30, 24, and 17 mM with *E. agglomerans*, *K. pneumonia*, and *C. freundii*, respectively, during glycerol (760 mM) fermentation [71].

In the process using resting cells of *L. reuteri*, active cells harvested from cultivation media, are resuspended in aqueous solution of glycerol for conversion to 3HPA [87], (Paper I-III).

The application of using a two-stage process is well suited for production of 3HPA due to less by-products formed. Production of active cells by anaerobic fermentation of glucose for 8 hours and using them as biocatalyst for conversion of glycerol (200 mM) in deionised water resulted in production of 170 mM 3HPA [55, 77]. However, the bioconversion of glycerol to 3HPA is discontinued within 2

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hours due to product inhibition [90]. The highest yield of 3HPA of 85% from 18.4 g/l glycerol using 30 g cell dry weight /l *L. Reuteri* cells has been reported [28].

The lowest yield reported is 2 mol% from 70 g/l glycerol using *C. freudii* [91]. The reaction is also inhibited by the substrate glycerol. The use of a fed-batch operation by adding glycerol gradually showed an improvement in biocatalyst activity to 5 hours and increase in 2.6 times higher 3HPA production than the batch system per gram cell dry weight (Paper I).

## 4.5. *In situ* removal of 3HPA

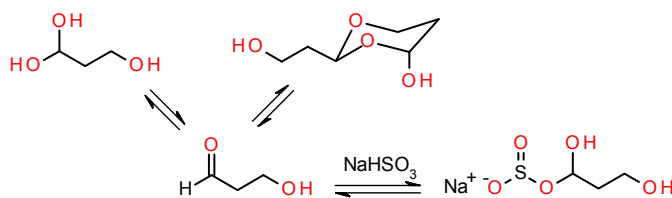
The highly reactive carbonyl group of 3HPA is used for different kind of reactions including nucleophilic addition reactions which produce adducts, alcohols, diols, cyanohydrins and amines [92], and condensation (addition - elimination ) reactions which produce oxime or semicarbazone [93]. These two reactions are used mainly for identification, protection, purification and removal of unwanted aldehyde from the reaction mixture [92]. One of the nucleophilic addition reactions is reversible bisulfite adduct formation which is used in beer and wine production. Oxidation of acetaldehyde to form acetic acid is inhibited by formation of bisulfite-acetaldehyde adduct [92]. Condensation reaction of aldehydes with semicarbazide to form semicarbazone derivatives is another well-known reaction for the isolation, purification and characterization of aldehydes [94].

*In situ* complex formation of HPA system with bisulfite, semicarbazide [79] and carbohydrazide [74] have been investigated for the production of HPA system and have resulted in increased yield and productivity of 3HPA, and biocatalyst life time (Paper I-III).

### 4.5.1. Adduct formation of 3HPA with sodium bisulfite

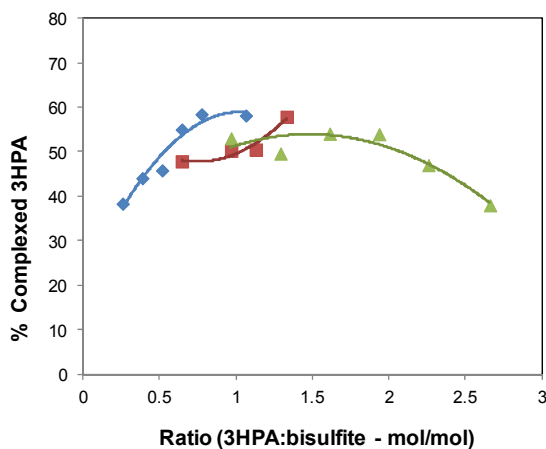
Bisulfite adduct formation with 3HPA is a useful procedure for removal of the aldehyde, since the reaction is reversible and regeneration of 3HPA is possible using sodium hydroxide, sodium chloride or bicarbonate [95]. An equilibrium is formed between the adduct and the free aldehyde (Fig. 4.7).

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**Figure. 4.7. Reversible formation of 3HPA-bisulfite adduct from 3HPA and bisulfite**

The position of this equilibrium is changed by change in the concentration of HPA system or bisulfite in the mixture, pH of the mixture and temperature. Increase in the concentration of bisulfite is not desirable because of its inhibitory effect on the biocatalyst (at the concentration of 50 mM) [95]. A maximum of 40-60 % adduct was formed at 3HPA:bisulfite molar ratio in the range of 0.8-2 (Fig. 4.8).



**Figure. 4.8. Effect of molar ratio of (3HPA : bisulfite) on the formation of 3HPA -bisulfite adduct at the bisulfite solution concentration of 100mM (♦), 80 mM(■) and 40 mM (▲) of bisulfite solution**

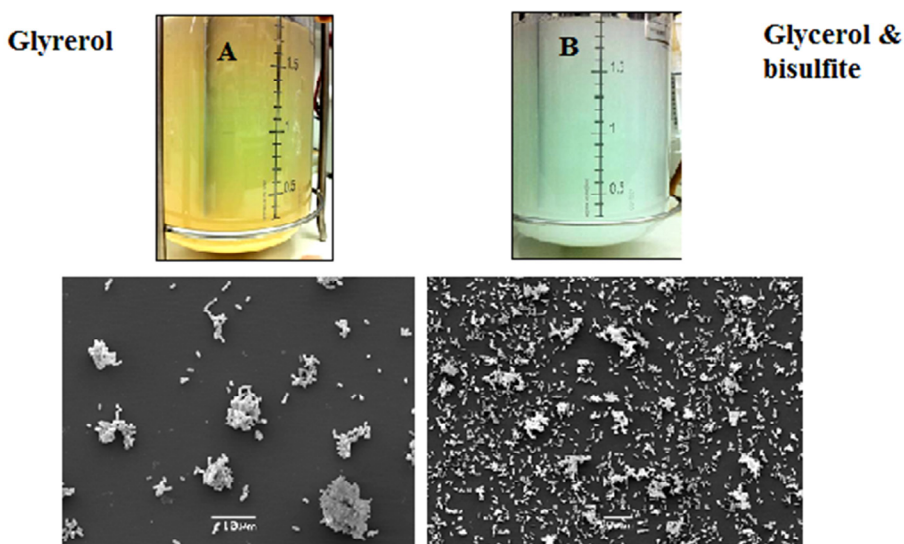
pH is another important parameter for 3HPA - bisulfite adduct formation.  $\text{SO}_2$ ,  $\text{HSO}_3^{-1}$  and  $\text{SO}_3^{-2}$  are the different forms of ions at pH less than 2, between (2-7) and higher than 7, respectively [96, 97]. pH 5 was seen to be optimum for the adduct formation and also for the production of 3HPA (Paper I).

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#### 4.5.1.1. Biotransformation of glycerol to 3HPA using *L. reuteri* in a fed batch system with integrated *in situ* complexation with bisulfite

*In situ* complex formation of 3HPA with bisulfite during biotransformation of glycerol using *L. reuteri* in a fed-batch mode reduced the toxicity of 3HPA to the biocatalyst. As a result, the biocatalyst life time was up to 18h and 3HPA produced per gram biocatalyst was 5.7 times and 2.2 times higher than that in the batch system and fed-batch system without *in situ* adduct formation, respectively (Paper I).

Scanning electron microscopy of *L. reuteri* cells in the solution without bisulfite showed the ability of 3HPA to aggregate the cells by cross-linking (Fig. 4.9) [82].



**Figure. 4.9.** Appearance of the reaction mixture and scanning electron microscopy of *L. reuteri* in: (A) fed-batch process, and (B) fed-batch process with *in situ* adduct formation of HPA system with bisulfite

Since the 3HPA-bisulfite adduct formation is not a complete reaction, the 3HPA concentration is increased in the solution with time, and the productivity was decreased from 1.5 g/h during the first 5.5 hours to 0.5 g/h between 5.5 and 18 hours (Paper I).

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The possibility to trap the 3HPA-bisulfite adduct such as by binding to a resin, would shift the equilibrium to formation of more adduct as well as separation of 3HPA from the by-products, glycerol, 1,3PDO and 3HP.

#### 4.5.1.2. Recovery of 3HPA by complexation with bisulfite and adsorption to Amberlite IRA-400 (Cl form)

Downstream processing of aldehydes in chemical industry is done using different methods such as distillation [98], pervaporation [99], solvent extraction [100], gas stripping [101]. These techniques are energy consuming and are not applicable for 3HPA due to its high sensitivity to heat [95] and high boiling point (168 °C). 3HPA is also highly soluble in water and highly hydrophilic which limits its removal by organic solvents.

Adsorption to a resins, is a common method for separation of several products from a complex mixture [43].

Free 3HPA has no binding to different resins such as Amberlite IRP-69, Amberlite XAD-16, and Amberlite IRA-400 (Cl form) and an irreversible binding to Amberlite CG-400 (OH form). Rutti et al. (2011) have earlier reported quantitative binding and desorption of 3HPA to Amberlite IRA-400 (Cl form) with bisulfite as a ligand [95].

##### 4.5.1.2.1. Binding capacity of Amberlite IRA-400 (Cl form) for 3HPA

In **Paper II**, different resins such as Amberlite IRA-400 (Cl form), Amberlite IRA-400 (OH form), Amberlite IRA-78 (OH form), and Dowex 66 (free base) were evaluated for binding the equilibrium mixture of 3HPA and 3HPA-bisulfite adduct in a batch system. Amberlite-400 (Cl form) showed good adsorption with a maximum binding capacity of 2.9 mmol 3HPA-bisulfite adduct /g by evaluation with Langmuir model. By performing adsorption in a column and determining a breakthrough curve, binding capacity of 2 mmol 3HPA and adduct/g resin was determined.

A highly pure mixture of 3HPA-bisulfite adduct and free 3HPA at a ratio of 0.77 mol/mol, free from glycerol, 1,3-PDO and 3-HP was obtained by elution from the resin using 0.2M NaCl (**Fig. 4.10**).



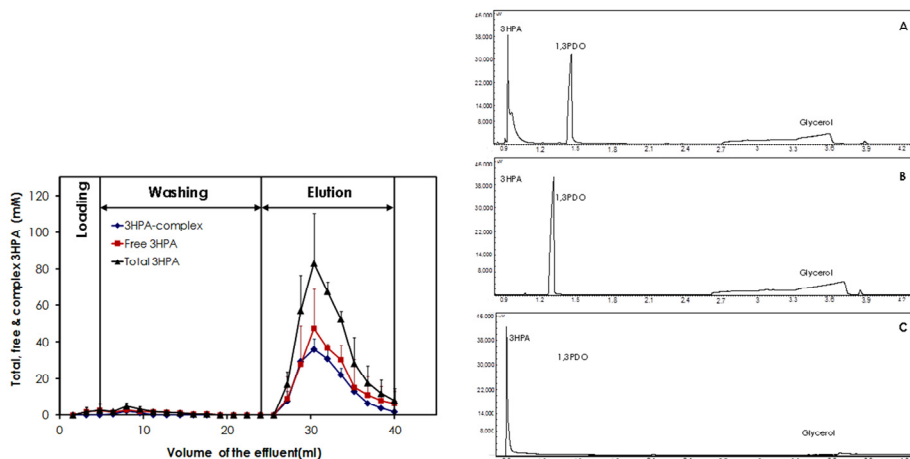
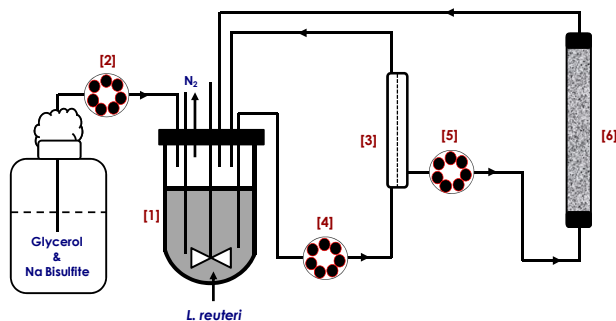


Figure. 4.10. Chromatographic profiles of 3HPA-bisulfite complex (♦), free 3HPA (■) and total 3HPA (▲) on loading 5 mL solution containing 3HPA-bisulfite complex and free 3HPA over 1g Amberlite IRA-400 (Cl) packed in a column, followed by washing with deionized water, and elution with 0.2 M NaCl, and gas chromatogram of (A) the reaction mixture containing 3HPA, 1,3PDO, and remaining glycerol prior to passing over the column of Amberlite IRA-400 (Cl) resin, (B) 3HPA containing solution eluted from the resin by 0.2 M NaCl, and (C) unbound fraction containing 1,3-PDO, glycerol and very low amount of 3HPA

#### 4.5.1.2.2. *In situ* removal of 3HPA and 3HPA-bisulfite adduct by integration of binding to Amberlite IRA-400 (Cl form) to fed-batch bioconversion by *L. reuteri*

An integrated system was set up as shown in Fig. 4.11 in which a packed bed column of Amberlite IRA-400 was coupled to the bioreactor intercepted by a membrane filter for retaining the cells in the bioreactor. The bioreactor containing *L. reuteri* cells with 21.7 mM glycerol and 10.86 mM sodium bisulfite was fed after 1h of batch biotransformation with a mixture of glycerol (802mM) and sodium bisulfite (401 mM) at molar ratio of 2:1, which is in the range of optimum ratio for 3HPA-bisulfite adduct formation considering the conversion of 1 mol glycerol to 1 mol 3HPA.

The reaction suspension was re-circulated over the membrane filter, and the cell-free permeate was then passed over the packed bed of the resin.



**Figure. 4.11.** Bioreactor system used for biotransformation of glycerol to 3HPA using resting cells of *L. reuteri*, with *in situ* adduct formation of 3HPA and recovery on an anion exchange resin. The cells were retained by a tangential flow microfiltration module (3) integrated with the bioreactor (1). Pump (2) was used to feed the mixture of glycerol and sodium bisulfite to the bioreactor at a rate of 0.92 mL/min. The reaction suspension with the cells was circulated between the reactor and the microfiltration unit at a rate of 30-40 mL/min using pump (4). The cell free permeate was circulated to the chromatographic column packed with 50g Amberlite IRA-400 (Cl) resin pre-functionalized with sodium bisulfite (6) using pump (5) at a rate of 6 mL/min

Using Amberlite 400 (Cl form) without prior pre-functionalization with saturated bisulfite solution, resulted in suppressed production of 3HPA due to release of chloride from the resin into the reactor resulting in the formation of NaCl that has an inhibitory effect on the production of 3HPA [90]. Bisulfite – functionalized resin is almost free of chloride and overcomes the toxicity of free 3HPA through binding to the Amberlite-400 ( $HSO_3$ ). As shown in figure Fig. 4.12 the consumption rate of glycerol for production of HPA was initially higher (3.77 g/h between 2 to 9 hours) and was reduced to 1.21 g/h towards the end.

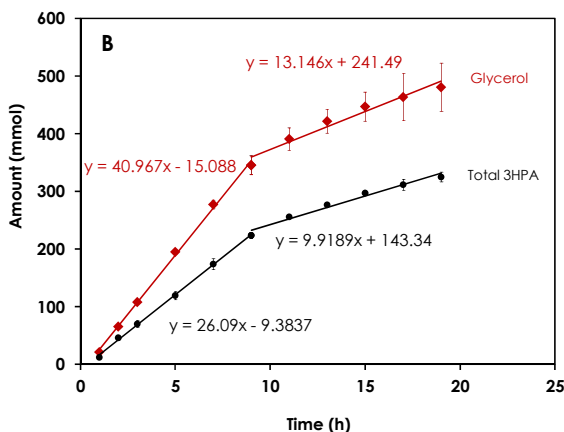


Figure. 4.12. Kinetics of glycerol consumption ( $\blacklozenge$ ) and 3HPA production ( $\bullet$ ) in the process of production of 3HPA from glycerol using *L. reuteri* in a fed-batch system with *in situ* complexation using sodium bisulfite and separation through binding to 50 g Amberlite IRA-400 ( $\text{SO}_3\text{H}$ )

The total amount of 3HPA per gram biocatalyst produced in this system was 1.75 times higher than just with *in situ* adduct formation.

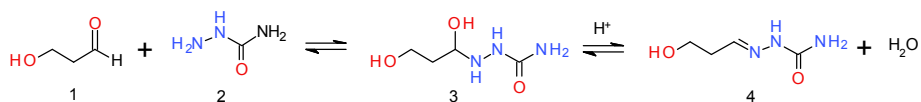
#### 4.5.2. Complex formation of 3HPA with semicarbazide and carbohydrazide

Using semicarbazide and carbohydrazide as scavengers has considerably enhanced the productivity of 3HPA [69, 74, 79, 102].

*In situ* removal of 3HPA using semicarbazide during production by growing cells of *K. pneumonia* in a batch fermentation of glycerol (333mM) resulted in accumulation of 3HPA at a concentration of (177 mM) as a complex with semicarbazide with a yield of 0.54 (mole 3HPA/ mole glycerol) [69, 102].

Bioconversion of glycerol in a batch system using resting whole cells of *K. pneumoniae* under oxygen limiting conditions resulted in the accumulation of 3HPA (474 mM) that was higher than that in anaerobic conversion (398 mM) [79]. Moreover, higher concentration of 3HPA (729 mM) was achieved by addition of semicarbazide in a fed-batch two-step process with oxygen limiting condition [79].

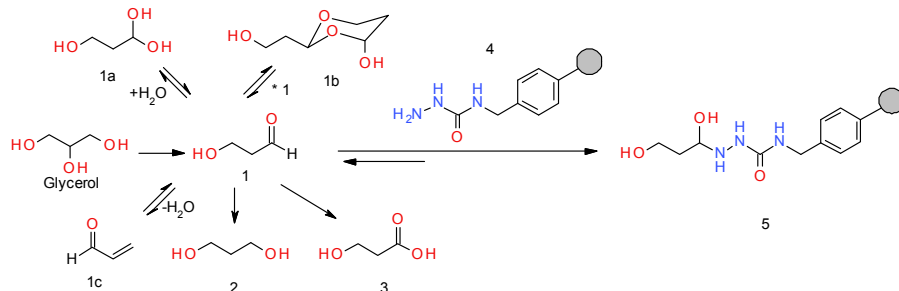
The reaction of semicarbazide or carbohydrazide with 3HPA is a condensation reaction resulting in a rapid formation of an intermediate which is then converted to semicarbazone precipitate by loss of water molecules in a slow reaction at high temperature (Fig. 4.13) [93]. The semicarbazone is very stable and its cleavage to liberate the 3HPA needs harsh conditions.



**Figure. 4.13. Complex formation of 3HPA with semicarbazide. The compounds shown are 3HPA (1), semicarbazide (2), 3HPA-semicarbazide complex (3) and 3HPA-semicarbazone (4)**

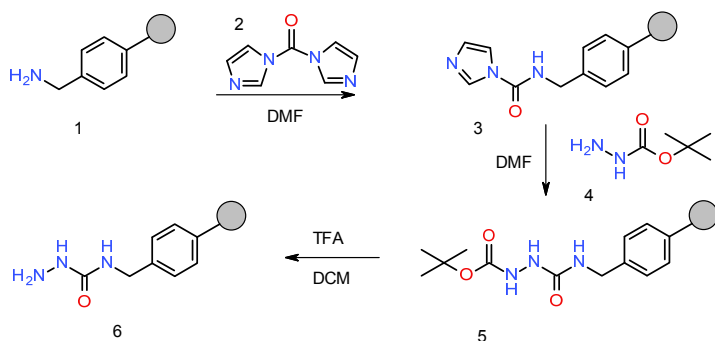
Studies by Krauter et al. (2012) have shown very high productivity of 3HPA (291.57 mM/h) from glycerol using *L. reuteri* by *in situ* complexation of 3HPA with carbohydrazide in a single fed-batch biotransformation [74]. Moreover, *in situ* complexation of 3HPA with semicarbazide and carbohydrazide in repeated batch biotransformation of glycerol resulted in production of 30g 3HPA (in seven batches) and 67g 3HPA (in ten batches) in complex form, respectively [74], and also led to increased biocatalyst life time. One carbohydrazide molecule has two hydrazide groups and can bind with two 3HPA molecules instead of one at semicarbazide and consequently increase the removal of 3HPA by complexation. However, recovery of 3HPA from these complexes needs harsh conditions and has not reported yet.

In Paper III, another approach employing a semicarbazide functionalized resin was used to bind 3HPA from the solution to form an intermediate as shown schematically in Fig. 4.14.



**Figure. 4.14.** Biotransformation of glycerol and complex formation of resulting 3HPA with semicarbazide resin. The compounds shown are 3HPA monomer (1), 3HPA hydrate (1a), 3HPA dimer (1b), acrolein (1c), 1,3PDO (2), 3HP (3), semicarbazide functionalized resin (4), and 3HPA-semicarbazide complex (5)

Preparation and development of the semicarbazide functionalized resin has been reported for solid-phase preparation of peptide ketones and aldehydes [99]. This resin was prepared by activation of aminomethyl polystyrene resin with 1,1'-carbonyldiimidazole (CDI) followed by functionalization with tert-butyl carbazate (Fig. 4.15).



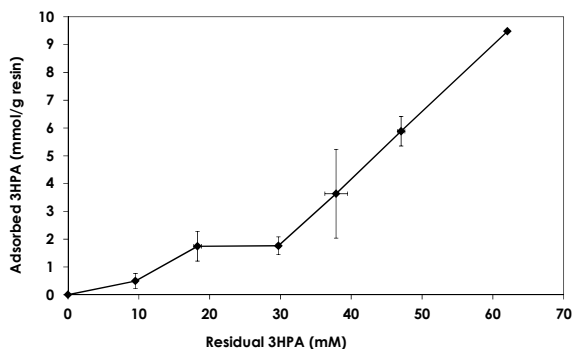
**Figure. 4.15.** Preparation of semicarbazide functionalized resin. The compound shown are aminomethyl polystyrene resin (1), 1,1'-carbonyldiimidazole (2), carbonyldiimidazole-aminomethyl complex (3), tert-butyl carbazate (4), Semicarbazide functionalized resin (6)

#### 4.5.2.1. Capture of 3HPA using a semicarbazide-functionalized resin

##### 4.5.2.1.1. Binding capacity of semicarbazide-functionalized resin for 3HPA

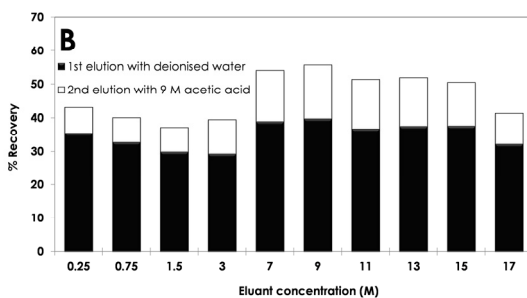
The maximum binding capacity of the resin for 3HPA in a batch system was found to be 9.47 mmol/g dry resin for the initial 3HPA concentration of 71 mM. Increase in the concentration of 3HPA increased the adsorbed amount. However, the maximum binding capacity of the resin for 3HPA molecules was not reached under the experimental conditions. In a packed-bed continuous flow system the maximum binding capacity was only 1.3 mmol/g dry resin for the initial HPA concentration of 107 mM. According to the initial slope and the overall pattern of the adsorption curve in the batch system (Fig. 4.16) which generally characterizes binding of the solute to the matrix occurs via two different mechanisms [103]. The first is mostly the complexation occurring between the carbonyl group of 3HPA and the semicarbazide ligand, “chemisorption” [104]. The short plateau indicates a first degree saturation of the semicarbazide-ligands on the resin surface [103]. The 3HPA-adduct will form a hydrophilic layer around the resin which facilitates the

adsorption and/or intermolecular attraction with other 3HPA molecules from the solution at higher 3HPA concentration, i.e. “physical adsorption/interaction”. This is known as co-operative adsorption [103].



**Figure 4.16.** Adsorption equilibrium between the amount of 3HPA bound to the resin and the concentration of residual 3HPA in solution (equilibrium concentration)

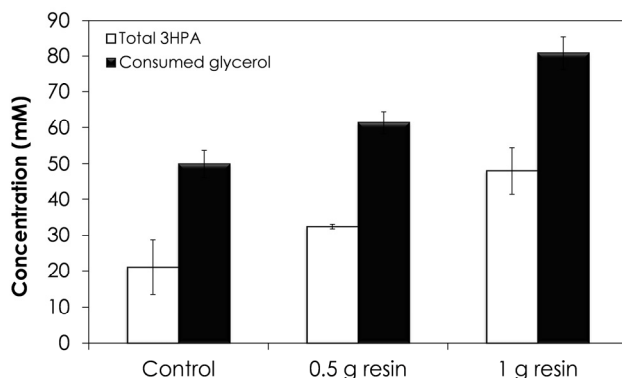
The elution of 3HPA bound to the resin was evaluated using deionised water, different acids and organic solvents. The total 3HPA recovery by 1<sup>st</sup> elution using deionized water and 2<sup>nd</sup> elution using acetic acid (9M) was over 55% (Fig. 4.17).



**Figure 4.17.** Total recovery of 3HPA (%) from the resin by 1<sup>st</sup> elution with deionised water and 2<sup>nd</sup> elution with acetic acid (9M)

#### 4.5.2.1.2. *In situ* removal of 3HPA through binding to a semicarbazide-functionalized resin.

Biotransformation of glycerol to 3HPA using *L. reuteri* with *in situ* removal by semicarbazide resin resulted in improved production of 3HPA and the resin had no inhibitory effect to the cells. Fig. 4.18 shows the concentration of consumed glycerol and total 3HPA produced (free in solution and bound to the resin) after 3 h of biotransformation.



**Figure 4.18. Biotransformation of glycerol to 3HPA using *L. reuteri* with and without *in situ* removal using semicarbazide-functionalized resin (0, 0.5 and 1 g)**

In case of production without *in situ* recovery, the produced 3HPA represented 42 mol% of the consumed glycerol. Addition of 0.5 g resin to the reaction resulted in enhanced 3HPA production with volumetric productivity of 10.81 mM/h, and molar yield of 53 mol%, respectively. Increasing the amount of the resin to 1 g was accompanied by 50% increase in total 3HPA concentration and volumetric productivity over that obtained with 0.5 g resin, and the 3HPA yield was increased to 59 mol% caused by reduced metabolic flux towards 3HP and 1,3PDO. In the presence of the resin, the concentration of the free 3HPA in the solution represented a minute fraction (2-3% of the total 3HPA produced), indicating the high efficiency of the resin as 3HPA scavenger (Table. 4.1).

**Table 4.1. Batch biotransformation of glycerol to 3HPA using resting cells of *L. reuteri* with and without *in situ* 3HPA removal using semicarbazide-functionalized resin.**

	Consumed glycerol (mmol)	Free 3HPA <sup>(a)</sup> (mmol)	Bound 3HPA (mmol)	Total 3HPA <sup>(b)</sup> (mmol)	Yield (mol %)	Volumetric Productivity (mM/h)
0	0.46 ± 0.037	0.19 ± 0.07	--	0.19±0.07	42	7.01
0.5	0.56 ± 0.02	0.008	0.29±0.005	0.3	53	10.81
1	0.74 ± 0.04	0.009	0.43±0.06	0.44±0.05	59	16.01

<sup>(a)</sup> Free 3HPA in solution

<sup>(b)</sup> Total 3HPA = Free 3HPA in solution + Bound 3HPA





## 5. 3-Hydroxypropionic acid from 3-hydroxypropionaldehyde

3-Hydroxypropionaldehyde formed by dehydration of glycerol by *L. reuteri* and other organisms like *Klebsiella pneumonia*, *Bacillus welchii*, *Citrobacter freundii*, *Enterobacter agglomerans*, and *Clostridium butyricum* are further metabolized by the microorganisms to 1,3-propanediol (1,3PDO) and 3-hydroxypropionic acid (3HP). Two moles of glycerol give 2 moles of 3HPA which is converted to 1 mole 1,3PDO and 1 mole 3HP in reductive and oxidative pathways, respectively [28, 74]. Both 1,3PDO and 3HP are important chemicals with a wide range of applications in chemical and polymer industries [105].

### 5.1. 3-Hydroxypropionic acid

3HP is a potential biobased C3 platform chemical for production of acrylic acid, 1,3-propanediol, methyl acrylate, acrylamide, ethyl 3HP, malonic acid, propiolactone and acrylonitrile. It can be used for production of crosslinking agent for polymer coatings, metal lubricants, antistatic agents for textiles and biodegradable polymers (Fig. 5.1) [25, 29].

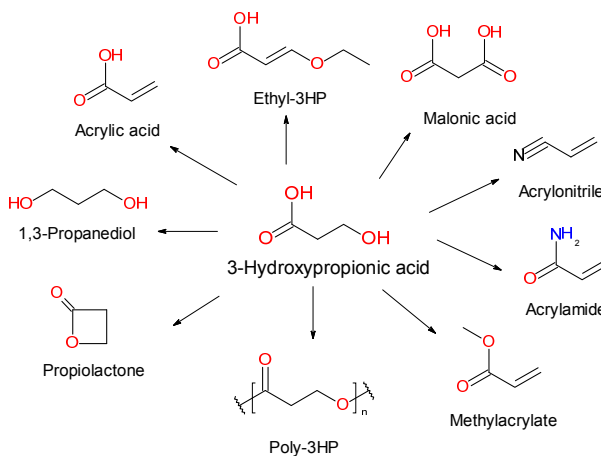


Figure.5.1. Potential products from 3-hydroxypropionic acid

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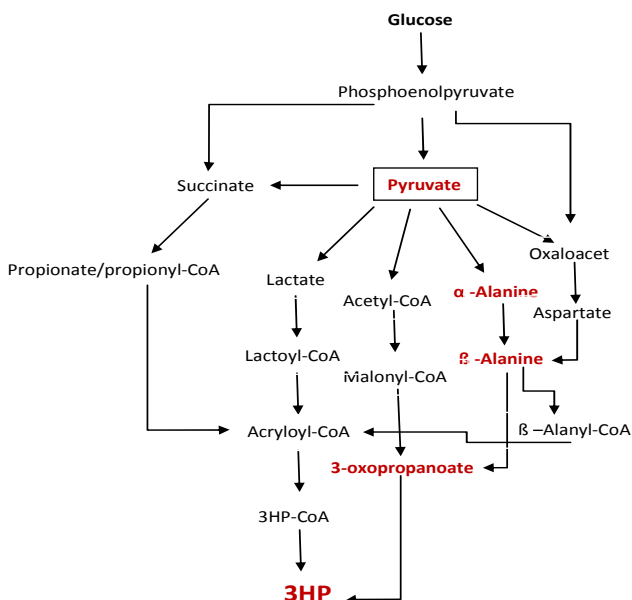
3HP contains a carboxyl group and a  $\beta$  hydroxyl group which can be used for different chemistries, hence making it a highly reactive compound [106]. Production of 3HP can be achieved by chemical synthesis or biotechnological route [25].

The chemical synthesis of 3HP can be achieved via different chemical routes such as hydration of acrylic acid, oxidation of 1,3PDO or allyl alcohol, and hydrolysis of  $\beta$ -propiolactone or 3-hydroxypropionitrile. Acrylic acid and 1,3 PDO are two important chemicals for the polymer industry[29]. 3HP can be produced in high yield (79-83)% by oxidation of allyl alcohol using a gold catalyst. However, the reusability of the catalyst decreases the yield markedly [107]. 3-Hydroxypropionitrile is produced by chemical reaction between 2-chloroethanol and sodium cyanide, which is highly toxic. Also, the carcinogenicity of  $\beta$ -propiolactone is the main bottleneck in the process of 3HP production [107].

3HP can also be produced in microorganisms as an intermediate of 3-hydroxypropionate and 3-hydroxypropionate/4-hydroxybutyrate pathways. These two pathways belong to the six pathways for autotrophic carbon dioxide fixation. The 3-hydroxypropionate pathway is functional in *Chloroflexus aurantiacus*, *Acidianus brierleyi*, *Metallosphaera sedula*, *Acidianus ambivalens*, *Sulfolobus species* [108, 109]. 3-Hydroxypropionate/4-hydroxybutyrate pathway was discovered in *Metallosphaera sedula* [110]. The starting materials for both pathways are acetyl-CoA and CO<sub>2</sub>. Some microorganisms use degradative pathway for production of 3HP. *Byssoschlamys sp.*, *Geotrichum sp.* and *Trichoderma sp.* can degrade acrylic acid for production of 3HP [111, 112]. *Alcaligenes faecalis* is able to use acrylic acid as an intermediate by degradation of dimethylsulfoniopropionate [113]. Uracil, also can be degraded to 3HP by *Saccaromyces kluyveri* [114].

For large-scale production, different metabolic routes have been constructed in recombinant microorganisms for production of 3HP from glucose and glycerol. Recombinant microorganisms have the advantages of using cheap and available substrates as well as providing efficient processes with high yield and productivity compared to the natural ones. Hence, development of a bioprocess using recombinant microorganisms seems to be essential for the commercial production of 3HP [29].

There are seven biochemical pathways for 3HP production from glucose suggested by Cargill (the US-based agricultural company)(Fig. 5.2)[25, 115-120].



**Figure. 5.2. Metabolic pathways for the production of 3HP from glucose by Cargill [115]**

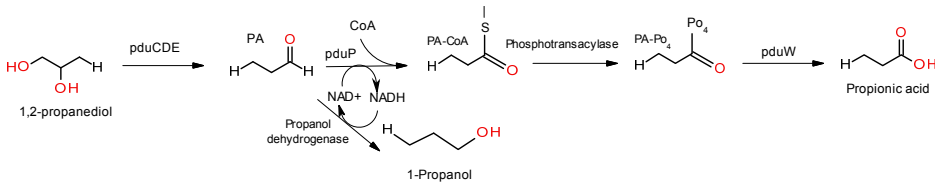
The main intermediates in all pathways are pyruvate and / or phosphoenolpyruvate. A highest possible yield of 2 moles 3HP/mole glucose which is equal to the ratio of the number of carbon atoms in glucose to the number of carbon atoms in 3HP, is achieved using all the pathways that consume no ATP and utilize NADH as an electron donor. The pathway of (pyruvate  $\rightarrow$   $\alpha$ -alanine  $\rightarrow$   $\beta$ -alanine  $\rightarrow$  3-oxopropanoate  $\rightarrow$  3HP) is both ATP producing and thermodynamically favourable as well as being redox neutral. Hence, the maximum 3HP yield in this pathway is 2 mole/ mole glucose [115].

The production of 3HP from glycerol is via two pathways; CoA-dependent and CoA-independent pathways. 3HPA is oxidized to 3HP via 3HP-CoA as an intermediate and produces ATP in the CoA-dependent pathway [115, 121] while in the CoA-independent pathway, 3HPA is converted directly to 3HP by an aldehyde dehydrogenase and no ATP is produced [29].

In (Paper V) the production of 3HP from 3HPA was investigated through enzymes of the propanediol utilization pathway from *L. reuteri* in a recombinant *E. coli*.

## 5.2. The propanediol utilization pathway

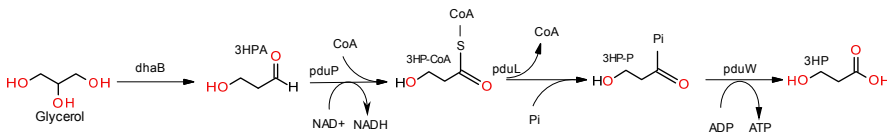
A CoA-dependent pathway in *Salmonella enterica* has been shown in which 1,2-propanediol is converted to propionic acid in a series of reactions catalyzed by CoA-acylating propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL), and propionate kinase (PduW) and 1-propanol catalyzed by propanol dehydrogenase via propionaldehyde as an intermediate (Fig. 5.3) [122].



**Figure. 5.3. CoA-dependent pathway of *Salmonella enterica* for conversion of 1,2-propanediol**

This pathway is an ATP generating pathway and the redox balance is achieved by production of 1-propanol. 1,2-Propanediol utilization (*pdu*) operon which contains 21 genes, encodes enzymes responsible for 1,2-propanediol metabolism. Some steps in the dismutation of 1,2-propanediol occur in a bacterial microcompartment (metabolosome or carboxysome) and have been observed in *K. pneumoniae*, *L. reuteri*, and *L. collinoides* [29, 123, 124]. The metabolosomes contain lots of enzymes and proteins which help direct transfer of intermediates between enzymes, and subsequently increase the catalytic efficiency of the reactions. They also have a barrier to isolate the toxic aldehyde intermediates [125].

The 3HPA produced from glycerol in a reaction catalysed by glycerol dehydratase in resting cells of *L. reuteri*, is converted to 1,3PDO catalysed by 1,3propanediol oxidoreductase (*pduQ*) in which  $\text{NAD}^+$  is regenerated from  $\text{NADH}$  inside the cells. Extra  $\text{NAD}^+$  leads the conversion of 3HPA to 3HP through CoA-dependent pathway using *pduP*, *pduL*, and *pduW* (Fig. 5.4) [126].



**Figure. 5.4. CoA-dependent pathway of *L. reuteri* for production of 3-hydroxypropionic acid from glycerol**

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The first enzyme, PduP is responsible for conversion of 3HPA to 3HP-CoA. During this conversion NADH is formed from NAD<sup>+</sup> [127]. PduP of *L. reuteri* JCM 1112 described by Luo et al. (2012) was expressed in *E. coli* and showed highest activity of 0.197 U/mg with propionaldehyde as substrate [121].

In (Paper IV), pduP gene from *L. reuteri* DSM 20016 was expressed in *E. coli* BL21(DE3), and the enzyme had a specific activity of 28.9 U/mg using propionaldehyde as substrate. The activity of *L. reuteri* PduP was (18 U/mg) with 3HPA as substrate which is the highest specific activity reported up to date.

The tertiary structure of PduP was proposed using homology modelling of known CoA-acylating aldehyde dehydrogenases and resulted in a model based on the crystal structure of probable aldehyde dehydrogenase from *Listeria monocytogenes* which has similarity above 30% to PduP with sequence coverage above 75%. Identification of PduP cofactor binding sites, NAD<sup>+</sup> and HS-CoA, was performed by *in silico* bonding of PduP and cofactors and was supported by replacement of four amino acids. Cys-277 plays a role in the binding of substrate and catalytic mechanism of the enzyme. Ile275 is involved in a van der Waals interaction with the nicotinamide ring of NAD<sup>+</sup>. Thr145 is essential for an efficient acylation. The three mutants (PduP-C277A, PduP-I275A, and PduP-T145A), were found to be inactive. The oxygen of a hydroxyl group of the NAD<sup>+</sup> ribose forms a hydrogen bond with the hydroxyl group of the (Ser417) side chain. PduP-S417A showed activity with a two-fold increase in  $K_m$  and 3.5 fold lower  $k_{cat}/K_m$  with NAD<sup>+</sup>.

### 5.3. 3HPA conversion to 3HP using the pdu pathway engineered in *E. coli*

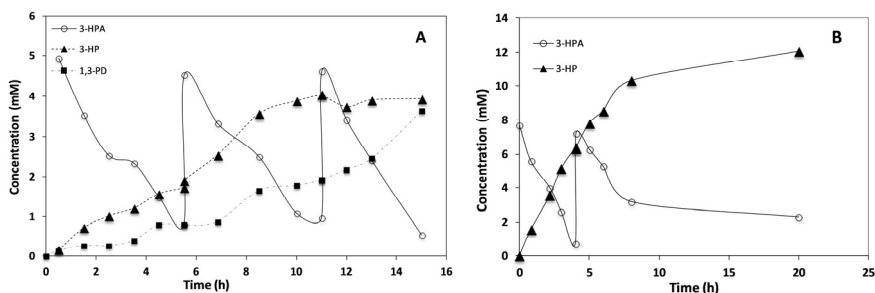
The PduP together with PduL and PduW from *L. reuteri* DSM 20016 were introduced in *E. coli* BL21 (DE3). To confirm the role of the pathway in conversion of 3HPA to 3HP, crude cell lysates of *E. coli* expressing functional PduP, PduL and PduW and mutants in which one of the enzymes was missing and a control without any of these enzymes, were used to catalyse the transformation of 3HPA (Paper V). Production of 3HP was observed in the case of strain the pdu:P:L:W, which was equimolar to consumption of 3HPA. The strain lacking expression of functional PduP could not consume 3HPA while consumption of

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3HPA was seen in the mutants lacking PduL and PduW. Also, the mutant lacking PduW could produce 3HP equimolar to 3HPA consumed which might be due to an enzyme, acetate kinase, similar to PduW, present natively in *E. coli*.

Subsequently, the bioconversion of 3HPA to 3HP was done using whole cells of *E. coli* pdu:P:L:W in growing as well as resting state. The bioconversion was started by adding 3HPA to the medium and after its complete consumption, another batch of 3HPA was added. Addition of 3HPA was continued until it could no longer be consumed.

Production of 3HP was observed in both experiments. The yield of 3HP was 0.5 mole 3HP/mole 3HPA with the growing cells (Fig. 5.5A) and 1 mole 3HP/mole 3HPA with the resting cells (Fig. 5.5B). The growing cells show the formation also of 1,3PDO that is ascribed to the activity of 1,3-propanediol oxidoreductase, present natively in *E. coli*. In the control *E. coli* strain without the Pdu enzymes, 3HPA was converted to 1,3PDO only.



**Figure 5.5. Bioconversion of 3HPA to 3HP by (A) growing cells and (B) resting cells of *E. coli* pdu:P:L:W**

As described in **Paper I** and **II**, the production of 3HPA is improved by complexation with bisulfite and then adsorption on to the Amberlite resin. Hence, a solution of 3HPA-bisulfite complex and free 3HPA eluted from the resin by 0.2 M NaCl, was also tested as the substrate for production of 3HP using resting cells of *E. coli* pdu:P:L:W. Even in this case, complete conversion of free and complexed 3HPA was achieved with a yield of 1 mole 3HP/mole 3HPA, however with a relatively lower productivity compared to the solution containing 3HPA.

The studies in **Paper V** thus show that it is possible to use the Pdu pathway for production of 3HP and that the resting cells are better catalysts than the growing cells but are limited by the cofactor limitation. However, a bottleneck with the use

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of 3HPA as the substrate is its antimicrobial activity on the whole cells thus limiting the concentration at which it can be used. This pathway can however be complemented with expression of glycerol dehydratase that would allow the production of 3HP from glycerol.





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## 6. Conclusions & Future Perspectives

Both 3HPA and 3HP are ranked among the top 10 target platform chemicals for the biobased chemical industry, and have lately attracted increasing interest. The central focus of this thesis was primarily 3HPA production from glycerol and secondly its conversion to 3HP.

3HPA is perhaps the first example of an aldehyde to be produced using a microbial process. Its production via single step dehydration of glycerol appears extremely simple, however because of its severely inhibitory property, 3HPA production turns out to be very challenging. *Lactobacillus reuteri* is an attractive biocatalyst for industrial production; the organism is already produced industrially for use as a probiotic. Its use for 3HPA production, as reported in this thesis, would involve two steps - one for production of the cells mass and the other for bioconversion of glycerol.

The known reactions of the aldehydes with bisulfite and semicarbazide, respectively, were used in novel configurations for *in situ* complexation and adsorption of 3HPA with concomitant lowered inhibition in contrast to the previous reports [74, 79, 128], which resulted in increased productivity. While dissociation of the 3HPA-bisulfite complex was achieved under relatively mild conditions, the need for separating the free aldehyde remains to be seen in the light of its eventual application; the complex could be used directly as substrate for further transformation of the aldehyde. Considering the problem of separating 3HPA from its complex with semicarbazide, the work in this thesis has shown the possibility to release a large fraction of the aldehyde from a semicarbazide-functionalized resin. There is of course need for further improvements in order to achieve high product recovery and -economy.

This thesis further demonstrates that 3HPA formed could be successfully used as a substrate for further conversion to 3HP by the enzyme system available in *L. reuteri* itself. While the first enzyme, PduP has been characterized to some extent, work is needed on characterization of the other two enzymes, PduL and PduW, and eventually optimization of the whole system.

The thesis has hopefully shown that *L. reuteri* is an interesting organism with a potentially important role in industrial biotechnology. The organism has been used in other studies in our laboratory for the co-production of 3HP and 1,3PDO [6]. Production of the *L. reuteri* cell mass is one of the major costs as it requires a rich

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medium for growth; production in other cheaper media such as cheese whey, potato juice, etc. needs to be evaluated. The possibility of using a recombinant organism for production of 3HPA and 3HP would depend on the successful expression of glycerol dehydratase that requires the machinery for producing Vitamin B<sub>12</sub> used as a cofactor by the enzyme. A B<sub>12</sub>-dependent enzyme from *Clostridium butyricum* is an alternative enzyme that is being considered, and that has been used earlier for 1,3PDO production [72], but this enzyme requires S-adenosylmethionine as a cofactor.

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