Microbial Production of Bio-Based Chemicals: A Biorefinery Perspective

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Microbial production of bio-based propionic acid, 3-hydroxypropionic acid (3HP) and 3-hydroxypropionaldehyde (3HPA) using glycerol, a by-product of biodiesel production process, as raw material. Different strategies to overcome the bottlenecks in bioprocesses for production of chemicals including high cell density cultivations, fed-batch biotransformation, continuous biotransformation with cell recycling, and in situ product complexation and removal were studied.
Microbial Production of Bio-Based Chemicals
A Biorefinery Perspective

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Lund University

Doctoral Dissertation
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Academic thesis, which by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Tuesday June 11 at 1:30 p.m., in Lecture Hall B at the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

The Faculty opponent is Prof. Shang-Tian Yang, Department of Chemical and Biomolecular Engineering, Director, Ohio Bioprocessing Research Consortium, The Ohio State University, USA.
To my beloved family
A shift from fossil- to renewable biomass feedstock for the emerging bio-based economy requires the development and adoption of new sustainable technologies that are more suited for transformation of biomass components to chemicals, materials and energy. This thesis presents investigations on the development of processes based on industrial biotechnology as a key element for the production of chemicals from agro-/industrial by-products. The chemicals of interest are the ones that could potentially serve as building blocks, platforms, for other chemicals and polymers. Glycerol, a by-product of biodiesel production, was used as raw material for the production of propionic acid, 3-hydroxypropionaldehyde (3HPA) and 3-hydroxypropionic acid (3HP), while methacrylic acid (MA) was produced from 2-methyl-1,3-propanediol, a by-product of butanediol production. Different strategies to overcome the bottlenecks such as product inhibition existing in the bioprocesses for production of the chemicals were studied.

Fermentation of glycerol to propionic acid was studied using Propionibacterium acidipropionici. High cell density cultivations were used to overcome the low production rate caused by slow microbial growth and product-mediated toxicity. Increasing the cell density by immobilization and sequential batch recycling improved the production rates by 2- and 6-fold, respectively, over that obtained using conventional batch fermentation. Potato juice, a by-product of potato starch processing, was shown to be a promising, inexpensive nitrogen/vitamin source for the growth of the organism and propionic acid production.

Lactobacillus reuteri was employed as a whole cell biocatalyst for the conversion of glycerol to 3HPA and 3HP in aqueous solution. Production of 3HPA using glycerol dehydratase activity of the cells, limited by substrate inhibition and product toxicity, was performed in a fed-batch mode with in situ complexation of the hydroxyaldehyde with bisulfite, and subsequent removal through binding to an anion exchanger. This resulted in increase in production of 3HPA from 0.45 g/g biocatalyst in a batch process to 5.4 g/g. 3HP is formed as an oxidation product of 3HPA, however its accumulation as a product of glycerol metabolism in wild-type L. reuteri has not been reported earlier. The metabolic fluxes through the glycerol reductive and oxidative pathways were calculated using variable volume fed-batch operation. The glycerol feeding strategies were optimized to yield complete conversion of 3HPA into equimolar mixture of 3HP and 1,3PDO, the products that can be easily separated from each other.

MA was quantitatively produced at high purity from 2-methyl-1,3-propanediol by a novel process involving integrated biological and chemical catalysis. Whole resting cells of Gluconobacter oxydans were used for selective oxidation of the substrate to the corresponding hydroxycarboxylic acid, which upon dehydration over TiO$_2$ at 210 °C yielded MA. This process offers a potential, significantly greener alternative to the acetone-cyanohydrin process used for MA production, involving highly toxic substrates, large amounts of waste and greenhouse gas emissions.
Abstract

A shift from fossil- to renewable biomass feedstock for the emerging bio-based economy requires the development and adoption of new sustainable technologies that are more suited for transformation of biomass components to chemicals, materials and energy. This thesis presents investigations on the development of processes based on industrial biotechnology as a key element for the production of chemicals from agro-/industrial by-products. The chemicals of interest are the ones that could potentially serve as building blocks, platforms, for other chemicals and polymers. Glycerol, a by-product of biodiesel production, was used as raw material for the production of propionic acid, 3-hydroxypropionaldehyde (3HPA) and 3-hydroxypropionic acid (3HP), while methacrylic acid (MA) was produced from 2-methyl-1,3-propanediol, a by-product of butanediol production. Different strategies to overcome the bottlenecks such as product inhibition existing in the bioprocesses for production of the chemicals were studied.

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MA was quantitatively produced at high purity from 2-methyl-1,3-propanediol by a novel process involving integrated biological and chemical catalysis. Whole resting cells of Gluconobacter oxydans were used for selective oxidation of the substrate to the corresponding hydroxycarboxylic acid, which upon dehydration over TiO₂ at 210 °C yielded MA. This process offers a potential, significantly greener alternative to the acetone-cyanohydrin process used for MA production, involving highly toxic substrates, large amounts of waste and greenhouse gas emissions.
Popular Summary

A large variety of microorganisms are present in nature and have indispensable roles in recycling carbon, remediation of the environment, agriculture, and also in providing health benefits to humans. Microorganisms are microscopic versatile living factories that can consume several different natural as well as synthetic compounds for their growth and also produce different useful chemicals. Microorganisms have long been used for production of cheese, wine, antibiotics such as penicillin, fuels such as ethanol, amino acids and other chemicals such as citric acid and acetic acid. However, majority of the chemical products we use in our daily lives are made from fossil resources in petrochemical refineries. These chemicals are used in foods, animal feed, pharmaceuticals, cosmetics, agriculture, paints, coatings, plastics and many more applications.

The increasing price of mineral oil and gas during the past few decades has led to a concern that fossil resources will come to an end and will not sustain the needs of the growing population. On the other hand, the fossil products are said to have a negative effect on the environment, and have contributed to global warming, pollution of soil and waters, and climate change. With the increased concern about the environment and sustainability, there is growing demand for alternative renewable raw materials for chemicals and energy and for cleaner processes that do not burden the environment. Organic biomass, e.g. from plants and trees, and also side products from different industries provide useful renewable raw materials for production.

This thesis provides evidence that microorganisms can be used to produce important industrial chemicals from side-products of existing industrial processes. In some cases, they provide an alternative route for an existing chemical, while in the others the chemicals not yet available in the market are produced. The target chemicals would serve as building blocks for other chemicals and polymers in the industry based on renewable raw materials. The focus in the thesis has been to overcome the bottlenecks in the microbial systems so as to make them economical for industrial production of chemicals.

Examples of the industrial side-products used in the present thesis are glycerol (glycerine), which is formed during production of biodiesel from vegetable oil, and potato juice, a liquid stream produced during extraction of starch from potatoes. Glycerol is used as a source of carbon by different bacteria and is converted to different important chemicals. Among the bacteria used in the work are Propionibacteria used in cheese making, and lactobacillus used as a probiotic.
List of Publications

The thesis is based on the following papers, which will be referred to by their roman numerals in the text.


IV Sardari, R.R.R., Dishisha, T., Pyo, S.-H. & Hatti-Kaul, R. Biotransformation of glycerol to 3-hydroxypropionaldehyde: improved production by *in situ* complexation with bisulfite in a fed-batch mode and separation on anion exchanger. 2013 (Submitted)

V Dishisha, T., Pyo, S.-H. & Hatti-Kaul, R. Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from biodiesel-derived glycerol using *Lactobacillus reuteri* cells. 2013 (Manuscript)


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Paper VI is reproduced by permission of The Royal Society of Chemistry
My Contribution to the Papers

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

I I was involved in experimental design with Dr. Alvarez and performed the entire experimental part and data analysis. Also, I coordinated the writing and was involved in major part of the manuscript writing and editing.

II I designed and performed all the experiments, data analysis and writing of the first draft of the manuscript. Dr. Ståhl and Dr. Lundmark provided the information regarding the propionic acid, glycerol and potato juice market prices and specifications.

III Roya Sardari (PhD student) has done the experimental part with complexation studies. I designed the integration with fed-batch operation for in situ complexation and performed the experimental section with Roya. Both were involved in data analysis and writing the manuscript under supervision of Dr. Pyo and Prof. Hatti-Kaul.

IV Roya Sardari has done the experiments on 3HPA purification and recovery. I designed the integration into fed-batch operation with cell recycling for in situ recovery and performed the experiments with Roya. I participated in data analysis and writing of the manuscript with Roya under supervision of Dr. Pyo and Prof. Hatti-Kaul.

V I designed and performed all the experiments, data analysis and writing of the manuscript which was revised by Prof. Hatti-Kaul and Dr. Pyo.

VI The idea was initiated by Dr. Pyo. Jargalan Gerelsaikhan, Secil Dayankac (Master students) and I performed the experimental part with regarding enzyme kinetics under supervision of Dr. Pyo and Prof. Hatti-Kaul. I designed the continuous biotransformation experiment with cell recycle and performed the experiment and data analysis with Secil. Dr. Pyo performed the dehydration step and structure elucidation. I participated in writing of the manuscript.
Abbreviations

\( Q_P \)  
Volumetric production rate (g/L.h)

\( Q_S \)  
Volumetric consumption rate (g/L.h)

\( \mu_{\text{max}} \)  
Maximum specific growth rate (1/h)

\( \text{CDW} \)  
Cell dry weight (g)

\( Y \)  
Yield (mol/mol) or (g/g)

\( q_S \)  
Specific substrate consumption rate (g/g\text{CDW}.h)

\( q_P \)  
Specific product formation rate (g/g\text{CDW}.h)

\( D \)  
Dilution rate (1/h)

\( \text{PA} \)  
Propionic acid

\( 3\text{HP} \)  
3-Hydroxypropionic acid

\( 3\text{HPA} \)  
3-Hydroxypropionaldehyde

\( 1,3\text{PDO} \)  
1,3-Propanediol

\( 2\text{M1,3PDO} \)  
2-Methyl-1,3-propanediol

\( 3\text{H2MPAL} \)  
3-Hydroxy-2-methylpropionaldehyde

\( 3\text{H2MPA} \)  
3-Hydroxy-2-methylpropionic acid

\( \text{Gly} \)  
Glycerol

\( \text{HTPJ} \)  
Heat-treated potato juice

\( \text{SA} \)  
Succinic acid

\( \text{AA} \)  
Acetic acid

\( \text{nPOH} \)  
n-Propanol

\( \text{DHA} \)  
Dihydroxyacetone

\( P. \text{acidipropionici} \)  
Propionibacterium acidipropionici

\( L. \text{reuteri} \)  
Lactobacillus reuteri

\( G. \text{oxydans} \)  
Gluconobacter oxydans

\( K. \text{pneumonia} \)  
Klebsiella pneumonia

\( \text{ICR} \)  
Immobilized cell reactor

\( \text{PQQ} \)  
Pyrroloquinoline quinine

\( \text{Ti} \)  
Titanium

\( \text{TiO}_2 \)  
Titanium dioxide
## Contents

1. **Introduction** 1

2. **Biorefineries & Industrial Biotechnology** 5
   2.1 Biomass and biorefineries 5
   2.2 Industrial by-products as raw materials 7
      2.2.1 Glycerol (Gly) 7
      2.2.2 2-Methyl-1,3-propanediol (2M1,3PDO) 8
      2.2.3 Potato juice 9
   2.3 Industrial biotechnology 9
      2.3.1 Indicators for bioprocess efficiency 10
      2.3.2 Bioprocess limitations 11
   2.4 Systems biology for bioprocess development 11
   2.5 Metabolic engineering & synthetic biology 12
   2.6 Bioprocess engineering 13
      2.6.1 Cell immobilization 13
      2.6.2 Cell recycling 14
      2.6.3 *In situ* product complexation and removal 15
   2.7 Integrated refineries 16

3. **Propionic Acid Production by Microbial Fermentation of Glycerol** 17
   3.1 Propionic acid 17
   3.2 Microbial route for propionic acid production 18
      3.2.1 Propionibacteria 18
         3.2.1.1 Metabolism 18
         3.2.1.1 Carbon and nitrogen/vitamin sources 19
         3.2.1.1 Fermentative production 20
      3.2.1 A potential biorefinery 21
      3.2.2.1 Production using immobilized cells 22
      3.2.2.2 Production using recycled cells 23

4. **3HPA & 3HP Production by Biotransformation of Glycerol** 25
   4.1 3-Hydroxypropionaldehyde 25
      4.1.1 Microbial production of 3HPA 26
   4.2 3-hydroxypropionic acid 26
      4.2.1 Microbial production of 3HP 27
   4.3 *Lactobacillus reuteri* as a production host for 3HPA & 3HP 28
      4.3.1 *Lactobacillus reuteri* 29
      4.3.2 3HPA accumulation and removal 30
   4.4 Biotransformation of glycerol using *L. reuteri* to 3HPA with *in situ* product removal 32
      4.4.1 Complex formation of 3HPA with sodium bisulfite 32
4.4.2 Binding of 3HPA-bisulfite complex to Amberlite® IRA-400
4.4.3 Fed-batch biotransformation: A step towards optimization
4.4.4 In situ complex formation and removal
4.5 Co-production of 3HP and 1,3PDO
4.5.1 Production of 3HP and 1,3PDO using L. reuteri

5. Methacrylic Acid Production by Integrated Bio- & Chemo Catalysis
   5.1 Methacrylic acid (MA)
   5.2 Acetic acid bacteria
      5.2.1 Gluconobacter oxydans
   5.3 Combined bio- and chemical catalysis
      5.3.1 Biotransformation of 2M1,3PDO to 3H2MPA
      5.3.2 Catalytic dehydration of 3H2MPA into MA

6. Conclusions & Future Perspectives
Acknowledgement
References
1. Introduction

Prior to the industrial revolution in mid-19th century, mankind has relied mainly on renewable resources for fulfilling the needs for food as well as non-food products. Subsequently, fossil resources, first coal and then mineral oil and gas, became the base material for the production of energy, chemicals and materials. The rapid growth of the petrochemical refineries has transformed the world completely by providing us with innumerable number of products for all aspects of our lives. As a result, the use of renewable raw materials decreased substantially, accounting for only 10% of the current chemicals production.

The recurring oil crisis in 1973, 1979 and 2008, however led to the concern about the finite nature of the fossil resources that would not suffice for the increasing demands of the growing population. Added to this, is the increasing awareness of the negative environmental impact of the fossil-based production seen as global warming, acid rain, smog, and recalcitrant wastes. Increasing public pressure and policy regulations are driving for the search for alternative resources for providing clean energy, green chemicals and materials that can be biodegraded when released into the environment after their useful lifespan [1-3]. These resources, besides being renewable, should be cheap, readily available, and not interfere with the food chain. The increased awareness and demand for sustainability in the modern society has made terms such as eco-friendly, environment-friendly, nature-friendly, green, bio-based and renewable quite popular for marketing of many products and increasing the profit of industries.

While several alternative sources for renewable energy exist, biomass is the only alternative to fossil resources for the production of chemicals and materials besides energy. In fact, the recent developments on the bio-based production have been targeted to the production of only biofuels, such as biodiesel and bioethanol. Several lessons have been learned from this, the important ones being that:

- the right choice of the raw materials is highly crucial for sustainability, the non-food residues from agriculture and forestry and related industries being the most suitable, and

- the production of biofuels alone is not economical but has to be combined with the production of chemicals and materials, as in a petrochemical refinery.

In a petrochemical refinery, the crude oil is fractionated into naphtha, gas, gasoline, kerosene, and oil residues. Naphtha and gas are further processed to yield a limited number of molecules including olefins such as ethylene and propylene, and aromatics such as benzene, toluene and xylene, which in turn serve as building
blocks for many chemicals [4]. The concept of biorefineries, analogous to the traditional refineries, has now emerged wherein the biomass can be transformed to different biochemical and chemical intermediates that are further fed into a variety of downstream product lines [5]. The shift from hydrocarbon raw material to oxygen rich biomass resource, however requires development of new technologies and routes. The technology area that is of utmost importance for bio-based production is industrial biotechnology using microbial cells and their enzymes as catalysts, which is the theme underlying this thesis.

The work presented in the thesis was performed within the framework of two research projects involving academic-industrial collaboration: “Industrial Biotechnology for Production of Platform Chemicals” (BioVINN), and “Development of Process Technologies for Immobilized Biocatalysts”, and an offshoot project “Acrylic Acid Production from Glycerol by Integrated Bio- and Chemo-Catalysis”. These projects were financed by The Swedish Governmental Agency for Innovation Systems [VINNOVA].

Scope of the Thesis

The aim of the present thesis is the development of environmental-friendly, economically feasible solutions, with industrial biotechnology as the main element for production of a number of platform and secondary chemicals in a biorefinery perspective (Fig. 1.1). Different techniques such as fermentation, biotransformation and chemical catalysis were studied, employed, integrated and optimized to achieve the goals.

The thesis is based on six papers, four of which are published:

**Paper I** and **II** deal with production of propionic acid from glycerol by *Propionibacterium acidipropionici* DSM 4900. Retaining high cell density by immobilization or cell recycle was investigated. Also utilization of potato juice, a by-product of potato starch processing was evaluated as cheap renewable nitrogen source for microbial growth.

**Paper III** and **IV** report the production of 3-hydroxypropionaldehyde (3HPA) from glycerol using resting cells of *Lactobacillus reuteri* DSM 20016. To avoid the product-mediated toxicity to the producer microorganism, complex formation of 3HPA with sodium bisulfite and its interaction with an ion exchange resin were studied *in vitro*. The optimum conditions were utilized for *in situ* complex formation and simultaneous recovery through binding of the complex to an ion exchange resin.
In Paper V, a process for conversion of glycerol to 1,3-propanediol (1,3PDO) and 3-hydroxypropionic acid (3HP) simultaneously using resting cells of Lactobacillus reuteri DSM 20016 was developed.

Paper VI presents a combined biological and chemical catalysis using Gluconobacter oxydans for selective oxidation of 2-methyl-1,3-propanediol (2M1,3PDO) to 3-hydroxy-2-methylpropionic acid (3H2MPA) followed by TiO₂ catalyzed dehydration to methacrylic acid (MA). The conversion was done in aqueous solution, yielding MA at high purity and conversion rates.

The following chapters will give a background of the area covered in the papers and will summarize the outcome and potential of the investigations. Chapter 2 provides a brief introduction to biorefineries and the area of industrial biotechnology with a focus on bioprocess engineering and integrated refineries. The different industrial by-products utilized as raw materials in this thesis are also covered in this chapter. Chapters 3 to 5 present different case studies performed, within the scope of the thesis, on the development of industrial biotechnology routes for production of several chemicals, majority of which are organic acids and one aldehyde. The different microorganisms, metabolic pathways and enzymes involved in the production of these chemicals are also presented. The final Chapter VI summarises concluding remarks and future perspectives based on the results generated within the scope of the thesis.
Fig. 1.1 Industrial biotechnology for the production of bio-based chemicals in a biorefinery perspective

Schematic representation for the utilization of biomass residues as renewable raw materials for the production of different platform and secondary chemicals within the scope of the present thesis. Biomass provides C- & N-sources that were converted by fermentation, biotransformation and chemical catalysis into 3-hydroxypropionaldehyde (3HPA), 3-hydroxypropionic acid (3HP), 1,3-propanediol (1,3PDO), methacrylate and propionate. The global production market (million tons/year) and the applications of the different chemicals are shown. The integration of anaerobic digestion for production of biogas from the generated waste is also presented.
2. Biorefineries & Industrial Biotechnology

2.1 Biomass and biorefineries

Significant efforts are being made around the world to move from the current fossil-based economy to a more sustainable economy based on biomass. Biomass has been best described as anything that grows and is available in non-fossilized form including arable crops, trees, bushes, animal by-products, human and animal waste, waste food and any other waste stream that rots quickly and it can be replenished on a rolling time frame of years or decades [6]. The annual production of plant biomass on Earth is estimated at 1.7 billion tons, constituting 75% carbohydrates, 20% lignin, and 5% proteins, lipids, vitamins, flavours, terpenes and dyes [2, 7]. Only 3.5% of this amount is used for human needs (63% food; 33% wood as energy, paper, constructions, etc.) [2].

The most important bio-based renewable raw materials include: Sugar, glucose, molasses and starch (sugars and starch sector), oleochemical intermediates and glycerol (oil and fat processing sector), and cellulose, cellulose derivatives, hemicelluloses and lignin (wood processing sector). For the conversion of biomass into chemicals and materials, four different concepts are considered [2]:

- natural production of the required structures,
- one step (bio-)chemical modification of naturally produced structures,
- several-step modifications, or
- back to C1 chemistry through conversion of biomass into CO and hydrogen (synthesis gas) by gasification, bio-oil by liquefaction at high pressure, or biomass to liquid (BtL) by thermo-chemical treatment, and rebuilding of molecules.

Currently, the largest non-food bio-based products are the biofuels. Production of first generation biofuels, bioethanol and biodiesel, over the past decade has raised a number of critical social, economic and ecological issues, such as competition for food and -land for cultivation of plants, and sub-optimal use of the biomass without any value addition. In order to be sustainable and competitive, the most efficient strategy for the bio-based economy is to utilize biomass residues and wastes of low or no value, and to develop biorefineries in which the biomass
components are utilized optimally to produce value-added food- and non-food products.

Biorefinery is a facility, analogous to a petrochemical refinery, centered on an agricultural or forest base which integrates biomass conversion technologies and equipment to produce fuels, power, chemicals and materials from biomass without compromising food production and availability [8, 9]. Biorefinery is also defined as “the sustainable processing of biomass into a spectrum of marketable products and energy” [10] (Fig. 2.1).

![Fig. 2.1 Biorefinery](image)

Utilization of renewable raw materials (biomass) as starting materials for production of fuels, chemicals and materials.

The rapid expansion of the biofuels market and the need to derive increased value from the co-products is leading to the development of a variety of biorefineries. The feedstock can be processed in various ways to a range of platforms, which include single carbon molecules such as biogas and syngas, and C3 molecules such as glycerol to mixed C5-C6 carbohydrate streams originating from hemicellulose, and C6 streams from cellulose, sucrose, and starch, etc., lignin, plant- and algal-based oils, etc. [11].

In order to match the efficiency and flexibility of the petrochemical industry, the bio-based industry needs to develop a set of versatile building blocks, or platforms from which a range of products can be derived [11]. Taking this into consideration, the Department of Energy, USA has identified 30 platform chemicals composed of one to six carbon atoms as potential candidates for bio-based production [12]. Polyols and organic acids constitute the majority of these
chemicals; examples are glycerol, propionic acid and 3HP that constitute raw material and products in this thesis.

2.2 Industrial by-products as raw materials

The processes investigated in this thesis for production of different chemicals were based on the use of by-products of agricultural and/or industrial processing, as raw materials. These raw materials include glycerol and 2-methyl-1,3-propanediol (2M1,3PDO) as carbon sources, and potato juice as a nitrogen/vitamin source.

2.2.1 Glycerol (Gly)

Glycerol (1,2,3-propanetriol) is a polyhydric alcohol with the molecular formula C₃H₅(OH)₃. The presences of three hydroxyl groups beside its physical and chemical characteristics have made it a versatile compound for a wide range of applications, and a building block for a vast array of compounds. It is used for preventing drying and extending the shelf life of many products, as emollient, solvent, lubricant and humectant in personal care products, as humectant, laxative and softener in pharmaceuticals, and as sweetener, solvent and preservative in food products [4, 13, 14]. Moreover, glycerol can be used as a precursor for several chemicals, materials and energy carriers through biological or chemical transformations (Fig. 2.2) [12, 13, 15-21] (Papers I-V).

The commercial production of glycerol is divided between fossil and renewable resources. The first process for the production of glycerol was the manufacture of soap via hydrolysis of fats and oils. Subsequently, a peak in glycerol production occurred during the World War I and was obtained through fermentation of glucose by yeast. Later on, the petrochemical refineries became the major supplier of synthetic glycerol, where propylene obtained from steam cracking of heavy oil is converted to glycerol via allyl chloride or allyl alcohol as intermediates [4]. Currently, the petrochemical route represents only 10% of the current glycerol production [14]. The remaining 90% is covered mainly by renewable glycerol obtained as a by-product of biodiesel production process.

With the expansion in liquid biofuels production, especially biodiesel through transesterification of animal or vegetable oil, substantial amount of glycerol is accumulated as a by-product (0.1 ton glycerol per ton biodiesel) [22, 23]. The biodiesel production in 2011 was estimated at 8.6 and 4 million tons in Europe and USA, respectively [24, 25]. Glycerol is also accumulated from sugar fermentation by yeast for the production of bioethanol [26]. It is anticipated that
by 2020, the global glycerol production will exceed the demand by approximately 6 fold [14]. At present, the fluctuation in glycerol market prices could influence the establishment of glycerol-based biorefineries. However, for the near future, glycerol will remain an attractive substrate. Glycerol was used as the raw material for the production of propionic acid, 3HPA and 3HP in Papers I-V.

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**Fig. 2.2 Derivatives of glycerol**

2.2.2 2-Methyl-1,3-propanediol (2M1,3PDO)

2M1,3PDO is a dihydric alcohol with two primary hydroxyl groups. It is used as an ingredient in liquid-saturated polyesters, powder polyesters, alkyd resins, unsaturated polyesters and plasticizers [27]. Industrially, 2M1,3PDO is obtained as a by-product from 1,4-butanediol production process through catalytic hydroformylation of propylene-based allyl alcohol by CO/H₂ gas mixture [28]. It can also be produced by the fermentation of glycerol and thin stillage by *Citrobacter freundii* [29]. 2M1,3PDO was used as the raw materials for the production of MA in Paper VI.
2.2.3 Potato juice

Potatoes are among the main sources for starch worldwide, containing 75% water, 18% starch, 1% fibre, 2.2% protein, 1% ash, 0.1% fat and some sugars. The processing of potatoes for the extraction of starch yields an enormous amount of liquid (3.5 tons/ton potato starch), called potato juice that is rich in proteinaceous compounds and other nitrogen and mineral salts (Fig. 2.3). Upon heat treatment of this juice, the protein fraction is precipitated, collected and used as animal feed. The remaining juice termed here heat-treated potato juice (HTPJ), contains 3.5% dry matter. The typical analysis shows the presence of 48 mg nitrogen, 120 mg potassium, 10 mg phosphorous, 11.6 mg sulphur, 4.4 mg calcium, 7.9 mg magnesium and other elements (per Kg dry matter). The HTPJ, being cheap (approximately 15 USD/m³), easily available, and rich in nitrogenous compounds and other salts essential for microbial growth, represented an interesting nitrogen source for microbial growth and production of chemicals and fuels in a biorefinery system [30, 31] (Paper II).

Fig. 2.3 Potato starch production process
The steps from potato tuber to heat-treated potato juice and starch.

2.3 Industrial biotechnology

For the successful production of bio-based chemicals and energy in biorefineries, development of effective conversion technologies is required. Industrial biotechnology provides an important technology base for biomass pre-treatment and transformation to different products. It is based on the use of microorganisms and enzymes as catalysts in processes like fermentations and biocatalysis, which provide cleaner, energy-efficient and less toxic routes than conventional chemical processes.
Microbial diversity in nature provides access to a vast array of metabolic networks for the production of a wide variety of molecules from natural as well as synthetic molecules. Microbial fermentations are used for production when the formation of products is associated to the growth of microorganisms. When the raw material can be converted to the product by a single or a limited number of steps, resting cells can be used. However, the use of wild-type microorganisms, which are adapted to thrive in natural ecosystems, can have limitations when used for industrial production. Therefore, strategies are required at organism and/or process level in order to fulfil the requirements for process feasibility.

2.3.1 Indicators for bioprocess efficiency

The production process goes through two main stages, upstream processing (USP) and downstream processing (DSP). The USP is concerned with the conversion of the raw materials to the final product. All the steps following the USP with regards to cell/biocatalyst separation, product concentration, recovery, purification, polishing, formulation, and packaging form the core of the DSP. The type, market size and application of the product determine the requirements for industrialization. Several values are important as indicators for the possibility of large-scale production (Fig. 2.4), however the final decision is dictated by process economics [1].

Fig. 2.4 Indicators for the bioprocess efficiency

Volumetric production rate \( (Q_p) \) is defined as “units of product formed per volume and unit time” [32]. Maximizing the volumetric productivity minimizes the reactor size, and hence lower the production cost [33]. For bulk chemicals, a productivity of 2 g/L.h or higher is desired for scaling up [12].
Product yield ($Y_p$) measures how much of the substrate is converted to the desired product. It determines the amount of substrate required, which in the case of bulk chemicals has the most share in the production cost [8, 34]. Additionally, the cost and availability of the substrate are major concerns [8].

Product concentration: Dilute product streams in bioprocesses present a major bottleneck for industrial biotechnology. Lower production cost is to a large extent influenced by the product concentration and the availability of an efficient separation technique with high selectivity and capacity for the desired product. Even the nature and concentration of by-products are crucial factors to be considered.

2.3.2 Bioprocess limitations

Microbial production of chemicals presents a number of challenges in terms of:

- overcoming the product-mediated toxicities for the producer organism that limits productivity and product yields,
- avoiding formation of other side-products that lead to reduction in the yield of the desired product and increase purification costs,
- minimizing the nutrient and energy costs of fermentation processes, and
- achieving efficient and economical recovery of the pure chemicals without leading to wastes and high costs.

2.4 Systems biology for bioprocess development

The steps from making a decision on a target product till reaching the industrial production are divided into three major phases (Fig. 2.5) [35]. The choice of microorganism for a bioprocess is usually based on the desired product, available substrate, and growth requirements, which affect fermentation design and downstream processing [1]. The efficiency of the microorganism determines whether to go for metabolic engineering (Second round) or for direct process optimization (Third round).
2.5 Metabolic engineering & synthetic biology

Wild-type microorganisms are in many cases not capable of producing the desired products at high enough efficiencies. This is where metabolic engineering and synthetic biology come to the stage [36]. Metabolic engineering “is about designing, engineering, and optimizing pathways for the production of a variety of products” [37]. The increasing availability of microbial genome sequences has been the driving force for development and growth in metabolic engineering [38]. According to Stephanopoulos 2012, synthetic biology, pathway engineering and protein engineering are components forming the overall scheme of metabolic engineering (Fig. 2.6). Synthetic biology deals with DNA and mRNA, protein engineering is concerned with proteins and enzymes, while pathway engineering deals with the different metabolites, reactions and fluxes within the cell. Through these components, enhanced production of current products, introduction of novel pathways for current products and introduction of novel products have been achieved [37]. Accordingly, metabolic engineering has become a platform for bio-based economy, as well as for discovery and production of new therapeutics [39].
2.6 Bioprocess engineering

Batch, fed-batch and continuous modes of operations have traditionally been the common routes utilized in microbial production. Limited productivity and product yield are major concerns in biotechnological processes that may be attributed to factors such as the substrate being partly used for biomass formation and inhibition of growth and activity by the product. Uncoupling biomass accumulation from production by retaining high cell density within the bioreactor has shown to lessen this problem, e.g. by cell immobilization and cell recycling. \textit{In situ} removal of the product during the process is a concept proposed for alleviating product inhibition.

2.6.1 Cell immobilization (Paper I)

Cells can be immobilized by a variety of techniques, however adsorption over a solid support is the simplest and most commonly applied. It was used successfully in wastewater treatment (AnoxKaldnes\textsuperscript{TM} MBBR Technology), where different bacteria, ciliates and rotifers are established in a biofilm over plastic carriers; and for industrial production of vinegar [40-42]. Immobilized cell reactors (ICR) are simple to operate, offer higher productivities, yield, and product concentration. Also, improved specific production- and consumption rates and isolation of tolerant mutants have been reported [42, 43]. Continuous fermentations can be
operated at high dilution rates without cell washout. It also allows easy handling and recovery of the immobilized cells [42].

The interaction between the microorganisms and the matrix is mediated by Van der Waal forces, ionic and/or hydrogen bonds [42]. Hence, the properties of the matrix, surface area available for immobilization, roughness, and nature of the surface (hydrophobicity, charge, ability for chemical modification) are important factors to be considered [44, 45]. Chemical modification for increasing the hydrophobicity or the positive charge also enhances the immobilization efficiency. Polyethylenimine (PEI), a cationic, branched polymer with primary, secondary and tertiary amine groups is widely used for treatment of matrices so that the positive charge of the polymer interacts with the negative charge on the cell surface leading to a stronger attachment [46, 47], Paper I. Even other physical parameters (pH, temperature, shearing) around the microorganism also determine the immobilization efficiency [44].

Packed-bed, fluidized-bed, stirred-tank and air-lift are the different forms of ICR, which can be operated under batch, fed-batch and continuous modes of operation.

2.6.2 Cell recycling (Papers II & IV - VI)

In this process, the biomass is retained within the bioreactor via filtration or centrifugation. Hence, the cell separation is integrated with the production step, yielding cell-free broth ready for product recovery and purification. In addition, the high cell density inside these reactors made them less prone to microbial contamination.

Bioprocesses with cell recycle can be operated using different modes. The continuous fermentation/biotransformation with cell recycle was the most investigated and resulted in substantial increase in volumetric productivities. Examples are the production of:

- ethanol (the industrial Biostil®2000 process, Chematur Engineering, Sweden), where the yeast cells are separated by centrifugation and returned to the bioreactor while the cell-free broth is sent to distillation column for the recovery of ethanol [48],
- lactic acid (Leibniz-Institute for Agricultural Engineering Potsdam-Bornim) (450 L), where the cells of Lactobacillus were retained via a tangential-flow hollow fibre membrane and the cell-free permeate containing 60 g/L lactic acid was sent for DSP [49],
- acetic acid, gluconic acid, propionic acid and 1,3-propanediol [34, 50-52].
It is highly useful when non-growing cells (resting cells) are used, where at low dilution rates cell washout would occur. Nevertheless, the membrane fouling by the high cell density, the high maintenance cost, the low product concentration if compared to batch and fed-batch operations have limited its application industrially.

Batch/fed-batch operation with cell recycle, sums the advantages of continuous operation that gives high volumetric productivity and the free cell batch operation which is a well-understood system, easy to handle and yields high product concentration. Also, the cell separation and recycle is done once at the end of each batch, which lowers the maintenance and operational costs. It allows cleaning, regeneration and sterilization of the membrane during the fermentation run.

The major limitations are membrane fouling, reduced space available for cell growth, increased culture viscosity accompanied by mass-transfer limitations, other effects related to changes in water content of the cells, high shear stress on the cells entering the filtration unit, and difficulties in scaling up [53].

2.6.3 *In situ* product complexation and removal (Paper III & IV)

Several end products of bioprocesses such as organic acids, aldehydes, ketones, alcohols and antibiotics are highly toxic to the producer microorganisms, which have a considerable effect on the bioprocess efficiency. To avoid product-mediated toxicity, *in situ* product complexation or removal can be applied so as to:

- convert the product to a non-toxic form, and
- maintain the product concentrations at non-inhibitory levels.

Some modes of *in situ* product recovery applied include integration of extraction, adsorption, electrodialysis, precipitation, and gas stripping (volatile compounds) [48, 54-57]. Considerable enhancement in volumetric production rates has been reported. Moreover, the recovered product could be collected at high concentration and purity. Hence, this technique combines both product recovery and purification, which shortens the production time.

However, the integrated recovery system is an additional constraint, which might complicate the process. For example, many organic solvents, complexing agents, charged ion exchange resins interfere with microbial metabolism and enzymatic activity. For instance, ion exchange resin, bisulfite, and semicarbazide used for complexation of 3HPA were reported to repress biotransformation of glycerol using *Lactobacillus reuteri* and *Klebsiella pneumonia* [58-60]. Production of propionic acid was halted by the organic solvent used for extractive fermentation [61]. This could be solved by compartmentalization of the different steps of production and product removal (Paper IV).
2.7 Integrated refineries (Paper VI)

The shift in the raw material and technologies for chemicals production necessitates the integration of the new technologies in the already established industry or development of a completely new process involving integration of a bioprocess and a chemical process. Chemical catalysis is a well-known technology. It has offered several economically feasible and environmental-friendly solutions for synthetic chemistry that is difficult to replace using biocatalysis at the present stage.

Some examples of integrated processes include the production of vitamin B12, vitamin C, 1-deoxynojirimycin and miglitol. For the production of vitamin B12, the methyl/hydroxy/adenosyl cobalamin produced from fermentation of sugars using *Propionibacterium freudenreichii* or *Pseudomonas denitrificans* is reacted with potassium cyanide (KCN) to yield the stable commercial form, cyanocobalamin (Eq1) [62]. *Glucunobacter oxydans* is used as a biocatalyst for the conversion of D-sorbitol into 2-keto-L-gluconic acid through a three-step catalyzed reaction by three different dehydrogenases. The resulting acid is then converted chemically to the active form of vitamin C (L-ascorbic acid) (Eq2) [63]. In case of 1-deoxynojirimycin and miglitol which are used for the treatment of non-insulin-dependent diabetes, *G. oxydans* catalyzes a regioselective oxidation step between two chemical reactions (Eq3,4) [63]. In Paper VI, bio- and chemical catalytic reactions were integrated for achieving a greener production of MA (Eq5). Similarly, the production of acrylic acid from the biologically-produced 3HP or fumaric acid includes chemical dehydration or cross-metathesis with ethylene, respectively (Eq6,7)[64, 65].

\[
\text{Sugars} \rightarrow \text{methyl-, hydroxy- \& adenosyl Cobalamin} \rightarrow \text{Cyanocobalamin (Eq1)}
\]

\[
\text{D-sorbitol} \rightarrow \text{L-sorbose} \rightarrow \text{L-sorbosone} \rightarrow \text{2-Keto-gluconic acid} \rightarrow \text{L-ascorbic acid (Eq2)}
\]

\[
\text{D-glucose \rightarrow 1-amino-D-sorbitol \rightarrow 6-amino-L-sorbose \rightarrow 1-deoxynojirimycin (Eq3)}
\]

\[
\text{D-glucose} \rightarrow \text{N-hydroxyethyl-1-amino-D-sorbitol} \rightarrow \text{N-hydroxyethyl-6-amino-L-sorbose} \rightarrow \text{miglitol (Eq4)}
\]

\[
\text{2M1,3PDO} \rightarrow \text{3H2MPAL} \rightarrow \text{3H2MPA} \rightarrow \text{Methacrylic acid (Paper VI) (Eq5)}
\]

\[
\text{Sugar/gly} \rightarrow \rightarrow \rightarrow \text{3HP} \rightarrow \text{Acrylic acid (Eq6)}
\]

\[
\text{Sugar} \rightarrow \rightarrow \rightarrow \text{Fumaric acid} \rightarrow \text{Acrylic acid (Eq7)}
\]

(Red arrows indicate biological steps – Multiple arrows indicate multiple steps).
3. Propionic Acid Production by Microbial Fermentation of Glycerol

3.1 Propionic acid

Propionic acid is a C3 organic acid currently produced by petrochemistry. The global production capacity for propionic acid was estimated at 349 thousand tons in 2006, with estimated market growth of 2.5% per year till 2010. The market growth is mainly in Asia and the Middle East due to the planned expansion in feed and food preservation [66]. BASF is the main production with a production capacity of 110 thousand tons/year followed by Dow Chemical, Eastman and Perstorp with production capacities of 90, 70 and 50 thousand tons/year, respectively. The main production route of propionic acid is the oxo-synthesis through hydroformylation of ethylene with carbon monoxide yielding the intermediate propionaldehyde, which gives propionic acid on oxidation. It is also obtained as a by-product of the chemical production of acetic acid by liquid phase oxidation of n-butane [67].

Various food products, animal feed, cosmetics, and pharmaceuticals are easily contaminated with microorganisms due to their content of carbohydrates, peptides, proteins and lipids. Propionic acid and its calcium, sodium and potassium salts are recognized as GRAS “Generally Regarded As Safe” by FDA (Food and Drug Administration), is used for preventing the spoilage of these products, and hence increasing their shelf life. Since being in a direct contact with humans and animals, naturally-produced preservatives are preferred over synthetic ones. Therefore, economical production of propionic acid from bio-based resources using microorganisms would be desirable [52]. As well, propionic acid is integrated in cellulose acetate propionate (CAP) plastics, which are used for the manufacture of textiles, fibres, and reverse osmosis membranes. It is also used in the production of anti-arthritic and antibiotic drug preparations, herbicides, flavours and perfumes [66-68]. Propionic acid can potentially serve as a platform chemical in the bio-based industry for production of other important chemicals. It can be converted to acrylates which are used in paints, resins and other products [12]. Hasegawa et al. (1982) showed the possibility of conversion of propionic acid to 3HP, another important platform chemical, using a mutant strain of *Candida rugosa* [69].
3.2 Microbial route for propionic acid production

The production of propionic acid by fermentation of different bio-based carbon sources has been under study since 1920 with the aim of developing a bio-based, environmental-friendly route, however it never exceeded pilot scale [52]. A recent life-cycle assessment for the propionic acid production by fermentation of glycerol showed a possible reduction of greenhouse gas (GHG) emissions by 60% compared to the fossil-based route (1.4 vs. 3.5 Kg CO2 eq/Kg propionic acid). However, the energy requirement was higher, which could be lowered by further development in fermentation and downstream processing technologies [31].

Several microorganisms can produce propionic acid including Propionibacterium spp., Veillonella (parvula and alcalescens), Clostridium propionicum, Selenomonas (ruminantium and sputigena), Megashaera elsdenii and Fusobacterium necrophorum. Among them, propionibacteria have been mostly used as hosts for propionic acid production [70] (Papers I and II).

3.2.1 Propionibacteria

Propionibacteria are Gram-positive, non-spore forming, non-motile, facultative anaerobic, pleomorphic, rod-like microorganisms (Fig. 3.1) [71]. Based on their natural habitat, propionibacteria are classified into: classical (dairy) propionibacteria isolated from cheese and milk and include P. freudenreichii, P. jensenii, P. thoenii, P. acidipropionici, P. coccoides, P. cyclohexanicum, and cutaneous propionibacteria isolated from human skin and include P. acnes, P. avidum and P. granulosum [71]. Beside propionic acid, propionibacteria also produce a number of other industrially important products including vitamin B12, trehalose, bacteriocins and exopolysaccharides [72-75]. The whole cells of propionibacteria are even used/or can be potentially used as starter cultures in cheese manufacture, unicellular protein for animal feed, leavening additive for baking, and in preparation of ensilage [71].

3.2.1.1 Metabolism

Propionibacteria can metabolize different carbon sources and produce propionic acid as the main product with acetic acid and CO2 as major by-products. The steps from pyruvate to propionate through succinate as intermediate are represented in a
closed cycle known as Wood-Werkman cycle or dicarboxylic acid cycle (Fig. 3.2) [52, 70]. Within this cycle, shifting between C3 molecules and C4 molecules is achieved by recirculating a single carbon atom continuously. S-methylmalony-CoA acts as a carbon donor and pyruvate as an acceptor in a reaction catalyzed by methyl-malonyl CoA transcarboxylase. Generally, closed cycles are known to provide a higher amount of energy and a tool for co-factor regeneration within the microbial cells [71].

Fig. 3.2 Propionic acid fermentation pathway
The metabolic pathway for propionic acid production from different carbon sources, showing the Wood-Werkman cycle, different intermediates and the metabolic end products.

3.2.1.2 Carbon and nitrogen/vitamin sources
The type and the degree of reduction (γ) of the carbon source influences the pattern of metabolic by-products obtained [76]. With the majority of carbon sources including glucose, lactose, xylose, sucrose and lactate, acetic acid is obtained as a major by-product (Eq8, 9)1. Glycerol, in contrast, triggers a

---

1 The degrees of reduction (γ) of: glucose (4), lactose (4), xylose (4), fructose (4), lactate (4), acetate (4), glycerol (4.667), propionate (4.67), succinate (3.5) and n-propanol (6).
homopropionate fermentation pattern leading to a high propionate yield (Eq10) [52, 77-79]. Since glycerol is more reduced than other carbon sources, its conversion to pyruvate generates higher amount of reducing equivalents, driving the metabolic flux towards propionic acid to achieve the required co-factor balance. Glucose, on the other hand, generates lower amount of reducing equivalents, and hence acetate production is preferred as a source for ATP, yielding higher cell density. Consequently, the molar ratio of propionic acid to acetic acid (PA/AA) is greatly affected by the carbon source and could vary between 2:1 when lactate is utilized to 30:1 in case of glycerol [77].

\[
\begin{align*}
1.5 \text{ glucose} & \rightarrow 2 \text{ propionic acid} + 1 \text{ acetic acid} + 1 \text{ CO}_2 + 1 \text{ H}_2\text{O} \quad \text{Eq8} \\
3 \text{ Lactic Acid} & \rightarrow 2 \text{ propionic acid} + 1 \text{ acetic acid} + 1 \text{ CO}_2 + 1 \text{ H}_2\text{O} \quad \text{Eq9} \\
1 \text{ Glycerol} & \rightarrow 1 \text{ propionic acid} + 1 \text{ H}_2\text{O} \quad \text{Eq10}
\end{align*}
\]

With regards to the nitrogen source, many strains of propionibacteria can grow on basal medium, however a considerable improvement in growth was observed in presence of amino acids [80, 81]. Other stimulatory factors for growth and propionic acid production were isolated from potato- and yeast extracts [80, 82, 83]. The nutritionally-rich yeast extract represents an excellent medium for the cultivation of propionibacteria and propionic acid production. However, it would be an additional burden to the process cost. Replacement of yeast extract with cheaply available agricultural or industrial by-products such as potato juice, fish hydrolysate and de-lactose whey have been evaluated [80, 82, 84, 85] (Paper II).

Most propionibacteria can cover their own needs of vitamins, except for biotin (vitamin B7) and pantothenic acid (vitamin B5) that should be supplemented [86, 87]. Biotin serves as a co-factor for methylmalonyl-CoA transcarboxylase catalyzing the transfer of (COO) from S-methylmalonyl-CoA to pyruvate with simultaneous production of propionyl-CoA and oxaloacetate. However, pantothenic acid is required for the synthesis of coenzyme-A utilized in several enzymatic reactions [71].

3.2.1.3 Fermentative production

Production of propionic acid is highly restricted by the low growth rate of propionibacteria and the strong product-mediated inhibition to the microorganism [88]. In order to overcome these problems, several approaches have been evaluated including in situ product removal via extractive fermentation or binding to ion exchange resin [56, 57, 89], development of mutant strains with improved tolerance to propionic acid [90-92], optimization of the type and concentration of nitrogen source [93], and retaining high cell density by immobilization [43, 85, 92, 94-98] (Paper I), or cell recycling [50, 51, 53, 99] (Paper II). Fig. 3.3 presents the current status for propionic acid production from glycerol using propionibacteria in batch/fed-batch operations.
Fig. 3.3 Current status of propionic acid production from glycerol

The figure shows different propionic acid concentrations and volumetric productivity obtained by glycerol fermentation. The requirement for industrialization is 2-3 g/L.h and 100 g/L [13]. The data obtained for batch/fed-batch operations using free and immobilized cells are from the following references [19, 50, 56, 77-79, 91, 94, 100-102], Papers I & II.

The low propionate yield and concentration, and high amounts of acidic by-products formed also increase the costs for downstream processing. Besides the proper choice of the carbon source, other strategies have been evaluated to reduce the by-product formation. Metabolic engineering for lowering the acetate production through knocking out the gene encoding acetate kinase mediating the conversion of acetyl-phosphate to acetic acid resulted in the reduction of acetate production. However, a concomitant reduction in microbial growth was reported [103]. Fermentation of co-substrates such as glycerol/glucose has led to enhanced microbial growth, volumetric productivity and decreased acetic acid production [102, 104].

3.2.2 A potential biorefinery

From the perspective of product yield, less by-products, downstream processing, and absence of substrate-mediated inhibition, glycerol was chosen as a carbon source (Paper I & II). However, due to the narrow gap between glycerol and propionic acid prices [105], a cheap nitrogen/vitamin-rich source was required. HTPJ was thought to meet these demands and was evaluated in Paper II.
Analysis of conventional propionic acid batch fermentation shows that approximately 39% of the total fermentation time is spent without significant consumption of glycerol or production of the acid (non-productive phase). Furthermore, at propionic acid concentrations exceeding 15 g/L, glycerol consumption- and propionic acid production rates drop by approximately 58%. As a consequence, substantial reduction in volumetric productivity occurs. In Papers I & II, the effect of high cell density cultivations on propionic acid volumetric productivity, yield, and fermentation kinetics using glycerol as a carbon source, yeast extract and HTPJ as nitrogen sources was evaluated.

3.2.2.1 Production using immobilized cells

Cell retention via immobilization was evaluated in Paper I. Two matrices of different materials and distinct surface properties were used for immobilization: Poraver*, a foamed highly porous recycled glass, and Luffa, a fibrous mature dried fruit of *Luffa cylindrica* (Fig. 3.4). For improving the immobilization efficiency, the matrices were treated with the cationic polymer, polyethyleneimine (PEI). The immobilized cells were used in a packed-bed bioreactor operated in a recycle-batch mode for propionic acid production. PEI-treated Poraver attached over 30 times higher amount of cells than PEI-treated Luffa, which in turn led to complete omission of the non-productive phase. The inhibitory effect of propionic acid on its production was also decreased. Consequently, the volumetric productivity in fermentation from 40 g/L glycerol was increased by a factor of 2 over free cells and reached 0.86 g/L.h. Using 60 and 90 g/L glycerol, the volumetric production rates were 0.43 and 0.35 g/L.h, respectively (Fig. 3.5). Under continuous mode of operation using cells immobilized on PEI-treated Poraver at a dilution rate of 0.1 1/h, propionic acid volumetric productivity reached 1.44 g/L.h, while cell washout occurred in the free cell continuous stirred tank reactor.

Despite the high cell density immobilized on PEI-poraver, the reactor operated at 8.3% of its theoretical capacity. This could be due to the highly packed cell mass resulting in diffusion limitation of the nutrients.
Fig. 3.5 Propionic acid production from glycerol by immobilized *P. acidipropionici*

The cells immobilized to polyethylenimine-treated Poraver were used for 9 consecutive recycle-batches with intermittent washing using saline solution (Paper I). The figure shows the concentration of glycerol (grey bars), and propionic acid (white bars), volumetric productivity (▲), propionate yield (■) and PA/AA molar ratio (x) for each recycle-batch.

3.2.2.2 Production using recycled cells

In Paper II, cell recycling was used to achieve high cell density in the bioreactor and to avoid the mass transfer limitation observed with immobilized cells. HTPJ supplemented with 0.5 mg/L biotin was used as an alternative nitrogen source to yeast extract. The production was done in sequential batches where the cells were collected at the end of each run and used for the subsequent batch (Fig. 3.6). The glycerol consumption- and propionic acid production- rates were dependent on the initial biomass concentration over 11 sequential batches. The equations describing these relations were \( Q_s = 0.0304 \cdot OD + 0.2722 \) and \( Q_p = 0.0189 \cdot OD + 0.168 \), indicating an increase of the corresponding rates by 0.2 and 0.3 g/L.h when the initial cell density was increased by a factor of 10. The maximum biomass concentration achieved during such an operation was 23.4 gCDW/L. At this initial cell density glycerol consumption- , total acid- and propionic acid production rates were 2.3, 1.7 and 1.4 g/L.h, respectively, from 50 g/L glycerol; representing a 6-fold increase over those obtained in the first batch. The yields of propionic acid and other metabolic by-products were independent of the initial cell density. However, acetate and succinate were reduced by 40 and 30% respectively, when the concentration of HTPJ and biotin were reduced by 50% in batches 10 and 11. Consequently, the molar ratio of propionic acid to total organic acids was increased from 79 mol% to 87 mol%. This would lower the cost for nitrogen source and downstream processing. At higher glycerol concentrations of 85 and 120 g/L, respectively, 43.8 and 50.8 g/L propionic acid were obtained at a rate of 0.88 and 0.29 g/L.h and an average yield of 89.5 mol%. 

![Graph showing glycerol and propionic acid concentrations, volumetric productivity, propionate yield, and PA/AA molar ratio for each batch.](image-url)
Fig. 3.6 Production of propionic acid from glycerol by sequential batch fermentation

Production of propionic acid from glycerol and heat-treated potato juice as nitrogen source by free cells of *P. acidipropionici* DSM 4900 for 11 sequential batches with cell recycle (Paper II). The figure shows the concentration of glycerol (●) and propionic acid (★) during consecutive batches.
4. 3HPA & 3HP Production by Biotransformation of Glycerol

3HPA and 3HP are not commercially available products, but have attracted interest as potential platform chemicals in a bio-based industry for industrially important chemicals such as acrolein, acrylic acid, malonic acid, 1,3PDO, acrylamide, acrylonitrile, methyl acrylate, and ethyl-3HP [13, 106, 107].

4.1 3-Hydroxypropionaldehyde

Aldehydes are important versatile compounds that are widely used in organic synthesis as starting materials for other compounds. They can undergo several reactions such as addition, reduction to alcohols, and oxidation to carboxylic acids. The extra β-hydroxyl group in 3HPA adds to the molecular flexibility. In aqueous solution, 3HPA is present in an equilibrium mixture with its hydrate and dimer, known as reuterin (Fig. 4.1) [108]. This mixture possesses antimicrobial activity against a wide range of pathogens and food spoilage organisms [13, 109-111], which allows its application in functional foods and healthcare sector [108]. In tissue fixation, 3HPA can be used as a cross-linking agent, as a less cytotoxic alternative to glutaraldehyde [112].

![3-Hydroxypropionaldehyde](image)

**Fig. 4.1 Concentration-dependent equilibrium of 3HPA with its dimer and hydrate**

Industrially, 3HPA is formed as an intermediate during the production of 1,3PDO from fossil-based propylene and ethylene via the Degussa and Shell processes, respectively [113, 114]. However, separation of 3HPA in pure form is
not feasible [108]. 3HPA can be efficiently produced from renewable glycerol via chemical or biotechnological means. Chemically, glycerol is initially thermally dehydrated to acrolein which upon rehydration in presence of sulphuric acid yields 3HPA [115, 116]. However, acrolein is a highly toxic and explosive compound, the process requires harsh conditions of temperature and pH, and large amounts of gypsum are accumulated during the neutralization of the sulphuric acid with calcium salts. On the other hand, using a microbial biocatalyst, 3HPA production from glycerol is achieved in a single step enzymatic reaction catalyzed by glycerol/diol dehydratase at 37 °C and neutral pH [117].

4.1.1 Microbial production of 3HPA

3HPA is formed as an intermediate of glycerol metabolic pathway by several members of the Lactobacillus, Klebsiella, Enterobacter, Clostridium, and Citrobacter spp. L. reuteri was chosen for 3HPA production since it is a probiotic (in contrast to Klebsiella), able to tolerate high concentrations of 3HPA, able to accumulate and excrete 3HPA even in absence of scavenger, and doesn’t utilize glycerol as a carbon source for growth which lowers by-products formation and increase product yield [60, 108, 118] (Fig. 4.2).

4.2 3-Hydroxypropionic acid

3-Hydroxypropionic acid is a β-hydroxycarboxylic acid and a structural isomer of lactic acid (2-hydroxypropionic acid). It is categorized among the topmost platform chemicals for potential bio-based production [7, 12, 119]. The bifunctionalities, hydroxyl- and carboxyl groups, make it a versatile compound for organic synthesis. Besides serving as a raw material for several other chemicals, 3HP is also a starting material in cyclization reactions for the synthesis of propiolactone [107] (Fig. 4.3). Poly-3-hydroxypropionate (Poly-3HP), a biocompatible and biodegradable polymer with excellent glass transitions, and high melting point can also be obtained by self-condensation of 3HP monomers [120, 121]. It is also incorporated as a cross-linking agent in coatings, lubricants and antistatic agents for textiles [106]. Other applications are expected within the field of food industry, cosmetics and fertilizers [122].
Fig. 4.3 Derivatives of 3-hydroxypropionic acid

However, in contrast to lactic acid which is presently produced at industrial scale, no industrial process, neither chemical nor biological, for production of 3HP has been established yet. This is due to lack of economic feasibility and/or environmental compatibility for most of the suggested production routes [106]. Nevertheless, the high expectation for 3HP as a platform chemical gives a projected market volume of 3.6 million ton/year [8].

Different chemical processes for production of 3HP rely on utilization of acrylic acid, 1,3PDO, acrolein and propiolactone as starting materials, which are in fact expected products of 3HP with a higher market value [106, 107].

4.2.1 Microbial production of 3HP

Several microorganisms such as L. reuteri, L. collinoides, Alcaligenes faecalis can produce 3-HP at low concentrations as an end product of glycerol and acrylic acid metabolism [106]. However, the feasibility for these production routes has not been sufficiently investigated. So far, approximately all studies have focused on the production of 3HP using genetically engineered microorganisms. A number of companies (including Cargill, Novozymes, OPXBO, DuPont) and research groups are developing recombinant microorganisms to produce higher levels of 3HP from bio-based glucose and glycerol. Jiang et al. (2009) and Henry et al.
(2010) suggested different metabolic pathways for 3HP production [123, 124]. Though many routes were redox neutral, only two were thermodynamically favorable. The first pathway starts with glucose, through pyruvate, α-alanine, β-alanine and malonate semialdehyde as intermediates. The second starts with glycerol through 3HPA, 3HP-CoA, 3HP-phosphate (3HP-P) as intermediates with simultaneous production of 1,3PDO for achieving the redox balance [123] (Fig. 4.4).

![Fig. 4.4 Metabolic routes for 3HP production](image)

A number of suggested metabolic pathways for the production of 3HP from glucose and glycerol [123-125]. The pathway from glycerol through 3HP-CoA and 3HP-P (dotted line) was studied in Paper V.

### 4.3 Lactobacillus reuteri as a production host for 3HPA & 3HP

*L. reuteri* is a member of lactic acid bacteria (LAB) that are a group of Gram positive bacteria, non-spore forming, cocci or rods, producing lactic acid as the major product of their metabolism. The core members of this group are *Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus* [126].

LAB can metabolize different carbohydrates efficiently and based on the products of sugar metabolism, they are classified as homofermentative and heterofermentative. In homofermentative LAB, pyruvate is the main electron acceptor with formation of lactate as the main end product. Examples are *L. lactis*
and *L. delbreuckii*. On the other hand, in heterofermentative LAB, lactate and ethanol are the main products for regeneration of NAD⁺ from NADH, in addition to acetic acid coupled with ATP formation and CO₂. Examples are *L. brevis* and *L. reuteri* [126].

LAB can utilize different compounds as electron acceptors including oxygen, citrate, glycerol, 1,2-propanediol, 1,2-ethanediol and fructose (Fig. 4.5). Oxygen is reduced to water in a reaction catalyzed by NADH oxidase (NOX). Glycerol, 1,2-propanediol and 1,2-ethanediol are initially dehydrated by glycerol/diol dehydratase to 3HPA, propionaldehyde and acetaldehyde, respectively, and then further reduced to the corresponding alcohols with generation of NAD⁺ from NADH [127]. Citrate is split into acetate and oxaloacetate, which is converted to lactate through pyruvate as intermediate. Fructose is reduced to mannitol in a single-step reaction catalyzed by mannitol dehydrogenase [128]. Co-metabolism of these electron acceptors with the main substrate results in higher cell density, as most of the acetyl-phosphate that was initially reduced into ethanol is converted to acetate yielding ATP [127, 129].

![Fig. 4.5 Mechanisms for electron transport in lactic acid bacteria (LAB)](image)

**Fig. 4.5 Mechanisms for electron transport in lactic acid bacteria (LAB)**

Utilization of 1,2-propanediol, citrate, fructose and oxygen as external electron acceptors by LAB and formation of n-propanol, lactic acid, water and mannitol as end products.

### 4.3.1 *Lactobacillus reuteri*

*L. reuteri* is an obligate heterofermentative lactic acid bacterium [126]. It can grow on several carbon sources, but not glycerol due to the lack of dihydroxyacetone kinase. However, the presence of small quantities of glycerol, 1,2-propanediol or 1,2-ethanediol in the cultivation medium induces the expression of the genes encoding enzymes and structural proteins required for glycerol metabolism and
utilization as an electron acceptor. The expression of these genes is under the control of the propanediol-utilization operon (pdu). This operon is located in a gene cluster adjacent to cob, cbi and hem operons responsible for the biosynthesis of cobalamin (vitamin B12). The structural proteins form cytoplasmic microcompartments known as metabolosomes. These protein shells act as a native cellular machinery to mitigate the toxicity of the intermediate aldehyde against the producer microorganism (Fig. 4.6) [130, 131]. Also they form an optimum environment with concentrated enzymes and co-factors for efficient degradation of the aldehyde [131].

![Fig. 4.6 Protein microcompartments for the metabolism of glycerol](image)

Protein microcompartments for degradation of glycerol into 3HPA and further conversion to 1,3PDO or to 3HP through 3HP-CoA and 3HP-phosphate at intermediates.

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PduCDE</td>
<td>Glycerol/diol dehydratase</td>
</tr>
<tr>
<td>PduQ</td>
<td>1,3-Propanediol oxidoreductase</td>
</tr>
<tr>
<td>PduP</td>
<td>Propionaldehyde dehydrogenase</td>
</tr>
<tr>
<td>PduL</td>
<td>Phosphotransacylase</td>
</tr>
<tr>
<td>PduW</td>
<td>Propionate kinase</td>
</tr>
</tbody>
</table>

The glycerol metabolic pathway is made up of five enzymes. Glycerol/diol dehydratase (PduCDE) is the first and most important enzyme, catalyzing the dehydration of glycerol to 3HPA. This is followed by a reductive and an oxidative pathway. The flux of 3HPA through these pathways is determined by the level of NADH inside the microbial cell. Higher NADH levels would result in reduction of 3HPA to 1,3PDO by 1,3-propanediol oxidoreductase (PduQ) (normal case with growing cells). On the other hand, accumulation of NAD⁺ would result in oxidation of 3HPA to 3HP via 3HP-CoA and 3HP-phosphate as intermediates. The corresponding enzymes are propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW). Production of 3HPA using purified glycerol/diol dehydratase is not feasible at the present stage due to the high sensitivity of the enzyme to oxygen, glycerol and 3HPA, the requirement for adenosyl-cobalamin as a co-factor, and the requirement for an additional enzyme for reactivation of inactivated glycerol/diol dehydratase [132-135].

### 4.3.2 3HPA accumulation and removal

In order to prevent further conversion of 3HPA to 1,3PDO by the cells, depletion of the intracellular NADH is crucial. This is tricky in case of growing cells.
However, when resting cells were used as biocatalyst for transformation of glycerol in aqueous solution, substantial amounts of 3HPA were produced at a yield of 85-87 mol% [16, 60]. Yet, this production route was restricted by the high toxicity of 3HPA to the enzymatic system causing cessation of biotransformation within 2 hours [118]. The 3HPA toxicity is mediated by interaction of the reactive carbonyl group with amino- and thiol- groups on proteins and other intracellular molecules [111, 112, 136]. Chemical blocking of the carbonyl group, \textit{in situ} removal of 3HPA from the reaction media, and engineering of glycerol/diol dehydratase for elevating the tolerance to 3HPA have been investigated for enhanced 3HPA production [58, 137-139].

Aldehydes are known to form stable adducts with bisulfite, semicarbazide and carbohydrazide, respectively. These adducts lower the 3HPA-mediated toxicity and resulted in extension of the biocatalyst activity, improved 3HPA concentration and volumetric production rate [138]. The breakdown of semicarbazone and carbohydrazone hasn’t been reported yet. On the other hand, 3HPA-bisulfite complexes can be easily broken for liberation of the free aldehyde using NaCl. Moreover, capture of 3HPA from the reaction mixture through binding to a bisulfite-functionalized anion exchanger and subsequent elution in purified form was achieved [58] (Fig. 4.7). However, \textit{in situ} complexation of 3HPA with bisulfite and its removal using bisulfite-functionalized resin resulted in repression of glycerol biotransformation by bisulfite at concentrations exceeding 50 mM and by the ion exchange resin, separately.

![Fig. 4.7 Complex formation of 3HPA with (A) bisulfite and (B) semicarbazide](image)

The compounds shown are glycerol (1), 3HPA monomer (2), 3HPA hydrate (2a), 3HPA dimer (2b), acrolein (2c), 1,3PDO (3), 3HP (4), 3HPA-bisulfite complex (5), 3HA-bisulfite-ion exchange resin (6), semicarbazide (7), intermediate (8) and 3HPA-semicarbazone (9).
4.4 Biotransformation of glycerol using *L. reuteri* to 3HPA with *in situ* product removal

The complex formation of 3HPA with bisulfite and subsequent removal of the complex using anion exchange resin were investigated with an aim to enhance the specific biocatalyst productivity (Papers III and IV).

### 4.4.1 Complex formation of 3HPA with sodium bisulfite

The complexation of 3HPA with bisulfite follows a thermodynamic equilibrium. The amount of 3HPA complexed did not exceed 60% of the initial amount irrespective of the molar ratios of the two components. The optimum complexation was obtained at molar ratios of 0.8-2:1 mol$_{3HPA}$/mol$_{bisulfite}$. At higher ratios, the reaction is no longer in equilibrium due to the depletion of free bisulfite ions (Fig. 4.8) (Paper III). The thermodynamic equilibrium allows easy breakdown of the complex using sodium chloride, however the limitation for *in situ* product complexation is that 50% of the 3HPA remains in the free toxic form. Continuous removal of the free aldehyde and/or the complex from the reaction mixture, e.g. using anion exchange resin, should lower the amount of free aldehyde.

![Fig. 4.8 Complex formation of 3HPA with bisulfite](image)

**Fig. 4.8 Complex formation of 3HPA with bisulfite**

Effect of 3HPA to bisulfite molar ratio on the remaining free bisulfite (A) and the amount of 3HPA complexed (B) (Paper III). The concentration of bisulfite in the reaction mixture was 100 mM (♦), 80 mM (■), 40 mM (▲).
4.4.2 Binding of 3HPA-bisulfite complex to Amberlite®IRA-400

Chromatographic separation is one of the most frequently used techniques for separation of molecules on industrial scale. Anion exchange resins exhibit low capacity for adsorption of 3HPA, however when the resin was pre-functionalized with bisulfite, its binding capacity showed a dramatic increase [58]. However, among the three different forms of 3HPA (monomer, dimer and hydrate), only the monomer form with free carbonyl group is able to interact with bisulfite ions. The concentration dependent distribution of 3HPA with its dimer and hydrate showed that the monomer represented small mole fraction of 15-27% over a concentration range up to 5 M [16]. Pre-complexation of 3HPA with bisulfite converts at least 50% of the aldehyde into complex form ready to interact with the anion exchanger. The residual un-complexed 50% are expected to have a distribution with approximately 15-27% still in the monomer form. Hence, pre-complexation would increase the mol fraction of species that can be adsorbed to the ion exchange resin from 27% to 77%. Using this strategy, the binding capacity was determined under batch and dynamic operation (Fig. 4.9) to be 2.56 and 2.19 mmol/g resin, respectively. Subsequent elution with 0.2 M NaCl resulted in quantitative recovery of 3HPA at high purity as a mixture of complex and monomer (0.73 mol/mol) (Paper IV).

![Fig. 4.9 Breakthrough of 3HPA mixture on Amberlite® IRA-400 (Cl)](image)

The breakthrough curve of free 3HPA, 3HPA-bisulfite complex, and total 3HPA on loading a solution (50 mL) containing 106.4 mM total 3HPA (▲) [63.7 mM 3HPA-bisulfite complex (♦) and 42.7 mM free 3HPA (■)], at a rate of 0.16 mL/min in a downward direction over a column packed with 1 g of Amberlite® IRA-400 (Cl) (Paper IV).
4.4.3 Fed-batch biotransformation: A step towards optimization

Due to the high toxicity of 3HPA and also glycerol mediated inhibition of glycerol/diol dehydratase activity, the amount of 3HPA produced per gram biocatalyst was too low. In a batch transformation of 20 g/L glycerol using resting *L. reuteri* cells, the amount of 3HPA produced per gram cell dry weight was 0.48 g<sub>3HPA</sub>/g<sub>CDW</sub>. In order to minimize the inhibitory effects, a variable-volume fed-batch biotransformation process was designed in which glycerol is fed at a low rate allowing gradual accumulation of the aldehyde (Paper III). Although the concentration of 3HPA obtained was lower than in the batch system, the amount of 3HPA produced was increased to 1.26 g<sub>3HPA</sub>/g<sub>CDW</sub>. Moreover, the activity of the cells was maintained for 5 hours. The cells showed a tendency to aggregate most likely due to glutaraldehyde-like cross-linking activity of 3HPA [112].

4.4.4 *In situ* complex formation and removal

Since bisulfite concentration exceeding 50 mM inhibits the biotransformation, *in situ* complex formation of 3HPA was performed by feeding the bisulfite together with glycerol in a fed-batch operation [58]. Using this system, the activity of the cells was maintained for at least 18 hours, and 3HPA production per gram cell dry weight was increased by 5.7 and 2.2 times, respectively, compared to that under batch and fed-batch processes without *in situ* complex formation. The molar yield of 3HPA from glycerol was 0.74 mol<sub>3HPA</sub>/mol<sub>Gly</sub>. The glycerol consumption- and 3HPA production rates were decreased from a maximum of 2.6 and 1.5 g/h during the initial 5.5 hours to 0.56 and 0.47 g/h, respectively, for the remaining 13 hours (Paper III).

To further shift the equilibrium towards complex formation, the 3HPA was removed using bisulfite-functionalized resin. In order to avoid the inhibitory effect of the ion exchange resin to the biocatalyst [58], the resin was placed in an external loop where only cell-free permeate will circulate and back to the reactor (Fig. 4.10).

As a consequence, partial removal of 3HPA was achieved and the amount of 3HPA produced per gram cell dry weight reached 5.4 g<sub>3HPA</sub>/g<sub>CDW</sub>, which is approximately 11 times higher than that for batch operation. As a result of saturation of the resin as well as accumulation of free bisulfite in the reaction mixture, the glycerol consumption and 3HPA production rates were decreased after 9 and 10 hours, respectively (Paper IV).
Fig. 4.10 Reactor design for 3HPA production with \textit{in situ} complex formation and removal

Bioreactor system used for biotransformation of glycerol to 3HPA using resting cells of \textit{L. reuteri}, with \textit{in situ} complexation 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 1 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 50 g Amberlite® IRA-400 (Cl) resin pre-functionalized with sodium bisulfite (6) using pump (5) at a rate of 6 mL/min (Paper IV).

Table 4.1 Production of 3HPA from glycerol

Comparison of the different fed-batch processes used for 3HPA production from glycerol using resting cells of \textit{L. reuteri} (Paper III and IV).

<table>
<thead>
<tr>
<th>E</th>
<th>Condition</th>
<th>Residual Glycerol (g)</th>
<th>3HPA (g)</th>
<th>( Q_0 ) (g/h)</th>
<th>( Y ) (g/g)</th>
<th>3HPA g/g cdw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycerol</td>
<td>NaHSO₃</td>
<td>Free</td>
<td>Complex</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>1(α)</td>
<td>52</td>
<td>0</td>
<td>38.35</td>
<td>6.3</td>
<td>6.3</td>
<td>0.35</td>
</tr>
<tr>
<td>2(α)</td>
<td>52</td>
<td>45</td>
<td>29.06</td>
<td>6.7</td>
<td>7.03</td>
<td>13.7</td>
</tr>
<tr>
<td>3(α)</td>
<td>27</td>
<td>23</td>
<td>0</td>
<td>4.5</td>
<td>6.95</td>
<td>11.5</td>
</tr>
<tr>
<td>4(β)</td>
<td>56.5</td>
<td>45</td>
<td>17.81</td>
<td>9.53</td>
<td>9.1+8.4(γ)</td>
<td>27.0</td>
</tr>
</tbody>
</table>

(α) E (1,2 & 3) from Paper III
(β) E (4) from Paper IV – \textit{in situ} complex formation and removal on an anion exchange resin
(γ) Complex = 3HPA complex in solution + 3HPA attached to the ion exchange resin
4.5 Co-production of 3HP and 1,3PDO

3HP is formed in small quantities as a by-product during 3HPA accumulation using resting cells of wild-type *L. reuteri* [109, 138], Paper III & IV. Trials for its production as a main product using wild-type strain have reached a closed end [140, 141]. The production was only possible after knocking out the gene encoding glycerol dehydrogenase catalyzing the conversion of glycerol to dihydroxyacetone (DHA). Although the accumulation of DHA by *L. reuteri* was not reported earlier, the authors claimed that it interferes with 3HP production pathway [141].

For the conversion of 3HPA to 3HP through the glycerol metabolic pathway, one mole of NADH is accumulated per mole acid formed. This step is unlikely to happen under growing conditions. Even depletion of NAD⁺ when resting cells are used will drive the reaction to an end “Challenge 1”. To resolve this challenge, the oxidative pathway should be coupled to another pathway that utilizes NADH. In Paper V, the simultaneous production of 3HP and 1,3PDO was investigated. The simple separation of these chemicals made this route more attractive [123].

The co-production of these two chemicals has been extensively studied using recombinant *K. pneumonia*. However, the ability of the microorganism to grow on glycerol gave rise to several by-products including lactic acid, ethanol, butanol, succinic acid, acetic acid and others which lower the product yield and increase the production cost. Also, the high structural similarity between the desired products and by-products would complicate the downstream processing [106, 142-144]. Subsequent production using lactate dehydrogenase deficient mutant led to complete removal of lactic acid, however the cumulative yield of 1,3PDO and 3HP was only 0.77 mol/mol glycerol [145].

The second challenge was noticed after kinetic characterization of PduP, the first enzyme in the oxidative pathway, for the conversion of 3HPA to 3HP-CoA. The study revealed 3HPA-mediated inhibition at a concentration exceeding 8 mM (0.59 g/L) [146]. Hence, accumulation of high amounts of 3HPA is unfavorable for 3HP production. For overcoming this problem, the metabolic fluxes through the different branches of glycerol metabolic pathway were determined for resting cells. Feeding the glycerol at a rate below that required for conversion of 3HPA to 3HP and 1,3PDO will ensure the absence of the inhibitory aldehyde and drive the reaction to completion. Under optimal conditions, the cumulative yield of 1,3PDO and 3HP from glycerol is expected to reach 100%.

The utilization of wild-type *L. reuteri* instead of recombinant *K. pneumonia* would ensure by-products- and antibiotics-free process, and simple downstream processing. No or minor metabolic engineering is required compared to the multiple engineering which at certain level could be a burden for microbial growth.
and production of the desired products. Additionally, fewer constraints are expected for industrial production in contrast to *K. pneumonia* which is an opportunistic pathogen (risk group 2 microorganism).

According to a recent simulation studies for acrylic acid production from glucose via 3HP using recombinant *E. coli*, the main concerns were the estimated high cost for downstream processing and the cost of nitrogen source required for microbial growth and maintenance [147]. So avoiding or minimizing these two factors would be advantageous. In *Paper V*, the production was done in aqueous solution of biodiesel-derived glycerol using resting cells of *L. reuteri* DSM 20016.

### 4.5.1 Production of 3HP and 1,3PDO using *L. reuteri*

For efficient conversion of glycerol to 3HP and 1,3PDO, the activity of the biocatalyst is a crucial factor. The quantity of *L. reuteri* cells were enhanced by increasing the concentration of glucose in the growth medium. Additionally, the medium was supplemented with 1,2-propanediol as an electron acceptor and stimulator for expression of the genes encoding enzymes and structural protein for glycerol metabolism. The dehydration of 1,2-propanediol by glycerol/diol dehydratase yields propionaldehyde which is less toxic to *L. reuteri* [136].

Following biocatalyst production step, the whole resting cells were used for biotransformation of glycerol. The metabolic flux through the different pathway branches was determined by a variable-volume fed-batch operation. By using a glycerol feeding strategy that allows gradual accumulation of the intermediate 3HPA, the maximum flux through the oxidative and reductive pathways were 58.3 mg$_{3HP}$/gCDW-h and 49.3 mg$_{1,3PDO}$/gCDW-h, respectively. Subsequently, using a lower glycerol feeding rate, complete consumption of 41 g/L glycerol was achieved in 55 hours yielding 19.8 g 3HP and 17.5 g 1,3PDO at a rate of 0.35 g/h and 0.3 g/h, respectively ([Fig. 4.11](#)). The cumulative molar yield was 1 mol/mol glycerol and the molar ratio between the two products was 1:1.

In order to prevent the accumulation of 1,3PDO and 3HP to inhibitory levels, continuous mode of operation was evaluated. As the cells are in a resting condition, they were retained using a tangential-flow microfiltration module. A feeding solution containing 10 g/L glycerol was fed at rate of 0.35 g$_{gly}$/h, where consumption of the entire glycerol was achieved yielding 4.9 g/L 3HP and 4.1 g/L 1,3PDO.

The co-production was further evaluated using growing cells under continuous mode of operation with cell retention, through feeding a mixture of (MRS medium, glucose and glycerol). Varying the ratio between the glucose and glycerol caused variation in 1,3PDO and 3HP molar ratios, and volumetric productivities
of 1.86 g/L.h for 1,3PDO and 0.87 g/L.h for 3HP were achieved. However, high amounts of by-products including lactic acid, ethanol, acetic acid and succinic acid were also obtained.

![Graph showing simultaneous production of 3HP and 1,3PDO from biodiesel-derived glycerol using resting cells of L. reuteri DSM 20016](image)

The compounds shown are the consumed glycerol (■), 1,3PDO (x) and 3HP (▲).

\[
y = 0.347x + 0.7452 \\
y = 0.3018x + 1.0231 \\
y = 0.7243x + 1.8386
\]
5. Methacrylic Acid Production by Integrated Bio- & Chemo Catalysis

5.1 Methacrylic acid (MA)

Methacrylic acid (MA) and methyl methacrylate (MMA) are important monomers for a range of polymer products; the major product is poly(methyl methacrylate) (PMMA), with an annual global consumption of 2.1 million tons (Fig. 5.1) [148]. Approximately 80% of the global production of MMA is achieved using the well-known acetone-cyanohydrin (ACH) process, in which acetone and hydrogen cyanide are reacted with excess concentrated sulfuric acid to produce the methacrylamide acid sulfate [149], followed by treating with excess aqueous methanol, and hydrolysis and esterification of the amide to yield a mixture of MMA and MA. However, there are serious problems with this route due to the use of highly toxic raw materials, high process cost, and formation of significant amounts of by-products, which have to be disposed [148, 149].

![Fig. 5.1 Production of Poly(methyl methacrylate) from methacrylic acid](image)

The ACH process is estimated to result in emission of 5.5 kg CO$_2$ eq/kg MMA, which is much higher than that from other platform chemicals such as propylene (1.1 kg CO$_2$ eq/kg), ethylene (1.1 kg CO$_2$ eq/kg), and vinyl chloride monomer (1.5 kg CO$_2$ eq/kg) [150].

Therefore, among various important chemicals, a greener production route of MMA can be expected to be highly beneficial for the environment. Different alternative processes have been proposed since 1980s to replace the ACH technology. Some of them are close to commercialization, while others, though attractive from the environmental and economic points of view, are still in the research stage [149].
5.2 Acetic acid bacteria

Acetic acid bacteria are Gram-negative, obligate aerobic rods. They are well-known for their industrial applications such as acetic acid production from ethanol, glucouronic acid from glucose, dihydroxyacetone from glycerol and others [151]. Acetic acid bacteria are divided with regards to their ability to perform complete oxidation of acetate to CO₂ into: Suboxydans (Gluconobacter genus) and Peroxydans (Acetobacter, Gluconoacetobacter) [63, 152]. Gluconobacter shows a strong ketogenesis from polyols and prefers habitats rich in sugars. They are not able to perform complete oxidation of acetate to CO₂ due to the lack of TCA cycle as well as enzymes required for the glyoxylic acid shunt.

5.2.1 *Gluconobacter oxydans*

*Gluconobacter* is a group of Gram negative, α-proteobacteria, rods or oval shaped bacteria. The bacteria are isolated from flowers, fruits, garden soil, alcoholic beverages, cider and soft drinks. They can grow at a high concentration of sugars and low pH. The full potential of *G. oxydans* is unresolved yet. Bioinformatic analysis of the whole genome of *G. oxydans* 621H has shown the presence of more than 75 genes encoding for potential oxidoreductases/dehydrogenases, mostly with unknown substrate specificities [153]. Several dehydrogenases/oxidoreductases are membrane-bound facing the periplasm and are PQQ- or flavin-dependent enzymes [151]. This location allows a faster conversion of substrates into products. Besides the membrane-bound enzymes, there are soluble NADP-dependent dehydrogenases in the cytoplasm, which were reported to participate partially in the oxidation processes [154].

5.3 Combined bio- and chemical catalysis

In Paper VI, an environmental-friendly route for the production of MA was presented through combined bio- and chemical catalysis. Fig. 5.2 shows the process scheme, where resting cells of *G. oxydans* were used for selective oxidation of 2M1,3PDO into 3-hydroxy-2-methylpropionic acid (3H2MPA). The resulting acid was dehydrated into MA using TiO₂.
5.3.1 Biotransformation of 2M1,3PDO to 3H2MPA

2M1,3PDO was quantitatively oxidized with over 95% conversion resulting in 3H2MPA with over 95% selectivity. The optimum conditions for bioconversion were pH 6 - 7.5, 25-30 °C, 5-10 g substrate and 2.6 g cell dry weight per litre. Under optimal conditions, 100 % conversion of 5 g/L substrate and 98% with 7.5 g/L substrate were achieved within 9 hours. Increasing the concentration of either substrate or biocatalyst resulted in accumulation of higher aldehyde concentrations and incomplete oxidation process. For repeated batch reaction, oxidation of the entire substrate was achieved in the first batch, while lower conversion was observed in the following batches (Fig. 5.3).

Process optimization through continuous feeding of 20 g/L substrate with removal of produced acid to avoid product inhibition was investigated. The resting *G. oxydans* cells were retained inside the bioreactor using tangential-flow microfiltration module connected to the bioreactor. After initial batch for 7 hours, continuous mode was started. 3H2MPA concentration increased gradually reaching a maximum of 7.3 g/L followed by a gradual decrease with rising 2M1,3PDO concentration in the outflow. Cell activity was maintained for long
periods and small amount of aldehyde was observed at the beginning of the feeding.

**Fig. 5.3 Repeated batch biotransformation of 2M1,3PDO using resting cells of G. oxydans**
The compounds shown are 2M1,3PDO (▲), 3H2MPAL (■) and 3H2MPA (♦) over three repeated batches (Paper VI).

### 5.3.2 Catalytic dehydration of 3H2MPA into MA

Titanium (Ti) is the ninth most abundant element in earth and TiO₂ is considered a green catalyst. 3H2MPA was dehydrated using packed column with TiO₂. The effects of residence time and reaction temperature are presented in **Fig 5.4**. Over 95% conversion of 3H2MPA was achieved with over 85% selectivity at 210 °C.

**Fig. 5.4 Catalytic dehydration of 3H2MPA over TiO₂**
Effect of flow rate and temperature on production of MA by dehydration of 3H2MPA (Paper VI).
Conclusions & Future Perspectives

The present thesis has focused on the production of several chemicals using wild-type microorganisms. The collaboration with industry has widened the perspective and made it clear that for a process to be only environment-friendly is not enough, it should also be economical. The main lessons from the present study are summarised in the following points:

**Strategic products**
For the development of biorefineries as an alternative for petroleum refineries, the choice of the target molecules to be produced is crucial. Among the different chemicals, versatile molecules that act as building blocks for other chemicals and materials are of immense need. Propionic acid, 3HPA, 3HP and MA were strategic targets within the frame of this thesis. Their global market covers a range of industries including food, feed and pharmaceuticals (as preservatives); plastic, coating and painting industries (as monomers for homo- and hetero- polymers); and as precursors for a vast array of chemicals [66, 106, 108, 155, 156]. Studies on the microbial production of these chemicals allowed us to address the challenges that are common to most bioprocesses. The important factors to be considered are summarised below:

**Starting cheap – ending cheap**
Carbon- and nitrogen sources: The proper choice of the starting materials has a great impact on the downstream processing and process economics. For example, using glycerol as a C-source for propionic acid increases the product yield and minimizes by-products formation. The narrow gap between the prices of the C-source and the products particularly in case of bulk chemicals underscores the need to find cheap N-sources. The ultimate target would be N-source-free process, which was achieved in the case of 3HPA, 3HP and MA. For propionic acid, the N-source was essential. Therefore, heat-treated potato juice was used for achieving economical feasibility.

**Bioprocess engineering as a technical support for natural-producer strains**
Reducing the processing costs and finding alternative solutions for enhancing the titres, yields and productivities will ensure the long-term growth of industrial biotechnology. Some promising approaches are:

High cell density fermentation/biotransformation provides a tool for enhancing the productivities and titres of the desired products. The advantages of using
immobilized cells and cell recycling for fermentations characterized by product inhibition were demonstrated. The choice of the matrix for immobilization is important for achieving the desired efficiency. Even the mode of operation and the process design have significant impact. The author expects that further development in cell separation technologies, by making them more suitable and available at low cost for industrial scale, will ensure the economical feasibility for several bio-based products.

Whole-cell biocatalysis presents a solution for many hurdles, especially when the conversion of substrate to the desired product can be done through few enzymatic reactions. It fills the gap between fermentation (where the whole metabolic pathway of the microorganism is essential for conversion of the substrate to the products) and enzyme-based catalysis (where purified/crude enzymes catalyze such reaction). Handling whole cells is much easier than enzymes particularly when expensive co-factors and co-factor recycling are required. Additionally, the high purity of the products obtained decreases the burden on the downstream processing.

Co-factor recycling: The cofactor balance within the microbial cells is essential for achieving the continuous flow of the desired products. However, in many cases simultaneous production of undesired products occurs which decreases the product yield. A promising approach in this area is co-factor engineering by introducing enzymatic reactions for co-factor recycling [157]. These reactions should preferably utilize external substrates and yield products that can be easily separated from the desired product. An interesting example is NADH oxidase, an enzyme that utilizes oxygen as electron acceptor with the production of water, which ensures a by-product-free process.

In situ product complexation and removal is a must during the production of compounds such as 3HPA that are highly toxic to the producer microorganisms. In addition to alleviating product toxicity, it allows integration of product concentration and purification steps with the production, which shortens the whole process time.

Integrated refineries: A considerable development in the chemical synthesis has been achieved over the past decades. Making use of the accumulated knowledge in this field as a support for industrial biotechnology is recommended for shifting towards a bio-based economy. Through integrated refineries, strategic products such as acrylates and methacrylates could be produced at low cost from renewable feedstocks. The incompatibility of bio- and chemical processes in terms of handling of the large amount of water needed for the bioprocesses should be overcome both by engineering of the microorganisms and bioprocesses. Energy-
efficient means for recovery of the product from water and recycling of the water are essential.

**Microbial diversity**
Several wild-type microorganisms possess innumerable enzymes and metabolic pathways that can be employed for the production of a vast array of chemicals and materials. However, these abilities are obscured by the limited conditions applied to them. A systems approach is essential for optimising the microorganisms. A deep understanding of these microbial factories at the levels of the genome, transcriptome, proteome, etc. and optimized bioprocessing conditions would save time and money spent on developing novel production hosts. Further metabolic engineering of these microorganisms for enhanced production is possible. Recombineering (recombination mediated genetic engineering) is a promising approach, where the expression of genes could be regulated *in vivo* by simple modification of the promoter, the enzyme properties could be improved by mutating the native gene, etc. [158].

**Transfer of knowledge to the chemical industry**
Industrial biotechnology poses a significant opportunity for the chemical sector by developing new, efficient and sustainable ways for production of chemical and material providing economic, environmental and societal benefits. The collaboration between academia and chemical industry increases the know-how and experience concerning industrial biotechnology in the industry. This is expected to increase the interest in integrating biotechnology in the current chemical production processes. Also it provides a platform for smooth shift towards the bio-based economy.

**Life-cycle assessment (LCA)**
Evaluation of the environmental impact of the developed processes in comparison to the current production routes has been carried out for propionic acid and 3HPA using the data generated at the lab scale. LCA has been a useful tool for addressing the steps that exert higher environmental impact, and has helped in taking decisions with regards to upstream and downstream processing. It also highlighted the potential of industrial biotechnology for valorisation of agro/industrial by-products.
Future perspectives

Trials to develop an industrial process for production of propionic acid have been conducted since the 1920s, however with limited success. Most of the available studies have focused on optimizing a single parameter. Combination of these optimized parameters in a single process could be interesting. The requirements for successful process would be: tolerant mutant strain, high cell density fermentation, glycerol or glycerol/glucose mixture as carbon source, *in situ* product removal, and optimized nitrogen source concentration.

With regards to 3HPA, so far the achieved productivities and product concentrations are reasonable when *in situ* complex formation is applied. The actual success in this process is foreseen by further optimization of bisulfite and ion exchange resin process, or ability to liberate 3HPA from semicarbazones or carbohydrazones under mild conditions. Increasing the biocatalyst activity via metabolic engineering would also lower the production cost.

The developed process for the co-production of 3HP and 1,3PDO would be more promising when a conversion of the entire glycerol to 3HP is achieved. Preliminary evaluation showed the possibility to get highly pure 3HP and acrylic acid from glycerol. Yet, the basket is full of ideas for strain and process development, which are under investigation.

The first step for production of MA where a selective oxidation of 2M-1,3PDO occurs using *G. oxydans* has a great opportunity for further improvement. The industrial production of gluconic acid, dihydroxyacetone or acetic acid can be taken as a roadmap.
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