Microbial and enzymatic syntheses of polymer building blocks through selective transformations of polyols and furans

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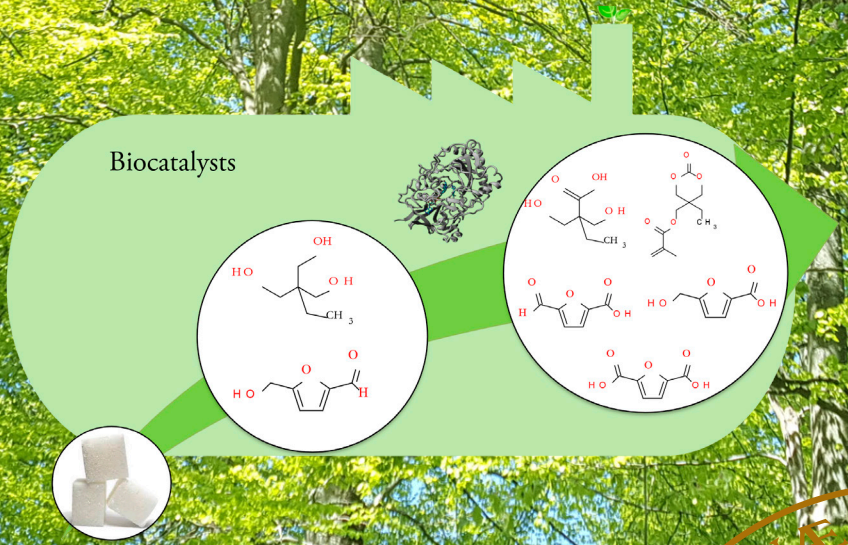

Microbial and enzymatic syntheses of polymer building blocks through selective transformations of polyols and furans

Mahmoud Sayed Ali Sayed

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Abstract

Transition from fossil- to bio-based economy is a critical step towards reduction of greenhouse gas emissions and climate change, and hence for achievement of sustainable communities and environment. In order to be fossil-free, the chemical and material industry is in need of carbon-neutral building blocks from renewable resources for the diverse array of products that are currently produced from olefins and aromatics. Hence, new pathways for producing the same or novel chemical structures are needed. Industrial biotechnology offers a key technology area for transformation of biomass components or derivatives to chemical building blocks by the use of microorganisms or their enzymes.

The thesis introduces new routes for microbial and enzymatic biotransformations of trimethylolpropane (TMP) and 5-hydroxymethyl furfural (HMF) to building blocks for polymers. TMP is an important industrial polyol produced from butyraldehyde and formaldehyde that can be potentially biobased, while HMF, a dehydration product of sugar, is totally biobased. The building block molecules produced from TMP include 2,2-bis(hydroxymethyl)butyric acid (BHMB), six membered cyclic carbonates, and from HMF are 5-hydroxymethyl-2-furan carboxylic acid (HMFCA), 5 formyl-2-furan carboxylic acid (FFCA) and 2,5- furan carboxylic acid (FDCA).

Growing cells of *Mycobacterium* sp. MS1601 (previously *Corynebacterium* sp. ATCC 21245) was the only bacteria that showed the ability to selectively oxidize two hydroxyl groups of TMP to form BHMB at high yield. After optimization of the process parameters and employing high cell density cultivations in a sequential batch mode with cell recycling and cell bleeding, the volumetric productivity of BHMB was improved from 0.02 g/L.h to 0.2 g/L.h to yield 21 g/L BHMB, the highest amount reported so far. Moreover, BHMB was recovered from the reaction medium by anion exchange resin at 78% yield. Transesterification of TMP with methacrylic acid and its derivatives including methyl, ethyl vinyl and dimethyl carbonate (DMC) was investigated using immobilized lipase (Novozym®435) in solvent free medium in order to produce methacrylate functionalized six-membered cyclic carbonates. The results obtained from the experimental part and *in-silico* analysis indicate that methyl and ethyl methacrylate are preferable substrates for the enzyme to give the product with 61.3 % yield and 73% selectivity after 9h reaction. Also, the functionalized cyclic carbonate was purified from the reaction solution using silica chromatography at 60.5% yield. Even the production of bio-based TMP under mild conditions was demonstrated by condensation of bio-based butyraldehyde with formaldehyde produced by oxidation of the corresponding alcohols using *Gluconobacter oxydans* cells and alcohol oxidase, respectively.

Oxidation of crude 5-HMF to HMFCA at 100 % selectivity and yield was achieved using resting cells of *G. oxydans* DSM 50049. The bacteria show the ability to oxidize 31.5 g/L of crude HMF completely to HMFCA after only 6 h of the reaction, indicating that the bacteria is tolerant to the antimicrobial activity of HMFCA. The product was recovered from the reaction with 98% purity using a simple liquid-liquid extraction step. On the other hand, *Mycobacterium* sp. MS1601 cells activated by growth in glycerol, oxidized HMF to FDCA and HMFCA with 60 and 40% yield, respectively. A gene sequence encoding HMF oxidase (HMFO) like enzyme was identified in the genome sequence of *Mycobacterium* sp. MS1601, cloned and expressed in *E.coli* BL21 (DE3), and the enzyme was purified and characterized. The enzyme oxidized HMF to diformyl furan (DFF) followed by conversion to FFCA at 100 % yield without further conversion to FDCA. *In-silico* analysis of the HMFO-Myc1with substrate docking indicated that tyrosine 444 and 443 residues, which are directing the substrate into the right position in the active site, hinders FFCA from being accommodated in the right position, which motivates further studies on engineering the enzyme to enable conversion of FFCA to FDCA.

Key words: *Mycobacterium* sp. MS1601, *G. oxydans*, oxidase, lipase, trimethylolpropane (TMP), 2,2-bis(hydroxymethyl)butyric acid (BHMB), 5-hydroxymethyl furfural (5-HMF), 5-hydroxymethyl-2-furan carboxylic acid (HMFCA), 2,5-furandicarboxylic acid (FDCA), cyclic carbonate , estrification, Biotransformation, polymer building blocks, product recovery, selective oxidation, bioprocess engneering, cell recycling, cell bleeding, DNA recombinant technology, in-silico analysis

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MADE IN SWEDEN

Dedicated to

My parents

My brothers and sisters

My wife, Shimaa

My daughters, Aasha, Hagar and Sara

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Abstract

Transition from fossil- to bio-based economy is a critical step towards reduction of greenhouse gas emissions and climate change, and hence for achievement of sustainable communities and environment. In order to be fossil-free, the chemical and material industry is in need of carbon-neutral building blocks from renewable resources for the diverse array of products that are currently produced from olefins and aromatics. Hence, new pathways for producing the same or novel chemical structures are needed. Industrial biotechnology offers a key technology area for transformation of biomass components or derivatives to chemical building blocks by the use of microorganisms or their enzymes.

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Popular summary

Microorganisms are the most abundant life form on the planet Earth, and have an important role in the environmental sustainability due to their wide distribution and ability to recycle the carbon, and break down the complex biomass to small molecules, which can be reused by the soil and other living organisms. They have been used for the production of food, feed, and cosmetic products by the ancient people, and are even used to produce antibiotics and clean up wastes. Due to their ability to adapt and survive in the environment they inhabit, it is possible to find microorganisms that can perform almost any kind of chemical reaction for which they make use of enzymes, the biological catalysts.

In more recent times, microorganisms and their enzymes have attracted attention as important tools to enable a shift from fossil based economy to one that relies on renewable resources such as biomass, i.e. bioeconomy. Regarding their great potential in the industry, a Nobel-laureate physical chemist, Sir Cyril Hinshelwood has nicely said: "Bacteria are capable of bringing about chemical reactions of amazing variety and subtlety in an extremely short time…. Many bacteria have great importance to industry where they perform tasks which would take much time and trouble by ordinary chemical methods". Moreover, the microorganisms perform transformations in a selective manner under ambient conditions without the need of harsh reaction conditions often needed for the chemical processes.

Despite the progress in the application of microorganisms and enzymes for the production of diverse bio-based products including food, feed, fuels, chemicals, and materials, majority of the products we use in our daily lives are still coming from fossil oil and gas, and less than 10% are substituted by biomass-based products. Also, owing to the increase of population, the consumption of fossil-based products has increased and raised serious environmental concerns such as emission of greenhouse gases, global warming, water-, land- and air pollution. Therefore, the development of biotechnology by using biocatalysts including microorganisms and enzymes becomes a critical issue and a good alternative for the chemical processes in order to achieve a sustainable society and environment.

This thesis introduces different examples for the production of bio-based chemicals that can provide new value chains around two potential biobased platform chemicals, trimethylolpropane (TMP) and 5-hydroxymethylfurfural (HMF). These chemicals were transformed to value-added chemicals with high selectivity and yield using either a microorganism or an enzyme in a bioprocess or as an integrated process with a chemical reaction. The chemicals produced have potential applications as components in coatings, plastics, pharmaceuticals, etc.

Populär sammanfattning

Mikroorganismer är den mest omfattande livsformen på planeten Jorden och har en viktig roll i miljöhållbarheten på grund av deras stora förekomst och förmåga att återvinna kolet och bryta ner den komplexa biomassan till små molekyler, som kan återanvändas av jorden och andra levande organismer. De har använts för produktion av mat, foder och kosmetiska produkter av forntida människor, och används till och med för att producera antibiotika och städa upp avfall. På grund av deras förmåga att anpassa sig och överleva i sin livsmiljö, är det möjligt att hitta mikroorganismer som kan utföra nästan vilken typ av kemisk reaktion eftersom de använder sig av enzymer, de biologiska katalysatorerna.

På senare tid har mikroorganismer och deras enzymer dragit till sig uppmärksamhet som viktiga verktyg för att möjliggöra ett övergång från en fossilbaserad ekonomi till en som bygger på förnybara resurser som biomassa, dvs bioekonomi. När det gäller deras stora potential i branschen har en nobelpristagare fysisk kemist Sir Cyril Hinshelwood fint sagt: "Bakterier kan genomföra kemiska reaktioner på otroligt varierande och rafinerat sätt på extremt kort tid .... Många bakterier har stor betydelse för industrin där de utför uppgifter som skulle ta mycket tid och skapa problem med vanliga kemiska metoder ". Dessutom utför mikroorganismerna transformationer på ett selektivt sätt under existerande omgivningsbetingelser utan att det behövs svåra reaktionsbetingelser som ofta behövs för kemiska processer.

Trots utvecklingen i tillämpning av mikroorganismer och enzymer för produktion av olika biobaserade produkter, inklusive mat, foder, bränslen, kemikalier och material, kommer flertalet av de produkter vi använder i våra dagliga liv fortfarande från fossil olja och gas, och mindre än 10% är ersatta av biomassbaserade produkter. På grund av ökningen av befolkningen har konsumtionen av fossila produkter ökat och givit upphov till allvarliga miljöproblem som utsläpp av växthusgaser, global uppvärmning, vatten-, mark- och luft-förorening. Därför blir utvecklingen av bioteknik genom att använda biokatalysatorer, inklusive mikroorganismer och enzymer, en kritisk fråga och ett bra alternativ till rent kemiska processer för att uppnå ett hållbart samhälle och en miljö.

Denna avhandling introducerar olika exempel på produktion av bio-baserade kemikalier som kan ge nya värdekedjor, runt två potentiella biobaserade plattformskemikalier, trimetylolpropan (TMP) och hydroxymetylfurfural (HMF). Dessa kemikalier omvandlas till mättade kemikalier med hög selektivitet och utbyte med användning av antingen en mikroorganism eller ett enzym, i en bioprocess, eller som en integrerad process med en kemisk reaktion. De kemikalier som produceras har potentiella tillämpningar som komponenter i ytbeläggningar, plastmaterial etc.

الملخص العربى

الكائنات الدقيقة هي أكثر أشكال الحياة وفرة على كوكب الأرض ، ولها دور مهم في الاستدامة البيئية بسبب توزيعها الواسع وقدرتها على إعادة تدوير الكربون ، وتكسير الكتلة الحيوية المعقدة إلى جزيئات صغيرة ، والتي يمكن إعادة استخدامها بواسطة التربة والكائنات الحية الأخرى. وقد تم استخدامها لإنتاج الأغذية والأعلاف ومستحضرات التجميل من قبل القدماء ، وتستخدم ايضا لإنتاج المضادات الحيوية وتنظيف النفايات. بسبب قدرتها على التكيف والبقاء في البيئة التي تعيش فيها ، فمن الممكن العثور على الكائنات الدقيقة التي يمكنها أن تؤدي تقريبا أي نوع من التفاعلات الكيميائية مستخدمة فى ذلك الإنزيمات الموجودة بها،والتى يطلق عليها المحفزات البيولوجية.

في الآونة الأخيرة ، جذبت الكائنات الدقيقة وأنزيماتها الانتباه كأدوات مهمة لتمكين التحول من الاقتصاد الأحفوري إلى اقتصاد يعتمد على الموارد المتجددة مثل الكتلة الحيوية كبقايا النباتات والمخلفات الصناعية وثانى اكسيد الكربون وغيرها ، في مجال الاقتصاد الحيوي. وفيما يتعلق بإمكانياتهم الكبيرة في الصناعة ، قال السير سيريل هينشلوود ، وهو كيميائي فيزيائي حائز على جائزة نوبل ، "إن البكتيريا قادرة على إحداث تفاعلات كيميائية ذات تنوع ودقة مدهشة في وقت قصير للغاية ... العديد من البكتيريا لها أهمية كبيرة للصناعة حيث تقوم بأداء المهام التي تستغرق الكثير من الوقت والمتاعب من خلال الطرق الكيميائية العادية ". علاوة على ذلك ، فإن الكائنات الحية الدقيقة تقوم بتحولات بطريقة انتقائية في الظروف المحيطة دون الحاجة إلى ظروف تفاعل قاسية ؛ والتى غالباً ما تكون مطلوبة للعمليات الكيميائية.

على الرغم من التقدم في تطبيق الكائنات الحية الدقيقة والإنزيمات لإنتاج منتجات حيوية متنوعة بما في ذلك الغذاء والأعلاف والوقود والمواد الكيميائية والبوليمرات ، فإن غالبية المنتجات التي نستخدمها في حياتنا اليومية لا تزال تأتي من النفط والغاز الأحفوري ،وفقط أقل من 10 ٪ من المنتجات القائمة على النفط تم استبدالها بمنتاجات قائمة على الكتلة الحيوية. ايضا ، بسبب زيادة عدد السكان ، ازداد استهلاك المنتجات القائمة على الوقود الاحفورى وأثار مخاوف بيئية خطيرة مثل انبعاث الغازات السامة والاحتباس الحرارى وتلوث المياه والارض والجو. ولذلك ، فإن تطوير التكنولوجيا الحيوية باستخدام المحفزات الحيوية بما في ذلك الكائنات الدقيقة والإنزيمات يصبح قضية حاسمة وبديل جيد للعمليات الكيميائية من أجل تحقيق مجتمع وبيئة مستدامين.

تقدم هذه الأطروحة أمثلة مختلفة لإنتاج المواد الكيميائية الحيوية القائمة على الكتلة الحيوية من خلال الاكسدة الميكروبية والانزيمية لمادتين أساسيتين وذات قيمة اقتصادية وهما ثلاثى هيدروكسي ميثيل بروبان (TMP) ، و هيدروكسى ميثيل فيرفيرال (HMF) وتحويلها إلى مواد كيميائية ذات قيمة مضافة عالية الانتقائية والعائد وذلك باستخدام إما كائن دقيق أو إنزيم في عملية حيوية أو كعملية متكاملة مع تفاعل كيميائي. المواد الكيميائية المنتجة لها تطبيقات محتملة كمكونات في الطلاء والبلاستيك والمستحضرات الصيدلانية ، إلخ.

List of publications

The thesis is based on the following papers, which are listed and referred on the thesis by their roman number.

I. **Mahmoud Sayed**, Tarek Dishisha, Waiel F. Sayed, Wesam M. A. Salem, Hanan A. Temerk, and Sang-Hyun Pyo. Selective oxidation of trimethylolpropane to 2,2-bis(hydroxymethyl)butyric acid using growing cells of *Corynebacterium* sp. ATCC 21245. *Journal of Biotechnology*, 2016, 221, 62-69.

II. **Mahmoud Sayed**, Tarek Dishisha, Waiel F. Sayed, Wesam M. Salem b, Hanan M. Temerk and Sang-Hyun Pyo. Enhanced selective oxidation of trimethylolpropane to 2,2-bis(hydroxymethyl)butyric acid using *Corynebacterium* sp. ATCC 21245. *Process Biochemistry*, 2017, 63, 1-7.

III. **Mahmoud Sayed**, Yasser Gaber, Amin Bornadel, and Sang-Hyun Pyo. Multi-steps green process for synthesis of six-membered functional cyclic carbonate from trimethylolpropane by lipase catalyzed methacrylation and carbonation, and thermal cyclization. *Biotechnology Progress*, 2016, 32, 83-88.

IV. **Mahmoud Sayed**, Hossameldeen Elsabaa, Waiel F. Sayed, Wesam M. Salem, Hanan A. Temerk, and Sang-Hyun Pyo. Sustainable synthetic route of a bio-based polyol, trimethylolpropane, from renewable resources by integration of biotechnology and chemical process (Manuscript)

V. **Mahmoud Sayed**, Sang-Hyun Pyo, Nicola Rehnberg, and Rajni Hatti-Kaul. Selective oxidation of 5-hydroxymethylfurfural to 5-hydroxymethyl-2-furancarboxylic acid using *Gluconobacter oxydans.* (Manuscript)

VI. **Mahmoud Sayed**, Yasser Gaber, Eric Valdés, Sang-Hyun Pyo and Rajni Hatti-Kaul. Biocatalytic oxidation of HMF using *Mycobacterium* sp. MS1601 and a derived HMF oxidase. (Manuscript)

My contribution to the papers

The overall idea was provided by Dr. Sang-Hyun Pyo and Prof. Rajni Hatti-Kaul.

I. I planned and performed all the experimental part and wrote the first draft of the manuscript and was involved in the editing of the final draft with Dr. Tarek, under the supervision of Dr. Sang-Hyun Pyo.

II. I planned and performed all the experiments, data analysis and writing the first draft of the manuscript and was involved in editing of the final version. Dr. Tarek Dishisha provided the information about cell recycling technique and helped in editing of the manuscript. All work was done under the supervision of Dr. Sang-Hyun Pyo

III. The idea and experimental design was provided by Dr. Sang-Hyun Pyo and I performed the experimental part, data analysis and contributed to editing of the final draft of the manuscript. *In silico* analysis was done by Dr. Yasser Gaber.

IV. I planned the experimental part and monitored Hossameldeen for doing the experiments for butyraldehyde production, and I performed the experiments on formaldehyde and TMP production. Also I did the data analysis and wrote the first draft of the manuscript under supervision of Dr. Sang-Hyun Pyo.

V. I designed, performed all experiments, data analysis, writing the first draft of the manuscript under supervision of Dr. Sang-Hyun Pyo and Prof. Rajni Hatti-Kaul. Also, I was involved in editing of the final draft.

VI. The idea was initiated by Prof. Rajni Hatti-Kaul; I planned the experiments and performed the experimental part, data analysis, writing the first draft of the manuscript under supervision of Dr. Sang-Hyun and Prof. Rajni Hatti-Kaul. The *in-silico* analysis was carried out by Dr. Yasser Gaber.

Publications not included in the thesis

I. Amin Bornadel, Mohamed Ismail, **Mahmoud Sayed**, Rajni Hatti-Kaul, Sang-Hyun Pyo. Six-membered cyclic carbonates from trimethylolpropane: Lipase-mediated synthesis in a flow reactor and in silico evaluation of the reaction. *Biotechnology progress*, 2017, 33, 375-382.

II. **Mahmoud Sayed**, Waiel F Sayed, Rajni Hatti-Kaul, Sang-Hyun Pyo. Complete Genome Sequence of *Mycobacterium* sp. MS1601, a Bacterium Performing Selective Oxidation of Polyols. *Genome Announcements*, 2017, 5, e00156-17.

III. Pengrui Wang, Ji Hoon Park, **Mahmoud Sayed**, Tae Sun Chang, Amy Moran, Shaochen Chen, Sang-Hyun Pyo. Sustainable synthesis and characterization of bisphenol A-free polycarbonate from six-membered dicyclic carbonate. *Polymer Chemistry*, 2018, 9, 3798-3807.

IV. Luis Romero Soto, Eoin Byrne, Ed WJ van Niel, **Mahmoud Sayed**, Cristhian Carrasco Villanueva, Rajni Hatti-Kaul. Hydrogen and polyhydroxybutyrate production from wheat straw hydrolysate using *Caldicellulosiruptor* species and *Ralstonia eutropha* in a coupled process. *Bioresource Technology*, 2019, 259-266

V. Sang-Hyun Pyo, **Mahmoud Sayed** and Rajni Hatti-Kaul. Batch and continuous flow production of 5-hydroxymethylfurfural from high concentration of fructose using acidic ion exchange catalyst. (Manuscript)

Abbreviations

TMP	Trimethylolpropane
BHMB	2,2-bis(hydroxymethyl)butyric acid
5-HMF	5-Hydroxymethyl furfural
HMFCA	5-hydroxymethyl-2-furan carboxylic acid
DFF	2,5-diformyl furan
FFCA	5-formyl-2-furan carboxylic acid
FDCA	2,5-furan dicarboxylic acid
BHMF	2,5-bis(hydroxymethyl)furan
TMP-mMACC	Trimethylolpropane mono-methacrylate cyclic carbonate
TMP-mMA	Trimethylolpropane mono-methacrylate
CDW	Cell dry weight
Q_p	Volumetric production rate (g/l.h)
μ_{max}	Maximum specific growth rate (h^{-1})
Y	Yield (g/g) or (mole/mole)
G. oxydans	*Gluconobacter oxydans*
E. coli	*Escherichia coli*
P. pastoris	*Pichia pastoris*
DoE	US Department of Energy
3-HPA	3-hydroxy propionic acid
OECD	Organization for Economic Cooperation and Development
PEF	Polyethylene furanate
PET	Polyethylene terephthalate
HMFO-Myc1	HMF oxidase from *Mycobacterium* sp. MS1601
RAST	Rapid Annotations using Subsystems Technology
DMC	Dimethyl carbonate
ISPR	*In-situ* product removal
IER	Ion exchange resin

1 Introduction

The chemical industry produces enormous variety of chemicals and materials, which are used in different applications such as fibres, resins, composites, construction materials, plastics, pharmaceuticals, agrochemicals, food products, sports, furniture, etc., that are used in our everyday lives. All of these products afford the backbone of the present economy [1].

Majority of the chemicals and polymers besides energy are still produced from fossil resources. Over 97% of the consumer products of specialty, commodity and pharmaceutical chemicals, and personal care products, are produced by chemical processes and only 3% are obtained through bioprocesses [2]. The fast growth of global population, limitation of arable land, climate change, and finite nature of fossil resources are the major challenges for the achievement of sustainable societies [3]. Thereby, over the entire world many steps have been taken for the transition from fossil based economy to bio-economy. Bio-economy is defined by European Commission as "the economy including the production of renewable resources and their conversion into food, feed, bio-based products and bioenergy" [3]. In a recently updated Bioeconomy strategy [4,5], it is highlighted that a sustainable European bioeconomy is necessary to build a carbon neutral future in line with the Climate objectives of the Paris Agreement.

An important and major sector of bio-based products is the chemicals that can be obtained from renewable feedstocks. For example, many platform chemicals including alcohols, diols, carboxylic acids, short chain olefins, etc. can be produced from biomass, and used as starting chemicals for the production of other bio-based chemicals, materials, fuels, polymers [6,7]. In 2004, the US Department of Energy (DoE) identified 30 chemicals as potential building blocks or platform chemicals from which many value-added chemicals can be produced. Of these, 12 chemicals derived from sugars, include 1,4-diacids, 2,5-furan dicarboxylic acid (FDCA), 3-hydroxypropionic acid (3-HPA), aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, and polyols such as glycerol, sorbitol, xylitol and arabinitol, were identified as potential platform chemicals [8]. Currently, the bio-based chemicals represent only around 10 % of the worldwide production of chemicals, which is expected to reach around 17% with a value of €175-420 billion in 2025 [9,10].

Hence, the development of new technologies for processing of biomass and production of bio-based chemicals has attracted great attention in both academia and industry. Industrial biotechnology is regarded as one of the key technology pillars for the biobased economy. Industrial biotechnology, also known as white technology, is defined as the sustainable production of chemicals, materials, food, feed, pharmaceutical, cosmetics, textile, paper, pulps and energy using biological systems and their components microorganisms and enzymes [11]. The use of microorganisms and enzymes for industrial applications has several advantages including mild conditions, selective process, less generation of industrial waste and emissions, and lower cost for cleanup, compared to the use of conventional chemical processes, which involve harsh conditions, expensive materials and facilities, multi-steps reactions, toxic solvents and wastes [12]. Nevertheless, only few industrial biotechnology processes for chemicals production have been developed in industrial scale. Therefore, development of efficient tools in terms of microbial product hosts and enzymes, and bioprocesses, and their integration into the chemicals and materials industries is required in order to make a transition to bio-economy.

1.1.1 Scope of the thesis

The aim of the thesis is to explore the development of biotechnology processes for production of bio-based chemicals and their derivatization to novel products. The focus has been on the development of microbial and enzymatic biotransformation for production of potential bio-based polyols and furan derivatives including trimethylolpropane (TMP), 2,2-bis(hydroxymethyl)butyric acid (BHMB), TMP cyclic carbonate, hydroxymethyl furfural carboxylic acid (HMFCA), formyl furan carboxylic acid (FFCA) and furan dicarboxylic acid (FDCA), that can serve as platforms and building blocks for chemicals and materials. The biotransformations involved selective oxidation and transesterification reactions using whole cells and enzymes. Moreover, the development of both bioprocess and microbial cell factories was achieved through bioprocess engineering, integrated catalysis, molecular biology, and bioinformatics techniques.

The thesis includes 6 papers three of which are published.

Paper I & II deal with the selective oxidation of TMP to 2,2-bis(hydroxymethyl)butyric acid (BHMB) using the growing cells of *Mycobacterium* sp. MS1601 (previously known as *Corynebacterium* sp. ATCC 21245). The selective oxidation was enhanced through optimization of the cultivation medium and reaction conditions, cell recycling, and cell recycling with cell bleeding. Also, the recovery of the product from the reaction medium has been achieved by adsorption to ion exchange resin.

Paper III shows the application of TMP as a platform chemical for the production of six-membered cyclic carbonate functionalized with methacrylic acid through transesterification. The resulting product, TMP-mono-methacrylate (TMP-mMA) was used for transesterification reaction with dimethyl carbonate (DMC) to TMP-mMA-mono-carbonate. Subsequently, six-membered cyclic carbonate was obtained by thermal cyclisation of the TMP-mMA-mono-carbonate. The transesterification reactions were carried out by immobilized lipase (Novozyme 435) in a solvent free medium.

Paper IV describes the production of the polyol, TMP from biobased feedstock using an integrated biological and chemical process. Particularly, the biological process including the production of butyraldehyde and formaldehyde was achieved by incomplete oxidation of bio-butanol and bio-methanol using *Gluconobacter oxydans* DSM 2343 cells and alcohol oxidase from *Pichia pastoris*, respectively, followed by the aldol condensation and Cannizzaro reactions of bio-formaldehyde and bio-butyraldehyde for the production of biobased TMP. Recovery of TMP from the reaction medium has also been achieved.

Paper V deals with selective oxidation of 5-hydroxymethyl furfural (HMF) to 5-hydroxymethyl-2-furfural carboxylic acid, HMFCA, using the resting cells of *G. oxydans* DSM 50049 in aqueous medium. Efficient recovery of HMFCA from the reaction medium by liquid/liquid extraction was also demonstrated.

Paper VI reports the oxidation of HMF to furan dicarboxylic acid (FDCA) using *Mycobacterium* sp. MS1601. Further, it includes the identification, cloning, expression and characterization of an enzyme responsible for the oxidation of HMF.

The following chapters provide the background of the research area and our contribution in this area through the results obtained from the thesis work. Chapter 2 describes the concept of bio-economy, renewable resources, biorefinery and industrial biotechnology. Also it gives a brief background of industrial polymers and some established examples of potential bio-based chemicals from renewable resources. Chapter 3 introduces the area of biotransformations using whole cells and enzymes, including those used in the thesis. In addition, the development of industrial biotechnology for the production of bio-based chemicals through microbial cell factories and process engineering is discussed. Chapters 4 and 5 are case studies in the thesis on the development of a new value chains around TMP and HMF, respectively, for industrial applications. Chapter 6 is the conclusion and future perspectives for the work done in the thesis.

2 Bio-economy

Over the past century, our society has become increasingly dependent on fossil sources as a raw material for energy, chemicals and materials. This has come at a great cost to the environment in the form of greenhouse gas emissions and toxic wastes. One of the drastic consequences that we face today is that of climate change, which has led to a Paris agreement accepted by most countries in the world proposing actions to limit the annual temperature increase to below 2 °C [4,5]. As fossil based production is a major cause of greenhouse gases emissions and other health and environmental impact, replacing the fossil oil and gas with an alternative cleaner, renewable resource is essential. The urgent need for transition from a linear fossil based economy towards the sustainable usage and development of renewable bio-based resources, have prompted the definition of a concept of bioeconomy, which has been defined by OECD (Organization for Economic Cooperation and Development) as "the transformation of life science knowledge into new, sustainable, coefficient, and competitive products" [13]. A similar definition was given by the White House, USA without emphasizing the sustainability aspect as "the use of research and the innovations in the biological science to produce an economic activity and benefits for the society" [14]. Moreover, according to OECD, bioeconomy will involve three major elements of the advanced biotechnological knowledge including the knowledge of genes and whole cell processes, renewable biomass, and the integration of biotechnology applications in different sectors [13]. Therefore, many countries are establishing biotechnology, bio-based products and industries under the bioeconomy term, in order to investigate the effect on the economy, society, and environment [15].

The main challenge for the development of bio-economy is the need of the integration between the environmental and the sustainability components. The sustainable use of renewable resources means that the production of bio-resources should be connected with their and their products consumption pattern [15]. According to the European Union, bioeconomy involves different industries and sectors including chemicals and polymers, food and feed, agriculture, wood industry and forestry, paper and pulp, biofuels, aquaculture, enzymes and others [16,17]. **Figure 2.1** shows the relation between volume and the price of the different bio-based products in the market. The transition to bioeconomy requires re-thinking of production both with respect to a different raw materials and generating minimal emissions and environmental impact.

Hence applying green chemistry principles and industrial biotechnology based processes are favored.

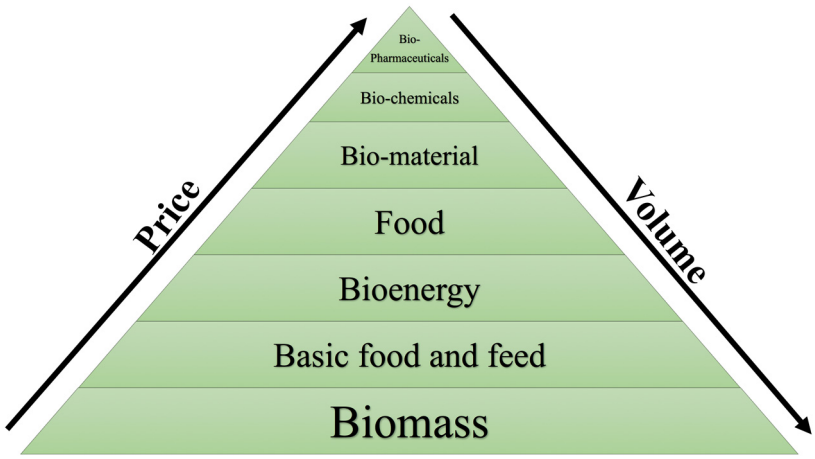


Fig 2.1
price and volume of bio-products

2.1 Renewable resources and biorefinery

Renewable resources refer to any organic matter, which is available in renewable or reoccurring form including dedicated energy crops and trees, food and feed crops residue, wood and wood residue, aquatic plants and seaweeds, animal wastes, bio-industrial wastes, and other waste materials. Majority of the biomass is obtained from agriculture, forest and forest industries and by microbial system [18,19]. Renewable resources are the main players for the shift from fossil based economy into bio-economy, where they are used as starting materials for many industries including paper and pulp, cardboard, chemicals, materials, fuels, pharmaceuticals, food and feed, and others. The importance and wide applications of the renewable resources comes from the complex composition of the biomass which consists of different components including carbohydrates, proteins, lipids, lignin and fats besides other substances such as vitamins, flavors, hormones, enzymes and dyes. Another benefit compared to fossil resources is that the renewable resources do not require expensive extraction steps and some can be used directly for transformation into industrial intermediates and final products [20]. The complexity of biomass provides opportunities to valorise the different components but also poses challenges for pretreatment and fractionation of the biomass. As in petrochemical refineries, bio-based production requires the production of multiple products in a so-called biorefinery to generate economic value. Biorefinery is thus sustainable transformation of the biomass into marketable bio-

products and energy, as indicted in (**Figure 2.2**) [17,21,22]. Biorefineries involve a combination of different methods and processes such as physical, chemical, biological, and thermal, in order to pretreat, fractionate and transform the raw material components to several products with added value. Plant biomass on an average consists of 75% carbohydrates which can be converted biochemically into intermediate platform sugars, which are further transformed into more valuable bio-products [23].

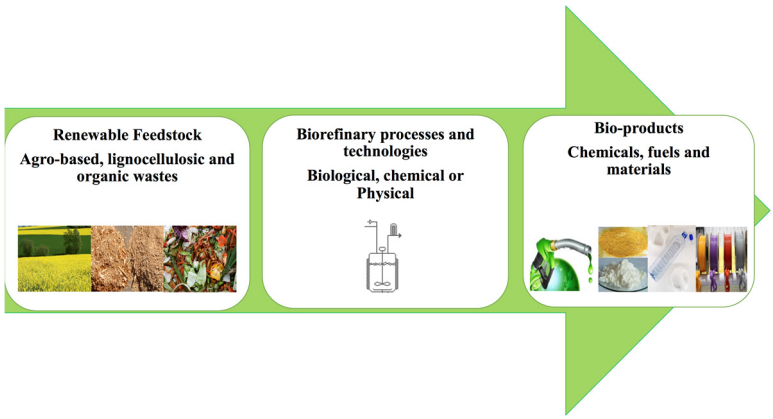


Figure 2.2
Principles of biorefinery concept

2.2 Biobased Polymers

Biobased Polymers represent around 1% of the global polymer's market[24]. Bio-based polymers include three classes called natural polymer, bioengineered polymers and synthetic polymers (**Table 1.1**). Natural polymers like starch, cellulose, cellulose derivatives, and chitin, are derived directly from biomass and used without purification. Bioengineered polymers are the polymers produced naturally by microorganisms and are used without any modification, e.g. PHA. The third class is the synthetic polymers that are produced from biomass through production of their monomers using biological and chemical process. Synthetic polymers include bio-polyolfines and polyethylene furanoate (PEF), and others. The third class is the most promising between bio-based polymers due to their potential as alternative to chemical based polymers, and flexibility of their chemical structure for design compared to other bio-based polymers. The flexibility of the polymers is linked to the flexibility of their monomer to be modified into different derivatives[25]. Therefore, production of new building block chemicals from biomass is a critical step in bio-based polymer industry in order to introduce new synthetic bi-based polymers with new properties.

Table 1.1
Examples of industrial bio-based polymers, their building blocks and their applications [24,25]

Biopolymers	Monomers	Applications
Cellulosic Polymers	Glucose, Cellobiose	synthetic detergents, sheet, sizing and finishing, food, cosmotics and pharmaceutical, Membranes, Chromatographic applications
Polylactic acid	Lactic acid	Packaging, electronic automotive applications, commodity containers, floor mats, and spare parts
Polyhydroxyalkanoates	Hydrocarbons	Commodity applications, shampoo and cosmetic bottles, cups and food containers, and food additive
Polyethelne furanoate	Ethylyne glycol and 2,5 furan carboxylic acid	Bottels, films, pakaging, and beverage industry

2.3 Production of bio-based chemicals

In the chemical industry, more than 70 000 products are produced with more than US$ 4 trillion of the global sales, and it is expected to reach US$ 5 trillion by 2020 [26,27]. The chemical industry has four subsectors including commodity chemicals, specialty chemicals, fine chemicals, and personal care products. The worldwide production of platform chemicals and bulk chemicals is around 250-330 million tons/year. Besides the finite nature of fossil materials, there are challenges and problems associated with the fossil based process as below [27]:

- Harsh conditions represented by high temperature, high pressure and toxic solvents
- Low selectivity and regioselectivity of chemical catalysts
- High energy costs
- Low safety due to the possibility of leakage and explosions
- Low sustainability as a result for the emission of greenhouse gases
- Difficulty of chemical waste disposal resulting in hazardous level of pollution and contamination

Only less than 10 % of fossil based chemicals have been substituted by bio-based chemicals from renewable feedstock [28]. Developments over the past decades in the production of biofuels have shown the need for integration of chemicals production to achieve sufficient value addition. According to USDA (US Department of Agriculture) the share of bio-based chemicals in the chemical market will increase from 3-4 % in 2010 to around 17 % in 2025 [7,8].

2.3.1 Examples of biobased chemicals

The 12 sugar based chemicals were proposed by US DoE based on four criteria including 1) the production of these chemicals from renewable resources, 2) the value of these chemicals as a replacement for fossil-based chemicals or as novel chemicals, 3) the complexity of the biotechnological pathways for the production of these chemicals and their transformation to other building blocks, and 4) their potential as building block and as platform chemicals for production of various building block chemicals. These chemicals include mainly diacids, hydroxy acids and polyols as indicated in **Table 2.1** [8].

Table 2.1
Top 12 chemicals from sugars [8]

Top chemicals
1,4-diacids (succinic acids, malic acid and fumaric acid)
2,5-furan dicarboxylic acid
Levulinic acid
Aspartic acid
Itaconic acid
Glucaric acid
Glutamic acid
3-hydroxy propionic acid (3HP)
3-hydroxybutyarolactone
Glycerol
Sorbitol
Xylitol

Alcohols, polyols and organic acids represent categories of chemicals covering a large share of the chemicals market. Besides being used themselves, they serve as platforms for many other chemical products.

2.3.2 Alcohols

- Bio-methanol has wide applications as biofuels, hydrogen storage compound, automotive fuel, solvent, inhibitor, and as a platform for production of formaldehyde, acetic acid, methyl methacrylate and dimethyl terephthalate [29-31]. Biomethanol is produced mainly from biomass including agriculture and forestry biomass at 55% yield, specifically, from rice bran and lignocellulosic materials. Pyrolysis and gasification are the most common processes suitable for the production of methanol from renewable resources at large scale [30]. Biosynthesis of methanol from methane occurs by the

methanotrophic bacteria, e.g. *Methylosinus trichosporium* [32,33]. Also, biosynthesis technique is used to produces methanol from organic wastes, sewage sludge, and agriculture wastes trough conversion of the obtained methane [30,32,33]. Moreover, biomethanol is produced by electrolysis and photo electrochemical techniques from lab scale [34,35]. In this study biomethanol was used as a platform for the production of bio-formaldehyde using commercial alcohol oxidase from *P. pastoris*, in order to use it for the production of bio-based TMP (**Paper IV**).

- Bio-butanol includes the two isomers, *n*-butanol and isobutanol, which are produced commercially by fermentation of sugars with a capacity of 90 ktons/year [36] and 55 ktons/year [37], respectively. Also, n-butanol is produced from ABE fermentation [38,39]. Bio-butanol is used as biofuel and an important platform chemical. In this work, bio-butanol is used as starting chemical for the production of bio-based trimethylolpropane (TMP) through butyraldehyde (**Paper I**)

2.3.3 Polyols

Polyols, also known as sugar alcohols or polyhydric alcohols, are produced by hydrogenation of monosaccharides and disaccharides. Polyols are also used as sweeteners e.g. xylitol, as replacement of conventional sugars in food industry. Polyols and their derivatives are also used as components in pharmaceuticals, cosmetics, medicine, lubricants, coatings, polymers and platform chemicals [40].

- Trimethylolpropane (TMP) is a polyol (triol), with a number of industrial applications. It is mainly used as a precursor for alkyd resins, its acrylated and alkoxylated and allyl ether derivatives serve as functional monomers for various coatings, while epoxides are used for production of flexible polyurethanes [41]. It is currently produced by condensation of fossil based butyraldehyde and formaldehyde followed by Cannizzaro reaction[41,42]. In the present study, a bio-based route for the production of TMP from glucose was conceived by integration of biological and chemical processes (**Paper V**). It was further used as platform chemical for the production of 2,2-bis(hydroxymethyl)butyric acid and six- membered cyclic carbonate (**Papers II, III & VI**).

2.3.4 Organic acids

Organic acids constitute a major chemical segment produced from biobased raw materials. Besides their applications in food and feeds, organic acids are used as functional monomers in polymer and bioplastic industries [43]. Examples of the most

potential bio-based organic acids are glycolic acid (C2), 3-hydroxypropionic acid (C3), succinic acid (C4), and FDCA (C6)

- 2,5-Furan dicarboxylic acid (FDCA), a C-6 chemical, is used as a monomer for the production of polyethylene-furanate (PEF), which is a replacement for the petroleum based polymer, polyethylene- terephthalate (PET) produced from terephthalic acid. Additionally, FDCA is used as a monomer for other polymers such as poly (butylene furandicarboxylic acid), polyamides, polyester polyols, and polycarbonates. In particular, FDCA based polyamide has been developed by Avantium and Solvay companies [44]. FDCA is produced by oxidation of hydroxymethyl furfural (HMF), which is obtained by dehydration of mono-sugars such as glucose and preferably fructose [45-47]. In **Paper VI**, FDCA was shown to be produced from HMF by whole cells of *Mycobacterium* sp. MS1601 and recombinant *E.coli* under mild conditions.
- 5-Hydroxymethyl-2-furancarboxylic acid (HMFCA) is a partially oxidized derivative of HMF, possesses a hydroxyl and a carboxylic groups. These functional groups give its potential as a monomer for synthesis of different polyesters. HMFCA has also been shown to have antitumor activity and is a promising interleukin inhibitor. HMFCA is a potential platform chemical for FDCA. However, the production of HMFCA from HMF oxidation is still very limited compared to the production of DFF and FDCA [48-50]. In this study HMFCA was produced with 100 % yield and selectivity from the selective oxidation of the crude HMF that was produced in our lab, using resting cell of *G. oxydans* DSM50049 (**Paper V**)

2.4 Industrial biotechnology

Biotechnology processes have been used over several decades for industrial production of several high value products with applications mostly in foods and pharma sectors, and even in environmental remediation. Even before discovering the existence of microorganisms, they were used in ancient times for making cheese, yoghurt, and bread. Despite the long history of biotechnology and the knowledge of its potential, biotechnology has been perceived as expensive for production of commodity products. It is only in relatively recent times that biotechnology processes are being developed for large scale production of biobased fuels and chemicals. Industrial biotechnology makes use of biological systems, primarily microbial cells and their enzymes as catalysts for transformation of renewable resources. The process is based either on microbial fermentations, biotransformation using resting cells, single enzyme system or enzymatic cascade reactions. The global market of the industrial biotechnology products was estimated as 414.5 USD billion with growth of 11.6% from 2012 to 2017 [11].

Industrial biotechnology offers potentially cleaner processes compared to the conventional chemical processes. Additionally, the need for chirality and functionality, especially in pharmaceutical industry, and the need for sustainability are the main driving forces for the application of biotechnology in industrial scale [49, 50]. The following points represent advantages of the industrial biotechnology techniques compared to conventional chemical processes [51]:

- Microorganisms and enzymes act as non-toxic biocatalysts
- High selectivity – chemo-/regio-/enantio-selectivity of biocatalysts results in high product purity, less by-products and fewer process steps compared to chemical catalysis
- Low waste production
- Operation under moderate conditions such as ambient temperature, pressure, which lead to low energy consumption
- Biotransformation reactions are often performed in aqueous medium and organic solvents.

The increasing interest and use of industrial biotechnology is attributed to rapid advancements that have been made during the past decades in life science technologies including genome sequencing, bioinformatics, synthetic biology, metabolic engineering, protein engineering, high throughput screening, process engineering, etc. Such developments help to gain access to the vast biocatalytic potential available in the enormous microbial diversity in nature, ability to produce non-natural complex molecules, improvements in microbial host systems for expression of heterologous enzymes and metabolic pathways, improvements in enzyme activity, selectivity and stability, etc. On the basis of various established biotechnological processes and the new developments in the various tools, industrial biotechnology promises to become an important part for the achievement of green industry and bioeconomy, and even facilitate expansion of the applications of microorganisms and enzymes (**Figure 2.3**). Thereby, the development of industrial biotechnology provides alternative, competitive and sustainable processes for the production of bio-based chemicals from biomass.

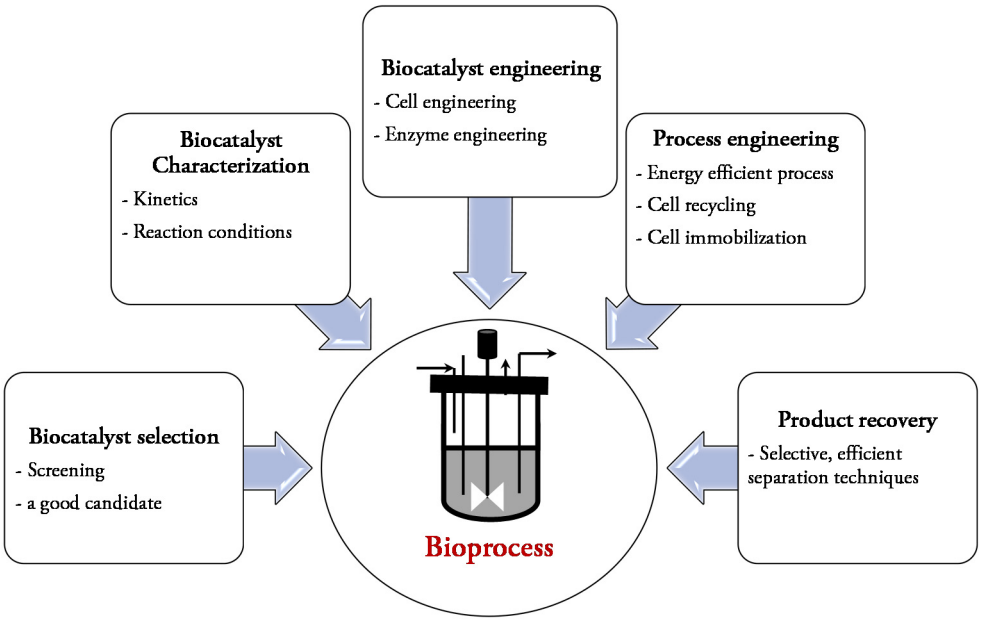


Figure 2.3
Development cycle of industrial biotechnology

3 Microbial and enzymatic transformations

Biotransformation is the conversion of a chemical compounds (natural or synthetic) into a desired product through the use of a biological catalyst such as microbial cells (growing or resting), cellular extract or pure enzyme. Biotransformations involve a variety of chemical reactions including oxidation, reduction, isomerization, condensation, hydrolysis, C-C bond formation, halogenation, etc., which are very important for different industries [52,53]. Hence, many chemical companies are used microbial and enzymatic biotransformation for production in large scale ranging from 100-10000 tons/year. Examples of microbial transformations include a number of simple and complex reactions like conversion of ethanol to acetic acid by *Acetobacter acetii*, production of acrylamide from acrylonitrile by nitrile hydratase of *Rhodococcus* sp. [54], penicillin by *Penicillium chrysogenum* [55], and Vitamin B12 produced industrially by propionic acid bacterium such as engineered *Propionibacterium freudenreichii* [56,57]. Besides several chiral products such as R-mandelic acid using nitrilases, enantiopure alcohols by lipase, non-proteinogenic L-amin acid by amidases and many others [52,58,59]. Nowadays, more than 134 biotransformation processes for chemicals production are already established at the industrial scale [60,61].

3.1 Oxidation reactions

Oxidation reactions represent a very important category of biotransformations, and oxidative enzymes are widespread in all living organisms ranging from bacteria to humans in order to achieve the oxidative metabolism of many organic compounds [62,63]. Oxidation reactions represent around 30% of organic chemical industry and more than 60% of synthesized chemicals and intermediates obtained by oxidation process [64]. However, oxidation of many chemicals such as inert hydrocarbons or alcohols to other important chemicals such as carbonyl and epoxide compounds is a major challenge for chemical industry. Also, the use of traditional chemical oxidation methods, which use expensive, toxic and waste generating catalysts like, sodium hypochlorite, Potassium peroxymonosulfate (KHSO5), and the expensive transition

metal catalysts (based on Pd, Ru, Pt, Au, Rh, etc.), and afford low selectivity, yielding mixtures of products that affect negatively on the downstream processes [64-69]. Thereby, the use of oxidative biocatalysis by microorganisms and enzymes, as alternative clean and new catalysts, has attracted the attention of academia and industry.

3.1.1 Selective oxidation

The transformation of (1) methanol to formaldehyde, (2) propene to acrolein (3), and butane to malic anhydride are examples of selective oxidations reactions achieved in the chemical industry. Alcohol selective oxidation to aldehydes and ketone is an important example of oxidation reactions for the production of essential building blocks and many valuable compounds. Therefore, the applications of biocatalysts, whole cells and enzymes, for the selective oxidation of alcohols, aldehyde, and polyols under mild conditions with high chemo-, regio-, and enantio-selectivity are highly desirable. Oxidoreductases are the group that possess most oxidative enzymes. These enzymes include dehydrogenases, monooxygenases, dioxygenases, oxidases, peroxidases and others. The action of this group depends on the cofactors such as $NAD(P)^+$ for dehydrogenases, molecular oxygen for oxidases, monooxygenases and dioxygenases, and hydrogen peroxide in case of peroxidases. Owing to the high cost of some cofactors like $NAD(P)^+$, which was estimated as €1500 and €6000 per kilogram for NAD^+ and $NADP^+$, respectively, the regeneration of these cofactors is very critical step for the enzymatic process [70]. Regeneration of cofactors can be achieved through enzymatic strategies include enzyme coupled system or substrate coupled system (**Figure 3.1**). However, many criteria should be considered to choose the right regeneration system. These considerations include the low price, stability and high specificity of the chosen enzyme, the simplicity of the reagents, hence they will not interfere with the product of interest or the enzyme stability, and the equilibrium between the coupled enzymes [70]. Therefore, the whole cell systems provide cheap and efficient solution for the continuous cofactor regeneration using different enzymes inside the cell [67,71,72]. Thus, several bacterial species such as *G. oxydans*, *Rhodococcus erythropolis*, *Pseudomonas putida*, *Acetobacter pasteurianus,* and many others have been used as whole cells as well as having a source for oxidative enzymes for selective oxidation of alcohols, diols and polyols [73]. In this work, many selective oxidation reactions were carried out using whole cell and pure enzymes. In particular, resting cells of *G oxydans* DSM 50049 & DSM 2343 were used for selective oxidation of HMF to HMFCA and butanol to butyraldehyde, respectively, (**Paper IV & V**) with high selectivity and high yield. Especially, 100 % selectivity and yield of HMFCA from HMF was obtained by the use of resting cells of *G. oxydans* DSM 50049 (**Paper V**). Another example is the growing cells of *Mycobacterium* sp. MS1601 used for selective oxidation of TMP to BHMB with 100% selectivity and yield (**Paper I & II**). Moreover, the resting cells of *Mycobacterium* sp. MS1601 have been used for the oxidation of HMF to HMFCA, FFCA, and FDCA

(**Paper VI**). An HMFO oxidase like enzyme (HMFO-Myc1) has been used for the oxidation of HMF (**Paper VI**). Yet another example used in **Paper IV** is the selective oxidation of methanol to formaldehyde by pure alcohol oxidase in buffer.

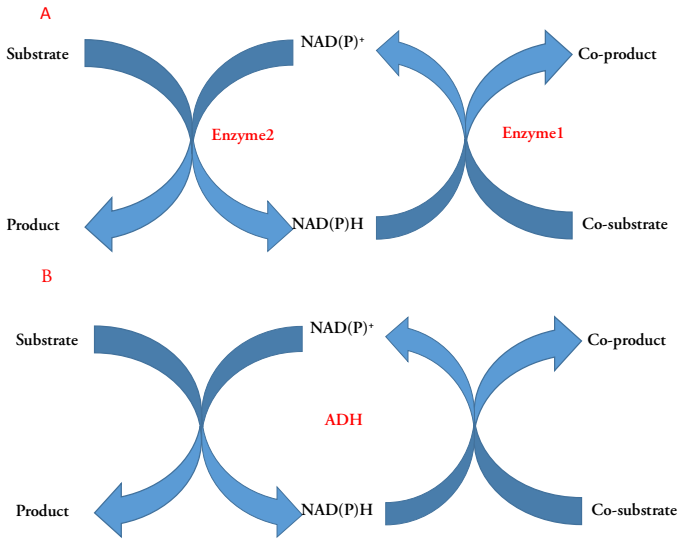


Figure 3.1
Enzymatic strategies for cofactors regeneration using coupled enzymes (A) or coupled substrate (B)[70]

3.2 Esterification and transesterification reactions

Esterification and transesterification reactions are reactions between an alcohol and acid/ester in which the -OH of a carboxylic acid or alkoxy group of an ester are exchanged with the alkoxy group of the alcohol to form an ester as the final product and water or another alcohol as the co-product. Besides the ester/alcohol reaction (alcoholysis), transesterification reactions include the reaction of ester/carboxylic acid (acidolysis), and ester/ester reaction (interestrification) (**Figure 3.2**). Esterification and transesterification reactions are used for the production of biodiesel, monomers, oligomers, polymers and chemicals for different applications in pharmaceutical, personal care and materials industries [74-76].

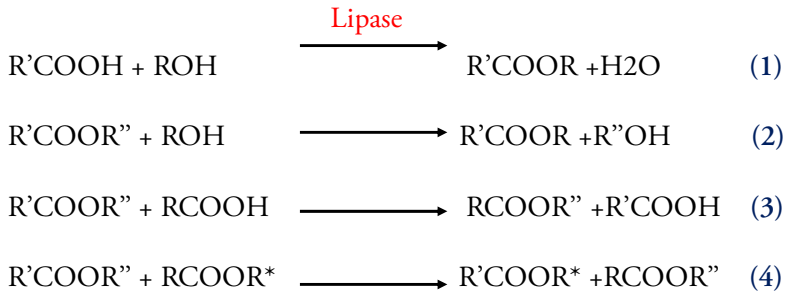


Figure 3.2
Esterefication and transesterification reactions by lipase enzyme

Many examples of esterification and transesterification reactions catalyzed by enzymes like lipases and esterases are available in the literature, which include the synthesis of biodiesel, fats, and polymers [77,78]. Lipase-based transesterification reactions for production of the valuable monomer, methacrylated TMP-cyclic carbonate, a potential monomer, for the production of bisphenol-free polycarbonates and isocyanate-free polyurethane, are reported in **Paper III.**

3.3 Whole cell biotransformation

Whole cell biocatalysis has been used in many bioprocesses for the production of bulk chemicals from renewable resources and highly selective synthesis of fine chemicals for pharmaceuticals, polymers and other products [7,79-81]. Whole cell biocatalysts include both wild type and genetically modified microorganisms, and have many advantages compared to the use of a pure or immobilized enzyme, which include:

- The use of whole cell biocatalyst in large scale is more economical than the use of pure enzyme [79,82]. The pure enzyme is 10 fold more expensive than using the whole cells.
- The ability of whole cells to generate the cofactor for the cofactor dependent reactions, especially redox reactions using oxidoreductase enzymes, without adding external cofactors [79,82].
- Whole cells carry out multi-steps reactions, which save time and money required for the isolation of the undesired intermediates. For example, multi-steps biotransformation reactions for production of heteroaromatic carboxylic acid in large scale by *Pseudomonas putida* and *Agrobacterium* sp. DSM 6336 have been reported [81,83,84].

- Whole cells provide protective environment for the enzymes, thus enzymes are more stable for reaction under harsh conditions [81,82].
- Whole cell biocatalysts are able to perform biotransformation processes for different compounds using a single strain having different metabolic pathways with vast number of enzymes. These pathways are controlled through the systematic alteration of cultivation parameters that include aeration, temperature, pH, medium composition, and enzyme inhibitors or through engineering of the microorganism by manipulation or knocking out specific genes according to the desired compound [82,85].

An example of the large scale whole cell biocatalysis is the use of whole cells of acetic acid bacteria for oxidation of carbohydrates and their derivatives [86]. Other examples large scale productions of different bio-products using whole cell are indicated in **Table 3.1**[87].

Table 3.1
Industerial whole cell biocatalysts for the production of different potential bio-products [87]

Whole cell biocatalyst	Product	Reference
Rhodococcus rhodochrous J1	Acrylamide and Nicotinamide	[87,88]
Pseudomonas denitrificans	Vitamin B12	[89,90]
Propionibacterium shermanii	Vitamin B12	[90,91]
Corynebacterium glutamicum	L-lysine and L-glutamic acid	[92,93]
Agrobacterium	L-Carnitine	[88,94]
Rhizobium HK1349	L-Carnitine	[88,94]
E.coli	L-aspartic acid	[59]

Other bacterial whole cell biocatalysts, which are used in this thesis, are explained in detail in the following sections:

3.3.1 *Mycobacterium* sp. MS1601 (previously, *Corynebacterium* sp. ATCC21245)

The genus of Mycobacterium consists of 188 species including environmental species as well as human pathogens. Most of environmental mycobacteria are saprophytic and fast growing bacteria, however, human pathogens are obligate parasitic and slow growing bacteria [95]. Mycobacteria represents the large group of Actinobacteria phylum besides other industrially important bacteria such as *Propionibacterium* spp. that are used for production of propionic acid by sugar fermentation [96], *Corynebacteria glutamicum* for production of glutamic acid and other amino acids [97], *Streptomyces* spp. for production of antibiotics as secondary metabolites, *Rhodococcus* spp., and *Arthrobacter* spp. Actinobacteria include most common bacteria in soils, water, and plants, and are recognized for their ability of production of primary and

secondary metabolites, which represent 45% of the discovered bioactive microbial metabolites. In particular, Actinobacteria play an important role in the decomposition of environmental organic matter such as cellulose, chitin and fats [98,99]. Moreover, they have the ability for biotransformation of aliphatic and aromatic compounds, of interest to the chemical industry. In particular, some *Mycobacterium* species have the ability of degradation and biotransformation of several organic compounds, for example, *Mycobacterium smegmatis* shows high ability for cholesterol degradation [100]. Also, the biotransformation of -sitosterol to 4-androstene-1, 17-dion has been achieved by immobilized *Mycobacterium* sp. NRRL B-3805 in an organic medium [101,102]. Epoxidation of allyl phenyl ether to phenyl glycidyl ether has been achieved by *Mycobacterium* M156 in biphasic system [103,104]. We have reclassified *Corynebacterium* sp. ATCC 21245 to *Mycobacterium* sp. MS1601 using 16S rRNA. Further confirmation was done by comparing the whole genome to those available on the online database using online server, rapid annotation using subsystem technology (RAST, version 2). In this work, *Mycobacterium* sp. MS1601 has been used as whole cell biocatalyst for the selective oxidation of TMP to BHMB using growing cells, and oxidation of HMF to FFCA and FDCA using resting cells (**Paper, I, II &VI**). Additionally, the assembly and annotation of the whole genome sequencing have contributed in the identification of the enzymes responsible for the oxidation of HMF (**Paper VI**). As a result of the huge number of oxidoreductases that include dehydrogenases, oxidases and oxidoreducatses enzymes[105], *Mycobacterium* sp. MS1601 can be a potential platform biocatalyst for oxidation of variety of substrates to target products (**Paper VI**).

3.3.2 *Gluconobacter oxydans*

G. oxydans is one of the popular bacteria in the field of industrial biotechnology. It is gram-negative, rod-shaped, obligatory aerobic and acidophilic bacteria. Also, it has a huge number of membrane bound dehydrogenases enzymes, which help bacteria to adapt and survive in rich environments with nutrients. Therefore, *G. oxydans* has high ability to perform incomplete oxidation of polyol, alcohols, sugars and their related compounds (**Figure 3.2**). For example, *G. oxydans* is used for production of vitamin C, keto-gluconic acid, acetic acid, L-ribulose, D-tagatose, chiral acids and aldehydes [106,107]. **In paper V**, *G. oxydans* DSM 50049 was used in the form of resting cells for incomplete oxidation of HMF to HMFCA in one liter scale with 100% selectivity and yield. Also, *G. oxydans* DSM2343 was used in this thesis for production of bio-butyraldehyde for the selective oxidation of bio-based butanol

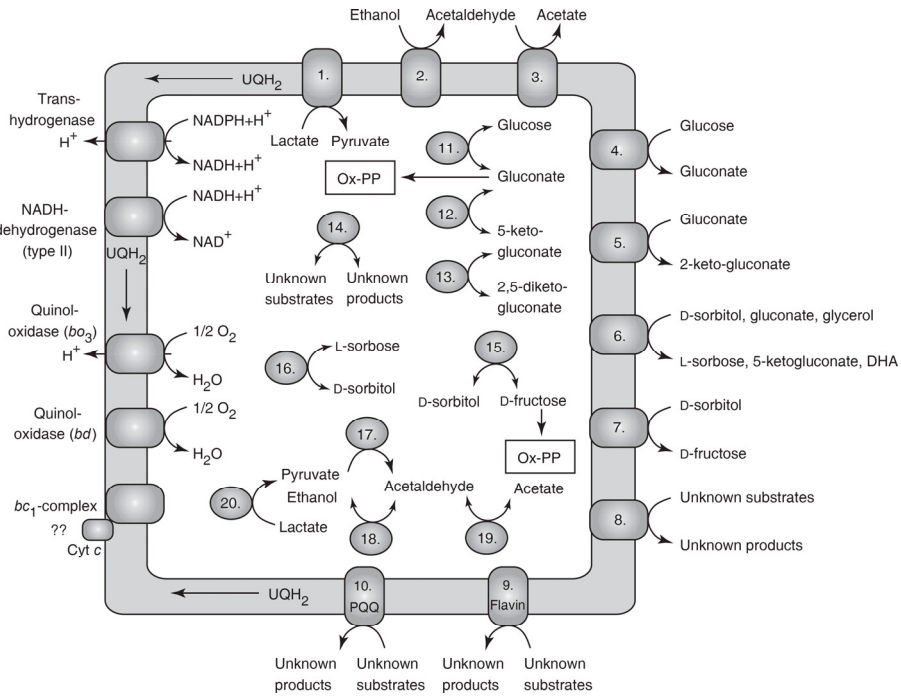


Figure 3.3
Oxidoreductase enzymes in *G. oxydans* bacteria. (1) Membrane bound lactate dehydrogenase (GOX1253). (2) PQQ-dependent alcohol dehydrogenase (GOX1067–1068). (3) Membrane-bound acetaldehyde dehydrogenase (GOX0585–0587). (4) PQQ-dependent glucose dehydrogenase (GOX0265). (5) Membrane-bound gluconate-2- dehydrogenase (GOX1230–1232). (6) Glycerol/ sorbitol dehydrogenase (SldAB; GOX854–855). (7) Sorbitol dehydrogenase (fructose forming; GOX2094–2097). (8) Uncharacterized membrane-bound oxidoreductases. (9) Uncharacterized flavin-containing oxidoreductases. (10) Uncharacterized PQQ-containing oxidoreductases (GOX0516, GOX1441, GOX1857, GOX1969). (11) Soluble glucose dehydrogenase (GOX2015). (12) Soluble gluconate dehydrogenase (GOX2187). (13) 2,5-diketogluconate reductase (GOX0644). (14) Uncharacterized cytoplasmic oxidoreductases. (15) Soluble sorbitol dehydrogenase (fructose forming; GOX1432). (16) L-sorbose reductase (GOX0849). (17) Pyruvate decarboxylase (GOX1081). (18) Soluble acetaldehyde dehydrogenase (GOX2018). (19) Soluble alcohol dehydrogenase (GOX0313)[106].

3.4 Enzymatic biotransformation

Enzymes were classified according to their function into six major categories including oxidoreductases, transferases, lyases, hydrolases, ligases and isomerases [108,109]. As a result of the versatile functions of the enzymes, they have wide applications in different industries such as paper and pulp, food and feed, leather, detergents and textile, chemicals, bioenergy, cosmetics and pharmaceuticals. Therefore, many enzyme-based processes for production of valuable bio-products have been scaled up and commercialized [110,111]. Despite of that the use of native enzymes still suffers from many limitations, which hinder industrial applications. These limitations include the low stability outside the host cell, narrow specificity, low catalytic efficiency, substrate

and product inhibition, and the problem of cofactor regeneration for some enzymes. To overcome these limitations many techniques have been developed for improvement of the native enzymes or development of new enzymes such as by genetic modifications and immobilization. The genetic modification techniques consist of two major ways including rational design and direct evolution [111-113]. In rational design the native enzyme is improved through site-directed mutagenesis of one or more target amino acids that are selected based on computational modelling [111]. Directed evolution approaches, on the other hand, are based on random mutagenesis e.g. by error prone PCR, and screening and selection of the mutants with desired properties for the reaction conditions [111,112,114]. The problem of cofactor regeneration is overcome through integrating a cofactor recycling system based on the same or alternative enzyme and an additional substrate. Lipases are among the most used enzymes in biocatalysis for esterification and transesterification reactions and also epoxidation. In this thesis, lipase and oxidase enzymes have been used for the production of valuable bio-products (**Paper I & VI**).

3.4.1 Lipases

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are abundantly present in plants, animals, algae, fungi and bacteria. Lipases are used in biodiesel, food, detergents and cosmetics, polymer, chemicals, pharmaceuticals and paper industries etc. Lipases are normally quite stable, and hence maintain their activity under different reactions conditions [74,115]. The catalytic triad of lipases comprises of serine, histidine and glutamate or aspartate and the conserved sequence is represented by G-X1-S-X2-G, where G is glycine, X1 is histidine, S is serine and X2 is glutamate or aspartate [116]. Identification of the catalytic triad in the purified lipases helps in predicting the 3D structure of the enzymes, which is important for improvement and engineering of the enzyme. In particular, lipases carry out hydrolysis reaction of fats, oils and other esters in water and transesterification and esterification reactions in non-aqueous medium such as organic solvent or solvent free media. Additionally, they are employed for versatile industrial applications with their enantio-, chemo-, and regio-specificity in catalysis of hydrolysis and esterification reactions. The immobilized lipases provide more advantages such as the high reusability and stability, easy separation from the reaction medium, and convenient application in a continuous operation through the use of packed column with lipase [74,117]. immobilized *Candida antarctica* lipase B, Novozym® 435 (N435) is a potential example of immobilised lipases that used widely in esterification reactions in nonconventional media for production of monomers, and polymers [118,119] .In **Paper VI,** Novozym® 435 (N435) was used for transesterification of TMP with methacrylic acid and it's derivatives (methyl, ethyl and vinyl methacrylate) for production of TMP-mono methacrylate (TMP-mMA), and followed by the transesterification of TMP-mMA with dimethyl carbonate, DMC to

obtain TMP-mMA-mono-carbonate (TMP-mMA-CC) in solvent free medium. Lipase mechanism of action in TMP transesterification reactions starts with activation of serine in active site by histidine and aspartic acid/glutamic acid. The substrate, like methacrylic acid or its ester derivatives and dimethyl carbonate, forms a tetrahedral acyl–enzyme intermediate by reaction with the OH group of the catalytic serine residue. The resulting negative charge is stabilised by the oxyanion hole [120]. The tetrahedral intermediate I forms a serinate ester with elimination of water molecule. Subsequent nucleophilic attack of alcohol, as TMP, to the acyl–enzyme intermediate leads to tetrahedral intermediate II. Finally, the product ester is released, and enzyme is free for the next molecule to attack.

3.4.2 Oxidases

Among oxidoreductase family, oxidases enzyme are more advantageous compared to other oxidative enzymes because of their ability to use the cheap and green molecular oxygen as an oxidant, which is reduced to hydrogen peroxide (H_2O_2) on water. The production of H_2O_2 is a limitation step to the enzyme activity due to the toxicity of H_2O_2. Therefore, coupling oxidases with peroxidases or catalases helps to breakdown H2O2 through its oxidation to oxygen and water (**Figure 3.4**). To transfer electrons from the substrate to the molecular oxygen, the oxidases employ different metals and cofactors as mediators [71,72]. Oxidases have wide ranging applications for oxidation of vast range of substrates for production of corresponding aldehydes or imines [72]. A number of oxidases from different organisms are commercially available. Some examples are alcohol oxidase from *Pichia pastoris*, which is used in **Paper IV** for selective oxidation of bio-methanol to formaldehyde under ambient temperature, glycerol oxidase from *Aspergillus niger*, galactose oxidase from *Dactylium dendroides,* and glycolate oxidase from sugar beet [71]. Other examples like polyphenol oxidase and pyranose oxidase were drawn great attention due to their biocatalytic potentials, wide substrate range and different potential products. These products include polyphenol polymers, antioxidants, and m -glycosides and others [121-124]. Furthermore, 5-hydroxymethyl furfural oxidase (HMFO) like enzyme from *Mycobacterium* sp. MS 1601 was identified and used for the selective oxidation of HMF to FDCA (**Paper VI**).

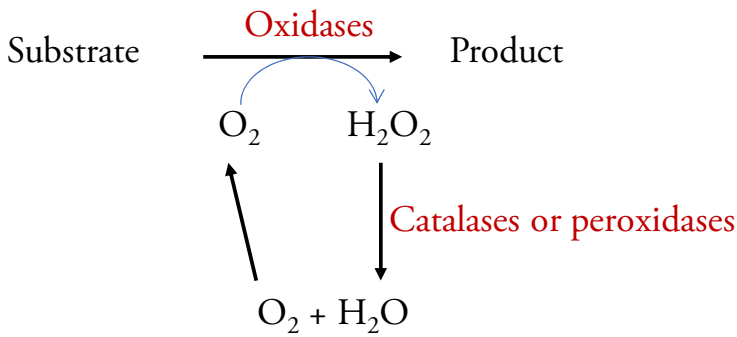


Figure 3.4
Coupled reaction system of oxidases with peroxidases or catalases

3.5 Bioprocess development

The cost of biotransformation processes is controlled by the productivity, yield, product concentration, stability, and reusability of the biocatalyst. Availability of robust and effective microbial strains and enzymes and well established means for their recovery and downstream processes are the key factors for the success of the biotransformation process [125]. Thereby, the cell development technologies such as metabolic engineering and synthetic biology, enzyme engineering technologies, and bioprocess engineering increases the impact of biotransformation in the synthesis of organic chemicals [61,125].

3.5.1 Development of microbial cell factories

The complex pathways, which are evolved in connection with the adaptation of the cells to their environments, provide many difficulties to engineer the cell metabolism without mapping the regulatory structures and recognizing the genes encoding of the proteins and identifying the correct engineering strategies for sufficient improvement of the cells. Hence, the system biology tools including high throughput analysis tools such as genomic, transcriptomics, proteomics, metabolomics analysis, and bioinformatics analysis, are used for this purposes [126]. Genome sequence of microorganisms has gained a great interest for well understanding and development of new cell factories through providing valuable genetic information and identification of genetic map for the microbial cells [27,127,128]. In addition, the development of the second generation sequencing and the ability to manipulate microbial genomes have developed the field of biotechnology for more applications and innovations [129]. The whole genome sequence, assembly and annotation of *Mycobacterium* sp. MS 1601 were

achieved, [105], in order to provide the genetic map for identifying the enzymes responsible for the oxidation of TMP and HMF, and also for further applications of this bacteria in other bioprocess.

3.5.1.1 Recombinant DNA technolog

Recombinant DNA technology has become a commonly used tool in laboratories for cloning expression of genes and altering the enzyme features. It is imperative to improve the bioprocess through the overexpression of genes, use of different and new expression hosts, overproduction of the target proteins, enhancement of the target proteins with new properties using mutagenesis methodology and manipulation of the selected pathways, and providing a new pathways for the production of different products [130]. In this thesis, genes responsible for the oxidation of HMF have been identified, cloned and overexpressed in *E. coli* Bl21 (DE3), which is a fast growing host compared to *Mycobacterium* sp. MS 1601 (**Paper VI**). Thereby, a new route for the production of formyl furan carboxylic acid (FFCA) and furan dicarboxylic acids (FDCA) via new biocatalysts in environment friendly bioprocess can be achieved.

3.5.2 Bioprocess engineering

Besides the importance of the development of industrial microorganisms, engineering of the bioprocess is an important factor for optimizing the capital costs and operating costs, for scaling up of a bioprocess[131,132].

*3.5.2.1 Experimental design (ED) **(Paper I&II)***

Experimental design in the bioprocess helps to investigate the effect of the input factors including medium constituents, pH, aeration and temperature through evaluation of the output factors from the bioprocess such as biocatalysts activity, product yield and productivity and the consumption and change rate of input factors (**Figure 3.5**). The experimental design also reveals how the interaction of the input factors enhances the responses of the output factors. The carbon and nitrogen sources have high potential in activating and deactivating of microorganisms, and metabolism, which consequently influence the outcome of the whole biotransformation process, positively or negatively [133]. For example, in **Paper I** the combination of glucose, xylose and sodium acetate as carbon sources with the ratio of 1:1:0.4 was optimal for the selective oxidation of TMP to BHMB by cells of *Mycobacterium* sp. (previously, *Corynebacterium* sp. ATCC 21245). Moreover, replacement of glucose, xylose and sodium acetate with glycerol and sodium acetate at the ratio of 1:0.4 shows significant improvement in the reaction time and productivity of BHMB from 0.02 to 0.06 g/l.h (**Figure 3.5**). Additionally, increasing the concentration of yeast extract in the cultivation medium enhanced the activity of *Mycobacterium* sp. MS 1601 against TMP (**Paper II**). The optimum values of the other input factors such as pH values and aeration were 8 and 1 vvm, respectively

(**Paper I**). Hence, experimental design of the selective oxidation of TMP using *Mycobacterium* sp. MS1601 was a good step towards scaling up the biotransformation process. In addition, it helps to find a suitable starting carbon source, glycerol, which is available as a byproduct from biodiesel production. However, the low productivity, long reaction time and requirement of high concentration of the expensive nitrogen sources (yeast extract) are still major limitations, which can potentially be overcome by further engineering techniques.

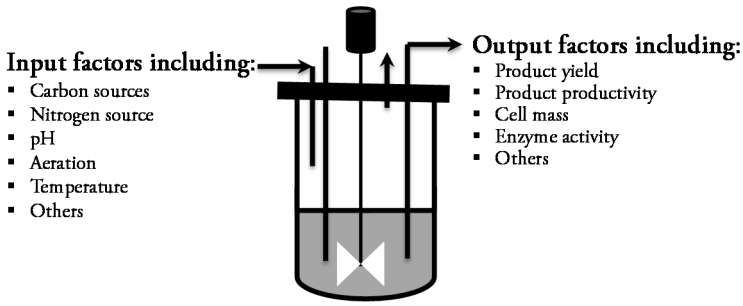


Figure 3.5
Principle of input factors and output responses of a bioprocess based on Experimental Design (ED)

3.5.2.2 Cell recycling and cell bleeding (Paper II)

High cell density with cell recycling has been used for the production of many bio-based chemicals such as ethanol [134], butanol [135], acetone-butanol-ethanol, (ABE) [136], and lactic acid [137], produced using continuous fermentation with cell recycling, and propionic acid through sequential batch fermentation and cell recycling [96,138,139]. Cell recycling technology has many advantages including:

- Achievement of high cell density and high productivity
- Recyclability of the produced cells
- Avoid the toxic effect of the product and other byproducts on the cells through continuous removal of the old broth
- Availability of the cells in different stages of growth
- Reduction in the process cost through minimizing the reaction time and using the biocatalysis several times
- Facilitating downstream process through providing cell free broth which eliminates the cell recovery step

In **Paper II**, selective oxidation of TMP to BHMB was significantly improved through the use of sequential batch cultivation with cell recycling and cell bleeding. BHMB productivity was increased from 0.06 g/L.h in batch to 0.2 g/L.h in sequential batch.

The cell recycling further reduced the reaction time from 7 days in batch cultivation to only 1 day for the oxidation of 5 g/l TMP. This improvement is due to the oxidation of TMP by cells in late exponential phase and early stationary phase. Therefore, cell recycling provides the cells in different growth stages through the continuous growth of new cells in the new batch with the availability of the old cells from the previous batch. Moreover, the recycling of cells along with cell bleeding assisted to oxidize higher concentration of TMP, around 21 g/l (**Paper II**). Also, the cell bleeding technology through fixing the amount of cells at a certain concentration depend on the reactor volume, helped to overcome the limitations generated by cell recycling technique such as the high viscosity due to cell accumulation that lead to air limitation (**Figure 3.6**).

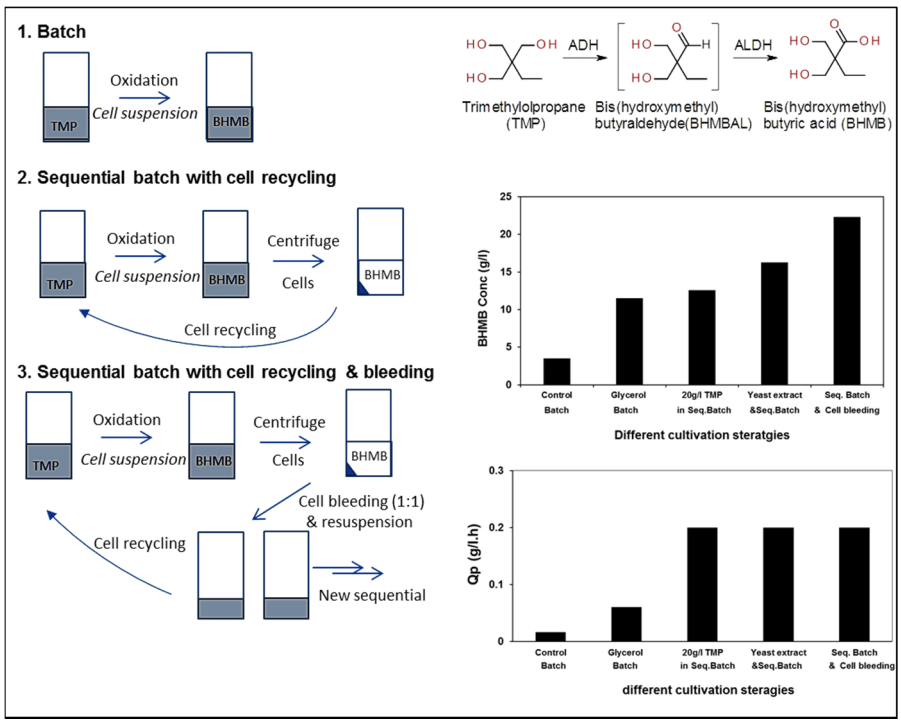


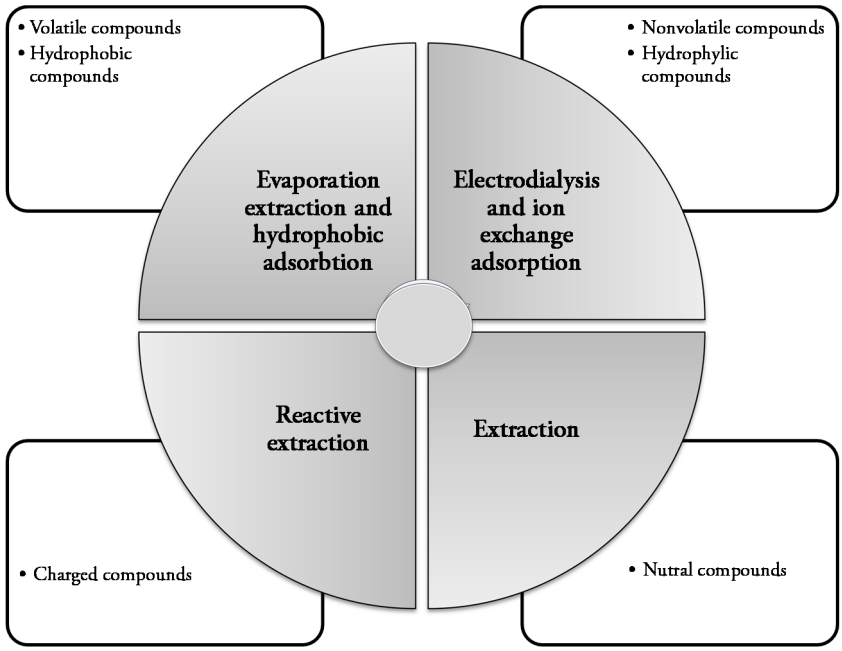
Figure 3.6
Comparison of the selective oxidation of TMP using different cultivation strategies of Mycobacterium sp. MS1601

3.5.2.3 Product recovery

Downstream processing accounts for the major cost of a bioprocess. Bioprocesses often suffer from low productivity and yield due to product inhibition and side reactions. These limitations lead to increase in the cost of the upstream and downstream processes, waste water treatment, and eventually the cost of the whole bioprocess. Therefore, product recovery represents a critical step for improvement of the whole bioprocess through elimination of product inhibition and side reaction by the selective

removal of the product from the fermentation processes. Thus, the integration of *in-situ* product removal (ISPR) technology with the bioprocess leads to improvement of productivity and product yield, and also the reduction of waste volume and downstream cost. ISPR includes different separation techniques, which depend on the nature of the product (**Figure 3.7A**) [**140**]. An example is the adsorption to ion exchange resin (IER), which is cationic and anionic depending on the ligand (negatively and positively charged, respectively) linked to a polymer matrix. The ligands have oppositely charged groups bound to them (**Figure 3.7B**). Ion exchange adsorption involves interchange of ions with the same charge from the solution, and it has wide application for water treatment and softening, environment remediation and biomolecule recovery from the reaction medium [141]. Furthermore, the application of IER for product recovery from the fermentation medium has many advantages such as the insolubility of resin, which allows easy recovery of resin by filtration, and also provides continuous separation through using solid resins in columns. Additionally, IER is highly recyclable through loading/regeneration/reloading cycles. In **Paper I** anion exchange resin, Ambersep® 900 (OH-), has been used for the recovery of BHMB from the fermentation broth with the overall yield of 77.6% (**Table 3.2**). The separation efficiency of biomolecules using IER depends on several factors such as the concentration of biomolecules in the solution, pH, temperature, and binding capacity of the adsorbent. Also, **Paper V** show another example of the application of anion exchange resin for the recovery of HMFCA form the reaction solution (**Figure 3.8**), which will contribute in the development of the selective oxidation of crude HMF using resting cells of *G. oxydans* DSM 50049 through the reduction of the toxicity of the product by combination of ISPR with the selective oxidation of HMF.

A



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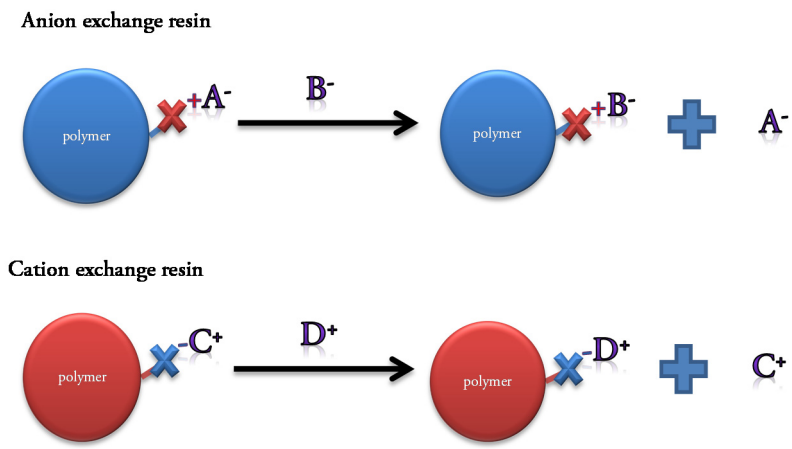


Figure 3.7
ISPR techniques for different molecules according to their physiochemical properties (A) and the exchange mechanism of an ion exchang resin in the solution according to the type of resin and product (B)

Table 3.2
BHMB recovery from fermentation broth by anion exchange resin

		Purity (%)	Concentration (mg/ml)	Volume (ml)	Amount (mg)	Step yield (%)	Overall yield (%)
Starting soln.		64.4	3.39	10	33.9	100	100
Residual			0.46	10	4.6		
Adsorbed					29.3	86.4	86.4
Washing solution			0	30	0	0	0
Elution	1st elution	89.9	2.38	10	23.8	81.2	
	2nd elution	77.3	0.25	10	2.5	8.5	
	Total elution		1.32	20	26.3	89.7	77.6

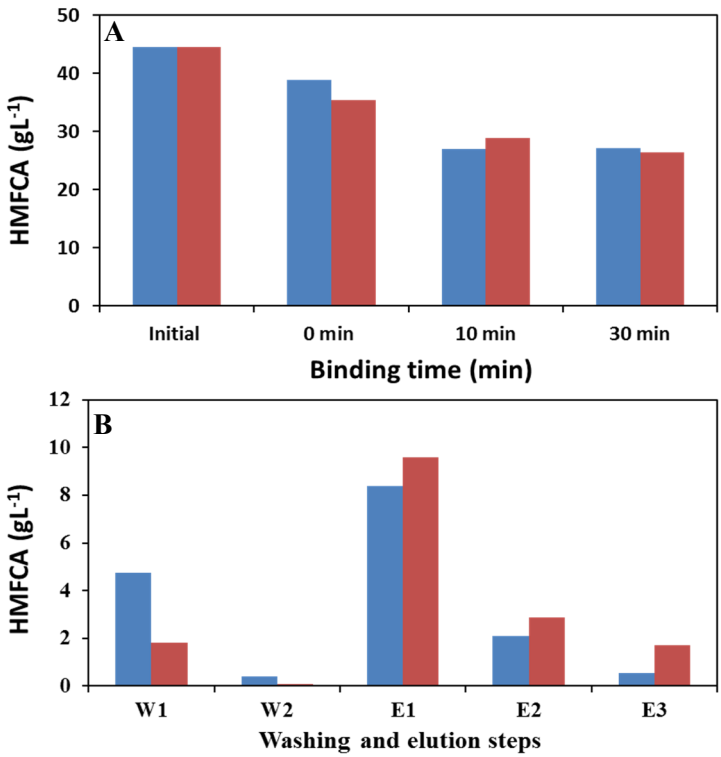


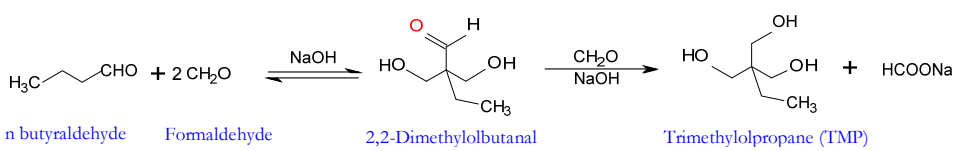
Figure 3.8
Binding profile of HMFCA from 1 mL reaction solution to 100 mg Amberlite IRA-400 (Cl form) (red) and Ambersep® 900 (OH- form) (blue). (A) Concentration of unbound HMFCA during adsorption step, and (B) concentration of HMFCA desorbed during washing (W) and elution (E) steps.

4 A new value chain around TMP

4.1 Trimethylolpropane (TMP)

TMP is a polyol with three functional hydroxyl groups, which contribute to the wide applications in alkyd resin polymers, coatings, foams, and lubricants industries. TMP enhances the outdoor durability, thermal stability and resistance of the resins. TMP diallyl ether (TMPDE) and TMP monoallyl ether (TMPME) are used in the production of personal care products and ion exchange resins. Moreover, TMP derivatives, propoxylated and ethoxylated TMP are used in the production of polyurethane foams, elastomers and sealants [142].

TMP is produced by Cannizzaro reaction, involving condensation of n-butyraldehyde with formaldehyde in the presence of stoichiometric amount of a base catalyst, followed by the reaction of the produced intermediate, 2,2-dimethylolbutyraldehyde with excess amount of formaldehyde using the same catalyst in an aqueous medium (**Scheme 1**) [143].



Scheme 4.1
Cannizzaro reaction for production of TMP ,

4.1.1 Synthesis of bio-based TMP

TMP is produced by several companies including BASF, Lanxess, Perstorp, Polioli, Oxea, Mitsubishi Gas, and PetroChina [142]. Only a few articles discuss the production of bio-based TMP. For example, the production of TMP by integration of the enzymatic hydrolysis of starch into mono and di- oligosaccharide and the hydrogenation of oligosaccharides into pentaerythritol or TMP has been reported [144,145]. However, the hydrogenation reactions required harsh conditions and toxic solvents. In **Paper IV**, production of biobased TMP has been achieved with high selectivity and high yield under mild reaction conditions from renewable resources

using integrated biological and chemical processes. In particular, the integrated process includes the production of bio-based n-butyraldehyde and formaldehyde, and their condensation to TMP using sodium hydroxide at room temperature. TMP was recovered from the reaction medium using liquid/liquid extraction and distillation, and and structure were elucidated using HPLC, GC, NMR.

4.1.1.1 Synthesis of bio-based n-butyraldehyde

n-Butyraldehyde is a very useful chemical, being used as an intermediate for the production of C4-C8 alcohols, esters and carboxylic acids. In addition *n*-butyraldehyde is a monomer for polyvinyl butyral polymer used in laminated glass. n-Butyraldehyde is produced chemically by hydroformylation of propylene at a production volume of 7 million tons/year [146,147]. Also, bio-based butyraldehyde is produced chemically by dehydration of 1,2-butanediol that can be produced from glucose, fructose or mannose. The dehydration reaction was carried out at 280–400 °C and 25–34 MPa using 5-20 mmol/L H_2SO_4 as a catalyst [145,148]. Besides the harsh conditions used, most aldehydes are highly toxic and reactive making their biological production highly challenging. n-butyraldehyde was produced at low concentrations of 0.6 and 1.6 g/L from glucose fermentation by the engineered *E. coli* and *Clostridium acetobutylicum* respectively [147,149]. In **Paper IV,** resting cells of *G. oxydans* DSM 2343 were used for selective oxidation of bio-butanol to n-butyraldehyde in 0.1M buffer phosphate at pH 5 and 30 °C in 500 ml reaction medium. After 36 h reaction, 15 g/l bio-based butanol was completely converted to 75 % butyraldehyde and 25 % butyric acid (**Figure 4.1**). The resulting n-butyraldehyde was used to produce biobased TMP without further purification.

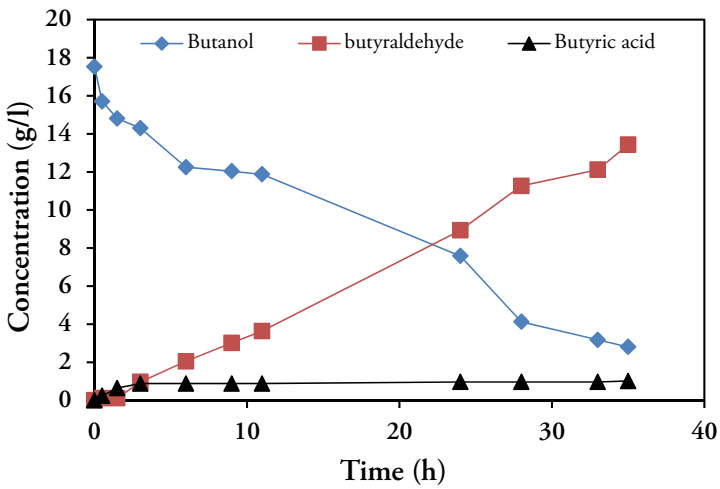
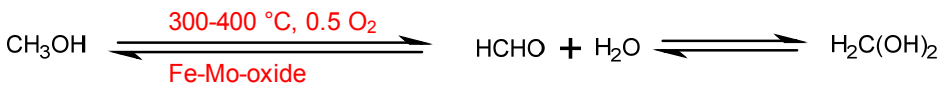


Figure 4.1
Production of n-butyraldehyde by selective oxidation of biobased butanol using resting cells of *G. oxydans* DSM 2343

4.1.1.2 Synthesis of bio-based formaldehyde

Formaldehyde is one of the most abundant platform chemicals with annual demand of 30 megatons/year in different industries. In particular, formaldehyde is used as a building block for pharmaceuticals, polymers, resins, adhesives, cosmetics and other products. Moreover, formaldehyde is used as a liquid carrier for the organic hydrogen as well as preserving agent for biological samples [150,151].

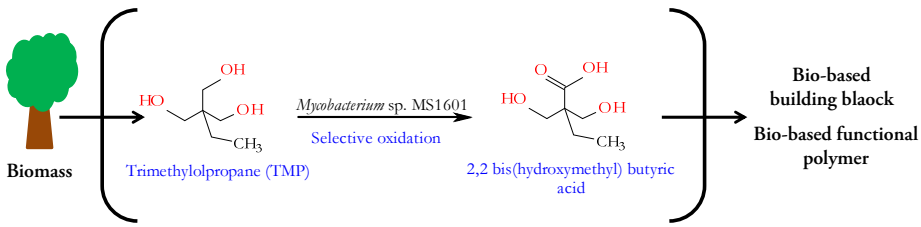
Formaldehyde is produced commercially by partial oxidation or dehydrogenation of methanol, which is produced by an energy-intensive gas phase process as indicated in (**Scheme 4.2**). Also, many studies have been published about the possibility of formaldehyde production by reduction of CO_2. On the other hand, bio-based formaldehyde is abundant in nature, being formed as a result of natural and artificial decomposition pathways of organic matter [151]. Also, the enzymatic production of bio-based formaldehyde from carbon dioxide has been achieved using formate dehydrogenase (EC 1.2.1.2) and formaldehyde dehydrogenase (EC 1.2.1.46) [151,152]. In **Paper IV**, bio-based formaldehyde was produced by selective oxidation of bio-methanol using pure alcohol oxidase from *Pichia pastoris* in 0.1 M buffer phosphate pH 8 with 52 % yield and 100 % selectivity. The resulting formaldehyde was used to produce bio-based TMP. However, no TMP was observed with using bioformaldehyde due to the low productivity. Therefore, the improvement of formaldehyde production is under investigation through two strategies. First strategy is increasing the biocatalyst resistance by using the whole cell, enzyme immobilisation, or enzyme mutagenesis. The second is coupling the *in situ* product removal along with the production bioformaldehyde in order to reduce its toxicity.



Scheme 4.2.
Formox process for the production of formaldehyde from methanol oxidation

4.1.2 Selective oxidation of TMP (Papers I & II)

Selective oxidation of alcohols to the corresponding carbonyl compounds is one of the important reactions in organic synthesis. Many alcohols, diols and some polyols are oxidized selectively by different microorganisms such as *Acetobacter*, *G. oxydans*, *pseudomonas* sp., *Rhodococcus* sp. and others [153], nevertheless, all of them show no activity against branched polyols such as pentaerythritol and TMP. Unlike other bacteria the growing cells of *Mycobacterium* sp. MS1601 (previously *Corynebacterium* sp. ATCC 21245) catalyzed the selective oxidation of TMP to BHMB (**Scheme 4.3**) (**Paper I**).



Scheme 4.3. Selective oxidation of TMP to BHMB using whole cell biotransformation

BHMB consists of three functional groups including two hydroxyl groups and one carboxylic acid group. Such a multi-functional molecule is potentially a versatile building block for the polymer industry, including polyesters, and hyper-branched polyesters [154], unsaturated polyesters, alkyd-resins, and polyurethanes. Also, BHMB is an important platform chemical for the production of cyclic carbonates that are used as monomers for the production of isocyanate free polyurethanes [155]. BHMB is produced by oxidation of TMP using hydrogen peroxide as a catalyst. However, there is no prior report on the biotransformation of TMP to BHMB. In **Papers I & II**, growing cells of *Mycobacterium* sp. MS1601 were used as biocatalyst for the oxidation of TMP into BHMB with high selectivity and yield (**Table 4.1**, **Paper I**).

Table 4.1 TMP biotransformation conditions and kinetics

Carbon source	Co-substrate (g/l)	Airflow (v/v/m)	pH	Mixing (rpm)	TMP (g/l)	μmax (1/h)	Qp (g/l.d)	BHMB (g/l)	Conversion %	Vol (l)	t (d)
	Shake flask										
Glucose (G)	10	-	-	300	1	0.072	0.073	0.73	66.3	0.05	10
Glucose	10	-	-	300	3	0.070	0.097	0.97	29.2	0.05	10
Glucose	10	-	-	300	5	0.068	0.122	1.22	22.2	0.05	10
Glucose	10	-	-	300	7	0.069	0.094	0.94	12.2	0.05	10
Glucose	10	-	-	300	10	0.068	0.151	1.15	10.5	0.05	10
Fructose	10	-	-	300	4.6	0.11	0.09	0.86	16.9	0.05	10
Xylose (X)	10	-	-	300	4.8	0.11	0.17	1.71	32.3	0.05	10
Lactose	10	-	-	300	4.7	0.07	0.03	0.33	6.4	0.05	10
Acetate (A)	2	-	-	300	4.5	0.10	0.13	1.33	26.7	0.05	10
Lactate	2	-	-	300	4.3	0.10	0.11	1.11	23.4	0.05	10
X+A	5+2	-	-	300	4.6	0.082	0.09	0.88	17.4	0.05	10
G+A	5+2	-	-	300	4.6	0.080	0.15	1.53	30.2	0.05	10
G+X+A	5+5+2	-	-	300	4.4	0.090	0.17	1.70	34.9	0.05	10
	Bioreactor										
G+X+A	5+5+2	1	6	500	5	0.005	0.00	0.00	0	0.5	10
G+X+A	5+5+2	1	7	500	4.5	0.098	0.13	1.30	26.6	0.5	10
G+X+A	5+5+2	1	8	500	4.2	0.084	0.28	2.79	61.4	0.5	10
G+X+A	5+5+2	1	9	500	4.6	0.083	0.02	0.22	4.5	0.5	10
G+X+A	5+5+2	0	8	500	4.6	0.029	0.01	0.06	1.3	0.5	10
G+X+A	5+5+2	0.5	8	500	5.0	0.069	0.02	0.19	3.6	0.5	10
G+X+A	5+5+2	1.7	8	500	4.6	0.085	0.23	2.25	45.3	0.5	10
	Optimum conditions										
G+X+A	5+5+2	1	8	500	4.8	0.095	0.37	3.74	70.6	0.6	10

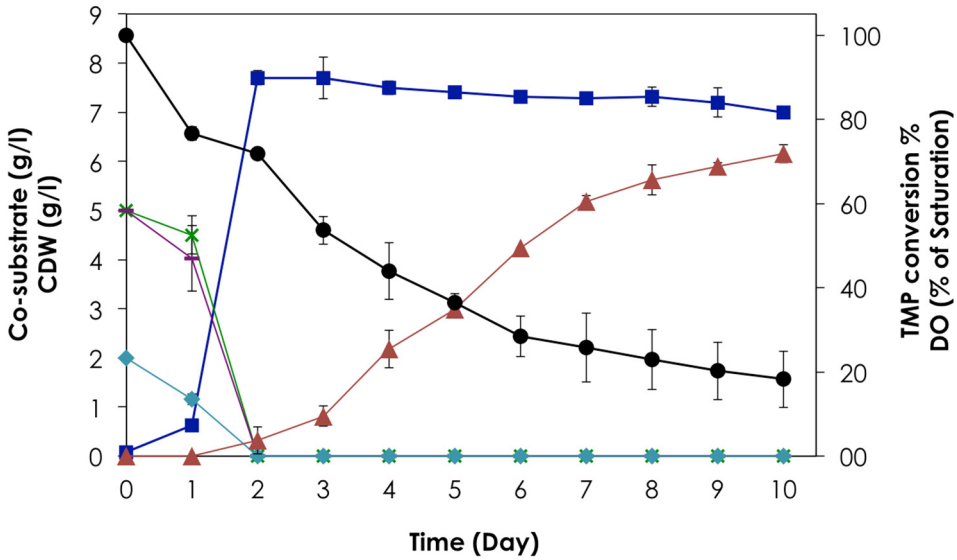


Figure 4.2
Biotransformation of (5 g/l) TMP to BHMB at pH 8, 1.0 v/v/m airflow, 31ºC and 500 rpm mixing speed using growing cells of *Corynebacterium sp.* ATCC 21245 (reclassified as *Mycobacterium* sp. MS1601). The production medium contained (×) glucose (5 g/l), (-) xylose (5 g/l), and (♦) acetate (2 g/l) as co-substrates. Cell growth shown as cell dry weight (g/l) (■), percentage dissolved oxygen (DO %) (●), and percentage conversion of TMP (▲).

In **Paper II**, the reaction rate and productivity were enhanced through (1) replacement of glucose, xylose and acetate with glycerol and acetate as carbon source, (2) increase of nitrogen source concentration, and (3) process engineering by cell recycling, and cell recycling with cell bleeding techniques. Firstly, the replacement of glucose and xylose with glycerol can reduce the whole process cost through the reduction of reaction time from 10 days to only 4 days with 100 % conversion of 5 g/L TMP. Additionally, 100 % and 50% conversion of 10 and 20 g/L TMP were achieved after 7 day reaction time, respectively, (**Figure 4.3**). Also, the productivity of BHMB was improved from 0.02 g/L.h with glucose and xylose to 0.06 g/L.h using glycerol as alternative carbon source in the medium.

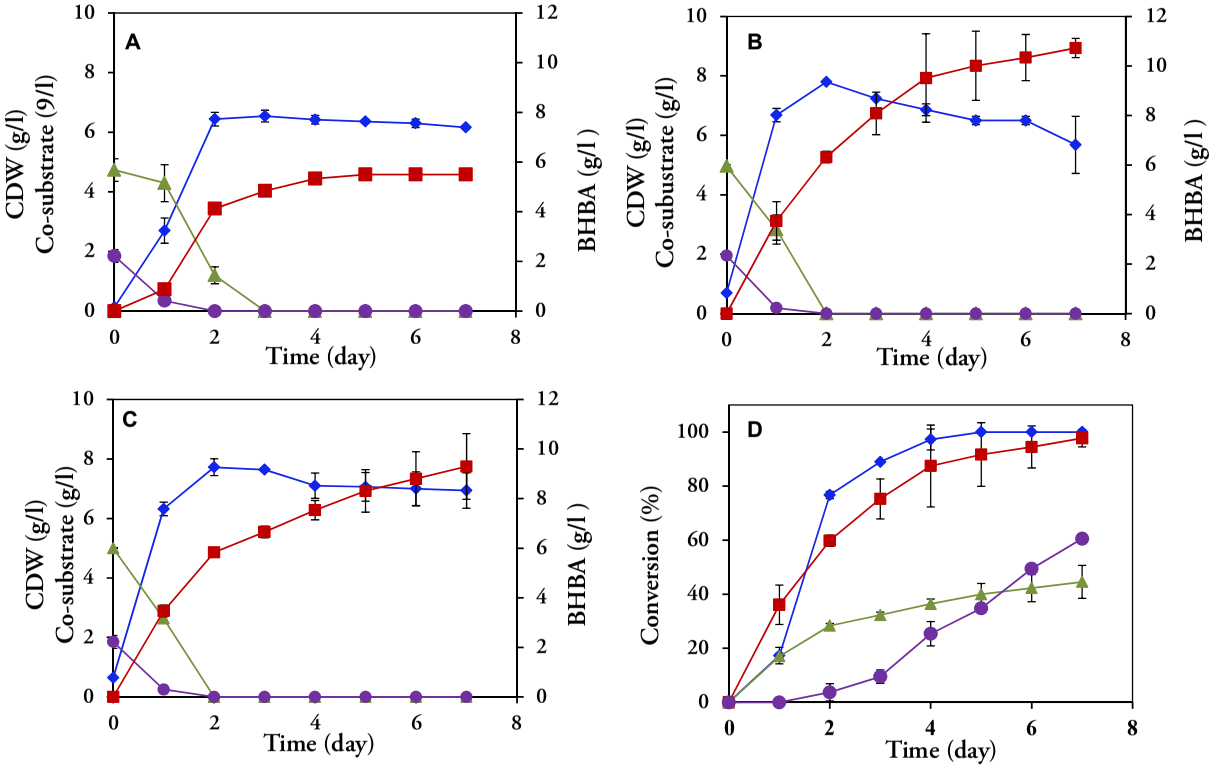


Figure 4.3
Effects of glycerol and sodium acetate as co-substrates on the selective oxidation of TMP using Corynebacterium sp. ATCC 21245(reclassified as *Mycobacterium* sp. MS1601) . Biotransformation of (A) 5, (B) 10 and (C) 20 g/L TMP; BHMB (●), cell mass (♦), glycerol (▲) and sodium acetate (■). (D) Conversion (%) of 5 (♦), 10 (■) and 20 g/L TMP (▲) in the presence of 5 g/L glycerol and 2 g/L sodium acetate, and 5 g/L TMP (●) in the presence of 5 g/L glucose, 5 g/L xylose and 2 g/L sodium acetate instead of glycerol.

Moreover, the conversion rate of 5, 10 and 15 g/L TMP was significantly enhanced by cell recycling and increasing the amount of nitrogen source. This enhancement might be caused by use of cells collected at late exponential phase and early stationary phase (**Paper I**) **Table 4.2 B**. Hence, the cell recycling with cell bleeding technique was employed to overcome the limitations through maintaining a certain amount of active cells, which increase cells viability and prevent the problems of air limitation and mass transfer for oxidation at higher TMP concentration (**Table 4.2 C**). Additionally, the cell activity was significantly improved by increasing the concentration of nitrogen source, e.g. yeast extracts (**Table 4.2 B &C**). Yeast extract is rich in vitamins and cofactors, which may have a positive effect on the enzyme activity compared to other nitrogen sources [156].

Table 4.2
Kinetics of sequential batch biotransformation of TMP with cell recycling using (A) increasing concentration of TMP, with (B) increasing yeast extract concentration, and (C) cell bleeding and increasing the concentration of yeast extract as well.

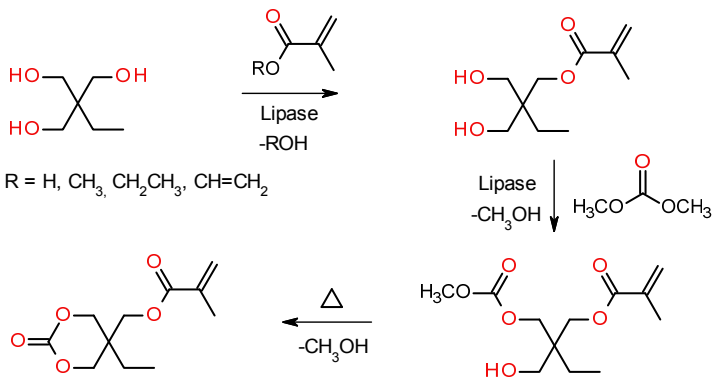
Exp.	Parameter	Batch number			
		1	2	3	4
A	Initial TMP (g/L)	5	10	15	20
	Final BHMB (g/L)	5.5	9.8	12.1	12.7
	Conversion (%)	100	90	75.1	60
	Cultivation time (h)	26	44	75	72
	QP (g/L.h)	0.21	0.22	0.16	0.18
	Yp/s (g/g)	1.10	1.10	1.10	1.10
	qp (g/g dw.h)	0.024	0.019	0.011	0.010
	Final cell mass (g dw/L)	7.546	11.8	15.32	18.68
B	Initial TMP (g/L)	5	10	15	
	Final BHMB (g/L)	5.2	11.0	16.3	
	Conversion (%)	95.4	100	99.1	
	Cultivation time (h)	23	22	82	
	QP (g/L.h)	0.23	0.50	0.20	
	Yp/s (g/g)	1.10	1.10	1.10	
	qp (g/g dw.h)	0.015	0.024	0.024	
	Final cell mass (g dw/L)	15.4	20.8	8.34	
C	Initial TMP (g/L)	5	10	17.15	21.35
	Final BHMB (g/L)	5.4	10.0	18.3	22.3
	Conversion (%)	97.3	91.7	97.3	95.3
	Cultivation time (h)	23	45	136	171
	QP (g/L.h)	0.23	0.22	0.13	0.13
	Yp/s (g/g)	1.10	1.10	1.10	1.10
	qp (g/g dw.h)	0.029	0.019	0.011	0.013
	Final cell mass (g dw/L)	8.08	11.82	12.62	10.3

A. Yeast extract = 10 g/L in all batches.
B. Yeast extract = 10, 15 and 20 g/L for batches 1–3, respectively.
C. Yeast extract = 10, 15, 20 and 25 g/L for batches 1–4, respectively.

4.1.3 Synthesis of functional cyclic carbonate from TMP

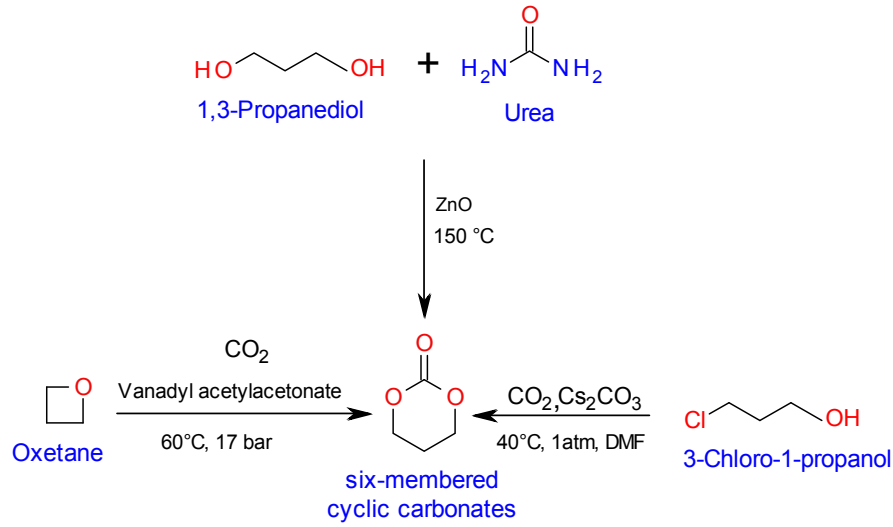
4.1.3.1 Six-membered cyclic carbonates

In **Paper III**, a methacrylated TMP cyclic carbonate was produced by lipase-catalyzed transesterification of TMP with methacrylic acid, followed by lipase-catalyzed carbonation with dimethyl carbonate (DMC) and thermal cyclization (**Scheme 4.4**). One of the three primary alcohol groups of TMP can be functionalized, and the resulting functional diol can be converted to cyclic carbonate. The polymerization, application and polymer properties can be varied using acrylated or methacrylated TMP cyclic carbonate. TMP cyclic carbonate functionlized with methacrylate group can be subjected to UV curing polymerization to obtain different properties of polymers.



Scheme 4.4.
Reaction pathway for production of TMP cyclic carbonate functionalized with methacrylate by lipase-catalyzed reaction and thermal cyclization

Besides the use of cyclic carbonates as intermediates in polymer industry, they are used as electrolytes in lithium batteries, plasticizers, reactive diluents, and as green alternatives for the conventional toxic solvents [157-159]. However, production of six-membered cyclic carbonates using chemical routes (**Scheme 4.5**), is more complicated, and has many drawbacks including the production of polycarbonates as byproducts, and the use of toxic catalyst and solvents [157,158,160].



Scheme 4.5.
Chemical routes for synthesis of six-membered cyclic carbonates

On the other hand, six-membered cyclic carbonates were synthesized and functionalized through transesterification of TMP with dimethyl carbonate (DMC) and diethyl carbonate (DEC) using immobilized lipase (Novozym® 435) as a catalyst in solvent free medium, followed by thermal cyclization [119,155]. In **Paper III** the methacrylate functionalized-six membered cyclic carbonates was synthesized by 2-steps transesterification catalysed by immobilized *Candida antarctica* lipase B, Novozym®435 (N435) followed by thermal cyclization in a solvent-free medium. TMP-mMA was produced from the transesterification of TMP with methacrylic acid and esters of methyl, ethyl and vinyl in solvent free medium using immobilized lipase (Novozym® 435). For the different acyl donors, the reaction rates were in the order: methyl > ethyl > vinyl > acid. The initial rate constants (k_d) at 6 h for methyl and ethyl esters were 0.1122 and 0.1144, respectively, which were about 2.5 times higher than that of vinyl ester, while very low reaction rate (0.0001 at 36 h) was observed with methacrylic acid (**Figure 4.4**).

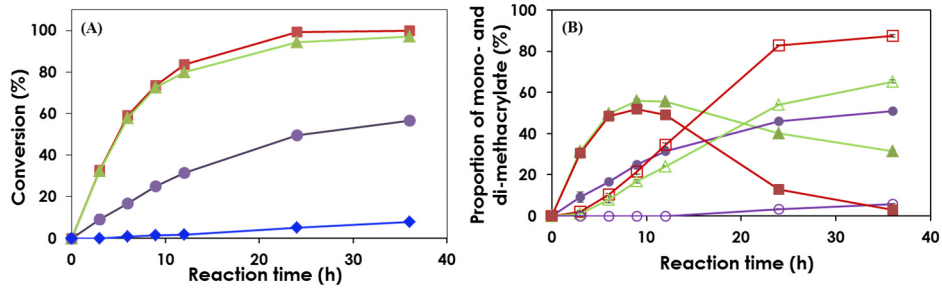


Figure 4.4.
Lipase-catalyzed transesterification of TMP with methacrylic acid and its esters. (A) Conversion (%) of TMP with methacrylic acid (◆), methyl ester (■), ethyl ester (▲) and vinyl ester (●) with respect to time. (B) Proportions (%) of monomethacrylate (filled simbols) and dimethacrylate (empty symbols) formed from reaction with methyl ester (■), ethyl ester (▲) and vinyl ester (●).

Then TMP-mMA was purified from 10 g scale reaction with 82% conversion of TMP. The purification was carried out by precipitation of remaining TMP in ethyl acetate, followed by silica chromatography for separation of TMP-mMA from TMP-diMA. The purified TMP-mMA was used as starting substrate for the production of TMP-mMA-monocarbonte in a transesterification reaction with DMC using immobilized lipase (Novozym®435) in a solvent free medium. The maximum conversion of TMP-mMA was obtained with 61% yield and around 73% selectivity of TMP-mMA-mono carbonate after 9 h. Thereafter, TMP-mMA-mono carbonate was cyclized to cyclic carbonates through thermal cyclisation reaction at 90 ˚C (**Figure 4.5**). The irreversible reaction of cyclization was prevented by applying molecular sieves, which removed the by-product methanol from the reaction.

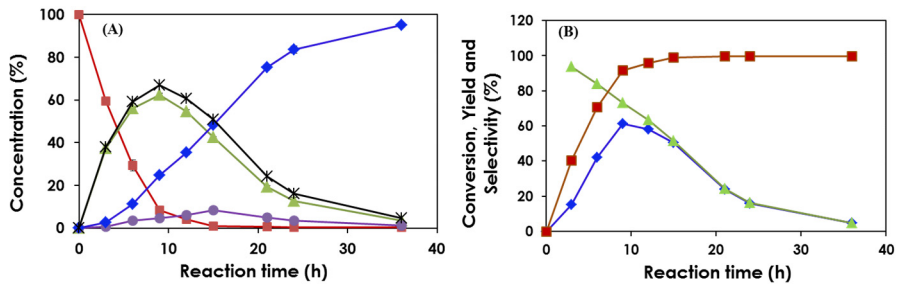


Figure 4.5.
Time-dependent profile of lipase-catalyzed transesterification of TMP-mMA with DMC. (A) Profile of substrate and products formation. TMP-mMA (■), TMP-mMA monocarbonate (▲), TMP-mMA cyclic carbonate (●), TMP-mMA di-carbonate (◆), and sum of mono- and cyclic carbonate (*). (B) Conversion of TMP-mMA (■), selectivity of mono- and cyclic carbonate (▲), and yield of mono- and cyclic carbonate (◆).

Moreover, the esterification of TMP with methacylates was evaluated by *in silico* modeling of the interaction between acyl donors (methyl and vinyl methacrylate) and the active site of lipase. The difference in the reaction rate was explained by molecular interaction between carbonyl oxygen and amino acid residues (Thr^{40} and Gln^{157}) in the active site of lipase. **Figure 4.6** shows more favorable interaction and formation of the acyl donor in the active site of lipase in the formation of the first tetrahedral intermediate with hydrogen bonding between oxygen of the carbonyl group and amino acid residues. Methyl methacrylate formed the bonding with Thr 40 hydroxyl group and NH of Gln 157, properly (**Figure 4.6a**), while the hydrogen bonds were not formed with vinyl ester (**Figure 4.6b**) (**Paper III**). The method developed is of high significance for synthesis of a functionalized six-membered cyclic carbonate to be used in the polymer industry. Furthermore, the reaction was carried out without any solvents; methyl methacrylate ester and DMC used in excess served as the solvent, which is easily recovered by distillation.

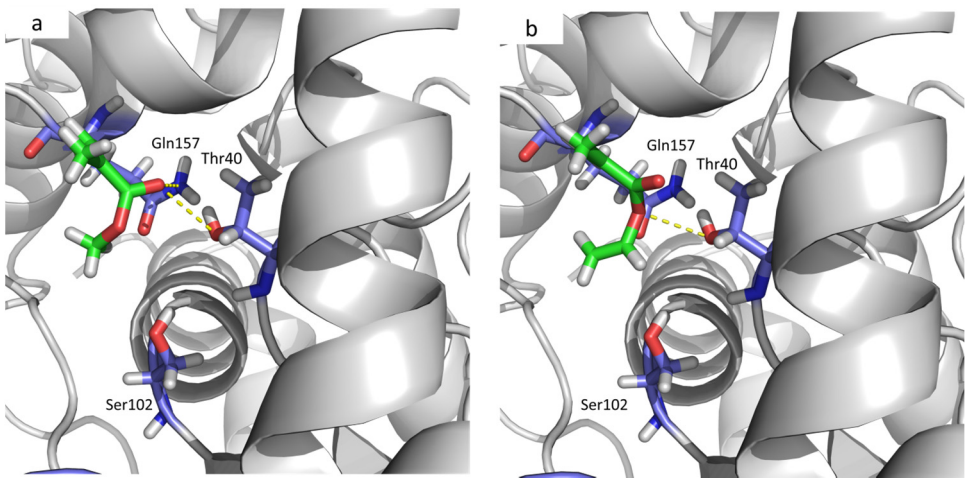
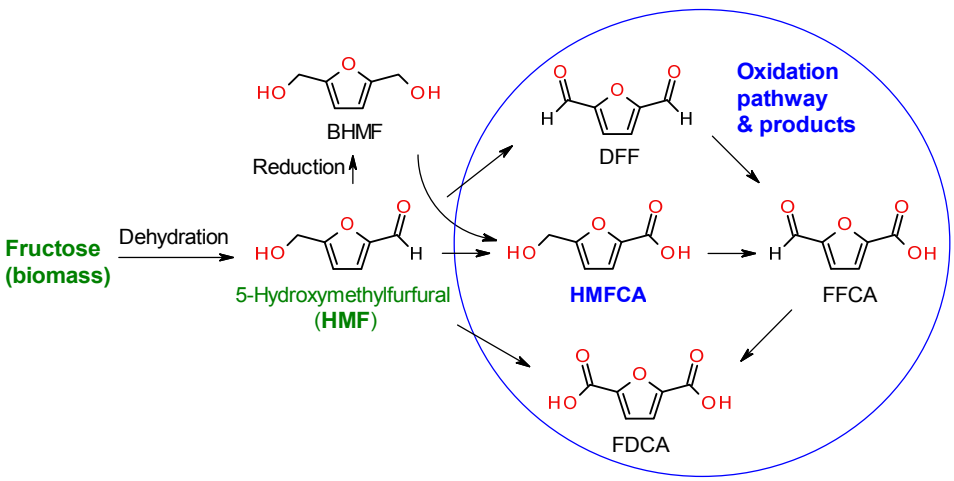


Figure 4.6.
Methyl methacrylate (a) and vinyl methacrylate (b) docked into CalB structure (PDB: 1TCA). Docking structures do not fulfill near attack conformation in both cases, however, for methyl ester, the carbonyl is coordinated by two hydrogen bonds to Gln157 and Thr40. These results indicate that initial conformation of esters can be the limiting catalytic step.

5 Selective oxidation of 5-HMF

5.1 5-Hydroxymethyl furfural (HMF)

Regarded as a valuable platform chemical for biobased chemicals and fuels, HMF production and transformation has become a hot research topic. HMF is obtained by dehydration of mono-sugars including glucose and fructose, which can be obtained from the most abundant carbohydrates in the biomass. Oxidation of HMF produces multi-functional chemicals such as 2,5-diformyl furan (FFA), 5-formyl-2-furancarboxylic acid (FFCA), 5-hydroxymethyl-2-furan carboxylic acid (HMFCA) and 2,5-furandicarboxylic acid (FDCA) for the polymer industry (**Scheme 5.1**).



Scheme 5.1
HMF as potential platform chemical from renewable resource

Oxidation of 5-HMF using different chemical catalysts has been reported, but generally encounters drawbacks related to selectivity, safety and cost [161,162]. Hence, evaluating the potential of biocatalysis using whole microbes or isolated enzymes for selective oxidation of 5-HMF becomes an interesting area for research [163,164]. In this thesis, oxidation of HMF to HMFCA with high yield and high selectivity using *G. oxydans* DSM 50049 was demonstrated (**Paper V**). Also in **Paper VI,** oxidation of HMF

to HMFCA, FFCA and FDCA was investigated using resting cells of *Mycobacterium* sp. MS1601.

5.1.1 5-Hydroxymethyl-2-furancarboxylic acid (HMFCA) (Paper V)

HMFCA can be produced by the oxidation of formyl group in HMF, and used as a bifunctional monomer for the synthesis of various polyesters. Besides, it also shows antimicrobial and antitumor activities and has thus potential for pharmaceutical applications. Also, it is used as a building block for production of FDCA [48-50].

The selective (incomplete) oxidation of HMF is complicated because of the possibility of the over oxidation to FFCA and FDCA [165], hence, only few reports are found on the oxidation of HMF to HMFCA. The majority of these reports involve the use of chemical catalysts such as MnO_2, Pt/C and Au-Pd, which gave poor yields of 14, 18 and 36%, respectively [161,165,166]. Recently, some systems with high HMF conversion and HMFCA yields have been reported. For example, total HMF conversion with 86.9% HMFCA yield was obtained using a montmorillonite K-10 clay immobilized molybdenum acetylacetonate complex in toluene [48], and 97.2% HMF conversion with 72.9% HMFCA yield were obtained using Cs-substituted tungstophosphate-supported ruthenium nanoparticles 16. Also, selective photocatalytic oxidation of HMF to HMFCA at 90–95% yields under ultraviolet and visible light in aqueous Na_2CO_3 solution by Au/TiO2 has been achieved [167]. However, the problems associated with the use of chemical catalysts such as the low selectivity, toxicity of the catalysts, as well as the harsh conditions required for both upstream and downstream processes, are major challenges.

Biocatalytic oxidation offers a more selective and mild alternative to chemical processes, and has lately received some attention. Lipase-mediated Baeyer-Villiger oxidation of HMF to HMFCA using H_2O_2 as the oxidant for *in situ* generation of peracid from ethyl acetate or ethyl butyrate gave HMFCA with a yield of approximately 80%. Also, the oxidation of HMF using xanthine oxidase from *E. coli* produced HMFCA with a yield of 94% and a selectivity of >99% after 7 h [50,168,169]. Since whole cells provide a more protective environment for the enzymes, allow regeneration of cofactors, and are also less expensive, the use of whole cell oxidation is generally more preferable to pure enzymes [79,87]. The main challenges could however be the inhibitory effect of HMF on the microorganisms as for the enzymes, and the over oxidation of HMF by other oxidative enzymes present in the cells. Only one report is found on the selective oxidation of HMF to HMFCA using *Comamonas testosteroni* SC1588; product yield of 98% was obtained from 20 g/L substrate in the presence of histidine as an additional enhancer. However, the cell viability, HMF conversion, and HMFCA yield were negatively affected by just a slight increase in HMF concentration. Also, bis(hydroxymethyl)furan (BHMF) and FDCA were observed as coproducts with

HMFCA in case of using *C. testosteroni* SC1588 . In **Paper V**, resting cells of *Gluconobacter oxydans* DSM 50049 showed high efficient oxidation of HMF to HMFCA at 100 % yield and selectively (**Figure 5.1**). Also, scaling up the oxidation of the crude HMF , which is produced in our lab and recovered with 75% purity using only liquid/liquid extraction technique, was done by resting cells of *G. oxydans* DSM 50049 in 50 and 500 ml of 100 mM sodium phosphate supplemented with 24 and 43.5 g/L of crude HMF, respectively, under controlled conditions. The results obtained in (**Figure 5.2**) indicate that *G. oxydans* DSM 50049 has an efficient ability in both experiment for the production of HMFCA at 100 % yield and selectivity. Particularly, in 50 ml scale around 91% of 24 g/L was converted to HMFCA after 33 h of reaction by *G. oxydans* DSM 50049 with controlling pH at 7 every 3h. However, complete oxidation of 31.5 g /L crude HMF to HMFCA with an initial volumetric productivity of (Q_p),10 g/L.h, after only 6h of reaction was achieved under continuous control of pH at 7 in a bench scale bioreactor (**Figure 5.2 B**). Additionally, *G. oxydans* cells oxidized 94% of an extra 12 g/L of crude 5-HMF fed after 6h of the reaction, and the productivity was reduced to 2 g/L. h due to the accumulation of HMFCA with high concentration as indicted in **Figure 5.2 B** . The results was oxidation of 43.5 g/L crude 5-HMF to HMFCA at 100 % selectivity and 94% conversion after 23h using whole cell biotransformation in buffer without adding any enhancer.

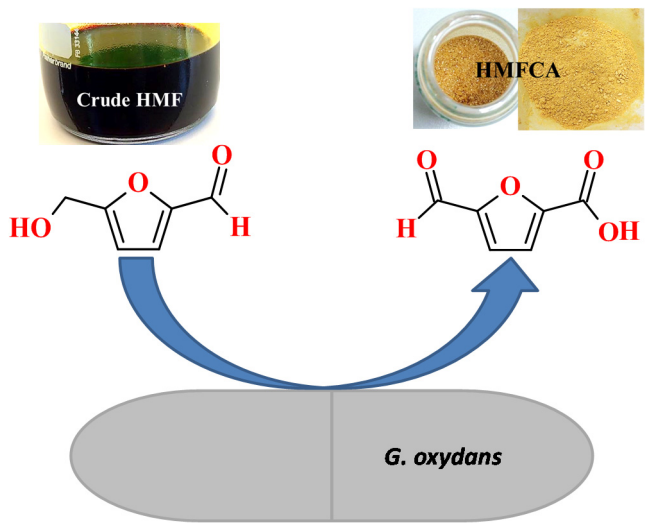


Figure 5.1
Selective oxidation of crude 5-HMF to HMFCA using resting cells of *G. oxydans* DSM 50049

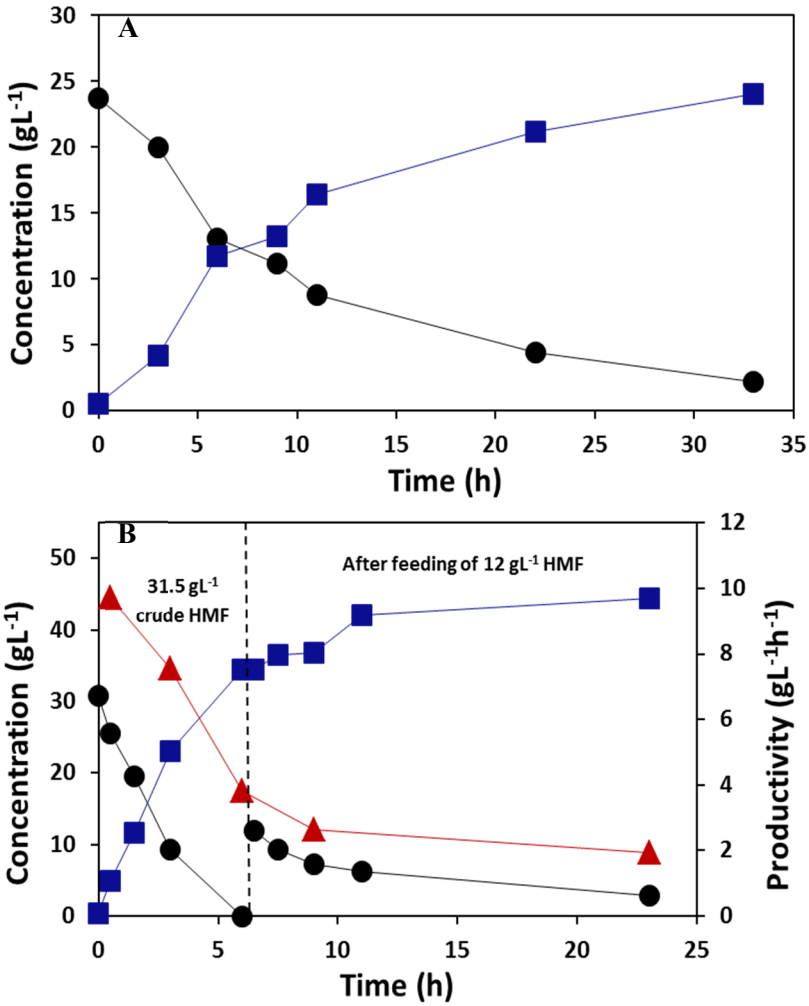


Figure 5.2
Profiles of selective oxidation of HMF (●) to HMFCA (■) using *G. oxydans* DSM 50049 cells on scaling up the reaction. Reactions with (A) 24 g L-1 HMF in 50 mL scale with intermittent pH control, and (B) with 43.5 g L-1 HMF in 250 mL solution in a bioreactor with continuous pH control. HMFCA productivity (▲) was calculated at different time points to evaluate the performance of the reaction.

The problem of the negative effect of the HMFCA high concentration can be overcome through *in situ* removal technique using anion exchange resin. In **Paper V,** preliminary experiments on the recovery of HMFCA from the reaction solution were performed using 100 mg/mL of the anion exchange resins, Amberlite IRA-400 (Cl form) and Ambersep® 900 (OH- form). The binding capacity of the resin after 30 min was 169 and 161 mg/g resin of Amberlite IRA-400 (Cl form) and Ambersep® 900 (OH- form),

respectively. The anion exchange resin (Cl form) showed higher binding efficiency, and also exhibited much less loss of the bound HMFCA during washing (1.8 mg/mL versus 5 mg mL^{-1}) compared to the (OH- form) resin. Moreover, 83.6% of the bound HMFCA to (Cl form) resin was recovered after 3 elution steps using 1 mL each of 2 M HCl, while only 68.3 % was recovered from (OH form) under similar conditions. The results suggest that a large amount of the resin, ~265 g/L, would be required for capturing all HMFCA of 43.5 g/L concentration from one liter solution. The elution of the bound HMFCA, using 2M HCl makes the product suitable for further purification by liquid/liquid extraction. Furthermore, HMFCA with a purity of 98% was obtained on liquid-liquid extraction from the reaction medium at pH 1.5 using ethyl acetate followed by concentration of the solution (**Figure 5.3**).

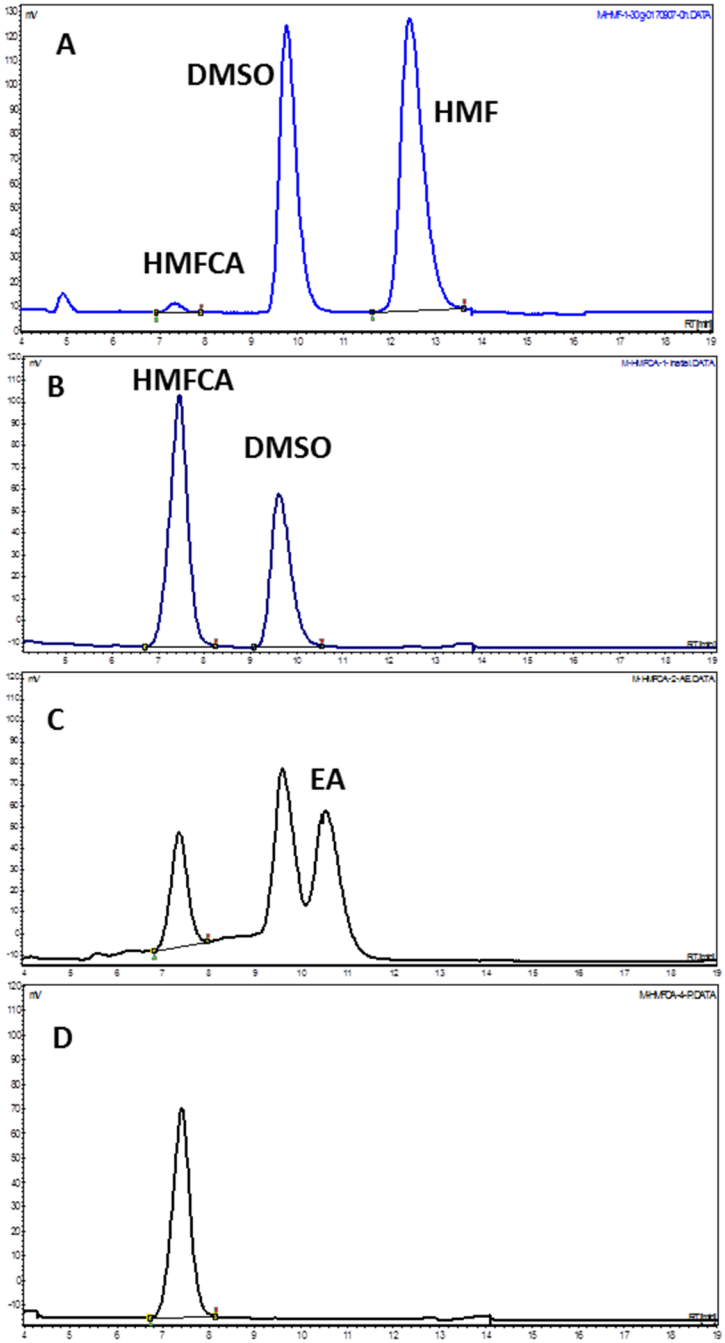
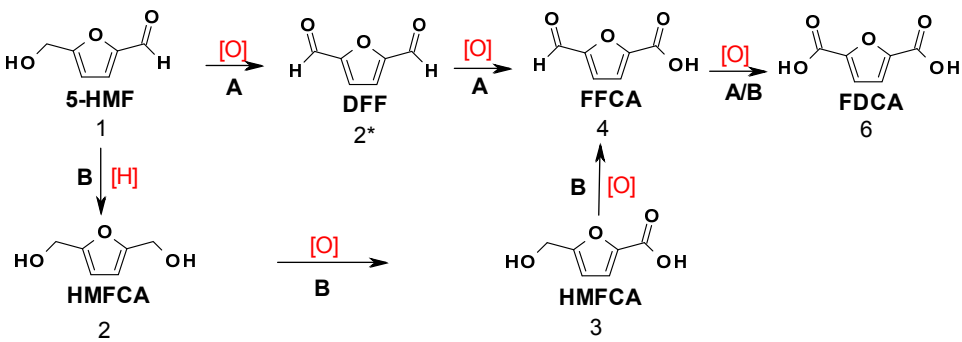


Figure 5.3
HPLC chromatograms of samples of (A) initial HMF solution used for oxidation by *G. oxydans* DSM 50049; (B) end product of the oxidation reaction (C), remaining HMFCA after mixing with ethyl acetate; and (D) recovered HMFCA from ethyl acetate.

5.1.2 2,5-Furan dicarboxylic acid (FDCA) (Paper VI)

Production of FDCA by both chemical and biological oxidation of HMF has drawn great attention [45,170]. Currently, two industrial consortia, Synvina and Total-Corbion are in the process of scaling up the production of FDCA.

FDCA is generally produced chemically from biomass sugars through HMF as an intermediate [171]. HMF is oxidized to FDCA through two pathways. In the first pathway, the alcohol group of HMF is oxidized to an aldehyde, 2,5-diformylfuran (DFF), followed by further oxidation of the two aldehyde groups in FFCA. In the second pathway, the aldehyde group of HMF is oxidized to HMFCA, and then the hydroxyl group of HMFCA is oxidized to an aldehyde group yielding 5-formyl-2-furoic acid (FFCA), which is further oxidized to carboxylic acid, FDCA (**Scheme 5.2**) [172].



Scheme 5.2
Pathways for production of FDCA from the oxidation of HMF

Notwithstanding many chemical processes using homogeneous and heterogeneous catalysts were achieved, but still many drawbacks are related to the use of chemical processes [**173**]. The bio-production of FDCA by selective oxidation of HMF requires the combination of two enzymes, and different enzymatic systems have been used. For example, galactose oxidase was employed for the oxidation of HMF to DFF, followed by lipase-based oxidation of DFF to FDCA with productivity of 0.6-0.8 g/L.h [**174**]. In the same context, McKenna et al., (2015) have reported an enzyme cascade reactions using oxidase enzymes including galactose oxidase M3-5 variant that oxidizes HMF to DFF and an aldehyde oxidase ABC, which oxidizes DFF to FFCA which converted directly to FDCA by the same enzyme [163]. Another example is the combination of alcohol oxidase with per-oxygenase from *Agrocybe aegerita* in one pot reaction. Similarly, the combination of galactose oxidase with xanthine oxidoreducatses in one-pot reaction produces FDCA with 18 g/L.d. Moreover, the whole cell biotransformation using the engineered *Pseudomonas putida* S12 for the production of FDCA has been achieved only after expression of HmfH enzyme. Also, the recovery of

FDCA still presents as challenge with the whole cell system due to the formations of other coproducts from HMF oxidation [170].

To our knowledge, only HMF oxidase (HMFO) shows the ability for the oxidation of HMF to FDCA at very low concentration. HMFO belongs to glucose-methanol-choline (GMC) oxidoreductase family, which includes enzyme members with two conserved domains including flavin adenine dinucleotide (FAD) binding domain and a C-terminal domain that contains the active site residues [175,176]. Only few members of GMC family can perform double oxidation of alcohol to carboxylic acid. HMFO has the ability to oxidize alcohol to carboxylic acid, however, the enzyme is able to oxidize only hydrated form of substrate, hence high conversion rate is achieved with DFF compared to FFCA which is less hydrated compared to DFF [177]. Therefore, many mutations were carried out in order to improve the enzyme activity against FFCA [178]

In **Paper VI,** the oxidation of HMF to 60% FDCA and 40% HMFCA was observed by whole cell biotransformation using resting cells of *Mycobacterium* sp. MS1601 grown for 48h using glycerol as carbon source (**Figure 5.4 A**). However, no FDCA, and only HMFCA and BHMF were observed with the strain using glucose and sorbitol as carbon source (**Figure 5.4 B &C**). The accumulation of FFCA in the initial stage of the reaction, and its further oxidation to FDCA implied that the production of FDCA from HMF by *Mycobacterium* sp. MS1601 might follow the pathway A (**Scheme 5.2**). This pathway is a typical pathway used by HMFO from *Methylovorus* sp. MP688 [177]. The above observations highlight the presence of a number of useful enzymes in *Mycobacterium* sp. MS 1601, the activities of which could be triggered by the choice of the carbon source used for activation.

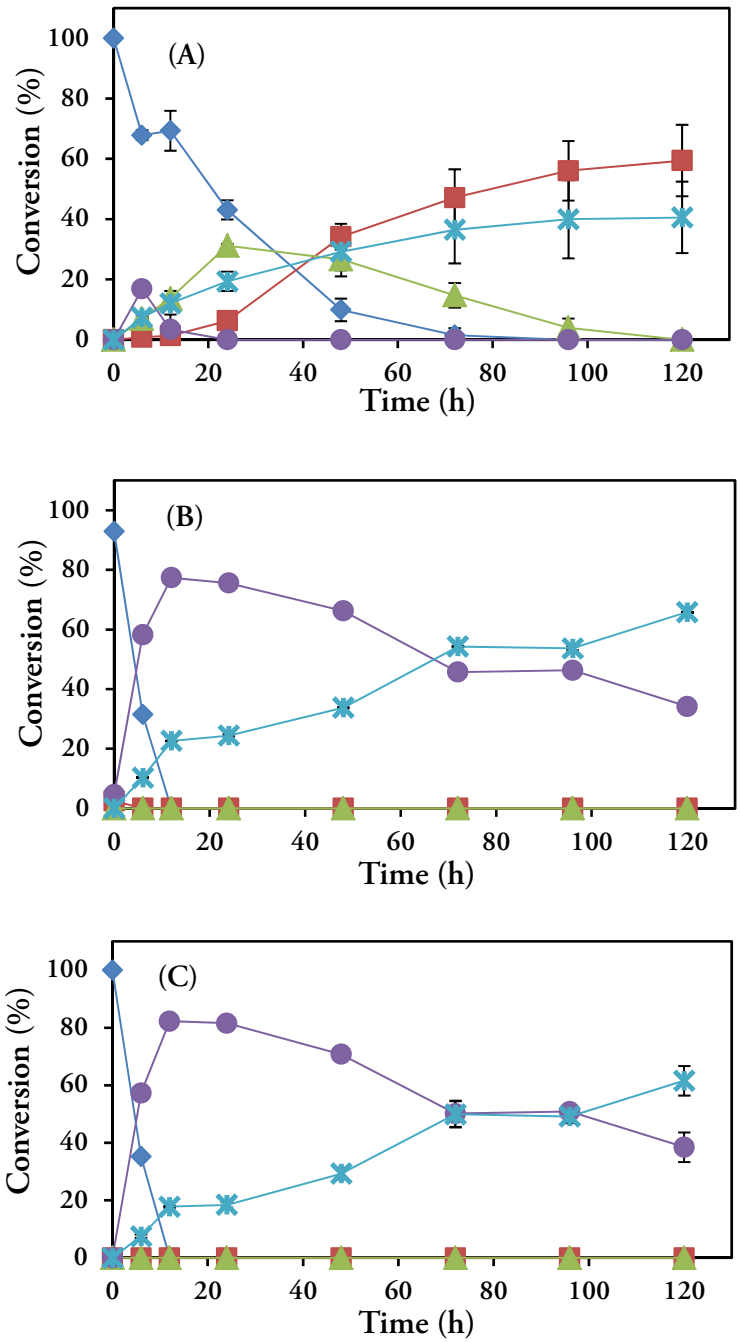


Figure 5.4
Selective oxidation of HMF using resting cells of *Mycobacterium* sp. MS1601, which were grown in a medium with different carbon sources (5g/L): (A) glycerol, (B) sorbitol, and (C) glucose

The putative HMF-oxidizing enzymes in *Mycobacterium* sp. M1601 were identified by searching its genome using the previously studied HMF-oxidase by Dijkman et al. using BLAST (UniProt accession code E4QP00) [179]. The search resulted in 3 main candidates namely: A0A1P8X5Y5, A0A1P8XFW1, and A0A1P8XIE5 with sequence identities 33%, 29% and 25%, respectively. Therefore, the DNA sequence of A0A1P8X5Y5 entry was synthesized, and codon optimized for expression in *E. coli*. The expression conditions were optimized, and 200 µg/mL of pure protein was purified using a metal chelate chromatography column (HiTrap Chelating HP), charged with Ni ions. Moreover, oxidation of HMF using the purified enzyme under mild conditions led to the accumulation of FFCA with 100 % yield and selectivity (**Figure 5.5**). The purified enzyme showed the same behavior as HMFO from *Methylovorus* sp. strain MP688 in terms of the inability of the enzymes to oxidize the non-hydrated aldehyde group of HMF [177,180].

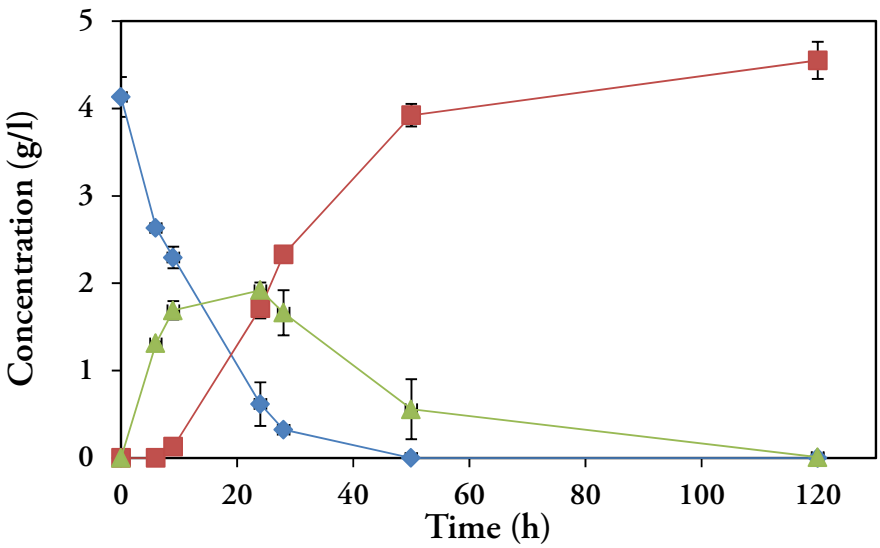


Figure 5.5
The selective oxidation of HMF using purified HMFO –Myc1

Furthermore, the homology modeling of HMFO myc1 (**Figure 5.6**) were evaluated based on the crystal structure of HMFO [178]. The generated 3D model of HMFO-Myc1 (**Figure 5.5A**) was superimposed on the PDB 4udp in order to gain information about the residues shaping the active site configuration. The first observation was the absence of the conserved histidine responsible for covalent binding of FAD molecule in HMFO-Myc1 in the position 91, instead Ile is found. This position is equivalent to

V101 in 4udp. The contribution of hydrophobic residues in the active site of 4udp has been reported and similarly in the present case the residues M337, A358, V360 and L298 are contributing to the guidance of the substrate orientation in the active site to face the FAD molecule (**Figure 5.5B**). In addition, the active site of HMFO-Myc1 was found to be generally smaller in volume, the active site pocket volume of 4udp using CASTp analysis was 919 compared to 536 in HMFO-Myc1 using 1.4 Å probe[181]. Upon investigation of the active site of HMF-Myc1 based on the model structure generated in our study, we noticed tight active site due to the presence of two tyrosine residues Tyr443 and Tyr444 in the substrate binding site facing the FAD molecule. Docking results showed that HMF-Myc1 can accommodate the FFCA in a productive mode where the α -carbon is facing FAD. However, upon docking the gem-diol form of FFCA (Hydrated form of FFCA) using the same docking settings a non-productive binding mode was obtained. The obvious reason is the bulkiness of the gem-diol structure that hindered the proper orientation of the substrate in the active site and consequently no enzymatic activity. We therefore, docked manually the gem diol in the active site of the HMF-Myc1 and run molecular modeling simulation for 2 pico seconds. Visual analysis of the snapshots taken during the molecular modeling showed significant displacement in the side chain of Tyr443 that highlights the possible interference of this residue in the proper binding of the gem-diol form of the FFCA. Therefore, the introduction of a positive charge residue in the beta sheet facing the FAD molecule could be beneficial in the directing the proper binding of the gem-diol substrate in the active site, this is based on the observation by Dijkman et al., 2015 [178]. From multi sequence alignment the structurally equivalent residue to Val367 in HMF-Myc1 is Ala358 (**Paper VI**) and a mutation to a positively charged residue such as Arg is thought to be a good candidate to improve the HMF-Myc1 activity toward production of the dicarboxylic acid. Experimental validation of this observation is currently under investigation.

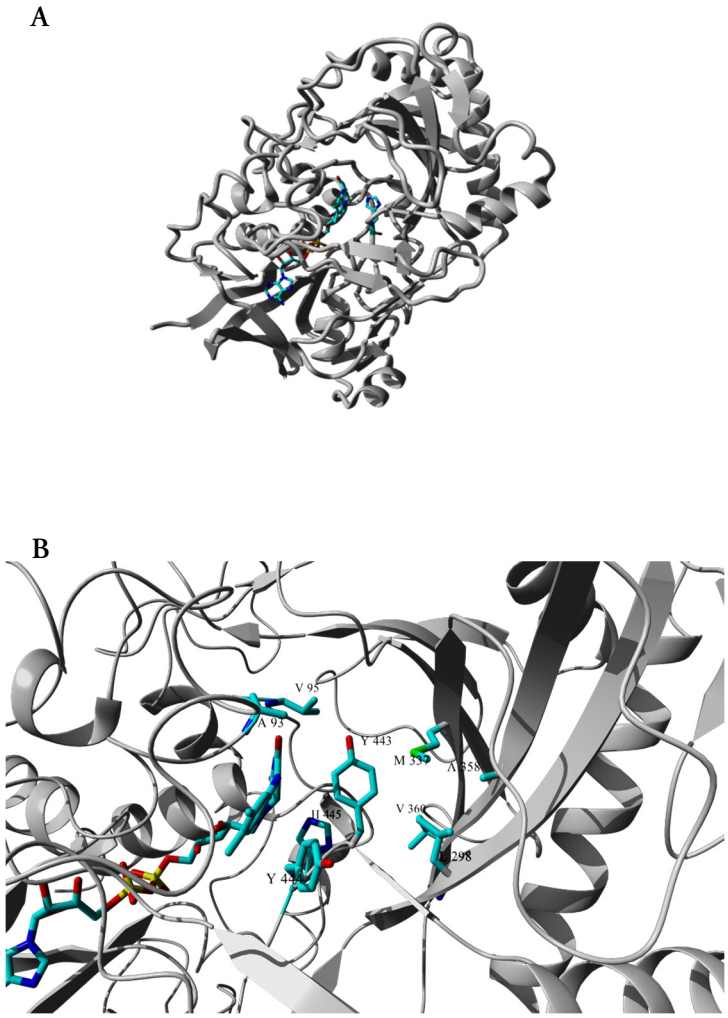


Figure 5.5
(A) The homology model obtained for HMF-Myc1, the FAD molecule and catalytic histidine is shown in stick representation. (B) Active site of HMF-Myc1 3D model. The conserved catalytic histidine is positioned at 445, the hydrophobic residues M337, A358, V360 and L298 contribute to the shaping of the active site, presence of two tyrosine residues 443 and 444 tightens the space available for the substrate to bind in front of the oxidizing FAD molecule. Figure is generated using YASARA structure

6 Conclusion and future perspective

This thesis has demonstrated the feasibility of utilizing the whole cells of microorganisms and enzymes as selective biocatalysts for transformation of bio-based chemical structures to new products that can be difficult to achieve by chemical catalysis, and would be interesting building blocks for novel polymers production. The reactions were performed under mild conditions either in an aqueous medium or in a solvent-free medium. It was also shown that the characteristic limitations of low productivity of the biocatalytic systems due to product inhibition or other factors could be alleviated to a great extent by reaction engineering. Furthermore, the biocatalytic step was integrated with a chemical reaction whenever necessary to achieve the final product.

The polyol TMP was converted into two different products, 2,2-bis(hydroxymethyl)butyric acid (BHMB) and a six membered cyclic carbonate with a methacrylate functionality.

– 2,2-bis(hydroxymethyl)butyric acid (BHMB) (Paper I&II)

The whole cells of *Mycobacterium* sp. MS1601 catalysed selective oxidation of one of three hydroxyl groups of TMP at 100% selectivity and yield. BHMB production was improved after medium, pH and aeration optimization. Significant enhancement of BHMB production was achieved by process optimization and by increasing the cell density in the reactor through recycling the cells after each batch and also introducing cell bleeding to maintain the active group of cells at optimal concentration for the reaction. Moreover, BHMB was successfully recovered from the reaction medium using anion exchange resin with 77.6 % yield.

– Methacrylate functionalized TMP-cyclic carbonates (TMP-mMA-CC) (Paper III)

TMP was functionalized with a methacrylate group by a transesterification reaction using an immobilized lipase followed by reaction with dimethyl carbonate and cyclization in a rather simple reaction to give TMP-mMA-CC. Six membered cyclic carbonates are usually produced using multi-step reactions generating a lot of waste. In the system developed in the present study, all the catalysts and residual reactants can be potentially recycled to improve the environmental and economic potential of the process.

– Bio-based trimethylolpropane (bio-based TMP) (Paper IV)

It was further shown in the thesis that TMP, which is currently produced only partly from feedstock of renewable origin, can be made fully bio-based using integrated biological and chemical processes under mild conditions.

The other set of products, 5-hydroxymethyl-2-furan carboxylic acid (HMFCA), 5-formyl-2-furan carboxylic acid (FFCA) and 2,5-furan carboxylic acid (FDCA) were produced by oxidation of HMF by microorganisms and their enzymes.

– 5-Hydroxymethyl-2-furan carboxylic acid (HMFCA) (Paper V)

Resting cells of *G. oxydans* DSM 50049 was found to be a robust and highly selective biocatalyst for total conversion of the crude HMF to HMFCA under mild conditions in a buffer. No other co-products were observed at any point during the reaction. Hence the recovery of pure HMFCA was facilitated using a simple separation procedure.

– 2,5-Furan dicarboxylic acid (FDCA) (Paper VI)

In contrast to *G. oxydans*, the *Mycobacterium* sp. MS1601 cells used a different set of enzymes for oxidation of HMF to FFCA and FDCA, the latter being currently under focus as a substitute for fossil based terephthalic acid. From the genome of the microorganism that was recently sequenced by us [105], the gene encoding a HMF oxidase like enzyme was identified, cloned, expressed in *E. coli* Bl21 (DE3). The free enzyme was shown to oxidize HMF only to FFCA via DFF, indicating that perhaps another enzyme is involved in the cells for complete oxidation to FDCA.

6.1.1 Future perspectives

Both *G. oxydans* and *Mycobacterium* sp. are a storehouse for a large variety of oxidative enzymes. Hence, identification of the enzyme(s) catalysing the specific transformation is like looking of a needle in a haystack. For further development, it will be of scientific interest to pinpoint the enzymes for the oxidative reactions in the study to understand their structure, mechanism and modify their activity and stability through mutagenesis. We have already started this journey with the HMFO like enzyme in *Mycobacterium* sp., and even located a residue Tyr443 that could obstruct further conversion of FFCA to FDCA. It is indeed possible that the microorganism possesses other enzymes with similar catalytic mechanism. Even identifying the enzyme catalysing TMP oxidation is of interest. The same goes for the enzyme system in *G. oxydans* providing extraordinary selectivity to HMF oxidation.

There are also process considerations, both technical and economical, for further development of the systems reported here. This will include also the choice of the biocatalyst form, i.e. wild type whole cells or recombinant microorganism containing

the enzyme of interest or isolated enzyme, free or immobilized, and biocatalyst recyclability. Other important aspects are the reactor design and downstream processing. For example, biotechnological processes are characterized by the presence of a large amount of water, removal of which is highly energy consuming and may contribute significantly to process costs. Also a very important aspect would be to perform life cycle assessments taking into account a whole value chain from bio-based feedstock to the product(s) to motivate the implementation of the proposed routes.

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Transition from fossil- to bio-based economy is a critical step towards reduction of greenhouse gas emissions and climate change, and hence for achievement of sustainable communities and environment. In order to be fossil-free, the chemical and material industry is in need of carbon-neutral building blocks from renewable resources for the diverse array of products that are currently produced from olefins and aromatics. Hence, new pathways for producing the same or novel chemical structures are needed. Industrial biotechnology offers a key technology area for transformation of biomass components or derivatives to chemical building blocks by the use of microorganisms or their enzymes

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