Bio-catalytic cascades and molecular oxygen-accessing amines and nitriles

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Summary

Reaching the finish line, never walking, and enjoying the race. These three, in this order, are my goals.

Haruki Murakami
Biocatalysis has proven its value and potential over last decades by finding its way into chemical industry. The pursuit of cleaner and more sustainable methodologies put forward a utilization of molecular oxygen as cheap and abundant oxidant. **Chapter 1** serves as an introduction that describes aerobic biocatalytic methods for C-H activation/hydroxylation, Bayer-Villiger oxidation, oxidations of alcohols, aldehydes, amines, iminines and alkene oxidation reactions. However, a major advantage of biocatalysis lies in the possibility to combine multiple enzymes in one pot. The utilization of such multi-enzymatic cascades allows skipping the tedious purification of intermediates and thus performing the reactions in a more economic fashion. One of the most sought-after biocatalytic reaction of last years is reductive amination yielding amines from carbonyl compounds. The cascade process, entailing reductive amination, often starts from alcohols being oxidized by an oxidoreductase and subsequent amination of the resulting carbonyl compound using some of available aminating enzymes occur. Therefore, **Chapter 2** describes our attempts to combine two classes of alcohol oxidases and amine dehydrogenases. We made surprising observations that created significant challenges to be tackled in the future. Notably, the main hurdle was related to the catalytic promiscuity of alcohol oxidases. In detail, the flavin-dependent alcohol oxidases exhibited the ability to catalyze the over-oxidation of alcohols to carboxylic acids, whereas the variant of copper-dependent galactose oxidase yielded nitriles from aldehydes in presence of ammonium ions. Further exploitation of flavin-dependent alcohol oxidases was limited by the low reproducibility of the methodology until now (i.e., considerable variation of analytical yields of carboxylic acids in independent experiments). Moreover, we attempted to use the copper-dependent alcohol oxidases in the flow and—while the oxidation of alcohols to aldehydes proceeded to a certain extent—we were unable to fully combine the copper dependent alcohol oxidases with the amine dehydrogenases in flow. However, the investigation into flow-biocatalysis prompted us the development an agarose-based hydrogel entrapping amine dehydrogenase and formate dehydrogenase, the latter for the recycling of the co-enzyme. This research was described in **Chapter 3**. One of the most interesting and innovative observations about the alcohol oxidases is the aforementioned ability of a copper-dependent galactose oxidase variant from *Fusarium*
sp. to catalyze the formation of nitriles from aldehydes in presence of ammonium ions in aqueous medium. The development of this serendipitous observation into a synthetically applicable methodology for cyanide-free ammoxidation of alcohols or aldehydes using ammonia and molecular oxygen was described in Chapter 4. Regarding the aims of the thesis described in Chapter 1, Chapter 5 steps outside from the overall focus on aerobic biocatalysis, and it deals with the protein crystallography. In detail, it provides an insight into the crystallographic work aimed at elucidating the structure of the engineered amine dehydrogenase LE-AmDH-v1. The variant was previously engineered by using an in silico generated homology model as the base for the structure determination. This work provided insights into the differences between the engineered amine dehydrogenase, its homolog from the archaeon Pyrococcus horikoshii and a saccharopine reductase from Magnaporthe grisea. These data will put the basis for further engineering of novel AmDHs possessing complementary substrate scope. Finally, Chapter 6 describes an attempt to combine five enzymes into a one-pot modular cascade for the transformation of cyclohexanol into the open-chain form of nylon-6 monomer. Initial design consisted in a combination of alcohol dehydrogenase and Baeyer-Villiger monooxygenase in Module 1, and a hydride-borrowing (H-B) combination of an alcohol dehydrogenase and amine dehydrogenase in Module 2. These two modules were connected by a hydrolase reaction. However, the H-B step had to be abandoned and an alternative design of the cascade utilizing alcohol oxidase instead of the alcohol dehydrogenase was devised. Interestingly, the use of the alcohol oxidase in the final cascade is connected to Chapter 2 of this thesis, since the combination of the alcohol oxidases and amine dehydrogenases was indeed one of the aims of the PhD project. Furthermore, the alternative design allowed us the completion of the cascade using seven enzymes in one pot. Moreover, different reaction set-ups for the maximization of product yields were investigated. In summary, we investigated the combination of alcohol oxidases and amine dehydrogenases. Furthermore, we capitalized on unexpected observations and challenges that we have encountered and, in turn, we developed: 1) a 3-D printing based agarose system that enables (co)entrapment of enzymes; 2) the first enzymatic method for the direct synthesis of nitriles from alcohols using ammonia; 3) we
elucidated the crystal structure of novel amine dehydrogenase; 4) we demonstrated the applicability of alcohol oxidase/amine dehydrogenase system in a modular multi-enzymatic cascade, thereby yielding 6-aminohexanoic acid (open-chain nylon-6 monomer) from cyclohexanol.
Samenvattig
Biokatalyse heeft zich de afgelopen decennia bewezen in waarde en potentie en zijn weg gevonden naar de chemische industrie. Het streven naar schonere en duurzamere methoden bracht het gebruik van moleculair zuurstof naar voren als efficiënt en overvloedig beschikbaar oxidatiemiddel. **Hoofdstuk 1** dient als een inleiding in de aerobe bio-katalytische methodes voor C-H activering en hydroxylatie, Bayer-Villiger oxidatie, oxidaties van alcoholen, aldehyde, amines, imines en alkeen oxidatiereacties. Het grote voordeel van biokatalyse is echter de mogelijkheid om meerdere enzymen in één pot te combineren. Het gebruik van dergelijke multi-enzymatische cascades maakt het mogelijk omschijn van tussenproducten over te slaan en zo de reacties op een meer economische manier uit te voeren. Een van de meest gewilde bio-katalytische reacties van de afgelopen jaren is de reductieve aminering, die amines oplevert uit carbonylverbindingen. Het cascadeproces, dat reductieve aminering omvat, begint vaak met de oxidatie van alcoholen door een oxido-reductase en vervolgens vindt de aminering van de resulterende carbonylverbinding plaats met behulp van aminerende enzymen. **Hoofdstuk 2** beschrijft onze pogingen om de twee klassen van alcohol oxidases en amine dehydrogenases te combineren. We hebben verrassende waarnemingen gedaan die voor nieuwe toekomstige uitdagingen hebben gezorgd. De belangrijkste hindernis was gerelateerd aan de opmerkelijke katalytische reactiviteit van alcohol oxidases. De flavine-afhankelijke alcohololoxidases vertoonden namelijk het vermogen om de over-oxidatie van alcoholen tot carbonzuren te katalyseren. Echter, de koperafhankelijke galactose-oxidase leverde, in de aanwezigheid van ammoniumionen, nitrillen op vanuit aldehyde. Verder gebruik van flavine-afhankelijke alcohol oxidases werd beperkt door de lage reproduceerbaarheid van de methodologie tot nu toe (d.w.z. aanzienlijke variatie van analytische opbrengsten van carbonzuren in onafhankelijke experimenten). Bovendien hebben we geprobeerd koper-afhankelijke alcohol oxidases in een flowproces te gebruiken. Terwijl de oxidatie van alcoholen tot aldehyde kon worden waargenomen, slaagden we er niet in om de koper-afhankelijke alcohol oxidases volledig te combineren met amine dehydrogenases in dit flowproces. Het onderzoek naar flowbiokatalyse bracht ons echter tot de ontwikkeling van een op agarose gebaseerde hydrogel die zowel een amine dehydrogenase als een formate
dehydrogenase bevat. Dit laatste enzym dient voor de recycling van de cofactor. Het onderzoek is beschreven in hoofdstuk 3. Een van de meest interessante en innovatieve observaties van alcohol oxidases is het bovengenoemde vermogen van een koperafhankelijke galactose-oxidase variant van Fusarium sp. om de vorming van nitrillen te katalyseren vanuit aldehyden in de aanwezigheid van ammoniumionen en in waterig medium. De ontwikkeling van deze verrassende observatie tot een synthetisch toepasbare methodologie voor cyanide-vrije amino-oxidatie van alcoholen (of aldehyden) met ammoniak en moleculaire zuurstof is beschreven in hoofdstuk 4. Met betrekking tot de doelstellingen van het proefschrift beschreven in hoofdstuk 1, hoofdstuk 5 stapt buiten de algemene focus op aerobe biokatalyse en behandelt eiwitkristallografie. Het hoofdstuk geeft inzicht in kristallografische methoden gericht op het ophelderen van de structuur van amine dehydrogenase variant LE-AmDH-v1. De variant was eerder ontworpen door een in-silico gegenereerd homologiemodel te gebruiken als basis voor de structuurbepaling. Dit werk leverde inzicht in de verschillen tussen de ontworpen amine dehydrogenases, i.e. de homoloog van de archaeon Pyrococcus horikoshii en een sacccharopine-reductase van Magnaporthe grisea. Deze gegevens zullen de basis vormen voor de verdere ontwikkeling van nieuwe AmDH's met vergelijkbare reactiviteit. Hoofdstuk 6 beschrijft, tenslotte, een poging om vijf enzymen te combineren tot een één-pots modulaire cascade voor de transformatie van cyclohexanol naar de open-keten vorm van het nylon-6-monomeer. Het oorspronkelijke ontwerp bestond uit een combinatie van een alcohol dehydrogenase en een Bayer-Villiger mono-oxygenase in module 1 en een hydrogen-borrowing (H-B) combinatie van een alcohol dehydrogenase en een amine dehydrogenase in module 2. Deze twee modules waren verbonden door een hydrolase reactie. De H-B-stap moest echter worden opgegeven en er werd een alternatief ontwerp van de cascade ontwikkeld waarbij een alcohol oxidase werd gebruikt in plaats van een alcohol dehydrogenase. Interessant is dat het gebruik van de alcohol oxidase in de laatste cascade verband houdt met hoofdstuk 2 van dit proefschrift, aangezien de combinatie van de alcohol oxidases en amine dehydrogenases inderdaad één van de doelstellingen van het doctoraatsproject is. Bovendien zorgde het alternatieve ontwerp ervoor dat we de cascade konden voltooien met zeven enzymen in één pot. Daarnaast werden
verschillende reactieopstellingen voor het maximaliseren van productopbrengsten onderzocht. Samenvattend hebben we de meest efficiënte combinatie van alcohol oxidases en amine dehydrogenases onderzocht. We profiteerden van onverwachte waarnemingen en hebben uit de uitdagingen die we tegenkwamen het volgende ontwikkeld: 1) een op 3D-printen gebaseerd agarose systeem dat (co-)immobilisatie van enzymen mogelijk maakt; 2) de eerste enzymatische methode voor de directe synthese van nitrilen uit alcoholen met ammoniak; 3) we hebben de kristalstructuur van een nieuw amine dehydrogenase opgehelderd; en 4) we hebben de toepasbaarheid van een alcohol oxidase/amine dehydrogenase systeem aangetoond in een modulaire multi-enzymatische cascade, waardoor 6-aminohexaanzuur (open-keten nylon-6-monomeer) uit cyclohexanol kan worden verkregen.
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It took exactly five years and two weeks from the time that I have first set foot into the buildings of Science Park, as a still slim baby scientist, to the date of the defense. I have gained centimeters around my waist and some facial hair but more importantly, grown as a person and a scientist and it would not be possible without the people that I have met along the way. This section is a brief tribute to those people.

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Interestingly, I have also managed to make some Dutch friends. Gaston, our bike rides (90 km on a city bike? Easy!), Little Collins campaigns and countless “Choufs” all over the Amsterdam were simply extraordinary. On the similar note, Marissa, you are just remarkable, it is always a pleasure to chat with you. Most importantly, you got me to learn salsa and bachata. I would have never guessed how much I would like it, really thank you! And Roel, I think took a bit of time to become friends, but when we did, you were always up for grabbing a beer or a gin-thonic (especially under the Rhodian sun).

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Děkuju.
List of publications

Publications (part of the thesis)


**Publications (outside the thesis)**


