Foreword

I am proud to present the final publication of the IBOS programme of ACTS (Advanced Chemical Technologies for Sustainability). You will find an overview of ten years of IBOS: the objectives we had and the results we accomplished. You will also find a look ahead to the future.

Integration of processes plays an increasingly important role during the development of sustainable and cost-effective production processes in the chemical and life sciences industry and is the central theme for the future of (bio)organic synthesis. Integrated processes contain elements from the triangle organic chemistry, biocatalysis and process technology. The researchers in IBOS aimed for innovation within this challenging triangle.

At the start of the programme, we dreamt about a (patented) toolbox for sustainable, efficient processes with which natural and non-natural substances desired by industry and society could be produced. About reaching new dimensions for both organic synthesis and bio-synthesis. For example new catalytic methods for organic syntheses, making a number of bonds simultaneously. Once-through concepts and in-situ product removal would become routine, while protective group chemistry, elaborate isolations and purifications would become obsolete. Also a much wider scope for fermentation was looked for, including non-natural products ranging from simple bulk-molecules to complex medicinals such as antibiotics, and finally new hybride syntheses integrating molecular biology and chemistry. Ten years, 24 projects and 13.5 M€ later, IBOS contributed considerably to reach these goals and to make traditional organic synthesis faster and cheaper and with less impact on the environment. We should be proud of these results. However, the scientific community should not stop dreaming, as there is still a lot of work to be done to make chemistry more sustainable.

The programme was special in another way. Not only the large chemical companies had the opportunity to participate, but we especially focused on Small and Medium Enterprises (SMEs) to join. We foresaw that, by including these companies, the focus in the research projects would shift from more basic research towards pure innovation. And as you can read in the interviews with Hans Kierkels (Isobionics) and Richard Blaauw (Chiralix), this is exactly what happened. Impressive results were obtained, which could be implemented directly.

A toolbox as such has not been developed yet within the limits of the programme, but I am sure that you will be impressed by the scientific creativity by academics, the expertise of chemical companies and the collective results they achieved. I believe that the results of IBOS will encourage you to make chemistry yet more effective and sustainable. Be inspired!

Dr Louis B.J. Vertegaal
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Let’s consider utilizing the complexity of molecules in biomass as feedstock

Alle Bruggink, retired professor and former DSM employee, was actively involved in founding ACTS and the IBOS-programme, of which he was the first chairman. During IBOS he saw several of the initial wishes coming true. In the meantime new dreams arose: to exchange traditional reductionalist thinking for the holistic approach as seen in nature.

During the nineties, DSM was the first in the world to apply enzymatic processes for the production of antibiotics. This resulted in both lower costs and less environmental issues. “The inspiration for the IBOS programme was the wish to apply this in a generic way”, Bruggink recalls, “and to integrate chemo-catalysis and bio-catalysis in fine chemistry. When IBOS was launched in 2002, the use of catalysis in fine chemistry was still in its infancy, as fine chemistry was dominated by stoichiometric processes.”

IBOS aimed to create a catalyst for every conversion and a micro-reactor for each catalyst. Bruggink: “These should be integrated into a cascade of synthesis steps. The ultimate goal was to cut production costs by half and to speed up development of complex molecules for the pharmaceutical industry. We envisaged ‘one time right’ processes, in which the desired compound is produced in a set of micro-reactors, after it is designed on screen. This involves far-reaching automation and miniaturisation: after giving the right command, the computer automatically selects the right catalysts and reactors to produce the first micrograms. This substance can subsequently be tested for its capabilities as a lead compound.”

Dramatic improvements

So did this dream come true? “Yes, to a surprising extent it did”, says Bruggink, “within ten years we have seen catalysis becoming fully integrated into fine chemistry. For a lot of conversions we now have a catalyst. Miniaturisation, chemistry-on-a-chip has become reality on the factory floor. Even the first cascade processes have been reported, including catalytic reactions without intermediate isolation or purification steps. The efficiency in feedstock utilization has improved dramatically, resulting in a similar dramatic fall in the amount of waste that, moreover, is far less harmful.”

He would not suggest this to be only the merit of IBOS, as elsewhere huge progress was made as well. “But IBOS certainly stood in the middle of these events. The Netherlands were first ten years ago, and still are on the forefront of academic and industrial developments.”
The excellent ‘Dutch school of catalysis’ still exists in petrochemicals as well as in fine chemistry.”

Holistic approach
At this point, research is approximately halfway from having a micro-reactor for each catalyst, and cascade development is even further from completion. “There still is much work to be done for 2020 and beyond”, Bruggink says. “We still need a full programme to make lead compounds in the way described above.
In the meantime, new dreams have come up. An important goal is to embrace a holistic approach in chemistry, in order to get a grip on the complexity of biomass. During our 150 years of crude oil tradition, we have become drenched in reductionalist thinking. We have grown accustomed to reducing the feedstock to a limited amount of molecules as building blocks. From there we built end products like a Lego building. We followed the same approach towards biomass. There is some logic in that, as long as we focus on using the same installations.”

“In order to become fully fledged conversation partners it would be good if companies and universities would work on the same research topics. Competition between project partners should be allowed.”

Nature shows another option, says Bruggink. “Are we able to utilize the functionality of complex molecules in the feedstock to convert it directly into end products? Nature can achieve this in four or more enzymatic steps. The agro-food industry, possibly our new partner, inhabits a world without reductionalism. A farmer starts with highly complicated seeds and grows complicated crops from these. Nature is able to manage this complexity; we can also aim for that. Therefore the next phase could be chemical synthetic biology.”

New ways of cooperation
To get to the next phase, Bruggink proposes an extra dimension in cooperation between academia and industry. “Industrial participants in PPP-projects should work in-house on the same research topics as they do in the collaboration with the academia. This in-house research could be capitalized and count as in-kind contribution to the PPPs. So far only SMEs are allowed to pay their contributions partly in kind. But in kind contributions should be preferred over cash contributions. This will stimulate interaction and development speed. Results would bubble up all around, as several examples have already shown us.”
Semi-Synthetic Cephalosporins (SSCs) are an important class of antibiotics. The current production methods rely on a combination of a biological fermentation with a fungus followed by a series of complex, noxious chemical steps. The costs and environmental impact of this process are significant. Now, using fungi and their enzymes, a new ‘biological factory’ for the fermentative production of a novel cephalosporin has been developed, which is much less polluting than classical chemical synthesis routes. Further optimization of this process will result in new production methods significantly more sustainable and cost-effective than the currently used technologies.
Dick Kellogg, scientific coordinator at Syncom BV and the current chairman of the IBOS programme committee, observes that significant changes have occurred in both the scientific landscape and science policies. Despite these developments, IBOS was still able to reach the majority of its goals.

“I am very content with the results of the projects I was involved in as a member of the programme committee.” Kellogg states. “These were the Industrially Relevant Heterocyclic Compounds project, led by professor Rutjes in Nijmegen, and the BIOMOX project, led by professor Fraaije in Groningen. The scientific content of these projects was impressive, which is essential for the success of the programme. The link with industry was credible. This has not led to direct financial returns for Syncom as industrial participant. We hoped, of course, for short term benefits but realised that these were less likely than long term ones. Participation in these kind of programmes entails long term knowledge building and investment in talent. This is indispensable for knowledge driven companies: knowledge is power. We were happy to see a patent application by DSM based on an IBOS discovery.”

Syncom was not the only one to benefit: “All the participating parties have gained knowledge and insight. The IBOS programme has scored well in terms of publications in high-rated scientific journals, although the actual intellectual and economic value of this knowledge can be assessed probably only after about ten years. At a maximum only about ten per cent of new technology eventually ends up in an economic process. That is about the best one can expect. It is possible to attempt to measure future impact, but extrapolating the results remains a highly subjective process. For this reason, I am sceptical about these kind of impact measurements.”

Difficult times and what to do
Recent developments seem to be detrimental for organic biosynthesis and pharmaceutical research, Kellogg observes. “The pharmaceutical chemistry in the Netherlands has in recent years become smaller owing to negative economic developments. Current policy is aimed more at strengthening the strong, hence little aid in the pharmaceutical sector may be expected. Knowledge remains, however, a key to success. It is noteworthy that, despite unfavourable economic developments, IBOS has managed to educate young people for a future career in either
companies or universities. In the Netherlands, we have a solid university infrastructure and Dutch chemistry has an excellent reputation. Let us hope that the existing industry will be able to offer sufficient employment, so that the country will benefit from the PhD students now being educated. It would be a pity if they had to go abroad owing to lack of job openings in the Netherlands, taking their skills with them.”

**From multinational to start-ups**

“If we had known then what we know now, probably there would not have been a second phase in the IBOS programme. Perhaps it was better we did not know. Otherwise we would never have achieved some impressive results. The second phase of IBOS was set up with Organon, DSM and Syncom as industrial partners. Organon does not exist anymore, Syncom had to reorganize because of the recession, and DSM changed its priorities. All in all, organic biosynthesis and pharmaceutical research in the Netherlands suffered a serious blow, and, of course, this had its effect on IBOS.” However, there is still hope for pharmaceutical research in the Netherlands: “It is a promising development that several Organon employees decided to join forces and formed SMEs to continue their research. Maybe they can find inspiration in the outcome of the dismantling of Fokker. This was expected to be the end of Dutch aerospace. Actually, new, specialized companies have been founded based on Fokker knowledge. Now this sector is doing better than before. I think that start-ups in pharmaceutical chemistry would benefit from support in programmes such as IBOS, although in the current environment it will be difficult to start such programmes again.”

**Commercial harm or profit?**

So what makes a public-private-partnership (PPP) like IBOS successful? Kellogg: “The success for participants in a PPP largely depends on sound project tuning before starting the project. Although this research is in principle pre-competitive, direct competition is not always avoided and this makes cooperation far more difficult. I do not feel that we had problems of direct competition in the second round of IBOS. The planning was sensible and we avoided that problem. For me a ‘credible link’ means that contacts were good. Programme committee meetings were well organized, PhD-students were motivated and well prepared for high level discussions. Knowledge transfer was smooth via open and easy communication and not complicated by political games. Contacts between university and academia were also pleasant, based on mutual understanding of each other’s roles.”

For Kellogg, the value of PPP collaboration is clear. He is concerned, however, by the blurring of the line between university and business. “Universities have educational and research tasks that are different from business considerations. During university study young people should have the opportunity to think openly, unreservedly and fundamentally. Making some mistakes is part of learning. So is learning how to make fewer mistakes. A good scientist should learn how to think, how to ask questions and how to dream. The university needs to be able to provide an opportunity for free thought. This is, in my opinion, crucial both in terms of personal development and in terms of future capabilities. When young talent is limited to purely commercial research from the start, the added value that distinguishes academic training from higher vocational education may be lost. Only after their university training, should talented young people, who are used to original thinking, start work in companies and perform commercial research. I know that this is a minority standpoint, but I do not like the idea of the ‘entrepreneurial university’. Mixing of tasks will probably do more harm to the present cooperation than to stimulate it. Advocates of the entrepreneurial university often refer to MIT and Harvard. They tend to forget that these universities for the greater part invest the income generated by commercial activities in free, purely fundamental research. They use their profits to buy academic freedom. When freedom is sacrificed for commercial research, you do exactly the opposite.”
Ideally, the synthesis of a complex molecule can be achieved in a single step starting from renewable sources and without producing any waste. This can be achieved by broadening the scope of enzymes that are able to catalyze the formation of C-C bonds, and by integration of synthetic steps into one-pot cascade processes. Using a one-pot, four enzyme-cascade process, it is possible to prepare several enantio- and diastereomERICALLY pure carbohydrate derivatives, which in turn can be converted into biologically relevant compounds. All these cascades are carried out in water, make minimal use of protecting groups and proceed in a highly selective manner, thereby fulfilling important green chemistry requirements.
Added value works both ways

“The starting point for IBOS is knowledge of corporate interest”, Marco Fraaije of the University of Groningen explains. “This means that companies have in-depth knowledge of the field and that they are actively involved in the project.” In addition to that, according to Ron Wever of the University of Amsterdam, they add specialized knowledge: “It often concerns knowledge that no one else in the Netherlands has. Corporate specialists can discuss matters thoroughly and indicate bottlenecks. The input from the companies is therefore highly important.”

“It is all about applying scientific knowledge. From the start there is an interaction with the routines in a company”, as Wever describes the IBOS-approach. Fraaije sees the corporate involvement also as an opportunity to get a glimpse of the other end of the knowledge chain. “Our role lies at the start of the chain and it is relevant for us to hear about corporate research issues and about daily production practice.” Moreover, corporate partners can carry out certain analyses that are not easily accessible to the university - and the other way round. The same is true for materials: partners can provide compounds that are hard to obtain. Wever confirms: “In our first IBOS-project, we were able to get a hand on certain steroids for our experiments. In the latest project with DSM and Syncom we were supplied with plasmids harbouring genes that we needed.”

Stimulating contacts
It can be highly motivating for young researchers to contribute to present or future production processes. Wever: “It can be beneficial to their career. A joint research programme puts researchers in the middle of things, the contacts are already there.” That is convenient when there is a job opening.

“Collaboration with people who apply scientific knowledge in practice is stimulating”, says Fraaije. “Gradually, young researchers get to know what working in a company is like. Several of our former researchers are now employed at biotech companies, both IBOS-partners and non-IBOS-partners. Both for us and for the companies, this definitely is an added value. One can say that about added value in general; it works both ways.”
Companies are curious to know what research is performed in the academia. Fraaije: “They pursue their own research strategy, which is not always about paying attention to promising new developments. In academic research we can elaborate on these developments to our mutual benefit and sometimes this even offers opportunities for further research outside the scope of the programme.”
This mechanism works the other way round as well, Wever knows: “Through our participation in IBOS-II
we got acquainted with other groups and learned about the activities within DSM. IBOS-SME brought us, via the group of professor Floris Rutjes at the Radboud University Nijmegen, useful contacts with several groups at Wageningen University.”

**Mutual understanding**

Success and efficiency in PPPs depend on adaptability, say both Fraaije and Wever. “As a research group, we are application-oriented”, says Wever, “Via STW-projects we have accumulated a lot of experience in collaborating with companies like DSM and Unilever, which is useful. Programmes like IBOS have a clearly targeted approach, that make it stand out from other research. You hold on to the original idea; this can prove to be a benefit as well as a drawback.” Fraaije: “From a practical point of view, we have exchanged protocols and materials. However, both parties should understand the working routine of the other party and should respect each other’s interests. For instance, publications have to be planned carefully, because the companies have to read and approve of the draft publication first. When you understand that, there’s also the chance of a bonus such as a patent. We had a patent in IBOS-II and are currently investigating a further patenting opportunity.”

**Trusting each other**

Wever remarks that pleasant contacts are not the only success factor. For instance, the latest project alone resulted in four publications. “The value of contacts can be a deliverable, but it does not equal scientific success. In the SME-project we were involved in, there was not much contact with the SME-partner. The assignment was to investigate an alternative route for a synthetic process. We found an enzymatic route that turned out to be successful and publishing these results was an obvious scientific success.”

Within the scope of a programme, trust is very important. Fraaije: “Of course a company cannot inform you about all its activities. But trust in a good, dynamic interaction more or less defines how far you can get together.” Wever: “That is exactly my experience. It does not work in all projects, but sometimes you can bring each other a lot further. Therefore I am very happy with IBOS. It has contributed to making the academic life more interesting.”
Artemisinin is an important anti-malarial drug, which has the potential to also be developed into drugs against other diseases such as schistosomiasis and cancer. However, the supply of artemisinin is troublesome as total synthesis is not feasible and the only plant species known to produce artemisinin, *Artemisia annua*, contains only low amounts of this compound. The suitability of using alternative plant sources for the production of artemisinin, such as industrial chicory and the tobacco species *Nicotiana benthamiana*, has been investigated. It was shown that a precursor of artemisinin could indeed be produced in *N. benthamiana* and this process will be transferred to chicory.
Collaboration is the name of the game

“There were various degrees of success in the IBOS projects”, says Rinus Broxterman, corporate scientist at DSM and member of the IBOS programme committee. “...as might be expected in any scientific programme”, he adds. For Broxterman, the measure of success lies not only in the number of patents or direct potential financial value, but mostly in the soft deliverables. “These are not always obtained either, but collaboration is what we are good at. This is what we need to maintain and to improve, to the benefit of the Dutch knowledge economy.”

DSM has gained useful scientific insights from the IBOS programme, but according to Broxterman it is too early to comment on actual applications. “One does not always realize that the proof of principle is not the end, but rather the start of a long and winding road. The invention is only ten per cent of the process. Ninety per cent of the work still has to be carried out, before the application is a commercial success”, Broxterman comments. “This fact is by no means an underestimation of the value of inventions. They create the options you need: there is no route without a beginning.”

Another popular misconception involves clearly negative scientific results. As opposed to expectations, this can be a very positive project result for a company. Broxterman explains: “The exploration of a certain synthetic route could indicate that this is not the right direction to go. The concept of multi-enzyme cascade synthesis in the ‘Industrially relevant heterocycles through biocatalytic cascades’ project is an example of this. The concept proved to be viable on a lab scale, which is a great scientific achievement. However, aligning and matching the activities and stabilities of all enzymes in the cascade of four enzymatic steps is just too complicated for use in a routine manner. For the researcher this might be disappointing, but it allows us to close this concept for the short term and to save further R&D investments. A perfect outcome on the basis of good science!”

On the other hand there is the ‘Biocatalytic exploitation of mono oxygenases’, in short the BIOMOX project, where Broxterman considers parts of it, as successful. “The concept of smart libraries of enzymes for our work on bio-oxidation was reinforced within DSM as a result of this project. The post-doc that carried out the project is now working at DSM. Both projects are successes, but in a different way.”

Real collaboration

“This was the science part, but pre-competitive PPP programmes should not be judged solely by counting patents or direct cash value”, Broxterman emphasizes. “It is all about collaboration within a community, on the individual intellectual and personal level. It is about soft skills, cooperation on a basis of trust. In this respect, we have seen different situations. With one
research group we are having a difference of opinion on the ownership of certain strains that were developed in the project. We still haven’t got them. On the other hand, another research group thought along very well with us during the IBOS project, was flexible and well-aware of our position. We were so satisfied with the collaboration, that we are now involving some of the researchers from the ‘Chemo-enzymatic peptide synthesis’ project as consultants in a confidential internal programme. This shows quite a difference in attitude. We both know that these researchers cannot use their work with us on their citation indices. We agree though that this approach leads to added value for the academia too.”

Broxterman believes that, in general, a consortium will lead to better results than solitary research: “Real collaboration is pivotal for the Dutch knowledge economy in the long run.” He also points out another network aspect: PhD-students getting to know companies and vice versa. “It all contributes to a flexible knowledge chain. In the Netherlands, we can be proud of the PPP infrastructure that has been created. This unique cooperation holds high value; we should be keen on preserving this achievement. There will be no sequel to the IBOS-programme, but fortunately there will be the new opportunities provided in the Topsector Chemistry and the TKI (Top consortium for Knowledge and Innovation) New Chemical Innovations.”

Natural law

However, Broxterman is convinced that there is a certain optimal size to a consortium: a maximum of three academic partners with no more than three industrial partners. “It is almost a natural law: the larger the consortium, the more important politics will be. There has to be something in it for every partner. The larger the group, the harder it gets to attain that goal. Also, the time management of work packages is more difficult to manage in larger consortia. A project plan can be well thought through, but reality is harsh. So you might end up with a PhD-student who has to work with an enzyme that does not exist yet, because another PhD-student is behind on schedule, or has not been appointed yet.”

Consortia do not only revolve around collaboration between industry and academia, but also on collaboration between academic research groups. “This point deserves attention”, Broxterman remarks. “The future lies in integration of multidisciplinary research lines; real inventions occur on the interfaces between scientific disciplines. Therefore it would not be such a bad idea to include the ability to collaborate as a selection criterium for scientists. Neither universities nor companies have put enough emphasis on this competence yet.”
Carvone is used as anti-sprouting agent for potatoes. The current production method, extracting carvone from caraway oil, has several drawbacks: it is very inefficient, requires significant cropland and large amounts of fertilizer are needed. Therefore, carvone is an interesting target for an industrial biotechnological process. The aim was to develop a bacterial strain that is capable of producing (+)-carvone from D-limonene, a waste product from the orange juice industry and the main component of the oil in orange peels. A recombinant Pseudomonas strain has been shown to efficiently convert limonene into carveol, which can be further converted to (+)-carvone. This can be used in a biotechnological process for carvone production.
Eric Keller is a product manager in metabolite synthesis at Syncom in Groningen. He qualifies the collaboration with the academia in his project as relatively limited. “Overall results have been disappointing for us. We were very interested in the central IBOS-theme, the integration of enzymatic routes in organic chemistry. However, in practice we have not seen much of this because the original research plan was changed after disappointing results. For a small contract research organisation like us that is a pity, because we have to be picky about our external research investments. Scientific results have to be closely related to practical application. Larger organisations have a less limited approach towards deliverables. For them knowledge on a ‘hot topic’ can be an important deliverable.”

Pleasant and high level
To senior scientist Bernard Kaptein from DSM this remark is spot-on. “My colleague Martin Schürmann was involved in the successful BIOMOX project, which resulted in a patent application. Together, we were also involved in the Heterocycles-project, in which carbon-carbon bond formation through enzymatic reactions is followed by ‘conventional’ organic synthesis. This is a vastly complex multi-disciplinary project. The main topic involves five different enzymatic reactions, of which four reactions, with three different enzymes, are on the synthetic path to the product. From an organisational point of view the project was interesting too. Four research groups from various disciplines in three universities participated in the project. With their different backgrounds, the specialists involved were able to turn the project into a successful collaboration.”

Both scientific content and organisation of research asked for careful fine tuning, says Kaptein: “Collaboration and discussions were pleasant and high level. Unfortunately, the overall conclusion was that, because of the different characteristics of the individual enzymes, it is still too difficult to adjust all necessary enzymatic activities to get satisfactory yields. Currently the whole process is still too complicated for practical application. But we knew from the start that we were embarking on a highly challenging, exploratory subject. It was too high risk to include it in our in-house research. Also, four years is a too long a period
for in-house exploratory projects. That made it an almost ideal subject for PPP research."
So did the Heterocycles project generate economic value like BIOMOX did? Kaptein: "No, but it was definitely worthwhile. We gained important insights and we learned in which direction we should find the solution. This indirect deliverable is important enough."

Sideways
On the other hand, Keller explains why his project was disappointing: "Our influence on the research was rather limited. We are partly to be blamed for this. We were clear about our deliverables right from the start, but we did not define the route towards them. Personally, I became involved at a late stage. Research was well under way by then, and the university researchers were on a certain track."
One research group was involved in very fundamental biology research, far from Syncom’s own expertise. “There was no connection with organic chemistry”, Keller recalls. “The other group had a more interesting research topic for us. We supplied them with the relevant p450 molecules, and it turned out that the conversion into the desired active metabolites was too low. After that, we did some recommendations for a sequel, but the researcher probably changed the original research plan once problems arose. This is understandable when you know how PhD-research works. When the original idea does not work, you bend the mission in a direction more likely to succeed. However, the new research plan was not of use for Syncom. Nature can make the desired conversion in one step, but it appears that the same process is still too complicated to accomplish in the lab.”

Focus on communication
If Syncom would participate again in such a programme, things would be managed differently. Keller: “We would connect our deliverables to clear deadlines and go/no go-decisions. We learned that it is hard to succeed when there is no link between the research interest of participants and our own activities. That is the main difference between our project and more successful projects.”
Kaptein agrees. “It is important that we perform comparable research, but preferably not in such complex routes.” DSM was actively involved and made sure that the research proceeded in a favourable direction. “When you keep your distance, the project will find its own direction and nothing will come out for the industrial party. To prevent this, we had three to four annual project meetings, organised by one of the participating groups. All along, we could communicate with each other very well. That is exactly what is necessary for PPP research and for this reason I consider IBOS to be an example for other PPP projects.”
Nature is much more efficient than man in the preparation of complex molecules, as it can perform a series of different reaction steps in the right order, and without problems of interference of other reactive sites. Nature’s possibility to perform multistep cascade reactions in one reactive environment can be used to develop new catalytic processes for the production of industrially relevant compounds. Bearing this in mind, several new concepts for synthetic chemistry have been developed, such as two-step, one-pot cascade reactions, the construction of compartmentalized reaction environments for the positional assembly of (bio) catalysts and the modification of enzymes to immobilize them non-covalently on supramolecular supports.
Chiralix in Nijmegen is a contract research company specialized in chiral synthesis. The company participated in a regular project and in a SME project. “The integration of biocatalysis in organic synthesis is our core activity, so it was a logical step to participate”, Managing Director Richard Blaauw explains. “We have high expectations of these kind of projects, but we do not expect that we can apply the results directly. The value lies in acting on the forefront of academic development, in building up expertise and in expanding and sustaining your knowledge network. Results from both projects lived up to these expectations.”

Isobionics, a production organisation in biotechnological fermentation of flavours and fragrances, started as a spin-off from DSM in 2008. “Just as for Chiralix, the IBOS goals are core business for us”, says Project Manager Biotechnology Hans Kierkels. His academic partners are located in Wageningen and Amsterdam. He is straightforward about the project deliverables: “Intellectual Property (IP) is not our main goal. It is nice when IP is generated, and in our case it was. Unfortunately, in the present setting a patent was not an option. Still, the IBOS knowledge is worth ten to fifteen times our initial investment. Not immediately, of course; it is now up to us to further develop the results”

Chemistry in collaboration

Blaauw notices a difference between collaboration with Nijmegen University and with the NKI in Amsterdam. “In both projects we contributed materials for the researchers to use. The NKI project involved beautiful technology, with special molecules built into peptides, that bind to tumours. They are used in immune therapy for cancer patients. But there was no face-to-face contact with the researchers as compared to the Nijmegen project; we were intellectually less involved.” On the contrary, communication in the Nijmegen project was open, frequent and effective. Contacts with a Nijmegen group were intensified and new contacts with a Wageningen group were built up. “Even in a small country like the Netherlands, it appears to be important being closely located to your cooperation partners.” For Isobionics on the Geleen Chemelot Campus,
Wageningen was not that close. But collaboration was close, even with Isobionics’ research partners in Switzerland and Austria. This collaboration will be continued. Kierkels: “Effectiveness in partnerships is a two way street. Something works best when both parties put in the same amount of effort. When you are only absorbing knowledge, it is not half as effective. The better your relationship and the better the project is embedded in your organisation, the higher the chance of success. We got an outstanding scientific output, so we continue research on a bilateral basis. It explains why we praise the networking function of IBOS.”

Chiralix was also closely involved in the research, Blaauw comments. “We presented our vision and mentioned the potential added value in certain aspects. But we also wanted to respect the unbiased character of scientific research and tried to influence the researchers as little as possible.” Kierkels agrees: “Our influence was satisfactory. But I think it should only be deployed for the sake of scientific argumentation and not for dominant commercial reasons.”

Highly relevant knowledge

“The decision of NWO to stimulate SME participation is a big step forward”, Blaauw concludes. “Innovation happens much faster in smaller companies. Large companies have more financial power, but the focus of NWO lies on knowledge building. In that respect, consortia of universities and SMEs are valuable. More projects like these would be highly appreciated, preferably in a bilateral setting without complex contracts. This way, the added value of the innovation drive at SMEs could be mobilized. In large companies research often supports production. On the other hand, research driven SMEs strive for new technologies to realize added value in the future. This fits perfectly to academic goals.”

Kierkels fully agrees. “For large companies the staffing angle is the most important asset in PPP-projects. They often complain about the gap between students and business. Therefore, I would like to appeal to these companies: take your responsibility and participate in PPP-programmes. They are a fantastic instrument to bridge the gap.”

Stimulation of the innovation capacity of SMEs and of academic research can go hand in hand, Kierkels: “The knowledge yield and added value for us has a lot more impact than for large companies, where deliverables might become relevant at some point. For us the knowledge gained was highly relevant right from the start. It led to better production processes and an entirely new product. Our scientific partners had a fundamentally different view at enzymes than we had. In one or two years’ time, we will be able to profit from this collaboration. And we noticed this works for researchers too. The economic relevance of their work is highly motivating for scientists.”
Facts & Figures

Number of projects

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Total budget

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Output (October 2012)

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At the IBOS Open Conference, almost a hundred researchers witnessed the latest developments in enzymatic and organic synthetic methods for peptides, chiral amines and alcohols. With these synthetic methods, complex products can be synthesized that contribute to a sustainable future with less waste generated from their production.

NWO-ACTS compiled a very attractive programme, consisting of renowned international scientists, among which Pere Clapés from Barcelona, Roderich Süssmuth from Berlin and Kurt Faber from Graz. The Dutch research in this field was represented by Jan van Hest, Hermen Overkleeft and Alan Rowan.
Patent (applications)

1. Chemoenzymic coupling reactions (resulted from a project of Jan van Hest ‘Nanosized multicatalytic objects for cascade reactions in multistep synthesis’)
2. Improved Cephalosporin Production (resulted from a project of Arnold Driessen ‘CefFerm’)
3. P450 BM3 mutants and their use for regio- and stereoselective hydroxylation of alpha-ionone (resulted from a project of Marco Fraaije ‘Biocatalytic exploitation of monooxygenases (BIOMOX)’)

Participating companies

- Applikon B.V.
- Chiralix B.V.
- CLEA Technologies B.V.
- Dafra Pharma International
- Diosynth B.V.
- DSM
- Encapson B.V.
- Enzyscreen B.V.
- Friesland Coberco Dairy Foods
- ICI
- Isobionics B.V.
- MSD
- ProSensa B.V.
- ProteoNic B.V.
- Sanquin Reagents
- Solvay Pharmaceuticals
- SyMO-Chem B.V.
- Syncom B.V.
- Synthon B.V.

Project meetings

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Chairs programme committee

- Prof. J.A.M. (Jan) de Bont (2003 - 2007)
- Dr M. (Marcel) Schreuder Goedheijt (2007 - 2011)
- Prof. R.M. (Dick) Kellogg (2011 - 2012)
Nanosized multicatalytic objects for cascade reactions in multistep synthesis

The aim of this research program was to combine nature’s possibility to perform multistep cascade reactions in one reactive environment, with synthetic chemistry to develop new strategies for the production of industrially relevant compounds.
Results
The aim of this research program was to combine nature’s possibility to perform multistep cascade reactions in one reactive environment, with synthetic chemistry to develop new strategies for the production of industrially relevant compounds. Two model two-step, one-pot cascade reactions were investigated. The first system combined an enzyme-catalyzed halogenation of aromatic substrates (using vanadium haloperoxidases) with a Pd coupling reaction. The second cascade consisted of an enzymatic stereoselective amidation, combined with a Pd-catalyzed amination or Heck coupling to an aryl compound. Especially the latter cascade reaction proved to be very interesting, since an unexpected synergistic effect was observed between the two reaction steps, which led to a strongly enhanced reaction rate and selectivity in reaction order. Furthermore, it was observed that different optimal conditions were obtained when the two reactions were performed separately, as compared to when they were combined in a one pot reaction. This line of research was an intensive collaboration between the UvA and RU groups, and has led to a patent application (with Synthon), the ACTS innovation award (in 2005), and a publication in Org Biomol Chem.

A second line of research focused on the construction of compartmentalized reaction environments for the positional assembly of (bio) catalysts. In Nijmegen a new method was developed that enabled the controlled positioning of enzymes in the interior or in the bilayer of polymeric vesicles. This methodology has been patented and published in Angewandte Chemie International Edition. As a final line of research, enzymes have been modified to immobilize them non-covalently on supramolecular supports, in order to bring them into close contact with transition metal catalytic systems. This was a collaborative effort between Nijmegen and Eindhoven and this subproject was the final part of the program, and was finished by the end of 2005.

In conclusion, it can be stated that this fundamental program has succeeded in developing novel concepts that can be applied in synthetic chemistry, as is shown by the two patent applications and the ACTS innovation award.

Project leader
- Prof. J.C.M. (Jan) van Hest (RUN)

Co-applicants
- Prof. J.N.H. (Joost) Reek (UvA)
- Prof. A.E. (Alan) Rowan (RUN)
- Dr N.A.J.M. (Nico) Sommerdijk (TU/e)
- Prof. R. (Ron) Wever (RUN)

Researchers
- Alfonso Caiazzo (Postdoc, UvA)
- Mark Lambermon (Postdoc, RUN)
- Paula Leandro Garcia (Postdoc, RUN)
- Nicholas Millot (Postdoc, UvA)

Duration
- 2003-2007

Budget
- €379

Highlights
- ACTS innovation award in 2005.
Nitrogen heterocycles from aldehydes via biocatalytic cascades

Integration of oxidizing and C-C-bond forming enzymes in a single reaction flask leads to cascade processes that bring us somewhat closer to realizing efficient and sustainable processes. In this way, synthetically versatile so-called cyanohydrins have been prepared, which served as starting materials for a variety of biologically relevant heterocycles.
> Results
The production of new chemical entities has to proceed in an increasingly cleaner, faster and cheaper way due to economic and environmental constraints. Ideally, that would lead to the synthesis of a complex molecule in a single step, starting from renewable sources and without producing any waste. But it is obvious that this goal cannot be easily reached.
However, by broadening the scope of oxidizing enzymes, and of enzymes that are able to catalyze the formation of C-C-bonds using directed evolution techniques, we might be able to develop novel, sustainable and industrially viable synthetic pathways to versatile enantiomerically pure heterocyclic compounds. These are relevant for the fine chemical and pharmaceutical industry. Finally, by integrating the oxidizing and C-C-bond forming enzymes in a single reaction flask, cascade processes will occur that could bring us closer to the ideal one step synthesis.
In this project, novel enzymes have been identified that can catalyze the oxidation of organic molecules and other enzymes that are capable of catalyzing the formation of C-C-bonds with high selectivity on a variety of substrates. Both enzyme classes have been combined to realize one-pot cascade reactions: on one hand a proof concept was successfully realized in this way, but on the other hand it also has become clear that several hurdles still need to be overcome to turn these cascades into efficient processes.

> Project leader
- Prof. F.P.J.T. (Floris) Rutjes (RUN)

> Co-applicants
- Dr M.C.R. (Maurice) Franssen (WUR)
- Prof. A.P.G. (Tom) Kieboom (UL)
- Prof. J. (John) van der Oost (WUR)

> Researchers
- Rutger van den Berg (Technician, WUR)
- Maud Cabrières (PhD, WUR)
- Marloes Schurink (Postdoc, UU)
- Arjan Siebum (Postdoc, UL)
- Marloes Wijdeven (PhD, RUN)
- Roel Wijtmans (PhD, RUN)

> Duration
- 2003-2009

> Budget
- k€ 866

> Highlights
- Presentations at various national and international meetings, eg: Marloes Wijdeven, Chemoenzymatic Routes to Bioactive Natural Products, University of California, Davis, USA, October 10, 2007.
- The results of this project were instrumental in realizing an NWO/WOTRO financed collaboration with Prof. Chibale (University of Cape Town, South-Africa) on febrifugine based antimalarial compounds.
Use of sulfatases in the production of sulfatated carbohydrates and steroids

An enzymatic method has been developed which allows the synthesis of a variety of sulfated steroids, phenols and non-phenolic alcohols. The unique sulfating enzyme is a promising tool in biotransformation processes, providing a green and simple method to specifically sulfate compounds, without need for side group protection.
Results
Sulfated compounds are of great importance to many physiological processes and many sulfated compounds are produced as drugs. A well-known example is heparin sulfate, a sulfated polysaccharide that plays an important role in blood coagulation. Because of the great biological relevance, there is growing interest in the synthesis of sulfated molecules. Sulfated compounds are in general synthesized by the pharmaceutical/chemical industry using complexes of sulfur trioxide with tertiary amines or amides. However, use of these reactive reagents suffers from numerous disadvantages, such as the harsh reaction conditions and lack of reaction selectivity. Additionally, in order to prevent side reactions of labile functionalities and to enhance the chemo- or regio-selectivity of the overall reaction, these functionalities have to be protected adding complexity to the synthesis. Nature uses enzymes to sulfate compounds under mild conditions and the possibility to use enzymes to specifically sulfate compounds may have clear advantages.

In this project the use of an arylsulfotransferase in the synthesis of sulfated carbohydrates, alcohols and steroids was explored. A suitable enzyme was found in the bacterium Desulfitobacterium hafniense and after expression in a host and purification the enzyme was successfully used in the sulfation of many compounds using p-nitrophenylsulfate as a cheap sulfate donor. Because of its stability and its resistance towards organic solvents this unique enzyme is a promising tool in biotransformation processes, providing a green and simple method to specifically sulfate compounds without the need for side group protection.

Project leader
- Prof. R. (Ron) Wever (UvA)

Co-applicants
- Dr F.L. (Floris) van Delft (RUN)
- Prof. H. (Henk) Hiemstra (UvA)

Researchers
- Martijn Huibers (PhD, RUN)
- Johan van Lieshout (Postdoc, UvA)
- Harald Ruijssenaars (Postdoc, UvA)

Duration
- 2003-2008

Budget
- k€ 379
Highly functionalised enantiopure epoxides and epoxide derived products by cascade bio- and homogeneous catalysis in table-top reactors

Using centrifugal separators to perform two-phase catalytic reactions allows continuous recycling of the expensive catalyst, thus making the reaction more economic. The concept works both for metal catalysts and enzymes. A combination of these catalysts was also used to produce intermediates for pharmaceuticals.
Results
Modern drugs tend to be very expensive as a result of their complicated structures. The production process often consists of many steps. On average it takes ten synthetic steps to make a drug; syntheses of twenty steps are not exceptional either. Mostly stoichiometric chemistry is used, leading to a lot of waste. An additional problem is the occurrence of many drugs into two forms that are mirror images of each other. Since only one of these is active and the other one may even be harmful, they need to be separated using complicated crystallization procedures.

In this project we wanted to solve these problems by developing new catalytic methods for the production of drug intermediates, that immediately give the desired form and not the mirror image. A problem with the use of catalysis in fine chemicals is often that the catalysts are slow because of the complicated structures of the reactants. This makes their use too expensive. We have been able to solve this problem by performing these reactions continuously in two phases – catalyst in one phase, reactants and product in the other- that are first mixed very well to allow a reaction and then rapidly separated by a centrifuge. This enables a continuous reuse of the catalyst and leads to more turnover numbers. New enzymes and metal catalysts were also developed, that were used in a single reaction to make epoxides, important pharmaceutical building blocks in only the desired form without its mirror image. Another class of drug intermediates could be produced by using “designer” cells that contained two different enzymes. They were used in a process in which three consecutive reactions occurred.

Project leader
• Prof. J.G. (Hans) de Vries (RUG)

Co-applicants
• Prof. B.L. (Ben) Feringa (RUG)
• Prof. H.J. (Erik) Heeres (RUG)
• Prof. D.B. (Dick) Janssen (RUG)
• Prof. A.J. (Adri) Minnaard (RUG)

Researchers
• Florian Berthiol (Postdoc, RUG)
• Arnaud Gayet (Postdoc, RUG)
• Robert Haak (Postdoc, RUG)
• Gerard Kraai (PhD, RUG)
• Vincent Ritleng (Postdoc, RUG)
• Chiara Tarabiono (PhD, RUG)

Duration
• 2003-2009

Budget
• € 983

Highlights
• Discovery, cloning and expression, and crystallographic structural analysis of a new sugar oxidase. This enzyme, alditol oxidase, now is the best characterized flavoprotein oxidase on the market.

• Discovery and detailed analysis of several new oxidative enzymes by database genome mining and environmental gene library screening, illustrating the relevance of genomics for biocatalysis.

• Several well-cited reviews and primary research publications were published.
Selective redox biocatalysts for oxidative activation of biomolecules (BIOREDOX)

Discovery of new enzymes for green chemistry and food technology, all the way from sequence to structure and application properties.
Results
The BIOREDOX project targeted the development of new biocatalytic platforms to perform selective redox reactions. Chemical redox reactions often show poor selectivity, may require tedious and costly blocking and deblocking steps, and are typically catalyzed by heavy metals. Therefore, enzymes such as monooxygenases and oxidases represent highly attractive alternatives as they are able to catalyze a huge variety of redox reactions while exhibiting a remarkable selectivity. Exploitation of these biocatalysts would afford effective environment-friendly synthesis routes (“green chemistry”) that have several advantages over chemical routes. Selective oxidations catalyzed by enzymes are potentially also highly relevant in food chemistry. For example, when introducing carbonyl functions, they provide a handle for unique cross-linking reactions. Oxidative reactions can also cause disulfide bond formation between peptides, which is equally important in food technology.

The BIOREDOX project focused on flavoproteins. Examples of target reactions are:

• regio- and enantioselective alcohol oxidations, which indeed are difficult to accomplish by chemocatalysis;
• reactions that lead to cross-linking of food-relevant biomolecules (e.g. protein-carbohydrate coupling).

Both reaction types can be catalyzed by flavoprotein oxidases, which therefore were intensively investigated in this research. For example, we discovered new flavoprotein monooxygenases, and were able to develop the system from scratch to a well-characterized overexpressed biocatalyst. The scope of new enzymes was carefully investigated in Wageningen and Leiden. Thus, the project provided a toolbox of new enzymes with well-described characteristics, ready for further development and application tests by industry.

Project leader
• Prof. D.B. (Dick) Janssen (RUG)

Co-applicants
• Prof. W.J.H. (Willem) van Berkel (WUR)
• Prof. M.W. (Marco) Fraaije (RUG)
• Prof. A.P.G. (Tom) Kieboom (UL)

Researchers
• Erik van Hellemond (PhD, RUG)
• Dominic Heuts (PhD, RUG)
• Vivi Joosten (Postdoc, WUR)
• Adrie Westphal (Technician, WUR)
• Arjan van Wijk (Postdoc, UL)

Duration
• 2003-2008

Budget
• k€ 713

Highlights
• Discovery, cloning and expression, and crystallographic structural analysis of a new sugar oxidase. This enzyme, alditol oxidase, now is the best characterized flavoprotein oxidase on the market.
• Discovery and detailed analysis of several new oxidative enzymes by database genome mining and environmental gene library screening, illustrating the relevance of genomics for biocatalysis.
• Several well-cited reviews and primary research publications have appeared.
Cell and enzyme engineering for the construction of integrated biocatalysts performing sterol-steroid biotransformations

Biocatalytic removal or modification of the sterol side-chain provides an environmentally friendly method for the production of important pharmaceutical compounds. The current project explored enzymes that can be used for these attractive reactions.
Results
The project explored various enzymatic conversions and microbial transformations for application in the synthesis of steroids from cheap precursors. Traditionally, steroids are prepared from cholesterol or plant sterols by multistep pathways that employ chemical catalysis and lead to large amounts of waste, such as toxic heavy metals and salts. There is a need in industry for cleaner and shorter routes that start from cheap and renewable plant-derived resources.
Reactions that were investigated are the removal of the side chain from the cholesterol and sitosterol molecule by the bacterium Rhodococcus and by a one-step conversion with a cytochrome P450 type of enzyme. Furthermore, based on tedious genomic and transcriptomic analysis, the Rhodococcus bacteria were modified in such a way that they no longer degrade sterol molecules completely but instead accumulate useful intermediates that may serve as a starting point for preparing bioactive steroids.
Examination of the enzymes involved in bacterial steroid conversion yielded valuable knowledge about their properties and led to the discovery of promising steroid modification reactions that would be difficult to achieve chemically. Industrial implementation of the results will require further metabolic and enzyme engineering experiments, especially in case of direct side chain cleavage by cytochrome P450, because conversion rates must be elevated.

Project leader
- Prof. D.B. (Dick) Janssen (RUG)

Co-applicants
- Prof. L. (Lubbert) Dijkhuizen (RUG)
- Dr R. (Robert) van der Geize (RUG)

Researchers
- Roga Kembaren (PhD, RUG)
- Kamila Rosloniec (PhD, RUG)
- Maarten Wilbrink (PhD, RUG)

Duration
- 2004-2009

Budget
- k€ 565

Highlights
- The steroid degradation pathway of Rhodococcus was explored by a combination of genomics, transcriptomics, and biochemical techniques. This provides all the information required to engineer blocks in the degradation pathway that should enable intermediate product accumulation.
- The use of cytochrome P450ccc, expressed in Escherichia coli, for conversion of the cheap precursors cholesterol and sitosterol to pregnolone was demonstrated.
- Mutants of cytochrome P450 BM3 were also capable of selective steroid hydroxylation, which expands the toolbox of well-characterized steroid modification enzymes.
For sustainable production of semi-synthetic antibiotics, newly engineered fungal host strains are needed that are capable of producing these compounds from a renewable feedstock, thereby circumventing polluting chemical routes. Using natural enzymes, a novel biosynthetic route including product secretion has been developed for a non-natural cephalosporin.
Results
Antibiotics are widely used drugs to treat common bacterial infections (like those causing dental pains or pneumonia), and have significantly contributed to worldwide increase in life expectancy of human beings. Especially the penicillins and cephalosporins that are produced by fungi are very effective, even though some resistance may occur. Obviously, the large-scale use of these compounds requires that the chemical industry has effective ways to produce them. The work in Groningen and Delft, together with DSM, was aimed at improving methods for the biosynthesis of cephalosporins using fungi and their enzymes. Such green processes are much less polluting than classical chemical synthesis routes and may yield purer and safer products. We have especially investigated how we can improve the capacity of fungi to make large amounts of stable cephalosporin variants. To achieve this, several new enzymatic steps and a transporter for cephalosporins were introduced into the fungus to yield a novel cephalosporin that can function as a synthon for semi-synthesis of cephalosporins that are used in the clinic. In addition, we have investigated competing pathways in the cell that reduce the economy of the newly developed production process, and increased the number of subcellular compartments in the fungal cell to improve the efficiency of cephalosporin production. The project has yielded a new biological factory for the fermentative production of cephalosporin that now needs to be further optimized toward a commercial feasible industrial process.

Project leader
- Prof. A.J.M. (Arnold) Driessen (RUG)

Co-applicants
- Prof. D.B. (Dick) Janssen (RUG)
- Prof. J.T. (Jack) Pronk (TUD)
- Prof. M. (Marten) Veenhuis (RUG)

Researchers
- Iza Czartoryska (PhD, RUG)
- Diana Harris (PhD, TUD)
- Martijn Koetsier (PhD, RUG)
- Wieb Meijer (PhD, RUG)
- Jeroen Nijland (Technician, RUG)

Duration
- 2003-2009

Budget
- k€ 716

Highlights
- Engineered fungal strain for the production of a novel non-natural cephalosporin, that can act as synthon for the production of a range of cephalosporins used in the clinic.
- Improved secretion of cephalosporins by the introduction of a transporter.
- Identification and peroxisomal localization of CoA ligases involved in side chain activation for β-lactam production.
Catalytic cascade reactions for selective oxidations with benign oxidants

Environmentally friendly, sustainable processes for the oxidation of alcohols, a pivotal reaction in industrial organic synthesis.
Results
From an environmental viewpoint, it is of vital importance to develop clean processes that have an efficient utilisation of (preferably renewable) raw materials. In particular, the oxidation of alcohols, a pivotal reaction in industrial organic synthesis, often involves processes that employ stoichiometric amounts of heavy metal oxidants and/or environmentally undesirable solvents, and generate copious amounts of toxic effluent. In this project the Delft University of Technology has succeeded in developing two alternative processes for the oxidation of alcohols employing the green oxidants oxygen (air) or hydrogen peroxide (bleach) as the oxidant and an enzyme or a polymeric hypervalent iodine reagent as a catalyst. The only by-product is water. One method involves the use of the enzyme laccase, in conjunction with a mediator as a cocatalyst and oxygen (air) as the oxidant, for the environmentally attractive production of fragrances. In the second method a polystyrene attached hypervalent iodine reagent is employed as a catalyst in the selective oxidation of alcohols with hydrogen peroxide. The catalyst can be readily recycled after simple filtration and regenerated with hydrogen peroxide. This second method is particularly suitable for the oxidation of steroidal alcohols in the synthesis of steroid pharmaceutical products. In both methods the by-product is water.

Project leader
- Prof. R.A. (Roger) Sheldon (TUD)

Co-applicants
- Prof. I.W.C.E. (Isabel) Arends (TUD)
- Prof. W.R. (Wilfred) Hagen (TUD)
- Prof. S. (Simon) de Vries (TUD)

Researchers
- Aleksandra Kotlevska - Miernovska (PhD, TUD)
- Inga Matijosyte (PhD, TUD)

Duration
- 2004-2008

Budget
- k€ 377
High throughput screening of micro organisms in *Fed batch on a chip*

Complete biological production of chemicals and pharmaceuticals from renewable feed stocks through large scale fermentation becomes more and more important as alternative for traditional chemical processes. The research conducted in this project was aimed at developing a platform for high throughput selection of suitable production organisms under industrially relevant conditions.
Results
The research carried out within this project was aimed at developing a platform of parallel micro-bioreactors with working volumes of approximately 100 microliters, which can be applied to cultivate microorganisms under industrially relevant, substrate limited fed-batch conditions.

Such a technological platform, which allows to carry out large numbers of parallel cultivations of microorganisms under controlled environmental conditions and substrate limitation, will greatly enhance the effectiveness of the screening and selection of industrially relevant micro-organisms for the production of chemicals and pharmaceuticals. In addition, for fundamental research such a platform can accelerate research on the functioning of microorganisms at the level of nutrients and on the genetic and molecular level.

To monitor and control the processes in each individual micro-bioreactor, each reactor needs to be equipped with microsensors for the important cultivation parameters, such as temperature, acidity and concentration of dissolved oxygen, as well as with sensors that allow online monitoring of the performance of the cells, such as growth rate and rate of product formation. To this end, a sensor chip was developed for the online measurement of these parameters in each reactor. Additionally, microsensors were constructed for the measurement of the oxygen consumption and carbon dioxide production rates of the cells.

Furthermore microfluidics was integrated for controlled feeding of substrates and to keep the acidity of each culture within certain limits. An important design criterion for the individual components is the possibility of parallelization and the ability to integrate with existing formats (e.g. microtiter plates) and (robot) platforms.

The research conducted has successfully resulted in a "proof of principle" of such a platform of micro-reactors where virtually all important components were developed and tested and have been assembled to result in a working system. However, additional research is needed to develop a production-ready platform.

Project leader
- Prof. J.J. (Sef) Heijnen (TUD)

Co-applicants
- Prof. A. (Albert) van den Berg (UT)
- Prof. G.W.K. (Gjjs) van Dedem (TUD)
- Prof. J.G.E. (Han) Gardeniers (UT)
- Dr W.M. (Walter) van Gulik (TUD)
- Prof. L.A.M. (Luuk) van der Wielen (TUD)

Researchers
- A.Y. Bosma (PhD, TUD)
- Erik Krommenhoek (PhD, UT)
- Michiel van Leeuwen (PhD, TUD)
- Xiaonan Li (PhD, TUD)

Duration
- 2003-2008

Budget
- k€ 798

Highlights
- An integrated electrochemical sensor array for measurement of pH, dissolved oxygen, and viable biomass and was developed, built and successfully applied for the online monitoring of yeast fermentations in micro bioreactors.
- It was demonstrated that cultivations carried out in the developed micro reactors with volumes of 100 microliter were closely comparable with similar cultivations in bench scale bioreactors with volumes of 4 liters.
- The project has resulted in twelve publications in international, peer reviewed, scientific journals, three PhD theses and several oral and poster presentations on (inter)national conferences.
- The project has resulted in several follow-up projects, e.g. on microfluidics and microsensor development and fruitful cooperation with different (inter)national research groups.
A cell factory for the biosynthesis of complex peptides (CELLPEP)

Filamentous fungi have the unique property that they can synthesize peptides using special enzymes, the non-ribosomal peptide synthetases (NRPS). Penicillin production by Penicillium chrysogenum is an example of such a process. We improved this cell factory by genetic engineering for peptide production and identified novel NRPSs and their products.
> Results

There is a growing need for the efficient and sustainable synthesis of complex peptides for pharmaceutical applications. Complex peptides can be applied as antibiotics (e.g. penicillin) or anti-cancer drugs. The use of fungal cell factories has major advantages above chemical synthesis, as the desired complex peptides can be produced in a sustainable manner using renewable raw materials. Natural complex peptides are typically synthesized by special enzymes, called non-ribosomal peptide synthetases (NRPS). One of the best-studied NRPS is ACVS, an enzyme essential for the production of penicillin by the fungus Penicillium chrysogenum. Interestingly, analysis of the genetic information (DNA) of this fungus revealed that it is capable to make several additional NRPSs. However, for most of them it was unknown which peptide is produced.

This project aimed to characterize unknown P. chrysogenum NRPSs in order to elucidate whether they are involved in the biosynthesis of peptides with potentially interesting bioactivities. A second objective was to improve the P. chrysogenum cell factory for the production of NRPSs, which will contribute to obtaining higher yields of the desired products and hence a more sustainable production process.

At the cellular level we have genetically engineered quality control processes that keep the cell factory in a better shape during the fermentation process and hence result in higher yields of peptides. Growth conditions were established at which the novel NRPS enzymes are produced by the fungus. Together with a combination of genetic engineering and chemical analysis of peptides produced by cells, novel NRPS enzymes and their products were characterized. NRPSs were identified that produces the yellow pigment chrysogen, the toxin roquefortin and hydrophobic cyclic peptides. The latter may aggregate yielding the so-called “witte puntjes” that can disrupt an industrial fermentation process. Finally, experiments were performed that generated knowledge to design an improved fermentation process for the production and subsequent downstream processing of commercially interesting peptides.

> Project leader

- Prof. I.J. (Ida) van der Klei (RUG)

> Co-applicants

- Prof. A.J.M. (Arnold) Driessen (RUG)
- Prof. J.J. (Sef) Heijnen (TUD)
- Prof. J.T. (Jack) Pronk (TUD)

> Researchers

- Rinse de Boer (Technician, RUG)
- Amit Deshmukh (PhD, TUD)
- Annemarie Kralt (PhD, RUG)
- Marta Samol (PhD, RUG)
- Tânia Veiga dos Inocentes (PhD, TUD)

> Duration

- 2007-2012

> Budget

- k€ 901

> Highlights

- Elucidation of the biosynthetic pathways towards the production of the toxin roquefortin, the yellow pigment chrysogen and hydrophobic cyclic peptides including the definition of the substrate specificity of the A-domains of the respective NRPSs.
- Elimination of the production of oxalate, an undesirable byproduct of the antibiotic-producing fungus Penicillium chrysogenum, by metabolic engineering.
- Identified pathway for degradation of adipate, an important side-chain precursor for production of cephalosporin precursors, in P. chrysogenum and improved efficiency of side chain incorporation by metabolic engineering.
- Publication of the characterization of a novel peroxisomal Lon protease in Penicillium chrysogenum in the Journal of Biological Chemistry (287:27380-95).
Results

The industrial production of existing and new chemical entities has to proceed in an increasingly cleaner, faster and cheaper way due to economic and environmental constraints. Ideally, the synthesis of a complex molecule can be achieved in a single step starting from renewable sources and without producing any waste. It will be obvious that this goal cannot be easily reached. However, by broadening the scope of enzymes, in particular enzymes that are able to catalyze the formation of C-C-bonds, we might be able to develop novel, sustainable and industrially viable synthetic pathways to versatile enantiomerically pure heterocyclic building blocks. Finally, integration of the separate steps into one-pot cascade processes could bring us somewhat further in approaching the ultimate goal.

In this project, the following results were obtained:

- It was shown that a cascade reaction involving glucose, sodium pyrophosphate and three naturally occurring enzymes can be used to synthesize glucosides in an environmentally friendly manner. The first enzyme transfers a phosphate group from pyrophosphate to the primary hydroxyl of glucose. The second enzyme isomerizes this compound to glucose-1-phosphate, and the third enzyme couples the thus activated glucose moiety to the desired compound, forming the glucoside and releasing phosphate. Using this cascade, five glucosides were prepared. It is possible to perform the enzymatic
cascade in a one-pot fashion, but yields are higher when the reaction steps are performed separately.

- Several enzymes that can be applied in cascade reactions were cloned, produced and characterized, including dihydroxyacetone phosphate (DHAP)-dependent aldolases (fuculose-1-phosphate, fucA) and glycerolphosphate oxidase (GlpO). These enzymes were screened on their activity on glycerol and glycerol derivatives.

- A one-pot four-enzyme cascade reaction was developed, starting from glycerol, involving acidphosphatase (PhoN-Sf), glycerol-3-phosphate oxidase (GPO), catalase, and fructose-1,6-biphosphate aldolase (RAMA).

- This cascade reaction has been optimized with Alloc-protected 3-aminopropanal leading to a synthesis of the natural product D-fagomine in only two steps. D-Fagomine and related azasugars that were synthesized in a similar manner can act as inhibitor of glucosidases and may therefore be considered as leads for the treatment of diabetes and diseases connected to the metabolism of carbohydrates.

- To be able to use the aforementioned cascade in a continuous flow process for the phosphorylation of primary alcohols, immobilization of PhoN-Sf and RAMA was investigated and optimized. This has resulted in a packed-bed reactor system with immobilized PhoN-Sf and RAMA, with which certain non-natural carbohydrates could be produced on a gram scale. A computational model is being produced to optimize the process.

> Project leader
- Prof. F.P.J.T. (Floris) Rutjes (RUN)

> Co-applicants
- Dr M.C.R. (Maurice) Franssen (WUR)
- Prof. J. (John) van der Oost (WUR)
- Prof. R. (Ron) Wever (UvA)

> Researchers
- Lara Babich (PhD, UvA)
- Aleksandra Bury (Technician, UvA)
- Pierpaolo Falcicchio (PhD, WUR)
- Lieke van Hemert (PhD, RUN)
- Michal Rachwalski (Postdoc, RUN)
- Rokus Renirie (Postdoc, VU)

> Duration
- 2007-2012

> Budget
- k€ 1043

> Highlights
- Lieke van Hemert won the 1st poster award with the poster she presented at the NWO/CW study group meeting Design & Synthesis, October 19th – 21st 2009, Lunteren.
- Many presentations of researchers at international meetings, e.g. Lara Babich at Zing Biocatalysis Conference in Cancun, Mexico, dec 2010.
Biocatalytic exploitation of monooxygenases (BIOMOX)

The BIOMOX project has resulted in the identification and production of a large number of new oxidative enzyme which can be used as effective catalysts in industrial processes.
Results

The fine chemicals industry traditionally is responsible for a large part of the total chemical waste production, although the production volumes are much smaller than with the bulk chemistry. This is mainly due to the use of polluting chemicals for hydrolysis, oxidation and substitution reactions rather than catalytic steps. The use of biocatalysts will make a major contribution to reducing production of industrial wastes. Moreover, these catalysts will allow “green chemistry” routes to enantiomerically pure key building blocks which appear to become of utmost importance for manufacturing biologically active products. The BIOMOX-project aimed at generating a portfolio of new oxidative enzymes: monooxygenases. Such biocatalysts do not contain heavy-metal cofactors, thus also are suitable for replacing heavy-metal containing chemical catalysts.

The BIOMOX-project aimed specifically at the development of new biocatalysts and (bio)catalytic routes towards synthesis of fine chemicals and steroid-related compounds. These are important targets for the participating Dutch industries. The work has generated a wide range of new biocatalytic tools: a large set of newly identified flavoprotein monooxygenases, a new expression system for the production of biocatalysts, and a collection of newly engineered P450 monooxygenases. The availability of such large and well-defined toolbox of biocatalysts will facilitate the industries to more quickly respond to rapid changes in customer needs for specific chemical intermediates. The generation of an industrially relevant monooxygenase also resulted in a patent application by industrial partners of the BIOMOX-project.

Project leader

- Prof. M.W. (Marco) Fraaije (RUG)

Co-applicants

- Prof. W.J.H. (Willem) van Berkel (WUR)
- Prof. L. (Lubbert) Dijkhuizen (RUG)
- Prof. N.P.E. (Nico) Vermeulen (VU)

Researchers

- René de Jong (Postdoc, VU)
- Stefania Montersino (PhD, WUR)
- Evelien te Poele (Postdoc, RUG)
- Anette Riebel (PhD, RUG)
- Harini Venkataraman (PhD, VU)

Duration

- 2008-2012

Budget

- k€ 1043

Highlights

- The project resulted in a patent concerning a specific monooxygenase-catalyzed conversion.
- The project yielded a totally new and unique bacterial expression system for protein production.
- The project resulted in a large collection of new oxidative enzymes.
- Through this project five young researchers were trained in industrially oriented research. One of them is currently working at a Dutch biotech company and the others are still performing research at academia.
Chemoenzymatic peptide synthesis

This project aims to develop chemoenzymatic strategies and tools for low-cost and versatile synthesis of peptides, which is done either by changing the properties of coupling enzymes (Groningen), by modification of the target substrates (Nijmegen) or by influencing process conditions (Wageningen).

Results

The use of enzymes for peptide bond formation in peptide synthesis has important advantages over chemical synthesis. For example, due to the regioselectivity of enzymes protection of side chains is not needed, and unlike with chemical copupling racemisation of amino acids is not observed. However, although enzymatic peptide synthesis has been commercialized in some cases, there are still problems that need to be solved to make the technology generally applicable. Most coupling enzymes do not accept a broad range of amino acids and peptides. Furthermore, since they are proteases used in the reverse direction, the coupling enzymes can also catalyze hydrolysis of the acyl donor or internal peptide bonds in the products, which complicates their use in water. To address these issues, the project focused on:

- an enzyme discovery and engineering approach aimed at finding better enzymes for coupling, deprotection, and substrate activation (RUG);
- a bioprocess engineering approach aimed at optimizing reactions conditions, including enzyme formulation and water activity (WUR);
- an investigation of alternative substrate-activation strategies, incl. substrate mimetics, to allow wider acceptance of substrates (RUN).

The results of the project should contribute to the development of low-cost versatile chemoenzymatic strategies for the synthesis of peptides by C-terminal...
coupling, that can be implemented in industrial peptide synthesis. Therefore the research was carried out in close collaboration with DSM, including mutual exchange of experimental protocols, peptide samples, and enzyme preparations.

Results that were obtained include:
• Cloning, expression and characterization of a novel plant peptide amidase that can catalyze peptide amide hydrolysis as well as peptide methylester formation. The catalytic scope of the enzyme and of a bacterial counterpart includes several new peptide modification reactions that are of synthetic relevance.
• Novel thermostable subtiligase-like enzymes have been constructed and tested in synthetic applications, both in water and in organic solvents.
• Several novel thermostable and organic solvent-resistant proteases have been discovered by genome mining and expression in E. coli. They are evaluated for application in peptide coupling in collaboration with DSM.
• The effects of enzyme formulation and water activity on peptide coupling synthetic reactions were established in Wageningen. Immobilisation protected coupling enzymes against organic solvents that are required for substrate solubilisation in synthetic reactions. The effects of biocatalyst recycling and mechanical stress were described, as well as the kinetics of the coupling reaction and of the dehydration of the enzyme due to the presence of molecular sieves.
• The substrate mimetics approach was carefully explored for a range of alternative enzymes (papain, chymotrypsin, trypsin, subtilisin) and activating groups for peptide coupling. This greatly enhanced the possibilities of the coupling reactions. The work in Nijmegen also showed that computational methods may be used to understand enzyme selectivity in peptide coupling, and led to a detailed understanding of the scope and limitations of substrate activation methods.

Project leader
• Prof. D.B. (Dick) Janssen (RUG)

Co-applicants
• Prof. F.P.J.T. (Floris) Rutjes (RUN)
• Prof. J. (Hans) Tramper (WUR)

Researchers
• Irfan Arif (PhD, RUG)
• Roseri de Beer (PhD, RUN)
• Ana Toplak (PhD, RUG)
• Petra Vossenberg (PhD, WUR)
• Bian Wu (Postdoc, RUG)

Duration
• 2008-2012

Budget
• k€ 1007

Highlights
• In her PhD thesis, Roseri de Beer (Nijmegen) showed that the substrate mimetics approach should be regarded as a generic peptide activation method, and may be used in a much broader way, including with several proteases and activating groups. Computational methods can predict and explain reactivity of substrates in enzymatic coupling reactions.

Petra Vossenberg (Wageningen) has discovered that immobilization may be used to prevent enzyme incompatibility, and may also strongly enhance organic solvent tolerance and stability of the coupling enzyme. The process conditions, with regard to the water activity, were optimized for the coupling reaction.

Ana Toplak and Bian Wu (Groningen) discovered that a calcium-free variant of subtilisin that carries multiple mutations in the active site can be used for peptide coupling in water, especially in case of longer peptides. Enzymes with improved properties were also obtained by genome mining.

Irfan Arif and Bian Wu discovered a new plant amidase that can be used for peptide amide deprotection. Such amidases also allow a number of carboxylate modification reactions of synthetic relevance.
Development of conditional protein-ligand exchange applied to immune technology

Biocatalysis and immunology merged: a short chemo-enzymatic route to reagents for characterisation of T cell reactivity and for screening of immunomodulatory therapeutics.

Results

Biocatalysis, organic synthesis and immunology come together in this project with the goal to develop immune technology for diagnostic and therapeutic applications. This project aimed at generating novel conditional ligands that can be chemically triggered to dissociate from their respective binding partners. Such ligands are valuable to terminate macromolecular interactions. The utility of this strategy was previously shown using UV-sensitive ligands. Systems in which interactions can be disrupted by mild chemical triggers offer greater versatility and robustness. To show proof-of-concept major histocompatibility complex (MHC class I) molecules and ligands thereof were used as a model system. MHC complexes present antigenic peptides on the cell surface. T-lymphocytes scan body cells for the presence of such aberrant peptides, indicating cancer or infection. Selective binding of T cells to MHC complexes forms the molecular basis of the ability of T cells to recognize and destroy infected cells and tumor cells. This specific protein-protein interaction can be exploited for the characterization and purification of desired T cell populations and multivalent recombinant MHC reagents have become a core technology for both diagnostic and therapeutic applications.

The design of the cleavable linker was based on the periodate reactivity of a vicinal amino alcohol moiety, which is hypersensitive to oxidative cleavage. The synthesis of a building block suitable for solid phase peptide synthesis involved a short chemo-enzymatic route.
“green chemistry” route featuring L-threonine aldolase which led to L-α,γ-diamino-β-hydroxybutanoic acids. Incorporation of such amino acids into the backbone of a peptide generated conditional peptides that were rapidly cleaved by sodium periodate. This cleavable peptide was used in the generation of MHC exchange reagents for the detection of antigen specific T cells. The extremely low concentration of periodate required to trigger MHC peptide exchange, precluded problematic side reactions, such as co-oxidation of methionine and disulfide residues. When T cells recognize self peptides as aberrant and start destroying healthy cells, one speaks of an autoimmune disease. Blocking the MHC complex will inhibit interaction with the T cell and prevent the autoimmune response. Using the developed MHC exchange technology >23,000 compounds were screened for MHC blocking capacity. Such compounds can form the foundation of a novel class of pharmacological agents to treat autoimmune disease, and solid organ transplant rejection and screening hits are currently being evaluated.

Project leader
- Dr H. (Huib) Ovaa (NKI)

Co-applicants
- Dr B. (Boris) Rodenko (NKI)
- Prof T.N.M. (Ton) Schumacher (NKI)

Researchers
- Alessia Amore (Postdoc, NKI)
- Rieuwert Hoppes (PhD, NKI)
- Remco Merkx (Postdoc, NKI)
- Farid El Oualid (Postdoc, NKI)
- Nienke van Rooij (Technician, NKI)
- Jos Urbanus (Technician, NKI)

Duration
- 2008-2012

Budget
- k€ 888

Highlights
- Publication submitted: A. Amore et al., Development of a hypersensitive periodate cleavable amino acid that is methionine and disulfide compatible and its application in MHC exchange reagents for T cell characterisation.
Enzymatic synthesis of enantio- and diastereomerically pure unsaturated β-hydroxy-α-amino acids and synthetic elaboration into biologically relevant derivatives

A sustainable, chemoenzymatic approach involving threonine aldolases was developed to synthesize biologically active threonine derivatives via the direct coupling of aldehydes with the amino acid glycine.
Results

β-Hydroxy-α-amino acids comprise an important compound class owing to the biological activity of many of its members and therefore it is important to study sustainable methods to synthesize such molecules. In this proposal we developed – in accordance with the initial goal – a new chemoenzymatic strategy to form a selected set of β-hydroxy-α-amino acids. Some of these amino acids contained functional groups that were used as a handle for further catalytic functionalization into biologically and industrially relevant heterocyclic structures. The integration of biocatalytic and metal-catalytic steps is a key element in this chemoenzymatic route and strongly contributes to the environmentally benign character of the approach.

Project leader
• Prof. F.P.J.T. (Floris) Rutjes (RUN)

Co-applicants
• Prof. J. (John) van der Oost (WUR)
• Prof. R. (Ron) Wever (UvA)

Researchers
• Teunie van Herk (Postdoc, RUN)

Duration
• 2007-2010

Budget
• k€ 138

Highlights

• After the project, Teunie van Herk was awarded the ‘Van Marum prijs’ for her scientific work on phosphatases and threonine aldolases.

• The spin off involved, Chiralix, can use the newly developed methods to produce amino acids to increase their product portfolio.
Catharanthus plants produce terpenoid indole alkaloids (TIAs) with anticancer properties; yet, the supply remains too limited. As alternative, we aim to synthesize TIAs using Catharanthus cell cultures. As geraniol was recognized to be a limiting precursor for TIA production in cell-cultures, we aimed to determine whether its synthesis is the limiting factor and/or its transport and availability to down-stream steps. Cell-line selection, and feeding & elicitation approaches were employed successfully to enhance production of TIA-precursors. Continued efforts towards over-expression of geraniol synthase, building metabolic bridges by diversifying the enzyme’s localization and silencing competing steps are being pursued further to reach project aims.

> Results

It is our goal to realize new production systems for bio-active plant metabolites based on plant-cell cultures. In this project our target is the medicinal plant Catharanthus roseus that is known for its bio-active terpenoid-indole-alkaloids (TIAs), e.g. the anticancer compounds vinblastine and vincristine. In this project we aim to improve the production of catharanthine and vindoline; by chemical-modifications and enzymatic-coupling a modified vinblastine can be derived with improved uptake-kinetics and reduced side-effects in patients.

The terpenoid route is limiting the biosynthesis of TIAs in the Catharanthus cell-cultures, which relates to a lack of the early precursor geraniol. We aimed to increase geraniol availability by

- over-expressing geraniol synthase (GS) in the chloroplast;
- by changing GS localization to the cytosol to overcome geraniol transport while creating a metabolic-bridge with the mevalonate pathway, and;
- by blocking conversion of precursors to other terpenoids, such as carotenoids or sterols.

We established analytical methods to determine TIAs, carotenoids and sterols in our Catharanthus cell-lines. This revealed one particular cell-line producing highest levels of TIAs and carotenoids with strictosidine-levels equal to total TIA-levels in some Catharanthus plants! Subsequently, we optimized geraniol-feeding and jasmonate-elicitation strategies to overcome geraniol
limitations while boosting the TIA pathway genes. These approaches increased accumulation of the down-stream intermediates loganic acid by 7x and secologanin up to 28x in a time-dependent way; however, production of down-stream TIA remained limited and needs to be solved beyond the scope of this project.

In the meanwhile, we cloned a Basilicum GS and the Catharanthus GS into several expression vectors with different leader peptides (for chloroplast, peroxisomes and with no leader) and with GFP as a marker gene. Experiments are ongoing to create transgenic cell-lines and to determine the effect of GS over-expression and localization on TIA production. In addition, we tried to decrease the gene expression of geranylgeranyldiphosphatase synthase (GGPPS) and squalene synthase (SQS) in a targeted manner to reduce the competition for GPP and thus favor the conversion of GPP to geraniol. The results look promising and further optimization is ongoing. The overall results obtained so far are thus promising that the project will be pursued to bring the proposed activities and their concomitant results to their full potential.

- **Project leader**
  - Prof. R. (Rob) Verpoorte (UL)

- **Co-applicants**
  - Prof. J. (Johan) Memelink (UL)
  - Dr A.E. (Annelies) Schulte (UL)

- **Researchers**
  - Natali Mustafa (Postdoc, UL)
  - Marianne Verberne-Wójciech (Postdoc, UL)

- **Duration**
  - 2008-2011

- **Budget**
  - k€ 195

- **Highlights**
  - Analytical tools established to measure Catharanthus terpenoid indole alkaloids (TIAs) in the context of other major terpenoid groups like carotenoids and sterols.
  - Screening for a high producing cell-line delivered one Catharanthus cell-line that accumulated the TIA precursor strictosidine at equal levels to total TIAs in plants!
  - Feeding the early precursor geraniol increased accumulation of the down-stream intermediates loganic acid by 7x and secologanin up to 28x in a time-dependent way.
  - Proof-of-principle delivered for silencing of competing genes by application of antisense oligonucleotides; 40-60 per cent reduction of gene expression achieved!
  - Identification and cloning of geraniol synthase from Catharanthus achieved; transformation experiments ongoing.
Combining Cross Linked Enzyme Aggregates with Multi-Component Reactions for the efficient preparation of fine chemicals

Enzymes work in dry solvents, however, this is often due to the fact that the carrier modifies the reaction conditions.
Results
Most enzymes work in water or in mixtures of organic solvents and water. Only very few specialized enzymes work in dry organic solvents. In this project we tried to immobilise enzymes so that we could use them in dry organic solvents, essentially under conditions of a chemical factory. In this way we hoped to combine enzymes with organic chemistry all in one pot. Results turned out to be different to what we expected. The enzymes could be immobilised but behaved in an unexpected way. Together with the enzyme a lot of water was immobilised and this water changed the reaction conditions. Originally we intended to work in water-free organic solvents, but this was impossible because of the water in the immobilised enzyme. We then investigated the influence of water on enzymes when the enzymes are used in organic solvents. This gave us new, fundamental insights that we can use to design the next generation of immobilised enzymes. Essentially CLEAs of the enzymes we used shed water into the reaction mixture (see picture). The enzyme used was MeHNL, the hydroxynitrile lyase from Manihot esculenta.

Project leader
Prof. U. (Ulf) Hanefeld (TUD)

Co-applicants
Prof. R.V.A. (Romano) Orru (VU)

Researchers
Monica Paravidino (Postdoc, TUD)

Duration
2008-2010

Budget
k€ 133

Highlights
- When using enzymes in dry solvents all components should be dry.
- MeHNL works best in toluene.
- For tertiary alcohols there is no “Rule of Kazlauskas”. 
Industrial chicory as fine-chemical production platform

Artemisinin is the most important anti-malarial drug currently available and is extracted from Artemisia annua which contains only low amounts. We cloned artemisinin pathway genes into smart vectors and have shown that we can produce a conjugate of the artemisinin precursor artemisinic acid in transient expression. We will transfer this approach to the industrial crop chicory to create a more efficient anti-malarial drug production platform.
> Results
Artemisinin is the most important anti-malarial drug currently available. It is extracted from Artemisia annua which contains only low amounts. In this project we have investigated the possibility to establish industrial chicory for the production of this important anti-malarial drug. Industrial chicory is a suitable crop for this approach because the existing inulin production chain can be used. Additionally, chicory contains a number of cytochrome P450 enzymes that were shown to catalyse important conversions. In the project we first successfully established transient expression of terpene biosynthetic genes in Nicotiana benthamiana (Van Herpen et al., 2010). This provides a valuable tool for the quick evaluation of constructs and engineering strategies and is currently used on a large scale in our group for other projects. For stable transformation the introduction of several genes into plants is quite a challenge. Therefore we have made a genetic fusion construct of 3 artemisinin pathway genes: AMS, FPPS and tHMGR in which the genes were connected by ribosomal skipping sequences (“2A-peptides”), and the whole, single open reading frame is expressed from a single 35S promoter. This construct was functional in transient expression in N. benthamiana and resulted in high production of the artemisinin precursor amorphadiene. The construct was transformed to chicory. However, even though we have shown that the introduced genes are expressed, we have not been able to detect new products. In transient expression in N. benthamiana the combination of this construct, with a P450 gene from A. annua resulted in formation of a novel compound. This was identified as a diglucoside conjugate of artemisinic acid, which can be transformed in only one step to the preferred artemisinin precursor dihydroartemisinic acid. In the mean time we have shown that also production of dihydroartemisinic acid (also as its glycoside conjugates) is feasible in N. benthamiana and we are studying the best strategy to transfer this to chicory.

> Project leader
- Prof. H.J. (Harro) Bouwmeester (WUR)

> Co-applicants
- Prof. H.J. (Dirk) Bosch (UU)
- Dr M.C.R. (Maurice) Franssen (WUR)

> Researchers
- Jules Beekwilder (Postdoc, WUR)
- Teun van Herpen (Postdoc, WUR)

> Duration
- 2008-2011

> Budget
- k€ 219

> Highlights
- Transient expression of terpene biosynthetic genes in Nicotiana benthamiana was established and published (Van Herpen et al., 2010).
- A genetic fusion construct of 3 pathway genes was made. This construct was functional in transient expression and resulted in production of the precursor amorphadiene in N. benthamiana. The construct was transformed to chicory. This construct, and the addition of a P450 gene in transient expression in N. benthamiana resulted in formation of a novel compound, which was identified as a precursor of dihydroartemisinic acid.
Dynamic assemblies by dynamic kinetic resolution

In this research, we set out to study in detail a ‘tandem catalysis’ process, in which an enzymatic reaction is combined with a polymerization reaction. The insights we obtain will be used to prepare benzene-1,3,5-tricarboxamides (BTAs), a class of compounds that is an interesting candidate for application as MRI contrast agents.

> Results

This proposal aimed to combine the research themes of iterative tandem catalysis and supramolecular polymerizations. With those, we designed a simple, modular approach to functional benzene-1,3,5-tricarboxamides (BTAs) by using a biocatalytic transformation in combination with non-covalent synthesis to arrive at functional assemblies.

BTAs based on peptides are interesting candidates for applications in the life sciences, as water soluble non-toxic dynamic assemblies are attainable, of which the aggregation behaviour can be controlled, and that can be used with respect to various functions (e.g. targeting, drug delivery, imaging). Our specific interest lies in the development of imaging agents such as MRI contrast agents.

With respect to the synthetic part of the research, some setbacks were encountered. Despite the very successful development of tandem catalysis to prepare enantiomerically pure molecules; unwanted degradation of synthesized C3-symmetrical oxazolone derivatives and synthetic products and difficulties in the threefold substitution of the C3-symmetrical core molecule, caused by increased steric hindrance of a C3-symmetrical core. As a result, we abandoned our desire to design a modular, biocatalytic approach for C3-symmetrical, functional molecules that can be applied as supramolecular MRI agents and followed traditional synthetic procedures. With these “traditional” procedures, a library of BTA based molecules was prepared to investigate in detail the
effect of molecular structure on the self-assembly behaviour of BTAs. On the other hand, we studied independently the successful preparation of covalent polymers by enzymatic transformation. The different BTAs comprising Gd(III) complexes, prepared according to traditional procedures, were evaluated in detail for their potential as supramolecular MRI contrast agents. We first focused on the self-assembly behaviour and performed detailed physical characterisations of the systems applying UV and CD spectroscopy, SAXS, cryo-TEM and NMR techniques. The combination of these different techniques allowed to gain detailed insight in the self-assembly behaviour of the different BTA based compounds, which was reported in a number of publication including in PNAS. Remarkable and intriguing properties emerged from their self-assembly behaviour, and the exact balance between the different non-covalent interactions and the charges on the Gd(III) complex determined the nature and stability of the aggregates formed. We can now turn our attention to the development of multimodal and target specific MRI contrast agents. Attaching peptides for target-specific imaging and making use of multivalency makes the supramolecular contrast agents very competitive against conventional macromolecular constructs, with the added advantage of maintaining control over in vivo excretion rates based on the reversible nature that forms the self-assembled scaffold.

Project leader
• Prof. E.W. (Bert) Meijer (TU/e)

Co-applicants
• Dr A.R.A. (Anja) Palmans (TU/e)

Researchers
• Pol Besenius (Postdoc, TU/e)
• Martijn Veld (Postdoc, TU/e)

Duration
• 2008-2011

Budget
• k€ 144

Highlights
• Novel Gd(III) containing self-assembling supramolecular compounds were obtained which might serve as the next generation of target-specific contrast agents.
• The incorporation of fluorinated building blocks into BTAs will open the opportunity towards 19F MRI detection.
• The development of hydroxyl-group containing BTAs by SyMO-Chem provides a new class of organogelators and hydrogelators.
Polymersome nanoreactors for enzymatic screening and biotransformation

Different methods have been developed based on the self-assembly of amphiphilic block copolymers to construct porous nanocapsules that can be used to encapsulate enzymes in their active form while protecting them from undesired effects in the environment.
> Results
Enzymes are catalysts that facilitate the synthesis of molecules in nature. They are highly selective and efficient and therefore also very useful for the fine chemical industry. One important drawback that limits their applicability is the fact that enzymes are also quite fragile and easily lose their activity. To make them better compatible and to protect them from undesired interactions we can encapsulate enzymes in nanometer sized polymer capsules, called polymersomes. However, in order for the enzymes to be useful, the molecules they have to transform still have to be able to reach the enzymes inside the polymersomes. These polymer capsules therefore have to be permeable. Until now porosity in the polymeric shell was introduced using complicated polymer materials or specific pore forming proteins which made this entire process hardly commercially viable.

In this project, we developed an elegant method to create pores, with a high level of control over the amount of porosity that can be introduced. The polymers used are standard polymersome forming molecules and this now opens up the way to further commercial evaluation of polymersomes as enzyme nanoreactors.

> Project leader
- Prof. J.C.M. (Jan) van Hest (RUN)

> Co-applicants
- Prof. J.L.M. (Jeroen) Cornelissen (UT)
- Dr M. (Madhavan) Nallani (RUN)
- Prof. R.J.M. (Roeland) Nolte (RUN)
- Prof. A.E. (Alan) Rowan (RUN)

> Researchers
- Kyoung Kim (Postdoc, RUN)

> Duration
- 2008-2011

> Budget
- k€ 127

> Highlights
- K.T. Kim, J.J.L.M. Cornelissen, R.J.M. Nolte, J.C.M. van Hest, A Polymersome Nanoreactor with Controllable Permeability Induced by Stimuli-Responsive Block Copolymers. Advanced Materials (2009), 21, 27, P2787+
Cytochrome P450 BM3 mutants: Towards Highly Efficient Stereoselective Biocatalysts (BM-STEREO)

A cascade reaction of two enzymes, genetically engineered bacterial Cytochrome P450 BM3 and Aldehyde DeHydrogenase (ADH) has resulted in a major step forward in the regio- and stereoselective production of Nootkatone from (+)-Valencene.
Results
Nootkatone is a fragrance and flavoring compound that provides the characteristic flavor to grapefruit. It is, for example, added in small amounts to Coca-Cola. Nootkatone is difficult to prepare synthetically due to the formation of unwanted side-products. Currently, this compound is extracted from grapefruits, which is a very intensive and relatively expensive process. Therefore, new biotechnological strategies are needed to produce nootkatone. As a starting point for such an approach, we used a cytosolic Cytochrome P450 enzyme from Bacillus megaterium, i.e. Cyt P450 BM3, since it has shown a number of advantages for the production of interesting chemical compounds. The wildtype Cyt P450 BM3, however, showed the same disadvantages as the traditional production, i.e. too many side-products. New genetically engineered versions of Cyt P450 BM3 resulted in a better efficiency and a purer product. Subsequently, a cascade of an engineered Cyt P450 BM3 mutant and a second enzyme, namely Aldehyde DeHydrogenase (ADH) was established. With this cascade of two enzymes, it was possible to produce 40 times more nootkatone when compared to the wildtype Cyt P450-BM3. Moreover, the regio- and stereo-selectivity was at least 10-fold higher then at the start of our project. Finally, we rationalized several improvements, amongst others concerning product inhibition and low substrate solubility. Overall, this project has resulted in a major step forward to the final goal, namely the production of nootkatone from (+)-valencene.

Project leader
- Prof. N.P.E. (Nico) Vermeulen (VU)

Co-applicants
- Dr J.N.M. (Jan) Commandeur (VU)

Researchers
- Rokus Renirie (Postdoc, VU)

Duration
- 2009-2011

Budget
- € 185

Highlights
- Novel screening methodologies for regio- and stereoselective hydroxylations of (+)-Valencene and (+)-Methoxy-valencene by Cyt P450 BM3 mutants were developed.
- An in-house library of Cyt P450 BM3 mutants was screened for the desired hydroxylation reactions.
- Selected Cyt P450 BM3 mutants were genetically engineered to optimize the conversion of (+)-Valencene to Nootkatone.
- A cascade reaction by a combination of a Cyt P450 BM3 mutant and Aldehyde dehydrogenase (ADH) resulted in a major step forward in the conversion of (+)-Valencene to Nootkatone.
Nootkatone is a sesquiterpene ketone highly appreciated for its typical grapefruit-like aromatic properties. We isolated and characterised two novel cytochrome P450 enzymes for the enzymatic synthesis of nootkatone from two different plant species. Upon expression in yeast fermentative production of (+)-nootkatone was demonstrated for the first time.
Results

Plant sesquiterpenes are appreciated for their medicinal and aromatic properties. The main objective of the project was to establish an enzymatic route to produce (+)-nootkatone, the most important aroma component of grapefruit. Natural nootkatone is produced by extraction from grapefruit and has limited availability. The alternative chemical routes for nootkatone production require use of environmentally critical reagents, may result in formation of side products and the final product cannot be marketed as a natural flavour. In plants nootkatone is produced by cytochrome P450 enzymes from its precursor valencene by two-step oxidation via a nootkatol intermediate. This project aimed to isolate and express a novel plant valencene oxidase gene that will oxidise valencene specifically into nootkatone. This novel fermentation process will enable sustainable production of “natural” nootkatone at a reduced cost. Chicory (Cichorium intybus) and Alaska cedar (Callitropsis nootkatensis) were identified as possible plant sources for the valencene oxidase gene. Large scale sequencing approach was used to analyse the transcriptome of the sesquiterpene producing tissues. Cytochrome P450 candidates were functionally characterised in yeast and a chicory valencene oxidase and Alaska cedar valencene oxidase were successfully identified by this approach. Upon co-expression of the chicory valence oxidase with a valencene synthase in yeast, predominantly nootkatol and a small amount of nootkatone were produced de novo. The expression of C. nootkatensis valencene oxidase in yeast resulted in an improved conversion of valencene into predominantly nootkatone. For the first time a fermentative production of (+)-nootkatone was demonstrated in yeast.

Project leader
- Prof. H.J. (Harro) Bouwmeester (WUR)

Co-applicants
- Dr. M.J. (Jules) Beekwilder (WUR)
- Prof. H.J. (Dirk) Bosch (WUR)

Researchers
- Katarina Cankar (Postdoc, WUR)

Duration
- 2009-2011

Budget
- € 144

Highlights
- Discovery of two novel cytochrome P450 enzymes that mediate the production of important flavour sesquiterpene nootkatone.
- First demonstration of fermentative nootkatone production in yeast.
- The project resulted in two scientific papers.
A biotechnological process for the production of (+)-carvone

In this project a production strain has been developed for a new biotechnological process for producing carvone, a highly valuable anti-sprouting agent.
Results
Carvone is an interesting target for an industrial biotechnological process:
- The current carvone extraction from caraway oil is very inefficient, which results in high costs;
- The cultivation of caraway for carvone production requires significant cropland;
- Caraway cultivation requires large amounts of fertilizer with a substantial negative environmental impact.
Clearly, a biotechnological process for the production of carvone would be advantageous. This project aimed at developing a process for carvone production using limonene as source, a waste product from orange processing industry. For this, a recombinant organism would be engineered that is capable of the required limonene to carvone transformation.
For the project it was crucial to identify a suitable enzyme, i.e. the corresponding gene, which is capable of regioselective hydroxylation of limonene. For this, several bacteria were studied with respect to their capability of limonene hydroxylation. Also sequence genomes were searched for putative monooxygenases that may suitable. Using a bacterium from the collection of Enzyscreen, the desired activity was verified. For this specific bacterium, chromosomal DNA was used to determine the respective genome sequence. By PCR-methods, a gene cluster was amplified that was found to encode the desired enzyme system. The genes were cloned in several hosts. Expression in a Pseudomonas strain has resulted in a recombinant host that efficiently converts limonene into carveol. Gratifyingly, the recombinant Pseudomonas converts limonene only into one product: carveol.
The project resulted in a recombinant system which effectively convert limonene into carveol. This is the key step to a biotechnological process for carvone production, using limonene as substrate. However, for implementing this system in an industrial setting, more research will be necessary and for this a follow-up project will be needed.

Project leader
- Prof. M.W. (Marco) Fraaije (RUG)

Researchers
- Maarten Groeneveld (Postdoc, RUG)

Duration
- 2010-2012

Budget
- k€ 164

Highlights
- Oral presentation at the Biotrans 2011, Sicily, Italy, October 2011 “A biotechnological process for the production of (+)-carvone”.
- Oral presentation at the ACTS symposium 2012, Lunteren, The Netherlands, February 2012 “A biotechnological process for the production of (+)-carvone”.
- The involved researcher has been trained in biotechnological research and is now working at a biotech company in The Netherlands.
Novel laccases and their enhanced expression in Aspergillus niger

New laccase enzymes were discovered in the filamentous fungus Aspergillus niger. The production of four of them was optimized by means of translation enhancing elements, that had a positive effect in their final production yields. In addition, one the newly characterized laccases has promising potential for industrial application.
Results

The project aimed the identification, characterization and the expression optimization of novel laccases from A. niger. On the basis of a bioinformatics analysis, ten genes potentially encoding new laccases from A. niger were selected to be studied. Interestingly, some of them belong to laccase families whose catalytic properties had never been investigated in depth. These genes were cloned and expressed in A. niger. Through enzyme activity assay screenings in A. niger growing plates it could be shown, in all cases, that the proteins encoded by these genes display laccase activity. The fact that four different laccase substrates were used in these simple and fast screenings allowed to observe remarkable differences in their catalytic properties. The three enzymes featuring more interesting catalytic properties in the screening assays were successfully purified and further characterized. As predicted, the three newly obtained oxidases displayed strongly different activities towards the array of substrates tested for the characterization of their catalytic properties, that included aromatic compounds and synthetic dyes. Based on the catalytic properties observed for the three enzymes it could be concluded that one of them, named McoB, showed promising potential industrial applications, in processes concerning waste waters pre-treatment and dye decolorization.

Expression optimization of five of the ten new laccases from Aspergillus niger was also studied. For this purpose, the ability of different translation enhancement elements (TEE) to increase the extracellular yields of these enzymes was tested. The production of four of the five enzymes was clearly improved by the three different TEEs tested in this study.

In summary, the described project objectives have been amply achieved. As a result, new laccase enzymes were found and their production was optimized. Furthermore, at least one of them has promising potential industrial applications.

Project leader
- Dr L.H. (Leo) de Graaff (WUR)

Researchers
- Juan Tamayo Ramos (Postdoc, WUR)

Duration
- 2009-2011

Budget
- k€ 157

Highlights
- Poster presentation at OxiZymes Conference in Leipzig, Germany (June 2010).
- Poster presentation at 26th Fungal Genetics Conference in Asilomar, USA (March 2011).
- Three publications are expected to be published in SCI journals of good impact factor (first quarter 2013) in the area of Biotechnology and Applied Microbiology.
Colophon

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