Greenchem

Speciality Chemicals from Renewable Resources

ANNUAL REPORT 2009

LUND UNIVERSITY

FUNDED BY MISTRA

The Greenchem programme

The Swedish research programme Greenchem focuses on development and application of clean processes based on biocatalysis for production of chemical products from renewable raw materials. The programme aims to demonstrate the feasibility of robust biotechnology tools and processes to establish a technology link between the renewables sector and the chemical producer/user industry. Greenchem has its academic base at **Lund University**, Sweden.

A range of industries, representing renewable raw material providers as well as producers and users of chemicals, participate in the programme, and help to identify the products for which the environmental effect is critical from the manufacturing and application perspective. In some cases, it implies the replacement of an existing manufacturing process while in the others that of the final product. The participating industries are:

- **AarhusKarlshamn/Binol**
- **ACO HUD NORDIC**
- **Akzo Nobel Industrial Coatings**
- **Akzo Nobel Surface Chemistry**
- **AstraZeneca**
- **IKEA of Sweden**
- **Perstorp**
- **Protista**

The programme further involves the evaluation of the environmental impact of the products from a life-cycle perspective and also the analysis of critical factors for introduction of new technologies in the chemical industry. The chemicals chosen for study within Greenchem are of interest to both Swedish and international industry, and are targeted for application in environmental-friendly surface coatings, lubricants, consumer care and cleaning. The product examples include:

- **Epoxides** (fatty epoxides, glycidyl ethers)
- **Acrylates** (acrylated polyesters and oils)
- **Polyol esters**
- **Alkyl glycosides**
- **Alkanolamides**

Greenchem entered its second phase of existence in 2007 after a Phase 1 of 3.5 years. The second phase is regarded as an implementation phase, i.e. the results achieved will be transformed into application with an environmental benefit.

PHOTOS AND ILLUSTRATIONS:
Cover by Anna Rosenquist and Ulrika Törnvall
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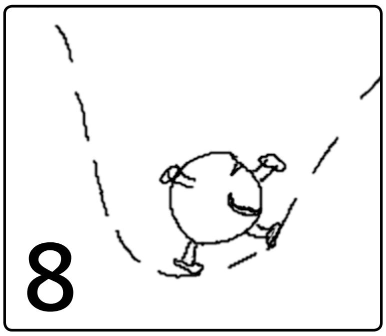

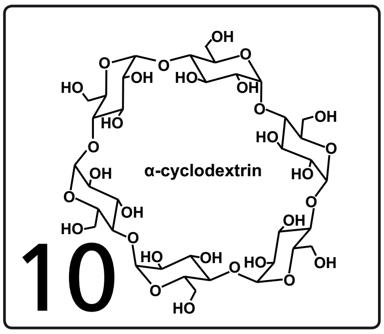

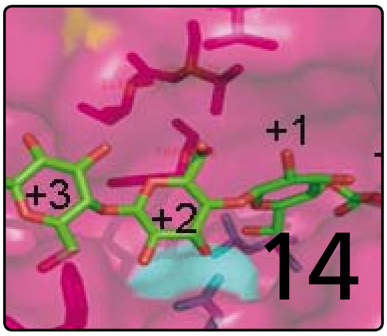

A few words from...

THE BOARD

The year 2009 was a year of financial crises when companies would not look further than to the closing of the books for the year. This had an impact also on the climate meeting in Copenhagen were several countries where afraid that climate measures would be a threat against necessary growth. Contrary to this WWF presented a report showing that by 2030 a saving of 1-2,5 billion tons of CO_2 per year would result from the application of industrial biotechnology in various ways and for various purposes, a saving giving also competitiveness and work and providing benefits for sustainability and the environment. The Greenchem programme provides the means for some of these savings. It also initiates support for industrial biotechnology in general which will provide further savings in CO_2.

Areas in which environmental gains can be achieved by industrial biotechnology are in the production of fuels, materials and chemicals, in the use of wastes and by-products, in using enzymes for making various human activities more efficient and for different purposes in the chemical, pulp-and paper, food and textile industries as well as in agriculture. Only some of these applications are a direct part of the Greenchem programme. The use of enzymatic technology however may imply use of fermentation for the production of enzymes and for the production of raw materials for the enzymatic transformations. Frequently technologies based on renewable raw materials also include both enzyme processing and fermentation as well as the taking care of byproducts and wastes.

The Greenchem programme was originally set to initiate a paradigm shift in the chemical industry. By the use of enzyme technology production methods would be more benign, products be biologically decomposable, less solvents and toxic materials be used and new products replacing environmentally harmful ones be produced. In addition the productions should also be sustainable. This last factor requires the use of renewable raw materials which builds the connection to the greenhousegas problem which is the prime driving force for the global interest in industrial biotechnology. Typically European conferences on Industrial Biotechnology are totally concentrated on technologies with a CO_2 -profile i.e. biofuel, biopolymers and platform chemicals. For the type of products, however, which are part of the Greenchem programme, the saving of greenhouse gas is marginal and a different driving force is therefore required to achieve the paradigm shift. The environmental improvements are thereby a driving force primarily if there are legal demands for these. The other important driving force is current or future profitability and competitiveness. The rising price and future scarcity of fossile raw materials are then the most important economic factors determining the interest to use industrial biotechnology in the chemical industry. Increasingly a competitive edge may be achieved by providing an environmentally benign product. It normally does not fetch a premium price, however.

The Greenchem Programme in its original form has another year to go. A review of where we stand in relation to the aims set by Mistra is then appropriate. The development of at least one product with a proven environmental profile bound to reach the market is one goal. In order to fulfill this goal the development has to provide profitability for the product at the current price of fossil raw materials or to have a value as an environmentally superior product. Of the successfully developed products, enzymatic production of one normally chemically produced surfactant, did meet the environmental criteria but it did not provide profitability, so it was rejected by the company. For another product (a biolubricaant) the environmental advantage of the enzymatic production may be such that this route will be used although there is no cost advantage. For another one of the developed products (a coating product) using renewable raw material has been a criteria for success as defined by the major customer. This can be said to be an environmental criteria and it is not yet clear if the customer will accept a price providing profitability for the producer. Another product in development has a strong environmental profile but is a new product and may be hampered by the Reach legislation. Finally a group of new surfactants where some may be used with environmental benefits but the initial uses will be in small quantities for special uses without such benefits. These products will form the basis for Devenz AB.

With only one year to go for the Programme the mentioned products will be the ones that may reach the Mistra goal of one product bound to get to the market. We shall therefore in 2010 focus our efforts on these projects to bring them to successful ends or at least to a point where the respective companies will continue the development on their own or together with e.g. Devenz.

The main task for the Board in 2010 will be to plan the great final of the Programme and to spread the results and the experience therefrom as widely as possible. Any possibilities for some kind of prolongation shall also be investigated.

Harald Skogman
Chairperson of the Board

THE INDUSTRIAL PARTNERS

A program passes through a number of phases, each one with its own characteristics and charm. With now only one more year to go the thrill is to use the knowledge and experience gained so far in order to stretch and reach the final goals. In the iterative process of enzyme preparation, biocatalytic synthesis, scale up, testing and feedback, each cycle has a purpose in the sense that it builds knowledge and speeds up future cycles. Cycles resulting in a well performing product are easy to identify as successful, but not even then the activities stop. Further optimization is necessary in order to achieve a commercially viable product and this activity can be just as challenging as those of the first cycle.

The Greenchem projects span all the way from fundamental research to product on the market. At the Autumn Meeting some of the industrial representatives noted that this situation is rare, judging from other projects we participate in. The wide scope makes the work of Greenchem inspiring as well as challenging.

A variety of ongoing or planned industrial activities were brought up at the Autumn Meeting; at ANIC application testing and price level information, training of academic personnel at Perstorp, investigation of possibilities for scale up at AAK/Binol, preparations for cell cultivation at Protista, skin irritation study performed at ACO, preparations at ANS for application testing, Astra Zeneca presenting conclusions of product evaluation so far and plans for a showcase, IKEA raising questions contributing to application focus and quality. The groups within Greenchem are working on the details, just as it should be, each contributing with their piece to the big picture.

Johanna Brinck
Chairperson of the Industrial group

THE MANAGEMENT GROUP

Over the past decade or so, the society has focused on the production of biofuels, primarily to replace a fraction of the fossil fuels and also to reduce the carbon footprint. Now as we leave the year 2009, the bio-based industry has realized that in order to have economy in the use of biomass, production of chemicals and materials also needs to be integrated with that of biofuels. Having started already in 2003, Greenchem has hence been a forerunner in production of bio-based chemicals and also in the use of processes based on green chemistry principles.

It is with mixed feelings that we enter the final year for the programme. Greenchem has become kind of a brand for chemistry for a better environment and many of us who have been associated with it wonder how to continue the work in the future. We can however already see the Greenchem spirit has proliferated into several other projects including the development of biorefineries that are supported by other funding agencies. Moreover, the students who graduated from the programme have found places where they can utilize their knowledge and experience and multiply the effect.

During 2009, the collaboration between the researchers and industrial partners of Greenchem had excellent collaboration through the two Application Management Groups. There has indeed been a focus on product development and a common interest to solve the problems. It is our hope that the industry will take up the products that show promise and help to take them to the market.

Rajni Hatti-Kaul
Programme director

The future is not what it used to be

At the end of year 2009 all kinds of journals and newspapers were filled with articles that took a retrospective look at the first decade of the new millennium. Ten years of highlights in the popular culture, the economy, the political situation, and many other issues, were analysed and judged by journalists and analysts. From the financial point of view, 2000 – 2009 was more or less a lost decade. From the perspective of public policy and human rights we saw some catastrophic situations, but also some hope for the future. From the environmental point of view the past decade demonstrated an unexpected, but very urgent focus, on the climate change issues. What will the future look like and will there be a place for industrial biotechnology in the future society? Let us take a look in the crystal ball and reflect over role of technical innovations in the shaping of the future.

The role of technical innovations

The idea that technical innovations result in an improved environment has not always been a generally held view. In the 1970s and 1980s, a large number of people had little faith in the potential of technology. There were real concerns that population growth, famine and environmental destruction would affect the world's population to an ever-increasing extent in the future. Some of these problems have, however, been reduced. From a global perspective, poverty is falling, average life expectancy is increasing and a growing number of decision-makers, and ordinary citizens, have realised the significance of environmental issues. One interesting observation is that commercial companies put more and more effort to reduce their environmental impact, and a relevant question is: What makes companies implement stricter environmental policies and develop new environmentally-friendly products and services? I believe that the following driving forces are of great importance:

- Increased environmental awareness in society, by consumers and business partners.
- More stringent environmental legislation.
- Economic control measures.
- International agreements.
- Commercial threats and opportunities.
- Technical innovations.

Will the next megatrend be about cleantech?

Modern life is dependent on innovations from the past two or three centuries. The steam engine, electric power, the internal combustion engine, petrochemicals and computer technology have all resulted in strong growth and have created completely new economic and social patterns in society. Such development patterns are referred to as megatrends, and make a significant impression on history. If we regard megatrends as "waves in economic activity" (Kondratieff cycles or waves), they appear to be becoming increasingly short. The circumstances showed below will probably become important technical development factors that will influence the next Kondratieff wave:

- Globalisation with a more closely-linked world. The "global village", that Marshall McLuhan forecasted in the 1960s, is actually already here.
- The world's largest economies will be situated in Asia. For example, China Daily reported in January 2010 that by 2015 China is forecast to overtake the US as the world's largest chemical producer.
- An ageing population in many countries will lead to changes in consumer patterns, new views on working life and increased demands for more and improved healthcare.
- New lifestyles and values will take root. Ethics, individual solutions and personal wellbeing will become increasingly important.
- A more widespread understanding of how humans affect the environment will make sustainable development and growth, as well as social responsibility, more significant components.
- The significance of climate issues has recently permeated the public debate. If human beings take the threat of climate change seriously, new production and consumer patterns will emerge.

The figure shows an illustration of five, and a possible sixth, Kondratieff waves. It is obvious that we are currently surfing at the top of a gigantic "communications wave", but also that we are highly impacted of what was created during the previous waves in economic activity. However, there is considerable evidence to suggest that we are heading for a sixth cycle. We don't yet know if this trend will consist of some kind of "new economy", or something else. At present, there are clear indications that clean technologies (cleantech), and other activities that support a sustainable future, could be important components in the sixth wave.

Cleantech

Clean technology or ”cleantech” is a concept which is rapidly gaining momentum. The cleantech concept covers more than the environmental technology, or green technology concepts, used in the 1970s and 1980s in particular. Back then, environmental technology was associated with technology which responded to regulations, or ”end-of-pipe” solutions, for example emission abatement equipment for air pollutants. Cleantech is a broader concept where new technological solutions and business models provide solutions to global environmental challenges, and meet the requirements to provide a competitive yield on investments.

Venture capital is looking for suitable candidates

There are many companies with exciting technology and good future prospects. Venture capital companies all over the world are therefore searching for suitable candidates for investment. The reason behind their growing interest in cleantech is, as we have seen before, due to several parallel driving forces, such as climate change, high energy prices, oil dependence, more stringent environmental legislation and a growing understanding of the importance of environmental issues. These factors seem to have triggered a trend break in the appreciation of the commercial opportunities in environmental technology. Not only do investors look for companies with environmental technology as their core business, but also for companies which develop applications for the technology. A sound approach to the environment involves a combination of organisational management systems, conservation of natural resources and the application of various technical solutions. Venture capitalists have taken note of this fact.

Greenchem and the sixth Kondratieff wave

So far, the Greenchem project has generated both useful scientific information, and technical insights, concerning the role of industrial biotechnology in a future society. If we take a look at the definition of cleantech, Greenchem certainly meets the criteria of providing technical solu-tions to tackle important environmental issues. What so far has been more difficult for us to achieve, according to the cleantech definition, is to provide a quick pay-back time on financial investments and expenditures. However, according to my opinion, there must be an exciting future for a concept that will result in reduced use of fossil fuels and petrochemicals, and that utilises energy efficient biotechnical processes with less toxic waste products and emissions.

The future is not what is used to be – and will probably never be what we expect it to be. However, as society (hopefully) is heading for a more sustainable future, we should reap the harvest of the results of the Greenchem project, but also create new results. This will give us a good op-portunity to surf on the coming sixth Kondratieff cleantech wave.

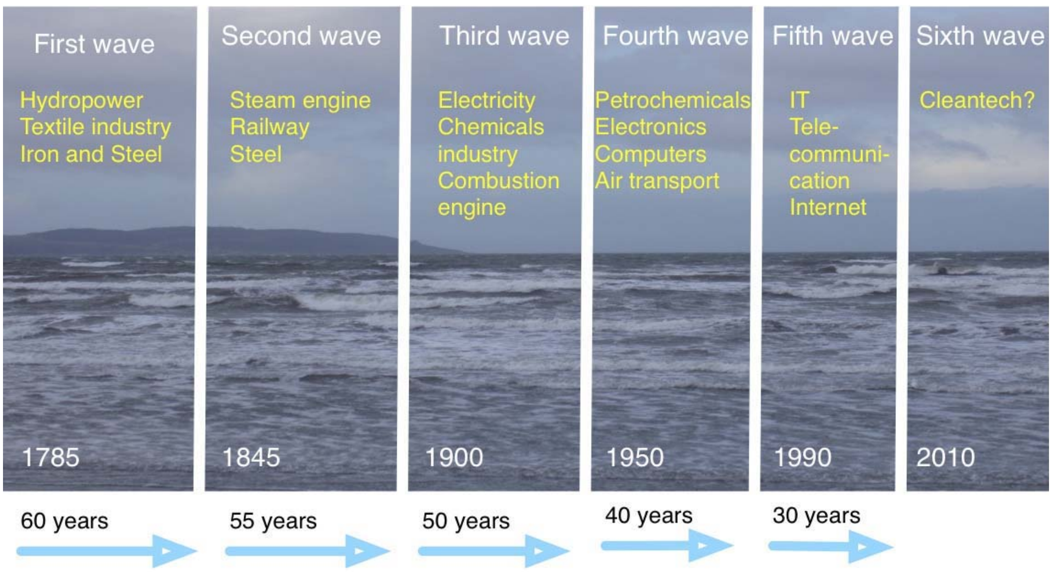

Figure. The figure shows an illustration of five, and a possible sixth, Kondratieff waves.

Torbjörn Brorson, Lund University, member of the Greenchem Board

The Biotechnology dream

The biotechnology boom has long been spoken of and biocatalysis is often expected to play a main role in the paradigm shift from depleting to renewable feedstocks for chemical production. Almost every biotechnologist working with characterization of new enzymes, or improvement of old ones, dreams of seeing an industrial process (so be it one kg product or thousands of tons a year). The expected boom is easy to understand thinking about the fantastic potential offered by the about 3000 enzymes discovered so far, especially considering enzyme reaction efficiency and specificity. However, the biotechnologists' dream fits less well with the fact that only 134 industrial processes were based on any kind of biocatalysis in 2002. The figure was probably still the same one year later when Greenchem was started with the vision to amplify this number. The mix of slightly over-optimistic enzyme technologist with hardcore chemistry industrial realists has over the years endeavored one main new insight for each group. Or to quote a representative from the latter group: "You can do pretty damn cool stuff with enzymes". While the people in the former group have come to realize that you do not get far from the lab with your new process unless you accomplish to keep the account on the same, or preferably bigger, plus as with the current method.

Steeplechase challenges

Although reported life times for some immobilized biocatalysts are several years, stability remains a major hurdle for the broad front implementation of industrial biotech. And hurdle is here not to be understood as the light portable barrier over which competitors like Susanna Kallur must leap in certain races. No, to decrease the contribution of the enzyme to the overall production cost of bulk chemicals is yet in most cases rather a steeplechase. One of the biggest remaining tasks for the industrial biotechnologists (and hence also for research funding distributors) is a joint effort on improving the stability of process enzymes. Enzyme stability in this case reflects the extent to which an enzyme retains its structural conformation, and its activity, when subjected to process conditions. The reasons for deactivation can be almost anything, for example heat, oxidation, extreme pH and mechanical distortion due to stirring. Probably the single most devastating to industrial enzymes is heat, hampering the re-use of many an interesting enzymes. Imagine for example that you are frying an egg, after a while you will obviously notice coagulation of the egg in the pan, which is due to conformational changes of the egg protein caused by the heat. Would you ever consider using the same coagulated egg for a second round of egg-frying? Probably not. So what can be done? The most obvious approach would be to create a more stable enzyme by well-known stabilizing methods for example by introducing extra disulphide bridges, increasing internal hydrogen bonds, improving internal hydrophobic packing or increasing surface salt bridges. In fact, with such methods almost any enzyme could probably be engineered to withstand over 100 degrees. But since enzyme stability is in a more or less opposite relationship to activity, introducing such rigidity to the enzyme makes it about as useful as a waterproof teabag (Figure 1). Increasing the stability of enzymes is certainly a tricky issue, needing more attention.

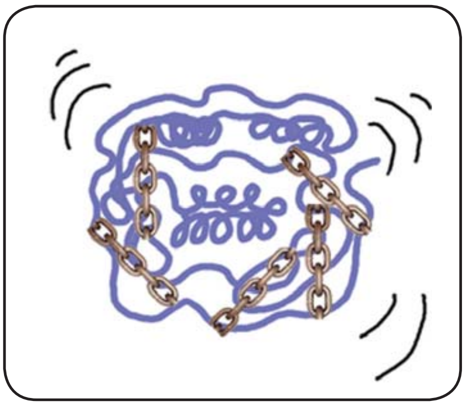

Figure 1. Using molecular biology, it is quite easy to make an enzyme more stable. There is however a fine line between increased stability and decreased activity.

Flipping the coin versus the direct approach

One approach for enhanced stability is to use random mutagenesis; no background information is needed since huge libraries of mutants are produced and thereafter screened for improved properties. Using this method, even the thermostability of the Greenchem work horse *Candida antarctica* lipase B have been increased. A prerequisite for the random approach is that a screening method distinguishing between good and bad candidates of the right property is at hand. Instead of letting the PCR machine flip the coin for which position to mutate, the molecular biologist could spend a few hours company with a well-programmed computer and end up with a dozen promising mutations ready for the lab. Sensitive amino acids can be detected using analysis methods such as bottom-up proteomics, i.e. determination of the amino acid sequence and post-translational modifications by proteolytic digestion of the protein and subsequent analysis by LC/MS/MS. Once the sensitive amino acids are known, these can be exchanged using site-directed mutagenesis. Enzymes have become remarkably resistant towards hydrogen peroxide just by replacing one key methionine to alanine or serine.

In other words; there are remarkable enzymes that can (at least for a while) withstand oxidizing conditions strong enough to turn the darkest black hair into pale white. There are several methods to improve them even further. Let's take a renewed joint effort to make the biotechnology dream come true!

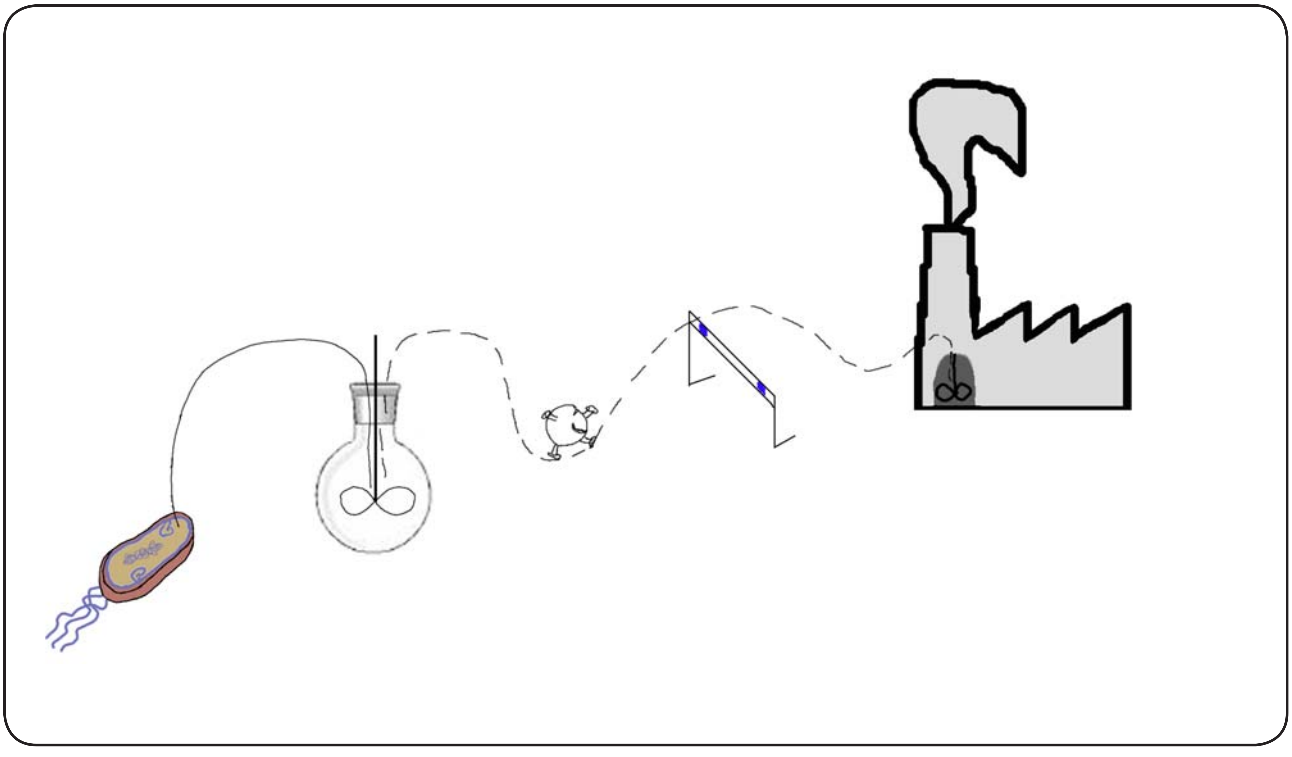

Figure 2. The path of an enzyme from a microorganism into our lab reactors, which is already accomplished, and further into the large scale industrial reactor, which in most cases still needs more attention.

Ulrika Törnvall, PhD, Lund University

Are we looking at a paradigm shift from chemical to biocatalysis within the synthesis of surfactant?

The vision of Greenchem is to initiate a paradigm shift within chemical industry from classical chemistry to chemistry based on biotechnology to develop clean, sustainable technologies to effect the production of “green” chemical products from renewable raw materials. Have we succeeded within the field of surfactants? The answer to this question is both yes and no.

Looking at the product segment “alkyl glycosides”, which has become a very important group of sugar based surfactants especially within areas such as detergency and personal care, can be roughly divided into two groups: technical grade (alkyl polyglycoside, (APG)) and “high-end” (enantiomerically pure) alkyl glycosides.

APGs are produced on very large scale (several thousands of tonnes annually) in a chemical process called Fischer glycosylation. This is an established very efficient process using, already today, to a large extent renewable feedstock. However, there is still a barrier to overcome - high energy consumption! This is not only because the reaction itself is run at high temperatures (100 °C or higher) but there is also a need for removal of excess fatty alcohol (one of the starting materials). The larger the alcohol is the more energy is required! The same goes for the making of high-end alkyl glycosides, but in this case another dimension is added to the chemistry – selectivity. In classic synthetic chemistry this is very difficult and time consuming to achieve and has to involve extensive protection/deprotection schemes (will be described in more detail below). This is because carbohydrates contain several hydroxyl groups with similar reactivity. However, if one could introduce biotechnology or more specifically enzymes into these chemical routes, it would be a great leap towards a paradigm shift to produce “greener” products. The use of enzymes will bring about several advantages; lowered energy consumption (lower reaction temperature and no or reduced need for removal of alcohol) and above all avoiding protection/deprotection chemistry. Within Greenchem we started working with the high-end (high-price!) alkyl glycosides, especially the challenging ones having both long carbohydrate and long alkyl chain. Previously, preparation of such products has been reported using synthetic chemistry based on protection/deprotection strategies involving reactive reagents and organic solvents. The initial step in preparing alkyl glycosides is to protect the hydroxyl groups of the carbohydrate chain (final size hydrophilic part). There are several methods of doing this but the most common is acetylation using alkali metal acetate in acetic anhydride at high temperatures (140 - 170 °C). The protected carbohydrate is then reacted with an appropriate fatty alcohol (alkyl part of the surfactant) using a catalyst like a weak Lewis acid (typically $SnCl_4$ or $FeCl_3$) or when needed hetero-polyacids (phosphomolybdic and phosphotungstic acids) in an organic solvent (typically toluene) at moderate temperatures (~80 °C). The final step is eventually to remove the protection (acetyl group) of the remaining hydroxyl groups using sodium methoxide in methanol. Even if this seems pretty straight forward, it is however tedious work since every step involves product work-up and the use of solvents and catalyst is great (50 to 100 wt% catalyst per added carbohydrate). It is not particularly hard to appreciate the need for improvements. The biggest obstacles in classical synthetic chemistry are above all to efficiently bring the substrates together and to introduce selectivity. This is where enzyme technology has the opportunity to accomplish deed and to play a main role in the initiation of a paradigm shift towards production of “greener” and more efficient surfactants. Enzymes are remarkable catalysts that can, as we have proven, perform very useful chemistry under very mild condition, which sometimes is impossible to accomplish with other means. There is though a tiny little hold-fast, one has to find a suitable enzyme to do the job! As is, the enzymes suitable for this assignment do not particularly enjoy being in contact with the alcohol, they rather stick to water (these enzymes are naturally active on carbohydrate that is poorly soluble in other solvents than water). Since larger fatty alcohols are poorly or not at all soluble in water, it is impossible to produce alkyl glycosides at satisfactory yields with appropriately long alkyl chain (coming from the fatty alcohol) using these types of enzymes. However, one should not be too pessimistic because the battle is far from lost! It is very important to realise that only a tiny number of nature's own enzymes have been identified and that we always have the possibility to modify these enzymes to agree with the specificities needed for the job. In our case, the solution was to use a combination of a new synthesis strategy and a new type of enzyme.

Instead of letting the enzymes synthesize the target molecules from fatty alcohol and carbohydrate, we chose to improve already existing alkyl glycosides by adding glucose residues. Luckily, nature has provided us with a very useful group of enzymes for doing this job: the cyclodextrin glycosyltransferases (CGTases). They can in one step, without the use of protection and in water, add several glucose residues with selectivity and high efficiency to give us a new range of highly desirable surfactants (Figure 1). For these high-end products, the enzymatic method is clearly superior, both concerning technical simplicity and environmental impact and we can say that a paradigm shift has been initiated in this field. During the final part of the Greenchem program we will focus on making the reaction more efficient to make it competitive also for the production of less sophisticated surfactants which are used in larger volumes.

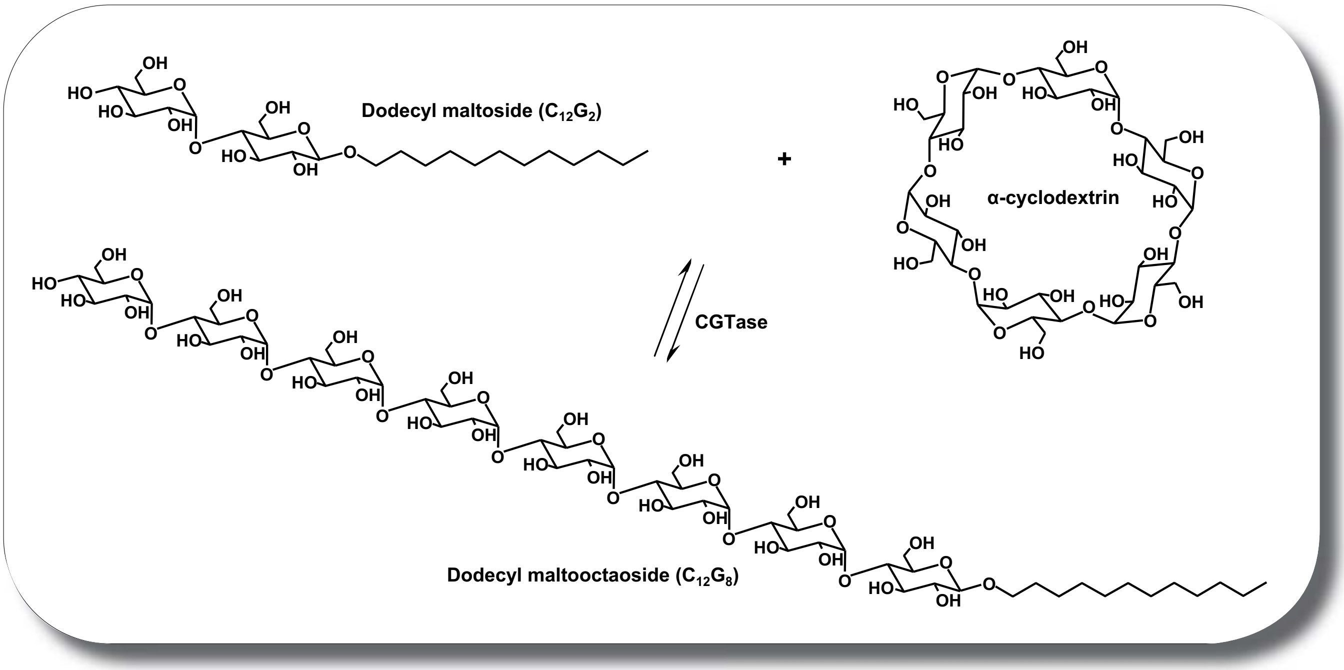

Figure. Reaction scheme for the CGTase catalysed coupling reaction between dodecyl maltoside (an alkyl glycoside) and α-cyclodextrin (cyclic carbohydrate with six glucose residues) producing dodecyl maltooctaoside (the target alkyl glycoside).

Patrick Adlercreutz, Project leader, Lund University
David Svensson, PhD, Lund University

Pentose based surfactants: new generation alkylglycosides?

Surfactants are amphiphilic compounds assembled from hydrophilic (water loving) and hydrophobic (water avoiding) components. Although surfactants can be synthesized from naturally occurring renewable resources, petrochemicals or synthetic compounds, the growing trend towards sustainable production favors the synthesis of surfactants from renewable resources. However, the interest in surfactants from renewable resources goes beyond sustainability. A good example could be the alkyl glycosides, surfactants produced from sugars and fatty alcohols, which are widely used in the food, pharmaceutical, hygiene and cosmetic industries. The primary driving force behind the success of these surfactants is not sustainability but rather, their quality, i.e. the good performance and mildness. However, as has always been, there is still an interest in producing efficient alkyl glycosides which are amenable to existing and emerging applications. There may be different approaches that can be employed to produce efficient alkyl glycosides and varying the sugar component seems a possible alternative.

The majority of alkyl glycosides known so far are based on glucose molecules. However, at least from the chemical point of view it is clear that glucose is not the only carbohydrate that can be used in the synthesis of alkyl glycosides. There is a wide variety of carbohydrates that can potentially be used to generate enormously diverse alkyl glycosides from which novel surfactants can be identified for various applications. Constituting up to one-third of plant biomass and being remarkably diverse, hemicelluloses, which have pentoses (five carbon sugars) as primary building blocks, are definitely interesting sources of sugars for synthesis of surfactants. Among hemicelluloses, xylan is the most abundant and accounts roughly for 30% of the total plant biomass. It can be easily extracted from woods, agricultural residues (such as bran and straw) and pulp mill black liquor for synthesis of pentose based surfactants, specifically alkyl xylosides (figure 1).

Synthesis of alkyl xylosides has been achieved by both chemical and enzymatic routes. The enzymatic synthesis that uses xylanases (xylan degrading enzymes) has the advantage of providing a cleaner process involving fewer steps due to the high selectivity of enzymes, and is accomplished under mild reaction conditions. However, it has its own challenges. The yield is low and it disappointingly decreases further as the carbon chain size of the hydrophobic tail (the alcohol) increases. Work within Greenchem has led to a better yield of alkyl xylosides than what has been reported in literature for similar systems, by the use of a thermostable xylanase from Thermotoga neapolitana, a hyperthermophilic bacterium that optimally grows around 80°C. Moreover, an integrated system of online product recovery that separates the alkyl xylosides from the reaction medium has also been developed and proven useful in improving the alkyl xylosides yield. But, are these surfactants better than the existing alkyl glycosides? Well, let us have a look at the properties of one of the alkyl xylosides which may possibly give us some clue about the performance of these surfactants.

Octyl xylobioside, the major alkyl xyloside in the project, has been purified and characterized. Compared to the glucose based octyl maltoside, octyl xylobioside has shown remarkable foaming properties. The same amount of octyl xylobioside gives higher foam volume than octyl maltoside, with long lasting, small, wet and spherical foam bubbles. Nonionic surfactants are known to be mild towards skin, hair and eyes, but they normally exhibit poor foaming properties. Thus, alkyl xylosides, being nonionic but with superior foaming properties, provide an attractive alternative. As consumers often equate rich and long lasting foam products with high quality, xylose based surfactants could be very attractive in the formulation of luxurious hair shampoos, body gels, dentifrices and shaving creams. Moreover, due to the high water retaining nature of xylose, skin and hair care products containing alkyl xylosides are expected to prevent the skin and hair from drying and problems related to dryness such as weak or brittle hair formation. Derivatives of xylose such as pro-xylane are known to have skin anti-aging activity, and have proven ef-

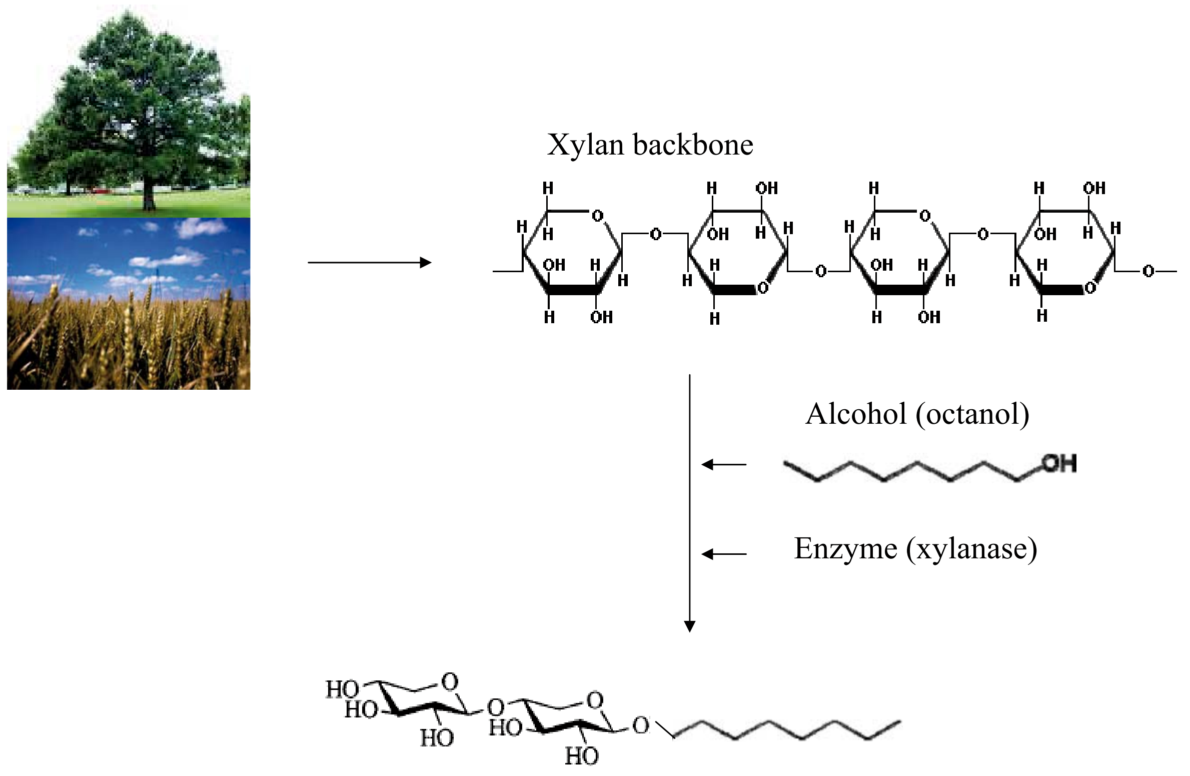

Figure. Schematic presentation of production of alkyl xylosides from renewable resources

ficient to alleviate age related wrinkles, sagging and pigmentation. However, it is unclear whether alkyl xylosides have a similar 'rejuvenation' effect or not.

The other character determined for octyl xylobioside was the critical micelle concentration (CMC), which is the concentration of surfactants in the bulk at which micelles start forming. Often, lower CMC is indicative of a more efficient surfactant. The wetting property, which is the ability of surfactants to make liquids spread across or penetrate the surface of a solid more easily, has also been studied by us. High wetting surfactants are desirable in cleaning detergents because, in their presence, dirt is more easily loosened and more efficiently removed. Moreover, due to the efficient spreading, better adhesion and reduced run-off properties they impart to solutions, high wetting surfactants are more amenable in the formulation of spray solutions such as foliar sprays. Octyl xylobioside has remarkably much lower CMC and higher wetting property than octyl maltoside. Due to favorable features of xylose, a similar trend of excelling properties is expected for other alkyl xylosides as well and this may possibly lead to an increase in alkyl xyloside containing products in the market. Thus, it is tempting to say that these pentose based surfactants are potentially the 'next generation' alkyl glycosides. Who knows?

Gashaw Mamo, Post doc, Lund University

Challenges in directed enzyme engineering and production

Since the beginning of this millennium we are witnesses of a change, which can be described as a paradigm shift, in exploitable enzymology. We have come from species collection, purification and laboratory experimentation to the mining of bioinformatics databases followed by selection of organisms hosting interesting activities. Now when we find such candidates, we can continue to design (on DNA level) individual template enzymes based on rational design or randomised development, screening or selection to obtain candidates, which in a following step are produced in recombinant host systems, to get access to the developed enzyme.

The rational design approach is based on structural evaluation of a target enzyme followed by specific changes in its coding sequence, leading to defined changes in the DNA-codon encoding a defined amino acid residue. This method is frequently used to evaluate the importance of individual defined interactions in the catalytic site of an enzyme. Using this method you produce a limited number of predetermined mutations. These mutations are obtained as the single products after mutagenesis, and you do not need further selection. The limitation in this method is of course that you will only get the mutants that you design, and interesting changes not immediately positioned in the catalytic site are easy to overlook.

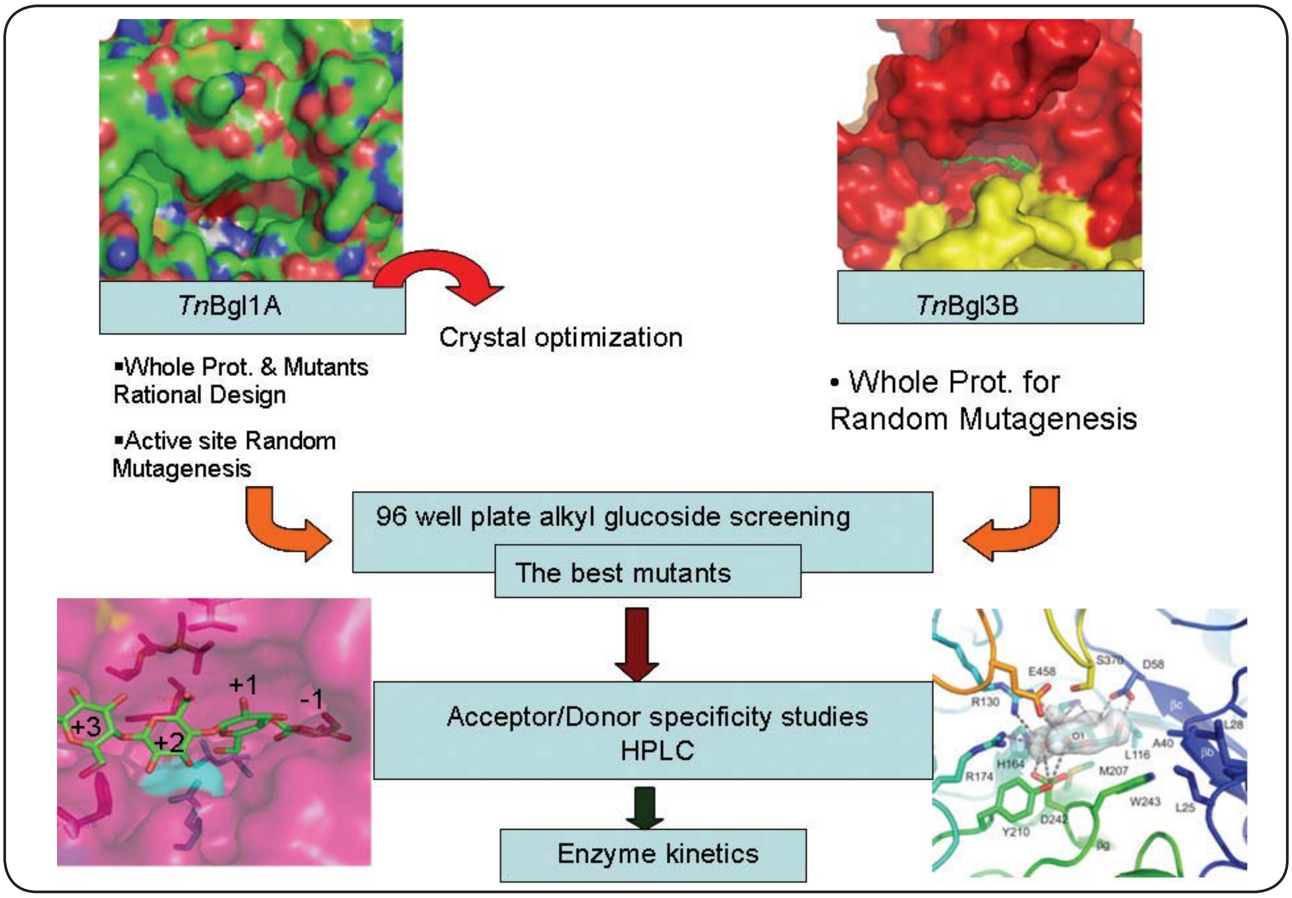

Figure 1. Development strategies for the beta-glucosidases Bgl1A and Bgl3B utilized in Greenchem for production of alkyl glucosides

Directed evolution is a method developed as an approach to expand natural diversity, thus being able to introduce changes not prioritized by nature, and gain desired new activities among biocatalysts /enzymes. This process begins with the generation of a large and diverse DNA library, using different molecular techniques, followed by screening, identification of the mutants with the desired activity and ending with further structural and biochemical studies of the successfully improved biocatalyst. The bottlenecks with this approach are the need of automated equipment for screening of large libraries, combined with efficient screening methods, to be able to find the changes that are beneficial for your specific purpose. Apart from the immediate possibility to analyse a large amount of variation, another advantage is that you can find changes, indirectly affecting the catalytic activity, and those residues do not necessarily have to be located at the catalytic site, but can have an indirect effect via other residues in the enzyme molecule.

Development of screening methods is thus a crucial step as soon as a large number of variants is to be evaluated, and the development of assays that can use microtiter plates for detection by various methods is crucial for success.

With such a method in place, this can be utilized in screening both the results of a random mutagenesis of an individual gene, but of course also for screening of natural diversity, if you have the access to a number of different microorganisms that are natural producers of a specific type of enzyme activity. After the initial discovery of such an enzyme with potentially interesting characteristics for biotechnological application, the next major challenge is to obtain sufficient amounts of the protein for further study, including structure determination or reaction optimization, and eventually for industrial scale enzyme production.

While isolation of enzymes from the original organism producing the protein is still possible, production of the enzyme is often facilitated by expression in a heterologous host, most often *Escherichia coli*. Other expression systems include yeast such as *Pichia pastoris*, which may improve expression of eukaryotic proteins and can include postranslational modifications, but which may require optimization of growth and expression induction conditions. Other bacterial systems, such as Bacillus-based expression, as well and insect and mammalian expression systems, are also commercially available. However, *E. coli* is often the organism of choice for initial attempts at optimizing enzyme expression, since the organism is easy to work with and the genetics and molecular techniques are well established.

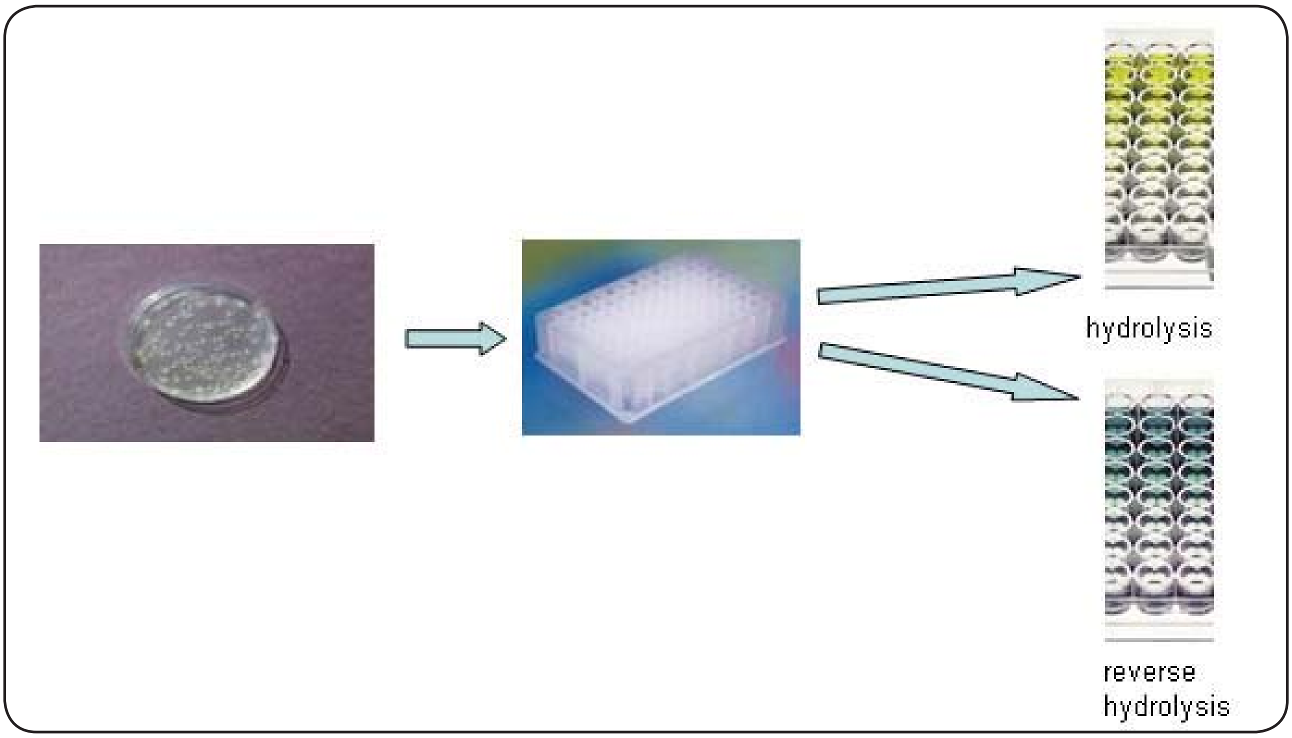

Figure 2. A new screening method for hexylglucoside production, developed within the Greenchem-program

Due to the unusual origin of many enzymes with interesting biotechnological properties, such as thermophilic archaeal, it is often a challenge to obtain sufficient amounts of protein, even in recombinant systems.

With the extensive molecular biological knowledge of the steps involved in protein expression, including transcription, translation and folding, in *E. coli*, one option is to choose different host strains with different genetic backgrounds that may permit increased expression of the desired enzyme. Strains are available that: provide rare codons that are not usually available in high quantities in *E. coli*; assist in incorporation of disulphide bonds in the target enzyme; stabilize the mRNA transcript; and, co-express additional chaperone proteins to accommodate the additional folding required in the cell following induced protein expression. Strains also exist that allow for tuning of inducer concentration. Optimization of growth temperature, induction timing and media components for expression of the target enzyme in any of these *E. coli* strains may result in increased recombinant protein expression.

In addition to controlling which heterologous host, *E. coli* or otherwise, is chosen for expression, another option is to alter the gene sequence to increase protein expression. As the genetic code contains redundancy, mutations can be introduced into the DNA sequence that will affect expression, without altering the protein sequence. These could include exchanging rare codons for codons suited to the expression host, optimization of promoter sequences, alteration of mRNA secondary structures impeding translation, and removal of alternate start codons. In some cases, a synthetic gene may be constructed using the host codon distribution that will still express the protein sequence of the desired enzyme. It is often the case that optimal expression of an enzyme must be empirically determined requiring a systematic investigation of any or all of the options mentioned above. While this can be laborious, the numerous options available increase the possibility that optimal expression of the desired enzyme is possible, once the correct conditions are determined.

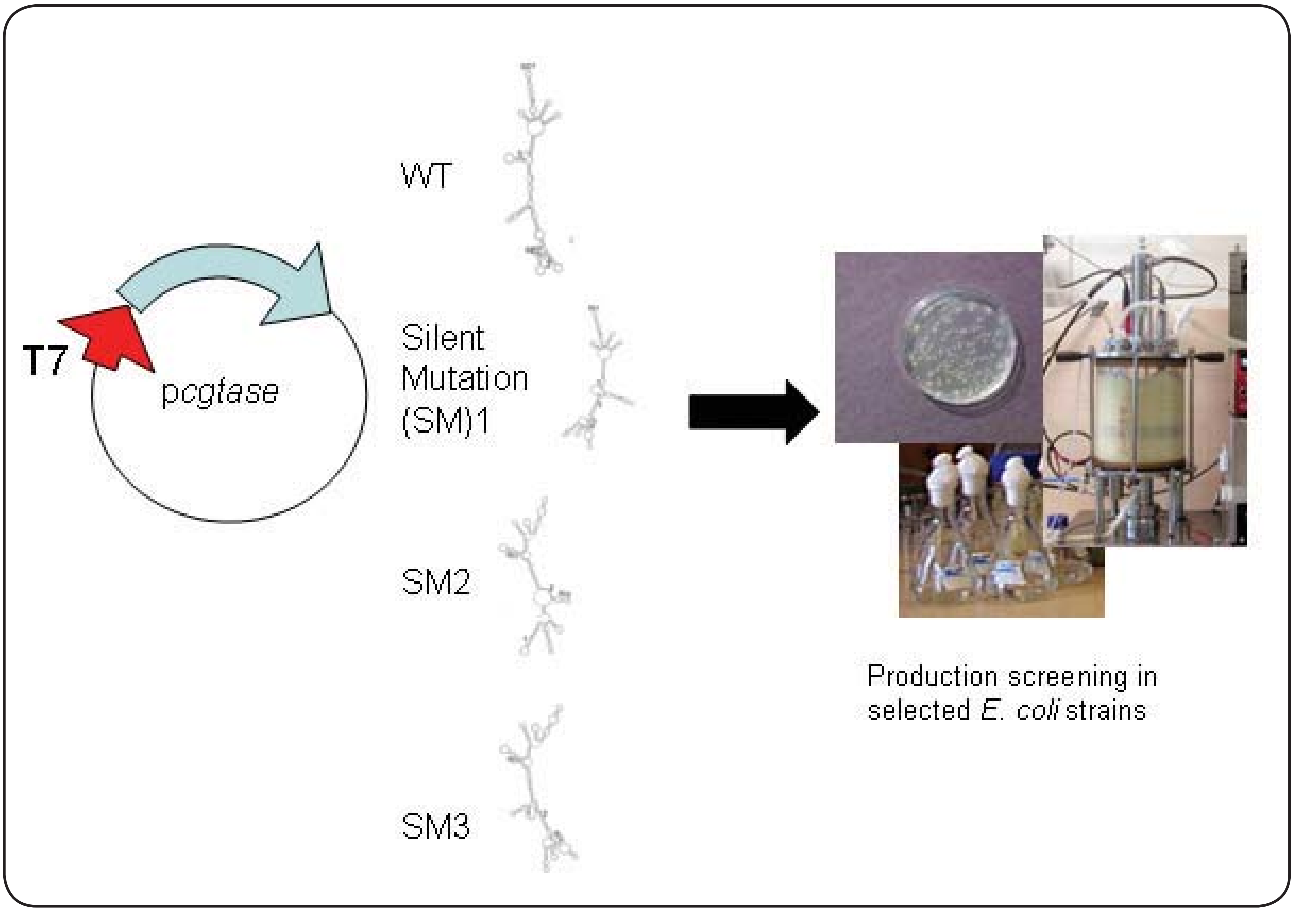

Figure 3. Recombinant production of an archaeal glycosyltransferase in *E. coli*

Catherine J Paul, Post doc , Lund University
Tania Pozzo, PhD , Lund University,
Eva Nordberg-Karlsson Project leader, Lund University

Environmental assessment of biolubricants

Why biolubricants?

Lubricants are necessary in almost all mechanical equipment regardless of size; they are used to reduce friction and wear, to keep moving parts apart, transfer heat and power as well as for several other applications. Lubricants have been used for thousands of years and until a few decades ago most lubricants were made of vegetable oils or animal fat. Today, the majority of lubricants that are used are derived from fossil oil. Some attempts to produce lubricants from vegetable raw materials have been made after the oil crisis in the 1970s but these biolubricants consisted of native fats and had very poor oxidation stability and technical properties.

Today increased environmental concerns have created a new demand for environmentally acceptable lubricants. The concern about climate change and depletion of fossil resources contribute to the interest in biolubricants but another important factor is the concern of the ecological fate of the lubricants when released in the environment. For example, in forestry spills and leakages of lubricants with high ecotoxicity and low biodegradability can cause great damages to the ecosystems exposed. For lubricating applications such as hydraulic fluids in hydraulic systems the spills of lubricants to the environment are extensive. In some cases 60-80% of the hydraulic fluids used are released in the environment. Thus, an increased use of vegetable oil based hydraulic fluids in forestry will be a gradually more important measure to fulfil the sustainable criteria established in the forestry certification schemes.

Vegetable oil based lubricants have shown to have better ecological properties than more conventionally used lubricants of fossil origin. The development of vegetable oil based lubricants in the last two decades has created products which have good oxidative stability and are technically equal to mineral oil based lubricants but are more biodegradable. One strategy to improve the properties of vegetable oil based lubricants is to create esters between the fatty acids of the

An increased use of biobased hydraulic fluids in forestry is an important environmental measure.
Photo by Stana, Photo Xpress.

oil and a polyol i.e. TMP (trimethylolpropane) or pentaerythritol. The tri-ester between TMP and rapeseed oil fatty acids, mainly oleic acid (TMP oleate), is commonly used as a base fluid for hydraulic fluids. This product has also been successfully produced by biocatalytic methods within the Greenchem programme.

But are the biolubricants really as environmentally superior as they are said to be? The environmental properties of a typical biolubricant and a mineral oil based lubricant were compared by Life Cycle Assessment (LCA). The biolubricant in this study was TMP oleate derived from rapeseed oil, produced either by an enzyme or chemically catalysed production method. The functional unit was 1 litre of base fluid for hydraulic fluids and the LCA was cradle to gate including waste disposal. Rapeseed was assumed to be cultivated in Denmark which is representative for average northern European conditions. Production of lubricant was assumed to take place at an arbitrary location in Europe.

The environmental impact was assessed in the categories Global Warming Potential (GWP), Primary Energy Consumption (PE), Acidification Potential (AP), Eutrophication Potential (EP) and Photooxidant Creation Potential (POCP). The main contributions in all impact categories were due to the raw materials. TMP oleate had lower environmental impact than the mineral oil based lubricant in the categories GWP, PE and POCP. However the contributions to AP and EP were higher for TMP oleate than the mineral oil based hydraulic fluid. The main contribution to EP came from nutrient leaching in rapeseed cultivation. There was no significant difference in environmental impact from the chemical process and the biocatalysed process. The yield from the chemical process is as high as the yield from the enzymatic process and none of the processes include organic solvents or other environmentally harmful substances. Although the lower reaction temperature in the biocatalysed process was not favourable from an energy consumption perspective due to longer reaction times. However there are still possibilities to improve and optimise the biocatalytic process to be competitive in the future. Looking at the environmental impact from the entire production chain for TMP oleate the impact from production was minor.

A few years ago, an EU-labelling for environmentally compatible lubricants was established. To obtain this label the lubricant shall contain a certain amount of renewable raw material, it can not be toxic to humans or ecosystems ant it must be easily biodegradable. In forestry application biodegradability is perhaps the most important environmental aspect since spills of lubricants come in immediate contact with sensitive forest ecosystems. In several studies using both standardised and modified biodegradability tests, biolubricants have proven to be more easily biodegradable than mineral oil based lubricants. Within our work a biodegradability test is also being performed to investigate if biocatalytic production will increase this additional important property of a biolubricant.

Biolubricant is a typical example of a renewable product showing several environmental benefits over a product based on fossil raw materials. The performance would be even better if the spills could be reduced and the amount of oils recovered after use could be increased.

Anna Ekman, PhD, Lund University
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Challenges and opportunities for renewal in the Swedish chemicals sector

The Innovation System Study which focused on challenges and opportunities in the Swedish chemicals sector was finished in 2009. This study will be complemented by an international outlook in 2010 to allow for comparisons concerning conditions for industrial (white) biotechnology in different countries (including the Netherlands, Germany, Finland, Japan and Canada) and different sectors (chemicals and forest & paper industry).

The current state of white biotechnology in Sweden was investigated by interviewing representatives from universities, industry and governmental agencies in connection to the field of biotechnology. All in all 38 people have expressed their views and the information has been analyzed from an innovation systems perspective. An innovation system is defined as "network(s) of agents interacting in a specific industrial area under a particular institutional infrastructure that are involved in the generation, diffusion and utilization of technology". This approach then gave a tool for understanding the basic preconditions for innovation and acceptance of change in industry and society at large. The definition used of industrial biotechnology in these contacts was "the process of converting biomass feedstocks into various types of chemical products by means of fermentation or enzymatic conversion". By using a wide definition all potential applications like organic chemicals, bioplastics and biofuels could be included.

It was no big surprise to find a chemical industry in Sweden that generally was weakly involved with industrial biotechnology. Partly this can be explained by the fact that most of the production units belong to international business groups with headquarters and R&D units in different European countries or in the US. The knowledge about industrial biotechnology and the freedom to work actively in cooperation with e.g. universities is limited due to the structure of the industry. Some exceptions exist, however, with Perstorp usually mentioned as the most promising industrial partner for further development.

The main driving force put forward by the interviewees for exploiting white biotechnology is that the fossil raw materials used today for chemicals in the long term must be substituted to renewable ones. Therefore starch, sugar and lignocellulose and other biomass sources must gradually be used as chemical raw material in the future.

However, a paradigm shift tends to be evolutionary, rather than revolutionary, in its nature where long-term, global developments need to be taken into account.

From an innovation system perspective two fundamental system failures were noticeable from our study: organizational thinness and fragmentation.

Organizational thinness is due to the lack of critical mass of actors dedicated to industrial biotechnology in Sweden. There are a handful of excellent research groups at some universities but these are fairly small and marginalized within their internal organizational structures. Therefore it is rather difficult to attract financial and other resources to build up capacity and sustain strong relations with industrial actors.

The other characteristic feature found was fragmentation which means lack of strong interconnection between actors. Few collaborative projects or exchange of knowledge across the boundaries could be found.

Society push is at present strongly towards ”green” energy of all kinds which also might promote more funds directed to industrial biotechnology albeit in competition with other technologies like gasification. Our interviewees stressed that there are enough well trained researchers in this country to do more in the biotechnological field but there is an obvious risk that other countries (like Denmark) with more viable projects will attract some of these.

Some niche products based on industrial biotechnology are already established on the Swedish market. Most interesting from a chemical point of view is probably polylactic acid (PLA) which is produced from corn using a combination of biotechnological and chemical methods and has some unique properties like biodegradability and therefore has got an image of ”sustainable” and ”environmentally benign”. This product has got a sizeable niche market in many countries in spite of a much higher consumer price compared to the traditional polymers.

Mobilizing financial resources will probably remain a crucial problem for white biotechnology, especially in the aftermath of the global financial crisis. Without a strong network of actors including industry and government a general breakthrough of biotech methods for use in chemical industry is remote. In some niches, however, like production of biogas, Sweden can be regarded as a major player in the world.

Another important aspect frequently brought up by the interviewees was the severe doubt that mature industries (e.g. petrochemicals and pulp & paper) are ready to adopt biotechnological thinking. Several believe that typical entrepreneur driven firms outside the present structure must play a more important role as has been the case for red (medical) biotechnology.

In conclusion, the Innovation System Study provides a challenging picture for industrial biotechnology in Sweden, However, a paradigm shift tends to be evolutionary, rather than revolutionary, in its nature where long-term, global developments need to be taken into account. The Innovation System Study in 2010 is going to explore international best practices in terms of industrial activity and policy to complement the above findings on the Swedish innovation system. This will allow for a roadmap to identify and asses possible future pathways for industrial biotechnology.

Lars Coenen, Post doc, CIRCLE, Lund University
Gunnar Modig, Senior researcher, Lund University
Jerker Moodysson, Senior researcher, CIRCLE, Lund University

Publications

Ph.D. THESES

Svensson, D. (2009) Enzymatic synthesis of alkyl glycosides: New applications for glycoside hydrolases, Lund University, Sweden

Törnvall, U. (2009) Chemo-enzymatic epoxidation: Activity and stability of *Candida antarctica* lipase B, Lund University, Sweden

PEER-REVIEWED PAPERS

Published 2009

Börjesson P. (2009) Good or bad ethanol from a greenhouse gas perspective – what determines this? Applied Energy, 86, 589-594

Hagström, A.E.V., Nordblad, M. and Adlercreutz, P. (2009) Biocatalytic polyester acrylation - process optimization and enzyme stability. Biotechnology and Bioengineering 102, 693-699

Leak, D.J., Sheldon, R.A., Woodley, J.M. and Adlercreutz, P. (2009) Biocatalysts for selective introduction of oxygen. Biocatalysis and Biotransformation 27, 1-26

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Törnvall, U., Melin Furst, C., Hatti-Kaul R. and Hedström, M. (2009) Mass spectrometric analysis of peptides from an immobilized lipase: focus on oxidative modifications. Rapid Communications in Mass Spectrometry 23, 2959-2964

David Svensson defended his thesis "Enzymatic synthesis of alkyl glycosides: New applications for glycosyl hydrolases" on the 24th of September 2009. The faculty opponent was Dr. Pere Clapés, from the Spanish Research Council (CSIC), Catalonian Institute for Advanced Chemistry (IQAC), Barcelona, Spain.

On the 29th of October 2009 Ulrika Törnvall was defending her thesis "Chemo-enzymatic epoxidation: Activity and stability of *Candida antarctica* lipase B". The faculty opponent was Professor Uwe T. Bornscheuer, Greifswald University, Germany.

Accepted

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Graeber, M., Rundback, F, Pozzo, T., Andersson, M., Nordberg Karlsson, E. and Adlercreutz, P. A Novel direct screening method for evaluation of alkyl glucoside producing ß-glucosidases. Journal of Biotechnology.

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Pozzo, T., Linares Pasten, J., Nordberg Karlsson, E., and Logan, D. Structural and functional analysis of β–glucosidase 3B from *Thermotoga neapolitana*: a thermostable 3-domain representative of glycoside hydrolase family.

Orellana Coca C., Noraini N., Lämsä M. and Hatti-Kaul R. Synthesis of biolubricants by heterogeneous catalysis.

Törnvall, U., Hedström, M., Schillén, K. and Hatti-Kaul, R. Structural, functional and chemical changes of *Candida antarctica* lipase B on exposure to hydrogen peroxide.

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Adlercreutz D., Tufvesson P., Annerling A. and Hatti-Kaul R. Technical, economic and environmental evaluation of enzymatic alkanolamide production.

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Tufvesson P., Törnvall U., Carvalho J., Karlsson A.J. and Hatti-Kaul R. Cost effective immobilization of lipase B from *Candida antarctica* on Accurel MP1000 for synthesis of specialty chemicals.

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Börjesson P. Are biofuels better than fossil fuels for the reduction of climate change? Plantekongres, Herning, Denmark, 13-14 January 2009. Oral presentation

Börjesson P. God or bad biofuels from a GHG perspective – what determine this? Beyond Kyoto, Århus, Denmark, 5-6 March 2009. Oral presentation

Börjesson P. Bioenergi – hållbarhet, tillgång och konkurrens? NoBio Conference, Oslo, Norway, 2 June 2009. Oral presentation

Börjesson P. How sustainable are biofuels? World Bioenergy & Clean Vehicles and Fuels, Stockholm, Sweden, 16-18 September 2009. Oral presentation

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Hatti-Kaul, R. Biorefineries - a path to sustainablility? Science Forum 2009, Wageningen, The Netherlands, 16-17 June 2009. Oral presentation. Published in Crop Science 50, 2, 2010

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Nordberg Karlsson, E. Glycoside hydrolases from extremophiles for sustainable processing or processes. SASM_Bio2Biz-meeting 2009, Durban, South Africa, Sept 20-23, 2009. Keynote speaker

Paul, C.J., Rask, E., Pozzo, T., Sousa, C. and Nordberg Karlsson, E. Expression and characterization of a GH 57 cyclodextrin glycosyltransferase from the hyperthermophilic archaeon, *Archeoglobus fulgidus*. CBM8, Ischia, Italy, May 10-13, 2009. Abstract and poster presentation

Stiba, K. Perhydrolysis acitivity of *Candida antarctica* lipase B. 14th European Congress on Biotechnology - SYMBIOSIS - Science, Industry and Society" (ECB 14), Barcelona, Spain, Sept 13-16, 2009. Poster

Svensson, D. and Adlercreutz, P. A novel enzymatic method for the synthesis of long carbohydrate chain alkyl glycosides catalysed by cyclodextrin glycosyl transferase (CGTase). Biotrans 2009, Bern, Schweiz, July 5-9, 2009. Poster 136

INVITED LECTURES

Hatti-Kaul, R. Universiti Putra Malaysia (UPM) in Selangor, May 4, 2009 and Universiti Teknologi Malaysia (UTM) in Johor Bahru, Malaysia, May 11, 2009

Nordberg Karlsson, E. Greenchem – speciality chemicals from renewable resources. JSPS colloquium, AlbaNova University Centre, KTH, Stockholm, Sweden, May 25, 2009

Nordberg Karlsson, E. Microbial glycoside hydrolases from extremophiles. Sweden/Japan Cellwall workshop, Stensnäs Conference Center, Stockholm, Sweden, May 26-27, 2009

Nordberg Karlsson, E. Chemicals and Byproducts from renewable resources. Graduate course "Processing Biomass" (3 ECTS) given within the Bioenergy graduate school at the NL faculty, SLU Uppsala, Sweden, October 2, 2009

MASTER THESES

Melin Fürst, C. (2009) Exploring the oxidative inactivation of lipase B from *Candida antarctica*, Lund University

Rask, E. (2009) Exchanging rare codons in the 5'-end of the *Archaeoglobus fulgidus* cyclodextrin glucanotransferase gene affects expression in *Escherichia coli*, Lund University

Greenchem in media

JOURNALS AND MAGAZINES

Green biotechnology attracts financiers, an interview with Rajni Hatti-Kaul
Original title: Grön bioteknik lockar finansiärer, LUM, Nr. 2, 2009

Biotechnology for a better environment
Universitas 21 Newsletter, Issue 14, March 2009

Finally things have started to happen, a chronicle by Harald Skogman
Original title: Äntligen börjar det hända någonting, Kemivärlden Biotech med Kemisk Tidskrift, Nr. 5, May 2009

Almedalen new arena for Mistra
Original title: Almedalen ny arena för Mistra
Mistra Newsletter, Nr. 5, 2009

Hard times for Green Chemistry, interview with Rajni Hatti-Kaul, Jakob Hansen och Pär Tufvesson
Original title: Trögt för grön kemi, Kemivärlden Biotech med Kemisk Tidskrift, Nr. 10, 2009

Programme meetings during 2009

SPRING MEETING, Lund

The Old Bishop´s House, Lund, 20-21 April, 2009

Lectures

Per Simonsson
Deputy Managing Director, Sustainable Business Hub

Shukun Yu
Senior Scientist, Danisco

Per Eriksson
Vice-Chancellor, Lund University

AUTUMN MEETING, Åhus

Åhus Gästgifvaregård, 6-7 October, 2009

Lecture

Olle Wikström
Director, Senior Scientific Advisor, Lyckeby

Olle Wikström, Scientific Advisor, Lyckeby gives a lecture during the autumn meeting 2009.

Greenchem participants at Åhus Gästgifvaregård in Åhus during the autumn meeting 2009.

Members

The Board

The Greenchem board at the program meeting in autumn 2009

Britt Marie Bertilsson
Programme administrator at Mistra

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Cecilia Orellana-Coca
Post doc, Biocatalysis Development

Catherine Paul
Post doc, Biocatalyst Development and Production

Tania Pozzo
Ph. D. Student, Biocatalyst Development and Production

Anna Rosenquist
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Ph.D. Student,Process Technology

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MISTRA

Research with practical benefits

The Swedish Foundation for Strategic Environmental Research – Mistra - supports research of strategic importance for a good living environment and sustainable development.

It invests in research groups who, working alongside users, are able to contribute to solving major environmental problems.

Mistra's programmes cut across disciplinary boundaries, and the results are intended to find practical applications in companies, public agencies and non-governmental organizations.

Mistra provides funding for some twenty major programmes, each extending over six to eight years. All of them have the aim of building bridges, both between disciplines and between researchers and users.

The Foundation's strategy is to seek to ensure that its funds produce a threefold return: strong research environments that create value for users, asset management in support of sustainable development, and active communication to make the results known.

Further information can be found on Mistra´s web site: **www.mistra.org.**

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Speciality Chemicals from Renewable Resources

ANNUAL REPORT 2009

Editor and layout: Josefin Ahlqvist/Anna Rosenquist. Printing: Ljungbergs tryckeri AB, Klippan, 2010.