LentiKat's BIOTECHNOLOGIES

Lentikats Bulletin

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Into 2011 with a New Strategy

LentiKat's is about to enter its sixth year of existence, which is a good time to review the achieved results and evaluate the set strategy.

Considerable effort has been invested so far in the development and commercialisation of the solutions for wastewater treatment (WWT). This tremendous effort has been paid off in form of several full-scale installations (see previous issues) and establishment of a strong network with WWT companies and operators in the Czech Republic, enabling LentiKat's to deliver full solution packages to majority of customers. For 2011, it has been decided to continue strengthening our position on the WWT market in central Europe and carry out necessary steps towards establishment of new partnership with selected engineering companies in Korea, China, US and Brazil.

The end of 2010 found our biofuels team chin-deep in the preparation of two major trials (page 4), the first tests carried out beyond a laboratory scale in this field. Understandably, these trials have become the main focus of the biofuels team for 2011. Moreover, due to the excellent marketing work of our North American partner Houston Holdings, our technology caught the eye of some key players in the field, several of which expressed the interest in preliminary testing. Hopefully, we will be able to share more information on that in one of the next issues.

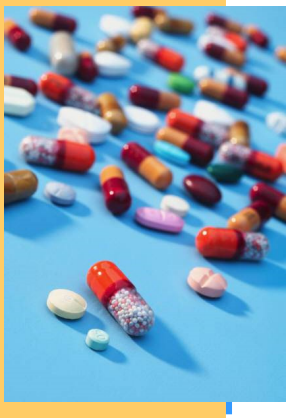
Despite being the youngest, our Pharma-Food segment caused something of a stir among our higher management during the annual meeting. The speed with which this segment has grown over the last year shows not only the great effort and excellent qualities of the team, but also indicates the great potential our technology has in the pharmaceutical and food industry. This segment has been given the green light to go and fully exploit its potential via extensive marketing activities world-wide, full R&D support and new members for the team.

Once again, allow us to take you through our new exciting projects and discoveries on the following pages of our Bulletin.

Lactose-Free Milk Trials

Following some extensive laboratory research into the lactose hydrolysis using Lentikats Biocatalyst (LB) with lactase activity, LentiKat's have installed a 15 litres test reactor in a dairy plant in the Czech Republic at the beginning of 2011. Hydrolysis of lactose in real pre-pasteurised milk using LB has been carried out in order to optimise the main operating parameters, such as temperature, conversion rate, microbial stability, sanitation and LB replacement procedure. A positive effect of process temperature on LB activity and microbial stability was determined, indicating that the future full-scale process would be best operated at 50°C with a batch conversion not exceeding 3 hours. Successful development of a suitable storage solution and handling procedure shall be verified in early March, whereupon the testing will be transferred into a larger, 150 litres reactor. The dairy plant intends to use the milk processed in the 150 litres reactor to produce their first ever lactose-free yogurt. Final scale-up to 6000 litres reactor shall take place already within the first half of 2011.

The cooperating dairy company will not only be one of the first producers of lactose-free dairy products in the Czech Republic, but will also be the first user of this groundbreaking technology in the food processing industry. Majority of the world's lactose-free milk production is based on the application of free soluble enzymes. LentiKat's is prepared to demonstrate that this nutritionally important product can be produced in a more economically and environmentally efficient way.



PGA Biocatalyst On its Way to First Customers

Successful finish of one of our very first projects, a development of Lentikats Biocatalyst with Penicillin G acylase (PGA) activity, is drawing closer every day. Development of this product has been a joint effort of the research teams of LentiKat's, Fermenta Biotech Ltd (India) and of the Institute of Microbiology in Prague.

What seems to have been a long way led the research team over various hurdles. Firstly, the preparation of cross-linked enzyme aggregates (CLEA) had to be optimised with the view of future CLEA immobilisation into the PVA matrix of Lentikats Biocatalyst. Secondly, a reason for inconsistency in samples' dry matter content had to be identified and dealt with. At last, a selection of suitable preservation agent and optimisation of storage and transport conditions was carried out to allow seamless transportation of the produced Biocatalyst from the production plant to customers.

The optimised production procedure resulted in a development of Biocatalyst with characteristics meeting market requirements in terms of activity (~ 625 U/g dw), operational stability (750 conversions) and microbial stability during transport. First 150 kg of the Biocatalyst will be prepared and dispatched from the manufacturing plant in March to reach potential customers in India for first evaluation. Given to optimised characteristics, Lentikats Biocatalyst with PGA activity shall be a strong competition to the PGA biocatalysts currently available on the market.

Moreover, Fermenta Biotech have already commenced preparation for trials of our lactose hydrolysis system (page 1). To the delight of LentiKat's management team, we seem to have found an equally enthusiastic partner for the commercialisation of our superb technology!

Promising Future for Industrial Encapsulation

Industrial Encapsulation is a new service that has been launched on the market at the beginning of last year. The idea is to let our customers choose their own process where they identify the immobilisation step will bring the highest benefits. We are able to offer such service only thanks to the comprehensive nature of Lentikats Biotechnology, which allows us to immobilise any kind of biomass (free enzymes, CLEA, cells, crude cellular extracts, fungal spores, etc.) and our extensive experience with the technology. Basic initial immobilisation phase is usually followed by a more advanced optimisation of the immobilisation procedure to obtain Biocatalyst's activity and stability matching our customer's requirements.

As opposed to bulk production, such tailor-made solutions may often be specific to one-reaction only. On the other hand, the customer's involvement in the early-stage identification process ensures that the benefits, i.e. enhanced process efficiency and stability and operating costs savings, will often be of much larger extend and significance than in processes requiring large amounts of Biocatalyst.

In 2010 we have started testing the industrial encapsulation with several companies all over the world (EU, China, India, RSA, etc.). While several projects are still running to optimise crucial parameters of the immobilised enzymes, one of the projects have been brought to a successful end, i.e. development of Lenti-CLEA Alcalase in cooperation with CLEA Technologies. The industrial encapsulation service is currently being tested by 4 new companies, two top US pharmaceuticals manufacturers and two European renowned enzyme producers. We hope to bring you more information in the next issue of our Bulletin.

3 IN 1 = MULTIENZYMATIC BIOCATALYST

Extreme selectivity and high efficiency of enzymes is being recognised and appreciated in various field of industry, including remediation technologies. 1,2,3-trichlorpropane (TCP) has been indentified as an emerging contaminant in 2009, typically found at industrial or hazardous waste sites. It is exclusively a man-made chemical that has been used as an industrial solvent, cleaning and degreasing agent and in the production of pesticides. TCP is currently used as a chemical intermediate in the polymers production. High toxicity and carcinogenetic properties raised the concern of world's health organisations over the presence of TCP in drinking and surface water.

Following the modern trends of sustainable and mild treatment technologies, scientists considered biological degradation of TCP. Unfortunately, to date, no microorganism capable of natural TCP degradation has been identified due to the lack of appropriate enzymes.

Our colleagues from Enantis Ltd. took up somewhat different approach. They first identified the three main enzymes that can facilitate the complete five-stages degradation of TCP to harmless glycerol. Then they synthesised these enzymes, cloned and expressed them using cells of *E. coli*. Purified enzymes were analysed separately for their substrate selectivity and activity, before being applied altogether in a buffer solution. The multienzymatic degradation concept proved possible. In the upcoming month, Enantis Ltd. will optimise the immobilisation procedure for these enzymes using Lentikats Biotechnology and the final Biocatalyst will be evaluated in terms of degradation efficiency. The immobilisation of whole cells engineered for the production of the appropriate enzymes will be tested and evaluated at the last stage of the project.

This research project presents a breakthrough science in the field of biodegradation of persistent organic pollutants, but more importantly, the ability to immobilise several enzymes within one pellet would open new opportunities for Lentikats Biotechnology.

Dr. Jiří Damborský from Enantis Ltd. presented the latest findings at the international conference Recent Trends in Developing Bioremediation Strategies for Hexachlorocyclohexane (HCH) & Other Chlorinated Contaminants in Indian Delhi in early February 2011.

TCP has been identified as an emerging contaminant of drinking water sources.

LB PROVES STABLE IN ORGANIC SOLVENTS

An increasing interest has been paid to the performance of biotransformations using or aided by organic solvents. This is because the organic solvent itself may be of interest as a substrate, or it may be needed to shift the equilibrium composition in a favourable direction, and in particular because many of the substrates of interest in modern biotransformations are essentially insoluble in aqueous media, and/or are highly toxic to whole cells.

Testing of the stability of PVA matrix of Lentikats Biocatalysts in various organic solvents was an apparent necessity. Isopropanol, acetone, chloroform, diethyl ether, benzene and toluene were selected for the preliminary evaluation.

With the exception of acetone, which had a detrimental effect on the PVA matrix, the aqueous phase has been successfully replaced with all selected solvents. It was discovered that a direct transfer from water to isopropanol is possible, while transfers into the other solvents had to be carried out via at least one intermediate step (via isopropanol). After that, they had no negative effect on the stability of the matrix.

This finding further expanded the group of potential applications of Lentikats Biotechnology and proved once again its comprehensiveness.

ONE STEP CLOSER TO FULL SCALE BIOETHANOL PRODUCTION

Fermenta Biotech Ltd, our long-term business partner in the pharmaceutical industry (see page 2) has come to realise the potential our technology offers for other sectors of biotech industry and expressed their intention to introduce our biofuels solutions to the Indian market. They decided to invest into this activity and via a lab-scale demonstration and dedicated workshop disprove the ever present scepticism of biofuels professionals about the applicability of Lentikats Biotechnology for ethanol production. Based on the process design and operation experience Lenti-Kat's team provided, Fermenta Biotech has constructed a laboratory reactor for alcohol fermentation from molasses and sugar beet juices.

Following the workshop demonstration (first half of 2011), a pilot-scale trial will be set-up at a selected distillery to prove a possible scale-up of the technology. The pilot plant shall take the form of a by-pass stream of the plant, hence a real substrate will be processed during the trial.

New Applications for LB in Medicine

A novel application for Lentikats Biotechnology in applied medicine is currently being tested by the research group of Dr. Petr Kačer from the Institute of Chemical Technology, Department of Organic Chemistry. Their main area of interest is medical diagnosis, specifically the diagnosis of oxidative stress or bronchial asthma. During the first year of testing, the researchers optimised the procedure for efficient immobilisation of relevant antibodies. Reaching this crucial milestone, the group is now investigating the most suitable application in the field of modern medicine. At present, the intention is to develop extremely sensitive biomarkers of pulmonary or oncogenes diseases, or markers for detection of drugs residues using immobilised antibodies in the form of disposable strips. Preliminary marketing analysis suggests a high potential of such product due to its simple production procedure, high sensitivity and selectivity and competitive price.

Re-Discovering America

LentiKat's seeks partnership with North American biofuels companies!

In 2007, LentiKat's made an attempt to introduce their biofuels solutions on the market in North America. This effort has been interrupted by the sharp turn in the society's perception of biofuels following concerns over the use of food commodities for fuels production. However, via an increasing awareness and continuing effort to replace food commodities with waste materials in the near future, majority of developed countries have managed to introduce biofuels into their energy saving programmes for the upcoming years. And so did the countries of North America. The US currently stands as the second largest fuel ethanol producer with corn being their main substrate. Canada is gradually making its way among the top market leaders with substantial governmental support into the sector.

The higher management of LentiKat's decided that it is the right time again to approach this market. Having 3 more years of R&D under our belt, LentiKat's and their North American representative Houston Holdings started seeking potential partnership within the North American ethanol producers. We are looking in two directions. The first objective is to commercialise our technology for ethanol production from starch or sugar based substrates. The second and equally important objective is to engage ourselves in the current development of lignocellulosic ethanol.

The quest for a suitable partner is on and so is the search for alternative funding sources through governmental or private support programmes. As negotiations with big players, such as NREL, Greenfield Ethanol or Cargill are taking place at present, we are becoming increasingly positive about the future successful completion of our mission.

Where Can We Meet?

Although this year's busy conferencing season has already kicked off, there are still plenty of opportunities to meet LentiKat's representatives before the end of this year.

⇒	February 23-24	Industrial Biotech World Europe, Amsterdam, Netherlands
⇒	May 4-5	IWA Water & Industry, Valladolid, Spain
⇒	June 16-17	Biotech 2011, 5th Czech-Swiss symposium
⇒	June 27-30	BIO International Convention, Washington DC, USA
⇒	October 2-6	BIOTrans 2011, Sicilly, Italy
⇒	October 25-27	CPhi Worldwide, Frankfurt, Germany

Relief for Biogas Plants

Biogas plants are designed to convert (waste) organic matter into an alternative energy source, biogas. Apart from this valuable product, biogas plants produce digested sludge, which being rich in nitrogen and phosphorus, is often applied on land as fertiliser. To minimise the cost for sludge handling, the sludge is dewatered prior to its disposal or reuse, which creates yet another residue, reject water. In ideal case, the reject water would be recycled to the front of the plant and used for diluting of the incoming material. However, often very high concentrations of ammonia (2-3 $g \cdot L^{-1}$ $N\text{-}NH_4$, a result of anaerobic conversion process) in the reject water hinder this action, as the ammonia would inhibit the biogas production process. For the same reason, reject water is extremely difficult to treat by conventional biological methods (nitrification by activated sludge), since concentrations above 1000 $mg \cdot L^{-1}$ $N\text{-}NH_4$ are reported to be inhibiting to common nitrification bacteria.

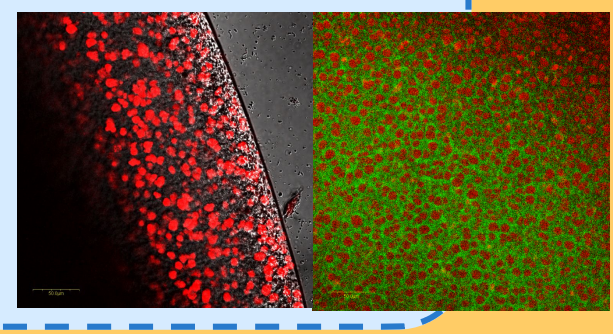
Lentikats Biotechnology has in numerous cases demonstrated the ability to enhance the robustness of the encapsulated biomass towards unfavourable operating conditions, which made us believe that nitrification bacteria, immobilised into the PVA matrix, will be able to handle ammonia concentrations even beyond the inhibition limit. A simple lab-scale reactor was set-up to treat synthetic reject water. A short initial period with an input concentration of 600 $mg \cdot L^{-1}$ $N\text{-}NH_4$ was followed by a one-step increase in the input concentration to 2500 $mg \cdot L^{-1}$ $N\text{-}NH_4$. The Biocatalyst exhibited a short decrease in activity to about 30% of its previous maximum over 3 days, which was however followed by a steep increase in the activity to finally almost double its previous maximum level. The Biocatalyst maintained this increased activity for the following 15 days, upon which the input nitrogen concentration was lowered again to its previous level of 600 $mg \cdot L^{-1}$ $N\text{-}NH_4$. These results clearly demonstrate the ability of Lentikats Biotechnology to work under very extreme conditions, well beyond the operating capacity of traditional technologies. Taking into account the environmental nature and financial viability, the technology fully deserves to be considered a leading state-of-the-art alternative for biogas plants.

How is Life Inside the Biocatalyst?

Frequent comparisons of Lentikats Biocatalysts with biofilm carriers within the wastewater field made us look closer into the cells distribution within our PVA carrier. The biofilm technologies are based on natural growth and development of unspecified bacterial consortium on the surface on a solid carrier. Two general drawbacks of that technology is a lack of control over the composition of the bacterial film and a reduced diffusion of substrate and oxygen to the downmost bacterial layers once the biofilm exceeds a certain thickness. Lentikats Biotechnology, on the other hand, encapsulates pure cultures of a selected bacterium and the specific shape and porosity of the carrier allows unlimited transport of substrates to any part of the carrier. But, how can we be sure that the colonies growing closer to the carrier's surface or even on the surface itself do not outgrow the inner ones, building a "shield" disabling the diffusion? The proof is fairly simple, you have to look inside the carrier.

Confocal microscopy coupled with fluorescent labelling technigue was used to study colonisation of two types of Biocatalyst - a freshly manufactured and cultivated LB with immobilised *Paracoccus denitrificans* and a the same type of Biocatalyst obtained from our full-scale installation already operating for more than a year. *Paracoccus denitrificans* is an heterotrophic fast growing bacterium reliant on the presence of suitable electron acceptor and organic matter. Due to its fast growth rate and short reproduction period, it is exactly the type of bacteria most likely to incline towards uneven distribution throughout a carrier.

The longer operating Biocatalyst showed to carry slightly larger colonies in comparison to the freshly cultivated one, yet in both cases the bacteria were evenly and densely distributed throughout the carrier. No difference in size was observed between the colonies detected in the central areas of the Biocatalysts and colonies close to the carriers surface. More investigation is going to be carried out, yet the already achieved results have clearly proven the ingenious structure of Lentikats Biocatalysts.



Pictures: Confocal microscopy images of *Paracoccus denitrificans* colonies from a longer-term operation scanned in the centre (right) and on the edge (left) of a PVA carrier. Green fluorescence—PVA, Red fluorescence—colonies.

Team Lentikats - Michal Norek

This time, we would like to introduce one of the oldest members of the team, Michal Norek, who has been with LentiKat's since early 2008 as a project manager for biofuels applications.

Michal was born in 1982 in a historic town of Pelhřimov. Shortly after that his family moved to Jihlava, where Michal spent most of his childhood. As fate would have it, they lived in a close proximity of the local brewery, which seemed to have played a large role in Michal's future profession. Following the completion of his high-school education at the Gymnasium of Jan Masaryk, Michal enrolled in the Institute of Chemical Technology in Prague for the post-graduate programme of Fermentation Chemistry and Technology . He completed his MSc. studies with a thesis on optimisation of cultivation conditions for protein production by genetically modified yeast *Pichia pastoris* in 2007. During his university studies Michal worked as a laboratory assistant at the brewery and dairy plants in Jihlava .

Following his education, Michal started his professional carrier with a short employment as a microbiology specialist at Coca-Cola Beverages Ltd. before finding his way to the offices of LentiKat's a.s. The main line of his work is within the biofuels segment, where he is responsible for project management from process design, administration of contracts and business processes, operation of lab– and pilot-scale test units to supervision and administration of R&D projects. Although biofuels are his main area of interest, Michal has often demonstrated a great flexibility, when he managed to step in to the operation of our wastewater and pharma-food projects and test units with an incredible easy and fast adaptation. He has got several start-ups of our wastewater pilot unit under his belt as well as a substantial involvement in the currently operating lactose-free trials. Michal is currently busy supervising the construction and operation of the bioethanol tests in India and Brazil.

Michal is a very proud and happy husband and a father of one-year old twin girls. To relax from his working and parenting activities, Michal enjoys a good game of squash, badminton, tennis or soccer. Among his favourite summer activities is canoeing or rafting on rivers with a group of friends and his family. On his table one can find a mix of technical literature and thrillers. And as a true fermentation specialist Michal takes pride in his homemade fruit wines of various tastes and flavours.

Michal is a very valuable member or our team, a reliable colleague always prepared to help and a positive and kind person.

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