DOM – Domestication of Microorganisms

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Introduction

Microorganisms and their metabolic activities play a paramount role for both mankind and the environment. Some microbes have been domesticated for human service for thousands of years, for example in preservation of animal feed and the production of food and beverages. Moreover, microorganisms are important for the well-being of both humans and animals by establishing a balanced and functioning microbial community in the gastrointestinal tract. Other bacteria and fungi play a major role in the mineralisation of organic matter in soils and thereby supply agricultural plants with available nutrients.

Due to their collective wealth of metabolic capabilities, microorganisms can also be useful in a variety of novel applications, including efforts to reduce environmental problems. For instance, antagonistic organisms can be used as biological control agents to reduce the use of chemical pesticides, or efficient degraders can be applied as bioprophylactics to minimise the spread of chemical pollutants. Microorganisms can also be used for the biological clean-up of polluted soil or as plant growth-promoting bacteria that stimulate nutrient uptake. The use of microorganisms as producers of biofuels (ethanol, butanol, biogas, hydrogen gas) is also rapidly expanding.

Many microbial applications require large-scale cultivation of the organisms. The biomass production must then be followed by formulation steps to ensure long-term stability and convenient use. Moreover, safe use of the organisms demands a careful consequence analysis, which for some applications must be followed by a pre-market authorisation procedure. Lack of necessary knowledge in these areas has seriously hampered the development of microbiological solutions to environmental problems, and their transformation into a corresponding range of commercially available products. Thus, there remains an unfulfilled potential for growth of biotechnology industries based on such applications

Knowledge about fermentation of microorganisms has largely focused on traditional model organisms, e.g. *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Pichia pastoris*, and to some extent also lactic acid bacteria. Many of those organisms are used as producers of specific compounds, mainly for pharmaceutical purposes, where there is no interest in maintaining the viability of the cells after fermentation. However, there still remains a need to further develop knowledge on how to optimise fermentation of ”non-conventional microorganisms” for environmental applications involving the intact living cells.

The subsequent formulation of the organisms can follow a variety of paths that are often specific for individual organisms. Research within this area of ”galenic microbiology” has been lagging behind, and existing know-how on formulation of microorganisms is largely based on trial and error with traditionally used organisms, (Melin 2007a). One consequence of this is that in formulation science, like that of fermentation, some groups of microbes have been seriously neglected, the gram-negative bacteria in particular (Bjerketorp 2006).

As the microorganisms will be produced in large amounts and concentrated forms, it is essential that risks to human health during production, manufacture, storage and application are minimised.

Today, standardised methods to determine the safety profile of microorganisms do not exist. Where the organisms are released into the environment, it must be evaluated to what extent this may result in undesirable side effects. Presently, this assessment is hampered by poor knowledge of the fundamental ecology of the organisms, for example their natural distributions.

Material and methods

An overall DOM goal is to develop fermentation and formulation methodology for neglected groups of organisms with major biotechnical potential, *e.g.* gram-negative bacteria and non-conventional yeasts. Development of efficient and cost-effective media and process parameters giving high cell yields are important priorities. This also involves establishing fermentation parameters yielding cells well adapted to subsequent formulation procedures. Collectively, these strategies will deliver a high proportion of viable cells with good long-term survival.

Within formulation, DOM provides new fundamental knowledge by combining general formulation science (*i.e.* pharmacology and galenics) with microbiology. The main focus is on development of more efficient drying techniques for micro-organisms, particularly vacuum drying, freeze-drying, and fluidised bed-drying. The advantages of dry formulations are that storage and delivery costs are much lower than for liquid formulations and that long-term survival can be very high if initial packaging is carefully optimised. DOM also improves and optimises formulations through the addition of various kinds of excipients that have beneficial effects on the viability of the organisms and the storage stability of the product.

The DOM programme (www.mistra.org/dom) is funded by Mistra – The strategic environmental research foundation, the Swedish University of Agricultural Sciences (www.slu.se) and several industrial partners. The overall aim of the programme is to provide new fundamental knowledge that is used to develop more relevant and efficient methods to determine the safety profile of microorganisms used in environmental or biotechnical applications. A new assessment system/package encompassing the collection and evaluation of relevant scientific literature and other information, *in vitro* testing of potential production of toxic substances, determination of antibiotic resistance pattern, and temperature range for growth, has been developed and evaluated. We also refine and adapt assays based on biosensor organisms, for determination of potential pathogenicity and production of toxic compounds in non-conventional microorganisms.

The assessment of environmental safety also has to be made case-by-case. In DOM, strain-specific genetic markers are developed with SCAR methodology (Sequence Characterized Amplified Region). These markers are used to determine persistence and spread of introduced organisms, but they can also give information about the organisms' natural distribution. Such knowledge provides background information when evaluating whether the organism will have any adverse effects in the environment. Important information is also collected from literature surveys, toxicity tests, and greenhouse and field trials.

Results and Discussion

Novel encapsulation technique s- In collaboration with the Chemistry department at SLU, Uppsala, DOM has evaluated a new technique for encapsulation of biomaterials in inorganic sol-gels that are based on common metal oxides. Data on the fundamentals of the sol-gel chemistry behind the invention have been published (Kessler 2006). The work has resulted in a full PCT patent application and publication of data on encapsulation of biological materials has been submitted for

publication. All subsequent results of this project are presented at this meeting in a separate contribution (Kessler et al.)
Improved survival rates of dried microorganisms - Previously, we have published our results on the impact of parameters such as cell density, concentration of dry protectant and freezing rate on freeze drying survival of biopreservative *Lactobacilli* strains (Schoug 2006). Efforts has been made to investigate the impact of preconditioning stress, such as high and low temperatures and pH, on membrane lipid fatty acid composition and freeze-drying survival (Schoug 2008).

Comparative studies on the biocontrol yeast *P. anomala* were performed using several different dry formulations techniques. Freeze-, vacuum-, fluidized bed drying, as well as liquid formulation was found to yield products with very good stability that can be stored for one year without significant losses (Melin 2006 and 2007b).

Vacuum drying and addition of sugar-based drying protectants have been evaluated for the development of dry formulations of biocontrol and plant growth promoting gram-negative bacteria. The drying protectants trehalose and polyvinylpyrolidone gave the highest initial survival rates and the most stable formulations, without significant losses of viability after storage for 1 month at 4ºC, (Levenfors in prep.).

The tolerance of different microbial species to the less expensive, but harsher fluidized bed drying technique is also investigated. Together with the MASE research programme we are developing functional fluid bed-based formulations of plant growth promoting *Pseudomonas* spp. Initial data indicate that direct survival can be high and that the specific plant growth promoting effect is retained. More work is needed to improve long-term storage stability, and this is currently a focus in the DOM laboratory.

Genetic markers for specific detection and quantification of the biocontrol bacterium *Pseudomonas brassicacearum* MA250 in samples from the environment are now in place. They were successfully applied in a greenhouse experiment with winter wheat inoculated with MA250 and an article on these results will be submitted in spring 2008. Next, the markers are used to study the persistence and spread of MA250 in field experiments with this bacterium. Field samples have been collected and are currently under analysis in the laboratory.

In collaboration with BINAB Bio-Innovation AB we have developed strain-specific markers for their *Trichoderma* isolates that form part of the company's products for biocontrol of fungal plant diseases. During 2007, candidate markers for the *Trichoderma* fungi were identified using genotyping. This typing also yielded new insight into their classification, and the two product strains have now been reclassified as *Trichoderma atroviride* and *Trichoderma parapiluliferum*. Currently, we are using the markers for detection of the fungi on golf greens and in greenhouses where BINAB's products have been applied. Real-Time PCR methods allowing quantitative determination of the two *Trichoderma* strains will be set up during spring 2008.

A co-formulation concept based on the simultaneous addition of a chemical pesticide and a degrading microorganism is being developed by DOM. We found that degradation of MCPA by a *Sphingomonas* strain isolated from the former production site of phenoxyalkanoic acids in a polluted site in Sweden was dependent on the simultaneous presence of another bacterium. A pilot study on railway embankments was performed in summer 2007. Enhanced degradation of MCPA after inoculation with the *Sphingomonas sp.* was found, but a high variability, particularly in the control plots, complicated the evaluation. Follow-up field trials will be done after further investigations of the viability of the inoculated strain. In addition, studies are under way on the need for adapting the bacteria to certain conditions before field application.

In recognition of our formulation and safety assessment expertise, DOM has been invited to join a new EU-project, BACSIN (Bacterial Abiotic Cellular Stress Improvement Network), which will start in March 2008. The aim is to understand the control of catabolic gene expression for pollutant degradation in contaminated environments under stress conditions, and to understand the capacities of bacteria to adapt or evolve to abiotic stresses that they face in the environment. This will provide rational strategies for application of stabilised bacterial formulations for targeted pollutant degradation (www.unil.ch/bacsin).

Conclusions

DOM research on safety assessment, fermentation technology and formulation of microorganisms contributes to bridging the gaps between academic research, regulatory policy making, industrial developments and market requirement. This will aid in the implementation of sustainable solutions for a knowledge driven bioeconomy.

References

Å. Schoug et al. (2008) *Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of Lactobacillus coryniformis Si3*. J. Ind Microbiol Biotech. 35(3):175-81

P. Melin et al. (2007a) *Biological preservation of plant derived animal feed with antifungal microorganisms – Safety and formulation aspects*. Biotech. Letters 29:1147-1154

P. Melin et al. (2007b) *Optimisation and comparison of liquid and dry formulations of the biocontrol yeast Pichia anomala J121*. Appl. Microbiol. Biotechnol. 73:1008–1016

Å. Schoug et al. (2006) *Freeze-drying of Lactobacillus coryniformis Si3 – effects of sucrose concentration, cell density, and freezing rate on cell survival and thermophysical properties*. Cryobiol. 53(1): 119-127

VG. Kessler et al. (2006) *New insight in the role of modifying ligands in the sol-gel processing of metal alkoxide precursors: A possibility to approach new classes of materials*. J. Sol-Gel Sci. Techn. 40:163–179.

P. Melin et al. (2006) *Survival of the biocontrol yeast Pichia anomala after long-term storage in liquid formulations at different temperatures, assessed by flow cytometry*. J. Appl. Microbiol. 100(2):264-271.

J. Bjerketorp et al. (2006) *Advances in preservation methods: keeping biosensor microorganisms alive and active*. Curr. Opin. Biotechnol. 17:1-7