Applications for Synthetic Biology in Industrial Biotechnology

Friday, 29 November 2013
Cineworld: The O2, London, SE10 0DX, UK
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With their provenance as an excellent source of pharmaceutical, neutraceutical and health promoting chemistries, plant natural products are an attractive target for biotechnological development for industrialization so as to make them more widely available. To realize this potential, two strategies are currently being employed whereby the associated metabolic pathways are engineered *in planta*, or are ectopically expressed in microbial hosts and produced through fermentation. In both cases it is clear that recent developments in genetics, and our understanding of metabolism are providing us with unprecedented tools to fast-track these ambitions. In addition, the advent of synthetic biology, where approaches more commonly employed in engineering are applied to design and optimize bioprocesses has much to offer industrial biotechnology in the future.

In this meeting a series of metabolic engineering programs representing each of the differing strategies for natural product biosynthesis will be presented and the potential merits of plant Vs microbial industrial biotechnology discussed, along with projections as to how each might benefit from synthetic biology based approaches. In addition to reviewing the latest development in plant natural product biochemistry and molecular biology, the meeting will be formative in shaping thinking as to how and where new approaches like synthetic biology can be best applied in industrial biotechnology in the coming years. This meeting will also include a meeting of the **High Value Chemicals from Plants Network.**

This event has CPD accreditation and is part of
The 2013 BioProcessing Summit - www.BioprocessingSummit2013.com

Meeting Chairs: *Professor Robert Edwards*, Chief Scientist, The Food and Environment Research Agency, UK. *Professor Ian Graham,* CNAP Director and Weston Chair of Biochemical Genetics, University of York, UK. *Professor Gary Loake*, The University of Edinburgh, Scotland, UK.

9:00 – 9:25 **Registration**

9:25 – 9:30 **Introduction by the Chair**: *Professor Robert Edwards*, Chief Scientist, The Food and Environment Research Agency, UK

9:30 – 10:00 **Building novel isoquinolines with synthetic plant enzymes**
Professor John Ward, University College London, UK
Alkaloids are a large and diverse family of nitrogen-containing compounds, many of which are used as pharmaceuticals and they represent a huge repository of functional chemical space. We are using the enzyme (S)-norcoclaurine synthase (NCS) which carries out the key first coupling step in the pathway that generates the tetrahydroisoquinolines. The enzyme catalyses a Pictet-Spengler reaction between arylethylamines and aldehydes, generating a chiral centre. Synthetic versions of the enzyme from several plant species have been designed and expressed in *E. coli*. The mechanism of the recombinant NCS is being elucidated and mutations of the *Coptis japonica* and *Thalictrum flavum* NCS enzymes have been used to make novel benzyl and aliphatic tetrahydroisoquinolines.

10:00 – 10:30 **Cultured cambial meristematic cells as a source of plant natural products**
Professor Gary Loake, The University of Edinburgh, Scotland, UK
A plethora of important, chemically diverse natural products are derived from plants. In principle, plant cell culture offers an attractive option for producing many of these compounds. However, it is often not commercially viable because of difficulties associated with culturing dedifferentiated plant cells (DDCs) on an industrial scale. To bypass the dedifferentiation step, we isolated and cultured innately undifferentiated cambial meristematic cells (CMCs). Using a combination of deep sequencing technologies, we identified marker genes and transcriptional programs consistent with a stem cell identity. This notion was further supported by the morphology of CMCs, their hypersensitivity to γ-irradiation and radiomimetic drugs and their ability to differentiate at high frequency. Suspension culture of CMCs derived from *Taxus cuspidata*, the source of the key anticancer drug, paclitaxel (Taxol), circumvented obstacles routinely associated with the commercial growth of DDCs. These cells may provide a cost-effective and environmentally friendly platform for sustainable production of a variety of important plant natural products.

10:30 – 10:55 **Speakers' photo then mid-morning break and trade show**
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10:55– 11:00 **Introduction by the Chair**: *Professor Ian Graham,* CNAP Director and Weston Chair of Biochemical Genetics, University of York, UK

11:00 – 11:30 **Engineering flavonoid metabolism in yeast**
Professor Robert Edwards , Chief Scientist, The Food and Environment Research Agency, UK
Phenylpropanoids are simple aromatic natural products found in all plants which are used as the building blocks for a wide range of polyphenols including a diverse array of flavonoids with activities as diverse as dietary cytoprotectants, colourants and flavour enhancers. Using polyprotein technology we have engineered bakers' yeast to transform readily available phenylpropanoids left over from brewing and biofuel production into high value flavonoids, including glycosylated derivatives with uses as artificial sweeteners. The approach adopted shows the value of effectively transferring plant metabolic pathways into non-natural hosts to extend the diversity of end products which can be generated in useful quantities.

11:30 – 12:00 **Metabolic engineering of high value lipids in transgenic plants**
Professor Johnathan Napier, Rothamsted Research Limited, Hertfordshire, UK
Using genetic engineering it is now possible to generate transgenic plants which have the capacity to synthesise high value fatty acids such as the omega-3 long chain polyunsaturates.

12:00 – 12:20 **Oral Presentation:**
METABOLIC ENGINEERING OF MICROALGAE FOR ENHANCED PRODUCTION OF OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS.
O. Sayanova, M. Hamilton, R. P. Haslam, J. A. Napier.
Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK

12:20– 13:30 **Lunch and trade show**

13:30– 14:30 **High Value Chemicals from Plants Network Meeting**

14:30– 15:00 **Engineering polyphenols in tomatoes**
Professor Cathie Martin, Theme Leader Plant Natural Products, John Innes Centre, UK
Understanding the complex relationship between diet and health has become key to developing preventive strategies to reduce the rising incidence of chronic disease, globally. As an example of how this new experimental field can work I will describe our work on enrichment of tomatoes with different polyphenolic bioactives, for comparative nutritional studies. We have produced tomatoes enriched in anthocyanins, flavonols, resveratrol and isoflavones. Experiments using animal disease models have shown the anthocyanin-enriched tomatoes to have anticancer and anti-inflammatory properties. Cell-based assays have provided insight into the mechanisms of action of anthocyanin bioactives. Ongoing preclinical studies address how well other polyphenols compare to anthocyanins (in a common food matrix) in a cardiovascular disease model. Human intervention studies are also about to begin.

15:00 - 15:25 **Afternoon Tea/Coffee and trade show**
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15:25 – 15:30 **Introduction by the Chair**: *Professor Gary Loake*, The University of Edinburgh, Scotland, UK

15:30 – 16:00 **A ten gene cluster responsible for synthesis of the anticancer alkaloid noscapine in opium poppy**
Professor Ian Graham, CNAP Director and Weston Chair of Biochemical Genetics, University of York, UK
Noscapine is an antitumor alkaloid from opium poppy that binds tubulin, arrests metaphase and induces apoptosis in dividing human cells. We recently discovered a cluster of 10 genes encoding five distinct enzyme classes that are responsible for noscapine production in poppy (Winzer et al., Science, 2012). Virus induced gene silencing resulted in accumulation of pathway intermediates allowing a novel biosynthetic pathway to be proposed. This advance adds to our knowledge of gene clusters in plants and will enable improvement in commercial production of noscapine and related bioactive molecules.

16:00 – 16:30 **Genome mining and metabolic engineering for triterpene synthesis**
Professor Anne Osbourn, Associate Research Director, John Innes Centre, UK
Plants produce a huge array of natural products, many of which are specialised metabolites associated with particular species.These secondary metabolites often have important ecological functions. Although the ability of plants to perform in vivo combinatorial chemistry by mixing, matching and evolving the genes required for different secondary metabolite biosynthetic pathways is likely to have been critical for survival and diversification of the Plant Kingdom we know very little about the mechanisms underpinning this process. This talk will focus on plant natural product function and synthesis, the origins of metabolic diversity and potential for metabolic engineering, drawing on our research on triterpene synthesis in crop and model plants. Triterpenes have important ecological and agronomic functions, contributing to pest and pathogen resistance and to food quality in crop plants. They also have a wide range of commercial applications in the food, cosmetics and pharmaceutical sectors.

16:30 - 17:00 **Chairmen's summing up and Close of Meeting**

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About the Chairs:

Robert Edwards is the Chief Scientist at the Food and Environment Research Agency (Fera) and also runs a research group at the University of York in the Centre for Novel Agricultural Products, where he has a Chair in Crop Protection. He was formerly Head of Biology at Durham University, with 26 years of postdoctoral experience as a Plant Biochemist working in the public and private sectors in the UK and USA. As co-ordinator of the cross Research Council-funded network 'Synthetic Plant Products for Industry' he has been working with industrialists and academics on applications for synthetic biology in the improvement and utilisation of crop plants in biorefining. Research interests in metabolic engineering include manipulating high value flavonoid production and the biotransformation of synthetic chemicals. Expertise in natural products at Fera includes state-of-the-art facilities in measuring a wide range of analytes in food and environmental samples and research programmes in biorenewables.

Ian Graham holds the Weston Chair of Biochemical Genetics and is the Director of the Centre for Novel Agricultural Products (CNAP) at the University of York. His research interests focus on seed biology and metabolic engineering of novel oils and other high value chemicals. Current projects range from the development of novel oilcrops such as Jatropha curcas to medicinal plants such as Artemisia annua that produces the anti-malarial compound artemisinin and opium poppy that produces analgesics and other compounds for the pharmaceutical industry. Funding for Ian's research comes from a range of sources including industry, UK Government, EU and various charities.

Gary Loake's research aims to understand the molecular mechanisms underpinning plant disease and resistance.
1996 Joined University of Edinburgh, 1995-1996 Senior Postdoctoral Fellow, The Plant Laboratory, University of York, UK, 1991-1994 Salk-Noble Plant Biology Fellow, Salk Institute, California, USA, 1990-1991 Salk-Noble Plant Biology Fellow, Samuel Robert Noble Foundation, 1990 Ph.D. University of Durham, Durham, UK

About the Speakers:

John Ward is Professor of Synthetic Biology for Bioprocessing in the Advanced Centre for Biochemical Engineering at University College London. He and colleagues at UCL have used enzymes such as transaminases, transketolase, Bayer-Villiger monooxygenase, cytochrome P450 and norcoclaurine synthase to make chiral compounds. He has developed biocatalysis and synthetic biology routes to chiral compounds such as aminodiols and tetrahydroisoquinolines.

Johnathan A. Napier's research on the biosynthesis of polyunsaturated fatty acids has delivered some of the key advances in the last 15 years. He obtained his BSc from the University of Nottingham, followed by a PhD in plant biochemistry from King's College, London. He carried out post-doctoral research in the Department of Plant Sciences, University of Cambridge, then taking up a position at Long Ashton Research Station in Bristol. His research group relocated to Rothamsted Research in 2003 where he is currently Institute Assistant Director and Programme Leader. Johnathan is also an Affiliated Lecturer at the University of Cambridge.

Cathie Martin's interests span the entire spectrum of plant biology, from the fundamental to the applied ends. Recently, she has engineered phenylpropanoid metabolism using transcription factors to improve foods and demonstrated that elevated dietary anthocyanin levels in food extend the life span of cancer prone mice by 30% and afford cardioprotection. Cathie has co-ordinated two EU projects, FLORA and ATHENA, which linked the activities of plant geneticists, metabolic engineers, chemists, nutritionalists, food technologists, cardiologists, epidemiologists to develop model foods with defined levels of flavonoid bioactives and to investigate the health promoting effects of these foods.

Anne Osbourn is Associate Research Director of the John Innes Centre, Norwich. Her research focuses on plant natural products - function, synthesis and metabolic diversification. She is an author of over 100 peer-reviewed scientific publications and recently co-edited a comprehensive textbook on plant-derived natural products [Lanzotti V & Osbourn A. (2009) Plant-derived natural products – Synthesis, function and application. Springer, New York, USA]. She has also developed and co-ordinates the Science, Art and Writing (SAW) initiative, a cross-curricular science education programme for schools (www.sawtrust.org).

POSTER PRESENTATIONS

METABOLIC ENGINEERING OF MICROALGAE FOR ENHANCED PRODUCTION OF OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS

O. Sayanova, M. Hamilton, R. P. Haslam, J. A. Napier
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It is now well established that omega-3 long chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA; 20:5Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6Δ4,7,10,13,16,19) are essential constituents of human nutrition and have key roles in growth and development of infants and children and in maintaining health through their effects on immune system. Although marine fish is the main dietary source of EPA and DHA, the depletion of fish stocks and pollution of the marine environment indicate an urgent need for an alternative and sustainable source of LC-PUFAs. Marine microorganisms are the primary producers of LC-PUFAs in the aquatic food chain and EPA- and DHA–rich microalgae have been demonstrated to be a promising alternative source to fish oils. Commercial production of high value algal products like omega-3 LC-PUFAs is well-established, with the production (primarily for infant formula and nutraceuticals) of DHA through auxotrophic fermentation representing a multi-billion dollar industry in which supply can barely keep pace with demand. However, commercial production of highly valuable products like omega-3 LC-PUFAs is expensive to maintain and represents a substantial technological challenge. Therefore, there is increasing interest in the metabolic engineering of microalgae and genetic modification of algal strains is considered to be one of the most promising strategies to produce sustainable omega-3 oils. *Phaeodactylum tricornutum* is an unicellular diatom which accumulates up to 30% EPA and only traces of DHA and is considered a good source for the industrial production of EPA. We have engineered the *P.tricornutum* strain to accumulate elevated levels of DHA by overexpressing heterologous genes encoding enzyme activities of the LC-PUFA biosynthetic pathway. Our data demonstrate the efficient channelling of DHA into neutral lipids with several novel triacylglycerol species being detected in the transgenic strains. This study provides novel evidence for the potential of using metabolic engineering to optimize omega-3 LC-PUFAs content in transgenic microalgae.

MOLECULAR DISPLAY SYSTEMS DEVELOPED USING *SACCHAROMYCE CEREVISIAE* PROVIDE A SYNTHETIC TOOL FOR BIOPRODUCTION

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The yeast *Saccharomyces cerevisiae* has been used in the process of bioproduction or fermentation, as well as in biochemical or molecular biological studies. It plays an important role in both upstream and downstream processes of biotechnology. We developed a molecular display system by using yeast cells and a cell wall protein for immobilization of foreign proteins (1, 2). α-Agglutinin, a mannoprotein in the yeast cell wall, was exploited to anchor proteins of interest on the cell surface. At present, other types of molecular display systems, e.g. phage, *Lactobacillus*, or *Escherichia coli* cell surface display systems, are available for biotechnological studies. Among these systems, the yeast molecular display system has advantages in bioproduction because foreign proteins derived from both eukaryotes or prokaryotes can be displayed and the cell surface size is larger than that of other microorganisms. In addition, *S. cerevisiae* has long been used in the food industry and is classified as a generally recognized as safe organism. Therefore, the yeast molecular display system is suitable for the production of bioactive compounds and related molecules. In this presentation, we introduce the applications of the above-mentioned system and describe the results of bioproduction of isoflavone and lipase (3, 4). Fermentation properties of xylose isomerase-displaying yeast have also been introduced (5). *References:* 1) Ueda M., *J. Mol. Catl. B*, **28**, 139-143 (2004), 2) Shibasaki S. et al., *Anal. Sci.*, **25**, 41-49 (2009), 3) Shibasaki S., *Yakugaku Zasshi*, **130**, 1437-1444 (2010), 4) Shibasaki S. et al., *Appl. Microbiol Biotechnol.*, **75**, 821-828 (2007), 5) Ota M. et al., *Biotechnol. Prog.*, **29**, 346-351 (2013).

STATISTICAL EXPERIMENTAL DESIGN GUIDED OPTIMIZATION OF A ONE-POT BIPHASIC MULTIENZYME TOTAL SYNTHESIS OF AMORPHA-4,11-DIENE

X Chen[1], CQ Zhang[1], RY Zou[1], K Zhou[1,3], G Stephanopoulos[1,3], HP Too [1,2*]
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In vitro synthesis of chemicals and pharmaceuticals using enzymes is of considerable interest as these biocatalysts facilitate a wide variety of reactions under mild conditions with excellent regio-, chemo- and stereoselectivities. A significant challenge in a multi-enzymatic reaction is the need to optimize the various steps involved simultaneously so as to obtain high-yield of a product. In this study, statistical experimental design was used to guide the optimization of a total synthesis of amorpha-4,11-diene (AD) using multienzymes in the mevalonate pathway. A combinatorial approach guided by Taguchi orthogonal array design identified the local optimum enzymatic activity ratio for Erg12:Erg8:Erg19:Idi:IspA to be 100:100:1:25:5, with a constant concentration of amorpha-4,11-diene synthase (Ads, 100 mg/L). The model also identified an unexpected inhibitory effect of farnesyl pyrophosphate synthase (IspA), where the activity was negatively correlated with AD yield. This was due to the precipitation of farnesyl pyrophosphate (FPP), the product of IspA. Response surface methodology was then used to optimize IspA and Ads activities simultaneously so as to minimize the accumulation of FPP and the result showed that Ads to be a critical factor. By increasing the concentration of Ads, a complete conversion (~100%) of mevalonic acid (MVA) to AD was achieved. Monovalent ions and pH were effective means of enhancing the specific Ads activity and specific AD yield significantly. The results from this study represent the first *in vitro* reconstitution of the mevalonate pathway for the production of an isoprenoid and the approaches developed herein may be used to produce other isopentenyl pyrophosphate (IPP)/ dimethylallyl pyrophosphate (DMAPP) based products.

COMBINING GENOTYPE IMPROVEMENT AND STATISTICAL MEDIA OPTIMIZATION FOR ISOPRENOID PRODUCTION IN *E. COLI*

Congqiang Zhang[1], Xixian Chen[1], Ruiyang Zou[1], Kang Zhou[1], Gregory Stephanopoulos[1, 3], Heng-Phon Too[1, 2, *]
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Isoprenoids are a large and diverse class of compounds that includes many high value natural products and are thus in great demand. To meet the increasing demand for isoprenoid compounds, metabolic engineering of microbes has been used to produce isoprenoids in an economical and sustainable manner. To achieve high isoprenoid yields using this technology, the availability of metabolic precursors feeding the deoxyxylulose phosphate (DXP) pathway, responsible for isoprenoid biosynthesis, has to be optimized. In this study, phosphoenolpyruvate, a vital DXP pathway precursor, was enriched by deleting the genes encoding the carbohydrate phosphotransferase system (PTS) in *E. coli*. Production of lycopene (a C40 isoprenoid) was maximized by optimizing growth medium and culture conditions using factorial design and Response Surface Methodology (RSM). In optimized conditions, the lycopene yield from PTS mutant was seven fold higher than that obtained from the wild type strain. This resulted in the highest reported specific yield of lycopene produced from the DXP pathway in *E. coli* to date (20,000 µg/g dry cell weight). Both the copy number of the plasmid encoding the lycopene biosynthetic genes and the expression were found to be increased in the optimized media. Deletion of PTS together with a similar optimization strategy was also successful in enhancing the production of amorpha-1,4-diene, a distinct C15 isoprenoid, suggesting that the approaches developed herein can be generally applied to optimize production of other isoprenoids.

SYNTHETIC BIOTECHNOLOGY FOR RECOMBINANT PRODUCTION OF POLYUNSATURATED FATTY ACIDS FROM MYXOBACTER

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Polyunsaturated fatty acids (PUFAs) are straight chain fatty acids that contain more than one double bond. For omega-3 PUFAs, and in particular for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), beneficial health effects have been reported. As an attractive alternative to oceanic fish and fish oil products, microbial fermentation offers a new source for industrial-scale production of PUFAs. Different marine microorganisms are known to produce PUFAs under strictly anaerobic conditions. The involved pathways employ polyketide synthase (PKS)-like enzymes known as PUFA synthases for *de novo* synthesis of PUFAs from acyl-CoA precursors [1]. Interestingly, large amounts of EPA and DHA were also found in the novel myxobacterial genus *Aetherobacter*, recently isolated from terrestrial soil samples [2,3]. To gain deeper insights into the molecular basis of PUFA production in myxobacteria, the corresponding biosynthetic gene clusters were identified and completely sequenced. As the native producer strains grow slowly, are difficult to handle and genetic modification has proven difficult, synthetic biotechnology approaches have been developed to transfer and express myxobacterial PUFA pathways in suitable host strains. At a first attempt, PUFAs could be successfully heterologously produced in the myxobacterial model strain *Myxococcus xanthus* DK1622 [4]. The subsequent work mainly focused on the establishment of the GRAS certified microbes *Pseudomonas putida* KT2440 and *Yarrowia lipolytica* as heterologous PUFA producers due to their fast growth characteristics, amenability to genetic modifications, and vast potential for industrial applications. However, efficient heterologous expression of biosynthetic pathways represents several limitations: The construction of expression vectors for complex pathways is technically very challenging and the functionality of native DNA sequences is often restricted in heterologous hosts. Such limitations were addressed by synthetic biology approaches involving the redesign and subsequent gene synthesis of the PUFA biosynthetic pathway. The codon composition and other functional elements were adapted to *P. putida* KT2440 and *Y. lipolytica*. Through this approach, the PUFA yield could be significantly increased. *References:* [1] J. A. Napier, *Trends Plant Sci.* 2002, *7*, 51-54, [2] R. Garcia, D. Pistorius, M. Stadler, R. Müller, *J. Bacteriol.* 2011, *193*, 1930-1942, [3] M. Stadler, E. Roemer, R. Müller, R. Garcia, D. Pistorius, A. Brachmann, international patent application 2010, WO/2010/063451, [4] S. C. Wenzel, S. Rachid, K. Gemperlein, R. Müller, international patent application 2011, WO/2011/151298.

THE IMPACT OF DESENSITIZING MUTATIONS IN *ESCHERICHIA COLI thrA* GENE ON THREONINE PRODUCTION

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The aspartokinase I-homoserine dehydrogenase I (AK I-HDH I) of *Escherichia coli* is the first bifunctional enzyme that has been studied as early as 1965. The desensitizing mutation in *thrA* gene encoding AK I-HDH I is obligatory constituent of all threonine producing strains. In spite of the fact that such mutations have been utilized in threonine manufacture, exact position of mutations was being remained undisclosed for many years. Herein we compare three the most prominent previously known desensitizing mutations in *thrA* gene of *E. coli*. In 2003 the first mutation in *E. coli* enzyme has been published that corresponded to substitution Ser345Phe. Subsequently, it has been successfully applied for construction of threonine producing strain by South Korean company Cheil Jedang. Another mutation has been originally isolated in the strain *E. coli* β-101 which is parental for the well-known strain βIM-4 (ATCC 21277). This mutation has been used for the construction of threonine producers in many laboratories including United States manufacturer Archer Daniels Midland Company. The substitution induced by this mutation is Gly433Arg that has been disclosed in 2008. The third mutation known as *thrA*^{442} primarily has been isolated in the strain MG442 and then applied to construct the strain VKPM B-3996 that has been industrialized by Japanese company Ajinomoto. We found that the plasmid pVIC40 being carried by this strain encoded essentially the same mutation (Glu253Lys) as had been disclosed in 2011 through whole genome sequencing of the industrial *E. coli* strain XH001 of unknown origin. We also constructed *E. coli thrA* gene encoding AK I-HDH I with substitution Ser352Gln. As previously shown, this substitution desensitized both activities of AK I-HDH I in the greatest extent when has been tested for closely related enzyme from *Serratia marcescens*. Each mutant gene as well as wild type *thrA* were assembled under the control of strong promoter in the integrity of *thrABC* operon and placed onto chromosome of TDH6 strain. TDH6 is the plasmidless derivative of the strain VKPM B-3996 that is meaningful threonine producer. The genetic ambience of that strain is favorable as allows comparing mutations *in vivo* at high production rate. The substitution Gly433Arg was the most prominent among examined. The strain carrying the Gly433Arg substitution accumulates threonine as much as 70 g per liter under feedbatch cultivation conditions. Strains carrying Ser345Phe and Glu253Lys substitutions produce 30 and 60 g per liter, respectively. However, the effect of Ser352Gln only slightly differs from that of wild type gene.

INDUSTRIAL BIOTECHNOLOGY OF MICROALGAE: GENETIC ENGINEERING OF COMMERCIALLY IMPORTANT CHLORELLA SPECIES

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Chlorella is a genus of industrially important microalgae, belonging to the phylum Chlorophyta. Together with *Spirulina*, they dominate the present microalgal market mainly because they are easy and fast to grow; they have high nutritional value as food and feed, and have applications in the cosmetic industry. As a result, *Chlorella* spp have been the focus of an increasing number of publications over the last few years in the area of algal biotechnology. Despite numerous efforts, however, reports of successful genetic manipulation and stable transgenic expression remain somewhat elusive with very few studies showing convincing and solid evidence. Here we present a simple and robust protocol we have developed for *Chlorella vulgaris* and, for the first time, for *Chlorella sorokiniana* based on transformation by *Agrobacterium tumefaciens.* We have also developed a two-marker system allowing a quick and convincing identification of *bona fide* transformant lines. The activity of such markers in transformants is detected after more than a year suggesting stable integration and expression of the transgenes. The tools and techniques presented here pave the way for further work on genetic manipulation of *Chlorella* with the potential to develop it as a new platform for algal biotechnology.

Keywords: High value products; metabolic engineering; non-food crops; biorefining; natural products, monoclonal antibodies, hybridoma, recombinant protein, monoclonal antibody, production, hollow fiber., hollow fiber, double membrane, cell culture; viral vaccine; gene therapy; stem cell; scale-up, Biorefining, Design of Experiments, process scale-up, industrial biotechnology, biorenewable chemicals, enzymes, Phenylpropanoids, cytoprotectants, flavonoid, yeast, Transgenic plants, omega-3 polyunsaturates, metabolic engineering, Metabolic engineering; terpenes; synthetic biology; plant protection; anti-cancer drugs, Alkaloids, Papaver somniferum, gene cluster, biochemical pathway, Polyphenols, tomato, metabolic engineering, cancer, preventive therapy

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