Business Interaction Vouchers Public Summaries of Project outcomes – Projects funded by Metals in Biology Network

ID number	**BIVMiB001**
Title	Metal demands during protein overexpression in bacteria
Academic (lead) Partner	Peter Chivers, Durham University
Industrial Partner	Mark Blight, Biocatalysts Ltd
Public summary	Protein and enzyme overexpression is a major facet of industrial biotechnology. The capacity of host organisms for protein overexpression is not naturally optimized. Transition metals are key components of the cellular machinery required for protein synthesis. The effects of protein overexpression on metal allocation within the cell have not been examined. This project will explore the effects of protein overexpression on metal utilization in *Escherichia coli*, a widely used platform for biologics production. The objective is to gain insight into the cellular response to the metal demands of protein overexpression. Metal allocation and utilization will be studied using RNASeq to monitor changes in gene expression that are metal-regulated or encode metal-requiring enzymes and proteins related to protein synthesis.
Start date	16 February 2015
Public summary of project outcomes	Transcript levels and metal content of E. coli cells were measured at different time points post-induction during a representative fermenter run. Increased transcript levels were observed in genes important for Mg, Fe, Mn, and Ni acquisition at and beyond the midway point of overexpression (≥ 9 h post-induction). These increases correlated with decreases in total cellular metal content for each metal, consistent with metal deficiency sensed by metal-responsive transcriptional regulators. These deficiencies have potential effects on translational efficiency (Mg), synthesis of non-natural amino acids that affect the fidelity of tRNA charging (Ni), and posttranslational processing of newly synthesized polypeptides (Fe/Mn). No evidence for Zn-deficiency, or Cu-stress, was detected based on transcript levels and metal content.

ID number	**BIVMiB002**
Title	Optimising metal acquisition by commercial metalloenzymes
Academic (lead) Partner	Kevin Waldron, Newcastle University
Industrial Partner	Stuart West, Biocatalysts Ltd
Public summary	Biocatalysts Ltd produce a number of metalloenzymes of commercial value through expression in bacteria and fungi. However, metal supply to these 'foreign' enzymes may not be optimal in these protein production hosts, so that a proportion of the commercial product is either bound to the 'wrong' metal, or lacks a bound metal ion altogether. Increasing the proportion of the enzyme that is correctly metal-loaded can directly increase profitability of metalloenzyme products. We will analyse the metal content of samples of the metalloenzymes produced by Biocatalysts. Where metal occupancy is found to be sub-optimal, we will work with the Biocatalysts technical team to test the effect of altering the bacterial/fungal growth conditions during enzyme production in order to increase the proportion of the target protein bound to the 'correct' metal ion. We will analyse the resulting enzyme samples to determine the effect of these changes on metal occupancy.
Start date	5 January 2015
Public summary of project outcomes	The aim of the project was to perform analyses of the elemental composition of the enzyme products that are produced by Biocatalysts Ltd. for the commercial market to determine whether the metal occupancy of these enzymes was sub-optimal. If so, the expression conditions could be adjusted in an effort to improve the metal supply to the enzymes.

	A single class of enzyme was selected for analysis, a set of three variants of magnesium-dependent galactosidase. We hypothesised that magnesium's position at the bottom of the Irving-Williams series would make it likely that these enzymes could wrongly acquire a non-native metal ion from further up the Irving-Williams series, either during heterologous expression or during sample preparation. Such association with the non-native metal cofactor is likely to lead to inhibition of the enzyme activity. The project partner, Biocatalysts Ltd. supplied the PI (Newcastle University) with a set of samples of these three enzymes, manufactured over an ~18 month period, to enable a longitudinal comparison of the metal content of these preparations. Each sample was solubilised and then analysed for total metal content by inductively coupled plasma mass spectrometry (ICP-MS). As a positive control, this confirmed the presence of abundant magnesium in each of the preparations (the product is exchanged into a buffer containing elevated magnesium concentration prior to drying). Importantly, in addition all preparations were found to contain significant trace quantities of (in order of amount detected) iron, zinc, copper and nickel. As the target enzymes in these samples are not homogenous however, as demonstrated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, further analysis was required in order to determine the amount of this trace metal detected in the preparations that was associated with the enzyme. Each sample was resolved by liquid chromatography, and the resulting fractions were again analysed for elemental composition by ICP-MS and for protein by SDS-PAGE. This method allowed accurate quantitation of the enzyme-associated metal, by separating it from unbound metal ions and from metals bound to contaminating proteins. The results of these analyses were that small amounts of copper, zinc and nickel (though not iron) were found to be present on the target enzyme in the preparations. The quantity of contaminating metals varied slightly between the longitudinal samples, and interestingly also varied between the three enzyme variants suggesting possible differences in their metal-binding properties. Importantly, though, the absolute quantity of these contaminating metals was low as a percentage of the total enzyme present (ranging from 3.8 - 12.8% occupancy), suggesting that they would make only a minor diminution of the total enzyme activity of the enzyme preparations.

ID number	**BIVMiB003**
Title	Optimisation of heme incorporation into a commercially important enzyme
Academic (lead) Partner	Nick Le Brun, University of East Anglia
Industrial Partner	Jon Wood, Biocatalysts Ltd
Public summary	Metals as cofactors of proteins and enzymes are essential for all of life. Many metalloproteins have properties that are useful outside of the cell, for example in the synthesis of useful materials or medicines. However, to exploit this the metalloprotein must be purified with the metallo- cofactor fully incorporated, requiring a matching of protein and cofactor synthesis/insertion. In this project, the academic partners, who are experts in metalloprotein chemistry, will provide analyses of commercially generated metalloprotein samples to assist the commercial partner in solving a problem of highly variable cofactor insertion.
Start date	1 February 2015
Public summary of project outcomes	The aims of the project were met in that it was determined that: • Cytochrome P450BM3 samples contained heme with properties characteristic of the P450 environment; • Samples were only ~50% loaded with heme. The poor protein expression and truncated form(s) of the protein were unexpected outcomes and point to some fundamental problems with the expression system.

ID number	BIVMiB004
Title	Metalloenzyme system for Hydrogen-driven NADPH recycling in pharmaceutical synthesis
Academic (lead) Partner	Kylie Vincent, University of Oxford
Industrial Partner	David Tew, GSK
Public summary	Bacterial cells are miniature chemical factories, with enzymes as the key machinery for making molecules. Pharmaceutical companies have recognised the benefits of using enzymes instead of traditional chemical routes to make drug molecules. Enzymes generate less waste, make purer chemical products, and allow chemistry to be carried out in water rather than in toxic, polluting solvents. However, there are challenges to overcome before enzyme approaches can be widely adopted. Many enzymes only work in the presence of special helper-molecules called cofactors, which are used up during the production of chemicals. The cofactors are expensive and for enzyme processes to be economically viable, it is essential to have some way of recycling the cofactors. We have demonstrated a novel approach for recycling a common cofactor called NADH. In collaboration with GSK, this project extends the approach to a related cofactor, NADPH, and demonstrates NADPH recycling for enzyme synthesis of pharmaceutically-relevant molecules.
Start date	1 November 2014
Public summary of project outcomes	Introduction. Biocatalysis is gaining increasing importance in synthesis of pharmaceuticals and other speciality chemicals, yet barriers remain - in particular the dependence of many redox enzymes on the expensive nicotinamide cofactor, NADH, and more costly NADPH. We have previously demonstrated a novel H2-driven NADH recycling system, and in this Business Interaction Voucher project we have extended this system to recycle NADPH. The project exploits genetic variants of an NAD+ reductase generated in the lab of Dr Oliver Lenz, Technical University of Berlin, which are designed to have higher affinity for the related cofactor NADP+. We have identified a suitable NADP+ reducing variant, and we have demonstrated NADPH recycling by coupling the cofactor recycling beads with a C=C bond reductase, PETNR, supplied by industrial collaborator GSK. Project outcomes: During the project we have recorded KM values for the most promising NAD+ reductase variants and identified one which functions as an NADP+ reductase with a KM of 1 mM NADP+, compared with >8 mM for the wild type. Data on the affinity constants for a series of variants will form the basis for the publication to be submitted in the coming months. The selected NADP+ reductase was then incorporated into cofactor recycling beads. A nickel-iron hydrogenase oxidises H2 and transfers electrons, via its internal relay chain of iron-sulphur clusters, to the electrically-conductive carbon bead. Electrons are then transferred through the bead to the co- immobilised NADP+-reductase which also possesses a relay chain of iron-sulphur clusters for fast electron transfer to its flavin active site. Thus H2 oxidation is coupled efficiently to NADP+ reduction. The imine reductase and ene reductase enzymes supplied by GSK were examined in biochemical assays with product detection by HPLC or GC. The C=C bond reductase PETNR was selected as the best enzyme for a first demonstration of NADPH recycling. In the presence of H2, NADPH was supplied to PETNR which was co-immobilised on the beads or handled in solution, for the reduction of cyclohexenone to cyclohexanone. Near-complete conversion (>99%) of the alkene to the alkane was observed by GC. Thus we have generated promising results on the feasibility of coupling H2-driven NADPH recycling to a NADPH-dependent hydrogenation. These results will be prepared for publication later this year as part of the demonstration of the modularity of the H2- driven cofactor recycling system which can be adapted for

	operation under different conditions by varying the hydrogenase or the NAD(P)+ reductase. Conference presentations: Dr Holly Reeve presented a Flash Talk and Poster that included these findings at the European Symposium on Biological and Organic Chemistry, Gregynog, Wales, 15th-17th May 2015. Prof Kylie Vincent gave an invited lecture at the Academic Day of GSK's Global Technologies Conference, Cambridge, 25 March 2015: 'Heterogeneous biocatalysts for H2-driven chemical synthesis', and included some of these results in her presentation.

ID number	**BIVMiB007**
Title	Developing an ultra-compact integrated hyperspectral monolithic fluorescence biosensing system
Academic (lead) Partner	Po-Wah So, King's College London
Industrial Partner	Joanna Coote, Zinir Ltd
Public summary	We are using magnetic resonance imaging (MRI) to look into the distribution of iron in the brain without surgery or biopsy, to learn whether iron accelerates/promotes ageing or disease. However, changes in the MRI signal can also be due to changes in cell structure and/or biological molecules in the brain. We aim to determine the specifications for a new ultra-compact, easily transportable device for simultaneously visualising a number of cells/biological molecules, individually labelled to emanate a characteristic type of light. We will assess the feasibility of incorporating such a device within a MRI system such that we can see cells/molecules using the new device but in a 3D anatomical/structural context provided by MRI. This readily transportable and unique device will monitor specific biological processes in living systems used in industrial biotechnology, enhancing efficiency (production) and improving manufacturing methods, and able to perform in environments that other devices cannot operate.
Start date	8 December 2014
Public summary of project outcomes	KCL and ZiNIR collaborated on a project to identify the technical specifications of an ultra- compact, easily transportable hyperspectral imaging system for detecting a range of biomolecules in vivo. The system would employ arrays of novel miniature solid-state spectrometer chips developed by ZiNIR, and could potentially be incorporated into a MRI system for multimodal imaging. A literature review and patent search were conducted to assess the current status of hyperspectral fluorescence imaging and multimodal MRI-optical imaging, both in biomedical applications and in industrial biotechnology. A number of parallels were found between the requirements for fluorescence imaging in preclinical studies and chlorophyll fluorescence imaging, a technique used in a wide variety of plant biology and biotechnology applications. Accordingly, a set of technical specifications for the imaging system was defined to satisfy the requirements of both application areas.

ID number	**BIVMiB008**
Title	Metallo-enzymes for production of nootkatol, a potential new citrus flavour
Academic (lead) Partner	Luet Wong, University of Oxford
Industrial Partner	Jason King, Oxford Biotrans Ltd
Public summary	The sought after grapefruit flavour compound nootkatone is biosynthesised by air oxidation of valencene catalysed by a haem enzyme, firstly to nootkatol and then nootkatone. Haem enzymes are involved in the biosynthesis of numerous natural products, including flavours such as menthol and nootkatone but also medicinal compounds such as antibiotics, the antimalarial artemisinin and the anticancer drug taxol. The industry partner is developing commercial scale biocatalytic synthesis of nootkatone from valencene. The academic partner will

	modify the haem enzyme used in this process to produce nootkatol, which is found in minute quantities in grapefruit, to explore it's potential as a novel flavour. This primer-project and the nootkatone process will underpin future collaborative work within the metals-in -biology community (1) to test strains optimised (by others) in haem production to further enhance nootkatone/nootkatol synthesis, (2) to manipulate the primary and secondary coordination sphere of the haem moiety to further enhance nootkatol synthesis.
Start date	1 January 2015
Public summary of project outcomes	Samples of the pure nootkatol isomers were produced and delivered to the industry partner. The potential market of these novel flavours is being assessed. Enzyme variants that gave increased proportions of either nootkatol isomer were generated. Process optimisation also led to improved yields of the nootkatone production process. New strains from the NIBB MiB network can be applied to the systems and processes developed in this BiV project to benefit the UK industrial biotechnology sector.

ID number	**BIVMiB009**
Title	Adding Value to Galactomannan Polysaccharides with Cu Enzymes
Academic (lead) Partner	Julea Butt, University of East Anglia
Industrial Partner	Seth Hartshorne, Schlumberger Gould Research
Public summary	Concerns over fuel security are frequent news headlines and the rising costs of fuel are a daily reminder of the challenges faced by a global society with ever increasing energy demands. Medium- to long-term solutions to these challenges will require effective access to renewables alongside the development of infra-structures that enable such energy to be delivered to the point of need with the ease that is presently enjoyed with our use of fossil fuels. Improved access to the oil and gas reserves that are found in shale present an attractive option for the short- to medium-term. Here we aim to investigate opportunities to improve the recoveries of shale oil/gas through the use of copper-containing enzymes that can modify the properties of a natural biopolymer, guar gum, that plays a key role in the shale extraction process.
Start date	1 January 2015
Public summary of project outcomes	Galactomannans are naturally occurring polymers with high solubility in water. The resulting solutions have high viscosity at relatively low polymer concentration such that they are widely used as industrial colloids. Galactomannans find application as thickening agents in pharmaceutics, cosmetics, food products and water-based fracturing fluids. However, the properties of galactomannans can be augmented by chemical modification. The traditional routes to modification require protecting groups and the use of non-aqueous solvents. However, enzymatic modification offers prospects of more sustainable routes to modified galactomannans. The galactomannan polymer is comprised of covalently linked sugar molecules, namely, mannose and galactose. Polymer hydrolysis resulting in chain shortening was demonstrated using a commercially available enzyme cellulase. Oxidation of the galactose sidechains within the polymer in aqueous solution was demonstrated using a commercially available enzyme, galactose oxidase. In addition, electrochemical oxidation of the galactose and mannose sugars was demonstrated by oxidation of aqueous guar suspensions at a graphite electrode. The results demonstrate that commercially available enzymes offer routes to controlled modification of guar in aqueous solution and this offers prospects for the development of more sustainable routes to industrial scale galactomannan modification.

ID number	**BIVMiB011**
Title	Assessing the bioavailability of metal ions accumulated by DRAM® filters
Academic (lead) Partner	Louise Horsfall, University of Edinburgh
Industrial Partner	Leigh Cassidy, Epona Technologies Ltd

Public summary	The environmental quality standard for copper in groundwater in the UK is set at 1 - 28 µg/l, due to its toxic nature, but copper compounds are one of the few pesticides allowed for organic food production. They are also used to control fungal diseases in vineyards and coffee plantations; as molluscicides and insecticides; as a disinfectant in the farming and fishery industries and to prevent algal blooms. Whilst the spirits industry produce whisky, vodka and gin in a process which involves a universal step of distillation in copper pot stills, producing waste contaminated with solvated copper ions. Consequently this has led to increasing concern over environmental copper levels, their toxicity and the adverse effects on humans and wildlife. Epona Technologies Ltd has developed DRAM® filters, which are able to accumulate polluting copper ions from industry and agriculture but, without steps to allow its reuse, the problem is just being transferred elsewhere. With the finite supply of copper and its ever increasing cost, employing bacteria for the recycling of such contaminants to allow their reuse may provide a long-term sustainable solution. The Horsfall Lab is currently examining the process of copper nanoparticle production by bacteria. We have already determined that *Morganella* sp. can transform copper ions from whisky waste into nanoparticles of solid, zero-valent copper in addition to the reported biotransformation of model solutions and we would like to determine whether the copper ions accumulated by DRAM® filters could be transformed into copper nanoparticles too. This would be dependent upon the bioavailability of the copper ions accumulated and whether the conditions of bioavailability coincide with the conditions under which transformation occurs. It would also be of significant importance and impact if the nanoparticles were retained by the DRAM® filters, allowing for a separate elution step.
Start date	1 January 2015
Public summary of project outcomes	This project assessed the potential to recover copper(II) from DRAM® media filters and its bioconversion to metallic nanoparticles harnessing the ability of *M.psychrotolerans* to reduce Cu(II) to Cu(0). Using a CuSO4 solution, copper(II) was accumulated on these filters through filtration or stirred incubation. Both methods resulted in 1g of DRAM® capturing about 2.4 mg Cu(II). Approximately 20% of this bound Cu(II) can be desorbed into the surrounding medium and made available for biotransformation into copper(0) nanoparticles. These particles were visualised and positively identified by electron microscopy. Thus *M.psychrotolerans* can form nanoparticles from copper(II) accumulated on DRAM® filters. As DRAM is a biological material non-uniform in size its presence was a significant obstacle in the positive identification of metal nanoparticles. To facilitate electron microscopy visualisation of nanoparticles in this study DRAM® media was isolated from the culture medium containing *M.psychrotolerans* cells using a copper-permeable membrane. It can however be reasonably assumed that reduction of copper(II) can take place when bacterial cells are incubated together with DRAM® in the absence of a membrane. Moreover, a culture of *M.psychrotolerans* cells with a DRAM® media suspension might increase bioconversion through improved availability of copper(II). In this study, approximately 12% of Cu(II) ions are reduced by *M.psychrotolerans* to metallic Cu(0) nanoparticles after overnight incubation in LB medium containing 5 mM CuSO4. It is expected that the gradual release of copper from the DRAM® media may alleviate copper toxicity to live cells and thereby increase productivity. However, while the proof of principle study was successful with model solutions and controlled copper solutions, the industrially used DRAM® media with its unknown contaminants is currently beyond our methods of nanoparticle isolation and analysis, and therefore no nanoparticles could be identified from this source.

ID number	**BIVMiB014**
Title	Metal interactions with a novel disulphide folding catalyst: a new strategy to improve antibody fragment production

Academic (lead) Partner	Mark Shepherd, University of Kent
Industrial Partner	David Humphreys, UCB- New Medicines
Public summary	The production of biotherapeutics has a total market value of around £100 Billion per year. This project has the potential to develop a new system to improve the production of antibody fragments in *E. coli*, a product with wide ranging applications in diagnostics (*e.g.* pregnancy tests), human therapeutics and as fundamental research tools. In addition, future consumer applications might include the use of antibodies in shampoos to prevent the formation of dandruff or in toothpaste to protect against tooth decay. This project explores the impact of copper upon a novel protein folding catalyst (ScsC), with the overall aim of improving the quality of antibodies produced in *E. coli*.
Start date	1 October 2015
Public summary of project outcomes	The production of biotherapeutics has a total market value of around £100 Billion per year. This project aimed to test the efficacy of a new bacterial system to improve the production of antibody fragments, which have wide-ranging applications in diagnostics (e.g. pregnancy tests), human therapeutics and as fundamental research tools. This project explored the impact of copper upon novel machinery from Salmonella (the Scs system) that is proposed to facilitate the introduction of disulphide bonds, which are key structural features of antibodies. The main hypothesis was that the Scs system and/or copper could improve the synthesis of antibodies in E. coli (copper is redox active metal that has previously been implicated in Scs function). This hypothesis was tested via co- expression of the Scs proteins alongside an antibody fragment based on Herceptin, a breast cancer therapeutic. The abundance of antibody fragment was assessed using standard immunological detection procedures, and the main outcomes are listed below: •Antibody fragments were detected in whole cells as intact disulphide-bonded dimers •Preliminary data suggests that the Scs system and copper deliver a synergistic enhancement of antibody fragment yield.

ID number	**BIVMiB015**
Title	Analysis of ferritin iron in pea flour
Academic (lead) Partner	Janneke Balk, John Innes Centre
Industrial Partner	Patrick Mitton, AgriTopics
Public summary	Peas provide a rich source of proteins and nutrients for human diets. They also contain relatively high levels of iron in a special form which is very easy for the body to absorb. To extract this high value component or use pea flour directly in food products, the first step is to mill the dry peas. Together with the industrial partner AgriTopics, we will evaluate different milling procedures, milling fractions and particle sizes to optimize this first step for producing nutritional flour and iron extraction. The project will provide the basis for the development of new iron supplements, which could also be used clinically to treat iron deficiency anaemia, and specialist flours for the food industry. Our application fits within the remit of the Metals in Biology BBSRC NIBB as it investigates the industrial processes for refining peas to produce a bio-available iron nanoparticle for the health (and food) industries.
Start date	1 October 2015
Public summary of project outcomes	Peas are rich in nutrients and protein, but highly undervalued as a healthy part of our diet. Janneke Balk's laboratory has developed a method to extract ferritin, an iron-rich protein nanoparticle, from dried peas. It has further shown in collaboration with Prof Susan Fairweather-Tait (UEA Med School) that purified pea ferritin has excellent iron bio-availability in cultured intestinal cells. AgriTopics is a specialist agribusiness developing high value products from processed pea flours for the "free-from" (gluten-free) and health food markets. In this project we tested the effect of different milling techniques on the extraction of ferritin. We also investigated the nutritional profile of pea flour

	before and after ferritin extraction. We found that a specialised milling process, developed and optimised by the Industry partner, is as good, or even better than a range of alternative milling processes for the extraction of ferritin. The extraction procedure removes sugars from the pea flour, but these are only 3 g / 100 g to start with. Fatty acids, protein and micronutrients remain in sufficient quantities for the flour to be used for food products.

ID number	**BIVMiB026**
Title	Microbial recovery of metals from contaminated Miscanthus used in the industrial remediation of degraded landscapes.
Academic (lead) Partner	Chris Chuck, University of Bath
Industrial Partner	Michal Mos, Terravesta Limited
Public summary	Metal leaching from mining and further industrial activity has the potential to degrade landscapes across the globe. However, recently a range of techniques have been trialled and brought to market to restore the natural capital of these areas. One of the most promising is growing Miscanthus x giganteus, an energy crop that can remove metal contamination while being used as a biofuel feedstock. However, the processing of the contaminated Miscanthus x giganteus remains an issue. In this BIV we will explore using the oleaginous yeast M. pulcherrima, which produces metal chelators such as pulcherriminic acid, as a method of valorising the Miscanthus biomass into a range of products including a palm oil substitute and removing the metal waste into a smaller containable volume. This method will then be compared to hydrothermal processing of the Miscanthus waste
Start date	1 February 2016
Public summary of project outcomes	The project demonstrated that the most suitable technique for metal recovery from MIscanthus grown on contaminated land was hydrothermal liquefaction (HTL). The HTL of the Miscanthus showed a reasonable bio-oil yield and in addition the majority of metals from the MIscanthus partitioned in the aqueous phase or the solid residue and could be recovered / recycled easily. Further work would need to be invested in increasing the bio-oil content, further optimisation to partition the metals into the solid residue while decreasing the carbon content.

ID number	**BIVMiB020**
Title	Novel disposable cell culture systems for microbial growth in metal-regulated environments
Academic (lead) Partner	Robert Poole, University of Sheffield
Industrial Partner	Kelly Davidge, Kirkstall Ltd
Public summary	Growing cells and tissues in biotechnology requires a well-defined growth environment. Important factors include design of the culture apparatus and growth medium, which must provide all nutrients including metals in biologically accessible forms, but not in excess. Most growth vessels are metal or glass, but these can leach or adsorb metal ions. Synthetic materials, however, may be biologically inert and interact little with dissolved metals. This project will test the suitability for microbial growth of miniaturised growth chambers (Quasi-Vivo® by Kirkstall) that were developed for culturing mammalian cells. These chambers are made from biocompatible materials and, under flow conditions, mimic conditions in the body. We will grow bacteria in such chambers and test their ability to provide environments in which the metal concentrations available for growth will be varied from 'trace' to toxic. The work has potential for developing improved methods of cell culture in industrial biotechnology.
Start date	1 January 2016
Public summary of project outcomes	This project aimed to test the suitability for microbial growth of miniaturised growth chambers (Quasi Vivo® by Kirkstall) that were developed for culturing mammalian cells. These chambers are made from biocompatible materials and, under flow conditions, mimic conditions in the body. We grew bacteria in such chambers and

	tested their ability to provide environments in which the metal concentrations available for growth could be varied from 'trace' to toxic (Hubbard et al., 1990; Hubbard et al., 1986). The work has potential for developing improved methods of cell culture in industrial biotechnology. We conclude that these vessels are suitable for growths involving metal-controlled conditions.

ID number	**BIVMiB013**
Title	Development of new refolding methodologies for expression of heme protein targets
Academic (lead) Partner	Emma Raven, University of Leicester
Industrial Partner	Andreas Kuglstatter, Roche Innovation Center, Switzerland
Public summary	Heme (iron)-containing enzymes are a mainstay of industrial biotechnology, and the industry depends on fundamental improvements in methodology emerging from academic groups around the world to harness the potential of their investments in biopharmaceuticals, bioenergy, biocatalysis and drug design. For a number of complex reasons, the interactions between industry/biotechnology and academic laboratories are often less facile and less extensive than they could be, so that new (often Specialist and/or unpublished) information is not transferred fluently to industrial partners. Our overall objective is to use this project to develop new refolding methodologies for expression of difficult (insoluble) heme protein targets, and to set up an on-going dialogue between industrial and academic partners with mutual cognate interests in specific heme enzymes targets. The methodologies that we develop will open up new avenues for industry partners in cases where they have intractable (insoluble) protein targets.
Start date	1 February 2016
Public summary of project outcomes	In this project, protocols for expression methods were developed for a range of different heme enzymes that are not readily expressed using conventional methodologies in *E. coli*. Early stage targets from the Raven laboratory were used as a "test bed" for other heme systems.

ID number	**BIVMiB023**
Title	Maximising Biomarker Detection Sensitivity through Metal Enhanced Fluorescence
Academic (lead) Partner	Lu Shin Wong, University of Manchester
Industrial Partner	Stephen Kilfeather, Aeirtec Ltd
Public summary	Fluorescence-based immunosorbent assays have become a key technology for detection and quantification of microbial contamination (in water, chemical, food and drug production), as well as for molecular biomarkers in agriculture, drug discovery, industrial biotechnology, medical diagnostics and cellular imaging. Here, the selectivity of antibody binding combined with fluorescence spectroscopy, has led to huge advances in sensitivity, selectivity and speed. Typically, these assays employ Fluorescence-based immunosorbent assays have become a key technology for detection and quantification of microbial contamination (in water, chemical, food and drug production), as well as for molecular biomarkers in agriculture, drug discovery, industrial biotechnology, medical diagnostics and cellular imaging. Here, the selectivity of antibody binding combined with fluorescence spectroscopy, has led to huge advances in sensitivity, selectivity and speed. Typically, these assays employ an immobilised antibody to capture the target molecule from the test sample, followed by the immobilisation of a second antibody bearing a fluorescent label. To maximise the fluorescence output from the label, many researchers have started to harness metal enhanced fluorescence (MEF) as a means to improve diagnostic sensitivity. Here, the co-location of the antibody in the vicinity of a metallic nanoparticle results in a large enhancement of fluorescence output (in the order of 100-fold). This project aims at improving the sensitivity of these MEF-based assay systems by the application of state-of-the-art bioconjugate methods to

	control the orientation of the immobilised antibodies with respect to the nanoparticle.
Start date	1 July 2016
Public summary of project outcomes	This interaction voucher was used to develop a collaborative relationship on the production of protein-metallic nanoparticle conjugate materials, for use in Aeirtec's diagnostics platform. It particular, it concentrated on the bioconjugate chemistries needed for the immobilisation of these materials. Specifically, comparative analyses were conducted with various diamine linker molecules using a variety of protecting groups. Robust and quantifiable methods for linking fluorescence marker materials were developed and delivered to Aeirtec. From an academic perspective, the voucher was used to part-fund the research undertaken by students in Dr. Wong's laboratory. In particular, it provided the basis for a mini-project for one of our MSc students. It also part-funded two undergraduate summer internships and provided them with further research experience. Aeirtec has benefited from this interaction through access to chemistry in relation to our capacity to generate a metallic-protein microparticle surface. The interaction has now set a direction for incorporation of metals alongside proteins and microparticle identifying dyes.

ID number	**BIVMiB018**
Title	Investigating platinum group metals in wastes from roadside verges
Academic (lead) Partner	Neil Bruce, University of York
Industrial Partner	Richard Bate, Yorwaste Ltd
Public summary	Platinum group metals (PGMs) are rare elements that are essential components in a widening number of industrial applications and particularly used in catalytic converters on road traffic vehicles. Over time, these metals are lost from the converters via exhaust fumes, with significant levels being deposited onto roads and verges. This increasing dispersal and dilution into the environment is of growing concern. Plants can take up PGMs, and our work at the University of York has demonstrated that the subsequently harvested, PGM-rich plant biomass has catalytic activity. This project aims to measure the levels of palladium and platinum in roadside verge wastes from rural, inner city and highway road stretches. Plants will be grown on the wastes to see if they can take up PGMs. This study will enable us to understand if significant levels of PGMs exist in road sweeping waste and whether a phytoextraction process is a viable technology to recover it.
Start date	1 July 2016
Public summary of project outcomes	We have achieved our objectives to investigate NP formation using synthetic peptides and analyse catalytic activity in the subsequently pyrolysed NP-containing plant biomass. Our promising ICP-OES, TEM and catalysis results demonstrate that the expression of synthetic peptides in plants can be used to alter gold NP size and subsequent catalytic activity in planta. As part of our third objective, to determine if plants could be used to selectively take up PGMs from sweeper wastes, we have shown that sweeper wastes contain detectable levels of valuable metals. However, our studies show that further work is needed to understand the phytotoxicity behind these wastes so that they can be optimised to allow plant growth. Our wider studies indicate that synthetic biology could be used to develop plants that can selectively take up PGMs from sweeper wastes.

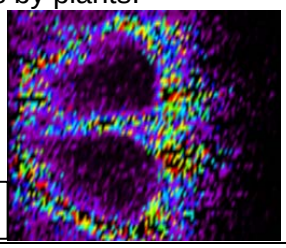
ID number	**BIVMiB012**
Title	Chelation Therapy in the Washing Machine
Academic (lead) Partner	Nick Jakubovics, Newcastle University
Industrial Partner	Adam Hayward, Procter and Gamble
Public summary	Biofilms are a major problem in all sorts of industrial settings, including bioprocessing facilities. Mechanical biofilm removal is not always possible due to the chemical and physical properties of the contaminated surface and the use of

	chemical agents is the most appropriate approach for biofilm control. This proposal involves working with an industrial partner (P&G) to develop novel chemical technologies for biofilm removal at low temperatures on a complex surface (laundry). We envisage that successful outcomes can be translated to biofilm control in many different settings including bioprocessing plants.
Start date	1 March 2016
Public summary of project outcomes	Quantitative analysis of anti-biofilm effects of chelators: Following initial screening, three strains of bacteria were selected as representatives of important laundry-relevant microbes: Corynebacterium propinquum FH1, Micrococcus luteus NCTC 2665 and Staphylococcus aureus NCTC 6571. Biofilms were formed on the surface of plastic 12-well plates in a static growth culture model. Following biofilm formation, a range of chelating agents were applied to biofilms under vigorous shaking conditions and in the presence of detergent, to mimic the conditions encountered in a washing machine. Biofilms were washed and residual biofilm was stained using crystal violet. The dye was dissolved and quantified spectrophotometrically as a measure of biofilm remaining. Despite extensive efforts at optimizing biofilm formation, C. propinquum and M. luteus biofilms were very weak and chelating agents did not reduce the biofilm biomass further. S. aureus biofilms were stronger, but were not significantly reduced by chelating agents. Imaging of biofilms: To obtain more detailed information on the effects of chelators on biofilms, M. luteus biofilms were cultured on the surface of washing machine grade stainless steel under aeration (shaking). Biofilms were stained with a membrane dye, FM5-95, and DNA-specific dye YOYO1. These images showed that biofilms were relatively thin and patchy across the surface. Qualitatively, the biofilm architecture did not appear to be affected by treatment with chelating agents. Higher resolution field emission scanning electron microscopy images demonstrated that microbial cells were present in patches, and that there were other areas where cells were absent, but the residue from previous biofilm growth was clearly apparent. This residue may have been extracellular matrix material or lysed microbial cells.

ID number	**BIVMiB030**
Title	Investigating the antimicrobial properties of copper infused fabrics
Academic (lead) Partner	Bill Keevil, University of Southampton
Industrial Partner	Rory Donnelly, Copper Clothing Ltd
Public summary	The University of Southampton have been testing the antimicrobial effects of copper for many years, and have shown that copper can kill bacterial cells in several ways, including rupturing the cell, and destroying the DNA. Copper Clothing Ltd has begun investigating new processes for incorporating copper into their antimicrobial fabrics. Initial work will investigate the antimicrobial properties of fabrics infused with copper using novel technologies. The new fabrics developed by Copper Clothing Ltd. will be tested for levels of antimicrobial kill in a range of bacteria, both by culture and by using fluorescence microscopy to determine the presence of living but non culturable organisms – bacterial that are not killed by the copper, but survive in a stressed form. Future work plans to use bio-produced nano-copper for incorporation into the fabrics and determine their improved efficacy against superbugs compared to the current novel fabric chemistries.
Start date	1 August 2016
Public summary of project outcomes	Of the fabric samples tested with three different bacteria, the thin, single layered bamboo viscose and nylon fabrics with impregnated copper showed a greater than 3 –log reduction of bacteria at 24 h. In contrast, the more absorbent and thicker tea towel fabrics impregnated with silver or copper showed no reduction of bacteria after 24 h. It is suggested that the main difference between the two types of fabrics were the thickness and absorbency. We recommend that future tea towel fabrics be made with a higher percentage of copper-impregnated

	threads to allow bacteria to come into contact with the copper, and thus allow the copper to act. By training the bacterium *Morganella psychrotolerans* to grow on various concentrations of copper sulphate (CuSO4) agar, the industrial partner was able to select for variants that could survive in the presence of this usually bactericidal chemical. By adding CuSO4 to the growth media of these variants, the pellet of bacteria that grew took on a brown colour indicating the presence of copper particles within. This suggested that copper nano-particles were present inside the bacteria. However, while we are as yet unable to visualise bacteria containing BioCuNPs inside their membranes, we have managed to image nano-particles of various sizes present in the supernatant of the growth media, the largest of which are well defined hexagonal nanoparticles.

ID number	**BIVMiB024**
Title	Investigating uptake and catalytic potential of miscanthus grown on palladium mine wastes
Academic (lead) Partner	Neil Bruce, University of York
Industrial Partner	David Stone, AgriKinetics Ltd; Mike Cooper, Miscanthus Nursery Ltd
Public summary	We have shown that Arabidopsis plants grown in liquid culture can take up palladium, depositing it as nanoparticles. Following a processing step, the nanoparticle-containing biomass can be used directly as a catalyst for industrially important reactions. Palladium mining wastes contain significant levels of palladium which are uneconomical to recover using conventional methods; mining areas also need to be re-vegetated. Phytoextraction using field-relevant species could be a viable and environmentally sustainable method to re-green these sites while generating a catalytic product of higher value than the bulk metal. Our preliminary studies at the Centre for Novel Agricultural Products (CNAP) have demonstrated that the grass, miscanthus, can take up palladium from synthetic mine wastes, but not yet at levels that confer catalytic activity. A major limitation to uptake is the insolubility of palladium in the wastes. This project will enhance palladium availability, measure accumulation in miscanthus and provide biomass for catalysis testing.
Start date	1 April 2016
Public summary of project outcomes	We have achieved our objective to test the effect of multiple KCN treatments on palladium uptake by miscanthus. While repeated applications of KCN did not significantly increase palladium concentrations in the aerial tissues we know, from earlier experiments, that miscanthus can tolerate higher levels of palladium. Although our studies have demonstrated that plant material containing greater than 12 g/kg palladium can be used as a commercially-comparable catalyst, in practice, achieving this target is difficult. However, our recent studies suggest that during controlled, low-energy pyrolysis, the presence of lower levels of palladium in plant biomass can catalyse the production of novel, high value, compounds from the plant biomass. Towards this, the ground miscanthus samples are now being investigated by the GCCE. Miscanthus is now an established biomass crop in the UK, with existing agricultural infrastructure and agronomy. It has a vigorous growth habit, and tolerates poor soils, and toxic metals. As such, it is well-placed for studies to investigate the remediation of valuable metals from waste sources. While this project focused on the use of KCN as the lixiviant, this is not the method of choice for solubilisation. In addition to environmental concerns, cyanide treatments solubilise a number of other metals, out of reach from the root zone, with metals then lost as leachate. We are currently investigating techniques whereby synthetic biology can be used to solubilise metals specifically around plant roots.

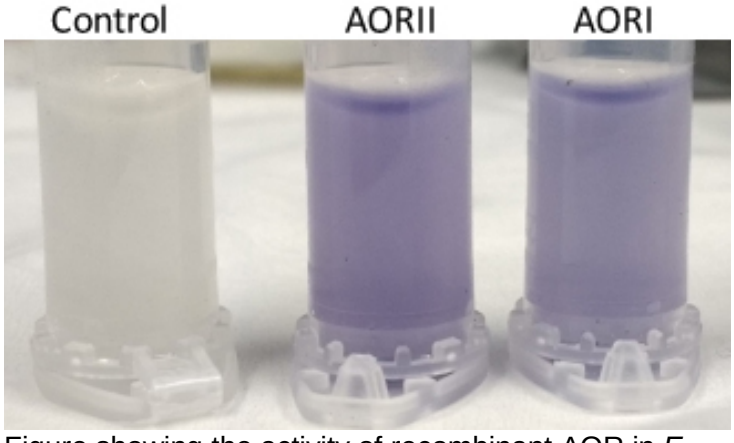
ID number	BIVMiB019
Title	Studies into the Uptake and Distribution of Metal Oxide Nanoparticles in Plants
Academic (lead) Partner	Neil Bricklebank, Sheffield Hallam University
Industrial Partner	Hannah Griffiths, Croda Europe Ltd
Public summary	The uptake of metals is essential for the growth and development of healthy plants. Plants obtain the metals they need from soil or from fertilizers applied to the growing plant. One of the most important metals is zinc which is found in many metalloenzymes. Zinc is also essential for humans who gain it from the grains and vegetables which they eat. In this project we will study the effect of zinc, in the form of a formulation containing zinc oxide, on the growth of plants and use a new analytical tool, known as Laser Ablation-Inductively Coupled Plasma-mass spectrometry (LA-ICP-MS), to study the uptake and distribution of zinc in plants. This project fits within the remit of the BBSRC-NIB because it will investigate metal availability, uptake and assimilation into biomolecules required for bio-energy production. It will also enable us to develop new tools and technologies for studying metals in biological systems.
Start date	18 May 2016
Public summary of project outcomes	All plants require metals for healthy growth and development which they obtain as micronutrients from soil or from fertilizers applied to the growing plant. Amongst the most important micronutrients is zinc, which is also crucial for the health of humans and animals, who acquire it from the grains and vegetables that they eat. Zinc deficiency is a major problem in soil fertility and plant nutrition throughout the world and zinc-containing fertilizers have been developed to correct deficiencies. The aim of the BIV project was to study the uptake and distribution of zinc by barley, using a analytical tool know as Laser Ablation-Inductively Coupled Plasma-mass spectrometry (LA-ICP-MS). LA-ICP-MS is capable of quantifying trace elements and can be used in bio-imaging to determine the distribution of metals in samples. Interest in studying trace metal distribution in biological material is increasing and there are several reports into the use of LA-ICP-MS in metallomic studies of human and animal tissues. However, there are fewer reports of its application to plant material. The zinc was applied to the barley before cultivation as a seed coating. The coating is a chemical solution designed to be retained by a seed before planting. It is made up of surfactants and inert carriers for micronutrients, growth promoters, antimicrobial agents and fungicide. Seed coatings are an efficient way of delivering micronutrients to plants and help protect the seed before and during germination. Two samples of barley seeds coated with zinc oxide particles and different seed treatments were prepared by Croda. Seeds from each sample were germinated at Sheffield Hallam and harvested at different growth points. The selected germinated seeds were embedded in gelatin, cryosectioned, and then analysed by LA-ICP-MS. Untreated, germinated, seeds were used as the control. The results provide a two-dimensional 'map' (figure 1) showing the location of the zinc within the seed at different time points throughout its germination. The results clearly show that as the seed is germinated the zinc is transported from the coating applied to the surface and into the shoot of the seedling. The results are being used by Croda to assess the effectiveness of their products, including surfactants, adjuvants and formulation aids, which are used in commercial seed coatings, on the uptake of metals by plants.  a b  c

Metals IN BIOLOGY

	Figure 1. Image of (a) un-germinated seed, (b) section of treated seed tissue, and (c) LA-ICP-MS image showing distribution of zinc in seed tissue.

ID number	**BIVMiB017**
Title	Extracting mercury from industrial waste using microalgae
Academic (lead) Partner	Mark van der Giezen, University of Exeter
Industrial Partner	Tonnie Schuijl, Reym
Public summary	Affordable and sustainable energy is an important global challenge. Biofuels are seen as possible solutions; however, currently they have limited environmental benefits and put pressure on land and water. Algal biofuels could overcome many drawbacks of terrestrial plant-based biofuels but are currently more energy-intensive and expensive. Water pollution is another global problem and causes over 14,000 deaths each day. By 2025, 1.7 billion people are faced with absolute water scarcity and two-thirds will have drinking water shortages. Unfortunately, many essential industrial activities contribute greatly to water pollution and more sustainable production methods or waste management practices are required to support the global demand for products but protect water sources. With relevance to both challenges, we will grow algae on metal contaminated industrial waste streams followed by hydrothermal liquefaction. This process will separate waste into four fractions: water, gas, oil and solids, the latter containing the metal waste, thereby valorising waste.
Start date	1 July 2016
Public summary of project outcomes	Mercury contaminated petrochemical waste is routinely stored in abandoned mineshafts. Considering the volume of these wastes (several metric tonnes/day in the Netherlands alone), compaction of the toxic mercury alone would save the industry money and space. Mercury contaminated sludge from 6 different locations in the Netherlands was processed. To assess the microbial diversity associated with this material, sludge was diluted and incubated under various growth conditions. DNA was isolated from individual morphologically distinct colonies. In addition, total environmental DNA was isolated directly from sludge to assess the metagenome of this material. Several complete genomes were assembled from these experiments. Analysis indicated a metabolic potential to deal with heavy metals, in particular mercury. Another strand of research investigated the possibility to remove metal contaminants using hydrothermal liquefaction (HTL) with or without biomass in the form of algae or alginate beads. Different HTL temperatures were tested. Our results show that algae alone are not capable of removing the high mercury load from the sludge. Alginate beads shows better removal capabilities that algae but the combination of alginate beads loaded with algae shows excellent mercury removal capabilities with all mercury removed from the water phase. HTL under different conditions indicated that algae alone with the sludge were least effective with removing 96.9% of mercury. HTL with sludge alone was most effective with 100% of the mercury ended up in the solid fraction. The alginate beads conditions resulted in 99.9% of mercury removed. It is clear that we have demonstrated the ability of HTL to massively reduce the volume of the mercury containing sludge. We have successfully demonstrated the potential of HTL to remove and concentrate metal contaminants from industrial waste streams. Similar to our successful removal of metal contaminants from mine waste, we have now also clearly demonstrated that our approach can be successfully applied to petrochemical waste as well.

ID number	**BIVMiB022**
Title	Cloning and metal analysis of recombinant aldehyde ferredoxin oxidoreductase (AOR)
Academic (lead) Partner	Martin Warren, University of Kent
Industrial Partner	Dr Michelle Gradley, ZuvaSyntha

Public summary	The aim of the project is to enhance the recombinant production of a key enzyme of biotechnological importance with a view to the production of metal-cofactor complete protein. Specifically, the aim is to find optimal conditions that allow for overproduction of recombinant aldehyde ferredoxin oxidoreductase (AOR), an enzyme that allows the transformation of carboxylic acids into aldehydes. AOR has an unusual metal requirement in that it contains an oxo-tungsten centre bound to a pair of molybdopterin cofactors and a 4Fe-4S cluster. We will clone two mesophilic forms of AOR from *Clostridium ljungdahlii* and another thermophilic form of the enzyme from *Pyrococcus furiosus*. The enzyme will be produced with a His-tag to allow for easy purification. Moreover, the protein will also be produced with a tag to allow for the protein to be targeted to a bacterial microcompartment (BMC). BMCs are utilised by nature to help accommodate enzymes that produce aldehydes as pathway intermediates.
Start date	1 September 2016
Public summary of project outcomes	Bio-based alternatives to petrochemical commodities, which are required as inputs into a variety of industrial applications such as polymers, coatings and surfactants, are predicted to rise from 2% to 25% of global production over the next ten years. The international chemical market is currently worth around $4 trillion with bio-production offering advantages such as environmental sustainability, cheaper production costs and protection from oil-price volatility. Approaches such as metabolic engineering and synthetic biology can be used to make bio-products and processes more efficient and cost competitive, and are fuelling innovation in the chemical industry. In this project we have explored the potential of bacterial microcompartments (BMCs) as a way to enhance the accumulation of acetaldehyde through the incorporation of a key enzyme, aldehyde ferredoxin oxidoreductase (AOR). This enzyme is able to convert acetate into acetaldehyde and we have shown that it is possible to produce this enzyme recombinantly in *E. coli* and also to target in to BMCs. In this respect, we have achieved the main objective of the BiV. It is now our intention to enhance this project by transferring this enzyme activity to specific bacteria that accumulate acetate, a group of bacteria called the acetogens. By redesigning and engineering some of the metabolic pathways in these organisms, bacteria that are able to grow on waste products such as gas emissions, it will be possible to convert these waste materials into commodity chemicals.  Figure showing the activity of recombinant AOR in *E. coli*. An increase in the blue colour demonstrate activity. Two (AOR) genes from *Clostridium ljungdahlii* were cloned into pET14b and transformed into *E. coli*. The second AOR, AOR II, appears to be slightly more active. Current approaches for enhancing bio-based commodity production are restricted to known biosynthetic pathways and limitations to metabolite toxicity. However, many key bio-commodities are made via aldehyde-intermediates such as acetaldehyde, lactaldehyde and propionaldehyde and their production is often limited because of the inherent toxicity of their chemical reactivity. Ways to reduce this toxicity, for instance by the use of

	BMCs, offer a significant advantage to the commercial production of these materials. The results from this project are part of a strategy that show promise in being able to reduce the toxicity of key metabolic intermediates.

ID number	**BIVMiB031**
Title	Bioaccumulation of platinum from waste
Academic (lead) Partner	Helen Carney, Teesside University
Industrial Partner	Pattanathu Rahman, TeeGene Biotech Ltd
Public summary	Platinum is a scarce metal, being one of the least abundant elements in the earth's crust and as such has a high material value. This research will focus on the recovery of platinum from wastewaters where it is in its soluble, ionic form. Platinum is present in wastewater from a diverse range of sources such as metal refining, chemical industries and hospital waste where it can be found as a component of chemotherapy drugs. Bacteria have been found to take up and accumulate platinum using both active and passive methods, often referred to as biosorption and bioaccumulation. The proposed research, a collaboration between TeeGene, Teesside University, and University of York, will investigate the potential of bacteria to recover platinum from industrial wastewater with the aim of re-using the recovered metal. The project aims to identify a suitable bacterium to be used in a waste refining process and identify any physicochemical factors that influence platinum recovery.
Start date	1 September 2016
Public summary of project outcomes	The results obtained add to knowledge in an important area for waste management. Finding an easier and cheaper way to reclaim of platinum which would otherwise be lost to the environment is important for global resource management. There are two broad categories of waste – high volume, low concentration (e.g. sewage waste or mining waters) or low volume, high concentration (e.g. electroplating discharge). The former needs a biosorbants with a high affinity for platinum, the latter needs a high uptake capacity (Vijayaraghavan & Yun, 2007). Determining the ideal conditions for each scenario is a logical next step including testing at a variety of salt concentrations, differing temperatures and lengths of time. Considering contaminants and mixed metal solutions could inhibit S. algae or lead to a mixed recovery process. Work on this project has confirmed the findings of other studies that reported Shewanella algae as being capable of taking up platinum ions from solution (Konishi, et al., 2007). Subsequent work could confirm the enzymes involved are hydrogenase enzymes followed by identifying the genetic sequence of said enzymes. The hypothesis that there is an upregulation of the enzyme when exposed to platinum could be explored. However, if on further experimentation, the time was reduced to only a few seconds or minutes, then it is more likely that there has been a passive uptake rather than an enzyme mediated uptake. Using a scanning electron microscope would help confirm that the platinum nanoparticles are in the periplasmic space as previous researchers have found and further work could be carried out looking at the Pt concentration in the cell pellets alongside the loss from solution.

ID number	**BIVMiB037**
Title	Exploiting the commercial potential of novel biometallic catalysts
Academic (lead) Partner	Jon Lloyd, University of Manchester
Industrial Partner	Nigel Powell, Johnson Matthey plc

Public summary	This project brings together biotechnologists from the University of Manchester and experts in industrial catalysis at Johnson Matthey, a leading multinational specialty chemicals and sustainable technologies company headquartered in the United Kingdom. This Business Interaction project will facilitate collaborative discussions required to underpin the development and exploitation of a new generation of “biometallic” industrial catalysts. They are based on naturally occurring metal- reducing bacteria that are able to accumulate metals from process environments, as catalytically active nanoparticles, while also expressing enzymes that are able to extend the range and complexity of industrial reactions that can be produced from these novel microorganisms. This novel extension of “synthetic biology” has the potential to transform several sectors of UK industry including those of industrial biotechnology and makers and users of catalysts, simplifying current processes, underpinning novel reactions and extending the range of available products.
Start date	1 June 2017
Public summary of project outcomes	Metal-reducing bacteria are able to accumulate metals from process environments in the form of catalytically active nanoparticles, offering a simple, green method for high-value nanoparticle production. These nanoparticles have a wide-range of applications, including in the production of fine and speciality chemicals such as pharmaceutical intermediates, fats and oils, and upgrading of fuels and biorenewables. Working with Johnson Matthey, a world leader in supplying catalysts for speciality chemical production, we aimed to develop the commercial potential of novel biometallic nanocatalysts. Bimetallic nanoparticles offer a number of advantages over their monometallic counterparts due to the combined properties of the two metals present, and through new properties created from the synergy between these metals. Using a metal-reducing bacterium we investigated the potential for producing bimetallic nanoparticles from metal solutions containing a range of metals supplied in combination. Metallic nanoparticles were biosynthesised in Manchester and Johnson Matthey's STEM facilities were then used to characterise the products. Here we found that the pattern of distribution of the metallic nanoparticles was highly dependent on the combination of metals supplied to the cells. Evidence was provided for the formation of bimetallic nanoparticles in some examples, and these nanomaterials are the focus of future work.

ID number	**BIVMiB042**
Title	Creating new starch active copper LPMOs through the generation of loop libraries
Academic (lead) Partner	Jonathan Worrall, University of Essex
Industrial Partner	Johannes de Bie, WeissBioTech
Public summary	The efficient deconstruction of plant biomass into biofuels and other chemicals is a key challenge to secure a low carbon economy. In nature, many microorganisms secrete enzymes that can break down recalcitrant biomass that is composed mostly of lignocellulose into soluble substrates. Harnessing the catalytic power of these enzymes to treat biomass outside of their natural habitats is challenging and a major goal of industrial biotechnology. Recently, a new class of enzyme that drastically increases the efficiency of biomass conversion has been identified. These enzymes contain a copper ion and are called lytic polysaccharide monooxygenases (LPMOs). The aim of this project is to assess whether second generation LPMOs with enhanced substrate activities can be created. As a proof of principle, we will use a starch degrading LPMO as a template to design and synthesize DNA libraries that will then be screened for substrate activity.
Start date	23 October 2017

Public summary of project outcomes	Copper containing lytic polysaccharide monooxygenases (LPMOs) greatly aid the deconstruction of plant biomass to value added products and the production of "renewable" bioenergy. The Business partner, WeissBioTech has a vested interest in using enzyme cocktails for the liquefaction and saccharification of starches. To improve the economic outputs of such processes the creation of second generation enzymes with better substrate interaction and more efficient substrate turnover is desirable. The long-term aim of this project is to create second generation starch active LPMOs that are more efficient through better substrate interaction and higher substrate turnover.To create second generation starch active LPMOs, loops that form the active surface surrounding the catalytic copper ion were targeted. In total, there are five active surface loops that may be considered important for interaction and specificity with starch. Active surface loop libraries have been designed in silico with saturating mutations in selected amino acid positions in each loop based on sequence variability within the starch active LPMO family. This Business Interaction Voucher has allowed for the synthesis, through combining Site Evaluation Library and Combinatorial Library technology, of two out of the five loop libraries in a starch active LPMO. By using an in vivo assay these two loop libraries will be screened for enhanced activity relative to the wild type LPMO.

ID number	**BIVMiB044**
Title	Evaluation of the potential of the molybdenum-containing enzyme DMSO reductase as an oxygenation catalyst
Academic (lead) Partner	Gary Black, Northumbria University
Industrial Partner	Robert Holt, Piramal Healthcare UK Ltd
Public summary	Over the past 30 years science has made huge advances in understanding how biological systems work and this understanding is now being translated into valuable tools for the manufacture of products. This new technology, referred to as biotechnology, can bring many advantages over more traditional methodology. For example, pharmaceuticals are usually very complex molecules that have traditionally been manufactured using conventional chemistry techniques that rely on reactive and sometimes difficult to handle materials; in contrast, biotechnological approaches make use of Nature's own catalysts (called enzymes) to carry out reactions under very benign conditions and unlike conventional catalysts enzymes are biodegradable and non-toxic. This project is exploring the potential of enzymes to carry out complex oxidation chemistry which can be applied in the manufacture of pharmaceuticals and other important products. Emphasis will be placed on getting the enzymes to work effectively in the non-natural environment of a chemical reactor.
Start date	1 November 2017
Public summary of project outcomes	Over the past 30 years science has made huge advances in understanding how biological systems work and this understanding is now being translated into valuable tools for the manufacture of products. This new technology, referred to as biotechnology, can bring many advantages over more traditional methodology. For example, pharmaceuticals are usually very complex molecules that have traditionally been manufactured using conventional chemistry techniques that rely on reactive and sometimes difficult to handle materials; in contrast, biotechnological approaches make use of Nature's own catalysts (called enzymes) to carry out reactions under very benign conditions and unlike conventional catalysts enzymes are biodegradable and non-toxic. This project explored the potential of enzymes to carry out complex oxidation chemistry which can be applied in the manufacture of pharmaceuticals and other important products. In total six DMSO reductase enzyme preparations were produced and their capacity to perform complex oxidation chemistry was determined.

ID number	**BIVMiB035**
Title	Metal utilisation in *Clostridium* microbial biocatalysts

Academic (lead) Partner	Peter Chivers, Durham University
Industrial Partner	Liz Jenkinson, Green Biologics Ltd
Public summary	*Clostridium* is an exemplar for microbial fermentations that convert biomass to renewable chemicals, such as butanol (with diverse uses including consumer fuels, paints and coatings and food additives) and acetone (used in cosmetics, plastics and numerous other markets). These fermentations depend upon metalloenzymes to convert starting material to product. The optimisation of metal supply is therefore critical for cost-efficiency. Currently, little is known about the metal requirements of industrial *Clostridium* species, or the metal circuitry important for maintaining metal supply to these pathways. We will identify the metal requirements during different fermentative processes and in different environments (batch vs. continuous; lab vs. industrial). Results will be applied to commercial operations for immediate outcomes including reducing waste and water use impacts. To further the development of *Clostridium* in a variety of IB processes, the metal sensor components of the metal circuitry will be identified to enable fine-tuning of metal supply pathways.
Start date	1 April 2017
Public summary of project outcomes	Clostridium is an exemplar for microbial fermentations that convert biomass to renewable chemicals, such as butanol (with diverse uses including consumer fuels, paints and coatings and food additives) and acetone (used in cosmetics, plastics and numerous other markets). Little is known about the metal-demands of Clostridium strains during solvent production. GBL collected biomass from a range of timepoints during standard Research and Development fermentation protocol. Interestingly, the ICP-MS analysis revealed a previously unknown and large change in the metal composition and content of the cells coincident with the onset of solvent production. This change in content was essentially complete by the next time point (12 h later), so the exact timing of this change remains unclear. The link between metal content increase, butanol production, and competing physiological changes in the culture (sporogenesis) is not known. In parallel, bioinformatics analysis of publicly available Clostridium genomes was used to identify candidate metal sensor genes. The analysis used a combination of sequence homology searches (BLAST) using representatives of the known families of metalloregulators combined with manual genome context analysis (Microbial Genome content Viewer) to link candidate regulators with metal-specific function. A total of ten genes from four metalloregulatory families (ArsR, DtxR, Fur, and RcnR) were identified, in some cases in proximity to genes encoding metal homeostasis proteins (transporters). Synthetic genes optimized for overexpression in E. coli were obtained, and nine of the ten genes showed visible overexpression by SDS-PAGE analysis. The tenth gene appeared to be toxic to E. coli due to slower growth and poor overexpression under standard laboratory induction conditions. The identification of these genes will facilitate studies of their metal specificity and their regulons. This information will be useful to understand the regulatory mechanisms linked to changes in metal content and butanol production. A visit by the PI to GBL allowed broader communication about the role of metals in microbial physiology to the GBL R&D team. The visit also facilitated planning of a follow-up application based on the results of this project.

ID number	**BIVMiB043**
Title	Investigating the link between metal homeostasis, sporulation, and solvent production in the Clostridial ABE fermentation process
Academic (lead) Partner	Peter Chivers, Durham University
Industrial Partner	Liz Jenkinson, Green Biologics Ltd
Public summary	Clostridia are exemplars of fermentative microbes that convert biomass to renewable chemicals. Green Biologics Limited use this process commercially to produce the platform solvent chemicals butanol (with diverse uses including consumer fuels, paints and coatings and food additives) and acetone (used in cosmetics, plastics and numerous other markets). Solvent yield is limited by the

	physiology of the microbes under production conditions, including the onset of sporulation. Endospore formation diverts energy to generate biomass, which does not contribute to solvent production. A detailed understanding of the link between metal ions and Clostridium metabolism and physiology during solvent production will provide the means to improve strains for greater solvent yields, an important factor in the economic viability of the process.
Start date	1 November 2017
Public summary of project outcomes	Clostridia are exemplars of fermentative microbes that convert biomass to renewable chemicals. Solvent yield is limited by the physiology of the microbes under production conditions, including the onset of sporulation. Endospore formation diverts energy to generate biomass, which does not contribute to solvent production. A detailed understanding of the link between metal ion homeostasis and Clostridium metabolism and physiology during solvent production can be applied by GBL to commercial processes. This project focused on obtained detailed information on metal content and changes in gene expression within a 12-h window of growth identified in previous study (BIVMiB035 – Metal utilisation in Clostridium microbial biocatalysts). Biomass samples were collected by GBL at 3-h intervals for metal content analysis and RNA isolation. The metal content analysis (Durham) demonstrated that the previously identified increase in metal content occurred over the 12 h window, rather than more rapidly within a shorter time window. An aporogenic mutant did not show the changes in metal content and composition. Replicate RNA samples at the same 3 h intervals were isolated (Durham) from a wild-type strain and an asporogenic strain for RNASeq analysis. Because of the short time frame for the project, the RNASeq results and their analysis are not complete. The project was beneficial for knowledge transfer – the metal content and RNA sample analysis were carried out at Durham by a GBL scientist. The informal interactions that occurred during the 3-week visit will enhance future work at both sites.

ID number	**BIVMiB041**
Title	Exploiting a copper-dependent chaperone system to improve bioprocessing of therapeutic antibodies
Academic (lead) Partner	Mark Shepherd, University of Kent
Industrial Partner	FUJIFILM Diosynth Biotechnologies
Public summary	The production of biotherapeutics has a total market value of around £100 Billion per year. This project expands the repertoire of a novel system that has been shown to improve the production of antibody fragments in E. coli, a product with wide ranging applications in diagnostics (e.g. pregnancy tests), human therapeutics and as fundamental research tools. In addition, future consumer applications might include the use of antibodies in shampoos to prevent the formation of dandruff or in toothpaste to protect against tooth decay. This project focusses upon copper-dependent protein folding catalysts (Scs proteins), with the overall aim of improving the yield and quality of antibody fragments produced in E. coli. Previous antibody targets include the cancer therapeutic heceptin, whereas the current work has a particular focus on Lucentis, a therapeutic antibody used to treat macular degeneration.
Start date	1 September 2017
Public summary of project outcomes	The production of biotherapeutics has a total market value of around £100 Billion per year. This project expands the repertoire of a novel system that has been shown to improve the production of antibody fragments in E. coli, a product with wide ranging applications in diagnostics (e.g. pregnancy tests), human therapeutics and as fundamental research tools. In addition, future consumer applications might include the use of antibodies in shampoos to prevent the formation of dandruff or in toothpaste to protect against tooth decay. This project focusses upon copper-dependent protein folding catalysts (Scs proteins), with the overall aim of improving the yield and quality of antibody fragments produced

	in E. coli. Previous antibody targets include the cancer therapeutic heceptin, whereas the current work has a particular focus on Lucentis, a therapeutic antibody used to treat macular degeneration.

ID number	**BIVMiB040**
Title	A pilot study to characterise plant-derived compounds that promote the synthesis of copper nanoparticles from contaminating copper ions in waste water
Academic (lead) Partner	Keith Lindsey, Durham University
Industrial Partner	Andrew Moore, Northumbrian Water
Public summary	Contamination of land and waterways by toxic metals is a serious environmental problem particularly in areas of the UK where mineral mining was once widespread. However, if the polluting metal can be sequestered into bioactive metal nanoparticles (NPs) then these NPs have important commercial values e.g. Copper NPs have diverse uses ranging from industrial catalysts to antimicrobials in food packaging. Previous work has established that crude plant extracts when mixed with solutions of metal ions extracts stimulate the synthesis of metal nanoparticles. The aim of this project is to determine the identity of the bio-active molecules within the plant extracts that are required for Copper NPs synthesis and their mode of action. The results from this pilot study will inform future use of plants, plant cell cultures or specific plant-produced compounds to remove contaminating copper, and other trace metal ions from waste water in order to synthesize commercially valuable metal nanoparticles for further exploitation in a specific collaboration with our industrial partners.
Start date	1 August 2017
Public summary of project outcomes	Contamination of land and waterways by toxic metals is a serious environmental problem, particularly in areas of the UK where mineral mining was once widespread. However, if the polluting metal ions can be sequestered into bioactive metal nanoparticles (NPs), these NPs would have important commercial value. This project was initiated in partnership with Northumbrian Water Ltd (NWL) to investigate the "green synthesis" of metal NPs from contaminated water using total plant extracts. Phytoremediation, the use of plants and plant-derived compounds to decontaminate land and water, is widely perceived as a sustainable, eco-friendly approach. It has previously been shown that active ingredients within plant extracts are effective in stimulating the formation of NPs (reviewed by Makarov *et al.*, 2014). The majority of the work has been carried out with noble metals, principally silver and gold, however NWL are particularly interested in the removal and recovery of lead, zinc and copper ions from waste water. These toxic metal ions accumulate in water treatment plants as a result of leaching from mining spoil. Many regions of the UK with a history of coal and mineral mining have been left with a legacy of water and land contamination, and innovative ways to decontaminate the waste water prior to release into the river systems are being sought. In this project, we investigated how copper ions can be removed from solution and formed into copper nanoparticles (CuNPs) by the addition of plant extracts. The mechanism by which plant compounds can precipitate metal ions from dilute solutions to form metal nanoparticles (NPs) is not fully understood, but it is believed to occur through reduction of the metal ions into metal atoms that coalesce into nanoparticles. A schematic representation of the proposed process is shown in Figure 1. Previous work has suggested that there are several possible plant compounds that can act as bio-reductants and these include flavonoids, terpenoids, sugars and proteins. Many of the extracts that have significant bio-reductant activity are from plants that contain relatively high levels of aromatic compounds e.g. cinnamon (Sathiskumar et al, 2009) basil, mint and coriander (Kulkarni and Kulkarni, 2013) and geranium (Shankar *et al.*, 2003). This observation perhaps indicates the importance of secondary metabolites as bio-reductants. Cell wall components including polysaccharides and glycoproteins and proteins that are rich in histidine, tryptophan and tyrosine, are also

	candidate molecules (Makarov *et al.*, 2014). The experimental system that was used in this project was the proposed formation of CuNPs from a copper sulphate (CuSO4) solution following the addition of plant leaf extract from either mint (*Mentha spicata*) or coriander (*Coriandrum sativum*). The aim was to gain a better understanding of how this process occurs, characterise the nanoparticles produced and the bioactive constituents within the plant extracts. It was demonstrated that plant proteins play a crucial role in the formation of the CuNPs. Proteomics analysis identified 105 proteins associated with the CuNPs formed with both the mint and coriander extracts, however, in the time available, it was not possible to identify which if any of the proteins in isolation are sufficient to form the CuNPs from the CuSO4 solution. Future research to identify key plant molecules required for the formation of CuNPs will inform biotechnological processes that could be implemented for the phytoremediation of waste water and the capture of industrially valuable metal nanoparticles.

ID number	**BIVMiB032**
Title	Embedding technical expertise in the optimisation of trace metal supplementation strategies for successful biomethane production
Academic (lead) Partner	Yue Zhang, University of Southampton
Industrial Partner	Michael Mason, Tropical Power Ltd
Public summary	Transformation of waste biomass into bioenergy and useful resources is a key component in 21st century industrial biotechnology. It is increasingly clear that successful biomethanisation of mixed biomass requires complex enzyme systems that are produced by both natural and engineered synthetic microbial communities. Trace quantities of metals play a role in certain essential metallo-enzymes, and thus in ensuring that microbial communities function in the most effective and productive way. There is a growing commercial market in trace metal supplements, but these are often generic rather than based on specific requirements. The current project involves transfer of knowledge and expertise in determining trace metal requirements to a UK company using novel waste feedstocks in Africa for renewable biomethane production. This will enable the company to formulate specific trace metal mixtures for optimum plant performance, while the scientific knowledge gained will contribute to creating future markets for UK suppliers of tailored supplements.
Start date	16 January 2017
Public summary of project outcomes	Trace element supplements for anaerobic digestion are available commercially, but are expensive, rarely tailored to specific feedstock requirements, and can consume unnecessary resource. This is particularly an issue where these supplements need to be shipped and imported to a developing country as no local supplies are available. The current project has developed methods that are helping a UK company, which has built and is currently operating Africa's first grid connected anaerobic digester, to determining more precisely the trace element requirements for optimum digestion of their novel agricultural waste feedstocks. The work involved developing a method using simple apparatus to test which trace elements are actually required. This is done using simple multi-purpose equipment, and is suitable for use in Africa. The methodology was made available to the company in the form of a training video and a detailed description of the procedures. In addition the scientists at the University of Southampton have helped the company in interpreting historical data from the digestion plant and provided them with a simple spreadsheet-based calculator to allow them to maintain steady state concentrations of essential elements in the digester in proportion to the feed added. The work also added tantalisingly to growing evidence that minor trace elements such as tungsten may play a critical role in these microbial systems.

ID number	**BIVMiB036**
Title	Improving biocatalytic processes by enzyme stability enhancement

Academic (lead) Partner	Stuart Ferguson, University of Oxford
Industrial Partner	Jason King, Oxford Biotrans Ltd
Public summary	The industry partner has licensed biocatalytic oxidation technology from the University of Oxford for the production of fine chemicals such as flavours, fragrances, agrochemicals and active pharmaceutical ingredients. The first product is nootkatone, the high-value grapefruit flavour compound. This industrial biotechnology approach not only replaces classical, more energy-demanding and polluting chemical processes, it also enables non-fossil fuel, sustainable feedstocks to be used. A key improvement to the process is enhancement of the stability of the enzyme catalyst that lies at the heart of the technology, making it applicable to a wider range of products.
Start date	1 July 2017
Public summary of project outcomes	Amino acid residue side chains with their b carbons within 5 Å of the terminal carbon of the two heme vinyl groups in P450BM3 were identified from the crystal structure. The side chain of three residues (L272, L322 and A406) are close to one vinyl group while F405 is close to the other. Cysteine and histidine substitutions were introduced at each residue, together with the double substitution combinations Cys/Cys, Cys/His, His/Cys and His/His to explore whether both vinyl groups could be covalently linked to the polypeptide. A total of 20 single and double mutants were generated by site-directed mutagenesis. Expression trials revealed that all nine mutants with His substitutions at F405 and A406 did not give holoprotein. The double mutants F405C/A406C and L272H/F405C were produced at a low level. The remaining nine mutants were tested in parallel with the wild type enzyme for the oxidation of lauric acid, a natural substrate of the enzyme, and the unnatural substrate propylbenzene. The parameter of interest is the total turnover number (TON) of the enzymes with these substrates. The results showed that the wild type was the most active for lauric acid oxidation, with a TON of ~30,000; the mutants showed 63% to 90% of the wild type activity, with the L272C and L322C mutants being the most active. All mutants showed very similar product profile to the wild type. The product profiles were also virtually identical for propylbenzene oxidation, with the dominant product being from oxidation at the benzylic position. The wild type enzyme was surprisingly active, with TON reaching 15,000, and again the mutants showed lower TON, with the highest activity (82% of the wild type) observed for the L322C mutant. The wild type and mutant P450BM3 enzymes were treated with dithinoite in a glove box (<20 ppm O2) for 16 hours, according to the literature protocol. Excess dithionite was removed, and the buffer exchanged, by ultrafiltration. The FeII(CO) assay revealed high retention of P450 content, with no evidence of the inactive P420 form. The lauric acid and propylbenzene oxidation activity and selectivity tests showed very similar patterns as the untreated enzymes, with the mutants being less active (i.e. lower TON) than the wild type enzyme. The dithionite-treated variants were assayed by staining for the presence of retained heme after SDS-PAGE. The untreated and treated wild type enzyme, with no covalent heme attachment, were used as negative controls while cytochrome *c* was the positive control. Gels were run in duplicate; one gel was stained with Coomassie Brilliant Blue to reveal the presence of enzyme of the correct MW while the other was stained for retained heme. Cytochrome *c* gave a strong distinct band under the heme stain assay but none of the P450BM3 enzymes showed retained heme despite strong bands in the normal gel stain. Clearly there was no covalent attachment of the heme vinyl groups to the introduced Cys and His residues under the reaction conditions, and the introduced mutations lowered the activity of the enzyme.