

The Potential of Metabolomics Tools in Bioremediation Studies

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ABSTRACT

As a post-genomics tool, *metabolomics* is a young and vibrant field of science in its exponential growth phase. *Metabolome analysis* has become very popular recently, and novel techniques for acquiring and analyzing metabolomics data continue to emerge that are useful for a variety of biological studies. The *bioremediation* field has a lot to gain from the advances in this emerging area. Thus, this review article focuses on the potential of various experimental and conceptual approaches developed for metabolomics to be applied in bioremediation research, such as strategies for elucidation of *biodegradation pathways* using *isotope distribution analysis* and *molecular connectivity analysis*, the assessment of *mineralization* process using *metabolic footprinting* analysis, and the improvement of the biodegradation process via *metabolic engineering*. We demonstrate how the use of metabolomics tools can significantly extend and enhance the power of existing bioremediation approaches by providing a better overview of the biodegradation process.

INTRODUCTION

THE WORD METABOLOME was first used less than a decade ago to refer to all low molecular mass compounds (metabolites) produced and modified by a living organism (Oliver, 1998). Due to the genome-wide requirements of functional genomics studies we have witnessed a tremendous advance in methodologies for global detection and quantification of cellular metabolites during the past few years (Gavaghan et al., 2000; Roessner et al., 2000, 2001; Soga and Heiger, 2000; Soga et al., 2002a, 2002b; Allen et al., 2003; Castrillo et al., 2003; Dunn et al., 2005; Villas-Bôas et al., 2005a; and others). Therefore, metabolomics has emerged as a new field in biology with the promise to speed up the functional analysis of genes with unknown function (Villas-Bôas et al., 2005b). However, the concept of an unbiased and non-targeted analysis of cellular metabolites in a system-wide hypothesis-driven approach has changed the methodological strategies in different areas of life sciences dramatically.

Today metabolomics approaches and tools are of interest in a variety of areas such as human and animal nutrition (Whitfield et al., 2004; Gibney et al., 2005; Rist et al., 2006), cancer diagnosis and therapy

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(Hartmann et al., 2006; Malhi and Gores, 2006), biomarker discovery (Goodacre, 2005; Schlotterbeck et al., 2006), toxicology (Robertson, 2005; Gerner et al., 2006), obesity studies (Hochberg, 2006), enzyme discovery (Saito et al., 2006; Villas-Bôas et al., 2006), drug discovery (Harrigan, 2006), transplantation (Wishart, 2005), agriculture (Bender, 2005; Dixon et al., 2006), and bioremediation (Singh, 2006).

The analytical techniques developed for metabolomics studies allow the screening of hundreds of metabolites from complex biological samples with relative high-throughput rate, and the data generated by these techniques provide very useful information regarding the metabolism of living organisms associated with the samples. Being the intermediates of biochemical reactions, metabolites play a very important role in connecting the many different pathways that operate within a living cell. Therefore, the level of metabolites in a cell or tissue represents integrative information of the cellular function and, hence, defines the phenotype of a cell or tissue in response to genetic or environmental changes (Villas-Bôas et al., 2005c).

But how can this new field of science and its techniques be used in environmental bioremediation studies? We believe metabolomics can be very useful in bioremediation studies and, therefore, we discuss here the major aspects of environmental bioremediation that can largely benefit from making use of metabolomics tools. To begin, we comment briefly on the available metabolomics tools to date in order to familiarize the reader and review the terms used subsequently.

METABOLOMICS TOOLS

Metabolome analysis covers the identification and quantification of all intracellular and extracellular metabolites with molecular mass lower than 1000 Da, using different analytical techniques (Villas-Bôas et al., 2005c). As with the transcriptome and the proteome, the metabolome is context-dependent, the levels of each metabolite depending on the physiological, developmental, and pathological state of a cell, tissue, or organism. However, an important difference is that, unlike with mRNA and proteins, it is difficult or impossible to establish a direct link between genes and metabolites. The convoluted nature of cell metabolism, in which the same metabolite can participate in many different pathways, complicates the interpretation of metabolite data (Villas-Bôas et al., 2005c).

In addition, the genome, transcriptome, and proteome analyses are based on target chemical analyses of biopolymers composed of four different nucleotides (genome and transcriptome) or 22 amino acids (proteome). Those compounds are highly similar chemically, and facilitate the development of high-throughput analytical approaches. Within the metabolome, however, there is a large variance in chemical structures and properties. The metabolome consists of extremely diverse chemical compounds, from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids, and complex natural products (Villas-Bôas et al., 2005c). This complexity makes it virtually impossible to simultaneously determine the complete metabolome. Furthermore, the very rapid turnover of metabolites adds to the complexity of metabolome analysis; many metabolites are present in fairly low concentrations and there are very high fluxes through the metabolite pools. It is therefore important to quench the metabolism rapidly, which calls for efficient methods of quenching and extracting metabolites from living cells. Thus, metabolite analysis encompasses sampling, sample preparation, metabolite separation and detection, and data analysis and interpretation. The textbook published by Villas-Bôas et al. (2007a) covers all aspects of the metabolomics field, and guiding the novice through each step of metabolome analysis. In addition, there are several articles that review the analysis of metabolites within a metabolomics context (Buchholz et al., 2002; Fiehn, 2002; Dunn et al., 2005; Villas-Bôas et al., 2005c, 2007b; and others). Therefore, a detailed discussion on techniques for metabolome analysis is not presented here.

Nonetheless, there is no single analytical method appropriate for analysis of all metabolome of a cell or organism, and different terms are often used in the field of metabolomics referring to different analytical approaches. The diverse terminology currently in use has been controversial (Villas-Bôas et al., 2005b), but there is a general consensus that the term *metabolome* describes the total sum of metabolites a given biological system can either use or form by its metabolism. In addition, the metabolome is often divided into *exometabolome* and *endometabolome*, where the former represents metabolites outside the cell and the lat-

ter, intracellular metabolites (Villas-Bôas et al., 2007a). Although this distinction between exo- and endometabolome is quite useful for microbial systems in which it is easy to separate the cells from the extracellular medium, it is less useful for multicellular systems where it may be difficult to isolate the cells from complete tissues. However, it is still conceptually important to differentiate between these two as the exometabolome often plays a very different physiological role than the endometabolome and the strategies for analyzing them can be substantially different (Villas-Bôas et al., 2007a).

Two important terms that are often used to describe analysis of a part of the metabolome are *metabolite profiling* and target analyses. Metabolite profiling is defined empirically as the set of all metabolites or derivative products (identified or unknown) detected by analyzing a sample using a particular analytical technique, together with an estimation of quantity (Villas-Bôas et al., 2005b). Target analysis, on the other hand, is defined as the analysis of a list of metabolites ignoring all non-targeted compounds. Whether profiling or target analysis is used, what is new in metabolomics methodology is the robustness of the analytical techniques developed, which permit the simultaneous analysis of hundreds of compounds using a single method. Within this context, mass spectrometry and nuclear magnetic resonance are the most frequently employed methods of detection in the analysis of the metabolome, which very often is combined with efficient chromatographic techniques (Villas-Bôas et al., 2005c, 2007a).

ELUCIDATING BIODEGRADATION PATHWAYS USING METABOLOMICS

It is well recognized today that metabolome analysis is a powerful approach for discovering novel metabolic pathways and characterizing metabolic networks (Weckwerth and Fiehn, 2002; Villas-Bôas et al., 2005c, 2007a). Metabolomics data represent physiological responses to developmental, genetic, or environmental changes (Weckwerth and Fiehn, 2002). Although metabolomics data are perhaps the most difficult and complex to interpret and integrate with other OMICS-related data, which limit a direct interpretation of metabolic networks generated from metabolic snapshots (or commonly named metabolite profiling), there are different analytical strategies available that facilitate the interpretation of metabolomics data towards elucidation of metabolic pathways. Two of these are of special interest for elucidating biodegradation pathways of xenobiotics compounds: isotope distribution analysis of metabolites and molecular connectivity analysis using ultra-high mass accuracy techniques.

Isotope distribution analysis

Isotope distribution analysis exploits the relationships between metabolites using stable isotope-labeled substrates. These are classical techniques long employed for the characterization of metabolic pathways (Brown and Sampathkumar, 1977; Brazier et al., 1980; Williams et al., 1985) and, more recently, for metabolic flux analysis (Szyperski, 1998; Christensen and Nielsen, 1999). However, with the recent advances in methodologies for analysis of metabolites as part of analytical platforms developed for metabolomics, it became considerably easier to analyze the isotopic distribution in different metabolites using stable isotope-labeling substrates.

The most commonly used isotope-labeled atoms are ^{13}C , ^{14}C , ^{15}N , ^2H , ^3H , and ^{36}Cl . A wide range of isotope-labeled chemicals is commercially available nowadays. Currently, two experimental approaches can be employed to determine the relative abundances of specific isotope isomers (isotopomers) in metabolite pools: nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). The merits of both approaches have been discussed extensively elsewhere (Szyperski, 1998).

The use of isotope-labeled xenobiotics in bioremediation studies is not entirely new to the field. For example, Kubatova and co-workers (1998) investigated the biodegradation of polychlorinated biphenyls (PCB) in soil using uniformly ^{14}C -labeled dichlorobiphenyl. The PCB compound used in this work was assumed to be readily degraded by microorganisms, but the isotope-labeling experiments showed that its mineralization was lower than 0.4% after 2 months, and the metabolism of a major biodegradation product, 3-chlorobenzoic acid, was found to be the bottleneck step in the mineralization process. The same research group has employed ^{14}C -labeling to study the biodegradation process of other xenobiotics, such as

the commonly used herbicide trichloroacetic acid (Forczek et al., 2001; Matucha et al., 2003, 2006; and others).

On the other hand, an emerging technique using isotope-labeled substrates is now available, which is focused on the analysis of the phospholipid fatty acids (PLFA) profile of environmental samples in order to gain quantitative and chemotaxonomic information for a group of microorganisms utilizing a given substrate (Evershed et al., 2006). Although some prefer not to include lipids in the metabolome realm, due to the large molecular mass of some molecules, it is worth mentioning this approach here because of its great potential for bioremediation studies. According to Evershed and co-workers (2006), the analysis of isotope-labeling PLFAs provides an important new culture-independent approach for the study of environmental microorganisms and their ability to metabolize certain substrates. These substrates can be all sorts of organic compounds, including xenobiotics that must be isotopic-labeled, in order to determine which microorganisms are utilizing them as a carbon source. Accordingly, this methodology offers several advantages: (1) isotope-labeled microbial PLFAs confirm the presence of an active microbial community in the environment, which cannot be confirmed by genetic probes; (2) isotope-labeling of a specific subset of PLFAs, as a result of incubation with specific substrates, highlights members of the microbial community consuming that substrate; (3) quantified isotope-labeled PLFA profiles can be searched against existing PLFA databases of cultured microorganisms to provide taxonomic information; and (4) variations in isotope-labeled PLFA profiles between environments following treatment with a substrate indicate population-level differences (Evershed et al., 2006).

Therefore, by using metabolite profiling techniques coupled with MS or NMR detection, it is considerably easier today to follow the biodegradation steps of xenobiotic compounds either in the environment or in laboratorial assays. Usually, the xenobiotic compound to be studied is isotopically labeled and either obtained commercially or via organic synthesis. However, another alternative never explored is to cultivate the bioremediation agent itself (the microbial cells) in uniform labeled substrates, such as $U^{13}C$ -glucose, and keep the xenobiotic molecules unlabeled. Thereby, all metabolites genuinely formed by microbial metabolism will be isotope-labeled, except the catabolic products of the xenobiotic molecules. This is an option when there is no commercially available isotope-labeled molecules of a given xenobiotic. However, this alternative is only suitable for laboratory assays and requires that the cultivated microorganisms be able to grow in defined, ideally minimal mineral media.

Molecular connectivity analysis using ultra-high mass accuracy techniques

Molecular connectivity analysis is based on the principle that metabolites in a biological sample are related to each other, which means they share biochemical ancestry and are linked with a closely connected metabolic network (Breitling et al., 2006). Ultra-high mass accuracy mass spectrometers (mass accuracy of ~ 1 ppm and resolution $> 100,000$) are now within the reach of many researchers. They have the ability to identify molecular formulae based on exact masses and the inference of biosynthetic relationships between masses directly from the mass spectrum (Breitling et al., 2006). There are currently two types of mass analyzer able to routinely achieve high resolution and mass accuracy in the 1 ppm range: the Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) (Marshall et al., 1998) and the Orbitrap (Hu et al., 2005). The merits of each instrument are well discussed by Breitling and colleagues (2006).

Ultra-high mass accuracy also implies ultra-high mass difference accuracy. Accurate mass differences in turn contain information about chemical relationships between molecules. According to Breitling et al. (2006), a limited repertoire of chemical transformations account for the majority of biochemical reactions. For example, the hydrogenation/dehydrogenation reactions result in metabolites presenting mass difference of ± 2.01565 Da (H_2), oxidation reactions in mass difference of 15.9949 Da (O), and phosphorylation in mass difference of 79.9663 Da (PO_3H). Detecting a difference corresponding to a common transformation between two metabolites implies that they are at least chemically related and may well be connected directly by an enzymatic reaction. Breitling et al. (2006) proposed that a systematic screen for common mass differences can create a comprehensive metabolic map of a sample in order to determine the molecular connectivity of metabolic intermediates, which is an extremely useful tool to enhance elucidation of biodegradation pathways. Although molecular connectivity is not a new approach in mass spectrometry, the recent

advances in ultra-high accuracy mass spectrometers make this task more easily achievable and may change the way we approach metabolomics.

MINERALIZATION ASSESSMENT VIA METABOLIC FOOTPRINTING ANALYSIS

Recently, the measurement of extracellular metabolite profiles alone has been recognized as a powerful tool in the classification of microbial mutants for functional genomics; this was given the name *metabolic footprinting* analysis (Allen et al., 2003). Metabolic footprinting analysis is focused on the measurement of chemical and biochemical alterations provoked by an organism on its environment (e.g., a laboratory culture medium) (Villas-Bôas et al., 2006). A living cell secretes enzymes and excretes metabolites to the extracellular medium, and these enzymes and metabolites interact with and modify the components of the medium, resulting in metabolic profiles that are highly specific to species and/or genetic backgrounds (Allen et al., 2003; Kell et al., 2005; Villas-Bôas et al., 2006).

The measurement of extracellular metabolites presents several advantages over the analysis of intracellular compounds for microbial cultures. For instance, it is easier to carry out this measurement because the metabolites do not need to be extracted from inside the cells and the turnover of extracellular metabolites is much slower than that of intracellular ones, which results in more reproducible results. In addition, samples containing extracellular metabolites are often more concentrated, which enhances the detection of different metabolic intermediates.

Thus, metabolic footprinting analysis can be a very useful approach to assess the mineralization process of xenobiotics in the environment or in the laboratory. Analytical techniques developed for metabolic footprinting analysis aim to be an unbiased and non-targeted analysis of low molecular mass compounds in a broad hypothesis-driven approach. Therefore, this is a highly recommended approach to ensure that a polluting compound is being totally degraded to CO₂ and water or that the biodegradation process is resulting in the accumulation of hazard and recalcitrant catabolic products that are bottlenecking the mineralization process.

IMPROVEMENT OF BIODEGRADATION PROCESS VIA METABOLIC ENGINEERING

The central aim of metabolic engineering (ME) is to improve cellular properties or introduce new ones through the use of recombinant DNA technology (Stephanopoulos et al., 1998). Thus, the ME approach is the alternative to classical mutagenesis for the improvement of industrial microorganisms. However, the use of ME tools and principles are also relevant for bioremediation due to the shortcomings of natural microbial population to degrade recalcitrant xenobiotics such as chlorotoluens, PCBs PAH, and others (Urgun-Demirtas et al., 2006; Pieper and Reineke, 2000; Rui et al., 2004). Accumulation of these compounds in the environment represents a potential pollution problem, since many of them are highly toxic, mutagenic, and/or carcinogenic.

Both the introduction of novel biodegradation pathways, as well as the modification and extension of a substrate range of enzymes in existing degradation pathways, are examples of metabolic engineering for the development of microorganisms with improved bioremediation properties (Dua et al., 2002). One major limitation with *in situ* field experiments is the very low survival rate of the introduced organisms vs. the indigenous population, since laboratory strains seem to lose their environmental adaptation properties during laboratory cultivation. One solution would be to develop novel degradation pathways and improved properties of existing pathways in laboratory strains and transfer these traits to environmental isolates with a limited number of laboratory cultivations. This was done for a psychrotolerant *Pseudomonas fluorescens* strain that was genetically modified for degradation of 2,4-dinitrotoluene by transferring the *Burkholderia* sp. strain DNT pJS1 megaplasmid containing the *dnt* genes for degradation of 2,4-dinitrotoluene (Monti et al., 2005). However, a stable 2,4-dinitrotoluene-degrading phenotype was first achieved by integrating the *dnt* genes into the *P. fluorescens* chromosome. Studies in soil revealed survival and degradation of 2,4-dinitrotoluene at 10°C of *P. fluorescens* while the *Burkholderia* sp. strain did not. The latter strain exhibits plant

and human pathogenic properties, also rendering it inappropriate for use in *in situ* environmental biotechnological applications, while the *P. fluorescens* strain is nonpathogenic.

Metabolomics tools are essential for metabolic engineering. The analysis of metabolites is important for exploring and understanding the stresses that are caused by the substrates, including intermediates and toxic dead-end products, and other environmental conditions. PCBs are resistant to degradation but various PCB-degrading microorganisms have been isolated. There are several factors limiting PCB degradation, such as solubilization and entry into cells, narrow substrate range, and expression of PCB-degrading enzymes. Genetic and metabolic engineering have been used to improve the PCB-catabolic properties of microorganisms (e.g., introduction of PCB-degrading enzymes in biosurfactant-producing microorganisms, replacement of native regulatory systems of PCB-degrading genes with more sensitive and wider specificity transcriptional regulatory circuits, and constitutive promoters [Ohtsubo et al., 2004]).

Between 20 and 60 of the 209 theoretical congeners of PCB are present in commercial products. The high number of possible substrates for degradation and intermediates in the degradation pathways calls for comprehensive analytical protocols to separate and quantify all congeners in the sample. But the same protocols can be used to study the fate of the PCBs in the environment and in the laboratory experiments designed to gain insight into the degradation mechanisms, as well as through metabolic engineering development of novel microbial PCB degraders suitable for *in situ* bioremediation. Due to its excellent resolution, gas chromatography coupled with electron capture and/or mass spectrometry detection has been the standard analytical choice for detection and quantification of PCBs and other organochlorine pesticides (Muir and Sverko, 2006). The unbiased and non-target approach of metabolomics by analyzing as many metabolites as possible, from detailed analysis of the substrates and degradation intermediates to all other intracellular metabolites, can provide new critical knowledge for successful PCB bioremediation. For instance, very complex samples can largely benefit from the new ultra-high resolution GCxGC-MS instrumentation.

Even the simpler chloroaromatic compounds such as 2-chlorotoluene are very recalcitrant to natural degradation. Several studies have addressed this challenge by creating hybrid pathways. Haro and Lorenzo (2001) combined two catabolic segments from TOL and TOD pathways of *Pseudomonas putida* to create an upper hybrid pathway for bioconversion of 2-chlorotoluene into 2-chlorobenzoate. This pathway was integrated into the pathway of two 2-chlorobenzoate degrading *Pseudomonas* strains, expecting to provide complete degradation of 2-chlorotoluene. The two strains were able to co-metabolize 2-chlorotoluene to 2-chlorobenzoate with citrate as co-substrate but failed to grow on 2-chlorotoluene alone. Analysis of the cultures with GC-MS showed that citrate-grown cells accumulated 2-chlorobenzoate and other intermediates in the upper pathway during exposure of 2-chlorotoluene. But no reason was found for the lack of degradation of 2-chlorobenzoate. This clearly emphasizes that an unbiased and non-target analysis of the performance and physiology of the biological model system via metabolomics is necessary. Toxic intermediates that were not detected with the applied metabolite target analysis may accumulate. Therefore, a comprehensive metabolomics approach to monitor the changes in all (detectable) intracellular metabolites may provide suggestions for further metabolic engineering improvements by highlighting the active metabolic routes and bottlenecks. In this regard, it might be sensible to evaluate the use of several different analytical separation techniques (e.g., LC and GC with and without derivatization agents) (Halket et al., 2005).

Metabolomics and other genome-wide methodologies as transcriptomics, proteomics, interactomics, and fluxomics have become important tools for describing how a phenotype is generated from its genotype and the environmental conditions. These technologies became important both for metabolic engineering and system biology since the focus is on integrated networks of metabolic pathways and not on individual isolated reactions (Friboulet and Thomas, 2005). The strategy of inverse metabolic engineering is through comparisons between mutated strains and reference strains to gain insight into the metabolism (Bro and Nielsen, 2004). In bioremediation, this approach is also relevant (e.g., comparison of reference strains with recombinant strains modified with a new degradation pathway). But also important is the exploration of the response of the microorganisms to pollutant exposure. A metabolome analysis can supply information regarding which pathways are activated under expression of the new heterologous pathway and under pollutant exposing conditions. The strength of metabolome analysis is that it points the pathways that need to be targeted by metabolic engineering (Trethewey, 2001). Since the other OMICS-related approaches provide invaluable complementary information, the future of metabolic engineering is to integrate the different sets

of data to develop optimal strains for use both in bioprocessing and bioremediation (Park et al., 2005). Lee et al. (2006) used a quantitative proteomics approach to provide important insights into the metabolic and physiological changes that occur upon *cis*-dichloroethylene degradation by engineered *Escherichia coli* strains. Because the number of identified central proteins in the degradation pathways was less than the number of genes, a transcriptome analysis may complement the proteomics approach as would the metabolomics and fluxomics analyses on the physiological characterization.

CONCLUSION

In 1999 the physicist Freeman Dyson said, “the surprises in science generally happen from the development of new tools and new technologies and not from new concepts” (Madou, 2002). As a post-genomics tool, metabolomics is a young field but in its exponential growth phase. As discussed in this review, the bioremediation field has much to gain from the advances in this emerging area of science. We believe that explains why metabolomics has become so popular—because the analysis of small organic molecules is important to all aspects of life science.

Today it is well recognized that the future bioremediation research projects must focus on transferring the remediation strains from the test tube to the environment. It might be that microorganisms different than those chosen today are better suited for *in situ* bioremediation simply because the microbes isolated through traditional enrichment procedures might not be the ones performing the bulk biodegradation (Cases and De Lorenzo, 2005). Post-genomics tools will certainly play a central role in the achievement of this task, and metabolomics is likely to be the approach of major impact in this field.

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