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Microbial degradation of model compounds of coal and production of metabolites with potential commercial value

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Abstract

Due to the need of new strategies for improving the economical performance of coal technologies, efforts were undertaken to develop techniques of solubilizing coal by the action of microorganisms. Because of the poor information in the beginning of this research, the microbial metabolism of some monomeric structural elements of coal was investigated first. Dibenzofuran, for example, was chosen as it represents a model structure frequently found in many coals, i.e. the cyclic biarylether moiety. It was found to be degraded by many different organisms isolated from soil via a new degradative mechanism called "angular" dioxygenation. Fluorene, a model compound for dibenzo-cyclopentane structures in coal, surprisingly followed essentially the same metabolic steps. Additional compounds which were integrated in the research program like naphthalenes, biphenyls, biarylethers and carbazoles also exhibited an oxygenase-dependent mode of initial attack. Since all enzymes involved were not active outside the cells, there seems to be no way to employ them in biological depolymerisation of untreated coals. Current work therefore, by employing special selection substrates, concentrates on the detection of new enzyme systems which follow non-oxygenase dependent mechanisms.

In a second line of research the enzyme systems mentioned above are used to synthesize new organic compounds from coal-derived substances. Due to the relaxed substrate specifity of the initial dioxygenases many structural analogues of dibenzofuran are metabolized. Several optically active compounds of the dihydrodiol-type were isolated and characterized by spectroscopic methods. We recently developed a preparative technique to produce these potentially valuable metabolites at a gram-scale. In addition, methods of genetic engineering are currently being adopted to create stable high expression organisms with improved productivity.

Keywords: Coal biodegradation; Coal bioliquefaction

1. Introduction

In the last years the biological attack on coal has found increasing attention [1–4]. This is due to the overwhelming amounts of coal waiting to be employed in energy

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production or serving as raw material for chemical industries. Despite that strong interest the knowledge in the basic principles of biological coal degradation is still very scarce. The advantages of biological over chemical or physical methods are obvious. Biological processes are characterized by low temperatures, low pressures and biodegradability of unwanted byproducts. Therefore, employing biotechnological methods, strong disadvantages of thermochemical processes can be avoided.

These advantages, however, are counterbalanced by severe intrinsic constraints of biological methods. Analysis of many structurally different types of coals reveals some common features, which have to be taken into consideration, if one aims at a biological solution of coal mining problems. First, coal is essentially derived of lignin, which is in itself a biologically generated polymer of considerable recalcitrance i.e. of moderate biodegradability only. This is due to its complex polymeric structure. During extensive structural modification in the diagenesis of this coal-precursor, the accessability for biochemical reactions is even further reduced. Thus, all attempts to directly investigate the biodegradation of coal are very difficult and time consuming. Although many investigations on the attack by fungi on lignin and on some types of brown coal have been reported, the bacterial attack on this compounds is much less understood. In order to gain basic knowledge on the mechanisms of bacterial coal degradation, a strategy employing simple model compounds of rather low molecular weight was chosen. Each of these substances represents a certain type of structural "building block" of the coal molecule. The bacteria degrading this model structures were purified and analysed for their reaction mechanisms. A major goal consisted in a possible adoption of these bacteria to degrade high molecular weight coal structures. Secondly, careful attention was paid to the products produced by these bacteria during degradation of coal model structures. These biotransformations often yield regio- and stereospecific hydroxylated compounds representing useful synthons for organic synthesis [5]. Most of these compounds are valuable because of their specific substitution pattern often not available by chemical synthesis (e.g. hydroxylation, dihydroxylation, cis-dihydrodiol structure). Some of these compounds may therefore contribute to amelioration of low price products from coal. Model compounds chosen for the investigation were, amongst others, dibenzofuran, fluorene, biphenyl, naphthalene and phenylcyclohexane.

2. Biological degradation of coal-substructures

It has been frequently demonstrated (these proceedings) that the biarylether linkage represents an integral part of the coal structure. Therefore, the degradation of arylalkyl- and biarylethers has been thoroughly studied in order to elucidate the biochemical mechanisms of their bacterial degradation. Also other linkages between phenyl rings are found in coal (C–C, C–N, C–S) and their reaction with ether-cleavage oxygenases has been recently studied.

2.1. Degradation of simple alkaryl- and biarylethers

Previous investigations have shown that besides the known mechanisms of cleavage of alkarylethers i.e. cleavage of alkoxyl groups after monooxygenation other alternatives do exist [6]. In anoxic environments, methane-forming organisms have been shown to remove the methylgroup of alkoxyl ethers with concomitant formation of methane and a free hydroxy-group. In another investigation, 2-methoxybenzoate was demonstrated to be dioxygenated with concomitant liberation of methanol from the aromatic nucleus [7]. The substrate range of the respective *Pseudomonas* strain CLB250 could be extended up to 2-hexoxybenzoate. A dioxygenase reaction is responsible for this ether cleavage, as has been demonstrated by $^{18}O_2$-experiments and cooxidation of 2-methylbenzoate, which yielded 3,5-cyclohexadiene-2-methyl-1,2-diol-1-carboxylic acid as a dead end product.

The same principle of initial attack was found for another model compound, 4-carboxybiphenyl ether in *Pseudomonas pseudoalcaligenes* POB310 (Fig. 1). After initial angular dioxygenation, an unstable hemiacetal is produced which chemically liberates phenol and 3,4-dihydroxybenzoate [8]. Further degradation proceeds via well known pathways. The gene for the initial dioxygenase was cloned and sequenced

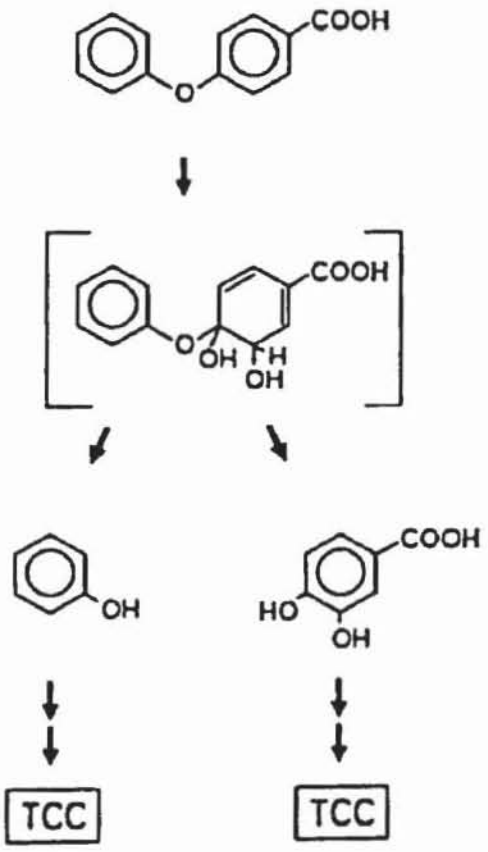

Fig. 1. Metabolism of 4-carboxybiphenylether in *Pseudomonas pseudoalcaligenes* POB310 proceeds via an unstable "angular" dihydrodiol. TCC: further degradation proceeds via reactions of the Krebs-cycle.

in collaboration with the team of K.N. Timmis, GBF, Braunschweig, representing the first example of a well characterized "angular" dioxygenase.

2.2. Degradation of dibenzofuran and fluorene

The degradation of dibenzofuran (Fig. 2), has been thoroughly investigated as this molecule represents a direct substructure of coal, namely the cyclic biarylether moiety. Interestingly, its degradation in Gram-positive bacteria was initiated following the same principle as has been demonstrated for the example given above [9]. The action of an angular dioxygenase has been unequivocally demonstrated by the cometabolism of fluorene (Fig. 3). This compound is structurally analogous to dibenzofuran (the ether oxygen atom has been replaced by a carbon atom).

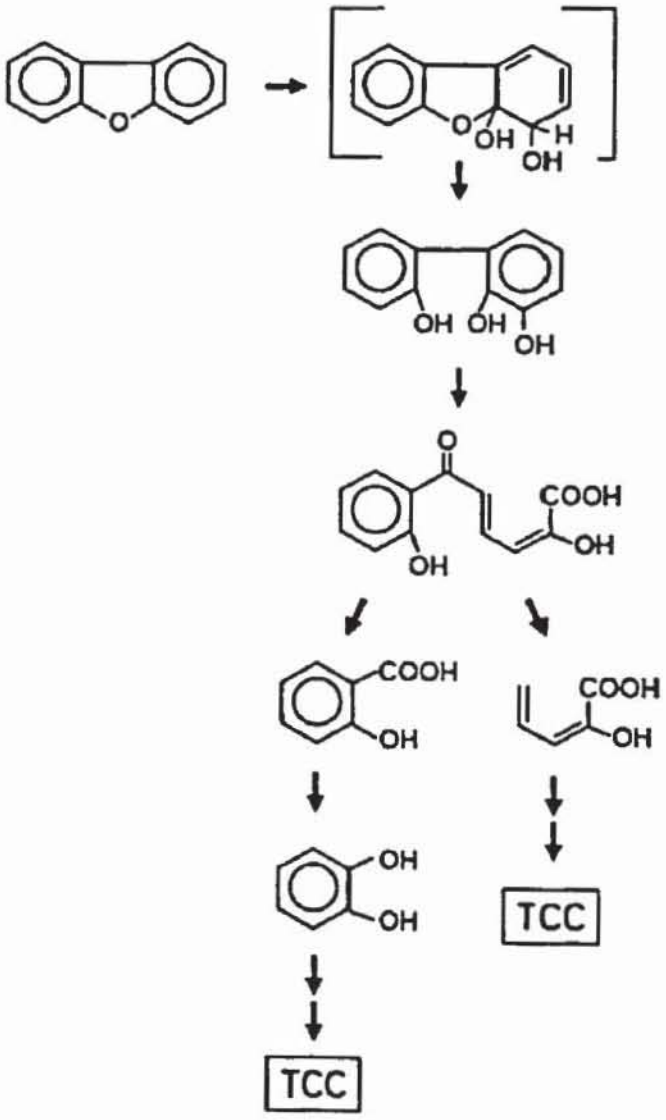

Fig. 2. Metabolism of dibenzofuran in *Brevibacterium sp.* strain DPO1361 after initial "angular" attack. TCC: further degradation proceeds via reactions of the Krebs-cycle.

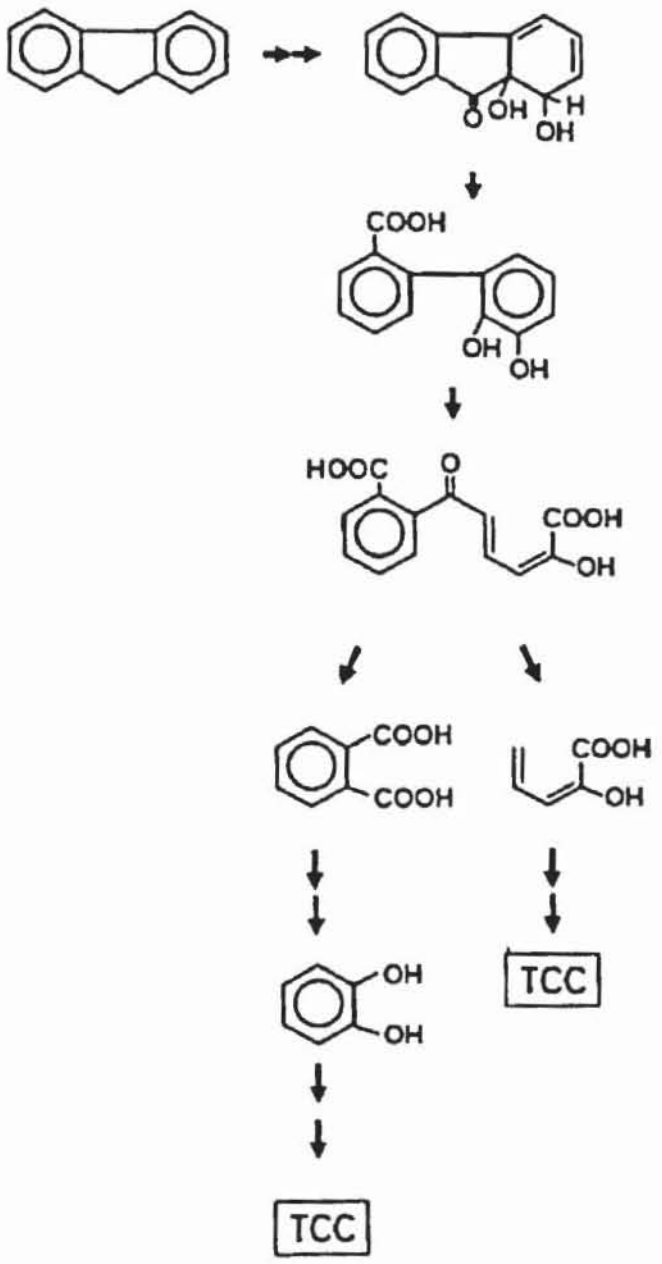

Fig. 3. Metabolism of fluorene in *Brevibacterium sp.* strain DPO1361 after initial "angular" attack. TCC: further degradation proceeds via reactions of the Krebs-cycle.

Consistingly, 1,10-dihydro-1,10-dihydroxyfluoren-9-one was excreted into the medium, a compound which can hardly be produced by chemical methods. Involvement of an initial angular dioxygenase in dibenzofuran degradation was therefore unambiguous [10]. This new type of initial attack was also indicated by inhibition of the ring cleavage enzyme, which cleaves 2,2'-3-trihydroxybiphenyl in a meta fashion. Accumulation of this biphenyl derivative during the metabolism of dibenzofuran was in total coincidence with the degradation pathway outlined in Fig. 2. The strong analogy to the pathway of 2-hydroxybiphenyl degradation, which will not to be discussed here, is obvious [11].

2.3. Possible application of initial dioxygenases

These and other results strongly indicate that the common mechanism of biarylether cleavage is a dioxygenolytic one. As in all cases investigated the responsible enzymes proved to be unstable outside the cell, a severe constraint is visible with respect to their possible use in degradation of coal. According to accepted structural models of coal, it is a macromolecular structure not suspected to enter the bacterial cell. Therefore there seems to be no way of employing these enzymes in biological liquification of coal. However, an interesting application is feasible in using them for specific transformations of coal derived compounds to special mono- and dihydroxylated products so far not synthesized by chemical methods (Figs. 4 and 5)

2.4. Enrichment of non-oxygenase dependent strains

As conventional enrichment techniques only yielded bacterial strains with oxygenase-dependent metabolic pathways, a different strategy of isolation had to be developed in order to enrich bacteria which employ enzymes potentially soluble in cell free environments. This represents the "conditio sine qua non" of a possible applica tion in biosolubilization of coal. As these enzymes may be very rare in nature and not readily selected, special enrichment- or detection-substrates like electron-deficient

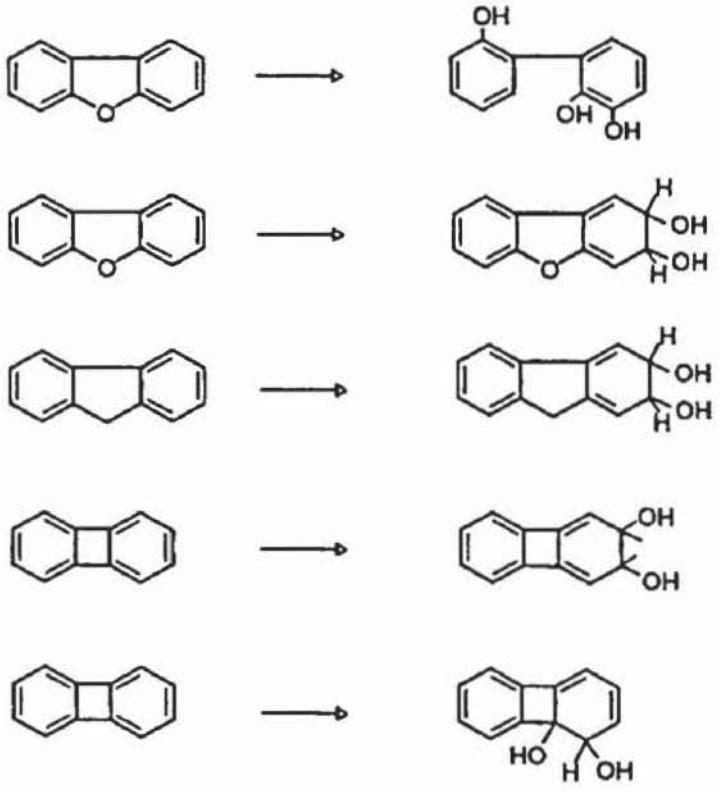

Fig. 4. Examples for hydroxylated compounds from biotransformations performed with biarylether, naphthalene and biphenyl degrading bacteria [15].

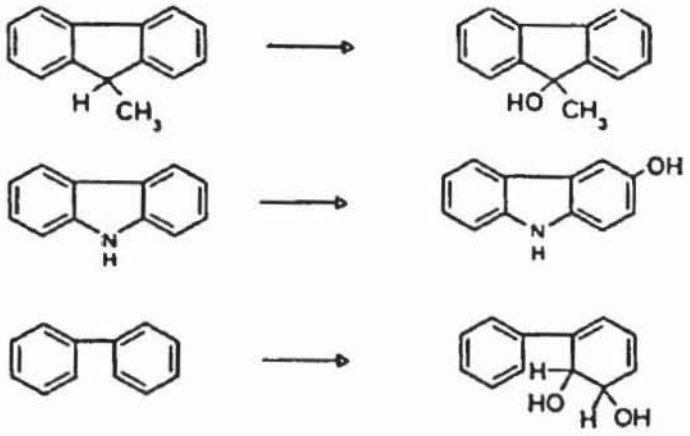

Fig. 5. Examples for hydroxylated compounds from biotransformations performed with dibenzofuran and biphenyl degrading bacteria [15].

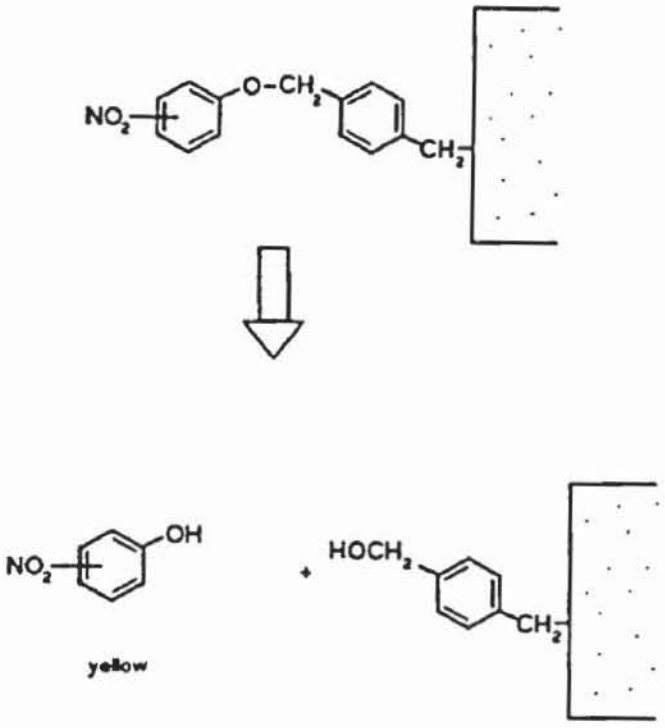

Fig. 6. Polymer attached enrichment substrate for detection of non-oxygenolytic ether cleaving enzymes.

halogen and nitro-substituted biarylethers may be of great value. First experiments employing special selection substrates are in progress that will clarify whether strains can be selected cleaving biarylether bonds according to a non-oxygenolytic mechanism (Fig. 6). The use of chromogenic selection substrates attached to a polymeric matrix should allow detection of lytic enzymes outside the cells.

3. Production of compounds hydroxylated regio- and stereospecifically

The enzymes isolated so far are likely to be of limited value for the purpose of bioliquification of coal, as only the mobile phase with low molecular weight is potentially degradable. These enzymes, however, can be employed to produce valuable metabolic intermediates from compounds derived from coal processing. Profound preparative work has been performed with compounds of the dihydrodiol-type [5]. They are employed as building blocks and in the synthesis of natural products [12] because they often display substitution patterns inaccessible in high yields by chemical synthetic procedures. For this reason a future commercial application is imaginable [13, 14]. Examples for products from our laboratory are given in Figs. 4 and 5. Biotechnology does provide these new bacterial intermediates as potential synthons for organic chemistry: a typical biotechnological procedure for producing for example 2,3-dihydro-2,3-dihydroxybiphenyl by non-engineered bacteria is initiated by growing the cells with a cheap carbon source. After induction with biphenyl the substrate is transformed to 2,3-dihydro-2,3-dihydroxybiphenyl. When the amount of accumulated metabolite will not increase further, cells and unchanged substrate are separated from the culture broth and the product is absorbed on a solid phase matrix. After washing and desorption with appropriate eluents it is crystallized and characterized (yield ca. 1 g/l culture broth [15]).

Amelioration of bulk products like coal should be possible due to the fact that many bacterial metabolites mentioned above are optically active compounds. Sometimes, they are dead end products of bacterial metabolism and may therefore be isolated easily. Frequently, for example in the case of 1,10-dihydro-1,10-dihydroxyfluoren-9-one, only transient accumulation of compounds is observed, followed by further metabolisation. In addition, many potential production strains have been found to be unstable, loosing the capability to transform one or more compounds of interest. We also observed the amount of enzyme decreasing with time or having never been sufficiently high. In these cases it may be advantageous to employ genetic engineering techniques and e.g. clone the enzymes of choice and express them in a suitable background strain not exhibiting any product metabolising enzymes. A further advantage consists in the possibility to separate mixtures of enzymes responsible for undesired production of mixtures of products. This avoids complex, time consuming and expensive purification procedures. As the initial dioxygenase enzymes mentioned above are not soluble in crude extracts of bacteria, normal enzyme purification techniques will not be successful. Once having cloned the corresponding gene, the expression of these enzymes i.e. the amounts of enzyme synthesized by the cells may be further elevated. Up to now, two biphenyl dioxygenases and a carboxy biphenyl ether dioxygenase have been cloned and are currently manipulated in order to increase expression levels. Work is in progress to clone and stably express genes for oxygenase enzymes transforming e.g. dibenzofuran in an angular fashion.

4. Summary

Many bacteria have been isolated metabolizing "building block" compounds of coal. Due to the instability of initial dioxygenases outside of the cell, an application in biological depolymerisation of coal does not seem feasible. For this purpose the need for non-oxygenase dependent enzyme systems has become obvious. First results employing new enrichment substrates to detect such enzymes are looking promising. Secondly, the capability of oxygenative enzyme systems to produce specifically hydroxylated or optically pure metabolites from coal-derived substances represents a powerful synthetic method. Further research will concentrate also on their application as synthons or fine chemicals.

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