CLASSICAL AND NEW ASPECTS IN DEGRADATION OF AROMATIC XENOBIOTICS

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Abstract: Organic chemical mixtures are prevalent in waste waters from industrial and municipal sources as well as in contaminated groundwater. Phenols are pollutants found in wastewaters from oil refineries, chemical plants, explosives, resins and coke manufacture, coal conversion, pesticide and textile industries. The main contaminants of refinery wastewater include phenols, polycyclic aromatic hydrocarbons (PAHs) as well as heavy metals. Among these toxic pollutants, phenols are considered to be the most hazardous ones, and they are certainly the most difficult to remove. Phenolic compounds are toxic at relatively low concentration. Because of these low concentrations the most suitable methods for their removal are the microbial ones. The present work is a review of biodegradation of phenol. Degradation of phenol occurs as a result of the activity of a large number of microorganisms including bacteria, fungi and actinomycetes. There are reports on many microorganisms capable of degrading phenol through the action of variety of enzymes. These enzymes may include oxygenases, hydroxylases, peroxidases, tyrosinases, oxidases etc. Batch experiments were carried out in a different bioreactors. Biodegradation of organic chemicals by microbes using pure cultures can produce toxic intermediates. This problem may be overcome by the use of mixed cultures that have a wider spectrum of metabolite degradation properties.

In this review we described also some new technology for biodegradation of phenol like: different immobilization, FISH (Fluorescence *in-situ* hybridization) analysis, nanobiotechnologies and etc.

Key words: phenol, biodegradation, microorganisms, enzymes, immobilization, FISH analysis, nanobiotechnologies

INTRODUCTION

Phenol is one of the main pollutants in industrial waste water. Phenol and its vapors are erosive to the respiratory tract, the skin and the eyes. With the development of industrial production and continuous demand for chemicals, a large volume of wastewater containing phenols was discharged into the aquatic environment. Moreover, chemical leakage further increased the emission of phenols into aquatic systems. Phenol and its methylated derivative (cresols) were selected due to their extensive use in industry and ecotoxicity to freshwater and marine organisms. The review from Duana et al. (2018) [1] focused on the ecotoxicity of phenol and m-, o-, and p-cresol on aquatic systems. The mechanism of action of phenols was also discussed. The aim of literature review [1] was to summarise the knowledge of the behaviour, and toxicity on marine and freshwater organisms, of phenols as well as to try to select a series of sensitive biomarkers suitable for ecotoxicological assessment and environmental monitoring in aquatic environments.

Phenols are typically classified into two main types on the basis of the number of phenol units, including polyphenols and simple phenols. Phenols are not only synthesized industrially, but also produced by plants and microorganisms [2, 3]. They could accumulate in surface water, ground water and soil when released into the environment [4]. Generally, microorganisms can be applied to water treatment with

phenol pollution, such as *Pseudomonas sp.* and *Acinetobacter sp.* [5].

Accidental spills of phenol, which happened in the Port of Gothenburg (Sweden) [6] and Xin'an River (China), caused leakage of a significant amounts of phenol, posing a threat to water quality and aquatic systems (China Chemical Safety Association (2011) [7]; HELCOM (2002) [8]). Due to their high water-solubility (phenol, 8.28 g/100 mL; cresols, 2.15–2.60 g/100 mL), phenol and cresols can persist at a high concentration in aquatic environments [9].

BIODEGRADATION STUDIES

MECHANISM OF AEROBIC PHENOL DEGRADATION

It was investigated detoxification potential and rehabilitation of activated sludge (AS) after shock loading of Sofia's wastewater treatment plant 'Kubratovo' with mazut [10]. The studied enzymological indicators for the detoxification of the aryl-containing contaminants in mazut are the key enzymes of the preparatory pathways of the cleavage of the benzene ring. Parallel to this authors studied the succinate dehydrogenase activity, which is an indicator of the functioning of the cycle of tricarboxylic acids (CTC) on the one hand, and the infusion of the metabolic products of detoxification in the central metabolic pathways, on the other (Fig. 1).

Fig. 1. Phenol biodegradation pathways and key enzymes in different stages [11]

The results show that despite the elapsed period of 16 days, activated sludge (AS) is still in a phase of damage due to the experienced intoxication shock. The detoxification potential, indicated by the activity of the oxygenases and succinate dehydrogenase (SDH), in this first phase of adaptation shows a reduction of the oxygenase activity as phenol 2-monooxygenase (P2MO), catechol 1,2-dioxygenase (C12DO), protocatechuate 3,4-dioxygenase (P34DO), catechol 2,3-dioxygenase (C23DO) and total dioxygenase TDO. In first adaptation phase the meta-mechanism for cleavage

of the benzene ring is slightly activated, which pathway is shorter, more effective for detoxification, while the enzymes are driven by coordinated synthesis or are predominantly constitutive. The cleavage is catalyzed by specific metalloenzymes – dioxygenases, divided into intradiol dioxygenases which cleave the ring on ortho-position to the hydroxyl substituent and extradiol dioxygenases which cleave on meta-position [12,13] Orthopathway is the oxidation of catechol to cis, cis-muconate in a reaction catalyzed by C12DO, and meta-pathway is the oxidation of catechol to 2-

hydroxymuconic semialdehyde catalyzed by C23DO. The cleavage of the benzene ring of protocatechuate is carried out by P34DO, with the product β -carboxy-cis, cis-muconate [14,11]. These processes can be realized at the conditions of denitrification at low oxygen concentration. In the denitrification zone, the rate of detoxification processes are lower. The enzyme mechanisms of this low rate detoxification potential present applied and scientific interest.

MECHANISM OF ANAEROBIC PHENOL DEGRADATION

The efficiency of biodegradation of aryl-containing xenobiotics is influenced by the type of the organic pollutant, the nature of the organism, the enzyme involved, the mechanism of degradation and the nature of the influencing factors.

An anaerobic phenol metabolism [15] in a denitrifying *Pseudomonas* sp. is shown in Fig. 2

Fig. 2. Intermediates and enzymes involved in the initial steps of anaerobic phenol metabolism in the denitrifying *Pseudomonas* strain K 172. (1) and (2), Phenol carboxylase system; (3), 4-hydroxybenzoate-CoA ligase (AMP forming); (4), 4-hydroxybenzoyl-CoA reductase (dehydroxylating); (5), benzoyl-CoA reductase (aromatic ring reducing).

Although strictly anaerobic conditions prevail in several environmental compartments, up to now, biodegradation studies with emerging organic contaminants (EOCs), such as pharmaceuticals and personal care products, have mainly focused on aerobic conditions. One of the reasons probably is the assumption that the aerobic degradation is more energetically favorable than degradation under strictly anaerobic conditions. Certain aerobically recalcitrant contaminants, however, are biodegraded under strictly anaerobic conditions and little is known about the organisms and enzymatic processes involved in their degradation.

Ghattas et al. (2017) [16] provide a comprehensive survey of characteristic anaerobic biotransformation reactions for a variety of well-studied, structurally rather simple contaminants (SMOCs) bearing one or a few different functional groups. Furthermore it summarizes anaerobic degradation studies of more complex contaminants with several functional groups (CMCs), in soil, sediment and wastewater treatment. While strictly anaerobic conditions are able to promote the transformation of several aerobically persistent contaminants, the variety of observed reactions is limited, with reductive dehalogenations and the cleavage of ether bonds being the most prevalent. Thus, it becomes clear that

the transferability of degradation mechanisms deduced from culture studies of SMOCs to predict the degradation of CMCs, such as EOCs, in environmental matrices is hampered due the more complex chemical structure bearing different functional groups, different environmental conditions (e.g. matrix, redox, pH), the microbial community (e.g. adaptation, competition) and the low concentrations typical for EOCs.

Yonga and Zhong (2010) [17] provide an up-to-date review on progress in biodegradation research in China. They describe include the isolation of extremophilic microorganisms for pollutant degradation in extreme conditions and the study of genes and enzymes related to biodegradation pathways. Progress has been achieved in these fields, including the isolation of novel biodegradation microorganisms, elucidation of biodegradation pathways, innovation in biodegradation engineering, and generation of bioenergy from environmental waste. Biodegradation engineering has potential as an interesting and powerful platform, where genetic engineering, process engineering and signal transduction engineering are applied together. In addition, pollutant treatment combined with the production of renewable sources of bioenergy by microorganisms is attractive.

ENZYMES INVOLVED IN THE BIODEGRADATION OF PHENOLIC COMPOUNDS

Generally aromatic compounds are broken down by natural bacteria. Derivatisation of aromatic nuclei with various substituents particularly with halogens

makes them more recalcitrant. There are reports on many microorganisms capable of degrading phenol through the action of variety of enzymes. These enzymes may include oxygenases, hydroxylases, peroxidases, tyrosinases, oxidases etc. (Table 1).

Table 1. Enzymes involved in the biodegradation of phenolic compounds.

Type of Phenol	Enzyme	Reference	
Phenol	Phenol hydroxylase	Gurujeyalakshmi and Oriel (1988)	[18]
Phenol	Polyphenol Oxidase	Burton et al. (1993)	[19]
Phenol	Polyphenol Oxidase	Cano et al. (1997)	[20]
Phenol	Phenol Oxidase	Okeke et al. (1997)	[21]
Phenol	Polyphenol oxidase	Shashirekha et al. (1997)	[22]
Phenol	2,3 dioxygenase	Ali et al. (1998)	[23]
Phenol	Laccase	Bollag et al. (1998)	[24]
Phenol	Polyphenol oxidase	Garzillo et al. (1998)	[25]
Phenol	Peroxidase	Ghiourelitis and Nicell (1999)	[26]
Phenol	Horseradish peroxidase	Wu et al. (1998)	[27]
Phenol	Horseradish peroxidase	Zahida et al. (1998)	[28]
Phenol	Polyphenol oxidase	Edwards et al. (1999)	[29]
Phenol	Laccase	Kadhim et al. (1999)	[30]
Phenol	Laccase`	Schneider et al. (1999)	[31]
Methoxyphenol	Laccase	Setti et al. (1999)	[32]
Phenol	Laccase	Hublik and Schinner (2000)	[33]
Phenol	Laccase	Robles et al. (2000)	[34]
Phenol	Catechol 1,2oxygenase	An et al. (2001)	[35]
Phenol	Polyphenol oxidase	Luke and Burton (2001)	[36]
Phenol	Peroxidase	Sakurai et al. (2001)	[37]
Phenol	Polyphenol oxidase	Steffens (2002)	[38]
Phenol	Phenol oxidase	Johjima et al. (2003)	[39]
Phenol	Tyrosinase	Xiangchun and Zhang(2003)	[40]
Lignophenols	Peroxidase	Xia et al. (2003)	[41]

MICROORGANISMS IN THE BIODEGRADATION OF PHENOLIC COMPOUNDS

Microorganisms are an important part of natural ecosystems. They are found in industrially contaminated soils and water as a result of their ability to survive in restrictive conditions. In this respect, their ability to rapidly and efficiently purify the environment of phenolic contamination is important, with a view to protecting the living environment and human health directly. A large number of aromatic compounds including phenol and phenol derivatives are difficult to dissolve and very dangerous due to their high toxicity. The study of key enzymes involved in aromatic catabolism is important from both a theoretical and a technological point of view since it can be aimed at

the development of modern and effective treatment facilities [42, 43]. The application of molecular methods of analysis to the study of strains able to degrade hazardous chemical contaminants can lead to new approaches that will optimize the currently used technologies. The investigations on specificity of phenol biodegradation by different microbial strains are meaningful for the invention of effective remediation technologies for industrial wastes where the phenolic substrates are a common occurrence. Most of the current research has been directed at the isolation and study of microbial species of potential ecological significance.

In the review from Krastanov et al. (2013) [43], some of the best achievements in degrading phenolic compounds by bacteria and yeasts are presented, which draws attention to the high efficiency of strains of

Pseudomonas, *Candida tropicalis*, *Trichosporon cutaneum*, etc. The unique ability of fungi to maintain their degradation potential under conditions unfavorable for other microorganisms is outstanding.

Degradation of phenol occurs as a result of the activity of a large number of microorganisms including bacteria, fungi and actinomycetes [5]. These microorganisms are presented in Table 2.

Table 2. Microorganisms in the biodegradation of phenolic compounds

Type of phenol	Microorganisms	Reference	
Phenol	<i>Basidiomycota</i>	Martinkov et al. (2016)	[44]
Phenol	<i>Rhodococcus</i>	Martinkova et al. (2009)	[45]
Phenol	<i>Bacillus stearothermophilus</i>	Gurujeyalakshmi and Oriol(1988)	[18]
Phenol	<i>Pseudomonas putida</i>	Allsop et al. (1993)	[46]
Phenol	<i>Agaricus bisporus</i>	Burton et al. (1993)	[19]
Pentachlorophenol	<i>Lentinus bisporous</i>	Okeke et al. (1993)	[47]
Phenol	Aerobic consortium	Ambujam and Manilal(1995)	[48]
Phenol	<i>Acinetobacter johnsonii</i>	Hoyle et al. (1995)	[49]
2- chlorophenol	<i>Pseudomonas putida</i>	Over and Rehon (1995)	[50]
Phenol	<i>Pseudomonas sp</i>	Bodzek et al. (1996)	[51]
Phenol	<i>Pseudomonas sp</i>	Gotz and Reuss(1997)	[52]
Penta, chlorophenol	<i>Lentinula edodes</i>	Okeke et al. (1997)	[21]
Phenol	<i>Ochromonas danica</i>	Semple and Cain(1997)	[53]
Phenol	<i>Bacillus sp</i>	Shashirekha et al. (1997)	[22]
Phenol	<i>Phormidium valderianum</i>	Ali et al. (1998)	[23]
Phenol	<i>Rhizoctonia praticola</i>	Bollag et al. (1988)	[24]
Phenol	<i>Trametes trogii</i>	Garzillo et al. (1998)	[25]
Phenol	<i>Pseudomonas putida</i>	Loh and Wang (1998)	[54]
Phenol	<i>Pseudomonas fluorescens</i>	Torres et al. (1998)	[55]
Phenol	<i>Pseudomonas putida</i>	Mordocco et al. (1999)	[56]
Phenol	<i>Coriolus versicolor</i>	Kadhim et al. (1999)	[30]
Phenol	<i>Ralstonia eutropha</i>	Leonard et al. (1999 a,b)	[57, 58]
Phenol	<i>Coprinus cinereus</i>	Schneider et al. (1999)	[31]
Phenol	<i>Pseudomonas putida</i>	Wang and Loh (1999)	[59]
Phenol	<i>Pseudomonas putida</i>	Zumriye and Gultac (1999)	[60]
Phenol	<i>Pseudomonas pictorium</i>	Annadurai et al. (2000a)	[61]
Phenol, Nitrophenol	<i>Nocardioideis</i>	Cho et al. (2000)	[62]
Phenol	<i>Phanerocheate chrysosporium</i>	Garcia et al. (2000)	[63]
Phenol	<i>Pleurotus ostreatus</i>	Hublik and Schinner (2000)	[33]
Phenol	<i>Pseudomonas putida</i>	Loh and Tar (2000)	[64]
Phenol	<i>Acinetobacter calcoaceticus</i>	Nakamura and Sawada (2000)	[65]
Phenol	<i>Chalara paradoxa</i>	Robles et al. (2000)	[34]
Phenol	<i>Streptomyces setonii</i>	An et al. (2001)	[35]
Phenol	<i>Alcaligenes sp</i>	Baek et al. (2001)	[66]
Phenol	<i>Pseudomonas sp</i>	Gonzalez et al. (2001)	[67]
Phenol	<i>Pseudomonas putida</i>	Loh and Liu (2001)	[68]
Phenol	<i>Pseudomonas putida</i>	Petruschka et al. (2001)	[69]
Bisphenol	<i>Coprinus cinereus</i>	Sakurai et al. (2001)	[37]
Phenol	<i>Acinetobacter sp</i>	Hao et al. (2002)	[70]
Phenol	<i>Rhodococcus erythropolis</i>	Prieto et al. (2002)	[71]
Phenol	<i>Trichosporon cutaneum</i>	Godjevargova et al. (2003)	[72]
Phenol	<i>Termitomyces albuminosus</i>	Johjima et al. (2003)	[39]
2, 4 dichlorophenol	Mixed culture	Quan et al. (2003)	[73]

Chlorophenol	<i>Pseudomonas putida</i>	Farighian et al. (2003)	[74]
Chlorophenol	<i>Achromobacter sp</i>	Xiangchun et al. (2003)	[40]
Phenol	Mixed Fungi	Atagana (2004)	[75]
Phenol	<i>Pseudomonas putida</i>	Hamed et al. (2004)	[76]
Phenol	<i>Alcaligenes sp</i>	Nair and Shashidhar (2004)	[77]
Phenol	<i>Fusarium sp</i>	Santos and Linardi (2004)	[78]
Pentachlorophenol	<i>Sphingomonas chlorophenolica</i>	Bielefeldt and Cort (2005)	[79]
Dichlorophenol	<i>Pseudomonas putida</i>	Kargi and Eker (2005)	[80]
Phenol	<i>Pseudomonas sp</i>	Prpich and Dougliis (2005)	[81]
Phenol	<i>Bacillus brevis</i>	Arutchelvan et al. (2006)	[82]
Nonylphenol	<i>Clavariopsis aquatica</i>	Moeder et al. (2006)	[83]
Phenol	<i>Aspergillus awamori</i>	Stoilova et al. (2006)	[84]
Phenol	<i>Candida tropicalis</i>	Krastanov et al. (2013)	[43]
Phenol	<i>Pseudomonas</i>	Belouhova et al. (2017)	[85]
Phenol	<i>Bacillus laterosporus</i>	Topalova et al. (2011)	[86]
Phenol	<i>Pseudomonas rhodesiae</i>	Satchanska et al. (2009)	[87]
Phenol	<i>Bacillus subtilis</i>	Satchanska et al. (2015)	[88]
Phenol	<i>Candida tropicalis</i>	Hristov et al. (2012)	[89]
Phenol	<i>Trichosporon montevidense</i>	Hristov et al. (2012)	[89]
Phenol	<i>Pseudomonas sp.</i>	Yotinov et al. (2016)	[11]

The phylum *Basidiomycota* include organisms with enormous bioremediation potential. A variety of processes were proposed at the lab scale for using these fungi and their phenol oxidases in the degradation of phenolics. Martinkov et al. (2016) [44] present a survey of this topic using literature published mostly over the last 10 years. *Basidiomycota* have a high potential for environmental technologies. Here the focus is on their use in the biodegradation of phenolic pollutants. The new trends in the production of their phenol oxidases are summarized.

Various forms of the catalysts (enzymes, whole cells) and reactors are compared. The perspectives of their industrial applications are discussed. Fungi of the phylum *Basidiomycota* are prominent tools for the degradation of recalcitrant pollutants [90] such as phenols, naphthols, bisphenols, phenolic or non-phenolic textile dyes, halogenated aromatics, polyaromatic hydrocarbons, etc. The focus of a review [44] is on the potential of these organisms, and especially their phenol oxidases, to degrade phenolics, which cause serious pollution of surface water, groundwater and soils [91].

A large number of aromatic compounds and organic nitriles, the two groups of compounds covered in this review from Prieto et al. (2002) [92]. They are intermediates, products, by-products or waste products of the chemical and pharmaceutical industries, agriculture and the processing of fossil fuels. The majority of these synthetic substances (xenobiotics) are

toxic and their release and accumulation in the environment pose a serious threat to living organisms. Bioremediation using various bacterial strains of the genus *Rhodococcus* has proved to be a promising option for the clean-up of polluted sites. The large genomes of rhodococci, their redundant and versatile catabolic pathways, their ability to uptake and metabolize hydrophobic compounds, to form biofilms, to persist in adverse conditions and the availability of recently developed tools for genetic engineering in rhodococci make them suitable industrial microorganisms for biotransformations and the biodegradation of many organic compounds.

The genus *Rhodococcus* was soon regarded as one of the most promising groups of organisms suitable for the biodegradation of compounds that cannot be easily transformed by other organisms [93]. So far only pseudomonads and related bacteria have been reported to possess comparable biodegradation abilities.

Rhodococci are non-sporulating, aerobic bacteria classified into mycolate-containing nocardioform actinomycetes [94]. The genus *Rhodococcus* is comprised of genetically and physiologically diverse bacteria, which have been isolated from various habitats, for example soils and sea water. Since they are equipped with a large number of enzymatic activities, unique cell wall structure and suitable biotechnological properties, *Rhodococcus* strains may be utilized as industrial organisms, primarily for biotransformations and the

biodegradation of many organic compounds [95]. *Rhodococci* can thus be applied in environmental remediation and in the pharmaceutical and chemical industries [96, 97]. The cell wall of rhodococci contains mycolic acids, like that of the genera *Mycobacterium*, *Nocardia* and *Corynebacterium*.

Cells of *R. erythropolis* immobilized on inorganic carriers (diatomaceous earth, zeolite) were found to be a suitable basis for an efficient phenol biodegradation process [92, 98].

Many studies on biodegradation of phenol come from bacteria. Bacterial species include *Bacillus sp*, *Pseudomonas sp*, *Acinetobacter sp*, *Achromobacter sp* etc. The genus *Pseudomonas* is widely applied for the degradation of phenolic compounds. These bacteria are known for their immense ability to grow on various organic compounds. Phenol biodegradation studies with the bacterial species have resulted in bringing out the possible mechanism and also the enzyme involved in the process. The efficiency of the phenol degradation could be further enhanced by the process of cell immobilization [61, 99].

Fusarium sp, *Phanerocheate chrysosporium*, *Corious versicolor*, *Ralstonia sp*, *Streptomyces sp* etc are also proved to be efficient fungal groups in phenol biodegradation. However, these microorganisms suffer from substrate inhibition at higher concentration of phenol, by which the growth is inhibited [92].

The mycelium (or conidia) of *Aspergillus awamori* NRRL 3112 is investigated for its ability to degrade phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol in high concentrations. The biodegradation studies were performed in a liquid medium with the phenolic compounds as a sole carbon and energy source [84]. The organism had mineralized phenol concentration of 0.3 g/l in 60 h, 0.6 g/l in 72 h and 1.0 g/l in 7–8 days. Successfully simulated phenols degradation profiles in all studies are obtained by Haldane-type kinetics. Besides, such mathematical models could successfully be used to predict the rate of phenols biodegradation under industrial conditions. The values of endogenous or decay and yield coefficients for all phenols at different concentrations are also determined.

IMMOBILIZATION

IMMOBILIZATION IN POLYACRYLONITRILE (PAN) GRANULES

The ability of *Trichosporon cutaneum* R57 to grow on and utilize some toxic compounds (phenol, acetophenone, acetone, amethylstyrene, benzoic

acid, dimethyl phenyl carbinol, methanol and isopropylbenzene) as sole carbon and energy sources is studied. The growth of *T. cutaneum* R57 in a model solution including all the contaminants mentioned above is studied and results are compared with growth curves in two types of waste water taken from the basin of the biopurification station with active sludge, treating water from oil refinery (Lukoil-Neftochim Co. (Burgas, Bulgaria)) and some accompanying organic products facilities, including waste water from industrial phenol production [72]. The strain is found to grow well in both types of waste water (inlet and outlet of the biobasin) and in the model solution. The results showed that strain *T. cutaneum* R57 can utilize a wide range of toxic compounds simultaneously.

For the study of immobilized cells [100], 2 g porous polyacrylonitrile (PAN) granules are prepared by dissolving 18 g PAN (product of Neftochim, Bulgaria), 1 g LiNO₃ and 3 g glycerol in 78 ml of dimethylformamide (DMFA). The homogenized mixture was pipetted and precipitated in water. The granules obtained are washed with distilled water and immersed in a 30% aqueous solution of glycerol for 24 h and they dried at 70°C to constant weight.

The immobilized system had high vitality and adaptivity into waste water from phenol production. The highest degradation after 24 h is observed for acetone (50-60%), benzoic acid is reduced by 25-/40% and phenol and dimethyl phenyl carbinol approximately by 30-45% depending on the type of the cells. Immobilized cells are showed a higher ability to degrade the more toxic compounds compared to free cells [72]. The results obtained indicate the ability of *T. Cutaneum* R57 to degrade some of the most toxic contaminants present in waste water.

IMMOBILIZATION IN POLYETHYLENE OXIDE CRYOGELS (PEO-CRYOGEL)

In recent years different organic carriers for bacterial immobilization were investigated. Among the synthetic polymers, poly(ethylene oxide) hydrogels are excellent candidates because they are nontoxic biocompatible materials which meet all of the requirements of strength, absorbency, exibility and adhesiveness. In the study by Satchanska et al (2009) [87] macroporous poly(ethylene oxide) PEO cryogels were investigated as carriers of xenobiotics degrading bacteria and the obtained PEO-biofilms were investigated for their ability for phenol biodegradation.

CRYOGELS PREPARATION:

High molecular weight poly(ethylene oxide) (Union Carbide; MW = 2.106 g/mol) was dissolved in distilled water (5 wt.%) and then a photo initiator (4-benzoylbenzyl) trimethylammonium chloride (Aldrich; 2 wt.% with respect to the polymer) was added under stirring at room temperature. The resulting homogeneous solution was poured into Teflon dishes (50 mm diameter) forming a 2.5 mm thick layer, which was then kept in a freezer at a temperature of -20°C for 2 h. The dishes were quickly placed in a thermostatic open chamber connected with a Julabo cryostat apparatus. The frozen system was irradiated with full spectrum UV-VIS light at -20°C with a Dymax 5000-EC UV curing equipment with 400W metal halide flood lamp for 2 min (dose – 11.4 J/cm^2 ; input power – 93 mW/cm^2). The PEO cryogels were extracted in distilled water for seven days, quickly frozen in liquid nitrogen and freeze dried in an Alpha 1-2 Freeze Drier (Martin Christ) at -55°C and 0.02 mbar for 24 h. The gel fraction (GF) yield was calculated by the following equation:

GF yield [%] = (weight of dried sample/initial weight of polymer) $\times$ 100. 2.

The study by Satchanska et al. (2015) [88] show the phenol-degradation performance of a promising new environmentally friendly biomaterial that was constructed and investigated for its biodegradation capability. The biofilter is cheap, highly effective and could be potentially useful in industrial wastewater treatment. Two xenobiotic-degrading bacteria are isolated from industrial areas polluted with heavy metals and aromatics. The studied microbial isolates were cultured from polluted soil near the Pb-Zn smelter called KCM (strain KCM R5) and from groundwater collected near the pesticide producing factory AGRIA (KCM RG5). Both manufactories are situated at a distance of about 6km from the town of Plovdiv, Bulgaria. The Pb-Zn smelter is the biggest one on the Balkan Peninsula. AGRIA factory is manufacturing more than 50 various pesticides.

Sequencing of xenobiotic-degrading bacteria 16S rDNA classified them as *Pseudomonas rhodesiae* (denoted as KCM R5) and *Bacillus subtilis* (denoted as KCM RG5).

The cryogels demonstrated non-toxicity, high biocompatibility with bacteria and excellent mechanical characteristics. PEO cryogels showed high biocompatibility with xenobiotics degrading bacterial strains KCM R5 and KCM RG5. The PEO-

KCM R5 biofilter was found to remove phenol at a concentration of 1000 mg/L^{-1} , while the PEO-KCM RG5 system was unable to degrade phenol at a concentration of about 600 mg/L^{-1} . After four weeks of biodegradation, the PEO biofilms remained compact, porous and elastic, while containing compact microbial biofilm as shown by scanning electron microscopy analysis of the cryogels.

Immobilization of the *Bacillus laterosporus* BT-271 microbe culture is described by Topalova et al. (2011) [86]. A long (20 days) model process of phenol biodegradation was performed in biofilters in sequencing batch regime and with active biological system – polyethylene oxide cryogels (PEO-cryogel) with immobilized bacteria *Bacillus laterosporus* BT-271. The model waste water, containing mineral medium and phenol as the only source of carbon and energy with 300 mg/l in the effluent, was used. One of the key investigated parameters was the manner of immobilization of the bacteria in polyethylene oxide cryogels. The two variants of the experiment were carried: direct immobilization of the inoculation material (SBB-D) and inoculation of the biomass after preliminary separation and further concentration (SBB-S) [86]. The implantation of the gel with the immobilized microorganisms in a sequencing batch biofilter showed good biodegradation, kinetic, enzymological characteristics, as well as self-renovating adequate biofilm structure in laboratory conditions. The two biofilters functioned well and the efficiency of phenol elimination reached 100% in 15 days. The variant SBB-D showed a more stable process, as well as an easier way of preparing the initial PEO-plate biofilm. The SBB-D system is suitable for scaling and future implementation of the water purification processes in practice.

All obtained results show that bacterial culture *Bacillus laterosporus* BT-271 is suitable for immobilization in PEO-gel. The formed biofilm is stable and after a 15-day adaptation period shows steady biodegradation activity. More stable variant for immobilization of the microbe biomass is the direct soaking of the PEOplate in the preliminarily multiplied bacterial culture, variant SBB-D. The implantation of the gel with the immobilized microorganisms in a sequencing batch biofilter shows good biodegradation, kinetic, enzymological characteristics, as well as self-renovating adequate biofilm structure in laboratory conditions. The SBB-D system is suitable for scaling and future implementation of the water purification processes in practice.

IMMOBILIZATION OF YEAST ASSOCIATION

The study by Hristov et al. (2012) [89] demonstrated the phenol degrading potential of yeast association consisting of two strains identified as *Candida tropicalis* and *Trichosporon montevidense*. At initial phenol concentration of 0.5 g.l⁻¹ the process was carried out in semicontinuous mode using cells of microbial association, free and immobilized on three different carries (Ca-alginate, zeolite and polyether-polyurethane (PUF)). The removal efficiency of phenol by free and immobilized cells, the influence of a mixture of five heavy metals (Cu, Mn, Co, Ni, Zn) on the biodegradation process were reported. The experimental results indicated that both cells, free suspended and immobilized in Ca-alginate, showed high phenol degrading efficiencies within 144 h, but the immobilized cells showed better performance in the presence of heavy metals compared to that of the suspended cells.

IMMOBILIZATION OF PENOPOLYSTYROL PELLETS, COVERED WITH ACTIVE CARBON

Two steps of enhancement of phenol detoxification by *Bacillus laterosporus* BT-2 71 by means of increasing of the rate of aeration and adding in an appropriate moment a carrier (penopolystyrol pellets, covered with active carbon) were studied from Topalova et al. (1995) [101]. The investigated bacterium was isolated from phenol polluted soil in the region of the petrochemical plants near Bourgas (Bulgaria). The strain was subjected to laboratory selective adaptation towards high phenol concentrations. Phenol was added after sterilization in a concentration 6.96 mmol. The inoculation of the reactor was being done with a 23-hour inoculation material. The carrier (30 % vol.) was put in bioreactor at a chosen optimum aeration regime. It was in the form of penopolystyrol pellets foamed to a density of 500 kg/m³, diameter - 2.0±0.2 mm, whose surface was activated by covering with layers of active carbon. Comparative study of the oxygenase activities of the freely suspended cells at the two steps of speeding up of the phenol elimination to elucidate the mechanisms of the processes was carried out. The effectiveness of phenol biodegradation was enhanced with 42%.

NEW TECHNOLOGY

FISH (FLUORESCENCE *IN-SITU* HYBRIDIZATION) ANALYSIS

The main governing factors in the biotransformation technologies are the concentration of xenobiotic pollutants in dependence of critical one as well as the way of its inflow in the system – gradually or shock loading. The aim of the study from Belouhova et al. (2017) [85] is to investigate the key differences in adaptation mechanisms of 1/ sediment biofilm, subjected to *shock loading* of toxic pollutant (phenol)/2/ biofilm community, that is purposefully adapted to the increased xenobiotic concentration (amaranth) The adaptive changes in two biofilm communities from lab-scale biotransformation technologies were estimated and compared on the base of functional and microbiological parameters. The processes were studied in two analogous models – case studies. *Case study 1* - bioremediation of sediments from Iskar river with shock phenol loading in concentration three times higher than critical one, that is about 250 mg/g sediment; *Case study 2* – laboratory sand biofilter - treating amaranth polluted wastewater. The inoculation material was specially treated activated sludge, taken from Sofia WWTP (wastewater treatment plant). The biofilter was supplied with highly specialized adaptive algorithm for gradually increase of xenobiotic concentration (from 5 to 50 mg/L amaranth).

Two detoxification technologies have been compared according to: key technological and microbiological parameters, the micro-distribution and the role of unculturable microorganisms as well as the microbial relationships in genus *Pseudomonas*. The special attention has been paid on the analysis of the polyphosphates as an indicator for the alternative energetic sources – polyhydroxyacetate and polyhydroxybutyrate. In the study new fluorescent method FISH (Fluorescence *In-Situ* Hybridization) was used. The fluorescence *in-situ* hybridisation is one of the most widely used methods for studying the biofilms, involved in water purification and detoxification processes This molecular techniques gives information about the quantitative and qualitative composition and spatial distribution of microorganisms in communities.

A fluorescence *in-situ* technique, particularly suitable for the study of biofilms, is coloring of the polyphosphate granules with DAPI (4',6-diamidino-2-phenylindole). It is known that when DAPI bonds to the polyphosphate granules the maximum of

emission of the dye changes, and its color instead of blue looks yellowgreen [102]. In the biological purification of waters with microbial elimination of phosphates the technologies are based on the accumulation of polyphosphates (metachromatin) in the bacterial cells. These bacterial inclusions are directly connected with the synthesis and using of polyhydroxybutyrate and polyhydroxyacetate granules. These granules can be used as alternative sources of energy. In the extreme difficult conditions of availability of high concentration of xenobiotics the using of the alternative sources of energy by microorganisms can play an indicative role for an adaptation to the biodegradation of the xenobiotics. The reason is that the xenobiotics affect negatively the electron transport and oxidative phosphorylation and inhibit the synthesis of ATP [103, 104]. By investigation of the amount and localization of polyphosphates and the related use of reserve carbon sources it is possible to detect the degree of adaptation of the microbial community to xenobiotic biodegradation.

The results from Belouhova et al. 2017 [85] showed that the technology with shock xenobiotic load reached the effectiveness of xenobiotic elimination 28%, while the technology with adaptive algorithm – 89%. As a general conclusion the adaptation mechanisms towards xenobiotic biodegradation were different on the basis of ways of inflow of the toxicants in the technology. After the shock xenobiotic loading in concentration three times higher than critical the biofilm developed the most simple mechanism – the multiplying of the microorganisms, but the biodegradation effectiveness was low. After application of the purposely constructed adaptive algorithm, microbial communities developed complex mechanisms of azo-detoxification, included the increased role of unculturable bacteria and synergistic relationships between them. This led to the increased effectiveness of xenobiotic detoxification. At concentrations of the toxicant exceeding the critical ones the microbial community was dependent to a larger extent on reserve sources of energy and adaptation took place mostly on the basis of an increase in the number of culturable biodegraders.

NANOBIOTECHNOLOGIES

Nanobiotechnologies are a rapidly growing field that offers new opportunities thus far unknown including regulation processes at a nano level. The biotransformation and mechanisms of degradation of many xenobiotics have been studied and are well

documented. There remains the important issue of the impact of nanomodulators on biotransformation processes and their potential to optimize and regulate biodegradation of recalcitrant xenobiotics. Carbon nanoparticles, which include nanodiamonds (ND), are used in a number of technological and medical applications. It is investigated the effect of ND on the biodegradation of phenol in model conditions [11]. The process of degradation was realized with a microbial culture of *Pseudomonas sp.* isolated from contaminated sediments in the small reservoir Lakatnik. It is a part of the Middle Iskar cascade (Bulgaria). It was found that ND have a stimulating effect on the initial phase of biodegradation of phenol. ND in concentrations of 100 ppm added at the start of the process led to an increase in a lot of important parameters of biological detoxification – specific rate of phenol biodegradation, effectiveness of phenol biodegradation and oxygenase enzyme activities and shorten the biotransformation. The results show that the ND affected in a different way the activity of inductive and constitutive oxygenases. The application of ND has a high potential for the management of microbial biodegradation and enhancement of the effectiveness of detoxification technologies for sediments and wastewater contaminated with xenobiotics.

The purpose of the study by Yotinov et al. (2017) [105] is to clarify in comparative terms the effect of carbon nanoparticles (single-walled nanotubes (SWNT) and nanodiamonds (ND)) on these processes. It is a new mechanism for regulation of detoxification processes included in the industrial wastewater treatment. In order to achieve this objective analogous modeling of biodegradation processes is performed. The experiment is conducted in simplified conditions, using a microbial culture of *Pseudomonas sp.* [105]. It was observed the influence of ND and SWNT on the basic kinetic parameters and key oxygenase enzymes of the bacteria from the genus *Pseudomonas*. ND have a positive regulating effect regarding the detoxification of phenol at the initial stages of the process. SWNT show weaker augmentation effect but lasting during the whole detoxification process, which is seen from the high values of the inductive enzyme. ND increase the activities of phenol-2-monooxygenase and catechol-2,3-dioxygenase respectively by 63,91% and 63,94% in comparison to the control variant. Under the same conditions SWNT have positive influence on the catechol-1,2-dioxygenase activity by 30,12% in comparison to the control. Depending on the

conditions of the wastewater treatment process both nanomodulators can be used purposely for regulating the biological detoxification. ND are more applicable in the risk situation of shock loading of industrial waters with xenobiotic pollutants to overcome the initial high toxic effect of phenol and its derivatives. The SWNT are more appropriate for long lasting detoxification processes to sustain the constant rate of the detoxification.

MOLECULAR DOCKING

Molecular docking is a method which finds out the preferred orientation of one or more molecules in the active sites of proteins [106]. Liu et al. (2018) [107] wrote a review for the application of molecular docking between organic pollutants and enzymes. They summarize the fundamental knowledge of molecular docking, such as its theory, available softwares and main databases. Moreover, five types of pollutants, including phenols, BTEX (benzene, toluene, ethylbenzene and xylenes), nitrile, polycyclic aromatic hydrocarbons (PAHs) and high polymer (e.g., lignin and cellulose), are discussed from molecular point of view. Different removal mechanisms are also explained in detail via docking technology. Even though this method shows promising application in the research of biodegradation, further studies are still needed to relate with actual condition.

Moreover, usually one kind of microorganism such as *Pseudomonas sp.*, *Acinetobacter sp.*, *Nocardia globerula sp.*, *Bacillus sp.*, *Phanerochaete sp.* and *Trametes sp.* could convert and decompose several organic compounds due to the secretion of a variety of enzymes. Actually, enzymes are the main reactants in the process of biodegradation. In order to study the interactions between enzyme and phenol at the molecular level, some simulation technology has been adopted. Nowadays, with the development of science and software technology, molecular docking approach has been used to analyse the interactions between substrates and enzymes, such as laccase with phenol [91].

CONCLUSION

Commercial enzymes have been useful for designing the basic principles of potential industrial processes, but the large-scale use of these preparations seems to be prohibitive because of their high costs. For this purpose, inexpensive fungal biomasses from solid-state cultures are more

attractive. The combination of fungal mycelia and enzymes with other physico-chemical or biological agents such as chemical oxidation and activated sludge are also promising for scale-up. The published studies also demonstrate the necessity of analysing the reaction products, assessing their potential toxicity and working out the processes for their efficient post-treatment removal.

During the past few years, researchers have focused on the removal efficiency of contaminants by controlling and adjusting reaction condition, less mentioned deeper reason such as the transformation of enzymes.

The immobilized system had high vitality and adaptivity into waste water from phenol production. In recent years different organic carriers for bacterial immobilization were investigated. Among the synthetic polymers, poly(ethylene oxide) hydrogels are excellent candidates because they are nontoxic biocompatible materials which meet all of the requirements of strength, absorbency, exhibility and adhesiveness. The immobilized cells showed better performance in the presence of heavy metals (Cu, Mn, Co, Ni, Zn) compared to that of the suspended cells.

FISH (fluorescence *in-situ* hybridization) analysis led to explain the increased effectiveness of xenobiotic detoxification. At concentrations of the toxicant exceeding the critical ones the microbial community was dependent to a larger extent on reserve sources of energy and adaptation took place mostly on the basis of an increase the number of culturable biodegraders.

Nanobiotechnologies are a rapidly growing field that offers new opportunities thus far unknown including regulation processes at a nano level. There remains the important issue of the impact of nanomodulators on biodegradation processes and their potential to optimize and regulate biodegradation of recalcitrant xenobiotics. Nanodiamonds are more applicable in the risk situation of shock loading of industrial waters with xenobiotic pollutants to overcome the initial high toxic effect of phenol and its derivatives.

The review [20] has mainly demonstrated that molecular docking is a promising method. Molecular docking was widely used in many research fields by virtue of its convenience and low cost. Especially, molecular docking is able to predict and account for the mechanism of biological reaction.

FUTURE RESEARCH

Over the years, our understanding of biodegradation has been improved significantly. All of the studies outlined above are significant for exploring efficient and sustainable biological processes for the clean up of pollutants. The optimization of the substrate concentration in phenol biodegradation is particularly important since it inhibits the growth of the organism at higher concentrations. Biotechnology for hazardous waste management involves the development of biological systems that catalyse the detoxification, degradation or decontamination of environmental pollutants.

It is important to understand the behaviour and ecotoxicity of phenols. Future studies should focus on four aspects as follows: firstly, research into the ecotoxicity of phenols to marine systems should be strengthened given the current lack of data and its irreplaceability by data pertaining to freshwater organisms. Secondly, it is necessary to isolate marine organisms that have the ability of degrading phenols of high solubility in water. Thirdly, joint toxicity of mixed chemicals needs more attention for the lack of data. Finally, the mechanisms of action of phenols on freshwater and marine organisms warrants further study. Sensitive biomarkers should be screened, as this is deemed conducive to improved ecotoxicological assessment and monitoring in aquatic systems.

More effort should be made to explore new microorganisms, enzymes, genes, and to elucidate new biodegradation pathways, mechanisms and applications of process engineering, as well as the effects of signal transduction. Investigation of bioenergy from wastes is at an early stage, and more attention should be paid to investigation of its mechanisms and to its field-scale application.

In summary, biodegradation is still attracting technology for environmental pollutant treatment; the successes of biodegradation will encourage us to further unveil these processes and to explore more efficient and cost-effective biodegradation processes.

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КЛАСИЧЕСКИ И НОВИ АСПЕКТИ В РАЗГРАЖДАНЕТО НА АРОМАТНИ КСЕНОБИОТИЦИ

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Резюме: Органичните химични съединения са широко разпространени в отпадъчните води от промишлеността, минералните извори, както и в замърсените подпочвени води. Фенолите се срещат в петролните рафинерии и химическите заводи, при производството на взривни вещества, смоли и кокс, при преработката на въглища, пестициди и текстилна индустрия. Основните замърсители в отпадъчните води на рафинериите включват феноли, полициклични ароматни въглеводороди, както и тежки метали. Сред тези токсични замърсители, фенолите са считани за най-опасни и са доста трудни за отстраняване. Тези съединения са токсични при относително ниски концентрации. Разграждането им се осъществява в резултат от метаболитната активност на голяма група микроорганизми като бактерии, гъби и дрожди. Съществуват съобщения за много микроорганизми, способни да разграждат фенол благодарение на активността на множество ензими. Тези ензими включват: оксигенази, хидроксилази, пероксидази, тирозинази и оксидази. Експерименти с подхранване са провеждани в различни биореактори за осъществяване на биоразграждането на фенола. При отстраняването на органични замърсители се използват чисти култури, но това може да доведе до създаването на токсични междинни съединения. Този проблем може да се преодолее при използването на консорциум от микроорганизми, които имат широк спектър от метаболитни свойства. В този обзор ние описваме също така и някои нови технологии за биоразграждане на фенол като: различни видове имобилизации, флуоресцентен *in situ* хибридизационен анализ, нанобиотехнологии и др.

Ключови думи: фенол, биоразграждане, микроорганизми, ензими, имобилизации, флуоресцентен *in situ* хибридизационен анализ, нанобиотехнологии

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