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POSSIBILITIES OF BIODEGRADATION OF PHENOL-CONTAINING WASTES ORIGINATING FROM THE OIL-SHALE BASIN IN NORTHEASTERN ESTONIA

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Abstract. The ecological situation of Northeastern Estonia was studied using experimental and literature data. The most critical issues are the waste waters from chemical plants that contain phenols and other xenobiotical compounds, especially the waste waters from the regional waste water treatment plant of the AS Kiviter with insufficient and variable purification rate, and the waters from the ash mounds of the chemical industry with a very high content of mineral and organic pollutants which are discharged into the environment without any treatment.

Methods of increasing the efficiency of bioremediation processes, especially under sharp changes in pollution loads were examined. Possibilities of the bioremediation of polluted sites were tested. The microflora in the polluted water streams and soil was examined to create a collection of microorganisms adapted to the conditions of the region. The growth parameters of the isolated strains were studied using various substrates (among them xenobiotical compounds), temperatures, and concentrations of the carbon source. The feasibility of the purification of ash waters and polluted soil using different technologies (immobilized cell bioreactors, solid state fermentations, etc.) was analysed.

Key words: oil-shale phenols, ecology, biodegradation, Northeastern Estonia.

INTRODUCTION

The ecological situation of Northeastern Estonia was studied using our own experimental data and data from literature [1–8]. Serious environmental problems are associated with the waste waters from chemical plants containing phenols and other xenobiotical compounds, especially the waste waters from the regional waste water treatment plant of the AS Kiviter with insufficient and variable purification rates and the waters from the ash mounds of the chemical industry with a very high content of mineral and organic pollutants which are led into the environment without any treatment.

The Regional Waste Water Treatment Plant discharges annually 16 million tons of partially cleaned waters containing various phenolic compounds (mostly resorcinols), polyaromatic hydrocarbons (residual

content 7–10 µg/l), etc. into the environment. The annual emission of phenols is 550 tons. Every year 3 million tons of waste waters from the ash mounds with a very high content of mineral and organic pollutants and high pH values (11–12) pollute the Purtse River and the Gulf of Finland. They contain various (more than ten different compounds) phenols (400–500 mg/l), polyaromatic hydrocarbons (52 µg/l), and other contaminants [6]. The purification of ash waters is complicated because of their complex chemical composition and high content of contaminants. So, they are still directed into the environment without any treatment. Various studies carried out with the aim to elaborate methods of biopurification of ash waters have shown that their biodegradation requires dilution with municipal waste water or addition of huge amounts of easily degradable compounds whereas the content of water from the ash mound cannot exceed 10–30% [1, 6, 8].

The key problems complicating the waste water treatment in the region are the variability in the pollution loads both in the water purification plant and in the outflow of the phenol-containing waste waters from the ash mounds due to the meteorological conditions (precipitation amounts etc., which cause changes in the quantities of polluted water) and because of the industrial faults (disorders in the functioning of the dephenolizing plant and separator of resins at Kohtla-Järve Chemical Plant, which lead to the extermination of the activated sludge process in the aeration tanks of the Regional Waste Water Treatment Plant).

To solve the ecological problems described efficient and reliable chemical and biological water treatment technologies should be developed. It has been shown that most of the tested bacteria with phenol-degrading capacity are able to utilize phenols in concentrations not higher than 200 mg/l. Strains with especially good biodegradable properties can degrade phenol concentrations of about 500–1000 mg/l [9, 10]. Successful biodegradation of phenolic wastes from the coal industry containing up to 1000 mg/l and even more phenols has been reported [11, 12]. However, because of the different chemical composition of the oil-shale waters the treatment effects did not coincide.

The aim of this study was to examine the methods that should help increase the efficiency of bioremediation processes, especially in the conditions of sharp changes of pollution loads. Possibilities of the bioremediation of the polluted waste waters in the case of Northeastern Estonia were also tested.

The microflora in the polluted water streams and soil was examined; a collection of microorganisms, adapted to the conditions of the region was created; and the growth parameters of the isolated strains were studied using various substrates (among them xenobiotical compounds), temperatures, and concentrations of the carbon source. The feasibility of the purification of the waste waters from the ash mounds and polluted soil using different technologies (immobilized cell bioreactors, solid state fermentations, etc.) was analysed.

EXPERIMENTAL

1. Study of consortia of microbes inhabiting the polluted sites

The microbiological monitoring of consortia was carried out using the plate count method (spread plate and pour plate techniques) [13–15] and various rich and selective media (meat peptone agar, malt agar, starch ammonium agar; mineral media based on the solution M9 with yeast extract and sodium acetate as a source of carbon for the determination of general microflora; mineral medium M9 with the addition of phenol, resorcinol, or the ash water in concentrations of 2.5 mM and the same media with yeast extract for the assessment of xenobiotics-degrading microorganisms).

Study of consortia of microbes in the aeration tank of the regional waste water treatment plant

The composition of consortia in aeration tanks varies depending on the season but also on the stage of the treatment process. The number of general microflora in the samples, taken at different times, was similar (10^9–10^{10}) but the composition of consortia (moulds predominated in autumn 1993) and the content of the xenobiotics-degrading microflora varied depending mainly on the working state of the plant (10^4–10^6–10^8). This caused also large variability in treatment effects. Aerobic strains were predominant in the samples.

"Ash water" and polluted soil

The cell count and the composition of the consortia in "ash water" and polluted soil samples varied depending on the season of the year (in winter the growth on the plates was very low) and due to the environmental conditions (moulds predominated in autumn 1993 after a cold and rainy summer, probably only spore-forming organisms were able to survive in the polluted water because of hard environmental conditions). The number of outgrown microorganisms from the samples taken in summer and in early autumn was found to be 10^4–10^5 (general microflora) and up to 10 (xenobiotical strains) cells/ml in water samples or cells/g in soil samples. Aerobic microorganisms (mostly psychrophilic strains of pseudomonads) were predominating in the consortia.

The microbial consortia formed in the ash water and polluted soil samples are too weak to ensure self-cleaning of the polluted water. This makes the development of a technology for the treatment of ash water and polluted soil necessary.

2. Identification of microorganisms isolated

The identification of microorganisms was carried out using the Gram reaction and various morphophysiological tests, e.g. the microscopic study of the shape and motility, examination of growth on different selective

media (King's media A and B, Winogradsky's nitrite medium, etc.) as described in the literature [13, 14, 16–18].

More than twenty strains found from the samples of polluted water and soil were isolated into pure cultures and examined. The strains isolated from the oil-shale waste waters and polluted soil samples were determined to belong to the genera *Rhodococcus* and *Pseudomonas*. Strains of *Pseudomonas aeruginosa* were also identified.

3. Determination of the growth characteristics of selected strains

The **examination of the growth rate** was carried out using the streak plate method and a mineral medium based on the solution M9 with various (more than 20) sources of carbon (carbohydrates, hydrocarbons, phenolic compounds, pesticides, etc. in 2.5 mM concentration). A mesophilic strain of *Rhodococcus* sp. found was able to degrade a wide range of xenobiotics (various phenolic compounds, hydrocarbons, pesticides, etc.).

The **growth rates of mesophilic strains** on the phenol-containing medium (0.48 $g \cdot l^{-1}$) in batch cultures were as follows:

Pseudomonas aeruginosa	0.2–0.7 h^{-1}
Rhodococcus sp.	0.5 h^{-1}
Pseudomonas sp.	0.53 h^{-1}

No growth of the microorganisms was observed on substrates of higher concentrations, except *Rhodococcus* sp., which was able to grow on the substrate containing 2.5 $g \cdot l^{-1}$ phenols (growth rate 0.14 h^{-1}).

The **determination of the growth characteristics of isolated microorganisms at various temperatures** (+4 °C in refrigerator, +30 °C, and +40 °C in thermostat) showed that pseudomonads were able to grow both at low (+4 °C) and high (+40 °C) temperatures. The other isolated mesophilic strains had very slow growth both at low and high temperatures while psychrophilic strains did not grow at +40 °C.

The **quantitative growth characteristics** of the mesophilic strain of *Rhodococcus* sp. isolated from oil-shale waste waters were determined **using the A-stat method** of cultivation (computer-controlled continuous cultivation with a smooth increase in the dilution rate, i.e. the acceleratostat) [19–24]. The A-stat cultivation experiments were carried out using different accelerations of the dilution rate: 0.025 h^{-2}, 0.01 h^{-2} (commonly used in the work with *Escherichia coli* and pseudomonads) [19–24], and 0.0025 h^{-2}. A solution of M9 with phenol as a source of carbon was used as the reaction medium. The initial concentration of the substrate by the pre-growth in batch culture was 0.48 $g \cdot l^{-1}$ and in continuous cultivation 7.6–7.7 $g \cdot l^{-1}$ (the residual concentration in chemostat conditions was 0%). The strain was able to grow at relatively low dilution and acceleration rates and the culture was very sensitive to the changes in conditions (possible changes in pollution loads etc. in

purification plants). The study of these peculitarities is continued currently.

4. Solid state fermentation

The aim of the experiments was to develop a continuous solid state fermentation system. The preliminary study was carried out in a batch culture with the mesophilic strain of *Rhodococcus* sp. in a thermostat reactor with a mechanical stirrer. Washed and sterilized sand was impregnated with a solution of sodium benzoate and the mineral medium M9. The moisture content, the residual concentration of the substrate, and the count of living cells obtained with the plate count method and luciferin-luciferase method [25] were monitored. The reaction temperature was 30 °C, pH was 6.9±0.2, the initial content of moisture was 11.2%, and the initial content of the substrate in the film of moisture covering the particles of the sand was 20 mM.

It was observed that the substrate was intensively utilized in the beginning of the cultivation: the content of sodium benzoate decreased 3.7 times during 24 h and all of the substrate was utilized in 2 days. However, the experiments also showed that the technique of the experiment needed essential improvement.

5. Immobilization

It is known from the literature that immobilization increases the stability of microorganisms to shock loads [26–28]. Immobilized microorganisms are able to degrade phenol in batch cultivation experiments in concentrations up to 4 $g \cdot l^{-1}$ (sintered glass system) and even 17 $g \cdot l^{-1}$ (activated carbon system) [29]. Our experiments were carried out with cells of *Pseudomonas putida* (*P. putida* PaW 85 – the biodegradation of benzoate with PVA-cryogel, or the plasmid strain of *P. putida* – the degradation of phenol with preparations immobilized by the adsorption method). The reaction temperature was 30 °C and pH was 6.8–7.0. For the cultivation of microorganisms the mineral salt medium M9 and some source of carbon (2.5 mM sodium salt of benzoic acid or phenol whose concentration varied in different experiments from 2.5 to 10 mM) were used. The inoculum was pregrown in the medium M9 containing glucose and microelements.

Analyses

The **pO_2** and **pH** values were measured continuously using oxygen- and pH-electrodes. The **concentrations** of phenol and Na-benzoate were determined quantitatively by a spectrophotometrical method using Uvicord II on $\lambda = 280$ nm (continuous determination) or Specord UV-VIS (Germany) on $\lambda = 230$ nm (Na-benzoate) or $\lambda = 270$ nm (phenol) after every 1–3 h. The samples were dissolved using distilled water. The suspended **biomass** was measured by dry weight using the centrifugation method (daily), and through measuring the optical density at $\lambda = 540$ nm

using a photoelectric colorimeter KFK-2MP after every 1–3 h. The **number of viable cells** in the reactor was measured using the plate count method.

Results

The **selection of a carrier** usable in bioremediation processes

Porous rubber with a density similar to that of water was found to be suitable in fluidized-bed bioreactors. Using pieces of porous rubber as a carrier, a homogenous distribution of the particles of the carrier in the reactor was achieved. The carrier was mechanically stable, easily regenerated, and with a sufficient catalytic activity.

The carriers heavier (sand, coke) or lighter (various kinds of porous plastics) than porous rubber did not mix properly in the examined types of fluidized bed reactors. Their use required an improvement of the construction of the reactor or additional energy expenditure for the mechanical stirring of the reaction mixture and the particles of the carrier.

The particles of PVA-cryogel with quite a good initial catalytic activity (the specific utilization rate of the substrate per unit of carrier weight at a dilution rate D=0.5 was 70 $mM \cdot ml \cdot g^{-1}$ and the residual concentration of the substrate was low, whereas the specific utilization rate of the substrate for the porous rubber at the same dilution rate was 6 $mM \cdot ml \cdot g^{-1}$ and the steady state residual concentration of the substrate was 55%), however, decomposed after some days of incubation.

The growth characteristics of the carriers (PVA-cryogel and porous rubber) were studied under different **dilution loads**. The experiments carried out in chemostat conditions in a fluidized bed bioreactor showed that under low dilution loads both free and immobilized cells were utilizing the substrate, but at higher dilution rates mainly immobilized cells were responsible for the utilization of the substrate. In comparison with suspended cells a higher biodegradation step was shown with immobilized cells at the same dilution rates.

The residual concentration of the substrate increased rapidly with increasing dilution rate (Table).

Steady state residues of the substrate (%) for various substrate dilution rates

Carrier	Dilution rate, h^{-1}					
	0.2	0.5	0.7	1.0	1.5	2.0
PVA-cryogel	-	0	50	65	-	74
Porous rubber	0	55	57	71	78	82

However, the relative utilization rate of the substrate per unit of carrier remained at the same level, showing that the immobilized cells were utilizing xenobiotics also at high concentrations. In case porous rubber

was used the following **kinetic parameters of phenol degradation**, calculated from the steady-state conditions, were obtained: monod constant $Ks = 519\ mg \cdot l^{-1}$; steady-state substrate concentration in the culture liquid Sst = 5.52 mM and $\mu m = 0.28\ h^{-1}$.

The **examination of various types of fluidized-bed bioreactors** filled with porous rubber showed that the **fluidized-bed column** with 5×5×5 mm particles of carrier created working conditions

– with a homogenous distribution of the particles of the carrier,
– with a good reliability: the column was working for months, and
– with a low residual concentration of the substrate (0% at $D = 1.5\ h^{-1}$).

By the use of the **fluidized-bed reactor with thin filling** the residual concentration of the substrate is higher (78%) due to the relatively low concentration of immobilized preparation. The utilization rate of the substrate per unit of carrier was, however, similar in both cases. The **column** filled **with small particles of carrier** had a low reliability: the flow was clogged after some days of incubation.

Abrupt changes in the concentrations of the substrate were studied with the aim to establish the sensitivity of the cells immobilized onto porous rubber to the fluctuations in pollution loads. The experiments were carried out in a continuous culture. The investigations showed that the consumption of the substrate increased in the case of relatively slight fluctuations (from 2.5 to 5.0 mM). Successive fluctuations from 5.0 (residual concentration 35%) to 10 mM (65%) and anew to 5.0 mM (the residue of substrate 75%) caused stress and growth inhibition.

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KIRDE-EESTI FENOOLE SISALDAVATE HEITMETE MIKROBIOLOOGILISE LAGUNDAMISE VÕIMALUSTEST

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Kirjanduse põhjal ja *in situ* on uuritud Kirde-Eesti ökoloogilist olukorda. Ilmneb, et üks olulisemaid valupunkte on fenoole ja muid ksenobiootilisi ühendeid sisaldavad keemiatööstuse heitmed, eeskätt ebapiisava ja varieeruva puhastusastmega heitveed AS Kiviter'ile kuuluvast rajooni puhastusseadmest ning tuhamägede nõrgveed, mille puhastamine on nende kõrge ksenobiootikumide sisalduse tõttu komplitseeritud ja mis juhitakse looduslikesse veekogudesse ilma mingi töötluseta.

Põlevkivikeemiatööstuse heitvete puhastamisel on põhiprobleem reostuskoormuse suur kõikumine nii puhastis kui ka tuhamägede vees. Kõikumine on tingitud meteoroloogilistest oludest (puhastatava vee kogus muutub vastavalt sademevee hulgale) või tööstuslikest möödalaskmistest (defenolaatorist ja vaigueraldusseadmest väljuva vee puhastusastme kõikumised kahjustavad puhastis tekkinud aktiivmuda). Kujunenud ökoloogiliste probleemide lahendamine nõuab tõhusate ja töökindlate keemiliste ning bioloogiliste puhastusprotsesside väljakujundamist.

Käesoleva uurimistöö eesmärk on bioremediatsiooniprotsesside efektiivsuse suurendamise meetodite väljatöötamine, eeskätt reostuskoormuste ulatuslike ja järskude muutuste puhul; samuti saastekollete bioremediatsiooni võimaluste hindamine.

Vastava piirkonna tingimustega kohanenud mikroorganismidest koosneva kollektsiooni loomiseks uuriti saastunud vete ja pinnase mikrobioloogilist kooslust. Suurema kasvukiiruse või psührofiilsuse alusel eraldatud tüvede kasvuparameetrid määrati eri (sh. ksenobiootilistel) substraatidel, temperatuuridel ning ksenobiootikumi kontsentratsioonidel.

Põlevkivifenoolidega saastunud vete ja pinnase bioremediatsiooni tõhustamise võimaluste uurimiseks kasutati mitmesuguseid meetodeid (immobiliseeritud rakupreparaadid, erinevad reaktoritüübid, tahkefaasiline fermentatsioon jne.).

ВОЗМОЖНОСТИ БИОЛОГИЧЕСКОЙ УТИЛИЗАЦИИ ФЕНОЛСОДЕРЖАЩИХ СТОЧНЫХ ВОД В СЕВЕРО-ВОСТОЧНОЙ ЭСТОНИИ

Тийна РАНДЛА, Алексей РОЖКОВ, Арво КЯЭРД, Райво ВИЛУ

Одной из самых болевых точек в экологической обстановке в Северо-Восточной Эстонии являются фенолсодержащие сточные воды Кохтла-Ярвеского химкомбината и дренажные воды с отвалов сланцевого полукокса, которые отводятся в р. Пуртсе и Финский залив без всякой очистки.

Основной проблемой при биологической очистке фенолсодержащих сточных вод Кохтла-Ярвеского химкомбината являются большие колебания объемов сточных вод и концентраций содержащихся в ней ксенобиотических веществ, вызванные нарушениями работы дефеноляторов. Токсическое действие больших концентраций фенолов вызывает субстратное ингибирование роста активного ила и нарушение работы очистных сооружений.

Исходя из этого перед нами стояли две задачи: выработать методы повышения эффективности работы очистных сооружеий, прежде всего в условиях резких изменений объемов и концентраций загрязняющих веществ, а также оценить возможности применения биологических методов очистки других источников загрязнения.. Из загрязненных ксенобиотиками воды и почвы были выделены микроорганизмы с высокой способностью к биодеградации. Исследовали состав бактериальных сообществ. У микроорганизмов, обладающих наиболее высокой деградационной активностью, определяли параметры роста на различных субстратах и в различных условиях. Исследовали методы повышения эффективности работы очистных сооружений (иммобилизацию бактерий на носителе, различные типы аэротенков и др.).