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Enzymatic Activities in Petroleum Wastewater Purification System by an Activated Sludge Process

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Abstract The enzymology of an activated sludge system for a petroleum wastewater purification process was investigated. Leucine-aminopeptidase (L-AMP), β-glucosidase (β-GLC), and lipase (LIP) were selected for the study. It was found that more than 81.7% of enzymatic activity was associated with microbial cells in the activated sludge floc. The metabolic response of a mixed microbial population to increased phenol concentration showed that L-AMP activity increased in the activated sludge, whereas activities of β-GLC and LIP decreased, due to the inhibitory effect of the phenol which varied from 100 mg/l to 500 mg/l.

Key words: Enzymatic activity, activated sludge, phenol, petroleum wastewater

Petroleum refining wastewaters have been an important environmental problem with growing yield and variety in the petroleum industry [2, 4, 11, 13]. The main constituents of aqueous effluents during the process of refining are confirmed to be phenol and its derivatives, which are toxic to aquatic life when more than 50 ppb of phenol are present in the water, and are fatal to human if 1 g of phenol is ingested into the body [8, 15]. Effluents containing such compounds are usually treated in an activated sludge process, where complex organic pollutants are removed by a mixed population of microorganisms [12].

Traditional methods to investigate the activity of activated sludge, such as mixed liquor suspended solids (MLSS), biochemical oxygen demand (BOD), and chemical oxygen demand (COD), are shown to be practical, but do not give much insight into the factors that control the proper biological process [10]. It has been found that the enzyme activities reflect microbial activities in wastewater and activated sludge. The enzymological approach has received particular attention in characterizing the system of biological wastewater treatment [3, 6, 9].

In this study, leucine-aminopeptidase (L-AMP), β-glucosidase (β-GLC), and lipase (LIP) were selected, since proteins, polysaccharides, and lipids are the major organic matters in a complex wastewater [7, 14]. L-AMP is an exopeptidase that hydrolyzes the peptide bond adjacent to a free amino group in a protein molecular, β-GLC breaks the β-1,2 glucosidic linkage in disaccharides to release glucose, and LIP is a carboxylesterase that is responsible for degradation of triglycerides. The objectives of this study were to investigate the microenvironment of the enzymes in the petroleum wastewater purification system, establish whether they were immobilized in activated sludge flocs or dissolved in bulk solutions, and study the impact of phenol concentration on the activities of the selected enzymes. In addition, a relationship between dissolved organic carbon (DOC) and activities of studied enzymes was also investigated.

Materials and Methods

Reactor Set-Up

A lab-scale reactor, consisting of a 2,500 cm^3 aeration tank and a 590 cm^3 sedimentary chamber, was operated under an ambient condition. The seed sludge, obtained from Orlen Petroleum Company in Płock, Poland, had a MLSS of 2–4 g/l and settleability (30 min) of 200–400 ml/l, and was continuously grown in the model reactor of activated sludge wastewater purification system in the laboratory. The initial feed at the start-up phase had the ratio of 1:5 (seed sludge:simulated wastewater). The flow of influent was maintained at 4 l/d by peristaltic pump, with the hydraulic retention time of 6–8 h. The dissolved oxygen in the aeration tank was controlled at 2–3 mg/l. The components

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of simulated wastewater were as follows: 10 ml of phenol (50 g/l) were added to 4 l real wastewater from the petroleum plant to reach a COD load between 1,500 mg/l and 2,000 mg/l. Phenol in the continuously fed reactor ranged normally from 100 to 120 mg/l. After the reactor reached a steady state (COD concentration in the treated effluent was less than 100 mg/l and no phenol could be detected in the effluent), the mixed liquor of wastewater and activated sludge from the aeration tank and effluent from the sedimentation chamber were sampled for enzyme assays.

Enzyme Assays

The enzymatic activities in activated sludge and effluent were measured spectrophotometrically (Shimadzu UV-1202, Japan) in 5-cm cuvettes. Working solutions (0.5 ml) of substrates were thoroughly mixed with 2.5 ml of the samples studied by using a vortex mixer (UMH-5, Wigo) and incubated in dark at 25°C for a desired time interval (h). Activated sludge samples in the reaction tubes were centrifuged at 6,000 rpm for 15 min (Hettich centrifuge, Germany). Absorbance of enzyme-produced chromophore in the supernatants and reaction effluent samples was measured for enzyme activities. Control samples were assessed immediately after substrate addition. For the activated sludge sample, blanks were prepared by adding substrate to centrifuged supernatant just before reading of absorbance. All tests and controls were carried out in triplicate.

Enzyme activity assays were based on the release of a chromophore (p-nitrophenyl or p-nitroaniline) after the hydrolysis of the following synthetic substrates: L-leucine-p-nitroanilide (Sigma L9125), p-nitrophenyl-β-D-glucopyranoside (Sigma N7006), and p-nitrophenyl-palmitate (Sigma N2752). The absorbance of p-nitroaniline (λ_{max}=380 nm) and p-nitrophenol (λ_{max}=415 nm) was measured after 4–24 h of incubation for activated sludge samples and 24–48 h for effluents (Table 1). Stock solutions (30 mM) were prepared in 96% ethanol, and working solutions of enzyme substrates (except p-nitrophenyl-palmitate) were prepared by dilution with deionized sterile water. Lipase substrate stock solution (10 mM) and working solutions were prepared in 2-propanol.

Enzyme activity (EA) was calculated from absorbance of samples and controls at 380 nm or 415 nm. To quantify the amount of hydrolysis product in the enzyme reaction, calibration curves with known amounts of p-nitrophenol or p-nitroaniline were prepared. Hence, 1 EA unit was defined as the amount of enzyme to produce 1 μmol/l product in one hour, i.e., μmol/l/h.

Other Analyses

MLSS of activated sludge and COD of wastewater were analyzed according to Standard Methods [1]. DOC was determined with a Shimadzu TOC 5050 organic carbon analyzer.

RESULTS AND DISCUSSION

Distribution of Enzyme Activities Studied

Enzyme assays were carried out to define the distribution of the enzyme activities studied, using the samples of untreated activated sludge to measure the total enzyme activities associated with microbial cells+extracellular (cell free) enzymes. The effluent of the reactor (purified wastes) was used to measure free dissolved extracellular enzyme activity. High enzyme activities were found in the petroleum wastewater purification reactor (Fig. 1). The average activities of L-AMP and β-GLC in the activated sludge were 27.8 μmol/l/h and 32.9 μmol/l/h, respectively. LIP activity was outstandingly highest among the enzymes selected, and more than 79.6% higher than the other enzymes. These results are coincident with the composition of organic constituents in petroleum wastewater; i.e., more lipid and less protein and carbohydrate are available for microorganisms in their environment. In contrast to the overall activities, only 4.3 μmol/l/h of proteolytic enzyme (L-AMP) and 3.7 μmol/l/h of glycolytic enzyme (β-GLC) activities were found in the cell-free extracellular fraction. Similarly, lipolytic enzyme (LIP) activity accounted for 18.3% of the total in the purified waste samples.

The ratio of different enzymatic activities between activated sludge and bulk solutions reveals the location of these enzymes and participation to the overall activity. An 81.7%–88.7% of the extracellar enzymes studied were found to be associated with microbial cells or immobilized in the extracellular polymer matrix of the flocs in the petroleum wastewater system. This immobilization of enzymes seemed to be an advantage for microorganisms to obtain energy and nutrient from organic matter. The extracellular matrix of activated sludge flocs contributed to

Table 1. Enzyme assay.

Enzyme	Substrate	Product of reaction	Incubation time (h)		Termination
			Activated sludge	Effluent	
Leucine aminopeptidase (L-AMP)	L-Leucine-p-nitroanilide	p-Nitroaniline	4	24	3.0 ml 0.1 M NaOH
β-Glucosidase (β-GLC)	p-Nitrophenyl-β-D-glucopyranoside	p-Nitroaniline	4	48	3.0 ml 0.1 M NaOH
Lipase (LIP)	p-Nitrophenyl-palmitate	p-Nitroaniline	24	24	3.0 ml 96% ethanol

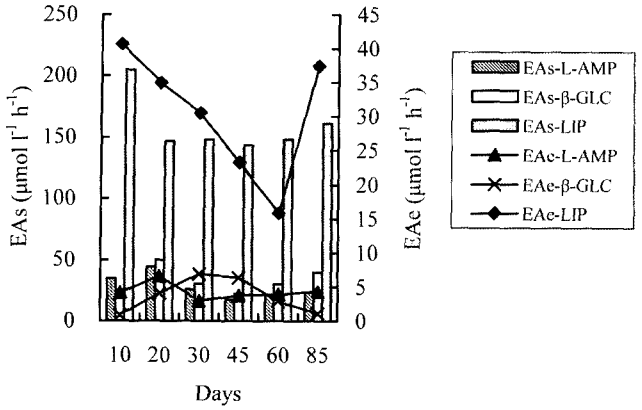

Fig. 1. Distribution of extracellular enzyme activities.
Abbreviations: EAs, enzymatic activity in activated sludge; EAe, enzymatic activity in effluent.

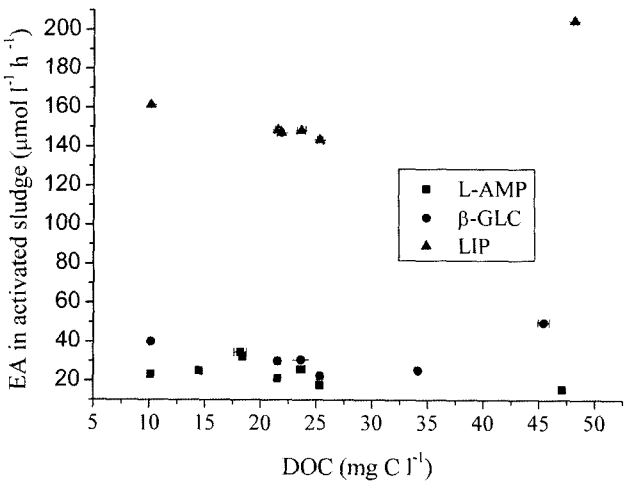

Fig. 2. Relationship between the enzyme activities selected and DOC concentrations in activated sludge.

the retention, accumulation, and stabilization of the associated enzymes, in support of the reports by Cadoret *et al.* [5] and Wingender *et al.* [17].

The changes of specific enzyme activities of L-AMP, β-GLC, and LIP over a 90-day experimental period are shown in Fig. 1. Although an attempt was made to maintain a stable operation, and samples were identically taken from the center of the reactor for the routine test, changes of enzyme activities were found in the reactor, indicating that the enzyme activities tend to be influenced by environmental conditions, such as fresh feed, temperature, and concentration of microorganisms. The decrease of enzyme activity observed from days 45 to 60 could be due to the fact that 100 ml of activated sludge was taken away from the reactor, resulting in the decreases of microbial concentration and enzyme synthesis. The investigation on the chemical, biological, and operating effects on enzyme activities would be worth future research.

In all cases, the activity of each enzyme of microorganisms tested was further characterized by measuring the hydrolysis rate of various substrates in different wastewater reactors. It should be noted that controls (blank samples) constituted the enzyme activity not because of the interaction of substrate with the assay components, but because of an effect on the enzyme activity.

Enzymatic Activities and DOC

DOC concentrations were measured to find out the relationship between the changes of each enzymatic activity tested and the increase of DOC concentration. Figure 2 shows no significant relationships between them. The observed DOC concentration varied from 10.2 mg/l to 47.1 mg/l in the activated sludge, and between 16.0 mg/l and 41.8 mg/l in the effluent. Similar DOC concentration was found in the effluent and activated sludge, although the majority of enzymes were immobilized in the activated sludge matrix. Lower DOC concentration was found in the reactor, indicating that complex organic compounds could be degraded by extracellular enzymes, if there was moderate level of loading rather than high or saturated shock load in the system. The change of DOC concentration implied that the performance of biodegradability of wastewater could be improved by the effect of enzymes secreted from microbes.

The Effect of Phenol on the Selected Enzyme Activities

The effect of phenol on the enzyme activities was studied by inoculation of samples with phenol in the reaction, ranging from 0 to 500 mg/l. Incubation was started by the addition of substrate, whose final concentration in the assay was 50 μmol/l.

L-AMP activity increased with the increase of phenol concentration in activated sludge; however, the opposite tendency was found in the effluent samples (Fig. 3). A 39.8% increase of L-AMP activity occurred after the

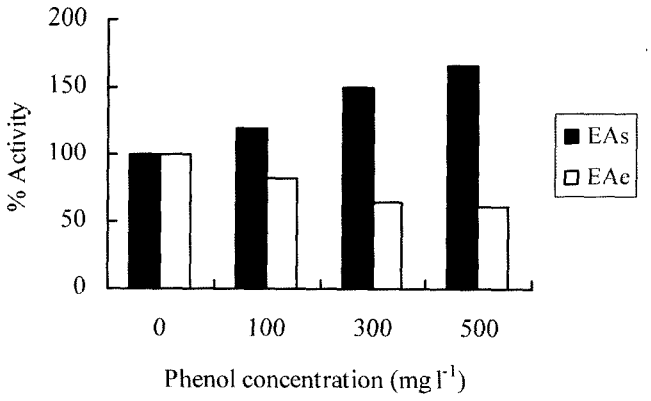

Fig. 3. Effect of phenol on L-AMP activity.
Abbreviations are as in Fig. 1.

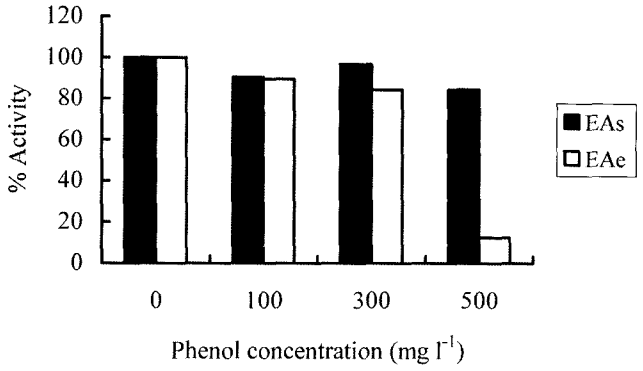

Fig. 4. Effect of phenol on β-GLC activity.
Abbreviations are as in Fig. 1.

addition of 500 mg/l phenol in the activated sludge floc, suggesting that "added" phenol may damage or even destroy the structure of the protein molecular network. When more substrate was exposed to microorganisms, enhanced activity of proteolytic enzyme resulted, similar to the mechanism found in literature [16].

Changes of β-GLC and LIP activities by changing phenol concentration are shown in Fig. 4 and Fig. 5. Both enzyme activities decreased, when phenol concentration increased from 100 mg/l to 500 mg/l. However, very little change of enzymatic activities was observed in the activated sludge samples by the change of different phenol concentration. A concomitant drop in the effluent was noticed, especially for β-GLC: Only 12.6% of glycolytic enzyme activity remained in the bulk solution at phenol concentration of 500 mg/l. Lower activities of β-GLC and LIP could most likely be explained by the fact that high phenol concentration inhibited these two extracellular enzyme activities in the petroleum wastewater purification system.

In conclusion, the major fractions of L-AMP, β-GLC, and LIP activities were found associated with microbial cells in the activated sludge matrix flocs, and LIP activity was the highest in the purification system. The DOC assay showed that these enzymatic activities displayed an irregular change. An increase of L-AMP activity was found in the activated sludge sample, when phenol concentration was increased. Conversely, "added" phenol decreased the β-GLC and LIP activities owing to inhibition.

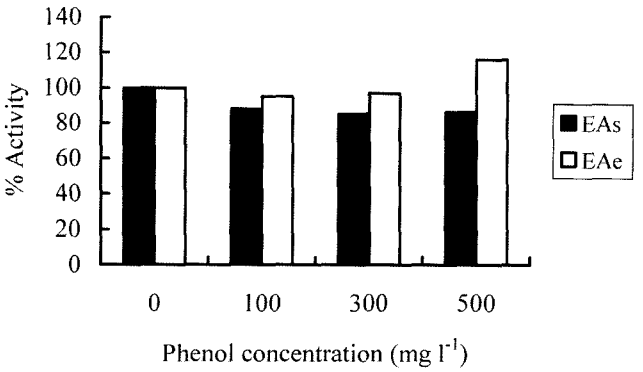

Fig. 5. Effect of phenol on LIP activity.
Abbreviations are as in Fig. 1.

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