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ENZYMATIC ACTIVITY STUDIES OF BIOLOGICAL WASTEWATER TREATMENT

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ABSTRACT. Biological wastewater treatment is the most common technology for municipal wastewater purification, where different consortia of microorganisms aggregated in sludge flocks degrade organic matter in wastewater; activity and distribution of enzymes in flocks reflects microbial activities. A lab-scale sequencing batch reactor (SBR) was built and used for the treatment of synthetic wastewater to investigate the activity and distribution of hydrolytic enzymes (α-amylase, α-glycosidase and alkaline phosphatase) in sludge flocks. The enzymatic activity parameters of the enzymes were analyzed in function of chemical oxygen demand values of samples to determine the activity and distribution in the course of the whole process, aiming in the future to a better understanding of biological processes that occur during wastewater treatment.

Keywords: extracellular enzymes, enzyme activity, enzyme distribution, sequencing batch reactor, activated sludge flocs

INTRODUCTION

The activated sludge treatment is today's most popular type of biological wastewater treatment. In its over 100 years of history, the initial aerobic oxidation process, developed for organic carbon removal, and has been completed with other biological nutrient removal processes to meet the more and more severe emission limits and to deal with the increasing magnitude and complexity of wastewater loads. The success of the activated-sludge process is dependent upon establishing a mixed community of microorganisms that will remove and consume organic waste material, that will aggregate and adhere in a process known as bioflocculation, and that will settle in such a manner as to produce a concentrated sludge for recycling. Organic matter carried by urban wastewater is a complex mixture of single carbohydrates, amino acids, alcohols and volatile fatty acids mixed with polymers and heteropolymers including proteins (1/3 of chemical oxygen demand, COD),

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polysaccharides (1/5 of COD), and lipids (1/3 of COD) [1]. A large fraction of this wastewater organic matter (30–85%) consists of particles larger than 0.1 μm [2] preventing them from being directly assimilated by microorganisms. In most of the cases only monomers and oligomers (MW < 1000) are able to cross the bacterial membrane via specific active transports [3]. For example, in seawater only small peptides (MW < 600–700) are imported directly into the cell [4]. It is well known that before bacteria can assimilate high molecular weight compounds, the compounds are usually hydrolysed by extracellular enzymes. These extracellular enzymes are either bound to cell surface (ecto-enzymes) or released into the medium (exo-enzymes) in the free form [5] prior to form complexes with high molecular weight substances [6]. Enzymes like α-amylase, α-glycosidase and alkaline-phosphatase play essential role in the biological wastewater treatment processes. The amount of extracellular enzymes in the bulk solution of activated sludge is negligible, indicating that almost all extracellular enzymes are immobilized in flocks [7]. The contact probability of enzymes whit proteins and polysaccharides is determined by the distribution of enzymes in the sludge flocks [8].

Microbial cells can produce extracellular polymeric substances (EPS) which lead to floc formation by agglomeration of bacteria. In other words, these exopolymers are responsible for increased bridging flocculation that helps create good settling [9]. Enzymatic activities distributions were studied in the EPS sludge matrix by activity measurement. EPS in sludge flocs were characterised as described by Guang-Hui *et al*. 2007 [10], based on their properties to exhibit a dynamic double-layer structure, composed of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) [10]. The LB-EPS fraction is considered to easily exchange substances with the bulk solution, having greater impact to numerous sludge processes like coagulation and dewatering [10, 11]. The goal of this paper is to investigate extracellular enzymatic activities and distribution in activated sludge samples collected during the aerobic period from a lab-scale sequencing batch reactor (SBR). To the author's best knowledge, no work has been done to characterise enzymatic activities and distribution on a lab-scale sequencing batch reactor in three different samples taken every hour of the 3 hour aeration phase for each enzyme to characterises enzymatic activities and distribution in the course of the whole process period.

RESULTS AND DISCUSSION

Samples taken from the lab-scale SBR reactor in the course of the whole process period, at the beginning, in the middle and in the end of the aerobe phase were met to investigate extracellular enzyme activity and distribution in activated sludge flocs in the entirely whole process. Figure and Tables 1, 2, 3 presents enzyme activities and distribution of each studied enzyme in every sample period and fraction of the sludge floc.

Table 1. Alpha-amylase activities

COD [mg/L]	120		72.8		32	
Fraction	EA [u/ml]	Stand. Dev	EA [u/ml]	Stand. Dev	EA [u/ml]	Stand. Dev
LB-EPS	0.49	±0.13	1.25	±0.15	1.26	±0.51
TB-EPS	0.61	±0.17	1.25	±0.077	0.71	±0.26
Pellets	0.15	±0.09	0.01	±0.009	0.19	±0.06
Total	1.2533857		2.508418		2.155	
Efficiency %	**60**		**75.73333**		**89.33333**	

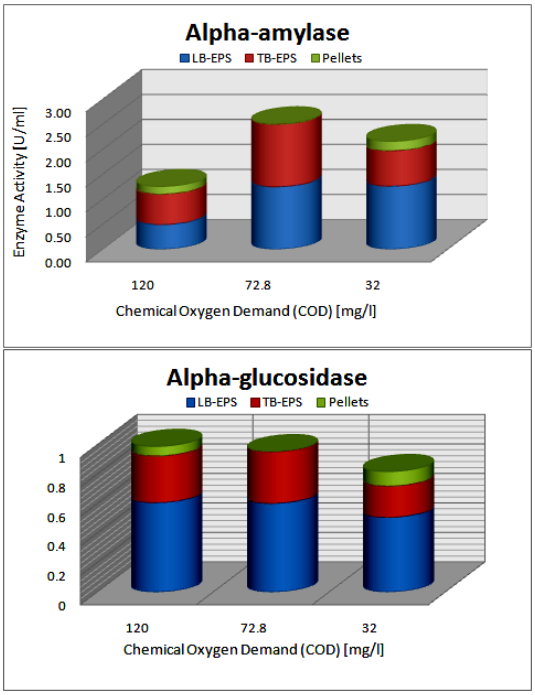

Figure 1. Alpha amylase, alpha glucosidase distribution in sludge flocs.

Significant alpha amylase and alpha glucosidase enzyme activity was measured in the LB and TB-EPS fractions of the activated sludge flocs in every sample, therefore we can infer that these enzymes are immobilized in the EPS matrix in the free form, i.e. exo-enzymes [10]. From Figure 1 we can conclude that enzymatic activity values may vary from time to time (Table 1, 2) due to the substrate diffusion or other parameters but distribution, location of enzyme is ‘specific’, or is an ecto-enzyme (bound to the cell surface of the bacteria), exoenzyme or ‘universal’ that can be found in the whole sludge sample like alkaline phosphatase shown in Table 3, and Figure 2.

Table 2. Alpha-glucosidase activities

COD [mg/L]	120		72.8		32	
Fraction	EA [u/ml]	Stand. Dev	EA [u/ml]	Stand. Dev	EA [u/ml]	Stand. Dev
LB-EPS	0.7107107	±0.15	0.31395	±0.091	1.077441	±0.18
TB-EPS	0.6515607	±0.1	0.42952	±0.08	1.021931	±0.14
Pellets	0.3339703	±0.04	0.584221	±0.014	1.696242	±0.1
Total	1.6962417		1.327691		3.795614	
Efficiency %	60		75.73333		89.33333	

Table 3. Alcaline phosphatse activities

COD [mg/L]	120		72.8		32	
Fraction	EA [u/ml]	Stand. Dev	EA [u/ml]	Stand. Dev	EA [u/ml]	Stand. Dev
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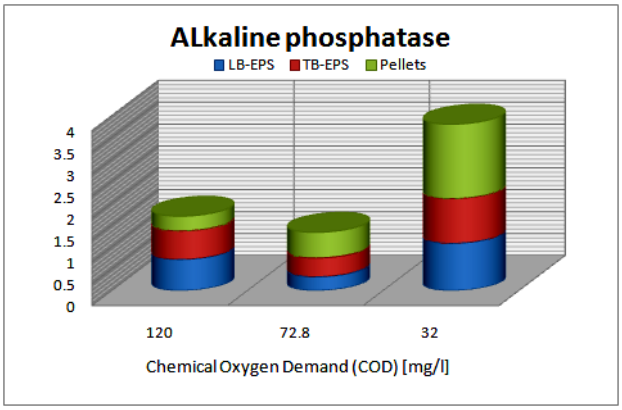

Figure 2. Alcaline Phosphatase distribution in sludge flocs.

Alcaline phosphatase enzyme activity and distribution is significant in all the tree fractions of the sludge flocs, concluding that this enzyme can be found in whole sludge floc, as a 'universal' enzyme. All the tree studied enzymes can be characterized, localized, in sludge floc because during the whole treatment period of the batch system activities values fluctuated but the enzyme localization were the same. Further research is needed to investigate under different process parameters enzymatic behavior of the sludge floc, and to find a correlation between floc and enzyme characteristics.

CONCLUSIONS

Enzymatic activities and distribution in activated sludge flocks can be detected and analyzed; it should also be considered physical and chemical characteristics of the flocks in enzyme activities and distribution. It is very important to understand the enzymatic distribution in the sludge flocks because enzyme activities reflect their microbial activities when degrading organic matter in wastewater [10]. This work denotes that there are different fractions of activated sludge flocks containing different types of enzymes with different activities. Hereinafter our goal is to characterize enzymes at 'standardized' process parameters and investigate parallel activated sludge morphology characterization to find correlation between sludge morphology and enzyme distribution. A better understanding of the distribution of enzymes in the different fractions, of the activated sludge flocks should offer a more precise understanding of biological processes, and should lead to higher removal efficiencies, better control and lower wastewater treatment costs.

EXPERIMENTAL SECTION

Sequencing batch reactor (SBR)

The SBR activated-sludge systems differ from continuous activated-sludge plants because they combine all of the treatment steps and processes into a single basin, or tank, whereas conventional facilities rely on multiple basins [12]. The SBR reactor used for this research is shown in Figure 1, with a total volume of 21 liter.

Table 1. Specifications of the synthetic wastewater used in the experiments, modified from Nopens I. *et al*. 2001 [13]

	mg/l	COD mg/l	N mg/l	P mg/l
Chemical Compounds				
Urea	91.74	23.22	42.81	0
NH_4Cl	12.75	0	3.52	0
o Na-acetate . $3H_2O$	131.64	79.37	0	0
Peptone	17.41	17.41	0.67	0
$MgHPO_4.3H_2O$	29.02	0	0	5.14
KH_2PO_4	23.4	0	0	3.14
$FeSO_4.7H_2O$	5.80	0	0	0
Food ingredients				
Starch	122.00	122.00	0	0
Milk powder	116.19	116.19	6.95	1.14
Yeast	52.24	52.24	6.28	0
Soy oil	29.02	29.02	0	0
Total	**631.21**	**439.45**	**60.23**	**9.42**

Trace Metals		
	mg/l	mg metal/l
$Cr(NO_3)_3.9\ H_2O$	0.770	0.100
$CuCl_2.2\ H_2O$	0.536	0.200
$MnSO_4.\ H_2O$	0.108	0.035
$NiSO_4.6\ H_2O$	0.336	0.075
$PbCl_2$	0.100	0.075
$ZnCl_2$	0.208	0.100

The seed sludge was taken from the Miercurea Ciuc wastewater treatment plant activated sludge basin. For this work, synthetic wastewater has been used (which composition listed in Table 1). The COD/N/P ratio of the synthetic waste water was around 100:17:5, its theoretical BOD_5 was 300 mg/l, assuming a COD to BOD conversion factor of 0.65. [13] The reactor full cycle operation, with fill, react, settle, draw was 3 hour and 45 minutes as presented in Figure 1 where the sampling periods are also presented in the aerob phase.

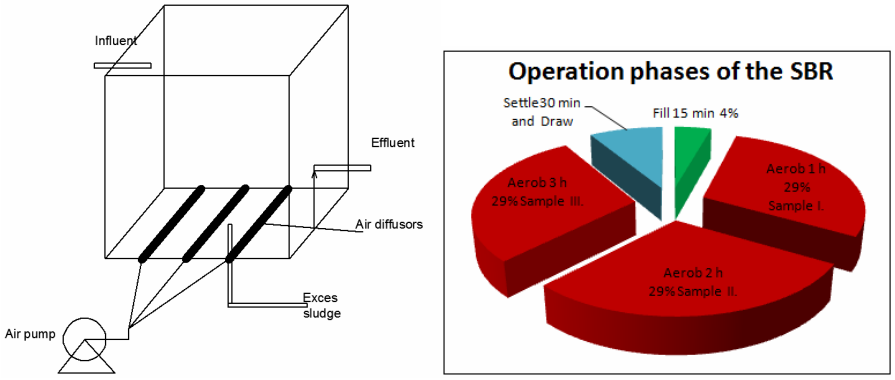

Figure 1. Lab-scale SBR reactor and the operation phases for a full cycle.

EPS extraction

Activated sludge samples were collected from the SBR lab scale reactor at steady-state, and were settled for 30 min. at 4°C temperature. The sludge sediments were then centrifuged (Beckman Oulter Allegra 64R) at 2,000 x g for 15 min, and the supernatant was decanted. As Guang-Hui *et al.* [10] described in their paper, the discarded fraction of the sludge flocs was taken as slime that contained few enzymes. The collected sediments were then resuspended in 0.05% w/w NaCl solution to its original volume and centrifuged again at 5000 x g for 15 min. The organic matter in the supernatant gives the LB-EPS of the sludge samples. Collected sediments were resuspended again with 0.05% NaCl solution to the original volumes

for further extraction of TB-EPS. After resuspension the sludge was sonicated for 2 min and centrifuged at 20000 x g for 20 min. The extraction process is summarized in Figure 2. The pellets give the residues after centrifugation. The organic matter of the supernatant gives the TB-EPS while the enzymes in the pellet were released as Gessesse et al.[14] described in their paper. The pellets of samples were first mixed in 10 mmol l^{-1} Tris buffer at pH 8 in ice bath, and sonicated for a total of 30 min, after 20000 x g centrifugation for 15 min the supernatant was decanted and analysed further for enzyme activity measurement.

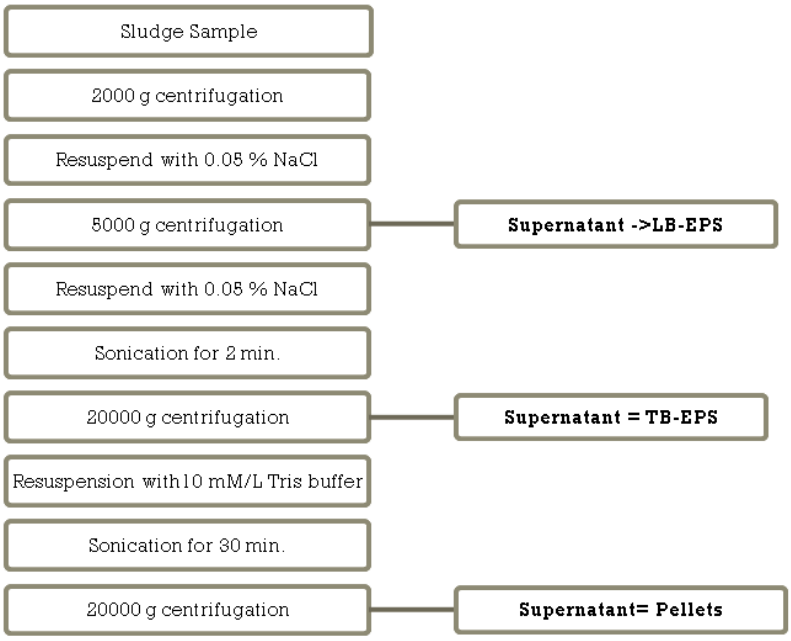

Figure 2. Extraction protocol used for enzyme extraction.

Enzyme Assay

Membranes of 0.45 µm cellulose membranes (Teknokroma, S. Coop. Ltda., OlimPeak Filters with M.E. Celullose Membrane) were used to remove particulates in the supernatant, LB-EPS, TB-EPS and Pellets. The activities of α-amylase were analyzed with starch-iodine method as described by Xiao Z. *et al.* 2006 [15], alkaline-phosphatase and α-glucosidase activities were measured as Goel R. *et al* [16] discussed in their paper, p-nitriphenylphosphate di sodium salt (Sigma N 4646) substrate for alkaline-phosphatase and for α-glucosidase p-nitrophenyl α-D glucopyranoside (Sigma N 1377) was used as substrate. Activated sludge enzymes were measured triplicated in microplate reader (Fluostar Optima, BMG Labtech) to check data reproducibility.

Enzyme activities for alkaline-phosphatase and α-glycosidase were calculated as eq.(1) where activated sludge volume where V [ml] is the volume of the cuvette, ε [1/mmol·cm] is the extinction coefficient, v [ml] the volume of the enzyme sample and l [cm] is the optical path length.

$$EA = \frac{\Delta A / \min \cdot V \cdot 1000}{\varepsilon \cdot l \cdot v} \text{ [U/ml]} \quad (1)$$

For α-amylase enzyme activity calculation we had used the formula as described in Xiao Z. *et al.* 2006 [15] where one unit (U) for the microplate-based starch-iodine assay is defined as the disappearance of an average of 1 mg of iodine binding starch material per min in the assay reaction. U/ml was calculated for amylase using the formula:

$$EA = \frac{(A_{580control} - A_{580sample})}{A_{580/mg.starch} \cdot 30\min \cdot 0.04ml} \text{ [U/ml]} \quad (2)$$

Where $A_{580\ control}$ is the absorbance obtained from the starch without the addition of enzyme, $A_{580\ sample}$ is the absorbance for the starch digested with enzyme, $A_{580/mg\ starch}$ is the absorbance for 1 mg starch derived from the standard curve, 30 min is the assay incubation time and 0.04 ml is the volume of the enzyme used in the assay.

Other analyses

Mixed liquor suspended solids (MLSS) of activated sludge, chemical oxygen demand (COD) and SVI of wastewater, Table 2, were analyzed according to *Standard Methods* [17]. The efficiency (E) of COD removal (in %) was calculated as follows: E (%) = [(C influent – C effluent) / C influent] x 100, where C influent is the concentration in the influent stream, C effluent is the concentration in the effluent stream

Table 2. Parameters of the system analyzed according to Standard Methods

Parameter	Value
Chemical oxygen demand (COD)	300 [mg/l]
Mixed liquor suspended solids(MLSS)	556 [mg/l]
Sludge volume index (SVI)	78 [ml/g]
Dissolved Oxygen (DO)	1.8 ±0.2 [mg/l]

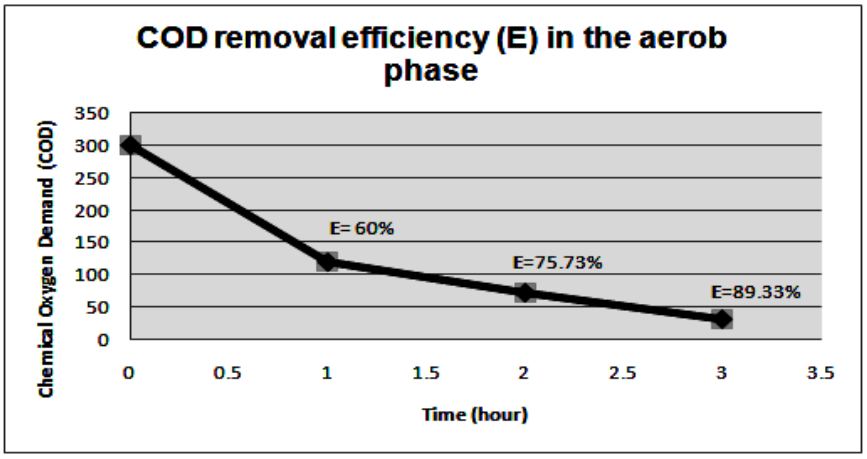

Figure 3. COD removal efficiency of the SBR.

ACKNOWLEDGMENTS

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