ENZYME ACTIVITIES AND DISTRIBUTION IN ACTIVATED SLUDGE IN A LAB-SCALE SEQUENCING BATCH REACTOR (SBR)

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Activitatea şi distribuţia enzimelor în flocoane de nămol activ reflectă activitatea microbiană în procesul degradării materiei organice. Pentru tratarea apei uzate şi pentru investigarea distribuţiei enzimelor hidrolitice (α-glucosidază, protează şi fosfataz alcalină, în substanţe polimerice extracelulare şi în pelet) a fost construit un reactor pilot discontinuu (SBR). Flocoanele de nămol au fost împărţite în fracţiuni, loosely bond (LB) şi tightly bond (TB). Parametrii cinetici a reacţiilor enzimatice au fost identificate pentru o descriere mai bună a proceselor biochimice prezente în epurarea apelor uzate.

Activity and distribution of enzymes in activated sludge flocks reflect microbial activities when degrading organic matters in wastewater. A lab-scale sequencing batch reactor (SBR) was build and used for the treatment of synthetic wastewater to investigate the location and distribution of hydrolytic enzymes, α-glycosidase, protease and alkaline phosphatase, in the extracellular polymeric substances (EPS) and in pellets. The aerobic flocks were categorized into loosely bond (LB) and tightly bound (TB) fractions. Kinetic parameters of enzymatic reactions were determined to identify new biological parameters to describe processes that occur during biological wastewater treatment.

Keywords: activated sludge, enzymes, sequencing batch reactor, extracellular polymeric substances

1. Introduction

The activated sludge treatment is today's most popular type of biological wastewater treatment. In it's over 100 years of history, the initial aerobic oxidation process, developed for organic carbon removal, and has been completed

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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 modern activated sludge processes are very reliable, produce high quality effluent and are considered to be the one of the cost-effective way to remove organic materials from domestic wastewater. 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. Any of several types of activated sludge solids separations problems indicate an imbalance in the biological component of this process. The organic load of wastewater is mainly made up of proteins, polysaccharides and lipids which are high molecular weight compounds. The organic compounds are usually hydrolyzed by extracellular enzymes to be assimilable for bacteria. These extracellular enzymes are either bound to cell surface (ecto-enzymes) or released into the medium (exo-enzymes) in the free form [1] prior to form complexes with high molecular weight substances [2]. Protease, α-amylase, α-glucosidase 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 [3]. The contact probability of enzymes whit proteins and polysaccharides is determined by the distribution of enzymes in the sludge flocks [4].

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 [5]. 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 [6], based on their properties to exhibit a dynamic double-layer structure, composed of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) [6]. 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 [6, 7]. The goal of this paper is to investigate the EPS ultrasound extraction protocol proposed by Guang-Hui *et al*. 2007 [6] on activated sludge samples collected from a lab-scale sequencing batch reactor (SBR). During the degradation of organic pollutants kinetic parameters of enzymatic reactions were determined in order to identify new biological parameters to describe processes that occur in biological wastewater treatment.

2. Materials and methods

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 [8]. The SBR reactor used for this research is shown in Fig.1, with a total volume of 21 liter. 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. [9]

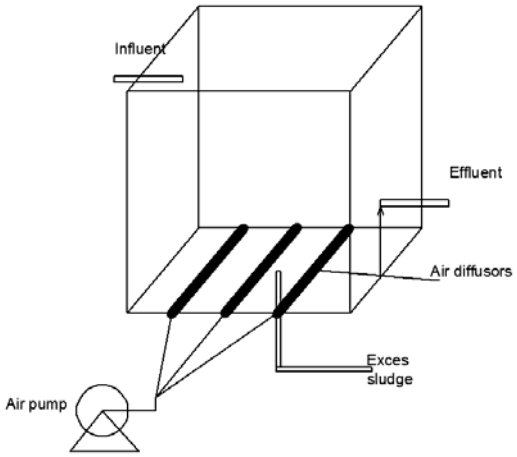

Fig.1. Lab-scale SBR reactor.

Table 1.

Specifications of the synthetic wastewater used in the experiments, modified from Nopens I. *et al.* 2001 [9]

Chemical compounds	mg/l	COD mg/l
Urea	30	8
Peptone	160	160
NaCl	7	0
$MgSO_4 \cdot 7H_2O$	2	0
$CaCl_2$	4	0
KH_2PO_4	23,4	0
Food ingerdients		
Starch	70	70
Meat extract	110	110
Milk powder	65	65
Yeast	52	52
Total	523,4	465

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. [6] 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 (applying ultrasound energy to tear up floc structure) for 2 min and centrifuged at 20000 x g for 20 min. The extraction process is summarized in Fig.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.[12] described in their paper.

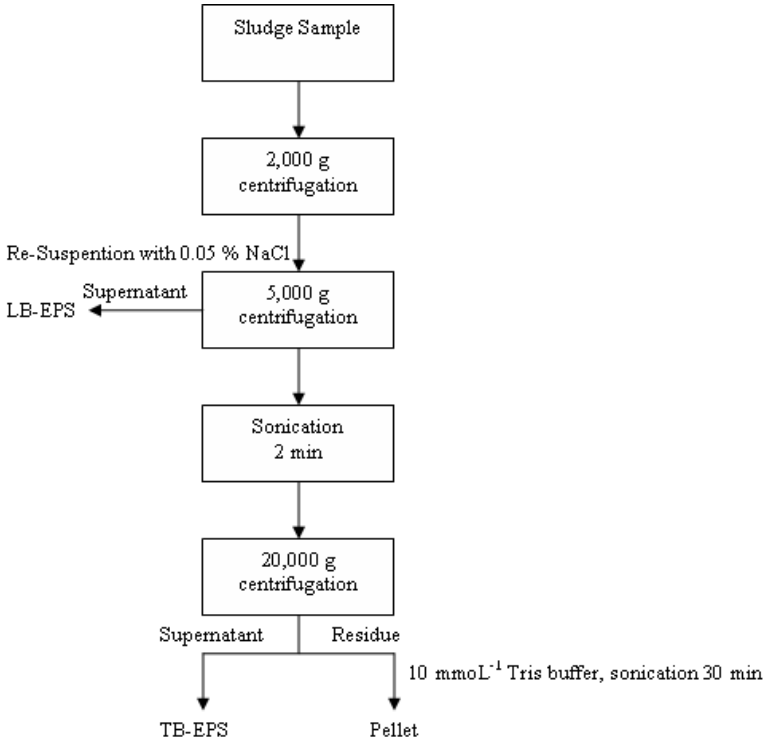

Fig.2. Extraction protocol used for enzyme extraction.

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.

3. Enzyme Assay

Protease activity was measured with N-α-CBZ-l-Arginine-p-nitrolamide (Sigma C 4893) as substrate, alkaline-phosphatase and α-glucosidase activities were measured as Goel R. et al [11] 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 duplicated in microplate reader (Fluostar Optima, BMG Labtech), at five different substrate concentrations to define the kinetic parameters for active enzymes.

Enzyme activities were calculated in mU/ml 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.

$$mU/ml = \frac{\Delta A/\min \cdot V \cdot 1000}{\varepsilon \cdot l \cdot v} \tag{1}$$

Kinetic parameters were defined for enzymes that showed acceptable activities in different fraction of the wastewater and calculated with the help of the double reciprocal/Lineweaver-Burk plot and Michaelis Menten kinetics.

$$\frac{1}{V_0} = \frac{K_m}{V_{\max} \cdot [S]} + \frac{1}{V_{\max}} \tag{2}$$

4. Result and discussion

Extraction effectiveness and distribution of enzymes

Figures 3,4 presents the activities of protease, α-glucosidase and alkaline-phosphate in different fractions of activated sludge flocks. The sonication extraction protocol proved to be reliable for enzyme extraction. Enzyme activities for each investigated enzyme were found in those fractions of the activated sludge flocks, that are reported also in the literature [3, 5, 6, 10].

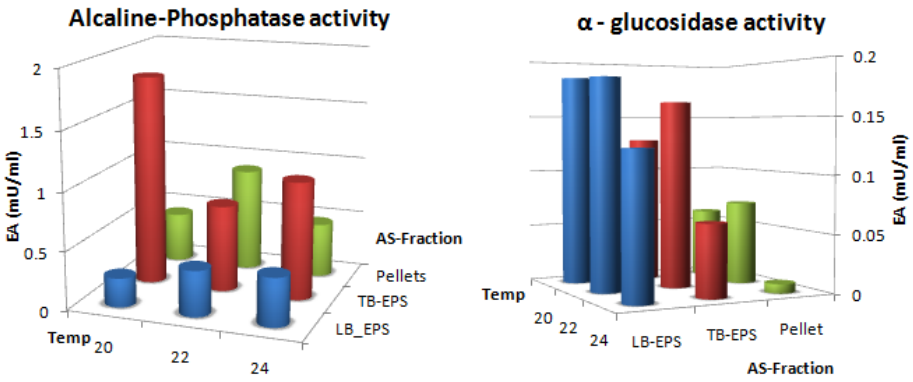

Fig.3. Enyme activities in the different fractions of the flocs and temperature.

α-glucosidase enzyme were found to be most active in the LB and TB-EPS (figure 3.) fraction of the flocs, protease activities were found to be significant in the pellets and also in the TB-EPS fraction (figure .4) and alkaline phosphatase activities were found in both LB and TB-EPS fraction and also in the pellets (figure.3).

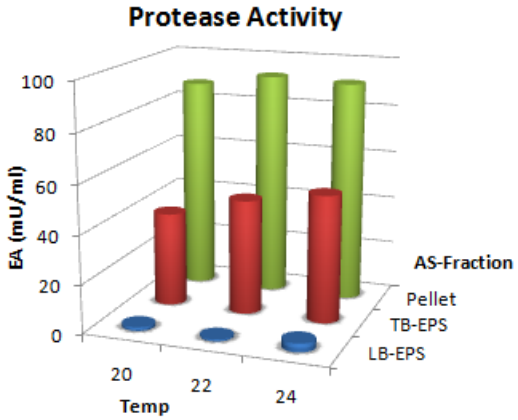

Fig.4. Protease activity in the different fractions of the flocks, and temperature.

Kinetic parameters for the studied enzymes

The highest enzyme activity was found for the protease enzyme in the pellets, even if alkaline-phosphatase and alfa-glucosidase also displayed a significant activity. Protease activity value is significant high comparing whit the other to enzyme activities, protease has minim ten times order of magnitude activity, this points to the fact that during sonication intracellular cell substances were also released and measured. The V_{max} and K_m values correlate with the calculated enzyme activity values as shown for each investigated enzyme in tables 2, 3, 4.

Table.2

Alcaline-phosphatase activity values
R^2 – linear regression correlation coefficient for enzyme kinetic determination

Fraction	**LB-EPS**				**TB-EPS**			
Temp.	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2
20	0.25	17.672	0.021	0.948	1.81	556.124	2.095	0.999
22	0.395	24.734	0.092	0.847	0.747	55.861	2.86	0.994
24	0.417	28.609	0.197	0.886	1.018	85.021	6.18	0.978

Fraction	**Pellets**			
Temp.	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2
20	0.43	29.205	0.07	0.846
22	0.8927	140.029	0.81	0.991
24	0.4696	41.237	0.227	0.913

According to kinetic parameters of the studied enzymes, alkaline-phosphatase enzyme had higher values in the LB and TB fraction of the sludge. By increase of temperature activity, kinetic parameters had shown value increase, in the TB fraction the M-M constant K_m from 2.095 [mmol/l] had increased to 6.18 [mmol/l], so as the activity in the LB from 0.25 to 0.417 [mU/ml]. Alfa-glucosidase enzyme activity and kinetic parameter values were found significant at temperature 20 and 22 C^o of operating conditions and for protease enzyme activities had increased during temperature increase, but kinetic parameters had shown instability, probably due to the cell lysis caused during sonication of sludge samples.

Table.3

Alfa-glucosidase activity values
R^2 – linear regression correlation coefficient for enzyme kinetic determination

Fraction	**LB-EPS**				**TB-EPS**			
Temp.	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2
20	0.1842	12.771	0.0044	0.807	0.128	8.838	0.0033	0.715
22	0.183	13.882	0.038	0.867	0.162	9.733	0.0041	0.803
24	0.124	10.216	0.0018	0.927	0.062	5.213	0.0075	0.796

Fraction	**Pellets**			
Temp.	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2
20	0.06	No calc	No calc	-
22	0.072	No calc	No calc	-
24	0.008	No calc	No calc	-

According to the kinetic information about different enzymes types at BRENDA [13] database, the comprehensive enzyme information system, for phosphatase enzyme from *Escheria Coli* the K_m value is 0.021, for alfa-glucosidase enzyme in *Bacillus subtilis* is 0.21, for purified protease enzyme described by Towatana *et. all* [14] at N-α-CBZ-l-Arginine-p-nitrolamide substrate the K_m value is 600 mmol/l. Comparing the Michaelis Menten constant gathered from the database and literature whit our values we can find significant differences between them, just in case of the LB fraction of phosphatase K_m values show acceptable value resemblance. The difference between constant values is due, because in the database each kinetic parameter corresponds for a typical bacteria, and in our case we must take in to account that we are dealing whit consortia's of bacteria, and the intercellular substance released under sonication were not taken into account.

Table.4.

Protease activity values.
R^2 – linear regression correlation coefficient for enzyme kinetic determination

Fraction	**LB-EPS**				**TB-EPS**			
Temp.	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2
20	1.45	No calc	No calc	-	38.9	5439.31	1115.61	0.889
22	0.76	No calc	No calc	-	47.46	6937.05	2799.34	0.823
24	3.53	No calc	No calc	-	52.641	8299.21	222.48	0.971

Fraction	**Pellets**			
Temp.	EA mU/ml	Vmax mmol/l*h	Km mmol/l	R^2
20	87.8	8479.84	2395.57	0.992
22	92.51	6851.03	4575.28	0.912
24	91.309	5136.72	507.33	0.956

Kinetic parameters for activated sludge models [15] are estimated trough respirometric batch tests for readily biodegradable fraction of wastewater not taking in to account the characteristics of sludge flocks, the diffusivity of substrate, oxygen and also the location of hydrolytic processes under organic matter utilization. By analyzing enzymatic activities and distribution in sludge flocks we could extend our view about biological processes occurring in wastewater treatment, and also could help to describe more accurately the degradation kinetics, and to help to evaluate more precise kinetic parameters which are needed for wastewater modeling, thus for this concept to be true, acceptable we need further and more precise research.

6. Conclusions

Enzymatic activities and distribution in activated sludge flocks can be detected and analyzed, however further research is needed for an exact extraction protocol where intracellular compounds cannot affect the measurement and also for analyzing intracellular component presence in the samples. It should also be considered physical and chemical characteristics of the flocks in enzyme activities and distribution and furthermore also the flocculation and substrate, oxygen diffusion process should be also taken into account under hydrodynamic factor influence in the aeration tank [16]. 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. This work denotes that there are different fractions of activated sludge flocks containing different types of enzymes with different activities. 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.

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