INFLUENCE OF Ca$^{2+}$, Mg$^{2+}$ AND Fe$^{3+}$ ON THE ACTIVITY OF AMYLASES AND PROTEASES DURING AEROBIC GRANULATION

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Abstract. The article presents the results of studies on amylase and protease activity in different fractions of extracellular polymeric substances of granular sludge. The research showed that most of the enzymes are stored in the fraction of tightly-bound extracellular polymers and in pellets. The activity of enzymes is influenced by the presence of divalent and trivalent cations. The highest protease activity of 4 μmol x min$^{-1}$ was observed in the presence of Ca$^{2+}$ and the lowest of 0.5 μmol x min$^{-1}$ in the presence of Fe$^{3+}$. Protease activity in the system without cation addition was almost two times lower than in the all-cation-fed system. The highest activity of extracellular enzymes was observed at day 7 of granulation. The maximum value of amylase activity was 30 μmol$_{maltose}^{-1}$ x min$^{-1}$. The presence of Ca$^{2+}$ is crucial for the processes of cell adhesion. Ca$^{2+}$ plays a role in the regulation of proteolytic enzyme activity and thus on the growth rate of granules. The study also showed a change in enzyme activity during a single cycle of operation in the granular sludge sequencing batch reactor. The highest enzyme activity was observed at the end of the aeration cycle.

Keywords: aerobic granule, enzymes, extracellular polymeric substances, cations

Introduction

Due to numerous advantages, such as reduced space and, low energy requirements as well as a high efficiency in wastewater treatment (Pranik et al., 2015; Nancharaiah et al., 2019), the technology based on aerobic granular sludge is becoming increasingly popular (Li and Tay, 2004). The results of researches to date indicate that the process of sludge granulation is initiated by physical factors such as increased aeration intensity, reduced sedimentation time, or increased rate of sewage discharge from the reactor (Morgenroth et al., 1997; Arrojo et al., 2004, 2007; Liu et al., 2010b; Wojnowska-Baryła et al., 2010). All these factors cause microbial stress and therefore affect the increased secretion of extracellular polymeric substances (EPS) by microorganisms to protect themselves in activated sludge. Extracellular polymeric substances (proteins, polysaccharides, eDNA) take part in the processes of bacterial cell adhesion (Zhang et al., 2018) and biofilm and granule formation (Liu et al., 2010a, 2020).

The physical strength of the sludge granules is a key factor that provides the long-term stable operation of granular biomass reactors (Zhu et al., 2015). There are very few literature reports in which the strength of granules is analysed (Nor-Anuar et al., 2012; de Graff et al., 2018; Czarnota et al., 2020). Kończak et al. (2014) stated that an increase in the density of granules and improvement of their sedimentation properties can be achieved by dosing Ca$^{2+}$ and Mg$^{2+}$ cations. However, the mechanisms that determine this process are unknown.

In turn, Hamiruddin et al. (2019) reported that extracellular polymeric substances (EPS) play a key role in maintaining the granular form and stability of the granules. Felz (2019) indicated that EPS play multiple roles in granules, ranging from the initiation of
granulation processes to the protection of granule co-forming micro-organisms and as a source of nutrients and stores of extracellular enzymes. As extracellular polymeric substances are a mixture of many compounds, therefore are difficult to analyse and quantify (Jiao et al., 2010). Due to that the knowledge about the structure of EPS matrix are still largely unknown.

The functioning of the EPS matrix also depends on the bivalent and trivalent metal cations present in the wastewater, especially Ca$^{2+}$ and Mg$^{2+}$ cations (Kończak and Miksch, 2011, 2012; Felz et al., 2016).

Therefore, this article focuses on the study of changes in the activity of extracellular enzymes in the presence of bivalent and trivalent metal cations. The research is a contribution to the development of knowledge on the processes occurring in the EPS matrix under the influence of extracellular enzymes. The available literature reports mostly concentrated on the role of extracellular enzymes in the process of maintaining granule form and activity of microorganisms during the storing process at 4°C (Adav et al., 2008). It was observed that a high activity of proteolytic enzymes caused the degradation of extracellular proteins, whereas only slight degradation of polysaccharides occurred. Subsequent research has shown that polysaccharides are a structural material, whereas proteins are a carbon and energy reservoir released due to the activity of proteolytic enzymes (Bae et al., 1995; Ng, 2002; Martinez et al., 2004; Zeng et al., 2007; Wang et al., 2007, 2008; Caudan et al., 2014; Ding et al., 2015). Due to the digest of protein by proteolytic enzymes, the low molecular weight polypeptides are found in the supernatant. This allows smaller fragments of proteins to diffuse into the granule, feeding the micro-organisms in deeper layers with nutrients, which allows them to remain active (van der Berg et al., 2020). Hydrolytic enzymes can also actively participate in cell adhesion processes (Burgess and Pletschke, 2008), similarly to the invasion of bacterial cells in the human body and the creation of a biofilm that covers the tissues. The hydrolytic enzymes digest the tissues, revealing the receptor areas, which makes it possible for bacteria to adhere to the tissues of the infected organism (Lipka and Boratynski, 2010). The role of enzymes in the regulation of biomass growth had previously been studied for the conventional sewage treatment plant and wastewater treatment system based on biofilm (Weissbrodt et al., 2013; Hassard et al., 2016) but never for the aerobic granular sludge system.

The estimation of enzyme activity during biogranulation could help us to clarify the hypothetical roles of enzymes in aerobic granules formation process and their role in the biological distribution of excess extracellular polymeric substances.

The paper presents innovative results of studies concerning changes in protease and amylase activity during the formation of aerobic granular sludge in the presence of Ca$^{2+}$, Mg$^{2+}$ and Fe$^{3+}$ cations.

**Materials and experimental methods**

*Laboratory tests*

Laboratory tests were carried out in six sequential reactors (GSSBR, Granular Sludge Sequencing Batch Reactor) with a capacity of 2 L and the following dimensions: height: 565 mm, internal diameter: 83.5 mm. The sludge inoculum was the activated sludge taken from the sewage treatment plant in Gliwice (Poland). Synthetic wastewater of the following system was cyclical introduced to the reactor (3 cycles per day): 0.75 g/l CH$_3$COONa (Stanlab, p.a.), 0.1 g/l NH$_4$Cl (Stanlab, p.a.) and 0.075 g/l KH$_2$PO$_4$. 

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Individual or mixed compound doses were fed to the reactors: 0.016 g/l CaCl\(_2\) (Stanlab, p.a.), 0.05 g/l MgCl\(_2\) (Stanlab, p.a.), 0.02 g/l FeCl\(_3\) (Stanlab, p.a.). The reactors’ operating cycle included: feeding (3 min), aeration (170 min), sedimentation (3 min), discharge (3 min) and idle period (1 min). The air bubbles for aeration were supplied through an air diffuser at the reactors bottom.

**EPS extraction and concentration measurement**

The methodology described in previous studies was applied for the EPS extraction (Kończak et al., 2014). The samples were used to extract the loosely bound extracellular polymeric substances (LB-EPS) and the fraction of tightly bound extracellular polymers marked as TB-EPS. The LB-EPS fraction was obtained by centrifuging granules at 2,000 g for 15 minutes and filtering the separated liquid through a cellulose acetate filter with 45 µm pore diameter. The TB-EPS was obtained by extraction with cation exchange resin, followed by centrifugation at 20,000 g for 20 minutes and filtration of the resulting liquid. The residue from the centrifugation (a fraction of pellets) was also used in the research.

The content of polysaccharides was determined using the Dubois method (Dubois et al., 1956). The protein content was determined by using Quant-iT™ Protein Assay Kit (Thermo Fisher Sc.).

**Enzyme activity analyses**

Measurements of enzyme activity were conducted by the colorimetric method using the Genesys 5 Spectronic spectrophotometer. Enzyme activity was measured in three repetitions and the obtained values were averaged. The blank was prepared simultaneously with the samples, adding the homogenate after the enzyme reaction was interrupted. The enzyme activity was tested on the same day that the extraction of extracellular polymers was performed. This study examined the activity of enzymes contained in the supernatant fraction (LB-EPS), the tightly-bound granule fraction (TB-EPS), and pellets (P-EPS). The measurement of the rate of the catalysed reaction enables the determination of the enzyme activity. The Enzyme Commission of the International Union of Biochemistry has introduced the concept of an international standard unit ‘U’ to ease the comparison of the activity of enzyme preparations. This unit represents the amount of enzyme that catalyses the transformation of 1 µmole of the substrate or a specific part of it, in 1 minute, under optimal conditions. The standard unit is µmol/min (Worsfold, 1995).

**Protease activity determinants**

General proteolytic activity was determined on the basis of the methodology proposed by Stygar (2008). 0.15 ml of azocasein solution and 0.05 ml of the sample were introduced into 0.2 ml of Tris-HCl buffer. Samples were incubated for one hour at 30°C. Enzymatic reactions were interrupted by adding 0.3 ml TCA. After 15 minutes of protein precipitation, the samples were centrifuged for 10 minutes at 10,000 g, then extinction at 366 nm was measured within 10 minutes.

The total proteolytic activity for a given sample was expressed in standard Units of Activity (U), with the difference of 0.001 in the value of absorbance reading between the reference and the test sample being considered as 1 U.
In the case of a reference sample, an appropriate amount of buffer was added instead of the enzyme. The blank test for the enzyme was prepared by mixing the enzyme, TCA, and azocasein in that order. The unit of activity (U) is the amount of the enzyme that causes a change in absorbance of 0.01 under given reaction conditions.

**Amylase activity determinations**

Amylase activity was determined using the modified Bernfeld method (Bernfeld, 1955). Tubes containing 0.5 ml of 1% starch solution were preincubated for 5 minutes at 30°C. Then 0.1 ml of the sample was added, mixed with Vortex, and incubated for one hour at 30°C. Termination of the enzymatic reaction was performed by adding 1 ml of 3,5-dinitrosalicylic acid reagent and boiling it in boiling water for 10 minutes. Samples were cooled and 1.5 ml of distilled water was added. After a minimum of 10 minutes (but maximum before 1 hour) extinction at 540 nm light wavelength was read. The calibration curve for maltose with a 3,5-dinitrosalicylic acid reagent was prepared for solutions of maltose at concentrations between 0.2 and 2.0 mg in the sample. Using the calibration curve for maltose, the concentration of the released maltose was determined, and the specific α-amylase activity was then calculated according to the formula (Eq. 1), expressed in μmoles of released maltose per 1 g of starch and per 1 minute.

$$A_{amylose} = \frac{c \cdot V}{B \cdot t} \cdot \left[\frac{\mu{mol} {maltose}}{g \ {starch} \cdot min}\right]$$  

(Eq. 1)

where:

A – activity (μmol\textsubscript{maltose}×g\textsubscript{starch}×min\textsuperscript{-1}),

c – concentration of released maltose (μmol\textsubscript{maltose}×ml\textsuperscript{-1}),

V – volume of reaction mixture (ml),

B – amount of starch in the reaction mixture (g),

t – incubation time (min)

**Statistical analysis**

Statistical analyses were performed in Statistica 13 (TIBCO Software) (p ≤ 0.05). Averages and standard deviations were calculated.

**Results and discussion**

The results showed that during granulation the activity of proteases contained in the supernatant is low and remains in the range of 0.5 – 1 μmol x min\textsuperscript{-1}. No correlation was found between changes in protease activity in the LB-EPS fraction (loosely bound EPS) and the type of bivalent or trivalent cation dosed (Fig. 1a). The proteases contained in the TB-EPS fraction (tightly bound EPS) showed much higher activity. Zhang et al. (2015) reported that a fraction of tightly-bound extracellular polymeric substances (TB-EPS) can also capture extracellular catalytic enzymes and keep them close to cells (Li et al., 2008; Zhang et al., 2015). Similar results were presented by Dube and Guiot (2019), who reported that catabolic enzymes are stored in the EPS matrix, which makes the extracellular envelope capable of extracellular catabolic reactions, which is an advantage for both conventional activated sludge and anaerobic granular activated sludge.

This study has shown that Ca\textsuperscript{2+} and Mg\textsuperscript{2+} cations have a significant role in binding enzymes to the TB-EPS matrix. In all tested fractions of EPS matrix proteases showed
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The highest activity in the presence of Ca\(^{2+}\) cations. In the case of Fe\(^{3+}\) cation, no increase in protease activity was observed. At the same time, it was found that the variability of protease activity is related to the initiation of granules formation and further development process of granules. In the first days of granulation (up to the 7th day), it was observed the increase in protease activity from 3 \(\mu\)mol x min\(^{-1}\) to 6 \(\mu\)mol x min\(^{-1}\) in the Ca-fed reactor. In the system, where Mg\(^{2+}\) cation was dosed on the 7th day, an inconsiderable increase of activity was observed, whereas in the system where Fe\(^{3+}\) was dosed, no increase in protease activity was observed. From the 20th day, the protease activity stabilized at the level of 4 \(\mu\)mol x min\(^{-1}\) and 3.5 \(\mu\)mol x min\(^{-1}\), respectively for the system where Ca\(^{2+}\) and Mg\(^{2+}\) were dosed (Fig. 1b).

The research showed no statistically significant differences between the activity of protease in the cation-fed systems and in the control system (without cations addition) during the first 7th days of granulation (p<0.05). The statistically significant differences were observed from the 13th days of granulation till the end of granulation process, except Mg-fed system (p<0.05). In the Mg-fed system the statistically significant differences in the protease activity was observed from 15th days of granulation (Fig. 1d) (p<0.05).
Previous results show that the time of granule formation in laboratory conditions is about 20-30 days and granules reach from 3 mm to even 29 mm (Kończak et al., 2014). Changes in the activity of extracellular proteases in the initial days of the granulation process are the result of the self-immobilisation of microorganisms form the activated sludge. From the 20th day of the process, when the granules reach their maximum size, the enzyme activity stabilises and remains at a high level close to the maximum. The diffusion of substrates into the granule is limited by the size of the granules (Liu et al., 2005; Li and Chróst, 2006; Li et al., 2008; Dube and Guiot, 2019). The storage of enzymes in the EPS matrix helps to maintain the stability of the sludge granules (regulation of granule size through degradation of excess organic compounds) and to nourish the microorganisms inside the granule (diffusion of low-molecular substrates into the granule).

Moreover, it was found that proteases in the TB fraction are most active in the presence of Ca$^{2+}$ and in the system where all cations were dosed. The activity of proteases in the system where Ca$^{2+}$ cation was dosed on the 7th day was 6 μmol $\times$ min$^{-1}$, then on the 15th day it decreased to 1 μmol $\times$ min$^{-1}$, which was related to changes in the structure of forming granules. From the 30th day, it remained at 5-5.5 μmol $\times$ min$^{-1}$. The fluctuations of protease activity in the all cations-fed system ranged from 1.3 μmol $\times$ min$^{-1}$ on the 13th day to 7.5 μmol $\times$ min$^{-1}$ on the 20th day of the granulation process. It was found that bivalent and trivalent cations participate in the activation of extracellular proteases. The protease activity in the control system, where no cations were dosed, was almost twice as low as in the system to which all cations were dosed (Fig. 1d).

The main function of extracellular proteases is an enzymatic digestion of the intercellular matrix components. The results of the research indicate that the extracellular matrix proteases are involved in the process of its reconstruction.

As in the case of proteases, the amylase activity was lower in the LB-fraction than in the other fractions (Figure 2a). The highest amylase activity was observed in TB-fraction and pellets (Fig. 2b and c). From the 3rd day of the biogranulation process, the amylase activity started to increase rapidly, reaching a peak on the 7th day. From this point, the activity dropped quickly and started to remain stable at the initial value (Figure 2).

Additional, in the previous research, it was found that polysaccharides are not degraded during granulation (Kończak et al., 2014) so that they can form a three-dimensional network to make stable of the granular form of sludge.

The amylase activity was statistically significant highest in the presence of Mg$^{2+}$ ($p \leq 0.05$). On the 7th day of the process, in the control system to which all cations were dosed the activity of amylases reached 30 μmol$_{\text{maltose}}$ $\times$ g$_{\text{starch}}^{-1}$ $\times$ min$^{-1}$. However, in the system without cations, the maximum amylase activity was only 6 μmol$_{\text{maltose}}$ $\times$ g$_{\text{starch}}^{-1}$ $\times$ min$^{-1}$. Starting from the 13th day of the process, it was observed that amylase activity in the TB-fraction, in the presence of Ca$^{2+}$, was higher than in the presence of other cations. Therefore, it was found that Ca$^{2+}$ and Mg$^{2+}$ cations, as well as Fe$^{3+}$ cations, affect the increase in amylase activity (Fig. 2d).

The research showed no statistically significant differences between the activity of protease in the cation-fed systems and in the control system (without cations addition) during the first 3rd days of granulation ($p<0.05$). The statistically significant differences were observed from the 7th days of granulation till the end of granulation process, except Mg and Fe-fed systems ($p<0.05$). In the Mg and Fe-fed systems no statistically significant differences in the protease activity was observed in 20th and 23th days of granulation (Fig. 2d) ($p<0.05$).
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The analysis of the activity of proteases and amylases during a single cycle of operation of a reactor with granulated biomass showed the increase of the activity of pellets amylase (P-amylase) and TB-amylase under substrate deficiency conditions, i.e. from 34 minutes of aeration. At the same time, it was observed that the amount of secreted polysaccharides in the TB-fraction (PS-TB) increased from ca. 5 mg/g\(\text{VSS}\) (VSS – volatile suspended solids) to 15 mg/g\(\text{VSS}\) and the content of PS in LB-fraction of EPS was almost the same (ca. 2 mg/g\(\text{VSS}\)). It was therefore concluded that under substrate deficiency conditions, microorganisms activate amylases, which participate in extracellular catabolic processes (Fig. 3a).

The activity of proteases stored in the TB-EPS fraction and pellets (P-EPS) increased from 94 minutes of the wastewater aeration process. No changes in protease activity were observed in the LB-EPS. The concentration of polypeptides in both LB-EPS and in the TB-EPS fraction increased with the aeration time (Fig. 3b).

Figure 2. The influence of Ca\(^{2+}\), Mg\(^{2+}\) and Fe\(^{3+}\) on the change of amylase activity a) in LB-EPS fraction, b) in TB-EPS fraction, c) in P-EPS fraction (pellets) and on the change of the total amylase activity (d) (vertical bars – standard deviation, * - no statistical significance, p<0.05)
Conclusion

The extracellular hydrolytic enzymes are stored mainly in the fraction of tightly-bound extracellular polymeric substances and play an important role in regulating the granulation process. The research has shown that under substrate deficiency conditions, microorganisms activate amylases, which participate in extracellular catabolic processes.

The extracellular enzymes are responsible for regulating the content of proteins and polysaccharides in granules. The activity of enzymes is related to the presence of bivalent and trivalent metal ions, which are activators of proteases and amylases present in fractions of tightly-bound polymers.

Extracellular enzymes show the highest activity in the presence of Ca\(^{2+}\) cation and in systems to which Ca\(^{2+}\), Mg\(^{2+}\) and Fe\(^{3+}\) cations are simultaneously dosed. Fe\(^{3+}\) cation is the weakest activator of extracellular enzymes.
The results show that there is a need to further expand the knowledge on the role of extracellular enzymes in the processes of active sludge granulation under aerobic conditions, which will contribute significantly to understanding the mechanisms of this process.

The research is a contribution to the knowledge of the processes occurring in the EPS matrix under the influence of extracellular enzymes. An increase in protease activity in the first phase of granulation (up to the 7th day), especially in the presence of Ca$^{2+}$, showed the importance of this cation in the processes of regulation of proteolytic enzymes activity. Ca$^{2+}$ play the special role in the processes of cell adhesion and control of granule growth. The research need to be continued to determine the relationship between cations addition and the process of cell adhesion during aerobic granulation process.

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REFERENCES

[1] Adav, S. S., Lee, D. J., Tay, J. H. (2008): Extracellular polymeric substances and structural stability of aerobic granule. – J. Wat. Res. 42: 1644-1650.

[2] Arrojo, B., Mosquera-Corral, A., Garrido, J. M., Mendez, R. (2004): Aerobic granulation with industrial waste-water in sequencing batch reactors. – Wat. Res. 38: 3389-3399.

[3] Arrojo, B., Vazquez Padin, J. R., Figueroa, M., Mosquera-Corral, A., Campos, J. L., Mendez, R. (2007): Reactors of biomass granular – more efficiency in less space (in spanish: Reactores de biomasa granular - más capacidad en menos espacio). – Tecnologia de agua 287: 31-39.

[4] Bae, B.-U., Shin, H.-S., Paik, B.-Ch., Chung, J.-Ch. (1995): Re-activation characteristics of preserved anaerobic granular sludges. – Bioresour. Technol. 53: 231-235.

[5] Bernfeld, P. (1955): α-, β-amylases. – In: Colwick, S. P., Kaplan, N. O. (eds.) Methods in enzymology. Academic Press, New York, pp. 149-158.

[6] Burgess, J. E., Pleitschke, B. I. (2008): Hydrolytic enzymes in sewage sludge treatment: A mini-review. – Water SA 34(3): 343-349.

[7] Caudan, C., Filali, A., Spérandio, M., Girbal-Neuhauser, E. (2014): Multiple EPS interactions involved in the cohesion and structure of aerobic granules. – Chemosphere 117: 262-270.

[8] Czarnota, J., Masłoń, A., Zdeb, M., Łagód, G. (2020): Powdered Ceramsite and Powdered Limestone Use in Aerobic Granular Sludge Technology. – Molecules 25(2): 386.

[9] de Graaf, D. R., van Dijk, E. J. H., van Loosdrecht, M. C. M., Pronk, M. (2018): Strength characterization of full-scale aerobic granular sludge. – Env Technol. 41(13): 1637-1647.

[10] Ding, Z., Bourven, I., Guibaud, G., van Hullebusch, E. D., Panico, A., Pirozzi, F., Esposito, G. (2015): Role of extracellular polymeric substances (EPS) production in bioaggregation: application to wastewater treatment. – Appl. Microbiol. Biotechnol. 99(23): 9883-9905.

[11] Dube, Ch. D., Guiot, S. R. (2019): Characterization of the protein fraction of the extracellular polymeric substances of three anaerobic granular sludges. – AMB Express 9: 23.

[12] Dubois, M. K., Gilles, J. K., Hamilton, J. K., Rebers, P. A., Smith, F. (1956): Colorimetric Method for Determination of Sugars and Related Substances. – Anal. Chem. 28: 350-352.

[13] Felz, S., Al-Zuhaify, S., Aarstad, O. A., van Loosdrecht, M. C. M., Lin, Y. M. (2016): Extraction of structural extracellular polymeric substances from aerobic granular sludge. – J Vis Exp. 115: 54534.
[14] Felz, S., Vermeulen, S., van Loosdrecht, M. C. M., Lin, Y. M. (2019): Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). – Water Res. 157: 201-208.

[15] Hamiruddin, N. A., Awang, N. A., Shaaban, M. G. (2019): The performance of extracellular polymeric substance (EPS) on stability of aerobic granular sludge (AGS). – CEER 29(3): 060-069.

[16] Hassard, F., Biddle, J., Cartmell, E., Stephenson, T. (2016): Biosolids recycling impact on biofilm extracellular enzyme activity and performance of hybrid rotating biological reactors. – Process Saf. Environ. Prot. 103: 69-75.

[17] Jiao, Y., Cody, G. D., Harding, A. K., Wilmes, P., Schrenk, M., Wheeler, K. E., Banfiled, J. F., Thelen, M. P. (2010): Characterization of Extracellular Polymeric Substances from Acidophilic Microbial Biofilms. – App. Environ. Microbiol. 76(9): 2916-2922.

[18] Kończak, B., Miksch, K. (2011): Influence of Ca\(^{2+}\), Mg\(^{2+}\) and Fe\(^{3+}\) on granular sludge formation. – Chem. Eng. 90/11: 2002-2005.

[19] Kończak, B., Miksch, K. (2012): Distribution of extracellular polymeric substances and their role in aerobic granule formation. – Chem. Process Eng. 33(4): 679-688.

[20] Kończak, B., Karcz, J., Miksch, K. (2014): Influence of Calcium, Magnesium, and Iron Ions on Aerobic Granulation. – Appl. Biochem. Biotechnol. 174: 2910-2918.

[21] Li, Y., Chróst, R. J. (2006): Microbial enzymatic activities in aerobic activated sludge model reactors. – Enzym. Microb. Technol. 39: 568-572.

[22] Li, Y., Liu, Y., Shen, L., Chen, F. (2008): DO diffusion profile in aerobic granule and its microbiological implications. – Enzyme Microb. Technol. 43(4-5): 349-354.

[23] Lipka, D., Boratyński, J. (2010): Metalproteinase MMP. The structure and function. (in polish: Metaloproteinazy MMP. Struktura i funkcja). – J. Postępy Hig. Med. Dośw. 62: 328-336.

[24] Liu, Y., Tay, J. H. (2004): State of the art of biogranulation technology for wastewater treatment. – Biotechnol. Adv. 22: 533-563.

[25] Liu, Y.-Q., Liu, Y., Tay, J.-H. (2005): Relationship between size and mass transfer resistance in aerobic granules. – Lett. Appl. Microbiol. 40(5): 312-315.

[26] Liu, L., Gao, D. W., Zhang, M., Fu, Y. (2010a): Comparison of Ca\(^{2+}\) and Mg\(^{2+}\) enhancing aerobic granulation in SBR. – J. Hazard. Mater. 181: 382-387.

[27] Liu, T.-Q., Moy, B., Kong, Y.-H., Tay, J.-H. (2010b): Formation, physical characteristic and microbial structure of aerobic granules in pilot-scale sequencing batch reactor for Real wastewater treatment. – Enzyme Microb. Technol. 46: 520-525.

[28] Liu, Y., Shan, R., Chen, G., Liu, L. (2020): Linking flow velocity-regulated EPS production with early-stage biofilm formation in drinking water distribution systems. – Water Supply 20(4): 1253-1263.

[29] Martinez, F. O., Lema, J., Mendez, R., Cuervo-Lopez, F., Gomez, J. (2004): Role of exopolymeric protein on the settleability of nitrifying sludges. – Bioresour. Technol. 94: 43-48.

[30] Morgenroth, E., Sherden, T., van Loosdrecht, M. C. M., Heijnen, J. J., Wilder, P. A. (1997): Aerobic granular sludge in a sequencing batch reactor. – Wat. Res. 31: 3191-3194.

[31] Nancharaiah, V. Y., Sarvajith, M., Krishna Mohan, T. V. (2019): Aerobic Granular Sludge: The Future of Wastewater Treatment. – Current science 117(3): 395-404.

[32] Ng, P. H. (2002): Storage stability of aerobic granules cultivated in aerobic granular sludge blanket reactor. – Master Thesis Nanyang Technological University, Singapore.

[33] Nor-Anuar, A., Ujang, Z., van Loosdrecht, M. C. M., de Kreuk, M. K., Olsson, G. (2012): Strength characteristics of aerobic granular sludge. – Water Sci Technol 65(2): 309-316.

[34] Pronk, M., de Kreuk, M. K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M. C. (2015): Full scale performance of the aerobic granular sludge process for sewage treatment. – Water Res. 1(84): 207-17.
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[35] Stygar, D. (2008): Digestive and glucose metabolism enzymes in the caterpillars of successive generations Cameraria Ohridella (Lepidoptera: Gracillariidae). – PhD Thesis, The Silesian University, Katowice, Poland.

[36] van der Berg, L., Kirkland, C. M., Seymour, J. D., Codd, S. L., van Loosdrecht, M. C. M., de Kreuk, M. K. (2020): Heterogeneous diffusion in aerobic granular sludge. – Biotechnol. Bioeng. 117(12): 3809-3819.

[37] Wang, Z. W., Liu, Y., Tay, J.-H. (2007): Biodegradability of extracellular polymeric substances produced by aerobic granules. – Microbiol. Biotechnol. 74: 462-466.

[38] Wang, X., Zhang, H., Yang, F., Wang, Y., Gao, M. (2008): Long-term storage and subsequent reactivation of aerobic granules. – Bioresour. Technol. 99: 8304-8309.

[39] Weissbrodt, D. G., Neu, T. R., Kuhlicke, U., Rappaz, Y., Holliger, Ch. (2013): Assessment of bacterial and structural dynamics in aerobic granular biofilms. – Front. Microbiol. 4(175): 1-18.

[40] Wojnowska-Baryła, I., Cydzik-Kwiatkowska, A., Sztatkowski, M., Gutowski, Ł. (2010): Granulation of activated sludge in SBR reactor. – Biotechnologia 1(88): 161-169.

[41] Worsfold, J. (1995): Classification and chemical characteristics of immobilized enzymes. – Pure & Appl. Chem. 67(4): 597-600.

[42] Zeng, P., Zhuang, W. Q., Tay, S. T. L., Tay, J. H. (2007): The influence of storage on the morphology and physiology of phthalic acid-degrading aerobic granules. – Chemosphere 69: 1751-1757.

[43] Zhang, P., Shen, Y., Guo, J. S., Li, C., Wang, H., Chen, Y. P., Yan, P., Yang, J. X., Fang, F. (2015): Extracellular protein analysis of activated sludge and their functions in wastewater treatment plant by shotgun proteomics. – Sci Rep. 5: 12041.

[44] Zhang, H., Jia, Y., Khanal, S. K., Lu, H., Fang, H., Zhao, Q. (2018): Understanding the Role of Extracellular Polymeric Substances on Ciprofloxacin Adsorption in Aerobic Sludge, Anaerobic Sludge, and Sulfate-Reducing Bacteria Sludge Systems. – Environ. Sci. Technol. 52(11): 6476-6486.

[45] Zhu, L., Zhou, J., Lv, M., Yu, H., Zhao, H., Xu, X. (2015): Specific component comparison of extracellular polymeric substances (EPS) in flocs and granular sludge using EEM and SDS-PAGE. – Chemosphere 121: 26-32.