Influence of calcium, magnesium and iron ions on the molecular mass of exoproteins during biogranulation

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In this study, we performed the qualitative analysis of exoproteins during granule formation in the presence or in the absence of cations. The staining of thin granule cryosections showed that nucleic acids, proteins, polysaccharides and calcium cations were the dominant components of the granules. Proteins are the structural components associated with calcium ions. We determined changes in the proteomic profile and tightly bound extracellular polymeric substances (EPS) of the slime. The exopolymeric matrix containing the proteins was extracted using the Dowex resin method. Proteomic profile was analysed by SDS-PAGE method (sodium dodecyl sulphate polyacrylamide gel electrophoresis) using Coomassie blue staining in the samples of the aerobic granule matrix formed in the presence of multivalent cations and compared with that of the aerobic granules cultivated without cations. The results indicate that the granule matrix is predominantly composed of large and complex proteins that are tightly bound within the granular structure. The tightly bound extracellular polymeric substances (TB-EPS) may play a role in improved mechanical stability of aerobic granules. In the supernatant fraction of the sludge, only a small amount of free proteins in the medium molecular mass range was detected. The protein with high molecular mass ($\geq$ 116 kDa) produced in the reactors with added Ca^{2+}. Ca^{2+} had a considerable regulatory influence on production of extracellular proteins during aerobic granulation.

Keywords: aerobic granular sludge, extracellular protein, SDS-PAGE, divalent cations, multivalent cations

1. INTRODUCTION

In recent years, the aerobic granular sludge technology has become increasingly popular. The technology is suitable for large municipal wastewater treatment plants as well as for small and compact industrial wastewater treatment plants with special requirements (Chen et al., 2019). Aerobic granules are spherical and dense aggregates of microorganisms are cultivated in a granular sludge sequencing batch reactor (GSSBR) (Arrojo et al., 2004; Li et al., 2014; Morgenroth et al., 1997). The process of formation of the aerobic granules is very similar to the process of biofilm growth (Vu et al., 2009; Xuan et al., 2010).

Extracellular polymeric substances (EPS), especially polysaccharides and proteins, play a special role in the process (Huan et al., 2010; Li et al., 2018; McSwain et al., 2005). Some studies detected eDNA in the extracellular polymeric matrix (Cheng et al., 2011; Xiong et al., 2019). There are two hypotheses

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clarifying the presence of eDNA. One possibility is that the eDNA originated due to cell lysis. Another possibility is that microorganisms can actively excrete DNA that plays a structural role in the biofilms (Cheng et al., 2011).

Polysaccharides and proteins play a functional role in the aerobic granules by creating a network between their functional groups and the cells. The specific EPS-EPS interaction is considered to be an adhesive that holds the microorganisms and organic-mineral sludge compounds together.

Extracellular proteins play a special role in the adhesion of the cells of microorganisms (Ma et al., 2013). These proteins modify the impact of electrostatic and Van der Waals forces and hydrophobic interactions in particular. Moreover, due to binding of water molecules by extracellular proteins, cell dehydration can occur and thus surface adhesion becomes easier (Czaczyk, 2004).

Neilsen at. al. (1997) observed that proteins are the dominant component of the EPS matrix in biofilms. Our previous study (Miksch and Kończak, 2012) demonstrated that the protein levels were five-fold higher than those of polysaccharides. Additionally, we reported that an increase in the protein content was correlated with the growth of cell hydrophobicity and this was an important factor in the development of aerobic granules in the reactor. Moreover, the reactive groups of proteins are involved in direct interaction forming electrostatic bonds with cations. It should be also taken into account their potential for iron-protein interactions by forming coordinate bonds.

Crosslinking of these proteins with multivalent cations forms a three dimensional extracellular matrix (Patrauchan et al., 2005). Similarly, Zhang et al. (2015) reported that proteins originating from the microorganism cells play an important role in binding of multivalent cations and organic molecules. Apart from that, their major functions included catalysis and degradation. These authors also reported that in an aerobic activated sludge system, proteins are more abundant than in the anaerobic wastewater systems. Proteins facilitate adhesion of sludge flocs to each other.

A recent study was focused on the mechanism of aerobic granulation with regard to excretion of extracellular polymeric substances. However, little is known about the interaction between the multivalent cations and extracellular polymeric substances during aerobic granulation (Manas et al., 2012; Yu et al., 2000; Yu et al., 2009). Therefore, this study was conducted to examine the roles of Ca^{2+}, Mg^{2+} and Fe^{3+} in the sludge granulation mediated by cation binding to the exoproteins. The diversity of the proteins and their molecular weights were investigated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The changes in protein profile in the presence of divalent (Ca^{2+}, Mg^{2+}) and trivalent (Fe^{3+}) cations were assayed to better understand a possible role of extracellular proteins in the formation and stability of aerobic granules. Another objective of this work was to evaluate the relationship between Ca^{2+} concentration and exoprotein secretion. This information is expected to improve our understanding of the aerobic process of granule formation in the presence of divalent and trivalent cations.

2. MATERIALS AND METHODS

2.1. Reactor configuration

The methodology used in the present investigation is similar to the one used in our previous study (Miksch and Kończak, 2012; Kończak et al., 2014). The present work is divided into two stages: Assessing the influence of Ca^{2+}, Fe^{3+}, and Mg^{2+} ions on the secretion of exoproteins during granulation (stage I); Assessing the influence of the doses of calcium ions on the secretion of exoproteins during granulation (stage II).

During stage I, the experiment was performed in six 1l sequencing batch reactors (SBR 1–6) with an internal diameter of 40.5 mm each. The height-to-diameter ratio (H/D ratio) was approximately 6.9. Aeration was effected using an air diffuser at the bottom of the reactors. The experiment was continued for 30 days. During stage II, the experiment was carried out in three sequencing batch rectors of the same size as described in the preceding paragraph. This stage was continued for 30 days as well.

Activated sludge obtained from the Wastewater Treatment Plant "Środmieście" (Zabrze, Poland) were used as an inoculum. All reactors were operated at a time cycle of 3 h that included 3 min feeding, 168 min aeration, 5 min sedimentation, 3 min of effluent pumping and 1 min of idling. The volumetric exchange ratio was 50%.

2.2. Medium

The microorganisms of the activated sludge were fed with synthetic wastewater. The organic loading rates were approximately 2.4 kg COD/(m^3 d). Synthetic wastewater was prepared by adding sodium acetate (0.543 g/l), $(NH_4)_2SO_4$ (0.075 g/l) and KH_2PO_4 (0.075 g/l). The synthetic wastewater was enhanced by a trace solution containing: $MnSO_4·H_2O$ (0.05 g/l); $CoCl_2·6H_2O$ (0.05 g/l); H_3BO_3 (0.05 g/l); $AlCl_3$ (0.05 g/l); $ZnCl_2$ (0.05 g/l); $NiCl_2$ (0.05 g/l); $(NH_4)_6Mo_7O_{24}·4H_2O$ (0.05 g/l); $CuCl_2$ (0.03 g/l). pH of the feeding medium was adjusted to 7.2.

Table 1 lists the additions of Ca^{2+}, Fe^{3+} and Mg^{2+} to the reactors during the first stage of the study. The feeding dose was 0.125 mmol/l for Ca^{2+} and Mg^{2+} cations and 0.075 mmol/l for Fe^{3+} cation. The value of cation dose was chosen as for other microelements.

Table 1. Scheme of the multivalent cation additions into the reactors during the first stage of the study

Cation	SBR1	SBR2	SBR3	SBR4	SBR5	SBR6
Mg^{2+}	-	-	×	×	–	×
Fe^{3+}	-	-	-	–	×	×
Ca^{2+}	-	×	-	×	–	×

During the second stage of the study, the influence of the dose of calcium on aerobic granulation was assessed. For measuring the influence of calcium on granulation, the dose of Ca^{2+} was increased 4-fold and 8-fold compared to the initial value. The tested dose of Ca^{2+} was 0.5 and 1 mmol/l.

2.3. Extraction of EPS

Frølund et al. (1996) reported the possibility of EPS extraction from the sludge using a cation exchange method. In the present work, the sludge fractionation method was used according to the method previously described by Kończak et al. (2014). First, sludge samples were centrifuged, the supernatants were collected and used as the samples of soluble EPS (SOL-EPS). Then, the remaining biomass (the sediments) was re-suspended in 0.1M PBS (pH 7.4) buffer solution and the cation exchange resin (Dowex 50×8, Fluka, USA) at a dose of 60 g/g VSS was added to extract TB–EPS (tightly bound extracellular polymeric substances). Finally, the samples were centrifuged and the supernatant containing TB-EPS was collected. The remaining sediment was not used in these studies.

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2.4. Confocal laser scanning microscopy (CLSM)

Distribution of calcium ions, DNA, polysaccharides and proteins in the aerobic granules was determined using confocal laser scanning microscopy (CLSM). First, mature granules were cryosectioned into 20 μm sections (Cryostat, Leica). The cryosections from the middle part of the granules were stained for EPS with calcofluor white (β-D-glucopyranose polysaccharides), fluorescein isothiocyanate (protein), SYTO 63 (nucleic acids) and calcium green (calcium ions). The staining procedure was performed according to Adav et al. (2010). The sample was imaged by a Zeiss LSM500 microscope with a 405 nm diode laser Ar458/477/488/514 nm and a 633 nm HeNe laser. Images were analysed using the ZEN 2008 software (Zeiss).

2.5. Protein precipitation

Proteins were precipitated by adding 100% (m/v) TCA (Trichloroacetic acid) to protein samples (1:4) (Sanchez et al., 2001). Then, the samples were centrifuged for 5 min at 14,000 rpm to collect the protein pellet. The protein pellet was washed with ethanol/ethyl acetate and centrifuged again at the same conditions. The procedure was repeated three times. Finally, the protein pellet was dried in a thermal block at 95 °C for 5–10 min. Samples were stored at -20 °C after being dissolved in 2X Laemmli buffer (Sigma Aldrich, Germany).

2.6. SDS-PAGE method (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

The analysis of protein samples was performed using a MINI-Protean 3-System (Life Science - Bio-Rad Polska, Warsaw, Poland) SDS-PAGE as described previously by Laemmli (1970). The concentration of polyacrylamide in the separating gel was adjusted according to the molecular mass of the separated proteins. The 15% polyacrylamide separation gel solution contained 3.4 ml H_2O, 7.5 ml acrylamide (30%), 3.8 ml Tris-HCl (1.5 M, pH 8.8), 150 μl SDS (10%), 150 μl ammonium persulfate (APS) (10%), and 10 μl tetramethylethylenediamine (TEMED). The 5% stacking gel solution contained 4.1 ml H_2O, 1 ml acrylamide (30%), 750 μl Tris-HCl (1.5 M, pH 6.8), 60 μl SDS (10%), 60 μl APS (10%), and 10 μl TEMED. Then, the gel casting chamber was filled with the separation gel and overlaid with Milli-Q water. After polymerization, the stacking gel was poured on top. Proteins were incubated at 96 °C for 3 min, centrifuged and mixed with the loading buffer (1×). The samples were loaded in the lanes. The electrophoresis was performed at a constant 30 mA current for 2 h.

Coomassie blue was used to stain the protein bands for 1–1.5 h.

3. RESULTS

The experiment started with bioreactor inoculation. Activated sludge with a typical flocculent morphology was used as an inoculum. The core of the flocs was built from the filamentous microorganisms and bacteria. The granulation was effectively only in the cation-feeding reactors and was apparent due to the changes in the sludge structure. In the Ca^{2+}–Mg^{2+}–Fe^{3+} – fed reactor, suspended flocs gradually disappeared from the reactor and the intense growth of biomass in granular form was observed. The granulation process was evaluated by measuring the diameter of aggregates and examining the granule structure using scanning electron microscopy. The cation-fed granules had a more rigid structure compared with non-cation-fed granules. In the control reactor without addition of cations, the biomass was almost completely washed-out after 30 days of reactor operation. The specific weight of cation-fed granules increased and they could be

retained inside the reactor. A detailed report of granule development and performance in the presence of Ca^{2+}, Mg^{2+} and Fe^{3+} was published (Kończak et al., 2014).

Figure 1 shows the fluorescent staining of a 20 μm granule cryosection. The fluorescence intensity of the EPS matrix components indicated that the dominant components of the granules include nucleic acids (live and dead cells, eDNA), protein and, interestingly, calcium cations. On the outskirts of the granules (0–500 μm deep into the granule), proteins and calcium ions were predominant. The surface layer of the granules (500–750 μm deep) was dominated by bacterial cells and the central part of granules (750–1250 μm deep) was filled by proteins and cells. The least intense fluorescence was noted in the case of β-polysaccharides primarily at the depth of 50–125 μm into the granules.
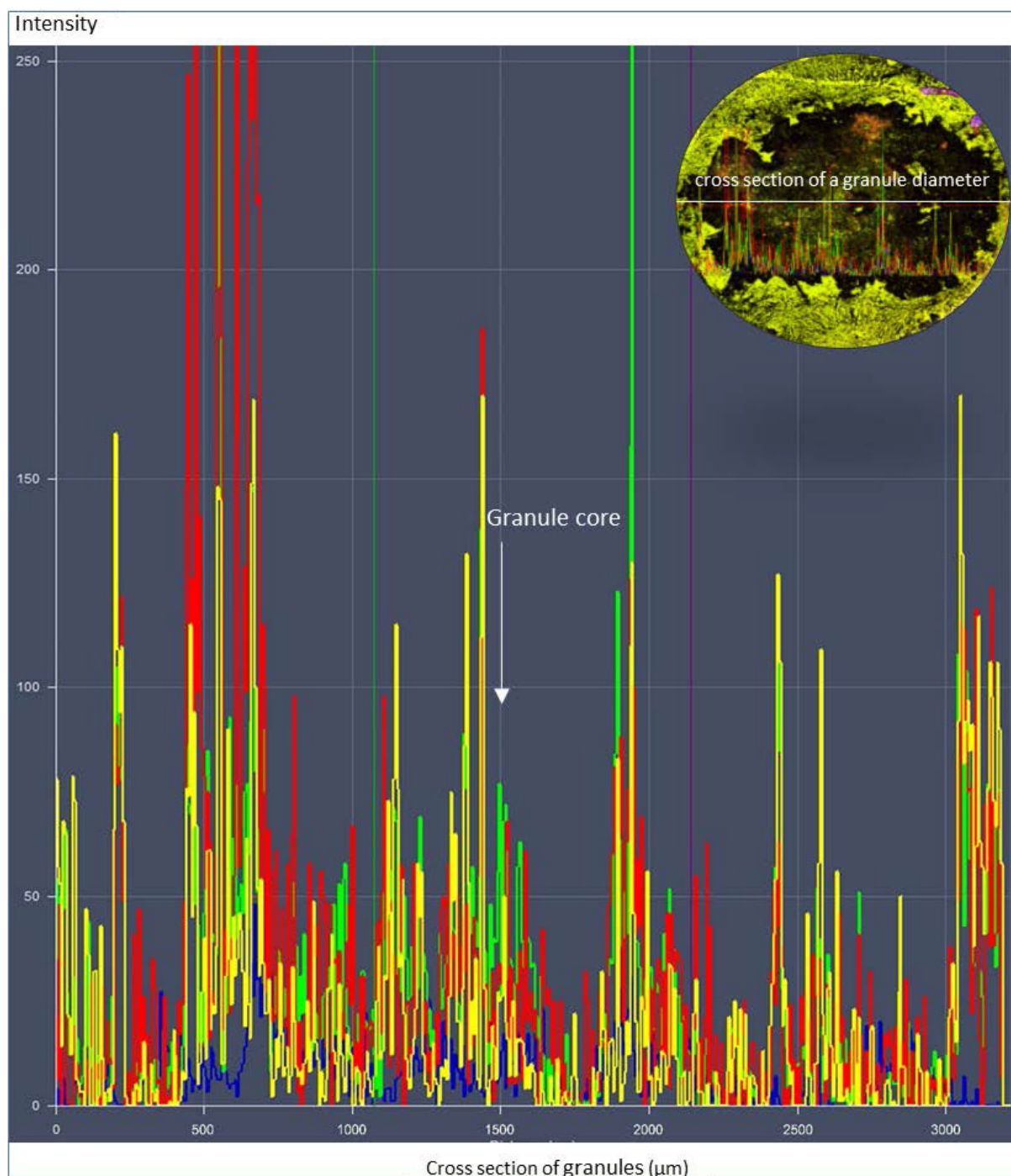
Fig. 1. Fluorescence spectra of various EPS in a 20 μm aerobic granule cryosection (blue line, β-polysaccharides (calcofluor white); red line, cells (SYTO 63); yellow line, Ca^{2+} ions (calcium green); green line, proteins (FITC))

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High protein concentrations in the outer and inner parts of the granule were associated with high levels of calcium ions suggesting their important role in the stabilization of the three-dimensional network of the granules. Subsequent investigation was focused on the changes in molecular mass of the proteins during granulation in the presence of cations and specifically in the presence of calcium ions.

The protein samples were characterized by SDS-PAGE. The analysis included the qualitative assessment of the proteins in the fraction of tightly bound EPS (TB-EPS) and in the supernatant samples (SOL-EPS).

The protein samples from different reactors were run on the same gel and the protein profiles are shown in Fig. 2.
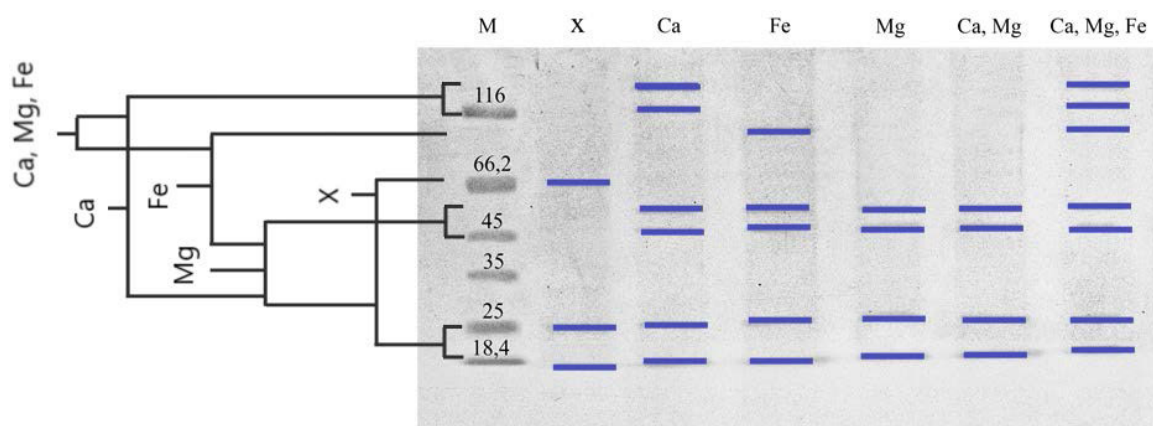
Fig. 2. Secretion of exoproteins in the presence of Ca^{2+}, Fe^{3+}, and Mg^{2+} after 23 days of granulation during the 1st stage of experiment (TB-EPS fraction). The position of the molecular weight markers (in kDa) is indicated on the left. M – markers, X – without cations

In the reactor without added cations, molecular weight of the exoproteins produced by microorganisms and tightly bound to the cells (TB-protein) were 18.4 kDa, 25 kDa and 66.2 kDa. The addition of Ca^{2+}, Mg^{2+}, and Fe^{3+} cations or their combinations to the granular sludge reactor changed the protein profiles manifested as appearance of the proteins with medium molecular mass including 45 kDa and 47 kDa proteins. In the samples from the cation-supplemented reactors, a 66.2 kDa protein was not detected. In general, the protein profiles obtained from aerobic granules formed in the presence of cations were similar. However, a new protein with high molecular mass ($\geq$ 116 kDa) was produced in the reactors with added Ca^{2+} and in the reactor supplemented with all tested cations (Ca^{2+}, Mg^{2+}, and Fe^{3+}).

The results of the second stage of the study showed that microorganisms secreted TB-proteins with higher molecular weight concomitant to an increase in the dose of Ca^{2+} (Fig. 3). The protein profiles obtained
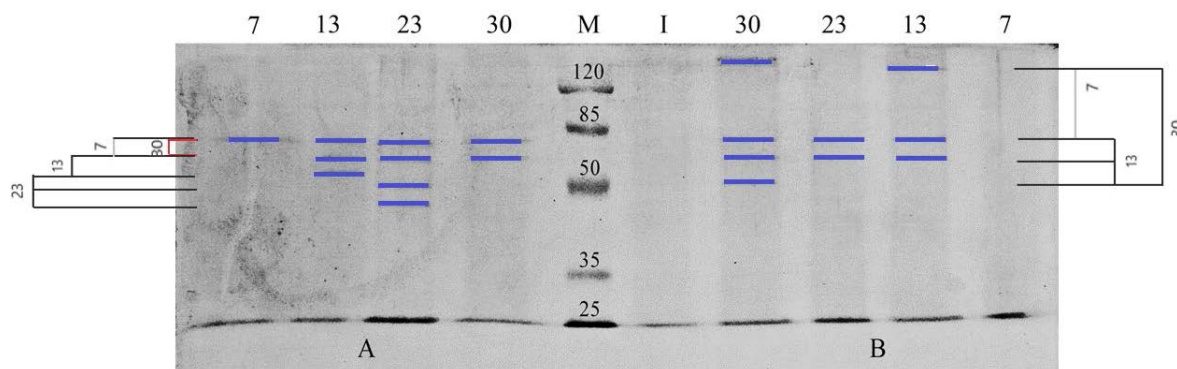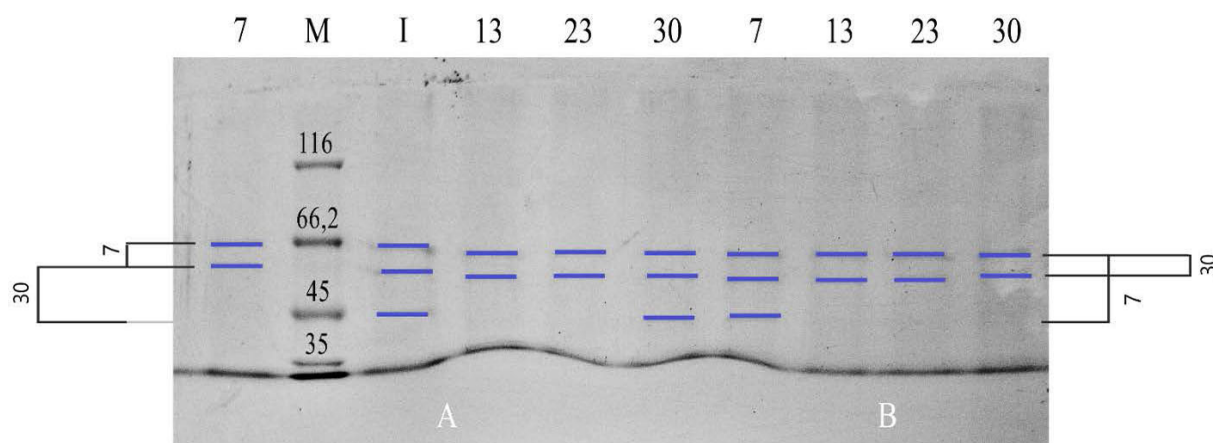

Fig. 3. Expression and secretion of exoproteins from day 7 to day 30 of granulation during the 2nd stage of experiment in the presence of calcium ions (TB-EPS fraction). A – Ca^{2+} (0.5 mmol/l); B – Ca^{2+} (1 mmol/l). M – markers; I – inoculum

in the samples from the Ca^{2+}-supplemented reactor at 1 mmol/l Ca^{2+} included the proteins with apparent molecular mass of 51 kDa, 67 kDa, 85 kDa and over 120 kDa. The addition of 0.5 mmol/l Ca^{2+} to the reactor caused the secretion of the 47 kDa, 51 kDa, 67 kDa and 85 kDa proteins.

The presence of cations in the reactor influenced only the TB protein secretion (Fig. 4). The results showed that the protein profiles in the supernatant (SOL-EPS) were identical in the samples prepared with and without cation additions. In all analysed samples, proteins with molecular mass of 51 kDa and 66.2 kDa were detected. From day 1 to day 30 of biogranulation, these proteins were observed in all samples. Moreover, the changes in the Ca^{2+} concentration did not significantly influence the protein profiles of the samples. The protein with molecular weight of 45 kDa was observed mostly in the samples where the sludge was supplemented with 0.5 mmol/l Ca^{2+}. Surprisingly, on days 13 and 23, this protein disappeared. In the reactor supplemented with 1 mmol/l Ca^{2+}, the 45 kDa protein was observed only on day 7 of biogranulation.

Fig. 4. Expression and secretion of exoproteins from day 7 to day 30 of granulation during the 2nd stage of experiment in the presence of calcium ions (SOL-EPS fraction). A – Ca^{2+} (0.5 mmol/l); B – Ca^{2+} (1 mmol/l). M – markers; I – inoculum

The SOL-protein profile of the samples from the reactors supplemented with Mg^{2+} or Fe^{3+} had only two proteins with molecular mass of 66.2 kDa and approximately 51 kDa (Fig. 5).
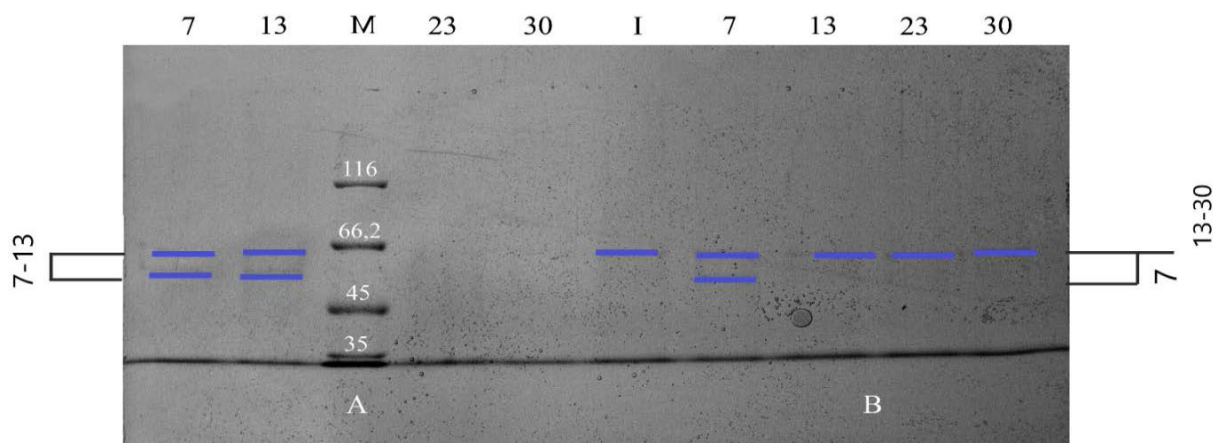

Fig. 5. Expression and secretion of exoproteins from day 7 to day 30 during the 2nd stage of experiment of granulation in the presence of Mg2+ and Fe3+ (SOL-EPS). A – Mg^{2+} (0.125 mmol/l); B – Fe^{3+} (0.075 mmol/l). M – markers; I – inoculum

After the granulation process had finished, the supernatant was free of the low molecular weight proteins.

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4. DISCUSSION

This study provides information on global changes in protein profiles resulting from a combination of two parameters, formation of aerobic granules and the presence of cations, separately or in combination in the feeding medium.

According to Garnier et al. (2005), the molecular weight of extracellular proteins extracted from the activated sludge varied from small (10 kDa) to large (600 kDa) sizes. Our results indicate that microorganisms produced proteins of various molecular weights during granulation. The molecular weight of tightly bound proteins varied from 18.4 kDa to over 116 kDa. In the supernatant, only medium molecular weight proteins were observed.

These results reveal that the proteins closely connected to the cells of the microorganism are more important for the granulation process than the freely suspended proteins. Initially, microorganisms produced the TB-proteins of low molecular mass that may help them to change their cell surface and initiate the contact. The connection of the cells in an aggregate induced secretion of proteins with higher molecular mass by the microorganisms. These proteins are used to form the three-dimensional structure of EPS and to bind the cells together. The mature granules (between days 23 and 30 of granulation) have the highest diversity of proteins with various molecular weights. Thus, it appears that the aerobic granulation can be closely associated with an increase in the extracellular TB-proteins secreted by the sludge.

Interestingly, TB-EPS extracted from the mature granules had an additional high molecular weight band compared to that extracted from the activated sludge (inoculum). We suggest that the changes in the TB-EPS profile can contribute to the synthesis of extracellular polymeric substances in response to high stress conditions during granulation. Moreover, a high molecular weight band detected in TB-EPS may play a role in the improved mechanical stability of aerobic granules.

Contrary to other authors (Hao et al., 2016), our research has shown that, apart from Ca^{2+}, the influence of other cations on the formation of granule proteins has not been apparent. Most proteins were formed in the Ca-feed reactor, and only in the reactor with all cations the effect was similar. It should be noted that this conclusion is valid for the bioreactor values and parameters tested. The Ca^{2+} cations play a special role in the formation of aerobic granules. Additional protein bands were observed at high Ca^{2+} concentration. These proteins were not observed in the aerobic granules generated in the absence of Ca^{2+} cations indicating that Ca^{2+} has considerable regulatory influence on production of extracellular proteins during aerobic granulation.

According to Morais et al. (2018) and Hao et al. (2016), the addition of calcium ions enhanced the uniformity of the structure of the granules and increased their mechanical strength. Ca^{2+} ions have a positive effect on the process of microbial attachment that influences the settling velocity of the granules. Binding of proteins to Ca^{2+} ions helps to stabilize their structure and to fold into a unique three-dimensional shape.

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