Production of Extracellular Polymeric Substances by Isolate Consortia Obtained from Mesophilic Aerobic Granules from the Treatment of Paper Mill Effluent

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Aerobic granules are large, compact microbial aggregates when compared to flocculent sludge, and they can be used in wastewater treatment. The application of aerobic granular sludge in bioreactors for the treatment of industrial effluents is still considered innovative and has been the subject of several recent studies. In the present study, 19 microbial isolates from mesophilic aerobic granules, obtained from a previous study, were evaluated in co-aggregation tests. The extracellular polymeric substances (EPS) produced, such as carbohydrates, proteins, and humic acids, were determined. The aim was to evaluate the relationship between the amount of EPS produced and the contribution of each isolate in the granulation process. The results of EPS production were used to analyze the polysaccharide / protein (PS / PN) ratio. The consortia with an absence of isolates 4, 8, 11, 14, 19, and 25 presented a PS / PN ratio <0.5. These isolates, identified as belonging to the genera Staphylococcus, Agrobacterium, *Enterobacter*, and *Rhodococcus*, were considered effective for the production and stability of the mesophilic aerobic granules.

Keywords: Aerobic granular sludge; Extracellular polymeric substances; Granule formation; Stability

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INTRODUCTION

The aerobic granular sludge process has increased the efficiency and reduced costs of effluent treatment (Li *et al.* 2014). The development of aerobic granular sludge treatment has demonstrated that the process can present a highly efficient removal of organic matter and nutrients (Morais *et al.* 2016a; Wei *et al*. 2014). The larger size, compact structure, and high biomass retention of aerobic granular sludge (AGS) results in a higher settling velocity, high surface area, dense and porous microbial structure, better filterability, and a high tolerance to organic loads and toxicity when compared to conventional activated sludge (Tay *et al.* 2002; Toh *et al.* 2003; Li *et al.* 2014; Morais *et al.* 2016a). These advantages in relation to conventional activated sludge have led to an increase of AGS treatment research due to the great potential for municipal and industrial wastewater application (Xie *et al*. 2019).

The formation of aerobic granules occurs by microbial aggregation, so aerobic granules can be defined as a mobile biofilm with a gel-like consistency (Weissbrodt *et al.* 2013).

The production and composition of EPS are important to determine the characteristics of the microbial aggregates in effluent treatment processes (Tay *et al.* 2002; Xuan *et al.* 2010). They are essential for the formation of microbial aggregates, since the bacteria are distributed in an EPS matrix. These substances have a structural and protective function for the aggregates and influence porosity, density, surface electric charge, hydrophobicity, mechanical stability, and other biofilm properties (Liu and Tay 2007; Wei *et al*. 2015).

EPS are a complex high-molecular-weight mixture of polymers, consisting mainly of polysaccharides (PS), proteins (PN), humic-like substances, nucleic acids, lipids, and glycoproteins, which involve bacterial cells and influence the formation and stability of aerobic granules (Gao *et al*. 2011, Wei *et al*. 2016). The cohesion of the granular sludge is obtained by increasing both the protein and polysaccharide EPS content (Caudan *et al.* 2014). The strength of the granules depends on the substrate and growth conditions. Denser granules are formed as a consequence of the growth of microorganisms with a slow growth rate and due to specific interactions involving the EPS of the granule matrix (Caudan *et al.* 2014).

Aerobic granules can take several weeks to form from activated sludge. Like other treatments, granule formation can be obtained by selecting microbial cultures. The inoculation of microorganisms into the bioreactor can contribute to accelerating granulation and increase granule strength (Ivanov *et al.* 2006; Adav *et al.* 2010; Wan *et al.* 2015). The mixture of a pure culture with high self-aggregation capacity and the activated sludge resulted in the formation of aerobic granules with a mean diameter of 446 μm after only 8 days of cultivation (Ivanov *et al.* 2006). Bacteria of the genus *Sphingomonas* sp. were present at the beginning of granulation in sequential batch reactors (SBR) fed with synthetic effluent, contributing to the maintenance of granule structure and functioning (Wan *et al.* 2016).

Interactions between aggregated bacteria facilitate metabolic interactions, intercellular communication, and genetic exchanges between cells (Maszenan *et al.* 2011). Studies have suggested that the physical characteristics of granules depend on the diversity of the microbial communities and the composition of the EPS associated with these communities (Caudan *et al.* 2014). Although the characteristics and dynamics of EPS are well-known in conventional activated sludge systems, information about these characteristics and dynamics in AGS systems is scarce (Xie *et al*. 2019; Zhang *et al*. 2019). Such information may contribute to a stable operation of AGS process.

In a previous study, the microorganisms found in the aerobic granular sludge that contributed to microbial aggregation and the increase of the mechanical resistance of the granules were determined. Nineteen strains were isolated from an aerobic granular sludge formed in an SBR treating paper mill effluent. The formation of the granules was obtained by microbial selection. Co-aggregation tests indicated that some isolates (2, 7, 9, 13, and 25) improved granule formation, while others (10, 14, 18, and 26) inhibited granulation (Morais *et al.* 2016b).

The objectives of the present study were to quantify the main components of the extracellular polymeric substances produced by microorganism consortia isolated in the aerobic mesophilic granulation process and to identify microorganisms that produce EPS that favor the formation of aerobic granule mesophilic cells.

EXPERIMENTAL

Methodology

Bacterial isolates from mesophilic granules

The mesophilic aerobic granules were obtained in a sequential batch reactor (SBR), fed with recycled paper mill effluent (Morais *et al.* 2016b).

Aliquots of 5 mL of granular sludge were submitted to three centrifugation steps at 650 g for 2 min. After each centrifugation, the supernatant was discarded and the pellet resuspended in 0.85% saline. At the end of the third centrifugation, the samples were subjected to ultrasonic pulses (20 kHz, 4 s) for sludge disintegration using the Ultrasonic homogenizer-Cole Palmer Instrument Company 4710 series Chicago Illinois 60648.

The samples disaggregated using the ultrasound were centrifuged (650 g, 2 min), and aliquots of 100 μL of the supernatant were removed to obtain serial dilutions of 10^{-1} to 10^{-9}.

Samples of 100 μL of each dilution were inoculated into Petri dishes containing solid R2A medium (0.5 g of hydrolyzed casein, 0.5 g of yeast extract, 0.5 g of peptone, 0.5 g of dextrose, 0.3 g of Dipotassium phosphate, 0.024 g of magnesium sulfate, 0.3 g of sodium pyruvate, 15.0 g of agar, and 1000 mL of distilled water). The plates were maintained at room temperature, and the resulting colonies were picked, spread on solid R2A media, and the purified colonies were isolated. Each pure culture was inoculated into liquid R2A medium and stored in ultra-freezer at -80 °C.

Identification of the isolates: DNA extraction

The isolates were inoculated into microtubes (2.0 mL) containing 1 mL of liquid TY medium (5g of Tryptone, 3 g of yeast extract, 0.9 g of calcium chloride dihydrate and 1000 mL of distilled water). The DNA extraction was performed after 24 h of incubation, according to the methodology described by Kennedy *et al.* (2008), with modifications. One mL of the lysis buffer (100 mM EDTA, 100 mMTris-HCl, 2% CTAB, 1% SDS, and 1.5 M NaCl) was added to each culture. The reaction mixture was homogenized in a vortex and heated for 20 min in a water bath at 65 °C (mixed every 5 min). The mixture was then centrifuged for 20 min at 2800 g. The aqueous phase was transferred to another microtube (2.0 mL) and the phenol: chloroform: isoamyl alcohol (25:24:1) solution was added to the same volume as that collected and vortexed for 1 min, followed by centrifugation for 10 min at 2800 g. The supernatant was transferred to another microtube (2.0 mL), and 0.7 volume of isopropanol (100%) and 0.1 volume of 3 M sodium acetate were added. The tubes were shaken 10 times and incubated at -20 °C for approximately 12 hours. The tubes were centrifuged three times for 10 min at 2800 g, discarding the supernatant, followed by washing the pellet with 70% alcohol and further centrifugation. The pellet was allowed to dry and then resuspended in 40 μL of sterile Milli-Q water.

DNA amplification

DNA from each isolate was amplified with the primers 27F (5'-AGAGTTT-GATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3'). The PCR mixture contained 0.3 μL of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.5 μL of each primer (10 μM), 2.5 μL of the 10X reaction buffer, 0.5 μL Of dNTP's (10 mM), 1.5 μL MgCl 2 (50 mM), 1 μL of the DNA sample, and 18.2 μL of sterile Milli-Q water per sample. The amplification was performed with a thermocycler (Mastercycler Personal, Eppendorf, Hamburg, Germany) under the conditions: 94 °C for 5 min, followed by 14

cycles (94 °C for 30 s, 65 °C for 40 s, and 72 °C for 1 min), 15 cycles (94 °C for 30 s, 50 °C for 40 s, and 72 °C for 1 min), and finished with extension at 72 °C for 7 min.

The amplicons were sequenced by ACTGene Laboratory (Biotechnology Center, Federal University of Rio Grande do Sul, Porto Alegre, Brazil) using an ABI-Prism 3100 Genetic Analyzer with 50 cm capillaries and POP6 polymer (Applied Biosystems 2016).

Sequence analysis was performed with the MEGA (V. 6.0) and Bioedit, and compared to the GenBank database using BLAST (NCBI 2015) for isolate identification.

Co-aggregation index with absence of an isolate

Twenty co-aggregation assays were carried out from consortia of the isolates, one containing all 19 isolates and used as the control, and 19 other consortia lacking one particular isolate at a time. The consortium with all the isolates was called the control and the others were identified by "-n" which indicates the isolate absent from the consortium. For example, the consortium "-1" signifies the combination of 18 isolates but lacking isolate 1, and so on.

Each isolate was initially inoculated into liquid R2A medium and incubated at 30 ± 1 °C under shaking (110 ± 10 rpm) for 48 h.

Aliquots of the liquid cultures for each isolate, with optical density (OD) equal to 1.00 mL^{-1} (λ = 600 nm), were inoculated into 75 mL of liquid R2A medium in 250 mL Erlenmeyers. The consortia were incubated at 30 ± 1 °C with shaking (110 ± 10 rpm) and 5 ml samples of each Erlenmeyer were taken after 2, 4, 8, 24, 48, and 72 h of incubation. The OD were measured (λ = 600 nm) and the samples stored for EPS extraction and analysis.

EPS extraction and analysis

One mL of each sample was transferred to microtubes (2.0 mL), centrifuged (11200 g, 4 °C, 15 min), and the supernatant stored in another microtube for quantification of free EPS. The pellets were resuspended in 20 mL of phosphate buffer. The pH was adjusted to 11 by the 1M NaOH, followed by heating (80 °C, 30 min). After this extraction step, the samples were centrifuged (11200 g, 4 °C, 10 min) and the supernatant stored for further analysis of the bound-EPS (McSwain *et al.* 2005).

The chemical characterization of the EPS was carried out through the carbohydrate, protein and humic acid content analysis.

The EPS content of the aggregates was measured in each consortia of isolates and performed in triplicate.

Carbohydrate quantification

The phenol-sulfuric acid dosing method was used in which 0.25 mL of phenol (5%) and 1.25 mL of sulfuric acid were added in 0.5 mL of each sample. After 10 minutes, the tubes were shaken and placed in an ice bath for 20 minutes. The calibration curve using sucrose (0-1.0 $mg.mL^{-1}$) was used as the standard. Absorbance values were measured in a spectrophotometer at 490 nm (Albalasmeh *et al.* 2013).

Proteins and humic acids quantification

The modified Lowry method was used (Frolund *et al.* 1996), with two reagents (A and B) for the reactions. Reagent A was produced by the addition of three solutions in the ratio of 1:1:100 (1-35 $g.L^{-1}$of CuSO4, 2-70 $g.L^{-1}$ of $KNaC_4H_4O_6$:$4H_2O$ solution and 3-70 $g.L^{-1}$ of Na_2CO_3). Reagent B was produced with a composition similar to reagent A except

for replacing the CuSO4 solution by deionized water. In the reactions, the Folin-Ciocalteau reagent (2N) diluted 10 times was also used.

The solutions were added in the following sequence: sample: Reagent A: Folin Reagent in the ratio 1:1:1. The reaction mixture was shaken, and after 30 min absorbance was measured in a spectrophotometer at 750 nm. The same procedure was carried out with Reagent B. A calibration curve of 0-1.0 mg.mL^{-1} of albumin was used as the control.

For the protein determination the Eq. 1 was used:

$$Abs_{proteins} = 1.25 \cdot (Abs_A - Abs_B) \quad (1)$$

The determination of humic acids was performed using Eq. 2:

$$Abs_{humic\ acids} = Abs_B - (0.2 \cdot Abs_{protein}) \quad (2)$$

Total Organic Carbon (TOC) determination

TOC was determined by the catalytic oxidation method (Xuan *et al.* 2010) with a carbon analyzer (Shimadzu® 2016).

RESULTS AND DISCUSSION

Isolate identification

Nineteen bacterial isolates were obtained and identified (Table 1), belonging to five bacterial genera. Only isolate 3 could not be identified.

Table 1. Identification of the Isolates Obtained from the Mesophilic Aerobic Granules formed in the Treatment of Recycled Paper Mill Effluents

Isolates	Genus
1, 4, 5, 10, 15, 18 and 23	*Acinetobacter*
2, 13 and 19	*Agrobacterium*
3	----------
7, 8, 11, 14, 24 e 26	*Enterobacter*
9	*Staphylococcus*
25	*Rhodococcus*

Coaggregation Assay

The importance of each isolate in the granule formation process and its ability to coaggregate with the others was evaluated in a prior study (Morais *et al.* 2016b).

Combinations in which the isolates 4, 10, 14, 18, and 26 were absent formed larger aggregates than the control. This could indicate that these isolates inhibit granulation and, in their absence, the important bacteria for granule formation can increase in number, leading to the development of larger aggregates.

The absence of isolates 2, 7, 9, 13, 19, and 25 resulted in small aggregates or an absence of visible aggregates, indicating that these isolates may be important in the process and their presence may contribute to the formation of aerobic granules.

Optical Density of Consortiums

Optical density (OD) is one of the techniques commonly used to estimate cell concentration in liquid cultures (Myers *et al.* 2013). The consortia showed minimal alterations in OD between 48-72 h (Fig. 1), demonstrating the continuation of microbial growth, which can lead to the production or release of EPS that may favor the formation of mesophilic aerobic granules.

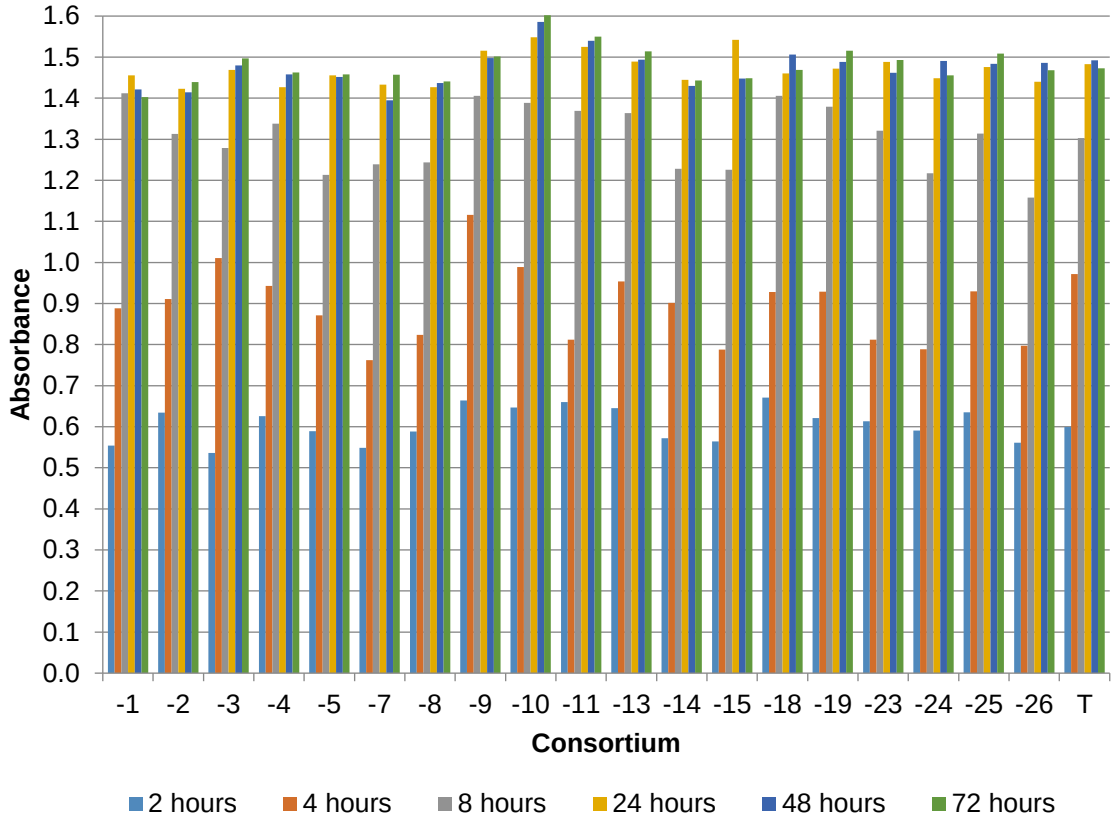

Fig. 1. Absorbance of samples of each consortium during co-aggregation experiment. Optical density (λ = 600 nm). (-n = consortium with absence of the n^{th} isolate, T = control consortium with all isolates).

Optical density measurement is a fast and reliable method, but it is based on the assumption that bacteria grow as single cells of equal size and that the cells are uniformly dispersed in the liquid culture (Haaber *et al.* 2016). In the case of cultures forming clusters of cells with a three-dimensional structure, the correlation between the optical density data and the number of cells must be verified, since part of the biomass can cover internal layers of cells (De Carvalho *et al.* 2005). Other challenges that may potentially affect the measurement of optical density include viable but uncultured cells or non-viable but intact cells present in the culture as well as cells in the dividing process (Haaber *et al.* 2016).

In a bioreactor, the optical density is correlated with the concentration of volatile suspended solids in the aeration tank (SSVTA). The OD followed the variations of the SSVTA during the process of aerobic granule formation in an SBR for the treatment of 2,4-dichlorophenol (Khan *et al.* 2011). Additionally, the presence of toxic substances inhibits growth and reduces OD. The addition of 2 $mg.L^{-1}$ and 3 $mg.L^{-1}$ of Cr^{6+} caused a

reduction in OD of 1.0 to 0.8 and 0.3, respectively (Yang *et al.* 2016), while the addition of 3.5 mM of sodium dodecyl sulfate caused an OD decrease from 1 to 0.2 within only 30 minutes of incubation (Klebensberger *et al.* 2006).

EPS Determination

Carbohydrate content

The consortia with the absence of isolates 5, 7, 13, 15, 19, 24, 25, and 26 presented carbohydrate production between 48 and 72 h (Fig. 2).

In the studies developed by (Morais *et al.* 2016b) isolates 2, 7, 9, 13, 19, and 25 were indicated as being favorable for aggregate formation. In general, the consortia with an absence of isolates indicated as contributing to aggregate formation maintained or increased carbohydrate production between 48 and 72 h (Fig. 2).

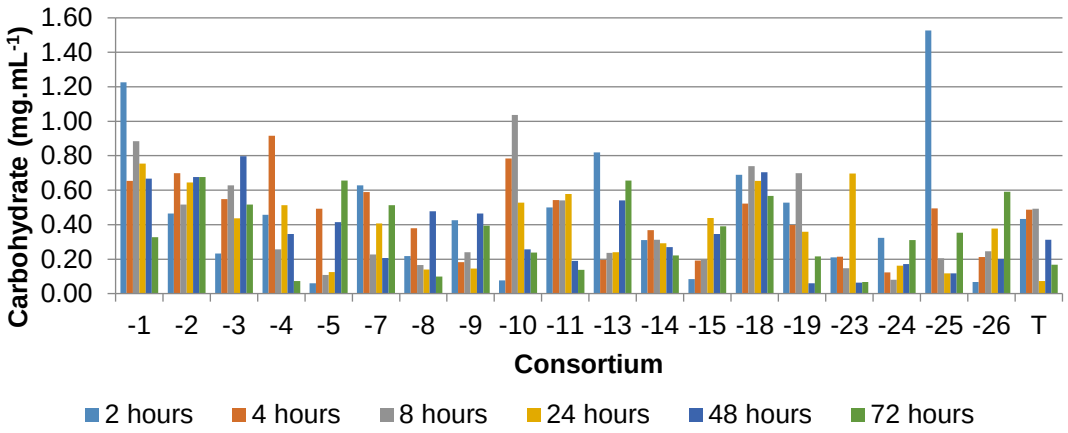

Fig. 2. Carbohydrate concentration (mEq.mL^{-1}of sucrose) during co-aggregation experiment. (-n = consortium with absence of the nth isolate, T = control consortium with all isolates)

Therefore, these isolates can impair the formation of granules, because the polymeric polysaccharides act, mainly, in the adhesion of the bacteria favoring the formation of micro-colonies. Polysaccharides serve as bridges or connections between smaller granules, favoring the formation of larger granules (Jiang *et al.* 2002; Liu *et al.* 2004). On the other hand, carbohydrates are hydrophilic components of EPS and excess carbohydrate production (especially when coupled with a low protein/carbohydrate ratio) results in an increase in the absolute value of zeta potential, impairing the formation of granules (Tu *et al.* 2010). Thus, the isolates indicated as harmful to the granulation process may have reduced the formation of aggregates due to their higher production of carbohydrates.

The reduction in carbohydrate content observed in some consortia may be associated with their use as a carbon source by the bacteria present in the consortia for their metabolic activity (Zhang *et al.* 1999). The consortia -4, -10, -14, and -18, which lack the aggregates harmful to the granulation process (aggregates 4, 10, 14, 18, and 26), showed a rapid increase in carbohydrate content at the initial stage (reduction of microbial growth after 48 h, as presented in the OD results). This behavior is similar to that observed by Xie *et al.* (2019). The reduced carbohydrate content observed in some consortia may be associated with its use as a carbon source by the bacteria present in the consortia for their metabolic activity (Zhang *et al.* 1999). At the beginning of the granulation process, the

sludge tends to produce more EPS as a way of increasing the aggregate's size and reducing the selective sludge washout pressure. Polysaccharides play an important role in this process of formation of large-sized aggregates and, therefore, these substances increase significantly. However, after aggregate formation and maturation, oxygen and substrate deficiency in the core regions of the granules leads to the utilization and reduction of the more easily biodegradable EPS compounds, mainly polysaccharides (Xie *et al.* 2019).

According to Seviour *et al.* (2012), aerobic granules contain a multispecific microbial community, which synthesizes a great diversity of exopolysaccharides that play a structural role and are distinguished from the conventional activated sludge by the presence of gelatinous exopolysaccharides.

The granular sludge settleability is related to the amount of EPS produced (Kim *et al.* 2014). Polysaccharides are important for granule formation, but the stability of the granules depends on the protein core (Zhu *et al.* 2015). The amount of EPS depends, among other factors, on the microbial community present in the granule and on the reactor operating conditions. The decrease in the sedimentation time caused the loss of the microorganisms by means of slow sedimentation and an increase in the microbial community with good flocculation, which resulted in the rapid increase of protein (PN) and polysaccharides (PS) levels. However, with granular sludge maturation, the PS content decreased due to the consumption of the polysaccharides by the microorganisms during the starvation phase (Zhu *et al.* 2012).

Protein content

The consortia that presented an absence of the isolates 2, 4, 5, 8, 23, 25, and 26, showed protein production between 48 and 72 h (Fig. 3).

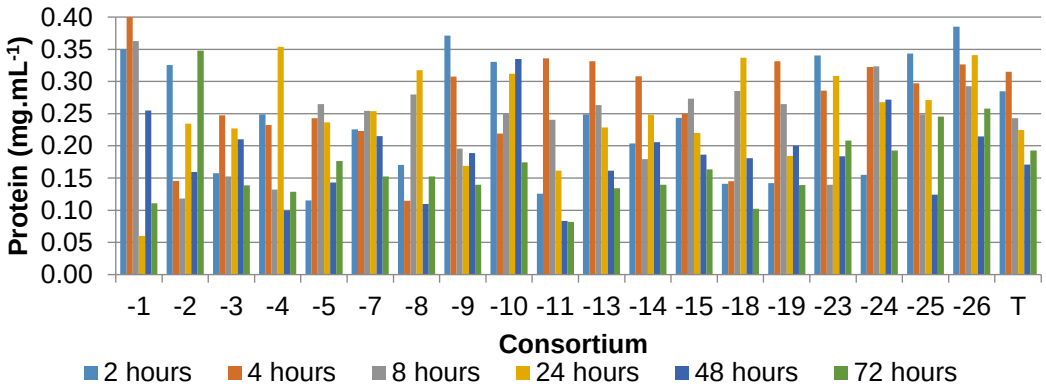

Fig. 3. Concentration of proteins (mEq.mL^{-1}of albumin) during co-aggregation experiment. (-n = consortium with absence of the nth isolate, T = control consortium with all isolates)

Consortia lacking four isolates (7, 9, 13, and 19) (Fig. 3), indicated as contributors to the formation of aggregates in the previous study (Morais *et al.* 2016b), showed a decrease in protein content. This may indicate that these isolates can act by maintaining or increasing protein concentration and favoring the formation of more stable aerobic granules.

The absence of these isolates can inhibit the formation of granules with greater stability, since the proteins are responsible for the formation and structural stability of the granules (McSwain *et al.* 2005; Di Laconi *et al.* 2006; Tu *et al.* 2010).

The protein content of aerobic and anaerobic granular sludge is higher than observed in flocculent sludge and presents a negative linear correlation with sludge surface loading, favoring the formation of granules (Zhu *et al.* 2015). The amount of polysaccharides and proteins in the EPS of the granular sludge is approximately 1.3 to 2.8 times greater than the conventional activated sludge content (Wei *et al*. 2018).

The reduction of the protein content observed in some consortia may also be associated with their hydrolysis and partial utilization by microorganisms present in the consortia for their metabolic activity, especially in the endogenous decay phase (Zhang *et al.* 1999; Wei *et al.* 2018).

The proteins are hydrophobic constituents of the EPS and contribute to a decrease in the zeta potential, favoring formation, as well as increasing the structural stability of the granules (McSwain *et al.* 2005; Di Laconi *et al.* 2006; Tu *et al.* 2010). The high content of negatively charged amino acids of the proteins contributes to the greater amount of electrostatic bonds with the multivalent cations in stabilizing aggregate structures (Wei *et al*. 2018). Thus, tryptophan and protein-like substances are the main EPS components in stable granules (Zhang *et al*. 2019).

EPS production of the granular sludge in a sequential batch reactor increased during the formation of the granules and the PN were the main component of the EPS in the whole bio-granulation process (Wei *et al*. 2018). The amount of PN was 3.9 times higher than that of PS and contributed to the improvement of the structural stability of the granules (Wei *et al*. 2018).

Humic acid quantification

The consortia lacking isolates 1, 3, 9, 10, 11, 14, 15, 18, 19, and 24, presented increased humic acid production in the period between 48 and 72 h, related to growth stabilization (Fig. 4).

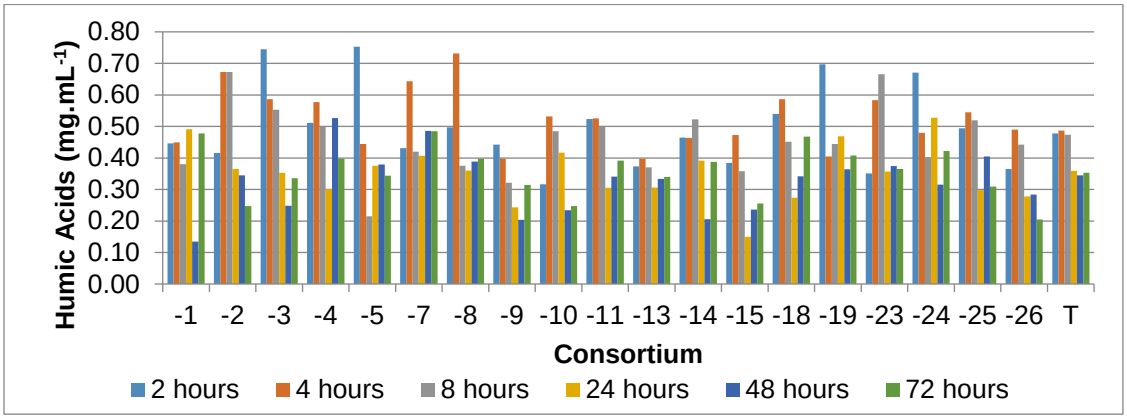

Fig. 4. Concentration of humic acids (mEq.mL^{-1} of albumin) during co-aggregation experiment. (-n = consortium with absence of the nth isolate, T = control consortium with all isolates)

Consortia with an absence of four isolates (2, 7, 13, and 25), indicated as contributing to aggregate formation (Morais *et al.* 2016b), showed a reduction or maintenance of humic acid content. Such behavior may indicate that these isolates can act by maintaining or increasing protein concentration, which may favor the formation of more compact and stable aerobic granules.

On the other hand, the consortia -10 and -26, with absence of isolates harmful to aggregate formation, had lower concentrations of humic acids in the stage of the microbial growth stabilization (after 48h of incubation). Humic acids are mainly derived from the adsorption of compounds present in the medium and hydrolysis of other biopolymers, such as proteins (Zhang *et al*. 2018). The study by Zhang *et al*. (2018) indicated that the humic acid-like substances can be detrimental to the stability of the granules. Thus, removal of isolates 10 and 26 may have contributed to improved aggregation.

Humic acids are components of the EPS that are produced during the formation of the aerobic granules. The function of humic acids and proteins, is to act on the granule structure (Gao *et al.* 2011).

According to the EPS quantifications, the PN/PS rate was determined, which evaluates the relationship between nitrogenous (PN) and polysaccharide (PS) content.

The PN/PS ratio can positively influence hydrophobicity and zeta potential, contributing to the increase in aggregative ability (Zhang *et al.* 2015). Additionally, granules formed with a PN/PS ratio of less than 1 are susceptible to disaggregation, since cell-cell interactions are weak (Jiang *et al.* 2002; Liu *et al.* 2004; Zhang *et al.* 2015).

The consortia -4, -10, -14, -18, which do not contain the isolates harmful to granulation, were absent, presented, except for -26, a PN/PS ratio ≥ 1 (Table 2). A higher PN/PS ratio enhances hydrophobicity and contributes to greater aggregation and stability of the aggregates (Zhang *et al.* 2015, 2018). In addition, the higher PN content may be related to the stabilization of aggregates and PS consumption due mainly to the reduction of substrate in the internal layers of the granules, which leads to the consumption of the easily biodegradable EPS components, especially polysaccharides.

Table 2. Nitrogenous (PN)/polysaccharide (PS) Substance Ratios from the EPS Extracted from the Samples after 72 h of Consortia Growth, in Liquid R2A Medium, (aeration at 30 ± 1 °C in the dark)

PN/PS	Consortia
1.80	-1
0.88	-2
0.92	-3
7.19	-4
0.79	-5
1.24	-7
5.56	-8
1.15	-9
1.77	-10
3.45	-11
0.72	-13
2.38	-14
1.07	-15
1.00	-18
2.35	-19
8.43	-23
1.97	-24
1.57	-25
0.78	-26

The PN/PS ratio of the granules is higher than that of the activated sludge (Sajjad and Kim, 2015; Zhu *et al.* 2015). Aerobic granules presented higher protein content (PN/PS between 1.4 and 1.6), while flocs had higher polysaccharide content (PN/PS = 0.5) (Basuvaraj *et al.* 2015). The PN/PS ratio may vary throughout the granule formation process. During simultaneous nitrogen and phosphorus removal in a reactor with granular sludge, the PN and PS content was 60.2 and 12.5 $mg.g^{-1}$ VSS, respectively, resulting in a PN/PS ratio of 4.8. During granule formation there was an increase in the concentration of PN and PS and in the PN/PS ratio. The matured granules presented concentrations of 101.1 $mg.L^{-1}$ PN and 15.8 $mg.L^{-1}$ PS after 150 days of reactor operation, corresponding to a PN/PS ratio of 6.4 (Wei *et al*. 2014). In addition, the chemical composition of EPS was influenced by the feeding COD/N ratio. In the sludge used as inoculum and in the system with COD/N equal to 6, there was a predominance of tryptophan PN-like substances and aromatic PN-like substances, respectively, whereas in systems with COD/N equal to 7 and 9 there was a predominance of humic acid-like substances (Wei *et al*. 2014).

The PN also present higher affinity for cations such as Ca^{2+} and Mn^{2+} and facilitate the binding of EPS with these ions, reducing surface charge and promoting cell adhesion (Zhu *et al.* 2015). The higher affinity of the proteins for cations was also observed in a study of Zn sorption onto aerobic granular sludge. PN-like substances were also more susceptible to Zn (II) adsorption than humic-like substances (Wei *et al*. 2016). In addition, increasing the doses of Zn (II) from 0 to 600 $mg.L^{-1}$ reduced the PN/PS ratio from 3.98 to 2.32, as well as PN concentrations from 23.55 to 9.55 $mg.g^{-1}$ VSS and PS from 5.92 to 4.11 $mg.g^{-1}$ VSS (Wei *et al*. 2016). These results indicate the importance of EPS in ion binding and granule formation.

The isolates favoring the formation of the granules (2, 7, 9, 13, 19, and 25) belong to four of the five genera identified (Table 3). Bacteria of these four genera are described as producing EPS with characteristics that favor the formation of biofilms and granules (Lv *et al.* 2014).

Table 3. Identification of the Isolates Favorable to Formation of Aerobic Granules

Isolate	Identification
2	*Agrobacterium* sp.
7	*Enterobacter* sp.
9	*Staphylococcus* sp.
13	*Agrobacterium* sp.
19	*Agrobacterium* sp.
25	*Rhodococcus* sp.

Bacteria of the genera identified in Table 3 are described as producing EPS with characteristics that favor the formation of biofilms and granules.

Enterobacter strains have been reported as producing intracellular adhesion polysaccharides, which are primary substances directly involved in the formation of biofilms and granules (Limoli *et al.* 2015). An *Enterobacter* strain demonstrated better EPS production by maintaining the pH at 7.0 and the temperature at 30 °C (Torres *et al.* 2012). The growth curve of *Enterobacter aerogenes* showed that EPS secretion was parallel to cell growth, with the highest EPS release observed in the initial stationary phase (Salehizadeh and Yan 2014).

The EPS produced by *Rhodococcus* strains have biofilm protection properties, and some species have been widely used in bioremediation processes to reduce contaminants

in water and soil (Pen *et al.* 2015; Rodrigues *et al.* 2006). Rhodobacteraceae and other organisms with high EPS production, especially tryptophan and protein-like substances, contribute to microbial aggregation (Zhang *et al*. 2019). *Rhodobacter* spp., *Thauera* spp., and *Pseudoxanthomonas* spp. were the main microrganisms for stable granules in two bioreactors with different C/N ratios. These microorganisms were related to high EPS secretion and the increased hydrophobicity of the granules (Zhang *et al*. 2018).

Isolates from *Agrobacterium sp.* were observed on the surface of granules and generated large amounts of protein and flocculants. In addition, the use of phosphorus in the production of EPS of *Agrobacterium* strains was evaluated, and it was found that phosphorus limitation increases the production of substances that help in the formation of biofilms and granules by species of this bacterial genus (Huang *et al.* 2012). The use of phosphorus in the EPS production of *Agrobacterium* strains was evaluated by Ma *et al.* (2016). The limitation of phosphorus increases the production of substances that contribute to the formation of biofilms and granules by species of this bacterial genus.

Hydrophobic interactions are important for the coaggregation of *Acinetobacter sp*. and other sludge-forming bacteria (Phuong *et al.* 2009).

Isolates from *Flavobacterium croceum* and *Agrobacterium sp.* were observed on the surface of granules and generated large amounts of protein and flocculants that contribute to the formation of biofilms and granules (Huang *et al.* 2012).

Cationic exopolysaccharides such as intercellular adhesin and poly-N-acetylglucosamine found in biofilms of *Staphylococcus epidermidis* and *Staphylococcus aureus*, respectively, play an important structural role in biofilms (Seviour *et al.* 2012). *Staphylococcus aureus* is a human pathogen that causes a large number of diseases ranging from benign skin infections to fatal conditions such as bacteremia, infective endocarditis, and chronic infections (Haaber *et al.* 2016). The pathogenicity of *S. aureus* is related to its high capacity for self-aggregation and to form biofilms. Additionally, *S. aureus* aggregates efficiently in human plasma, a condition that is related to virulence, and can form planktonic aggregates that protect cells against antibiotics (Haaber *et al.* 2016).

CONCLUSIONS

1. Nineteen cultivable isolates were obtained from the mesophilic aerobic granules produced during the proposed treatment. Based on the extracellular polysaccharides (EPS) results, it was noticed that there are differences in the effectiveness of the influence of each isolate on the production of EPS that contributes to granule formation. Among the isolates obtained, six (2, 7, 9, 13, 19 and 25) were indicated as important for the formation and maintenance of the mesophilic aerobic granules.
2. The isolates indicated as important for granule formation were identified as belonging to the genera *Agrobacterium, Enterobacter, Rhodococcus* and *Staphylococcus* and are reported in the literature as being high EPS producers, helping in the formation / maintenance of biofilms and granules.
3. The coaggregation test, associated with EPS analysis and quantification, could be used to determine the isolates that effectively contribute to the formation and maintenance of mesophilic aerobic granules.

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