Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2016.1192490

57 (2016) 28537–28550
December

Aerobic granular sludge to treat paper mill effluent: selection of ideal strains that contribute to the formation of strong aggregates

Ismarley Lage Horta Morais[a], Claudio Mudadu Silva[a,*], Antônio Galvão do Nascimento[b], Natalino Perovano Filho[c], João Carlos Teixeira Dias[c]

[a]*Depto Eng. Florestal, Universidade Federal de Viçosa, Campus UFV, DEF, Viçosa, MG, 36.570-000, Brazil, Tel. +55 31 3899 2717; Fax: +55 31 3899 2490; emails: ismarley.morais@ufv.br (I.L.H. Morais), mudado@ufv.br (C.M. Silva)*
[b]*Depto de Microbiologia, Universidade Federal de Viçosa, Campus UFV, DEF, Viçosa, MG, 36.570-000, Brazil, email: agalvao@ufv.br*
[c]*Programa de Pós-Graduação em Biologia e Biotecnologia de Microrganismos, Universidade Estadual de Santa Cruz, Campus Soane Nazaré de Andrade, Rod. Jorge Amado, km 16, Salobrinho, Ilhéus, BA, 45662-900, Brazil, emails: npfilho@uesb.edu.br (N.P. Filho), dias.jct@gmail.com (J.C.T. Dias)*

Received 9 December 2015; Accepted 13 May 2016

ABSTRACT

The formation of aerobic granules after the treatment of paper mill effluents was feasible. Granular sludge presented a lower fouling potential than flocculent sludge, allowing a higher flux through the membranes in a membrane bioreactor (MBR). However, it was observed that after filtration many of the biogranules disrupted, which increased the membrane fouling. Therefore, the formation of aggregates that are physically and mechanically more resistant to decomposition would be beneficial to an MBR process. This work determines which of the microorganisms found in the aerobic granular sludge contribute to microbial aggregation, thereby increasing the mechanical resistance of aggregates. Nineteen strains were isolated from granular aerobic sludge. Tests for coaggregation showed that some isolates (E2, E7, E9, E13, and E25) were able to improve the formation of granules, while others (E10, E14, E18, and E26) inhibited the aggregation. The extracellular polymeric substances analyses indicated which substances contributed to the granule formation. The mechanical resistance of the aggregates was determined and the results showed that isolate E19 substantially improved the resistance of the aggregates to disruption.

Keywords: Aerobic granular sludge; Paper mill; MBR; Aggregate mechanical resistance; Wastewater treatment

1. Introduction

Microorganisms are found in a wide variety of ecosystems where they form biofilms that can be composed of several species. Biofilms are functional consortia among cells and have a higher metabolic activity than the isolated species. They are commonly found attached to substrates or dispersed in a liquid medium from which they obtain nutrients [1]. The strength and specificity of the interactions in the biofilm environment favor the survival of bacteria in adverse conditions (such as shear forces and deficiency of nutrients), allowing them to survive and

*Corresponding author.

1944-3994/1944-3986 © 2016 Balaban Desalination Publications. All rights reserved.

proliferate in conditions where nonaggregated cells would exhibit reduced growth [1].

In wastewater-activated sludge treatment processes, microorganisms in the aeration tank tend to be organized in the form of biological flocs. Flocculation is the result of microbial metabolism and tends to occur when the food availability is limited or the microbial community is subjected to some other type of stress [2]. Good flocculation and settling characteristics of the sludge are fundamental to the performance of the wastewater treatment process.

Recent studies have shown that under certain conditions the microbial community can organize itself into the form of granules. The morphology of aerobic biogranules is completely different from other types of biofilms, such as those found in activated sludge flocs. This new sludge morphology is still under investigation and currently has little use in wastewater treatment systems.

In order to use aerobic granular sludge in the treatment of wastewater, it is essential to form and maintain the aerobic granules in the reactor. An inoculum from a conventional activated sludge system can take weeks to start forming granules. Several factors can interfere with the formation of granules, such as the type of substrate used, organic load, shear forces, sedimentation speed, and, above all, the microbial cultures present in the environment [3,4].

The formation of granules can be achieved by the selection of microbial cultures. The addition of selected strains or the enrichment of cultures with particular characteristics can be advantageous to the treatment system. When microorganisms are inoculated and incorporated into the sludge, they can remain in the aggregates for a long time, contributing to aggregate formation while maintaining resistance to degradation [3]. [3] cited various studies that demonstrated a significant reduction in the time required for the formation of dense granules with low values of Sludge Volumetric Index after the addition of certain strains.

One strategy used to select bacterial cultures that form aggregates and examine their ability to accelerate the formation of granules is to select some aggregates, isolate the cultures, and then study their re-aggregation [3].

In a recent study [5], the formation of aerobic granules after the treatment of paper mill effluents was observed. The authors compared the treatment of paper mill effluent with the aerobic granular sludge to treatment with the conventional flocculent sludge and concluded that both types of treatment are highly effective at removing chemical oxygen demand. During the membrane bioreactor (MBR) process, the potential for clogging of the polymeric membranes by granular or flocculent sludge was also compared. Granular sludge presented a lower fouling potential, allowing a higher flux through the membranes. However, it was observed that after filtration many of the biogranules brokedown, which increased the membrane fouling and consequently reduced the fluxes. Thus, the formation of aggregates that are physically and mechanically more resistant to decomposition would be beneficial to an MBR process using granular sludge.

2. Objectives

The objective of this study was to determine which of the microorganisms found in the aerobic granular sludge during the treatment of a paper mill effluent contribute to microbial aggregation, thereby increasing the mechanical resistance of aggregates.

The specific objectives were as follows:

(1) To isolate the microorganisms present in the aerobic granular sludge.
(2) To perform coaggregation tests to determine which isolates contribute to the formation of aggregates.
(3) To quantify the extracellular polymeric substances (EPS) of the best consortia which formed the largest granules.
(4) To evaluate the aggregates' resistance to disruption by ultrasound.

3. Methodology

3.1. Experimental plan

The experiments were conducted in five steps in order to understand the process of granule formation, explore the potential for a reduction in the time required for the formation of granules, and form aggregates more resistant to disruption.

Step 1 consisted of the breakdown of granules obtained from a sequential batch reactor and the isolation of pure cultures through plating and serially streaking isolated colonies. Twenty-six pure cultures were obtained and named Isolate E1, Isolate E2, and so forth until Isolate E26. The stock vials from the 19 remaining isolates were stored at –80˚C.

In Step 2, different consortia of isolates were prepared, one containing all isolates and used as the control in the following steps, and 19, each with a different isolate excluded. In some of these consortia, the aggregates formed were more numerous and larger in size than those in the control sample, indicating that the absent isolate may inhibit the formation of granules. Therefore, the absence of that particular

isolate may allow the growth of other isolates that are important to the process of aggregation. On the other hand, in some consortia, the aggregates formed were smaller in size and in quantity than the control, indicating that the missing isolate may be important to the formation of granules.

In Step 3, the isolates were paired to further investigate their importance to the formation of granules. During this step, the E19 isolate was notable because its presence was correlated with the formation of larger aggregates showing higher resistance to breakage than those from other consortia. However, these aggregates floated and remained on the surface of the culture medium instead of settling.

Consequentially, Step 4 consisted of selecting pairs of isolates showing high levels of co-aggregation and good sedimentation properties, and the subsequent testing of these consortia in the presence of the E19 isolate to verify if this isolate would cause the combined isolates to float.

Finally, in Step 5, aggregates formed in the presence of the E19 isolate were tested for the resistance to disruption with ultrasound. Fig. 1 presents a flowchart of the steps performed in this study.

3.2. Bacterial isolates

The bacterial isolates were obtained from the biological sludge from an aerobic granular reactor fed with the effluents from a recycled paper mill (old corrugated cardboard). Biological treatment was performed in a sequencing batch reactor. The system had a 6-L aeration tank, and 3 L of treated effluent were removed and replaced with new effluent for each cycle. The reactor was maintained at room temperature. Oxygen was injected in the reactor by air pump connected to diffusers to maintain the dissolved oxygen over 2 mg L^{-1}. The cycle was 12 h and the hydraulic retention time was 24 h (3 L was replaced in each cycle and the reactor volume was 6 L). Minutes before completing this 12-h period, the aeration was ceased to allow sedimentation of the biological sludge.

About 5 mL of granular sludge was collected and centrifuged three times at 650× *g* for 2 min. The supernatants were discarded and the pellets were resuspended in a 0.85% saline solution after each centrifugation. The final re-suspended material was passed through a syringe several times and exposed to ultrasound to breakdown the aggregates. Ultrasound pulses of 20 kilohertz (kHz) for 4 s were applied four times to the sample using a 4710 series Ultrasonic homogenizer from the Cole Parmer Instrument Company, Chicago, Illinois, USA.

The broken down suspension was centrifuged one more time at 650× *g* for 2 min to remove the remaining aggregates. An aliquot of 0.1 mL, collected from the supernatant, was used to prepare ten-fold serial dilutions from 10^{-1} to 10^{-9}.

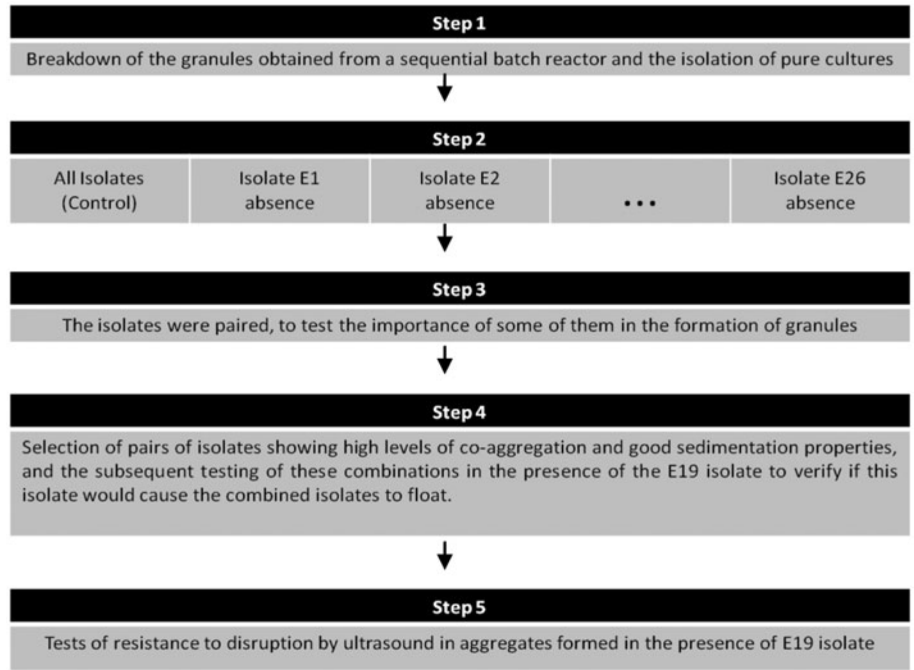


Fig. 1. Flowchart with the steps performed in this study.

Each dilution was spread on four different solid media in order to broaden the possibility of isolation of different microorganisms, including the following: R2A agar, 1/10 LB (10-fold diluted LB), mineral and the autoclaved effluent itself (solidified in 1.5% agar). The plates were kept at room temperature. The diluted LB was used in an attempt to select microorganisms with lower growth rates over the ones that have higher growth rates in a richer medium.

The isolated colonies were serially streaked on fresh R2A agar plates to obtain pure cultures. Subsequently, each of the pure cultures was grown in liquid medium (autoclaved effluent) and their stocks were stored at –80˚C.

3.3. Co-aggregation in the absence of a single isolate

In order to determine the influence of each of the 19 isolates in the formation of aggregates, 20 consortia were prepared, one containing all 19 isolates and used as the control, and 19 other consortia lacking one particular isolate at a time.

The isolates were first plated on nutrient agar medium directly from the frozen stocks; one isolated colony was subsequently used to inoculate 20 mL of nutrient broth. These liquid cultures were incubated at room temperature for 1 h and 30 min in a shaking incubator rotating at 150 rpm. The incubation time was determined based on previously established growth curves showing that all isolates reached the exponential growth phase by 1 h and 30 min.

The optical density (OD) of each culture was measured, and the volume needed to inoculate and obtain 50 mL cultures with 0.1 OD was calculated. The 50 mL cultures were incubated in a shaking incubator with rotation at 150 rpm. Visual observations were performed for each consortium over the incubation time.

3.4. Extracellular polymeric substance extraction and quantification

The quantification of EPS was performed with only the consortia that formed the largest granules in the co-aggregation tests. For extraction of EPS, samples (5 mL) were collected from the cultures in medium R2A, of each consortia at different time (2, 4, 8, 24, 48, and 72 h). Initially, 1 mL was submitted to centrifugation (15,000 *g*, 4˚C, 15 min) and the supernatant was discarded. The pellet were resuspended in milli-Q water and centrifuged again. The supernatant of the second centrifugation was stored for analysis of free EPS. The pellet was resuspended in phosphate buffer, completing the volume to 20 mL. After adjusting pH to 11 with NaOH, the samples were heated at 80˚C for 30 min. After cooling, the samples were centrifuged for 1 min and the supernatant were centrifuged again for 10 min. The final supernatant and the stored sample was used for analysis of carbohydrate content [6], proteins [7], humic acids [7], and total organic carbon [8]. One sample with phosphate buffer at pH 11 was used as the standard.

3.5. DNA extraction method

The isolates were inoculated in Eppendorf tubes containing 1 mL of TY medium. After 24 h of incubation, the DNA was extracted. For each culture, it was added 1 mL of lyse buffer (EDTA 100 mM, Tris–HCl 100 mM, CTAB 2%, SDS 1% e NaCl 1.5 M). After homogenization in vortex, the mixture was heated at 65˚C for 20 min. Each tube was inverted every 5 min. After that procedure, the samples were centrifuged for 20 min at 5,000 rpm. The aquous phase, upper fase, was transferred to a new sterile Eppendorf tube and the samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1, pH 6.7; Sigma-Aldrich). After each extraction, samples were centrifuged at 5,000 rpm for 10 min at 4˚C, and supernatants were transferred in wells containing 0.7 volume of isopropanol (Sigma-Aldrich) and 0.1 volume of 3 M sodium acetate. DNA was precipitated at –20˚C overnight. Three ethanol washes were performed by adding 70% (v/v) ethanol to each sample and centrifuging for 10 min at 5,000 rpm. Supernatants were discarded after each ethanol wash. DNA pellets were air-dried prior to being resuspended in 40 μL of water Milli-Q.

3.6. DNA amplification

The extracted DNA samples were used in PCR amplifications using the sequences of the primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1525R (5´-AAGGAGGTGATCCAGCC-3´). Amplification was conducted using a 50 μL (total volume) mixture containing 0.25 U/μL of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 12.5 pmol of each primer, 2.5 μL of reaction buffer 10X, 100 μM dNTPs, 1,8 mM $MgCl_2$, 1 μL of DNA and Milli-Q sterile water (Purelab-Ultra). Amplification was conducted with a Mastercycler (Eppendorf AG, Hamburg, Germany). The amplification conditions for the PCR amplification were as follows: initial denaturation at 95˚C for 5 min followed by 35 cycles of incubation at 95˚C for 90 s, annealing at 55˚C for 90 s and incubation at 72˚C for 90 s. A final elongation at 72˚C for 5 min was also included.

The amplicons were sequenced at ACT Gene Laboratory (Biotechnology Center, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) using ABI-Prism 3100 Genetic Analyzer with 50 cm capillary and POP-6 polymer (Applied Biosystems, Foster City, CA, USA).

3.7. Coaggregation among consortia of two or three isolates

The results of the coaggregation in the absence of a single isolate identified isolates that were potentially important to understanding co-aggregation. Thus, consortia of two or three isolates were chosen to verify whether they contributed to the formation of aggregates that were more resistant to disruption.

The isolates were pre-cultivated as stated previously. The isolates were first plated in agar nutrient medium directly from the frozen stocks; one isolated colony was subsequently used to inoculate 20 mL of nutrient broth. These liquid cultures were incubated with shaking at 150 rpm at room temperature for 1 h and 30 min, at which point they had reached the logarithmic growth phase. The OD of each culture was measured and the volume needed to inoculate and obtain 6 mL cultures with 0.1 OD was calculated. These 6 mL cultures were incubated in glass tubes and shaken at 150 rpm. Each consortium was observed visually over the incubation time.

After 31.5 h of incubation, samples taken from the consortia were smeared on glass slides and then fixed and stained with fuchsin.

3.8. Criteria used to determine the co-aggregation index

The criteria used to determine the coaggregation index by a semiquantitative method based on visual observations was previously proposed by [9]:

(1) (0) or no score: the absence of visible aggregates in the cell suspension;
(2) (1): small and uniform aggregates in a turbid suspension;
(3) (2): easily visible aggregates in a turbid suspension;
(4) (3): easily visible aggregates that quickly settle, however, turbidity in the supernatant is still visible;
(5) (4): large aggregates that settle instantly leaving a clear supernatant.

In some of the aggregation tests, the formation of aggregates that floated instead of settled, remaining on the surface of the liquid medium, was observed. Because this characteristic has not been reported in the literature and it was important to differentiate aggregates that floated from those that settled, a negative sign was used to identify the consortia in which the aggregates floated.

Therefore, the coaggregation index corresponds to the size of the aggregates, and the positive/negative sign indicates whether the aggregates floated or settled to the bottom.

3.9. Test of resistance to disruption by ultrasound

The resistance to disruption of the consortia aggregates was compared by ultrasound exposition. Ultrasound pulses of 20 kHz for 4 s were applied to which consortia sample until the aggregates were completely disrupted using a 4710 series Ultrasonic homogenizer from the Cole Parmer Instrument Company, Chicago, Illinois, USA.

4. Results and discussion

4.1. Bacterial isolates

The strains were identified by DNA extraction and amplification. The genus of each nineteen isolate was presented in Table 1.

4.2. Evaluation of the absence of each isolate

Contrary to what was expected, the formation of aggregates was observed in all consortia in which only

Table 1
Identification of the isolates

Isolate	Identification (genre)
1	*Acinetobacter*
2	*Agrobacterium*
3	–
4	*Acinetobacter*
5	*Acinetobacter*
7	*Enterobacter*
8	*Enterobacter*
9	*Staphylococcus*
10	*Acinetobacter*
11	*Enterobacter*
13	*Agrobacterium*
14	*Enterobacter*
15	*Acinetobacter*
18	*Acinetobacter*
19	*Agrobacterium*
23	*Acinetobacter*
24	*Enterobacter*
25	*Rhodococcus*
26	*Enterobacter*

one isolate was absent, and the phenomenon of aggregation could not be assigned to the presence of one or a few isolates. Similar to what has been reported in previous studies, the formation of aggregates was dependent on the cultivation time. Particular consortia showed an increase in the coaggregation index over time, virtually constant indexes throughout the period of observation, or an initial increase followed by the loss in the ability to coaggregate. The different patterns of aggregation and the temporal variation in aggregation could be the consequence of multiple interactions among the 19 isolates in each consortium. The 19 isolates were incubated in a complex medium (nutrient broth). Thus, the possibility that the different bacteria grew in different cultivation periods, changing the medium composition over time, must be considered. It is then necessary to consider two variables: the bacterial species that are able to coaggregate could have grown at different times or simultaneously, and their cell surface could have changed over time due to variations in the composition of the medium. Therefore, the preliminary results suggest that some isolates may form aggregates and some may hamper the aggregation process. Despite the complexity of the bacterial interactions, the results of the following experiments support this hypothesis, confirming that the approach used in this study facilitates understanding of these complex interactions in natural aggregates.

Different aggregation phenotypes were also observed by [10] in a study of the coaggregation between strains of aquatic bacteria. *Blastomonas natatoria 2.1* and *Micrococus luteus 2.13* formed aggregates during the exponential phase which reached a maximal level in the stationary phase. Similar results were obtained in studies by [11,12]. On the other hand, *M. luteus 2.13* and *B. natatoria 2.8* formed aggregates in only the stationary phase, while *B. natatoria 2.1* and *B. natatoria 2.8* formed coaggregates only after 144 h of incubation, when already in the late stationary phase [10]. Similarly, the percentage of coaggregation consortia selected by [13] increased with time. The maximum coaggregation percentage was more rapidly obtained in some consortia than in others, and the progression of coaggregation over time was different for the different pairs of isolates. The coaggregation patterns differed primarily because of changes in the composition and density of the biofilms, as a function of the nutrient composition and the chemical characteristics of the media [13].

The absence of the E4 isolate resulted in greater aggregate formation in comparison to the control after 21.25 h of incubation, followed by a loss in the co-aggregation levels. This result suggests that the E4 isolate grows rapidly and thereby inhibits the growth of granule-forming bacteria. A greater formation of aggregates compared to the control was also observed in the consortia in which the E10, E14, E18, and E26 isolates were absent. This suggests that these isolates inhibit the process of aggregation, and, in their absence, the bacteria that are important for the process grow, leading to the increased formation of aggregates.

On the other hand, the absence of the E7 isolate resulted in the absence of visible aggregates. Additionally, the consortia lacking the E2, E9, E13, and E25 isolates had reduced aggregate formation similar to the control (including all nineteen isolates). These results suggest that these isolates may be important to the process of aggregation and their presence can contribute to the formation of aerobic granules.

Table 2 shows the EPS content of the consortia that produced the largest granules.

As can be observed in Table 2, all the consortia presented higher polysaccharides concentration than proteins except for the –E26 consortium (PS/PN > 1). This can explain the higher aggregation index of the –E26 consortium once polymeric polysaccharides in the EPS serve as bioglue to facilitate cell-to-cell interactions [14,15].

The formation of granules in the consortia –E10 and –E14 was related to nitrogen-based substance. The stability of these granules tended to be low because of the low protein content and the granule stability is directly related to the EPS protein content [16,17]. This explains the lower aggregation index of the –E10 and –E14 consortia. In previous study [18] also confirmed that proteins are the main components of EPS, while polysaccharides constitute a higher portion of the SMP. Proteins are hydrophobic constituents of EPS, while carbohydrates are hydrophilic. With the implement of regulation measures in period 2, the P/C ratio increased gradually and resulted in the rising of relative hydrophobicity and reduction of zeta potential. This finally promoted the formation of aerobic granular sludge and slowed down membrane fouling [18].

Table 2
Quantification of EPS produced and coaggregation index

	Combinations			
EPS	–E10	–E14	–E18	–E26
Polysaccharides (mg/L)	0.24	0.22	0.57	0.59
Proteins (mg/L)	0.17	0.14	0.10	0.26
Humic acids (mg/L)	0.25	0.39	0.47	0.20
Rate PS/PN	0.57	0.41	1.00	1.28
Coaggregation index	2	2	2	3

4.3. Pairwise consortia between the E2, E7, E9, E13, and E25 isolates and all the other isolates

The results obtained in the first experiment suggest that some isolates may be important to aggregate formation, and, when these isolates are absent, the formation of aggregates does not occur or occurs to a lesser degree. To confirm the importance of the E2, E7, E9, E13, and E25 isolates in the process of aggregate formation, consortia of each of these isolates with the nineteen obtained isolates were evaluated.

Table 3 presents the results obtained from the experiment. The consortia that showed the formation of aggregates, regardless of the incubation period, received a positive sign, "+", whereas the consortia in which visible aggregates were not observed during the entire period of the experiment received a negative sign, "–".

According to the visual observations, all pairs containing the E11 isolate had easily visible aggregates and a good settling speed. The formation of these visible aggregates was observed early in the incubation in most cases (Table 3).

The consortia that contained the E19 isolate tended to form aggregates that remained on the surface of the liquid medium; these aggregates floated and returned to the surface after manually shaking the culture (Table 3). This phenomenon was sporadically observed in other consortia; however, only the E19 isolate presented this phenotype consistently when combined with any of the five isolates tested.

According to Table 3, the tested isolates E2, E7, E9, E13, and E25 showed a high percentage of aggregation with the other isolates, ranging between 73.68% in the consortia including the E13 isolate and 89.47% in the other consortia. These percentages were obtained by determining the ratio between the numbers of positive consortia divided by the total number of consortia in which the isolate was present. For example, the E13 isolate was present in 19 consortia (including a consortium with itself, i.e. auto-aggregation), and the formation of aggregates was observed in 14 of them.

This high percentage of consortia in which aggregates were observed confirms the importance of the E2, E7, E9, E13, and E25 isolates in the aggregation process, either through their aggregation with other isolates or their role as a bridge organism facilitating the association of other species that do not autoaggregate.

The E10 isolate did not form aggregates with any of the five tested isolates, and the E4 isolate only formed aggregates in consortium with E25. Auto-aggregation was observed for the E2, E7, E9, E13, and E25 isolates (i.e. aggregates were observed in the consortia E2 + E2; E7 + E7; etc.). The absence of aggregates when E4 and E10 were present indicates that these isolates do not form aggregates with the other isolates. On the other hand, the absence of aggregates in the consortia that showed auto-aggregation when E4 and E10 were also present indicates that these isolates inhibit the auto-aggregation processes of the other isolates. This supports the conclusion that these isolates can inhibit the formation of granules, first formed after evaluation of cultures lacking each of these isolates.

4.4. Evaluation of the effect of the E19 isolate in the formation of aggregates

The E19 isolate generated outstanding results for the formation of larger aggregates, which were noted

Table 3
Combinations that formed or did not form aggregates during the experiment

	Isolates																			
Isolates	1	2	3	4	5	7	8	9	10	11	13	14	15	18	19	23	24	25	26	(%)[a]
2	+	+	+	–	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	89.47
7	+		+	–	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	89.47
9	+		+	–	+		+	+	–	+	+	+	+	+	+	+	+	+	+	89.47
13	–		+	–	–		–		–	+	+	+	+	+	+	+	+	+	+	73.68
25	+		+	+	+		+		–	+		–	+	+	+	+	+	+	+	89.47
(%)[a]	80		100	20	80		80		0	100		80	100	100	100	100	100		100	

[a]Represents the percentage of combinations that showed the formation of aggregates at some time during the experiment. Example: seventeen combinations presented visible aggregates out of the 19 combinations containing the E2 isolate; one combination presented visible aggregates out of the five combinations containing the E4 isolate.

[b]The combinations that showed the formation of aggregates, regardless of the incubation period, received a positive sign, "+", whereas the combinations in which visible aggregates were not observed during the entire period of the experiment received a negative sign, "–".

through the visual observations in all consortia where this isolate was present. However, these cultures consistently showed aggregates that floated rather than settled (Fig. 2). In addition to the increased size of the aggregates, these cultures were resistant to disruption after manual agitation of the flasks.

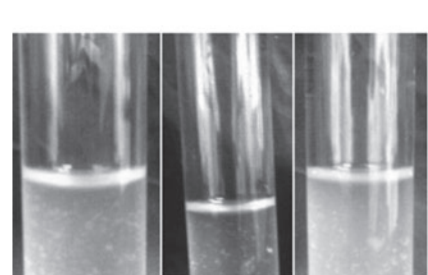
In previous studies, aerobic aggregates (granules) from the sludge produced in the biological treatment of paper mill effluents broke apart when subjected to high pressure during treatment in membrane bioreactors [5]. This rapidly fouled the membranes, reducing the permeate flux. The formation of granules that are more resistant to disruption may be beneficial to the function of the membrane bioreactors by reducing the rupture of the granules and decreasing the membrane fouling. This would result in a steadier, higher flux over longer periods. The flotation of the aggregates formed in the presence of the E19 isolate should be less important in a membrane bioreactor than in a conventional activated sludge system, where good sludge sedimentation is critical.

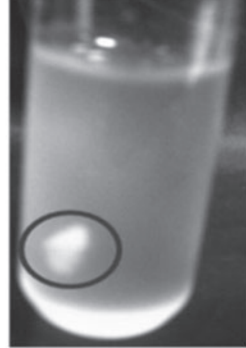
In order to assess whether the presence of the E19 isolate causes flotation of aggregates even in the presence of other isolates that formed aggregates with good sedimentation properties, a few pairs from the second experiment were selected, and the E19 isolate was introduced as a third isolate in these consortia. The pairs without the E19 isolate were used as controls in this experiment. The size of the aggregates and their sedimentation properties (settling or floating) were evaluated. The results are presented in Table 4.

The results presented in Table 4 show that the presence of the E19 isolate caused the formation of floating aggregates in almost all tested consortia, except in E13 + E15 + E19 and E7 + E13 + E19. In addition, for all consortia in which flotation was observed, large aggregates with co-aggregation indexes equal to four were formed; in the consortia including E13 + E13 + E19 and E13 + E23 + E19, the aggregates formed were larger than in the controls (E13 + E13 and E13 + E23, respectively). The co-aggregation indexes do not necessarily indicate the relative strength of the interactions between different bacterial cells. The size and density of the aggregates depends on the size and morphology of the bacteria involved and the intensity of the interactions on the bacterial surface. Smaller co-aggregation indexes do not necessarily indicate weak interactions between cells [13].

Therefore, the fact that aggregates formed in consortia containing the E19 isolate did not break after their flasks were manually shaken may indicate that the interactions involving this isolate are strong. This may contribute to the formation of granules as large as those used in MBRs, which are not easily broken down.

The consortia including E13 + E15 + E19 and E7 + E13 + E19 lacked large aggregates and flotation similar to the controls. These consortia differ from the E13 + E13 + E19 consortium in the presence of the E15 and E7 isolates. Because E13 + E13 + E19 produced larger aggregates that were more resistant to disruption, it is possible that the presence of the E7 and E15 isolates inhibits either the growth of the E19 isolate or the interactions at the bacterial surface, affecting the size of the aggregates. These results can be observed in Fig. 3. The curves presented in the graphs in Fig. 3 from these consortia show values below those observed for other consortia. Based on this, a modification of the classification system of [9] is proposed: the aggregates which float should receive negative values, showing that they have a different phenotype from those that settle. In the system of [9], there is an increasing tendency of the aggregates to settle when





Combinations in the presence of *E19*

Fig. 2. Formation of aggregates in the presence and absence of the E19 isolate.

Table 4
Results from the introduction of isolate E19 in combination with paired isolates that previously formed large aggregates with good sedimentation properties

Combination	Incubation time						
	3.00 h	5.00 h	6.50 h	8.83 h	21.25 h	54.00 h	76.60 h
E13 + E11 + E19		1	2	2	4[a]	4	4
E13 + E11	2	4	4	4	4	4	3
E13 + E13 + E19		1	2	2	4[a]	4[a]	4[a]
E13 + E13[b]					2	2	1
E13 + E23 + E19		3	3	3	4	4[a]	4[a]
E13 + E23		3	3	3	3	2	1
E13 + E15 + E19					1	1	2
E13 + E15					2	2	2
E7 + E13 + E19				1	2	2	1
E7 + E13		1	1	1	2	2	2
E25 + E11 + E19		1	2	2	4[a]	3[a]	3[a]
E25 + E11	2	4	4	4	4	4	4
E2 + E11 + E19		3	3	2	4[a]	4[a]	4
E2 + E11		3	3	3	4	4	4

[a]Observed the presence of aggregates that remained on the surface of the liquid medium (floating).
[b]Autoaggregation. Twice as much of the calculated volume for the E13 isolateinoculum was added in the culture medium to obtain the same initial optical density value from the other combinations.

moving from class 1 through class 4. Thus, the indexes for the consortia that had floating aggregates were represented in the negative axis. The index score represents the size of the aggregates and the positive/negative axis indicates the flotation/sedimentation phenotype. Therefore, a consortium with an index of "–4" indicates the presence of easily visible aggregates in a culture medium with low turbidity (similar to the consortia with an index of "+4"), but the aggregates floated when the culture was standing still.

The results presented in Table 5 confirmed the phenotype shown in Fig. 3. Specifically, the consortia with the higher coaggregation index had more cells than the other consortia. For example, the images of the E13 + E13 + E19 consortium showed a greater number of cells and greater aggregation compared with the images of the E13 + E13 consortium. Similarly, the images of the E7 + E13 + E19 and E7 + E13 consortia confirmed the phenotype shown in Fig. 3, with little difference between these consortia.

4.5. Tests on the aggregates' resistance to disruption by ultrasound

Techniques for measuring granule strengths can be derived from those developed for measuring floc strength. Floc strength has been largely studied using various methods.

Floc strength may be estimated through floc size analysis by examining its break-up consecutively by exposing to known stresses. Other techniques, such as ultrasonic methods, have also been adapted to test aggregate strength but are more suitable for application to mineral flocs than to bioaggregates due to the effect of ultrasound on bacterial components [19]. In this study, ultrasound was used to indicate a better granule stability, even though resistance to disruption with ultrasound of aggregate is quite different from granular stability maintenance.

In the previous experiments, it was observed that the aggregates formed in the presence of the E19 bacteria had greater resistance to disruption when the flask was subjected to manual agitation. However, manual agitation is not a standardized or reliable method for comparison of the resistance to disruption between different aggregates. Thus, a standardized test using ultrasound equipment was performed in order to compare the resistance to disruption among these consortium aggregates and the granules initially used for the isolation of the strains. Some of the consortia containing the E19 isolate were used in this test after 21.25 h of incubation. Pulses of 20 kHz for four seconds each were applied until the aggregates were completely disrupted. The consortia used and the co-aggregation rates achieved during the incubation (before the ultrasound testing) are presented in Table 6.

According to the results presented in Table 6, after 21.25 h of incubation, all selected consortia showed large aggregates that did not break after manual

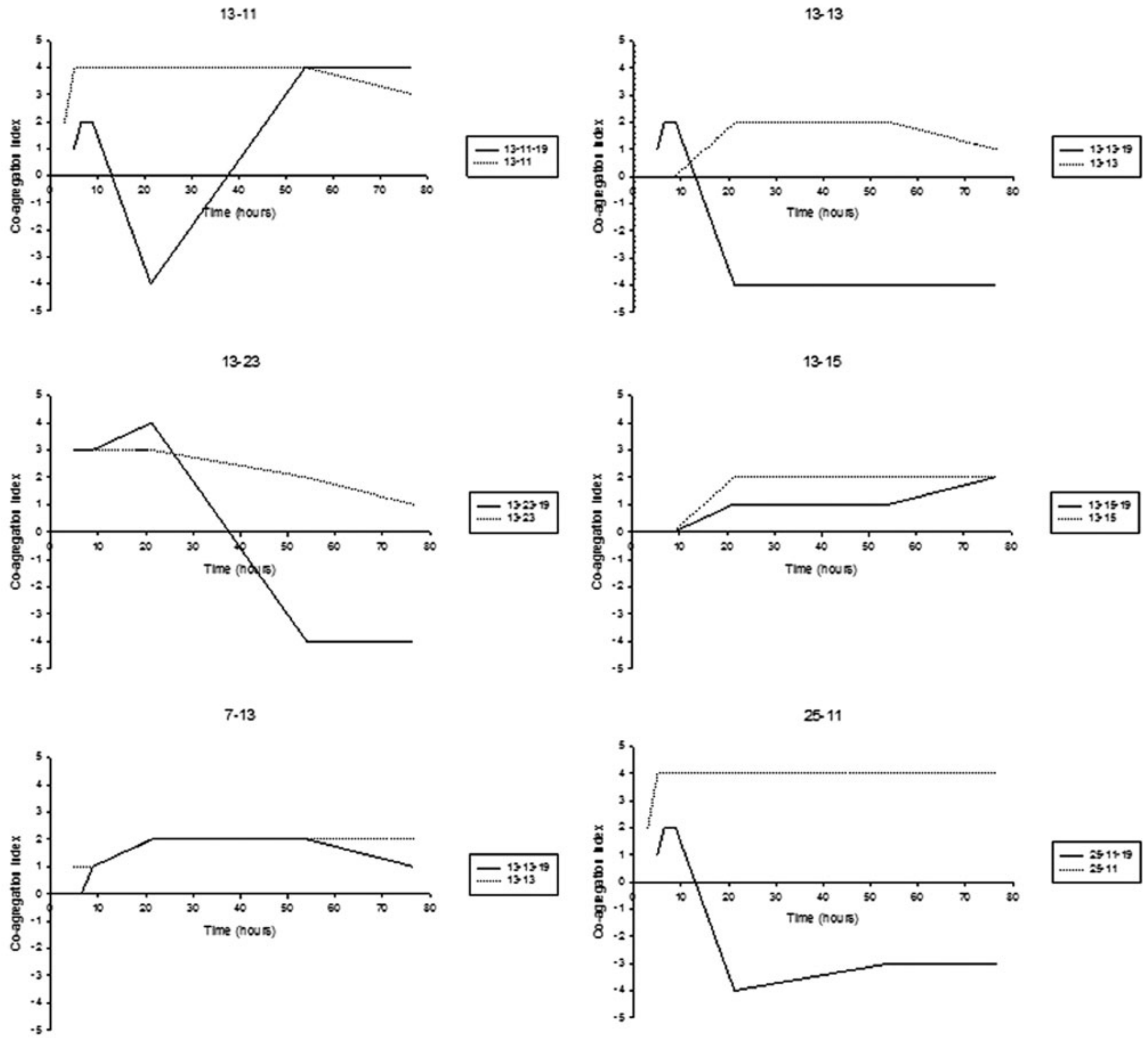


Fig. 3. Morphological and sedimentary characteristics vary over time in combinations of isolates with the presence and absence of the E19 isolate.
Notes: Index—(0): absence of visible aggregates in the cell suspension; (1) small and uniform aggregates in a turbid suspension; (2) easily visible aggregates in a turbid suspension; (3) easily visible aggregates that quickly settle or float, however, turbidity in the supernatant is still visible; (4) large aggregates that settle or float instantly leaving a clear supernatant. The index module is related to the size of the aggregates and the positive/negative axis with the flotation/sedimentation aspect, respectively.

shaking. However, the aggregates did not float in the E13 + E23 + E19 consortium.

The samples were transferred to plastic tubes with conical bottoms and ultrasound pulses were applied to the tubes to test the resistance of the aggregates formed from the consortia shown in Table 6. Ultrasonic pulses were applied to the material as described above until no aggregates were visible. The test results are presented in Table 7.

The results in Table 7 indicate that the consortium requiring the highest average number of pulses for the complete disruption of visible aggregates was the E13 + E13 + E19 consortium. However, considering the standard deviation, there is no significant difference

Table 5
Microscopic images of the isolate combinations in the presence and absence of isolate E19 after 31.5 h of incubation. 1,000× magnification

Combination			
E13 + E11 + E19			
E13 + E11			
E13 + E13 + E19			
E13 + E13			
E13 + E23 + E19			
E13 + E23			
E13 + E15 + E19			

(Continued)

Table 5 (*Continued*)

Combination			
E13 + E15			
E7 + E13 + E19			
E7 + E13			
E25 + E11 + E19			
E25 + E11			
E2 + E11 + E19			
E2 + E11			

Table 6
Combinations containing the E19 isolate before the test of disruption by ultrasound

	Incubation time				
Combination	3.00 h	5.00 h	6.50 h	8.83 h	21.25 h
E13 + E13 + E19		1	1	1	4[a]
E13 + 23 + E19	3	3	3	3	4
E25 + E11 + E19	2	2	2	2	4[a]
E2 + E11 + E19	3	3	3	3	4[a]

[a]Observed the presence of aggregates that remained on the surface of the liquid medium (floating).

Table 7
Average and standard deviation of the number of pulses needed for the complete disruption of the aggregates formed by the indicated combinations

Combination	Average (± standard deviation)
E2 + E11 + E19	6.33 (±1.53)
E25 + E11 + E19	6.67 (±2.08)
E13 + E13 + E19	10.00 (±2.00)
E13 + E23 + E19	8.67 (±1.15)

Note: Average and standard deviation of the samples in triplicate.

between this consortium and the E25 + E11 + E19 and E13 + E23 + E19 consortia.

Before beginning the aggregation tests, a sample containing granules was collected from the biological reactor. The sample was centrifuged, and ultrasound pulses were applied under the same conditions used in the above experiment. Four pulses were required for the complete disruption of the granules present in this sample (this procedure was carried out during the step in which strains were isolated). Therefore, for all the consortia presented in Table 6, the average number of pulses required for the complete disruption of the aggregates was higher than that required for the disruption of granules present in the biological sludge. This indicates that the aggregates formed by these consortia are more resistant to disruption by ultrasound than the granules from which the strains were initially isolated.

Therefore, the E19 isolate may contribute to the formation of granules that are more resistant to disruption, preventing degradation of the granules during the membrane filtration process. However, the mechanisms involved in the disruption of aggregates by ultrasound are quite different from the mechanisms that cause the breakdown of granules during filtration. According to [20], the ultrasound waves remove cell wall surface structures that are important to the co-aggregation.

During the coaggregation studies, the isolates were mixed when in the exponential growth phase and further incubated together in culture, unlike procedures used in other studies in which the isolates were grown separately, mixed in saline solution after the achievement of the desired growth, and then used to measure aggregation. The coculture of the isolates presents a different dynamic, in which some isolates may be inhibited or benefit from the growth of the others. This environment resembles the bioreactor environment in which the different strains grow together.

Several factors can interfere with the process of aggregate formation, such as the composition of the medium and the culture conditions (shaking speed, which affects the amount of dissolved oxygen, pH, etc.). These factors were not evaluated in this study and could be addressed in future studies.

It is important to consider that old granules would show lower cohesion, even if it were formed by isolates that contribute to the granules strength, because of deterioration of the internal structure. The centers of granules may became porous, showing an empty internal heart after long-term starvation. This can be attributed to the fact that the internal part of the aggregate (exopolymers) could be partly biodegraded, i.e. used as a secondary substrate in such famine conditions [19].

5. Conclusions

In this research, 19 strains were isolated from granular aerobic sludge used to treat paper mill effluent. Tests for coaggregation showed that some isolates were able to improve the formation of granules while others inhibited the aggregation. The mechanical resistance of the aggregates was determined by manual shaking, and the results showed that few isolates substantially improved the resistance of the aggregates to disruption. Further investigation of the mechanical resistance of the aggregates including Isolate E19, the most promising strain, to disruption was conducted using ultrasound. The results confirmed that this isolate has the capacity to improve aggregation.

Acknowledgements

The authors thank the FAPEMIG and CNPq for scholarships granted. They also thank the Cataguases de Papéis for supply of effluents and PAM Membranes for offering the filtration system.

References

[1] A.H. Rickard, P. Gilbert, N.J. High, P.E. Kolenbrander, P.S. Handley, Bacterial coaggregation: An integral process in the development of multi-species biofilms, Trends Microbiol. 11 (2003) 94–100, doi: 10.1016/S0966-842X(02)00034-3.

[2] M. Von Sperling, Princípios de Tratamento Biológico de Águas Residuárias–Lodos Ativados, second ed., Universidade Federal de Minas Gerais, Belo Horizonte, 2005.

[3] V. Ivanov, X.H. Wang, S.T.L. Tay, J.H. Tay, Bioaugmentation and enhanced formation of microbial granules used in aerobic wastewater treatment, Appl. Microbiol. Biotechnol. 70 (2006) 374–381, doi: 10.1007/s00253-005-0088-5.

[4] A.H. Rickard, P. Gilbert, N.J. High, P.E. Kolenbrander, P. Handley, Bacterial coaggregation: An integral process in the development of multi-species biofilms, Trends Microbiol. 11 (2003) 94–100, doi: 10.1016/S0966-842X(02)00034-3.

[5] I.L.H. Morais, C.M. Silva, C.P. Borges, Aerobic granular sludge to treat paper mill effluent: Organic matter removal and sludge filterability, Desalin. Water Treat. 1 (2015) 1–8, doi: 10.1080/19443994.2015.1022803.

[6] A.A. Albalasmeh, A.A. Berhe, T.A. Ghezzehei, A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry, Carbohydr. Polym. 97 (2013) 253–261, doi: 10.1016/j.carbpol.2013.04.072.

[7] B. Frølund, R. Palmgren, K. Keiding, P.H. Nielsen, Extraction of extracellular polymers from activated sludge using a cation exchange resin, Water Res. 30 (1996) 1749–1758.

[8] W. Xuan, Z. Bin, S. Zhiqiang, Q. Zhigang, C. Zhaoli, J. Min, L. Junwen, W. Jingfeng, The EPS characteristics of sludge in an aerobic granule membrane bioreactor, Bioresour. Technol. 101 (2010) 8046–8050.

[9] J.O. Cisar, P.E. Kolenbrander, F.C. Mcintire, Specificity of coaggregation reactions between human oral Streptococci and strains of Actinomyces viscosus or Actinomyces naeslundii, Infect. Immun. 24 (1979) 742–752, doi: 0019-9567/79/06-0742/11$02.00/0.

[10] A.H. Rickard, S.A. Leach, C.M. Buswell, N.J. High, P.S. Handley, Coaggregation between aquatic bacteria is mediated by specific-growth-phase-dependent lectin-saccharide interactions, Appl. Environ. Microbiol. 66 (2000) 431–434, doi: 10.1128/AEM.66.1.431-434.2000.

[11] A.H. Rickard, A.J. McBain, R.G. Ledder, P.S. Handley, P. Gilbert, Coaggregation between freshwater bacteria within biofilm and planktonic communities, FEMS Microbiol. Lett. 220 (2003) 133–140, doi: 10.1016/S0378-1097(03)00094-6.

[12] A. Malik, M. Sakamoto, S. Hanazaki, M. Osawa, T. Suzuki, M. Tochigi, K. Kakii, Coaggregation among nonflocculating bacteria isolated from activated sludge, Appl. Environ. Microbiol. 69 (2003) 6056–6063, doi: 10.1128/AEM.69.10.6056.

[13] C.M. Buswell, Y.M. Herlihy, P.D. Marsh, C.W. Keevil, S.A. Leach, Coaggregation amongst aquatic biofilm bacteria, J. Appl. Microbiol. 83 (1997) 477–484, doi: 10.1046/j.1365-2672.1997.00260.x.

[14] H.L. Jiang, J.H. Tay, S.T.L. Tay, Aggregation of immobilized activated sludge cells into aerobically grown microbial granules for the aerobic biodegradation of phenol, Lett. Appl. Microbiol. 35 (2002) 439–445.

[15] Y.Q. Liu, Y. Liu, J.H. Tay, The effects of extracellular polymeric substances on the formation and stability of biogranules, Appl. Microbiol. Biotechnol. 65 (2004) 143–148.

[16] C. Di Iaconi, R. Ramadori, A. Lopez, Combined biological and chemical degradation for treating a mature municipal landfill leachate, Biochem. Eng. J. 31 (2006) 118–124.

[17] B.S. McSwain, R.L. Irvine, M. Hausner, P.A. Wilderer, Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge, Appl. Microbiol. Biotechnol. 7 (2005) 1051–1057.

[18] X. Tu, S. Zhang, L. Xu, M. Zhang, J. Zhu, Performance and fouling characteristics in a membrane sequence batch reactor (MSBR) system coupled with aerobic granular sludge, Desalination 261 (2010) 191–196, doi: 10.1016/j.desal.2010.03.034.

[19] J. Wan, I. Mozo, A. Filali, A. Liné, Y. Bessière, M. Spérandio, Evolution of bioaggregate strength during aerobic granular sludge formation, Biochem. Eng. J. 58–59 (2011) 69–78, doi: 10.1016/j.bej.2011.08.015.

[20] H. Ekmekci, B. Aslim, S. Ozturk, Characterization of vaginal lactobacilli coaggregation ability with *Escherichia coli*, Microbiol. Immunol. 53 (2009) 59–65, doi: 10.1111/j.1348-0421.2009.00115.x.