Structural Stabilization of Granular Sludge by Addition of Calcium Ions into Aerobic Bioreactors

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Granulation is a gradual process that makes flocculent sludge granular through the simultaneous densification and selection of aggregates *via* sedimentation. The damage to the granule structure over time in a bioreactor operation is one of the most severe barriers to the practical application of the process. The addition of metal ions may increase aggregation rates and granular structure stability. Four sequential batch reactors fed with pulp mill effluent were operated and monitored. Three reactors contained aerobic granular sludge and one contained flocculent sludge. One granular sludge SBR received the addition of 100 mg·L^{-1} of Ca^{2+}, the second 200 mg·L^{-1} of Ca^{2+}, and the third received no intentional addition of calcium. The fourth SBR was operated with conventional flocculent sludge. The efficiency of the organic matter removal and the effect of calcium on the morphological characteristics of the granules formed were evaluated. The removal efficiency of the COD and the BOD was similar among all SBR, *i.e.*, 60% and 90%, respectively. The addition of calcium did not interfere with granule size. The addition of 100 mg·L^{-1} of Ca^{2+} increased the uniformity and the mechanical strength of the granules. It also increased approximately 36% of the settling velocity of the granules.

Keywords: Aerobic granular sludge; Calcium; Pulp mill effluent; Sludge settling velocity

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INTRODUCTION

Aerobic granulation is a promising technique for wastewater treatment and has significant potential for organic matter and nutrient removal, including recalcitrant and toxic effluents for aquatic organisms (Chen *et al*. 2016; Morais *et al*. 2016). Granulation can be initiated by the adsorption and bacterial adhesion to inert materials and inorganic precipitates and also through the adhesion of microorganisms to other cells by physicochemical interactions. Filamentous bacteria increase the granulation process, forming structures that support the adhesion of new cells (Yu *et al*. 2001).

Microorganisms produce organic biopolymers that contain construction materials for microbial aggregates and are very important to the granulation process. These extracellular polymeric substances (EPS) are composed mainly of proteins, polysaccharides, humic acids, nucleic acids, and lipids (Ren *et al*. 2009; Sajjad and Kim 2015a). These compound can form a matrix involving the microorganisms, facilitating their aggregation. EPS interactions and their characteristics are crucial for bioflocculation mechanisms because they constitute a major part of the sludge mass (Sobeck and Higgins 2002).

Biofloc surface is often negatively charged due to the presence of functional groups of EPS (Sobeck and Higgins 2002). Carboxyl groups of uronic acids are deprotonated at usual pH values found in activated sludge systems (between 6.5 and 8.5) and contribute to the negative charge of bioflocs (Sobeck and Higgins 2002; Seviour and Nielsen 2010). In addition, they contribute to the negative charge of the bioflocs by having exocellular proteins rich in amino acids containing carboxyl groups, such as glutamic and aspartic acids (Sobeck and Higgins 2002).

In the presence of Ca^{2+}, Mg^{2+}, and Fe^{2+} cations, the ionic bonds between the carboxyl and the phosphate groups are reduced and granulation is increased (Yu *et al*. 2001; Sajjad and Kim 2015b). Metallic cofactors improve the action of enzymes in the metabolism of viable cells (Yu *et al*. 2001). Bivalent cations bind negative functional groups with EPS stabilizing the biopolymer microorganism matrix, which favors aggregation. The addition of Ca^{2+} increased the polysaccharides content in relation to the protein content of EPS (reduced PN/PS ratio), while the addition of Mg^{2+} increased de PN/PS ratio of EPS. The granulation rate, particle size, and sludge settleability were rapidly increased by Ca^{2+} addition, whereas the sludge dewaterability was significantly enhanced by increasing Mg^{2+} (Sajjad and Kim 2015b). These ions are probably constituents of polysaccharides or extracellular proteins. Although monovalent ions can also neutralize EPS functional groups, the extracellular polymers mainly bind to bivalent ions forming more stable complexes. The formation of calcium carbonate and the linkage of calcium to EPS and cells are non-specific processes controlled by the calcium ion gradient in the aqueous phase and by the granules. The addition of calcium or simply the replacement of sodium bicarbonate ($NaHCO_3$) by calcium hydroxide ($Ca(OH)_2$) for the neutralization of pH in the bioreactor can improve the rate of granulation (Cail and Barford 1985; Dolfing *et al*. 1985; Mahoney *et al*. 1987; Thiele *et al*. 1990).

Various kinds of metal ions are often detected in wastewaters and many of them are harmful or toxic to microorganisms (Hao *et al.* 2016; Pal and Paul 2008; Wang and Teng 2010). On the other hand, some metals and some levels can be beneficial, as in the case of metallic cofactors that were mentioned earlier (Yu *et al.* 2001). Calcium also probably plays a role in multiplication of the cells and development of the granules acting not only to facilitate cell–cell bridging but also to promote growth of aggregates indirectly (Yu *et al.* 2001).

Metal ions affected the microbial attachment aerobic granular sludge through inducing changes in the quorum sensing system, EPS, and relative hydrophobicity of the sludges (Hao *et al.* 2016). The cations Ca^{2+}, Mg^{2+}, and K^+ could enhance the microbial attachment ability, while Cu^{2+}, Fe^{2+}, and Zn^{2+} could inhibit the microbial attachment at lower concentrations (Hao *et al.* 2016).

Bivalent cations, such as Mg^{2+} and Ca^{2+}, could influence biofilm formation directly through their effect on electro-static interactions and indirectly *via* physiology-dependent attachment processes, by acting as important cellular cations and enzyme cofactors. Interestingly, K^+ exhibited a positive impact on the attachment, and had no negative effect even at the high concentration of 80 mg/L, which may be because K^+ is the major intracellular cation in bacteria as well as in eukaryotic cells, and bacteria accumulates K^+ by a number of different transport systems that vary in kinetics, energy coupling, and regulation. The addition of Cu^{2+} or Fe^{2+} reduced the protein content of EPS, *i.e.*, a very low protein/polysaccharide (PN/PS) ratio was produced and led to the detachment of biomass

(Hao *et al.* 2016).

Quorum sensing (QS) allows bacteria to communicate with each other through the production and perception of small extracellular signal molecules. Three kinds of QS systems have been identified according to the different types of signal molecules (oligopeptides, Acylated Homoserine Lactone (AHLs), autoinducer-2) (Hao *et al.* 2016). With 32 mg/L Cu^{2+} and Fe^{2+}, the content of AHLs was significantly decreased. The cations became too high and the quorum sensing system was depressed, dropping the biomass attachment. This indicated that the metal ions could affect the microbial attachment through regulating the quorum sensing system (Hao *et al.* 2016).

Calcium precipitates neutralize the negatively charged cell surface and function as an inert support for bacteria. This phenomenon is important for the initial adsorption and initiation of the aggregation process. Thus, calcium deficiency or loss may decrease resistance or cause granule disruption (Yu *et al*. 2001).

The presence of phosphate can be detrimental to granule formation and can even inhibit the action of calcium in the granulation process. Phosphates can form calcium precipitates inside the granules, damaging the environment required for maintenance of the granular structure and bacterial activity (Yu *et al*. 2001).

Higher concentrations of calcium may have a negative effect on the granulation process. Most studies, with concentrations between 100 and 200 $mgCa^{+2}{\cdot}L^{-1}$, presented a positive effect on granulation, whereas those above 300 $mgCa^{+2}{\cdot}L^{-1}$ formed precipitates and had a negative influence on granulation (Yu *et al*. 2001).

Added sodium and excess calcium can reduce floc properties by replacing bivalent cations. The ratio between the sums of the monovalent cations (MC) ($meq{\cdot}L^{-1}$) divided by the sum of the bivalent cations (BC) with values greater than two (MC/BC > 2) predicts deterioration of the floc properties (Sobeck and Higgins 2002).

Some studies have investigated the effects of cations on aerobic granules formation (Kończak *et al*. 2014; Ye *et al*. 2016). However, the role of calcium addition on physical characteristics of granules was not satisfactorily explored, mainly for treatment of industrial wastewaters. There is still a lack of knowledge about the effects of cations on aerobic granulation and granules' performances.

The objective of this study was to evaluate the characteristics of aerobic granular sludge of a system treating bleached kraft pulp mill effluent. Aerobic granular sludge characteristics such as size, settling velocity, strength, and organic matter removal efficiency and filtrability were evaluated using the addition of calcium chloride ($CaCl_2$).

EXPERIMENTAL

Materials

Biological treatment

The biological treatment was performed in four (R1, R2, R3, and R4) sequential batch reactors (SBR) operated in parallel (Table 1) in the course of 156 days. Each system had an aeration tank with a functional volume of 1.6 L (height/diameter ratio of 4). The temperature of the reactors was maintained at 35 °C by controllers connected to electric heaters. At the bottom of these reactors, fine bubble aerators were set to introduce air to supply oxygen to the biomass. The reactors were operated in 12-h cycles with 10-min filling, 60-min settling (R1) or 1-min settling (R2, R3, and R4), 1 min of discharge, and 59 min of idle period (R2, R3, and R4). This led to 649 min of aeration in one cycle. The volume exchange ratio was 50%, resulting in a 24-hour hydraulic detention time (HDT). Dissolved oxygen (DO) was maintained above 2 mg·L^{-1}. Influent pH was kept between 6.5 and 7.5, and nitrogen and phosphorus were added in the proportion of COD:N:P equal to 200:5:1. The reactors were inoculated with biological sludge collected in the recirculation stream of an activated sludge treatment plant of a kraft pulp mill. The systems were fed with effluent from the pulp mill. Two SBR received $CaCl_2$ as an extra calcium source (beyond the calcium content of the pulp mill effluent). The addition of an extra amount of calcium (100 mg·L^{-1} and 200 mg·L^{-1}, respectively) in reactors R3 and R4 aimed to evaluate the effect of this element on the aerobic granulation process.

Table 1. Experimental Plan

Treatments			
Reactor R1	Reactor R2	Reactor R3	Reactor R4
Treatment comparison		Granular sludge comparison	
Organic matter (COD and BOD_5) removal efficiency among the four treatments.		The effect of calcium addition on granular sludge reactors (R2, R3 and R4) was evaluated. The diameter, sedimentation velocity, mechanical rupture strength, specific growth rate and filterability of the granular sludge were also evaluated.	

Table 2. Differences among the Four SBR for the Treatment of Kraft Pulp Mill Effluent

Treatment	Biological sludge	Feeding Effluent
Reactor R1	Flocculent	Pulp mill effluent
Reactor R2	Granular	Pulp mill effluent
Reactor R3	Granular	Pulp mill effluent + 100 mg·L^{-1} Ca^{2+}
Reactor R4	Granular	Pulp mill effluent + 200 mg·L^{-1} Ca^{2+}

Methods

Sludge morphology and calcium concentration in the SBR

Biological sludge (granular or flocculent) and the addition of a calcium source ($CaCl_2$) in the feed (0, 100, and 200 mg·L^{-1} of Ca^{2+}) differed among the SBR (Table 2).

Physical, chemical, and biological analyses

Soluble COD, soluble BOD, pH, and total suspended solids (TSS) in the treated effluents were analyzed following the standard methods (APHA 2012). The COD and TSS analyses were carried out three times a week, BOD twice a month, and pH measurement was performed daily. Calcium was determined by atomic absorption spectroscopy according to TAPPI Standard T266 om-02 (2006). The calcium determination was performed six times (every three weeks) after each effluent sample collection in the kraft pulp mill.

The size and the circularity of the granules were determined (Lochmatter *et al*. 2013) by the ImageJ program (Rasband 1997) with measurements after SBR stabilization, *i.e.*, when the reactor reached steady state conditions and mature granules were observed. The diameters were calculated as circular equivalent diameters (the diameter of a circle with the same area as the biofilm particle) by taking only particles with a diameter > 0.2 mm into account. The average diameters of the granules were determined to evaluate whether the addition of calcium affected their size. The diameters of the granules were compared among Reactors R2, R3, and R4. The distribution frequency of the granule diameters was evaluated throughout the test period after a steady-state was achieved.

The specific growth rate of the granules (μ) was calculated based on the kinetic model (Yang *et al*. 2004). The growth of bacteria (or other microorganisms) in batch culture can be modeled with four different phases: lag phase, log phase, stationary phase, and death phase. The log phase is a period characterized by cell doubling. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. The growth of aerobic granules after the initial cell-to-cell self-attachment is the result of interaction between bacterial growth and detachment. Under given growth and detachment conditions, the equilibrium size (D_{eq}) of aerobic granules exists when the growth and detachment forces are balanced, i.e. the size of aggregate (D) would gradually approach the equilibrium size, D_{eq}. The specific growth rate of aggregate can be determined by the following equation,

$$dD/dt = \mu(D_{eq} - D) \quad (1)$$

where D is the size of microbial aggregate at time t, D_{eq} is the size of the microbial aggregate at equilibrium, and μ is the specific growth rate of aggregate by size (day^{-1}).

The settling velocity and the mechanical strength (integrity coefficient) of the granules were determined (Ghangrekar *et al*. 2005). The average settling velocity was determined using a column of 7.5 cm diameter and height 75 cm filled with tap water. The fraction of the granules settled in 1 min was used for determination of strength. The shear force was introduced, albeit indirectly and approximately by placing these settled granules in conical flask containing tap water making total volume of 150 mL on a platform shaker. Each sample was subjected to the same degree of agitation (200 rpm for 5 min). After agitation the sample was allowed to settle for 1 min in a 150 mL measuring cylinder, followed by decanting of the supernatant and weighing of SS (mg) in the supernatant. In addition, weight of residual granular sludge (mg of SS) was determined. The results are expressed in terms of an integrity coefficient defined herein as, the ratio of solids in the supernatant to the total weight of the granular sludge, expressed in percent.

Scanning electron microscopy

Granules were fixed for 12 h in 3.7% paraformaldehyde. After rinsing three times with phosphate-buffered saline (PBS), the aggregates were dehydrated in an ethanol series (30, 50, 70, 80, 90, and 100%, 10 min per step) and subsequently critical point dried with CO_2. After gold sputter coating, the aggregates were examined on a Scanning Electron Microscope model JEOL JSM 5510.

RESULTS AND DISCUSSION

Characterization of the industrial effluent

The characteristics of the industrial effluent were determined (Table 3). The effluent pH remained neutral. The electrical conductivity and sodium concentration were typical of bleached kraft pulp mills (Ketep *et al*. 2013; Patel *et al*. 2016). Total suspended solid levels were low because they had been previously removed by primary sedimentation. The BOD and COD were similar to those reported for typical bleached kraft pulp mills (Ketep *et al*. 2013; Patel *et al*. 2016). The organic matter in the effluent originates from wood (lignin, extractives, and carbohydrates) during the wood pulping process (Kamali and Khodaparast 2015).

Calcium concentrations in SBR R1 and R2 correspond to that of the industrial effluent used without added calcium from an external source (Table 3). The higher calcium concentration in SBR R3 and R4 compared to SBR R1 and R2 is due to the intentional addition of calcium chloride in these SBR.

Table 3. Feeding Effluent* Physical-Chemical Parameters

Parameter	Unit	Results (mean ± standard deviation)		
pH	-	7.08 ± 0.25		
BOD_5	$mg \cdot L^{-1}$	500 ± 13		
COD	$mg \cdot L^{-1}$	1142 ± 140		
BOD/COD	-	0.44 ± 0.09		
Electrical conductivity	$mS \cdot cm^{-1}$	2.31 ± 0.26		
TSS	$mg \cdot L^{-1}$	44 ± 8		
Sodium	$mg \cdot L^{-1}$	622 ± 102		
Calcium	$mg \cdot L^{-1}$	R1 and R2	R3	R4
		170 ± 7	283 ± 2	385 ± 20

* Bleached eucalypt kraft pulp mill effluent.

Organic matter removal

The removal of organic matter was evaluated for soluble COD and soluble BOD_5 (Table 4). Similar organic matter removal efficiency was achieved in the four SBR. A COD removal of 60% and a BOD_5 of 90% were obtained, which agrees with reported activated sludge systems treating effluents from pulp mills (Kamali and Khodaparast 2015).

Table 4. Soluble COD and BOD_5 Removal

Treatments	COD (%)	BOD_5 (%)
Reactor R1 (FS)	60 ± 7	91 ± 4
Reactor R2 (GS)	61 ± 6	90 ± 5
Reactor R3 (GS100)	64 ± 6	90 ± 5
Reactor R4 (GS200)	62 ± 6	91 ± 4

* FS: flocculent sludge; GS: granular sludge; GS100: granular sludge with addition of 100 $mg \cdot L^{-1}$ Ca^{2+}; GS200: granular sludge with addiction of 200 $mg \cdot L^{-1}$ Ca^{2+}
Data are shown as mean ± standard deviation.

The granular aerobic sludge SBR R2, R3, and R4 achieved similar effectiveness to the flocculent sludge SBR R1 (Fig. 1). This agrees with the organic matter removal obtained from SBR in the treatment of recycled paper mill effluent (Morais *et al*. 2016).

The addition of calcium to SBR R3 and R4 did not reduce the efficiency of the removal of organic matter. No negative effect or reduced microbial activity was observed, demonstrating that the presence of calcium in these concentrations was inert to biological degradation. Nevertheless, over the long term, the addition of calcium may increase the removal efficiency of organic matter due to improved formation and stability of the granules (Ding *et al*. 2015a; Ye *et al*. 2016).

Physical characteristics of the granules

The physical characteristics of the granules formed in SBR R2, R3, and R4 were evaluated considering diameter size, settling velocity, and mechanical strength (Table 5).

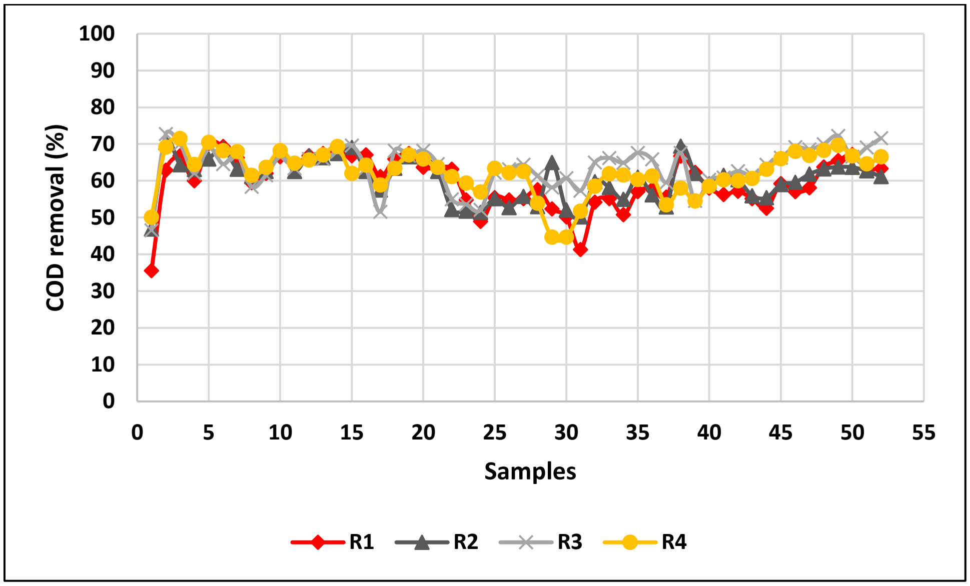

Fig. 1. Soluble COD removal. COD analyses were carried out three times a week (52 samples during 156 days).

The addition of calcium did not affect the size of the granule diameter, which presented an average of 11.16 to 11.33 mm, similar in the three SBR R2, R3 and R4. In addition, the granules of the three SBR achieved larger diameters than those observed by other authors with and without the addition of bivalent Ca^{2+} and Mg^{2+} cations (Yu *et al*. 2001; Adav *et al*. 2007, 2010; Tay *et al*. 2007; Zitomer *et al*. 2007; Sajjad and Kim 2015b; Wan *et al*. 2015). The formation of granules with diameters larger than 10 mm is greater than typical granules reported in the literature that are found to have a range from 0.8 to 4 mm (Long *et al*. 2014; Liu and Tay 2015; Pronk *et al*. 2015; Yan *et al*. 2015; Long *et al*. 2016). The larger diameter of the granules could be the result of the characteristics of the industrial effluent that has a significant portion of recalcitrant organic matter (low biodegradability) and by the SBR operational conditions. Compared to small granules, mass transfer and diffusion limitation of large granules are more obvious, which can lead to EPS consumption by the cell core of the granules that would weaken the granular structure (Zhang *et al*. 2015).

Table 5. Diameter, Settling Velocity, and Integrity Coefficient of the Granules

Treatments	Diameter (mm)	Settling Velocity ($m \cdot h^{-1}$)	Integrity Coefficient
Reactor R2 (GS)	11.31 ± 0.80	23.53 ± 6.90	16.97 ± 9.67
Reactor R3 (GS100)	11.16 ± 1.01	32.73 ± 6.25	0 ± 0
Reactor R4 (GS200)	11.33 ± 0.98	37.34 ± 8.53	1.56 2.21

* FS: flocculent sludge; GS: granular sludge; GS100: granular sludge with addiction of 100 $mg \cdot L^{-1}$ Ca^{2+}; GS200: granular sludge with addiction of 200 $mg \cdot L^{-1}$ Ca^{2+}
Data are shown as mean ± standard deviation.

The diameter of the granules was greater than 9 mm in all SBR (Fig. 2). Reactor R2, without calcium addition, presented 38.9% of the granule diameters between 10 and 11 mm, 27.8%, between 11 and 12 mm, and 27.8% greater than 12 mm. In SBR R3 and R4, with added calcium, granule diameters remained mostly (50% of the observations) within a range of 11 and 12 mm, presenting a smaller size variation and indicating greater stability. Approximately, 86% of the granules formed in a bioreactor with the addition of Mn^{2+} had a diameter size in a range of 2.5 and 3.5 mm, whereas less than 58% of those were in the same range without the addition of Mn^{2+}. Thus, the addition of Mn^{2+} promoted a narrower range and a greater similarity of the particle sizes of the granules (Huang *et al*. 2012). The addition of low concentrations (0 to 1.05 mM) of a chelating agent, nitrilotriacetate (NTA), reduced the growth rate and homogeneity of the granule size. The results showed that granules with a diameter equal to 0.5 mm were 20% in the reactor with the addition of 1.05 mM NTA and 60% in the reactor without NTA (Nancharaiah *et al*. 2008).

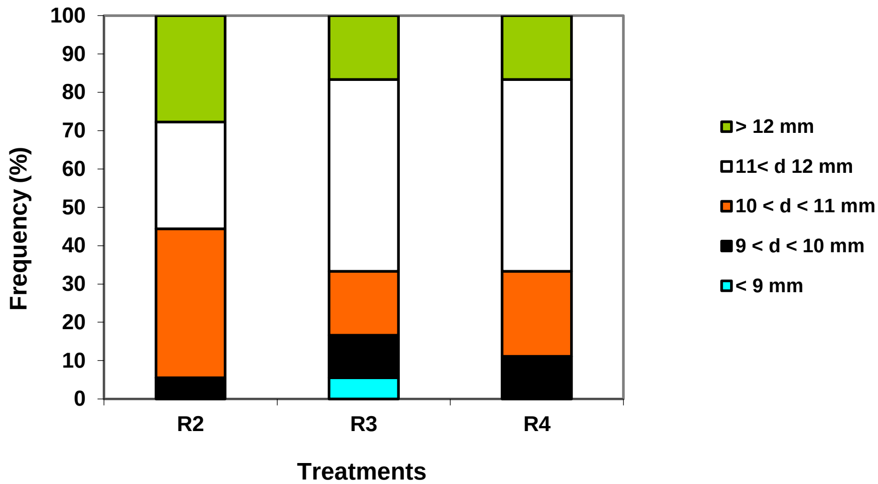

Fig. 2. Frequency of the diameter of the granules produced in reactors R2, R3, and R4

Granules produced in reactors R3 and R4 were more uniform and larger than those of reactor R2, which had no added calcium. The granules from reactor R3 were denser and more compact than those from the other reactors (Fig. 3).

The average coefficient of integrity of the granules was 16.97, 0.00, and 1.56 in reactors R2, R3, and R4, respectively (Table 5). The strength of the granules of reactors R3 and R4, with added calcium, was higher than that of reactor R2, considering that the higher the integrity coefficient, the lower the physical strength of the granules (Ghangrekar *et al*. 2005). This is in agreement with the increase in strength of the granules in the presence of 100 $mg \cdot L^{-1}$ of Ca^{2+} due to the calcium precipitation within the granules and the increase of the polysaccharide content, forming a resistant core (Lee *et al*. 2010). Visually, the granules of reactor R2 had a more fragile appearance than those from reactors R3 and R4.

The integrity coefficient of reactor R3 was zero in all the tests, indicating absence of rupture and demonstrating the importance of increasing the resistance of the granules in the presence of calcium.

The separation efficiency of the granules of the treated effluent depends on the sedimentation velocity of the granules. The sedimentation velocity of the granules produced in reactors R2, R3, and R4 was 23.5, 32.73, and 37.34 $m \cdot h^{-1}$, respectively (Table 5) showing a 36% and 59% increase in the settling velocity of the granules of the reactors R3, and R4 with the addition of calcium compared to those of the reactor R2 without added calcium. Positive bivalent and trivalent ions can bind to negatively charged cells to form microbial nuclei, improving settling and strength properties (Lee *et al*. 2010).

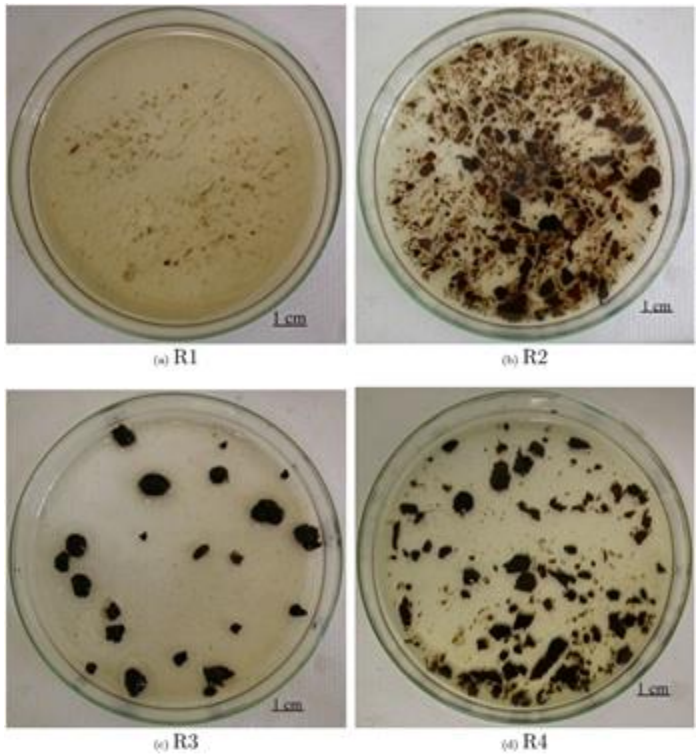

Fig. 3. Uniformity and granule size of sludge produced in reactors R1, R2, R3, and R4

Sedimentation velocities between 24.2 and 36.4 m·h^{-1}, similar to those of reactors R2, R3, and R4 were reported for granules with diameters between 1.2 and 1.8 mm (Liu *et al*. 2005). Velocities higher than 40 m·h^{-1} were reported in granules with added calcium or magnesium (Sajjad and Kim 2015a). The sedimentation velocity of flocculent sludge varies from 3 to 5 m·h^{-1} (Wang *et al*. 2006).

Specific growth rate of aggregates

Growth of granule size was initially slow (lag phase), followed by a more marked growth phase (log phase), and stabilizing at a steady state condition. The particle diameter was initially 0.6 to 0.8 mm, gradually increasing to values greater than 11 mm after equilibrium. These values allowed the calculation of the growth rate, which presented higher values in reactor R2 than in reactors R3 and R4 (Table 6).

Table 6. Specific Growth Rate of Granules Produced in Reactors R2, R3, and R4

Treatments	D_0 (mm)	D (mm)	D_{eq} (mm)	μ (10^{-3}·d^{-1})
Reactor R2 (GS)	0.61	1.26	11.31	2.09
Reactor R3 (GS100)	0.78	1.38	11.16	1.99
Reactor R4 (GS200)	0.75	1.23	11.33	1.55

* Size of microbial aggregates at lag phase (D_0), exponential (D), and at equilibrium (D_{eq}); GS: granular sludge; GS100: granular sludge with addiction of 100 mg·L^{-1} Ca^{2+}; GS200: granular sludge with addiction of 200 mg·L^{-1} Ca^{2+}

The growth rate of the granules can vary from 0.02 to 2.4 d^{-1} (Yang *et al.* 2004; De Carvalho *et al.* 2005; De Liu and Tay 2007; Adav *et al.* 2008). The lower growth rates of the granules observed in reactors R3 and R4 are important and may be related to the presence of calcium. Because of the lower growth rate, the mechanical strength of the granules was higher. High growth rates are related to a rapid proliferation of microorganisms, increasing the granule size and deteriorating granule structure and density (Lee *et al*. 2010). The specific growth rate and the granule size increase when the cycle time decreases (Adav *et al.* 2008). A lower growth rate increases the stability of the granules. Thus, a shorter cycle time and a higher organic loading rate (OLR) should be avoided (Liu and Tay 2007). On the other hand, a very low OLR may favor filamentous growth, causing loss of granule stability (Adav *et al.* 2008).

The size of the granules and the growth rate reduced with the increase of the shear force and the Nitrogen/COD ratio (Yang *et al.* 2004). The higher shear force lead to high detachment force, resulting in small size of aerobic granules (Yang *et al*. 2004). High N/COD ratio would encourage the growth of nitrifying populations, that grow much more slowly than heterotrophs (Yang *et al.* 2004). Heterotrophic and autotrophic bacteria in aerobic granules had specific growth rates of 3.18 and 1.52 d^{-1}, respectively. The fast-growing heterotrophs contribute to an increase of EPS, which is important for the maintenance of the integrity and stability of the structure of the matured granules (Cui *et al*. 2013). The size of granules and their specific growth rates decreased from 2 to 0.45 mm and from 0.085 to 0.065 d^{-1}, respectively, due to the increased ratio of nitrogen to COD (N/COD) (Yang *et al.* 2004).

Extracellular polymeric substances (EPS)

The amount of EPS produced by the sludge in each reactor was determined, and the polysaccharide (PS) and protein (PN) contents and the PS/PN ratio were quantified (Table 7). The PS production in the systems with granular sludge in reactors R2, R3, and R4, was higher than in reactor R1 with flocculent sludge (Table 7). A large amount of PS facilitates adhesion between cells and strengthens the microbial structure by forming a strong, thick polymer matrix. The greater the microbial activity, the higher the PS, which increases the initial granule resistance (Cao *et al*. 2014). The amount of EPS, especially polysaccharides, showed a positive correlation with the sedimentability of the sludge (Chen *et al*. 2010; Kim *et al*. 2014). Thus, the higher PS content and the PS/PN>1 ratio of the granules formed in reactors R3 and R4 may have contributed to the higher sedimentation velocity of these granules in relation to the granules formed in reactor R2.

Table 7. Polysaccharides (PS), Proteins (PN), and PS/PN Ratio in Extracellular Polymeric Substances (mg g^{-1}VSS) of Reactors R1, R2, R3, and R4

Treatments	PS	PN	PS/PN
Reactor R1	61	86	0.71
Reactor R2	105	179	0.59
Reactor R3	91	60	1.53
Reactor R4	80	70	1.14

The amount of protein was higher in the system with granular sludge without added calcium, in reactor R2. In addition, the specific growth rate of the granules was higher in reactor R2 (Table 6). The increase in particle size is faster with high protein content (Sajjad and Kim 2015b).

The total amount of EPS (PS + PN) was higher in reactors R2, R3, and R4, with granular sludge, than in reactor R1, with flocculent sludge. EPS influences granulation by changing surface characteristics such as surface charge and hydrophobicity. A lower surface charge and greater hydrophobicity favors cell aggregation (Zhu *et al*. 2015). Reactor R2 produced about 90% more EPS (PS + PN) than the others and had a higher growth rate of the granules (Table 6). A large amount of EPS facilitates adhesion between cells due to the presence of functional groups such as hydroxyl, carboxyl, and starch in the molecules of the extracellular polymer substances, rapidly increasing the size of the granules (Sajjad and Kim 2015b).

Scanning electron microscopy (SEM)

Scanning electron microscopy was used to evaluate the morphology and structure of the granules formed in the reactors (Fig. 4). The granules observed in reactors R2, R3, and R4 presented a great amount of filamentous bacteria and extracellular polymeric substances, besides the presence of mold spores. Channels (3a, 3d, and 3h) that allow the oxygen and nutrients transfer and favor the microbial growth inside the granules (De Sanctis *et al*. 2009) were observed. The EPS matrix can be observed distributed uniformly inside the granules, which indicates a uniform bioactivity of the aggregates formed in the three reactors (Yang *et al*. 2014).

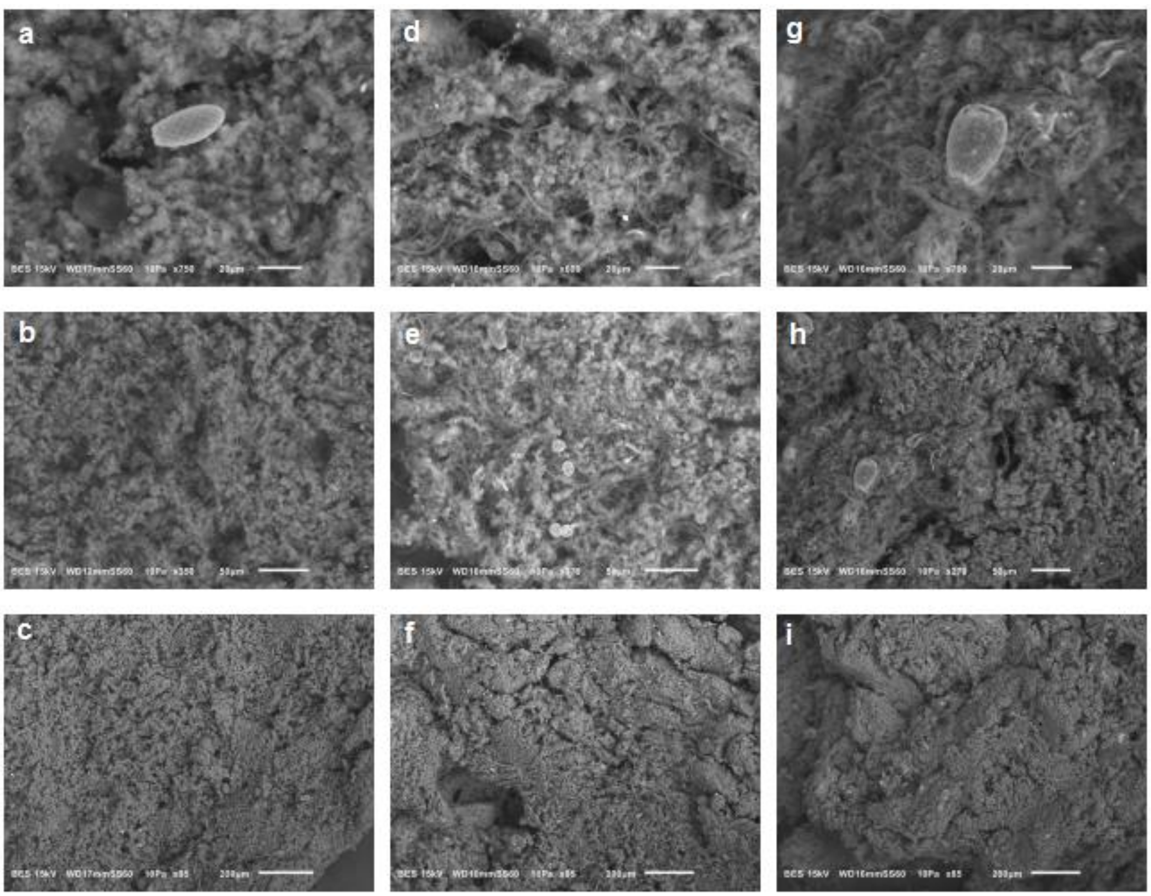

Fig. 4. Scanning electron microscopy of the granules from reactors R2 (a, b, c), R3 (d, e, f), and R4 (g, h, i). The images differ from each other due to the scale 20 µm (a, d, g), 50 µm (b, e, h), and 200 µm (c, f, i).

The granules formed in the reactors R3 and R4 with calcium addition showed a denser and more compact structure than in the reactor R2 without addition of Ca^{2+} (3c, 3f, 3i). Calcium allows the connection between anionic proteins and among other macromolecules, maintaining the structure of the granule (Caudan *et al*. 2014; Ding *et al*. 2015b). The addition of Ca^{2+} increases the granules strength and cohesion (Caudan *et al*. 2014). The precipitation of calcium and its accumulation inside the granules contributes to increase the strength and settleability of the granular aerobic sludge (Chen *et al*. 2016).

CONCLUSIONS

1. The addition of calcium improved the physical characteristics of aerobic granular sludge, but it did not affect the efficiency of the removal of organic matter by the reactors.
2. The addition of 100 and 200 $mg \cdot L^{-1}$ of Ca^{2+} increased the settling velocity of the granules formed with calcium addition and increased its strength, decreasing the integrity coefficient by 36% and 59%, respectively.
3. The mean diameter of the granules was similar in reactors R2, R3, and R4, but with lower variation in R3 and R4, with added calcium.
4. The higher settling velocity, the higher mechanical strength, and the high organic matter removal efficiency of the reactor R3, suggest that the addition of 100 $mg \cdot L^{-1}$ of Ca^{2+} guarantees greater stability of the bleached kraft pulp mill effluent through the treatment process using granular aerobic sludge.

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