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Research Article

Developing Aerobic Granular Sludge with Sesame like Biodegradable Support

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Abstract

Aerobic granular sludge (granules) is an innovative wastewater treatment, where dense biomass is in granular form. The problem of applying this technology at full scale is the granulation process time, which takes several months. In this paper, in an attempt to quicken this process, a biofilm was tested using several candidates as biodegradable support (BDS) for granulation. In this study, it was found that not only the granulation process was accelerated using sesame seeds as BDS (10, 15 and 21 days, compared to 2 months proposed by other studies), but also a new phenomenon was observed where new granules were generated from mature granules, enhancing the granulation process. This paper offers an enhanced option for granulation process.

Keywords: Aerobic granular sludge; Granulation process; Biodegradable carrier; Biodegradable support.

Introduction

Since Lettinga et al. 1980 discovered the granular sludge formation on the Up-flow Anaerobic Sludge Blanket (UASB) reactor [1], researchers have been constantly striving to improve this technology by applying granules to wastewater treatments. Granular sludge is an innovative wastewater treatment which presents various advantages. Among these advantages we can find the settling time, biomass concentration in small volumes, easy manipulation as it can be dried and stored, and the small amount of space required for application [2-4]. These advantages have made both organic elimination [2,5,3] and nutrient elimination (i.e., nitrogen and phosphorus, N and P,

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respectively) possible during sewage treatment [6-8], obtaining satisfactory results. At full scale, two granular processes have been performed: The CANON process and the Nereda process. The CANON process consists of autotrophic consortia among ammonium oxidizer bacteria (AOB) and ANAMMOX bacteria, which can be found inside the granules [6]. AOB oxidizes ammonia to nitrite while ANAMMOX bacteria utilizes nitrite and ammonia to produce N2 and both types of bacteria can use this specific Nitrogen forms as an energy source [9]. The Nereda process consists of consortia among AOB, nitrite oxidizing bacteria (NOB), phosphorus accumulating bacteria (PAO), denitrifying-phosphorus accumulating bacteria (DPAO), fermenters and denitrifying bacteria which are inside the granules [10,7]and aimed successfully removing nitrogenous and phosphorus.

Despite the advantages which granules offer, they also have some disadvantages compared to other wastewater treatment: slow granule generation time (granulation time) which can take from 2-8 months [3]. This proves problematic in the application of the granules, as the sewage comes to the Wastewater Treatment Plants (WWTP) every day and input volumes are as high as several hundred m3 per day. In order to fight this problem, micro-carriers have been introduced as a support to form biomass. Interestingly, some researchers have suggested using zeolites [2] or a water absorbent polymer (WAP) [5], reducing the start-up time of granular biolfilm from 1.5 months to 3 weeks [2,5,11]. However, Yoda et al., observed that between 10-40% of the microcarrier was colonized, which could be a setback and could also result in an economic loss. These materials must be slightly denser than water to avoid floating [12,11,]. For example, Wenjie et al. used gel pearls with 1.03g/cm3 density as microcarrier of polyvinyl [11]. On the other hand, the utilization of biodegradable supports (BDS) for granulation has not been found in the literature. However, BDS application for denitrification treatment is documented; in this studies BDS provide an organic resource for heterotrophic microorganism growth and promotes removing nitrate by these microorganisms [13,14]. The vast majority of papers about BDS on wastewater treatments have registered a biofilm form over BDS, but there are no references that confirm granule formation with these. According to this, a question could be asked; if microorganisms need a core to compact themselves and form granules, why shouldn´t this phenomenon be encouraged by removing BDS?

For this reason, the present essay aims to show whether it is possible to form granules from BDS, mainly focusing on biological organic matter removal. To this end, it is necessary to use a BDS whose composition would be a source of carbon for heterotrophic microorganisms, this way, this essay could be applied to different wastewater treatments. In addition, BDS must be a low-cost material, with the added difficulty of being able to be incorporated into a WWTP.

The phenomena which our team was expecting are the following: (1) Adhesion of biomass to BDS due to the attraction of microorganisms by the support as a carbon

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source. (2) BDS hydrolysis by heterotrophic microorganisms as a consequence of extracellular enzymes released by them. Thus, the BDS would be reduced by hydrolytic action. (3) Granulation process as a consequence of the biofilm formed on BDS; after the BDS is completely removed, the biofilm will substitute the space which was previously occupied by the BDS. Removal of organic material on the media. We wait which present paper helps future research into wastewater treatment.

Materials and methods

Selection of biodegradable support candidates

For these experiments, several characteristics of BDS were considered. These characteristics were classified on criteria variables and secondary variables. Criterion variables included physical and chemical properties such as density, hardness, water solubility, specific surface and toxic compound absence (Table 1). In agreement with other studies about microcarriers, BDS density should be slightly higher than water density [12,11]. In terms of water solubility, this property should be negative because if the BDS is soluble in water, the granulation will be unstable. This might be due to the pasting phenomenon [15], where solid materials form a gel structure in contact with water, loosing initial stability and having water solubility. Hardness is a strict quality, because the BDS will be applied with different shaking methodsin the WWTP. In the same way, specific surface area is an important property because a support whose surface area is higher than volume is useful in biofilm formation to treat waste water [16]. Lastly, toxic compound absence is also a criterion, due to the fact that it can affect both bacterial growth and wastewater treatment negatively.

On the other hand, secondary variables were mainly chemical properties such as percentage of lipids, carbohydrates, proteins, phosphorus and calcium contents, economic cost and size of BDS (Table 1).

These variables were classified as secondary because they are not as important as the criterion variables. Carbohydrate content had a negative effect on PAO growth; since this bacterial group does not have the ability to intake glucose, in contrast with other competitive groups like GAO [17,18]. This is important for phosphorus removal systems. As for protein content, we consider two possible scenarios: 1- protein with high cysteine content and 2- protein with low cysteine content. These possible scenarios were considered in this study, due to a higher sensitivity of PAO compared to sulfide ion [19] and the proteolytic activity of PAO [20]. As far as lipids are concerned, a BDS with a lipid content greater than 50% is desirable. The reason for this is that when lipids are hydrolyzed, they produce fatty volatile acids, which would be intaken by the PAO and GAO groups [10]. This is important, because these microorganisms promote the granulation process [4]. Moreover, to enhance PAO growth, a high phosphorus level is needed. As for granule stability, a high calcium support should be applied to help the interaction between calcium ion and the functional groups which promotes stability [21,22,4].

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Characteristic	Value	Unit
Size (length or diameter)	0.1-1	mm
Density	1.2>rho>0.9	g/cm3
Hardness	120-250	N
Specific surface	>1	mm^2/mm^3
Porosity	>40	%
Water solubility	no	-
Lipids	>25	%
Proteins (low cystein)	<25	%
Proteins (high cystein)	<10	%
Carbohydrates	<50	%
Phosphorus	500-1500	mg/100g
Calcium	500-1500	mg/100g
Toxic compound	no	-
Cost	<8	€/kg

Table 1: Criterion and second desirable variables of biodegradable supports.

With these BDS characteristics in mind, 35 candidates were researched in the literature references according to physical and chemical properties [Table A1]. We mainly focused on plant seeds because they are readily available, economical and their chemical compositions are known.

A database was set up by us, where different characteristics were exposed and evaluated with points and scores. Five candidates were chosen due to their scores and properties. On the one hand, these BDS candidates showed variable criterion within the required ranges. On the other hand, several candidates did not have an adequate range of secondary variables. The selected candidates included sesame, flax, poppy, millet, and clover seeds (Table 2).

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BDS candidates	Specific surface (mm2/ mm3)	Density (g/cm3)	Hardness (N or N/mm2)	Watersolubility	Toxiccompounds	Size (mm)	Porosity (%)	Lipids (%)	Proteins (%)	Carbohydrates (%)	Phosphorus (mg/100 g)	Calcium (mg/100g)	Economiccost (€/Kg)
Sesameseed	8.76	1.19	No reference; this is hard	No	No	2-Jan	53.58	75.38	22.3	1.8	774	1227	7.92
Poppyseed	5	1	226N/mm2	No	Alkaloids (lowconcentration)	<1	No reference	43	13	30	1276	1070	13
Milletseed	5.44	1.3-1.5	161N	No	No	1.4	36-46	3.9	12	65.4	599	910	7.8
Cloverseed	8.48	1.2	No reference; this is hard	No	No	1.4-1.6	31-32	8.7	32.5	45	No reference	185	7.25
Flaxseed	9.64	1	No reference; this is hard	No	No	4	27-57	45	21	10	530	212	5

Table 2: Characteristics of selected candidates as biodegradable supports.

Settling velocity of biodegradable support

To calculate the settling speed of the BDS candidates, 10 seeds of each BDS candidate were collected. Then a 1Lvolume test tube with a height of 21.4cm was filled with 1 L of water. Each seed was left to sink down into the water column and the elapsed time from water surface to the bottom was measured.

Culture media

During this experiment, wastewater from San Román WWTP (Santander, Spain) was used as culture medium. As this wastewater has a medium organic load (250mg COD/L), 8mL anaerobic sludge was applied, with an organic load of 31600mg COD/L, to set an organic load with 500mg COD/L value. Moreover, phosphate and ammonia values for this mix were 2.13mg $P\text{-}PO_3^{3-}$/L and 9.6mg $N\text{-}NH_4^+$/L.

Microscopic observation

For sample observation, we used an optical microscope and magnifying glass from the Zeiss Company in Germany. The Samples were observed each week, blending the two optical techniques to obtain the best results and to have microscopy and macroscopy perspectives.

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Sludge seed and operational conditions

For this experiment, active sludge was collected from San Román WWTP. Six test tubes of 1L were used as a sequencing batch reactor (SBR), with a height of 41.9cm and a diameter of 6.5cm, each SBR having an H/D rate of 6.4. Then, 100mL of active sludge were added to each SBR. We named these SBR as follows: SBR-S (sesame seed), SBR-P (poppy seed), SBR-F (flax seed), SBR-M (millet seed), SBR-C (clover seed) and SBR-CS (control sand); this last SBR was used as a control with siliceous sand which size was 0.5-2mm. Afterwards; the different seeds were introduced into their respective SBR. To obtain homogeneous conditions, the number of seed collected for this experiment was 300 seeds for each SBR. Then, wastewater was introduced into each SBR to make up to 500ml. An air pump with a stone diffusor at the bottom of the SBR and a 2,5L/min airflow capacity was used as a shaking mechanism. In this study, we have applied a shorter version of the granulation cycle carried out by Kiram Kumar Reddy et al. [23], and its phases were the following: 3min to fill, 23h in aeration, 5min for settling, 3min to wash out 250 mL, and 45min in idle. Apart from idle time, this cycle was repeated over several days.

Chemical analyses

For chemical analyses, a compact photometer PF-12^{Plus} (Macherey-Nagel Company, Germany) was used to determine P-PO3, N-NH4 on influents. For COD determination on sludge and San Román wastewater Merk COD kit (Merk Company, Germany) was used. OxiTop® heads (WTW, Xylem Analytics Company, UK) were used to determine BOD5.

Biomass Weight

The granules were weighted both wet and dry. To this end, 10 old sludge granules and 10 young sludge granules were introduced into porcelain capsules (the weight of these was measured before). Then, each capsule with each granule was weighed onGRAM© FC laboratory balance. The capsules were introduced into Selecta© Conterm oven at 105ºC for 24h. Then, granule weight was taken again.

Biomass density and concentration

Once the granules were weighted, distilled water was introduced into two 10 ml test tubes to fill 5 ml. Then the summation of the 10 granules of each type was carried out and, knowing this, the same 10 granules were introduced into the test tubes and the change in volume was measured. Applying Archimedes' principle, the density for each type of granule was determined. To determinate granular biomass concentration, the same principle was applied to this.

Statistical analyses

Statistical analysis was carried out in this study. For this, the statistical packet SPSS version 15.0 for Windows was used. The Krustal-Wallis non-parametric test was used to determine BOD removal and settling velocity [24]. The confidence interval to refuse hypothesis was 0.05.

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Results and Discussion

Dimension and settling velocity of biodegradable support candidates

Dimensional means was carried out on each selected BDS. In ellipsoidal shapes, length, width and height were measured while in spherical shapes, only width, which corresponds to the diameter of spherical-like seeds, was measured (Table 3). Moreover, average weight was calculated by measuring 100 seeds of each species before the experiments with the following results: 0.04g poppy/100seeds, 0.31g sesame/100seeds, 0.3g millet/100seeds, 0.06g clover/100seeds and 0.54g flax/100seeds. In contrast with the results of other studies made with inert supports [12,11], the BDS which was colonized did not have minimal size as these studies report, and a new viewpoint regarding BDS is opening up, suggesting a new perspective (Table 4). According to statistical analyses, a significant difference between settling velocity of sesame seed without biofilm and colonized sesame seed has not been found (Krustal-Wallis test; χ^2=0.562; p-value=0.453>0.05). This may be due to mass balance between BDS and biofilm, where loss of BDS and biofilm growth will be in equilibrium, with no increase in settling velocity

BDS (N=6)	L (mm)	W (mm)	H (mm)	Stand. Dev. L	Stand. Dev. A	Stand. Dev. H
Sesame seed	3	2	1	0.07	0	0
Poppy seed	-	1.02	-	-	0.04	-
Millet seed	-	1.9	-	-	0.22	-
Clover seed	-	1.04	-	-	0.05	-
Flax seed	4.04	2.02	0.88	0.05	0.04	0.04

Table 3: Dimensions of selected biodegradable supports.

BDS (N=8)	SV (m/h)	Stand. Deviation (m/h)
Sesame seed	57.528	17.82
Colonized sesame seed	61.452	9.612
Poppy seed	62.1	5.616
Millet seed	188.748	13.968
Clover seed	114.156	10.656
Flax seed	114.156	10.656

Table 4: Settling velocity of selected biodegradable supports

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Removal and behavior of biodegradable support candidates for granulation

Throughout the all experiments, two plant seeds had shown the best qualities as BDS: sesame and poppy seeds. In contrast about this, other candidates as BDS were worse options because different motives. During first week, clover and flax seeds were removed from the experiment, due to germination percentage was higher than 80%. While millet seeds were disintegrated by shaking mechanism throughout the experiment.

However, laminar-like flocs were presents on SBR-M from the third week, despite biofilm was not observed about this candidate. On the other hand, we found a particular problem with the sample collection of SBR-CS, as the sand grains settled immediately when the sample was shaken.

Despite extracting all the water from the SBR, those grains remained at the bottom of the reactor. So, the formation of biofilm was observed on the sand grains from second week and granules were not observed on SBR-CS. Although good results were showed about poppy seeds during two weeks, due to thin layer biofilm observed about edge of seeds at this period of time, this candidate was retired because that biofilm was not colonized other surface on this support.

A compactness biofilm was provided about sesame seeds, being the best candidate as BDS. Brown sphere-like structures were observed about sesame at first week, presenting a progressive growth throughout following weeks. As a result of this, 50% seeds were colonized at second week to form granular biofilm (e.g., granules) from 21 days; the following days, colonization was higher 95% sesame seeds (Figure 1).In contrast with other studies, colonization of BDS was approximately 100% while these studies were not aimed high percentage of colonization with inert microcarriers [2,5]. Also, granulation process was accelerated with sesame seed like BDS such as shown the present results, overcoming start-up with other microcarriers whose granulation process was 27 days [11].

However, breakage of most of the granules was observed around 5th weeks. That happens as a consequence of biofilm growth when surpassing a critical measure and shears force break biofilm stability, and detaches it [16]. For this reason, we repeated the experiment with sesame seeds on two SBR-S with diluted wastewater from gin distillery (10mL/L) [25], because its composition is more stable than the wastewater from San Román WWT.

Hence, influent composition was as follows: 820mg BOD/L, <0.4mg N-NH_4/L and 1.3mg P-PO_4/L; as exchanging volume was 50%, concentration stayed like following: 410mg BOD/L, <0.2mg N-NH_4/L and 0.65mg P-PO_4/L. Furthermore, during this repetition of the experiment under the same conditions as the first experiment, 40mL of young and old activated sludge were introduced (the latter being stored in anaerobic conditions for one month), when the experiments lasted for 40 days.

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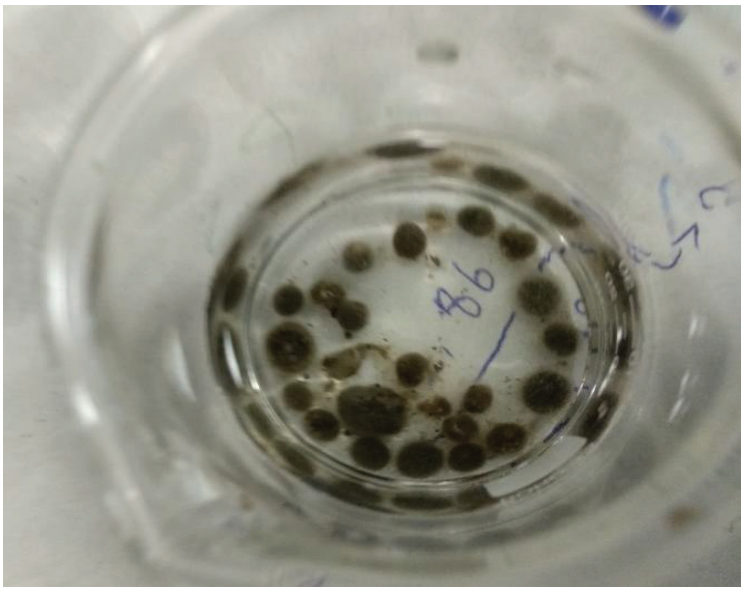

Figure 1: Developed granules after 28 days of experiment.

Interestingly, after 10 days granules were observed on young sludge experiment (Figure 2a). After 15 days in this experiment, a curious phenomenon was observed in young sludge experiment: granules were broken but little granules were observed together with sesame fractions (Figure 2b). In addition, these granules continued to grow throughout next week's (Figure 2c) and started to look like black filamentous granules with bad settling velocity.

To avoid the growth of these black filamentous granules, 4g $NaHCO_3$ was added to the media, in order to increase pH (6.7-6.8 from 7.1) and it was thought that this could be due to fungal growth; the growth of these granules was kept under control. On the other hand, granules were not seen until 15 days with old sludge experiment (Figure 2d). In contrast with young sludge experiment, these granules were rapidly not broken and BDS fractions inside granules and globular granules without BDS (previously had it) have been observed at next days (Figure 2e). This second experiment confirmed not only sesame seeds promote granulation process, but accelerate this process.

Moreover, confirms that not only can BDS be used in biofilm formation, as other studies suggested [13,14], but to carry out BDS granulation process and develop a new granulation process focusing on the rapid growth of the microorganism's core. Comparing the granulation process presented in this paper with other studies [2, 5,11], offers a fast start-up achieving granules at 10 days and 15 days, opening a gate for rapid transformation from aerobic granules to other physiological types of granules.

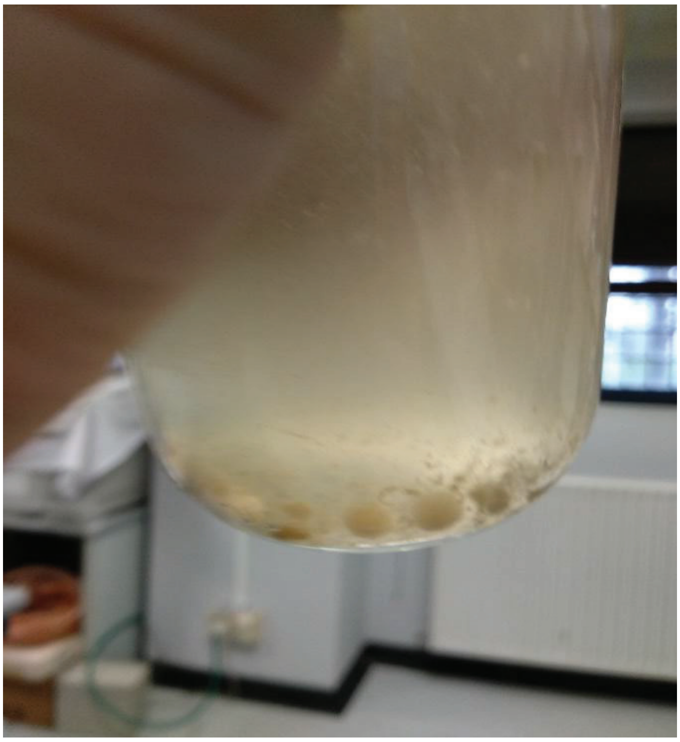

Figure2 (a): Young sludge granules with BDS after 10 days.

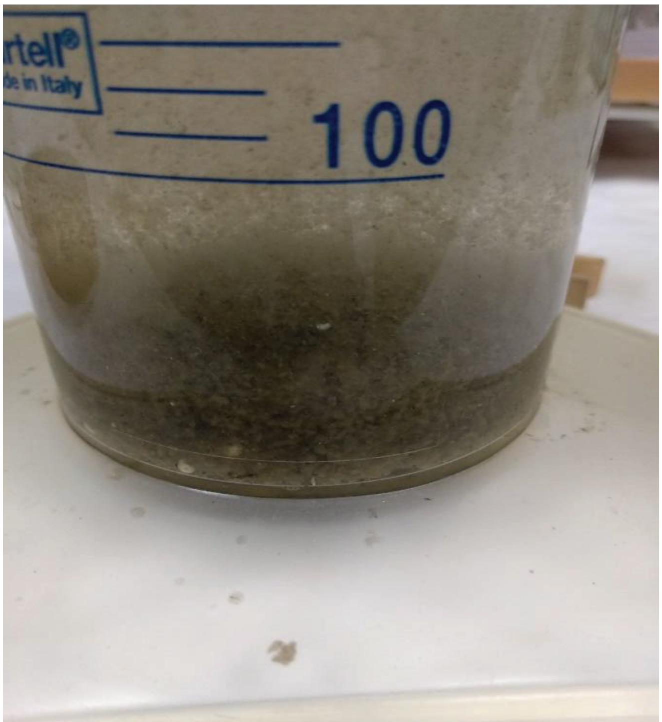

Figure 2(b): New formation of granules from BDS granules after 15 days with young sludge.

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Figure 2(c): New granules growth after 21 days with young sludge.

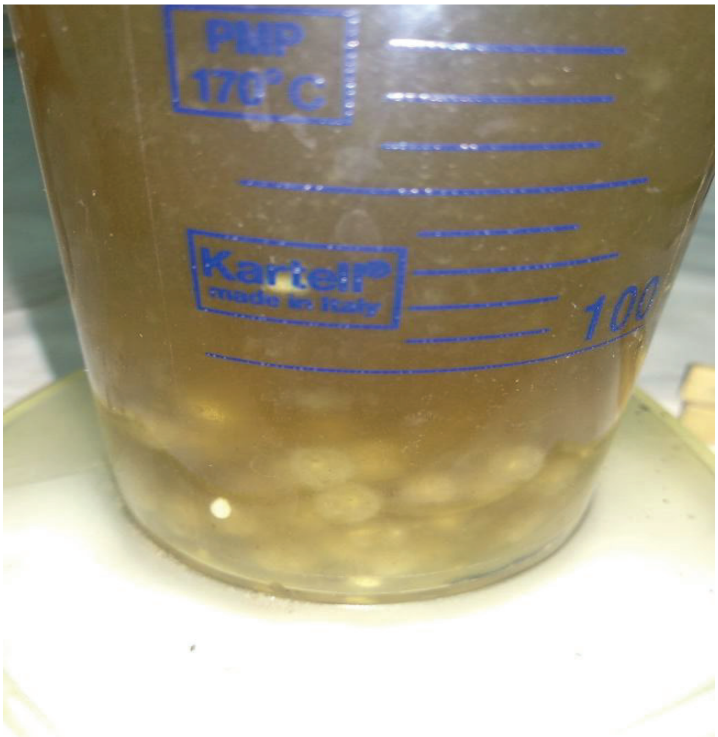

Figure 2(d): BDS granules with old sludge.

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Figure 2(e): Different types of granules on old sludge experiment. Red arrow: broken granules with BDS fractions; green arrow: globular granules.

Proof of granulation from granules

Throughout the second experiment, differences among the granules developed on BDS could be observed on both young and old sludge. Part of these differences refers to the structural stability of the granules. The old sludge BDS granules presented better stability than the young sludge BDS granules during granular biofilm formation. However, with young sludge, once the BDS granules were broken, new small and compact granules formed. An explanation was not found in this case. In contrast, small granules were found in both the broken and unbroken granules of the old sludge experiment on day 27. To find out what was occurring, a globular granule was examined. Interestingly, pre-granular structures were found inside the globular granule. After 34 days, new granules could be observed both externally and internally on mature granules (Figure 3a). Curiously, filamentous black granules could also be observed after 34 days (Figure 3b). These granules began to appear on the young sludge granules after 27 days, and their density was less than other granules. Finally, different types and sizes of granules were observed at the end of the experiment.

Interestingly, we could not find evidence what occurred in the young sludge experiment while was could see what happened in the old sludge experiment. A possible explanation for that may be that the young sludge had more growth and, as a consequence by this, more pre-granules

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could develop themselves from inside mature granules with BDS.

Then pre-granules were released when mature granules with BDS broke themselves. This hypothesis is based on observation of old sludge granules, because pre-granules were observed inside this type of granules. On the other hand, we can say sesame seed as BDS might act as a mechanism of double selective pressure:

1. adhesion of biomass with hydrolytic activity on BDS surface, removing another biomass that could not fix onto or hydrolyse this surface and
2. enhancing compacted biomass development.

The second point can be explained by the inner biomass hydrophobicity of BDS granules. Hydrophobicity is a factor which enhances granulation, due to hydrophobic biomass being potentially granular [3,4]. In addition, since BDS is slowly degraded, this might form hydrophobic biomass on middle layers, promoting new granulation inside BDS granules. Moreover, the microorganisms in the lower layers of the BDS granules will be like a feast-famine cycle, as the nutrient gradient is different throughout the layers of the granules, with fewer nutrients in the lower layers.

In agreement with other papers, a feast-famine cycle promotes biomass hydrophobicity due to microorganisms remembering these phases and then they reserve materials which are synthetized by them, increasing their hydrophobicity and density [26].The present study concurs with a recently published review undertaken by Aqeel et al. 2019. In this review, the authors propose that when granules break down, new flocs are formed, and these, in turn, originate new granules [27].

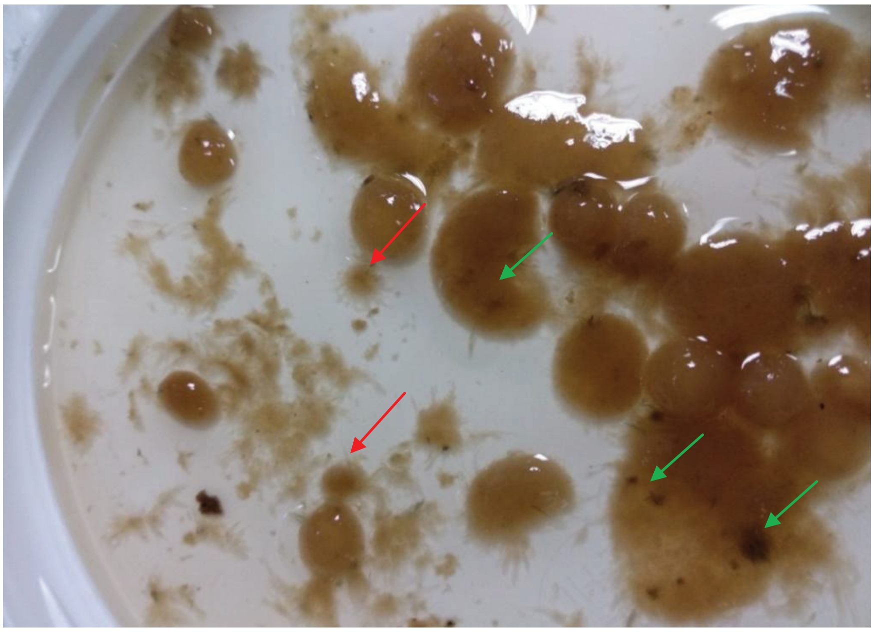

Figure 3(a): External and internal new granules formation from other granules.

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Figure: 3(b): Filamentous black granules. Red arrow: external new granules; green arrow: internal new granules; blue arrow: filamentous black granules.

Carbon source removal and global semi-saturation constant calculation of young and old sludge granules

First BOD5 proof was carried out young sludge granules, due to appearance of these that seems granules of other papers. According to statistical analyses, significant differences have been found among initial, 2h and 4h BOD5 results (Krustal-Wallis test; χ^2=21.196; p-value=0.000<0.05; means: 410±0mg BOD/L (n=9), 95.555 ± 48.762mg BOD/L (n=9) and 35.555 ± 34.318mg BOD/L (n=9)). As can be seen in Figure 4, BOD5 at 2h and 4h shown that approximately 5h, such BOD5 would be remove (Figure 4). Second BOD5 proof was carried out to compare organic source removing of young and old sludge granules, and to determinate

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global Ks both granule types. Then, another eight samples of BOD5 were taken (4 young sludge and 4 old sludge) to calculate global semi-saturation constant. Biomass and density were measured before and the operation of the substrate material in the SBR was assumed to follow a kinetic of type Monod, expressed as follows:

$$\frac{V\Delta S}{\Delta t} = -V * \left(\frac{K*X*So}{Ks+So}\right) \quad \text{Eq. (1)}$$

$$Ks = So * \left(\frac{\Delta t*K*X}{\Delta S} - 1\right) \quad \text{Eq. (2)}$$

Where ΔS: substrate variation (mg BDO/L), Δt: time variation (d), Q: affluent (L/d), Qw: effluent (L/d), So: initial substrate, V: reaction volume (L), K: maximum consumption rate (mg BOD/mg VSS·d^{-1}), X: biomass concentration (mg VSS/L) and Ks: semi-saturation constant (mg BOD/L). The values taken were Q=0.25L/d, V=0.5L, K=μmax/Yh (being μmax: maximum growth rate= 6mg STT/mg VSS and Yh: cellular yield=0.625mg BOD/mg Xh), Δt=0.166d, ΔS= 330mg BOD/L (young sludge granules); 220mg BOD/L (old sludge granules), So=410mg BOD/L and X=4680mg VSS/L (young sludge granules); 3780mg VSS/L (old sludge granules). As Ks had a negative value, absolute value for Ks was taken. ΔS was taken from the average value of BOD5 over 4h on each granule type.

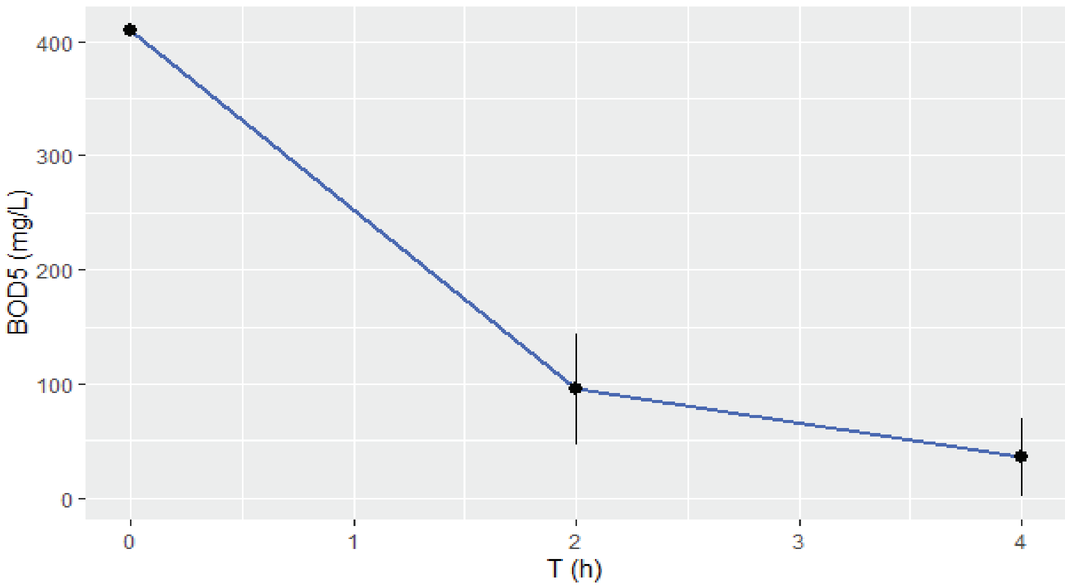

Figure4: BOD5 taken on initial, 2h and 4h from wastewater of second experiment with developed granules.

The results show that both biomass and density were higher in granules developed from young sludge than in those developed from old sludge. In addition, Ks value was lower in young sludge granules than in old sludge granules (Ks YG=8893.272mg BOD/L, Ks OG=10861.272mg BOD/L; YG: young sludge granules, OG: old sludge granules). One possible explanation for this could be that the old sludge was subjected to a stand-by situation before granulation with BDS. Once Ks was calculated, an Excel simulation was carried out under the same operational conditions using one day as the simulation

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time-frame [Appendix A]. As the simulation results show, BOD removal velocity was slightly higher in the young sludge granules (Figure S.1.A.) than in the old sludge granules (Figure S.1.B.). Also, the time range between young and old sludge granules can be appreciated, showing that carbon source removal inside the system took an hour longer with the first granules than with the second.

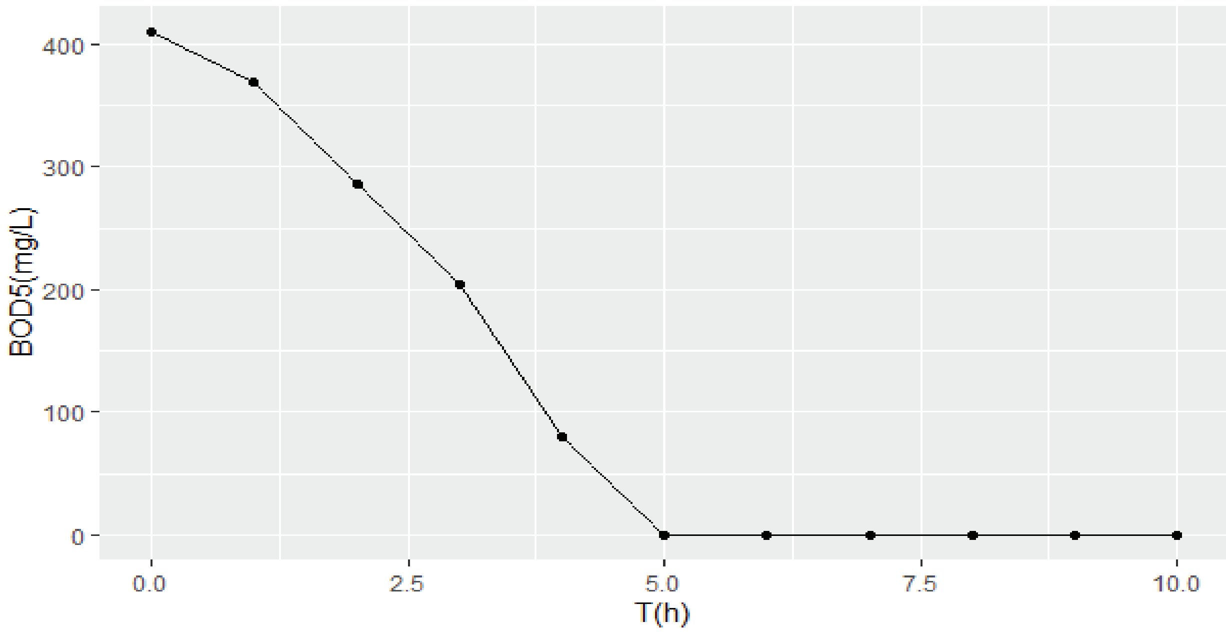

Figure S1 (A): Removing BOD from Ks calculation for developed granules young sludge.

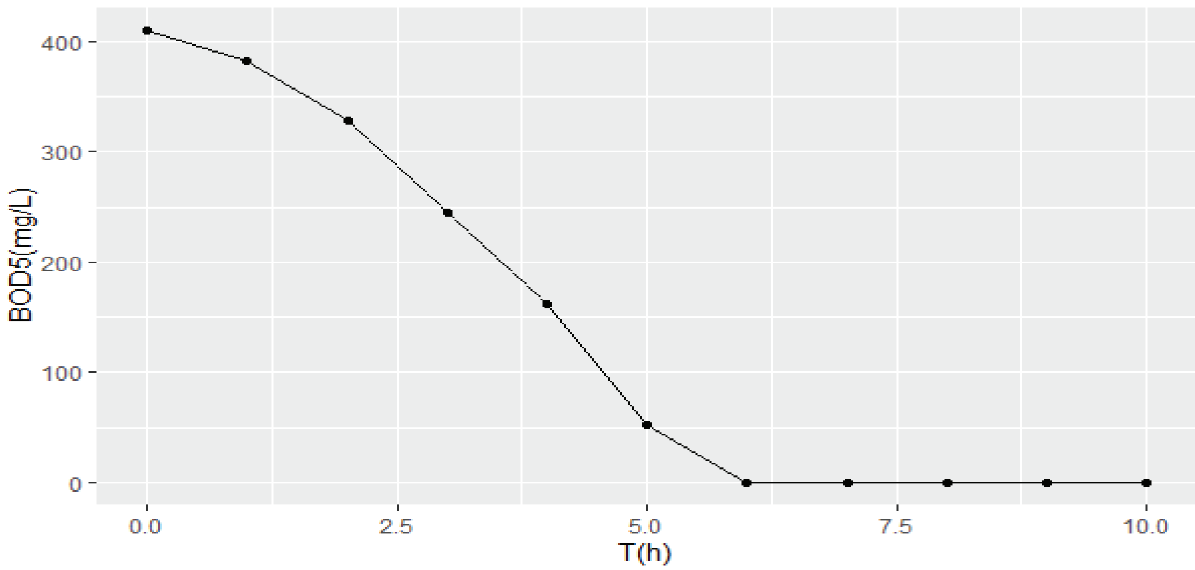

Figure S1 (B): Removing BOD from Ks calculation for developed granules old sludge.

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Conclusion

A rapid granulation process with sesame seeds as BDS has been shown in this paper with a start-up at 10 days, 15 days and 21 days. Our research is potential methods for granulation which can be applied for full scale wastewater treatment, granules transformation, opening the application of granules in different treatment. In addition, the application of sesame seeds as BDS produced the development of new granules from mature granules, concluding that BDS as nuclei for granulation is a great option to accelerate this process. We recommend to future research the use of fresh activated sludge (e.g., young sludge) because this presents better results than old sludge. On other hand, organic matter removing was efficient by these granules, promoting the application of these for domestic wastewater treatments.

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