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Biopolymers recovery from aerobic granular sludge treating industrial wastewater: preliminary analysis of different carbon routes for organic carbon utilization

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Abstract: Valorization of excess sludge through the recovery of high-value products, such as biopolymers, could be a crucial step to implement circular economy principles in wastewater treatment plant (WWTP). In this frame, the present study was aimed at evaluating the simultaneous production of polyhydroxyalkanoates (PHA) and extracellular polymeric substances (EPS), obtainable from the treatment of an agro-industrial wastewater. Two biological systems, one implementing aerobic granular sludge (AGS) and the other a conventional activated sludge operating as a sequencing batch reactor (SBR) were operated for 204 and 186 days, respectively. Both the systems involved a three-stage process for mixed microbial culture enrichment and biopolymers accumulation. The maximum biopolymers accumulation capacity was close to 0.60 mg $gVSS^{-1}$ in the AGS when the enrichment reactor was operated at 3 $kgCODm^{-3}d^{-1}$, whereas in the SBR it was slightly more than half (0.35 mg $gVSS^{-1}$). Biopolymers extracted from the AGS were mainly constituted by EPS (>70%), which percentage increased with the organic loading rate applied in the enrichment reactor up to 95%. In contrast, SBR enabled to obtain a higher PHA production (50% of the biopolymers). Results suggested that organic carbon was mainly channeled toward metabolic pathways for extracellular storing in AGS, likely as a consequence of metabolic stressors (e.g., hydraulic selection pressure, shear forces) applied for promoting aerobic granulation.

Keywords: Aerobic granular sludge; Biopolymers; Circular Economy; Extracellular Polymeric Substances; Polyhydroxyalkanoates; Sewage Sludge; Wastewater Treatment.

1. Introduction

During the last years, the efforts for the application of the biorefinery concept to the field of wastewater treatment plant (WWTP) are constantly growing. The wastewater biorefinery approach aims to overcome the view of the WWTPs for remediating wastewater to an acceptable quality only, but also to facilities aimed at enhancing energy and resource recovery. According to this approach, wastewater is considered a renewable resource from which treated water itself, but also energy and secondary raw materials can be recovered for several purposes [1].

Recent EU regulations (Directive 2018/851/EC) emphasized that overcoming criticalities in operational management of WWTP by exploiting environmental-friendly solutions for resource recovery could a valuable approach in line with sustainability and circularity concepts. In this frame, one of the main criticalities in WWTP is that related to sludge management and disposal, which alone can account for about 50 per cent of total operating costs (OPEX) [2]. Therefore, the valorization of sewage sludge (excess sludge) could be a sustainable solution to address both the issues related to its management and to implement circular economy principles for the recovery of high-value products [3]. Among

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these, the recovery of polyhydroxyalkanoates (PHAs) from sewage sludge has seen growing interest due to their wide application as precursors to bioplastics and, thus, as environmental-friendly material [4]. PHA are biodegradable, biocompatible and non-toxic biopolymers synthesized by several microbial species as a carbon and energy reserve generally under nutrient-unbalanced conditions [5].

The current industrial production of PHA is linked to the use of pure bacterial cultures that, although enabling to obtain very high PHA productivity (up to 90% cell dry weight), requires selected substrates, sterile conditions and a high oxygen demand [6], thereby leading to a high economic burden [7]. Using mixed bacterial cultures (MMC) from the activated sludge process is largely considered a sustainable solution to overcome the above issues. Indeed, MMC does not require sterile conditions and allows using organic matter contained in wastewater as feedstock. PHA production by MMC is carried out in a three-stage process involving the fermentation of the organic feedstock to produce volatile fatty acids (stage 1), the enrichment of the MMC with PHA-accumulating organisms (stage 2) and the intracellular accumulation of PHA (stage 3). Generally, this is obtained in a side-stream line operating in parallel with the main one dedicated to wastewater treatment. Despite the above advantages over the use of pure microbial cultures, PHA production by MMC results in lower PHA accumulation yields [8]. Effluents generated by agro-food industries were recently exploited as secondary feedstocks to increase PHA productivity, as these are characterized by a higher organic substance content than municipal wastewaters [9]. Nevertheless, recent study indicated that the breakeven price for the process must be reduced in order to make it more attractive [10], for instance by exploiting additional pathways for resources recovery.

To increase the cost-effectiveness of the process and maximize resources reclamation, the possibility of recovering other value-added co-products such as EPS (extracellular polymeric substances) together with intracellular polymers was recently proposed [11]. EPS are products of microbial metabolism and are mainly composed of proteins (PS), carbohydrates (PN) and in a minor quantity of nucleic acids, lipids and various heteropolymers [12]. EPS are functional substances that protect cells from external environmental agents such as toxic substances and, similarly to PHAs, they can be used as a source of carbon and energy under nutrients-lack condition [13]. EPS represent a potential recoverable resource that can replace alginate in the pharmaceutical, food, pharmaceutical and textile industries [14,15].

Simultaneous recovery of EPS and PHA was recently investigated in the literature. For instance, Kopperi and coauthors [16] demonstrated that is possible to achieve the simultaneous production of PHA and EPS by isolated *Providencia sp*. Similarly, in a recent study EPS and PHA were successfully produced by a MMC treating municipal wastewater [17]. In this frame, a suitable technology for maximizing biopolymers recovery from wastewater treatment, while implying smaller footprint and less energy requirement when compared with conventional activated sludge system is aerobic granular sludge (AGS). In AGS system, microbial adhesion of microorganisms in large particles (> 1 mm) occurs, and EPS constitutes the structural elements of these bio-aggregates [18]. Pronk et al. [19] demonstrated that different types of biopolymers can be extracted from AGS treating municipal wastewater depending on the operating conditions applied. Indeed, given that the key element leading to the synthesis of PHA and EPS is carbon, it was postulated that based on operating conditions a regulatory mechanism in carbon utilization could switch in pathway from PHA polymerization to EPS synthesis and vice versa [16,20]. However, the low organic carbon concentration in municipal wastewater could be the trigger of such a process limitation.

To the best of the authors' knowledge, any relevant studies are available in the literature on the recovery of biopolymers (EPS+PHA) from AGS treating industrial wastewater with high-carbon content. Considering this, the present study was aimed at evaluating the simultaneous production of biopolymers, PHA and EPS, obtainable from the treatment of an agro-industrial wastewater by implementing AGS. In more detail, the effect of the organic loading rate (OLR) on the PHA and EPS yields was studied, and the

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results were compared with that of a conventional activated sludge plant operating in parallel with the AGS system.

2. Materials and Methods

2.1. Wastewater characterization

The wastewater used for the experiments was collected from a citrus processing industry located in Palermo (Italy). Citrus wastewater was generated by the processing of different fruits such as oranges, tangerines, and lemons, as well as by the machineries washing and essential oil extraction. Citrus wastewater was characterized by a low pH equal to 4.5, on average, high chemical oxygen demand (COD) ranging between 4.5-5 gCOD L^{-1} and unbalanced nutrients (carbon to nitrogen ratio greater than 200). Table 1 summarizes the average values of the main characteristics of citrus wastewater.

Table 2.: Main values of the principal characteristics of citrus wastewater.

Parameter	Value
Total COD [mg L^{-1}]	4486±391
Soluble COD [mg L^{-1}]	3281±195
Total nitrogen [mg L^{-1}]	21±7
Total phosphorous [mg L^{-1}]	12.1±4.3
pH [-]	4.2±0.3

2.2. Experimental setup

The experiment was carried out using a three-stage process consisting of a fermenter (i), a sequencing aerobic granular sludge (AGS) reactor for MMC enrichment (ii) and a fed-batch reactor (FBR) (iii) to maximize the production of EPS and PHA. The bioreactor for the enrichment of the MMC was seeded with activated sludge from an industrial WWTP treating the same wastewater used as organic feedstock. This unit consisted of a 4-L poly-methyl-methacrylate column-reactor (700 mm of height, 60 of inner diameter) equipped with an internal riser according to a sequencing-batch-airlift-reactor (SBAR) configuration [21]. This reactor operated aerobically under the typical feast/famine (F/f) regime. Continuous aeration (3.5 LPM, corresponding to 2.4 cm sec^{-1} of flux velocity) was provided by an air stone-diffuser placed at the bottom of the reactor and coaxial with the riser, connected to an air blower.

The raw citrus wastewater was stored into a refrigerated storage tank and after pH neutralization it was fed to the fermenter. From this, wastewater was feed in upflow mode to the reactor, by means of a peristaltic pump connected to the fermenter. The effluent was discharged at different heights to change the volumetric exchange ratio (VER) according to operational needs by means of a dedicated pump. All electrical devices were connected to a programmable logic controller (PLC) that handled cycle operations. The AGS was operated in cycles of 12 h, consisting of 10 minutes of influent feeding, 690 minutes of aeration, 5 minutes of settling, 10 minutes of effluent discharge and lastly 5 minutes of idle. The volume of wastewater fed and discharged in each cycle changed according to the operating period (see section 2.3), thus resulting in different hydraulic retention times (HRT). An additional stream contained nitrogen and phosphorus (2 gCH_4N_2O L^{-1} and 1 gK_2HPO_4 L^{-1}) was added at the beginning of the cycle to obtain a ratio between carbon (as total chemical oxygen demand - COD)/nitrogen/phosphorous equal to 100: 5: 1.

Another enrichment SBR (22 L) with flocculent sludge was operated in parallel with the AGS. The SBR operated under the same conditions of the AGS apart from the settling time that was of two hours. For additional information about the SBR, the reader is referred to the literature [22].

The storage capacity of the enriched MMC was assessed by the performances of several accumulation assays performed in a 1.5 L FBR seeded with the sludge of both the

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AGS and SBR. An automatic dissolved oxygen control system provided for the calculation of the oxygen uptake rate (OUR) in order to monitor the accumulation capacity of the MMC.

Figure 1 shows the plant layout.

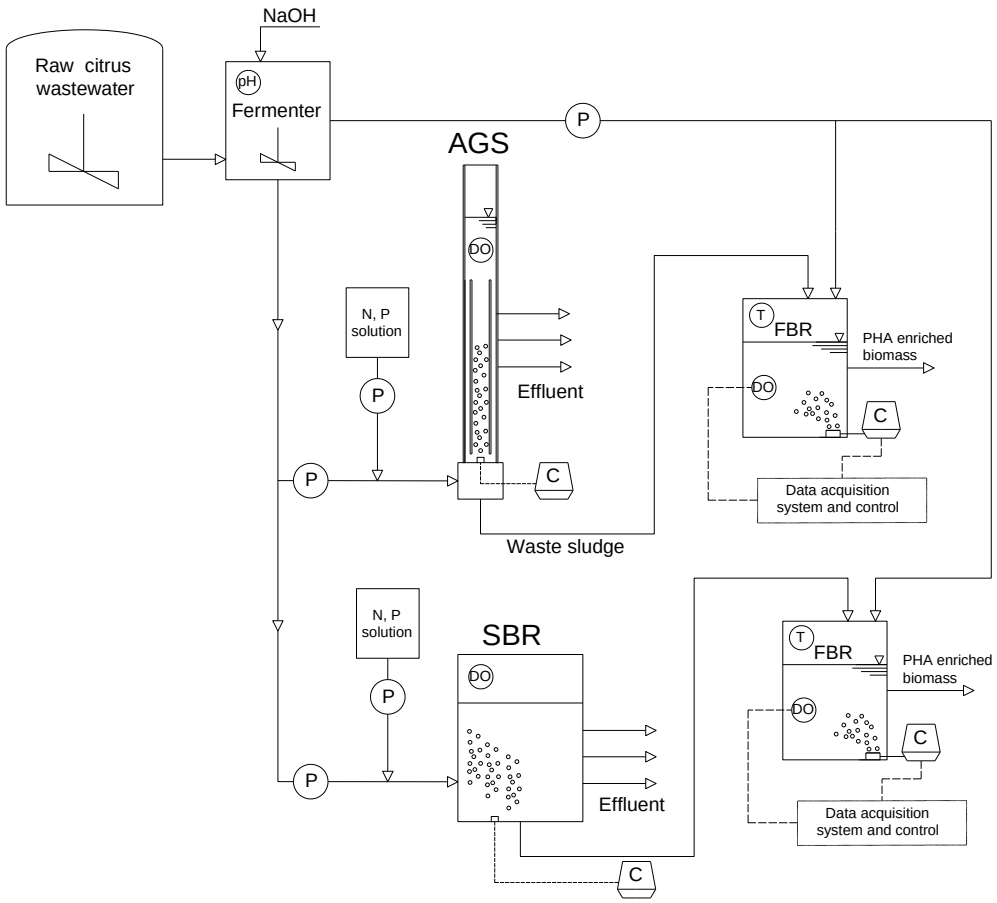

Figure 1. Pilot plant layout. Legend: C: Air blower; P: Pump; pH: pH controller; DO, dissolved oxygen sensor; T: temperature sensor.

2.3. Operational strategy of the enrichment reactors

The enrichment AGS was operated for 204 days with its operation subdivided into three periods, namely Period 1 (P1), Period 2 (P2) and Period 3 (P3), having a duration of 126, 37 and 41 days, respectively. These periods were characterized by three different OLRs, equal to 1 kgCOD $m^{-3}d^{-1}$ (P1), 2 kgCOD $m^{-3}d^{-1}$ (P2) and 3 kgCOD $m^{-3}d^{-1}$ (P3), obtained by varying the VER of the reactor.

Period 1 was aimed at achieving complete granulation of the activated sludge. This occurred within approximately 70 days, after that a period equal to three time the sludge retention time (SRT) was passed before increasing the OLR. All the other periods lasted at least a period equal to three SRT to obtain steady state. The total suspended solids (TSS) concentration was maintained at approximately 5.0±0.4 gTSS L^{-1} by daily purging the excess sludge according to the biomass growth yield. Accordingly, the food to microorganism's ratio (F/M) resulted equal to 0.20 kgCOD $kgTSS^{-1}d^{-1}$ during Period 1, 0.41 kgCOD $kgTSS^{-1}d^{-1}$ during Period 2 and 0.64 kg COD $kgTSS^{-1}d^{-1}$ during Period 3, on average.

Operating conditions of the two enrichment reactors are shown in Table 2.

Table 2.: Main operating parameters of the AGS and SBR.

	P1		P2		P3	
	AGS	SBR	AGS	SBR	AGS	SBR

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Duration [d]	126	78	37	32	41	62
Daily flow [L d^{-1}]	0.90	5	1.80	10	2.72	15
VER [-]	0.11	0.11	0.22	0.23	0.33	0.34
Biomass concentration [gTSS L^{-1}]	4.89±0.21	4.56±0.12	5.11±0.09	4.42±0.09	5.06±0.11	4.46±0.15
OLR [kgCOD $m^{-3}d^{-1}$]	1.02±0.06	1.07±0.03	2.08±0.04	2.04±0.09	3.12±0.08	3.05±0.15
F/M [kgCOD $kgTSS^{-1}d^{-1}$]	0.20±0.03	0.22±0.08	0.41±0.06	0.43±0.04	0.62±0.08	0.63±0.05
SRT [d]	23±1	22±2	10±3	7.0±1.5	9±1.6	5.1±0.9

At the end of each period, at least two accumulation assays were performed in order to evaluate the maximum EPS and PHA achievable by the enriched granular and flocculent sludges.

The accumulation reactor was operated aerobically by maintaining the dissolved oxygen (DO) concentration between 2-4 mg L^{-1}, and according to the aerobic dynamic feeding (ADF) regime [23], obtained by adding the substrate (fermented citrus wastewater) in pulses of 50 mL. To maximize the biopolymers accumulation and prevent biomass growth no nutrients source was fed. The maximum accumulation capacity was assumed when the DO remained close to saturation value after adding a substrate pulse.

2.4. Analytical methods

All the physical-chemical analyses for the assessment of total suspended solids (TSS), volatile suspended solids (VSS), Chemical Oxygen Demand (COD), total nitrogen (TN) and total phosphorus (TP) were carried out according to the standard methods [24].

COD measurements were performed on both the influent and effluent samples of the enrichment AGS and SBR reactors, without any pretreatment in order to assess the systems' purification performances. The settling properties of the granular sludges in the enrichment reactors was assessed by calculating the SVI_{30} and SVI_5. These were calculated by dividing the volume occupied by the sludge inside a 1 L graduated cylinder after a 30 min, or 5 minutes in the case of SVI_5, of static settling by the concentration of TSS in the sample. A unit ratio between SVI_5 and SVI_{30} was considered as an indicator to assume the achievement of complete granulation in the enrichment AGS. Moreover, the average particle size and the particle size distribution (PSD) of granular sludge was measured by an optical granulometer (QICPIC - Sympatec). The percentage of granules in the enrichment AGS was assumed to be equal to the percentage of particles with a size greater than 400 μm [25].

PHA and EPS were measured in the FBR at the beginning of the assay and after that the maximum accumulation capacity was achieved. At the same time, TSS, VSS and COD were measured on the same samples to assess carbon mass balance. Specifically, PHA were extracted from the sludge samples by applying the procedure suggested by Fiorese and co-authors [26] using 1-2 propylene carbonate as solvent. The purity of the extracted PHA was measured by a spectrophotometer using commercial standards as blanks (PHB-HV 88-12% and PHB by Sigma Aldrich). EPS were extracted according to the heating method [27]. Then, the extracted EPS were characterized by measuring the protein [28] and carbohydrates concentrations [29] using bovine serum albumin and glucose as standards, respectively.

DO, pH and temperature measurements in the enrichment and accumulation reactors were performed by on-line sensors (WTW).

2.5. Calculation

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The percentage of biopolymers produced (EPS or PHA) expressed in dry weight (%wt) was calculated by dividing the mass of PHA and EPS by the mass of VSS present in the medium (equation 1.1 and 1.2):

$$PHA\ (\%) = \frac{gPHA}{gVSS}\ x\ 100 \qquad (1.1)$$

$$EPS\ (\%) = \frac{gEPS}{gVSS}\ x\ 100 \qquad (1.2)$$

Mass balance for organic carbon (as total COD) in FBR was assessed according to equation 2:

$$COD_{d(gCOD)} = PHA_{p(gCOD)} + EPS_{p(gCOD)} + X_{p(gCOD)} + COD_{r(gCOD)} \qquad (2)$$

- COD_d: total mass (g) of COD dosed during the entire accumulation assay until the maximum accumulation capacity was obtained;
- PHA_P: mass of PHA produced (g), obtained as the difference between the final PHA mass at the end of the accumulation assay and that measured at the beginning. The following stochiometric coefficients were assumed for referring PHA mass as COD (1,67 gCOD $gPHB^{-1}$ e 1,92 gCOD $gPHV^{-1}$);
- EPS_P: the mass of EPS (g) produced, which was obtained as the sum of the proteins (PN) and carbohydrates (PS) produced during the assay multiplied by the respective stoichiometric coefficients obtained experimentally (1,36 gCOD gPS^{-1} e 1,40 gCOD gPN^{-1});
- COD_r: the residual mass of COD obtained from the product of the reactor volume by the COD concentration measured in the supernatant at the end of the accumulation assay;
- X_P: the new biomass produced during the assay, measured as the volatile suspended solids produced during the accumulation assay as COD (1,42 gCOD $gVSS^{-1}$), minus the mass of biopolymers (EPS+PHA) as COD and the residual COD at the end of the assay.

3. Results

3.1. Characteristics of aerobic granular sludge

The granulation process of the flocculent inoculum was monitored by means of the PSD and the ratio SVI_5/SVI_{30}. Figure 2 depicts the average PSD obtained in each experimental period and that of the seed sludge (Fig. 2a), and the trend of the SVI_5/SVI_{30} and SVI_5 (Fig. 2b).

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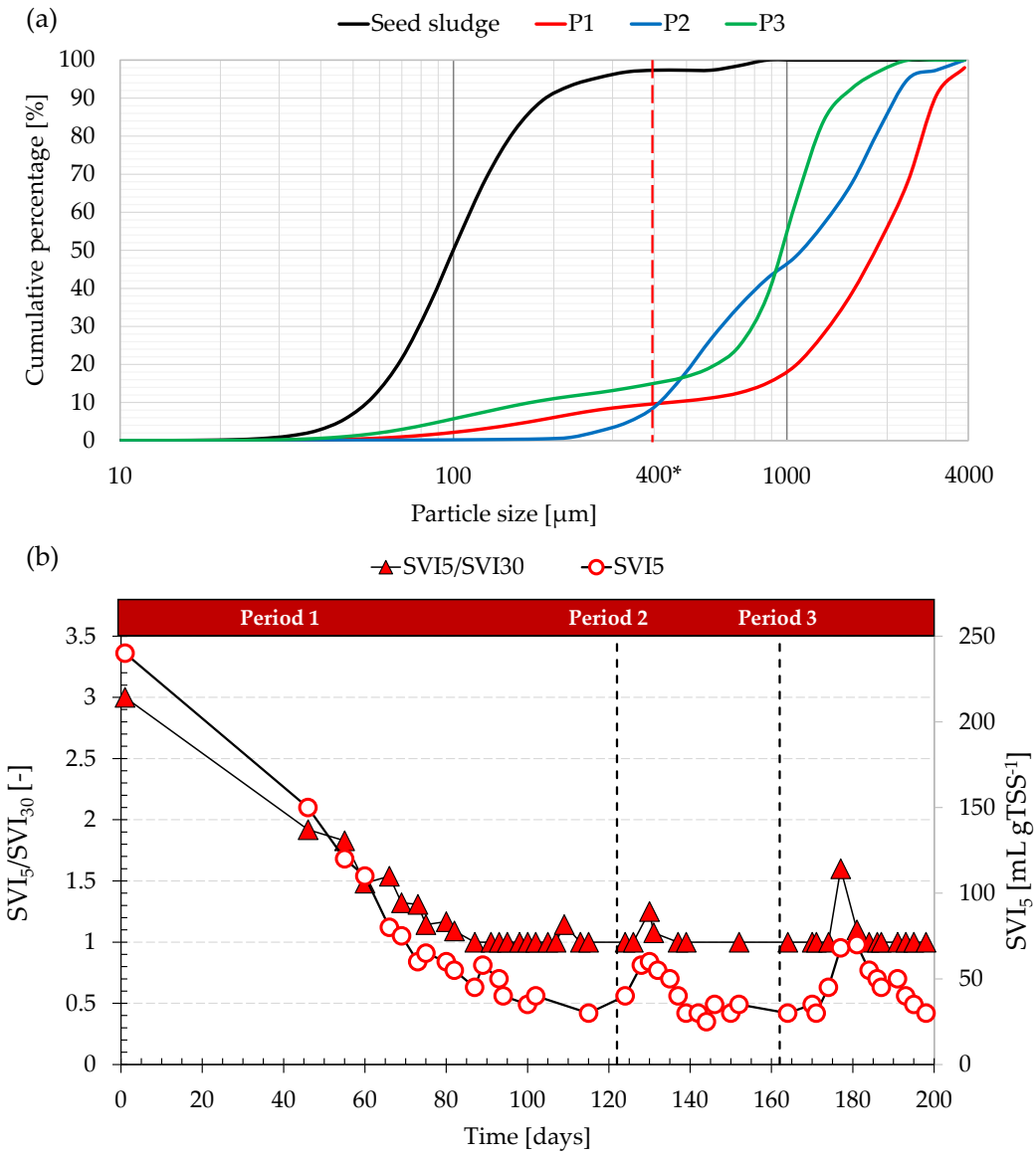

Figure 2. Particle size distribution of aerobic granular sludge during the experiment (a); trends of SVI_5/SVI_{30} ratio and SVI_5 (b). 400*µm represent the cut-off value between flocculent and granular sludge assumed in this study.

The seed sludge was characterized by an average floc size (particle size corresponding to 50% of cumulative distribution) close to 100 µm and a SVI_5/SVI_{30} close to 3. According to the SVI_5/SVI_{30} ratio, complete granulation was obtained after 80 days, although the percentage of sludge particles assimilated to granules (>400 µm) was not 100% but about 90%. This suggested the coexistence of granules for the most part and residual flocculent sludge in the medium of the enrichment AGS reactor. At steady state, the SVI_5 was close to 40 mL $gTSS^{-1}$, while the average particle size of granules was 1.8 mm.

In Period 2, the SVI_5/SVI_{30} remained at approximately 1 during the entire period, as well as the SVI_5 that remained close to 40 mL $gTSS^{-1}$. In contrast to the previous period, the average size of granules decreased to approximately 1.2 mm, although the percentage of granular sludge was similar (90%). A significative disappearance of flocculent sludge was noted as suggested by the negligible percentage of particles with a size smaller than 300 µm.

In Period 3, the average size of granules still decreased, reaching a value of approximately 0.90 mm and suggesting the occurrence of some degranulation. Contextually, the

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percentage of flocculent sludge slightly increased, thereby resulting in a small reduction of the granules percentage to 85%. Nonetheless, apart from a short period around the 180th day, the SVI_5/SVI_{30} was maintained at 1 and the SVI_5 was on average close to 50 mL $gTSS^{-1}$, indicating the excellent settling performances of AGS.

The achieved results were in line with previous literature in which an increase instability and related deflocculation was observed at increasing the OLR [30]. Indeed, high OLRs result in fast granules formation but the developed granules are unstable due to the large size resulting from excessive microbial growth. Moreover, since no anaerobic feeding was performed in the present study, it was assumed that the growth of fast-growing bacteria prevailed over slow-growing thus resulting in an albeit modest granules instability at increasing of the OLR [31].

3.2. COD removal performances in the enrichment AGS and SBR reactors

Figure 3 depicts the average COD removal efficiencies obtained in the enrichment reactors during the three experimental periods.

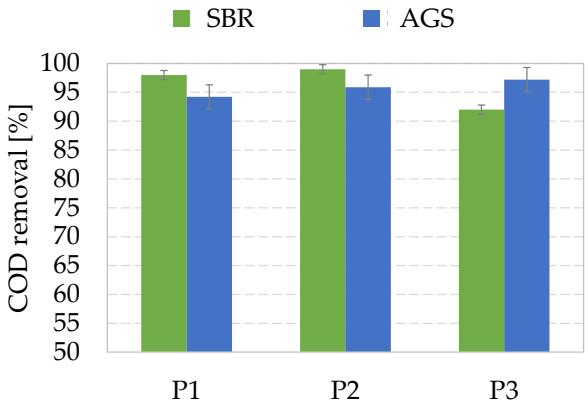

Figure 3. Average COD removal efficiencies in the enrichment ASG and SBR.

Both the enrichment AGS and SBR exhibited very high carbon removal, higher than 90% in general. In more detail, SBR showed a slightly decreasing trend with the OLR, although the effluent COD concentration resulted always lower than 400 $mgO_2\ L^{-1}$, in compliance with EU regulations for industrial activities [32]. In contrast, AGS showed greater reliability in COD removal at increasing OLR. Indeed, even in Period 3 the average COD removal was higher than 95%, thereby resulting in effluent COD concentration lower than 200 $mgO_2\ L^{-1}$.

In a previous study it was pointed out that the simultaneous enrichment of MMC in biopolymers-storing populations via the feast/famine strategy and achievement of pollutant removal in compliance with regulations could be challenging in conventional activated sludge systems with flocculent biomass [22]. This is because at high OLR occurrence of filamentous bulking decrease the sludge settling properties that worsen the effluent quality [33]. Therefore, this indicated that the enrichment and the wastewater treatment should be performed in different stages when using conventional systems, thereby involving an increase of the facilities required and plant footprint [34].

In the present study, it was noted that the MMC enrichment strategy did not cause significant decrease of the AGS performances, thus suggesting that granular sludge systems could perform the enrichment and the wastewater treatment phases simultaneously. Moreover, the possibility to operate under higher OLR result in a further decrease of the treatment/enrichment reactor and of plant footprint as well.

3.3. Assessment of biopolymers accumulation capacity

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Accumulation assays were performed in the FBR once the enrichment AGS and SBR became stable in each period. More precisely, a ratio between the length of feast and famine phases lower than 0.20 was assumed as indicator for the selection of microorganisms with storage capacity [35].

The operation of the enrichment AGS became stable after the 90th, 140th and 181st day in Period 1, Period 2 and Period 3, respectively. Thus, the accumulation assays were performed in two replicates on 120th - 121st (P1), 160th – 161st (P2) and 123rd – 124th days (P3).

Figure 4 shows the maximum biopolymers content (as a sum of PHA and EPS) obtained on average from the two assays of each period, in comparison with that obtained from the enriched SBR.

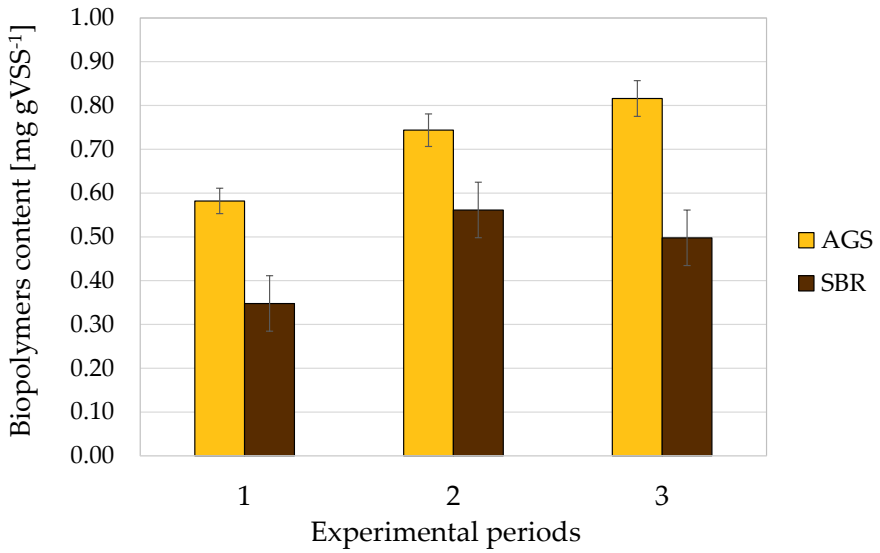

Figure 4. Average biopolymers content (as a sum of PHA and EPS) obtained at the end of the accumulation assays in the FBR from the AGS and SBR systems.

The measured biopolymers content referred to the unit of VSS resulted higher in the AGS in all the periods. In Period 1, the maximum accumulation capacity in the AGS was close to 0.60 mg gVSS^{-1}, whereas in the SBR it was slightly more than half (0.35 mg gVSS^{-1}). When increasing the OLR in the enrichment reactors, the MMC of AGS and SBR showed a different behavior. Indeed, while a positive linear relationship between the OLR in the enrichment reactor and the maximum biopolymers accumulation in the FBR was noted in the AGS, a decrease of the maximum biopolymer accumulation after a certain OLR was observed in the SBR. This result indicated that up to a certain OLR, the selection of biopolymers accumulating microorganisms in the enrichment reactor is less effective in the SBR, whereas this was maintained in the AGS, although it cannot be excluded the existence of a such threshold value also for the aerobic granular sludge systems.

In previous studies, it was already observed that too high substrate availability was not favorable to select microorganisms able to convert organic carbon into intracellular or extracellular storage polymers [36,37]. The feast/famine selection strategy is based on the capacity of the accumulating-population to growth on the stored substrates produced during the feast phase, when external substrate is no longer available. This allows to washout from the system those microorganisms not able to produce storage compounds, thereby resulting in the enrichment of the MMC with biopolymers storing populations [38]. Indeed, when organic substrate availability increases, the length of the feast phase is higher, while reducing that of starvation. Consequently, microorganisms with internal storage capacity are no longer favored over non-storing populations during famine phase. In AGS systems, because of the higher microbial density of the bio-aggregates respect to the flocculent sludge in conventional SBR, substrate uptake during the feast phase is faster [39,40]. This, under the same operating conditions (e.g., cycle length, OLR, aeration rate, VER, etc.), could result in a longer famine phase, which guarantees for a more effective

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selection of biopolymers-accumulating microorganisms. In the present study, it was noted that the length of the feast phase was shorter in the AGS than the SBR in all the periods. Indeed, in Period 1 the feast phase lasted about 35 minutes at steady state, whereas in the SBR it was close to 45 minutes. Similarly, at steady state in Period 2 and Period 3, the feast phase resulted shorter in the AGS, being equal to 45 minutes (P2) and 60 minutes (P3), respectively, whereas in the SBR it was 55 minutes (P2) and 75 minutes (P3) [22]. This result pointed out that feast/famine selection strategy is more effective in AGS systems than conventional SBR with flocculent sludge.

Another possible reason to explain the higher biopolymers accumulation in the AGS could be related to the better settling properties of granular sludge. In other studies, it was reported that high OLR caused the worsening of the sludge settleability and wash-out of polymers-accumulating microorganisms occurred [41]. More precisely, overgrowth of filamentous bacteria was noted in the SBR when the OLR increased, and this was associated to a slight loss in polymers accumulation capacity by the MMC. For further details on the SBR, the reader is referred to the literature [22]. In the present study, the improvement of sludge settling properties due to aerobic granulation enabled to avoid significant loss in biopolymers accumulation capacity as observed in the enrichment reactor with flocculent sludge. Overall, AGS has proved to be more efficient than flocculent sludge for biopolymers recovery from wastewater treatment.

3.4. Biopolymers composition

Results reported in the previous section showed the effectiveness of using AGS as enrichment technology for biopolymers production. In Figure 5, the average compositions of the produced biopolymers in terms of EPS and PHA achieved in the FBR using the enriched AGS and SBR sludges are reported.

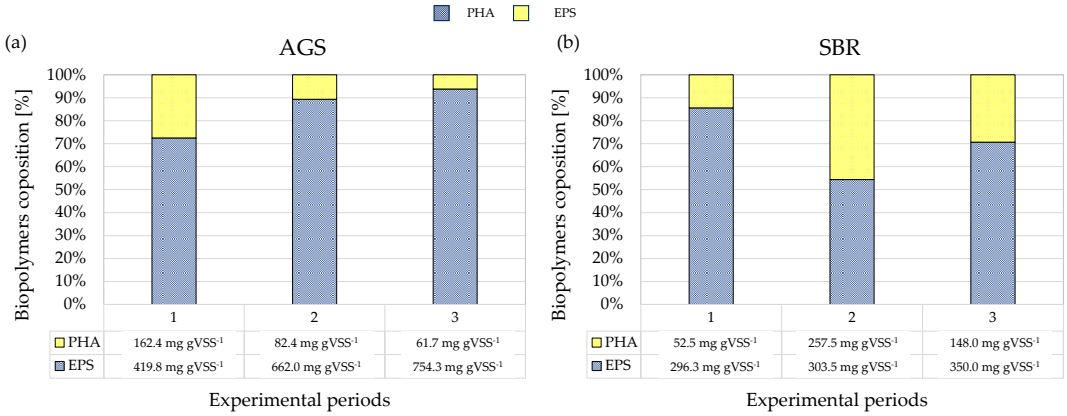

Figure 5. Percentages of PHA and EPS in the extracted biopolymers at the end of the accumulation assays performed in the FBR from the AGS and SBR systems.

In Period 1, the biopolymers extracted from the AGS were mainly constituted by EPS, accounting for approximately 70% (419.8 mg $gVSS^{-1}$), whereas the PHA content was almost 30% (162.4 mg $gVSS^{-1}$). A similar result was observed in the SBR, where EPS were the main compounds in the extracted biopolymers (83%, 296.3 mg $gVSS^{-1}$) and PHA content resulted lower (17%, 52.5 mg $gVSS^{-1}$).

The abundance of EPS in biopolymers from granular sludge significantly increased in Period 2 at the expense of PHA content. Indeed, the percentage of EPS increased up to 90% (662 mg $gVSS^{-1}$), whereas that of PHA decreased to 10% (82.4 mg $gVSS^{-1}$) and by almost 50% respect to the previous period (82.4 mg $gVSS^{-1}$ *vs* 162.4 mg $gVSS^{-1}$). This result was in opposite to what noted in the SBR, in which the EPS and PHA content was quite similar. Specifically, the EPS content was similar to that observed in the previous period (296 mg $gVSS^{-1}$ *vs* 303 mg $gVSS^{-1}$), whereas the increase of PHA content was statistically significant (52 mg $gVSS^{-1}$ *vs* 257 mg $gVSS^{-1}$).

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A similar tendency was noted in the AGS during Period 3. The percentage of EPS still increased up to 94% (754.3 mg $gVSS^{-1}$), whereas that of PHA reduced to 6%, although its reduction in terms of absolute value was lower than what observed passing from Period 1 to Period 2 (82.4 mg $gVSS^{-1}$ *vs* 61.7 mg $gVSS^{-1}$). In the SBR, slight but statistically significant increase in EPS content and decrease in that of PHA were observed.

Overall, results obtained indicated that AGS enabled to achieve higher EPS yield than SBR independently of the OLR in the enrichment reactor. Indeed, the EPS content in the extracted biopolymers from granular sludge resulted mostly twice than that measured in the flocculent sludge. Previous study reported that the amount of EPS produced by AGS treating municipal wastewater was about 300 mg $gVSS^{-1}$ [42,43], although only a fraction of such polymers, namely structural EPS (alginate-like exopolysaccharides) has a relevant market [44].

Referring to PHA, results evidenced that conventional SBR enabled to obtain a higher PHA production than AGS. The average PHA content measured in the FBR of the SBR system was almost double than that in the AGS, except for Period 1. Results obtained in the SBR were in line with previous studies carried out with industrial agro-based wastewaters [45], whereas that of AGS pointed out a considerable gap respect to the average values reported in the literature. Consequently, the enrichment stage of the process in AGS system needed to be optimized to improve culture selection and maximize PHA content.

In this respect, it was observed that when increasing the OLR in the enrichment reactor, the capacity of the MMC to produce PHA noticeably decreased in favor of EPS. In contrast, operating under low OLR (< 1 kgCOD $m^{-3}d^{-1}$) resulted in a better PHA yield than SBR although the achieved value (162.4 mg $gVSS^{-1}$) was lower than the reference ones reported in the literature [46]. Concerning the AGS technology, there are still few published studies on the impact of operating conditions on PHA synthesis. Generally, high OLR (> 3 kgCOD $m^{-3}d^{-1}$) are reported to be beneficial to maximize PHA production in AGS systems, whereas low OLR (< 2 kgCOD $m^{-3}d^{-1}$) are more indicated for conventional activated sludge [47]. Indeed, the effect of OLR on activated sludge was confirmed in this study, whereas opposite results were obtained referring to the AGS. A possible reason could be associated with substrate diffusion resistance observed in AGS, as the protection created by the AGS structure limited carbon concentration within these clusters [48]. Thus, a possible substrate limitation could be the reason of the lower PHA productivity observed in the AGS.

3.5. Analysis of carbon utilization in the FBRs

To examine how the organic matter was used by the enriched MMC of the AGS and SBR in the FBRs, mass balances were assessed to evaluate the conversion of the COD into intracellular biopolymers (PHA), extracellular biopolymers (EPS) and new biomass. All these elements were expressed in terms of COD using the respective conversion coefficients reported in the section 2.5. The achieved results are shown in Figure 6.

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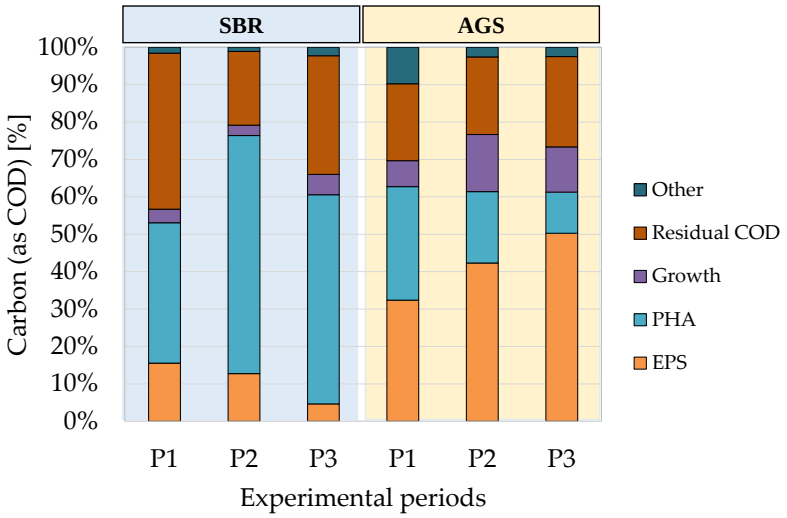

Figure 6. COD mass balance results over the three periods.

The enriched MMC of the SBR and AGS showed a different behavior in terms of carbon channeling into PHA and EPS. The organic carbon available for the MMC in the FBRs was mainly driven toward PHA in that of SBR and EPS in the AGS. More precisely, a maximum PHA storage yield close to 70% ($gPHA_{(as\ COD)}/gCOD$) was obtained in the SBR when the enrichment reactor was operated with an OLR of 2 kgCOD $m^{-3}d^{-1}$, whereas the maximum value obtained in the AGS was slightly lower than 30% in Period 1. In contrast, more than 50% ($gEPS_{(as\ COD)}/gCOD$) of the COD was used to synthetize EPS in the AGS in Period 3 (maximum value), whereas only 15% was the maximum EPS yield obtained in the SBR (Period 1).

A clear relationship between the fraction of COD channeled toward EPS and the OLR in the enrichment reactor was observed. When the OLR increased, the mass of COD used to synthetize EPS increased in the AGS (30% to 50%), whereas it decreased in the SBR (15% to 5%). An opposite trend referring to PHA was noted in the AGS, where COD driven toward PHA synthesis decreased from 30% to 11% as the OLR increased.

Referring to the other elements of the mass balance, any differences statistically significant were found. Although a slightly higher yield referred to new biomass synthesis was found in the AGS, ANOVA test did not highlight any statistically significant difference among the results (p-value > 0.05). Similarly, the residual COD resulted slightly lower in the FBR of the AGS system, although even in this case no statistical significance was observed.

The above results clearly indicated that MMC enrichment in the SBR was more favorable to the selection of PHA storing-population, and the OLR constitutes a key-control parameter to maximize the process yield. On the other hand, enrichment of MMC using AGS by applying similar operating strategies than conventional SBR led to a selective enrichment of EPS-storing microorganisms, such as to favor metabolic pathways for storing organic carbon extracellularly rather than intracellularly. This different behavior was more evident when operating at higher OLR.

A possible reason to explain why carbon was preferentially channeled toward EPS in AGS could be a side effect of the higher hydraulic selection pressure and shear forces applied in AGS system to promote aerobic granulation. Short settling time and high aeration rate corresponding to high shear stress are well recognized as key factors for transition from flocculent activated sludge to aerobic granules [49]. Several studies in the past decades have demonstrated that an increase of both the hydraulic selection pressure and shear forces determined a noticeable excess of EPS secretion by bacteria [50]. Specifically, it was demonstrated that such metabolic stressors activated quorum-sensing signaling among microorganisms and a positive correlation between the signal activity and EPS production was found [51]. Therefore, when AGS operated in presence of metabolic

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stressors, it was likely that carbon was channeling toward EPS formation as key element to enable microbial aggregation to withstand external disturbances.

In conclusion, the results obtained in this study suggested that production of EPS from AGS could be considered the most effective process for sludge valorization through the biopolymers recovery pathway. Contrarily, PHA production is currently more convenient in conventional SBR. It could be also stressed that PHA production was so far optimized for activated sludge systems rather than granular sludge. Consequently, to maintain the same effectiveness, operational of AGS would be adequately modified. Therefore, the effect of other operational parameters that could drive microbial metabolism toward the preferential production of EPS of PHA, without affecting the granulation process, should be investigated in future studies.

In particular, the effect of microbial community composition (prevalence of glycogen accumulating or phosphate accumulating organisms), or the role of the feeding strategy (full aerobic, upflow-anaerobic, etc.), as well the shear forces and hydraulic selection pressure should be elucidated.

5. Conclusions

This study evaluated the feasibility to use AGS as enrichment reactor for biopolymers production by MMC. The obtained results indicated that AGS enabled to obtain higher accumulation capacity respect to conventional activated sludge in flocculent form. However, organic carbon was mainly channeled toward metabolic pathways for extracellular storing, resulting in a significant prevalence of EPS in the extracted biopolymers. The presence of metabolic stressors (e.g., hydraulic selection pressure, shear forces) as key factors for promoting aerobic granulation, were supposed as the main forcers for organic carbon channeling toward EPS rather than PHA. In contrast, flocculent sludge was more favorable to the selection of PHA storing-population and the OLR applied in the enrichment reactor was found a key operating factor to drive the process toward PHA recovery. Based on the achieved results, it was concluded that AGS could be considered as a suitable enrichment technology for EPS recovery from excess valorization. Future studies are necessary to optimize PHA productivity by AGS focusing on the role of the main operating parameter affecting aerobic granulation.

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