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Impact of operating cycle type on alginate-like exopolysaccharide and tryptophan production in aerobic granular sludge systems

Impacto do tipo de ciclo operacional na produção de exopolissacarídeos semelhantes ao alginato e de triptofano em sistemas de lodo granular aeróbio

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ABSTRACT

As wastewater treatment advances, there is a growing need to remove pollutants and recover valuable resources. This study focuses on the optimization of the aerobic granular sludge process, exploring the impact of varying the anaerobic period on the production of bioresources, i.e., on the synthesis of extracellular polymeric substances (EPS), alginate-like exopolysaccharides (ALE — like exopolysaccharides), and tryptophan (TRP). To this end, two sequential batch reactors (SBRs) were used, R1 and R2, fed with acetic and propionic acid, respectively, and subjected to different durations of the anaerobic phase (100, 70, 35, and 0 min) in the total cycle time of 6 h. The results were similar regarding COD, N, and P removal. However, R2 showed greater nitrate accumulation. Statistical analyses highlighted significant variations in SPE concentrations in the different phases ($p<0.05$) in both systems. ALE production in R1 was highest in the initial phase, decreasing with the reduction of the anaerobic period. However, this trend was not observed in the R2 system ($p \approx 0.13$). Tryptophan production remained stable across all phases for both systems. The results reveal that the duration of the anaerobic period significantly influences bioresource production, emphasizing the importance of defining optimal operational conditions for efficient resource recovery in wastewater treatment.

Keywords: bioresource recovery; high-added-value products; sequential batch reactors; wastewater treatment.

RESUMO

À medida que o tratamento de águas residuárias avança, há uma crescente necessidade de não apenas remover poluentes, mas também recuperar recursos valiosos. Este estudo concentrou-se na otimização do processo de lodo granular aeróbio, explorando o impacto da variação do período anaeróbio sobre a produção de biorrecursos, ou seja, na síntese de substâncias poliméricas extracelulares (SPE), exopolissacarídeos semelhantes ao alginato (*alginate-like exopolysaccharides* — ALE) e triptofano (TRP). Para tanto, foram utilizados dois reatores em batelada sequenciais (RBS), R1 e R2, alimentados com ácido acético e propiônico, respectivamente, e submetidos a diferentes durações da fase anaeróbia (100, 70, 35 e 0 minutos) no tempo total de ciclo de 6 horas. Os resultados foram semelhantes em termos de remoção de demanda química de oxigênio (DQO), N e P, porém R2 apresentou maior acúmulo de nitrato. Análises estatísticas destacaram variações significativas nas concentrações de SPE nas diferentes fases ($p<0,05$), em ambos os sistemas. A produção de ALE em R1 foi mais alta na fase inicial, decrescendo com a redução do período anaeróbio, contudo essa tendência não foi observada no sistema R2 ($p \approx 0,13$). A produção de triptofano permaneceu estável em todas as fases para ambos os sistemas. Os resultados revelam que a duração do período anaeróbio exerce influência significativa na produção de biorrecursos, enfatizando a importância de definir condições operacionais ótimas para a recuperação eficiente de recursos no tratamento de águas residuárias.

Palavras-chave: recuperação de biorecursos; produtos de alto valor agregado; reatores em batelada sequencial; tratamento de água residuárias.

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Introduction

The continuous development of the wastewater industry is steering wastewater treatment plants (WWTPs) toward removing pollutants and recovering valuable resources. This shift toward resource recovery, especially high-value products, supports implementing circular economy practices in sanitation (Feng et al., 2021; Chen et al., 2022). For instance, sludge produced during treatment serves as a raw material for recovering various resources such as biogas, cellulose, bioplastics, phosphorus (P), alginic acid, and tryptophan (Schambeck et al., 2020).

In this regard, aerobic granular sludge (AGS) stands out among emerging biotechnologies for its efficiency and several advantages over conventional flocculent-activated sludge. These advantages include efficient nutrient removal, rapid sludge settling, high biomass retention, and enhanced removal of total suspended solids and pathogens (Zheng et al., 2018; Sarvajith and Nancharaiah, 2023). A key feature of AGS is its extracellular polymeric substances (EPS), which are primarily composed of proteins, polysaccharides, nucleic acids, glycoproteins, lipids, and humic acids. These components are essential for granular sludge formation and structural integrity (Liu S et al., 2023).

The gel-like properties of the AGS extracellular matrix are enriched with alginate-like exopolysaccharides (ALE), which form a significant part of the EPS (Campo et al., 2022). Being primarily characterized as polysaccharides, ALE have been found in concentrations nearly double those in activated sludge flocs (Lin et al., 2013). Unlike commercial alginates derived from algae, recent studies suggest that bacterial processes can also produce ALE (Gao et al., 2018; Chen et al., 2022).

The gel-forming ability of alginate is influenced by its chemical structure, including polyguluronic acid (GG), polymannuronic acid (MM), and heteropolymeric (MG) blocks. ALE from AGS is noted for a high proportion of GG blocks, which have a strong affinity for divalent cations like Ca^{2+}, enhancing the biopolymer's mechanical stability and viscoelastic properties (Sarvajith and Nancharaiah, 2023). The concentrations of GG blocks in alginate can vary based on carbon sources and operational configurations (Li et al., 2022).

Alginate's versatility and beneficial properties have made it a valuable material across various industries, including food, medical, pharmaceutical, biotechnology, horticulture, agriculture, construction, and paper (Pronk et al., 2017; Chen et al., 2022; Zahra et al., 2022; Sethi et al., 2023). ALE-based materials, which are noted for their biodegradability and recyclability, offer a sustainable alternative to conventional fossil-based materials (Zeng et al., 2023).

Significant industrial-scale production has already been demonstrated. For instance, pilot reactors treating municipal sewage have achieved ALE production rates of 219 and 236 mgALE·gVSS^{-1} (Rollemberg et al., 2020a; Schambeck et al., 2020). Field tests in Zutphen, the Netherlands, showed that 18 kg of bio-ALE could be produced from 80 kg of Nereda granular sludge, equating to a 22.5% bio-ALE recovery (van Leeuwen et al., 2018). The production facilities in Zutphen and Epe, Netherlands, can produce up to 500 tons of Kaumera annually. "Kaumera" represents the various biopolymers extracted from AGS from the Nereda wastewater treatment process, including ALE. With Dutch production estimated to reach 85,000 tons/year by 2030, the market value of these biopolymers is projected at €170 million annually by the same year (van Leeuwen et al., 2018).

Moreover, tryptophan (TRP), which is another key compound in the EPS matrix, has been identified as one of the dominant organic materials in the EPS matrix of aerobic granules (Luo et al., 2014; Hamza et al., 2018). Its production occurs through biochemical synthesis and microbial fermentation processes (Guo et al., 2020; Carvalho et al., 2021). Initially, the enzyme tryptophanase is produced by bacteria and isolated and then used to synthesize tryptophan from substrates such as indole, pyruvate, and ammonia. Alternatively, bacteria capable of synthesizing tryptophanase can directly produce tryptophan using additional substrates like glucose and xylose (Carvalho et al., 2021).

Rollemberg et al. (2020a) demonstrated TRP production in a pilot-scale AGS system treating sanitary wastewater, achieving about 50 mg·gVSS^{-1}. This suggests significant industrial potential for TRP in various sectors, including its use as a preservative and dietary supplement in animal feeds and as an active ingredient in psychiatric drugs (Carvalho et al., 2021).

Numerous studies have explored the factors influencing the production of bioresources like ALE and TRP, including cycle distribution, sludge discharge, shear stress, temperature, and microbial diversity. Variations in these factors, such as the organic loading rate and C:N ratio, significantly affect ALE and TRP production (Yang et al., 2014; Rollemberg et al., 2020b; Chen et al., 2022). Saline stress and the addition of cations have been favorable for granulation and bioresource production. Conversely, prolonged cellular retention times can decrease ALE and TRP content (Liu et al., 2010; Cui et al., 2021; Frutuoso et al., 2023). In addition, literature has indicated that successful development of granules successful development of granules increases the production of biological resources (Schambeck et al., 2020; Frutuoso et al., 2023), with the reactor configuration, particularly the cycle type, playing a fundamental aspect on system efficiency (Rollemberg et al., 2020b). Strategies such as staggered feeding improve denitrification and system stability and cycles with a short anoxic phase can stimulate ALE and TRP productions (Rollemberg et al., 2020b; da Silva et al., 2021; Frutuoso et al., 2023).

In this context, this study aimed to evaluate how different cycle types in AGS systems impact the production of EPS and bioresources (ALE and TRP). Despite numerous studies, the search for system optimization is essential as the literature still needs a comprehensive understanding of the mechanisms underlying the production of these bioresources. Information concerning the impacts of varying the anaerobic period is crucial in defining the optimal duration of this reaction phase over the total cycle to maximize bioresource production efficiency.

Materials and methods

Experimental system configuration

The experimental system involved two sequential batch reactors (SBRs) with conventional configurations, operated at ambient temperature (28±2°C). Both reactors functioned under identical operational conditions but differed in their carbon sources: acetic acid for reactor R1 and propionic acid for reactor R2. These specific carbon sources were selected based on their potential to enhance resource production, as reported by Santos et al. (2022). Each reactor was constructed from acrylic, had a working volume of 7.6 L, and measured 100 cm in height and 10 cm in internal diameter, yielding a height-to-diameter (H/D) ratio of 10. Figure 1 provides a schematic overview of these systems.

The operating cycle of the reactors included anaerobic feeding, reaction (anaerobic and aerobic periods), sedimentation, and withdrawal, lasting 6 h total cycle. During the experiment, the anaerobic reaction period was systematically reduced on a weekly basis, and the saved time was reallocated to the aerobic reaction phase, as detailed in Table 1. The experiment was structured into four phases: in Phase I, the anaerobic period lasted 100 min; in Phase II, it was reduced to 70 min; in Phase III, to 35 min; and in Phase IV, it was completely eliminated. This gradual reduction aimed to explore the impact of anaerobic period duration on bioresource production and to determine the optimal duration for maximizing outputs.

The volumetric exchange ratio was set at 50%, achieving a hydraulic retention time (HRT) of 12 h. During the anaerobic period, sludge mixing was facilitated by intermittent aeration pulses lasting 1 min every hour. Aeration was supplied by an air compressor (model ACO-003, Sunsun, China) at a rate of 10.0 $L \cdot min^{-1}$, achieving a superficial air velocity of 2.1 $cm \cdot s^{-1}$. This setup maintained the dissolved oxygen (DO) levels between 2 and 4 $mg \cdot L^{-1}$. The sludge retention time (SRT) was not controlled in this study.

Inoculum sludge and synthetic influent

The systems had already been operating for about 230 days, cultivating mature AGS with synthetic wastewater. They were installed at the Laboratory of Environmental Sanitation (LABOSAN), located in the Department of Hydraulic and Environmental Engineering (DEHA) at the Federal University of Ceará (UFC), Fortaleza, Ceará, Brazil. R1 started the research with a mixed liquor volatile suspended solids (MLVSS) concentration of 1.8 $g \cdot L^{-1}$ and a sludge volume index in 30 min (SVI_{30}) of 55 $mL \cdot g^{-1}$, while R2 had 6.0 $g \cdot L^{-1}$ and 41 $mL \cdot g^{-1}$, respectively. Therefore, the experiment did not aim to compare the efficiencies and operational stability of the systems, as the initial conditions were not the same.

The feeding solution was prepared with potable water, a carbon source, a basal medium (macro and micronutrient solution), and a

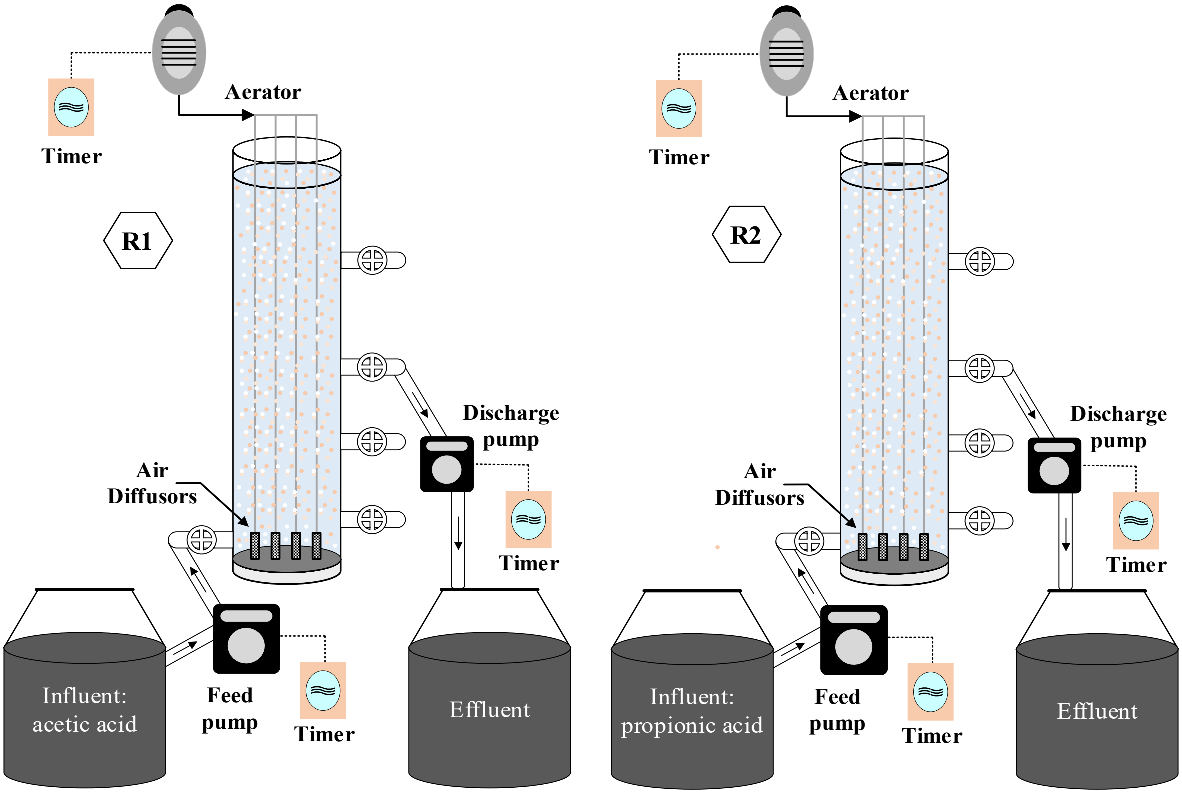

Figure 1 – Schematic of the conventionally configured SBR-AGS experimental system.

Table 1 – Operational cycle applied in each phase of the experiment.

Phase	Anaerobic feeding (min)	Anaerobic period (min)	Aerobic period (min)	Sedimentation (min)	Withdrawal (min)
I	20 (6%)	100 (28%)	235 (66%)	5	<1
II	20 (6%)	70 (20%)	265 (75%)	5	<1
III	20 (6%)	35 (10%)	300 (84%)	5	<1
IV	20 (6%)	0 (0%)	335 (94%)	5	<1

buffer. The composition of the synthetic wastewater was as follows: 1000 mgCOD·L^{-1}, 50 mgNH$_4^+$-N·L^{-1} (NH_4Cl), 10 mgPO$_4^{3-}$-P·L^{-1} (KH_2PO_4), 2 mgCa^{2+}·L^{-1} ($CaCl_2·2H_2O$), 5 mgMg^{2+}·L^{-1} ($MgSO_4·7H_2O$), and 1 mL·L^{-1} of micronutrient solution (50 mg·L^{-1} of H_3BO_3; 2.7 g·L^{-1} of $FeCl_2·4H_2O$; 50 mg·L^{-1} of $ZnCl_2$; 500 mg·L^{-1} of $MnCl_2·4H_2O$; 38 mg·L^{-1} of $CuCl_2·2H_2O$; 50 mg·L^{-1} of $(NH_4)_6Mo_7O_{24}·4H_2O$; 90 mg·L^{-1} of $AlCl_3·6H_2O$; 2 g·L^{-1} of $CoCl_2·6H_2O$; 92 mg·L^{-1} of $NiCl_2·6H_2O$; 162 mg·L^{-1} of $NaSeO_3·5H_2O$; and 1 g·L^{-1} of EDTA). The feeding solution was buffered with 2.5 g·L^{-1} of sodium bicarbonate ($NaHCO_3$) to maintain a neutral pH.

The applied organic load was 2.0 g·L^{-1}·day^{-1}. The C:N ratio was kept at 20, which is considered favorable for producing ALE and tryptophan (Rollemberg et al., 2020a).

Analytical methods

Duplicate sequential cycle tests were performed to understand the mechanisms of production and consumption of bioresources. For this purpose, mixed liquor samples were collected from the reactor at 1-h intervals to extract and quantify EPS, ALE, and TRP.

For the phase with the best results in terms of ALE and TRP content, duplicate cycle tests were also conducted to evaluate the system's performance in terms of organic matter and nutrient removal, with analyses of mixed liquor samples for chemical oxygen demand (COD), nitrogen in the form of ammonia (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^--N), and phosphate (PO_4^{3-}-P). All analyses followed the methods described in the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). DO and pH in mixed liquor samples were measured weekly using a probe (YSI Pro1020, YSI Inc., USA).

Physicochemical characterization of biomass was conducted weekly by analyzing MLVSS concentration and sludge volume index at 5 and 30 min (SVI_5 and SVI_{30}, respectively), according to APHA (2012).

Granulometry analysis was performed using three sieves with openings of 0.2, 0.6, and 1.0 mm. The percentage of granules larger than the sieve opening was calculated as the ratio between the mass of granules passing through the sieve and the total sample mass (de Kreuk et al., 2007). The average granule diameter was determined from optical microscope images (Opton) processed using the Image-Pro Plus software.

The structure of mature granules was analyzed by scanning electron microscopy (SEM) combined with energy-dispersive X-ray spectroscopy (Inspect S50, FEI Company, USA). The pre-treatment involved fixation, washing, and lyophilization, following the methodology described by Motteran et al. (2013).

Extraction and identification of bioresources

Extracellular polymeric substances

EPS were extracted using a modified heat extraction method proposed by Yang et al. (2014). Protein (PN) content was determined using the modified Lowry method, while polysaccharide (PS) content was analyzed using a sulfuric acid-phenol method (Long et al., 2014). Total EPS was considered the sum of PN and PS.

Alginate-like exopolymers

The method for extracting ALE from AGS followed the procedure described by Lin et al. (2010) with some modifications. Initially, a 50 mL aliquot of the mixed liquor was subjected to centrifugation at 3850 rpm for 20 min, after which the supernatant was removed. Subsequently, the sample underwent freeze-drying (lyophilization) for approximately 36 h using a Freeze Dryer L101 from Liotop, Brazil. Notably, 0.71 g of Na_2CO_3 and 100 mL of Mili-Q water were added to the resulting dry biomass. This suspension was then heated in a water bath at 80°C, with continuous stirring at 400 rpm, for 30 min to facilitate the release of ALE into the medium. Following this, the sample was once again centrifuged at 3850 rpm for 20 min, and the resulting supernatant was collected. ALE was precipitated by the addition of 4 M HCl while adjusting the pH to a range of 2.0–2.5. The solution was centrifuged to obtain ALE in an acidic form as a precipitate, which was subsequently frozen, lyophilized, and weighed. The results were expressed in accordance with the recommendations of Felz et al. (2016).

Tryptophan

The pH of the EPS aliquots was adjusted to the range of 6.5–7.5 using 4 M HCl, and the amount of acid added was recorded to calculate the dilution. For sample doping, a 50 ppm tryptophan solution was added in a 1:1 ratio to achieve a minimum concentration of 25 ppm tryptophan. The quantification of tryptophan content was conducted through high-performance liquid chromatography (HPLC CTO20A, Shimadzu Corporation, Japan), following the methodology described in previous research (Wang et al., 2014; Peltre et al., 2017; Rollemberg et al., 2020b). The HPLC system was equipped with a Hypersil BDSC-18 column (250×4.6 mm, 5 µm), UV 280 nm detection, and a UV/VIS detector with an injection volume of 20 µL. The chromatographic analysis employed an isocratic elution with a mobile phase consisting of a methanol–water mixture in a molar ratio of 3:10 and a flow rate of 1 mL/min. Tryptophan concentration was determined by calculating the difference between the sample's peak areas and Milli-Q water enriched with tryptophan (used as a blank).

Statistical analysis

The Kruskal-Wallis and Mann-Whitney tests were applied to compare bioresources production between operational phases. In addition, Pearson correlation was also used to assess the degree of correlation between two variables when possible. The test results were evaluated based on the p-value. If $p \leq 0.05$, the null hypothesis is rejected, meaning that the data groups are considered statistically different.

Results and discussion

Extracellular polymeric substances production dynamics

EPS are distributed around the granule and make up a significant portion of the dry mass in biological wastewater treatment systems. In AGS systems, EPS play an essential role in maintaining the granular structure and providing greater physical strength (Feng et al., 2021). The recovery of EPS from the discarded sludge is favorable for retrieving high-value-added resources from WWTPs, potentially promoting the development of a circular economy (Karakas et al., 2020).

Statistical analysis revealed significant differences in the EPS content during all phases of both systems, with $p<0.05$. The total EPS production in Phase I (anaerobic period of 100 min) was higher in both reactors (226.4 mg EPS·g MLVSS^{-1} in R1 and 139.4 mg EPS·g MLVSS^{-1} in R2), as shown in Table 2. It is known that EPS production mainly occurs during the *feast* phase, while its consumption occurs during the *famine* phase as an endogenous carbon source to maintain cellular metabolic activities (Deng et al., 2016). In long anaerobic stages, the available substrate tends to be slowly consumed by heterotrophic microorganisms, remaining available in the medium for a longer time (Wu et al., 2012). As a result, the famine period is reduced, which may have resulted in the high EPS content in Phase I.

In a similar study conducted by Rollemberg et al. (2020b), in which different cycle configurations were explored, including variations in the distribution of anaerobic/aerobic and anaerobic/aerobic/anoxic periods, it was found that cycles with a short anoxic phase favored the production of bioresources. As observed in this study, higher production was associated with a shorter famine period, in other words, lower endogenous activity, which could induce microorganisms to use EPS as an electron donor. Furthermore, the authors identified a reduced production of EPS in the reactor with a shorter anaerobic period.

Phases II (anaerobic period of 70 min) and III (anaerobic period of 35 min) show EPS being continuously consumed as aeration increases. On the contrary, in Phase IV (0 min), there was an unexpected increase in EPS in both reactors (196.6 mg EPS·g MLVSS^{-1} in R1 and 134.6 mg EPS·g MLVSS^{-1} in R2). In this case, the stress caused by the aeration conditions probably prevailed, stimulating EPS production in the systems, even after a longer starvation period (Liu et al., 2010).

The trend described above was reflected in PN and PS results, the main components of EPS, as shown in Table 2. PN was the dominant component in the EPS secreted in all phases of both systems (between 136.7 and 160 mgPN·gMLVSS^{-1} in R1 and between 76 and 105.1 mgPN·gMLVSS^{-1} in R2). Due to its charging properties, this protein portion plays an important role in AGS formation and stabilization (Liu et al., 2022).

The PS content in R1 sludge in Phase I (66.4 mgPS·gMLVSS^{-1}) was higher than in the other phases, resulting in a slightly lower PN/PS ratio (2.4), which is probably closely related to the functionality and stability of R1 in Phase I (He et al., 2018). In R2, the PN/PS ratio remained stable at around 3.0.

Although R1 (between 185 and 230 mgEPS·gMLVSS^{-1}) presents production greater than R2 (between 100 and 150 mgEPS·gMLVSS^{-1}) in all phases, indicating that the carbon source interferes with EPS production (Santos et al., 2022), the size of the granules and solid retention time (SRT) should also be considered (Table 3). In this regard, previous research has suggested that higher SRT may induce the consumption of biopolymers as carbon source and decrease in their production in granule diameters greater than 1.5 mm, possibly due to a reduction in EPS production caused

Table 2. Polysaccharide and protein content in operational phases of R1 and R2.

Phase (anaerobic period)	R1		R2	
	PS (mgPS/gMLVSS)	PN (mgPN/gMLVSS)	PS (mgPS/gMLVSS)	PN (mgPN/gMLVSS)
I (100 min)	66.4±3.2	160.0±5.5	34.3±0.2	105.1±3.8
II (70 min)	55.1±0.8	154.7±7.9	31.6±0.2	96.7±9.8
III (35 min)	49.4±0.7	136.7±10.7	27.3±0.1	76.0±8.1
IV (0 min)	48.4±1.3	148.2±2.4	33.4±0.6	101.2±5.9

PS: polysaccharides; PN: proteins; MLVSS: mixed liquor volatile suspended solids.

by diffusion limitations of carbon (Rollemberg et al., 2020b; Frutuoso et al., 2023). Therefore, a direct comparison is not applicable as the systems had different initial conditions, SRT, and granule sizes.

It should be noted that excess EPS can cause problems for the granule structure. Some studies reported that the cause of the granule breakage is likely related to the mineralization of their core (Lemaire et al., 2008), and the porosity clogging is related to an excess of EPS production. Therefore, if, on the one hand, a low EPS content does not allow granulation, on the other hand, an excess of EPS production could limit the aerobic granules maintenance in the long term (Corsino et al., 2016).

Bioresource recovery: alginate-like exopolysaccharide and tryptophan

The operational cycle configuration also had a direct effect on ALE production. Figure 2A and Table 4 show that for R1, the ALE content

Table 3 – Physical characteristics of mature aerobic granules in the experimental systems with optimal aeration Phase I (anaerobic period of 100 min).

Parameter	R1	R2
MLVSS (g·L^{-1})	2.0±0.4	5.6±1.9
SVI$_{30}$ (mL·g^{-1})	55.0±14.6	41.8±6.1
SVI$_5$/SVI$_{30}$	0.82	1.00
Average Diameter (mm)	1.2	1.8
MLTSS > 1 mm (%)	99.7±0.1	99.6±0.1
SRT (days)	4±2	33±1

MLVSS: mixed liquor volatile suspended solids; SVI$_{30}$: sludge volume index in 30 min; SVI$_5$: sludge volume index in 5 min; MLTSS: mixed liquor total suspended solids; SRT: sludge retention time.

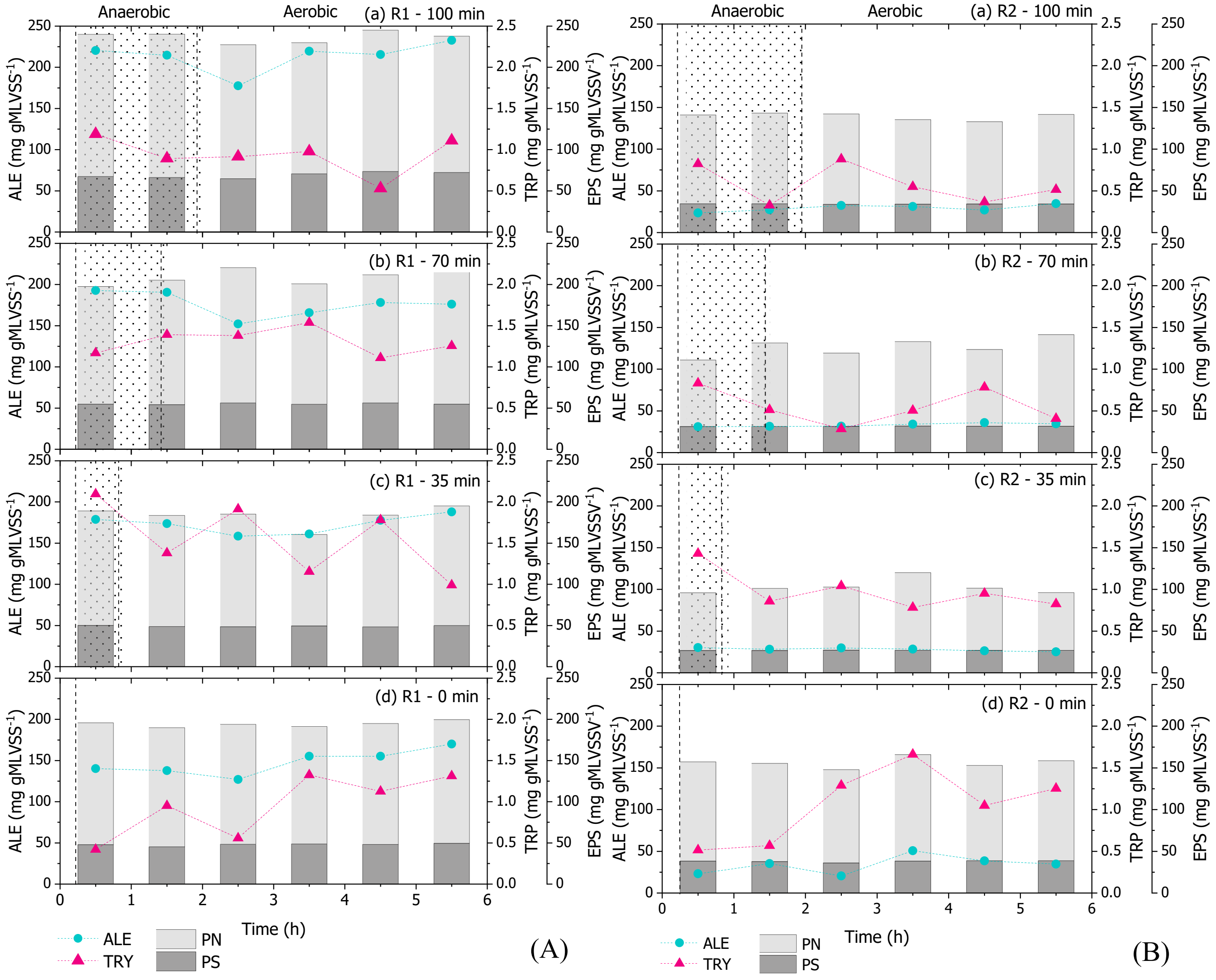

Figure 2 – Alginate-like exopolysaccharide and tryptophan productions throughout the cycle in each operational phase in systems R1 (A) and R2 (B).

was, on average, higher in Phase I (213.4±27.1 mgALE·gMLVSS^{-1}) and decreased with the anaerobic period shortening. This may be due to the extended famine period, which could induce microorganisms to consume EPS as electron donors (Wu et al., 2012), as explained in the previous section. The ALE content was similar to the results reported in other studies (Meng et al., 2019; Rollemberg et al., 2020b; Schambeck et al., 2020), considering operational differences.

However, this trend was not observed in the R2 system phases. As depicted in Figure 2B, ALE production remained constant despite the reduction in the anaerobic period (approximately 31.1±2.4 mgALE·gMLVSS^{-1}), with no statistically significant differences noted ($p \approx 0.13$). The high SRT in R2 likely had a greater impact on the production of this bioresource, such that a prolonged famine period only resulted in a reduction in EPS material.

Santos et al. (2022) conducted a study where five reactors were evaluated with varying SRTs and substrate sources. ALE yield was higher in the reactor with a shorter SRT, suggesting that increasing SRT predominantly leads to endogenous biomass consumption of ALE. Similarly, Pronk et al. (2017) observed a yield of only 1% of ALE in systems with SRTs ranging from 51 to 24 days.

As part of the EPS, ALE is a special hydrogel material with a complex structure composed mainly of polysaccharides but also contains proteins and humic substances (Schambeck et al., 2020). Pearson's statistical test assessed whether the ALE content changed simultaneously with the EPS content. The results indicated that the variables were statistically correlated ($p<0.05$) in R1 with a positive and strong correlation ($r=0.70$) but not in R2 ($p \approx 0.07$) (Figueiredo Filho and Silva Júnior, 2009).

Due to the complexity of these compounds, the literature has presented different results and relationships. Schambeck et al. (2020) did not find a correlation between ALE and EPS production, suggesting that the lack of correction for EPS with environmental factors might be the reason, indicating that EPS content is more resistant to environmental and operational factors in real treatment systems. On the contrary, Frutuoso et al. (2023) proposed that the correlation between ALE and EPS production is predominantly influenced by endogenous consumption in systems with SRT exceeding 20 days.

Moreover, the gel-forming ability of ALE relies on its chemical composition, particularly the abundance of GG blocks, which are crucial for mechanical stability and viscoelasticity (Sarvajith and Nancharaiah, 2023). The varying concentration of GG blocks due to different carbon resources and operational configurations influences the properties of formed ALE (Li et al., 2022). The consensus in the literature is that the content of bioresources can vary due to various factors, such as operational configuration, wastewater characteristics, SRT, F/M ratio, C:N ratio, DO, bioaggregate type, microbial community complexity, and extraction protocol (Yang et al., 2014; Rollemberg et al., 2020b; Schambeck et al., 2020; Carvalho et al., 2021; Frutuoso et al., 2023). The ALE's properties, influenced by this variety of factors, underscore the complexity and applicability of these biopolymers in diverse industrial sectors.

Regarding TRP, the production trend was similar for both systems in all phases (Figure 2) and intensified and statistically different ($p<0.05$) with the anaerobic period reduction. TRP contents were, on average, 1.6±0.4 mgTRP·gMLVSS^{-1} in Phase III (35 min) of R1 and 1.1±0.4 mgTRP·gMLVSS^{-1} in Phase IV (0 min) of R2. Pearson's statistical test was also performed to evaluate a correlation between TRP content and EPS content, but this analysis did not show a statistically significant correlation ($p>0.05$) for either system.

It is known that tryptophan can be produced in three distinct ways. First, through controlled chemical synthesis, it is artificially manufactured from organic compounds such as indole. The second option involves biochemical synthesis, where the enzyme tryptophanase is produced by bacteria, isolated, and subsequently used to generate tryptophan from indole, pyruvate, and ammonia. Finally, the third approach is microbial fermentation, in which genetically modified bacteria like *Escherichia coli* and *Corynebacterium glutamicum* directly produce tryptophan as part of their metabolism, utilizing precursors such as glucose and xylose (Watanabe and Snell, 1972; Konosuke and Mitsugi, 1975; Niu et al., 2019).

Therefore, it is believed that reducing the anaerobic period in AGS systems could increase tryptophan production due to a combination of factors. These include 1. greater availability of precursors directed toward tryptophan synthesis, as there would be less competition between different metabolic processes that utilize the same precursors or intermediaries, 2. inducing bacteria in the system to activate biosynthetic pathways leading to tryptophan production, and 3. responses to

Table 4 – Alginate-like exopolysaccharide and tryptophan content in operational phases of R1 and R2.

Phase (anaerobic period)	R1		R2	
	ALE (mg·gMLVSS^{-1})	TRP (mg·gMLVSS^{-1})	ALE (mg·gMLVSS^{-1})	TRP (mg·gMLVSS^{-1})
I (100 min)	213.4±27.1	0.9±0.2	29.4±4.1	0.6±0.2
II (70 min)	175.9±24.5	1.3±0.1	33.1±2.8	0.6±0.2
III (35 min)	173.0±20.9	1.6±0.4	28.0±2.4	1.0±0.2
IV (0 min)	147±21.1	0.9±0.4	33.8±3.1	1.1±0.4

ALE: alginate-like exopolysaccharide; TRP: tryptophan; MLVSS: mixed liquor volatile suspended solids.

periods of famine or substrate scarcity, during which bacteria might activate genes related to tryptophan production as part of an adaptive strategy in challenging environments. However, it is important to note that various variables can influence tryptophan production in AGS systems, and it is a complex subject that requires further studies for a more comprehensive understanding.

The results obtained in this study were much lower than those found in previous studies, which reported TRP contents between 50 and 60 mgTRP·gMLVSS^{-1} (Rollemberg et al., 2020a, 2020b). On the contrary, they are similar to the findings of Santos et al. (2022) and Frutuoso et al. (2023), which observed values between 1.9 and 4.1 mgTRP·gMLVSS^{-1}. A possible explanation for this is the different quantification methods used for TRP in previous studies, as such methods still need to be well-established in the literature (Ferreira et al., 2021).

Bioresource productions varied throughout the operational cycles. However, in most observations, they were higher during the aerobic period, both for ALE and TRP, which is consistent with what was reported by Rollemberg et al. (2020a). Therefore, performing sludge discharge during the aerobic period is more advantageous.

Assessment of the operational cycle for optimal resource production

Although the operational cycle for optimal ALE production (Phase I) differs from that for TRP (Phases III and IV), we chose to assess in Phase I (anaerobic period of 100 min) the aerobic granules' physical characteristics and system performance because the ALE results appeared more promising than those of TRP.

Aerobic granules characteristics

When AGS samples were collected for the current study, both laboratory-scale SBRs had been operating stably for over 230 days.

The physical characteristics of the aerobic granules were recorded in Phase I (anaerobic period of 100 min), and the main information is presented in Table 4. The aerobic granules from R1 showed a more irregular shape with an average diameter of 1.2 mm, while the granules cultivated in R2 were mostly spherical with an average diameter of 1.8 mm (Figure 3). The biomass from both reactors showed a high proportion of granules with diameters greater than 1 mm (>99%).

In the study conducted by Rollemberg et al. (2020a) with laboratory-scale SBRs fed with acetate, granules with diameters between 1.0

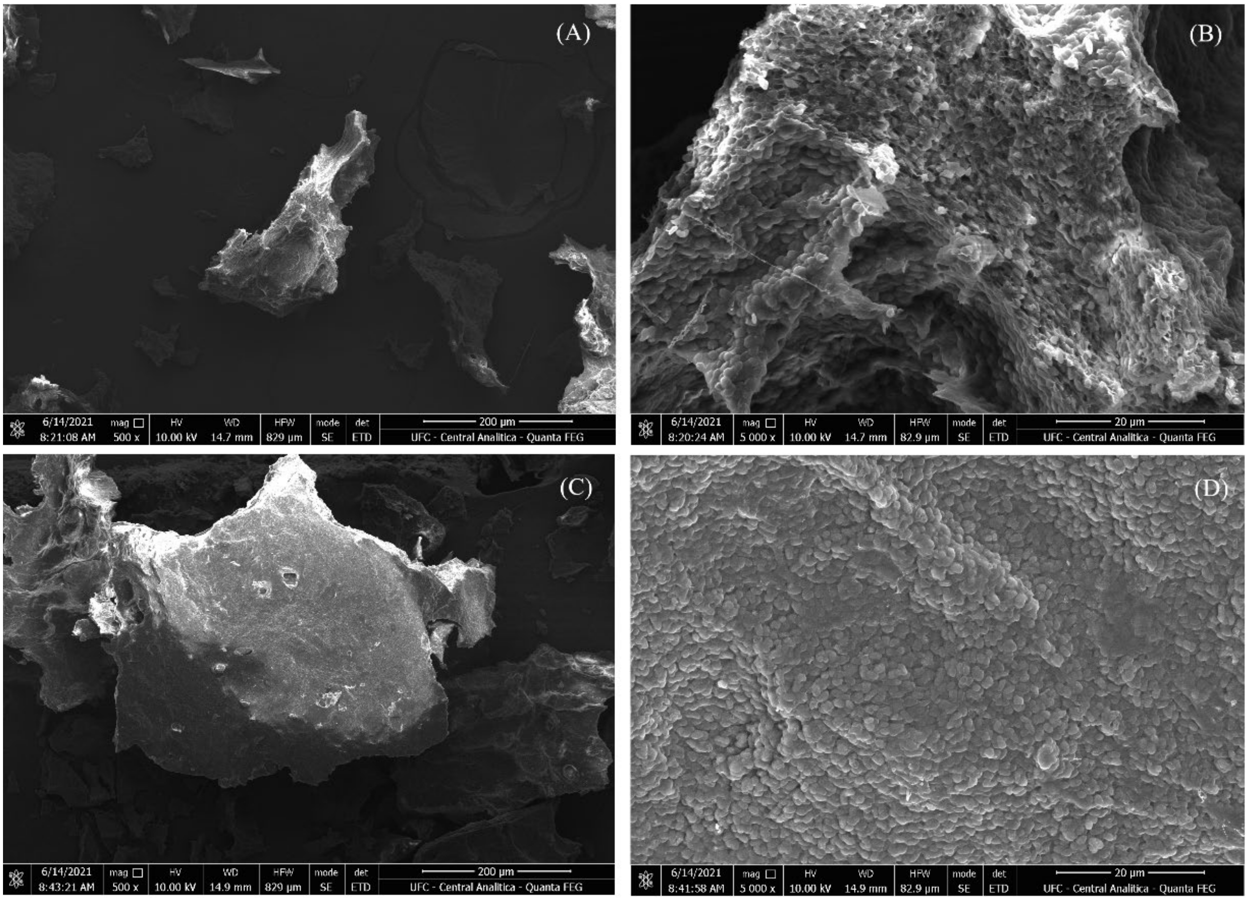

Figure 3 – Scanning electron microscopy images (right) of aerobic granules: (A) R1 – 100 min, at 200 µm; (B) R1 – 100 min, at 20 µm; (C) R2 – 100 min, at 200 µm; and (D) R2 – 100 min, at 20 µm.

and 1.5 mm showed a higher content of ALE, about 285 mgALE·g-MLVSS^{-1}, similar to the results obtained in this study. Furthermore, the authors also reported a decrease in ALE and TRP content above a critical diameter (greater than 1.5 mm), possibly due to a reduction in EPS production caused by carbon diffusion limitations. Similarly, dos Santos et al. (2022) observed lower EPS production in AGS systems and associated it with the increase in granule diameter caused by oxygen limitation and carbon diffusivity. Thus, granule size may partially explain the low bioresource production in R2.

Although not controlled, the SRT may have interfered, especially in the bioresource concentration, as there is a higher rate of endogenous respiration at longer SRT (above 20 days), which can induce the consumption of EPS as a carbon source (Rollemberg et al., 2020b; Frutuoso et al., 2023). Polyphosphate-accumulating organisms (PAOs) have been implicated as microorganisms involved in producing ALE. The decrease in SRT was reported to be beneficial for the growth of PAOs, resulting in higher ALE production, as seen in R1 (Zahra et al., 2022). Moreover, the highest amount of VSS in mixed liquor indicates that organic matter was used for cellular metabolism and microbial proliferation instead of biopolymer synthesis (Li et al., 2022).

Thus, considering the physical characteristics of the granule, the lower biopolymer content in R2 is likely explained by the greater granule diameter and SRT, which influence carbon diffusivity and consumption. Regarding TRP, the lower production in both reactors can be justified by the granule's SRT and size (Santos et al., 2022).

Chemical oxygen demand, nitrogen, and phosphorus removal efficiency

The profile of organic matter and nutrient (N and P) consumption was observed during the cycle of highest bioresource production, with an extended anaerobic period, to understand how organic matter and nutrient conversions can influence bioresource production in each reactor (Figure 4).

COD was significantly consumed during the anaerobic period in all reactors, especially in R2, where the concentration at the end of the anaerobic period was below 200 mgCOD·L^{-1}. This probably indicates a higher rate of endogenous respiration.

Two hours after the aerobic period began, there was a sharp drop in ammonium with the production of nitrite (NO_2^-) and nitrate (NO_3^-), which were subsequently consumed. However, nitrate was not entirely consumed due to the lack of organic matter to enable complete denitrification (Frutuoso et al., 2023).

Despite the slight release of phosphorus in the first minutes of the anaerobic phase, a complete removal of phosphorus was not achieved (≈50%). A possible explanation for the lower phosphorus removal performance in R2 is the extended SRT, which was approximately 33 days. Saturated PAOs persist without controlled sludge discharge, interfering with phosphorus removal (Bassin et al., 2012). On the contrary, despite the low SRT in R1 (4 days), phosphorus removal efficiency remained around 50%, likely due to competition between PAOs and glycogen-accumulating organisms (GAOs) (Carvalheira et al., 2014). Furthermore, the pres-

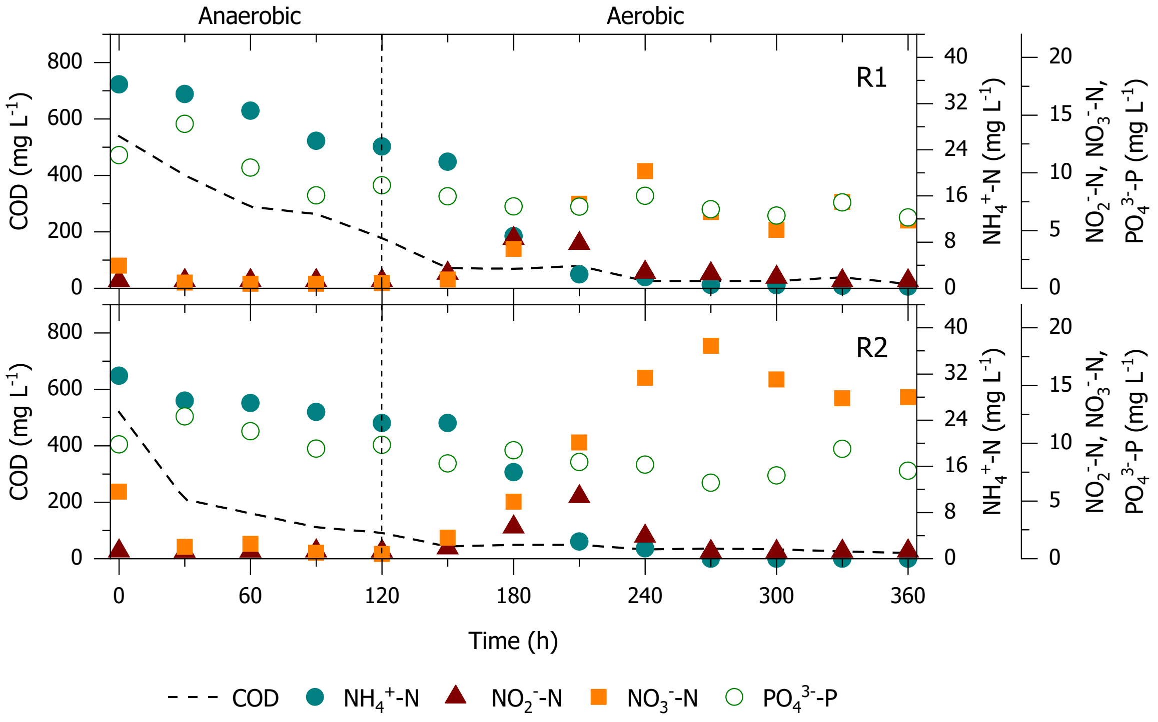

Figure 4 – Chemical oxygen demand, nitrogen, and phosphorus removal profile throughout the operational cycle (6 h).

ence of high NO*x* concentrations (>10 $mg{\cdot}L^{-1}$) associated with limited substrate availability in the aerobic phase can inhibit phosphate uptake by PAOs due to competition with heterotrophic denitrifying bacteria, resulting in reduced removal capacity (Cheng et al., 2023; Liu X et al., 2023).

Cheng et al. (2023) investigated various influent ammonia loads in an AGS system and observed that influent concentrations exceeding 60 $mg{\cdot}L^{-1}$ led to the accumulation of NOx, inhibiting PAOs' activity. This led to a reduction in the phosphorus removal rate to approximately 49.7%. Barros et al. (2020) reported a similar behavior, relating the kinetic advantage of heterotrophic denitrifying microorganisms compared with PAOs. Da Silva et al. (2021) also observed a comparable trend, although they did not explicitly discuss this behavior in their study. Notably, as noted in their work, the systems with higher NOx accumulation did not exhibit a significant phosphate buildup. Additionally, during the anaerobic period, the systems initially displayed a slight phosphate release, followed by a modest accumulation.

Therefore, owing to the complexity of the various metabolic pathways in an AGS system, there may have been a slight phosphate release in the first half-an-hour, coinciding with the feeding period when organic matter is available. This organic matter is utilized by denitrifying microorganisms, GAOs, and PAOs, as evidenced by the rapid initial organic matter decay. In a subsequent phase, specific electron acceptors likely enabled DPAOs and DGAOs to initiate the phosphate and glycogen accumulation process.

Practical implications of this study

The maximum values obtained in this study for TRP recovery were approximately 1.6 (±0.4) $mg{\cdot}gMLVSS^{-1}$. The value was lower than the one obtained (30–40 $mg{\cdot}gMLVSS^{-1}$) by Rollemberg et al. (2020b), who had already indicated the low feasibility of recovering this bioresource. On the contrary, R1 and R2 showed promising ALE production results, estimated to be approximately 11.8 and 2.73 $gALE{\cdot}m^{-3}{\cdot}day^{-1}$, respectively. The literature has reported various ALE production rates, such as 4.9 $gALE{\cdot}m^{-3}{\cdot}day^{-1}$ (Schambeck et al., 2020), 4.5 $gALE{\cdot}m^{-3}{\cdot}day^{-1}$ (Rollemberg et al., 2020a), and 8 $gALE{\cdot}m^{-3}{\cdot}day^{-1}$ (Rollemberg et al., 2020b). The yield of biopolymers can be affected by various factors, such as the operational cycle, carbon source, solid retention time, granule size, organic loads, inoculum type, and salinity.

Currently, sludge produced from wastewater treatment processes, including granular sludge, is considered a waste product. The cost of handling/disposing of the waste sludge represents up to 50% of the wastewater treatment costs. If biomaterials can be recovered from the waste sludge and applied, the sustainability and economics of wastewater treatment can be strongly increased.

van Leeuwen et al. (2018) estimated that WWTPs in the Netherlands will produce approximately 85,000 tons of bio-ALE per year by 2030, resulting in revenue of around €170 million (€2,000 per ton). However, Rollemberg et al. (2022a) argue that the ultimate revenue from bio-ALE recovery is expected to be between €1,000 and €2,000 per ton, considering the other associated costs.

Additionally, studies have reported that implementing ALE recovery in the WWTP may lead to a 50% reduction in operational expenditure (OPEX) (Murujew et al., 2021). In Kaumera Nereda, ALE recovery decreased sludge generation by 20–35%, leading to lower energy consumption and fewer CO_2 emissions.

Therefore, given the higher production levels and greater added value of ALE when compared with TRP, it is recommended that further research be directed toward enhancing ALE production and recovery.

Conclusions

The cycle configuration, specifically the distribution of anaerobic and aerobic times in AGS systems, affects the production of ALE, EPS, and TRP. While the extended anaerobic period favored ALE production in the R1 system, possibly due to a reduced famine period, the high SRT in R2 exerted a more substantial influence, maintaining ALE levels despite cycle changes. Rather than AGS concentration, the size of the granules plays a role in the EPS and ALE productions.

Regarding tryptophan production, reducing the anaerobic period can increase its yield, offering opportunities for future optimization. Considering the complexity and variability of these biological resources, this study can also be a basis for more comprehensive studies to uncover the nuances of their production in full-scale treatment systems.

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FRUTUOSO, F.K.A.: conceptualization, data curation, formal analysis, acquisition, investigation, methodology, project management, writing – original writing. BARROS, A.N.: conceptualization, data curation, formal analysis, writing – review & editing. SANTOS, A.F.: conceptualization, data curation, formal analysis, acquisition, investigation, writing – original writing. BARROS, A.R.M.: acquisition, investigation, methodology, supervision, validation. ROLLEMBERG, S.L.S.: supervision, validation, writing – review & editing. SANTOS, A.B.: conceptualization; funding, resources, supervision, validation, visualization, writing – review & editing.

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