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RESEARCH PAPER

Evaluation of the production of alginate-like exopolysaccharides (ALE) and tryptophan in aerobic granular sludge systems

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Abstract

The engineering and microbiological aspects involved in the production of alginate-like exopolysaccharides (ALE) and tryptophan (TRY) in aerobic granular sludge systems were evaluated. The inclusion of short anoxic phase (A/O/A cycle–anaerobic, oxic, and anoxic phase) and the control of sludge retention time (SRT ≈ 10 days) proved to be an important strategy to increase the content of these bioproducts in granules. The substrate concentration also has a relevant impact on the production of ALE and TRY. The results of the microbiological analysis showed that slow-growing heterotrophic microbial groups (i.e., PAOs and GAOs) might be associated with the production of ALE, and the EPS-producing fermentative bacteria might be associated with the TRY production. The preliminary economic evaluation indicated the potential of ALE recovery in AGS systems in decreasing the OPEX (operational expenditure) of the treatment, especially for larger sewage treatment plants or industrial wastewaters with a high organic load.

Keywords Aerobic granular sludge (AGS) · Resource recovery · Alginate-like exopolysaccharides (ALE) · Tryptophan (TRY)

Introduction

The aerobic granular sludge (AGS) system is considered one of the most promising biological wastewater treatment technology of the twenty-first century [1] due to the high capacity of pollutants removal in a single reactor, good settling ability of the developed biomass, strong and compact microbial structure, resource recovery possibilities, etc. [2].

These systems have been implemented in new wastewater treatment plants (WWTPs) and even in constructed facilities (upgraded) by using the existing structures [3]. Compared to activated sludge, AGS presents a significant reduction in footprint (50–75%), power consumption (20–50%), and reduction in operating costs (20–25%). Concerning other compact treatment options, such as membrane bioreactor (MBR), the energy demand of AGS is about 35–70% lower [2].

However, a yield coefficient (Y) of 0.35 g VSS/g COD was found for aerobic granules [4], indicating an excess sludge production six times higher than the values found in anaerobic reactors [5]. Moreover, recent papers are reporting the low digestibility of discharged aerobic granular sludge, impairing the anaerobic digestion of the biomass [6]. In this context, studies evaluated the possibilities of resource recovery from the discharged aerobic biomass.

The early works evaluated the phosphorus recovery as struvite and polyhydroxyalkanoate (PHA) recovery as bioplastics [7, 8]. Afterward, Lin et al. [9] showed the possibility of utilizing polysaccharide-based biomaterial from AGS as a coating material for paper and fabrics. Pronk et al. [10] demonstrated that various polymers could be extracted from AGS. Recently, some studies have focused on other value-added products generated in AGS systems, such as alginate-like exopolysaccharides (ALE), tryptophan, and glycosaminoglycans, which can be extracted from the EPS [11–14].

Commercial alginate is a group of linear polysaccharides that can be used in many industries, such as paper, food,

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cosmetic, medical, pharmaceutical, and textile, among others. Depending on its characteristics, alginate is used as a thickener, emulsion and foam stabilizer, encapsulation agent, gelling agent, and film and synthetic fiber-forming agent, among other possibilities [15, 16]. According to the literature [17], ALE need to achieve some specific characteristics (e.g., high gel-forming capacity) to compete with the commercial alginate.

Lin et al. [12] showed that ALE present in the AGS was different from the ALE present in the activated sludge (AS) flocs. The ALE found in the granules had chemical and mechanical properties (gel-forming capability) that allowed industry applications, unlike ALE found in the AS flocs that presented different blocks, making its commercial application rather tricky. The high potential of ALE recovery from AGS provided a new Nereda® project through RoyalHaskoning HDV, called Kaumera Nereda® Gum, aiming at the production of bio-based resources to a variety of oil-based materials. The first large-scale Kaumera production unit is currently in operation in Zutphen, The Netherlands [18].

On the other hand, tryptophan is a hydrophobic amino acid (protein), and hydrophobicity is the main driving force of cell adhesion [19], which is why it is usually present in AGS [13]. Substances similar to tryptophan probably accumulate in EPS (protein fraction) under high shear stress and selective discharge pressure, contributing to the enhancement of the adhesion capacity of the biomass to achieve sludge granulation and its granular structure maintenance [11]. Besides having a fundamental role in granulation, tryptophan also has several applications in the chemical industry, agriculture, and especially in the pharmaceutical industry. Tryptophan is considered an essential component of the human diet, as it cannot be synthesized in the human body. Besides, it is the precursor of serotonin and melatonin [20].

Up to date, just a few studies have evaluated ALE recovery from AGS [12, 21, 22], and, to the best of the authors' knowledge, none of them have investigated tryptophan recovery. Besides the little information about the recovery of these products from AGS systems, none of the reported papers assessed the effects of the operational conditions and the microbial groups involved in the production of ALE and tryptophan. Therefore, this paper aimed to evaluate the engineering and microbiological aspects involved in the production of alginate-like exopolysaccharides (ALE) and tryptophan (TRY) in aerobic granular sludge systems.

Material and methods

The investigation was divided into three different experiments focusing on ALE and TRY recovery in AGS systems. For the first experiment (90 days of operation), five identical reactors were operated under the same conditions (SRT ≈ 15 days), only changing the cycle configuration (Table 1). In the second experiment (40 days of operation), three identical reactors were operated with the best cycle observed previously to assess the effect of different solids retention times (SRT) (*R*6–10 days; *R*7–15 days; *R*8–20 days). In the third experiment (40 days of operation), three identical reactors were operated with the best SRT identified previously to evaluate the effect of different COD:*N* ratios (*R*9–COD: 500 mg/L and NH_4^+-N: 50 mg/L; *R*10–COD: 1000 mg/L and NH_4^+-N: 50 mg/L; and *R*11–COD: 1500 mg/L and NH_4^+-N: 50 mg/L).

Set-up and operating condition of SBRs

Experiments were performed in column-type sequencing batch reactors (SBRs) made of acrylic and operated at simultaneous fill/draw (constant volume regime) [23]. All reactors had a working volume of 7.6 L, internal diameter (D) of 10 cm, and 100 cm height (H), resulting in a H/D ratio of 10.

The air was injected from the bottom (air compressor Yuting SUN, China) of the reactors by using a fine bubble porous diffuser. The dissolved oxygen (DO) concentration varied from 1 to 3 mg/L, and the aeration velocity was 1.2 cm/s. The volumetric exchange ratio per cycle was 50%, and the hydraulic retention time (HRT) was 12 h. The

Table 1 Different cycle time distributions for the SBRs operated during the Experiment I

Phases	A/O		A/O/A		
	*R*1	*R*2	*R*3	*R*4	*R*5
Feeding (anaerobic)	60 min (16.5%)	60 min (16.5%)	60 min (16.5%)	60 min (16.5%)	60 min (16.5%)
Anaerobic	30 min (8.5%)	60 min (16.5%)	60 min (16.5%)	60 min (16.5%)	60 min (16.5%)
Aerobic	265 min (75%)	235 min (65%)	225 min (62.5%)	215 min (60%)	205 min (57.5%)
Anoxic	–	–	10 min (2.5%)	18 min (5%)	36 min (7.5%)
Settling	20–10–5 min	20–10–5 min	20–10–5 min	20–10–5 min	20–10–5 min
Decanting	1 min	1 min	1 min	1 min	1 min

In all systems, the idle time was the remaining value to complete the total cycle of 6 h

A/O, anaerobic/oxic cycle; A/O/A, anaerobic/oxic/anoxic cycle

reactors were subject to mechanical stirring (Magnetic stirrer, WEA, 30 rpm) through the anaerobic, oxic, and anoxic phases to prevent settling. Sludge discharge took place three times a week, aiming at the removal of filamentous sludge (biomass with low sedimentation capacity) [24].

Seed sludge and wastewater

The SBR seeded sludge was collected from a domestic activated sludge treatment plant located in Fortaleza, Ceará, Brazil. The reactors were inoculated with the same sludge and concentration in all experiments. Approximately 3.5 L was introduced into the SBRs, resulting in an initial concentration of mixed liquor volatile suspended solids (MLVSS) of about 1450 mg/L. The sludge volume index at 30 min (SVI_{30}) during start-up was 170 mL/g. Initially, some sludge was washed out from the reactor due to the reduced settling capacity of the biomass.

The feeding took place from the bottom of the reactors during the anaerobic phase. The synthetic wastewater was composed as follows (per liter): 500 mg/L COD of carbon source (provide by sodium acetate), 50 mg/L of NH_4^+-N (provided by ammonium chloride, NH_4Cl) as nitrogen source and 5 mg/L of PO_4^{3-}-P (provided by potassium phosphate monobasic KH_2PO_4) as phosphorus source, and 1 mL of a trace elements solution as described by Rollemberg et al. [25]. The pH of influent wastewater was adjusted to about 7.5 using sodium bicarbonate. In the third experiment, the reactors were operated with different COD concentrations (500, 1000, and 1500 mg/L), aiming at testing different COD: *N* ratios.

Analytical methods

Effluent samples were collected three times a week for the analysis of COD, ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and phosphate (PO_4^{3-}-P). In contrast, mixed liquor samples were collected once a week for the analysis of mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and sludge volume index (SVI). COD, pH, NH_4^+-N, NO_3^--N, NO_2^--N, phosphate (P-PO_4^{3-}), MLSS, and sludge volume index at 10 and 30 min (SVI_{10} and SVI_{30}) were determined according to APHA [26], whereas DO was measured by a YSI 5000 m. Total inorganic nitrogen (TIN) was regarded as the sum of NH_4^+-N, NO_3^--N, and NO_2^-N [27].

EPS were extracted by a modified heat extraction method proposed by Yang et al. [28]. The carbohydrate and protein concentrations were determined according to the phenol–sulfuric acid method with glucose as the standard [29] and by the Folin method with bovine serum albumin as the standard [30], respectively. Regarding the analysis frequency for EPS and bioproducts determinations, a sludge sample of the mixed liquor was collected once a week, and analyses were done in duplicate. For results discussion, it was considered the average of the period.

Optical microscopy (Inspect S50—FEI) was used to monitor the granule formation and shape throughout the operating period. Size profiles of the mature granules were also obtained. For this, sieving was carried out with sieves of 0.2, 0.5, 1, 1.5, 2.0, 2.5, and 3.0 mm openings [27].

Alginate-like exopolysaccharides (ALE) extraction and identification

The method described by Lin et al. [21] was used to extract the ALE from the aerobic granular sludge. A dried biomass (0.5 g) was lyophilized for 5 min (Freeze Dryer L 101, Liotop, Brazil), and the ALE were extracted by using 80 mL of a 0.2 M Na_2CO_3 solution at 80 ºC for 1 h. After centrifugation at 15,000 rpm for 20 min, the pellet was discarded. The dissolved ALE were centrifuged (3850×*g*, 20 min) and the supernatant was collected. Then, the ALE were precipitated by the addition of HCl 4 M to adjust the pH to a value between 2.0 and 2.5. The solution was centrifuged, and the pellet obtained is the ALE in the acid form, which was then frozen, lyophilized, and weighed. The mass value was expressed following the recommendations of Felz et al. [31] and Lin et al. [21].

Tryptophan extraction and identification

TRY determination followed a heat extraction method proposed by Yang et al. [28]. TRY content was measured by high-performance liquid chromatography (HPLC CTO-20A, Shimadzu Corporation, Japan), considering some previous reports [32, 33]. The HPLC was equipped with a Hypersil BDSC-18 column (250 mm × 4.6 mm, 5 mm), UV 280 nm, and UV/VIS detector (Injection volume 20 µl, run of 6 min, isocratic elution). The mobile phase was a molar rate of methanol and water of 1:1, and the flow rate was 1 mL/min.

Cycle test

To understand the mechanisms of ALE and TRY consumption/production, a cycle test was conducted in duplicate at the end of the investigation I, using the reactor that showed the best results in terms of products' content.

Microbiology

Samples from the mixed liquor (at the end of the aeration reaction) were collected (end of the experiment), and the DNA was extracted using the PowerSoil® DNA isolation kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions. All analytical procedure is described

elsewhere [25]. The sludge samples collected for analysis were the inoculum and the mature granules obtained in reactor R3 at the end of the first investigation. This choice was based on the best results in terms of ALE and TRY content in the granules.

The libraries were sequenced using the 300-cycle MiSeq Reagent Kits v2 chemistry (Illumina, 2013) with a MiSeq Desktop Sequencer (Illumina) at the Center for Genomics and Bioinformatics (CeGenBio) of the Drug Research and Development Center (NPDM), at the Federal University of Ceará, Brazil. The data obtained by the sequencing were analyzed with bioinformatics tools as follows. All reads were clustered into OTUs using QIIME script pick_open_reference_otus.py with 99% identity, using the Greengenes 16S rRNA database.

Good's coverage (C) was calculated according to the formula $C = 1 - n/N$, where n is the number of OTUs and N is the number of all tags in that sample [23]. The diversity of microbial communities aiming the evaluation of the species richness and diversity estimators of microbial populations in samples was evaluated using the richness index ACE.

Statistical methods

Statistical analyses were performed with Statgraphics Centurion XV computer software applying the Mann–Whitney Rank Sum and Kruskal–Wallis ANOVA on Ranks tests to compare the performance of the reactors. The results of the tests were evaluated according to the p value. If $p \leq 0.05$, the null hypothesis is rejected, i.e., the data groups are considered statistically different.

Results and discussion

Granule formation

As shown in Table 1, $R1$ and $R2$ had an A/O cycle, while $R3$, $R4$, and $R5$ had an A/O/A cycle. The motivation to use different cycles was due to the influence of the cycle configuration on the production of EPS, which can be directly related to the content of ALE and TRY. The evolution of mixed liquor volatile suspended solids (MLVSS) concentration and SVI_{30} of the reactors are shown in Fig. 1.

The reactors showed the same MLVSS (1.45 g/L) and SVI_{30} (170 mL/g) during start-up, even though the MLVSS increased differently among the five reactors tested. The MLVSS concentrations (around 3.3 g/L) of the reactors $R1$, $R2$, and $R3$ were very close over the operational period ($p \approx 0.10$). On the other hand, the values for the reactors $R4$ and $R5$ were lower, i.e., 2.7 and 2.5 g MLVSS/L, respectively. The reactors with a more extended aerobic phase produced a higher amount of biomass, which is in agreement with

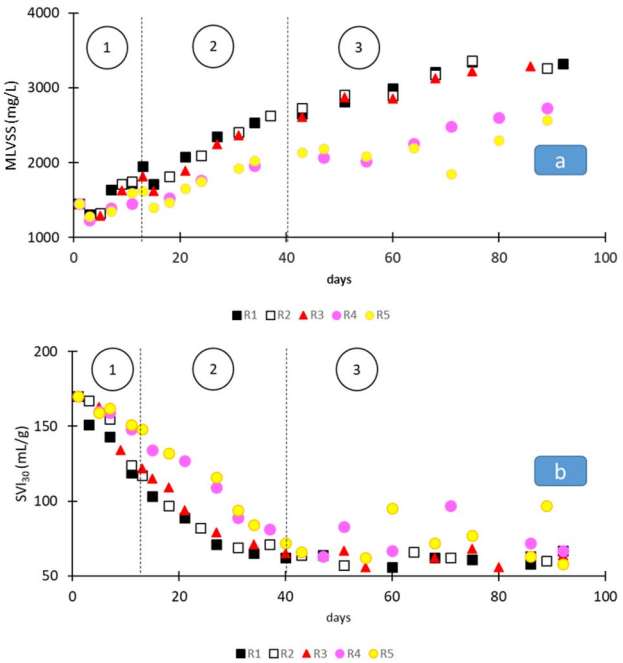

Fig. 1 MLVSS concentration profile during the operational period (**a**) and evolution of the sludge volume index at 30 min (**b**). Settling times: (1) 20 min; (2) 10 min; (3) 5 min

previous literature [34]. However, the short anoxic phase in $R3$ did not affect the MLVSS concentration, unlike what occurred in $R4$ and $R5$, where the longer anoxic phase resulted in a lower concentration of volatile solids, as also observed previously [35].

In terms of settleability, the SVI_{30} was statistically similar among $R1$, $R2$, and $R3$ ($p \approx 0.09$). However, the granules of $R4$ and $R5$ showed higher SVI_{30} values, possibly related to the prolonged anoxic and smaller aerobic phases, which resulted in a lower shear stress, favoring the growth of filaments on the external surface of the granules, thus reducing the settling capacity [36].

SBR cycle affects the production of ALE and TRY

The variation of PS, PN, ALE, and TRY contents in all reactors tested are shown in Table 2. As expected, the PS and PN content increased during the granulation process, since aerobic granular biomass is reported to have a higher amount of EPS when compared to the activated sludge flocs, which were used as inoculum [2]. However, a reduction in the PS content was observed when the granules were in the maturation phase, likely due to the lower production of PS when the granules have a larger diameter [37].

The concentrations of TRY (60 mg/g VSS) and ALE (230 mg/g VSS) during the formation of the aerobic granules were significantly higher than those found for activated sludge flocs, increasing sixfold the TRY content and

Table 2 Content of polysaccharides (PS), proteins (PN), alginate-like exopolysaccharides (ALE), and tryptophan (TRY) throughout the granulation process of the Experiment I

Period	Biomass	PS (mg/g VSS)	PN (mg/g VSS)	ALE (mg/g VSS)	TRY (mg/g VSS)
Start-up	Inoculum	43 ± 7	28 ± 5	12 ± 3	7 ± 1
Formation	*R*1	140 ± 44	142 ± 30	214 ± 47	59 ± 14
	*R*2	151 ± 52	151 ± 28	232 ± 52	61 ± 14
	*R*3	153 ± 50	159 ± 24	238 ± 49	63 ± 14
	*R*4	155 ± 60	156 ± 30	227 ± 52	63 ± 17
	*R*5	147 ± 43	139 ± 29	190 ± 44	56 ± 16
Maturation	*R*1	114 ± 15	163 ± 9	226 ± 19	48 ± 2
	*R*2	120 ± 17	161 ± 5	234 ± 13	47 ± 2
	*R*3	129 ± 16	167 ± 11	252 ± 17	50 ± 3
	*R*4	128 ± 24	150 ± 26	251 ± 24	50 ± 4
	*R*5	113 ± 21	143 ± 19	241 ± 23	34 ± 6

15.3-fold the ALE content. During the maturation phase, ALE content continued to increase. However, the TRY content decreased, showing the variation of these substances along the granulation process.

The cycle configuration also directly affected the content of the studied substances, in which cycles with a short anoxic phase favored the production of ALE and TRY. Such a finding was possibly due to the shorter famine period, which could induce microorganisms to use EPS as an electron donor [38]. Long anoxic phases can provide a more significant accumulation of EPS, but forming unstable granules that cause problems of settleability and loss of biomass (Fig. 1), possibly due to the low shear stress imposed [34]. The A/O/A operation and cycle conditions used in R3 showed the highest content of ALE and TRY in the biomass (Table 2), as well as the possibility of achieving high removals of COD (> 90%), NH_4^+-N (> 90%), total nitrogen (> 85%), and total phosphorus (> 75%). Moreover, this cycle configuration can provide a reduction in energy demand due to the shorter aeration period.

Relationship between granule diameter and bioproducts content

The relationship between the average granule diameter and ALE and TRY contents was also evaluated (Table 3). Granules with a diameter between 0.5 and 1.0 mm had a higher TRY content. On the other hand, granules with a diameter between 1.0 and 1.5 mm had a higher ALE content. While the higher production of TRY is mainly associated with newly formed granules (smaller diameters), the higher production of ALE is associated with the maturation of the biomass, with granules with an average diameter between 1.0 and 1.5 mm. However, too large granules (diameter higher than 2.0 mm) had a low ALE content, possibly due to the reduction in the EPS production caused by the carbon diffusion limitation [25]. Given the biomass stratification in full-scale AGS reactors, it is possible to determine collection points targeting granules with a higher content of the desired resources. Ali et al. [39] showed that the larger diameter granules were in the bottom (bed) and the smaller granules in the blanket. Therefore, it is possible that the discharge of sludge from the blanket has a higher content of by-products. However, studies are needed to define the composition of resources in full-scale reactors.

Table 3 Relationship between the diameter of the granules and the contents of alginate-like exopolysaccharides (ALE) and tryptophan (TRY)

Diameter	ALE (mg/g VSS)	TRY (mg/g VSS)
< 0.2 mm	7.3 ± 0.9	12.4 ± 3.2
0.5–1.0 mm	217.9 ± 23.2	58.1 ± 4.6
1.0–1.5 mm	284.5 ± 17.7	52.3 ± 5.2
1.5–2.0 mm	232.7 ± 9.8	48.7 ± 7.0
2.0–2.5 mm	213.4 ± 20.6	39.1 ± 2.8
> 3.0 mm	194.6 ± 15.3	32.8 ± 6.5

Production and consumption of bioproducts throughout SBR cycle

Considering that reactor *R*3 provided the highest production of ALE and TRY, cycle analyses were carried out, aiming at monitoring the variation of the studied resources along the cycle (Fig. 2). The maximum production of the compounds varied along the cycle. While the granules showed the highest ALE content after 90 min of aeration, the highest TRY content occurred after 180 min of aeration. These results suggest that during a full-scale operation aimed at resource recovery, the sludge discharge must occur before the end of the aerobic period.

It is important to note that the highest ALE and TRY content may vary due to several factors, such as characteristics of the influent, concentration of biomass, and dissolved oxygen, among others. Thus, a monitoring plan must be

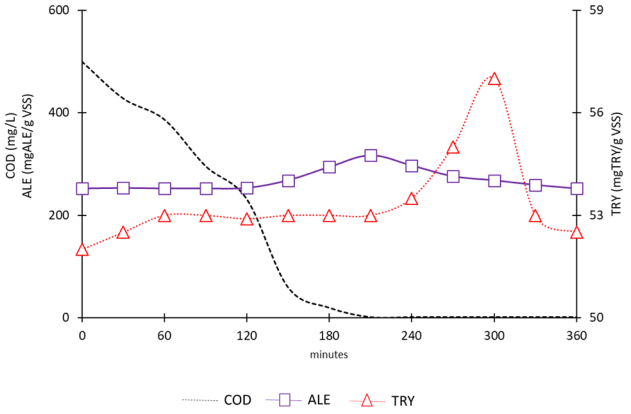

Fig. 2 Evaluation of alginate-like exopolysaccharides (ALE) and tryptophan (TRY) contents over a cycle

conducted to combine a good AGS operational stability, its efficiency to simultaneously remove C, N, and P, and also achieve the highest productivity of the desired resources to be recovered.

Sludge retention time (SRT) affects the content of ALE and TRY in the granules

Sludge discharge is an essential operational procedure in aerobic wastewater treatment systems [40]. In AGS reactors, sludge discharge assists in phosphorus removal, granule stability, and reduction of suspended solids in the treated effluent [25, 37]. In this investigation, three identical reactors inoculated with the same concentration of AGS (MLVSS = 3 g/L) were operated with different SRTs (Table 4), using the same A/O/A cycle and conditions of reactor R3. However, during the experimental investigation with R3, there was no SRT control.

In terms of TRY content, no significant difference was found among the reactors *R*6, *R*7, and *R*8 ($p \approx 0.07$), and also there were no significant differences in relation to *R*3 ($p \approx 0.07$). However, a high SRT reduced the ALE content in the granules of *R*8, which was statistically different from *R*3, *R*6, and *R*7 ($p \approx 0.04$). Such a finding was likely related to the higher rate of endogenous respiration for high SRTs [25, 40], inducing EPS consumption as a carbon source. Another possible explanation may be related to polyphosphate-accumulating organisms (PAOs), as preliminary studies have pointed out these microorganisms as responsible for ALE production [41]. The reduction of the SRT could benefit the growth of PAOs, allowing a higher production of ALE. Therefore, AGS operation with a low SRT could favor a higher ALE content on the granules.

This observation can be used in future studies aiming at evaluating the ALE content in granules enriched with PAOs, a process called EBPR (enhanced biological phosphorus removal). In this way, it would be possible to cultivate granules capable of removing nutrients (wastewater treatment) associated with higher production of ALE (resource recovery) in a single system.

COD:*N* ratios affect the content of ALE and TRY in the granule

The COD:*N* ratio in the influent is deterministic in the formation of stable granules. High values of this ratio can cause the disintegration of the AGS by the growth of filaments in the granule. On the other hand, the decrease in the COD:*N* ratio to values close to 1 causes significant changes in the microbial community and a reduction in the content of EPS, also impacting on the nitrification and resistance of the granule [42, 43].

In order to evaluate the effect of the COD:*N* ratio on the production of ALE and TRY, three identical reactors inoculated with aerobic granular sludge at the same concentration (MLVSS = 3 g/L) were operated (Table 5). No statistical difference was observed between reactors *R*9 and *R*10 concerning the TRY content ($p \approx 0.08$), but there was a significant positive difference in terms of ALE content ($p \approx 0.04$). When comparing reactors *R*10 and *R*11, significant positive differences were found for ALE ($p \approx 0.04$) and TRY ($p \approx 0.04$) contents. Thus, the influent concentration, and subsequently, the organic loading rate (OLR), directly influences the production of resources, especially ALE production.

The usual higher production of ALE and TRY when increasing the COD:*N* ratio may be related to the higher amount of EPS produced on the granule. Literature reports

Table 4 Relationship between sludge retention time (SRT) and the contents of alginate-like exopolysaccharides (ALE) and tryptophan (TRY)

Phase	Parameters	*R*6 (SRT = 10 d)	*R*7 (SRT = 15 d)	*R*8 (SRT = 20 d)
Startup	Diameter (mm)	0.8–1.3	0.8–1.3	0.8–1.3
	ALE (mg/g VSS)	218.9	218.9	218.9
	TRY (mg/g VSS)	49.6	49.6	49.6
End of investigation (40 days)	Diameter (mm)	0.8–1.2	0.8–1.2	1.1–1.7
	ALE (mg/g VSS)	237.5 ± 11.8	244.1 ± 15.2	203.9 ± 27.8
	TRY (mg/g VSS)	58.3 ± 4.6	52.9 ± 5.7	48.1 ± 7.0

Table 5 Relationship between COD:*N* ratios in the influent and the contents of alginate-like exopolysaccharides (ALE) and tryptophan (TRY)

Phase	Parameters	*R*9 (COD:*N* = 10)	*R*10 (COD:*N* = 20)	*R*11 (COD:*N* = 30)
Startup	Diameter (mm)	0.8–1.2	0.8–1.2	0.8–1.2
	ALE (mg/g VSS)	241.5	241.5	241.5
	TRY (mg/g VSS)	50.8	50.8	50.8
End of investigation (40 days)	Diameter (mm)	0.8–1.2	1.3–1.9	1.6–2.3
	ALE (mg/g VSS)	243.6 ± 14.4	289.7 ± 33.2	236.6 ± 57.8
	TRY (mg/g VSS)	51.8 ± 5.3	54.1 ± 8.0	37.9 ± 22.7

that EPS production is directly proportional to the organic load increase [2, 44]. However, when COD:*N* ratio is larger than 20, the nitrogen may be deficient, and this might stimulate the growth of filaments or too much EPS production, thus making the granules unstable [36].

In this study, when the COD:*N* ratio was around 30 (reactor R11), a reduction of both ALE and TRY contents was observed. In addition to nitrogen deficiency, another problem associated with an increase of COD:*N* ratio is the excessive increase of the aerobic granules, which became (on average) larger than 2.5 mm, likely making the carbon diffusion difficult. Franca et al. [36] presented cases where the granules disintegrated due to high OLR (> 15 kg COD/m^3 day). Similar results regarding granules disintegration were also reported by Kocaturk and Erguder [45]. Therefore, this parameter must be optimized to achieve both operational stability and resource recovery.

Possible microbial groups involved in the production of ALE and TRY

To evaluate which microbial groups present in the aerobic granular sludge could be involved in resource recovery, the inoculum was compared with the granules obtained in reactor R3 at the end of the investigation (Fig. 3). A total of 500,163 and 838,613 sequence tags were retrieved from the inoculum and aerobic granules, respectively, which were assigned to 13,481 and 15,086 OTUs, respectively. The species richness and diversity indicators of microbial populations in the AGS and the inoculum were also evaluated. The Good's Coverage index was 99.6% and 98.6% for AGS and inoculum, respectively, indicating that almost all OTUs were covered during sequencing. The ACE index was 2005 and 1929 for AGS and inoculum, respectively, suggesting that the microbial abundance in the granules was greater than the one found for the inoculum.

Initially, a reduction in the abundance of the Rhodocyclaceae family was observed in the aerobic granules. Most of the species in this group are related to ordinary heterotrophic organisms (OHOs) and ordinary heterotrophic denitrifying

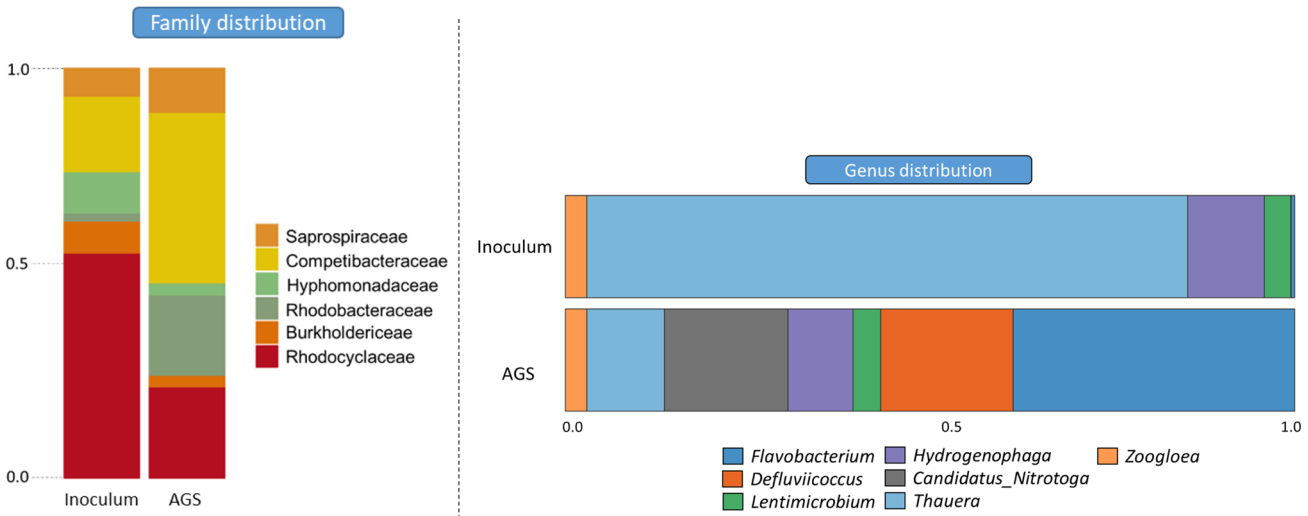

Fig. 3 Taxonomic affiliation at the family level and at the genus level of the activated sludge flocs (ASF) used in the inoculum and aerobic granular sludge (AGS) of the R3 collected in the maturation phase of investigation I

organisms (OHDOs). Therefore, microorganisms without the capacity to store polyhydroxyalkanoates (PHA) [46].

The abundance of Burkholdericeae also decreased in AGS compared to the inoculum (Fig. 3). This family is associated with OHDOs, also found in activated sludge systems that are facing the bulking sludge problem [47]. The reduction of Rhodocyclaceae and Burkholdericeae serves as an indicator to show that the selection pressure imposed on the system was able to remove/reduce microorganisms that could harm the aerobic granule, i.e., rapidly growing heterotrophic microorganisms [2].

At the genus level, *Flavobacterium* was the most abundant genus in AGS followed by *Defluviicoccus*, although both were absent in the seed sludge. The literature reports that the *Flavobacterium* genus is one of the most important EPS producers, justifying the high presence of biopolymers in the cultivated AGS [48]. Studies demonstrated that some species of this genus might be associated with the production of TRY. Therefore, the greater presence of fermentative microorganisms of the *Bacteroidetes* group indicates the greater production of TRY in the biomass [49].

As shown in Fig. 3, Rhodobacteraceae and Competibacteraceae were the two families that showed the highest growth in AGS in relation to the activated sludge used as inoculum. These results are significant because they are associated with PAOs and glycogen-accumulating organisms (GAOs), respectively [50]. Thus, this study shows that the production of ALE and TRY can be likely associated with these groups. Schambeck et al. [22] observed that the production of ALE is linked with PAOs abundance. This hypothesis may explain the increase in the content of ALE when the SRT was controlled since this parameter is directly related to the higher activity of PAOs [3, 37]. Nonetheless, other detailed studies are needed, evaluating the microbial groups responsible for the production of ALE and TRY.

Economic and technical aspects involved in the recovery of ALE and TRY in WWTPs

Throughout the granulation process, the TRY content reaches its highest value during the formation of the granules. In this period, the ALE/TRY ratio content was below 3. However, after the granules have matured, this ratio reached values close to 5. Considering the commercial values of ALE (US$ 80–140/kg) and the higher content of this product, the results revealed that the recovery of this substance appears to be much more viable, likely indicating the reason why Kaumera Nereda® Gum is focusing on ALE recovery from the aerobic granular biomass (Fig. 4). Some studies reported that the OPEX (operational expenditure) could be reduced by 50% in the WWTP when recovery of ALE is implemented [51]. In the Kaumera Nereda, the ALE recovery provided a reduction of sludge generation by up to 35%, less CO_2 emissions, and energy saving.

Regarding the evaluation of ALE and TRY recovery in WWTPs, Table 6 shows the comparison of the values found in the production of these products in different papers involving AGS with those achieved in the current

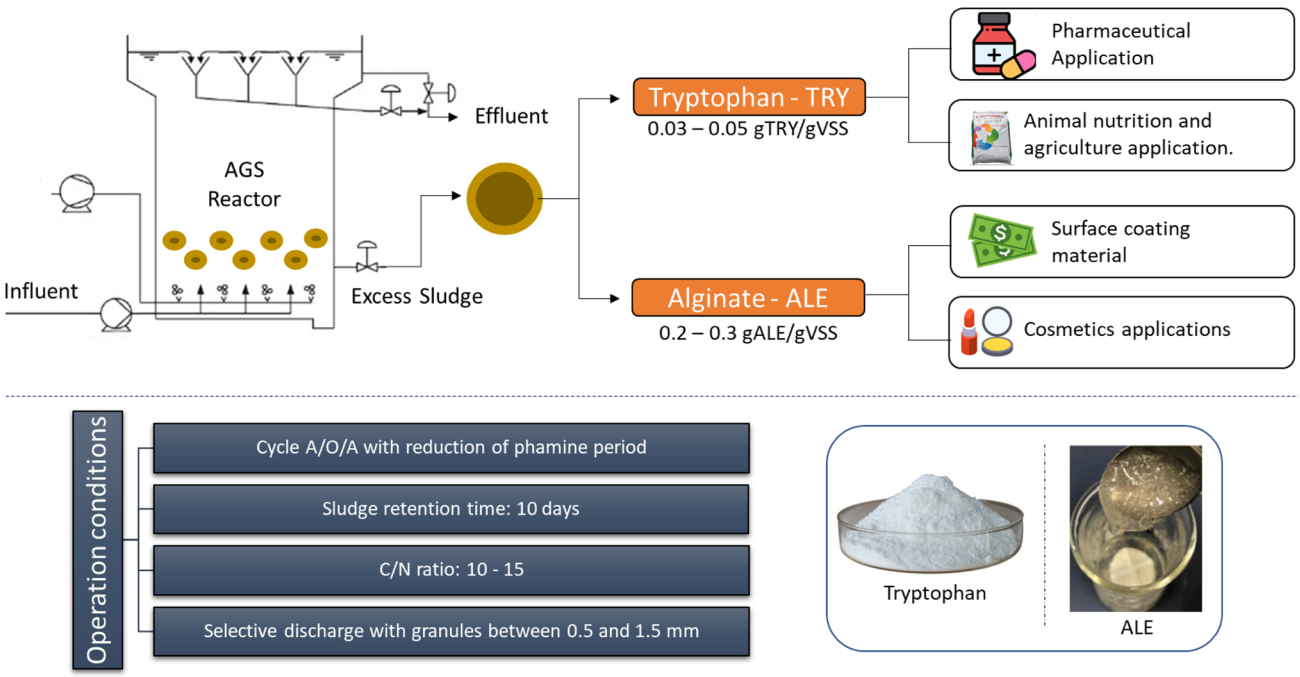

Fig. 4 Resource recovery from AGS: system optimization

Table 6 Comparative evaluation of alginate-like exopolysaccharides (ALE) and tryptophan (TRY) production values

Parameter	This Research	Rollemberg et al. [24]	Schambeck et al. [22]	Meng et al. [52]
COD influent (mg/L)	500	460	513	600
SBR cycle (h)	6	6	6	4
Volumetric exchange rate (%)	50	60	50	50
SBR working volume (L)	7.5	140	110	2
Number of SBR cycles per day (cycles/day)	4	4	4	6
Volume of wastewater treated (L/day)	15	336	220	6
Organic loading rate (gCOD/L·day)	100	110.4	102.6	180
MLVSS (g/L)	3	4	3.5	7
F/*M* ratio (kgCOD/kgVSS)	0.03	0.03	0.03	0.03
Yield coefficient (gVSS/gCODr)	0.3[a]	0.3[a]	0.3[a]	0.3[a]
Sludge age (days)	10	12	15	20
COD effluent (mg/L)	50	23	92	60
Endogenous decay coefficient–kd (day^{-1})	0.1[b]	0.1[b]	0.1[2]	0.1[b]
Biodegradable fraction of MLVSS –fb (Xb/Xv)	0.67	0.65	0.62	0.57
Sludge production (gVSS/day)	0.53	7.92	4.07	0.17
ALE content (g/gVSS)	0.23	0.21	0.24	0.24
TRY content (g/gVSS)	0.06	0.05	–	–
Daily ALE production (gALE/day)	0.12	1.66	0.98	0.04
Volumetric ALE production (gALE/m^3 day)	8.05	4.95	4.44	6.88
Daily TRY production (gTRY/day)	0.03	0.40	–	–
Volumetric TRY production (gTRY/m^3 day)	2.10	1.18	–	–

[a]Adopted from Rollemberg et al. [4]

[b]Adopted from Von Sperling [53]

investigation. Rollemberg et al. [24] and Schambeck et al. [22], evaluating the presence of ALE in pilot reactors treating municipal wastewater, found ALE production rate of 4.9 gALE/m^3 day and 4.5 gALE/m^3 day, respectively. These values were significantly lower than those observed in this research (8 gALE/m^3 day).

This difference may be related to the carbon source because the works that used acetate presented higher content of ALE in the granules than the studies that used domestic wastewater. Meng et al. [16] cultivated AGS in a lab-scale reactor using synthetic sewage (a mixture of acetate and glucose), obtaining values of 6.9 gALE/m^3 day. Therefore, it is possible that the substrate has a significant impact on ALE production. As it is known, the carbon source impacts on the abundance of microbial groups [25]. Acetate, for example, is known to favor the presence of PAOs and GAOs [37]. In this sense, studies using propionate are recommended in order to assess whether the formed granules have a higher ALE content because this substrate is known to be the best to support PAOs growth [2].

TRY production also appears to be impacted by the carbon source. In this work, production rates of 2.1 gTRY/m^3 day were observed. On the other hand, Rollemberg et al. [24], cultivating AGS with municipal wastewater, found a production rate of 1.2 gTRY/m^3 day. There are still few studies evaluating the production of TRY in AGS systems. Therefore, studies are needed to assess the effect of high fermentable substrates (dairy wastewater and similar) on TRY production [49].

In economic aspects, ALE recovery from AGS systems appears to be a trend. As it is known, alginates are produced from seaweeds, and the availability and costs of alginate seaweeds are beginning to be a concern of alginate producers. Higher costs have been driven by higher energy, chemicals, and seaweed costs, reflecting seaweed shortages [54]. In a field test in Zutphen, The Netherlands, it was demonstrated that 18 kg bio-ALE could be produced from 80 kg of Nereda granular sludge, i.e., 22.5% bio-ALE recovery [55], being in accordance with the results obtained in this work (23% ALE recovery from the sludge).

It is expected that an industrial Nereda bio-ALE factory, also located in Zutphen, will produce about 400 tons of bio-ALE per year [55]. The total Dutch production is estimated at 85,000 tons per year from 2030. The market price depends on the quality and subsequent application. However, the total value in the Dutch market is currently estimated at € 170 million per year from 2030.

The analysis of the values reported above leads to the conclusion that one ton recovered from ALE (after extraction and refining costs) generates a final revenue of € 1000–2000. These results show that a WWTP with AGS technology treating domestic wastewater (COD ≈ 600 mg/L), with a flow rate of approximately 3.0 m^3/s, would be able to produce approximately 1 ton of ALE/day, generating a revenue of approximately € 365,000–730,000/year, which could decrease the OPEX of the treatment plant.

These values may be even higher if strategies to increase the production of ALE in the granules are adopted. Among these strategies, this work showed that a more prolonged fermentation period (anaerobic time in the SBR cycle), the reduction of the famine period, the SRT control, and the correct sludge discharge can positively affect the higher ALE content and, therefore, make it even more viable ALE recovery in AGS treatment plants.

Conclusion

The operational conditions influence the production of ALE and TRY, in which the use of a short anoxic phase (A/O/A cycle) and a short SRT (around 10 days) is recommended. During the maturation phase, ALE content continued to increase, but TRY content decreased. COD:*N* ratio also showed a positive effect on the production of the compounds. However, when the ratio reached a value of 30, a production decrease was verified.

The results of the microbiological analysis showed that the PAOs and GAOs might be associated with the production of ALE, and some fermentative bacteria of the Bacteroidetes group might be associated with the production of TRY.

The preliminary economic evaluation indicated the potential of ALE recovery in AGS systems in decreasing the OPEX of the treatment, especially for larger sewage treatment plants or industrial wastewaters with a high organic load.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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