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Short Communication

The second messenger (cyclic diguanylate and autoinducer-2) promotes N-acylated-homoserine lactones-based quorum sensing for enhanced recovery of stored aerobic granular sludge

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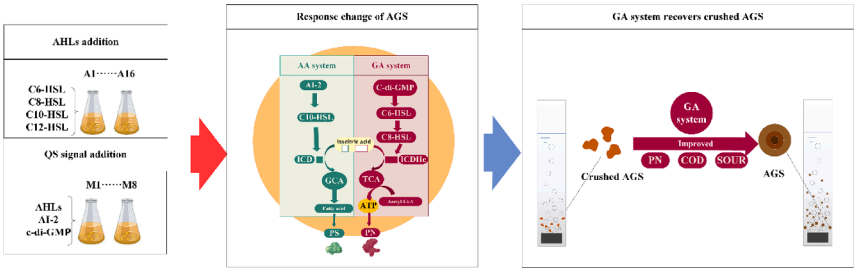
HIGHLIGHTS

- Crosstalk between the secondary messenger and AHLs in granulation is studied.
- c-di-GMP + AHLs accelerate the tricarboxylic acid (TCA) cycle for yielding excess proteins.
- AI-2 + AHLs accelerate the glyoxylate (GCA) cycle for stimulating polysaccharides secretion.
- c-di-GMP and AI-2 compete with the AHLs to decline the EPS secretion.
- c-di-GMP + AHLs recovered PN-rich granules from refrigerated-stored and dried-stored AGS.

GRAPHICAL ABSTRACT



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ABSTRACT

Efficient quorum sensing (QS) response is the premise for recovering the activities of stored aerobic granular sludge (AGS). This study aims to explore the crosstalk between the secondary messenger and the N-acylated-homoserine lactones (AHLs) to yield protein-rich granules efficiently from stored AGS by enhancing its QS efficiency selectively. 80 nmol/L cyclic diguanylate (c-di-GMP) with 20 nmol/L AHLs could increase the activity of isocitrate lyase activity (ICD) by 89 % and isocitrate dehydrogenase activity (ICDHc) by 113.5 %, to accelerate the tricarboxylic acid (TCA) cycle for yielding excess proteins by 166.4 %. In contrast, 80 nmol/L autoinducer-2 (AI-2) with 20 nmol/L AHLs could increase the activities of ICD and ICDHc by 485 % and 54.5 %, respectively, accelerating the glyoxylate (GCA) cycle to activate fat acid synthesis for stimulating polysaccharides (PS) secretion by 137.9 %. The strategy with c-di-GMP successfully recovers the refrigerated-stored and dried-stored AGS into proteins-rich AGS, with enriched functional strains for the PN secretion.

1. Introduction

Aerobic granular sludge (AGS) is a promising wastewater treatment process with excellent settling performance and enriched functional consortia (**Ran *et al.*, 2022**). Quorum sensing (QS) is a bacterial communication mechanism that can regulate microbial activities in an

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AGS system (Zhang et al., 2023). The general signal molecules N-acyl-homoserine lactones (AHLs) can assist the EPS secretion in the AGS (Shuai et al., 2021): the N-hexanoyl-l-homoserine lactone (C6-HSL) and N-octanoyl-l-homoserine lactone (C8-HSL) can increase the abundance of genes related to the tricarboxylic acid (TCA) cycle, glycometabolism, and nucleotide metabolism (Liu et al., 2021), positively correlate with the EPS concentrations (**Zhang *et al.*, 2022**); decanoyl-l-homoserine lactone (C10-HSL) correlates significantly with EPS concentrations, density and the surface hydrophobicity of the AGS (Gao et al., 2021); and N-(3-oxododecanoyl)-l-homoserine lactone (C12-HSL) can affect the microbial community structures of the granules (Zhao et al., 2018). The AHLs-based QS on granulation processes has been widely explored (Zhang et al., 2023).

The second messenger, cyclic diguanylate (c-di-GMP) and autoinducer-2 (AI-2) in AGS can stimulate cells to secret extracellular polymeric substances (EPS) to enhance cells' aggregation (Wang et al., 2017). The crosstalk of signal molecules can affect the microbial processes in the AGS (Wang et al., 2017); however, the crosstalk between the AHLs and the second messengers on AGS performance is rarely investigated (Gao et al., 2023), its roles in AGS cultivation remains unclear. The stored AGS can be applied to accelerate the start-up of AGS reactors (Gao et al., 2021). The proteins (PN)-rich AGS are of high structural stability, revealing superior stability compared to polysaccharide (PS)-rich AGS (Chen et al., 2016).

This paper aims to highlight the use of crosstalk and cooperation between AHLs and second messengers to enhance the recovery rate of the stored AGS, with the novel capacity of enriching selectively the interior with secreted proteins. The stimulation of TCA cycle for promoting polysaccharide (PS) production and of the glyoxylate cycle for enhancing proteins (PN) production is the discussion basis.

2. Experimental

2.1. Setup and batch tests operation

This study utilized sequential batch reactors (SBRs) of identical diameter (60 mm) and working volume (2.2 L) for all tests. The reactor was seeded by the waste-activated sludge collected from a local wastewater treatment plant in Shandong, China. The SBRs were operated at 5-min feeding, 195-min aeration, 5-min settling, and 5-min withdrawal mode (0.75 vol exchange ratio). The adopted four-hr SBR cycle corresponds to practical SBR operations. The testing temperature was at 30 ± 1 °C. The air was fed at the reactor's bottom at a superficial velocity of 3.0 cm/s. The wastewater is at 1500 mg/L chemical oxygen demand (COD) with 2/1 propanol and acetate, 400 mg/L peptone, 250 mg/L yeast exact, 50 mg/L NH_4Cl, 4.85 mM KH_2PO_4, 0.27 mM $CaCl_2$, 0.21 mM $MgSO_4$, 0.13 mM $FeSO_4$, 0.51 mM $NaHCO_3$. The mature granules were cultivated from SBRs operated for long time as fresh AGS (N), part of which was stored according to Lv et al., (2018b) in the dried (D) or refrigerated (F) modes over one year to yield the stored AGS.

2.2. Batch tests

2.2.1. Activity tests

Two batches of activity tests were performed: Group A is to determine the interactions between different signal components of AHLs, while Group M is to determine the interactions between specific second messenger and AHLs.

In Group A, sixteen batch tests (A1-A16) were conducted with the fresh granules mixed at 160 rpm and 30 °C with AHLs added at prescribed compositions (Table 1). Each test lasted for four hr, and then the supernatant and the granules were collected separately for analysis. In Group M, a total of eight batch tests (M1-M8) were conducted, added with the best AHL composition identified in the Group A test that can peak the total EPS production by the fresh granules and an extra 80 nmol/L of the second messenger c-di-GMP or AI-2 (Table 1). Group A's shaking conditions and temperature were applied in the Group M tests. This test measured and recorded COD, AHLs, ATP, ICD, ICDHc, and EPS.

Table 1
The compositions of signal molecules were added in the Group A and Group M. The Group M is with AHLs in A15. The recovery tests were with signals M5.

ID	C6-HSL	C8-HSL	C10-HSL	C12-HSL
A1	0	0	0	0
A2	20	0	0	0
A3	0	20	0	0
A4	0	0	20	0
A5	0	0	0	20
A6	10	10	0	0
A7	10	0	10	0
A8	10	0	0	10
A9	0	10	10	0
A10	0	10	0	10
A11	0	0	10	10
A12	6.65	6.65	6.65	0
A13	6.65	6.65	0	6.65
A14	6.65	0	6.65	6.65
A15	0	6.65	6.65	6.65
A16	4	4	4	4

ID	AHLs (A15)	c-di-GMP	AI-2
M1	0	0	0
M2	20	0	0
M3	0	80	0
M4	0	0	80
M5	20	80	0
M6	20	0	80
M7	0	80	80
M8	20	80	80

2.2.2. Recovery tests

The recovery tests of fresh (N), refrigerated-stored (F), and dried-stored (D) AGS were conducted in six reactors (NC, NR, FC, FR, DC, DR; C for control with 0 nM signal added, and R for M5 signals added: 20 nM for AHLs and 80 nM for c-di-GMP) (Table 1). The recovery tests were performed for 5 days, with the signal molecules being supplied at the start of the tests. This test measured COD, SOUR, and EPS.

2.3. Analysis

2.3.1. EPS measurements

The EPS in the AGS samples was extracted using a heat extraction method (He et al., 2019). The PN and PS contents in the supernatants after 0.45-μm membrane filtration were analyzed using the Coomassie brilliant blue method and phenol–sulfuric acid colorimetric method. Their total amounts are the total EPS.

2.3.2. Extraction and detection of AHLs

Lv *et al.* (2021) devised a method to extract the aqueous AHLs. 0.5 ml sample was taken from reactors, and 1.5 ml ethyl acetate was used to separate the supernatant. The AHLs of the granules were extracted based on Chen et al. (2019): samples with 5 ml of ethyl acetate were ultrasound in an ice bath for 3 s, 30 times. Then, the sample was centrifuged at 12,000 rpm for 10 min to collect the supernatant. The AHL detection protocol was from Lv et al., (2018a) with liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (Waters, USA).

2.3.3. ICDHc, ICD, and ATP contents

The contents of isocitrate lyase activity (ICD), isocitrate dehydrogenase activity (ICDHc), and adenosine triphosphate (ATP) were analyzed by ICD Content Determination Kit of Ruixin Biotechnology Co., Ltd (Fujian, China), the ICDHc Content Determination Kit of Sangon Co., Ltd (Shanghai, China), and ATP Content Determination of Kit Grace Biotech. Co., Ltd (Jiangsu, China), respectively. The isocitrate lyase contents correlate with the contributions by the glyoxylate cycle, while

isocitrate dehydrogenase, the TCA cycle.

2.3.4. Microbial community analysis and metabolite analysis

The extraction and sequence of DNA samples were analyzed by Novogene Ltd. (Beijing, China). The DNA extracted from the samples was used as a template, and the V3-V4 region of the bacterial 16S rRNA genes was amplified with the primers (341F 5′-ACTCCTACGGGRSGCAGCAGCAGCAGCAG-3′; 806R 5′-GGACTACVVGGGTATCTAATC-3′). The Illumina NovaSeq platform was used for high-throughput sequencing, following a 2 × 300 bp pairing protocol. Novogene Ltd. (Beijing, China) analyzed the untargeted metabolisms of AGS samples. All resulting sequencing data have been deposited in the NCBI SRA database under accession code PRJNA1070261.

2.3.5. Other analyses

The COD, specific oxygen uptake rate (SOUR), mixed liquid suspension solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) of the AGS were determined according to the Standard Methods. Statistical analysis for all variables was conducted by the *t*-test using SPSS (SPSS 18.0). $p < 0.05$(*) is statistically different, and $p < 0.01$(**) indicates significantly different. The values reported in **Supplementary Materials** were examined.

3. Results and discussion

3.1. Activity tests

The degradation time of COD ranges from 3.38 to 4.80 hr, with that for A15 (with C8 to C12-HSL) being the shortest in Group A; the M5 with A15-AHLs and 80 nmol/L c-di-GMP has the shortest degradation time than those in Group M tests (**Supplementary Materials**).

The dosed AHLs are rapidly consumed in the Group A tests (Fig. 1). The C8-HSL can be rapidly stimulated by C6-HSL (A2 and A6 in Fig. 1**b**). The presence of C12-HSL (A5 and A11) also increases the quantity of C8-HSL. High C8-HSL negatively correlates with that of C10-HSL. As Fig. 1**d** shows, the concentration of C6-HSL in M5 is increased by 119.5 nmol/g, much higher than in M2. At the same time, the concentration of C10-HSL is decreased by 8.89 nmol/g. Conversely, the concentration of C6-HSL in M6 is decreased by 15.9 nmol/g, whereas that of C10-HSL is increased by 34.4 nmol/g (Fig. 1**d**). The c-di-GMP and AI-2 oppositely impact the concentrations of C6-HSL and C10-HSL in the AGS.

The increase ratio of ΔPN/ΔPS is 12.5 in A2, much higher than in A3 (1.26) and A4 (2.86) (Fig. 1**e**). The A15 (mix of C8-C12 AHLs) has the maximum ΔPN and ΔPS, being 1.3 (198.5 %) and 1.5 mg/g (114.1 %), respectively (Fig. 1**e**) in the Group A tests. Meanwhile, the ΔPN in M5 is increased by 2.3 mg/g (166.4 %), and the ΔPS in M6 is increased by 2.0 mg/g (137.9 %) (Fig. 1**f**). The PN is stimulated by c-di-GMP instead of by the AI-2. Since A15 generates the highest total EPS, favoring granulation, Group M utilizes the mixed AHLs in A15 to study the crosstalk of AHLs with the second messengers in AGS activities.

The ΔICD of all Group A tests is positive (Fig. 2**a**). Considering the tests generating a high ΔICD, the C10-HSL can enhance the ICD more than C8-HSL. The ΔICDHc in A9 and A15 increase by 12.9 times and 10.6 times, respectively (Fig. 2**c**). The high C8- and C10-HSL correlates with high ICDHc. The contents of ΔATP of A9 and A15 increased by 142.5 % and 142.8 %, respectively (Fig. 2**e**). No clear, direct correlation between the type of AHLs on the increased ATP quantities.

The ΔICD in M5 decreased by 0.89 times compared with M2, while that in M6 increased by 4.85 times (Fig. 2**b**). The Group M tests show a positive correlation between the presence of AI-2 and the ICD. The ΔICDHc in M6 is increased by 54.5 % compared with M2, whereas that in M5 has a significant increase of 113.5 % (Fig. 2**d**). The c-di-GMP has positively correlated with the ICDHc. The ΔATP in M5 increases by 3.41 times, but in M6, almost no increase is noted (Fig. 2**f**). Adding AI-2 minimally impacts ATP in the AGS.

The comparable relative abundances of *Brachymonas* are M2 (4.09 %), M5 (4.84 %), and M6 (7.61 %) (**Supplementary Materials**). Significant upregulation of *Brachymonas* was seen in M6 (**Supplementary Materials**). *Flavobacterium*_sp_enrichment_culture_clone_SA_NR2_1, *Chryseobacterium*_sp_A1-ST2, and *Bdellovibrio*_sp_ETB in M6 are decreased by 10.8 %, 26.2 %, and 21.3 % compared with M2, respectively. However, in M5, the corresponding increases are 18.2 %, 19.5 %, and 85.1 %, respectively (**Supplementary Materials**). The c-di-GMP and AI-2 have opposite effects on the microbial community structures in the M1-M8 AGS.

The above results reveal that the best recipe for the AHL-second messenger pair for AGS stability is M5, which is used in the subsequent recovery test for cultivating PN-rich granules.

3.2. Recovery tests

Fig. 3 shows the samples (N, F, D) crushed before tests, with or without M5 signals, and reveals that the granulation with signals is much faster than those without M5.

The COD degradation rates for granules are low on day 1, 12–31 %. However, those with signal molecules recover more rapidly than those without signal molecules (**Supplementary Materials**). The freezing or drying deteriorates the microbial structures of the granules, leading to slower recovery than the fresh granules. However, the M5 signals enhance the recovery process, reaching plateau COD degradation in two days of cultivation.

The EPS concentration, PN/PS (**Supplementary Materials**), and SOUR (**Supplementary Materials**) in the signals-added tests are all higher than the pristine tests, with those for dried, stored sludge more extensive in enhancement than in the N and F AGS.

A few functional strains for EPS secretion are detected. *Sphingobacterium*_mizutaii, *Bdellovibrio*_sp_ETB, and *Chryseobacterium*_sp_A1-ST2 in fresh granules are enriched with M5 signals from 0.31 to 1.46 % to 0.92–1.73 %. The M5 signals enrich the relative abundance for *Chryseobacterium*_sp_A1-ST2 in refrigerated stored AGS from 2.32 % to 2.96 %, and *Flavobacterium*, *Sphingobacterium*, *Chryseobacterium*_sp_A1-ST2, and *Bacteroidetes*_bacterium_RBE2CD-54 in dried stored AGS from 12.8 % to 22.66 % and 9.43 % to 11.97 %, respectively (**Supplementary Materials**).

The fatty acid synthesis mechanisms in M5 significantly ($p < 0.05$) increased using M2 as a comparison (**Supplementary Materials**). The purine metabolism and synthesis of phenylalanine, tyrosine, and tryptophan in M5 are upregulated (**Supplementary Materials**). In comparison, the arachidonic acid (fatty acid) metabolic in recovered dried stored AGS with signals is significantly($p < 0.01$) promoted (**Supplementary Materials**).

3.3. Discussion

The A15 has the highest COD removal rate and a marked increase in EPS and ATP contents, by 142.8 % and 142.2 %, respectively. The 20 nmol/L C8-HSL, C10-HSL, and C12-HSL at w/w/w 1:1:1 have the peak QS modulation efficiency. The ΔEPS and ΔATP for c-di-GMP additions are 139.1 % and 3.41 times, respectively. Therefore, 80 nmol/L c-di-GMP promotes the QS modulation efficiency with 20 nmol/L AHLs.

Complicated crosstalk and cooperation occur between the AHLs and the second messengers: c-di-GMP promotes secretion of C6-HSL and C8-HSL and inhibits C10-HSL, while AI-2 affects it oppositely. A competitive inhibition between C8-HSL and C10-HSL with c-di-GMP is noticeable. Also, the C8-HSL promotes ICDHc, and the C10-HSL likely promotes ICD. The tests in M1-M8 show that competitive inhibition of ICDHc and ICD for isocitrate leads to the noted crosstalk with c-di-GMP or AI-2. The c-di-GMP has increased the ATP contents by 3.41 times, but the AI-2 has a minimal impact. Moreover, c-di-GMP and AHLs promote the secretion of PN, and the AI-2 and AHLs activate PS secretion. An independent test adding c-di-GMP and AI-2 to A15 decreases PN and PS by 8.9 % and 11.0 %, respectively (not shown for brevity's sake). The microbial

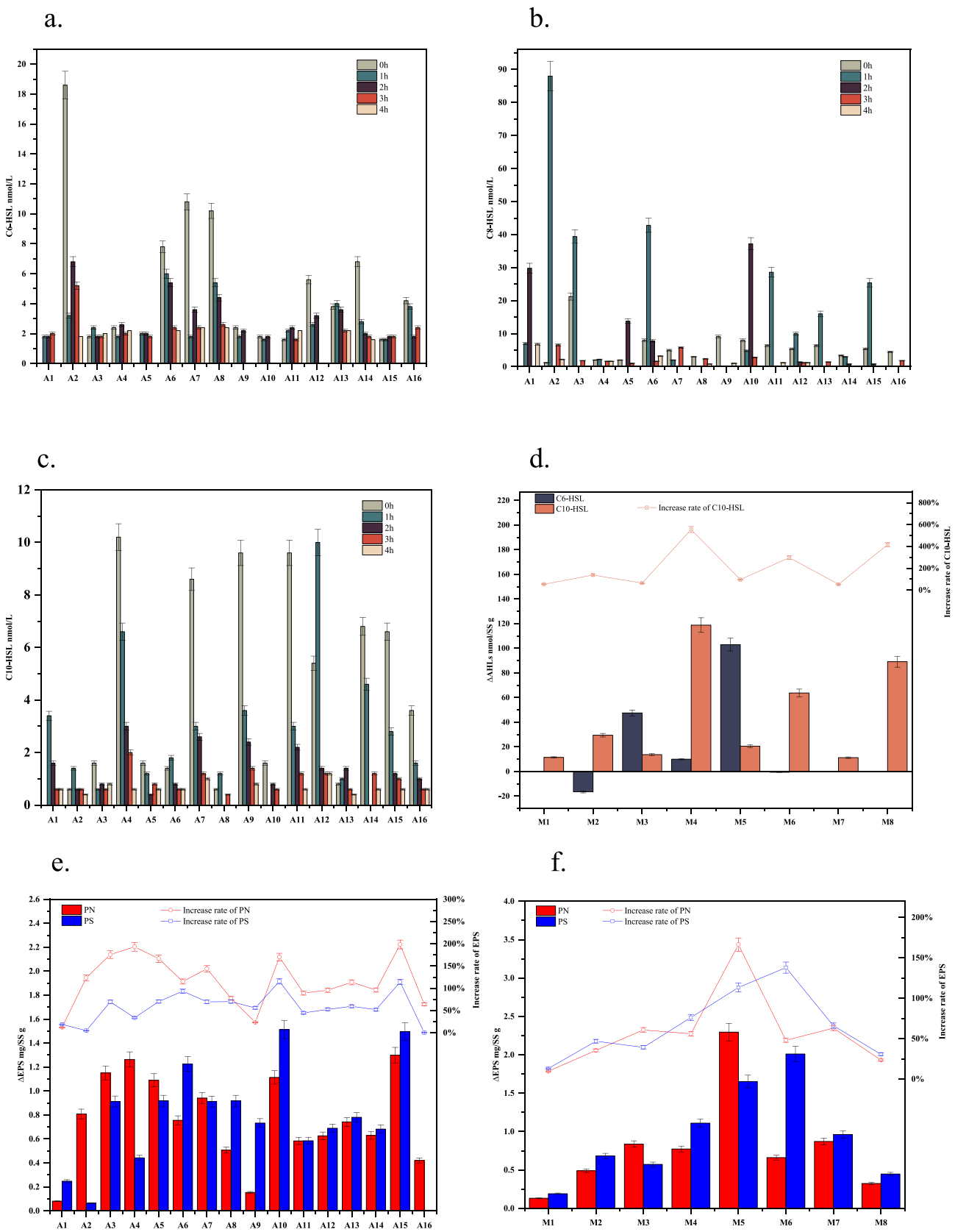

Fig. 1. AHLs concentration of aqueous phase and ΔEPS in activity tests (a: C6-HSL in A1-A16 b: C8-HSL in A1-A16 c: C10-HSL in A1-A16 d: C6-HSL & C10-HSL in M1-M8 e: ΔEPS in A1-A16 f: ΔEPS in M1-M8).

Fig. 2. The changes of ICD, ICDHc and ATP (a: ΔICD in A1-A16 b: ΔICD in M1-M8 c: ΔICDHc in A1-A16 d: ΔICDHc in M1-M8 e: ΔATP in A1-A16 f: ΔATP in M1-M8).

community data also support these findings. Therefore, it proposes the mechanisms for the c-di-GMP and AI-2 to the A15 systems for regulating EPS secretion as follows (Fig. 4). The c-di-GMP can diffuse into cells and activate the synthesis of C6-HSL, enhancing C8-HSL to improve ICDHc. The TCA cycle is then activated to consume acetyl-CoA, generating excess ATP to synthesize PN. Conversely, cells respond to AI-2 stimulation to excrete C10-HSL, which enhances ICD to activate the GCA cycle to synthesize fat acid, stimulating PS secretion. This mechanism can realize a novel way to synthesize PN- or PS-rich AGS from fresh or stored AGS.

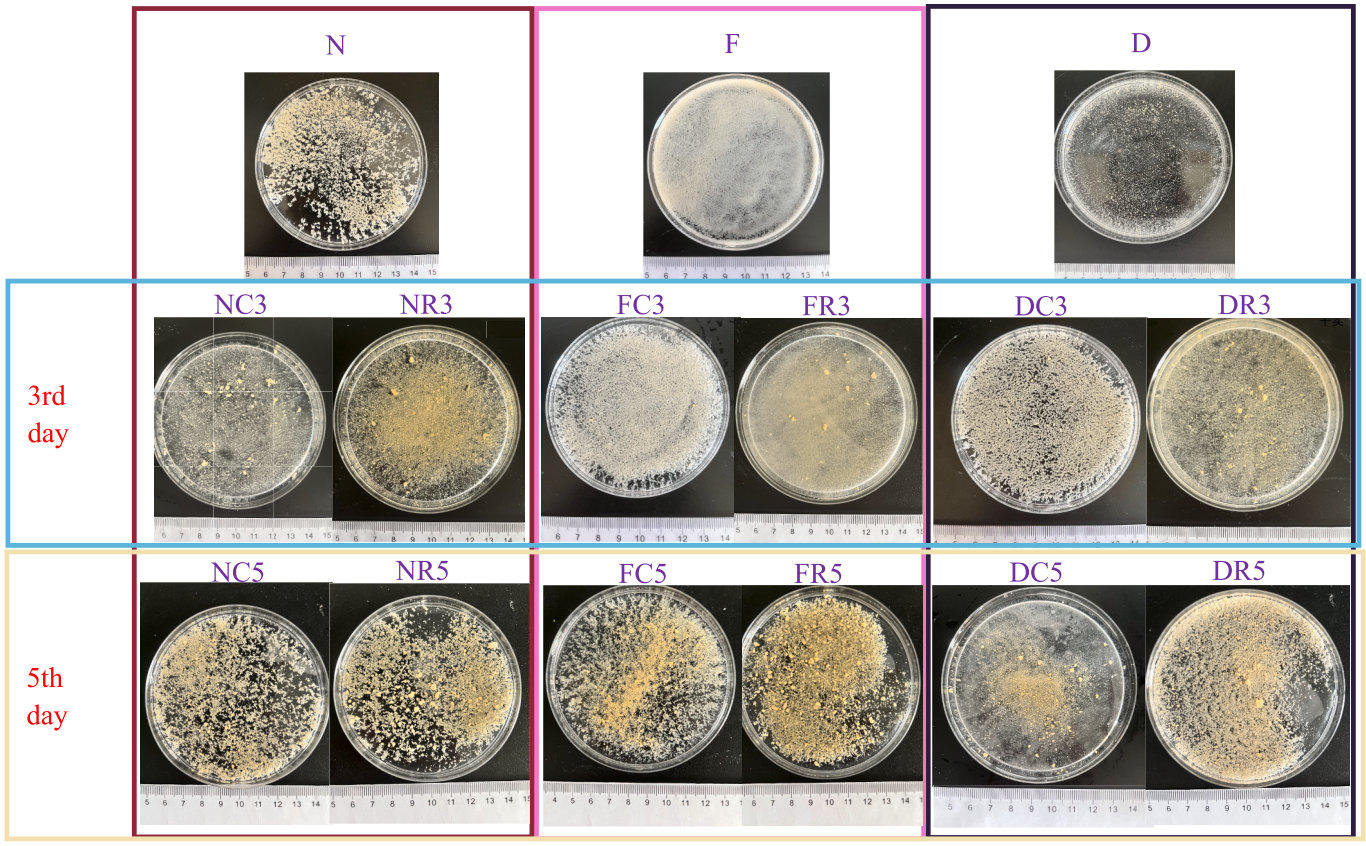

Fig. 3. The recovery observation of stored AGS.

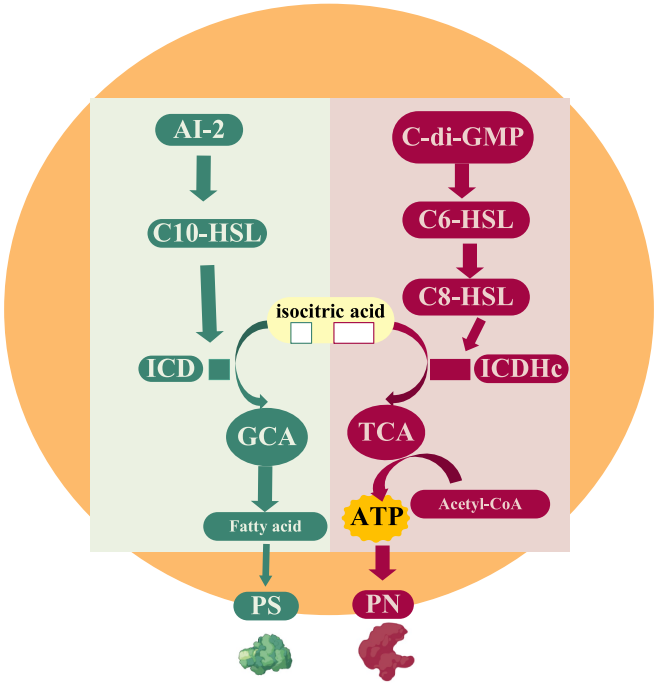

Fig. 4. The mechanisms for yielding PN-rich or PS-rich.

4. Conclusions

This study concludes the following. 20 nmol/L AHLs, including C8-HSL, C10-HSL, and C12-HSL (1:1:1 w/w/w), inducts efficient QS. 80 nmol/L c-di-GMP and 20 nmol/L AHLs induce the highest QS efficiency. With A15 AHLs, the ci-di-GMP promotes PN production, and the AI-2 promotes PS production, yielding a selective cultivation strategy for AGS. The dried-stored AGS could be recovered effectively to produce the PN-rich granules using the c-di-GMP and AHL addition.

CRediT authorship contribution statement

Yi Lv: Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Xin Huang:** Investigation, Formal analysis, Data curation. **Duu-Jong Lee:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DJL is the editorial board member of this journal.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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