Development of a reversible regulatory system for gene expression in the cyanobacterium *Synechocystis* sp. PCC 6803 by quorum-sensing molecules *N*-Acyl-homoserine lactone (AHLs)

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Development of a reversible regulatory system for gene expression in the cyanobacterium *Synechocystis* sp. PCC 6803 by quorum-sensing molecules *N*-Acyl-homoserine lactones (AHLs)

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Abbreviations

AHLs: *N*-Acyl homoserine lactones

AP: Alkaline phosphatase

ANOVA: Analysis of variance

CAPS: *N*-cyclohexyl-3-aminopropanesulfonic acid

HSL: Homoserine lactone

HK: Histidine kinase

IPTG: Isopropyl β-D-1-thiogalactopyranoside

LB: Lysogeny broth

MES: 2-(N-morpholino) ethanesulfonic acid

OD: Optical density

PCR: Polymerase chain reaction

PNP: *p*-nitrophenol

PNPP: *p*-nitrophenyl phosphate

QS: Quorum sensing

QQ: Quorum quenching

RR: Response regulator

TM: Transmembrane

Abstract

The two-component system, involving a histidine kinase and a response regulator, is a common mechanism in microorganisms to sense and adapt to environmental changes. In response to a cognate environmental stimulus, response regulator proteins can alter the gene expression. The transcriptional regulation in microalgae is an important approach to control the response and production of useful target compounds. The transcriptional regulation in microalgae is important to control the response and production of useful target compounds. However, in most cases, induction of gene expression by heavy metal ions or chemical inducers makes it difficult to remove them from the medium, and transcription is limited to only once per culture period. Hence, the development of a reversible gene regulatory system is important to produce useful, targeted compounds without affecting the cellular growth profiles. Therefore, in this study development of a reversible gene regulation system was focused by fusion of quorum-sensing (QS) sensors, LuxN and VanN obtained from *Vibrio harveyi* and *Vibrio anguillarum*, respectively, with the kinase domain of SphS, a phosphate-deficiency sensor from the cyanobacterium *Synechocystis* sp. PCC 6803. The response of developed chimeric sensors, LuxN_SphS and VanN_SphS, after expressing in S*ynechocystis* cells to the application of various *N*-acyl-homoserine lactones (AHLs) was assessed by measuring the alkaline phosphatase (AP) activity. The addition of OHC6-HSL resulted in repression of AP activity in VanN_SphS chimeric sensor, while the introduction of AHL-degradation enzyme, Aii20J from a marine bacterium, degraded the OHC6-HSL, and AP was recovered to its original level. The use of AHLs for transcriptional regulation in *Synechocystis* sp. PCC6803 was demonstrated for the first time in this study, which can be utilized to produce useful, targeted compounds in the cyanobacterium in the future.

Keywords: Chimeric sensor, Quorum sensing, Sensory domains, VanN, LuxN, Histidine kinase, SphS, gene regulation, AHLs

General Introduction

Cyanobacteria are model single-cell microorganisms to study photosynthetic processes. Their capability to produce valuable chemical products directly from carbon dioxide (CO_2) makes them ideal hosts for industrial production of these value-added products (Abed et al., 2009; Angermayr et al. 2014; Kobayashi et al. 2019). Various synthetic biological techniques have been utilized to regulate gene expression and control the production of chemicals in cyanobacteria (Deng and Coleman 1999; Atsumi et al. 2009; Dexter and Fu 2009; Oliver et al. 2013; Savakis et al. 2013; Osanai et al. 2015). The conventional approaches have been previously applied in cyanobacteria to regulate gene expression using chemical inducers including IPTG, theophylline, anhydrotetracycline, toluene, heavy metal ions (Geerts et al. 1995; Huang et al. 2010; Guerrero et al. 2012; Nakahira et al. 2013; Ma et al. 2014; Maeda et al. 2014; Inaba et al. 2018). But the limitations with such chemical induction are difficulty removing these inducers from the culture medium, and regulation can be achieved only once per culture period. Hence the development of a toggle switch system for gene regulation is required to effectively regulate the production of value-added compounds and to regulate gene expression more robustly and cost-effectively.

The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) has been used to regulate gene expression by the application of two-component system based chimeric sensors and exogenous physical signals (Shimura et al. 2012; Kotajima et al. 2014; Inaba et al. 2018). This study is also based on the utilization of chimeric sensors and exogenous molecules for gene regulation in *Synechocystis*. For the development of chimeric sensors, the transmembrane (TM) region of quorum sensing (QS) sensory domains (LuxN and VanN) was used as signal input domains, and for the kinase domain SphS, a phosphate-deficiency sensor from *Synechocystis* was used. The exogenous application of QS-signaling

molecules, *N*-acyl-homoserine lactones (AHLs), was carried out to regulate the gene expression for the alkaline phosphatase (AP) activity. This study involves the development of a toggle gene switch by the addition of AHLs and then their subsequent degradation through quorum quenching (QQ) enzyme.

The bacteria can communicate to regulate several physiological processes through extracellular signaling molecules, autoinducers and this phenomenon is known as quorum sensing. Among different signaling molecules or autoinducers, *N*-acyl-homoserine lactones (AHLs) are the most common and well-studied autoinducers used by bacteria (Fuqua et al. 1994; Fuqua et al. 2001; Bassler and Losick 2006). The AHL-based QS processes to regulate gene expression is commonly observed among *Vibrio* species. The QS-sensory kinases used in this study, LuxN and VanN were obtained from different *Vibrio* species, and despite their structural similarities, both the sensory kinases can respond to different AHLs (Freeman et al. 2000; Milton et al. 2001; Henke and Bassler 2004; Buchholtz et al. 2006; Timmen et al. 2006; Jung et al. 2007; Bonneau 2008).

The chimeric sensors LuxN_SphS and VanN_Sphs were constructed by fusing the transmembrane (TM) regions of LuxN and VanN, respectively, with the C-terminal of SphS from *Synechocystis*. The effect of various AHLs along with the cognate AHLs of LuxN and VanN were evaluated by measuring the AP activity. The administered AHLs in the culture of chimeric sensors may stay for a longer period of time as the *Synechocystis* genome does not have any enzymes or other built mechanisms to degrade the AHLs. Therefore, the quorum quenching (QQ) phenomenon was applied in this study. QQ is a process of enzymatic degradation of AHLs and enzymatic class, AHL-lactonase provides broader substrate specificity and effective degradation of both short and long-chain AHLs (Dong et al. 2001; Ulrich 2004; Mayer et al. 2015; Romero et al. 2015; Huang et al. 2016).

Ultimately, this study was designed to develop a toggle gene switch system in *Synechocystis* sp. PCC6803, where the effect of QS-signaling molecules, AHLs was evaluated on the chimeric sensors, LuxN_SphS and VanN_SphS, by measuring the AP activity, and the degradation of these applied AHLs was achieved by the introduction of a QQ enzyme.

Construction of a toggle gene switch (OFF/ON) system using chimeric sensors in *Synechocystis* sp. PCC6803

1. Introduction

A two-component signal transduction system (TCS) composed of an N-terminal histidine kinase (HK) and a C-terminal response regulator (RR) protein can detect and respond to specific stimuli in the microorganism. The perception of an extracellular stimulus by the signal input domain of HK autophosphorylates the conserved histidine residue, and the phosphate group is transferred to the RR domain (Grimshaw et al. 1998; Stock et al. 2000; Bretl et al. 2011; Zschiedrich et al. 2016). TCS presents an essential role in controlling many physiological activities in different microorganisms, including antibiotic-resistance, virulence, competence, sporulation, bacterial survival, and utilization of essential elements like phosphate, nitrogen, and carbon (Barrett et al. 1998; Barrett and Hoch 1998; Krell et al. 2010). Different TCSSs has been characterized in bacteria to regulate gene expression for various physiological processes such as the CheA-CheY system responsible for chemotaxis in *Escherichia coli* and *Bacillus subtilis* (Kirby 2009), the EnvZ-OmpR, to respond to osmotic stress in *E. coli* (Egger and Inouye 1997), and the PhoQ-PhoP to respond to magnesium concentrations in *Salmonella enterica* (Kato and Groisman 2008).

Chimeric HKs has been developed in various bacteria to regulate the gene expression for certain genes in response to a specific stimulus, including chimeric sensor DcuSZ to detect fumarate in *E. coli* (Ganesh et al. 2013), Tar-EnvZ, in *E. coli* to regulate expression of enzymes using aspartate as a stimulus (Yoshida et al. 2007), and using light as a stimulus for the regulation of phycocyanobilin production by developing chimeric HK sensor in *E. coli* (Sugie et al. 2016; Hori et al. 2017) has been demonstrated.

The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) is a model unicellular photoautotrophic organism that utilizes TCSSs to respond and regulate its gene expression by detecting environmental stimuli (Hoch 2000; Alves and Savageau 2003;

Marin et al. 2003; Hsiao et al. 2004; Suzuki et al. 2004). *Synechocystis* can adapt to phosphate limiting environments by using TCSSs composed of HK SphS, a phosphate-deficiency sensor, and its cognate RR, SphR (Aiba et al. 1993; Nagaya et al. 1994; Hirani et al. 2001; Kimura et al. 2009) by regulating the pho regulon expression. *PhoA* gene expresses the alkaline phosphatase (AP) activity under phosphate starvation (Ray et al. 1991; Aiba et al. 1993; Hirani et al. 2001), resulting in the synthesis of periplasmic or extracellular phosphates to increase phosphate uptake by the cells (Grillo and Gibson 1979; Moore et al. 2005). For the development of chimeric sensors in cyanobacterium *Synechocystis*, HK SphS has been fused with various signal-input domains, and induction of gene expression via exogenous stimuli was studied, like regulation of AP gene expression via a chimeric sensor, TodS_SphS by the application of chemical, toluene (Inaba et al. 2018), Hik33_SphS for the regulation of *phoA* gene (Shimura et al. 2012) using various exogenous stimulus and measurement of AP activity using Cl^- as a stimulus for the chimeric sensor Hik2_Hik7 (Kotajima et al. 2014).

The bacterial communication process to regulate gene expression and diverse physiological functions based on the accumulation of extracellular signaling molecules is known as Quorum sensing (QS). These signaling molecules are denoted as autoinducers or Acyl-homoserine lactones (AHLs) or homoserine lactones (HSLs), and they can freely diffuse across the cell membrane. Bacterial population density is directly proportional to the autoinducer accumulation (Fuqua et al. 1994; Fuqua et al. 2001; Bassler and Losick 2006). Under the suitable cell numbers amount and autoinducers accumulation density, bacteria can alter their gene expression and control numerous physiological phenomena, including biofilm formation, bioluminescence, production of extracellular polymeric substance (EPS), and virulence (Kaplan and Greenberg 1985; Pearson et al. 1999; Galloway et al. 2011). QS is a common phenomenon in both Gram-positive and Gram-negative bacteria. Gram-

positive bacteria use oligopeptides to control gene expression through QS, while in the case of Gram-negative bacteria, acyl-homoserine lactones (AHLs) are the most common autoinducers that can easily diffuse through the bacterial cell membrane (Taga and Bassler 2003; Waters and Bassler 2005; Papenfort and Bassler 2016). The process of inhibition of these AHLs by enzymatic degradation is known as quorum quenching (QQ), and the most common enzyme to degrade these AHLs are denoted as AHL-lactonases that can degrade several AHLs (Dong et al. 2001; Ulrich 2004; Mayer et al. 2015; Romero et al. 2015; Huang et al. 2016). Among various AHL-lactonases, Aii20J presents a wider range of AHL degradation activity and can degrade various carbon chain lengths, unsubstituted and substituted AHLs effectively (Mayer et al. 2015).

QS-sensor domain LuxN and its homolog VanN from the marine bacteria *Vibrio harveyi* and *Vibrio anguillarum* respectively show similarity in their structures comprising nine transmembranes (TM) regions by each (Bonneau 2008). but still, they can detect specific AHL molecules to regulate gene expression. The cognate signal molecule for LuxN is *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL) (Freeman et al. 2000; Henke and Bassler 2004; Timmen et al. 2006; Jung et al. 2007) while VanN can detect *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) (Milton et al. 2001; Buchholtz et al. 2006). These QS-sensor LuxN and VanN were fused with the HK SphS from the *Synechocystis* to construct two chimeric sensors LuxN_SphS and VanN_SphS, in the present study. The developed chimeric sensors were evaluated against various AHLs along with the cognate signaling molecules of these sensory domains, and the regulation of expression of the AP activity was measured. Additionally, to degrade these AHLs via a QQ enzyme, an additional construct of the Aii20J enzyme was introduced to develop a reversible gene regulation system in *Synechocystis* sp. PCC6803.

2. Materials and Methods

2.1. Bacterial strains, plasmid construction and growth conditions

The strains, wild-type (WT) *Synechocystis* sp. PCC 6803 (glucose tolerant strain) (Williams 1988), ΔSphS (SphS deleted strain) developed by Kimura et al. (2009), LuxN_SphS, VanN_SphS, and VanN_SphS::Aii20J (*Synechocystis* transformants developed in this study) were grown in BG-11 medium (Stanier et al. 1971). A 20 mM 2-(N-morpholino) ethanesulfonic acid (MES)-NaOH (pH 6.5) buffer was added to the BG-11 medium. The cells were grown by aeration of 1 % (*v/v*) CO_2-enriched air, a continuous illumination (70 µmol photons m^{-2} s^{-1}) at a temperature of 34 °C (Wada and Murata 1989). All the transformants were maintained on solidified BG-11 medium combined with 1.5 % (*w/v*) Bacto agar (Japan Becton Dickinson, Tokyo, Japan). Antibiotics were added at a final concentration of 25 µg mL^{-1} spectinomycin dihydrochloride salt and chloramphenicol as required. Lysogeny broth (LB) with 30 µg mL^{-1} Kanamycin was used to grow the AHL biosensor strain, *Chromobacterium violaceum* CV026, at 30 °C (Romero et al. 2015). Competent cells of *E. coli* JM109 (Takara Bio, Ohtsu, Japan) were used to construct the plasmids. LB medium provided with suitable antibiotics (ampicillin, spectinomycin, and chloramphenicol) at a concentration of 50 µg mL^{-1} was used to screen and maintain these cells harboring the engineered plasmids at 37 °C and shaking at 180 rpm.

2.2. Selection of chimeric histidine kinases (HKs)

The chimeric sensors LuxN_SphS and VanN_SphS were constructed by fusing the QS domains, LuxN and VanN with the kinase domain, SphS. The sensor LuxN was obtained

from the marine bacteria, *V. harveyi* BB120, while its homolog VanN was identified from *V. anguillarum* 90-11-287 to be used as signal input domains. These sensory domains can recognize the QS signaling molecules AHLs or autoinducer, where LuxN recognizes the OHC4-HSL, and VanN detects OHC6-HSL (Buchholtz et al. 2006; Swem et al. 2008). An inorganic phosphate (Pi)-deficient sensory kinase from *Synechocystis* sp. PCC6803, SphS (Hik7, sll0337) was used as the kinase domain in this study. The HK SphS regulates the expression of AP activity under phosphate limiting conditions.

2.3. Development of chimeric sensors

The SphS-expression vector, pSK05ΔPAS, developed by Kimura et al. (2009), was used to integrate the DNA fragments from the signal-input domains of LuxN and VanN by substituting the signal input domain of SphS using In-Fusion® HD cloning Kit (Takara Bio) to develop chimeric sensors, LuxN_SphS and VanN_SphS. The amino acid regions of LuxN (M1-R460) and VanN (M1-C460) were connected to the C-terminal of SphS (G197-P430), respectively. In detail, nearly 1 Kbp upstream and downstream regions of the kinase domain of the SphS gene, spectinomycin resistance gene cassette, and pUC vector were amplified using the expression vector, pSK05ΔPAS (Kimura et al. 2009). Similarly, through polymerase chain reaction (PCR), the DNA fragments for the signal-input domains of LuxN and VanN were amplified using the primers, LuxN_F and LuxN_R, VanN_F and VanN_R (Table 1). Double homologous recombination was used to insert the LuxN_SphS and VanN_SphS genes into the native loci of the *sphS* gene in the *Synechocystis* chromosome via the substitution of the coding sequence of the *sphS* gene (Shimura et al. 2012; Kotajima et al. 2014). This resulted in the expression of the chimeric genes from the native promoter of the *sphS* gene.

The synthetic plasmids, pSK05LuxN::SphS and pSK05VanN::SphS, were transformed into the *Synechocystis* strain ΔSphS. The cells of ΔSphS were grown in the BG-11 medium until the logarithmic growth phase and centrifuged to collect the cell pellet, which was resuspended with fresh BG-11 along with the plasmids, for the overnight incubation under low light with shaking at 30 °C. The transformant colonies showing resistance to spectinomycin were screened through the agar-solidified BG-11 medium containing 5 µg mL^{-1} spectinomycin dihydrochloride salt, later transferred and maintained on BG-11 medium supplemented with a higher concentration of 25 µg mL^{-1}spectinomycin dihydrochloride.

2.4. Expression of quorum quenching (QQ) enzyme (Aii20J)

AHL-lactonase, Aii20J, from the marine bacterium *Tenacibaculum* sp. 20J was introduced in the *Synechocystis* cells harboring the VanN_SphS chimeric sensor to degrade the administered AHLs as the *Synechocystis* genome lacks any system to quench the AHLs. For that purpose, the codon-optimized sequence of Aii20J, obtained by Codon Optimization OnLine (COOL) (https://cool.syncti.org) (Supplementary Fig. 1), and synthesized by the Eurofins Genomics (Tokyo, Japan) was introduced into the vector, pTHCT2031V, which has the homologous sequences for the neutral site, p*Trc* promoter and chloramphenicol resistance gene by PCR (Ishizuka et al. 2006).

The primers βLAC_F and βLAC_R, pTCHT_slr2031_F and pTCHT_slr2031_R were used for the amplification (Table 1). The resultant plasmid pTHCT2031V::Aii20J obtained through In-Fusion was then introduced into *Synechocystis* cells harboring the VanN_SphS chimeric sensor at the neutral site (slr2031) via homologous recombination. The strain was denoted as, VanN_SphS::Aii20J.

2.5. Alkaline phosphate (AP) activity

The rate of degradation of *p*-nitrophenyl phosphate (PNPP) was measured to detect the AP activity in the cells (Aiba et al. 1993). The AP activity in the LuxN_SphS and VanN_SphS strains was measured by inoculating the cells (OD_{730} = 0.2) in 50 mL BG-11 medium. The AP activity was measured from the harvested cells with and without addition of AHLs following time course experiments. Briefly, 200 µL culture was mixed with 700 µL of 285 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS)-NaOH buffer (pH 9.5). Then 100 µL of 36 mM PNPP was added to start the AP activity reaction. At 5 and 20 min intervals in individual tubes, 100 µL of 4 M NaOH was added to inhibit the reaction. The absorbance of the supernatant was measured at 397 nm after centrifugation at 10000 × *g*, for 1 min, and *p*-nitrophenol (µmol mL^{-1}) was measured from the standard curve of *p*-nitrophenol in CAPS-NaOH buffer (pH 9.5). The Chl*a* amount in mg mL^{-1} was measured following the methodology described by Tandeau de Marsac and Houmard (1988).

2.6. Alkaline Phosphatase (AP) activity using cognate signaling molecules

The response of newly developed chimeric sensors, LuxN_SphS and VanN_SphS, was evaluated by the application of cognate signaling molecules of LuxN and VanN. The LuxN sensor can respond to *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL), and VanN can respond to *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL). The AP activity was measured for 24 h using WT *Synechocystis* and ΔSphS strains as control cultures.

2.7. Effect of various Acyl homoserine lactones (AHLs)

The chimeric sensors developed in this study were evaluated against various unsubstituted and substituted AHLs, which were purchased from Sigma Aldrich, Japan. The unsubstituted-AHLs comprised of *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-hexanoyl-DL-homoserine lactone (C6-HSL racemic), *N*-dodecanoyl-L-homoserine lactone (C12-HSL) whereas, substituted-AHLs were *N*-(*β*-ketocaproyl)-L-homoserine lactone (OC6-HSL), *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL), and *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) (Fig. 3A). The AHLs were diluted to acetonitrile to be used as 10 µM working concentrations.

2.8. Effect of different concentrations of OHC6-HSL on VanN_SphS

The effect of various concentrations of OHC6-HSL was assessed on the VanN_SphS cultures. For that purpose, 0 µM, 0.1 µM, 1 µM, 2.5 µM, 5 µM, and 10 µM concentrations were applied in VanN_SphS cultures, and AP activity was measured until 24 h.

2.9. CV026 plate bioassay

C. violaceum strain CV026 was used for the AHL-degradation bioassay (Romero et al. 2010) using the *N*-hexanoyl-L-homoserine lactone (C6-HSL) added in the cultures of VanN_SphS and VanN_SphS::Aii20J as CV026 can detect only C6-HSL but not OHC6-HSL (McClean et al. 1997). The C6-HSL was added to the cultures of VanN_SphS and VanN_SphS::Aii20J to a final concentration of 10 µM. The samples were taken until 48 h of cultivation, and cultures were centrifuged at 5000 × g for 3 min, and the presence of OHC6-HSL in the

supernatant was evaluated by the *C. violaceum*-based plate bioassay. In detail, 1 mL concentrated overnight culture of CV026 mixed with 4–5 mL of liquid soft LB (0.8 % (*w/v*) (Bacto-agar) was added to the LB plates to obtain a homogenous layer and after solidification wells were created on that with the help of a sterilized glass tube (ϕ = 6 mm). The supernatants (100 µL) of VanN_SphS and VanN_SphS::Aii20J containing C6-HSL were added to these wells, and production of the Violacein (purple halo) by C6-HSL was observed. The size of these purple halos was used to measure the AHL concentration in comparison to the control concentration of OHC6-HSL.

2.10. Special growth technique for the AP recovery

To achieve the AP recovery, a special growth technique was applied to keep the cultures optical density constant (OD_{730} = 1), as continuous cultivation over time results in dense cultures, and the light become a limiting factor for the cells, a phenomenon also observed by Asada et al. (2019). The following formula was used to achieve this unique growth technique in such a manner that cultures optical density remained constant to 1, and OHC6-HSL concentration did not get affected throughout the experiment.

$$\mathbf{Y = \left(a - \frac{a}{n}\right) x V}$$

Where,

Y = the specific amount of culture (mL) to be removed for centrifugation

a = the required optical density (OD_{730} = 1)

n = the difference in optical density every 24 h

V = the volume of the culture (mL) remaining in the tubes after every 24 h of aeration

Following the above formula, the specific volume of culture was subjected to centrifugation at 5000 × *g* for 3 min. The supernatant was decanted to the stock culture tubes

in order to maintain the same concentration of OHC6-AHL and OD_{730} to 1, while the resulting cell pellet was used for estimation of AP activity after resuspending with an equal amount of new BG-11 medium as acquired from the above calculation.

2.11. Extraction of total RNA

The total RNA from the VanN_SphS cultures supplemented with (10 µM) and no OHC6-HSL was extracted using Invitrogen TRIzol Max Bacterial RNA Isolation Kit (Thermo Fisher Scientific, Tokyo, Japan) and purified by RNeasy Mini Kit (Qiagen, Hilden, Germany), respectively (Kotajima et al. 2014). To fix the cells, equal volume (50 mL) of cultures and ice-cold 10 % phenol/ethanol (*v/v*) were mixed and centrifuged (2800 × *g* for 5 min at 4 °C) to discard the supernatant. In the cell pellet, 0.5 g zirconia beads (0.1 mm in diameter) and 1 mL TRIzol was added and vortexed using MINI-BEADBEATER™ (BioSpec Products, Bartlesville, OK) for 5 min. The samples were supplemented with 200 µL iced chloroform after robust mixing by shaking the samples were kept at room temperature for 5 min. After centrifuging (15000 × *g* for 20 min at 4 °C), 400 µL of the upper aqueous phase after shifting to a new tube was mixed with 300 µL of 100 % Ethanol, and RNA was purified according to the user's manual of RNeasy Mini Kit.

2.12. Expression of phoA transcripts by real-time PCR analysis

The PrimeScript RT reagent Kit with a genomic DNA (gDNA) Eraser (Perfect Real Time) (Takara Bio) was used to produce complementary DNA (cDNA) from the1 µg purified total RNAs. The Real-time PCR was done using GOTaq qPCR Master Mix (Promega, Madison, WI) on a PikoReal 96 Real-Time PCR system (Thermo Fisher Scientific).

The primers set PhoA_RT_F (5'-CAGTGGCTTTGCCTTCAGTTT-3'), and PhoA_RT_R (5'-ACCATTACGCACAACAACATCC-3') were used to measure the expression levels of the *phoA* gene. The reference gene *rnp*B was amplified by the primers, rpnB_RT_F (5'-GTAAGAGCGCACCAGCAGTATC-3') and rpnB_RT_F(5'-CAAGCGGTTCCACCAATC-3') and *rnp*B. The threshold cycles (C_T) values were calculated using PikoReal software ver. 2.2 (Thermo Fisher Scientific) and relative expression were measured $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

2.13. Data analysis

All the data were statistically analyzed by using the statistical software R (version 3.6.1) (R Development Core Team 2011). Two-way variance (ANOVA) with interaction was performed, and post-hoc analysis was done using Holm's sequential Bonferroni correction (Holm 1979). A *p*-value ($p \leq 0.05$) was considered statistically significant.

3. Results and Discussion

3.1. Construction of chimeric sensors

The QS-sensory kinases LuxN and VanN were obtained from the marine bacteria, V. harveyi BB120 and V. anguillarum 90-11-287, respectively. Both the sensory kinases show structural similarities comprising nine TM helices (Bonneau 2008; Milton et al. 2001). To develop the chimeric sensors LuxN_SphS and VanN_SphS the TM regions from the sensory kinases LuxN and VanN were fused with the C-terminal of HK, SphS obtained from Synechocystis. SphS comprises of a TM and PAS domain and regulates the expression of AP under phosphate limiting environments. The TM regions of LuxN (M1-R460) and VanN (M1-C460), were combined with the kinase domain of SphS (G197-P430) and resulting chimeric sensors were donated as LuxN_SphS and VanN_SphS, respectively (Fig. 1). The chimeric genes were the introduced into the Synechocystis chromosome by double homologous recombination. The insertion was confirmed through sequencing and transformant strains were analyzed by the application of AHLs and measurement of AP assay.

3.2. Alkaline Phosphatase (AP) activity using cognate signaling molecules

The QS sensory domains LuxN and VanN detect and respond to *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL) and *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL), respectively (Freeman et al. 2000; Henke and Bassler, 2004; Milton et al. 2001). Therefore, the response of chimeric sensors LuxN_SphS and VanN_SphS was evaluated against these two cognate signaling molecules. The chimeric sensors LuxN_SphS

and VanN_SphS along with wild-type (WT) *Synechosystis* PCC 6803 and ΔSphS cells (as controls) were assessed against exogenously applied AHLs by measuring the AP assay. A final concentration of 10 µM for both the AHLs was applied in aeration culture tubes of all the strains. After 24 hours of exposure of the cells to these two cognate AHLs molecules, AP activity was measured.

The cells showed a clear decrease in the AP activity as compared to the cells without any addition of AHLs. In the case of the cells with no addition of AHLs, AP activity reached a value of 1.23 ± 0.16 while, for the cells added with OHC4-HSL and OHC6-HSL, the AP amount was 1.00 ± 0.03 and 0.35 ± 0.02 respectively, in the case of VanN_SphS strain, after 24 h. Moreover, the response to the applied AHLs was more apparent in the case of the chimeric sensor VanN_SphS, specifically responding to its cognate signaling molecule OHC6-HSL and presented a significant decrease in AP activity until 24 h by the addition of this AHL. However, for WT, ΔSphS, and chimeric sensor LuxN_SphS, the alkaline phosphatase activity was almost negligible (Fig. 2). After this result, it was speculated that VanN_SphS may function as a negative regulator and may suppress the AP activity after the addition of exogenous AHLs. Hence, the response of these two chimeric sensors LuxN_SphS and VanN_SphS were assessed against various other AHLs along with their cognate signaling molecules following time-course experiments.

3.3. Effect of different carbon chain AHLs on the chimeric sensors

To investigate the effect of different carbon length AHLs on the chimeric sensors, the AP activity was measured with and without the addition of various AHLs and evaluated the response of LuxN_SphS and VanN_SphS to them (Fig. 3). The AHLs used in these experiments were *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-hexanoyl-DL-

homoserine lactone (C6-HSL racemic), *N*-dodecanoyl-L-homoserine lactone (C12-HSL), *N*-(*β*-ketocaproyl)-L-homoserine lactone (OC6-HSL), *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL), and *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) (Fig. 3A).

Firstly, the AP assays were conducted by using the unsubstituted-AHLs, including C6-HSL, C6-HSL racemic, and C12-HSL for LuxN_SphS and VanN_SphS strains till 2 days (Fig. 3a, b, c). The chimeric sensor LuxN_SphS did not respond to these AHLs. In the case of VanN_SphS, it responded to the applied unsubstituted-AHLs, and a decrease in the AP activity was observed by the addition of these AHLs as compared to no addition, but the difference was not significant (Fig. 3a, b, c). Similarly, in the case of the substituted-AHLs, OC6-HSL, OHC4-HSL, and OHC6-HSL, LuxN_SphS could not respond to any of them, including the cognate signaling molecule (OHC4-HSL) for LuxN, while VanN_SphS did respond to these AHLs. Still, again the difference between addition and no addition of these AHLs was not significant (Fig. 3d, e), except for its cognate signaling molecule (OHC6-HSL) (Fig. 3f), where the addition of this AHL molecule significantly decreased the AP activity reaching to zero in VanN_SphS cultures as compared to without addition of this AHL till 2 d. The AP activity in VanN_SphS cultures with no addition of OHC6-HSL was 1.20 ± 0.215 (μmol PNP mg^{-1}Chla min^{-1}) and showed a significant decrease in AP activity up to 0.08 ± 0.009 (μmol PNP mg^{-1}Chla min^{-1}) in cells added with OHC6-HSL until 2 d (Fig. 3f). Statistical analysis applying two-way ANOVA suggested significant differences for the application of OHC6-HSL ($p = 0.00$), application time ($p = 0.00$), and for the interaction effect of OHC6-HSL × time in the VanN_SphS cultures. Additionally, post-hoc analysis by Holm's sequential Bonferroni correction (Holm 1979) showed AP activity was significantly decreased ($p = 0.00$) by the application of OHC6-HSL in VanN_SphS cultures compared to no addition of this molecule (Fig. 3f).

In summary, LuxN_SphS did not respond to any of the applied AHLs along with OHC4-HSL, which is the cognate signaling molecule for the LuxN sensor (Cao and Meighen 1989) whereas, VanN_SphS responded to the OHC6-HSL, which is the cognate signal for VanN sensor (Milton et al. 2001; Buchholtz et al. 2006). The AP activity was significantly suppressed by the addition of OHC6-HSL in VanN_SphS cultures whereas, Inaba et al. (2018) found higher AP activity by the exogenous application of toluene (gaseous stimuli) in their developed chimeric sensor, TodSS_SphS in *Synechocystis* sp. PCC6803.

The possible reason for the chimeric sensor, VanN_SphS, to be a negative regulator of AP activity by the addition of OHC6-HSL and another chimeric sensor, LuxN_SphS, not to be functional even for its own cognate signaling molecule, OHC4-HSL, can be the alteration in the linker region, a short region that connects the HK to the RR domain, as previously described by Möglich et al. (2009) and Bhate et al. (2015). The rotation of the linker region connected to the HK activates the autophosphorylation of the His residue. The different transformants developed by altering the linker region in *Synechocystis* sp. PCC6803 either did not respond to the light signal for gene regulation or resulted in a reverse response for the gene expression to the light signals as compared to the wild-type cells (Nakajima et al. 2016). These results are in line with the response of LuxN_SphS, which did not induce AP activity by the cognate molecule and VanN_SphS responded by the repression of expression of AP activity.

3.4. Effect of different concentrations of OHC6-HSL on VanN_SphS

The cultures of VanN_SphS were subjected to different concentrations of OHC6-HSL till 24 h in order to evaluate the effect of concentrations lower than 10 µM to suppress the AP activity. The concentrations used for this experiment were 0, 0.1, 1, 2.5, 5, and 10 µM where

0 µM (No OHC6-AHL) and 10 µM concentrations were used as negative and positive controls, respectively. The aeration cultures of VanN_SphS were supplemented with these different concentrations of OHC6-HSL after 12 h of culture, and then the changes in the alkaline phosphatase activity were observed at every 4 h intervals till 24 h. In this trial, the only concentration that significantly reduced the AP activity to 0.38 ± 0.002 (µmol PNP mg^{-1}Chla min^{-1}) after 24 h of culture was 10 µM (Fig. 4). At the same time, all other concentrations could not repress the AP activity significantly. Hence, after this experiment, it was verified that 10 µM is the only concentration of OHC6-HSL that may significantly respond to the VanN_SphS transformants and AHL concentrations lower than that are not so effective in suppressing AP activity. So, in further experiments, only 10 µM concentration of OHC6-AHL was used.

3.5. Recovery of AP activity

The addition of OHC6-HSL in the cultures of chimeric sensor VanN_SphS showed repression of AP activity with time. As *Synechocystis* has no built-in system to degrade the AHLs so this repression of AP activity in VanN_SphS cells by the addition of OHC6-AHL may continue for a long time until the added OHC6-AHL is degraded through some system. Therefore, in the *Synechocystis* cells harboring the VanN_SphS chimeric sensor, an additional construct expressing Aii20J was introduced and denoted as VanN_SphS::Aii20J. Aii20J is a lactonase enzyme from the marine bacterium, *Tenacibaculum sp*. 20J, which can degrade a wide range of AHLs, including OHC6-AHL (Mayer et al. 2015). The OHC6-HSL was added to the VanN_SphS and VanN_SphS::Aii20J cultures at a final concentration of 10 µM. The VanN_SphS culture was used as a control in this experiment. The AP assay was performed till 5.5 d. The OHC6-HSL addition resulted in the decline of AP activity in both

the strains till 2 d with a value of 0.045 ± 0.002 and 0.049 ± 0.004 (µmol PNP mg^{-1}Chla min^{-1}) for VanN_SphS and VanN_SphS::Aii20J respectively. Although the after 2 d, the VanN_SphS::Aii20J strain, showed initiation of AP recovery, the rate of recovery was rather low to reach to initial AP level till 5.5 d with a value of 0.0975 ± 0.009 (Supplementary Fig. 2). In previous studies, it has been demonstrated that in cyanobacteria, sufficient light supply is important for the induction of gene expression (Mironov et al. 2012), and insufficient light supply in dense cultures results in repression of AP activity in *Synechosystis* (Asada et al. 2019). These findings lead to the postulation that limited light intensity inhibited the full recovery of AP activity in the case of VanN_SphS::Aii20J cultures due to increased culture density with time. Hence, a special growth technique was applied to prevent light from being a limiting factor in the cultures.

3.6. Unique growth technique for the AP recovery

The unique procedure to grow the cultures of VanN_SphS and VanN_Aii20J was applied to keep the cell density constant (OD_{730} = 1) in such a way that the concentration of applied OHC6-HSL (10 µM) in the cultures does not get affected (Supplementary Fig. 3 a, b). By using the formula and procedure described in materials and methods, a specific culture volume was withdrawn and centrifuged. The supernatant was returned back to the stock tubes of both strains whereas, and the cell pellet was resuspended with fresh BG-11and used for the measurement of AP activity (Supplementary Fig. 3 a, b).

At 0 d (before the addition of OHC6-HSL), both the strains VanN_SphS and VanN_SphS::Aii20J showed nearly an equal amount of AP activity (0.67 µmol PNP mg^{-1}Chla min^{-1}). After addition of OHC6-HSL in the cultures, until 2 d both strains showed a decrease in AP activity (0.09 ± 0.002 for VanN_SphS and 0.10 ± 0.023 for VanN_SphS::

Aii20J). For the VanN_SphS::Aii20J cultures, an initiation of AP recovery was started after 2 d, and a full recovery was achieved until 7 d, with a value of 0.69 ± 0.09 (µmol PNP mg^{-1}Chla min^{-1}) (Fig. 5). However, the VanN_SphS (control culture) did not show any recovery and its AP activity value was 0.15 ± 0.01 (µmol PNP mg^{-1}Chla min^{-1}) until 7 d. A significant difference (p = 0.00) was observed for the strain VanN_SphS::Aii20J and OHC6-AHL application time by two. The interaction effect (strain × time) was also significant with a value p = 0.00 as compared to the control cultures (Fig. 5). Significant effect (p = 0.00) for the VanN_SphS::Aii20J in degrading the OHC6-HSL and to recover the AP activity in comparison to the control strain of VanN_SphS was also observed by post-hoc analysis (Fig. 5). Hence, the introduction of the QQ enzyme, Aii20J, effectively degraded the OHC6-HSL, and AP activity was restored to its initial level gradually.

Previously, effective degradation of different short and long-chain AHLs by AHL-lactonases has been studied. Different AHL-lactonases, including MomL (Tang et al. 2015), HqiA (Torres et al. 2017), Aii810 (Fan et al. 2017), and Aii20J (Mayer et al. 2015), showed successful degradation of various short and long-chain AHLs like C4-HSL, OC6-HSL, C8-HSL, OC12-HSL, C10-HSL, and OC10-HSL.

3.7. AHL-degradation assay

To validate the degradation of the AHL, *C. violaceum* strain CV026 based plate bioassays were performed. For that purpose, C6-HSL (10 µM) was used instead of OHC6-HSL, which is the cognate signaling molecule of sensory kinase VanN and also functional for chimeric sensor VanN_SphS. However, the strain CV026 can detect only C6-HSL but not OHC6-HSL (Romero et al. 2010). The C6-HSL was added to the cultures of VanN_SphS and VanN_SphS::Aii20J to a final concentration of 10 µM and bioassay was done up to 48 h.

The plate bioassay was performed using the supernatants of VanN_SphS and VanN_SphS::Aii20J.

The strain VanN_SphS showed a gradual decrease in the concentration of C6-HSL and after 6 h the AHL concentration was decreased to 8.26 µM. The concentration of C6-HSL reached to half (5.22 µM) till 48 h in the supernatants of VanN_SphS (Table 2).

However, VanN_SphS::Aii20J expressing the QQ enzyme, Aii20J showed a much faster degradation of C6-HSL by reducing its concentration 26 to half (4.86 µM) in 24 h, and complete degradation of 10 µM concentration of C6-HSL occurred till 48 h (Table 2). This indicated that the introduction of QQ enzyme, Aii20J in our system could successfully degrade the AHL and resulted in the AP recovery in case of VanN_SphS::Aii20J. Previously, the enzyme Aii20J has been demonstrated to degrade various short and long-chain AHLs actively compared to other AHL-lactonases (Romero et al. 2014; Mayer et al. 2015).

3.8. Relative expression of the phoA mRNA by real-time PCR

The real-time PCR was performed to measure the relative expression of the *phoA* mRNA in the cultures of VanN_SphS by application (+OHC6-HSL) and without (-OHC6-HSL) application of it (control samples) until 60 min. The relative expression of *phoA* decreased with time in the VanN_SphS cultures (+OHC6-HSL) and reached a value of 0.14 ± 0.15 as compared to the control (-OHC6-HSL), which has relative expression of 1.27 ± 0.28 until 60 min (Fig. 6). A highly significant effect ($p = 0.00$) was observed for time × AHL and OHC6-HSL application by two-way ANOVA, while Bonferroni correction suggested a significant effect ($p = 0.004$) of OHC6-HSL application to decrease the *phoA* expression compared to the control (Fig. 6). These results suggested successful gene expression

regulation where the application of OHC6-HSL successfully repressed the *phoA* mRNA expression in comparison to the control VanN_SphS cells without the addition of OHC6-HSL. The higher relative expression of *phoA* mRNA was detected by applying exogenous stimuli, toluene, in the chimeric TodSS_SphS cells in the *Synechocystis* sp. PCC6803 (Inaba et al. 2018).

4. Conclusions

In this study, gene expression for AP activity was successfully regulated by the exogenous application of AHLs. Among the two chimeric sensors developed in this study, LuxN did not respond to any of the applied AHLs, including its cognate signal molecule, OHC4-HSL. The possible reason for that can be the alteration/rotation in the linker region that connects the HK to the RR in a two-component signaling transduction system, this phenomenon has been described in previous studies. The chimeric sensor, VanN_SphS, effectively responded to its cognate signaling molecule, OHC6-HSL, and its application resulted in the repression of AP activity, through this gene expression was switched OFF, while the introduction of QQ enzyme Aii20J successfully degraded the applied OHC6-HSL and AP activity was recovered to its original level, switching the gene expression ON. Hence, this way a reversible gene regulatory system was developed that can be used as a toggle switch OFF/ON system for gene expression in cyanobacterium Synechocystis sp. PCC 6803. This is the first study on the application of QS molecules, AHLs, along with QS-sensory domains to construct a successful gene switch OFF/ON system that can be used. I further research in *Synechocystis* for the controlled production of targeted compounds.

5. General Discussion

This study involves the utilization of quorum sensing (QS) sensors (LuxN and VanN) from marine bacteria as signal input domains and phosphate deficiency sensor (SphS) from the cyanobacterium *Synechocystis* sp. PCC6803 is the kinase domain to develop chimeric sensors LuxN_SphS and VanN_SphS (Fig. 1). The aim of this study was to regulate the gene expression in *Synechocystis* by exogenous application of QS-signaling molecules (AHLs) by measuring the alkaline phosphatase (AP) activity.

The cognate AHLs for LuxN and VanN, *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL) and *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL), respectively, were added to the chimeric sensors LuxN_SphS and VanN_SphS to assess their response (Fig. 2). Besides that, different other substituted and unsubstituted AHLs were also used in this study to assess their effect on developed chimeric sensors (Fig. 3A). The results showed that the chimeric sensor, LuxN_SphS, could not respond to any of the applied AHLs, including the cognate signaling molecule of LuxN, OHC4-HSL, whereas the only significant response for the chimeric sensor VanN_SphS was observed for the AHL, OHC6-HSL which is the cognate signaling molecule of VanN. It was also observed that the effect of OHC6-HSL on the chimeric sensor VanN_SphS resulted in the repression of AP activity with time, and a clear decrease in AP was observed within two days (Fig. 3f). The successful repression of AP activity by the addition of OHC6-HSL for the chimeric sensor VanN_SphS resulted in the development of a gene switch OFF mechanism in *Synechocystis*, where the expression can be turned off by the addition of this exogenous compound.

In this study, the non-functionality of the chimeric sensor LuxN_SphS and the functioning of the chimeric sensor VanN_SphS as a negative regulator of AP activity may be attributed to any possible changes in the linker region, which a connecting region between

the sensory kinase and response regulator in a two-component system. In different studies, the importance of stability/instability of the linker region was described to regulate the signaling mechanism, and it was shown that any change in the linker region results in the alteration of the signaling mechanism (Möglich et al. 2009; Bhate et al. 2015; Saita et al. 2015). Similarly, truncated liker region to construct transformants in *Synechocystis* sp. PCC6803 for photo-regulation responded differently to light signals as compared to the wild-type, where one group of transformants could not respond to the light signal for gene expression (similar to LuxN_SphS in this study, no response to AHLs), whereas another group of transformants regulated the gene expression oppositely as to the wild-type *Synechocystis* (similar to VanN_SphS in this study, repression of AP by OHC6-HSL) (Nakajima et al. 2016). These results suggest that rotation in the linker region or any other changes may affect the response of signals, and a similar phenomenon might happen in this study.

After the successful development of a gene switch off system by VanN_SphS and OHC6-HSL, the next step was to restore the expression back to its original level. The lack of presence of an enzyme that can degrade the AHLs or any other mechanism that can interfere with these signaling molecules, the repression achieved by the addition of OHC6-HSL in the chimeric sensor VanN_SphS might stay for an unlimited time. Therefore, degradation of this AHL was required by some mechanism to restore the AP expression. For that purpose, enzymatic degradation of AHLs (quorum quenching) was used, and an AHL-lactonase with broader efficiency to degrade various chain length AHLs, Aii20J from the marine bacterium *Tenacibaculum* sp. 20J was introduced in the *Synechosystis* cells harboring the VanN_SphS cells. In the first attempt to recover the AP activity, the increasing cell density with culturing time makes the light a limiting factor for the cells, and despite the

initiation of AP recovery, full recovery to the original level could not be achieved (Supplementary Fig. 2).

A unique growth technique was applied to address the light limitation factor for the cells, and by this special growth technique, not only the culture optical density was maintained to a lower level during the experiment, but also the AHL concentration was kept the same (Supplementary Fig. 3). In the later experiment, the recovery of AP activity to its original level was attained, where the QQ-enzyme, Aii20J, degraded the OHC6-HSL in the culture and until seven days AP activity was restored (Fig. 5). The successful restoration of AP activity through degradation of OHC6-HSL by the QQ-enzyme, Aii20J, allowed the development of a gene switch ON system in *Synechocystis*, where the expression was turned on by the degradation of the exogenous compound.

In this study, the gene expression for the AP activity was successfully regulated, and the development of a toggle switch OFF/ON system was achieved. The use of QS and QQ phenomena for the development of chimeric sensors and utilization of the QS-signaling molecules AHLs was demonstrated for the first time in *Synechocystis* sp. PCC6803. This study may contribute to the development of artificial gene regulatory systems and the production of useful compounds in cyanobacteria.

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Table 1 List of the primers used in this study for Infusion and PCR to develop the chimeric sensors LuxN_SphS, VanN_SphS, and VanN_SphS::Aii20J.

Primers Name	Sequences (5’ to 3’)
LuxN_F	GGCAACTGCTAGAACATGTTTGATTTTAGCCTAGA
LuxN_R	AGCTTGATCCCTACCTCGAATACGGCGATCCGCTT
VanN_F	GGCAACTGCTAGAACATGCTTAACCTCAACTTAGA
VanN_R	AGCTTGATCCCTACCTCGAATACGGCGATCCGCTT
βLAC_F	ACGATAAGGATCATACATATGAAAAAGATTTTCTTACTAG
βLAC_R	TGAGGTTAACAGATCAGATCTTATTTCTTCAAGAGATTC
pTCHT_srl2031_F	AGATCTGTTAACCTCACATTGG
pTCHT_srl2031_R	TATGATCCTTATCGTCATCGTC

Table 2 Change in the concentration of C6-HSL in the supernatants of VanN_SphS and VanN_SphS::Aii20J using the CV026 plate bioassay till 48 h.

Time	VanN_SphS	VanN_SphS::Aii20J
–	10 µM	10 µM
6 h	8.26 µM	7.43 µM
24 h	6.96 µM	4.86 µM
48 h	5.22 µM	n.d

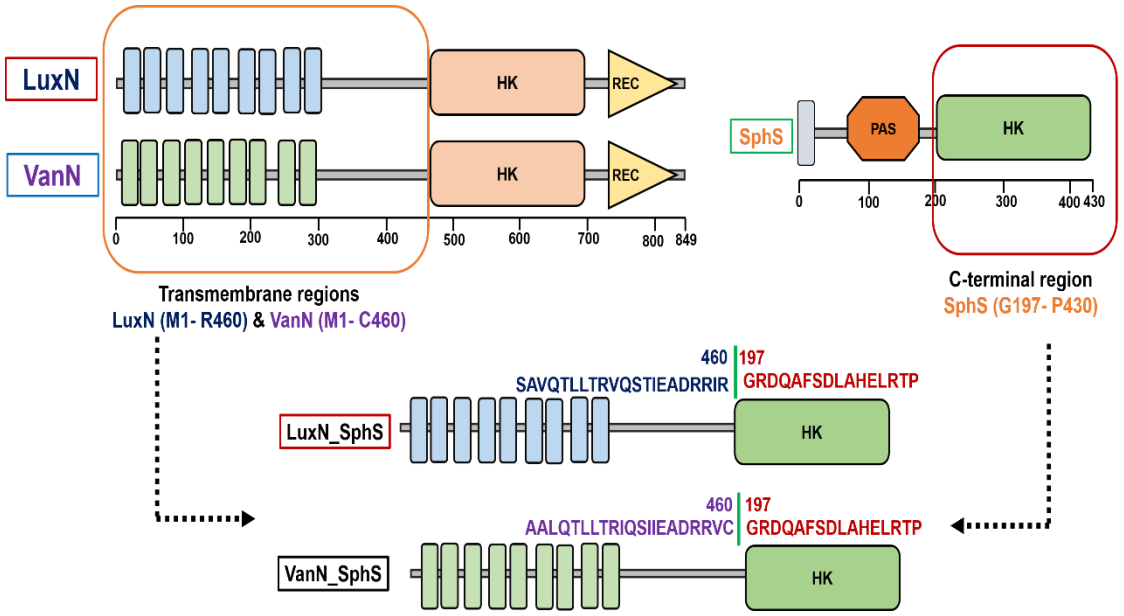


Fig. 1 Graphical representation of construction of chimeric sensors LuxN_SphS and VanN_SphS. The fusion of transmembrane regions (orange rectangle) of sensory kinases LuxN (M1-R460) and VanN (M1-C460) with the C-terminal region (red rectangle) of SphS (G197-P430) to develop the chimeric sensos LuxN_SphS and VanN_SphS, respectively. The quorum- sensing sensory LuxN and its homolog, VanN obtained from marine bacteria *Vibrio harveyi* and *Vibrio anguillarum*, while Pi-deficiency sensory kinase SphS, from cyanobacterium *Synechocystis* sp. PCC 6803.

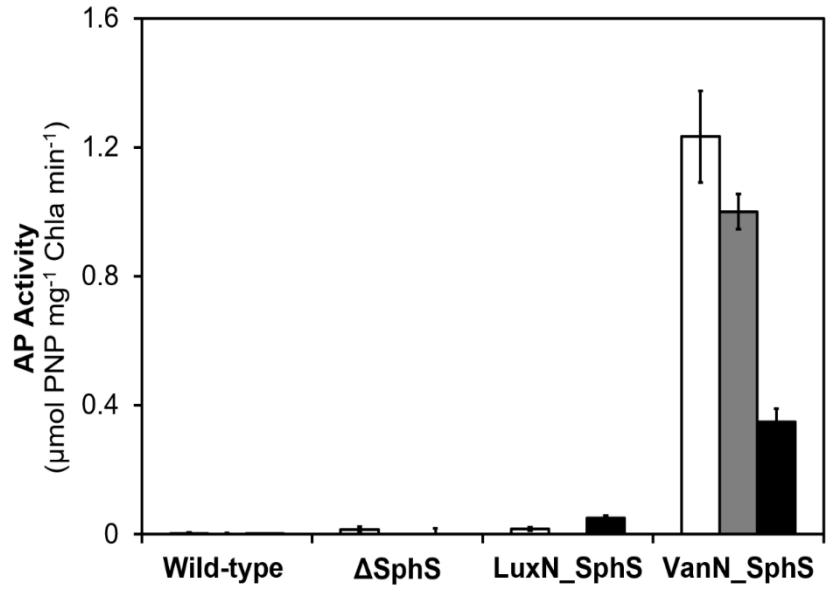


Fig. 2 Effect of cognate signaling molecules (AHLs) on chimeric sensors. The activity of alkaline phosphatase (AP) in the wild-type, ΔSphS, LuxN_SphS, and VanN_SphS strains with the addition of AHLs (10µM), OHC4-HSL (grey bar), OHC6-HSL (black bar) and without AHLs addition (white bar) till 24 h. The data represents the means of three independent biological replicates along with ± standard deviation.

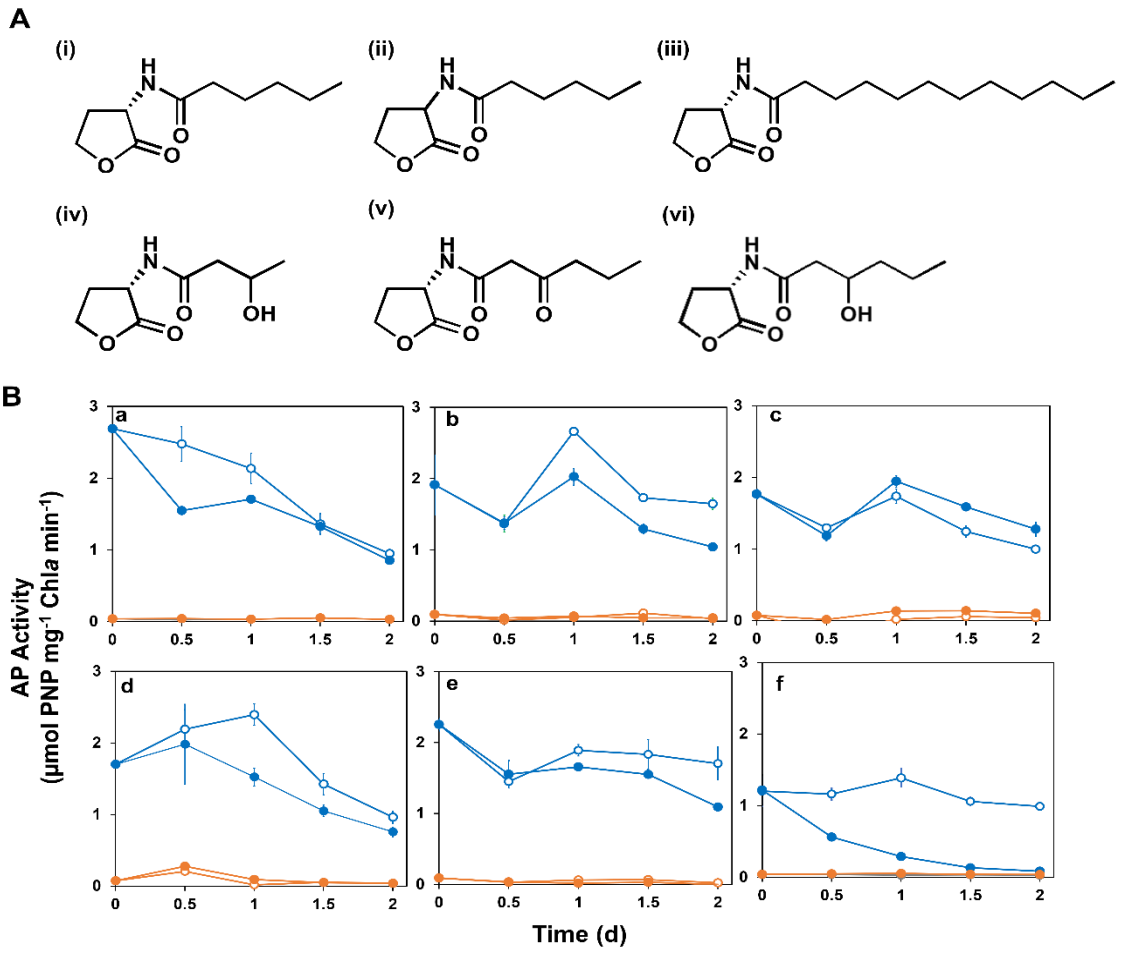


Fig. 3 Effect of different carbon chain AHLs on the chimeric sensors. A) The structures of different carbon chain AHLs (i). *N*-hexanoyl-L-homoserine lactone (C6-HSL) (ii). *N*-hexanoyl-DL-homoserine lactone (C6-HSL racemic) (iii). *N*-dodecanoyl-L-homoserine lactone (C12-HSL) (iv). *N*-(β-ketocaproyl)-L-homoserine lactone (OC6-HSL) (v). *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL) (vi). *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL). B) The effect of different AHLs with addition (filled circles) and no addition (open circles) on chimeric sensors LuxN_SphS (orange line) and VanN_SphS (blue line) for AP activity. (a). C6-HSL (b). C6-HSL racemic (c). C12-HSL (d). OC6-HSL (e). OHC4-HSL (f). OHC6-HSL. The data represents the means of three independent biological replicates along with ± standard deviation.

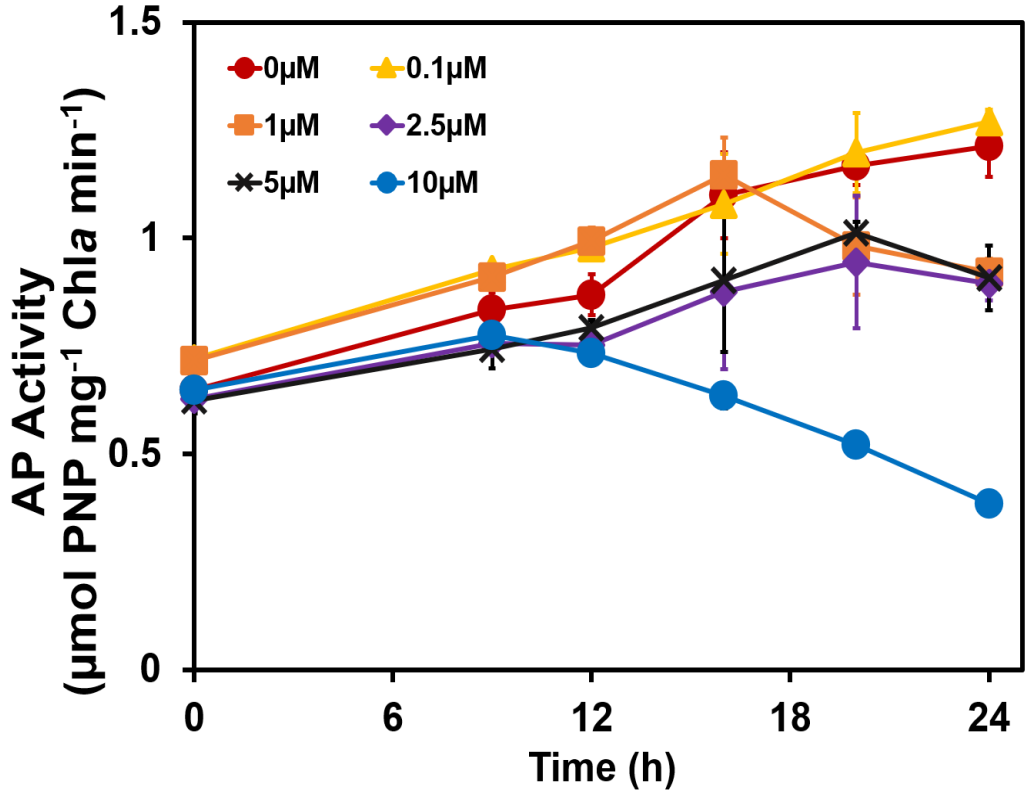


Fig. 4 The AP activity in VanN_SphS cultures using different concentrations of OHC6-HSL. The data represents the means of three independent biological replicates along with ± standard deviation.

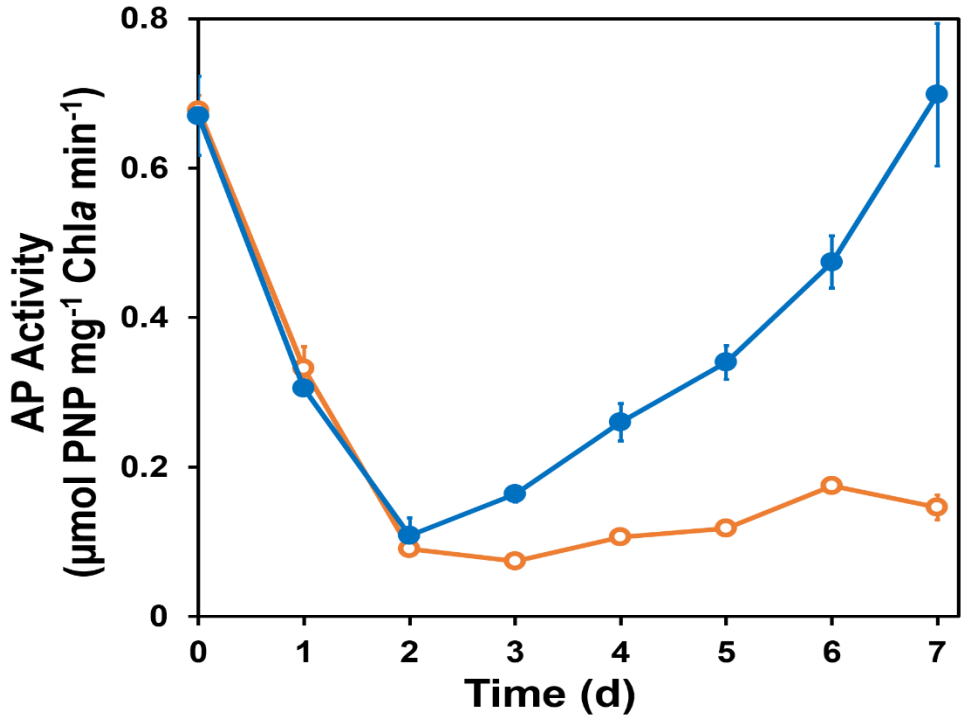


Fig. 5 AP Recovery in the cultures of VanN_SphS cultures (orange line) and VanN_SphS::Aii20J cultures (blue line) under unique growth method by the degradation of *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) through Aii20J until 7 d. The data represents the means of three independent biological replicates along with ± standard deviation.

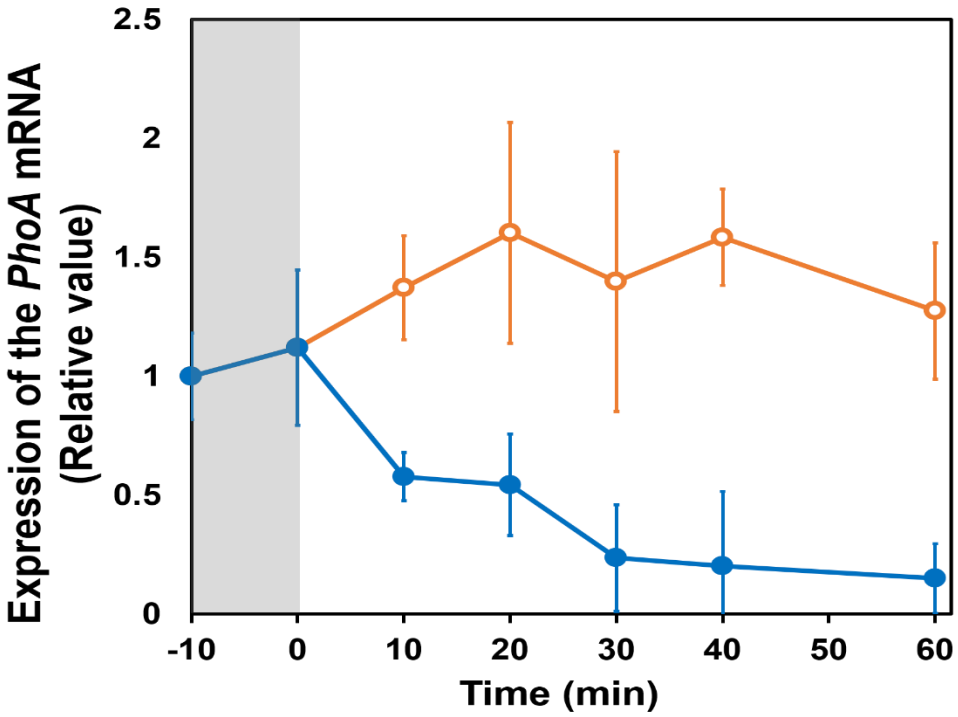
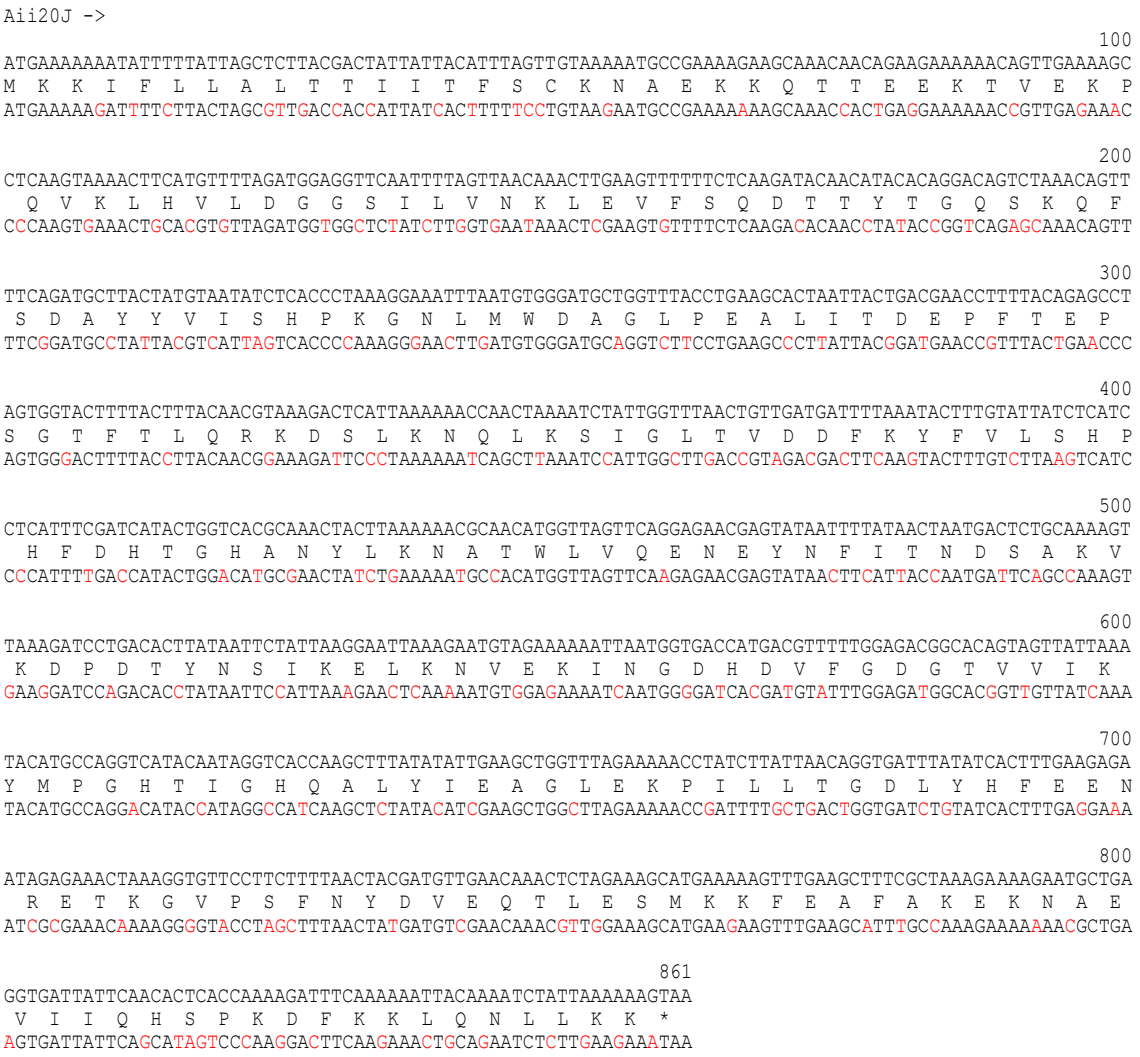
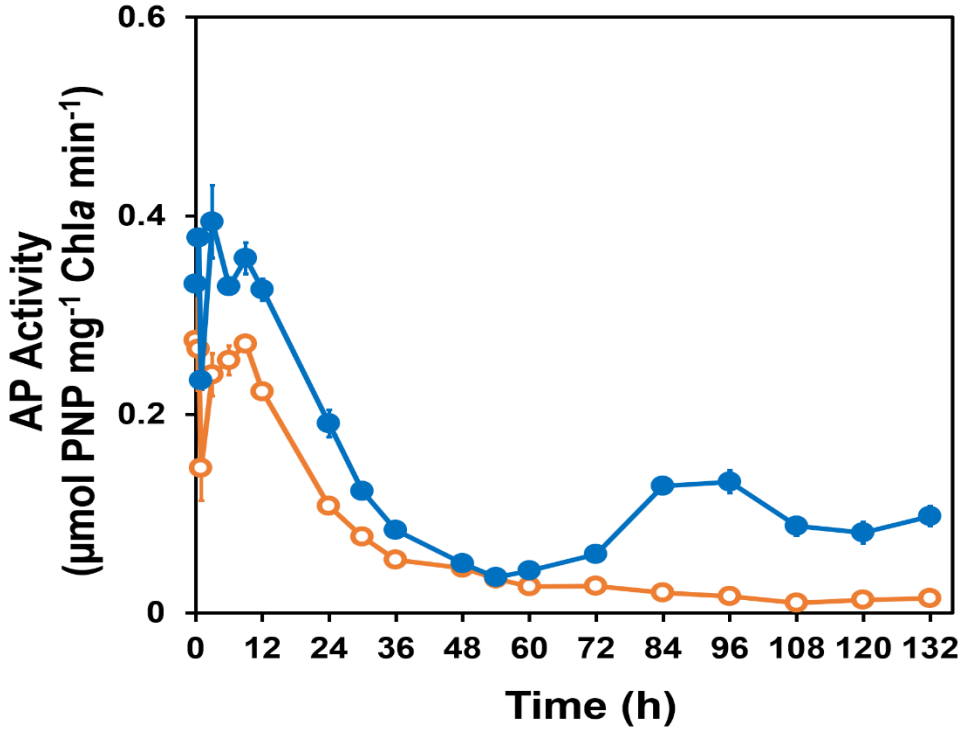


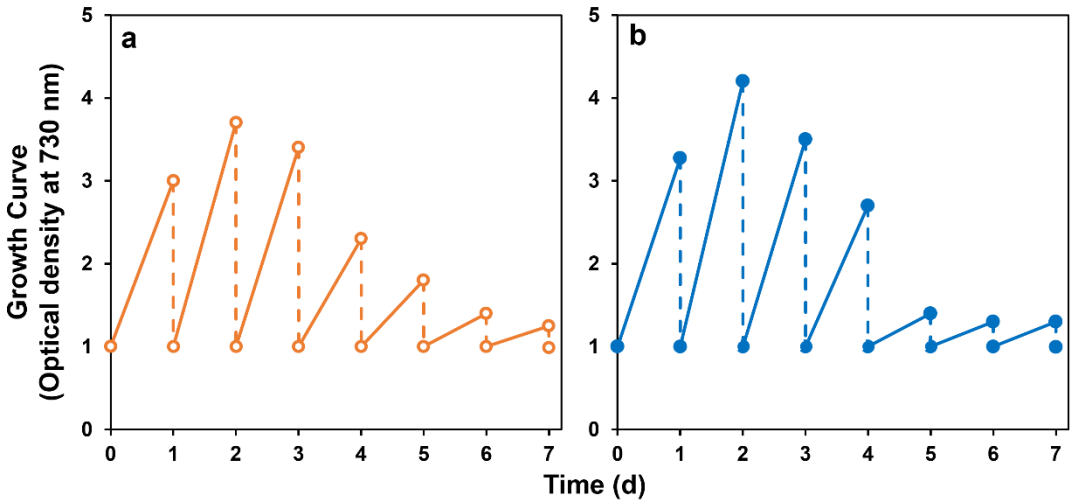
Fig. 6 Relative expression of *phoA* mRNA. The VanN_SphS cultures with addition (blue line) and no addition (orange line) of 10 μM of *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) until 60 min. The shaded gray part represents the time before the addition of OHC6-HSL. The data represents the means of three independent biological replicates along with ± standard deviation.



Supplementary Fig. 1 The codon-optimized sequence of QQ enzyme, Aii20J. The upper lines represent the original sequence of Aii20J, whereas the lower lines are the codon-optimized sequence of Aii20J. The modified nucleotides are shown as red characters. The numbers represent the nucleotide position from the 5’ end.



Supplementary Fig. 2 AP recovery by Aii20J enzyme by degrading *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) under continuous growth till 132 h. The control strain VanN_SphS (orange line) and VanN_SphS::Aii20J (blue line). The data represents the means of three independent biological replicates along with ± standard deviation.



Supplementary Fig. 3 AP recovery through a unique growth technique by dilutions to maintain the optical density of cultures constant to OD_{730} = 1.0. (a) Variations in the growth of VanN_SphS (orange) cultures (Solid line) and dilutions (dotted line) to keep the cell density constant (b) Variations in the growth of VanN_SphS::Aii20J (blue) cultures (solid line) and dilutions (dotted line) to keep the cell density constant.

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