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ENGINEERED CYANOBACTERIA AS A POTENTIAL ORGANIC DEGRADER FOR WASTEWATER TREATMENT

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Abstract: During the secondary wastewater treatment, bacteria in the activated sludge could break down organic matter into carbon dioxide (CO_2), a greenhouse gas resulting in global warming. It then will be ideal if the process can assimilate the released CO_2 to control its emission. Previous studies indicated that cyanobacteria would play an indispensable role in controlling climate change due to their vigorous ability of capturing and fixing CO_2 through CO_2 Concentrating Mechanism (CCM). It is thus promising if cyanobacteria can be integrated with the secondary wastewater treatment system. However, most existing cyanobacteria are photoautotrophy and can hardly metabolize organic carbons directly. To achieve the target of using cyanobacteria for organics degradation in wastewater, efforts are needed to obtain engineered cyanobacteria to utilize simple organics like sugars as carbon sources. In this study, the cyanobacteria strain *Synechococcus elongates* PCC7942 (*S. elongates*) was chosen due to its high genetic manipulation capacity. Three types of sugars (i.e., glucose, xylose and arabinose) were selected as carbon sources, respectively. The pivotal modules including the sugar transporters and peripheral metabolic processes were introduced into the genome of *S. elongates*. Results showed that the engineered *S. elongates* strain led to organics (i.e., sugars) consumption for biomass formation successfully. In addition, based on the exploration of the growth state of engineered *S. elongates* under diurnal light condition (with the light/dark cycle), the sugar consumption could be carried out when using natural light. The research outputs paved a road for future applications of cyanobacteria for CO_2 emission control during the secondary wastewater treatment.

1. INTRODUCTION

The activated sludge technique has been widely adopted to remove organic matter during the secondary wastewater treatment (Dignac et al. 2000). Heterotrophic bacterium are key components in the microbial community of activated sludge, which can break down organic waste into CO_2 (Gupta et al. 2012). However, the emission of CO_2, a greenhouse gas, will eventually cause an environmental issue like global warming (Cherubini et al. 2011). Thus, it is ideal if the CO_2 emission can be alleviated, or the system can initially assimilate the released CO_2.

Cyanobacteria can play an indispensable role in efficiently sequestrating CO_2 through CO_2 Concentrating Mechanism (CCM) (Kumar et al. 2011). During the process, key reactions occur in carboxysome containing CA (i.e., Carbonic Anhydrase) and RubisCO (i.e., Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase). In these reactions, CO_2 enters cells through cell membrane in the form of HCO_3^- (Bicarbonate), and CA converts HCO_3^- to CO_2, which is then fixed by RubisCO and ultimately utilized through Calvin Cycle (Price

et al. 2007). The CCM of cyanobacteria can elevate up to 1000-fold CO_2 around the active site of RubisCO so as to eventually achieve the inorganic carbon fixation (Badger and Price 2003). Cyanobacterial species have been widely applied in multiple fields including production of biofuels (Nozzi et al. 2013, Quintana et al. 2011), as food supplements (Dufossé et al. 2005, Saini et al. 2018) and soil fertilizers (Prasanna et al. 2008, Jangir et al. 2015). It is promising to identify the potential of cyanobacteria for integration with secondary wastewater treatment system for reducing the emission of CO_2.

However, many existing cyanobacteria are photoautotrophy and can hardly degrade organic carbon directly due to the lack of organic metabolic modules in genome (Zhang et al. 1998). Luckily, the breakthroughs of molecular biology especially synthetic biology have led to more functional genetic modules and genetic manipulation tools, which can strategically modify model microorganisms for desired purposes via introducing and functioning heterogeneous genetic modules in model species genomic DNA (Hartwell et al. 1999, Andrianantoandro et al. 2006). It makes rebuilding organics metabolic modules in model cyanobacterial species possible, and the organic degradation can then be achieved.

To employ cyanobacteria for organic degradation during the secondary wastewater treatment, efforts should firstly be made to obtain engineered cyanobacteria strains to utilize simple organics like sugars as carbon sources. In this study, model cyanobacterial species, *Synechococcus elongates* PCC7942 (*S. elongates*) was selected due to its high genetic manipulation capacity (Brahamsha 1996). Three types of sugars (i.e., glucose, xylose and arabinose) were chosen to represent the simple organics in wastewater. *S. elongates* was modified to metabolize these simple sugars, respectively, and the associated microbial growth and carbon usage were tracked. The research outputs can help to generate an option for CO_2 emission control and pave a road for various complex organics degradation during the secondary wastewater treatment.

2. METHODOLOGY

2.1 Strains and culture conditions

The cyanobacteria strain *S. elongatus* PCC7942 was obtained from ATCC (American Type Culture Collection). Each cyanobacterial strain before and after modification was cultured in a 250 mL conical flask with a total volume of 100 mL BG-11 medium and the culture temperature was set at 30°C (Rippka et al. 1979). The lumination was set to 2,000 - 3,000 Lux. Both the continuous lighting and light-dark sequencing treatments were applied during culturing. Spectinomycin (20 μg/mL) was added into the medium for culturing each mutant strain while the wide cyanobacterial strain grew without the addition of spectinomycin. Growth assays were conducted using cells in exponential phase. The cells were diluted to OD_{730} of 0.2 with the addition of 0.1 mM inducer Isopropyl-β-D-thiogalactopyranoside (IPTG), and the growth rate was monitored by measuring OD_{730}. All assays were conducted in triplicates.

Two *E. coli* strains were also applied. The *E. coli* MG1655 was used as template to extract the genomic DNA and the *E. coli* DH5α was used for cloning and manipulation of plasmids. All *E. coli* strains were cultivated in the Luria-Bertain (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) at 37°C with shaking at 150 rpm. Spectinomycin (100 μg/mL) was added in the LB medium for the maintenance of the plasmids containing the corresponding spectinomycin resistance marker.

2.2 Plasmid construction

All plasmids used in this study were constructed based on *S. elongatus* PCC7942 genomic neutral site I (NSI) specialized plasmid pAM2991 (Addgene #40248) (Ivleva et al. 2005). Integrating genes into NSI of *S. elongates* would no longer change the other characters (Thomas et al. 2004). All genes were amplified from *E. coli* MG1655 genomic DNA. All primers and plasmids used in this study were summarized in Tables 1 and 2.

To construct pYQ1 plasmid, the *galp* gene from *E. coli* MG1655 genomic DNA was amplified using primers Y1 and Y2, digested with restriction enzyme MfeI and BglII, then ligated with pAM2991, which was digested using EcoRI and BamHI.

To construct pYQ2 plasmid, the Gibson Assembly procedure (Siuti et al. 2013) was applied to fuse four genic fragments onto pAM2991. Particularly, *xylE* gene was amplified by primers Y3 and Y4. The *xylAB* genes were amplified by primers Y5 and Y6 (since they belong to an operon, both genes were amplified together). Two fragments of pAM2991 template were amplified by primers Y7, Y8 and primers Y9, Y10, respectively.

The Gibson Assembly procedure was also implemented for pYQ3 plasmid construction. Three genic fragments, *araBAD* operon fragment and the other two fragments from pAM2991 template, were amplified and fused together. Particular, *araBAD* operon was amplified by primers Y11 and Y12, and two parts of pAM2991 template were amplified by primers Y8, Y13 and primers Y9, Y14, respectively.

Table 1: Primers used in this study

Primer name	5' - 3' sequence
Y1	ctaacaattgatgcctgacgctaaaaaacaggggcg
Y2	ctatagatctttaatcgtgagcgcctatttcgcgcagtt
Y3	atttcacacaggaaacagaccatggaattcaatacccagtataattccagttatatattttcga
Y4	gcgatcgagctggtcaaaataggcttgcatttacagcgtagcagtttgttgtgttttct
Y5	agaaaacacaacaaactgctacgctgtaaatgcaagcctattttgaccagctcgatcgc
Y6	cttctgcgttctgatttaatctgtatcaggttacgccattaatggcagaagttgctgatagagg
Y7	atcagcaacttctgccattaatggcgtaacctgatacagattaaatcagaacgcagaagcgg
Y8	tgattctgtggataaccgtattaccgcctttgagtg
Y9	cactcaaaggcggtaatacggttatccacagaatca
Y10	tcgaaaatatataactggaattatactgggtattgaattccatggtctgtttcctgtgtgaaat
Y11	atttcacacaggaaacagaccatggaattcatggcgattgcaattggcctcgattttggc
Y12	gactctagaggatccccgggtaccgagctcttactgcccgtaatatgccttcgcgccatg
Y13	catggcgcgaaggcatattacgggcagtaagagctcggtacccggggatcctctagagtc
Y14	gccaaaatcgaggccaattgcaatcgccatgaattccatggtctgtttcctgtgtgaaat

Table 2: Plasmids used in this study

Plasmid name	Genotypes	Source
pAM2991	NSI targeting vector; P_{trc}; Sp^R	Addgene #40248
pYQ1	pAM2991, but P_{trc}: *galp*	This study
pYQ2	pAM2991, but P_{trc}: *xylE-xylA-xylB*	This study

pYQ3	pAM2991, but P_{trc}: *araB-araA-araD*	This study

2.3 Transformation of *S. elongates*

Transformation of pAM2991, pYQ1, pYQ2 and pYQ3 were carried out following the natural transformation procedure to build strain YQ0, YQ1, YQ2 and YQ3, respectively (Golden et al. 1987). Particularly, the harvested and concentrated mid-log phase *S. elongates* cells (OD_{730} = 0.4 – 0.7) was incubated with 2 μg/mL plasmid DNA with gentle shake at 30°C in the dark overnight. The mixed culture was then spread on BG-11 agar plates (containing 20 μg/mL Spectinomycin) and cultivated for nearly 2 weeks to select the transformants. The correct strains were confirmed through DNA sequencing by BGI (Beijing Genomics Institute). All *S. elongatus* strains used were listed in Table 3.

Table 3: *S. elongatus* strains used in this study

S. elongatus strains	Relevant Genotypes	Source
Wide-Type (WT)	*S. elongatus* PCC7942	ATCC #33912
YQ0	WT with pAM2991	This study
YQ1	P_{trc}: *galp* integrated at NSI	This study
YQ2	P_{trc}: *xylE-xylA-xylB* integrated at NSI	This study
YQ3	P_{trc}: *araB-araA-araD* integrated at NSI	This study

2.4 Sugar consumption assay

Samples were prepared by centrifuging and filtering the BG-11 broth with 0.22 μm cellulose acetate membrane. High-performance liquid chromatograph (RID, Refractive Index Detector) was used to measure the glucose and xylose concentrations in the culture medium. The arabinose concentration was determined by orcinol methods (Tomoda 1963). Particularly, dissolving 0.1% orcinol in concentrated HCl-AcOH (1:3, v/v), and 99 mL of this solution was mixed with 1 mL $FeCl_3$ (1 M) to prepare the stick solution. Reaction mixture containing 1 mL sample and 4 mL stock solution was then put in boiling water bath for 0.5 h and cooled to reach room temperature for wavelength measurement. The detective wavelength was set to 665 nm.

3. RESULTS AND DISCUSSION

3.1 Construction of organic degrading *S. elongates* strains

It was previously reported that *S. elongates* lack efficient capacity of metabolizing organic carbons due to its impermeable membrane and deficiency of converting them to central metabolites (McEwen et al. 2013). Besides, based on KEGG (Kyoto Encyclopedia of Genes and Genomes) database, we found that *S. elongates* does not contain the xylose and arabinose peripheral metabolic pathways. Introduction of heterogeneous sugar transporters and corresponding enzymes to the associated genomic DNA would facilitate sugars enter core metabolic pathway of *S. elongates* so as to achieve sugar degradation.

Though *S. elongates* can hardly transport glucose inside a cell, it can degrade intracellular glucose through both Oxidative Pentose Phosphate (OPP) and Embden–Meyerhof–Parnas (EMP) Pathways (Kanno et al. 2017). McEwen et al. (2013) compared the expression of three heterogeneous glucose transporters from *E. coli* BW25113 (GalP), *Homo sapiens* (Glut1), and *Synechocystis* sp. PCC 6803 (GlcP) in *S. elongates* respectively, and found that only GalP expression in *S. elongates* could lead *S. elongates* exponentially growth rate increase, suggesting that introducing GalP should be the first choice for *S. elongates* to

assimilate glucose. Based on this, we integrated GalP into the NSI (neutral site I) of *S. elongates* genomic DNA to construct strain YQ1 (Figure 1A).

To modify *S. elongates* for xylose degrading, not only the transporter, but also the peripheral metabolic pathways were taken into consideration. When only introducing heterogeneous xylose transporter (XylE) from *E. coli* into *S. elongates* genome, the growth rate of *S. elongates* was not increased but decreased, indicating that *S. elongates* can hardly metabolize excess intracellular xylose, resulting in the metabolic stress (McEwen et al. 2013). In *E. coli*, intracellular xylose can be converted by XylA (Xylose Isomerase) to xylulose, which is then phosphorylated by XylB (Xylulokinase) to xylulose-5-phosphate that can eventually enter Pentose Phosphate Pathway (PPP) (David and Weismeyer 1970). Thus, the operon including *xylE*, *xylA* and *xylB* genes from *E. coli* was integrated into the NSI of *S. elongates* to construct YQ2 (Figure 1B).

We have previously verified that *S. elongates* exhibited efficient capacity of transporting arabinose with its native transporters (Cao et al. 2017). To further modify *S. elongates* to degrade arabinose, heterogeneous corresponding enzymes were introduced to lead intracellular arabinose to enter pentose phosphate pathway (PPP). In *E. coli*, intracellular arabinose can be converted by AraA (Arabinose Isomerase) to ribulose, and AraB (Ribulokinase) subsequently phosphorylates ribulose to ribulose-5-phosphate, which is then converted into xylulose-5-phosphate by AraD (L-Ribulose-5-Phosphate Epimerase) to enter PPP (Englesberg et al. 1969). Therefore, the operon including *araA*, *araB* and *araD* from *E. coli* was introduced into the NSI of *S. elongates* to construct YQ3 (Figure 1C) in this study.

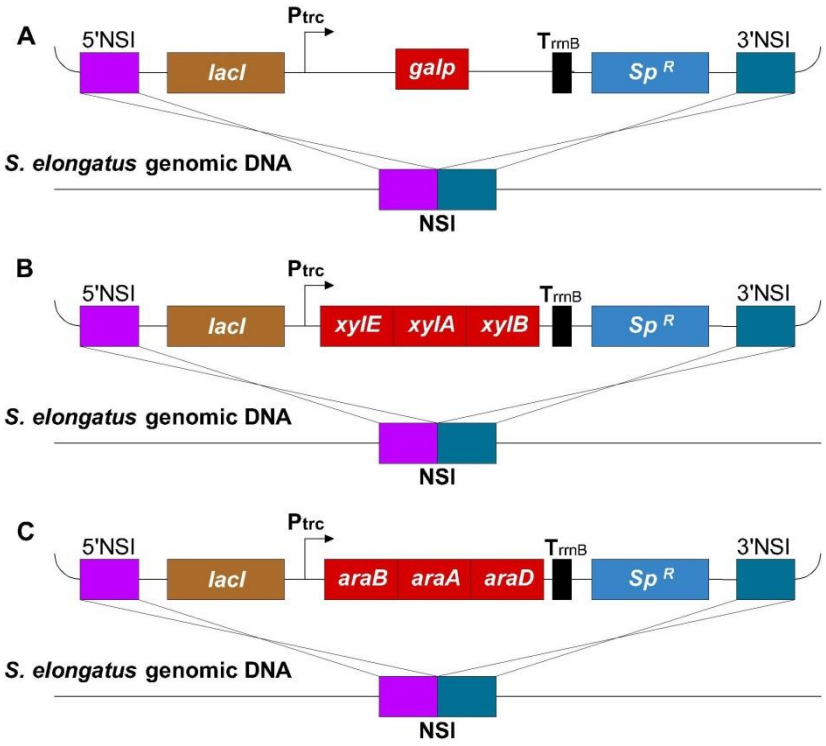

Figure 1: The designing scheme of glucose transporter gene (A), xylose degradation pathway (B), and arabinose degradation pathway (C) integration into the *S. elongates* genomic DNA, respectively.

3.2 Organic degradation for biomass increase

Performance of these engineered *S. elongates* strains for degrading different organics was evaluated. Initial sugar concentration was set around 5 g/L in this study. As expected, engineered *S. elongates* strains led

to respective sugar degradation, while the strain YQ0 could hardly degrade sugars and the concentration of sugars slightly increased just due to water evaporation in the culture medium (Figure 2). Particularly, the strain YQ1 could degrade 4.40 g/L glucose in 8 days, and YQ2 and YQ3 could degrade 2.76 g/L xylose and 4.54 g/L arabinose, respectively (Figures 2A, 2B and 2C). Eventually the engineered *S. elongates* strains achieved relative higher removal rate for glucose and arabinose than xylose, indicating their robust capacity for glucose and arabinose degradation.

It is promising to demonstrate the use of natural light for organic degradation and to conserve the energy for light supply (McEwen et al. 2016). To imitate natural light, *S. elongates* strains were cultured in a cycle with 12-h diurnal light and 12-h dark conditions for a duration of 8 days. Sugar degradation rates in diurnal light condition are similar as those obtained in continuous light condition, with relative higher removal (1.77 g/L and 2.01 g/L removal, respectively) of glucose and arabinose than xylose removal (0.80 g/L) (Figures 2D, E and F). However, under diurnal light condition, the corresponding sugar degradation rates were decreased comparing with those under continuous light condition. It may be because the decreased light supply resulted in less cell density for sugar degradation.

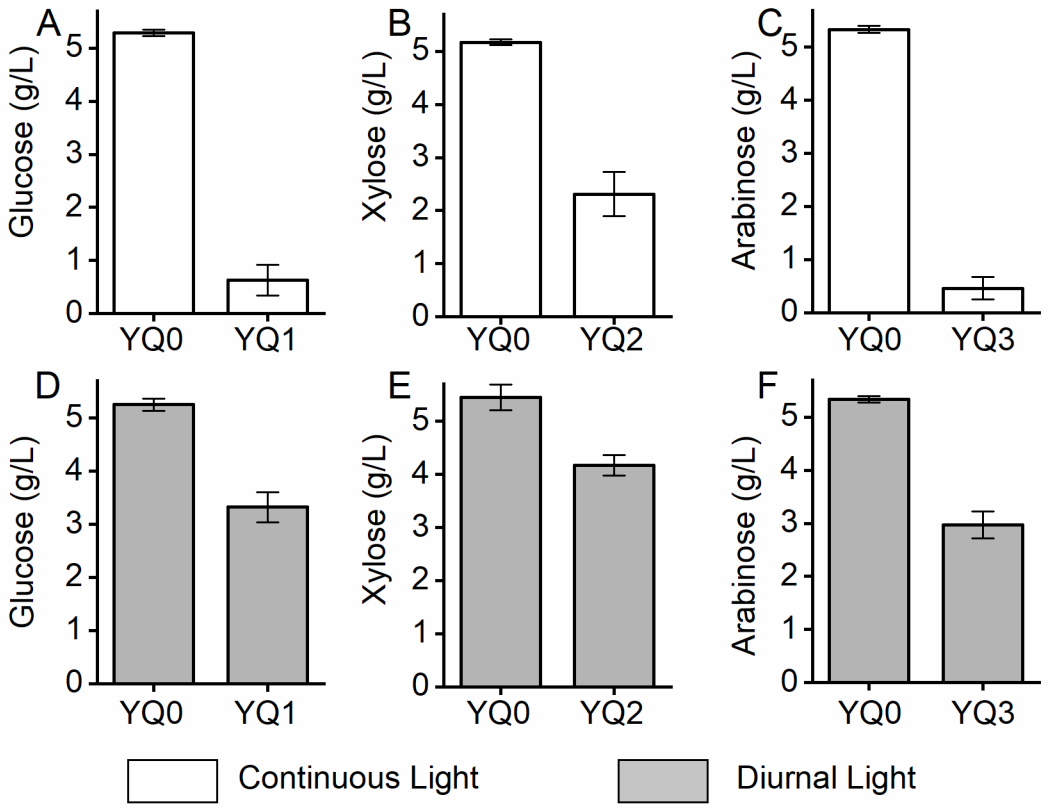

Figure 2: Glucose (A), xylose (B), and arabinose (C) degradation in continuous light condition for a duration of 8 days. Glucose (D), xylose (E), and arabinose (F) degradation in diurnal light condition for a duration of 8 days.

Organic degradation eventually led to the increase of biomass. The growth curve with the addition of respective sugars was also measured, with the growth rate of these engineered sugar degrading *S. elongates* strains remarkably increased (Figure 3). The stronger sugar degradation capacity resulted in the higher cell density. When using glucose for photomixotrophic cultivation, the growth rate of YQ1 was observed to be the highest with OD_{730} achieved 4.40 on the 8th day comparing with the utilization of xylose (OD_{730} = 1.69) and arabinose (OD_{730} = 4.00) (Figures 3A, 3B and 3C). It should be mentioned that *S.*

elongates could initially utilize a bit glucose for growth (Figure 3A) and hardly degrade pentose. In the presence of xylose and arabinose (Figures 3B and 3C), the growth rate of *S. elongates* decreased due to the intracellular metabolic stress.

When using diurnal light, the growth rate of strain YQ0 could hardly increase with the addition of different sugars in the dark (Figures 3D, 3E and 3F) because of no light energy supply, suggesting that it could be the only energy for biomass increase. Engineered sugar degrading *S. elongates* strains could achieve continuous growth in the presence of respective sugars, indicating that sugar degradation could happen in the dark (Figures 3D, 3E and 3F). However, the growth rate of engineered *S. elongates* under diurnal light condition is lower than that under continuous light condition. It is due to less light energy supplement under diurnal light condition, leading to less cell quantity and less sugar degradation. In general, these results demonstrated that natural light could be the ultimate energy for degrading organic matters.

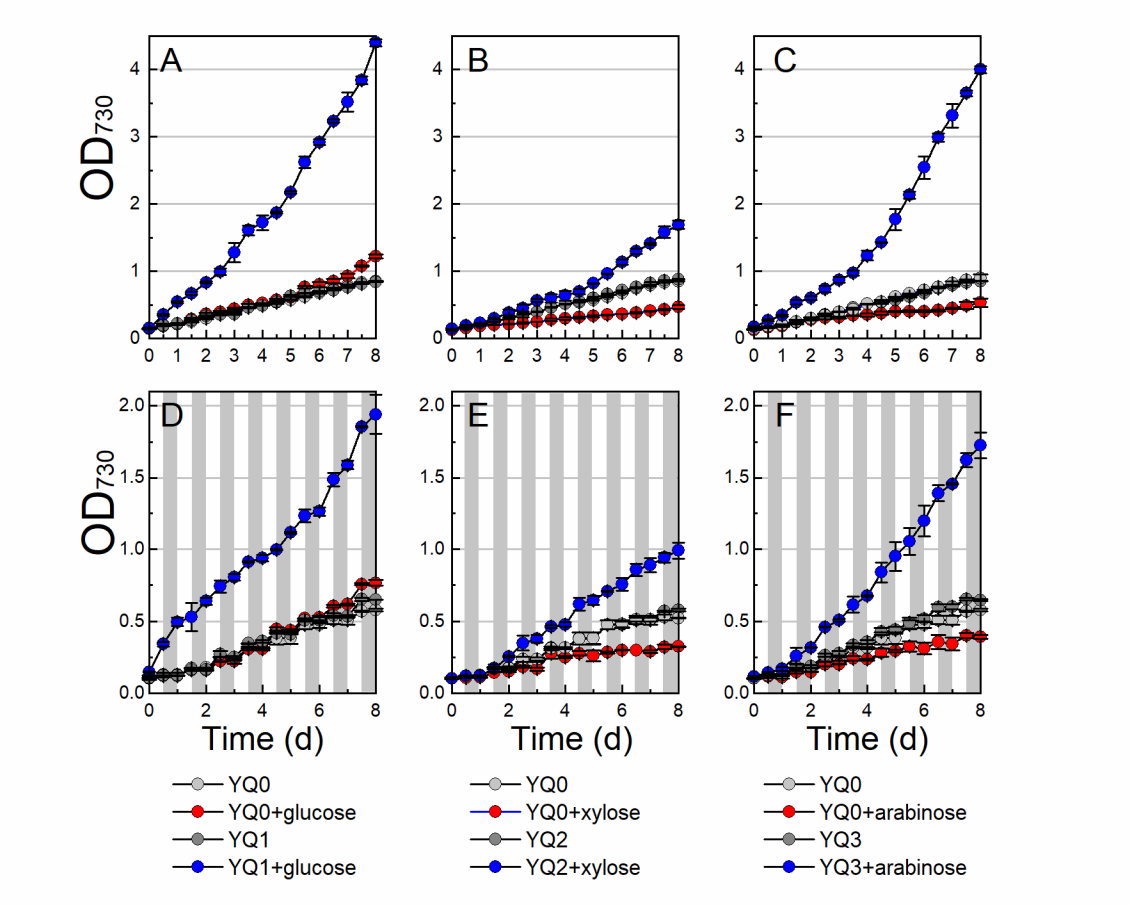

Figure 3: Growth curve of engineered *S. elongates* strains for degrading glucose (A), xylose (B), and arabinose (C) in continuous light condition. Growth curve of engineered *S. elongates* strains for degrading glucose (D), xylose (E), and arabinose (F) in diurnal light condition

4. CONCLUSION

In this study, a novel strategy for organic removal during secondary wastewater treatment was presented via rewriting the metabolic flux of *S. elongates*. The engineered *S. elongates* were constructed based on transplanting corresponding sugar transporters and degrading enzymes from *E. coli*. Engineered *S. elongates* led to respective sugar degradation and natural light could serve as the energy supply for organic consumption. In general, the engineered cyanobacteria could be an ideal organic degrader with great

potential for controlling CO_2 emission during wastewater treatment. Future study based on modifying cyanobacteria would be conducted for degrading more complex organics and for demonstrating their applicability for treating organic in actual wastewater streams.

5. ACKNOWLEDGEMENTS

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