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FINE-TUNING CHROMOPROTEIN EXPRESSION FOR BIOPRODUCTION IN HALOMONAS SPP

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Abstract

As bioproduction, especially microbial production, of substances became spotlighted, attention has been raised on developing tools to combat the unpredictable cellular synthetic activities that are often posed by the microbial cell factories. Fine-tuning tools, as one way to ameliorate cell factories for production, have become popular and effective for improving biosynthesis processes by achieving relatively stable regulatory purposes. In this study, a tunable expression system was created and utilized to control the level of expression of five single genes in the non-model bacteria *Halomonas bluephagenesis*, a member of *Halomonas spp*. and a sustainable and low-cost bioengineering and biomanufacturing platform for "next-generation industrial biotechnology" (NGIB). To better visualize results of the inducible flux regulation of this multiple inducible expression system which is designed to control five genes simultaneously, chromoprotein single genes were expressed and regulated through various combinations of induction concentration. The results demonstrated the system's ability to regulate expression of multiple individual genes at the same time. The intensely visible chromoprotein production of varied colors and the colorful bacteria paintings from the visual results also proved the viability of this system in *Halomonas bluephagenesis*, the utilization of which contributes to a more cost-effective and waste-reducing solution to resource waste and cost issues of current industrial biotechnology. Overall, this study reveals the feasibility of the desirable tuning system for efficacious expression regulation of target genes and allows for further exploration and metabolic flux optimization in the bioproduction of other substances to allow more stable bioproduction processes in synthetic biology.

Keywords: *Chromoproteins, Halomonas bluephagenesis, Next generation industrial biotechnology, Metabolic flux engineering, Synthetic Biology*

Introduction

Many elements of our nature present fascinating and multitudinous colors, and marine coral reefs being a significant one of them in adding hues to the oceans. Coral reefs derive their colors from both fluorescent proteins (FPs) and chromoproteins (CPs) which are homologous to the green fluorescent protein (GFP) (1). Thanks to their macroscopic luminescence properties,

chromoproteins absorb visible ambient light and give off strong colors visible to the naked eye (2). Therefore, CPs play crucial parts as biomarkers in finding food contaminants, landmines and biowarfare agents, and biosensors were adapted. They are also popular being used as dye or paint replacements for artistic creations in BioArt, While FPs are broadly utilized in *in vivo* bioimaging of molecules, live cells, organelles, and manufactured compounds, CPs, as a subset of the fluorescent protein family, are also used for bioimaging purposes and have advantages over FPs such as its ability to be detected in an instrument-free way (3–4). Whereas the detection of FPs usually require fluorometers, flow cytometers, or ultraviolet light (UV) lamps which can result in issues such as background fluorescence, photobleaching, and UV damage of the sample (2). The utilization of CPs leaves out these possible concerns that FP imaging can raise and the necessity of expensive lab equipment, making CPs attractive for a wide range of applications such as visual detection of gene expression without equipment in synthetic biology development and industrialized biomanufacturing where costs, production resources and required experimental proficiency of labor are major considerations (2, 5). Therefore, CPs are selected as visual identifiers of bioproduction in this study.

In modern days, most CPs require expression in bacteria such as *Escherichia coli* (2). While such model bacteria have been extensively used for recombinant protein production (6), relatively fewer efforts have been made to explore the tuning of multiple inducible genes in other engineered and improved non-model bacteria like *Halomonas bluephagenesis*. Although various attempts have been made to explore intense production of novel CPs and general bioengineering tools in model bacteria like *E. coli* (Bao et al., 2020; Meyer et al., 2019; Pang et al., 2020), bioprocesses in these bacteria often necessitates the use of stainless steel bioreactors, strict sterilization, and costly separation procedures and practiced engineers who are able to perform the processes under sterile conditions, often diminishing competitiveness of resultant industrial biotechnology products (Chen & Jiang, 2018). On the other hand, *Halomonas bluephagenesis*, an extremophilic non-model halophile possessing tolerations for high salt and pH condition, has been continuously engineered and improved in the last few decades as a chassis (Ye & Chen, 2021). Its strong growth rate under high salt and pH levels enables efficacious biosynthesis and fermentation processes under non-sterile conditions (Tan et al., 2011), particularly characterized in the Next Generation Industrial Biotechnology (NGIB), greatly reducing the difficulties led by microbial contamination, the consumption and possible waste of energy and freshwater resources for sterilization, slow growth of production organisms, and the expensive separation, which are existing issues of current industrial biotechnology (CIB) that does not make it more competitive in contrast to the chemical engineering industry (Chen & Jiang, 2018). Thus the use of *Halomonas* as a biological chassis can raise the cost-effectiveness of production and contribute to the sustainable and low-consumption development of synthetic biology research in the long run (Yu et al., 2019). Therefore, it is crucial to create and establish tools for efficacious control and tuning of biosynthesis in this previously scarcely explored non-model bacteria, *Halomonas bluephagenesis*.

Research objective

This study aimed to explore the feasibility of inducible production regulation systems in this non-model chassis bacteria *H. bluephagenesis* by constructing and testing an induction system containing multiple inducible operons that enables the simultaneous regulations of the expression of five independent chromoprotein genes, and using the direct visualization of the color and intensity resulted from the targeted chromoprotein genes' expressions as indication of its level of throughput controlled by the system. The hypothesis is that by changing the concentration of inducers added to induce each of the five genes encoding for CPs, the color intensity of the produced will vary, suggesting the successful fine-tuning of the gene expressions of multiple genes in *H. bluephagenesis*.

Materials and methods

Plasmid Design

For the purpose of this study, the plasmid 'CP' was constructed by Gibson Assembly. The plasmid holds inducible operons with promoters, terminators, the five corresponding open-source chromoprotein genes, *spisPink*, *fwYellow*, *amajLime*, *amilCP*, and *gfasPurple*, as target sequences expressing distinct colors of pink, yellow, green, blue, and purple under the induction with corresponding inducers IPTG, OHC14, OC6, arabinose, and vanillic acid, respectively.

Figure 1. *Schematic plasmid design illustration of the inducible chromoprotein expression flux regulation pathways. Characterization of constitutive promoters, insulators, ribosomal binding sites (RBS), and genes of interest (GOI), and terminator, presented in order from left to right, for the five independently regulated pathways, each labeled in a different color*

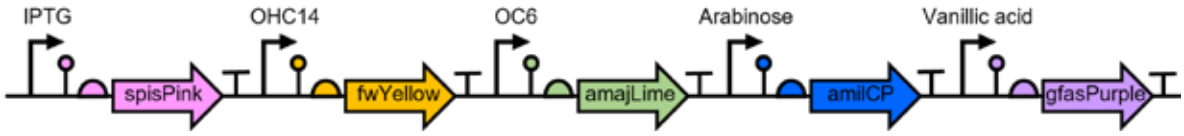
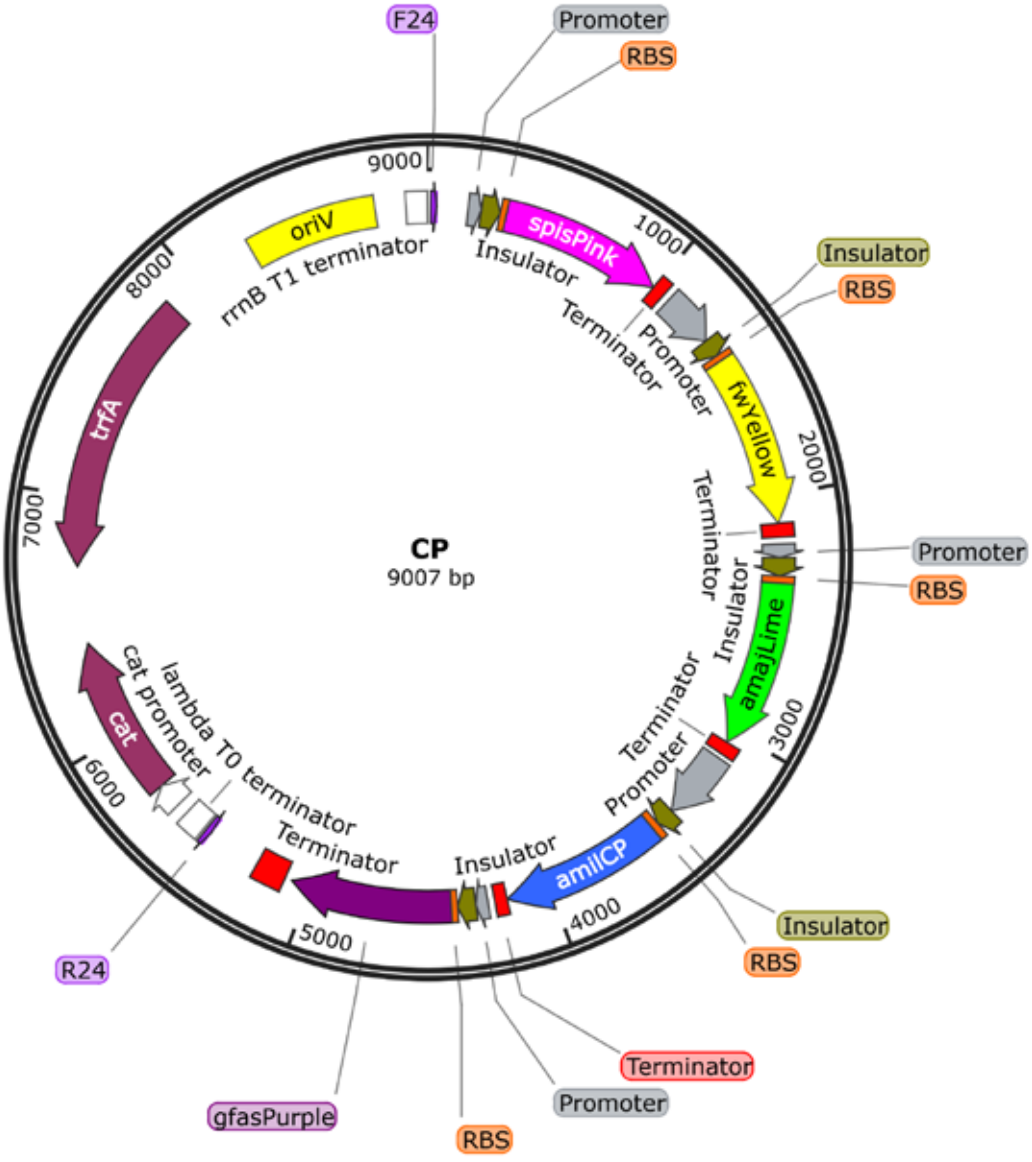


Figure 2. *Vector design illustration of the plasmid 'CP'*



Plasmid Transformation and Cell Proliferation

The plasmid 'CP' was transformed into the chemically competent *E. coli* cells of strain s17–1. The *E. coli* s17–1 cells holding the CP plasmid and the chassis *H. bluephagenesis* strain TDR2 cells were incubated at 37 °C and 200 rpm in a 20mL LB medium of 10g/L NaCl concentration and in a 20mL LB medium of 60g/L NaCl added with 20μL chloramphenicol antibiotic, respectively. The conjugated *E. coli* cells with the designed plasmid were grown in a 20mL LB liquid medium at 37 °C with the addition of 10g/L NaCl and 20μL liquid chloramphenicol antibiotic. The halomonas strain was grown in a 20mL LB medium with 60g/L NaCl at 37 °C for 12 hours with no additional antibiotic.

Conjugation of the CP Plasmid into H. bluephagenesis TD cells

The two bacteria cultures of *H. bluephagenesis* TDR2 and *E. coli* s17–1 harboring plasmid CP, respectively, are then centrifuged at 5000 rpm and the liquid supernatant was discarded. The precipitations from both tubes were mixed and added into 50 μL of LB liquid medium of 10g/L NaCl concentration. The mixture is then dropped on a petri dish and grown at 37 °C for 8–12 hours to allow conjugation of CP plasmid into the TDR2 cells.

Afterwards, recombinant *H. bluephagenesis* TDCP cells harboring the CP plasmid were scrubbed up and evenly spread on three separate agar plates. In order to purify the remaining bacteria mixture and to sift out the *E. coli* cells and halomonas cells that do not contain the designed plasmid, the bacteria culture that we suppose contains the designed plasmid were evenly spread on the three petri dishes with agar-solidified LB medium with 60g/L NaCl and chloramphenicol addition. The bacteria on these agar plates were grown at 37 °C for 48 hours.

Monoclonal purification and Conjugation Verification

The monoclonals grown on agar plate were then picked up using sterile wooden toothpicks and purified on agar plates with chloramphenicol and of 60g/L NaCl concentration using the streaking method and incubated at 37 °C. Each clone was numbered on the petri dish to make sure that we use the clones of correct DNA length for verification and induction later performed in this study.

In order to verify that the monoclonal cells were successfully conjugated, PCR was performed to amplify the recombinant TDCP cells and a gel electrophoresis was done to visualize the size of the target conjugated TD DNA. The clone bands showing lengths of about 6000 bp, the desired DNA length of the conjugated TD cells, indicate the presence of successful conjugation of CP plasmid with TDR2 cells.

Pipette tips were used to transfer such clones from the agar plates into the wells of a 96-wells deep-well plate. In each well, the pipette tip attaching the TDCP cells were put into 1mL LB medium of 60g/L NaCl concentration with chloramphenicol antibiotic added.

Chromoprotein (CP) Induction in TD cells for novel colors and microbial paintings

The recombinant *H. bluephagenesis* TDCP cells were induced to produce chromoproteins in three 96-wells deep-well plates. On each plate, 32 wells were designed to hold different multiple protein combinations (**Table 1**) and the other 15 wells show single protein induction for the five proteins, each at induced three different inducer concentrations (**Table 2**). The rest of the wells were left empty.

Table 1. *32 combinations generated from the following inducer concentrations: 10, 0 mg/L IPTG, 10^{-6}, 0M OHC14, 10^{-6}, 0 M OC6, 10^{-3}, 0M Ara (Arabinose), 10^{-5}, 0 M Van (Vanillic acid). Each cell in the bolded perimeters of this table corresponds to one of the 32 wells where combined expression of colors occur*

	IPTG = 10 mg /L			
	OHC14 10–6 M		**OHC14 0M**	
	1	**2**	**3**	**4**
A	OC6 10–6M Ara 10–3M	OC6 10–6M Ara 0M	OC6 10–6M Ara 10–3M	OC6 10–6M Ara 0M

	IPTG = 10 mg /L			
	OHC14 10–6 M		**OHC14 0M**	
	1	**2**	**3**	**4**
	Van 10–4M	Van 10–4M	Van 10–4M	Van 10–4M
	OC6 10–6M	OC6 10–6M	OC6 10–6M	OC6 10–6M
B	Ara 10–3M	Ara 0M	Ara 10–3M	Ara 0M
	Van 0M	Van 0M	Van 0M	Van 0M
	OC6 0M	OC6 0M	OC6 0M	OC6 0M
C	Ara 10–3M	Ara 0M	Ara 10–3M	Ara 0M
	Van 10–4M	Van 10–4M	Van 10–4M	Van 10–4M
	OC6 0M	OC6 0M	OC6 0M	OC6 0M
D	Ara 10–3M	Ara 0M	Ara 10–3M	Ara 0M
	Van 0M	Van 0M	Van 0M	Van 0M
	IPTG=0mg /L			
	OHC14 10–6M		**OHC14 0M**	
	1	**2**	**3**	**4**
	OC6 10–6M	OC6 10–6M	OC6 10–6M	OC6 10–6M
E	Ara 10–3M	Ara 0M	Ara 10–3M	Ara 0M
	Van 10–4M	Van 10–4M	Van 10–4M	Van 10–4M
	OC6 10–6M	OC6 10–6M	OC6 10–6M	OC6 10–6M
F	Ara 10–3M	Ara 0M	Ara 10–3M	Ara 0M
	Van 0M	Van 0M	Van 0M	Van 0M
	OC6 0M	OC6 0M	OC6 0M	OC6 0M
G	Ara 10–3M	Ara 0M	Ara 10–3M	Ara 0M
	Van 10–4M	Van 10–4M	Van 10–4M	Van 10–4M
	OC6 0M	OC6 0M	OC6 0M	OC6 0M
H	Ara 10–3M	Ara 0M	Ara 10–3M	Ara 0M
	Van 0M	Van 0M	Van 0M	Van 0M

Table 2. *Three different inducer concentrations each added to induce each of the five individual CPs. Each shaded cell in the table corresponds to each of the 15 wells designed for individual chromoprotein expression*

Chromoprotein name	**Inducer**	**Concentration (inducer/ddH_2O)**		
spisPink	IPTG	10 mg/L	5 mg/L	0 mg/L
fwYellow	OHC14	10^{-6}M	10^{-8}M	0M
amajLime	OC6	10^{-6}M	10^{-8}M	0M
amilCP	Arabinose	10^{-3}M	10^{-5}M	0M
gfasPurple	Vanillic acid	10^{-4}M	10^{-5}M	0M

In each well, the induction mix was prepared by adding 1mL LB medium of 60 g/L NaCl concentration, 5µL liquid medium containing recombinant TDCP cells, each inducer solution of different concentrations, and chloramphenicol antibiotic to see the effects of the simultaneous induction of multiple colors that result in 32 mixed colors in total (**Figure 4**).

The deep-well plates were put into a micro-perforated plate thermostated oscillator and incubated at 37 °C and 1000rpm. After 48 hours, the plates were centrifuged for 15 minutes at 4700 rpm. The liquid supernatants were discarded and TDCP cells presenting visible colors as a result of chromoprotein expression were precipitated at the bottom of each well. Pictures were taken from the bottom-view of the deep-well plates to gain a vi-

sual of the level of production of chromoproteins centrifuged at the bottom of wells.

Several colors were chosen for microbial paintings imitating the starry sky, flowers, and Chinese landscape painting on agar plates. Because the area where colors should cover vary in sizes, the appropriate amount of inducer needed to bring out these colors at desired intensities were calculated by approximating the volume of solidified LB medium with 60g/L NaCl carried in sections of the agar plate and the corresponding volume of inducer. The calculated amounts of each inducer needed were spread on plain agar plates first, then TDCP bacteria mediums were evenly spread in designated areas after the inducer solutions were fully absorbed. The cells were grown at 37 °C.

Cell lysis and Protein Concentration Measurement

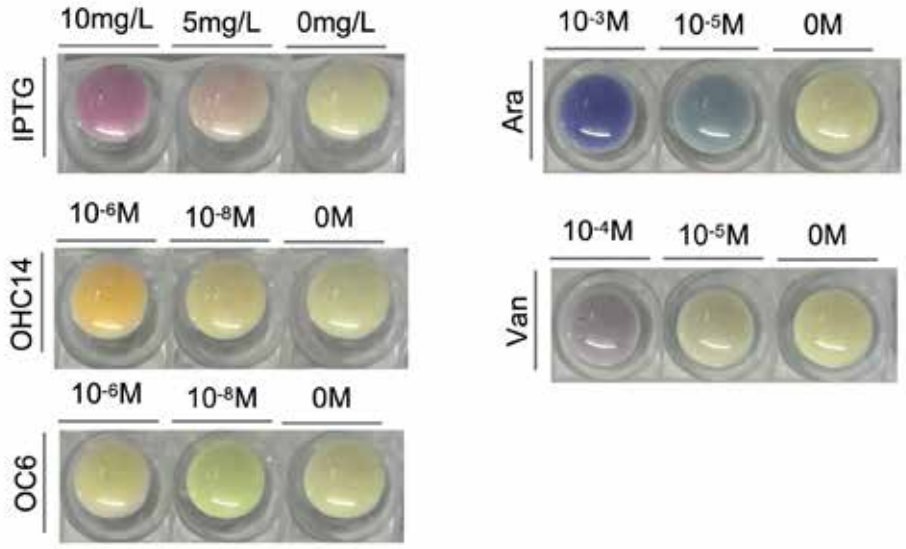
To further investigate the effects of the constructed induction system, the cells in the 15 wells of each deep-well plate that contains single chromoprotein induction were lysed and the proteins were extracted to measure the group protein concentration generated. The lysis solution includes 500μL 0.1M EDTA, 500μL 100X protease inhibitor cocktail for bacterial cell extracts, 1mL 50mg/mL lysozyme, and 50mL Beyo Lytic™ Bacterial Active Protein Extraction Reagent. 1mL of the lysis solution was added to the TDCP cells in wells where only the genes of one chromoprotein were induced in order to see the effect of different inducer concentrations on the color intensity produced. The lysate solution and cell mixture were centrifuged at 25 °C and 4700 rpm for 20 minutes after being incubated at 37 °C and 1000 rpm for 10 minutes. 2μL of the protein precipitate from each well selected was measured using the Scopes method in the Nanodrop A280 program with baseline correction of 340 nm. The protein concentrations were recorded and analyzed by calculating the percentage differences in group protein concentrations between the samples in which different levels of inducer were added.

Results and discussion

Overall, the direct visualization of the visibly and intensely colored recombinant *H. bluephagenesis* TDCP cells suggests that the constructed tunable expression system was successful in regulating the expression of multiple genes in this non-model chassis bacteria.

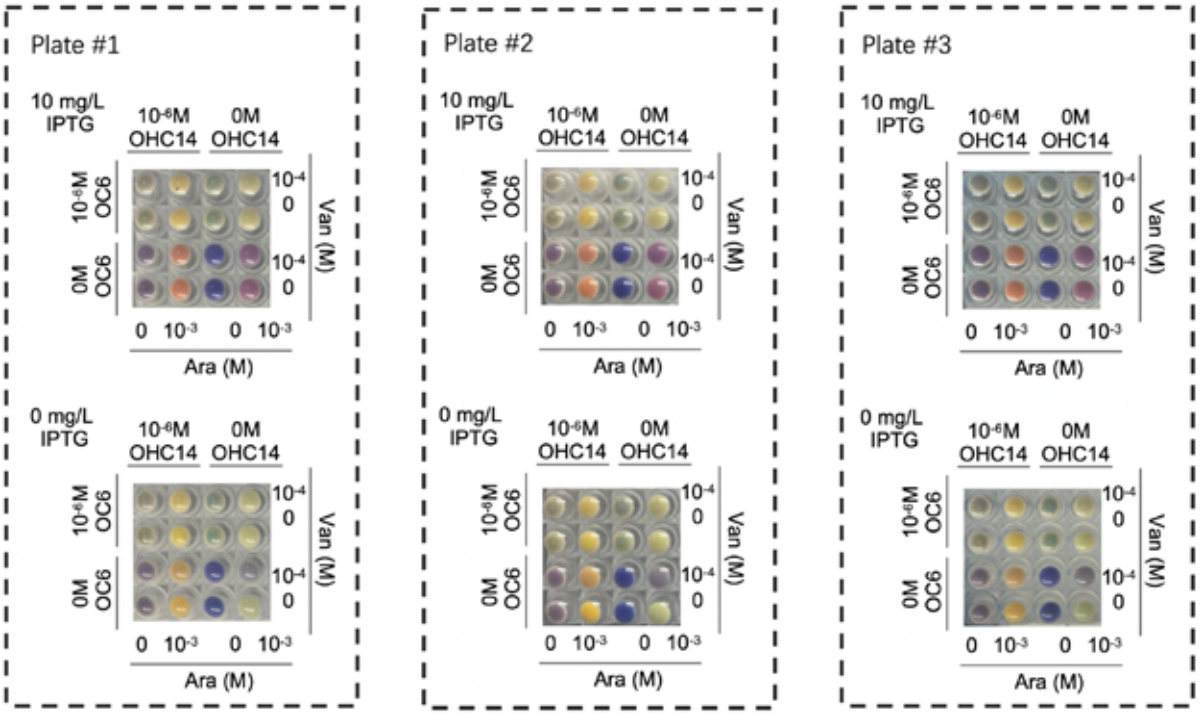
This induction tuning system constructed for multiple inducible expressions in this non-model bacteria produced vibrantly-colored bacteria after induction, resulting in the successful expression regulation of five chromoproteins in combinations and individually, as shown in the figures below.

Figure 3. *Matrix of recombinant H. bluephagenesis TDCP in which each of the five chromoproteins were induced individually and induced by three distinct concentrations of inducer*



While the wells with 0M inducer concentration acted as negative controls of no chromoprotein production, it is observed that the product cells present a general increase in color intensities along with the increase in concentration of the separately added inducers (**Figure 3**). This visualization directly suggests that the production of chromoproteins is regulated by the induction system as hypothesized, demonstrating the effectiveness of the systems in controlling the relative degree to which each of the genes are independently expressed. It is also observed, however, that the results of the OC6-induced expression stands as an exception to this trend. It is worth noticing that the observed color intensity and the measured group protein concentration of the *amajLime* gene induced by OC6 both show a lower chromoprotein production under a higher-concentration (10^{-8} M) inducer induction than that under lower-concentration (10^{-8} M) inducer induction (**Figure 3**). One possible explanation for this low production of *amajLime* chromoprotein that resulted from a relatively high-level induction with OC6 inducer is metabolic burden, a longstanding issue in engineered recombinant cells in biotechnology.

Figure 4. *Matrix of tuned recombinant H. bluephagenesis TDCP with expressed chromoprotein induced by 10, 0 mg/L IPTG, 10^{-6}, 0M OHC14, 10^{-6}, 0 M OC6, 10^{-3}, 0M Ara (Arabinose), 10^{-5}, 0 M Van (Vanillic acid), resulting in 32 colors of combination on each of the three deep-well plates*



In the visual results of inductions in the deep-well plates and agar plates (Figure 4), it is also shown that the mixed induction of the CPs in each well produced a well-blended mix of colors that complies with the common-sense resultant colors of mixing colored pigments. The various mixed colors presented proves the successful activation of multiple CP gene expressions simultaneously, further establishing the induction system's effect in regulating gene expression in *H. bluephagenesis*. Furthermore, the colors produced on the agar plates are the same colors desired by researchers, who selected and used the corresponding inducer concentrations that, in liquid culture in the deep well plates, produced those specific desired colors as reference. The successful production of colored proteins on the agar plates proves the recombinant cells' consistency in responding to the different inducers and their respective varying concentrations in both liquid and solid culture (Figure 4 and Figure 5).

Figure 5. *Cell paintings as results of chromoprotein expression in H. bluephagenesis on agar plates with different inducers concentrations in select parts of the plate that made shapes with various colors constituting the paintings*

In summary, the results described above lead to the conclusions of 1) a trend showing the general increased in color intensity resulted from the augmentation of inducer concentration thus the successful fine-tuning of chromoprotein expression flux, and 2) the feasibility of the induction systems in expression of multiple chromoprotein genes at the same time and in producing various mixed colors. Overall, the fine-tuning of genes for biosynthesis of substances like chromoproteins in the extremophilic but previously seldomly investigated non-model halophile *H. bluephagenesis* were made possible in this study.

The fine and precise simultaneous regulation of multiple gene expressions in the extremophilic halophile *H. bluephagenesis* bacteria consequently widens the door for future biological exploration of the behaviors and capabilities of this non-model bacteria. This study, thus, endows the prospect of using this bacteria for controllable bioproduction of other products other than chromoproteins, shedding light on further research in possible recombinant protein production in this bacteria for medical, industrial, or academic applications. Most importantly, the possibility successful application of this non-model bacteria as chassis for recombinant engineering, which is explored in this study, would have significant contribution to the sustainability of prospective scientific research with this bacteria, especially in terms of saving laboratory consumables and water and energy resources, allowed by its extremophilic and environmental toleration characteristics and its needlessness and unnecessity for sterile experimental conditions (Ye & Chen, 2021).

Besides its applications in biological research, the bacteria has been proven useful in producing producing colorful agar paintings that allow for countless number of colors that can be produced by the inducing multiple chromoprotein genes simultaneously for the artistic community (Liljeruhm et al., 2018), as combinations of inducer concentrations are shown to be able to produce novel mixtures of colors made up of combinations of chromoproteins.

Conclusion

This study successfully constructed and tested an induction system of five independently inducible genes by demonstrating and proving the hypothesis that the induction of this newly designed tunable expression system in the non-model extremophilic bacteria chassis *H. bluephagenesis* is viable and concentration-dependent, as shown in the resulting levels of chromoprotein production. Thus, a new inducible flux control system feasible is provided for this previously scarcely explored but valuable and environ-

mentally beneficial bacteria for synthetic biology research.

Future Direction

This experiment can be improved, on one hand, by inserting epitope tag genes, which were absent in the design of this study, onto the target plasmid so that the resultant chromoproteins can be purified and separated from the bacteria's group protein, for easier and precise quantification of the chromoproteins than the naked eye can distinguish. The quantification data can then be used to more accurately compare protein concentrations caused by each specific concentration of inducers, allowing for clearer determinations of flux yield as a result of various levels of induction. Although group protein measurements were done in this study, they were not accurate indicators of chromoproteins alone; thus, it is important to extract single proteins to determine the exact effects the tuning system has on bioproduction for future efforts.

In addition, future expansions on the success of these induction systems can be explored to regulate and hopefully optimize the flux of the bioproduction of other substances other than chromoproteins. Along with the benefits of reducing sterilization cost and waste, allowed by its extremophilic characteristics, investigating production using chassis could be a significant step in achieving the goals of Next Generation Industrial Biotechnology (NGIB) and a more sustainable and environmentally friendly future of biological research (Chen et al., 2022; Liljeruhm et al., 2018).

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