frontiers in Environmental Science

MINI REVIEW
published: 24 November 2021
doi: 10.3389/fenvs.2021.778260

Synthetic Biology-Based Approaches for Microalgal Bio-Removal of Heavy Metals From Wastewater Effluents

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Heavy metal polluted wastewater from industries is currently one of the major environmental concerns leading to insufficient supply of clean water. Several strategies have been implemented to overcome this challenge including the use of microalgae as heavy metal bio-removers. However, there are still limitations that prevent microalgae to function optimally. Synthetic biology is a new biological discipline developed to solve challenging problems via bioengineering approaches. To date, synthetic biology has no universally affirmed definitions; however, it is uncontroversial that synthetic biology utilizes a constructive library of genetic standardized parts to create new biological systems or to redesign existing ones with improved characteristics. In this mini-review, we present state-of-the-art synthetic biology-based approaches that can be used to enhance heavy metal bio-removal from wastewater effluents by microalgae with a narrative synthetic biology workflow (Design-Build-Test-Learn cycle) to guide future developments of more advanced systems. We also provide insights into potent genes and proteins responsible for the bio-removal processes for stepwise developments of more advanced systems. A total of 49 unique genes and proteins are listed based on their eight heavy metals (Mn, Fe, Cu, Zn, As, Cd, Hg, and Pb) bio-removal functions in transport system, cellular tolerance, synthesis of key players in heavy metal bio-removal, biotransformation of heavy metals, and gene expression regulation. Thus, with our library, genetic parts are ready to be recruited for any synthetic biology-based designs. Thereby, this mini-review identifies potential avenues of future research and maps opportunities to unleash more potential of microalgae as heavy metal bio-removers with synthetic biology.

Keywords: synthetic biology, heavy metal, microalgae, bio-removal, wastewater treament

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Edited by:
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Specialty section:
This article was submitted to Water and Wastewater Management, a section of the journal Frontiers in Environmental Science

Received: *16 September 2021*
Accepted: *08 November 2021*
Published: *24 November 2021*

Citation:
Sattayawat P, Yunus IS, Noirungsee N, Mukjang N, Pathom-Aree W, Pekkoh J and Pumas C (2021) Synthetic Biology-Based Approaches for Microalgal Bio-Removal of Heavy Metals From Wastewater Effluents. Front. Environ. Sci. 9:778260. doi: 10.3389/fenvs.2021.778260

INTRODUCTION: OVERVIEW OF SYNTHETIC BIOLOGY AND WASTEWATER TREATMENT

With recent advances in biological field, it is relatively more feasible to create purposeful synthetic systems through careful designs and engineering. This rapidly emerging discipline is referred to as "synthetic biology" and, since its birth in the year 2000, has demonstrated a number of major achievements (Meng and Ellis, 2020). With its widely applicable scope, synthetic biology has been

used to overcome several challenges that our world has been facing including the development of CO_2 fixing heterotrophic bacteria, CRISPR-based systems including for rapid diagnostics and, particularly for algal engineering (Gleizer et al., 2019; Krishnan et al., 2020; Meng and Ellis, 2020; Kaminski et al., 2021). Synthetic biology adopts the philosophy of bioengineering and aims at creating new biological systems or redesigning existing ones for particular purposes (Deplazes, 2009). This discipline builds up on constructive libraries of genetic parts ready to be recruited for any designs.

Water pollution is one of the global concerns affecting people in certain parts of the world. To alleviate this problem, microalgae have been used and developed extensively as a biological system for wastewater treatment (Wollmann et al., 2019). Heavy metals (HMs) are often referred to metal elements that are potentially toxic to human and the environment (Duffus, 2002; Rahman and Singh, 2019). Sources of HM contamination are varied, yet mostly industrial-based (Selvi et al., 2019). Heavy metal-contaminated water is conventionally treated via several approaches including the use of microalgae as a bio-remover that takes away HMs from liquid phases (Cheng et al., 2019).

Interestingly, previous reviews on similar topics only focus on 1) Genetic engineering tools and techniques for microalgae 2) Synthetic biology approaches for HM bio-removal in all potential microorganisms 3) Synthetic biology in microalgae for biomass and chemical production and 4) The use of customized algae for HM adsorption. To give some examples, a recent review by Fajardo et al. (2020) focuses on genetic engineering techniques and tools for microalgae ranging from classical to advanced ones (Fajardo et al., 2020) without any discussions on HM bio-removal. This may indicate that genetic engineering in microalgae is still considered under development. There are a few reviews on the use of genetically modified microorganisms (GMOs) to bio-remove HMs; however, they focus on all potential microorganisms, not particularly microalgae as a host in question (Diep et al., 2018; Capeness and Horsfall, 2020). Many reviews on the development of synthetic biology toolkits for microalgae only focus on biomass and chemical production (Wang et al., 2012; Jagadevan et al., 2018; Fabris et al., 2020; Kumar et al., 2020; Naduthodi et al., 2021) but not from the HM bio-removal perspective. Although, one review aims to emphasize the use of customized algae for HM bio-removal and discusses several approaches used to enhance algal performances including biological/molecular and chemical strategies, the review only focuses on adsorption as a removal mechanism (Cheng et al., 2019). Altogether, understandings on synthetic biology-based approaches applied to all microalgal HM bio-removal mechanisms is still missing. Potential genes/proteins involved in the process and their potential are yet to be collectively reported. To fill the knowledge gap, here, synthetic biology-based strategies for microalgal HM bio-removal are comprehensively discussed based on a synthetic biology concept (Design-Build-Test-Learn cycle) and all possible mechanisms used by microalgae to bio-remove HMs 1) biosorption 2) bioaccumulation and 3) biotransformation). A collective genetic part library of genes/proteins involved in and/or have been engineered for such purposes is also constructed to enable future developments of synthetic microalgal systems for HM bio-removal. Therefore, our work fills up the missing aspect on this topic and serves as a guide for stepwise developments. For instance, if a highly effective microalga is of interest as a host to bio-remove HMs, one can utilize the synthetic biology workflow presented in this mini-review to develop such systems by which they can select a strategy and handpick the most suitable genes/proteins listed here to start off the development. Specifically, if HM transporters of a microalga are observed to be ineffective, which limits the bio-removing performances, potential HM transporters from the library provided here can be handpicked and engineered.

MICROALGAL MECHANISMS TO REMOVE HEAVY METALS FROM WASTEWATER EFFLUENTS

There are several mechanisms that microalgae use to remove HMs from contaminated sources. Here, we classify these mechanisms into 1) biosorption, 2) bioaccumulation, and 3) biotransformation.

Microalgae can be used as biosorbents by which HMs are absorbed onto their cell surface, or to be specific, onto the functional groups (e.g., $-COO^-$, $-OH^-$ and $-PO_4^{3-}$) or associated proteins present on their cell walls (Monteiro et al., 2012; Kumar et al., 2015; Mantzorou et al., 2018; Salama et al., 2019). In general, these functional groups are present in negatively charged forms, thereby, attribute to the overall anionics and attract positively charged metal ions. This mechanism is called biosorption. In this regard, the components of cell wall are functionally important key players and the binding affinity of cell surface to HMs in question is a key parameter determining the effectiveness of this mechanism. Modification of cultivation conditions to alter surface properties has been demonstrated to improve Hg biosorption in *Scenedesmus obtusus* XJ-15 (Huang et al., 2019). Moreover, increased accumulation of extracellular polymeric substances (EPSs) such as exopolysaccharides (Xiao and Zheng, 2016; Naveed et al., 2019) have been reported as a HM stress response mechanism in *Chlamydomonas reinhardtii* (Li et al., 2021) presumably because HMs can immobilize onto these substances.

Some may refer to biosorption and the second mechanism presented here, bioaccumulation, as the same process since they both ultimately result in free HMs taken away from liquid phases. However, in this mini-review, we propose that they are two different mechanisms to simplify the principles and lay out plain understandings toward synthetic biology-based approaches. Bioaccumulation is defined as a process that cells uptake metal ions and accumulate them intracellularly in a so-called "import-storage system" (Diep et al., 2018). Thus, it is important to state that in biosorption, HMs can be retrieved via simple methods such as washing with chelators;

however, bioaccumulation requires cell disruption (Diep et al., 2018). Bioaccumulation is divided into 2 sequential steps: 1) cellular uptake of HMs, and 2) binding of the HMs to metal-biding peptides or polyphosphates (polyP) and sequestering in inactive forms. Microalgae uptake HMs via various transporters (Blaby-Haas and Merchant, 2012). Once HMs enter the cells, they can bind to designated biomolecules. In this section, metal-binding peptides (phytochelatins and metallothioneins) and polyphosphate bodies are discussed. Phytochelatins (PCs) are one of the metal-binding peptides (Cobbett and Goldsbrough, 2002) synthesized by biosynthetic enzymes (glutamate-cysteine ligase (GCL), glutathione synthetase (GSHS), and PC-synthase (PCS)) (Balzano et al., 2020; García-García et al., 2020). Typically, HM-PC complexes are sequestered in compartmentalized vacuoles, chloroplasts, and cytosol (Nagel et al., 1996; Zitka et al., 2011). Interestingly, glutathione—the final intermediate in PC synthesis—is also involved in HM-PC interactions (Balzano et al., 2020). Thus, all PC biosynthetic enzymes play a major part in HM bio-removal as they can determine intracellular availability of PCs (see **Section 3**). Metallothioneins (MT) are another well-known group of metal-binding peptides allowing the same process (Balzano et al., 2020). The main difference between PCs and MTs is that MTs are proteins translated directly from encoded gene, unlike PCs, which are enzymatically synthesized from intracellularly available precursors (Cobbett and Goldsbrough, 2002). Lastly, HMs can also bind to intracellular polyphosphates (polyP) because of the anionic nature of the phosphate groups (PO_4^{3-}) making up the polymer. PolyP accumulation was shown to increase upon HM stress conditions (Sanz-Luque et al., 2020) emphasizing its unrealized potential in HM-bio-removal.

Biotransformation of toxic HMs into less or non-toxic forms is another strategy used by microorganisms (Chaturvedi et al., 2015). Evidently, arsenic (As) is most toxic in the forms of As (III) and As (V), respectively. These forms can be detoxified by means of oxidation [for As (III)], reduction [for As(V)], methylation and conversion to arsenosugars/arsenolipids in different diatom species (Wang et al., 2015; Papry et al., 2019). Similarly, Hg and Cr reduction was reported to be catalyzed by intracellular reductases in microalgae (Kelly et al., 2007; Lee et al., 2017; Leong and Chang, 2020), which facilitates the bio-removal of these metals. Previously with synthetic biology, Sattayawat and colleagues described a novel concept "bioderivatization" in cyanobacteria where a toxic compound produced by the cells was biotransformed into a less toxic form. This successfully alleviated the deleterious effects on microbial cells and resulted in higher production yields (Sattayawat et al., 2020).

Intriguingly, we also reported that *Pediastrum duplex* AARLG060, a HM bio-removing microalga, performed a combination of mechanisms to remediate Mn (Thongpitak et al., 2019). This observation suggests that several engineering strategies could be implemented concurrently to achieve more impact.

SYNTHETIC BIOLOGY-BASED APPROACHES AS STRATEGIES TO ENHANCE HEAVY METAL BIO-REMOVAL FROM WASTEWATER EFFLUENTS

Although the native bio-removal mechanisms in microalgae are promising, there is still room for improvement. For instance, the use of fast-growing microalgae is preferable as this could increase total surface area for biosorption and metal uptake. However, such fast-growing microalgae are usually sensitive to high concentrations of HMs.

Several synthetic biology toolkits for microalgae have been developed over the last few years (Gimpel et al., 2016; Crozet et al., 2018; Poliner et al., 2018, 2020; de Grahl et al., 2020; Mehrshahi et al., 2020; Südfeld et al., 2021; Windhagauer et al., 2021) with a recent comprehensive review on synthetic biology-based approaches for enhancement of biomass accumulation and carbon flux partitioning (Naduthodi et al., 2021). Construction of biosynthetic pathways for attractive chemicals has also been reported (Wichmann et al., 2018, 2021; Yunus et al., 2018). Recently, a work on ground-breaking CRISPR technology in *Picochlorum celeri* (Krishnan et al., 2020) emphasizes the rise of toolkit development even more.

Synthetic Biology Workflow—Design

Identification of desirable characteristics is a fundamental key toward engineering success that can facilitate the first step in synthetic biology workflow—design (**Figure 1**). Firstly, different species or even strains of microalgae exhibit different performances as biosorbents (Kumar et al., 2015; Salama et al., 2019). This suggests that the degree of anionics plays an important role in determining microalgal performances. From this observation, modification of anionic moieties on cell walls could be the first strategy. Physically, acid treatment could alter surface functional groups and increased HM bio-removal efficiency (Almomani and Bohsale, 2021). However, this strategy also requires an extra acid treatment step. Certainly, an alternative approach is to address this through synthetic biology. The next question is, how can microalgae be genetically modified to increase the binding affinity between microalgal cell surface and HMs? A recent work by Ma and colleagues suggested that biofilm formation facilitated *Scenedesmus* Cd bio-removal efficiency (Ma et al., 2021). Given that this process is a stress response mechanism toward HMs (Li et al., 2021; Ma et al., 2021) and extracellular polymeric substances (EPSs) forming up the biofilm are generally negatively charged, engineering microalgae for enhanced biofilm production can be implemented to facilitate HM bio-removal. The genetic control of biofilm formation in microorganisms has been studied and several reports on manipulation of biofilm formation in bacteria have been discussed elsewhere (Perni et al., 2013; Benedetti et al., 2016). Mutagenesis studies of cyanobacteria for enhanced EPS production have also been investigated (Pereira et al., 2019; Yadav et al., 2020). However, these remain to be explored in microalgae.

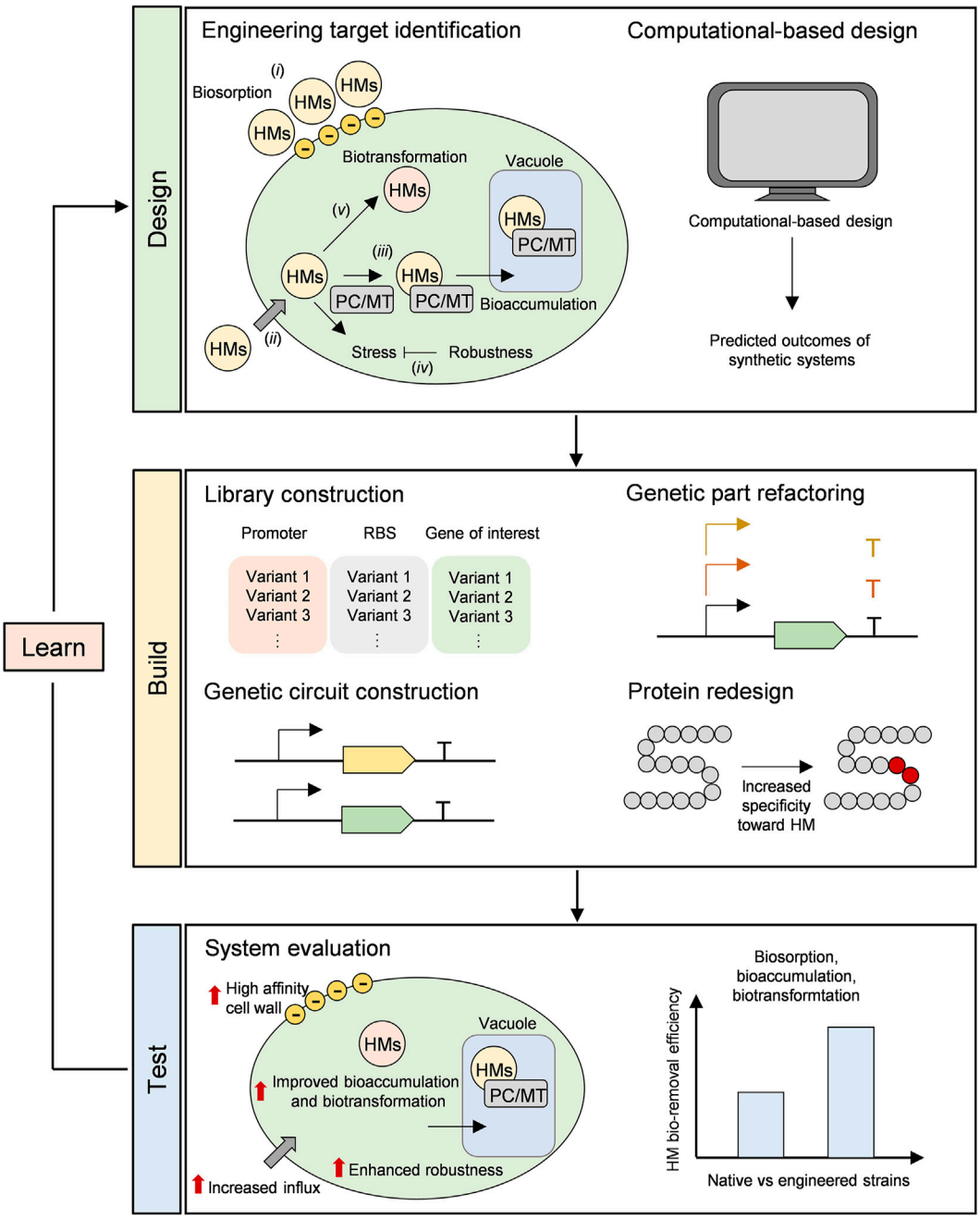

FIGURE 1 | Design-Build-Test-Learn (DBTL) cycle of synthetic biology for HM bio-removal. Design: (*i*) Biosorption (*ii*) Cellular uptake of HMs (*iii*) Bioaccumulation (*iv*) Native cellular responses to HM-induced stress (*v*) Biotransformation.

Increasing cellular uptake of HMs is another strategy employed to enhance bio-removal efficiency. Mostly, this is carried out by increasing a number or activity of HM active transporters (Zagorski and Wilson, 2004; Deng et al., 2007; Deng and Jia, 2011; Sone et al., 2013). Heterologous expression and overexpression are one of the prime synthetic biology-based approaches leading to increased protein level and activity. For example, expression of a plant transporter for Zn and Cd (AtHMA4 C-terminal domain protein) in *C. reinhardtii* increases cellular tolerance and bioaccumulation of these HMs compared with the wild-type (Ibuot et al., 2020).

Expression and overexpression of genes involved in metal-binding peptide synthesis have also been reported to promote HM bio-removal. As to metal-binding peptides, PCs and MTs are the prime candidates. PCs are enzymatically synthesized from naturally available precursors (Hirata et al., 2001; Bräutigam et al., 2011; Filiz et al., 2019; Balzano et al., 2020); therefore, heterologous expression and overexpression of PC biosynthetic

enzymes can increase their availability and capacity (Clemens et al., 1999; Bae et al., 2001; Pal and Rai, 2010; Singh et al., 2010). The expression of MT gene from chicken in *C. reinhardtii* was first reported in 1999 and showed to promote Cd bioaccumulation (Cai et al., 1999). Later, other works have also shown that the expression of MT enhanced binding affinity and cellular tolerance in the same host (Sihai et al., 2008; He et al., 2011). This is also the case when expressed in *E. coli* (Singh et al., 2008; Jafarian and Ghaffari, 2017; Shahpiri and Mohammadzadeh, 2018). Furthermore, overexpression of metal-tolerant proteins, CrMTP4, has shown to increase Cd tolerance in *C. reinhardtii* by 2.29 folds and resulted in a 3.09-fold increase in Cd bioaccumulation (Ibuot et al., 2017). In a more advanced level, synthetic phytochelatins were expressed and showed to enhance the ability of HM bioaccumulation in bacteria (Bae et al., 2000, 2002). Metalloregulatory proteins also play an important role in determining the efficiency of HM bioaccumulation and homeostasis (Quinn et al., 2003; Silver and Phung, 2005; Sommer et al., 2010; Reyes-Caballero et al., 2011; Merchant et al., 2020) with examples of engineering in *E. coli* (Sato and Kobayashi, 1998; Yang et al., 2013).

Highly tolerant microalgae tend to remove HMs more effectively due to their ability to withstand HMs at high concentrations. Several enzymes and proteins have been reported to play a role in cellular tolerance toward HMs. For example, increased superoxide dismutase (SOD) expression was observed during Hg exposure as a defense system in *Halamphora veneta* (Mu et al., 2017). Glutathione peroxidase (GPX), an enzyme responsible for cellular responses to oxidative stress from HM exposure, has also been identified in *C. reinhardtii* (Fu et al., 2002; Dayer et al., 2008; Ma et al., 2020). Another example, thioredoxins in *C. reinhardtii* are also induced upon Cd exposure (Lemaire et al., 1999). On an engineering point of view, heterologous expression of *C. reinhardtii* Fe-assimilating protein 1 (FEA1) was shown to alleviate HM toxicity in yeast and plants (Narayanan et al., 2011). Sequestration also plays a role in HM tolerance. For instance, CrMRP2 (a member of the ATP-binding cassette (ABC) transporter subfamily) in *C. reinhardtii* functions in cellular sequestration of PC-Cd complex. It was shown that Cd bioaccumulation and sequestration were higher in the wild-type compared with the CrMRP2 mutant. In the same study, expression of CrMRP2 could complement Cd-sensitive yeast mutant lacking a vacuolar glutathione (GSH)-conjugate ABC transporter (Wang and Wu, 2006).

As mentioned in the previous section, reductases have been shown to facilitate HM bio-removal. Expression of mercuric reductase from *Bacillus megaterium* MB1 in eukaryotic microalga *Chlorella* sp. DT was previously studied and shown to promote Hg bio-removal (Huang et al., 2006). For more exploratory features, construction of biotransformation pathways could be an option and has previously been implemented in bacteria (Dvořák et al., 2017).

Computational-based analysis to predict the outcomes is another approach facilitating the design step. Though, computational-based approaches for HM bio-removal have not been widely used in microalgal engineering. Molecular dynamics of PCs have been studied (Pochodylo and Aristilde, 2017) to gain better understandings on the HM-PC complexes and computational approaches for redesigning metal-binding sites were reviewed (Akcapinar and Sezerman, 2017). A web tool to facilitate the design of intron-containing transgene expression in microalgae has also been established (Jaeger et al., 2019). Note that gene expression or overexpression could burden overall cellular performances; hence, *in silico* modelling that can predict a balance state between protein expression and energy production (or metabolic flux) could be beneficial. Recently, this principle has been applied to microalgae for bioproduction purposes (Tibocha-Bonilla et al., 2018; Zuñiga et al., 2018), but not yet for HM bio-removal.

Synthetic Biology Workflow—Build

Variant libraries as a collection of genetic parts ready to be recruited are helpful in the second step of synthetic biology workflow—build (**Figure 1**). With Next Generation Sequencing (NGS) technology, more whole genome sequences are available for putative gene discovery. Previously, a list of metal transporters in microalgae has been reported based on a whole-genome analysis (Hanikenne et al., 2005). Similarly, metagenomic analysis allowed the discovery of a new MT family with confirmed ability to chelate Cu(I), Zn(II) and Cd(II) (Ziller et al., 2017). Moreover, putative HM tolerance genes from *C. acido*phila—an extremophilic green alga—are also reported based upon transcriptomic analysis (Puente-Sánchez et al., 2018).

To emphasize the potentials of employing synthetic biology in microalgal bio-removal of HMs and facilitate future designs of such systems, we accumulatively search for genes taking part in HM bio-removal and summarized in **Table 1**. Subsequently, we group the genes based on their functions: 1) HM transport system, 2) cellular tolerance, 3) synthesis of key players, 4) biotransformation reactions of HMs, and 5) regulation of gene expression. However, it should be noted that these genes are listed based on their reported potential in HM bio-removal mechanisms, some of them have never been confirmed to enhance the process experimentally.

In synthetic biology, genetic circuits can be designed and constructed in a manner that would maximize system performances. In this sense, refactoring native genetic parts—promoters, terminators—is a useful approach to enhance protein expression. Promoter and terminator engineering in *C. reinhardtii* have been studied and reported (Einhaus et al., 2020). To boost up the engineered constructs even further, fine-tuning of regulatory systems could be implemented. This strategy has been illustrated in microalgae, though in the area not related to HM bio-removal (Sun et al., 2018). Additionally, protein redesign based on computational predictions could be implemented experimentally in this step.

Synthetic Biology Workflow—Test and Learn

The final two steps in synthetic biology workflow are to test and to learn from the newly designed system (**Figure 1**). In these steps, system performances are assessed and factors affecting the system

TABLE 1 | List of genes involved or reported on heavy metal (HM) bio-removal.

Gene	HM	Original host	Heterologous expression host	Type of function	Maximum improvement (if available)	References
Cyanobacteria, microalgae, and related examples						
mntA (Mn transport system ATP-binding protein)	Mn	*Synechocystis* sp. PCC 6803	—	HM transport system	—	Kizawa et al. (2016)
mntC (Mn transporter)	Mn	*Synechocystis* sp. PCC 6803	—	HM transport system	—	Kizawa et al. (2016)
AtHMA4-CT4 (Cd/Zn transporter)	Cd	*Arabidopsis thaliana*	*C. reinhardtii*	HM transport system	~2.8-fold[a] (bioaccumulation)	Ibuot et al. (2020)
AtHMA4-FL2 (Cd/Zn transporter)	Zn	*A. thaliana*	*C. reinhardtii*	HM transport system	~3-fold[a] (bioaccumulation)	Ibuot et al. (2020)
FSD (Fe superoxide dismutase (FeSOD))	Fe	*C. reinhardtii*	—	Cellular tolerance (antioxidant activity)	—	Dudley Page et al. (2012)
MSD (Mn superoxide dismutase (MnSOD))	Mn	*C. reinhardtii*	—	Cellular tolerance (antioxidant activity)	—	Dudley Page et al. (2012)
gpx5 (glutathione peroxidase (GPX))	-	*C. reinhardtii*	—	Cellular tolerance (antioxidant activity)	—	Ma et al. (2020)
SOD (superoxide dismutase (SOD))	Hg	*Halamphora veneta* (Kützing) Levkov	—	Cellular tolerance (antioxidant activity)	—	Mu et al. (2017)
CrAPX4 (ascorbate peroxidase (CrAPX))	-	*C. reinhardtii*	—	Cellular tolerance (antioxidant activity)	—	Kuo et al. (2020)
H O -1 (Heme oxygenase-1)	Hg	*C. reinhardtii*	*C. reinhardtii*	Cellular tolerance (Suppress reactive oxygen species generation)	48.2% increase (in cell number)	Wei et al. (2011)
P5CS-1 (Δ^1-pyrroline-5-carboxylate synthetase)	Cd	Mothbean	*C. reinhardtii*	Cellular tolerance (Cd tolerance + proline accumulation)	4.16-fold (bioaccumulation) and enhanced growth	Siripornadulsil et al. (2002)
H43 (Fe-deficiency-inducible gene)	Fe	*Chlorococcum littorale*	*S. cerevisiae* Fe-uptake mutant	HM uptake system or cellular tolerance	2-fold (bioaccumulation) and partially rescued growth	Rubinelli et al. (2002)
CrMTP4 (Metal-tolerant protein)	Cd	*C. reinhardtii*	*C. reinhardtii*	Cellular tolerance	2.29-fold (bioaccumulation)	Ibuot et al. (2017)
CrMTP4 (Metal-tolerant protein)	Mn	*C. reinhardtii*	*C. reinhardtii*	Cellular tolerance	2.48-fold (bioaccumulation)	Ibuot et al. (2017)
Not specified (plasma membrane-anchored MT polymer)	Hg	Not specified	*C. reinhardtii*	Synthesis of key players	~1.45-fold[a] (biosorption/ bioaccumulation)	He et al. (2011)
CrPTC1 (P_i transporter)	-	*C. reinhardtii*	Yeast mutant YP100	Synthesis of key players	P_i uptake	Wang et al. (2021)
CrVTC4 (PolyP polymerase)	-	*C. reinhardtii*	—	Synthesis of key players	—	Wang et al. (2021)
gshA (γ-glutamylcysteine synthetase)	Cd	Synthetic gene from Uniprot ID P0A6W9	*C. reinhardtii*	Synthesis of key players	32.2% increase (bioaccumulation)	Piña-Olavide et al. (2020)
MT gene	Cd	Chicken	*C. reinhardtii*	Synthesis of key players	1.64-fold (bioaccumulation)	Cai et al. (1999)
PC4 (PC synthase (PCS))	Zn	*Dunaliella tertiolecta*	—	Synthesis of key players	—	Hirata et al. (2001)
CaPCS (PC synthase (PCS))	Cd	*C. acidophila*	—	Synthesis of key players	—	Nishikawa et al. (2006)
CaECS γ-glutamylcysteine synthetase	Cd	*C. acidophila*	—	Synthesis of key players	—	Nishikawa et al. (2006)
CrPCS (PC synthase (PCS))	Cd	*C. reinhardtii*	—	Synthesis of key players	—	Bräutigam et al. (2011)
γ-GT (γ-glutamyl transpeptidase (γ-GT))	Cd	*C. reinhardtii*	—	Synthesis of key players	—	Bräutigam et al. (2011)
cDNA of MT-like gene (Accession No. U96646)	Cd	*Festuca rubra* cv. Merlin	*C. reinhardtii*	Synthesis of key players	324.83% increase (bioaccumulation) and 55.43% increase (tolerance)	Sihai et al. (2008)
MerA (mercuric reductase)	Hg	*Bacillus megaterium* MB1	*Chlorella* sp. DT	Biotransformation reactions	~2.08-fold[a] (bioaccumulation) and ~1.98-fold[a] (biotransformation)	Huang et al. (2006)

(Continued on following page)

TABLE 1 | (*Continued*) List of genes involved or reported on heavy metal (HM) bio-removal.

Gene	HM	Original host	Heterologous expression host	Type of function	Maximum improvement (if available)	References
pcc7942_1134 (gene involved in biofilm formation)	-	*Synechococcus elongatus*	—	Synthesis of key players (biofilm formation)	—	Schatz et al. (2013)
pcc7942_1133 (gene involved in biofilm formation)	-	*Synechococcus elongatus*	—	Synthesis of key players (biofilm formation)	—	Schatz et al. (2013)
AztR (ArsR/SmtB Metalloregulator)	Zn	*Anabaena* PCC 7120	*E. coli*	Regulation of gene expression (tolerance)	Enhanced growth	Liu et al. (2005)
AztR (ArsR/SmtB Metalloregulator)	Pb	*Anabaena* PCC 7120	*E. coli*	Regulation of gene expression (tolerance)	Enhanced growth	Liu et al. (2005)
AztR (ArsR/SmtB Metalloregulator)	Cd	*Anabaena* PCC 7120	*E. coli*	Regulation of gene expression (tolerance)	Enhanced growth	Liu et al. (2005)
Bacteria, yeasts, and related examples						
mntA (Mn(II) and Cd importer)	Cd	*Lactobacillus plantarum*	*E. coli*	HM transport system	~5.3-fold[a] (bioaccumulation)	Zagorski and Wilson (2004)
copA (Cu(I) importer)	Cu	*Enterococcus hirae*	*E. coli*	HM transport system	~2.42-fold[a] (bioaccumulation)	Zagorski and Wilson (2004)
MerT and *MerP* (Hg^{2+} transport protein)	Hg	*E. coli*	*E. coli*	HM transport system	~2.71-fold[a] (bioaccumulation co-express with EC20)	Bae et al. (2001)
Ars (Metal-resistant gene)	As	Bacteria	—	Cellular tolerance	—	González Henao and Ghneim-Herrera (2021)
Cad (Metal-resistant gene)	Cd	Bacteria	—	Cellular tolerance	—	González Henao and Ghneim-Herrera (2021)
Pbr (Metal-resistant gene)	Pb	Bacteria	—	Cellular tolerance	—	González Henao and Ghneim-Herrera (2021)
CrMRP2 (ABC transporter)	Cd	*C. reinhardtii*	*S. cerevisiae*	Cellular tolerance	—	Wang and Wu (2006)
ArsR (Metalloregulatory protein)	As	*Bacillus subtilis*	*E. coli*	Regulation of gene expression (tolerance and transport)	3.4 to 5.6-fold (biosorption/ bioaccumulation)	Yang et al. (2013)
gMT (MT protein)	Pb	*Corynebacterium glutamicum*	*E. coli*	Synthesis of key players	~1.81-fold[a] (bioaccumulation)	Jafarian and Ghaffari (2017)
gMT (MT)	Zn	*Corynebacterium glutamicum*	*E. coli*	Synthesis of key players	~3.06-fold[a] (bioaccumulation)	Jafarian and Ghaffari (2017)
fMT (MT)	As	*Fucus vesiculosus*	*E. coli*	Synthesis of key players	26 to 30-fold (bioaccumulation)	Singh et al. (2008)
OsMT1 (MT)	Hg	Rice	*E. coli*	Synthesis of key players	~10-fold[a] (bioaccumulation)	Shahpiri and Mohammadzadeh (2018)
ec8 (synthetic PC $(Glu\text{-}Cys)_{20}Gly$))	Cd	Synthesized	*E. coli*	Synthesis of key players (Surface expression)	~4.75-fold[a] (bioaccumulation)	Bae et al. (2000)
ec11 (synthetic PC $(Glu\text{-}Cys)_{20}Gly$))	Cd	Synthesized	*E. coli*	Synthesis of key players (Surface expression)	~7-fold[a] (bioaccumulation)	Bae et al. (2000)
ec20 (synthetic PC $(Glu\text{-}Cys)_{20}Gly$))	Hg	Synthesized Bae et al. (2000)	*E. coli* (expression of cell surface anchored EC20)	Synthesis of key players (Surface expression)	~19-fold[a] (bioaccumulation)	Bae et al. (2001)
ec20 (synthetic PC $(Glu\text{-}Cys)_{20}Gly$))	Hg	Synthesized Bae et al. (2000)	*E. coli*	Synthesis of key players (Surface expression)	~7-fold[a] (bioaccumulation)	Bae et al. (2002)
ec20 (synthetic PC $(Glu\text{-}Cys)_{20}Gly$))	Hg	Synthesized Bae et al. (2000)	*Moraxella* sp	Synthesis of key players (Surface expression)	~6-fold[a] (bioaccumulation)	Bae et al. (2002)
GFP-hMT2A (MT)	Cu	Human	*S. cerevisiae*	Synthesis of key players	2.1-fold (bioaccumulation)	Geva et al. (2016)
Pho84p (As importer)	As	*S. cerevisiae*	*S. cerevisiae*	HM transport system	~1.4-fold[a] (bioaccumulation)	Shen et al. (2012)
PpMT1.1a (Metallothionein-like genes)	Cu	*Physcomitrella patens* (Moss)	*S. cerevisiae* (*Δcup1*)	Synthesis of key players	~1.78-fold[a] (bioaccumulation)	Pakdee et al. (2019)
MT4b (MT)	Cu	*Arabidopsis thaliana*	*S. cerevisiae* (*Δcup1*)	Synthesis of key players	~2-fold[a] (bioaccumulation)	Guo et al. (2008)
MT4a (MT)	Zn	*Arabidopsis thaliana*	*S. cerevisiae* (*Δzrc1, Δcot1*)	Synthesis of key players	~4.88-fold[a] (bioaccumulation)	Guo et al. (2008)

(Continued on following page)

TABLE 1 | (*Continued*) List of genes involved or reported on heavy metal (HM) bio-removal.

Gene	HM	Original host	Heterologous expression host	Type of function	Maximum improvement (if available)	References
Combination of strategies						
GlpF (As(III)-specific transporter) and *fMT*	As	*E. coli* and *Fucus vesiculosus*	*E. coli*	HM transport system and synthesis of key players	43.05-fold (bioaccumulation)	Singh et al. (2008)
MerP and *MerT* with synthetic *ec20*	Hg	*E. coli* and synthesized	*E. coli*	HM transport system and synthesis of key players	~19-fold[a] (bioaccumulation)	Bae et al. (2001)

[a]*Data obtained by estimation from reported graphs.*

are taken into consideration for the second round of the Design-Build-Test-Learn (DBTL) cycle. The goal of each cycle is to achieve a more optimal system, thus observations made from the previous cycle essentially put each piece of jigsaws together toward better performances. For instance, a bacterial system expressing synthetic phytochelatins showed enhanced HM bioaccumulation (Bae et al., 2000); however, it was learnt that HM uptake was a rate-limiting step of the process. Thus, the same system was improved in the second round of DBLT cycle by expressing anchored synthetic phytochelatins on the cell surface or expressing the synthetic phytochelatins together with HM transporters to overcome the limitation (Bae et al., 2001).

PROSPECTS OF SYNTHETIC BIOLOGY-BASED APPROACHES FOR MICROALGAL HEAVY METAL BIO-REMOVAL

Synthetic biology builds solely on genetic manipulations. Thus, legislations that are in effect to control the use of genetically modified organisms (GMOs) in each country are the major restrictions. Moreover, relatively few numbers of genetic parts and tools compared with that of other microorganisms also limit the development of microalgal chassis for synthetic biology, though the knowledge is building up (Tran and Kaldenhoff, 2020; Sproles et al., 2021). Omics information is also required for accurate computational predictions. This allows us to foresee cellular states after modifications and facilitates the system design. Altogether, despite such limitations, we expected that synthetic biology will gain more attention as a tool to improve microalgal HM bio-removal from wastewater effluents in the near future.

CONCLUSION

In microalgae, synthetic biology is often investigated as a tool to improve biomass and chemical production. Here, we discuss synthetic biology from another point of view—for heavy metal bio-removal from wastewater. A descriptive Design-Build-Test-Learn synthetic biology cycle is also presented for the first time to serve as a direction for straightforward implementations of synthetic biology for microalgal HM bio-removal. To facilitate this even further, we construct a genetic part library of genes and proteins involved in the process and/or reported to be engineered in a synthetic biology manner that in future, each part can be handpicked directly from our library.

AUTHOR CONTRIBUTIONS

PS conceptualized and drafted the manuscript. IY, NN, NM, WP-A, JP ,and CP revised the manuscript critically and approved the final draft.

FUNDING

This research work was partially supported by Chiang Mai University.

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