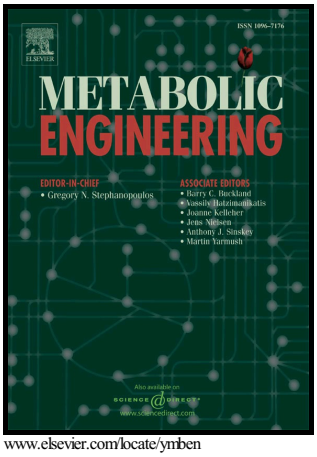

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One-Pot Two-Strain System Based on Glucaric Acid Biosensor for Rapid Screening of Myo-inositol Oxygenase Mutations and Glucaric Acid Production in Recombinant Cells

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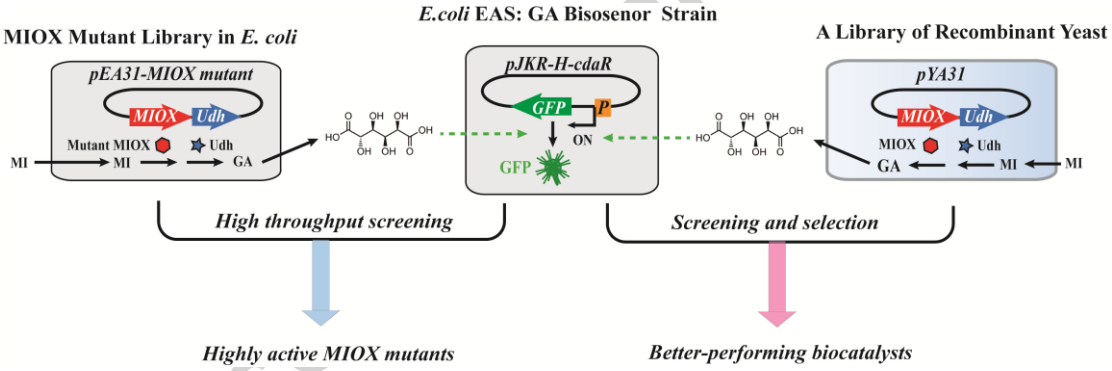
Abstract

The development of D-glucaric acid (GA) production in recombinant cells has leapt forward in recent years, and higher throughput screening and selection of better-performing recombinant cells or biocatalysts is in current demand. A biosensor system which converts GA concentration into fluorescence signal in *Escherichia coli* was developed in 2016, but its application has rarely been reported. Herein, an

effective high-throughput screening approach independent of special-purpose devices such as microfluidic platforms was established and tentatively applied. In this one-pot two-strain system, GA producers—bacterial or yeast cells containing the GA biosynthetic pathway—were sorted with the help of another *E. coli* strain acting as a GA biosensor. The identification of highly active mutants of myo-inositol oxygenase through this system validates its effectiveness in sorting *E. coli* cells. Subsequently, accurate ranking of the GA synthesis capacity of a small library of *Saccharomyces cerevisiae* strains containing distinct GA synthesis pathways demonstrated that this optimized one-pot two-strain system may also be used for eukaryotic producer strains. These results will assist in research into metabolic engineering for GA production and development of biosensor applications.

Graphical Abstract



Keywords

Biosensor; D-Glucaric acid; Screening; *Escherichia coli*; *Saccharomyces cerevisiae*; Myo-inositol oxygenase

1. Introduction

D-Glucaric acid (GA), otherwise known as saccharic acid, is an organic acid found widely in plants and mammals (Walaszek et al., 1996). GA has been identified as one of the "top value-added chemical from biomass" by the United States Department of Energy because of its potential applications as a versatile platform chemical for making synthetic polymers such as nylons and plastics (Werpy and Petersen, 2004). Therapeutic uses of GA have also been investigated, including adjuvant treatment in cancer chemotherapy and cholesterol lowering, as has its use as a food additive (Walaszek et al., 1996).

Marketed GA is currently produced chemically by nonselective oxidation of D-glucose. The production requires harsh reaction conditions, costly catalysts and complicated purification steps. Furthermore, this method is not environmentally friendly because of toxic byproducts generated during the nitric acid oxidation (Pamuk et al., 2001). It is highly desirable to develop environmentally friendly and cost-effective biosynthetic strategies to produce GA. For this, metabolic engineering is an effective strategy.

The development of GA producers based on recombinant *Escherichia coli* cells has leapt forward in recent years, and the titer and yield have increased rapidly (Moon et al., 2009, 2010; Shiue and Prather, 2014; Brockman and Prather, 2015; Reizman et al., 2015; Doong et al., 2018; Qu et al., 2018). However, once the GA titer reaches around 5 g/L, it is difficult to further increase the production because of pH-caused acid toxicity (Shiue and Prather, 2014; Gupta et al., 2016). Thus, yeast is an attractive alternative host cell for GA production because of its high acid resistance (Weber et al., 2010). A synthetic pathway for GA has been ported into *Saccharomyces*

cerevisiae (Gupta et al., 2016; Chen et al., 2018) and *Pichia pastoris* (Liu et al., 2016), and these engineered yeast cells show the ability to synthesize GA *de novo*. It should be noted that several critical points have been identified by Prather's group. Firstly, a positive correlation exists between the final GA yield of recombinant *E. coli* cells and the catalytic activity or stability of myo-inositol oxygenase (MIOX) that is the least active enzyme in the heterogeneous pathway (Moon et al., 2010; Shiue and Prather, 2014). Secondly, dynamic control of metabolic fluxes that redirect flux into the desired pathway is useful to enhance the GA titer in *E. coli* (Brockman and Prather, 2015; Reizman et al., 2015). Lastly, in 2018, Prather's group developed a pathway-independent quorum sensing system, as well as constructed a myo-inositol (MI) biosensor, and successfully modulated the timing of MIOX expression, which improved the GA metabolic pathway in *E. coli* (Doong et al., 2018).

The lack of an effective high-throughput *in vivo* screening approach remains one of the bottlenecks in research into GA biosynthesis. As well as chemical sensors (Yang et al., 2003), biosensors allowing real-time control of expression of a fluorescent protein or antibiotic resistance gene by responding to GA are an effective solution to this issue, and of high value in metabolic engineering applications (Rogers et al., 2015). In 2016, Church's group developed a series of biosensor systems in *E. coli*, including a GA biosensor based on the co-expression of the transcription factor *cdaR* and a green fluorescent protein reporter gene (*gfp*) under the control of a 521-bp promoter (Rogers and Church, 2016). The link between GA concentration and GFP expression provided by GA biosensor systems allows researchers to screen desirable cells or enzyme mutants in a high-throughput manner. However, surprisingly, few examples of applications of this fluorescent-type biosensor have been reported so far. Herein, an effective high-throughput screening approach (one-pot two-strain system)

independent of special-purpose devices was established and tentatively applied. In this system, GA producers—bacterial or yeast cells containing the GA biosynthetic pathway—were sorted with the help of another *E. coli* strain acting as a GA biosensor.

2. Materials and methods

2.1 E. coli *and yeast strain construction*

The plasmids, the *E. coli* and *S. cerevisiae* strains used in this study are listed in Table 1. More details are presented in the supplemental files.

2.2 Comparison of effectiveness of fluorescence-activated GA assays based on one-pot one-strain system and one-pot two-strain system

For the one-pot one-strain system, 5 μL seed culture of EA31S, a recombinant *E. coli* strain containing the GA biosynthetic route and biosensor system, was inoculated in deep 96-well culture plates (Sangon Biotech, Shanghai, China; Catalog No. F600580) with a working volume of 495 μL fresh Luria-Bertani (LB) broth medium with 34 μg/mL chloramphenicol and 100 μg/mL ampicillin. After incubation at 37 °C with shaking at 225 rpm for 3 h, log-phase cells were exposed to 60 mM MI and 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and continued to grow for an additional 80 min, followed by addition of extra GA into each well in the final concentration range 0 to 0.8 mM. After cultivation for another 40 min, the cell density and GFP fluorescence intensity (excitation 485 nm, emission 528 nm) were determined. Each data point represents the average of 24 values from individual wells.

With respect to the one-pot two-strain system, 5 mL of an overnight culture of EASH or EASL—biosensor-containing DH5α—was transferred into 995 mL of fresh LB medium containing 100 μg/mL ampicillin and incubated at 37 °C for 8 h. Then,

EASH (or EASL) cells were pelleted at 3000 × *g* for 5 min and resuspended in fresh LB medium with culture optical density at 600 nm (OD_{600}) of 5. Meanwhile, *E. coli* strain EA31 expressing MIOX and Udh was cultured in deep 96-well culture plates. Growth medium was 500 μL LB with 34 μg/mL chloramphenicol. To allow for meaningful well-to-well comparisons of results, all growth samples were inoculated from the same seed culture of EA31 using 5 μL per well. After incubation at 37 °C with shaking at 225 rpm for 3 h, EA31 cells were exposed to 60 mM MI and 0.2 mM IPTG and continued to grow for additional 80 min, followed by addition of extra GA into each well in the final concentration range 0 to 0.8 mM. Then, the OD_{600} values of each well were determined. Subsequently, 180 μL supernatant of individual wells was transferred into the wells of a fresh plate and respectively mixed with 120 μL biosensor cell culture (OD_{600} = 5). The plate was exposed for 40 min, and the fluorescence intensity of GFP was determined.

2.3 Mutant library preparation

A mutant library of Small Ubiquitin-like Modifier (SUMO)-MIOX fragments (up to 10^{11} variants) was created by GenScript (GenScript Inc., Nanjing, China) with advanced degenerate oligonucleotide techniques. A peer group of 192 individual transformants was sequence-verified, suggesting that the mutation frequency was 1 to 2 nucleotide mutations per kilobase-pair. The library was subcloned into pACYCDuet-MIOX-Udh by the Gibson method (Gibson, 2011) to replace the wild-type *SUMO-MIOX* fragment. Electrocompetent *E. coli* BL21 (DE3) cells were transformed with the resulting library and cells were grown on LB-agar plates supplemented with 34 μg/mL chloramphenicol overnight. Approximately 2000 single colonies were picked and separately grown overnight at 37 °C in deep 96-well plates

containing 500 μL/well LB medium (34 μg/mL chloramphenicol). For storage at −80 °C, 100 μL glycerol was added into each well.

2.4 MIOX mutation screening in one-pot two-strain system

EA31 variants were inoculated in parallel in 500 μL fresh LB medium containing 34 μg/mL chloramphenicol for 3 h at 37 °C. Cells were grown to an OD_{600} of 0.6–0.7 and expression of the enzymes (MIOX variants and uronate dehydrogenase [Udh]) was induced by the addition of 0.2 mM IPTG. Cells were incubated with 60 mM MI for 2 h at 37 °C. Afterwards, the OD_{600} values of each well were detected, following which the supernatants were collected by centrifugation (3000 × *g* for 10 min). For the purpose of screening, the responses of the GA biosensor were tested according to the procedures described in section 2.2. Briefly, these supernatants containing GA synthesized by EA31 variants were inoculated in parallel into fresh plates, and respectively mixed with EASH cells. The mixture in each well contained 180 μL of supernatant and 120 μL concentrated EASH culture (OD_{600} = 5). After an incubation time of 40 min at 37 °C, fluorescence intensities were determined.

2.5 Coupling of Udh to compare the activity of MIOX and its mutants in vitro

Plasmids isolated from 12 selected EA31 variants were sequenced (Biosune Bioscience, Shanghai, China) followed by the determination of the mutation sites in each plasmid. MIOX variant activities were checked using a Udh-coupled assay *in vitro*. Recombinant proteins preparation and activity assay details are presented in the supplemental information.

2.6 GA production by S. cerevisiae *strains sorted by coupling with* E. coli *GA biosensor*

SG, recombinant strain carrying a biosynthetic route consisting of *MIOX* and *Udh* genes, was used as the model strain to determine the ability of the one-pot two-strain system to discriminate GA production by *S. cerevisiae* cells. Briefly, strain SG was cultured in the wells of deep 96-well culture plates. The growth medium was 500 μL Yeast Nitrogen Base medium without histidine and leucine, and with 60 mM inositol. Incubation was at 30 °C for 12 h, followed by addition of extra GA into each well in the final concentration range 0 to 0.8 mM. Meanwhile, EASH culture was prepared according to the procedures described in section 2.2. Subsequently, 180 μL supernatants of individual SG samples were transferred into the wells of a fresh plate and respectively mixed with 120 μL EASH culture (OD_{600} = 5). The plate was exposed to 37 °C with shaking at 225 rpm for 2 h, and fluorescence intensity of GFP was determined. CEN.PK102-5B (Entian and Kötter, 2007) was used as the negative control strain and was treated the same as SG, except that GA was not added.

Afterwards, the GA production of six recombinant yeast strains (SG, SGI, SGTN, SGGN, SGTNI and SGGNI) and 102-5B were sorted by the optimized one-pot two-strain system according to the procedures described in the previous paragraph.

3. Results and Discussion

3.1 One-pot two-strain system is better for monitoring GA production by recombinant E. coli *than one-pot one-strain system*

EA31, a recombinant *E. coli* strain heterologously expressing SUMO-tagged mouse MIOX (accession no. NP_064361) and *Pseudomonas syringae* Udh (accession no. NP_790889), was constructed. As expected, mass spectrometry (MS) analysis identified GA production in the medium of strain EA31 (Supplemental Fig. S1A).

At first, a one-pot one-strain screening system, based on a recombinant *E. coli*

strain containing both the GA biosynthetic pathway and the biosensor system of Church's group (Rogers et al., 2015; Rogers and Church, 2016) was established and tentatively applied to rapid sorting. The plasmid pJKR-H-*cda*R (Addgene plasmid no. 62557) (Rogers and Church, 2016), carrying the transcription factor *cdaR* and a green fluorescent protein reporter gene (*gfp*) under the control of a 521-bp promoter, was transformed into strain EA31, resulting in strain EA31S. Firstly, the impacts of intracellularly synthesized GA and the supplemented GA in extracellular environment on activating the reporter gene expression were distinguished. GA titers assays of EA31S culture induced for 2 h under 0.2 mM IPTG, 60 mM MI and 37 ℃ showed that the concentration of endogenous GA in the medium was approximate 0.4 mM. Thus, 0.4 mM GA was added into EA31S culture without IPTG and MI at 37 ℃, followed by the determination of AU/OD_{600} after 40 min. The AU/OD_{600} of EA31S activated by 0.4 mM GA supplemented in extracellular environment is about 90% of the one from the cells containing similar GA titer synthesized intracellularly (Fig. S2). This result demonstrated that it could effectively simulate GA biosynthesis by the supplement of GA in extracellular environment.

As shown in the schematic of the screening approach (Figure 1A), a total of 120 EA31S cultures were grown in parallel in deep-well culture plates to the log-phase followed by the induction of MIOX and Udh. A gradient of extra GA was also added to some wells, to simulate the improvement in GA synthetic ability that might be expected for engineered strains. After cultivation for another 40 min, the cell density and GFP fluorescence intensity of each well were determined using a microplate reader. The fluorescence intensity of each sample is equal to the signal value of GFP produced by each cell multiplied by the cell density.As expected, the median values showed a good correspondence with GA concentrations (Figure 1B). However, the

analysis showed that the values obtained for parallel samples were greatly affected by the cell density, which would cause inaccurate selection when a library is subjected to high-throughput screening. In such screening, a series of EA31S variants would be activated in parallel wells and then inoculated into fresh plates. It is difficult to maintain consistency of the density of bacteria in each sample due to the individual differences as well as the inevitable procedural errors, making the one-pot one-strain screening system hard to implement effectively.

To avoid the high data errors caused by the difference in bacterial density between wells in the one-pot one-strain system, a one-pot two-strain system can be used. In this approach, GA producers—*E. coli* cells containing the GA biosynthetic route—were sorted with the help of an additional biosensor-containing strain (Figure 2 A). Herein, the GA biosynthetic route (plasmid pEA31) was introduced into *E. coli* strains BL21 (DE3); the resulting strains were named EA31. It should be noted that a low copy pET-L-cdaR plasmid was constructed with the same way as pJKR-H-cdaR (Rogers et al., 2015), except that the pUC origin of pJKR-H was replaced by the f1 origin from pET28a(+). DH5α introduced with low-copy pET-L-cdaR plasmid and high-copy pJKR-H-cdaR were named as EASL and EASH, respectively. To determine whether rapid screening would be possible using the combination of biosensor-containing strains, a one-pot two-strain system was established and optimized. Briefly, equal amounts of EASH (or EASL) bacteria and the GA-containing supernatant of EA31 culture from which the bacteria had been removed were mixed, followed by the detection of GFP fluorescence intensity after cultivation for another 40 min. Forty minutes is too short to cause significant differences of EASH (or EASL) cell density in the parallel wells. Therefore, fluorescence intensity detected in this way is directly related to the GA concentration

in the EA31 culture. Analysis of the ratio of GFP signal intensity to EA31 OD_{600} was performed using the GA yield simulation strategy (i.e. GA supplementation) described above. As shown in Figure 2 B and C, the median values (AU/OD_{600}) showed good correspondence with the GA concentrations for both the high-copy and low-copy systems. For the high-copy biosensor, the ratios of GFP signal intensity to EA31 OD_{600} were higher than the ones obtained in the low copy system. Between the groups to which the same concentration of extra GA was added, the coefficient of variation and the interquartile range normalized by Z-score (Turyn, 2013) obtained in the low or high copy systems show similar reproducibility of parallel wells, but were generally less than those from the one-pot one-strain system (Table 2), suggesting that AU/OD_{600} from the one-pot two-strain system shows better reproducibility of parallel wells.

Above, two *E. coli* screening systems (one-pot one-strain *vs.* one-pot two-strain) based on GA biosensors were established and tentatively applied in simulated test scenarios. The data from parallel samples was greatly affected by the cell density in the former approach; it is difficult to maintain the consistency of the density of each variant when a library is subjected to high-throughput screening, which would result in inaccurate selection by one-pot one-strain system. Droplet microfluidic technology could be adopted to eliminate the interference from different bacterial densities (Joensson and Andersson Svahn, 2012). With this technology, co-cultivating the metabolite producer and its biosensor cells in droplets makes it possible to couple the extracellular concentration of the metabolite of interest to the fluorescence intensity (Agresti et al., 2010). The one-pot two-strain screening system described here is a practical alternative when the complicated microfluidic screening platform is not available, and it works better than the one-pot one-strain system. The identification of

highly active mutants through high-throughput screening of MIOX mutants described in section 3.2 validates the effectiveness of this system.

3.2 Application of the GA biosensor in one-pot two-strain system and rapid screening of a MIOX mutant library in E. coli *strains*

We wanted to validate whether the one-pot two-strain system described above could be applied in the directed evolution of enzymes involved in GA biosynthesis. It should be noted that a positive correlation between GA production and MIOX activity has been observed in previous work (Moon et al., 2010; Shiue and Prather, 2014), making MIOX an attractive target for investigation to improve GA producers. A mutant library of SUMO-MIOX fragments (up to 10^{11} variants) with 1 to 2 nucleotide mutations per kilobase-pair was created and cloned into the GA biosynthesis vector. *E. coli* BL21 cells were transformed with the mutant library. Approximately 2000 EA31 variants were screened based on the approach shown in Figure 3 A. In this experiment, we cultured one EA31 variant per well, following which their supernatants were mixed with biosensor cells and sorted. The variants with the highest fluorescence intensity (top 5%) were selected in the first round and sorted in the second round. Among the screened mutants, we identified nine showing at least 15% improvement of the AU/OD_{600} value in comparison to the control (EA31, wild-type MIOX) (Figure 3 B). Meanwhile, to confirm the ability of the one-pot two-strain system to screen for downregulated mutants, three variants in which fluorescence signals were significantly lower than the ones of EA31 were selected (Fig. 3C). Liquid chromatography-mass spectrometry (LC-MS) analysis indicated that the GA concentration in the medium of each selected mutant was consistent with their AU/OD_{600} value (Figure 3B, Supplemental Fig. S4 and Supplemental table1), which

demonstrated that the one-pot two-strain system based on the GA biosensor was an effective procedure to identify improved producers in high-throughput screening.

Subsequently, the *MIOX* genes of the three selected mutants with the highest GA production were sequenced, revealing that these hits represented three different variants: single mutants D82Y and S173N, and triple mutant Q62H/S173N/L240M (the amino acid numbering is relative to the untagged MIOX). To probe the functions of these specific amino acid residues in MIOX, four single-site mutants (Q62H, D82Y, S173N and L240M) were expressed and purified (Supplemental Fig.S5). Their activities in converting MI to glucuronic acid (GlcA) were afterwards checked using a Udh-coupled assay *in vitro* (Figure 4, Supplemental Fig. S6-S7 and Table 3). Compared with the wild-type MIOX, the D82Y and S173N mutants displayed significant decreased K_m values and better k_{cat}. Thus, double mutant D82Y/S173N was prepared, and its activity also showed a drastic improvement compared with the wild-type but no statistical difference compared with the D82Y variant (Figure 4, Table 3). Additionally, homology modeling of mutants D82Y and S173N based on the structure of MIOX (Protein Data Bank ID code 2HUO) (Brown et al., 2006) and their induced-fit docking with MI were performed. The mutation sites are not among the amino acid residues lining the proposed substrate-binding pocket (Brown et al., 2006), but result in minor changes to the tertiary structure of the protein compared with wild-type MIOX (Supplemental Fig. S8). Thus, critical amino acid sites which are not located near the MI-binding pocket, usually overlooked in rational evolution based on the crystal structure or computational modeling, could be successfully identified from a random mutation library using the one-pot two-strain GA production screening approach.

3.3 The one-pot two-strain biosensor system is applicable for monitoring GA production by S. cerevisiae

As previously reported, it is difficult to increase the GA production of recombinant *E. coli* cells above 5 g/L (Shiue and Prather, 2014). Yeast is an alternative host cell for GA production because of its high acid resistance (Weber et al., 2010). Encouraged by the application of the one-pot two-strain system in the screening of the MIOX mutant library, we also tried to sort *S. cerevisiae* strains with the help of *E. coli* GA biosensor cells.

Firstly, a recombinant *S. cerevisiae* strain (SG) expressing MIOX and Udh was constructed and used as the model cell to determine the ability of the one-pot two-strain system to discriminate GA production by *S. cerevisiae*. MS analysis showed that this strain could synthesize GA (Supplemental Fig. S1B). As shown in the schematic of the screening approach (Figure 5A), additional GA was added into SG cultures, similar to the GA yield simulation strategy described for *E. coli* (section 3.1), following which the supernatant of the yeast samples was mixed with EASH culture. The plate was exposed for 2 h, and the fluorescence intensity of GFP was then determined. The response time required for the EASH strains was prolonged to 2 h after the addition of *S. cerevisiae* culture supernatant, whereas for the *E. coli* sorting system the time required was 40 min. Reproducibility between parallel samples with the same GA concentration, as well as the trend of fluorescence intensity with GA concentration, proved that rapid screening of *S. cerevisiae* strains would be possible using the *E. coli* GA biosensor (Figure 5B).

Subsequently, a library of six yeast strains (SG, SGI, SGTN, SGGN, SGTNI and SGGNI) that contained distinct pathways for GA biosynthesis were constructed

(Figure 5C). Firstly, *MIOX* and *Udh* were transformed into CEN.PK102-5B to construct strain SG; Then, *MIOX*, *Udh* and the endogenous gene *Ino1* were co-overexpressed in recombinant SGI; Heterologous gene *NOX* encoding a NADH oxidase from *Lactococcus lactis* under the regulation of constitutive promoter TEF and the NADH-dependent inducible promoter GPD were respectively introduced into SG, and the resulting strains were named SGTN and SGGN. Lastly, SGI was respectively transformed with pRS315-TEF1-nox and pYX242-GPD2-nox, resulting in the strains SGTNI and SGGNI. Then these strains were sorted in the optimized one-pot two-strain system alongside wild-type yeast (strain CEN.PK102-5B) (Figure 5D). After screening, these yeast cells were grown and the titers of GA were analyzed by LC-MS after 48 h (Figure 3D). The AU/OD_{600} values displayed the same trend as the GA concentration determined by LC-MS. This result demonstrated that the one-pot two-strain system including the *E. coli* GA biosensor is applicable for monitoring GA production in different production strains, such as *S. cerevisiae.* As a consequence of co-overexpression of inositol-3-phosphate synthase (INO1), which is important for endogenous inositol synthesis in yeast, the extracellular concentration of GA increased by 25% in strain SGI relative to SG (Figure 5D). It should be noted that GlcA was oxidized to GA by NAD^+-dependent Udh. Thus, excessive consumption of NAD^+ during GA synthesis in recombinant cells, resulting in cofactor imbalance, may be one of the factors restricting the improvement of GA production yields in recombinant cells. Herein, we introduced NADH oxidase (NOX) from *Lactococcus lactis* into a GA producer, regulated respectively by the GPD2 promoter that is induced under conditions of excess of NADH or by the constitutive TEF1 promoter. The expression of NOX under the control of the GPD2 and TEF promoters in the recombinant Ino1-MIOX-Udh background (SGNI) increased the GA yield by 58%

and 19% respectively compared with SGNI. This interesting result is direct proof that GA production can be increased by fine-tuning the NAD^+-NADH balance, and supports the previous hypothesis that cofactor engineering strategies could increase the yield of redox dependent metabolites (Hou et al., 2014).

Higher throughput screening and selection of better-performing biocatalysts in eukaryotes is currently in demand. Biosensors developed based on bacterial transcriptional regulators are straightforward and powerful components. The direct transplantation of these transcriptional activators into eukaryotes is hampered by the differences in transcriptional mechanisms between eukaryotic and prokaryotic organisms. Thus, only a few examples have been reported so far (Skjoedt et al., 2016). This work shows that sorting eukaryotic strains with the help of prokaryotic biosensor cells is feasible when a microfluidic platform is not available.

4. Conclusions

Two screening systems (one-pot one-strain *vs.* one-pot two-strain) based on a GA biosensor have been established and tentatively applied in simulated scenarios. The one-pot two-strain system shows better reproducibility between parallel samples, suggesting that this system is more apt than the one-pot one-strain system in high-throughput screening for GA yield. The identification of highly active MIOX mutants through high-throughput screening validates the effectiveness of this one-pot two-strain system. Accurate ranking of GA synthesis ability by a small library of yeast strains containing distinct GA synthesis pathways using the optimized one-pot two-strain system demonstrates that this approach may be applied to eukaryotic strains such as *S. cerevisiae*. These results will assist in research into metabolic engineering for GA production and the development of biosensor applications.

Additionally, we made two interesting findings related to GA production as consequences of the screening undertaken: (i) D82Y and S173N mutations result in increased activity of MIOX; (ii) cofactor engineering strategies, such as fine-tuning the NAD^+-NADH balance, could increase the production of redox dependent GA.

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Conflict of Interest

A patent has been published relating to this article.

Author Contributions

JS and JH designed and coordinated the work. SZ carried out the experiments. SZ and TW carried out the screening. YZ and HF conducted the model analysis of MIOX. SZ and FL conducted MIOX assay in vitro. JS and SZ wrote the manuscript. All authors have read and approved the final manuscript.

References

Agresti, J.J., Antipov, E., Abate, A.R., Ahn, K., Rowat, A.C., Baret, J.-C., Marquez, M., Klibanov, A.M., Griffiths, A.D., Weitz, D.A., 2010. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. Proc. Natl. Acad. Sci. 107, 4004–4009. https://doi.org/10.1073/pnas.0910781107

Brockman, I.M., Prather, K.L.J., 2015. Dynamic knockdown of *E. coli* central metabolism for redirecting fluxes of primary metabolites. Metab. Eng. 28, 104–113. https://doi.org/10.1016/j.ymben.2014.12.005

Brown, P.M., Caradoc-Davies, T.T., Dickson, J.M.J., Cooper, G.J.S., Loomes, K.M., Baker, E.N., 2006. Crystal structure of a substrate complex of myo-inositol oxygenase, a di-iron oxygenase with a key role in inositol metabolism. Proc. Natl. Acad. Sci. 103, 15032–15037. https://doi.org/10.1073/pnas.0605143103

Chen, N., Wang, J., Zhao, Y., Deng, Y., 2018. Metabolic engineering of *Saccharomyces cerevisiae* for efficient production of glucaric acid at high titer. Microb. Cell Factories 17. https://doi.org/10.1186/s12934-018-0914-y

Chen, Y., Daviet, L., Schalk, M., Siewers, V., Nielsen, J., 2013. Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab. Eng. 15, 48–54. https://doi.org/10.1016/j.ymben.2012.11.002

Doong, S.J., Gupta, A., Prather, K.L.J., 2018. Layered dynamic regulation for improving metabolic pathway productivity in *Escherichia coli*. Proc. Natl. Acad. Sci. 201716920. https://doi.org/10.1073/pnas.1716920115

Entian, K.-D., Kötter, P., 2007. 25 Yeast Genetic Strain and Plasmid Collections, in: Stansfield, I., Stark, M.J. (Eds.), Methods in Microbiology, Yeast Gene Analysis. Academic Press, pp. 629–666. https://doi.org/10.1016/S0580-9517(06)36025-4

Gibson, D.G., 2011. Chapter fifteen - Enzymatic Assembly of Overlapping DNA Fragments, in: Voigt, C. (Ed.), Methods in Enzymology, Synthetic Biology,

Part B. Academic Press, pp. 349–361. https://doi.org/10.1016/B978-0-12-385120-8.00015-2

Gupta, A., Hicks, M.A., Manchester, S.P., Prather, K.L.J., 2016. Porting the synthetic D-glucaric acid pathway from Escherichia coli to *Saccharomyces cerevisiae*. Biotechnol. J. 11, 1201–1208. https://doi.org/10.1002/biot.201500563

Hou, J., Suo, F., Wang, C., Li, X., Shen, Y., Bao, X., 2014. Fine-tuning of NADH oxidase decreases byproduct accumulation in respiration deficient xylose metabolic *Saccharomyces cerevisiae*. BMC Biotechnol. 14, 13. https://doi.org/10.1186/1472-6750-14-13

Joensson, H.N., Andersson Svahn, H., 2012. Droplet Microfluidics—A Tool for Single-Cell Analysis. Angew. Chem. Int. Ed. 51, 12176–12192. https://doi.org/10.1002/anie.201200460

Liu, Y., Gong, X., Wang, C., Du, G., Chen, J., Kang, Z., 2016. Production of glucaric acid from myo-inositol in engineered *Pichia pastoris*. Enzyme Microb. Technol. 91, 8–16. https://doi.org/10.1016/j.enzmictec.2016.05.009

Moon, T.S., Dueber, J.E., Shiue, E., Prather, K.L.J., 2010. Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*. Metab. Eng. 12, 298–305. https://doi.org/10.1016/j.ymben.2010.01.003

Moon, T.S., Yoon, S.-H., Lanza, A.M., Roy-Mayhew, J.D., Prather, K.L.J., 2009. Production of Glucaric Acid from a Synthetic Pathway in Recombinant *Escherichia coli*. Appl. Environ. Microbiol. 75, 589–595. https://doi.org/10.1128/AEM.00973-08

Pamuk, V., Yılmaz, M., Alıcılar, A., 2001. The preparation of D-glucaric acid by oxidation of molasses in packed beds. J. Chem. Technol. Biotechnol. 76, 186–190. https://doi.org/10.1002/jctb.356

Qu, Y.-N., Yan, H.-J., Guo, Q., Li, J.-L., Ruan, Y.-C., Yue, X.-Z., Zheng, W.-X., Tan, T.-W., Fan, L.-H., 2018. Biosynthesis of D-glucaric acid from sucrose with routed carbon distribution in metabolically engineered *Escherichia coli*. Metab. Eng. 47, 393–400. https://doi.org/10.1016/j.ymben.2018.04.020

Reizman, I.M.B., Stenger, A.R., Reisch, C.R., Gupta, A., Connors, N.C., Prather, K.L.J., 2015. Improvement of glucaric acid production in *E. coli* via dynamic control of metabolic fluxes. Metab. Eng. Commun. 2, 109–116. https://doi.org/10.1016/j.meteno.2015.09.002

Rogers, J.K., Church, G.M., 2016. Genetically encoded sensors enable real-time observation of metabolite production. Proc. Natl. Acad. Sci. 113, 2388–2393. https://doi.org/10.1073/pnas.1600375113

Rogers, J.K., Guzman, C.D., Taylor, N.D., Raman, S., Anderson, K., Church, G.M., 2015. Synthetic biosensors for precise gene control and real-time monitoring of metabolites. Nucleic Acids Res. 43, 7648–7660. https://doi.org/10.1093/nar/gkv616

Shiue, E., Prather, K.L.J., 2014. Improving d-glucaric acid production from myo-inositol in *E. coli* by increasing MIOX stability and myo-inositol transport. Metab. Eng. 22, 22–31. https://doi.org/10.1016/j.ymben.2013.12.002

Sikorski, R.S., Hieter, P., 1989. A System of Shuttle Vectors and Yeast Host Strains Designed for Efficient Manipulation of DNA in Saccharomyces Cerevisiae. Genetics 122, 19–27.

Skjoedt, M.L., Snoek, T., Kildegaard, K.R., Arsovska, D., Eichenberger, M., Goedecke, T.J., Rajkumar, A.S., Zhang, J., Kristensen, M., Lehka, B.J., Siedler, S., Borodina, I., Jensen, M.K., Keasling, J.D., 2016. Engineering prokaryotic transcriptional activators as metabolite biosensors in yeast. Nat. Chem. Biol. 12, 951. https://doi.org/10.1038/nchembio.2177

Turyn, L., 2013. Advanced Engineering Mathematics. CRC Press.

Walaszek, Z., Szemraj, J., Hanausek, M., Adams, A.K., Sherman, U., 1996. d-Glucaric acid content of various fruits and vegetables and cholesterol-lowering effects of dietary d-glucarate in the rat. Nutr. Res. 16, 673–681. https://doi.org/10.1016/0271-5317(96)00045-0

Weber, C., Farwick, A., Benisch, F., Brat, D., Dietz, H., Subtil, T., Boles, E., 2010. Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. Appl. Microbiol. Biotechnol. 87, 1303–1315. https://doi.org/10.1007/s00253-010-2707-z

Werpy, T., Petersen, G., 2004. Top Value Added Chemicals from Biomass: Volume I -- Results of Screening for Potential Candidates from Sugars and Synthesis Gas (No. DOE/GO-102004-1992). National Renewable Energy Lab., Golden, CO (US). https://doi.org/10.2172/15008859

Yang, W., Yan, J., Fang, H., Wang, B., 2003. The first fluorescent sensor for d

-glucarate based on the cooperative action of boronic acid and guanidinium groups. Chem. Commun. 0, 792–793. https://doi.org/10.1039/B300098B

Figure 1. Fluorescence-based glucaric acid (GA) assays in the one-pot one-strain system. (A) Scheme for the one-pot one-strain system. Strain EA31 cells heterologously express Small Ubiquitin-like Modifier (SUMO) -tagged mouse myo-inositol oxygenase (MIOX) and *Pseudomonas syringae* uronate dehydrogenase (Udh). The high-copy GA biosensor plasmid pJKR-H-cdaR (Rogers and Church, 2016), carrying the transcription factor *cdaR* and a green fluorescent protein reporter gene (*gfp*) under the control of a 521-bp promoter, was transformed into *Escherichia coli* EA31, resulting in strains EA31S. For the one-pot one-strain system, a total of 120 EA31S cultures were grown in parallel in deep well plates. A gradient of extra GA was added to simulate the improvement of GA production ability expected from engineered strains. (B) Data and variance were analyzed and compared for 120 samples in the one-pot one-strain system. The cell density (OD_{600}) and green fluorescent protein (GFP) fluorescence intensity (excitation 485 nm, emission 528 nm) of each well were determined. $AU/(OD_{600\ EA31S})^2$ among groups are presented as boxplots showing the median (inner line) and interquartile range (box height), and the whiskers represent the 95% confidence interval (n = 24). Additionally, the coefficient of variation and the interquartile range normalized by Z-score are listed in Table 2.

Figure 2. Fluorescence-based GA assays in the one-pot two-strain system. (A) Scheme for the one-pot two-strain system. The high-copy GA biosensor plasmid pJKR-H-cdaR and low-copy plasmid pET-L-cdaR were transformed into DH5α,

resulting in strains EASH and EASL, respectively. EASH or EASL bacteria were mixed with the GA-containing supernatant of EA31 culture from which the bacteria had been removed. A gradient of extra GA was also added to simulate the improvement of GA production ability expected from engineered strains. Data and variance were analyzed and compared for 120 samples in the one-pot two-strain system of EASH (B) and of EASL (C). $AU/OD_{600\ EA31}$ among groups are presented as boxplots showing the median (inner line) and interquartile range (box height), and the whiskers represent the 95% confidence interval (n = 24). The coefficient of variation and the interquartile range normalized by Z-score are listed in Table 2.

Figure 3. Rapid screening of a MIOX mutant library in *E. coli* strains via application of the one-pot two-strain system. (A) Scheme for the screening. Each EA31 variant was cultured in one well, following which the culture supernatants were respectively mixed with strain EASH and sorted. Approximately 2000 EA31 variants were screened. (B) Nine variants showed at least 15% improvement of AU/OD_{600} in comparison to the control (EA31). (C) Three variants of which the fluorescence is similar to EA3 which could only express Udh. Data points are the average of quadruplicate measurements.

Figure 4. D82Y and S173N mutations result in increased activity of MIOX and GA production in recombinant *E.coli*. (A) Determination of the kinetic parameters of MIOX mutants and wild-type MIOX. The mutants—signal site mutants Q62H, D82Y, S173N and L240M and double mutant D82Y/S173N—were prepared by site-directed mutagenesis. The activity of purified proteins (Supplemental Fig. S5) was identified by a Udh-coupled assay as described in the supplemental information (n=3). (B) The GA production of the EA31 variants containing MIOX mutants. Data points are the

average of quadruplicate measurements.

Figure 5. Application of the one-pot two-strain system based on the *E. coli* GA biosensor to monitor GA production by *S. cerevisiae.* (A) Scheme for the sorting with the help of *E. coli* biosensor cell. (B) Determination of the ability of the one-pot two-strain system to discriminate GA production by *S. cerevisiae*. SG heterologously expressing mouse MIOX and *P. syringae* Udh was used as the model recombinant cell to determine the ability of the one-pot two-strain system including the *E. coli* GA biosensor to discriminate the GA yield of *S. cerevisiae*. A total of 120 samples were inoculated from the same seed culture of SG, followed by addition of additional GA to simulate different GA synthesis levels. Subsequently, supernatants of individual SG samples were respectively transferred into the wells of a fresh plate and mixed with EASH culture (OD_{600} = 5). Fluorescence intensity of GFP was determined. Strain CEN.PK102-5B was used as the negative control and treated the same as SG except GA was not added. AU/OD_{600} among individual groups (n=24) is presented as boxplots showing the median (inner line) and interquartile range (box height). (C) Schematic overview of GA biosynthesis in *S. cerevisiae*. The endogenous gene *Ino1* and heterologous gene *NOX* encoding a NADH oxidase from *Lactococcus lactis* were overexpressed to improve GA production. NOX was respectively regulated by the GPD2 promoter that is induced under conditions of excess of NADH or the constitutive TEF1 promoter. (D) Fluorescence assays based on the one-pot two-strain system and GA titers assays of yeast cells. Data points are the average of quadruplicate measurements.

Table 1 Strains and plasmids

Strain or plasmid	Characteristics	Source
***E. coli* strains**		
DH5α		Thermo Fisher
BL21 (DE3)		Novagen
EASH (DH5α)	pJKR-H-cdaR	This work
EASL (DH5α)	pET-L-cdaR	This work
EA3 (BL21 [DE3])	pACYCDuet-Udh	This work
EA31 (BL21 [DE3])	pACYCDuet-MIOX-Udh	This work
EA31S (BL21 [DE3])	pJKR-H-cdaR and pACYCDuet-MIOX-Udh	This work
S. c. strains		
CEN.PK102-5B	MAT*a ura*3-52 *his*3Δ1 *leu*2-3/112	(Entian and Kötter, 2007)
SG	pIYC04-MIOX-Udh	This work
SGI	pIYC04-MIOX-Udh and pRS304-URA-INO1	This work
SGGN	pIYC04-MIOX-Udh and pYX242-GPD2-nox	This work
SGTN	pIYC04-MIOX-Udh and pRS315-TEF1nox	This work
SGGNI	pIYC04-MIOX-Udh, pYX242-GPD2-nox and pRS304-URA-INO1	This work
SGTNI	pIYC04-MIOX-Udh, pRS315-TEF1nox and pRS304-URA-INO1	This work
Plasmids		
pJKR-H-cdaR	High-copy number GA biosensor plasmid, Ap^R	(Rogers and Church, 2016)
pET28a(+)	Vector of pET-L-cdaR, Kan^R	Novagen
pET-L-cdaR	Low-copy number GA biosensor plasmid, Kan^R	This work
pACYCDuet	Expression vector, Cm^R	Novagen
pACYCDuet-MIOX-Udh	pRS315 carrying the N-His-SUMO-tagged *MIOX* gene from mice and the N-His-tagged Udh gene from *P. syringae*, Cm^R	This work
pIYC04	Expression vector, $Ap^R$and HIS3	(Chen et al., 2013)
pIYC04-MIOX-Udh	pIYC04 carrying the N-His-SUMO-tagged Mus *MIOX* gene and the *P. syringae Udh* gene, Ap^R and HIS3	This work
pRS304	Expression vector, Ap^R and TRP1	(Sikorski and Hieter, 1989)
pJFE3	The source of gene *URA*, Ap^R and URA3	(Shen et al., 2012)
pRS304-URA-INO1	pRS304 without the *TRP1* gene carrying the *Ino1* gene from *S. cerevisiae* and the *URA3* gene, Ap^R and URA3	This work
pRS315-TEF1nox	pRS315 carrying the *TEF1-nox* gene, Ap^R and LEU2	(Hou et al., 2014)
pYX242-GPD2-nox	pYX242-WS carrying the *GPD2-nox* gene, Ap^R and LEU2	(Hou et al., 2014)

Table 2 Dispersion degree of one-pot one-strain system and one-pot two-strain system

		GA (mM)[a]				
		0	0.2	0.4	0.6	0.8
CV[b]	EA31S[d]	0.081	0.083	0.114	0.119	0.127
	EA31 & EASH[e]	0.107	0.089	0.063	0.048	0.067
	EA31 & EASL[e]	0.079	0.054	0.073	0.070	0.055
IQR[c]	EA31S[d]	1.190	1.399	1.681	1.322	1.390
	EA31 & EASH[e]	0.805	0.761	1.116	1.496	1.302
	EA31 & EASL[e]	1.317	1.355	1.343	1.583	1.324

[a]gradient of extra GA was added respectively to simulate the improvement of the GA synthetic ability of the engineered strains

[b]coefficient of variation

[c]interquartile range of data normalized by Z-score (Turyn, 2013)

[d]AU/OD_{600}: the value of fluorescence intensity divided by the cell density of EA31S

[e]AU/OD_{600}: the value of GFP signal intensity to EA31 OD_{600}

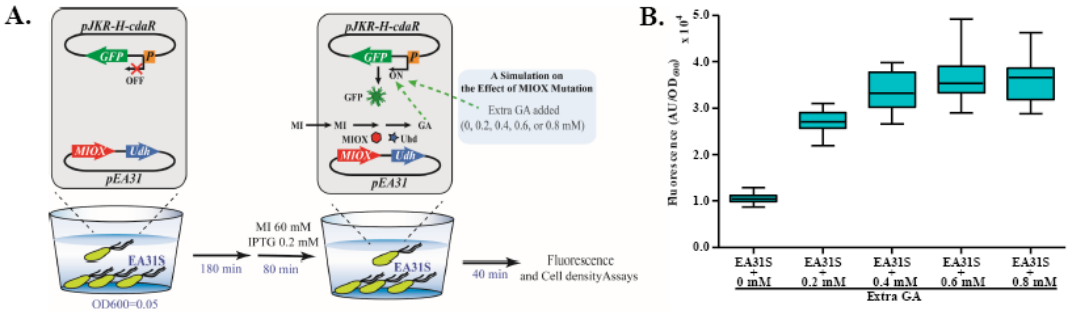
Table 3 Kinetic parameters of MIOX and its mutants.

	MIOX and its mutants					
	WT	Q62H	D82Y	S173N	L240M	D82Y/S173N
K_m (mM)	4.559	2.905	3.573	2.105	7.629	2.516
V_{max} (mM/min)	2.673	2.072	4.505	2.963	3.826	3.855
k_{cat} (s^{-1})	76.8	59.5	129.4	85.1	109.9	110.8

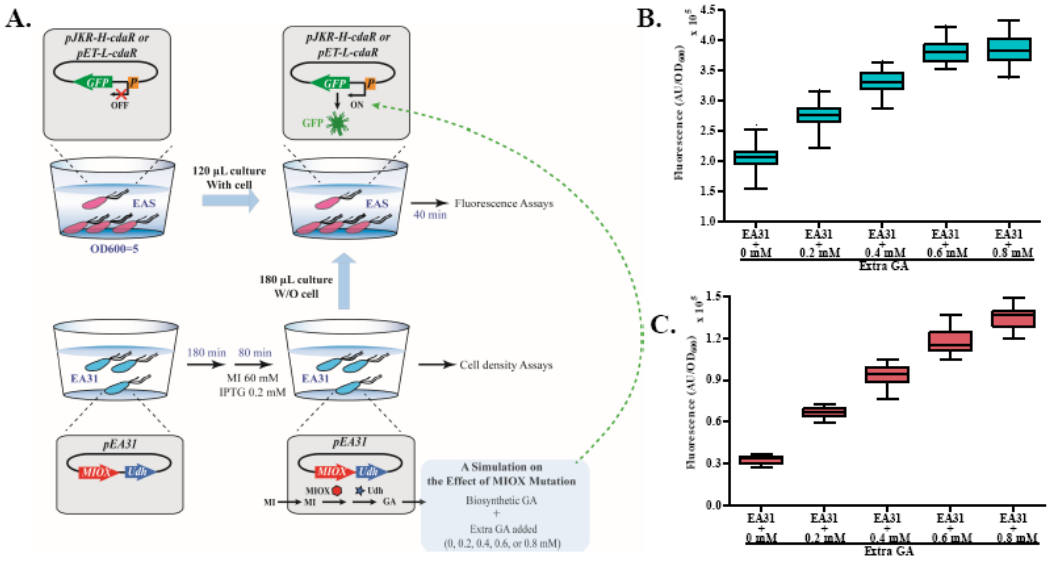
Highlights:

- An effective rapid screening approach based on D-glucaric acid (GA) biosensor in *E.coli* or yeast.
- GA producers were sorted with the help of another *E. coli* biosensor cell.
- D82Y and S173N mutations result in increased activity of MIOX.
- Fine-tuning the cofactor balance could increase the GA production of yeast.

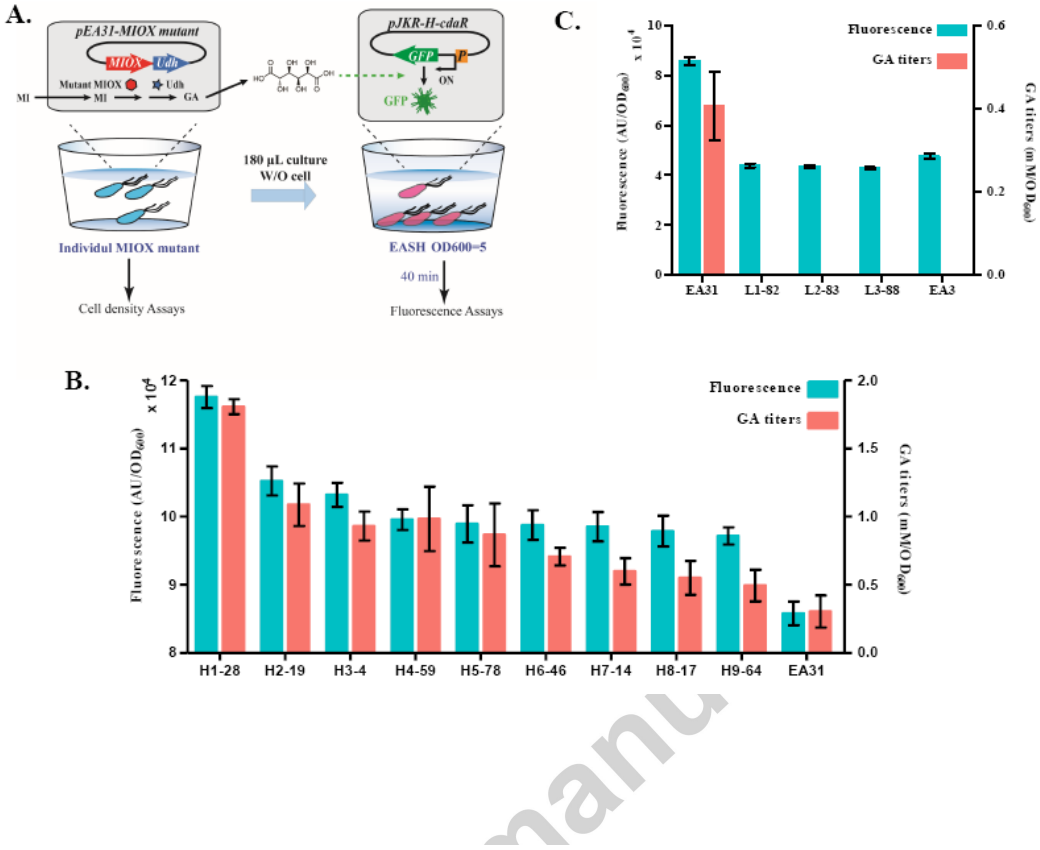
Zheng et al., Figure 1



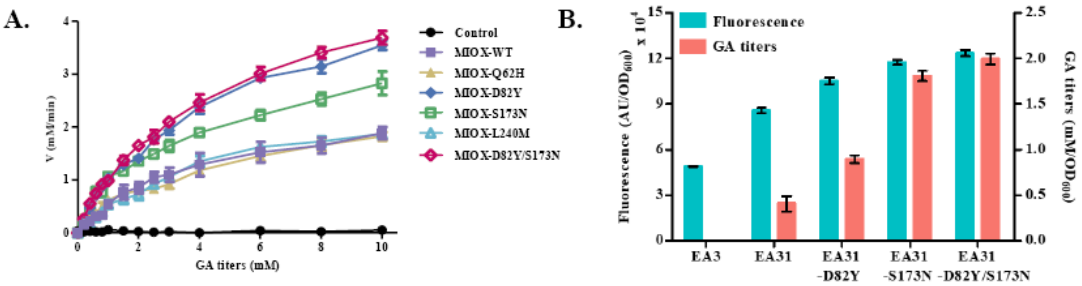
Zheng et al., Figure 2



Zheng et al., Figure 3



Zheng et al., Figure 4



Zheng et al., Figure 5