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# Directed evolution of glyoxal oxidase and high-throughput activity screening for biocatalytic valorization of furan derivatives

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## Abstract

Harnessing fungal lignocellulose-degrading enzymes offers sustainable pathways for converting biomass into value-added chemicals. Glyoxal oxidases (GLOX), copper metalloenzymes involved in lignin degradation, can catalyze the oxidation of several furan derivatives into 2,5-furandicarboxylic acid (FDCA), a bioplastic precursor. However, natural GLOX enzymes display limited catalytic activity, necessitating optimization via protein engineering. This study applied directed evolution to the *Trametes versicolor* glyoxal oxidase gene (*tvglox*), generating mutants via error-prone PCR and evaluating activity using a high-throughput, colorimetric, agar-based HRP-ABTS assay. In addition, both episomal and genome-integrated expression systems in *Pichia pastoris* were assessed. While no activity was detected from episomal expression variants, 2.5% of the genome-integrated mutants, along with wild-type controls, exhibited activity on methylglyoxal. Notably, three mutants displayed enhanced activity towards 5-hydroxy-2-furaldehyde carboxylic acid (FFCA), suggesting improved biocatalytic potential for FDCA synthesis. This study represents the first comparison of episomal and genome-integrated GLOX expression in *P. pastoris*, and highlights a rapid, cost-effective screening platform for furan-active GLOX variants.

## Introduction

The increasing impact of fossil fuel-dependent chemical production on climate change has prompted industries to adopt more sustainable practices through white biotechnology. In this context, the biotechnological conversion of lignocellulosic biomass into value-added compounds provides a sustainable strategy for producing key chemicals. Furan derivatives sourced from lignocelluloses, which constitute the majority of biomass, serve as a precursor molecule for synthesis in various industries. (Carro *et al.*, 2015; Daou *et al.*, 2019; Wohlschlager *et al.*, 2021).

5-Hydroxymethylfurfural (HMF) is a renewable building block that can be converted into valuable derivatives for the polymer industry (Figure S1-1). Its derivatives, such as 2,5-diformylfuran (DFF), 5-hydroxymethyl-2-furaldehyde-2-carboxylic acid (HMFCA), FFCA, and FDCA, hold significant potential for biotech applications (Rosatella *et al.*, 2011). Some studies have focused on exploring the bioconversion of HMF into FDCA, a precursor for bioplastics, using GLOX (EC 1.2.3.15), a copper radical oxidase from the auxiliary activity family AA5_1. However, the yield remains insufficient for industrial applications and requires

improvement through protein engineering of the enzyme or through optimization of the bioconversion process (Daou *et al*., 2019; Wohlschlager *et al*., 2021; Alpdağtaş *et al*., 2024).

Directed evolution, including both targeted and random mutagenesis, is a powerful tool in protein engineering for generating enzymes with improved activity and specificity (Cohen *et al*., 2001). Error-prone PCR (epPCR) has been widely recognized as a reliable method for introducing random mutations into enzymes (Viña-Gonzalez *et al*., 2015). A major challenge of this approach is the labor-intensive process of screening large mutant libraries. According to the literature, the use of episomal expression systems and agar-based assay techniques offers effective strategies to address this bottleneck and facilitate high-throughput screening (Lee *et al*., 2005; Jankowski & Koschorreck, 2022).

Directed evolution studies using genome-based cloning in *P. pastoris* (now reclassified as *Komagataella phaffii*) are known to make the screening of mutant libraries more labor-intensive. Contributing factors include the need for plasmid linearization for transformation, the low transformation efficiency of linear vectors, and the challenges associated with recovering the inserted gene from the genome for sequencing—factors that collectively result in a significantly prolonged screening process. To address these limitations, Lee *et al*. (2005) introduced an episomal plasmid (pBGP1) to facilitate the cloning and screening of variant libraries in *P. pastoris* (Lee *et al*., 2005). Nevertheless, during episomal expression, the high copy number may lead to the accumulation of misfolded and insoluble proteins, potentially reducing the amount of soluble and active enzyme.

On the other hand, as is well established, directed evolution studies typically generate thousands of enzyme variants. Consequently, a high-throughput activity screening process is essential. This is commonly performed using 96-well microtiter plate assays, a method known to be labor-intensive and time-consuming due to the sequential steps of cultivation, expression, and harvesting. To overcome these challenges, colorimetric plate-based assays have been proposed as a more efficient and cost-effective alternative (Alexeeva *et al*., 2002; Peña-García *et al*., 2016; Weiß *et al*., 2017). Jankowski and Koschorreck (2022) developed a multifunctional agar-based assay designed to screen *P. pastoris* mutant libraries for hydrogen peroxide ($H_2O_2$)-producing aryl-alcohol oxidase variants (Jankowski & Koschorreck, 2022). Their results show that this method reduces costs, minimizes manual workload, does not require specialized equipment, and can be applied to screen other enzymes.

This study presents the application of error-prone PCR to the *tvglox* gene from *Trametes versicolor* for the construction of a random mutant library, followed by a screening strategy based on a colorimetric agar assay to identify TvGLOX variants with enhanced activity on furan derivatives. The genomic integration of mutant *tvglox* gene (*mtvglox*) resulted in improved substrate affinity for FFCA, as indicated by the formation of a greenish halo surrounding the corresponding colonies. To the best of our knowledge, this study is the first to compare episomal and genome-integrated expression systems for mutant GLOX enzymes, while also presenting a rapid detection of randomly mutated GLOX variants that are active on furan derivatives.

## Materials and Methods

### Episomal cloning and screening

#### *Cloning of mutant genes into the episomal vector pBGP1*

The *pPICZA_wtvglox* plasmid used as a template in this study was previously constructed in our laboratory, as described by Alpdağtaş *et al*. (2024). Here, it was then subjected to epPCR to generate mutated *tvglox* genes, using a modified protocol based on Viña-Gonzalez *et al*. (2015). To control the number of mutations, five different concentrations of $MnCl_2$ were used in each PCR reaction. The reaction mixtures were prepared in a final volume of 50 µL, containing ThermoPol reaction buffer (1X), DNA template (*pPICZA_wtvglox*, 1 ng/µL), 0.2 µM forward/reverse primers, 0.2 mM dNTPs, increasing concentrations of $MnCl_2$ (0.01, 0.02, 0.04, 0.06, and 0.08 mM), and 1.25 U of Taq DNA polymerase (New England Biolabs, Inc.). The PCR reaction parameters and primer pairs were shown in Table S1. To determine the mutational load of the gene, two PCR products (0.04 mM and 0.08 mM $MnCl_2$) were selected and designated as A RM and B RM, respectively. PCR products from A RM, B RM, and the pBGP1 episomal plasmid were digested with Fast Digest BstBI (Bsp119I FD) and Fast Digest EcoRI restriction enzymes. The ligation reaction was then performed using T4 DNA ligase to produce the *pBGP1_mtvglox* (*mtvglox*: mutated *tvglox*) construct. Chemically competent *E. coli* DH5α cells were transformed with the resulting plasmid, and transformants were selected on LBLS agar plates containing 25 µg/mL Zeocin (Invitrogen, USA). Up to ten randomly selected colonies from each plate were used for colony PCR. Following PCR, 5 mL of LBLS liquid medium with 25 µg/mL Zeocin was inoculated with positive colonies and incubated overnight (37 °C, 180 rpm). The plasmids were isolated from colonies using the Plasmid Miniprep Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's instructions. Four different clones from each cloning stage (A RM and B RM) were sequenced to verify the mutation load by DNA sequencing (Eurofins Genomics, Germany). To compare the expression via enzyme activity of the mutated and wild-type enzymes, the wild-type *tvglox* gene was also cloned into the pBGP1 plasmid using the same primers and standard PCR amplification. The amplified wild-type *tvglox* gene was inserted into pBGP1 using the same restriction and ligation cloning procedures. Mutated and wild-type constructs were used to transform electrocompetent *P. pastoris* X-33

cells. Transformants were selected on YPDS agar plates supplemented with 100 µg/mL Zeocin and incubated for 3-5 days at 30 °C.

***Agar assay for screening activity of mutants from episomal expression***

Several *P. pastoris* transformants carrying the recombinant constructs were used for gene expression on the BMG agar assay. The inoculated plates were incubated at 30 °C for 3–5 days. Methylglyoxal, glyoxylic acid, and furan derivatives were individually used as substrates in the agar assay. Agar-based screening was further validated through a liquid culture activity assay, designed to compare the expression levels of *pBGP1_wtvglox* and *pPICZA_wtvglox*. Pre-cultures for episomal and genome-based expression were grown overnight (30 °C, 200 rpm) in 10 mL of BMGY and YPD, respectively. These pre-cultures were used to inoculate 10 mL of BMMY and YPD to an initial optical density at 600 nm ($OD_{600}$) of 1. The cultures were incubated for 96 h (25 °C, 200 rpm), with 0.5% (v/v) methanol added every 24 h for the AOX induction. Optical density ($OD_{600}$) and volumetric activity in the cell-free supernatant towards glyoxylic acid (the main subtrate of TvGLOX) were measured daily.

**Genome-based cloning stage and screening**

***Cloning of mutated genes into pPICZA vector for genomic integration into P. pastoris***

For genome integration, mutated inserts were cloned into pPICZA using the same restriction sites. Initially, the *pPICZA* plasmid was digested with Bsp119I FastDigest and EcoRI FastDigest enzymes to generate the *pPICZA* backbone. The mutated *tvglox* inserts were obtained by digesting the *pBGP1_mtvglox* plasmid with the same restriction enzymes. Ligation of the *mtvglox* insert into *pPICZA* was carried out using T4 DNA ligase according to the manufacturer's instructions. The resulting *pPICZA_mtvglox* construct was linearized at the 5'AOX1 region using MssI FastDigest (Thermo Fisher Scientific, Waltham, USA) and subsequently used for electroporation into *P. pastoris* X-33 cells. Recombinant transformants were selected on YPDS plates supplemented with 100 µg/mL Zeocin and incubated at 30 °C for 3–5 days.

***Agar assay for screening activity of mutants from genome-based expression***

Several *P. pastoris* transformants carrying mutant constructs were used for gene expression on BMM agar assay. The plates were incubated at 30 °C for up to 96 hours. Clones exhibiting activity towards methylglyoxal were selected for a subsequent agar assay incorporating HMF and its derivatives to identify mutants with activity on these substrates. To eliminate background activity and validate the screening procedure, *P. pastoris* colonies harbouring the empty vector (*pPICZαA*) and the wild-type construct (*pPICZA_wtvglox*) were included as negative and positive controls, respectively. All medium and assay components were listed in Table S2.

Subsequently, a comparison of volumetric activity between the new mutants and the wild-type was conducted using shaking flask cultivation. Pre-cultures for genome-based expression were grown overnight at 30 °C and 200 rpm in 50 mL of BMGY medium. These pre-cultures were then used to inoculate 200 mL of BMMY medium to an initial optical density at 600 nm($OD_{600}$) of 0.5. The cultures were incubated for four days at 25 °C and 200 rpm, with daily measurements of $OD_{600}$ and volumetric activity in the cell-free supernatant towards glyoxylic acid. The cell-free supernatant activity was determined using an ABTS–HRP coupled colorimetric assay. Each reaction was performed in a total volume of 200 µL containing 30 mM sodium phosphate buffer (pH 6.5), 0.006 mg/mL horseradish peroxidase (HRP), 0.5 mM ABTS, 1 mM glyoxylic acid, and 0.5 µM $H_2O_2$ at their final concentrations. The reaction was initiated by adding 20 µL of the enzyme-containing supernatant. ABTS oxidation was monitored spectrophotometrically at 420 nm at room temperature.

## Results and Discussion

During the developmental stages of certain fungi, GLOX can be found in the secretome alongside other oxidoreductase enzymes. By producing $H_2O_2$, GLOX facilitates lignin degradation by supporting enzymes like manganese peroxidase and unspecific peroxygenase (Whittaker *et al.*, 1996; Duran *et al.*, 2023). The involvement of GLOX in these processes has been highlighted in multiple studies, underscoring its potential in the biotechnological production of value-added chemicals (Daou *et al.*, 2019; Wohlschlager *et al.*, 2021, Koschorreck et al., 2022). However, the inherent limitations of native enzymes necessitate their optimization for industrial applications. To enhance enzyme efficiency in the conversion, reaction conditions can be optimized, or the enzyme itself can be engineered for improved functionality. For the reaction optimization part, a *P. pastoris* strain expressing the wild-type TvGLOX enzyme was previously optimized for the bioconversion of HMF to FDCA in our group (Alpdağtaş *et al.*, 2024). The second option is to enhance the properties of the target enzyme using protein engineering methods; a library of mutants derived from the target enzyme can be generated through targeted or random evolution. In particular, random libraries target the entire gene of interest and can be applied even without detailed information about the enzyme of interest (Nirantar, 2021). In the present study, wild-type *tvglox* gene was subjected to directed evolution through error-prone PCR. Both episomal and genome-integrated expression systems were evaluated in *P. pastoris*. Active strains were then identified efficiently using a colorimetric assay targeting HMF derivatives (Figure 1).

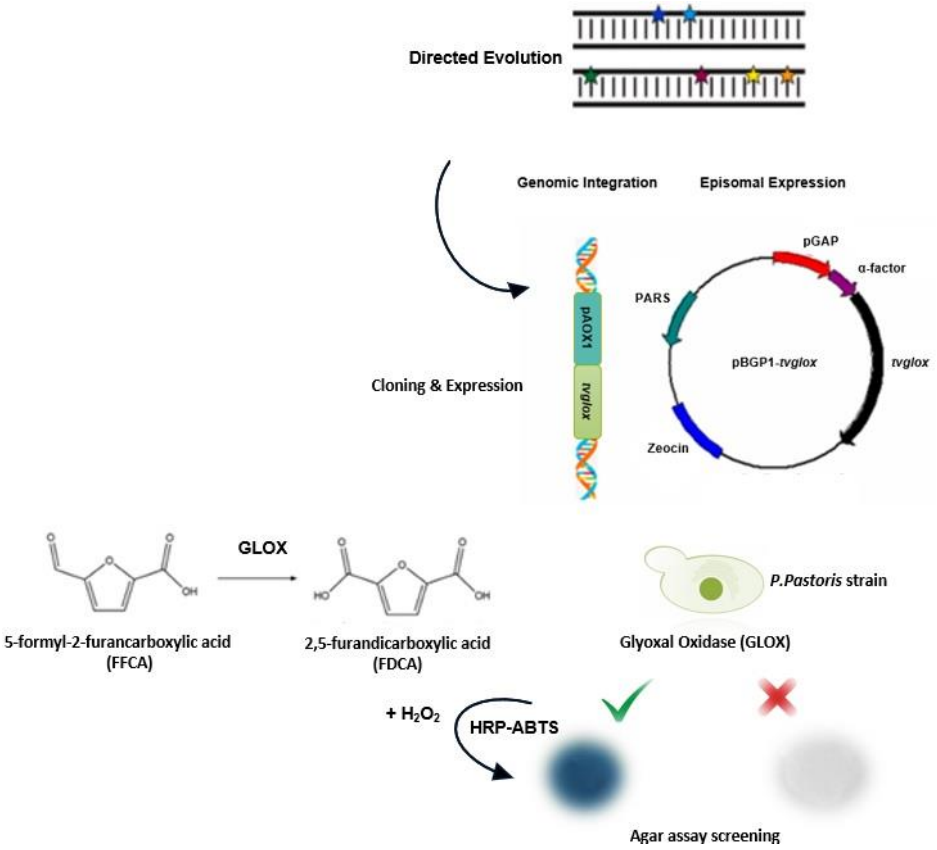


**Figure 1.** Schematic representation of the experimental workflow.

**Episomal cloning stage and screening**

This study employed two different *P. pastoris* expression systems to express mutant and wild type GLOX. As is well known, gene expression in *P. pastoris* can be achieved through two distinct systems: genome-integrated expression and episomal expression. Genomic integration refers to the incorporation of heterologous genes into the yeast genome via specific homologous sequences, whereas in episomal plasmids, heterologous genes are located in the cytoplasm of yeast cells and remain as a separate genetic element from the genome without integration (Xia *et al.*, 2021). Genomic integration typically leads to stable strains; however, the stability can be influenced by the nature of the recombination event and the type of promoter or signal peptide used (Geier *et al.*, 2015; Vogl *et al.*, 2018). Moreover, due to the frequent use of the inducible alcohol oxidase promoter (pAOX1) in genome-integrated expression systems, the requirement for methanol induction and the potentially hazardous nature of methanol—being both toxic and flammable—pose significant safety concerns (Arnau *et al.*, 2011; Cámara *et al.*, 2019). Consequently, alternative expression strategies independent of methanol induction—whether genome-integrated or episomal—continue to be actively explored. In particular, in the context of directed evolution studies, the use of multicopy episomal plasmids has been proposed as an effective strategy, as it facilitates the rapid analysis of mutations, increases transformation efficiency, and allows for the assessment of their impact on expression (Cregg *et al.*, 1985; Weiss *et al.*, 2024). Therefore, the use of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (GAP) in expression systems eliminates the need for methanol induction. However, previous studies have shown that episomal expression driven by GAP generally yields lower expression levels compared to pAOX1 (Li *et al.*, 2013; Zhu *et al.*, 2014). To verify the validity of this outcome in the case of GLOX, the episomal plasmid pBGP1—containing the constitutive GAP promoter—was used for cloning mutant *tvglox* genes, resulting in the construction of *pBGP1_mtvglox* plasmids. For this purpose, a library of randomly mutated *tvglox* genes was generated, and five distinct PCR products were obtained using increasing concentrations of $Mn^{2+}$. Since excessive mutagenesis can generate a high proportion of nonfunctional variants, while very low concentrations risk producing products with few or no mutations, we selected intermediate (0.04 mM) and upper–intermediate (0.08 mM) $MnCl_2$ concentrations for subsequent cloning. Of these, only two PCR products—those generated in the presence of 0.04 mM and 0.08 mM $MnCl_2$, hereafter referred to as A RM and B RM, respectively—were digested with the same restriction enzymes as above (Figure S1-2). These digested inserts and vectors were then ligated to generate the *pBGP1_mtvglox* constructs (Figure S1-3a). Following transformation of the recombinant plasmids, colony screening was performed using colony PCR to verify gene insertion. Plasmids from four positive clones were isolated and subjected to sequencingSequence alignments for A RM were

provided (S2 Appendix) According to the alignment data, constructs derived from A RM and B RM contained 2–8 and more than 8 point mutations, respectively (Figure S1-3b,c, S2 Appendix). It is well known that once $Mn^{2+}$ concentration increases, polymerase fidelity decreases, resulting in a higher frequency of nucleotide misincorporation (Dymond, 2013); this relationship was confirmed here experimentally. Due to the lower mutation load, the A RM-derived constructs were selected for expression and advanced to the next stage of the study. This was because our aim was to evaluate the functional consequences of a limited number of substitutions which is a strategy widely used to avoid confounding effects arising from excessive overall destabilization (Cadwell & Joyce, 1992).

Additionally, to compare the expression levels via activity between mutant and wild-type TvGLOX, similar cloning steps were performed for wild-type *tvglox* into *pBGP1*, and the colony PCR results are presented in Supplementary Figure S1-4a. The successful gene insertion was confirmed through sequencing.After generating a library through directed evolution, high-throughput screening systems are required. These options are continuously being explored as alternatives to the time-consuming and costly methods, such as gas chromatography, HPLC, and automated microplate screening. Of these methods, particularly colorimetric tests on solid media, aim to enhance screening efficiency by bypassing laborious inoculation, sample preparation, and spectral reading processes. In this context, a colorimetric agar assay detects $H_2O_2$ produced by active GLOXs, facilitating the rapid assessment of enzyme activity. Therefore, this plate-based screening method not only facilitates the identification of GLOX mutants but also enables efficient screening of similar copper radical oxidase enzymes for biotransformation potential through enzyme mining.

The expression of the relevant variant and its conversion into a detectable signal without the need for purification can be achieved by detecting $H_2O_2$ through an agar assay that involves the HRP-substrate pair (Weiß *et al.*, 2017). To perform a rapid activity screening of mutants, we used the HRP-ABTS agar assay procedure (Viña-Gonzalez *et al.*, 2015; Viña-Gonzalez *et al.*, 2016). In this assay, GLOX activity is visualized by the appearance of green zones around the GLOX-secreting transformants due to oxidized ABTS. To assess episomal expression, *pBGP1_mtvglox* and *pBGP1_wtvglox* were transformed into *P. pastoris* X-33 competent cells and grown on BMG and YPD agar plates, respectively. However, no detectable activity was obtained via colorimetric assay (Figure S1-4b).

To validate the lack of detectable expression on agar plates, volumetric enzyme activities in liquid culture were compared between episomally expressed wild-type enzyme (*pBGP1-wtglox*) and genome-integrated wild-type TvGLOX *(pPICZA-wtvglox)*. While the genome-integrated strain showed activity, the episomal expression did not exhibit any detectable signal over four days. The absence of any visible colour change on the agar plate is supported by spectral measurement. This could be attributed to factors such as protein misfolding, insolubility, or expression levels that were too low to be detected by the assay. Because the episomal system did not yield detectable activity, quantitative comparison with the genome integrated system was not feasible and is therefore not included. Consequently, we discontinued further screening of episomal expression via agar assays, and proceeded through the genome integrated expression for the mutants.

**Genome-based cloning stage and screening**

Owing to the absence of detectable activity via episomal expression, the mutated *tvglox* genes were subsequently subcloned into the pPICZA vector for genome-integrated expression. The recombinant plasmid, *pPICZA_mtvglox*, was introduced into *P. pastoris* X-33 cells by electroporation. Transformants containing *pPICZA-wtvglox* and the empty *pPICZαA* vector were used as positive and negative controls, respectively. A library comprising approximately 1,000 colonies with genome-integrated *tvglox* genes was selected and screened for activity towards methylglyoxal using the aforementioned modified agar plate assay (Figure S1-5a) (Jankowski & Koschorreck, 2022). Following 48 hours of incubation, active mutant clones expressing GLOX, under the control of the methanol-inducible *AOX1* promoter, were observed to be surrounded by green-coloured zones, attributed to the formation of the cationic ABTS oxidation product. Among all mutants, approximately 2.5% of transformants exhibited catalytic activity towards methylglyoxal (Figure S1-5b). The positive control (*wtglox*) also demonstrated activity towards methylglyoxal, whereas the negative control did not.

The primary objective of this study is to identify the most effective mutant capable of converting furan derivatives into FDCA via agar assay. As is known, the chemical synthesis of FDCA typically requires harsh conditions, including toxic catalysts, elevated pressures and temperatures, and the use of organic solvents, which are both energy-intensive and environmentally burdensome (Sajid *et al.*, 2018). However, previous studies have demonstrated that FDCA can also be produced from lignocellulosic biomass via more sustainable enzymatic routes involving various biocatalysts (Karich *et al.*, 2018; Daou *et al.*, 2019; Lappe *et al.*, 2021; Alpdağtaş *et al.*, 2024). To evaluate whether the isolated mutants possess such a conversion potential, the obtained strains were tested using separate agar plate assays containing HMF and its derivatives, including HMFCA, DFF, and FFCA. As shown in Table 1, these active clones did not exhibit activity on any of the HMF derivatives, except for FFCA. Interestingly, three mutant clones (M5, M10, and M20) showed activity toward FFCA (Table 1, Figure S1-6),

suggesting that these mutants may have a higher affinity for FFCA compared to other furan derivatives.

**Table 1**. Evaluation of active mutants in various expression systems with specific substrates

| Expression System | Substrate | Active mutant (with 2–8-point mutation) | Result (Colony appearance) |
|---|---|---|---|
| Episomal | Methylglyoxal, Glyxoylic acid, HMF, HMFCA, DFF, FFCA | None (mutant / wild type) | |
| Genomic Integration | Methylglyoxal, Glyxoylic acid | 2.5% of all mutants and wild type | |
| Genomic Integration | HMF, HMFCA, DFF | None (mutant / wild type) | |
| Genomic Integration | FFCA | 3 mutants | |

To validate the agar assay, the active strains were used for expression in shaking flask experiments conducted over 96 hours and the resulting supernatants were analyzed via spectrophotometric measurements to compare enzymatic activity. Due to the lag phase of TvGLOX enzyme towards FFCA, the activity assays were performed using glyoxylic acid, the main substrate of TvGLOX. As observed, M10 exhibits 1.2 times higher activity than the wild-type towards glyoxylic acid within the first 48 hours (Figure 2). This result may be attributed to several mechanistic differences in *P. pastoris* expression systems. For instance episomal plasmids in *P. pastoris* are unstable and prone to loss during cultivation, resulting in heterogeneous populations with lower expression (Karbalaei et al., 2020; Pan et al., 2022). This instability likely contributed to the absence of detectable GLOX activity under the GAP promoter. In addition, the constitutive GAP promoter may cause a continuous burden on the folding and secretory machinery, which can be detrimental for secreted, cofactor-dependent oxidases. In contrast, the AOX1 promoter enables high-cell-density cultivation before induction, which known to improve production of difficult or toxic proteins. Furthermore, the relative performance of episomal expression through GAP promoter is protein-dependent. For instance, Lee et al. (2005) demonstrated that, even under identical episomal expression conditions, different variants of the same enzyme can yield markedly different activity levels. This clearly demonstrates that episomal expression in *P. pastoris* is highly dependent on the specific enzyme variant being expressed. (Lee et al., 2005). Similarly, Várnai et al. (2014) demonstrated that when the expression context was fully constant, the differences in expression clearly arose from intrinsic properties of the enzymes themselves (Várnai et al., 2014). The successful secretion of active GLOX under the AOX1, therefore, suggests that inducible expression is compatible with correct folding, copper loading, and secretion of this enzyme. In addition, genomic integration provides stable inheritance and allows isolation of clones with favorable gene dosage. Screening many transformants, as commonly recommended (Ahmad et al., 2014), enabled the identification of clones producing GLOX mutants at higher levels than the wild type—an outcome not achievable with unstable episomal vectors. Although the exact mechanism requires further investigation, the improvement is likely due to a combination of mutational effects and differences in expression mechanisms."

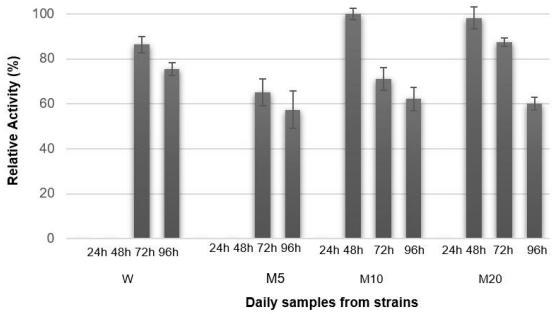


**Figure 2.** Comparison of the relative activities of genomically integrated mutants (M5, M10, M20) and wild-type strains toward glyoxylic acid.

When the same spectrophotometric assay was performed for the best mutant (M10) using FFCA as the substrate, overoxidation took time and it was observed within 20 hours. Although FFCA oxidation in the agar assay occurred over approximately 48 hours and in the deep-well plate assay over 20 hours—likely due to differences in enzyme concentration, substrate availability, and oxygen transfer between solid and liquid medium—the obtained mutant can be used as an auxiliary enzyme for FFCA bioconversion reactions (Jankowski & Koschorreck, 2022). This suggests that random mutation plus genomic integration contribute to the FFCA substrate affinity enhacement in mutants, therefore these mutants may have the potential to be further optimized for FDCA production in a cascade reaction and in addition, the point mutations in the gene and genomic integration site can be evaluated in another study for structure-function relationship.

## Conclusion

Directed evolution remains a promising approach for tailoring enzyme functionality, although its application is often constrained by the labor-intensive and time-consuming nature of mutant screening. In this study, we applied error-prone PCR to generate a random mutant library of *tvglox* and employed a rapid, agar-based colorimetric assay—modified from existing ABTS–HRP detection methods—for high-throughput activity screening. This platform enabled efficient identification of three mutants with activity toward FFCA, one of which (M10) exhibited improved catalytic performance relative to the wild-type enzyme. Additionally, we demonstrate that episomal expression of TvGLOX in *P. pastoris* under the GAP promoter was insufficient or insoluble for detectable enzyme activity,

while genomic integration under the AOX1 promoter supported functional expression. The combined approach presented here provides a scalable and cost-effective platform not only for evolving GLOX activity but also for evaluating substrate specificity across lignin-derived compounds. Future work may focus on expanding the mutagenesis strategy and elucidating structure–function relationships to enable the efficient enzymatic production of FDCA from furan derivatives.

## Ethical Statement

This study did not involve human participants, animals, or any data requiring ethical approval. Therefore, ethical approval was not required.

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## Author Contributions

SA: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – Original Draft, Writing – Review & Editing, Funding Acquisition. Katja Koschorreck and Vlada Urlacher: Conceptualization, Supervision.

## Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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