Efficient whole-cell oxidation of α,β-unsaturated alcohols to α,β-unsaturated aldehydes through cascade biocatalysis of alcohol dehydrogenase, NADPH oxidase and hemoglobin

Yan Qiao  
Zhejiang University of Technology

Can Wang  
Zhejiang University of Technology

Yin Zeng  
Zhejiang University of Technology

Tairan Wang  
Zhejiang University of Technology

Jingjing Qiao  
Zhejiang University of Technology

Chenze Lu  
China Jiliang University

Zhao Wang  
Zhejiang University of Technology

Xiangxian Ying  
Zhejiang University of Technology  https://orcid.org/0000-0002-2885-2657

Research

**Keywords:** α,β-Unsaturated aldehydes, Alcohol dehydrogenase, NADPH oxidase, NADP+ regeneration, Enzyme fusion

**DOI:** https://doi.org/10.21203/rs.3.rs-29963/v2

**License:** ☝️  This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background: α,β-unsaturated aldehydes are widely used in as organic synthesis of fine chemicals such as flavor, fragrances and pharmaceuticals. The selective oxidation of α,β-unsaturated alcohols to the corresponding α,β-unsaturated aldehydes remains challenging to overcome poor selectivity, over-oxidation and low atom efficiency in chemical routes.

Results: An *E. coli* strain co-expressing NADP⁺-specific alcohol dehydrogenase YsADH and oxygen-dependent NADPH oxidase TkNOX was constructed, which enabled the NADP⁺ regeneration and catalyzed the oxidation of 100 mM 3-methyl-2-buten-1-ol to 3-methyl-2-butenal with the yield of 21.3%. The oxygen supply was strengthened by introducing the hemoglobin VsHGB into recombinant *E. coli* cells and replacing the atmosphere of the reactor with pure oxygen, which increased the yield up to 51.3%. To further improve catalytic performance, the *E. coli* cells expressing the multifunctional fusion enzyme YsADH-(GSG)-TkNOX-(GSG)-VsHGB were achieved, which totally converted 250 mM 3-methyl-2-buten-1-ol to 3-methyl-2-butenal after 8 h of whole-cell oxidation. The reaction conditions of the cascade biocatalysis were optimized, in which the supplement of 0.2 mM FAD and 0.4 mM NADP⁺ was essential for maintaining high catalytic activity. Finally, the established whole-cell system could serve as a platform for the synthesis of valuable α,β-unsaturated aldehydes from selective oxidation of various α,β-unsaturated alcohols.

Conclusions: The construction of the strain expressing the fusion enzyme YsADH-(GSG)-TkNOX-(GSG)-VsHGB fulfilled efficient NADP⁺ regeneration and selective oxidation of various α,β-unsaturated alcohols to the corresponding α,β-unsaturated aldehydes. With the scope of redox enzymes, the fusion enzyme YsADH-(GSG)-TkNOX-(GSG)-VsHGB has become the latest successful example to improve catalytic performance in comparison with separated counterparts.

Background

α,β-unsaturated aldehydes serve as important intermediates in the organic synthesis of an extended range of fine chemicals such as flavor, fragrances and pharmaceuticals [1-3]. For example, citral (3,7-dimethyl-2,6-octadien-1-al) is in high demand for the production of ionones, vitamins A and E, as well as carotenoids [4]. α,β-unsaturated aldehydes can be obtained through the selective oxidation of the corresponding α,β-unsaturated alcohols via either chemical catalysis or biocatalysis [3, 5-7]. Traditional chemical oxidation methods have required the use of equimolar amounts of oxidizing reagents, which atom efficiency is relatively low. Moreover, practical application of chemical routes is often limited by over-oxidation, poor selectivity and the use of organic solvents and toxic compounds [8]. Alternatively, biocatalytic oxidation has raised great interest because of its excellent selectivity and environment-friendly nature as well as mild reaction conditions [9-11].

Both alcohol oxidases and alcohol dehydrogenases are attractive biocatalysts used for selective oxidation of α,β-unsaturated alcohols. Alcohol oxidases utilize the cheap and mild oxidant - molecular oxygen and generally form highly reactive hydrogen peroxide as by-product [9]. In most cases, the toxicity of hydrogen peroxide can be partially avoided by catalytic dismutation of H₂O₂ into O₂ and water using catalase. A prominent example was the recombinant aryl alcohol oxidase from *Pleurotus eryngii* (PeAAOx) used for the selective oxidation of *trans*-2-hexen-1-ol to *trans*-2-hexenal, which turnover number was greater than 2.2 million [3]. PeAAOx in active form was obtained from inclusion bodies of the cell extract, which prevented from the use of whole-cell biotransformation [12]. Compared with isolated enzymes, the use of whole-cell catalysts simplifies the procedure, reduces the cost and benefits the enzyme stability. Despite great potentials, the number of characterized alcohol oxidases was very limited in contrast to a large array of alcohol dehydrogenases with various substrate specificities [7, 13-16]. Alcohol dehydrogenases catalyze reversible oxidation reactions and require NAD(P)⁺, suggesting that efficient NAD(P)⁺ regeneration is needed to shift the reaction equilibrium for the product formation [11, 17].
The approaches of NAD(P)$^+$ regeneration could be substrate-coupled or enzyme-coupled, the latter of which required no use of excess amount of co-substrate to ensure the efficient oxidation [11]. Among enzyme-coupled approaches, the cascade bio-transformation of alcohol dehydrogenase and NAD(P)H oxidase is much more atomically efficient for NAD(P)$^+$ recycling, where direct oxidation of NAD(P)H by molecular oxygen formed hydrogen peroxide or water [18]. In particular, water-forming NAD(P)H oxidase is an ideal one because there is no by-product [19, 20]. In multiple enzymatic cascade reactions, the possibilities and advantages of enzyme fusions have been explored for various enzyme types, including the fusions of redox enzymes [21-26]. With this approach, the enzymes can be produced simultaneously and are co-localized in the cells. The fusion of an ADH with a NOX for performing alcohol oxidation supported the rapid regeneration of NADP$^+$, enabling the cascade reaction more efficient than the separate enzymes [27].

Our previous work reported that the NADP$^+$-specific YsADH, an $\alpha\beta$-unsaturated alcohol dehydrogenase from Yokenella sp. WZY002, demonstrated high activity and stability in the selective oxidation of crotonyl alcohol to crotonyl aldehyde [7]. In this work, the selective oxidation of 3-methyl-2-buten-1-ol to 3-methyl-2-butenal was chosen as the model reaction, considering that both 3-methyl-2-buten-1-ol and 3-methyl-2-butenal serve as important organic synthesis intermediates. NADPH oxidase TkNOX together with hemoglobin VsHGB capable of binding and releasing oxygen, was explored on the regeneration of NADP$^+$ and catalytic efficiency of selective oxidation [28-30] (Scheme 1). Moreover, the fusion protein of YsADH-(linker)-TkNOX-(linker)-VsHGB was constructed to increase the catalytic performance, affording to the efficient whole-cell oxidation of various $\alpha\beta$-unsaturated alcohols to the corresponding $\alpha\beta$-unsaturated aldehyde.

**Results And Discussion**

**Cascade catalysis of alcohol dehydrogenase, NADPH oxidase and hemoglobin**

The alcohol dehydrogenase from Yokenella sp. WZY002 (YsADH) was highly active in the oxidation of various $\alpha\beta$-unsaturated alcohols to the corresponding $\alpha\beta$-unsaturated aldehyde [7]. To be compatible with YsADH activity (e.g., temperature and pH optima), a thermostable NADPH oxidase from Thermococcus kodakaraensis (TkNOX) was chosen to catalyze the oxidation of NADPH, predominantly converting $O_2$ to $H_2O$ [29]. The recombinant strain expressing YsADH alone and the strain co-expressing YsADH and TkNOX were successfully constructed and induced, in which the formation of inclusion bodies was not observed (Fig. 1, Fig. S1). It was noted that the co-occurrence of YsADH and TkNOX significantly affected the expression level of YsADH. Particularly, the activity of YsADH was reduced from 3568 U/g (expression of YsADH alone) to 650 U/g (coexpression of YsADH and TkNOX) (Table S1). The whole-cell catalyst expressing YsADH alone catalyzed the oxidation of 100 mM 3-methyl-2-buten-1-ol to 3-methyl-2-butenal with the yield of 11.75% (Table 1). The yield of 3-methyl-2-butenal was increased up to 21.3% when the cells co-expressing YsADH and TkNOX were used as biocatalyst, indicating the alcohol oxidation benefited from the NADP$^+$ regeneration.

Whole-cell oxidation catalyzed by oxygen-dependent enzymes, might be restricted by oxygen supply because of the limited solubility of oxygen in water [30]. To improve the supply of oxygen, the gene encoding hemoglobin from Vitreoscilla stercoraria (VsHGB) was further introduced into the recombinant cells co-expressing YsADH and TkNOX, giving the strain co-expressing YsADH, TkNOX and VsHGB [28]. The cell-free extracts of the cells co-expressing YsADH, TkNOX and VsHGB were prepared and the activities of YsADH and TkNOX were determined to be 613 U/g and 1542 U/g, respectively. The use of the strain co-expressing YsADH, TkNOX and VsHGB increased the yield of 3-methyl-2-butenal up to 35.48%. Besides that, the atmosphere of the reactor was replaced by pure oxygen to strengthen the supply of oxygen, at which the yield of 3-methyl-2-butenal reached up to 51.3% (Table 1). To determine whether the over-oxidation of the product occurred, the possible side-products isovaleraldehyde, isovaleric acid and 3,3-dimethylacrylic acid were verified by gas-chromatography. The results indicated that none of them was detectable, demonstrating that the reaction conditions were mild enough for the oxidation of 3-methyl-2-buten-1-ol to 3-methyl-2-butenal.
Construction of the YsADH/TkNOX/VsHGB fusion proteins and their catalytic performance

In redox reactions, the enzyme fusions have been approved to an efficient approach to support the rapid regeneration of NADP⁺ [27]. To test whether the fusion of YsADH and TkNOX could improve the selective oxidation of 3-methyl-2-buten-1-ol, the fusion YsADH-TkNOX with the linker GGGGS was initially constructed and successfully induced (Fig. 2). Whole-cell catalysis of the cells expressing YsADH-(GGGGS)-TkNOX indicated that the yield of 3-methyl-2-butenal was significantly increased from 51.3% to 80.57% (Fig. 3). It was suggested that the length of the flexible linkers might affect the activity of the fusion proteins [31]. Then, the fusion genes were constructed and compared by linking the YsADH and TkNOX with GGGGS as well as other peptide linkers (GGGGS)₂, GSG and (GSG)₂. All the resulting four fusion genes were transformed into E. coli and then induced, in which the formation of inclusion bodies was not observed (Fig. 2, Fig. S2). In contrast to the strain co-expressing separated enzymes, all the fusion proteins exhibited much higher catalytic efficiency (Fig. 3). The fusion enzyme with the linker GSG, (GSS)₂ or GGGGS showed similar activity for the substrate 3-methyl-2-buten-1-ol, which was higher than that with the linker (GGGGS)₂. Using the linker GSG, the cells expressing the fusion protein YsADH-(GSS)-TkNOX-(GSS)-VsHGB or VsHGB-(GSS)-TkNOX-(GSS)-YsADH were finally achieved (Fig. S3), both of which totally converted 100 mM 3-methyl-2-buten-1-ol to 3-methyl-2-butenal after 2 h whole-cell oxidation. The functional expression of both enzymes YsADH and TkNOX was also verified by measuring their activities in cell-free extracts comprising YsADH-(GSS)-TkNOX-(GSS)-VsHGB or VsHGB-(GSS)-TkNOX-(GSS)-YsADH. The specific activities of YsADH and TkNOX in the cell free extracts comprising YsADH-(GSS)-TkNOX-(GSS)-VsHGB were determined to be 801 U/g and 983 U/g, respectively, meanwhile those in the cell free extract comprising VsHGB-(GSS)-TkNOX-(GSS)-YsADH were 752 U/g and 1064 U/g (Table S1). In the previous case of the fusions of alcohol dehydrogenase with cyclohexanone monooxygenase, the orientation of alcohol dehydrogenase-cyclohexanone monoxygenase showed low to no activity of alcohol dehydrogenase [25]. The organization of the fusion protein YsADH-(GSS)-TkNOX-(GSS)-VsHGB or VsHGB-(GSS)-TkNOX-(GSS)-YsADH was not only maintain high level of activity of both YsADH and TkNOX, but also the ratio of YsADH and TkNOX activities was more reasonable than that of the cells co-expressing YsADH, TkNOX and VsHGB separately.

Evaluation of factors affecting catalytic efficiency of whole-cell catalyst expressing YsADH-(GSG)-TkNOX-(GSG)-VsHGB

During whole-cell cascade biocatalysis, multiple reactions are run in the same pot and each reaction can often not meet for the optimal conditions [32]. To achieve optimal catalytic efficiency, it is essential to orchestrate the catalytic performance of YsADH, TkNOX and VsHGB in the fusion protein YsADH-(GSG)-TkNOX-(GSG)-VsHGB. Hence, various factors such as temperature, pH, rotation, the concentration of FAD and NADP⁺ and the substrate concentration were investigated when the cells comprising YsADH-(GSG)-TkNOX-(GSG)-VsHGB was chosen as whole-cell catalyst. To magnify the differences of catalytic performances, the biocatalyst loading was reduced to be 0.1 g lyophilized cells in 5-ml reaction mixture. The influence of the reaction temperature was determined over a range of 40-65 °C, and the highest yield of 3-methyl-2-butenal (70.35%) was observed at 45 °C (Fig. 4). When the temperature was greater than 45 °C, the product yield decreased as the temperature rose. Since TkNOX was highly thermostable and thermoactive, the thermal optima might be mainly attributed to that of YsADH [7, 29]. To determine the optimal pH, the reaction was carried out at different pH values ranging from 6.0 to 8.5 at 45 °C. The highest production was detected at pH 8.0 (Fig. 5), which was consistent with pH optima of both YsADH and TkNOX [7, 29]. Similarly, the rotation was optimized to be 600 rpm (Fig. 6), giving consideration to both mass transfer and shear force. It has suggested that the expression of FAD-dependent NOXs in E. coli might result in poor activity due to the missing of FAD [33]. The yield of 3-methyl-2-butenal (53.1%) in the presence of 0.2 mM exogenous FAD was 6.56 times higher than that without the addition of exogenous FAD. When the FAD concentration was set as 0.2 mM, 0.4 mM NADP⁺ was sufficient for maintaining high activity in the oxidation of 3-methyl-2-buten-1-ol (Fig. 7). When the substrate concentration was increased stepwise, typical time courses with the biocatalyst loading of 0.3 g lyophilized cells in 5 ml reaction mixture were shown in Fig. 8. The times for completely
converting 50, 100, 150, 200 and 250 mM 3-methyl-2-buten-1-ol to 3-methyl-2-butenal were 1.5, 2, 4, 6 and 8 h, respectively. A further increase in substrate to 300 mM resulted in 80.1% yield of 3-methyl-2-butenal within 12 h, which decrease of catalytic efficiency might be attributed to cell disintegration from the accumulation of hydrophobic compounds and/or the enzyme inactivation [12].

**Oxidation of various α,β-unsaturated alcohols to α,β-unsaturated aldehydes**

To expand applicability of the established whole-cell oxidation, various α,β-unsaturated alcohols were examined to produce α,β-unsaturated aldehydes, some of which are of great industrial interests (Table 2). For example, the oxidation of retinol was performed to form retinal, which might be further converted to carotene [34]. The oxidation of farnesol to farnesal was a key step in the synthesis of vitamin E using farnesol as a starting material [35, 36]. Similar to the case of 3-methyl-2-buten-1-ol, it was noted that 200 mM crotyl alcohol was totally converted to crotonaldehyde after 6 h oxidation. The yield of crotonaldehyde (96.70%, 8 h) with the initial 300 mM crotyl alcohol was even greater than that (80.1%, 12 h) with the initial 300 mM 3-methyl-2-buten-1-ol. The 8 h oxidation of trans-2-Hexenol, geraniol and nerol showed similar catalytic performances (83.35%~93.46% yields), whereas that of cinnamyl alcohol exhibited relatively lower yield of cinnamyl aldehyde (47.49%). The results suggested that the catalytic efficiency might be associated with the substrate solubility and/or its molecule size. In addition, the enzyme inactivation through covalent modification could not be ignored since the carbonyl group of α,β-unsaturated aldehydes (e.g., crotonaldehyde) could form a Schiff base with the lysine side chain and then the cysteine thiol group of the enzyme attacked the Cβ atom of the C=C band of α,β-unsaturated aldehydes [37]. The efforts of protein engineering toward mitigating activity inhibition are currently being implemented in our laboratory.

**Conclusions**

Rather than the use of alcohol oxidase and catalase, this study developed the whole-cell oxidation of α,β-unsaturated alcohols to α,β-unsaturated aldehydes based on the combination of alcohol dehydrogenase YsADH and NADPH oxidase TkNOX. Both NADP⁺-dependent YsADH and oxygen-dependent TkNOX were highly active and compatible on temperature and pH optima. The catalytic efficiency as well as NADP⁺ regeneration in the whole-cell oxidation was enhanced through the improvement of oxygen supply, the fusion over-expression of YsADH, TkNOX and VsHGB, and the orchestration of reaction conditions. In the case of 3-methyl-2-buten-1-ol oxidation, the whole-cell cascade catalysis system enabled efficient alcohol oxidation, but also overcame the frequently-occurred poor selectivity and over-oxidation in chemical synthesis. The established whole-cell system could be tuned to fulfill the synthesis of various α,β-unsaturated aldehydes from the selective oxidation of α,β-unsaturated alcohols.

**Methods**

**Chemicals, enzymes, plasmids and strains**

The standards α,β-unsaturated alcohols and aldehydes were obtained from Sigma-Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China). Other chemicals of analytical grade were purchased from Sangon Biotech Co. Ltd (Shanghai, China) or Shanghai Jingchun Reagent Co., Ltd (Shanghai, China). The kits and the enzymes for gene manipulation were obtained from Takara Biomedical Technology Co., Ltd. (Beijing, China). The vectors pET28a and pACYCDuet-1 were used for the over-expression of the enzymes, and the E. coli strain BL21(DE3) was used as the host. E. coli cultures were grown routinely in Luria Bertani (LB) medium at 37 °C for 12 h.

**Cloning and over-expression of YsADH, TkNOX and VsHGB in E. coli**
The DNA sequences encoding YsADH (GenBank accession number: KF887947), TkNOX (BAD85488) and VsHGB (AAA27584) were codon-optimized and synthesized at Vazyme Biotech Co., Ltd (Nanjing, China) (Figure S4). The gene encoding YsADH was introduced into the sites \textit{NcoI}/\textit{HindIII} of the vector pACYCDuet-1, yielding the recombinant plasmid pACYCDuet-1-YsADH. Moreover, the gene encoding TkNOX was further inserted into the sites \textit{NdeI}/\textit{XhoI} of the plasmid pACYCDuet-1-YsADH, yielding the recombinant plasmid pACYCDuet-1-YsADH-TkNOX. In addition, the gene encoding VsHGB was introduced into the sites \textit{EcoRI} and \textit{HindIII} of the vector pET28a, offering the recombinant plasmid pET28a-VsHGB.

The recombinant plasmids pACYCDuet-1-YsADH and pACYCDuet-1-YsADH-TkNOX were transformed into the host strain \textit{E. coli} BL21(DE3), giving the recombinant strains \textit{E. coli} BL21(DE3)/pACYCDuet-1-YsADH and \textit{E. coli} BL21(DE3)/pACYCDuet-1-YsADH-TkNOX, respectively. Furthermore, the recombinant plasmid pET28a-VsHGB was transformed into the strain \textit{E. coli} BL21(DE3)/pACYCDuet-1-YsADH-TkNOX, leading to the strain \textit{E. coli} BL21(DE3)/pACYCDuet-1-YsADH-TkNOX/pET28a-VsHGB.

The recombinant \textit{E. coli} strains were routinely grown in LB medium containing 50 \(\mu\)g/ml chloramphenicol and/or 50 \(\mu\)g/ml kanamycin at 37 °C until the OD\textsubscript{600} of 0.6–0.8. Specifically, chloramphenicol was used for the cells containing the vector pACYCDuet-1, kanamycin was used for the cells containing the vector pET28a, meanwhile both chloramphenicol and kanamycin were supplemented for the cells containing the vectors pACYCDuet-1 and pET28a. The strains were induced by adding 0.3 mM IPTG and cultured at 20 °C for 12 h. The cells were washed twice using 50 mM Tris-HCl buffer (pH 8.0) and then harvested by 8000 \(g\) centrifugation at 4 °C for 10 min. Finally, lyophilized cells were obtained by freeze-drying and stored at -20 °C for further use.

Construction and over-expression of the fusion enzymes YsADH-(linker)-TkNOX and YsADH-(linker)-TkNOX-(linker)-VsHGB in \textit{E. coli}

The fusion genes encoding YsADH, TkNOX and VsHGB were constructed by multiple overlap extension PCR [38]. To assembly four YsADH–(linker)–TkNOX fusion genes, the stop codon of the YsADH gene was removed, and the linkers of different lengths, (GSG)\textsubscript{n} (n=1, 2) or (GGGGS)\textsubscript{n} (n=1, 2), were introduced between the open reading frames of the YsADH and TkNOX genes via two rounds of PCR. The first round of the PCR introduced the linkers (GSG)\textsubscript{n} (n=1, 2) and (GGGGS)\textsubscript{n} (n=1, 2) into the YsADH gene using four pairs of primers. Simultaneously, the complementary linkers (GSG)\textsubscript{n} (n=1, 2) and (GGGGS)\textsubscript{n} (n=1, 2) were introduced into the TkNOX gene using four other pairs of primers (Table 3). Each PCR product was purified and served as a template in the second round of PCR. The PCR program included a 4 min period at 98 °C, 32 cycles at 98 °C (10 s), 58 °C (10 s) and 72 °C (30 s), and a final 5 min extension at 72 °C. The gel purified PCR products were ligated into a pACYCDuet-1 vector. Next, the PCR products of the YsADH and TkNOX genes were joined by overlapping extension PCR. The PCR program included a 4 min period at 98 °C, 32 cycles at 98 °C (10 s), 58 °C (10 s) and 72 °C (30 s), and a final 5 min extension at 72 °C. The gel purified PCR products were ligated into pACYCDuet-1 vector. The four fusion genes were confirmed by sequencing. Finally, the four fusion genes were ligated to pACYCDuet-1 between \textit{Ncol} and \textit{HindIII} sites, yielding pACYCDuet-1-YsADH(GGGGS)-TkNOX, pACYCDuet-1-YsADH(GGGGS)\textsubscript{2}-TkNOX, pACYCDuet-1-YsADH(GSG)-TkNOX and pACYCDuet-1-YsADH(GSG)\textsubscript{2}-TkNOX. Using the construction of the plasmid pACYCDuet-1-YsADH(GGGGS)-TkNOX as an example, the procedure was depicted in Fig. S5. Each expression construct pACYCDuet-1-YsADH(GGGGS)-TkNOX, pACYCDuet-1-YsADH(GGGGS)\textsubscript{2}-TkNOX, pACYCDuet-1-YsADH(GSG)-TkNOX or pACYCDuet-1-YsADH(GSG)\textsubscript{2}-TkNOX, was transformed into \textit{E. coli} BL21(DE3) grown in LB medium containing 50 \(\mu\)g/ml chloramphenicol.

During the construction of YsADH-(linker)-TkNOX-(linker)-VsHGB fusion gene, the linker was chosen set as GSG. Similar to the construction of YsADH–(linker)–TkNOX fusion genes, the fusion gene encoding YsADH(GSG)-TkNOX(GSG)-VsHGB or VsHGB(GSG)-TkNOX(GSG)-YsADH was obtained via two rounds of PCR and confirmed by sequencing. The fusion
gene was ligated to pACYCDuet-1 between Ncol and HindIII sites and the resulting recombinant plasmid pACYCDuet-1-
YsADH(GSGr)-TkNOX(GSG)-VsHGB or pACYCDuet-1-VsHGB(GSG)-TkNOX(GSG)-YsADH. Using the construction of the
plasmid as an example, the procedure was depicted in Fig. S6. Then, recombinant plasmid pACYCDuet-1-YsADH(GSGr)-
TkNOX(GSG)-VsHGB or pACYCDuet-1-VsHGB(GSG)-TkNOX(GSG)-YsADH was transformed into E. coli BL21(DE3) grown
in LB medium containing 50 μg/ml chloramphenicol. Following the same procedure for the induction mentioned above,
the cells expressing the fusion enzyme YsADH-(linker)-TkNOX, YsADH(GSG)-TkNOX(GSG)-VsHGB or VsHGB(GSG)-
TkNOX(GSG)-YsADH were induced, harvested and lyophilized.

Enzyme assays

The lyophilized cells were re-suspended in the 50 mM Tris-HCl buffer (pH 8.0) and disrupted through ultrasonication for 10
min. After that, the cell-debris pellet and cell-free extract were separated by 17000 g centrifugation at 4 °C for 10 min.
Then, the cell-debris pellet was re-suspended to the same volume of cell-free extract using the 50 mM Tris-HCl buffer (pH
8.0). Finally, the cell-debris pellet and cell-free extract samples were run by SDS-PAGE (12% acrylamide in the resolving
gel) and stained with Coomassie Brilliant Blue R-250 [39]. Activities of TkNOX in cell-free extracts were determined according to the previously-reported procedure [20]. Activities of
YsADH in cell-free extracts were measured at 45 °C by monitoring the change of the absorbance at 340 nm. The enzyme
assay for alcohol oxidation was carried out at 45 °C in triplicate in a reaction mixture (2.5 ml) composed of 20 mM crotyl
alcohol and 1 mM NADP\(^+\) in 50 mM Tris-HCl (pH 8.0) buffer. The reaction was started by the addition of the enzyme. One
unit of the activity is defined as formation or oxidation of 1 μmol NADPH per min. The protein concentrations of all
samples were determined using the Bradford reagent with bovine serum albumin as the standard protein [40]. In addition,
the determination of H\(_2\)O\(_2\) was conducted according to the previously-reported procedure [33].

The reaction mixture of α,β-unsaturated alcohol oxidation and its optimization

The set up of the reactor with hot plate/magnetic stirrer was shown in Fig. S7, in which the three-neck flask with magnetic
stirring bar was used as reaction vessel and the balloon was used to fill the atmosphere of reactor with oxygen. The
standard reaction mixture (5 ml) contained 100 mM α,β-unsaturated alcohols, 0.3 g lyophilized cells, 0.4 mM NADP\(^+\), 0.2
mM FAD, 50 mM Tris-HCl buffer (pH 8.0). The reaction was carried out at 45 °C and 600 rpm for 2 h. The optimal
temperature of alcohol oxidation was determined at a series of temperatures ranging from 40 to 65 °C. The optimal pH
was determined over a range of pH 6.0 to 8.5 at 45 °C. The buffers used were piperazine-1,4-bisethanesulfonic acid
(PIPES, pH 6.1-7.5), Tris-HCl (pH 7.5-9.0). The optimal rotation was determined over a range of 400 to 900 rpm. The
concentrations of FAD and NADP\(^+\) were explored within the range of 0 to 1 mM. After alcohol oxidation, the reaction
mixture was extracted by 5 ml of ethyl acetate under strong vibration. The organic phase in samples was separated by
8000 g centrifugation at room temperature for 10 min and dehydrated by anhydrous sodium sulfate, and then 1 μl
dehydrated sample was applied for GC analysis.

Determination of substrates, products and possible by-products by gas chromatograph

α,β-unsaturated alcohols/aldehydes and possible by-products were determined by GC (Agilent 6890N) equipped with an
FID detector and chiral capillary BGB-174 column (BGB Analytik, Böckten, Switzerland, 30 m × 250 µm × 0.25 µm). The
flow rate and split ratio of N\(_2\) as the carrier gas were set as 1.38 ml/min and 1:100, respectively. Both injector and detector
were kept at 250°C. The injection volume was 1 μl.

For crotyl alcohol/crotonaldehyde, 3-methyl-2-buten-1-ol/3-methyl-2-butenal, trans-2-hexenol/trans-2-Hexenal and
cinnamyl alcohol/cinnamaldehyde, the column temperature program was listed as follows; initial temperature of 75 °C for
3 min, 10 °C/min ramp to 120 °C for 3 min, and 30 °C/min ramp to 180 °C for 3 min. For geraniol/geranal and nerol/neral,
the column temperature program was listed as follows; initial temperature of 75 °C for 3 min, 4 °C/min ramp to 120 °C for 3 min, and 30 °C/min ramp to 180 °C for 3 min. The retention times of the above-mentioned substrates and products were summarized in Table 4.

For possible by-products isovaleraldehyde, isovaleric acid and 3,3-dimethylacrylic acid, the column temperature program was listed as follows; initial temperature of 60 °C for 5 min, 10 °C/min ramp to 120 °C for 3 min, and 30 °C/min ramp to 180 °C for 3 min. The retention times for isovaleraldehyde, 3,3-dimethylacrylic acid and isovaleric acid were 6.987 min, 7.839 min and 9.612 min, respectively.

**HPLC-based determination of retinol and retinal**

Retinol and retinal were determined by HPLC (Waters 2010) equipped with an UV detector and C-18 column (Welwich, 30 m × 250 µm × 0.25 µm). The HPLC conditions were listed as follows: Temperature, 40 °C; the wavelength of UV detector, 340 nm; flow rate, 1 ml/min; mobile phase, methanol: acetonitrile=95: 5. The retention time for retinol and retinal were 5.237 min and 5.618 min, respectively.

### Additional Files

Table S1. The activities for YsADH and TkNOX in the cell-free extracts of whole-cell catalysts; Figure S1. SDS-PAGE analyses of cell-free extract and cell-debris pellet from the same whole catalysts comprising YsADH, TkNOX and/or VsHGB; Figure S2. SDS-PAGE analyses of cell-free extract and cell-debris pellet from the same whole catalysts comprising the fusion enzyme of YsADH and TkNOX; Figure S3. SDS-PAGE analyses of cell-free extract and cell-debris pellet from the same whole catalysts comprising the fusion enzyme of YsADH, TkNOX and VsHGB; Figure S4. The codon-optimized nucleotide sequences encoding YsADH (a), TkNOX (b) and VsHGB (c); Figure S5. The construction of the pACYCDuet1-YsADH-(GSG)-TkNOX; Figure S6. The construction of the pACYCDuet1-YsADH-(GSG)-TkNOX-(GSG)-VsHGB; Figure S7. The reactor with hot plate/magnetic stirrer (a) and its key components (b).

### Declarations

**Author's contributions**

XY designed the study. YQ, CW, YZ, TW and JQ carried out the experiments. CL, ZW and XY analyzed and interpreted the data. YQ and XY wrote the manuscript. All authors read and approved the final manuscript.

**Author details**

1 Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China. 2 College of Life Sciences, China Jiliang University, Hangzhou 310018, China; .

**Acknowledgements**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**
The gene sequences in this study are available in the GenBank with accession number of KF887947 for YsADH, BAD85488 for TkNOX and AAA27584 for VsHGB.

Funding

This work was supported by The Natural Science Foundation of Zhejiang Province, China (No. LY17B020012) and Xinmiao Talents Program of Zhejiang Province (No. 2020R403072).

Abbreviations

YsADH: alcohol dehydrogenase from *Yokenella* sp. WZY002; TkNOX: NADPH oxidase from *Thermococcus kodakaraensis*; VsHGB: hemoglobin from *Vitreoscilla stercoraria*; FAD: flavin adenine dinucleotide; NADP: nicotinamide adenine dinucleotide phosphate; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; IPTG: isopropyl α-d-1-thiogalactopyranoside; LB: Luria-Bertani; GC, gas chromatograph; FID, flame ionization detector; HPLC, high performance liquid chromatography.

References


Tables

Table 1 Selective oxidation of 3-methyl-2-buten-1-ol to 3-methyl-2-butenal catalyzed by the cells expressing YsADH, TkNOX and/or VsHGBa.

<table>
<thead>
<tr>
<th>The protein(s) expressed in E. coli cells</th>
<th>Yield of 3-methyl-2-butenal (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>YsADH</td>
<td>11.75 ± 0.22</td>
</tr>
<tr>
<td>YsADH and TkNOX</td>
<td>21.3 ± 0.79</td>
</tr>
<tr>
<td>YsADH, TkNOX and VsHGB</td>
<td>35.48 ± 1.47</td>
</tr>
<tr>
<td>YsADH, TkNOX and VsHGBc</td>
<td>51.3 ± 2.03</td>
</tr>
</tbody>
</table>

a The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.3 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reaction was carried out at 45 °C and 600 rpm for 2 h. The values are represented with standard deviation from triplicate measurements.

b The yield of 3-methyl-2-butenal was calculated by the following equation: yield (%) = product forming (mM)/(product forming (mM) + substrate remaining (mM)) × 100%.

c The atmosphere of the reactor was replaced with pure oxygen.

See Table 2 in the supplementary files.
Table 3 The primers used for the construction of the fusion enzyme YsADH-(linker)-TkNOX, YsADH-(GSG)-TkNOX-(GSG)-VsHGB or VsHGB-(GSG)-TkNOX-(GSG)-YsADH
<table>
<thead>
<tr>
<th>Linker</th>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGGS</td>
<td>ADH</td>
<td><strong>F</strong> 5'-TAACTTTATAAAGGAGATATACCATGGGCCATGTCTATTATAAAAAGCTATGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-ACCACCGGTTTTAGGTCTTCCATGCTGCCGCCGCCGCCAAAGTCGGCTTTGAGATAC-3'</td>
</tr>
<tr>
<td></td>
<td>TkNOX</td>
<td><strong>F</strong> 5'-TGTTTACTGCAAGCCGACTTTGGCAGCCGATGGAACCTAAAACGCGTGTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-ACTTAAGCATTAGCGGCCCAAGCTTTCAAATTTCAAGACACGTGCC-3'</td>
</tr>
<tr>
<td>GSG</td>
<td>ADH</td>
<td><strong>F</strong> 5'-TGTTTATAAAGGAGATATACCATGGGCCATGTCTATTATAAAAAGCTATGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-GCCCCAAGCCGACTTTGGCAGCCGATGGAACCTAAAACGCGTGTT-3'</td>
</tr>
<tr>
<td></td>
<td>TkNOX</td>
<td><strong>F</strong> 5'-GTGTTACTGCAAGCCGACTTTGGCAGCCGATGGAACCTAAAACGCGTGTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-ACTTAAGCATTAGCGGCCCAAGCTTTCAAATTTCAAGACACGTGCC-3'</td>
</tr>
<tr>
<td>(GGGGS)</td>
<td>ADH</td>
<td><strong>F</strong> 5'-TAACTTTATAAAGGAGATATACCATGGGCCATGTCTATTATAAAAAGCTATGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-CACCACCGGTTTTAGGTCTTCCATGCTGCCGCCAAAGTCGGCTTTGAGATAC-3'</td>
</tr>
<tr>
<td></td>
<td>TkNOX</td>
<td><strong>F</strong> 5'-GTGTTACTGCAAGCCGACTTTGGCAGCCGATGGAACCTAAAACGCGTGTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-ACTTAAGCATTAGCGGCCCAAGCTTTCAAATTTCAAGACACGTGCC-3'</td>
</tr>
<tr>
<td>(GSG)</td>
<td>ADH</td>
<td><strong>F</strong> 5'-TAACTTTATAAAGGAGATATACCATGGGCCATGTCTATTATAAAAAGCTATGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-CACCACCGGTTTTAGGTCTTCCATGCTGCCGCCAAAGTCGGCTTTGAGATAC-3'</td>
</tr>
<tr>
<td></td>
<td>TkNOX</td>
<td><strong>F</strong> 5'-GTGTTACTGCAAGCCGACTTTGGCAGCCGATGGAACCTAAAACGCGTGTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-ACTTAAGCATTAGCGGCCCAAGCTTTCAAATTTCAAGACACGTGCC-3'</td>
</tr>
<tr>
<td>GSG</td>
<td>YsADH-(GSG)-TkNOX</td>
<td><strong>F</strong> 5'-TAACCTTTATAAAGGAGATATACCATGGGCCATGTCTATTATAAAAAGCTATGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-GGTCTGCTGGTCCAGCATGCCGCCAAATTTCAGAACACGTGCC-3'</td>
</tr>
<tr>
<td></td>
<td>VsHGB</td>
<td><strong>F</strong> 5'-GGCACGTGTTCTGAAATTTGGCAGCGGCATGCTGGACCAGCAGACC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-ACTTAAGCATTAGCGGCCCAAGCTTTCAAATTTCAAGACACGTGCC-3'</td>
</tr>
<tr>
<td>GSG</td>
<td>VsHGB</td>
<td><strong>F</strong> 5'-TAACCTTTATAAAGGAGATATACCATGGGCCATGTCTATTATAAAAAGCTATGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-ATAACCCACCCGGTTTTAGGTCTTCCATGCTGCCGCCAAATTTCAGAACACGTGCC-3'</td>
</tr>
<tr>
<td></td>
<td>TkNOX</td>
<td><strong>F</strong> 5'-ATCTGTACGCTAGCCGCTCTGAAATTTGGCAGCGGCATGGAACCTAAAACGCGTGTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>R</strong> 5'-CGGCATAGCTTTTTATAATAGACATGGCCGCCCTGCCCAAATTTCAGAACACGTGCC-3'</td>
</tr>
</tbody>
</table>
Table 4 The retention times of substrates and products in GC analyses

<table>
<thead>
<tr>
<th>Entry</th>
<th>Alcohols</th>
<th>Time (min)</th>
<th>Aldehydes</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crotyl alcohol</td>
<td>3.509</td>
<td>Crotonaldehyde</td>
<td>3.777</td>
</tr>
<tr>
<td>2</td>
<td>3-Methyl-2-buten-1-ol</td>
<td>5.320</td>
<td>3-Methyl-2-butenal</td>
<td>7.752</td>
</tr>
<tr>
<td>3</td>
<td>trans-2-Hexenol</td>
<td>6.115</td>
<td>trans-2-Hexenal</td>
<td>13.151</td>
</tr>
<tr>
<td>4</td>
<td>Nerol</td>
<td>17.619</td>
<td>Neral</td>
<td>18.858</td>
</tr>
<tr>
<td>5</td>
<td>Geraniol</td>
<td>18.562</td>
<td>Geranial</td>
<td>19.504</td>
</tr>
<tr>
<td>6</td>
<td>Cinnamyl alcohol</td>
<td>14.851</td>
<td>Cinnamaldehyde</td>
<td>14.117</td>
</tr>
<tr>
<td>7</td>
<td>Farnesol</td>
<td>19.662</td>
<td>Farnesal</td>
<td>19.471</td>
</tr>
</tbody>
</table>

For possible by-products isovaleraldehyde, isovaleric acid and 3,3-dimethylacrylic acid, the column temperature program was listed as follows; initial temperature of 60 °C for 5 min, 10 °C/min ramp to 120 °C for 3 min, and 30 °C/min ramp to 180 °C for 3 min. The retention times for isovaleraldehyde, 3,3-dimethylacrylic acid and isovaleric acid were 6.987 min, 7.839 min and 9.612 min, respectively.

Figures
Figure 1

SDS-PAGE analysis of YsADH, TkNOX and/or VsHGB in the cell-free extracts. Lane M, marker; lane 1, expression of VsHGB (15.5 kDa) alone; Lane 2, expression of YsADH (33.2 kDa) alone; Lane 3, co-expression of YsADH (33.2 kDa) and TkNOX (43.4 kDa); Lane 4, co-expression of YsADH (33.2 kDa), TkNOX (43.4 kDa) and VsHGB (15.5 kDa); Lane 5, no induction of the cells co-expressing YsADH, TkNOX or VsHGB as the control. The percentage of acrylamide in the resolving gel was 12%. The value in the bracket represents the apparent molecular mass of YsADH, TkNOX or VsHGB.
Figure 1

SDS-PAGE analysis of YsADH, TkNOX and/or VsHGB in the cell-free extracts. Lane M, marker; lane 1, expression of VsHGB (15.5 kDa) alone; lane 2, expression of YsADH (33.2 kDa) alone; lane 3, co-expression of YsADH (33.2 kDa) and TkNOX (43.4 kDa); lane 4, co-expression of YsADH (33.2 kDa), TkNOX (43.4 kDa) and VsHGB (15.5 kDa); lane 5, no induction of the cells co-expressing YsADH, TkNOX or VsHGB as the control. The percentage of acrylamide in the resolving gel was 12%. The value in the bracket represents the apparent molecular mass of YsADH, TkNOX or VsHGB.
Figure 2

SDS-PAGE analysis of the fusion proteins YsADH-(linker)-TkNOX and YsADH-(linker)-TkNOX-(linker)-VsHGB in the cell-free extracts. Lane M, marker; Lane 1, expression of YsADH-(GSG)-TkNOX-(GSG)-VsHGB (99.7 kDa); lane 2, expression of YsADH-(GSG)-TkNOX (80.7 kDa); lane 3, expression of YsADH-(GSG)2-TkNOX (80.7 kDa); lane 4, expression of YsADH-(GGGGS)-TkNOX (80.7 kDa); lane 5, expression of YsADH-(GGGGS)2-TkNOX (80.7 kDa); lane 6, expression of TkNOX (42.7 kDa); lane 7, expression of YsADH (32.7 kDa); lane 8, no induction of the cells co-expressing YsADH, TkNOX and VsHGB as the control. The percentage of acrylamide in the resolving gel was 12%. The value in the bracket represents the apparent molecular mass of the fusion enzyme, YsADH or TkNOX.
Figure 2

SDS-PAGE analysis of the fusion proteins YsADH-(linker)-TkNOX and YsADH-(linker)-TkNOX-(linker)-VsHGB in the cell-free extracts. Lane M, marker; Lane 1, expression of YsADH-(GSG)-TkNOX-(GSG)-VsHGB (99.7 kDa); lane 2, expression of YsADH-(GSG)-TkNOX (80.7 kDa); lane 3, expression of YsADH-(GSG)2-TkNOX (80.7 kDa); lane 4, expression of YsADH-(GGGGS)-TkNOX (80.7 kDa); lane 5, expression of YsADH-(GGGGS)2-TkNOX (80.7 kDa); lane 6, expression of TkNOX (42.7 kDa); lane 7, expression of YsADH (32.7 kDa); lane 8, no induction of the cells co-expressing YsADH, TkNOX and VsHGB as the control. The percentage of acrylamide in the resolving gel was 12%. The value in the bracket represents the apparent molecular mass of the fusion enzyme, YsADH or TkNOX.
Effect of the linker types on the catalytic performances of the fusion enzymes YsADH-linker-TkNOX. The control represented the E. coli cells co-expressing YsADH, TkNOX and VsHGB separately. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.3 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reaction was carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 3

Effect of the linker types on the catalytic performances of the fusion enzymes YsADH-linker-TkNOX. The control represented the E. coli cells co-expressing YsADH, TkNOX and VsHGB separately. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.3 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reaction was carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 4

Effect of temperature on the catalytic performances of the cells expressing the fusion enzyme YsADH-(GSG)-TkNOX-(GSG)-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reactions were carried out at 40~65 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 4

Effect of temperature on the catalytic performances of the cells expressing the fusion enzyme YsADH-(GSG)-TkNOX-(GSG)-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reactions were carried out at 40~65 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Effect of pH on the catalytic performances of the cells expressing the fusion enzyme YsADH-GSG-TkNOX-GSG-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM PIPES (pH 6.0~7.0) or 50 mM Tris-HCl (pH 7.5~8.0). The reactions were carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 5

Effect of pH on the catalytic performances of the cells expressing the fusion enzyme YsADH-GSG-TkNOX-GSG-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM PIPES (pH 6.0~7.0) or 50 mM Tris-HCl (pH 7.5~8.0). The reactions were carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 6

Effect of rotation on the catalytic performances of the cells expressing the fusion enzyme YsADH-GSG-TkNOX-GSG-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reactions were carried out at 45 °C and 400~900 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 6

Effect of rotation on the catalytic performances of the cells expressing the fusion enzyme YsADH-GSG-TkNOX-GSG-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reactions were carried out at 45 °C and 400~900 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 7

Effect of coenzyme FAD and NADP+ on the catalytic performances of the cells expressing the fusion enzyme YsADH-GSG-TkNOX-GSG-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0~1.0 mM NADP+, 0~1.0 mM FAD, 50 mM Tris-HCl (pH 8.0). The effect of FAD concentration was investigated at a NADP+ concentration of 0.2 mM, and that effect of NADP+ concentration was then evaluated at a FAD concentration of 0.2 mM. All the reactions were carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 7

Effect of coenzyme FAD and NADP+ on the catalytic performances of the cells expressing the fusion enzyme YsADH-GSG-TkNOX-GSG-VsHGB. The reaction mixture (5 ml) contained 100 mM 3-methyl-2-buten-1-ol, 0.1 g lyophilized cells, 0~1.0 mM NADP+, 0~1.0 mM FAD, 50 mM Tris-HCl (pH 8.0). The effect of FAD concentration was investigated at a NADP+ concentration of 0.2 mM, and that effect of NADP+ concentration was then evaluated at a FAD concentration of 0.2 mM. All the reactions were carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 8

Time courses of alcohol oxidation at higher substrate concentrations. The reaction mixture (5 ml) contained 50~300 mM 3-methyl-2-buten-1-ol, 0.3 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reaction was carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).
Figure 8

Time courses of alcohol oxidation at higher substrate concentrations. The reaction mixture (5 ml) contained 50~300 mM 3-methyl-2-buten-1-ol, 0.3 g lyophilized cells, 0.4 mM NADP+, 0.2 mM FAD, 50 mM Tris-HCl (pH 8.0). The reaction was carried out at 45 °C and 600 rpm for 2 h. Standard deviations are indicated in the diagram (n=3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.png
- Scheme1.png
- Revision1106S.docx
- Revision1106S.docx