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Green conversion of 5-hydroxymethylfurfural to furan-2,5-dicarboxylic acid by heterogeneous expression of 5-hydroxymethylfurfural oxidase in *Pseudomonas putida* S12

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Summary

Transforming petrochemical processes into bioprocesses has become an important goal of sustainable development. The chemical synthesis of 2,5-furandicarboxylic acid (FDCA) from 5-hydroxymethylfurfural (HMF) is expensive and environmentally unfavourable. The study aims to investigate a whole-cell biocatalyst for efficient biotransformation of HMF to FDCA. For the first time, a genetically engineered *Pseudomonas putida* S12 strain expressing 5-hydroxymethylfurfural oxidase (HMFO) was developed for the biocatalytic conversion of HMF to FDCA. This whole-cell biocatalyst produced 35.7 mM FDCA from 50 mM HMF in 24 h without notable inhibition. However, when the initial HMF concentration was elevated to 100 mM, remarkable inhibition on FDCA production was observed, resulting in a reduction of FDCA yield to 42%. We solve this substrate inhibition difficulty by increasing the inoculum density. Subsequently, we used a fed-batch strategy by maintaining low HMF concentration in the culture to maximize the final FDCA titre. Using this approach, 545 mM of FDCA was accumulatively produced after 72 hs, which is the highest production rate per unit mass of cells to the best of our knowledge.

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Introduction

Reducing the utilization of fossil resources is one of the most prominent direction for sustainability and has prompted the search for more suitable sources for fuels and chemicals. Lignocellulosic biomass is recognized as the most attractive alternative due to its availability and significant amounts. Furthermore, the utilization of lignocellulose does not compete with the food as traditional sugar-based bio-production would. It is also readily presented in various waste streams (Delidovich *et al*., 2016). Therefore, new processes and technologies have been developed in an effort to switch from petroleum-based chemical production to that of biomass-based (Manzer, 2006; Lin *et al*., 2016; Yew *et al*., 2019; Khoo *et al*., 2019).

5-Hydroxymethylfurfural (HMF) is a valuable chemical obtained from lignocelluloses. It is synthesized by dehydration of monosaccharides, generally fructose (Bao *et al*., 2008). Alternatively, the direct conversion of glucose has also been demonstrated recently (Zhang *et al*., 2017). It is a structurally attractive raw material for various chemical applications, including use preparation of organic solvents or polymer building blocks (Rosatella *et al*., 2011). Among the building blocks made from HMF, 2,5-furandicarboxylic acid (FDCA) is one of the most value-added platform chemicals to be produced. FDCA has been identified as one of the top 12 value-added chemicals from biomass. It is a platform chemical that can be used for the synthesis of polyethylene furanoate (PEF) (Bozell and Petersen, 2010; DeJong *et al*., 2011). However, the chemical synthesis of FDCA from HMF is expensive and environmentally unfavourable as it requires high pressure, high temperature, metal salts and organic solvents. In contrast, the biological conversion of HMF to FDCA is less energy-intensive and is more environment-benign (Wierckx *et al*., 2015). However, biocatalytic production of FDCA is insufficiently studied, potentially due to HMF toxicity to most cells in fermentation (Jonsson *et al*., 2013).

Biocatalytic conversion of HMF to FDCA can be achieved using purified enzymes as well as whole cells. In general, whole cells are relatively more preferable for FDCA production because they do not require tedious enzyme purification and complicated cofactor regeneration processes (Wachtmeister and Rother, 2016). However, to date, only a few reports on whole cell-catalysed oxidation of HMF into FDCA are available. Koopman *et al.* (2010) expressed an HMF/furfural oxidoreductase, encoded by *hmfH* gene, in *Pseudomonas putida* S12 and achieved FDCA from HMF with a yield of 97%. Subsequently, Yang and Huang (2016) isolated a *Burkholderia cepacia* H-2 strain that was able to transform 2 g l^{-1} of HMF to 1.276 g l^{-1} FDCA under pH of 7 and 28°C with a yield of approximately 50%. Recently, Hossain *et al.* (2017) genetically engineered a newly isolated strain *Raoultella ornithinolytica* BF60. Through inhibition of FDCA degradation, removing undesired HMF catabolism and overexpressing an aldehyde dehydrogenase (ALDH), conversion of HMF to FDCA was achieved at 89.0% yield.

In another study, Dijkman and Fraaije aimed to express HmfH in *Escherichia coli*. However, they reported that HmfH could not express in *E. coli* due to inclusion body formation. As an alternative, they expressed an HmfH homologue from *Methylovorus* sp. strain MP688 with a 46% sequence identity to that of HmfH and showed its functional expression. However, FDCA yield was only 8% as the majority of the production was 5-formylfuroic acid (FFA) (Dijkman and Fraaije, 2014). Subsequently, by increasing the enzyme to substrate ratio and adding flavin adenine dinucleotide (FAD) as a cofactor, the enzyme was able to produce FDCA at a yield of 95% (Dijkman *et al*., 2014). Therefore, it was proposed that this HmfH homologue is a FAD-dependent HMF oxidase (HMFO) that can be potentially used for the production of FDCA from HMF.

Heterologous FDCA producer was developed by overexpressing HMFO. Yuan *et al.* (2018) expressed HMFO in *R. ornithinolytica* BF60 and produced 16.5 mM of FDCA from 20 mM of HMF. When using high biomass density to overcome the toxicity of HMF, FDCA production was increased to 76.9 mM, corresponding to a molar conversion ratio of 76.9 %. However, the reported production rate of 14.29 ± 0.07 μM/(gCDW•h) was lower than that of the HmfH expressing *P. putida* S12.

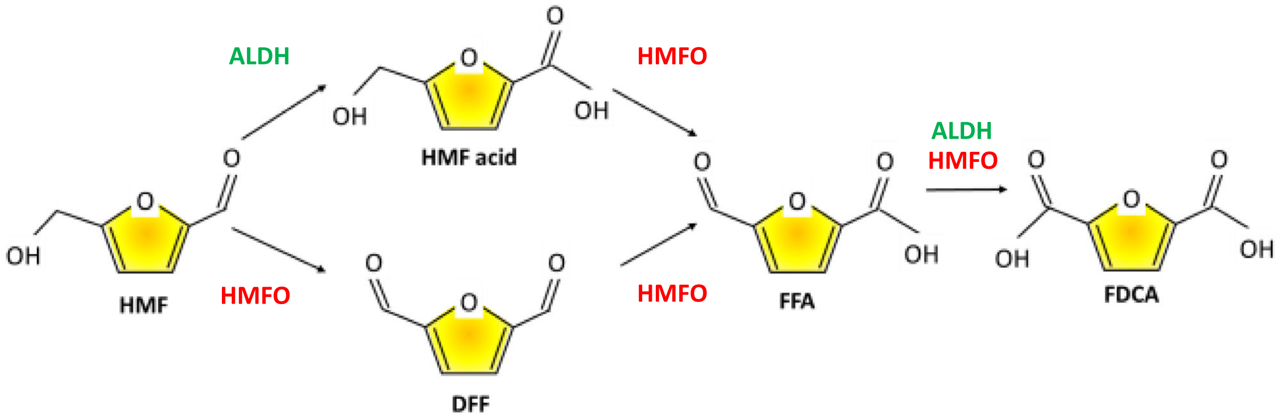
P. putida is potentially a superb host for FDCA production from HMF. It is well known for its remarkable tolerance to a range of chemical stressors. its endogenous aldehyde dehydrogenases can act synergistically in converting HMF to FDCA (Koopman *et al*., 2010; Ramos *et al*., 2015). In addition, it is natively rich in the necessary cofactors, such as FAD and NADH, for the biocatalysis process (Duetz *et al*., 2001). Although the FDCA production rate of *R. ornithinolytica* BF60 expressing HMFO was lower than that of *P. putida* S12 expressing HMFH, the product titre was promising. While no one has ever successfully expressed HMFO in *P. putida* S12, in this study we investigated the feasibility of expressing HMFO in *P. putida* S12 for constructing a whole-cell biocatalyst for efficient biotransformation of HMF to FDCA and explore the biocatalytic properties of this strain.

Results and Discussion

Functional expression of HMFO in P. putida S12

Recently, an FAD-dependent enzyme HMFO active towards HMF has been successfully identified from *Methylovorus* sp. Strain MP688 (Dijkman and Fraaije, 2014). As depicted in Scheme 1, it performs the three consecutive oxidation steps of HMF to FDCA. The alcohol group of HMF is first oxidized to the corresponding aldehyde to generate diformylfuran (DFF). This compound then undergoes spontaneous hydration to the *gem*-diol, which is oxidized by the enzyme to formylfuroic acid (FFA). Finally, this monocarboxylic intermediate is oxidized by HMFO to form the dicarboxylic product FDCA. Through expression in *E. coli*, the purified recombinant HMFO showed a remarkable capability of production FDCA from HMF with high yield at ambient temperature and pressure (Dijkman *et al*., 2014). Although the results were promising, the requirement of FAD addition and the weakness of substrate tolerance (2–4 mM) have trimmed its broad use as an enzymatic biocatalyst. Therefore, we examined the effects of heterologously expressing HMFO in *P. putida* S12 for the construction of an effective whole-cell biocatalyst for FDCA production from HMF.

First, we showed the functional expression of HMFO in *P. putida* S12 strain by demonstrating the conversion of HMF to FDCA using the engineered strain. As showed in Fig. 1A, the control strain harbouring an empty pBR122 plasmid (P. putida_Cntl) was able to oxidize HMF to HMF acid. This process was believed to be mediated by aldehyde dehydrogenases in *P. putida* S12 (Koopman *et al*., 2010). Unfortunately, the strain harbouring the p122HMFO plasmid (*P. putida*_Ocat), which expresses HMFO under the control of cat promoter, was unable to produce FDCA. Similar to the control strain, only HMF acid was observed (Fig. 1B). Next, we replaced the cat promoter with that of HEC promoter. Encouragingly, the resulting strain P. putida_Ohec showed FDCA production (Fig. 1C). The appearance of FDCA as result from the three-step oxidation of HMF indicated functional expression of HMFO. Here, with the addition of 50 mM HMF, P. putida_Ohec strain was able to produce 35 mM of FDCA with 15 mM HMF acid as



Scheme 1. Pathway for the oxidation of HMF to FDCA. Abbreviations and their corresponding full names are as follows. ALDH, Aldehyde dehydrogenase (native); DFF, Diformylfuran; FDCA, Furandicarboxylic acid; FFA, Formylfuroic acid; HMFO, HMF oxidase (foreign); HMF, Hydroxymethyl furfural.

the by-product within 24 h. Clearly, the expression of HMFO in *P. putida* S12 was significantly affected by the promoter used for unspecified reasons.

Although the conversion of HMF to FDCA was only approximately 70%, this result is encouraging because it represents the first functional expression of HMFO in *P. putida* S12. In addition, the substrate concentration used in this conversion was 50 mM, which is 12.5–25 times higher than that used with purified HMFO enzyme (Dijkman *et al*., 2014). Furthermore, the use of HMFO-expressing cells as biocatalysts to produce FDCA could substantially reduce the cost for practical use because no external FAD cofactor was used. Lastly, only HMF acid was identified as the major by-product, instead of FFA as produced using purified HMFO (Dijkman and Fraaije, 2014), indicating that aldehyde dehydrogenases in *P. putida* S12 worked synergistically with HMFO in converting HMF to FDCA. Together, these results indicated that HMFO-expressing *P. putida* S12 may be preferred over using purified HMFO for FDCA production.

Environmental settings on FDCA production

While microorganisms can only survive under a certain range of environmental settings, identifying the optimal condition for a microorganism to achieve its best catalytic activities is important for industrial applications. To establish the optimal condition for FDCA production, the effects of pH and temperature on FDCA production were investigated.

The optimal pH for P. putida_Ohec strain to produce FDCA was determined with 50 mM of HMF as the substrate and 50 mM of glycerol as the carbon source in the mineral medium. The pH of the mineral medium was adjusted by adding different amounts of KH_2PO_4 and Na_2HPO_4. Rates of FDCA production at different pH levels were monitored. As is shown in Fig. 2A, the optimal pH for P. putida_Ohec strain to produce FDCA was 8, which is similar to the results of the two previous reports (Dijkman and Fraaije, 2014; Hossain *et al.*, 2017). Interestingly, the strain was able to retain at least 80% of its best activity within the pH range (Bao *et al.*, 2008; Bozell and Petersen, 2010; Rosatella *et al.*, 2011; Zhang *et al.*, 2017) tested herein, suggesting its broad pH tolerance for practical applications.

To find the optimal temperature of HMF to FDCA conversion, P. putida_Ohec strain having OD600 of 2 was exposed to the mineral medium containing 50 mM of HMF and 50 mM of glycerol at different a temperature ranging from 20 to 60°C (Fig. 2B). At temperatures lower than 30°C, the frequency of collisions between molecules and enzyme decreases, resulting in a slow reaction rate and FDCA productivity. As the temperature increased to 40°C, frequency of collisions between molecules and enzyme increases, speeding up the metabolism and resulting in a higher FDCA production rate. However, when the temperature increased above 40°C, FDCA production rate dropped. In contrast to the FDCA production using HMFO with the optimal activity of 55°C [17], the optimal temperature of FDCA production by the whole-cell biocatalyst in this study is lower at 30–40°C. This may be attributed to the fact that other enzymes within the cell started to denature, or the cell stress was increased substantially, rendering the total effect to be detrimental. Therefore, the optimal temperature for FDCA production is a compromise between HMFO and cellular growth.

The maxima substrate (HMF) concentration for purified HMFO that has been reported so far was 4 mM (Dijkman *et al.*, 2014). Here, we showed that the engineered strain expressing HMFO could convert 50 mM HMF to 35 mM FDCA. To assess the effect of substrate

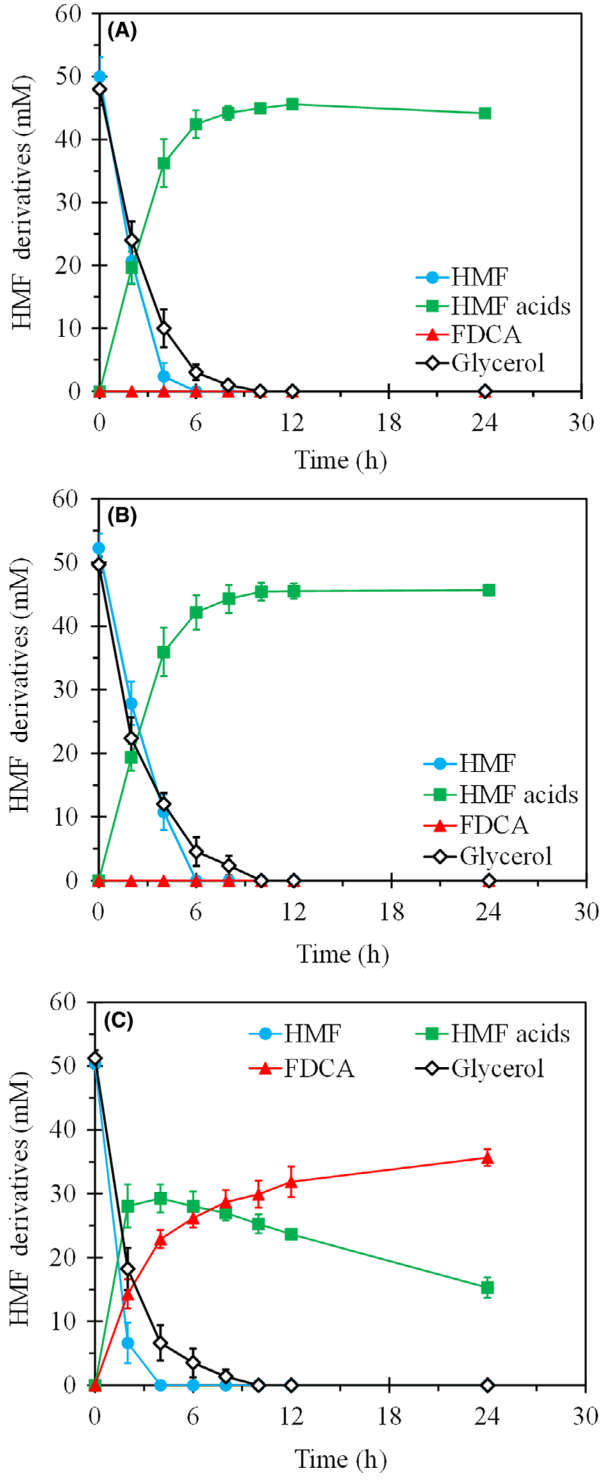


Fig. 1. Time course of HMF conversion by (A) the wild *P. putida* S12 harbouring an empty pBR122 plasmid, (B) the engineered *P. putida* S12 expressing HMFO using cat promoter and (C) the engineered *P. putida* S12 expressing HMFO using HEC promoter.

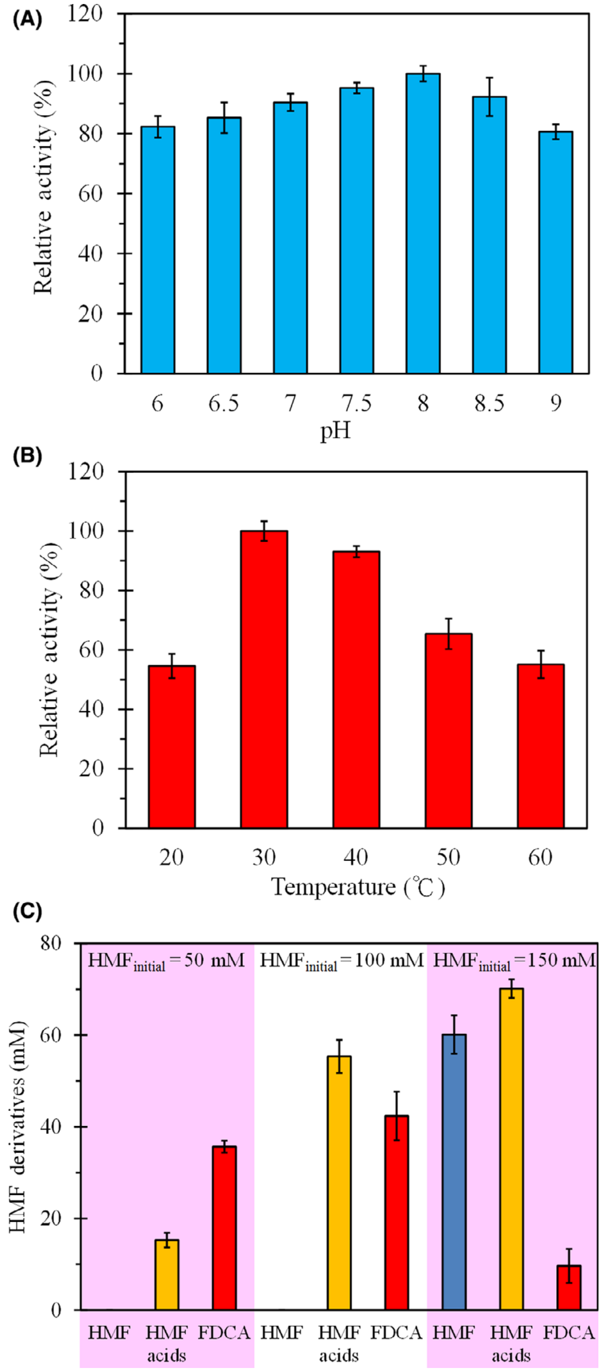


Fig. 2. Environmental settings on HMF conversion. (A) pH effect on HMF conversion by the engineered *P. putida* S12 expressing HMFO. (B) Temperature effect on HMF conversion by the engineered *P. putida* S12 expressing HMFO. (C) HMF conversion by the engineered *P. putida* S12 expressing HMFO with different initial HMF concentration. Data obtained at the 24th hour. Complete time courses of the fermentation results are available in Fig. S1 of the supplementary information.

concentrations on FDCA production, 50 mM to 150 mM of 5-HMF was tested. As shown in Fig. 2C and Fig. S1, the yield of FDCA production based on the amount of HMF fed decreased with the increasing amount of substrate. When 100mM of HMF was fed, the time required to oxidize HMF was increased to 8 h. The HMF acid was accumulated to 55 mM accompanied with 42 mM of FDCA production in 24 h. However, when the amount of HMF fed reached 150 mM, a remarkable inhibition on HMF oxidation was observed. HMF could not be fully oxidized even after 24 h. Furthermore, less than 10 mM of FDCA was produced. Based on these results, 50 mM of HMF was used as the substrate for the following experiments as it has a relatively high FDCA conversion yield of around 70%.

Effects of biomass concentrations on FDCA production

Many microorganisms reduce or oxidize furanic compounds to their alcohols, or carboxylic acids to mitigate their toxic effect (Almeida *et al.*, 2008; Almeida *et al.*, 2009; Wierckx *et al.*, 2011). This cellular process typically leads to the depletion of cofactors required for these conversions, rendering an inhibition of enzymes in primary metabolism and an increase in the lag phase. It has been demonstrated that increasing biomass density could be a suitable way to overcome HMF toxicity (Wierckx *et al.*, 2011; Yuan *et al.*, 2018). Therefore, we evaluated the effects of three differential initial biomass concentrations (O.D.= 20, 50, or 100) on FDCA production.

As shown in Fig. 3, the inhibition of HMF on FDCA conversion was notably mitigated with increasing biomass. With an initial O.D. of 20, the conversion of 100 mM HMF to FDCA reached around 75% within two days. Furthermore, the conversion rate for the first 12 h could be 0.2 mM/[(O.D.)·h] (Fig. S2A). When the biomass concentration was further increased to O.D.=50, the conversion yield of 100 mM HMF to FDCA reached almost 90%. The conversion yield for the culture containing 150 mM starting HMF concentration was 43% (Fig. 3B and Fig. S2B). These results compare favourably to other previous studies. The metabolically engineered *R. ornithinolytica* BF60 achieved an 89% conversion yield at 100 mM starting concentration of HMF (Hossain *et al.*, 2017). Although achieved similar conversion yield as our strain in this study, the reaction time was around 1 week, which is significantly longer than the reaction time demonstrated in this study. In another study, newly engineered *R. ornithinolytica* BF60 strain expressing HMFO achieved full conversion in 120 h with an 80% conversion yield (Yuan *et al.*, 2018). Here, we showed that *P. putida* offers the advantage of having faster reaction time and achieving high conversion yields.

To further enhance the final FDCA yield at 150 mM of HMF, we employed even higher biomass density. As depicted in Fig. 3C and Fig. S1C, when the initial cell density was increased to O.D.=100, the conversion efficiency of 100 mM and 150 mM of HMF to FDCA were 96% and 75% respectively. Besides, the reaction rates for 100 mM and 150 mM of HMF were 71.11 and 42.92 μM/[(O.D.)·h] respectively. This remarkable biocatalytic activity was by far the best reaction rate at high substrate conditions.

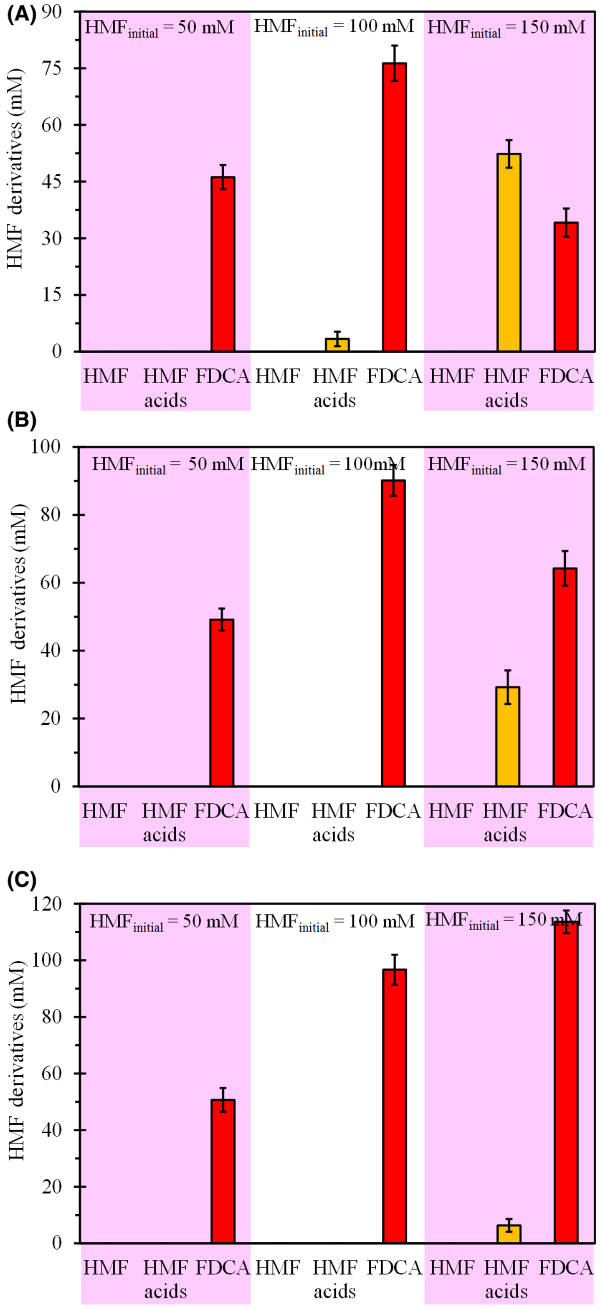


Fig. 3. Effects of different inoculum density on HMF tolerance and FDCA conversion. (A) initial O. D. = 20; (B) initial O. D. = 50; and (C) initial O.D. = 100. Data obtained at the 48th hour. Complete time courses of the fermentation results are available in Fig. S2 of the supplementary information.

Our results in this study showed that HMF toxicity on *P. putida* can be effectively overcome by increasing the biomass density. This result was in good agreement with the notion that endogenous ALDH in *P. putida* S12 oxidizes HMF to HMF acid, which is a significantly less toxic substance to cells (Wierckx *et al.*, 2015). Since resting cells were used as biocatalysts in this study, higher initial biomass concentration implies that more ALDH is present initially. Consequently, more HMF is converted into the less toxic HMF acid, resulting in apparently increased HMF tolerance. This explanation also could be supported by the fact that a large number of transient HMF acids were accumulated at high HMF concentration and biomass density.

Apart from using high cell density, it is worthy of mention that researchers recently have also demonstrated that improving the oxygen transferring using compressed oxygen supply-sealed and stirred tank reactor (COS-SSTR) could also achieve the same goal for detoxification of furfural (Zhou *et al.*, 2017). Therefore, this strategy can be a potential alternative for growing cells with slow growth rates in the future.

Fed-batch production of FDCA

Fed-batch fermentation is a commonly used technique in biotechnological processes. It allows for the addition of nutrients to the reaction continuously or intermittently to control the metabolic activity of the cells and generate high cell densities or product concentrations. A high concentration of HMF could be harmful to *P. putida* S12. Although we showed that high initial biomass density can overcome HMF toxicity, increasing the initial substrate concentration indefinitely is unlikely to be able to achieve efficient conversion and obtain high final FDCA titre. Therefore, we next aimed to increase the overall FDCA titre through fed-batch culture with a low concentration of, but constantly fed, HMF to avoid substrate inhibition. Here, we analysed the effect of two different HMF feeding rates (2 and 5 ml h^{-1}) on the overall conversion to FDCA performance.

With a 2 ml h^{-1} feeding rate, FDCA was cumulatively produced and reached a final concentration of around 323 mM in 72 h (Fig. 4A). In the meantime, around 19 g l^{-1} of biomass was produced. During the operation, HMF acid was progressively increased as the major intermediate and reached around 48 mM at the 48th hour. The accumulation of HMF acid lowers the FDCA productivity and causes negative effects on the downstream polymerization processes. Therefore, to minimize its accumulation, HMF feeding was terminated at the 48th hour, allowing the remaining HMF acid to be converted to FDCA. As expected, HMF acid was completely consumed in the following 24 h. Obviously, this approach avoided the accumulation of HMF and HMF acid. It also further elevated the overall final FDCA titre.

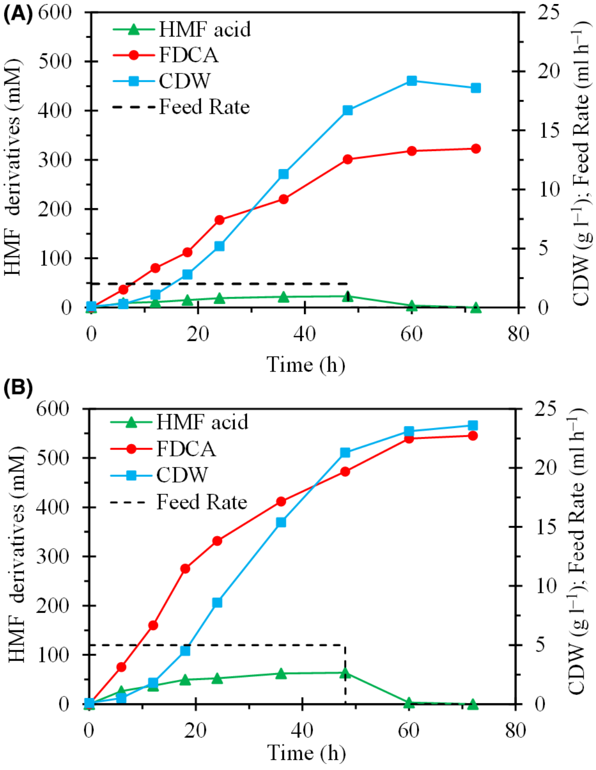


Fig. 4. Fed-batch cultivation of the engineered *P. putida* S12 expressing HMFO for the production of FDCA. The feed liquid contained 2X mineral media supplied with 5 M glycerol, 0.1 M $MgCl_2$ and 1 M HMF. Feed rate was set at (A) 2 ml h^{-1} and (B) 5 ml h^{-1}. Glycerol was also measured periodically. However, no detectable glycerol was observed during the fermentation.

To further push the final FDCA titre, an increased feed rate of 5 ml h^{-1} was employed (Fig. 4B). In this case, the titre of FDCA was successfully increased to 482 mM with 48 h. However, the fed HMF also could not be fully converted into FDCA. In particular, the accumulation of HMF acid was even severe (63 mM) than that of the slower HMF feeding rate described above. Therefore, we used the same strategy as described above. Feeding was terminated after 48 h. The reactor was allowed for an additional 24 h to react HMF acid to FDCA. At the end of the reaction, an FDCA concentration of around 545 mM was obtained in 72 h. This result showed that the higher feeding rate is effective in increasing the overall FDCA titre. Koopman *et al.* used fed-batch cultivation of a genetically engineered *P. putida* S12 and produced 30.1 ± 0.7 g l^{-1} of FDCA after 144 h (Koopman *et al.*, 2010). Later on, the same group further added HmfT1 and Aldh into the engineered *P. putida* S12, and successfully produced approximately 150 g L^{-1} of FDCA after 90 h (Wierckx *et al.*, 2017). Therefore, it is clear that the fed-batch method could certainly make useful additions to overcome the limitation of HMF toxicity on

Table 1. Primers used in this study for the construction of HMFO expression plasmid. Restriction sites are shown in bold.

Name	Sequence
F122His	5′-GGG**GCTAGC**CACCACCACCACCACCACTAATCTAG ATTTTTTTAAGGCAGTTATTGGTGC-3′
R122MCS	5′-CCC**GCTAGC**ACTAGTGTCGACGGATCCCATTTTAGC TTCCTTAGCTCCTG-3′
F122HMFO	5′-GGG**GGATCC**ATGACGGACACCATCTTCGATTACGT GATC G-3′
R122HMFO	5′-GGG**GCTAGC**CCTGCGTCAGGATGGCGTCGGCGG-3′
FpHCE	5′-GGG**GGATCC**AGAAGGCCATCCTGACGGATGGCCTT TTACAGATTCCCAATCTCTTGTT-3′
RpHCE	5′-CCC**GGATCC**TGGTTCCAGCTCCTTTT-3′

engineered *P. putida* S12 and make biological FDCA production more attractive for industrial applications.

Conclusions

In this work, a genetically engineered *P. putida* S12 strain capable of converting HMF to FDCA was successfully developed. The engineered strain expressing HMFO was able to produce 35 mM of FDCA from 50 mM of HMF in 24 h, which was corresponding to 70% of the theoretical yield. HMF acid of 15mM was the main intermediates during the process. High HMF concentration inhibited the conversion of HMF to FDCA. The issue of HMF inhibition was solved by either increase the cell density or fed-batch cultivation. Using a low amount but constantly fed HMF to avoid substrate inhibition, around 545 mM of FDCA was produced after 72 h of fed-batch operation, which corresponding to the 100% theoretical yield. The results of this study could be an important basis for further development of efficient biological processes for the production of FDCA from renewable resources. Since HMF tolerance is significantly a major bottleneck of the process, developing a strain with superior HMF tolerance is very important for future studies.

Experimental procedures

Plasmid construction and transformation

The synthetic gene that has been codon-optimized for the expression of HMFO (YP_004038556.1) in *P. putida* S12 was purchased from GeneDireX (New Taipei City, Taiwan). Primers F122His and R122MCS were used to introduce new restriction sites (*Bam*HI, *Sal*I, *Spe*I, *Nhe*I, and *Xba*I) and the hexahistidine tag into the multiple-copy plasmid pBR122 (Boca Scientific Inc., Westwood, MA, USA) to extend the manipulability. After PCR amplification, the resulting product that retains the *cat* promoter but no chloramphenicol acetyltransferase ($\mathrm{Cm^R}$) gene was re-cyclized. The modified plasmid was named as pBR122N. To construct a vector capable of expressing HMFO in *P. putida* S12, plasmid p122HMFO encoding the HMF oxidase under the control of the *cat* promoter was constructed. Briefly, the synthetic gene encoding HMFO was amplified with F122HMFO and R122HMFO primers. The resulting fragment was cloned into the *Bam*HI-*Nhe*I linearized pBR122N to form p122HMFO. To replace the *cat* promoter of the constructed plasmid p122HMFO, a DNA fragment (226-bp) containing a strong constitutive HCE promoter (Poo *et al.*, 2002) and the Shine–Dalgarno sequence upstream DAAT gene of *Geobacillus toebii* (NBRC 107807) was amplified using primers FpHCE and RpHCE. The amplified fragment was digested and ligated into the *Bam*HI linearized p122HMFO to generate pHECHMFO. All constructed plasmids were transformed into *E. coli* NEB5α strain [*fhuA2 Δ(argF-lcZ) U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*] for genetic manipulations and then subcloned into *P. putida* S12 (ATCC 700801) (Tao *et al.*, 2012) for HMFO expression and FDCA production using electroporation (Dower *et al.*, 1988; Iwasaki *et al.*, 1994). Primers used for gene manipulation are given in Table 1.

Whole-cell biocatalytic conversion

Engineered *P. putida* S12 cells were grown overnight at 30°C on a rotary shaker in Luria-Bertani (LB) medium ($10.0\ \mathrm{g\ l^{-1}}$ tryptone, $5.0\ \mathrm{g\ l^{-1}}$ yeast extract, $10.0\ \mathrm{g\ l^{-1}}$ NaCl) supplemented with $50\ \mu\mathrm{g\ ml^{-1}}$ kanamycin and $100\ \mu\mathrm{g\ ml^{-1}}$ ampicillin. The pre-cultures were washed twice with the mineral medium (Table 2) and then subinoculated into 500 ml Erlenmeyer flasks containing 100 ml mineral medium, 50 mM of glycerol, $50\ \mu\mathrm{g\ ml^{-1}}$ of kanamycin and $100\ \mu\mathrm{g\ ml^{-1}}$ of ampicillin. The initial $\mathrm{OD_{600}}$ of the subculture was around 0.5 unless stated otherwise. To assess the HMF biocatalytic activity of the engineered *P. putida* S12 cells, 50 mM to 150 mM of 5-

Table 2. Composition of the mineral medium used for *P. putida* S12 growth and FDCA production.

Compound	Conc. ($\mathrm{g\ l^{-1}}$)	Compound	Conc. ($\mathrm{mg\ l^{-1}}$)
K_2HPO_4	5.230	$ZnSO_4 \cdot 7H_2O$	2.00
NaH_2PO_4	2.460	$CuSO_4 \cdot 5H_2O$	2.00
NH_4Cl	0.800	$CaCl_2 \cdot 2H_2O$	1.00
Na_2SO_4	0.200	$MnCl_2 \cdot 4H_2O$	1.00
$MgCl_2$	0.140	$CoCl_2 \cdot 6H_2O$	0.40
EDTA	0.040	$Na_2MoO4 \cdot 2H_2O$	0.20
$FeSO_4 \cdot 7H_2O$	0.015		

HMF was introduced into the mineral medium containing 50 mM of glycerol (as carbon source) and reactions were undertaken in the rotary shaker at 30°C for 24 h. Samples were taken periodically and subjected to high-performance liquid chromatography (HPLC) analysis of HMF, HMF acid and FDCA as described below. All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation.

Effects of pH and temperature on FDCA production

To test the relative activity of cells expressing HMFO at different pH, culture media were supplied with different amounts of KH_2PO_4 and Na_2HPO_4, or H_3BO_3 and NaOH to give a pH of 6 to 9. Cells after overnight culturing in LB were washed twice with the mineral medium of the desired pH. Thereafter, cells were sub-inoculated into 500 ml Erlenmeyer flasks containing 100 ml mineral medium at different pH, 50 mM of glycerol, 50mM of HMF and the antibiotics. After 6 h of incubation at 30°C, the one with the highest FDCA yield was defined as 100% and the relative activities of others were compared. For testing the temperature effect, cells after pre-culturing in LB were washed twice with the mineral medium subject to different temperatures. Thereafter, cells were sub-inoculated into 500 ml Erlenmeyer flasks containing 100 ml mineral medium at different temperatures, 50 mM of glycerol, 50 mM of HMF and the antibiotics. Similarly, after 6 h of incubation at different temperatures, the one with the highest FDCA yield was defined as 100% and the relative activities of others were compared.

Fed-batch production of FDCA

To test the production of FDCA in a fed-batch process, the engineered *P. putida* S12 cells were grown overnight in the LB medium supplemented with 50 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin on a rotary shaker at 30°C. The cells were then washed twice with mineral medium and inoculated into a one-litre fermenter. The stirring speed was set at 150 rpm, and filtrated oxygen was sparged. The feed liquid was prepared with modified 2X mineral media supplied with 5 M glycerol, 0.1 M $MgCl_2$ and 1 M HMF. The pH of the reactor was maintained to 8 by the addition of NH_4OH until its accumulation was above the inhibition concentration (4 g l^{-1}), after that it is replaced with NaOH. Samples of 3 ml were taken periodically to determine the concentrations of biomass, HMF and its derivatives.

Analytical methods

The optical cell density at 600 nm (OD_{600}) was measured by a UV/Vis spectrophotometer (JASCO V-630, JASCO International, Tokyo, Japan). In this study, an OD_{600} of 1.0 corresponds to 0.43 ± 0.07 g l^{-1} of dried *P. putida* S12. Glycerol was analysed by a glycerol assay kit (Sigma-Aldrich Co. LLC, Saint Louis, MO, USA). Furan derivatives (HMF, HMF acid, FFA and FDCA) were analysed by HPLC (Jasco 4000 series) equipped with a photodiode array detector (PAD). The column used was a Zorbax Eclipse XDB-C8 (pore size of 80 Å, Agilent), and its temperature was controlled at 25°C in a column oven. As the mobile phase, 20 mM KH_2PO_4 (A) and acetonitrile (B) were used at a flow rate of 1.2 ml min^{-1}. After feeding 100% of 20 mM KH_2PO_4 for 1 min, the amount of acetonitrile in the eluent was gradually increased to 40% in 15 min and then kept for 1 min. Thereafter, the eluent was returned to 100% of 20 mM KH_2PO_4 and maintained for 10 min. Calibration curves were made with 0.1, 0.5, 1.0, 5.0, 10, 20 and 50 mM solutions.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Time course of HMF conversion by the engineered *P. putida* S12 expressing HMFO with different initial HMF concentration.

Fig. S2. Time courses of the effects of different inoculum density on HMF tolerance and FDCA conversion. (A) initial O. D. = 20; (B) initial O. D. = 50; (C) initial O. D. = 100.