# Solvent tolerance mechanisms in Pseudomonas putida

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# CHAPTER 2 Introduction

## Solvent Tolerance in Bacteria: Fulfilling the Promise of the Biotech Era?

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## Abstract

The challenge of sustainably producing highly valuable chemical compounds requires specialized microbial cell factories because the majority of these compounds can be toxic to microbial hosts. Therefore, solvent-tolerant bacteria are promising production hosts because of their intrinsic tolerance towards these compounds. Recent studies have helped to elucidate the molecular mechanisms involved in solvent tolerance. Advances in synthetic biological tools will enable further development of streamlined solvent tolerant production hosts and the transfer of solvent tolerant traits to established industrial strains. In this review, we outline challenges and opportunities to implement solvent tolerance in bacteria as a desired trait for industrial biotechnology.

## Solvent tolerant bacteria are efficient biocatalysts

The transition of a fossil raw materials-based economy to a biobased economy is characterized by complex and ambitious systems innovations. Recent breakthrough developments in green chemistry and biotechnology are major drivers enabling production of biobased chemicals (1–4). Today, in the new Biotech Era, increased demands for bio-based “green” chemicals and pharmaceuticals are met with rapid product development benefitting from years of research in the microbial physiology and metabolic engineering fields. Biobased production of these compounds is becoming economically competitive with petrochemical-based production. Both environmental considerations and the need to further improve the competitiveness of the chemicals industry, promise to drive continued biotechnology developments and innovation in the production of biobased chemicals.



Biobased production of valuable chemicals and biopolymer compounds puts a challenge on the choice of microbial host strains (3–6). Many of these chemicals have hydrocarbon-solvent properties and thus exhibit toxicity towards the microbial hosts (7, 8). Furthermore, the production of more complex biobased products, such as o-cresol and 3-methylcathecol, requires toxic solvent-like compounds as substrates or intermediates (9, 10). Therefore, solvent tolerance becomes an essential trait for microbial host in the biobased production of valuable chemicals and biopolymer compounds. Several species of bacteria can grow and survive in the presence of hydrocarbon solvents (11) and can therefore be identified as promising and advantageous platforms for the production of such potentially toxic compounds, or for bioremediation. These bacteria can efficiently withstand or degrade various toxic solvent-like compounds (12, 13). Therefore, the application of solvent-tolerant bacteria in the biocatalytic production of (new) chemical building blocks is rapidly increasing (1–4). Using these solvent-tolerant bacteria in biotechnological production processes, however, requires a thorough understanding of solvent tolerance mechanisms involved. With recent advances in genome sequencing and omics studies of solvent-tolerant bacteria, unique clusters of genes have been identified that confer solvent tolerance traits (14–18). Better understanding of these solvent tolerance traits in combination with modern synthetic biology tools will enable further development of specialized biocatalysts, new applications, and improved production processes of high value compounds (19–27). In this review, we discuss recent findings in solvent tolerance mechanisms and new advances in synthetic biology tools that can help

to design microbial hosts and processes in industrial productions for a plethora of new and valuable compounds.

**Table 2.1. Hydrocarbon solvents and their industrial relevance**

| Hydrocarbon solvent | Solvent class | Industrial relevance | $LogP_{o/w}$ | Refs. |
|---|---|---|---|---|
| acetone | ether | solvent in cosmetic, pharmaceutical, medical, and domestic uses | -0.24 | - |
| ethyl acetate | ester | solvent in coating formulation for epoxies, urethanes, acrylics, and vinyls. | 0.73 | - |
| n-butanol | short chain alkanol | biofuel | 0.88 | (28) |
| phenol | aromatics | precursor for plastics | 1.5 | (29) |
| butyl acetate | ester | product co-solvent (vanillin) | 1.78 | (3) |
| benzene | aromatics | substrate for the production of 3-methylcatechol | 2 | (30) |
| toluene | aromatics | substrate for the production of 3-methylcatechol, o-cresol, & p-hydroxybenzoate | 2.69 | (9, 30, 31) |
| styrene | aromatics | substrate for the production of (S)-styrene oxide | 2.9 | (32) |
| 1-octanol | long chain alkanol | product co-solvent (phenol) | 3 | (29) |
| ethylbenzene | aromatics | production of paints, varnishes, and lacquers | 3.3 | - |
| cyclohexane | cyclic alkane | precursor to nylon, adipic acid, caprolactam | 3.4 | - |
| m-xylene | aromatics | substrate for the production of 3-methylcatechol | 3.46 | (10) |
| n-hexane | alkane | extraction solvent for vegetable oil, cleaning agent | 3.9 | - |
| 1-decanol | long chain alkanol | product co-solvent (p-hydroxystyrene) | 4.57 | (6) |

## Current understanding of solvent tolerance mechanisms

Since the first discovery of solvent-tolerant bacterium *Pseudomonas putida* IH-2000 by Inoue and Horikoshi (12), the number of known solvent-tolerant strains has been rapidly expanding. Despite this growing number of identified solvent-tolerant bacteria, the current knowledge and understanding of solvent tolerance mechanisms has mostly been obtained from studying various strains of *P. putida* (14, 17, 18). But solvent-tolerant traits are not restricted to *P. putida*,

as exemplified for instance by *Exiguobacterium* sp., *Pseudoalteromonas* sp., *Vibrio* sp., *Marinomonas* sp., *Paracoccus denitrificans*, and *Halomonas* sp. (33–36). The discovery of new solvent-tolerant strains and their unique features may help to better understand the molecular and physiological mechanisms underlying bacterial solvent tolerance.

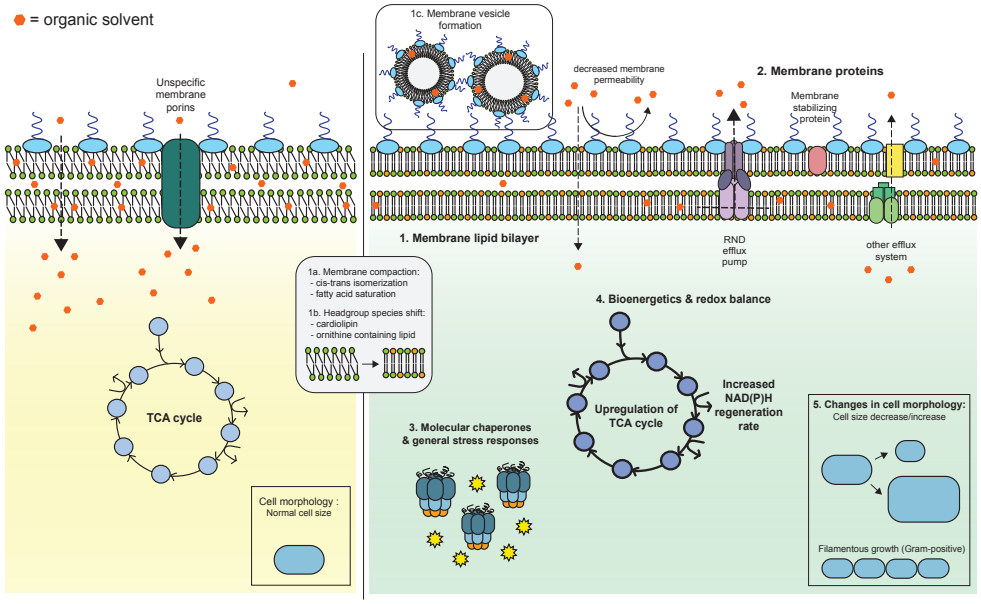


**Fig. 2.1.Current understanding on solvent tolerance mechanism of bacteria**

The left panel represents the state of a bacterium upon the addition of solvent and before solvent tolerance mechanisms are induced, and the right panel represents the state of the bacterium after solvent tolerance mechanisms are induced. Gram-negative and Gram-positive bacteria employ similar strategies to compensate for solvent exposure. The increase in membrane compaction [1] is a consensus for solvent tolerance mechanism between Gram-positive and Gram-negative bacteria by using multiple strategies [1a & 1b]. Resistance, nodulation, and division (RND) efflux pumps [2] and general stress responses [3] play important roles in both groups. Several mechanisms are also unique to certain species, such as the increase of bioenergetics and NAD(P)H regeneration rate in *Pseudomonas putida* [4], membrane vesicle formation in *P. putida* [1c], and filamentous growth in Gram-positive bacteria [5].

Hydrocarbon solvents with a log $P_{o/w}$ value in the range of 1 to 4 (Table 2.1) are toxic to microorganisms at very low concentration because these solvents bind and penetrate the cell membrane and severely affect cell permeability (37). Solvents with log $P_{o/w}$ value lower than 1, like short-chain alkanols (C2-C4), exhibit toxicity in high concentration. Short-chain alkanols directly interact with the phospholipid headgroups, while longer-chain alkanols (e.g.

C8) accumulate within the lipid bilayer of the membrane, 'competing' with the fatty acid acyl chains (38). Solvent-invoked membrane damage inhibits various important membrane functions, such as the permeability barrier function and the structural matrix scaffold for many metabolic and enzymatic reactions (39). Consequently, this membrane damages leads to disrupted cellular metabolism, growth inhibition, and eventually, cell death (11, 38).



Tolerance to hydrocarbon solvents is a multifactorial trait. Bacterial cells employ various strategies to change their physiology and gene expression to circumvent cellular damage caused by these solvents (Fig. 2.1). Tolerance mechanisms have been more extensively studied in gram-negative bacteria than in gram-positive bacteria, but similar mechanism have been observed for both groups (40, 41).

## Membrane fluidity

In the presence of a hydrocarbon solvent, tolerant Gram-negative bacteria respond by changing their cell membrane composition towards saturated and trans-unsaturated fatty acids (7, 42). The formation of trans-unsaturated fatty acid is catalysed by a periplasmic, haem-containing cis-trans isomerase (Cti) (43). In *P. putida* DOT-T1E, Cti is constitutively expressed at a constant level during log-growth and stationary-phase cells and moderately upregulated in the presence of toluene (42). Recently, a working model of Cti activity was proposed by Eberlein and colleagues (44): initially, Cti activity is regulated by the limited accessibility to cis fatty acid under nonstressed condition due to membrane rigidity. The membrane bilayer becomes more fluid upon interaction with hydrocarbon solvents, enabling hydrophilic Cti to reach cis fatty acids and isomerize them into trans fatty acid. Saturated and trans-unsaturated fatty acids increase membrane rigidity, exemplified by a higher phase-transition temperature. This rigid membrane structure provides resistance to hydrocarbon solvents by decreasing solvent influx and accumulation in the membrane. Similarly, Gram-positive bacteria also shift their membrane composition towards a more rigid structure in presence of hydrocarbon solvents by a concentration-dependent decrease in anteiso/iso branched fatty acid ratio. This modification in branched fatty acid promotes a more compact membrane structure, resulting in reduced accumulation of hydrocarbon solvents (43, 45).

## Phospholipid headgroup species

The phospholipid headgroup constituents found in Pseudomonads are phosphatidyl-ethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Those phospholipid headgroups, especially CL, appear to play an important role in aiding Pseudomonads in their adaptation against hydrocarbon solvents (46). Recently, an increase in CL-containing lipids was reported in strains of *P. putida* S12 and *Pseudomonas taiwanensis* VLB120 grown in the presence of n-butanol (35). Accordingly, CL-containing lipids are important for the function of the efflux pumps in *P. putida* DOT-T1E (42).

Recent metabolomic analyses of *P. putida* DOT-T1E showed that the intracellular ornithine concentration increases in response to toluene exposure (47). Ornithine-containing lipids are known to play an important role in stabilizing the outer membrane and the negative charge of lipopolysaccharides (LPS), as well as in the stress response towards abiotic conditions such as elevated temperature and acidic environment (48).

## Membrane vesicle formation

Outer membrane vesicle (OMV) is a spherical compartment released from the outer membrane of bacteria (consisting phospholipids, LPSs, and small amounts of outer membrane proteins) as a response to various stress condition encountered in the environment (49). Encapsulation of hydrocarbon solvents by the formation of membrane vesicles is an effective defence mechanism in solvent tolerant *P. putida* strains in the presence of toluene (50). By forming these membrane vesicles, the cells effectively discard toluene adhering to the outer membrane. In *P. putida* DOT-T1E, the formation of OMVs contributes to a rapid and extreme rise in cell surface hydrophobicity, which prepare the cells for biofilm formation as a protective response towards solvent-induced stress (51, 52). Membrane vesicles also play a role in releasing lipids with lesser degrees of saturation, enabling rapid lipid turnover as a response to the presence of hydrocarbon solvents (52).

## Resistance, nodulation and division (RND) efflux pump and membrane proteins

Adaptive cell membrane properties constitute a robust mechanism against toxic hydrocarbon solvents. However, decreased membrane permeability does not necessarily generate sufficient tolerance in the presence of hydrocarbon solvent (53). Therefore, cells need an effective mechanism to actively extrude accumulating toxic solvents.



In both Gram-positive and Gram-negative bacteria, the most important membrane proteins in terms of solvent tolerance are the RND efflux pumps (35, 53–55). The RND efflux pumps can extrude a broad range of compounds with little chemical resemblance to each other. They are frequently associated with resistance to a broad spectrum of antibiotics and heavy metals (54, 56). Some RND efflux pumps are specifically induced by (and only extrude) hydrocarbon solvents and are not induced by, for example, hydrophobic antibiotics. Illustrative examples are SrpABC from *P. putida* S12 and TtgDEF from *P. putida* DOT-T1E (53, 55). Recent knowledge and advances in the field of these efflux pumps, their role, control mechanisms, and cross-resistance with antibiotics and efflux properties have recently been extensively reviewed (57, 58).

Novel recent findings have pointed to differential expression of membrane porins and other secretion systems in solvent-tolerant *Pseudomonads* exposed to solvents (14, 15, 59). Unspecific outer membrane porins are downregulated in the presence of toluene to prevent influx of toluene (14, 15, 59). A membrane protein OprH is found to be upregulated to stabilize cell membrane and decrease the uptake of toluene (15, 59). Hence, alongside the RND efflux pumps, other membrane proteins may play important roles in constituting solvent tolerance.

## Molecular chaperones and general stress responses

The presence of hydrocarbon solvents invokes similar stress responses in both Gram-positive and Gram-negative bacteria (15, 16, 60). In several bacterial species confronted with hydrocarbon solvents, general stress response regulators such as the heat shock protein and the cold shock protein are upregulated (15, 16). Other members of the general stress response system may be induced by the presence of toluene, such as molecular chaperones, oxida-

tive stress response components, and other resistance proteins in Gram-negative *P. putida* DOT-T1E and *P. putida* S12 as well as in Gram-positive *B. subtilis* (16, 60). Accordingly, the toluene-repressed gene (*trgI*) of *P. putida* S12 was found to control a large number of protein modification and chaperone genes (18).

## Bioenergetics and redox balance



Several studies in *P. putida* have indicated that in the presence of hydrocarbon solvents, tricarboxylic acid (TCA) cycle components are upregulated, the NAD(P)H regeneration rate is increased, and growth is reduced (14–16, 18, 61). Differential expression of TCA cycle-related proteins modulates the NAD(P)H concentration, and therefore the redox balance, throughout the solvent stress (15). Upregulation of the TCA cycle and concomitant increase of the NAD(P)H regeneration rate enable the cells to cope with the energetic potential loss connected with rapid solvent extrusion through the efflux pumps (15, 61). As a representative illustration, the ATP content, cellular concentration of potassium and adenine nucleotides, and the adenylate energy charge were all similar in cells of *P. putida* DOT-T1E grown in the presence or absence of 1-decanol (51). These findings reflected the efficient metabolic and energetic adaptation of solvent-tolerant bacteria during their exposure to toxic hydrocarbon solvents.

## Changes in cell morphology

Both Gram-positive and Gram-negative bacteria exhibit changes in cell morphology and in cell size as a response to the presence of hydrocarbon solvents (62–65). For example, decrease in cell size was observed in *P. aeruginosa* and *Enterobacter sp.* upon the exposure to hydrocarbon solvents (63, 65). However, conflicting observations were reported in *Bacillus lichineformis* S-86, *P. putida* P8, and *Enterobacter sp.* VKGH12 which have shown increases in cell volume in the presence of hydrocarbon solvents (41, 64). Additionally, in the presence of 0.6% 3-methylbutan-1-ol, *B. lichineformis* S-86 was reported to exhibit filamentous growth (62). By decreasing cell-size, cell surface-to-volume ratio increases, contributing to a more efficient uptake of nutrient. With the decreased cell surface-to-volume ratio, cell surface exposure is reduced and solvent extrusion can be more effective.

## Applications of solvent-tolerant bacteria in biocatalysis of valuable compounds



Employing bacteria for biocatalysis is currently a preferred method for industrial synthesis of various biochemicals, pharmaceuticals, and enantiomerically pure intermediates. Indeed, such synthesis routes require co-enzymes and co-factors, and stepwise/multiple enzymatic reactions that may be readily available within the microorganism of choice (38). In the bio-production of industrial chemicals, the production process is often hampered by the toxicity of the substrate or the product, which may severely affect the product yield (3, 6). Solvent-tolerant bacteria are favored for the biocatalytic production of many valuable compounds, since they are far less prone to inhibition by toxic compounds, so the desired yields can be better achieved. Valuable compounds that can be readily produced through the use of solvent-tolerant bacteria include simple aromatic compounds such as phenol or p-hydroxybenzoate, as well as more complicated compounds such as 2,5-furandicarboxylic acid (FDCA), enantiomerically specific (s)-2-octanol, and pharmaceutically active 15β-hydroxytestosterone (Table 2.2). Recently, the biobased production of a major building-block chemical FDCA, a promising 'green' alternative to terephthalate in the production of polyesters, from 5-hydroxymethyl-furfural (HMF) was achieved in the noted solvent-tolerant strain *P. putida* S12. Hence, solvent-tolerant traits of microbial production strains can enable the use of hydrocarbon solvents and solvent-like compounds as substrate and intermediates for the production of high-value compounds. In addition, the unique features of solvent-tolerant bacteria allow tolerance towards a broad range of potentially toxic compounds and make them highly suitable for implementation in two-phase bioreactors production set-up (3, 66). The main challenges that arise in using solvent-tolerant bacteria in biocatalysis are maintaining product yield and system complexity.

Solvent-tolerant bacteria are well suited for biocatalytic production in two-phase biocatalysis systems, as reviewed previously (38). This system can significantly improve production yield by reducing substrate and/or product toxicity (3, 6). The use of a hydrocarbon solvent as the second phase has several advantages, including reduced reaction inhibition, reduced toxicity towards the microbial host and the prevention of product hydrolysis (3). Moreover, the second hydrocarbon phase acts as a simultaneous extraction step, thus simplifying downstream processing and purification and increasing the yield of poorly water-soluble products (29). Hydrocarbon solvents having log $P_{o/w}$ values in the range of 1 to 4 are considered

suitable for product extraction and substrate reservoir in a two-phase biocatalysis system, and solvent-tolerant bacteria can survive and exhibit biocatalytic activity under these circumstances. Known bacterial index values have been extensively listed in previous articles (11, 67). Predominantly Gram-negative bacteria have index values in the ideal two-phase biocatalysis range from 1 to 4.



Several examples demonstrate increased product titre and optimized production of valuable chemicals in two-phase biocatalysis system (3, 6, 68). Production of p-hydroxystyrene in *P. putida* S12 was established with by introducing the *pal* (L-phenylalanine/L-tyrosine ammonia lyase) and *pdc* (p-coumaric acid decarboxylase) genes in combination with inactivating the *fcs* gene (6). A product titre of 4.5 mM with a yield of 6.7% (C-mol p-hydroxystyrene/C-mol glucose) and maximum volumetric productivity of 0.4 mM $h^{-1}$ was initially achieved. However, due to the toxicity of p-hydroxystyrene, cell growth and production was inhibited. Using decanol as a second phase, the toxicity of the product p-hydroxystyrene was significantly reduced, which resulted in a p-hydroxystyrene titer of 147 mM (17.6 g $l^{-1}$), a fourfold increase compared with a standard fed-batch production. The maximum volumetric productivity was also increased to reach 0.75 mM $h^{-1}$. Similarly, production of p-hydroxystyrene from p-coumaric acid from corn cob hydrolysate using recombinant *Escherichia coli* and simultaneous extraction by n-hexane as the second phase clearly improved product titre (68). Another example is the bioproduction of vanillin from isoeugenol, which can be inhibited by two major phenomena: the toxicity of isoeugenol and vanillin to microbial host, and the low solubility of isoeugenol in water (3). The solvent-tolerant Gram-positive bacterium *Brevibacillus agri* 13 can produce vanillin from 2 g $l^{-1}$ isoeugenol with a yield of 7.6% (Cmol vanillin/Cmol isoeugenol) in a single-phase system. Using butyl-acetate (30% v/v) as an second-phase with 10 g $l^{-1}$ isoeugenol increases the production yield to 17.2% with product titer of 1.7 g $l^{-1}$ after 48 hours of fermentation. Here, the reduction of isoeugenol and vanillin toxicity in combination with the simultaneous extraction of vanillin by the second phase result in increased product formation.

**Table 2.2. Biocatalysis using solvent tolerant bacteria**

| Product | Biocatalyst | System | Challenge(s) in production process | Product titer (mM) | Yield ($Cmol_p/Cmol_s$) | Productivity | Comparison | Ref. |
|---|---|---|---|---|---|---|---|---|
| p-Hydroxybenzoate | *P. putida* S12 expressing *pal* gene from *Rhodosporium toruloides* | Fed batch whole-cell biocatalysis system | Toxic aromatic product | 12.9 | 8.5% | 0.168 (mmol $h^{-1}$ $gCDW^{-1}$) | - | (5) |
| FDCA (2,5-furandicarboxylic acid) | *P. putida* S12 expressing hmfH gene from *Cupriavidus basilensis* HMF14 | Fed batch whole-cell biocatalysis system | Toxic aromatic substrate | 192.83 | 97% | 0.096 ± 0.004 (mmol $h^{-1}$ $gCDW^{-1}$) | - | (4, 97) |
| Anthranilate | *P. putida* KT2440 expressing *trpDC* with futher optimization of anthranilate production pathway | Fed batch whole-cell biocatalysis system | Toxic aromatic product | 11.23 | 3.6 ± 0.5% | N/A | 1.83 mM, without further optimization of the anthranilate production pathway | (2) |
| (S)-2-Octanol | *P. putida* DSM 12264 expressing CYP154A8 | Fed batch whole-cell biocatalysis system | Hydrocarbon solvent as product | 15.7 (87% ee) | N/A | 0.172 (mmol $h^{-1}$ $gCDW^{-1}$) | 2.2 mM, 58% ee in *E. coli* system expressing CYP154A8 | (66) |
| 3-Methylcathecol | *P. putida* DOT-T1E containing pWW0 plasmid from *P. putida* KT2440 | Two phase batch whole-cell biocatalysis system with aliphatic alcohol as the second phase | Second-phase for product reservoir | 70 | N/A | 4.83 (mM $h^{-1}$) | 3 mM, using the same strain without the two phase system | (10) |
| Phenol | *P. taiwanensis* VLB120 with minimal genomic modification and expressing *tpl* gene from *Pantoea agglomerans* | Fed batch whole-cell biocatalysis system | Toxic product | 3.62 | 18.5 ± 0.2%, | 0.09 ± 0.00 (mM $h^{-1}$) | 1.5 mM in *P. putida* S12 | (1) |
| p-Hydroxystyrene | *P. putida* S12 expressing *pal* gene from *Rhodosporium toruloides* and *pdc* gene from *Lactobacillus plantarum* | Two phase fed batch whole-cell biocatalysis system with decanol as the | Toxic product and second-phase for product reservoir | 147 | 4.1%, | 0.75 (mM $h^{-1}$) | 21 mM, using the same strain without the two phase system | (6) |
| Vanillin | *B. agri* 13 | Two phase batch whole-cell biocatalysis system with butyl acetate as the second phase | Second phase for toxic product and substrate reservoir | 11.17 | 27.8% | N/A | < 6.5 mM $l^{-1}$, on *Bacillus subtilis*, *Pseudomonas chlororaphis*, and recombinant *E. coli* without two phase system | (3) |
| 1-Naphthol | *E. coli* TG1 pBS(kan)TOM-Green expressing srpABC operon from *P. putida* S12 | Two phase batch whole-cell biocatalysis system with lauryl acetate as the second phase | Toxic aromatic product | 9.91 | N/A | 3.81 (mmol $gCDW^{-1}$) | 0.27 mM , without two phase system | (83) |

## The case of FDCA

A recent study identified and characterized a fully biobased enzymatic route for the production of 2,5-furandicarboxylic acid (FDCA) directly from 5-hydroxymethylfurfural (HMF) [86]. HMF, like furfural, is an intrinsically toxic furanic aldehyde occurring in lignocellulosic hydrolysates (Fig. 2.2). FDCA has been proclaimed by the USA Department of Energy as 1 of 12 priority chemicals for the realization of a biobased green chemistry industry (69). It is regarded an important platform compound for the synthesis of a variety of aromatic chemical building blocks, including as a biobased alternative for the monomer terephthalic acid in polymeric polyethylene terephthalate (PET) (70–73). Polymerisation of ethylene glycol and FDCA yields polyethylene furanoate (PEF), which has improved barrier, thermal and mechanical properties compared with PET (72).

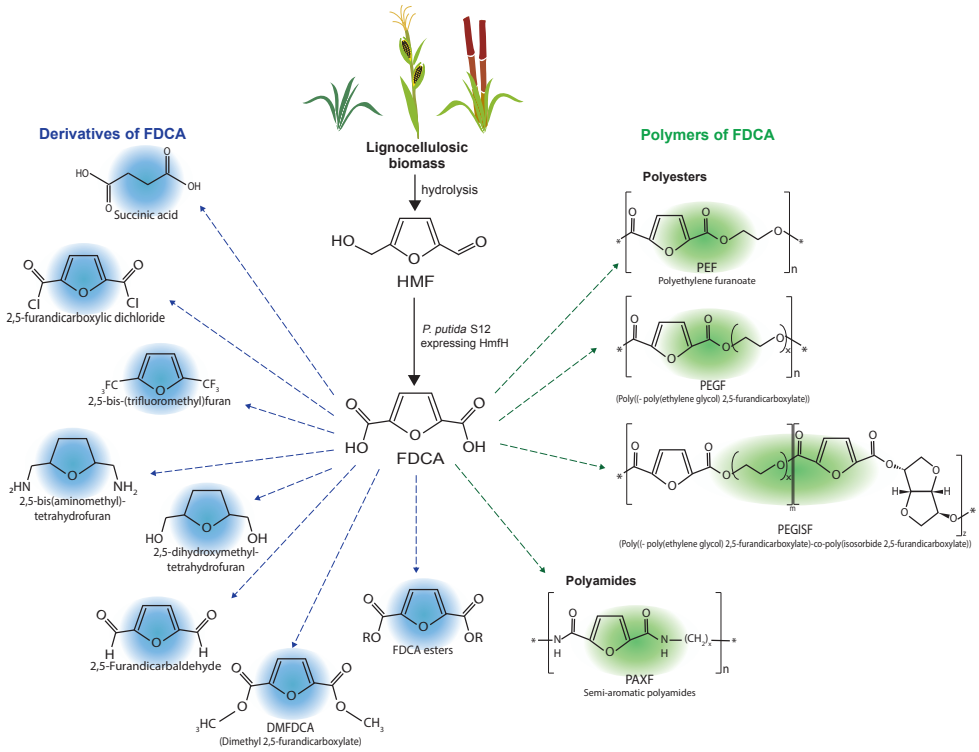


**Fig. 2.2. Potential applications of FDCA, from lignocellulosic biomass to biopolymers**

Expression of a novel, specific HMF/furfural oxidoreductase from the soil bacterium *Cupriavidus basilensis* in the solvent-tolerant industrial host bacterium *Pseudomonas puti-*

*da* enabled efficient high-yield production of FDCA from HMF (4). In this process, *P. putida* proves to be an efficient whole-cell biocatalyst. The company Corbion is currently developing a cost-effective production route for FDCA, based on this novel enzymatic route (http://www.corbion.com/bioplastics/products/fdca-for-pef).



### Challenges in biocatalysis using solvent tolerant bacteria

The primary complication with using solvent-tolerant bacteria in industrial biotechnology is the unpredictable product yield caused by host interference issues (19, 74, 75). Adapting native biocatalytic pathways often provokes imbalances in pathway flux, the accumulation of toxic intermediates, and reduced cellular fitness, again causing unpredictable product yields (76). Genome streamlining reduces host interference, resulting in increased biomass and growth rate and subsequently leading to optimizing production chassis (19). Imbalances in pathway fluxes, bottlenecking enzymes, and accumulation of toxic intermediates can be mitigated by optimizing metabolic pathways (5, 29, 76, 77). And the development of synthetic biology tools is becoming crucial to support the implementation of solvent-tolerant bacteria in biocatalysis (20, 78).

Another advantage of solvent-tolerant bacteria is their easier implementation in two-phase bioreactor systems. However, other challenges may arise in two-phase bioreactor systems, such as increased system complexity, problems with waste disposal, and the hazardous risk of using flammable solvents (79). By applying heat treatment or a continuous-plate centrifuge, a solvent emulsion in an aqueous phase can be degraded or, preferably, be avoided, resulting in a clear solvent that can be processed by further downstream treatment. Distillation may be applied in the downstream process specifically to purify volatile product from its volatile substrate. Schmid and colleagues developed a safe and efficient pilot-scale two-phase bioreactor containing flammable solvent (79). Finally, exogenously supplemented glycerol provides effective protection and thus improves bacterial growth in a two-phase bioreactor system (80).

## Synthetic biology and engineering towards advanced biocatalysts

Host interference issues can be overcome by reducing the complexity of the genome in the

microbial chassis by genome streamlining (74). Genome streamlining is widely used in engineering industrial bacterial strains (75, 81). This approach has resulted in increased biomass formation, reduced doubling times, increased product yield, and ultimately optimized production systems (19). Metabolic pathway optimization can resolve imbalances in pathway fluxes and reduce accumulation of toxic intermediates to restore cellular fitness (76, 82). Transferring solvent-tolerant traits to a preferred industrial host strain is also a plausible strategy (83). In combination, these strategies comprise promising approaches to exploit the solvent tolerance features of bacteria for producing a wide range of valuable compounds with a high degree of predictability and robustness (Fig. 2.3). Existing and novel tools for synthetic biology and the rapidly accumulating genome sequencing data of solvent-tolerant bacteria, drive the opportunities to implement these strategies.

## Molecular synthetic tools for improving solvent-tolerant process design and application

Synthetic molecular tools are crucial aspects for developing a robust industrial bacterial strains. BioBricks was developed as flexible exchangeable DNA fragments that can be combined to fully synthesized biological tools suitable for common industrial strains like *E. coli* (78). The Standard European Vector Architecture (SEVA) established a reliable and efficient vector repository accompanied by a simple and user-friendly database mainly implemented in solvent-tolerant *P. putida* and other industrial strains (20). A plasmid system, GeneGuard, was constructed to overcome the safety concerns including unwanted horizontal gene transfer by host-mutual dependency, based on using SEVA plasmids (24).

Optimizing a robust bacterial chassis requires both precise genome editing tools and the ability to incorporate new features into its genome. The CRISPR/Cas system has become a standard tool in editing bacterial genomes (84). Using SEVA plasmids as its backbone, a recombination event between free homologous DNA sequences, allowing an accurate genome editing, was developed for a wide variety of Gram-negative bacteria (21).

Transposon tools for specific transgene introduction, in combination with promoter libraries for *P. putida* cell factories, have been developed as an alternative to the use of multi copy plasmids (22, 23, 85). The developed mini-Tn5 vector offers the advantages of maintaining introduced genes without selective pressure, construct stability, recurrent use of the

system, and introducing a relatively large DNA sequence (22). The mini-Tn7 transposon system can integrate with a high frequency in a specific location as a unidirectional single copy of gene that is suitable in various studies for gene expression, characterization of certain genes, and gene complementation (23). Finally, a novel broad range system for the transfer and expression of biosynthetic pathways (TREX) was developed using this transposon system to include all functional elements that are essential for efficient introduction and expression of pathway clusters in different bacteria (86).

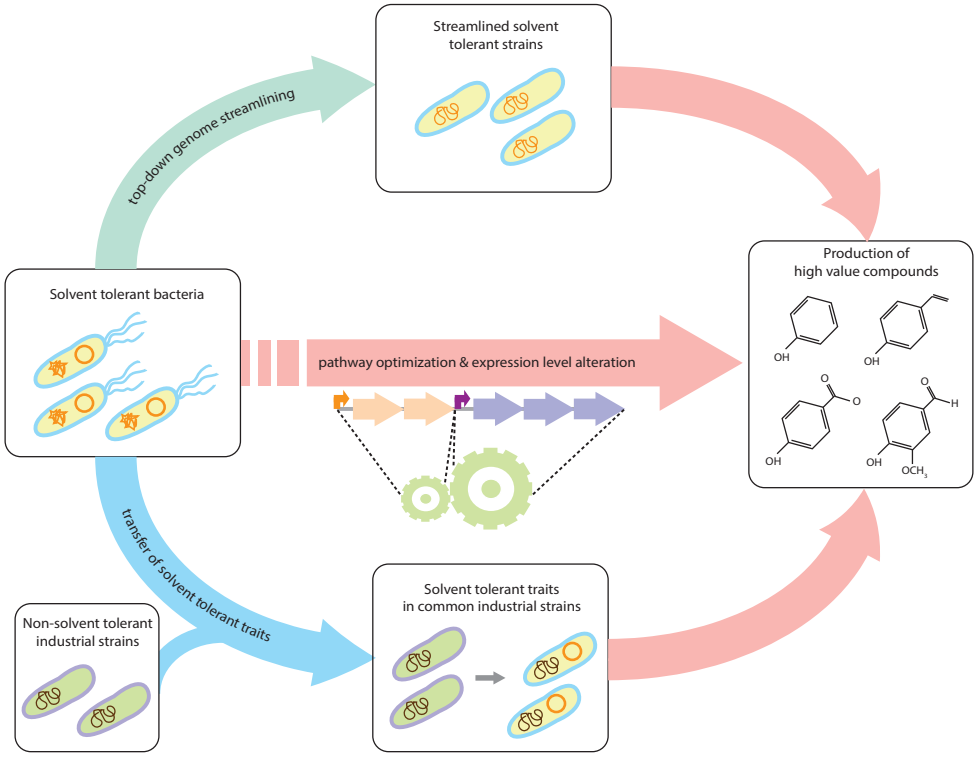


**Fig. 2.3. Synthetic biology and engineering towards advanced solvent-tolerant biocatalysts**

Three optimization strategies on biocatalysis using solvent-tolerant bacteria are employed to solve problem of host interference, which can cause an unpredictable yield of products. Pathway flux imbalance can be reduced by identifying bottlenecking enzymes and altering the expression level of the bioproduction pathway (red arrows). Genome streamlining can be applied to reduce the genome complexity of solvent-tolerant strains (green arrow). Introducing solvent tolerance-related genes into an existing industrial strain is also proven to be a promising approach (blue arrow). Synthetic biology tools are useful in pathway optimization, altering the expression level of bioproduction, strain optimization, and conferring solvent-tolerant traits.

## Pathway optimization and adaptation of enzyme expression

Metabolic pathways can be optimized by characterizing enzyme expression, identifying bottlenecking enzymes, and subsequently optimizing the expression and activity of enzymes through modulation of transcription, translation and specific enzyme characteristics (76, 82). As an example, transcriptomics and proteomics studies of p-hydroxybenzoate-producing *P. putida* S12 identified critical components of the tyrosine degradation pathway (5, 77). Subsequent deletion of the *hpd* gene involved in p-hydroxybenzoate degradation led to a 22% increase of p-hydroxybenzoate production. In another case, by overproducing the pyruvate dehydrogenase subunit gene *acoA* or deleting the glucose dehydrogenase gene *gcd* to overcome bottlenecking, production of polyhydroxyalkanoate (PHA) in *P. putida* KT2440 was increased by 33% and 121%, respectively (87).

In combination with rapidly emerging synthetic biology tools, pathway optimization is a powerful strategy in designing optimized bacterial strains for application in industrial biotechnology. The highest yield in microbial phenol production reported so far was achieved by implementing pathway optimization on solvent-tolerant *P. taiwanensis* VLB120 (1). To optimize phenol production, catabolic routes toward aromatic compounds and shikimate pathway intermediates are inactivated. This inactivation is accomplished by the deletion of five genes: *pobA*, *hpd*, *quiC*, *quiC1*, and *quiC2,* along with the subsequent loss of megaplasmid pSTY. This process yields *P. taiwanensis* VLB120Δ5, which is unable to grow on 4-hydroxybenzoate, tyrosine, and quinate. The introduction of a codon-optimized tyrosine-phenol lyase (TPL) gene from *Pantoea agglomerans* facilitates tyrosine transformation into phenol. Metabolic flux towards phenol production is further increased using forward- and reverse-engineering from leads indicated by previous mutagenesis of phenol-producing *P. putida* S12 (88) and the addition of bottlenecking enzymes AroG and TyrA. *P. taiwanensis* VLB120Δ5-TPL36 achieved the yield of 15.6% and 18.5% (Cmol/Cmol) of phenol in minimal medium from glucose and glycerol, respectively, without requiring additional complex nutrient.

Synthetic promoter libraries can optimize the expression of several modules in a metabolic pathway (23). Using synthetic promoters, the production of rhamnolipids in *P. putida* KT2440 was significantly increased, reaching a yield of 40% rhamnolipids on sugar (89, 90). These examples present further proof that pathway optimization is a highly promising

approach to resolving pathway flux imbalance and improving biomass and product yield in solvent-tolerant bacterial industrial host strains.

## Top-down strategies in genome streamlining



Genome streamlining has been implemented in various industrial host strains, such as *E. coli* and *Streptomyces* species (75, 81). Top-down genome streamlining deletes from microbial chassis multiple genes or gene clusters that are predicted to be inessential for the microbes, consume high amount of energy, contribute to the degradation of products or intermediates, or reduce metabolic flux towards the product of interest (75). Alternatively, the bottom-up strategy attempts to design a production chassis from scratch based on minimum requirements for a functioning microbial chassis. The top-down strategy significantly increased the biomass yield and the maximum specific rate for protein synthesis in the streamlined hosts *P. putida* EM329 and *P. putida* EM383, compared with the parental strain *P. putida* KT2440 (19, 91). One early example was *Pseudomonas arvilla* mt-2, described by Murray and colleagues in 1972 as a fascinating strain of *Pseudomonas* able to grow on benzoate, m-toluate (3-methylbenzoate) or p-toluate (4-methylbenzoate) as its sole carbon source (92). A derivative of this strain, *P. putida* KT2440, has been cured of the endogenous megaplasmid pWW0 present in the parental strain *P. putida* mt-2. Since then, *P. putida* KT2440 has proven to be a suitable host for gene cloning due to its deficiency in endogenous DNA restriction, so it can efficiently receive plasmid DNA for gene cloning purposes (93). *P. putida* KT2440 is a generally regarded as safe (GRAS) strain of *P. putida*[1]. The genome of *P. putida* KT2440 comprises of a 6,181,873-bp single circular chromosome (25).

In the process of optimizing *P. putida* KT2440 towards a robust industrial chassis, 11 chromosomal regions comprising 300 genes, including mobile elements, were found to be responsible for genetic instability or massive energy spillage (19). Together, these genes comprise a 170 kb genome segment encoding two transposons (Tn7 and Tn4652), prophages, two type I DNAses (*endA-1* and *endA-2*), an operon encoding type I DNA restriction-modification system (hsdRMS operon), and the 69-kb complete flagellar operon. Mobile elements play a significant role in the adaptation during solvent exposure, but mobile elements are

[1] **Correction:** *P. putida* KT2440 is classified by the FDA as HV1 certified, indicating it is safe to use in a P1 or ML1 environment. (98)

also responsible for genetic instability (94). Removing all of these genes resulted in a new optimized strain of *P. putida* EM42. To further diminish the probability of genetic instability, *recA* was deleted, resulting in *P. putida* EM383. This streamlined *P. putida* EM383 was shown to be superior to *P. putida* KT2440, as it exhibited a reduced lag phase, increased biomass formation, and increased redox charge, leading to exceptional tolerance against redox stress and reactive oxygen species damage.



## Optimization of industrial host strains with solvent tolerance traits

Improving tolerance against toxic compounds is an important step towards developing a robust bacterial chassis for the industrial production of a wide range of valuable compounds. Using a modular semisynthetic system, overexpression of heat shock proteins GrpE, GroESL, and ClpB in *E. coli* generated a stress response that increased tolerance towards ethanol, n-butanol, and other toxic compounds (95). An engineered *E. coli* TG1-derived strain expressing the solvent efflux pump SrpABC from *P. putida* S12 was employed for 1-naphthol production in a two-phase fermentation (83). Although 1-naphtol production did not reach the same levels as in *P. putida* S12, this result demonstrated the successful transfer of the *Pseudomonas* solvent extrusion pump gene cluster, providing the engineered *E. coli* strain with a genuine solvent-tolerant trait.

The introduction of multiple efflux pumps may promise further advantages, but overexpression of efflux pumps may severely inhibit cell growth (96). As demonstrated by Turner and Dunlop, certain combinations of different efflux pumps can be highly toxic, even at basal expression levels of the pump proteins. Another successful example of optimizing solvent tolerance relates to bacterial fatty acid modification. Introducing cyclopropane fatty acid synthase (Cfa) from the solvent-tolerant strain *Enterococcus faecalis* CM4A into *E. coli* clearly increased tolerance towards n-butanol (34). Cfa activity maintains the fluidity of the cell membrane upon exposure to toxic hydrocarbon solvents. Further understanding of the roles of and interplay between solvent-tolerant mechanisms will enable the transfer of solvent-tolerant traits into suitable industrial host strains.

## Concluding Remarks and Future Perspectives

Increased insight into solvent tolerance mechanisms is an important basis for biotechnological production of challenging compounds. An increasingly wider variety of compounds will be produced in microbial hosts due to the transition to a biobased economy. However, biobased production of added-value compounds, many of which are aromatics, is still challenging because of the inherently toxic nature of most of these compounds. Solvent-tolerant strains indeed represent a promising solution to this problem. A deeper understanding of the interplay in solvent tolerance mechanisms is still required to further increase the applicability of solvent-tolerant traits in industrial production.



With the help of modern synthetic biology tools, top-down genome streamlining of solvent-tolerant strains is essential to reduce host interference and increase production yields. In this approach, the challenge is to identify minimal gene clusters required for solvent tolerance and biosynthetic capacity which should not be disrupted. Implementing specific synthetic biological tools, like efficient gene editing for introducing heterologous genetic feature, or adjustable transcriptional regulators for pathway optimization, will enable the rapid generation of optimized production strains.

Transferring solvent tolerance traits into existing industrial strains may be a promising alternative strategy to optimize biobased production. The required synthetic biology tools are already available for established industrial strains. The challenge in this strategy is in obtaining the desired expression level of exogenous gene clusters in their new hosts. Once again, this highlights the necessity for thorough analysis and understanding of solvent tolerance mechanisms and the interplay of these mechanisms that orchestrate the tolerance toward solvents.

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