Acta Scientific Biotechnology

Volume 1 Issue 4 April 2020

Research Article

Engineered *Pseudomonas putida* for Biosynthesis of Catechol from Lignin Derived Model Compounds and Biomass Hydrolysate

Priya Upadhyay[1] and Arvind Lali[1,2]*

[1]*DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai, Maharashtra, India*

[2]*Department of Chemical Engineering, Institute of Chemical Technology, Mumbai, Maharashtra, India*

***Corresponding Author:** Arvind Lali, DBT-ICT Centre for Energy Biosciences and Department of Chemical Engineering, Institute of Chemical Technology, Mumbai, Maharashtra, India.

Received: March 02, 2020

Published: March 14, 2020

Abstract

Catechol is one of the industrially relevant chemicals with myriad applications. Its production via chemical route suffers from several drawbacks the major being a non-green and non-selective route. Currently, bio-based products using biocatalyst are gaining attention due to the growing awareness about the environmental and health hazards over the use of petroleum derived feedstock. Lignocellulosic biomass serves as a promising sustainable and renewable feedstock wherein celluloses have found successful ways to a variety of fuel and biochemical molecules but concomitantly results in surplus lignin being produced. Lignin valorization is the demand of the current scenario to make the cellulosic bio-refineries viable. This task is complicated by complexity, heterogeneity and diversity of lignin structures posing limitations towards lignin valorization via chemical routes. There are several microorganisms that possess the ability to metabolize lignin monomers via their central metabolic pathways and this paves a way to synthesis of a number of products. *Pseudomonas putida* KT2440 is one such organism and was chosen for genetic manipulations for catechol biosynthesis using lignin derived model compounds and biomass hydrolysate stream comprising of various lignin fragments/monomers. Catechol production was engineered by diverting various lignin monomers and addressing the identified metabolic bottlenecks particularly vanillic acid accumulation towards catechol biosynthesis. The engineered strain could convert the model lignin monomers as well as monomers in the biomass hydrolysates to catechol and vanillic acid in more than 60% and 90% molar yields, respectively.

Keywords: *Pseudomonas putida*; Catechol; Lignin Valorization; Lignin Derived Model Compounds; Biomass Hydrolysates

Abbreviations

PCR: Polymerase Chain Reaction; SOE: Splicing for Overlap Extension; LBNS: Luria Bertini No Salt medium; RBS: Ribosome Binding Sequence; SDS PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; GCMS: Gas Chromatography Mass Spectroscopy; HPLC: High Performance Liquid Chromatography; LBM: Lignocellulosic Biomass Hydrolysate.

Introduction

Catechol and its derivatives are important chemical precursors for several applications such as carbamate insectidices (carbofuran and propoxur), polymerization inhibitors (4-tert-butylcatechol), photographic developers, tanning agents, perfumes, cosmetics, biomaterial and therapeutic agents [1,2]. Annually, 2.5 × 10^7 kg of catechol is produced commercially by synthetic/chemical routes

[3]. The various chemical routes for catechol production includes hydrolysis of 2-chloro phenol with copper as a catalyst at elevated temperature; hydroxylation of phenol in presence of peroxide along with catalyst; dehydrogenation of 1,2-cyclohexanediol with palladium as a catalyst at 300°C; and oxidation of salicylic aldehyde and demethylation of guaiacol [1,4,5]. Chemical synthesis of catechol is a catalyst based energy intensive process, resulting in co-production of compounds such as hydroquinone and resorcinol. This compound along with un-reacted phenol mixtures makes separation and scale up a tedious job [1,6,7]. Moreover, catechol is derived from petroleum based feedstock leading to several environmental issues thereby necessitating focusing our attention towards catechol synthesis from sustainable and renewable starting material using microbes [3,8]. Lignocellulosic biomass is one such sustainable and renewable feedstock giving rise to bio-refinery concept.

Bio-refineries have gained tremendous attention worldwide for the production of biofuels and allied chemicals due to growing environmental concerns, zeal towards rural development and energy security [9]. These bio-refineries, co-produce substantial amount of lignin which is treated as waste while only a part of it is used for unit operational processes and rest is simply relegated for combustion [10]. Moreover, the lignin generated in this process is wet and has lower calorific value compared to coal limiting its application in operation of cellulosic biorefinery, accounting to its market value lower than $50/dry ton [11]. Thus, lignin valorization towards high value end products such as bulk or fine chemicals is mandatory for the sustainability and economic viability of these cellulosic bio-refineries [12].

Lignin is a complex heterogeneous poly-aromatic molecule with potential to serve as a raw material for the production of wide array of aromatic chemical building blocks [13]. Huge amount of research and efforts has been undertaken to address lignin up-gradation by tackling the issues of recalcitrant nature, heterogenous and complex structural chemistry [11]. Success in lignin valorization depends upon its effective separation; depolymerisation to its monomer components and finally up-gradation of these components towards the targeted product [10,14]. The up-gradation can be carried out via a chemical route; where catalysts assist selective bond cleavage of the lignin molecules resulting in production of more than 20 individual compounds, thereby making their effective separation and purification to a single or a few useful products economically nonviable [12,15]. On the other hand, nature has devised several microbial pathways that can assimilate and metabolize these compounds. It is therefore advisable to use such organisms that possess these pathways and possibly engineer them for targeted chemical synthesis from lignin.

Pseudomonas serve to be an ideal host for lignin up-gradation via biological route as it posses the versatile metabolism and can tolerate the stress of inhibitory compounds generated from the lignocellulosic biomass [16,17]. Catechol is an intermediate metabolite of β-ketoadipate pathway through which *Pseudomonads* catabolises a plethora of aromatic compounds [18,19]. Catechol biosynthesis can be carried out by generation of the mutant defective in catechol metabolism [19]. There are reports wherein mutants *Pseudomonas* species Ba-0511 and *Pseudomonas* species B3-1TB were engineered for catechol biosynthesis using sodium benzoate as substrate [19,20]. Sodium benzoate however, remains the only lignin derived model compound reported for catechol biosynthesis using *Pseudomonas sp*.

In this study, *Pseudomonas putida* KT2440 was genetically modified for the biosynthesis of catechol from different lignin-derived aromatic model compounds such as sodium benzoate, p-coumaric acid, p-hydroxy benzoic acid, ferulic acid, vanillic acid and protocatechuic acid. These aromatics were taken into consideration as different varieties of biomass such as poplar, corn stover, switch grass and empty fruit bunch upon chemical pre-treatment process result into a range of aromatic monomers such as p-hydroxy-phenyl monomers (hydroxybenzoic acid and p-hydroxybenzaldehyde), vanillic acid, vanillin, p-coumaric acid and ferulic acid [21,22]. The biomass hydrolysate stream generated in our laboratory also resulted in generation of range of lignin monomers; majorly comprising of aromatic monomers that form natural substrates and are also metaloic intermediates of the protocatechuate branch of β-ketoadipate pathway. Catechol biosynthesis using lignin-derived model compounds and lignocellulosic biomass hydrolysates was attempted by the engineered *Pseudomonas putida* KT2440 via creating the gene specific targeted mutant defective in catechol and protocatechuate metabolism; while also diverting the protocatechuate branch aromatics towards catechol (Figure 1).

Figure 1: β-Ketoadipate pathway engineering in *Pseudomonas putida* KT2440 for the biosynthesis of catechol.

Catechol biosynthesis involved knocking of the genes *cat*A/A2 encoding catechol 1,2-dioxygenase (PP_3713; PP_3166; E.C. 1.13.11.1) and *pca*HG encoding protocatechuate 3,4-dioxygenase (PP_4656; PP_4655; E.C. 1.13.11.3). Protocatechuate branch aromatics were diverted towards the catechol via the heterologous expression of the codon optimized protocatechuate decarboxylase gene from *Enterobacter cloacae* encoded by *aroY*. Vanillic acid accumulation was addressed by the overexpression of codon optimized *van*AB gene encoding vanillate-O-demethylase from *Acinetobacter sp*. ADP1.

Materials and Methods

Chemicals and reagents

T4 DNA ligase, restriction enzymes (EcoRI, HindIII, kpnI, BamHI, PstI), shrimp alkaline phosphatase were purchased from New England Biolabs USA. PrimeSTAR Max DNA polymerase (2×) high fidelity PCR master-mix was purchased from Clontech (DSS TaKaRa Bio India Pvt Ltd.). Dream Taq Green PCR Master Mix (2×) was procured from ThermoFisher Scientific (India). NucleoSpin® Gel and PCR Clean-up kit was purchased from Macherey-Nagel (MN, India). Miniprep kit was purchased from GeneAll USA. Primers were procured from Eurofins Genomics, India.

Standard aromatic chemicals such as vanillin, vanillic acid, protocatechuic acid and p-coumaric acid were purchased from Sigma-Aldrich, India; ferulic acid, p-hydroxybenzoic acid and sodium benzoic acid was procured from S. D. Fine Chemicals, India while Pyrocatechol was procured from Loba Chemie, India. All HPLC and GCMS grade reagents were from Sigma-Aldrich, India. Maize bran hydrolysates were provided by Privi Organics Ltd. Mumbai, India

Bacterial strains, plasmids and growth conditions

Escherichia coli DH5α was used for cloning while all the genetic manipulations were carried out in *Pseudomonas putida* KT2440 (ATCC47054). *Escherichia coli* DH5α was cultivated in Luria Bertani broth (Himedia, India) at 37°C while *Pseudomonas putida* KT2440 was grown in M9 minimal medium (Na_2HPO_4: 6 g/L, KH_2PO_4: 3 g/L, NaCl: 0.5 g/L, NH_4Cl: 1g/L, $MgSO_4.7H_2O$: 10mM, $CaCl_2$: 100 µM and trace element solution containing $MnSO_4$: 10 mg/L, $CuSO_4.5H_2O$: 10 mg/L, $FeSO_4$.7H2O: 50 mg/L, H_3BO_4: 10mg/L, Na_2MoO_4: 10 mg/L) supplemented with glucose and aromatics at 30°C under agitation of 180 rpm in 250mL flask in an incubator. Plasmid pk18mobsacB (ATCC 87097) was used for the knockout by gene replacement via

homologous recombination method while plasmid *p*SEVA 234 (Dr. Victor de Lorenzo's lab, CNB-CSIC, Madrid, Spain) was used for the gene expression. These plasmids were maintained in *E. coli* DH5α. *p*SEVA 234 is a medium copy number plasmid with trc based IPTG inducible promoter. When required, kanamycin (Himedia) was used at a concentration of 50 µg/ml for *Pseudomonas putida* KT2440 while 25 µg/ml in case of *Escherichia coli*. Plasmid pk18mobsacB was used for the knockout and maintained in *E. coli* DH5α. When required, kanamycin was used at the concentration of 50 µg/ml for *Pseudomonas putida* KT2440 and at 25 µg/ml for *Escherichia coli.* Sucrose was used at a concentration of 25% (w/v) for counter selection. Growth was monitored by colorimeter at OD_{600}.

Construction of the knockout mutant *P. putida* KT2440 for catechol biosynthesis

The mutant *Pseudomonas putida* KT2440 was created by gene knockouts in a stepwise manner using allelic exchange protocol [23]. The genes *cat*A/A2 encoding catechol 1,2-dioxygenase and the gene *pca*HG encoding protocatechuate 3,4-dioxygenase were knocked out by counter selection based homologous recombination. The knockout protocol involved in-vitro synthesis of truncated variant of wildtype gene with homologous end to the gene of interest to be replaced. Truncated gene was synthesized from *Pseudomonas putida* KT2440 genomic DNA by PCR amplification of the gene fragments using high-fidelity PrimeSTAR Max DNA polymerase with the pairs of primers as described in table 1. These gene fragments with the overlapping ends were spliced together *via* SOE PCR to obtained truncated allele/gene. The truncated variant of gene *cat*A, *cat*A2 and *pca*HG were synthesized in-vitro; cloned into the suicidal vector pk18mobsacB between restriction site EcoR1 and HindIII using the standard molecular biology techniques to obtain the knockout plasmid [24]. Each gene knockout was carried out one after the other in the clone bearing previous mutation (non-functional gene) subsequently in a step wise manner by introduction of the knockout plasmid containing the truncated variant of the gene to be replaced (1 to 3 µg) in *Pseudomonas putida* KT2440 via electroporation [25]. Transformants were selected onto the cetrimide agar plate supplemented with kanamycin (50 µg/mL). This was further subjected to multiple passages in LBNS (10 g/L tryptone, 5 g/L yeast extract) medium after every 12 hours and thereafter, counter selected on LBNS agar (1.5% bacteriological agar) plates supplemented with sucrose (25% w/v). Colonies from these plated were further screened by patching these colonies onto various selection plates in the order of a) cetrimide agar plate containing kanamycin (50 µg/mL); b) LBNS agar plate with sucrose (25% w/v) and c) finally onto the cetrimide agar plate (master plate). The screened colonies were finally subjected to colony PCR to evaluate the correct knockout mutants using the forward FP (A) and the reverse RP (D) primers as mentioned in Table 1. These clones were further confirmed by using internal sets of PCR primers as mentioned in table 2. The gene sequence were confirmed by sequencing using Sanger sequencing performed by SciGenome (India).

Gene	Primer name	Primer sequences
*cat*A	FP (A)	agagtc***GAATTC***ATGACCGTGAAAATTTCCCACA
	SOE RP (B)	**GGCGTGCCACAGGTCGACGGT**GATACCCAGGCCAGCAGCCAGCA
	SOE FP (C)	**TGCTGGCTGCTGGCCTGGGTATC**ACCGTCGACCTGTGGCACGCC
	RP (D)	aaatca ***AAGCTT***TCAGCCCTCCTGCAACGCC
*cat*A2	FP (A)	attaaa***GAATTC***ATGACCGTGAACATTTCCCATACT
	SOE RP (B)	**CAGGTTGTACGCCGACTG**GAGGAAGTGTTCGATGCC
	SOE FP (C)	**GGCATCGAACACTTCCTC**CAGTCGGCGTACAACCTG
	RP (D)	agatca***AAGCTT***TCAGGCCTCCTGCAAAGCT
*pca*HG	FP (A)	attaaa***GAATTC***ATGCCCGCCCAGGACAAC
	SOE RP (B)	**CCGTCATACACCTGGCCGAG**GGCGTTGTTCATGTCGAGCTTGGCG
	SOE FP (C)	**CGCCAAGCTCGACATGAACAACGCC**CTCGGCCAGGTGTATGACGG
	RP (D)	agatca***AAGCT***TTCAGAAGTCGAAGAAGACGGT

Table 1: Primers for the construction of the truncated gene/allele.

Primers: 5′ to 3′ end

Extra nucleotide: Lowercase

Restriction site: Bold and italics

Priming gene sequence: Uppercase

Overlapping gene sequence: Bold and underline.

Gene	Primer name	Primer sequences
*cat*A	FP (A)	ATGACCGTGAAAATTTCCCACA
	RP (D)	ATCGGTGATGATACGCCGA
*cat*A2	FP (A)	AAACGCATCGTGCAGCGCCTG
	RP (D)	ATAGCCCGATGGCACGATGGA
*pca*HG	FP (A)	ATGCCCGCCCAGGACAACA
	RP (D)	GGCATCCTGATACTCGCCATT

Table 2: Internal set of PCR primers for confirmation of the mutant.

Primers: 5′ to 3′ end.

Pathway modulation by gene expression for catechol production

Synthetic codon optimised *aro*Y gene encoding protocatechuate decarboxylase from *Enterobacter cloacae;* and *van*AB gene encoding vanillate-O-demethylase from *Acinetobacter sp*. ADP1 were procured from GenScript. GenScript provided the synthesized nucleotide sequences in multiple cloning site of pUC57 plasmid, containing codon optimized gene along with the additional sequence such as restriction enzyme site; RBS along with the spacer as GAATTCAGAGGAGGGAGA; and the stop codon as TGA. Homologous gene *van*AB encoding vanillate-O-demethylase from *Pseudomonas putida* KT2440 as well as from *Pseudomonas putida* S12 (ATCC 700801) was PCR amplified from respective genomic DNA using the primer pairs mentioned in table 3. The *van*AB gene from *Acinetobacter sp*. ADP1; *Pseudomonas putida* KT2440 or *Pseudomonas putida* S12 was cloned along with the RBS in pSEVA 234 vector between the restriction site EcoRI and BamHI. Vanillic acid accumulation problem was addressed by the expression of *van*AB gene from one of the organisms (*Acinetobacter sp*. ADP1; *Pseudomonas putida* KT2440 or *Pseudomonas putida* S12) in the clone containing Δ*pca*HG knockout.

The *aro*Y gene and *van*AB gene from *Acinetobacter sp*. ADP1 along with the RBS was cloned in between the restriction site EcoR1 and KpnI; XbaI and PstI in an operonic arrangement in pSEVA 234 to obtain the final construct. The plasmid construct was transformed in the strain bearing Δ*cat*A/A2; Δ*pca*HG mutation and the transformants were selected on the cetrimide agar plate containing kanamycin (50 µg/mL). The plasmid bearing culture was induced at the O.D. of 0.6 to 0.8 at 600 nm with 1mM IPTG and further subjected to SDS PAGE for protein analysis.

Phenotypic evaluation of the mutant after genetic manipulation

The mutant strain of Δ*cat*A and Δ*cat*A/A2 were assessed for catechol accumulation from the supernatant by cultivating the mutant along with the wildtype strain in M9 minimal medium supplemented with sodium benzoate (1g/L) as a sole carbon source. Similarly, mutant strain Δ*pca*HG phenotype was assessed by evaluating protocatechuate formation. The protocatechuate accumulation were analysed using different aromatic substrates (p-hydroxy benzoate, ferulic acid, vanillic acid and p-coumaric acid) belonging to the protocatechuate branch of β-Ketoadipate pathway. The Δ*pca*HG mutant was cultivated in M9 minimal medium supplemented with aromatic (p-hydroxy benzoate; ferulic acid; vanillic acid or p-coumaric acid at 1g/L) along with glucose (1g/L) at 30 °C in a rotary shaker set at 180 rpm.

Protocatechuate branch aromatics were to be further diverted for catechol biosynthesis. The Δ*cat*A/A2;Δ*pca*HG mutant wherein plasmid containing *aro*Y gene was expressed was evaluated for catechol production using M9 minimal medium supplemented with protocatechuic acid (1g/L), glucose (3g/L) and kanamycin (50 µg/mL). Since substantial vanillic acid accumulation was observed, an attempt was made to metabolise it through expression of *van*AB genes from *Acinetobacter sp*. ADP1; *Pseudomonas putida* KT2440 or *Pseudomonas putida* S12 in the Δ*pca*HG knockout strain. The effectiveness of the gene expression was evaluated by quantification of the protocatechuate formation using M9 minimal medium supplemented with vanillic acid (1 g/L), glucose (3g/L) and kanamycin (50 µg/mL) at 30°C in a rotary shaker set at 180 rpm. All the plasmid bearing cultures were induced at 0.8 $O.D_{600\ nm}$ using 1mM IPTG and expressed. Growth was monitored by colorimeter at OD_{600} along with metabolite analyses at regular time interval. Samples were drawn periodically and cells were pelleted by centrifugation at 10,000 rpm for 10 min while the supernatant was subjected for metabolite analysis and stored at -20°C till further analyses. Product verification was done on GCMS and HPLC using authenticated standards.

Metabolite analysis

Metabolites verification was done on GCMS while quantitative analysis was done on HPLC with authenticated standards. For GCMS, the supernatant was subjected to ethyl acetate extraction (1:1 v/v) wherein the organic layer was separated and derivatized

using BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) (Sigma-Aldrich, India). The sample was analyzed on Agilent Technologies (Santa Clara, CA, USA) 7890A GC (gas chromatography) system equipped with Agilent 5975C mass selective detector using Agilent 19091J-413 HP-5 column (30 m × 320 µm × 0.25 µm). The GC oven temperature was held at 90°C for 1.5 min, ramped to 185°C to 205°C at 10°C/min, ramped to 250°C at 10°C/min and held for 5 min. Sample peaks were identified by comparing with authentic standards, and by using the NIST Mass Spectral Search Program (version 2.0 f). HPLC analyses of the metabolites from supernatant were carried out by reverse-phased chromatography on Agilent Eclipse Plus C18 RP column (3.5 µm; 4.6 × 100 mm). The mobile phase A was methanol while phase B was 1% acetic acid. The solvent B gradient program (time [min], % B, flow rate [mL min^{-1}]) was employed as: 15,95,0.8; 20,80,0.8; 25,50, 0.8; 30,0.0,0.8 and 35,100,0.8; considering absorbance at 254nm at 40°C.

Catechol biosynthesis using standard lignin monomers and biomass hydrolysate

The final construct bearing Δ*cat*A/A2;Δ*pca*HG knockouts along with plasmid containing *aro*Y gene from *Enterobacter cloacae* and *van*AB gene from *Acinetobacter sp*. ADP1 was used for catechol biosynthesis. Catechol production was carried out using M9 medium supplemented with various aromatic compound (p-coumaric acid; p-hydroxy benzoic acid; protocatechuate; ferulic acid; vanillic acid or sodium benzoate at 1 g/L), glucose (3 g/L) and kanamycin (50 µg/mL) at 30°C in 250mL in a rotary shaker set at 180 rpm. Similarly, catechol biosynthesis was also attempted using various lignocellulosic biomass hydrolysates such as cotton stalk biomass hydrolysates, empty fruit bunch hydrolysate and maize bran hydrolysate diluted with M9 medium (50% v/v) containing glucose (1 g/L) and kanamycin (50 µg/mL). The culture was induced at the O.D. of 0.8 at 600 nm with 1mM IPTG. At regular time intervals, growth was monitored at the $O.D_{600\ nm}$ while the metabolites were analysed from the supernatant using HPLC as described above. For LBM, metabolites were analysed using GCMS.

Results and Discussion

Construction of the knockout mutant *Pseudomonas putida* KT2440 for catechol biosynthesis

Pseudomonads such as *Pseudomonas putida* GB-1 (GenBank CP000926), *Pseudomonas putida* F1 (GenBank CP000712) and *Pseudomonas putida* KT2440 (GenBank CP000712) metabolise catechol via catechol 1, 2- dioxygenase encoded by two genes present onto the chromosomal DNA [20]. For catechol biosynthesis a mutant deficient in catechol metabolism was obtained by gene inactivation via homologous recombination. Catechol is metabolized in *Pseudomonas putida* KT2440 via orthologous genes *cat*A and *cat*A2 encoding catechol 1,2-dioxygenase activity where 95% of the activity is due to *cat*A gene [26].

Pseudomonas putida KT2440 mutant deficient in catechol and protocatechuate metabolism were generated by gene inactivation/knockout via homologous recombination protocol. The *cat*A gene was first knocked out followed by *cat*A2 and *pca*HG in *P. putida* KT2440. For e.g., Δ*cat*A knockout was carried out as depicted in Figure 2. It involved construction of the gene replacement plasmid containing truncated *cat*A gene corresponding to 726 bp, which upon introduction into the host organism resulte in site specific integration of the entire cassette leading to merodiploid formation. Merodiploid clone contains two copies of a gene, one corresponding to wildtype *cat*A gene with 936 bp while another one corresponding to the truncated *cat*A gene with 726 bp as indicated in Figure 2a. Later upon second homologous recombination, mutant clone was obtained wherein the wildtype gene was replaced with the truncated variant making the gene nonfunctional. This was further confirmed by using internal set of primers where the wildtype PCR product corresponds to 500 bp while the mutant corresponds to 300 bp as indicated in Figure 2c.

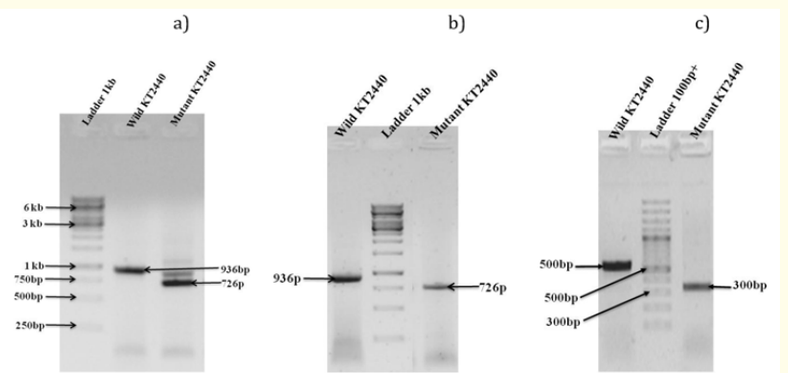

Figure 2: Representative agarose gel image for catA gene knockout.

a) Colony PCR after first homologous recombination leading to merodiploid formation in P. putida KT2440; b) Colony PCR after second homologous recombination event resulting in truncated catA gene mutant; c) Confirmation of the mutant with the second set of internal PCR primers.

Phenotypic evaluation of the mutant after genetic manipulations

Mutant *Pseudomonas putida* KT2440 was obtained by gene knockout of *cat*A and *cat*A2 gene encoding catechol 1,2-dioxygenase activity. The mutant phenotype was assessed for catechol biosynthesis by using sodium benzoate as a carbon source. The catechol biosynthesis was evaluated by monitoring the growth and the catechol accumulation profile of the mutant Δ*cat*A and wildtype on sodium benzoate (0.7g/L) as a sole carbon source. The growth and product profile are depicted in Figure 3a, where growth profile is represented by the solid line (red colour) while the catechol accumulation profile in the dotted line (blue colour). Mutant showed the maximum catechol (0.202g/g) accumulation at 8 hours after which it starts declining while wildtype did not show any catechol accumulation. This might be due to the activity of counterpart of catechol 1,2-dioxygenase encoded by *cat*A2 gene. Hence, this gene was knocked out using the same strategy leading to stable catechol accumulation (0.507g/g). The Δ*cat*A mutant resulted in 27.47% catechol yield whereas Δ*cat*A/A2 mutant resulted in 57.68% catechol yield on molar basis under growing condition. The specific growth rate (μ) on sodium benzoate as a sole carbon source decreased after each knockout, where for wildtype it wass 0.27 h^{-1}, for Δ*cat*A 0.22 h^{-1} and for Δ*cat*A/*cat*A2 0.18h^{-1}. There are two reports on use of *Pseudomonads* for the biosynthesis of catechol from sodium benzoate using *Pseudomonas* sp. B3- and *Pseudomonas* sp. Ba-0511. These strains showed catechol 1,2-dioxygenase activity in spite of *cat*A gene knockout leading to decreased catechol yield [19,20]. However, our mutant *Pseudomonas putida* KT2440 strain resulted in 58% catechol yield on sodium benzoate under growing conditions. It happens to be the highest catechol yield reported using sodium benzoate as a carbon source from *Pseudomonas putida* under growth conditions.

In order to also activate the protocatechuate branch aromatics for catechol production the mutant deficient in protocatechuate metabolism was obtained by knocking out protocatechuate 3,4-dioxygenase encoded by *pca*HG gene. This manipulation was carried out in the strain bearing Δ*cat*A/A2 mutation. The mutant Δ *pca*HG phenotype was evaluated for protocatechuate accumulation by using various aromatic compounds belonging to protocatechuate branch of β-Ketoadipate pathway such as p-coumarate; p-hydroxy benzoate; ferulate; vanillic acid as a carbon source. The percentage yield of protocatechuate on p-coumaric acid was 99%; ferulic acid was 11.21% and on vanillic acid was 1.3%. The yield of protocatechuate on vanillic acid and ferulic acid was low due to substantial accumulation of vanillic acid as depicted in Figure 3d and 3e. Vanillic acid accumulation seemed to be a major hurdle towards protocatechuate synthesis and thereby catechol production.

Figure 3: Phenotypic evaluation of the mutant on respective carbon source.

a) Growth and catechol accumulation profile of the wildtype and mutant ΔcatA on sodium benzoate as a sole carbon source; b) Growth and catechol accumulation profile of the mutant ΔcatA and mutant ΔcatA/catA2 on sodium benzoate as a sole carbon source; c) Protocatechuate accumulation profile after ΔpcaHG knockout on p-Coumaric acid; d) Metabolite profile analysis after ΔpcaHG knockout on ferulic acid; e) Metabolite profile analysis after ΔpcaHG knockout on vanillic acid.

Protocatechuate branch aromatics account for major aromatics obtained in lignin depolymerised stream. Thus, these aromatics needed to be diverted towards catechol biosynthesis via heterolo-

gous gene expression since the native strain lacked this pathway. Therefore, heterologous gene *aro*Y encoding protocatechuate decarboxylase from *Enterobacter cloacae* was chosen rather than *Klebsiella pneumoniae* as it has higher substrate affinity [27]. To divert the protocatechuate branch aromatic towards catechol, plasmids with *aro*Y gene bearing culture was induced and subjected to SDS PAGE for the protein analysis (data not shown). The expression of this gene resulted in 60% catechol yield from protocatechuate when used as a carbon source. Further catechol production was carried out using protocatechuate branch aromatics (p-coumarate; ferulic acid; p-hydroxy benzoate and vanillic acid) which though led to substantial accumulations of vanillic acid. Thus, *aro*Y gene expression had no effect upon driving the vanillic acid reaction towards catechol biosynthesis thereby posing it to be a major road block.

Vanillic acid accumulation issue was addressed by the overexpression of different vanillate-O-demethylase genes e.g. from *Pseudomonas putida* KT2440; *Pseudomonas putida* S12 or *Acinetobacter sp.* ADP1 in the clone containing Δ*pca*HG knockout to assess which gene source perform better and can be taken ahead. The expression of *van*AB from *Acinetobacter sp.* AP1 resulted in 52.21 ± 0.622% protocatechuate yield from vanillic acid; thereby leading to promising result and was considered for further work in the final construct.

Catechol biosynthesis

Catechol biosynthesis was evaluated using the genetically modified strain of *Pseudomonas putida* KT2440 containing Δ*cat*A/A2/*pca*HG knockouts, along with plasmid pSEVA 234 containing *aro*Y gene from *Enterobacter cloacae* and *van*AB gene from *Acinetobacter sp.* AP1 under trc based promoter. Various lignin model compounds such as ferulic acid, vanillic acid, protocatechuic acid, p-coumarate, p-hydroxy benzoate and sodium benzoate were taken into consideration based upon the monomeric lignin stream profile generated in our laboratory. The catechol yields from all these monomers are represented in table 4. Promising yields were obtained on the lignin model compounds, and hence catechol biosynthesis was also attempted on lignocellulosic biomass (LBM) hydrolysate.

LBM hydrolysates such as cotton stalk biomass hydrolysate; empty fruit bunch hydrolysate and maize bran hydrolysate were used for the biosynthesis of catechol. Catechol bioaccumulation was studied qualitatively using GCMS and was observed to be produced in 36 to 48 hours under growing conditions as indicated in the GC chromatogram of figure 4. Catechol yields on the biomass hydrolysates were very low, as the lignin monomers in the streams obtained and specific to the pathway were in low concentrations and hence could not be quantified. This is largely attributed to the fact that in the course of pretreatment of biomass, the lignin was not completely hydrolysed to most of the aromatic monomeric compounds that could be metabolized by the mutant strains.

Gene	Substrate	Percentage Yield
aroY	*p-Coumarate*	64 ± 1.9%
	p-Hydroxy benzoate	56 ± 3.6%
	Protocatechuate	60 ± 4.1%
	Ferulic acid	0.42 ± 0.05%
	Vanillic acid	22 ± 3.5%
	Sodium Benzoate	60 ± 0.27%
aroY + vanAB	*Ferulic acid*	6 ± 0.7%
	Vanillic acid	56 ± 3.5%

Table 4: Catechol yield on various lignin monomers.

Figure 4: GC chromatogram showing catechol biosynthesis from lignocellulosic biomass hydrolysates.

a) Catechol biosynthesis on cotton stalk biomass hydrolysates upon nitric acid pretreatment; b) Catechol biosynthesis on empty fruit bunch biomass hydrolysates upon nitric acid pretreatment and passed through 300 Da membrane; c) Catechol biosynthesis on maize bran derived ferulic acid stream upon alkaline alcoholic treatment.

However, the maize bran hydrolysate was analysed to contain 5% w/w of lignin of which 2% w/w was observed to be ferulic acid and another 1% was p-coumaric acid. Experimental catechol yields on maize bran derived lignin hydrolysate was found to be 36.3% while on purified maize bran stream (comprising majorly of ferulic acid along with p-coumaric acid) was 57.5%. Since the catechol yield on both bran hydrolysate streams were comparable to the yield on model p-coumaric acid, it is deduced that bulk of the catechol production used p-coumaric acid as substrate while diverted ferulc acid to vanillic acid production to some extent. This is important result which indicates that the mutant engineered strain designed in this work is able to consume both significant components of the maize bran hydrolysate namely, ferulic acid and p-coumaric acid, while produces vanillic acid and catechol in good yields of 90% and 60%, respectively.

Conclusions

Lignin monomers such as protocatechuate, p-hydroxybenzoate, p-coumarate, vanillic acid, vanillin and benzoic acid can be utilized and diverted towards catechol biosynthesis using the genetically modified *Pseudomonas putida* strain KT2440. Promising yields were obtained using lignin derived aromatic monomers. Our report claims the highest molar yield of catechol (58%) from sodium benzoate as a carbon source. This report also attempts to address the issue of vanillic acid accumulation to a certain extent leading to the catechol yield of 53 to 56% on protocatechuate branch lignin monomers.

Catechol biosynthesis was also demonstrated as a proof-of-concept on hydrolysed lignin containing streams of lignocellulosic biomass hydrolysates thereby paving the way for scalable catechol biosynthesis from renewable biomass derived feedstock.

Maize or corn bran hydrolysate gave promising results showing high yields of catechol and vanillic acid from its ferulic acid and p-coumaric acid components. Our efforts to channel the vanillic acid to catechol did were not fully successful and this forms the subject matter for further studies.

Acknowledgements

The authors thankfully acknowledge the financial support to the project from Department of Biotechnology, Ministry of Science and Technology, Government of India.

Bibliography

1. Fiege H., *et al*. "Phenol Derivatives". Ullmann's Encyclopedia Of Industrial Chemistry (2000).

2. Ryu J., *et al*. "Bio-inspired adhesive catechol-conjugated chitosan for biomedical applications: A mini review". *Acta Biomaterialia* 27 (2015): 101-115.

3. Li W., *et al*. "Benzene-Free Synthesis of Catechol : Interfacing Microbial and Chemical Catalysis". *Journal of the American Chemical Society* 127.9 (2005): 2874-2882.

4. Young J. "Catechol". *Journal of Chemical Education* 82.1 (2005): 31.

5. Yang L. "Green and efficient synthesis route of catechol from guaiacol". *Journal of Molecular Catalysis A: Chemical* 368-369 (2013): 61-65.

6. Ivanov D., *et al*. "New Way of Hydroquinone and Catechol Synthesis using Nitrous Oxide as Oxidant" *Advance Synthesis and Catalysis* 344.9 (2002): 986-995.

7. Huang C., *et al*. "Synthesis of Catechols from Phenols via Pd-Catalyzed Silanol-Directed C-H Oxygenation". *Journal of American Chemical Society* 133.44 (2011): 17630-17633.

8. Balderas-hernandez V., *et al*. "Catechol biosynthesis from glucose in Escherichia coli anthranilate-overproducer strains by heterologous expression of anthranilate 1,2-dioxygenase from Pseudomonas aeruginosa PAO1". *Microbial Cell Factories* 13 (2014): 136.

9. Kille L. "Biofuels, energy security, the environment and human health: Research roundup". *Journalist's Resource* (2014).

10. Ragauskas A., *et al*. "Lignin valorization: Improving lignin processing in the biorefinery". *American Association for the Advancement of Science* 344.6185 (2014): 1246843.

11. Wyman C., *et al*. "Lignin Bioproducts to Enable Biofuels". *Bioproducts and Biorefining - Wiley Online Library* (2015).

12. Huang Y., *et al*. "Lignin-first biorefinery: a reusable catalyst for lignin depolymerization and application of lignin oil to jet fuel aromatics and polyurethane feedstock". *Sustainable Energy and Fuels* 3 (2018).

13. Sun Z., *et al*. "Bright Side of Lignin Depolymerization: Toward New Platform Chemicals". *Chemical Reviews* 118.2 (2018): 614-678.

14. Abdelaziz O., *et al*. "Biological valorization of low molecular weight lignin". *Biotechnology Advances* 34.8 (2016): 1318-1346.

15. Li C., *et al*. "Catalytic Transformation of Lignin for the Production of Chemicals and Fuels". *Chemical Review* 115.21 (2015): 11559-11624.

16. Dias F., *et al*. "Exploring the Microbial Production of Aromatic Fine Chemicals to Overcome the Barriers of Traditional Methods". *Advances in Applied Science Research* 8.1 (2017): 94-109.

17. Nelson E., *et al*. "Complete genome sequence and comparative analysis of the metabolically versatile Pseudomonas putida KT2440 ". *Environmental Microbiology* 4 (2002): 799-808.

18. Jr T., *et al*. "Biotechnological opportunities with the β -ketoadipate pathway". *Trends in Biotechnology* 30.12 (2012): 627-637.

19. Wang C., *et al*. "Isolation of a benzoate-utilizing Pseudomonas strain from soil and production of catechol from benzoate by transpositional mutants". *Microbiological Research* 156 (2001): 151-158.

20. Xiong W., *et al*. "Mutagenesis of catA from Pseudomonas Sp. B3-1 to Enhance Catechol Accumulation." *Advanced Materials Research*, Trans Tech Publications, Ltd (2012): 1615-1620.

21. Fenske J., *et al*. "Comparison of aromatic monomers in lignocellulosic biomass prehydrolysates". *Journal of Industrial Microbiology and Biotechnology* 20.6 (1998): 364-368.

22. Tang P., *et al*. "Production of Monomeric Aromatic Compounds from Oil Palm Empty Fruit Bunch Fiber Lignin by Chemical and Enzymatic Methods". *BioMed Research International* 2015 (2015).

23. Hmelo L., *et al*. "Precision-engineering the Pseudomonas aeruginosa genome with two-step allelic exchange". *Nature Protocols* 10.11 (2105): 1820-1841.

24. GM Sambrook J. Molecular Cloning: A Laboratory Manual. 4 ed, 1 (2012).

25. Choi k., *et al*. "A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells : Application for DNA fragment transfer between chromosomes and plasmid transformation". *Journal of Microbiological Method* 64 (2006): 391-397.

26. Jimenez J. "A second chromosomal copy of the catA gene endows Pseudomonas putida mt-2 with an enzymatic safety valve for excess of catechol". *Environmental Microbiology* 16 (2014): 1767-1778.

27. Payer S., *et al*. "Regioselective para-Carboxylation of Catechols with a Prenylated Flavin Dependent Decarboxylase". *Angewandte Chemie International Edition* 56.44 (2017): 13893-13897.