[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).

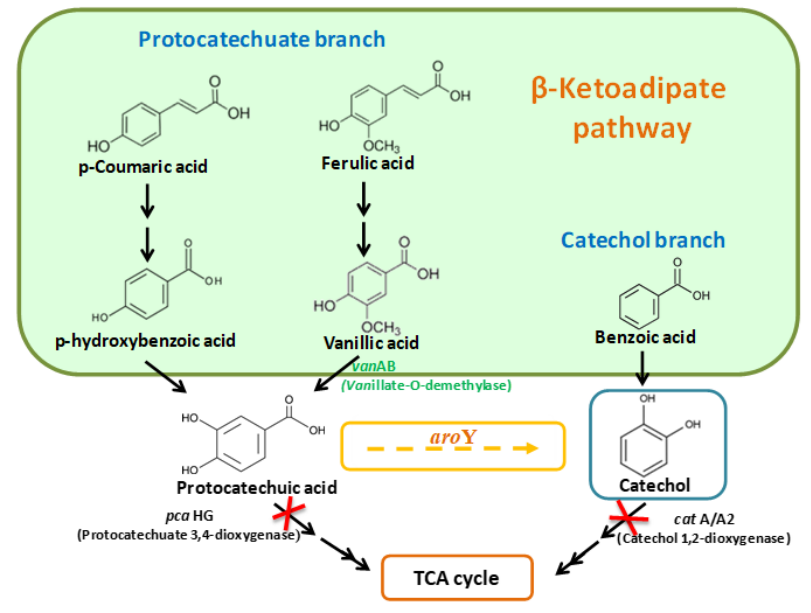

Figure 1: β-Ketoadipate pathway engineering in *Pseudomonas putida* KT2440 for the biosynthesis of catechol.

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, FeSO4.7H2O: 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

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 were not fully successful and this forms the subject matter for further studies.

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