Current Trends in OMICS (CTO)
Volume 4 Issue 1, Spring 2024
ISSN$_{(P)}$: 2790-8283 ISSN$_{(E)}$: 2790-8291
Homepage: https://journals.umt.edu.pk/index.php/cto

Article QR

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DOI: https://doi.org/10.32350/cto.41.03

History: Received: September 13, 2023, Revised: October 19, 2023, Accepted: November 14, 2023, Published: December 26, 2023

Citation: Fatima M. Diversity analysis of catechol 2, 3-dioxygenase in POPs metabolizing bacteria using in-silico approach. *Curr Trend OMICS*. 2024;4(1):36–54. https://doi.org/10.32350/cto.41.03

Conflict of Interest: Author(s) declared no conflict of interest

A publication of
The Department of Life Sciences, School of Science
University of Management and Technology, Lahore, Pakistan

Diversity Analysis of Catechol 2, 3-Dioxygenase in POPs Metabolizing Bacteria using *in Silico* Approach

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ABSTRACT

The persistent nature of persistent organic pollutants (POPs), their lipophilicity and volatility, resulted in their high concentration, not only in environmental resources but also in living organisms. Their complete removal is possible through mineralization using enzyme-based strategies. Catechol 2, 3-dioxygenase has been reportedly involved in the degradation of a wide variety of POPs. This study was designed to find out the diversity of this enzyme among highly efficient bioremediating bacteria. A total of 07 bacteria belonging to the genera Acinetobacter, Pseudomonas, Burkholderia, Stutzerimonas, and Paraburkholderia were targeted. The sequences of enzyme were retrieved from Uniprot database and analyzed via ProtParam, CELLU, and SOPMA tools and AlphaFold database. The enzyme was found to be cytoplasmic. Its physicochemical properties were recorded as pI 4.75 – 5.50, aliphatic index (73.41 – 88.55), instability index (24.98 – 43.37), and GRAVY (-0.209 – 0.511). Secondary structure attributes were recorded to be α-helix (30.13 – 37.30), extended strand (18.27 – 21.54), β-turn (5.14 – 6.95), and random coil (38.33 – 42.95). All the proteins showed complex folding except in Pseudomonas sp. strain EST1001. These properties might be exploited during the selection, purification, manipulation, and cloning of catechol 2, 3-dioxygenase enzyme for efficient bioremediation.

Keywords: AlphaFold, catechol 2, 3-dioxygenase, configuration, persistent organic pollutants (POPs), physicochemical properties

1. INTRODUCTION

Persistent Organic Pollutants (POPs) are resistant to degradation. Hence, they undergo biomagnification in food chain due to their accumulation in the fatty tissues of living organisms and long range of transport. Moreover, they also persist in environmental resources, namely

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soil, air, and water for a longer period of time [1]. This long stay enhances the exposure of plants and animals to their hazardous effects. It not only results in polluting the environment but also causes human health hazards [2]. Being a part of chemicals and textiles, pulp ans paper, petrochemicals, printing, chloralkali, cooking, herbicides, cement, pharmaceutical and pigment industries, it is not possible to prevent the distribution of POPs to the environment [3]. Health hazards caused by POPs include endocrine disruption, various cancers such as testicular, breast, prostate, uterine, and ovarian cancer, obesity, diabetes, as well as reproductive and cardiovascular problems including high cholesterol, increase in blood pressure, and ventricular systolic and diastolic dysfunction [2, 4].

The most notorious forms of POPs that exist in the environment include hexachlorobenzene (HCBs), polychlorinated biphenyls (PCBs), benzene, toluene, ethylbenzene, and xylene (BTEX) [5]. POPs remediation from the environment is a critical need at present. An emerging mitigation approach is the use of bioremediating agents, such as bacteria. Bacteria are able to tolerate a wide range of physical conditions including high and low temperature, alkaline and acidic pH, and aerobic and anaerobic environments. Further, they also exhibit mechanisms that break POPs into hydrophilic metabolites. Thus, they are the best bioremediating agents. Hydrophilicity decreases the toxicity and persistence of these compounds [6]. This sound bioremediation potential of bacteria is induced by the unusual enzymes possessed by them, such as cytochrome P450, toluene dioxygenase, toluene p- and m-oxygenases, naphthalene dioxygenase, 1, 2-dihydroxynaphthalene dioxygenase, naphthalene dihydrodiol dehydrogenase, 2-hydroxy-2-H-chromene 2-carboxylate isomerase, salicylaldehyde dehydrogenase, 2-hydroxymuconate tautomerase, 4-oxalocrotonate decarboxylase, 4-hydroxy 2-oxovalerate aldolase, 2-oxo-4-pentanoate hydratase, 2-hydroxymuconic semialdehyde dehydrogenase, 2-hydroxybenzalpyruvate aldolase, catechol 2, 3-dioxygenase, catechol 1, 2-dioxygenase, salicylate 5-hydroxylase, 4-chlorobenzoyl-CoA dehalogenase, tetrachlorohydroquinone dehalogenase, chlorobenzene dioxygenase, muconatecycloisomerase and biphenyl dioxygenase [7].

Catechol 2, 3-dioxygenase is also known as 2, 3-pyrocatechase, metapyrocatechase, pyrocatechol 2, 3-dioxygenase, and xylE. It belongs to the oxidoreductase class of enzymes. It catalyzes the conversion of 2-hydroxy-cis, cis-muconate semialdehyde [8]. During this reaction, it adds

two oxygen atoms to catechol and causes the cleavage of extradiol (Figure 1). It uses Fe^{3+} as a co-factor and initiates the meta-cleavage pathway of catechol. The degradation of multiple POPs including phenanthrene, 2-methylaniline, BTEX and PCBs involves the formation of catechol as an intermediate [9, 10].

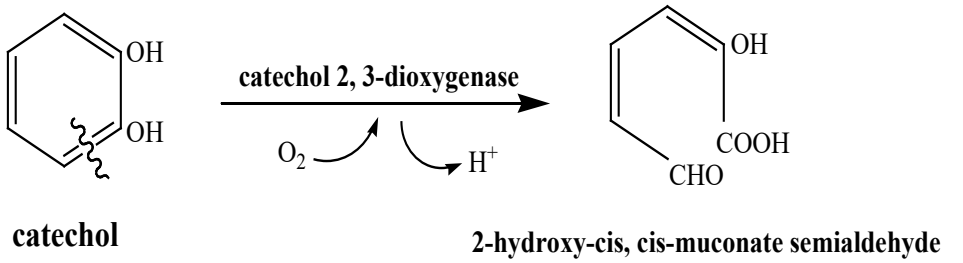

Figure 1. Reaction Catalyzed by Catechol 2, 3-Dioxygenase During Meta-Ring Cleavage Pathway

Pseudomonas sp. HA10, *Pseudomonas* sp. HA12 and *Pseudomonas* sp. HA14, *Pseudomonas* sp. ZP2, *Sphingomonas* sp. ZP2, *Bacillus cereus* UKMP-6G, *Rhodococcus ruber* UKMP-5M, *Burkholderia cepacia*, *Burkholderia* sp. AA1, *Stenotrophomonas maltophilia, Brevibacillus agri*, *Bacillus paralicheniformis*, *Gordonia polyisoprenivorans*, *Bacillus cereus* IrC2, *Achromobacter* sp. BP3, Bacillus macerans, Sphingomonas xenophaga QYY, Rhodococcus sp. R04 and Thauera sp. K11, Paraburkholderia xenovorans [11–18].

Acinetobacter, *Pseudomonas*, *Burkholderia lata, Stutzerimonas,* and *Paraburkholderia* are active bioremediating agents for POPs [19–26]. So, the current study targeted the catechol 2, 3-dioxygenase enzyme from these bacteria.

Catechol 2, 3-dioxygenase is associated with the degradation of the majority of POPs. So, the cloning of its gene can be useful for simultaneous bioremediation of a wide range of pollutants. For the effective use of an enzyme in remediation, it is important to get an insight into its structure, sub-cellular localization, and physiochemical attributes. Therefore, the current project was initiated to perform the diversity analysis of catechol 2, 3-dioxygenase enzyme among the 07 POPs metabolizing bacteria.

2. METHODOLOGY

In the current study, 07 variants of catchol 2, 3-dioxygenase from different POPs degrading bacteria were compared using various *in silico* tools.

2.1 Uniprot Database

Sequences of catchol 2, 3-dioxygenase variants were retrieved from the Uniprot database [27]. Sequences, along with their accession IDs, are shown in Table 1.

Table 1. Sequences and Accession IDs of Catechol 2, 3-dioxygenase retrieved from Uniprot database

#	Bacterium	Accession ID	Sequence
1	*Acinetobacter lwoffii*	O33948	MSIKVFGTKEVQDLLKAATNLEGKG GNARSKQIVHR LLSDLFKAIDDLDITPDEVWAGVNYL NKLGQDGEAT LLAAGSGLEKYLDIRLDAADKAEGIE GGTPRTIEGPL YVAGATVHDGVSKIDINPDEDAGPLV IHGTVTGPDG KPVAGAVVECWHANSKGFYSHFDPT GAQSDFNLRG AVKTGADGKYEFRTLMPVGYGCPPQ GATQQLLNVL GRHGNRPAHVHFFVSSDSARKLTTQF NIEGDPLIWDD FAYATREELIPPVTEKKGGTALGLKA DTYKDIEFNLT LTSLVKGKDNQVVHRLRAEVAA
2	*Pseudomonas* sp. (strain EST1001)	P31019	MTVKIYDTPEVQDFLKIVAGLDQEGG NDRGKQIIHR ILSDLYRTIDDFDITAEQYWSAVSLLN ALGQASQFGL LSPGLGFDHYMDMRMDAADAEAKR TGGTPRTIEGP LYVAGAPEAEGFARMDDDPDTDGET MWLHGQVRD TAGKPIPGAKVEIWHCNSKGGYSFFD KSQTPYNLRR TIIADNEGYYRARSVIPSGYGVPEGAP TDQVLKLLGR

#	Bacterium	Accession ID	Sequence
			HGERPAHIHYFISAPGHQHLTTQINLA GDPYTYDDFA FATRQDLAAEGKRVENHPAAQQYGV EGTVTEVIFNI ELSPTAEEELQARP
3	*Burkholderia lata* strain DSM 23089	Q393C6	MNKQAIDALLKTFDDAAEQPGNPRV RAIVNRIVKDI CYTIEDFDVQPSEFWTALNYLNEAGK ELGLIAAGLG LEHFLDVRMDEAEAKAGIQGGTPRTI EGPLYVAGAP ESVGHARLDDGTDPGQTLIMRGQVL GHDGAPVANA LVEVWHANHLGNYSYFDQSQPAFNL RRSIRTDADG RYSFRSVLPVGYSVPPGGKTEQLLDQ LGRHGHRPAH IHFFVSADGYRKLTTQINIDGDPHLW DDFAFATREGL IPAVKQAEGAEGKPYGVDGQFALIDF DFSLLKDKQD VPGSEVERARAQA
4	*Acinetobacter baumannii* (strain AB307-0294)	A0A5K6CQP8	MNRQQIDALVKQMNVDTAKGEVDA RVQQIVVRLL GDLFQAIEDLDIQPSEVWKGLEYFTD AGQANELGLL AAGLGLEHYLDLRADEADAKAGITG GTPRTIEGPLY VAGAPESVGFARMDDGTETGKIDTLII EGTVTDTDG NIIENAKVEVWHANSLGNYSFFDKSQ SDFNLRRTIFT DADGKYVALTTMPVGYGCPPEGTTQ ALLNKLGRHG NRPSHVHYFVSAPGYRKLTTQFNIEG DEYLWDDFAF ATRDGLVATAVDVTDPAEIQRRGLD HAFKHITFNIEL VKEAAAAPSTEVERRRASA
5	*Stutzerimonas stutzeri* DSM 5190	F8H6B9	MTVKISHTNDVQQFFKEASGFNNDA GSSRLKTVINR VLTDTARIIEDLEITQDEFWKAVDYIN RLGGRHEAGL

#	Bacterium	Accession ID	Sequence
			LVAGLGLEHYLDLLQDAKDEQEGLV GGTPRTIEGPL YVAGAPIAQGIARMDDGSEDDVATV MFLQGRVFDP SGKPLAGAVVDLWHANTKGNYSYFD KSQSEYNLRR RIVTDENGYYRARSIVPSGYGCSPDGP TQEVLDMLG RHGQRPAHIHFFISAPGHRHLTTQINL AGDKYLWDD FAYATRDGLVGDIRFIDDAEAARARG VQGRFAEVD FDFQLQKAPAPQAEQRSKRPRALQQA
6	*Paraburkholderia* sp. WP4_3_2	A0A7W5IGH0	MSVKVFDTQEVQDLLKAAANIGADS GNARLQQIVH RLLGDLFEAIDDLDITPDEVWAGVNY LNKLGQDGEA ALLAAGIGLEKYLDVRLDAADKAAG TDGGTPRTIEG PLYVAGAPLREGVSRIDINEDADAGP LVIRGKVTDTD GKPVANAIVECWHANSKGFYSHFDP TGAQSSFNLRG AVKSGPDGAYAFHTLMPVGYGCPPQ GATQQLLNGL GRHGNRPAHVHFFVTSAAHRKLTTQ FNIEGDPLIWD DFAYATREELIPAVVEKTGGAALGLK DDAYKDIEFN VTLTPLVQGKDNQIVSRPRAAATA
7	*Pseudomonas putida*	A0A0C5RTA7	MTVKISHTADIQAFFNKVAGLDHAEG NPRFKQIILRV LQDTARLVEDLEITEDEFWHAIDYLN RLGGRNEAGL LAAGLGIEHFLDLLQDAKDAEAGLSG GTPRTIEGPLY VAGAPVAQGEARMDDGTDPGVVMF LQGQVFDADG KPLAGATVDLWHANTQGTYSYFDST QSEYNLRRRII TDAEGRYRARSIVPSGYGCDPQGPTQ ECLDLLGRHG QRPAHVHFFISAPGHRHLTTQINFEGD KYLWDDFAY

#	Bacterium	Accession ID	Sequence
			ATRDGLIGELRFIEDAAAARDRGVQG ERFAELAFDF HLQGATAVEAEARSHRPRALQEG

2.2 CELLO

To predict the sub-cellular localization of enzyme variants in bacterial cells, CELLO: sub-cellular localization prediction system was employed [28].

2.3 ProtParam

For the prediction of physicochemical properties of the enzyme, ProtParam tool was employed [29]. The properties computed included molecular weight, isoelectric point (pI), aliphatic index, instability index, and GRAVY.

2.4 SOPMA

SOPMA secondary structure prediction method was consulted [30]. Different attributes computed using this tool included α-helix, extended strand, β-turn, and random coil.

2.4 AlphaFold

To compare the variants of catchol 2, 3-dioxygenase at the level of three dimensional (3D) configuration, AlphaFold database of protein structures [31].

3. RESULTS

3.1 Sub-cellular Localization

Catechol 2, 3-dioxygenase enzyme was found to be localized in cytoplasm in all the POPs metabolizing bacteria. The highest score value obtained via CELLO tool was in the case of cytoplasm. The score value was recorded in the range of 4.000-4.882 (Table 2).

Table 2. Prediction of Sub-cellular Localization of Catechol 2, 3-Dioxygenase in Present Study Bacteria Predicted using CELLO Tool

Bacterium	Sub-cellular Localization			
	Cytoplasm	Extracellular	Membrane	Cell Wall
A. lwoffii	4.665	0.193	0.096	0.047
Pseudomonas sp. strain EST1001	4.593	0.257	0.141	0.009

Bacterium	Sub-cellular Localization			
	Cytoplasm	Extracellular	Membrane	Cell Wall
B. lata strain DSM 23089	4.882	0.080	0.035	0.003
Acinetobacter baumannii strain AB307-0294	4.850	0.092	0.035	0.023
S. stutzeri DSM 5190	4.601	0.206	0.180	0.013
Paraburkholderia sp. WP4_3_2	4.091	0.013	0.032	0.020
P. putida	4.000	0.207	0.012	0.01

3.2 Physicochemical Properties

The physicochemical properties of catechol 2, 3-dioxygenase assessed on the basis of ProtParam tool revealed the highest molecular weight (34624.62) in *S. stutzeri* DSM 5190, while the lowest value (32833.69) was observed in *B. lata* strain DSM 23089. The pI ranged between 4.75 and 5.50. Less variation was recorded in pI and aliphatic index. The aliphatic index ranged between 73.41 and 86.27. The instability index values of 24.98, 27.91, 34.17, 38.24, 40.25, 41.80, and 43. 37 were found in *A. lwoffii*, *Paraburkholderia* sp. WP4_3_2, *B. lata* strain DSM 23089, *Pseudomonas* sp. strain EST1001, *P. putida, S. stutzeri* DSM 5190, and *Acinetobacter baumannii* strain AB307-0294, respectively (Table 3). The enzyme showed negative GRAVY values (-0.300 to -0.511) in all the bacteria documented in the current study.

Table 3. Prediction of Physicochemical Properties of Catechol 2, 3-Dioxygenase using ProtParam Tool

#	Bacteria	Mol. wt. (Da)	pI	Aliphatic index	Instability index	GRAVY
1	*A. lwoffii*	33377.58	5.50	86.27	24.98	-0.300
2	*Pseudomonas* sp. strain EST1001	33362.07	4.80	73.41	38.24	-0.511
3	*B. lata* strain DSM 23089	32833.69	5.11	82.33	34.17	-0.380
4	*Acinetobacter baumannii* strain AB307-0294	33493.37	4.75	84.22	43.37	-0.318
5	*S. stutzeri* DSM 5190	34624.62	5.50	78.85	41.80	-0.485
6	*Paraburkholderia* sp. WP4_3_2	33114.12	5.01	88.55	27.91	-0.209
7	*P. putida*	34263.09	5.07	80.13	40.25	-0.393

pI: isoelectric point, GRAVY: grand average of hydropathy

3.3 SOPMA tool

SOPMA tool was used to predict the four properties of the 2D structure of catechol 2, 3-dioxygenase in the selected bacteria. The properties included α-helix, extended strand, β-turn, and random coil (Table 4, Figure 2). Regarding all these properties, it was found that catechol 2, 3-dioxygenase showed very limited variation in all the bacteria. The α-helix, extended strand, β-turn, and random coil values ranged between 30.13% to 34.97%, 18.27% to 20.90%, 5.14% to 6.95%, and 38.33% to 42.95%, respectively.

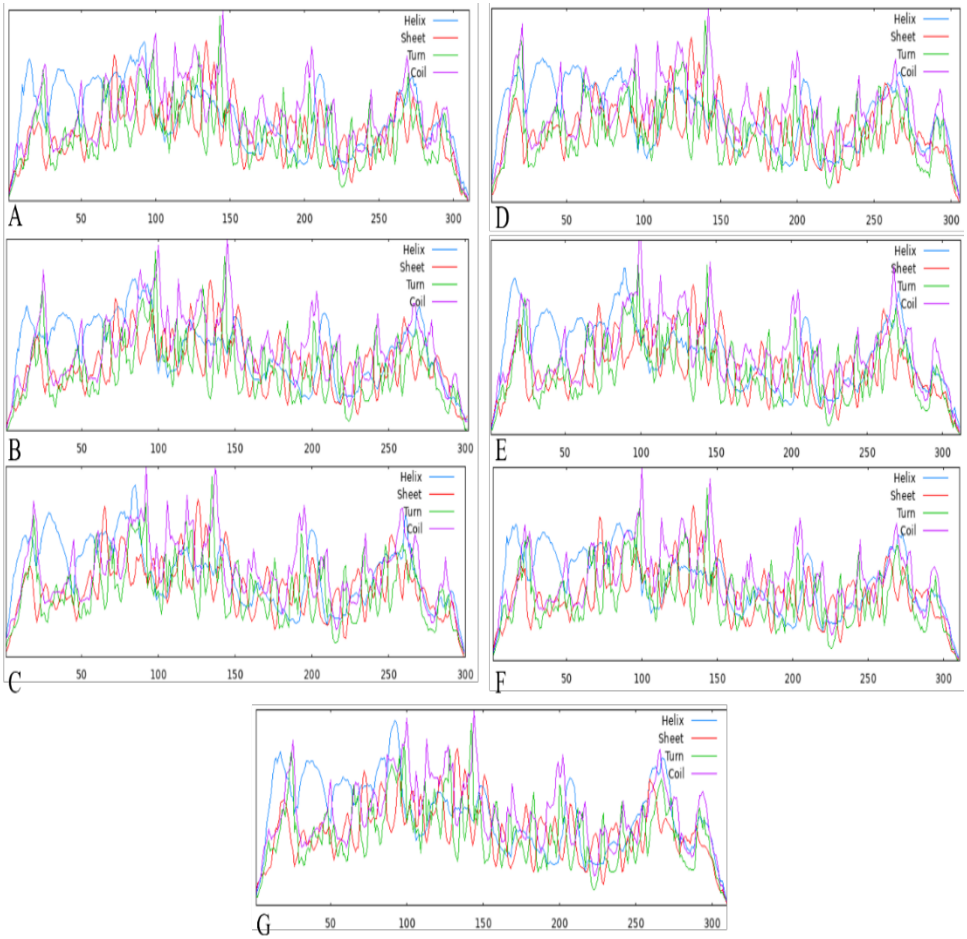

Figure 2. 2D Configuration of Catechol 2, 3-Dioxygenase Isoforms Based on SOPMA Tool

Table 4. Prediction of Properties of Catechol 2, 3-dioxygenase in Selected Bacteria using SOPMA Tool

#	Bacterium	α-helix (%)	Extended strand (%)	β-turn (%)	Random coil (%)
1	*A. lwoffii*	34.41	20.90	5.14	39.55
2	*Pseudomonas* sp. strain EST1001	30.13	20.53	6.95	42.38
3	*B. lata* strain DSM 23089	33.67	21.33	6.67	38.33
4	*Acinetobacter baumannii* strain AB307-0294	34.97	18.95	5.88	40.20
5	*S. stutzeri* DSM 5190	33.33	18.27	5.45	42.95
6	*Paraburkholderia* sp. WP4_3_2	34.08	21.54	5.79	38.59
7	*P. putida*	37.30	18.33	5.47	38.91

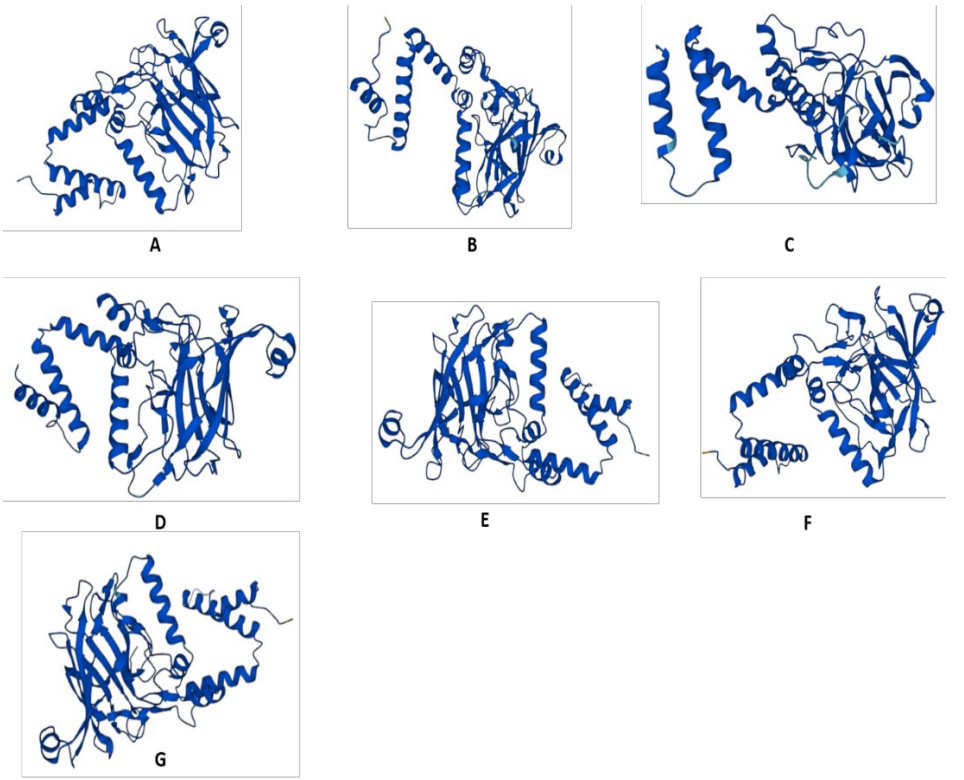

Figure 3. Assessment of Diversity in 3D Configuration of Catchol 2, 3-Dioxygenase using AlphaFold. A: *Acinetobacter lwoffii*, B: *Pseudomonas* sp. strain EST1001, C: *Burkholderia lata* strain DSM 23089, D: *Acinetobacter baumannii* (strain AB307-0294), E: Stutzerimonas stutzeri DSM 5190, F: *Paraburkholderia* sp. WP4_3_2, G: *Pseudomonas putida*

3.4 AlphaFold Database

The 3D configuration was slightly similar among all the isoforms of catechol 2, 3-dioxygenase documented in the current study. Less complex folding was observed in the enzyme of *Pseudomonas* sp. (strain EST1001). Structures of *Acinetobacter lwoffii*, *Acinetobacter baumannii* (strain AB307-0294), and *Paraburkholderia* sp. WP4_3_2 shared a high level of similarity, as compared to others (Figure 3).

4. DISCUSSION

The current study attempted to characterize the catechol 2, 3-dioxygenase in different POPs degrading bacteria to determine its diversity and to find out the best isoform for bioremediation.

Catechol 2, 3-dioxygenase reportedly plays a significant role in the degradation pathways of various organic pollutants categorized as POPs by Environment Protection Agency (EPA) including benzene, toluene, ethylbenzene, benzoate, and salicylate (Figure 4). In *Pseudomonas putida* G7, the salicylate is reportedly converted into catechol via salicylate dehydrogenase (NahG) enzyme. *Cupriavidus metallidurans* (CH34) catalyzes the breakdown of benzene into catechol via phenol formation as an intermediate step. The reaction is catalyzed by benzene phenol monooxygenase [32]. According to the literature, toluene is metabolized into catechol in *Pseudomonas pickettii* PKO1. During this transformation, toluene is transformed into benzyl alcohol by methyl monooxygenae. Benzyl alcohol is then converted into benzaldehyde via benzoyl alcohol dehydrogenase. This step is followed by the formation of benzoate in the presence of benzaldehyde dehydrogenase. Afterwards, cis-benzoate dihydrodiol and catechol are formed by benzoate 1, 2-dioxygenase and dihydrocyclohexadiene dehydrogenase, respectively [33]. A pathway for ethylbenzene transformation into catechol was reported in *Mycobacterium cosmeticum* by f-4. In this pathway, ethylbenzene is first converted into 1-phenylethanol and then into acetophenone in the presence of ethylbenzene dehydrogenase and 1-phenylethanol dehydrogenase, respectively. Acetophenone is then transformed into catechol via acetophenone carboxylase [28]. Catechol is degraded via ortho ring cleavage pathway [15]. These transformations strengthen the role of catechol 2, 3-dioxygenase in POPs degradation.

Literature reports the purification and characterization of catechol 2, 3-dioxygenase form bacteria [23]. Studies have reported the kinetic properties and catalytic properties of this enzyme [34]. However, a study involved the characterization of this enzyme in *Rhodococcus pyridinivorans*. This study used the ProtParam tool, JPred4 tool, and ASSP2 server for 2D structure prediction, CPH Model and PHYRE2 tool for 3D structure prediction, and STRING for protein interactions prediction. When the findings of this study were compared with the current findings, consistency was found in pI values and GRAVY. In fact, GRAVY was found negative in all cases, pI ranged between 5.01 to 5.50 and instability index for *Acinetobacter baumannii* strain AB307-0294, *S. stutzeri* DSM 5190 and *P. putida* was also compared. However, pI values in case of *Pseudomonas* sp. strain EST1001 (4.80) and *Acinetobacter baumannii* strain AB307-0294 (4.75) and instability index in case of *A. lwoffii, Pseudomonas* sp. strain EST1001, *B. lata* strain DSM 23089, and *Paraburkholderia* sp. WP4_3_2 were not found in accordance with the previous literature.

The values of pI showed that all the variants exhibited acidic pI. Hence, all of them can be crystallized effectively by using buffer with acidic pH. pI value shows the point of the minimum solubility of a protein [35]. Aliphatic index is directly proportional to enzyme thermostability. In all cases, values are higher, that is, 73.41 to 88.55 [36]. Instability index below 40 is an indicator of protein stability *in vitro* [37]. Except *Acinetobacter baumannii* strain AB307-0294 and *Acinetobacter baumannii* strain AB307-0294, all were found to be thermostable. A negative GRAVY value shows the non-polar nature of the protein. In the current study, all the proteins were found to be non-polar [38].

As far as the sub-cellular localization of catechol 2, 3-oxygenase is concerned, it was found to be cytoplasmic which is in accordance with the previously reported work [39]. A study modelled the catechol 2, 3-dioxygenase in *Bacillus stearothermophilus* on the basis of X-Ray configuration. The level of complexity of folding predicted in the current study is consistent with the findings in *B. stearothermophilus* [8].

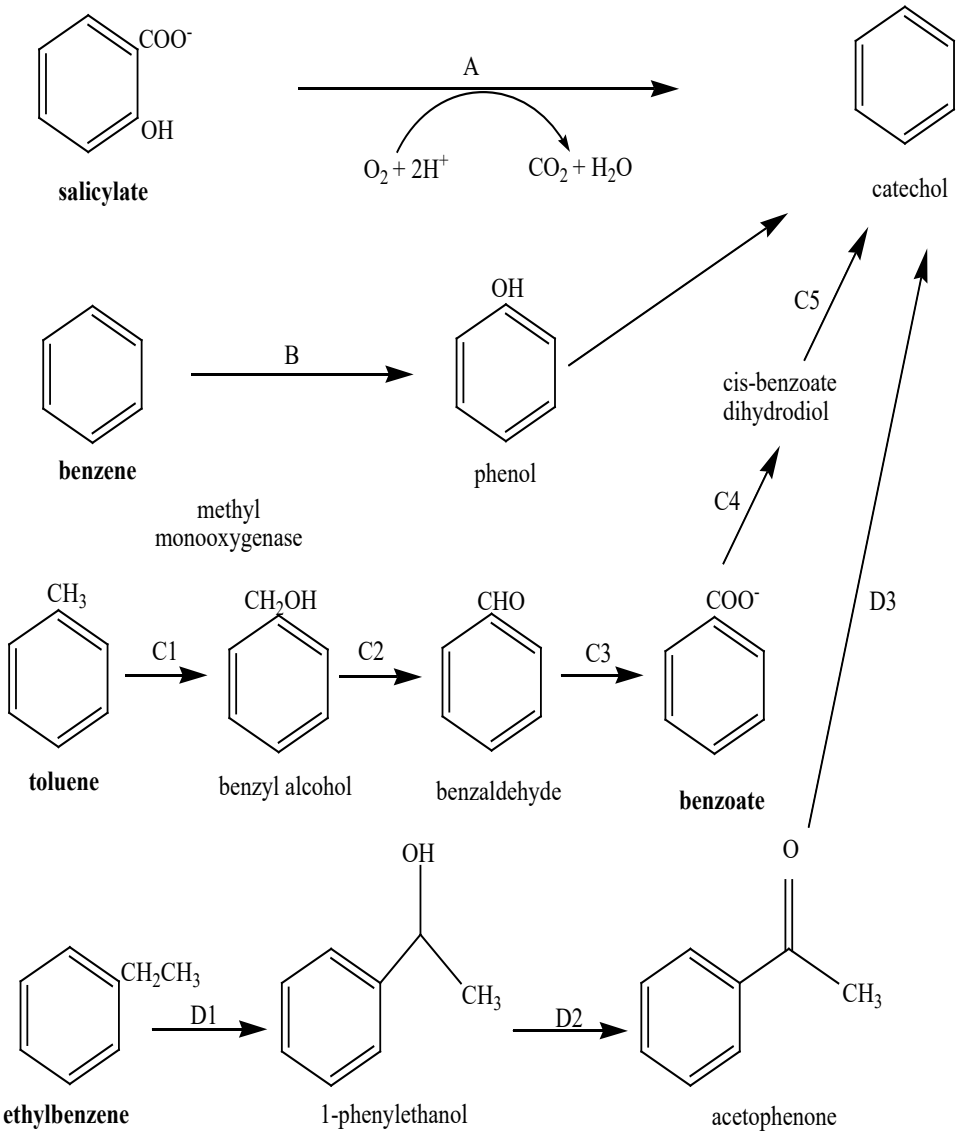

Figure 4. Reported Pathways for the Degradation of Benzene, Toluene, Phenol, Ethylbenzene and Salicylate into Catechol**.** A: Salicylate Dehydrogenase, B: Benzene Phenol Monooxygenase, C1: Methyl Monooxygenase, C2: Benzoyl Alcohol Dehydrogenase, C3: Benzaldehyde Dehydrogenase, C4: Benzoate 1, 2-Dioxygenase, C5: Dihydrocyclohexadiene Dehydrogenase, D1: Ethylbenzene Dehydrogenae, D2: 1-Phenylethanol Dehydrogenase, D3: Acetophenone Carboxylase

4.1. Conclusion

Diversity analysis of catechol 2, 3-dioxygenase revealed greater similarity among the variants of catechol 2, 3-dioxygenase than differences. The enzymes in *A. lwoffii, Pseudomonas* sp. strain EST1001, *B. lata* strain DSM 23089, and *Paraburkholderia* sp. WP4_3_2 were found to be stable *in vitro.* So, they can be used in the laboratory for cloning purposes and for the synthesis of enzyme-based nanoparticles. They can also be immobilized easily. As enzymes are least soluble at pH close to their pI, so the use of acidic conditions might help to stabilize them during their purification and use for bioremediation.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVALIABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

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