Glucose-6-Phosphate Dehydrogenase, ZwfA, a Dual Cofactor-Specific Isozyme Is Predominantly Involved in the Glucose Metabolism of Pseudomonas bharatica CSV86T

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ABSTRACT Glucose-6-phosphate dehydrogenase (Zwf) is an important enzyme in glucose metabolism via the Entner-Doudoroff pathway and the first enzyme in the oxidative pentose-phosphate pathway. It generates NAD(P)H during the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone, thus aiding in anabolic processes, energy yield, and oxidative stress responses. Pseudomonas bharatica CSV86T preferentially utilized aromatic compounds over glucose and exhibited a significantly lower growth rate on glucose (0.24 h⁻¹) with a prolonged lag phase (~10 h). In strain CSV86T, glucose was metabolized via the intracellular phosphorylative route only because it lacked an oxidative (gluconate and 2-ketogluconate) route. The genome harbored three genes zwfA, zwfB, and zwfC encoding three Zwf isoforms. The present study aimed to understand gene arrangement, gene expression profiling, and molecular and kinetic properties of the purified enzymes to unveil their physiological significance in the strain CSV86T. The zwfA was found to be a part of the zwfA-pgl-eda operon, which was proximal to other glucose transport and metabolic clusters. The zwfB was found to be arranged as a gnd-zwfB operon, while zwfC was present independently. Among the three, zwfA was transcribed maximally, and the purified ZwfA displayed the highest catalytic efficiency, cooperativity with respect to G6P, and dual cofactor specificity. Isozymes ZwfB and ZwfC were NADP⁺-preferring and NADP⁺-specific, respectively. Among other functionally characterized Zwfs, ZwfA from strain CSV86T displayed poor catalytic efficiency and the further absence of oxidative routes of glucose metabolism reflected its lower growth rate on glucose compared to P. putida KT2440 and could be probable reasons for the unique carbon source utilization hierarchy.

IMPORTANCE Pseudomonas bharatica CSV86T metabolizes glucose exclusively via the intracellular phosphorylative Entner-Doudoroff pathway leading the entire glucose flux through Zwf as the strain lacks oxidative routes. This may lead to limiting the concentration of downstream metabolic intermediates. The strain CSV86T possesses three isoforms of glucose-6-phosphate dehydrogenase, ZwfA, ZwfB, and ZwfC. The expression profile and kinetic properties of purified enzymes will help to understand glucose metabolism. Isozyme ZwfA dominated in terms of expression and displayed cooperativity with dual cofactor specificity. ZwfB preferred NADP⁺-, and ZwfC was NADP⁺ specific, which may aid in redox cofactor balance. Such beneficial metabolic flexibility facilitated the regulation of metabolic pathways giving survival/fitness advantages in dynamic environments. Additionally, multiple genes allowed the distribution of function among these isoforms where the primary function was allocated to one of the isoforms.

KEYWORDS glucose-6-phosphate dehydrogenase, Zwf, isozymes, transcription analysis, metabolic flexibility, redox cofactors, dual cofactor specificity, kinetic characterization, cooperativity
Glucose metabolism is one of the key metabolic pathways evolved in bacteria to yield energy and biomass. There are two major routes for glucose metabolism, the Embden-Meyerhof-Parnas (EMP) and the Entner-Doudoroff (ED) pathway (1). In *Escherichia coli* and *Bacillus subtilis*, glucose is channelized mainly via the EMP pathway. However, in *Pseudomonas* spp., glucose is metabolized through the ED pathway because they lack the key glycolytic enzyme, 6-phosphofructokinase (6pfk) required for the functional EMP pathway (2). Compared to the EMP pathway, glucose metabolism via the ED pathway yields a half amount of ATP. However, it is economically balanced by fewer metabolic steps, thus offering a beneficial trade-off for enzyme synthesis versus energy yield (3, 4). In *Pseudomonas* spp. glucose is transported and metabolized majorly (80% to 90%) through the oxidative (glucuronate and 2-ketogluconate) route while the remaining is metabolized through ATP-dependent glucose transport followed by intracellular phosphorylation pathway. Metabolic steps further involved NAD(P)H production by the action of glucose-6-phosphate dehydrogenase (Zwf or G6PDH) (Fig. 1) (5–8). All metabolic routes converge at 6-phosphogluconate (6PG). Organisms also followed the cyclidal ED-EMP pathway where glucose-6-phosphate (G6P) generated via the gluconeogenic EMP route (upper EMP pathway) was funneled into the ED pathway to increase NAD(P)H production (Fig. 1) (9, 10). Zwf is an important enzyme in ED as well as the pentose phosphate (PP) pathway. The conversion of G6P to 6PG in the PP and ED pathways share common metabolic steps. In the PP pathway, 6PG was metabolized to ribulose-5-phosphate by decarboxylating 6-phosphogluconate dehydrogenase (encoded by *gnd*) along with the generation of NADPH (Fig. 1). The PP pathway is majorly responsible to produce essential metabolites/anabolic precursors, including C4, C5, and C7 sugars and reducing equivalent NADPH. Thus, during glucose metabolism, the redox cofactor balance is maintained by flux distribution between Zwf (phosphorylative ED, PP) and non-Zwf (EMP, oxidative ED) routes (Fig. 1).

In *Escherichia coli*, the Zwf is majorly involved in the PP pathway while the ED pathway remains inactive (11). The deletion of glucose-6-phosphate isomerase (Δpgi) diverts glucose metabolism through an NADP+–preferring Zwf resulting in the cofactor imbalance and negative effect on the growth (12). The deletion of Zwf in *E. coli* (Δzwf) led to increased metabolic flux via isocitrate dehydrogenase and malic enzyme to fulfill the metabolic demands of NAD(P)H (13). However, in *P. putida*, where multiple copies of *zwf* are reported, the deletions of *zwfs* resulted in poor growth on glucose (14), indicating the importance of Zwf in the ED pathway-dependent bacteria. The *zwf* copies varied from one (e.g., *P. douguensis* and *P. vranovensis*), two (e.g., *P. fluorescens* and *P. aeruginosa*), three (e.g., *P. japonica*, *P. putida*, and *P. alkylphenolica*), or greater than or equal to four copies (e.g., *A. vinelandii* and *M. methanica*). However, in most of them, only one of the Zwf isoforms has been functionally characterized. Among these kinetically characterized Zwfs, most of them were NADP+–preferring/specific while some are dual cofactor specific or NAD+–preferring (14–18) Additionally, few enzymes were also reported to have cooperativity for the substrate, G6P (15, 16, 19, 20). However, the information about kinetic properties, catalytic efficiencies, expression patterns, and significance of multiple isoforms from a single bacterial strain is scanty.

*Pseudomonas bharatica* CSV86® metabolized glucose via an intracellular phosphorylative route and lacks oxidative routes (glucuronate and 2-ketogluconate route) (Fig. 1). Strain CSV86® displayed a unique carbon source utilization hierarchy, where aromatics and organic acids were preferred over simpler carbon sources, such as glucose and glycerol, while aromatics and organic acids are co-metabolized (21–23). Enzymes involved in the metabolism of glucose are suppressed in the presence of aromatics. Besides these unique properties, the strain displays various ecophysiological properties (like heavy metal resistance, plant growth-promoting traits, etc.) which makes it an ideal candidate for bioremediation (22). On glucose, the strain displayed poor growth with a prolonged lag phase. Genome analysis revealed the lack of genes responsible for oxidative routes and the presence of three *zwf* genes. Therefore, it is of significance to study their involvement in glucose metabolism, their arrangement with respect to other glucose metabolism/transport genes, and their molecular and kinetic properties.
FIG 1 Glucose metabolic pathway in *Pseudomonas* spp. ATP-dependent glucose transport and intracellular phosphorylation route are depicted by blue color arrows, oxidative routes (gluconate and 2-ketogluconate routes, which were absent in strain CSV86T) are depicted by red color arrows, the upper EMP pathway by green color arrows, the downstream ED pathway in black color arrows while PP pathway is depicted by orange color arrows.
The present study attempted to understand the expression profiling and catalytic efficiencies of multiple Zwfs in the glucose metabolism in strain CSV86. This will help to understand the underlying mechanism of slower glucose metabolism and unique carbon source utilization hierarchy.

RESULTS AND DISCUSSION

Genome analysis revealed the presence of three copies of zwf in P. bharatica CSV86. The draft genome analysis reveals the presence of three zwf gene copies annotated as zwfA (1473 nt, 490 aa), zwfB (1503 nt, 500 aa), and zwfC (1443 nt, 480 aa) (Fig. 2A). zwfA and zwfC were located on the contig 8 (NCBI accession no. NZ_AMWJ02000002.1), while zwfB was present on the contig 1 (NCBI accession no. NZ_AMWJ02000001.1). The zwfA was found to be a part of the glucose metabolic cluster zwfa-pgl-eda, where pgl and eda encoded 6-phosphogluconolactonase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase enzymes, respectively. The hexR, which encoded a putative transcriptional regulator of glucose metabolic genes was found to be located divergently upstream of zwfA.

Genes involved in the glucose transport (gfb-glcf-glcG-glcK-oprB) (24, 25) and metabolism (edd-egl-gllklr-hkr) (26) were present as two independent transcription units (operons) upstream to the zwfA encompassing the total length of ~16 kb (Fig. 2A). The zwfC was found to be located ~1200 kb away from zwfA and next to hexR-like transcriptional regulator annotated as hexR1. The zwfB was found to be a part of a gnd-zwfB cluster where the gnd encoded the decarboxylating 6-phosphogluconate dehydrogenase enzyme involved in the PP pathway (Fig. 2A).

BLASTN/P and genome analysis were performed for Pseudomonas spp. harboring multiple zwf copies. In P. putida KT2440 (genome accession number NC_002947) and P. japonica NBRC (NZ_BBIR00000000), three copies of zwf were found to be present with similar gene arrangement as observed in strain CSV86. In P. fluorescens Pf0-1 (NC_007492.2) and P. aeruginosa PAO1 (NC_002516.2), two copies of zwf were present, among which one of the zwf was associated with the glucose transport and metabolic genes, which was similar to zwfA of strain CSV86. The other copy of zwf from P. fluorescens Pf0-1 showed a gene arrangement similar to zwfB, while in P. aeruginosa PAO1, the arrangement was found to be similar to zwfC of strain CSV86. Apart from Pseudomonas spp., multiple zwf copies were also found to be present in most of the ED pathway-dependent microbes such as Xanthomonas, Azotobacter, Methylobacterium, and Acinetobacter, among others.

The pairwise amino acid sequence alignment of Zwf isozymes from strain CSV86 showed ~40% to 50% identity among each other. The ZwfA, ZwfB, and ZwfC of strain CSV86 shared 84.5, 82, and 84.1% identity with respective Zwf of P. putida KT2440, and 94.1%, 89.8%, and 95.6% identity with respective Zwf of P. japonica (unpublished data). The multiple amino acid sequence alignment of Zwf from CSV86 with structurally and functionally characterized Zwf from Leuconostoc mesenteroides (27) and functionally characterized Zwfs from P. putida (14), Zymomonas mobilis (28), E. coli (18), and Thermotoga maritima (29) depicted in Fig. 2B revealed that (i) the conserved active site residues involved in the nucleotide binding, the (G/A)X, GXX(G/A) motif with Rossmann fold (βαβ) at N-terminal region, the arginine residue at β2-domain, and (ii) substrate binding, i.e., the nonapeptide motif RIDHLYGKE and the catalytic dyad (His240-Asp177) where His240 acted as the general base abstracting a proton from the C1-hydroxyl group of G6P and Asp177 helped in stabilizing the positive charge of His240 in the transition state (27, 30–32). Most of the reported Zwf preferred NADP⁺ as a cofactor and possessed a conserved arginine residue at β2-domain. This arginine residue formed four hydrogen bonds with the phosphate group of NADP⁺ (18, 27, 30, 33).

In strain CSV86, ZwfA, and ZwfC were found to possess conserved arginine residue at position 49 and 54 in the β2-domain, respectively, while ZwfB had histidine at corresponding position 52. Besides arginine in the β2-domain, the lysine residue in the glycine-rich β1α1-loop has been reported to be involved in the interaction with NADP⁺. The mutation of this lysine to alanine resulted in a reduced affinity to NADP⁺ (18).

However, the lysine residue was not predominantly conserved across the bacterial species and was often found to be substituted with leucine, threonine, serine, or
asparagine. In strain CSV86T, the residue found to be present at the position corresponding to β1α1-loop lysine was leucine (L17) in ZwfA, serine (S20) in ZwfB and threonine (T14) in ZwfC, which could alter the affinity of these isozymes for NAD(P)⁺.

The rooted phylogenetic analysis revealed separate clades for ZwfA, ZwfB, and ZwfC, suggesting that three isozymes might share a distant common ancestor (Fig. 3A).

**FIG 2** In silico analyses of glucose-6-phosphate dehydrogenases (Zwf) from *Pseudomonas bharatica CSV86T*. (A) Arrangement of zwf genes in the CSV86T genome. Genes are depicted by pentagonal boxes labeled with names and its length (bp). The numbers below the boxes indicate intergenic spaces (bp). Gene annotations: contig 8 – eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; pgI, 6-phosphogluconolactonase; zwfA, glucose-6-phosphate dehydrogenase; hexR, transcriptional regulator; opb, carbohydrate-selective porin; gliK, ATPase component of glucose ABC transporter; gliG and gliF, transmembrane components of glucose ABC transporter; gliP, periplasmic glucose binding protein; insR, two-component sensor histidine kinase; gltRII, response regulator; glk, glucokinase; edd, 6-phosphogluconate dehydratase; hexRI, transcriptional regulator; zwfC, glucose-6-phosphate dehydrogenase. Contig 1, mobA, molybdenum cofactor guanylyl transferase; marR, MarR family transcriptional regulator; zwfB, glucose-6-phosphate dehydrogenase; gnd, decarboxylating 6-phosphogluconate dehydrogenase; duf, DUF6026 family protein. (B) Multiple sequence alignment of Zwfs from *P. bharatica CSV86T* with other structurally/functionally characterized Zwfs. Residues involved in the nucleotide-binding are highlighted in green; substrate binding in blue and catalytic dyad in yellow color.
Phylogenetic analysis of glucose-6-phosphate dehydrogenases (Zwf) from *Pseudomonas bharatica* CSV86⁷. (A) Rooted phylogenetic tree depicting the distribution of Zwf isozymes into distinct clades. The functionally and kinetically characterized Zwfs were NADP⁺-specific in blue font, NADP⁺-preferring in pink font, and dual cofactor-specific in green font. Functionally uncharacterized Zwfs are in black font. Numbers at the nodes indicate the bootstrap values. (B) Consensus logo representing the conserved nucleotide binding motif and residues responsible for cofactor specificity in clades ZwfA, ZwfB, ZwfC, and other Zwf.

**FIG 3** Phylogenetic analysis of glucose-6-phosphate dehydrogenases (Zwf) from *Pseudomonas bharatica* CSV86⁷. (A) Rooted phylogenetic tree depicting the distribution of Zwf isozymes into distinct clades. The functionally and kinetically characterized Zwfs were NADP⁺-specific in blue font, NADP⁺-preferring in pink font, and dual cofactor-specific in green font. Functionally uncharacterized Zwfs are in black font. Numbers at the nodes indicate the bootstrap values. (B) Consensus logo representing the conserved nucleotide binding motif and residues responsible for cofactor specificity in clades ZwfA, ZwfB, ZwfC, and other Zwf.
Zwf from *Pseudomonas* members possessing single copy were observed to cluster with ZwfA. Exceptionally, out of four Zwfs in *Azotobacter vinelandii* DJ, three were found to cluster with ZwfA while the fourth Zwf with ZwfB. Zwf from non-*Pseudomonas* members were found to cluster in different clades. It was also observed that ZwfA and ZwfC were more closely related, while ZwfB displayed early diversification in the phylogenetic tree and, thus, distantly related to ZwfA and ZwfC. It was observed that the Zwf in each clade shared >69% amino acid sequence identity with the respective Zwf of strain CSV867 (unpublished data). Such high amino acid sequence identities among Zwfs suggested probable similar functional features of these enzymes in the respective organism.

The key amino acid residues/motifs involved in the cofactor specificity were analyzed in each clade, revealing the presence of conserved nucleotide binding glycine-rich motif at β1α1 fold in all Zwfs. Interestingly, an anomaly was found at a position corresponding to the β2-arginine residue, which was majorly involved in NADP⁺ binding. All Zwfs except for the ZwfB group showed conservation of this arginine residue thus indicating a probable preference for NADP⁺, whereas ZwfB showed conservation of histidine residue at the corresponding position (Fig. 3B). Furthermore, to support these observations and clustering pattern, the cofactor preference of the functionally reported Zwfs was derived using the ratio of catalytic efficiency (kcat/Km) with NADP⁺ to NAD⁺. In the case where kcat was not determined, the ratio of affinity constant (Km) for NADP⁺ to NAD⁺ was used. Based on the comparison of kinetic properties, the Zwfs were classified into three categories: NADP⁺-specific if the ratio was >10-fold; NADP⁺-preferring if the ratio was 2 to 10-fold; and dual-cofactor specific if the ratio was in between 0.5 to 2-fold (Fig. 3A). It was observed that most of the Zwfs were NADP⁺-preferring or NADP⁺-specific, whereas only one Zwf have been reported among ZwfB (from *P. putida* KT2440), which was observed to display dual cofactor specificity.

**Cotranscription analysis revealed the operonic organization of zwf loci.** The gene arrangement, promoter prediction, and ribosome binding sites are depicted in Fig. 4A. In S'-zwfA-pgl-eda-3' cluster (zwfA locus), promoters were predicted upstream to zwfA and pgl genes with linear discriminant function (LDF) scores of 1.47 and 1.08, respectively. At the S'-gnd-zwfB-3' cluster (zwfB locus), a promoter upstream to gnd with an LDF score of 2.34 was detected, while the zwfC was found to be present independently possessing an upstream promoter with an LDF score of 4.10 (Fig. 4A). Based on gene arrangements, the cotranscription analyses were performed for zwfA and zwfB loci using specific intergenic primers (Table 1). At the zwfA locus, due to the presence of a weak pgl promoter, the forward primer designed for zwfA-pgl was upstream to the pgl promoter to demonstrate the polycistronic nature of mRNA for zwfA-pgl-eda. The electrophoretic analysis of various PCR products is depicted in Fig. 4B. The PCR products obtained using the intergenic primer pairs for zwfA-pgl, pgl-eda, zwfA-pgl-eda, and gnd-zwfB were ~750, 650, 1350, and 900 bp lengths, respectively. These amplicons corresponded to their expected length (Table 1), which were further gel purified and sequence confirmed (unpublished data). These results supported the gene arrangement at loci zwfA and zwfB and their cotranscription, suggesting transcription units (operons). Observed results were in accordance with previous reports for the zwfA locus from *P. putida* strains H and KT2440 (9, 34) and zwfB locus from *P. fluorescens* (35).

**ZwfA was the key player in glucose metabolism.** *P. bharatica* CSV867 prefers aromatic and organic acids over glucose by suppressing genes involved in glucose metabolism (21, 22). The specific activity of Zwf in the cell-free extract (CFE) was found to be ~5-fold higher in glucose-grown cells (450 ± 21 nmol min⁻¹ mg⁻¹) compared to naphthalene-grown cells (84 ± 8 nmol min⁻¹ mg⁻¹).

qPCR analyses revealed the maximum expression of zwf in the glucose-grown cells compared to benzoate or succinate (Fig. 4C). However, the relative expression of zwfA, zwfB, and zwfC was observed to be variable depending on the carbon sources used for the growth (Fig. 4C). In glucose-grown CSV867 cells, the gene zwfA displayed ~35- or 72-fold higher expression, while zwfB showed ~3 or 5.5-fold, and zwfC 2- or 2.5-fold higher expression compared to benzoate or succinate grown cells, respectively.
These results confirmed the inducible nature of glucose metabolic genes, and all three isoforms were found to display higher expression in glucose-grown CSV86T cells. Furthermore, the zwfA expression in the glucose-grown cells was found to be \( \sim 75 \) and 4500-fold higher than zwfB and zwfC, respectively (Fig. 4C). The expression analyses suggested that ZwfA was the predominant isozyme involved in glucose metabolism in strain CSV86T. In \( P. \) putida KT2440, the deletion of zwfA showed poor growth in glucose, while the deletion of zwfB and/or zwfC had a negligible effect on the growth (14). qPCR analyses for zwf in strain CSV86T and deletion mutant analyses in strain KT2440 corroborated the significant role of ZwfA in glucose metabolism.

In \( P. \) bharatica, glucose metabolic genes are regulated by HexR, the transcriptional regulator (36, 37). It binds specifically to the conserved pseudopalindromic DNA sequences at the promoter region suppressing the target genes (38). In \( P. \) bharatica CSV86T, hexR was
located divergently upstream to zwfA. Similarly, a hexR-like gene annotated as hexR1 was found to be present divergently upstream to zwfC (Fig. 2A). HexR and HexR1 shared 62% amino acid sequence similarity. Furthermore, the sequence alignment of the consensus binding regions and its comparison with *P. putida* KT2440 revealed the presence of HexR binding sites each for zwfA and zwfC genes but were absent for zwfB (unpublished data).

Interestingly, the gene zwfB was found to be a part of the transcription unit with *gnd* (the PP pathway gene), indicating its exclusion from HexR binding and regulation. This might aid in fine-tuning metabolic processes to meet the cellular requirement of ATP, NAD(P)H, or pentose sugars under glucose repression conditions.

**Kinetic properties supported ZwfA as the key enzyme.** Three isozymes were cloned into the pET vector (pET28a-zwfA, pET28a-zwfB, and pET28a-zwfC) and overexpressed individually into *E. coli* BL21(DE3). The CFE prepared from optimally-induced *E. coli* cells showed the specific activity of ~18.5 μmoles min⁻¹ mg⁻¹ for ZwfA, which was ~8 and 23-fold higher than that observed for ZwfB (~2.4 μmoles min⁻¹ mg⁻¹) and ZwfC (~0.8 μmoles min⁻¹ mg⁻¹), respectively. ZwfA and ZwfB utilized both NAD⁺ and NAD⁺ as cofactors while ZwfC utilized only NAD⁺ as a cofactor and displayed negligible enzyme activity in the presence of NAD⁺. The activity of ZwfA was found to be stable (100%) compared to ZwfB (50% loss) and ZwfC (20% loss) in 48 h at 5°C in the CFE.

**Purification and kinetic properties of Zwf isozymes.** The overexpressed ZwfA, ZwfB, and ZwfC isozymes were purified from optimally induced *E. coli* using NINTA affinity chromatography. The concentration range for imidazole to elute bound isoenzymes was 180 to 250 mM (for ZwfA), 100 to 175 mM (ZwfB), and 50 to 120 mM (ZwfC). ZwfA and ZwfC were obtained in the pure form while ZwfB retained impurities in the preparation (Fig. 5A). ZwfA was purified to 4.1-fold, 71% yield and specific activity of 76.4 μmoles min⁻¹ mg⁻¹, while ZwfC was purified to 9.9-fold, 21% yield and specific activity of 7.6 μmoles min⁻¹ mg⁻¹ using NINTA column chromatography. From

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**TABLE 1** List of primers used in the present study

| Gene                  | Primer name¹    | Sequence (5’→3’)                       | Length of amplicon (bp) | Annealing temp. (°C) |
|-----------------------|-----------------|----------------------------------------|--------------------------|----------------------|
| *zwf*-pgl             | zwfA_pgl_FP     | TGAATCACCAAGAAAGCG GC                  | 752                      | 60                   |
|                       | zwfA_pgl_RP     | GGAAACAGGAGCAGGAGTGT                   | 630                      | 65                   |
| *pgl*-eda             | pgl_eda_FP      | TCTATGCACCCGCGCGCGCA                   | 1382                     | 60                   |
|                       | pgl_eda_RP      | TACCGGCCCGATGCAAC                     | 208                      | 60                   |
| *zwf*-pgl-eda         | zwfA_eda_FP     | TGAATCACCAAGAAAGCG GC                  | 1382                     | 60                   |
|                       | zwfA_eda_RP     | TACCGGCCCGATGCAAC                     | 208                      | 60                   |
| *gnd*-zwfB            | gnd_zwfB_FP     | AGCGAACGGTTAGAATCGCGGCC             | 925                      | 60                   |
|                       | gnd_zwfB_RP     | GCTCCCGAGACCTGTGAAACA                   | 60                      |                       |

¹FP, forward primer; RP, reverse primer; restriction enzyme sites are underlined.
SDS-PAGE analyses, the subunit molecular weights were found to be ~56, 52, and 57 kDa for ZwfA, ZwfB, and ZwfC, respectively. The native molecular weight was determined by monitoring the elution profiles of standard molecular weight proteins on size exclusion chromatography. The elution profile for purified ZwfA was monitored using Enrich SEC650 column (CV = 24 mL, Bio-Rad), and for purified ZwfC using Superdex 300 increase 10/300GL column (CV = 24 mL, GE Healthcare [Cytiva]). From the semilog plot of $V_e/V_0$ (elution/void volume) on the x-axis and log$_{10}$(molecular weight) on the y-axis, the native molecular weight of ZwfA and ZwfC were determined.
ZwfB showed optimal activity in citrate-phosphate buffer 50 mM pH 6.5. At concentration, 4 mM G6P and 200 μM NADP+ or NAD+, the specific activity (μmoles min⁻¹ mg⁻¹) of ZwfA was 128 ± 11 with NADP+ and 140 ± 26 with NAD+. For ZwfB, it was 3.6 ± 3.5 with NADP+ and 1.64 ± 1.4 with NAD+. For ZwfC, it was 8.4 ± 1.5 with NADP+ and 0.61 ± 0.2 with NAD+. The kinetic constants were determined at the optimum pH and are summarized in Table 2. ZwfA showed a sigmoidal substrate saturation profile for G6P in the presence of NADP+ (Hill’s coefficient, n = ~1.84, Fig. 5B) as well as with NAD+ (Hill’s coefficient, n = ~2.1, Fig. 5B). ZwfB and ZwfC displayed hyperbolic substrate saturation profile for G6P in the presence of either NADP+ or NAD+ (unpublished data). At a fixed concentration of G6P, all isozymes displayed hyperbolic substrate saturation profiles for NADP+ or NAD+ (unpublished data). The ZwfA showed an equal affinity for G6P irrespective of NADP+ or NAD+ but at a fixed concentration of G6P, it showed ~3-fold higher affinity for NADP+ (Table 2). The ZwfB displayed ~2-fold higher affinity for G6P in the presence of NADP+ and ~9-fold higher affinity toward NADP+ at a fixed concentration of G6P (Table 2). While ZwfC showed ~5-fold higher affinity for G6P in the presence of NAD+ and ~175-fold higher affinity toward NADP+ at the fixed concentration of G6P (Table 2). The catalytic efficiency (kcat/Km) was ~2 and ~120-fold higher for NADP+ for ZwfA and ZwfC, respectively (Table 2). The ZwfA appeared to be catalytically more efficient (higher kcat/Km) compared to ZwfC (Table 2). These results suggested that ZwfA was dual cofactor specific, ZwfB was NADP+-preferring, and ZwfC was an NADP+-specific isozyme. The majority of the Zwf displayed a preference for NADP+, although few dual cofactor-specific or NAD+-preferring were reported (14–18). Notably, the dual cofactor specificity of Zwf was linked to the presence of its multiple isoforms, where this feature was observed in at least one of the isoforms. The presence of multiple zwf genes and, thus, probable dual cofactor utilization characteristic was found majorly in ED pathway-preferring organisms. The higher catalytic efficiency of ZwfA with positive cooperativity (n = 2, allosteric property) further supported it to be a key enzyme involved in the metabolism of glucose under dynamic substrate concentration conditions. The dual-cofactor specificity of ZwfA might further aid in conserving the intracellular NAD(P)+/NAD(P)H ratios, and its allosteric nature assisted in regulating the utilization of glucose-6-phosphate. Such fine-tuning of metabolic processes may help the organism to attain physiological robustness and ultimately yield survival benefits. Such allosteric property has been previously reported in P. aeruginosa, A. vinelandii, and P. fluorescens with ‘n’ ranging from 1.6 to 2.4 (15, 16, 20).

In strain CSV86, isoforms displayed different kinetic properties and cofactor utilization patterns. ZwfA with dual cofactor specificity and cooperativity played a major role, while ZwfB and ZwfC had a minor role in glucose metabolism. ZwfB probably participated in the PP pathway and ZwfC with low catalytic efficiency potentially evolved to function differently. ZwfA displayed ~2- to 120-fold lower affinity for G6P (higher Km), as well as significantly lower catalytic efficiency, compared to Zwf, reported from other organisms (Table 3). The strain displayed a lower specific growth rate on glucose (0.24 h⁻¹, a prolonged lag phase of ~10 h and stationary phase at ~20 h) compared to organic acids (~0.6 h⁻¹ and a lag phase of ~2 h for succinate, fumarate, pyruvate, or α-ketoglutarate) and aromatics (~0.55 h⁻¹ and a ~2 h lag phase for naphthalene or benzoate) (21–23). The absence of the oxidative routes and lower catalytic efficiency of...
| Enzyme | G6P with fixed NADP⁺ conc. | G6P with fixed NAD⁺ conc. | NADP⁺ with fixed G6P conc. | NAD⁺ with fixed G6P conc. |
|--------|----------------------------|--------------------------|---------------------------|--------------------------|
|        | Kₘ (µM) | Vₘ₉ (µmol min⁻¹ mg⁻¹) | kₘₙ/Kₘ | n | Kₘ (µM) | Vₘ₉ (µmol min⁻¹ mg⁻¹) | kₘₙ/Kₘ | n | Kₘ (µM) | Vₘ₉ (µmol min⁻¹ mg⁻¹) | kₘₙ/Kₘ | n | Kₘ (µM) | Vₘ₉ (µmol min⁻¹ mg⁻¹) | kₘₙ/Kₘ |
| ZwfA   | 1827 ± 235 | 191 ± 12 | 177 ± 11 | 0.096 ± 0.016 | 1.84 ± 0.3 | 1697 ± 284 | 248 ± 42 | 230 ± 37 | 0.16 ± 0.04 | 2.1 ± 0.17 | 110.5 ± 20 | 201 ± 28 | 187 ± 26 | 17 ± 0.3 | 366 ± 70 | 329 ± 74 | 306 ± 69 | 0.86 ± 0.15 |
| ZwfB   | 5827 ± 94 | ND⁣² | ND | ND | NA | 9958 ± 2716 | ND | ND | ND | NA | 67.5 ± 3 | ND | ND | ND | 577 ± 34 | ND | ND | ND | ND |
| ZwfC   | 1940 ± 172 | 114 ± 2.7 | 109 ± 2.6 | 0.0056 ± 0.0014 | NA | 3.64 ± 22 | 11.9 ± 1.6 | 11.4 ± 1.6 | 0.03 ± 0.0043 | NA | 18 ± 4.4 | 8.9 ± 1.6 | 8.5 ± 1.5 | 0.48 ± 0.05 | 310 ± 285 | 12.4 ± 3.3 | 11.9 ± 3.2 | 0.0041 ± 0.0014 |

*Kₘ, µM; Vₘ₉, µmol min⁻¹ mg⁻¹; kₘₙ, s⁻¹; kₘₕ/Kₘ, µM⁻¹ s⁻¹.

†Enzyme displayed cooperativity for G6P binding (see Fig. 5B).

‡Kinetics were performed using a partially purified enzyme.

§ND, not determined due to impure ZwfB preparations.

∥NA, not applicable because enzyme displayed a hyperbolic Michaelis-Menten saturation profile under these conditions.
| Organism          | G6P with fixed conc. of NADP⁺ | G6P with fixed conc. of NAD⁺ | NAD⁺ with fixed conc. of G6P | NAD⁺ with fixed conc. of G6P | kcat/Km | Quaternary structure |
|-------------------|-------------------------------|-------------------------------|-----------------------------|-----------------------------|---------|----------------------|
|                   | Kₘ(a) | kcat | kₘ/Km | SS² | Kₘ(a) | kcat | kₘ/Km | kₘ/Km | SS² | kₘ(Kₐ₃₄(NAD⁺))/kₘ(Kₐ₃₄(NAD⁺)) | Subunit mol. wt. | pH | References |
| P. bharatica CSV86T (A) | 1827 | 191  | 0.10  | S (1.84) | 1697 | 230  | 0.16  | S (2.1) | 110 | 187 | 1.7  | 366  | 306  | 0.86  | Homotetramer | 55.8 | 8.25 | Current study |
| P. bharatica CSV86T (B) | 5827 | NA² | NA² | M | 9968 | NA² | NA² | M | 67 | NA² | NA² | 577 | NA² | NA² | Homodimer | 51.5 | 6.5 |
| P. bharatica CSV86T (C) | 1940 | 11.4 | 0.0056 | M | 364 | 11.4 | 0.03 | M | 18 | 85  | 0.48 | 3100 | 11.9 | 0.004 | Homotetramer | 57.5 | 8.25 |
| T. maritima        | 200  | 35000 | 117  | S (1.0) | 875 | 1125 | 12000 | 9.2 | 955 | Homodimer | 60 | 7.4 | 29 |
| E. coli            | NA² | NA² | NA² | M | NA² | NA² | NA² | M | 7.5 | 174 | 23.2 | 5090 | 28.8 | 0.06 | 410 | NA² | 8.2 | 18 |
| P. aeruginosa      | 280  | 44  | 0.16 | M | NA² | NA² | NA² | M | 26 | 43  | 1.6  | 740  | 43  | 0.06 | 28 | Homodimer | 54.5 | 7.4 | 42 |
| L. monilestandii   | 114  | 523  | 4.6 | M | 69 | 1125 | 163 | M | 8 | 522 | 65.3 | 162 | 11.25 | 6.9 | 94 | Homodimer | - | 7.6 | 39 |
| A. aeolicus 40°C   | 15   | NA² | NA² | M | 58 | NA² | NA² | M | 9.1 | 47  | 5.1  | 230 | 58  | 0.25 | 20 | Homodimer | 55 | 7 | 41 |
| A. aeolicus 70°C   | 63   | NA² | NA² | M | 180 | NA² | NA² | M | 161 | 894 | 5.6  | 2096 | 2012 | 0.96 | 5.8 | Homodimer | 52 | 8 | 28 |
| Z. mobilis         | NA² | NA² | NA² | M | NA² | NA² | NA² | M | 40 | 338 | 8.5  | 210 | 589 | 2.8 | 3 | Homodimer | 52 | 8 | 28 |
| P. fluorescens     | 2700 | NA² | NA² | S (1.65) | 2330 | NA² | NA² | S (1.59) | 360 | 1117 | 3.1 | 150 | 1383 | 9.2 | 0.34 | NA² | 8.9 | 15 |
| P. putida KT2440 (A) | 946  | NA² | NA² | M | 1137 | NA² | NA² | M | 14 | 102 | 7.3  | 127 | 227 | 1.8 | 4.1 | NA² | 8 | 8 |
| P. putida KT2440 (B) | 940  | NA² | NA² | M | 291 | NA² | NA² | M | 165 | 113 | 0.68 | 151 | 120 | 0.8 | 0.86 | NA² | 8 | 14 |
| P. putida KT2440 (C) | 944  | NA² | NA² | M | 2030 | NA² | NA² | M | 3.2 | 0.54 | 0.17 | 9500 | 0.77 | 8.1 × 10⁻³ | 2082 | NA² | 8 |
| P. aeruginosa      | 499  | NA² | NA² | S (1.99) | 1146 | NA² | NA² | S (2.38) | 57 | 540 | 9.5  | 527 | 1017 | 1.9 | 4.9 | Homotetramer or octamer | 55 | 8 | 20 |
| A. vinlandii       | 530  | NA² | NA² | S (1.66) | 690 | NA² | NA² | S (1.76) | 50 | 36.7 | 0.73 | 220 | 91  | 0.41 | 1.8 | Homotetramer | 52 | 8.5 | 16 |

²Kₘ, μM; kcat, s⁻¹; kₘ/kₙ, μM⁻¹ s⁻¹.
²SS, substrate saturation profile; S, sigmoidal saturation profile with Hill's coefficient (n) value in the bracket indicating the allosteric nature of enzyme; M, Michaelis-Menten hyperbolic saturation profile.
²Zwf A, B, or C isoforms.
²NA, data not available.

**TABLE 3 Comparison of kinetic properties of glucose-6-phosphate dehydrogenases**
Zwf were probable reasons for the slower growth of strain CSV86T on glucose. In P. putida KT2440, which has intracellular phosphorylative as well as both oxidative routes for glucose metabolism, displayed faster growth on glucose (0.25%) with a specific growth rate of \( \sim 0.56 \text{ h}^{-1} \) (growth profiles are unpublished data).

The presence of multiple genes might be the result of segmental duplication (paralogs) or horizontal gene transfer events (homologs). The gene duplication is either the cause (Ohno model) or consequence (IAD model) of the promiscuous side function (43). In E. coli, the divergence of three isozymes of 3-deoxy-7-phosphohexulonate synthase after duplication allows the acquisition of differential feedback inhibition. Where each isozyme is inhibited by one of the aromatic amino acids thus controlling the flux into each pathway (43). In E. coli, a point mutation of Glu383 to Ala in \( \lambda \)-gamma-glutamyl phosphate reductase (ProA, enzyme involved in proline synthesis) increased its promiscuous ability to participate in arginine biosynthesis by catalyzing the reduction of \( N \)-acetyl-\( \lambda \)-gamma-glutamyl phosphate while decreasing its original activity as ProA (44). In eukaryotes, differential cellular localization of two NADP\(^+\)-dependent isocitrate dehydrogenase isozymes has been reported where one is localized in both cytoplasm and peroxisome while the other is found in mitochondria. The NADPH-produced aids in different functions based on the location (45, 46).

\textit{Pseudomonas bharatica} CSV86T metabolized glucose solely via the intracellular phosphorylative ED route but with a low growth rate. The enzyme G6PDH (Zwf) was one of the key enzymes to generate redox cofactors, NAD(P)H during glucose metabolism. Genome analysis revealed the presence of three copies of \( zwf \) with 40% to 50% amino acid sequence identity and clustering into distinct phylogenetic clades. \( zwfA \) and \( zwfB \) were organized as individual transcription units as \( zwfA-gpl-edA \) and \( gnd-zwfB \), respectively, whereas \( zwfC \) was present independently. The \( zwfB \) was found to be devoid of the consensus sequence for binding of transcription repressor HexR, indicating its probable role in the PP pathway. \( zwfA \) was expressed abundantly compared to \( zwfB \) and \( zwfC \) in CSV86T. All three isozymes were purified and kinetically characterized. Among them, \( zwfA \) displays allosteric nature for substrate binding and higher catalytic efficiency. However, the catalytic efficiency was significantly low compared to other reported \( Zwfs \). With respect to cofactor utilization, \( zwfA \) exhibited dual-cofactor specificity, \( zwfB \) displayed a preference for NADP\(^+\) while \( zwfC \) was NADP\(^+\) specific. The substitution of key amino acids lysine by leucine (\( ZwfA \)), serine (\( ZwfB \)), or threonine (\( ZwfC \)) at 81\( \alpha \)-1-loop glycine-rich motif and replacement of arginine with histidine at the \( \beta2 \)-domain in \( ZwfB \) might be responsible for the observed altered cofactor specificity. The varied cofactor preferences and allosteric nature might enable balance in glucose utilization and cofactor production. However, the low catalytic efficiency of \( Zwfs \) and lack of oxidative routes might lead to limiting concentration of downstream pathway intermediates, such as KDPG (one of the proposed modulators of the metabolic pathway through HexR (36)), thus affecting the growth on glucose. Such defective glucose metabolism might enable strain CSV86T to preferentially utilize substrates, such as aromatics. The unique hierarchy of carbon sources hence can be further explored for its application in the field of bioremediation of toxic aromatic pollutants and aids in environmental cleanup.

**MATERIALS AND METHODS**

\textit{Microorganisms, growth, and culture conditions.} \textit{Pseudomonas bharatica} CSV86T, \textit{Pseudomonas putida} KT2440, and \textit{Escherichia coli} strain DH5\( \alpha \) and BL21(DE3) were used in the present study. Strain CSV86T was grown on 150 mL minimal salt medium (MSM) pH 7.5 (47) supplemented aseptically (wt./vol.) with naphthalene (0.1%), benzoate (0.1%), succinate (0.25%), or glucose (0.25%) in 500 mL baffled Erlenmeyer flasks at 30°C on a rotary shaker (200 rpm). The \textit{E. coli} strains DH5\( \alpha \) and BL21(DE3) (Novagen) were grown on Luria-Bertani broth (LB) at 37°C on a rotary shaker at 200 rpm (48). Growth experiments for strains CSV86T and KT2440 were performed on MSM medium supplemented with 0.25% glucose by measuring optical density at 540 nm using a spectrophotometer every 2 h.

\textit{Genome mining for glucose metabolic genes and bioinformatic analyses.} The advanced version of the genome sequence of \textit{Pseudomonas bharatica} CSV86T was retrieved from NCBI (accession no. AMWJ02000000) (49). The nucleotide and amino acid sequences of glucose metabolic genes were extracted using BLASTN/P. The amino acid sequences of \( Zwf \) isozymes were retrieved from NCBI and
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**Protein Purification.** All protein purification steps were performed at 4°C.

For the purification of ZwfA, the protein was purified using FPLC (NGC quest plus, Bio-Rad, USA) at a constant flow rate of 30 mL h⁻¹. The CFU prepared from *E. coli* cells overexpressing ZwfA was loaded on to Ni-NTA affinity chromatography matrix (~3 mg/mL of matrix, column volume, CV 5 mL, Qiagen, Germany) pre-equilibrated with Buffer A. The column was washed with Buffer A (4 × CV) followed by 2 × CV each of 50 and 100 mM imidazole solution in Buffer A. Bound ZwfA was eluted using a linear gradient of imidazole (100 to 350 mM, 5 × CV) in Buffer A. The active and pure fractions were pooled, concentrated using 30K centricon (PALL Corporation, USA) and chromatogramed on the size exclusion chromatography (CV = 24 mL, Enrich SEC 650 column, Bio-Rad, pre-equilibration and developed with Buffer A).

For the purification of ZwfB, the CFU (~ 5 mg protein/mL matrix) prepared from *E. coli* cells overexpressing ZwfB was loaded on to Ni-NTA affinity chromatography matrix (5 mL, Qiagen, Germany) pre-equilibrated with Buffer B. The column was washed with Buffer B (4 × CV) and bound protein was eluted using a linear gradient of imidazole (0 to 300 mM, 10 × CV) in Buffer B. Active fractions were pooled and concentrated using 10K centricon (30K) and chromatogramed on the size exclusion chromatography (CV = 24 mL, Enrich SEC 650 column, Bio-Rad, pre-equilibration and developed with Buffer B).

For the purification of ZwfC, the protein was purified by using the protocol as described for ZwfB using Buffer A. Active and pure fractions were pooled and concentrated using 10K centricon (30K) and chromatogramed on the size exclusion chromatography (CV = 24 mL, Superdex 200 Increase 10/300 GL column (GE Healthcare [Cytiva], USA) pre-equilibrated and developed with Buffer A).

To determine the native molecular weight by size exclusion chromatography the molecular weight markers used were: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa) and oryzalin (30 kDa). The molecular weight of native ZwfC was determined to be ~ 34 kDa by size exclusion chromatography.
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(66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa).

The purity of enzyme preparations was monitored using SDS-PAGE and Native-PAGE (57, 58). Subunit molecular weight was determined by SDS-PAGE using molecular weight markers: lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase b (97.4 kDa).

Kinetic characterization. For ZwfA, the kinetic constants were determined using 1.3 µg of purified ZwfA per assay in Tris-Cl buffer (50 mM, pH 8.25), spectrophotometrically. The substrate saturation profiles were generated using (i) various concentrations of substrate, G6P (5 to 1500 µM) and fixed concentration of cofactor NADP⁺ (0.5 mM) or NAD⁺ (1 mM); and (ii) fixed concentration of G6P (5 mM) and various concentrations of NADP⁺ (2.5 to 1500 µM) or NAD⁺ (1 to 2000 µM).

For ZwfC, the kinetic constants were determined using 5 µg of partially purified ZwfC was used per enzyme assay in citrate-phosphate buffer (50 mM, pH 6.5), spectrophotometrically. The substrate saturation profiles were generated using (i) various concentrations of G6P (50 to 2 × 10⁵ µM) and fixed concentration of NADP⁺ (0.5 mM) or NAD⁺ (6 mM); and (ii) fixed concentration of G6P (5 mM) and various concentrations of NADP⁺ (2 to 5000 µM) or NAD⁺ (5 to 7500 µM).

For ZwfC, the kinetic constants were determined using 5 µg of purified ZwfC per assay in Tris-Cl buffer (50 mM, pH 8.25), spectrophotometrically. The substrate saturation profiles were generated using (i) various concentrations of G6P (20 to 5 × 10⁴ µM) and fixed concentration of NADP⁺ (0.25 mM), and various concentrations of G6P (20 to 10⁶ µM) and fixed concentration of NAD⁺ (15 mM); and (ii) fixed concentration of G6P (5 mM) and various concentrations of NADP⁺ (0.5 to 1000 µM) or NAD⁺ (50 to 3 × 10⁵ µM).

The kinetic constants and Hill’s coefficient (n) were determined by plotting specific activity data using Sigmaplot software (version 12) as Michaelis-Menten plot and Hill’s plot, respectively. Each experiment was performed at least in triplicates and each reading was in triplicates.

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