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Research Article
Biochemical Leaning of Phosphoglucose Isomerase is More Towards Gluconeogenesis in *Pseudomonas aeruginosa* PAO1

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Abstract

Background: *Pseudomonas aeruginosa* is an opportunistic and highly versatile in metabolism. Whole genome sequence showed, the organism does not follow the normal glycolysis rather Entner-Doudoroff pathway for energy production. Whole genome sequence annotation shows the presence of glycolytic enzyme. It is important to study biochemical properties of glycolytic enzyme which may reveal the information for its role in other cellular processes. **Methodology:** Phosphoglucose isomerase, considered as a moonlighting protein by showing the role as an autocrine motility factor, cytokine, neuroleukin, differentiation and maturation factor. **Results:** The biochemical study revealed that the enzyme is most active under alkaline conditions with optimum pH of 8.0. The higher working temperature is 40°C and it does not require metal ions to initiate isomerization however, metal ion stabilizes the enzyme as compared to apoenzyme. The enzyme showed k_{cat}/K_m for glucose 6-phosphate and fructose 6-phosphate is ≈0.078 and ≈1.0 sec^{-1} mM^{-1}, respectively, which indicates that it has a role in gluconeogenesis rather than glycolysis. **Conclusion:** Amino acid sequence analysis establishes the evolutionary enzyme by showing conserved active site residues and share ≈48.13% identity with *Homo sapiens*.

Key words: *Pseudomonas aeruginosa*, phosphoglucose isomerase, moonlighting protein, glycolysis, gluconeogenesis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pseudomonas aeruginosa shows high metabolic versatility organism with the ability to survive in different habitats[1]. It is an opportunistic pathogen and causes cystic fibrosis, necrotising anemia, meningitis, nosocomial urinary tract infection is common in hospitalized patients and claims millions of lives annually by such infections[2,3]. Complete genome sequence of *P. aeruginosa* PAO1 has been studied[4] and have been focused on its virulence mechanism[2,5] and strategy to design the therapeutic targets.

Whole genome sequencing has given deeper insight of metabolic versatility of PAO1. In the of glucose catabolism, functional Embden-Meyerhof pathway is absent. Rather, glucose is stabilized through Entner-Doudoroff (ED) pathway[6,7]. The ED flux of *P. aeruginosa* provides a major benefit for this pathogen, which must counteract oxidative stress imposed by the host as a response to infection. There are possibilities that such normal catabolic pathways are shown anabolic purpose rather than the energy production. As in the case of the pentose phosphate pathway, both oxidative and non-oxidative reactions are produced ribose 5-phosphate, erythrose 4-phosphate and fructose 6-phosphate which is used as a starting material for other biomolecules.

Phosphoglucose isomerase (PGI) carries the reversible reaction between glucose 6-phosphate (G6P) to fructose 6-phosphate (F6P). Fructose 6-phosphate is one of the important precursor for many biomolecules (Fig. 1). The PGI (EC 5.3.1.9) plays a central role in the sugar metabolism, both in glycolysis and in gluconeogenesis, where the enzyme operates in the reverse direction[8,9]. Apart from housekeeping function, it is considered as a "Moonlighting protein" by showing different activities. It acts as a tumor autocrine motility factor[10], neuroleukin[11,12], differentiation and maturation factor[13], antigens involved in rheumatoid arthritis[14], sperm agglutination[15], serine-protease inhibitor[16], cytokines in mammalian[17] and lysyl amino peptidase activity in vibrios[18]. It is consider as a marker for the progression of various cancers[19,20] also, as it is a specific determinant of cell survival and contributes to tumor cell progression[21,22]. In human PGI deficiency can be associated with hydrops fetalis, neurological impairment and immediate neonatal death[23]. The PGI is found as adhesive moonlighting protein considered to show enhanced release in acid and bile stress response in human gut colonizing *Lactobacillus* spp.,[24,25] which indicates that the surface architecture of bacterium is modified upon contact with the central innate immunity system of human host. The PGI is involved in pathogenicity[26,27] and also found to promote angiogenesis, enhances metacestode growth and parasite-host cell interactions[28]. The planktonic cells of *P. aeruginosa* requires exopolysaccharide for adherence and antibiotic resistance[29]. The PGI has been assumed to play a key role in governing carbon availability in the biosynthesis of EPS[30].

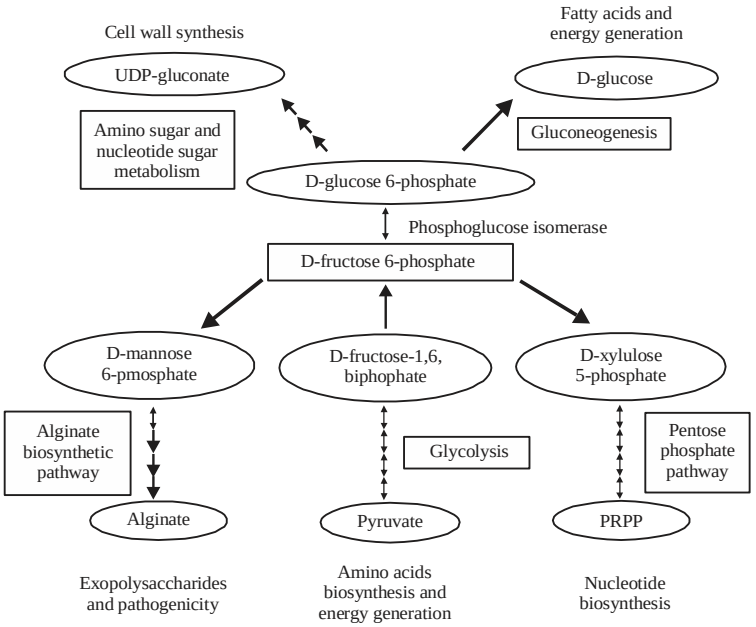

Fig. 1: Various routs of fructose 6-phosphate utilization for the synthesis of essential metabolites, virulence factors and cell components in *Pseudomonas aeruginosa*

The PGI from a variety of pathogens such as *Vibrio vulnificus*[18], *Mycobacterium tuberculosis*[31,32], *Plasmodium falciparum*[33-35], *Listeria monocytogenes*[36], *Trypanosoma brucei*[31], *Leishmania mexicana*[37] and *Echinococcus multilocularis*[28] have been studied and revealed that the PGI possess additional biological action with involvement in bacterial virulence. Considering the significant biological role of PGI, in this study, the sequence analysis and biochemical characterization of PGI from *P. aeruginosa* PAO1 (Pa-PGI) was reported.

MATERIALS AND METHODS

Bacterial strains and materials: The *Pseudomonas aeruginosa* PAO1 (MTCC 741) strain was purchased from Microbial Type Culture Collection and GenBank, Chandigarh. All the molecular techniques were performed according to methods described by Sambrook and Russell[38]. All the chemicals and glucose-6 phosphate dehydrogenase were procured from Sigma (St. Louis, MO), media from Himedia (Mumbai, India) and DNA polymerase from Takara (Otsu, Japan). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs (UK). The Ni-nitrilotriacetic acid (NTA) agarose beads was purchased from Invitrogen (Carlsbad, CA).

Cloning and expression of Pa-PGI in *Escherichia coli*: The *P. aeruginosa* was grown overnight in nutrient broth at 37°C with revolution 120 rpm. The DNA was isolated using the method described by Chen and Kuo[39]. The PGI of *P. aeruginosa* PAO1 is encoded by the gene PA4732 as indicated by the Pseudomonas Genome Database (http://www.pseudomonas.com/). The Pa-pgi (1665 base pair) was amplified using primers 5′ CTG TTG GAT BamHI ATG AAG CAC CAC CTC ACT CCG and 3′ TCA ATT TGG XhoI TCA GCC GCG ATG GCG GCC GCG. The amplified product of 1665 bp was cloned into 6x-His tagged pRSET-A vector and the sequence was confirmed. *Escherichia coli* BL21 (DE3) was transformed by pRSET-A expressing Pa-PGI for protein production. A colony was used to inoculate 5 mL Luria Broth (LB) containing 100 μg mL^{-1} of ampicillin and incubated overnight at 30°C. The overgrown culture was used to inoculate 400 mL LB broth and incubated at 30°C. The cells were harvested by centrifugation and suspended in cell suspension buffer (50 mM tris-HCl, 300 mM NaCl, 20 mM imidazole, pH 8.0). It was lysed by sonication. The lysate was clarified by centrifuging at 12,000 rpm for 30 min and loaded on a Ni-NTA agarose bead column for protein purification. After extensive washing with wash buffer (50 mM tris-HCl, 300 mM NaCl, 40 mM imidazole, pH 8.0), the lysate was finally eluted with an elution buffer containing 250 mM imidazole (pH 8.0) and dialyzed against 50 mM tris-HCl (pH 8.0) using a 10 kDa ultra filter (Merckmillipore, Germany). Finally, purified protein was quantified using Bio-Rad protein assay reagent (Bio-Rad, CA) against bovine serum albumin as the standard protein[40]. The purified protein was subjected to 10% SDS-PAGE and visualized with coomassie brilliant blue R-250 stain.

Protein expression and purification levels were monitored by enzyme activity using the substrate G6P. One unit of activity was defined as the amount of enzyme required to produce 1 mM min^{-1} of F6P under standard assay conditions. Activities were expressed in unit per milligram of protein.

Enzyme assay: The enzyme Pa-PGI catalyses the conversion of G6P from the substrate F6P. The F6P production was analysed as described earlier by Kulka method[41] with some modifications. Briefly, the assay was performed in 50 mM tris-HCl (pH 8.0) with 2 mM G6P and 5 μg μL^{-1} of enzyme in a reaction mixture of 100 μL. The reaction mixture was incubated at 40°C and the reaction was stopped by adding 250 μL of solution A and solution B[41]. The reverse reaction (formation of F6P from the substrate G6P) by Pa-PGI was monitored by the coupling assay with glucose 6-phosphate dehydrogenase performed as described earlier[42]. The rate of isomerization, for the formation of G6P was monitored for 10 min and determined from the linear reaction phase in a thermostatically regulated spectrophotometer (UV-visible recording spectrophotometer, Shimadzu).

Physicochemical characterization

Effect of pH: Effect of pH on the Pa-PGI was checked by incubating the purified enzyme with G6P as a substrate. The reaction mixture was prepared in the following buffers (50 mM): Sodium phosphate buffer (pH 6.0-7.0), tris-HCl (pH 8.0-9.0). After 15 min of incubation at 40°C, activity was measured. The relative percentage of activity was calculated and compared.

Effect of temperature: To compare the effect of temperature, Pa-PGI was incubated in a standardized reaction mixture at various temperatures (20-80°C). After 15 min of incubation, samples were subjected to activity analysis and data was compared in percentage of relative activity.

Thermal stability: To determine the thermal stability of the enzyme, Pa-PGI was incubated at 40°C. At regular interval of 60 min, samples were drawn and residual activity was measured. Stability was compared and the half-life was determined accordingly.

Effect of metal ions: Effect of metal ions on the Pa-PGI activity was checked by incubating the purified enzyme with G6P as a substrate in tris-HCl buffer 8.0. The reaction mixture was prepared with the following metal ions (1 mM): $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $MgCl_2$, $CuSO_4.5H_2O$, $CaCl_2$, $FeCl_3$ and apoenzyme. After 15 min of incubation at 40°C, activity was measured. The percentage relativity of activity was calculated and compared.

Kinetic parameters of Pa-PGI: The efficiency of Pa-PGI was analysed at 40°C using G6P at a range of concentrations (1-20 mM). Each concentration was treated with enzyme containing 1 mM Mn^{2+} in 50 mM tris-HCl, pH 8.0. Reactions were stopped after 15 min and the reaction mixtures were analysed for F6P production. The Michaelis constant Km (mM) and the turnover number k_{cat} (min^{-1}) were determined by arranging the data according to Michaelis-Menten equation.

Various sugars were analysed for specificities towards Pa-PGI. Fifty millimolar D-glucose, D-fructose, G6P and F6P were treated with Pa-PGI with or without 1 mM Mn^{2+} under conditions optimized for a reaction time of 15 min. The isomerized product was checked by the methods described earlier. Specific activity was defined as the amount of aldose or ketose produced per enzyme amount per reaction time.

RESULTS AND DISCUSSION

Sequence analysis: The PCR was performed with gene specific primers and resulted amplicon was cloned into pRSET-A expression vector. The sequence analysis showed 1665 bp amplicon of Pa-pgi encoding for 555 amino acids. It shares 59.0% nucleotide identity with human PGI (Hu-PGI). The amino acid sequence alignment score of Pa-PGI with characterized PGI of human, *Mycobacterium tuberculosis*, *Plasmodium falciparum* showed 48.13, 44.05 and 32.04% identity, respectively[31,34,43]. The conserved residues in the deduced sequence of Pa-PGI are shown in the box (Fig. 2). Other species also have shown to be two conserved sequences-[DENS]-X-[LIVM]-G-G-R-[FY]-S-[LIVMT]-X-[STA]-[PSAC]- [LIVMA]-G- and [GS]-X- [LIVM]-[LIVMFYW]-XXXX-[FY]-[DN]-Q-X-G-V-E-X-X-K-, known as signature sequence of the PGI superfamily[44,45]. The amino acid content comparison indicated the great dissimilarity in their properties with human and other pathogens. A group of active residues were (Glu 359, His 390 and Lys 518 in Pa-PGI) found to be conserved throughout the family (Human, Mouse, Rabbit, Pig, Fly, Plant, Bacteria, Archea and Yeast). The conserved active-site residues included the side-chain hydroxyl groups of Ser 160, Ser 210, Thr 215 and Thr 218. Other residues including Lys 211, Glu 217, Gln 354, Glu 359, Gln 511, Glu 515 and Lys 522 were also found to be conserved[46].

The PGI is a moonlighting protein which also showed Autocrine Motility Factor (AMF) activity. The study of AMF revealed that the substrate binding is similar to PGI as it shares the same substrate and inhibitors[18,47,48]. The Hu-PGI and Pa-PGI shares similar active site and hence probably follow the same mechanism of action. However, the residues nearby active site revealed variation as compare to Hu-PGI which may results in to the difference in catalytic efficiency of enzyme. Sequence similarity of other characterized PGI with Pa-PGI showed higher identity percentage which reveals that the protein may be a dimer. Histidine residue is structurally unique in dimeric form. Phosphoglucose isomerase Histidine from one monomer (His 387 of *M. tuberculosis*) is found to form the active site with its partner monomer in dimeric protein[49]. The sequence alignment between Mt-PGI and Pa-PGI showed that His 389 of Pa-PGI (His 387 of *M. tuberculosis*) is extending its side chain for activity between two monomers. The highly specific contents between two subunits make PGI a tight dimer. It was also observed that the hook like structure between Arg 438 and Glu 469 corresponds to Leu 439 and Pro 470 of Pa-PGI, respectively with C-terminal extension, wrap around both subunits and make stable dimer[43].

Cloning, expression and purification of Pa-PGI: The protein was expressed in soluble form. Nickel affinity chromatography purified protein was detected around 62 kDa molecular mass on SDS-PAGE analysis (Fig. 3). Total 72 units of specific activity were obtained from 400 mL of culture.

Physicochemical characterization of Pa-PGI: The determination of the optimal conditions for Pa-PGI activity reveals that it is an alkaline, mesophilic and metal independent protein. The optimum pH was found to be 8.0 (Fig. 4a). The pH profile of Pa-PGI followed the bell shaped curve and found to be active over the range of pH 6.0-9.0. The medium working temperature of Pa-PGI was 40°C. The enzyme lost its activity completely at 60°C (Fig. 4b).

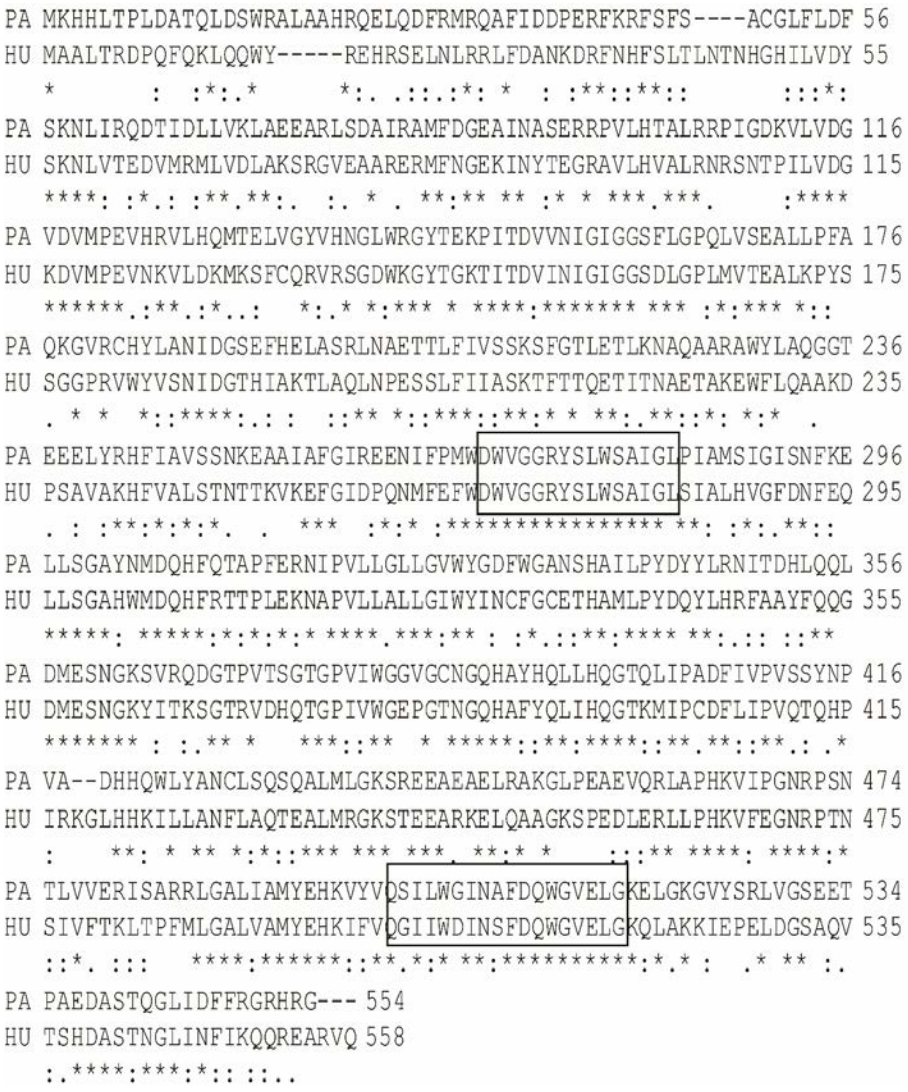

Fig. 2: Multiple sequence alignment of deduced amino acid sequence of Pa-PGI with human PGI using ClustalW: The deduced amino acid sequence of Pa-PGI was compared for its homology with Hu-PGI (Accession No. AAH04982). Single fully conserved residues are represented by instinct mark, the black coloured instinct mark represents active site residues and strongly conserved and weakly conserved residues between the two PGIs are denoted by double dots and single dot, respectively. Box region represent highly conserved sequence of the PGI superfamily

The enzyme followed first order kinetics in thermal inactivation. The half-life of Pa-PGI was found to be about 150 min at 40°C (Fig. 4d). However, it showed high thermal stability (half-life 573 min) in the presence of divalent metal Mn^{2+} (Fig. 4d).

The Pa-PGI was found as a metal independent enzyme and did not display any increase in isomerase activity, which is similar to other PGI superfamily. Among all tested metals, Cu^{2+} demonstrated some inhibitory effect on isomerization (Fig. 4c). Crystal structure of Rabbit PGI (Ra-PGI) showed absence of metal binding site[46]. The PGI family is metal independent in its action similar to triose phosphate isomerase in contrast to the isomerization of xylose/glucose by xylose isomerase, which requires metal ion for substrate ring opening and catalysis[50,51]. Moreover, it was observed that the Arg 272 of Ra-PGI correspond to Arg 274 in Pa-PGI which provides potential electrostatic field to the substrate and stabilize the negative charge to stabilize the enediolate transition state.

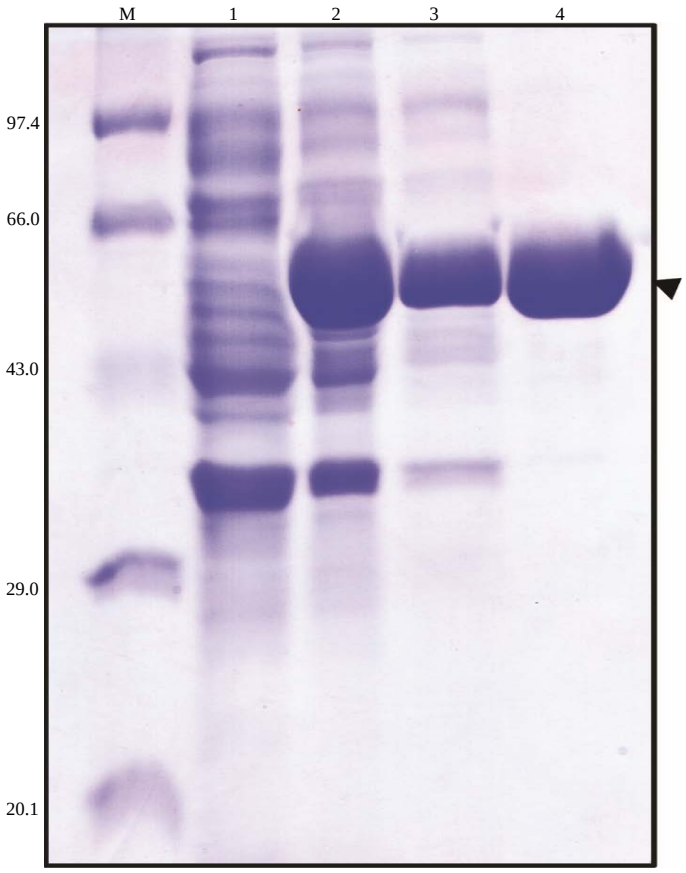

Fig. 3: SDS-PAGE analysis of localization of recombinant Pa-PGI. The recombinant PGI was expressed in *Escherichia coli* strain BL21 (DE3) and subjected to SDS-PAGE (10%). The gel was stained with coomassie brilliant blue. Lane M: Molecular size markers, lane 1: Control BL21 cells, lane 2: Pa-PGI BL21 cells, lane 3: Crude extracted Pa-PGI, lane 4: Ni-NTA purified concentrate of Pa-PGI. The arrow points to the Pa-PGI

Table 1: Substrate binding efficiency of Pa-PGI

Substrate	Specific activity (U mg^{-1})[c]
F6P	7.661±2.14
G6P	2.147±1.89
D-glucose	1.714±3.03
D-fructose	3.596±2.17

[c]Data are means of three separate experiments. The reaction was run in 50 mM tris-HCl (pH 8.0) containing 50 mM of each sugar with or without 1 mM Mn^{2+} metal ions at 40°C with 5 µg of Pa-PGI

Table 2: Comparison of kinetic properties of Pa-PGI

Substrate	K_m	k_{cat}	k_{cat}/K_m
G6P	167.81 mM	13.23 sec^{-1}	0.08 sec^{-1} mM^{-1}
F6P	2.34 mM	2.36 sec^{-1}	1.09 sec^{-1} mM^{-1}

Kinetic properties of Pa-PGI on G6P and F6P, data are means of three individual experiments

Substrate specificity and catalytic efficacy: The Pa-PGI was evaluated with substrate specificity for both phosphorylated and non-phosphorylated sugars. The enzyme exhibited highest specific activity towards F6P followed by fructose, G6P and glucose. The catalytic efficacy also indicated that the kinetic efficiency ratio (k_{cat}/K_m) is higher for F6P as compared to G6P (Table 1 and 2).

The Pa-PGI followed Michaelis-Menten kinetics. The substrate specificity of Pa-PGI for F6P was significantly higher compared to G6P (Table 2). The data are in agreement with the previously reported values for *Listeria monocytogenes*[36]. Kinetic efficiency clearly indicates that the enzyme is not for glycolysis but it is for gluconeogenic and some other biological purpose. Berger *et al.*[6] have demonstrated that, in the glucose flux analysis, *P. aeruginosa* directs the flow towards ED pathway rather than glycolysis. Moreover, the glycolytic and unidirectional enzyme phosphofructo kinase which converts F6P to fructose 1,6-biphosphate is absent while class-I type fructose 1,6-biphosphatase in this bacteria which allows glucose to flux for gluconeogenesis. Based on its kinetic efficiency and of genome sequence analysis, Pa-PGI appears to be a gluconeogenic enzyme rather than glycolytic one.

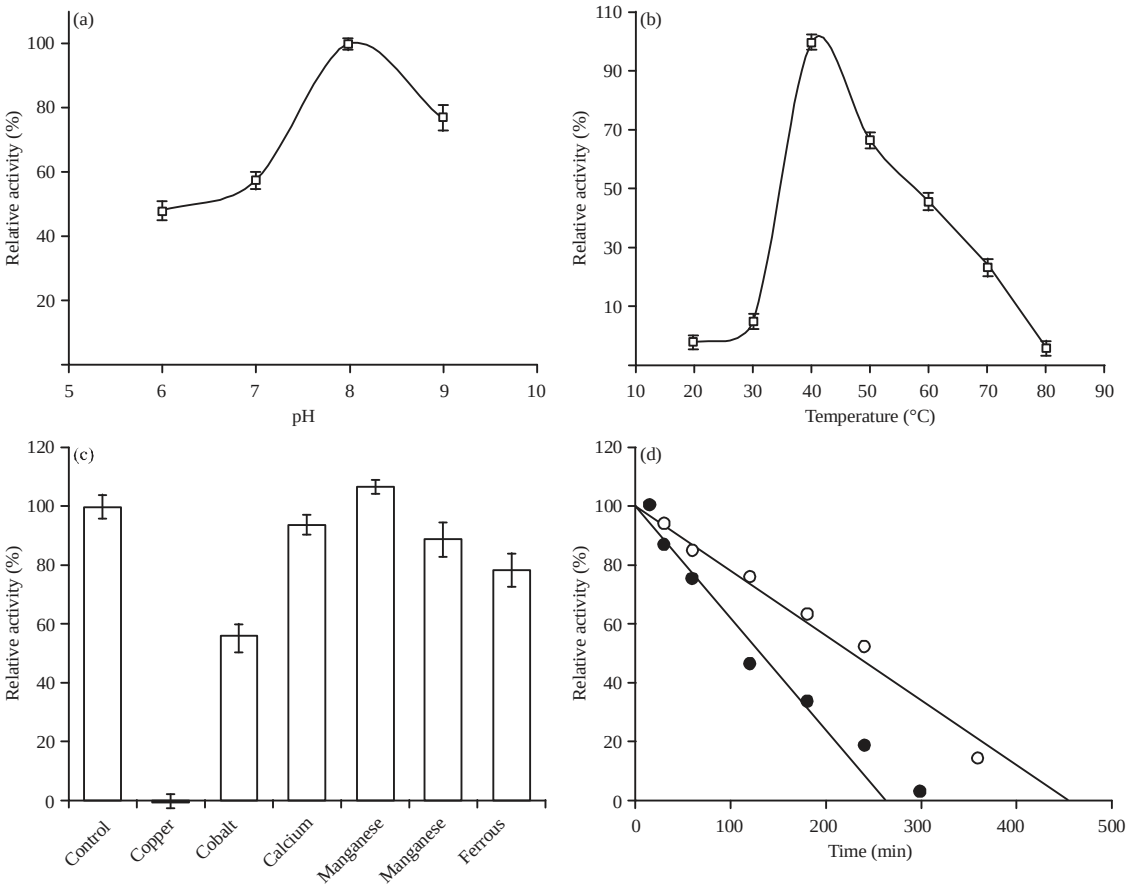

Fig. 4(a-d): Effect on the specific activity of Pa-PGI (a) pH optima. Enzyme activity was measured in the pH range of 6-9 in different buffers (pH 6.0-7.0 sodium phosphate buffer; pH 8.0-9.0, tris-HCl buffer), (b) Temperature optima. The reaction was carried out at different temperatures (20-80°C), (c) Effects of metal ions and (d) Effect of Mn^{2+} on thermal inactivation. The residual activity was determined by incubating Pa-PGI with (filled circles) or without 1 mM $MnCl_2$ (circles). The reaction was run in 50 mM tris-Cl (pH 8.0) containing 5 µg Pa-PGI and 2 mM D-glucose 6-phosphate at 40°C. Data are means of three experiments

CONCLUSION

In this study, it was observed that the enzyme PGI from *P. aeruginosa* is active under alkaline condition and mesophilic in nature. Like other bacterial phosphoglucose isomerases, it does not require metal ion for its activity. Catalytic efficiency establishes its gluconeogenic role rather than as a glycolytic enzyme. Further work to establish it moonlighting function will shed more information regarding its importance in pathogenicity of the organism.

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