

Updates on Glucose 6-Phosphate Dehydrogenase (G6PD): From Prokaryotes to Human

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Abstract: *Glucose 6-phosphate dehydrogenase (G6PD) is the rate determining enzyme that catalyzes the first step in pentose phosphate pathway (PPP) which produces NADPH that serves as reducing agent for reductive biosynthetic reactions such as steroids, fatty acids and nucleotides syntheses. Been ubiquitous, it has been found in wide range of organisms, ranging from prokaryotic organisms to even higher animals like human. Numerous G6PD isoenzymes have been isolated, purified and well characterised. However, obtained information such as its kinetic parameters and biological properties have not been judiciously appropriated for biotechnological/medical applications. This review thus concisely showcases G6PD, focusing on some sources where it has been isolated, purified and characterized, physicochemical/biochemical properties, the benefits and adverse effects its evolution may confer, and the possible biotechnological applications/prospects for the enzyme.*

Keywords: Anaemia, Haemoglobin, NADPH, Oxidative stress, Pentose Phosphate Pathway (PPP), Reactive Oxygen Species (ROS)

1. Introduction

Enzymes are biochemical substance that mediate biochemical reactions by binding to specific substrates, enabling them to either slow down or increase the rate of reactions without been used up [1]. Also recognised as biological catalysts, they are classified as been proteinous with the exception of ribozymes. Enzymes can be categorized into six different classes, which include oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase [1].

Glucose 6 phosphate dehydrogenase (G6PD; E.C. 1.1.1.49) belongs to the oxidoreductase classes of enzymes and are peculiar to Pentose Phosphate Pathway (also called the Hexose Monophosphate Shunt or the phosphogluconate pathway) [2, 3]. The Pentose Phosphate Pathway (PPP) is involved in the generation of reducing power, NADPH (reduced nicotinamide adenine dinucleotide phosphate), common to non-photosynthetic cells and organisms [2, 3]. This pathway is also involved in the synthesis of ribose 5-phosphate which is highly essential for diving cells to produce RNA, DNA and coenzymes such as ATP, NADH and coenzyme A [2, 3].

1.1 Pentose Phosphate Pathway

The Pentose Phosphate Pathway (Fig 1) provides an alternate route of carbohydrate catabolism to glycolysis [4]. The major difference between glycolysis and the Pentose Phosphate Pathway is that the latter produces only NADPH, whereas glycolysis generates NADH [5]. It is also found in all prokaryotes and eukaryotes, both heterotrophic and photosynthetic [6]. It is the pathway that supplies the

majority of reducing power in form of NADPH for reductive biosynthesis. It consists of two stages:

- The oxidative phase; and,
- The non-oxidative (regenerative) phase

The first step of the oxidative phase, which is the limiting step, is catalysed by G6PD [4]. G6PD catalyses dehydrogenation of β -D-glucose 6 phosphate (G6P) at carbon 1, leading to the formation of 6-phosphogluconolactone. This molecule is then hydrolysed by a lactonase to give 6-phosphogluconate. 6-phosphogluconate is oxidatively decarboxylated to form ribulose-5 phosphate. In the first two steps, NADP⁺ is the electron acceptor and as result, NADPH is produced. These NADPH molecules provide reducing power for the prevention of oxidative stress and for biosynthesis. Ribulose 5 phosphate is then isomerised to ribose 5 phosphate (R5P). This ribose 5 phosphate is used in nucleotide and nucleic acid synthesis. The oxidative phase which is well regulated and also irreversible produces two molecules of NADPH, one molecule of CO₂ and ribulose 5 phosphate [6, 7, 8].

In the non-oxidative phase, transaldolase and transketolase catalyse the inter-conversion of three, four, five, six and seven carbon sugars [8]. Carbon inputs may be recycled in the regenerative phase of the PPP and recycled back into the oxidative portion of the pathway, by the activity of hexose phosphate isomerase [9]. The sugars are reversely converted into glucose 6 phosphate which starts the cycle all over again, making this stage the regenerative phase [10].

The expression of the reactions in PPP can be illustrated using the equation:



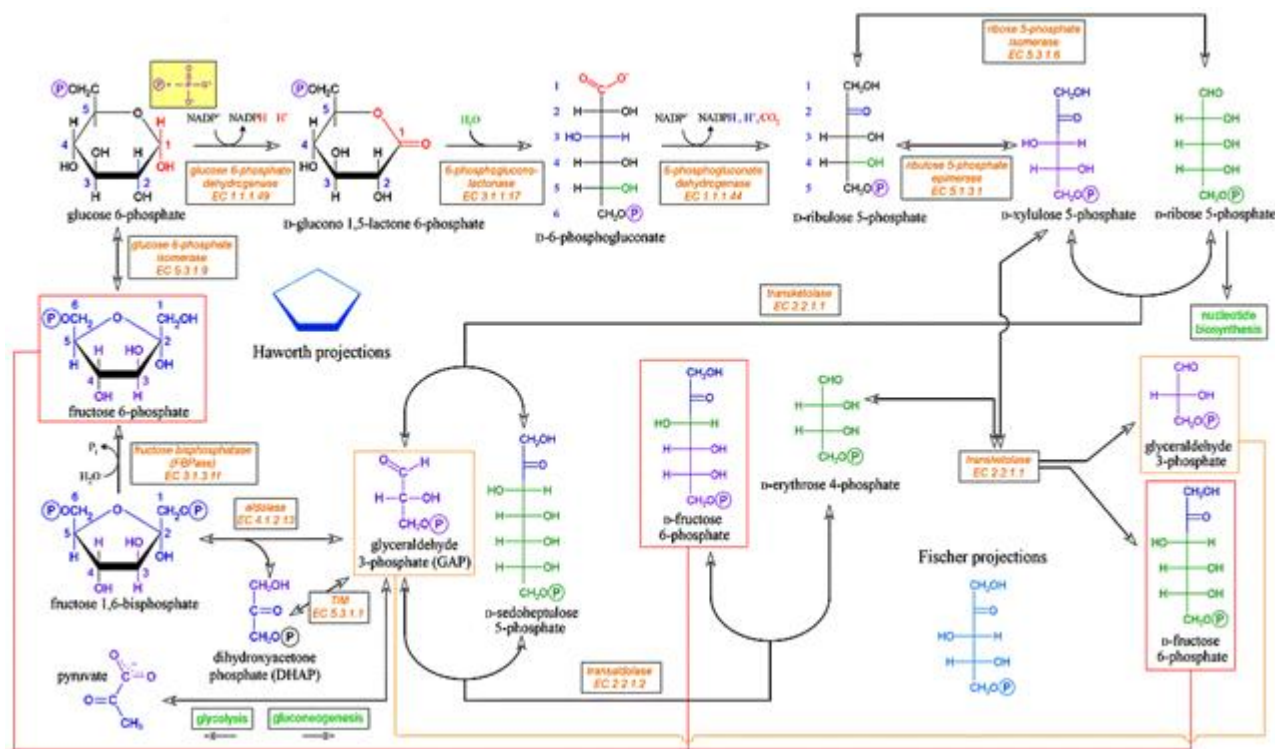


Figure 1: Pentose phosphate pathway. Adapted from Cronk [11]

1.2 Glucose 6-Phosphate Dehydrogenase (G6PD)

The entire antioxidant system is dependent on NADPH; as a result, the production of reduced glutathione is dependent on the production of NADPH which is also overly dependent on G6PD activity. G6PD activity is induced during oxidative stress and is the only source of protection against oxidative stress in red blood cells [12, 13].

Aside from G6PD, other enzymes like isocitrate dehydrogenase, glutamate dehydrogenase and malic enzyme have also showed ability to produce NADPH in the mitochondria; however G6PD is the chief producer of NADPH [13]. There are many other systems dependent on NADPH including enzymes involved in lipid production such as 3-hydroxy-3-methylglutaryl-CoA reductase and the NADPH-cytochrome P450 oxidoreductase that is required for drug metabolism, steroid biosynthesis, and many other functions [8].

G6PD is a cytosolic homeostatic enzyme that is encoded by a housekeeping X-linked gene, and the structural and functional features of G6PD promoter conform to those of a typical housekeeping gene [14]. The G6PD gene is present on the long arm of the X chromosome (Xq28) and consists of 13 exons with a length of 18 kb. Its active form is either a dimer or a tetramer of a single polypeptide subunit of about 59 kD [15].

Amongst the two molecule of NADPH produced during PPP, the first molecule reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) which is used to prevent

oxidative stress, while the second molecule provides reducing power for fatty acid metabolism, cholesterol and steroid hormones biosynthesis in the liver and other organs [12].

1.3 Historical Background & Distribution of Glucose 6-Phosphate Dehydrogenase

D-G6PD (*Zwischenferment*) was first identified in 1931 by Warburg and Christian [16] in horse erythrocytes. It was characterised in 1962 by Kirkman and Henderickson in human erythrocytes [4]. By 1979, G6PD was isolated and characterised in red blood cells and yeast cells [3]. Ever since then it has been identified in various organisms ranging from prokaryotes to humans, including bacteria, algae, fungi and ferns. It is present in all cells, but its concentration varies in tissues [11]. Even with its ubiquitous nature, it was discovered that G6PD is absent in some microorganisms. This could be attributed to the fact that some organisms are not exposed to oxidative stress [14].

In animal tissues, G6PD is found in the mitochondria and cytosol and in plant tissues, it is found in the cytosol and chloroplast [17]. In mammals, it shows the highest activity in mammary glands, adipose tissues and the liver [13]. The increased activity in these organs and tissues is probably due to the fatty acid biosynthesis. Activity of this enzyme has also been discovered in the kidneys, though not as much as the abovementioned organs [18]. Some of the organisms that have been discovered to produce G6PD are shown in Table 1.

Table 1: Showing Sources of Identified and Characterised G6PD

| ORGANISMS | EXAMPLES | REFERENCE(S) | ORGANISMS | EXAMPLES | REFERENCE(S) |
|-----------|-----------------------------------|--------------|---------------|---|--------------|
| BACTERIA | <i>Cyanobacteria anabaena</i> | [19] | HIGHER PLANTS | Plastidic stroma, cytosol and peroxisome of higher plants | [38] |
| | <i>Escherichia coli</i> | [20] | | Spinach | [39] |
| | <i>Leuconostoc mesenteroides</i> | [21, 22] | | Pea leaves | [40, 41] |
| | <i>Bacillus licheniformis</i> | [23] | | Tobacco | [42] |
| | <i>Acetobacter henseni</i> | [18] | | Wheat | [43] |
| | <i>Zymomonas mobilis</i> | [24] | | Castor oil seeds | [44] |
| | <i>Azotobacter vinelandii</i> | [25] | | Potato tubers | [45] |
| | <i>Corynebacterium glutamicum</i> | [26] | | Sycamore seeds and cauliflower buds | [46, 47] |
| ALGAE | <i>Chlamydomonas reinhardtii</i> | [28] | INSECTS | <i>Plasmodium falciparum</i> | [48] |
| | <i>Chlorella vulgaris</i> | [29] | | <i>Drosophila melanogaster</i> | [48] |
| FUNGI | <i>Aspergillus aculeatus</i> | [30] | MAMMALS | Rabbit luminal endoplasmic reticulum | [51] |
| | <i>Aspergillus niger</i> | [31] | | Mouse liver | [53] |
| | <i>Aspergillus nidulans</i> | [32] | | Rat liver microsome | [54] |
| | <i>Penicillium notatum</i> | [33] | | Dog liver | [55] |
| | <i>Penicillium dupontis</i> | [33] | | Pig liver and sheep lens | [13] |
| | <i>Aspergillus parasiticus</i> | [34] | | Buffalo erythrocytes | [56] |
| | <i>Neurospora crassa</i> | [35] | | Bovine erythrocyte | [57] |
| | <i>Candida tropicalis</i> | [36] | | Goat erythrocyte | [58] |
| | <i>Agaricus bisporus</i> | [37] | | Human erythrocyte | [9, 59 – 64] |
| FISH | Rainbow trout liver | [49] | BIRD | Chick brain | [52] |
| | Lake van fish erythrocytes | [50] | | | |
| | Rainbow trout erythrocytes | [17] | | | |

1.4 Purification and Characterisation of Glucose 6-Phosphate Dehydrogenase

With the importance of G6PD in various organisms, more studies and experiments beyond the works of Warburg and Christian [65] have been carried out to further explain its structure and function (See Table 2 and 3).

Yoshida [59, 66] revealed that G6PD could be purified from human erythrocyte, however, when purified from plant tissue, it was observed to be unstable [68]. This unstable nature of G6PD in general has been the major setback in its purification from various sources, which consequently hinders its successful elucidation and as a result many research work involve the use of crude extracts or partially purified samples. Table 2 shows some of the sources G6PD have been well purified, while Table 3 shows some biochemical properties of the enzyme.

2. Structural Characteristics of G6PD

The structure of G6PD can be described in three categories, which includes primary, secondary and tertiary structure.

2.1 Primary Structure of G6PD

It involves the arrangement of the amino acids linked by peptide bonds in a linear sequence [78]. Though G6PD is found in many organisms, the primary structures as well as their amino acids composition slightly varies between organisms due to the enzyme's heterogeneous nature as shown in Table 4 and 5.

64.2% of the amino acid residues of G6PD are highly conserved in most organisms with the homo-dimer of the enzyme having a unique structure due to each amino acid residue occurring twice in symmetrical positions [14]. As example, the complete amino acid sequence of human erythrocytes G6PD is made up of 531 amino acid residues [79, 80] containing a reactive lysyl residue that further explains its catalytic function with C-terminal and N-terminal sequence [81], even though it was previously assumed that the N-terminus of the human G6PD is blocked [82]. The N-terminal was identified to be pyroglutamic acid, while that from *Saccharomyces cerevisiae* was tyrosine [83, 84]. The N-terminals from the two subunits of *C. utilis* are glycine and alanine [3]. The C-terminals of the two *C. utilis* subunits are the same though; they consist of glycine [80]. Just like human G6PD, the G6PD of *L. mesenteroides* also contains a region which controls catalytic activity in the primary sequence. This suggests that the G6PD in both organisms are structurally related despite functional and structural differences [3, 81].

2.2 Secondary Structure of G6PD

The secondary structure of G6PD involves the arrangement of the α -helical and the β -pleated sheet structure expressed in various organisms. G6PD monomer is made up of two domains which are connected by α -helix [16, 95 – 97]. The larger domain has a β - α fold predominantly with nine antiparallel β -sheet strands, while the smaller domain has a typical β - α - β dinucleotide binding fold [80]. According to Rowland et al. [80], conservation in the β - α - β domain is minimal with only 17 of the 177 residues totally conserved. Recent studies have shown that *L. mesenteroides* G6PD has a higher level of the α -helical structure than the β -pleated

structure as determined at 2.0Å resolution as shown in Figure 2 [16, 80]. However, studies on the secondary structure of G6PD from *S. cerevisiae* showed a low level of α -helical structure [3].

Table 2: Organisms where G6PD have been successfully purified

| SOURCE | PURIFICATION PROCESS | REFERENCE |
|----------------------------------|---|-----------|
| <i>Neurospora crassa</i> | Ammonium sulphate precipitation, calcium phosphate gel adsorption, DEAE-Cellulose, hydroxyl apatite and bio-gel A column chromatography | [35] |
| Human erythrocyte | DEAE Cellulose fractionation, Ammonium sulphate fractionation, Affinity and anion exchange chromatography | [9] |
| Potato tuber (higher plants) | Ammonium sulphate fractionation and affinity chromatography | [68] |
| <i>Chlamydomonas reinhardtii</i> | SDS-PAGE, Ammonium sulphate fractionation, immunoblotting, Bradford method | [28] |
| Lamb kidney cortex | $2''$, $5''$ -ADP-Sepharose 4B affinity gel chromatography and DEAE Sepharose fast flow | [69] |
| Rainbow trout | Ammonium sulphate precipitation and $2''$, $5''$ -ADP-Sepharose 4B affinity gel chromatography | [17] |
| <i>Aspergillus aculeatus</i> | Ammonium sulphate fractionation, Reactive Blue 2 Agarose affinity and Sephacryl S-200 Gel filtration chromatography | [30] |

Table 3: Some biochemical properties of G6PD isolated from various organisms

| Organisms / Extraction Buffer | Examples | K _m for NADPH (μM) | K _m for G6P (μM) | V _{max} (U/mg) | Optimum pH | Optimum temperature (°C) | References |
|-------------------------------|----------------------------------|-------------------------------|-----------------------------|-------------------------|------------|--------------------------|------------|
| Bacteria/ Tris.HCl | <i>Thiobacillus ferrooxidans</i> | 24.3 | 53.9 | 20 | 7.8-7.9 | 50 | [70] |
| | <i>Acetobacter hansenii</i> | 340 | 89 | | [71] | | |
| | <i>Leuconostoc mesenteroides</i> | 5.69 | 81 | | [72] | | |
| | <i>Acetobacter hensenni</i> | 340 | 89 | | 7.0-8.5 | | [70] |
| | <i>Azotobacter vinelandii</i> | 50 | 30 | | [73] | | |
| | <i>Thermotoga maritima</i> | 50 | | | 8.5 | | 92 |
| Algae/ Tris.HCl | <i>Chlamydomonas reinhardtii</i> | | | | 7.0-9.5 | 25 | [28] |
| Fungi/ Tris.HCl | <i>Neurospora crassa</i> | 12 | 37 | | 7.4-8.2 | 55 | [35] |
| | <i>Aspergillus aculeatus</i> | 6 | 75 | | 7.5 | | [30] |
| Higher plants | Pea leaves | 500 | 2000 | | 8.0 | | [68] |
| | Potato tubers | 6 | 260 | | 7.5-9.0 | | [39] |
| | Spinach | | 400 | | 9.0-9.5 | | [75] |
| Mammals/ Glycine/NaOH | Dog erythrocyte | 18 | 36 | 28.23 | | 38 | [7] |
| | Lamb kidney cortex | 4.8 | 41 | | | | [69] |
| | Buffalo erythrocyte | | | | 8.0 | | [56] |
| | Rat small intestine | | | | 8.1 | | [75] |
| Birds/ Tris.HCl | Goose erythrocyte | 7.4 | 24.3 | | 7.0 | 50 | [13] |
| | Turkey erythrocyte | 17.1 | 49.7 | | | 50 | [77] |
| Fish/ Tris.HCl | Rainbow trout erythrocyte | 166 | 500 | 1.352 | 8.9 | 45 | [17] |

Table 4: Variations of amino acid residues among organisms

| Organisms | Amino acid residues | References |
|----------------------------------|---------------------|------------|
| <i>Leuconostoc mesenteroides</i> | 488 | [85] |
| <i>E. coli</i> | 490 | [86] |
| <i>Plasmodium falciparum</i> | 994 | [48] |
| <i>Saccharomyces cerevisiae</i> | 504 | [87] |
| Rattus sp. (rat) | 513 | [88] |
| Rodentia sp. (mouse) | 514 | [89] |
| Human Erythrocytes | 531 | [3, 80] |

Table 5: Amino acid composition of G6PD in various organisms

| ORGANISMS | AMINO ACIDS | | | | | | | | | | | | | | | | | | REFERENCES |
|-------------------------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------------|
| | Asx | Thr | Ser | Glx | Pro | Gly | Ala | Cys | Val | Met | Ile | Leu | Tyr | Phe | Lys | His | Arg | Trp | |
| <i>L. mesenteroides</i> | 144 | 48 | 44 | 104 | 30 | 70 | 116 | 0 | 60 | 18 | 52 | 72 | 44 | 64 | 74 | 12 | 36 | 16 | [3] |
| <i>B. subtilis</i> | 130 | 52 | 54 | 108 | 54 | 60 | 76 | 4 | 58 | 20 | 68 | 86 | 50 | 40 | 62 | 26 | 44 | 10 | [3] |
| <i>C. utilis</i> (As-75) | 134 | 44 | 67 | 106 | 49 | 83 | 67 | 14 | 57 | 15 | 47 | 81 | 32 | 42 | 89 | 12 | 35 | 10 | [90] |
| <i>C. utilis</i> (As-95) | 103 | 47 | 64 | 110 | 42 | 72 | 70 | 8 | 51 | 14 | 47 | 78 | 34 | 42 | 88 | 23 | 42 | 10 | [90] |
| <i>C. utilis</i> (Sigma) | 118 | 50 | 64 | 130 | 29 | 85 | 51 | 10 | 85 | 8 | 42 | 82 | 40 | 48 | 76 | 18 | 41 | 9 | [91] |
| <i>S. cerevisiae</i> | 96 | 30 | 44 | 80 | 35 | 50 | 59 | 4 | 50 | 12 | 36 | 60 | 28 | 39 | 54 | 14 | 36 | 8 | [92] |
| <i>N. crassa</i> | 101 | 43 | 59 | 100 | 45 | 60 | 52 | 32 | 51 | 34 | 56 | 76 | 39 | 40 | 71 | 21 | 57 | 14 | [35] |
| Human erythrocyte (variant B) | 99 | 41 | 50 | 114 | 63 | 72 | 58 | 18 | 55 | 23 | 41 | 84 | 35 | 46 | 51 | 24 | 47 | 13 | [3] |
| Bovine adrenal cortex | 111 | 59 | 74 | 134 | 48 | 96 | 75 | 16 | 85 | 24 | 59 | 83 | 45 | 49 | 55 | 29 | 65 | 19 | [93] |
| Mouse liver | 122 | 62 | 60 | 138 | 52 | 96 | 80 | 12 | 84 | 28 | 60 | 104 | 42 | 56 | 78 | 24 | 68 | ND | [94] |

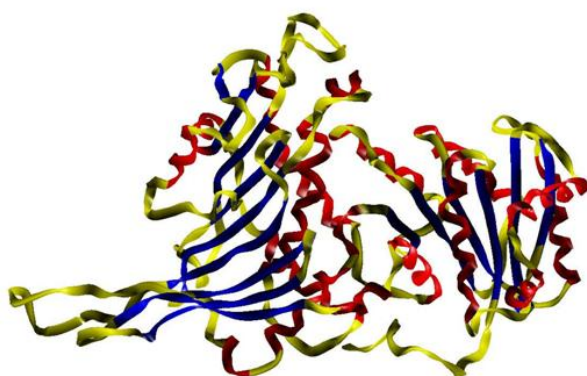


Figure 2: Three dimensional secondary structure of *L. mesenteroides* G6PD showing α -helix (in red) and β -sheet (in blue). Adapted from Rowland [80]

Kotaka et al. [98] elucidated the secondary structure of the human G6PD dimer. It was found to contain β - α binding domain with a high degree of α -helical structure (Figure 3). Similarly, mouse liver G6PD also showed a high degree of α -helical structure [94]. However isozymes of *C. utilis* As-75 and As-95 G6PDs were discovered to have low α -helical structure [99]. Thus according to Kotaka et al. [98], the human G6PD is similar to the *L. mesenteroides* G6PD; a dimer having a $\beta + \alpha$ domain interface.

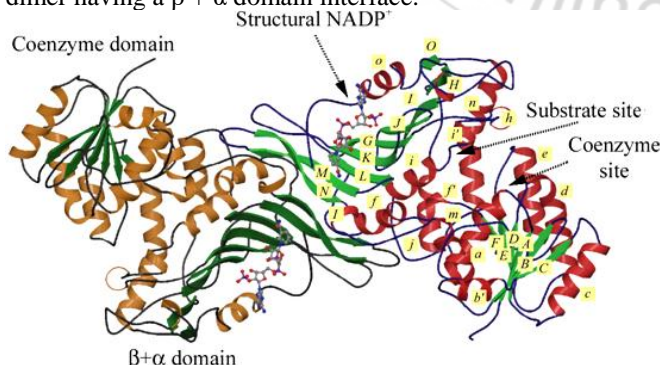


Figure 3: Human G6PD dimer enzyme, showing α -helix (in red) and β -sheet (in green). Adapted from Kotaka et al. [98]

2.3 Tertiary Structure of G6PD

Most isolated G6PD has a single dimeric structure as the minimum structural requirement for catalytic activity, although some occurs also as monomer, trimer, tetramer or as

hexamer [3, 35]. Some of the various forms of G6PD as revealed in several organisms are illustrated in Table 6.

Table 6: Various forms Catalytically Active G6PD

| Organisms | Nature | References |
|---|--|-------------------------|
| Human erythrocyte; <i>A. aculeatus</i> ; <i>L. mesenteroides</i> | Dimer | [30, 80, 100] |
| <i>A. nidulans</i> and <i>A. niger</i> | Trimer | [101] |
| <i>N. crassa</i> | Occurs mostly as tetramer, may also occur as dimer | [35] |
| <i>Zymomonas mobilis</i> , <i>Pseudomonas multivorans</i> , <i>S. cerevisiae</i> , <i>C. utilis</i> , <i>A. parasiticus</i> , Chloroplasts, Cytosol of pea leaves and potato tubers, Plant plastids, Beef and Bovine adrenal cortex | Tetramer | [7, 39, 56, 68, 75, 76] |

2.4 Structure of the Active Site

With the catalytic form been a dimer, G6PD portrays different configuration of their active site between human and microbes [80]. As observed in *L. mesenteroides*, the dimer interface is close to the active site, located at the edge and more stable than monomeric form when in aqueous solutions [21]. It also reveals one NADP^+ molecule is present in every subunit of tetramer, away from the active site but close to dimer interface [102].

On the other hand, G6PD in humans contains two NADP^+ binding site in each subunit. The first is the catalytic site and the second is the structural site. However, NADP^+ which is required to aid folding and monomer hybridization is bound at the structural site and not involved in the catalysis of the enzyme [16, 103, 104]. The auxiliary cofactor helps to maintain the stability and integrity of the enzyme when it is in its active form [105]. Generally, the structural site of NADP^+ is found and conserved in higher organisms [98].

2.5 Coenzyme Binding Site

The coenzyme associated with G6PD in most organisms is NADPH, although some organisms like *L. mesenteroides* could exhibit either NADH or NADPH [106]. In addition,

the coenzyme binding domain in *L. mesenteroides* contains 177 residues which consist of dinucleotides folds that can be aligned with the folds in other dehydrogenases present in the PPP [80]. Two major differences in the dinucleotide binding fold in dimeric G6PD which are likely to affect the niceties of coenzyme binding are found in the β A- α loop and at the initial of α loop [71]. The first difference is the insertion of Gly-13 in the fingerprint and the second is the main chain conformation when proline (Pro-24) is present at the start of the helix α loop [102]. Proline tends to distort the helix which causes the residue's 19-21 side chains to stretch towards the cleft at the boundary of the coenzyme bounding site making it possible for the coenzyme to bind by creating opportunity for conserved arginine to interact with the adenine ring and 2'phosphate of NADP [80]. On the other hand, a monomeric G6PD contains a coenzyme-binding domain at the amino terminus and a β + α domain at the carboxyl terminus, a second domain usually exist which indicates a new fold [102].

Sequenced G6PD has also shown that Gly-12, Gly-15, Arg-16 and Leu-17 are conserved at the coenzyme binding site. Further work also shows that glycine might be also conserved at region 219, although its use has been deemed irrelevant [80].

2.6 Substrate Binding Site

The substrate most associated with G6PD is glucose 6-phosphate (G6P). G6P is dehydrogenated at C-1 to give 6-phosphogluconolactone (Figure 1). In *L. mesenteroides*, the substrate binding site has been assumed to be in the pocket which is located at the domain boundary with nine highly conserved peptide [25, 80, 102].

G6P tend to bind in the position where its phosphate can make contact with each monomer, close to the conserved residues [78], as a result five residues which form ligands with the phosphate exist and are fully conserved in all species [80]. Lys-182 (in *L. mesenteroides*), which interacts with the second phosphate in subunit A, is similar to the lysine in the human G6PD and important for catalytic activity. The side chain of Lys-148 is less tightly bound in subunit A than in subunit B as a result of the different conformations of the two proline residues (A149 and B149) in the two subunits. This leads to a tighter binding of phosphate in subunit B since only one phosphate binds in subunit B; although limited knowledge is provided on this [104, 105]. A theoretical reason might be that the specificity of the site of phosphate is greater if Pro-149 exist in a Trans in subunit B (Figure 3).

Also at the entrance of the active site cleft, a band of basic residues (Lys-19, Arg-20, Lys-21 and Lys-32) draw protons from the hydroxyl group of G6P in the absence of a metal ion and help the substrate bind in correct orientation [80].

2.7 Substrate Specificity

Naturally, G6P is the substrate for all G6PD enzymes, having the highest V_{max} in all G6PD enzymes [3, 107]. However, recently phosphorylated monosaccharides like galactose 6-phosphate and 2-deoxyglucose 6-phosphate were identified

to be oxidizable by G6PD [16]. Research for further clarifications, revealed that substrates without OH on C-2 were oxidised by G6PD, an example is 2-deoxyglucose-6-phosphate [107]. Even substrates with alteration such as replacement of OH with $-NH_2$, $-F$ or $-Cl$ at C-2 were found to be tolerated by some forms of G6PD [16]. However, substrates that possessed bulky groups such as $-OCH_3$ at C-2 were not oxidized. Some substrate like galactose 6-phosphate that showed alteration at C-4 were weakly oxidized by G6PD, it thus implies that changes at C-4 is not as determining factor as changes in C-2 [3, 16].

Non-phosphorylated sugars, like isoesteric phosphonate, sulphate and glucose have also shown little compatibility with G6PDs [73], though some changes in substrate structure can be tolerated, activity and binding of G6PD are reduced, showing that G6PD has a high level of substrate specificity for G6P [30]. Apart from the substrate specificity, G6PD from various sources have displayed non-consistent affinity for coenzymes (Table 7), making it possible to classify them based on nucleotide specificity.

Table 7: Classes of G6PD based on nucleotide specificity

| G6PD from these organisms react only with NADP ⁺ | EXAMPLES | REFERENCES |
|---|------------------------------|------------|
| | <i>E. coli</i> | [20] |
| | Brewers' yeast | [65] |
| | <i>N. crassa</i> | [35] |
| | Spinach leaves | [39] |
| | <i>Aspergillus aculeatus</i> | [30] |
| | Goose erythrocytes | [13] |
| G6PD from these organisms react with NADP ⁺ , but also show a low activity with NAD ⁺ | <i>A. vinelandii</i> | [108] |
| | <i>A. suboxydas</i> | [108] |
| G6PD from these organisms react with both NADP ⁺ and NAD ⁺ | <i>L. mesenteroides</i> | [71, 109] |
| | <i>Pseudomonas W6</i> | [110] |

2.8 Regulatory Properties of G6PD

G6PD is regulated at transcriptional, translational, posttranslational levels and also at intracellular levels by various factors such as nutrition, hormone and oxidative stress [16].

2.8.1 Nutritional Factors

Studies on liver cells showed that excess dietary carbohydrate induced G6PD whereas starvation repressed it. This was identified as normal since an enzyme is only activated when its specific substrate is readily available [3]. Apart from carbohydrate availability, G6PD activity was also found to be regulated by the type of carbohydrate present in the diet, as work done on rats fed with diets high in glucose and starch had increased levels of G6PD activity [99], whereas high fat and low carbohydrate diets inhibit G6PD activity [111]. More nutritional regulatory is polyunsaturated fatty acids (PUFAs) which inhibit G6PD gene expression through a posttranscriptional mechanism whereby the splicing efficiency of pre-mRNA is decreased [112]. Insulin activation of phosphatidyl inositol 3-kinase pathway also tend to increases G6PD expression, while essential polyunsaturated fatty acids like arachidonic acid stops the accumulation of G6PD by inhibiting insulin signalling [16].

2.8.2 Hormonal Factors

Insulin and dexamethasone have been revealed as an important hormone in upregulating G6PD expression at transcription level [16]. Glucocorticoids have also been identified to activate G6PD in rat liver cells [113]. Epidermal growth factor similarly has an effect on G6PD by activating G6PD mRNA [114]. Norepinephrine particularly induces G6PD mRNA activity in the heart [116] and Vitamin D in the epithelium of the gut [116].

2.8.3 Oxidative Stress

Substances that produce oxygen radicals tend to activate G6PD by the use of SoxR regulon, thereby upregulating the enzyme and other genes required to protect cells from free radical damage [12]. An example was the exposure of type II pneumocytes to hyperoxia which resulted to a high level of G6PD activity in mammalian neonatal rats' lungs [16]. G6PD in the liver could also be induced by chemicals like diquat and ethanol [117].

In higher plants, G6PD is regulated by transcriptional and translational processes. In plants like alfalfa, the root nodules containing fungi have been observed to induce G6PD transcription and activity [118]. Light also tend to induces plastidic G6PD expression in light deprived potato shoots since plastidic G6PD can be activated by a low level of NADPH to NADP⁺ ratio [119].

2.9 Inhibition of G6PD

NADPH is an effective inhibitor of NAD-linked G6PD and a competitive inhibitor of NADP-linked G6PD [18]. Studies involving *L. mesenteroides* showed that when G6P was the substrate of varied concentration, inhibition was linearly non-competitive but when NADP⁺ was the substrate of varied concentration, linear competitive inhibition was obtained [80]. Random inhibitors of G6PD include phosphoenolpyruvate, ATP, fructose 6 phosphate and Co²⁺ [30], although some inhibitors are peculiar to some organisms (Table 8).

Table 8: Possible G6PD inhibitors in some organisms

| ORGANISMS | G6PD INHIBITOR | REFERENCES |
|--|---|--------------|
| <i>Pseudomonas</i> W6 | NADH | [120] |
| <i>Methylobionans</i> M15 and <i>S. aureofaciens</i> | NADPH and NADH | [121] |
| Human placenta | NADPH | [122] |
| <i>Anabaena</i> spp | NADPH | [19] |
| Yeast, bacteria, fungi and plants | NADH and NADPH | [30, 55, 70] |
| Brewers' yeast | D-glucosamine 6 phosphate, Pregnenolone and dehydroisoandrosterone at concentrations $\leq 10^{-6}$ | [123] |
| Red blood cells | Cyanate, some antibiotics, Aluminium, nickel and cadmium | [56] |

2.10 Multiple Variants of G6PD

Isozymes or variants of G6PD usually occur as a result of genetic mutations and results to wide ranging levels of enzyme activity (Table 3, 4 and 5) and associated clinical symptoms. According to Notaro et al. [14], 122 mutations of human G6PD have been identified and 114 of these mutations are as a result of missense mutations. Of these 114 mutations, 106 have single mutations, 7 have two mutations and 1 has three mutations. Six mutations contain in-frame deletions (four single amino acid deletions, one double amino acid deletions and one eight-amino acid; deletions). 1 of the variant is due to nonsense mutations and another due to a splicing site mutation. Of all human variants of G6PD, three have no effect on enzyme activity, while the others cause some form of the enzyme deficiency [14]. 57 mutations are associated with nonspherocytic haemolytic anaemia (WHO class I severe phenotype) and 58 mutations have a high risk of acute haemolytic anaemia (WHO classes II and III, mild phenotype) [14].

Notaro et al. [14], emphasised that most of the human mutations responsible for G6PD mutations are genetic and 'null' (includes early nonsense mutations; mutations in the coenzyme or substrate binding regions; mutations destroying

the reading frame; and yet to be identified mutations). In human G6PD, mutations occur throughout the amino acid sequence and are completely conserved [61]. Partially conserved amino acids are usually not represented properly in G6PD-deficient mutants unlike fully and moderately conserved amino acids which are over-represented in these variants. Usually, 83% of all microorganisms' G6PD don't exhibit a replacement of amino acid sequence peculiar to human isozymes and therefore do not alter enzymatic activity. In some cases, the residues that are altered are found normal in other organisms. Comparing the distribution of mutations in a human gene is very useful in elucidating the function of domains and even individual amino acids in a protein [14, 16]. To do this various range of potential, clinical consequences of the several mutations of G6PD are considered, some of them include:

- Mutations such as null mutation which is deleterious and can't support life [61]. An experiment performed by [14] on mice, proved that G6PD null mutations didn't support the survival past the embryonic stage of development.
- Mutations which causes G6PD protein molecules to lack stability. This tends to make G6PD deficient in erythrocytes, affecting the normal activity of red blood cells.

- Mutations that affect coenzyme and substrate binding. This affects the catalytic mechanism and hence the cell that contains them tends to cluster. However, these clusters are yet to be identified. The only cluster of mutations occur between amino acid residue 380 and 410 and this is similar to the subunit interface in the G6PD dimer which is enzymatically active [87, 96, 124].

The unique pattern of mutation of G6PD therefore reveals that more than two thirds of the amino acid alteration that cause G6PD deficiency are present in highly and moderately conserved amino acids with a few in poorly conserved amino acids [96]. This shows that the more the number of conserved amino acids that are mutated the more the effect; therefore mutation of fully conserved amino acid will tend to be deleterious while mutation of moderately conserved amino acids may cause instability but not lethal, whereas mutation of poorly conserved amino acids may go undetected.

G6PD is the only housekeeping enzyme in which most of the identified mutations are both pathogenic and polymorphic [60]. Some G6PD variant include, G6PD_{canton}, peculiar to South China, which was observed to be as an alteration in nucleotide position 1376 of the cDNA (from G to T) which results in a genomic mutation in amino acid position 459, from arginine to leucine [95]. Other G6PD variants cause nonspherocytic anaemia, which is as a result of deletion or alteration around exon 10 necessary for substrate binding. It has been shown in some situations that G6PD mutation may cause the individual to be less susceptible to malaria [125].

3. Biological Importance of G6PD

The major role of the pentose pathway is for the production of NADPH, which provides reducing power to aid various reductive biosynthetic processes and serve as electron donor for many enzymatic reactions. In mammals, NADPH from the PPP is used in tissues like the liver for fatty acid synthesis and cholesterol metabolism, in the testes for steroid synthesis, adipose tissues for fatty acid synthesis, ovaries for steroid synthesis, mammary glands for fatty acid synthesis (during lactation and development of embryo) adrenal gland for steroid synthesis and red blood cells for the maintenance of reduced glutathione which is used to fight against oxidative stress [25, 99]. G6PD is also useful for cell survival as it is involved in the production of ribose-5-phosphate which is a precursor for nucleic acid synthesis [10].

As G6PD variant exist amongst organisms, they possess various role in their cellular environment as described in Table 9.

Generally, the antioxidant system depends on NADPH production to function well. For instance, the generation of GSH by glutathione reductase is dependent on NADPH production. Catalase also needs NADPH for its maintenance in its active conformation in the allosteric binding site [12]. Although, superoxide dismutase doesn't use NADPH to convert superoxide to hydrogen peroxide, but superoxide needs to be reduced by catalase or glutathione which are dependent on NADPH. The nitric oxide synthase (NOS) needs NADPH to be able to convert arginine to citrulline and nitric oxide [12].

Also, NADPH oxidases are dependent on NADPH from G6PD which are involved in cell growth and white blood cell function. They are also involved in pathological situations like diabetes and cardiovascular diseases, which are a major source of reactive oxygen species [130]. Many other systems dependent on NADPH include lipid production such as 3-hydroxy-3-methylbutyryl-CoA reductase and NADPH cytochrome P450 oxidoreductases which is required for drug metabolism and steroid metabolism [131].

3.1 The Deficiency of G6PD

G6PD deficiency is the most common enzyme deficiency; affecting more than 400 million people worldwide (Figure 4) with more cases in malaria prone zones [8]. G6PD deficiency was first identified in 1956, with most cases in Africa, Middle East, Mediterranean regions and Indochina; with few cases in some part of northern Europe, North and South America [8, 132].

G6PD deficiency was initially identified in black Americans as primaquine's sensitivity to anaemia peculiar to the Caribbeans and the Mediterranean region when patients were treated with 6-methoxy-8-aminoquinoline drugs against malaria and haemolytic anaemia was diagnosed [132]. To determine why some antimalarials caused acute haemolysis, an experiment using ⁵¹Cr technique was carried out using volunteer prisoners at Illinois State Penitentiary in Chicago. It revealed that that the sensitivity to primaquine was because of dome internal defect of the red blood cells attributed to G6PD deficiency, eventually causing haemolytic anaemia [8, 133].

Table 9: Specific role of G6PD in some organisms

| ORGANISMS | RELEVANCE OF G6PD | REFERENCE |
|--|--|-----------|
| Fungi like <i>N. crassa</i> and <i>A. flavus</i> | Provide NADPH by PPP for fatty acid biosynthesis for growth and development. | [35] |
| Bacteria | Reduce oxidative stress and assist in the production of ribonucleotides used as precursors for DNA synthesis. Some bacteria like <i>L. mesenteroides</i> use the activity G6PD to create products of fermentation | [109] |
| Higher Plants | Provides NADPH for steroid biosynthesis in the absence of light, as the photosystem I&II are readily unavailable when ultraviolet light is limited. | [126] |
| Mammary glands | Provides NADPH for milk fat biosynthesis by PPP which is suspended after weaning | [127] |
| Mammalian embryo | Helps to supply the embryo with maternal blood glucose | [128] |
| Fish | G6PD activity is necessary during the period of blood system development, hatching and completion of resorption. | [129] |
| Human | Apart from several functions, G6PD is highly important in erythrocyte since they have no alternative source of NADPH as they lack mitochondria. The NADPH supplied help to form reduced GSH in order to curb free radicals, as red blood cells tend to liberate several free radicals due to their conversion of oxyhaemoglobin to deoxyhaemoglobin and other cellular activities. Therefore, NADPH prevents haemoglobin denaturation, preserves the integrity of erythrocyte cell membrane sulphydryl groups and detoxifies oxygen radicals and hydrogen peroxide in and on the erythrocytes. If G6PD is limited the haemoglobin sulphydryl groups will no longer be maintained in its reduced form, making molecules of haemoglobin to cross link with one another forming aggregates called Heinz bodies on the cell membranes which accumulate and cause lysis | [17] |

G6PD deficiency is an X-linked hereditary defect prevalent in males that occurs as a result of G6PD gene mutations. This leads to variant forms of the gene with various biochemical and clinical manifestations with a 10% reduction in general

enzymatic activity [125], G6PD deficiency creates the formation of different proteins with varying levels of enzyme activity [8], with clinical manifestations such as neonatal



Figure 4: Map showing world distribution of G6PD deficiency. Adapted from Cappellini and Fiorelli [8].

jaundice (NNJ), acute haemolytic anaemia, chronic haemolysis which leads to chronic nonspherocytic haemolytic anaemia (CNSHA) [104]. These clinical manifestations are usually triggered by external factors such as drugs and fava beans [133].

3.2 Classes of G6PD Deficiency

The physical manifestations of G6PD are heterogenous with more than 140 gene mutations identified [8]. In 1962, WHO characterised G6PD deficiency into five classes based on enzyme activity, electrophoretic mobility of the enzyme, kinetic parameters (K_m for NADP and G6P, substrate analogues and dependence of pH), physicochemical properties and clinical manifestations [8]. G6PD could also be classified as polymorphic or sporadic [14]. These classes include:

- Class I: Highly deficient with chronic non-spherocytic haemolytic anaemia;
- Class II: Highly deficient (1-10% residual activity). It is associated with acute haemolytic anaemia;
- Class III: Moderately deficient (10-60% residual activity). Haemolysis is self-limiting;
- Class IV: Normal activity (60-150%); and,
- Class V: Increased activity (>150%).

3.3 Clinical Manifestations of G6PD Deficiency

Although, most G6PD deficient individuals don't exhibit any symptoms of this defect nor a reduction in life span, the most common manifestation is haemolysis which occurs usually when erythrocytes undergo oxidative stress induced by drugs, fava beans or infection [133].

Some of the clinical manifestations of G6PD include neonatal jaundice, favism, drug-induced haemolytic anaemia, infection-induced haemolytic anaemia and chronic non-spherocytic haemolytic anaemia [104]. Sometimes, heart

attack and diabetes could also induce haemolysis in individuals who are G6PD deficient [14]. Associated with the clinical manifestations are tiredness, anaemia, back ache and jaundice. Other markers include lactate dehydrogenase and unconjugated bilirubin [104].

3.3.1 Infection - Induced Haemolytic Anaemia

Infections such as hepatitis A and C, typhoid fever and pneumonia could lead to anaemia in G6PD deficient individuals [8]. Severe haemolysis and kidney failure may affect viral patients who are G6PD deficient, leading to death. Studies have shown that drug metabolism might be distorted by hepatitis which could result in increased number of metabolites known to cause haemolysis [14]. Generally, hepatitis increases the total concentration of bilirubin and haemolysis [8].

3.3.2 Favism

Favism was first identified in the 20th century, although the relationship between favism and G6PD deficiency was not

established until much later. It was first discovered in the Mediterranean region [134]. It is specific to G6PD deficient individuals and involves an onset of haemolytic anaemia due to the ingestion of beans (like broad beans) or exposure to the bean pollens [8]. This could be attributed to the presence of divicine, isouracil, pamaquine and convicine in the beans that stimulates PPP activity and hence haemolysis [134]. Favism presents as haemolytic anaemia 24 hours after the beans were ingested. This is usually more serious than drug-induced or infection-induced haemolytic anaemia and it could lead to kidney failure [134].

3.3.3 Drug - Induced Haemolytic Anaemia

Certain drugs such as primaquine and sulphonamides induce haemolysis in G6PD deficient individuals (Table 10). This is due to the fact that metabolism of drugs involves the production of reactive oxygen species, and with limited NADPH available to tackle the oxidants, haemolysis will thereby occur [112]. Some common symptoms of such cases may show brown to black urine (haemoglobinuria), jaundice, fever and anaemia. Although, the patient begins to recover over time since G6PD deficiency is a self-limiting process [8].

Table 10: Some drugs associated with G6PD deficiency.
Adapted from Cappellini and Fiorelli [8]

| Drugs | Biological Action | Drugs | Biological Action |
|---------------------------------------|-------------------|-----------------|-------------------|
| Primaquine | Antimalarials | Aspirin | Analgesics |
| Pamaquine | | Phenazopyridine | |
| Sulphonamides/ Furazolidone | Antimicrobials | Doxorubicin | Chemotherapy |
| Quinolones/ Dapsone/ Niridazole | | Flutamide | |
| | | | |

3.3.4 Neonatal Jaundice

Most male neonates, diagnosed with neonatal jaundice are usually G6PD deficient [8, 133]. Neonatal jaundice occurs later than normal physiological jaundice of ABO or Rh incompatibility. Kernicterus can occur and this could lead to severe neurological damage [133].

3.3.5 Congenital Non - Spherocytic Haemolytic Anaemia (CNSHA)

Chronic haemolysis caused by G6PD deficiency leads to CNSHA. The variants of G6PD that cause CNSHA are non-sporadic [8]. CNSHA is classified into two:

- Type I: When the raised level of haemolysis can be corrected by ATP; and,
- Type II: When the raised level of haemolysis cannot be corrected by ATP.

Most G6PD deficient people with CNSHA usually fall into the type I category. CNSHA usually occurs during childhood with elevated bilirubin and lactate dehydrogenase with occurrence of extra vascular haemolysis [133].

3.3.6 Malaria *Plasmodium Falciparum* & G6PD Deficiency

Though G6PD deficiency has so many deleterious, ranging from anaemia to jaundice and even to death, it has an

advantage under some certain conditions [133]. In G6PD deficient people, G6PD deficiency is used as a mechanism in the defence against *Plasmodium falciparum* since the plasmodium requires NADPH to produce reduced glutathione and ribonucleotides (for growth and development) in the host [95, 104].

3.4 Prospects of G6PD in Biotechnology Applications

The promising nature of biotechnology in the last two decades has lead to a great interest in possible ways to make it more effective in product development and production [135], thereby out phasing other cultural means of production [136]. Biotechnology stresses on the use of nature products with genetic modification in order to increase yield and quality of products [137].

One of the major biomaterials used in production are enzymes which tend to transform substrates to desired products [138]. However, some enzymes like G6PD show little implementation in biotechnology unless for laboratory studies [138, 140].

Although G6PD has been well researched in terms of the elucidation of its structure and properties; thus its biochemistry is very well understood. However, the prospects of this enzyme are yet to be well enact as literatures involving its applications are limited. Though it is know that G6PD is necessary for the synthesis of reductive potential (NADPH) and it is necessary for steroid and nucleotide biosynthesis and a host of other important functions such as its role in reducing oxidative stress; its applications industrially and in other areas are still being deciphered. Hence this section therefore provides proposed prospective uses and/or development of G6PD in biotechnology applications.

3.4.1 In Fermentation

Fermentation involves the use of microbes acting on solid or liquid substrate in anaerobic condition [140]. They are highly employed in production with the most popular been alcohol brewing and the commonest microorganism been *Saccharomyces cerevisiae* [141].

S. cerevisiae like any other organism thrive and multiply, thereby making use of the sugars in the fermenters releasing carbon dioxide and alcohol as by-products [140]. However, on accumulation, alcohol becomes lethal, giving the reason why most fermentation generally end with alcohol concentration of 10% - 15%, although some strains of *Saccharomyces cerevisiae* could survive up to 21% of alcohol [141]. Alcohol tends to eliminate the microbe by scavenging structural and functional molecules, causing oxidative stress [142, 143]. Oxidative stress usually prevented by various molecules like glutathione, catalase, peroxidase and superoxide dismutase is dependent on the activity of G6PD (previously discussed section 3.0). Lojudice et al. [138] showed how G6PD could be over expressed in *Saccharomyces cerevisiae* by genetic modification. Therefore, the increase in the expression of G6PD in *Saccharomyces cerevisiae* during fermentation may tend to increase the quantity of alcohol production by increasing the survival rate of the organism.

3.4.2 In Bioremediation

The task of wastewater treatment still remains an issue in the safekeeping of the environment. Several methods have been employed in managing wastewater effluents in the society, however, with the most effective means been microbial remediation; several setbacks are however still encountered [144]. Among several impediments, a vital issue is the brief life span of microbes employed in the remediation process [144]. Major lethal pollutants that tamper the efficiency of these microbes are reactive oxygen species (ROS) that destroys the cellular structure of these microorganisms [145]. Although, ROS are essential in tertiary remediation with abilities to decolourize and eradicate pathogenic microbes, they tend to impede the initial activities of microbes necessary for wastewater treatment [146].

Studies performed by Lojudice et al. [138] proved that G6PD which plays a role in combating ROS can be over expressed in microbes. Hence, genetic amplification of G6PD in remediating microbes may have a tendency to increase their shelf life and therefore prolong their activity.

3.4.3 In Enzyme Immobilization for Biofuel Production

The use of immobilized enzymes has proven to be more effective and advantageous than free enzymes as these enzymes could be recycled and has greater thermal and operational stability.

Among several demands of fuel, the request of hydrogen gas is becoming highly recognised, and this has led to the development of several means of hydrogen gas production to match up with the demand [147]. Amongst several ways is the implementation of biotechnology in the production of hydrogen gas through fermentation process [140].

Research shown by Woodward et al. [148], proved that hydrogen gas been the main product from G6PD purified from *Pyrococcus furiosus* had a yield of 11.6 mol H₂ per mol glucose-6-phosphate. Also, studies done by Simons et al. [149] revealed that G6PD could be efficiently immobilized via genetically engineered cysteine, producing higher enzyme activity than the regular immobilized G6PD. Therefore, implementation of the improved technique of immobilized G6PD from *Pyrococcus furiosus* may tend to increase the yield and lower cost of constantly isolating G6PD.

3.4.4 Cellulose Production

Industrially, glucose 6 phosphate dehydrogenase is important in the production of bacterial cellulose in organisms like *Acetobacter spp* [150]. This bacterial cellulose is a gelatinous membrane that is produced at the surface of liquid culture by this organism. It could be eaten as "Nata de Coco" or used as a thickener, disperser, stabilizer, emulsifier, in paper, artificial skin, medical supplies and for cosmetics [150]. Thus, the higher activity of this enzyme in *Acetabacter spp*, the higher the yield of the bacteria cellulose. This therefore implies that increasing G6PD activity in this organism could boost its economic value.

3.4.5 In Plant Defence Strategies

The inhibition of G6PD in tobacco leaves has proved to be advantageous as it improves plant defence by preventing

oxidative burst and formation of hypersensitive lesions induced by *Phytophthora nicotianae* infection, and was also shown to improve tolerance to drought [151]. Thus transgenic technologies may be applied to plants susceptible to infection by *Phytophthora nicotianae* and plants in the arid regions, making them more resistant and likely to survive harsh weather conditions like drought. Inhibition of G6PD activity could therefore be exploited as a means of biological control as insecticides, pesticides and herbicides.

3.4.6 In Disease Diagnosis

Due to the fact that G6PD is important for cell growth, G6PD activity could be used in the detection of diseases. Studies suggest that decreased G6PD activity may lead to the development of diabetes on the basis of correlations of increased frequency of diabetes mellitus in G6PD deficient subjects compared to individuals in the same population who have normal G6PD activity [16, 111]. Decreased activity of G6PD was shown to adversely affect pancreatic cells, as these cells already have low level of antioxidant production. Thus the level of G6PD may be used as a marker in the diagnosis of some ailments.

Furthermore, the over expression G6PD was found to be required for prostate cancer cell proliferation [152] as its expression was increased upon androgen treatment in hormone sensitive prostate cancer cell lines. Thus it was suggested that G6PD activity could be used as a clinical marker for prostate cancer exams as its activity were found to be four times higher in carcinomas than in benign prostatic hyperplasia [152].

4. Conclusion

Although the biochemistry of the G6PD that catalyzed the most essential step in the pentose phosphate pathway, producing the reducing power in form of NADPH and which is essential for numerous biosynthetic pathways from the least microorganism to the highest in the plant and animal phyla is well understood; its huge potentials is still not been fully utilized for biotechnological applications. Thus we anticipate this review will be a gateway for more critical reasons and innovations into how G6PD enzyme could be judiciously used in solving or overcoming biotechnological applications challenges.

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Authors Profile



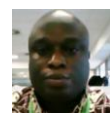
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