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# Oxy-haemoglobin protein engineering: An automated design for hotspots stability, site-specific mutations and smart libraries by using HotSpot Wizard 2.0 software

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***Abstract:*** Oxy-haemoglobin is an important metallo-protein, which helps in oxygen binding and transporting to the tissues. The objective of the present study was to detect hot spots and design of smart libraries for engineering protein stability, substrate specificity, tunnels and cavities as well as suitable mutability position of oxy-haemoglobin protein by using a software, HotSpot Wizard, version 2.0., is a free online software. The prediction results were obtained in output interface for functional hot spots, stability hot spots (structural flexibility), correlated hot spots and stability hot spots (sequence consensus). In conclusion, pocket identification and mutability prediction of oxy-haemoglobin can lead to detect structural alternation mainly in disease diagnosis and space for ligand binding pocket in new drug development for disease therapy. This computational prediction is suggesting to compare with experimental hotspots for oxy-haemoglobin in relation to therapeutic efficacies and druggability assessment.

***Keywords:*** oxyhaemoglobin; metallo-protein; protein engineering; HotSpot Wizard; computational biology; druggability

## I. INTRODUCTION

The oxy-haemoglobin protein is an important metallo-protein, which play a vital role in transport of oxygen [1-3]. It is well known that each subunit of haemoglobin (Hb) contains globular protein along with heme group. The protein tetramer comprises of two α- and two β-chains assembled to form symmetrical (αβ) dimers. In the centre of each heme group is a $Fe^{2+}$. De and Girigoswami [4] and Furuyama et al. [5] have described that the haemoglobin exists in functionally important two isomeric forms the R form (oxy/ligand bound state), which helps in proper coordination between upload of oxygen. Lack of this protein lead to anaemia in human.

Generally, protein-protein interaction, which indicated residues of $\Delta\Delta G \geq$ 2kcal/mol, is termed as hot spot [6]. In other words, certain residues in protein-protein interactions, termed as hot spots. These residues have unique and variety of energetic properties, can be designed an important target of protein-protein complex [7]. Several experiments resulted that only a small subset of contact residues showed significance binding free energy. These residues have been termed 'hot spots' and if mutated then they can disrupt the interaction [8]. Most conserved amino acids are found in hot spot residues. As per experimental study, Hb variants determined through physical examination and/or routine laboratory testing, which found in the patients of diabetes, anaemia, cyanosis, etc. [6; 9-10]. Globin gene mutation causes the structural globin proteins or Hb variants, and these are associated with deletions, multiple amino acid substitutions, anti-termination mutations, and altered post-translational processing. It is well known naturally occurring Hb mutations cause biochemical abnormalities, some of which employ clinically significant symptoms [6].

Bloom et al. [11] have emphasized that stability of proteins is of great concern, those who are working on protein engineering research for the implementation of enzymes in industrial sector. Now-a-days the study of protein stability has led to develop in future utilization of biomolecules in different sectors viz. biocatalyst, disease diagnosis and therapy, nanoscience etc. [12]. In general, stability means protein gets unfold and refold during unfavourable environmental conditions as temperature or solvent, etc.

It is interesting to note that all proteins are simplest form and suitable example of evolvable biological systems as per their potent biochemical functions in which alterations can be noted due to few mutations [13]. Wagner [14] has revealed that evolvability is robustness to mutations, and proteins are often quite mutationally robust. It was found in experimental study that several proteins are retaining their native functions due to more than half number of single mutant [11; 15-17].

Since decades, the function and properties determination have developed through automated simulation by several researchers to know molecular mechanisms of any protein but still unclear the sequences of protein encode the exact function [18-19]. Generally, enzyme is known as biocatalyst, which has specific substrate binding ability as lock and key strategy for maintaining biochemical reactions in an organism. In recent trend of research, several computational tools for protein engineering have been developed by researchers mainly detection for tunnel and cavity, smart libraries, mutation positions, functions etc. [12; 20-27].

In the present study an attempt was done for oxy-haemoglobin protein to detect of hot spots and design of smart libraries for engineered protein stability, substrate specificity, tunnels and cavities as well as suitable mutability position through computational prediction by using HotSpot Wizards, version 2.0 and the protein was used oxyhaemoglobin because this is an important protein for blood related disease identification and also prevention.

## II. MATERIALS AND METHODS

The oxy-haemoglobin, the crystal structure of protein, .pdb files as PDB ID: 1hho were selected and incorporated separately in the input interface of HotSpot Wizard (version

2.0) online software. In this automated prediction study, chains were not specified manually.

HotSpot Wizard 2.0 is free online software for automatic detection of hot spots and design of smart libraries for engineered proteins' stability, cavity and tunnels, catalytic activity, substrate specificity and enantioselectivity [20; 27-28]. On the other hand, this present tool can be utilized for the annotation of protein structures. This tool is modified version of previous software launched in 2009 [20]. This present online server comprises sequence, structural and evolutionary information obtained from 3 databases and 20 computational tools. According to Bendl et al. [27] and Sebestova et al. [29], this online tool integrates annotated residues, which can be known easily for mutagenesis and designed for suitable codons for each implemented strategy. Ultimately, this software helps in comprehensive annotations of protein structures and engineering with the stable design of site-specific mutations and targeted libraries.

In the present study, this software was calculated automatically hotspots for function, stability, correlated and consensus sequences for oxy-haemoglobin (Fig. 1). Bendl et al. [27] have developed the workflow steps in HotSpot Wizard, the calculation is based on the particular protein annotations, mutagenesis hot spots and smart library design as first, second and final phases respectively.

For statistical analysis, Z scoring values were obtained for each computational tools such as DCA (Direct Coupling analysis), ELSC (Explicit Likelihood of Subset Variation), McBASC (McLachlan Based Substitution correlation), MI (Mutual Information), aMIc (All Microarray Clustering), OMES (Observed Minus Expected Squared) and SCA (Statistical Coupling Analysis).

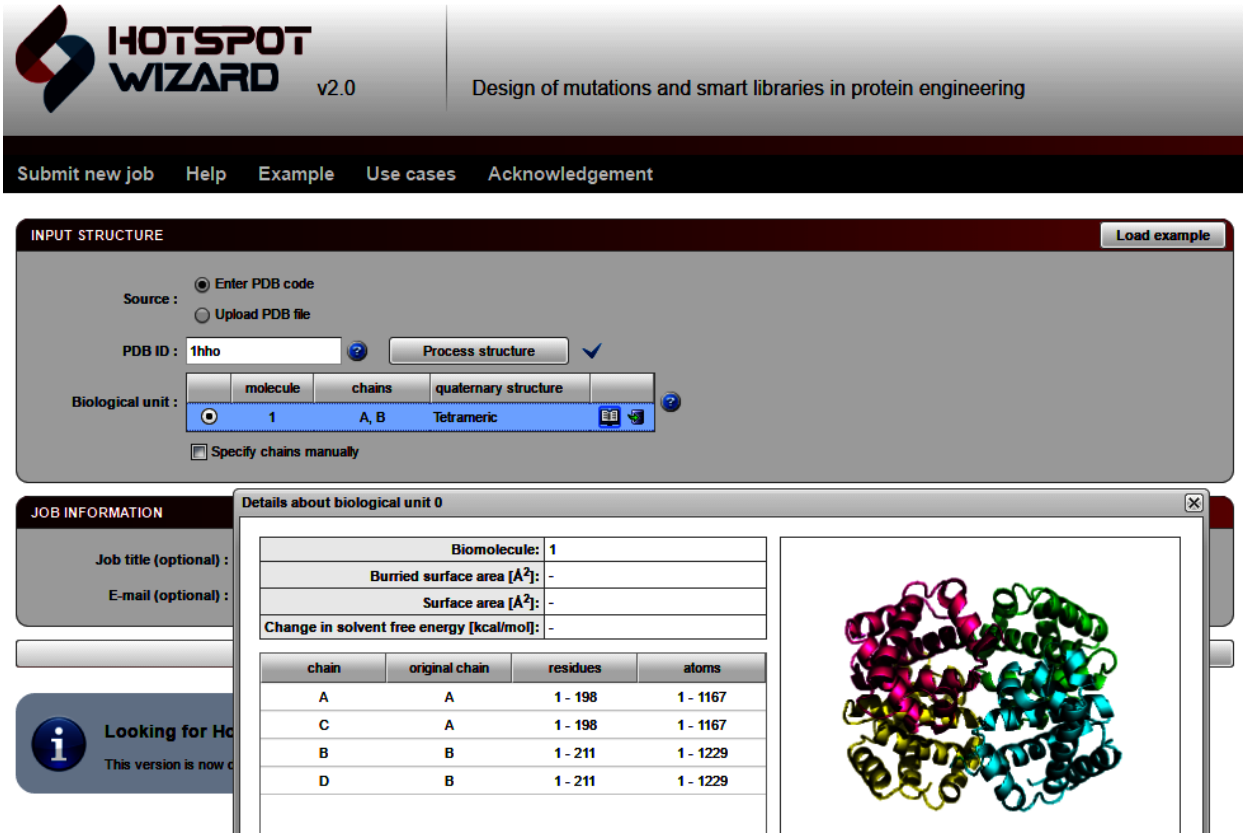



Figure 1. Hotspot wizard input interface for oxy-haemoglobin (1hho)

## III. RESULTS AND DISCUSSION

In the present results, the oxy-haemoglobin engineering strategies through automated computational prediction were observed. Fig. 2. showed results as output interface through Hotspots wizard for four separate prediction data such as functional hot spots, stability hot spots (structural flexibility), correlated hot spots and stability hot spots (sequence consensus).

In functional hot spots, the data were obtained for activity, substrate specificity and selectivity and also this step identified residues, which were forming catalytic pocket or accessible tunnel that were not directly participated in the catalysis or located at the evolutionary-conserved position.

For stability hot spots (structural flexibility), the prediction was done to identify the residues in flexible structure, which is observed mainly residues with highest B-factors.

In case of the study of correlated hot spots, the data were obtained same as functional hot spots along with the identification of correlated position through consensus approach resulted data from other computational tools viz. DCA (Direct Coupling analysis), ELSC (Explicit Likelihood of Subset Variation), McBASC (McLachlan Based Substitution correlation), MI (Mutual Information), aMIc

(All Microarray Clustering), OMES (Observed Minus Expected Squared) and SCA (Statistical Coupling Analysis). For stability hot spots (sequence consensus), consensus design is an important strategy for the stabilization of proteins. It helps amino acid conservation in sets of homologous protein to identify likely beneficial as well as deleterious mutations of the target protein.

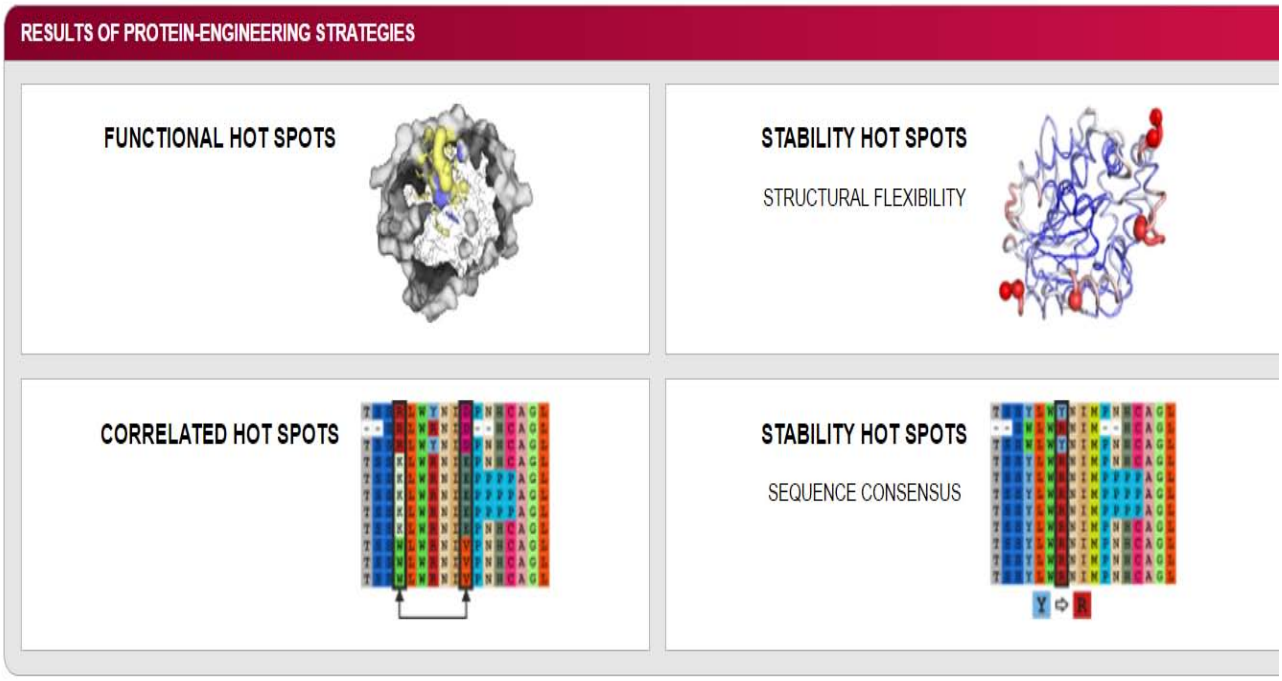
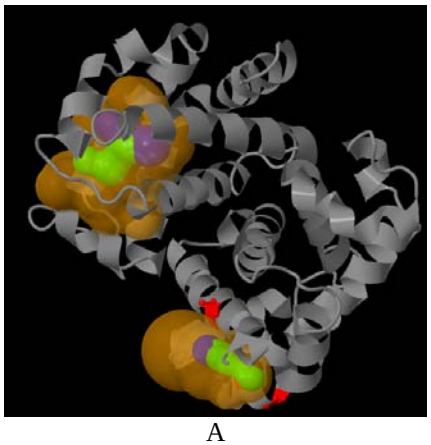


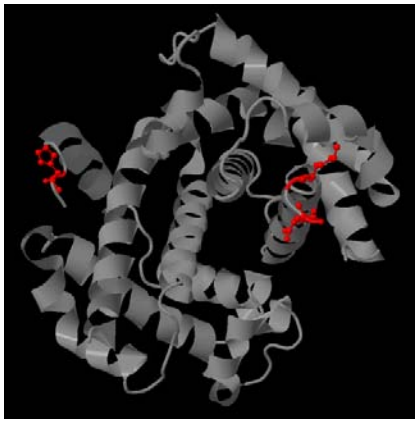

Figure 2. Protein engineering strategies of oxy-haemoglobin

Fig. 3 (A, B and D), revealed that oxy-haemoglobin showed different hotspots through HotSpot Wizard tool. In general, hot spot determines the energy distribution along with the interface region without homogenous in nature, where certain residues do not contribute majorly for free energy binding [30-35]. The hot spot prediction detects the exact protein binding sites, which helps for designing specific therapeutic agents in protein interactions [34]. In Fig. 3 (C), sequence consensus was obtained for oxyhaemoglobin. In this observation, wild-type and mutated consensus sequences were obtained based on hot spots (Richter et al., 2007). Bendl et al. [27] supported the concept of molecular mechanisms of any protein, still unclear to researchers, how the sequences of protein encode the exact function? Still have not yet answered [18-19]. It was documented that experimental evolution work suffered major problems when occurred by several irregular study of mutagenesis and detecting of large sequence libraries to evaluate the mutational landscape and proteins showed important structural and functional properties [19; 27; 37-39].

Table I, describes the functional hotspot of oxyhaemoglobin where only chain B attached to residues like Ala at 135 position, Gly at 83 position and Leu at 81 position while correlated residues like Gly at 136 position, Leu, Ser, Gly, met, Gly, Asn at 3, 9, 29, 55, 136, 139 positions respectively. The pockets and tunnels were obtained in 14 and 1, 2 (from pocket 16), 0 and & 2 (from pocket 16) and 16 (catalytic) & 1, 2 (from pocket 16) in which B-factor values 22.67, 49.39 and 46.80 $Å^2$ respectively. The B-factor values mainly influenced by crystal contacts and solvent conditions, various theoretical methods have used to predict flexible regions, which help to determine the targets for stabilization [40-41]. The mutability rate was observed high and score values were 9, 8 and 6 respectively. According to Weinkam and Salia [42], haemoglobin is a protein of complex system, which undergoes conformational changes in response to oxygen, allosteric effectors, mutations, and environmental changes. It was observed in previous study that haemoglobin has evolved with complex allosteric mechanism, which showed point mutations at different sites [43]. Weinkam and Salia [42], predicted and suggested naturally occurring mutations can be tolerated due to structural symmetry of several types of haemoglobins.



A



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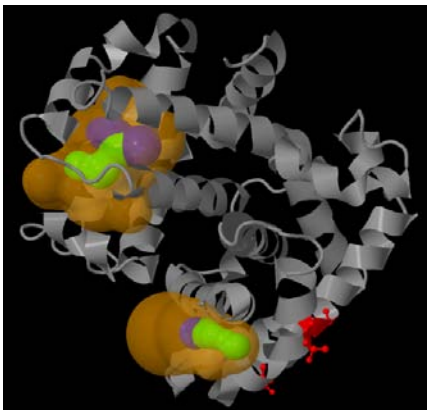

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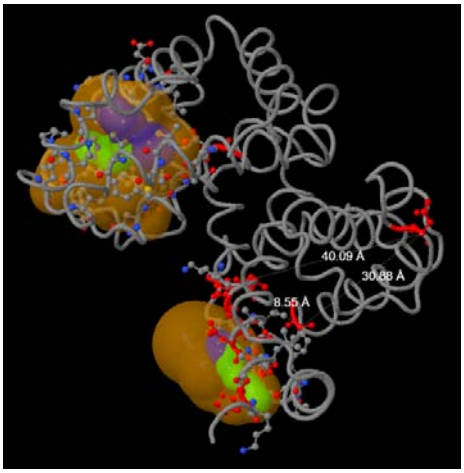



D.

Figure 3. Oxy-haemoglobin protein (1hho): A = functional hotspot; B = stability hotspot; C = sequence consensus and D = correlated hotspot

Table I. Study of functional hotspots

| ***Studied Protein*** | ***Chains*** | ***Residues & position*** | ***Secondary structures*** | ***Pockets & tunnels*** | ***Average B-factor (in Å$^2$)*** | ***Mutability rate & score*** | ***Correlated residues & position*** |
|---|---|---|---|---|---|---|---|
| 1hho | B | Ala & 135 | Alpha helix (H) | 14 & 1, 2 (from pocket 16) | 22.67 | High & 9 | Gly & 136 |
| | B | Gly & 83 | Alpha helix (H) | - & 2 (from pocket 16) | 49.39 | High & 8 | --- |
| | B | Leu & 81 | Alpha helix (H) | 16 (catalytic) & 1, 2 (from pocket 16) | 46.80 | High & 6 | Leu, Ser, Gly, Met, Gly, Asn & 3, 9, 29, 55, 136, 139 |

Table II. Values obtained from different tools for functional hot spots

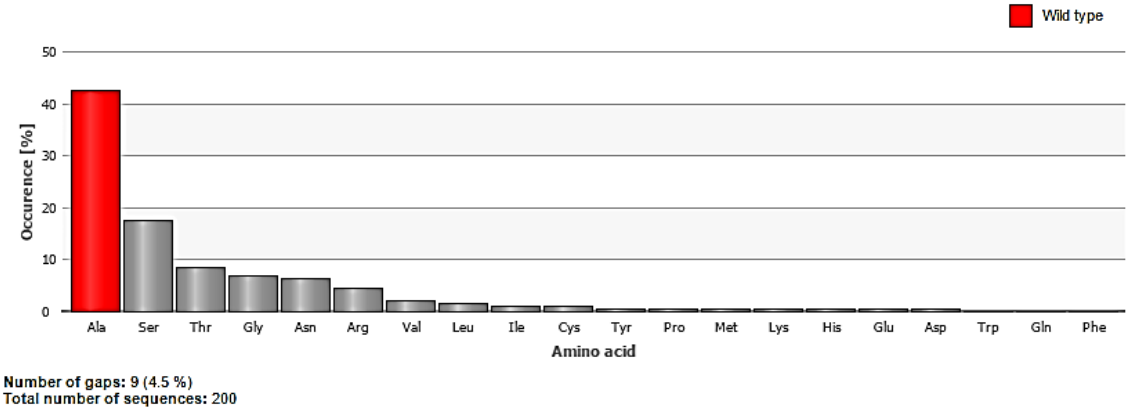
| ***Chains*** | ***Consensus z-scoring values*** | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***aMIc*** | ***DCA*** | ***ELSC*** | ***McBASC*** | ***MI*** | ***OMES*** | ***SCA*** |
| B | 0.42 | 4.37 | 4.12 | 1.01 | 5.26 | 4.05 | 7.57 |
| B | - | - | - | - | - | - | - |
| B | 2.93 | 1.70 | 4.69 | 1.02 | 5.77 | 9.24 | 7.84 |
| | 2.55 | 1.90 | 4.51 | 0.88 | 6.14 | 7.45 | 6.34 |
| | 2.28 | 1.10 | 7.94 | 1.47 | 3.40 | 7.89 | 4.32 |
| | 2.21 | 0.96 | 9.12 | 1.43 | 4.42 | 6.11 | 5.98 |
| | 3.43 | 1.58 | 11.26 | 1.57 | 5.29 | 9.58 | 4.74 |
| | 1.98 | 1.42 | 9.76 | 0.42 | 4.82 | 7.81 | 3.18 |

In Table II, consensus z-scoring value was obtained for different parameters such as aMIc 0.42 Gly, 2.93 Leu, 2.55 Ser, 2.28 Gly, 2.21 Met, 3.43 Gly, 1.98 Asn; DCA 4.37 Gly, 1.70 Leu, 1.90 Ser, 1.10 Gly, 0.96 Met, 1.58 Gly, 1.42 Asn; ELSC 4.12 Gly, 4.69 Leu, 4.51 Ser, 7.94 Gly, 9.12 Met, 11.26 Gly, 9.76 Asn; McBASC 1.01 Gly, 1.02 Leu, 0.88 Ser, 1.47 Gly, 1.43 Met, 1.57 Gly, 0.42 Asn; MI 5.26 Gly, 5.77 Leu, 6.14 Ser, 3.40 Gly, 4.42 Met, 5.29 Gly, 4.82 Asn;

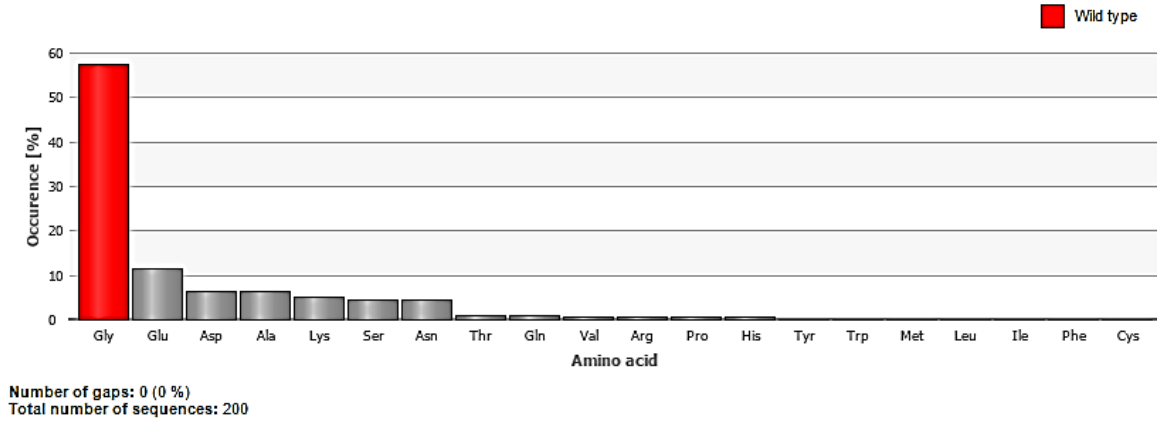
OMES 4.05 Gly, 9.24 Leu, 7.45 Ser, 7.89 Gly, 6.11 Met, 9.58 Gly, 7.81 Asn and SCA 7.57 Gly, 7.84 Leu, 6.34 Ser, 4.32 Gly, 5.98 Met, 4.74 Gly, 3.18 Asn were obtained through this tool for oxy-haemoglobin as per correlated residues.

In Fig. 4, it was obtained that the amino acid residues fulfilling the criterion of minimal frequency in the multiple sequence alignment. The wild type variety was observed Ala (42%), Gly (58%) and Leu (52%) as per positions of different amino acids frequencies of oxy-haemoglobin (Fig. 4A, B and C). Fig. 5 states that mutational landscape, which mainly showed the estimation of the probability in relation to preservation of protein function for individual substitution at a particular site of oxy-haemoglobin. It was obtained that higher deleterious mutation in Fig 5 C, followed by Fig 5 B and Fig 5A. In the present computational study, the results were obtained for β subunits, which indicated a strong linking with the quaternary transitions than the α subunits for human oxy-haemoglobin, which is supported by previous molecular dynamic simulation of unliganded haemoglobin for quaternary and tertiary T to R transitions (Hub et al., 2010). It has been documented that molecular docking study for both T-state haemoglobin and oxy-haemoglobin with different ligands of natural origin showed allosteric effects [45-46].

It was reported that mutability scale is ranged between 1 to 9 i.e. lower to higher rate. In the present study high mutability rate was observed 9, 8 and 6 respectively (Table I). In Fig. 5A, amino acids viz. Trp, Tyr (47%), Glu, Pro, Arg (45%) and Asp (43%), Fig. 5B, Cys, Phe, Thr, Trp (43%), Pre (39%), Ile, Tyr (31%), Val (27%) and Fig. 5C Phe (47%), Met, Val (43%), Ala, Cys, Tyr (27%), Gln, Trp (25%), Ser, Thr (23%), Gly, His, Asn, Pro (18%), Lys (12%), Asp, Glu, Arg (10%) were observed deleterious mutation of amino acids. It was reported when diseases occur then amino acid of haemoglobin undergoes mutation. According to Thom et al. [6], there are several haemoglobin variants that occurred amino acid substitutions and diseases viz. Ala>Pro, Tyr>Phe and Val>Phe (haemolytic anaemia and reticulocytosis); Val>Ala, His>Arg and Ala>Asp (haemolytic anaemia); Arg>Ser (anisocytosis and hypochromia), His>Tyr (anaemia), Phe>Ser (microcytosis); Pro>Ser (haemolytic anaemia and microcytosis); Phe>Leu (Heinz body haemolytic anaemia), Leu>Arg (Heinz body haemolytic anaemia and dominant inclusion body thalassemia), etc. found due to gene mutation in globin protein that lead to structural abnormalities of globin protein by single amino acid substitution while Pro>Arg, Lys>Glu and Lys>Asn have been detected as normal (without disease). The present prediction of oxy-haemoglobin with an evidence of high mutability score in oxyhaemoglobin (PDB ID: 1hho), which may be a clinical symptom in future research. According to Weinkama and Salia [42], haemoglobin is not a simple system and easily allows conformational changes in relation to oxygen, allosteric effectors, mutations, and environmental changes.




A.

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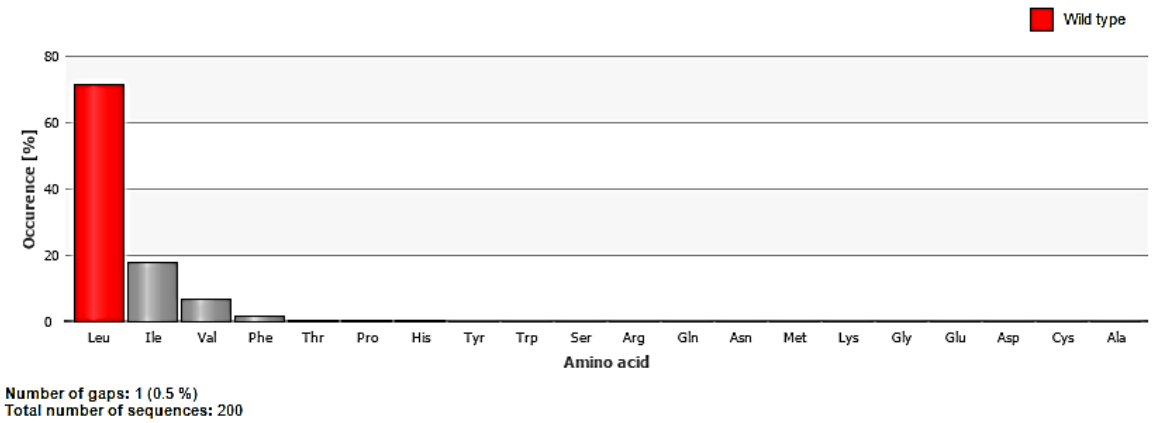
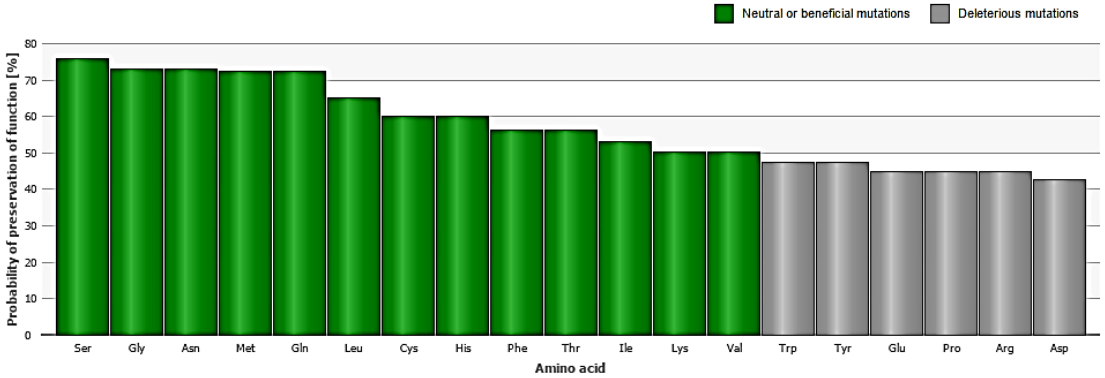



C.

Figure 4. Amino acids frequencies as per positions

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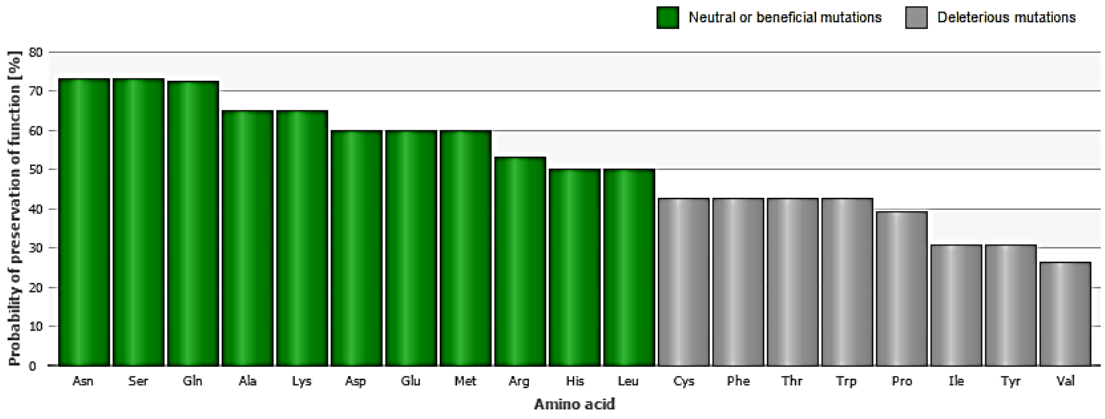


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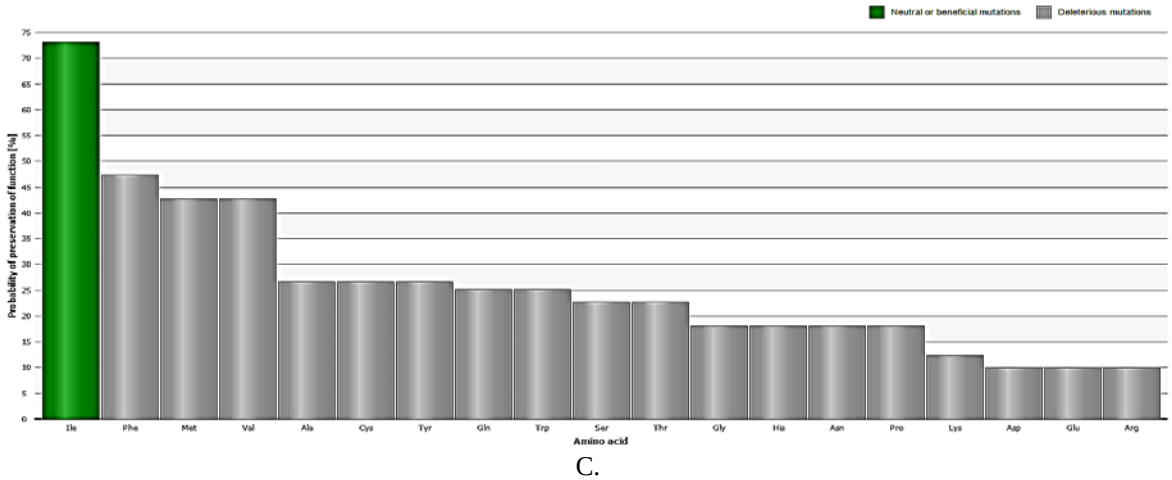


C.

Figure 5. Amino acids mutability landscape

However, the prediction of different hotspots can be facilitated drug designing and development. It was suggested that the starting point of a binding site of a receptor in the hotspots may be granted to analyse docking of ligands [47]. On the other hand, rigid docking lead to an achievement the comparatively least flexible hotspots, which lead to an upgradation in protein docking has been performed by creating dominant conformation of the hotspot side chains resulted through molecular dynamics probing rather than the unbound X-ray conformation [7; 48-49]. Thus, the prediction of hotspots is a suitable tool to identify exact functional mechanisms of particular protein to identify mutant residue(s) in relation to cause of disease and new drug development.

## IV. CONCLUSIONS

In conclusion, HotSpot Wizard (version 2.0) is an online computational tool, which helped easily to obtain results for oxy-haemoglobin through protein engineering protocol by the integration of several inbuilt databases derived from other bioinformatics tools and all the data generated within short duration to prevent laborious jobs of experiment [27]. This software also helped to incorporate only .pdb file as an input of studied protein without prior knowledge of computational biology to set up input interface. The parameters like pocket identification and mutability prediction of oxyhaemoglobin can lead to know structural alternation of particular in disease diagnosis as well as space for ligand binding pocket in new drug discoveries [42-43]. The present prediction work is suggesting to compare with experimental hotspots for oxy-haemoglobin in relation to therapeutic efficacies and druggability assessment.

## V. ACKNOWLEDGEMENT

Authors are thankful to the developers of online software HotSpot Wizards (Version 2.0), European Protein Databank for crystal structure (1hho), used in the present study.

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest for the present study.

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