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In-Silico Side-Directed Mutagenesis of Oxidoreductase from *Anoxybacillus sp.* SK3-4

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Abstract: Precisely, mutagenesis can introduce mutations into the target gene by using mismatch primers which are partially complementary to the template strand of DNA using polymerase chain reaction (PCR). Oxidoreductase enzymes are generally proteins that involved in oxidation-reduction reactions in biological systems. For this study, primary sequence analyses of oxidoreductase protein from *Anoxybacillus sp.* SK3-4 was conducted with the aim of generating functional information and theoretically improve catalytic stability of the protein by *in-silico* mutagenesis. The primary sequence of a novel protein with 386 amino acid residues was analyzed using Expasy-tool for translation of the amino acid sequence into a nucleotide gene sequence. Important catalytic binding sites of the protein were predicted using 3DLigandSite program, Pheres2 and Protein Bioedit servers for generating functional information of the protein. Site-directed mutagenesis (SDM) was used against the novel protein (oxidoreductase), in which two site mutations were created based on rational design. Amino acids; leucine (L) and histidine (H), involved in substrate and metal binding sites in the protein were substituted for isoleucine (I) and arginine (R) i.e. L138I and H280R, to check for significant change in the functional stability of the protein, thereby increasing the efficiency of the enzyme to help speed up the rate of chemical reactions.

Keywords: *Anoxybacillus sp.* SK3-4, Oxidoreductase, Catalytic Stability, Site-Directed Mutagenesis

1. Introduction

Mutagenesis of proteins is of great importance for understanding the relation between the protein structure and function. The functional and structural roles of amino acid residues in a desired protein can be studied by comparing the mutant protein carrying changes in amino acid residues to the wild-type protein. To obtain samples of a specific mutant protein, the mutant gene must be created. Before site-directed mutagenesis (SDM) has begun, the only way to obtain a mutation was to isolate naturally occurring mutants with phenotypic screening due to the reason being that the rate at which naturally occurring errors in DNA synthesis was very low, and such screening was usually done after treatment with mutagens [1].

Mutagenesis, now achieved by either polymerase chain reaction (PCR) or non-PCR, has revolutionized the means, by which mutants are obtained [2, 3]. Mutations can now be created precisely at a specific residue with a specific codon change to produce the desired amino acid substitution (SDM), which has allowed alteration of any amino acid residue in a protein without extensive screening [4, 5]. Thus, the SDM approach is much more efficient, yielding desired mutations in 50–100% of the molecules produced, than that of phenotypic screening (<1%).

In rational redesign, precise changes in amino acid sequence are preconceived based on a detailed knowledge of protein structure, function and mechanism, and are then

introduced using site- directed mutagenesis [6, 7]. This technology holds strong promise for optimizing the desired properties for commercial applications. It also greatly enhances our basic understanding of enzyme binding and catalytic mechanisms, thus increasing the success of future enzyme engineering efforts and laying the foundation for functional prediction of new protein sequences in databases [5].

Anoxibacillus sp. SK3-4 is a thermophilic, rod-shaped, Gram positive and endospore-forming bacterium isolated firstly from hot spring in Malaysia [8]. Being relatively new genus as compared to the well documented *Geobacillus or Bacillus,* most of the data reported have shown that *Anoxybacillus sp.* produce interesting enzymes that are both thermostable and alkaline tolerant [9]. Thus, their potentials need to be explored for possible biotechnological applications being one of the recently discovered bacterium. At present, enzymes are being vigorously and systematically developed, as economically viable and industrial biocatalysts along with the fast advancement and expansion in Modern Science and Biotechnology [10, 11]. One of the uniqueness of oxidoreductases is that they are widely distributed among microbes, plants and animals. They employ various redox active centers [12].Some of these functions include residue binding, catalysis, structural stability and regulation.

To date, all members of the iron-activated dehydrogenase family are microbial and have been identified on the basis of primary structure homology. It includes ADHs with subunit size around 40 kDa, such as ADH II from *Zymomonas mobilis*, ADH IV from *Saccharomyces cerevisiae*, methanol dehydrogenase(MDH) from *Bacillus methanolicus*, two butanol dehydrogenase (BDH A and BDH B) from *Clostridium acetobutilicum* and propanediol oxidoreductase (POR) from *Escherichia coli* [13]. Three multifunctional dehydrogenases, ADH Es from *E. coli* and from *C. acetobutilicum* and ADH 2 from *Entamoeba hystolitica*, with a molecular mass of 96 kDa, are also included in this group [14-16]. Oxidoreductases can be classified according to their sequence or three-dimensional structure, which is very informative for the study of structure-function relationship, enzyme relationship and functional genomics [14].

As described earlier about the function of oxidoreductase in which the divalent metal ions help it maintains its activity and stability under physiological conditions, a DNA manipulation can be done to specific amino acids in the sequence so that the enzyme can yield high quality products for industrial purposes [17]. Many scientists also do manipulate genes specifically for characterization and catalytic functional determination of some important proteins.

There was also a report that some amino acid residues involved in iron-containing dehydrogenase family were characterized by the site-directed mutagenesis of selected candidate residues of propanediol oxidoreductase from *E. coli* based on the findings that mutations H263R, H267A, and H277A resulted in iron-deficient propanediol oxidoreductases without catalytic activity, and identified three conserved Histidine residues as iron ligands which also bind Zinc [18]. Therefore, Site-directed mutagenesis of DNA plays a vital role in protein engineering technology in which change in genetic sequence of the DNA can facilitate the study of the structure–function relationships of DNA, RNA, or protein coded by the DNA sequences. Various techniques have been applied for the introduction of specific changes at predetermined sites in DNA sequence [19, 20]. In view of the foregoing, site-directed mutagenesis of oxidoreductase from *Anoxybacillus sp.* SK3-4 was carried out based on rational design with the aim of improving the functional stability of the protein.

2. Materials and Methods

>Primary sequence of Oxidoreductase
MENFIFHNPTKLIFGRGQIEHLKKELHSYEHILIVYGGGSIKKNGV
YDDVVSILRSLNKSWSELAGVEPNPRLSTVQKGIHICREEKVDFIL
AVGGGSVIDCAKAIAAGALYDGEAWFISRKATVERALPIGTVLTL
AATGSEMNANSVITNWETKEKYGWSSPAVFPQFSILDPVYTTT
VPKDHTVYGIVDIMSHVLEQYFHHAPNTPLQDRMCEAILRTVIE
TAPKLIEDLQNVDHRETILYCGTMALNGILRMGLRGDWATHNI
EHAVSAVHDIPHAGGLAILFPNWMKHVLDEHIDRFKQLAVRVF
DVYPEGKGDREIALEGIEKLRAFWNRLGAPCRLADYHIGESLPII
VEKAMAFGPFGNFKKLHHDDVMTILQASL

Figure 1. *Primary sequence of oxidoreductase from Anoxybacillus sp. SK3-4.*

2.1. Determination of Active / Substrate Binding-Site of the Target Protein

Active or substrate binding sites can provide information on functional stability of the protein molecules. For this study, *3DLigandSite software* and *Protein Bioedit server* was used for the prediction of important active or substrate binding sites from the amino acid template used (oxidoreductase). Blasting was performed using computer system by copying, pasting and submitting the primary sequence of the template (query sequence) as an input to which after some few minutes the results delivery will be retrieved as output.*Phres2 server* was also used to generate model that will show the active/ binding site residues of the novel protein using PyMol viewer.

2.2. Site-Directed Mutagenesis (SDM) of the Target Protein

Desired mutations can be introduced into the target gene by using mismatch primers which are partially complementary to the template strand of DNA using PCR approach. For this study, Overlap-extension PCR (OE-PCR) approach was used because, double point mutations were designed which are not possible in mega-PCR approach [1, 21]. Mutation at two different sites were introduced into the target gene sequence of the protein in which amino acids; leucine (L) and histidine (H), involved in substrate and metal binding sites in the protein were substituted for isoleucine (I) and arginine (R) i.e. L138I

and H280R for mutagenesis. Six primers were carefully designed from the open reading frame of the gene nucleotides sequence [22]. Four sequential PCR rounds were performed for the amplification of the whole gene to generate the desired mutagenic amplicons. Two external forward and reverse primers as 'a' and 'f' were designed (i.e. 5'-GGAAAACTTTATTTTTCATAACCCGAC-3' and 5'-CAGGCTCGCCTGCAGAAT-3'). Two internal mutagenic forward primers as 'c' and 'e' carrying substitution codons were re-designed (i.e. 5'-GTGCTGACCATCGCGGCG-3' and 5'-GATATTCCGCGTGCGGGC-3'). Another two internal mutagenic reverse primers as 'b' and 'd' (i.e. 5'-CGCCGCGATGGTCAGCAC-3' and 5'-GCCCGCACGCGGAATATC-3') were also re-designed carrying substitution codons for the two amino acid residues at the appropriate positions marked with red color (see Figure 3).

SDM was carried out by substituting 138 leucine with isoleucine (L138I) and 280 histidine with arginine (H280R). During the first PCR round, the external forward primer 'a' and mutagenic internal reverse primer 'b' were used to amplify the first portion of the gene at sequence position 138 of the amino acid residue. For the second PCR round, the mutagenic internal forward primer 'c' at sequence position 138 was paired with mutagenic internal reverse primer 'd' at sequence position 280 to amplify the second portion of the gene. For third PCR round, the mutagenic internal forward primer 'e' was paired with external reverse primer 'f' at sequence position 280 to amplify the last portion of the gene (Figure 4). Finally, the mutant amplicons with overlapping sequences produced from all the three PCR rounds were mixed, denatured, and annealed in a PCR-ready buffer to generate mutant DNAs with complete desired two sites mutation in the fourth PCR round where two external forward and reverse primers 'a' and 'f' were used again for the complete amplification (see Figure 4).

Expasy translate tool was used for translation of the primary sequence into nucleotide sequence (figure 2). *Oligocal* software was also used for primer design. According to Goh *et al.* [20], certain factors to prove the good qualities of the designed primers were also considered such includes, the length of the primer, melting temperature (*Tm*), GC content, GC clamp, and self complementarity (see Table 1).

3. Results and Discussion

>Gene sequence of oxidoreductase from *Anoxybacillus sp.* SK3-4

ATGGAAAATTTTATTTTTCATAATCCAACGAAATTAATTTTCGGTAGAGGACAA
ATTGAACATTTGAAAAAGGAACTTCATTCGTATGAACACATTTTAATTGTATAT
GGCGGAGGAAGCATTAAGAAAAACGGCGTTATGATGATGTCGTATCTATTTTG
CGTTCATTGAATAAGTCTTGGTCCGAGCTAGCGGGTGTCGAACCAATCCTCGTT
TATCTACTGTTCAAAAAGGAATTCACATTTGCCGTGAAGAGAAAGTCGATTTTA
TTTTAGCAGTAGGCGGAGGAAGTGTCATCGATTGTGCCAAAGCCATTGCAGCA
GGTGCATTATATGATGGAGAGGCATGGGATTTTATTTCGCGAAAGGCGACGGT
TGAGCGAGCGCTGCCAATTGGAACAGTATTAACGCTAGCTGCGACAGGATCGG
AAATGAATGCAAATTCCGTTATTACAAATTGGGAAACGAAGGAGAAATACGGA
TGGAGTAGTCCAGCTGTTTTCCCTCAATTTTCGATTTTAGATCCTGTTTATACAA
CAACTGTTCCGAAAGATCATACAGTGTATGGCATCGTTGACATCATGTCGCACG
TATTAGAACAATATTTTCACCATGCGCCAAATACGCCATTACAAGATCGAATGT
GCGAAGCCATTTTACGAACAGTCATTGAAACCGCACCAAAACTCATAGAAGATT
TACAAAATGTTGATCATCGTGAAACGATTTTATATTGTGGAACAATGGCTTTAA
ATGGAATTTTACGCATGGGGCTACGCGGTGATTGGGCGACACATAATATTGAG
CATGCTGTGTCAGCAGTGCATGACATTCCACACGCAGGTGGGCTAGCTATTTTA
TTCCCAAATTGGATGAAGCACGTGCTTGATGAACATATCGATCGTTTTAAGCAG
CTAGCTGTTCGGGTGTTTGATGTTTATCCAGAAGGCAAAGGAGATCGAGAAAT
TGCGCTAGAAGGAATTGAAAAACTTCGGGCGTTTTGGAACAGATTAGGTGCTC
CTTGTCGATTAGCAGATTATCATATCGGTGAAGAATCATTGCCAATAATAGTAG
AAAAAGCGATGGCGTTCGGCCCATTTGGTAATTTTAAAAAGTTACATCATGATG
ATGTCATGACGATTTTACAAGCCTCATTGTAA

Figure 2. *Translated gene sequence of oxidoreductase from Anoxybacillus sp. KS3-4 obtained from Expasy translate tool.*

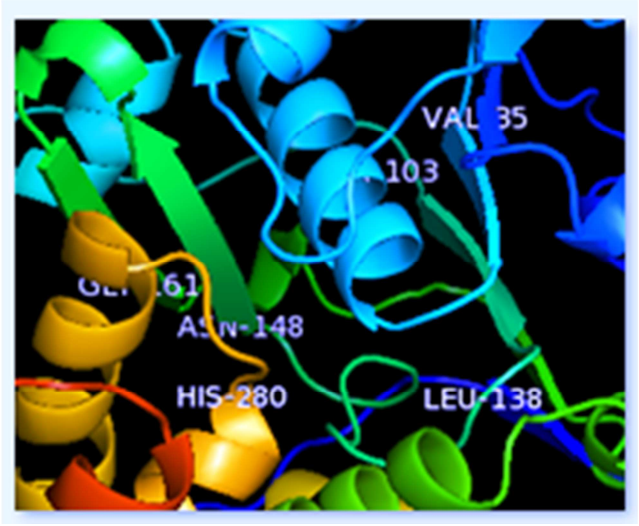

Figure 3. *Phres2-model generated by PyMol viewer indicating the active/ binding residues of the novel protein.*

3.1. Active / Substrate Binding-Sites of the Target Protein

The active site residues were predicted to be at positions 138 and 280 which correspond to Leucine (Lue) and Histidine (His) as the nucleophiles. His was found to have metal-ion binding activity (Fe or Zn) for enzyme structure stability as predicted by Protein Bioedit server with accuracy over 90%. The active sites are usually found in a 3- D groove or pockets of the enzyme, lined with amino acid residues. Other important amino acid residues that form the protein binding sites predicted by 3DLigandSite program include; Leu, His, Asp, Glu, Asn, Val, Pro, Ser, Thr, Tyr, etc (see Figure 3). The binding site residues of a protein participate in binding with enzymes' substrate and therefore important for catalytic activity of the protein. Therefore, the function of this novel protein may be attributed to the presence of these active or binding sites.

3.2. Mutagenesis of the Target Protein

Site-directed mutagenesis (SDM) is a powerful tool for introducing changes at a desired position in a gene of DNA sequence. SDMwas carried out by substituting 138 leucine with isoleucine (L138I) and 280 histidine with arginine (H280R). The two site mutations designed for the study were carried out at the sites important to the protein sequence as they were found to be actively engaged for substrate binding or ligand binding thus, important for catalytic stability of the protein.

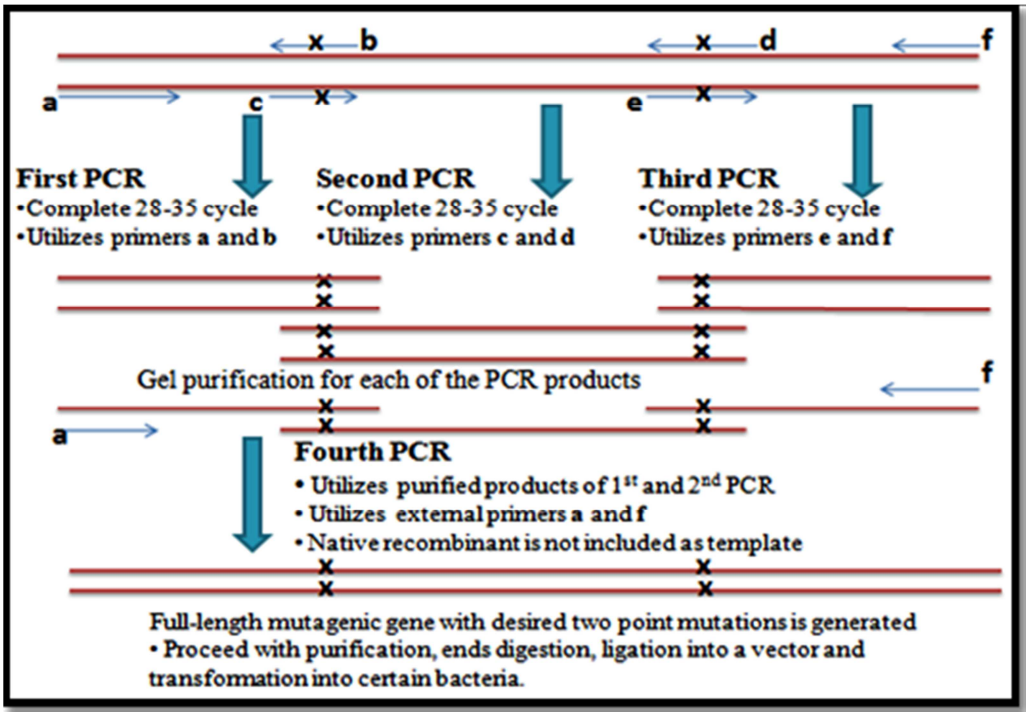

Figure 4. *Schematic diagram showing two (2) sites mutation using Modified OE-PCR.*

Table 1. *Primers designed for the study and their quality requirements of being theoretical "good primers".*

S/N	PRIMER	LENGTH (BP)	GC-CONTENT (%)	Tm (^{O}C)
1	External forward primer 'a' 5'-GGAAAACTTTATTTTTCATAACCCGAC-3'	27	33	62.1
2	Mutagenic internal reverse 'b' 5'-CGCCGCGATGGTCAGCAC-3'	18	72	62.9
3	Mutagenic internal forward 'c' 5'-GTGCTGACCATCGCGGCG-3'	18	72	62.9
4	Mutagenic internal reverse 'd' 5'-GCCCGCACGCGGAATATC-3'	18	61	58.4
5	Mutagenic internal forward 'e' 5'-GATATTCCGCGTGCGGGC-3'	18	61	58.4
6	External reverse primer 'f' 5'-CAGGCTCGCCTGCAGAAT-3'	18	67	60.8

Note: b, c, d and e are the mutagenic primers used for the study carrying substitution codons for the two amino acid residues at the appropriate positions.

3.3. Discussion

Multiple mutations are sometimes required in the same gene for studies of whether a second mutation site modulates another [23]. Multiple mutations can be obtained simply when several mutagenic primers are utilized one at a time for several PCR rounds of mutagenesis [24]. Sometimes, desired multiple mutations can be obtained even when all mutagenic primers are placed at the same time in the same reaction [25]. Alternatively, a few DNA fragments, each carrying mutations, can be connected to generate a joined product with multiple mutations [26]. Literature search has revealed that a number of novel proteins belonging to oxidoreductase family have been engineered previously with a view to enhance their catalytic properties for industrial applications. Oxidoreductase from *Anoxybacillus sp.* SK3-4 is a unique enzyme with no record of previous engineering work been documented. However, a similar enzyme (oxidoreductase) from *Rhodococcus erythropolis*has shown to have been previously mutated at different gene sequence positions to improve its catalytic activity [27].

For this study, amino acid residues, 138L and 280H, were selected due to reason being that they were found to be worth for mutagenesis like aspartic acid [18]. Although arginine is a basic amino acid, its protonation in the reaction medium may enhance the catalytic function of the protein as the way histidine does. Hence, it may serve as nucleophile for electron donation thus, may help in increasing the stability of the enzyme. Therefore, leucine and histidine residues were chosen for mutagenesis as they are part of the important residues found conserved on domain of our unique protein (oxidoreductase) as active residues responsible for active site and metal-ion binding. Therefore, by mutating these sites through substitution, there is every possibility for an increase in enzyme reactivity by increasing the catalytic stability of the protein. However, the activity, function, or even the stability of the enzyme may either be totally lost or disrupted depending on the successful achievement in the process [3, 28]. Because, mutation will only show two possibilities, either increase or decrease in stability of the enzyme [1, 21].

Oligocal soft ware was used to check for all the properties described in Table 1, especially the GC-content (%) and melting temperature (Tm/°C) for each of the primer used. GC-clamp and self-complementarity (presence or absence of a hair-pin or stem loops) properties were not included in the table. GC-clamp and PCR temperatures are the most important determining factors for the efficiency of the new products being generated in site-directed mutagenesis technique [8]. Therefore, our GC-clamp (%) was good enough for each of the primers to function effectively in the PCR reaction. Because for all the primers, except for primer 'a', their nucleotide sequences started with either G to end with C, or started with C to end with G to prevent "breathing" at both ends.

The choice of mutation sites for the present study was based on the multiple sequence alignment with oxidoreductase from thermo-tolerant *Anoxybacillus sp*. Finally, Overlap-extension PCR (OE-PCR) approach was chosen for the deliberate alteration of the protein (i.e. oxidoreductase) because two point mutations from different sites of the protein's primary sequence were created [29]. Therefore, apart from its use in single or double point mutations, OE-PCR approach can also be used to create multiple point mutations, residue insertion or deletion, and fusion fragment mutagenesis which are not possible in mega-PCR approach [21, 30].

4. Conclusion

From the current study, leucine and histidine residues were chosen for mutagenesis as they are part of the important residues found conserved on domain of our unique protein (oxidoreductase) as active residues responsible for active site and metal-ion binding. Therefore, by mutating these sites through substitution with isoleusine and arginine, there would be a possibility for an increase in enzyme reactivity by increasing the catalytic stability of the protein. However, the activity, function, or even the stability of the protein may either be totally lost or disrupted depending on the successful achievement in the experimental procedure. Also, the predicted active or binding site residues may provide information about the functional stability of the novel protein (oxidoreductase).

Therefore, mutating gene at a specific point via the use of site-directed mutagenesis approach has been enormously found to be effective engineering technique for enhancing the properties of protein molecule in one way or the other, thereby increasing the efficiency of protein to help speed up the rate of chemical reactions for industrial applications.

Conflict of Interest

We declared no conflict of interest.

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