PCR Based Random Mutagenesis Approach for a Defined DNA Sequence Using the Mutagenic Potential of Oxidized Nucleotide Products

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Abstract: Oxidizing conditions have not been explored well for the *in vitro* random mutagenesis in directed evolution. The mutagenic potential of diverse range of oxidized products is well reported in literature. Incorporation of errors during PCR in the presence of oxidized nucleotides can be a very effective alternative to error prone PCR as the transversion mutation frequency is higher in the former case. Earlier reports used a single purified oxidized nucleotide for introducing mutations during polymerase chain reaction. This could be further improved using the entire range of oxidized nucleotides to widen the mutation spectrum. The highlight of the present work lies in the fact that the oxidized nucleotides used in this study were generated by incubating the mixture of all the four nucleotides (dATP, dCTP, dTTP and dGTP) with an oxidizing agent, ferrous sulphate. This oxidized nucleotide mixture was then directly used without purification in polymerase chain reaction to introduce random mutations. The 100 μM oxidized nucleotides mixture treated with 5 mM $FeSO_4$ for 10 minutes along with 200 μM nucleotides are the optimized parameters for PCR amplification of a desired gene. The effect of manganese and magnesium ions over the incorporation of oxidized nucleotides was also investigated. An optimized PCR based approach which can be an efficient alternative to error-prone PCR for introducing random mutations in a defined gene sequence has been successfully developed.

Keywords: Random mutagenesis, oxidized nucleotides, error prone PCR.

1. INTRODUCTION

Oxidative stress has been shown to be involved in biological processes such as mutagenesis, carcinogenesis and ageing [1-3]. Reactive oxygen species (ROS) produced in cells react with DNA and its precursors, and the oxidative DNA lesions formed causes mutational events. One of the oxidative DNA lesions is 8-hydroxydeoxyguanosine (8-OH-dG; 7,8-dihydro-8- oxodeoxyguanosine) [4-7] and it pairs with dA as well as dC in *in vitro* DNA synthesis and induces mainly G → T transversions in cells [8-16]. Moreover, 2 hydroxydeoxyadenosine (2- OH-dA) and 5-hydroxydeoxycytidine (5-OH-dC), which are produced by ROS, are miscoding and mutagenic in nature [17-21]. Major products reported are 2-hydroxydeoxyadenosine (2-OH-dA), 8, 5'-cyclodeoxyadenosine (cyclo-dA), 5-hydroxydeoxycytidine (5-OH-dC), 8-hydroxydeoxyguanosine (8-OH-dG), 5-formyldeoxyuridine (5-CHO-dU) and glyoxal [22]. These results suggested that triphosphates of 2-OH-dA, cyclo-dA, 8-OH-dA, cyclo-dG, 5-CHOdU, 5-OH-dC, and glyoxal-dG as well as 8-OH-dG may be produced in cells with different ratio by various types of oxidative stress and involved in mutagenesis and carcinogenesis. Glyoxal is a major product of DNA oxidation in which Fenton-type oxygen free radical-forming systems are involved. It had been reported that the yield of glyoxal was much higher (17-fold) than that of 8- hydroxydeoxyguanosine (8-OH-dG). Moreover, the formation of glyoxal was estimated to be 13- fold more than that of 8-OH-dG when mixtures of deoxynucleosides were treated [22]. Glyoxal is known to be mutagenic in *Salmonella typhimurium* strains TA100, TA102 and TA104 [23-25]. It was further reported that glyoxal induces mutations at G:C base pairs, in a study using a set of seven *S. typhimurium* strains (TA7001–TA7006 and TA98) [26]. Moreover, glyoxal induces mutations mainly at G:C base pairs in wild-type *Escherichia coli* [27]. It was found that glyoxal induced predominantly G:C→T:A transversions, followed by G:C→C:G, A:T→T:A and G:C→A:T mutations. Oxidation of the methyl group of thymine produced 5-hydroxymethyl uracil (5-hmU) and as major products. One of the attractive approaches for random mutagenesis is the addition of a mutagenic nucleotide analog during PCR, to enhance the mutation frequency. 6-(2-Deoxyβ-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-C][1,2]oxazin-7-one-5-triphosphate (dPTP) and 8-oxo-2'-deoxyguanosine triphosphate (8-oxodGTP) were previously used to create mutations. The former induces A:T→G:C and G:C→A:T transitions, and the latter elicits A:T→C:G transversions [28]. The 2-hydroxyadenine base in DNA induces A:T→G:C and A:T→C:G mutations in living cells and 2-OH-dATP has the potential to elicit G:C→A:T and G:C→T:A mutations [29]. It was reported that a 2- substituted purine nucleotide analog, 2-hydroxy-2-deoxyadenosine 5-triphosphate (2-OHdATP), was used for the random PCR mutagenesis [30]. It was also reported by them that PCR with 8-OH-dGTP, after error-prone PCR with Mn^{2+} induced A:T→G:C and G:C→A:T transitions and A:T→T:A and A:T→C:G transversions with similar frequencies. These results indicated

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that the combination of the Mn^{2+}-PCR and 8-OH-dGTP PCR may be useful to generate random mutant libraries of proteins or functional nucleic acids [31].

It has been very well demonstrated that all these oxidized products have diverse mutagenic capacity. Oxidizing conditions have not been explored well for *in vitro* random mutagenesis in directed evolution experiments. Herein, we report the development and optimization of a PCR based system where the entire oxidized nucleotide products were used to have a highly efficient random chemical mutagenesis approach. This may be an effective alternative method for the error prone PCR and could be used for inducing wide spectrum of transition and transversion mutations with higher frequencies.

2. MATERIALS AND METHOD

2.1. Random Mutagenesis with Oxidized Nucleotides

Pseudomonas aeruginosa lipase encoding gene was amplified by the specific forward 5' GCCATATGATGACACACAAGAGGTGTGGCCCGC 3' flanked with *NdeI* restriction site and reverse oligonucleotides 5' CGGATGTCAGAGGAGATAAATCTGTCAGTAGAC 3' flanked with *XhoI* restriction site in 1X Taq buffer with KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.1-0.5 µM primer (forward and reverse), 1-2 units *Taq* polymerase and 100 ng genomic DNA. DNA molecular weight marker and all the PCR components except primers were from MBI fermentas GMBH, Germany. PCR was done in Eppendorf Master cycler gradient (Eppendorf AG, Germany) under the following conditions: initial denaturation at 94°C for 10 min followed by cycling conditions, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1 min 30 sec (repeated for 30 cycles). Final extension at 72°C for 10 min was used to complete the reaction. The PCR amplification of lipase encoding gene was done in the presence of oxidized nucleotides (dNTP). dNTP mixture was incubated for various time intervals in the presence of 5-10 mM $FeSO_4$. Mannitol (0.5 M) was used to stop the oxidation reaction. This oxidized nucleotide mixture was added to the normal polymerase chain reaction mixture and the PCR was run. The oxidized nucleotide mixture was used in combination with normal dNTP in the polymerase chain reaction. Except oxidized dNTP, all other components of a normal polymerase chain reaction were added before the incubation of the nucleotides with the oxidizing agent was over. Oxidized dNTP was always the last component added in the reaction mixture in all the experiments. *Taq* polymerase was added just a minute before the incubation of nucleotides was over. Care was taken to be quick in setting the reaction. All other components were kept same as in any normal polymerase chain reaction. The densitometry analysis was performed using the Quantity one software associated with the gel documentation unit (Biorad, India).

The amplification product obtained under the optimised oxidised condition was purified by gel extraction using a QIAquick PCR purification kit (Qiagen) and ligated to a linear, pDrive (T-A based) cloning vector (Qiagen). The ligation mixture was then transformed to *E.coli* DH5α cells. The sequencing of the mutants was performed at Bangalore Genie, India.

2.2. Effect of Concentration of Oxidizing Agent

To observe the effect of concentration of oxidizing agent, dNTP were incubated with various concentrations of oxidizing agent ($FeSO_4$) for 10 minutes at 37ºC. All other components were kept same as in any normal polymerase chain reaction.

2.3. Effect of Incubation Time with Oxidizing Agent ($FeSO_4$)

To observe the effect of incubation time with the oxidizing agent, dNTP was added with the oxidizing agent and incubated for various time intervals. This oxidized dNTP mixture was then added to the polymerase chain reaction mixture.

2.4. Effect of Manganese Ions on PCR Fidelity

Manganese (500 µM) in a normal polymerase chain reaction results in the incorporation of errors. The effect of manganese in the incorporation of oxidized nucleotides was checked during polymerase chain reaction. It was checked by adding 500 µM $MnCl_2$ to the polymerase chain reaction mixture in the presence and absence of oxidized nucleotides.

2.5. Effect of Oxidized Nucleotide Concentration on Taq Polymerase Activity

To observe the effect of oxidized nucleotide concentration, they were added to the polymerase chain reaction mixture in varying concentrations. All other components were kept same as in any normal polymerase chain reaction.

2.6. Effect of Concentration of Nucleotides and $MgCl_2$ over the Incorporation of Oxidized Nucleotides

To observe the effect of untreated dNTPs and $MgCl_2$, untreated dNTP was varied with and without $MgCl_2$ in the reaction mixture.

2.7. Effect of $MgCl_2$ over the Incorporation of Oxidized Nucleotides

Increase in $MgCl_2$ concentration in the polymerase chain reaction results in the decreased fidelity of *Taq* polymerase. This allows misincorporation of nucleotides in the growing DNA template in the polymerase chain reaction. To observe the effect of $MgCl_2$ over the incorporation of oxidized nucleotides, 7.5 mM $MgCl_2$ was added in the polymerase reaction mixture in the presence of 50 and 100 µM oxidized nucleotides keeping all the other components constant.

3. RESULTS AND DISCUSSION

3.1. Effect of Concentration of Oxidizing Agent

Treatment of nucleotides with an oxidizing agent in *in vitro* condition and using the oxidized nucleotides for random mutagenesis is an approach which is very less explored till now. Unlike to the earlier reports which involved the use of single purified oxidised nucleotide for introducing the errors in defined DNA sequence, the present work employed the use of non-purified oxidised nucleotide mixture for incorporation of the mutations [30, 31]. Instead of using the oxidizing agent in the PCR reaction mixture, we oxidized the nucleotide mixture by incubating it with $FeSO_4$ (Fig. **1a**). The polymerase chain reaction mixture containing all the components in optimized concentration resulted in an intense

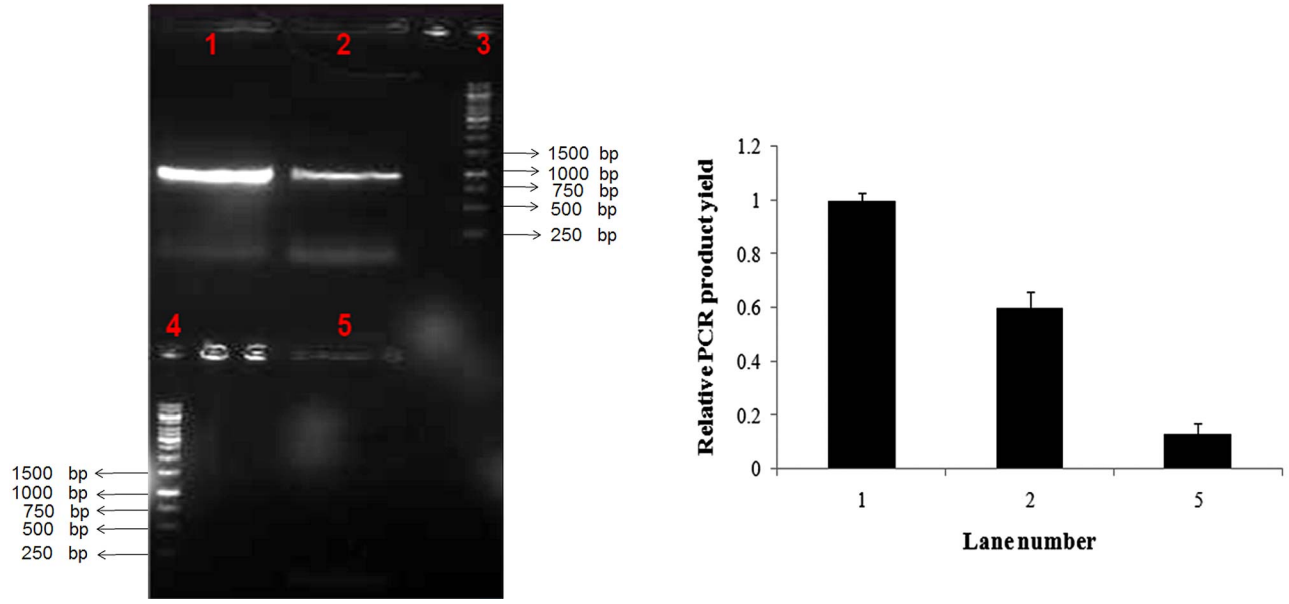
933 bp band. This PCR reaction mixture when supplemented with only the oxidized nucleotides without any untreated dNTPs, resulted in no amplification of lipase encoding gene. Treated dNTP mixture when supplemented with untreated dNTPs, resulted in the appearance of a 933 bp band. It was observed that when the dNTPs were treated with increasing concentration of $FeSO_4$, the PCR yield was decreased. Kamiya *et al*. used the 5 mM $FeSO_4$ concentration for their studies with single nucleotide oxidation [22]. In the present work, sizeable band intensity was obtained with 5 mM $FeSO_4$ while amplification was negligible with 10 mM $FeSO_4$. Densitometry analysis was also performed to compare the relative PCR product yield (Fig. **1b**). The probable explanation behind such an observation may be the increase in the concentration of oxidized nucleotide products which resulted due to the higher concentration of oxidizing agent. This higher concentration of oxidized nucleotide products seems to stall the *Taq* polymerase enzyme for which oxidized nucleotides products are not the natural substrates.

3.2. Effect of Incubation Time with Oxidizing Agent ($FeSO_4$)

The effect of increasing concentration of oxidizing agent led us next to investigate the role of incubation time with oxidizing agent (Fig. **2**). Increasing of incubation time from 10 to 20 minutes led to no amplification of 933 bp lipase gene. This observation indicated the difficulty faced by *Taq* polymerase in incorporating oxidized nucleotides to the template. The increased time of incubation had a direct proportionality with the number of oxidized nucleotides in the solution. The effects observed with the increased incubation time might be due to the number of oxidized nucleotides in the solution, oxidation state of the nucleotides and type or chemical nature of the oxidized nucleotides. Since *Taq* polymerase doesn't have a natural affinity towards oxidized nucleotides, the enzymes seems to be stalled when faced with a high concentration of oxidized nucleotides. This observation suggested that to induce mutation with oxidized nucleotides, the ideal incubation time should be the one at which the concentration of the oxidized nucleotide does not inhibit the polymerizing activity completely. The incubation time was not increased further beyond 20 minutes due to the disappearance of desired band on increasing the incubation time.

3.3. Effect of Manganese Ion on the PCR Fidelity

Introduction of manganese in the polymerase chain reaction mixture is one of the mostly used approaches in introducing mutations in a desired DNA sequence. Earlier reports establishing the role of manganese in decreasing the fidelity of *Taq* polymerase led us to investigate the cooperative role of manganese ion in inducing mutations in case of oxidized nucleotides. Mutagenic PCR involving the combination of the Mn^{2+}-PCR and 8-OH-dGTP PCR has been exploited for inducing the various mutations [31]. In the present work, introduction of manganese in the presence of oxidized dNTPs did not result in any PCR amplification of the desired gene (Fig. **3**). Though presence of manganese ions decreased the fidelity of *Taq* polymerase, the incorporation of oxidized nucleotides was not visible when these two conditions were included in a normal polymerase chain reaction mixture. The probable reason behind such an observation might be the stalling of *Taq* polymerase in the presence of excess of oxidized nucleotides.



a **b**

Fig. (1). a. Effect of concentration of oxidizing agent on the amplification. The lipase encoding gene was amplified in the presence of different mixtures of nucleotides treated for either 10 minutes or not: 200 µM nucleotides (lane 1); 100 µM of 5 mM $FeSO_4$ treated-nucleotides + 200 µM nucleotides (lane 2); 100 µM of 10 mM $FeSO_4$ treated-nucleotides + 200 µM nucleotides (lane 5) and visualized by agarose gel electrophoresis and ethidium bromide staining. DNA molecular weight markers are given in lanes 3 and 4.

b. Densitometry analysis to compare the relative PCR product yield. The intensity of the control reaction was designated as 1 (Lane 1) and relative intensity of other lanes were quantified against the control reaction.

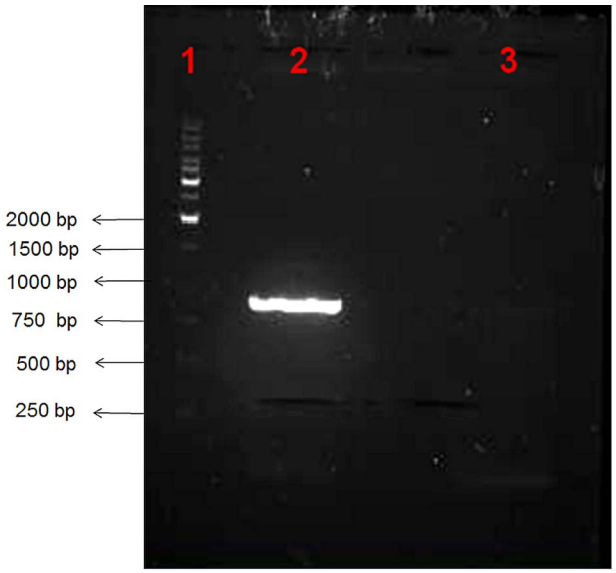


Fig. (2). Effect of incubation time with oxidizing agent on the amplification of the gene of interest. 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane 2); 100 µM nucleotides treated with 5 mM $FeSO_4$ for 20 minutes + 200 µM nucleotides (lane 3). DNA molecular weight marker is shown in lane 1.

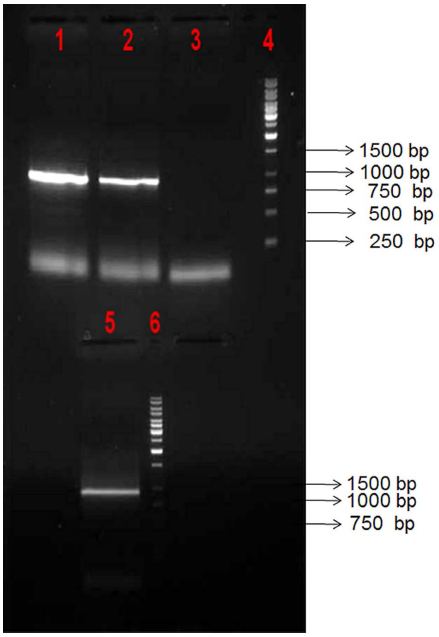


Fig. (3). Effect of manganese ions on the amplification of the gene of interest in the presence of oxidized nucleotide products. 200 µM nucleotides (lane 1); 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane 2); 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides + 500 µM $MnCl_2$ (lane 3); 200 µM nucleotides + 500 mM $MnCl_2$ (lane 5). DNA molecular weight marker is shown in lane 4 and 6.

3.4. Effect of Treated Nucleotide Concentration on Taq Polymerase Activity

It was clear from the previous experiment that concentration of oxidizing agent affected PCR yield. Further, the effect of varying treated nucleotide concentration (keeping the untreated dNTP concentration constant) was checked (Fig. **4a**). It was observed that the treated dNTP concentration when decreased from 100 to 50 µM resulted in nearly the same PCR yield while the increased dNTP concentration (200 µM) resulted in no amplification of lipase encoding gene. The relative PCR product yield was also quantified for individual lane by densitometry (Fig. **4b**). From the above observation it could be concluded that the concentration of treated dNTPs in the reaction mixture is a crucial factor in polymerase chain reaction in the presence of oxidized nucleotides. The amount of oxidized nucleotides in the reaction mixture may or may not affect the *Taq* polymerase fidelity depending on whether the amount present in the reaction is enough to induce mutations or to stall the *Taq* polymerase. The excess of oxidized nucleotides in the reaction mixture leads to complete inhibition of *Taq* polymerase activity as the oxidized nucleotides are not the natural substrates of *Taq* polymerase. So, a critical concentration of oxidized nucleotides in the reaction mixture is required for polymerase chain reaction in the presence of oxidized nucleotides.

3.5. Effect of Untreated dNTPs Concentration over the Incorporation of Oxidized Nucleotides

The importance of a critical concentration of oxidized nucleotides in the reaction mixture led us to investigate the role of concentration of untreated nucleotides in the polymerase chain reaction mixture (Fig. **5**). It was observed that amplification was being done by *Taq* polymerase when 100 µM treated nucleotides were added with 200 µM untreated nucleotides. On decreasing the concentration of untreated nucleotides to 100 µM keeping the concentration of treated nucleotides at 100 µM (constant), it was observed that there was no amplification of the desired gene. This observation indicated that a proper ratio of oxidized as well as normal dNTP was required for amplification to take place. In such amplification, there were chances for oxidized dNTPs to get incorporated in the desired gene, which in further amplification cycles base pairs with normal bases and thus resulted in introduction of point mutations at random places. It is well established that increased concentration of $MgCl_2$ leads to the decreased substrate specificity of *Taq* polymerase. In order to investigate the effect of decreased substrate specificity of *Taq* polymerase on the incorporation of oxidized nucleotides, 7.5 mM $MgCl_2$ was added to the above two reaction mixture. It was found that increased concentration of $MgCl_2$ in the reaction mixture resulted in no amplification in both the conditions. Though increased concentration of $MgCl_2$ decreased the fidelity of *Taq* polymerase, the incorporation of oxidized nucleotides was not visible when these two conditions were included in a normal polymerase chain reaction mixture. The probable reason behind such an observation might be the stalling of *Taq* polymerase in the presence of excess of oxidized nucleotides which resulted due the acceptance of oxidized nucleotides by *Taq* polymerase in the presence of higher concentration of $MgCl_2$.

3.6. Effect of $MgCl_2$ over the Incorporation of Oxidized Nucleotides

The role of $MgCl_2$ on the fidelity of *Taq* polymerase led us to investigate the effect of $MgCl_2$ over the incorporation of oxidized nucleotides during polymerase chain reaction in

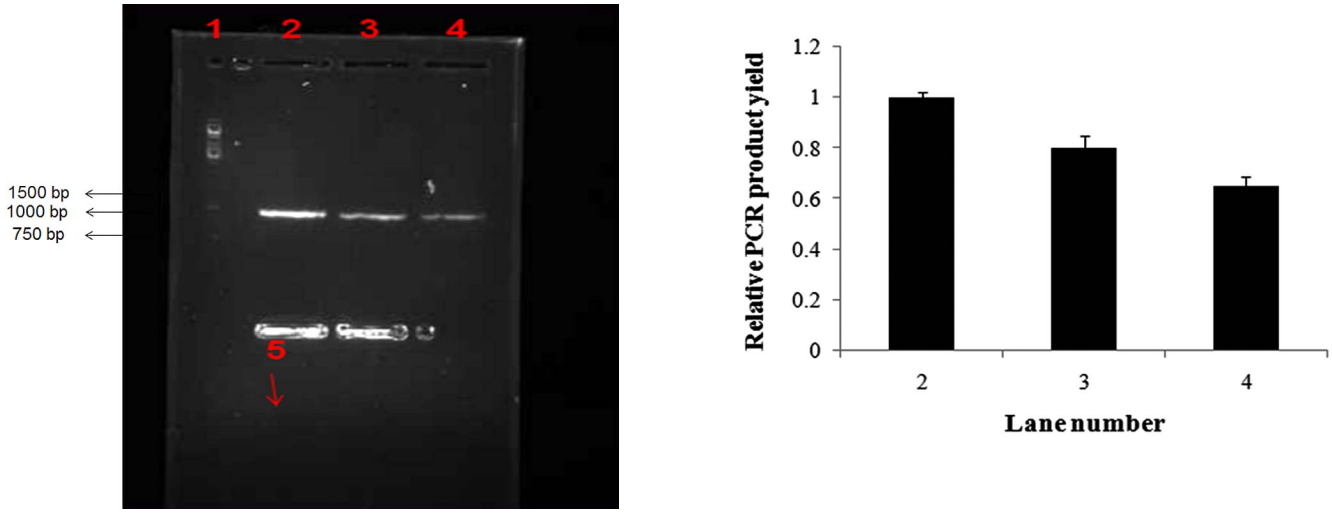


Fig. (4). a. Effect of treated nucleotide concentration on the amplification of the gene of interest. 200 µM nucleotides (lane 2); 50 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane 3); 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane 4); 200 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane 5). DNA molecular weight marker is shown in lane 1.

b. Densitometry analysis to compare the relative PCR product yield. The intensity of the control reaction was designated as 1 (Lane 2) and relative intensity of other lanes were quantified against the control reaction.

the presence of oxidized nucleotide mixture (Fig. **6**). Reports are available showing the use of increased $MgCl_2$ concentration with oxidised nucleotides for the incorporation of errors in defined DNA sequence [32]. In present case, $MgCl_2$ concentration when increased to 7.5 mM keeping all other components of PCR reaction same, there was a decrease in the band intensity and amplification of non specific fragments took place (Fig. **6**, lane 3). The effect of $MgCl_2$ in the presence of oxidized dNTPs was investigated. The significant reduction in amplification of the desired gene was observed when oxidized dNTP (100 µM) was supplemented with 7.5 mM $MgCl_2$ (Fig. **6**, lane 6). There was no amplification even when the $MgCl_2$ concentration was decreased to 4.5 mM (Fig. **6**, lane 7).

3.7. Sequencing of Products of PCR Amplification with Oxidized Nucleotides

All the above results led to the conclusion that 100 µM oxidized nucleotides mixture (treated with 5 mM $FeSO_4$ for 10 minutes) along with 200 µM nucleotides are the optimized parameters for PCR amplification of a desired gene in the presence of oxidized nucleotide products. The PCR-amplified lipase encoding gene obtained using 100 µM oxidized nucleotides mixture (treated with 5 mM $FeSO_4$ for 10 minutes) and 200 µM nucleotides were cloned and sequenced. Both the transition and transversion mutations were observed in the sequenced clones (Table **1**). Sequencing of mutants resulted in the similar pattern of mutations that was earlier reported by Kamiya *et al*. [30, 31].

CONCLUSIONS

The entire studies on the oxidized nucleotides led us to conclude that to incorporate errors with oxidized dNTPs; it was necessary to adjust the ratio of the treated and untreated dNTPs in the reaction mixture, concentration of oxidizing agent and the time of incubation. The polymerizing activity of Taq polymerase was observed to be affected in the presence of an excess oxidized nucleotides. It stalls when the

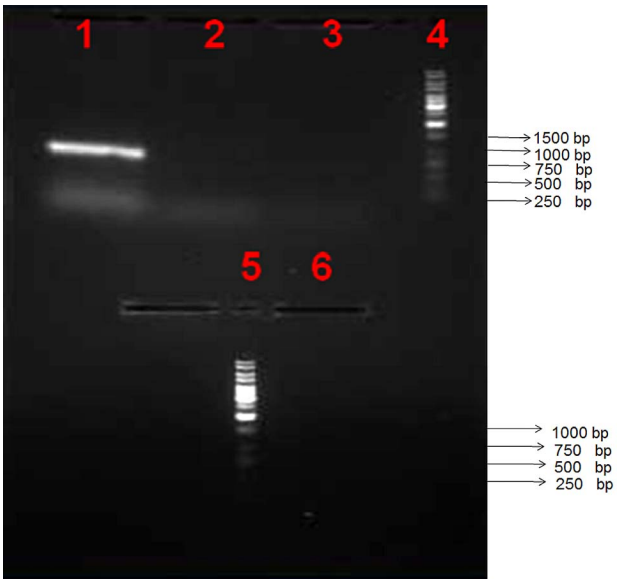


Fig. (5). Effect of untreated dNTPs concentration on the amplification of the gene of interest in the presence of oxidized nucleotide products. 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane 1); 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 100 µM nucleotides (lane 2); 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides + 7.5 mM $MgCl_2$ (lane 3); 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 100 µM nucleotides + 7.5 mM $MgCl_2$ (lane 6). DNA molecular weight marker is shown in lane 4 and 5.

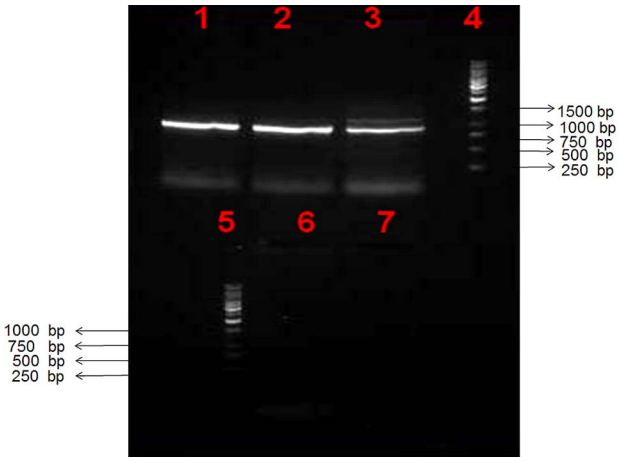


Fig. (6). Effect of $MgCl_2$ on the amplification of the gene of interest in the presence of oxidized nucleotide products. 100 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane1); 50 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides (lane 2); 200 µM nucleotides + 7.5 mM $MgCl_2$ (lane 3); 50 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides + 7.5 mM $MgCl_2$ (lane 6); 50 µM nucleotides treated with 5 mM $FeSO_4$ for 10 minutes + 200 µM nucleotides + 4.5 mM $MgCl_2$ (lane 7). DNA molecular weight marker is shown in lane 4 and 5.

Table 1. Point Mutations in the Variant Generated by PCR in the Presence of Oxidized Nucleotides

Base Pair Change	Nucleotide Position	Base Pair Change	Nucleotide Position
C → T	210	C → T	303
A → G	245	A → T	419
G → C	259	A → G	441
C → T	280	A → G	500

number of unnatural nucleotides crosses a critical concentration. Earlier reports used a single pure oxidized nucleotide for introducing mutations [30, 31] during polymerase chain reaction. This was a serious drawback as one requires tedious purification of oxidized nucleotide product before using it in the PCR reaction to induce point mutations. Moreover, the diverse range of oxidized nucleotide products in a single polymerase chain reaction has never been utilized to induce the random mutations. The highlight of the present work lies in the fact that the oxidized nucleotides used in this study were generated by incubating the mixture of all the four nucleotides (dATP, dCTP, dTTP and dGTP) with an oxidizing agent. This oxidized mixture was then used in polymerase chain reaction to introduce random mutations. The diverse range of oxidized products of these four dNTPs (which can induce a wide spectrum of mutations: both transitions and transversions) [10, 17, 21, 26, 29, 30, 31] when used together in a polymerase chain reaction could constitute an efficient alternative to error-prone PCR for introducing random mutations in a defined gene sequence.

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