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Amplification and Structure Analysis of Dioxygenase of *Anoxybacillus* sp. DT3-1

A. Emami[1], H.Yari[1]*, H. Hosseinipour Nasirmahaleh[1], S. Malekshahi[1], A. Fouladvand[1], A. Hosseinnia[1] and H. Sadegh[2]

[1]Universiti Teknologi Malaysia, Faculty of Bioscience and Medical Engineering, Johor, Malaysia.
[2]Department of Biology, Science and Research branch, Islamic Azad University, Tehran, Iran.

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Dioxygenase hasfundamental role in degradation of aromatic compounds and is able to cleavearomatic rings. This enzyme can catalyze the interpolation ofboth atoms of molecular oxygen into substrates during the several reactionmechanisms. This work aims to amplify the dioxygenase of *Anoxybacillus* sp. DT3-1 and to analyze the different structures. The genomic DNA was extracted in order to isolate and amplify the full length gene of dioxygenase using Netprimer program (to design the primers) and Polymerase Chain Reaction (PCR) method. According to the Gradient PCR (GPCR), 50 ºC was determined as the optimum annealing temperature in this study. Three softwares (EsyPred, I-Tasser and CPH Model) andone template chain (2pw6 chain 'A')were employed to predict the tertiary structure of dioxygenase. The predicted tertiary structure by CPH Model showed the most accurate result in comparison with EsyPred3D and I-Tasser. Also, four conserved regions and their locations were determined based on the predicted 3D structure by CPH Model. According to the obtained phylogenetic tree, there are three main branches A, B and C which rationally categorize the highly related species based on the dioxygenase which was highly related to*Anoxybacillus*flavithermus WK1 and *Anoxybacillus* sp. SK3-4.

Key words: *Anoxybacillus* sp. DT3-1, CPH Model, Dioxygenase, Signal P.

Aromatic compounds can be described as organic molecules containing one or more aromatic rings, including benzene rings[1]. The most important aromatic compounds are polycyclic aromatic hydrocarbons (PAHs), because they are known as strong mutagenic, carcinogenic and toxic compounds and they belong to the group of persistent organic pollutants (POPs)[2]. These pollutants have a high durability in environment and have the potential to cause significant unpleasant environmental effects[3]. PAHs are weakly dissolve and volatile in water and their solubility would decrease with an increase in the number of aromatic rings[4].

* To whom all correspondence should be addressed.
Tel.: 0166949252, Fax: 00608843232,
Email: yhamed2@live.utm.my

There are some enzymes which are involved in the degradation of PAHs including dioxygenase, dehydrogenase and lignolytic enzymes[5]. In fact, dioxygenase is one of the most important enzymes fordegradation of aromatic compounds[6], and isa member of extradiol-ring-cleavage dioxygenase, class III enzymes. The class III enzymes have two subunits (α and β). The crystal structure of dioxygenase discovered that the molecule is a α2β2 tetramer[7]. The active site contains a non-heme iron coordinated by His12, His61, Glu242, and a water molecule located in a deep cleft of the beta subunit, which is covered by the alpha subunit[8]. Dioxygenases are multicomponent enzyme systems containing an electron transport chain and a terminal dioxygenase which is composed of small (β) and large (α)subunits[9]. The large subunit is a catalytic

doamin and also surrounds two conserved regions: the [Fe2-S2] catalyticcenter and the mononuclear iron binding domain, which are involved in the successiveelectron transfer to the dioxygen molecule[10]. Dioxygenase can be categorized into two cardinal groups based on thescission of the aromatic ring[11,12].

The applications of naturally engineered and improved microbes for bioremediation have been considered and developed since last decade[13]. *Anoxybacillus* is a thermophile gram-positive bacteria found in forms of rod and coccus, and its optimum growth temperature is between 45 and 75 °C [14] and optimum growth PH is 7. Also these bacteria are aerotolerant anaerobe, facultative anaerobe, alkaliphilic, alkalitolerant and heterotrophic, and they are capable to ferment a much variety of carbon sources including D-glucose, sorbitol, L-rhamnose, sucrose, starch, L-arabinose and glycogen[15]. *Anoxybacillus* sp. DT3-1 is one of the thermophilic isolated and characterized rod-shaped andgram-positive bacteria from hot springs with the growth temperature of 75 °C. *Anoxybacillus* sp. DT3-1 was used as a bacterial model in this study in order to isolate the full length gene of dioxygenase and analyze the structure of both gene and enzyme using wet laboratory and computational procedures.

MATERIALS AND METHODS

Preparation of bacteria

First, solid thermus medium was used to culture the *Anoxybacillus* sp. DT3-1 and obtain a single colony, then broth thermos medium was used to suspend and prepare the bacteria for isolation of the genomic DNA. The pH of the media was adjusted to 7 with adding NaOH. In both cases, all media were incubated at 50 °C for 24 hours (along with 200 rpm rotation speed for broth media) and the growth of bacteria was examined by UV-visible spectrophotometer at 600 nm[16].

Genomic DNA Isolation

Some 1.5 mL of cultured bacteria was transferred into 1.5 mL microcentrifuge tube and was spun at 14,500 g for 2 min. Discarding the supernatant, the pellet was suspended in 480 µL of 50 mM EDTA. As the next step, 120 µL of lytic enzyme (lysozyme solution) was added into the tube and mixed gently by pipette. The cells were incubated in 37 °C in water bath for 45 min. The tube was centrifuged again as mention above and supernatant was discarded. Then, 600 µL of nuclei lytic solution was added to the pellet and gently was mixed. The mixture was incubated for 5 min at 80 °C for lysing the cells then it was cooled in room temperature. Subsequently, 3µL of RNase solution was added into the tube and was mixed by inverting gently 3-5 times. Then it was incubated in 37 °C for 45 min and afterward was cooled in room temperature. Also 200 µL of Protein Precipitation Solution was added to the micro-centrifuge tube and was spun at 20,000 g. Then the mixture was incubated in ice for 5 min and was centrifuged at 14,500 g for 3 min. In order to precipitate the DNA, the supernatant was transferred into a microcentrifuge tube which contained 600 µL of room temperature isopropanol then mixed by pipette. The tube was centrifuged at 14,500 g for 2 min and the supernatant was then discarded. The tube was drained on sterile absorbance paper. Then, 600 µL of room-temperature 70% ethanol was added into the tube and was mixed by using the pipette. Afterwards, it was centrifuged at 14,500 g for 2 min. Using clean absorbance paper, the pellet was left to air dry for 10 min. As the last step, 100 µL of rehydration solution was added to the pellet and incubated in water bath at 65 °C for 1 hour.

Polymerase Chain Reaction

For the PCR amplification, design of primerswere done by using the Netprimer program. The PCR was carried out using Promega kit and MyCycler Thermal Cycler machine (Bio-Rad). The PCR tubes were prepared as mentioned in table 1. The Gradient PCR (GPCR) was carried out using different annealing temperatures 47, 48, 49, 50 and 51 °C.The PCR condition was set up as follow: initial denaturation at 98 °C for 1 min followed by 30 cycles (denaturation at 98 °C for 10 s, annealing at 50 °C for 20 s and extension at 72 °C for 25 s) and a 5-min final extension. PromegaWizardTM gel extraction kit was used for purifying the amplified gene fragments.

Analysis of PCR Product

For analyzing the PCR product, NanoDropand Gel Electrophoresis was carried out as described elsewhere[17]. Briefly, Gel Electrophoresis was performed at 80 V, 45 W for 60

min. Lastly, the UV Gene flashwas used for observing the DNA bands from the Gel Electrophoresis.

Computational analysis

Primary structure of dioxygenase was studied using Expasy program for Isoelectric point (pI)value analysis and SAPS program for molecular weight (MW) prediction[18]. Signal peptide of dioxygenase was analyzed with signal Psoftware[19]. Secondary structure prediction for dioxygenase was carried out using GOR IV program[20]. Also using 2pw6 chain 'A' as a template model, three softwares (EsyPred, I-Tasser and CPH Model) were used to predict the tertiary structure of the dioxygenase. Furthermore, Ramachandran plot and ERRAT softwares were triggered to validate the predicted tertiary structure[21]. Moreover, NCBI CDART (conserved domain Architecture Retrieval Tool) and InterProScansoftwares were used for identifying the conserve domains in dioxygenase[22].The phylogenetic tree was prepared using Mega 6 software as described elsewhere[23].

Table 1. Component 50 µL Reaction Final Concentration.

Component	50 µL Reaction	Final Conc.
Nuclease-free water	30 µL	30 µL
5X Phusion GC Buffer	10 µL	1X
10 mMdNTPs	1 µL	200 µL
10 µL Forward Primer	2.5 µL	0.5 µL
10 µL Reverse Primer	2.5 µL	0.5 µL
Template DNA	2 µL	< 250 ng
DMSO	1.5 µL	3%
Phusion DNA Polymerase	0.5 µL	1.0 unit/ 50 µL PCR

RESULTS AND DISCUSSION

Genomic Isolation and PCR Amplification

The genomic DNA of *Anoxybacillus* sp. DT3-1 was isolated by using the Promega genome extraction kit. Using Gel Electrophoresis and NanoDrop machines, the isolated genome was validated and as can be seen in figure 1 (A) the isolated genome was intact, the band was clear which, in turn, indicates that there is no RNA in the samples and the genomic DNA is pure. Based on NanoDrop machine, the average concentration of genomic DNA was 175 ng/µL in the samples.

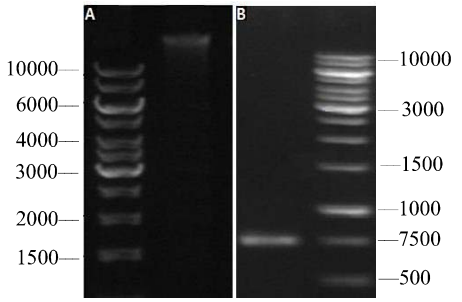


Fig. 1. The isolated bands; (A) genomic DNA, (B) Dioxygenase gene of Anoxybacillus sp. DT3-1.

Table 2. Templates and Template identities via different softwares for prediction of 3D structure of dioxygenase.

Enzyme	Model	EsyPred3D	I-Tasser	CPH Model
Dioxygenase	Template	2pw6 chain 'A'	2pw6 chain 'A'	2pw6 chain 'A'
	Template Identity	50.40%	56.96%	63.11%

Table 3. Tertiary structure validation of dioxygenase.

Tertiary Structure Prediction tools	Ramachandran Plot			ERRAT
	Favored Region (%)	Allowed Region (%)	Outliers (%)	Overall Quality (%)
EsyPred3D	87.6	7.2	5.2	64.3
I-Tasser	84.4	7.6	8.0	41.2
CPH Model	89.4	5.9	4.7	69.8

In this study, 50 °C was determined as the optimum annealing temperature using GPCR. Based on the Gel Electrophoresis method, the best bands were obtained when 50 °C was set as the annealing temperature in GPCR. After optimization of annealing temperature and design of primers, the full-length gene of dioxygenase was amplified (figure 1, B) from *Anoxybacillus* sp. DT3-1 and sequenced (figure 2). Finally, a 759 bp dioxygenase gene was obtained and used for further analysis.

ComputationalAnalysis of the dioxygenase

Based on the primary structure of the dioxygenase, Isoelectric point (pI) value was 5.66 and molecular weight was 28.5 kDa. Most protein molecules have a hydrophobic core, which is not accessible to water and a polar surface in interaction with the aqueous environment[24]. Based on the composition analysis of dioxygenase, 72 residues (28.6%) of the enzyme are hydrophobic while 46 residues (18.3 %) are charged amino acids. Moreover, the frequency of the Alanine and Leucine were measured 9.9 and 9.5 %, respectively, as the maximum numbers of amino acids, and also Cytosine and Asparagine were considered as the minimum numbers of amino acids 0.0 % and 1.2 %, respectively, in the dioxygenase primary sequence.

Thesignal peptide (signalP) is necessary for protein targeting to the membrane-embedded export machinery in Prokaryotes, Archaea and Eukaryotes. According to the results obtained from signalP software, signal peptide in the dioxygenase of*Anoxybacillus* sp. DT3-1 was not detect because S-score and Y-score have not crossed D-score and there is no cleavage site between them (figure 4), therefor it suggests that the studied dioxygenase is an intracellular enzyme[25].

Based on the secondary structure prediction of dioxygenase, the most fragments of the secondary structure of this enzyme were made up of random coil (46.83%, orange sequences), α-helix (36.11%, blue sequences) and β-sheet (17.06%, red sequences) respectively.

ATG ACA GCT **TTA TTT CTT GCG CAC GGA TCA CCC ATG TTA GCT ATT** GAA GAC AGC GCA TAT ACA TCG
M T A L F L A H G S P M L A I E D S A Y T S

TTT TTA ACG ACG TTC GGA AAA CAC ATT CAT CCA AAA GTA ATC GTC GTC **TTC ACG GCA CAT TGG** ACA
F L T T F G K H I H P K V I V V F T A H W T

ACA CGC CAA CCG ACC GTT TCA TCA ATC GCT GGA ACA TAT GAA ATG ATT TAT GAT TTT TCC GGC TTC CCA
T R Q P T V S S I A G T Y E M I Y D F S G F P

CGT GAA TTG TAT GAA GTG ACC TAT GCT GCA CAA GGT TCA GTC GAA TGG GCA AAG CGC GTC CAA CAA
R E L Y E V T Y A A Q G S V E W A K R V Q Q

CAT CTA AAA CAC GTT TCC CCC ATC GTT CAT ATC GAT GAG ACG **CGG GGG CTT GAC CAC GGT TCA TGG**
H L K H V S P I V H I D E T R G L D H G S W

GTC TTG TTA AGC CGA CTA TTT CCA GAA GCA AAC ATT CCC ATT GTC CAA GCA TCC GTC GTT CCG TGG TGG
V L L S R L F P E A N I P I V Q A S V V P W W

ACG CCA AAA CAG CTC TTT CAA CTT GGT GAG GCG TTA C GT CCG TTG CGT GAC GAA GAT GTC ATG ATT CTC
T P K Q L F Q L G E A L R P L R D E D V M I L

GGC AGC GGT GGT **ACA GTG CAT AAT TTA ATG GCG CTT** CGT TGG GAG GAA CAG ACA AAA GCA GAT TCT TGG GCT
G S G G T V H N L M A L R W E E Q T K A D S W A

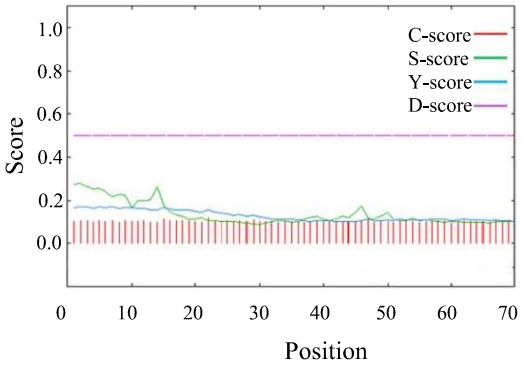
GTT GCC TTC GAT GAT TGG TTG CTT GCC CAG TCA TCC GCC CAT AGT GAA GAC GTG TTT CAC TAT GAA
V A F D D W L L A Q S S A H S E D V F H Y E

GAA AAA GCC CCT TAT GCC AAA CAA GCC GTT CCA ACA CCT GAA CAT TTA GCA CCG TAC TGG ATT
E K A P Y A K Q A V P T P E H L A P Y W I

GCG TAC GGA GCA GGC GAT CGC TCG CCA CAT GTT TTA TTC CGA GAA TAC GAA TAC GGA ACT TAA
A Y G A G D R S P H V L F R E Y E Y G N L

GCC TTT TAG CGT GTA TCA TTT TAA
S L L A V S F *

Fig 2. Full-length gene and amino acid sequence of Anoxybacillus dioxygenase. Four conserved regions are shown in bold characters.



Measure	Position	Value	Cutoff	Signal Peptide?
Max. C	22	0.120		
Max. Y	15	0.166		
Max. S	2	0.279		
Mean S	1-14	0.233		
D	1-14	0.202	0.450	NO

Network = SignalP-noTM

Fig. 3. Signal peptide prediction of dioxygenase.

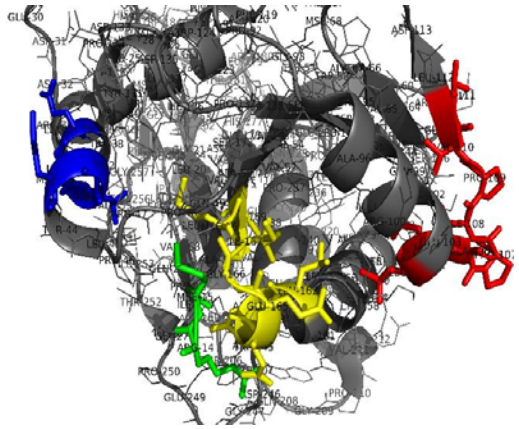


Fig. 4. Locations of conserved regions in *Anoxybacillus* dioxygenase on predicted tertiary structure based on the CPH Model. The picture was obtained by Pymol software. Four conserved domains (I to IV) are shown in green, blue, red and yellow, respectively.

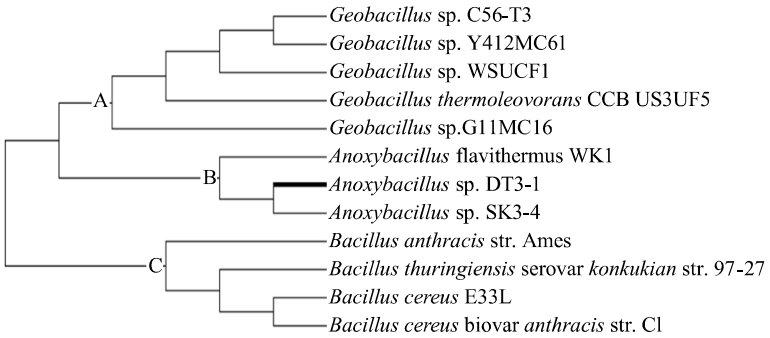


Fig. 5. Phylogenic tree of dioxygenase of*Anoxybacillus* sp. DT3-1. The three main branches are shown by A, B and C.

For tertiary structure prediction of the dioxygenase,2pw6 chain 'A' was selected as the template for tertiary structure prediction in all the used programs (EsyPred, I-Tasser and CPH Model), while the sequence of template identities predicted via these three softwareswere different. Base on the template identities in Table 2, the most accurate predicted tertiary structure was obtained from CPH Model (63.11%) in comparison with EsyPred3D (50.40%) and I-Tasser (56.96%). Also the accuracy of the obtained results were validated using two softwares Ramachandran plot and ERRAT. As can be seen in Table 2, the CPH Model was the most precise software for the prediction of tertiary structure of the dioxygenase.

Multiple Sequence Alignment and Conserved Regions

The obtained sequence of the dioxygenase was blasted through NCBI Blastp service to determine the most relevant sequences. *Anoxybacillus* sp. DT3-1 dioxygenase showed the highest similarities with the dioxygenases of *Anoxybacillus flavithermus*WK1 (92%) and *Anoxybacillus* sp. SK3-4 (90%). Four conserved regions and their locations were determined based on the predicted 3D structure by CPH Modeland shown in Figure 4.

Phylogenic Analysis

Phylogenic tree is known as a diagram which describes the lines of evolutionary descant

organisms or species from common ancestor (26). According to the obtained phylogenetic tree, there are three main branches A, B and C which rationally categorize the highly related species based on the dioxygenase of *Anoxybacillus* DT3-1. The studied dioxygenase was highly related to that of *Anoxybacillus* flavithermus WK1 and *Anoxybacillus* sp. SK3-4.

CONCLUSION

The dioxygenase of *Anoxybacillus* sp. DT3-1 was amplified and, for the first time, its primary, secondary and tertiary structures were analyzed using wet laboratory and computational procedures. Sequencing showed a gene of 252 amino acids with four conserved regions. Also this enzyme was found to be a member of extra cellular enzymes. Future studies on this enzyme may reveal more detail on its structure and function.

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