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In *silico* Characterisation of Conserved Functional Domain and Isolation of Coniferyl Aldehyde Dehydrogenase Gene from *Pseudomonas nitroreducens* Jin1

Praveen P.Balgir*, Dinesh Kalra

Department of Biotechnology, Punjabi University, Patiala, Punjab. INDIA.

E-mail : balgirbt@live.com Contact No : +91 .....

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Abstract

Coniferyl aldehyde dehydrogenase gene (*calB*) oxidises coniferyl aldehyde into ferulic acid, the last step in the metabolic pathway of eugenol to ferulic acid. It occurs in various bacterial strains like *Pseudomonas nitroreducens* Jin1, *Ralstonia eutropha, Escherichia coli, Pseudomonassp.* HR199*, Rhodococcus opacus. C*oniferyl aldehyde produced as the intermediate product by the biotransformation of eugenol, is oxidised to ferulic acid, NADPH and H^+. Aldehyde dehydrogenase-related bacterial sequences were aligned and using *insilico* methodology analysed to determine functionally important conserved residues in this protein. A total of 16 residues have been identified to be conserved in this protein. *calB* gene *from P.nitroreducens* Jin1 located in region 107290-108732 (1442bp) was found to contain all the functionally important residues and hence was selected for isolation.

INTRODUCTION

Coniferyl aldehyde dehydrogenase enzyme is member of the family oxidoreductase, which acts on the aldehyde or oxo group of donor with NAD+ or NADP+ as acceptor. The systematic name is aldehyde:NAD^+ aldehydeoxidoreductase. NAD-dependent aldehyde dehydrogenase,NAD-linked aldehydedehydrogenase, propionaldehyde dehydrogenase and aldehydedehydrogenase (NAD)[1]. Aldehyde dehydrogenase belongs to class of NADPH dependent enzymes which catalyze reduction of various phenylpropenyl aldehyde derivatives. These enzymes mainly catalyse the oxidation (dehydrogenation) of aldehydes to carboxylic acid [2]. Aldehyde dehydrogenase gene is found in many organisms from prokaryotes such as bacteria, fungus to eukaryotes such as mammals. They belong to aldehyde dehydrogenase domain (Aldehyde dehydrogenase domain (IPR015590).

Aldehyde dehydrogenase family which consists of enzymes involved in conversion of a broad variety of aliphatic and aromatic aldehydes to their respective carboxylic acids [2-4]. Dehydrogenase enzymes may be dimers or tetramers of identical subunits [3]. It is clear from crystal structures of enzymes that subunits of different ALDHs contain very similar domains like each has catalytic, nucleotide binding and oligomerization domains [5-11].Aldehyde dehydrogenases participate in different metabolic pathways [12] such as glycolysis/ gluconeogenesis[13], ascorbate and aldarate metabolism[14], bile acid synthesis[15],

$$\text{Aldehyde} + NAD^+ + H_2O \rightleftharpoons \text{an acid} + NADH + H^+$$

It is an enzyme that catalyses the oxidation of aldehyde to ferulic acid. Coniferyl aldehyde, NAD^+ and H_2O are the 3 subtrates of this reaction giving rise to 3 products ferulic acid, NADH and H^+. Structurally the enzyme consists of three domains which are: a NAD(P)+ cofactor-binding domain, a catalytic domain and a bridging domain. The catalytic mechanism of enzyme involve cofactor (NAD(P)+) binding, which results in a conformational change and activation of an invariant catalytic cysteine nucleophile. It has been found that ALDH reaction is completed in two steps, acetylation and deacetylation [6,8,16,17]

In first step invariant active site cysteine makes a nucleophillic attack on the carbonyl carbon of the aldehyde molecule (Fig.1) and thus thio-hemiacetal intermediate is formed. From this intermediate hydride ion is transferred from aldehyde to the C4 atom of the nicotinamide ring of the $NAD(P)^+$. This causes a collapse of the thio-hemiacetal to thioester intermediately. In second step an activated water, hydrolyses the thioester intermediate which releases the product. Deprotonation of catalytic cysteine before nucleophillic attack on the substrate, is an essential step in this mechanism. It may be caused by either glutamate 268 or 399[6,8,18,19,20]. The resulting thiolate ion is likely stabilized by the positively charged nicotinamide ring of the coenzyme and/or adjacent main chain amide groups. The proton abstracted by Glu-268 may then go to bulk water[11,21]. In addition, Glu-268 may be most likely residue which activates water molecule in the second step of the reaction [6, 8, 11, 17, 22]. Beside cysteine and glutamate many other residues interact with the $NAD(P)^+$to hold it in place. In reaction, the aldehyde enters the active site through a channel located on the outside of the enzyme. The active site contains a Rossman fold. Interactions between the cofactor and the fold allow the isomerization of the enzyme while keeping the active site functional [5]. In aldehyde dehydrogenase enzyme active site is largely conserved throughout the different classes of the enzyme and although the number of amino acids present in a subunit can change but there is little change in overall function of the site[17,22].

Different organisms have several distinct ALDH genes. In *E. Coli* in one species the largest number of sequences of ALDH present are 13. In human eleven sequences of ALDH are present, excluding the ALDH8 protein [23]. In *Bacillus subtilis* 10 sequences and 14 from various species of *Pseudomonas* are known. Enzyme is used in the bacteria for degradation of toxic aromatic compounds. During degradation, the enzyme's crystal structure shows that intermediates are shuttled directly between the aldolase active site and the acetaldehyde dehydrogenase

Table 1. Protein Sequences used for alignment and functional analysis

	NCBI Accession No.	Name	Organism
1.	ACP17965.1	putative coniferyl aldehydedehydrogenase	*P. nitroreducens* Jin 1
2.	YP_340637.1	aldehyde dehydrogenase	*Pseudoalteromonas haloplanktis*TAC125
3.	NP_783904.1	aldehyde dehydrogenase	*Lactobacillus plantarum* WCFS1
4.	ZP_01625132.1	coniferyl aldehyde dehydrogenase	*marine gamma proteobacterium* HTCC2080
5.	ABO11525.2	Aldehydedehydrogenase	*Acinetobacter baumannii* ATCC 17978
6.	YP_298830.1	aldehyde dehydrogenase	*Ralstonia eutropha* JMP134
7.	YP_001135593.1	aldehyde dehydrogenase	*Mycobacterium gilvum PYR-GCK*
8.	YP_001501975.1	aldehyde dehydrogenase	*Shewanella pealeana* ATCC 700345
9.	ZP_01102424.1	coniferyl aldehyde dehydrogenase	*Congregibacter litoralis* KT71
10.	ZP_01914134.1	aldehyde dehydrogenase	Limnobacter sp. MED105
11.	O86447.3	coniferyl aldehyde dehydrogenase	*Pseudomonassp*. HR199
12	YP_001342112	aldehyde dehydrogenase	*Marinomonas sp.* MWYL1
13.	YP_765012.1	coniferyl aldehyde dehydrogenase	*Rhizobium leguminosarum bv. viciae* 3841
14.	sp|Q9A777.1|	Probable coniferyl aldehyde dehydrogenase	*Caulobacter crecentus* CB15
15.	YP_001241859	coniferyl aldehyde dehydrogenase	*Bradyrhizobium sp.* BTAi1
16.	|ZP_01104105.1|	aldehyde dehydrogenase	*Congregibacter litoralis* KT71
17.	ZP_01625971.1	coniferyl aldehyde dehydrogenase	*marine gamma proteobacterium* HTCC2080
18.	|ZP_01039823.1|	coniferyl aldehyde dehydrogenase	*Erythrobacter sp.* NAP1
19.	|YP_497575.1|	aldehyde dehydrogenase	*Novosphingobium aromaticivorans* DSM 12444
20.	YP_001171157	coniferyl aldehyde dehydrogenase	*Pseudomonas stutzeri* A1501
21.	YP_001155443.	aldehyde dehydrogenase	*Polynucleobacter necessarius subsp. asymbioticus* QLW-P1DMWA-1

active site. Such communication between proteins allows for the efficient transfer of substrates from one active site to the next [24].

P. nitroreducens Jin 1 is an aerobic, gram-negative soil bacterium, first isolated from oil brine in Japan. It was used to isolate bacteria capable of growing on eugenol and isoeugenol [25]. A soil bacterium isolate was found to be capable of utilizing eugenol and isoeugenol as a sole source of carbon and energy and was named jin1 [26]. Based on 16S rRNA analysis, *P. nitroreducens* Jin 1 has been placed in the *P. aeruginosa* group [27]. *P. nitroreducens* Jin 1 is having genomic DNA of size 1, 37,693 bp. *calB* is located in the region 107290-108732 (1442bp) of the genom [26].

Present study involved comparative analysis of various prokaryotic aldehyde dehydrogenase genes available in the NCBI database. The conserved domains present in them point to the functionally active sites and domains of the enzymes. The analysis was utilized for selection and isolation of the gene for further cloning. The analysis revealed *calB* gene sequence from *P. nitroreducens* Jin 1 to be the gene of interest.

Table 2. Sixteen Conserved residues and their putative functions

Residue	Position	Function
Arg(R)	38 (25)	Catalytic activity
Gly(G)	117 (105)	
Asn(N)	126 (114)	Stabilazation during catalysis
Pro(P)	128 (116)	
Gly(G)	143 (131)	
Lys(K)	149 (137)	H-bond
Gly(G)	199 (187)	Binding NAD
Gly(G)	223 (211)	Dipeptide formation
Gly(G)	252 (240)	Position the catalytic nucleophile
Cys(C)	255 (243)	Catalytic activity
Glu(E)	354 (333)	Help in binding NAD
Phe(F)	356 (335)	Binding NAD
Pro(P)	358 (337)	
Gly(G)	404 (383)	
Asn(N)	409 (388)	terminate strand β-12.
Gly(G)	424 (403)	Involved in U-turn region

MATERIALS AND METHODS:

Insilico analysis:

calB sequence was obtained from NCBI. Protein sequence was determined using expasy translate software (www.expasy.org). The sequence was analysed for functional domains using Conserved Domain Database. (www.ncbi.nlm.nih.gov). Domain analysis of translated sequence was done to find conserved residue/s. Different conserved residues as found in the member of SDR (Short dehydrogenase Reductase) family were found in this sequence. Multiple sequence alignment has been carried out for selected members of SDR family. These selected members included Aldehyde dehydrogenase-related sequences of members of Domain family cd07133 (Table No 1), along with *calB* from *P. nitroreducens* Jin 1. The selected sequences were aligned to find the presence of conserved residues amongst these proteins using clustalW software (www.ebi.ac.uk /tools /msa/clustalw2). The available sequences at NCBI database from bacterial genomes have been downloaded as per accession numbers given in Table No.1

Wet Lab Experiment

Further wet lab experiment for isolation of the gene from *P. nitroreducens* Jin 1 was carried out. All chemicals including Nutrient Broth, TNE Buffer, Triton-X, Lysozyme, Proteinase-K, Chloroform-Isoamyl alcohol, absolute alcohol, TE Buffer were procured from Hi-Media.

DNA Isolation from P. nitroreducens Jin 1 *was* carried out by modified protocol given by Goldberg [28]. Inoculated 50 ml nutrient media with 1.% P. nitroreducens Jin 1 culture. The culture was grown at 30°C on shaker for 24 h. Next day transferred 2 mL culture from overnight culture into microfuge. Pelleted the cells by centrifugation at 8000 rpm for 7 minutes. Resuspended and washed the bacterial pellet in 1 mL TNE buffer pipetting gently up and down until the pellet is completely resuspended. Again pelleted the cells by centrifugation at 8000rpm for 7 minutes. Resuspended the pellet in 135 µl TNE buffer. *Then,* added 135 µl of TNE buffer containing 2% Triton X-100. Added 30 µl freshly prepared lysozyme (5 mg/mL), mixed well by tapping the tube. Incubated it in a 37°C water bath for 30 minutes. Added 15 µl proteinase K solution (20 mg/mL). Mixed well by inverting the tube several times. Incubated in a 65°C waterbath for 2 hours. Added Choloroform Isoamyl alcohol in 24: 1 ratio was added in equal amount to the mixture. After mixing, the upper aqueous layer was isolated and again added the Choloform Isoamyl alcohol solution in equal amount. Took upper layer and added chilled ethanol in equal amount. Kept it overnight at 4°C. Next day centrifuged at 12000 rpm for 10 minutes. Washed pellets *with 80% alcohol.* Centrifuged at 8000 rpm for 8 minutes. Discarded the supernatant and stored pellet in 50 µl TE buffer at

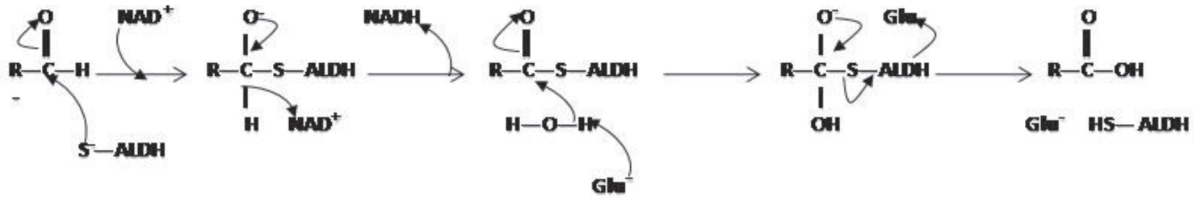

Fig. 1. Molecular mechanism of Aldehyde dehydrogenase Activity.

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gi|227015868|gb|ACP17965.1|         --------MSILGLNGAPVGAEQLGSALERMKKAHLEQGPANLELRLSRL
gi|20137265|sp|O86447.3|            --------MSILGLNGAPVGAEQLGSALDRMKKAHLEQGPANLELRLSRL
gi|152997277|ref|YP_001342112.      --------------MTPTNAHSDLLNTLNQMKQHNMAEGPAGAELRKDRL
gi|116249171|ref|YP_765012.1|       --------MNTITAIATDTSPDVMKALLDRQRASFLKEGPPSIDTRIDRI
gi|20137447|sp|Q9A777.1|            --------MNAPVNLSSDAHKAVMNDVLQRQKAAHLRDGPPSAEKRIQWL
gi|148257274|ref|YP_001241859.      --------MNMTAIPSTEATKARMQATLHRQKAEQTRLGPPSLSLRRDRI
gi|88706400|ref|ZP_01104105.1|      -------MNAPQTATNVADAEMLMQSTLEAQRSDYISEGSVSAATRRDRL
gi|119503889|ref|ZP_01625971.1      -------MTAPEPQLTTADVLTQLNATLNSQKQSYLAEGAVSAEVRMDRL
gi|85708757|ref|ZP_01039823.1|      ---------------MADDKREELERLLDLQRSAFTASRPESMKMRKDRI
gi|87200318|ref|YP_497575.1|        ---------------MKDTTRGEMLAQLERQKAAFTQARPEPLSTRNDRL
gi|146281004|ref|YP_001171157.      --------MSSSQQPDAESTIARMRDVLKRQQAAFIAEGPPSVELRIARI
gi|193076803|gb|ABO11525.2|         -------MESSTLKEYSGSATEHMELIFSKQRANFEADPYPSLEARRTKL
gi|73538463|ref|YP_298830.1|        -------------MVPNTAQEKDLQRWLGKQRAAFMASPFPSLGERRDTL
gi|149925870|ref|ZP_01914134.1      ----------------------MQDVFDCQQRTYRANPHPSKQERISNL
gi|77361062|ref|YP_340637.1|        ---------------MNSKDSGSMAALLQHQQQAHKLQMLVTAAQRRERI
gi|157961941|ref|YP_001501975.      ----------------MTTPINSLTQLLTQQKQAHLASPNPSASERKKRI
gi|119503047|ref|ZP_01625132.1      -------MGAAEHQNLTGFDTQDLDALIKLQRSKFRAEGEVTYATRIDRL
gi|145224915|ref|YP_001135593.      ----------MTTTEQVTVTAESMSSMLERQRAAFVADGPPDVALRRNRI
gi|145588846|ref|YP_001155443.      --------------------MNRFTIQLDEIKAAFAAEPNPTLEVRLERI
gi|88704711|ref|ZP_01102424.1|      MVSVVIATLHHEGEPTVQTTIKTMNAILEQQKAAFQKNPMPTLKERTEWL
gi|28377012|ref|NP_783904.1|        ----------------MMTTINEMHQIFNLQKIQYDRAQPMTYEERVTVL

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gi|20137265|sp|O86447.3|            DRAIAMLLENREAIADAVSADFG-NRSREQTLLCDIAGSVASLKDSREHV
gi|152997277|ref|YP_001342112.      QRAIKLIKENHRDLSEAMSKDFG-HRSVYQSSTADIATTIKLLKDAIENL
gi|116249171|ref|YP_765012.1|       DRVIALLVDHKDAIAAALSEDFG-SRSVEASLLLDVFTCVGSLKYAKAHL
gi|20137447|sp|Q9A777.1|            NRCIDLLVGHQAEIAKAVNQDFG-SRSPEATSLTDVAGSIGPLKHAREHL
gi|148257274|ref|YP_001241859.      ARAISMVLTHKNELLDAMNTDFG-ARSRDMGMLYDIVGSVQPLREAHKNV
gi|88706400|ref|ZP_01104105.1|      QRGIDVCVKYQDKMIDALNTDFS-CRPREVTLLTDVAASITPMKHCMKRL
gi|119503889|ref|ZP_01625971.1      DRALDILVRHAERISEAMNADFV-CRPRQINMMTDVAGSIDCIKHNKKHL
gi|85708757|ref|ZP_01039823.1|      RRAMALLKDHGENLCKAMSADFG-NRAMHQSMITDIDGTINAGKNALSNM
gi|87200318|ref|YP_497575.1|        ERCARLLLQHGEDFARAMSTDFG-HRSHEQSMLTDIMPALSLVRYSQKRM
gi|146281004|ref|YP_001171157.      DKVIALLQDNQQLLCEALASDFS-WRSHDQSLMTDVLLPIAGLKYARKHL
gi|193076803|gb|ABO11525.2|         HQLKKQIIRYQDALAAAINADFS-SRSLDESKLLDLLGSVLEADHAIHHL
gi|73538463|ref|YP_298830.1|        RCLRTAIRGHAHRLAAAAQRDFS-ARAEAETLMVDVLPSVMHINHLLRGL
gi|149925870|ref|ZP_01914134.1      ELLEEIILRNEGELLDAMSEDFG-HRSRHESTMLDIVSSLSAVRHSRKHV
gi|77361062|ref|YP_340637.1|        QQVINLLVEHHQAFAEAIEADFN-GRSQGFSIMNDVLGSLASLKYTRDNL
gi|157961941|ref|YP_001501975.      QQVIDVLVANHSALVTAMEADFG-GRSQGFSLMNDVLGSLGSLKHTRDNL
gi|119503047|ref|ZP_01625132.1      KRLKALILENKVEFANTTKREFNGARSYEFSLFSEFGSKVEAIDYSIKHL
gi|145224915|ref|YP_001135593.      DRLMALVLDNVDDFVDAMGRDFG-TRPKQGSLFTEIMGMISVIEHTRSHV
gi|145588846|ref|YP_001155443.      GRISKMIEVNEEKICKALVADFG-VRDSIETRLVEFQKIYQACAYARKHL
gi|88704711|ref|ZP_01102424.1|      DAIIAMMLEYRPKILDALNTDFG-THPTLQGDFIEVFGMLARAEYNKTKL
gi|28377012|ref|NP_783904.1|        NQIEALIRDNIPELITALQADFG-QRDPLQILSADLTGPLATIAYIKKHL
                                                      117     126 128
gi|227015868|gb|ACP17965.1|         AKWMEPEHHEAMFP-----GAEARVEFQPLGVVGVISPWNFPIVLAFGPL
gi|20137265|sp|O86447.3|            AKWMEPEHHKAMFP-----GAEARVEFQPLGVVGVISPWNFPIVLAFGPL
gi|152997277|ref|YP_001342112.      SNWMQDQPVEAPEP-----GMKAWIQQQPLGVIGIISPWNFPINLSFGPL
gi|116249171|ref|YP_765012.1|       AEWLKPEQHEAL-----FPDAVAEVVYQPKGVVGILSPWNFPYQLALAPL
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gi|88706400|ref|ZP_01104105.1|      EKWMRPEKRPTMFP-LNLLGGRSSIEYQPLGVVGVISPWNFPVNLTFGPL
gi|119503889|ref|ZP_01625971.1      KRWMKSESRPSKFP-LGLLGSRSKIEYQPKGVVGIIAPWNFPVAMVFEPL
gi|85708757|ref|ZP_01039823.1|      DKWAKPEKRKVQFP-LGLMGAKAEVRYEPKGVIGILSPWNFPVNLAFSPL
gi|87200318|ref|YP_497575.1|        KAWSKPEKRHVNFP-LGLLGARAEVRYEPKGVIGIVAPWNFPVGLTLAPL
gi|146281004|ref|YP_001171157.      RQWMKPERRRAELG-AGLLGAKAEIHYQPLGSIGVMTPWNFPVAIAFGPL
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gi|28377012|ref|NP_783904.1|        RKWILPAKQRSGLM--ALTGTKQYVYNEPLGVVGIMSPFNAPIDLALDPA
                                        143    149
gi|227015868|gb|ACP17965.1|         AGIFAAGNRAMLKPSELTPRTSTLLAELIARYFDETELTTVLGDAEVGAL
gi|20137265|sp|O86447.3|            AGIFAAGNRAMLKPSELTPRTSALLAELIARYFDETELTTVLGDAEVGAL
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                                                199                   223

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gi|88704711|ref|ZP_01102424.1|    FPALPFDHLLYTGNPGVARQVAAAAAPNLTPLTLELGGKSPTVFTPGGVT
gi|28377012|ref|NP_783904.1|      FSALDWDKLVFTGGSETGKKVLSAAAPNLTPVVLELGGKNPAVVLPDANK
                                                252 255

gi|227015868|gb|ACP17965.1|       ADVAQRVLTVKTFNAGQICLAPDYVLLPEESLDSFVAEATRFVAAMYPTL
gi|20137265|sp|O86447.3|          ADVAQRVLTVKTFNAGQICLAPDYVLLPEESLDSFVAEATRFVAAMYPSL
gi|152997277|ref|YP_001342112.    VQAAERIMTVKTFNVGQICISPDYLLLPEDKVETFVEAAKSFVQSSFPTM
gi|116249171|ref|YP_765012.1|     ADAARRVMTVKTLNAGQICLAPDHVYVPEESVEAFAGHAVAAVGAMYPAL
gi|20137447|sp|Q9A777.1|          ATAAARVMNGKTLNAGQICLAPDYVLAPQEDVEAFVKEAQAAVSRYFPTI
gi|148257274|ref|YP_001241859.    GKTAARIMMGKMINAGQVCVAPDYVLAPDDRVEPFITAATTAVAKMFPTL
gi|88706400|ref|ZP_01104105.1|    KMAAQRIMLGKTLNAGQICLAPDYLMVPEERLSEVVETLKAVTEEMYPKL
gi|119503889|ref|ZP_01625971.1    ELSLERIMSGKCLNAGQICIAPDYLMVPEESLDEVIKITQSVVGQMYPTL
gi|85708757|ref|ZP_01039823.1|    AKAGERIALGKMMNAGQICLAPDYMYVPEESEDEAIAGVTNAASTMYPTV
gi|87200318|ref|YP_497575.1|      EQAAQRIALGKMMNAGQICLAPDYMLVPEDMEERAIGAVSASVAQMYPTL
gi|146281004|ref|YP_001171157.    DNAARRIWGGKGISSGQACIAPDYVLVHEDDRERLLVSMQAELPRMFPTI
gi|193076803|gb|ABO11525.2|       ADAAKRITHGKATNSGQICVAPDYALVPKESIDEFVDAAKSSFIKMFGQS
gi|73538463|ref|YP_298830.1|      RTAATRIAHGKTVNGGQICVAPDYALVPAEDAEPFAQAVLDAASQMHPQ-
gi|149925870|ref|ZP_01914134.1    AQAAACAAHGKMINAGQVCVAPDYAMVHESQLRRFISEVKRASHKQYPTI
gi|77361062|ref|YP_340637.1|      SESCKRLALAKSNNGGQVCVSPDIVYVPRNKLDQVAALLLENFSHFYPEI
gi|157961941|ref|YP_001501975.    DQACYRLAIAKGTNGGQICVSPDIIYLHQENLEQGISTLKQVFSSLYPSI
gi|119503047|ref|ZP_01625132.1    ELAGTRLTFSKFLNGGQLCLSPDYVLVPRELEEQLVARVIHEAQSMYPNV
gi|145224915|ref|YP_001135593.    RRAATRIAQGRMINGGQVCVCPDYVFVPAARIDAFVAVARETLQAMFPTI
gi|145588846|ref|YP_001155443.    KEVASSIAYGKLLNSGQTCIAPDYVLIEQEQRDTFIQELQAAAQKQFSDP
gi|88704711|ref|ZP_01102424.1|    AANVADCLRIKMIKNGQMCVSPDYALVPRADLENFVSLAKAFISKEAPDY
gi|28377012|ref|NP_783904.1|      QMTAAKIARSRSGNSGQICLDTDYVLLPTDQLDEYVGLITAQLAQVFPTY

gi|227015868|gb|ACP17965.1|       L-DNPDYTSIINARNFDRLHRYLTDAQAKGGRVIEINPAAEELGDS-GIR
gi|20137265|sp|O86447.3|          L-DNPDYTSIINARNFDRLHRYLTDAQAKGGRVIEINPAAEELGDS-GIR
gi|152997277|ref|YP_001342112.    Q-ESPDYTSIISDNHYKRLIGLLEDAKEKGANVISLAAEGEVDYDE-SSR
gi|116249171|ref|YP_765012.1|     Q-ENPDYTSIVNARHLARIEGLIDDARAKGARIVEINPAAENFSQQ-SAH
gi|20137447|sp|Q9A777.1|          K-DNPDYTAVVAQRHYDRVKGYVDDARAKGARVIEINPAGEDLSQQ-EHR
gi|148257274|ref|YP_001241859.    K-DNPDYTSIITQRHYERLQMLIDDARAKGARIVEINPAKENFSQQ-QFR
gi|88706400|ref|ZP_01104105.1|    L-DNPEYTSIVNERHFQRLNSYLADAAERGVTATPINPANEDFSTQNGTF
gi|119503889|ref|ZP_01625971.1    L-DNPQYTSVINQRHHDRLSGYLADAESQGLQVIPINPADESFETS-TSM
gi|85708757|ref|ZP_01039823.1|    L-ENDDYASIVSDRHFERLQGLVEDARDKGAEVIEVNPAGENFASA-NQR
gi|87200318|ref|YP_497575.1|      L-ANDDYTSVINRRHRDRLVGLVDDAVAKGAEAIVVNPGGENFEGS-NGN
gi|146281004|ref|YP_001171157.    R-NNPDYTAMATPGQYQRLQSYLGDAEALGVRCIEINPAGEDLSSV---R
gi|193076803|gb|ABO11525.2|       IENNENYTSIVNDRHLKRIQDILTDAQEKGARIIPC--DTYSFDQQ--GR
gi|73538463|ref|YP_298830.1|      --GSDDYTAVIHDGALARQRALLDDARALGARVFSA--AMPSG-----GR
gi|149925870|ref|ZP_01914134.1    R-NNPDVSCVINSKHMARLRDLVEDARKKGAEIIEINPAHEVFEPS--AK
gi|77361062|ref|YP_340637.1|      T-HNPDFVPSVNRQHCQRVVDLVEQARDAGCQVLSSHEVANLAELVTSDK
gi|157961941|ref|YP_001501975.    S-GNQDLVPIVNQAHFERVESYINDAKDKGVRVESSHQTS----ADAMTR
gi|119503047|ref|ZP_01625132.1    T-ENEDYAGVINERHFARLQNYIDDAVAKGAKLTIVGADQTR--ASEDNR
gi|145224915|ref|YP_001135593.    V-ANDDFCSSVNEANFERVVGLIEDARAKGARVESVAPAGESLPDP-GTR
gi|145588846|ref|YP_001155443.    K----ELTGPIDDEQLQYWQHLVSDAIDRGAKVIPLLNNPSAG-----AR
gi|88704711|ref|ZP_01102424.1|    V-AGDDITGIINDRHYARIESILDDARTRESQIEQLTGESDAAARR----
gi|28377012|ref|NP_783904.1|      I-NNSEYSAIINDRSYDRILGMLTEARQRGTRLITINPNNELVPYV-KRR
                                                  354 356 358

gi|227015868|gb|ACP17965.1|       KIAPTLIVNVSDEMLVLNEEIFGPLLPIKTYRDFDSAIDYVNSKQRPLAS
gi|20137265|sp|O86447.3|          KIAPTLIVNVSDEMLVLNEEIFGPLLPIKTYRDFDSAIDYVNSKQRPLAS
gi|152997277|ref|YP_001342112.    KIAPFLIFNVNDDMAIMQEEIFGPLLPVKTYARPEETVAYINSNPRPLAA
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Fig. 2. Multiple sequence alignment conserved residue are shown in red colour

-20°C. *Qualitative analysis of DNA was carried out by loading in Agarose gel. Quantity of genomic DNA was analysed spectrophotometrically.*

PCR Primers have been designed using Invitrogen primer designing (www.invitrogen.com) tool Oligoperfect Designer and checked for their properties using Gene Runner V3.05 (www.generunner.net). For amplification of *calB* gene the primers synthesized by Geneart, Germany were used.

calB Forward primer 5'**AAGCTT** CCTCTACCAC GGTTCTAATCACTCC 3'

calB Reverse Primer 5'**CTGCAG** TAAGGAGGT GATAAGCGATGAGCATTCTTGGTTT

GA3'.Bold alphabets denote HindIII and Pst1 restriction sites. Underlined sequence denotes Shine-Dalgarno sequence. Using primers and genomic DNA the gene was amplified. Annealing was done at 60°*C.* After amplification 5µl of PCR product was loaded on agarose gel for confirmation of size.

RESULTS

Typically ALDH range in size from 450 to 520 amino acids. For sequence similarity studies multiple-alignment using clustalW included full length sequences starting with N terminal to C terminal. ClustalW alignment has been shown in Fig.2 The alignment reveals (Table No.2) sixteen invariant residues which are Arg38(25), Gly117(105), Asn126(114), Pro128(116), Gly143(131), Lys149(137), Gly199((187), Gly223(211),

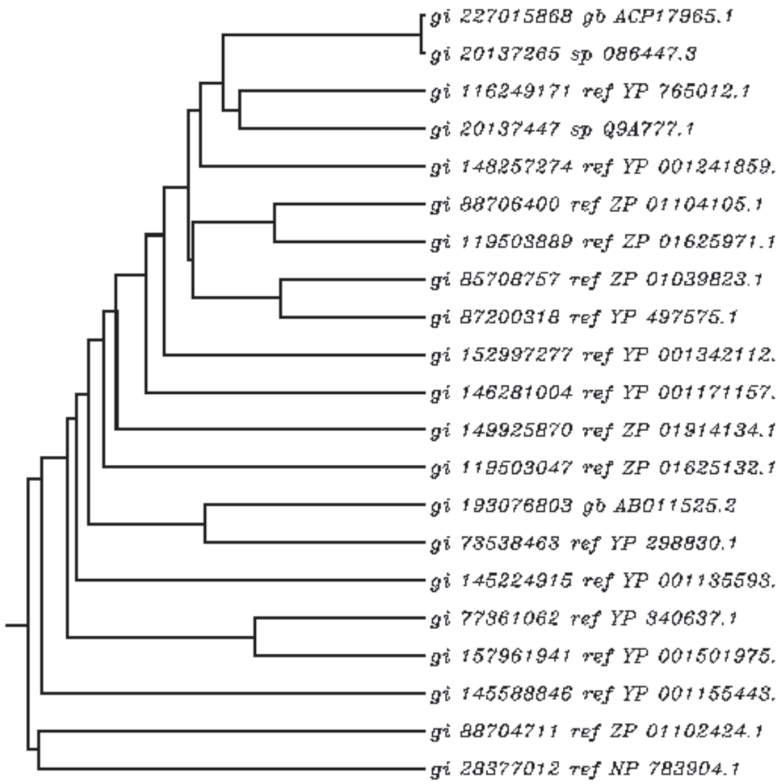

Fig. 3. Phylogenetic Tree of Protein Domain cd07133 and *P. nitroreducens* Jin 1

Gly252(240), Cys255(243), Glu354(333), Phe356(335), Pro358(337), Gly404(383), Asn409(388), Gly424(403). A phylogenetic tree was generated from alignment of sequences obtained from ClustalW. Tree has been shown in Fig.3. This tree has been formed by multiple alignment of sequences from some members of domain family cdo7133 and *P.nitroreducens* Jin1 which aligned with members of this domain. The PCR amplification product obtained was 1467 bp in size and was eluted from agarose for further restriction digestion and cloning (Fig.4).

DISCUSSION

There are 16 invariant residue in ALDH. Out of 16, 14 residue are conserved in all the genes analysed. Lys is replaced by Val in only one and Pro337 is conserved in 80%. Number within bracket is according to model protein whose PDB structure is available.[29] The glycines and prolines, represent 9 of the 16 highly conserved and invariant residues, all lie at critical turns in the class 3 ALDH structure. [29] For example, Gly223 is the first glycine of a Gly-Gly dipeptide that marks the boundary between the coenzyme-binding and catalytic domains. Also, Gly424 is involved in the "U-turn" region.[5] Glycines are over represented among the conserved residues in ALDHs. Similar observations have been made in other enzyme families, such as short- and long-chain alcohol dehydrogenases and "Rec-A like" proteins [30-32]. The cysteine 255 that provides the catalytic thiol and its closest neighbor in space, an asparagine residue, are conserved in all ALDHs with demonstrated dehydrogenase activity. Cephalopod V-crystallins have arginines present at this position, but have been reported to lack ALDH activity. [33]

Gly199 and Phe356 are integral for binding the nicotinamide

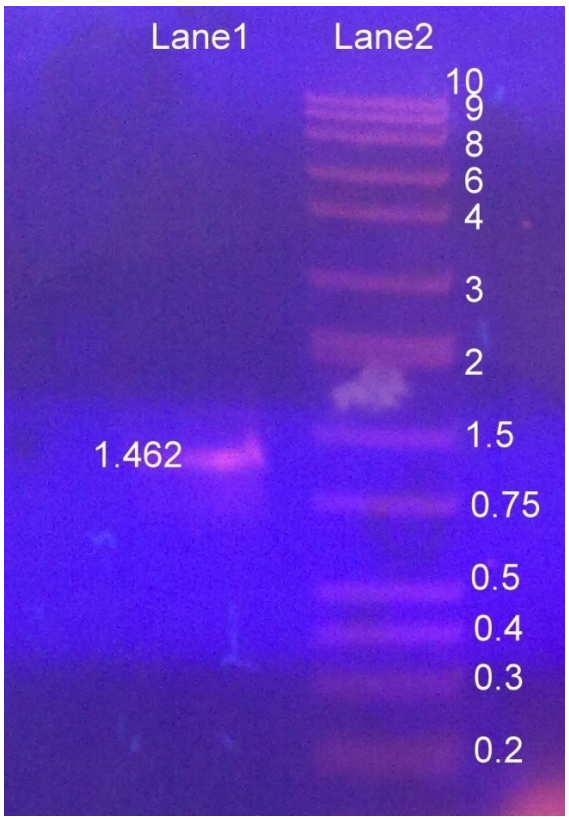

Fig. 4. PCR product of 1467 bp of calB gene was observed on 1% Agarose gel.

ring of NAD. By its extreme Ramachandran angles,[5] Gly252 appears necessary to position the catalytic nucleophile,Cys255. Glu354 has been proposed to act in binding NAD [6,34]. Asn126 has been proposed to stabilize the carbonyl oxygen of the substrate aldehyde during catalysis [6, 35]. Only two sequences from molds lack this residue, with Glu present instead. Alignment of these residue has been shown in Fig.2. Coloured letters show conserved residues.

In addition to the 16 residues noted above, 37 residues, including all of the remaining invariant residues identified previously [36] were conserved in at least 80% of the sequences. Thus, only about 10% (53 out of 500) of all residues in ALDHs are conserved above the 80% level. Twelve of the 16 residues has been clustered into 7 of the 10 most conserved motifs in ALDH. These motifs cluster around the active site of the enzyme. Motif 4 covers the essential NAD-binding turn of the Rossmann fold, between ß -4 and α -D in the class 3 ALDH PDB structure. The first glycine in this turn, Gly199 is invariant in ALDHs, as well as in the Rossmann folds of several other dehydrogenase families [37]. The only motif with multiple invariant residues, Glu354and Phe356 is Motif 8 that helps in binding NAD.

Motif 5 contains both Glu221 conserved in just less than 95% of the 145 ALDHs and proposed to act as a general base [22,38], and also the GlyGly (423424) dipeptide boundary between the coenzyme-binding and catalytic domains. Motif 6 includes the invariant Gly252 and the catalytic thiol, Cys255. The residue nearest to the catalytic thiol, Asn126 discussed above, is centered in Motif 1, the most conserved motif in ALDHs. The intriguing "U-turn" spanning ß -12 and α -14 is encoded in Motif 10 [5]. Overall, the 10 motifs reside at or near the active site of the molecule. A large portion of the ß -sheet structure is highly conserved vs. very little helical structure. Nearly all motifs contain a turn or loop with a well-conserved small amino acid residue such as glycine, proline, aspartic acid, or asparagine. The well-conserved large hydrophobic amino acid side chains in these motifs often point away from the rest of the motif and appear to anchor these elements to the core of the protein.

The phylogenic tree which has been shown in Fig.3. displayed two main trunks. *Lactobacillus plantarum* WCFS1 (NP_783904.1) and marine *gamma proteobacterium* HTCC2080 (ZP_01102424.1) are members of one group and are different from all other bacteria; but they also have all conserved residues of aldehyde dehydrogenase domain. The phylogenetic Tree represents protein sequence similiarity between *P.nitroreducens* Jin1 (ACP17965.1) and *Pseudomonassp* HR199 (086447.3).

The primers were designed not only to amplify the gene but to have the desired gene ready for cloning. Keeping this in view the primers were designed with restriction enzyme sites at the ends. In forward primer Hind III restriction site and in reverse primer Pst1 restriction site is present. Further for facilitating expression of the gene insert the Shine -Dalgarno sequences were appropriately positioned

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