British Biotechnology Journal
4(6): 684-695, 2014

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Domain Analysis and Isolation of Coniferyl Alcohol Dehydrogenase Gene from *Pseudomonas nitroreducens* Jin1

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Authors' contributions

This work was carried out in collaboration between both authors. Author PPB designed the study. Author DK analyzed the data. Both authors contributed equally to the study. Both authors read and approved the final manuscript.

Short Research Article

Received 14th November 2013
Accepted 20th April 2014
Published 7th June 2014

ABSTRACT

Aim: The Aim of present study is to analyse conserved functional Short Dehydrogenase Reductase (SDR) domain from bacteria. Based on the domain analysis selection of coniferyl alcohol dehydrogenase gene for isolation from *Pseudomonas nitroreducens* Jin1.
Place and Duration of Study: Department of Biotechnology, Punjabi University, Patiala. From July, 2012 to November, 2012.
Methodology: Bioinformatics tools were used to analyse various *calA* genes from bacteria based on the presence of conserved domain in members of SDR family. Based on insilico analysis, *Pseudomonas nitroreducens* Jin1 *calA* was selected. PCR was used for amplification of the gene from the genome of *Pseudomonas nitroreducens* Jin1.
Result: Multiple sequence alignment results for conserved domains amongst members of SDR family identified presence of all domains of Short Dehydrogenase Reductase members in *Pseudomonas nitroreducens* Jin1 *calA* gene. Amongst the various sequences compared the *P. nitroreducens* Jin1, *calA* was found to be the smallest in size. The locus of *calA* in the genome resides at 103513-104280 bases. It was amplified from the genome of *Pseudomonas nitroreducens* Jin1. The *calA* gene that was amplified is of size 768bp.
Conclusion: *calA* gene isolated from *Pseudomonas nitroreducens* Jin1 is a small gene with all the functional domains and can be used for biotransformation of coniferyl alcohol to coniferyl aldehyde.

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Keywords: Coniferyl alcohol dehydrogenase; Pseudomonas nitroreducens Jin1; coniferyl alcohol; coniferyl aldehyde.

1. INTRODUCTION

A group of isozymes that catalyses the oxidation of primary and secondary alcohol to aldehyde and ketones respectively are known as alcohol dehydrogenase (ADH). ADH can also catalyse the reverse reaction. These are found in both prokaryotic and eukaryotic organisms. Reduction/oxidation (Redox) reaction involves the coenzyme nicotinamide adenine dinucleotide. ADH also catalyzes the reverse reaction which is a part of fermentation reaction to ensure a constant supply of NAD^+ in yeast [1], plants [2] and many bacteria. Alcohol dehydrogenase (ADH) was first isolated and purified by Negelein and Wuluff from *Saccharomyces cerevisiae* (baker's yeast) [1]. Animal and bacterial ADH showed different properties such as the yeast enzyme is double the size of animal ADH and its activity is 100 times more. The yeast enzyme is more specific than mammal ADH [3].

Different ADH have been divided between short-chain dehydrogenase, medium- chain dehydrogenases and long-chain dehydrogenases/reductases on the basis of distinct sequence motifs, protein chain length, mechanistic features and structural comparisons [4,5,6]. From study of different genomes, it has been found that about 1/4 of all dehydrogenases found are Short Dehydrogenase Reductase (SDRs). This superfamily was found to be the largest, having more than 60,000 non-redundant sequences (over 30,000) of the 'classical' type and close to 30,000 of the 'extended' type [7]. Currently more than 47,000 primary structures are found in this superfamily. These sequences are available in sequence databases and over 300 crystal structures are now deposited in the Protein Data Bank [8]. All the members of SDR show early divergence, most of family members show only low pair-wise sequence identity (typically 20–30%), but have several properties in common [9,10].

SDR superfamily is present in all domains of life. Of the large number of completely sequenced bacterial genomes (close to 400 in January 2007), about 3/4 of all known SDR forms are of bacterial origin [11]. The members of SDR family belong to domain cd05328 of NCBI Conserved Domain Database (www.ncbi.nlm.nih.gov). The short name of this domain is alpha_HSD_SDR_c. Pair-wise sequence identity amongst different SDR enzymes is typically 15-30%. All available 3D structures (27 deposited in PDB at present) show a high similarity of α/ß folding pattern with the Rossmann-fold being present in all. The Rossmann fold is characterised by presence of a typical central ß-sheet which is flanked by α-helices. The fold is also common to other subdivision of oxidoreductases. The active site is constituted from a triad of catalytically important Ser, Tyr, Lys residues. Out of these three, Tyr is found to be the most conserved residue within the whole family [12]. NNAG around position 86-89, singular Asp around position 60, another Asn around position 111 and an IRVN sequence preceding a P-G motif around position 180, followed by a conserved Thr around position 188 [12,13]; are other conserved sequences found in members of SDR. The functions of all these residues have been found by combinations of chemical modifications, sequence comparisons, their structure analyses and site-directed mutagenesis [5,6,13-15]. In the 3D SDR structure, all these sequence motifs are assigned to three different regions. Namely (i) The coenzyme binding region with the central ß-sheet, (typical of a Rossmann-fold) (ii) The active site and (iii) Sections proximal to the substrate binding region. A core structure in most of SDR enzymes is 250-350 residues [12] in length, which have N - or C - terminal trans - membrane domains or signal peptides, or form parts of multi-enzyme complexes [11].

There are three major classes of microbial alcohol dehydrogenases depending on categorised specificities [16].

1. NAD (P) dependent enzyme which are futher subdivided in to three classes; Zn-dependent alcohol dehydrogenase. Zn-independent alcohol dehydrogenase. Iron activated alcohol dehydrogenase.
2. NAD (P) independent alcohol dehydrogenase which are pyralloquinoline quinine, heme or cofactor F420.
3. FAD dependent Alcohol Dehydrogenase (ADH).

Coniferyl alcohol dehydrogenases (CADH) are mostly NAD dependent. It occurs in many prokaryotic as well as eukaryotic species. In eukaryotes, it is mainly found in the plants such as sorghum, sweet potato, oryza, populous, *Populus, Aspergillus nidulans* etc [17] and known as Cinnamyl alcohol dehydrogenase (CAD). CAD participates in phenyl-propanoid biosynthesis and catalyses the reversible conversion of p-hydroxy cinnamaldehydes to its corresponding alcohol, which further leads to the biosynthesis of lignin in plants [17,18]. Cinnamyl alcohol dehydrogenase plays a role in synthesis of coniferyl alcohol in tabacco xylem of *Eucalyptus gunnii* [18]. As CAD was isolated from periderm tissue of *Eucalyptus gunnii***,** it was used for production of monolignols used in synthesis of lignin [19]. In *Aspergillus nidulans* [20], it is involved in conidial germination and cell wall morphogenesis. Its role is not very well defined beyond plants and its molecular mass is yet to be established.

Sequential degradation pathway of eugenol has been found in different species of bacteria such as *Acinetobacter* [21], *Corynbacterium* [22], *Streptomyces* [23,24] and *Helicobacter pylori* [25]. Coniferyl alcohol dehydrogenase (CADH) has been studied in many bacteria such as *Rhodococcus erythropolis* [26], *Pseudomonas sp*. HR199 [27] and plants [2]. From *Helicobacter pylori,* the gene for cinnamyl alcohol dehydrogenase (HpCAD) was cloned in *Escherichia coli*. The recombinant enzyme was characterized for substrate specificity (HpCAD) and found to be a monomer of 42.5kDa located predominantly in the cytosol of the bacterium [25]. This enzyme uses NADP (H) specifically as cofactor and has abroad substrate specificity for alcohol and aldehyde substrates. The enzyme was found to be capable of catalysing the dismutation of benzaldehyde to benzyl alcohol and benzoic acid. This dismutation reaction has not been shown previously for this class of alcohol dehydrogenase and provides the bacterium with a means of reducing aldehyde concentration within the cell [25]. In *Streptomyces* NL15-2K, two isoform of CADH have been found which are CADH1 and CADHII. CADH1 has high substrate affinity for cinnamyl alcohol than coniferyl alcohol. It also favours aromatic alcohols without 4-hydroxy-3-methoxy groups. CADHII acts on only coniferyl alcohol, cinnamyl alcohol and 3-(4- hydroxyl-3-methoxyphenyl)-1-propanol. Molecular mass of native CADH from *Streptomyces* (CADHII), *Rhodococcus* and *Pseudomonas* was found to be 151kDa, 200kDa and 54.9kDa respectively. CADH from *Pseudomonas* is a dimer composed of two identical subunits with identical molecular mass of 27 kDa (27). CADHII is tetrameric. Thisshows molecular mass and quartenary structure vary among bacterial enzymes. In *Rhodococcus* sps., subunits are not identified. Coniferyl alcohol is the best substrate for enzymes from both *Streptomyces* and *Rhodococcus*.

Coniferyl alcohol dehydrogenase (calA) gene has been isolated and characterized from many prokaryotes such as *Escherichia coli*, *Pseudomonas sp.* HR199 [28] and *Ralstonia eutropha* [29]. While in some others it is known but not cloned for e.g. *P. nitroreducens* Jin1. So present study has selected *calA* from *P. nitroreducens* Jin1 for cloning. A comparative

analysis of the conserved domains present in various *calA* genes occurring in prokaryotes, was planned and carried out. It was an indirect way of assessing the functionality of the enzyme. The smallest sized protein with all the requisite functional residues was then selected for isolation. The comparative analysis of various prokaryotic CADH gene sequences helped in selection of gene *calA* from the genome of *P. nitroreducens* Jin1 for isolation using PCR.

First of all, *P. nitroreducens* Jin1 was isolated from oil brine in Japan. It is an aerobic, gram-negative soil bacterium and was capable of growing on eugenol and isoeugenol [30] as a sole source of carbon and energy and was named Jin1 [31]. Based on 16S rRNA analysis, *P. nitroreducens* Jin1 has been placed in the *P. aeruginosa* group [32]. *P. nitroreducens* Jin1 is having genomic DNA of size 1,37,693 bp. It has different genes like *lolC, lolD, lolE, gpd, calB, calA.* Coniferyl alcohol dehydrogenase (*calA*) gene under study is located in region 103513-104280 and is constituted of 768 bp [32]. *calA* codes for coniferyl alcohol dehydrogenase which carries out conversion of coniferyl alcohol to coniferyl aldehyde in the presence of NADH (Fig. 1).

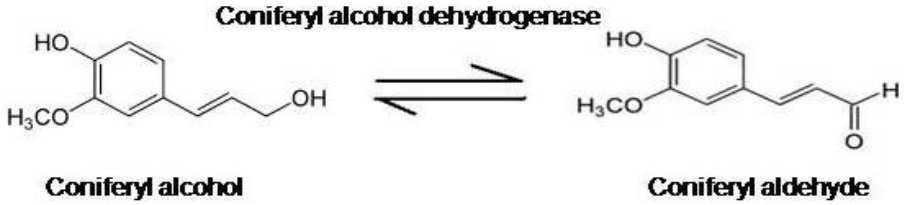

Fig. 1. Bioconversion of coniferyl alcohol into coniferyl alcohol aldehyde

This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with NAD^+ or $NADP^+$ as acceptor. The systematic name of this enzyme is coniferyl- alcohol:$NADP^+$ oxidoreductase. Coniferyl alcohol is the main product in the biotransformation of eugenol to ferulic acid. Eugenol can be used as a source for the production of ferulic acid and *calA* gene product oxidises coniferyl alcohol to coniferyl aldehyde, which is then oxidized to ferulic acid [33].

2. MATERIALS AND METHODS

2.1 In silico Analysis

calA sequence was obtained from NCBI. Using expasy translate software, protein sequence was obtained (www.expasy.org) (Fig. 2).

The functional domains analysis using Conserved Domain Database (www.ncbi.nlm. nih.gov) was carried out to find conserved residue/s. Different conserved residues as found in the members 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 Alcohol dehydrogenase-related sequences of members of Domain family cd05328 (Table No 1), along with *calA* from *P. nitroreducens* Jin1. 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.

MQLTNKKIVV**TGVSSGIG**AETARVLRSHGATVIGVDRNMPSLTLDA
FVQADLSHPEGI**D**KAISQLPEKIDGLCNIAGVPGTADPQLVANV**NYL**
GLKYLTEAVLSRIQPGGSIVNVSSVLGAEWPARLQLHKELGSVVGF**S**
EGQAWLKQNPVAPEFC**YQYFK**EALIVWSQVQAQEWFMRTSVRM**N**C
IA**PG**PVF**T**PILNEFVTMLGQERTQADAHRIKRPAYADEVAAVIAFMC
AEESRWINGINIPVDGGLASTYVStop.

Fig. 2. Translation Product of *calA* gene from *P. nitroreducens* Jin 1 (Bold letter shows conserved residue)

Table 1. Protein Sequences used for alignment and functional analysis

S. No.	Accession no	Name	Organism
1.	ACP17962.1	Putative coniferyl aldehyde dehydrogenase	*Pseudomonas nitroreducens* Jin1
2.	NP_737721.1	3-alpha-hydroxysteroid dehydrogenase	*Corynebacterium efficiens* YS-314
3.	YP_764993.1	3-alpha-hydroxysteroid dehydrogenase	*Rhizobium leguminosarum* bv. viciae 3841
4.	ZP_00650764.1	Short-chain dehydrogenase/reductase	*Xylella fastidiosa*
5.	YP_952831.1	Short-chain dehydrogenase/reductase	*Mycobacterium vanbaalenii* PYR-1
6.	114793713	3-alpha-hydroxysteroid dehydrogenase	*Pseudomonas sp. B-0831*
7.	13096535	3-alpha-hydroxysteroid dehydrogenase	*Comamonas Testosteroni*
8.	13096537	3-alpha-hydroxysteroid dehydrogenase	*Comamonas Testosteroni*

2.2 Gene Isolation

Further wet lab experiments for isolation of the gene from *P. nitroreducens* Jin1 were carried out. All chemicals including Nutrient Broth, TNE Buffer, Triton-X, Lysozyme, Proteinase-K, Chloroform-Isoamyl alcohol, absolute alcohol, TE Buffer were of molecular biology grade, procured from Hi-Media.

DNA Isolation from *P. nitroreducens* Jin1 was carried out by modified protocol given by Goldberg in 2002 [34]. Culture was grown in NB media at 30°C overnight. 50ml nutrient media was inoculated with *P. nitroreducens* Jin1 culture. The culture was incubated at 30ºC on roller for 24h. Next day, 2ml of culture was transferred from this overnight culture into 2ml microfuge. The cells were pelleted by centrifugation at 8000rpm for 7 minutes. Resuspended and washed the pellet in 1ml 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 135µl TNE buffer. 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 at 37°C in water bath for 30 minutes. Added 15 µl proteinase K solution (20mg/mL). Mixed the solution well by inverting the tubes several times. Incubated in a 65ºC waterbath for 2 hours. After incubation, added Choloroform Isoamyl alcohol in 24:1 ratio in equal amount. After mixing, took the upper aqueous layer and 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. Centrifuged at 12000rpm for 10 minutes. Pellet was formed. Discarded the supernatant. Washed pellet with 80% alcohol and stored the pellet of genomic DNA in 50µl TE buffer at - 20ºC. Qualitative analysis of DNA was carried out by loading in Agarose gel. Quantity of genomic DNA was analysed spectrophotometrically. PCR Primers have been designed for amplification of *calA* gene, using Gene Runner V3.05. The primers were synthesized by Geneart, Germany.

Forward Primer 5'-CTGCAGGAGCATGCAACTGACCAACAAG-3'
Reverse Primer 5' GGATCC**TAAGGAGGTGA**GAACTTACACGTAGGTCGATGCC-3'

RE sites (green colour) for Pst1 and BamH1 were placed 5' to primer sequences in order to facilitate expression. Shine-Dalgarno sequence (red colour) have been inserted in the reverse primer to ensure translation of the insert. Using these primers and genomic DNA the gene was amplified. The PCR protocol followed included denaturation at 95ºC initially for 5 mins and during 25 subsequent cycles for 30 secs annealing at 60ºC for 30 secs and extension at 72ºC for 60 secs. After amplification, 5µl of PCR product was loaded on agarose gel for qualitative analysis.

3. RESULTS

3.1 Conserved Domain Analysis

Domain analysis on NCBI shows that cd05328 is a conserved domain. This is known as alpha-hydroxy steroid dehydrogenase. Dehydrogenase catalyses the NAD-dependent oxido-reduction of alpha hydroxyl steroids. Coniferyl alcohol dehydrogenase belongs to this group. Multiple sequence alignment for conserved domain analysis is done of members of cd05328 domain and *P. nitroreducens* Jin1 (Fig. 3).

All the conserved residues and sequence motifs which are found in members of SDR are also found in *calA* gene of *P. nitroreducens* Jin1 (Table 2). The criterion for SDR membership is found to be the occurrence of typical sequence motifs which are arranged in a specific manner. These motifs comprise Rossmann-fold, elements for nucleotide binding and other specific residues for the active site. These specific residues also include its highly conserved triad of Ser, Tyr and Lys residues (Table 2).

N-Terminal amino acid sequence of *Pseudomonas sp.* HR199 is found to be MQLT NKIVVV. Same N-terminal sequence has been found by translation of *calA* gene from *P. nitroreducens* Jin1 using expasy translate software. Table 2 shows all the conserved residues to be present in *P. nitroreducens* Jin1. The position of different motifs however differ slightly from those found in 17 ß-hydroxysteroid dehydrogenase (3 ß /17 ß –HSD) of *C. testosteroni* or to homologous positions (PDB code 1hxh). Positions within bracket refer to residue numbering of *C.testosteroni* starting with N-terminal TGXXXGXG (12-19)11-18, Aspartic acid (60)59, NXXG (86-89)91-94, Asparigine (111)114, S-Y-K triad (138,151,155)

140,157,161, Asparigine (179)184, Proline (183)188, Glycine (184)189, Threonine (188)193. The positions outside bracket are those of the CADH of *P. nitroreducens* Jin1. A phylogenetic tree shown in Fig. 4 has been obtained for members of domain cd05328 and *P. nitroreducens* Jin1.

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gi|227015865|gb|ACP17962.1          -----MQLTNKKIVVTGVSSGIGAETARVLRSHGATVIGVDRNMPSLTLD
gi|71274476|ref|ZP_00650764.1       -----MNLQNKTVVITGVASGIGSEVARLARFHGATVIGIDCRPVTITLH
gi|116249152|ref|YP_764993.1        MRKVTFMLFGKTILVTGVASGIGARTAELAGQMGAEVIGVDVREPVGGSA
gi|120403002|ref|YP_952831.1        -----MQVEKRRIVVVGAGSGIGAAVAAHFHDNGDHVLAVDLRPNDTPAA
gi|13096535|pdb|1FJH                ---------MSIIVISGCATGIGAATRKVLEAAGHQIVGIDIR--DAEVI
gi|13096537|pdb|1FK8                ---------MSIIVISGCATGIGAATRKVLEAAGHQIVGIDIR--DAEVI
gi|114793713|pdb|2DKN               ---------MSVIAITGSASGIGAALKELLARAGHTVIGIDRG--QADIE
gi|25027667|ref|NP_737721.1         -----MVFMSRTIVVTGAASGIGQATAQALEDAGDRVIRVDLK--EGDVT

gi|227015865|gb|ACP17962.1          AFVQADLSHPEGIDKAISQLPEKIDGLCNIAGVPGTADPQ-LVANVNYLG
gi|71274476|ref|ZP_00650764.1       GFFHADLGDPASIDALIAQLPERIDVLANIAGVPGTVAAD-LVAKVNYLG
gi|116249152|ref|YP_764993.1        AFIKGDLSTAAGVAEIVAQLPARLDALANVAGLSGSTGVV-STLAVNFYG
gi|120403002|ref|YP_952831.1        AHAQCDLRDPQSIAGLLDDIGADWDVLAHVAGVPGTAPAD-DVLTVNYLG
gi|13096535|pdb|1FJH                ADLSTAEGRKQAIADVLAKCSKGMDGLVLCAGLGPQTKVLGNVVSVNYFG
gi|13096537|pdb|1FK8                ADLSTAEGRKQAIADVLAKCSKGMDGLVLCAGLGPQTKVLGNVVSVNYFG
gi|114793713|pdb|2DKN               ADLSTPGGRETAVAAVLDRCGGVLDGLVCCAGVGVTAANSGLVVAVNYFG
gi|25027667|ref|NP_737721.1         ADLGTKEGVAAAIEKITGLSDGVLDGLVANAGVS---HPTELALKINYFG

gi|227015865|gb|ACP17962.1          LKYLTEAVLSRIQPG---GSIVNVSSVLGAEWPARLQLHKELGSVVGFSE
gi|71274476|ref|ZP_00650764.1       LRHLTQALLPRLAPG---GNVINMTSLLGAEWQQRLELHKQLAATPDFAA
gi|116249152|ref|YP_764993.1        LRALSEAVAPRLREG---GAIVNVASIAGYGWRANLERAKSLTAIEGFPE
gi|120403002|ref|YP_952831.1        MRKMVEGMLPRMRRG---GSVVAVASIAGIGWEQRLDELSELLAATDPEA
gi|13096535|pdb|1FJH                ATELMDAFLPALKKGHQPAAVVISSVASAHLAFDKNPLALALEAGEEAKA
gi|13096537|pdb|1FK8                ATELMDAFLPALKKGHQPAAVVISSVASAHLAFDKNPLALALEAGEEAKA
gi|114793713|pdb|2DKN               VSALLDGLAEALSRGQQPAAVIVGSIAATQPGAAELPMVEAMLAGDEARA
gi|25027667|ref|NP_737721.1         TVALIEGLRPLLEKSEAPRITVTSSAATLQGNDPELVDLLLGGNYDAAME

gi|227015865|gb|ACP17962.1          G-QAWLKQNPVAPEFCYQYFKEALIVWSQVQAQE-WFMRTSVRMNCIAPG
gi|71274476|ref|ZP_00650764.1       G-LDWLAAHPVPASTCYQYFKEALIVWTMLRSRD-WFAGHGVRVNSVAPG
gi|116249152|ref|YP_764993.1        V-AAVVTEHGLKDEEGYPLSKELLLLWTMRAAHQPLFKNRGIRVNAVSPG
gi|120403002|ref|YP_952831.1        V-AAWQSRQ-DPSYPVYTTSKQAVILYAKRLAGT-ALSRYGVRVNTVSPG
gi|13096535|pdb|1FJH                R-AIVEHAGEQGGNLAYAGSKNALTVAVRKRAAA--WGEAGVRLNTIAPG
gi|13096537|pdb|1FK8                R-AIVEHAGEQGGNLAYAGSKNALTVAVRKRAAA--WGEAGVRLNTIAPG
gi|114793713|pdb|2DKN               I-ELAEQQGQ--THLAYAGSKYAVTCLARRNVVD--WAGRGVRLNVVAPG
gi|25027667|ref|NP_737721.1         RGVALAEQGPQIGYANYSSSKRAISRWIRRMAPTPEYAGAGIGINGVGPG

gi|227015865|gb|ACP17962.1          PVFTPILNEFVTM-LGQ-ERTQADAHRIKRPAYADEVAAVIAFMCAEESR
gi|71274476|ref|ZP_00650764.1       PVFTPILGDFVSM-LGP-DRVQADASKMKRPSYADEVAEAVIFLASDAAR
gi|116249152|ref|YP_764993.1        PVETPILKQFRAV-LGD-ARVDSDITRVGRAGTSADIAPAVLFLCSDGAR
gi|120403002|ref|YP_952831.1        PVETPILADFEQT-MGK-EILDMLRDTYGRHGTVDDIVPVIAFLASDAAQ
gi|13096535|pdb|1FJH                ATETPLLQAGLQD-PRYGESIAKFVPPMGRRAEPSEMASVIAFLMSPAAS
gi|13096537|pdb|1FK8                ATETPLLQAGLQD-PRYGESIAKFVPPMGRRAEPSEMASVIAFLMSPAAS
gi|114793713|pdb|2DKN               AVETPLLQASKAD-PRYGESTRRFVAPLGRGSEPREVAEAIAFLLGPQAS
gi|25027667|ref|NP_737721.1         QVYTGMTTELFSTEEGRKMAADAMPTPYNGIAEAEHIAAVHAFLVSADNA

gi|227015865|gb|ACP17962.1          WINGINIPVDGGLASTY--------
gi|71274476|ref|ZP_00650764.1       WINGVNLPVDGGLAATTI-------
gi|116249152|ref|YP_764993.1        WINGANLAADGGLEASINAEVLGF-
gi|120403002|ref|YP_952831.1        WINGQDVHVDSGFVTSMTAGTPIAL
gi|13096535|pdb|1FJH                YVHGAQIVIDGGIDAVMRPTQF---
gi|13096537|pdb|1FK8                YVHGAQIVIDGGIDAVMRPTQF---
gi|114793713|pdb|2DKN               FIHGSVLFVDGGMDALMRAKTF---
gi|25027667|ref|NP_737721.1         RMTGQIIFVDGGYDALSRGDDIW-
```

Fig. 3. Multilpe Sequence Alignment of cd05328 of seven bacterial coniferyl alcohol dehydrogenases along with *Pseudomonas nitroreducens* Jin1 CADH

Table 2. Conserved residues present in coniferyl alcohol dehydrogenase

Conserved residues	Position	Function
TGXXXGXG	11-18	Coenzyme binding region, maintenance of central *β* sheet
D	59	Stabilization of adenine ring pocket, weak binding to coenzyme
NXXG	91-94	Stabilization of central *β* sheet
N	114	Active Site
S-Y-K	140,157,161	Active Site
N	184	Connection of substrate binding loop and active site
PG	188-189	Reaction direction
T	193	H- bonding to carboxamide of nicotinamide ring

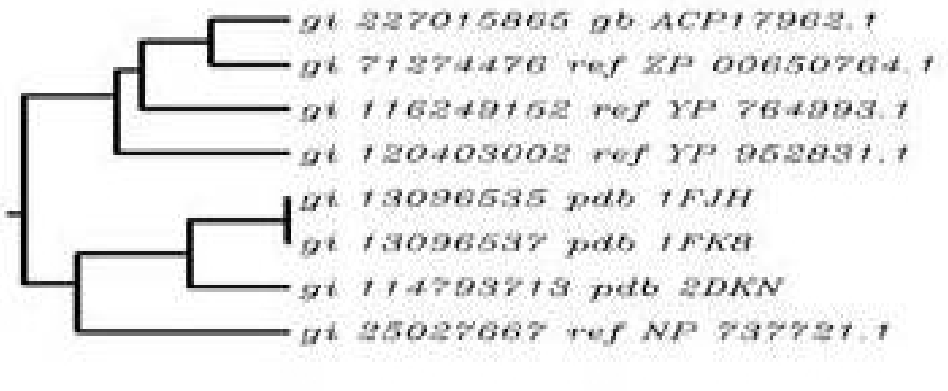

Fig. 4. Phylogenetic tree for members of domain cd05328 and *P. nitroreducens* Jin1 (ACP17962.1) All the sequence identities are as per Table 1

A Phylogenetic tree shown in Fig. 4 has been obtained for members of domain cd05328 and Jin1. Eight genes analysed separated into two major branches with four members each. The first group includes *P. nitroreducens* Jin1, *M. vanbaalenii, Xylella fastidiosa and Rhizobium.* The second group includes *Pseudomonas sps.* B0831, *Corynebacterium efficiens* YS-314, *Comamonas testosterone* belonged to other trunk. All the bacteria in first group show characteristized by xenobiotic metabolism and biodegradation with the exception of *Xylella* which is a plant pathogen and fastidious in its survival in the plant xylem.

3.2 Gene Isolation

The bioinformatic analysis shows the gene under study to be the smallest in size with all the requisite conserved functional domains. Thus the gene was selected for isolation. The PCR product of 768bps was obtained and stored for further cloning into *E. coli*. 1% agarose gel was run and PCR product of size 768bp was found in gel Fig. 5.

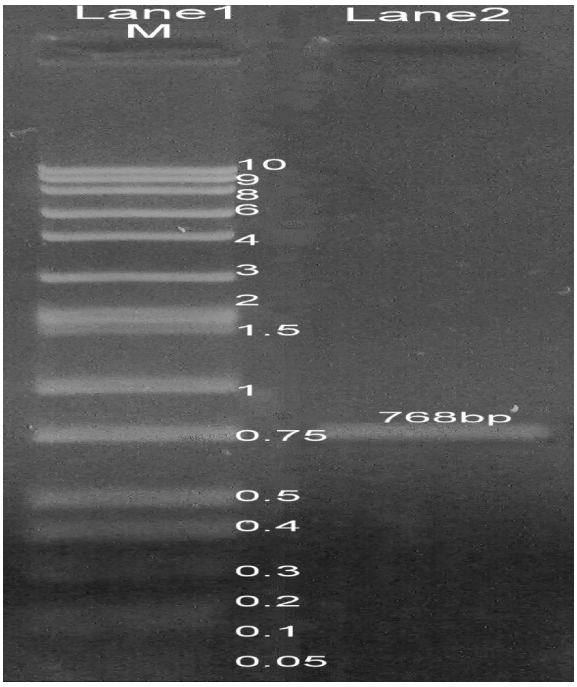

Fig. 5. PCR product of 768 bp of *calA* gene was observed on 1% Agarose gel

4. DISCUSSION

Coniferyl alcohol dehydrogenase is an NAD dependent enzyme with cd05328 conserved domain. The N-terminal Domain TGXXXGXG positions 11-18; NXXG positions 91-94; YXXXK positions 157-161; N at 179; P at 183; G at 184 and T at 188 are conserved in 100% of the CADH sequences analysed (Table No. 2). Aspartic acid at 59 is conserved in only two members. Asparigine at 114 is conserved in only three members. Serine at position 140 is conserved in only *P. nitroreducens* Jin1. Fig. 2 shows that all conserved residues are found in *P. nitroreducens* Jin1. At N-terminal end of CADH, the conserved sequence regions is found to be a variable N-terminal TGXXXGXG which is around position 12 (all numbering refers to 3 ß /17 ß–HSD- PDB code 1hxh) motif. This motif is part of the nucleotide binding region. The glycine-rich motif TGXXXGXG and the NNAG help to maintain the discrete structure of the central ß–sheet. Maintainence of discrete structure is essential for coenzyme binding and determination of reaction direction which was shown by several mutational and structural studies [4,13]. Other conserved residues involved in cofactor interactions are Asp60, Ala88, Thr188, and presumably Pro or Gly in the P-G motif [4,13,35]. The triad of S, Y, K residues (positions 138,151,155 in 1hxh) which is catalytically active constitute the active site [12]. Recently it has been found that addition of one more essential and conserved residue Asn111 to this triad forms a tetrad of N-S-Y-K residues in most SDR forms [13]. Studies have shown that the Tyr functions acts as the catalytic base, Ser stabilizes the substrate and Lys interacts with the nicotinamide ribose and which helps to lowers the pKa of the Tyr-OH [12,13,35].

In the classical SDRs the coenzyme-binding region typically contains a TGXXXGXG motif and an NNAG motif which is approximately 70 residues downstream. TGXXGXXG and HXAS patterns are usually found in the extended SDRs instead. These motifs and the active

site pattern YXXXK helps to discriminate between SDR members and non-members. As TGXXXGXG sequence is present in coniferyl alcohol dehydrogenase so they are classical SDR type. The least conserved regions in SDR molecules are those conserved residues and motifs which are located proximal to the variable substrate binding loop are and these are distal to helix α [4,13,16,35]. Least conserved region in SDR family are P-G motif and a conserved Thr188. In some SDR proteins, a sequence of polar and hydrophobic IRVN (176-179) residues which precedes the ‘‘P-G’’ motif, is also least conserved. Strikingly, the surrounding sequences resemble the eukaryotic N-glycosylation signal N-X-T/S [13]. This motif is also found in 11ß-hydroxysteroid dehydrogenase type1 (11ß-HSD1) in *Homo sapiens.* In *Homo sapiens,* the presence of the Asn is found to be necessary for its activity [36]. Role of this residue has been explained by replacing the homologous Asn179 in 3 ß /17ß -HSD [11]. In 3ß/17ß-HSD Asn179 appears to be essential for its activity as mutation to Ala completely abolishes activity. Study of the crystal structure has shown the interactions of the Asn side-chain which links the substrate loop and active site via a conserved water molecule. Homologous interactions in the majority of SDR structures highlight the importance of these sequence motifs in SDR enzymes.

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 Pst1 restriction site and in reverse primer BamH1 restriction site is present. Further for facilitating expression of the gene the Shine-Dalgarno sequences were inserted and appropriately positioned. The PCR amplification product obtained was 768bp in size and was eluted from agarose for further restriction digestion and cloning.

5. CONCLUSION

calA gene sequence from *P. nitroreducens* Jin1 have all the functional domains which are present in domain cd05328. It can be used for biotransformation of coniferyl alcohol to coniferyl aldehyde.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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