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Cloning, Expression and Purification of Putative Isovaleryl-CoA Dehydrogenase from *Paracoccus denitrificans* Pd1222

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Abstract

In *Paracoccus denitrificans* Pd1222 bacterium, *Pden*_3633 encoding gene has been nominated to encode for Isovaleryl CoA dehydrogenase (IVDH) [1], the enzyme which involve in leucine catabolism pathway. In this study, this putative IVDH was investigated. IVDH encoding gene from *P. denitrificans* Pd1222 in addition to desired features for cloning, expression and purification have been designed and synthesized. The synthetic coding sequence was expressed in *Escherichia coli*. The enzyme was purified as a *Strep*-Tagged protein with a total protein 220.5 mg. An apparent molecular weight of 42.9 kDa was determined on SDS gel. Amino acid alignment showed a very high similarity (91-96%) with corresponding IVDH from several other *Paracoccus* species. As for genera other than *Paracoccus*; *Roseovarius mucosus*, *Catellibacterium nectariphilum* and *Oceanicola nanhaiensis* recorded the highest similarity (85-86%), Suggesting that these mentioned species all have similar IVDH.

Keywords: Cloning, Acyl-CoA Dehydrogenase, *Paracoccus denitrificans*

أستنسال وتعبير وتنقية الانزيم المفترض Isovaleryl-CoA dehydrogenase من بكتريا *Paracoccus denitrificans* Pd1222

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الخلاصة:

بعد تحديد تتابع القواعد النتروجينة لجينوم بكتريا *Paracoccus denitrificans* Pd1222 وجد ان الجين Pden_3633 هو احد الجينات المرشحة للتشفير عن انزيم Isovaleryl−CoA dehydrogenase (IVDH) [1]، احد الانزيمات المشاركة في مسار هدم الحامض الاميني الليوسين. في هذه الدراسة تم التحري عن هذا الانزيم المفترض، اذ تم تصميم وتصنيع جين *IVDH* من *P. denitrificans* Pd1222 ليشتمل على الصفات المرغوبة للأستنسال والتعبير والتنقية. تم الحصول على ناتج تعبير التتبعات الصناعية في بكتريا *E. coli*. نقي الانزيم كبروتين مرتبط *Strep*−Tagged IVDH بقيمة بروتين كلي 220.5 ملغم، كما اظهر الانزيم وزنا جزيئيا قدر ب 42.9 كيلو دالتون على هلام SDS. أظهر تطابق الأحماض الأمينية تشابهات عالية جدا مع انزيمات IVDH المفترضة في العديد من أنواع *Paracoccus* (96−91 %)، اما بالنسبة للأنواع من غير جنس *Paracoccus* فقد سجلت كلا من *Roseovarius mucosus* و *Catellibacterium nectariphilum* و *Oceanicola nanhaiensis* أعلى نسب تشابهات (86−85 %) مما يوحي بأن جميع هذه الانواع المذكوره لها IVDH متشابه.

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Introduction

Paracoccus denitrificans is a beta-proteobacteria and denitrifying organism that caused environmental emissions of the powerful greenhouse gas nitrous oxide (N_2O) from agricultural soils. Also, the bacterium is a model for the study of the oxidative phosphorylation because it has respiratory transport chain analogous to that in the eukaryotic mitochondrion [2-3]. Most of studies were focused on nitrite respiration in *P. denitrificans* and other bacteria. Not far, the attention turned to the way in which the bacteria use low molecular weight methyl-branched carbon compounds such as citronellyl-CoA or leucine as a sole carbon source rather than the short, medium and long chain-fatty acids that are available in the environment. Catabolism of these compounds is complicated by bacteria because β-methyl group hinder β-oxidation. Therefore, the bacteria require a functional methyl-branched/ utilization pathway to circumvent this problem [4-5]. Isovaleryl CoA dehydrogenase is one of these enzymes that involve in the catabolism of methyl-branched compounds. The enzyme catalyzes the conversion of methyl branched isovaleryl-CoA, the compound outputted by the second step of leucine catabolism pathway, to 3-methylcrotonyl-CoA. IVDH is one member of the Acyl CoA dehydrogenases (ACADs) family, They are all highly similar homotetramers with a subunit mass 43 kDa except the very long chain acyl-CoA dehydrogenase [6] and acyl-CoA dehydrogenase-9 [7] are homodimers with 70 and 65 kDa subunit mass respectively. All members of ACADs family use flavin adenine dinucleotide (FAD) cofactor as electron acceptor and they all employ the same catalytic mechanism in the first step of the β-oxidation and transfer electrons from acyl-CoA substrates to the electron transfer flavoprotein (ETF). ACADs have been identified in bacteria [8], Human [9-11], plants [12], rat [13] and many other organisms. In addition to IVDH, isobutyryl-CoA dehydrogenase and 2-methyl branched chain acyl-CoA dehydrogenases are two other members of ACADs family that involve in catabolism of methyl-branched compounds, they involve in valine and Isoleucine catabolism pathways respectively. While the other members of this family concerned in fatty acids β-oxidation and showed different in substrate specificity according to the length of fatty acid chain, those are short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD) and very long-chain (VLCAD) acyl-CoA dehydrogenases respectively [14-15].

Materials and Methods

Construct the Synthetic P*aracoccus denitrificans* IVDH (P. d IVDH)

A project was designed, using Gene Designer 2.0 software, to construct the synthetic *IVDH* gene. Briefly, nucleotide sequences for *Nco* I restriction site and *Strep*-Tag peptide, to facilitating cloning and purification respectively, were inserted at the 5' of an open reading frame of *P. denitrificans* PD1222 *IVDH* gene (Pden_3633). While a sequence for *Bgl* II restriction site, to facilitating cloning, was inserted at the 3' of the synthetic gene. The designed project was then sent to GeneScript Company (New Jersey, USA) which optimized the sequences for expression in *E. coli* Figure-1 and the project was conducted by incorporating the synthetic gene into a pUC57 vector harbored selectable marker for ampicillin resistance.

Construction of expression plasmids

The delivered synthetic *Strep*-Tag IVDH/ pUC57 vector was amplified by transformation into *E. coli* DH5α competent cells. Clones selection was done on LB-plates supplemented with ampicillin (50 µg/ml). The vector was extracted from transformed cells and double digested with *Nco* I plus *Bgl* II. After fixing the band of the synthetic gene on agarose gel (the gene with left *Nco* I and right *Bgl* II sticky ends), the gene was purified from gel, cloned into double digested pET24d (*Bam* HI plus *Nco* I) and transformed into *E. coli* DH5α. DNA sequencing by Eurofins Genomics GmbH, UK, was done to check the correctness of the synthetic gene sequences using T7 promoter primer. The resulting constructs was transformed into *E. coli* BL21 (DE3) for expression experiment.

Expression of synthetic IVDH

A Single colony of the overnight transformed *E. coli* BL21 (DE3), that harbor the constructed expression plasmid, was used to inoculate 5 ml of LB broth supplemented with 50µg/ml kanamycin. The culture was incubated overnight at 37°C in shaker (200 rpm). 2ml of the overnight culture was then added to100ml of LB broth containing 50µg/ml kanamycin. The cultures in several flasks (8-10) were incubated at 37°C with 200 rpm shaker until optical density (OD_{600}) reach approximately 0.5-0.6. And then the expression of the synthetic *Sterp*-Taggad IVDH was induced by adding 100µl of 1M stock IPTG, 1mM as a final concentration. Also, growth media was supplemented with 1µg/ml riboflavin for the stability of protein. After that, the flasks were incubated overnight in shaker (200

rpm) at 24°C. The cells of the flasks were then harvested by spin down at 6800 g for 20 min at 4°C. Next, the pellets were resuspended with the lysis buffer (50mM Tris-HCl, 1mM EDTA and 0.1% Triton X-100) and then one quick-dissolving tablet of complete-Mini Protease Inhibitor Tablets (Roche Biotech, USA) was added for each 10 ml of sample. The sample was then French pressed three times using 1280 psi (pound per square inch). A little volume of produced cell lysate was kept for further use (as whole cell lysate fraction). The lysate was then spun down at 12000 rpm for 30 min at 4°C, the supernatant was carefully separated and kept as a soluble fraction, while the pellet was resuspended in the lysis buffer and kept as insoluble fraction.

Purification of recombinant IVDH

The N-terminal *Strep*-Tagged IVDH was purified by using *Strep*-Tactin superflow plus cartridge, a kit from Qiagen, UK. After expression experiment, soluble extract fraction of *E. coli* BL21 (DE3) was loaded on *Strep*-Tactin superflow plus column (1 ml bed volume) to purify recombinant protein by affinity chromatography. The column was washed with 10 column volumes of 50 mM $NaH_2PO_4.H_2O$, 300 mM NaCl, pH 8.0. Elution was performed using 4 column volumes of 50mM $NaH_2PO_4.H_2O$, 300 mM NaCl, 2.5 mM desthiobiotin, pH 8.0. SDS-PAGE was then used to analysis the fractions.

CCATGGCAAGCTGGAGCCACCCGCAGTTTGAAAAATTTAATGCTGGTATGCAGTTTGACCTGGGCGAAGATGTGAATGCTCTG
CGTGAAACCGTGCATCGTTGGGCGCAGGAACGCGTGAAACCGATTGCGGCCGAAGTTGATCGTAAAAACGCCTTTCCGAATGA
ACTGTGGCGCGAAATGGGTGACCTGGGCCTGCTGGGTATCACCGTTAGTGAAGAACTGGGCGGTTCCGGCATGGGTTATCTGG
CGCATGTGGTTGCCACCGAAGAAATTGCACGTGCTAGCGCGTCTGTTAGTCTGTCCTACGGCGCACACAGTAACCTGTGCGTC
AATCAAATCAAACTGAACGGTACCGATGAACAGCGCGCGAAATATCTGCCGAAGCTTTGTTCCGGCGAACACGTGGGTGCCCT
GGCAATGTCAGAAGAAGGCGCCGGTTCGGATGTCGTGGGCATGAAACTGCGTGCAGAAAAACGTAACGACCGCTATGTTCTGA
ACGGTAATAAATACTGGATTACCAATGCTCCGGATGCGCATACGCTGGTTGTCTATGCTAAAACCGACCCGGAAGCGGGCTCT
AAAGGTATTACGGCCTTCATCGTGGAACGTGGCATGAAAGGTTTTTCAACCTCGCCGCACTTCGATAAACTGGGCATGCGCGG
TAGCAACACGGGCGAACTGATCTTTGAAGACTGCGAAGTCCCGTTCGAAAATGTGCTGGGCGCGGAAGGCAAAGGTGTCCGTG
TGCTGATGAGCGGTCTGGATTATGAACGCCTGGTGCTGTCTGGCATTGGTACCGGCATCATGGCAGCTTGTCTGGATGAAGTG
ATGCCGTACGTTAAAGAACGCAAACAGTTTGGCCAACCGATTGGTAGTTTCCAGCTGATGCAAGGCAAAATCGCCGATATGTA
TGTTGCACTGAACACGGCTCGTGCGTATGTTTACGAAGTCGCCAAAGCATGCGATGCGGGTAAAGTTACCCGTCAAGATGCCG
CAGGTGCAGTGCTGTACGCTAGCGAACAGGCGATGGTTCAGGCCCATCAAGCAGTCCAGGCTCTGGGCGGTGCTGGTTTTCTG
AATGATAGCGTGGTTTCTCGTCTGTTCCGCGACGCAAAACTGATGGAAATT
GGCGCTGGCACCTCTGAAATTCGTCGTATGCTGATTGGTCGTGAACTGCTGGGTCTGGCGTGAGATCT

While:-

CCATGG *Nco* I for cloning into pET24d

TGGAGCCACCCGCAGTTTGAAAAA encodes *strep* tag for purification

AAGCTT *Hind* III for restriction mapping of cloned *IVDH* gene.

AGATCT *Bgl* II for cloning into pET24d

Figure 1-Optimized Sequence of synthetic *IVDH* gene (Optimized Sequence Length:1198 bp, GC%: 53.23).

Results and discussion

Cloning of *IVDH*

The delivered pUC57 vector that harbor the synthetic P. d *IVDH* gene was amplified by transformation into *E. coli* DH5α. The transformed clones were selected based on their Amp resistant (pUC57 selectable marker). Thus, it never need to hold PCR for amplify gene of interest. To investigate whether the synthetic vector carrying the gene of interest, the vector was double digested with *Nco* I and *Bgl* II. A band of a predicted full length synthetic *Strep*-Tag P. d *IVDH* gene (1198 bp) was resulted from the double digestion Figure-2 which indicates success of construction of the synthetic vector as well as the restriction sites are in the correct positions, in flanked of the synthetic gene. The pET24d vector was used for cloning of the synthetic *IVDH*. The vector has two sites for restriction enzymes; *Bam* HI (locus; 198) and *Nco* I (locus; 234) appeared to be complementary with *Bgl* II and *Nco* I sticky ends of the synthetic gene respectively. pET24d was double digested with *Bam* HI and *Nco* I Figure-3 to obtain a linearized vector with non-complementary sticky ends, the length of truncated segment (36 bp) resulted from double digested pET24d is too small and cannot be seen on 0.8% agarose gel because *Bam* HI and *Nco* I are close located. To construct the expression plasmid, the double digested fragments of the synthetic *Strep*-Tag P. d *IVDH* and pET24d vector were ligated together and the resulted DNA used to transform *E. coli* DH5α and only transformed clones are able to grow in present of kanamycin (pET24d selective marker). Separately, each of the pET24d and the synthetic *Strep*-Tag *IVDH* gene have only one restriction site for *Hind* III. Therefore, the recombinant DNA will has two sites for *Hind* III. One located within the *Strep*-Tag *IVDH* gene, at the predicted position 6076 of the recombinant DNA, and the other located within the pET24d, at the

predicted position 5242 of the recombinant DNA. Two bands were produced as a result of mapping of the recombinant DNA with *Hind* III, and this an evidence for success of cloning as it shown in Figure-4. There are poorly Knowledge about bacterial acyl-CoA dehydrogenases in contrast to these from Eukaryote. Few biochemical studies conducted on bacterial branched and short-chain acyl-CoA dehydrogenases in 70s and 90s of last century [16-20] but acyl-CoA/ isovaleryl-CoA dehydrogenases from bacteria was first purified and studied in more details by Förster-Fromme and Jendrossek [21] in addition to Förster-Fromme et al [22]. They used pET28a and *E. coli* JM 109 for cloning IVDH from *Pseudomonas aeruginosa* PAO 1. Other study by Liu et al [23] they also used pET28a for cloning IVDH from rat and produce it in *E. coli* strain BL21 (DE3).

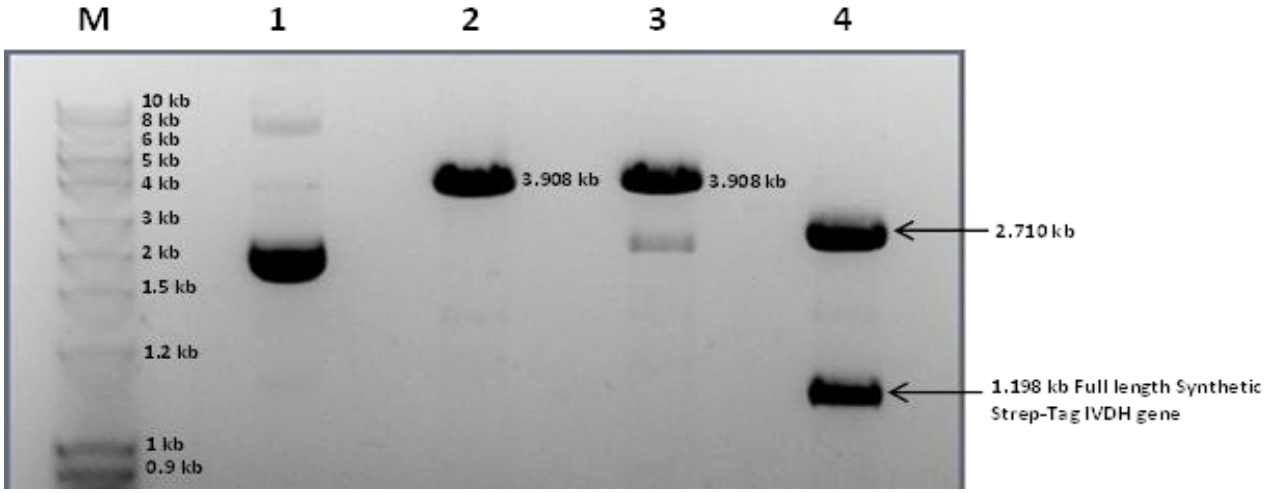

Figure 2-Analysis of the double digested synthetic Strep-Tag P. d IVDH/pUC57 on 1.2 % agarose gel electrophoresis. The gel was run for 1 hour at 100-120 volts. M: 2-Log DNA Ladder (0.1-10.0 kb); Lane 1: Uncut synthetic Strep-Tag P. d IVDH/pUC57; Lane 2: Single digested with *Nco* I; Lane 3: Single digested with *Bgl* II; Lane 4: Double digested with *Nco* I/ *Bgl* II

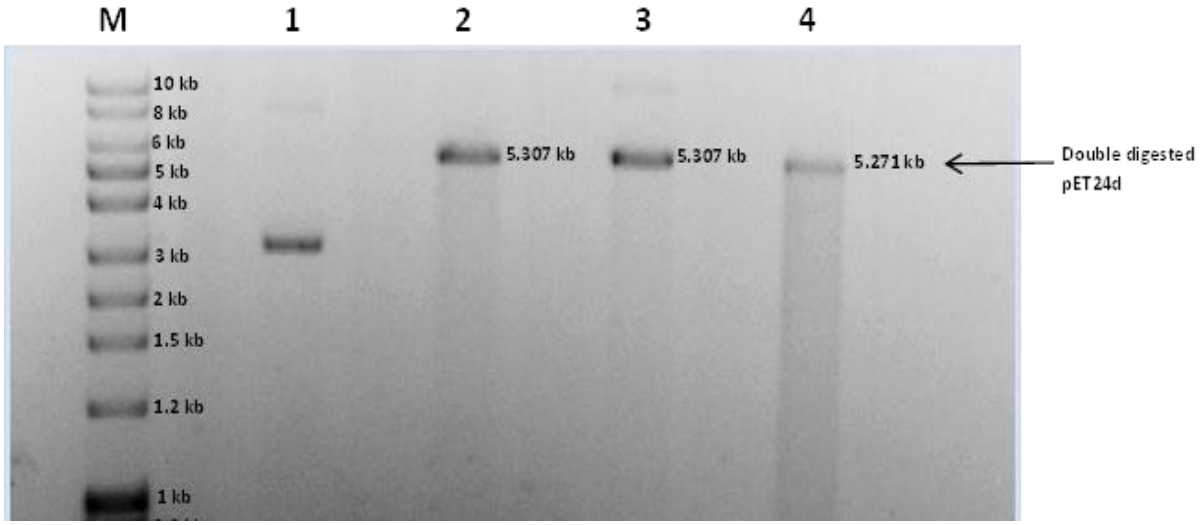

Figure 3-Analysis of the double digested pET24d vector on 0.8 % agarose gel electrophoresis. The gel was run for 1 hour at 100-120 volts. M: 2-Log DNA Ladder (0.1-10.0 Kb); Lane 1: Uncut pET24d vector; Lane 2: Single digested with *Nco* I; Lane 3: Single digested with *Bam* HI; Lane 4: Double digested with *Nco* I plus *Bam* HI

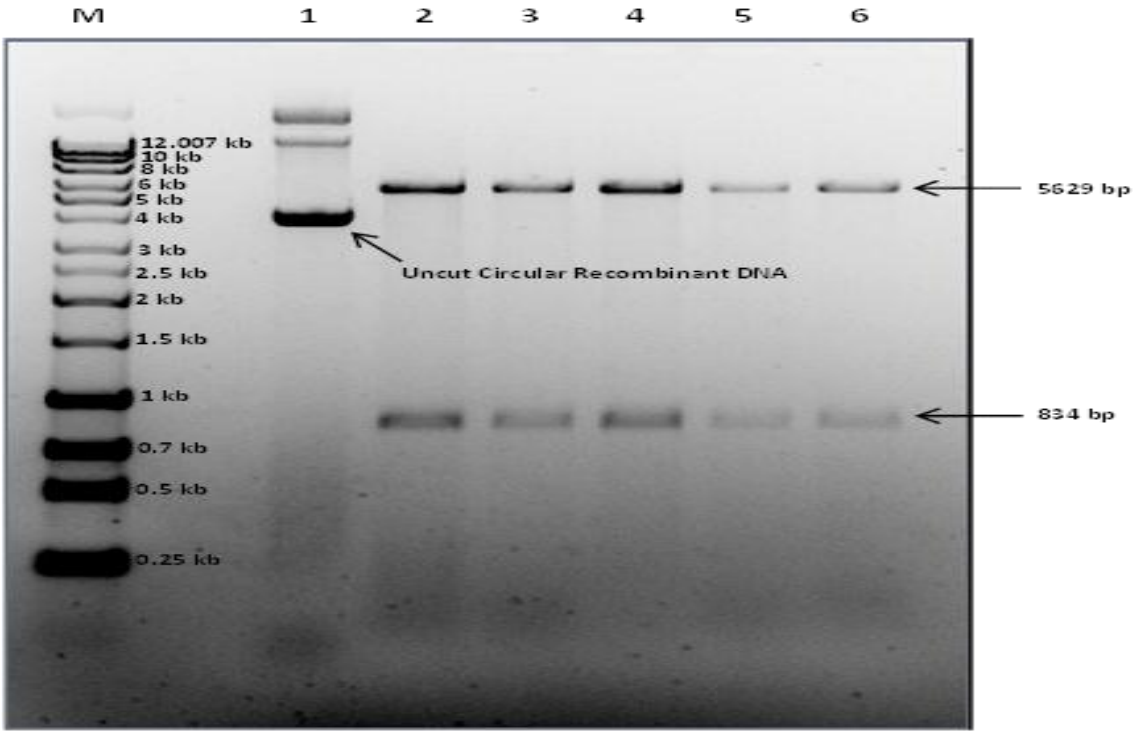

Figure 4- Mapping analysis of the Strep-Tag IVDH/ pET24d on 0.8 % agarose gel electrophoresis. The gel was run for 1 hour at 100-120 volts. M: HyperLadder™ 1kb Plus; Lane 1: Uncut circular Strep-Tag IVDH/ pET24d (bands marked with asterisk indicate isoforms of the Strep-Tag IVDH/ pET24d); Lanes 2 to 6: Single digested with Hind III.

Expression and purification of recombinant IVDH

The expression experiment performed by transform *E. coli* BL21 (DE3) with expression plasmid and the expression was induced by IPTG. The results revealed that the most of the recombinant IVDH was existed as a soluble fraction and less was insoluble aggregates or inclusion bodies in which the protein is un-active or denatured. These aggregates consisted because the formation of the recombinant protein occurs in a foreign environment which differs than its origin environment [24-26]. It was easy in current study to obtain a good yield of the recombinant IVDH by purification of the soluble extract, the bands of the synthetic Strep-Tag P. d IVDH were visible around 43 kDa in an un-purified soluble extract and also in the eluted fractions resulted from purification of the soluble extract. While no recombinant protein band was viewed in a wash which demonstrate the highly binding affinity between recombinant protein and *Strep*-Tactin column Figure-5. Purification by using *Strep*-Tag was very successful and the enzyme was purified with a total protein 220.5 mg from 63 ml of soluble extract *E. coli* BL21 (DE3). Current study is in good agreement with many studies which mentioned that IVDH from *P. aeruginosa* [21], Human [27], Rat [28], Nematode [29], has molecular mass subunit around 43 kDa.

Amino acids alignment

Amino acids alignment of the synthetic IVDH from *P. denitrificans* PD1222 (Query) with those from Rat, Human, *Pseudomonas aeruginosa* PAO1 and putative IVDH of *P. denitrificans* PD1222 was conducted by using ClustalW Omega software from European Bioinformatics Institute (EMBL), www.ebi.ac.uk, and the results showed a high degree of homology (100% identity) with the putative IVDH from *P. denitrificans* PD1222 Figure-6 and this a conclusive proof which confirmed that the putative *IVDH* gene from *P. denitrificans* PD1222 is an intact gene, fidelity of the reading frame, and has ability to express for a mature protein. Also, the synthetic IVDH showed 64.77, 59.07 and 58.81% identity with IVDH from *P. aeruginosa* PAO1, Rat and Human respectively, percent identity matrix created by Clustal2.1. BLASTP analysis of the recombinant P. d IVDH (using NCBI genebank) revealed a very high values of similarity 96%, 95%, 92% and 91% to the putative IVDHs (not confirmed yet) from *Paracoccus pantotrophus*, *P. versutus*, *P. halophilus* and *P. zeaxanthinifaciens* respectively. While the highest similarity values to a predicted proteins from genera not belong to *Paracoccus* were seemed to be a putative Isovaleryl-CoA dehydrogenase from *Roseovarius mucosus*

(86% identity), *Catellibacterium nectariphilum* (86% identify) and *Oceanicola nanhaiensis* (85% identify). Accordingly, it can be concluded that the species of the genera *Paracoccus*, *Roseovarius*, *Catellibacterium* and *Oceanicola* sharing similar IVDH.

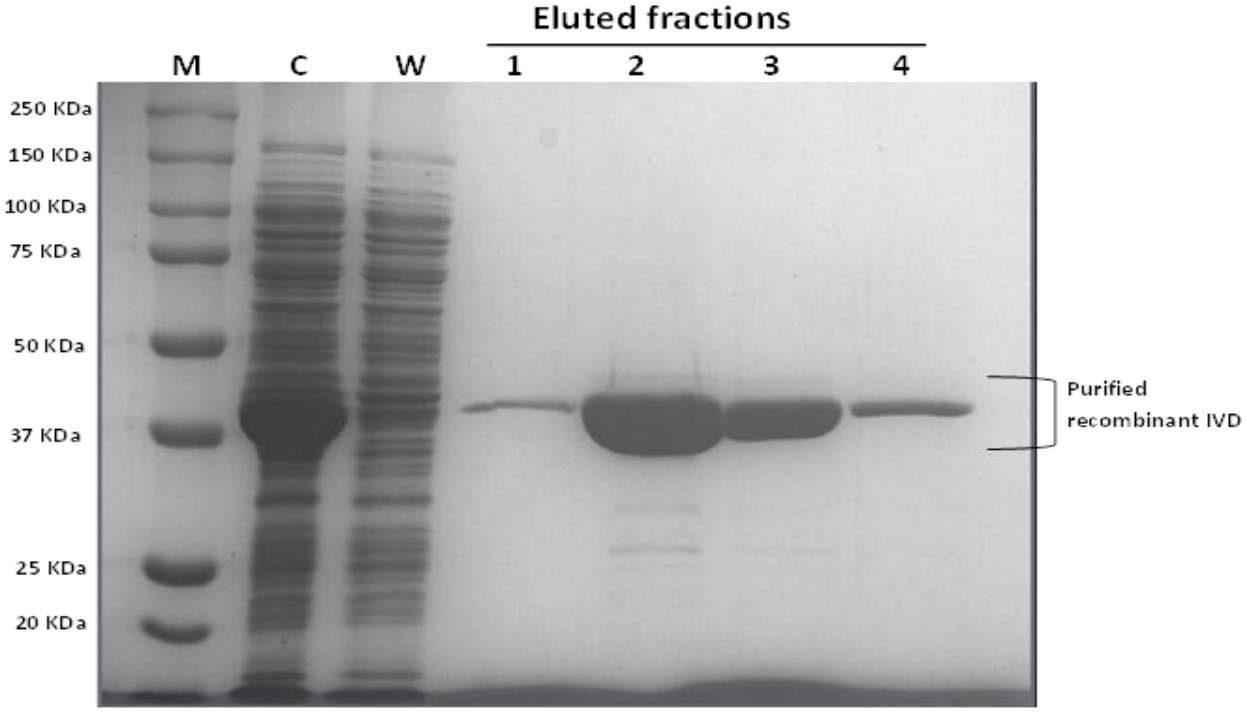

Figure 5-SDS gel electrophoretic pattern of the purified synthetic *Strep*-Tag P. d IVDH. M: Precision plus proteinTM standard marker; C: soluble extract of *E. coli* BL21 (DE3), that harbor recombinant plasmid, before loaded onto Strep-Tactin column (Control); W; Wash fraction; lanes 1-4 are eluted fractions of the purified recombinant IVDH. The SDS-PAGE was run on 12% and 4% separating and stacking gels respectively using 110 Volt for 1-1.5 hrs, followed by Coomassie blue staining.

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Rat IVDH                ATAVRLLGRRVSSWRLRPLPSPLAVPQRAHSMLPVDDDINGLNEEQKQLRHTISKFVQEN 60
Human IVDH              ----------------------------HSLLPVDDAINGLSEEQRQLRQTMAKFLQEH 31
Query                   ---------------------------------FNAGMQFDLGEDVNALRETVHRWAQER 27
Putative P. d IVDH      ---------------------------------FNAGMQFDLGEDVNALRETVHRWAQER 27
P. aeruginosa IVDH      ---------------------------------TYPSLNFALGETIDMLRDQVRGFVAAE 27
                                                          * *    **. :  :   .

Rat IVDH                LAPKAQEIDQSNDFKNLREFWKQLGSLGVLGITAPVQYGGSGLGYLEHVLVMEEISRASA 120
Human IVDH              LAPKAQEIDRSNEFKNLREFWKQLGNLGVLGITAPVQYGGSGLGYLEHVLVMEEISRASG 91
Query                   VKPIAAEVDRKNAFPN--ELWREMGDLGLLGITVSEELGGSGMGYLAHVVATEEIARASA 85
Putative P. d IVDH      VKPIAAEVDRKNAFPN--ELWREMGDLGLLGITVSEELGGSGMGYLAHVVATEEIARASA 85
P. aeruginosa IVDH      LQPRAAQIDQDNQFPM--DMWRKFGEMGLLGITVDEEYGGSALGYLAHAVVMEEISRASA 85
                         : * * ::*:.* *    ::*:::*.:*:****.  : ***.:*** *.:. ***:***.

Rat IVDH                AVGLSYGAHSNLCINQIVRNGNEAQKEKYLPKLISGEFIGALAMSEPNAGSDVVSMRLKA 180
Human IVDH              AVGLSYGAHSNLCINQLVRNGNEAQKEKYLPKLISGEYIGALAMSEPNAGSDVVSMKLKA 151
Query                   SVSLSYGAHSNLCVNQIKLNGTDEQRAKYLPKLCSGEHVGALAMSEEGAGSDVVGMKLRA 145
Putative P. d IVDH      SVSLSYGAHSNLCVNQIKLNGTDEQRAKYLPKLCSGEHVGALAMSEEGAGSDVVGMKLRA 145
P. aeruginosa IVDH      SVALSYGAHSNLCVNQIKRNGNAEQKARYLPALVSGEHIGALAMSEPNAGSDVVSMKLRA 145
                         :*.**********:**:  **.  *: :*** * ***.:*******  ******.*:*:*

Rat IVDH                EKKGDHYVLNGNKFWITNGPDADVLVVYAKTDLTAVPASRGITAFIVEKDMPGFSTSKKL 240
Human IVDH              EKKGNHYILNGNKFWITNGPDADVLIVYAKTDLAAVPASRGITAFIVEKGMPGFSTSKKL 211
Query                   EKRNDRYVLNGNKYWITNAPDAHTLVVYAKTDPE--AGSKGITAFIVERGMKGFSTSPHF 203
Putative P. d IVDH      EKRNDRYVLNGNKYWITNAPDAHTLVVYAKTDPE--AGSKGITAFIVERGMKGFSTSPHF 203
P. aeruginosa IVDH      DRVGDRFVLNGSKMWITNGPDAHTYVIYAKTDAD--KGAHGITAFIVERDWKGFSRGPKL 203
                         ::  ::::***.* ****.***.. ::***** .::********:  *** . ::

Rat IVDH                DKLGMRGSNTCELVFEDCKVPAANILSQESKGVYVLMSGLDLERLVLAGGPLGIMQAVLD 300
Human IVDH              DKLGMRGSNTCELIFEDCKIPAANILGHENKGVYVLMSGLDLERLVLAGGPLGLMQAVLD 271
Query                   DKLGMRGSNTGELIFEDCEVPFENVLGAEGKGVRVLMSGLDYERLVLSGIGTGIMAACLD 263
Putative P. d IVDH      DKLGMRGSNTGELIFEDCEVPFENVLGAEGKGVRVLMSGLDYERLVLSGIGTGIMAACLD 263
P. aeruginosa IVDH      DKLGMRGSNTCELIFQDVEVPEENVLGAVNGGVKVLMSGLDYERVVLSGGPVGIMQACMD 263
                         ********** **:*:* ::*  *:*.    ** ******* **:**:*   *:* * :*

Rat IVDH                HTIPYLHVREAFGQKIGQFQLMQGKMADMYTRLMACRQYVYNVARACDEGHITAKDCAGV 360
Human IVDH              HTIPYLHVREAFGQKIGHFQLMQGKMADMYTRLMACRQYVYNVAKACDEGHCTAKDCAGV 331
Query                   EVMPYVKERKQFGQPIGSFQLMQGKIADMYVALNTARAYVYEVAKACDAGKVTRQDAAGA 323
Putative P. d IVDH      EVMPYVKERKQFGQPIGSFQLMQGKIADMYVALNTARAYVYEVAKACDAGKVTRQDAAGA 323
P. aeruginosa IVDH      VVVPYIHDRRQFGQSIGEFQLVQGKVADMYTALNASRAYLYAVAAACDRGETTRKDAAGV 323
                          .:**:: *. *** ** ***:***:****. * :.* *:* ** *** *. * :*.**.
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Rat IVDH              ILYTAECATQVALDGIQCLGGNGYINDFPMGRFLRDAKLYEIGGGTSEVRRLVIGRAFNA 420
Human IVDH            ILYSAECATQVALDGIQCFGGNGYINDFPMGRFLRDAKLYEIGAGTSEVRRLVIGRAFNA 391
Query                 VLYASEQAMVQAHQAVQALGGAGFLNDSVVSRLFRDAKLMEIGAGTSEIRRMLIGRELLG 383
Putative P. d IVDH    VLYASEQAMVQAHQAVQALGGAGFLNDSVVSRLFRDAKLMEIGAGTSEIRRMLIGRELLG 383
P. aeruginosa IVDH    ILYSAERATQMALDAIQILGGNGYINEFPTGRLLRDAKLYEIGAGTSEIRRMLIGRELFN 383
                      :**::* *   * :.:* :** *::*:   .*::***** ***.****:**::*** :

Rat IVDH              DFR 423
Human IVDH            DFH 394
Query                 LA- 385
Putative P. d IVDH    LA- 385
P. aeruginosa IVDH    ETR 386
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Figure 6-Amino acid alignment of the synthetic *P. denitrificans* IVDH (Query) with putative *P. denitrificans* IVDH (Accession No. ABL71700) and other IVDH from Rat (Accession No. AAH88401), Human (Accession No. 1IVH-D) and *P. aeruginosa* (Accession No. NP_250705). An (*) indicates fully conserved residue; A (:) indicates conservation between groups of strongly similar properties; A (.) indicates conservation between groups of weakly similar properties; active side catalytic residues shadowed by yellow color.

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