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IN SILICO CHARACTERIZATION OF NITRATE REDUCTASE INVOLVED IN GOLD NANOPARTICLES SYNTHESIS IN *PSEUDOMONAS DENITRIFICANS*

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ABSTRACT

Various studies suggest the involvement of nitrate reductase in gold nanoparticle synthesis from different bacterial sources. The present study focuses on the *in silico* characterization of four subunits namely alpha, beta, gamma and delta of respiratory nitrate reductase enzyme using online tools and softwares in *Pseudomonas denitrificans* .CodonW and CAIcal server are used to perform codon usage biasness study on the respiratory nitrate reductase genes of *P. denitrificans.* Further different parameters of the physicochemical characterization were analysed by CLC Protein workbench, Expasy ProtParam, BLAST and COBALT. The analysis of various above parameters shows that the expression of gamma subunit is more out of all four subunits, hence suggesting the major involvement of it in gold nanoparticle synthesis. This may help in future to find out ways to enhance gold nanoparticle production in *P. denitrificans* and other related organisms.

KEYWORDS: Gold nanoparticles, *Pseudomonas denitrificans*, nitrate reductase, Codon usage biasness.

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INTRODUCTION

Recently nanobiotechnology has received increasing attention due to a growing need to develop environment friendly techniques to synthesise nanoparticles. Biological methods are gaining impetus because under normal conditions they enable us to control the size of the nanoparticles without producing any toxic bio hazardous waste [1]. Biological methods for nanoparticle synthesis use microorganisms, enzymes, and plants or plant exudates [2-3]. The biological synthesis of metal nanoparticles acts as an intersection point between nanotechnology and biotechnology. Different applications of nanoparticles in the area of electrical and medical has already been reported in various publication. It has been experimentally proved that by incubating the bacterial cells with Au+3 ions, particles of nanoscale dimensions may be readily precipitated within the bacterial cells [4-6]. *Thiobacillus ferroxidans* [7], *Bacillus subtilis* as well as Lactobacillus, intra-cellularly reduce Au3+ions to gold nanoparticles (GNP's). GNP's of a size range of 5–25 nm is produced inside the cell walls and plasma membrane. *Pseudomonas aeuroginosa* ATCC 9027 exploits NADH-dependent reductase to extra-cellularly synthesize GNP [8]. *Adathoda vasica* has also been studied and exploited to know the reduction potentials which in turn help to predict parameters such as pH and temperature, for GNP synthesis [9]. *Ixora coccinea* [10] and *Helianthus annuus* [11] (sun flower) flowers are studied to know their antimicrobial activity along with gold nanoparticle biosynthesis. This study focuses on *Pseudomonas denitrificans* which is a gram negative facultative anaerobic bacterium that performs denitrification and is involved in gold nanoparticle biosynthesis employing the enzyme nitrate reductase [11-12]. The enzyme converts nitrate to nitrite and an electron shuttle is induced thereby reducing the incoming gold ions to gold nanoparticles. Nitrate reductase comprises of four subunits namely alpha, beta, gamma and delta. The exact mechanism for the extracellular formation of gold nanoparticles by micro organism is not fully understood. But the fact that nanoparticles are formed can be justified by the proposed hypothesis: the gold ions were first trapped on the surface of the microbe cells via electrostatic interaction between the ions and negatively charged cell wall from the carboxylate groups in the enzymes. Next, the enzymes reduced the metal ions to form gold nuclei, which subsequently grow through further reduction and accumulation.

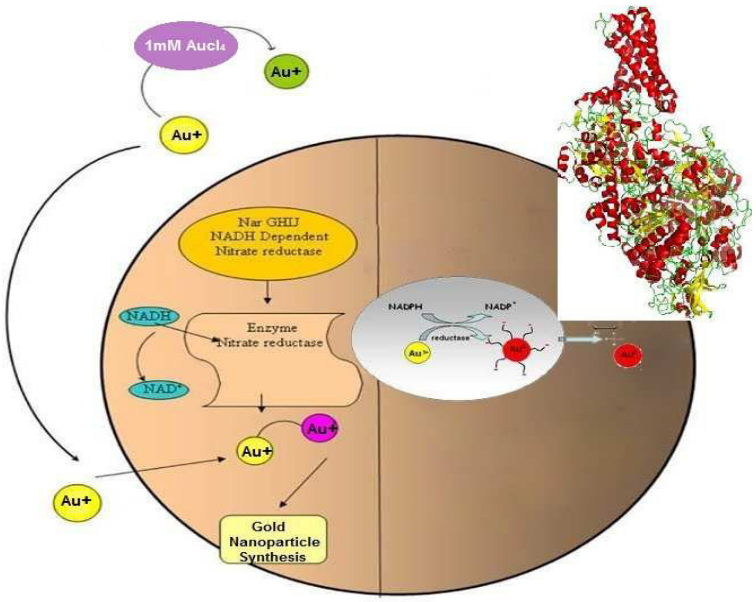

Figure 1
Possible mechanism of gold nanoparticle biosynthesis using enzyme nitrate reductase

Further with the use of online softwares and tools, *in silco* analysis of all the four subunits of enzyme nitrate reductase was done. Four subunits namely alpha, beta, gamma and delta were characterized and the results were used to predict the extent of possible involvement of nitrate reductase subunits in gold nanoparticle biosynthesis. In this study, synonymous codon usage bias patterns were determined. Information on the codon usage pattern can provide significant insights pertaining to the prediction, classification, and molecular evolution of genes and design of highly expressed genes. Codon usage biasness is the probability that a given codon will be used to code for an amino acid over a different codon which codes for the same amino acid. Codon adaptation index (CAI) is used as a measure of synonymous codon bias [14-18]. The most common factor that determines codon usage is a mutational bias that contributes to the genome GC composition. It concludes that species with high GC content use more G- and C-ending codons than species with low GC content. In order to study physicochemical parameter computational tools are used for the prediction and characterization of protein, which gives us various statistics about its sequence. The physico-chemical properties [19] of proteins such as molecular weight, GRAVY, aliphatic index, instability index [20-22] etc were computed using the Expasy's ProtParam (http://us.expasy.org/tools/ protparam.html). The amino acid sequence provides most of the information required for determining and characterizing the molecule's function, physical and chemical properties [23-25]. As physico-chemical characterization of nitrate reductase involved in GNP synthesis has not been done so far. In this paper, we report the *in silico* analysis and characterization studies on various subunits of enzyme nitrate reductase. Also, our comparative analysis of codon and amino acid usage patterns of enzyme nitrate reductase of *P. denitrificans* strains will provide an insight into the divergence and compositional similarities within and across their genes for nitrate reductase and also to predict which functional unit among the four shows maximum expression level. Further BLAST and multiple sequence alignment are also done to predict and identify the percentage similarity and closely related species to *P. denitrificans.* They also help us to infer phylogenetic relationships and evolution of organisms and can lead us to elucidate biological facts about proteins since most conserved regions are biologically significant.

MATERIALS AND METHODS

(i) Retrieval of Nucleotide and Protein Sequences

The nucleotide and protein sequences of nitrate reductase of *P. denitrificans* were retrieved from primary databases of NCBI. Individual sequences of all the four subunits namely alpha, beta, gamma and delta were downloaded in FASTA format.

(ii) Codon Usage Biasness Study

The probability of using one codon over another codon for the same amino acid is termed as codon usage biasness. The program CodonW 1.4.4 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::codonw) and CAIcal SERVER (http://genomes.urv.es/CAIcal) were used to run the gene of nitrate reductase subunits. Different parameters like gene length, CAI value, % GC content and Nc value were tabulated (Table 1).

(iii) In silico Characterization

In the present study all the four subunits of nitrate reductase of *Pseudomonas denitrificans* were computed using the tool CLC free Workbench (http://www. clcbio.com/index.php?id=28). The protein charge plot (Fig.1), percentages of hydrophobic and hydrophilic residues (Fig. 2) and the amino acid composition (Fig.3) were evaluated. Expasy's ProtParam (http://us.expasy.org/tools/ protparam.html) prediction server was also used to compute the physico-chemical parameters, theoretical isoelectric point (pI), and molecular weight, total number of positive and negative residues, instability index and grand average hydropathy (GRAVY).

(iv)Multiple Sequence Alignment and Phylogenetic Analysis

BLASTp (http://blast.ncbi.nlm.nih.gov/) was performed on the gamma subunit of nitrate reductase to retrieve more number of similar sequences from different organisms. Identity was kept up to 70% with 97% query cover and total score up to 300. Thereafter 20 best hits of different pseudomonas species were chosen and multiple sequence alignment was performed on them using COBALT. A rooted phylogenetic tree was constructed to study evolutionary relationship of gamma subunit among the pseudomonas species.

(v) Structure Prediction

SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) and CFSSP (http://www.biogem.org/tool/chou-fasman/) was used for the secondary structure prediction. The SOSUIs server (http://harrier.nagahama-i-bio.ac.jp/sosui/) performed the identification of transmembrane regions. Further Expasy's Swiss Model (http://swissmodel.expasy.org/) was used to predict the 3D structure of gamma subunit of nitrate reductase in *P.denitrificans.*

RESULTS AND DISCUSSIONS

Nitrate reductase protein sequences were retrieved from the primary NCBI. The sequences were retrieved in FASTA format and used for analysis. Initially gene level study was done on the basis of codon usage biasness. Codon usage biasness is an unavoidable phenomenon in organisms where one codon is preferred over the other coding for the same amino acid. Various factors such as GC content, nucleotide distribution, translational selection, protein hydropathy and protein secondary structure, are reported to contribute to codon usage preference. The synonymous codon usage pattern is helpful in identifying the expression pattern of genes as well as the evolutionary relationship between the sequences. CAI value shows that higher the value is more is the level of expression of genes. As per the result of codon biasness mentioned in Table 1 and taking the CAI value as the basis, it brings us to a close that in *P. denitrificans* gamma subunit has highest value i.e. 0.984. The CAI index ranges from 0 to 1 being if gene always uses the most frequently used synonymous codon in the reference set and so it is the most expressed genes among the four subunits. Also the above result gives us the gene length, total %GC content and %GC content at position 1, 2 and 3 along with NC (effective number of codon) which provides measure of the extent of codon preference in a gene. Highest Nc value is of delta subunit i.e. 28.6 and lowest happen to be of beta subunit i.e. 27.3. Nc can take values from 20, in the case of extreme bias where one codon is exclusively used for each aa, to 61 when the use of alternative synonymous codons is equally likely. GC content shows tighter constraints and divergence control in coding regions. For instance, among the positions of codons in a gene, the dispersion can vary 50-fold in the 1st position, 20% for 2nd position, and 10% in the 3rd position. For many genomes, the 3rd codon is highest in GC content; the 1st is greater than the 2nd, with the order 3-1-2. As per table1 highest %GC 3 happens to be 96.9% of gamma subunit. Usually in bacteria, the 3rd is much higher than the 2nd and 1st (3rd position has a 3.6 slope on a least sum squared plot versus 2nd and 1st). These freedoms and constraints provide a framework for the codon bias table specificity [25].

Table 1
Gene Parameters in P. denitrificans

Name	Gene Length	CAI	%GC	%GC 1	%GC 2	%GC 3	Nc
gi_alpha	3756	0.980	66.7	61.6	45.0	93.6	27.5
gi_beta	999	0.979	61.9	55.3	39.6	90.7	27.3
gi_gamma	684	0.984	64.5	58.3	38.2	96.9	27.8
gi_delta	759	0.977	69.7	77.5	41.5	90.1	28.6

Computational tools provide researchers to understand physico- chemical and structural properties of proteins. A large number of computational tools are available for making predictions regarding the identification and structure prediction of proteins. Here ProtParam tool of Expasy is used to evaluate and determine various parameters. Isoelectric point (pI) is the pH at which the surface of the protein is covered with charge but the net charge of the protein is zero. Proteins are found to be stable and compact at their pI. The calculation of the protein charge does not include knowledge about any potential post-translational modifications the protein may have. The protein charge plot (Fig. 2) here shows that in the pH range of 6-8, the isoelectric point of all the four subunits of the enzyme lie. The computed pI value of alpha, beta and delta subunits (pI < 7) indicates that these subunits are acidic and the pI of gamma subunit (pI > 7) reveals that it is basic in character. The computed isoelectric point (pI) will be useful for developing buffer systems for purification by isoelectric focusing method.

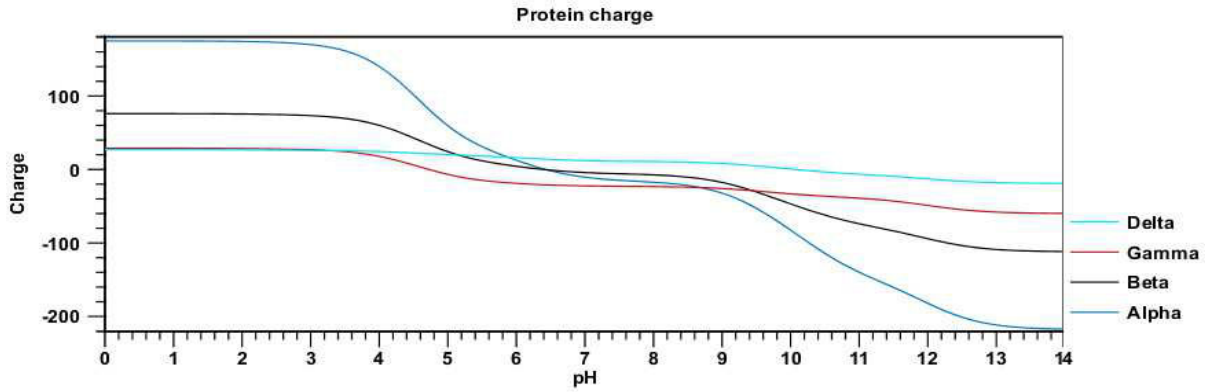

Figure 2
Protein Charge Plot of subunits of nitrate reductase in P. denitrificans

The results of primary Hydrophobicity analysis suggest that most all the four subunits are hydrophobic in nature due to the presence of high non-polar residues content (table 2). The protein charge plot is particularly useful for finding the net charge of the protein at a given pH. This knowledge can be used in relation to isoelectric focusing on the first dimension of 2D-gel electrophoresis.

Table 2
Hydrophilic and hydrophobic residues content of Sub units of nitrate reductase in P. denitrificans

Subunits of Nitrate Reductase	Length of Sequence	Number of Hydrophobic residues	Number of Hydrophilic residues	Other residues	Net Hydrophobic residues content
Alpha subunit	1259	622	311	326	Very high
Beta Subunit	518	246	129	143	Very high
Gamma Subunit	228	142	53	33	Very high
Delta Subunit	252	131	46	75	Very high

The instability index assigns a weight value of instability to the proteins and provides an estimation of the stability of protein. Instability index (II) is computed using these weight values. A protein with instability index smaller than 40 is predicted as stable whereas a value above 40 is predicted as unstable protein. On the basis of instability index Expasy's ProtParam [20] classifies the alpha and gamma subunits as stable (Instability index < 40) whereas beta and delta subunits as instable (Instability index > 40). The aliphatic index (AI) which is defined as the relative volume of a protein occupied by

aliphatic side chains (A, V, I and L) is regarded as a positive factor for the increase of thermal stability of proteins. The aliphatic index (AI) is defined as the relative volume of a protein occupied by aliphatic side chains (A, V, I and L). It is considered as a positive factor for the increase of thermal stability of globular proteins [21]. The very high aliphatic index of gamma and delta subunits (Table 2) infers that these may be stable for a wide range of temperature as compared to alpha and beta subunits with low aliphatic index.

Table 3
Parameters computed for subunits of nitrate reductase in P. denitrificans using Expasy's ProtParam tool

Name of subunit	Sequence Length	Mol. weight (K Da)	pI	-R	+R	Others	Instability Index (II)	Stability status	Aliphatic index (AI)	GRAVY
Alpha	1259	141,109	6.20	136	152	971	34.18	Stable	72.13	-0.497
Beta	518	59,015	6.16	68	62	388	40.63	Unstable	76.24	-0.454
Gamma	228	25,283	4.47	7	18	203	41.80	Unstable	101.94	-0.271
Delta	252	28,339	10.6	47	24	181	31.44	Stable	127.32	0.750

Molecular weight (Mol. Weight) ; Isoelectric point (pI) ; Number of negative residues (-R) ; Number of positive residues(+R) ; aliphatic and aromatic residues (Others) ; Instability index(II) ; Aliphatic index (AI) ; Grand Average Hydropathy (GRAVY) Average hydropathy (GRAVY) is calculated by adding the hydropathy value for each residue and dividing by the length of sequence. GRAVY Index ranges from –0·1 to 0·9. Negative GRAVY value signifies hydrophilic nature whereas positive GRAVY value hydrophobic nature [22]. Thus out of all four subunits only gamma subunit is hydrophobic in nature and hence shows least interaction with water.

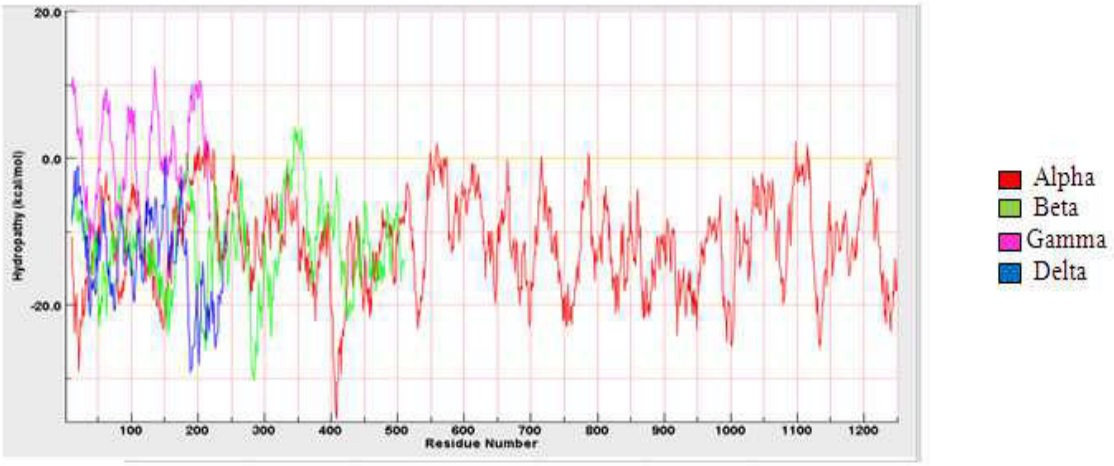

Figure 3
Hydropathy graph of all the subunits of nitrate reductase in P. denitrificans

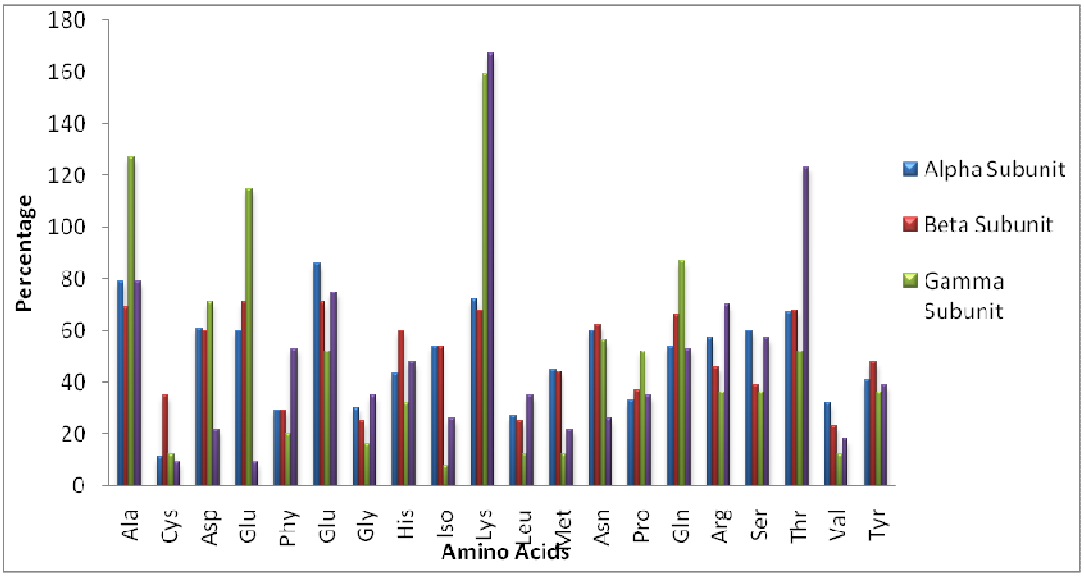

Figure 4
Bar Chart showing amino acids distribution in subunits of nitrate reductase in P. denitrificans

The Gamma subunit has least content of aliphatic amino acids, namely glycine, Valine, leucine and Isoleucine thus reducing the flexibility of the gamma subunit as compared to other three. Also, it has rich content of hydrophobic cyclic proline amino acids. Proline has a special property of creating kinks in polypeptide chains and disrupting ordered secondary structure [19-21]. It has a considerably high content of lysine, which is basic in nature, hence imparting its basic characteristics. Further protein BLAST was performed taking the gamma subunit sequence as the template against the non redundant protein sequences keeping the other parameters default. Putative domains were detected in the given BLAST result which on further analysis showed presence of three domains, out of which Nitrate_red_gam was query specific hit whereas Nar I and nar I happens to be non specific hits. The Nitrate_red_gam is a b-type cytochrome that receives electrons from the quinone pool. It then transfers these via the iron-sulphur clusters of the beta subunit to the molybdenum cofactor found in the alpha subunit. The nitrate reductase enzyme, EC: 1.7.99.4 catalyses the conversion of nitrite to nitrate via the reduction of an acceptor. The presence of domain may help us to enhance GNP production by targeting it directly and increasing its level of expression.

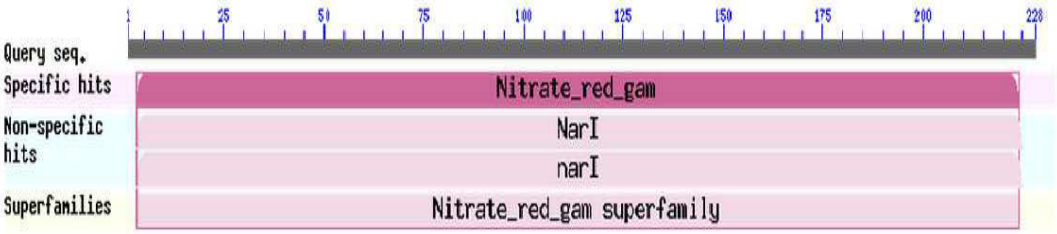

Figure 5
Conserved domains on gamma chain of nitrate reductase in P. denitrificans

The protein BLAST result generated multiple hits out of which 20 sequences of different Pseudomonas species were taken on the basis of percentage similarity more than 70% and coverage area 97%. These 20 sequences were used to perform multiple sequence alignment using COBALT. COBALT is a multiple sequence alignment

tool that finds a collection of pair wise constraints derived from conserved domain database, protein motif database, and sequence similarity, using RPS-BLAST , BLASTp and PHI-BLAST. Pair wise constraints are then incorporated into a progressive multiple alignments. Motif scan was also done using PROSITE SCAN but no hits were found. Further phylogenetic tree was drawn using COBALT, of same 20 sequences keeping the identity up to 70. From the tree it can be easily concluded that *Pseudomonas nitroreducens* shares the closest similarity with our study organism *P. denitrificans* which happens to be 99% as per the BLAST result. So we can expect major involvement of the gamma subunit of nitrate reductase in GNP biosynthesis in *P. nitroreducens* as well. Sequences with more than 0.85 differences are removed from the tree view. All these 20 species of pseudomonas lie in category of g-proteobacteria.

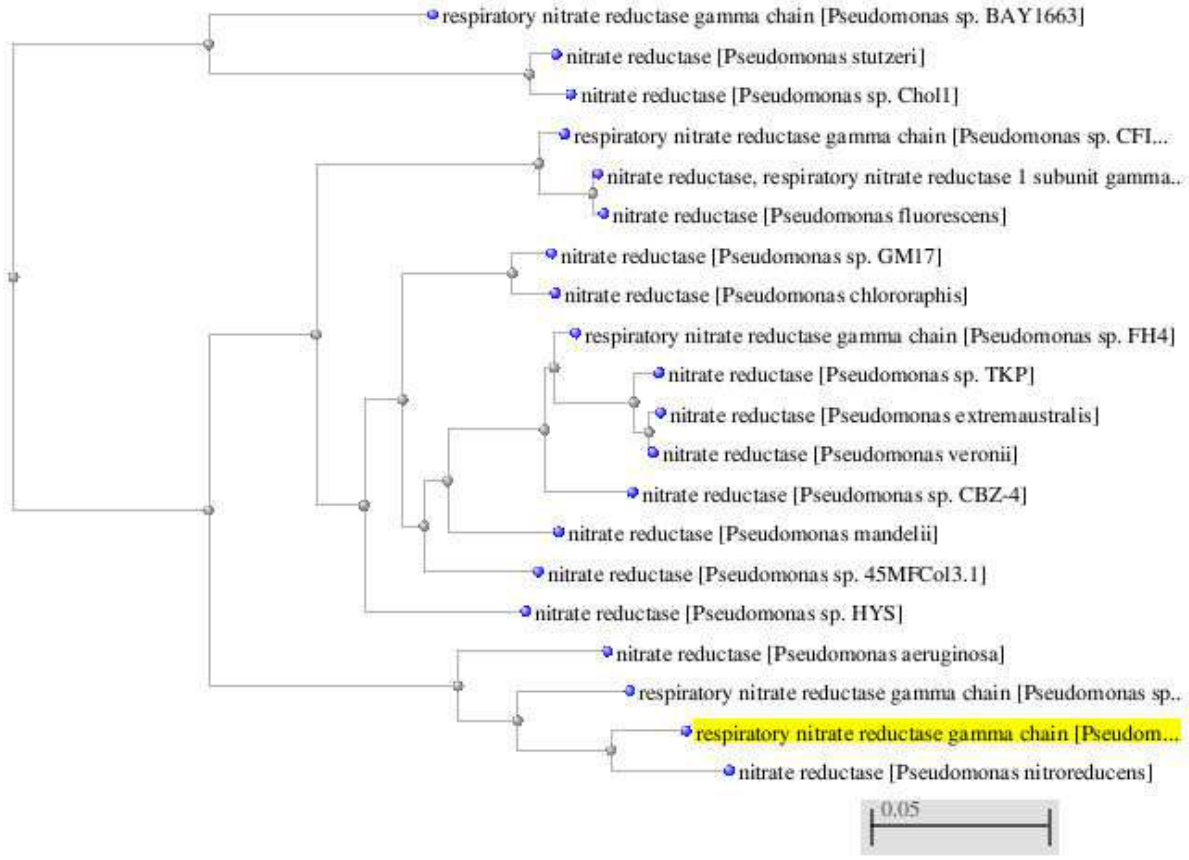

Figure 6
COBALT Phylogenetic Tree View for 20 sequences

The structure of gamma subunit of nitrate reductase in *P. denitrificans* was also studied using online tools and servers. The server SOSUI classified gamma subunit as membrane protein which has 6 transmembrane helix. The transmembrane regions and their length are tabulated in table 4.

Table 4
Gamma subunit of P. denitrificans is a membrane protein which has 6 transmembrane helices

No.	N terminal	Transmembrane region	C terminal	Type	Length
1	4	NLLVFGVYPYVALLICLVGSWAR	26	PRIMARY	23
2	50	YSNLFHVGVLFILAGHFVGLLTP	72	SECONDARY	23
3	88	LAMVSGGFFGVLCFIGLTGLIL	109	SECONDARY	22
4	123	ASDLMILLVLYVQLILGLSTIVA	145	SECONDARY	23
5	156	VMLANWAQSIVTLQPMAAADAIA	178	SECONDARY	23
6	192	LTLFVLFPFTRLVHIVSAPVWYF	214	PRIMARY	23

The secondary structure prediction called "SOPMA" which means Self Optimised Prediction from Multiple Alignment was used to further analyse gamma subunit. The sequence length is 228 amino acid and the result is given in table 5. As per the SOPMA results these regions are found to be rich in alanine (A), glutamic acid (E), leucine (L), and methionine (M) and poorer in proline (P), glycine (G), tyrosine (Y), and serine (S) as these amino acids tend to form an α helix. Considering the results of Table 5 we can say that the highest percentage happens to be of alpha helix followed by random coils and then extended strands. Beta turns contributes the least in gamma subunit structure.

Table 5
Composition and distribution of various secondary structures in gamma subunit of nitrate reductase in P. denitrificans

Types of Secondary Structure	Number (out of 228)	Percentage
Alpha Helix (Hh)	97	42.54%
Extended Strand (Ee)	60	26.32%
Random Coil (Cc)	64	28.07%
Beta Turn (Tt)	7	3.0%

Online server CFSSP was also used to analyse and predict the secondary structure of gamma subunit using amino acid sequence. Regions richer in alanine (A), glutamic acid (E), leucine (L), and methionine (M) and poorer in proline (P), glycine (G), tyrosine (Y), and serine (S) tend to form an α-helix. The secondary structure transitions into two categories. *Partial helix-to-strand transition* contains at least one dipeptide unit with an α-helix in one protein and a β-strand, for the same residues, in another (e.g., CCHHH in one protein and TCCEE in another protein, where H represents helix, E strand, C coil, and T turn) ; while for *complete helix-to strand transition* one peptide of a pair should contains only an α-helix structure and the other only a β -strand structure (e.g., HHHHH in one protein and EEEEE in another protein) . While the greater percentage of alanine (A), leucine (L), and methionine (M) explains the region of greater percentage of alpha helix or vice versa.

>gi|505291478|ref|WP_015478580.1| respiratory nitrate reductase gamma chain [Pseudomonas denitrificans]

MSLNLLVFGVYPYVALLICLVGSWARFDLSQYTWKAGSSQMLSKKGMRVYSNLFHVGVLFILAGH
FVGLLTPHAVYEHLISTEQKQLLAMVSGGFFGVLCFIGLTGLILRRLTNDRVRATGNASDLMILLVL
YVQLILGLSTIVASTHHLDGSVMVMLANWAQSIVTLQPMAAADAIAPVSLVYKLHVTLGLTLFVLFP
FTRLVHIVSAPVWYFGRRYQVVRTKRRAV

The random coil is not a true secondary structure, but is the class of conformations that indicate an absence of regular secondary structure. So on the basis of given data and the combine result of both SOPMA and CFSSP, the predicted structure of gamma subunit is considered in category of partial helix-to-strand category.

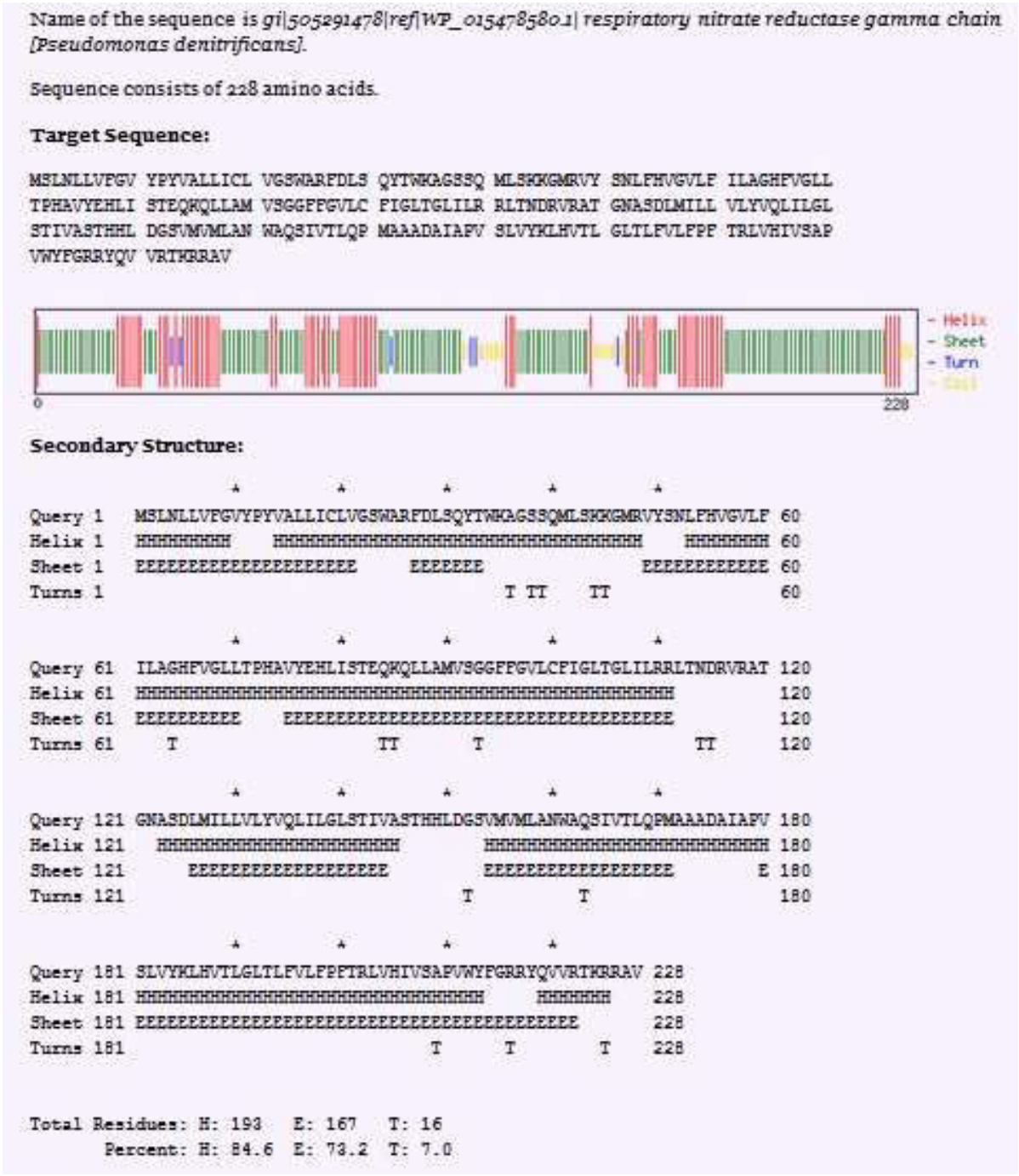

Figure 7
CFSSP result of gamma subunit of nitrate reductase in P.denitrificans

Further using Expasy's Swiss Model, 3D structure of gamma subunit was predicted as the X-ray crystallography structure of nitrate reductase in *P.denitrificans* is not reported. It uses nitrate reductase of *E.coli* as template sequence [27]. The result gave 3 probable models with their GMQE value i.e. Global Model Quality Estimate. GMQE calculates a quality estimate on the basis of a single model or derive a score from the information contained in the ensemble of all the models generated for a given sequence. Here in this case, GMQE of model 1, Model 2 and model 3 respectively were 0.80, 0.77 and 0.78 out of which model 1 with highest GMQE 0.80 was chosen as the best probable model [28].

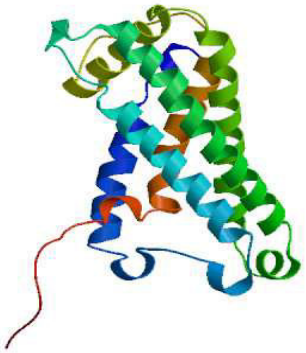

Figure 8
Expasy Swissmodel 3D structure of gamma subunit of nitrate reductase in P.denitrificans

CONCLUSION

Nitrate reductase being the main enzyme involved in gold nanoparticle biosynthesis in *P. denitrificans* is taken for study of codon biasness and other parameters like physico-chemical properties of *Insilco* analysis. Physico-chemical characterization studies give a good idea about the properties such as pI, Aliphatic Index, GRAVY and Instability Index that are essential and vital in providing data about the four subunits. The BLAST and MSA result also leads to showing close relatedness of gamma subunit of nitrate reductase in between *P. denitrificans* and *P. nitroreducens*. The result of secondary structure prediction using SOPMA, SOSUI and CFSSP leads to conclude that gamma subunit lies in the category of partial helix-to-strand category which contains major proportion of α-helix. 3D structure was also predicted using Expasy Swissmodel. The presented work emphasizes that we must target the gamma subunit in *P. denitrificans*, as it has the highest CAI value which shows the maximum level of expression and hence we must try to enhance its level of expression in order to increase the production rate of gold nanoparticle.

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