Original Article

Cloning, Expression and Characterization of NAD Kinase from *Staphylococcus aureus* Involved in the Formation of NADP (H): A Key Molecule in the Maintaining of Redox Status and Biofilm Formation

U. Venkateswara Prasad, D. Vasu, R. Rishi Gowtham, Ch. Krishna Pradeep, V. Swarupa, S. Yeswanth, Abhijit Choudhary[1], P. V. G. K. Sarma

From the Departments of Biotechnology and [1]Microbiology, Sri Venkateswara Institute of Medical Sciences, Tirupati, Andhra Pradesh, India

Abstract

Background: *Staphylococcus aureus* has the ability to form biofilms on any niches, a key pathogenic factor of this organism and this phenomenon is directly related to the concentration of NADPH. The formation of NADP is catalyzed by NAD kinase (NADK) and this gene of *S. aureus* ATCC 12600 was cloned, sequenced, expressed and characterized. **Materials and Methods:** The NADK gene was polymerase chain reaction amplified from the chromosomal DNA of *S. aureus* ATCC 12600 and cloned in pQE 30 vector, sequenced and expressed in *Escherichia coli* DH5α. The pure protein was obtained by passing through nickel metal chelate agarose column. The enzyme kinetics of the enzyme and biofilm assay of the *S. aureus* was carried out in both aerobic and anaerobic conditions. The kinetics was further confirmed by the ability of the substrates to dock to the NADK structure. **Results:** The recombinant NADK exhibited single band with a molecular weight of 31kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the gene sequence (GenBank: JN645814) revealed presence of only one kind of NADK in all *S. aureus* strains. The enzyme exhibited very high affinity for NAD compared to adenosine triphosphate concurring with the docking results. A root-mean-square deviation value 14.039Å observed when NADK structure was superimposed with its human counterpart suggesting very low homology. In anaerobic conditions, higher biofilm units were found with decreased NADK activity. **Conclusion:** The results of this study suggest increased NADPH concentration in *S. aureus* plays a vital role in the biofilm formation and survival of this pathogen in any environmental conditions.

Keywords: *Adenosine triphosphate, biofilms, NAD kinase, NADPH, root-mean-square deviation*

Introduction

Staphylococcus aureus is a Gram-positive cocci and an important cause of nosocomial infections. Appearance of multidrug resistance strains of *S. aureus* including resistant to vancomycin all over the world in such strains conspicuous variations are observed in colony morphology, physiology and growth characteristics due to high reductive conditions with poor acetate metabolism.[1-4] Studies have shown that high anaerobic conditions favor accumulation of NADPH and NADH in bacteria and these molecules inhibit NAD kinase (NADK).[5]

The numerous pivotal functions of NAD(P) in metabolism, transcription, signaling pathways and detoxification reactions makes nicotinamide dinucleotide a central molecule for cell viability, which means that its concentration is firmly regulated. The biosynthesis of NAD(P) have been elucidated in detail[6,7] in *Escherichia coli*, *Salmonella enterica serovar* and *Salmonella typhimurium*, a multifunctional protein NadR is reported as NAD-dependent repressor of transcription of genes implicated in NAD biosynthesis,[8,9] thus explains this pathway is regulated at the transcriptional level.

NAD is synthesized through *de novo* or pyridine salvage pathway and there are many differences exists between prokaryotes and eukaryotes.[6,7] The main metabolite in *de novo* NAD biosynthesis in all living organisms is quinolinic acid (QA). Eukaryotes synthesize QA via tryptophan degradation while prokaryotes obtain QA through the condensation of imino aspartate with dihydroxyacetone phosphate which is catalyzed by quinolinate synthetase system.[6,7] QA is

How to cite this article: Prasad UV, Vasu D, Gowtham RR, Pradeep K, Swarupa V, Yeswanth S, *et al.* Cloning, Expression and Characterization of NAD Kinase from *Staphylococcus aureus* Involved in the Formation of NADP (H): A Key Molecule in the Maintaining of Redox Status and Biofilm Formation. Adv Biomed Res 2017;6:97.

Received: March, 2015. **Accepted:** June, 2015.

Address for correspondence:
Dr. P. V. G. K. Sarma, Department of Biotechnology, Sri Venkateswara Institute of Medical Sciences, Tirupati - 517 507, Andhra Pradesh, India.
E-mail: sarmasvims@gmail.com

Access this article online

Website: www.advbiores.net

DOI: 10.4103/2277-9175.211833

Quick Response Code:

transformed into nicotinic acid mononucleotide (NaMN) by QA phosphoribosyltransferase afterward NaMN adenylyltransferase catalyzes adenylation of NaMN to nicotinic acid adenine dinucleotide.[6,7,10] The *nadD* gene encoding NaMN adenylyltransferase was shown to be essential for survival in *S. aureus* and *Streptococcus pneumonia* that are fully dependent on niacin salvage pathway.[11] Finally, nicotinic acid adenine dinucleotide is changed into NAD through the reaction catalyzed by NAD synthetase encoded by gene *nadE,* this reaction is followed by the synthesis of NADP catalyzed by magnesium dependent ubiquitous enzyme NADK[6,7] - encoded by gene NADK. Since this reaction is the only biochemical step in the synthesis of NADP from NAD, NADK is therefore, key enzyme for NADP synthesis and for the NADP-dependent anabolic and biosynthetic pathways in the cell. NADKs show homo oligomer structures but differ in the molecular size and number of subunits.[12] The molecular size of subunit from prokaryotes is approximately 30–35 kDa, almost all known NADKs are oligomeric proteins consisting of 2-8 identical subunits of 30–60 kDa.[12]

NADK is a ubiquitous, allosteric enzyme, catalyzes the formation of NADP using adenosine triphosphate (ATP) as phosphoryl donor and plays a central role in coupling oxidative - reductive conditions. It dictates whether the system is in oxidative or reductive conditions based on the concentration of [NAD^+/NADH] ratio near 1000, favors the oxidative conditions whereas [$NADP^+$/NADPH] ratio near 0.01, favors the reductive conditions[13] and defense against oxidative stress[14] by providing electrons for reductive repair. This could be one of the most crucial growth-limiting stimuli to control the pathogenesis.[15] As, NAD(H) and NADP(H) participate in more than 300 different oxidative-reductive reactions[16] their importance in substance metabolism and energy metabolism has long been known in tricarboxylic acid (TCA) cycle and *de novo* biosynthesis. NAD(H) is primarily involved in oxidative catabolic reactions, whereas NADP(H) participates in reductive anabolic reactions.[13] Thus, owing to the importance of NADK which is essential for the survival of microorganisms[17] the present study is focused on cloning, expression and characterization of NADK of *S. aureus* ATCC 12600.

Materials and Methods

Bacterial strains and conditions

S. aureus ATCC 12600 and *E. coli* DH5α were obtained from Merck Biosciences. Pvt Ltd. *S. aureus* was grown on modified Baird Parker media at 37°C. A clear isolated colony exhibiting distinct zone and shiny black color was picked and inoculated in brain heart infusion (BHI) broth and grown for overnight at 37°C and this grown *S. aureus* ATCC 12600 culture was used to characterize NADK. *E. coli* DH5α were used in the expression of *S. aureus* NADK gene cloned in pQE 30 vector.[18]

Biofilm assay

The biofilm assay was carried out for *S. aureus* ATCC 12600 grown in Luria-Bertani and BHI broths following earlier explained method.[19]

Kinetic study of NAD kinase

The cytosolic fraction was collected from the *S. aureus* to perform the enzyme assay and kinetics for NADK. The reaction mixture contains 10 mM $MgCl_2$, 5 mM NAD, 10 mM ATP, 5 mM glucose-6-phosphate, 10 mM Tris-HCl pH 7.5, pure glucose-6- phosphate dehydrogenase, crude or pure NADK of *S. aureus*. The reaction mixture was incubated for 4-5 min at 37°C and absorbance was taken at 340 nm. The maximum velocity of the enzyme catalyzed reaction was calculated by taking varying concentrations of substrate NAD from 0.25 to 10 mM. K_M and V_{max} for NADK were determined using Hanes–Woolf plot by taking [S_o] on X-axis and [S_o/V_o] on Y-axis. From Y-intercept value obtained in the graph, K_M was calculated.[20] Bradford method was applied to estimate the concentration of proteins.[21]

NAD kinase gene amplification and sequencing from *Staphylococcus aureus* ATCC 12600

Chromosomal DNA was extracted from late log phase culture of *S. aureus* and NADK gene was amplified by using forward primer: 5'-CATGCGTTATACAATTT-3' and reverse primer: 5'-TCATCGTTCTTCATCAC-3' which were designed from the NADK gene sequence of *S. aureus* Mu 50 strain.[22] The cocktail reaction mixture of 50 µl contained 0.5 µg of chromosomal DNA, 100 µM of dNTP's mixture, 100 picomoles of forward primer, and reverse primer, 1 unit of Taq DNA polymerase (Merck Biosciences. Pvt Ltd). Amplification parameters included an initial denaturation step for 10 min at 94°C; 40 cycles of each having denaturation at 94°C for 60 s, annealing at 37.35°C for 90 s and amplification at 72°C for 120 s which was followed by a final extension step at 72°C for 5 min in a master cycler gradient Thermocycler (Eppendorf). Nanoparticle-polymerase chain reaction (PCR) cleansing kit (Taurus Scientific, USA) was used to purify the PCR products which were then subjected to sequencing using dye terminating method at MWG Biotech India Ltd. Thus, obtained NADK gene sequence was deposited at GenBank (www.ncbi.nlm.nih.gov/genbank).

Cloning, expression and purification of NAD kinase

NADK gene was cloned in the Sma I site of pQE 30 and followed by transformation in *E. coli* DH5α. Thus formed clone was called as NADK-1. The insert in the clone was sequenced and on confirming the sequencing in the clone the gene was over-expressed with 1mM IPTG. The recombinant NADK [rNADK] was purified from the cytosolic fraction of clones by passing through nickel metal chelate agarose column (by following QIA express expression system

protocol) and protein was eluted using 300 mM imidazole hydrochloride the product was analysed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[18,23] The enzyme kinetics of purified rNADK was performed as described earlier in the section.

Sequence and structural analysis of NAD kinase

The three-dimensional structure of *S. aureus* NADK was built by using modeler 9v8 tool. The stereochemistry of the final model was verified by submitting to PROCHECK and ProSA-web servers. The structural alignment of *S. aureus* NADK and human NADK structures were carried out using PyMol software.[24-26] ATP and NAD docking to NADK of *S. aureus* and human was performed to find out the mode of binding and affinity variations using Molecular Operating Environment MOE version 2011.10 software (Chemical Computing Group, Canada).

Results

Characterization of NAD kinase

NADK is the most prominent enzyme whose products detects whether the system is in oxidative or reductive conditions. In the present study, we have cloned, sequenced, expressed, and characterized NADK gene from *S. aureus* ATCC 12600. The sequence of NADK (GenBank Accession number: JN645814) showed complete homology with NADK gene of all the strains of *S. aureus* reported in the database. The NADK gene expressed from NADK-1 clone was purified by passing through nickel metal chelate affinity column showed a molecular weight of 31 kD on 10% SDS-PAGE [Figure 1a and b].

The NADK identified in the cytoplasm of *S. aureus* ATCC 12600 demonstrated an enzyme activity of 1.65 ± 0.05 mM/ml/min and K_M 0.64 ± 0.5 mM [Table 1] for NADP substrate and for ATP as substrate the enzyme exhibited 1.2 ± 0.04 μM/ml/min as enzyme activity with K_M 1.01 ± 0.4 mM. Similar results were observed with pure rNADK [Table 2], signifying the presence of only one NADK gene in *S. aureus* corroborating with the basic local alignment search tool and docking results. NADK docking of *S. aureus* results showed highest docking score with NAD (−13.9069 kcal) compared to ATP (−13.7903 Kcal) [Supplementary Table 1 and Supplementary Figure 1a and b]; however, human NADK showed highest docking score with ATP (−12.7409) compared to NAD (−9.7059) [Supplementary Table

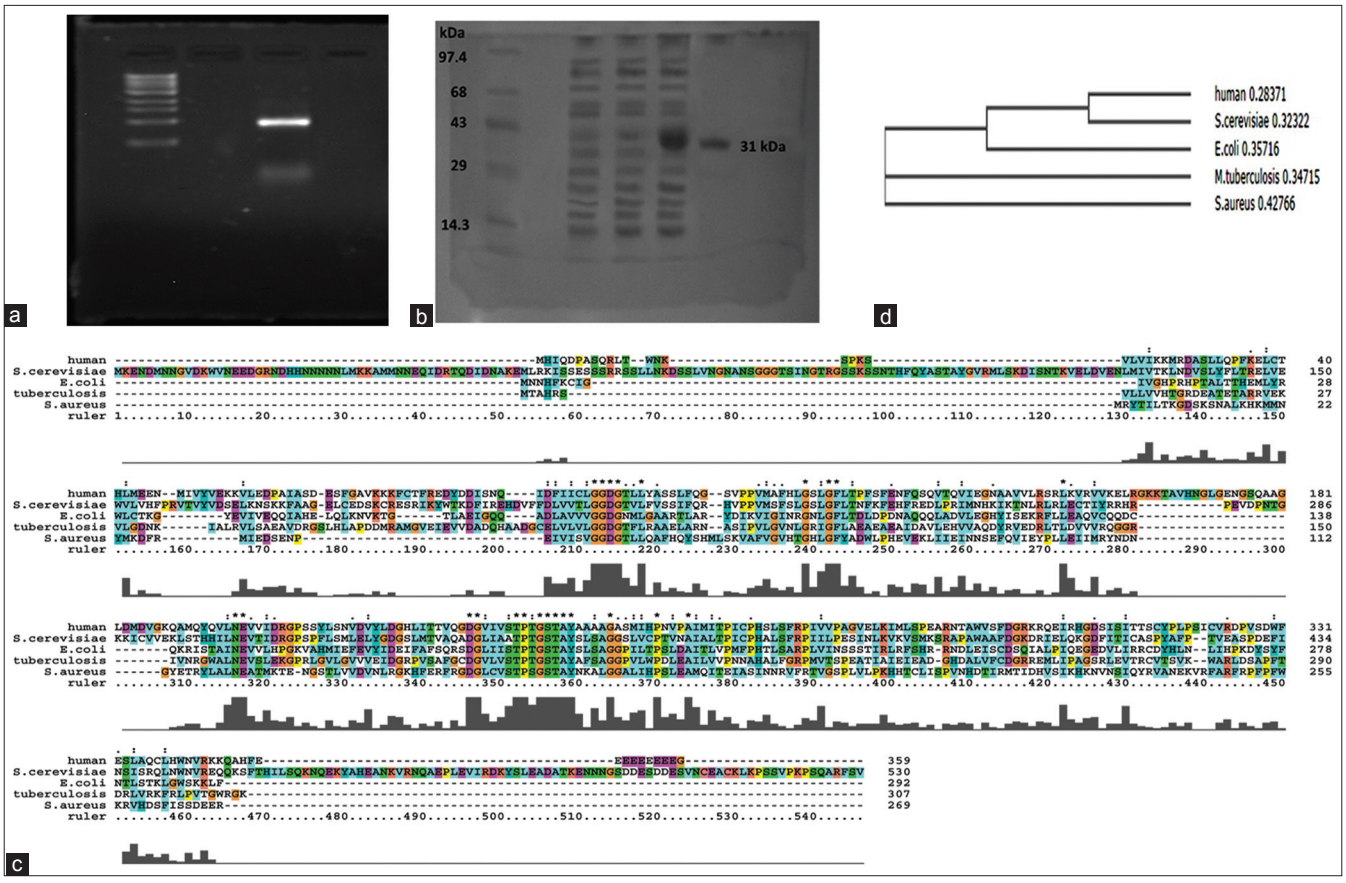

Figure 1: (a) Amplification of NAD kinase gene using NAD kinase 1 and NAD kinase 2 primers from the chromosomal DNA of *Staphylococcus aureus*. M Lane showing DNA ladder L1 lane showing amplified product of NAD kinase gene, (b) electrophoretogram showing the expression of recombinant NAD kinase clone in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane M: Molecular weight marker. Lane 1 and 2: Uninduced cell lysate of NAD kinase 1 clone. Lane 3: Induced cell lysate of NAD kinase 1 clone Lane 4: Purified recombinant NAD kinase. (c) Multiple sequence alignment of NAD kinase. (d) Phylogenetic tree based on NAD kinase sequence of *Staphylococcus aureus* with other bacterial NAD kinase sequences and Human NAD kinase

Table 1: The enzyme kinetics of NADK and rNADK for NAD substrate

Cytosolic fraction	Enzyme activity (NADPH mM/ml/min)	V_{max} (NADPH mM/mg/min)	K_M (mM)
S. aureus ATCC 12600	1.65±0.05	331±0.7	0.64±0.5
rNADK	1.7±0.03	329.49±0.6	0.66±0.2

Values±SD from three determinations. NADK: NAD kinase, V_{max}: Maximum velocity, SD: Standard deviation, *S. aureus: Staphylococcus aureus*, rNADK: Recombinant NADK

Table 2: The enzyme kinetics of NADK and rNADK for ATP substrate

Cytosolic fraction	Enzyme activity (NADPH mM/ml/min)	V_{max} (NADPH mM/mg/min)	K_M (mM)
S. aureus ATCC 12600	1.2±0.04	147.4±0.8	1.01±0.4
rNADK	1.1±0.03	169.49±0.6	0.99±0.2

Values±SD from three determinations. SD: Standard deviation, NADK: NAD kinase, V_{max}: Maximum velocity, ATP: Adenosine triphosphate, *S. aureus: Staphylococcus aureus*, rNADK: Recombinant NADK

2 and Supplementary Figure 1c-d]. The buildup of NADPH in the bacteria allosterically inhibits NADK[5] and in the present study the pure rNADK activity was inhibited by the NADPH [Supplementary Figure 2]. The enzyme kinetics when compared with other organisms such as *Saccharomyces cerevisiae*,[27] *Mycobacterium tuberculosis*,[28-30] *E. coli*[29-31] and human[32] showed significant differences [Supplementary Table 3] correlating with the differences observed in the multiple sequence alignment of NADK gene sequence [Figure 1c and d].

Increased NADK activity was observed in *S. aureus* ATCC 12600 grown in aerobic broth compared with anaerobic conditions indicating increased buildup of NADPH in the organism corroborating the high reductive conditions favoring high rate of biofilm formation [Table 3].

Comparative structural analysis of *S. aureus* NAD kinase with human NAD kinase

The structural alignment of *S. aureus* ATCC 12600 NADK [Figure 2a] and human NADK [Figure 2b] structures revealed an identity of 9.1% that is, distributed randomly throughout the conformation and when both structures were superimposed [Figure 2c], the root-mean-square deviation (RMSD) value 14.039Å indicates the extensive structural variations in both domain and nondomain regions. This value also implies distantly aligned variation between the Cα atoms of the backbones of two aligned structures.

Discussion

There is a growing recognition that NADP (H) is the crucial coenzyme for many cellular processes in living organisms,

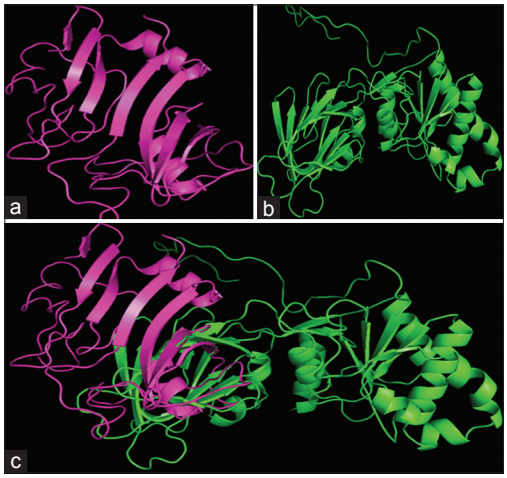

Figure 2: Structural comparison of *Staphylococcus aureus* NAD kinase (magenta) and human NAD kinase (Green) using PyMol. (a) *Staphylococcus aureus* NAD kinase; (b) human NAD kinase; (c) super imposed structures of human NAD kinase (green), staph NAD kinase (magenta)

such as NADPH-dependent reductive anabolic pathways; signal transduction, cellular defense against stress.[33,34] Studies have indicated that NADK is an essential enzyme for the survival of microorganisms in varied environmental conditions;[17] therefore, the present study is focused on cloning, expression and characterization of NADK from *S. aureus* ATCC 12600. In *S. aureus* and in *M. tuberculosis*, the activity of NADK is inhibited intensively by $NADP^+$[29,31] whereas in *E. coli*[35] and *S. enteric*[5] NADH and NADPH are potent allosteric negative modulators of NADK.

NADK mainly concerned with these reactions and dictates whether the system is in oxidative or reductive conditions. This could be one of the most crucial growth-limiting stimuli to control the pathogenesis.[15] The ratio of NAD/$NADP^+$ and NADH/NADPH controls many pathways in *S. aureus* such as TCA cycle, *de novo* biosynthesis and this regulation has profound effect on the redox status which is a key factor in *S. aureus* for the production of toxins, virulence factors and biofilm formation.[19,26,36,37] In the present study, the anaerobic conditions favored biofilm formation with decreased NADK activity which explains there is a greater build-up of NADPH in the bacteria leading to increased synthetic phase [Table 3].

The comparison of amino acid sequences of several known prokaryotic and eukaryotic NADKs revealed a general structural organization consisting of a conserved catalytic domain within the C-terminus and variable N-terminal parts[12] but the regulatory patterns of NADKs differ distinctively among microbes. These conspicuous differences throw light on the pathogenesis of *S. aureus* in the human host as compared with other bacteria.

The structural analysis of NADK revealed that it has a very low homology with human NADK as indicated from

Table 3: NADK activity in *S. aureus* ATCC 12600 grown in aerobic and anaerobic media

Source of enzyme	Aerobic medium (LB)				Anaerobic medium (BHI)			
	Enzyme activity (NADPH mM/ml/min)	V_{max} (mM/mg/min)	K_M (mM)	Biofilm units	Enzyme activity (NADPH mM/ml/min)	V_{max} (mM/mg/min) NADPH to mM/mg/min	K_M (mM)	Biofilm units
NADK in the cytosolic fraction of *S. aureus* ATCC 12600	4.17	286	0.66±0.4	0.04±0.02	1.65±0.05	331±0.7	0.64±0.5	0.08±0.02

Values±SD from three determinations. SD: Standard deviation, NADK: NAD kinase, V_{max}: Maximum velocity, BHI: Brain heart infusion, LB: Luria-Bertani, *S. aureus: Staphylococcus aureus*

the RMSD value 14.039Å. The results correlated with the enzyme kinetic data[28-32] which indicated that human NADK has very high affinity for the substrate compared to *S. aureus* NADK[32] [Supplementary Tables 1-3 and Supplementary Figures 1 and 2]; which is understandable owing to the fact that high oxidative conditions prevails in human tissues compared with *S. aureus*. However, it is very well known that this human pathogen can colonize in any anatomical locales in the host[38] for which *S. aureus* must be influencing the cellular redox state thus regulating metabolic, signaling and transcriptional processes in the cell,[2,19,33,34,37] this probably facilitates in the colonization and biofilm formation.

Conclusion

In the present study, NADK which catalyzes the synthesis of NADP in *S. aureus* was cloned, sequenced, expressed and characterized. The results of the present study indicated the buildup of NADPH in *S. aureus* in reductive conditions favors higher biofilm formation and this phenomenon is vital in the survival and spread of its infection in both hospital settings and community-acquired conditions.

Acknowledgments

We sincerely acknowledge Sri Venkateswara Institute of Medical Sciences University for providing funds and facilities under SBAVP scheme (SBAVP/Ph. D/02) to carry out this work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: Molecular docking of *S. aureus* NADK

Compound	Docking score (kcal/mol)	Number of hydrogen bonds	Interacting residues	Hydrogen bond length in Å
NAD	−13.9069	2	Val 201	2.2
			His 206	2.4
ATP	−13.7903	1	Arg 141	2.8

NADK: NAD kinase, ATP: Adenosine triphosphate, *S. aureus: Staphylococcus aureus*

Supplementary Table 2: Molecular docking of human NADK

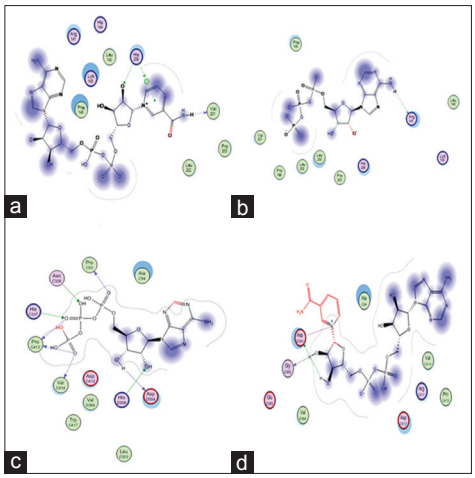
Ligand	Docking score	Interacting residues	Number of hydrogen bonds	Distance in Å
NAD	−9.7059	Asp (304)	1	2.2
		Gly (293)	1	2.4
		Asp (304)	1	2.6
ATP	−12.7409	Pro (93)	1	2.3
		Asn (339)	1	2.5
		His (337)	1	2.6
		Pro (413)	1	2.2
		Val (414)	1	2.1
		His (306)	1	2
		ASP (304)	1	2.1

NADK: NAD kinase, ATP: Adenosine triphosphate

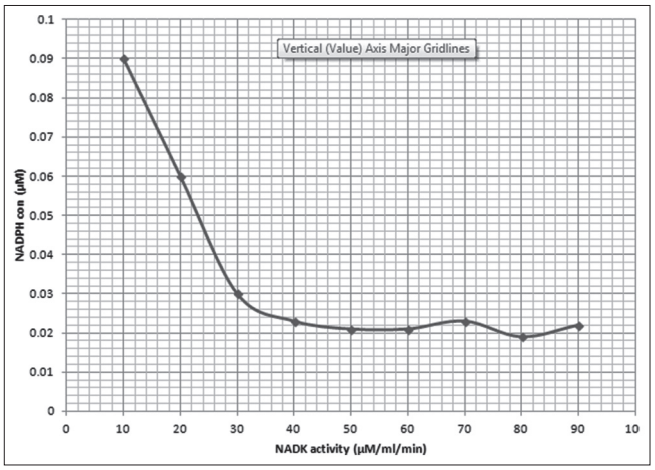
Supplementary Table 3: The comparative NADK kinetics with other bacteria and human

Organism	K_M	References
S. aureus	0.66 mM	In the current study
S. cerevisiae	0.32 mM	[27]
Human	0.54 mM	[32]
E. coli	2 mM	[30]
M. tuberculosis	0.9-3.3 mM	[31]

NADK: NAD kinase, *S. aureus: Staphylococcus aureus, S. cerevisiae: Saccharomyces cerevisiae, E. coli: Escherichia coli, M. tuberculosis: Mycobacterium tuberculosis*



Supplementary Figure 1: (a) Docking of NAD with *Staphylococcus aureus* NAD kinase. (b) Docking of adenosine triphosphate with *Staphylococcus aureus* NAD kinase. (c) Docking of NAD with human NAD kinase (d) Docking of NAD with human NAD kinase



Supplementary Figure 2: Graph showing the inhibition of NAD kinase with NADPH