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Gene cloning and characterization of NADH oxidase from *Thermococcus kodakarensis*

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The genome search of *Thermococcus kodakarensis* revealed three open reading frames, Tk0304, Tk1299 and Tk1392 annotated as nicotinamide adenine dinucleotide (NADH) oxidases. This study deals with cloning, and characterization of Tk0304. The gene, composed of 1320 nucleotides, encodes a protein of 439 amino acids with a molecular weight of 48 kDa. Expression of the gene in *Escherichia coli* resulted in the production of Tk0304 in soluble form which was purified by heat treatment at 80°C followed by ion exchange chromatography. Enzyme activity of Tk0304 was enhanced about 50% in the presence of 30 µM flavin adenine dinucleotide (FAD) when assay was conducted at 60°C. Surprisingly the activity of the enzyme was not affected by FAD when the assay was conducted at 75°C or at higher temperatures. Tk0304 displayed highest activity at pH 9 and 80°C. The enzyme was highly thermostable displaying 50% of the original activity even after an incubation of 80 min in boiling water. Among the potent inhibitors of NADH oxidases, silver nitrate and potassium cyanide did not show any significant inhibitory effect at a final concentration of 100 µM. Detergents, ionic as well as nonionic, did not display any notable effect on the enzyme activity. The presence of salts in the reaction mixture enhanced the enzyme activity. The K_m and V_{max} values toward NADH were calculated as 80 µM and 110 µmol min^{-1} mg^{-1}.

Key words: NADH oxidase, *Thermococcus kodakarensis*, flavoenzyme, thermostable, hyperthermophile, archaea.

INTRODUCTION

Nicotinamide adenine dinucleotide oxidases that catalyze the oxidation of nicotinamide adenine dinucleotide (NADH) by the reduction of molecular oxygen (Condon, 1987) are widely distributed in nature. They have been found in a variety of organisms including bacteria (Ahmed and Claiborne, 1989; Zoldak et al., 2003), archaea

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Abbreviations: **NADH**, Nicotinamide adenine dinucleotide; **FAD**, flavin adenine dinucleotide; **NADPH**, nicotinamide adenine dinucleotide phosphate; **DNA**, deoxyribonucleic acid; **BLAST**, basic local alignment search tool; **PCR**, polymerase chain reaction; **SDS**-**PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

(Ward et al., 2001; Kengen et al., 2003), yeast (Heux et al., 2006) and mammals (Xia et al., 2003). These enzymes mediate the direct transfer of electrons from NADH to molecular oxygen and depending on the enzyme, water, hydrogen peroxide, or superoxide may be formed as products. Among archaea homologues of NADH/ nicotinamide adenine dinucleotide phosphate (NADPH) oxidase have been found in *Pyrococcus horikoshii* (Harris et al., 2005), *Pyrococcus furiosus* (Ward et al., 2001), *Thermococcus profundus* (Jia et al., 2008), *Archaeoglobus fulgidus* (Kengen et al., 2003), and *Methanocaldococcus jannaschii* (Case et al., 2009). This is surprising because most of these organisms are strict anaerobes, a class of organisms that have not been expected to possess NADH oxidases. *Thermococcus kodakarensis* KOD1 is a hyperthermophilic archaeon, isolated from a solfatara at a wharf on Kodakara Island, Kagoshima, Japan (Morikawa et al., 1994; Atomi et al.,

2005). The strain is a strict anaerobe and grows on a variety of organic substrates including starch, pyruvate, amino acids, and peptides (Atomi et al., 2004; Rashid et al., 2004). The genome search of *T. kodakarensis* revealed three open reading frames namely Tk0304, Tk1299 and Tk1392 annotated as NADH oxidases (Fukui et al., 2005). All the three NADH oxidase homologues are needed to be studied in order to shed light on their function in this hyperthermophilic archaeon. Tk1392 contains a distinct C-terminal domain and we have cloned and characterized the gene product (Rashid et al., 2011). In this study, we examined the enzymatic properties of Tk0304, revealing that it is a true NADH oxidase in *T.kodakarensis*.

MATERIALS AND METHODS

Chemicals, enzymes and reagents

Chemicals used in this study were purchased from Sigma (St. Louis, Mo., USA) and were of analytical grade. Restriction enzymes, deoxyribonucleic acid (DNA) extraction and ligation kits, protein standards and DNA markers were obtained from Fermentas Life Sciences (Hanover, MD, USA).

Sequence analyses

DNA and amino acid sequence analyses were performed using DNASIS software (Hitachi Software, Tokyo, Japan). Database homology searches were executed by using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990). Multiple-alignment and phylogenetic analyses were performed by using CLUSTAL W program (Thompson et al., 1994) provided by DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/top-e.html).

Primer designing

Complete genome sequence of T. kodakarensis *is available under the accession number NC_006624. The gene sequence of Tk0304 was obtained from GenBank and restriction map of the gene was constructed using software WEBcutter (http://rna.lundberg.gu.se/cutter2/). Primers for gene amplification by polymerase chain reaction (PCR) were designed using Primer 3.0 software (http://primer3.sourceforge.net/). An* NdeI *site was introduced at the 5'-end of the gene in order to clone it in pET-21a expression vector. Melting temperature, guanine-cytosine (GC) content, 3'-end complementarity and potential hairpin formation of the selected primers were checked by Oligonucleotide Properties Calculator available at http://www.unc.edu/~cail/biotool/oligo/.*

Gene cloning

For cloning of Tk0304 gene, forward (Tk0304F 5'-CATATGAAAATCGTCGTGGTCGGTTC) and reverse (Tk0304R 5'-GGGATAGAGGTTCAGCGGAGC) primers were designed based on the DNA sequence of the gene. Tk0304 gene was amplified by PCR using these primers as priming strands and genomic DNA of *T. kodakarensis* as template. PCR product was resolved on 1% agarose gel and purified. The purified PCR product was ligated in pTZ57R/T and *E. coli* DH5α cells were transformed. The resulting plasmid was named as pTZ-Tk0304.

Gene expression in *E. coli* and purification of recombinant Tk0304

For expression, the gene fragment was cleaved from pTZ-Tk0304 utilizing the *Nde*I and *Bam*HI restriction enzymes and ligated in pET-21a expression vector digested with the same restriction enzymes. The resulting plasmid was named as pET-Tk0304. This recombinant plasmid was used for the expression of Tk0304 gene in *E. coli* BL21 (DE3). Cells carrying recombinant vector were grown overnight at 37°C in Luria broth (LB) medium containing ampicillin (100 μg/mL). The preculture was inoculated (1%) into fresh LB medium containing ampicillin and cultivation was continued until the optical density at 660 nm reached 0.4. The gene expression was induced with 0.5 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside and incubation was continued for another 4.5 h at 37°C. Cells were harvested by centrifugation at 6,000 × *g* for 10 min and washed with 50 mM Tris-HCl buffer (pH 8.0). The cell pellet was resuspended in the same buffer, and the cells were disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation at 13,000 × *g* for 15 min. The recombinant Tk0304 in the soluble fraction was purified by heat treatment at 80°C for 20 min followed by ion exchange column chromatography by using Resource Q (GE Healthcare). Protein pattern analysis was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Estimation of protein contents

Protein contents of the samples were estimated by the Bradford assay. Bovine serum albumin was used for the standard curve construction. Absorbance was taken at 595 nm after incubating the samples with Bradford reagent for 10 min at room temperature (Bradford, 1976).

Activity measurements

NADH oxidase activity of Tk0304 was spectrophotometrically assayed by using a Shimadzu ultraviolet (UV)-160A spectrophotometer equipped with a thermostat. The standard assay mixture consisted of 50 mM Tris-Cl pH 9.0, 2 mM NADH and Tk0304 reconstituted with 1.0 mM FAD in a final volume of 1.0 ml. The reaction mixture was incubated at 60°C. The oxidation of NADH was monitored at 340 nm by recording the decrease in absorbance at 340 nm for 5 min. A control experiment contained all the contents except for Tk0304. The decrease in absorbance in the control was subtracted from the experimental value.

Temperature and pH effect on the enzyme activity

Optimal temperature of recombinant Tk0304 was determined by incubating the standard reaction mixture at various temperatures ranging from 50 to 90°C. A control reaction containing all the reagents except for Tk0304 was always incubated along with the experiment at each temperature. In order to determine the thermostability of Tk0304, the recombinant enzyme, in 50 mM Tris-Cl buffer (pH 8.0), was incubated at 80 and 100°C for various intervals of time, and the residual activity was determined by the standard assay method at 60°C. Optimum pH for the NADH oxidase activity of recombinant Tk0304 was determined by incubating the assay mixture at various pH at 60°C using 50 mM each of sodium acetate buffer (pH 6.0 to 6.5), sodium phosphate buffer (pH 6.5 to 7.5), Tris-HCl buffer (pH 7.5 to 9.5) and glycine-NaOH buffer (pH 9.5 to 10.5).

Kinetic parameters

For examination of the kinetic parameters, various concentrations of NADH ranging from 20 to 200 µM and fixed amount of enzyme were used and assay was conducted at 60°C.

RESULTS AND DISCUSSION

Primer designing and PCR amplification of Tk0304 gene

In order to clone the gene encoding Tk0304, a set of forward (Tk0304F) and reverse (Tk0304R) primers was used to amplify the gene. PCR by using these two oligonucleotides as priming strands and genomic DNA of *T. kodakarensis* as template resulted in the amplification of a 1.3 kb DNA fragment (data not shown) exactly matching the size of Tk0304 gene.

Cloning of Tk0304 gene

When pTZ-Tk0304 was used to transform *E. coli* DH5α competent cells, a total of 34 white and eight blue colonies appeared on the selection plates. White colonies were further screened for the presence of Tk0304 gene by colony PCR (data not shown). Plasmid DNA was isolated from two of the positive clones and digested with *Nde*I and *Bam*HI which resulted in the liberation of a 1.3 kb DNA fragment from vector (data not shown) in both cases. When pET-Tk0304 was used to transform *E. coli* DH5α competent cells, a total of 29 colonies appeared on the selection plate. Positive clones were selected by colony PCR and restriction enzyme digestion analysis (data not shown). One of the positive clones was sequenced in duplicate from both the strands.

Sequence analysis

Tk0304 gene consisted of 1,317 nucleotides encoding a protein of 439 amino acids having a molecular mass of 47,571 Da and an isoelectric point (pI) of 8.51. Sequence analysis indicated that amino acid residues with non polar side chains, involved in hydrophobic interactions, are present abundantly in Tk0304 (data not shown). NADH oxidases in the family Thermococcaceae have been characterized from three organisms; *P. furiosus*, *P. horikoshii* and *T. profundus* (Ward et al., 2001; Harris et al., 2005; Jia et al., 2008). When multiple sequence alignment of Tk0304 was performed using the amino acid sequence of these three proteins along with one from *M. jannaschii*, we could identify the nucleotide binding domains (Figure 1). Similarly, archaeal specific NADH oxidase amino acids (Case et al., 2009) were completely conserved in Tk0304 (Figure 1). A phylogenetic tree was constructed by comparing the amino acid sequence of

Tk0304 and NADH oxidases from archaea and bacteria. In the phylogenetic tree, Tk0304 clustered with other uncharacterized archaeal NADH oxidases (data not shown). Among the NADH oxidases that have been characterized, Tk0304 displayed the highest homologies of 85 and 44% (identities) with that originated from *P. furiosus* and *M. jannaschii*, respectively (Table 1). Surprisingly, it displayed only a 36% identity with NADH oxidase from *T. profundus* that has recently been characterized (Jia et al., 2008).

Production in *E. coli* and purification of Tk0304

In order to examine whether the Tk0304 gene product exhibits the NADH oxidase activity, the encoding gene was expressed by utilizing the T7 promoter expression system in *E. coli* BL21 (DE3). Heterologous gene expression resulted in the production of 48 kDa Tk0304 protein in the soluble form (Figure 2). When the soluble fraction containing the recombinant Tk0304 protein was incubated at 80°C for 20 min, most of the host proteins got precipitated and were removed by centrifugation (Figure 2). After removal of the heat denatured *E. coli* proteins by centrifugation, Tk0304 was further purified by ion exchange column chromatography. Recombinant Tk0304 was eluted from Resource Q column when salt gradient was 0.3 M. Analysis of the sample after ion exchange chromatography demonstrated a single protein band when analyzed by SDS-PAGE (Figure 2).

Effect of temperature, pH, detergents and salts on the enzyme activity of Tk0304

When we examined the enzyme activity of Tk0304 at various temperatures, we found that the activity increased with the increase in temperature from 50 to 80°C. Highest activity was found when the reaction mixture was incubated at 80°C (Figure 3). The enzyme activity started decreasing beyond 80°C and a 50% activity could be detected at 90°C compared to 100% at 80°C. The optimal temperature for Tk0304 enzyme activity (80°C) is a little lower than the optimal growth temperature of the strain (85°C) which is not unusual. NADH oxidases from hyperthermophiles bacteria and archaea, have lower optimal temperature for their activities compared to optimal growth temperatures of these hyperthermophiles. NADH oxidase from *Thermotoga maritima* and *Thermotoga hypogea* (optimal growth temperature of 90°C) exhibited highest activities at 80°C (Yang and Ma, 2007) and 85°C (Yang and Ma, 2005), respectively. Similarly NADH oxidases from *T. profundus* (optimal growth temperature 80°C) and *P. furiosus* (optimal growth temperature 100°C) displayed optimum activities at 70°C (Jia et al., 2008) and 85°C (Ward et al., 2001), respectively. Thermostability of

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                        FAD-binding                          Active-site
                          domain I                               Cys
Tk0304        ---------------MKIVVVGSGTAGSNFALFMRKLDRKAEITVIGKEPTMQYSPCALP 45
P.furiosus    ---------------MRIVVIGSGTAGSNFALFMRKLDRKAEITVIGKEETMQYSPCALP 45
M.janachii    MVNRKPNNPNKNGEEMRAIIIGSGAAGLTTASTIRKYNKDMEIVVITKEKEIAYSPCAIP 60
T.profundus   -----------MERKRVVIIGGGAAGMSAASRVKRLKPEWDVKVFEATEWVSHAPCGIP 48
P.horikoshii  ---------MGENMKKKVVIIGGGAAGMSAASRVKRLKPEWDVKVFEATEWVSHAPCGIP 51
                             * * **    *                  *            ** *

Tk0304        HVVSGTIEKPEDIIVFPNEFYEKQK-INLMLNTEAKAID-RERKVVVTDKG----EVPYD  99
P.furiosus    HVISGVIEKPEDVIVFPNEFYEKQR-IKLLLNTEAKKID-RERKVVVTDKG----EIPYD  99
M.janachii    YVIEGAIKSFDDIIMHTPEDYKRERNIDILTETTVIDVDSKNNKIKCVDKDGNEFEMNYD 120
T.profundus   YVVEG-ISPKEKLMHYPPEVFIKKRGIDLHMKAEVIEVE--QGRVRVREPDG-EHTYEWD 104
P.horikoshii  YVVEG-LSTPDKLMYYPPEVFIKKRGIDLHLNAEVIEVD--TGYVRVRENGG-EKSYEWD 107
                * *        *       *        *                             *
                                                             NAD-binding
                                                               domain
Tk0304        KLVLAVGSKAFIPPIKGVENEGVFTLKSLDDVRRIKAYIAERKPKKAVVIGAGLIGLEGA 159
P.furiosus    KLVIATGSKAFVPPIKGVENEGVFTLKSLEDVRKIKEFIKKRNPKNAVVIGAGLIGLEGA 159
M.janachii    YLVLATGAEPFIPPIEGKDLDGVFKVRTIEDGRAILKYIEENGCKKVAVVGAGAIGLEMA 180
T.profundus   YLVFANGASPQVPAIEGCHLEGVFTADLPPDAVAITEYMEKHDVKNVAVIGTGYIAIEMA 164
P.horikoshii  YLVFANGASPQVPAIEGVNLKGVFTADLPPDALAIREYMEKYKVENVVIIGGGYIGIEMA 167
                ** * *      * * *    ***       *   *            * * *   * *

Tk0304        EAFAKLGMEVLIVELMDRLMPTMLDKDTAKLVQAEMEKYGVSFRFGVGVSEIIG-SPVRA 218
P.furiosus    EAFAKLGMKVTVVELLEHLLPTMLDKDIAKIVEENMRKYGVDFKFGVGVDEIIG-DPVEK 218
M.janachii    YGLKCRGLDVLVVEMAPQVLPRFLDPDMAEIVQKYLEKEGIKVMLSKPLEKIVGKEKVEA 240
T.profundus   EAFVERGKNVTLIGRSERVLRKTFDKEITEVVEGKLREN-LNLRLEELTMRFEGDGRVEK 223
P.horikoshii  EAFAAQGKNVTMIVRGERVLRRSFDKEVTDILEEKLKKH-VNLRLQEITMKIEGEERVEK 226
                 *   *          *        *                    *     *
                                                            FAD-binding
                                                              domain II
Tk0304        VKIGDEEVPADLVLVATGVRANTDLAKQAGLEVNR--GIVVNEHLQTSDPEVYAIGDCAE 276
P.furiosus    VKVGEEEIDADIVLVATGVRANVELAKEAGLEVNR--GIVVNEYLQTSDPDIYAIGDCAE 276
M.janachii    VYVDGKLYDVDMVIMATGVRPNIELAKKAGCKIGF-AIEVNEKMQTSIPNIYAVGDCVE 299
T.profundus   VITDAGEYPADLVIVATGIKPNTELARQLGVRVGETGAIWTNDKMQTSVENVYAAGDVAE 283
P.horikoshii  VVTDAGEYKAELVILATGIKPNIELAKQLGVRIGETGAIWTNEKMQTSVENVYAAGDVAE 286
              *          *  ***   * **   *        *   *   ***   ** **  *

Tk0304        VIDAVTGKRTLSQLGTSAVRMAKVAAEHIAGKDVSFRPVFNTAITELFGLEIGTFGITEE 336
P.furiosus    VIDAVTGKRTLSQLGTSAVRMAKVAAENIAGRNVKFRPVFNTAITEIFDLEIGAFGITEE 336
M.janachii    VIDFITGEKTLSPFGTAAVRQGKVAGKNIAGVEAKFYPVLNSAVSKIGDLEIGGTGLTAF 359
T.profundus   TKRMITGRRVWMPLAPAGNKMGYVAGSNIAGKEVHFPGVLGTSITKFLDLEIGKTGLTEA 343
P.horikoshii  TRHVITGRRVWVPLAPAGNKMGYVAGSNIAGKELHFPGVLGTAVTKFMDVEIGKTGLTEM 346
                 **              **  ***   * *        *** ***   * *

                            ••••                          •
Tk0304        RAKKEDIEVAVGKFKGSTKPEYYPGGKPITVKLIFRKSDRKLIGGQIVGGERVWGRIMTL 396
P.furiosus    RAKKEEIEVVVGKFRGSTKPEYYPGGKPIVVKLIFRKEDRRLIGAQIVGGERVWGRIMTL 396
M.janachii    SANLKRIPIVIGRAKALTRARYYPGGKEIEIKMIFN-EDGKVVGCQIVGGERVAERIDAM 418
T.profundus   EAIKEGYDVRTAFIKAGTKPHYYPGSRTIWLKGVVDNETNKLLGVQAVGAE-ILPRIDTA 402
P.horikoshii  EALKEGYDVRTAFIKASTRPHYYPGGREIWLKGVVDNETNRLLGVQVVGSD-ILPRIDTA 405
                *            * ****     * *            * * **      **
                            ••••          •
Tk0304        SALAQKGATVEDVAYLETAYAPPISPTIDPITVAAEMAQRKLR--               439
P.furiosus    SALAQKGATVEDVVYLETAYAPPISPTIDPITIAAEMAMRKL---               438
M.janachii    SIAIFKKVSAEEELANMEFCYAPPVSMVHEPLSLAAEDALKKLSNK              463
T.profundus   AAMLTAGFTTKDAFFTDLAYAPPFAPVWDPLIVLARVLKF-----               442
P.horikoshii  AAMLMAGFTTKDAFFTDLAYAPPFAPVWDPLIVLARVLKF-----               445
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Figure 1. Multiple sequence alignment. Alignment was performed using CLUSTAL W programme. The accession numbers of the enzymes analysed are as follows: *T. profundus* (CAQ43117); *P. furiosus* (AAL81656); *M. jannaschii* (AAB98641); *P. horikoshii* (BAA29661); Tk0304 (BAD84493). Archaeal specific NADH oxidase amino acids are shown with close circle at the top. Identical sequences in all the three proteins are shown by asterisks at the bottom.

Table 1. Homology comparison of Tk0304 and related homologues.

Source	Accession number	Identity (%)
Thermococcus gammatolerans	YP_002958730	94
Thermococcus onnurineus	YP_002307656	92
Pyrococcus abyssi	NP_126342	87
Pyrococcus horikoshii	NP_143369	87
Pyrococcus furiosus	NP_579261	85
Thermococcus barophilus	ZP_04877884	84
Thermococcus sibiricus	YP_002994724	79
Methanocaldococcus jannaschii	NP_247633	44
Methanothermus fervidus	YP_004003969	42
Methanopyrus kandleri	NP_614164	40
Archaeoglobus fulgidus	NP_069231	39
Archaeoglobus profundus	YP_003400359	38
Methanosarcina mazei	NP_633320	38
Geobacillus thermoglucosidasius	ZP_06811015	37
Thermococcus profundus	CAQ43117	36
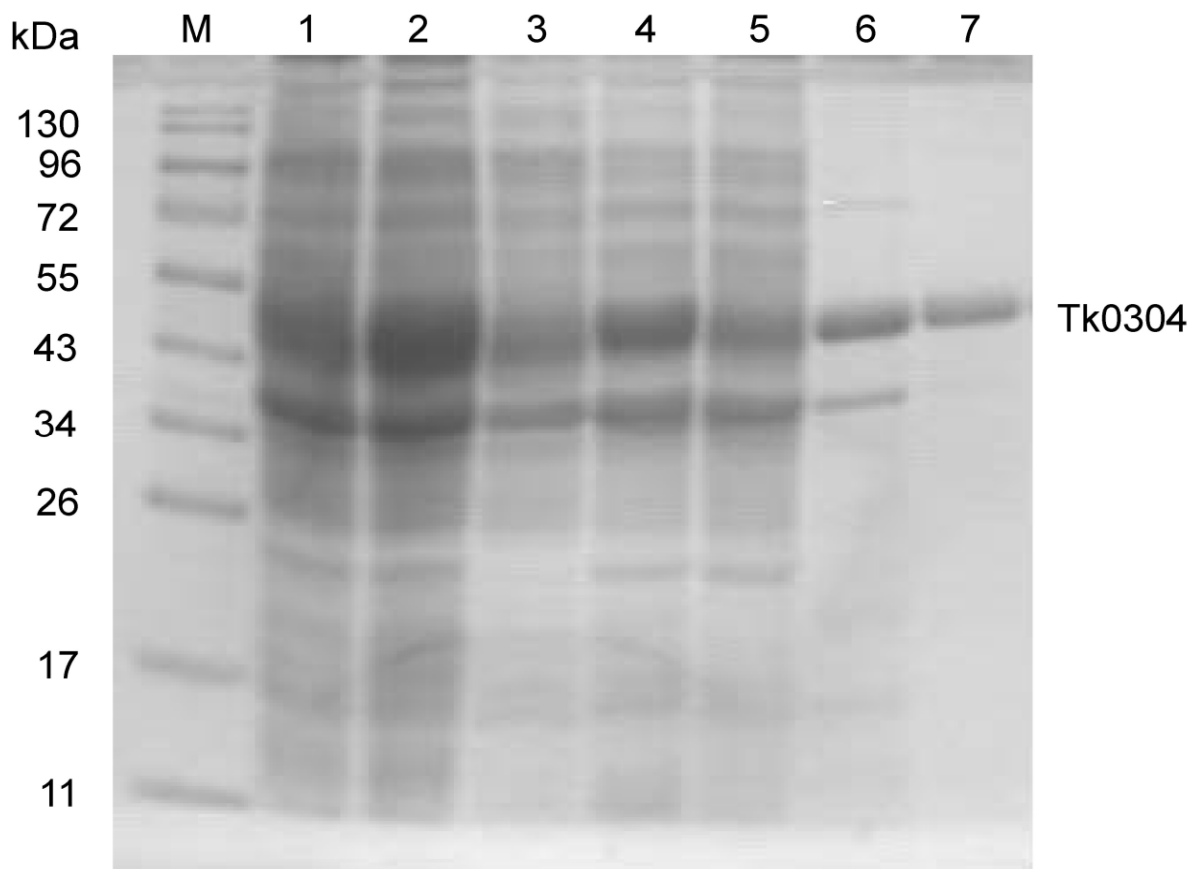
Figure 2. Coomassic brilliant blue stained SDS-PAGE demonstrating the production of TK0304. Lane M, protein marker; lane 1, cells carrying pET- 21a vector; lane 2, cells carrying pET-Tk0304 plasmid; lane 3, insoluble fraction of the sample in lane 2; lane 4, soluble fraction of the sample in lane 2; lane 5, insoluble fraction after heat treatment of sample in lane 4; lane 6, soluble fraction after heat treatment of sample in lane 4; lane 7, purified recombinant Tk0304 after resource Q column.
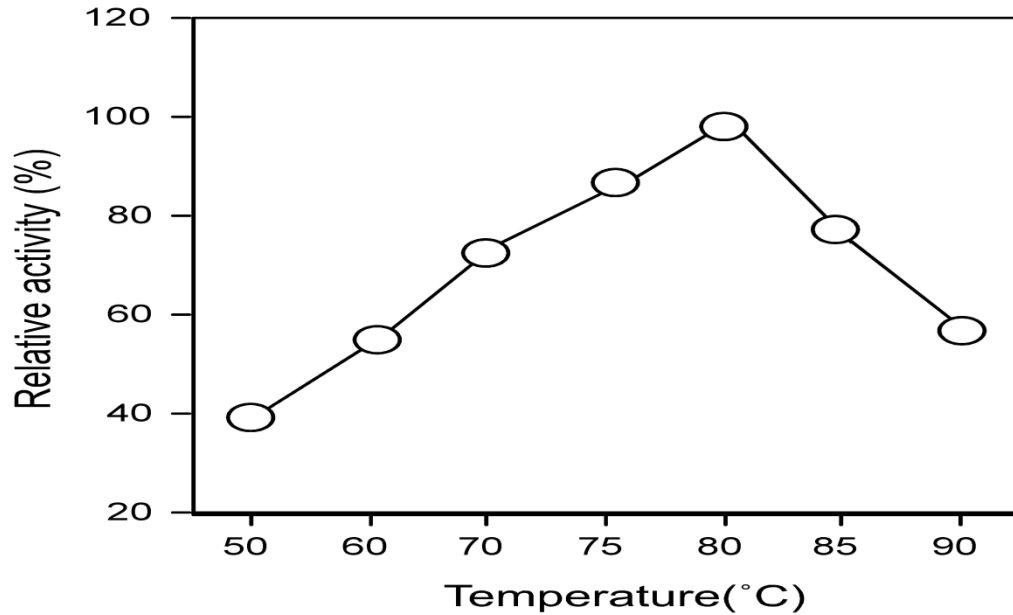
Figure 3. Effect of temperature on the enzyme activity of recombinant Tk0304. The enzyme activity was examined at various temperatures at pH 9.0.
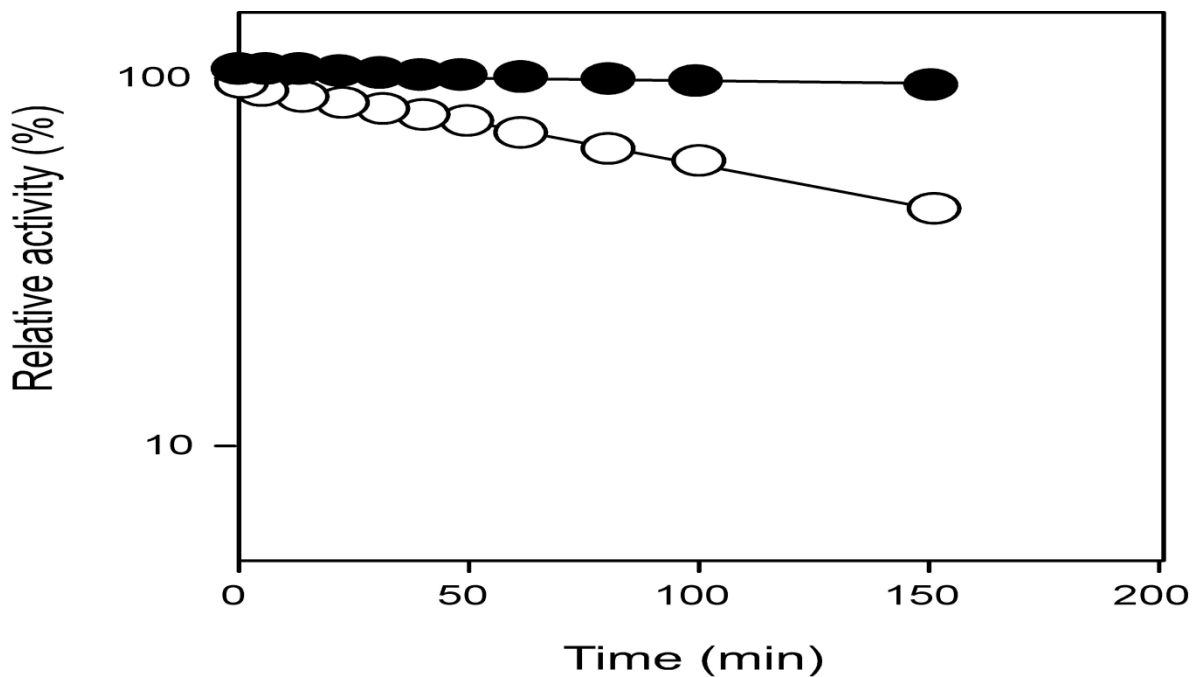

Figure 4. Effect of temperature on the stability of Tk0304. The recombinant enzyme was heated in sodium phosphate buffer (pH 7.0) at 80°C (close circles) and 100°C (open circles) for various time intervals and the residual activity was examined at 60°C.

Tk0304 was examined at 80°C (the optimal temperature for enzyme activity) and 100°C. The enzyme was quite stable at 80°C displaying more than 95% of the enzyme activity even after incubation of 150 min. The half-life of the enzyme was 80 min at 100°C (Figure 4).

The effect of pH on the enzyme activity was examined by measuring the enzyme activity at various pH in different buffers. The enzyme was active in a wide pH range. Highest activity was observed at pH 9.0 in Tris-HCl buffer (Figure 5) which indicated that NADH oxidase
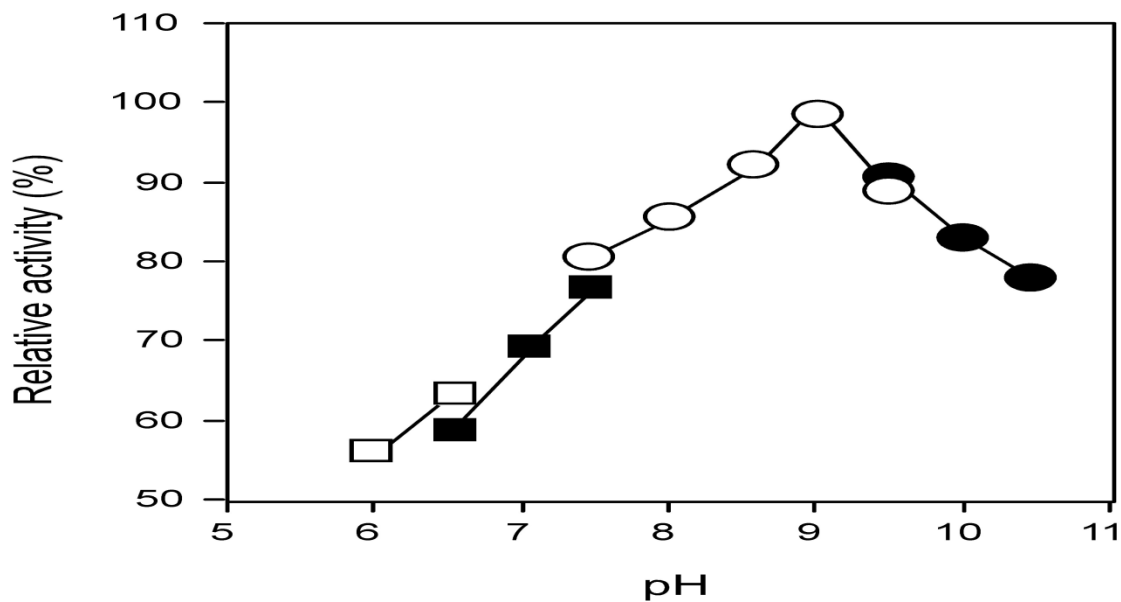
Figure 5. Effect of pH on the enzyme activity of Tk0304. Activity was examined at various pH and 60 °C. The following buffers were used: sodium acetate buffer (open squares), sodium phosphate buffer (closed squares), Tris-HCl buffer (open circles) and glycine NaOH buffer (closed circles).

functioned optimally under alkaline conditions in contrast to other archaeal NADH oxidases. The NADH oxidases from acidophilic archaea including *Acidianus ambivalens* and *Sulfolobus solfataricus* have optimal activity at highly acidic pH similar to physiological conditions of these microorganisms (Gomes and Teixeira, 1998; Masullo et al., 1996). Redox enzymes catalyze industrially important reactions and these reactions often require organic solvents (Ward et al., 2001). We, therefore, examined the effect of organic solvents such as ethanol and dimethyl sulfoxide and we found that there was no significant change in enzyme activity when both the solvents were independently added at a final concentration of 30% (v/v). Beyond 30%, there was a slight decrease in enzyme activity (data not shown). Silver nitrate and potassium cyanide are reported to inhibit the NADH oxidase activity of NADH oxidases (Kim and Kim, 2004) by tightly binding to these oxidases (Kao et al., 2007). When we examined the enzyme activity of Tk0304 in the presence of 100 µM of silver nitrate or potassium cyanide we could not observe a significant decrease in enzyme activity (data not shown) probably due to lack of binding pockets for these compounds. When we examined the effect of detergents, ionic as well as non-ionic, we found that enzyme activity was slightly increased in the presence of 10% each of Triton X-100 and Tween 20. Interestingly, the presence of 1% sodium dodecyl sulphate in the reaction mixture also resulted in a slight increase in the enzyme activity (data not shown). No data are available in literature for comparison of effect of detergents on NADH oxidases. The presence of salts in the reaction mixture also enhanced the enzyme activity of Tk0304.

There was a 33% increase in enzyme activity when NaCl was added in the reaction mixture at a final concentration of 300 mM. In the presence of same concentration of LiCl, there was a 17% increase in the enzyme activity of Tk0304. Enhancement of enzyme activity of NADH oxidase from *Bacillus cereus* has also been reported (Kim and Kim, 2004).

Specific activity and kinetic parameters

Tk0304 displayed a specific enzyme activity of 95 U mg^{-1}. The specific activity of Tk0304 was quite high compared to NADH oxidases from other archaeal sources. For example, NADH oxidases (NOXA-I and NOXB-1) from *A. fulgidus* displayed specific activities of 5.8 U mg^{-1} and 4.1 U mg^{-1}, respectively (Kengen et al., 2003). Similarly, NADH oxidases from *T. profundus*, *P. furiosus* and *M. jannaschii* have been reported to exhibit specific activities of 7 U mg^{-1} (Jia et al., 2008), 2 U mg^{-1} (Ward et al., 2001) and 16 U mg^{-1} (Case et al., 2009), respectively. On the other hand, NADH oxidases from bacterial origin displayed higher specific activities. For insistent, NADH oxidase from *Lactobacillus brevis* displayed a specific activity of 116 U mg^{-1} (Hummel and Riebel, 2003). When we examined the kinetic parameters, we found that Tk0304 followed the Michaelis-Menten equation (Figure 6). K_m and V_{max} values were found to be 80 µM and 112 µmol min^{-1} mg^{-1}, respectively. The k_{cat} value, calculated on the basis of monomeric form of Tk0304, was found to be 88 s^{-1} whereas k_{cat}/K_m valve was 1.1 µM^{-1} s^{-1}. Role of NADH oxidase in *T. kodakarensis* is not very clear. *T.*

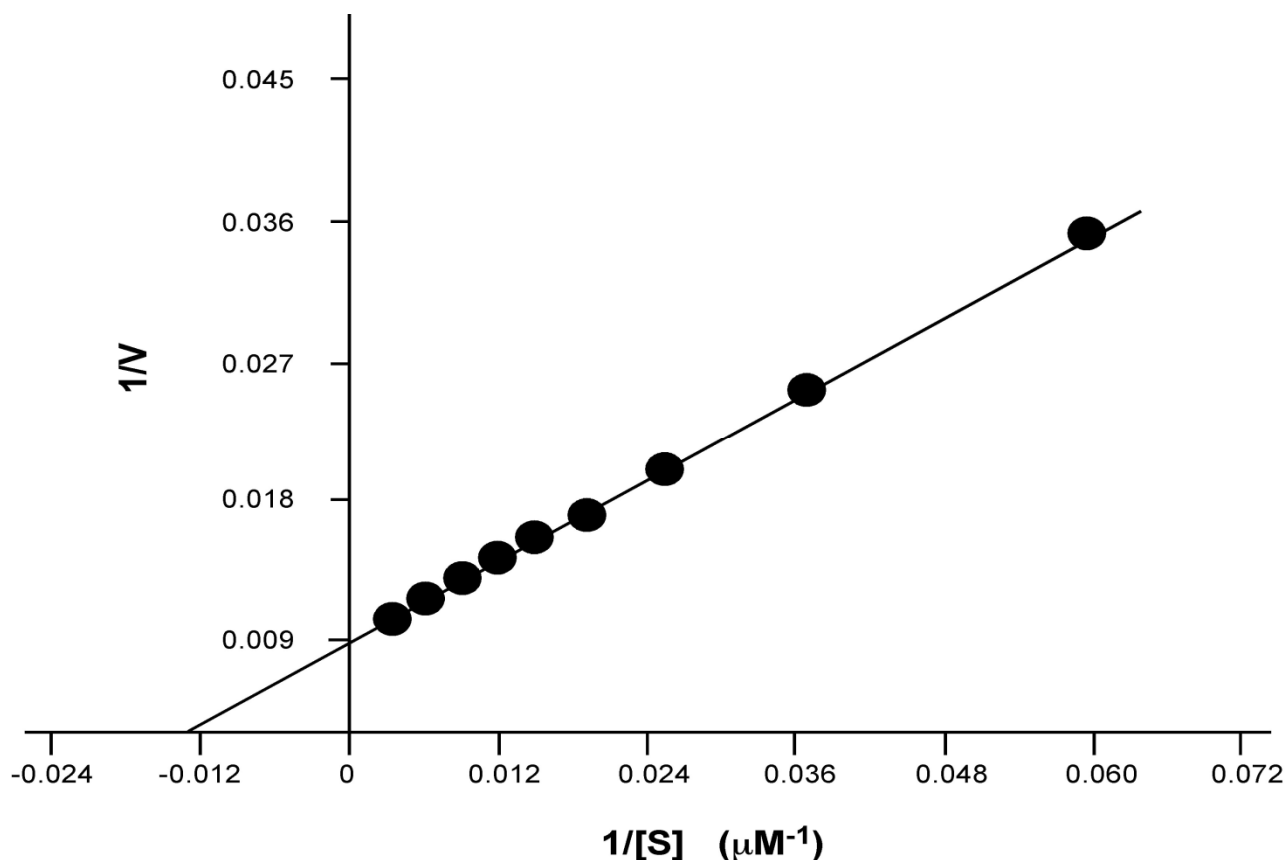**Figure 6.** Lineweaver-Burk plot obtained by taking the inverse of the substrate concentrations (mM) along X-axis and velocities (µmol min^{-1} mg^{-1}) along Y-axis.

kodakarensis is a strict anaerobe and it is unlikely that NADH oxidase acts in the classic sense to transfer electrons to O_2 for the purpose of regenerating NAD$^+$. It may instead play a protective role against oxygen stress by direct reduction of O_2 to water. Higher enzyme activities of Tk0304 and NADH oxidases from other hyperthermophilic archaeal strains at temperatures lower than their optimal growth temperatures further support this hypothesis as the dissolved oxygen concentrations are higher at low temperatures. Gene disruption studies are needed to determine the physiological role of NADH oxidases in this microorganism.

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