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Human DT-diaphorase expression in *Escherichia coli*: optimization, purification and structural stability

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Abstract

Expression and purification of human DT-diaphorase, also referred to as NAD(P)H quinone oxidoreductase 1 (NQO1; EC. 1.6.99.2), which is a flavoprotein belongs to the family of oxidoreductases are optimized. The DT-diaphorase plays an important role in biosensor design for laboratory analysis and also developing biosensor for measurement of glucose level in blood. The aim of this study was to investigate various parameters regarding the expression of DT-diaphorase in *Escherichia coli* BL_{21} (DE3) and thermal stability of DT-diaphorase activity at different temperatures in the presence of sucrose. Expression conditions of DT-diaphorase in *E. coli* were optimized with an induction time (22.00 hr), induction temperature (18.00 ˚C) and also lactose (5.00 mM) and isopropyl ß-D-1-thiogalactopyranoside (1.00 mM) concentrations as inducers. The *Km*, *Vmax* and *kcat* values for NADH as a substrate were 25.50 µM, 357 µM per min and 446.40 µM mg^{-1} per min, respectively. Results of our research revealed that different concentrations of sucrose at 40.00 ˚C did not have any significant effect on enzyme structure; while, relatively significant changes, especially in the presence of sucrose (0.75 M) at 50.00 ˚C were observed. The results presented show that sucrose causes DT-diaphorase inactivation rate reduction and relatively little increases in thermal stability and thus, sustains its conformation against thermal unfolding.

Introduction

Endophytic Nicotinamide adenine dinucleotide quinone oxidoreductase 1 (NQO1; EC.1.6.99.2) is categorized as flavo-protein enzyme being able to catalyzes the conversion of quinones into hydroquinone using nicotinamide adenine dinucleotide as its cofactor.[1,2] The NQO1 is a homodimer enzyme and each monomer has a molecular weight of 30.00 kDa.[3,4] The NQO1 enzyme is mostly found in humans and animals. Besides NQO1, several commercial diaphorase enzymes with diaphorase activity have been extracted from various prokaryotes and eukaryotes sources.[5]

Catalytic characteristics of enzymes were first reported by Ernster and Navazio, and Ernster *et al.*[6,7] The NQO1 has a broad range of useful applications such as metabolism of vitamin K and activation of anti-cancer drugs such as diaziquone, mitomycin C and dinitrophenyl aziridine.[1,8] It also acts as a protectant against some undesired actions such as mutagenesis as well as carcinogenic and cytotoxic effects of quinone and its derivations. The NQO1 has also potential application for colorimetric measurements of dehydrogenase enzymes. Due to the crucial role of NQO1 in prokaryotes and eukaryotes and its instability, using compatible osmolytes has drawn widespread attention in biological fields.[5,9,10]

Osmolytes are small molecules accumulated by organisms, microorganisms, plants and animals under stress conditions to protect the structure and molecular function. They can have increasing, decreasing or no effects on stability of proteins. Osmolytes based on the functional activity of the enzymes are divided into two groups that may have compatible and incompatible effects on proteins. The compatible groups such as amino acids and polyols do not have significant effect on enzymes activity; but the incompatible groups including methyl-amines, arginine, lysine and histidine may affect the enzyme function. Organic osmolytes are widely used to protect cellular proteins against adverse conditions such as dehydration, hyper-tonic states and denatured metabolites. However, not all osmolytes protect cell components.[11] There are various

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osmolytes that stabilize proteins under *in vivo* and *in vitro* conditions. Unfortunately, the mechanism by which this stabilization occurs is not fully understood; but, generally three major mechanisms such as surface tension, preferential hydration and increasing the solvency system have been described for the effects of additives on protein stability and how proteins are stabilized by osmolytes.[10,12,13] However, no survey regarding the characterization of conformational changes and thermal stability of DT-diaphorase in presence and absence of sucrose has ever been introduced. The purpose of this study was expression of DT-diaphorase and optimization of DT-diaphorase activity at different temperatures by sucrose. We also compared temperature dependent conformational changes of DT-diaphorase in presence and absence of sucrose.

Materials and Methods

Materials. Nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), kanamycin and lactose (Sigma-Aldrich, St. Louis, USA), Ni-NTA Sepharose affinity column (Novagen; Merck Millipore, Darmstadt, Germany) and sucrose and isopropyl ß-D-1-thiogalactopyranoside (IPTG; Merck, Darmstadt, Germany) were purchased. Liquid media were used for the cultivation of *Escherichia coli* including Luria–Bertani (LB; 10.00 g L^{-1} peptone, 5.00 g L^{-1} yeast extract and 5.00 g L^{-1} NaCl; Merck) and 2xYT medium (2xYT; 10.00 g L^{-1} yeast extract, 16.00 g L^{-1} tryptone and 5.00 g L^{-1} NaCl; Merck).

Expression and purification of NQO1 enzyme. The procedure for transforming of *E. coli* BL21, which plasmid pET-28a contains NQO1 coding gene with His tag at N-terminal, was described before.[14] To determine the influence of different culture media on the yield of active NQO1, types of media were investigated (LB and 2xYT). To induce NQO1 enzyme, *E. coli* BL21 clones were grown overnight in 10.00 mL of LB medium supplemented with kanamycin (50.00 mg mL^{-1}) at 200 rpm shaking speed and 37.00 ˚C. Then, 1.00 mL of culture was added to 125 mL of fresh 2xYT medium and incubated under same condition at 220 rpm shaking speed and 37.00 ˚C. Afterwards, to optimize the expression condition, time and inducer concentration were investigated. Hence, when the optical $density_{600}$ (OD) ~ 0.60 reached (after 4 - 5 hr), protein expressions at different concentrations of IPTG (0.60, 0.80 and 1.00 mM) and lactose (2.00, 3.00, 4.00 and 5.00 mM) at 18.00 ˚C and 220 rpm shaking speed were measured. To optimize the effect of time on protein expression, incubation was performed for 14, 16, 18 and 22 hr with 1.00 mM IPTG at 18.00 ˚C and 220 rpm shaking speed. Cells were harvested by centrifugation (MPW-260 R; MPW Med. Instruments, Warsaw, Poland) at 6,000 *g* for 10.00 min at 4.00 ˚C. The cell pellet was resuspended by lysis buffer (50.00 mM Tris-HCl, NaCl 300 mM and imidazole 250 mM; pH: 7.80, Merck). Finally, the suspension was lysed by sonication on ice, and supernatant was separated by centrifugation at 14,000 *g* for 20.00 min at 4.00 ˚C. To protein purification, the supernatant was loaded onto Ni-Sepharose column affinity chromatography. After washing the column with Tris-HCl 50.00 mM, NaCl 300 mM and imidazole 20.00 mM, recombinant protein was eluted with Tris-HCl 50.00 mM, NaCl 300 mM and imidazole 250 mM. The protein concentration was estimated by Bradford method[15] and its purity was monitored by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS – PAGE; Cleaver Scientific, Warwickshire, UK).

Enzyme assay. The calorimetric method was performed for determination of NQO1 enzyme assay in solution. In this method, the NADH is oxidized by coupling with a tetrazolium salt/diaphorase system. The assay was based on measuring the color of a formazan dye. To optimize the NBT value, different concentrations of NBT (0.02 - 0.40 mM) have been investigated in the presence of 0.50 mM NADH and 13.00 µL enzyme in Tris-HCl buffer (50.00 mM; pH: 8.00). Briefly, the reaction mixture in a total volume of 320 µL consisting of 270 µL Tris-HCl buffer (50.00 mM; pH: 8.00), 26.00 µL of NBT (0.406 mM), 11.00 µL of NADH (0.51 mM) and suitably diluted enzyme was prepared. The reaction was initiated by adding 13.00 µL of the NQO1 enzyme (0.80 mg mL^{-1}) to the reaction mixture and reading the change in absorbance at 585 nm for 1 min by visible spectrophotometer (model 721; Shanghai Yuefeng, Shanghai, China). The kinetic parameters such as *Km*, *Vmax* and *Kcat* values of diaphorase enzyme against different concentrations of NBT (0.406, 0.325, 0.243, 0.162, 0.81, 0.040, and 0.020 mM) and NADH (0.51, 0.275, 0.137, 0.06, 0.03, 0.017, 0.008, 0.004, and 0.002 mM) as substrates were also determined.

Optimum temperature and thermal stability. To determine optimum temperature of NQO1 in presence and absence of sucrose (0.50 M), purified NQO1 enzyme was incubated in the range of 0.00 - 100 ˚C for 5 min and then, enzyme activities were measured. Rates of inactivation were measured by incubating of NQO1 enzyme in the absence and presence of sucrose (0.50 - 1.00 M) at 30.00, 40.00, 50.00 and 55.00 ˚C for 60 min. Samples were then placed on ice for 3 min and the remaining activity was determined. To determine thermal stability of NQO1 in absence and presence of sucrose (0.50 - 1.00 M), purified NQO1 enzyme was incubated in the range of 0.00 - 100 ˚C for 5 min. After that, the remaining activity was recorded as a percentage of the original activity after incubation for 3 min on ice.

Fluorescence spectroscopy. Intrinsic fluorescence emission spectroscopy of enzyme in absence and presence of different concentrations of sucrose (0.50, 0.75 and 1.00 M) and temperatures (40.00 ˚C and 50.00 ˚C) was obtained by fluorescence spectrometers (LS 55; PerkinElmer Inc., Waltham, USA). The excitation wavelength of NQO1 was 295 nm and the intrinsic emission spectra were recorded between 250 - 500 nm. All the fluorescence measurement

were carried out at room temperature in final volume of 60.00 µL. The excitation emission slit widths were set at 5.00 and 10.00 nm, respectively and purified NQO1 concentration was 0.80 mg mL^{-1} for each assay.

Results

NQO1 expression, enzyme activity and kinetic properties. To identify the effect of different concentrations of IPTG and lactose on expression of the enzyme, purification by affinity chromatography (Ni-NTA-Sepharose) and SDS - PAGE (12.00%) and ImageJ Software (National Institutes of Health, Bethesda, USA) analyses were carried out. According to the data obtained from SDS - PAGE and ImageJ Software, protein band density at 1.00 mM IPTG concentration was higher than 0.60 and 0.80 mM IPTG concentrations (Fig. 1). Based on the results obtained from ImageJ Software by comparing the concentration of the purified proteins, protein expressions with IPTG 1.00, 0.80 and 0.60 mM were 42.00, 29.00 and 27.00%, respectively (Fig. 2A). According to our results, as depicted in Figure 1, induction by 5.00 mM lactose was more suitable for protein expression in comparison with the other lactose concentrations. The results data from ImageJ software suggested that the protein concentrations from lactose 2.00 mM, 3.00 mM, 4.00 mM and 5.00 mM were 28.00%, 9.00%, 25.00% and 37.00%, respectively (Fig. 2B). The highest incubation time was obtained at 22 hr; so that, according to the data from ImageJ software, protein concentrations at 14, 16, 18 and 22 hr were 26.00%, 21.00% and 30.00%, respectively (Fig. 2C).

The activity of DT-diaphorase was determined by calorimetric method. Our result revealed that the best and most suitable concentration of nitro blue tetrazolium (0.02 - 0.40 mM) for measurement of diaphorase activity was 0.24 mM. Kinetic properties of enzyme were studied after drawing Lineweaver-Burk plot for recombinant DT-diaphorase. Kinetic constants of the recombinant purified enzyme for NADH substrate were determined. The *Km*, *Vmax* and *Kcat* values for NADH were estimated to be 25.50, 357 and 446.40 µM mg^{-1} per min, respectively.

Stability of diaphorase enzyme activity in presence of sucrose. In order to investigate the temperature effect on enzyme stability, the enzyme was incubated at different temperatures (0.00 - 100 ˚C) for 5 min and after that, the remaining activity was recorded as a percentage of the original activity after incubation for 3 min on ice. Our results indicated that the activity of the enzyme in the presence and absence of sucrose (0.50 - 1.00 M) had no significant effect on the remaining activity of the enzyme, and with increasing temperature, the enzyme activity decreased in the presence and absence of osmolyte sucrose. In order to investigate the stability of enzyme activity, the enzyme was placed at 30.00 ˚C for 1 hr and every 5 min after cooling on ice-cold water for 3 min, the enzyme activity was measured. According to Figure 3A - D, illustrating the stability of enzyme activity in the presence and absence of sucrose 0.50 M, it can be concluded that 0.50 M sucrose at 30.00 ˚C and 55.00 ˚C had no significant effect on activity of the enzyme. Stability of enzyme in presence of 0.50 M sucrose at 40.00 ˚C showed that during 10 - 60 min, the remaining activity of the enzyme was

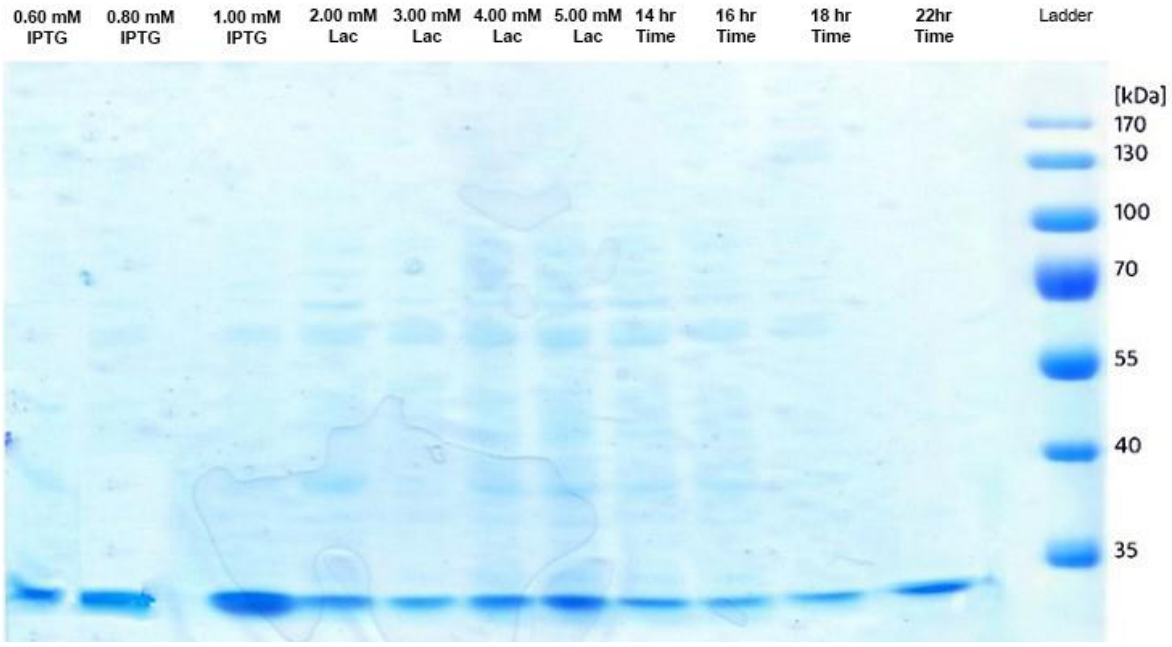

Fig. 1. The SDS-PAGE profile of the NQO1 expression at the different conditions. Profile of the NQO1 purified enzyme in 12.50% SDS - PAGE: NQO1 enzyme was expressed in different concentrations of IPTG (0.60 mM, 0.80 mM and 1.00 mM) and lactose (2.00 mM, 3.00 mM, 4.00 mM and 5.00 mM) and purified via affinity Ni-NTA column chromatography.

sustained rather than free enzyme (Fig. 3B). Also, at 50.00 ˚C and in the initial 35 min, the enzyme activity in presence of sucrose 0.50 M was higher than free enzyme (Fig. 3C). As depicted in Figures 4A and 4D, sucrose (1.00 M) at 30.00 ˚C and 55.00 ˚C had no significant effect on activity of the enzyme. Stability of the enzyme at 40.00 ˚C showed that in the first 15 min and the last 30 min, the activity of the enzyme in presence of 1.00 M sucrose was higher than free enzyme (Figs. 4B and 4C) and also at 50.00 ˚C in the last 20 min, it was higher than free enzyme (Fig. 4D).

Intrinsic fluorescence changes of DT-diaphorase in presence of sucrose. Intrinsic fluorescence spectra of enzyme in the absence and presence of sucrose in different concentrations (0.50 - 1.00 M) at 50.00 ˚C were measured (Fig. 5A). These results revealed that the enzyme in presence of sucrose as an osmolyte lost its fluorescence intensity rapidly at 50.00 ˚C and so the inactivation of the enzyme occurred. In the presence of 0.75 M sucrose and at 50.00 ˚C in comparison with other concentrations of sucrose, fluorescence intensity was left and the enzyme maintained its structural stability to some extent. Emission spectra in the absence and presence of sucrose in different concentrations (0.50 - 1.00 M) were measured at 40.00 ˚C (Fig. 5B). The results revealed that there were no significant changes in the structural stability of the enzyme in presence of 0.50, 0.75 and 1.00 M of sucrose at 40.00 ˚C. In other words, the stability of enzyme in presence of sucrose was observed at this temperature.

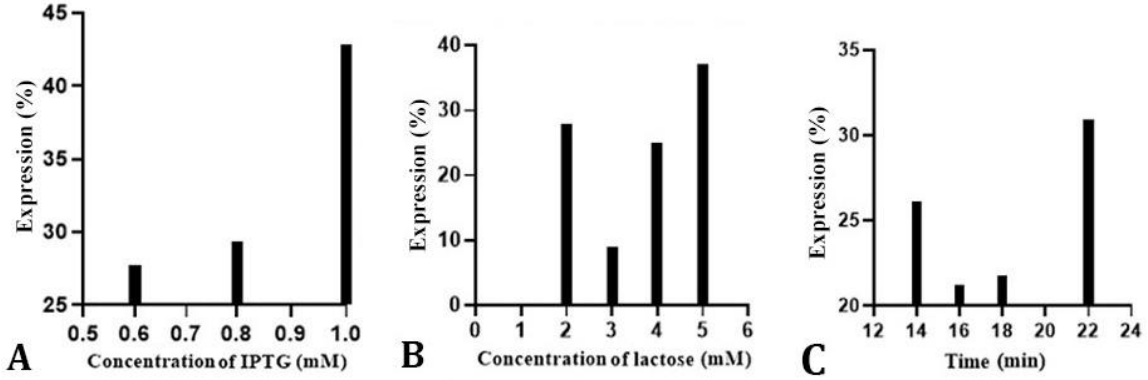

Fig. 2. Comparison of protein expression with change of concentration of inducers and incubation time: **A)** Concentration of inducers IPTG, **B)** lactose, and **C)** incubation time.

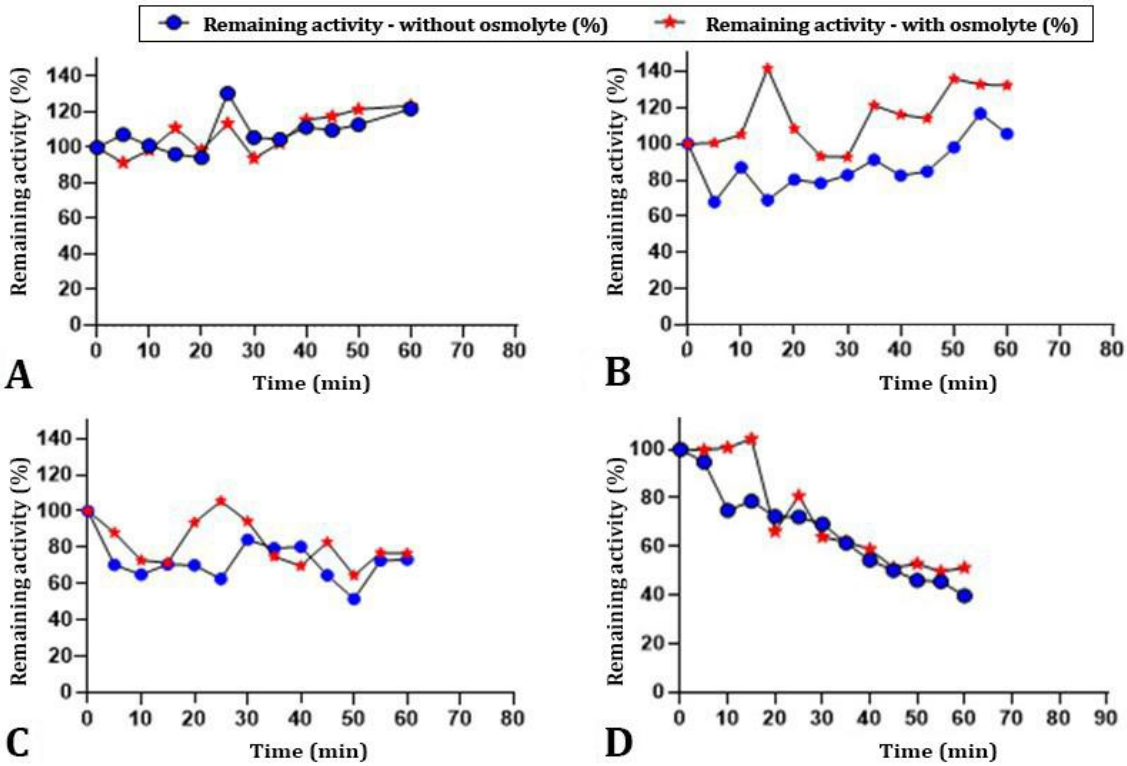

Fig. 3. Comparative diagram of diaphorase activity in the presence and absence of sucrose 0.50 M at temperature of **A)** 30.00 ˚C, **B)** 40.00 ˚C, **C)** 50.00 ˚C, and **D)** 55.00 ˚C.

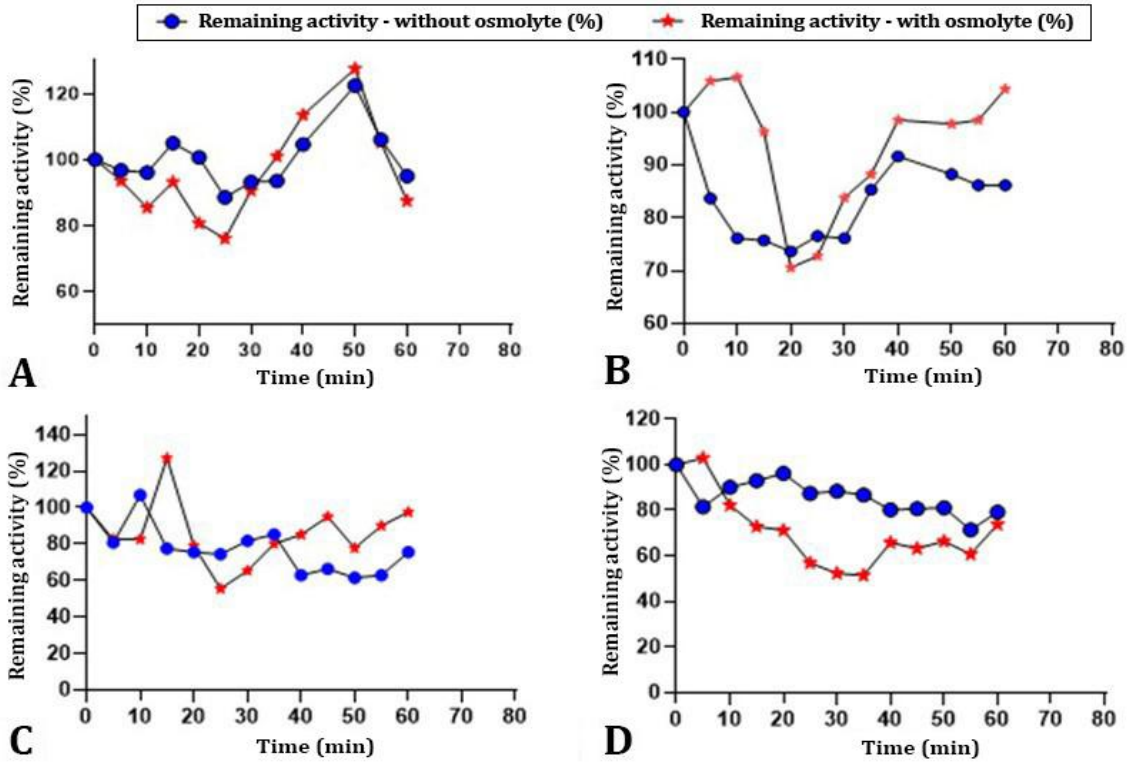

Fig. 4. Comparative diagram of the stability of diaphorase in the presence and absence of sucrose 1.00 M at a temperature of **A)** 30.00 ˚C, **B)** 40.00 ˚C, **C)** 50.00 ˚C, and **D)** 55.00 ˚C.

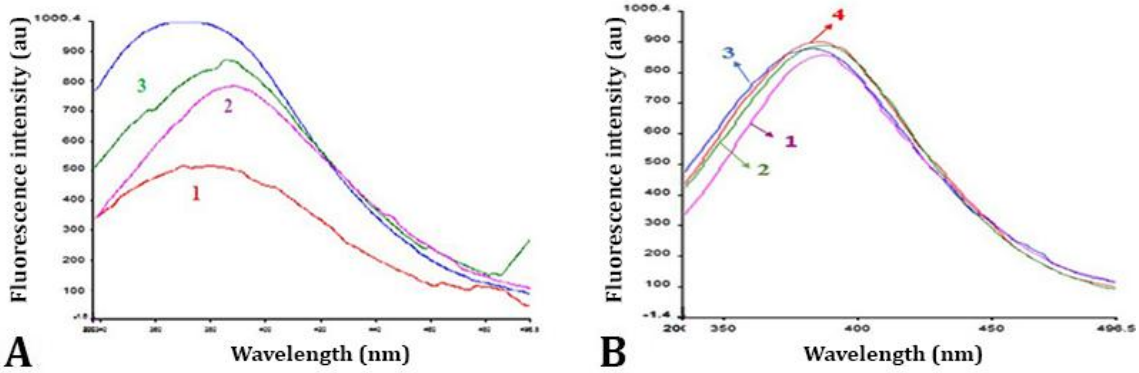

Fig. 5. Intrinsic fluorescence changes of DT-diaphorase in presence of different concentrations of sucrose. **A)** Intrinsic fluorescence changes at 0.50 M (1), 1.00 M (2), 0.75 M (3) and absence (4) of sucrose, at 50.00 ˚C; **B)** Intrinsic fluorescence changes at 1.00 M (1), 0.75 M (2), 0.50 M (4) and absence (3) of sucrose at 40.00 ˚C.

Discussion

Today, the importance of utilizing recombinant technology in various fields such as drug, vaccine and treatment and diagnosis of diseases has drawn significant attention. The choice of an expression system for generating high amounts of recombinant proteins is dependent on many factors including cell growth characteristics, expression levels, intra-cellular or extra-cellular expressions, post-translational changes and bioavailability of the protein, particularly in the production of protein. In addition, the expression system requires cost saving processes and other economic considerations.[16,17] Some of the important parameters affecting the level of expression of the recombinant protein are concentration of inducers such as lactose and IPTG and induction time.[18,19] Optimization of expression is an important factor for better expression of DT-diaphorase in *E. coli* BL21 (DE3) and pET system (plasmid for expression by T7 RNA polymerase) with various conditions. Various researchers have reported expression of DT-diaphorase in *E. coli* using pET-22b plasmid.[5] In this work, maximum amount of enzyme production was obtained under the best conditions (1.00 mM IPTG, 5.00 mM lactose, incubation time of 22 hr, incubation temperature of 18.00 ˚C and 150 rpm of shaking speed). As compared to our study, various

researchers have used 1.00 mM IPTG for 5 hr at 37.00 ˚C [5,9] and 2.00 mM IPTG and then incubated the NQO1 for 17 hr,[3] as an optimal condition for protein expression. Previous studies have revealed that the recombinant diaphorase being produced in *E. coli* BL21 (DE3) exhibits its optimal activity at temperature of 30.00 ˚C and pH of 8.50. The *Km* and *Vmax* values with NADH were determined to be 0.025 mM and 275.8 U mL^{-1}, respectively.[5] In the present study, the *Km*, *Vmax* and *Kcat* values for NADH as a substrate were 25.50 µM, 357 µM min^{-1} and 446.40 µM mg^{-1} per min, respectively.

Technical applications of enzymes in the industry are feasible only if the enzymes are stabilized against temperature and pH extremes and in the presence of additives such as salts, osmolytes, alkalis and surfactants. Most of the reports regarding enzyme stabilization are focused on the effect of additives on protein stability. Using additives is a good approach towards enhancing enzyme storage stability. However, some of these stabilizing additives may interfere with the final use of the enzyme due to incompatibility with the reaction system.[10] In this study, we investigated the extent of stabilization and increasing the enzymatic activity of DT-diaphorase at higher temperatures by sucrose and its conformational changes upon addition of sucrose. Stability of enzyme was slightly retained at 40.00 ˚C in presence of 0.50 M sucrose; while, at 50.00 ˚C and 55.00 ˚C, no effect on the stability of the enzyme was observed. In presence of 1.00 M sucrose, stability of enzyme at 40.00 ˚C in various times was higher than that in absence of sucrose. The best result was obtained with 0.75 M sucrose, keeping almost 90.00% of original activity after 5 min at 50.00 ˚C.

Polyols such as sucrose and trehalose by increasing surface tension and preferential hydration, increase the stability of enzyme against abnormal conditions. Mehrabi *et al.* have shown that osmolytes such as trehalose, sucrose and sorbitol increase remaining activity of firefly luciferase, thermal stability and optimum temperature by increasing the concentration of osmolytes (0.40 - 1.50 M).[20] Rasouli *et al.* have exhibited that the stability of enzyme improves in the presence of different concentrations of sucrose and trehalose or a combination of both osmolytes.[21] The effect of osmolyte on DT-diaphorase structure was monitored by fluorescence spectroscopy at two different temperatures. Structural stability of DT-diaphorase at 40.00 ˚C retained in the presence and absence of different concentrations of sucrose. The results of fluorescence studies at 50.00 ˚C showed that sucrose did not affect the structural stability of the enzyme as decrease in fluorescence emission at different concentrations of sucrose compared to the absence of sucrose was observed. Decrease of fluorescence peak intensity represents partially unfolding of protein structure against temperatures and the lack of effect of sucrose on stability of enzyme. Result of this study is indicative of the fact that with increase of temperature, a change in the tertiary structure of the protein can result in the exposure of buried aromatic residues to the polar solvent level. Thus, sucrose through none of the mechanisms such as surface tension and preferential hydration has not been able to increase the stability of enzyme against temperature.

As seen from the discussion, a great deal of research has been devoted to the development of evaluation of stabilized enzyme with the aim of additives like osmolytes. The results showed that they may have positive, negative or even no effects at all. Besides that, optimized stabilizing parameters will not only lead to a more efficient enzyme but also increase the economic potential in existing enzymatic processes and in novel areas where enzymes have not been used till now because of their instability.

Acknowledgments

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Conflict of interest

The authors have declared that no competing interests exist.

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