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RESEARCH ARTICLE

Identification and Purification of Membrane-Bound NAD (P) $^{+}$ - Dependent D-Glucose Dehydrogenase (nGDH) and Thiosulfate Dehydrogenase (TDH) from Cattle Manure Inhabitant Sulphur-Oxidizing *Pseudomonas* PRK786.

[1]PERIYASAMY RAMESHKUMAR, [1]SHANMUKHANAND POTHANA, [2]KOPPULAN VIJAYARAJ [3]GOVINDASAMY MANIVANNAN AND [3]PRABHU

1.Department of Biotechnology, GITAM Institute of Science, GITAM University, Visakhapatnam, Andhra Pradesh, India.

2. Research Department of Chemistry, NMSSVN College, Madurai, Tamilnadu, India

3. Departments of Microbiology and Biochemistry, NMSSVN College, Madurai, Tamilnadu, India.

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.***Corresponding Author***

PERIYASAMY RAMESHKUMAR

Abstract

Novel membrane bound-enzymes such as NAD (P) $^{+}$- dependent D-glucose dehydrogenase (nGDH) and thiosulfate dehydrogenase (TDH) were identified and separated from cattle manure compost inhabitant sulphur oxidizing *Pseudomonas* PRK 786. The unique bacterial strain belongs to chemolithotrophic oxidizer due to that presence of dual characters - A) oxidation of glucose B) oxidation of inorganic sulphur compounds. In membrane preparation, the optimum level (membrane of *Pseudomonas* PRK 786) of sonication parameters is 40 % duty cycle; power - 10; total time - 30 min. 7.2µg of phospholipids were estimated in1ml of cured membrane sample. The total activity of NAD (P) $^{+}$- dependent D-glucose dehydrogenase and thiosulfate dehydrogenase were assayed at three levels (Cured preparation, Heat precipitation and Desalting) the results 28.93 µmol min^{-1}, 20.25 µmol min^{-1}, 35.69 µmol min^{-1}, 103.54 µmol min^{-1}, 86.28µmol min^{-1} & 150.37 µmol min^{-1} respectively. An effect of catalytic processes was assayed with and without substrates, enzymes and electron acceptor. 80% of proteins were precipitated at 50^{0}C, ~80 kDs and ~48 kDs protein bands were identified by SDS-PAGE and two different peaks were observed by HPLC at retention time -1.390 min and retention time -2.170.Its clearly indicates purified sample contains of nGDH and TDH.

INTRODUCTION

Outer membranes of gram-negative bacteria have possessed variety of proteins[1]. Moreover, the D-Glucose dehydrogenase (mGDH) is one of the member of membrane bound enzyme in aerobic gram-negative bacteria[2]. Various experimental evidence clearly indicated that *Pseudomonas*[10], *Klebsiella*[3], *Acinetobacter*[4], *Erwinia* sp.34-1[5] and *Serratia*[6] are capable to oxidize various substrates like sugar and sugar alcohol to corresponding oxidative products. The membrane-bound D-Glucose dehydrogenase (mGDH) that located on the outer surface of the cytoplasmic membrane catalyzes these oxidation reactions. In addition, the cattle manure compost inhabitant sulphur-oxidizing *Pseudomonas* sp.PRK786 was characterized[7].

Generally, glucose oxido-reductases are exist in three forms namely; acceptor dependent D-glucose dehydrogenase, quinoprotein dependent glucose dehydrogenase (qGDH) and NAD (P)$^{+}$ dependent glucose dehydrogenase (nGDH). They are oxidized the glucose into glucono-1, 5-lactone based on the dependency. nGDH and qGDH both are membrane bound enzymes in most of the gram-negative bacteria. In addition, membrane-bound glucose dehydrogenase has either dependent on nicotinamide adenine dinucleotide[8] dependent on pyrroloquinolinequinone (PQQ) or flavin adenine dinucleotide (FAD)[9]. The outer and cytoplasmic membrane bound proteins of *Pseudomonas aeruginosa* was characterized[10]. Especially, D-Glucose dehydrogenase purified from the

membrane of *Pseudomonas fluorescens*[11], the soluble form of Ca^{2+} and temperature dependent quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*[12], Quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* L.M.D. 79.41[13], D-glucose dehydrogenase from *Bacillus thuringiensis* M15[14] and new glucose dehydrogenase from vegetative cells of *Bacillus megaterium*[15] were also noted. In contrast, Glucose dehydrogenase has been widely used for direct enzymatic determination of glucose in diagnostic, food laboratories. Besides PQQ-GDH based biosensors has been developed for detection of heavy metals[16 & 17]. More recently, structure and mechanism of soluble quinoprotein glucose dehydrogenase was proposed[18], Glucose-inducible, and aromatic compounds-repressible (even in the presence of glucose) outer-membrane protein (OprB -40 kDa) of P. *putida* CSV86 was reported[19]. Antibody can also rise against D-glucose dehydrogenase purified from *Pseudomonas fluorescens*[20]. The substrate specificity of quinoprotein glucose dehydrogenase was reported [21] and mechanism of interaction between the ubiquinone and membrane – bound glucose dehydrogenase were studied[22].

However, the oxidation of sulfur compounds was not unique character for the sulfur bacteria. This process also occurs in heterotrophic bacterial isolates from soil and marine environment [23]. Most of the aerobic sulphur-oxidizing bacterial genera belong to *Pseudomonas* sp[24]. Sulphur oxidizing bacteria can oxidize the thiosulfate in to varying amounts of tetrathionate, elemental sulphur and sulfate. The structure and fatty acid composition of sulphur-oxidizing bacterial membranes were more are similar to *Pseudomonas stutzeri*[25]. Most of the obligate heterotrophic bacteria oxidized thiosulfate to tetrathionate both aerobically and anaerobically in the presence of N_2O [26]. *Pseudomonas* sp. was a sulphur oxidizer in unique mangrove ecosystem of Bhitarkanika, Odisha[27]. Highest sulphur oxidase producer [11.6 to 126.83 U/ ml/ min.] were isolated form mangrove soil. Moreover, soluble thiosulfate oxidase from *Pseudomonas aeruginosa* was purified [28]. The present study was aims to identify and purify the membrane-bound NAD $(P)^+$ - dependent D-Glucose dehydrogenase (nGDH) and thiosulfate dehydrogenase (TDH) from cattle manure inhabitant sulphur-oxidizing *Pseudomonas* PRK786.

MATERIALS AND METHODS

Bacterial Growth Condition

Pseudomonas PRK786 was cultured in thiosulphate broth and medium (with 1% (w/v) of agar-agar) at 37°C and stored at 4^0C. To obtain the biomass, the freshly prepared thiosulphate broth was inoculated with 200 µl of log phase culture of *Pseudomonas* PRK786 (1ml of log phase culture (OD_{440} nm=1.802) and incubated at 37^0C in an orbital shaker (120rpm) for 24-48 hr. The cells were harvested by centrifugation at 6000 rpm for 10 min.

Preparation of Total Cell envelopes from *Pseudomonas* PRK786

The collected 20g of wet biomass were washed with distilled water and centrifuged at 8000 rpm for 10 minutes and then, suspended with 10 mM sodium phosphate buffer (pH 7.2) and 5 mM $MgCl_2$ and stored at- 20^0C until use. The crude membrane preparation was done by according to method[29] with slight modification. The cells were subjected to sonication (equipped with a medium tip (40 % duty cycle; power - 10; total time - 30 min) at 4^0C using 10 mM sodium phosphate buffer (pH 7.2). Unbroken cells and large cell debris were removed by centrifugation at 10,000 g for 30 min. The supernatant was centrifuged at 19,000 rpm for 90 min to collect the bacterial cell envelope. The collected supernatant was washed twicely with the same buffer and considered as sample.

Quantification of Total Phospholipids

2ml of supernatant was suspended with 5ml of ethanol than the blends allow sonicating for 5min and added 5ml of chloroform to the mixture after allow to stands for 10 min. mixture again sonication for 2min and centrifuged at 2000 rpm for 5 min with 20^0C. The above experiment was repeated to get the clear supernatant then it was transfer in another tube and added 2ml of KCl and vortexes thoroughly. Supernatant was collected by centrifugation 2000 rpm for 5min. Dry over the pellet after discarded the supernatant. The pellet resuspended with 0.4ml of chloroform and 0.1 ml of chromogenic solution. According to method[30] the mixture was read at 750nm against the reagent blank after the 5 min incubation.

Quantification of Total Membrane Bound Proteins.

5ml sample was suspended with 5ml of acetone and subjected to vigorous shaking for 15min and allow to centrifuge at 3000 rpm for 10min. Discarded the supernatant and pellet was suspended in to 1ml of distilled water, and determination of proteins concentration by Barford's method [31].

Assay of NAD $(P)^+$- Dependent Glucose Dehydrogenase

The assays of NAD $(P)^+$- dependent D-glucose dehydrogenase was performed according to method[32] using spectrophotometer UV-1800 (Shimadzu) at 37^0C, pH 8.0.The reaction mixture contains tris HCl buffer [200 mM], freshly prepared $NADP^+$ [5-10mM], BSA [10% (w/v)], Triton X-100 [10% (v/v)]β-D-Glucose [250 mM] and cured enzyme solution (0.1 mg protein /ml of cured preparation). The blank contains all above reagents without enzyme and triton X-100 solutions and mixed thoroughly to read at A_{340nm}.The reaction was started by addition of enzyme

solution and absorbance was record against A_{340nm} after 15 min of incubation and addition 1ml of triton X-100. Each steps was repeated thrice for the confirmation. The one units of enzyme activity is equal to one µmol of $NADPH^+$ formed per min the calculations were based on

$$\text{Relative activity } (\mu mol/min^{-1}) = \frac{(A_{340nm}/min\ Test - A_{340nm}/min\ Blank)\ (3)\ (DF)}{(6.22)\ \ (0.1)} \quad (1)$$

3 = Total volume (in ml) of assay, DF = Dilution factor, 6.22 = Extinction co-efficient for $NADPH^+$ [mM] at 340 nm, 0.1 = volume (in ml) of enzyme used.

Specific activity (Unit/mg) = Test OD × Protein concentration – 1 × Reaction time -1 (2)

Assay of Thiosulfate Dehydrogenase

The method adopted by [33&34] with slight modification, the assay of thiosulfate dehydrogenase activity was individually measured by the amount of reduction of ferricyanide. The reaction mixture contained 50 mM $(NH_4)_2SO_4$ buffer with low pH 2.0, 0.9 mM $K_3Fe\ (CN)_6$, 15 mM$Na_2S_2O_3{\cdot}5H_2O$, and the cured enzyme solution (0.1 mg protein /ml of cured preparation). The reaction was initiated by adding thiosulfate at 37^0C. After a period (10 min) of time, reaction was stopped by the tubes were allow to boiling water bath for 5 min. The reduction of ferricyanide was monitored at $A_{420\ nm}$. One unit of activity (U) was defined as one µmol of ferricyanide reduced per min.

$$\text{Relative activity } (mM/\ min^{-1}) = \frac{(A_{420nm}\ Test - A_{420nm}\ Blank)\ (3)\ (DF)}{(1.043)\ \ (0.1)} \quad (3)$$

3 = Total volume (in ml) of assay, DF = Dilution factor is 2, 1.043 = Extinction co-efficient for Potassium ferricyanide at 420 nm and 0.1 = volume (in ml) of enzyme used.

Precipitation and Desalting of Protein from Cured membrane preparation

20ml of crude enzyme extracts were heated at nearly 50ºC for 20 minutes, the tube was immediately cooled in ice bath and centrifuge at 5500 rpm at 4^0C for 10 minutes. The precipitated protein pellets were removed and subjected in to pretreated (membrane soaked with mixture of 250ml of 2% of $NaHCO_3$ and 10mMEDTA and boiled for 20min) dialysis membrane (5-10 cm length) and immersed into 10mM phosphate buffer (pH 7.2) for 12 hours change of buffer every 3-4 hours.

Determination of Protein Molecular Weight by SDS-PAGE:

Polyacrylamide separating gel (10%) of pH 8.8 and stacking gel (4%) of pH 6.8 was prepared[35]. The standard molecular weight marker proteins were made by using bovine albumin, ovalbumin, and lysozyme of different molecular weight of 66.5, 43.0, 14.0, KDa respectively. The precipitated protein was mixed with sample loading buffer was loaded in gel and run for 2 hours in increasing 50V and 100V. The gel was directly placed on staining solution (commassie brilliant blue R-250 -150 mg, methanol-40ml, glacial acetic acid-10ml and ddH_2O-10ml) for 1 hour, and detained with distaining solution (methanol-150ml, glacial acetic acid-50ml and ddH_2O-200ml) several times until clear band observed, photograph while in wet, dried and kept for comparison.

RESULTS AND DISCUSSION

Pseudomonas is an oxidative bacterium, which oxidizes D-glucose into D-Glucono- δ-lactone along with reduction of NAD^+ to $NADH^+{+}H^+$. The gram-negative bacteria are usually bounded by two membranous structure the inner one (IM), called the plasma membrane, that bounds the bacterial protoplasm and it composed of a phospholipids bilayer. The outer membrane consists of proteins, including porins, receptors, and an asymmetric distribution of lipids. The outer leaflet was composed of primarily lipopolysaccharide (LPS) and lipoproteins [36]. Generally, gram- negative cell membranes were localized of several enzymes and it's essential for their growth.

Fig.1 *Pseudomonas* PRK786 in thiosulphate broth (Right- Control and Left-Culture contains *Pseudomonas* PRK786)

Sonication was best method for crude membranes preparation because the protein and peptides were less disturbed than the membrane treated with lysozyme-EDTA. The speed of sonication 40 % duty cycle; power - 10; total time - 30 min was optimum level for disturb the gram-negative cell membranes even the separation the membrane bound proteins and phospholipids with aid of $MgCl_2$.

Quantification of Total Phospholipids

7.2µg phospholipids were estimated in 1ml of cured sample. Ethanol and chloroform were phospholipids solubilizing agents. The Hundrieser method of phospholipids estimation range is 0-12µg/ml, the formation of Prussian blue-phospholipids-chromogenic complex directly propositional to amount of phospholipids present in the sample solution. Especially, chromogenic solution response to all types of phospholipids.

Quantification of Total Membrane Bound Proteins

Determination of proteins concentration by Bradford's method exhibited the sample contains 0.1 mg protein /ml that was revealed the membrane has highly composed of proteins because *Pseudomonas* membrane consists of variety of proteins[37] like peptidoglycan and other proteins including porins, receptors, unique electron transport system-linked gluconate dehydrogenases[1], D-Glucose Dehydrogenase[11], Bacteriocins (pyocins)[38].

Assay of NAD (P) $^+$- Dependent Glucose Dehydrogenase

The strains of *Pseudomonas aeruginosa* and *P. fluorescens* are suitable sources of D-glucose dehydrogenase[39]. The assay performed by formation of $NADPH^+$ in the reaction mixture, the results showed in table.1 the formation $NADPH^+$ depends on amount glucose reduced by NAD^+ dependent glucose dehydrogenase. From this view, cured preparation has significant total activity (12-µmol min^{-1}) because *Pseudomonas* PRK786 was belongs to heterotrophs [40].

Table.1 Activity of NAD (P) $^+$- dependent D-Glucose dehydrogenase from *Pseudomonas* PRK786

Steps	Total proteins (mg/ml)	Relative activity (µmol min^{-1})	OD at 340nm		Specific activity Units/mg of protein
			T	B	
Cured preparation	2.3	28.93	3.2	0.2	220.80
Heat precipitation	1.98	20.25	2.2	0.1	130.68
Desalting	1.87	35.69	3.8	0.1	213.18

Note: Assay condition- Buffer [200 mM of Tris HCl], NADP [12µM], Triton X-100 [10% (v/v)], β-D-Glucose [250 mM] and 0.1 of enzyme solution (2.3 mg protein /ml) at 40^0C, pH 8.0 for 30min.

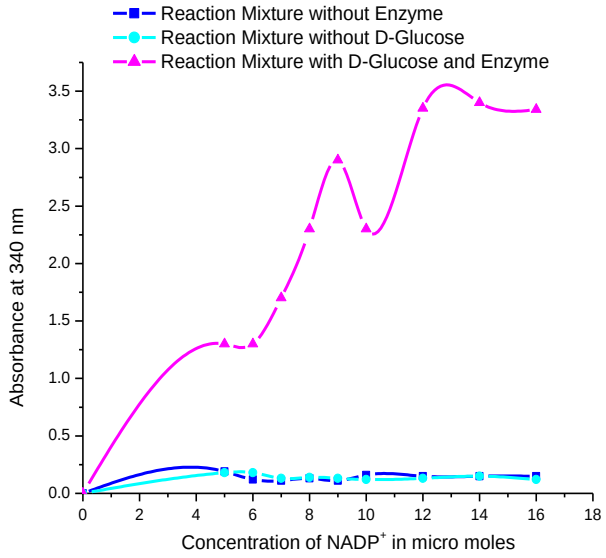

Fig 2 .The effects of NAD (P) $^{+}$- dependent D-glucose dehydrogenase in presence and absence of D-glucose with different concentration of NADP^{+}

The results from the Fig.2, combination of D-glucose and enzyme in the reaction mixture shows significant absorbance due to that glucose oxidized by membrane bound D-glucose dehydrogenase.

Assay of Thiosulfate Dehydrogenase

Thiosulfate and other reduced sulphur compounds as sole energy sources for many chemolithtrophic bacteria[41]. However, we attempt the assay of thiosulfate dehydrogenase (TSD) in cured membrane preparation evident that thiosulfate dehydrogenase inhabitant of periplasmic space[42]. TSD activity was assayed with or without of ferric cyanide and thiosulfate dehydrogenase contains sample and the results was tabulated at table.2.

Table. 2 Activity of thiosulfate dehydrogenase from *Pseudomonas* PRK786

Steps	Total proteins (mg/ml)	Relative activity (mM/ min^{-1})	OD at 340nm		Specific activity Units/mg of protein
			T	B	
Cured preparation	2.3	103.54	2.2	0.4	25.30
Heat precipitation	1.98	86.28	1.7	0.2	16.83
Desalting	1.87	150.37	2.8	0.2	26.18

Note: Enzyme activity was measured at pH 4.0 and 40°C for 5min, a mixture containing 1 mM ferricyanide and 15 mM thiosulfate.

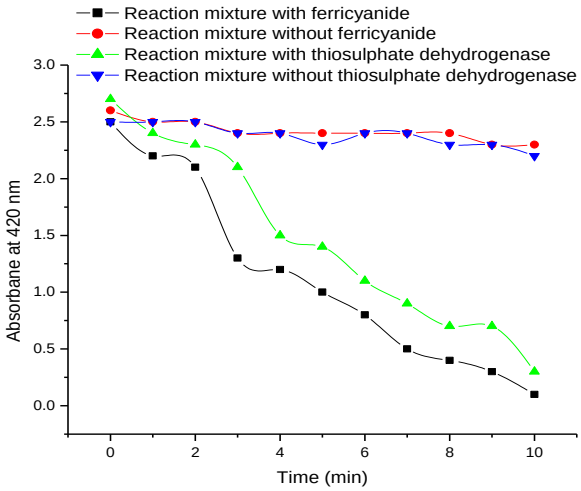

Fig 3 .The effects of thiosulfate dehydrogenase in presence and absence of thiosulfate and ferricyanide

All steps of enzyme activity were carried out at pH of 4.0. Since thiosulfate is unstable at a pH of<4.0. And results from the Fig.3 shows, the reaction mixture contains ferricyanide and thiosulfate dehydrogenases (supernatant of cured sample) have gradually decreases the absorbance due to the formation of reduced ferricyanide. In view of the fact that ferricyanide as final electron acceptors and thiosulphate were reduced by thiosulfate dehydrogenase respectively after the formation of tetrathionate.

Precipitation and Desalting of Protein from Cured Membrane Preparation

From the crude preparation, 80% of the proteins were precipitated at 50^0C. Since it might more stable in 60^0C [43]. Enzymatic activity showed over a broad after heat precipitation displayed in table .1 and 2. However, the results revealed that heat (50^0C) not influenced to their activity. After 12 hours dialysis, the catalytic activity of sample was significantly improved due to the removal of sodium, magnesium, chloride, and ammonium and sulphate ions from the sample was also displayed in table .1 and 2.

SDS-PAGE Analysis

The dialysate samples were loaded in lane 2, lane 3, and lane 4. Molecular weight of the dialysate samples were estimated to be ~80 kDs and ~48 kDs against the standard molecular weight marker proteins were made by using bovine albumin (66.5 KDa), ovalbumin (43.0KDa), and lysozyme (14.0 KDa), of different molecular weight were loaded in lane 1. After running time of 2 hours, two clear band were seen in lane 3, and 4 and it coincides with[2, 44, 43]and [34, 42, 33] respectively. These bands were suggesting that the dialysate sample contains D-Glucose dehydrogenase and thiosulfate dehydrogenase enzymes and some other proteins. Lane 2 was not clear.

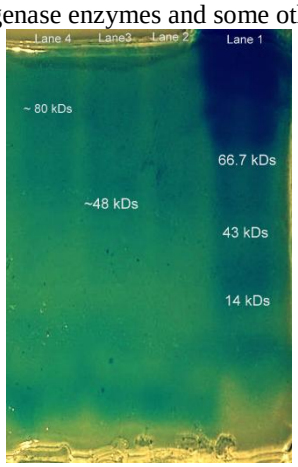

Fig.4 SDS-PAGE of partially purified membrane proteins from *Pseudomonas* PRK786: Lane 1- Standard molecular weight marker proteins (30µl), lane-2 (25µl), lane-3 (25µl), and lane-4 (25µl) dialysate membrane proteins samples.

HPLC Analysis

Chromatography for the separation of enzymes routine by high-performance liquid chromatography (HPLC)-based procedures, this allowing to reproduced higher results, precision, and linearity. HPLC provided an effective method to separate and analyze membrane bound enzymes shown Fig.5 by this way the dialysate membrane proteins sample (20µl) analyzed. The results were showed at table3 and Figure 5.Two significant peaks were observed at retention time (Rt) of 1.39 min and 2.170 min. The 2.170 Rt dialysate membrane proteins residues that area (%) occupied high than the 1.39 Rt due to the of their molecular weight and amount fraction. 1.390 Rt and 2.170 Rt might be thiosulfate dehydrogenase and D-glucose dehydrogenase respectively.

Table.3 HPLC Data of dialysate membrane proteins residues from *Pseudomonas* PRK786

S.No	Reten.time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	**1.390**	**13.950**	**2.192**	**48.1**	**69.0**	**0.08**
2	**2.170**	**15.065**	**0.983**	**51.9**	**31.0**	**0.25**
	Total	**29.015**	**3.175**	**100.0**	**100.0**	

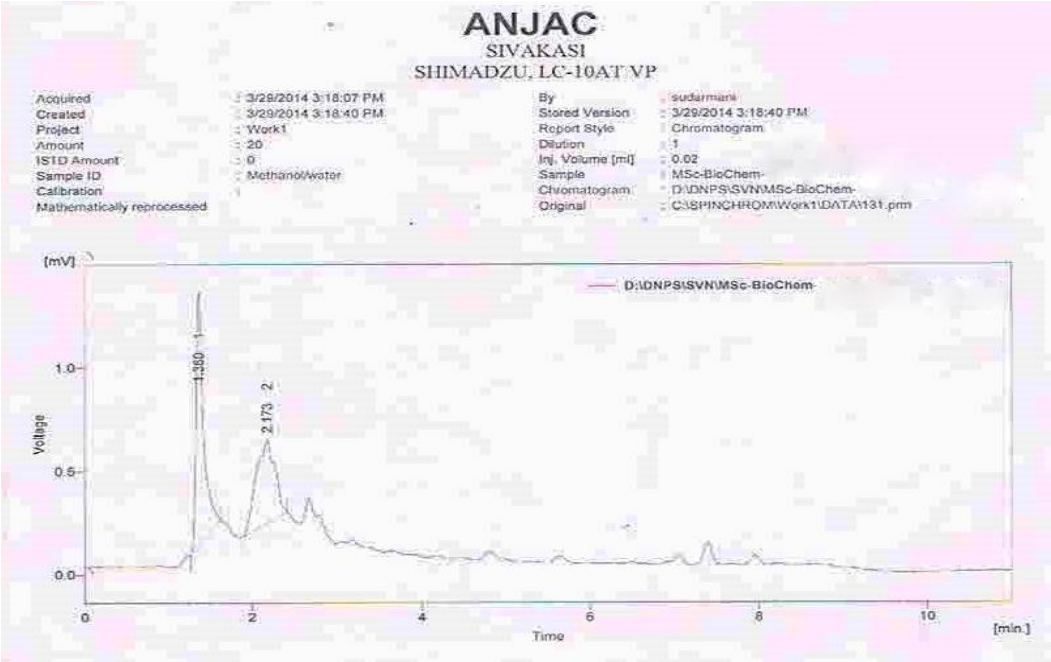

Fig 5. HPLC chromatogram of dialysate membrane proteins fractions from *Pseudomonas* PRK786

CONCLUSION

Present findings to suggest that *Pseudomonas* PRK786 was suitable sources for NAD (P) $^{+}$- dependent D-glucose dehydrogenase and thiosulfate dehydrogenase. D-glucose dehydrogenase was wieldy used enzyme in diagnostic, food laboratories, and recently used in development of biosensors for detection of heavy metals in various sources since thiosulfate dehydrogenase has little benefits. *Pseudomonas* PRK786 membrane highly composed both enzymes. They are importantly required for its survival because *Pseudomonas* PRK786 bacteria belongs chemolithoheterotrophic sulphur-Oxidizer. In future goes to find the amino acid composition and applications.

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NO CONFLICT OF INTEREST

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