The 6-phospho gluconate dehydrogenase activity from isolated thermotolerant *Bacillus* strains under different environmental conditions.

Aqel, H.[1,2] Al Quadan, F.[3] and Boulenouar, N.

[1]Clinical Laboratory Sciences, King Saud Bin Abdulaziz University for Health Sciences, P.O. Box 2490, Riyadh 11426, Mail code: 3129, Saudi Arabia. Tel: 00966612520088/Ext. 51069. E-mail: aqelh@ksau-hs.edu.sa

[2]Department of Medical Laboratory Sciences. Hashemite University; P.O. Box 330093. Zarqa 13133. Jordan. Tel.: 05-3826600/Ext. 4177. Fax: 05-3826613. E-mail: hazemm@hu.edu.jo.

[3]Department of Biological Sciences; Hashemite University; P.O. Box 330093. Zarqa 13133. Jordan. E-mail: fquadan@yahoo.com

Running Title: Activity of 6-phospho gluconate dehydrogenase of thermotolerant *Bacillus* strains.

Key Words: 6-phospho gluconate dehydrogenase; thermotolerant *Bacillus*; thermal stability; isoenzymes**.**

Corresponding Address:
Dr. Hazem Aqel
Clinical Laboratory Sciences, King Saud Bin Abdulaziz University for Health Sciences, P.O. Box 2490, Riyadh 11426, Mail code: 3129, Saudi Arabia. Tel: 00966612520088/Ext. 51069. E-mail: aqelh@ksau-hs.edu.sa

Abstract

Nine new isolated thermotolerant *Bacillus* strains were used to evaluate the effect of growth temperatures, pH-values and NaCl concentrations on intracellular 6-phospho gluconate dehydrogenase (6PGDH) activity. The optimal growth condition of temperature, pH-value and NaCl concentration for most studied strains was 43^{o}C, 7 and 3%, respectively. All new and known strains were able to produce 6PGDH to different levels. The growth temperature showed the most effect on 6PGDH activity; in addition, the combined effect of temperature/NaCl had more effect than the combined effect of temperature/pH and pH/NaCl. In addition, thermal stability at 53^{o}C and electrophoretic mobility was investigated. Electrophoretic study demonstrated that 6PGDH was composed of one isoenzyme for all strains except HUTB 41 that had two isoenzymes.

Key words: 6-phospho gluconate dehydrogenase; thermotolerant *Bacillus*; thermal stability; isoenzymes**.**

Introduction

The 6-phosphogluconate dehydrogenase (6PGDH) is the third enzyme of the pentose phosphate pathway (PPP). It catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate, with the concomitant production of NADPH (Reginald and Charles, 1995; Emmanuel *et al*., 1999).

The mechanism of action of the enzyme has been studied in several species and crystal structures are known for the enzyme from various sources. The 6PGDH has been proposed as a potential target for anti-microbial chemotherapy, since accumulation of its substrate, 6PG, is toxic to eukaryotic cells, causing a decrease in glycolysis due to its inhibitory effect of phosphoglucose isomerase. Understanding more about the structure and function of the enzyme from many sources will assist in developing inhibitors, which may be of specific antimicrobial value. The enzyme has been subject to detailed analysis from *African Ttrypanosomes* which cause the disease sleeping sickness. Several Gram-positive bacteria have

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been shown to contain NAD^+ dependent 6PGDH (Emmanuel *et al.*, 1999). It has been suggested that the activity of 6PGDH is subjected to a two-way regulation: NADPH, which regulates the pentose phosphate pathway, inhibits the enzyme, while 6-phosphogluconate, levels of which rise when NADPH inhibition is removed, acts as an activator ensuring that 6-phosphogluconate is rapidly removed (Rippa *et al.*, 1998).
Several studies showed that the pentose phosphate pathway activity increased during stress. The hypothesis that the pentose phosphate pathway is involved in the response of bacteria to temperature, pH and NaCl changes was tested in this study.
The purposes of this study were to: (a) Evaluate the effect of temperature, pH and NaCl concentration on 6PGDH activities, (b) Determine the number and types of isoenzymes for 6PGDH present in these thermotolerant *Bacillus* strains, and (c) Evaluate the thermal stability of 6PGDH by measuring the residual activity after incubation at 53ºC for different period of time.

Materials and Methods

Bacillus Strains

The *Bacillus* strains used in this study were isolated from Jordanian hot springs at the Hashemite University, Jordan. The isolates had been classified on the basis of morphological, physiological, biochemical, antimicrobial susceptibility and genotypic characteristics as described by Akel and Atoum (2003).
Nine thermotolerant *Bacillus* strains were used in this study. They were new isolated *Bacillus* form Jordanian hot-springs, designated: HUTB17, HUTB19, HUTB20, HUTB26, HUTB41, HUTB42, HUTB53, HUTB55 and HUTB71.

Growth and Harvesting of Bacteria

One liter of nutrient broth contains: 5g peptone, 5g yeast extract and (1%, 3%, 5% or 7%) NaCl; components were dissolved in 1000 ml distilled water. The pH of nutrient broth was adjusted to: pH 5, pH 7 or pH 9 by NaOH or HCl. To prepare approximately 22 petri dishes of thick nutrient agar plates 18g agar was dissolved in 1000 ml nutrient broth. Nutrient broth and nutrient agar were autoclaved (15 min at 121ºC) before use.
The nine strains were grown at different conditions of temperature (37ºC, 43ºC, 53ºC and 63ºC), pH-values (5, 7 and 9) and NaCl concentrations (1%, 3%, 5% and 7%).
Inoculations were carried out in a safety cabinet 'Class II'. For each experiment, 35 ml of nutrient broth were inoculated with one of the *Bacillus* strains and incubated for 24h at 37ºC, 43ºC, 53ºC or 63ºC. At the end of incubation period, samples were centrifuged at 3500 rcf for 10 min at 4ºC in a refrigerated centrifuge; pellets were washed in 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, then centrifuged for a second time at 6400 rcf for 10 min. Pellets were stored in deep freeze at -70ºC until use.

6PGDH Extraction

Extraction solution used consists of: 100 mM Tris-HCl (pH 7.8), 2 mM EDTA disodium and 30 mM β-Mercaptoethanol. The stored pellets were suspended in the extraction solution (1 ml/pellet); bacterial cells were disrupted using one cycle freeze-thaw and homogenized for 4 min using homogeniser at 25000 rpm to release its content of intracellular 6PGDH. During homogenisation the sample was cooled in ice. Homogenates were centrifuged at 24000 rcf for 10 min. The supernatant was stored at -70ºC until use for 6PGDH assay.

6PGDH Assay

The 6PGDH activity was measured at 43ºC using the method of Hohorst (1965) with slight modification, which depends on the reduction of $NADP^+$ to NADPH by 6PGDH. The activity measurement was made by monitoring the increase in absorbance at 340 nm, and the calculation of enzyme unit per litre (U/L) was done by assuming a molar extinction coefficient of 6270 U/L/Mole as described by Wei-Ying and

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Tang (1999) for NADPH. One enzyme unit (U) was defined as the reduction of 1 µmol of NADPH per minute at 43ºC, pH 7.6 (Dennis and Bernlohr, 1973).

Each assay consisted of 850 µl Tris-HCl buffer (100 mM, pH 7.6), 100 µl $MgCl_2$ (67 mM), 10 µl 6-phosphogluconic acid (6 mM) and 10 µl $NADP^+$ (5 mM); mixture was incubated at 43ºC for 2 minutes; then 30 µl of supernatant were added to assay mixture (the total volume was 1 ml) and incubated further for 1 min at 43ºC. Absorbance at 340 nm was followed for 3 minutes using spectrophotometer (Clima, Spain).

Protein Determination

Dye stock solution was prepared by dissolving 100 mg Coomassie brilliant blue G-250 in 50 ml methanol, this solution was added to 100 ml of 85% H_3PO_4. The mixture volume was completed to 200 ml with distilled water. Final concentrations of stock assay mixture were: Coomassie brilliant blue G-250 (0.5 mg/ml), methanol (25%) and H_3PO_4 (42.5%). The protein assay reagent was prepared by diluting the dye stock 1:4 with distilled water. The protein assay reagent was filtered just before assays using Whatman filter paper (Whatman paper Nº1, England).

One ml assay reagent was mixed with 20 µl sample. The mixture was incubated at room temperature for 5 minutes, and then absorbance at 595 nm was recorded. The absorbance values were converted to protein concentrations using the standard curve. A convenient standard curve was constructed using BSA with concentrations of: 0, 0.25, 0.5, 1, 1.5 and 2 mg/ml.

Residual Activity

Thermal stability of 6PGDH was determined after incubation of the crude extract at 53ºC for 5 minutes to 6 hours. Then, the crude extract was cooled to assay temperature of 43°C and used for 6PGDH activity measurement as described before. Residual activity for 6PGDH was calculated and compared to the untreated sample.

Isoenzymes Study

Polyacrylamide gel electrophoresis was carried out according to the method of Ornstein and Davis (1962), the crude extracts from each *Bacillus* strains were analysed for 6PGDH isoenzyme components using 5% and 7.5% polyacrylamide for stacking gel and separating gel, respectively.

The sample buffer contains: 5.55 ml deionised water; 1.25 ml Tris-HCl (0.5 M, pH 6.8), 3 ml glycerol and 0.2 ml 0.5% (w/v) bromophenol blue. Bromophenol blue was added to serve as a tracking dye. Sample was mixed with buffer at 1:1 ratio. Electrophoresis runs were carried out in vertical gel electrophoresis apparatus at constant voltage of 120 volts as described by Richard and Frank (1979). At the end of electrophoresis run, isoenzymes were visualized according to the procedure described by Schnarrenberger and Tolbert (1973) and Raymond *et al.* (1975) by placing the gels for 30 min at 43ºC in solution containing 40 mM Tris-HCl (pH 8.8), 5 mM $MgCl_2$, 250 µM $NADP^+$, 500 µg/ml nitro blue tetrazolium, 25 µg/ml phenazine methosulfate and 1 mM glucose-6-phosphate for G6PDH. After staining, bands were photographed using digital camera (Olympus, Japan) interfaced to a computer [Dell, USA].

Experimental Design

We have studied the effect of three variables: temperature, with 4 levels (37, 43, 53 and 63ºC); pH, with 3 levels (5, 7 and 9); and NaCl concentrations, with 4 levels (1, 3, 5 and 7%) on 6PGDH activity. The experimental design used in this study was the factorial experiment. Then, the combinations between the three variables had given 48 experiments for each *Bacillus* Strain.

Statistical Analysis

Experimental mean values were analysed using the analysis of variance (ANOVA) test; one-way ANOVA test was used to determine the level of significance within the single *Bacillus* strain regarding the effect of temperature pH and NaCl on 6PGDH activities. Two-ways ANOVA test was used to determine the level

of significance between the different *Bacillus* strains regarding 6PGDH activities. Significance of differences was accepted when $p<0.05$.

Results

Effects of growth conditions (temperatures, pH-values and NaCl concentrations) on the activities of intracellular 6PGDH

The 6PGDH activities for nine isolated thermotolerant *Bacillus* strains were measured as mU/mg protein and the results are presented in *Tables 1-4*. All studied isolates showed that the temperature had a significant effect on 6PGDH production ($p<0.005$). The pH-value had a significant effect on 6PGDH production ($p<0.005$) for HUTB20, HUTB26, HUTB55 and HUTB7. Sodium chloride had significant effect on 6PGDH production ($p<0.005$) for only HUTB20 and HUTB42 (*Table 5*).

The combined effect of (pH and NaCl) was not significant for all tested strains, whereas combined effect of (temperature and NaCl) was significant ($p<0.005$) for HUTB19 and HUTB41; the combined effect of (temperature and pH) was significant for HUTB20 and HUTB26 (*Table 5*)

Relationship between growth conditions and production of 6PGDH

Conditions for high growth and high 6PGDH production are presented in *Table 6*. The variation was seen between the high growth and high 6PGDH under different conditions for all tested strains except HUTB26. Both HUTB55 and HUTB71 showed high growth and high 6PGDH production at neutral pH-value. The HUTB17, HUTB19, HUTB20 and HUTB53 showed high growth at neutral pH-value but high 6PGDH production at acidic pH-value. The HUTB41 and HUTB42 showed high 6PGDH production at pH 9 (alkaline condition) and high bacterial growth at pH-values 5 and 7, respectively. For NaCl, only HUTB17 showed high bacterial growth and high 6PGDH production in the presence of 7%NaCl. The production of 6PGDH was higher than high bacterial growth in the presence of 7% NaCl was observed for HUTB53, HUTB55 and HUTB71.

Residual activity of 6PGDH crude enzyme after incubation at 53^{o}C

The time at which 50% of 6PGDH activity remains is presented in *Table 7*. The only two strains (HUTB55 and HUTB26) had high thermostable 6PGDH compared with the other strains 16 and 14 minutes, respectively.

Isoenzymes of 6PGDH crude enzyme

The isoenzyme patterns of 6PGDH from new isolates are presented in *Fig. 1*. It had been determined that 6PGDH was composed of only one isoenzyme for all tested strains except HUTB41 with two isoenzymes.

Discussion

Results obtained from this study are important as they add to current understanding of temperature, pH and NaCl effect on pentose phosphate pathway in thermotolerant *Bacillus* strains and the possible interactions between these three parameters. In addition, it adds to our knowledge certain characteristics on these *Bacillus* strains such as thermostability and isoenzyme components of 6PGDH.

Analysis of variance has demonstrated that 6PGDH production was most strongly stimulated by temperature change than pH and NaCl. One interpretation of this observation was that 6PGDH production is more sensitive to temperature range used (37 to 53^{o}C) than to pH-values range (5 to 9) and NaCl concentration (1 to 7%), or temperature plays a better signal of energy flow through PPP than pH and NaCl. The pH and NaCl had shown effect in most cases less than temperature, may be their effects give undetectable values for the tested enzyme.

At growth temperature of 37^{o}C, most strains had given a detectable 6PGDH activity except for some strains at pH 5 and 7 and NaCl 1 and 7%, suggesting that some strains were able to utilize a wide range of

temperature, pH and NaCl which may reflect their great metabolic flexibility as described by Elena *et al.* (1999) but others don't tolerate high salt concentration and alkaline pH-value.

For most studied strains, the best growth temperature, pH-value and NaCl concentration were respectively 43^{o}C, 7 and 3% approve that unknown strains used in this study were isolated from similar natural sources. Conditions given minimum or no growth were for temperature 53^{o}C and 63^{o}C, for pH 9 and for NaCl 3, 5 and 7%.

The similarity in conditions (temperature, ph-value and NaCl) between high growth and high 6PGDH as seen for HUTB62, suggesting that metabolism via PPP was mainly used for cell proliferation as described by Wei-Ying and Tang (1999). Whereas, The differences between best growth conditions and 6PGDH activity suggesting that PPP was mainly used to manage stress caused by non-optional temperature, pH and/or NaCl as described by Tuttle *et al.* (2000) that during stress the glucose flux through PPP increased by as much as 200-folds over basal levels to protect cells against death caused by change in the environmental condition.

Study of pH effects on glucose metabolism had demonstrated that its effect was not significant for 6PGDH for many strains might be either due to the ability of these strains to tolerate the pH range used or effect was not detected. For others, flux of glucose via the pentose phosphate pathway had shown significant difference by decrease in 6PGDH activity when passing from pH 7 to 5, this might be due to optimum growth pH equal to 7 so high growth need high PPP activity, as shown for *Neisseria gonorrhoeae* increased in flux through PPP by passing from pH (7.2-8.0) to pH 6.0 this might be related to that pH 6 gave high growth or was stressful (Morse and Hebeler, 1978).

NaCl effect was not significant for most strains, suggesting that used NaCl wasn't so effective or had given undetermined effect.

Among numerous environmental factors affecting the vital functions of an organism, temperature is one of the most important and thoroughly investigated. Thermotolerant organisms have various biochemical devices to minimize the damage caused by temperature alterations. Traditionally, special attention in this field is paid to proteins and to enzymes first of all, because the enzyme systems ensure the necessary metabolism intensity during the temperature changes. In addition, the enzyme activity is a convenient marker for experimental measurement. Thermostability (thermoresistance) of enzymes has often been used to characterize the adaptative biochemical events. Biochemical systems of organisms usually respond to alterations in ambient temperature conditions by both modifications and genetic variations (Yulia and Alexander, 2001).

The thermostability of 6PGDH after incubation at 53^{o}C for different period of time expressed as $T_{50\%}$. It is noticeable from the data that the time for 50% inactivation ($T_{50\%}$) of 6PGDH after incubation at 53ºC for all strains was low compared with G6DHP results for the same strains by Aqel *et al.* (2012).

For 6PGDH, all tested strains were composed of only one isoenzyme except HUTB41 with two isoenzymes. Same result has been shown for 6PGDH from *Streptococcus faecalis* (Raymond, 1975), pea peroxisomes (Corpas *et al.*, 1998) and *Corynebacterium glutamicum* (Bernd *et al.*, 2000; Bianchi *et al*. 2001) that was composed of only one isoenzyme.

Metabolism is too complicated process to be controlled by one or two factors, it is the combination of thousands of enzymes affected by external and internal factors; this work has focused may be on one corner of the extended process of metabolism. Most important, it has been founded that different thermotolerant *Bacillus* strains may have different or similar metabolic characteristics. Regarding our study; many strains had given interest on the basis of their 6PGDH production and thermostability. These strains may be used for industrial purposes to produce high stable 6PGDH with high quantities. Finally, further works are needed to study the mechanisms of temperature, pH, and NaCl effect on metabolism flux through PPP especially in microorganisms.

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Table 1. Effect of 37^{o}C at different pH-values and NaCl concentrations on 6PGDH production.

Bacillus Strains	Specific activity of 6PGDH (mU/mg protein)											
T	37ºC											
pH	5				7				9			
NaCl	1%	3%	5%	7%	1%	3%	5%	7%	1%	3%	5%	7%
HUTBS 17	24.98	17.15	4.63	29.76	19.89	16.55	14.48	25.01	21.44	13.12	11.02	ND
HUTBS 19	73.64	39.90	20.67	30.12	53.76	21.72	16.17	24.09	43.22	ND	0.63	9.30
HUTBS 20	13.80	20.36	16.92	22.10	9.01	12.25	10.11	14.90	5.00	ND	6.16	ND
HUTBS 26	13.25	14.97	9.10	10.08	17.91	20.00	15.11	17.02	9.00	ND	5.00	ND
HUTBS 41	7.38	5.02	2.90	2.11	10.13	6.92	4.88	4.09	15.00	ND	ND	ND
HUTBS 42	12.94	10.91	5.97	11.97	10.02	5.00	3.00	6.92	15.17	ND	5.03	ND
HUTBS 53	12.12	19.18	15.00	36.11	6.00	21.93	16.90	30.30	3.00	43.40	2.48	63.66
HUTBS 55	11.68	8.32	13.73	18.00	ND	6.03	9.50	10.75	3.36	1.51	ND	ND
HUTBS 71	5.30	4.56	4.18	ND	17.00	15.40	5.36	22.25	15.32	ND	ND	ND

Where, ND: No detection of 6PGDH.

Table 2. Effect of 43°C at different pH-values and NaCl concentrations on 6PGDH production.

Bacillus strains	Specific activity of 6PGDH (mU/mg protein)												
	T	43ºC											
	pH	5				7				9			
	NaCl	1%	3%	5%	7%	1%	3%	5%	7%	1%	3%	5%	7%
HUTB 17		32.20	28.05	22.73	38.55	26.94	14.44	9.89	31.45	24.63	ND	13.99	25.87
HUTBS 19		29.28	26.46	8.91	ND	15.84	8.44	7.98	10.03	13.10	ND	ND	ND
HUTBS 20		24.88	30.00	27.29	ND	19.00	22.68	21.09	25.21	13.08	ND	14.94	ND
HUTBS 26		19.86	22.20	16.12	ND	28.10	29.82	21.00	22.99	15.00	ND	11.04	ND
HUTBS 41		24.91	15.00	8.80	ND	40.23	30.00	25.10	ND	71.03	ND	33.15	ND
HUTBS 42		25.00	19.96	14.00	ND	20.11	12.10	10.90	15.00	35.12	ND	15.89	ND
HUTBS 53		3.00	27.38	18.00	84.38	2.50	12.94	12.96	29.00	15.08	ND	20.01	ND
HUTBS 55		34.98	18.12	22.00	ND	20.09	9.55	14.95	41.05	21.96	ND	17.00	ND
HUTBS 71		4.50	4.03	2.52	ND	13.00	5.30	4.00	16.01	5.42	ND	3.20	ND

Where, ND: No detection of 6PGDH.

Table 3. Effect of 53°C at different pH-values and NaCl concentrations on 6PGDH production.

Bacillus strains	Specific activity of 6PGDH (mU/mg protein)												
	T	53ºC											
	pH	5				7				9			
	NaCl	1%	3%	5%	7%	1%	3%	5%	7%	1%	3%	5%	7%
HUTBS 17		10.22	9.02	7.11	ND	ND	5.78	4.20	9.11	ND	3.78	2.01	ND
HUTBS 19		6.63	3.32	1.17	ND	7.20	ND	2.93	5.79	1.62	ND	0.04	ND
HUTBS 20		5.86	10.21	9.12	ND	2.01	5.07	3.96	7.23	0.50	1.00	0.78	ND
HUTBS 26		ND	6.89	3.00	ND	7.90	10.99	6.00	7.07	3.57	6.00	ND	ND
HUTBS 41		5.00	2.00	0.98	ND	8.00	ND	1.99	0.91	ND	6.21	4.00	ND
HUTBS 42		15.08	12.00	5.92	ND	13.13	7.00	4.90	10.02	21.00	13.99	9.00	ND
HUTBS 53		11.00	26.11	11.90	ND	2.33	ND	2.01	6.20	ND	6.00	ND	ND
HUTBS 55		17.89	ND	15.11	21.94	16.00	ND	6.00	17.15	ND	ND	ND	ND
HUTBS 71		1.82	1.50	0.98	ND	4.61	3.50	2.00	7.03	ND	ND	ND	ND

Where, ND: No detection of 6PGDH.

Table 4. Effect of 63°C at different pH-values and NaCl concentrations on 6PGDH production.

Bacillus strains	Specific activity of 6PGDH (mU/mg protein)											
	T	63ºC										
	pH	5				7				9			
	NaCl	1%	3%	5%	7%	1%	3%	5%	7%	1%	3%	5%	7%
HUTBS 17		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTBS 19		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTBS 20		ND	ND	ND	ND	ND	ND	0.17	ND	ND	0.06	ND	ND
HUTBS 26		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTBS 41		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTBS 42		ND	ND	0.59	ND	ND	ND	0.51	ND	**1.32**	ND	ND	ND
HUTBS 53		ND	ND	ND	ND	1.19	ND	**1.00**	ND	ND	ND	ND	ND
HUTBS 55		ND	ND	0.75	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTBS 71		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Where, ND: No detection of G6PDH.

Table 5. *p*-values of ANOVA test for all tested thermotolerant *Bacillus* strains.

Bacillus strain	6PGDH					
	One Way			Two Ways		
	T	**pH**	**NaCl**	**T/pH**	**T/NaCl**	**pH/NaCl**
HUTBS-17	<0.05	NS	NS	NS	NS	NS
HUTBS-19	<0.05	NS	NS	NS	<0.05	NS
HUTBS-20	<0.05	<0.05	NS	<0.05	NS	NS
HUTBS-26	<0.05	<0.05	NS	<0.05	NS	NS
HUTBS-41	<0.05	NS	NS	NS	<0.05	NS
HUTBS-42	<0.05	NS	<0.05	NS	NS	NS
HUTBS-53	<0.05	NS	NS	NS	NS	NS
HUTBS-55	<0.05	<0.05	NS	NS	NS	NS
HUTBS-71	<0.05	<0.05	NS	NS	NS	NS

Where, NS: not significant; <0.05: significant.

Table 6. Conditions for high growth and high 6PGDH production for all tested thermotolerant *Bacillus* strains.

Bacillus strains	**Conditions for High Growth**			**Conditions for High 6PGDH Production**		
	T (°C)	pH	NaCl (%)	T (°C)	pH	NaCl (%)
HUTBS-17	53	7	7	43	5	7
HUTBS-19	37	7	7	37	5	1
HUTBS-20	43	7	3	43	5	3
HUTBS-26	43	7	3	43	7	3
HUTBS-41	43	5	3	43	9	1
HUTBS-42	43	7	3	43	9	1
HUTBS-53	43	7	3	43	5	7
HUTBS-55	43	7	3	43	7	7
HUTBS-71	37	7	5	37	7	7

Table 7. $T_{50\%}$ for 6PGDH crude enzyme after incubation at 53^{o}C for different period of time.

***Bacillus* strains**	$T_{50\%}$ **(minute)**
HUTBS 17	< 5
HUTBS 19	9
HUTBS 20	< 5
HUTBS 26	14
HUTBS 41	8
HUTBS 42	< 5
HUTBS 53	7
HUTBS 55	16
HUTBS 71	6

Where, $T_{50\%}$: time at which remains 50% of the 6PGDH activity.

17 19 20 26 41 42 53 55 71

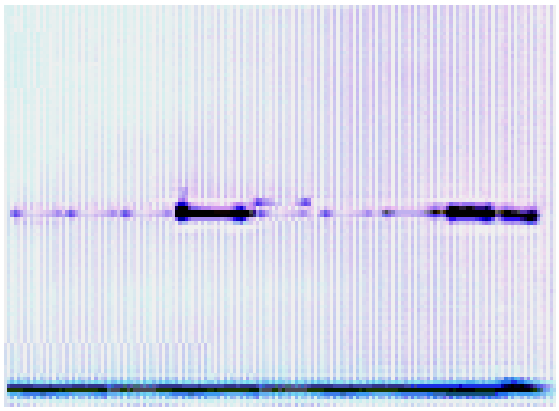

Figure 1. Polyacrylamide gel electrophoresis of 6PGDH crude enzyme.