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Effects of temperature, pH-values and sodium chloride concentrations on the glucose-6-phosphate dehydrogenase activity by thermotolerant *Bacillus* strains

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ABSTRACT

Thirteen new isolated thermotolerant *Bacillus* strains and four known *Bacillus* species were used to evaluate the effect of growth temperature, pH-values and NaCl concentrations on the intracellular glucose-6-phosphate dehydrogenase (G6PDH) activity. Results had shown a significant difference in G6PDH production among all species at all used temperatures ($p<0.05$). The response of individual new isolates and controls for production of G6PDH under growth conditions was variable. The optimal growth conditions did not correspond to the optimal cultivation conditions for maximum G6PDH production. The growth temperature showed the most significant effect on G6PDH activity. The combined effect of temperature and NaCl on the G6PDH activity was strongly pronounced in comparison with the combined effect of temperature and pH or pH and NaCl. Thermal stability at 53ºC and electrophoretic mobility were also investigated. G6PDH from HUTB41 was the most thermostable G6PDH enzyme with $T_{50\%}$ of more than 360 minutes. Electrophoretic study demonstrated that G6PDH was composed of two isoenzymes for all strains except *B. marinus* and *B. schlegelii* that had three isoenzymes.

Key words: *Bacillus*, glucose-6-phosphate dehydrogenase, temperature, sodium chloride, *pH*-values

Introduction

Glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49) is an ubiquitous enzyme that catalyzes the initial step in the pentose phosphate pathway (PPP) and regulates its oxidative branch (Kindzelskii et al., 2002). G6PDH is a cytosolic enzyme whose main function is to produce NADPH, a key electron donor in defense against oxidizing agents and in reductive biosynthetic reactions (Beydemir et al., 2003; Boulenouar et al., 2006).

The glucose metabolized through the pentose pathway has been estimated in a number of *Bacillus* species. Russell et al. (1989) studied the carbohydrate metabolism in mosquito pathogenic strain *B. sphaericus* 2362. This bacterium was found to be unable to transport glucose or sucrose into the cell and it lacked glucokinase and hexokinase activities. Blumenthal (1965) reported that 2% of the glucose in *Bacillus cereus* was utilized via the pentose pathway during logarithmic growth and 15% during the outgrowth of the spore. However, *Bacillus subtilis* utilized the pentose pathway to catabolize 25 to 41% of the glucose that it metabolizes (Blumenthal, 1965). On the other hand, Pepper & Costilow (1964) were able to demonstrate that the use of this pathway could be regulated in *Bacillus popilliae*.

The aim of the present study was to determine the activities of G6PDH from different new isolates thermotolerant *Bacillus* strains compared with four known strains under variable growth conditions (different temperature, pH-values and NaCl concentrations). The number of isoenzymes of G6PDH in each of the new isolates was also determined.

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Materials and Methods

Bacillus strains

Seventeen thermotolerant *Bacillus* strains were used in this study. Thirteen of them were new isolated *Bacillus* strains designated: HUTB17, HUTB19, HUTB20, HUTB26, HUTB41, HUTB42, HUTB53, HUTB55, HUTB71, HUTB77, HUTB82, HUTB83 and HUTB84. For comparison were used four known thermotolerant *Bacillus* species: *Bacillus circulans* (ATCC 4513), *Bacillus sphaericus* (ATCC 14577), *Bacillus marinus* (ATCC 29841) and *Bacillus schlegelii* (DSM 2000).

Growth and harvesting of bacteria

One liter of the nutrient broth contained 5 g peptone, 5 g yeast extract and NaCl (1, 3, 5 or 7%). All components were dissolved in 1000 ml distilled water. pH of the nutrient broth was adjusted to pH 5, pH 7 or pH 9 by NaOH or HCl. The nutrient broth and nutrient agar were autoclaved for 15 min at 121ºC before use.

The seventeen strains were grown at different temperature conditions (37, 43, 53 and 63ºC), different pH-values (5, 7 and 9) and different 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* species and incubated for 24 h at 37, 43, 53 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.

G6PDH extraction

An extraction solution consisting of 100 mM Tris-HCl (pH 7.8), 2 mM EDTA disodium and 30 mM β-mercaptoethanol was used. The stored pellets were suspended in the extraction solution (1 ml/pellet). Bacterial cells were disrupted using one cycle freeze-thaw and homogenization for 4 min using homogenizer at 25000 rpm to release its content of intracellular G6PDH. During homogenization the sample was cooled in ice. Homogenates were centrifuged at 24000 rcf for 10 min. The supernatant was stored at -70ºC until use for G6PDH assay.

G6PDH assay

G6PDH activity was measured at 43ºC using the method of Hohorst (1965) with slight modification, method depends on the reduction of $NADP^+$ to NADPH by G6PDH. 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 Kuo & 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 (Opheim & Bernlohr, 1973).

Each assay consisted of 850 µl Tris-HCl buffer (100 mM, pH 7.6), 100 µl $MgCl_2$ (67 mM), 10 µl glucose-6-phosphate (10 mM) and 10 µl $NADP^+$ (5 mM). The mixture was incubated at 43ºC for 2 minutes and 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

Quantitative protein determination was performed spectrophotometrically at 595 nm by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

Residual activity

Thermal stability of G6PDH was determined after incubation of the crude extract at 53ºC for 5 minutes to 6 h. Then, the crude extract was cooled to assay temperature of 43°C and used for G6PDH activity measurement as described before. Residual activity for G6PDH was calculated and compared to the untreated sample.

Isoenzymes study

Polyacrylamide gel electrophoresis was carried out according to the method of Ornstein & Davis (1962). The crude extracts from each *Bacillus* strain were analysed for G6PDH isoenzyme components using 7.5% polyacrylamide.

The sample buffer contained 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. Sample was mixed with sample buffer at ratio 1:1. Bromophenol blue was added to serve as a tracking dye. Electrophoresis runs were carried out in vertical gel electrophoresis apparatus at constant voltage of 120 Volts as described by Wolf & Shew (1979). At the end of electrophoresis run, isoenzymes were visualized according to the procedure described by Schnarrenberger et al. (1973) and Bridges 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-

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phosphate for G6PDH. After staining, bands were photographed using digital camera (Olympus, Japan) interfaced to a computer [Dell, USA].

Experimental design

Seventeen *Bacillus* strains were divided into two groups: controls (n=4) and new isolates *Bacillus* species (n=13). We have studied the effect of three variables - temperature, with 4 levels (37ºC, 43ºC, 53ºC and 63ºC), pH, with 3 levels (5, 7 and 9) and NaCl concentrations, with 4 levels (1%, 3%, 5% and 7%), on the G6PDH 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* species.

Statistical analysis

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 the G6PDH activity. Two-ways ANOVA test was used to determine the level of significance between the different *Bacillus* strains regarding the G6PDH activities. The statistical significance was accepted when $P<0.05$.

Results

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 Hazem & Manar (2003).

Effects of the growth conditions (temperature, pH-values and NaCl concentrations) on the activity of intracellular G6PDH

The thirteen new isolated thermotolerant *Bacillus* strains (HUTB17, HUTB19, HUTB20, HUTB26, HUTB41, HUTB42, HUTB53, HUTB55, HUTB71, HUTB77, HUTB82, HUTB83, HUTB84) and four known strains (*B. circulans*, *B. marinus*, *B. schlegelii* and *B. sphaericus*), were incubated at variable growth conditions (temperature, pH-values and NaCl concentrations) as indicated above, and the G6PDH activity was measured as mU/mg protein. Results are presented in Tables 1-4.

Table 1. *Effect of 37°C at different pH-values and NaCl concentrations on G6PDH production. ND = No detection of G6PDH production.*

***Bacillus* strains**	**Specific activity of G6PDH (mU/mg protein)**											
	T	**37°C**										
	pH	**5**				**7**				**9**			
	NaCl	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**
HUTB17		2.2	2.86	2.01	3.4	2.01	2.6	1.81	3	1.8	2.33	1	ND
HUTB19		13.89	6.2	3.05	4.19	10.13	6.08	1.95	4.15	8.92	ND	1	1.99
HUTB20		1.02	1.33	1.1	2.8	0.6	0.79	0.55	1.01	0.81	ND	0.69	ND
HUTB26		6.52	7.2	3.05	3.92	6.02	6.54	2	3.49	4.11	ND	1.49	ND
HUTB41		1.72	1.21	1.1	1	2.91	2.2	1.46	0.97	5.87	ND	ND	ND
HUTB42		1.49	2.49	1.1	0.8	1.09	1.42	0.78	0.46	1.95	ND	0.99	ND
HUTB53		1.95	2.5	0.62	7.8	2.98	3.8	2.57	7.95	2.1	4.61	1	7.52
HUTB55		1.28	2	0.79	1.9	ND	2.5	1.78	2.49	0.9	1.81	ND	ND
HUTB71		1.5	3.41	1.9	ND	1.3	2.95	1.55	2	1.35	ND	ND	ND
HUTB77		1.46	4.85	2.04	2.76	1.59	7.13	3.11	3.55	0.57	3.29	1.2	ND
HUTB82		1	2.1	1.1	2.76	1.8	2.5	1.87	5.01	1.51	2.3	1.6	ND
HUTB83		0.65	3.3	1	2.55	1.1	5.2	1.43	4.01	1.3	5.99	1.81	5.05
HUTB84		1.51	2.81	1.64	3.33	2.53	3.19	2.73	7.06	1.99	3.11	2.15	ND
B. circulans		0.2	1.98	0.3	0.41	2	20.05	2.99	4.01	0.69	ND	0.99	1.3
B. marinus		1.49	2.8	1	3	2.55	4.76	1.7	5.1	2.25	4.21	1.5	4.5
B. sphaericus		4.9	2.1	3.6	2.5	2.45	1	1.81	1.25	2.7	ND	ND	1.38
B. schlegelii		1.67	2.9	0.67	4	3.7	6.4	1.5	19.36	0.97	1.7	0.39	ND

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Table 2. *Effect of 43°C at different pH-values and NaCl concentrations on G6PDH production. ND = No detection of G6PDH production.*

***Bacillus* strains**	**Specific activity of G6PDH (mU/mg protein)**											
	T	**43°C**										
	pH	**5**				**7**				**9**			
	NaCl	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**
HUTB17		1.93	3.03	0.65	3.40	0.61	0.85	0.41	1.00	1.70	ND	0.20	2.31
HUTB19		6.10	5.26	1.99	ND	2.59	1.12	0.62	0.91	2.40	ND	ND	ND
HUTB20		3.08	3.96	2.99	ND	1.28	2.80	1.20	3.00	2.58	ND	3.72	ND
HUTB26		7.30	7.98	4.02	ND	6.00	7.41	3.00	4.89	4.51	ND	2.50	ND
HUTB41		9.00	5.12	3.12	ND	13.00	8.91	7.06	ND	18.00	ND	8.90	ND
HUTB42		3.47	5.00	2.47	ND	2.51	4.13	1.80	1.50	3.83	ND	3.00	ND
HUTB53		1.10	2.00	0.80	3.06	1.31	2.29	1.01	3.50	1.04	ND	0.50	ND
HUTB55		4.94	5.29	3.00	ND	5.13	5.80	3.50	5.63	4.00	ND	1.99	ND
HUTB71		2.41	5.38	2.61	ND	1.69	4.40	3.32	2.89	1.50	ND	1.70	ND
HUTB77		3.51	5.16	3.80	4.02	3.70	8.33	4.11	4.50	2.99	4.80	3.51	ND
HUTB82		0.80	1.70	0.81	2.20	1.30	2.21	1.39	3.00	1.00	ND	1.11	ND
HUTB83		0.75	3.41	1.20	2.71	1.29	5.50	1.60	4.49	1.61	7.00	1.81	6.70
HUTB84		0.99	2.30	1.10	2.90	1.90	3.00	2.00	4.80	1.51	2.61	1.60	4.01
B. circulans		0.04	0.42	0.06	ND	0.43	4.01	0.60	0.80	0.14	ND	0.20	ND
B. marinus		0.88	1.65	0.59	ND	1.45	2.76	0.95	2.95	1.30	ND	0.88	ND
B. sphaericus		3.50	1.50	2.57	1.78	7.00	3.00	5.00	ND	ND	ND	1.50	ND
B. schlegelii		1.61	2.80	0.65	ND	3.59	6.20	1.45	18.82	0.94	ND	0.39	4.85

Table 3. *Effect of 53°C at different pH-values and NaCl concentrations on G6PDH production. ND = No detection of G6PDH production.*

***Bacillus* strains**	**Specific activity of G6PDH (mU/mg protein)**											
	T	**53°C**										
	pH	**5**				**7**				**9**			
	NaCl	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**
HUTB17		0.42	0.51	0.41	ND	ND	0.50	0.31	0.65	ND	0.20	0.31	ND
HUTB19		1.11	0.59	1.00	ND	1.48	ND	0.32	0.77	1.32	ND	0.64	ND
HUTB20		0.65	0.91	0.72	ND	0.40	0.52	0.39	0.70	0.52	0.72	0.39	ND
HUTB26		ND	6.50	2.00	ND	2.92	4.49	1.50	2.50	2.75	3.52	ND	ND
HUTB41		1.40	1.14	0.51	ND	2.04	ND	1.10	0.80	ND	2.00	1.60	ND
HUTB42		1.00	1.50	0.79	ND	0.90	1.02	0.70	0.49	1.20	2.04	1.00	ND
HUTB53		0.80	1.29	0.40	ND	1.10	ND	0.51	2.72	ND	1.00	ND	ND
HUTB55		3.05	ND	1.80	3.50	3.51	ND	1.90	3.71	ND	ND	ND	ND
HUTB71		1.29	2.10	1.51	ND	0.99	1.81	1.30	1.60	ND	ND	ND	ND
HUTB77		2.49	4.49	3.20	ND	3.09	5.01	3.49	3.70	1.32	4.00	ND	ND
HUTB82		0.50	1.03	0.68	2.03	0.90	1.90	0.95	2.41	0.71	1.49	0.80	ND
HUTB83		0.49	2.91	0.71	2.01	0.60	ND	0.90	2.41	0.65	3.26	ND	ND
HUTB84		0.30	0.60	0.40	ND	0.61	ND	0.70	1.70	0.51	1.00	ND	ND
B. circulans		0.08	0.81	0.12	ND	0.82	ND	1.21	1.60	ND	2.79	ND	ND
B. marinus		1.32	2.48	0.88	2.64	2.18	ND	1.42	4.42	ND	3.70	ND	ND
B. sphaericus		2.90	1.25	2.10	ND	5.81	2.50	4.16	2.90	ND	ND	ND	ND
B. schlegelii		0.11	0.20	ND	ND	0.26	0.44	0.10	1.34	ND	0.12	0.03	0.35

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Table 4. *Effect of 63°C at different pH-values and NaCl concentrations on G6PDH production. ND = No detection of G6PDH production.*

***Bacillus* strains**	**Specific activity of G6PDH (mU/mg protein)**											
	T	**63°C**										
	pH	**5**				**7**				**9**			
	NaCl	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**	**1%**	**3%**	**5%**	**7%**
HUTB17		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTB19		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTB20		ND	ND	ND	ND	ND	ND	10	ND	ND	0.14	ND	ND
HUTB26		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTB41		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTB42		ND	ND	0.08	ND	ND	ND	0.70	ND	0.19	ND	ND	ND
HUTB53		ND	ND	ND	ND	0.56	ND	0.20	ND	ND	ND	ND	ND
HUTB55		ND	ND	0.09	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTB71		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTB77		ND	ND	0.16	0.38	ND	ND	ND	ND	ND	0.06	ND	ND
HUTB82		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HUTB83		ND	ND	ND	ND	ND	ND	0.05	ND	ND	ND	ND	ND
HUTB84		ND	ND	ND	ND	ND	ND	0.04	ND	ND	0.05	ND	ND
B. circulans		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B. marinus		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B. sphaericus		ND	ND	4.78	ND	11.39	ND	ND	ND	ND	ND	ND	ND
B. schlegelii		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

The individual response of the new isolates and controls for production of G6PDH under growth conditions was variable. Temperature had significant effect on G6PDH production ($p<0.05$) for all studied new isolates and controls except *Bacillus circulans* and *Bacillus sphaericus*. The pH had significant effect on G6PDH production ($p<0.05$) for HUTB55, HUTB71, HUTB77, HUTB82, *Bacillus sphaericus* and *B. schlegelii*; NaCl had significant effect on G6PDH production ($p<0.05$) for HUTB19, HUTB41, HUTB77, HUTB83 and *Bacillus sphaericus* (Table 5).

The combined effect of temperature and pH was not significant ($p>0.05$) for all strains, while the combined effect of temperature and NaCl was significant ($p<0.05$) for HUTB19, HUTB41, HUTB53 and HUTB83. The combined effect of pH and NaCl was significant ($p<0.05$) only for *Bacillus sphaericus* (Table 5).

Relationship between growth conditions and production of G6PDH

Conditions for high growth and high G6PDH production are presented in Table 6. The optimal growth conditions did not correspond to the optimal cultivation conditions for maximum G6PDH production for all studied strains except HUTB55. The optimal temperature of 37°C was equal for both high growth and high G6PDH production for HUTB19, HUTB55 and HUTB84. Moreover, the temperature of 43°C was the same for both high growth and high G6PDH for HUTB20, HUTB26, HUTB41, HUTB42, HUTB55 and HUTB77. On the other hand, the temperature for high growth was higher than for high G6PDH production for HUTB53 (43°C and 37°C, respectively), HUTB-17 (53°C and 37°C, respectively) and *Bacillus circulans* (43°C and 37°C, respectively). In contrast, the temperature for high growth was lower than for high G6PDH production for HUTB71 and HUTB83 (both 37°C and 43°C, respectively), and for *Bacillus sphaericus* (43°C and 63°C, respectively). The pH-value and NaCl concentration for high growth and high G6PDH production for most strains were found to be variable.

Residual activity of G6PDH crude enzyme after incubation at 53°C

The thermostability of G6PDH crude enzyme was evaluated by incubation at 53°C for different period of time

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Table 5. *P-values of ANOVA test for all tested thermotolerant Bacillus strains. NS = not significant; <0.05 = significant.*

ANOVA test	One way			Two ways		
Variables / *Bacillus* strains	T	pH	NaCl	T/pH	T/NaCl	pH/NaCl
HUTB17	<0.05	NS	NS	NS	NS	NS
HUTB19	<0.05	NS	<0.05	NS	<0.05	NS
HUTB20	<0.05	NS	NS	NS	NS	NS
HUTB26	<0.05	NS	NS	NS	NS	NS
HUTB41	<0.05	NS	<0.05	NS	<0.05	NS
HUTB42	<0.05	NS	NS	NS	NS	NS
HUTB53	<0.05	NS	NS	NS	<0.05	NS
HUTB55	<0.05	<0.05	NS	NS	NS	NS
HUTB71	<0.05	<0.05	NS	NS	NS	NS
HUTB77	<0.05	<0.05	<0.05	NS	NS	NS
HUTB82	<0.05	<0.05	NS	NS	NS	NS
HUTB83	<0.05	NS	<0.05	NS	<0.05	NS
HUTB84	<0.05	NS	NS	NS	NS	NS
B. circulans	NS	NS	NS	NS	NS	NS
B. marinus	<0.05	NS	NS	NS	NS	NS
B. sphaericus	NS	<0.05	<0.05	NS	NS	<0.05
B. schlegelii	<0.05	<0.05	NS	NS	NS	NS

Table 6. *Conditions for high growth and high G6PDH production for all tested thermotolerant Bacillus strains.*

Conditions	Conditions for high growth			Conditions for high G6PDH production		
Variables / *Bacillus* strains	T (°C)	pH-values	NaCl (%)	T (°C)	pH-values	NaCl (%)
HUTB17	53	7	7	37	5	7
HUTB19	37	7	7	37	5	1
HUTB20	43	7	3	43	5	3
HUTB26	43	7	3	43	5	3
HUTB41	43	5	3	43	9	1
HUTB42	43	7	3	43	5	3
HUTB53	43	7	3	37	7	7
HUTB55	43	7	3	43	7	3
HUTB71	37	7	5	43	5	3
HUTB77	43	5	1	43	7	3
HUTB82	37	7	3	37	7	7
HUTB83	37	7	3	43	9	3
HUTB84	37	7	3	37	7	7
B. circulans	43	5	3	37	7	3
B. marinus	53	5	5	37	7	7
B. sphaericus	43	7	3	63	7	1
B. schlegelii	43	7	3	37	7	7

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(0, 5, 10, 15, 20, 30, 60, 120, 180, 240 and 360 minutes). The times of half-life of G6PDH activity at different temperatures are listed in Table 7. It was found that some new isolates had very thermostable G6PDH. For example HUTB41 had $T_{50\%}$ of more than 360 minutes, HUTB55 had $T_{50\%}$ of 332 minutes, HUTB83 had $T_{50\%}$ of 258 minutes, HUTB77 had $T_{50\%}$ of 252 minutes. On the other hand, *B. sphaericus* had the lowest $T_{50\%}$ of 8 minutes (Table 7).

Table 7. *$T_{50\%}$ for G6PDH crude enzyme after incubation at 53°C for different periods of time.*

***Bacillus* strains**	**$T_{50\%}$ (minute)**
HUTB17	25
HUTB19	236
HUTB20	104
HUTB26	12
HUTB41	>360
HUTB42	184
HUTB53	148
HUTB55	332
HUTB71	16
HUTB77	252
HUTB82	148
HUTB83	258
HUTB84	72
B. circulans	17
B. marinus	28
B. sphaericus	8
B. schlegelii	212

$T_{50\%}$: time at which remains 50% of the G6PDH activity.

Isoenzymes of G6PDH crude enzyme

Isoenzyme patterns of G6PDH from new isolates and control strains are presented in Figure 1. All strains had two G6PDH isoenzymes except *Bacillus marinus* and *Bacillus schlegelii* that appear to have three. The isoenzymes of HUTB71 have different motilities than those isoenzymes of other studied species.

Discussion

The activities of G6PDH of the thermotolerant *Bacillus* strains isolated from Ma'in hot springs, Jordan, have been shown to have specific characters. The differences in activity, thermoresistance and electrophoretic mobility are retained under different growth conditions. These differences reflect biochemical diversity among these thermotolerant *Bacillus* species with regard to pentose phosphate pathway.

At growth temperatures of 37°C, 43°C and 53°C, the majority of studied species showed appreciable G6PDH activity suggesting that studied species were able to grow at wide range of pH-values and NaCl concentrations at these temperatures, which may reflect their great metabolic flexibility (Ivanova et al., 1999).

The cultivation temperature affects intracellular G6PDH activity of the studied strains, strongly than pH and NaCl concentration.

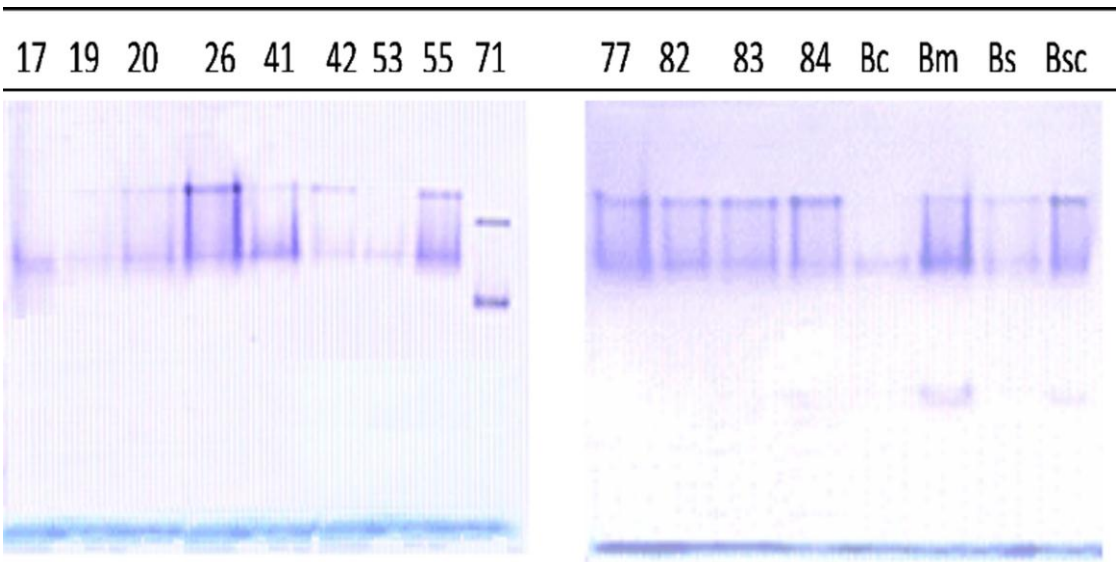

Figure 1. *Polyacrylamide gel electrophoresis of G6PDH crude enzyme.* ***Bsc:*** *Bacillus schlegelii;* ***Bs:*** *Bacillus sphaericus;* ***Bm:*** *Bacillus marinus;* ***Bc:*** *Bacillus circulans.*

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Temperature may be a better signal of energy flow through pentose phosphate pathway than pH and NaCl. Membré & Burlot (1994) demonstrated that temperature has a stronger effect on growth and pectinolytic enzymes from *Pseudomonas marginalis*.

Comparing the growth conditions with G6PDH activity should be able to reveal the stress level as compared to optimum growth. Heyer & Woodward (2001) described the optimal temperature (30°C) and pH (7.8) for G6PDH production from *Leuconostoc mesenteroides*. The similarity between conditions for high growth and conditions for high G6PDH activity suggested that metabolism via the pentose phosphate pathway was mainly used for cell proliferation (Kuo & Tang, 1999; Zhi et al., 2003). In growth conditions that give high G6PDH activity but not high growth, the pentose phosphate pathway is used mainly to manage the stress, caused by non-optimal temperature, pH, and/or NaCl. Tuttle et al. (2000) found that during stress the glucose flux through pentose phosphate pathway increased by as much as 200-folds over basal levels to protect cells against death, caused by change in the environmental conditions. Moreover, Lord-Fontaine & Averill-Bates (2002) found that hyperthermia (42°C to 43°C) increases NADPH levels and basal activity of the pentose phosphate pathway in ovary cells. Rizhsky et al. (2002) concluded from study on tobacco that the heat shock could influence G6PDH activity.

The effect of variation in growth medium pH on pentose phosphate pathway demonstrated less variability with regard to G6PDH for the majority of the studied strains. This might be due to the ability of these strains to tolerate the range of used pH. Earlier studies on *Neisseria gonorrhea* showed a decrease in G6PDH activity upon increasing or decreasing pH over optimum growth pH. This might be related to change in growth or to the stress effect that affects the pentose phosphate pathway (Morse & Hebeler, 1978).

Addition of NaCl to the growth medium was not so effective on the pentose phosphate pathway for most studied strains. We suggest that these strains have a pentose phosphate pathway that is not sensitive to the used NaCl range (1%, 3%, 5% and 7%).

Thermostability of the enzymes has often been used to characterize the adaptation to biochemical events. Biochemical systems respond to alterations in ambient temperature conditions by both modifications and genetic variations (Podlipaeva & Yudin, 2001). It is noticeable from our data that the time for 50% inactivation ($T_{50\%}$) of G6PDH for HUTB41 was more than 360 minutes and for HUTB55 it was 355 minutes. Menezes et al. (1989) demonstrated that *Lactobacillus casei* G6PDH had similar thermostability. On the other hand, Heyer & Woodward (2001) demonstrated that mesophilic G6PDH possess $T_{50\%}$ of 2.88 minutes at 60ºC. G6PDH extracted from vegetative cells of *Bacillus cereus* had $T_{50\%}$ of 10 minutes at 47ºC (Warth, 1980). Thermostability of G6PDH is provided by different mechanisms and one of them is the conformational lock formed by two intersubunit contacts in the hydrophobic region of protomers (Zaitseva et al. 2000; Podlipaeva, 2003).

The number of G6PDH isoenzymes varied among different studied species from two to three. G6PDH isoenzymes isolated from *Corynebacterium glutamicum* had two isoenzymes (Moritz et al., 2000), while that isolated from pea peroxisomes had three isoenzymes (Corpas et al., 1998). On the other hand, one isoenzyme was found for *Bacillus licheniformis* (Opheim & Bernlohr, 1973), in the liver of *Colonorchis sinensis* (Park et al., 2000), and in the sheep liver (Çiftçi et al., 2002).

This study has demonstrated that different thermotolerant isolates may have different or similar metabolic characteristics. It was found that G6PDH from HUTB41 was the most thermostable enzyme with $T_{50\%}$ more than 360 minutes. Moreover, results obtained from this study add to the current understanding of temperature, pH and NaCl effect on the pentose phosphate pathway in thermotolerant *Bacillus* strains and possible interactions between these three parameters.

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