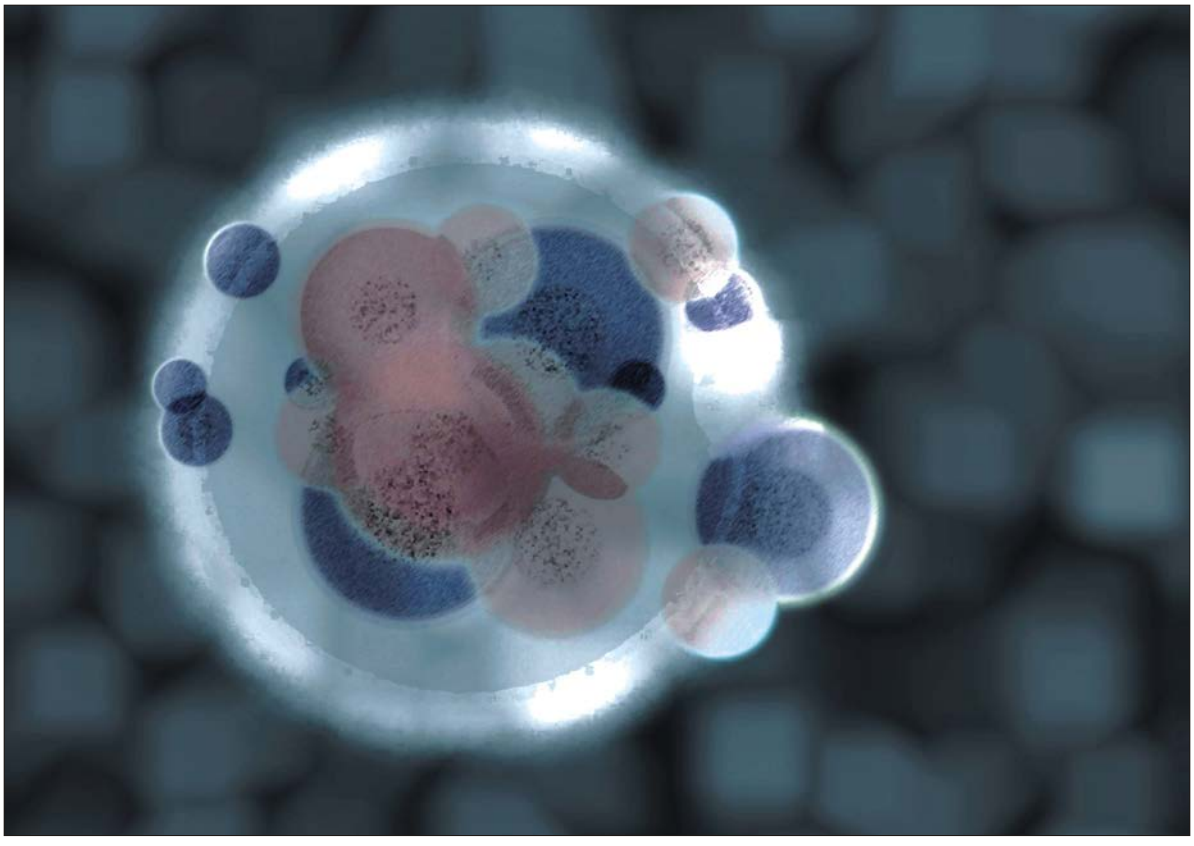

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# Effect of Various Combinations of Growth Temperature, pH and NaCl on Intracellular Activities of G6PDH and 6PGDH from Four *Bacillus* strains isolated from Jordanian Hot springs

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**Abstract:** Intracellular glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) from *Bacillus schlegelii*, *Bacillus sphaericus*, *Bacillus marinus* and Bacillus *circulans* were variably sensitive to growth conditions (temperature, pH and NaCl) and so were G6PDH and 6PGDH within the same strain. Thermostability of 6PGDH and G6PDH vary among the studied species; the difference in thermostability between the more stable G6PDH and the less stable 6PGDH from the same species may compensates the lower G6PDH and the higher 6PGDH activity found in this study. These results may indicate the existence of separate regulatory pathways for the two enzyme activities; one pathway may be related to thermostability and other pathways may be related to other growth conditions of pH and NaCl.

**Key words:** G6PDH, 6PGDH, *Bacillus schlegelii*, *Bacillus sphaericus*, thermostability, *Bacillus marinus*, *Bacillus circulans*

## INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PDH E.C 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH E.C 1.1.1.44) are key enzymes in the pentose phosphate pathway (PPP), an alternative metabolic pathway for glucose metabolism (Andrei *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 (Fiükrü *et al*., 2003). A complete absence of G6PDH activity is lethal and has never been reported in mammals (Dorota and Anna, 2003). G6PDH activity was reported in all organisms and cell types. Ludmila *et al*. (2002) found that heat shock influence G6PDH activity; Tuttle *et al*. (2000) found that pentose phosphate pathway increased by as much as 200-folds during stress. Stress response in *Bacillus subtilis* relies on regulation of a transcription factor, sigma(B) through a complex genetic and biochemical processes (Holtmann *et al*., 2004) ; nutritional stress , ATP, proton motive force and redox state, were involved (Zhang and Haldenwang , 2005).

In the present study, four thermotolerant *Bacillus* strains grown under combined growth conditions (temperature, pH and NaCl) were analysed for adaptational responses of intracellular G6PDH and 6PGDH activities (a) to optimize G6PDH and 6PGDH production (b) to evaluate whether or not adaptational responses were unified for G6PDH and 6PGDH (c) to evaluate activity and thermal stability of both enzymes from each strain (d) and to determine the number of isoenzymes present of both enzymes.

## MATERIALS AND METHODS

***Bacillus* species:** *Bacillus sphaericus*, *Bacillus schlegelii*, *Bacillus marinus* and *Bacillus circulans* were originally supplied by Dr. Amjad Khalil (Department of Bioagriculture, Al-Balq'a University, Salt, Jordan) who had isolated them from Jordanian thermal ponds (Khalil, 2002).

**Bacterial growth and harvesting:** *Bacillus* species were cultured (in our lab at the Hashemite University),

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aerobically at [37, 43, 53 or 63°C] with vigorous aeration using Basal Salt [5 g peptone, 5 g yeast extract and 0.1% NaCl in 1000 mL distilled water, pH 7.4 medium. The temperature, pH and NaCl optimum of the two enzymes was determined using combination of different temperatures, different pH-values [5, 7 or 9] and different NaCl concentrations [1, 3, 5, or 7%]. After 12 h of cultivation, bacterial cells were centrifuged at 3500 rpm for 10 min at 4°C in a refrigerated centrifuge; pellets were washed in 100 mM Tris-HCl buffer (pH 7.6) containing 10 mM $MgCl_2$, then centrifuged for a second time at 6400 rpm for 10 min. Pellets were weight and stored in deep freeze at -70°C until use.

**G6PDH and 6PGDH extraction:** Extraction solution was composed of: 100 mM Tris-HCl (pH 7.6), 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 of freeze and thaw and then homogenized at 25000 rpm (using KIA homogeniser) for 4 min. During homogenisation the samples were immersed in ice bath. Homogenates were centrifuged at 24000 rpm for 10 min. The supernatants were used for G6PDH and 6PGDH assays or stored at -70°C until use.

**G6PDH and 6PGDH assays:** G6PDH or 6PGDH activity was measured at 43°C using the method of Hohorst (1965); the method depends on the increase in absorbance at 340 nm upon reduction of $NADP^+$ to NADPH by G6PDH or 6PGDH. Absorbance was followed for 3 min using spectrophotometer (Clima, Spain) at 340 nm.

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

**Residual activity:** The thermostability of G6PDH and 6PGDH crude enzyme was evaluated by incubation of the crude preparation at 53°C for different period of time (0, 5, 10, 15, 20, 30, 60, 120, 180, 240 and 360 min). Then, the crude extract was cooled to assay temperature of 43°C and used for enzyme activity measurement as described before. Rresidual activity for G6PDH or 6PGDH was calculated and compared to the untreated sample. The time at which 50% of activity remains ($T_{50\%}$) is an index for thermostability.

**Electrophoretic mobility studies:** Polyacrylamide gel electrophoresis was carried out according to the method of Ornstein and Davis (1962), using Bio-Rad vertical gel electrophoresis at constant voltage of 120 volts. At the end of electrophoresis run, isoenzymes were visualized as described by Schnarrenberger *et al.* (1973). After staining, bands were photographed using digital camera (Olympus, Japan) interfaced to a computer [Dell, USA]

**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* species regarding the effect of temperature, pH and NaCl on G6PDH or 6PGDH activity. Significance of differences was accepted when $p<0.05$. Two-ways ANOVA test was used to determine the level of significance regarding the effect of (Temperature and pH), (Temperature and NaCl) and (pH and NaCl) on G6PDH and 6PGDH production. Significance of differences was accepted when $p<0.05$.

## RESULTS

**Effects of Growth Parameters on Intracellular G6PDH and 6PGDH Activities:** G6PDH and 6PGDH specific activities produced from *Bacillus sphaericus*, *Bacillus schlegelii*, *Bacillus marinus* and *Bacillus circulans* grown at various combinations of temperature, pH and NaCl concentrations (Table 1-4). The results of our study indicated that pH or NaCl had significant effect on G6PDH ($p<0.05$) from *Bacillus sphaericus*, as indicated by analysis of variance (Table 5), 6PGDH on the other hand, was significantly affected by pH alone; the combined effect of (pH and NaCl) was significant on G6PDH but not 6PGDH. The combined effect of either (temperature or pH), or (temperature and NaCl) were not significant for both enzymes ($p>0.05$) in *Bacillus sphaericus*.

*Bacillus schlegelii* growth temperature and pH had significant effect on intracellular G6PDH and 6PGDH activities ($p<0.05$), while the effect of combined (temperature and pH) was significant only on 6PGDH ($p<0.05$). On the other hand, the effect of NaCl concentration, or combined (Temperature and NaCl) was not significant for G6PDH and 6PGDH ($p>0.05$) from *Bacillus schlegelii*, however the combined effect of (Temperature and pH) was significant for 6PGDH only from this strain (Table 5).

*Bacillus circulans* growth temperature showed significant effect on 6PGDH activity alone ($p<0.05$), while the effect of pH, NaCl concentration, (temperature and pH), (Temperature and NaCl) and (pH and NaCl) was not significant for the two enzymes ($p>0.05$) from *Bacillus circulans*.

Table 1: *Bacillus sphaericus* G6PDH and 6PGDH specific activities produced from strain grown at experimental conditions described

| | | G6PDH (mU mg$^{-1}$) | | | | 6PGDH (mU mg$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Temperature (°C) | | | | Temperature (°C) | | | |
| pH | NaCl (%) | 37 | 43 | 53 | 63 | 37 | 43 | 53 | 63 |
| 5 | 1 | 4.9 | 3.5 | 2.9 | ND | 13.6 | 9.8 | 5.8 | ND |
| | 3 | 2.1 | 1.5 | 1.3 | ND | 3.8 | 2.7 | 1.5 | ND |
| | 5 | 3.6 | 2.5 | 2.1 | 71.0 | 14.4 | 10.3 | 6.1 | 31.9 |
| | 7 | 2.5 | 1.7 | ND | 71.0 | 10.3 | 7.3 | ND | ND |
| 7 | 1 | 2.4 | 7.0 | 5.8 | 11.4 | 19.1 | 13.6 | 8.0 | ND |
| | 3 | 1.0 | 3.0 | 2.5 | ND | 5.3 | 3.8 | 2.3 | ND |
| | 5 | 1.8 | 5.0 | 4.2 | ND | 19.1 | 14.4 | 8.4 | ND |
| | 7 | 1.3 | ND | 2.9 | ND | 14.4 | ND | 6.1 | ND |
| 9 | 1 | 2.7 | ND | ND | ND | 7.3 | ND | ND | ND |
| | 3 | ND | ND | ND | ND | ND | ND | ND | ND |
| | 5 | ND | 1.5 | ND | ND | ND | 5.6 | ND | ND |
| | 7 | ND | ND | ND | ND | 5.6 | ND | ND | ND |

Specific readings are average of 3 reading. ND: Not detected

Table 2: *Bacillus schlegelii* G6PDH and 6PGDH specific activities produced from strain grown at experimental conditions described

| | | G6PDH (mU mg$^{-1}$) | | | | 6PGDH (mU mg$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Temperature (°C) | | | | Temperature (°C) | | | |
| pH | NaCl (%) | 37 | 43 | 53 | 63 | 37 | 43 | 53 | 63 |
| 5 | 1 | 1.7 | 1.6 | 0.1 | ND | 9.4 | 6.7 | 1.2 | ND |
| | 3 | 2.9 | 2.8 | 0.2 | ND | 15.1 | 10.6 | 1.9 | ND |
| | 5 | 0.7 | 0.7 | ND | ND | 3.8 | 2.7 | ND | ND |
| | 7 | 4.0 | ND | ND | ND | 18.8 | ND | ND | ND |
| 7 | 1 | 3.7 | 3.6 | 0.3 | ND | 19.8 | 14.2 | 2.6 | ND |
| | 3 | 6.4 | 6.2 | 0.4 | ND | 31.5 | 22.5 | 4.1 | ND |
| | 5 | 1.5 | 1.5 | 0.1 | ND | 7.9 | 5.6 | 1.0 | ND |
| | 7 | 19.3 | 18.8 | 1.3 | ND | 39.4 | 28.2 | 5.0 | ND |
| 9 | 1 | 1.0 | 0.9 | ND | ND | 8.6 | 6.1 | ND | ND |
| | 3 | 1.7 | ND | 0.1 | ND | 13.8 | ND | 1.8 | ND |
| | 5 | 0.4 | 0.4 | 0.0 | ND | 3.4 | 2.5 | 0.4 | ND |
| | 7 | ND | 4.8 | 0.3 | ND | ND | 12.3 | 2.2 | ND |

Specific readings are average of 3 reading. ND: Not detected

Table 3: *Bacillus circulans* G6PDH and 6PGDH specific activities produced from strain grown at experimental conditions described

| | | G6PDH (mU mg$^{-1}$) | | | | 6PGDH (mU mg$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Temperature (°C) | | | | Temperature (°C) | | | |
| pH | NaCl (%) | 37 | 43 | 53 | 63 | 37 | 43 | 53 | 63 |
| 5 | 1 | 0.2 | 0.0 | 0.1 | ND | 3.0 | 0.5 | 2.7 | ND |
| | 3 | 2.0 | 0.4 | 0.8 | ND | 17.8 | 2.5 | 15.0 | ND |
| | 5 | 0.3 | 0.1 | 0.1 | ND | 6.5 | 0.9 | 5.7 | ND |
| | 7 | 0.4 | ND | ND | ND | 9.0 | ND | ND | ND |
| 7 | 1 | 2.0 | 0.4 | 0.8 | ND | 12.0 | 1.8 | 10.8 | ND |
| | 3 | 20.0 | 4.0 | ND | ND | 73.5 | 10.1 | ND | ND |
| | 5 | 3.0 | 0.6 | 1.2 | ND | 28.0 | 4.0 | 24.0 | ND |
| | 7 | 4.0 | 0.8 | 1.6 | ND | 37.0 | 5.1 | 30.1 | ND |
| 9 | 1 | 0.7 | 0.1 | ND | ND | 9.0 | 1.4 | ND | ND |
| | 3 | ND | ND | 2.8 | ND | ND | ND | 45.0 | ND |
| | 5 | 1.0 | 0.2 | ND | ND | 19.0 | 2.9 | ND | ND |
| | 7 | 1.3 | ND | ND | ND | 27.0 | ND | ND | ND |

Specific activities are average of 3 readings. ND: Not Detected

*Bacillus marinus* growth temperature had significant effect on G6PDH and 6PGDH ($p<0.05$), while pH, NaCl concentration, (temperature and pH), (Temperature and NaCl) and (pH and NaCl) effect was not significant for the two enzymes ($p>0.05$) from *Bacillus marinus*.

Table 4: *Bacillus marinus* G6PDH and 6PGDH specific activities produced from strain grown at experimental conditions described

| | | G6PDH (mU/mg) | | | | 6PGDH (mU/mg) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Temperature (°C) | | | | Temperature (°C) | | | |
| pH | NaCl (%) | 37 | 43 | 53 | 63 | 37 | 43 | 53 | 63 |
| 5 | 1 | 1.5 | 0.9 | 1.3 | ND | 10.2 | 14.4 | 8.1 | ND |
| | 3 | 2.8 | 1.7 | 2.5 | ND | 15.4 | 22.5 | 12.8 | ND |
| | 5 | 1.0 | 0.6 | 0.9 | ND | 5.0 | 7.3 | 4.2 | ND |
| | 7 | 3.0 | ND | 2.6 | ND | 8.9 | ND | 7.3 | ND |
| 7 | 1 | 2.5 | 1.4 | 2.2 | ND | 19.9 | 28.8 | 16.5 | ND |
| | 3 | 4.7 | 2.7 | ND | ND | 30.1 | 44.7 | ND | ND |
| | 5 | 1.7 | 1.0 | 1.4 | ND | 10.2 | 14.6 | 8.1 | ND |
| | 7 | 5.1 | 2.9 | 4.4 | ND | 18.0 | 25.9 | 14.9 | ND |
| 9 | 1 | 2.2 | 1.3 | ND | ND | 14.9 | 21.4 | ND | ND |
| | 3 | 4.2 | ND | 3.7 | ND | 22.5 | ND | 19.1 | ND |
| | 5 | 1.5 | 0.9 | ND | ND | 7.6 | 10.7 | ND | ND |
| | 7 | 4.5 | ND | ND | ND | 13.6 | ND | ND | ND |

Specific activities are average of 3 readings. ND: Not Detected

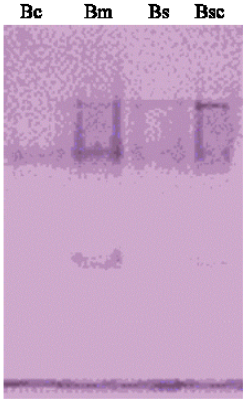


Fig. 1: Polyacrylamide gel electrophoresis of G6PDH crude enzyme. Bsc, *Bacillus schlegelii*; Bs, *Bacillus sphaericus*; Bm, *Bacillus marimus*; Bc, *Bacillus circulans*

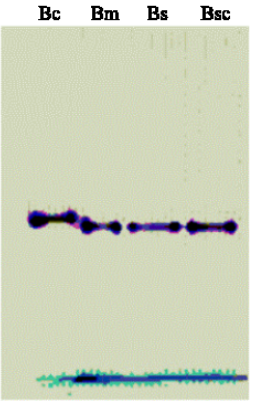


Fig. 2: Polyacrylamide gel electrophoresis of 6PGDH crude enzyme. Bsc, *Bacillus schlegelii*; Bs, *Bacillus sphaericus*; Bm, *Bacillus marimus*; Bc, *Bacillus circulans*

Table 5: Analysis of variance (ANOVA) for effects of temperature, pH and NaCl concentration on intracellular activities of G6PDH and 6PGDH

| | G6PDH | | | | | | 6PGDH | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | One way | | | Two ways | | | One way | | | Two ways | | |
| *Bacillus* strain | T | pH | NaCl | T/pH | T/NaCl | pH/NaCl | T | pH | NaCl | T/pH | T/NaCl | pH/NaCl |
| *B. sphaericus* | NS | <0.05 | <0.05 | NS | NS | <0.05 | NS | <0.05 | NS | NS | NS | NS |
| *B. schlegelii* | <0.05 | <0.05 | NS | NS | NS | NS | <0.05 | <0.05 | NS | <0.05 | NS | NS |
| *B. circulans* | NS | NS | NS | NS | NS | NS | <0.05 | NS | NS | NS | NS | NS |
| *B. marinus* | <0.05 | NS | NS | NS | NS | NS | <0.05 | NS | NS | NS | NS | NS |

NS: Not significant

Table 6: $T_{50\%}$ for G6PDH crude enzyme after incubation at 53°C for different period of time

| *Bacillus* strains | G6PDH $T_{50\%}$ (min) | 6PGDH $T_{50\%}$ (min) |
|---|---|---|
| *B. sphaericus* | 8 | <5 |
| *B. schlegelii* | 212 | 11 |
| *B. circulans* | 17 | 7 |
| *B. marinus* | 28 | 8 |

**Residual activity of G6PDH and 6PGDH crude enzyme:** G6PDH from *Bacillus schlegelii* was the most stable and had $T_{50\%}$ of 212 min, while that from *Bacillus sphaericus* had $T_{50\%}$ of 8 min, G6PDH from *Bacillus circulans* had $T_{50\%}$ of 17 min while that from *Bacillus marinus* had $T_{50\%}$ of 17 min. On the other hand 6PGDH from *Bacillus sphaericus* had $T_{50\%}$ of <5 min, while that *Bacillus schlegelii* had $T_{50\%}$ of 11 min, moreover, 6PGDH from *B. circulans* and *B. marinus* had $T_{50\%}$ of 7 and 8 min, respectively (Table 6).

**Isoenzymes of G6PDH and 6PGDH crude enzyme:** *Bacillus sphaericus* and *B. circulans* has two G6PDH isoenzymes however, *Bacillus schlegelii* and *Bacillus marinus* appeared to have an additional fast moving isoenzymes. 6PGDH for all *Bacillus* strains studied has only one isoenzyme (Fig. 1 and 2).

## DISCUSSION

Growth temperature has been considered as a signal for energy flow through pentose phosphate pathway (Membre *et al*., 2005); G6PDH and 6PGDH activities from all thermotolerant *Bacillus* strains studied were variably sensitive to growth conditions of temperature pH and NaCl; the variation in response to combined culture parameters on both enzymes reflected metabolic flexibility of the four thermotolerant strains (Elena *et al*., 1999). The difference in thermostability between the more stable G6PDH and the less stable 6PGDH from the same species compensates the lower G6PDH and the higher 6PGDH activity found in this study; moreover the alterations in growth conditions may reflect genetic variations for adaptation (Yulia and Alexander, 2001).

Results of combined culture parameters and thermostability in this study may indicate the existence of separate pathways for regulating activities of the two enzymes; one pathway may be related to abundance and/or thermostability of each enzyme ($T_{50\%}$ of as high as 212 min for *Bacillus schlegelii* and as low as 11 for 6PGDH) and other pathways may reflect cultural adaptations for pH and NaCl. The present study had demonstrated that G6PDH and 6PGDH from thermotolerant *Bacillus schlegelii*, *Bacillus sphaericus*, *Bacillus marinus* and *Bacillus circulans* were variably sensitive to growth conditions and so were enzyme activities within the same strain.

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