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Study of Varying Temperature and PH on Bacterial Cell Morphology of Wild Type (Py79) and Sporulation Defective Strains of *Bacillus*

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Abstract: The present study deals with the effect of temperature (37 and 45°C) in combination with varying pH (6, 7, 8, 9) after 24 and 96 h of incubation on cell morphology, staining behavior and growth rate of PY79 (wild type) and sporulation defective mutant strains (5, 19, 96, 99) of bacillus. Strains were isolated from local polluted area around Lahore. All the strains were gram positive spore former aerobic rods. In majority of strains the optimum temperature was 37°C and pH 7. Frequencies of different cell types differ at different temperatures and at varying pH. Cell degeneration and lysis was more in sporulation defective strains (5, 19, 96, 99). Spores which were formed by the bacterial isolates 5, 19, 96 and 99 were different from wild type and were unable to germinate.

Key words: *Bacillus subtilis*, sporulation, temperature, pH, cell morphology

INTRODUCTION

Bacillus subtilis is most extensively studied non pathogenic, aerobic, gram positive saprophytic rods found in soil. It belongs to industrially important group of organisms. There are over 150 genes involved in sporulation and germination that have been mapped on *B. subtilis* map (Dean and Zeigler, 1994). Sadaie and Kada (1985) have described the relationship between the initiation of cell division and initiation of sporulation, temperature, pH, starvation and enzyme action are the environmental factor which effects the cell division in *B. subtilis* (Chan *et al.*, 1998; Helmann *et al.*, 2001; Sabri and Hasnain, 1994, a, b; Kuile, 1994; Schut *et al.*, 1995; Periago, 2002; Sachaik *et al.*, 2004). In *B. subtilis* the non permissive temperatures involved the mutations and defects in cell division (Sato *et al.*, 1984; Sadaie *et al.*, 1991; Yanoun *et al.*, 1993; Butler *et al.*, 1993). pH response is important for growth and survival of bacteria in environment (Errington *et al.*, 2003). It not only affect the morphogenetic processes in bacteria but also regenerates the expression of several genes (Castanie-Cornet and Foster, 2001; Stanick *et al.*, 2002; Tucker *et al.*, 2003). A major reason which may be involved in spore dormancy is the pH in the spore core, which is rather low, 6.3 for *B. cerues* spores, 6.4 for *B. megaterium* and *B. subtilis* was found to be ~ pH (Setlow and Setlow, 1980; Setlow and Johnson, 2002). The objective of the present study was to isolate and characterized bacillus strains and to compare them with wild type PY79.

MATERIALS AND METHODS

The present study was conducted in the Microbiology and Molecular Genetics Research Laboratory, Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore-54590, Pakistan, in the year 2005.

Table 1: Strains used in the study

Strains	Type	Source
PY79	Wild type	Youngman <i>et al.</i> , 1983
5	Spore defective mutant	Microbial and Molecular Genetics Lab.
19	Spore defective mutant	Microbial and Molecular Genetics Lab.
99	Spore defective mutant	Microbial and Molecular Genetics Lab.
96	Spore defective mutant	Microbial and Molecular Genetics Lab.

Bacterial Strains

Bacterial strains used in this study were PY79, 5, 19, 96, 99 (Table 1).

Temperature and pH Range of Bacteria

The most suitable temperature for the growth of bacteria is 37°C at neutral pH 7. Eight test tubes of each bacterial strain were labeled for each temperature (37 and 45°C), pH (6, 7, 8, 9) and bacterial strains were taken in which 5 mL of nutrient broth were poured and then autoclaved. After autoclaving the 10 µL inoculums was given in each test tube from 24 h old fresh culture. The initial bacterial density (fixed at 0.5 optical density at 600 nm) was same for each strain.

Effect of Temperature and pH on Cell Morphology

Bacterial strains were incubated for different times (24, 96) at low different temperatures (37 and 45°C) at various pHs (6, 7, 8, 9). After gram staining the cell shapes and staining behavior was observed.

RESULTS

Gram Staining

All the strains used (PY79, 5, 19, 96, 99) were gram positive spore former rods.

Effect of Temperature and pH on Bacterial Growth

Bacterial isolates 19, 96, 99 preferred pH 7 (at 37 and 45°C) for maximal growth after 24 and 96 h. In strain PY79, after 24 and 96 h at both temperature (37 and 45°C) the optimum bacterial growth is recorded at pH 8.0 except after 96 h at 45°C the maximum growth is observed at pH 7 while in strain 5, after 24 and 96 h at different temperatures (37 and 45°C) at pH 7 there was maximum bacterial growth while after 96 h at temperature 37°C the bacterial growth is maximum at pH 8 (Table 2).

Effect of Temperature and pH on Cell Morphology

Strain-PY79

Alteration in the pH in combination of high temperature affected cell morphology as well as staining behavior after 24 h at 37 and 45°C with the isolated rods some rods were also visible in chains and pairs. Frequency of cells and staining behavior and sporulation of cells and staining behavior and sporulation property was not affected by extremes of pH and at high temperature. At temperature 37°C at pH 6 (~54%) isolated rods, (0.17%) in chains and (0.1%) in pairs were observed, (~45%) spores were also seen. at alkaline pH 9 (37°C) isolated rods were (~39%), (~7%) rods in chains and (~18%) rods in pairs and (~33%) spores were observed. at temperature 37°C rods were decreased from acidic to alkaline pH except at pH 8 (~45%). At temperature 45°C at pH 6 isolated rods (~21%), (~41%) rods in chains and (~18%) rods in pairs were observed. Spores were (~19%). At pH 9 (45%) isolated rods (~36%), (~7%) rods in chains, (~25%) rods in pairs and spores were (~30%). At 45°C the isolated rods were increased with the increase in pH from (6-9), rods in chains were decreased with the increase in pH while rods in pairs (~18-25%) and spores (~19-30%) were increased with the

Table 2: Effect of temperature and pH on bacterial growth of strains PY79, 5, 19, 96 and 99

Strains	Time (h)	Temperature (°C)	pH			
			6	7	8	9
PY79	24	37	0.419	0.555	0.649	0.509
		45	0.271	0.280	0.424	0.272
	96	37	0.763	1.065	1.117	0.833
		45	0.751	0.963	0.914	0.409
5	24	37	0.528	0.595	0.415	0.408
		45	0.419	0.672	0.398	0.287
	96	37	0.797	1.030	1.142	0.934
		45	0.766	1.028	0.706	0.628
19	24	37	0.507	0.596	0.486	0.464
		45	0.372	0.522	0.291	0.212
	96	37	0.583	0.936	0.841	0.733
		45	0.475	0.864	0.294	0.218
96	24	37	0.512	0.693	0.680	0.555
		45	0.436	0.491	0.293	0.222
	96	37	0.436	0.986	0.662	0.612
		45	0.510	0.758	0.646	0.607
99	24	37	0.388	0.510	0.458	0.369
		45	0.261	0.393	0.360	0.264
	96	37	0.806	0.822	0.482	0.256
		45	0.433	0.474	0.443	0.394

increase in pH in comparison of 45°C with 37°C isolated rods were more at 37°C as compared to 45°C. After 96 h of incubation only gram negative isolated rods and spores were observed at all pHs (6, 7, 8, 9) and at both temperatures (37 and 45°C). At pH 6 and 37°C (~30%) isolated rods and (~70%) spores were observed while at pH 9 with the (~22%) isolated rods, some (~5%) rods in pairs and (~73%) spores were observed.

Strain-SD5

At all pH (6, 7, 8, 9) and at both temperatures (37 and 45°C) all the rods were gram variable. After 24 h at temperature 37°C at pH 6 (~56%) isolated rods, (1.12%) in chains and (~25%) in pairs were observed. (~18%) spores were also seen.

At pH 9 (37°C) isolated rods (~52%), (~12%) rods in pairs and (~64%) spores were observed. After 24 h at temperature 45°C at pH 6 isolated rods (~53%), (~1%) rods in chains and (~27%) rods in pairs were observed. spores were (~19%) but these spores were defective in germination. after 24 h at temperature 45°C at pH 9 isolated rods (~53%), (~17%) rods in pairs and spores were (~30%) present. At 45°C rods in chains were increased with the increase in pH except at pH 9 where they were absent while rods in pairs (~16-27%) and spores were increased with the increase in pH. In comparison of 45°C with 37°C isolated rods and rods in chains were more at 37°C as compared to 45°C. Rods in pairs were more at 45°C then 37°C while spores were more at 37°C as compared to 45°C. After 96 h of incubation at pH 6 and 37°C (~34%) isolated rods, (~11%) rods in pairs and (~55%) spores were observed and at pH 9 (~26%) isolated rods, (~18%) rods in pairs and (~56%) spores were observed. At 45°C at pH 6 (~32%) isolated rods, (~105) rods in pairs and (~58%) spores were observed which were defective while at pH 9 (~20%) isolated rods, (~16%) rods in pairs and (~64%) defective spores were observed.

Strain-SD19

After 24 h of incubation at temperature 37°C and pH 6 (~24%) gram positive isolated rods, (~10%) in chains and (~11%) in pairs were observed. (~55%) spores were also seen. At pH 9 (37°C) isolated rods were (~45%), (~18%) rods in pairs and (~37%) spores were observed. at temperature 37°C rods were more at neutral pH then acidic and alkaline pHs. At temperature 45°C at pH 6 isolated

rods (~38%), (~10%) rods in pairs were observed. defective spores (~52%) at above pH and temperature. At pH 9 (45°C) isolated rods (~45%), (~13%) rods in pairs and defective spore were (~42%). At 45°C the isolated rods were more at alkaline pHs then at neutral and acidic pHs while rods in pairs less at extremes of pH and spores were same at all pHs. In comparison of 45°C with 37°C isolated rods were more at 37°C as compared to 45°C while spores were almost same at both temperature (37°C, 45°C) but at 45°C defective spores were observed as compared to 37°C. After 96 h of incubation at pH 6 (37°C), (~25%) isolated rods, (~11%) rods in pairs and (~64%) spores were observed. While at pH 9 with the (~33%) isolated rods, some (~15%) rods in pairs and (~52%) spores were observed.

Strain-SD96

After 24 h of incubation, at temperature 37°C and pH 6 (~27%) isolated rods, (~8%) in pairs were observed. (~65%) spores were also observed. At alkaline pH 9 (37°C) isolated rods were (~5%) and (~95%) spores were seen. At temperature 37°C rods were increased from acidic to neutral pH and then to alkaline except at pH 9 (~5%). At temperature 45°C at pH 6 isolated rods (~14%), (~30%) rods in chains and (~16%) rods in pairs were observed. spores were (~40%) which were defective in germination. At pH 9 (45°C) isolated rods (~27%), (~8%) (rods in chains, (~7%) rods in pairs and defective spores were (~58%). Rods in chains were decreased with the increase in pH while rods in pairs (~6-16%) and spores (~39-7%) increased with the increase in pH. In comparison of 45°C with 37°C isolated rods, rods in chains and rods in pairs were more at 45°C then 37°C while spores were observed more at 45°C. After 96 h of incubation gram negative isolated rods and spores were observed at all pHs (6, 7, 8, 9) and at both temperatures (37 and 45°C). At pH 6 (37°C) (~15%) isolated rods, (~8%) rods in pairs, (~6%) rods in chains and (~71%) spores were observed. While at pH 9 (37°C) (~23%) isolated rods and isolated rods and (~73%) spores were recorded.

Strain-SD99

After 24 h at temperature 37°C and pH 6 (~67%) (isolated rods, (~3%) rods in chains and (~20%) in pairs and (~10%) spores were observed. At alkaline pH 9 (37°C) isolated rods were (~45%), (~12%) rods in chains and (~28%) rods in pairs and spores (~15%) were observed. At temperature 37°C rods were more at acidic and neutral pH while they were less at alkaline pHs. At temperature 45°C at pH 6 isolated rods (~74%), (~14%) rods in pairs were observed. Spores were (~12%) which were defective. at pH 9 (45°C) isolated rods (27%), (195%) rods in chains, (~29%) rods in pairs and spores were (~25%) which were defective. in comparison of 45°C with 37°C isolated rods were less at 37°C as compared to 45°C. Rods in chain and in pairs were more at 45°C than 37°C while spores were more at 37°C as compared to 45°C. After 96 h of incubation gram negative isolated rods, rods in chains, rods in pairs and spores were observed at all pH (6, 7, 8, 9) and at both temperature (37 and 45°C). At pH 6 (37°C) isolated rods were (~29%), rods in pairs (~5%), rods in chains (~1%) and spores were (~65%) observed. While at pH 9 (~23%) isolated rods, some (~55) rods in pairs, rods in chains (~2%) and (~70%) spores were observed.

DISCUSSION

Bacterial growth rate was monitored at 600 nm after 24 and 96 h of incubation at 37 and 45°C with varying pH (6, 7, 8, 9). After 24 and 96 h of incubation the bacterial growth of strains PY79, 5 (except at pH 7, 37 and 45°C), 19, 96 (except at pH 9, 45°C) and 99 (except at pH 9, 45°C) was more at 37°C (at pH 6, 7, 8, 9) as compared to 45°C (Table 3). Sabri and Hasnain (1994a), Ahmed and Sabri (2004) reported that higher temperature and varying pH affected the growth rate of wild type as well as sporulation defective div mutant strains. The growth of sporulation defective *Bacillus* strains after

Table 3: Frequency of different cell types per 100 cells of bacillus strains(PY79, 5, 19, 96, 99) at different pH (6, 7, 8, 9) at two temperatures (37 and 45°C) after incubating for 24 and 96 h on nutrient broth

Bacterial strains	Time (h)	Cell types	pH 6		pH 7		pH 8		pH 9			
			37°C	45°C	37°C	45°C	37°C	45°C	37°C	45°C		
PY79	24	Rods (isolated)	54.92	21.03	42.27	37.61	45.00	36.20	39.21	36.30		
		■ Gram +ve	-	-	-	-	-	-	-	-		
		■ Gram -ve	-	-	-	-	-	-	-	-		
		■ Gram variable	-	-	-	-	-	-	-	-		
		■ Pairs	0.12	18.11	0.18	22.70	5.00	24.01	18.30	25.59		
		■ chains	0.17	41.20	0.09	16.21	2.00	10.16	7.89	7.16		
		Spores	45.00	19.56	42.00	26.48	48.00	29.63	33.98	30.95		
		PY79	96	Rods (isolated)	-	-	-	-	-	-	-	-
				■ Gram +ve	-	-	-	-	-	-	-	-
				■ Gram -ve	30.95	25.92	30.46	30.43	25.88	27.46	22.80	17.57
				■ Gram Variable	-	-	-	-	-	-	-	-
				■ Pairs	-	-	-	-	-	-	4.67	5.45
				■ chains	-	-	-	-	-	-	-	-
Spores	69.05			74.08	69.54	69.57	74.12	72.54	72.53	76.98		
5	24			Rods (isolated)	-	-	48.71	54.95	-	-	-	-
				■ Gram +ve	-	-	-	-	-	-	-	-
				■ Gram -ve	-	-	-	-	-	-	-	-
				■ Gram Variable	56.17	53.11	-	-	54.68	48.26	51.61	53.33
				■ Pairs	24.71	27.08	26.49	23.42	21.87	27.58	12.90	16.66
				■ Chains	1.12	1.04	10.25	2.70	3.12	3.44	-	-
		Spores	18.00	18.77*	14.55	18.93*	20.33	20.72*	64.51	30.01*		
		5	96	Rods (isolated)	-	-	-	-	-	-	-	-
				■ Gram +ve	-	-	-	-	-	-	-	-
				■ Gram -ve	33.70	32.37	32.67	33.06	26.17	24.79	25.21	20.06
				■ Gram Variable	-	-	-	-	-	-	-	-
				■ Pairs	11.32	9.04	14.00	10.75	14.90	11.78	18.14	15.69
				■ Chains	-	-	-	-	-	-	-	-
Spores	54.98			58.59*	53.33	56.19*	58.93	63.43*	56.65	64.25*		
19	24			Rods (isolated)	23.72	-	-	-	-	-	-	-
				■ Gram +ve	-	-	-	-	-	-	-	-
				■ Gram -ve	-	37.22	40.00	36.00	39.00	43.00	45.72	44.71
				■ Gram Variable	-	-	-	-	-	-	-	-
				■ Pairs	11.11	10.46	12.00	15.00	9.00	15.00	17.14	12.94
				■ Chains	9.62	-	-	8.00	-	-	-	-
		Spores	55.55	52.32*	48.00	41.00*	52.00	42.00*	37.14	42.35*		
		19	96	Rods (isolated)	-	-	-	-	-	-	-	-
				■ Gram +ve	-	-	-	-	-	-	-	-
				■ Gram -ve	25.30	29.74	31.00	31.33	34.49	36.69	32.52	26.49
				■ Gram Variable	-	-	-	-	-	-	-	-
				■ Pairs	11.07	10.24	15.27	15.66	11.79	13.38	15.26	14.48
				■ Chains	-	-	-	-	-	-	-	-
Spores	63.63			60.02*	53.73	53.01*	53.72	56.93*	52.22	59.03*		
96	24			Rods (isolated)	27.02	13.68	28.21	-	30.26	51.23	-	-
				■ Gram +ve	-	-	-	-	-	-	-	-
				■ Gram -ve	-	-	-	33.76	-	-	5.00	27.20
				■ Gram Variable	-	-	-	-	-	-	-	-
				■ Pairs	8.12	16.43	8.06	13.54	15.13	16.25	-	6.82
				■ Chains	-	30.13	5.67	4.78	6.48	6.25	-	8.16
		Spores	64.86	39.76*	58.06	47.92*	48.13	26.27*	95.00	57.82*		
		96	96	Rods (isolated)	-	-	-	-	-	-	-	-
				■ Gram +ve	-	-	-	-	-	-	-	-
				■ Gram -ve	14.99	17.31	21.42	25.43	26.71	32.66	24.79	23.35
				■ Gram Variable	-	-	-	-	-	-	-	-
				■ Pairs	7.51	10.33	8.40	8.05	-	-	-	-
				■ chains	6.20	3.64	3.60	2.68	-	-	-	-
Spores	71.30			68.72*	66.58	63.84*	73.29	67.34*	75.21	76.65*		
99	24			Rods (isolated)	-	-	-	56.75	-	52.72	44.73	26.91
				■ Gram +ve	-	-	-	-	-	-	-	-

Table 3: Continued

Bacterial strains	Time (h)	Cell types	pH 6		pH 7		pH 8		pH 9	
			37°C	45°C	37°C	45°C	37°C	45°C	37°C	45°C
96		■ Gram -ve	66.36	74.07	71.42	-	44.61	-	-	-
		■ Gram Variable	-	-	-	-	-	-	-	-
		■ Pairs	20.90	14.33	16.80	32.43	18.46	18.18	27.63	29.23
		■ Chains	3.63	-	6.72	-	12.30	10.27	11.84	19.48
		Spores	9.11	11.6 [*]	5.06	10.82 [*]	24.63	18.83 [*]	15.80	24.38 [*]
		Rods (isolated)	-	-	-	-	-	-	-	-
		■ Gram +ve								
		■ Gram-ve	28.48	21.19	31.90	30.72	32.22	25.72	23.15	26.21
		■ Gram Variable	-	-	-	-	-	-	-	-
		■ Pairs	5.06	8.55	6.74	6.77	9.19	7.27	5.38	3.98
		■ Chains	1.26	1.60	1.84	1.04	2.29	2.54	1.79	0.78
		Spores	65.20	68.66 [*]	59.82	61.47 [*]	56.30	64.47 [*]	69.68	69.03 [*]

* Defective spores

96 h of incubation was less as compared to PY79. The growth rate and sporulation processes are interrelated (Sabri and Hasnain, 1994b). Ahmed and Sabri (2004) also reported that div mutants have low growth rate as compared to PY79. In general growth is affected by extreme of temperature and pH (Bakermans and Nealson, 2004; Richard and Foster, 2004; Yuk and Mashall, 2004; Nascimento *et al.*, 2004). In general at acidic pH the bacterial growth was less as compared to neutral pH. In alkalophiles bacteria the heat treatment at lower pH destroy heat resistance mechanisms of spores and induce low survival rate, optical density decrease and DPA release (Kudo and Horikoshi, 1983). Extreme temperature and pH had adverse effects on cell morphology and cell lysis (Sabri and Hasnain, 1994 a, b). Lysis may be either due to affects of temperatures on biochemical reaction, membrane proteins, membrane lipids and septation (Chaloupka, 1985; Kubitschek, 1990; Intriago and FloodGate, 1991) or due to change in wall components, peptidoglycan and defect in cell envelope (Waximan and Strominger, 1983; Shohayeb and Chopra, 1987).

Strain PY79 after 24 h of incubation shows optimum growth at pH 8 (both at 37 and 45°C). After 24 h of incubation strain 5 showed optimum growth at pH 7 (both at 37 and 45°C). For bacterial strain 19, 96 and 99, pH 7 was favourable for maximal growth at both temperatures (37 and 45°C). pH could also affect the diversity of bacteria (Iqbal, 2000). Cell wall component are important in determining pH tolerance in the medium (Aono *et al.*, 1993).

After 24 h at all pH (6, 7, 8, 9) at both temperatures (37 and 45°C), the maximum number of isolated rods were observed in PY79 (54.92 % at pH 6, 37°C), 5 (56.17 % at pH 6, 37°C), 19 (45.72 % at pH 9, 37°C), 96 (51.23 % at pH 8, 45°C) and 99 (74.07 % at pH 6, 45°C). Rods in pairs were more in PY79 (24.01 % at pH 8, 45°C), 5 (27.08 % at pH 6, 45°C), 19 (17.14 % at pH 9, 37°C), 96 (16.43 % at pH 6, 45°C) and 99 (32.43 % at pH 7, 45°C). Rods in chains were also present in strains PY79 (24.59 % at pH 9, 45°C), 5 (10.25 % at pH 7, 45°C), 19 (9.62 % at pH 6, 37°C), 96 (30.13 % at pH 6, 45°C) and 99 (19.48 % at pH 9, 45°C). Cell growth is influenced by change in temperature (Leroy and Devuyt, 1999). Increase in pH cause variation in shape and size of cell (Lowe *et al.*, 1989). So from above discussion it is clear that cell degeneration and lysis was more in sporulation defective strains (5, 19, 96, 99). Spores, which were formed by the bacterial isolates 5, 19, 96 and 99, were different from wild type. They were unable to germinate.

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