ISSN No.: 2454- 2024 (online)

International Journal of Technical Research & Science

EFFECT OF POLLUTED ENVIRONMENT ON GERMINATION OF BACILLUS SPORES

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Abstract-The environment is extremely variable, since temperature, pH and osmolarity change rapidly and alter morphogenetic pathway of bacteria. In addition the supply of nutrients usually undergoes drastic fluctuations. Those conditions cause defects/alteration on sporulation and germination of these spores by creating changes in genetic makeup. The work being discussed here address sporulation and germination of Bacillus spores, isolated from polluted and saline areas (soils, plants). Initially 20 spore forming bacterial strains were isolated All the germination defective spore forming strains belonged to family Bacillacea.

Keywords: Bacillus, Spore, Polluted, Isolation

1. INTRODUCTION

Dormant spores are a survival stage of bacteria which are created as a response to the stress of starvation. The developmental pathway leading from a vegetatively growing bacterial cell to a spore is triggered by depletion from the bacterium's local environment of a readily metabolized form of carbon, nitrogen or phosphate [1, 2] . Spore formation thus represents a strategy by which the bacterial cell escapes temporally from nutritionally unfavorable local conditions via dormancy.

In the terrestrial (soil) environment where many spore forming bacteria are obtained such potentially lethal extreme conditions can include cycles of heat and cold, including freezing thawing, physical abrasion, extreme desiccation, exposure to corrosive chemicals, attack by other organisms and their extracellular degradative enzymes and prolonged exposure to solar radiation [3, 4]. These lethal conditions can affect spore formation in many ways to unable them to germinate by creating mutation in genetic or any morphogenetic pathway. Hasnain and Sabri [5] demonstrated that spores of div mutant of B. subtilis are defective in germination (gerB) pathway. It has been observed that bacteria from stress environment (saline, polluted with industrial effluents) could produce spores, but these spores were germination defective. In present chapter isolation of germination defective bacterial strains from polluted and saline stressed soils as well as different plants is being described.

2. MATERIAL AND METHODS

2.1 Field Survey

Samples were collected from saline, industrial and polluted area around (Narang Mandi, Muridkey, Kasur, Kala shah Kaku and Rai Wind) and from Lahore (Muslim Town, Zafar Ali road), different plant species which were grown in that area were also collected along with soil and water samples, for isolation of germination defective spore forming bacteria. Ten soil samples were selected and analyzed.

2.2 Isolation

2.2.1 From Plant

For isolation of bacteria from histoplane roots and leaves of plants were washed thoroughly and sterilized with 5% sodium hypochloride for five minutes followed by repeated gentle washings with glass distilled water. Sterilized plant parts were crushed in a small amount of sterilized glass distilled water (2-3 ml) transferred in 50 ml flasks. And volume was made to 10 ml. After 2 hours, plated on Schaeffer's media containing plates.

For isolation of bacteria from rhizoplane, roots and leaves, plant parts were rinsed gently first with tap H_2O then with glass distilled water and blot dried. Pieces of these parts were placed on plates containing Schaeffer's media.

2.2.2 From Soil

Polluted and Saline soil samples and soil associated with roots of plants were taken. Suspensions was made by adding 10 g of sieved soil in 100 ml of glass distilled water. Soil suspensions kept for 6-8 hours, dilutions of which were plated on plates containing Schaeffer's media were incubated at 37°C for 96 hours. From bacterial growth, dark brown and light brown coloured colonies were picked and purified for further studies.

2.3 Spore Staining

To check the ability of spore formation, 0.76% malachite green is used on smear providing steam. Gram safranine applied as secondary stain.

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2.4 Medium to Check Germination Potential

Strains were grown on tryptose blood base agar (TBBA) to check germination potential of bacterial spores. Pink color appearance showed positive result. The isolates which were unable to show pink coloration were unable to germinate [6].

3. RESULT

Different plants, soil and water samples (saline and polluted) were collected from the area within (Muslim town, Zafar Ali road, Raiwind road) and around (Moridke, Kala Shah Kaku, Kasur, Narang Mandi) Lahore for isolation of spore forming bacteria. Bacteria from rhizosphere, rhizoplane, histoplane and philloplane of different plants (Eucalyptus alba, Cana sp., Jatropha alba, Bumeu dentatus, Eucalyptus prostrata, Lycopersicon esculentum, Cynodon dectylon, Chenopodium album, Oryza sativa, Penicum antidolata, Chenopodium sp., Acacia nilotica, Convolvulus arvensis), soils and water samples of polluted and saline area were isolated on Schaeffer's medium. Apparently different and brown to light brown coloured colonies were selected for further studies. From 253 isolates, 111 were from soil samples, 70 were from water samples, 19 from rhizosphere of plants, 21 from rhizoplane, 16 from philloplane and 16 from histoplane of roots. Symbols of isolates along with sources are listed in table -3.1. Among the isolates, which gave positive result on Schaeffer's

Table-3.1 Soil Samples Used for Bacterial Isolations

S. No	SAMPLE LOCATION	SAMPLE NO.	SAMPLE CONDITION	ISOLATES OBTAINED FROM	
				Soil	Water
1	Moridke	Sample-2	Industrial area	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	04, 05, 06
2	Kala Shah Kaku	Sample-3	Polluted area	11, 12, 13, 14, 15, 16, 17, 18, 19	20

medium majority appeared brown, excluding 04, 05, 06, 1, 2, 3, 4, 5, 14, 16, 18, 19, which showed light brown appearance. All the isolates were resistant to $NiSO_4$, $CoCl_2$ (100 μgml^{-1}) and 0.5 M NaCl therefore these were further checked for spore formation on Schaeffer's media supplemented with stresses of $NiSO_4$, $CoCl_2$ (100 μgml^{-1}) and 0.5 M NaCl and behaviour of strains exhibiting growth and sporulation under stress were recorded. With $NiSO_4$ (100 μgml^{-1}) isolates gave brown appearance excluding 04, 05, 06, 1, 2, 3, 4, 5, 14, 16, 18, 19, bacterial strains, which had no change (light brown/offwhite) in appearance. With $CoCl_2$ (100 μgml^{-1}) isolates gave brown appearance With 0.5 M NaCl brown coloured colonies of strains 04, 05, 06, 1, 2, 3, 4, 5, 14, 16, 18, 19, produced no change (light brown/offwhite) in appearance on 0.5M NaCl containing Schaeffer's medium(Table-3.2).

Table-3.2 Appearance of Isolates on Schaeffer's Media With and Without Stress

Isolates	OBSERVATIONS ON SHAEFFER'S MEDIA			
	0	$NiSO_4$ (100μgml^{-})	$CoCl_2$ (100μgml^{-})	NaCl 0.5M
04	W	-	+	-
05	W	-	+	-
06	W	-	+	-
1	w	-	+	-
2	w	-	+	-
3	w	-	+	-
4	w	-	+	-
5	w	-	+	-
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+

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11	+	+	+	+
12	+	+	+	+
13	+	+	+	+
14	w	-	+	-
15	+	+	+	+
16	w	-	+	-
17	+	+	+	+
18	w	-	+	-
19	w	-	+	-
20	+	+	+	+

+ Brown w Offwhite/Light brown - /no change in colour

3.1 Confirmation of Spore Formation by Staining

Spore staining was performed to test the spore forming ability of isolates. Smears for spore staining were prepared from 72 hours incubated cultures on Schaeffer's medium and nutrient agar medium plates. Both with and without stress media were used. Spores of all isolates cultured on Schaeffer's and nutrient agar media (with and without relative stress on which they exhibited brown appearance) were stained by malachite green which showed the confirmation of spore forming ability of all isolates (Table-3.3).

Table-3.3 Spore Staining After Smear Preparation from 72 hours Old Culture on Schaeffer's and Nutrient Media With and Without Relevant Stresses (at Which Strains Isolated).

Isolates	**SHAEFFER'S MEDIUM**				**NUTRIENT AGAR**			
	0	**$NiSO_4$ (100)**	**$CoCl_2$ (100)**	**NaCl 0.5M**	**0**	**$NiSO_4$ (100)**	**$CoCl_2$ (100)**	**NaCl 0.5M**
04	+		+		+		+	
05	+		+		+		+	
06	+		+		+		+	
1	+		+		+		+	
2	+		+		+		+	
3	+		+		+		+	
4	+		+		+		+	
5	+		+		+		+	
6	+		+		+		+	
7	+		+		+		+	
8	+		+		+		+	
9	+		+		+		+	
10	+		+		+		+	
11	+		+		+		+	
12	+		+		+		+	
13	+		+		+		+	
14	+		+		+		+	
15	+		+		+		+	
16	+		+		+		+	
17	+		+		+		+	
18	+		+		+		+	
19	+		+		+		+	
20	+		+		+		+	

+ Spores stained, - Spores not stained, Blank column= staining not checked at that stress, stress=µgml^{-} units,

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Spores were detected in all strains both under stress as well as without stress culture conditions.

3.2 Spore Germination Potential

3.2.1 At Different Stresses

Tetrazolium overlay test was performed to check the germination potential of spores of isolates. As these strains were isolated from polluted (water, soil) and saline habitat, germination potential was checked on metal or salt stress media. Isolates were stabbed on tetrazolium blood base agar (TBB) (with and without respective stress) and incubated at 37^{o}C for 16 hours. After incubation stabbed culture plates were kept at 90^{o}C for 20 minutes to kill the vegetative cells. Plates were overlayed with soft agar containing tetrazolium, after solidifying agar plates were incubated at 37^{o}C. Change in colour was noted after 1 and 24 hours of incubation. No pink colour developed in any isolate during one hour incubation either with stress or without stress spores, while within 24 hours incubation in TBB pink colour developed in spores of majority of strains both with and without stress excluding 04, 05, 06, 1, 2, 3, 4, 5, 14, 16, 18, 19, (with $CoCl_2$ stress), where red color did not develop (Table-3.4).

Table-3.4 Observation of Germination Response of Bacterial Spores With Tetrazolium Overlay Method at Relevant Stresses (μgml^{-} at Which Isolated) As Well As With Different Incubation Periods (72 and 96 hours)on TBB Agar

Isolates	AT DIFFERENT STRESSESS (16 hours incubated culture)								WITH CULTURES OF DIFFERENT INCUBATION PERIOD(without stress)					
	After 1 hour				After 24 hour				72			96		
	0	$NiSO_4$ (100)	$CoCl_2$ (100)	NaCl 0.5M	0	$NiSO_4$ (100)	$CoCl_2$ (100)	NaCl 0.5M	OBSERVATION AFTER			OBSERVATION AFTER		
									2 hour	4 hour	6 hour	2 hour	4 hour	6 hour
04	-		-		+		-		-	-	-	-	-	-
05	-		-		+		-		-	-	-	-	-	-
06	-		-		+		-		-	-	-	-	-	-
1	-		-		+		-		-	-	-	-	-	-
2	-		-		+		-		-	-	-	-	-	-
3	-		-		+		-		-	-	-	-	-	-
4	-		-		+		-		-	-	-	-	-	-
5	-		-		+		-		-	-	-	-	-	-
6	-		-		+		+		+	+	+	+	+	+
7	-		-		+		+		+	+	+	+	+	+
8	-		-		+		+		+	+	+	+	+	+
9	-		-		+		+		+	+	+	+	+	+
10	-		-		+		+		+	+	+	+	+	+
11	-		-		+		+		+	+	+	+	+	+
12	-		-		+		+		+	+	+	+	+	+
13	-		-		+		+		+	+	+	+	+	+
14	-		-		+		-		-	-	-	-	-	-
15	-		-		+		+		+	+	+	+	+	+
16	-		-		+		-		-	-	-	-	-	-
17	-		-		+		+		+	+	+	+	+	+
18	-		-		+		-		-	-	-	-	-	-
19	-		-		+		-		-	-	-	-	-	-
20	-		-		+		+		+	+	+	+	+	+

+ Pink color with tetrazolium dye - No pink color with tetrazolium dye Blank column=not checked at that stress

3.2.3 Incubation Period

Germination potential of spores of isolates, which were incubated on tetrazolium blood base agar for 72 hours and 96 hours, were checked by overlaying soft agar containing tetrazolium dye after incubating at 90^{o}C for 20 minutes.

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The plates were incubated at 37°C and observations were recorded after 2, 4 and 6 hours. All isolates showed spore germination response excluding 04, 05, 06, 1, 2, 3, 4, 5, 14, 16, 18, 19, which showed no colour development after 2, 4 and 6 hours at 37°C (Table-4). There was no difference in germination response of spores obtained after incubation of 72 and 96 hours.

4. DISCUSSION

The main aim was to isolated germination defective spore forming bacterial strains which could be further utilized to explore the kind of mutation which affected their germination pathway. Spore forming strains were isolated from the polluted and saline stressed environments. Soil, water and plants were used to isolate such bacteria. Spore forming bacteria are rather wide spread within the low G+C subdivision of the gram positive bacteria and represent inhabitants of diverse habitats, such as aerobic heterotrophs (Bacillus and Sporosarcina spp.), halophiles (Sporosarcina halophila), microaerophilic lactate fermenters (Sporolactobacillus spp.), anaerobes (Clostridium and Anaerobacter spp.), sulfate reducers (Desulfotomaculum spp.) and even phototrophs (Heliobacterium and Heliophilum spp.). Despite the diversity exhibited by spore forming bacterial species, the spore formers about which most detailed information gleaned are common rod shaped soil inhabitants belonging to the genera Bacillus and Clostridium, and among them most work has concentrated upon the descendants of a single strain of Bacillus subtilis called strain 168. Bacillus subtilis has long been regarded as a model gram positive organism for various genetic and physiological studies. Because of resistance and longevity bacterial spores have also been studied as possible candidate for transfer of life between the planets as a result of impact processes. Studies of the resistance of Bacillus and Clostridium spores to a variety of treatments in the laboratory have identified a number of factors important in determining the level of spore resistance. These factors influenced by genetic makeup of the sporulating species, the precise sporulation conditions. Bacillus subtilis decide to sporulate and then germination of spore by integrating a wide range of environmental and physiological signals, culminating in the activation of a key transcriptional regulatory proteins. Any kind of mutation or change in environmental conditions during these process is ended with the formation of such spores which are unable to germinate or they might also required some specific germination initiating conditions and factors. But at this moment it is difficult to comment on specific requirements for spore germination.

The isolates from different environmental conditions were checked for spore forming ability and later on germinating property of those spores was analyzed. On Schaeffer's media (without stress) and spore staining all the isolates gave positive result i.e. brown colouration of Schaeffer's media and green stained spores but on Schaeffer's media with different stresses ($NiSO_4$, $CoCl_2$, NaCl) variable results observed with $NiSO_4$ strain No. 04, 05, 06, 1, 2, 3, 4, 5, 14, 16, 18, 19, gave negative result or no change in colour of growth on Schaeffer's media was observed.

Spore germination property was analyzed by tetrazolium overlay method with and without different stresses and with varying incubation periods. The stresses used to check tetrozolium overlay results were the same on which respective strains were isolated and gave brown coloured growth on Schaeffer's medium. All spore formers (16 hours old cultures) gave pink coloured appearance at without stress and variable results with stresses after 24 hours incubation. With $CoCl_2$ stress strain No. 04, 05, 06, 1, 2, 3, 4, 5, 14, 16, 18 gave no pink coloured appearance. These strains even with 72 hours and 96 hours old cultures gave no evidence of spore germination.

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