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SHORT COMMUNICATION

Sporulation and regeneration efficiency of phosphobacteria (*Bacillus megaterium* var *phosphaticum*)

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Abstract Sporulation in *Bacillus megaterium* var *phosphaticum* (PB - 1) was induced using modified nutrient media. This modified medium induced sporulation within 36 h. After spore induction the spores were kept under refrigerated (5°C) and room temperature (32°C) for five months and survival of spores was studied at 15 days intervals by plating them in nutrient agar medium. It was observed that there was not much variation in the storage temperature (5°C & 32°C). The spore cells of *Bacillus megaterium* var *phosphaticum* (PB – 1) were observed up to five months of storage under refrigerated (5°C) and room temperature (32°C). Regeneration of spore cells into vegetative cells was studied in tap water, rice gruel, nutrient broth, sterile lignite and sterile water at different concentrations of spore inoculum. The multiplication of sporulated *Bacillus megaterium* var *phosphaticum* culture was fast and reached its maximum (29.5 x 10^8 cfu ml^{-1}) in nutrient broth containing 5 per cent inoculum level.

Keywords *Bacillus megaterium* · Spores · Regeneration efficiency

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Introduction

The capacity to form endospore endows *Bacillus* sp. with the ability to survive in adverse environmental conditions. Bacterial sporulation is a sequence of integrated biochemical reactions, which are independent of its vegetative growth and may be interrupted at certain susceptible stages[3]. Sporulation of *B. thermoacidurans* in protease peptone agar was increased by the addition of Li^{++}, Mg^{++}, Ca^{++}, Fe^{++}, Zn^{++} or Mn^{++} [14]. Sporulation was rapid and remained at high level with progressive reductions were present in the NO_3-, $SO4^{--}$, Cl^-, Na^+, $Fe^{++(+)}$, and Zn^{++} [5]. Sporulating bacteria possess more adaptive power to establish itself in a new habitat[2]. A wide range of physical and chemical effectors can trigger germination of bacterial spores. However, the success of the survival strategy of spores depends on the presence of an efficient mechanism for returning to the vegetative state under favorable conditions. Germination is the process by which dormant bacterial spores resume metabolism and growth and it is generally triggered by the presence of nutrients, including amino acids, sugars and nucleosides. After germination of spores, the spore start to lose its spore specific properties in a sequential fashion for example, heat resistance and refractility[4].

Bacterial sporulation was induced by inoculating the *Bacillus megaterium* var *phosphaticum* (PB-1) culture in supplementary nutrient medium[15] containing (g L^{-1}) : nutrient broth, 13.0; glucose, 1.00; $MgSO_4$, 0.25; KCl, 1.00; $CaCl_2$. $2H_2O$, 0.15; $MnSO_4$. $4H_2O$, 3.96; $FeSO_4$. $7H_2O$, 278; pH 7.0, and incubated at 32°C, 500 rpm for about 24 h in an arbitrary shaker. Phosphobacterial broth culture having a cell load of 10^9 cfu ml^{-1} was inoculated into sterile supplementary nutrient medium in polybags aseptically using sterile plastic syringe fitted with hypodermic needle. The inoculant

Table 1. Survival of sporulated culture of *Bacillus* during storage.

Days of storage	Storage condition (Population cfu ml^{-1})	
	Room temperature(32°C)	Refrigerated temperature(5°C)
0th day	16.0 x 10^{11} (12.2041)	16.0 x 10^{11} (12.2041)
15th day	8.3 x 10^{11} (11.9191)	11.3 x 10^{11} (11.0530)
30th day	5.3 x 10^{11} (11.7242)	8.0 x 10^{11} (11.9031)
45th day	3.6 x 10^{11} (11.5563)	5.6 x 10^{11} (11.7481)
60th day	2.0 x 10^{11} (11.3010)	2.3 x 10^{11} (11.3617)
75th day	0.6 x 10^{10} (9.7782)	1.6 x 10^{10} (10.2041)
90th day	2.6 x 10^{9} (9.4149)	3.6 x 10^{9} (9.5563)
105th day	2.6 x 10^{8} (8.4149)	3.4 x 10^{8} (8.5314)
120th day	0.6 x 10^{8} (8.7781)	1.6 x 10^{8} (8.2041)
135th day	4.6 x 10^{7} (7.6627)	5.3 x 10^{7} (7.7242)
150th day	6.9 x 10^{6} (6.8388)	9.8 x 10^{6} (6.9912)
	C.D (P=0.05)	
Storage condition (S)	*0.0283*	
Days (D)	*0.0664*	
S x D	*0.0940*	

(Values in parenthesis indicate Log transformed values).

Table 2. Regeneration efficiency of sporulated *Bacillus* Culture.

Treatments	Population (cfu ml^{-1})				
	0th h	3rd h	6th h	9th h	12th h
Tap water					
1% inoculum	15.0 x 10^{2} (3.1761)	10.5 x 10^{4} (5.0211)	14.0 x 10^{6} (7.1461)	15.0 x 10^{7} (8.1761)	8.5 x 10^{7} (7.9294)
2% inoculum	20.5 x 10^{2} (3.3117)	15.5 x 10^{4} (5.1903)	19 x 10^{6} (7.2787)	19 x 10^{7} (8.2787)	12.0 x 10^{7} (8.0791)
5% inoculum	23.5 x 10^{2} (3.3710)	20 x 10^{4} (5.3010)	25 x 10^{6} (7.3979)	25.5 x 10^{7} (8.4065)	20 x 10^{7} (8.3010)
Rice gruel					
1% inoculum	18.0 x 10^{2} (3.2552)	15.5 x 10^{4} (5.1903)	20.0 x 10^{6} (7.3010)	15.0 x 10^{7} (8.1761)	9.0 x 10^{8} (7.9542)
2% inoculum	23.0 x 10^{2} (3.3617)	21.5 x 10^{4} (5.3324)	25.5 x 10^{6} (7.4065)	20.0 x 10^{7} (8.3010)	16.5 x 10^{7} (8.2174)
5% inoculum	28.5 x 10^{2} (3.4548)	30.0 x 10^{4} (5.4771)	30.0 x 10^{6} (7.3010)	28.0 x 10^{7} (8.4471)	25.5 x 10^{7} (8.4065)
Nutrient Broth					
1% inoculum	20.0 x 10^{2} (3.3010)	20.0 x 10^{4} (5.3010)	25.0 x 10^{6} (7.3979)	20.0 x 10^{7} (8.3010)	22.5 x 10^{8} (9.3521)
2% inoculum	25.0 x 10^{2} (3.3979)	28.0 x 10^{4} (5.4471)	30.0 x 10^{6} (7.4771)	25.0 x 10^{7} (8.3979)	26.5 x 10^{8} (9.4232)
5% inoculum	30.0 x 10^{2} (3.4771)	35.0 x 10^{4} (5.5441)	32.0 x 10^{6} (7.5051)	30.0 x 10^{7} (8.4771)	29.5 x 10^{8} (9.4698)
Sterile Lignite					
1% inoculum	15.0 x 10^{2} (3.1761)	17.0 x 10^{4} (5.2304)	15.0 x 10^{6} (7.1761)	17.5 x 10^{7} (8.2430)	10^{9} (10.0000)
2% inoculum	15.5 x 10^{2} (3.1903)	20.5 x 10^{4} (5.3117)	20.5 x 10^{6} (7.3117)	18.5 x 10^{7} (8.2671)	13.0 x 10^{8} (9.1139)
5% inoculum	22.5 x 10^{2} (3.3521)	25.5 x 10^{4} (5.4065)	24.5 x 10^{6} (7.3891)	26.0 x 10^{7} (8.4149)	17.0 x 10^{8} (9.2304)
Sterile water					
1% inoculum	15.0 x 10^{2} (3.1761)	22.5 x 10^{4} (5.3521)	18.0 x 10^{6} (7.2552)	18.0 x 10^{7} (8.2552)	12.0 x 10^{8} (9.0791)
2% inoculum	17.0 x 10^{2} (3.2304)	25.5 x 10^{4} (5.4065)	22.5 x 10^{6} (7.3521)	19.0 x 10^{7} (8.2787)	13.0 x 10^{8} (9.1139)
5% inoculum	25.0 x 10^{2} (3.3979)	28.5 x 10^{4} (5.4548)	25.5 x 10^{6} (5.4065)	24.5 x 10^{7} (8.3891)	18.0 x 10^{8} (9.2552)
	Period (P)		Treatments (T)		P x T
C.D (P=0.05)	*0.2650*		*0.4590*		*1.0280*

(Values in parenthesis indicate Log transformed values).

packets prepared were stored at refrigerated temperature (5°C) and room temperature (32°C). Bacterial population was estimated by pour plate technique[1]. The bacterial population of the culture was estimated at every 15 days interval up to 6 months period. Regeneration of *Bacillus* sp. spores was examined in tap water, sterile water, rice gruel, nutrient broth and lignite carrier. *Bacillus* spore inoculum of 8.7 x 10^7 cfu ml^{-1} was added into tap water, sterile water, rice gruel, nutrient broth and lignite carrier at one per cent, two per cent and at five per cent levels and incubated at 32°C temperature. Except the lignite carrier treatment, others were given with shaking @ 120 rpm for 12 h in an arbitrary shaker. The regeneration and multiplication of cells were examined by pour plate technique[1] at 3 h interval upto12 h.

Bacterial sporulation by certain gram positive bacteria such as *Bacillus* is resistant to serve physical and environmental conditions. Survival of sporulated cultures in supplementary nutrient medium was observed and recorded in Table 1. In this study, supplementary nutrient medium was used to induce sporulation of *Bacillus megaterium* and maximum sporulation was observed at 36 h. Similar observation has been reported for supplementary nutrient medium and the process of sporulation was almost over at the start of sporulation in other media such as modified sporulation media and sporulation media[16]. The results indicated that the survival of phosphobacteria *Bacillus megaterium* var *phosphaticum* (PB-1) under refrigerated (5°C) and room temperatures (32°C) was the same. Bacterial spores are dormant structures and they are not affected by the environmental conditions, there is not much variation in the survival of cells under room and refrigerated temperatures. Moreover these endospores are resistant to desiccation, antibiotics, disinfectants and other chemicals[12]. So bacterial spores remained as such and there is no difference in the survival of cells. The influence of storage temperature on the survival of bacteria depends on the purity of the culture and moisture loss during storage[13]

The results of regeneration of spores of *Bacillus megaterium* var *phosphaticum* (PB-1) are presented in Table 2. Among the inoculum densities, five percent inoculum level was found to be optimum when compared to one and two percent levels. The regeneration of spore into vegetative cells were slow up to 3rd h and it exhibited a positive up trend after 6th h and invariably all the treatments (except tap water and rice gruel) the population reached up to 10^8 cfu ml^{-1}. *Bacillus mycoides* germinated in the presence of glucose and acetate[8]. Different sugars trigger germination through different metabolic pathways. Glucose and L-asparagine were sufficient for complete germination of *B.subtilis* spores[6]. In this study, although regeneration was comparable in all the treatments, nutrient broth containing glucose and sterile lignite had triggered faster germination compared to other treatments. Germination of *Bacillus* spores was noticed higher in nutrient broth followed by rice gruel and sterile lignite as it had nutrients, which helped the bacterial spores to germinate. Similar observation was observed for phosphobacteria in nutrient broth[15] and its germination was not triggered in water alone[11]. Germination was obtained in strains of *B.megaterium* strains when glucose was added[7] in to the medium. Many gram-negative bacteria survived well in Phosphate Buffered Saline (PBS) for at least 30 weeks[10]. Post germinative development of the spore into a dividing vegetative cell requires sources of phosphorus, sulfur and nitrogen as well as a metabolizable carbon source, such as glucose[9].

It is concluded that neither a carbon source nor nitrogen source is specifically required for germination of spores to vegetative cells. By induction of sporulation can survive phosphobacteria for about 5 to 6 months. By supplying external carbon or nitrogen sources, the germination of bacterial spores can be triggered which can be used for specific inoculation progammes.

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