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EVALUATION OF SHELF LIFE OF *BACILLUS SUBTILIS*

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ABSTRACT

Rhizosphere bacteria are excellent agents to control soil-borne plant pathogens. Bacillus subtilis *is a nonpathogenic bacterium that lives in soil.* B. subtilis *cells are capable of forming dormant spores that are resistant to extreme conditions and thus can be easily formulated and stored.* B. subtilis *also produces a variety of biologically active compounds with a broad spectrum of activities toward phytopathogens and that are able to induce host systemic resistance. The major bottleneck of biological control agent is the shelf life and inconsistent performance. In order to overcome this problem, the present study was conducted to improve the shelf life of liquid formulation of B. subtilis strain BG42. Nutrient amendments like glycerol at 10mM, trahalose at 5mM, Sorbitol at 5mM, glycine at 10mM, and the stickers like polyvinylpyrollidone (PVP) at 2 per cent, starch 2 per cent, liquid paraffin 2 per cent and gum acacia 2 per cent and combinations of glycerol 10mM and starch 2 per cent were used. Based on the results, addition of glycerol along with starch is considered as best amendments for enhancing shelf life of B. subtilis strain BG42 than other treatments.*

Key words**:** *Bacillus subtilis,* shelf life, glycerol, starch

INTRODUCTION

Bacillus subtilis cells are rod-shaped, gram-positive bacteria that are naturally found in soil and vegetation. It grows in the mesophilic temperature range. The optimal temperature is 25-35 degrees Celsius. *B. subtilis* is an endospore forming bacteria and the endospore that it forms allows it to withstand extreme temperatures as well as dry environments. It is considered and obligate aerobe, but can also function anaerobicly when in the presence of nitrates or glucose. *B. subtilis* is an adept rhizobacterium and has gained global attention as a biopesticide (Edgecomb and Manker, 2006) for control of several plant diseases. The potential of this biocontrol bacterium has been reported to be effective against plant parasitic nematodes (Siddiqui and Ehteshamul, 2001) and other soil borne pathogens (Asaka and Shoda, 1996; Edgecomb and Manker, 2006). Because they are efficient root colonizers, have multiple modes of action and promising ability to sporulate (Kloepper *et al.,*, 2004). It is also produces several ribosomal and non-ribosomal peptides that act as antibiotics such as iturins, surfactins and zwittermycin (Asaka and Shoda, 1996; Stein, 2005) and it secretes also hydrolytic enzymes, i.e. protease, glucanase (Cazorla *et al.,*, 2007), chitinase (Manjula *et al.,*, 2004), lipase (Detry *et al.,*, 2006) and amylase (Konsoula and Liakopoulou Kyriakides, 2006). The composition and concentration of substrate are important in improving the performance of *B. subtilis* antagonists. In the present study, experiments have been conducted to improve the shelf life of microbial inoculants of *Bacillus subtilis* by the addition of suitable amendments**.**

MATERIALS AND METHODS

Isolation of *B. subtilis* strain BG42 from Rhizosphere of Gerbera

Ten *Bacillus* spp. isolates were obtained from rhizosphere region of gerbera grown in different districts of Tamil Nadu comprising of Coimbatore, The Nilgiris, Salem and Krishnagiri. Bioefficacy of *Bacillus* spp. isolates was assayed against root knot nematode by hatching and mortality tests (Shahnaz Dawar *et al.,*, 2008). Among the ten isolates screened, the highest inhibition in egg hatching and highest per cent mortality of *M. incognita* juveniles was observed in *Bacillus* isolate BG42. The partial 16SrDNA sequences of the isolated strains BG42 showed 99% per cent similarity to *B. subtilis* isolate and were deposited in the GenBank under accession numbers of KM588210 (Manju and Subramanian, 2015).

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Preparation of liquid based formulation of *B. subtilis* strain BG42 with different amendments for enhancing shelf life

For developing enhanced shelf life of *B. subtilis* strain BG42, nutrient broth was prepared in combination with different nutrient amendments and stickers to increase the survival of *B. subtilis* strain BG42 cells. The nutrient amendments *viz*., glycerol at 10mM, trehalose at 5mM, Sorbitol at 5mM, glycine at 10mM, and the stickers *viz*., polyvinylpyrollidone (PVP) at 2 per cent, starch 2 per cent, liquid paraffin 2 per cent and gum acacia 2 per cent and combinations of glycerol 10mM and starch 2 per cent were added to 1 litre of nutrient broth as per the method described by Tamilvendan and Thagaraju (2006). One ml of log phase culture was inoculated into each broth. An uninoculated control was also maintained. The flasks were incubated at room temperature. The broth cultures were analyzed for viable population at monthly intervals up to lag phase.

Enumeration of viable cell population

Nutrient Agar medium was prepared, sterilized and 10 ml of the medium was poured into sterile plates. The plates were incubated at room temperature for 48 h. Eight equal sectors on the outside bottom of the petridish were readily marked. Four sectors were used for replications of one dilution and four for another, allowing two dilutions per plate. Serial dilution was prepared upto 10^{-9th} dilution. From the dilutions, 10µl was pipette out and placed on the respective quadrant in the petri plate. The plates were incubated at 28±2°C without any disturbance for 24h and individual colonies were counted through drop plate method (Somasegaran and Hoben, 1994).

RESULTS AND DISCUSSION

The microbial inoculants *B. subtilis* strain BG42 used in the present study were isolated from rhizosphere soils of gerbera. The above strain was authenticated by performing morphological and biochemical tests. Based on the results *B. subtilis* strain BG42 was opaque, wrinkled and cream in Colour and showed gram positive reaction (Manju and Subramanian, 2015). The survival of *B. subtilis* strain BG42 in different carrier materials viz., glycerol (10mM) , trehalose (5mM) , sorbitol (5mM) and glycine (10mM) and the stickers *viz*., starch(2%), liquid paraffin (2%), PVP(2%) and gum acacia (2%) and combinations of glycerol 10mM and starch 2 per cent were estimated under controlled conditions over a period of seven months of storage period at room temperature by serial dilution technique on Nutrient Agar plate (Table 1).

Table 1: Population of *B. subtilis* strain BG42 in nutrient broth supplemented with different nutrient amendments and stickers

Days	Population (cfu/ml)									
	Nutrient amendments				Stickers				Glycerol + Starch	Nutrient broth (control)
	Glycerol	Trehalose	Sorbitol	Glycine	Starch	PVP	Gum acacia	Liquid paraffin		
0	50.5×10^9	50×10^9	50×10^9	50×10^9	50.5×10^9	50×10^9	50×10^9	50×10^9	51×10^9	50×10^9
2	75×10^9	73.5×10^9	68.5×10^9	54.5×10^9	62.5×10^9	60.5×10^9	57×10^9	51.5×10^9	78×10^9	51×10^9
5	96×10^9	85.5×10^9	82×10^9	62.5×10^9	75.5×10^9	72×10^9	64×10^9	58.5×10^9	96×10^9	5×10^9
15	78.5×10^9	73×10^9	67.5×10^9	7.5×10^8	61.5×10^9	57.5×10^9	12×10^9	3×10^8	79×10^9	2.5×10^8
30	44×10^9	39.5×10^9	33.5×10^9	6.5×10^6	29.5×10^9	24.5×10^8	7.5×10^8	3.5×10^5	47.5×10^9	3×10^5
45	9.5×10^8	6.5×10^8	5×10^8	3.5×10^5	6×10^8	7.5×10^7	6×10^6	1×10^3	12×10^8	1×10^3
60	2.5×10^8	9.5×10^7	4×10^7	2.5×10^3	2.5×10^8	2.5×10^6	1.5×10^5	1.5×10^2	5×10^8	1.5×10^2
90	9×10^7	3.5×10^7	2×10^7	2×10^2	2×10^7	4×10^5	3×10^4	0	13×10^7	0
120	4.5×10^7	6×10^6	6×10^6	0	5.5×10^6	2.5×10^2	2×10^2	0	5×10^7	0
150	2×10^7	2×10^6	1.5×10^6	0	3.5×10^5	0	0	0	2.5×10^7	0
180	6×10^6	4.5×10^5	4×10^5	0	2×10^5	0	0	0	6×10^6	0
210	3.5×10^5	5.5×10^4	2×10^4	0	1.5×10^3	0	0	0	6.5×10^5	0

Initially (at 0 days) all carriers revealed non-significant differences in colony forming units (cfu). The population of *B. subtilis* strain BG42 was increased up to 5 days of storage in all carrier materials on storage and there was slow decline in number of viable propagule after 5 days of storage. Addition of different amendments favoured the population level upto 7 months of storage whereas the control (without any chemical amendments) recorded the population level only upto one month. Glycerol and glycerol+starch treatments maintained the population level of 10^5cfu/ml upto 210 days. The initial population of 10^9cfu/ml was maintained upto 30 days and thereafter decreased gradually. In control, the population was at 10^9cfu/ml during 5 days of inoculation and declined sharply to 10^2cfu/ml during 60 days of inoculation. Among the chemical treatments, addition of glycerol along with starch recorded the higher level of population throughout the period of observation followed by glycerol and trahalose. This result was in accordance with the findings of Patino-Vera *et al.,* (2005) who developed the liquid formulation of yeast, *Rhodotorula minuta* by adding glycerol (20%) which preserved the cells of 10^7cfu/ml upto 6 months at 4°C.

Enhanced survival of *Bacillus* cells in the liquid medium may be due to the addition of amendments to the medium. Addition of glycerol (2%) increased the viability and virulence of *Verticillium lecanii* (Chavan and Kadam, 2009). *Rhizobium japonicum* showed complete viability when plated onto the medium supplemented with 0.2M Mannitol (Bassirou Ndoye *et al.,*, 2007). *B. subtilis* and *Escheretia coli* increased viability during storage at various temperature and relative humidity when formulated with gum acacia (April Krumnow *et al.,*, 2009). In the present study addition of chemical amendments glycerol and starch was found to be better in supporting the population of *B. subtilis* strain BG42 than the other amendments.

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