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Research Article

Standardization of physiological requirements, substrates and carrier materials for mass production of *Bacillus subtilis*

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ABSTRACT: Optimization of *Bacillus subtilis* growth conditions for mass production under laboratory conditions was investigated as part of a biological control programme. Aspects such as increasing yield using various culture media, pH, temperatures and carrier materials were studied. Nutrient broth (100×10^8) and 20 g molasses and 10 g yeast extract based media (98.60×10^8) gave the highest yield and 20 g molasses and 10g yeast extract broth was the most economical. pH of 7-8, temperature of 30-35°C and talc as carrier material were proved to be the best for mass production of *B. subtilis.*

KEY WORDS: *Bacillus subtilis,* carrier material, media, temperature

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INTRODUCTION

The genus *Bacillus* comprises a diverse and commercially useful variety of species widely distributed in nature (Harwood, 1989). Apart from their application in industry, *Bacillus subtilis* (Ehrenberg) Cohn. has been commonly utilized in biological control of plant diseases. For commercial production, the antagonist must first be cultivated and all processes involved, optimized. Optimization takes place under laboratory conditions before up scaling for mass production. Although there is considerable information available on laboratory-scale production *of Bacillus* spp, but available literature on culturing of *Bacillus* spp mainly describes selective media (Norris *et al.,* 1981), or laboratory-scale fermentations (Sharp *et al.,* 1989). Optimum conditions for culturing, harvesting, and storing of *Bacillus* antagonists for eventual use against plant pathogens, had to be determined in order to obtain maximum yield.

An important area of biological control is the development of formulations that would take care for viable microbial activity for long period of time. Mass multiplication of bioagent in a suitable medium and development of a powder formulation was first carried out in 1980. A dried powder formulation of bioagent is important for seed treatment and soil application. *Bacillus subtilis* is one of the most important biocontrol agents for the management of plant diseases. Commercial success of a biocontrol agent depends not only on its bioefficacy or shelf life but also ease with which it can be mass multiplied on a suitable substrate which is easily available and relatively inexpensive.

MATERIALS AND METHODS

There are different steps involved in formulation of *B. subtilis* such as standardization of medium, growth studies, method of harvesting, drying and ultimately developing the commercial formulation. Therefore, before starting mass multiplication of *B. subtilis,* studies related to standardization of media, pH and temperature requirements were carried out as per the details mentioned below.

Standardization of media

Medium (broth)	Dosage (g/l)
Nutrient broth (Check) Himedia	13
Sucrose + Yeast extract	10+5
Sucrose + Yeast extract	15+8
Molasses + Yeast extract	20+10
Molasses + Yeast extract	12+12
Molasses + Urea	20+2
Molasses + Urea	25+5

Above mentioned media were prepared by dissolving different contents (as per dosage) in one litre double distilled water. One hundred ml of media were taken in 250

ml flask, sterilized and inoculated with pure culture of *B. subtilis*. These flasks were kept for incubation at room temperature for two days. Three replications were maintained for each treatment. After incubation one ml suspension was taken from each media and serial dilution technique was performed up to 10^8 dilutions. An aliquot of 0.1 ml suspension was spread over pre sterilized and cooled down nutrient agar medium plates. The inoculated plates were incubated at 30±1 °C for 24-48h. The observations on colony forming units (cfu) were recorded and statistically analysed to find out the best medium.

Standardization of pH levels

The *B. subtilis* was grown on the best media selected from above experiment at different pH ranges such as 3, 4, 5,6,7,8 and 9. One hundred ml of selective media was taken in 250 ml flask and pH was adjusted to above mentioned range. After sterilization and inoculation with *B. subtilis* the flasks were kept for incubation for 1-2 days. Three replications were maintained for each treatment. After incubation one ml suspension was taken from each pH adjusted flask and serial dilution technique was performed up to 10^8 dilutions. An aliquot of 0.1 ml suspension was spread over pre sterilized and cooled down nutrient agar plates. The inoculated plates were incubated at 30±1 °C for 24-48h. The observation on cfu were recorded and statistically analyzed to find out the best pH range.

Standardization of temperature levels

The growth of *Bacillus subtilis* was observed at different temperature range such as 15, 20, 25, 30, 35, 40 and 45°C. One hundred ml of selective media was taken in 250 ml flask and pH was adjusted to standard range. After sterilization and inoculation with *B. subtilis* the flasks were kept for incubation at different temperature ranges for 1-2 days. Three replications were maintained for each treatment. After incubation one ml suspension was taken from each temperature range adjusted flask and serial dilution technique was performed up to 10^8 dilutions. An aliquot of 0.1 ml suspension from 10^8 dilution was spread over pre sterilized and cooled down nutrient agar plates. The inoculated plates were incubated at 30 ± 1°C for 24- 48h. The observations on cfu were recorded following standard procedure and statistically analysed to find out the best temperature range.

Standardization of carrier materials

To prepare commercial formulation of *B. subtilis,* the mass produced bacteria was mixed in a 1: 2.5 proportion with presterilized carrier materials such as fly ash, vermiculite, lignite, gypsum, vermicompost and FYM and talc as a standard check. For each treatment three replications were maintained. Viability of the bacteria was tested after 7 days and observations on cfu were recorded using plate count method.

RESULTS AND DISCUSSION

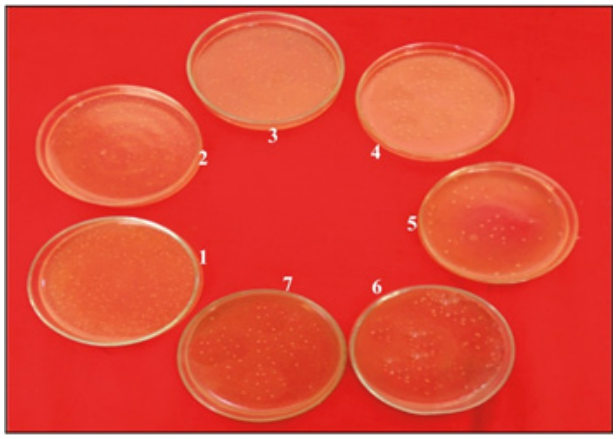
Among the seven liquid media used to grow *B. subtilis,* maximum cfu count was observed on nutrient broth (100.44×10^8 cfu) and 20 g molasses + 10 g yeast extract broth (98.60×10^8) whereas, least cfu count was recorded in 12 g molasses+12 g yeast extract broth (67.67×10^8 cfu) (Table 1 and Fig. 1). Cost of media/l was lowest with media containing 20 g molasses+10 g yeast extract broth (Rs. 49.80) compared nutrient broth (Rs. 69.40) indicating its superiority. Similar studies have been carried out by various workers and their results indicated suitability of different media for growth of *B. subtilis*. Peighmi- Ashnari *et al*. (2009) conducted the similar studies and found that molasses + yeast extract based media to be the most suitable for rapid growth and high cell yield of *B. subtilis* and their results are in tune with the results of the present study. However, results of studies conducted by Korsten and Cook (1996) and Nakkeeran *et al*. (2006) differed from present study and they opined that potato media and nutrient broth were superior for growth of *B. subtilis,* respectively.

Table 1. Effect of different media on population of *Bacillus subtilis*

Media	Dosage (g/l)	Mean cfu count (1×10^8)/ml	Cost of media /l (Rs)
Nutrient broth (ready mixture)	13	100.44	69.40
Sucrose + Yeast extract	10 + 5	067.67	40.50
Sucrose + Yeast extract	15 + 8	088.73	52.22
Molasses + Yeast extract	20 + 10	098.60	49.80
Molasses + Yeast extract	12 + 12	062.33	55.52
Molasses + Urea	20 + 2	071.67	31.40
Molasses + Urea	25 + 5	080.07	48.00
S. Em ±		001.06	
C.D. *P* = 1%		003.21	

Temperature and pH play important role among the external factors which influence the growth and reproduction of bacteria. All the bacteria have optimum temperature,

below which they cannot grow and above which they are inactivated or killed. Each bacteria has its optimum temperature and pH range for their growth and multiplication



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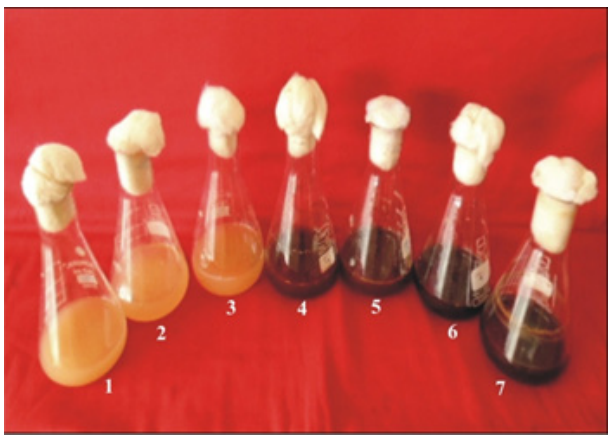

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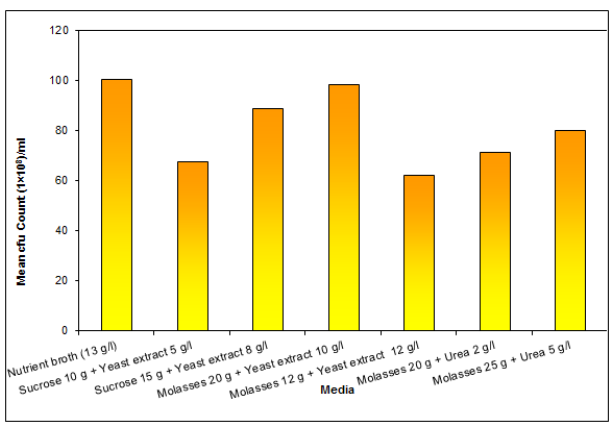

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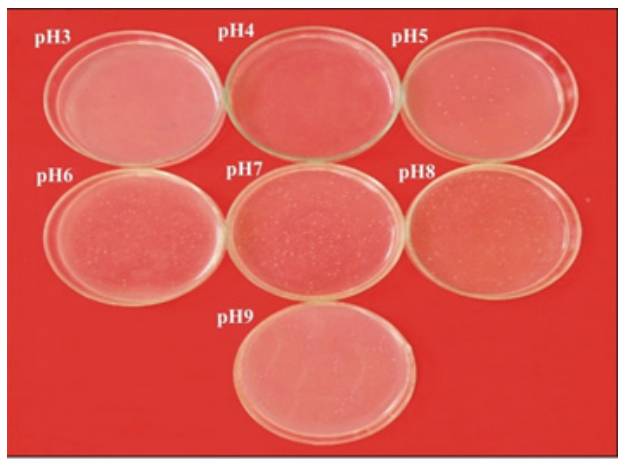
Fig. 1. (a-c) Effect of different media on population of *Bacillus subtilis*.

Table 2. Effect of different pH levels on multiplication of *Bacillus subtilis*

pH	Mean cfu count (1×108)/ml
3	0 (1.00)*
4	0 (1.00)
5	18.33 (4.40)
6	55.33 (7.50)
7	100.33 (10.07)
8	93.67 (9.73)
9	57.67 (7.66)
S. Em ±	0.11
C.D. at 1%	0.34

* $\sqrt{X+1}$ transformed values

In the present study, maximum cfu of *B. subtilis* was obtained at pH 7 (100.33 × 10^8 cfu) and no growth of bacteria was observed at pH 3 and 4 (Table 2 and Fig. 2). Similarly, maximum cfu was obtained at 30 and 35°C (103.6 × 10^8 cfu and 99.67 × 10^8 cfu respectively) and least growth was observed at 15°C temperature (Table 3 and Fig. 3). Similar results were obtained by Soleiman and Masoud (2013) while studying with the large scale production of *B. subtilis* strain UTB96.



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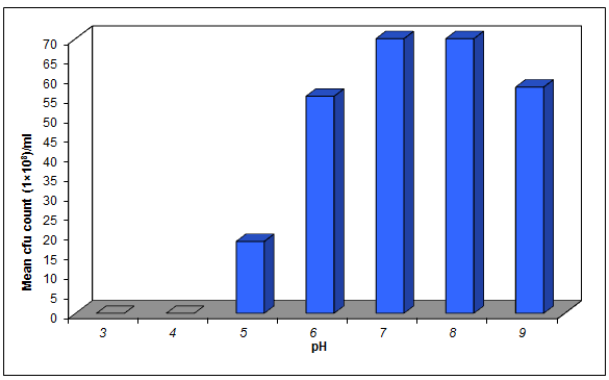

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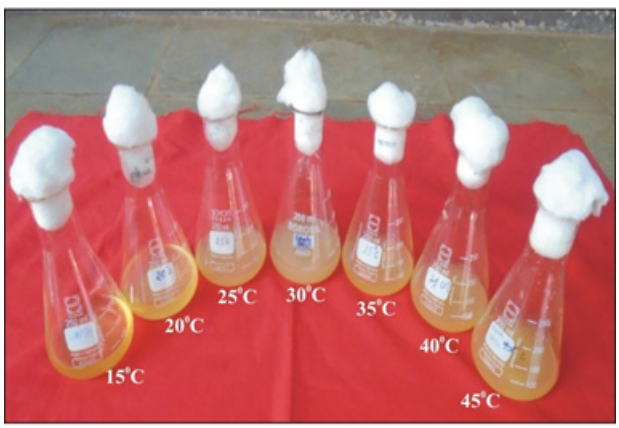
Fig. 2. (a-c) Effect of different pH on multiplication of *Bacillus subtilis.*



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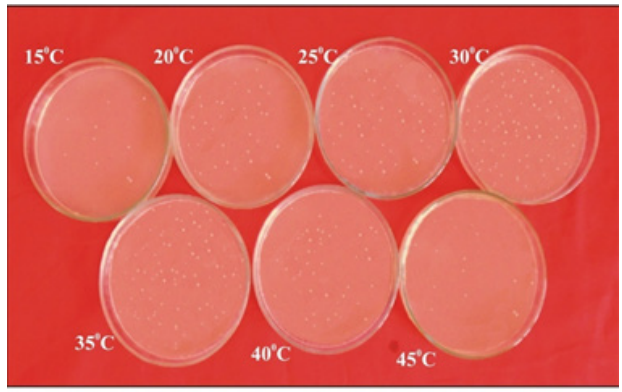

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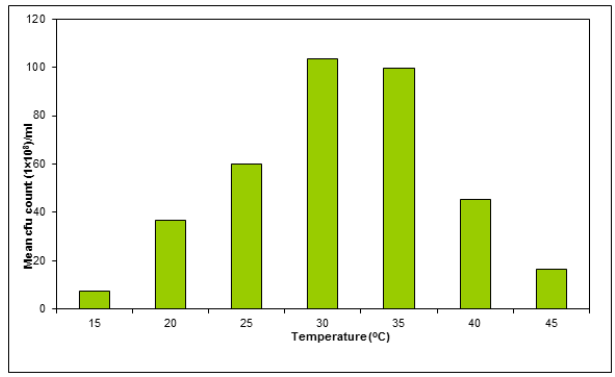


(c)

Fig. 3. (a-c) Effect of different tempearture on population of *Bacillus subtilis.*

Nakkeeran *et al*., (2006) reported that *B. subtilis* growth was good at temperature of 28±2 °C Whereas, Korsten and Cook (1996) reported that temperature of 30-37 °C and pH of 7-8 good for the growth and multiplication of *B. subtilis*.

Table 3. Effect of different temperature levels on population of *Bacillus subtilis*

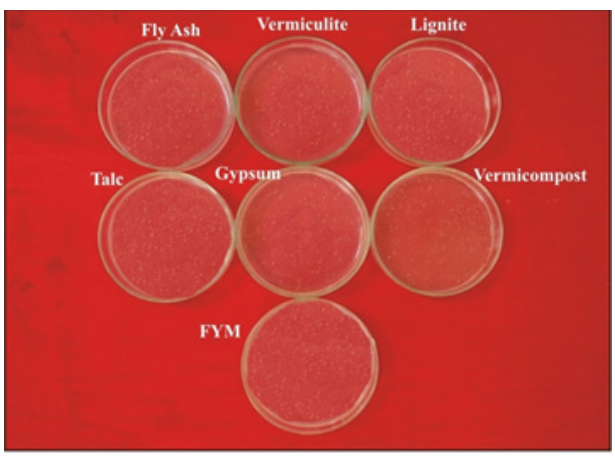
Temperature (°C)	Mean cfu count (1×10⁸)/ml
15	007.60
20	036.80
25	060.00
30	103.6
35	099.67
40	045.27
45	016.43
S. Em ±	001.43
C.D. at 1%	004.36

In the present study, different carrier materials were used to prepare commercial formulation of *B. subtilis*. All the carrier materials tested supported the multiplication of bacteria. When cfu count was observed after 7 days of incubation, talc based formulation was the best with highest count of 97.10 × 10 ^{8} cfu/g followed by FYM as a carrier material with 91.68 × 10 ^{8} cfu/g whereas, least growth was observed in gypsum (78.10 × 10 ^{8}) (Table 4 and Fig. 4) . Similar studies were carried out by Nakkeeran *et al*. (2006) and they reported that talc, FYM, vermiculite and lignite as a carrier material supported *B. subtilis* multiplication.

Table 4. Population of *Bacillus subtilis* as influenced by usage of different carrier materials

Carrier material	Mean cfu count (1×10^{8})/g
Fly ash	84.77
Vermiculite	89.78
Lignite	85.07
Talc	97.10
Gypsum	78.10
Vermicompost	87.83
FYM	91.68
S.Em ±	01.42
C.D. at 1%	04.31

(a)



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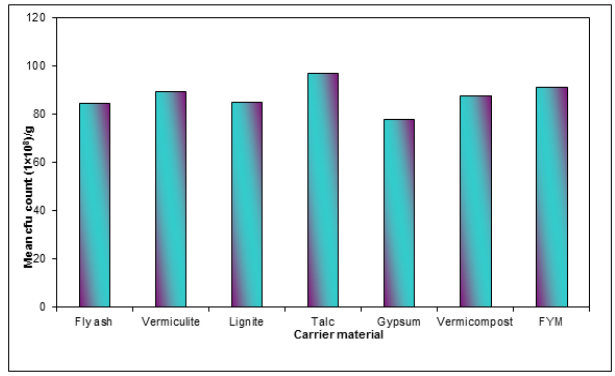

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Fig. 4. (a-c) Population of *Bacillus subtilis* as influenced by usage of different carrier.

B. subtilis was tested for its adoptability to different media, temperature, pH levels and carrier materials for its growth and survival. Results of the present study revealed that the nutrient broth and 20 g molasses + 10 g yeast extract broth, temperature of 30-35 ^{0}C, 7-8 pH and talc as a carrier material are good for the growth of *B. subtilis*.

REFERENCES

Harwood CR. 1989, Introduction to the biotechnology of *Bacillus.* In: C.R. Harwood (Ed.). *Biotechnology Handbooks,* vol 2: *Bacillus.* 1 - 4. Plenum Press, London.

Korsten L., Cook N. 1996. Optimizing culturing conditions for *Bacillus subtilis*. *South African Avacado Growers Association Yearbook* **19**: 54-58.

Nakkeeran S, Kavitha K, Chandrasekar G, Renukadevi P, Fernando WGD. 2006. Induction plant defense compounds by *Pseudomonas chlororaphis* PA23 and *Bacillus subtilis* BSCBE4 in controlling damping off of hot pepper caused by *Pythium aphanidermatum. Biocontrol Sci Technol.* **16** (4): 403- 416.

Norris JR, Berkeley RCW, Logan NA, Donnel AG. 1981. The genera *Bacillus* and *Sporolactobacillus. p 7111-1742.* In: M. Starr, H. Stolp, H.G. Truper, A. Balows and H. Starr M, Stolp H, Truper HG, Balows A, Schlegel H. (Eds) *The Prokaryotes: A handbook on habitats, isolation, and identification of bacteria,* vol 2: 1711 - 1742. Springer-Verlag, Berlin.

Peighmi-Ashnari S, Shariti-Tehrani A, Ahmadzadeh M, Behhoudi K. 2009. Interaction of different media on production and bioefficacy of *Psuedomonas fluorescens* P-35 and *Bacillus subtilis* B-3 against grey mold of apple. *J Pl Pathol.* **91** (1): 65-70.

Sharp J, Scawen MD, Atkinson T. 1989. Fermentation and downstream processing *oí Bacillus.* p 255-291 In: Harwood CR. (Ed.). *Biotechnology handbooks,* vol *2 Bacillus.* Plenum Press, London.

Soleiman G, Masoud A. 2013. Optimization of a cost effective culture medium for the large scale production of *Bacillus subtilis* UTB96. *Archives Phytopath Pl Prot* **46**: 1552-1563.