

Full Length Research Paper

## Screening of different media for sporulation of *Bacillus megaterium*

Neelam Verma<sup>1</sup>, Namita Ashish Singh\*<sup>2</sup>, Naresh Kumar<sup>2</sup> and H.V.Raghu<sup>2</sup>

<sup>1</sup>Department of Biotechnology, Biosensor Technology Laboratory, Punjabi University, Patiala -147002 (Punjab), India

<sup>2</sup>Dairy Microbiology Division, National Dairy Research Institute (NDRI), ICAR, Karnal- 132001 (Haryana), India

Accepted 21 March, 2013

Five different sporulation medium such as tryptone yeast extract (TYE) broth, tryptone glucose yeast extract (TGYE) broth, minimal media, Sucrose salt medium, Arret & Krishbaum (AK)sporulation agar were screened for spore production in *Bacillus megaterium* ATCC 9885. During this study, it was observed that out of different combinations of tryptone, yeast extract and dextrose sporulation was maximum (70%) in tryptone yeast extract broth. Effect of different salts on sporulation was studied by incorporation in TYE. It was observed that 41.7% sporulation in KCl followed by  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$  respectively. Maximum sporulation ( $90 \pm 0.6988\%$ ) was observed in AK sporulation agar with modified protocol followed by minimum sporulation in minimal medium and sucrose salt medium respectively. Spores and vegetative cells of ATCC 9885 was observed under Andor EMCCD (Electron Multiplying charge coupled device) camera showed that spores were arranged singly and small in size ( $2.8 \pm 0.2 \mu\text{m}^2$ ) compared to vegetative cells that were in chains and larger in size ( $4.0 \pm 0.2 \mu\text{m}^2$ ). Spores of *Bacillus megaterium* ATCC 9885 produced in A. K. Sporulation medium with modified protocol can be further utilized as bio-molecules for the development of bioassay for monitoring non-bacterial contaminants in milk (Aflatoxin M<sub>1</sub>).

**Key words:** *Bacillus megaterium* ATCC 9885, sporulation, A.K.agar, andor EMCCD camera, salts.

### INTRODUCTION

Sporulation, unique to two bacterial species, *Clostridium* and *Bacillus*, is a process induced by reduced levels of nutrients in the environment or in culture (Driks,2002). A specific sporulation medium is required for producing spores from specific group of spore forming bacteria. Spores of *Bacillus* species are dormant and extremely resistant to a variety of harsh environment, including extremes of heat and radiation as well as high levels of toxic chemicals (Setlow and Jhonson, 2011). Optimum temperature reported for the sporulation of *Bacillus megaterium* was 40°C (Luders et al., 2011). Sporulation occurred over a temperature range from 10 to 30°C and from pH 4.0 to 9.0 with *Phytophthora* isolates (Simpfendorfer, 2001). Optimum conditions of growth, sporulation and secondary metabolites occurred at 30°C

in *Aspergillus umbrosus* (Sood, 2011). Optimum spore formation was determined at 30°C pH 7.2 for *B. weihenstephanensis* and at 45°C pH 7.2 for *B. licheniformis* (Barilet et al., 2012).

Phosphoglyceratephosphomutase of *Bacillus subtilis*, *Bacillus cereus* and *Bacillus megaterium* required  $\text{Mn}^{2+}$  as cofactor, in the absence of  $\text{Mn}^{2+}$ , *B. subtilis* did not sporulate in normal sporulation media but it did sporulate if the proper ratio of glucose or glycerol and malate was used (Vasantha et al., 1978). A maximum spore percentage of *Bacillus megatherium* (89%) was recorded after 96 hrs of inoculation into a modified nutrient medium containing a mixture of 500 ppm of  $\text{MnSO}_4$ ,  $\text{CaCl}_2$ ,  $\text{ZnSO}_4$  and KCl (Omer 2010).

Despite their ruggedness, spores remain alert to specific environmental signals by triggering the spores to germinate rapidly into vegetative cells. This process may take 25-30 mins to break its dormancy and make metabolically active by realizing macromolecules such as

\*Corresponding author. E-mail: [namitas541@gmail.com](mailto:namitas541@gmail.com).

RNA's, proteins, enzymes and Calcium dipicolinic acid (Rotman, 2001). This process of sporulation and germination can be used as biosensing mechanism for screening the bacterial and non-bacterial contaminant in dairy food chain (Manju et al., 2012; Das et al., 2011; Gurpreet, 2012). Recently, *Bacillus* spores have been explored for the development spore based assay for monitoring of broad spectrum antibiotic, specific  $\beta$ -lactam residues & *Enterococci* in milk at dairy farm level, reception dock and manufacturing unit of dairy industry (Kumar et al., 2009; Kumar et al., 2012a; Kumar et al., 2012b) A one-step incubation/ detection based microbial biosensor by immobilizing *Bacillus subtilis* spores has been developed for detection of  $Zn^{2+}$  and bacitracin (Fantino et al., 2009).

For the exploration of *Bacillus* spores for the development of methods, assay and biosensors it is necessary to produce maximum number of spores from specific spore forming bacteria. In our present investigation, we were tried to produce maximum number of spores by developing specific sporulation medium under different optimized conditions.

## MATERIAL AND METHODS

### Procurement & maintenance of culture

*Bacillus megaterium* ATCC 9885 was procured from American type culture collection (ATCC). The procured strain was activated overnight in nutrient broth (NB) at 37°C and was streaked on nutrient agar (NA) followed by incubation at 37°C for 12 h.

### Effect of tryptone, yeast extract and dextrose on sporulation

Single colony of *B. megaterium* 9885 was inoculated in 5 mL NB and incubated at  $37 \pm 2^\circ\text{C}$  for  $24 \pm 2$  h. One hundred mL of individual tryptone (0.5% CR014 Hi Media, Mumbai), yeast extract (0.25% RM668 Himedia, TYE broth containing tryptone (0.5% CR014 Hi Media, Mumbai), yeast extract (0.25% RM668 Himedia), TGYE containing tryptone (0.5%), yeast extract (0.25%) and dextrose (0.1% RM077), having a pH 7.0 were inoculated at 1 mL.  $100 \text{ mL}^{-1}$  followed by incubation at  $37 \pm 2^\circ\text{C}$  for 48 h (Clements, 1968). The cell suspension was centrifuged at 8,994 g /10 min using Eppendorf centrifuge 2801R, (Germany) and pellet was washed, recentrifuged by using normal saline i.e., 0.85 % NaCl pH 7.0 (RM 031). The optical density of the final suspension was set to 0.3 at 595 nm by using microbiological plate reader (Perkin Elmer) and suspension was analyzed for total viable count (TVC) and spore count (SC) (Downes and Ito, 2001).

### Optimization of incubation time for sporulation in TYE medium

*B. megaterium* ATCC 9885 was grown in selected broth followed by incubation at  $37 \pm 2^\circ\text{C}$  for different period from 24-72 h to obtain maximum spores in minimum time and analysed for TVC and SC (Downes and Ito, 2001).

### Effect of different salts on sporulation

One hundred mL of tryptone yeast extract (TYE) broth was enriched with different salts such as 0.1% potassium Chloride (KCl) (RM 698), 0.015% calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) (RM 534), 0.278% ferrous sulphate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) (RM1377), 0.396 % manganese sulphate ( $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ ) (RM1381) and 0.025% magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) (RM 1281) having pH 7 (Gomathy et al., 2007) for their effect on sporulation of *B. megaterium* 9885 strain and analysed for TVC and SC (Downes and Ito, 2001). The maximum spores produced were taken as criteria for selection of particular salt for the development of sporulation medium.

### Screening of other sporulation media for *B. megaterium* 9885

*B. megaterium* ATCC 9885 strain was grown in different sporulation media like minimal medium (MM) containing 1% sucrose (RM 3063), 0.25%  $\text{K}_2\text{HPO}_4$  (RM168), 0.25%  $\text{KH}_2\text{PO}_4$  (RM 249), 0.1 %  $(\text{NH}_4)_2\text{HPO}_4$  (A.R. grade, Glaxo laboratories), 0.02%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (RM1281), 0.001%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (RM 1377) and 0.0007%  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$  (RM1381) having pH 6.8 (Todar's online textbook of bacteriology), Sucrose salt medium (SSM) containing 0.1% sucrose (RM 3063), 0.1 % NaCl (RM583), 0.24%  $\text{KH}_2\text{PO}_4$  (RM 249), 1.1 %  $(\text{NH}_4)_2\text{HPO}_4$  (A.R. grade, glaxo laboratories), 0.02%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (RM1281), 0.001 %  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (RM1377), 0.0007  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$  (RM1381), 0.001 %  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (RM695) and 0.0005 %  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (RM534) having pH 7.1 (Bruno et al. 1964) incubated at  $37 \pm 2^\circ\text{C}$  for 48 h. After sporulation, spores were harvested by centrifugation at 8,994 g/10 min using Eppendorf centrifuge 2801R, (Germany) and pellet was washed, recentrifuged by using normal saline (0.85 % NaCl, pH 7.0) and analyzed for TVC and SC.

*B. megaterium* ATCC 9885 strain was streaked on AKagar (M234) supplemented with extra 0.5 % ultra-pure agar (RM459, Himedia Mumbai) was incubated at  $37 \pm 2^\circ\text{C}$  for  $36 \pm 4$  h. Further, the culture was incubated at  $37 \pm 2^\circ\text{C}$  for 5 days followed by heating by using boiling water for  $100^\circ\text{C}/15$  mins. Heated suspension was washed with sterilized distilled water by centrifugation at  $5^\circ\text{C}/20$  mins/16000 rpm washed 3 times by 20 mL sterilized distilled water (Dey et al., 1998).

Suspension of vegetative cell was prepared by mixing

**Table 1.** Comparison of sporulation medium for *B.megaterium* ATCC 9885.

S. N.	Media	Composition	pH	Spore %
1	SSM	0.1% sucrose ,0.1 % NaCl , 0.24% KH <sub>2</sub> PO <sub>4</sub> , 1.1 % (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> , 0.02% MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.001 % FeSO <sub>4</sub> .7H <sub>2</sub> O, 0.0007MnSO <sub>4</sub> .7H <sub>2</sub> O,0.001 % ZnSO <sub>4</sub> .7H <sub>2</sub> O,0.0005 % CaCl <sub>2</sub> .2H <sub>2</sub> O	7.1 ± 0.2	1 ± 0.16
2	MM	1% sucrose,0.25% K <sub>2</sub> HPO <sub>4</sub> , 0.25% KH <sub>2</sub> PO <sub>4</sub> , 0.1 % (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> , 0.02% MgSO <sub>4</sub> .7H <sub>2</sub> O,0.001% FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.0007 % MnSO <sub>4</sub> .7H <sub>2</sub> O	6.8 ± 0.2	21.2 ± 0.88
3	TGYE	0.5 % tryptone, 0.25 % yeast extract,0.1% dextrose	7.0 ± 0.2	47.3± 0.13
4	TYE	0.5 % tryptone, 0.25 % yeast extract	7.0 ± 0.2	70.8±0.14
5	AKS	Pancreatic digest of gelatine (0.6%), casein enzymichydrolysate (0.4%), yeast extract (0.3%), beef extract (0.15%), dextrose (0.1%), manganoussulphate(0.03%), agar (1.5%)	6.6 ± 0.2	90.63 ±0.7

Note: SSM-Sucrose salt medium; MM-Minimal media; TGYE-Tryptone glucose yeast extract broth;TYE-Tryptone yeast extract broth; AKS – Arret & krishbaum sporulating agar No.2.

single colony of *B. megaterium* 9885 into 200 µL saline. 1µL vegetative cell suspension and 1µL spores sporulated in newly formulated medium was transferred on glass slide. The 0.5 µL of 105 nM fluorescein diacetate (RM 2094) was added on each slide and incubated at 37 ± 2°C for 15 mins. Slide was observed under Olympus BX61 model of EMCCD (electron multiplying charged coupled device) camera at an exposure time of 355 milliseconds with an excitation and emission spectra of 495nm and 535 nm respectively (Sujatha et al., 2010).

### Statistical analysis

Growth and sporulation experiments on *B. megaterium* ATCC 9885 in enriched sporulation medium were designed using CRD (Critical Random Design) with six replicates (n = 6) (Snedecor and Cochran, 1980).

## RESULTS AND DISCUSSION

### Effect of tryptone, yeast extract and dextrose on sporulation

During study of different components i.e. tryptone, yeast extract and dextrose on sporulation of *B. megaterium* ATCC 9885 it was observed that spore production was maximum in TYE broth (70%) followed by TGYE broth, tryptone and yeast extract that is 47, 22 and 13 % respec-

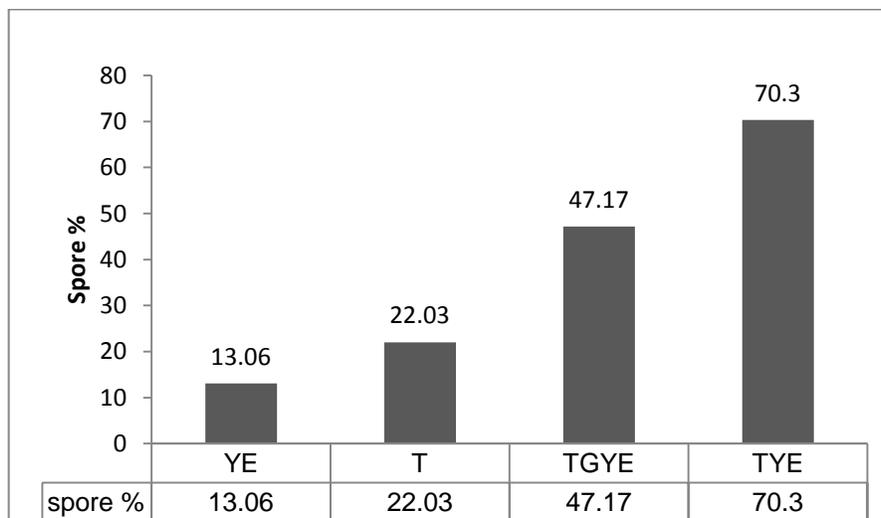
tively as depicted in Figure 1. Sporulation was maximum in TYE broth because glucose enhanced growth of all *Monascuspurpureus* strains tested but inhibited the sporulation rate (Ajdarriet al., 2011). Efficient sporulation ( $\geq 10^7$  spores per mL of *Bacillus larvae*) occurred in presence of 1.5 to 2.25% yeast extract (Dingman et al., 1983).

### Optimization of incubation time of sporulation in TYE broth

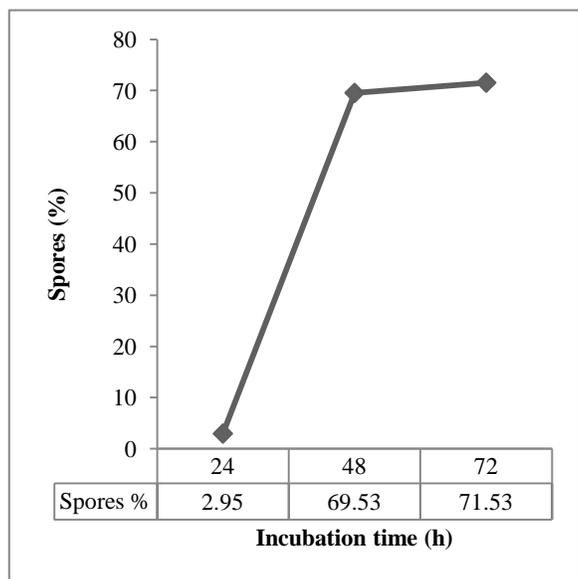
Incubation time for sporulation has been studied in TYE broth that is 24, 48, 72 h and it was observed that spore percentage increases from 2.95 - 69.53 % during 24 to 48 h incubation. Further, there is a slight increase in sporulation from 69.5 to 71.5% during incubation at 37°C for 48 to 72 h (Figure 2). On the basis of above results 48 h of incubation at 37± 2°C is selected for sporulation of *B. megaterium* ATCC9885 in TYE broth.

### Study of different salts on sporulation

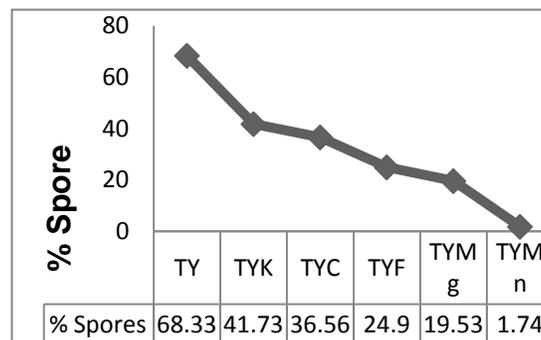
In the control medium i.e., TYE broth the spore percentage was 68.3% with a SC and TVC of 7.688 and 7.8444cfu/ mL, respectively. Effect of different salts on sporulation has been studied by adding individually in TYE broth. It was observed that spore percentage was drastically reduced in presence of manganese sulphate (MnSO<sub>4</sub>) i.e., 1.74 %. The addition of manganese to tryptic soy broth did not affect cell growth or spore form-



**Figure 1.** Effect of different media components on sporulation of *Bacillus megaterium* ATCC 9885. Note: YE-Yeast extract, T-Tryptone, TGYE- tryptone glucose yeast extract, TYE-tryptone yeast extract. No. of replicates n=6.



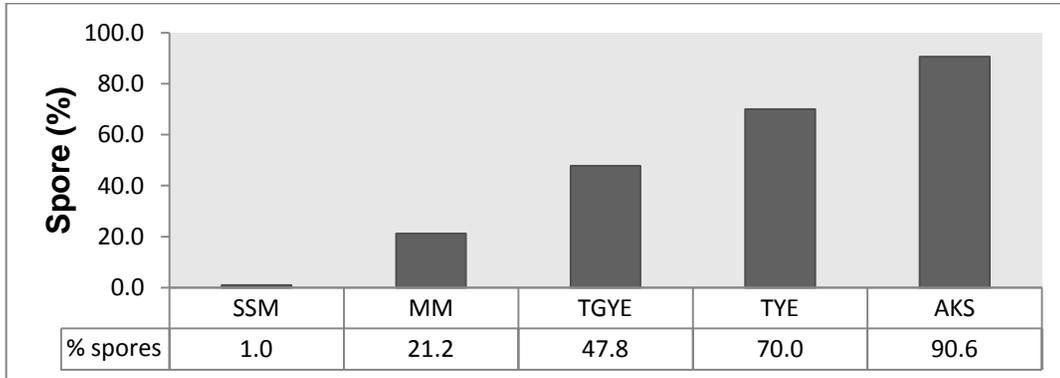
**Figure 2.** Spore (%) at different incubation period in Tryptone Yeast Extract (TYE) Broth. No. of replicates n=6.



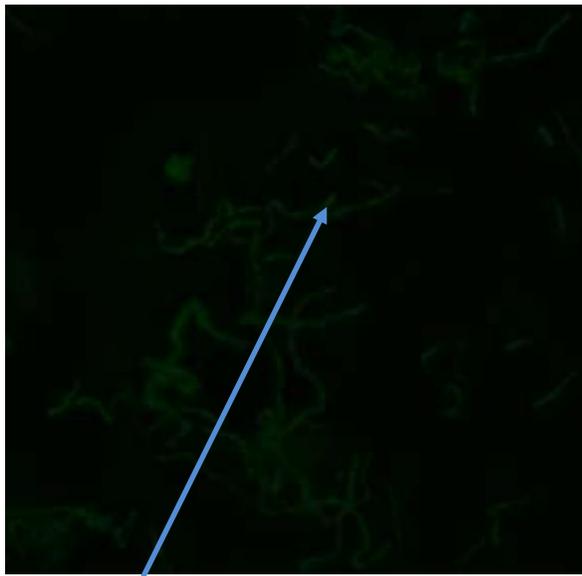
**Figure 3.** Effect of addition of different salts in TY broth on spore percentage of *Bacillus megaterium* ATCC 9885 at 37± 2 C for 48 h. TY-Tryptone Yeast extracts broth; TYK-Tryptone Yeast extract broth + KCl; TYC-Tryptone Yeast extracts broth+CaCl<sub>2</sub>; TYF-Tryptone Yeast extracts broth+FeSo<sub>4</sub>; TYMg-Tryptone Yeast extracts broth+ MgSO<sub>4</sub>; TYMn-Tryptone Yeast extracts broth+MnSO<sub>4</sub>. No. of replicates n=6.

ation, but manganese did enhance sporulation in diluted tryptic soy broth (Ryu et al., 2005). In case of iron sulphate and magnesium sulphate in TYE broth it decreased up to 24.9 and 19.5% respectively. In presence of KCl and CaCl<sub>2</sub> in TYE broth spore percentage decreased up to 41.7 and 36.5% respectively (Figure 3.). Calcium ion concentration (less than 2 microM) in the

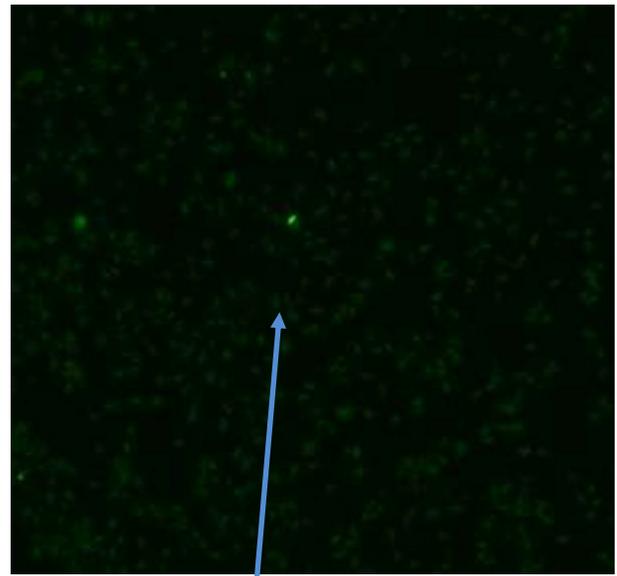
medium strongly inhibited the sporulation (98%) in *bacillus subtilis* (Hara and Hageman, 1990). Based on these observations it can be concluded that there was no significant effect of salts on sporulation of *B. megaterium* ATCC 9885 in TYE broth i.e., 68.3% as compared to broth added with different salts. Addition of Manganese chloride and iron chloride resulted in poor sporulation



**Figure 4.** Effect of different sporulation media on sporulation of *Bacillus megaterium* ATCC 9885  
 Note: SSM-sucrose salt medium, MM-Minimal media, TGYE-tryptone glucose yeast extract broth, TYE-tryptone yeast extract broth, A.K.S.- Arret& Krishnaum sporulating agar; No. of replicates n=6.



**Vegetative cells**



**Spores**

**Figure 5.** Comparison of vegetative cell & spore of ATCC 9885 observed under EMCCD system. The vegetative cells were observed in chains with larger in size compared to small size of spores with single arrangement.

while potassium chloride and magnesium chloride resulted in slight but not significant sporulation in *clostridium sporogenes* (Mahet al.,2008).

#### Effect of different sporulation media on sporulation of 9885

*B. megaterium* ATCC 9885 grown in 5mL nutrient broth 100 mL of minimal media containing sucrose,  $K_2HPO_4$ ,  $KH_2PO_4$ ,  $(NH_4)_2HPO_4$ ,  $MgSO_4 \cdot 7H_2O$ ,  $FeSO_4 \cdot 7H_2O$  and  $MnSO_4 \cdot 7H_2O$  and sucrose salt medium followed by incubation at  $37 \pm 2^\circ C$  for 48 hrs. The heat activated

spores were observed for sporulation it was observed that sporulation was minimum in minimal media i.e.,  $21.23 \pm 0.877\%$  while in case of sucrose salt medium sporulation was negligible i.e., only  $1 \pm 0.1591\%$  (Table 1). *B. megaterium* 9885 was sporulated on A.K. agar for approximately 7 days. Growth was harvested from bottles by 10-15 sterile glass beads and bacterial suspension was transferred into centrifuge tube & tubes were heated in boiling water for  $100^\circ C / 15$  mins. Heated suspension was washed with sterilized distilled water by centrifugation at  $5^\circ C / 20$  mins/16000 rpm washed 3 times by 20 mL sterilized distilled water. The grown spores were checked by heating at  $80^\circ C / 10$  mins. Spor-

lation in Arret & Krishbaum (A.K.) sporulating agar No.2 was maximum ( $90.63 \pm 0.6988$  %) compared to other sporulation media (Figure 4). Spore formation of *Bacillus megaterium* in A.K. agar medium is 7 days compared to 16 days in agar medium A & B (Clements, 1968) .A.K. agar used in current study with modified protocol was superior over existing A.K. agar protocol due to minimum steps that is 5 steps compared to 10 steps of existing protocol, low cost as there is no involvement of chemicals, homogenizer etc., less incubation time (one day less) and simplified protocol (centrifugation in only one stage & no homogenization while old protocol require centrifugation at 3 stages and homogenization) Spores and vegetative cells of *B. megaterium* 9885 were EMCCD camera morphologically Vegetative cells were large in size ( $4.0 \pm 0.2 \mu\text{m}^2$ ) with chain arrangement while spores were arranged in single with smaller in size ( $4.0 \pm 0.2 \mu\text{m}^2$ ) (Figure 5). Aiba et al.(1965) observed that the length of *B. megaterium* is  $0.9-1.7 \mu\text{m}$  and width  $0.6-1.2 \mu\text{m}$  with an area of  $0.54-2.04 \mu\text{m}^2$ . Bunk et al.(2010) reported the size of *B. megaterium megaterium* is  $4 \times 1.5 \mu\text{m}$ .

## CONCLUSION

During this study, it was observed that the spore production was maximum i.e.70% in TYE broth compared to tryptone, yeast extract and TGYE broth. There was no significant effect of salts on spore production compared to control TYE broth .Out of other medias screened for study, sporulation was  $90.63 \pm 0.6988$  % in A.K. agar No.2 compared to  $21.23 \pm 0.877$ % in minimal medium and only  $1 \pm 0.1591$ % in sucrose salt medium. So based on above findings it can be concluded that out of five sporulation media screened for *B. megaterium* ATCC 9885 A.K. agar was found satisfactory with maximum sporulation of  $90.63 \pm 0.6988$  %. Microscopic observation of *B. megaterium* ATCC 9885 under EMCCD camera by fluorescent staining technique revealed that larger size of vegetative cells in chains as compared to small size of spores with single arrangement. The finally selected A. K. Agar medium with modified protocol can be used for lab scale production of *Bacillus megaterium* ATCC 9885 spores.

## ACKNOWLEDGMENTS

The World Bank Funded National Agriculture Innovation Project (NAIP) is greatly acknowledged for supporting this research work. The Vice Chancellor, Punjabi University, Patiala is thankfully acknowledged for support for working at Department of Biotechnology, PU, Patiala to Mrs. Namita Ashish Singh. We wish to thank Dr. Naresh Kumar, Principal Scientist and Raghu H.V., Scientist, DM Division, NDRI, Karnal (India) for their

technical inputs and for conducting specialized work at NDRI, Karnal.

## REFERENCES

- Aiba S, Humphrey AE, Milis NF (1965). Biochemical Engineering (Book) New York, Academic Press, 159.
- Ajdari Z, Ebrahimpour A, Manan M A, Hamid M, Mohamad R , Ariff A B (2011). Nutritional requirements for improvement of growth and sporulation of several strains of *Monascus purpureus* on solid state cultivation. J. Biomed. Biotech., Article ID 487329: 1-9.
- Baril E, Coroller L, Couvert O, El Jabri M, Leguerinel I, Postollec F, Boulais C, Carlin F, Mafart P (2012). Sporulation boundaries and spore formation kinetics of *Bacillus* spp. as a function of temperature, pH and a(w). Food Microbiol.,32(1):79-86.
- Bunk B, Schulz A, Stammen S, Munch R, Warren MJ, Rohde M, Jahn D, Biedendieck R (2010). A short story about a big magic bug. Bioengineered Bugs. 1(2): 85-91.
- Clements NL (1968). Rapid confirmatory test for aflatoxin B<sub>1</sub> using *Bacillus megaterium*. J. A.O.A.C., 51:1192-1194.
- Das S, Kumar N, Raghu HV, Haldar L, Gaare M, Singh VK, Puniya AK (2011) Microbial based assay for specific detection of  $\beta$ -lactam group of antibiotics in milk. J Food Sci Tech.,doi: 10.1007/s13197-011-0609-4.
- Dey BP, White CA, Reamer RH, Thaker NH (1998). Detection of antimicrobial residues in meat and poultry tissue by screen tests. USDA/FSIS Microbiology Laboratory Guidebook. Chapter 33.3<sup>rd</sup> editions.
- Dingman D W and Stahly D P (1983). Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. Appl. Environ. Microbiol., 46( 4):860-869.
- Downes FP, Ito K (2001). Compendium of methods for the microbiological examination of foods,4th edn. In: American Public Health Association (ed) Frances Pouch Downes Keith Ito, Washington DC.
- Driks A (2002). Overview: development in bacteria: spore formation in *Bacillus subtilis*. Cell Mol .Life Sci., 59: 389-391.
- Fantino J-R, Barras F, Denizot F (2009). Sposensor: A Whole-Bacterial Biosensor That Uses Immobilized *Bacillus subtilis* Spores and a One-Step Incubation/Detection Process. J. Mol. Microbiol. Biotechnol.,17: 90-95.
- Gaare M, Kumar N, Raghu HV, Khan A, Singh VK (2012). Specific detection of  $\beta$ -lactam antibiotics in milk by spore based assay. Int. Res. J. Microbiol. 3(4): 168-173.
- Gomathy M, Thangaraju M, Gunasekaran S, Gopal NO (2007). Sporulation and regeneration efficiency of phosphobacteria (*Bacillus megaterium* var phosphaticum). Indian J. Microbiol.47:259-262.
- Hara M B O' and Hageman J H (1990). Energy and calcium ion dependence of proteolysis during sporulation

- of *Bacillus subtilis* cells. J Bacteriol., 172(8): 4161–4170.
- Kaur G (2012). Spore Germination-an innovative approach for detecting *Enterococci* in milk. M. Sc Thesis, National Dairy Research Institute (Deemed University), Karnal, India.
- Kolodziej BJ, Slepecky R (1964). Trace metal requirements for sporulation of *Bacillus megaterium*. J. Bacteriol., 88 (4):821-830.
- Kumar N, Das S, Manju G (2009). A kit for detection of  $\beta$ -lactam antibiotic group in milk using bacterial spore as biosensor. Indian Patent Reg No.115/ DEL/ 2009. Office of the Controller General of Patents, Designs & Trade Marks.
- Kumar N, Kaur G, Thakur G, Raghu HV, Singh N, Singh VK and Raghav N (2012a) Real time detection of *enterococci* in dairy foods using spore germination based bioassay. (IPR # 119/DEL/2012) Office of the Controller General of Patents, Designs & Trade Marks, Mumbai, India
- Kumar N, Raghu HV, Kumar A, Haldar L, Khan A, Rane S, Maik RK (2012b) Spore germination based assay for monitoring antibiotic residues in milk at dairy farm. World J. Microbiol. Biotechnol., 28(7):2559-66.
- Lüders S, David F, Steinwand M, Jordan E, Hust M, Dübel S, and Franco-Lara E (2011). Influence of the hydromechanical stress and temperature on growth and antibody fragment production with *Bacillus megaterium*. Appl. Microbiol. Biotechnol., 91:81–90.
- Mah J., Kang D, Tang J (2008). Effects of minerals on sporulation and heat resistance of *Clostridium sporogenes*. International J. food microbial., 128: 385-389.
- Omer AM (2010). Bioformulations of *Bacillus* spores for using as biofertilizer. Life Science Journal., 7(4):124-131.
- Rotman B (2001) Using living spores for real-time biosensing. Genetic Engineering News. 21(21): 33-57.
- Ryu JH, Kim H, Beuchat LR (2005). Spore formation by *Bacillus cereus* in broth as affected by temperature, nutrient availability, and manganese. J. Food Prot., 68(8):1734-8.
- Setlow P, Johnson EA (2011). Spores and their significance, in press. In Doyle MP, Buchanan R (ed), Food Microbiology, Fundamentals and Frontiers, 4<sup>th</sup> edition. ASM Press, Washington, DC.
- Simpfendorfer S., T. J. Harden., Murray (2001) G.M. Effect of temperature and pH on the growth and sporulation of *Phytophthora clandestina* Australasian Plant Pathology., 30(1): 1-5
- Snedecor GW, Cochran WG (1980) Statistical methods, 7<sup>th</sup> edition. The Iowa State University Press, Iowa.
- Manjulata Sood (2011). Effect of temperature of incubation on the growth, sporulation and secondary metabolites production of *Aspergillus umbrosus*. J. Phytol., 3(4): 35-37.
- Sujatha V, Sridhar B, Krishnamurthy S, Kumar VK, Kumar SK, Gautam P (2010). Use of tetra-ammonium tetrakis (4-Sulphonato) phenyl porphyrin for *Pseudomonas* and *Bacillus* cell imaging. Int. j. Analytical Chem., 1-3.
- Todar K (2013). Todar's online textbook of bacteriology, chapter genus *Bacillus*. [www.textbookofbacteriology.net/Bacillus.html](http://www.textbookofbacteriology.net/Bacillus.html). Accessed 17<sup>th</sup> February 2013.
- Vasantha N, Freese E (1978). The role of manganese in growth and sporulation of *Bacillus subtilis*. J. Gen. Microbiol., 112:329-336.