Bioformulations of Bacillus Spores for using as Biofertilizer

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Abstract: A maximum spore percentage of *Bacillus megatherium (B. megatherium)* (89 %) was recorded after 96 hours of inoculation into a modified nutrient medium containing a mixture of 500 ppm of MnSo4 , CaCl2, ZnSo4 and KCL. These spores were incorporated into 21 different talc, cellulose and clay based formulations and their viability were assessed over 6 months at room temperature. Of these bioformulations, Talc - glucose, Talc - yeast and Cellulose - clay based powder formulations were selected for additional in vivo testing because of their highest levels of viability . Field experiment was conducted to evaluate the efficiency of the treatment of bean seeds with selected powder bioformulations on the growth , yield parameters and root colonization ability of *B. megatherium* . The powder bioformulations as well as the free spore suspension effectively enhanced plant biomass ,increased the yield and accelerate the rhizosphere colonization by the bacterium under field condition . So, the commercially acceptable powder bioformulations of the *B. megatherium* which have a long storage life , aid product delivery, and promote the plant growth parameters were prepared to be used instead of the traditionally used free spore suspension.

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1. Introduction:

Formulations generally composed of the active material which must be preserved or maintained in viable condition to produce its biological effect, the carrier material may or may not include the incorporation of enrichment materials or additives.

Generally, amendments can be grouped as either carriers (fillers, extenders) or amendments that improve the chemical, physical, or nutritional properties of the formulated biomass (Schisler *et al*., 2004). The active material is mixed with carrier materials such as water, clay, talc, oil or others to make the formulation safer to handle, easier to apply and better suited for storage. In some formulations, enrichment materials comprising of nutrient-rich medium such as, molasses, trehalose, maltose and sucrose are incorporated to further enhance the viability of core (active) materials (Brar *et al*., 2006; Tu and Randall, 2005**).**

The commercial use of plant growth-promoting rhizobacteria requires inoculum that retains a high cell viability and easily be transported and applied to seed. The aims of formulating viable cells are to ensure that adequate cell viability is sustained to increase the efficacy of the cells and to facilitate the delivery and handling processes (Filho *et al.,* 2001). For commercialization, a long shelf-life is an advantage for any inoculant (Fages 1990, 1992).

This can be achieved by producing granular formulations, powder or dust formulations, microcapsules, or oil-emulsion formulations (Brar *et al.,* 2006).

A formulated microbial product, for purpose of this paper, is defined as a powder product composed of biomass of a phosphate dissolving bacteria and ingredients to improve the survival and efficient of the product.

Most often, dry formulations are generally preferred over wet formulations because they provide extended shelf life and are easier to store and transport (Lumsden *et al.,* 1995).

In powder formulation, the active material is preferred to be in spore form to increase the shelf life and efficiency of active material. Gram-positive microorganism that produce heat- and desiccation-resistant spores that can be formulated into stable, dry-powder products offer a biological solution to the problem of biofertilizer agent formulation (Caesart and Burr 1991)

A crucial initial step toward preserving biomass viability during formulation is to optimize fermentation protocols for not only maximal total biomass but also for maximal spores production.

When producing biomass of *Bacillus spp.,* in most instances fermentation protocols should be designed to maximize the production of efficacious spores rather than vegetative cells (Driks, 2004).

Different factors can enhance the sporulation process of *Bacillus spp.*It is well known that endospore formation in *B. subtilis* can be promoted by a high cell density (Grossman and

Losick, 1988), nutrient limitation (Schaeffer *et al.* 1965), high mineral composition and transition metals, especially Zn and Cu (Kihm, *et al.,* 1988).

Several investigators have shown that there is considerable variation in spore production depending on the mineral composition of the medium (Krueger and Kolodziej, 1977, Mallidis and Scholefield, 1987)

The spore forming bacteria *B. megatherium* serve as phosphate dissolving bacteria for solubilizing inorganic phosphatic compounds into soluble forms which is available for plant. It is well known that seed inoculation of phosphorus solubilzing microbes enhance P uptake and yield of economic parts (Gharib, et al., 2004). There is increasing evidence that phosphorus solubilizing bacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing available phosphorous and the solubilization effect is generally due to the production of organic acids by these organisms. They are also known to produce amino acids, vitamins and growth promoting substances like indole acetic acid (IAA) and gibberellic acid (GA3) which help in better growth of plants. (Ponmurugan and Gopi 2006) .

In this study, heat-resistant endospores of *B. megatherium* were formulated with various combinations of organic carriers with and without the incorporation of enrichment and additive materials to develop a formulation which has potential for large-scale applications.

2. Materials and Methods:

*Bacillus megatherium (*Phosphate dissolving bacteria) was grown on nutrient, Pikovskaya (Pikovskaya, 1948) .and modified Bunt and Rovira media (Abd El- Hafez, 1966) for 24h, 48 h, 72 h, and 96 h of cultivation on an orbital shaker at 150 rpm at 30^0C. Both viable and heat - resistant spore counts were determined. For heat - resistant spore counts, cultures were heated at 80°C for 15 min to kill any vegetative cells present. Spores were then subsequently enumerated by plating aliquots of serial dilutions onto nutrient agar media which were incubated for 3 days at 30°C.

Enhancement of sporulation by addition of different metals:

Nutrient broth medium was used as the basal medium to which different metals at a concentration of 500 ppm were added, total viable count, heat - resistant spore count and the spore percentage were determined after 72 h of cultivation as described before. Duplicate flasks were set up for each experiment.

The metals tested were: KCL , $MgSo_4$, $MgCl_2$, $FeSo_4$, $CaCl_2$, Na_2So_4, $MnSo_4$, $CuSo_4$ and K_2So_4 .

Preparation of B. *megatherium* spore yield:

B. megatherium were grown on a modified nutrient medium supplemeted with a mixture of MnSo4, $CaCl_2$, $ZnSo_4$ and KCL at a concentration of 500 ppm for 3 days on an orbital shaker at 150 rpm at 30^0C till the maximum spore yield was produced, these were harvested and subsequently washed by repeated centrifugation at 5,000 × *g* for 20 min at 4°C /resuspension in sterile distilled water (Warriner and Waites, 1999). Finaly, the spore pellet was re-suspended in sterile distilled water and used as active material in different formulations .The final spore titer was $\geq 10^8$ CFU/ml .

Formulation of B. *megatherium* :

The inert carriers used in the formulations were talc, clay and cellulose. For each carrier type, 1% carboximethylcellulose (CMC) as binder, traces of sodium benzoate as stabilizer ,15% $CaCo_3$ as buffer and 0.25% of different enrichment materials were incorporated .The enrichment materials incorporated to be tested were : glucose , sucrose, mannitol , yeast and peptone . Also, combinations of the three carriers (talc-cellulose, talc-clay, and cellulose-clay) were developed to be evaluated.

The inert carriers, enrichment and additive materials were mixed and sterilized by autoclaving. Twenty ml of spore suspension were added into them, mixed well under aseptic conditions, then the mixtures were air dried in a laminar flow chamber for 48 hours. After drying, a 1-g sample was removed for initial population counts. Powder formulations were then placed in plastic petri plates, sealed with parafilm, stored at room temperature, and sampled for viability assessment.

Viability assessment:

In the viability assessment, population counts of bacteria among various formulations were determined by serial dilutions from formulations and plated in triplicate on nutrient agar, and the CFU per gram of formulation were enumerated at intervals of 1 to 6 months.

Seed coating with bioformulation:

One gram of the selected bacterial formulation (Talc-glucose, Talc-yeast and Clay-cellulose) was added to 100 g bean seeds wetted with 1 ml sterile distilled water in a sterile plastic bag. The mixture was shaken until the seeds were thoroughly coated with the formulation. Also, 100 g bean seeds were mixed with free-spore suspension of *Bacillus megatherium* ($\geq 10^8$spore/ml) using CMC (1%) as sticker (Amran, 2006). Three corn seeds coated with the formulation were taken randomly and placed

separately into test tubes containing 10 ml sterile distilled water. With a sterile pipette, 0.1 ml from a 10^{-2} dilution was placed onto nutrient plates and spread using a sterile glass rod hockey. The plates were sealed and incubated at 30^0C. After 48 hours, bacterial colonies were counted.

Field evaluation of selected bioformulation on growth and yield parameters of bean plant:

A field experiment was conducted in a complete randomized design with three replicates at Maryut Experimental Station of the Desert Research Center (DRC), Alexandria. A standard plot size of 5 x 4 m^2 was maintained for all treatments. Soil in all treatments was amended with recommended dose of super phosphate (15.5% P_2O_5) at a rate of 250 kg/fed, ammonium nitrate (33.3% N) at a rate of 300 kg/fed and K-sulphate (48% K_2O) at a rate of 200 kg/fed .

Seeds of faba bean (Vicia faba cv Giza 40) purchased from agriculture ministry, Giza, Egypt. were treated with selected bacterial formulations (Talc-Glucose , Talc- yeast and Clay –Cellulose) at the rate of 1% (powder formulation : seeds) to give a bacterial population of $\geq 10^7$CFU/seed of formulation , For the free-spore suspension treatment, seeds were moistened in CMC solution (1%) before application of inoculum to get a thin, uniform coating of inoculum on seeds. Inoculated seeds were dried in shade before sowing (Samasegaran *et al.,* 1982), an untreated control was maintained. .

Plant height, weight of 100seeds, seed and straw yield (kg/fed.) of bean plants were recorded at the time of harvest for all treatments

Chemical analysis of bean seeds and straw was carried out after harvest to determine total phosphorus, nitrogen and protein in both straw and seeds

The plant materials were dried in an oven at 70 °C until a constant mass was reached and then they were grounded for chemical analysis. Total nitrogen was determined according to **(**Bremner and Mulvaney, 1982). and phosphorus was determined spectrophotometrically according to by the ascorbic acid method at 650 nm according to Watanabe and Olsen (1965).

The degree of rhizosphere colonization was estimated as following: After 30 days from sowing, roots and attached soil were divided into primary roots by cutting just below the seed. Each primary root was cut into thirds according to length, and the top section of each root was placed into sterile distilled water. Bacterial colonization was significantly higher in the uppermost section of the root compared to that of lower sections (Oliver, *et al*, 2004(Root sections and attached soil were sonicated for 5 min. The resulting bacterial suspensions were vortexed , serially diluted and grown on selective modified Bunt and Rovira agar plates for counting of phosphate dissolving bacteria and on nutrient media for total count . Colonies were counted after 48 h of incubation at 30°C, and the CFU per cm of the root system was estimated.

Statistical analysis:

Data were subjected to statistical analysis using the method described by (Snedecor, 1966). The least significant difference (L.S.D) was used to differentiate means according to (Waller and Duncan, 1969).

3. Results and Discussion:

From table (1), the highest values of TVC and HRSC detected were 400×10 ^{8}CFU/ ml and 100 × 10^8 spore/ml after 72 h of cultivation, respectively. Whilst the highest spore percentage (35 %) was recorded after 96 h of incubation on nutrient media although both total viable count and heat-resistant spore count decreased.

The nutrient media increased both the total viable count and spore yield over 1 and 2.5 folds compared to Bunt and Rovira medium and over 2.5 and 3.3 folds compared to Pikovskaya medium.

As the nutrient media enhanced the sporulation process and increased the sporulating percentage of *B. megatherium, so* it was selected to be a basal medium for the production of spore yield.

Table (1): Effect of different media used on total viable cell counts, spore count and spore percentage;

Time	Media used								
	Nutrient			Bunt and Roveira			Pikovskaya		
	TVC×10^8 CFU/ml	HRSC×10^8 spore/ml	%	TVC×10^8 CFU/ml	HRSC×10^8 spore/ml	%	TVC×10^8 CFU/ml	HRSC×10^8 spore/ml	%
24 h	70	5	7	20	2	10	10	1	10
48 h	180	18	10	45	6	13	60	8	13
72 h	400	100	25	200	40	20	160	30	19
96 h	80	28	35	120	30	25	20	5	25

TVC: total viable count HRSC: Heat-resistant spore count

As shown from table (2), incorporation of metals to the basal sporulating media generally increase the sporulation process except for Cu, and this may be due to increasing of osmotic pressure of media which have appositive effect on sporulation process , this is supported by the fact that certain transition metals including iron (Fe) and manganese (Mn) in a complex sporulation medium stimulated spore formation in certain strains of *Clostridium botulinum*, but sporulation was drastically decreased by the addition of copper (Cu) to the medium (David, *et al*., 1990).

Medium containing 500 ppm of $MgSo_4$,$MnSo_4$, K_2So_4 or $ZnSo_4$ showed the highest values of both total viable count and heat-resistant spore count compared with other concentrations tested and this is agree with a fact that there is a correlation between growth rate and spore yield. (Osadchaya, *et al*., 997).

Addition of a mixture composed of 500 ppm of $MnSo_4$, $CaCl_2$, $ZnSo_4$ and KCL (the metals gave the spore percentage over 75%) to the basal nutrient medium enhanced the sporulation process ,increased the spore yield to about 160 $\times 10^8$CFU $ml^{-1}$and increased the spore percentage to 89% (over 64% compared to control) .

The specific functions of metal ions in sporulation are probably that they act as activators of the various enzyme systems necessary for sporulation (Bruno and Ralph, 1964).

Varying the metal concentration in the sporulation media is known to influence the thermal-resistance spores due to inducuction of genes coding for the two small acid soluble proteins earlier during sporulation in the media that contained higher metal concentrations (Oomes and Brul**,** 2004).

Table (2): Effect of different metals on the enhancement of sporulation process (total viable cell counts, spore count and spore percentage):

Metals added at 500 ppm	Total viable count $\times 10^8$CFU ml^{-1}	Heat-resistant spore count $\times 10^8$ spore ml^{-1}	Spore percentage %
Control	80	20	25
KCL	96	72	75
$MgSo_4$	160	88	55
$MgCl_2$,	80	32	40
$MnSo_4$	120	96	80
$CaCl_2$	90	74	82
$FeSo_4$	55	30	55
Na_2So4	34	22	65
K_2So_4	190	80	42
$CuSo_4$	0, 002	0.0004	20
$ZnSo_4$	110	86	78
*Mixture	180	160	89

*Amixture composed of each of the following: 500 ppm of $MnSo_4$, $CaCl_2$, $ZnSo_4$ and KCL.

In all formulations, bacterial populations declined steadily over time, the bacteria survived even up to 180 days of storage with different percentage although the population declined from 30 days of formulation as shown from table (3) .

The type of carriers used influenced the viability of bacterial cells, the bacterial populations for both cellulose and talc--based formulations were generally higher than that of clay-based formulation.. All cellulose-based formulations with and without enrichments were able to maintain the highest viable cell count throughout the storage period, with a mean cell count of 81.7 x 10^8CFU/g of formulation compared to talc -based formulations and clay-based formulations, with 80 and 9.3 x 10^8CFU/g , respectively after 180days of storage . Recent studies on beneficial rhizobacteria have investigated the efficacy of powder formulations in combination with methylcellulose and xanthan gum (Kloepper and Schroth, 1981, Suslow and Schroth , 1982).

Among the formulations, enrichment materials proved to be the most useful as highest number of viable cells were recovered.

These are in agreement with previous research which showed that high-molecular-weight (C6 to C12) compounds such as sucrose and trehalose enhanced survival of bacteria in dried biopolymers (Ilyina *et al.*, 2000). Among these enrichment materials, glucose and yeast were the most efficient ones in preserving bacterial populations at different formulations.

Also, the formula composed of clay- cellulose was the most effective one among other formulations in survival of bacterial populations. This is may be due to the advantages of both clay and cellulose as carriers.

This showed that clay materials benefited the cells by providing large surface areas which act as an effective survival unit for nutrient absorption and protection (Lunsdorf *et al.*, 2000, Ting *et al.*, 2010).

In addition to the catalytic and shielding properties, clay also has good cation exchange capacity which enhances the bacterial metabolic activity (Adamis *et al.*, 2005). High cation exchange capacity enhances bacterial metabolic activity leading to higher viability (Beveridge, 1988),

The great value of porosity and capacity of cellulose to absorbe the hydrophobic and hydrophilic liquids may be useful for application as carrier for microorganisms for agriculture objectives. These properties are important for distribution of microorganisms in the granules and absorption of substrates limiting the growth of microbial population (Ilyina *et al.*, 2000).

This indicates the possibility of developing simple powder formulations with the capacity to provide long-term survival of beneficial rhizobacterial strains at high populations.

Among these formulations, five formulations (T-glucose, T-yeast, Ce -glucose, Cellulose - yeast and Cellulose-clay) were selected for additional testing because they had higher levels of viability amongst bacterial cell populations.

Table (3): Effect of different carriers and amendments on survival of bacteria in powder formulations

Formulation	x10^{8}CFU/g of formulation							*Survival
	0day	30day	60day	90day	120day	150day	180day	%
Talc	200	190	80	60	500	40	40	20
T-glucose	220	180	180	160	160	145	150	68
T-sucrose	190	190	160	90	70	50	50	26
T-.manitol	210	200	90	90	60	60	45	21
T-yeast	200	200	200	190	180	170	150	75
T-pepton	180	180	120	80	70	45	45	25
Clay	200	110	30	10	6	5	2	1
C-glucose	190	180	90	50	40	20	20	11
C-sucrose	180	150	40	15	12	4	3	1.6
C-manitol	210	200	170	35	20	10	7	3
C-yeast	210	170	150	70	60	30	18	8.5
C-pepton	200	140	35	30	20	10	6	3
Cellulose	210	210	200	160	150	140	70	33
Ce-glucose	230	230	180	140	140	120	120	58
Ce-sucrose	180	160	160	140	100	100	70	40
Ce-.manitol	190	160	130	120	120	100	70	37
Ce-yeast	200	200	190	190	160	140	110	55
Ce-pepton	200	180	160	90	90	80	50	40
Talc-Cellulose	190	180	145	100	74	60	57	30
Talc-Clay	210	200	175	140	130	105	105	50
Cellulose-Clay	200	200	200	190	170	170	160	80

Percent survival of each formulation was determined as follows: [(CFU/g of formulation at sampling)/CFU/g of formulation at beginning of experiment)] x 100

Results from table (4) showed that all seeds were successfully coated with bacterial spores of *B. megatherium* with different ranges , the highest bacterial population (2.3x 10^7CFU /seed) was on seed coated with the cellulose - clay based-formulation followed by talc based-formulations and the lowest was on seed with the cellulose - based formulations (0.8 x 10^7 CFU /seed). It has been proposed that a uniform coating of approximately 10^7 CFU of bacteria per seed is necessary for successful bacterization (Suslow, 1982).

Generally, bacterial populations on seeds treated with free cell suspension and CMC was higher than that of powder based-formulations except for cellulose - clay based-formulation which gave the highest value.

The ability of any PGPB to colonize its target plant roots and to produce growth effects is an ultimate test (Bashanand, 1997; Glick and Bashan 1997).

The obtained data from the table (6) generally showed that application of biofertilizer in the form of free-spore suspension or powder formulations considerably stimulates both total microbial and phosphate dissolvers counts in the rhizosphere of bean plants. Maximum numbers of inoculated

bacteria were recovered from the rhizosphere of free-spore suspension and cellulose - clay formulation treated plants after 4 weeks of growth.

These indicate that the colonization capacity of *B. megatherium* in free-spore suspension was superior to that of other powder formulations except for cellulose -clay formulation which gave the same colonization capacity.

Table (4): Bacterial populations on seed surface treated with different formulations :

Formulation	x10^{7}CFU/seed	Formulation	x10^{7}CFU/seed
FSS	2	**Ce-glucose**	0.8
T- glucose	1.5	**Ce - yeast**	0.9
T- yeast	1.3	**Ce - clay**	2.3

FSS: free spore suspension

Table (5): The effect of selected formulations on the degree of rhizosphere colonization:

Treatments	Total count x10^{6}CFU/cm	PDB count x10^{4}CFU/cm
Control	9	8
Fss	20	16
T-glucose	18	10
T-yeast	17	11
Ce-clay	21	16

Generally, data represented in table (6) indicated that seed inoculation with all bioformulations were found to enhance the plant height, weight of 100 seeds ,seed and straw yield of the faba bean plant over the control . no significant differences among bioformulations treatments were detected .

For plant height, the highest remarkable increase was recorded with half free spore suspension application followed by Cellulose-clay based formulation relative to control.

Free spore suspension recorded the significance increase over the control and other powder formulations for straw yield,, while Cellulose-clay based formulation record the heights significance value for seed yield .

Biofertilization treatments caused an effective action on N and P uptake by different parts of faba bean plants (seed and straw) relative to the control. Concerned to N % , there is no significant differences among formulations of *B. megatherium* in case of straw although the free spore suspension gave the maximum N % in seeds followed by other powder formulations.

However, the overall mean of both seeds and straw P % as affected by *B. megatherium inoculation* indicated a relative increase by about 18 % and 25 %, respectively over the untreated one.

Higher accumulation of P in seeds were recorded with inoculation of free spore suspension followed by other powder formulations where there is no significant differences among them .For P % in straw, there is no significant differences among different biotreatments. Of these treatments, the free spore suspension and Cellulose-clay based powder formulation were more effective than the other formulations and the spore application without a carrier as compared with other treatments.

Table (6): Evaluation of selected formulations on the bean growth and yield parameters:

Treatments	Plant height (cm)	Weight of 100 seeds(gm)	Yield kg/fed			Nitrogen %		Phosphorus %	
			Total	Seed	Straw	Seed	Straw	Seed	Straw
Control	48 c	80 b	2592c	1160c	1432b	2.9 b	1.5 a	0.66 b	0.5 b
Fss	62 a	95 a	3100a	1288b	1812a	3.5a	1.9 a	0.88 a	0.79 a
T-glucose	52 bc	90 ab	2760bc	1300b	1460b	3.2ab	1.9a	0.81ab	0.73 a
T-yeast	55abc	90 ab	2800b	1364b	1436b	3.2ab	1.7 a	0.8 ab	0.73 a
Ce-clay	60ab	93 a	2800b	1500a	1300b	3.4ab	1.8 a	0.85ab	0.76 a
LSD(0.5%)	7.6	11.1	174.5	89.04	183.7	0.56	0.77	0.139	0.173

- FS: Free spore suspension
- Values with same letter are not significantly different (P = 0.05).

4. Conclusion

This study reports the results of investigations on the different formulation combinations in maintaining the efficacy and viability of the *B. megatherium* endospores.

When heat-resistant endospores of *B. megatherium produced* from modified nutrient medium were formulated with different inorganic carriers, the dormancy of the endospores was maintained for 6 months at room temperature depending on the carrier type, also the enrichment materials were effective in enhancing the cell viability especially glucose and yeast .Of these bioformulations, cellulose-clay and talc based powder formulations were more effective than other formulations tested.

From the field application, it is indicated that seed treatment with powder formulations especially Cellulose –clay based formulation is an effective delivery system that can provide a conducive environment for *B. megatherium* to solubilize phosphate ,enhance growth and yield parameters of plants and has the potential for utilization in commercial field application .The application of powder formulation of phosphate solubilizer *B. megatherium* spores undoubtedly shows their advantages over traditionally used free spore inoculation as they increase the efficacy of the spore , enhance cell viability , facilitate the delivery and handling processes and promote the growth parameters of the plant.

Finally, the powder bioformulations have a potential for large-scale applications instead of the traditionally used free spore inoculation**.**

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