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Research Article

Additives in powder based formulation for enhanced shelf life of *Pseudomonas fluorescens* and *Bacillus* sp.

R. RANGESHWARAN, N. V. VAJID, B. RAMANUJAM, S. SRIRAM, T. V. BHASKARAN and SATENDAR KUMAR

National Bureau of Agriculturally Important Insects, Post Bag No. 2491, H. A. Farm Post, Hebbal, Bellary Road, Bangalore 560 024, Karnataka, India.

Corresponding author E-mail: rangeshw@rediffmail.com

ABSTRACT: Studies were carried out to evaluate the effect of adding nutrient additives to talc based formulations on the shelf life of bacteria stored under room temperature. Two organisms namely, *Pseudomonas fluorescens* (PDBCAB2) and *Bacillus* sp. (MTCC6534 – chickpea endophyte), a spore forming bacterium, were tested. A gradual increase in *P. fluorescens* population up to 90 days was observed in almost all the treatments that were amended with nutrients. At 90 days highest population of log 10.38 cfu g^{-1} was noticed in talc amended with 2 per cent tryptone and glycerol. Decline in the population was observed from 90 days onwards and was rapid from 150 days onwards. At 240 days highest count of log 1 x $10^{4.2}$ cfu g^{-1} was obtained with formulations amended with 2% tryptone and 2% glycerol and the results indicated that 2% peptone or 2% tryptone supplemented with 2% glycerol helped *P. fluorescens* in its better survival. The spore forming *Bacillus* sp. survived well throughout the study period. At 240 days all talc formulations amended with nutrient sources showed a population of log 9.0 cfu g^{-1} or above and the population declined to below log 7.0 cfu g^{-1} only in non amended treatments. Slow decline started from 240 days but high cfu of log 9.30 to 9.38 g^{-1} were noticed in yeast extract or tryptone treated treatments. Hence talc formulations that had yeast extract or tryptone supplemented with glycerol enhanced shelf life of *Bacillus* spp.

KEY WORDS: *Pseudomonas fluorescens, Bacillus* sp., Powder based formulation, talc, shelf life, gram positive, gram negative

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INTRODUCTION

In India *Pseudomonas fluorescens* and *Bacillus subtilis* are two important bacterial biocontrol agents exploited commercially and are being used by farmers as seed treatment or foliar spray in many crops. They are mostly available as talc based powder formulations. Development of powder based inorganic carrier (talc, vermiculite, perlite, ground rock phosphate, diatomaceous earth and calcium sulphate) and organic carrier based (charcoal, peat) powder formulations of bacteria have been reported by several workers (Fravel *et al.,* 1998; Sarma *et al*., 2009; Wiyano *et al.,* 2008). Evidence on the efficacy of talc based formulation of *P. fluorescens* is available (Vidhyasekaran *et al*., 1997; Rangeshwaran and Prasad, 2000). Formulation problems with Gram-negative biocontrol agents are similar to those that have been faced in developing rhizobia, which are sensitive to drying and heat. Peat and other carriers may be useful. Powder formulations of *P. fluorescens* with different carrier materials have been developed and the bacterium survived for five months in talc formulations (Vidhyasekaran and Muthamilan, 1995). Spore forming bacteria like *Bacillus* sp. can survive drying and heat and are usually well preserved in formulations.

Bacteria proliferating in normal culture media will reach an abrupt stationary phase upon exhaustion of nutrients. Cell proliferation is halted completely and some cell death occurs. In continuous cultures carbon source is replenished at the end of log phase (Madigan and Martinko, 2006). In any formulation log phase cultures are formulated wherein cell proliferation is arrested and slow death of cells will occur. In the presence of alternate carbon source slow cell proliferation will occur and can compensate for the loss of mature cells. Spore formers will sporulate under unfavorable conditions and loss of viable cells is minimized. One way to enhance the efficacy

of inoculation is to improve the growth of introduced microbial population using nutrient amendments (Devliegher *et al*., 1995). Amendments can stimulate the growth of bacterial inocula but a large amount of carbon source is often required (Acea *et al*., 1988). Talc is a to natural mineral and chemically it is referred to as magnesium silicate ($Mg_3Si_4O_{10}(OH)_2$ and available as powder form. It has very low moisture equilibrium, relative hydro-phobicity, chemical inertness, reduced moisture absorption and prevents the formation of hydrate bridges that enable longer storage periods. Owing to inert nature and easy availability as raw material from soapstone industries it is used as a carrier for formulation (Nakeeran *et al.,* 2005). In view of the above an attempt was made to study the effect of adding nutrient additives to talc based formulations on the shelf life of bacteria stored under room temperature. Two organisms, namely, *Pseudomonas fluorescens* (PDBCAB2) and *Bacillus* sp., (MTCC6534 – chickpea endophyte) a spore forming bacterium, were tested in the present study. The two organisms have been previously reported to be having biocontrol ability (Rangeshwaran and Prasad, 2000; Rangeshwaran *et al*., 2008).

MATERIAL AND METHODS

Bacterial Isolates

The pure cultures of *Pseudomonas fluorescens* (PDBCAB2) and *Bacillus* sp., (MTCC6534 – chickpea endophyte) were obtained from the culture collection of Project Directorate of Biological Control (PDBC), Bangalore.

Culturing of bacteria

Mass multiplication *P. fluorescens* was done by inoculating King's B broth with 0.1% of bacterial culture (from late log phase) in 250 ml medium in 500ml flasks and incubating for 48h on a rotary shaker at 200 rpm (28º C). *Bacillus* sp. was grown in nutrient broth under similar conditions.

Formulation and Treatments

A modified talc formulation was used. The formulation was initially prepared as per Vidhyasekaran *et al.* (1997) and modified with nutrients. Nutrients added included 1 or 2% bacteriological peptone or yeast extract or tryptone. Nutrients fortified with 1 or 2% glycerol were also evaluated (Table 1). The powder mix was sterilized by autoclaving at 15 lbs per square inch (121º C for 30 min). Autoclaving was repeated after 24h incubation. The formulations were prepared by mixing 200ml of culture broth containing a minimum population of 9×10^8 cfu ml^{-1} with 500g of sterile talc powder (pre-mixed with 15 g calcium carbonate + 10g of carboxy methyl cellulose (CMC) + nutrient). Calcium carbonate was used to adjust the pH to 7.0. Control treatments included i) talc + calcium carbonate + CMC + culture broth, ii) talc + culture broth. The resulting mixture was thinly spread over metal trays and dried overnight under sterile conditions (approximately 35% moisture content). The mixtures were packed in polypropylene bags, sealed and stored at room temperature (25 ± 2°C). One gram samples were drawn periodically under aseptic conditions in a laminar flow chamber. Moisture analysis was done using a moisture analyzer (Denver, Model 2000).

Population estimation

Viable populations in the formulations were determined (Kloepper and Schroth, 1981) by grinding the talc mixture in a mortar and pestle, removing 1.0g and mixing it with 10ml of sterile water for 20 min. Serial 10-fold dilutions were prepared, and 0.1ml aliquots of each were spread on King's B Agar (for *P. fluorescens*) or Nutrient Agar (for *Bacillus* sp.). Observations were recorded at 24h and thereafter at every 30 days and concluded at 240 days.

RESULTS AND DISCUSSION

The viable population of *P. fluorescens* and *Bacillus* sp. in powder based talc formulations amended with different nutrient or carbon sources was monitored over a 240 day period under room temperature conditions. The changes in population among the two test bacteria varied. There was a gradual increase in *Pseudomonas* population for up to 90 days in almost all treatments that were amended with nutrients. At 90 days highest population of log 10.38 cfu g^{-1} was noticed in talc amended with 2 per cent tryptone and glycerol. In non-amended talc the population started to decline after 30 days and lowest (log 7.0 cfu g^{-1}) was in talc alone. Decline in population started from 90 days onwards in all treatments. The decline was rapid from 150 day onwards; however, standard population of around log 7.0 cfu g^{-1} was seen in all nutrient amended treatments at 180 days but in standard talc it declined to log 6.0 cfu/g and in talc alone low count of log 2.0 cfu g^{-1} was observed. At 240 days viable cells started to decrease and highest count of $1x10^{4.2}$ cfu g^{-1} was again obtained with formulations amended with 2% tryptone and 2% glycerol but formulations amended with peptone and glycerol also showed almost equal number of viable cells. The results indicate that 2% peptone or 2% tryptone supplemented with 2% glycerol helped *P. fluorescens* survive better (Table 1). Decrease in viable cells from the eighth month onwards was noticed. Ozaktan and Bora (2004) showed that the population of *Pantoea agglomerans* (a gm-ve bacterium) survived well for six months in talc formulation amended with glycerol and population of 1×10^8 was maintained for up to 180 days. In our studies *P. fluorescens* population of above log

Table 1. Survival of *Pseudomonas fluorescens* in talc based powder formulation amended with additives

Treatments	Viable *P. fluorescens* population (log cfu g^{-1})								
	----------DAYS----------								
	24h	30	60	90	120	150	180	210	240
Talc + 1% peptone + 1% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	9.90	9.86	9.92	9.96	9.28	8.86	7.00	6.00b	3.00[f]
Talc + 2% peptone + 2% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	10.11	10.12	10.17	10.20	9.92	9.00	7.48	6.22[a]	4.10[b]
Talc + 1% peptone + 1% CaCO3 + 0.1% carboxymethylcellulose	9.76	9.79	9.79	9.78	9.20	8.78	7.10	6.20[a]	3.20[d]
Talc + 2% peptone + 1% CaCO3 + 0.1% carboxy methylcellulose	8.68	8.76	9.80	9.80	9.00	8.20	7.00	5.28[c]	3.00[f]
Talc + 1% yeast extract + 1% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	8.83	8.85	9.03	9.00	9.00	8.02	7.00	5.24[c]	3.10[e]
Talc + 2% yeast extract + 2% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	9.17	9.18	9.05	9.04	8.72	8.30	7.26	4.28e	3.00[f]
Talc + 1% yeast extract + 1% CaCO3 + 0.1% carboxymethylcellulose	8.68	8.72	9.00	9.26	9.00	8.02	7.00	4.00[f]	3.02[F]
Talc + 2% yeast extract + 1% CaCO3 + 0.1% carboxymethylcellulose	8.65	8.72	9.00	9.20	9.00	8.00	7.02	4.00[f]	3.00[f]
Talc + 1% tryptone + 1% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	9.83	9.86	9.90	9.92	9.20	8.44	7.46	6.06[b]	4.00c
Talc + 2% tryptone + 2% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	9.79	9.90	10.46	10.38	9.88	9.02	7.20	6.00[b]	4.20[a]
Talc + 1% tryptone + 1% CaCO3 + 0.1% carboxymethylcellulose	8.98	8.76	9.90	9.80	9.00	8.00	7.00	5.00[d]	3.00[f]
Talc + 2% tryptone + 1% CaCO3 + 0.1% carboxymethylcellulose	8.64	8.38	8.60	8.00	7.80	7.06	6.20	5.00[d]	2.00[g]
Talc + 1% CaCO3 + 0.1% carboxymethylcellulose	8.34	8.38	8.34	7.82	7.20	7.00	6.00	3.00[g]	2.00[g]
Talc alone	8.32	8.30	7.32	7.00	6.30	3.22	2.00	1.00[h]	ND*
CD ($P \geq 0.05\%$)	0.29	0.04	0.05	0.04	0.15	0.23	0.07	0.08	0.07

*ND=not detected

Table 2. Survival of *Bacillus* sp. in talc based powder formulation amended with additives

Treatments	Viable *P. fluorescens* population (log cfu g^{-1})								
	----------DAYS----------								
	24[h]	30	60	90	120	150	180	210	240
Talc + 1% peptone + 1% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	8.52[f]	8.85[d]	9.32	9.32[c]	9.22e	9.72[c]	9.20[d]	9.00[d]	9.00[d]
Talc + 2% peptone + 2% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	9.15[d]	9.97[a]	9.97	9.95[a]	9.90[b]	9.70[c]	9.28[c]	9.00[d]	9.10[c]
Talc + 1% peptone + 1% CaCO3 + 0.1% carboxymethylcellulose	8.40[h]	8.92[e]	9.36	9.34[c]	9.30[d]	9.20[d]	9.00e	9.00[d]	9.00[d]
Talc + 2% peptone + 1% CaCO3 + 0.1% carboxy methylcellulose	9.26[c]	9.98[a]	9.98	9.95[a]	9.90[b]	9.78[b]	9.22[d]	9.10[c]	9.00d
Talc + 1% yeast extract + 1% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	8.45[g]	8.86[d]	9.97	9.96[a]	9.92[b]	9.90[a]	9.90[a]	9.82[b]	9.30[b]
Talc + 2% yeast extract + 2% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	8.18[i]	8.65[d]	9.98	9.98[a]	9.96[a]	9.92[a]	9.90[a]	9.90[a]	9.38[a]
Talc + 1% yeast extract + 1% CaCO3 + 0.1% carboxymethylcellulose	9.00[e]	8.90[e]	9.36	9.34[c]	9.30[d]	9.20[d]	9.20[d]	9.00[d]	9.00[d]
Talc + 2% yeast extract + 1% CaCO3 + 0.1% carboxymethylcellulose	9.00[e]	9.98[a]	9.98	9.95[a]	9.90[b]	9.78[b]	9.20[d]	9.00[d]	9.10[c]
Talc + 1% tryptone + 1% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	9.58[a]	9.85[b]	9.89	9.89[b]	9.78[c]	9.90[a]	9.80[b]	9.90[a]	9.30[b]
Talc + 2% tryptone + 2% glycerol + 1% CaCO3 + 0.1% carboxymethylcellulose	9.54[ab]	9.91[c]	9.98	9.95[a]	9.95[a]	9.90[a]	9.90[a]	9.90[a]	9.28[b]
Talc + 1% tryptone + 1% CaCO3 + 0.1% carboxymethylcellulose	9.56[b]	9.90[c]	9.36	9.34[c]	9.30[d]	9.20[d]	9.20[d]	9.00[d]	9.00[d]
Talc + 2% tryptone + 1% CaCO3 + 0.1% carboxymethylcellulose	9.52[b]	9.92[c]	9.98	9.90[c]	9.90[b]	9.20[d]	9.20[d]	9.00[d]	9.10[c]
Talc + 1% CaCO3 + 0.1% carboxymethylcellulose	8.40[h]	7.85[f]	7.83	7.83[d]	7.58[f]	7.50[e]	7.20[f]	7.00[e]	6.90[f]
Talc alone	8.40[h]	7.48[g]	7.48	7.47[e]	7.20[g]	7.00[f]	7.00[g]	7.00[e]	6.80[g]
CD (P?0.05%)	0.04	0.04	NS*	0.03	0.03	0.05	0.06	0.10	0.05

*ND=not detected

1 x 10^6 cfu g^{-1} was maintained for up to 210 days indicating that nutrient amendments in talc formulations help *P. fluorescens* survive better. Further in non-amended talc formulations the population of *P. flourescens* was drastically reduced at 240 days and in formulations that had only the stock culture and talc, no viable cells were detected at 240 days. Talc as a carrier is used almost invariably to produce biopesticides as it is an inert material and does not promote multiplication of bioagents during storage but rather longer storage can cause decrease in the viability. Reports indicate varied survivability of *P. fluorescens* in talc – some claim only 45 days survival (Amar and Ukhede, 2000) but other reports indicate 6-8 months of survival (Vidhyasekharan *et al,* 1997; Bora *et al*, 2004). Our talc amended *P. fluorescens* formulations showed 8 months of survival. Wiyano *et al*. (2008) found that the survival of *P. fluorescens* decreased rapidly from an initial density of about 8 log cfu g^{-1} in dry diatomaceous earth and cotton flour and the antagonist did not survive storage of 4 months in diatomaceous earth and 6 months in cotton flour formulations. Bacterial survival in both types of dry wood flour declined considerably down to 2 log cfu g^{-1} after 7 months and no antagonist could be detected after 12 months.

The spore forming bacterium *Bacillus* sp. survived well throughout the study period. As shown in Table 2 at 240 days all talc formulations that were amended with nutrient sources showed a population of 9.0 cfu g^{-1} or above. The population declined to below 7.0 cfu g^{-1} only in non-amended treatments. Jayraj *et al*. (2005) tested the survival of *B. subtilis* in powder formulations of talc, lignite, lignite + fly ash, wettable powder, bentonite-paste, polyethylene glycol (PEG) paste and a water-dispersible tablet and found that populations of bacteria in the formulations were stable for up to 2 years storage at room temperature (28°C). However they also noticed that viability of *B. subtilis* decreased after one year in talc formulation. Whether talc is ideally suited for *Bacillus* needs to be further evaluated. Shelf life of spore forming bacteria in any formulation is always higher than that of non-spore formers. Our studies showed that at 240 days there was a slow decline in cell numbers in all treatments and high cfu of log 9.30 to 9.38 per gm were noticed in yeast extract or tryptone treated treatments (Table 2). It was concluded talc formulations that had yeast extract or tryptone supplemented with glycerol enhanced the shelf life of *Bacillus* sp. With the availability of alternate carbon/nitrogen source gradual multiplication of cells occurs. Spore formers sporulate under unfavorable conditions and loss of viable cells is minimized. Amendments can stimulate the growth of bacterial inocula but a large amount of carbon source is often required (Acea *et al*., 1988). Schmidt *et al*., (2001) reported that biocontrol activity of *B. subtilis* increased by adding peptone (0.25%) in medium and in *E. herbicola* by adding glucose (0.5%) or high concentrations of peptone (1–2%) to the medium. In this study the antagonistic activity of the test organisms were was established through evaluation of talc based formulations (Rangeshwaran and Prasad, 2000, Rangeshwaran *et al*., 2008).

In India *Pseudomonas* is available mostly as talc based formulations (0.5% W.P) based on the formulation adopted by Vidhyasekaran *et al*. (1997) and the quality check of biological products like microbial formulations is mostly carried out on fresh formulations which usually meet the quality requirements. But the product available to farmers may be six months old and the microbial population may have rapidly declined. Most of them claim six months shelf life and with the present regulation of a minimum of 10^7cfu g^{-1} it is difficult to maintain the population as the formulations will be invariably stored at room temperature. Under refrigerated conditions the shelf life can be doubled but this is not usually followed. The results indicate that powder based talc formulations fortified with 2% peptone or 2% tryptone supplemented with 2% glycerol enhanced the shelf life of *P. fluorescens* whereas 1 to 2% yeast extract or tryptone supplemented with glycerol improved the shelf life of *Bacillus* sp. Though a profound effect was not seen by addition of additives they at least helped the bacteria survive better. This information will be useful for large scale formulators as it will ultimately help in keeping quality of powder based formulations.

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