

Original Research Article

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Liquid Formulation Using Different Oils and Shelf Life Study of Effective Bacterial Bio-Agents

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ABSTRACT

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Liquid formulation of *Bacillus subtilis*-KK-9A, *Brevibacillus borstelensis*-BK-6, *Brevibacillus* sp-PM-2A, *Lysinibacillus xylanilyticus*-VK-6B and consortium were developed using distilled water, ground nut oil, nutrient broth, pongamia oil and sunflower oil to enhance the shelf life and efficacy of the biocontrol agent besides easing the delivery of bio-inoculants through micro-irrigation techniques. The highest population level of *Bacillus subtilis*-KK-9A, *Brevibacillus borstelensis*-BK-6, *Brevibacillus* sp-PM-2A and *Lysinibacillus xylanilyticus*-VK-6B were observed in pongamia oil amended with glycerol even after 3 months of storage. Pongamia oil based formulation was found best formulation followed by ground nut oil, sunflower oil, distilled water, nutrient broth based formulation which retains the highest per cent survival of *Bacillus subtilis* (45.05), *Brevibacillus borstelensis* (32.93), *Brevibacillus* sp (48.80), *Lysinibacillus xylanilyticus* (60.80), and consortium 31.12 per cent even after 90 days.

Introduction

Biological management of plant pathogen by antagonistic microorganisms is a potential non chemical means (Harman, 1991) and is known to be a cheap and effective, eco-friendly method for management of crop disease (Cook and Baker, 1983). Some of the bio-control agents like *Bacillus subtilis* and *Pseudomonas fluorescence* exhibit inhibition zone and found antagonistic to *Xanthomonas axonopodis* pv. *punicae* (Yenjerappa *et al.*, 2013).

In this context biological management of plant pathogen by antagonistic microorganisms is taking upper hand.

When the powder formulations were used for the management of plant diseases in horticultural and plantations crops through micro-irrigation techniques, the concerns are raised on blockage of nozzles and distribution of bio-inoculants. In addition, the process of bio-hardening in horticultural plantlets and tissue culture plants, necessitates the development of liquid-based bio-formulations. In addition, it has been demonstrated that the development of liquid formulation has several advantages including high cell count, zero contamination, longer shelf life, greater protection against environmental stresses and increased field efficacy (Hegde, 2002; Vendan and Thangaraju, 2006). In liquid formulation,

the microbial organisms are present in a dormant cyst form and after application in the field, the dormant form gives rise to active cells. This helps to increase the shelf life of liquid bioformulation for more than 1 year. So that current research has been conducted on formulation of four effective bio-agents (*Bacillus subtilis*-KK-9A, *Brevibacillus borstelensis*-BK-6, *Brevibacillus* sp-PM-2A and *Lysinibacillus xylanilyticus*-VK-6B) and consortium by using oils viz., distilled water, ground nut oil, nutrient broth, pongamia oil and sunflower oil.

Materials and Methods

Bacterial bio-agents

Four bacterial biocontrol agents viz., *Bacillus subtilis*-KK-9A, *Brevibacillus borstelensis*-BK-6, *Brevibacillus* sp-PM-2A and *Lysinibacillus xylanilyticus*-VK-6B which were previously identified as effective biocontrol agents against *Xanthomonas axonopodis* pv. *Punicae* (Ippikoppa, 2015) used in this study.

Efficacy of bacterial bio-agents against *X.a.p* by agar well diffusion method

The efficacy of these bio-agents were reconfirmed by agar well method it involves making of nutrient broth and before pour into petriplates add *X.a.p* culture into it, after solidification, an agar plate is punched aseptically with sterile cork borer and volume of the (20-100 μ l) of culture filtrate is introduced into well. Then, the culture filtrate of each bio-agent can be poured into well and it was incubated at 30°C for 48 hr.

Procedure for culture preparation and formulation

Bacterial antagonists (*Brevibacillus* sp-SM-1A, *Lysinibacillus xylanilyticus*-VK-6B,

Brevibacillus borstelensis-BK-6 and *Bacillus subtilis*-KK-9A) were grown on nutrient broth for 72 hrs, on an orbital shaker at 150 rpm at 30 °C, till the maximum spore yield was produced, these were harvested and subsequently washed by repeated centrifugation at 8,000 rpm for 20 min at 4 °C in sterile distilled water (Warriner and Waites, 1999). Finally, the spore pellet was re-suspended in sterile Nutrient broth and used as active material in different formulations. The final spore titer was adjusted to $\geq 10^8$ CFU/ml.

Different types of oils used in liquid formulation

Distill water, Ground nut oil, NA broth, Pongamia oil, Sunflower oil,

In case of liquid formulation the culture preparation was same in case of powder formulation. However in the preparation of 100 ml of liquid formulation 35 ml of glycerol, 8.5 ml of oil, and 0.1% Tritan -x as stabilizer and distill water and pellet mixture in the ratio 3:1 were added. The details were mentioned in the table 1.

Enumeration of CFU

The shelf life was determined by enumerating the population of bacterial antagonists by serial dilution Plate method using nutrient agar medium. The dilution was prepared by taking 1 ml of formulation in 9 ml sterilized water, shaken well on orbital spinax for 5 min and further diluted to get the final dilution of 10^7 .

From these dilutions, 0.1 ml was spread on sterilized Petri plates and sterilized media was poured over it. The plates were then given a gentle swirl clockwise and anti-clockwise to distribute the suspension uniformly in the medium. These plates were incubated at 25°C and observed regularly for the appearance of microbial colonies.

The population (cfu/g) was counted by average number of colonies (average of 10^5 , 10^6 and 10^7) developed in each treatment.

Results and Discussion

Shelf life of bioagents in liquid formulation

In case of liquid formulation of *Bacillus subtilis*, *Brevibacillus borstelensis*, *Brevibacillus sp* and *Lysinibacillus xylanilyticus* using distilled water, ground nut oil, nutrient broth, pongamia oil and sunflower oil, the best formulation was pongamia oil based formulation followed by ground nut oil, sunflower oil, distilled water and nutrient broth. The results were depicted in the Figure 1.

Bacillus subtilis

The shelf life of the *Bacillus subtilis* in the liquid formulations was tested and the data is presented in the table 2. Among the liquid carriers tested the pongamia oil was found best with the highest colony count even after the 90 days. The initial colony count at the time of first sampling was 45.50 which declined as the number of days proceeded. The colony count recorded was 44.50, 39.50, 33.00, 31.50, 25.75 and 20.50 at 15th, 30th, 45th, 60th and 75th day respectively.

The next best treatment among the tested formulations was groundnut oil, which significantly differed from the pongamia oil. The ground oil formulation recorded 14.50 colonies after the 90th day with the initial count of 45.00.

The sunflower oil formulation was to found significant with the 8.75 colonies at the 90th day where the initial count recorded was 44.25.

Though the distilled water and nutrient broth varied significantly they recorded the least

colony count of 5.75 and 1.75 respectively after the 90th day with the initial colony count of 43.50 and 46.50.

The highest survival per cent of 45.05 *Bacillus subtilis* at 90th day was observed in pongamia oil followed by ground nut oil (32.22per cent), sunflower oil (19.77per cent), distilled water (13.22per cent) and nutrient broth (3.76per cent).

Brevibacillus borstelensis

The shelf life of *Brevibacillus borstelensis* in different liquid carrier material were recorded and depicted in the table 3. Among the different liquid formulations tested, pongamia oil recorded the highest colonies at the 90th day (13.50), which was on par with ground nut oil, the initial count of 41.00 and 40.50 were recorded in pongamia oil and groundnut oil respectively. The decline in colonies was observed from initial count to 38.75, 32.25, 24.50, 18.25 and 18.75 respectively in case of pongamia oil.

The other treatment viz., sunflower oil, distilled water and nutrient broth recorded the 7.00, 3.50, 1.25 colonies at the 90th day and these values were found significantly different.

Among the different liquid formulation tested for *Brevibacillus borstelensis* survivability the pongamia oil recorded the highest survival percentage of 32.93 followed by groundnut oil (28.40 %), sunflower oil (18.30 %), distilled water (8.48per cent) and nutrient broth (2.96per cent).

Brevibacillus sp

The *Brevibacillus sp* tested for its shelf life in the liquid formulations and observations recorded at different intervals are depicted in the table 4.

Table.1 Treatment details of liquid formulation

Sl. No	Formulation	Composition for 100ml formulation
1	Distill water	Distilled water and pellet mixture in Nutrient broth 3:1(65ml) Glycerol (35ml); Tritan-x (0.1ml)
2	Ground nut oil based	Ground nut oil (8.5ml); Glycerol (35ml); Tritan-x (0. 1ml) Distilled water and pellet mixture in Nutrient broth (56.5m)
3	NA broth	Nutrient broth (8.5ml); Distilled water and pellet mixture in Nutrient broth 3:1 (56.5ml)
4	Pongamia oil based	Pongamia oil (8.5ml); Glycerol (35ml); Tritan-x (0. 1ml) Distilled water and pellet mixture in Nutrient broth 3:1 (56.5ml)
5	Sunflower oil based	Sunflower oil (8.5ml); Glycerol (35ml); Tritan-x (0. 1ml) Distilled water and pellet mixture in Nutrient broth 3:1(56.5ml)

Table.2 Shelf life *Bacillus subtilis* in different oil formulations

Sl. No.	Shelf life	x10 ⁶ CFU/g of formulation							Survival %
		0 day	15 th day	30 th day	45 th day	60 th day	75 th day	90 th day	
1	Distilledl water	43.50	26.25	21.00	17.75	14.50	11.25	5.75	13.22
2	Ground nut oil	45.00	39.50	33.50	26.75	22.50	18.25	14.50	32.22
3	Nutrient broth	46.50	39.25	18.50	11.50	6.00	2.25	1.75	3.76
4	Pongamia oil	45.50	44.50	39.50	33.00	31.50	25.75	20.50	45.05
5	Sunflower oil	44.25	32.25	23.25	21.75	18.50	15.25	8.75	19.77
SEm±			0.79	1.20	1.22	0.81	1.00	0.82	
CD (0.01)		NS	3.30	5.04	5.09	3.40	4.20	3.42	

Table.3 Shelf life *Brevibacillus borstelensis* in different oil formulations

Sl. No.	Shelf life	x10 ⁶ CFU/g of formulation							Survival%
		0 day	15 th day	30 th day	45 th day	60 th day	75 th day	90 th day	
1	Distilled water	41.25	21.75	17.50	15.75	13.50	9.50	3.50	8.48
2	Ground nut oil	40.50	31.00	28.75	20.50	18.25	13.75	11.50	28.40
3	Nutrient broth	42.25	29.50	16.25	11.50	4.75	2.75	1.25	2.96
4	Pongamia oil	41.00	38.75	32.25	24.50	18.25	18.75	13.50	32.93
5	Sunflower oil	38.25	24.25	22.25	18.75	15.00	12.75	7.00	18.30
SEm±			1.29	0.91	1.03	1.04	0.55	0.62	
CD (0.01)			5.39	3.83	4.33	4.36	2.32	2.62	

Table.4 Shelf life of *Brevibacillus* sp. in different oil formulations

Sl. No.	Shelf life	x10 ⁶ CFU/g of formulation							Survival %
		0 day	15 th day	30 th day	45 th day	60 th day	75 th day	90 th day	
1	Distilled water	40.50	20.75	14.25	11.50	11.00	7.25	4.00	9.90
2	Ground nut oil	41.25	24.50	21.75	17.25	15.75	14.50	8.50	20.60
3	Nutrient broth	41.75	18.75	10.50	9.50	8.00	6.50	2.00	4.80
4	Pongamia oil	41.50	33.25	31.00	25.75	25.00	21.50	20.25	48.80
5	Sunflower oil	40.25	22.25	18.25	14.75	12.00	12.00	6.50	16.10
SEm±			2.01	0.80	1.03	0.99	0.66	0.97	
CD (0.01)			8.38	3.36	4.32	4.16	2.76	4.06	

Table.5 Shelf life of *Lysinibacillus xylanilyticus* in different oil formulations

Sl. No.	Shelf life	x10 ⁶ CFU/g of formulation							Survival %
		0 day	15 th day	30 th day	45 th day	60 th day	75 th day	90 th day	
1	Distilled water	35.75	18.50	17.50	11.25	9.50	9.50	9.50	26.60
2	Ground nut oil	36.75	31.75	25.25	18.50	14.75	14.75	14.75	40.10
3	Nutrient broth	38.25	31.00	16.00	6.50	3.25	2.00	1.25	3.30
4	Pongamia oil	37.00	35.75	31.25	24.50	22.50	22.50	22.50	60.80
5	Sunflower oil	36.25	24.50	22.50	14.25	14.00	14.00	14.00	38.60
SEm±			0.81	0.86	1.14	0.88	0.87	0.88	
CD (0.01)			3.37	3.60	4.75	3.70	3.66	3.70	

Table.6 Shelf life Consortium in different oil formulations

Sl. No.	Shelf life	x10 ⁶ CFU/g of formulation							Survival %
		0 day	15 th day	30 th day	45 th day	60 th day	75 th day	90 th day	
1	Distilled water	48.75	23.50	19.50	18.50	13.50	11.50	7.75	15.90
2	Ground nut oil	49.00	40.00	35.25	31.75	22.75	16.00	13.50	27.55
3	Nutrient broth	49.50	40.50	26.50	16.00	10.75	3.50	1.50	3.03
4	Pongamia oil	49.00	46.25	41.00	36.00	30.25	19.50	15.25	31.12
5	Sunflower oil	48.50	34.50	31.00	26.70	19.00	14.50	12.25	25.26
SEm±			0.813	0.87	0.92	0.88	0.81	0.76	
CD (0.01)		NS	3.39	3.66	3.85	3.69	3.40	3.18	

Fig.1 Shelf life in different oil formulations

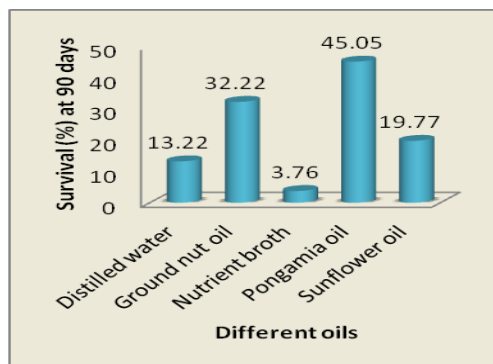


Fig.1a *Bacillus subtilis*

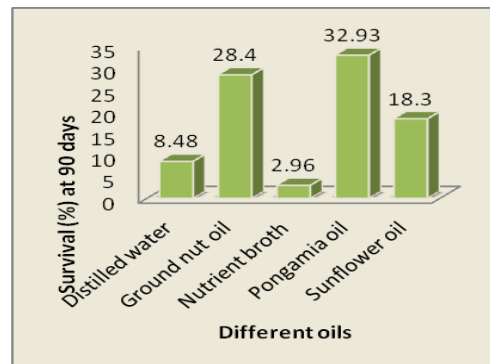


Fig.1b *Brevibacillus borstelensis*

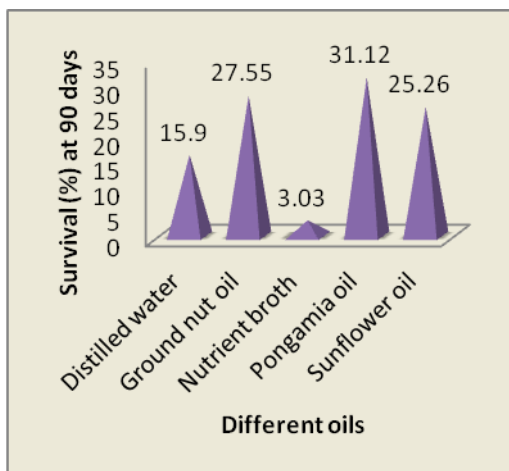


Fig.1c *Brevibacillus* sp.

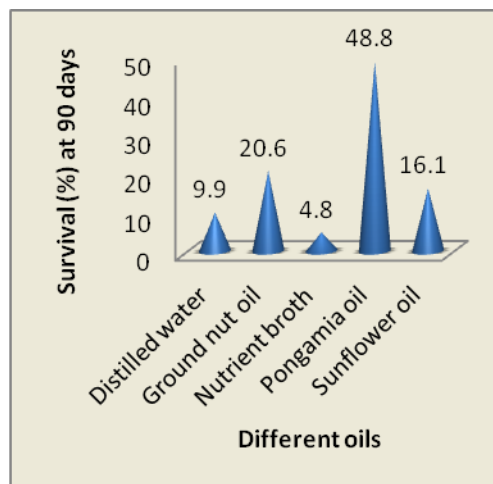


Fig.1d *Lysinibacillus xylanilyticus*

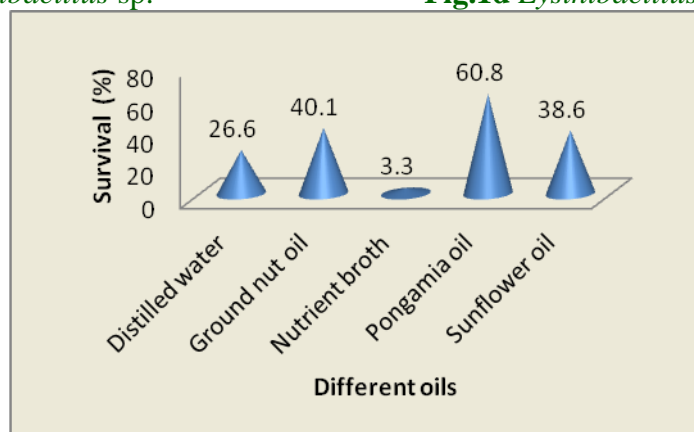


Fig.1e Consortium

Among the tested formulation the pongamia oil and ground oil were found significant. The highest number of colonies (20.25) was recorded in case of pongamia oil, where the initial count was 41.50. However the colony count declined from initial count to 33.25, 31.00, 25.75, 25.00, 21.50 and 20.25 at 15th, 30th, 45th, 60th and 75th day respectively.

The next best treatment groundnut oil recorded the 8.50 colonies @ 90th day where the initial count recorded was 41.25.

The sunflower oil recorded the 6.50 colonies at the 90th day and found on par with the ground nut oil and distilled water. The distilled water recorded the 4.00 colonies at the 90th day which was on par with the 2.00 colonies as observed.

The survival per cent was recorded for the *Brevibacillus* sp. on the he liquid formulations.

As per the recorded observations the pongamia oil showed highest survival (8.80 %) followed by ground nut oil (20.6per cent) sunflower oil (16.1per cent), distilled water (9.9per cent) and nutrient broth (4.8per cent).

Lysinobacillus xylaniticuIs

The *Lysinobacillus xylaniticuIs* when tested for the shelf life in different liquid formulations the pongamia oil recorded the highest CFU (22.50) at 90th day where the initial count recorded was 32.00 CFU, and the data presented in the table 5. The colony count declined from initial count to 35.75, 31.

25, 24.50, at 15th, 30th, 45th day respectively and after which no change was observed in CFU till the 90th day. The ground nut oil was proven as second best treatment with CFU of the 14.75 at 90th day and varied significantly from pongamia oil. However, the Sunflower oil was found on par with the ground nut oil where 14.00 CFU recorded at 90th day.

The distilled water and nutrient broth recorded the 9.50 and 1.25 CFU at 90th day respectively and varied significantly from each other. The pongamia oil showed the highest survival per cent of 60.8 followed by Groundnut oil (40 %), sunflower (38.6per cent) distilled water (26.6per cent) and nutrient broth (3.30 %).

Consortium

Shelf life of bacterial consortium was tested up to 90 days on different liquid formulations and observation recorded is presented in the table 6. With regard to consortium pongamia oil was found best with 15.25 CFU at 90th day where the initial count was 49.00.

The colony count declined from initial count to 46.25, 41.00, 36.00, 30.25, 19.50, 15.25 @ 15th, 30th, 45th, 60th, 75th, 90th day respectively.

The pongamia oil was found on par with Ground nut oil where the CFU recorded at 90th day was 13.50. The sun flower oil recorded the 12.25 CFU and was found to be on par with the ground nut oil. The treatments such as distilled water and nutrient broth showed 7.75 and CFU at 90th day respectively.

The highest survival per cent of consortium observed in the pongamia oil (31.27 per cent) followed by Ground nut oil (27.55 per cent), sunflower (25.26 per cent) distilled water (15.9 per cent) and nutrient broth (3.30 %).

Mainly researchers confirmed that the formulations of microbial biomass developed as biological control products grown in different liquid media of differing composition shown extended shelf life (Sathiyaseelan *et al.*, 2009 and Krishan and Greep, 2010).

Many workers while working with various bioagents have proved that their shelf life has improved significantly when these organisms are mixed with different oils/liquids in place of solid based substrates.

Moore *et al.*, (1995) proved that long term storability of *Metarrhizium flavoviride* in oils such as soya and groundnut or mineral oil retaining 47 to 68 per cent viability and was after 16 to 18 months of storage.

Similarly, Ramyabharathi *et al.*, (2016), formulated the *B. subtilis* using the chemical amendments glycerol and trehalose for increasing the shelf life, in that addition of glycerol to SOC broth recorded higher level of sporulation throughout the period of 365 days. The least sporulation was observed in broth alone.

Overall, our study was showed the liquid formulation helps in retaining higher count of viable population was found in pongamia oil followed by ground nut oil, sunflower oil, distilled water, nutrient broth upto 90 days. The pongamia oil retains the highest per cent survival of *Bacillus subtilis* (45.05), *Brevibacillus borstelensis* (32.93), *Brevibacillus* sp (48.80), *Lysinibacillus xylanilyticus* (60.00) and consortium (31.12).

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