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RESEARCH PAPER

Growth kinetics of *Pseudomonas fluorescens* in different media and evaluation of shelf life

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Abstract: Biopesticides open up a wide scope for restoration of environment that has been damaged through continuous use of chemicals. The study focused on assessing the growth kinetics of *Pseudomonas fluorescens* in various liquid media and evaluating their shelf life. Seven media were analysed for their ability to assist the growth and multiplication of the bacterium. Among the various media evaluated, Trypticase Soya broth (TSB) was considered as the effective basal medium owing to its ability to maintain high population, lesser generation time and lesser cost. Further, it was formulated with various amendments like glycerol and PVP (cell protectants), CMC (adjuvant) and Tween 20 (surfactant) and the formulation (TSB+ Glycerol 2%+ PVP 1%+ CMC 0.05%+ Tween-20 0.5%) was found to be promising in sustaining the higher population of *Pseudomonas fluorescens* up to 240 days of storage.

Key words: Biopesticide, Cell amendments, Liquid formulation, *Pseudomonas fluorescens*, Shelf life

Introduction

Integration of plant growth promoting bacteria in sustainable agriculture has been practiced over several decades. Even though there are ample of promising research regarding the antagonism of bioagents under lab conditions, the field efficacy offered by them is yet a puzzle to be solved. This is often attributed to the various environmental stresses they have to cope up with, the major ones being adaptation to introduced niche, competition of already established organisms and inability to multiply. This emphasizes the need of formulating the bioagents for better performance in field conditions (Bashan *et al*., 2014).

Majority of microbial formulations in India at present are mostly carrier based, and are widely used in that form. But these carrier-based formulations have many shortcomings, the prominent among them being the reduced shelf life of the produce, higher contamination, intolerance towards UV rays and high temperature, bulkiness, blockage of nozzles in drip irrigation and lower field performance. In this context, a better alternative is the liquid formulations that contain the cultures or cell suspensions of the microbial organisms, amended with surfactants, adjuvants, stabilizers, preservatives, *etc*., that can sustain the viability and support its growth by providing necessary nutrients (Vora *et al*., 2008; Teixidó *et al*., 2022). Apart from showing high cell count, they also exhibited increased shelf life of up to one year, zero contamination, improved protection against biotic and abiotic stresses which boosted the field efficacy compared to peat-based inoculants (Singleton *et al*., 2002).

Therefore, the aim of this research was to develop a formulation of *Pseudomonas fluorescens*, which is regarded as an efficient antagonist in curbing the pathogens (Panpatte *et al*., 2016; Mehmood *et al*., 2023), that can maintain the shelf life and potency.

Material and methods

Pseudomonas fluorescens (IOF) strain (NAIMCC-B-01981) from the Institute of Organic Farming, University of Agricultural Sciences, Dharwad, was used to develop the formulation.

Selection of suitable growth medium by developing the growth curve

Seven different media *viz.*, King's B broth (KB), Nutrient Broth (NB), Trypticase Soya Broth (TSB), Luria- Bertani Broth (LB), Pseudomonas (Fluorescein) broth (PF), Minimal salt medium (M9) and Potato Dextrose broth (PDB), were taken to evaluate the growth of *P. fluorescens*. One day old culture of *Pseudomonas fluorescens* was inoculated @ ten per cent to different media and were incubated at room temperature (100 rpm). Growth curve of *P. fluorescens* was developed by enumerating the number of viable cells by standard plate count technique on alternate hours for 24 h in different media. The petri plates were incubated at 30 ± 1°C for 48 h and the number of colonies were calculated using the formula: average no. of colonies × dilution factor/ Volume taken for dilution. Growth curve was obtained by plotting colony forming units as a function of time. The data were used to calculate the generation time of *Pseudomonas fluorescens* in different culture media, using the formula (Madigan *et al.,* 2015):

Generation time (g) = t/n

Where,

t- time interval in hour, between-two estimates

n-Number of generations ($n = 3.3 (\log N - \log N_0)$)

(N = the final cell count; N_0 is initial cell count)

The best basal medium for the formulation was selected based on criteria like minimum generation time, the highest population achieved and the time taken for the same, contamination level and cost economics.

Table 1. Population of *Pseudomonas fluorescens* in different media

Media	Population in log transfromed values (10^{10} cfu/ml)												
	0thh	2ndh	4thh	6thh	8thh	10thh	12thh	14thh	16thh	18thh	20thh	22ndh	24thh
KB	8.078	8.079	8.174	8.195	8.700	9.050	9.213	9.277	9.414	9.588	9.845	9.715	9.560
	(0.012)	(0.012)	(0.015)	(0.016)	(0.050)	(0.112)	(0.163)	(0.189)	(0.260)	(0.387)	(0.700)	(0.519)	(0.363)
NB	8.037	8.045	8.146	8.177	8.68	9.018	9.150	9.235	9.342	9.495	9.713	9.486	9.332
	(0.011)	(0.011)	(0.014)	(0.015)	(0.048)	(0.104)	(0.141)	(0.172)	(0.220)	(0.313)	(0.516)	(0.306)	(0.215)
TSB	8.082	8.090	8.185	8.198	8.710	9.152	9.499	9.595	9.699	9.889	10.175	10.209	10.300
	(0.012)	(0.012)	(0.015)	(0.016)	(0.051)	(0.142)	(0.316)	(0.393)	(0.500)	(0.770)	(1.496)	(1.618)	(1.990)
PF	7.940	8.017	8.028	8.056	8.293	8.491	8.572	8.653	8.776	8.890	9.157	9.390	9.588
	(0.009)	(0.010)	(0.011)	(0.015)	(0.019)	(0.031)	(0.037)	(0.045)	(0.059)	(0.078)	(0.143)	(0.245)	(0.387)
LB	8.024	8.053	8.093	8.154	8.572	8.999	9.123	9.225	9.374	9.500	9.623	9.704	9.743
	(0.011)	(0.011)	(0.012)	(0.014)	(0.037)	(0.099)	(0.132)	(0.168)	(0.236)	(0.316)	(0.420)	(0.506)	(0.553)
PDB	7.603	7.713	7.722	7.800	8.050	8.271	8.322	8.411	8.596	8.609	8.810	9.033	9.047
	(0.004)	(0.005)	(0.005)	(0.006)	(0.011)	(0.018)	(0.021)	(0.026)	(0.040)	(0.041)	(0.064)	(0.108)	(0.111)
M9	7.271	7.301	7.325	7.470	7.679	8.033	8.206	8.340	8.512	8.599	8.698	8.835	8.885
	(0.002)	(0.002)	(0.002)	(0.003)	(0.005)	(0.011)	(0.020)	(0.022)	(0.032)	(0.040)	(0.050)	(0.068)	(0.077)

Note: KB- King's B Broth; NB- Nutrient Broth; TSB-Trypticase Soya Broth; PF- Pseudomonas (Fluorescein) broth; LB- Luria Bertani Broth; PDB- Potato Dextrose broth; M9- Minimal salt medium *Values in parenthesis represent original CFU (Colony forming units×10^{10})

Preparation of liquid formulations of *Pseudomonas fluorescens* and assessment of shelf life

Once the best basal medium was selected, it was amended with two concentrations each of cell protectants (Poly vinyl pyrrolidone (PVP) (@ 1% and 2%) and glycerol (@ 2% and 1%), adjuvant (carboxy methyl cellulose (CMC) (@ 0.05 % and 0.10%) and surfactant (Tween-20 (@ 0.5% and 1.0%) (Santhosh, 2015; Biradar and Santhosh, 2018; Gopi *et al*., 2019). Thus, a total of 16 formulations with all the combinations of these amendments were evaluated and shelf life was determined by serial dilution and plate count method for following eight months.

Results and discussion

Among the seven media evaluated, the population observed in Trypticase Soy broth (1.99×10^{10} CFU ml^{-1}) was found to be highest at the end of 24th h of observation, followed by the population in Luria Bertani broth (0.553×10^{10} CFU ml^{-1}). In KB and NB broths, the population increased till 20th h in incubation, but there was a sudden decline in population afterwards.The least population at 24th h (0.077×10^{10} CFU ml^{-1}) was recorded with medium M9 (Table 1).

Trypticase Soya broth (TSB) exhibited the least generation time of 1.26 h, followed by King's B broth (1.41 h). It was comparable with that of Luria-Bertani Broth (1.42 h) and Nutrient Broth (1.43 h). The highest generation time for this bacterium was recorded in Pseudomonas (Fluorescein) broth (2.77 h). When the cost of the media was taken into consideration, Pseudomonas (Fluorescein) broth was the costliest, recording 15,706 ₹ /100 *l*, followed by King' s B broth (12,714 ₹ /100 *l*). Minimal salt medium (M9) was the least costly. (2,201.78 ₹ /100 *l*) (Table 2). Thus, Trypticase Soya broth was finally selected as the basal medium for shelf life studies, owing to its least generation time, lesser cost and ability to maintain higher population. The growth curves of the bacterium in different media are depicted in Fig 1. Vyas *et al*. (2014), revealed that the highest population of viable colonies of phosphate solubilizing bacteria, *Pseudomonas trivialis* BIHB 745 were maintained in Trypticase soy Broth, among the seven different media (King's B medium, Trypticase soya broth, TSB with 2% methanol, Nutrient broth, glucose yeast extract medium, *Pseudomonas* medium A and *Pseudomonas* medium 2) evaluated. The use of TSB for mass production of *P. fluorescens* was further authenticated by Rajeshwari and Appanna (2021), who found it ideal for maintaining the optimal growth of the bacteria.

Table 2. Properties of various media used in the study

Media	Generation time (h)	Cost of media required to prepare 100 *l* (₹)	Generation time, merits and demerits of the media
King's B (KB)	1.41	12,714	Generation time is short, but sudden decline in population, high cost of medium
Nutrient Broth (NB)	1.43	9175	Short generation time, but sudden decline in population
Trypticase Soya Broth (TSB)	1.26	9796	Maintained high population,shortest generation time, reasonable cost of medium
Pseudomonas (Fluorescein) the broth (PF)	2.77	15,706	High cost of the medium and highest generation time among all media
Luria- Bertani Broth (LBB) TSB	1.42	11,495	Short generation time, but lesser population and costlier as compared to
Potato Dextrose broth (PDB)	2.56	2608	It's a general medium used for the growth of fungi rather than bacteria, had higher generation time and lower population
Minimal salt medium (M9)	2.14	2201.78	Lesser population, and more generation time

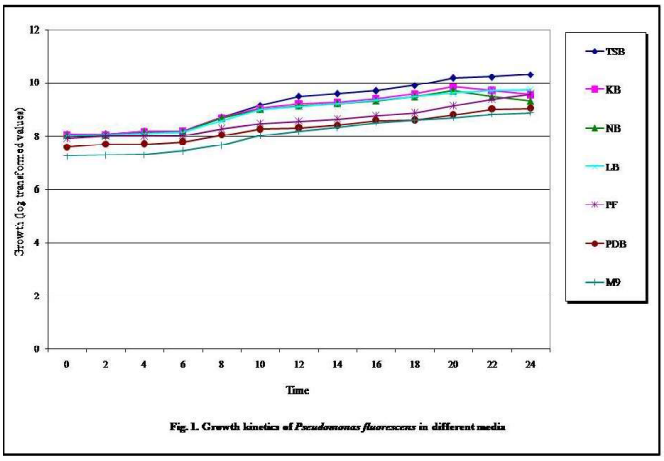


Fig 1. Growth kinetics of Pseudomonas fluorescens in different media

Preparation of liquid formulations of *Pseudomonas fluorescens* and assessment of shelf life

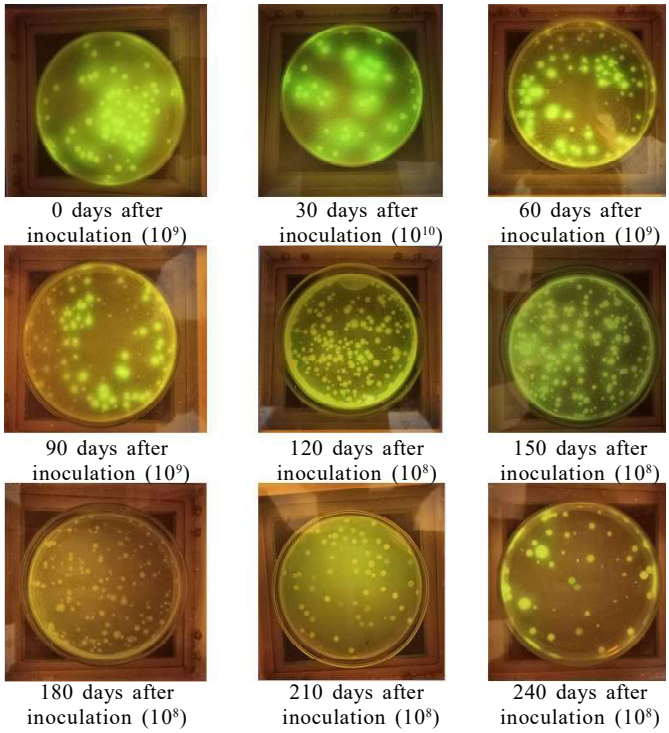
Various amendments were incorporated to the basal medium (TSB) at different concentrations and the population (CFU ml^{-1}) were estimated at monthly intervals (Table 3). The results revealed that, the viable count of colonies increased from 0^{th} day and reached a maximum by 30^{th} day of storage. The highest population observed (on 30^{th} day) was 48.33×10^{10} CFU ml^{-1}, in formulation 9. Later, the population declined gradually over the months of storage. Even at the end of eight months, the highest population was maintained in formulation 9 (TSB+ Glycerol 2%+ PVP 1%+ CMC 0.05%+ Tween-20 0.5%) (66×10^8 CFU ml^{-1}), followed by formulation 1 (TSB+ Glycerol 1%+ PVP 1 %+ CMC 0.05%+ Tween-20 0.5%) (52×10^8 CFU ml^{-1}). The lowest population among the formulations at 240^{th} day, was found in formulation 5 (TSB+ Glycerol 1%+ PVP 2%+ CMC 0.05% + Tween-20 0.5%) (3×10^8 CFU ml^{-1}). The population of bacteria in unamended (control) TSB (28×10^4 CFU ml^{-1}) was several folds lower as compared to the amended formulations. Among the checks, liquid based market sample of the antagonist maintained the population till 240 days (4×10^8 CFU ml^{-1}), while talc-based checks could not maintain requisite population beyond 180 days. The viable colonies obtained on different days of storage for formulation 9 is represented in Fig 2.

The amendments added to the medium had a positive effect in protecting the cells and prolonging the shelf-life when compared to the unamended broth and talc formulations. The cell protectants *viz.,* glycerol and PVP retained the water content, maintained the viability (Manikandan *et al*., 2010; Biradar and Santhosh, 2018). The adjuvant CMC retained the integrity of cells by maintaining a thick consistency of the medium, while Tween-20 reduced the surface tension of the medium, as a surfactant (Daniel *et al.*, 2013; Biradar and Santhosh, 2018). Manikandan *et al*. (2010) have documented the population count of *P. fluorescens* as 9.5×10^7 CFU ml^{-1}, in Nutrient broth amended with 10 mM glycerol. Similarly, shelf life was maintained till 210^{th} day of storage when nutrient broth

Table 3. Shelf life of *Pseudomonas fluorescens* at different days of storage in trypticase soya broth

Formulation No.	Composition of formulations	Colony forming units (CFU × 10^{10}) per ml of formulation at different DAI								
		0	30	60	90	120	150	180	210	240
1	TSB+ G(1%)+P(1%)+C(0.05%)+T(0.5%)	7.23	47.00	8.60	8.07	1.75	1.63	1.26	0.89	0.52
2	TSB+ G(1%)+P(1%)+C(0.05%)+T(1.0%)	6.77	40.67	7.43	6.24	1.51	1.33	0.90	0.38	0.11
3	TSB+ G(1%)+P(1%)+C(0.1%)+T(0.5%)	7.20	45.00	8.83	7.65	1.72	1.51	1.17	0.66	0.49
4	TSB+ G(1%)+P(1%)+C(0.1%)+T(1%)	6.63	36.00	6.90	5.43	1.28	1.15	0.70	0.10	0.07
5	TSB+ G(1%)+P(2%)+C(0.05%)+T(0.5%)	6.17	27.33	5.07	2.40	0.51	0.34	0.26	0.05	0.03
6	TSB+ G(1%)+P(2%)+C(0.05%)+T(1%)	6.13	29.33	5.27	2.73	0.61	0.47	0.29	0.05	0.04
7	TSB+ G(1%)+P(2%)+C(0.1%)+T(0.%)	7.07	42.67	7.93	7.06	1.63	1.37	0.99	0.46	0.26
8	TSB+ G(1%)+P(2%)+C(0.1%)+T(1.0%)	6.20	34.67	6.10	3.20	1.03	0.79	0.56	0.09	0.06
9	TSB+ G(2%)+P(1%)+C(0.05%)+T(0.5%)	7.43	48.33	9.57	8.50	1.97	1.84	1.36	1.05	0.66
10	TSB+ G(2%)+P(1%)+C(0.05%)+T(1%)	7.13	44.00	8.27	7.68	1.68	1.47	1.12	0.60	0.34
11	TSB+ G(2%)+P(1%)+C(0.1%)+T(0.5%)	6.87	42.33	7.57	6.54	1.57	1.42	1.04	0.53	0.29
12	TSB+ G(2%)+P(1%)+C(0.1%)+T(1%)	6.30	31.67	5.73	3.07	0.85	0.56	0.32	0.06	0.05
13	TSB+ G(2%)+P(2%)+C(0.05%)+T(0.5%)	6.73	39.33	7.32	6.10	1.47	1.29	0.85	0.32	0.09
14	TSB+ G(2%)+P(2%)+C(0.05%)+T(1%)	6.33	35.33	6.47	3.43	1.15	0.99	0.60	0.09	0.06
15	TSB+ G(2%)+P(2%)+C(0.1%)+T(0.5%)	6.50	37.33	7.19	5.87	1.36	1.25	0.76	0.25	0.08
16	TSB+ G(2%)+P(2%)+C(0.1%)+T(1%)	6.27	31.33	5.40	2.80	0.75	0.65	0.41	0.08	0.05
Unamended TSB broth		6.13	2.50	0.80	0.54	0.13	0.09	0.04	0.02	0.00
P. fluorescens- Talc based formulation (IOF strain) (check)		0.34	0.44	0.38	0.33	0.12	0.09	0.04	0.02	0.00
P. fluorescens- Talc based formulation (market sample) (check)		0.28	0.36	0.33	0.26	0.09	0.06	0.03	0.00	0.00
P. fluorescens- Liquid based formulation (market sample) (check)		0.85	0.88	0.60	0.56	0.15	0.12	0.10	0.04	0.04
S.Em±		0.07	0.06	0.10	0.07	0.01	0.01	0.01	0.01	0.01
C.D @ 1%		0.28	0.22	0.37	0.26	0.04	0.04	0.04	0.03	0.03
C.V. (%)		2.22	2.58	2.87	2.67	1.75	1.80	2.43	3.72	5.07

Note: TSB- Trypticase Soya Broth; G- Glycerol; P-Poly vinyl pyrrolidone; C- Carboxy methyl cellulose; T-Tween-20
DAI-Daysafter inoculation



amended with 2% PVP was used (Paranthaman and Chellappan, 2020) and till 12 months when canola oil-based formulation was used to grow *P. fluorescens* (Dobhal and Hegde, 2021). In all the above citations, the population observed at the end of storage period was highly significant over unamended broth or talc-based formulations, emphasizing the fact that, the liquid formulations are more efficient.

Conclusion

Formulating the bioagents is a crucial step in attaining the full potential of any antagonist. When this aim was kept at the fore, the investigation resulted in formulating a liquid biopesticide of *P. fluorescens* that could maintain the shelf life better than the talc formulations. Trypticase Soya Broth was selected as the basal medium and was amended with various additives to protect the cells from desiccation and to prolong the viability. The study concluded that the developed formulation of *P. fluorescens*(TSB+ Glycerol 2%+ PVP 1%+ CMC 0.05%+ Tween-20 0.5%) was highly efficient in maintaining the shelf life of the antagonist and thus, can be utilized in mass production.

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