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Evaluation of different growth media and carrier materials for Bio-formulation of probiotic *Lactiplantibacillus plantarum* NRB14 as novel biocontrol agent

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Abstract
Studies were carried to evaluate the effect of different growth media, temperature, pH on mass multiplication of probiotic bacterial biocontrol agent and different carrier based materials for bio formulation and to know the shelf life under different storage temperature conditions. Superior isolate *Lactiplantibacillus plantarum* NRB14 showing plant growth promotion and antifungal activity against chilli diseases under *in-vitro* and *in-vivo* condition were selected and evaluated to standardize media and carrier materials for bio-formulation. Among different growth media compositions highest biomass mass production was noticed in MRS broth with population count of 8.21 (log 10 cfu/ml) followed by molasses+ yeast extract with cell biomass of 8.10 (log10 cfu/ml) and lowest cell biomass was noticed in sucrose+ yeast extract 7.72(log10 cfu/ml) 48 h after incubation. Ideal temperature of 30-35 °C and pH of 6 to 7 was congenial for better biomass production. Amongst different carrier materials used, liquid based bio formulation amended with glycerol showed highest population density of (7.67 log10 cfu/ml) followed by talc based formulation (7.37 log 10 cfu/g) with shelf life period of 210 days at 28 °C storage temperature condition. Finally conclude that MRS broth and molasses + yeast extract broth showed good growth of bacteria for mass multiplication of biocontrol agent and liquid and talc based formulation showed shelf life period of 210 days at room temperature storage condition.

Keywords: Probiotics, *Lactiplantibacillus plantarum* NRB14, Bio formulation, carrier material, shelf life

1. Introduction

Probiotics are living microorganisms, which upon ingestion in certain numbers exert various health benefits beyond the inherent basic nutrition (Arora *et al.*, 2013) [1]. Probiotic organisms are generally recognized as safe microorganisms present in fermented food products, fruits, vegetables and soil hence they are ubiquitous in nature (Stiles and Holzapfel, 1997) [11]. Lactic acid bacteria strains (LABs) are known as probiotic organisms LABs cultures or their supernatants have been used as biological control agents on plant diseases in chilli, tomato and cucumber caused by the fungi *Colletotrichum capsici* (El-Mabrok *et al.*,2012) [5], *Fusarium oxysporum* and *Pythium ultimum*, respectively (Hamed *et al.*, 2011) [6] Several species of LABs have been recognized as producers of bioactive metabolites that act against a broad spectrum of undesirable microorganisms such as fungi, oomycetes and other bacteria (Axel *et al.*, 2012) [3]. LABs are reported to produce various antibacterial compounds, such as acetic acid, lactic acid (Ariyapitipun, Mustapha *et al.*, 1999) [2], hydrogen peroxide (Chang *et al.*, 1997) [4], several bacteriocins. Moreover, significant effects probiotic *Lactiplantibacillus plantarum* NRB14 chilli diseases were shown in our previous studies. For success of any biocontrol agent their application in field efficacy must be there for that cheap growth media and carrier material in which biocontrol agent applied to crop should be evaluated. For commercial production, the antagonist must first be cultivated and all processes involved, optimized. Optimization takes place under laboratory conditions before up scaling for mass production. Although, there is considerable information available on laboratory-scale production of *Lactiplantibacillus plantarum* NRB14, published literature has declined as processes became commercially more significant. Available literature on culturing *Lactiplantibacillus plantarum* NRB14 mainly describes selective media or laboratory-scale fermentations optimum conditions for culturing, harvesting and storing of *Lactiplantibacillus plantarum* NRB14 antagonists for eventual use against plant pathogens, had to be determined in order to obtain maximum yield.

An important area of biological control is the development of formulations that would take care for viable microbial activity for long period of time. Mass multiplication of bio agent in a suitable medium and development of a powder formulation was first carried out in 1980. A dried powder formulation of bio agent is important for seed treatment and soil application. *Lactiplantibacillus plantarum* NRB14 is one of the most important biocontrol agent for the management of plant diseases. Commercial success of a biocontrol agent depends not only on its bio efficacy or shelf life but also ease with which it can be mass multiplied on a suitable substrate, which is easily available and relatively inexpensive. Present investigation taken on objective to know growth media, growth conditions required for mass multiplication and different carrier based material for bio formulation. Thus which will help to best growth media and carrier material for multiplication and application of *Lactiplantibacillus plantarum* NRB14 biocontrol agent in field condition.

2. Material and Methods

Probiotic LAB Isolate: Pure culture of *Lactiplantibacillus plantarum* NRB14 were isolated from Nanjanagudu Rasabale local variety of banana, which showing good antifungal activity against chilli diseases in previous study were selected for the bio formulation study. There are different steps involved in formulation of probiotic LAB such as standardization of medium, growth studies, method of harvesting, drying and ultimately developing the commercial formulation therefore, before starting mass multiplication of probiotic LAB, studies related to standardization of media, pH and temperature requirements were carried out as per the details mentioned below.

2.1 Standardization of media

Media	Dosage (g)/l
MRS broth(de manos Sharpe)	56
SPY broth (soya peptone yeast extract)	20
Nutrient broth	25
Sucrose+ yeast extract	25+5
Molasses + yeast extract	20+10
Molasses + urea	25+5
Fructose + yeast extract	25+5

One hundred ml of different above mentioned media were taken in 250 ml flask, sterilized and inoculated with pure culture. These flasks were kept for incubation at room temperature for two days. Three replications were maintained for each treatment. After incubation one ml suspension was taken from each media and serial dilution technique was performed up to 10^8 dilutions. An aliquot of 0.1 ml suspension was spread over pre sterilized and cooled down MRS agar plates. The inoculated plates were incubated at 30±1 °C for 24-48 h. The observations on colony forming units (cfu) were recorded and statistically analyzed to find out the best medium and colony count was expressed as log10 cfu count/ ml.

2.2 Standardization of pH levels

The organism was grown on the MRS media selected from above experiment at different pH ranges such as 3, 4, 5, 6, 7, 8 and 9 by using 2N (NaOH, HCl and H_2SO_4). The inoculated plates were incubated at 30±1 °C for 24-48 h. The observation on cfu were recorded and statistically analyzed to find out the best pH range and colony count was expressed as log10 cfu count/ ml.

2.3 Standardization of temperature levels

The growth of probiotic LAB was observed at different temperature range such as 15, 20, 25, 30, 35, 40 and 45 °C. Hundred ml of selective media was taken in 250 ml flask and pH was adjusted to standard range. The inoculated plates were incubated at 30±1 °C for 24-48 h. The observations on cfu were recorded following standard procedure and statistically analyzed to find out the best temperature range and colony count was expressed as log10 cfu count/ ml.

2.4 Formulation and shelf life of superior isolate of probiotic *Lactiplantibacillus plantarum* NRB14

Talc powder, Lignite powder, Peat mass, Vermicompost, Areca compost, FYM were used as carrier materials for solid formulation and liquid based formulation also checked for survival of probiotic LAB.

2.5 Procedure for culture preparation and formulation

Mass culture of probiotic LAB superior isolate (*Lactiplantibacillus plantarum* NRB14) was obtained by adding 15ml of 24h old bacterial suspension, aseptically to 1000 ml MRS broth and incubated at 30 °C with 150 rpm for 72 h. 400 ml bacterial suspension (10^8 cfu/ml) was mixed with 1kg of carrier material plus 1% carboximethyl cellulose (CMC) as binder, 0.1% tritan-x as stabilizer was added and for liquid formulation 1% glycerol solution mixed thoroughly under laminar air flow chamber and packed in 120 guage polythene covers and stored at 4 °C, 27 °C. The study was to assess shelf life of probiotic LAB in different carrier materials at different intervals at different storage temperature. The shelf life was determined by enumerating the population of bacterial antagonists by serial dilution plate count method using MRS agar medium.

3. Results and Discussion

3.1 Standardization of different growth media for mass production of probiotic *Lactiplantibacillus plantarum* NRB14

For commercial production, the antagonist must first be cultivated and all processes involved are optimized. Optimization shall be standardized under laboratory conditions before up scaling for mass production. Available literature on culturing *Lactiplantibacillus plantarum* NRB14 mainly describes selective media or laboratory scale fermentations optimum conditions for culturing, harvesting and storing of *Lactiplantibacillus plantarum* NRB14antagonists for eventual use against plant pathogens, which had to be determined in order to obtain maximum yield.

Among the seven different liquid media used to grow probiotic *Lactiplantibacillus plantarum* NRB14, maximum cfu count was observed on MRS broth with log10 cfu value of 8.21 and 20 g molasses + 10 g yeast extract broth (log10 cfu 8.10) whereas, least cfu count was recorded in 25g sucrose+ 5 g yeast extract broth (log cfu 7.72) at 48 h of incubation period. Since media containing 20 g molasses + 10 g yeast extract broth showing on par results with MRS broth we recommend cheap growth media for mass production of lactic acid bacteria for commercial scale production compared to selective media. (Table 1).

Cost of media/l was lowest with media containing 20 g molasses+10 g yeast extract broth (Rs.49.80) compared to MRS broth (Rs.75.56) indicating its superiority. Similar studies have been carried out by various workers and their results indicated suitability of different media for growth of *B. subtilis*. Peighmi-Ashnari *et al*. (2009) [10] conducted similar studies and found that molasses + yeast extract based media to be the most suitable for rapid growth and high cell yield of *B. subtilis* and their results are in tune with the present study.

In the present study, maximum bacterial growth LAB was noticed from pH range of 3 to 9 but maximum growth of probiotic LAB was found in the pH 7 (log10 cfu of 8.82) and least population was noticed at pH 3 (log10 cfu of 7.6). Among different pH tested on growth of bacteria pH 7 was most suitable for maximum cell count of lactic acid bacteria (Table 2).

Study on effect of different temperature on growth of probiotic LAB using selected media showed that maximum bacterial population was noticed at temperature of 35 °C with population of log cfu count of 9.49 and at temperature of 20 °C, 25 °C, 30 °C, 40 °C mean population of count LAB was noticed log cfu count of 8.347, 8.653, 9.183, 9.19 respectively and least population was noticed at temperature of 15 °C with log10 cfu count of 8.037. It is concluded that temperature range of 20 °C to 40 °C suitable for production of good population of lactic acid bacteria. Temperature of 35 °C was most suitable for mass multiplication of the probiotic lactic acid bacteria (Table 3).

In the present study, maximum cfu of probiotic lactic acid bacteria was obtained at pH 7 (log10 8.82 cfu per ml) and least population count of bacteria was observed at pH 3 (log10 7.66 cfu per ml). Similarly, maximum cfu was obtained at 30 and 35 °C (log10 9.19 cfu per ml and log 9.49 cfu, respectively) and least growth was observed at 15 °C temperature. Similar results were obtained by Hadimani and Kulkarni (2017) [7] who studied the optimization of *Bacillus subtilis* growth conditions for mass production under laboratory conditions which was investigated as part of a biological control programme. Aspects such as increasing yield using various culture media, pH, temperatures and carrier materials were studied. Nutrient broth (100×10^8) and 20 g molasses and 10 g yeast extract based media (98.60×10^8) gave the highest yield and 20 g molasses and 10g yeast extract broth was the most economical. The pH of 7-8, temperature of 30-35 °C and talc as carrier material were proved to be the best for mass production of *B. subtilis*. Molasses is good source of nutrients especially carbohydrate available in sugar form in high quantity which will help in easy multiplication of bacteria since required more quantity of sugar for growth and reproduction, for growth of any bacteria neutral pH is most suitable and room temperature any bacteria multiply very fastley, since probiotic LAB multiplication at 30-35 °C very high because they thrive well at our gut body temperature (Hadimani and Kulkarni 2017) [7].

Table 1: Effect of different growth media on population of probiotic *Lactiplantibacillus plantarum* NRB14.

			(log10 cfu/ ml)					
Sl.no	**Media**	**Dosage(g/l)**	**12 h**	**24 h**	**36 h**	**48 h**	**Mean (log10 cfu/ ml)**	**Cost of media/ liter (Rs)**
1	MRS Broth	56	7.76	7.975	8.276	8.836	8.21	75.56
2	SPY Broth	20	7.426	7.817	8.118	8.72	8.02	40.50
3	Nutrient Broth	25	7.368	7.671	7.972	8.574	7.90	69.40
4	Sucrose+ yeast extract	25+5	7.099	7.531	7.832	8.437	7.72	52.22
5	Molasses + yeast extract	20+10	7.615	7.855	8.156	8.758	8.10	49.80
6	Molasses + urea	25+5	7.49	7.807	8.108	8.71	8.03	48.00
7	Fructose + yeast extract	25+5	7.325	7.567	7.869	8.471	7.81	31.40
		S. Em. ±	0.062	0.035	0.035	0.042	0.133	
		C.D.(P=0.01)	0.19	0.108	0.108	0.127	0.398	

Table 2: Effect of different pH levels on multiplication of probiotic *Lactiplantibacillus plantarum* NRB14.

Sl. no	pH	Mean log10 cfu count/ml
1	3	7.66
2	4	7.89
3	5	8.13
4	6	8.65
5	7	8.82
6	8	8.59
7	9	7.98
S. Em. ±		0.104
C.D.(P=0.01)		0.034

Table 3: Effect of different temperature level on multiplication of probiotic *Lactiplantibacillus plantarum* NRB14.

S.l. no	Temperature (˚C)	Mean log10 cfu count/ml
1	15	8.037
2	20	8.347
3	25	8.653
4	30	9.19
5	35	9.49
6	40	9.10
	S. Em. ±	0.042
	C.D.(P=0.01)	0.13

3.2 Survival of probiotic *Lactiplantibacillus plantarum* NRB14 in different carrier based formulations stored at 4 °C

The viable population of probiotic lactic acid bacteria in the first month in all treatments revealed that highest cfu count was noticed in liquid formulation log10 value 8.71 cfu per gram, followed by talc powder log10 value 8.62 cfu per gram, lignite powder log10 value 8.51 cfu per gram, peat log10 value 8.50 cfu per gram, vermi compost log10 value 8.35 cfu per gram and areca compost log10 value 8.30 cfu per gram and lowest population was noticed in FYM log10 value 8.10 cfu per gram at 4 °C.

When compared among the treatments, liquid formulation showed highest population and among different carrier materials tested talc based carrier material was next to liquid formulation in cfu count and FYM formulation having least population at 4 °C storage condition.

The viability and shelf life of probiotic lactic acid bacteria was from 30 days to 210 days decreased gradually (Table 4). But maximum survival rate of probiotic lactic acid bacteria was noticed in liquid formulation. Among carrier materials used talc based formulation showed viability of probiotic lactic acid bacteria next to liquid formulation with a shelf life period of 210 days.

3.2 Survival of probiotic *Lactiplantibacillus plantarum* NRB14 in different carrier based formulations stored at 28 °C

The viable population of probiotic antagonist was in the first month at 28 °C storage temperature which revealed that highest cfu count was noticed in liquid formulation log10 value 8.69 cfu per gram, followed by talc powder log10 value 8.62 cfu per gram, lignite powder log10 value 8.48 cfu per gram, peat log10 value 8.48 cfu per gram, vermi compost log10 value 8.26 cfu per gram and areca compost log10 value 8.21 cfu per gram and lowest population was noticed in FYM log10 value 8.09 cfu per gram depicted in Table 5. Nekkeran *et al*. (2006) reported that *B. subtilis* growth was good at temperature of 28±2 °C. Whereas, Korsten and Cook (1996) [8] reported that temperature of 30-37 °C and pH of 7-8 good for the growth and multiplication of *B. subtilis*.

After two ten days' storage period of different formulation the results depicted that MRS liquid formulation showed a viable count of log10 value 7.59 cfu per gram followed by talc based formulation log10 value 7.43 cfu per gram and least cfu count was noticed in FYM log10 value 0.0 cfu per gram. Among all treatments tested, viability of bacteria was noticed up to 210 days in liquid, talc, lignite, peat based formulation. In case of FYM and areca compost survival was up to 120 days and in case vermi compost 150 days' period at 28 °C storage condition. Similar studies were carried out by Nekkeeran *et al*. (2006) and they reported that talc, FYM, vermiculite and lignite as a carrier material supported *B. subtilis* multiplication.

Table 4: Population density and shelf life of probiotic LAB in different carrier based formulations stored at 4^0 C

Population density at different time intervals in days (log10 cfu/g)										Shelf life and population density	
S.I no	**Treatments**	**24h**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**Number of days**	**log10 cfu/g**
1	Talc powder	8.68	8.62	8.49	8.44	8.33	8.21	7.93	7.49	210	7.49
2	Lignite powder	8.57	8.51	8.42	8.34	8.27	8.09	7.79	7.37	210	7.37
3	Peat	8.59	8.50	8.44	8.38	8.23	7.93	7.69	7.12	210	7.12
4	Vermicompost	8.57	8.35	8.24	7.84	7.52	7.09	6.80	0.00	180	6.80
5	Areca compost	8.51	8.30	8.08	7.51	7.35	0.00	0.00	0.00	120	7.35
6	FYM	8.50	8.10	7.79	7.19	6.78	0.00	0.00	0.00	120	6.78
7	MRS Liquid formulation	8.79	8.71	8.57	8.46	8.36	8.36	8.08	7.67	210	7.67
	S. Em. ±	0.013	0.011	0.013	0.049	0.024	0.015	0.024	0.03		
	CD (P =0.01)	0.04	0.035	0.04	0.149	0.074	0.046	0.074	0.093		

Table 5: Population density and shelf life of probiotic LAB in different formulations stored at 28 ^{0} C.

Population density at different time intervals in days (log10 cfu/g)										Shelf life and population density	
S.I no	**Treatments**	**24h**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**Number of days**	**log10 cfu/g**
1	Talc powder	8.67	8.62	8.49	8.42	8.23	8.12	7.75	7.43	210	7.43
2	Lignite powder	8.55	8.48	8.42	8.32	8.15	7.94	7.47	7.31	210	7.31
3	Peat	8.54	8.48	8.44	8.35	8.07	7.83	7.10	7.12	210	7.12
4	Vermicompost	8.46	8.26	8.22	7.83	7.42	6.97	0.00	0.00	150	6.97
5	Areca compost	8.42	8.21	8.08	7.26	7.09	0.00	0.00	0.00	120	7.09
6	FYM	8.36	8.09	7.73	7.08	6.48	0.00	0.00	0.00	120	6.48
7	MRS Liquid formulation	8.72	8.69	8.57	8.44	8.26	8.26	7.85	7.59	210	7.59
	S. Em. ±	0.006	0.014	0.009	0.035	0.022	0.016	0.019	0.013		
	CD (P = 0.01)	0.019	0.043	0.029	0.108	0.066	0.048	0.059	0.039		

4. Conclusion

Probiotic *Lactiplantibacillus plantarum* was tested for its adoptability to different growth media, temperature, pH levels and carrier materials for its growth and survival. Results of present revealed that MRS broth and 20 g molasses+ 10 g yeast extract broth, 30-35^0 C temperature, 6-7 pH and liquid formulation and talc as carrier material are good for the growth *Lactiplantibacillus plantarum*. shelf life up to 210 days in talc based formulation found to be good at 28 °C and 4 °C.

5. References

1. Arora M, Sharma S, Baldi A. Comparative insight of regulatory guidelines for probiotics in USA, India and

Malaysia: A critical review. Inter J Biotechnol. Wellness Industry. 2013;2:51-54.
2. Ariyapitipun T, Mustapha A, Clarke AD. Microbial shelf life determination of vacuum packaged fresh beef treated with polylactic acid, and nisin solutions. J Fd. Protec. 1999;62:913-920.
3. Axel C, Zannini E, Coffey A, Guo J, Waters DM, Arendt EK. Ecofriendly control of potato late blight causative agent and the potential role of lactic acid bacteria: A review. Appl. Microbiol. Biotechnol. 2012;96:37-48.
4. Chang IS, Kim BH, Shin PK. Use of sulfite and hydrogen peroxide to control bacterial contamination in ethanol production. Appl. Environ. Microbiol. 1997;63:1-6.
5. El-Mabrok ASW, Hassan Z, Mokhtar AM, Hussain KMA, Kahar FKSBA. Screening of lactic acid bacteria as biocontrol against (*Colletotrichum capsici*) on chilli Bangi Research. J App. Scien. 2012;7:466-473.
6. Hamed HA, Moustafa YA, Abdel-Aziz SM. *In-vivo* efficacy of lactic acid bacteria in biological control against *Fusarium oxysporum* for protection of tomato plant. Life. Scien. J. 2011;8:462-468.
7. Hadimani B, Kulkarni S. Ideal growth conditions for mass production of biocontrol agent *Bacillus subtilis* (ehrenberg) cohn. Plant Archives. 2017;17:535-538.
8. Korsten LN. Cook Optimizing culturing conditions for *Bacillus subtilis*. South African Avacado Growers Association Yearbook. 1996;19:54-58.
9. Nakkeeran S, Kavitha K, Chandrasekar G, Renukadevi P, Fernando WGD. Induction plant defense compounds by *Pseudomonas chlororaphis* PA23 and *Bacillus subtilis* BSCBE4 in controlling damping off of hot pepper caused by *Pythium aphanidermatum*. Biocontrol Sci, Technol. 2006;16(4):403- 416.
10. Peighmi-ashnari S, Shariti-tehrani M, Ahmadzadeh, Behhoudi K. Interaction of different media on production and bio efficacy of *Pseudomonas fluorescens* P-35 and *Bacillus subtilis* B-3 against grey mold of apple. J Pl. Pathol. 2009;91(1):65-70.
11. Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. Internal. J Fd. Microbiol. 1997;36:1-29.