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Research Paper

A Novel Low-Cost Method for Liquid Bacterial Biofertilizer Production

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Abstract

This study was conducted to develop a low-cost technology to produce liquid bacterial biofertilizer. Sterilized Rice Porridge (SRP) was tested as a growth medium for mass multiplication of bacteria using fourteen bacterial isolates. Survival and bioactivity of seven growth promoting rhizobacterial isolates were determined by using sterilized distilled water (SDW) at room temperature (30 ± 2 °C) (RT) as a low-cost and convenient method for storage of bacteria. The novel biofertilizer production technology consisted of SRP and beneficial bacterial isolates storing in SDW at RT (30 ± 2 °C). Preparation of biofertilizer was done by mixing SRP and bacterial isolates in SDW at RT. Then the mixture was sealed immediately and incubated at RT for 24 hours before application. All the fourteen bacterial isolates produced more than 1x10^8 CFU/mL colony count after 24-hour incubation period in SRP at RT confirming the suitability of SRP for mass multiplication of bacteria. Tested seven growth promoting rhizobacterial isolates survived in SDW at RT without losing their bioactivity with a sufficient viable cell count in SDW up to 18 months when stored in SDW at RT. It revealed the suitability SDW at RT for storage of bacteria A biofertilizer produced by this technology using N-fixing rhizobacterial isolate *Priestia aryabhattai* (OP950582) was evaluated under field condition using two popular rice varieties. (At 362 & Bw 367). N biofertilizer developed using *Priestia aryabhattai* reduced chemical N fertilizer application by 50% when applied as a seed treatment at a rate of 1 x 10^8 CFU/mL of *P. aryabhattai.* This technology can be used to produce liquid bacterial biofertilizer at a low cost.

Keywords: Bacterial biofertilizer, Low-cost, Mass multiplication, Sterilized distill water, Sterilized rice porridge

Introduction

The exploitation of beneficial microbes as biofertilizers is of paramount importance in sustainable agriculture because of their potential role as alternatives for chemical fertilizers. Although there are several scientific evidences for identification of plant growth promoting bacteria through isolation and *in-vitro* screening processes, these useful bacteria rarely appear in the market as commercial biofertilizers (Nita *et al*., 2012). Delivery of identified beneficial microorganisms to the field is the most important aspect for their commercial exploitation (Anith *et al*., 2014). To formulate microbial biofertilizers, identified microorganisms should be multiplied at a large scale and delivered to the field either in solid form or in liquid form using a suitable carrier material (Princy *et al*., 2014). The major obstacle in developing bacterial biofertilizer products is the retention of survival and stable plant-growth promoting activity, as seen in freshly harvested cells and it has become a focus of research and industrial development of bacterial biofertilizers (Mitter *et al*., 2021). Therefore, biofertilizer formulations are designed to reduce metabolic activities of bacterial cells without killing them (Vassilev *et al*., 2020).

Liquid bio-fertilizers are special formulation containing the desired microorganisms. sufficient amount of nutrients and special cell protectants or chemicals that promote formation of resting spores or cysts for longer shelf life and tolerance to adverse conditions (Hegde, 2008). Commonly used commercial liquid growth media for multiplication of bacteria are expensive for industrial scale microbial inoculum production. Therefore, identification of suitable growth media for mass multiplication of bacteria is extremely important when considering the commercial production of bacterial inoculants. The media used for mass multiplication should support fast multiplication of beneficial bacteria, be cost effective and commonly available. Further, identification of a suitable technique to keep the bacterial cells in a dormant state instead of using costly cell protectants or chemicals is of paramount importance to reduce the cost of biofertilizer production and to minimize harmful effects on the environment.

Therefore, this study was initiated with the aim of developing a new liquid biofertilizer technology devoid of existing limitations of commercial liquid biofertilizer. The objectives of the study were, i. To study the suitability of sterilized rice porridge (SRP) as a medium for mass multiplication of bacteria, ii. To study the suitability of sterilized distilled water (SDW) at room temperature (RT) as a low-cost and convenient carrier medium to store bacterial inoculants without losing the bioactivity under room temperature (RT) condition, iii. To prepare new liquid biofertilizer formulation using a nitrogen (N)-fixing bacterial isolate in SDW and SRP, and iv. To study the effect of N biofertilizer developed by this technology on growth and yield of Bw 367 and At 362 rice varieties under field condition.

Materials and Methods

Experiment 1. Suitability of Sterilized Rice Porridge (SRP) as a low-cost growth medium for mass multiplication of bacteria

Bacterial isolates used for the study

Three phosphate (P)-solubilizing rhizospheric bacterial isolates (I_1, I_2 & I_3), four nitrogen (N) -fixing rhizospheric bacterial isolates (I_4, I_5, I_6 & I_7) and seven non-rhizospheric bacterial isolates (I_8, I_9, I_{10}, I_{11}, I_{12}, I_{13} & I_{14}), which were isolated from paddy fields in Low Country Wet Zone, Intermediate Zone and Dry Zone using soil dilution plate and spread plate techniques in a previous study were used for the study.

In a previous study, rice porridge was identified as a suitable medium for mass multiplication of phosphate solubilizing rhizobacteria (Sandamali *et al*., 2018). Therefore, in this study suitability of SRP as a low-cost growth medium for mass multiplication of bacteria was tested.

Rice porridge was prepared by boiling 250 g of commercially available red rice in 1 L of water and filtered using a muslin cloth. Next this prepared rice porridge medium was sterilized by autoclaving at 121 °C for 20 minutes. SRP medium was allowed to cool to room temperature (RT) and the medium (100 mL) was inoculated with a loop full of 24 hours old above bacterial cultures (each separately) grown on nutrient agar (NA) medium. The inoculated medium was incubated at RT for 24 hours. After the incubation period, viable bacterial cell count in the SRP was determined using serial dilution plate technique using NA medium (Naher *et al*., 2013). To obtain the colony count, 10^{-5} dilution was used. Three replicates were performed for each bacterial isolate.

Experiment 2. Suitability of sterilized distilled water (SDW) at RT as a carrier medium for the bacterial inoculants

Three P-solubilizing rhizospheric bacterial isolates (I_1, I_2 & I_3) and four N-fixing rhizospheric bacterial isolates (I_4, I_5, I_6 & I_7) which were used in the experiment 1, were used to test the suitability of SDW for the storage of bacterial inoculants without losing their viability and bioactivity.

Centrifuge tubes were filled with distilled water (10 mL) and sterilized at 121 °C for 20 minutes. They were allowed to cool down to RT and inoculated with a loop full of 24 hours old cultures of bacterial isolates grown on NA medium. Three tubes with SDW were inoculated with one bacterial isolate and considered as three replicates. The initial bacterial cell concentration in SDW in centrifuge tubes was determined using serial dilution plate technique on NA medium just after inoculation (Naher *et al*., 2013). To obtain the colony count, 10^{-5} dilution was used. Then these tubes were kept in the laboratory under normal conditions at RT for 18 months. RT in the laboratory was recorded throughout the period. The viability of each bacterial isolate in SDW was determined quantitatively in three-month intervals up to 18 months.

Bioactivity of each bacterial isolate in SDW at RT was also determined quantitatively in 3 months intervals up to 18 months by streaking a loopful of bacteria from the centrifuge tubes on relevant medium. Three biological replicates were performed for each bacterial isolate.

P-solubilizing isolates were streaked on fresh Pikovskya's medium, which contained insoluble $Ca_3(PO_4)_2$. The plates were incubated at RT for 24 hours. After the incubation period, P-solubilizing ability of isolates was visually detected by the formation of clear zones around the bacterial colonies by solubilizing $Ca_3(PO_4)_2$ (Pandey *et al*., 2007).

N-fixing bacterial isolates were streaked on fresh N free Burk's medium. The plates were incubated at RT for 24 hours. After the incubation period, N-fixing ability of isolates was detected by the growth of isolates on Burk's medium (Stella & Suhaimi, 2010).

Experiment 3. Formulation of liquid bacterial biofertilizer using bacterial isolate in SDW stored at RT and SRP

This formulation contained two solutions namely Solution 1 and Solution 2. SRP was considered as solution 1. Rice porridge can be kept in polypropylene bags or in glass

bottles, which are thermal resistant for sterilization at 121 °C. Beneficial bacterial isolates in SDW at RT was considered as solution 2. Solution 2 (beneficial bacterial isolate in 10 mL of SDW) was added to solution 1 (100 mL of sterilized rice porridge). Then, the glass bottle was sealed immediately to be airtight. This mixture was incubated for 24 hours at RT. Beneficial bacterial isolates were inoculated at approximately a rate of 1 x 10^8 CFU/mL.

Experiment 4. Evaluation of inoculation ability of bacterial isolate I_1 in SDW stored at RT with time

A pure culture of bacterial isolate I_1, with P-solubilizing ability, which was stored in 10 mL of SDW at RT for 6 months, was used for evaluation. After 6 months of storage, sterilized distilled water with I_1 was added to 100 mL of SRP and the mixture was incubated for 24 hours at RT to prepare the biofertilizer. After the incubation period, germinated rice seeds (50 g) were mixed with the biofertilizer at a rate of 1×10^8 CFU/mL and incubated for 6 hours at RT. Seeds (50 g), which were not inoculated with biofertilizer were considered as control. Seeds were planted in pots with sterilized soil. Rhizospheric soil samples were collected from these plants after one month. A soil dilution series was prepared using the SDW and 10^{-5} dilutions were used to isolate bacteria form the rhizosphere. Using the spread plate technique, 0.1 mL of each dilution was plated on NA medium and incubated at RT for 24 hours. After the incubation period, colonies of I_1 were identified using colony morphological characters, Gram staining and biochemical tests. Colony count of I_1 was obtained. Three replicates were performed. The experiment was conducted in six-month intervals up to 18 months using the I_1 stored in SDW in centrifuge tubes at RT (30 ± 2 °C).

Experiment 5. Evaluation of the growth promoting ability of nitrogen biofertilizer developed by this technology using N-fixing bacterial isolate I_4, *Priestia aryabhattai* (OP950582), under field conditions

Effectiveness of the developed N biofertilizer using I_4; *Priestia aryabhattai* (OP950582), was evaluated under field condition in a paddy field at the Rice Research Station, Bentota using Bw 367 rice variety in two consecutive seasons (2020 *Yala* and 2020/2021 *Maha* seasons) and in a paddy field at the Rice Research Station,

Ambalantota with At 362 rice variety in 2021/2022 *Maha* season. The experiment was carried out in a randomized complete block design with three replicates and five treatments. Size of each plot was 18 m^2. The treatments used were;

T_1- Department of Agriculture (DOA) recommended rate of urea (positive control), T_2 -50% of DOA recommended rate of urea only, T_3 - N biofertilizer only, T_4 – 50% of DOA recommended rate of urea + N biofertilizer, T_5 -No N fertilizer (negative control). Phosphorus and potassium fertilizer were applied for all the five treatments according to the DOA recommendations.

A pure culture of bacterial isolate I_4 which was stored in SDW ((10 mL) at RT for 12 months was used. Then it was added to SRP (100 mL) and the mixture was incubated for 24 hours at RT to prepare the biofertilizer. After the incubation period, the mixture was diluted using distilled water (3.586 L) and germinated rice seeds (180 g) were mixed with the biofertilizer (10^8 CFU/mL) and incubated for 6 hours at RT.

Plot yield was obtained at the end of the harvesting season and data were statistically analyzed using SAS 9.1.3 statistical software package.

Results and Discussion

Experiment 1. Suitability of SRP at RT as a low-cost growth medium for mass multiplication of bacteria

All the fourteen bacterial isolates used in the study showed growth in SRP and were able to produce sufficient colony counts (>1x10^8 CFU/mL) after 24-hour incubation period at RT revealing the suitability of rice porridge as a medium for mass multiplication of beneficial bacteria (Table 1). Initial number of bacterial cells in a loopful depends on the cell size of bacterial isolate. Further, growth of a bacterial isolate in a growth medium is determined by the initial number of bacterial cells inoculated to the medium and generation time of the bacterial isolate (Wang *et al*., 2015). Thus, colony count produced after the incubation period varied with the isolate (Table 1).

Experiment 2. Suitability of sterilized distilled water (SDW) at RT as a low-cost and convenient carrier medium for storage of bacterial inoculants

All the bacterial isolates showed growth with a sufficient viable cell count in SDW up to 18 months.

Table 1. Colony count of each bacterial isolate on SRP medium after 24 hours incubation period at RT

Bacterial isolate	Bioactivity	Colony count after 24 hours (x 10^8 CFU/mL)
I_1	P-solubilizing	3.0^{fg}
I_2	P-solubilizing	97.0^{a}
I_3	P-solubilizing	7.7^{ef}
I_4	N-fixing	33.6^{b}
I_5	N-fixing	21.7^{d}
I_6	N-fixing	35.8^{b}
I_7	N-fixing	27.8^{c}
I_8	-	9.2^{e}
I_9	-	22.0^{d}
I_{10}	-	8.6^{e}
I_{11}	-	8.6^{e}
I_{12}	-	33.3^{b}
I_{13}	-	17.4^{d}
I_{14}	-	1.9^{g}
CV% - 12.84		

(Values represent means of three replicates)
Means in column followed by the same letters are not significantly different from each other at p=0.05

Bacterial isolates I_1, I_2, and I_3 in SDW stored at RT showed P solubilizing ability on the Pikovskya's agar medium up to 18 months indicating existence of the P-solubilizing ability during storage. Bacterial isolates I_4, I_5, I_6, and I_7 in SDW stored at RT also showed N-fixing ability on Burk's medium up to 18 months (Table 2).

However, colony count of each isolate declined with time compared to the initial cell count and the decline of bacterial colony count in SDW at RT with time varied with the bacterial isolate (Table 3).

Table 2. Colony count and bioactivity of the bacterial isolates in SDW at RT with time

Bacterial Isolate		Just after inoculation	3 MAI	6 MAI	9 MAI	12 MAI	15 MAI	18 MAI
I_1	CC (x10^8 CFU/mL)	0.61	0.59	0.57	0.52	0.45	0.31	0.25
	Bioactivity	+	+	+	+	+	+	+
I_2	CC (x10^8 CFU/mL)	0.78	0.74	0.71	0.67	0.61	0.54	0.52
	Bioactivity	+	+	+	+	+	+	+
I_3	CC (x10^8 CFU/mL)	0.11	0.11	0.10	0.10	0.09	0.09	0.08
	Bioactivity	+	+	+	+	+	+	+
I_4	CC (x10^8 CFU/mL)	2.32	2.29	1.65	1.10	1.00	0.93	0.90
	Bioactivity	+	+	+	+	+	+	+
I_5	CC (x10^8 CFU/mL)	0.47	0.45	0.28	0.20	0.24	0.23	0.22
	Bioactivity	+	+	+	+	+	+	+
I_6	CC (x10^8 CFU/mL)	0.38	0.35	0.33	0.26	0.25	0.23	0.19
	Bioactivity	+	+	+	+	+	+	+
I_7	CC (x10^8 CFU/mL)	1.12	1.11	1.03	0.98	0.80	0.68	0.63
	Bioactivity	+	+	+	+	+	+	+

(Values represent means of three replicates)
(+) - Shows P-solubilizing Ability/ N-fixing Ability
(MAI - Months after Inoculation) (CC - Colony Count)

Table 3. Analysis of variance (ANOVA) of Colony count of the bacterial isolates in SDW at RT with time

Source of variation	DF	Means sum of squares	F value	p value	CD 5%
Treatments	48		160.117	0.000	
Factor A (Bacterial isolate)	6	45089346938775512.0	966.570	0.000	4183946.71
Factor B (Time)	6	6364775510204075.0	136.440	0.000	4183946.71
A×B	36	1383323129251701.2	29.654	0.000	11069682.49
Error	96				
Total	146				

According to the results, all the seven bacterial isolates could survive in SDW without losing their viability and bioactivity up to 18 months showing the potential of using SDW at RT as a low-cost and convenient method for storage of plant growth promoting bacteria. Iacobellis & Devay (1986) reported effectiveness of preservation of plant-pathogenic bacteria *Agrobacterium tumefaciens* and *Pseudomonas syringae subsp. Syringae* in sterile distilled water at 10 °C. The study revealed that a very high percentage (90 - 92%) survival of the isolates after 24 years of storage in distilled water. It showed that after an initial decrease of viable cells after 20 months, the remaining population underwent a much slower decline in viable cells and even after 24 years the bacterial populations of the isolates had not greatly decreased. Further, the bacterial isolates maintained their pathogenicity and their ability to produce syringomycin after 24 years indicating the retention of genetic stability of the bacteria for these traits during storage in sterilized distill water. Liao & Shollenberger (2003) also reported capability of preserving plant- and human-pathogenic bacteria in pure water stored in the dark and at room temperature for several years. Distilled Water does not contain nutrients (Ogunlesi & Okiei, 2003). Therefore, metabolic activity of bacterial cells is reduced to zero level. When it is mixed with the rice porridge, bacterial cells initiate metabolic activities again, begin to grow and reproduce in the rice porridge growth medium, which contains sufficient nutrients for bacterial growth.

Experiment 4. Inoculation ability of bacterial isolate I_1 in SDW at RT with time

Stored culture of bacterial isolate I_1 in SDW at RT showed inoculation ability up to 18 months and sufficient population of I_1 could be re-isolated from the rice rhizosphere compared to the control (Table 4). The results confirmed the survival of I_1 in rice rhizosphere and retention of inoculation ability of the bacterial isolate in SDW at RT with time.

Experiment 5. Growth promoting ability of N biofertilizer developed by this technology using a rhizospheric N-fixing bacterial isolate I_4 under field condition

N biofertilizer produced by the novel technology using rhizobacterial isolate I_4 *Priestia aryabhattai* (OP950582) was effective for rice cultivation. According to the results chemical N fertilizer usage could be reduced by 50% with the application of the N biofertilizer but without a reduction in grain yield. Grain yield obtained in each treatment in three seasons is shown in Table 5.

Table 4. Population of I_1 isolated from rice rhizospheric soil

Age of stored culture of I_1 culture in SDW at RT	Population of I_1 in rice rhizosphere (CFU/1 g x 10^5)
6-month-old culture of I_1	49
12-month-oldculture of I_1	44
18-month-oldculture of I_1	58

(Values represent means of three replicates)

Table 5. Effect of developed N biofertilizer by the novel technology on rice grain yield of two rice varieties under field condition

Treatment	Grain Yield (t ha^{-1})		
	Bw 367 rice variety		At 362 rice variety
	2020 *Yala*	2020/2021 *Maha*	2021/2022 *Maha*
Positive control (DOA recommended rate of urea)	2.856^{a}	2.835^{a}	4.122^{a}
50% of DOA recommended rate of urea	2.413^{b}	2.283^{b}	3.324^{b}
N bio fertilizer only	2.345^{b}	2.477^{b}	2.997^{bc}
N biofertilizer + 50% of DOA recommended rate of urea	3.057^{a}	2.884^{a}	3.951^{a}
Negative control (No N fertilizer)	1.137^{c}	1.628^{c}	2.794^{c}
CV	10.108	7.67	5.632

Means in each column followed by the same letters are not significantly different (p=0.05)

A biofertilizer production process consists of several steps. These include identification of efficient microorganisms and suitable media for mass multiplication of microorganisms, correct formulation to extend the shelf- life, mode of application and industrial consideration regarding production of commercial inoculants (Raimi *et al*., 2021). Liquid biofertilizers are more attractive than carrier based solid inoculants because of longer shelf life, higher tolerance to adverse conditions, ease of handling and application and free of contamination (Allouzi *et al*., 2022). Although liquid biofertilizers can be stored for a long time, lack of a continuous supply of nutrients required for vegetative bacteria and accumulation of toxic metabolites are the causes for loss of viability of bacterial cells in the stored product. However, designing a product, which could supply nutrients at even low levels of metabolic activity and devoid toxic metabolites over storage time period is difficult. Liquid biofertilizers contain desired microorganisms mass multiplied in a broth growth medium and cell protectants or chemicals, which induce the formation of latent spores or cysts for extended shelf life and tolerance to adverse environments (Adajar & Taer, 2021). Characteristics of a good bio formulation are effectiveness, less or no environment pollution, readily biodegradability with high water retention capacity and sufficient shelf life (Allouzi *et al*., 2022). According to the present technology, bacterial liquid biofertilizers were produced as two formulations, which could be kept under RT. This study revealed the suitability of SRP as a low-cost environmentally friendly growth medium for mass multiplication of beneficial bacteria. Princy *et al*. (2014) also reported the use of rice porridge for mass multiplication of phosphate solubilizing bacteria and potassium solubilizing bacteria isolated from tea plantation soils in southern India. Rice porridge is the liquid collected after boiling rice. It contains carbohydrate and minor amounts of phosphorus and potassium and used as a growth medium for microorganisms. (Princy *et al*., 2014). A suitable growth medium should give

more than 1 × 10^8 CFU/mL colony count of beneficial microorganism at a low cost, in large scale multiplication. Rice porridge is very cheap compared to commercially available bacteriological growth media such as nutrient broth and Luria Bertani broth (Devidas *et al*., 2014). The production cost for 1 L of commercial bacteriological growth media is approximately Rs. 1,500.00, whereas the production cost of 1 L of rice porridge is approximately Rs. 100.00. Most importantly, rice porridge does not exert an adverse impact on the environment. Production cost for SDW is very less and only for sterilization and storage in tubes. SRP (Solution 1) together with bacterial isolate in SDW at RT (Solution 2) could be provided to farmers on a commercial scale. Colony count of the bacterial isolates in SRP increased up to 10^{8-9} CFU/mL. In this study, bacteria inoculated culture media were incubated at RT (30 ± 2 °C) only for 24 hours. Higher colony count of bacteria can be obtained by incubating the bacteria inoculated media at 37 °C for longer time period or by increasing the initial population of bacterial isolate in SDW which is mixed with SRP. The mixture can be diluted before application and the dilution factor depends on the bacterial isolate in SDW. This can be applied as a seed treatment, a soil spray or a seedling dip, depending on the bacterial isolate in SDW and the same preparation could be further diluted and could be applied to plants in the form of as a seed treatment, a spray or a soil drench under field condition.

Short shelf-life of sterilized rice porridge is the major constrain when considering the commercial scale production. However, use of proper packaging materials could extend shelf-life of sterilized rice porridge.

New bacterial liquid bio-fertilizer production technology identified by this study has several advantages. The technique is very simple and inexpensive compared to other bacterial biofertilizer formulations. According to the present technology, bacterial liquid biofertilizeris produced as two formulations that can be kept under RT and does not require specific storage conditions. Further, biofertilizer produced by the present technology contains active bacterial cells at the time of applying, in contrast to the formulations with dormant cells. Most importantly, it contains only the natural substances and does not exert harmful effects on the environment. Sticky properties of rice porridge help bacterial inoculum to adhere to the seeds or roots, when the biofertilizer is applied as a seed treatment or seedling dip. Types of bacterial bio-inoculants that can be formulated using this technology are rhizobacteria with P-solubilizing ability or N-fixing ability, free-living N-fixing bacteria, bacteria with antagonistic activity against pathogens, bacteria with other growth promoting activities and compost degrading bacteria.

Conclusions

The novel low-cost technology developed by this study for liquid bacterial biofertilizer production consists of two formulations. SRP was identified as a low-cost growth medium for mass multiplication of bacteria and SDW at RT was identified as a low-cost and convenient carrier medium for storage

of bacterial inoculants without losing the viability and bioactivity. N biofertilizer developed by this novel technology using *Priestia aryabhattai* (OP950582) reduced chemical N fertilizer usage by 50% in Bw 367 and At 362 rice varieties without a reduction in grain yield under field condition. This technology can be used to produce liquid bacterial biofertilizer using N-fixing and P-solubilizing bacterial isolates.

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