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Formulation of New Growth Medium and Fermentation Conditions for *Paenibacillus mucilaginous, a* Potassium Releasing Bacterial Stain (KRB-9)

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ABSTRACT: Soil an important natural wealth comprises of microbes and minerals that construct the nutrient source and sink for biological uptake. Interactions between soil microbes and minerals play a major role in nutrient cycling processes and convert to available forms through solubilization, mineralization and mobilize nutrients from soil components. Biological source for potassium is very minimum compared to nitrogen fixation and phosphorus solubilization. An experiment was conducted to formulate a new medium for an efficient potassium releasing bacterial isolates (KRB). The growth rate, optical density, enumeration was analyzed and compared. Using the best carbon and nitrogen source a new medium was designed for mass production process. Sucrose and dextrose were the best carbon source and yeast extract and peptone were the two best nitrogen sources. For maximizing the population, new medium was formulated and compared with the two existing medium, Alexandrovs' and Sucrose minimal agar medium. Isolates KRB-1 and KRB-9 significantly had higher population in Modified KRB medium with the population load of 93.50 × 10^9cfu ml^{-1} and 95.48 × 10^9c fu ml^{-1} respectively. The same isolates had a population load of 48.90 × 10^8cfu ml^{-1} and 56.45 × 10^8cfu ml^{-1} in Alexandrovs' medium which had the least population than Sucrose minimal agar medium. In optimization of fermentation condition study, it was found that the cell load in terms of cfu ml^{-1} was increased with increase in incubation time and reached the maximum of 55.7 × 10^9 cfu ml^{-1}at 48 h and thereafter the microbial load was found to be static but the K dissolution was increased from 60 h (24.4 mg L^{-1}) and it was in increasing trend upto 96 h (29.8 mg L^{-1}) with a change in pH of 4.7. It was noticed that the cell load was static and at the end of incubation period, the pH decreased from an initial pH of 7.00 to 4.7 from the fermentation study. These findings can be employed for the mass production of potassium releasing bacterium at commercial scale.

Keywords: *Paenibacillus mucilaginous*, Potassium release bacterium, carbon and nitrogen source, medium, standardization.

INTRODUCTION

To overcome the steady demand of food supply, application of inorganic fertilizer is indispensable in modern agriculture. However, extensive use of such fertilizer and its imbalanced application also leads to serious environmental concerns. More emphasis is given to nitrogen and phosphorus fertilization than potash source leading to imbalanced nutrition. To deprive the chemical hazards caused by the application of fertilizers and to increase the production and productivity in a sustainable manner, novel microbial selection for specific nutrient delivery with an appropriate formulation is a need based one. Thus, many of these problems can be surmounted by utilization of biofertilizers, where microorganisms modify rates and mechanisms of weathering of minerals, transformations, and nutrient delivery in crop rhizosphere (Syed *et al*., 2018). The live cells with carriers and formulations are called as biofertilizers or bioinoculants (Dobereiner, 1997; Brahmaprakash and Sahu, 2012)

In India, there are many beneficial microbes, currently used as an alternate for fertilizers for mainly N fixation (Nayak *et al*.,1986; Gopalaswamy *et al.*,1989) and P solubilization (Nautiyal, 1999, Prajapati *et al.*, 2012) and microorganism for bio-dissolution of K very few *viz*., *B. subtilis*, *Frateuria aurantia*, (Chandra *et al.*, 2005); *Bacillus mucilaginosus* (Basak and Biswas, 2010) and mostly under research and yet in-depth research on application aspects has to be made. To fulfil this gap, intensive research was made to investigate an efficient potash bacterium, its characterization, dissolution potential, mechanism of K release. After a thoroughly study, potassium releasing bacteria were obtained and the isolates were abbreviated as KRB. Successful production process with these elite microbial strains (KRB) capable of solubilizing Si releasing K from soil and soil minerals, quickly in large quantity, no doubt, can conserve our existing resources and avoid environmental pollution hazards.

Microbes when introduced to the soil, sometimes fail to establish, or have minimal establishment in rhizosphere because of competition from native microbes, and difficult to proliferate new microenvironment. In order to exploit the benefits of the introduced microbes, the consistency of their performance must be improved by finding a novel production processes (Gao *et al*. 2018). Their success goes in hand with the efficient strain and its apt formulation. There is substantial interest in finding economical ways to produce the bioinoculants, to deliver them to the field in a bioactive form, to enhance and prolong their activity in the environment.

So, a medium formulation for enhanced bacterial population is important for inoculation preparation and bacterization. Hence, the present investigation aimed to standardize and optimize growth condition, and formulate a new medium for mass of potassium releasing bacterium.

MATERIALS AND METHODS

This study was conducted at Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The materials used in this study includes different carbon and nitrogen sources. The experiment was conducted using CRD with a set of treatments and replications which varies with the experiment. The results were statistically analyzed. Microbial populations were represented by log transformed value. The culture that possesses higher dissolution potential was used in this experiment. The best cultures employed were KRB-9 identified as *Bacillus mucilaginosus* (Brindavathy and Gopalaswamy, 2014) and now this culture is placed in Paenibacillus group (Zha *et al.*, 2011). KRB-1 was identified as *Bacillus flexis* by MALDI-TOF analysis (unpublished).

Experiment I. Effect of different carbon substrate on potassium releasing bacteria

The KRB isolates were analyzed both in plate assay and broth assay for its growth in different carbon substrates *viz.*, casein, dextrose, fructose, lactose, mannitol, sucrose and starch. These carbon substrates were substituted in Alexandrovs' broth (Baldani and Dobereiner, 1980) at 1 per cent (w/v) level. One ml of KRB-1 and KRB-9 were inoculated to the sterilized broth. Presence of growth was observed after 5 days of incubation at 30°C and compared with a control containing no added carbon source. The difference in carbon source was compared based on population in dilution plate technique and optical density reading. From the enumeration data obtained and OD value the best carbon source was selected for medium standardization (Table 2).

Experiment II. Effect of different nitrogen substrate utilization of potassium releasing bacteria

Similarly, the KRB isolates were analyzed for growth in different nitrogen substrates *viz.*, ammonium chloride, ammonium sulphate, beef extract, peptone, KNO_3 and yeast extract. These nitrogen substrates were substituted in Alexandrovs' medium (Sheng *et al.*, 2002) at 0.3 per cent (w/v) level and incubated at 30ºC for 5 days. Presence of growth was observed after 5 days of incubation and compared with a control containing no added nitrogen source in the medium. Based on the growth on plates and optical density reading of the grown liquid culture, the nitrogen source that supports the maximum growth was selected and the same was used for medium standardization.

Experiment III. Standardization of growth medium for potassium releasing bacteria

Standardization and formulation of new medium for KRB isolates was done with the best carbon and nitrogen substrates obtained from the experiment II and III. The new medium "modified KRB medium" was formulated with 1 per cent and 0.3 percent of carbon and nitrogen sources respectively and supplemented with one per cent mineral (powdered feldspar or Potassium alumino silicate). For medium standardization, the best carbon sources dextrose and best N sources peptone were added to formulate new medium. This was compared with the two standard medium selected *viz.*, Alexandrovs' medium and Sucrose minimal agar medium (Sheng and He, 2006). For this study three sets of 100 ml of each three medium were prepared in 250 ml flask and were sterilized. To this one ml of log phase grown cultures of five KRB isolates viz., KRB-9, KRB-1, KRB-3, KRB-7 and KRB-10 was inoculated and incubated at room temperature for 3-5 days. For microbial population assessment the 1ml of the broth of respective KRB isolates cultures were serially diluted, plated with each respective medium and parallelly the OD value was also observed at 620nm (Table 3 and Fig 1).

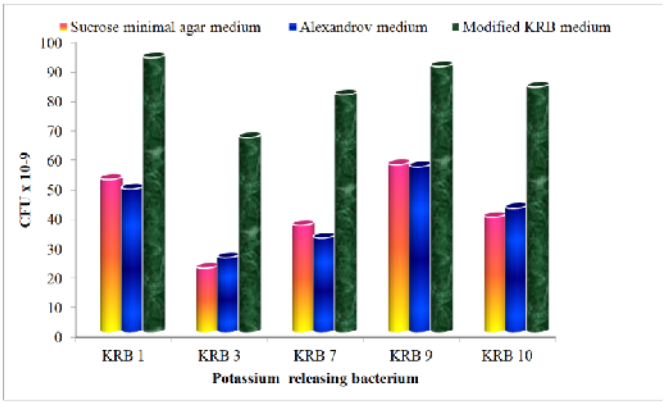

Fig. 1. Standardization of medium for potassium releasing isolates.

Experiment IV. Screening of optimum fermentation conditions for the selected KRB isolates:

This study was carried out for optimizing the fermentation condition to produce more bacterial population and to find out the phase at which maximum dissolution takes place was performed. The process was carried out in a 5.0 litre capacity fermenter with the working volume of 3.0 litre. Optimization of substrate concentration, pH and fermentation time were attempted and standardized. Modified KRB media was prepared and added to the fermenter. The medium pH was adjusted to 7.2 and sterilized. Ten percent inoculum of KRB-9 starter culture was inoculated. The samples drawn at every 12 hours intervals were used to find out the optimum fermentation conditions that supported the maximum population and cell density. Simultaneously the samples were subjected to analyses the K release using flame photometer. The concentration of potassium in the solution was deduced from the standard curve and the percentage of potassium in the sample was calculated (Jackson 1973). Population enumeration and extracellular polysaccharide production was studied by plate assay. EPS production was scored by Sambrook and Russell (1989) method. The plates were incubated at room temperature for 4-5 days. The amount of polysaccharide produced was observed visually and scored as no polysaccharide production (-), weak polysaccharide production (+), moderate polysaccharide production (++) and high polysaccharide production (+++).

RESULT AND DISCUSSION

A. Effect of different carbon and nitrogen sources on KRB-1 and KRB-9 isolates

Nutrient sources are an indispensable criterion for normal microbial metabolism and regulate the active centers in their physiological cycles. These provide the stability to the synthesis of macromolecules and maintaining cell structure. It supports the

cell by supplying energy while multiplication and also protects it while biointrusion in new environment (Ghasemi and Ahmadzadeh, 2013). Therefore, it is imperative to develop a large-scale culture of potash bacteria by optimizing C source and N source in the culture medium. The carbon and nitrogen substrate utilization profile of the KRB isolates tested, exhibited high divergence among the isolates. The differences in the ability to utilize various C and N sources may be mediated by specific operons and structural genes required for the breakdown of the specific substrate (Wang and Liu, 2008). The growth of KRB-1 and KRB-9 in medium supplemented with different carbon and nitrogen substrates was recorded after 4-5 DAI (Table 1). The isolate KRB-1 utilized all the carbon sources tested except lactose, which recorded very low population (9.8 $x10^6$ cfu ml^{-1}). Likewise, the isolate KRB-9 was able to utilize all the carbon tested except lactose with the population load of 10.2 $x10^6$ cfu ml^{-1}. The best carbon source that exhibited higher colonies was dextrose (40.8 x 10^6 cfu and 39.8 x 10^6 cfu per ml of KRB- and KRB-9 respectively). When nitrogen substrate is considered, both the isolates KRB-1 and KRB-9, showed very low population 7.6 $\times10^3$ and 8.8 $\times10^3$ cfu ml^{-1} respectively in ammonium chloride added medium. Higher cell load was produced in peptone supplemented medium (Table 2). In a similar study, Sheng *et al.*, (2002) studied the growth condition of two isolates of *Bacillus edaphicus* with different carbon and nitrogen sources at varied pH and found varied difference among the two isolates tested. Many researchers have attempted to find an effective protocol studies for large-scale production of new microbial cultures and best examples are with *Bacillus* and *Paenibacillus*, which supports this investigation, and it provides a suitable reference for their large-scale fermentation process (Ghasemi and Ahmadzadeh, 2013; Prabakaran and Hoti, 2008; Gao *et al.*, 2018a).

Table 1: Effect of different carbon sources on population and growth of efficient potassium releasing bacterium (KRB).

Carbon sources	KRB 1		KRB 9	
	Population ($x10^6$ cfu ml^{-1})	OD value (660nm)	Population ($x10^6$ cfu ml^{-1})	OD value (660nm)
Casein	12.3 (7.09)	0.79	18.8 (7.27)	0.32
Dextrose	40.8 (7.61)	1.72	39.8 (7.60)	0.76
Fructose	20.1 (7.30)	0.48	22.0 (7.34)	0.74
Lactose	9.8 (3.99)	0.33	10.2 (4.01)	0.43
Mannitol	36.6 (7.56)	0.77	39.0 (7.59)	0.29
Starch	22.0 (7.34)	1.85	30.4 (7.48)	0.12
Sucrose	42.6 (7.63)	1.77	38.9 (7.59)	0.76
SEd	0.09	0.005	0.10	0.008
CD (p=0.05)	0.21	0.012	0.23	0.018

Log transformed values in parenthesis

Table 2: Effect of different nitrogen source on the elite potassium releasing bacterial isolates.

Different N source	KRB -1		KRB -9	
	Population ($x10^6$ cfu ml^{-1})	OD value	Population ($x10^6$ cfu ml^{-1})	OD value
Peptone	36.6 (7.56)	1.96	33.2 (7.52)	1.19
Potassium nitrate	18.8 (7.27)	0.89	19.4 (7.29)	0.20
Ammonium chloride	7.6 (3.88)	0.18	10.8 (3.94)	0.48
Beef extract	19.0 (7.28)	0.83	18.8 (7.27)	0.34
Yeast extract	37.4 (7.57)	1.73	34.6 (7.54)	1.32
Ammonium sulphate	19.6 (7.29)	0.63	23.8 (7.38)	0.36
SEd	0.18	0.006	0.17	0.005
CD (p=0.05)	0.37	0.013	0.36	0.011

Table 3: Standardization of medium for potassium releasing isolates.

Isolates	Alexandrov medium ($x10^8$cfu ml^{-1})	Modified KRB medium ($x10^9$cfu ml^{-1})	Sucrose minimal agar medium ($x10^8$cfu ml^{-1})
KRB 1	48.90	93.50	52.20
KRB 3	25.55	66.40	22.00
KRB 7	32.24	80.90	36.52
KRB 9	56.45	95.48	57.13
KRB 10	42.36	83.53	39.33
SEd	0.212	0.334	0.231
CD	0.509	0.669	0.515

Table 4: Optimization of fermentation condition for KRB-9.

Time interval	Population (x cfu ml^{-1})	OD value (660nm)	pH	Biodissolution potential	
				Potassium (µg ml^{-1})	ECP production
12	52.0×10^8 (6.72)	0.12	7.0	0.3	+++
24	73.0×10^9 (9.86)	0.49	6.3	1.6	+++
36	37.67×10^9 (9.58)	0.92	6.0	3.7	+++
48	55.67×10^9 (9.75)	1.03	5.5	8.9	+++
60	38.33×10^9 (10.58)	1.40	4.9	24.4	+++
72	34.33×10^9 (10.54)	1.54	4.7	28.8	+++
96	33.67×10^9 (10.53)	1.53	4.7	29.8	+++
SEd	0.25	0.01	0.10	0.36	
CD	0.54	0.02	0.23	0.78	

Higher EPS production- + + +

B. Standardization of medium for KRB isolates

The aim of this experiment is to find the best medium for the growth of the isolate *Paenibacillus mucilaginosus* (KRB-9). Selection of an apt medium or formulation for obtaining maximum population favors for mass production technology (Jia *et al.,* 2017) and will be helpful for the dissemination of bioinoculants as biofertilizer, and further proliferation and colonization rhizosphere (Chinheya *et al.,* 2017). To select medium for production of higher cell load, the KRB-9 along with KRB-1, KRB-3, KRB-7, KRB-10 isolates were experimented. It was compared with Alexandrov's and sucrose minimal agar medium, which was commonly used for potassium solubilizing bacterium. Among the isolates tested KRB-1 and KRB-9 were on par with each other producing significantly higher population in Modified KRB medium with the population load of 93.50 $x10^9$cfu ml^{-1} and 95.48$x10^9$cfu ml^{-1} respectively (Table 4 & Fig. 1). Alexandrovs' medium had the least population than sucrose minimal agar medium. Modified KRB medium was selected as the best medium for KRB isolates for mass production. This is in accordance with the observations reported by Liu *et al.* (2006), where the population of *B. mucilaginosus* in sucrose minimal agar medium recorded higher population. Similar research was performed to study the population density of *Bacillus* strains using Alexandrovs' medium (Girgis *et al.,* 2008). Leaungvutiviroj *et al.* (2010) studied the mixed inoculum preparation for potassium solubilizing bacterium using sucrose minimal agar medium and obtained population upto 10^9 in culture medium when added with feldspar. Whereas in contrary the newly formulated Modified KRB medium produced higher population load than with sucrose minimal agar medium.

C. Optimization of fermentation condition

Optimization of fermentation condition was done to find out the suitable condition for the mass multiplication of the selected isolate. Initially the cultures were grown in small quantities in conical flasks in *invitro* conditions. For commercial scale production it has to be performed in fermenters. And the fermentation conditions should be familiar for further handling of cultures. Hence a fermentation study was carried out using KRB-9 isolates in fermenter by adopting the already optimized parameters. The amount of cell biomass, its optical density value, change in pH, polysaccharide production was analyzed at periodic. Chandra *et al.,* (2005) formulated a procedure for mass multiplication of liquid mixed culture of *F. aurantia*, *Azospirillum* and PSB and harvested maximum population and standardized the procedure. It was found that the cell load of KRB-9 (cfu ml^{-1}) was increased with increase in incubation time and reached the maximum of 55.7 $x10^9$ at 48 h and there after the microbial load was found to be static but the K dissolution rate was increased linearly from 60 h (24.4 mg L^{-1}) upto 96 h (29.8 mg L^{-1}) with a reduction in pH at 4.7. It was noticed that the cell load was static and from the initial stage to end of the incubation period, a steady decline of the pH from 7.00 to 4.7 was noticed.

Many studies have been documented for the pH reduction and acidolysis that reduces the pH and accelerates the release of K (Cameselle *et al.,* 2003; Li Sha., 2006). Their findings definitely support our study, and this might be the reason for the bio-dissolution of K from the supplemented mineral source. This reduction in pH was found to be higher after the log phase may be due to the production of biodissoluting compounds by the cells in stationary phase. Metabolites secreted by microbial leads mineral corrosion, causes reduction in pH and weathering of crystal structure of minerals by chelation and by the bacterial enzyme (Sun and Zhang, 2006). Acidolysis is the main accepted mechanism of microbes weathering silicate minerals (Jongmans *et al.,* 1997;). In the present study, there exist a correlation between organic acid production, EPS acidification and pH reduction in K release.

As far as extracellular polysaccharide (EPS) production is concerned, higher score was produced from its twelfth hour and continued to be seen upto 96th hour. Product of EPS extensively helps in dissolution of K. The acidic metabolite produce by the bacteria is absorbed by the EPS and forms acidic intermediate between the mineral and the cell, thereby supporting cationic release in the medium (Zhu *et al*., 2011). In a microbe-mineral study it was explained that organic acids produced are strongly absorbed by these polysaccharides and gets attached to the mineral surface. Thus, they create an acidic environment of high concentration of organic acids near the mineral that helps in release of K (Liu *et al*., (2006). In another microbe- mineral study with feldspar and B. *mucilaginous* leaching of K from feldspar had occurred, as a result of the participation of both EPS and organic acids (Girgis *et al.,* 2008). These findings are in accordance with the results of our study.

The promising cultures in *invitro* studies sometimes proves to be ineffective during larger scale mass multiplication process and further handling. So, a specific and productive protocol for scaling of bioinoculants is crucial for this hour. In this investigation maximum population was produced with dextrose and peptone as carbon and nitrogen source. When the same was implement in medium standardization, the newly formulated modified KRB medium recorded significant result. Simultaneously when these combinations were tested in fermenter, it recorded maximum population load, with production of metabolites that declined the pH of the growth medium. In this fermentation condition the KRB-9 isolate facilitated the release of K from the mineral added in the medium.

CONCLUSION

Bio-intervention of novel potassium releasing bacterial strain, KRB (*Paenibacillus mucilaginosus*) with its formulations could be another alternative and viable technology to solubilize insoluble K from soil minerals into plant-available pool. It can be used efficiently as a biological source of K nutrition to plants and in maintaining soil potassium. Hence these novel medium formulations obtained at this project end, would be an efficient and a flourishing delivery system for the proposed strain, KRB-9 and victory of these new bioinoculant technology lay concrete for eco-friendly approaches in crop production and would serve as a best commercial medium for mass production.

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