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Development of a Cost-effective Medium for the Mixed Fermentation of *Paenibacillus durus*, *Paenibacillus glucanolyticus* and *Bacillus megaterium*

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A B S T R A C T

In the recent years, plant growth promoting rhizobacteria (PGPR) are gaining much importance due to the drastic effects of chemical fertilizers. Growing these PGPR as an axenic culture is time consuming and also increases the production cost. The present study aims in the development of cost effective medium for the mixed fermentation of 3 PGP isolates i.e., *Paenibacillus durus*, *Paenibacillus glucanolyticus* and *Bacillus megaterium* and further optimization of the fermentation parameters. Among the 4 media developed, MC2 showed high CFU counts i.e., $1.2x10^9$/ml in *Paenibacillus durus*, $3.4x10^9$/ml in *Paenibacillus glucanolyticus*, $2.1x10^9$/ml in *Bacillus megaterium*. Sucrose optimization studies showed good results at 15 g/L and yeast extract optimization studies showed high microbial load at 2 g/L. Fermentation parameters were optimized viz. incubation temperature 30°C, pH 7.0 and inoculum percentage 8%. A batch trial was conducted by taking the optimized nutrient and fermentation parameters which showed good results in *P. durus* (final broth $6.2x10^9$/ml, cell biomass $2.8x10^{11}$/g), *P. glucanolyticus* (final broth $7.6x10^9$/ml, cell biomass $3.1x10^{11}$/g) and *Bacillus megaterium* (final broth $4.4x10^9$/ml, cell biomass $2.3x10^{11}$/g). Based on the above results, it can be stated that the optimized MC2 medium can be effectively used in the mixed fermentation in the industries.

***Key words*:** *Bacillus megaterium*, Mixed fermentation, Optimization, *Paenibacillus durus*, *Paenibacillus glucanolyticus*

Agriculture is one of the crucial sectors in most of the developing countries. Due to the increasing demand for the food and growing population an alternative method for increasing the crop production without disturbing the rhizosphere is much encouraged [1]. At present to attain this target, it is necessary to have fertile soil or else huge amounts of fertilizers or supplements need to be used in low fertile soil. In the current global scenario, three major chemical fertilizers viz. nitrogen, phosphorous and potash are playing a key role in increasing the crop productivity [2]. As per the FAO 2017 estimates, plants can utilize only 0.2% out of the total chemical fertilizers used. The excess chemical fertilizers used shows various adverse effects viz. acidification of soil, eutrophication, water pollution etc. In this scenario, plant growth promoting rhizobacteria (PGPR) play a key role in replacing the chemical fertilizers [3].

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Though several PGPR are notified till date, *Bacillus* genus is much focussed because of the aerobic endospore forming ability which enabled the industrial sector to use these microbes as biofertilizers and biopesticides. *Bacillus spp*. use quorum sensing which is a signal communication mechanism to initiate the process of sporulation. In the fermentation process, endospores are formed during the nutrient depletion or stress conditions when the density of the cells are too high i.e., $1.0x10^8$ cells/ml [4].

Commercialization and success of the plant beneficial microbes totally relies on the industries and scientific organizations. Whereas success of the PGPR strains in the market depends on market demand, consistency of the product, low capital cost and stability of the product [5]. In general, the nitrogen fixing bacteria, phosphate and potash solubilizing bacteria are grown individually as axenic cultures. This increases the production cost and takes much time for the fermentation process [6].

Mixed microbial culture fermentation is defined as the fermentation process in which the inoculum contains 2 or more microbial cultures. The mixed cultures may include group of bacteria/fungi or combination of both. Mixed cultures generally exist in the nature and the same rule has been implemented to culture various microbial cultures in a single medium in the same time. For example, rhizosphere contains various fungi, protozoa, bacteria and algae which totally depend on the

availability of the nutrients and the parameters like temperature and pH. Some of the microbes present in the rhizosphere may be parasites, some produce essential elements required for the growth of other microbes and some do not have co-relation with each other [7]. The present research work aims in optimizing a cost-effective medium for growing the aerobic endospore forming nitrogen fixing, phosphate and potash solubilizing bacteria in a single nutrient medium without effecting the prime plant growth promoting property.

MATERIALS AND METHODS

Collection of PGP microbial strains

The plant growth promoting rhizobacterial strains viz. *Paenibacillus durus* (Nitrogen fixing bacteria), *Bacillus megaterium* (Phosphate solubilizing bacteria) and *Paenibacillus glucanolyticus* (Potash solubilizing bacteria) were sourced from culture collection centre of Shivashakti Bioplanttec Limited, Hyderabad.

Development of seed inoculum

100 ml of seed inoculum for all the 3 PGPR were developed before carrying out the mixed culturing medium optimization studies. *Paenibacillus durus* was grown in the nitrogen free medium with composition g/L sucrose 20, K_2HPO_4 0.1, KH_2PO_4 0.4, NaCl 0.1, $MgSO_4$ 0.2, $FeCl_3$ 0.01 and Na_2MoO_4 0.002 [8]. *Paenibacillus gluconalyticus* inoculum was developed in the Aleksandrov medium with composition g/L sucrose 5, Na_2HPO_4 2, $MgSO_4$ 0.5, $FeCl_3$ 0.005, $CaCO_3$ 0.1, Potassium aluminium silicate 1.0 [9]. *Bacillus megaterium* inoculum was developed in the Pikovskaya medium with composition g/L dextrose 10, yeast extract 0.5, $Ca_3(PO_4)_2$ 5, $(NH_4)_2SO_4$ 0.5, KCl 0.2, $MgSO_4$ 0.1, $MnSO_4$ 0.0001, $FeSO_4$ 0.0001 [10]. After inoculation the medium flasks were kept on orbital shaker for 48 h at 120 rpm.

Medium optimization for mixed culturing

Medium optimization studies were carried out by taking 4 different media compositions with different ingredients and concentrations. After 48 h, liquid broth samples were collected and the total microbial cells were enumerated.

Optimization of carbon and nitrogen concentrations

Optimization of sucrose concentration

Various concentrations of sucrose were studied by taking other nutrients as constant. The study was carried out by using one factor at a time. Different sucrose concentrations viz. 5, 10, 15, 20, 25 and 30 g/L were studied. Based on the total microbial load (CFU) the best sucrose concentration was screened.

Optimization of yeast extract concentration

Different yeast extract concentrations viz. 0.5, 1, 1.5, 2.0 and 2.5 g/L were studied by keeping rest of the nutrients as constant. Best yeast extract concentration was screened based on the total microbial load.

Optimization of fermentation parameters

Optimization of incubation temperature

In order to optimize the incubation temperature, various temperature ranges viz. 20, 25, 30, 35 and 40°C were tested. Different medium flasks were taken and each flask was incubated at a specific desired temperature. Total viable cells were enumerated and CFU counts were calculated.

Optimization of pH

Various pH values (6.0, 6.5, 7.0, 7.5 and 8.0) were tested to arrive an optimized pH value. Total microbial load was assessed based on which the optimized pH value was set.

Optimization of inoculum percentage

Percentage of inoculum plays a key role in the high yield production of PGP. To optimize the inoculum percentage various percentages viz. 4, 5, 6, 7, 8, 9 and 10 were studied by taking different sets of medium flasks.

Batch trial

Batch trial was conducted by taking the optimized carbon, nitrogen concentrations and other nutrient ingredients were kept as constant. Optimized fermentation parameters were taken to conduct the batch trial. Total microbial load was assessed and the following parameters were estimated:

Estimation of nitrogen fixation

Estimation of the nitrogen fixation was estimated by using Kjeldahl method. It can be defined as the milligram of nitrogen produced by utilizing one gram of carbon source. Liquid broth samples were centrifuged at 1000 rpm at 4°C for 10 min. 10 ml of potassium dichromate solution and 20 ml of sulfuric acid were added to 2 ml of culture supernatant and heated for 1 min. 200 of water was added to the mixture and 4-5 drops of ferroin was added as an indicator. Titration was carried out against 0.5 N ferrous sulphate and the total carbon was calculated based on the total volume of ferrous sulphate solution used [11].

Phosphate solubilization assay

Phosphate solubilization assay was carried out by taking the liquid culture broth and inoculating on the Pikovskaya's agar plates with composition g/L dextrose 10, yeast extract 0.5, $Ca_3(PO_4)_2$ 5, $(NH_4)_2SO_4$ 0.5, KCl 0.2, $MgSO_4$ 0.1, $MnSO_4$ 0.0001, $FeSO_4$ 0.0001 and agar 15 [10]. Zone of solubilization was observed and calculated which resembles the phosphate solubilization ability of the PGP microbial strain.

Potash solubilization assay

The liquid culture broth was inoculated on the selective agar plates with composition g/L sucrose 5, Na_2HPO_4 2, $MgSO_4$ 0.5, $FeCl_3$ 0.005, $CaCO_3$ 0.1, potassium aluminium silicate 1.0 and agar 15. Potassium solubilization zone was observed and measured [9].

RESULTS AND DISCUSSION

Biofertilizers, due to their eco-friendly nature when compared to the chemical fertilizers is considered as a best choice for the sustainable agriculture [11]. Till date the chemical fertilizers (N, P, K) are playing a key role in supplying the adequate food required for the growing population. Though there are several disadvantages of chemical fertilizers, lack of competitive alternative source made the farming community to rely on the chemical fertilizers. From the past decade, researchers are focussing much on the plant growth promoting rhizobacteria as a prominent player in the sustainable agriculture. Nitrogen fixers, phosphate and potash solubilizers has the potential to reduce and avoid the chemical-based N, P, K fertilizers [2]. As on date, these nitrogen fixers, phosphate and potash solubilizers are grown individually in their respective selective media. This process is time consuming, costly and requires sophisticated laboratory facility. In this context, the present study is an attempt to grow the nitrogen fixers, phosphate and potash solubilizing bacteria in a single

culture medium without disturbing its efficiency. Mixed fermentation is an ancient technique which totally depends on the natures rule. During the course of time, the mixed fermentation technique was hindered focussing much on the axenic cultivation [7]. The present research focuses on optimization of medium for the mixed culturing of nitrogen fixer, phosphate and potash solubilizers. The PGP cultures were sourced from the culture collection centre of Shivshakti Bio planttec Limited, Hyderabad. These PGP strains are native strains isolated from the maize rhizosphere of Mallampet village, Telangana (18.0601° N, 78.0481° E). The PGP microbes are *Paenibacillus durus* which is a nitrogen fixing bacteria, *Paenibacillus glucanolyticus* which is a potash solubilizing bacteria and phosphate solubilizing *Bacillus megaterium*. Prior initiating the mixed culture medium optimization trials, seed/mother culture was prepared in the respective selective media and kept on orbital shaker (REMI CIS) for 48 h. Four different media compositions were designed as shown in the [8-10] (Table 1).

Table 1 Various media compositions for growing the mixed cultures

Ingredients	MC1	MC2	MC3	MC4
Dextrose	20			
Sucrose		20	10	20
K_2HPO_4		0.2	0.1	
KH_2PO_4		0.4	0.2	
NaCl		0.3		0.5
$MgSO_4$	0.1	0.5	0.1	0.1
$MnSO_4$	0.0001	0.1		0.1
$FeCl_3$		0.01		
Na_2MoO_4		0.002	0.001	0.001
Mica	1	1	2	1.5
Yeast extract	0.5	1.5	1	1
$Ca_3(PO)_4$	5	5	5	6
$CaCO_3$	0.0001	0.01	0.01	0.1

Enumeration of microbial count (CFU) by using the standard plate count assay showed the medium MC2 as the best suitable medium for mixed fermentation PGP bacterium. MC2 showed the CFU counts of $1.2x10^9$/ml in *Paenibacillus durus*, $3.4x10^9$/ml in *Paenibacillus glucanolyticus*, $2.1x10^9$/ml in *Bacillus megaterium*. MC2 was further taken for the optimization of carbon and nitrogen sources which plays a key role in the growth of the microbe (Fig 1).

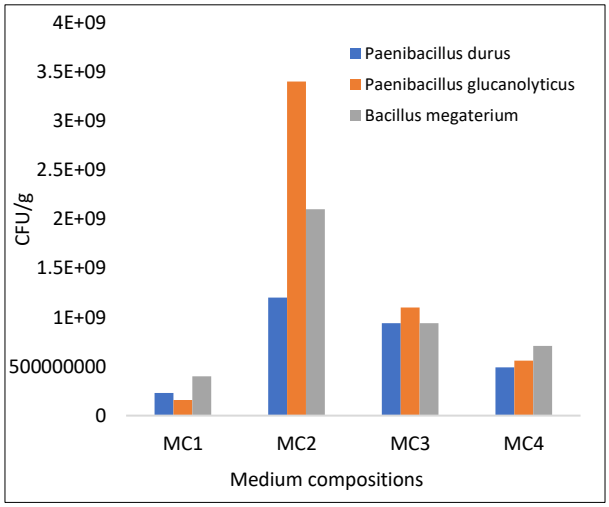

Fig 1 CFU counts of various medium compositions

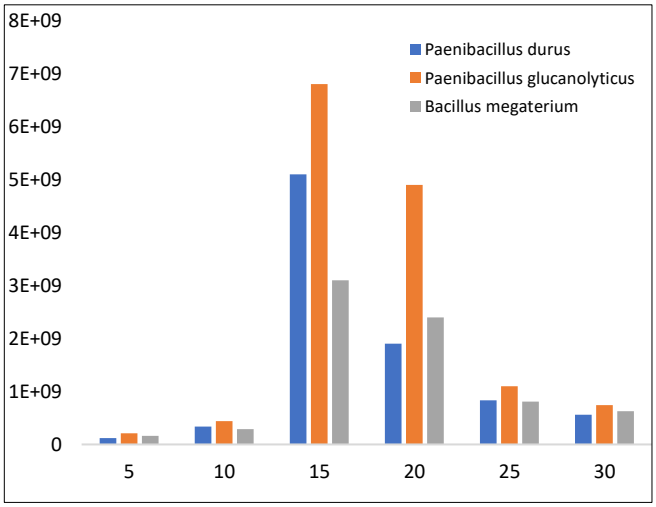

Fig 2 Sucrose concentration optimization studies

MC2 medium contains sucrose as the carbon source. With an aim to achieve high CFU count, the carbon source i.e., sucrose was further studied to get the optimized concentration. One factor at a time was used for the optimization studies in which only one variable was changed keeping the rest of the ingredients as constant. Various concentrations of sucrose viz. 5, 10, 15, 20, 25, 30 g/L were taken. The sterilized nutrient medium was inoculated with the *Paenibacillus durus*, *Paenibacillus glucanolyticus*, *Bacillus megaterium* and were kept on orbital shaker for 48 h. Sucrose with concentration 15 g/L showed high CFU count i.e., $5.1x10^9$/ml in *Paenibacillus durus*, $6.8x10^9$/ml in *Paenibacillus glucanolyticus*, $3.1x10^9$/ml in *Bacillus megaterium* (Fig 2).

Yeast extract concentration was further optimized by taking various concentrations viz. 0.5, 1, 1.5, 2.0 and 2.5 g/L. Among all the yeast extract concentrations taken, 2 g/L showed high CFU counts i.e., $4.8x10^9$/ml in *Paenibacillus durus*, $4.9x10^9$/ml in *Paenibacillus glucanolyticus*, $2.5x10^9$/ml in *Bacillus megaterium* (Fig 3). Balancing the carbon and nitrogen sources play a key role in improving the microbial loads [11].

Fermenter parameters optimization studies were carried out to achieve the high yield of PGP bacteria. Parameters like pH, temperature and inoculum percentage were optimized.

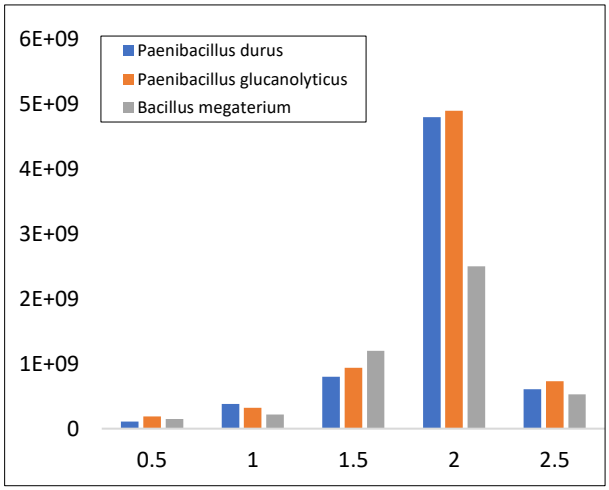

Fig 3 Yeast extract concentration optimization studies

pH optimization studies were carried out among which pH 7.0 showed high microbial load with CFU counts $4.2x10^9$/ml in *P. durus*, $3.6x10^9$/ml in *P. glucanolyticus*, $3.8x10^9$/ml in *Bacillus megaterium* (Fig 5).

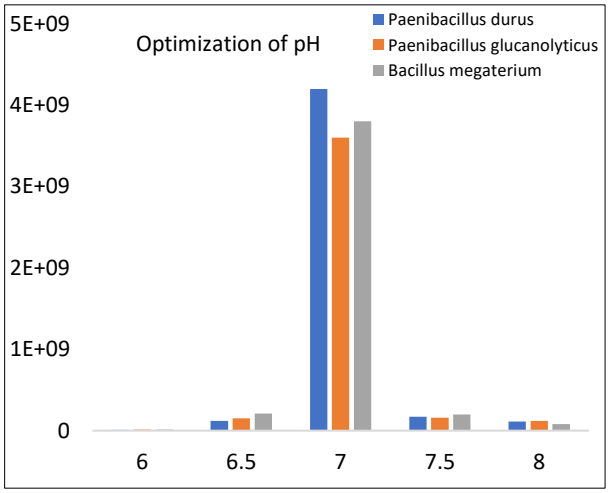

Fig 5 Optimization of pH

By using the optimized carbon and nitrogen sources, a batch trial was taken by using the medium composition g/L 15 sucrose, 0.2 K_2HPO_4, 0.4 KH_2PO_4, 0.3 NaCl, 0.5 $MgSO_4$, 0.1, $MnSO_4$, 0.01 $FeCl_3$, 0.002 Na_2MoO_4, 1 mica, 2 yeast extract, 5 $Ca_3(PO)_4$, 0.01 $CaCO_3$. Optimized fermentation parameters maintained were incubation temperature 30°C, pH 7.0, inoculum percentage 8%. Enumeration of microbial cells in the final broth and cell biomass were assessed which showed *P. durus* (final broth $6.2x10^9$/ml, cell biomass $2.8x10^{11}$/g), *P. glucanolyticus* (final broth $7.6x10^9$/ml, cell biomass $3.1x10^{11}$/g), *Bacillus megaterium* (final broth $4.4x10^9$/ml, cell biomass $2.3x10^{11}$/g). Similarly, nitrogen fixing estimation of *Paenibacillus durus* was carried out which showed 34.4 ± 2.6 mg/g of sucrose utilized. Phosphate solubilizing ability of *Bacillus megaterium* was assessed which showed 9.2 ± 4.2 mm zone of solubilization in 3 mm plate. *Paenibacillus glucanolyticus* showed 8.3 ± 2.1 mm zone of potash solubilization when tested. Based on the results, it can be stated that the optimized medium is not altering the actual bio efficacy

Temperature optimization studies showed good yield at 30°C with CFU counts $1.4x10^9$/ml in *P. durus*, $1.8x10^9$/ml in *P. glucanolyticus*, $2.3x10^9$/ml in *Bacillus megaterium* (Fig 4).

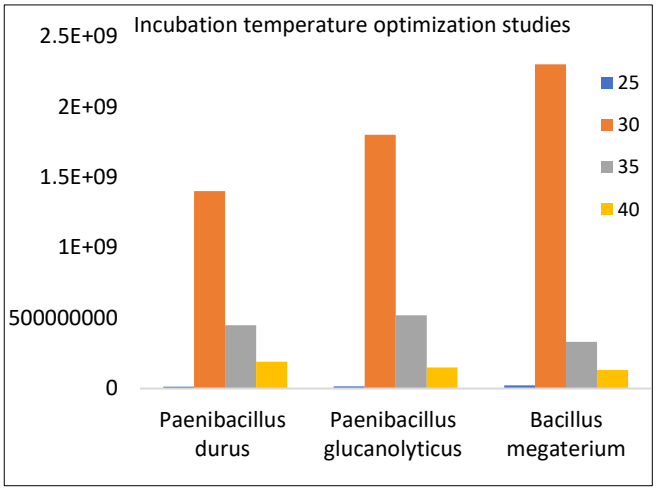

Fig 4 Optimization of incubation temperature

Optimization studies carried out on inoculum percentage showed high microbial load in 8% with CFU count $3.4x10^9$/ml in *P. durus*, $5.3x10^9$/ml in *P. glucanolyticus*, $4.7x10^9$/ml in *Bacillus megaterium* (Fig 6).

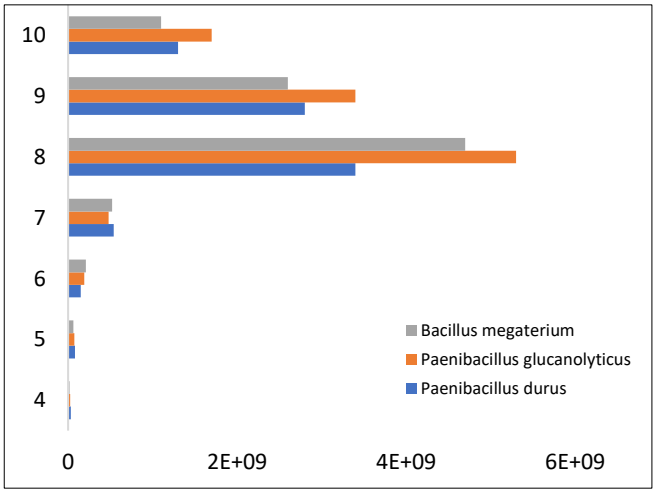

Fig 6 Optimization of inoculum percentage

of the PGP isolates. The optimized medium contains several key components like sodium molybdate, tricalcium phosphate and mica which helps the PGP isolates to retain their actual nature.

CONCLUSION

The research study was carried out to grow the *Paenibacillus durus*, *Paenibacillus glucanolyticus* and *Bacillus megaterium* by using mixed fermentation. Medium optimization studies showed the MC2 as the best suitable media for mixed fermentation. Further optimization of nutrients and fermentation parameters showed high microbial cell load. Based on the results obtained, it can be concluded that the MC2 medium can be effectively used for mixed fermentation in the industries which also helps the PGP isolates to retain its actual bio efficacy.

Conflict of interest: Authors have no conflict of interests

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