Development of Commercial Bioformulation of Plant Growth Promoting Rhizobacteria (PGPR) using Coir pith as carrier

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Project Title

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Introduction :

PGPR are Plant Growth-Promoting Rhizobacteria defined as root-colonizing bacteria that exert beneficial traits on plant growth and development. Root colonization comprises the ability of PGPR to establish on or in the root or rhizosphere to multiply, survive and colonize along the growing root in the presence of the indigenous microflora. PGPR are considered as efficient microbial competitors in the soil-root zone. The use of PGPR for reducing chemical inputs in agriculture is a potentially important issue. PGPR have gained world-wide importance and acceptance for sustainable agricultural benefits. The aim of the present study is to prepare a bioformulation from the bacterial strains having good PGPR properties using Coir Pith as substrate/carrier and to examine their effect on plant growth and productivity of some selected agricultural crops thereby developing a commercial product.

Objectives :

(i) Compatibility studies of various PGPRs to Coir pith as substrate/carrier

(ii) Colony establishment of PGPRs in coir pith

(iii) Field application of PGPR formulation with coir pith in specific agricultural crops for determining its ability to enhance crop production.

(iv) Influence of inorganic nutrients (MOP, Urea and Rock Phosphate) on PGPRs with coir pith as substrate.

(v) Developing a commercial PGPR formulation using Coir pith as carrier.

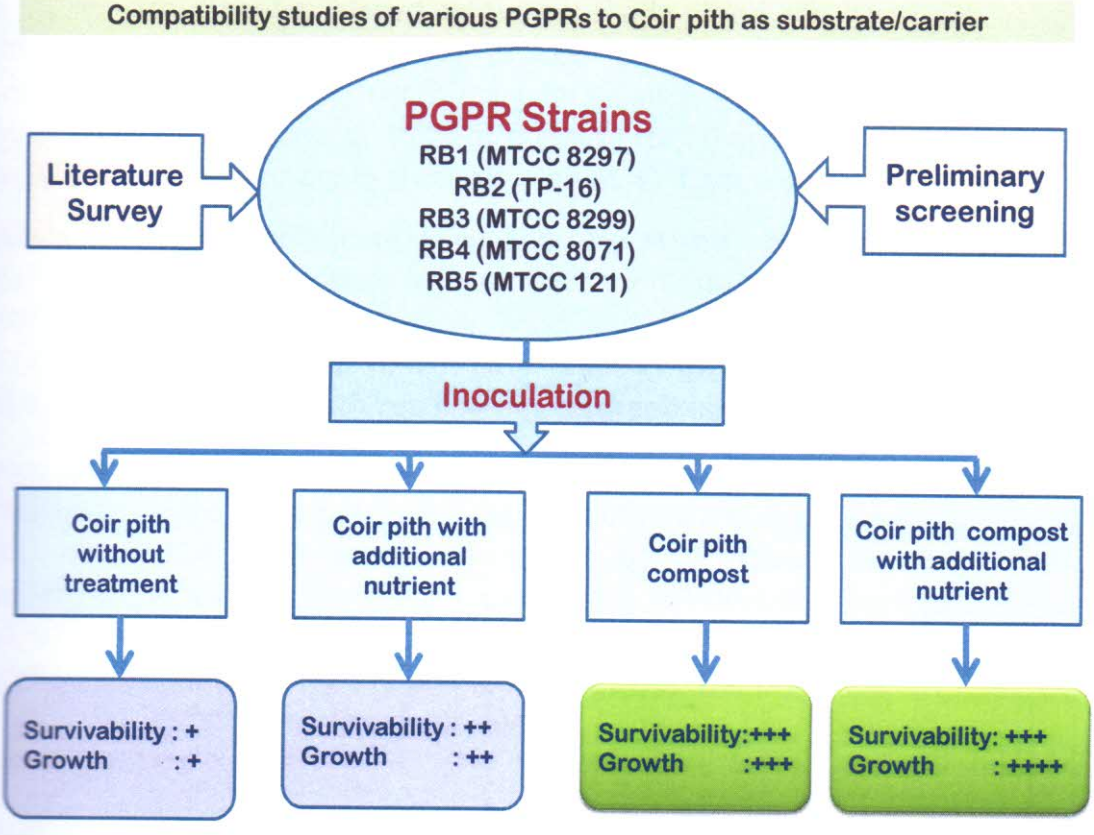
Objective wise achievement:

The project has been started on 26.03.2016 and following achievement have been made under each project objectives :

(1) **Compatibility studies of various PGPRs to Coir pith as substrate/carrier**

Collection of 5 different PGPR strains RB1 (MTCC 8297) *Bacillus cereus*, RB2 (TP-16) *Pseudomonas fluorescence*, RB3 (MTCC 8299) *Pseudomonas rhodesiae*, RB4 (MTCC 8071) *Chromobacterium violaceum* and RB5 (MTCC 121) *Bacillus subtilis* have been done and the culture is being maintained in laboratory for further studies.

- Compatibility studies of various PGPRs to Coir pith as substrate/carrier has been completed and found to be compatible to grow in coir pith medium.



(2) Colony establishment of PGPRs in coir pith

For colony establishment of PGPR in coir pith, Rhizobacteria strains are used for mass culture in coir pith in the present study. The strains were collected from IMTECH Chandigarh. All the media were purchased from Himedia, India. Kuhner shaker incubator was used for incubation of the bacteria. Optics

Technology Laminar Air Flow was used for all sterile experiments. Himedia limited Colony counter was used for counting all bacterial colony forming units [CFU].

Materials and methods:

Nutrient agar slants were prepared by weighing 2.8g in 100ml water. The media was autoclaved and left to solidify. Bacterial slants were then allowed to grow and maintained at 28±2 °C for 24 hours in an incubator. Single colony was picked up and inoculated in Nutrient broth as well as streaked on Nutrient Agar plates and incubated in a shaker incubator at 28°C for 24 hrs. After 24 hrs colony forming units on agar plates were counted with the help of a colony counter.

Coir pith was cleaned, sieved and then shade dried for 24 hrs at room temperature. After drying, 100g of coir pith was mixed with 1g CMC (carboxymethyl cellulose), transferred to 500ml Erlenmeyer flasks and then sterilized in an autoclave at 15lbs of pressure for 20 minutes. After that, the flasks were allowed to dry in a hot air oven at 40°C for 5 days.

100ml of nutrient broth containing $2.7x10^5$ CFU ml^{-1} of different bacterial strain are added separately to coir pith and mixed well under aseptic conditions of a Laminar Air Flow hood and then incubated in a shaker incubator for 48 hrs at 28°C. Till date readings of microbial population are recorded up to Day 90. Each reading was recorded in triplicate.

Serial dilutions were performed upto10^{-9}. 1g of the inoculated coir pith was transferred to the 10^0 tube containing 10ml of nutrient broth and then mixed well. 1ml of the broth was transferred to the 10^{-1} tube containing 9ml of nutrient broth and the process is repeated till 10^{-9} dilution. From each tube, 1ml of the broth is transferred to a sterile petri plate and then mixed with Nutrient agar media using the pour plate method. The plates were incubated at 28°C for 24 hrs in an incubator and then colonies were counted with the help of a colony counter.

Results:

It is observed from the microbial population have been significantly increased (increased CFU) up to day 90. The results are presented in the following tables :

(2) Colony establishment of PGPRs in coir pith

Readings of microbial population

1. ***Bacillus subtilis*** **(MTCC 121)**Dilution factor- 3, no. of replica= 3

Sl.no.	Inoculated Agar plate	No. Of CFU g^{-1} of coir pith							
		Day2 ($x10^6$)	Day5 ($x10^6$)	Day7 ($x10^6$)	Day10 ($x10^6$)	Day15 ($x10^7$)	Day30 ($x10^7$)	Day60 ($x10^7$)	Day90 ($x10^7$)
1	A	2.6	1.48	1.94	2.7	1.29	1.4	2.7	3.5
2	B	3.1	1.7	1.9	3.4	1.32	1.73	2.93	3.71
3	C	2.8	1.6	1.8	3.24	1.4	2.1	3.1	3.9

2. ***Bacillus cereus*** **(MTCC 8297)**

Sl. no.	Inoculated agar plate	No. Of CFU g^{-1} of coir pith							
		Day2 ($x10^6$)	Day5 ($x10^6$)	Day7 ($x10^6$)	Day10 ($x10^6$)	Day15 ($x10^7$)	Day30 ($x10^7$)	Day60 ($x10^7$)	Day90 ($x10^7$)
1	A	6.73	5.9	3.9	4.2	1.85	2.1	2.8	3.83
2	B	7	5.5	3.4	3.7	1.90	2.32	3.22	3.7
3	C	6.8	5.1	3.3	3.5	1.93	2.27	3.0	3.92

3. ***Pseudomonas rhodesiae*****(MTCC 8299)**

Sl. no.	Inoculated agar plate	No. Of CFU g^{-1} of coir pith							
		Day2 ($x10^6$)	Day5 ($x10^6$)	Day7 ($x10^6$)	Day10 ($x10^6$)	Day15 ($x10^7$)	Day30 ($x10^7$)	Day60 ($x10^7$)	Day90 ($x10^7$)
1	A	2.2	4.8	3.3	6.92	1.61	1.91	2.73	3.01
2	B	2.4	5.0	3.8	7.92	1.59	2.14	2.88	3.27
3	C	1.9	5.1	3.1	8.88	1.75	2.23	2.71	3.28

4. ***Chromobacterium violaceum*** **(MTCC 8071)**

Sl. no.	Inoculated agar plate	No. Of CFU g^{-1} of coir pith							
		Day2 ($x10^6$)	Day5 ($x10^6$)	Day7 ($x10^6$)	Day10 ($x10^6$)	Day15 ($x10^7$)	Day30 ($x10^7$)	Day60 ($x10^7$)	Day90 ($x10^7$)
1	A	1.8	3.7	4.1	7.81	1.3	2.1	2.63	3.44
2	B	2.1	4.4	5.2	8.13	1.26	1.91	2.54	3.61
3	C	1.9	3.32	4.6	8.4	1.38	2.3	2.81	3.53

5. *Pseudomonas fluorescens*(TP16)

Sl. no.	Inoculated agar plate	No. Of CFU g^{-1} of coir pith							
		Day2 ($x10^6$)	Day5 ($x10^6$)	Day7 ($x10^6$)	Day10 ($x10^6$)	Day15 ($x10^7$)	Day30 ($x10^7$)	Day60 ($x10^7$)	Day90 ($x10^7$)
1	A	1.12	2.9	4.28	7.33	1.27	2.51	3.2	3.7
2	B	1.36	3.36	4.54	7.65	1.33	2.6	2.9	3.66
3	C	1.41	3.51	4.84	7.91	1.81	2.72	3.1	3.8

Microbial colonies grown on Nutrient Agar

Colony establishment of PGPR B. subtilis on coir pith

Colony establishment of PGPRs in coir pith

Microbial colonies grown on Nutrient Agar

PGPR Formulation with coir pith

Colony establishment of PGPR on coir pith

Growth and survivability of different PGPR Strains in Coir pith Compost

Sl. No.	PGPR Strains	No. of CFU g^{-1} of coir pith compost							
		Day2 ($x10^6$)	Day5 ($x10^6$)	Day7 ($x10^6$)	Day10 ($x10^6$)	Day15 ($x10^7$)	Day30 ($x10^7$)	Day60 ($x10^7$)	Day90 ($x10^7$)
1	MTCC8297	3.77	5.50	3.53	3.80	1.89	2.23	3.01	3.82
2	TP 16	1.30	3.26	4.55	7.63	1.47	2.61	3.07	3.72
3	MTCC8299	2.17	4.97	3.40	7.91	1.65	2.09	2.77	3.19
4	MTCC8071	1.93	3.81	4.63	8.11	1.31	2.10	2.66	3.53
5	MTCC 121	2.83	1.59	1.88	3.11	1.34	1.31	2.91	3.70

Preparation of bioformulation of PGPR strains

Coir pith was cleaned, sieved and then shade dried for 24 hrs at room temperature. 100 kg of coir pith, 1 kg of urea and 400 gm of *Pleurotus sajor-caju* spawn is required to prepare one ton of coir pith compost. Sufficient moisture was applied for speedy decomposition in this composting process. It took nearly one month for complete decomposition of coir pith indicated when its colour changes to black. This was followed as per the method developed by CCRI, Coir Board, Kalavoor. 10 litre of bacterial inoculums containing 2.7x105 CFU ml-1 of different bacterial strains were added to 100 kg of decomposed coir pith and observed for bacterial colony establishment for 21 days, after which the formulation analysed for nutritive value and was ready for field application.

PGPR Formulation with coir pith

Coir Pith Composting Bed

Coir pith compost inoculated with PGPRs

Analysis of nutritional value of the PGPR formulation

Nutritional analysis of compost is done for estimation of their macro and micro nutrient composition using standard methods.

Sl. No.	Parameters	Raw Coir pith (%)	PGPR Formulation with coir pith as carrier (%)
1	Lignin	30	6.70
2	Cellulose	26.75	9.85
3	Carbon	28.50	25.34
4	Nitrogen	0.30	1.06
5	Phosphorous	0.01	0.06
6	Potassium	0.85	1.30
7	Calcium	0.45	0.58
8	Magnesium	0.32	0.42
9	Iron	0.10	0.16
10	Manganese	11.75	23.75
11	Zinc	6.32	13.26
12	Copper	2.90	5.34
13	C:N Ratio	95:1	24:1

(3) Field application of PGPR formulation with coir pith (CVCP Bioformulation) in specific agricultural crops for determining its ability to enhance crop production

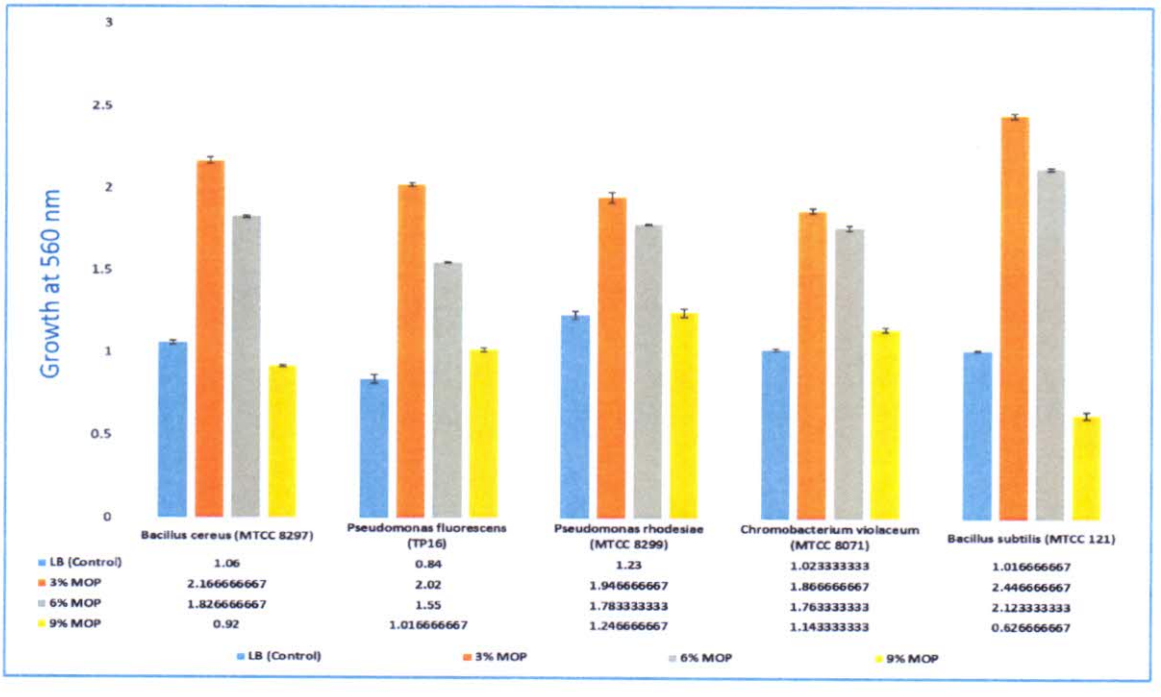
For field and pot experiments, mung bean (*Vigna radiata*), seasonal vegetables (Radish, knoll-khol, spinach, coriander spinach) were selected as the agricultural crop. 100mg of CVCP bioformulation was given near the root region of individual treated plants. The measurements of treated and untreated plants were taken before the application of bioformulation, continued at an interval of 10 days till the fruiting/maturity stage. Differences in height, number of leaves, early fruiting and total biomass after treatment were measured.

Effect of PGPR formulation with coir pith in Mung bean & seasonal vegetables

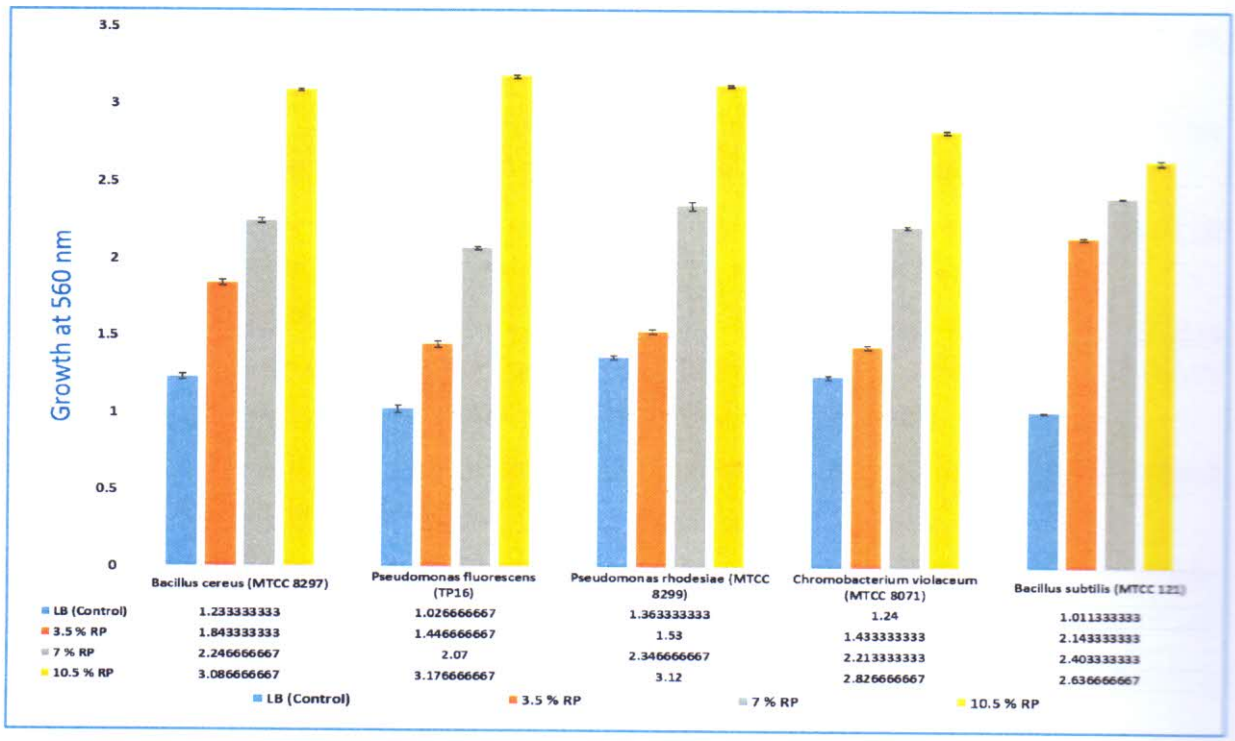
(4) Influence of inorganic nutrients (MOP, Urea and Rock Phosphate) on PGPRs with coir pith as substrate

Solutions of inorganic fertilizers such as Muriate of potash (MOP), Rock Phosphate (RP), and Urea were prepared. Different doses of all the three (NPK) minerals were amended in LB medium. PGPRs were inoculated in mineral extract amended LB broth and kept for incubation at 28°C for 48 hours. Uninoculated LB media was used as control for comparison. After incubation the growth or turbidity was measured at 560 nm wavelength using UV-Visible spectrophotometer. Three replicas were maintained in all the experiments.

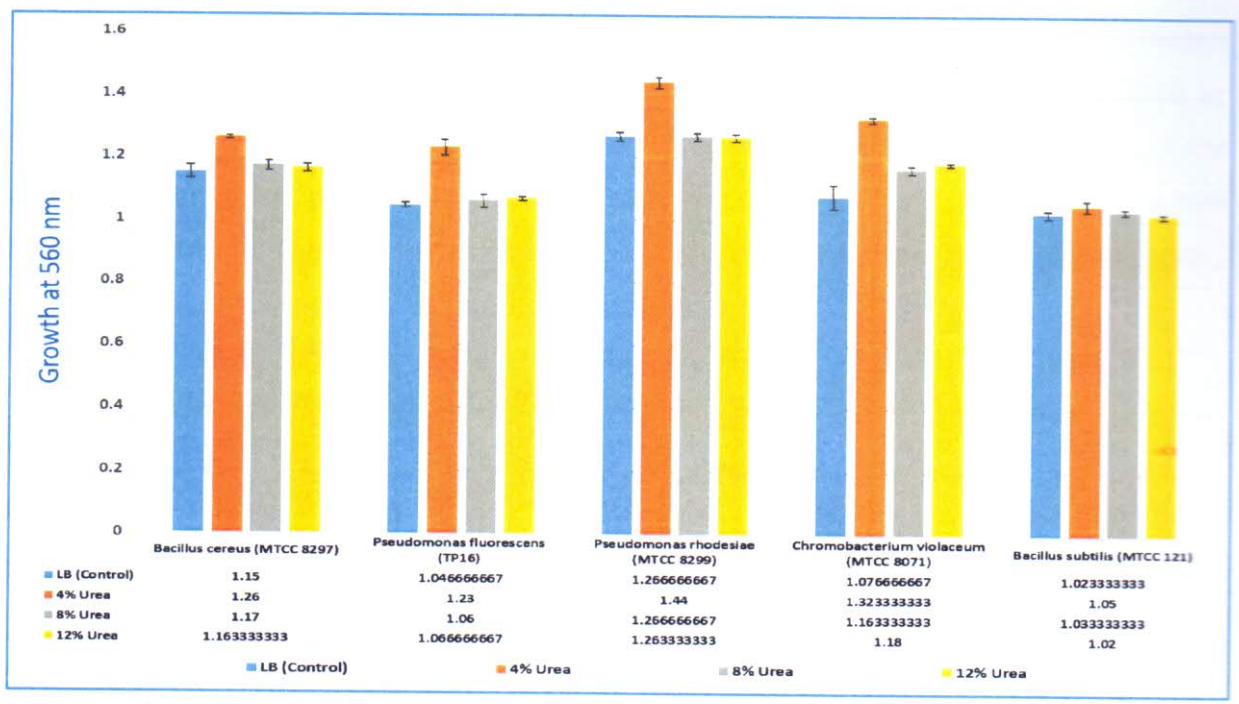
Results: 3% MOP showed optimum concentration. Among all *Bacillus subtilis* showed maximum growth. In case of RP, growth of PGPRs increased with increase in concentration. Growth was little at 4 %, less or no growth was observed at 8% and 12% concentration of Urea.



Interaction or Growth pattern of PGPRs in MOP dissolved in LB



Interaction or Growth pattern of PGPRs in RP dissolved in LB



Interaction or Growth pattern of PGPRs in Urea dissolved in LB

(5) Optimization and standardization of the process parameters for development of a commercial PGPR formulation using Coir pith as carrier (CVCP Bioformulation)

Out of all the PGPR strains *Chromobacterium violaceum* (MTCC 8071) was chosen for the commercial bioformulation. The reasons for the selection are as follows:

- Market survey reveals that this strain hasn't been used in a commercial bioformulation in the past, which makes it a novel product in the market.
- *Chromobacterium violaceum* grows in a wide variety of media (Jaggery solution, Nutrient agar, MacConkey agar, Blood agar, etc) so that manufacturers get a wide array of production options suited to their needs.
- The bacteria is reported to have beneficial biological activities like antioxidant & anticancer activities.
- The strain is readily found in the flora of soil and water in the tropical and sub-tropical regions.

Scale up studies

The scale up studies for CVCP Bioformulation in large scale was undertaken at the Chemical Engineering lab, CSIR-NEIST. Shake flask optimized data for maximized growth in terms of CFU was taken as a basis for bioreactor operation. Design expert 8.1 was used in designing of experiments using Central Composite Design and Response Surface Methodology. 10 no of experimental run were conducted at 1.2 L broth volume in NBS ST Bioreactor to optimize the operating conditions for the maximum growth in terms of input air volume, agitation and initial inoculum concentration. The optimized condition was further validated in a 10 L bioreactor. The results recorded are tabulated as follows:

Table : Scale up results of CVCP Bioformulation production in bioreactor

Sl. No.	*Expt. in*	*Capacity (Working vol.)*	*Temp. (°C)*	*Agitation (rpm)*	*LPM*	*Inoculum (mg/L)*	*Incubation period (hrs)*	*CFU (per ml)*
1	Shake flask	100 ml	30	180 shaking	-	1800	14	2.7×10^5
2	NBS Bio-reactor	1.2 L	30	150	0.90	1800	7	3.3×10^5
3	NBS Bio-reactor	10 L	30	150	1.85	1800	7	3.8×10^5

**Minimum required CFU value for field application is 1.5×10^5 per ml.*

Products/Formulation

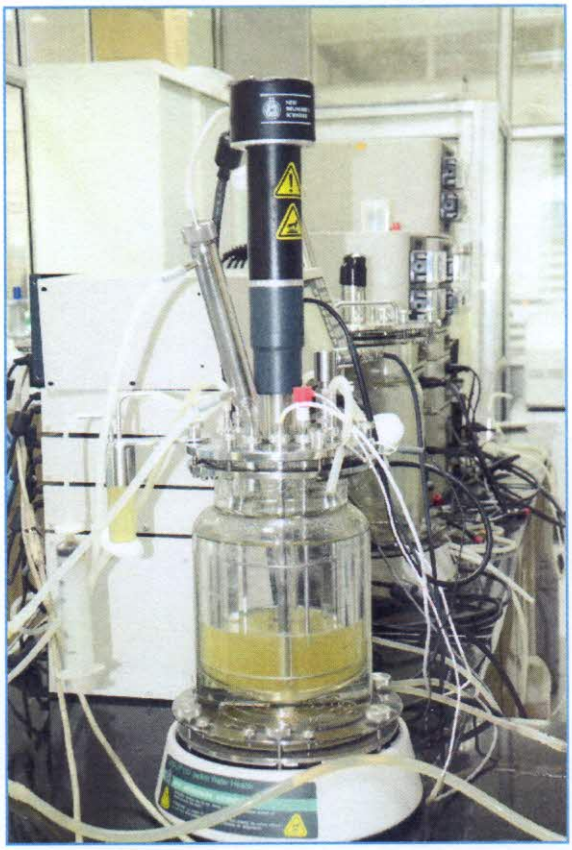
- 100-150µl of mother culture ($1X10^5$ CFU /ml approx.) is inoculated in 100 -150 ml of Jaggery (2%) and incubated for 7 hrs at 30° C with continuous shaking at 150 rpm. (Accordingly, 1.0 - 1.5 ml in 1L or 100 -150 ml in 100 L of Jaggery (2%).
- 400 ml of the bacterial suspension containing 2.7 x 10^5 CFU per ml of *Chromobacterium violaceum* (MTCC 8071) and 10 g CMC (carboxymethyl cellulose) is added to 1kg of sterilised composted coir pith and mixed well under aseptic conditions.
- The mixture is then incubated for 48 hrs at 28±2 °C.
- The product is now ready for application.

Application method of CVCP Bioformulation

- ***First application:*** Mix 100g of CVCP bioformulation per square meter of cultivation area just before sowing.
- ***Second application***: Application of bioformulation should be done in same schedule as recommended for inorganic fertilizer (NPK) of the respective crops, e.g. second application should be done after 45 days of first application.
- ***Third application:*** Third application (50% dose of the first application) can be done after 10-15 after second application.

Studies have proved that the CVCP bioformulation has a synergistic effect with fertilizers like Urea, Muriate of Potash (MoP) and Rock Phosphate (RP).So the bioformulation can also be used with other fertilizers as well.

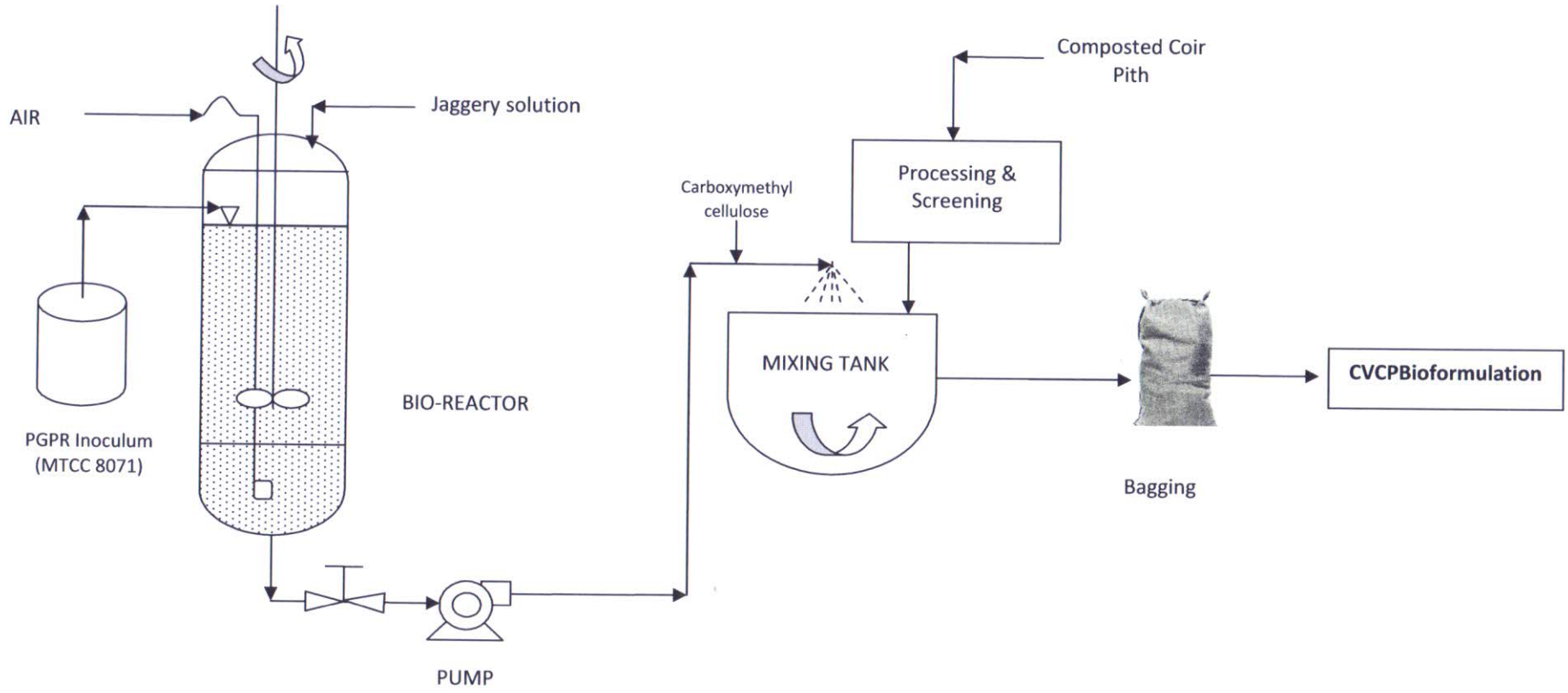
Scale up of Process of production of CVCP Bioformulation:



Bioreactor scale-up experiment in 1.2L & 10L NBS Bioreactor

Final product after the Scale-up process

PROCESS FLOW SHEET SHOWING UNIT PROCESSES FOR PRODUCTION OF CVCP BIOFORMULATION

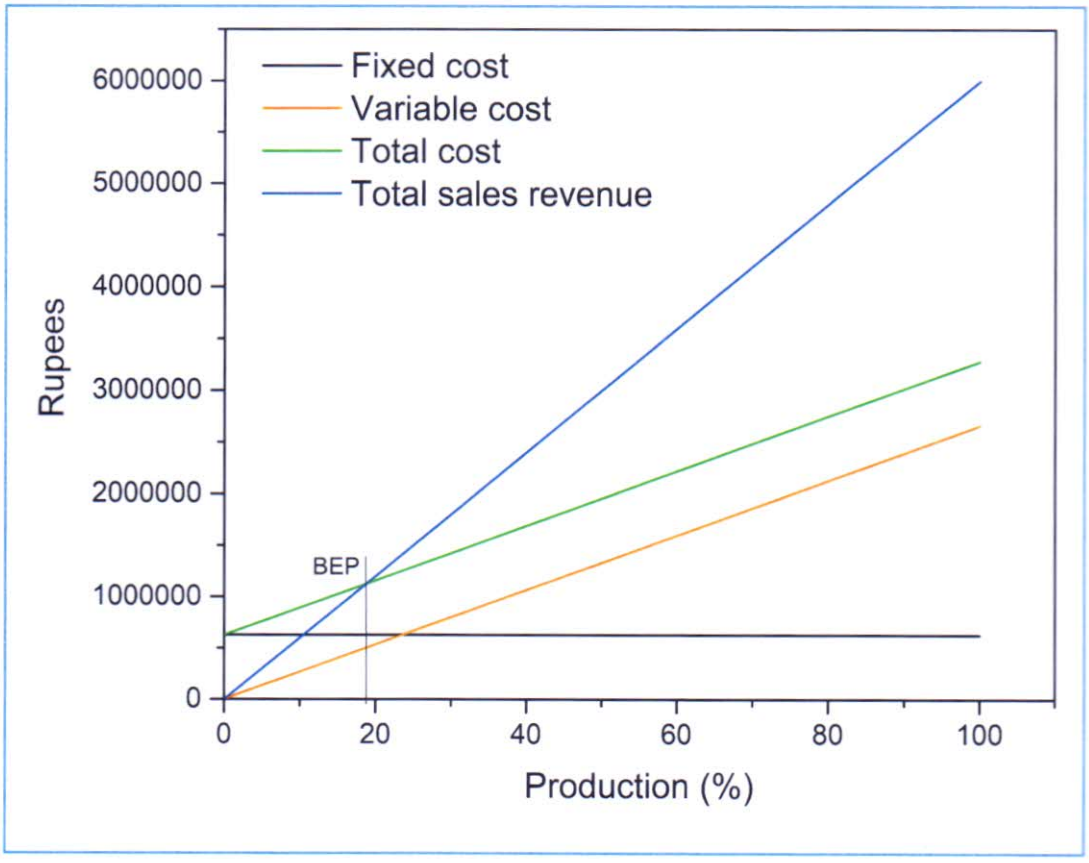


COST ANALYSIS

	COST OF PRODUCTION					
	Number of Working Days = 300					
	Plant Capacity CVCP Bioformulation= 1 tone per day					
Sl No.	Item	Requirement	Unit	Rate/ unit (Rs)		Amount per Annum (Rs)
1	Raw Material Cost					
	Coir pith	200	tones	8000		1600000
	Process water	100	tones	250		25000
	Jaggery	2	tones	40,000		80000
	CV*	75	litre	1000		75000
	HDPE Bag	30000	Nos.	10		300000
	Total Raw Material Cost					2080000
2	Utility					
	(i) Electricity	9780	KWh	7.5		73350
	(ii) Water	225000	litre	0.25		56250
	Utility Cost					129600
3	Maintenance & Repair 5% of Building & Plant & Machinery					67648
4	Manpower Cost					270000
	Depreciation @ 5% of building					25000
5	@ 10 % of Plant & Machinery					85297
	Total Depreciation cost					110297
6	Interest on fixed capital, 18% of project capital cost					297534
7	Miscellaneous Expenditure (Sale Promotion & other unforeseen)					34119
	TOTAL COST OF PRODUCTION					2989198
	SALES REVENUES					
	CVCP Bioformulation	300000	Kgs	20	per Kg	6000000
	Total Sales Revenue					**6000000**
	Profit per annum before tax					**3010802**

PROJECT CAPITAL COST OF CVCP BIOFORMULATION					
Sl. No.	**PARTICULARS**	**CAPACITY**	**UNIT**	**QTY**	**COST (Rs)**
	BUILDING				
1	FACTORY SHED				**500000**
	PLANT & MACHINERY				
2	SS Bio-Reactor	500	Litres	1	350000
3	Air compressor	2	HP	1	45000
4	Pump	0.75	HP	1	5000
5	Mixing Tank, 3HP	250	L	1	50000
6	Digital Platform Balance	20	Kg	1	10000
7	Platform Balance	100	Kg	1	22000
8	Water Tank	1000	Litres	2	20000
9	Electricals, @15% on 1-6				94800
10	Misc. Piping & Fittings				23000
11	Preoperative expenses				111980
12	Margin money for working capital @25%				130824
	Total				**852969**
13	Know-how fees & Consultancy Charges				300000
	GRAND TOTAL				**1652969**

BREAK-EVEN ANALYSIS



Break Even Point (BEP) analysis for production CVCP Bioformulation

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