3. MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. MEDIA USED (pH of the media were adjusted before autoclaving, ingredients (g/l) unless otherwise stated)

Nutrient-Broth

Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
pH	6.8±2

Pikovskaya's Broth (Modified by Sundara Rao & Sinha, 1963)

Glucose	10.0
$Ca_3 (PO_4)_2$	5.0
$(NH_4)_2SO_4$	0.5
KCl	0.2
$MgSO_4.7H_2O$	0.1
$MnSO_4$	0.006
$FeSO_4$	0.006
Yeast extract	0.5
pH	6.8-7.2

For solidification agar was added at the rate of 1.5% (w/v).

Medium for IAA production

Glucose	10.0
K_2HPO_4	5.0
$(NH_4)_2SO_4$	0.5
KCl	0.2
$MgSO_4.7H_2O$	0.1
$MnSO_4$	0.006
$FeSO_4$	0.006
Yeast extract	0.5
Tryptophan	0.1
pH	6.8-7.2

Starch Agar

Starch	20.0
Beef extract	3.0
Peptone	5.0
Agar	15.0
pH	7.0

3.2. REAGENTS USED

3.2.1. *Barritt's reagent (for Voges-Proskaur test)*

VP I Reagent (5% α-naphthol dissolved in absolute alcohol)
VP II Reagent (40 % KOH solution)

3.2.2. Gram Staining Reagents

Grams Crystal Violet, Grams Iodine, Grams decolouriser and Grams Saffranine were obtained as prepared solutions from HiMedia Laboratories, Mumbai, India

3.2.3. Ammonium Molybdate and ANSA Reagent (for detection of suluble phosphate)

Solution A: Dissolved 25 g ammonium molybdate in 400 ml distilled water.
Solution B: Dissolved 30g sodium metabisulphite, 6g sodium sulphite and 500mg ANSA (1-Amino 2-Naphthol 4-Sulphonic Acid) separately in small quantities of water and allowed to stand overnight and filtered. Stored refrigerated in an amber-coloured bottle. Fresh reagent prepared every fortnight.

3.2.4. Salokowski's ($FeHClO_4$) Reagent (For detection of IAA)

Solution A: 0.5 (M) $FeCl_3$
Solution B: 35% $HClO_4$
1 ml of 0.5 (M) $FeCl_3$ mixed with 50 ml of 35% $HClO_4$

3.2.5. Diphenylamine reagent for nitrate reduction

Reagents N, N-Dimethyl-1-naphthylamine and sulphanilic acid (0.8%) was obtained from Hi-Media Laboratories, Mumbai, India.

3.2.8. Catalase activity test reagent

3 % H_2O_2

(dilute 30% commercial solution . 1:10)

3.3. METHODOLOGY

3.3.1. Area of Study: Darjeeling District (27° 13" N to 26° 27" N and 88° 53" E to 87° 59" E), the northernmost District of West Bengal. The area under Darjeeling District is flanked by North Dinajpur district from South, Bangladesh from South-East, Bihar from South-West, Nepal from West, Sikkim from North and Bhutan from North-East.

The District has four Administrative Sub-divisions namely, Darjeeling, Kalimpong, Kurseong and Siliguri. The area of Darjeeling District falls under the Northern Hill Zone and Teesta is the most important river of this zone. It's principle tributaries are the Rangphu and Relli on the left bank; Rangeet, Reang and Sevoke on the right bank. In the west of Teesta, the Mahanadi, Balasan and Mechi merge into Ganges.

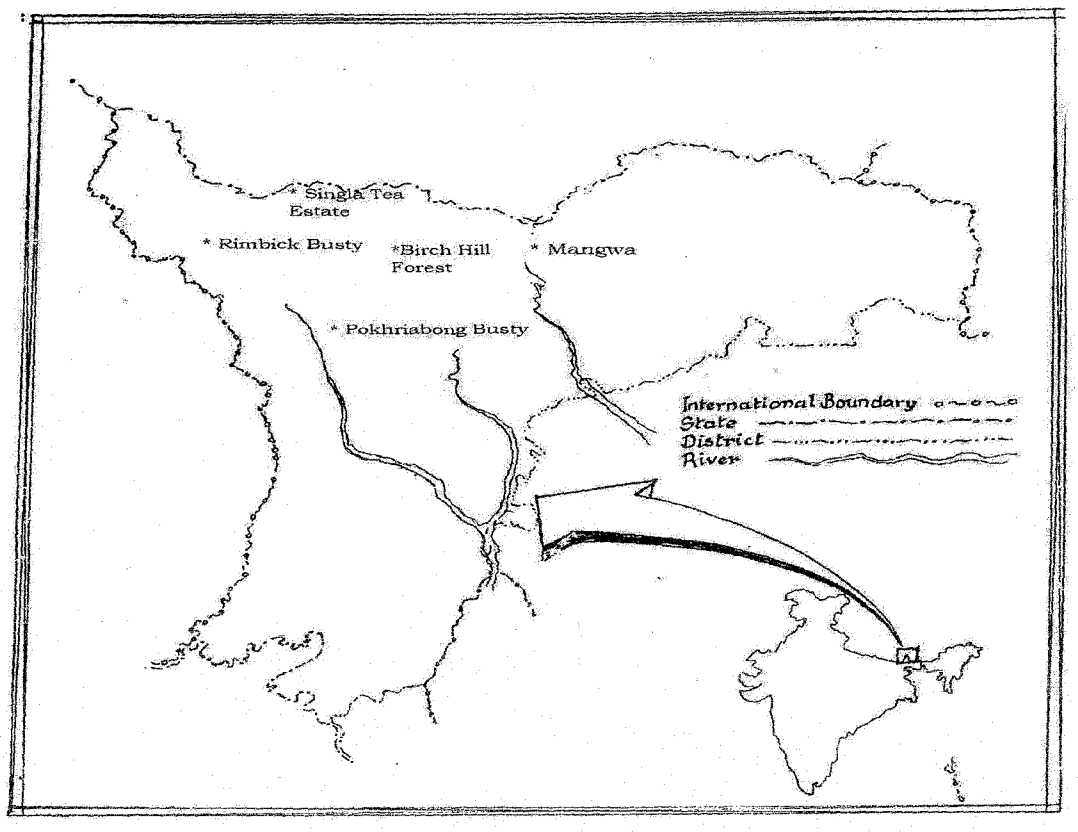

Figure 3.1. Area of study showing sampling sites

The geographical area of hill areas of Darjeeling District is 247800 hectare and the gross cropped area is 63786 hectare with cropping intensity of 171.92 %. The soil of this area is mostly categorised as brown forest soil due to their characteristic reddish brown colour, rich in organic carbon and potassium, but deficient in available phosphorus. The soil reaction varies from highly acidic to normal in nature. About 18% of the soil is highly acidic (pH below 4.9), 60% is moderately acidic (pH 5.0 to 5.9) and the rest 22% is normal (above 6.0). The rainfall varies from 1960-2941mm of which 80% is received during June to September. The average Maximum and Minimum temperature round the year records are 20°C and 2°C respectively. Details of climatic conditions are given in Table 3.1 and 3.2 respectively

Table 3.1. Climatic conditions of Darjeeling District- Average monthly rainfall for last five years

Month	rainfall (mm)				
	2008	2009	2010	2011	2012
January	0	0	0	0	0
February	0	0	0	39	0
March	18	21	0	14	0
April	60	54	51	48	204
May	96	59	127	123	81
June	265	175	447	629	339
July	648	574	861	961	529
August	587	596	573	667	284
September	256	199	284	438	505
October	102	323	88	14	18
November	0	0	12	8	0
December	0	4	0	0	0
Total	2032	2005	2443	2941	1960

Source: Deputy Director of Agriculture (Administrative), Darjeeling

Table 3.2.Climatic conditions of Darjeeling District- Average monthly temperature for last five years

Month	Temperature (°C)									
	2008		2009		2010		2011		2012	
	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini
January	13.4	2.9	15.2	2.8	14.1	3.2	9.8	1.3	9.5	2.0
February	10.4	4.0	11.4	4.2	13.9	4.2	15.1	4.3	10.5	4.0
March	17.0	5.6	18.0	6.6	11.1	7.1	19.1	7.6	10.5	6.0
April	18.9	8.6	18.3	8.7	21.0	11.8	19.7	8.0	22.3	9.0
May	19.7	10.2	20.2	9.9	19.8	11.8	20.0	12.3	21.5	8.8
June	19.6	14.1	20.3	13.3	20.9	13.8	19.4	14.5	20.0	16.0
July	19.2	12.3	19.4	11.2	19.9	15.2	18.5	15.0	20.0	15.5
August	20.1	14.5	20.7	15.3	20.3	15.1	19.9	14.8	22.0	15.6
September	19.8	14.8	20.6	13.9	19.2	13.9	20.2	14.2	21.5	15.0
October	19.6	11.5	20.5	10.8	21.0	12.0	18.8	12.7	20.1	12.0
November	16.5	5.9	18.6	7.4	17.1	7.8	12.0	6.8	17.0	6.5
December	12.2	3.5	12.6	3.3	16.5	3.9	12.5	4.5	14.0	5.5

Source: Deputy Director of Agriculture (Administrative), Darjeeling

3.3.2. Sampling sites: The sampling sites were selected on the basis of elevation and nature of crops grown (Table 3.3.). Three sampling sites located at sub tropical region are Singla Tea Estate, Pokhriabong Busty and Mangwa Busty and two other are located at temperate region [Birch Hill Forest and Rimbick Busty)] (Plate 3.1 – 3.5).

Table 3.3. Elevation and cropping practices of sampling sites

Sl no.	Sampling sites	Altitude (msl)	Cultivation practices
1.	Birch Hill Forest	2130	*Castonopsis*, *Schima*, *Alnus* forest
2.	Single Tea Estate	700	Tea garden, Rice, Wheat, Maize, Ginger
3.	Baramangwa Busty	1300	Orange, Beans, Rice, Gladioli flowers
4.	Pokhriabong	1550	Potato, Large Cardamom, Maize, Orchid
5.	Rimbick Busty	2600	Potato, Maize, Large Cardamom

3.3.3. Collection of soil samples: Soil samples were collected in sterilised polypacks from different sampling sites described in Table 3.3. Samples were collected from the rhizosphere of plants like tea, wild trees, orange, large cardamom, orchid etc. and brought to the laboratory on the same date. Soil samples collected from each site was composited before analysis.

3.3.3.1. Soil pH: Ten gram of fresh soil was suspended in 50 ml distilled water in a beaker. The soil water mixture was stirred for 20 minutes on a magnetic stirrer and pH of the suspension was read with electronic digital pH meter (Elico, India).

3.3.3.2. Soil organic carbon (%), available phosphorus (kg/ha), available potassium (kg/ha) and total nitrogen (%): Soil organic carbon (SOC) percentage was determined by Walkley and Black's rapid titration method (Walkley and Black, 1934). Available phosphorus (P) was determined colometrically through chloro-stannous molybdo-phosphoric blue method (Jackson. 1958), and exchangeable potassium (K) determined after leaching the soil with normal neutral ammonium acetate solution (Jackson, 1958) using Flame Photometer (Aimil, India). The total nitrogen was determined by colorometric technique. Nessler's reagent was added to digested solution (conc. H_2SO_4 + catalyst) of the soil sample and yellow product was measured in a Spectrophotometer (Systronics, India, Model-106) at 410 nm and the amount was calculated using calibration curve (Jackson, 1958).

3.3.3.3. Soil Moisture Content: The moisture content of the soil was determined by drying 10 g of freshly collected soil samples in a hot air oven at 105°C till the constant weight was observed and calculated using the following formulae:

$$\text{Moisture Content (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

Where, W_1 = weight of soil before drying
W_2 = weight of soil after drying

3.3.3.4. Water holding capacity of soil samples: Freshly collected soil samples were saturated with water and allowed to drain completely at room temperature (Misra, 1968). The water holding capacity was calculated using following formulae:

$$\text{Water Holding Capacity (\%)} = \frac{W_2 - W_1}{W_1} \times 100$$

Where, W_1 = weight of moistened soil
W_2 = weight of drained soil

3.3.4. Isolation of phosphate solubilizing microorganisms

Ten gram (10g) each of soil samples was suspended in 90 ml of sterile distilled water and 10^{-1} dilution was obtained. Serial dilutions were prepared by mixing 1 ml of the suspension made into 9 ml sterile water blanks, until the 10^{-7} dilution was obtained. The Pikovskaya's (PKV) agar was used for isolation, enumeration and maintenance of PSMs. The serially diluted soil suspensions were spreadplated on Pikovskaya's agar plates and incubated at 37°C for 7 days. Colonies causing clear zones around them were selected as phosphate solubilisers and further purified by replating on PKV agar medium.

3.3.5. Solubilisation Efficiency (SE) and Solubilisation Index (SI) of phosphate solubilising bacteria (PSB) isolates

Sterilized PKV agar was poured into sterilized petriplates, after solidification of the media, a pinpoint inoculation of bacterial strains positive in halo zone formation in the screening process was made on the plates under aseptic conditions. Isolates having the potential to solubilise insoluble phosphates on PKV medium by forming the halos were considered as

phosphate solubilisers. The growth and solubilisation diameter were measured after incubation at 37°C for seven days. On the basis of diameter of clearing halo zones, SI and SE (Gaur, 1990; Nguyen *et. al.*, 1992; Vazguez *et. al.*, 2000) were calculated using the following formula:

$$SE = \frac{\text{Solubilisation Diameter}}{\text{Colony diameter}} \times 100$$

$$SI = \frac{\text{Colony diameter + halozone diameter}}{\text{Colony diameter}}$$

3.3.6. *In vitro* quantification of phosphate solubilisation by PSB isolates

The phosphorus solubilising potential of PSB strains was tested *in vitro* by estimating available phosphorus in the Pikovskaya's broth amended with known amount of TCP as a substrate. A control without any inoculation was also maintained. The organisms were allowed to grow for 7, 14, 21, 28 days at 37°C and centrifuged at 10,000 rpm for 10 min in a cooling centrifuge (REMI-C30BL, Remi, India). Soluble phosphate was determined in supernatant following the procedure of Fiske and Subbarow (1925) as described below.

One ml of the culture supernatant was taken in a test tube to which 1 ml of ammonium molybdate solution was added and mixed thoroughly; 0.4 ml of ANSA reagent was added to each tube and mixed well. This reaction mixture was allowed to stand for 10 minutes and the volume was made upto 10 ml with distilled water. The blue colour thus developed was read in a UV-VIS Spectrophotometer (SHIMADJU UV-1700 Pharmaspec, Shimadju, Japan) at 660 nm against a reagent blank.

Simultaneously, a standard curve was prepared using various concentrations of standard 20 µg (20 ppm) KH_2PO_4 solution. The amounts of phosphorus solubilised by the isolates were calculated from the standard curve.

Preparation of standard curve: Dissolved 439 mg potassium dihydrogen phosphate (KH_2PO_4) in water. Ten ml of 10(N) H_2SO_4 was added to it and the volume was made upto one litre with distilled water and mixed thoroughly. Ten ml of the above stock solution was diluted further to 50 ml with distilled water to obtain 20 ppm solution and used for preparation of the standard curve (1ml = 20 ppm).

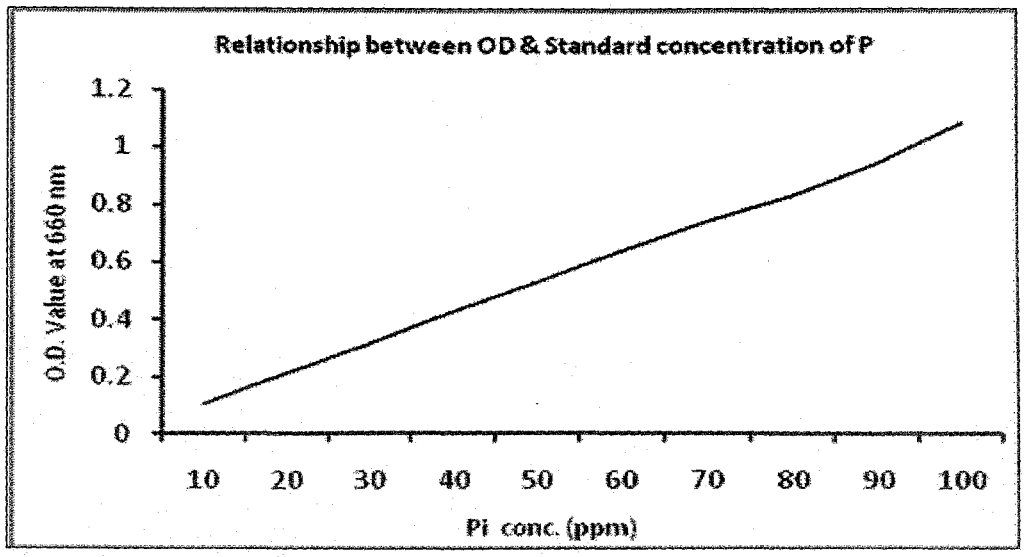

Fig. 3.2. Standard curve of soluble phosphate

3.3.7. Measurement of pH of the medium

A change in pH of the medium as a result of the growth of PSB was measured with a pH meter (Elico, India) after 7, 14, 21, 28 days of incubation.

3.3.8. Quantification of indole acetic acid (IAA) production

The production of IAA was determined according to the method of Bano and Mussaraat (2003). The tested bacterial strains was grown in liquid medium in the presence of tryptophan (100mg/l) and incubated at 30°C for 3 days. A 2 ml culture was removed from each tube and centrifuged at 10,000 rpm for 15 min in a cooling centrifuge (REMI-C30BL, Remi, India), 1 ml of supernatant fluid was transferred to fresh tube to which 100 µl of 10 mM orthophosphoric acid and 2 ml of reagent consisting of 1 ml of 0.5 % $FeCl_3$ in 50 ml of 35% $HClO_4$ were added sequentially. The absorbance of the developed pink color was read at 530 nm after 25 min in a UV-VIS Spectrophotometer (SHIMADJU UV-1700 Pharmaspec, Shimadju, Japan). IAA concentration in the culture was determined by using a calibration curve of pure IAA as standard.

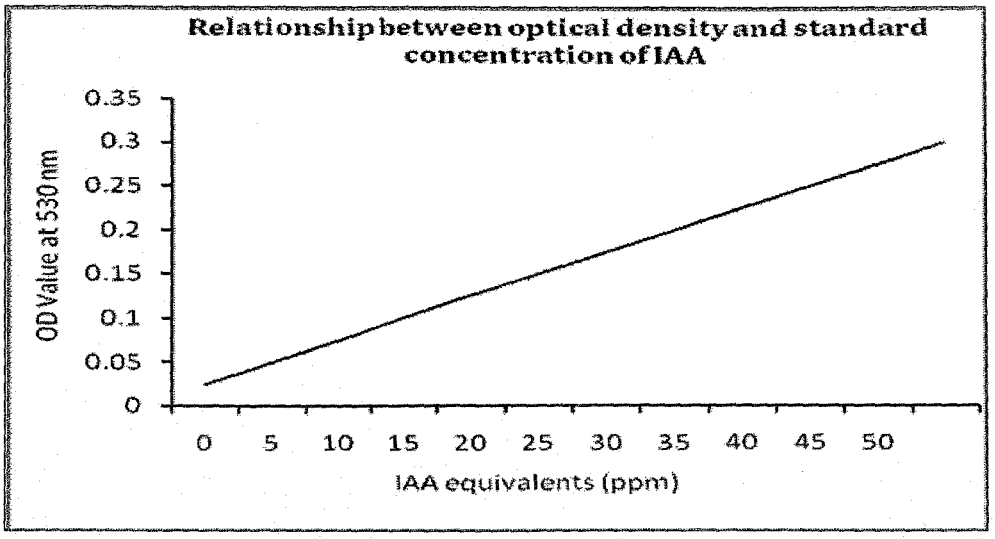

Figure. 3.3. Standard curve of IAA.

3.3.9. Effect of tricalcium phosphate concentration on phosphate solubilisation by selected PSB isolates

Selected PSB isolates were assessed for their capacity to release soluble phosphate in PKV broth with varying concentration of tricalcium phosphate i.e. 1,2,3,4 and 5 g/l. The soluble phosphate was read after 7, 14, 21 and 28 days of incubation at 37°C following the protocol described in section 3.3.6.

3.3.10. Effect of glucose concentration on phosphate solubilisation by selected PSB isolates

Selected PSB isolates were assessed for their capacity to release soluble phosphate in PKV broth with varying concentration of glucose i.e. 5, 10, 15 and 20 g/l. The soluble phosphate was read after 7, 14, 21 and 28 days of incubation at 37°C following the protocol described in section 3.3.6.

3.3.11. Biochemical activities of PSB isolates

Biochemical test kit obtained from HiMedia laboratories, India was inoculated with one drop each of confluently grown PSB isolates and incubated at 37°C for 24 hours and results thus obtained was compared and recorded as described in the kit.

Biochemical kit obtained from Hi-Media Laboratories, Mumbai, India which were designated as Biochemical test kit with product codes KB009 (Plate 4.2) and KBO13. The kit contained sterile media for Malonate, Voges-Proskaeur's, Citrate, ONPG, Nitrate reduction, Esculin hydrolysis, Catalase,

Arginine and 19 carbohydrates utilisation test Adonitol, D-Arabinose, Cellobiose, Dextrose, Dulcitol, Galactose, Glucose, Lactose, Maltose, Mannose, Mannitol, Melezitose, D-Methyl-D-Mannoside, Raffinose, Rhamnose, Sorbose, Sucrose, Trehalose and Xylitol. Supplied Reagents like N,N-Dimethyl-1-naphthylamine and sulphanilic acid (0.8%) were used for nitrate activity and Barritt Reagent A and Barritt Reagent B were used for VP test.

Overnight grown cultures of PSB isolates were utilised for the study and for procedure and interpretations of results manufacturers guidelines supplied with the kit was followed.

Starch hydrolysis test was done separately by streaking bacteria on the starch agar plates with selected bacterial isolates. The plates were incubated at 37°C for 48 hours. After 48 hours the plates were flooded with iodine solution with a dropper and were observed after 15 minutes. Transparent area around the colony against bluish background was interpreted as positive test.

3.3.12. Study of Gram Staining and morphological nature of PSB isolates:

Gram stained exponential phase growth cultures of PSB isolates were observed under the microscope and their nature of Gram-staining and morphology were recorded.

3.3.13. Antibiotic sensitivity pattern of PSB isolates

Overnight grown PSB isolates were spread plated on nutrient agar plates and antibiotic dodecadiscs (HiMedia Laboratories, India: impregnated with 20 different antibiotics) were placed on the plate and incubated at 37°C for 24 hours. The different antibiotics impregnated and their concentration were: Amikacin (AK-30µg), Ampicillin (AMP-10µg), Amoxycillin (AMX-10µg), Cefadroxil (CFR-30µg), Cefoperazone (CPZ-75µg), Ceftazidime (CAZ-30µg), Ceftriaxone (CTR-30µg), Chloramphenicol (C-30µg), Ciprofloxacin (CIP-05µg), Cloxacillin (COX-01µg), Co-Trimoxazole (COT-25µg), Erythromycin (E-15µg), Gentamicin (GEN-10µg), Nalidixic acid (NA-10µg), Netillin (NET-10 µg), Nitrofurantoin (NIT-300µg) , Norfloxacin (NX-10µg), Penicillin (P-10 units), Tobramycin (TOB-10µg) and Vancomycin (VA-30µg).

Observation of inhibition zone around the discs on the bacterial lawn was considered sensitive and diameter of which were recorded and represented in mm scale.

3.3.14. Native polyacrylamide gel electrophoresis (PAGE) profile of PSB isolates

Native PAGE profile of PSB isolates was prepared following the procedure of Bollag *et. al.*, (1996).

3.3.14.1. Requirements: Gel electrophoresis set, Power supply, Micropipette with disposable gel loading tip, Container for staining and destaining the gel, Nutrient Broth, PSB isolates, Distilled water.

3.3.14.2. Preparation of bacterial protein sample

i, PSB culture was inoculated to nutrient broth and incubated in a shaker at 37°C for 24hours.

ii, The culture was transferred to centrifuge tube (ice-cold) and centrifuged at 10000 rpm for 15 mins.

iii, The pellet was retained.

iv, The pellet was sonicated for cell lysis.

v, The cells were taken up in phosphate buffer (20-40µl) in Eppendorf tube.

vi, It was again centrifuged at 1000 rpm for 10 mins.

vii, The supernatant was retained as protein sample and stored in deep freeze for further analysis.

3.3.14.3. Working solutions

Solution A (Acrylamide stock solution) in distilled water

30% Acrylamide

0.8% Bis-acrylamide

Solution B (4X Separating gel buffer)

1.5 M Tris-HCl (pH 8.8)

Solution C (4X stacking buffer)

0.5 M Tris (pH 6.8)

10% Ammonium persulphate

Electrophoresis buffer, 1000 ml

3.0 g tris

14.4 g glycine

Distilled water to 1000 ml (final pH-8.8)

5X Sample buffer, 10 ml

3.1 ml 1M Tris-HCl (pH 6.8)

5 ml 1% bromophenol blue

1.4 ml distilled water

3.3.14.4. Preparation and pouring of separating gel

8% separating gel was prepared by mixing

4.8 ml distilled water

2.7 ml Solution A

2.5 ml Solution B

50µl 10% Ammonium persulphate

5µl TEMED

(This proportion was for 10 ml)

Separating gel was prepared by pouring above mixture in gel sandwich

3.3.14.5. Preparation and pouring of stacking gel

5% stacking gel was prepared by mixing

2.3 ml distilled water

0.67 ml Solution A

1.0 ml Solution C

30 µl 10% Ammonium persulphate

5 µl TEMED

(This proportion was for 4 ml)

Stacking gel was prepared by pouring above mixture above the separating gel in gel sandwich.

3.3.14.6. Sample preparation and loading in the wells:

Protein sample 20µl and sample buffer 5µl was mixed and introduced into well using a disposable gel loading tip.

3.3.14.7. Running the gel:

The power supply was turned on to 100-200 v (constant current) and electrophoresis was allowed to continue until the dye migrated within 5 mm of the bottom of the gel.

3.3.14.8. Staining the gel

Stain preparation

Coomassie Gel Stain

1.0 g Coomassie Blue R-250

450 ml methanol

450 ml distilled water

100 ml glacial acetic acid

Coomassie Gel destain

100 ml methanol

100 ml glacial acetic acid

800 ml distilled water

Staining procedure:

Wearing gloves, gel sandwich was dissembled and gel was picked up and transferred to a container containing Coomassie stain. The set was agitated for 10 mins on a slow rotary shaker. The stain was poured out and Coommasie destain was added and the shaking was continued till the gel was completely destained.

3.3.15. Effect of different pH on the survivality of selected PSB isolates

A loopful of bacteria from overnight grown broth cultures of six PSB isolates were inoculated to PKV broth adjusted to different pH i. e., 5, 7, 9 and incubated at 37°C. At each day interval 0.1 ml of the suspension was spread plated on nutrient agar plates and colony forming units (cfu/ml) were calculated up to 7 days.

3.3.16. Survivality pattern of PSB isolates in sterilised soil at different temperatures

Perforated polypacks of 2 kg capacity were filled with soil collected from actively engaged agricultural field and sterilised in an autoclave at 15 psi for 1 hour each day for 3 days. Each pot in duplicate was inoculated with broth cultures of PSB isolates and incubated at two temperature regimes i.e., room temperature and 37°C and cfu/g of soil was determined by serial dilution spreadplating technique on nutrient agar after one day and thereafter every fifteen days upto 60 days.

3.3.17. Effect of PSB isolates on seed germination of fenugreek *(Trigonella foenicum)*

Seeds of fenugreek were surface sterilized with 0.1% $HgCl_2$ (3 minutes) followed by successive washings with sterile distilled water (Shende *et al.*, 1977) and treated with 4 days old liquid cultures containing different PSB strains. The control seeds were treated with the sterilized medium alone. They were placed (50 each) on water soaked sterile filter paper in petridishes and incubated at room temperature. Germination was recorded each day upto 7 days.

3.3.18. Effect of PSB isolates on the yield of pea (*Pisum sativum*) and potato (*Solanum tuberosum*) in the field

3.3.18.1. Surface sterilization and bacterization of planting material:

Seeds of peas (*Pisum sativum)* was washed repeatedly with autoclaved distilled water, and soaked in distilled water for 10 min. Later, 150 g of seeds were coated with 15 % sugar solution. For uniform treatment of seeds, the flasks were kept in an orbital shaker (Macro Scientific Works, Delhi) for 2 h at 500 rpm. Seeds were bio-inoculated (bacterised) by 4 days grown PSB strains in PKV broth, allowed to dry in air. The average bacterial counts were about $1x10^4$ cfu/pea seed.

Potato tubers were cut to give uniform pieces with at least two eyes per piece, were washed repeatedly with autoclaved distilled water and coated with 15% sugar solution. Later, cut tubers were treated with four days old PSB cultures. The treated tubers were allowed to dry in air and later sown in the field.

3.3.18.2. Soil preparation and sowing: The soil in the field was brought to a fine tilth by ploughing and 4.0 m x 3.5 m plots were laid out. The soil in each plot was mixed well and perfect levelling was ensured in each plot. Rows were 30 cm apart. The seeds of pea treated with PSB strains were sowed in each row with a distance of 10 cm between the seeds. PSB strains were added to all the treatments except for the control. The treated potato tubers were planted at 60 cm interrow and 20 cm intrarow spacing in a plot size of 4.0 m X 3.5 m. The planting was done in November 2011 and harvested in February, 2012. Three replicates were set up for each treatment. The design used for the experiment was randomized complete

block design (RCBD). The plots were irrigated time to time to maintain the moisture level in the field. Pea pod yield was presented as pods per plant and quintals/ha. Similarly potato yield was represented as quintals/ha.

3.3.19. Effect of PSB isolates on growth of orange *(Citrus reticulata)* seedlings

Seeds of orange was collected from the fruits of same plant and germinated in sterilized soil contained in a polypack. Seedlings of uniform size (40 mm height) was selected, roots freed of soil, washed with sterile distilled water and treated with PKV broth having confluent growth of PSB isolates for 2 hours. Garden soil actively engaged in agriculture was filled in 2 kg capacity perforated black polybags and sterilized at 15 psi for 1 hour in an autoclave for consecutive 3 days. The treated seedling of orange was transplanted in the polypacks containing sterilized soil in April 2011 and kept in green house condition. Weeding and watering was done regularly. All the measurements were taken after one year of growth i.e. in April, 2012. Root length and plant height measured in mm scale.

Major chemicals used

(All the chemicals used were obtained from Hi-Media Laboratories, Mumbai, India)

Chemicals

Ammonium sulphate
Agar
ammonium molybdate
ANSA (1-Amino 2-Naphthol 4-Sulphonic Acid)
Beef Extract
Tricalcium phosphate
Ferric chloride
Ferrous sulphate
Glucose
Crystal Violet
Iodine,
Saffranine
Hydrogen peroxide
Perchloric acid
Dipotassium hydrogen phosphate
Potassium chloride
Potassium hydroxide
Magnesium sulphate
Mangenese sulphate
N, N-Dimethyl-1-naphthylamine
Peptone
Sodium chloride
Sodium metabisulphite
Sodium sulphite
Starch
Sulphanilic acid
Tryptophan
Yeast extract
α-naphthol

Plate 3.1. *Rhododendron* tree at Birch Hill Forest

Plate 3.2. Tea bushes at Singla Tea Estate

Plate 3.3. Orange orchard at Mangwa Busty

Plate 3.4. Vegetable cultivation at Pokhriabong Busty

Plate 3.5. Maize plantation at Rimbick Busty