CHARACTERIZATION OF PHYLLOSPHERE BACTERIA ISOLATED FROM LEAF OF PEPPER BETEL AND ITS INDUSTRIAL APPLICTION

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Abstract

The great nutritional importance in food is amino acids. In general, serious problems of food and nutrition deficiencies of the world at present is confronted. Researchers to search for unconventionally sources of proteins and amino acids in increased demand of proteins .some devastating biotic invasions are facilitated by microbial symbionts are the growing evidence . Bacterial community structure within the betel was isolated and its metabolites were evaluated. In this study the aminoacid, organic acid and indole acetic acid producing capacity of several bacterial strains were tested and *Streptomyce*s species was found to be novel isolates. Isolation and occurrence of *Streptomyce*s species in betelis first time reported in this study and also found to be three different amino acid producer. The present study concludes that the isolated microbial strain can be used for the production of amino acids at very economical rates followed by optimizing natural sources

Key: Phyllosphere bacteria,Amino acid,Organic acid ,Indole acetic acid

INTRODUCTION

PHYLLOSPHERE MICROBIAL DIVIRSITY

Phyllosphere is that leaf surfaces or total above-ground surfaces of a plant as a environment for microorganisms. The aerial part includes leaves, stems, buds, fruits and flowers that give a environment for microorganisms within the phyllosphere. The role in irregular and sometimes large changing of temperature, UV radiation and leaf humidity based on an exclusive and dynamic environment that is believed to play a (**Redford *et al*., 2010).**Archaea, filamentous fungi, and yeasts are considered to be significant microorga nism and are present as well most important microbial inhabitants in phyllosphere are bacteria (**Mukhtar *et al.,* 2010**).

These bacteria include pathogenic and non-pathogenic populations that contribute to the health status of the host plant and may play a global role in nitrogen and carbon cycles. (**Zhang *et al*., 2010).** The phylloxera is considered a hostile environment due to rapid changes in temperature and humidity, limited nutrients and solar radiation. Foreign exchange, many epiphytic bacteria prevent UV (UV) damage. Microbial communities primarily form around the nerves and trichomes and stomata, where nutrients leak from the plant surface. **(Agler *et al.,* 2016).**

Leaf surfaces can be densely populated by microorganisms. The most dominant group of microorganisms in the phyllosphere are bacteria,which reach a surprisingly dense population of on average104-105 bacteria mm^2 of leaf surface or up to 10^8 bacteria g^1 l eaf material **(Remus-Emsermann *et al*., 2014).**Fungi, algae, protozoa, and nematodes inhabit the leaf and stem surfaces, but the most abundant epiphytes are bacteria (averaging 10–10 cells cm). (**Mandolesi Pereira de Melo *et al*., 2009**). Polar regions to tropics are associated with by almost all major lineages of land plants distributed from taxonomically diverse phyllosphere fungi and can be subject to some ecological effects by these fungi, such as pathogenic damage (**Newton *et al*., 2010**) or benefits of enhancing tolerances against herbivores or pathogen.

PIPER BETEL AND THEIR LEAVES.

Potential medical specialty square measure gift in medicative plants square measure of proved price like the rise of resistant pathogens to normally used antibiotics and therefore the emergence of latest infectious diseases. Extracts of Piper beter leaf square measure shown to be effective against many human pahogens, though the mechanisms concerned haven't been elucidated. The treatment of the many diseases as a conventional medication in many countries square measure used numbers of natural merchandise. Treatment of asorted ailments since ages thanks to its essential properties like inhibitor, anticancer, antiallergic used the extracts of Piper betel. Piper betel belongs to the Piperaceae and has over 2000 species. The plant is autochthonal to Republic of India (Rajat Ghosh et al., 2014). The leaves of Piperbeter Linn have long been use in the Indian native system of drugs. Piper beterr leaves square measure thought of auspicious and square measure still extensively used throughout non secular functions in Asia and ancient Republic of India.

AMINO ACID PRODUCING BACTERIA:

Bacteria have been used commercially since 1950s for amino acid producing and strains have been subsequently improved by regulatory mutants.(Nadeem and Ahmad, 1999).The use of old wild type bacteria for the production of aminoacids such as L-glutamate, L-valine, L-alanine, L-glutamine, and L-proline relies on either inherent metabolic regulations or stimulation of secretion by environmental factors.

MATERIALS AND METHOD

Plant materials

Pyllospheric (epiphytic) bacterium were isolated from piper beetle. The higher than ground components parts of plants were collected from native market in a very sterile polythene bag and brought to the laboratory. Plant leaf samples that have been collected cut approximately 1 cm. The leaves were then disinfected with 70% ethanol for 5 minute.

Isolation and purification of epiphytic bacteria

The sterile plant segment was fed into 0.85% physiological NaCl solution and then kept on Petridish containing nutrient agar medium. It was then incubated at room temperature for

72 hours. Different bacterial colonies were inoculated on a Nutrient Agar medium streak plate and incubated for 24 hours at room temperature. Single colonies are inoculated on slant agar and labeled.

Phenotypic characterization

The microorganisms strains were known to the genus level supported the colony morphology (appearance, size, margin, form, elevation), microscopic examination (gram's and spore staining), and organic chemistry tests (catalase, oxidase, nitrate reduction, starch reaction, casein reaction, Voges Proskauer, citrate utilization, gelatin liquefaction, methyl red) by adopting standard procedures 16,28, 29. The strains were coded and started with B-1, and the last number was B-25.

Screening of bacterial isolates for amino acid production

Media formulation:For the assembly of amino acids, grape sugar salt fermentation media (DS media) were tested for the isolation of amino acid Producing bacteria. The ingredients for the 100 ml media were: grape sugar 1g, KH_2PO_4 (0.05g), K_2HPO_4 (0.05g), $MgSO_4.7H2O$ (0.025g), $(NH_4)2SO_4$ (2.00g) and $CaCO_3$ (2.00g). The pH of the media was kept within a range of 7.0-7.2. Sterilization was unbroken among a spread of seven . Sterilization was done through autoclaving the media at 121ºC at 15 lbs pressure for 15 min.

Fermentation: 10 ml of 24 ml old culture was injected into 100 ml fermentation medium in 250 ml Erlenmeier flask. The jar was sealed at 30oC in a rotary shaker. Tests to produce the amino acid of these bacterial strains were carried out at a rotary shaker at a speed of 120 rpm for a maximum of 96 hours. During this period, 3 ml of the sample was monitored every 24 h. Harvesting of each jar during the 96-hour incubation period was carried out at pH.

Analysis and identification of the fermented amino acids

About 5 ml sample of fermented broth was centrifuged at 15000 rpm for 10 min in order to collect the cell-free broth. Qualitative analyses of amino acids produced by bacteria in this fermented and cell-free broth were performed as detailed below

Qualitative analysis using thin layer chromatography

Lederer and Laderer (1957) technique was followed for chromatography, where 0.03M commonplace standard solutions of six amino acids viz., methionine, serine, leucine, proline, aminoalkanoic acid and glycine were ready. Solvent fabricated from n-butanol: acetic acid: water (at 4: 1: 1) was additional in a chromatographic rectangular glass jar. Standard amino acids and samples, 50 µl each, were loaded on to the silica chromatographic plate. The chromatographic tank papers were irrigated vertically within the solvent system for few hours until the solvent traveled the distance on plate up to a certain point. Then the papers were air dried and sprayed with 0.1% ninhydrin solution (0.1g.100 ml-1 ethanol), and later dried at 60-80ºC for 10 min to induce purple spots of the amino acids. The results were confirmed by examination the retention factors (Rf values) of the samples with that of the

standard amino acids. The *Rf* values were calculated by the subsequent formula:The distance was measured from the point where the amino acid was loaded to the purpose wherever solvent came to a halt.

Screening of organic Acid-Producing bacteria

Isolates obtained from phyllosphere region were assessed for their potential to supply aminoalkanoic acid during a basal medium. The microorganisms isolates were subjected for qualitative assay for acid production exploitation acid indicator medium (AIM) containing 0.04% of bromocresol purple (Das and Roy, 1998). Microorganisms culture was inoculated on broth medium contained (g/l) : Sodium nitrate 2.0, Dipotassium hydrogen phosphate 1.0, Magnesium sulphate 0.5, Potasssium chloride 0.5, Ferrous sulphate 0.01, sucrose 30, bromocresol purple 0.04 in distilled water and incubated for two days for the formation of yellow coloration of the medium which indicated production of acid.

Screening of phosphate solubilizing fungi

In order to notice phosphate-solubilizing microorganisms , each of the isolated of phyllosphere were inoculated on Pikovskaya's agar (PVK, containing per litre: 0.5 g yeast extract, 10 g dextrose, 5 g Ca3(PO4)2, 0.5 g (NH4)2SO4, 0.2 g KCl, 0.1 g MgSO4.7H2O, 0.0001 g MnSO4.H2O, 0.0001 g FeSO4.7H2O and 15 g agar, pH 7.2). The plates were incubated at 25±2° C for 10 days. Fungi capable of producing a halo/ clear zone due to solubilization were selected as potential phosphate solubilizers and used for further studies.

$$\text{Solubilization Index (SI)} = \frac{\text{colony diameter + halo zone diameter}}{\text{colony diameter}}.$$

Growth in liquid medium (Westerberg et al. 2000)

Selectedpositive isolate, were for growth in stripped medium below two different physiological condition was studied. Growth was followed by measuring the optical density at 600 nm (OD_{600}) as a function of time. Growth pattern at physiological factor such different Temperature at 28, 35, 50^{o} C and 5, 7,and 9 pH was monitored.

RESULTS AND DISCUSSION

Isolation of epiphytic bacteria from surface sterilized wild green betel leaves using nutrient agar medium (plate 1).Obtained by bacteria colonies were selected and subculture on agar slant (plate 2). Epiphytic bacterial isolates generally have moderate growth rates at 48 hours of incubation with a variety of colonial morphological characteristics such as shape, color, edges, colony elevation (table 1). Phyllosphere bacterial characteristics generally grow in isolation medium after two days of incubation (**Jalgaonwala *et al*., 2010**). Betel also contain Endophytic bacteria originating from the environment around the plant such as the area of rhizosphere and plant philosphere capable of penetrating into the plant tissue through stomata, lentikula or area of the emergence of lateral roots used forsecondary metabolites it produces (**Mano and Morasaki, 200**8). The results of screening of six green betel associated isolates showed that

50 % were Gram positive. Several authors have reported the presence of various bacteria species in plant samples, such as *Bacillus pumilus* (as dominant endophytes) in betel plants (**Araujo *et al.*, 2002**), *Stenotrophomonas* species in sweet potato plant and in the coffee seed, *Pseudomonas putida* in carrot, and *Seratia marcescens* isolated from rice

The production of amino acids was obtained by the Dextrose Salt (DS) media used in the present experiment. The amino acids produced by the isolates in the media were identified by using thin layer chromatography followed by calculating their specific R_f values. Results presented in Table.2 demonstrated that the bacterial strains PLS2, the amino acids cysteine, serine and methionine, respectively.The amino acid was separated by TLC and detected with ninhydrin. Pink and violet color band on TLC plates (plate 2) with R_f0.5, 0.6 and 0.2 corresponding to standard amino acid. Cysteine production by bacterial isolates in different fermentation media has been documented by Ali *et al*. (2011). Moreover, Chomini *et al*. (2015) demonstrated that four major amino acids *viz*., threonine, proline, glycine and alanine were released from phyllosphere.

Similarly out of 6 tested bacteria two isolates designated as PLS2, the UST were positive on organic acid (plate- 4) and three were (PLS1, PLS2, UST1) IAA. Based on their morphological and biochemical characteristics, the isolate PLS2 was identified to be Gram-positive, rod-shaped filamentous bacteria and belonging genus *Streptomyces* species respectively. All the tested isolates were failed to utilize inorganic phosphate (plate-5). The tested bacterial colonies were failed to grown on Pikovskaya agar.

STRAIN CODE	AMINO ACID	ORGANIC ACID	IAA
PLS 1	(-)	(-)	(+)
PLS 2	(+)	(+)	(+)
PL 3	(-)	(-)	(-)
PL 4	(-)	(-)	(-)
UST 1	(-)	(+)	(+)
SST 2	(-)	(-)	(-)

Isolated *Streptomyc*es species growth pattern was evaluated under minimal medium and the effect of pH that the isolates grew well at pH 7 followed by 5 and 8 (Fig. 1). The OD value of growth rate was 1.2 at 600 nm. Figure.2 reveals the optimum temperature for Streptomyces isolate was 35 and moderate growth was obtained at 28^{o} C. pH and temperature plays vital role in growth and production of primary metabolites (Yamada *et al*., 2006)

Table 1: COLONY MORPHOLOGY AND GRAM STAIN

Table 2 : QUALITATIVE SCREENING OF AMINO ACID, ORGANIC ACID, IAA

STRAIN CODE	COLONY MORPHOLOGY	GRAM STAIN
PLS 1	Circular	- rod
PLS 2	Irregular filamentous	+ long filamentous
PL 3	Yellow irregular	+ cocci
PL 4	Circular	-rod
UST 1	Circular	+ rod
SST 2	Circular	- rod

FIGURE 1:Effect Of pH on growth of *Streptomyces* species

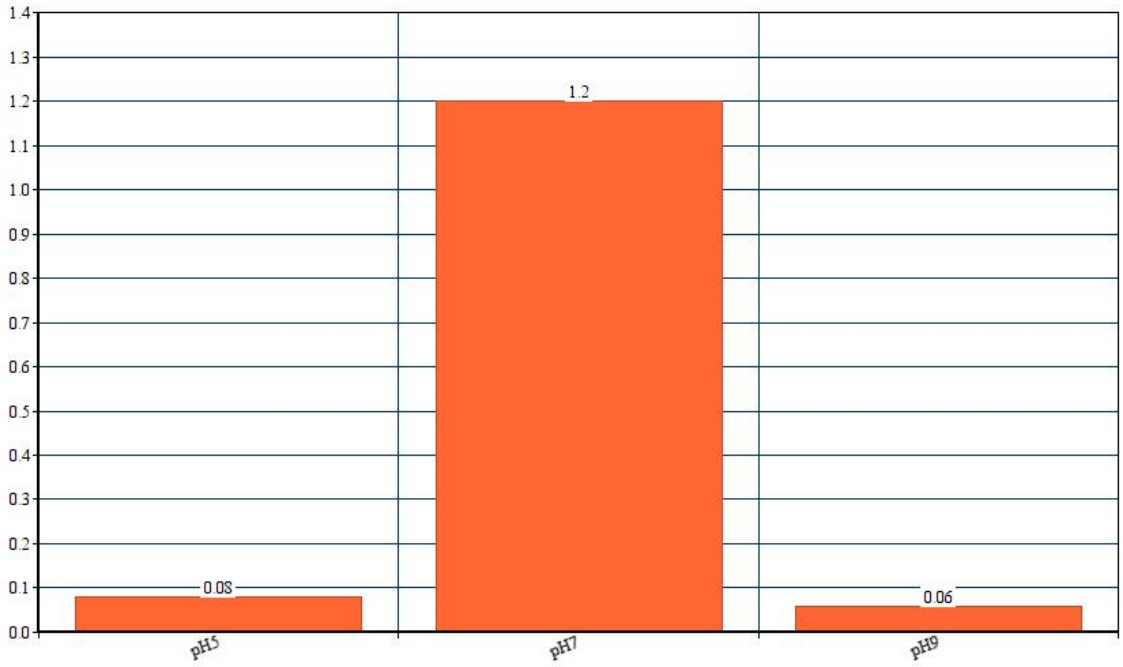


Figure 2: Effect Of Temperature on growth of *Streptomyces* species

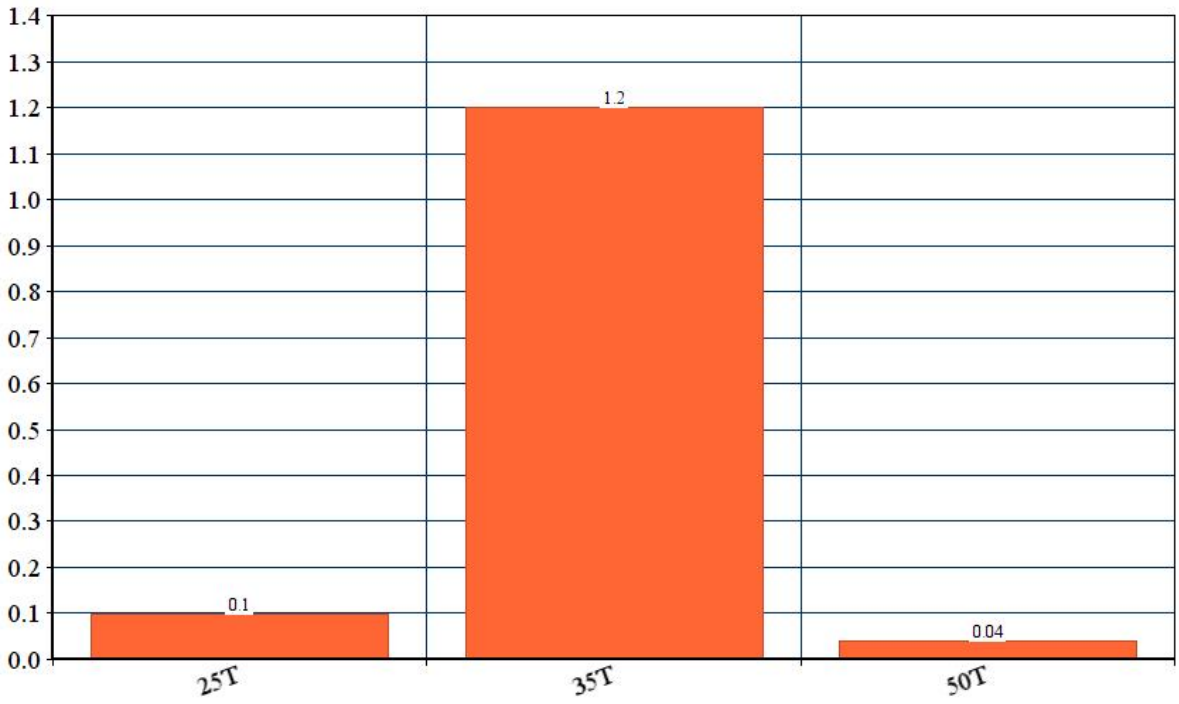


Photo plate 1: isolation of bacteria from phyllosphere

Plate 2: Isolated and Purified bacterial isolates

Plate 3 : a) screening and detection of amino acid **b) spore morphology PLS2**

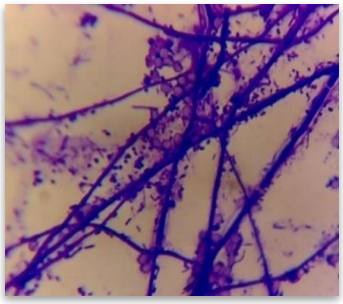
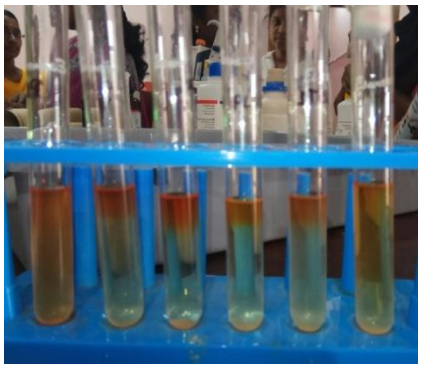
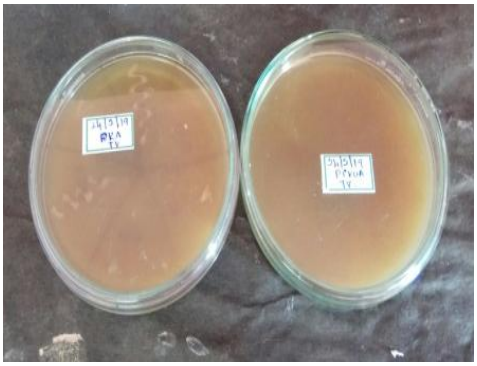


Plate 4: Organic acid production test **Plate 5: Phosphate solublization test**





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