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Developing Plant Probiotic Bacteria from Rohu Fish on Bioformulation by PGPR Activity

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Abstract

Probiotics are commonly used in health promoting "functional foods" for humans, as well as therapeutic; prophylactic and growth supplements in animal production and human health. It may be considered as an alternative to antimicrobials in disease control strategies of cultured fish. Plant Probiotic bacteria having both properties, Probiotic as well PGPR. Plant Probiotic is formulated to re-establish beneficial microbial populations that provide the soil with the necessary components to promote healthy growth and reduce plant stress. It is a unique complex of beneficial microorganisms. Fresh water fish Rohu from Chandola Lake Ahmedabad, Gujarat was used to screen the probiotic bacteria. After aseptic dissection, the gut extract sample was spread on nutrient agar plate containing 1.5% NaCl w/v and on other media like MacConkey's Agar Plate, MRS Agar Plate, Actinomycetes Agar Plate and Yeast Mannitol Agar Plate by Spread plate technique. Total 22 bacterial isolated were obtained and they further screened out as PGPR. The results had shown that from the Rohu fish, four potent plant probiotics were isolated, optimized and characterized on the basis of Morphological, Cultural and Biochemical characteristics. The four isolates ZF3, ZF7, ZF10 & ZF14 were gram positive *Bacillus spp*. They can be used as potent cultures at an industrial level due to its standard properties of plant probiotic. It might be a best as compare to PGPR.

Key words: Probiotic, PGPR

Introduction

Plant Probiotic is a unique complex of beneficial microorganisms. It promotes the establishment and enhances the growth of greenhouse, nursery crops and landscape plants in all types of soils and growing media. Probiotic products are good for our health, with benefits ranging from improved digestion to managing allergies and colds. Just as humans can benefit from the good bacteria of probiotics, plants can benefit from certain microbes. And that benefit is also good for the environment.

Beneficial microbe populations are essential to creating the ideal soil environment for healthy plant growth. The term "Plant Growth Promoting Rhizobacteria" (PGPR) was first used by Kloepper and Schroth (1978) to describe soil bacteria that colonize the roots of many plant species. PGPR have been applied to a wide range of crops and agricultural conditions for the purpose of enhancing plant growth and health, hence improving crop yields. The use of probiotic for aquatic animals is increasing with the demand for Environmental friendly sustainable aquaculture. Fish have a symbiotic ship with probiotic. Freshly fish shows the reflection of microbial quality of the waters from where they are harvested. Probiotics may be considered as an alternative to antimicrobials in disease control strategies of cultured fish. Fish get bacteria in the digestive tract from aquatic environment through water and food which are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers a favorable culture environment for the microorganisms. There are few probiotics in the gut as it is highly acidic. Research over the past few decades has shown that the nutrients and minerals in fish, and particularly the Omega 3 fatty acids found in fishes, are useful in brain development and reproduction. This has highlighted the role of fish in the functionality of the human body. The present work carried out on the Rohu species of fresh water fish. Bacillus spp. Isolated from digestive tract of the fish and another isolates from Pigeon Pea rhizospheric soil sample tested by morphologically, biochemically and bioformulating with PGPR properties which is more beneficial and economical. The benefits to crop growth attributed to PGPR, co-inoculation with these microorganisms might improve plant's performance. This approach is in agreement with modern demands of agricultural, economic, social and environmental sustainability (Chaparro et al. 2012). Current trends in Agriculture are focused on the reduction of the pesticide and inorganic fertilizers utilization, forcing the study for alternative ways to progress a more sustainable agriculture (Kloeper et al 1988). The current research will be focused on the exploitation of PGPR inoculants as Probiotic which is a promising healthier plant alternative to chemical fertilizers and pesticides. Probiotic PGPR inoculants can accelerate rehabilitation of degraded land and improvement of soil fertility, enhancing survival and growth of plants, increase grain yield, lowering malnutrition rates and reduce dependence on chemical fertilizers. Plant Probiotic could be exploited as effective a biofertilizer. This work was carried out to isolate Rhizobacteria and to develop Plant Probiotic bacteria as biofertilizer from fresh water Fish. The screening of the isolates for Plant Probiotic bacteria was carried out from Rohu fish sp. and studied on Pigeon Pea (*Cajanus cajan*) plant.

Materials and Methods

Sample collection

Fresh water fish (Rohu fish) was collected from latitude of 22.9870°N and longitude of 72.5870°E Chandola Lake, Ahmedabad, Gujarat, India. Fresh fish was collected in presterile screw cap container, transported to laboratory within an hour and processed immediately. Physical characterization of Fish sample was observed Rohu fish's length was 20cm and weight was 750g, Gut pH of fish was 2.5pH

Figure 1: Rohu fish

Morphological characterization

Morphological characterization of colony was done with the help of compound microscope. The characters observed include–colony size, shape, margin, elevation, colour, opacity, consistency, gram staining and motility.

Biochemical characterization

The biochemical characterization of the isolated bacteria include sugar utilization, respiration type, salt tolerance, pH tolerance, bile tolerance, lactose fermentation, amylase production, catalase production, oxidase production, citrate utilization, indole production, methyl red test and Voges prousker test.

Isolation and identification of Plant Probiotic bacteria

Procedure for Isolation of gut flora

Fresh fish was washed with distilled water then surface sterilized with alcohol (70%) and the gut was removed by dissection.

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The gut was immersed in sterile saline (1.5% NaCl).

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Heat on water bath at 80°C for 20 min

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1ml of sample was mixed in 9ml of 1.5% NaCl and same as serial dilution up to 10?6 was prepared.

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0.1ml of sample was spread on nutrient agar plate containing 1.5% NaCl w/v and on Hicrome Bacillus Agar Plate by Spread plate technique

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The plates were incubated at 30°C and 37°C for 24 h

↓

Isolates were purified by streaking on respective media for their Microscopic examination and Biochemical examination

↓

Screening of probiotic bacteria

↓

PGPR attributes

↓

Catalase test were used for identifying *Bacillus* species.

Identification based on their morphological and biochemical characteristics.

Graph 1: Isolation and identification of *Bacillus spp.* as Plant probiotic

Temperature tolerance

All 22 cultures were streaked on Nutrient Agar Plates and incubated at different temperature incubators like 30°C, 37°C, 45°C, 55°C, one plate put at room temperature (25°C±3) and one plate put in a refrigerator (7°C-8°C) for 24 h, with the control in each.

NaCl tolerance

All 22 cultures were streaked on Nutrient Agar Plates containing different concentration of NaCl 0.5% to 30.5% and incubated at 37°C for 24 h, with the control in each.

Bile tolerance

0% to 2.5% concentration of Bile salt containing Nutrient Agar Plates were prepared and on that all 22 cultures isolated from Rohu fish were streaked and incubated at 37°C for 24 h, with the control in each.

pH tolerance

0.1 ml respective culture suspension was inoculated in 5 ml Nutrient Broth containing different pH 2, 2.5, 3.0, 3.5,… up to 9.0 adjusted with 0.1N, 1N NaOH and 0.1N, 1N HCl, all tubes were incubated at 30°C for 24h and OD was taken at 420 nm in Spectrophotometer, uninoculated broth served as control.

Phosphate Solubilization

Phosphate solubilization was checked using tricalcium phosphate as insoluble phosphate. Spot inoculation of the isolates was done in the center of the Pikovskaya's agar medium amended with bromophenol blue. The zone of phosphate solubilization was checked in the form of a clear yellow colour halo formed around the colony representing the production of organic acids as a possible mechanism of the phosphate solubilization. Using Pikovskaya's agar plate measuring the zone of Phosphate Solubilization up to 6^{th} day of incubation at 37°C temperature.

IAA (Indole Acetic Acid) Production

Auxin production was checked in trypton yeast medium. Bacteria were grown in 50 ml yeast extract broth supplemented with 50 mgl^{-1} of L-Tryptophan and incubated in dark on orbital shaker at 200 rpm for 72 h. IAA production was checked in supernatant using Salkowsky's reagent method (Sarwer and Kremer 1995). One ml of culture supernatant was mixed with 1 ml of Salkowsky's reagent and incubated in dark for 30 min for development of pink colour, which was then estimated on spectrophotometer at 536 nm. The amount of IAA produced was calculated from the standard graph of pure indole acetic acid. Study was carried out every 24 h for up to 120 h and the pattern of IAA production was recorded. Using SIM's agar stab Medium also checked the production of Indole Acetic Acid.

Ammonia Production

Each strain was tested for the production of ammonia in peptone water. Overnight broth cultures

(100µl inoculum with approximately 3 x 10^8 c.f.u. ml^{-1}) were inoculated in 10 ml peptone water and these plates were then incubated at 37°C for 48 to 72 h. After Incubation Nessler's reagent (1 ml) was added to each tube. Development of brown to yellow color was recorded as a positive test for ammonia production (Cappucino and Sherman 1992).

Ammonia released by diazotrophs is one of the most important traits of PGPR's which benefits the crop (Kundu 1987). This accumulation of ammonia in soil may increase in pH creating alkaline condition of soil at pH 9-9.5. It suppresses the growth of certain fungi and nitrobacteria due to it potent inhibition effect. It also upset the microbial community and inhibits germination of spores of many fungi (Martin 1982). Christiansen et al. (1991) have reported that level of oxygen in aerobic conditions was same as the level of ammonia excretion under oxygen limiting conditions. However, Joseph et al. (2007) reported ammonia production in 95% of isolates of *Bacillus* followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45%).

Exopolysaccharide (EPS) production

Normally EPS production is studied in basal medium of all different organisms. As carbohydrate source 5% of sucrose is to be added as polysaccharide in to the medium (Modi et al.1989). 10 ml of culture suspension was collected after 5-6 days and centrifuge at 30,000 rpm for 45 min add thrice the volume of chilled acetone. EPS will be separated from the mixture in the form of a slimy precipitates. Precipitates were collected on a predried filter paper. Allow the precipitates to dry overnight at 50°C. Reweigh the dried filter paper after overnight drying. Note the increase in the weight of filter paper, is the EPS produced.

Carbohydrate Utilization

Carbohydrate utilization test of the isolates bacteria was carried out to identify them, following different carbohydrate were used for the utilization study are Arabinose, Dextrose, Fructose, Galactose, Lactose, maltose, Mannitol, sucrose and Xylose.

Pot experiments

The unsterilized farm soil was used for this experiment and clumps of soil were broken to remove concrete, debris, large stone and other plant or crop material. Soil was sieved with 10 mesh size sieve. Soil was initially sterilized at 200°C for 1 hr for 3 days consecutively in hot air oven. Then pots (7x10 cm) were filled approximately with sterilized 700g soil in each pot. Control having no bacterial culture was maintained throughout the study. For each treatment, three such pots were maintained. The soil was saturated with distilled water before sowing the seeds. *Pigeon Pea* (*Cajanus cajan)* seeds (pre sterile with 3% H_2O_2 for 20 min) taken from the Gujarat state corp. Marketing federation (L) Jamalpur Depo, Ahmedabad (GUJCOMASOL).

• Measurement of Biometric Plant Growth Parameters

The plants were washed through dipping into a vessel. Plant height (cm) and root length (cm) of each plant were recorded. Dry weights of shoot and root were recorded after drying in an oven for 1 day at 70°C. The experiment was repeated thrice. Observations were also recorded on rate of seedling emergence.

1. Length of the root and shoot (cm)
2. Wet weight of root and shoot (g)
3. Dry weight of root and shoot (g)
4. Number of leafs
5. Number of lateral roots
6. Number of branches

Vitek Identification

The VITEK 2 is an automated microbial identification system that provides highly accurate and reproducible results as shown in multiple independent studies. With its colorimetric reagent cards, and associated hardware and software advances, the VITEK 2 offers a state-of-the-art technology platform for phenotypic identification methods.

The BCL card was used for the automated identification of 38 taxa of the most significant aerobic endospore forming species of the family *Bacillaceae*. The BCL identification card is based on established biochemical methods and newly developed substrates (Atlas 1993, Claus and Berkeley 1986, Gordon et al. 1973, Logan and Berkeley 1981, Logan and Berkeley 1984, Logan et al. 1985, Logan et al. 2002, Logan and Turnbull 2003). There are 46 biochemical tests measuring carbon source utilization, enzymatic activities, inhibition, and resistance. Final identification results are available in approximately 14 hours. The list of test substrates is shown in Table 2

Results and Discussion

Isolation of Plant probiotic

From the nutrient agar and Hicrome bacilli agar total 22 isolates were obtained by serial dilution technique. Among them 10 isolates were gram positive bacilli, 8 isolates were gram positive cocci, 2 isolates were gram negative cocci, and 2 isolates were gram negative short rod. All 22 Cultures were spread on different media like Nutrient Agar, Ashby's Mannitol, Yeast Extaract Mannitol, Pikovskaya's agar and MacConkey's agar 0.1 ml of suspension from each dilution was spread on the above mentioned medium and incubated at 37°C for 24 hrs respectively. Among them 4cultures were selected for the further study. All cultures were maintained on Nutrient agar slant with monthly transfer and preserved at 4°C in a refrigerator

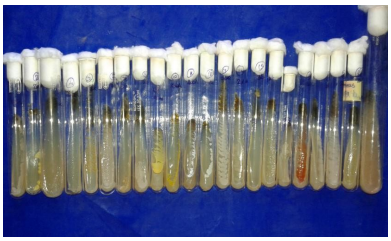

Figure 2: Bacterial isolates

All the organisms shown optimum growth at 37°C temperature, at 30°C temperature defined

excellent growth, at 20°C±5 temperature defined good growth, at 45°C temperature show poor growth and some organisms remained viable at 55°C temperature, no growth obtained at 7°C-8°C. From the isolates of Rohu fish isolate no. ZF22 was remaining viable growth up to 20.5% NaCl concentration, where all organisms show optimum growth upto 1.5% NaCl concentration. The Effect of Bile salt, organism ZF19 show tolerance up to 2.0% concentration of Bile salt and organism ZF7 and ZF15 show tolerance up to 1.5% concentration of Bile salt, where all organisms show 0.5 % tolerance to Bile salt. From the above results, only 9 organisms tolerate to high acidic pH and they define growth at 3 pH also. On the basis of that, these nine organisms considered as good probiotics, where all 22 organisms show optimum growth at 6.5 pH.

Using Pikovskaya's agar plate, measuring the zone of Phosphate solubilization up to 6th day of incubation at 37°C temperature. Maximum solubilization zone was observed with ZF7 (24mm) > ZF10 (23mm) > ZF3 (22mm) e" ZF8 (22mm) > ZF24 (16mm) > ZF14 (15mm). Qualitative production of Indole Acetic Acid by SIM's Agar medium was done along with motility. It turns medium brown to slightly black in colour where stab was injected. Semi solid stab were incubated at 37°C temperature observed up to 5 d. Estimation of IAA production by using Salkowsky's reagent method on TYM medium (Sarwer and Kremer 1995). One ml of culture supernatant was mixed with 1 ml of Salkowsky's reagent and incubated in dark for 30 min for development of pink colour, which was then estimated on spectrophotometer at 536 nm up to 5th d mentioned in table 1 and figure 3. Ammonia production was studied up to 8 d of incubation at 37°C temperature. Maximum concentration of ammonia production was observed in isolates ZF7 and ZF14 both. Qualitative production of Exopolysaccharide defines by EPS Agar plate. There were no quantitative production define by Basal medium containing carbohydrate source 5% of sucrose as polysaccharide (Modi et al.1989). Maximum EPS production was observed in ZF2, ZF3, ZF6, and ZF8. Total 12 different types of carbohydrates were tested. 1 % of 12 different carbohydrates was added in Nutrient agar selected 4 cultures were streaks and incubated all plates at 37°C for 24 to 28 h, all 4 culture show good utilization of carbohydrates.

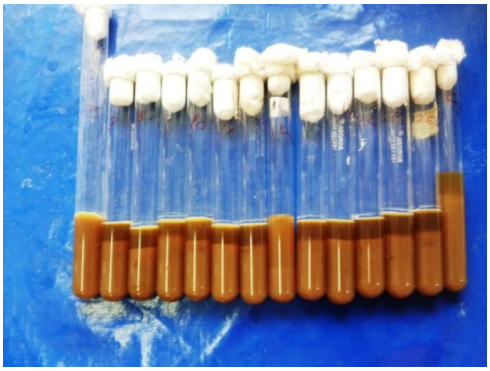

Figure 3: Results on SIM's media

Pot experiments were carried out in a net house (to prevent birds from damaging the crops) has proved useful for most of the year in warm climate prevailing in India. 30 days Study of *Pigeon Pea* plant growth under the influence of four selected isolates ZF3, ZF7, ZF10 and ZF14 from Rohu fish bacteria show increased growth of plants in terms of root length where maximum increase was observed in pot ZF14 (6.7cm), in pot ZF10 (6.5cm), in pot ZF3 (6.3cm), in pot ZF7 (4.7cm) was found and in control pot (4.5cm). In Shoot length where maximum growth was observed in pot ZF14 (14cm), in pot ZF10 (13.9cm), in pot ZF3 (13.1cm), in pot ZF7 (9.4cm) was found and in control pot (9cm). Number of leaves and fresh weight as well as dry weight also measured, ZF14 was found to be the most effective for *Pigeon Pea* plant.

Table 1: Probiotic and PGPR potential

Isolates	ZF3	ZF7	ZF10	ZF14	Control
% NaCl tolerence	20.5	10.5	15.5	20.5	-
% Bile salt tolerence	1	1.5	0.9	0.7	-
6th day zone of PO4 solubilizing	22mm	24mm	23mm	16mm	-
Ammoniya production of 8th day	++	+++	++	+	-
IAA Results as per Salkowsky's Reagents	+	+++	++	++	-
IAA Results as per SIM's Medium	++ Motile	+ Highly motile	+ ++ Non motile	++ Highly motile	-
Qualitative Analysis of EPS Growth	+++	+	++	++	-
1% Glucose	++	++	+	+++	-
1% Sucrose	+	++	+++	+++	-
1% Arabinose	-	-	-	-	-
1% Dextrose	+	++	-	++	-
1% Fructose	-	-	-	-	-
1% Galactose	-	+	-	-	-
1% Lactose	-	-	-	-	-
1% Maltose	++	++	+++	+	-
1%Mannitol	++	++	+	+	-
1% Xylose	+	-	++	+	-
1% Ribose	++	+	+	+	-
1% Mannose	+	+++	+	++	-
Catelase test	Positive	Positive	Positive	Positive	-
Gram's Reaction	Gram's positive	Gram's positive	Gram's positive	Gram's positive	-
Vitek identification	Bacillus subtilis	Bacillus megaterium	Bacillus amyloliqueficance	Bacillus atrophaeus	-

(Key: + = visible growth; ++= viable growth; +++ =maximum growth; - = no growth)

Table 2: Test Substrates on BCL Card and Results

Well	Test	Mnemonic	Amout/well	ZF3	ZF7	ZF10	ZF14
1	BETA-XYLOSIDASE	BXYL	0.0324mg	+	-	+	+
3	L-Lysine-ARMLA,IDASE	Lysa	0.0228mg	-	-	-	-
4	L-Aspartate ARYLAMIDASE	AspA	0.024mg	-	-	-	-
5	Leucin-ARYLAMIDE	LeuA	0.0234mg	+	+	+	+
7	Phenylalanine ARYLAMIDASE	PheA	0.0264mg	+	+	+	+
8	L-Proline ARYLAMIDASE	ProA	0.0234mg	-	-	-	-
9	BETA-GALACTOSIDASE	BGAL	0.036mg	+	+	+	+
10	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018mg	+	+	+	+
11	ALPHA-GALACTOSIDASE	AGAL	0.036mg	+	+	+	+
12	Alanine ARYLAMIDASE	AlaA	0.0222mg	-	+	-	-
13	TyROSIN ARYLAMIDASE	TyrA	0.0282mg	-	+	+	-
14	BETA-ACETYL-GLUCOSAMINIDASE	BNAG	0..0408mg	-	-	-	-
15	Ala-Phe-Pro ARYLAMIDASE	APPA	0.0384mg	+	+	-	+
18	CYCLODEXTRIN	CDEX	0.3mg	-	-	-	-
19	D-GALACTOSE	Dgal	0.3mg	-	+	-	-
21	GLYCOGEN	GLYG	0.1875mg	+	+	+	+
22	myo-INOSITOLMETHYL-A-D-GLUCOPYRANOSIDE	INO	0.3mg	+	-	+	+
24	Acidification	MdG	03mg	+	-	+	+
25	ELLMAN	ELLM	0.3mg	-	+	+	+
26	METHYL-D-XYLOSIDE	MdX	0.3mg	-	-	-	-
27	ALPHA-MANNOSIDASE	AMAN	0.036mg	-	-	-	-
29	MALTOTRIOS	MTE	0.3mg	+	+	+	+
30	Glysine ARYLAMIDASE	GlyA	0.012mg	+	-	+	-
31	D-MANNITOL	dMAN	0.3mg	+	+	+	+
32	D-MANNOSE	dMNE	0.3mg	+	-	+	+
34	D-MELEZITOSE	dMLZ	0.3mg	-	-	-	-
36	N-ACETYL-D-GLUCOSAMINE	NAG	0.3mg	-	+	-	-

(Key: + = test positive; - = test negative)

The bacterial isolates were sent to the Supratech Pathology Lab Paldi, Ahmedabad, Gujarat to confirm the identification of *Bacillus spp.* They identified as ZF3 *Bacillus subtilis*, ZF7 *Bacillus megaterium*, ZF10 *Bacillus amyloliquefaciens* and ZF14 *Bacillus atrophae* identification by BCD Card of Vitek machine.

Conclusion

This study revealed that these *Bacillus spp*. isolates having best characteristics of plant growth promoting potential that helps to increase the biomass of the plant *Pigeon Pea* (*Cajanus cajan).* They have properties of probiotic as well as PGPR hence, this plant probiotics good for human and animals as well as beneficial for plants and sustainable agriculture too.

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