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Full Length Article

Characterization and Cellulolytic Enzyme Potential of P Solubilizers from Pine Forests of Lower Himalaya

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Abstract

Phosphorus (P) present in large amounts in earth as rock phosphate is an important chemical compound for the soil quality and plant growth. In our previous work we isolated P solubilizing bacteria and fungi from pine forest of lower Himalaya, demonstrating their capabilities to solubilize P from tricalcium phosphate and exhibiting their various ecological characteristics. In this current study, we are reporting the multifunctional biochemical characterization of P solubilizing microorganisms with particular focus on biomass degrading cellulase enzyme. Our fungal strains are sequenced to be ascomycetes which showed exponential P solubilization during first 2–4 days of inoculation with steady performance afterwards. As the solubilization of phosphorus is normally governed by acidification, we detected acetic acid produced by our purified fungal strains. As for the biochemical characterization of P solubilizing bacteria (PSB), majority of the strains showed catalase enzyme activity while only one strain (PWB33) depicted indole acetic acid (IAA) production. Interestingly, 22% of the tested PSB showed cellulase enzyme activity as an extra ecological function. Quantitative analysis of cellulase enzyme activity verified the semi-quantitative cellulase enzyme assay showing PWB13, PWB 21 and PWB50 with maximum biomass degrading (hydrolase) enzyme production. In conclusion, the tested P solubilizing bacterial and fungal strains exhibited diversified biochemical functionality which may represent an attractive environment friendly alternative in future to solubilize the rock phosphate abundant in earth, thus, reducing the needs for chemical fertilizers and may also increase the carbon turnover *via* biodegradation through cellulase enzyme activity.

Keywords: Phosphorus; P solubilizing microorganisms; Biofertilizers; Cellulase; Himalaya

Introduction

The Himalayan forests represent a high-level biodiversity which is poorly understood, particularly the microbial diversity (Lovett *et al.* 2000; Myers *et al.* 2000; Kier and Barthlott 2001). On the other hand, soil microbial contributions in forest ecosystems play important role for sustainability (Vibha and Neelam 2006) which in turns depends on nutrient recycling, obtained from plant residues (fallen leaves, droppings and dead roots) and entering into the soil (Pandey *et al.* 2006). Soil microbes (*e.g*., fungi and bacteria) and their mutual interactions play a vital role in biogeochemical cycling, being major drivers for biodegradation of organic carbon pole (Vibha and Neelam 2006). However, elevated heterogeneity of diverse forest ecosystem and its richness limit our knowledge on soil microorganisms inhabiting in there.

Microorganisms are, though, ubiquitous in diversified habitats exhibiting a great diversity in their morphological characteristics, growth requirements and biochemical attributes (Pretty 2008). Majority of the microorganisms present in soil ecosystem are proficient mineralizers and thus are responsible for nutrients' cycling and environmental sustainability (de Bello *et al.* 2010; Bodelier 2011). These mineralizers play key roles in enhancing soil fertility and subsequent crop yields through their varying biochemical activities. In soil, the rhizosphere is considered as 'hot spot' for many active groups of microorganisms (Villacieros *et al.* 2003), commonly known as plant growth promoters (Kloepper *et al.* 1980). The plant growth promoting microorganisms are known to readily colonize the plant rhizosphere (Rangajaran *et al.* 2003) and affect the host plant growth *via* (1) enhanced nutrient availability *e.g*., phosphorus solubilization and nitrogen fixation (Hafeez *et*

al. 2019); (2) suppression of phytopathogens (Panth *et al.* 2020); (3) improving the plant's stress tolerance *e.g.*, salinity, drought, metal toxicity (Danish *et al.* 2020); and (4) production of phyto-hormone *e.g.*, indole-3-acetic acid (Gupta *et al.* 2000). The bacteria, for instance, providing at least one of these services are termed as plant growth promoting rhizobacteria (Danish *et al.* 2020).

Among essential plant nutrients, phosphorus (P) is a vital macro-nutrient available to plant roots through soil in limited soluble forms (Samreen *et al.* 2019), and basically is required for maximizing crop growth and production (Malviya *et al.* 2011). Phosphorus availability to plants contribute significantly in regulating metabolic pathways through activation of major enzyme functions, thus indicating its importance in plant metabolism (Padmavathi and Usha 2012). The greater pool of soil phosphorus contains total phosphorus (approximately 95–99%) but its availability to plant is very low due to its presence in the form of insoluble phosphates, thus becoming limiting factor for the plant growth (Vassileva *et al.* 2001; Mikanova and Novakova 2002). Therefore, P deficiency is reported as the most important chemical factor restricting the plant growth because this nutrient plays a vital role in biochemical and physiological functions of the plant (Rasool 2011). At the same time, P deficiency in soil may lead to the excessive use of chemical (P) fertilizers but again its availability is limited due to high binding capacity of phosphate anions in calcareous and alkaline soils resulting in insoluble products (Vessey 2003). As developing countries' economic stability depends on agricultural yield; hence to feed their rapidly increasing population, the excessive use of chemical fertilizers is therefore a big challenge (Antoun and Klfvoepper 2001).

In this regard, the application of phosphate solubilizing microorganisms in agricultural soil, is a promising approach to enhance P availability (van der Heijden *et al.* 2008; Wang *et al.* 2018). As a solution, Phosphorous Solubilizing Microorganisms (PSM) are employed, that play vital role in mineralizing organic phosphorus and rendering inorganic phosphorus availability to plants in soluble form, constricting the use of chemical fertilizers (Peix *et al.* 2001; Sodhi 2012). Both microbial groups *i.e.*, P solubilizing bacteria and P solubilizing fungi are of equal importance for plant growth promotion *via* solubilization and subsequent provision to plants through organic acid synthesis and plant growth promoting substances (Yadav *et al.* 2011). Along with this, such rhizosphere inhabiting microorganisms contribute in various other ecological functions like biomass degradation through catalytic enzymes (Egamberdieva *et al.* 2010). Consequently, the nutrient cycling and ecosystems stability is largely mediated by these soil microbes. For example, many recent studies have reported an extraordinary insight into the lignocellulolytic microorganisms for their metabolic potential and enzymatic expression (Maki *et al.* 2009; Jiménez *et al.* 2016; Behera *et al.* 2017; El-Barotty *et al.* 2019; Maravi and Kumar 2020). Isolation and the identification of cellulolytic microorganisms which enhance the plant growth and carry agricultural significance, are thus important for compost purposes as well (Schneider *et al.* 2009). In this regard, several bacteria and fungi have the ability to produce cellulase for the degradation of cellulose and to ultimately promote the carbon cycling (Roth *et al.* 2020). Soils under crop residues are rich cellulose sources (being the main constituent of plant fiber structure) and a substrate to numerous bacteria and fungi which excrete extracellular enzymes for degradation purposes (Hameeda 2006). While, in context to the above, northern areas of Pakistan (lower Himalaya) are abundant in biomass turnover and potentially have diversified microbiota with varied ecological functions. But very little knowledge is available regarding the multi-functionality of these lower Himalayan microorganisms. Therefore, the current study is focused to evaluate if P-solubizing bacteria and fungi isolated from Lower Himalayan soil have potential to perform crucial ecosystem functions. The particular objective is also to assess the cellulase enzyme activity of P-solubilizers as a potential ecological function to degrade plant biomass, ultimately contributing to natural carbon cycling.

Materials and Methods

Microbial isolation and growth reagents

Bacteria and fungi, used in this study, were isolated from Lower Himalayan region of Abbottabad, Pakistan (Latitude 34°N 73°S). The soil sampling and subsequent microbial purification was performed from rhizosphere of various plants. The 16S rDNA gene sequencing-based identification of these purified bacterial strains was previously reported (Nazir *et al.* 2017b). While the ITS sequences of fungal isolates are deposited *via* this report in NCBI-GenBank with accession numbers MK905458-MK905462 and MT622852.

For cellulase enzyme assay, P-solubilizing microorganisms (PSM) were inoculated in 10 ml of pre-sterilized LB Broth and incubated at 28°C for 24 h under shaking conditions *i.e.*, 180 r.p.m. Inoculums of saline washed bacterial cells were further used for various other tests (Nazir *et al.* 2013). At the same time, pre-sterilized glycerol was added to over nightly grown bacterial cultures (50% (v/v)) in order to preserve them at -80°C for future use (Nazir *et al.* 2017a).

Isolated fungi were stored aseptically in sterilized Eppendorf tubes as fungal plugs having even spores (El-Barotty *et al.* 2019). Fungal cultures on Barley flake agar (BFA) slants were also saved as hyphae + spores for prolonged preservation purposes. For experimentation, BFA plates containing fungi were refreshed after 2-weeks regularly (Hayat *et al.* 2017).

Standard microbial growth media, as explained in under, were prepared for various purposes and autoclaved (121°C for 20 min) in laboratory before use.

Luria-Bertani (LB) agar media constituted as peptone

5 g L^{-1}, NaCl 10 g L^{-1}, yeast extract 5 g L^{-1}, agar 12 g L^{-1} and pH 7.0. Chemical constituents of Pekovskaya's agar (PVK) medium were NH_4NO_3 1 g L^{-1}, KCl 8 mg L^{-1}, NaCl 1 g L^{-1}, $FeSO_4$ 0.25 g L^{-1}, $MgSO_4$ 1 g L^{-1}, Tricalcium Phosphate (TCP) 4 g L^{-1}, Glucose 10 g L^{-1}, Agar 12 g L^{-1} and pH 6.8. The Barley flake agar (BFA) medium was prepared *via* boiling 30 g barley flakes in deionized water for 15 min, followed by soup filtration through a sieve of 2 mm pore size. Subsequently, the volume was made up to one liter and the medium was then sterilized in autoclave after adding 15 g agar to the suspension. Carboxymethyl Cellulose (CMC) agar media was prepared in one liter *via* adding CMC 5.0 g, $NaNO_3$ 1.0 g, K_2PO_4 1.0 g, KCl 1.0 g, $MgSO_4$ 0.5 g, Yeast Extract Powder 0.5 g and Agar 15.0 g. Media pH was maintained at 6.8 before sterilization.

Evaluation of P-solubilizing fungal strains

Molecular identification: For identification purpose, fungal strains were prepared by culturing fresh colonies on BFA medium. The sequencing was subjected to conserved ITS region of fungi, a gene marker for molecular identification. PCR amplification from fungal DNA and subsequent sequencing was performed according to standard protocols (Lau *et al.* 2006). The subsequent sequences were manually evaluated *via* Chromas and aligned to infer their phylogenetic similarities by using MEGA. The neighbor joining phylogenetic tree was constructed, based on 'Maximum Composite Likelihood' model and Bootstrap method (1000 repeats), to cluster the strains in groups on the basis of similarities and differences which they have in their nucleotide sequences (Nazir *et al.* 2017b SR).

P-solubilization assessment: P solubilization of inorganic P from rock phosphate was assessed by inoculating fungal hyphae, on PVK agar. In brief, fresh hyphae of growing fungal strains were placed in the center of PVK agar plates (with sole P-source of TCP) and incubated as above. On fungal growth, the hallo developed on PVK's agar by different fungi was measured to assess P-solubilization potential over time (Elias *et al.* 2016). The PVK medium plates, having fungal growth on them, were carefully monitored after each 24-h for hallo zone procession, till the end of incubation (*i.e.*, 7-days). Moreover, the P-solubilization potential of test fungi was also calculated on the basis of hallo zone diameter developed by particular fungus (Elias *et al.* 2016). Multiple readings were, thus, recorded for replicated fungal systems and the data acquired is reported here as average of three replicates.

Headspace volatile collection

Fungal isolates were grown for 4-days in 250 mL Erlenmeyer flasks (E-flask) of barley flake medium. The mouth of the E-flask was closed with aluminum foil. Volatiles from the head space (HS) of fungal inoculated and control media were collected by adopting an earlier method (Azeem *et al.* 2015). HS volatile collection was performed by using 65 mm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME stable flex fiber (Supelco, U.S.A.). The same fiber was used for all samples and control media. Before using the fiber first time, it was conditioned for 30 min at 250°C. The SPME needle was immersed through a pin hole in aluminum foil covering, into the flask. The SPME fiber was thus exposed to HS volatiles for 30 min at ambient temperature. After volatiles' collection, the fiber was retracted into the SPME needle and immediately injected into a GC injector at 230°C. After desorption, the SPME fiber was cleaned at 230°C under helium gas stream for 5 min before using it for next collection (Azeem *et al.* 2015).

Identification of volatiles: Fungal volatiles were separated and identified according to Azeem and colleagues (2015) *via* using PerkinElmer Clarus 600 gas chromatograph (GC) connected with PerkinElmer Clarus 600°C mass spectrometer (MS). The GC was connected with a split/splitless injector (splitless mode, 30 s) and injection was performed in split mode (1 mL/min) in the presence of helium gas with a consistent flow through the column. Elite capillary column (5 MS) attached to GC was of 30 m length, 0.25 mm ID and 25 μm film thickness (PerkinElmer, U.S.A.). GC oven was operated at temperature of 40°C for 2 min followed by an increase of 5°C/min up to 240°C where final temperature was controlled for 8 min. The injector temperature was isothermally set at 235°C, while connection of GC to the MS by transfer line was isothermally maintained at 250°C. The MS ion source temperature was managed at 150°C. Mass spectra were obtained at 70 eV with a mass range of 30 to 400 m/z. Identification of separated compounds was initially carried out by comparing their mass spectra to the NIST-05 (National Institute of Standard and Technology, U.S.A.) MS library. The final authentication was made by injecting the pure acetic acid (purchased from Sigma-Aldrich) to the GC-MS using the same parameters used for the fungal samples and controls (Azeem *et al.* 2015).

Cellulose degradation by P-solubilizing bacteria

Qualitative assessment of cellulose degradation was done by Carboxymethyl Cellulose (CMC) agar plate assay *via* measuring the cellulase index (CI) for each test strain. To evaluate, CI was measured by inoculation of bacterial strains on CMC Agar. The inoculated CMC plates were incubated for 2 days at 28°C and subsequently cellulase index was measured. The test was performed by adopting an established protocol (Miranda *et al.* 2011). Briefly, the staining of CMC agar media containing bacterial colony was performed with Congo Red Dye (0.2%) for 20-min and then de-staining was done with 1 *M* NaCl solution for 10 min. The strain, having ability to degrade cellulose, exhibited de-coloration of the stain. The following formula was used to calculate CI for test strains:

CI = (Colony + Hallo zone) Diameter/Colony Diameter

Cellulase activity was further determined by evaluation of secreted cellulase enzyme (Amin *et al.* 2014; El-Barotty *et al.* 2019). For this purpose, the standard cellulase enzyme's stock solution was prepared as its concentration of 1200 UI. Subsequently, six sub-stock solutions (*i.e*., 20 UI, 40 UI, 60 UI, 80 UI, 100 UI, and 120 UI) were made *via* diluting it in distilled water by following the serial dilution.

The bacterial strains were grown in LB broth and subsequently the supernatants were analyzed in UV spectrophotometer at wavelength of 590 nm (Amin *et al.* 2014). Briefly, the samples were prepared by adding 0.5 mL of Azo-Cellulase enzyme substrate in an Eppendorf tube and then 0.5 mL of the bacterial culture-supernatant was vortex mixed and left for 30 min for enzyme-substrate reaction. After half hour incubation, 1ml ethanol was added to stop the reaction. Samples were then centrifuged for 10 min at speed of 10,000 r.p.m. Supernatant was collected and absorbance was measured (Amin *et al.* 2014). The samples were compared against standard curve prepared by above mentioned procedure using stock solutions of purified cellulase enzyme.

Biochemical characterization of P-solubilizing bacterial strains

Biochemical characterization of purified P-solubilizing microbial strains was carried out to assess their physiological potential by following Bergey's Manual of Systematic Bacteriology (adopted by Dhingani *et al.* 2013).

Starch hydrolysis: This test was executed for the efficacy of the isolated strains for starch degradation by production of amylase enzyme. On starch agar medium plates, bacterial strains were streaked and incubated as above for 48 h. Subsequently, the plates containing grown bacterial streaks were flooded with a solution of 1% iodine. The colorless zone appeared around bacterial growth indicated starch utilization by test bacterial strain while the appearance of the blue color means non-utilization of starch (Dhingani *et al.* 2013).

Catalase enzyme activity: Catalase/peroxidase enzyme assay was performed for the determination of microbial ability to degrade hydrogen peroxide (H_2O_2) by the secretion of catalase or peroxidase enzyme. In brief, streaking of bacterial culture (48 h old) was done on a clean glass slide followed by adding a drop of 3% H_2O_2. Catalytic activity of the bacterial culture was assessed as positive by the presence of emerging air bubbles (Farooq and Bano 2013).

Lactose fermentation test: The MacConkey agar was used for this test. Briefly, the bacterial strains were inoculated on MacConkey agar plates and incubated for 48 h. Subsequently, the color of grown bacterial colonies was evaluated *i.e.*, pink colored colonies represent lactose fermenters while non-lactose fermenters were identified by their yellow/orange color (Dhingani *et al.* 2013).

Indole production: Purified microbial strains were also examined to evaluate their ability to degrade amino acid (tryptophan) into indole. For analysis, isolated bacterial strains were inoculated in tryptone broth and incubated for 48 h. Subsequently, Kovac's reagent was added and a pink colored layer was assessed on top of the medium. The appearance of pink layer indicated the positive test *i.e.*, indole producers and vice versa (Dhingani *et al.* 2013).

Statistical analyses

The analyses reported in this work were performed with biological replicates (n = 3) and the results are presented in mean values. The variations are expressed by calculating the standard deviations which are presented in graphs as error bars while as numeric values in the text mostly reported in brackets. ANOVA, Tukey's tests and t-tests were performed for measuring the significant difference in our data wherever applicable.

Results

P-solubilizing fungal strains of Lower Himalaya

Molecular identification: For molecular identification of fungal strains, six out of eight purified fungal strains were sequenced (75%) and the sequence analyses of the ITS gene-region identified them as *Cladosporium* (2), *Penicillium* (2), *Trichoderma* (1) and *Cochliobolus* (1). The generated fungal sequences (deposited in GenBank database for fungal strains *i.e*., PWF1, PWF3, PWF5, PWF6, PWF8 and PWF9 with accession numbers MK905458, MT622852, MK905459, MK905460, MK905461, and MK905462 respectively) covered the heterogeneity regions in target gene. The sequence homologies of our strains are presented in Fig. 1. Based on differences and similarities in DNA sequences, the constructed phylogenetic tree showed inferred evolutionary relationships of our strains with various other similar fungal species. The phylogenetic tree for our P-solubilizing fungal strains and their close hits, was prepared *via* adopting the neighbor joining analysis to cluster the similar sequences in similar groups. There is, thus, a clear separation of basidiomycete (PWF8) from the rest of other fungal strains as ascomycetes (Fig. 1).

P-solubilization potential: The P solubilization potential for Himalayan microorganisms, showed that the four out of eight fungal strains (50% of purified fungi) were found to solubilize the inorganic P (tri calcium phosphate) when assessed on PVK growth medium. Subsequently we further evaluated the temporal potential of P solubilization by our isolated PSF. On evaluation of clear zone development over time in PVK medium, two strains (PWF-1 and PWF-2) showed a similar behavior and their potential was significantly better than the other two. When further assessed for a week on TCP medium, PWF-3 and PWF-6 showed a medium and low P-solubilization potential, respectively.

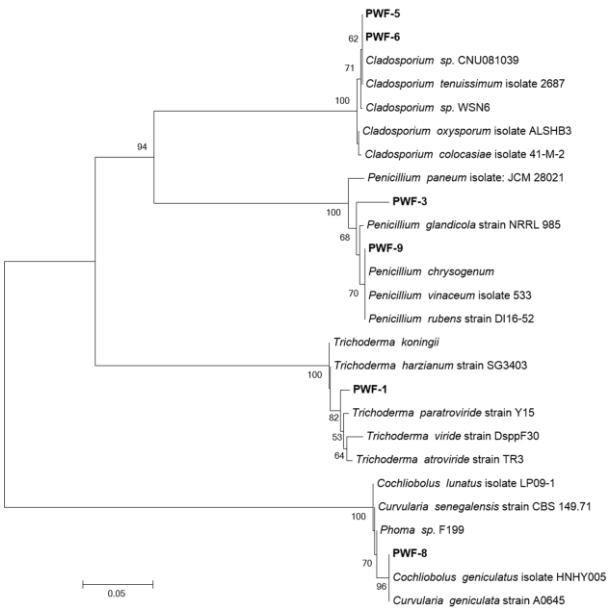

Fig. 1: Phylogenetic tree constructed after neighbor joining analysis for ITS sequences of the representative Himalayan fungal isolates. Maximum composite likelihood model was used with "Bootstrap method" using 1000 replications and the values < 50 is not included in tree

Unlike PWF-6, all other P-solubilizing fungal strains exhibited an exponential P-solubilization for the first 3–4 days of inoculation which was found steadily stable afterwards (Fig. 2). Moreover, the hallo zone development for PWF-3 was linearized about a day earlier than PWF-1 and PWF-2.

Organic acid production: For investigation of the organic acid production, the pH reduction was observed to be correlated with increased P-solubilization for Himalayan microbial strains (Hayat *et al.* 2017). Therefore, to evaluate the production of organic acids for these organisms, 2–3 days old cell culture (PVK's medium) of PWF-3 was analyzed by GC-MS. Interestingly, compared to control (medium without fungus), a differential peak (at 3.95 m) was observed in the fungal growth culture which was potentially identified as acetic acid (Fig. 3). In accordance, the similar peak was also verified by running the pure acetic acid under the same analytical conditions, which further signified the production of organic acids (acetic acid in this case) during P-solubilization by Himalayan microbial (fungal) strains.

P-solubilizing bacterial strains of lower Himalaya

Biochemical characterization: Considering the morphological resemblance, 24 bacterial P-solubilizers out of 53 PSB strains were selected for biochemical characterization *via* performing a range of tests (Table 1). Results revealed that all the tested strains were found positive for P-solubilization while eleven were detected for cellulase enzyme activity. However, only three isolates (12.5% *i.e.*, PWB-22, PWB-50 and PWB-52) could hydrolyze the starch when tested on agar plates. Among these PSB, most (83%) of the tested bacterial P-solubilizers were observed positive for catalase activity on agar media. It was also observed that eleven PSB strains (45.8%) were found positive for lactose fermentation. Surprisingly, only one isolate (PWB-33) out of 24 tested bacterial strains (*i.e.*, 4%) showed the positive result for indole AA production as confirmed by producing the red color layer at top of the bacterial culture (Table 1). This sole indole producing strain was originally isolated from 'None-Himalayan' surface soil and was earlier found to be a good P-solubilizer. In contrast, none of the isolated fungal strains showed positive signal for these assays in our test conditions.

Cellulose degradation potential: To assess the potential of isolated strains to degrade the lignocellulosic material (abundant in environment where these bacteria were isolated), an *in vitro* experiment was also conducted to evaluate the cellulase activity. When tested on CMC medium, no fungal strain showed positive signal for the cellulase. On contrary, 11 out of total 53 PSB strains were found positive for cellulase on CMC medium (Fig. 4). The potential cellulose degraders included six bacterial strains (54.5%) from *"Lower Himalayan Pine"* soil; four bacterial strains (36.3%) from *"Lower Himalayan None-Pine"* soil and one bacterial strain (9%) from *"None-Himalayan"* agricultural soil (Fig. 4).

In general, the highest Cellulase Index (2.25) was observed for PWB-13 isolated from *Lower Himalayan Pine* soil - and least cellulase index was observed for PWB-36 isolated from *None-Himalayan* agricultural soil, while all these (11) bacterial strains were also good P-solubilizers (Fig. 5a). Moreover, the quantitative analysis of cellulase concentration was also performed that verified the semi-quantitative cellulase indices of these bacterial strains (Fig. 5). For this purpose, the cellulase ability of these bacterial strains was compared with commercially available pure cellulase enzyme. The enzymatic activity by our P-solubilizers is shown in Fig. 5b. We observed that the overall enzyme activities of tested PSBs followed the trends of CMC-indices. In detail, the maximum cellulase concentration (117 UI) was shown by PWB-13 isolated from *Lower Himalayan Pine* rhizosphere and PWB-36 - isolated from *None-Himalayan* soil, showed minimum cellulase concentration (35 UI), though both strains are good P-solubilizers.

Discussion

Being the essential macronutrient, phosphorus (P) availability in soil is the prime need of growing plant and diversified microorganisms contribute in this natural phenomenon. And, during our lower Himalayan forest soil exploration, majority of our isolated bacteria and fungi showed proficient P solubilization capabilities. Another

Table 1: Characterization of P-solubilizing bacterial strains isolated from Lower Himalayan pine forest. LHPF, lower Himalayan pine forest; NHPA, non-Himalayan Punjab agriculture soil. Ag, *Agrobacterium*; B, *Bacillus*; O, *Ochrobactrum*; Ac, *Acinetobacter*; Ps, *Pseudomonas*; Pa, *Pantoea*; En, *Enterobacter*; St, *Staphylococcus*; Se, *Serratia*. N/A, not applicable; ND, not detected

Strain code	Strain Name	Strain origin	P-solubilization	Starch hydrolysis	Catalase enzyme activity	Indole AA production	Lactose fermentation	Cellulase activity
PWB-02	*Ag. tumefaciens*	LHPF	+	ND	+	ND	+	ND
PWB-04	*B. megaterium*	LHPF	+	ND	+	ND	ND	+
PWB-10	N/A	LHPF	+	ND	+	ND	+	+
PWB-12	*O. anthropic*	LHPF	+	ND	+	ND	+	ND
PWB-13	*Sphingobacterium* spp.	LHPF	+	ND	+	ND	+	+
PWB-15	N/A	LHPF	+	ND	+	ND	+	+
PWB-20	N/A	LHPF	+	ND	ND	ND	+	ND
PWB-21	N/A	LHPF	+	ND	+	ND	ND	+
PWB-22	N/A	LHPF	+	+	+	ND	+	+
PWB-23	N/A	LHPF	+	ND	+	ND	ND	ND
PWB-24	*Ac. Calcoaceticus*	LHPF	+	ND	+	ND	+	+
PWB-25	*Ps. Fluorescens*	LHPF	+	ND	+	ND	ND	ND
PWB-27	*Pa. agglomerans*	LHPF	+	ND	+	ND	ND	ND
PWB-28	*Ac. Calcoaceticus*	LHPF	+	ND	+	ND	+	+
PWB-33	N/A	NHPA	+	ND	+	+	ND	ND
PWB-34	*Ac. Calcoaceticus*	LHPF	+	ND	+	ND	+	ND
PWB-36	N/A	NHPA	+	ND	+	ND	ND	+
PWB-45	*En. ludwigii*	LHPF	+	ND	+	ND	+	ND
PWB-49	N/A	LHPF	+	ND	+	ND	ND	+
PWB-50	*Citrobacter* spp.	LHPF	+	+	+	ND	ND	+
PWB-52	N/A	LHPF	+	+	ND	ND	ND	ND
PWB-54	N/A	LHPF	+	ND	ND	ND	ND	ND
PWB-57	*St. warneri*	LHPF	+	ND	ND	ND	ND	ND
PWB-67	*S. multitudinisentens*	LHPF	+	ND	+	ND	ND	ND

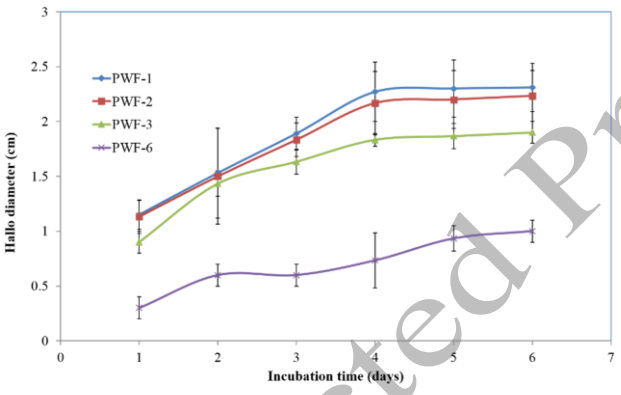

Fig. 2: Temporal potential of Himalayan fungal isolates for P solubilization exhibited by hyphal growth on TCP medium. Data is presented as averages (n=3) and standard deviations are shown as error bars in graph

similar exploration indicated 46.5% of diversified rhizospheric fungi to solubilize the inorganic source of phosphorus (Elias *et al.* 2016). The same study also reported their highest soluble-P being released in PVK broth medium after 10 days of fungal incubation (Elias *et al.* 2016). In continuity, our fungal strains reached their maximum solubilization potential in 4–5 days of their incubation on PVK agar plates (Fig. 2), exhibiting the acetic acid production (Fig. 3) as the potential mechanisms of P solubilization.

As a potential alternate to intensive chemical-fertilizers based farming that leads to severe environmental implications, the interests in sustainable agriculture are globally growing (Satterthwaite *et al.* 2010). Therefore, the microorganisms are very important for such sustainable alternatives to enhance the plant nutrition as well as to promote the biogeochemical cycling (Heijden *et al.* 2008). We, here, found a range of bacteria and fungi isolated from Lower Himalayan pine forest soil, capable of solubilizing tricalcium phosphate and also exhibiting the cellulase activity for potential carbon cycling. Such biochemical characterization of isolated microbial strains is very crucial to assess their potential for carrying a range of functions in natural ecosystems. Similar to our observations, a Gram-negative rod-shaped *Pseudomonas lurida* isolated from high-altitude Indian Himalayas was observed for simultaneously promotion of plant growth *via* P solubilization and for the production of Indole Acetic Acid (Mishra *et al.* 2009). Exploring such multi-functionality, another study was intended to develop commercial microbial agents exhibiting plant growth promoting abilities *e.g.*, P solubilization, efficient potassium release, degradation of crop straw cellulose to sustain organic matter in soil and suppression of plant pathogens (Wang *et al.* 2018).

In our work, the isolated strains, tested positive for biochemical characterization, showed the potential of plant growth enhancing functions along with P solubilization capability. Similarly, a very recent study has reported the fungal enzyme mechanisms to utilize cellulose and starch (Wang *et al.* 2020). Three out of our twenty-four tested bacteria were positive for starch hydrolysis, exhibiting the efficacy of bacteria for breakdown of starch by secreting the amylase enzyme with subsequent production of plant nutrients. Majority of these PSBs were catalase producers,

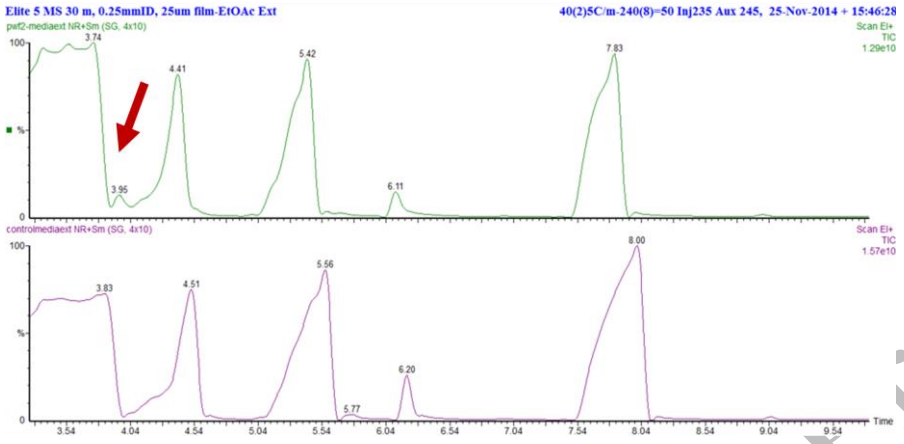

Fig. 3: High performance liquid chromatographic (HPLC) chromatogram of fungal (*e.g.*, PWF-3) exudates in the form of raw supernatant from growth medium (TCP). Arrow indicates the peak for acetic acid (AA) as verified by purified AA eluted through HPLC column under the same running conditions

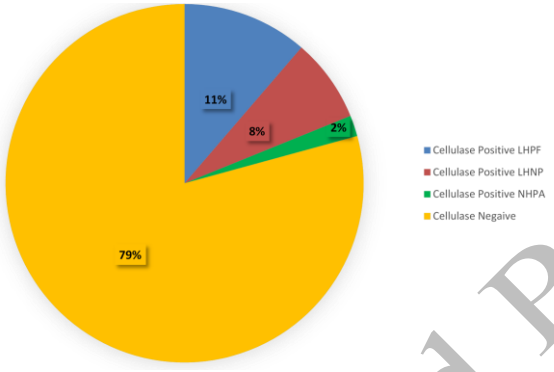

Fig. 4: Relative abundance of cellulase positive bacterial PSBs, in accordance to their source of isolation. LHPF, lower Himalayan pine forest; LHNP, lower Himalayan non-pine (agricultural) soil; NHPA, non-Himalayan Punjab agriculture soil

with potential protection of bacteria and host plants from toxic by-products of oxygen metabolism. In accordance, Sagervanshi *et al.* (2012) isolated P solubilizers from soils and observed one strain positive for starch hydrolysis, two for catalase and two for indole production. Similarly, Farooq and Bano (2013) observed 14 catalase positive strains while in another study Sahin *et al.* (2004) observed 3 PGPRs as catalase producers and starch hydrolysers along with P solubilization and N fixation for enhanced yield of sugar beet and barley (Sahin *et al.* 2004). During another functional characterization of 36 PGPRs, isolated from Junagadh India, 9 strains were found positive for catalase activity, 10 for indole production along with P solubilization, though all were negative for starch hydrolysis (Dhingani *et al.* 2013). Parani and Saha (2012) observed the indole acetic acid production by *Pseudomonas fluorescens* strains along with solubilization of tricalcium phosphate. On contrary, only one out of our 24 tricalcium phosphate solubilizing bacteria were found positive for indole production. Characterization of various phosphofungal strains presented a variety of microbial characteristics which contribute in improved soil and plant health (Wang *et al.* 2018). Though, the capability of isolated PSBs in our work showed a great range of functions carried out by the same strains but the mechanisms explaining the multi-functionality of these tested PSB should further be investigated. This may help to elucidate the detailed biochemical basis of these bacteria, warranting further studies for synergistic interactions including other bacteria with similar or different metabolic activities.

For multi-functionality of soil microorganisms in a sustainable ecosystem, the bioconversions of the biomass and particularly the cellullase enzyme activities are very important (Roth *et al.* 2020). In literature, the cellulase activity is largely connected with fungi but a variety of bacterial cellulases have also been reported, since last some years, from various environments (Behera *et al.* 2017; Islam and Roy 2018; Roth *et al.* 2020). Interestingly, some PGPRs have earlier been reported to secrete cellulase enzyme, observed by quantitative assay (Irfan *et al.* 2013). Therefore, the isolation and identification of cellulolytic microorganisms that may also enhance plant growth has great agricultural significance (Wang *et al.* 2018). In our work, the quantitative and semi-quantitative assays revealed that the 22% PSBs were found positive for cellulase enzyme activity. Cellulose is major constituent of plant biomass having insoluble nature and some microorganisms (like reported in this work) have potential to convert it into various valuable products (Lynd *et al.* 2002; Behera *et al.* 2017). We have analyzed the isolated bacterial strains for assessment (*via* semi-quantitative and quantitative assays) of their potential cellulose degradation through secretion of cellulase enzyme. Similar to our findings, Parani and Saha

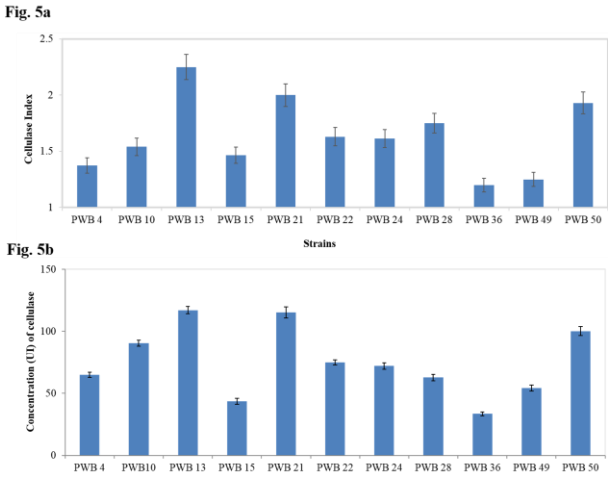

Fig. 5: Relative abundance of cellulase enzyme production exhibited by cellulase index (**a**) and cellulase enzyme activity (**b**). Data is presented as averages (n=3) and standard deviations are shown as error bars in graph

(2012) observed hallo zone indices ranging from 1.23 to 1.80 on CMC agar for their studied PSB. Another recent study has also provided an extraordinary insight of lignocellulose degrading microbial strains for cellulase like enzymes with great metabolic potential and expression (Jiménez *et al.* 2016). The PSBs isolated in our work from the lower Himalayan forest soils showed a significantly higher cellulase enzyme activities (Fig. 5a, b) as compared to other microbial strains. In brief, the lower Himalayan microbial strains tested in this work, present multi-functionality (Table 1) for any sustainable ecosystem. While, the potential of these tested strains should further be studied in detail with field applications for their plant protection and soil improvement capabilities.

Conclusion and Perspectives

A subset of our P solubilizing microbial strains isolated from lower Himalayan forest soils, have the metabolic potential to excrete the cellulase enzyme and thus to contribute in biomass degradation along with various other ecological functions. Phosphate solubilizing microorganisms (PSM) have, thus, the potential of making these phosphates available to plants. The results are promising to design the potentially active plant growth promoting microbial strains (solo and/or in consortia) which would also be beneficial for soil and plant health. More attention should also be paid to new combinations (microbial consortia) of P-solubilizing and N_2-fixing bacteria to improve the biofertilizer-efficiency along with biomass degradation. Moreover, the starch and cellulose degrading microorganisms can degrade these complex carbohydrates and subsequently increase the soil fertility, particularly if degraders are P-solubilizers as well. Ultimately, the use of these microorganisms as potential biofertilizers may represent an attractive environment friendly alternative thus reducing the needs for chemical fertilizers and decreasing the adverse effects on environment.

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