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Isolation, phenotypic and molecular characterization of *Burkholderia* sp. (strain, PCS1) from maize fields exhibiting starch hydrolysis ability

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ABSTRACT

The starch hydrolysing bacteria have important role in plant nutrient uptake. The aim of the present study is to isolate and characterize bacteria that are able to degrade starch from the rhizospheres of maize plants. Isolation and characterization were performed in the laboratory. The bacterial isolate (PCS1) was non-pathogenic and found to be positive for catalase, lipid hydrolysis and nitrogen fixation. The PCS1 also fermented sucrose, xylose, dextrose, galactose and mannose. The PCS1 strain showed halo-zone on starch medium and starch degrading index was found to be 2.14. The 16S rRNA gene sequence and phylogenetic tree analysis revealed that the PCS1 belonged to one of the strains of *Burkholderia cepacia* complex.

KEY WORDS: AMYLASE; PHYLOGENETIC ANALYSIS; RHIZOSPHERE; STARCH DEGRADING INDEX

INTRODUCTION

A wide range of ecological niches have been occupied by the members of the genus *Burkholderia* sp. ranging from soils to the respiratory tract of humans (van Elsas *et al*., 2002; Coenye and Vandamme, 2003; Salles *et al*., 2002, 2004; Janssen, 2006). Beside free-living rhizospheric association with plants, some of the *Burkholderia* strains and species are also epiphytic and endophytic, including obligate endosymbionts and phytopathogen (Coenye and Vandamme, 2003; Janssen, 2006). The research studies on maize rhizosphere have shown that the *Burkholderia* are the predominant bacterial groups in maize of Italian agricultural fields (Hebbar *et al*., 1992; Ramette *et al*., 2005). In addition, Viallard *et al*. (1998) have shown that the species *B. graminis* and *B. cepacia* genomovar III are

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abundant in maize fields of France. The most abundant isolates belonged to the genus *Burkholderia*. Silva *et al.* (2016) studied the soils of the Amazon basin and found that *Burkholderia* spp. associated with the maize plants carry *nifH* genes and able to synthesize indole-3-acetic acid (IAA) and solubilize calcium phosphate. Recently, Correa-Galeote (2018) have shown that the cultivation history is the main driver of endophytic colonization of *Burkholderia* spp. in maize plants

Bevivino *et al.* (1998) evaluated the metabolic and molecular profiles for some traits associated with biocontrol and plant growth promoting (PGP) activity in *Burkholderia cepacia* population. They showed that the *B. cepacia* strains displayed a wide antibiosis against the phytopathogenic fungi. Further studies on maize rhizosphere have shown the association of other species such as *Burkholderia unamae* (Caballero-Mellado *et al*., 2004) and *B. silvatlantica* (Perin *et al*., 2006a). The *Burkholderia vietnamiensis* was first described in the rice rhizosphere in Vietnam (Tran Van *et al*., 1994; Gillis *et al*., 1995) along with other *Burkholderia* species from the rhizosphere of maize and coffee in Mexico (Estrada-de Los Santos *et al*., 2001).

Several novel diazotrophic bacterial species belonging to the genus *Burkholderia* that may form both epiphytic and endophytic populations with the maize roots are known to be associated with N_2 fixation such as *B. vietnamiensis* (Estrada-de Los Santos *et al*., 2001; Estrada *et al*., 2002), *Burkholderia tropica* (Reis *et al*., 2004) and *Burkholderia cenocepacia* (Balandreau *et al*., 2001). Kost *et al.* (2014) demonstrated oxalotrophy as one of the vital traits of the genus *Burkholderia phytofirmans*. So far, the studies were focused on the diversity of *Burkholderia* spp. in maize soils, N_2 fixing ability and plant growth promotion traits. The starch solubilising ability of *Burkholderia* spp. from maize roots is still not reported from any part of India. The objective of the present research work is to identify and characterization of *Burkholderia* spp. associated with maize rhizosphere capable of starch solubilization.

MATERIALS AND METHODS

The research work was carried out in the Crop Research and Seed Multiplication Farm (CRSMF) of the University of Burdwan, West Bengal, located in the eastern part of India (latitude, 87°50'37.35' E and longitude, 23°15'7.29'N and average altitude of 30m above mean sea level) using one variety of *Zea mays* L. (var. *RE-55, Royal England*).The soil samples were collected at the depth of 10 cm using soil auger from the rhizosphere of maize plants in sterile polythene bags and were taken to the Microbiology and Parasitology Laboratory of the University of Burdwan for bacterial analysis. The soil was air-dried and diluted up to 10^{-3} using sterile distilled water. From this dilution 100µl portion was mixed with sterile nutrient agar medium (NA) (peptone: beef extract: NaCl: agar at 5:3:3:1 g l^{-1}) and was distributed in 5 sterilized plates. The plates were incubated at 30 ± 0.1 °C in the BOD incubator for 24 hours to obtain isolated colonies. The colonies were observed on the incubated plates and the most abundant colonies (S1) were purified on NA plates. The bacterial isolate was maintained on the NA slants at 4 ± 0.1° C for subsequent characterization and identification. The morphological characters such as shape, size, colour, margin and opacity of the isolated colonies were determined. The bacteria was streaked on different media including starch (Starch Agar, Himedia, India) and free-living nitrogen-fixing media (Mannitol, 10 g l^{-1}; K_2HPO_4, 0.5 g l^{-1}; $MgSO_4$. $7H_2O$, 0.2 g l^{-1}; NaCl, 0.2 g l^{-1}; $MnSO_4.4H_2O$, trace; $FeCl_3$, trace; Agar powder 10 g l^{-1}) and plates were kept at 30 ± 0.1 °C in the BOD incubator. After 24 hours, standard microbiological methods were followed for morphological and biochemical characterization of the isolate (Holt *et al*., 1994; Sneath and Holt, 2001; Forbes *et al*., 2007).

The Gram staining was performed using Gram staining kit (Himedia, India). Motility test was done by stabbing the bacteria into SIM agar (Czaban *et al*., 2007). The biochemical properties such as catalase, citrate utilization, nitrate reduction, indole production, Methyl-Red (MR), Voges-Proskauer (VP), urease, oxidase, and carbohydrate metabolism (acid-gas production) tests were performed. The pathogenicity test was done on Blood Agar and by DNase test (Benson *et al*., 2012). Carbohydrate fermentation test was performed using the sugar discs of maltose, adonitol, fructose, inositol, mannose sucrose, raffinose, dextrose, cellobiose, galactose, rhamnose, xylose, melibiose, lactose, salicin, ducitol, sorbitol, mannitol, trehalose and arabinose (Azmi *et al*., 2014).

In addition, the qualitative determination of enzymes such as starch hydrolysis, lipid hydrolysis, protein hydrolysis (gelatine) tests were also performed. The starch plates were flooded with Grams Iodine after 24 hours of incubation. The ability of the bacteria to degrade starch was described by Starch Degrading Index (SDI), which is the ratio of the total diameter of clear zone and colony diameter (Nusrat and Rahman, 2007). The sensitivity analysis of the isolate to the recommended doses of antibiotics was done following Brown *et al.* (2004). The strain was tested for tetracycline (tcn), amoxicillin (amx), gentamycin (gen), ciprofloxacin (cip), azithromycin (azi), nalidixic acid (nal), ofloxacin (ofx), neomycin (nm), rifampicin (rif), bacitracin (bac), chloramphenicol (chl), kanamycin (kan), erythromycin (ery), ampicillin (amp), penicillin (pcn) and doxycycline (dox). The zone interpretation was done following CLSI (2011).

Genomic DNA isolation kit was used to isolate the genomic DNA of the bacterial strain from the pure cul-

ture pellet (DNA-Xpress TM Kit, Himedia-MB501). The rDNA fragment was amplified by PCR and the product was sequenced by Genetic Analyzer (ABI 3130, Genetic Analyzer) bidirectionally using the forward and reverse primer. The bacterial 16S rDNA fragment was amplified with primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACG GCT ACC TTG TTA CGA CTT-3'). The following PCR cycling conditions were used in the study: 94°C for 300 s; followed by 36 cycles of 94°C for 30 s, 58°C for 60 s, and 72°C for 60 s; and then a final elongation step at 72°C for 7 minutes.

RESULTS AND DISCUSSION

The colony of the bacterial isolate (PCS1) was white, round with an average diameter of 6mm, opaque and flat (Fig. 1). The Gram staining of the bacteria showed rod-shaped and Gram-negative properties of this strain. The motility test of the strain indicated non-motile character. The bacterial isolate (PCS1) showed a positive result for catalase and negative for MR, VP, citrate reductase, nitrate reductase and indole production (Table 1). The Triple Sugar Iron (TSI) showed yellow slant and yellow butt, and also gas production. The pathogenicity test in Blood Agar and DNase agar showed no halo-zone formation. The carbohydrate fermentation results are shown in the Table 2. The strain (PCS1) was able to ferment sucrose, xylose, dextrose, galactose and mannose. The bacteria responded positively to the lipid hydrolysis and negatively to the protein (gelatine) hydrolysis test. The bacterial strain (PCS1) was able to grow on nitrogen-free medium.The starch hydrolysis test showed a positive result for the bacteria. The SDI calculated from the halo zone and colony ratio, which was found to be 2.14 in the present study.The bacterial strain was found to be sensitive to the recommended doses of the antibiotics used in the study except ampicillin and penicillin, which showed with no zone formation. The results of the zone of inhibition exhibited by the strain is shown in Fig. 2. Based on the morphological, biochemical and phylogenetic tree analysis, the PCS1 isolate was identified as one of the strains of *Burkholderia cepacia* complex. The restriction map of the 16S rRNA gene sequence of PCS1is shown in Fig. 3. The molecu-

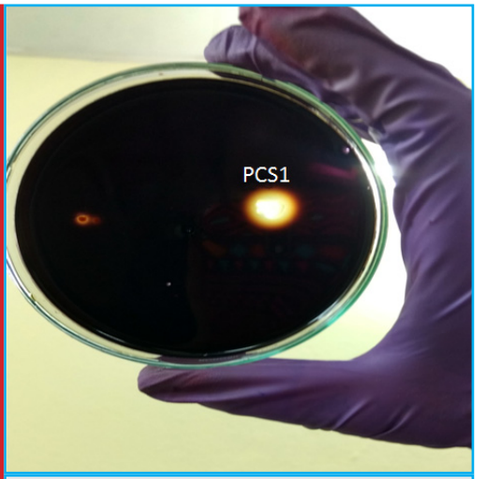

FIGURE 1. Halo-zone surrounding the PCS1 colony on starch agar plate.

Table 1. Biochemical test of PCS1 strain

Test	Results
Catalase	Positive
Indole	Negative
Citrate	Negative
MR	Negative
VP	Negative
TSI	Positive
Oxidase	Negative
Nitrate	Negative
Lipid hydrolysis	Positive
Protein (gelatine) hydrolysis	Negative
Starch hydrolysis	Positive

Table 2. Carbohydrate fermentation ability of PCS1

Carbohydrate	Response
Maltose	Negative
Adonitol	Negative
Fructose	Negative
Inositol	Negative
Mannose	Positive
Sucrose	Positive
Raffinose	Negative
Dextrose	Positive
Cellobiose	Negative
Galactose	Positive
Rhamnose	Negative
Xylose	Positive
Melibiose	Negative
Lactose	Negative
Salicin	Negative
Ducitol	Negative
Sorbitol	Negative
Mannitol	Negative
Trehalose	Negative
Arabinose	Negative

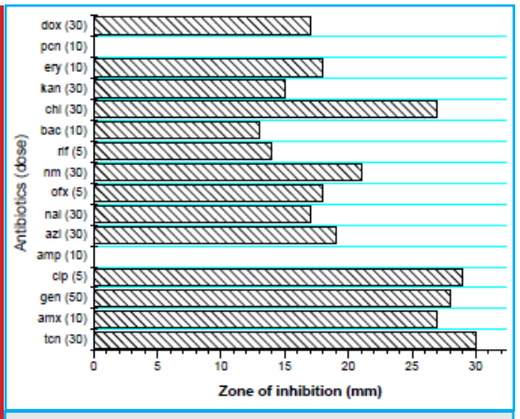

FIGURE 2. The sensitivity of PCS1 strain to the antibiotics.The size of zone of inhibition (mm) is represented in the X-axis. No zone formation is observed against penicillin (pcn) and ampicillin (amp). The antibiotics with recommended doses, in bracket are represented in Y-axis.

lar percentage of GC and AT content were 59.20% and 40.80 % respectively. The phylogenetic tree showed that the PCS1 was branched with *Burkholderia cepacia* ATCC 55792 (AY741359) (Fig. 4).

The bacterial communities are harboured by plant roots and shoots. In the rhizosphere, the root exudates are known to be the source of carbon and energy for the bacterial population. The secretion of root exudates varies with plant species and growth phases. These exudates are of highly diverse chemical nature ranging from small carboxylates to complex phenolic compounds. These exudates are utilized as substrates by the bacterial groups colonized in the rhizosphere. Brito *et al.* (2018) isolated *Burkholderia ambifaria* from the rhizoplane of the maize plants and showed that colonization of this bacterial isolate is responsible for an increase in shoot growth, root and root hair. The amylase is secreted extra-cellularly by starch degrading rhizobacteria and facilitate other different organic matters for plants to easily absorb and manufacture their food (Cordeiro *et al.*, 2003).

Smits *et al.* (1996) advocated that the rhizobacteria often show the high value of the starch degrading index, which belongs to Gram-positive and spore-forming bacterial groups; and the enzyme production from the microorganism is directly correlated to the time period of incubation. Another finding on starch degradability by amylase was shown by Wang *et al.* (2016), the authors observed that the ability to hydrolyse starch by amylase is independent of the diameter of the colony formed by Gram-negative, non-spore forming rhizobacteria. The calculated SDI value of PCS1 is quite high in comparison to some other gram-negative bacteria. The strains of *Burkholderia* spp. are capable of producing amylase enzymes to degrade starch into a soluble form and promote the maize plant growth. Dida (2018) studied the SDI of *Bacillus* spp. from the rhizosphere of various plants in Ethiopia and reported that the SDI falls between 1.23 and 2.15. The present findings in relation to SDI is comparable with *Bacillus* spp..

Draghi *et al.* (2014) reported two strains of *Burkholderia cordobensis* namely LMG 27620T and LMG 27621 from Argentina. They observed that the strains were well capable of starch hydrolysis. The DNA G+C content of the strain PCS1 was 59.20 mol %, which is within the range reported for members of genus *Burkholderia* (59-69.9 mol %; Gillis *et al.*, 1995). The present results are in agreement with the research works of Draghi *et al.* (2014) and Lim *et al.* (2012). The results of carbon compound utilization by the PCS1 strain of *Burkholderia cepacia* are corroborated with the findings of Yabuuchi *et al.* (1992).

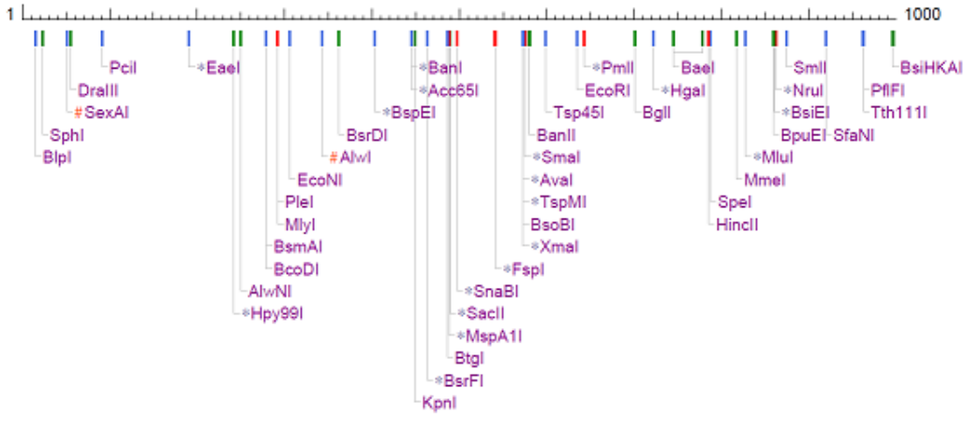

FIGURE 3. The figure showing the restriction map of the 16S rRNA gene sequence of the isolate PCS1.

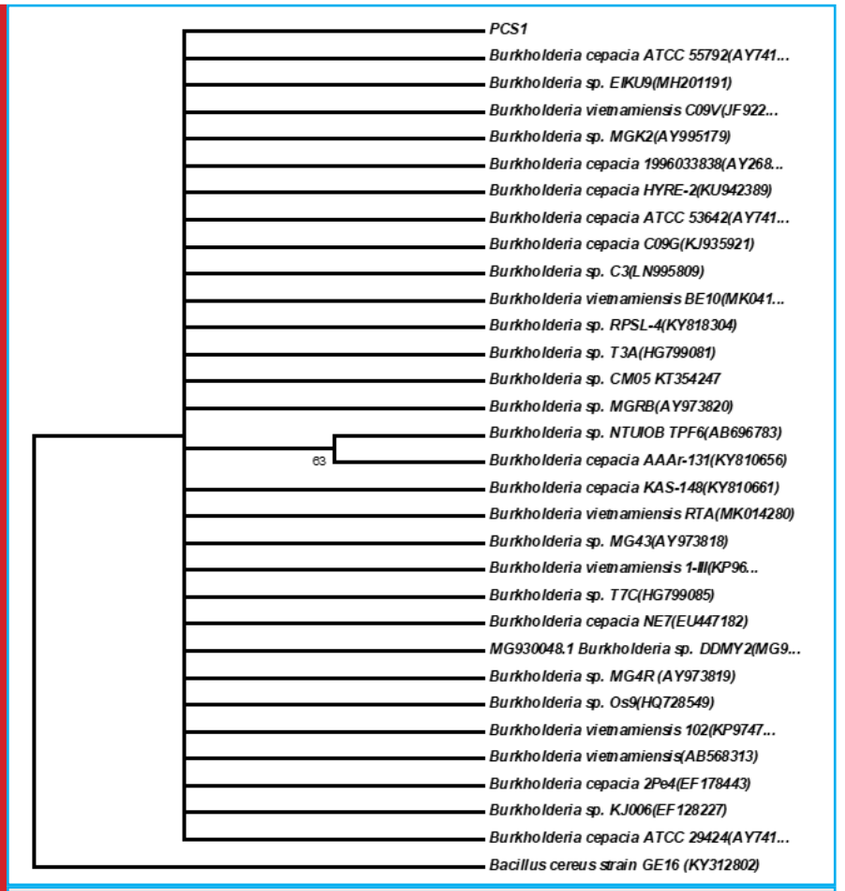

FIGURE 4. The neighbourhood joining tree of the PCS1 strain based on 16S rRNA gene sequence

Dalmastri *et al.* (1999) studied the effect of soil type, maize cultivar and root localization on the genetic diversity of *Burkholderia cepacia* populations and found that the degree of genetic diversity was regulated strictly by the soil types. Salles *et al.* (2004) added that the distribution of *Burkholderia* spp. in soil is attributed to the land use history of that area beside the maize cultivar and soil type. It is probably due to the soil type and maize cultivar, which were responsible for the deviation of the characteristics of the novel strain, PCS1 from the other strains of *Burkholderia cepacia*, reported from other parts of the world.

CONCLUSION

In addition to nitrogen fixation in soils, *Burkholderia* spp. also exhibit starch degrading property that is beneficial to maize plants. The DNA G+C content and the carbohydrate fermentation abilities of the novel strain, PCS1 supports the properties of the genus *Burkholderia*. The phylogenetic tree showed the clustering of PCS1 with *Burkholderia cepacia*. It can be said that the variation of the strains from the other strains of *Burkholderia* were due to the type of soil and maize cultivar used in the study.

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