



## **Biodegradation of maneb, a Fungicide by a $\gamma$ -proteobacterium *Pseudomonas psychrotolerans* Strain SDS18**

**Bhawna Vyas\* and Shanmugam Mayilraj**

Microbial Type Culture Collection & Gene Bank (MTCC), CSIR- Institute of Microbial Technology, Sector 39-A, Chandigarh, 160 036, India

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\*Corresponding author: [vyasbhawna@gmail.com](mailto:vyasbhawna@gmail.com)

### **Abstract**

The present study reports the isolation and identification of a  $\gamma$ -proteobacterium which is capable of degrading maneb and its photolytic product ethylene thiourea. The strain SDS18 was isolated from the surface of the most common weed *Parthenium hysterophorus* growing in agricultural field. Based on molecular systematics the strain SDS18 was identified as *Pseudomonas psychrotolerans*. Our study first time revealed that a single bacterial strain is capable of metabolising the toxic fungicide maneb and its carcinogenic photolytic product ethylenethiourea. We found that the strain SDS18 can tolerate upto 150ppm of maneb and 200 ppm of ethylene thiourea and ethylene urea as sole carbon sources. The optimum conditions for degradation were in the presence of ammonium sulphate as nitrogen source at 30°C at pH 7.0. Interestingly, the strain SDS18 exhibited activities like phosphate solubilisation, production and assimilation of ammonia, ACC deaminase activity, production of indole acetic acid and siderophores which are plant growth promoting activities and antifungal activities for *Alternaria citri* and *Cladosporium cladosporioides* indicating it could be beneficial for plant growth and maturation. The isolated strain can be used for bioremediation of maneb and its photolytic products.

**Keywords:** Degradation, maneb, ethylenethiourea, plant growth promoting

**1. Introduction:** Fungicides are important agrochemicals of present-day agriculture which are employed to kill, destroy, repel or mitigate fungi and fungal spores [1,2]. Dithiocarbamates (DTCs) based fungicides are widely used organosulfur molecules. DTCs are represented by a common structure  $(R_1R_2N)-(C=S) - SX$ , where R can be replaced with an alkyl, alkylene, aryl or related other groups and X is generally a metal ion [3,4]. World Health Organisation (WHO) estimates that approximately 25,000 to 30,000 metric tonnes of DTCs are used annually [5]. Ethylene bisdithiocarbamates (EBDCs) are extensively used DTCs based fungicides because of their wide range of antifungal activity [4,6]. EBDCs are complexes of dithiocarbamates in which the R group of two DTC molecules form an ethylene bridge [7]. Most commonly used EBDCs are mancozeb, maneb, metiram and nabam. They are effective in preventing fungal diseases of crop and ornamental plants [8]. Manganese ethylene bisdithiocarbamate (MANEB) is Mn containing EBDCs[9]. Approximately 2.5 million pounds of maneb are annually used on potatoes, almonds, walnuts, lettuce and peppers to inhibit fungal diseases [10]. Maneb is most effective against different species of *Fusarium* and *Alternaria*, the causal organism of early and late blight of potato and tomato [11]. Extensive agricultural application of maneb is a cause of concern as it is known to induce the production of nitric oxide, lipid peroxidation and Parkinson like disorder in mice [12]. There are reports for the increase in the incidence of Parkinson disease by 75 % when the agricultural workers of California were exposed to both maneb and paraquat [13].

Toxicity of maneb is primarily due to inhibition of mitochondrial complex III [14]; as a result of which Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are formed which lead to mitochondrial DNA damage and retarded ATP synthesis [15]. Among Mn containing organic and inorganic compounds, maneb exhibits greater cytotoxicity (about eight times more) due to the presence of manganese ions and dithiocarbamate moiety [16]. Maneb was ranked as the most hazardous pesticide for overall persistent health effects in research conducted at Yuma County, Arizona [17]. Under environmental conditions maneb undergoes photolytic degradation into Ethylene thiourea (ETU) [18]. ETU is known to cause thyroid disorder by impairing thyroid hormone synthesis and liver damage [19].

The bacteria which facilitate plant growth directly or indirectly are termed as “Plant growth promoting bacteria (PGPB)”. The use of PGPB in the agricultural field can lead to an increase in the productivity in an environment friendly manner. It is envisioned that in the near future PGPB can replace the chemical pesticides and fertilizers and can also be used for environmental cleanup practice [20]. Therefore, if a bacterial strain/consortium exhibits bioremediation and PGP potential, it could be a potential boon in agriculture. The present study explored the feasibility of isolating maneb and ETU degrading bacteria for developing biological means of eliminating their environmental toxicity. To the best of our knowledge there are no reports available for utilisation, biodegradation of maneb and ethylene thiourea; PGPR and antifungal activities by a single bacterial strain.

## 2. Materials and Methods

### 2.1. Chemicals and media

The chemicals, maneb and potential degradation intermediates were purchased from Sigma- Aldrich, Co. USA and other chemicals, microbiological growth media were procured from Hi Media, India. Mineral salt media(MSM)(pH=7±0.5) used in the present study was composed of Na<sub>2</sub>HPO<sub>4</sub>(4g/l); KH<sub>2</sub>PO<sub>4</sub> (2g/l); (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1g/l); MgSO<sub>4</sub> (0.8g/l); trace element solution consisting of Al(OH)<sub>3</sub>(0.1g/l); SnCl<sub>2</sub> (0.05g/l); KI (0.05g/l); LiCl (0.05g/l); MnSO<sub>4</sub>.4H<sub>2</sub>O (0.08g/l); H<sub>3</sub>BO<sub>3</sub>(0.05g/l); ZnSO<sub>4</sub>.7H<sub>2</sub>O(0.1g/l); CoCl<sub>2</sub>.6H<sub>2</sub>O(0.1g/l); NiSO<sub>4</sub>.6H<sub>2</sub>O(0.1g/l); BaCl<sub>2</sub> (0.05g/l); (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O (0.05g/l); FeSO<sub>4</sub> (0.001g/l). The media was autoclaved at 121°C temperature and 15lbs pressure. After autoclave, the media was supplemented with 50-200 parts per million (ppm) concentrations of maneb, ethylene thiourea and ethylene urea as mentioned in the text.

### 2.2. Sampling Site

Aerial parts of *Parthenium hysterophorus* plants were collected from agricultural fields located on outskirts of Chandigarh, India. The samples were collected at the end of month of June and were immediately transported to laboratory for analysis.

### 2.3. Isolation and selection of maneb and ethylenethiourea utilising bacteria

The plant parts were dipped in 5 ml of sterile normal saline for overnight and the saline was serially diluted and spread plated on mineral salt media (MSM) containing 50 ppm of maneb, ethylene thiourea and

ethylene urea respectively as sole carbon sources. The plates were kept at 30°C for further observation for the growth of bacterial colonies. The isolated colonies on these plates were further screened for utilisation of maneb as a Carbon source in MSM broth.

### 2.4. Identification of the isolated bacterial strain

The selected bacterial isolate strain SDS18, positive for utilisation of maneb as a Carbon source was identified by polyphasic taxonomy [21]. DNA extraction, amplification and sequencing were performed by methods as described by Mayilraj *et al* [22]. Using EZ Biocloud 16S database, 16SrRNA sequences of the species closely related to SDS18 were obtained and a phylogenetic tree was prepared using Mega 7.1 software [23].

### 2.5. Growth profile of strain SDS18 in presence of maneb and its intermediates

Growth profile of strain SDS 18 was studied in MSM containing maneb, ethylene thiourea (ETU) and ethylene urea (EU) as the sole sources of carbon respectively. The concentration of these compounds ranged from 50 ppm to 220 ppm. The strain was inoculated in ¼ diluted nutrient broth. After 24 hours, the cell suspension was pellet down and the cells were suspended in autoclaved double distilled water. For growth and degradation studies, 2% of the strain's inoculum was used for inoculating the MSM media and the flasks were maintained at 30°C at 180 rpm. The growth of the strain was monitored by measuring optical density of the culture broth in Spectrophotometer (UV 1800, Shimadzu) at a wavelength of 600 nm at different time intervals. The flasks without inoculums served as blank

or control.

## 2.6. Plant growth promoting activities of strain SDS18

The strain SDS18 was examined for several activities contributing to plant growth. The strain was checked for activities including catalase, ammonia, phosphate solubilisation, 4-amino-1-cyclopropane carboxylate (ACC) deaminase, siderophore, and for production of hormones like auxin. The methodology used for determination of activities was performed as methods described by Singh *et al* (24). Antifungal activity of SDS18 was checked against *Alternaria citri* (MTCC 4875), *Cladosporium cladosporioides* (ATCC 16022), *Fusarium oxysporum* (MTCC 7229) and *Alternaria alternata* (MTCC 2724). The bacterial strains *Pseudomonas aeruginosa* (MTCC 7903 and MTCC 4682) reported for antifungal activities were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. These strains and SDS18 were inoculated in Tryptic Soy Broth (TSB) for 48 hours.

1mM 0.5M EDTA was added in the culture broth and the cells were lysed by sonication in ultrasonics (Sonics Vibracell, VCX750) for 30 minutes at a pulse rate of 10 seconds on and 10 seconds off. The cell suspension was centrifuged at 9000 rpm for 5 minutes. The supernatant was made to concentrate in speed vacuum concentrator (Eppendorf AG,22331) for 4 hours till 2 ml of the solution remained. 100µl of fungal spore suspension ( $10^5$ - $10^6$ ) was spread plated on PDA plates (20ml). Wells were cut by use of micro tips. Approximately 150µl of the concentrated supernatant was added into each well. The plates were kept at 28- 30°C to observe the presence of zone of

inhibition around the wells.

## 2.7. Extraction of putative intermediates of maneb degradation

For degradation studies, nine amber colored flasks with 100 ml MSM (pH7±0.5) with 100 ppm maneb as Carbon source were prepared. The strain SDS18 was inoculated at 2% inoculum density as described above in all the flasks. The flasks were kept in rotary shaker (Innova44, Brunswick) at 180 rpm at 30°C. At a regular interval of 24 hours, 3 flasks were removed from shaker and the culture broth was combined and centrifuged at 10,000 rpm at 4°C (6K16, Sigma). The supernatant was extracted thrice with 100ml ethyl acetate in a separatory funnel. The layer of organic solvent was collected each time. The pH of the supernatant was made acidic (pH 2.0) and alkaline (pH10.0) by the addition of concentrated 10N HCl and 10N NaOH respectively. The supernatants were extracted three times with ethyl acetate. The organic layer was collected after every extraction. The collected ethyl acetate layers of different pH were mixed and concentrated in the rotary shaker (Nutronics, 1263) at 35°C. The concentrated residue was suspended in 1ml ethyl acetate and kept at 4°C for analysis. Heat killed cells and uninoculated media were taken as control.

## 2.8. Analytical techniques

### 2.8.1. Fourier Transform Infrared Spectroscopy (FTIR)

The intermediate extracted in the ethyl acetate was mixed with 100 mg of dried potassium bromide (KBr) powder and a thin pellet was formed. The pellet was subjected to FTIR analysis in the spectrometer Bruker Optik GmbH, Model

Vertex 70. The spectra were recorded in the transmittance mode within the range 400-4000  $\text{cm}^{-1}$ . The spectrum was processed with the Opus 6.5 software. The resolution of the measurement was 4  $\text{cm}^{-1}$ . The identity of the extracted intermediate was confirmed by comparison with the spectrum of the procured standards.

### **2.8.2. Electrospray Ionisation Mass Spectroscopy (ESI-MS)**

For determining the mass of the extracted intermediates of maneb degradation, the extracted intermediates were subjected to mass spectrometer (Waters, QTOF Micromass) in both positive and negative modes. The spectra were recorded in both positive and negative mode in the mass to charge (m/z) range of 50-500.

### **2.8.3. $^1\text{H}$ Nuclear Magnetic Resonance (NMR)**

The extracted intermediate dissolved in ethyl acetate was evaporated to dryness in the concentrator (Eppendorf AG, 22331). The concentrated residue was dissolved in 600  $\mu\text{l}$  deuterated chloroform ( $\text{CDCl}_3$ ) and subjected to Jeol ECX 300MHz NMR Spectrometer at 25°C. The results were processed with Delta 4.3.6 software.

### **2.8.4. High Performance Liquid Chromatography (HPLC)**

In order to confirm the structure of extracted intermediates/metabolites, HPLC analysis was performed with an Ultimate HPLC System (Thermo Fisher, USA) connected with a UV detector (Thermo Fisher, USA). The column used was Thermo Acclaim C18 (5 $\mu$ , 4.6x250 mm) while mobile phase was acetonitrile and water at a ratio of 10:90 (v/v) at a flow rate of 1ml/minute. The injection of samples at a volume of 20  $\mu\text{l}$  were done manually (Rheodyne) and detected at 232nm.

Identification of compounds was revealed by comparing the retention times with the external calibration curves prepared with authentic standard solutions between 62.5 ppb to 1 ppm.

### **2.8.5. Genome mining of strain SDS18**

Genomic DNA of the strain was isolated by fungal/ bacterial DNA isolation kit (Zymo research, USA) by using manufacturers' instructions. The isolated DNA of strain SDS18 was dispatched for sequencing at Genotypic Pvt Ltd (Bengaluru, India,). Following quality check, library preparation and further processes were carried out as methods described by [25]. Sequencing was done using the Illumina Miseq paired-end technology (2x300) and the obtained raw data was used for genome assembly with MaSuRCA [26] genome assembler version 2.3.2 The genome of the strain formed a total of 7,369,198 reads. The data was processed to trim and eliminate low quality sequences using CLC Bio Workbench v7.0.5 (CLC Bio, Aarhus, Denmark). A total of 7,368,052 high quality, vector filtered reads (~568 times coverage) were used for assembly (at word size of 45 and bubble size of 98). The sequence was deposited to NCBI. The final draft genome was used for genome annotation employing RAST server and RNAmmer 1.2server [27].

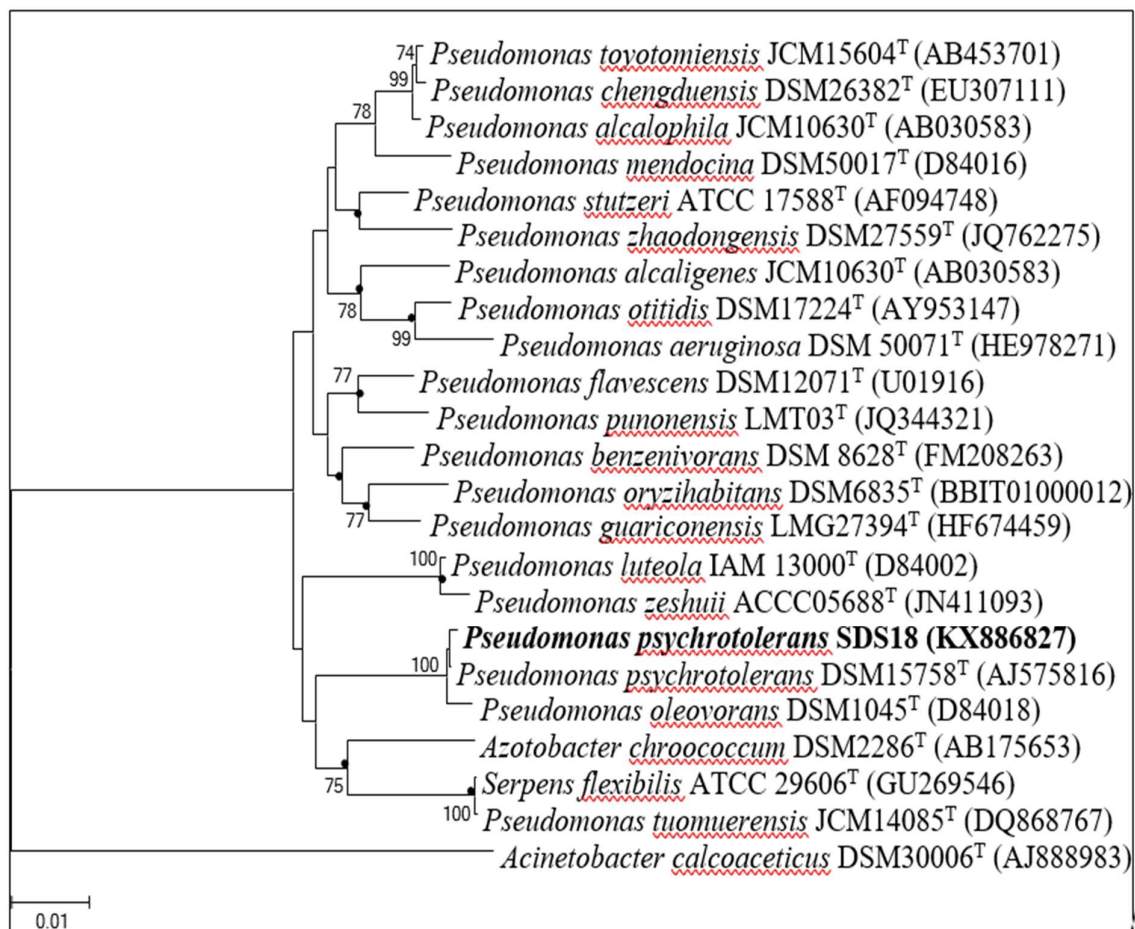
## **3. Results and Discussion**

### **3.1. Isolation of maneb and ethylene thiourea utilizing bacterial isolates**

The MSM plates were observed after 5 days of incubation. Based on morphology and pigmentation 10 different colonies were observed. The results of phylogenetic analysis of the isolates suggested that these isolates fall into two major groups

*Firmicutes* and *Proteobacteria* (Figure S1). The isolated strains were further screened for growth and degradation of

### 3.2. Identification of the isolated bacterial strain



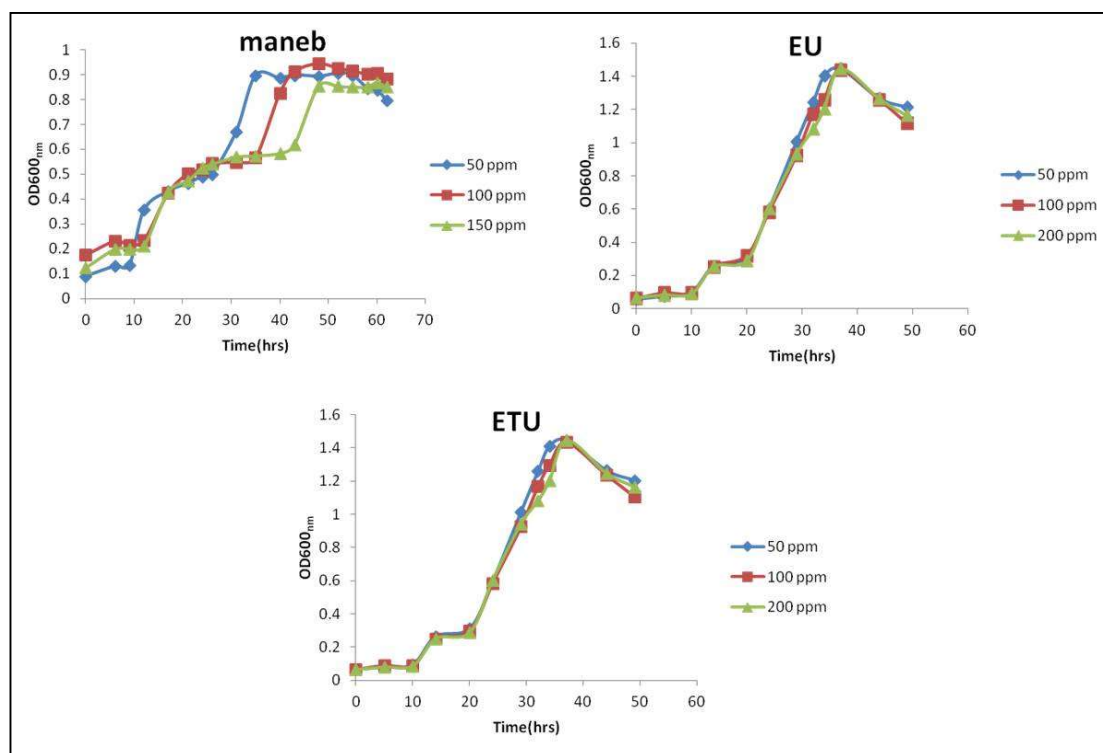
**Figure 1.** Phylogenetic neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain SDS18 and other closely related members of the genus *Pseudomonas*. *Acinetobacter calcoaceticus* DSM30006T (AJ888983) was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are given at nodes. Filled circles corresponding to the nodes were recovered in the tree generated with maximum parsimony and maximum likelihood algorithms. Bar, 0.01% sequence variation. Gen Bank accession numbers are given in parentheses

maneb. For screening, MSM broth supplemented with the maneb as sole Carbon source was used. The strain(s) showing growth in the presence of maneb were screened for utilisation of ethylene thiourea and ethylene urea as a Carbon source. One strain designated as SDS18 was able to utilise maneb, ethylene thiourea and ethylene urea respectively as Carbon sources. The strain SDS18 was used for further studies.

Microscopic studies suggested the bacteria to be Gram-stain negative, non-spore forming, motile and rod shaped. Biochemical and physiological tests indicated that the bacterium belongs to the genus *Pseudomonas*. Further, 16S rRNA gene sequence analysis (~1500bp) indicated the closest hit to be *Pseudomonas psychrotolerans* DSM 15758<sup>T</sup> with 99.93% similarity followed by *Pseudomonas*

*oryzihabitans* NBRC 102199<sup>T</sup>,  
*Pseudomonas oleovorans* IAM 1508<sup>T</sup>,

various biological activities including the  
 utilisation of several agrochemicals [28,



**Figure 2.** Growth curve of strain SDS18 on maneb, ETU and EU. The values represent an average value of three independent experiments

*Pseudomonas stutzeri* ATCC 17588<sup>T</sup>,  
*Pseudomonas toyotomiensis* HT-3<sup>T</sup> and  
*Pseudomonas chengduensis* MBR<sup>T</sup>. The  
 16S rRNA gene sequence was submitted to  
 the GenBank database under accession  
 number KX886827. The draft genome  
 sequence of strain SDS18 is deposited to  
 the NCBI database with accession number  
 MTLN 00000000.

The combined phylogenetic tree  
 constructed by Neighbor joining (NJ),  
 Maximum parsimony (MP) and Maximum  
 likelihood (ML) algorithms (Figure 1)  
 shows that the strain SDS18 forms a  
 distinct clade with *Pseudomonas*  
*psychrotolerans* (DSM 15758<sup>T</sup>) and  
*Pseudomonas oleovorans* (DSM 1045<sup>T</sup>).  
 The genus *Pseudomonas* is known for

29]. However, there are no reports for  
 degradation of ethylene  
 bisdithiocarbamates by the genus  
*Pseudomonas*.

### 3.3. Growth profile of strain SDS18 on maneb and ethylenethiourea

The growth of a bacterial strain is measured  
 by the increase in the turbidity and the  
 optical density of the inoculated liquid  
 broth [30]. Growth was absent in  
 uninoculated controls. The increase in the  
 optical density of MSM indicated the  
 growth of strain SDS18 in MSM with the  
 utilisation of maneb, EU and ETU as sole  
 sources of Carbon. The growth curve  
 revealed that the strain SDS18 utilises 50  
 ppm to 150 ppm concentration of maneb

and 50 ppm to 200 ppm concentration of EU and ETU (Figure 2). In case of maneb the lag phase was seen at (10-25 hours); log phase (28-40 hours) and stationary phase at 40-60 hours. In case of EU and ETU the strain showed lag phase at 10 hours followed by log phase at 12-35 hours and stationary phase at 35-40 hours. The possible reason for a long lag phase in case of maneb could be due to the toxic and recalcitrant nature of the compound. Maneb can inhibit microbial growth, activity and proliferation. Hence, the strain(s) requires comparatively longer duration to adapt and overcome its toxic effects before they can actively grow and reproduce. This observation correlates with the previous reports of pesticide degradation [31]. Growth in the presence of such toxic compounds as sole Carbon source indicates transformation of the molecules to simpler ones via metabolism. In case of maneb, OD reached a value of 0.945 in 62 hours at 100 ppm concentration while in case of ETU and EU, maximum OD (1.446) was attained in 48 hours at 50 ppm concentration. However, the strain does not show much variation in the growth curve at different concentrations of maneb, ETU and EU. No significant change in pH of media was observed because the concentration of  $\text{Na}_2\text{HPO}_4$  was quite high which acted as a buffer in the MSM.

### 3.4. Plant growth promoting activities of strain SDS18

There are some studies suggesting the isolation of bacteria conferring both pesticide degradation and plant growth promoting characteristics [32, 33]. The strains of *Pseudomonas psychrotolerans* are reported for plant growth promoting traits [34, 35]. The isolate SDS18 was

checked for plant growth promoting activities namely production of HCN, catalase, ammonia, siderophore, auxin, ACC deaminase activity and phosphorus solubilisation. The strain SDS18 was positive for all, except HCN production.

In plants, catalase helps to get rid of  $\text{H}_2\text{O}_2$ , which is generated under oxidative stresses like salinity, high or low temperature, strong light, drought and various chemicals including particulate matter of air causing air pollution and herbicides [36]. Microbial strains exhibiting catalase activity are resistant to different types of stress [37]. The strain SDS18 produced effervescence upon addition of  $\text{H}_2\text{O}_2$  indicating it to be positive for catalase production.

HCN is a gaseous secondary metabolite inducing negative effect on root growth and root metabolism. Thus, HCN producing bacteria are termed as 'deleterious rhizobacteria'. These are non-parasitic plant pathogens with the ability to secrete phytohormones and phytotoxins affecting the metabolism of plants negatively [38]. The strain SDS18 did not produce HCN even after 4 days of growth as colour of filter paper remained similar to the control. The unchanged colour of the filter paper indicates that the strain did not produce HCN, conferring plant growth promoting feature to the strain.

N is required to synthesise bio molecules like proteins and nucleic acids. Since atmospheric N is inaccessible to plants [39], PGPB converts N to ammonia (assimilable by plants) known as biological N fixation [40]. Ammonia production also hampers the growth of phyto pathogens [41,42] reported ammonia production in rhizospheric isolates belonging to *Bacillus*

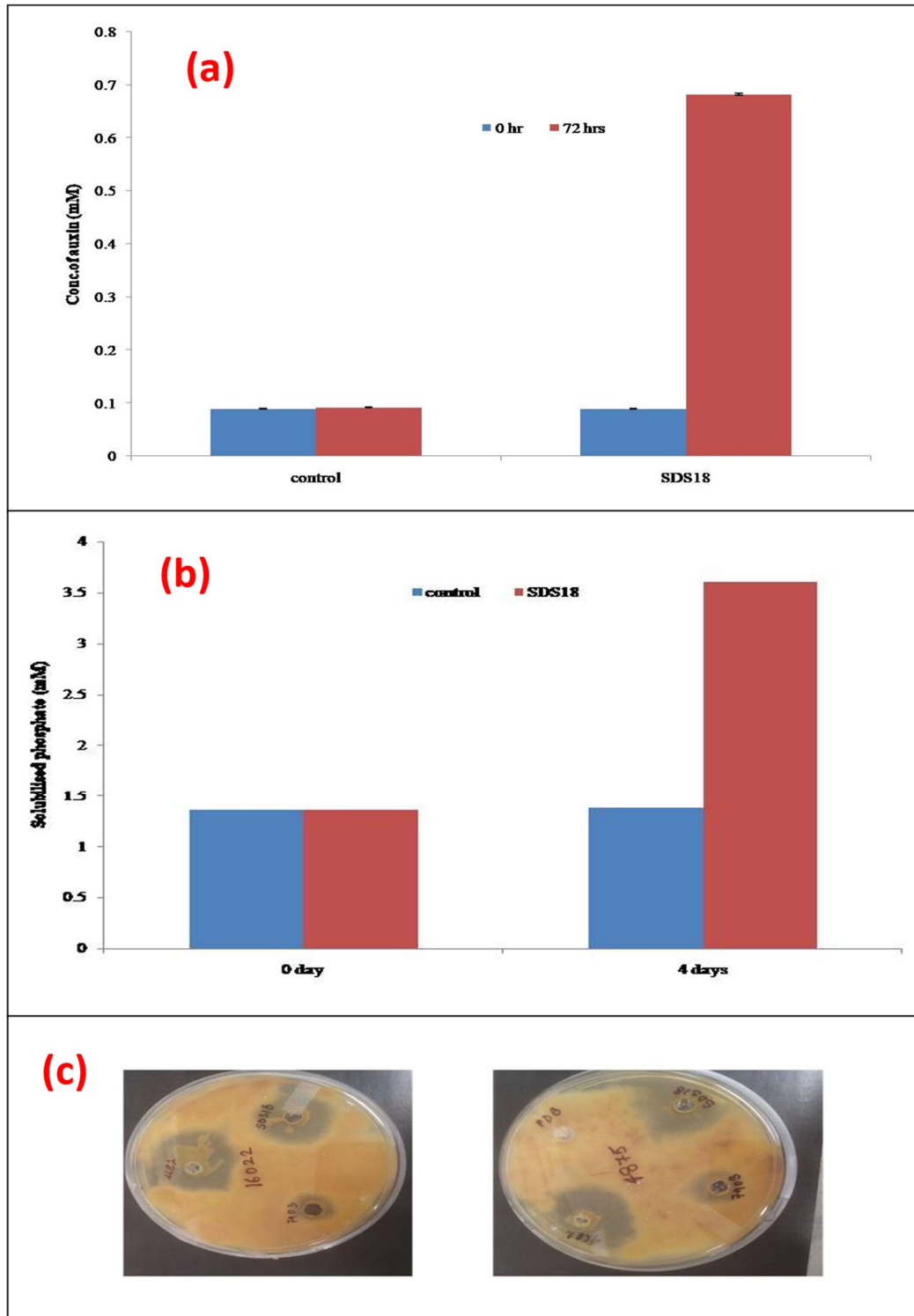
(95%) followed by *Pseudomonas* (94.2%). In fact, the endophytic strain *Pseudomonas psychrotolerans* PRS08-11306 possess 'turnerbactin' a biosynthetic gene cluster for nitrogen fixation [43]. In the present study, the strain SDS18 was able to produce ammonia in peptone water, as addition of Nessler's Reagent changed colour to brownish yellow.

Siderophores are small molecules having high affinity for iron which aid in scavenging iron by formation of soluble  $\text{Fe}^{3+}$  complexes which can be easily taken up by the mechanism of active transport [44]. CAS shuttle assay indicated that strain SDS18 was able to produce siderophores within 24 hours of growth. The percentage of siderophore production was found out to be 60.98%. Literature suggests the production of siderophores by *Pseudomonas* spp. Amongst the species of *Pseudomonas*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* produce pyoverdins which are fully characterized, while *Pseudomonas stutzeri* is known to produce ferrioxamine E, also called nocardamine [45,46]. Importance of siderophores producing bacteria in phytoremediation has been mentioned in numerous studies [47,48].

Phosphate solubilisation is one of the most important criterions for PGPB [49]. PGPB possess the ability of solubilising the inorganic phosphorus (P) such as calcium phosphate  $[\text{Ca}_3(\text{PO}_4)_2]$ , ferric phosphate  $[\text{FePO}_4]$  and aluminium phosphate  $[\text{AlPO}_4]$  of soil and make it available to the plants by secreting organic acids and acid phosphatases [50]. Members of genus *Bacillus* and *Pseudomonas* are reported as the most efficient phosphate solubilising bacteria [51]. The phosphate solubilising activity of strain SDS18 was checked by

growing the strain in Pikovaskaya's broth for 4 days and the amount of soluble phosphate was calculated (Figure 3a) as described by [52]. We found that the concentration of phosphate increased from 1.36mM to 3.682mM after 4 days of incubation, whereas in control the concentration remained unchanged.

Ethylene is the only gaseous phytohormone regulating the processes of plant growth, development and senescence. Under stress causing conditions like flooding, drought, action of heavy metals, phytopathogens, and high salt, the production of the hormone increases and negatively affects the development of a plant [53]. One of the mechanisms involving the regulation of ethylene production is the activity of an enzyme '1-aminocyclopropane-1-carboxylate (ACC) deaminase' found in bacteria. The enzyme ACC deaminase converts ACC which is the direct precursor of ethylene to  $\alpha$ -ketobutyrate and ammonia helping in the growth of plants [54]. The PGPB exhibiting ACC deaminase activity stimulates plant growth, mainly in unfavourable conditions [54]. When the strain SDS18 was cultured in the DF MSM medium with ACC as the sole N source, the strain was able to use ACC as sole nitrogen source implying its ACC deaminase activity. The activity was  $8.625 \pm 1.25 \mu\text{M}$  of  $\alpha$ -ketobutyrate/h/mg protein. Literature reports the existence of ACC activity in *Pseudomonas* spp [55]. Phytohormones are chemical compounds enacting as messengers and coordinating the cellular functions of plants. In plants, five major phytohormones namely auxin, gibberellins, cytokinin, ethylene and abscisic acid are present. The most abundant occurring auxin is indole-3-acetic acid (IAA). It is a major signaling molecule



**Figure 3.** Plant growth promoting traits of strain SDS18: (a) Phosphate solubilisation activity. The values represent an average value of three independent experiments. (b) IAA production, the values represent an average value of three independent experiments. (c) Antifungal activity of SDS18 on *Cladosporium cladosporioides* and *Alternaria citri*; MTCC4682-*Pseudomonas aeruginosa* IC230; ATCC16022- *Cladosporium cladosporioides*; MTCC 4875- *Alternaria citri*; MTCC7903-*Pseudomonas aeruginosa* PfD1.

since it regulates the process of plant development together with organogenesis and cellular responses like cell division, cell expansion, differentiation and gene regulation [56]. Interaction between IAA producing organisms and plants results in positive effects on the plant side, ranging from phytostimulation to pathogenesis. IAA producing bacteria use this hormone to interact with plants as a part of their colonization approach. L tryptophan is considered as the precursor of IAA synthesis in bacteria [57]. The strain SDS18 produced IAA in the presence of tryptophan (Figure 3b). The results presented in Figure 3b indicates that the concentration of auxin increased from 0.089mM to 0.682mM after 72 hours of growth in case of flasks inoculated with the strain SDS18, whereas the concentration of auxin remains unchanged in uninoculated control.

Several bacteria have been recognized as prospective biological control agent of fungal phyto pathogens due to their ability to guard plants against various important agronomical fungal diseases like black rot of tobacco, pea and wheat, damping off sugar beet. *Pseudomonas* secretes a variety of antibiotics like pyrrolnitrin, 2,4-diacetylphloroglucinol(2,4-DAPG) and Pyoluteorin [58]. Antifungal activity experiments confirmed that the strain

SDS18 was capable of inhibiting the growth of *Cladosporium cladosporioides* and *Alternaria citri* (Figure 3c). The zone of inhibition in case of *Alternaria citri* was 26 mm comparable with the positive control *Pseudomonas aeruginosa* IC230. The strain SDS18 showed 28 mm zone of inhibition in case of *Cladosporium cladosporioides*; while the positive control *Pseudomonas aeruginosa* showed 33 mm.

### 3.5. Comparative genomic analysis of the PGPR genes

*In silico* studies of plant growth promoting bacteria suggested the presence of genes in the selected bacterial strains conferring plant growth promoting traits [59]. The genome mining of the strain SDS18 revealed the presence of genes related to plant growth and development. Further to confirm the plant growth promoting ability of the strain SDS18, comparative genomic analysis between the  $\gamma$ -Proteobacteria strains reported for plant growth promoting traits was done. For analysis, recently reported plant growth promoting strains *Pseudomonas fluorescens* PS006 [60], *Klebsiella* sp. D5A [61] and *Pseudomonas putida* S11 [62] were chosen. The comparison (Table 1) shows that the strain SDS18 contains the necessary genes attributed for plant growth promoting characteristics.

**Table 1.** Comparative plant growth promoting genomic features of *Pseudomonas psychrotolerans* SDS18, *Pseudomonas fluorescens* PS006, *Klebsiellasp.*D5A, *Pseudomonas putida* S11.

S.No	Plant growth promotion traits	<i>Pseudomonas psychrotolerans</i> SDS18	<i>Pseudomonas fluorescens</i> PS006	<i>Klebsiellasp.</i> D5A	<i>Pseudomonas putida</i> S11	Genes with Potential for conferring PGP traits
1.	Phosphate solubilisation	Yes	Yes	Yes	Yes	Glucose dehydrogenase, <i>pqq</i> dependent
2.	IAA production	Yes	Yes	Yes	Yes	Indole-3-glycerol phosphate synthase
3.	Siderophore production	Yes (Achromobactin, Enterobactin)	Yes (Pyoverdine)	Yes (Enterobactin, Aerobactin)	No	<i>acsA</i> , <i>acsB</i> , <i>acsC</i> , <i>acsD</i> , <i>acsE</i> , <i>acsF</i>
4.	Catalase	Yes	Yes	Yes	Yes	Catalase gene homolog
5.	Acetoin & butanediol synthesis	Yes	Yes	Yes	Yes	Acetoin dehydrogenase cluster
6.	Peroxidases	Yes	Yes	Yes	Yes	
7.	Superoxide dismutase	Yes	Yes	Yes	Yes	
8.	Ammonia production/assimilation	Yes	Yes	Yes	Yes	Glutamate synthase gene cluster

### 3.6. Comparative genomic analysis of the stress response genes

Stress response is stimulated in bacteria when the bacterial cell encounters unfavourable conditions in the immediate environment. In order to cope with the stress, certain genes are activated which controls molecular pathways [63]. Maneb and ETU are toxic compounds. The compounds causes oxidative stress [64].

Literature suggests that increase in oxidative stress leads to Parkinson disease, Alzheimer's disease, heart failure, cancer, and atherosclerosis. In response to oxidative stress, cells are known to produce antioxidant enzymes like catalase, superoxide dismutase, heme oxygenase-1 and glutathione-S-transferases. These enzymes are known to protect the cells against oxidative stress/damage [65].

Literature suggests some bacteria like *Pseudomonas aeruginos* [66], *Micrococcus* sp. S2 [67] and *Bacillus subtilis* [68] produce superoxide dismutase especially manganese superoxide dismutase which plays a significant role in resistance towards oxidative stress.

The annotated genome of the bacterial strain *Pseudomonas psychrotolerans* SDS18 contains fifty six genes for oxidative stress. Some of the important genes are glutathione-S-transferase, NAD dependent glyceraldehyde-3-phosphate dehydrogenase, NADPH dependent glyceraldehyde-3-phosphate dehydrogenase, manganese superoxide dismutase, superoxide dismutase [Cu-Zn] precursor, catalase, hydrogen peroxide-inducible genes activator, ferroxidase and peroxidase. There are no reports for maneb and ETU degrading genes apart from the presence of cytochrome P<sub>450</sub>.

### 3.7. Analytical techniques for identification of putative intermediates of maneb degradation

There are some reports which state the transformation of maneb to ETU and EU [9]. The sample extracts obtained at 24 hours indicated no formation of metabolites. The sample extracts obtained at 48 hours and 72 hours indicated formation of ETU. Hence, the two sample extracts were combined. Further work of identification was carried out with this sample extract. No growth was seen in case of both the control flasks.

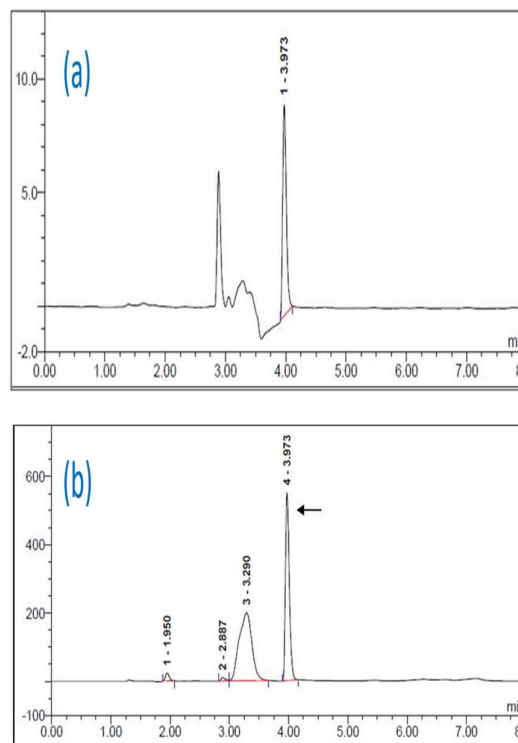
#### 3.7.1. HPLC Analysis

The extracted intermediate was subjected to HPLC in order to compare its retention time with that of the authentic standard ETU. The HPLC profile of the intermediate

showed the retention time of 3.973 minutes identical to that of the standard of 500 ppb ETU (Figure 4a and Figure 4b). The HPLC results suggest the extracted intermediate as ETU.

#### 3.7.2. FTIR Analysis

In order to determine the functional groups, present in the extracted intermediate, FTIR analysis was done. The similarities revealed between the FTIR spectra of the standard and extracted intermediate as shown in supplementary figure 2a and 2b indicates that the extracted intermediate is ETU. The medium peak within the range 3250-3400 cm<sup>-1</sup> represents the N-H stretch of ETU. C-N stretch is represented by the variable peak within the range 2210-2260cm<sup>-1</sup>. A strong peak within the range 3000-3100cm<sup>-1</sup> represents the C-H stretch



**Figure 4.** HPLC for structure determination of the extracted intermediate. (a) HPLC chromatogram of 500ppb ETU showing a peak at 3.973 minutes. (b)

of aromatic compounds [69]. The FTIR results finally revealed the presence of ETU in the extract.

**3.7.3. <sup>1</sup>HNMR Analysis:** To find out the position of protons, <sup>1</sup>HNMR is a useful technique. We performed and analysed the proton NMR spectra for both the standard and sample. The <sup>1</sup>HNMR spectra indicated that the compound ETU has one singlet at  $\delta$  4.86 due to the symmetrical environment (Figure 5a). Similar NMR spectrum is obtained for the extracted intermediate too (Figure 5b). The <sup>1</sup>NMR results revealed the compound to be ETU.

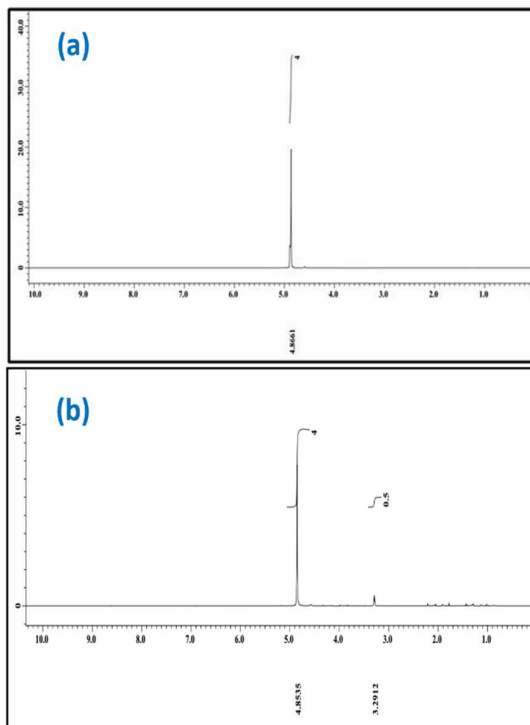
#### 3.7.4. LC/Q-TOF-MS Analysis

For finding out the percentage of transformation of maneb to ETU, LC/Q-TOF-MS studies of the culture supernatant were done at different time intervals. The m/z peak for maneb is at 265.01 (Figure 6a). After 75 hours the culture supernatant showed a reduction in m/z peak at 265.01 (Figure 6b). Hence, 35.78% reduction of maneb occurred.

The fungicide maneb is broken down to ETU. ETU is a well-known metabolite of dithiocarbamate fungicides. The molecular weight of ETU is 102.2. Since, the strain is able to utilise ETU as Carbon source, LC/Q-TOF-MS studies were performed to find out the percent reduction. The LC-MS spectrum of 0 hour (Figure 6c) indicates the m/z peak of ETU at 103.03 while the spectrum of 75 hours (Figure 6d) reveals the complete disappearance of the peak of ETU. This suggests the transformation of ETU to simple molecules. There are reports suggesting the transformation of ETU to CO<sub>2</sub>, H<sub>2</sub>O [70]. However, in our study we could not detect the formation of small molecules formed as

a result of ETU breakdown.

Further, identification of genes/enzymes involved in the biodegradation of maneb



**Figure 5.** <sup>1</sup>HNMR for structure determination of the extracted intermediate. (a) <sup>1</sup>HNMR spectrum of 1 ppm ETU, (b) <sup>1</sup>HNMR spectrum of the extract.

and ETU could lead to elucidation of the complete pathway. Maneb degradation ability and PGP traits of strain SDS18 suggest that bacteria can be used in field for eliminating fungicide as well as support growth of plants.

## 4. Conclusions

This study reports the characterization and biodegradation potential of a plant associated bacteria for maneb and its photolytic product. The plant associated bacteria mainly consisted of *Firmicutes* and *Proteobacteria*. Of all the isolates, the most promising strain SDS18 was characterized as *Pseudomonas*

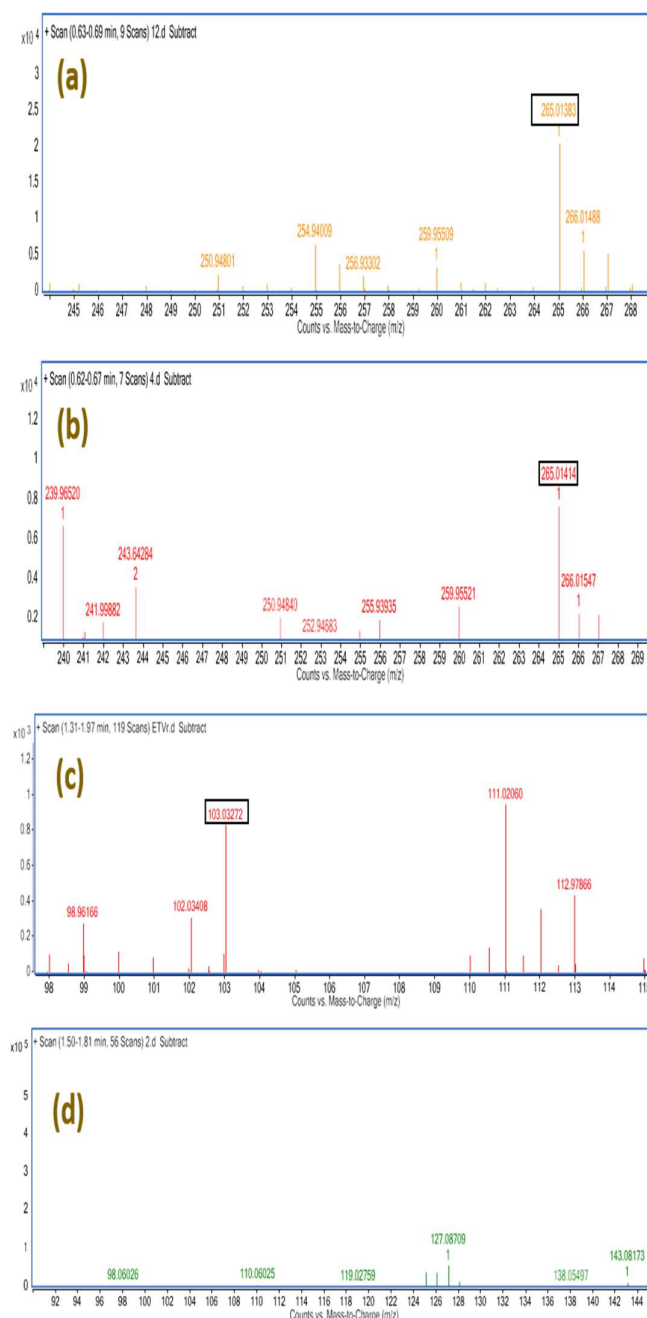
*psychrotolerans* by polyphasic taxonomy. The strain SDS18 was able to utilise upto 150 ppm of maneb and 200 ppm ethylene thiourea as sole Carbon sources. The isolate was unique in its attribute of exhibiting all plant growth promoting features and possessing antifungal activities against *Alternaria citri* and *Cladosporium cladosporioides*. *In silico* studies of the strain SDS18 revealed that its genome has genes for phosphate solubilisation, ammonia production and assimilation, IAA production, siderophore production, acetoin and butanediol synthesis, peroxidases and superoxide dismutase which are also present in other plant growth promoting bacteria. The genes to combat stress response generated due to the toxic maneb were also present. *In vitro* biodegradation studies had shown that the strain could degrade 35% maneb to ETU. Further, ETU was completely degraded. Hence, the plant growth promoting strain SDS18 can prove to be a potential candidate for maneb and ETU degradation.

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### Conflict of Interest

The authors declare that they have no conflict of interest.



**Figure 6.** LC/QTOFMS studies showing reduction of maneb and ETU. (a) LC-MS spectrum for 0 hour culture supernatant showing the m/z peak of maneb at 265.01; (b) LC-MS spectrum for 75 hours culture supernatant showing the reduction (35.78%) in the m/z peak of maneb at 265.01; (c) LC-MS of 0 hour culture supernatant

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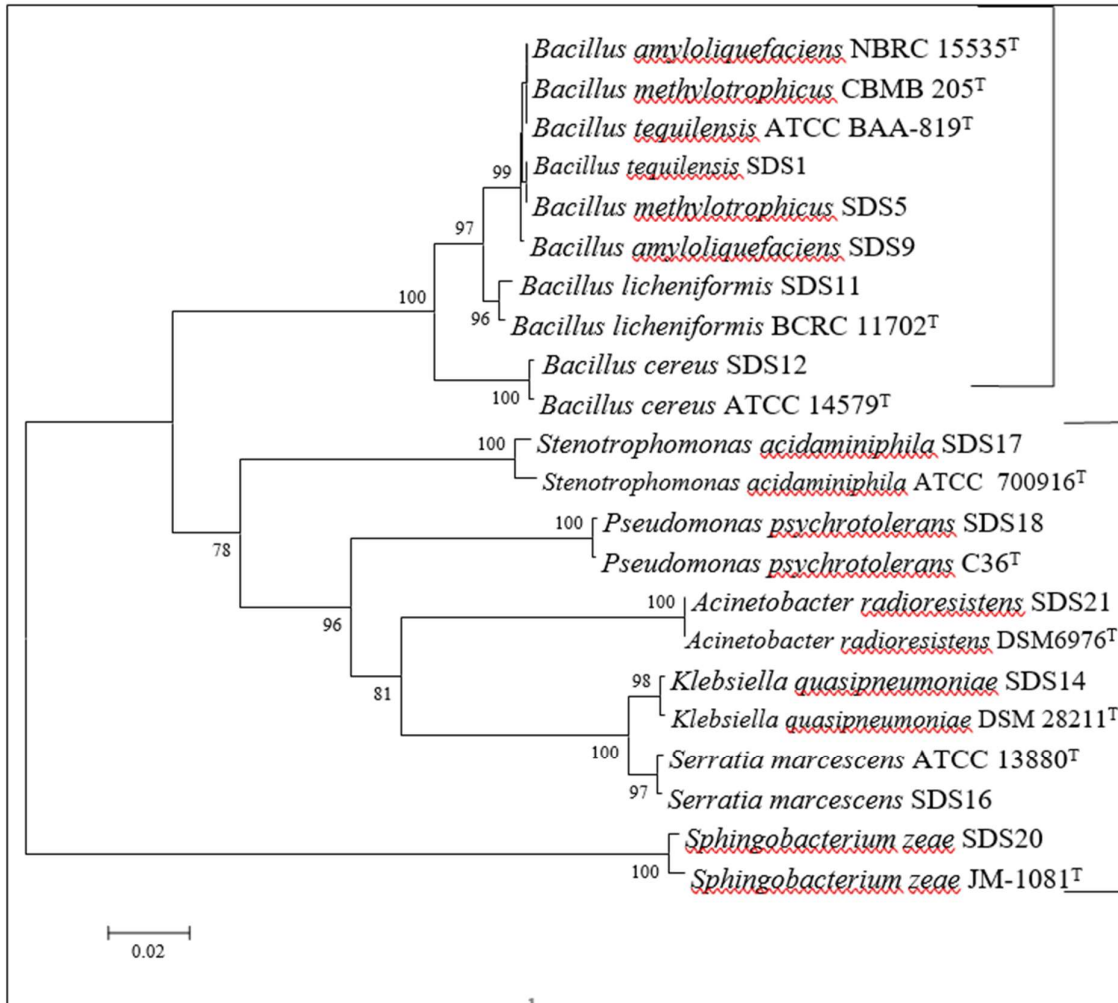
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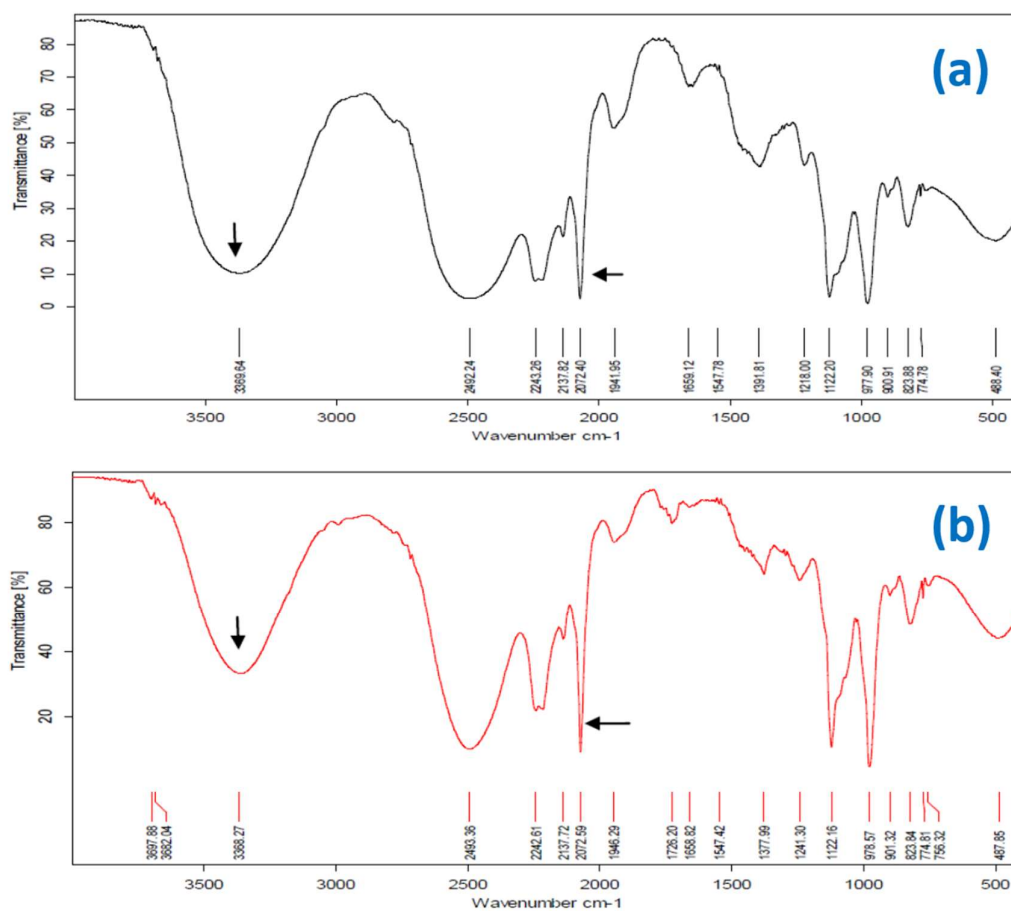
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**Supplementary Information**



**Supplementary Figure 1.** Neighbour joining based phylogenetic tree of the plant associated isolates.



**Supplementary Figure 2.** FTIR Spectrum. (a) 1 ppm ETU, (b) Extracted intermediate.