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Efficacy of Native *Bacillus subtilis* against Postharvest *Penicillium* Rot Pathogen *Penicillium* sp. of Khasi Mandarin Oranges in Meghalaya, India

Janshame Tariang*, D. Majumder and H. Papang

Department of Plant Pathology, School of Crop Protection, College of Post Graduate Studies, Central Agricultural University, Umiam - 793103, Meghalaya, India

**Corresponding author*

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A B S T R A C T

Khasi mandarin orange is an important horticulture crop in Meghalaya but several losses have been reported due to the post-harvest disease *Penicillium* rot caused by *Penicillium* sp. *B. subtilis* was evaluated for its efficacy against *Penicillium* sp. under *in-vitro*. A total of 260 isolates were isolated from samples collected from different habitats of Meghalaya. Twelve *B. subtilis* isolates were identified by morphological, biochemical and molecular identification and studied for their antagonistic properties against *Penicillium* sp. The habitats of these 12 *B. subtilis* isolates were recorded to befrom crop rhizosphere, mixed manure, fishery pond, mushroom compost and jhum area. Based on the antimicrobial traits, 6 *B. subtilis* isolates (Bs 167, Bs 197, Bs 217, Bs 219, Bs 256 and COB5Y1) were found with potential antagonistic properties as they were tested positive to chitinase and protease production. Bioassay showed that *B. subtilis* isolate COB5Y1 was recorded with the highest disease inhibition per cent against *Penicillium* sp. with 88.11% followed by Bs 167 (80.82%) and Bs 197 (54.82%). Four isolates COB5Y1, Bs 167, Bs 197 and Bs 217 were found with potential antagonistic properties as they could produce chitinase and protease and also were recorded with the maximum percentage of inhibition in dual test.

Introduction

Mandarin is a group name for a class of oranges with thin, loose peel which falls under members of a distinct species *Citrus reticulata* Blanco. Khasi mandarin in particular, is an important horticulture fruit crop in the North-Eastern (NE) parts of India. Meghalaya is the major state in the NE India for both area and production of Khasi mandarin (Singh, 2001). It is vastly cultivated in the outskirt and border areas of the state with an estimated area for its cultivation over 10,000 hectares and production of about 50,000 metric tonne (MT) annually. In India, the total area under cultivation for mandarin is 3.11 lakh hectare with the production of 29.06 lakh tons (Anonymous, 2013). However, Khasi mandarin constitutes about 43.6% of the total citrus fruits produced and occupies nearly 38.2% of the total citrus area in India (Anonymous, 2011).

Although Meghalaya is one of the largest producers of orange in the country, due to the problem of postharvest diseases, there are

considerable losses to harvested fruits and the average yield of orange in India is alarmingly low as compared to other countries. Several reports have been published world-wide during the last two decades regarding the green and blue mould infections of harvested citrus (*Citrus reticulata* Blanco), the causal agents identified as *Penicillium* sp.

Green mould caused by *P. digitatum* is the major postharvest disease of citrus, wherever it is grown and causes serious losses annually (Eckert and Brown, 1986). In the NE parts of India, upto 30–50% losses of citrus was reported to be due to *Penicillium* rot caused by *P. digitatum* (Selvakumar, 2011). In Meghalaya, 17.75-19.28% losses of Khasi mandarin was reported, due to *Penicillium* rot disease caused by *P. brevicompactum* (Barman, 2010).

B. subtilis is well known as one of the most active antagonist as it produces inhibitory substances and a large number of peptide antibiotics. The antibacterial-antifungal substances produced by *B. subtilis* has popularise its biological control potential against several phytopathogens that causes fruit decay. It is known to produce antibiotics such as iturin, surfactin, fengycin, enzymes that degrade fungal structural polymers such as chitinase, protease, β 1-3 glucanase and antifungal volatiles which are antagonictic properties (Fiddaman and Rossall, 1993; Knox *et al.,* 2000; Jiang *et al.,* 2001; Pinchuk *et al.,* 2002; Leelasuphakul *et al.,* 2008).

The use of synthetic fungicides is the primary means to control postharvest diseases which is of public concern due to the accumulation of chemical residues in the food chain and environmental (Eckert, 1990; Arul, 1994).An alternative biocontrol method was studiedby screening the efficacy of *B. subtilis*against *Penicillium* sp.

Materials and Methods

Collection of samples, isolation of bacterial isolates and maintenance

Samples were collected from different habitats of Meghalaya *viz*., root rhizosphere of different available crops, hot springs, coal mines, jhum area, manure compost, mushroom compost, forest, lime stone mines and fruit surfaces (Sneath 1986; Leelasuphakul *et al.,* 2008; Cihan *et al.,* 2011).

Each10g of thesample was added to 90 ml of sterile distilled water inconical flask and mixed thoroughly on a rotatory shaker for 5 min. Heat shock treatment was done for each sample *i.e*. heating the sample suspension in a water bath at 80°C for 10 mins (Kumar *et al.,* 2012). Isolation for *B. subtilis* isolates was done on Nutrient agar (NA) medium by serial dilution (Sushma *et al.,* 2012). The inoculated plates were incubated at 37±1°C for 24-48 h (Jamil *et al.,* 2007; Munich, 2014). Maintenance of isolates were stored for long term in Nutrient broth (NB) containing 20% (v/v) glycerol and at -20 °C (Munich, 2014) and subsequently sub-cultured over a period of 3months (Gomaa and Momtaz, 2007)

Identification of bacterial isolates as *B. subtilis*

Morphological identification

A 24 h old colony of each isolate was picked up (loopful), mixed with 10 ml of sterile water in a culture tube and vortex. Serial dilutions were made upto 10^{-7}cfu/ml and from the final dilution, 0.1 ml was pipetted out in Petri plates containing NA medium. Each isolates was recorded for their colony characters in terms of colony colour, colony elevation, colony edges, colony consistency and shape of the bacterium(Holt *et al.,* 1994).

Biochemical identification

Gram staining test, catalase test and oxidase test was done for each isolates so as to screen for the interested *B. subtilis* isolates only.

Gram staining: Gram staining was carried out for all the isolates to differentiate the bacteria as Gram positive or Gram negativeby using the gram staining kit following the manufacture's instruction (K00-1Kt, Himedia). Microscopically at 10X, 40X and 100X (using oil) was observed for Gram positive or negative reactions and the shape of the cells was recorded.

Catalase test: A loopful of the test bacterial culture (18-24 h) and a drop 3% hydrogen peroxide (H_2O_2) were mixed on a clean glass slide. Observation was made for the production of gas bubbles (Schaad, 1992).

Oxidase test: The test was carried out by using oxidase disc (DD018, Himedia), where 24 h old bacterial culture was picked up with the help of ainoculation loop and rubbed on the oxidase disc. A change in colour of the disc was observed and recorded.

Molecular identification

The isolates were identified for *Bacillus* sp. using *Bacillus* sp. specific primerand for *B. subtilis* using*B. subtilis* specific primer

DNA isolation

The genomic deoxyribonucleic acid (DNA) of each bacterial isolate was extracted from bacterial suspension (after 12 h incubation in LB) using DNA extraction kit (Himedia) following the manufacture's instruction.

Bacillus sp. specific primer

The isolates were identified for *Bacillus* sp. using *Bacillus* sp. specific primer BCF1-CGGAGGCAGCAGTAGGGAAT/ BCF2 – CTCCCCAGGCGGAGTGCT TAAT (Cano *et al.,* 1994). A PCR reaction mixtures (25µl) was prepared each containing 1X PCR buffer; 25mM MgCl2; 2.5mM dNTPs; each 10pmol primer; 3U Taq DNA polymerase and 100 ng bacterial DNA. The PCR in a volume of 25 µl was carried out with initial denaturation of 94 °C for 5 min, followed by 40 cycles of program 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min ending with 10 min extension at 72°C. PCR reactions were run on a 0.8% agarose gel in 1X TBE and the observation for amplicon size 546 bp was recorded using ultraviolet gel documentation system.

B. subtilis specific primer

Discriminating *B. subtilis* from *Bacillus* sp. isolates was then carried out using two sets of primers specific to *B. subtilis* EN1F (103–124 bp) 50 -CCAGTAGCCAAGAATGGCCAGC-30; EN1R (1,413–1,393 bp) 50 -GGAATAATCGCCGCTTTG TGC-30) (Ashe *et al.,* 2014). A PCR reaction mixtures (20µl) was prepared each containing 1X PCR buffer (10mM TRisCl, 50 mMKCl, 1.5 mM $MgCl_2$ and 0.01% gelatin); 100 µM dNTPs; each 10 pmol primer; 0.75U Taq DNA polymerase and 100 ng bacterial DNA).The touch down PCR in a volume of 20 µl was carried out with initial denaturation of 94 °C for 5 min followed by 10 cycles of touch down program (94 °C for 30 s, 70 °C for 20 s and 74 °C for 45 s, followed by a 1 °C decrease of the annealing temperature every cycle).

After completion of the touch down program, 25 cycles were subsequently performed (94°C for 30 s, 60°C for 20 s and 74°C for 45 s) and ending with a 10 min extension at 74°C. PCR reactions were run on a 0.8% agarose gel stained with ethidium bromide in 1X TBE. The observation for amplification at 1311 bp was recorded using ultraviolet gel documentation system.

Screening of *B. subtilis* isolates for their antimicrobial traits

Molecular detection for presence of biosynthetic gene coding for iturin

Molecular detection for presence of biosynthetic gene coding for iturin D was done against each *B. subtilis* isolates using Ituringene specific primers ItuD-F1 (TGAAYGTCAGYGCSCCTTT)/ ItuD-R1 (TGCGMAAATAATGGSGTCGT) (Chung *et al.,* 2008; Venkatesan *et al.,* 2015). A PCR reaction mixtures (25µl) was prepared each containing 1X PCR buffer with 1.5mM $Mgcl_2$; 200µM dNTPs; each 10pmol primer; 1U Taq DNA polymerase; bovine serum albumin (BSA) and 100 ng bacterial DNA. PCR reaction mixtures (25 µl) were transferred to a Mastercycler gradient (Eppendorf, Germany) with the following cycle conditions: initial denaturation at 95 °C for 15 min, 40 cycles of 95 C for 1 min, 52°C for 1 min and 72 °C extension for 1.5 min and a final extension at 72 °C for 7 min. EachPCR reaction was analysed by electrophoresis using a 0.8% agarose gel stained with ethidium bromide and visualized under ultraviolet light for amplification at 482 bp.

Molecular detection for presence of biosynthetic gene coding for fengyncins

Each *B. subtillis* isolates were screened for presence of biosynthetic gene coding for fengyncins using fengycin gene specific primers FenB-F1 (CCTGGAGAAAGAATAT ACCGTACCY)/ FenB-R1(GCTGGTTCAGT TKGATCACAT) (Chung *et al.,* 2008; Zokaeifar *et al.,* 2014; Venkatesan *et al.,* 2015). A PCR reaction mixtures (25µl) was prepared each containing 1X PCR buffer with 1.5mM $MgCl_2$; 200µM dNTPs; each 10pmol primer; 1U Taq DNA polymerase; bovine serum albumin (BSA) and 100 ng bacterial DNA. PCR reaction mixtures (25 µl) were run with the following cycle conditions: initial denaturation at 95°C for 15 min, 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C extension for 1.5 min and a final extension at 72°C for 7 min. Each PCR reaction was analysed by electrophoresis using a 0.8% agarose gel stained with ethidium bromide and visualized under ultraviolet light for amplification at 670 bp.

HCN production test

Hydrogen cyanide (HCN) production test was carried out for all the *B. subtilis* isolates. HCN production of bacterial bio-control agents was tested following the method of Bakker and Schipper (1987). The test bacteria were streaked into NA plates supplemented with glycine at 4.4g/l with simultaneous addition of filter paper (Watman No. 1) impregnated with 0.5% picric acid and 1% sodium carbonate (Na_2CO_3) in the upper lids of the plates along with uninoculated control. Petri plates were sealed with parafilm and incubated at 28±1 °C for 48 h. Discolouration of the filter paper from yellow to brown after incubation were co-ordinated as microbial production of cyanide. Brown or reddish-brown was recorded as weak (+), moderate(++), strong(+++) and negative (-) reaction respectively.

Chitinase production

The *B. subtilis* isolates were tested for chitinase production. Colloidal chitin was prepared from commercial chitin (HiMedia) by the method of (Mathivanan 1995; Shanmugaiah *et al.,* 2008). In the first step, acid hydrolysis of commercial chitin was done by suspending 5.0 g of chitin in 60 ml conc. HCl by constant stirring using a magnetic stirrer and stores at 4°C (refrigerator) overnight. Second step was the extraction of colloidal chitin by ethanol neutralization. To the resulting slurry (as obtained in step one),

200 ml of ice-cold 95% ethanol was added and kept at 26°C for overnight. It was then centrifuge at 3000 rpm for 20 min at 4°C. The pellet was washed with sterile distilled water by centrifugation at 3000 rpm for 5 min at 4°C. The washing of the pellets was done till the smell of alcohol vanished. Colloidal chitin thus obtained was stored at 4°C until further use.

The final chitinase detection medium consisted of a basal medium comprising of (all amounts are per litre) 10 g of 1% colloidal chitin, 6 g of NaH_2PO_4, 3 g of KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.05g Yeast extract, 15g of agar, pH was adjusted to 6.5 and then autoclaved at 121°C for 15 min. After cooling the medium was poured in to Petri plates and allowed to solidify. The fresh test bacterium was spot inoculated into the medium and incubated at 25±2 °C. Chitinase production was detected by observing clear zones around the colonies after growth for 48-72 h and recorded for diameter of the clear zones (Cihan *et al.,* 2012).

Protease production

B. subtilis isolates were tested for protease production. In screening for protease activity, the test bacterium was spot inoculated on Skim Milk agar (pH 7.0) plates and incubated at 37±1°C for 72 h. Isolates which gave a clear zone around their colonies due to the hydrolysis of skim milk were selected (Cihan *et al.,* 2012).

Bio-assay of *B. subtilis* against *Penicillium* sp.

B. subtilis isolates were screened for their antagonistic activity against *Penicillum* sp. isolated from Khasi mandarin (*Penicillium* rot infected fruit) by dual culture assay under *in-vitro.* A 0.1 cm agar plug from the margin of a growing fungal culture on a PDA plate was incubated centrally on a fresh PDA plate for 48 h at 24°C. The test bacterium culture was grown with PDB, shaken at 250 rpm for 24 h at 30°C then centrifuged at 4000rpm for 15 min and the pellet was streaked on the PDA plate 1 cm away from the fungal plug. Observations of the fungal reactions were recorded after 5 days and the radii of any zones of inhibition of the fungus were measured using a measuring scale. Three replicates were used for each *B. subtilis* isolate tested (Leelasuphakul *et al.,* 2008). The percentage of hyphal growth inhibition was calculated using the following formula (Gamliel *et al.,* 1989).

$$\mathbf{PI = 100-[(R^2/C^2)\times 100]}$$

Where,
PI = Percent percentage of hyphal growth inhibition
R = radius of treatment (cm) C = radius of control (cm)

Results and Discussion

A total of 15 habitats have been selected for sample collection *i.e.* crop rhizosphere, hot springs, pig manure, river bank deposits, citrus fruit surfaces, mixed manure, coal mines, limestone mines, forest, leave mould, vermi-compost, fishery ponds, oyster mushroom compost, jhum area and bio-extract. Majority of the isolates obtained, were isolated from samples collected from West Jaintia hills district (94) followed by East Khasi Hills (70), Ri-Bhoi district (51), West Garo Hills (20), North Garo Hills (10), West Khasi Hills (7), East Jaintia Hills (4) and South West Garo Hills (3). A total of 260 isolates were obtained from the different samples collected. Maximum isolates were obtained from crop rhizosphere (182), followed by limestone mines (14), coal mines (13), citrus fruit surfaces (10), river bank deposits (8), hot springs (5), forest (5), fishery ponds (5), oyster

mushroom compost (5), mixed manure (4), pig manure (2 isolates), leave mould (2), vermicompost (2), bio-extract (2) and jhum area (1). Reports have been made since the 1970s which supports the findings, that *Bacillus* sp. are micro-organisms that exist as soil inhabitant (Mundt and Hinckle, 1976; Walker *et al.,* 1998), inrhizosphere of plants (Zhang *et al.,* 1996; Maheswar and Sathiyavani, 2012) and many other environment (Sneath 1986; McSpadden Gardener 2004) such as *Brassica* leaves (Leifert*et al.,* 1992), compost (Phae*et al.,* 1990), citrus fruit surfaces (Obagwu and Korsten, 2003), textile wastewater (Gomaaand Azab., 2007), extreme environments such as hot, cold, acidic, alkali and saline areas (Cihan *et al.,* 2012), as their survival is aided by this ability to form endospores.

A total of 260 isolates were obtained from samples collected from 8 districts of Meghalaya Morphology character of the colony of the isolates were creamish white colony, large with irregular edges, non-fluidal, shiny with smooth texture and slightly raised (Plate 1), 253 isolates were Gram staining positive, 95 isolates were positive to catalase test, 6 isolates showed positive to oxidase test (Plate 2). Hence, only 95 isolates were studied further for molecular identification. The finding can be supported as per the findings of Saleh *et al.,* (2014) who reported that *B. subtilis* are straight or sometimes curved rods, Gram positive, catalase-positive bacilli. Similar findings were also reported by Yumoto *et al.,* (1998) that *B. subtilis* are found to exhibit positive to catalase and oxidase activity.

Molecular identification of the isolates using of 16S rRNA intervening sequence *Bacillus* sp. genus specific primer BC1R/BC1F revealed that 69 isolates were amplified at 546 bp and were designated as *Bacillus* sp. isolates (Plate3). Several reports have been made for identification of *Bacillus* sp. using primerBC1R/BC1F which supports the findings of the present investigation (Cano *et al.,* 1994; Rajendra and Samiyappan, 2008).

Table.1 *B. subtilis* isolates and habitat of origin

Sl no.	Isolates	Gram staining	Catalase test	Oxidase test	Shape	Habitat	Location
1	Bs 80	+	+	+	R	Radish rhizosphere	West Garo Hills, Tura.
2	**Bs 167**	+	+	+	R	Brinjalrhizosphere	West Jaintia Hills, Mookyndeng.
3	Bs 174	+	+	+	R	Forest	East Khasi Hills (EKH), Mawphlang.
4	Bs 190	+	+	+	R	Mint rhizosphere	EKH, Upper Shillong.
5	Bs 193	+	+	+	R	Mixed manure	EKH, Upper Shillong.
6	**Bs 197**	+	+	+	R	Chilli rhizosphere	EKH, Upper Shillong.
7	Bs 216	+	+	+	R	Fishery pond	Ri-Bhoi District, Sumer.
8	**Bs 217**	+	+	+	R	Fishery pond	Ri-Bhoi District, Sumer.
9	**Bs 219**	+	+	+	R	Oyster mushroom compost	EKH, Upper Shillong.
10	**Bs 256**	+	+	+	R	Fishery pond	Ri-Bhoi District, Umsning.
11	Bs 257	+	+	+	R	Mushroom compost	Ri-Bhoi District, Sumer.
12	**COB5Y1**	+	+	+	R	Jhum area	Ri-Bhoi District CPGS, SCP Laboratory.

(-) indicates negative to the test, (+) indicates positive to the test, (++) indicates strongly positive to the test, R= rod shaped

Table.2 Antimicrobial properties of *B. subtilis*

Isolates	Anti-microbial traits					Dual culture test of *B. subtilis* against *Penicillium* sp.	
	Fengycin B	**Iturin D**	**HCN test**	**Chitinase test**	**Proteaase test**	**Hyphal radial growth (cm)**	**Inhibition per cent (PI %)**
Bs 80	-	-	-	-	-	2.87±0.03[a]	2.26±2.26[ef]
Bs 167	-	-	-	++	+	**1.27±0.07[d]**	**80.82±2.06[a]**
Bs 174	-	-	-	-	-	2.17±0.07[bc]	44.07±3.49[bc]
Bs 190	-	-	-	-	+	2.57±0.28[ab]	19.74±1.47[de]
Bs 193	-	-	-	-	+	2.57±0.12[ab]	21.32±7.47[cd]
Bs 197	-	-	-	+	+	**1.93±0.18[c]**	**54.82±7.92[b]**
Bs 216	-	-	-	-	+	2.23±0.12[bc]	40.35±6.26[bc]
Bs 217	-	-	-	++	+	**2.23±0.15[bc]**	**40.19±7.81[bc]**
Bs 219	-	-	-	++	+	2.50±0.25[ab]	24.18±14.26[cd]
Bs 256	-	-	-	+	+	2.27±0.09[bc]	38.72±4.70[bc]
Bs 257	-	-	-	-	+	2.23±0.15[bc]	40.19±7.81[bc]
COB5Y1	-	-	-	++	+	**1.00±0.00[d]**	**88.11± 0.00[a]**
Control	-	-	-	-	-	2.90±0.00[a]	00.0±0.00[f]
SEM						0.14	7.83
CD at5%						0.41	22.77

Values in column with common letters do not differ significantly as determined by the one-way ANOVA at 5% level of significance.

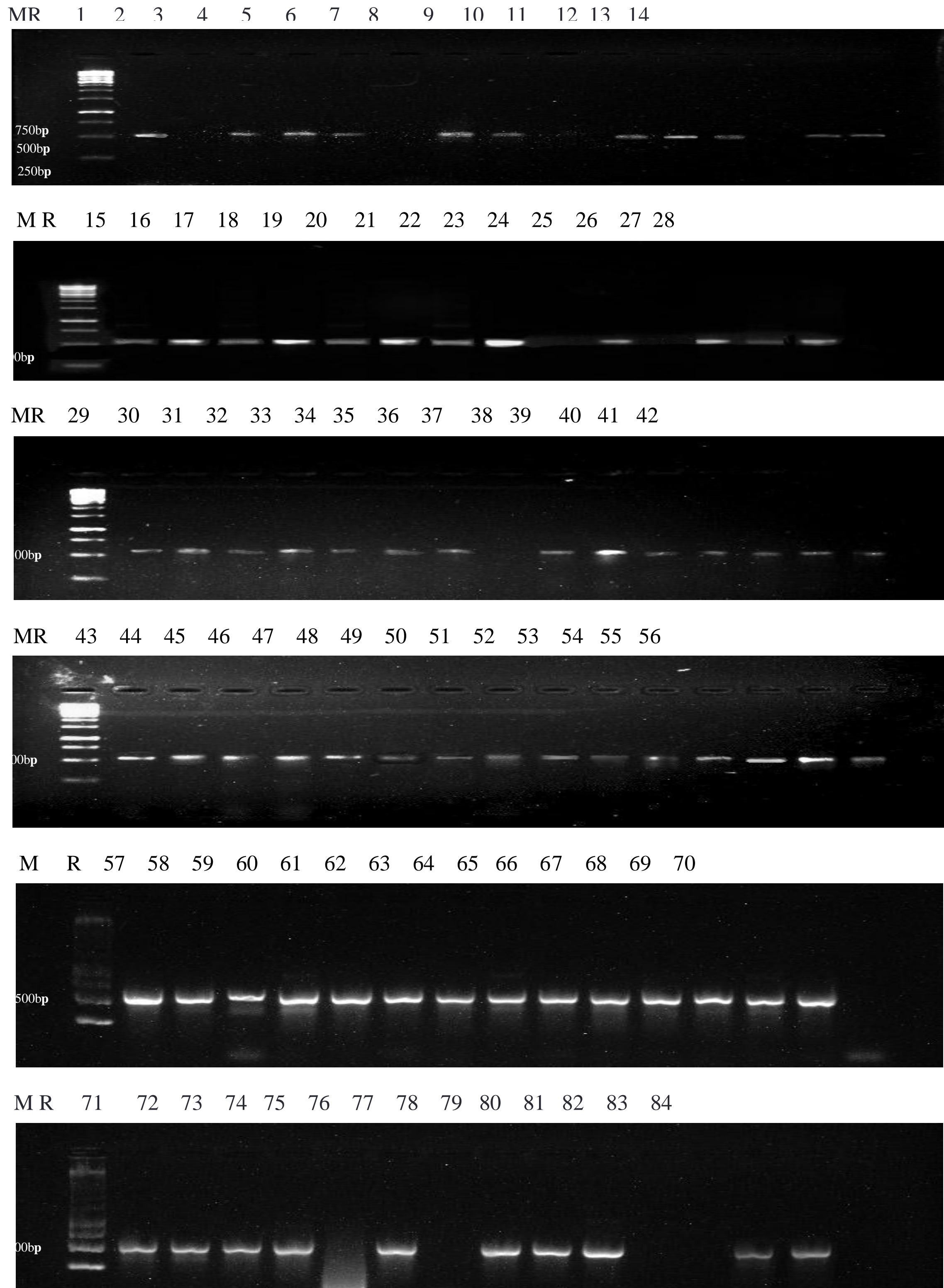
Plate.1 *B. subtilis* isolate

Colony morphology of *B. subtilis* isolate

Plates.2 Biochemical identification

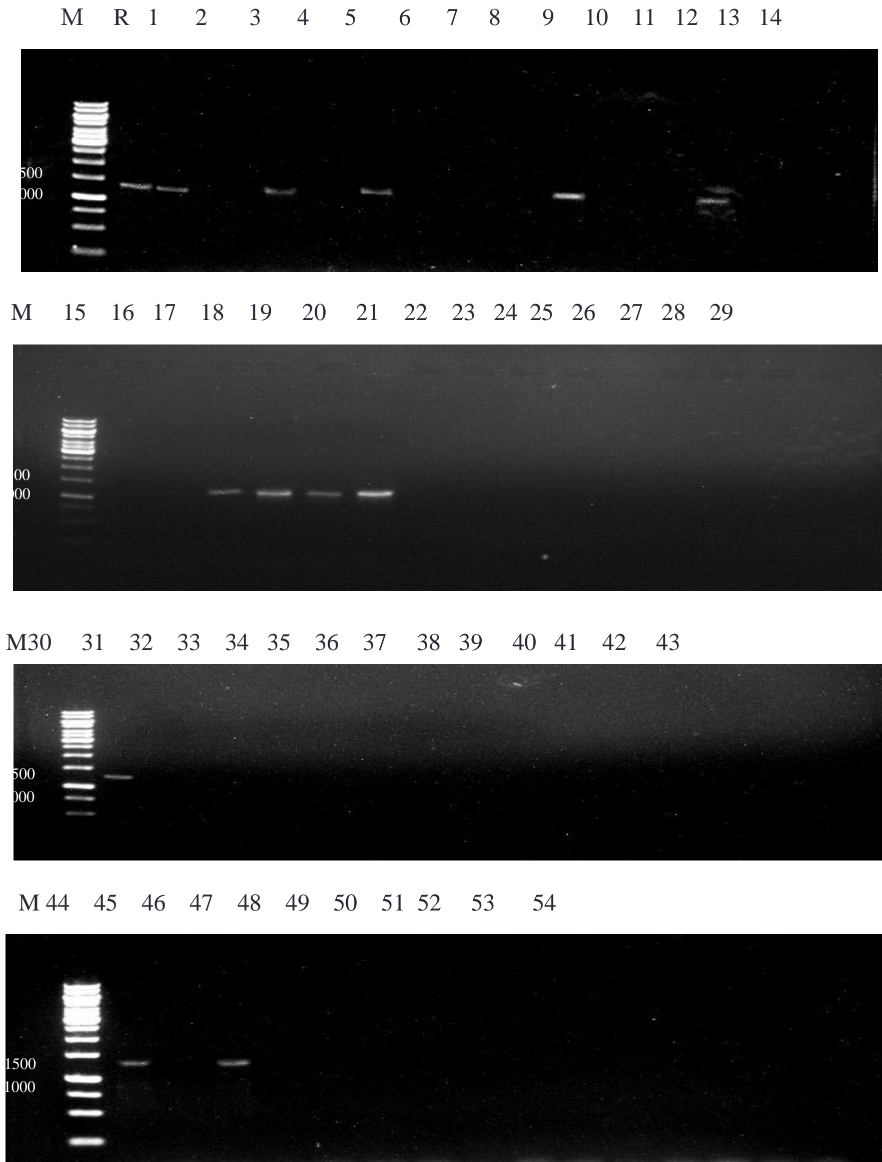
Gram staining test | Catalase test | Oxidase test

Plates.3 Molecular identification *Bacillus* sp. specific primer



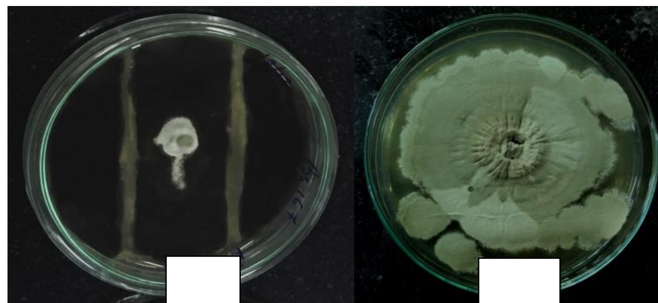
Bacillus sp. isolates showing amplification at 546bp to primer BCF1/BCR1.M=1kb ladder; R=positive reference; *Bacillus* sp. (+) isolates lane no.= 2-4, 6, 7, 9-11, 13-21, 23, 25-27, 29-34, 36-69, 71-73, 75, 77-79, 82-83

Plates.4 Molecular identification *Bacillus subtilis* specific primer



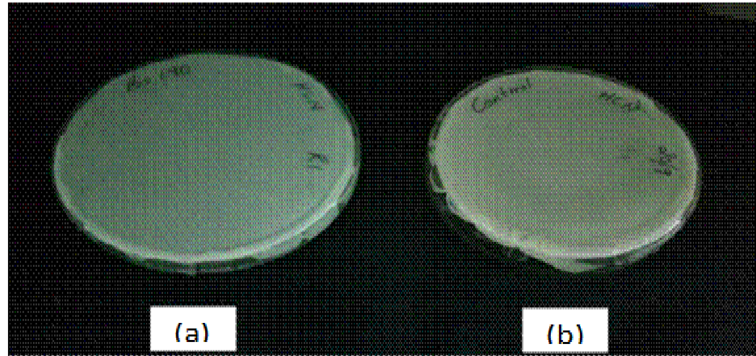
B. subtilis isolates showing amplification at 1311bp to primer EN1F/EN1R. M=1kb ladder; R=positive reference; *B.subtilis* (+) isolates lane no. 1=Bs 80, 3=Bs 167, 5=Bs 174, 9=Bs 190, 12=Bs 193, 17=Bs 197, 18=Bs 216, 19=Bs 217, 20=Bs 219, 30=Bs 256, 44=Bs 257 and 46=COB5Y1.

Plate.6 Bio-assay for antimicrobial activity against *Penicillum* sp.

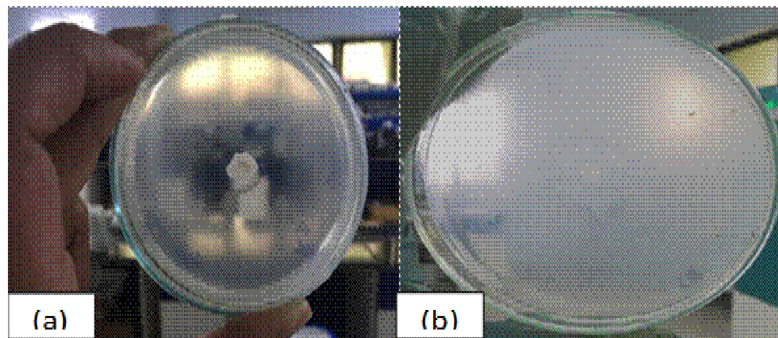


Dual culture test (a) test bacterium (b) Control

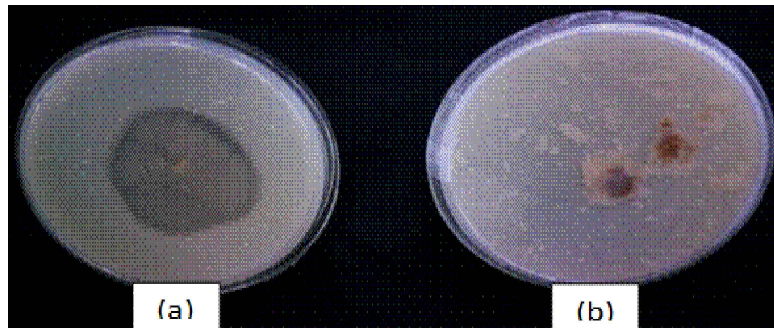
Plate.5 Antimicrobial tests of *B. subtilis* isolates



HCN production test (a) positive (b) negative



Chitinase production test (a) positive (b) negative



Protease production test (a) positive (b) negative

Bacillus sp. isolates were further confirm as *B. subtilis* isolates using species specific primer EN1F/EN1R which identified 12 isolates as *B. subtilis* (Bs 80, Bs 167, Bs 174, Bs 190, Bs 193, Bs 197, Bs 216, Bs 217, Bs 219, Bs 256, Bs 257 and COB5Y1) (Table 1, Plate 4). The findings can be supported as per the reports of Ashe *et al.,* (2014).

Antimicrobial traits studied against each of the 12 *B. subtilis* isolates revealed that all *B. subtilis* isolates were negative to biosynthetic antibiotic gene coding for iturinD and fengycinB. Twelve of the isolates were negative to HCN production, six isolates were positive to chitinase test and 10 isolates were positive to protease test (Table 2, Plate 5). Based on the antimicrobial traits, 6 *B. subtilis* isolates (Bs 167, Bs 197, Bs 217, Bs 219, Bs 256 and COB5Y1) were found with potential antagonistic properties as they were tested positive to chitinase and protease production.

Reetha *et al.,* (2014) reports supports the findings of the present investigation that none of the isolates of *B. subtilis* produce HCN but they showed efficacy against the pathogen *M.phaseolina* with 61.13- 63.36 per cent reduction over control. Production of chitinase and protease increase chitinase and protease inhibitory activities against the fungal pathogen (Vishwanathan and Samiyappan, 2002; Rajendra and Samiyappan, 2008; Liu *et al.,* 2010) and supports the findings of the present investigation.

Dual culture assay revealed that COB5Y1 had the smallest diameter of radial growth with only 1.00 cm followed by Bs 167 with 1.27 cm and Bs 197 with 1.93 cm. COB5Y1 was recorded with the highest disease inhibition per cent against *Penicillium* sp. with 88.11% followed by Bs 167 (80.82%) and Bs 197 (54.82%) (Table 2, Plate 6). Based on *in-vitro* evaluation, isolates COB5Y1, Bs 167 and Bs 197 were considered potential *B. subtilis* isolates amongst the 12 *B. subtilis* isolates as they showed maximum percentage of inhibition against the growth of *Penicillium* sp. The findings that *B. subtilis* could inhibit post-harvest fungal pathogen *Penicillium* sp. as well as other toxic fungus can be supported as per the findings of Yoshida *et al.,* (2001), Leelasuphakul *et al.,* (2008), Arrebola *et al.,* (2009) and Bharose *et al.,* (2017).

Judging by the anti-microbial traits and the bioassay of the 12 *B. subtilis* isolates,4 isolates COB5Y1, Bs 167, Bs 197 and Bs 217were found with potential antagonistic properties as they could produce chitinase and protease and also were recorded with the maximum percentage of inhibition in dual test**.**

In conclusion, *B. subtilis* is found to exist in different natural habitats other than the soil. *B. subtilis* has a great potential in the bio control management of the post-harvest *Penicillium* rot pathogen *Penicillium* sp. of Khasi mandarin. The four potential *B. subtilis* isolates COB5Y1, Bs 167, Bs 197 and Bs 217 could serve as potential bio-agent against post-harvest *Penicillium* rot of Khasi mandarin in Meghalaya which needs further post-harvest evaluation under field condition.

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