



E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(6): 449-455
Received: 19-09-2018
Accepted: 21-10-2018

Bhatt Hemangini D

Department of Biosciences,
Saurashtra University, Rajkot
and Vimal Research Society for
Agrobiotech, VEKO house,
Agiroad, Rajkot, Gujarat, India

Chudasama Kiran S

Department of Biosciences,
Saurashtra University, Rajkot
and Vimal Research Society for
Agrobiotech, VEKO house,
Agiroad, Rajkot, Gujarat, India

Thaker Vrinda S

Department of Biosciences,
Saurashtra University, Rajkot
and Vimal Research Society for
Agrobiotech, VEKO house,
Agiroad, Rajkot, Gujarat, India

Antimicrobial activity of *Bacillus paralicheniformis* SUBG0010 against plant pathogenic bacteria of mango

Bhatt Hemangini D, Chudasama Kiran S and Thaker Vrinda S

Abstract

Biocontrol using safe microorganisms is one of the best alternatives to hazardous and ecologically harmful chemical pesticides. In the present work, *Bacillus paralicheniformis* SUBG0010 was isolated from the rhizosphere of grass from the desert of India, as a potential antimicrobial producing strain. It was tested against six bacterial pathogens of mango. Four pathogens were strongly inhibited by the supernatant of the strain SUBG0010 after 48 h growth, revealed by co-culture and agar well diffusion methods. Antimicrobial protein isolated from the culture free supernatant is thermostable and proved to be a potential antimicrobial substance. The role of *B. paralicheniformis* SUBG0010 as a potential biocontrol agent against mango pathogens is discussed.

Keywords: Agar well diffusion method, *Bacillus paralicheniformis*, biocontrol, Co-culture method, *Mangifera indica*, Plant pathogens

Introduction

Mangifera indica, Mango, belongs to the family *Anacardiaceae*, is grown in the most tropical and subtropical regions for its delicious fruits. It also has many medicinal properties and used in the Ayurvedic and indigenous medical systems for over 4000 years (Shah *et al.*, 2010) [44]. India contributes around 56% of the total global production of mango (Rabari *et al.*, 2018) [35]. The major mango producing states are Andhra Pradesh, Maharashtra, Karnataka, Bihar, and Gujarat. Commercially grown mango varieties are Alphonso, Banganpalli, Chausa, Dashehri, Langra, Totapuri and Kesar. The mango tree is infected in the various stages of life by microorganisms including bacteria, fungi, algae and viruses (Chowdhury and Rahim, 2009; Litz, 2009) [9, 24] and worldwide annually crop loss is approximately 35-42% due to diseases, insects, and weeds (Rabari *et al.*, 2015). These organisms are responsible for developing symptoms including dieback, black spots, necrosis, mildew scab, blotch, anthracnose and rots in mango trees (Freeman *et al.*, 1999; Ploetz, 2003; Haggag and Abd El-Wahab, 2009) [15, 32, 19].

Fungal pathogen diseases and other pest diseases can be controlled at a certain level of infection but a bacterial black spot of mango is very difficult to control (Gagnevin and Pruvost, 2001) [16]. Commercial cultivars are highly sensitive to bacterial black spot disease and approximately 50 to 80% fruit infection is common on susceptible cultivars (Prakash and Misra, 1992) [33]. Symptoms developed on leaf and fruits are most common but the occurrence of twig and branch canker due to severe infection on highly sensitive varieties of mango (Gagnevin and Pruvost, 2001) [16].

In the era of green revolution, chemical fertilizers and pesticides have played the key important role to cope up with the demand of the ever-increasing population. Since last few years, it is realized that the chemical fertilizers or pesticides in excess cause severe damage to the agriculture land and crops (Aktar *et al.*, 2009) [2]. These agrochemicals are also responsible for an increased level of environmental pollution and hazardous pesticide residual effects on human health and on the ecosystem globally (Thind, 2012, Shafi *et al.*, 2017) [48, 43]. Therefore alternative should be not only helpful for the pathogen inhibition but also not harmful effects on human, soil and environment health. The demand for biocontrol agent with high specificity against plant pathogen, easily degradable after effective usage and economically beneficial for mass production is the need of a day. There are several rhizospheric plant growth promoters which help plants by producing antimicrobials or interfering with virulence factors of the pathogens (Rezzonico *et al.*, 2005) [38]. A certain group of bacteria which are supposed to

Correspondence**Bhatt Hemangini D**

Department of Biosciences,
Saurashtra University, Rajkot
and Vimal Research Society for
Agrobiotech, VEKO house,
Agiroad, Rajkot, Gujarat, India

know as a plant growth promoters are able to the synthesis of allochemicals such as iron chelating siderophores, antibiotics, lytic enzymes such as lysozymes and antimicrobial peptides (bacteriocins) which have particular significance for bacterial defense (Compant *et al.*, 2005; Thind, 2012) [13, 48]. *Bacillus* can have a potential of antagonistic activity, efficiency for root colonization and increasing host resistance (acts as an immune-stimulators) (Ongena and Jacques, 2008) [29]. *Bacilli* are omnipresent because of their growth characteristics such as temperature tolerance, rapid growth in liquid cultures and spore formation. They are considered as a safe therefore used as a biocontrol agent (Shoda, 2000) [45].

Considering the aforesaid, in search of the better biocontrol agent, in the present study, rhizospheric bacterial strain *Bacillus paralicheniformis* SUBG0010 obtained from the desert of Kutch, India was tested for antibacterial potential against the bacterial pathogens of mango.

Materials and Methods

1. Bacterial strains and growth conditions:

Bacillus paralicheniformis SUBG0010 was isolated and identified from the rhizosphere of *Sporobolus helvolus* of Kutch, Gujarat, India. Bacterial strain was grown overnight on nutrient agar medium (pH-7.0) at 37 °C. Bacterial isolates were grown routinely on nutrient agar medium (pH-7.0) at 37 °C for 24-48 h and preserved in glycerol at -20 °C. The plant pathogenic Gram's positive and negative bacteria used in this study were isolated from infected leaves of different varieties of *Mangifera indica* collected from the Mango orchard (Table 1). Primarily infected leaves of mango were washed continuously under tap water for 3 h then it was treated with 0.1% HgCl₂ for 15 mins. Further, it was washed thrice in sterile distilled water (Rakhashiya *et al.*, 2015) [36]. Infected part of the leaf was inoculated on N-agar plates and plates were incubated at 37 °C for 24-48 h.

Table 1: Isolation source of indicator strains, Gram's character and sequence accession number of NCBI Genebank

Plant Pathogen	Isolation Source	Gram's Character	Accession Number
<i>Exiguobacterium arabatum</i> (M1)	<i>Mangifera indica</i> cv. Jamadar	+ve	KP836352
<i>Bacillus</i> species (M4)	<i>Mangifera indica</i> cv. Betisha Andhra Tirupati	+ve	MF592796
<i>Pantoea ananatis</i> (M5)	<i>Mangifera indica</i> cv. Kesar	-ve	JF802201
<i>Micrococcus luteus</i> (M6)	<i>Mangifera indica</i> cv. Nylon	+ve	KM454981
<i>Microbacterium</i> species (M7)	<i>Mangifera indica</i> cv. Nil Franso	+ve	KM365269
<i>Enterobacter cloacae</i> (M8)	<i>Mangifera indica</i> cv. Manglori Badam	-ve	KP866103

2. Identification of bacterial strains

Bacterial strains were identified by morphological analysis, biochemical tests, and molecular technique. Gram's staining was performed for morphological analysis. Biochemical tests were performed as described in Bergey's Manual of Systematic Bacteriology. Molecular techniques: DNA isolation, 16S rDNA amplification, and 16S rRNA sequencing were carried out for the molecular level identification. Genomic DNA of mango pathogens and the antimicrobial producing strain was isolated using the protocol given by Chudasama and Thaker (2014) [11]. 16S rRNA gene was amplified using universal primer sets: 27F (5'-AGAGTTTGGATCCTGGCTCAG -3') and 1492R (5'-GGTTACCTTGTTACGACTT -3') was used for the amplification of strain SUBG0010 and universal primer set 8F (5'-AGAGTTTGGATCCTGGCTCAG -3') and 1525R (5'-ACGGCTACCTTGTTACGACTT -3') (Viriti™ Thermal Cycler, Applied Biosystems) was used for the amplification of pathogens. Genomic DNA used as a template for amplification of 16S rRNA gene (35 cycles, denaturation – 94 °C for 5 minutes, annealing - 47.5 °C (and 52 °C for 8F, 1525R) for 1 minute, final extension – 72 °C for 12 minutes). 16S rRNA gene sequencing was performed on 3130 Genetic Analyzer (Applied Biosystems, U.S.A.) (Chudasama and Thaker, 2012). These sequences were used for molecular identification with NCBI BLAST tool. 16S rDNA sequences of rhizospheric bacteria and plant pathogens used in this study were submitted to NCBI Genbank Database using BenkIt submission tool.

3. Antimicrobial activity assay

Two different methods were used for the antimicrobial activity against bacterial mango pathogens.

3.1 Co-culture method

Antimicrobial activity was tested initially by co-culture method against mango pathogens. Pure cultures of tested

organisms were inoculated in 10 ml sterile nutrient broth (HiMedia) and incubated for 24-48 h in an incubator shaker at 100 rpm at 37 °C. A pure culture of strain SUBG0010 was inoculated overnight in sterile nutrient broth at 37 °C in an incubator shaker at 100 rpm. Cells of strain SUBG0010 were removed by centrifugation at 10,000 g for 10 min. The 500 µl test culture (mango pathogens) and 500 µl supernatant of strain SUBG0010 were mixed in sterile 2 ml Eppendorf tube at 37 °C for 24-48 h while 500 µl individual test pathogen and/or 500 µl sterile nutrient broth has taken as a control. After incubation, nutrient agar plates were streaked from tubes and incubated at 37 °C for 24-48 h.

3.2 Agar well diffusion method for cell-free supernatant

Bacterial strain SUBG0010 was grown in nutrient broth medium at 37 °C in an incubator shaker at 100 rpm for 24 h. After incubation cell free supernatant was collected by centrifugation at 10,000 g for 10 min at room temperature, then passed through 0.4 µm sized sterile membrane filter. The 50 ml cell-free supernatant was sterilized by autoclaving (Autoclaved supernatant) at 120 °C, 15 lbs for 15 min. The antimicrobial activity was performed as described by Tiwari and Srivastava (2008) [49], filter sterilized supernatant and autoclaved supernatant used as an antimicrobial agent. In brief, 10 ml soft nutrient agar (0.8% agar) seeded with 24-48 h old 100 µl individual plant pathogenic indicator strain grown in nutrient broth was overlaid on nutrient base agar (2.5% agar). The wells cut out (19 mm) on only soft nutrient agar on plates, wells were filled with 200 µl of crude antimicrobial agents (filter sterilized or autoclaved supernatant). The inoculated plates were pre-incubated at a low temperature (in a refrigerator at 4 °C) (Rios *et al.*, 1988) [40] to retard the growth of pathogens before diffusion of an antimicrobial agent. Then the plates were incubated overnight at 37 °C. After incubation, the zone of inhibition was measured. A test was performed in duplicates and repeated two times.

3.3. Partial purification of antimicrobial protein and agar diffusion assay

Bacterial strain SUBG0010 was grown for 24 h at 37 °C in an incubator shaker at 100 rpm in sterile nutrient broth. The cells were removed by centrifugation at 10,000 g for 10 min at room temperature. Supernatant allowed precipitating overnight with 80% ammonium sulfate at 4 °C. From one liter of broth, the precipitates were collected by centrifugation at 10,000 g for 20 min and dissolved in 15 ml of 20 mM phosphate buffer (pH-6.8) and preserved at 4 °C.

Before using partially purified protein as an antimicrobial agent it was autoclaved at 120 °C, 15 lbs for 15 min. Antimicrobial activity of the partially purified protein was performed by agar well diffusion method as described above. The inoculated plates were pre-incubated at a low temperature (in a refrigerator at 4 °C) (Rios *et al.*, 1988) [40] to delay the growth of pathogens before diffusion of partially purified protein. Then the plates were incubated overnight at 37 °C. After incubation, the zone of pathogen growth inhibition was measured. A test was performed in duplicates. Protein content was determined according to the Lowry *et al.*, (1951) [25] using bovine serum albumin as a standard.

Results and Discussions

WHO banned many chemical pesticides which are causing pollution and damage to the ecosystem. It is strictly being pursued by some developed countries (Barnard *et al.*, 1997; Thind, 2012; Shafi *et al.*, 2017) [5, 48, 43] but it is desirable globally, to replace harmful chemical pesticides with the resources that are not the only alternative of chemical pesticide but also provide control of disease that cannot be managed by other strategies (Shoda, 2000) [45]. *Bacillus* species have become attractive to control a broad range of plant pathogens because of their ability to produce hard, resistant endospores and antibiotics (Cavaglieri *et al.*, 2005) [7].

In the present study, total six Gram positive and Gram negative bacterial mango pathogens were isolated from the infected leaves of *Mangifera indica* cultivars (Table 1). The strain SUBG0010 was isolated from the rhizosphere of *Sporobolus helvolus*, a high palatable grass species of Kutch. It is Gram positive; rod shaped bacteria and was able to produce antimicrobial substance. Strain SUBG0010 is positive to catalase, urease, oxidase activities and utilizes citrate.

The genomic DNA of SUBG0010 and pathogens was successfully isolated by using standard protocol and confirmed by 1% agarose gel electrophoresis. An approximately 1517 bp and 1465 bp DNA fragment of 16S rDNA of SUBG0010 and bacterial pathogens, respectively was amplified using universal primer set (27F, 1492R and 8F, 1525R, respectively). Amplified 16S rRNA gene region was sequenced. 16S rDNA sequence similarity determined using NCBI BLAST, strain SUBG0010 showed 98% identity with *B. paralicheniformis* strain LBT22. The sequence of strain SUBG0010 was submitted to NCBI under the accession KX094518. Sequences of 16S rDNA of plant pathogenic strains were submitted to NCBI and accession numbers in Table 1.

A number of methods are used to study antimicrobial activity *in vitro*; all the methods have their own principles, advantages, and limitations (Bhatt *et al.*, 2003) [6]. Further, the results obtained are also be greatly influenced by the method selected, microorganisms used for the test and the degree of solubility and rate of diffusion of each test compound. In this study, two different methods are used for the antimicrobial

activity i.e. co-culture method and agar well diffusion method (Fig.1).

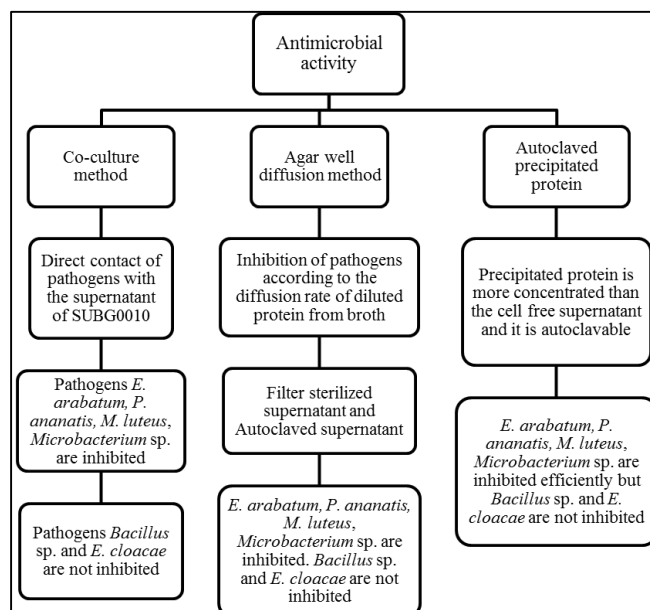


Fig 1: A schematic diagram of methods used for the antimicrobial activity against *M. indica* pathogens.

Co-culture method has used to study the interaction between cell populations and cell-cell interactions. It is expected that the bacterial ability to produce antimicrobial substances may be stimulated by an antagonistic interaction of bacteria with the pathogens that may enhance the production of new drugs against infectious diseases (Ueda and Beppu, 2016). Therefore, this method is used to engineer bacterial cells for the purpose of pathogen killing (Saeidi *et al.*, 2011) [42]. In the present study, antimicrobials resulted to give complete inhibition in co-culture method within broth medium because of direct contact of phytopathogens with antimicrobial agent produced by strain SUBG0010. In co-culture method (3.1), mango pathogens, *P. ananatis*, *M. luteus*, *Microbacterium* sp. *E. arabatum* were completely inhibited by *Bacillus Paralicheniformis* SUBG0010 which was confirmed on nutrient agar plates by streaking, showed pathogenic isolates were inhibited (Fig 2). *Bacillus* sp. (M4) and *E. cloacae* (M8) were not inhibited and the colonies of plant pathogens were observed on test nutrient agar plates.

The second method, the agar well diffusion is used to evaluate the antimicrobial activity of plants or microbial extracts (Valgas *et al.*, 2007; Magaldi *et al.*, 2004) [51, 26]. This assay offers many advantages over other antimicrobial test methods. It is simple, more economic, the ability to test many numbers of microorganisms, antimicrobial agents and provide easy to interpret the results. In this study, first, filtered sterile and/or autoclaved supernatant was used and tested against the mango pathogens using the agar diffusion method. The antibacterial activity was quantitatively assessed by measuring the diameter of the inhibition zone around agar well, which was measured in millimeter. In agar well diffusion method, organisms were inhibited up to the antimicrobial compound diffused. This may be due to the diffusion rate of the antimicrobial compound which might have limited the interaction with the pathogens. The highest growth inhibition was observed in *Microbacterium* sp. (15.8mm) followed by *E. arabatum* (10.25 mm), *M. luteus* (10.1 mm) and *P. ananatis* (9.5 mm). *E. cloacae* (M8) and *Bacillus* sp. (M4) are not inhibited by filter sterilized

and/or autoclaved supernatant (Fig 3). Many studies report that the *Bacillus* sp. secretes many metabolites including toxins, bacteriocins, antibiotics, and iron chelating siderophores (Peypoux *et al.*, 1980; Leong, 1986; Oscáriz *et al.*, 1999; Gong *et al.*, 2015; Zhi *et al.*, 2016) [31, 23, 30, 18, 55]. This output lead to two possibilities; (i) the supernatant has limited influence on pathogens and/or (ii) the concentration of the effective compound is less in the supernatant and hence *E. cloacae* (M8) and *Bacillus* sp. (M4) might have not inhibited. Two address the second possibilities; we have used precipitate protein, assuming one of the antibacterial compounds (may be bacteriocin?) of *B. paralicheniformis* SUBG0010.

In the second set of experiments, the protein was precipitated from the filter-sterilized supernatant using ammonium sulfate and the precipitates were dissolved in phosphate buffer (20 mM, pH-6.8). It was followed by agar well diffusion method to determine the antimicrobial activity against mango pathogens. Autoclaved precipitated protein showed high antimicrobial activity against *M. luteus* (13 mm) and *P. ananatis* (9.5 mm) than filter sterilized and autoclaved supernatant of strain SUBG0010 (Fig 4). The concentration of the precipitated protein was 4.89 mg/ml. It was autoclaved before using as antimicrobial properties against the pathogens. It showed marked inhibitions in the pathogens tested except the *Bacillus* strain (M4) and *E. cloacae* (M8). It is also

interesting to note that the protein used as an antibacterial agent can be autoclave and thus it is thermostable. Antibacterial activity by *Bacillus* sp. against *M. luteus* has been already described by Awais *et al.*, (2007) [3], Xie *et al.*, (2009) [53], Riazi *et al.*, (2009) [39], Mouloud *et al.*, (2013) [28], Ayed *et al.*, (2015) [4], Goh and Philip, (2015) [17], Collins *et al.*, (2016) [12] against pathogenic strains and culture collection center strains. *E. cloacae* and different species of genus *Bacillus* were inhibited by bacteriocin producing strain *B. thuringiensis* (Kamoun *et al.*, 2005) [20] but *B. paralicheniformis* showed no inhibition in this study (Fig.5).

Species of genus *Bacillus* are generally considered as a safe microbe and have ability to produce many biologically active compounds such as cyclic lipopeptides of the surfactin, iturin and fengycin families have potential that have been used in biotechnology, biopharmaceutical, agriculture and industrial purposes because of their surfactant properties as well as produced secondary metabolites which found to show antibacterial and antifungal activity against different plant pathogens (Katz and Demain, 1977; Yu *et al.*, 2002; Stein, 2005; Ongena and Jacques, 2008) [21, 54, 46, 29]. *Bacillus* species are recognized to produce a range of antimicrobials, either antibiotic (e.g. gramicidin, bacitracin) or bacteriocins (e.g. thuricin CD, mersacidin) (Katz and Demain, 1977; Chatterjee *et al.*, 1992; Rea *et al.*, 2010) [21, 8, 37].

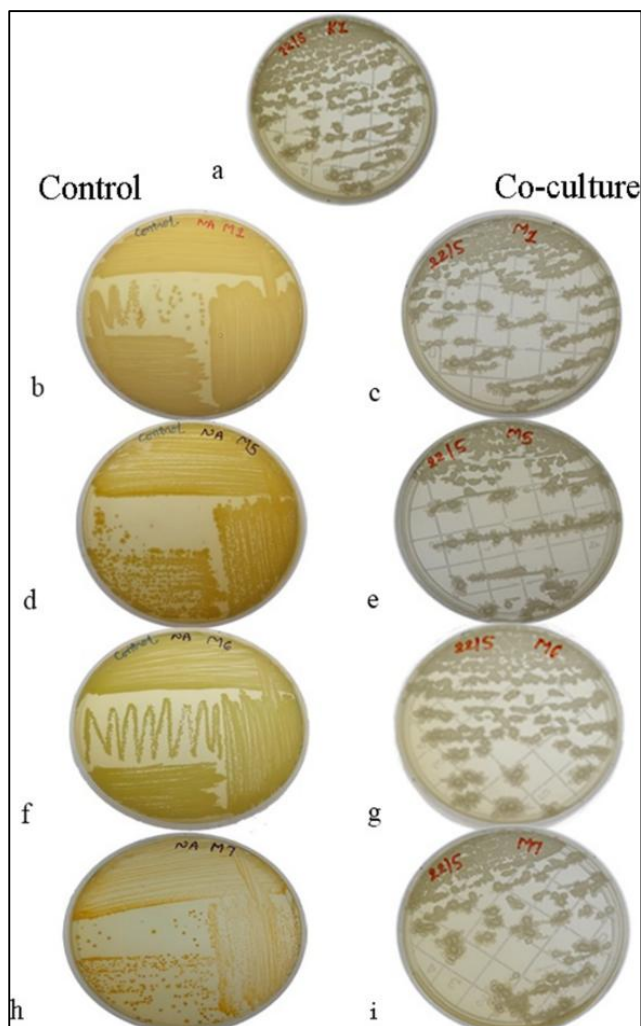


Fig 2: (a) Colony morphology of *B. paralicheniformis* SUBG0010; (b, c) *E. arabatum*, (d, e) *P. ananatis*, (f, g) *M. luteus*, and (h, i) *Microbacterium* sp., respectively. The antibacterial activity by co-culture method showed colonies of SUBG0010 against tested pathogens.

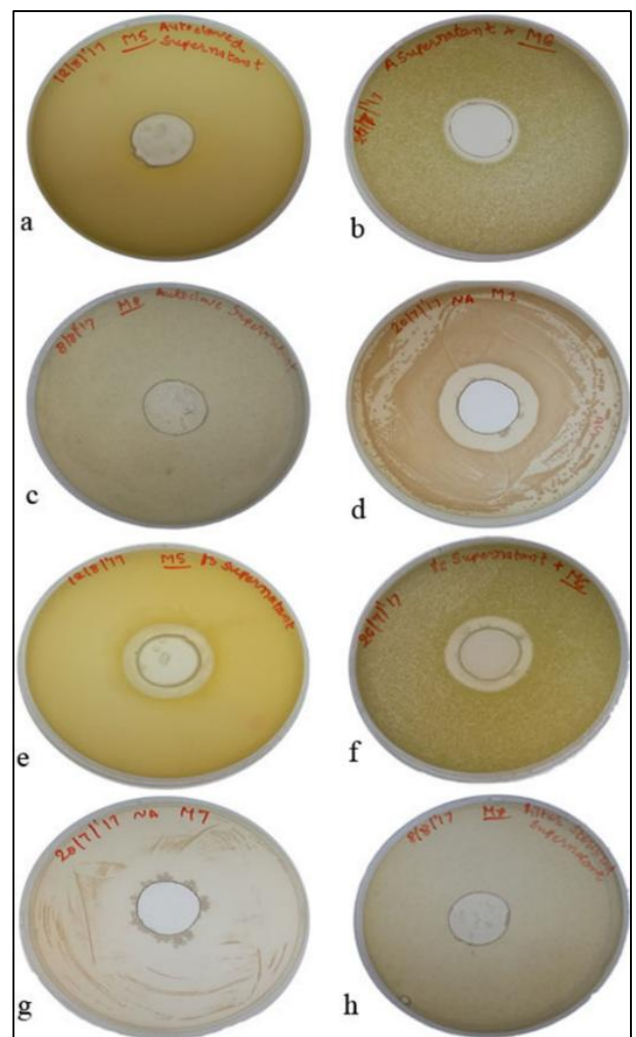


Fig 3: Inhibition of pathogens (a) *P. ananatis*, (b) *M. luteus* and (c) *E. cloacae* with filter sterilized supernatant; (d) *E. arabatum*, (e) *P. ananatis*, (f) *M. luteus*, (g) *Microbacterium* species and (h) *E. cloacae* by autoclaved supernatant of SUBG0010

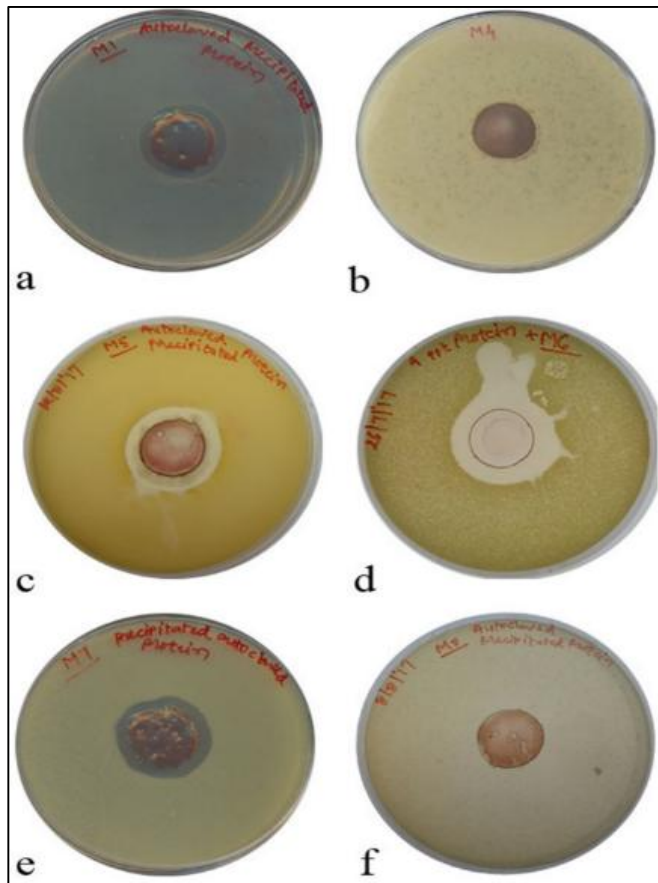


Fig 4: Antibacterial activity of autoclaved precipitated protein of *B. paralicheniformis* SUBG0010 against (a) *E. arabatum*, (b) *Bacillus* sp., (c) *P. ananatis*, (d) *M. luteus*, (e) *Microbacterium* sp., (f) *E. cloacae* pathogens.

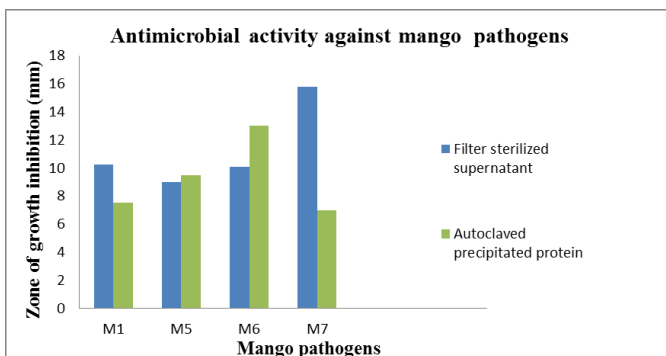


Fig 5: Antimicrobial activity of the filter sterilized supernatant and autoclaved precipitated protein of SUBG0010 against bacterial mango pathogens

Conclusions

The present study revealed that in co-culture method *Bacillus paralicheniformis* SUBG0010 may be secreting certain antimicrobial substance or antibiotic which directly affect the pathogen culture and inhibit the growth. The *Bacillus paralicheniformis* may be produced the bacteriocin or similar protein molecules that inhibit the bacterial growth. This strain showed a remarkable anti mango pathogen activity. Further research requires to identify the antimicrobial substance is bacteriocin or similar protein molecule responsible for inhibiting the growth of these plant pathogens.

Acknowledgement

Authors are thankful to UGC-CAS Department of

Biosciences, Saurashtra University, Rajkot and Vimal Research Society for Agro-biotech for lab facilities and support for this work.

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