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Exraction of Secondary Metabolites Using *Bacillus endopyticus* and Its Applications

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Authors' contributions

This work was carried out in collaboration among all authors. Author SK carried out the work. Author KTM designed the work and managed to analyse all the data. Authors JJ and RR assisted the work and managed the analyses of data. All authors read and approved the final manuscript.

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ABSTRACT

Bacillus Species was isolated from a soil sample, the isolate was cultivated, identified and its culture medium was extracted. Specific media called Gibbon's media used for the cultivation and confirmation of the bacteria. 16S rRNA study was done for the morphological identification of the isolate. The crude which was collected from *Bacillus endopyticus* was purified by column chromatography. The antibacterial activity of the crude extract was identified by agar diffusion test by measuring the minimum inhibitory concentration by dilution assay. The purified extract was active against *Staphylococcus aureus, Bacillus cerues, Escherichia coli*. The study suggest that Bacillus Sps have the potential to produce antibiotics and can be used to control the growth in future. The further work was able to bring the microbial strains under control. The percentage of cell death was calculated the cell death was 97.95%.

Keywords: Bacillus endopyticus; 16S rRNA; gibbon's media; antimicrobial activity.

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1. INTRODUCTION

Chemical food additives are known to cause extremely deleterious effects on the human health. Food additives are one of the major causes for allergic (hypersensitivity) reactions [1]. Food additives are also known to induce hyperactivity in young children [2]. Other known harmful effects of chemical preservatives include weakened heart tissue and cardiovascular diseases [3]. Butylhydroxyanisole (BHA) and Butylhydroxytoluene (BHT), commonly used antioxidants in food preservation, are known to be carcinogenic and toxic at higher doses [4]. The various harmful effects of the chemical additives used in the food industry have been widely reviewed throughout the scientific literature by many authors. Works by Sharma M et al. [5] and Anand SP et al. [6] are two among the noteworthy papers that provide exhaustive lists of food preservatives and their respective harmful effects on human health.

This being said about the chemical preservatives, research in the exploration of safer alternatives to chemical additives is expanding vastly. The use of bacterial secondary metabolites with anti-microbial potential as food preservatives is one proven alternative to chemical additives. Lactic acid bacteria (LAB) are one of the most widely used organisms in bio-preservation of foodstuffs. The use of LAB Bacteriocins like nisin and sonorensin in bio-preservation has been implied [7].

A wide range of anti-microbial peptides is known to be produced by *Bacillus sp.* that find many applications in bio-preservation [8,9]. *Bacillus sp.* are known for their survival in extreme environmental conditions and in response to these environments, they produce a host of secondary metabolites, which aid in their survival [10]. These metabolites are of great interest to researchers, due to their applications in various fields important to humans.

The present study is centered on the anti-microbial potential of secondary metabolites isolated from *Bacillis sp.* and their applications in the bio-preservation of foodstuffs. In this study, we have isolated and characterized bacteria from soil with possible anti-microbial potential. Three organisms belonging to the genus *Bacillus* were identified: *Bacillus subtilis, Bacillus endopyticus* and *Bacillus tequilensis.* These three bacteria have already been implicated in many fields of scientific enquiry.

Bacillus subtilis as an important organism [11]. An organism with both anti-microbial potential as well as a probiotic action is very valuable in the field of bio-preservation. This is because the organism can serve a two-way purpose of preserving the food material as well as enriching its nutritional composition as a probiotic.

Bacillus endopyticus is known for its anti-fungal activity and has been implicated in the control of fungal pathogens in potato [12]. This anti-fungal activity can also be exploited in the preservation of foodstuffs.

Bacillus tequilensis is known to produce a wide array of antibiotics [13] that can be effectively used against common food contaminating microorganisms. This paves the way for utilization of this bacterium as a bio-preservant.

2. MATERIALS AND METHODS

2.1 Apparatus

Orbital shaker (KEMI)
Incubator (TECHNICO)
Column chromatography (HIGH MEDIA MUMBAI)
Laminar air flow (TECHNICIAN INDIA)
Centrifuge (REMI INDIA)
UV Transiluminator (MEDICORP)
Colorimetry (ELICO)
Autoclave (LABTRONICS)
UV Spectrometer (LABTRONICS)

2.2 Collection of Samples

18 soil samples were collected in clean dry sterile container from 2-5 cm below the surface along with sterile spatula in different agricultural field in Coimbatore, Tamilnadu, India. All the samples were transferred aseptically to the laboratory.

2.3 PCR Amplification

PCR products characteristic for gene have been sequenced in order to confirm the identity of the organism and the obtained sequence was deposited in GenBank Database (Accession Number MK156388). Its comparison with existing sequences shows 99% identity. These findings fully confirmed that isolated strain was *Bacillus endopyticus* (Altschul et al., 1997).

2.4 DNA Sequencing

The BLAST search of Genbank for isolates provided the percentage similarity between the microorganism tested and those detected in Genbank(Rajapandi et al 2016). The gene bank accession numbers as follows. *Bacillus* subtilis (MK 156386), *Bacillus endopyticus* (MK 156388), and *Bacillus tequilensis* (MK 156389).

Among these, the three Bacillus was selected based on the screening methods and used

2.4.1 Isolation of soil bacteria using serial dilution

1g of soil was dissolved in 10ml of sterile distilled water and the suspension was serially diluted. The dilutions 10^{-2} to 10^{-4} were plated onto sterilized nutrient agar medium using the pour plate method.

2.4.2 Screening of soil bacteria

The suspected colonies (based on colony morphology) were sub cultured on Nutrient Agar, Casein Agar and Starch Agar. The zone of clearance around the colonies in the casein agar and starch agar confirms the identity of *Bacillus sp.*

2.5 Molecular Identification of *Bacillus sp*

2.5.1 DNA isolation

The genomic DNA of the bacteria was isolated using standard procedures described by Susan et al.

2.5.2 PCR amplification

The PCR mix was prepared by adding 1µl template DNA, 10µl 10X Taq DNA polymerase Assay Buffer, 1µl Taq DNA Polymerase Enzyme (3U/µl) and 2.5mM dNTPs. 400ng/µl each of forward (5'-AGAGTTTGATCMTGGCTCAG -3') and reverse (5'-TACGGYTACCTTG TTACGACTT -3') primers were added to the PCR master mix. The final volume was made up to 100µl with sterile distilled water. The PCR program was set as follows: Initial denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 30 sec (denaturation), 55ºC for 30 sec (Annealing), 72ºC for 1.30 min (Extension) and 72ºC for 5 min (Final Extension).

2.5.3 Extraction of crude metabolites from *Bacillus sp*

For the extraction of metabolites, the bacterial culture was inoculated into Gibbons Broth (containing 1.25g NaCl, 0.05g $MgCl_2$, 0.05g KCl, 0.5mg $FeCl_2$, 0.25g Casein Acid Hydrolysate and 0.5g Yeast extract per 100ml of sterile distilled water). The grown broth (24hr) was centrifuged at 10,000 rpm for 5 min. 5 ml of supernatant was added with 5 ml of diethyl ether (solvent) and was incubated at 37ºC for 24 hrs. This mixture was centrifuged again at 5,000 rpm for 5 min. To 2 ml of supernatant obtained, 2 ml of chloroform (solvent) was added. The mixture was incubated at room temperature for 24 hrs. The upper aqueous layer was collected into an Eppendorf tube.

2.5.4 Production of secondary metabolites

Inoculum was prepared and added in the Gibbons broth and the plates were incubated at 30ºC for 24 hrs. [14].

2.6 Production Media

Specific media called Gibbon's media used for the cultivation and confirmation of the bacteria. It consists of 25g of sodium chloride, 1g of Magnesium chloride, 1g of potassium chloride, 0.01g of Ferrus chloride, 5g of casine acid hydrolysate,10g of yeast extract.

2.6.1 Extraction of secondary metabolites

The crude extracts from *Bacillus subtilis*, *Bacillus endopyticus*, *Bacillus tequilensis* grown Gibbons broth and LB broth and Nutrient broth and kept on shaker for (24 hours) to mix well and incubated in incubator for 2 days. This three-tube culture were added with 5ml of Diethyl ether and incubated at 37ºC for 24 hrs. After incubation, these three-tube cultures were subjected to centrifuge at 5000 rpm for 5 minutes. 2ml of centrifuged supernatant was added with 2ml of chloroform transferred to fresh tube and the tube was covered and incubated at 37ºC for 24 hrs. After incubation 1ml of supernatant from this tube is transferred into Eppendorf tube. These broths were inoculated with 200µl of Escherichia coli broth culture and incubated at 37ºC for 24 hrs. These tube cultures were subjected to growth inhibition study and taken reading at 600nm in colorimeter.

2.7 Characterization of Compounds

2.7.1 UV-visible spectroscopy

The secondary metabolite extracts of the bacteria were analysed using a UV-Vis

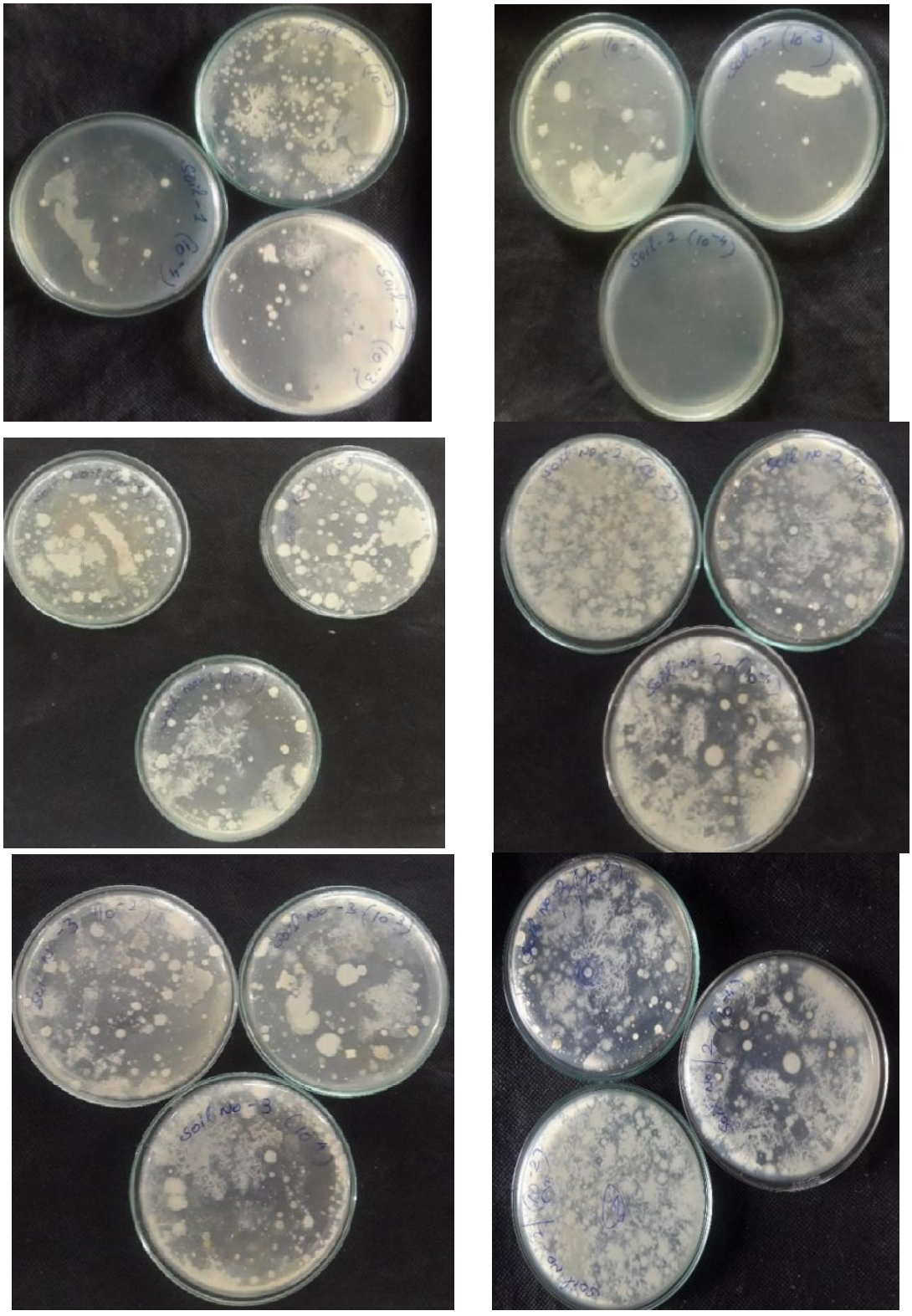

Fig. 1. The bacteria were identified

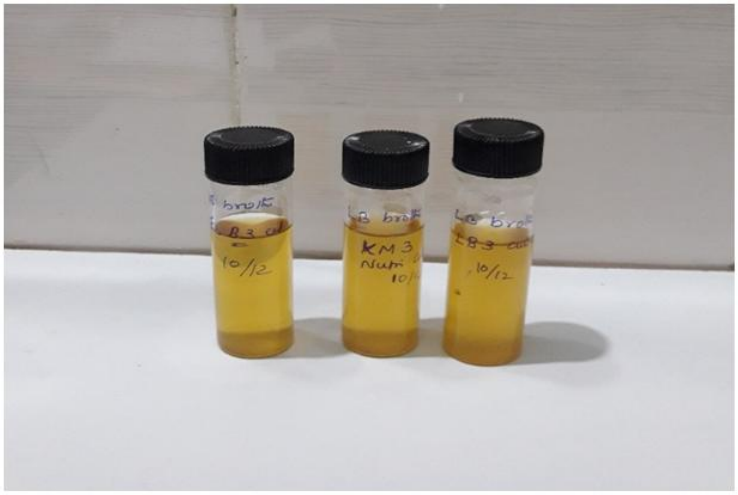

Fig. 2. Gibbon's media

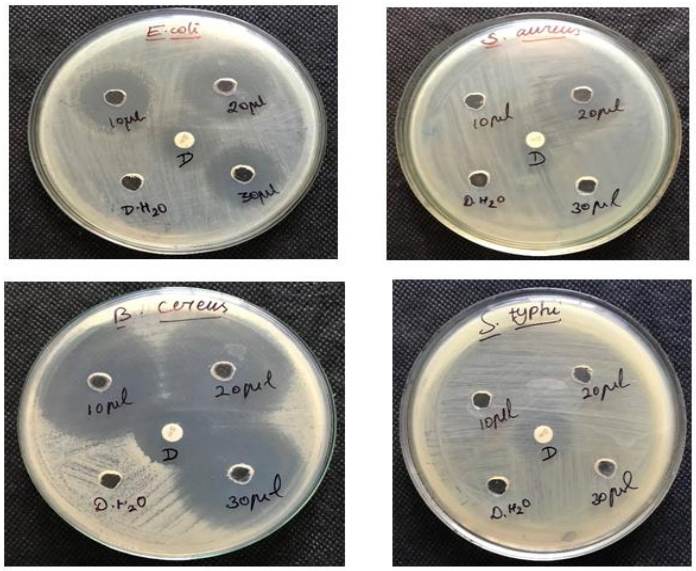

Fig. 3. Antimicrobial activity

spectrophotometer. The chloroform extracts were used for this analysis, and chloroform was used as blank. The absorption spectrum of each active extract was determined in the UV region (200-400nm). The absorption maxima values (peaks) were recorded [15].

2.7.2 Thin layer chromatography

The biologically active anti-microbial compounds present were analysed by using pre – coated thin layer chromatography silica gel TLC plates. The TLC was run with butanol: acetic acid: distilled water (3:1:1). The spots were developed with a ninhydrin spray after drying the plates for 5 – 10 min at 110°c. Retention values (Rf) were calculated for TLC ninhydrin positive bands [16].

2.7.3 Fourier transform infrared spectroscopy

FTIR helps to explore the functional groups and the chemical bonds present in the crude extract. The spectra were scanned in the range of 400-4000 cm^{-1}. The spectra were obtained using potassium bromide (KBr). The spectra were plotted as intensity versus wave number. The spectrum was studied to interpret the functional groups present in the extract [17].

Table 1. Zone of inhibition of extracts Bacillus endopyticus grown in Gibbons broth

Microorganism	Zone of inhibition (mm)				
	Dilutions				
	10 µl	20 µl	30 µl	H^2O	E15
E.coli	10mm	10mm	11mm	Nil	5mm
Staphylococcus aureus	Nil	6mm	Nil	Nil	3mm
Bacillus cereus	12mm	14mm	15mm	Nil	6mm
Salmonella typhi	Nil	Nil	Nil	Nil	Nil

2.7.4 GCMS Study

The diluted sample was heated at 60°C for 30 min and 1 ml of the headspace was injected using a gas syringe. The column was maintained at 40°C for 2 min, then at 250°C for 13 min after the temperature had been increased at 6°C per min. The injection port and interface were at 240°C and 200°C, respectively. The mass spectra of various compounds were compared with those in the NIST/EPA/NIH MS Library (version 2.0) [18].

2.8 Antimicrobial Activity

Three kinds of compound extracts are used by disk diffusion method were tested against pathogens including *E.coli, Staphylococcus aureus, Bacillus cereus, Salmonella typhi.*

3. RESULTS AND DISCUSSION

3.1 Isolation of Bacteria

3.1.1 Antimicrobial activity of *Bacillus endopyticus* against test pathogens

The crude extracts from *Bacillus endopyticus* grown Gibbon's broth was prepared and using two different solvents chloroform and ethyl acetate and was used for the antimicrobial assay. Compounds extracted from *Bacillus endopyticus* grown in Gibbons broth respectively. The extracts were active against *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* [13].

Antimicrobial activity of *Bacillus endopyticus* strain against test pathogens was observed in terms of clear zone around the well. This indicates the zone of inhibition measured in mm Table 1.

4. CONCLUSION

The newly isolated *Bacillus* species from soil was carried out to evaluate the production of antibiotic. The results showed that the obtained isolate have the potential for producing antimicrobial substances. Antimicrobial study showed inhibitory effects against some Gram negative and Gram-positive pathogenic bacteria.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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