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Isolation and Identification of Antimicrobial Compounds from *Bacillus Cereus* Obtained from Soil

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Abstract

Bacillus cereus cell-free culture filtrate from soil was highly antibacterial. Two bioactive substances were isolated from the bacterial culture filtrate ethyl acetate extract by silica gel column chromatography. The compounds' structure and stereochemistry were determined by spectroscopic investigations (UV and FTIR) and Marfey's technique. The molecules were cyclo(L-Pro-L-Val), and 3,5-dihydroxy-4-ethyl-trans-stilbene. Pseudomonas aeruginosa Staphylococcus aureus, and Escherichia coli were all inhibited by compounds both the isolated molecules. Best antibacterial activity was observed with 3,5-dihydroxy-4-ethyl-trans-stilbene (6 µg/ml) against Escherichia coli. We conclude that the Bacillus cereus strain from soil is a promising source of natural bioactive secondary metabolites that might be used to develop new medications in agriculture and pharmacology.

Keywords: *Antibacterial, Antimicrobial,Bacillus cereus, cyclo(L-Pro-L-Val), 3,5-dihydroxy-4-ethyl-trans-stilbene,*

Introduction

The term bacillus refers to a small rod, while cereus can be translated to mean wax-like from Latin (Marrollo, 2016). This name is aptly chosen as B. cereus has easily recognizable morphology when viewed in the microscope or on blood agar plates (Stenfors Arnesen et al., 2008). Bacillus cereus is a bacterium that has a large, rod-shaped structure that measures 1.0–1.2 µm by 3.0–5.0 µm (Zolock, 2002). Its colonies on ordinary agar media are large, with a diameter of 3-8 mm, and appear rather flat, greyish, and ground-glass in appearance, often with irregular borders (De Been, 2009).

Bacillus cereus is a peaceful soil dweller that can be found in a variety of settings and even in some animal gut flora (Pistone et al., 2021). Because it can produce endospores that are resistant to heat, dehydration, and other physical pressures, it can endure time and hard settings (Batt, 2000). Because of its extremely sticky endospores, which can cling to a wide variety of foods, Bacillus cereus is frequently found in settings where food is produced (Zhao et al., 2008). Bacillus cereus, B. thuringiensis, B. anthracis, B. weihenstephanensis, B. mycoides, B. pseudomycoides, and B. cytotoxicus are the seven closely related species that make up the Bacillus cereus group (Pfrunder et al., 2016). These species can be further classified into seven phylogenetic groups. The B. cereus group has a convoluted and unclear classification. B. pseudomycoides, B. weihenstephanensis, and B. mycoides strains clustering in group VII (B. cytotoxicus), and B. cereus, B. thuringiensis, and B. anthracis strains dispersed over groups II, III, IV, and V without forming clusters according to their "species" B. cereus, B. thuringiensis, and B. anthracis are all members of the same species, according to a comparison of housekeeping genes using multilocus sequence typing (MLST) and multilocus enzyme electrophoresis (MEE) (Lindbäck and Granum, 2019).

It is a bacterium that is commonly found in nature and occurs in different environments such as soils, sediments, dust, and plants (Ehling-Schulz et al., 2019). The bacteria are present in the form of spores, which can be passively spread outside their natural habitats (Majed et al., 2016). When the spores come in contact with organic matter, or an insect or animal host, they germinate, grow, and sporulate. B. cereus has a saprophytic life cycle and can also exist in a multicellular, filamentous mode of growth, which has been observed in soil and insect guts (Vilain et al., 2006; Bottone, 2010)).

Currently, several types of pathogenic bacteria detrimentally influence human health and cause diseases in them (Flandroy et al., 2018). The groups of pathogenic bacteria generally known to infect humans are E.coli, S.aureus and P. aeruginosa (Mancuso et al., 2021). Hence, various efforts are made to identify potential biological control agents that could potentially reduce infections caused by those pathogenic bacteria. *Bacillus cereus NC7401* is aspecies of bacteria isolated from soil regions in which no study has been reported so far regarding the antimicrobial potential of its isolates. Therefore, this study is specifically aimed to isolate the metabolites from *Bacillus cereus NC7401 and to* examine their potential against E. coli, S. aureus and P. aeruginosa.

Methodology

Media and Chemicals

Merck, located in Mumbai, India supplied high-performance liquid chromatography (HPLC) grade methanol, and all other chemicals utilized in the extraction and column chromatography processes were of analytical grade. Merck also supplied the precoated silica gel 60 GF254 plates for Thin Layer Chromatography (TLC) and the silica gel (230–400 mesh) used for column chromatography. Hi-Media Laboratories, located in Mumbai, India, provided the microbiological media. The other compounds employed in this investigation were of the \greatest purity, and every other reagent was analytical grade. Chemsketch was the program utilised for the chemical structure drawing.

Sample collection, primary screening, secondary screening and molecular identification of bacteria

In our previous paper we have mentioned methods for isolation and molecular identification of Bacillus cereus. The isolate *Bacillus cereus NC7401* were identified as Gram-positive by 16S rRNA studies. It showed antibacterial activity against all the mentioned human pathogens S. aureus, E. coli, and P. aeruginosa. They were found to be Gram-positive and showed positive activity for Catalase, Nitrate reduction, Casein hydrolysis, Voges-Proskauer, Salicin, Fructose and Sucrose. The 16sRNA was amplified from the isolated DNA sample using PCR. 1.2% of agarose gel was used to verify the amplified products which showed a fragment of 1.5 kb. The amplified 16s r RNA were subjected to purification and sequencing. The results confirmed the bacterial samples to be *Bacillus cereus NC7401*.

Extraction and fermentation

Tryptone 17 g/l, soytone 3.0 g/l, glucose 2.5 g/l, NaCl 5.0 g/l, beef peptone 10 g/l, water 1,000 ml were used for bacterial fermentation. The flask with 100 ml sterile media received one *Bacillus cereus NC7401* strain colony from the agar plate. For 24 h, the flasks were shaken (150 rpm) at 30 °C in the dark. With an optical density of 1.7 at 600 nm, the bacterial cultures were aseptically transferred into 400 ml sterile media and incubated in the dark for 96 h in the gyro rotatory shaker at 30 °C. After centrifugation (10,000 g, 20 min, 4 °C), the culture media were filtered through a 0.45-µm filter to extract cell-free filtrate. Neutralized with strong hydrochloric acid, 30 liters of cell-free culture filtrate were extracted three times with equal volume ethyl acetate. Ethyl acetate layers were mixed, dried over anhydrous sodium sulfate, and concentrated at 30 °C using a rotary flash evaporator. (Kumar et al., 2014)

Bioactive compound purification

After drying, the crude extract (9.3 g) was loaded onto a silica gel column (25 × 600 mm) and eluted with various solvents, including hexane and ethyl acetate. Two 100-ml portions were taken from each combination. Each fraction was tested for antibacterial activity against E. coli, the initial test bacterium, using agar well diffusion assay. To each well (6 mm), 50 µl of crude extract was applied and incubated at 35 °C for 24 hours.

The highly antibacterial methanol fraction was purified with a second column. 2.8 g of methanol fraction was loaded onto a silica gel column (10 × 300 mm) and eluted with 100 ml each of chloroform, linear gradient chloroform: acetone (v/v, 75:25 to 25:75), acetone, and linear gradient acetone: methanol (v/v, 75:25 to 25:75), and finally 100% methanol. Antibacterial activity against E.coli was tested on fractions. Compound purity was verified using TLC (silica gel) and HPLC. (Kumar et al., 2014)

Measurements by spectrum

FTIR-spectroscopy The powdered samples were examined by Fourier transform infrared spectrophotometer (IR Infinity, Shimadzu, Japan in the range of 4500–500 cm−1) (Sethi et al., 2019). An Indian Systronics twin beam spectrophotometer 2201 UV–VIS spectrophotometer and a Rudolph Research Autopol III polarimeter were used to capture UV spectra and optical rotations.

Marfey's approach for compound absolute configuration

Two compounds (1.5 mg) in 6 M HCl (1 ml) were heated to 120 °C for 24 h. After drying, the residue was redissolved in H_2O (100 µl), deposited in a 1-ml reaction vial, and treated with a 2% FDAA solution in acetone and 1.0 M $NaHCO_3$ (40 µl). After heating at 47 °C for 1 h, the reaction mixture was cooled to room temperature and acidified with 2.0 M HCl (20 µl). Similarly, typical D- and L-amino acids were derivatized independently. Derivatives of hydrolysates and standard amino acids were analysed using Shimadzu LC-20AD (C18 column, 5 µm, 4.6 × 250 mm; 1.0 ml/min) at 30 °C and a gradient programme: solvent A, water+0.2 % TFA; solvent B, MeCN; linear gradient 0 min 25% B, 40 min 60% B, 45 min 100% B; UV detection at 340 nm (Marfey 1984).

Determination of antibacterial activity

Minimum inhibitory concentration (MIC) MIC was measured using standard macro-dilution broth test against three test microorganisms. A stock solution of 2,000 µg/ml of the test compounds and standard antibiotics was generated, which was further diluted with methanol to give the requisite concentrations 1,000 to 1 µg/ml. The tubes were incubated at 35 °C for 24 h. The MIC value was determined as the lowest concentration of the drug displaying no observable development.

Minimum bactericidal concentration (MBC) MBC was measured according to the method of (Fyfe,1998) against all three test microorganisms. About 100 µl from the MIC tubes not indicating growth were serially diluted and plated on nutrition agar plates. The plates were incubated at 35 °C for 24 h. MBC is the lowest concentration at which bacteria failed to grow in nutrient agar injected with 100 µl of suspension.

Agar disc diffusion method

Compounds were tested for antibacterial and antifungal activity using agar disc diffusion assay against various bacteria and fungi. The sterile disks were infused with the minimum inhibitory concentration (MIC) of the test compounds. The antimicrobial activity was then assessed by measuring the zone of growth inhibition surrounding the disks. Positive reference standards for bacteria included ciprofloxacin. All assays were performed three times. (Eja et al., 2007)

Statistical analysis

All statistical analyses were done with SPSS. Statistical significance was assigned to $p < 0.05$.

Results

Purification and isolation of bioactive substances

Antibacterial activity against *E.coli* was demonstrated by the ethyl acetate extract of the bacterial cell-free culture filtrate. Two fractions were obtained from this extract's silica gel column chromatography in the first column. These fractions were then further refined by crystallizing the extract in hexane and benzene to produce two white-crystal compounds. Good antibacterial activity was found in the methanol fraction produced from the first column, which was then further purified using second-column chromatography to yield one pure compound.

Table 1: Isolation and purification of isolated compounds

Compound	Column Solvent	Yield (mg)	Rf value	Retention Time
1	28% ethyl acetate	10	0.32	2.765
2	35% ethyl acetate	8	0.31	2.459

Testing these chemicals against the indication test bacterium *E. coli* verified their initial bioactivity. The two compounds underwent reverse-phase HPLC analysis, and the chemicals were eluted as separate peaks (Table 1). The peak region indicates that the compounds' purity was more than 90%.

Finding the bioactive substance

The pure compounds were examined using a variety of spectroscopic techniques, including FTIR and UV. The chemicals that were found are 3,5-dihydroxy-4-ethyl-trans-stilbene as depicted in Figure 2 and cyclo (L-Pro-L-Val) as shown in figure 1

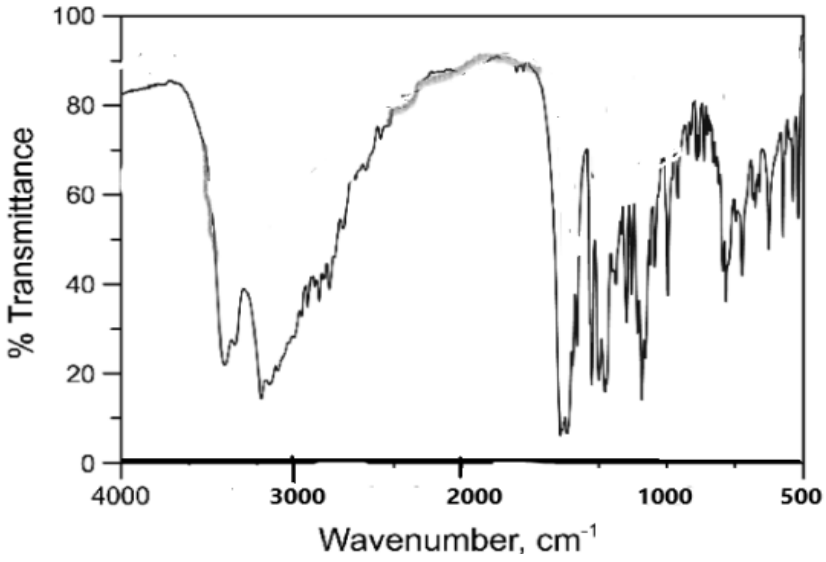


Figure 1: FTIR spectra of cyclo (L-Pro-L-Val)

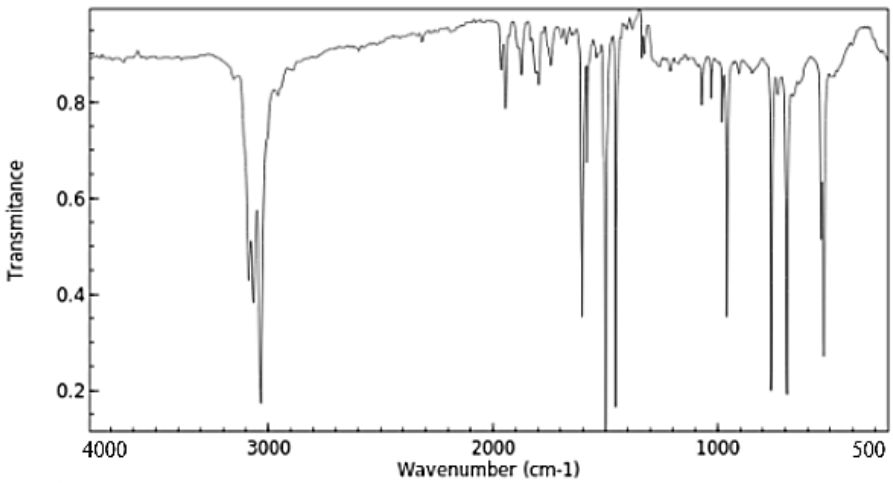
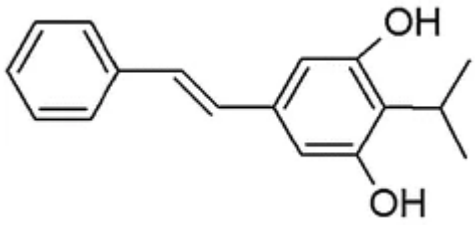
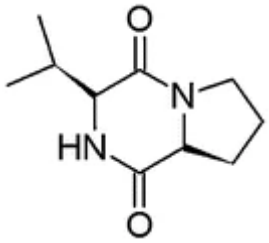


Figure 1: FTIR spectra of 3,5-dihydroxy-4-ethyl-trans-stilbene

Compounds' absolute configuration determination

The absolute configuration of compounds has been successfully determined using the modified Marfey's approach. (Table 2)

Table 2: Isolation and identification of antimicrobial secondary metabolites from *Bacillus cereus*

Compound	Structure
3,5-dihydroxy-4-ethyl-trans-stilbene	
cyclo (L-Pro-L-Val)	

Antimicrobial action

Using conventional techniques, the antibacterial activity of the obtained compounds was evaluated against four bacterial strains. Table 3 displays the results of the determination of the MIC and MBC values. Escherichia coli showed the highest sensitivity towards compound 1 as well as 2 with Staphylococcus aureus following closely after. It seems that the effective MIC of the microorganisms under test is also a good indicator of their effective bactericidal concentration. Compared to ciprofloxacin, the test compounds' activity was lower.

Table 3: MIC and MBC (µg/ml) of two isolated compounds B.cereus

Compound	*S. aureus*		*P. aeruginosa*		*E. coli*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
1	10	28	35	70	7	14
2	14	28	70	140	6	10

Diffusion test using Agar discs

The disc diffusion assay result is shown in Table 4. Compounds 1 had the highest efficacy against E.coli and compound 2 had highest efficacy against S.aureus.

Table 4: Antimicrobial activity of isolated compounds

Test organism	Compound 1 Zone of inhibition (mm)	Compound 2 Zone of inhibition (mm)
S. aureus	20 ± 0.57	25 ± 1.73
E. coli	27 ± 0.0	19 ± 1.06

P. aeruginosa	23 ± 1.7	17 ± 1.03

Discussion

Head-to-tail dipeptide dimers, or 2,5-diketopiperazines (DKPs), are frequent skeleton seen in nature (Dinsmore and Beshore, 2002). Microorganisms, sponges, and a range of bodily fluids and tissues have been shown to contain diketopiperazines that correlate to cyclic dipeptides (Bojarska, et al., 2021). For theoretical research as well as the creation of medicinal molecules, diketopiperazines are suitable models because of their relative simplicity (Farhadian et al., 2019)) and stability (Dinsmore and Beshore, 2002). Diketopiperazines exhibit a variety of biological actions, including antifungal (Kumar and Nambisan 2014), antibacterial (Ma et al. 2016), anticancer (Gomes et al., 2019), and antihyperglycemic (P de Carvalho and Abraham, 2012). They bind to a wide spectrum of receptors with high affinity because of their chiral, stiff, and functionalized architectures, providing a wide range of biological functions (Gomes et al., 2019). Diketopiperazines are hence appealing structures for the rational discovery of new lead compounds for the creation of novel medicinal medicines.

3,5-dihydroxy-4-ethyl-trans-stilbene DETS is a natural stilbene (first identified as bioactive bacterial secondary metabolite isolated from Bacillus cereus associated with a rhabditid entomopathogenic nematode) with potent antioxidant and anticancer properties. Its antioxidant activity surpasses that of two well-known antioxidants. DETS was studied for its cytotoxic activity towards five cancer cells and showed maximum cytotoxicity towards human melanoma cells. It down-regulates the expression status of major molecules contributing to melanoma progression and arrests the cells at S-G2 transition state of the cell cycle. (Nath et al., 2016). In another study, the antibiotic 3,5-dihydroxy-4-ethyl-trans-stilbene ES produced by Xenorhabdus luminescens was found to be effective against both gram-positive and some gram-negative bacteria. ES inhibits RNA and protein synthesis in susceptible bacteria and leads to the accumulation of an intracellular regulatory compound called ppGpp. The accumulation of ppGpp is correlated with the susceptibility of various bacteria to ES which suggests that this nucleotide plays a role in the regulation of RNA synthesis and growth in all these microorganisms. Therefore, inhibiting RNA synthesis by increasing ppGpp concentrations could be a mechanism that is common among most bacteria and could be explored for achieving rapid inhibition of bacterial growth. (Sundar and Chang1992The findings of this study are consistent with previous research. Confirmation of its potential as an antimicrobial agent and potent antibiotic was obtained through its effect on the growth of pathogens, specifically E. coli and S. aureus.

Cyclo-(L-Val-L-Pro) from Streptomyces sp., SUK 25, was particularly effective against MRSA. A DNA microarray was utilized to profile gene expression in MRSA treated with cyclo-(L-Val-L-Pro). Cyclo-(L-Val-L-Pro) upregulated unknown gene functions highest. This drug targets numerous biological pathways by downregulating genes encoding ribosomal proteins, cell membrane formation, DNA metabolism, citric acid cycle, and virulence in MRSA (Zin et al., 2020). In another study scientists extracted a considerable inhibitory component from Achromobacter xylosoxidans NFRI-A1 using high-performance liquid chromatography, thin-layer chromatography, and Diaion HP20 column chromatography. Physiochemical methods identified cyclo(l-leucyl-l-prolyl) with the inhibitor inhibitory dose of 0.20 mg ml−1 for aflatoxin production in A. parasiticus SYS-4 (NRRL2999). This study confirms the antimicrobial potency of cyclo(l-leucyl-l-prolyl) (Yan et al., 2004). Previous and current findings indicate that Cyclo-(L-Val-L-Pro) has potent antimicrobial activity against E.coli, S.aureus and P.aeurogeosa.

Hence, both the compounds Cyclo-(L-Val-L-Pro) and 3,5-dihydroxy-4-ethyl-trans-stilbene isolated from the *Bacillus cereus NC7401* confirmed their antimicrobial potential against harmful pathogens.

Conclusion

Several secondary metabolites from *Bacillus cereus NC7401* bacteria have been isolated and identified. Additionally, many EPN bacteria have not been explored for their bioactive metabolites. This study found that DKPs and stilbenes inhibited test bacterial strains. Thus, these microbial secondary metabolites may be

promising antibacterial agents and soil bacteria may be innovative medicinal sources. Thus, bacterial chemicals are promising probes for developing novel antimicrobial medicines from small molecules.

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