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# Isolation and Characterization of *Isolates of Bacillus cereus* Group and their Comparative Antibiotic Susceptibility Testing against Oxytetracycline

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**ABSTRACT**

***Background***: *Bacillus cereus* is a very important organism widely used for microbiological assays of pharmaceutical products especially in antibiotic susceptibility testing. The increasing emergence of antibiotic-resistant bacterial strains has emphasized the need for effective antibiotic susceptibility testing of pathogens for the management of patient's health.

***Methods:*** The present study isolated of 5 strains of *B. cereus* (R-01, R-02, C-01, C-03 & SS-01) from different samples collected from Ghaziabad, Uttar Pradesh, India. Pure cultures were then characterized based on microscopy, cultural and biochemical characteristics and also compared with standard reference strain *B. cereus* MTCC 430. Further, all the 5 isolates were subjected to MALDI-TOF analysis to confirm their identity. Finally, antimicrobial susceptibility of all the indigenous strains was tested in comparison with *B. cereus* MTCC 430 using the agar well diffusion method at various concentrations of Oxytetracycline (1.6, 2.0, 2.5, 3.12, 3.91, 4.0, 8.0, 12.0, & 16.0 µg/mL).

***Results:*** Morphological, cultural and biochemical characteristics confirmed the identity of all the bacterial isolates as *B. cereus* which was further authenticated by MALDI-TOF analysis. The antibiotic susceptibility tests revealed that out of 5 indigenous isolates, R-01 and R-02 were found to be susceptible at all tested concentrations of Oxytetracycline; whereas C-01, C-03 and SS-01 were found to be resistant at a concentration 2.5 µg/mL and below.

***Conclusion***: The antibiotics susceptibility test results displayed sensitivity profile of all 05 indigenous isolates of *B. cereus* against the Oxytetracycline and revealed that 02 isolates (R-01 and R-02) were more sensitive to Oxytetracycline compared to others.

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## Introduction

A wide range of microbiological assays or procedures were used to ensure the quality and safety of the different products from various industries such as food and pharmaceuticals (1). These assays/tests assist to identify and quantify microorganisms, including bacteria, yeasts, molds and viruses, which can affect the safety and quality of products (2). Different types of microbiological assays such as bacterial endotoxin tests (BET), sterility tests, microbial limit tests (MLT), and antibiotic susceptibility tests are widely used in different industries (3). Various microbes were used for these microbiological assays such as *Escherichia coli*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus aureus*, *Klebseilla pneumoniae*, *Salmonella enterica*, etc. (4, 5).

Among them, *Bacillus cereus* is commonly used in the microbiological assays of antibiotics (5). The standard strain of *Bacillus cereus* ATCC 11778 is commonly used in different microbiological quality control testing methods specifically antibiotic susceptibility testing (6, 7). *Bacillus cereus* is an aerobic or facultative anaerobic, endospore-forming, opportunistic, Gram-positive bacterium (8, 9). It is found in diverse environments such as soil, plants, contaminated food, insects, animal digestive tracts (10, 11). They can survive even in high temperature conditions because of high heat resistance (12).

Different species of *Bacillus* produce nutraceuticals such as vitamins and carotenoids which are utilized in manufacturing of health supplements for human use (13). *Bacillus cereus* is commercially used in the production of probiotics which enhances the intestinal microbial balance of the host animal (14, 15). Additionally, *B. cereus* strains can stimulate plant growth in various stress conditions such as salinity, drought, and heavy metal contamination (16). On the other side, as an opportunistic pathogen, *B. cereus* is capable of causing foodborne illness due to the synthesis of diarrheal toxin (enterotoxins) or emetic or cereulide toxin or non-ribosomal peptide synthetase (NPRS) toxin (17).

Antibiotics are still regarded the most effective treatment to treat *B. cereus* infections in people (18). Antimicrobial therapy is frequently utilized to treat food-borne illnesses, including those caused by *B. cereus*. However, the antimicrobial resistance has been found to be increasing worldwide as an outcome of extensive usage or improper treatment. The introduction of antibiotic-resistant *B. cereus* strains may result in treatment failure. Furthermore, a serious issue with patient care is currently the development of antibiotic resistance in *B. cereus* (19). Monitoring the antibiotic resistance profile of *B. cereus* is essential for understanding resistance patterns and devising suitable therapy options (20).

The present paper deals with isolation, identification and characterization of indigenous strains of *Bacillus cereus* group isolated from different samples in comparison with the standard strain of *Bacillus cereus* MTCC 430 (equivalent to ATCC 11778). It also comparatively studied the antibiotic susceptibility of the isolated strains against Oxytetracycline with standard reference strain of *B. cereus* MTCC 430.

## Materials and Methods

### *Sampling and Isolation*

Different types of samples (soil, contaminated food, air) were collected from different regions of Ghaziabad district. Soil sample was collected from garden area, whereas air sampling was done from the canteen area of IPC, Raj Nagar, Ghaziabad, India and contaminated food sample was collected from Modi Nagar, Ghaziabad, India.

One gram of soil sample was dissolved in the 10 mL of sterile normal saline and shaken vigorously for 2 minutes and then serial dilutions were performed from $10^{-1}$ to $10^{-6}$. A 100 µL for each

dilution was spread plated on soyabean casein digest agar (SCDA) medium (HiMedia) using spread plate method and then plate was incubated at 35 ± 2 °C for 24 hours.

For contaminated food samples, Freshly boiled rice was kept stored for 24 hours at room temperature for the incubation. After that, a spatula of rice sample was added in 25 mL test tube containing 3 mL of sterile normal saline and vortexed using a vortex mixer (Labman) and subjected to a serial dilution from $10^{-1}$ to $10^{-6}$ (21). A 100 µL of each dilution was plated on SCDA (HiMedia) plates using spread plate method and then plate was incubated at 35 ± 2 °C for 24 hours.

In case of air samples, passive air sampling method was used in which a petri plate containing SCDA media (HiMedia) was exposed to air for 2 hours. The plates were then taken to the laboratory and incubated at 35 ± 2 °C for 24 hours (22).

After incubation, all the plates were observed for the growth of bacterial colonies. Based on colony morphology, colonies resembling *Bacillus* sp. were picked and subcultured on fresh SCDA plate by four quadrant method. Plates were then incubated at 35 ± 2 °C for 24 hours. After incubation, pure bacterial cultures were transferred on SCDA slants and stored at 4 °C.

*Microscopic study by Gram staining*

All the 5 isolated bacteria and standard reference strain *B. cereus* MTCC 430 (equivalent to ATCC 11778) were subjected to Gram's staining by following standard regular procedure. In this method, a thin smear was prepared on clear glass slide, air dried and heat fixed. Gram's crystal violet was added on a smear for 1 minute and then slide washed under slow running tap water. After washing, Gram's iodine added for 1 minute. After that, slide was washed with slow running water. Then decolourized with Gram's Decolourizer until the blue dye no longer flows from the smear followed by washing with tap water. Then, counter strain (Gram's Safranin) was added for 20 seconds followed by washing the slide under tap water. Finally, slide was allowed to air dry and examined under microscope (Olympus).

*Motility test*

Isolated cultures were subjected to motility testing by tube motility method. For this method, pure cultures were inoculated in motility medium by piercing the center with a straight wireloop containing culture suspension and incubated for 18 to 24 hours at 35 ± 2 °C; the tubes were checked for diffuse growth away from the stab line (23).

*Cultural characterization*

Cultural characteristics of isolated bacteria on different media such as SCDA (HiMedia), differential and selective media including Mannitol egg yolk polymyxin agar (MYP) (HiMedia) and *Bacillus* agar (BA) (HiMedia) were comparatively studied with *B. cereus* MTCC 430. For this, pure cultures of isolated bacteria were streaked on SCDA, MYP and BA by four quadrant method and plates were then incubated 35 ± 2 °C for 24 hours. After incubation, plates were observed and observations were noted.

*Biochemical characterization*

The isolates of *B. cereus* were characterized by using biochemical tests such Indole test, Voges-Proskauer test, catalase test, oxidase test, triple sugar iron test, phenol red dextrose agar test, citrate utilization test, nitrate broth test, methyl red test and compared with *B. cereus* MTCC 430.

*Indole test*

A pure culture suspension was inoculated in a tube containing 3 mL of DEV tryptophan (HiMedia), incubated at 35 ± 2 °C for 24 hours. After the incubation Kovac's reagent (HiMedia) was added to the tube. The tubes were shaken gently and allowed

to stand for 1-2 min. Finally, the tube was observed for the formation of red ring at the top of media (24).

*Methyl red test*

A pure culture suspension was inoculated in a tube containing 3 mL of buffered glucose broth (MR-VP medium) (HiMedia), and incubated at 35 ± 2 °C for 24 hours. After the incubation a 2-3 drop of methyl red indicator (HiMedia) was added to the tube. Tubes were then checked for the appearance of bright red colour (24).

*Voges-Proskauer test*

A pure culture suspension was inoculated in a tube containing 3 mL of buffered glucose broth (MR-VP medium) (HiMedia), and incubated at 35 ± 2 °C for 24 hours. After the incubation a 2-3 drop of Barritt reagent A (HiMedia) and Barritt reagent B (HiMedia) was added and mixed well. Tubes were then observed for the formation of a pink colour (24).

*Citrate utilization test*

In this test, the culture suspension of isolated bacteria was streaked on Simmon's citrate agar (HiMedia) slants were then incubated at 35 ± 2 °C for 24 hours. Upon the incubation, slant was checked the for colour change from green to blue (25).

*Catalase test*

A pure culture was mixed with a drop of 3% hydrogen peroxide (Fisher Scientific) on a sterile clean glass slide. Then slide was observed for the formation of bubbles (26).

*Oxidase test*

In a sterile Petri plate, a loopful of pure culture was rubbed with a sterile wireloop on an oxidase disc (HiMedia). The dye is reduced to deep purple colour within 10 seconds was observed as a positive reaction (26).

*Triple sugar iron test*

For this test, a suspension of pure bacterial isolate was first stabbed through the center of the TSI agar medium (HiMedia) to the bottom of the tube and then streaked on the surface of the agar slant. Then the tubes were incubated for 24 hours at 35 ± 2 °C. After incubation, the tubes were checked for change in colour to yellow (acid production) or red (alkali production), cracks in butt (gas production), black colour at the bottom of butt ($H_2S$ production) (27).

*Nitrate reduction test*

In this test, a pure bacterial suspension was inoculated in test tube containing nitrate broth (HiMedia) and incubated at 35 ± 2 °C for 24 hours. After incubation, add 2-3 drops of α-naphthylamine (HiMedia) and add the 2-3 drops of sulphanilic acid (HiMedia) and observed for red colour development (26).

*Phenol red dextrose agar test*

In this test, a suspension of pure bacterial isolate was streaked on the slants of PRDA agar (HiMedia) and slants were incubated at 35 ± 2 °C for 24 hours. After the incubation the slants were observed for change in color (28).

*Identification using MALDI-TOF*

All the isolated bacterial strains (R-01, R-02, C-01, C-03 and SS-01), were subjected to MALDI-TOF analysis using MALDI mass spectrometry TOF/TOF 5800 system (AB Sciex) at NCCPF-PGIMER, Chandigarh, India to confirm the identity.

*Antibiotic susceptibility test*

The agar well diffusion method (cup plate method) was used to evaluate the antibiotic susceptibility profiles of 5 isolates of *B. cereus* R-01, R-02, C-01, C-03 & SS-01 and MTCC 430 strain. The antibiotic test was performed at various concentrations of Oxytetracycline (3.91, 3.12, 2.5, 2.0, 1.6 µg/mL) considering the median dose (2.5 µg/mL) as per IP procedure (5) and also tested for higher concentrations of Oxytetracycline (4.0, 8.0, 12.0 and 16.0 µg/mL). In this assay, a base layer was prepared in 90 mm petri plates by pouring around 21 mL of molten, antibiotic assay Media-F (HiMedia) and plates were allowed to solidify. Then, around 5 mL of bacterial suspensions were prepared from 24 hours old slants using normal saline and transmittance of these suspensions were adjusted to 25% at 530 nm using UV/VIS Spectrophotometer (Perkin Elmer). For the seed layer, 0.7 ml of culture suspension was transferred to the flask containing seed layer media (100 mL), mixed properly and poured around 4 mL seed layer media over the base layer and plates were kept for solidification at room temperature for 30 minutes. After solidification, wells were created by using sterile borer. Further, different concentrations of Oxytetracycline were prepared as mentioned above following standard procedure. A 100 µl of each antibiotic concentration were inoculated to the wells and plates were incubated at 35 ± 2 °C for 24 hours. After completion of incubation, zone of inhibitions (in mm) were measured for each test culture and standard reference strain of *B. cereus* by Vernier Caliper (Mitutoyo) and observations were recorded.

## Results

*Sampling and Isolation*

In this research, 3 samples were collected from various regions of Ghaziabad, Uttar Pradesh, India such as soil, contaminated food (cooked rice), and air (Table 1, Figure 1).

We have successfully isolated 5 bacterial isolates resembling *Bacillus* from collected samples which includes 2 isolates (R-01 & R-02) from cooked contaminated rice, 2 isolates (C-01 and C-03) from air sample and 1 isolate (SS-01) from soil (Figure 2).

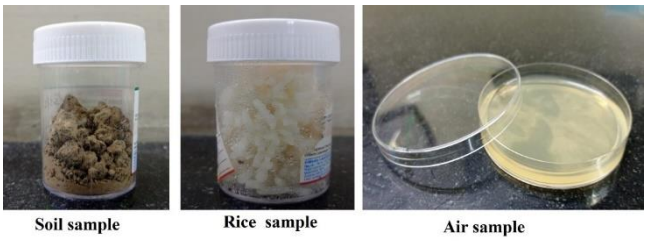



**Figure 1.** Collected samples from different regions of Ghaziabad.

*Microscopic study by Gram staining*

Upon microscopic analysis, all the bacterial isolates (R-01, R-02, C-01, C-03, & SS-01) appeared as Gram-positive, rod-shaped bacilli arranged in short to long chain similar to standard reference strain, *B. cereus* MTCC 430 (equivalent to ATCC 11778) (Figure 3).

*Motility test*

The isolated bacteria were found to be motile that showed diffuse, hazy growth through the motility medium similar to standard reference strain of *B. cereus* MTCC 430 (Figure 4).

*Cultural characterization*

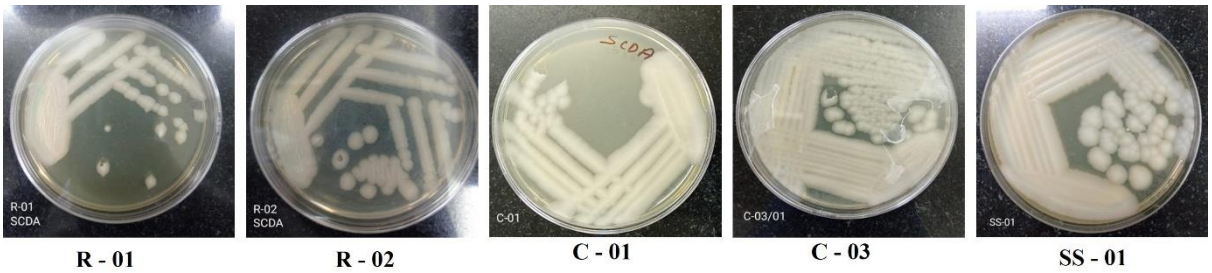
All the isolates (R-01, R-02, C-01, C-03, & SS-01) have shown circular, flat, off-white, colonies with irregular margin on SCDA media and crenated, blue coloured colonies on Bacillus agar; while reddish/pinkish colonies on MYP media similar to MTCC 430 (Figure 5).

**Table 1.** Details of collected samples.

| Samples | Sample Code | Place of collection |
|---|---|---|
| Cooked contaminated rice | R | Modi Nagar, Ghaziabad, India |
| Air | C | Canteen Area, IPC, Raj Nagar, Ghaziabad, India |
| Soil | SS | Garden area, IPC, Raj Nagar, Ghaziabad, India |

**Table 2.** Biochemical tests of isolated cultures along with standard reference strain.

| Sr. No | Biochemical test | MTCC 430 | R-01 | R-02 | C-01 | C-03 | SS-01 |
|---|---|---|---|---|---|---|---|
| **1.** | Catalase | +VE | +VE | +VE | +VE | +VE | +VE |
| **2.** | Oxidase | -VE | +VE | +VE | +VE | +VE | +VE |
| **3.** | Nitrate Broth | +VE | +VE | +VE | +VE | +VE | +VE |
| **4.** | PRDA | +VE | +VE | +VE | +VE | +VE | +VE |
| **5.** | TSI | A/A | A/A | A/A | K/A | K/A | K/A |
| **6.** | Citrate | -VE | -VE | -VE | -VE | -VE | -VE |
| **7.** | Voges – Proskauer | -VE | -VE | -VE | -VE | -VE | -VE |
| **8.** | Methyl Red | +VE | +VE | +VE | +VE | +VE | +VE |
| **9.** | Indole | -VE | -VE | -VE | -VE | -VE | -VE |




**R - 01** **R - 02** **C - 01** **C - 03** **SS - 01**

**Figure 2.** Isolated pure bacterial cultures from the collected samples.

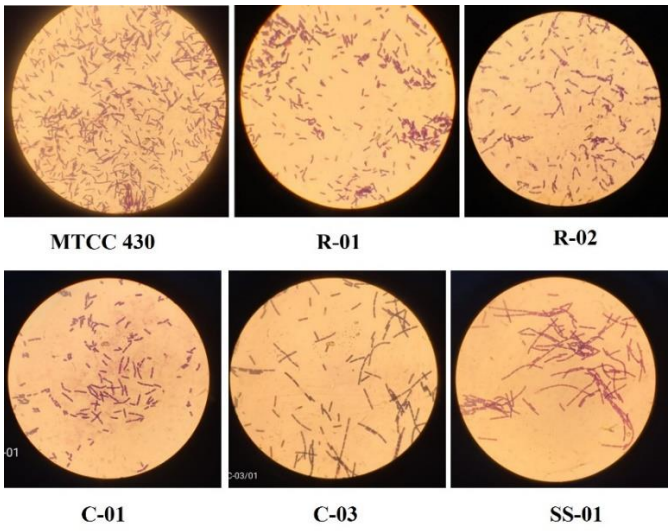



**Figure 3.** Gram staining of isolated cultures and standard reference strain.

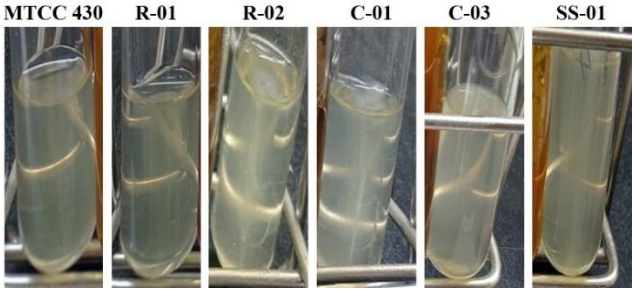



**Figure 4.** Motility test of isolated cultures and standard reference strain.

**Table 3.** Results of antibiotic susceptibility testing against Oxytetracycline.

| Test organisms | Diameter of zone of inhibition (in mm) against Oxytetracycline | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Concentrations as per IP | | | | | Higher Concentrations | | | |
| | 1.6 µg/mL | 2.0 µg/mL | 2.5 µg/mL | 3.125 µg/mL | 3.91 µg/mL | 4.0 µg/mL | 8.0 µg/mL | 12.0 µg/mL | 16.0 µg/mL |
| MTCC 430 | 21.0 | 22.0 | 22.4 | 23.1 | 24.2 | 25 | 25.9 | 27.2 | 29.0 |
| R-01 | 12.0 | 12.7 | 13.1 | 13.9 | 14.7 | 16.0 | 18.9 | 19.3 | 20.0 |
| R-02 | 13.0 | 13.7 | 14.0 | 14.6 | 15.1 | 15.5 | 17.9 | 19.0 | 20.6 |
| C-01 | - | - | - | 12.8 | 13.8 | 14.1 | 17.9 | 19.1 | 20.4 |
| C-03 | - | - | - | 11.9 | 13.0 | 13.8 | 17.2 | 19.3 | 20.4 |
| SS-01 | - | - | - | 11.9 | 13.0 | 13.1 | 17.0 | 18.2 | 19.8 |

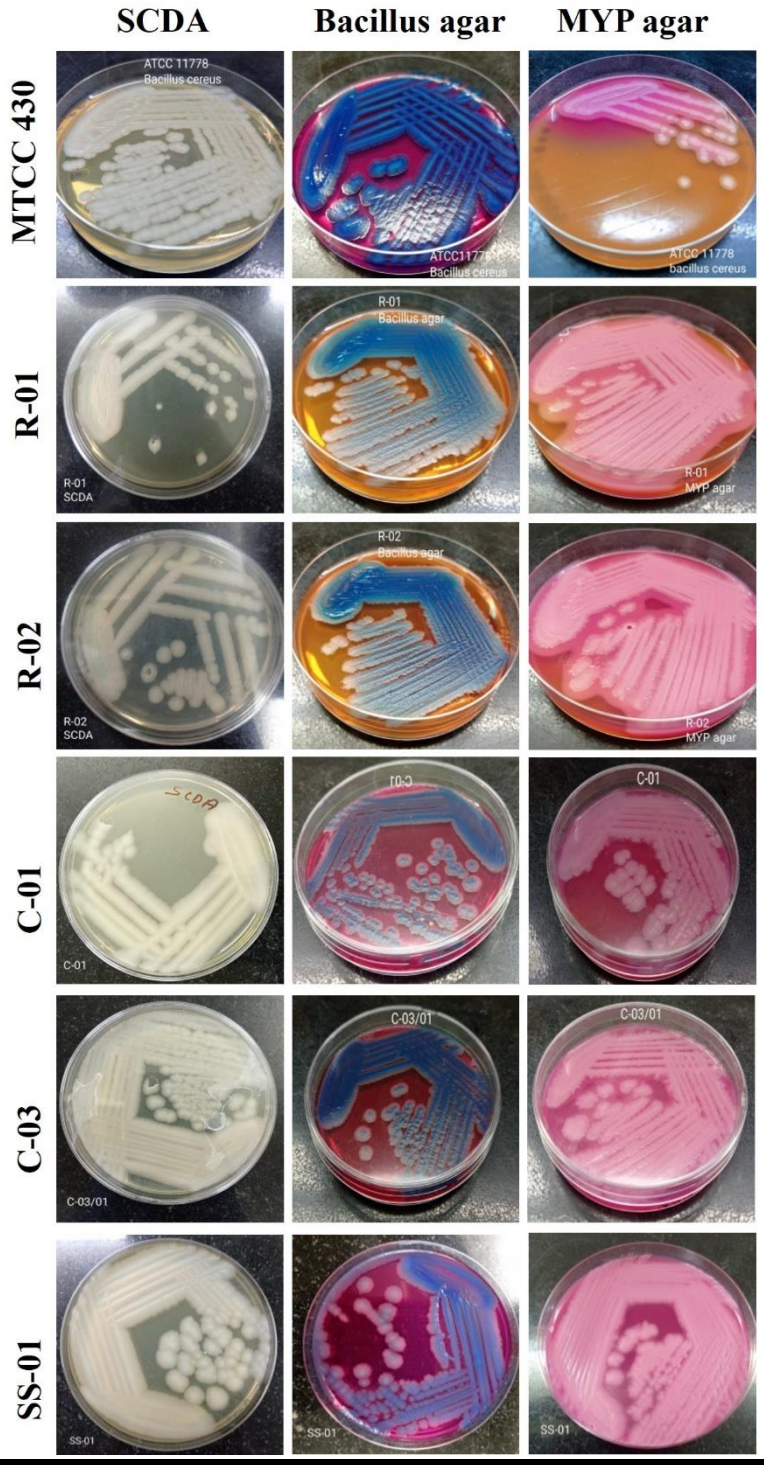



**Figure 5.** Colony characteristics of isolated cultures and standard reference strain.

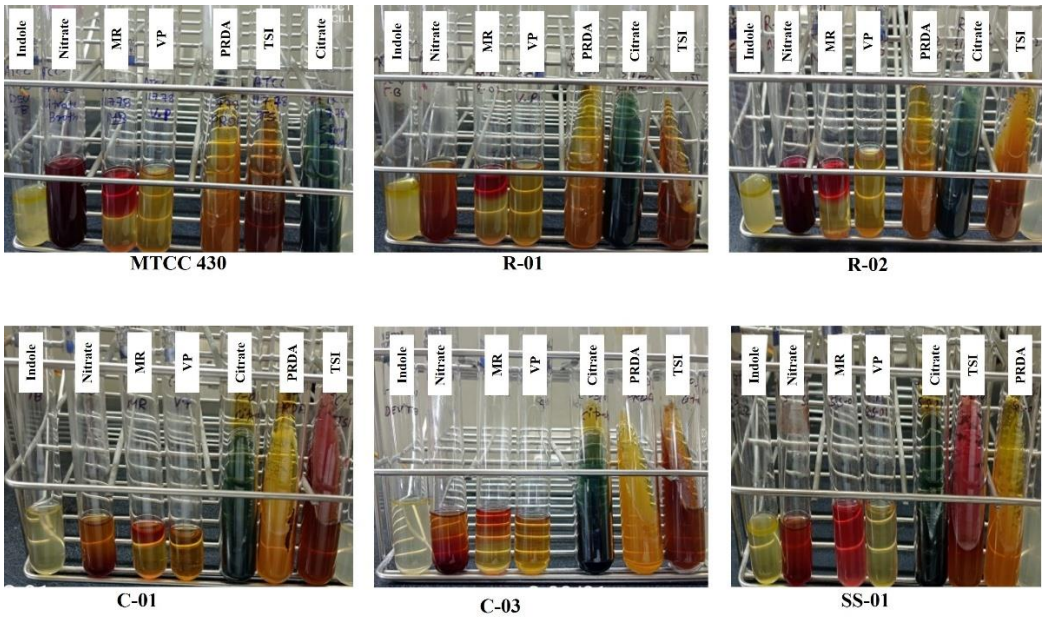



**Figure 6.** Biochemical characteristics of isolated cultures and standard reference strain.

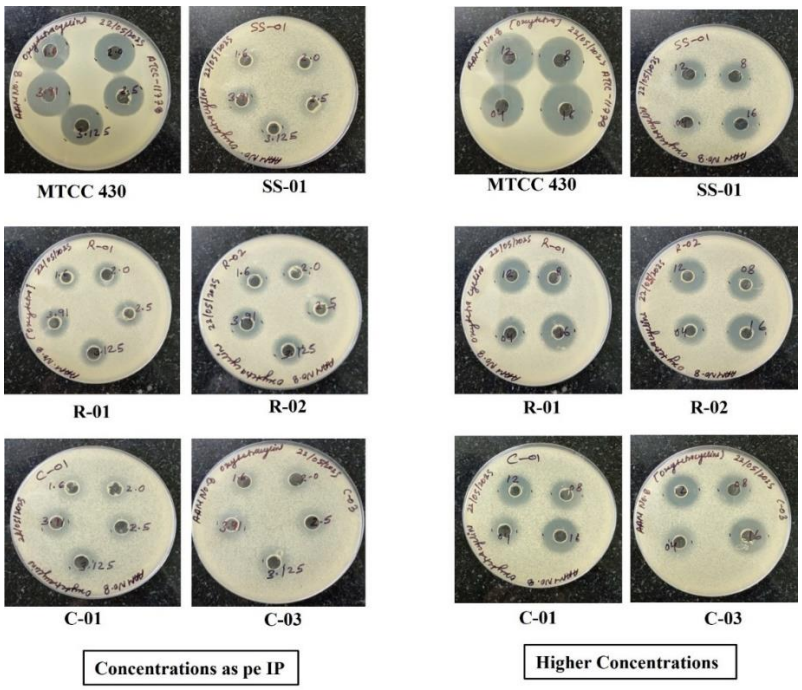



**Figure 7.** Comparative antibiotic susceptibility testing of isolated cultures.

*Biochemical characterization*

Among 05 isolates, 02 isolates (R-01 & R-02) have shown positive results in Catalase, Oxidase, Nitrate, PRDA, and Methyl red tests; while negative results were obtained in Indole, Voges-Proskauer, and Citrate, similar to MTCC 430 except for Oxidase. The other 03 isolates (C-01, C-03 & SS-01) have shown results similar to others, except for TSI (K/A) (Table 2, Figure 6).

*Identification using MALDI-TOF*

The MALDI-TOF analysis confirmed the identity of the isolated bacterial strains (R-01, R-02, C-01, C-03 and SS-01) as *Bacillus cereus* group.

*Comparative antibiotic susceptibility testing*

Comparative antibiotic susceptibility test results showed that, among 05 isolates, R-01 & R-02 reported prominent zone of inhibitions (Table 3) to all the tested concentrations of Oxytetracycline. Remaining 03 isolates, C-01, C-03 and SS-01 have shown no zone of inhibitions at 1.6, 2.0 and 2.5 µg/mL; while prominent zone of inhibitions were reported at 3.125, 3.91, 4.0, 8.0, 12.0, and 16.0 µg/mL of Oxytetracycline (Table 3, Figure 7).

## Discussion

Antibiotic resistance is currently emerging as a very serious problem for the public health globally (29). Especially, *B. cereus* was found to be resistant to different types of antibiotics such as Penicillin, Tetracycline, Ciprofloxacin, Streptomycin, Cloxacillin, and Erythromycin (17). One of the earlier studies has shown that, Oxytetracycline and Tetracycline resistance determinants are frequently observed in *B. cereus* isolates (30). The analysis of resistance of *B. cereus* to different antimicrobial drugs is vital during clinical problems or outbreaks (31). Antibiotic susceptibility testing is the important method used in clinical and pharmaceutical industry to identify the sensitivity and resistance profile of microorganisms against different antibiotics for the treatment of diseases or infections (32). Different antimicrobial susceptible organisms are very important in pharmaceutical susceptibility testing to assess the effectiveness of antibiotics against pathogens (4, 5).

In the present study, we have reported 05 indigenous isolates of *B. cereus* group from different samples including soil, air and contaminated food. Analysis of microscopic characteristics of all the isolates showed Gram positive, rods present singly or in chains resembling with the standard reference strain of *B. cereus* (MTCC 430). Appearance of blue-colored colonies on selective medium i.e. *Bacillus* agar showed the beta-glucosidase activity of the present isolates resulting into the formation of blue-colored colonies (33). Similarly, appearance of pink colonies on differential and selective medium (MYP agar) showed the lecithinase production which is characteristic of *Bacillus cereus* (34). In addition, all the isolates have shown similar results in biochemical tests (Indole, methyl red, Voges-Proskauer, Citrate utilization, phenol red dextrose test, catalase, and nitrate reduction test) identical to standard reference strain except oxidase and triple sugar iron test. Based on microscopic, cultural and biochemical characteristics, all the 5 isolates were phenotypically and biochemically confirmed as *Bacillus cereus*. Further, analysis of MALDI-TOF authenticated the identity of all the 5 isolates (R-01, R-02, C-01, C-03 & SS-01) as *B. cereus* group.

Some of the earlier investigations studied the antibiotic susceptibility of the *B. cereus* against Oxytetracycline (30, 34, 35). One of the studies has revealed that, *B. cereus* isolated from sub-clinical mastitis in sheep showed 100% susceptibility to Oxytetracycline (30 µg disks) with 15 mm zone of inhibitions (35). Likewise, another study reported that all the isolates of *B. cereus* were sensitive to 30 µg of Oxytetracycline disks showing susceptibility to Oxytetracycline (34). In the present study also we found similar

findings. The present comparative antibiotic susceptibility testing against Oxytetracycline revealed the sensitivity and resistance profile of all the 05 isolates in comparison with standard reference strain *B. cereus* MTCC 430 (equivalent to ATCC 11778). Screening of all the isolates against 5 different concentrations of Oxytetracycline (3.91, 3.12, 2.5, 2.0, 1.6 µg/mL) as per the IP standard procedure revealed that among 05 isolates, R-01 & R-02 were found to be sensitive to all the tested concentrations of Oxytetracycline showing prominent zone of inhibitions. Remaining 03 isolates, C-01, C-03 and SS-01 have shown sensitivity to Oxytetracyline from a concentration 3.125 µg/mL and above, while they found to be resistant at lower concentrations (1.6, 2.0, 2.5 µg/mL) of Oxytetracyline. Overall, the present comparative antibiotic susceptibility study reported 5 indigenous strains of *B. cereus* group and reported their sensitivity to Oxytetracycline in comparison with standard reference strain *B. cereus* MTCC 430 (equivalent to ATCC 11778).

## Conclusion

The present research work concludes that, indigenous isolates of B. *cereus* identified based on phenotypic and MALDI-TOF analysis, 2 isolates have shown sensitivity to Oxytetracycline in the antibiotic assay at all tested concentrations, suggesting their potential future use in microbiological assays of pharmaceutical products.

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## Ethics approval and consent to participate

Not applicable.

## Conflict of interest

No conflict of interest declared.

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