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BACTERIA ISOLATED FROM NATURALLY INFECTED CHILLI SEEDS USING MORPHOLOGICAL AND BIOCHEMICAL METHODS

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Abstract: This study focuses on identifying bacterial pathogens isolated from naturally infected chilli seeds using morphological and biochemical methods. A collection of infected chilli seeds was analyzed to identify the bacterial species responsible for their infection. Morphological methods, including colony characteristics, gram-staining and microscopic examination, were first employed to categorize the isolates based on their cellular structures and colony appearances. Subsequently, biochemical tests such as oxidase and catalase, sugar fermentation assays, and API tests were conducted to further characterize the bacteria and confirm their identities. The results indicated the presence of key bacterial pathogens, including *Pseudomonas syringae* and *Pseudomonas viridiflava,* which were linked to observed symptoms in the seeds. The findings underscore the importance of precise bacterial identification in developing strategies for disease management and enhancing crop productivity Their identification from infected chilli seeds suggests a potential role in seed borne transmission of disease. This dual approach of using both morphological and biochemical methods proved to be an effective and reliable strategy for bacterial identification. The findings emphasize the critical importance of early and accurate pathogen detection in seeds, which is essential for implementing effective disease management strategies, improving seed health, and ultimately enhancing agricultural productivity in chilli cultivation.

Keywords: Bacterial identification, *Capsicum annuum*, Plant-microbe interactions, *Pseudomonas*.

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INTRODUCTION

Chilli, a widely cultivated vegetable crop, is susceptible to various bacterial diseases that can lead to significant yield losses. It is commonly known as Red pepper in English and Mirchi in Hindi (Pandey *et al.,* 2012). Identifying bacterial pathogens affecting chilli seeds is crucial for effective disease management and ensuring healthy crop production. This study focuses on the morphological and biochemical methods

used for bacterial identification in naturally infected chilli seeds. Chilli (*Capsicum* spp.), a tropical fruit belonging to the Solanaceae family, has been widely used in cuisines and traditional medicines across the globe and various cultures. This pungent fruit, which includes species such as *Capsicum annuum* L. and *C. frutescens* L., contains several bioactive compounds with notable health benefits (Bosland and Votava, 2015). Capsaicinoids are acid amides of C_9 - C_{11} branched-chain fatty acids and vanillylamine. These compounds are responsible for the pungency of the *Capsicum* species and of cultivars regarded as hot peppers (Díaz *et al.,* 2004). Chilli peppers have gained immense popularity as a culinary ingredient, with their pungent and distinctive flavor serving as a key component in countless dishes across the globe. In addition to their culinary uses, chilli peppers are valued for their medicinal properties, primarily due to capsaicin, which has a variety of therapeutic applications.

The pungency of chilli peppers is attributed to capsaicin, a crystalline, acrid, and volatile compound located in the placenta and pericarp of chilli peppers, which is responsible for their spiciness. The capsaicin has been extensively studied for its preventive and therapeutic applications in both Allopathic and Ayurvedic medicine.

The name 'chilli' comes from the Mexican word chile. Christopher Columbus introduced chilli to Europe in 1493 after encountering it in tropical America. It is thought to have originated in Mexico and Peru and was commonly used by central and south Americans before Columbus' discovery. It expanded so swiftly that by 1542, three varieties of chilli had been imported into India.

Pathogens can have significant detrimental effects on red chilli plants (*Capsicum annuum* L.), impacting their growth, productivity, and overall health: Agricultural products can be exposed to microbial contamination through a variety of sources. Although vegetables are good examples of minimally processed foods, there is a high risk of contamination (Al-Mijalli, 2014). Soil borne pathogens like fungi, oomycetes, bacteria, and nematodes can adversely affect chilli plant growth and health by establishing parasitic relationships in the rhizosphere (Raaijmakers *et al.,* 2009). For example, *Leveillula taurica,* an obligate pathogen, causes powdery mildew on chilli pepper and is recognized as one of the most significant diseases affecting the crop (Hussein *et al.,* 2023).

Chilli plants are susceptible to various pathogens like *Phytophthora capsici, Rhizoctonia solani*, and *Pseudomonas syringae* that cause illnesses like wilt, root rot, and leaf blight. These infections destroy roots, stems, leaves and fruits, which results in lower yield and quality of the chilli. *P. syringae* is more widespread than the others, entering through wounds or natural openings and producing toxins that either destroy the plant cells or take away from plant health. Diseases like the one caused by *P. syringae* reduce both yield and market quality. There can be a high degree of susceptibility or tolerance; however, *P. syringae* is aerobic, gram-negative and is most robust because of its versatility. Once this pathogen is established well, the success of controlling it through chemical means becomes reduced having established plant tissue and is often unmanageable. Bacteria belong to Monera kingdom and show susceptibility to antibiotics (Verma and Prakash, 2020; Rane and Patel, 2021).

Understanding the behavior of *P. syringae* is therefore important in establishing control and/or management strategies. New avenues for further research on *P. syringae* are continuing to be developed: it is important to know that *P. syringae* colonies can be accurately identified, by way of their morphological and biochemical relationship-methodologies that can include gram staining, catalase test, indole test, citrate test, and the carbohydrate utilization test. While pathogens like *Pseudomonas syringae* represent a real threat to chilli cultivation, microbes like plant growth-promoting rhizobacteria (PGPR) can benefit sustainability efforts.

PGPRs can potentially suppress the effects of phytopathogens, provide fertility in the soil, and increase plant growth. In particular, linking fragmented pathogen DNA with other biological

control agents suggests reducing the severity of the disease would prove more beneficial in the future. Plant growth promoting rhizobacteria (PGPR) exhibiting multiple traits may be used in the development of new, eco-friendly, and effective bioformulations as an alternative to synthetic fungicides (Hyder *et al.,* 2020). These approaches are significant components of key eco-friendly approaches that could be integrated and with researcher thought and an eco-friendly strategy to reduce dependence on chemicals costs which offer long term success, especially in regions of high production like India. Early identification and listing of plant pathogens in an area allows for timely development of control and management strategies that goes a long way in avoiding epidemics and crop losses. It is also a means of checking the spread of many seed borne diseases and ensures the prevention of the spread of plant diseases to new areas (Chigoziri and Ekefan, 2013).

The purpose of this study is to isolate and identify the bacterial species in naturally-infested chilli seeds using morphological and biochemical procedures. This study will examine the differences in colony characteristics of the isolates (e.g. shape, size, color, and texture), which can be used for preliminary classification, and biochemical tests will be conducted to confirm their identity. The study will also establish a better understanding of the potential pathogenic impact of these bacteria on germination and overall plant health. This study will focus on early detection and identification of the bacteria to emphasize the importance of seed health in sustainable chilli production and provide helpful information for future disease management.

MATERIALS AND METHODS

In this examination, several materials and methods were employed to study the bacterial contamination of infected chilli seeds. The materials used included beakers, petri plates, filter paper, conical flasks, inoculation loops, cotton, forceps, burners, test tubes, and glass slides for microscopy. Two principal types of identification namely: morphological and biochemical characterizations were used in this research (Chauhan and Jindal, 2020). Growth requirements, colony characters on standardised culture media and a variety of biochemical and serologic characters help in the identification of bacteria up to genus and species level (Kootallur *et al.,* 2011). Investigating bacterial contamination on chilli seeds is critical because seed-borne pathogens are often the piscine infection centers for young plants. Contaminated seeds (with pathogenic bacteria) often lead to poor germination, diseases on seedlings, and lower crop yields (Chialva *et al.,* 2022). In crops, like chilli, that are economically relevant to a large part of our society and are heavily grown, the research and detection of harmful bacteria in infected seeds allow for containment through control long before any outbreaks at field level. This examination assists in seed quality assurance and fundamental management. Knowing the nature and identity of the bacteria found on these contaminated seeds assists with crop protection and food security support in the longer term.

Fig. 1: Bacterial isolation.

For sampling, infected chilli seeds were collected from various locations. Then seeds were surface sterilized prior to culturing using 0.1% $HgCl_2$ (Mercuric Chloride) solution. This crucial step was performed to eliminate external surface contaminants and ensure that only internal or truly seed-borne bacteria were isolated (fig. 1). The seeds were treated with $HgCl_2$ for few minutes, before being rinsed with sterile distilled water several

times to remove residual chemical. This sterilization process helped to provide conditions to maintain aseptic conditions for bacterial isolation and, also help the accuracy of results.

Nutrient agar medium was used for isolation and growing the bacteria in the study. The medium was prepared by dissolving 5.0g of 'peptone', 3.0g of 'beef extract', 5.0g of 'sodium chloride' and 15.0g of 'agar' in 1000 ml of distilled water. The pH was adjusted to 7.0 ± 0.2 for optimal growth. The medium was mixed, and then sterilized by autoclaving for 15 minutes, at 121°C, to provide aseptic condition before use. The bacterial contamination was assed using nutrient agar plates by placing in the medium, five dry seeds. The authors rolled the seeds while they pressed gently on the agar surface which allowed the microorganisms on seeds to grow. The plates were then incubated at 37°C for 24 - 48 hours and different colonies could be seen. The isolation of the colonies consisted of streaking with the streaking inoculum as desired to thin the bacteria on the required area.

This provided three colonies (fig. 2) designated as chilli white, chilli yellow, and chilli yellow and white.

Chilli white and yellow **Chilli yellow** **Chilli white**

Fig. 2: Isolated bacterial colonies.

A bacterial smear was prepared for a gram-stain. A small amount of bacterial growth was placed on a clean slide and mixed with water. The slide was set aside to dry. The gram-stain process used in this laboratory consisted of four reagents *namely*: crystal violet (primary stain), iodine (mordant), acetone-alcohol (decolorizer), and safranin (counterstain). The gram stain is useful for assessing bacterial contamination of tissue culture samples or for examining the gram stain status and morphological features of bacteria isolated from mixed or isolated bacterial cultures (Moyes *et al.,* 2009; Tripathi and Sapra, 2025). Finally, microscopic visualization determined whether or not the bacterium was from the three colonies: rod-shaped (bacilli), gram-negative, bacteria.

In the preliminary laboratory experiment, the bacterial smears were viewed on a compound light microcope at 1000x magnification, using an oil immersion lens. This allowed for a clear view of bacterial cell morphology, arrangement, and gram reaction. For each colony, the observations were made under sterile conditions to avoid contamination of the sample and microscope.

Bacterial staining allows for information about the shape, gram reaction, and the presence of bacterial structures such as capsules and endospores. Other than microscopic viewing, not a lot can be determined about a bacterium's genus or species. Therefore, amount of specific metabolic reactions a bacterium can produce can be tested biochemically. The amount of metabolic reactions each bacterium can process can be termed a 'thumb print' for identification.

To further characterize the bacteria, biochemical tests were performed, including the indole test, citrate test, cetrimide test, and carbohydrate utilization test.

Biochemical characterization

Based on the bacteria's metabolic pathways, several biochemical tests were carried out such as:

1. Catalase Test (Reiner and Tseng, 2010)

The catalase test is a biochemical characterization method used to identify bacteria based on their metabolic pathways. The test works on the idea that the enzyme catalase helps to break down hydrogen peroxide into oxygen and water. When a sample from an organism that produces catalase is introduced to hydrogen peroxide, it leads to the rapid generation of bubbles due to the release of oxygen, showcasing the enzyme's activity.To perform the test, pour 1-2 mL of hydrogen peroxide into a test tube. A sterile wooden stick or glass rod is then used to dip multiple colonies of the test organism, which have been cultured for 18 to 24 hours, into the hydrogen peroxide solution. Observations should be made for immediate bubbling. A positive result is indicated by copious bubbling, while a negative result shows no or very few bubbles.

2. Indole Test (Ljutov, 1959)

The indole test works on the concept that indole is created through the reductive deamination of tryptophan, which is aided by the enzyme tryptophanase, which removes the amine (-NH2) group from the tryptophan molecule, yielding indole pyruvic acid as an intermediary. To begin the indole test, sterilized test tubes with 4 mL of tryptophan broth are prepared. Growth from an incubated culture for 18 to 24 hours is then aseptically added to the broth. The tubes are incubated at 37°C for 24-28 hours. Following incubation, 0.5 mL of Kovac's reagent is added to the broth culture, and the tubes are checked to see if a color change occurs. A positive result is indicated by the formation of a red or pink ring at the top of the medium, whereas a negative result is the absence of color change following the addition of the reagent.

3. Citrate Test (MacWilliams, 2009)

The citrate test is intended to determine an organism's capacity to use citrate as the only source of carbon for metabolism, resulting in alkalinity.The principle behind the test involves the citrase enzyme, which hydrolyzes citrate to form oxaloacetic acid and acetic acid. The technique starts with dissolving the appropriate salts in deionized water and setting the pH to 6.9. Agar and Bromothymol blue are then added, and the mixture is slowly heated to boiling until the agar has completely dissolved.The solution is then dispensed into 16-mm tubes, autoclaved at 121 degrees Celsius under 15 psi pressure for 15 minutes, and cooled in a slanted position to form long slants with shallow butts. Following this, a light inoculum is streaked back and forth on the slant, using a loop picked from the center of an isolated colony. The tubes are incubated aerobically at 35 to 37 degrees Celsius for up to 24 hours, after which the color change of the media is observed. A positive result is indicated by a transition of the media's color from green to blue, while a negative result shows no change in color.

4. Cetrimide Test (Brown and Lowbury, 1965)

The cetrimide test is used to evaluate whether an organism, namely *pseudomonas aeruginosa,* can develop in the presence of cetrimide, a poisonous chemical that inhibits the growth of many other bacteria. In the process, a cetrimide agar slant is inoculated with either an 18-24 hour-old pure culture or straight from the specimen. The infected slant is streaked to provide isolated colonies, which are subsequently cultured at 35°-37°C for 4-6 days. Following incubation, the slant is checked for bacterial growth. A positive result is indicated by growth and a change in the color of the colonies, notably from yellow-green to blue, whereas a negative result reveals no development at all.

5. Carbohydrate Utilization Test (Whitworth *et al.*, 1991)

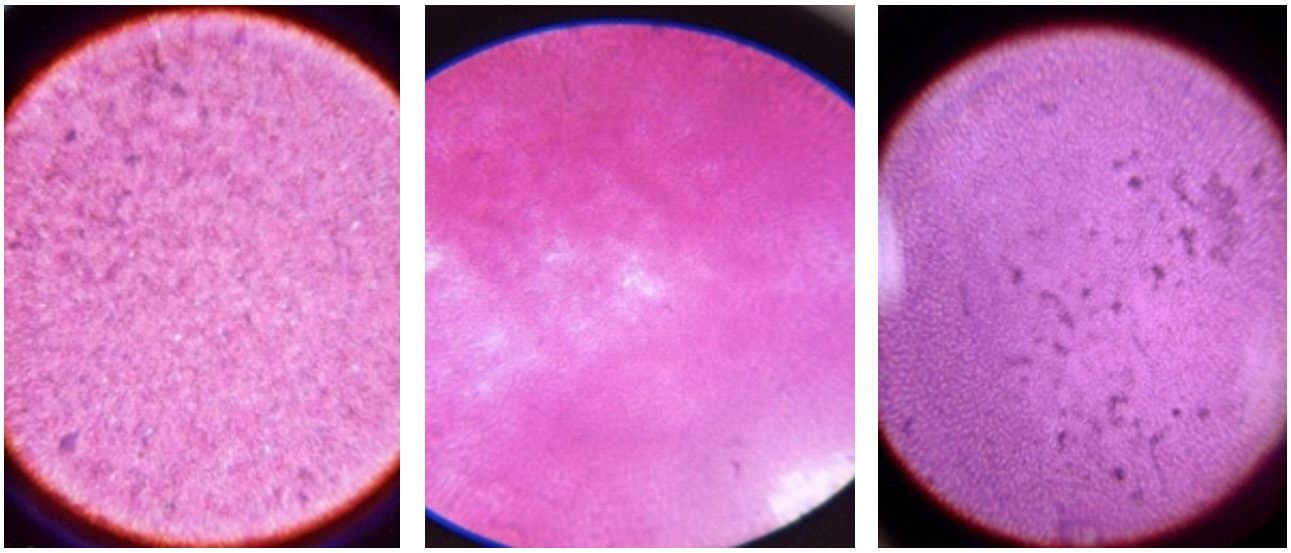
Carbohydrate utilization assays are used to determine a bacterium's ability to ferment a specific carbohydrate introduced into a basal medium, leading to the generation of acid or acid with visible gas. Prior to inoculation, the medium is allowed to reach room temperature. The purple broth is then inoculated with isolated colonies from an organism's 18-24 hour pure culture. To achieve reliable results, a control tube containing purple broth base is inoculated alongside the carbohydrate-based media. The infected media is incubated aerobically at 35-37ºC for 3-5 days.

Daily observations are done for the appearance of a yellow color in the medium, indicating a positive outcome. Conversely, a lack of yellow color development indicates a poor outcome.

In the earlier procedure, bacteria were inspected under a microscope. Staining offers useful information on bacterial shape, gram reaction, and the presence of structures like capsules and endospores. Beyond that, microscopic viewing provides little information about a bacterium's genus and species. Biochemical testing is essential for identifying bacteria. In fact, the type of metabolic reactions that each creature experiences, serves as a 'thumbprint' for its identification.

RESULTS AND DISCUSSION

A microscopic assessment of the three unknown bacteria, all demonstrated a rod-shaped morphology consistent with bacilli (fig. 3). Each species also showed a gram-negative reaction, which demonstrates thin peptidoglycan layer and an outer layer of lipopolysaccharides. This demonstrates that these bacteria may belong to the same genus or family designation from the gram-negative category. The designations suggest that bacteria have similar characteristics and biochemically there is a good economic starting place to begin. The last remaining thing to do is to test for specific characteristics using different biochemical and cultural methods of consideration.



Chilli White **Chilli Yellow** **Chilli White and Yellow**

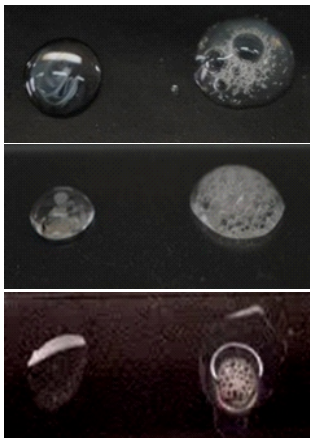
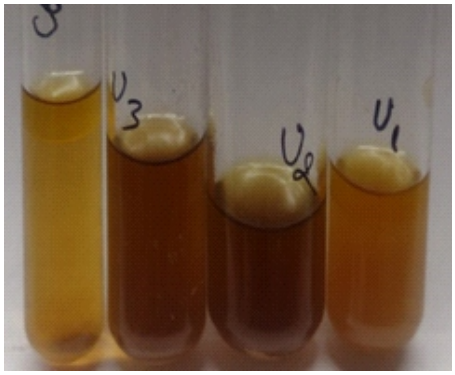
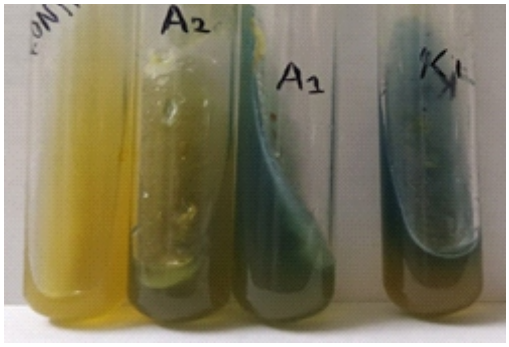
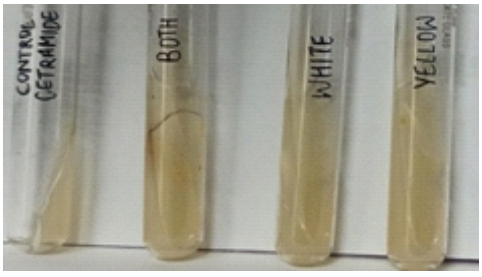
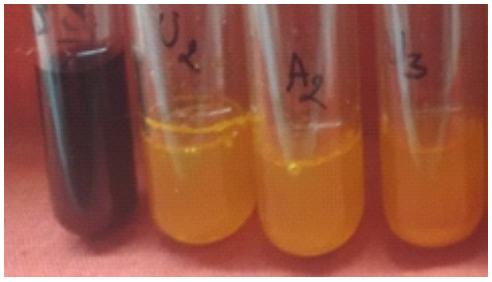
Fig. 3: Microscopic Identification of Pathogen.

Authors got valuable information for evaluation from their classification, especially their role in seed-borne diseases, and how to measure impairment in crop production. Various tests were performed to see the presence of various enzymes which are shown in Table 1 and 2.

Table 1: Biochemical Test Result (for each sample)

S. No	Sample/ Test	Chilli White	Chilli Yellow	Chilli White & Yellow
1.	Catalase test	+	+	+
2.	Indole test	-	-	-
3.	Citrate test	+	+	+
4.	Cetrimide test	-	-	-
5.	Carbohydrate utilization	+	+	+

Table 2: Biochemical Test Results.

Test	Observation	Result	Inference	Test
Catalase Test	Rapid bubbling was observed due to the release of oxygen.	+ve	Presence of catalase enzyme	
Indole Test	No color change after the addition of the reagent	+ve	No indole production	
Cetrimide Test	Intense Prussian blue color after 24 hours of incubation	+ve	Citrate is utilized as the sole carbon source	
Cetrimide Test	No color change observed after 4 days	+ve	Absence of *Pseudomonas aeruginosa*	
Carbo-hydrate Utilization	The medium turned yellow after 4 days of incubation.	+ve	Presence of *Pseudomonas syringae*	

The current study has identified the bacterium *Pseudomonas syringae* found as a key bacterial contaminant associated with naturally infected chilli seeds. This identification was based on the typical morphological characteristics, which included mucoid, irregular colonies, and a uniform biochemical reaction profile: catalase positive, indole negative, citrate positive and the carbohydrate fermentation positive. The microscopy results of rod-shaped gram-negative bacteria also supported identification of *P. syringae*. *Pseudomonas syringae* is a type of phytopathogen that spreads from plant to plant via injuries or stomatal openings and spreads inside the apoplast (space within the plant cells) by causing cell death and decay of tissue by releasing phytotoxins. When it infects chilli plants, it will cause the plants to become dwarfed, necrotic leaves and fruit quality deteriorated.

The result is a reduction in both yield and consumer value. This indicates the importance of identifying the pathogen at the seed level as the first option to prevent infection by destroying the lifecycle of the pathogen. This study also highlighted the opportunities for alternative materials such as biofertilizers, or herbal antimicrobials compared to synthetic chemicals to control the effect of *P. syringae* and to encourage sustainable farming of chilli peppers. This research confirmed the presence of *Pseudomonas syringae* in chilli seeds that were naturally infected through morphological and biochemical characterization including catalase positive, indole negative, citrate utilization and gram-negative rod morphology. The findings in this study are in agreement with previously research and confirm that *P. syringae* is a seed-borne pathogen, and has the potential to initiate field infections, as suggested by Alam *et al.* (2015).

CONCLUSION

In conclusion, *Pseudomonas syringae* has the potential to invade plants through both natural and artificial plant wounds and also secrete the phytotoxins which might impact seed germination, plant / vigor and fruit quality which can result in significant yield loss and economic loss. While chemical alternative treatments can suppress the disease, the consequences on both health and environment indicate a shift towards sustainable disease management. This study has identified the immediate need to search for environmentally friendly alternatives, such as PGPR and herbal antimicrobials which have previously identified the potential to manage bacterial pathogens with minimal disruption to the environment. The enhanced potential of biological fungicide treatments will promote early pathogen detection reducing dependence on chemical treatments, enhance resiliency in agriculture and will support sustainable development.

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