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Screening of Bacterial Isolates from Cow Manure with Potential as Antagonistic Agents against *Alternaria porri* (Ellis) Cif.

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Abstract

Environmentally friendly control of *Alternaria porri* (Ellis) Cif. can be carried out using antagonistic agents such as bacteria. Exploration of bacterial sources as antagonistic agents needs to be conducted continuously, including those from cow manure. This study aimed to screen bacterial isolates from cow manure with high antagonistic potential against *A. porri*. Samples were collected using a purposive sampling technique. Bacterial isolation was performed using the spread plate method from dilutions of 10^{-6} to 10^{-8} . Antagonistic activity was assessed using the food poisoning technique with three replications. This research obtained four isolates (PKa, PKb, PKc, and PKd) with cell counts ranging from 0.12×10^9 to 1.83×10^9 CFU g⁻¹. All isolates belonged to the Gram-positive group. Isolate PKa exhibited no catalase activity, whereas isolates PKb, PKc, and PKd showed catalase activity. PKa was presumptively identified as *Clostridium*, while PKb, PKc, and PKd were presumptively identified as *Bacillus* based on the presence of endospores and catalase activity. All isolates demonstrated potential as antagonistic agents, with antagonistic activity ranging from 82.00% to 90.00% (overall mean of $86.25 \pm 3.25\%$).

1. INTRODUCTION

Alternaria porri (Ellis) Cif. is a phytopathogenic fungus that kills host cells and utilizes nutrients from dead tissues to cause disease [1]. This fungus attacks plants in the Amaryllidaceae family, with the genus *Allium* being the most commonly infected [2]. Infection by this fungus results in reduced bulb size or the failure of bulb formation altogether, leading to yield losses of up to 57%, and potentially causing total crop failure if not effectively controlled [3].

Antagonistic microorganisms can be used to control this phytopathogenic fungus. The use of antagonistic microorganisms is a promising biocontrol alternative because they are easy to develop, effective in suppressing phytopathogens, pesticide-residue-free, environmentally friendly, and capable of reducing farmers' dependence on synthetic pesticides [4]. Bacteria are among the antagonistic microorganisms that can be utilized [5]. Research by [6] demonstrated that endophytic bacterial isolates exhibited antagonistic activity against phytopathogenic fungi in vitro. In addition, bacterial isolates derived from compost have been shown to inhibit the growth of *A. alternata* by up to 53%, a phytopathogen closely related to *A. porri* [7].

Although cow manure harbors diverse bacterial populations with high potential as antagonistic agents [8], current biocontrol research against *Alternaria porri* has largely focused on antagonistic fungi, particularly *Trichoderma* spp. Consequently, the specific potential of indigenous cow manure-derived bacteria to suppress *A. porri* remains underrepresented in the literature. Exploring these local bacterial isolates is crucial, as indigenous strains often demonstrate superior adaptability to local field conditions and distinct mechanisms of antagonism. Therefore, this study aims to screen bacterial isolates from cow manure with high antagonistic potential against *A. porri* [8].

2. MATERIALS AND METHODS

Materials and equipment

The materials used in this study included cow manure; a pure isolate of *A. porri* obtained from the Laboratory of Pathology, Entomology, Microbiology, and Soil Science, Faculty of Agriculture and Animal Science, Sultan Syarif Kasim State Islamic University of Riau. 300 g of cow manure fertilizer; nutrient agar medium (Merck®); potato dextrose agar medium (Merck®); distilled water; sterile distilled water; malachite green (Merck®); immersion oil (Merck®); safranin (Merck®); Gram staining kit (Himedia®); 96% alcohol (Bratachem®); physiological NaCl (0.85%) (Merck®); H₂O₂ (Merck®); alcohol/spirit (Bratachem®); and cotton wool. The equipment used included a Bunsen burner, measuring pipettes, beakers, microscope, autoclave, vortex mixer, Petri dishes, hot plate with magnetic stirrer, Erlenmeyer flask, analytical balance, laminar air flow cabinet, inoculating loop (ose needle), test tubes, incubator, glass slides, L-shaped spreader (L-rod), spatula, cotton, aluminum foil, marker, clip plastic bags, colony counter, stationery, and gloves.

Sample collection

Cow manure is obtained from the Tenayan Raya Agrotourism Park. The collection of cow manure fertilizer was carried out using a purposive sampling technique. Samples were taken from three different sacks, with 100 g collected from each sack. Each sample was placed in a sterile plastic bag and stored in a cool box (4°C) before being transported to the laboratory. Each sample was then composited for the isolation process.

Preparation of nutrient agar and potato dextrose agar

The preparation of nutrient agar medium was carried out by dissolving 23 g of the medium in an Erlenmeyer flask containing 1 L of distilled water. The solution was then heated to boiling and homogenized using a

hot plate with a magnetic stirrer. After the medium had completely dissolved and become homogeneous, the mouth of the Erlenmeyer flask was covered with cotton wool.

Potato dextrose agar was prepared by dissolving 39 g of the medium in an Erlenmeyer flask containing 1 L of distilled water. The subsequent steps were the same as those used in the preparation of the nutrient agar medium.

Sterilization

All heat-resistant materials and equipment were sterilized using an autoclave. Sterilization was carried out at a temperature of 121°C under a pressure of 15 psi for 15 minutes for glassware, while the culture media were sterilized for 20 minutes [9].

Isolation and purification of isolates

Isolation was carried out in triplicate. A total of 100 g of cow manure was dissolved in an Erlenmeyer flask containing 900 mL of physiological NaCl solution, then homogenized using a vortex mixer. The suspension was subsequently serially diluted up to 10⁻⁸.

Plating was performed on the last three dilution series (10⁻⁶ – 10⁻⁸) using the spread plate technique. This technique was conducted by pipetting 0.1 mL of the diluted sample onto sterile solid nutrient agar in a Petri dish, followed by evenly spreading it using an L-shaped spreader [10]. The Petri dishes were then covered and incubated in an inverted position for 24 hours at 30°C [11].

Bacterial colonies exhibiting distinct morphotypes-differentiated based on a combination of visual macroscopic characteristics including colony shape, margin, elevation, surface texture, and color were cataloged and quantified separately. The population density for each morphotype was expressed as Colony Forming Units per gram of sample (CFU g⁻¹), calculated using the formula:

$$CFU\ g^{-1} = \frac{N}{d \times V} \times \frac{1}{W}$$

Where:

N :colony count of a specific morphotype across replicates

d : dilution factor

V : volume plated

W : weight of the manure sample

Identical morphotypes observed across different dilutions or replicate plates were averaged at the most appropriate dilution level to avoid overestimation. Representative colonies of each distinct morphotype were subsequently purified using the four-way streak plate technique on Nutrient Agar (NA). Well-isolated single colonies were inoculated onto NA slants, incubated at 30°C for 24 h, and stored as working stock cultures in a refrigerator at 4°C for up to 1 month, with periodic subculturing to maintain viability.

Observation of microscopic characteristics

The purified isolates were subjected to phenotypic characterization to presumptively identify them to the genus level. Microscopic evaluations included cell morphology, Gram reaction, and endospore staining, while biochemical profiling was evaluated via catalase activity assays following standard procedures described by Cappuccino and Welsh [10].

Presumptive genus-level assignment was determined using the diagnostic key criteria outlined in Holt et al. [12]. Isolates were classified based on a combination of Gram-staining reaction, cellular shape, spore-forming capability, and catalase response (e.g., Gram-positive, endospore-forming, catalase-positive rods were presumptively assigned to *Bacillus* spp.). Furthermore, aerotolerance assays were conducted by culturing isolates under both aerobic and anaerobic conditions (using an anaerobic jar system) to differentiate obligate aerobes/facultative anaerobes from strict anaerobes such as *Clostridium* spp.

Observation of cell morphology and Gram grouping was carried out using the Gram staining method. Bacterial isolates aged 18-24 hours were aseptically collected using a sterile inoculating loop and thin smear preparations were made on clean glass slides. The smears were air-dried and heat-fixed by passing them over a flame 2-3 times.

Gram staining was performed by applying crystal violet for 1 minute, followed by rinsing with running distilled water. Iodine solution (mordant) was then added for 1 minute and rinsed again. Decolorization was carried out using 95% alcohol for approximately 10-20 seconds, immediately followed by rinsing with distilled water. The smear was then counterstained with safranin for 1 minute and rinsed again. The preparation was dried using absorbent paper and observed under a light microscope at 1000× magnification using immersion oil to determine cell shape (cocci, bacilli, or other forms) and Gram reaction (positive or negative).

Bacteria identified as Gram-positive were further examined for endospore formation. The presence of endospores was observed using the Schaeffer–Fulton endospore staining method. Smear preparations were made as described for Gram staining, then flooded with malachite green and heated over a water bath for approximately 5 minutes while ensuring the smear remained moist. After cooling and rinsing with distilled water, the smear was counterstained with safranin for 1 minute, then rinsed and dried. The preparation was observed under a microscope at 1000× magnification to identify the presence of endospores, which appeared as green structures either within or outside the red vegetative cells.

Catalase activity test

The catalase activity test was performed using the slide test method. A loopful of fresh bacterial culture (18–24 hours old) was placed on a clean glass slide, followed by the addition of 1–2 drops of 3% hydrogen peroxide (H_2O_2) solution. A positive reaction was

indicated by the rapid formation of oxygen bubbles due to the breakdown of H_2O_2 by the catalase enzyme, whereas the absence of bubble formation indicated a negative result [10].

Antagonistic Activity Assay

The antagonistic activity assay was conducted using the food poisoning technique, adapting the methods described by Gakuubi et al. [13] and Tawfik et al. [14] with modifications. Bacterial suspensions were prepared from 24-hour-old cultures and standardized to a concentration of 10^6 CFU/mL by adjusting the optical density at 600 nm ($OD_{600} \approx 0.1$) using a spectrophotometer, followed by verification via serial dilution plating.

A total of 1 mL of the standardized bacterial suspension (10^6 CFU/mL) was inoculated into a sterile Petri dish. Subsequently, 20 mL of molten PDA, cooled to 45-50°C to preserve bacterial cell viability, was poured into the dish and homogenized thoroughly. The mixture (total volume of 21 mL) resulted in a final bacterial cell concentration of approximately 4.76×10^4 CFU/mL in the solidified agar matrix.

Once the medium solidified, a 10 mm mycelial plug was taken from the actively growing margin of a 7-day-old *A. porri* culture and placed at the center of the Petri dish. The assay included three sets of controls: an untreated control (PDA without bacterial suspension), a negative control (PDA with sterile distilled water), and a positive control (PDA amended with 0.2% synthetic fungicide mancozeb). All treatments and controls were performed in triplicate.

Plates were incubated at 28°C for 7 days. Colony growth was monitored daily, and the final mycelial diameter was measured along two perpendicular axes using a digital caliper. The antagonistic activity (AA) was calculated using the formula [13- 15]:

$$AA = \frac{C-T}{C} \times 100\%$$

Where:

AA : antagonistic activity

C : negative control

T : treatment

3. Results

Bacterial isolation from cow manure resulted in four distinct isolates, designated as PKa, PKb, PKc, and PKd, each exhibiting different colony morphological characteristics (Table 1). The observed variations included colony shape, colony margin, and bacterial population density. In contrast, all isolates shared similar colony surface characteristics, exhibiting a flat elevation and a milky-white pigmentation.

Isolate PKa exhibited an irregular colony shape with lobate margins and a population density of 0.35×10^9 CFU g⁻¹. Isolate PKb produced punctiform circular colonies with smooth margins and showed the highest bacterial population among all isolates, reaching 1.83×10^9 CFU g⁻¹. Meanwhile, isolate PKc formed circular colonies with undulate margins and a population density of 0.93×10^9 CFU g⁻¹. In contrast, isolate PKd exhibited filamentous colonies with filamentous margins and the lowest bacterial population, measuring 0.12×10^9 CFU g⁻¹.

Microscopic characterization and physiological testing revealed that all bacterial isolates obtained from cow manure were rod-shaped, Gram-positive, and capable of forming endospores (Table 2). Despite exhibiting relatively uniform microscopic characteristics, the four isolates differed in their catalase activity and presumptive genus identification.

Isolate PKa exhibited a negative catalase reaction and was presumptively identified as belonging to the genus *Clostridium*. This isolate showed an antagonistic activity of 82%. In contrast, isolates PKb, PKc, and PKd exhibited positive catalase reactions and were presumptively assigned to the genus *Bacillus*. All three isolates demonstrated the same level of antagonistic activity, reaching 90%.

The results further showed that isolates PKb, PKc, and PKd exhibited numerically higher antagonistic activity than isolate PKa. This difference suggests variability in the inhibitory capacity among the bacterial isolates against the target microorganism. The observed antagonistic activity, ranging from 82% to 90%.

4. DISCUSSION

The observed morphological diversity among recovered culturable isolates highlights the role of cow manure as a favorable ecological niche for diverse bacterial communities with distinct phenotypic traits [16]. While macroscopic variation across the four isolates reflects phenotypic diversity under the specific growth conditions used, these results align with previous studies reporting similar phenotypic heterogeneities in manure-derived microbial populations [16]. Such morphological differences are often shaped by environmental adaptation, nutrient gradients, and structural dynamics during colony development rather than taxonomic divergence alone [16-17].

Isolate PKa exhibited an irregular colony shape with lobate margins, a flat surface, and a milky-white appearance, with a population density of 0.35×10^9 CFU g⁻¹. The observed lobate margins and irregular expansion are tentatively attributed to non-uniform colony expansion on solid media, a phenotypic trait that can be influenced by local nutrient gradients and mechanical constraints during growth [18]. However, further physiological and biophysical assays are required to determine whether these morphological features correlate with specific cell-level mechanisms in this strain [19].

Among all recovered isolates, PKb yielded the highest viable count on Nutrient Agar, reaching 1.83×10^9 CFU g⁻¹ [cite: 1]. While higher colony counts indicate good culturability under the specific isolation conditions used, this higher abundance does

not inherently prove superior ecological adaptability or competitive substrate utilization in vivo. The observed recovery levels can be influenced by multiple factors, including sampling variations, serial dilution steps, culture medium selectivity, incubation parameters, and the physiological state of the bacterial cells [18].

Isolate PKc produced circular colonies with undulate margins, a flat surface, and a milky-white color, with an abundance of 0.93×10^9 CFU g⁻¹ under the tested conditions. The circular shape with undulate margins describes the visual macro-morphology of the colony on Nutrient Agar. While variations in colony margins can be influenced by incubation conditions, moisture, or local nutrient gradients in solid media [20-21], specific cellular-level growth dynamics were not evaluated in this study.

In contrast, isolate PKd formed filamentous colonies with filamentous margins, a flat surface, and a milky-white color, yielding the lowest recovered density among the isolates at 0.12×10^9 CFU g⁻¹. It is important to distinguish this macroscopic colony morphology from cellular shape, as microscopic observation confirmed that PKd consists of standard rod-shaped vegetative cells rather than filamentous or branching individual cells. Filamentous colony margins on agar media are typically driven by multicellular growth patterns and cellular migration strategies during colony spreading, rather than cellular elongation. The lower recovered abundance on Nutrient Agar may simply reflect specific growth requirements or different culture dynamics under the standard laboratory conditions evaluated [22-23].

Although the isolates differed in colony shape and margin characteristics, they shared similar flat elevations and a milky-white visual appearance. Rather than indicating specific or comparable pigment production, this common appearance simply reflects similar non-pigmented visual characteristics under the given culture conditions. As noted, macroscopic colony morphology alone is

insufficient for reliable taxonomic assignment, since phenotypically similar colonies can belong to distinct bacterial taxa [24]. Consequently, microscopic examination, physiological assays, and molecular analyses (such as 16S rRNA gene sequencing) are essential to establish accurate taxonomic identities and relationships [25].

The total culturable bacterial load recovered from cow manure under the tested isolation conditions reached 3.23×10^9 CFU g⁻¹ across the four distinct morphotypes. While cow manure is rich in organic substrates, nitrogenous compounds, minerals, and moisture that support bacterial growth, these culturable counts reflect only the viable population capable of growing on Nutrient Agar rather than the entire microbial community [26-27]. Although cow manure microflora generally participate in organic matter decomposition and nutrient transformations in agroecosystems [17, 28-29], the specific metabolic contributions and nutrient cycling roles of these four isolates were not directly evaluated in this study.

Among the recovered isolates, isolate PKb represented the largest proportion of the culturable bacterial population under the isolation conditions tested. However, this numerical dominance in culture does not necessarily denote biological competitiveness or ecological dominance in the original manure environment, as relative recovery rates are heavily influenced by cultivation bias, media selectivity, and sampling variation [30-31]. Similarly, the lower abundance of isolate PKd merely reflects its lower recovery on the culture medium used, rather than lower intrinsic fitness. Overall, the observed variation in CFU values among the four morphotypes illustrates differences in recovery performance under standard laboratory culture conditions rather than full community dynamics in vivo.

The high in vitro inhibition rates observed in this study (82% to 90%) demonstrate the promising potential of cow manure-derived bacteria, specifically *Bacillus* and *Clostridium*

isolates as biocontrol agents against *Alternaria porri*. The observed antagonistic activity is comparable to, or exceeds, antifungal inhibition levels reported for compost-derived bacteria against related *Alternaria* species (such as *A. alternata* at 53%) [7] and endophytic strains evaluated in vitro [6]. While these results suggest potential mechanisms such as lipopeptide production, lytic enzyme secretion, or diffusible antifungal metabolites, these mode-of-action pathways remain speculative as they were not directly measured. Furthermore, a primary limitation of this study is the reliance on phenotypic and biochemical assays for presumptive genus identification, which requires validation through 16S rRNA gene sequencing. To establish the practical efficacy of these isolates, future research must extend beyond in vitro assays to evaluate bioactivity under greenhouse and field conditions, where plant-microbe-pathogen interactions and environmental variables heavily influence biocontrol performance [8,32].

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5. CONCLUSION

Four bacterial isolates were successfully isolated from cow manure, with recovered densities ranging from 0.12×10^9 to 1.83×10^9 CFU g⁻¹ under the tested conditions. All isolates were Gram-positive rods capable of forming endospores. Based on catalase reaction isolate PKa was presumptively assigned to the genus *Clostridium*, whereas isolates PKb, PKc, and PKd were presumptively assigned to *Bacillus*. Under in vitro conditions, all isolates demonstrated high antagonistic activity against *Alternaria porri*, ranging from 82% to 90% inhibition. However, without comparative statistical analysis, these numerical differences do not confirm superior efficacy among the isolates. Definitive taxonomic identification via molecular methods (such as 16S rRNA gene sequencing) and evaluation under greenhouse or field conditions are necessary to confirm their biocontrol potential in vivo

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