

ISOLATION AND CHARACTERIZATION OF HALOPHILIC ACTINOMYCETES FROM COW DUNG

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Abstract: This study is focused on the collection, isolation and characterization of mesophilic halophilic Actinomycetes from cow dung. Four different strains of the isolates designated 10³A, 103B, 104A, and 104B were isolated in which the four isolates stained pink, this indicates the isolates to be gram positive. The isolates were screened and confirmed to be Actinomycetes. The isolates were further characterized based on their carbon utilization ability, Melanin production, coverslip culture technique, Sodium Chloride (NaCl₂) tolerant test. The activity of α amylase was determined on solid medium supplemented with starch. The detection was based on the formation of clear zones around the colonies.

Keywords: Isolation, Characterization, Halophiles, Actinomycetes, Cow Dung.

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INTRODUCTION

Halophilic and mesophilic actinomycetes are widely distributed in nature and occur in diverse habitats ranging from ordinary soils and plant materials to highly specialized environments such as marine buoys, ant exoskeletons, salt lakes, and even Paleolithic cave paintings (Donadio et al., 2006). Mesophilic actinomycetes grow optimally at temperatures between 15 and 40°C, whereas other groups are adapted to extreme thermal environments. Actinomycetes are particularly notable for their ability to produce microbial volatile organic compounds (MVOCs), many of which contribute to the characteristic earthy or musty odor of soils and damp environments (Donadio et al., 2006). Their widespread occurrence in terrestrial and aquatic ecosystems highlights their ecological importance in nutrient cycling and biodegradation processes (Awari et al., 2023; Agu et al., 2022).

Cow dung has been utilized for centuries as an organic fertilizer in agricultural systems, particularly in the Indian subcontinent. Its application enhances soil fertility, improves plant resistance to

pests and diseases, stimulates plant growth, and promotes beneficial microbial activities such as sulphur oxidation and phosphorus solubilization (Periyasamy et al., 2014). Cow dung contains approximately 80% water and serves as a nutrient-rich substrate supporting diverse microbial communities, including bacteria, fungi, yeasts, and actinomycetes (Ware et al., 1988). Studies have demonstrated that organic substrates rich in microbial populations provide favorable conditions for the proliferation of beneficial microorganisms involved in biodegradation and nutrient recycling (Okafor et al., 2016; Orji et al., 2022). The lower gastrointestinal tract of cattle harbors beneficial microorganisms such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Enterococcus diacetylactis*, *Bifidobacterium* species, and *Saccharomyces cerevisiae*, many of which contribute to the microbial diversity of cow dung (Ware et al., 1988).

Actinomycetes constitute a diverse group of Gram-positive bacteria characterized by filamentous growth patterns that resemble

fungal hyphae. Members of the order Actinomycetales produce branching mycelia and, in some genera, spores that facilitate dispersal and survival under adverse environmental conditions (Manivannan et al., 2014). They are among the most abundant microorganisms in soil ecosystems and play crucial roles in the decomposition of organic matter, including cellulose, chitin, lignin, and other complex biopolymers (Kamal et al., 2014). Their metabolic versatility contributes significantly to environmental sustainability through the degradation of pollutants and the recycling of nutrients (Agu et al., 2015; Mbachu et al., 2014; Okafor et al., 2016).

Many actinomycetes are recognized for their ability to synthesize bioactive compounds, particularly antibiotics and extracellular enzymes. Several commercially important antibiotics, including streptomycin, tetracycline, and nystatin, are derived from actinomycetes (Cavaletti et al., 2006). In addition to antibiotic production, they secrete hydrolytic enzymes capable of lysing fungal and bacterial cell walls, thereby exhibiting antagonistic activity against numerous plant pathogens (Prepagdee et al., 2008; Kamal et al., 2014). These antagonistic properties have attracted considerable interest for their application in biological control and sustainable agriculture.

Actinomycetes also play an essential role during composting processes. Thermophilic and thermotolerant species dominate during the high-temperature phase of composting, where they facilitate the decomposition of recalcitrant organic matter and contribute to pathogen suppression (Cavaletti et al., 2006). Their activities enhance compost quality through the mineralization of organic substrates and the production of enzymes involved in the breakdown of cellulose and chitin. Similar degradative capabilities have been reported for other environmental microorganisms isolated from polluted soils and waste-contaminated environments (Anaukwu et al., 2016a; Anaukwu et al., 2016b; Ifediegwu et al., 2015; Ojiagu et al., 2018).

Despite their ecological and industrial significance, some actinomycetes are pathogenic to humans and animals. For example, *Nocardia asteroides* causes nocardiosis affecting the lungs, brain, and skin, while *Dermatophilus congolensis* is responsible for dermatophilosis in cattle, sheep, horses, and occasionally humans (Manivannan et al., 2014). Furthermore, inhalation of actinomycete spores has been associated with respiratory disorders such as hypersensitivity pneumonitis and farmer's lung, particularly among agricultural workers exposed to contaminated organic materials (Cavaletti et al., 2006).

Actinomycetes are cosmopolitan microorganisms capable of colonizing a broad range of habitats. Their filamentous hyphae form aerial mycelia bearing hydrophobic spores that can be dispersed by air, water, and soil invertebrates. Certain genera, such as *Actinoplanes*, have evolved specialized mechanisms for water-mediated dispersal through the production of motile zoospores enclosed within desiccation-resistant sporangia. Their adaptability to diverse environmental conditions, coupled with their remarkable metabolic capabilities, makes them valuable organisms for applications in agriculture, biotechnology, environmental remediation, and pharmaceutical development (Agu et al., 2022; Okeke et al., 2023; Oparaji et al., 2024).

This aim of this study is to isolate and characterize Halophilic Actinomycetes from cow dung.

MATERIALS AND METHODS

Different samples were collected from compost cow dung, soils where the hides and skins of animals were burnt in an Abattoir in Awka, aseptically in a sterile conical flask. The samples were homogenized and transported to Microbiology Laboratory for the evaluation of microbial analysis.

Sample Preparation

Ten gram of the cow dung sample was suspended in 100ml of sterile water, contained in a test tube and vigorously shaken for proper mixing. The diluted sample was then serially diluted in ten folds up to 10^{-8} dilution.

Isolation of Mesophilic Actinomycetes

Egorov mineral salt medium which had the following composition in g/l, 1g of K_2PO_4 , 1g of $(NH_4)_2SO_4$, 1g of $NaCl$, 1g of $MgSO_4$, 10g of soluble starch, 20g of Agar, supplemented with beef extract and one liter of distilled water was used as the isolating medium.

The Egorov medium was prepared with appropriate amount of components added, depending on the amount of medium required and autoclaved at $121^\circ C$ for 15mins. Thereafter, the medium was allowed to cool i.e. cheek friendly and poured into sterile Petri dishes and allowed to gel. Aliquots (0.1ml) from 10^4 and 10^5 dilutions of the sample were introduced in duplicates, into the prepared plates with the use of sterile pipettes and evenly spread on the plate using a sterile spreader. The inoculated plates were kept at room temperature for 2 – 4days.

Colony count

After about 2 – 4days, the average colony count was carried out on the plate and this showed discrete colonies and zones of concentration.

The colonies that show clear zones of starch hydrolysis characteristics of Actinomycetes were chosen and stored for further analysis.

Purification of isolates

Based on colony morphology, the Actinomycetes colonies were sub-cultured and purified by using streak plate method on Egorov medium using sterilized inoculating wire loop, slightly picked up the colony from the spread plate dragged the loop over the surface of another plate in a zigzag motion. Sterilized the loop over the flame, turned the plate to 90° and dragged the loop over the area streaked before in similar manner. Again, sterilized the loop over the flame and the same process was repeated again, all the plates were stored at room temperature for 2days. The heaviest growth was seen in the first sector, and the isolated colonies were in the third sector. This method was repeated several times until purified colonies were obtained.

The pure isolates were then stored on Egorov medium slants in Bijou bottles for further study.

Macroscopic Identification

This was done by observing cultural and morphological characteristics. The isolates cultured on the Egorov mineral salts were carefully observed by the nature of growth, rate of growth, colony colour, pigmentation, texture and size.

Carbon Utilization Test

This was examined using the modification of the method described by Shirling and Golieb (1986). The following sugars: Sucrose, Lactose, Glucose, Fructose and Maltose were used to replace starch in Egorov medium. The flasks were sterilized at 121°C for 15mins, with only the flask containing glucose sterilized at 115°C for 10mins.

After sterilization, the media were poured into plates and allowed to gel. The test organisms were then point inoculated into each plate and kept at room temperature for 5days.

NaCl₂ Tolerant Test

The normal composition of Egorov medium was prepared with different concentration of NaCl₂. The NaCl₂ composition includes 2.5g, 5.0g, and 7.5g. it was kept at room temperature for 5days.

Gram stain

The isolate was gram stained and observed with oil immersion lens for their gram reactions. Red or pink colour indicates gram negative organism while purple color indicated gram positive organisms. This also shows the structure of the organisms.

Covership Culture Technique

This was carried out as described by Williams *et al.*, (1968). Sterile coverslips were aseptically inserted at 45° into Egorov medium plates using a sterile forcep.

A loopful of the organism was streaked along base of the coverslip (at the point of where the upper surface of the coverslip meets the medium) and kept at room temperature for 7 days. After 7 days, the slips were removed, fixed with methanol and then stained with crystal violet for 2mins. The slips were viewed under the microscope using oil immersion (x100 lens).

Screening of isolates for amylase production

An inoculation of the isolate was made on the plate (Egorov medium) and kept at room temperature for 3-5 days. After 3-5

days, 1ml of iodine was flooded on the plate. Colonies that gave blue-black colour were classified tentatively as β -amylase producers, while α -amylase were noted by clear zone of starch hydrolysis on the starch agar plate.

RESULTS

Morphological Characteristics of the Isolates

Colonial morphology of the isolates were further characterized based on their shape, color, elevation, margin, texture, underneath color and aerial mycelium as shown in table 1.

Carbon (Sugar) utilization by the Isolates

The utilization of five sugars (Glucose, Sucrose, Fructose, Maltose and Lactose), were carried out on the Egorov (1964) mineral salt medium containing the isolates on a solid media and the results were recorded as shown in table 2.

Effects of NaCl₂ on the Isolates

The effects of various composition of NaCl₂ (2.5g, 5.0g, 7.5g) as recorded in table 3. With the composition of 2.5g having greater effect on the Egorov (1964) mineral salt medium containing Actinomycetes isolates. Followed by 5.0g, with a lesser or no effect on the Actinomycetes in 7.0g of NaCl₂.

Screening of Isolates for amylase production

Three of the isolates showed a clear zone when flooded with iodine as shown in table 4.

Gram staining

The gram stained isolates shows a characteristic pink color as recorded in table 5.

Covership Culture Technique

The isolates were viewed under microscope for aerial production and shown positive as recorded in table 6.

Table 1. The morphological Characteristics of the Isolates

Isolates	Shape	Colour	Elevation	Margin	Under color	Texture
A	Circular	White	Raised	Convex	Cream	Dry
B	Circular	White	Raised	Convex	Cream	Powdery
C	Circular	Grey	Raised	Convex	Cream	Powdery
D	Circular	White	Flat	Convex	Cream	Dry

Table 2. Carbon(Sugar) utilization by the Isolates

Sugars	A	B	C	D
Sucrose	+	+	+	+
Lactose	+	+	+	+
Glucose	+	+	+	+
Fructose	+	+	+	+
Maltose	+	+	+	+

Keys:

Positive = +

Negative = -

Table 3. Effect of the NaCl₂ on the isolates

NaCl ₂ (g)	A	B	C	D
2.5	+++	+++	+++	+++
5.0	++	++	+	-
7.5	+	-	-	-

Keys:

+++ = great tolerant

++ = better tolerant

+ = weak tolerant

- = no tolerant

g = gram

2.5, 5.0 and 7.5 = various NaCl₂ composition

Table 4. Screening of isolated Organism for amylase production

Isolates	Amylase production
A	+
B	-
C	+
D	+

Key:

+ = α – amylase

- = β- amylase

Table 5. Gram staining

Isolate	Gram staining
A	+
B	+
C	+
D	+

Key:

+ = Pink (Gram positive)

- = Purple (Gram negative)

Table 6. Coverslip Culture technique

Isolate	Mycelium production
A	+
B	+
C	+
D	+

Key:

+ = Present

- = Absent

DISCUSSION

A total of four Actinomycetes colonies were isolated and screened for amylolytic activity. Only three isolates A, C and D were found to be amylase producers as subsequently used for the production of amylolytic enzyme. It was observed that the Actinomycetes colonies were able to exhibit good cultural characteristics on the Egorov medium which is a nutritionally not very rich medium. More so, during the isolation of the organisms, the problems of the contaminants were reduced due to the deficiency of the medium. This further added an advantage during the isolation of the organisms. From the work carried out and the characterization undertaken in this project, majority of the organisms isolated are suspected to belong to the genera *Streptomyces*. Therefore, the isolates were further characterized. Among the criteria used for characterization were NaCl₂ tolerant test, utilization of five sugars were carried out and recorded. The four isolates were able to utilize all the sugars, which suggests good carbon utilization ability. This also suggests that these sugars may serve as good carbon sources for these organisms.

The isolates were able to grow on the Egorov (1964) mineral salts medium containing the different composition of NaCl₂, this show that the isolates are halophiles. During the identification for amylase production, the isolates D, C and A showed a clear zone of starch hydrolysis around the colonies when flooded with iodine. Thus, they were tentatively isolated and classified as α -amylase producing isolates. The amount of growth as they grew luxuriantly at 37°C (room temperature) shows that they are mesophilic halophilic organisms.

The characteristic pink color show that the isolates are gram positive organisms, also there was the presence of aerial mycelia showing that the isolates are Actinomycetes.

The isolates show the presence of aerial mycelia when stained with crystal violet and viewed under the microscope.

CONCLUSION

The ability of Actinomycetes to grow on deficient medium with the exclusion of contaminants makes these organisms readily available. From this work, it is observed that Actinomycetes are good amylase producers.

RECOMMENDATION

As in every industrial process, the consideration of cost is very important but the case of isolating Actinomycetes and its confirmed ability to produce amylase make the use of these organisms through genetic engineering create a huge frontier in the utilization of these organisms for the production of the enzyme. Considering the vast uses of amylase (finding immense application in various areas of industrial microbiology and biotechnology and being a very good biocatalyst).

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