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ISOLATION AND IDENTIFICATION OF MICROBES FROM BUFFALO (*BUBALUS BUBALIS*) DUNG

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Abstract

The domestic cattle's cows, bullocks, and buffalo are bovine animal species, which comprises domestic cattle (cows, bullocks, and buffalo), produce dung, also known as manure. Buffalo dung microbial community and its relationship to the rumen microbiome of the animal. The buffalo microbiome appears to have more capability for fibre degradation and less potential for methane production when compared to the rumen microbiomes of other cattle. Microbial load of buffalo dung having *Bacillus, Fibrobacter*, *Ruminococcus, Pseudomonas aeruginosa, Enterobacter xiangfangensis, Proteus mirabilus, Butyrivibrio* and *Prevotella*, capable of degrading non-cellulose plant fibres, is abundantly present in the rumen in buffalo digestive tract.

In the present study microbial load of buffalo dung was analyzed by using nutrient agar, blood agar and MacConkey agar, Sabouraud Dextrose Agar (SDA). The properties of the isolated bacteria's colonies and Gram's staining were observed. Buffalo dung microbial load was calculated using the number of cfu per gram of sample. The highest bacterial population was seen at dilution 10^{-3}, where it reached ranged from 155×10^{-4}cfu/ml. Total 20 bacterial isolates were isolated from buffalo dung including Gram Positive cocci, Gram Positive bacilli and Gram Negative bacilli. These bacterial isolates were identified as *Micrococcus* sp. *Bacillus* sp. and *Escherichia coli* respectively. Sabouraud Dextrose Agar (SDA) used for fungal isolation. The fungal colonies of *Rhizopus* sp. (F1) was exhibited in dilution 10^{-2} which ranged 30×10^{-3} cfu/ml. These microbes will be used forfurther research work.

Keywords: Buffalo dung, Microbial load, Bacteria, Fungi, Microbiome.

Introduction

The buffalo (*Bubalus bubalis*) often known as a water buffalo) is a domestic animal of great economic importance to humans, supplying milk, meat, and leather manufacturing, as well as draught power (Cockrill et al., 1975).

Since they are herbivores, buffaloes eat primarily plant fibre like forage grasses. Different processed products obtained from cow, such as milk, curd, ghee, urine, and by-product (dung), are frequently utilised in therapeutic formulations in Ayurveda, a system of traditional medicine with historical roots in the Indian Subcontinent (Sharma and Singh, 2015).

More than two billion people depend on the estimated 200 million buffaloes that live in the world, more than any other domesticated animal (Scherf and Scherf 2000). Even-toed, hoofed mammals belonging to the tribe Bovini, genus Bubalus and family Bovidae include buffaloes. There are two subspecies of` the domesticated water buffalo: swamp buffalo (*Bubalus bubalis* carabanesis, 2 N=48) and river buffalo (*Bubalus bubalis* bubalis, 2 N = 50) (Tanaka et al., 1996; Kumar et al., 2007).

Bovine animal species such as domestic cattle (cows, bullock and buffalo), yak and water buffalo produce dung or manure as a waste product. Water (80%), undigested residues (14.4%), and microbes (5.6%) are all present in buffalo dung (BD), which is the undigested plant matter that has gone through the animal's digestive system. In Asia and Africa, buffalo dung (BD) has long been utilised as an organic fertiliser (Sawatdeenarunat et al., 2016). Buffalo dung (BD) improves plant tolerance to pests and diseases, promotes plant growth, and solubilizes P and S in addition to providing nutrients to the soil (Sharma and Singh, 2015). Buffalo dung (BD) contains a variety of microorganisms that support water treatment and soil biogeochemical activities (Akinde and Obire, 2008).

The microbiome of different sites in the digestive tract has been connected for diverse studies. Numerous bacteria found in the gastrointestinal tract in ruminants having capacity to digesting cellulose (Liu, et al., 2019). Ruminococcus (Palevich et al., 2019) and *Butyrivibrio* (Derakhshani et al., 2017) similarly, *Prevotella*, a group of bacteria capable of degrading non-cellulose plant fibres, is abundant in the rumen (Stanislawski et al., 2019).

Microbial load of buffalo dung was calculated. Attempts were made to isolate different types of bacteria and fungi from buffalo dung by using serial dilution methods. Nutrient agar, Blood agar, Macconkey agar and Sabouraud Dextrose Agar (SDA) were used for culturing of microbes. Bacterial isolates were identified on the basis of their colony characteristics, morphology and Gram staining. The present study was aimed to study the microbial inhabiting in buffalo dung will be used for improvement in nutritional properties of soil and water treatment.

MATERIALS AND METHODS

Collection of Buffalo dung sample

Buffalo dung samples were collected aseptically in sterile container from Acharol village and transport to microbiology laboratory of the Department of Allied Medical Science and Technology, Nims University Rajasthan, Jaipur.

Preparation of Buffalo dung suspension

Buffalo dung suspensions were prepared in sterile distilled water, and serial dilution analysis was used to find out the number of bacteria present. In order to properly mix the collected sample and labelled. 1gm of buffalo dung samples was transferred to the 10 ml of sterilized distilled water and incubated in shaken for an hour. All bacterial samples were incubated at 37°C for 30-40 minute in an incubator for activation of microorganism.

Each sample was diluted using the traditional dilution process with the aid of a sterilized pipette. Each test tube contains 9 ml of sterilized distilled water. The labelled test tubes were placed in test tube stand then 1ml of activated standard solution was transferred in test tube number 1, and further 1ml of sample was transferred to number 2 and same procedure was repeated for further dilution. In our studies we used water to prepare buffalo dung suspension and in previous work two carrier materials, i.e. phosphate buffer and buffalo dung slurry, were chosen (Sharma and Singh 2015; Dhiman et al., 2022).

Microbial load of Buffalo dung

Isolation of bacteria and fungi from buffalo dung by using serial dilution method, sample were transfer on Nutrient agar, blood agar, Macconkey agar, Sabouraud dextrose agar (SDA). Calculate the number of microorganism and fungi per gram of buffalo dung by applying the formula.

$$\textit{Viable cells per gram buffalo dung} = \frac{Meanplatecount \times Dilutionfactor}{Dryweightofbuffalodung}$$

Isolation of bacterial and fungal colonies

Bacterial and fungal colonies of buffalo dung were study by using spread-plate and streak-plate method to separate the mixed culture of microbes.

Streak plate method

This technique is a quick and effective way to isolate things. It essentially involves spreading a loopful of culture over the top of an agar plate, which is a dilution technique. The four-way, or quadrant, streak is characterised despite the fact that numerous different operations are carried out. The sample was collected using a sterile inoculating loop and streaked onto the plate medium. The plates were then incubated for 24 hours at 37°C.

Spread plate method

In spread plate method the sample was picked from buffalo dung sample separately by the help of sterilized inoculating loop and transferred to Petri-plates separately. All plates were incubated in incubator at 37°C, for 24 hours and 27°C, for 72 hours for bacteria and fungi respectively.

Morphological and microscopically characterization of microbes

Unknown microorganisms were identified by combination of information from morphology and the microscopic observation. Cultural growth characteristics (culture characteristic such as colony diameter, growth, colour, form, elevation and margin and arrangements of cells) on agar media, Gram staining and morphological features cells shape, cells arrangement, microscopic were performed according to standard protocol (Cappuccino and Sherman, 2005).

Identification of an unknown bacterial and fungus

Streak the unknown bacterial and fungal culture on the surface of agar plates for isolation and identification. Inoculated petri-plates were incubated at 27°C and 37°C for bacteria and fungi for 24 and 48 hours for isolation respectively. Perform a Gram staining from the 24 hour old culture and Cotton blue lactophenol staining for fungi (Cappuccino and Sherman, 2005).

Biochemical Test

For the identification of bacterial isolates routine standard biochemical tests such as Indole, Triple sugar, Citrate, Urease and Mannitol motility were performed. Isolates were inoculated in these medium and incubated at 37^0C for 18-24 hours. Next day various biochemical reactions such as Indole production, urease hydrolysis, citrate utilization, fermentation of sugars and motility were tested.

Indole:

The Indole test evaluates an organism's capacity to break down the amino acid tryptophan and generate Indole. It is a component of the IMViC procedures, a set of diagnostic tests used to identify between members of the Enterobacteriaceae family. Indole is extracted from the medium into the reagent layer by the acidified butyl alcohol component and forms a complex with the *p*-dimethylaminobenzaldehyde, yielding the cheery red colour.

Triple sugar iron:

Triple sugar iron agar (TSI) is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate. The acid base indicator phenol red is also incorporated to detect carbohydrate fermentation that is indicated by a chance in colour of medium from orange red to yellow in the presence of acids with gas production.

Citrate test:

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. In middle of the growth, even if the color is not changed, is considered positive. We would see a color change in the medium if the test organism forms acid or alkali during its growth. The usual color change that is observed is from green (neutral) to blue (alkaline).

Urease test:

This test serves to rapidly distinguish members of this *Proteus* genus from lactose-nonfermenting enteric microorganisms. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms alkaline end product ammonia. The presence of urease is detectable when organisms are grown in a urea broth medium containing the pH indicator phenol red to turn to deep pink. This is a positive reaction for the presence of urease.

Mannitol motility:

Mannitol motility test media is designed to differentiated bacteria on the basis of their motility and the ability to ferment mannitol. Semisolid media contains 0.3 percent agar help to detect motility. Motile bacteria produce diffused growth throughout the media while non- motile bacteria grow only along the line of inoculation. Fermentation of mannitol produces acidity in the media. Phenol red used as a pH indicator, which detect acidity by exhibiting a visible colour change from yellow to red.

RESULTS AND DISCUSSION

Buffalo dung samples were collected from different location of Acharol village, aseptically in sterile poly bags and bring to microbiology laboratory of the Department of Allied Medical Science and Technology for isolation and identification of microbes.

Buffalo dung suspension and its characterization:

Buffalo dung suspension carrying large numbers of microbes, serial dilution technique used for microbial load analysis. That involves spreading a suspension over the surface of Nutrient agar for the isolation of bacteria and Sabouraud dextrose agar media (SDA) for fungus isolation from buffalo dung. Further characterisation of isolated bacterial and fungal colonies was performed according to standard protocols. Previous studies buffalo dung is one of the best sinks of microorganisms on the other hand, several bacterial genera present in dung (Dhiman et al., 2020).

Microbial load and isolation of bacteria by Serial dilution of buffalo dung

The collected samples of buffalo dung were enumerated for their total bacteria in microbial load of buffalo dung were calculating cfu/gm of samples. Serially diluted buffalo dung suspension was plated of nutrient agar plates. The maximum number (TNTC) of bacterial population was exhibited in initial dilution, then 10^{-3} to 10^{-4} showed which ranged from 155×10^{-4} to 50×10^{-5} cfu/ml and minimum concentration (TNFC) was exhibited in dilution 10^{-6}. Table 1 and Figure 1 shows the microbial count of buffalo dung in initial dilution large numbers of microbes are present as further dilution shows isolated pure colonies. Although bacteria and fungi are both important contributors to the composting process of cattle dung, bacteria are more abundant (Holman et al., 2016). The general microflora inhabitant of the cattle gut involves *Bacillus, Bifidobacterium,* and *Lactobacillus* (Teo and Teoh, 2013).

Table 1: Microbial load of the buffalo dung sample by serial dilution method

S.No	Dilutions	Methods used	Total bacteria count
1.	10^{-1}	Serial dilution method	TNTC
2.	10^{-2}	Serial dilution method	TNTC
3.	10^{-3}	Serial dilution method	155×10^{-4}
4.	10^{-4}	Serial dilution method	50×10^{-5}
5.	10^{-5}	Serial dilution method	TFTC
6.	10^{-6}	Serial dilution method	TFTC

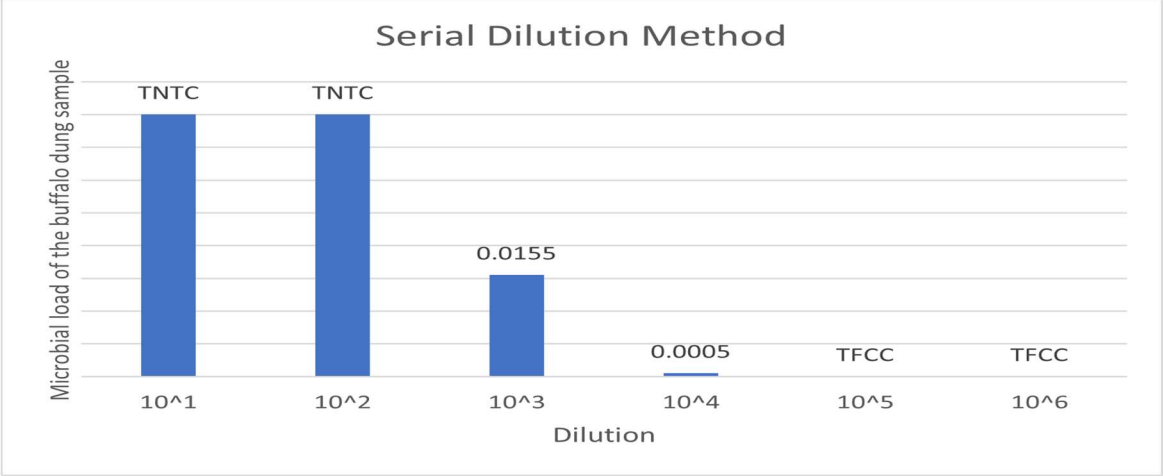

Figure 1: Microbial load of buffalo dung by serial dilution.

Similarly in previous studies of buffalo dung showed microbial population and mixture of strains growth (9.4×10^{-8} cfu/ml) for 120 DAI followed by molasses (9.1×10^{-8} cfu/ml) and rice gruel (7.9×10^{-8} cfu/ml). These useful strains were further applied for crop productivity and slurry-based formulation with mixture of strains exhibited incredible plant growth. This research disseminates a successful technology to develop an eco-friendly bioformulation of buffalo dung slurry augmenting the crop growth in an eco-friendly manner leading to sustainable agriculture (Dhiman et al., 2022).

Streak plate method

The sample was taken with sterilized inoculating loop and streaked on blood agar medium plates. Then the plates were incubated at 37° C for 24 hours. Haemolytic activity of buffalo dung bacteria was observed on blood agar plates. Mostly bacteria showed Haemolysis and similarly in previous studies (Russell et al., 2006; Dhiman et al., 2020).

The selected bacterial colonies were streaked from Nutrient agar plates to blood agar plates by using a sterile inoculating loop. Haemolysis was observed by the development of clear halo around the colonies after 24 h of incubation at 30°C.

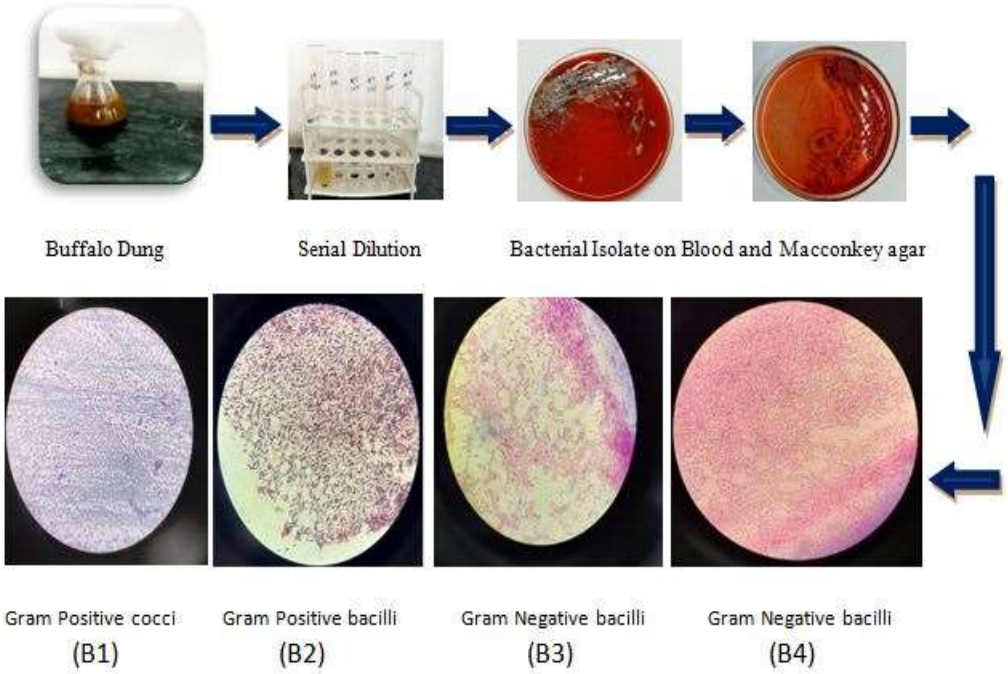

Figure 2: Isolation and identification of bacterial isolates from Buffalo Dung.

Table 2: Bacterial isolated from buffalo dung

Isolates	**Bacteria**
B1	*Micrococcus* sp.
B2	*Bacillus* sp.
B3	*Escherichia coli*
B4	*Escherichia coli*

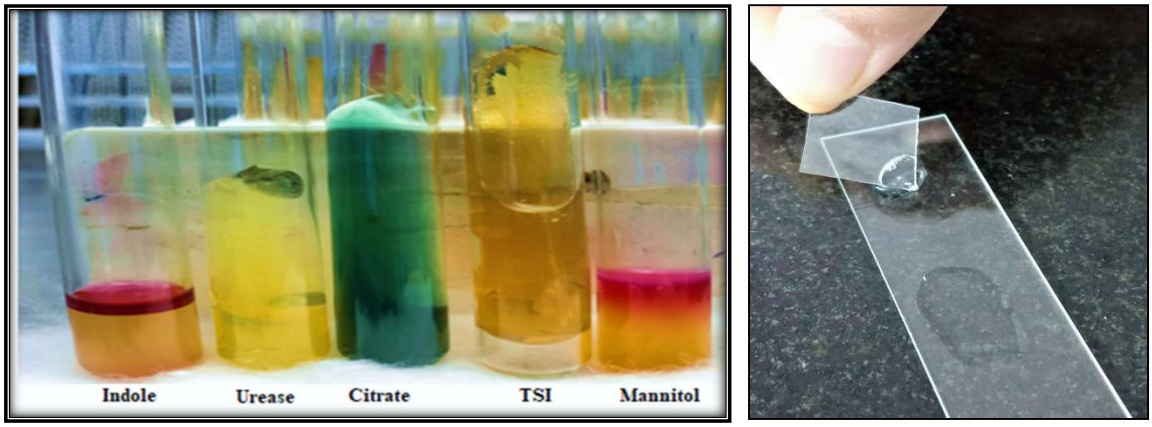

Figure 3: Biochemical tests for *Escherichia coli* and Catalase test for *Micrococcus* sp.

Morphological and Microscopically Characterization of bacteria

Microorganisms produce colonies with characteristics which could be seen by naked eyes that are called as cultural characteristics. The cultural characteristics were observed on Nutrient agar, Blood agar and Macconkey agar after incubation.

These morphological characteristics were observed in different forms such as colony form, colony elevation, surface of the colony and colony colour (Table 3). The morphological examinations of the isolates were determined by standard procedure of basic stain, gram stain and endospore stain (Cappuccino and Sherman, 2005).

These isolated bacteria were characterized on the basis of morphological and microscopically as showed in (Figure 2). On the basis of Gram's staining, bacteria were differentiated as Gram positive and Gram-negative bacteria shown in Figure 2. Gram-positive cocci, Gram-positive bacilli B1, B2, *Micrococcus* sp, *Bacillus* sp. were Gram positive bacteria and show purple colour after Gram's staining. Gram-negative bacilli, B3, B4 *Escherichia coli* show pink colour, rod shape after Gram's staining. B4 isolate was showing central endospore forming bacteria. Three beneficial bacteria *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Enterobacter xiangfangensis* were isolated from buffalo dung to evaluate for their effects individually as well as in consortium (Zhang et al., 2017; Dhiman et al., 2020; Tomar et al., 2020).

Table 3: Morphological characteristics of bacteria isolates from buffalo dung

Characteristics	Bacterial Isolates			
	B1	B2	B3	B4
Form of colony	Circular	Circular	Circular	Circular
Translucency and opacity	Opaque	Opaque	Opaque	Opaque

Elevation of colony	Convex	Fuzzy	Flat	Flat
Surface of colony	Smooth	Rough	Moist	Moist
Pigmentation	Creamy white	White or slightly yellow	Pink colour	Pink colour
Margin	Entire	Jagged edges	Entire	Entire

Microbial load and Isolation of fungi by Serial dilution of buffalo dung

The collected samples of buffalo dung were enumerated for their microbial load of total fungi. The serial dilution method was used to make suspension of buffalo dung in distilled water purpose to minimizing the fungi in the dung in each dilution. The dung sample was diluted six times and labelled as 10^{-1} to 10^{-6} dilution. Serially diluted buffalo dung suspension was plated of Sabouraud dextrose agar media (SDA). Microbial load of buffalo dung was calculating cfu/gm of sample. The maximum number (TNTC) of fungal population was exhibited in initial dilution, then 10^{-2} to 10^{-4} showed which ranged from 30×10^{-3} to 3×10^{-5} cfu/ml and minimum concentration (TNFC) was exhibited in dilution 10^{-6}. Table 4 shows the microbial count of buffalo dung in initial dilution fungal colonies were present.

Table 4: Microbial count of the fungal isolates

S.NO	**Dilutions**	**Method Used**	**Total Fungal Count**
			Sample
1.	10^{-1}	Serial dilution method	TNTC
2.	10^{-2}	Serial dilution method	30×10^{-3}
3.	10^{-3}	Serial dilution method	7×10^{-4}
4.	10^{-4}	Serial dilution method	3×10^{-5}
5.	10^{-5}	Serial dilution method	TFFC
6.	10^{-6}	Serial dilution method	TFFC

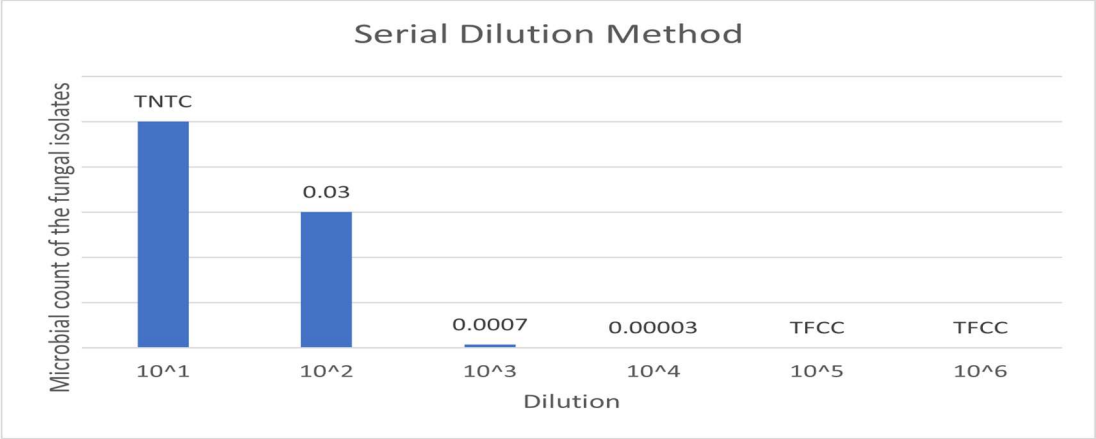

Figure 4: Microbial count of the fungal isolates by serial dilution method.

Streak plate method

The buffalo dung sample was taken with sterilized inoculating needle and transfer on Sabouraud dextrose agar media (SDA) agar medium plates. Then the plates were incubated at 27°C for 72 hours. Figure 5 shows the fungal colony appeared on the plate after incubation was then transfer to new plate. After incubation fungal colonial characteristic such as growth rate, texture, pigmentation on the surface and reverse side and the folds or ridges on the surface. The fungal isolates were identified up to genus level by standard protocol (Cappuccino and Sherman, 2005).

Morphological and Microscopically Characterization of fungi

The isolation of fungi from dung was carried out in order to identify each fungal species. The cultural characteristics of fungal colonies were observed on Sabouraud dextrose agar media (SDA) agar medium plates after incubation. The morphological examinations of the isolates were determined by standard procedure (Cappuccino and Sherman, 2005).

These morphological characteristics were observed in different forms such as colony form, colony elevation, surface of the colony and colony colour. It was observed that isolated different pure colony of same fungus (Figure 5).
This isolated fungus was characterized on the basis of morphological cultural characteristics such as form of colony, colour of colony, texture of colony, aerial hyphae, pigmentation, margin, sporangiospores circular, white cottony colony, with the black dots and covers the entire plate, and microscopically like mycelial non-septate, creamy white, entire and columella is present on the top of sporangiospore, root like rhizoids are present shown in Figure 5; Table 5. Only *Rhizopus* sp. was isolated from buffalo dung and evaluate for their effects for further work.

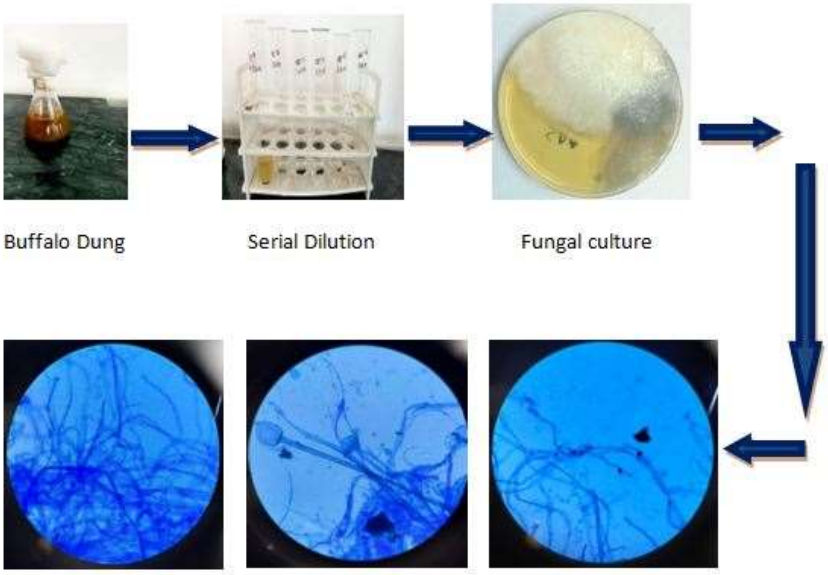

Figure 5: Isolation and identification of fungal isolate (*Rhizopus* sp.) from Buffalo dung.

Table 5: Morphological characteristics of fungal isolate isolated from buffalo dung

Characteristics	**Isolates**
	***Rhizopus* sp.**
Form of colony	Circular
Colour of colony	White colony mycelia, with the black dots and covers the entire plate.
Texture of colony	Cottony
Aerial Hyphae	Non septate
Pigmentation	Creamy white
Margin	Entire
Sporangiospores	Columella is present on the top of sporangiospore, root like rhizoids are present.

The buffaloes varied in terms of their breeds (three main breeds: river buffalo, swamp buffalo, and hybrid buffalo), developmental stages (calf, breeding, and adult), and sexes (oxen and cows). In addition to their roles in food digestion and nutrient absorption, the rumen and gut microbiota have been linked to more pronounced phenotypes, such as the milk production and quality of cattle.

Different processed products obtained from buffalo such as milk, curd, ghee, urine and by-product (dung) are widely used in medicinal formulations. Buffalo dung is an excellent fertilizer. Besides this, it also contains beneficial minerals, such as phosphorus, potassium and nitrogen that support the growth of soil microorganism. Some cultures are using buffalo dung for making paper and insect repellent. Microbial load of buffalo dung includes *Fibrobacter, Ruminococcus, Bacillus, Proteus mirabilus, Pseudomonas aeruginosa, Enterobacter xiangfangensis,* and *Butyrivibrio bacteria;* similarly, *Prevotella,* a group of bacteria capable of degrading noncellulose plant fibres, is abundantly present in the rumen in buffalo (Dhiman et al., 2020).

Conclusion

After searching the lots of literature, only few researches reported on microbial load of buffalo dung. In the present study, we analyzed the microbial load and isolate bacteria and fungi from buffalo dung. Isolated microbes were identified on the basis of their colony characteristics, morphology, Gram's staining, microscopically. The maximum number of bacterial population was exhibited in dilution 10^{-3} which ranged from 155×10^{-4} cfu/ml. Gram Positive cocci, Gram Positive bacilli, Gram Negative bacilli. 20 strains were isolated from buffalo dung. Among them, 4 isolates B1, B2, B3 and B4 were identify as *Micrococcus* sp. *Bacillu*s sp. and *Escherichia coli* respectively on the basis of morphology and microscopically. The maximum number of fungal population was exhibited in dilution 10-2 which ranged 30×10^{-3} cfu/ml different a fungus colony of Rhizopus sp. was observed. It was observed that spore forming bacillus were the predominant type of organism, which possibly present in buffalo digestive tract. These beneficial microbes will be used for further research work.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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