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Aspergillus niger grows faster than *Escherichia coli* in eosin methylene blue media and deter their growth by reducing the pH of the media

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ABSTRACT

Fungi is a kingdom that includes multicellular eukaryotic organisms such as yeast and mold; these organisms are heterotrophs (cannot make their own food) but have significant roles in nutrient cycling. To obtain nutrients from organic material, they use their hyphae, which elongate and branch off swiftly; using the mycelium quickly, they increase their size. Currently, a few media are suitable for fungal growth, such as sabouraud dextrose, malt extract and brain heart infusion medium. Bacterial eosin methylene blue (EMB) media is well-suited to fungi, which acts as selective media to differentiate Gram-negative bacteria. EMB, known as "Levine's formulation", is a selective and differential medium for Gram-negative bacteria. In EMB media, fungi even grow faster than Gram-negative bacteria. In addition to this faster growth of fungi, it deters the growth of Gram-negative bacteria by reducing the pH. The majority of the time, fungi require specific conditions to flourish. In this study, we observed fungal growth, especially mold (*Aspergillus niger*), in EMB media and its retardation activity of Gram-negative bacterial growth. For this new finding assurance, we performed the bacterial and fungal identification test further along with repeating the three times of the whole experiment, and we found the same result. The fungal species was *A. niger*, and the bacterial species was *Escherichia coli*.

INTRODUCTION

Fungi are group members of the eukaryotic family, including yeast mold and mushroom [1]. They obtain their food by taking dissolved molecules, typically by concealing digestive enzymes into their habitat [2, 3]. Fungi have a wide range of distributions and grow in almost all habitats, including extreme areas such as deserts or areas where high salt concentrations remain [4], deep-sea areas [5, 6] and ionizing radiation environments [7]. However, we know that most fungi cannot be adapted to bacterial media [8]. Due to their great plasticity and ability to adopt numerous shapes in response to adverse or unfavorable situations, fungi are extremely effective habitats [9]. Due to its capacity to create a wide range of extracellular enzymes, various organic materials can be breached, and soil components can be decomposed, and carbon and nutrient balance can be regulated [10]. Fungi transform dead organic materials to organic acids, carbon dioxide or biomass. A large number of fungal species can be efficient biosorbents in the fruiting bodies of poisonous metals such as cadmium, copper, mercury and zinc. These factors can impact their development and reproduction [11]. Various biotic (plants and other creatures) and abiotic (soil pH, humidity, salinity, structure and temperature) variables influence the variety and activity of the fungus [12, 13]. The variety and composition of the plant community

greatly influence fungal populations, which in turn impact plant development through mutualism, disease and the availability and cyclical effects of nutrients [14-16]. Fungi also participate in the fixation of nitrogen, hormone synthesis, biological root management and drought protection [17-19]. They serve an essential function in soil organic matter stability and residue breakdown [20].

Eosin methylene blue (EMB) is a differential microbiological medium that allows only Gram-negative bacteria by inhibiting the growth of Gram-positive bacteria and provides a color indicator distinguishing among organisms based on lactose fermentation [21]. EMB contains eosin Y and methylene blue, which are pH indicator dyes that are toxic to other Gram-positive bacteria without Gram-negative coliform bacteria [22]. It is also used to differentiate pathogenic microbes in medical science [23]. Without Gram-negative coliform bacteria, other microbes cannot grow on this medium, as they contain peptone, lactose, dipotassium phosphate, eosin Y (dye), methylene blue (dye), and agar [24]. Colic mastitis accounts for 20–80 percent of acute clinical mastitis and is an ongoing concern for US dairy producers due to the economic ramifications. Coliform mastitis pathogens include *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. and are Gram-negative, typically lactose-fermenting bacilli [25]. *Serratia, Pasteurella, Proteus* and *Pseudomonas* are other Gram-negative microbes that may be isolated from the mammalian gland. A quick and precautionary treatment strategy must be carried out if the causal organism is identified quickly [26]. Coliform bacteria usually grow rapidly when plated on 5% sheep agar and generally generate adequate bacterial growth for follow-up work after overnight incubation [27-29]. It is possible to identify *E. coli* with EMB agar based on the incidence on the surface of the bacterial colonies of a green-metallic sheen [30]. The dyes in EMB agar, eosin Y and blue methylene are pH indicators and Gram-positive inhibitors, combining green metallic precipitates with the formation of an acidic pH [31, 32].

Most fungi need special media for their growth. We usually believe that fungi cannot be well-adapted to bacterial media for their growth. However, in our findings, we found that the mold strain *Aspergillus niger* usually takes place in a small amount on the human mouth, belly and surface skin without causing any problems and can grow well in bacterial EMB media. In addition to their growth in EMB media, they also deterred bacterial growth. We examined the pH value before and after growing fungi in EMB medium to determine the cause of bacterial growth inhibition and discovered that the pH value decreased after mold development.

MATERIALS AND METHODS

We collected three samples from soil, washroom and canal water from the Bangabandhu Sheikh Mujibur Rahman Science and Technology University (BSMRSTU), Bangladesh.

For soil sample collection, we used a sterile spoon and Eppendorf tube. First, For the canal water sample, we used 400 µl canal water in a sterile Eppendorf tube. The samples were then vortexed for 1 min by a vortex mixer (Labnet, USA) in the lab. It employs a very simple mechanism to precisely agitate samples and stimulate reactions or homogenization. After settling down the soil sample debris into an Eppendorf tube, we took 50 µl supernatant using a sterile micropipette and poured it into the first EMB media petri plate. Then, using a one-time sterile spreader, we spread evenly over the surface smoothly as if all the surfaces of the petri plates were covered by the sample supernatant.

For washroom samples, we used sterile cotton bars and Eppendorf tubes. Then, we carefully swabbed tab handle with this sterile cotton bar. After the swabbing, the cotton bar was dipped into 400 µl sterile distilled water into Eppendorf tube and then cut the swab site using a scissor to drop off the collected sample in the Eppendorf tube. Then, we vortexed the sample containing an Eppendorf tube for 1 min by a vortex mixer (Labnet, USA) in the lab. Then, using a micropipette, a 50 µl sample was poured into a second EMB media petri plate, and using a readymade one-time sterile spreader, we spread evenly over the surface smoothly.

For the canal water sample, we used 400 µl of canal water in a sterile Eppendorf tube. After carrying the sample in the lab, we vortexed the sample containing an Eppendorf tube for 1 min. Then, 50 µl of micropipette sample was poured into third EMB media, and using a one-time sterile spreader, we spread evenly over the surface.

Before autoclaving the media, we measured the pH value to ensure the accuracy of our media preparation. After spreading out the samples on media plates, we kept all media containing the sample in an incubator at 25 °C. After 24 h, we did not find any colonies in the media, but after 48 h, we found fungal colonies in the plate. We again measured the pH of the media after 24 h and 48 h for bacterial growth retardation reasons.

Then, to confirm the growth time between Gram-negative coliform bacteria and fungi on EMB media, we individually cultured bacteria and fungi in two different EMB media. Additionally, we measured the pH value after 24 h and 48 h of incubation at 25 °C. Finally, we identified the fungal strain and bacterial strain by morphological and biochemical tests.

Confirmation of fungus

For fungal confirmation from the sample media that had grown in EMB media by inhibiting the bacteria, we separately collected the fungal colonies from EMB media and transferred them to separate fungal media petri plates for culture.

After separately growing fungi, we separately identified them through morphological and biochemical tests.

For each fungal colony identification, first, morphological identification of the specimen was performed using clean sterile glass slides, cotton blue lactophenol, wire loop, sterile coverslips and fluorescence microscopy. A sterile wire loop was used for gathering and placing a loop full of lactophenol cotton blue. The wire loop was passed over a flame and was used to collect colonies from microbial growth. The coverslip was then placed over the blade and afterward inspected for imagery under X40 amplification [33, 34].

The approach for representative colonies was implemented according to their physical characteristics (shape, size, and color of the colony; the shape of cells). Again, Penicillium insulate was cultivated with a liter of KH_2PO_{41} g, ammonium tartrate 0.5 g, $MgSO_{47}H_2O$ 0.01 g, $CaC_{122}H_2O$ 0.01 g, and yeast extract 0.001 g, $CuSO_{45}H_2O$ 0.001 g, $Fe_2(SO_4)_3$ 0.001 g, and $MnSO_4$ 0.001 g, and was incubated in the dark at 25 °C for 5 days to carry out the testing for biochemical activities. After that time, the agar disks with active fungus (6 mm indicator) were placed in solid media, which contains several substrates to detect beta-glucosidase, cellulase, laccases and tyrosinase [35-38].

Confirmation of bacteria

Inferential test

Differential media for the isolation of coliforms was MacConkey broth purple. Three broth tube series – the first series having 3 double strength broth tubes and the following two series comprising 6 single strength broth tubes – were infected with 10.0 ml, 1.0 ml and 0.1 ml of water (ratio 3:3:3).

Verification test

Eosin methylene blue (EMB) agar plates were infected with a sputhe in each positive presumptive broth tube throughout the agar surface to remove falsifying substances from non-coliform organisms. At 37 °C for 24 h, plates were incubated.

Accomplished test

Finally, MacConkey broth slants and nutrient agar tubes were inoculated with separate colonies selected from plates of EMB from cultivated isolates. Following 24 h of incubation at 37 °C, the cultivation of acid and gas was detected, and isolates were cultivated on agar slants with gram staining dye [39-41]. The multiple tube fermentation (MTF) method for coliform detection is illustrated in this simple diagram Figure 1.

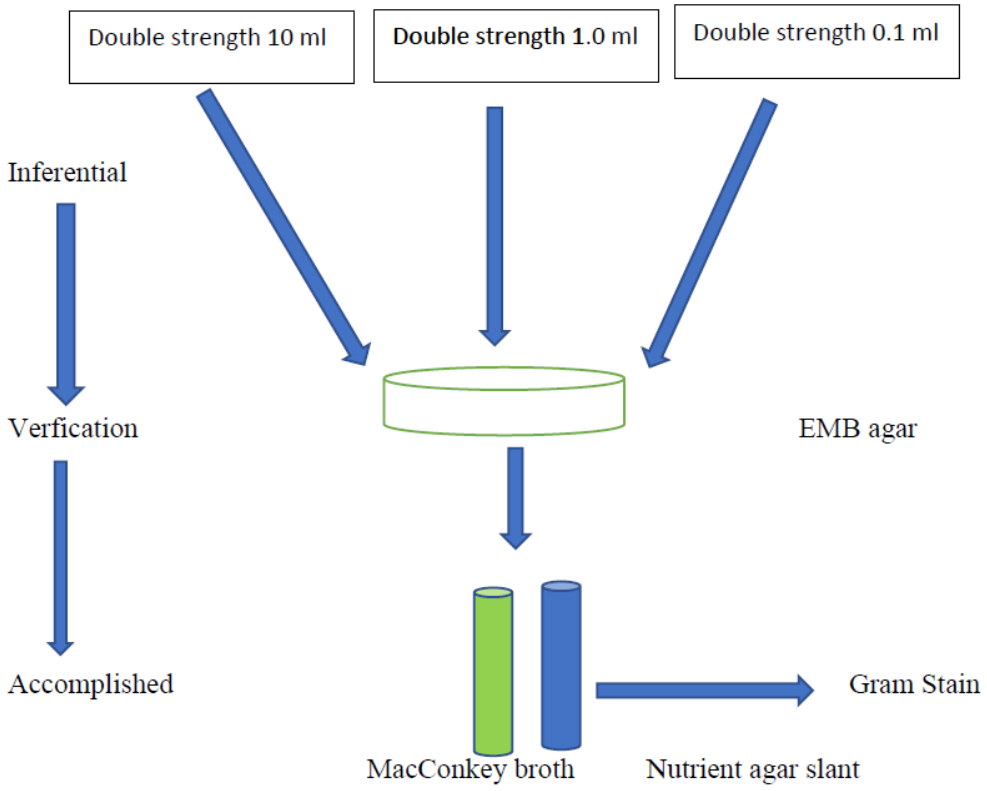

Figure 1. A simple illustration of the MTF technique for coliform identification.

Biochemical test

We used twelve common biochemical tests, including indole, methyl red, voges-proskauer, citrate, catalase, starch hydrolysis, gelatin liquefaction, mannitol, glucose, sucrose, lactose and inositol for bacteria identification.

Statistical analysis

There were three replicates in all experiments, and they were each conducted three times. The data are presented as the mean ± standard deviation of the three replicates. Differences among groups were evaluated by ANOVA using the Statistical Analysis Software (SAS) version 9.2 (SAS Inc., Cary, USA).

RESULTS

Combine observation of pH and colony

Before autoclaving the EMB media, we found a neutral pH value of this media of approximately 7.1. There were no bacterial or fungal colonies in the A, B and C petri plates.

After 24 h, we did not find any bacterial or fungal colonies in each petri plate. At that time, we measured the pH of each medium and showed that the pH value decreased compared with that before transferring the sample time. The pH values were 6.87, 7.01 and 6.61 for soil sample-A, washroom sample-B and canal sample-C, respectively.

However, after 48 h, we found fungal colonies on every medium plate at approximately 20+, 10, and 20+ from soil sample-A, washroom sample-B and canal sample-C (Figure 2). The colonies produced like dark spot into the agar. Then, again, we measured the pH value of these media containing fungal strains and found values of approximately 6.63, 6.52, and 6.33. We noticed that these pH values gradually decreased over time (Table 1).

Table 1. pH value and colony number of sample A, B, and C.

Sample title	pH			Number of colonies			Comment
	Before autoclave	After 24 h	After 48 h	Before autoclave	After 24 h	After 48 h	
Soil sample (A)	7.1	6.87	6.63	0	0	20+	pH value reducing from time to time and Fungal colony increasing
Washroom sample (B)	7.1	6.97	6.52	0	0	10	pH value reducing from time to time and Fungal colony increasing
Canal sample (C)	7.1	6.61	6.33	0	0	20+	pH value reducing from time to time and Fungal colony increasing

Individual observation of pH and colony

After culturing the Gram-negative *E. coli* bacteria and fungi separately to confirm growth on that medium, we noticed that fungi took less time than bacteria (Figure 2).

Before transferring the bacteria and fungi to the media, the pH value was 7.1, and there were no colonies on either medium.

After 24 h of incubation, the pH value was 7.1 in bacterial EMB media and 6.81 in fungal EMB media. Bacterial or fungal colonies were missing on both petri plates. After 48 h of incubation, the pH value was 7.0 in bacterial EMB media and 6.42 in fungal EMB media. Bacterial colonies were missing on bacterial EMB petri plates. However, there was a fungal colony on the fungal EMB petri plate at that time. Then, after 72 h of incubation, the pH value in bacterial EMB media was the same, and bacterial colonies were found on the media. The fungal colonies on the fungal EMB petri plate increased and differentiated, and the pH value was 6.40 (Table 2). pH value along with colony numbers of soil sample, washroom sample and canal sample and pH value along with colony numbers of *E. coli*, *A. niger* are depicted as column chart in Figure 3 (A-D).

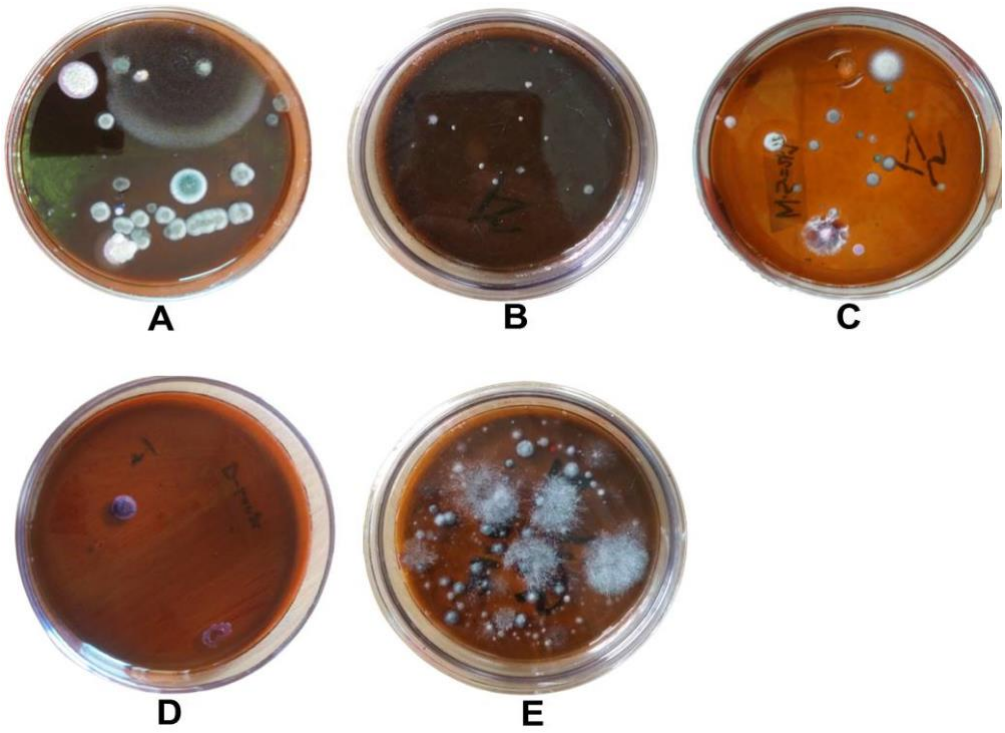

Figure 2. Sample growth after 48 h of incubation soil sample-A (A); Washroom sample-B (B); canal water sample-C (C); *E. coli* (non-spore forming, Gram-negative and rod shaped) bacteria after 72 h (D); *A. niger* (an infectious agent commonly found on mucosal surface, gastrointestinal tract and human skin) fungi after 72 h (E).

Table 2. pH and colony number of pure bacterial and fungal samples D and E.

Sample Title	pH				Number of colonies				Comment
	Before autoclave	After 24 h	After 48 h	After 72 h	Before autoclave	After 24 h	After 48 h	After 72 h	
Gram negative bacteria (D)	7.1	7.1	7.0	7.0	0	0	0	02	pH value remains same, and colony number increasing from time to time.
Fungal sample (F)	7.1	6.81	6.42	6.40	0	0	12+	15+	pH value reducing from time to time and fungal colony increasing

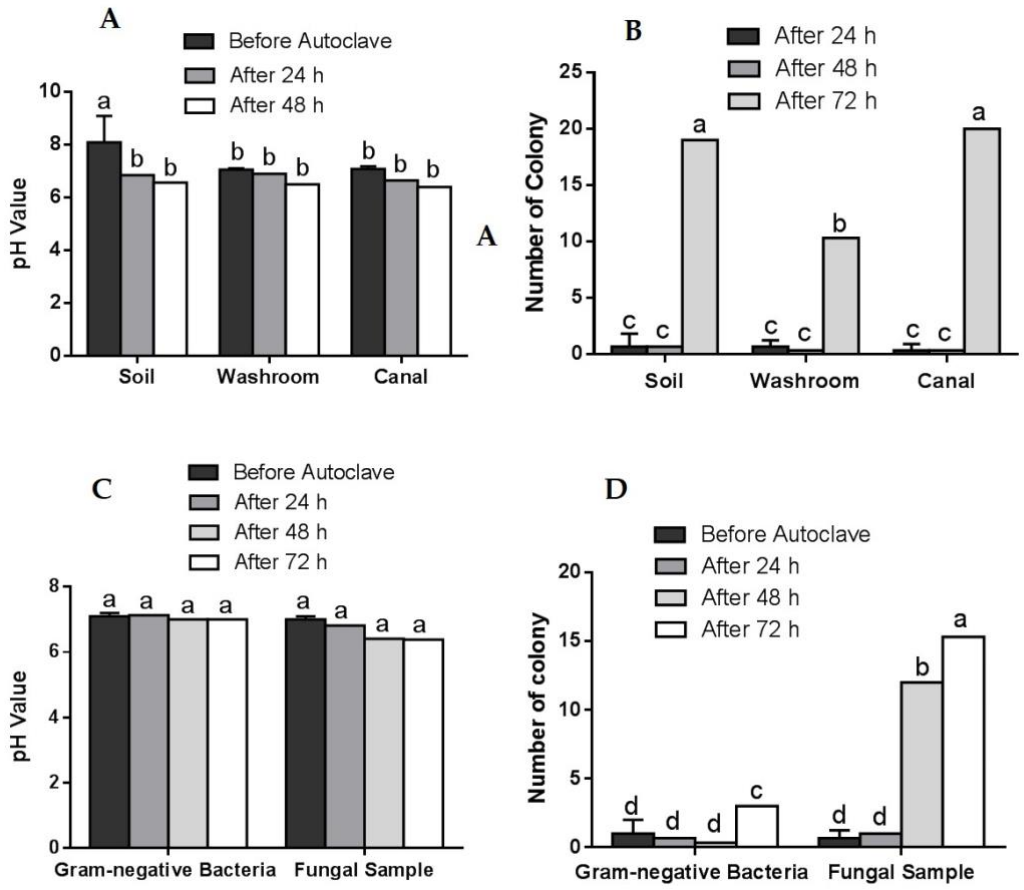

Figure 3. A) pH value of soil, washroom and canal samples. B) Colony numbers of soil, washroom and canal samples. C) pH value of Gram-negative bacteria and fungal samples. D) Colony numbers of Gram-negative bacteria and fungal samples. Different letters on bars indicate significant differences at $p < 0.05$.

Identification and confirmation of fungal isolates

Morphological and biochemical tests were used to describe the discovered fungal strain that thrived on EMB medium as a native, even suppressing the original population. A wet mount is a microbiological method that enables varied forms and cell and spore development to be identified. This is most like initially white to yellow and then turning black and morphological features for each strain are shown in Tables 3 and 4. The identified biochemical enzymatic analysis of the fungal isolates was performed for more authentic identification of fungal isolates (Table 4).

Table 3. Table of macroscopic characterization of identified fungi.

Macroscopic characterization	Identified isolates
Conidia seemed to be lighter, scarce green to parrot green, fluffy mycelium creamy white to dull white and exudates, to reverse uncolored to yellowish and wrinkled mycelial development. There were lacking soluble pigments.	*A. niger*
Growth in the substrates of white wool colonies felt like hyphae, becoming black, conidia formation brown. A yellow river pattern was the reverse of growth.	*A. niger*
The surface is thick cotton-like in texture; white on the surface becomes gray brown, frequently light white. Very fast growth.	*A. niger*

Table 4. Table of macroscopic characterization of identified fungi.

Morphological characterization	Identified isolates
Hyaline, grossly rugged and generally hefty walls are conidiophores apices become sub-phéric to spherical when young. One or two conidial-bearing series may be available.	*A. niger*
Sweet, hyaline, or tenderly brownish, conidiosphores are close to the apex. Sphere-like apples, although frequently very tiny. There are two series of conidium-carrying cells (cells and phialides that sustain them). Grows from white to blackish brown, typically colorless.	*A. niger*
Stolons are hyaline to nodes that can exist near a spectrum. Short, brown and occasionally missing are rhizoids. Sporangiosphores come from nodes on stolons alone or in small groups. They are dark, smooth, non-septate. Initially, white, but subsequently black, sporangia are sparkling. When dehisceous, the columellae are light-brown in shape of an umbrella. Sporangio-sphores are longitudinally striped, yellow to pale brown, round or oval.	*A. niger*

Table 5. Enzymatic activities of the identified molds isolates.

Item	Beta-glucosidase	Lypolytic activity	Laccase	Tyrosinase	Lignin Modifying enzymes	Pectolytic activity
A. niger	Positive	Positive	Negative	Negative	Negative	Negative

Table 6. Biochemical characteristics of *E. coli* isolates.

Item	Test	Result
E. coli	Indole	+
	Methyl red	+
	Voges-Proskauer	-
	Citrate	-
	Catalase	+
	Starch hydrolysis	-
	Gelatin liquefaction	+
	Mannitol	+
	Glucose	-
	Sucrose	+
	Lactose	+
	Inositol	-

Identification and confirmation of bacterial isolates

After the MTF technique, we found isolated bacteria were rod-shaped and Gram-negative. These strains are not motile, and we inferentially confirmed that is *E. coli* strain.

Here, we discuss the importance and consequences of observed biochemical differences about *E. coli* strain. Biotype I based on IMViC reaction patterns can be recognized as isolated *E. coli* [42-44]. Furthermore, considering that the vast majority of wild-type strains of *E. coli* cannot generate D-amylase for starch hydrolysis and that inositol may only be fermented in < 10 percent of the commensal and pathogenic strains of *E. coli* [45, 46].

DISCUSSION

Fungi have versatile habitats, as they can grow in various extreme places, including deserts, deep seas, buildings, etc. [47-49]. It includes yeasts, mildews, molds, rusts, smuts and mushrooms [50-52]. There are fungi such as slime molds and oomycetes, and some fungi are free-living in soil or water along with others and form parasitic or symbiotic associations with plants or animals [53-55]. Fungi cannot produce their own food; they must acquire nutrients from the animals, plants, or others in which they live [56-58]. However, most of the fungi do not grow in bacterial media except in some molds [59]. Filamentous fungi are sometimes characterized as molds as an artificial collection of a number of microfungal species with shared methods for existence [60]. They develop on the surface of objects, consume nutrients and energy sources that readily absorb substances and generate spores as scatter and survival units [61, 62]. These spores are generated in enormous quantities and are widely distributed in many settings. The spore germinates, and a tiny germ tube develops when suitable circumstances are available; if favored conditions predominate, hyphae are formed [63]. A hypha is a tubular cell structure near the tip [64]. The hyphae create a mycelium by their continual branching during growth. In the end, the hyphae form specialized structures (conidiophores), and they spur and distribute spores. The hyphae are responsible for the action of the fungus [65].

Selective and differential medium eosin methylene blue agar (EMB) is used to isolate fecal coliforms. At low pH, the pH indicator dyes eosin Y and methylene blue combine to create a dark purple precipitate, which inhibits the development of most Gram-positive bacteria [66]. Sucrose and lactose are fermentable carbohydrate sources that promote the development of fecal coliforms while also allowing them to be distinguished [67].

Lactose or sucrose fermenters that are active will generate enough acid to make the dark purple color complex. The growth of these organisms will be dark purple to black in color [68]. A green metallic sheen is frequently produced by *E. coli*, a strong fermenter [69]. Mucoid pink colonies are produced by slow or poor fermenters. Colonies that are normally colored or colorless suggest that the organism does not digest lactose or sucrose and is not a fecal coliform [70].

Although researchers believe that EMB media is only selective and allows the best growth for Gram-negative coliform bacteria, we found that EMB media is more suitable for *A. niger* than Gram-negative *E. coli*. Even *A. niger* takes a shorter time for their growth than *E. coli* and inhibits growth in EMB media.

In our research, we observed that on each EMB plate containing our three samples, *A. niger* grew well in EMB media by their morphological characteristics and took 48 h for their growth. An average of 20+ *A. niger* colonies grew on sample plate, but there were no bacterial colonies on that plate. Although we know that EMB media is a selective medium that allows only Gram-negative bacteria, in this research, we did not find them as they were retarded by fungal growth. To determine the reason for this retardation by faster fungal growth than bacteria, we measured the pH value of media before and after spreading out the sample. Then, we found that faster fungal growth inhibits bacterial growth on these media by reducing the pH value through producing acid. To confirm the growth time between Gram-negative bacteria and fungi on EMB media, we found that fungi took approximately 48 h for their growth, but bacteria took almost 72 h.

To identify all these isolates of culture medium, morphological characteristics were investigated. The morphological features of each isolate are detailed in Table 3 and 4.

The fungal strain identifies the *A. niger*. Organic component concentrations and other properties, such as pH, structure on the surface, etc. differ among materials, and the essential conditions predicted for moisture vary as well. For further confirmation, we performed various types of enzymatic activity tests in our lab. We described in Table 5 the found data and confirmed that this fungal isolate was mold *A. niger*.

Alongside the bacterial strain also characterized for more confirmation through the morphological test and the biochemical test is it *E. coli* strain (Table 6).

CONCLUSION

Fungi especially mold *A. niger* are eukaryotic organisms with versatile characteristics. Normally, researchers believe that fungi do not grow in bacterial media. However, in our study, we found that some molds, especially *A. niger*, grew faster in bacterial EMB media than Gram-negative coliform *E. coli* bacteria. Even after growing the mold *A. niger* on bacterial selective EMB media, it deterred the *E. coli* bacteria on EMB media by reducing the pH of the media.

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AUTHOR CONTRIBUTIONS

MIH and MSA designed and performed the experiments, analyzed and interpreted the data, and prepared the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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