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Research Article

Fungi Grow Faster than Gram-Negative Bacteria in Emb Media and Also Deterring the Growth of These Bacteria by Reducing the pH of that Media

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ABSTRACT

Fungus (singular) are a kingdom that includes multicellular eukaryotic organisms such as yeast, mould etc. These 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 have a few number media these are suitable for fungus growth such as Sabouraud dextrose, malt extract and brain heart infusion medium, etc. Although we decide that fungi cannot suit them to grow in bacterial media but fungi can suit them in bacterial EMB media which act as a selective media to differentiate Gram-negative bacteria. Eosin Methylene Blue (EMB) known as "Levine's formulation" is the selective and differential medium for Gram-negative bacteria. In EMB media fungi even grow faster than gram-negative bacteria. Besides this faster growing of fungi, it deterring the growth of gram-negative bacteria by reducing the pH.

The majority of the time, fungi requires specific conditions to flourish. Even in the scientific sector, we know that fungus does not grow well on bacterial media. But in this research, we observed the fungal growth specially mould (*Aspergillus Niger*) in EMB media along with its retardation activity of gram negative coliform bacterial growth. For this new finding assurance we performed the bacterial and fungal identification test further along with repeating the three times of whole experiment and we found the same result. The fungus was *Aspergillus Niger* and a bacterium was *E. coli*.

Keywords: Fungi; EMB media; Gram-negative bacteria; Retardation; Growth

INTRODUCTION

Fungus is the group member of eukaryotic family includes yeast mould and mushroom etc. [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 distribution and grow in almost all habitats, including extreme area such as deserts or areas where remain high salt concentrations [4], deep sea area [5] as well as ionizing radiation environment [6]. Although we know the most of the fungi can't suit them in bacterial media [7]. Due to its great plasticity and their ability to adopt numerous shapes in response to adverse or unfavorable situations, fungi are extremely effective habitants [8]. Due to its capacity to create a wide range of extracellular enzymes, all sorts of organic materials can be breached, soil components can be decomposed and carbon and nutrient balance regulated [9]. Fungi transform dead organic materials to organic acids, carbon dioxide or biomass. A large number of fungus species can be an efficient bio-sorbent in their fruiting body of poisonous metals like cadmium, copper, mercury and zinc. While these factors can impact their development and reproduction [10] Various biotic (Plants and other creatures) and abiotic (Soil pH, humidity, salinity, structure, temperature) variables influence the variety and activity of the fungus [11,12] 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 [13,14] Fungi also participate in the fixation of nitrogen, hormone synthesis, biologic root management and drought protection [15,16]. They serve an essential function in soil organic matter stability and

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residue breakdown [17,18].

EMB (Eosin Methylene Blue) is a differential microbiological medium which allows only Gram negative bacteria by inhibiting the growth of Gram positive bacteria and provides a colour indicator distinguishing among the organisms based on lactose fermentation [19-21]. EMB contain Eosin Y and methylene blue these are pH indicator dyes is toxic for the other the 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 can't grow on this media as they contains peptone, lactose, di-potassium phosphate, eosin Y (dye), methylene blue (dye), and agar [24]. Colic mastitis accounts for 20%–80% of acute clinical mastitis and, because of its economic repercussions, is a continuing worry of the US dairy farmers. Coliform mastitis pathogens include Escherichia coli, Klepsiella spp. and Enterobacter spp., and are gram negative, typically lactose fermenting bacilli [25]. The species of Serratia, Pasteurella, Proteus and Pseudomonas are other gram negative microbes that may be isolated from mammalian gland [26]. A quick and precautionary treatment strategy must be carried out if the causal organism is to be identified quickly [27]. Bacteria of coliform usually grow rapidly when they have been plated on 5 percent of sheep agar, and generally generate adequate bacterial growth for follow up work after overnight incubation [28]. It is possible to identify Escherichia coli with Eosin Methylene Blue (EMB) agar based on the incidence on the surface of the bacterial colonies of green metallic sheen [29]. 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 acid pH [30].

The most of the time fungus need to special media for their growth. Even in research field usually we believe that fungus can't suit in bacterial media for their growth. But in our findings, we found mould strain *Aspergillus Niger* usually take place in a small amount on human mouth, belly and surface skin without causing any problems, which can grow well in bacterial EMB media. Besides their growth in EMB media, they also deterring bacterial growth. We examined the pH value before and after growing fungus in EMB medium to determine the cause of bacterial growth inhibition and discovered that the PH value decreased after mold development [31-33].

MATERIALS AND METHODS

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

For soil sample collection, we used a sterile spoon and eppendorf tube. First of all we took 1 gm soil into 1000 µl size sterile eppendorf tube containing 400 µl distilled water. The samples were then vortexed for 1 min by vortex machine in the lab. After settle down of soil sample debris into the eppendorf tube, we took 50 µl supernatant using a sterile micropipette and poured into first EMB media petri plate. Then using readymade one time sterile spreader we spread evenly over the surface smoothly as if all the surface of petri plate cover by sample supernatant [34-36].

For washroom sample, we used sterile cotton bar and eppendorf tube. Then we carefully swab tab handle by this sterile cotton bar. After swab 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 sample containing eppendorf tube for 1 min by vortex machine in the lab [37,38]. Then using micropipette 50 µl sample poured into second EMB media petri plate and using readymade one time sterile spreader we spread evenly over the surface smoothly.

For canal water sample we used sterile 1000 µl size eppendorf tube and took 400 µl canal water. After carrying the sample in the lab we vortex sample containing eppendorf tube for 1 min. Then using micropipette 50 µl sample poured into third EMB media and using readymade one time sterile spreader we spread evenly over the surface.

Before autoclaving the media we measured the pH value for ensuring the accuracy of our media preparation. After spreading out the samples on media plate we kept all media containing the sample in an incubator at 25°C temperatures. After 24 hours we didn't find any colony in media but after 48 hours we found fungal colonies in the plate [39,40]. We again measured the pH of the media after 24 hours and 48 hours for findings of bacterial growth retardation reason.

Then for the confirmation of the growth time between Gram negative coliform bacteria and fungi on EMB media, we cultured individually bacteria and fungi in two different EMB media. And also we measured the PH value after 24 hours and 48 hours of incubation at 25°C temperature.

Finally we identified the fungus 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 colony from EMB media and transfer them to separate fungal media petri plate for the culture [41,42].

After separately growing fungus we separately identified them through morphological and biochemical test. For each fungal colony identification, first the morphological identification of specimen was done using clean sterile glass slides, cotton blue lactophenol, wire loop, sterile cover slips and fluorescence microscope [43]. 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 colony from a microbial growth. The cover slip was then put over the blade and

afterwards inspected for imagery under the ×40 amplification [44].

The approach for representative colonies was implemented according to their physical characteristics (shape, size, and color of the colony; shape of cells) again, Pencillium insulate was cultivated with a liter of KH2PO41 g, ammonium tartrate 0.5 g, MgSO47H2O 0.01 g, caCl22H2O 0.01 g, and yeast extract 0.001 g, CuSO45H2O 0.001 g, fe2(SO4)3 0.001 g, and MnSO4 0.000 g, and was incubated in the dark at 25°C during 5 days to carry out the testing for biochemical activities [45]. After that time, the agar disks with active fungus (6 mm indicameter) were placed in solid media, which contains several substrates to detect beta glucosidase, lypolitical activity, cellulase, laccases and tyrosinase.

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 ml, 1 ml and 0.1 ml of water (Ratio 3:3:3) respectively.

Verification test: Eosin methylene blue (EMB) agar plates have been infected with a spluthe in each positive presumptive broth

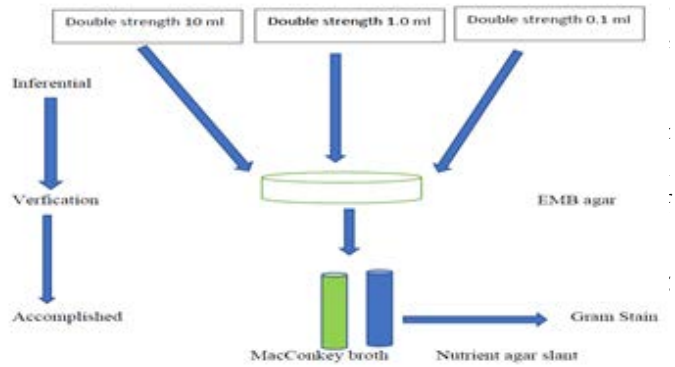

Figure 1: A simple illustration of MTF technique for coliform identification.

Biochemical test: The technique of Kirby-Bauer disk diffusion was utilized in Nigeria to determine the sensitivity of isolate to 13 popular antibiotics. Mueller-Hinton (Oxoid Code=CM0337) and two antibiotic multidisc type test types were used (Gram positive; MICRORING/DT-NEG and Gram negativ; MICRORING/DT-POS).

RESULTS

Before autoclave the EMB media we found a neutral pH value of this media around 7.1 and at that time there has no bacterial or fungal colony in the A, B, and C Petri plate.

After 24 hours we didn't find any bacterial or fungal colony in each petri plate. At that time we measured the pH of each media and have shown that the pH value decreased than before transferring the sample time [47]. The pH value was 6.87, 7.01 and 6.61 on the soil sample-A, washroom sample-B and canal sample C.

But after 48 hours we found the fungal colony on every media plate around 20+, 10, 20+ from the soil sample-A (Figure 1), washroom sample-B (Figure 2) and canal sample C (Figure 3) [48,49]. The colonies that produce a dark centre plunge into the agar. Then again we measured the pH value of these media containing fungi strain and found about 6.63, 6.52, and 6.33. We have noticed that these pH values were gradually decreasing from time to time (Table 1) [50].

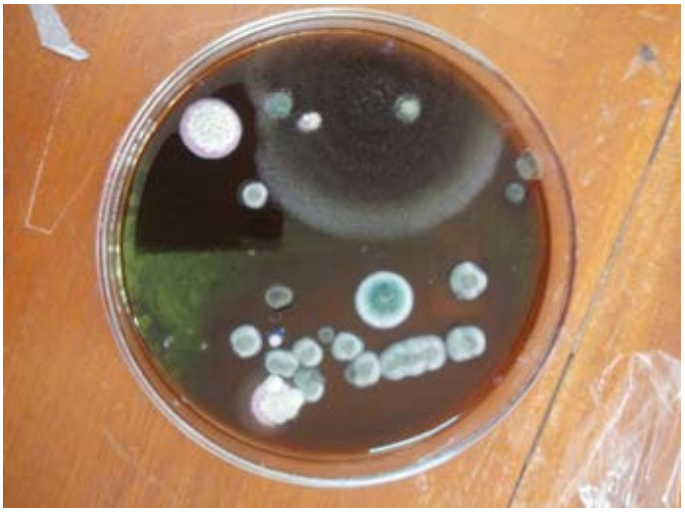

Figure 2: Sample growth after 48 hours of incubation soil sample-A.

Figure 3: Sample growth after 48 hours of incubation Washroom sample-B.

Table 1: PH value and Colony number of Sample A, B, and C.

Sample Title	pH			Number of colony			Comment
	Before autoclave	After 24 hours	After 48 hours	Before autoclave	After 24 hours	After 48 hours	
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

After culturing the gram negative *E. coli* bacteria and fungi separately for the confirmation of growth on that media, we noticed that fungi took less time than bacteria (Figures 4 and 5) [51,52]. Before transferring the bacteria and fungi on the media, the pH value was 7.1 and there's had no colony both of media.

Figure 4: Sample growth after 48 hours of incubation canal water sample-C.

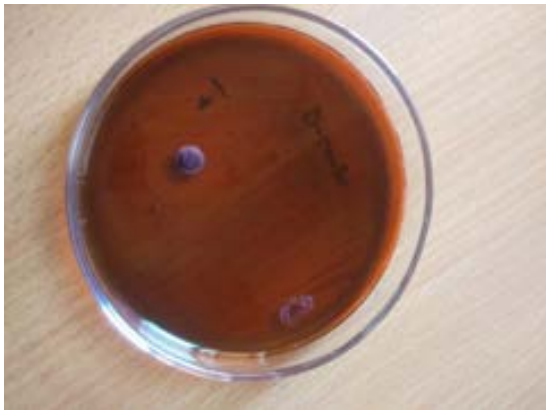

Figure 5: Coliform (non-spore forming, Gram-negative and rod shaped) bacteria after 72 hours.

After 24 hours of incubation, the pH value was in bacterial EMB media was 7.1 and in fungal EMB media was 6.81. The bacterial or fungal colonies were missing on both petri plates.

After 48 hours of incubation, the pH value was in bacterial EMB media was 7.0 and in fungal EMB media was 6.42. The bacterial colony was missing on the bacterial EMB petri plate. But there was a fungal colony on the fungal EMB petri plate at that time [53].

Then after 72 hours of incubation, the pH value was in bacterial EMB media was the same and found the bacterial colony on the media. The fungal colonies on the fungal EMB petri plate were increasing and differentiating and the pH value was 6.40 (Figure 6) (Table 2) [54,55].

Figure 6: *Aspergillus Niger* (an infectious agent commonly found on mucosal surface, gastrointestinal tract and human skin) fungi after 72 hours.

Table 2: PH and Colony number of Sample pure bacterial and fungal sample D and E.

Sample Title	PH				Number of colony				Comment
	Before autoclave	After 24 hours	After 48 hours	After 72 hours	Before autoclave	After 24 hours	After 48 hours	After 72 hours	
Gram Negative Bacteria (D)	7.1	7.1	7.0	7.0	0	0	0	02	PH value remain same and colony 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

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 form, cell and spore development to be identified. The most likely isolates and morphological features for each strain were shown in Tables 3 and 4 [56].

Table 3: Table of macroscopic characterization of identified fungus.

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

Table 4: Table of macroscopic characterization of identified fungus.

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. Philiades are present. Philiades are present.	*Aspergillus 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 colourless.	*Aspergillus 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 or smooth, non-septate. Initially white, but subsequently black, sporangia are speaking. When dehisceous, the columellae are light-brown in shape of an umbrella. Sporangio-sphores are longitudinally striped, yellow to pale brown, round or oval.	Aspergillus Niger

The identified biochemical enzymatic analysis of the fungal isolates was done for more authentic identification of fungus isolates (Table 5) [57].

Table 5: Enzymatic activities of the identified molds isolated.

Item	Beta glucosidase	Lypolytic activity	Laccase	Tyrosinas	Lignin	Pectolytic activity
Aspergillus Niger	Positive	Positive	Negative	Negative	Modifying enzymes	Negative

Identification and confirmation of bacterial isolates

After the MTF technique we found that isolated bacteria is rod shaped bacterium with gram negative (-ve). The volume is 1 × 0.40 µm and the volume is 0.6 µm-0.7 µm. It is individually or pairly placed [58]. The peritric flagella causes it to be motive. This strains which are not motile and we inferertially confirmed that is that Escherichia Coli.

We are discussing here the importance and consequences of observed biochemical differences of *E. coli* and *E. coli* ATCO reference. Biotype I based on IMViC reaction patterns can be recognized as the isolated *E. coli* [59]. Furthermore, considering that the vast majority of wild types of strains of *E. coli* cannot generate d-amylase for starch hydrolysis and that inositol [60] may only be fermented in <10% of the comensal and pathogensic strains of *E. coli* (Table 6) [61].

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

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

DISCUSSION

Fungus have versatile habitat as they can grow in various extreme place even desert, deep sea, buildings etc. It includes the yeasts, mildews, moulds, rusts, smuts and mushrooms. There have fungi like organism also such as slime moulds and oomycetes [62] and also have some are free living in soil or water along with others form parasitic or symbiotic associations with plants or animals [63]. Fungi cannot produce their own food; they must have to acquire nutrients from the animals, plants, or others in which they live [64]. But most of the fungi do not grow in bacterial media except some moulds. Filamentous fungi are sometimes characterized as molds as an artificial collection of a number of microfungi species with shared methods for existence [65]. They develop on the surface of objects, consume nutrients and energy sources readily absorbed substances and generate spores as scatter and survival units [66]. 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, a hypha is formed [61]. A hypha is a tubular cell structure near the tip. 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 [62].

The 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 [63]. Sucrose and lactose are fer-mentable carbohydrate sources that promote the development of fecal coliforms while also allowing them to be distinguished [64].

Lactose or sucrose fermenters that are active will generate enough acid to make the dark purple color complex. These organisms' growth will be dark purple to black in color [65]. A green metallic sheen is frequently produced by Escherichia coli, a strong fermenter. 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 [66].

Although researchers believe that the EMB media is only selective and allow the best growth growth for gram negative coliform bacteria, but we found that this EMB media is more suitable for *Aspergillus Niger* than gram negative *E. coli*. Even *Aspergillus Niger* takes shorter time for their growth than *E. coli* along with inhibiting the growth in EMB media.

In our research, we observed that on each EMB plate containing our three samples, *Aspergillus Niger* grew well in EMB media by their morphological characteristics and took 48 hours for their growing. Average 20+ *Aspergillus Niger* colonies grew on our sample plate but there have no any bacterial colony on that plate [67]. Although we know that EMB media is a selective media which allow only Gram negative bacteria in this research, we didn't find them as they were retarded by fungal growth. For seeking the

reason for this retardation by faster fungal growth than bacteria we measured pH value of media before and after spreading out the sample. Then we found that faster fungal growth inhibits the bacterial growth on these media by reducing the pH value through producing acid. For confirmation of the growth time between Gram negative bacteria and fungi on EMB media, we cultured individually and found that fungi took about 48 hours for their growth but bacteria took almost 72 hours [68-70].

In order to identify all these isolates of cultural medium, morphological characteristics were investigated. The morphological features of each isolate were detailed in Tables 3 and 4 [71]. The fungal strain identifies the Apsergillus Niger. Since organic component concentrations and other properties such as pH, structure on the surface, etc. differ among materials, the essential conditions predicted for moisture vary as well. For further confirmation we performed various types of enzymatic activity test in our lab. We described in Table 5 the founded data and confirmed that this fungal isolate was mould Apsergillus Niger [72-85].

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

CONCLUSION

Fungi specially mould Apsergillus Niger is eukaryotic organisms with their versatile characteristics. Normally researchers believe that fungus do not grow in bacterial media. But in our research, we found that some moulds specially Apsergillus Niger grow faster in bacterial EMB media than Gram negative coliform *E. coli* bacteria. Even after growing the mould Apsergillus Niger on bacterial selective EMB media, it deterring the *E. coli* bacteria on EMB media by reducing the PH of the media.

AUTHOR CONTRIBUTIONS

MIH and MSA performed the experiment, data collection, data analysis, data interpretation, and prepared the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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