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Aspergillus niger Culture Filtrate (ACF) Mediated Biocontrol of Enteric Pathogens in Wastewater

Suja Subhash †, Pradeesh Babu †, Amrutha Vijayakumar, Reshma Alookaran Suresh, Ajith Madhavan, Bipin Gopalakrishnan Nair and Sanjay Pal *

School of Biotechnology, Amrita Vishwa Vidyapeetham, Amritapuri, Kollam 690525, India; u2suja@gmail.com (S.S.); babupradeesh@gmail.com (P.B.); amruthavijayakumar123@gmail.com (A.V.); ammasdurga0124@gmail.com (R.A.S.); ajithmadhavan2003@gmail.com (A.M.); bipinnair03@gmail.com (B.G.N.)
* Correspondence: spal05@gmail.com; Tel.: +91-0476-2805316
† These authors contributed equally to this work.

Abstract: Robust control of pathogens in sewage facilitates safe reuse of wastewater rich in valuable nutrients for potential valorization through biological means. *Aspergillus niger* is widely reported in bioremediation of wastewater but studies on control of enteric pathogens in sewage are very sparse. So, this study aimed at exploring the antibacterial and nematicidal activity of *A. niger* culture filtrate (ACF). Antibacterial activity of ACF on enteric pathogens (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Salmonella enterica*, *Shigella dysenteriae*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella variicola*) was determined by spectrophotometric growth analysis, resazurin based viability assay and biofilm formation assay. ACF showed inhibition against all enteric pathogens except *Pseudomonas aeruginosa*. Nematicidal studies on *Caenorhabditis elegans* showed 85% egg hatch inhibition and 52% mortality of L1 larvae. Sewage treatment with ACF at 1:1 (*v*/*v*) showed 2–3 log reduction in coliforms, *Klebsiella*, *Shigella*, *Salmonella*, *S. aureus* and *Vibrio* except *Pseudomonas*, indicating significant alteration of complex microbial dynamics in wastewater. Application of ACF can potentially be used as a robust biocontrol strategy against infectious microbes in wastewater and subsequent valorization by cultivating beneficial *Pseudomonas*.

Keywords: wastewater; *Aspergillus niger*; enteric pathogens; biocontrol; biofilms

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1. Introduction

Poor sanitation worldwide is associated with severe enteric infections and malnutrition, resulting in a heavy burden of disease globally. The largest source of this infection is the untreated wastewater released into water bodies [1,2]. The most common pathogens usually present in wastewater include *Salmonella*, *Shigella*, *Escherichia coli*, *Streptococcus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium*, *Trichuris trichiura*, *Ascaris lumbricoides* and *Giardia lamblia* [3,4]. The increase in the discharge of human waste associated with the population growth resulted in the abundance of pathogenic bacteria in the environment, especially in water that deluges the natural processes of decomposition.

Traditional methods of wastewater disinfection including chlorination, radiation and ozonisation [5] are either inefficient for the elimination of many target pathogens or create hazardous disinfection by products (DBPs) as well as being operationally expensive.

It is well established that conventional wastewater disinfection strategies like chlorination have difficulty treating particle associated microbes, resulting in a phenomenon known as 'tailing' in which disinfection is not fully effective regardless of the amount of chlorine used [6]. Similarly, one of the limitations with UV radiation is that it consumes high energy and requires frequent maintenance, including UV lamp replacement [7].

Eco-friendly wastewater disinfection techniques could be one of the most exciting advances in this field [8]. Currently available eco-friendly techniques include bioremediation, bioleaching, bio flocculation, biosorption, and bioaugmentation, and commonly assessed

biological control agents for managing the wastewater pathogens include biofilm-forming bacteria in biofiltration units, microalgae, plants used in phytoremediation, phage-based biocontrol of infection and smell, and fungi capable of mycoremediation [9–11].

Fungi are ubiquitous in nature, exist with heterogeneity and biomass comparable to bacteria and in greater commensurate than archaea and viruses. Despite their metabolic and physiological differences, they accompany other groups of microorganisms controlling a wide variety of ecosystem functions in diverse environments. There is an increasing trend for the exploitation of fungi for its biocontrol potential. The attractiveness of fungi as biocontrol agents is due to their high magnitude of host specificity, destruction of the host, persistence, dispersal efficiency, ease of culture and maintenance in the laboratory [12]. *A. niger* is a multifaceted, rapidly growing filamentous fungus ubiquitous in soil, dead and decaying plant material and on many foods and feeds [13]. It is also suitable for the augmentation of compost because of their secretions of hydrolytic enzymes that target the plant materials in the compost [14] *A. niger* is also renowned for producing a huge diversity of secondary metabolites/mycotoxins [15,16] based on which most of the new clinically important antimicrobial agents have been developed [17].

A. niger has been the subject of industrial production of enzymes and acids [18]. One of the first acquired practical importance in the industry was its ability for production of citric acid, gluconic and fumaric acids [19–21] using cheaper carbohydrate substrates. *A. niger* is a safe organism for production and considered to be harmless under industrial conditions [16]. As a result, quite a few *A. niger* fermentations have been awarded the generally regarded as safe (GRAS) status by the US Food and Drug Administration [17] *A. niger* is commonly employed in biotechnological approaches to wastewater treatment as it plays a key role in remediation and bio-recovery of metals [22], biosorption of dyes from textile wastewater [23] and in bioleaching of wastewater sludge [24]. Most studies have focused on the effects of *A. niger* on the bioremediation process but neglected the responses of the pathogens present in the wastewater to the fungi.

In this study, we hypothesize that the culture filtrate obtained from the *A. niger* could be a cost-effective alternative for control of enteric pathogens in wastewater. We focused on exploring the ability of ACF to decrease the growth and viability of eight targeted enteric pathogens namely, *E. coli*, *S. dysenteriae*, *K. variicola*, *V. cholerae*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *S. enterica* and a nematode model worm *C. elegans*. We extended our study to wastewater treatment and our results clearly indicate that most of the pathogens are controlled except *Pseudomonas* demonstrating the biocontrol potential along with microbiome alteration properties of ACF.

2. Materials and Methods

2.1. Fungal Strain

A. niger, used in the study is a soil isolate collected from the vicinity of Amrita School of Biotechnology and deposited in GenBank (Accession No. KU987575.1). The fungal strain was maintained in Potato Dextrose agar (HiMedia, Mumbai, India) plates and stored at 4 °C [25].

2.2. Preparation of A. niger Culture Filtrate (ACF)

A. niger was cultured on potato dextrose agar plate and incubated at 28 °C. After 7 days, 5-mm diameter mycelial plug of the fungi was inoculated into Synthetic Minimal Media (SMM) per liter comprised of 5 g $(NH_4)_2SO_4$, 0.5 g KCl, 0.4 g KH_2PO_4, 0.01 g $FeSO_2.7H_2O$, 0.01 g $MnSO_4$, 0.2 g $MgSO_4.7H_2O$, 10 g Glucose in distilled water and pH 5.5 in a 500 mL Erlenmeyer flask and incubated at 28 °C for 7 days in shaking incubator. The culture filtrate was obtained by filtration using Whatman filter paper (Hyclone Life sciences, Bengaluru, India) followed by centrifugation of the cell free extract at 7000 *g* for 15 min. The ACF were further passed through a 0.22µm filter (Millex -GP, Millipore, Ireland) and stored at 4 °C for further analysis [26].

2.3. Bacterial Strains and Culture Conditions

Bacterial clinical strains *K. pneumoniae*, *P. aeruginosa*, *V. cholerae*, *S. enterica*, *S. dysenteriae*, a multidrug resistant *E. coli* (*E. coli* MDR), *S. aureus* obtained from Dr. Raja Biswas, Microbiology department, AIMS, Kochi, Kerala and *K. variicola* F2R9 a wastewater isolate collected from the vicinity of Amrita School of Biotechnology, Kerala and identified by 16S rDNA sequencing and deposited in Genbank with Accession number NR025635.1 were used in the study. All the strains were maintained in Luria Bertani Agar (HiMedia, Mumbai, India) and stored at 4 °C. All experiments with the bacterial strains were carried out in BSL -2 Safety Cabinet following strict biosafety regulations.

2.4. Antibiotic Sensitivity Profiling of Bacterial Strains

The susceptibility of the selected bacterial strains to antibiotics were determined by the Kirby–Bauer disk diffusion method [27]. 100 µL of 0.1 O.D bacterial culture was spread on Muller Hinton agar plate and the antibiotics discs (Himedia, Mumbai, India) Penicillin, Chloramphenicol, Methicillin, Tetracycline, Ceftazidime, Ampicillin, Ciprofloxacin, Amoxicillin, Cefoxitin were placed on the plate at proper distancePlates were incubated for 16–24 h at 37 °C prior to the determination of results.

2.5. Maintenance of C. elegans

C. elegans WT Bristol N2 strain were maintained on nematode growth medium NGM comprising 2.5 g peptone, 3 g NaCl, 17 g agar, 0.5% cholesterol, 1 mmol $CaCl_2$, 1 mmol $MgSO_4$, 25 mmol KPO_4 buffer in 1 litre of water) plates with *E. coli* strain OP50 as food and incubated at 20 °C. For isolation of eggs, worms from NGM plates were rinsed off with M9 Buffer comprising 3 g KH2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mmol $MgSO_4$ in litre of water] in 15 mL tubes at room temperature and washed twice by centrifugation for 2 min at 1000 *g* and then resuspended in M-9 buffer. After the last wash, only 1 mL of M-9 buffer was added in the tube. Then 5 mL of freshly prepared hypochlorite solution (5 mol NaOH and 1% hypochlorite in 1 litre) was added to the worms and the tube was shaken for 5 min. The lysis reaction was stopped by adding equal volume of M-9 buffer to the reaction mixture followed by centrifugation for 2 min at 1000 *g*. The supernatant was discarded, and the pellets of eggs were resuspended in 1 mL of M-9 buffer. These isolated eggs were counted under the microscope and used for further assays [28].

2.6. Collection of Wastewater Sample

Wastewater samples were collected in a sterile polypropylene falcon tube (Tarsons, Kolkata, India) from the wastewater treatment pilot plant (PP2) located in the University campus at Amritapuri, Kollam, Kerala on a sunny day to avoid dilution of the sample by rain [29]. Samples were mixed well and used for further treatment.

2.7. Determination of ACF Antimicrobial Activity

Routine spectrophotometric assay (OD 600 nm) and resazurin based fluorescence assay were performed to check the antimicrobial activity. All experiments were conducted in triplicate. Briefly, all the bacterial strains were cultured in Luria–Bertani broth (HiMedia, Mumbai, India) grown overnight at 37 °C and the optical density (OD) was adjusted to 0.4 with sterile LB broth in a Bio photometer (Eppendorf, Chennai, India). The suspension was used directly for all assays.

2.7.1. Spectrophotometric Assay

Antimicrobial activity testing by measuring the optical density (OD) was done as described elsewhere [30–32]. Briefly, 100 µL of bacterial suspension was mixed with ACF (100 µL) in a sterile 96-multiwell cell culture plate (Tarsons, Kolkata, India) and incubated at 37 °C. After 2 h, 24 h and 48 h of incubation, the optical density (OD) was measured at 600 nm using (BioTek Synergy™ HT Multi-Mode) Microplate Reader. Bacterial suspension without ACF served as control [33].

2.7.2. The Resazurin Based Fluorometric Assay

The cell viability assay was carried out as described by [34,35] with slight modifications. Briefly, 20 µL of 1 mM Resazurin (Sigma-Aldrich, Bangalore, India) was added to the wells containing the bacterial suspension, incubated with ACF at 37 °C for 2 h, 24 h and 48 h. Colour change (blue to pink) due to reduction of non-fluorescent resazurin to pink resorufin by the bacterial metabolic activity was recorded at 15 m of regular time interval until the colour disappeared [36–38]. The fluorescence was measured using the microplate reader with excitation/emission wavelengths of 530 nm and 590 nm, respectively.

2.8. Effect of ACF on Biofilm Formation

Inhibition of biofilm formation by ACF was accessed using the crystal violet biofilm assay with slight modifications. Briefly, overnight bacterial cultures were adjusted to 0.4 OD and treated with equal volume of ACF in a sterile flat bottomed 96-well polystyrene microtiter plate and incubated at 37 °C for 24 h. After incubation planktonic cells were removed and 0·1% crystal violet (HiMedia, Mumbai, India) was used to stain the remaining biofilms. The dye bound to the biofilm was eluted after two washes with 33% acetic acid (Merck). The eluted crystal violet was quantified by absorption at 600 nm using a plate reader [39]. Bacterial cultures without ACF served as control.

2.9. Nematicidal Activity

To choose the best methodology for detection of nematicidal activity of ACF the following methods were examined:

2.9.1. Egg Hatch Assay

Eggs were obtained as described above and approximately 30 eggs in a 100 µL volume of M9 were treated with 100 µL of ACF in a 96-well plate for 15 h at 20 °C. Unhatched eggs and L1 larvae were counted, and the percent of hatched eggs were calculated [40,41]. Eggs in the M9 buffer without ACF served as control. The rate of inhibition of egg hatching was assessed under an inverted microscope 15 h after treatment. Hatch inhibition (HI) was calculated according to the formula

$$HI = [(C - T)/C] * 100 \quad (1)$$

where C and T are the percentages of eggs hatched in the control and treatment respectively.

2.9.2. Larval Development Assay

Approximately 30 synchronized L1 larvae in 100 µL M9 buffer is incubated with 100 µL of ACF for 24 h at 20 °C [42]. After incubation L1 were observed and judged as dead if their body was straight without movement. L1 Larvae in the M9 buffer without ACF served as control. Mortality rates were calculated according to the formula [40]

$$M = [(Mt - Mc)/(100 - Mc)] * 100 \quad (2)$$

where Mt is the percentage of mortality in treatment and Mc is the percentage of mortality in control.

2.10. Reduction of Enteric Pathogens in Wastewater

Samples were collected from the wastewater treatment pilot plant (WTPP) as described earlier. The effect of ACF on potential and presumptive pathogens in wastewater was determined by incubating the sample with ACF at 1:1 ratio (v/v) for 1 h and colony forming units (CFU) were enumerated on selective and differential medium after 24 h incubation at 37 °C [43]. Sample incubated with sterile water served as control. The selective media used for the study were HiCrome *Klebsiella* Selective Agar (HiMedia, Mumbai, India) for isolation of *Klebsiella*, SS agar- *Salmonella Shigella* agar for enumeration of *Salmonella* and *Shigella*, EMB agar- Eosin Methylene blue agar for *E. coli* [44], TCBS –Thiosulfate citrate

bile salts sucrose agar for *Vibrio* [45], MSA-Mannitol Salt agar for *Staphylococcus* [46] and Cetrimide agar for *Pseudomonas* [47]. The effects of neutralised (N) ACF, heat-inactivated (H), and neutralised heat-inactivated (N+H) ACF on wastewater were also investigated to understand the antimicrobial nature of ACF. The acidic ACF was neutralised with 10 mmol NaOH, and heat-inactivated by exposing it to 100 °C for 10 min.

2.11. Statistical Analysis

GraphPad Prism 8.2 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis in this study. All experiments were conducted with three replicates and the experiments were twice repeated. The results of the experiments were expressed in terms of mean values and standard deviations. Statistical comparison was carried out using Students paired *t*-test and Two-way ANOVA (analysis of variance) followed by Sidak's multiple comparison test and Bonferroni test. Significance levels were at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

3. Results

3.1. Antibiotic Sensitivity Profile (Antibiogram) of the Host Bacteria

All the strains were characterized by their antibiogram to determine the antimicrobial resistance profile. Our results show that *E. coli* MDR was resistant to all the antibiotics, *K. pneumoniae*, and *S. dysenteriae* were resistant to 6 antibiotics, *V. cholerae* is resistant to five antibiotics, *S. enterica* and *K. variicola* were resistant to four antibiotics. This shows that all the bacterial strains used in the study were multidrug resistant strains except *S. aureus,* which was found to be resistant to only one antibiotic (Table 1).

Table 1. Antibiotic sensitivity profile (Antibiogram).

Bacterial Strains	Antibiotics								
	P	**C**	**MET**	**TE**	**CAZ**	**AMP**	**CIP**	**AMX**	**CX**
E. coli MDR	−	−	−	−	−	−	−	−	−
K. pneumoniae	−	+	−	−	+	−	+	−	−
K. variicola	−	+	−	+	+	−	+	−	+
P. aeruginosa	−	+	−	−	−	−	+	−	+
V. cholerae	−	+	−	+	−	−	+	−	+
S. enterica	−	+	−	−	+	+	+	−	+
S. dysenteriae	−	−	−	−	+	−	+	−	+
S. aureus	+	+	+	+	+	−	+	+	+

Penicillin–P, Chloramphenicol-C, Methicillin -MET, Tetracycline TE, Ceftazidime CAZ. Ampicillin-AMP, Ciprofloxacin–CIP, Amoxicillin-AMX, Cefoxitin-CX. Symbols: Antibiotic sensitive (+) and antibiotic resistant (−).

3.2. Antimicrobial Activity of ACF on the Growth of Target Organisms

ACF was obtained after seven days of incubation in minimal medium supplemented with glucose as a carbon source and used for spectrophotometric and fluorescence based resazurin cell viability assay to monitor the antimicrobial effects. Results obtained showed significant reduction in the growth of *E. coli*, *S. aureus*, *V. cholerae*, *Salmonella enterica*, *Shigella dysenteriae*, *Klebsiella pneumoniae* and *Klebsiella variicola* treated with ACF compared to untreated control after 2 h of incubation (Figure 1). On the contrary, *Pseudomonas* showed an increase in the growth after 2 h of incubation when compared with control.

The fluorometric analysis of the bacterial cell viability by resazurin based assay also showed the results similar to that of spectrophotometric assay (Figure 2). It was evident from both the assays that ACF shows antimicrobial activity against all the target pathogens used in the study with the exception of *Pseudomonas* as compared with control.

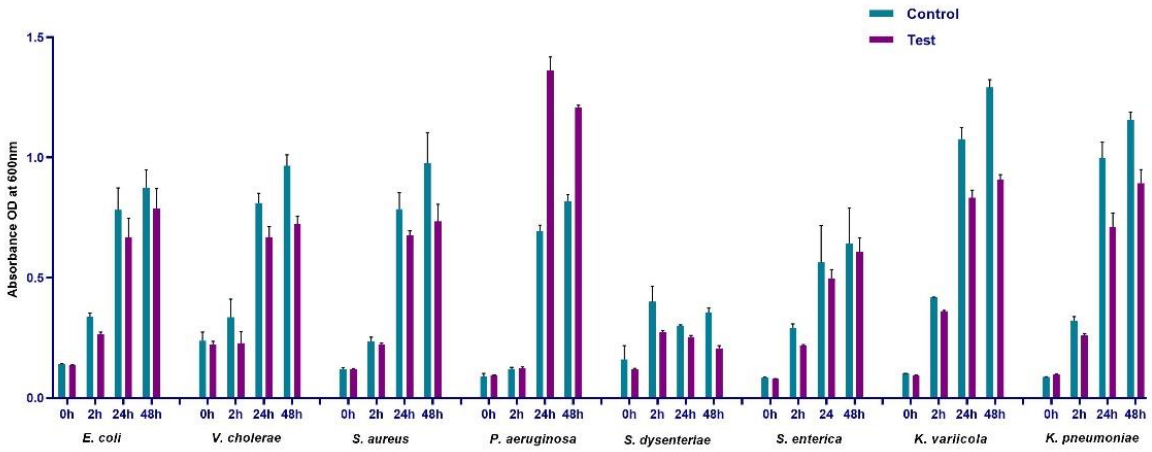

Figure 1. Effect of *A. niger* culture filtrate (ACF) on the growth of eight targeted enteric pathogens. The microtiter well plate with cultures (0.4 OD) treated with ACF at a ratio of 1:1 served as test and cultures without ACF were considered as control. Growth is marked as optical density (OD600 nm) measured after 0 h, 2 h, 24 h and 48 h of incubation. Values are the mean ± standard deviation of three replicates.

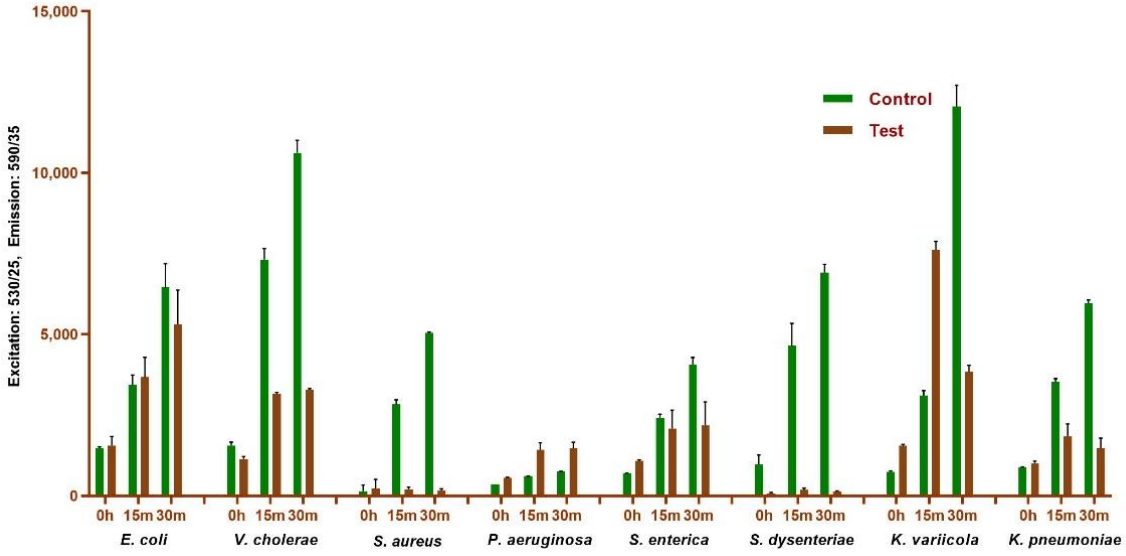

Figure 2. Effect of ACF on the viability of eight targeted micro-organisms by resazurin cell viability assay. The 96 well plate having cultures (0.4 OD) treated with ACF at a ratio of 1:1 served as a test. The cultures without ACF were considered as control. Both test and control were treated with resazurin (1 mmol) and the viability was measured at excitation and emission wavelength of 530/25, 590/35 respectively at an interval of 15 min. Values are means ± standard deviation of three replicates.

3.3. Effect of ACF on Biofilm Formation

The inhibitory effect of ACF on biofilm formation of the test organisms was quantitatively determined using the crystal violet assay. The ACF was found to inhibit the biofilm formation of selected pathogens. Biofilm being a more resistant structure compared to planktonic cells, their susceptibility varied greatly from the antimicrobial activity assays. Biofilm formation of *E. coli*, *V. cholerae*, *S. aureus* and *S. dysenteriae* were inhibited by ACF significantly when compared to untreated control whereas the biofilm formation in *S. enterica*, *P. aeruginosa*, *K. variicola* and *K. pneumoniae* were not inhibited on treatment with ACF (Figure 3). Our results show that ACF ability to eradicate Biofilm formation of pathogens is less compared to its effect on the planktonic cells.

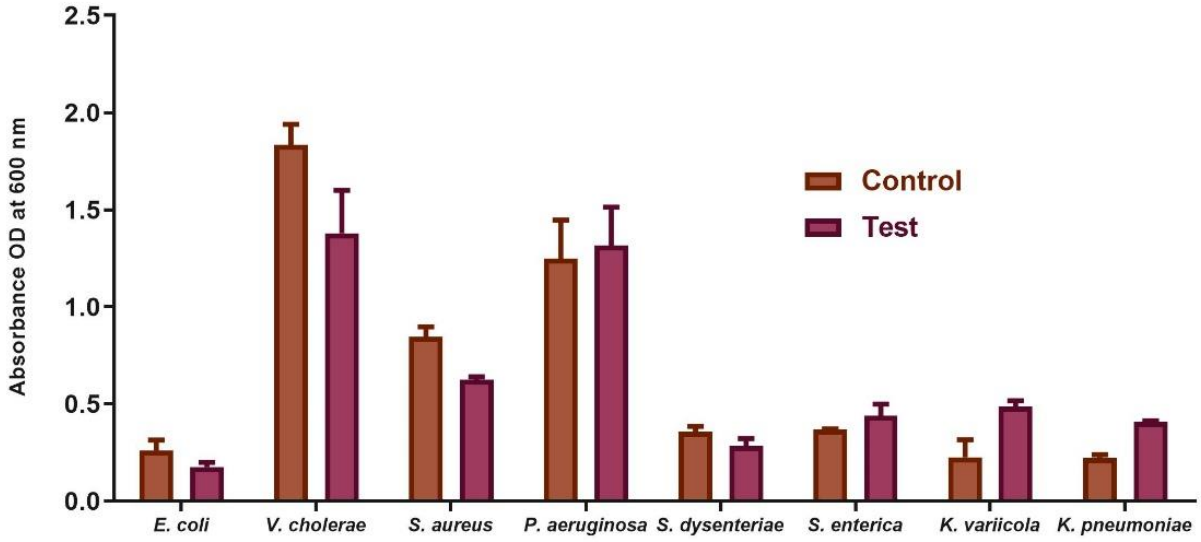

Figure 3. Effect of ACF on biofilm formation. All organisms grown in LB broth (0.4 OD) treated with ACF at 1:1 ratio is the test and the cultures without ACF served as a control. The absorbance was measured at OD 600 nm after crystal violet staining. Values are means ± standard deviation of three replicates.

3.4. Effect of A. niger Culture Filtrate on C. elegans Eggs and L1 Larvae

Treatment of eggs with ACF for 24 h showed 85% inhibition of egg hatching (Figure 4a) similarly, treatment of L1 larvae showed 52% mortality in L1 larvae as compared to the control after 24 h of treatment (Figure 4b). Significant reduction in hatching of eggsand mortality of L1 larvae (Figure 5) indicate that ACF also possesses nematicidal properties in addition to its antibacterial properties.

3.5. Effect of ACF against Potential and Presumptive Pathogens Present in Wastewater

Microorganisms in the wastewater were enumerated after treatment with ACF by plating in selective and differential media. After 24 h treatment of wastewater with ACF the samples were plated, the results were shown in (Figure 6), a 2–3 log reduction of presumptive coliforms, *Vibrio* spp., *Salmonella* spp., *Shigella* spp. and *Staphylococcus* spp. were observed when compared to control. The effect of ACF against *Pseudomonas* spp. remained the same as observed from the above experiments without any significant reduction. These results suggest that ACF could be a potent biocontrol agent specific against bacterial pathogens in wastewater.

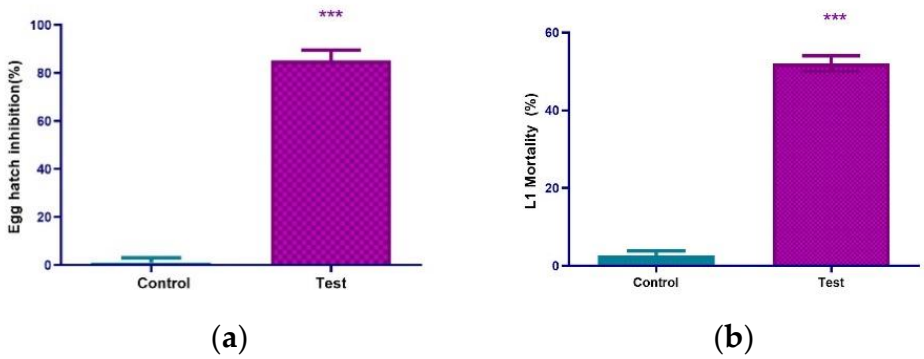

Figure 4. Effect of ACF on *C. elegans*. (**a**) Percentage of egg hatch inhibition was calculated 15 h after treatment of eggs in the M9 buffer with an equal volume of ACF; (**b**) Percentage of Mortality was calculated after 24 h of L1 larvae with ACF. Eggs and Larvae untreated served as control. Values are means ± standard deviation of three replicates. Significance at *** $p < 0.001$; Paired *t*-test.

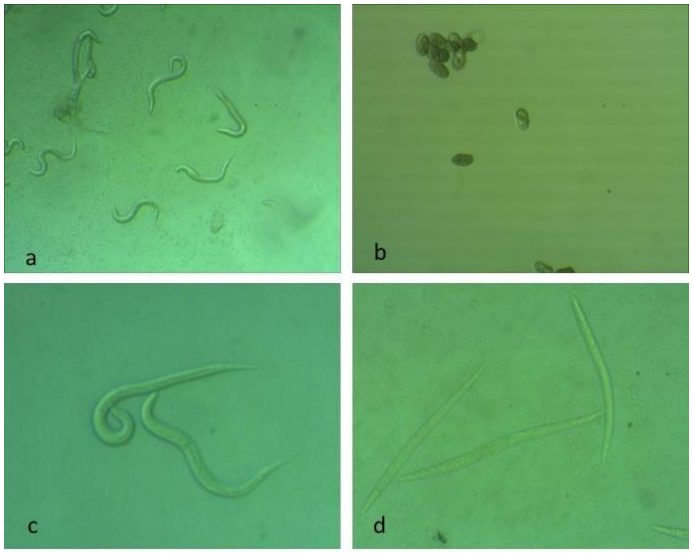

Figure 5. Microscopic images (Nematicidal assay) of *C. elegans.* The hatch inhibition assays of *C. elegans* eggs without ACF ((**a**). control) and with ACF ((**b**). test) were conducted and a number of unhatched eggs and hatched larvae were observed and counted under optical microscope after 24 h of incubation. Mortality assay of *C. elegans* L1 larvae without ACF ((**c**). control) and with ACF ((**d**). test) were conducted. After incubation for 24 h, L1 were observed and judged as dead if their body was straight without movement as compared to control.

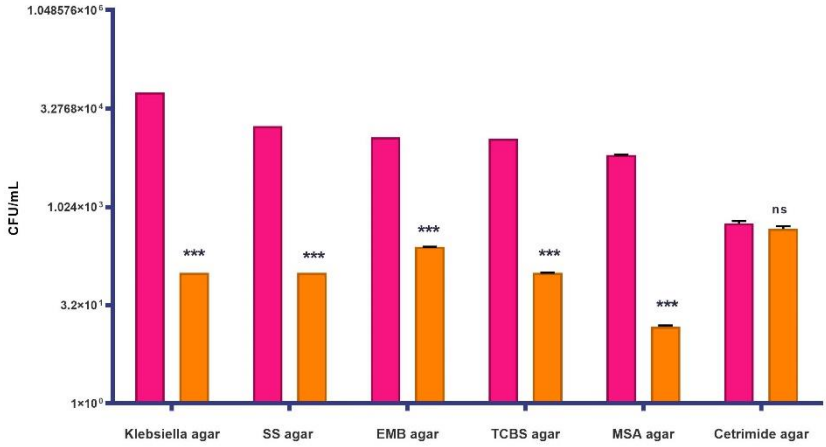

Figure 6. Effect of ACF on the reduction of enteric pathogens in wastewater. The raw wastewater treated with ACF in a 1:1 ratio served as test and the untreated wastewater served as control. The reduction of enteric pathogens was determined by CFU enumeration in selective and differential media (*Klebsiella agar*, SS agar- *Salmonella Shigella* agar, EMB agar- Eosin Methylene blue agar, TCBS –Thiosulfate citrate Bile Salts sucrose agar, MSA-Mannitol Salt agar) after 24 h of incubation. Values are means ± standard deviation of three replicates. Two-way ANOVA (analysis of variance) followed by Sidak's multiple comparison test *** $p < 0.001$, ns- not significant.

3.6. Effect of Inactivated ACF against Potential and Presumptive Pathogens Present in Wastewater

A. niger is a well-known producer of commercially and industrially valuable acids like citric acid, oxalic acid, fumaric acid etc., from glucose as carbon source [48,49]. The pH of ACF was found to be highly acidic (pH ~1). To enhance our understanding of the nature of the antimicrobial factors behind reduction of presumptive pathogens in wastewater by ACF, the changes in the antimicrobial activity were studied after neutralization (N), heat inactivation (H) and heat inactivation and neutralization (H+N) of ACF. We found an increase in CFU in the *Klebsiella* spp. in Klebsiella agar and *Salmonella* spp. and *Shigella*

spp. in SS agar when the sewage sample was treated with N and H however, there was no difference in CFU when treated with (N+H) compared to test (ACF). In the case of *E. coli* counted in EMB agar, we observed an increase in CFU after treatment with H, whereas N or (N+H) did not show any change in CFU when compared to test (ACF). There was no discernible difference in *Vibrio* spp. CFU after treatment with ACF, N, H, or (H+N) on TCBS agar. In MRSA plates, there was an increase in the *Staphylococcus* spp. CFU in samples treated with N whereas H or (N+H) did not show any change in CFU when compared to test (ACF). In Cetrimide agar, as observed in the previous results treatment of sample with ACF, N, H and N+H did not show decrease in the *Pseudomonas* spp. CFU compared to control; instead, an increase in the CFU was observed (Figure 7).

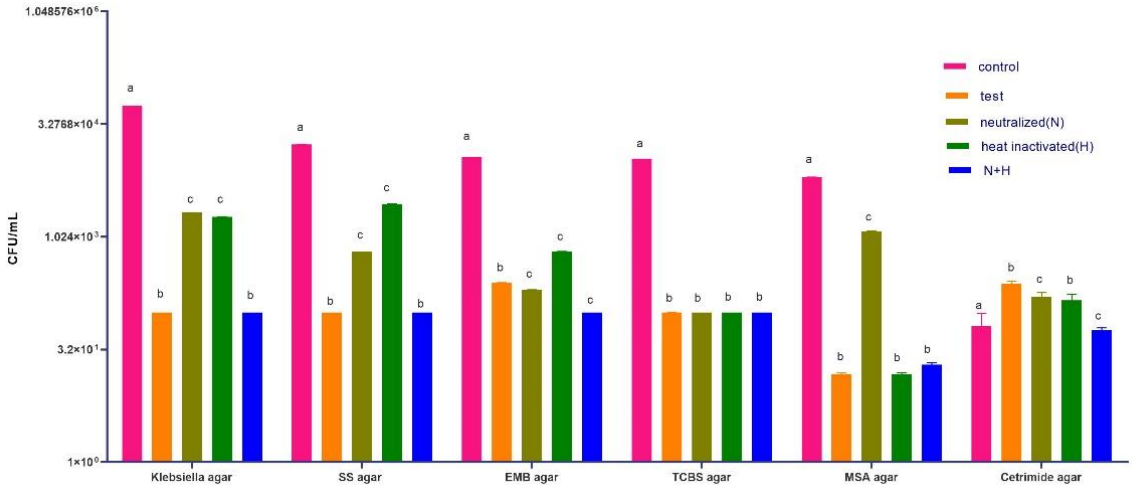

Figure 7. Effect of ACF on the reduction of presumptive pathogens in wastewater. The wastewater sample treated with ACF served as test and the untreated wastewater served as control. The ACF neutralized (N), heat-inactivated ACF(H) and ACF neutralized and heat-inactivated (N+H) were also used in the study. The reduction of presumptive pathogens was determined by CFU enumeration in selective and differential media (Klebsiella agar, SS agar- Salmonella Shigella agar, EMB agar- Eosin Methylene blue agar, TCBS –Thiosulfate citrate Bile Salts sucrose agar, MSA-Mannitol Salt agar and Cetrimide agar) after 24 h of incubation. Values are means ± standard deviation of three replicates. Different letters a, b and c indicate significant differences of treatment at $p < 0.001$ level (Bonferroni test) in their respective media.

4. Discussion

A. niger is generally regarded as safe by WHO and finds usage in many food industries to produce various enzymes and organic acids for food processing [17,19–21]. *A. niger* also plays an important role in the field of industrial and environmental biotechnology where it has been reported in the treatment of wastewater for removal of dyes, oils, and minerals [50,51]. The property of *A. niger* to grow as a bio-flocculant is widely used in wastewater treatment [52]. *Aspergillus* have received much attention as they are well known for their production of antimicrobial substances, several of which have formed the basis for the development of new antimicrobial agents [53]. Our group tried to study *A. niger* culture filtrate's ability to act as a biocontrol agent for removal of enteric pathogens in wastewater, as the pathogens associated with wastewater are one of the main causes of transmitted infections globally [7].

In this study we had tried to target bacteria and parasitic worms by using specific strains common in wastewater, especially enteric pathogens in case of bacteria and adopted a model nematode worm, *C. elegans*. Most of the bacteria in the environment exist either as planktonic, free-living organisms or attached matrix-enclosed communities called biofilm [54]. Bacteria in a biofilm can combat adverse environmental conditions like nutrient deprivation, temperatures, and pH changes and are more resistant to detachment,

disinfectants, and antibiotics than the individual planktonic cells [55]. A potent antibiofilm agent can either ease the dispersion of preformed biofilms or impede the formation of new biofilms [56]. While considering the nematodes, eggs are the infective forms in case of most of the parasites and they are adapted to long-term survival in the environment and are responsible for serious illness in both humans and animals [57] and their presence is the main limitation for the reuse of wastewater in agriculture or other purposes. Removal or inactivation of eggs from the wastewater is very important to control the spread of parasitic infections, and any agent that can inhibit the egg hatching to a greater extent acts as a good nematicidal agent. Most of the control strategies target the removal or inactivation of eggs [58]. The complex life cycle, lack of throughput screening techniques for parasitic nematodes makes it difficult to work with in the laboratory, and moreover, the majority of marketed anthelmintic proven efficacy against *C. elegans* makes it a useful model system [59].

Previously, antimicrobial activity of *A. niger* (mostly endophytic strains) against several bacterial pathogens was reported by growing *A. niger* in potato dextrose broth, and the culture filtrates obtained were extracted using organic solvents like ethyl acetate and further purified using chromatography [60–62]. The advantage of biological process is their high removal efficiencies and low operational cost [63]. Our group has been working in establishing cost-effective strategies for the control of enteric pathogens in wastewater. One of the strategies we experimented was the use of ACF obtained by growing *A. niger* in defined media with glucose as a sole carbon source. This filtrate obtained can be directly used as a biocontrol agent without any further purification. Various bioassays were employed to determine the antibacterial and anthelmintic properties of ACF. Though various endpoints were investigated the results of all methods were in good agreement.

A. niger is a good producer of acidic metabolites. This was also evident from our study, where the pH of culture filtrate obtained was nearly one, which may be attributed to the organic acids produced. To check whether the antimicrobial effect observed is related to the acid pH of the ACF, the neutralization studies were carried out. The interesting observation made was that the antibacterial effects of the ACF are not limited to the lower pH of the filtrate. Neutralization of ACF yielded similar results as that of the treatment with ACF alone with respect to control in most of the cases, thus nullifying the inhibitory effect associated with the pH as reported previously for certain organic acids [63]. Results obtained with neutralized, and heat-inactivated ACF emphasizes that its antibacterial activity could be due to the presence of other metabolites [53]. Even though low pH of ACF might inhibit bacterial growth, neutralization results indicated that pH may not be the significant factor. One critical issue with ACF is the organic acids released into wastewater may increase the overall organic content, that is biochemical oxygen demand (BOD) and chemical oxygen demand (COD). This needs to be addressed before this observation is put to practice in wastewater treatment. We have envisaged introducing some desirable species downstream to the treatment so that overall organic acids are consumed by them; thus, overall nutrient load is reduced. Our results indicated a selective increase in the growth of *Pseudomonas* when treated with ACF, which was earlier reported in disinfection strategies with UV has shown elimination of coliforms and resistant populations like *Pseudomonas* being unaffected [26]. The carbon catabolite studies of *Pseudomonas* showed its ability to use organic acids as the preferential carbon source rather than the glucose, which also gives them a competitive advantage over other organisms in a complex environment as our result suggested. Hence, we believe it might be possible to choose some non-pathogenic *Pseudomonas* species to grow in the acidic media. The choice of the non-pathogenic strain can vary depending on the end use of the treated wastewater. For example, probiotics to plant crops like *Pseudomonas fluorescens* can be cultivated so that treated water with the plant probiotics can help in crop growth during irrigation as well as help in reduction of BOD and COD.Moreover, volume of ACF to sewage at 1:1 (v/v) might be impossible to achieve in a real sewage treatment system. Hence, we envisage to deploy a biofilter with a layer of probiotic/commensal *Pseudomonas* (e.g., *P. flourescens*) where sewage and ACF will

be applied in 1:1 (*v*/*v*) periodically (once in a month). That would stabilise the layer of the probiotic and reduce the load of other enteric pathogens. We have previously designed a biofilter with a probiotic Lactobacillus & bacteriophage cocktail, which could reduce the coliform count by 2-log orders of magnitude [64]. We have also designed a novel lytics broadcasting system (LBS), which dispenses bacteriophage and other cell free lytic agents that could reduce the target bacterial load by 3 to 4 log orders of magnitude [65]. We believe that *Pseudomonas* being a very invasive and dominant species in their respective ecosystem, it would reduce the load even further [66]. The stability of the biofilm may reduce on continuous flow of sewage, but periodical application of ACF can increase the stability over time. However, it may still be necessary to develop an alternative strategy to eliminate possible enrichment of pathogenic *Pseudomonas (P. aeruginosa*). This could possibly be achieved by immobilization of the biofilter with high titre phage against the pathogenic *Pseudomonas*. Since our results showed that the reduction of pathogen load in wastewater is not attributed to the acid metabolites, we are in the process of finding out the metabolites in the ACF that are responsible for the activity. We need to characterize the individual components in the ACF by LC-MS and NMR, followed by their individual biological activity. There are reports of *A. niger* acting as a good source of several mycotoxins [16], and its toxicity studies in human/animal are known, but their effect against enteric pathogens are not well studied, so if we could find out the metabolite responsible for reduction of pathogens, we might be able to establish the mechanism of pathogen reduction.

5. Conclusions

Overall, our findings indicate that *A. niger* acts as a biocontrol agent with a potential role in microbial community shifting that may be attributed to the acidic nature of the culture filtrate or other metabolites present in the culture filtrate that inhibits the growth of most of the organisms except *Pseudomonas*. We need to find out appropriate microbiome engineering strategies by which beneficial *Pseudomonas* or other acid tolerant species can be cultivated in the media. There is active research going on in the field of microbial community modification in the agriculture sector by modifying pH [55]. Our work indicates a very interesting possibility of wastewater treatment and may be cultivation of useful bacteria (biofertilizer) by using the *A. niger* culture filtrate. In conclusion, to the best of our knowledge, this is the first study of application of *A. niger* culture filtrate on wastewater directly for the reduction of enteric pathogen-load, a potential eco-friendly, cost-effective technology to valorise wastewater.

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