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ORIGINAL ARTICLE

Isolation and Identification of Various Types of Microbes Present on Documents and the Inhibitory Effect of Ink on their Growth

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ABSTRACT

CONTEXT: Isolation and Identification of various types of microbes present on documents and the inhibitory effect of Ink on their growth.

AIM: The aim of the experiment is to study the inhibitory effect of ink on certain microorganisms prevalent on cellulose of paper.

SETTINGS AND DESIGN: An experimental setup was designed to study the effect of ink on different microbial growth on cellulose paper. For this purpose, pages old books were used as a sample on which well diffusion method was applied for determining the effect by ink.

MATERIALS & METHOD: Decaying book papers were collected from Ranchi city, Jharkhand, India. From these decaying paper samples four bacterial strains and one fungal strain was isolated and identified as Providentia stuartii, Serratia odorifera, Bacillus megaterium, Pseudomonas antimicrobica, and Aspergillus niger respectively. The effect of ink on these microorganisms were studied by agar well diffusion method.

RESULTS: Black, and blue gel pen inks showed maximum zone of inhibition against Providentia stuartii. Blue ball pen ink also showed maximum zone of inhibition against Providentia stuartii and Serratia odorifera. Red ball point ink showed maximum inhibition zone against Bacillus megaterium and black ball point pen ink showed maximum zone of inhibition for Pseudomonas antimicrobica. **CONCLUSIONS:** With printing ink no inhibitory effect was observed on the bacterial strain and zone of inhibition was completely absent. However, green colored printer's ink showed maximum inhibitory effect on fungus Aspergillus niger.

KEYWORDS | fragile document, decaying paper, preservation, isolation, microorganisms, agar well diffusion method, inhibitory effect, zone of inhibition

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INTRODUCTION

The first paper document was manufactured in 1901 from wood cellulose. This cellulose component of paper was tested against ageing factor, the result showed good stability of paper during the ageing process due to the presence of cellulose. During, mid of 19th century, other factors like: air contamination, SO_2 absorption and its effect on acidity on the paper surface were noticed. Similarly, environmental conditions like effect of temperature on paper during ageing process was also reported in the year 1931. Likewise in the 1960s, a relationship between acidity of paper and its breakdown during ageing process was studied. The study showed that acidity of paper is an important factor to persistence of

the paper.

In detail, cellulose is abundantly available on our planet. Since cellulose comprises of a group of fibrolytic enzyme, it hydrolyzes plant cell wall (made up of fiber) into oligosaccharides, and glucose.[1,2] Thus, hydrolysis process mainly involves three types of cellulase enzymes namely, carboxymethyl cellulase (CMCase), cellobiohydrolase and β-glucosidases or endoglucanase. These enzymes are mostly produced by microbes such as bacteria and paramecia. Despite this, cellulase enzymes are also produced by some plant materials and animal sources. Cellulase enzymes are inducible in nature as they grow on cellulosic materials during its synthesis from microorganisms.

In the current scenario, the cellulase enzymes are mostly used for industrial purposes. Therefore, there are large number of cellulase enzyme producers such as fungi (Cladosporium, Myrothecium, and Penicillium and Cladosporium) and bacteria (clostridium, micrococcus and streptococcus) are the most common producers of cellulase enzymes. However, in late 19th century researchers reported some cellulolytic bacteria namely, Cytophaga and some species of actinomycetes, which secretes strong organic acids and staining pigment that causes destruction of paper surface.[3,4,5] During 20th century, the growth of microbial species like fungi such as Saccharicola, Aspergillus and Trichoderma in humidity on paper surface were reported. Oftentimes, book binding are the first sufferers of microbial growth because they absorb air moisture. Owing its cellulolytic enzymatic property, some filamentous species of fungi is also observed during paper degradation which ultimately leads to the hydrolysis of cellulose fiber. Furthermore, it causes discoloration of ink on the paper in the form of stain or depigmentation. Theses, stains may be of different colors like red, yellow, purple and black.

In order to understand the whole process of microbial growth with reference to ink, the current work focuses on isolation and identification of various microorganisms such as bacteria and fungus on various papers and their growth.

METHODS AND MATERIALS

Since the aim has been identified, a working protocol has been formulated in order to achieve the objectives that have been framed. The materials and methods that have been adopted to achieve the objectives are given below.

Isolation of microorganisms: Book samples were weighed separately and serially diluted and cultured on NAM plates for isolation of bacterial species.

Dilutions of 10-1 to 10-3 were done and the diluents of 10-3 was employed for spreading onto the agar plates that were incubated at 37° C for about 24 hours. Further, Gram's staining and Gram nature of each isolated colonies were studied. Isolation of bacterial species: For the Isolation of bacterial species two books dated 1969 and 1958 as sample 1 and sample 2 respectively were used. Book samples were weighed separately and serially diluted and cultured on nutrient agar medium (NAM) plates for isolation of bacterial species.

Preparation of Media: Nutrient agar medium was prepared for isolation of microbes

- Nutrient agar (NAM)
- Nutrient Broth
- Potato Dextrose Agar (PDA)

Gram's staining: Bacterial smear was prepared on a slide and heat fixed. The cationic crystal violet dye is used to stain the microorganism for one minute. Afterward, it was washed using water and then Gram's iodine was added for upto 1 minute forming a purple complex. Acetone was used to decolorize. The obtained smear was washed again using water and then treated with saffranin for almost 45 seconds. Smear was washed and stained. Microorganisms were then observed under microscope and identified Gram positive (purple) and Gram negative as (red/pink).

Culture Preparation: The isolated microorganisms were cultured onto suitable media and incubated until colonies were

observed on the plates. Using agar plate each colony was firstly cultured, secondly collected, thirdly inoculated in nutrient broth and lastly the cultures were incubated.

Isolation of fungal species: Paper sample was taken from the old books. The paper sample was serially diluted and cultured on PDA plates for the isolation of fungal species.

Microscopic identification of fungus: Slides were prepared by using Lacto Phenol Blue for the microscopic identification of fungal species.

Morphological Characterization of Bacteria: Gram staining of isolated bacteria was done by using light microscope of Olympus Company and the bacteria were observed under 100X.

Biochemical Tests

Biochemical identification of isolates was done as per the procedure given by Aneja (2007).

Sugar Fermentation

By using sugar fermentation test, the strength of microbes for degrading various carbohydrates, glucose, lactose, fructose, sucrose and mannitol can be determined. For performing this, a test tube is filled with10 ml of basal media through inverted Durham's tube and then solution is autoclaved at 121°C for 15-20 minutes. Whereas, 1% concentration of carbohydrate is prepared in distilled water that placed for autoclaved at 10 lbs/inch.[2] Afterward, 10ml of basal media and 1ml of sugar is mixed with loopful organism. For control sample, an un-inoculated tube (without organism) was taken. Then all the final test tubes were place in incubator at 37°C for 24 hours. After incubation process, gas and acid examination was performed on the test tubes. When media coloration changes from purple to yellow, it shows the production of acid in the media, while no change, in color results is negative test. Whereas, gas production shows the accumulation of gas bubbles in Durham's tubes.

Indole Hydrolysis

The indole hydrolysis test is performed by using tryptone broth because it contains large quantity of tryptophan. On hydrolysis, this tryptophan is converted to indole and pyruvic acid in the presence tryptophanase. To perform this, isolated bacteria inoculated in tryptone broth. For control sample an un-inoculated tube (without organism) was taken. The control tubes along with inoculated tubes were placed in incubator at 37°C for 48 hours. After this process, about 5 drops of Kovac's reagent is added in the test tube. If a red layer form at the top of the broth it indicates the positive test and if no change in color observed then it indicates negative test.

Methyl Red Test

In this test, bacterial cultures were inoculated into sterilized glucose peptone, followed by incubation at 37°C for about 24 hrs. Then an incubated Methyl red pH indicator was added to the obtained solution, after addition of indicator if the color changes to yellow it indicates negative test (pH > 6), whereas if color of the media remains red it indicates positive test (pH < 4.4).

Voges Proskauer Test

The bacterial cultures were inoculated into sterilized glucose peptone broth, proceeded by incubation at 37°C for about 24 hours. Afterward, the VP (Voges Proskauer) reagent is added slowly to observe the change in color. The development of ruby pink color, indicates the positive test of VP reagent, whereas no change in the color of media represents negative test.

Citrate Test

This test is used to distinguish enteric bacteria, on the basis of their capability to use citrate, acting as a source of carbon and energy. In organisms, citrase enzymes are present to utilizes citrate. Therefore, this test involves two steps, first: inoculating of microorganism in citrate agar media. Second, addition of bromothymol blue (indicator) in the media. The appearance of green to blue color indicates the positive test, while no change in the coloration represents negative test.

Glycerol Test

This test is similar as the carbohydrate test, where capability of microorganism for degrading carbohydrates is determined. For

this 150ml glycerol broth was prepared and pH was maintained around 7.3 and autoclaved at 121°C for 15-20 minutes. After autoclaving one loopful of bacterial isolates were transferred in the tubes containing glycerol media and kept in incubator for 24 hours at 37°C. If the color of the solution changes from red to yellow, then acid is produced and glycerol test is positive and no color change shows that the test is negative.

Casein Test

Casein is a protein that is responsible for the white coloring of milk. For this test casein media was prepared and pH was maintained around 7.2 and autoclaved. Casein plates were made and streaked with the bacterial isolates and kept in incubator at 37oC for 24hrs. A clear zone around bacterial growth indicates that organism can utilize casein.

Urease Test

This test is performed for determining the capability of microorganisms for degrading urea in the presence of urease enzyme. For performing this, urease broth was prepared, and pH was maintained around 6.2 and autoclaved and loopful of bacterial isolates were transferred in the broth and kept in incubator at 37oC for 24hrs. If the color of media changes, from pink to dark pink the test is positive and bacterial isolates are able to utilize urease.

Starch Hydrolysis

This test is performed for determining the capability of microorganisms to utilize starch, like carbohydrate made by glucose and acting as source of energy for growth. Alpha-amylase is an enzyme used to accomplish starch. For this, starch agar media plates were prepared and streaked with the 5 isolated strains and kept in incubator at 37oC for 24 hrs and after incubation, grams' iodine is added to observe the presence of starch, if the clear zone is formed near the growth that bacterial isolates shows starch hydrolysis test positive, if no zone is formed then that bacterial isolates shows negative test.

Acetate Test

The test is performed to determine whether the organism is able to use acetate as an acting source of carbon. In that situation, breaking of sodium acetate effect the pH of the media which results in shifting of pH toward alkaline and the color of the indicator changes from green to blue.

Lecithinase Test

Egg yolk agar media is used for the determination of lecithinase and lipase enzyme activity. These enzymes (lecithinase and lipase) are most commonly found in microorganisms. The microorganisms which contain lecithinase enzymes, are breaks down into an insoluble phosphorylcholine, which results in the formation of white precipitation. Whereas, microorganisms which contain lipase enzyme, are hydrolyzed the fat of the egg yolk, which produces an iridescent sheen on media colony.

Salt tolerance test

For salt tolerance test, the bacterial isolates were inoculated with higher NaCl concentrations of 7.0%.

Catalase test:

Most of the microorganism uses oxygen to produce H2O2. But H2O2 is toxic for their enzymes. Therefore, to overcome this toxic effect, microorganisms are possessing catalase enzyme. Thesecatalase enzymes convert H2O2 to H2O and O2.

Determination of Inhibitory Effect of Different Inks on Bacterial Growth:

To perform the inhibitory effect of different ink samples against bacteria,well diffusion method was employed. For this, 3 different types of inks were collected i.e. Ball point pen ink, Printer ink and Gel pen ink and from all the 3 types, 4 different colors were used i.e. blue, black, green and red. Bacterial isolates were inoculated into nutrient broth. Anti-microbial activity was performed by well diffusion method against different isolated bacterial strains.

RESULTS

Growth of bacteria on Nutrient Agar after Serial Dilution: After the serial dilution of the paper sample the microbes isolated from book1 (sample1) and the book 2 (sample2) the growth

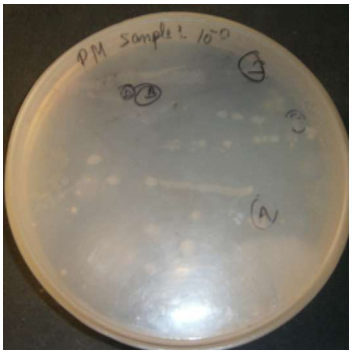

Figure 1(a): Growth of bacteria after serial dilution from sample1 (Book 1)

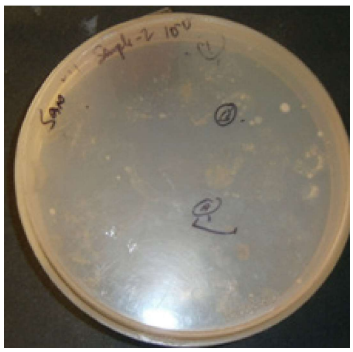

Figure 1(b): Growth of bacteria after serial dilution from sample1 (Book 2)

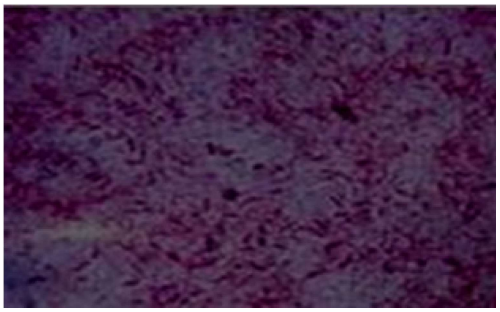

Figure 2(a): Strain 1

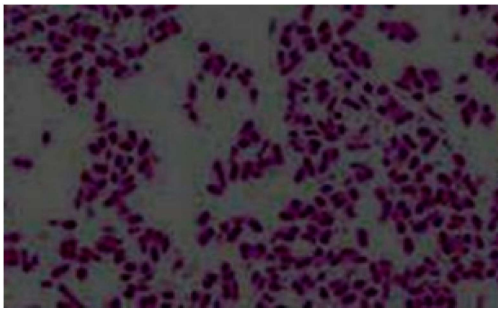

Figure 2(b): Strain 2

Figure 2(c): Strain 3

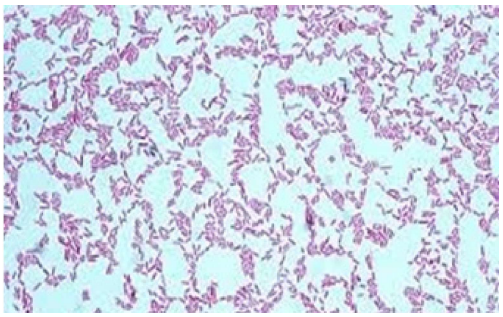

Figure 2(d): Strain 4

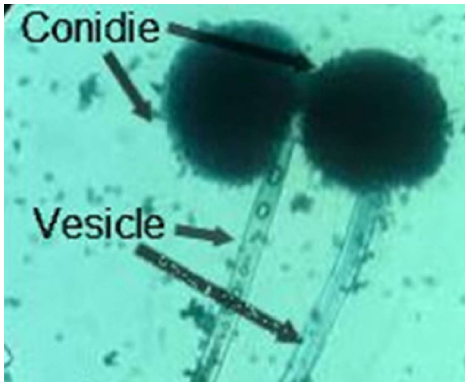

Figure 3: Microscopic view of Aspergillus Niger the entire surface

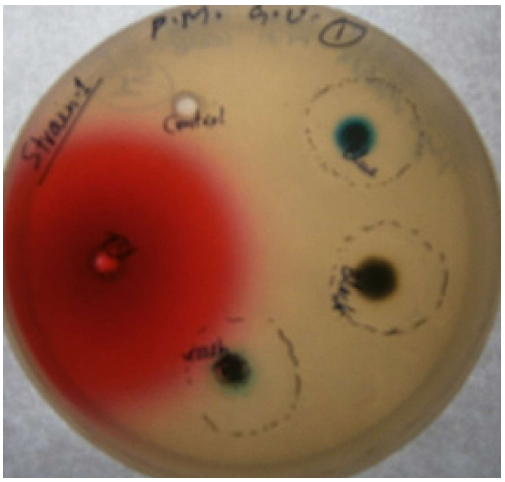

Figure 4(a): Effect of gel pen ink on strain1 well diffusion method

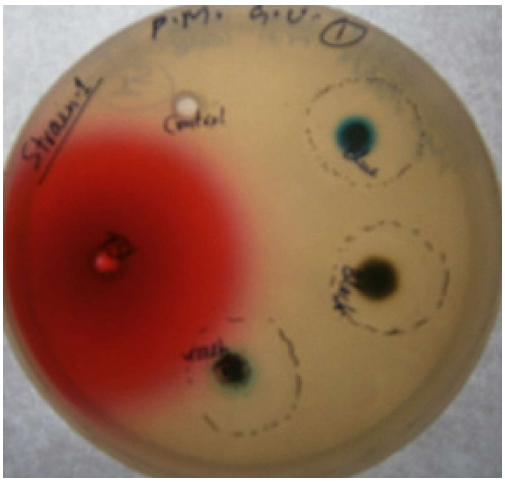

Figure 4(b): Effect of gel pen ink on strain 2 using agar-well diffusion method

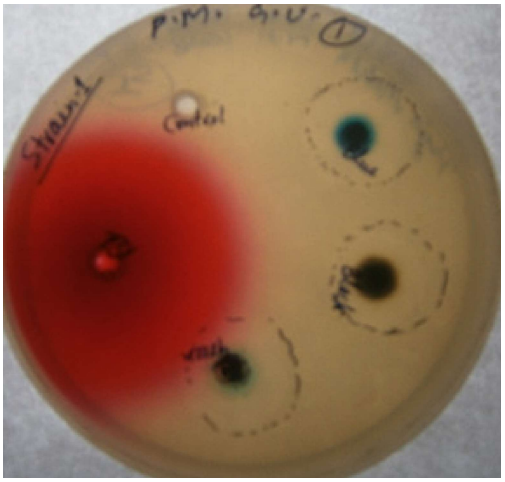

Figure 4(c): Effect of gel pen ink on stain3 using agar- well diffusion method

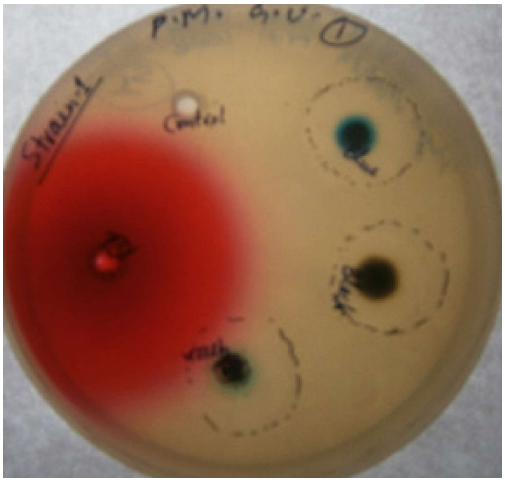

Figure 4(d): Effect of gel pen ink on strain 4 using agar -well diffusion method

of bacteria seen on the nutrient agar plates are as pictured in Fig 1(a): and Fig 1(b).

Morphological characterization of bacterial isolates by Gram Staining: The bacteria grown in the nutrient agar plates in figure 1(a) and 1(b) were then marked as colonies and subjected to Gram staining. These isolated bacteria were then observed under 100X objective lens of the microscope. The observation is as depicted in Fig. 2(a), Fig. 2(b), Fig. 2(c) and Fig. 2(d) which were named Strain1, Strain 2, Strain 3, Strain 4, Strain 5 respectively. Figures are shown below.

Microscopic Identification of Fungus:

Slides prepared by the method mentioned above were observed under the microscope. The slides of different fungal species showed different characteristic features when observed under microscope. (Fig. 3) shows the microscopic characters of different fungal species which were used for further studies. Lacto phenol blue staining of A. niger showed that the colonies were globose, brown to black in color, consists of smooth conidie with transparent vesicle and conidiophores over.

On the basis of biochemical tests along with sugar utilization test results. The detection

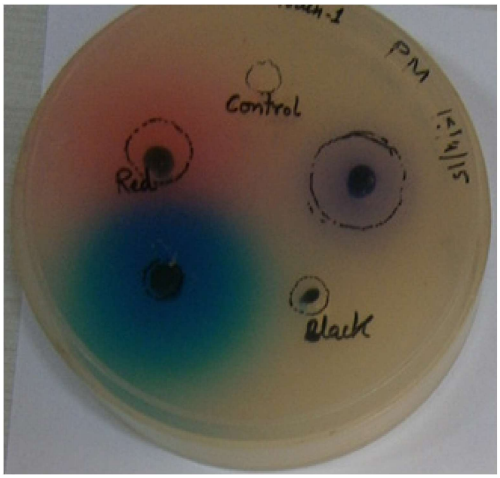

Figure 5(a): Effect of Ball pen ink on strain1using agar- well diffusion method

Figure 5(b): Effect of Ball pen ink on strain 2 using agar- well diffusion method

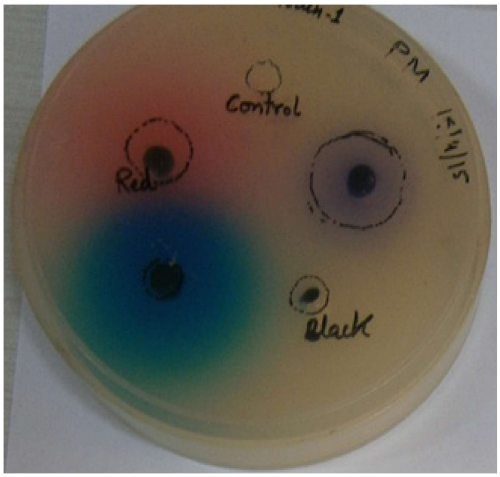

Figure 5(c): Effect of Ball pen ink on strain 3 using agar- well diffusion method

Figure 5(d): Effect of Ball pen ink on strain 4 using agar-well diffusion method

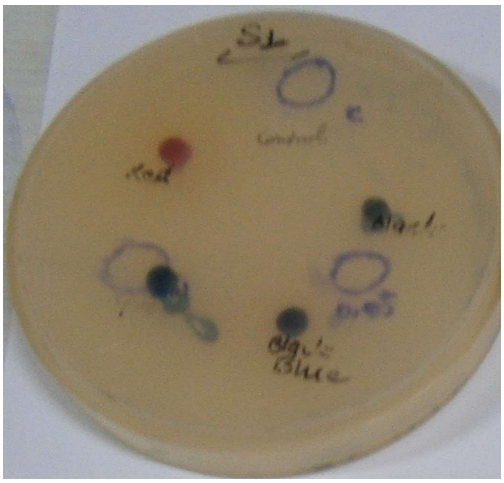

Figure 6(a): Effect of printer ink on strain1 using agar- well diffusion method

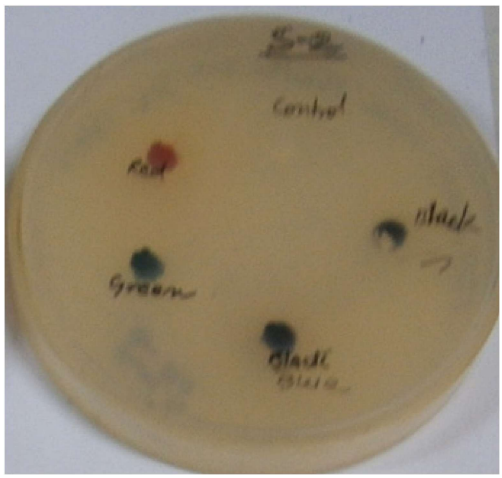

Figure 6(b): Effect of printer ink on strain using agar- well diffusion method

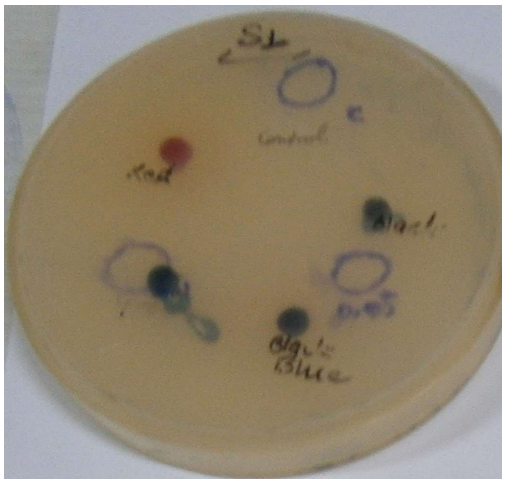

Figure 6(c): Effect of printer ink on strain 3 using agar- well diffusion method

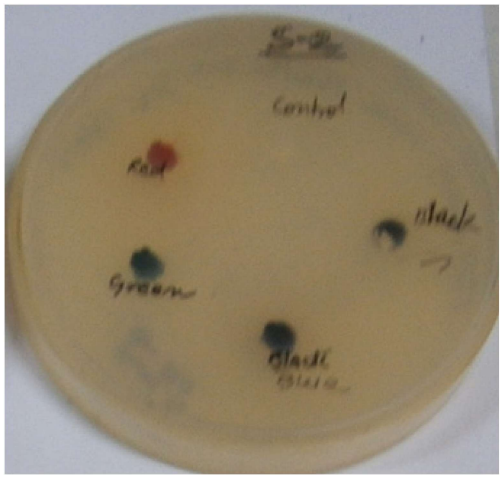

Figure 6(d): Effect of printer ink on strain 4 using agar- well diffusion method

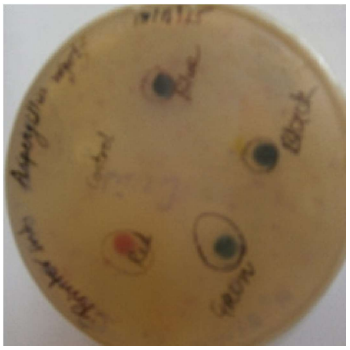

Figure 7:(a) Effect of Printer Ink

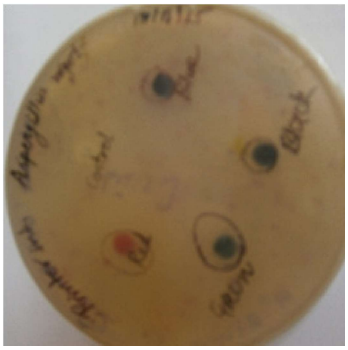

Figure 7:(b) Effect of Ball Pen ink

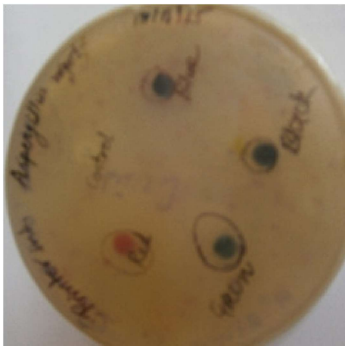

Figure 7: (c)

Sample Bacterial Strain	Morphological Identification	Name of Bacterial Strain after identification
Strain -1	Pink Color, Short rod	Gram -ve
Strain -2	Pink Color, Short rod	Gram -ve
Strain -3	Violet Color, Short rod	Gram +ve
Strain -4	Pink Color, Short rod	Gram +ve

Table 1: Results after gram staining and morphological identification of bacteria in 100x objective lens of microscope

	INDOLE	METHYL RED	VOGES-PROSKAUER	CITRATE
Strain- 1	-ve	+ve	-ve	+ve
Strain- 2	-ve	+ve	-ve	+ve
Strain- 3	-ve	+ve	-ve	+ve
Strain- 4	-ve	+ve	+ve	+ve

Table 2: (a) IMViC Test results.

Name of the Test	Strain-1	Strain -2	Strain-3	Strain- 4
Starch	+ve	+ve	+ve	-ve
Casein	+ve	-ve	-ve	+ve
Glucose	+ve	-ve	-ve	-ve
sucrose	-ve	+ve	+ve	-ve
Lactose	-ve	-ve	-ve	-ve
Glycerol	-ve	+ve	+ve	-ve
Maltose	-ve	+ve	+ve	+ve
Mannintol	-ve	+ve	+ve	+ve
Acetate	+ve	+ve	+ve	+ve
Urease	+ve	-ve	-ve	+ve
Salt tolerance	Low- growth	High -growth	High -growth	Low -growth
Lecithinase	+ve	-ve	-ve	+ve
Catalase	+ve	+ve	+ve	+ve

Table 3: Result of different types of Biochemical Test

	Ink	Zone of Inhibition Diameter in CM			
Strain	Sample Control	Blue	Black	Green	Red
S1 Providentia stuartii	0	2.2	2.0	1.8	1.5
S2 Serratia odorifera	0	1.5	1.1	0.9	1.3
S3 Bacillus megaterium	0	1.3	0.8	1.0	1.4
S4 Pseudomonas antimicrobica	0	1.0	1.5	1.1	1.2

Table 4: Zone of Inhibition in Gel pen ink by Well Diffusion method

	Ink	Zone of Inhibition Diameter in CM			
Strain	Sample Control	Blue	Black	Green	Red
S1 Providentia stuartii	0	2.1	0.7	1.1	1.85
S2 Serratia odorifera	0	1.9	1.0	1.7	1.6
S3 Bacillus megaterium	0	2.1	1.3	1.8	1.05
S4 Pseudomonas antimicrobica	0	1.5	0.85	1.9	0.9

Table 5: Zone of Inhibition in Pure Ball Point Pen Ink by well Diffusion Method

	Ink	Zone of Inhibition Diameter in CM			
Strain	Sample Control	Blue	Black	Green	Red
Printer Ink	0	1.2	1.0	1.1	1.5
Gel Pen Ink	0	1.9	1.4	1.55	1.7
Ball Pen Ink	0	2.2	1.5	1.9	2.0

Table 6: Zone of Inhibition when different types of ink on Aspergillus niger.

of unknown bacterial strains is performed by using Bergey's manual.

The results showed that Strain 1 – Providentia stuartii (97% similarity), Strain 2 – Serratia odorifera (88% similarity), Strain 3 – Bacillus megaterium (80% similarity), Strain 4 – Pseudomonas antimicrobica (85% similarity).

Results of effect of different types of ink on document microbes by well diffusion method:

1. Well diffusion method to study the effect of gel pen ink against bacterial isolates:

According to the effect of different colour gel pen ink, zone of inhibition of different diameter were observed after 24hr. of incubation which were described in figure 4(a), 4(b), 4(c)and4(d).

In reference to the above mentioned table, it

has shown that on strain 1, strain 2 and strain 3 blue color gel pen has maximum inhibitory effect, whereas, in case of strain 4 black color gel pen has shown maximum inhibitory effect and blue color has minimum inhibitory effect.

2. Well diffusion method to study the inhibitory effect of ball pen ink against bacterial isolates:

According to the effect of different color ball pen ink zone of inhibition of different diameter were observed after 24hr. of incubation which were illustrated in the following figures 5(a), 5(b), 5(c), 5(d) respectively:

In pure printer ink no inhibitory effect was observed on microbes. 100% Microbial growth was observed on petri plates and zone of inhibition were completely absent.

Effect of different type of ink on fungus aspergillus niger by well diffusion method:

These 3 plates were observed after 48 hours of incubation and different zone of inhibition were observed on different plates which were presented in figure 7(a), 7(b) and 7(c).

Table showed that on fungus blue ball pen and blue gel pen ink has more inhibitory effect followed by green color ink. Whereas, in case of printer ink green color ink has shown maximum zone of inhibition.

DISUCSSION

In a similar study conducted by Kamel *et al.,* [2014], Bacillus subtilis and Acrodictysfimicola[6] were isolated from decaying book and papers collected in Erbil city, Iraq. In the present study 5 strains of bacteria and 2 fungal strains were isolated and identified from book papers dated as old as 1958 and 1969 collected from the market of Ranchi city, Jharkhand, India. In the present study an attempt was made to study the effect of ink on the growth of the above mentioned bacteria Providentia stuartii, Serratia odorifera, Pseudomonas antimicrobica, Bacillus megaterium, Streptomyces species and fungus Aspergilllus niger. A similar study was done by Bragulat et. al, in 1991, reported an inhibitory effect of thirteen dyes on the growth of mycelium fungi (Deuteromycetes and Zygomycetes).[7] Other researchers reported that bacterial contamination of books can be identified by using genera of Pseudomonas, bacillus, micrococcus and clostidium.[8]

As per the literature survey, present study focuses on the isolation and identification of microorganism from decaying papers of books and to study the effect of ink on these microorganisms.[9,10] In this study, variety of dyes including dichloran methylene-blue, aura-mine, phenol-red, rose-bengal and gentian-violet were used. The result showed that the bacterial genera of Providentia, Serratia, Bacillus, Pseudomonas and fungus Aspergilllus niger were isolated. It has been found that species of Aspegillus can degrade cellulose and they are often associated by the holdings of library as found in the study of Konkol *et al.,* in 2009. In present study also Aspergillus was isolated from degraded book samples, so it can be said that Aspegillus degrade the documents. In the present study, the effect of ink was studied by Agar well diffusion method and result of Gel pen ink of blue and black color shows maximum zone of inhibition against Strain S1 – Providentia stuartii. Blue colour of ball pen ink shows maximum zone of inhibition against S1 – Providentia stuartii and S2 – Serratia odorifera , in S3 – Bacillus megaterium red colour ink showed maximum inhibition but in Strain-4 Pseudomonas antimicrobica black colour shows maximum zone of inhibition followed by green colour gel pen ink.In ball pen ink blue coloured ink showed maximum inhibition against S1, S2, S3 and green colour showed maximum inhibition against S4.In pure printer ink no inhibitory effect was observed on microbes. 100% Microbial growth was observed on petriplates and zone of inhibition were completely absent. Against Aspergillus niger inhibitory effect of all colour ball pen ink and Gel pen ink shows maximum result and inhibitory effect of Printer ink was also observed, whereas on bacteria inhibitory effect of pure printer ink was completely absent. On Fungi Aspergillus niger green colour printer ink showed maximum inhibition whereas Blue colour of gel pen ink

and ball pen ink showed maximum inhibition followed by green coloured ink Whereas, in case of printer ink green colour ink has shown maximum zone of inhibition.

CONCLUSION

Paper is a very fragile document and susceptible to attack by microbial and fungal growth. Hence, their preservation and storage is very important. In this regard, inks from the present study have shown that they are significant in inhibitory growth of certain microorganisms. In the present study five bacterial strains and two fungal strain were isolated from two different book samples collected from Ranchi City, Jharkhand (India). The strains which were isolated were identified as:

Bacterial Strains

Strain1 – Providentia stuartii (97% similarity); Strain2 – Serratia odorifera (88% similarity); Strain3 – Bacillus megaterium (80% similarity) ;Strain4 – Pseudomonas antimicrobica (85% similarity)

Fungal Strains

Aspergilllus niger Kavkler et al. in 2011 claim the fungi are the main cause for the degradation of cellulose and cellulose and cellulose containing items. Fungi apparently attack first the cuticle and then penetrate the lumen of the fibre degrading it from inside to out. In the present study, the effect of ink was studied by Agar well diffusion method and result of Gel pen ink of blue and black color shows maximum zone of inhibition against Strain1 – Providentia stuartii. Blue color of ball pen ink shows maximum zone of inhibition against Strain1 – Providentia stuartii and Strain2 – Serratia odorifera , in Strain 3 – Bacillus megaterium red colour ink showed maximum inhibition but in Strain 4 Pseudomonas antimicrobica black colour shows maximum zone of inhibition followed by green colour gel pen ink.In ball pen ink blue coloured ink showed maximum inhibition against S1, S2, S3 and green colour showed maximum inhibition against S 4. In pure printer ink no inhibitory effect was observed on microbes. 100% Microbial growth was observed on petriplates and zone of inhibition were completely absent. Against Aspergillus niger inhibitory effect of all colour ball pen ink and Gel pen ink shows maximum result and inhibitory effect of printer ink was also observed, whereas on bacteria inhibitory effect of pure printer ink was completely absent. On Fungi Aspergillus niger green colour printer ink showed maximum inhibition whereas Blue colour of gel pen ink and ball pen ink showed maximum inhibition followed by green coloured ink. In CFU, the quantitative analysis, maximum growth was observed in case of printer ink and in Pseudomonas strain that means very less inhibitory effect on microbial growth. Hence, based on the present study certain recommendations can be made on the type of ink that is most suitable for writing important documents such as wills which would have a storage value for a longer time. As these type of ink such as blue ball pen ink, green ink also tend to preserve the fibres of the paper cellulose. Care should also be taken that the strains of Pseudomonas should be clenched away from the document as and when possible.

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