

■ ORIGINAL ARTICLE

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

<sup>1</sup>Puja Mehta, <sup>2</sup>Sally Lukose, <sup>3</sup>Vandana Prasad

## 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

### Author's Credentials:

<sup>1</sup>Assistant Director, Toxicology Division, Forensic Science Laboratory, Patna, Bihar-800001, India, <sup>2</sup>Professor, <sup>3</sup>Assistant Professor, Forensic Department, School of Allied Health Sciences, Greater Noida, Uttar Pradesh-244001, India.

### Corresponding Author:

Vandana Prasad, Assistant Professor, Forensic Department, School of Allied Health Sciences, Greater Noida, Uttar Pradesh-244001, India.

### Email:

[pvdandanajhs@gmail.com](mailto:pvdandanajhs@gmail.com)



### How to cite this article

Puja Mehta. Isolation and Identification of Various Types of Microbes Present on Documents and the Inhibitory Effect of Ink on their Growth. *Indian J Forensic Med Pathol*.2021;14(3 Special):543-554.

## 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<sub>2</sub> 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.<sup>1,2</sup> Thus, hydrolysis process mainly involves three types of cellulase enzymes namely, carboxymethyl cellulase (CMCase), cellobiohydrolase and  $\beta$ -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.<sup>3,4,5</sup> 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.

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#### METHODS AND MATERIALS

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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<sup>-1</sup> to 10<sup>-3</sup> were done and the diluents of 10<sup>-3</sup> 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 with 10 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.<sup>2</sup> 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 37°C 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 37°C 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 37°C 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 H<sub>2</sub>O<sub>2</sub>. But H<sub>2</sub>O<sub>2</sub> is toxic for their enzymes. Therefore, to overcome this toxic effect, microorganisms are possessing catalase enzyme. These catalase enzymes convert H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub>.

#### **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.

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## RESULTS

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**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)

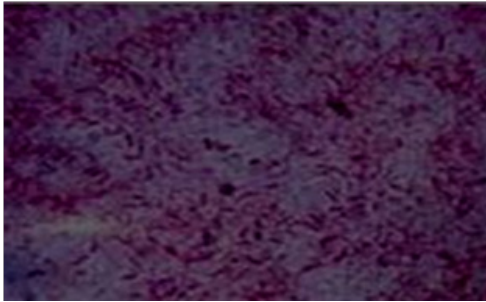


Figure 2(a): Strain 1

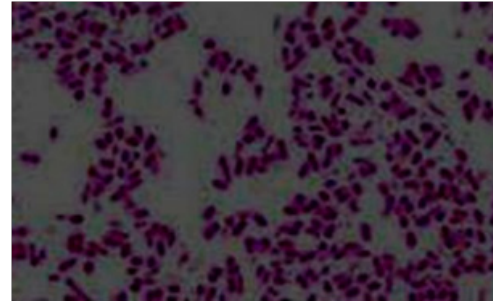


Figure 2(b): Strain 2



Figure 2(c): Strain 3



Figure 2(d): Strain 4

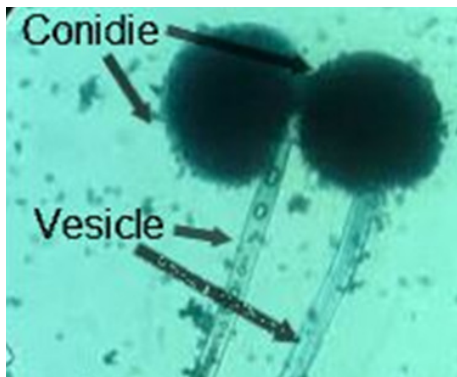


Figure 3: Microscopic view of Aspergillus Niger the entire surface

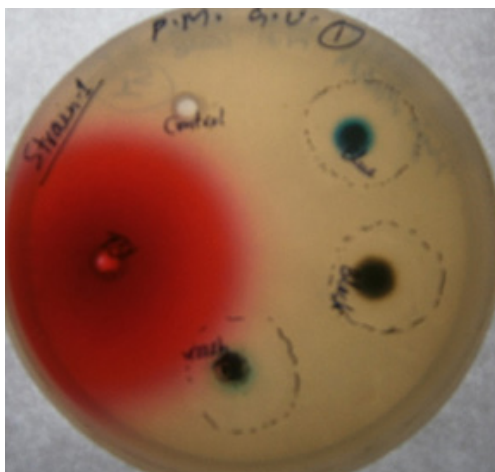


Figure 4(a): Effect of gel pen ink on strain1 well diffusion method

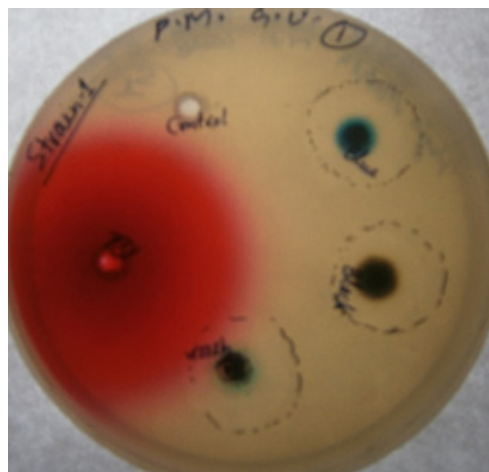


Figure 4(b): Effect of gel pen ink on strain 2 using agar-well diffusion method

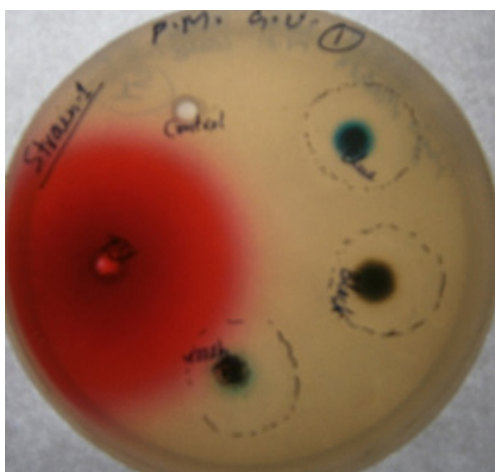


Figure 4(c): Effect of gel pen ink on stain3 using agar- well diffusion method

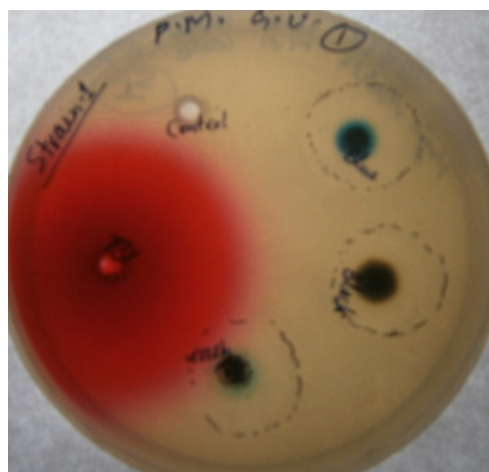


Figure 4(d): Effect of gel pen ink on strain 4 using agar -well diffusion method

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 conidia with transparent vesicle and conidiophores over.

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