THE ISOLATION AND CHARACTERISATION OF BACTERIA EXHIBITING DEINKING ACTIVITY FROM REGIONS IN SYLHET

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Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Abstract

The wastage of paper and ink is a major cause of pollution in Bangladesh. The ever-rising population demands an increased requirement for paper and ink that often end up occupying landfills or contributing to air pollution. However, the current methods of paper recycling involve paper de-inking, which is largely dependent on chemical means that further aggravates this problem. The objective of this research was to find an alternative biological solution to the chemical de-inking process. Bacteria capable of decolorizing ink were identified and isolated using media that contained ink as the only carbon source. Five samples of bacteria capable of efficient decolorization were found with decolorization percentages of 42.93%, 39.75%, 37.95%, 37.55%, and 30.02% for isolates RGS-1, SPS-1, RGS-2, SPS-2, and SPS-3, respectively. Isolates SPS-1, SPS-2, and SPS-3 also exhibited cellulolytic activity. Protein crystals from the bacterial culture supernatant were precipitated and purified using ammonium sulfate precipitation followed by snake-skin dialysis respectively, after which de-inking activity increased. The optimal de-inking was observed when extracellular and intracellular extracts were combined and added to ink; isolate SPS-2 showed a decolorization percentage of 70.75%. The bacteria provide an avenue of research for the sustainable recycling of paper, thereby alleviating the climate problem.

Keywords: De-inking, Effluent, Pollution, Paper industry

Dedication

This is dedicated to

our parents, partners, friends, and faculties

for their ever-lasting support and encouragement.

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List of Acronyms

BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
B&H	Bushnell & Haas
OD	Optical Density
VBNC	Viable But Non-Culturable
NA	Nutrient Agar
SS	Salmonella Shigella
TCBS	Thiosulfate Citrate Bile Salts-Sucrose
MIU	Motility Indole Urea
TSI	Triple Sugar Iron
XLD	Xylose Lysine Deoxycholate
EMB	Eosin Methylene Blue
CMC	Carboxymethylcellulose
MSA	Mannitol Salt Agar

Chapter 1

Introduction

1.1 Brief Overview of Ink and Paper Pollution in Bangladesh

Recycling waste paper is an important procedure to address the alarming levels of pollution and alleviate climate change due to rapidly depleting forests. A method closely associated with paper recycling is the process of de-inking which removes ink from the paper. The chemical process of de-inking paper involves the release of toxic materials such as formaldehyde, heavy metals, benzene, fine particulate matter, etc. (Chen et al., 2012). These materials contribute to air pollution and harm the environment and human health alike; additionally, effluent from printing industries adds to water pollution and harms aquatic life (Mia et al., 2019). While this is a global issue, the environmental impact that ink pollution has on Bangladesh remains a relatively undiscussed topic. Research showed that Karnaphuli Paper Mills, the oldest and largest paper manufacturing plant in Bangladesh, has been consistently dumping liquid waste, consisting of residual printing ink, into the Karnaphuli river, leading to its pollution (Ahmed, 2013; Nayeem, Majumdar & Hossain, 2018). Another study at Fakirapool, Dhaka, demonstrated that 6.82 kg of colored waste, i.e.- ink, was generated per month in a printing plant (Tabassum et al., 2017). From the limited data available in Bangladesh's context, it can be concluded that ink and paper waste leads to high levels of pollution in the country.

1.2 Chemical Means of Degrading Ink

Paper recycling, after collection and sorting, primarily involves shredding, pulping, and repulping the paper; this is followed by de-inking and drying the pulp to form sheets of recycled paper (van Velzen et al., 2021; Chaudhary, 2022). Pulping is the primary step of the entire operation; it disintegrates paper into separate, dispersed fibers and detaches ink from them. The fibers are firstly disaggregated by mechanical forces, aided by chemicals such as surfactants, hydrogen peroxide, sodium hydroxide, sodium silicate, sodium silicate, sodium carbonate, etc. (van Velzen et al., 2021; Borchardt, Miller & Azevedo, 1998; Shankar et al., 2018). After pulping, the dispersed ink particles have to be removed to prevent their redeposition onto paper (Borchardt, Miller & Azevedo, 1998). The processed pulp is then de-inked either by wash de-inking or more commonly by flotation de-inking. In conventional de-inking, chemicals such as sodium carbonate, magnesium sulfate, sodium hydroxide,

sodium silicate, hydrogen peroxide, chelating agents, surfactants, etc. are used. Sodium hydroxide is used as an alkaline agent that enhances cellulose fiber swelling, which reduces ink adhesion to fiber. Hydrogen peroxide can lead to an increase in brightness, while sodium silicate reduces the amount of ink in recycled paper, thereby, improving the whiteness of the paper (Biswas et al., 2019). Surfactants detach ink from the cellulose fiber by interacting with the ink (van Velzen et al., 2021; Borchardt, Miller & Azevedo, 1998; Biswas et al., 2019). However, the use of chemicals can affect the quality of paper, as well as confer detrimental effects on the environment (Singh et al., 2019). The use of alkaline chemicals increases the BOD and COD of the effluent, making treatment of the effluent expensive (Shankar et al., 2018; Singh et al., 2019).

1.3 Current Biological Methods to Degrade Ink

In recent years, biological de-inking of used paper has been emerging as a preferred treatment for recycling in the paper industries. Different biological methods of ink degradation have been employed to improve the efficiency of the procedure. Among these processes, the most common methods of biological de-inking either include the use of microbial cell cultures, cell-free culture supernatant, or a direct mixture of enzymes like lipase, pectinase, esterase, etc. (Bajpai, 1997). In a study conducted by Mohandass and Raghukumar (2005), both free and immobilized cells of the bacterium, *Vibrio alginolyticus*, isolated from marine sediments, were used to decolorize ink from inkjet-printed paper pulp.

Enzymatic de-inking is another sustainable alternative to traditional de-inking where specific chemicals are used to decolorize paper. It is cheaper, more effective, and more efficient compared to the chemical de-inking of paper (Singh et al., 2019). An enzyme mixture is used as different enzymes have varied specific functions, and this leads to a high synergistic output that ensures maximum decolorization (Bajpai, 1997). Commonly used enzymes are lipases, esterases, pectinases, hemicellulases, cellulases, ligninolytic enzymes (Bajpai, 1997; World Paper Mill, 2021), ∝-amylase (Shankar et al., 2018), laccase (Biswas et al., 2019), xylanase (Singh et al., 2019), pectinase, etc.

1.4 Enzymes in De-inking

Enzymes alter the surface of the fiber and bonds of the ink particles, allowing the removal of cartridge ink from paper - after which the ink particles are removed by washing or flotation. The most widely suggested mechanism of enzymatic de-inking is the removal of the surface layer of cellulose from fibers by the combination of enzymatic hydrolysis and shear forces (Shankar et al., 2018). The enzymes detach ink particles from fiber by partial hydrolysis of cellulose fibers at the fiber/ink inter-bonding regions, which allows ink to be detached (Lee et al., 2011). In particular, cellulase and hemicellulase enzymes can remove fines and microfibrils, which free pulp and facilitate ink removal by washing or flotation (Shankar et al., 2018).

Enzymatic de-inking reduces the number of chemicals and energy needed in addition to reducing waste for paper recycling (Biswas et al., 2019). It avoids the use of chemicals as well as the alkaline environment typically employed in chemical de-inking, ultimately reducing the COD content of the effluent. This lowers the expense for the treatment of the effluent and also results in decreased environmental pollution. Compared to chemical de-inking, enzymatic treatment of paper pulp results in less residual ink and improved physical and optical properties (Singh et al., 2019), as improved brightness, tensile strength, and cleanliness of pulp (Lee et al., 2011).

1.5 Objectives of Research and Research Gaps

The main aim of this study is to find a sustainable alternative to conventional chemical de-inking. This process focuses on using microbial culture from environmental samples to isolate organisms that can degrade ink. As tackling pollution is one of the biggest challenges in the country, the study was carried out to minimize the addition of further pollutants in the environment, which occurs during chemical de-inking (World Paper Mill, 2021).

In contrast, biological de-inking employs the use of microorganisms that can be easily mass-produced, making it more environmentally and economically feasible (Timmis et al., 2017). Furthermore, the process comparatively does not produce any harmful by-products, hence keeping the environment safe.

Currently, the use of bacteria for bioremediation purposes is increasing globally due to its many environmental and economical benefits (EmergenResearch, 2020). However, there is

still a lack of research that focuses on the full extent of using bacteria for de-inking purposes - for example, the exact metabolic process of separating ink from paper is yet to be elucidated. This is imperative to shed light on increasing the efficiency of the de-inking process and making it economically viable. More so, Bangladesh has a severe lack of accurate, recent data regarding levels of pollution in the country. Extensive research is required to assess the levels of pollution so that biological means of de-inking can be feasibly applied to alleviate the pollution problem.

In this study, bacterial cultures isolated from regions of Sylhet were hypothesized to be able to decolorize ink from paper effectively, thereby proving to be a useful avenue of research for the sustainable development of the paper industry in Bangladesh.

Chapter 2

Materials and Methods

2.1 Collection of samples

Environmental samples were collected from Sylhet in the form of soil, water, and mud. The samples from Sylhet were collected from Lalakhal (water), Sada Pathor (water and soil), and Ratargul (mud). Afterward, the samples were put into centrifuge tubes, labeled according to the location of extraction, stored at 4°C, and sent to the laboratory as soon as possible.

2.2 Sample processing

0.9% normal NaCl was prepared and sterilized. The environmental samples were then placed into the prepared test tubes and serially diluted up to a dilution of 10^{-5}.

2.3 Ink Extraction

The ink reservoir was taken out of the pen's barrel and cut open using a pair of scissors and washed out using a syringe filled with autoclaved distilled water. To prepare ink each time, 6 reservoirs were used for every 10 ml of distilled water. The ink used was Matador i-teen gel in the color black. The cartridge ink used was Canon Laser Print in the colour black.

2.4 Inoculation into B&H Agar plates

2.4.1 Spread Plate

Inside the biosafety cabinet under aseptic conditions, 100 µl of the serially diluted bacterial suspensions were dispensed onto B&H agar plates using autoclaved pipette tips. The bacterial suspension was inoculated on the media using the spread plate method (Aryal et al., 2022) using a glass spreader. The inoculated plates were incubated at 37°C until bacterial colonies exhibited growth on the media.

2.4.2 Streaking and Subculturing

After bacteria exhibited growth on B&H media containing ink, colonies with distinct, regular morphology were taken from each spread-plated sample. The chosen colonies were subcultured by streaking (Tankeshwar, 2022) onto B&H media containing 1% ink (v/v) and then incubated at 37°C for 48-72 hours. The bacteria were subcultured every week for 7 weeks to enrich the bacterial cultures and prevent them from returning to VBNC state.

2.4.3 Isolation of Single Colonies of Bacteria

Once the bacteria in each sample plate exhibited strong growth, each strain was taken and streaked onto B&H media plate containing 1% (v/v) ink. This was done to isolate the single colonies of the bacteria which were later used to inoculate the B&H broth.

2.5 Inoculation into B&H broth

2.5.1 Inoculation of Bacteria in B&H Broth

B&H broth was prepared and ink was added such that the concentrations of the ink in the broth were gradually increased during each subculture. Initially, 0.3% (v/v) B&H broth was prepared and 2-3 colonies of bacteria were inoculated into it. These were allowed to incubate for 144 hours at 37°C, after which optical density was measured. This process was repeated for broth concentrations of 0.6% (v/v), 0.8% (v/v), and 1.0% (v/v). Each concentration also had a blank prepared which contained ink of the same concentration and uninoculated B&H broth of the same volume.

2.5.2 Measuring the Optical Density of Broth

The initial optical density of the broth was measured immediately after inoculation. After the 7 days incubation period, the final optical density of the inoculated broth was measured. The absorbance reading for each sample was taken at a wavelength of 605 nanometers (Morais et al., 2020). The readings were all tabulated and compared. The optical density was used to calculate the decolorization percentage by using the formula (Shah et al., 2021; Varghese & Sebastian, 2015):

$$decolorization\ percentage = \frac{initial\ OD - final\ OD}{initial\ OD} \times 100$$

2.6 Culture Supernatant

2.6.1 Crude Culture Supernatant Extraction

Thirty milliliter of subcultured broth was taken into two centrifuge tubes. The tubes were balanced and centrifuged at 10,500 rpm at 4°C for 30 minutes. This ensured the bacteria and ink were sedimented at the bottom in the form of a pellet. The supernatant was then collected in a fresh centrifuge tube using a pipette to avoid

disrupting the pellet. This process was repeated for every sample in each set of subcultures.

2.6.2 Measuring Optical Density of Culture Supernatant

Two milliliters of crude culture supernatant and 1 ml of 1% (v/v) ink were put into autoclaved centrifuge tubes and incubated at 37°C in a shaker incubator for 7 days. The optical density before and after the incubation was recorded. The decolorization percentage was calculated using the formula (Shah et al., 2021; Varghese & Sebastian, 2015):

$$decolorization\ percentage = \frac{initial\ OD - final\ OD}{initial\ OD}\ x\ 100$$

2.7 Paper decolorization in broth

Bacteria were inoculated in 5 ml of B&H broth in test tubes. Square sections of paper with inkjet cartridge ink, measuring 1 cm by 2 cm, were placed in the test tubes. The tubes were sealed and labeled accordingly, then incubated in a shaker incubator at 37°C for 7 days.

2.8 Biochemical Testing for Putative Identification

All media for the biochemical tests were prepared following the manufacturer's instructions. All the media, except for SS agar, XLD agar, and TCBS agar, were autoclaved at 121℃ for 15 minutes for sterilization. The media were inoculated under a laminar hood. Before inoculation into biochemical media, the bacteria were incubated in Nutrient Agar for 24 hours. The tests were triplicated.

The biochemical tests were as follows:

2.8.1 Methyl Red Test

MRVP or glucose phosphate broth was prepared in test tubes and autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The cultures were incubated for 48 hours at 37 °C. After the incubation period, 1 drop per 1 ml MR reagent was added to inspect for color change.

2.8.2 Voges Proskauer Test

MRVP or glucose phosphate broth was prepared in test tubes and autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The

cultures were incubated for 48 hours at 37 °C. After the incubation period, alpha-naphthol and potassium hydroxide were added in a ratio of 3:1 and allowed to stand for 15 minutes to inspect for color change.

2.8.3 Citrate Utilisation Test

Simmon's citrate agar was dispensed into small vials and autoclaved, after which slants were created. The agar was inoculated by stabbing bacterial culture and then by streaking the surface of the agar slant. The cultures were incubated at 37°C for 48 hours. Bacterial growth was observed by inspecting for color change of media.

2.8.4 Indole Test

Peptone water containing the amino acid tryptophan was prepared in test tubes and autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The cultures were incubated for 48 hours at 37°C. Following incubation, a few drops of Kovac's reagent were added and then inspected for ring formation.

2.8.5 Nitrate Reduction Test

Nitrate broth was prepared in test tubes and autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The cultures were incubated for 24 hours at 37 °C. Following incubation, nitrite reagent A and reagent B were added in a ratio of 1:1 and inspected for color change.

2.8.6 Catalase Test

A loopful of sample culture was placed on a glass slide and 1 drop of 3% hydrogen peroxide was added; the slides were observed for immediate effervescence.

2.8.7 Oxidase Test

Whatman's filter paper was soaked in a freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride and dried. The paper was then placed in a petri dish and moistened with distilled water. The bacterial colony to be tested was picked up with an autoclaved cotton swab and smeared over the moist area and checked for the development of an intense purple hue within 15 minutes.

2.8.8 Starch Hydrolysis Test

Bacteria were streaked on the surface of the Starch agar using an aseptic technique under a laminar hood and incubated at 37℃ for 48 hours. After incubation, the plates were flooded with Gram's iodine and immediately checked for the development of zones.

2.8.9 SS Agar Test

SS agar plates were prepared and streaked with bacterial cultures and incubated at 37 ℃ for 48 hours. The plates were then checked for the growth of bacteria.

2.8.10 MSA

Bacteria were streaked onto sterile MSA agar plates under a laminar hood using an aseptic technique and incubated at 37 ℃ for 24 hours, and inspected for change in color of media and growth of bacterial colonies.

2.8.11 TCBS

Bacteria were streaked onto sterile TCBS agar plates under a laminar hood using an aseptic technique and incubated at 37 ℃ for 24 hours, and inspected for change in color of media and growth of bacterial colonies.

2.8.12 Cetrimide

Bacterial culture was streaked to get isolated colonies using an aseptic technique onto Cetrimide agar plates under a laminar hood and incubated at 37 ℃ for 24 hours, and inspected for growth of bacterial colonies and to check for color changes.

2.8.13 XLD

Bacteria were streaked onto sterile XLD agar plates under a laminar hood using an aseptic technique and incubated at 37 ℃ for 24 hours, and inspected for change in the color of media and growth.

2.8.14 Bile Esculin

Bile esculin agar was prepared in test tubes and autoclaved, after which slants were created in the test tubes before the media solidified. The slant was inoculated by stabbing with 1-2 colonies. The cultures were incubated for 48 hours at 37 °C. Next, growth was observed by inspecting for blackening of media.

2.8.15 MIU

MIU agar was prepared in test tubes and autoclaved. After cooling, 40% urea solution was added to the media and mixed well, and allowed to solidify in an upright position. The agar was inoculated by stabbing straight in the middle with 1-2 colonies. The cultures were incubated for 24 hours at 37 °C. After which, growth was observed by inspecting for color change of media.

2.8.16 TSI

TSI agar was prepared in test tubes and autoclaved, after which slants were created in the test tubes before the media solidified. The slant was first inoculated by stabbing with 1-2 colonies through the center and then by streaking the surface of the agar slant. The cultures were incubated for 24 hours at 37 °C. Next, growth was observed by inspecting for color change of media.

2.8.17 EMB

Bacteria were streaked onto sterile EMB agar plates under a laminar hood using an aseptic technique and incubated at 37 ℃ for 24 hours, and inspected for change in color of media and growth of bacterial colonies.

2.8.18 UTI Hi-Chrome Test

Bacteria were streaked onto sterile Hi-chrome UTI agar plates under a laminar hood using an aseptic technique and incubated at 37 ℃ for 24 hours, and inspected for growth of bacteria.

2.8.19 Gram Staining

A clean slide was used to prepare the smear of suspension using a loopful of the bacterial culture. The slide was air dried and heat fixed; next, crystal violet was poured and kept for about 30 seconds and then rinsed with water. The slide was flooded with the gram's iodine and kept for 1 minute before washing it with water. Then, 95% alcohol was used to wash the slide for 10-20 seconds and again rinsed with water. Lastly, safranin was added for about 1 minute and washed with water. The slide was then air-dried and observed under a microscope at x400 magnification under immersion oil.

2.8.20 Phenol Red Test

A. **Sucrose** - Phenol red sucrose broth was dispensed into test tubes containing Durham tubes and then autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The cultures were incubated for 24 hours at 37℃ and then inspected for acid and gas production.
B. **Lactose** - Phenol red lactose broth was dispensed into test tubes containing Durham tubes and then autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The cultures were incubated for 24 hours at 37℃ and then inspected for acid and gas production.
C. **Glucose** - Phenol red glucose broth was dispensed into test tubes containing Durham tubes and then autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The cultures were incubated for 24 hours at 37℃ and then inspected for acid and gas production.
D. **Maltose** - Phenol red maltose broth was dispensed into test tubes containing Durham tubes and then autoclaved. A loopful of bacterial culture was inoculated into the broth under the laminar hood. The cultures were incubated for 24 hours at 37℃ and then inspected for acid and gas production.

2.9 Inoculation onto CMC and Agar media

The bacterial isolates were inoculated into CMC agar, as well as plates only containing agar and salt. This was done to check for cellulolytic and agarolytic properties respectively. The zones of hydrolysis on CMC and agar, visualized after flooding both sets of plates with iodine, were recorded.

Next, the isolates that showed growth on CMC agar were inoculated into CMC broth to check for the ability to use cellulose as a sole carbon source, without the presence of agar.

2.10 Change in pH

To check if the ink was capable of acting as an indicator, HCl and NaOH solutions were alternatively added to the ink. Furthermore, the pH of the inoculated broth before the 48-hour incubation period and after was also recorded.

2.11 Ammonium Sulfate Precipitation of Culture Supernatant

The isolate SPS-2 was incubated in 300 ml of B&H broth with 1% (v/v) ink for 7 days. Upon decolorization, 100 ml of the broth was centrifuged to collect the culture supernatant. The supernatant was then transferred to a Duran bottle and 100 g of ammonium sulfate was added in small increments and gently stirred to dissolve. This was done for 8 hours. The solution was then left at 4°C overnight.

2.12 Snakeskin Dialysis

From the ammonium sulfate precipitation set-up, the aqueous layer, along with the crystals of hypothesized protein, were extracted using a pipette and collected into a centrifuge tube. This was then centrifuged at 100,000 rpm, 4℃, for 30 minutes. The salt and crystals that were deposited at the bottom were collected. A snakeskin membrane with a pore size of 16 mm inner diameter and a molecular weight cutoff of 10 K was taken, and the layer of salt and crystal was inserted into the membrane which was knotted at both ends. The tied membrane was then put into a 2 L beaker. Additionally, a 60-times concentrated B&H salt solution was prepared as the dialysis buffer. This was added to the beaker in increments of 20 ml, followed by the addition of 1200 ml of autoclaved distilled water to the beaker. The beaker was placed on top of a magnetic stirrer machine and rotation was allowed for 1.5 hours. The dialysis buffer was then discarded. The buffer was changed in this way 5 times, after which the beaker was kept overnight at 4℃.

2.13 Concentrated Culture Supernatant Activity

Two milliliters of the concentrated, purified culture supernatant was incubated with 1 ml of ink for 48 hours, and the initial and final optical densities were measured.

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2.14 Characterization of Culture Supernatant

2.14.1 Supernatant Extraction from Lysed Cells

The isolate SPS-2 was grown in B&H by the spread plate method. After the exhibition of heavy growth, the bacteria were collected into a microcentrifuge tube and 1 ml of distilled water. The cells were then lysed using a sonicator (Burden & Whitney, 1995) it was sonicated at 30 kHz for 10 seconds, after which the microcentrifuge tube was plunged into ice (Feliu & Villaverde, 1994). This was repeated 4 times. The tube was then centrifuged at 4℃, 7500 rpm for 10 minutes. The supernatant was then collected, and the pellet was discarded. 1% (v/v) ink was added to this, and the control was set up with distilled water instead of lysed bacterial cells. These were allowed to incubate for a week at 37℃. Their optical densities were then measured.

2.14.2 Lysed Cell Supernatant and Precipitated Culture Supernatant

One milliliter of the precipitated culture supernatant was added to the supernatant from lysed cells and allowed to incubate for 72 hours. Their optical densities before and after inoculation were measured.

2.15 Process Optimization

Fifty milliliters of B&H broth containing 1% (v/v) ink was inoculated with isolate SPS-2 and incubated at 25℃, 37℃, and 42℃ for 7 days at pH 7. Additionally, B&H broth of pH 5, 6, 7, 8, and 9 was inoculated and kept in the shaker incubator at 37℃ for 144 hours. Their optical densities before and after inoculation were recorded.

Chapter 3

Results

To conduct this research, different environmental samples were used to isolate nine different colonies with a distinct morphology. The isolates were then streaked in B&H salt agar plates containing ink as the sole carbon source. Initially, the growth of each isolate was checked to determine their ink degradation activity. The ink degradation activity was calculated by measuring the change in optical density for each isolate. It was concluded that the higher the percentage of decolorization, the greater the ink degradation activity. Isolates RGS-1, SPS-1, RGS-2, SPS-2, and SPS-3 showed the highest decolorization percentage and so were selected for further experimentation. Isolate SPS-2 exhibited the highest ink degradation activity, so was precipitated using ammonium sulfate, followed by snakeskin dialysis to obtain purified, concentrated culture supernatant. Live cell culture of isolate SPS-2 was sonicated and the bacterial lysate was mixed with concentrated supernatant. The decolorization percentage of this mixture was checked using 1% ink (v/v). The optimum pH and temperature for ink degradation were also checked using this isolate.

3.1 Growth on B&H Agar

3.1.1 Growth of environmental samples collected from different parts of Sylhet

fig 1(a)

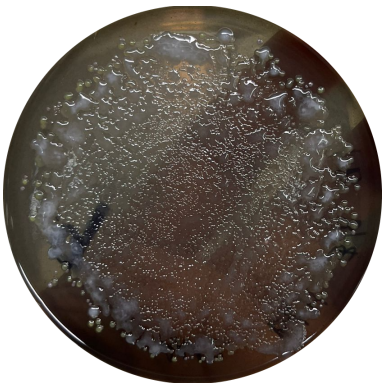

fig 1(b)

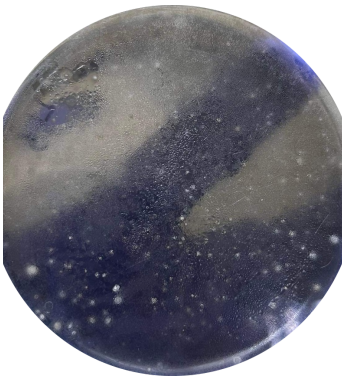

fig 1(c)

The figures 1(a) to 1(c) show the results of spread plating the processed environmental samples onto B&H media. Each plate contained samples from a different location. Figure 1(a) shows the sample from Shada Pathor soil processed in 0.9% NaCl, while figure 1(b) shows colonies from Shada Pathor water. Figure 1(c) shows colonies from Ratargul soil, which had visible signs of fungal contamination.

3.1.2 Streaking bacterial culture from the colony with different morphologies

fig 2(a)

fig 2(b)

fig 2(c)

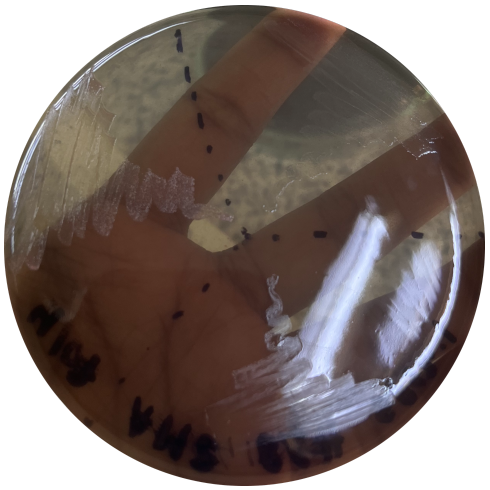

fig 2(d)

The figures 2(a) to 2(d) show the streaking of morphologically variant bacterial colonies selected from the spread plates according to the origin of the environmental samples. Figure 2(d) shows samples selected from Ratargul soil - since the spread plate contained fungal contamination, bacterial colonies were carefully selected and spread based on their morphology. Figures 2(a) to 2(c) show colonies streaked from Shada Pathor soil and Shada Pathor water. These samples were subcultured 6 times to bring bacteria out from the VBNC state and strengthen the strains.

3.1.3 Isolation of single colonies

fig: 3

Figure 3 shows the isolation of single colonies from the streaked plates in figures 2(a) - 2(d). The colonies with identical morphology were discarded.

3.2 B&H Broth

3.2.1 Inoculation into B&H Broth

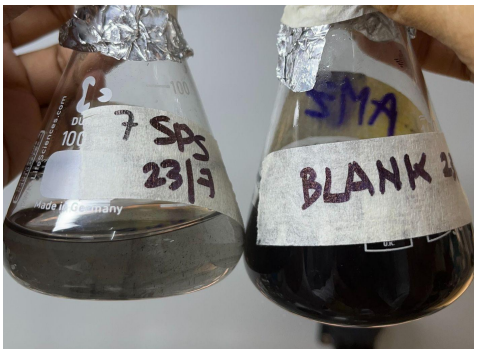

fig: 4(a)

fig 4(b)

fig: 4(c)

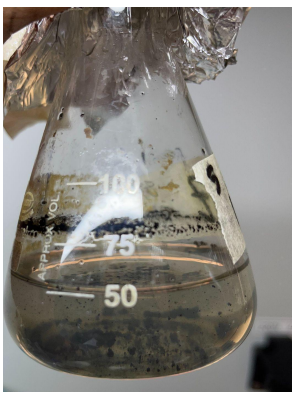

fig: 4(d)

Figures 4(a) to 4(d) show the decolorization of isolate SPS-2 in ink concentrations of 0.3% (v/v), 0.6% (v/v), 0.8% (v/v), and 1.0% (v/v) respectively.

3.2.2 Percentage of Decolorization by Various Soil Samples

Table 1: Percentage of decolorization of 5 bacterial isolates

Source of isolate	Isolate	Percentage Decolorization (%)					
		Ink concentration (0.3% v/v)		Ink concentration (0.6% v/v)		Ink concentration (0.8% v/v)	Ink concentration (1.0% v/v)
		1	2	1	2		
Ratargul Soil	RGS-1	14.90	37.28	24.08	20.64	37.67	42.93
Shada Pathor Water	SPS-1	43.87	49.94	27.13	36.05	37.11	39.75
Ratargul Soil	RGS-2	16.81	28.76	23.47	30.65	45.38	37.95
Shada Pathor Water	SPS-2	63.74	70.89	56.70	59.43	40.40	37.55
Shada Pathor Water	SPS-3	62.37	72.54	46.93	52.61	50.84	30.02

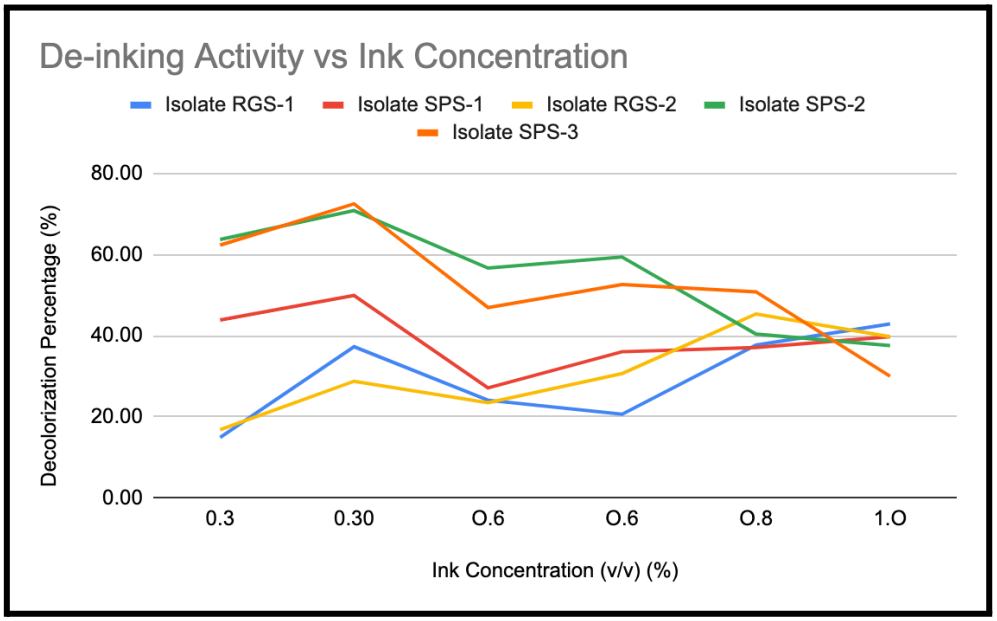

Fig 5: Relationship between the decolorization percentage against the ink concentration

Figure 5 shows the relationship between the de-inking activity of each isolate against an increasing (v/v) ink concentration. The isolates SPS-2 and SPS-3 showed the highest de-inking activity as they showed the highest decolorization.

The optical densities were measured at 605 nm and were tabulated as shown in table 1 according to the formula (Shah et al., 2021; Varghese & Sebastian, 2015):

$$decolorization\ percentage = \frac{initial\ OD - final\ OD}{initial\ OD}\ x\ 100$$

Isolate SPS-2 showed the most efficient decolorization, so was chosen as the optimal bacteria to work with.

3.2.3 De-inking Activity at 0.3% (v/v) Ink

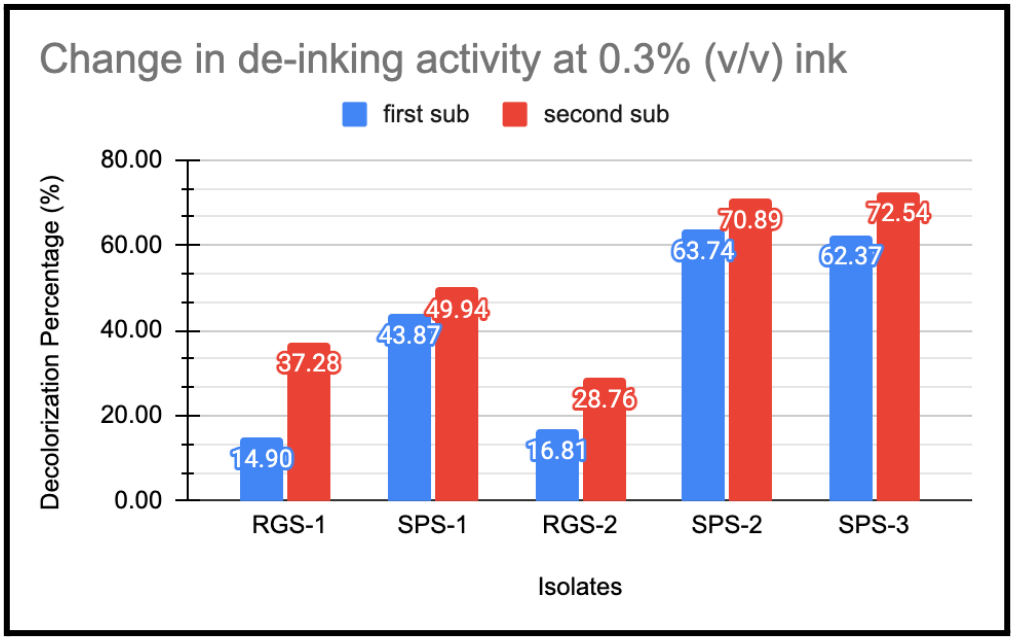

Fig 6: Change in de-inking activity for ink concentration 0.3% (v/v) between subcultures

The isolates demonstrated increased de-inking activity in the second subculture at ink concentration 0.3% (v/v) compared to the first subculture. Isolates SPS-2 and SPS-3 had the highest activity compared to all the samples with increased activity in the second subculture. Isolate RGS-1 had significantly higher activity in the second subculture than in the first.

3.2.4 Change in De-inking Activity at 0.6% (v/v) Ink

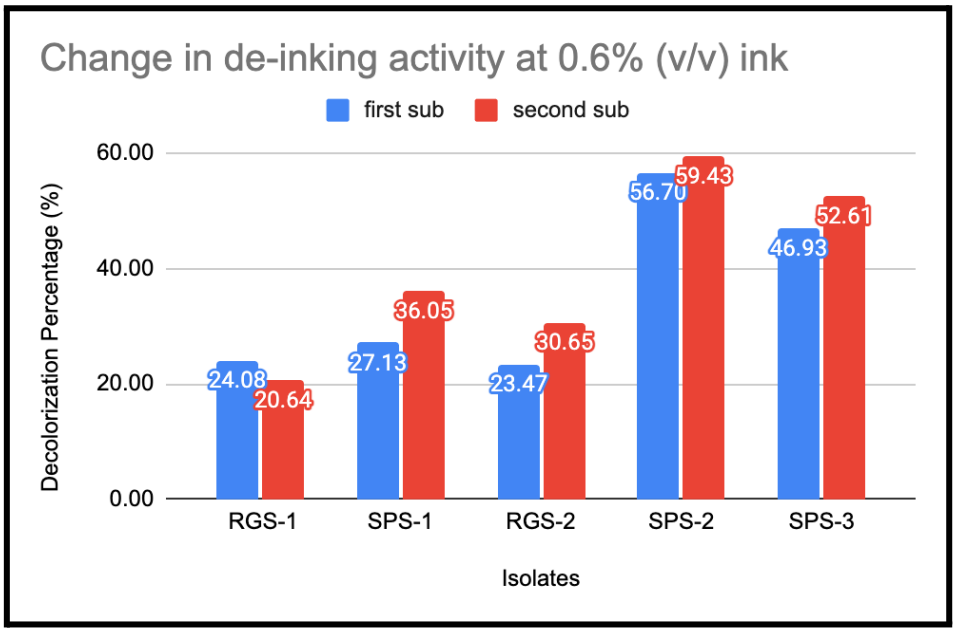

Fig 7: Change in de-inking activity for ink concentration 0.6% (v/v) between subcultures

There was an increase in decolorization percentage in the second subculture from the first subculture. The de-inking activity in all four isolates showed an increase except for isolate RGS-1 which had a slight decrease in activity. Isolate SPS-2 showed the highest activity in both subcultures at this ink concentration.

3.3 Culture Supernatant

3.3.1 Extraction of Culture Supernatant

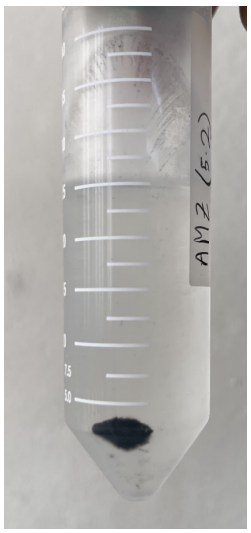

fig 8: Pellet containing residual ink and cells in culture supernatant after centrifugation

The figure 8 shows 25 ml of the culture supernatant that was collected; the pellet of residual ink and cells was discarded.

3.3.2 Culture supernatant showing decolorization activity

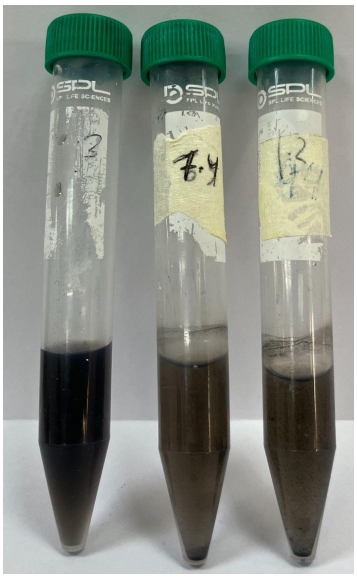

fig 9: Ink in the culture supernatant

The figure 9 shows the decolorization of ink using culture supernatant collected from isolate SPS-2 next to a control.

Table 2: Percentage of decolorization of culture supernatant

Isolate	Decolorization Percentage (%)
SPS-2	40.23
SPS-3	24.84

The optical densities for two of the most efficient isolates' culture supernatant decolorization is shown in table 2.

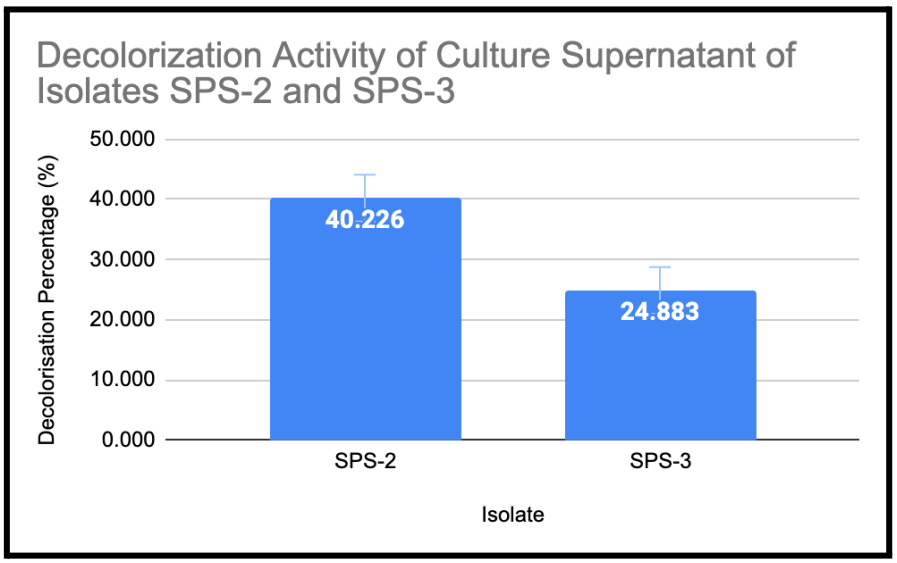

Fig 10: The relationship between the de-inking activity of isolates SPS-2 and SPS-3

The culture supernatant from isolates SPS-2 and SPS-3 showed 40% and 24% decolorization respectively. Isolate SPS-2 demonstrated higher de-inking capacity than isolate SPS-3, which aligns with culture de-inking at concentrations 0.3% (v/v), 0.6% (v/v), and 0.8% (v/v).

3.4 Activity on Paper and Further Justification

3.4.1 Printed paper

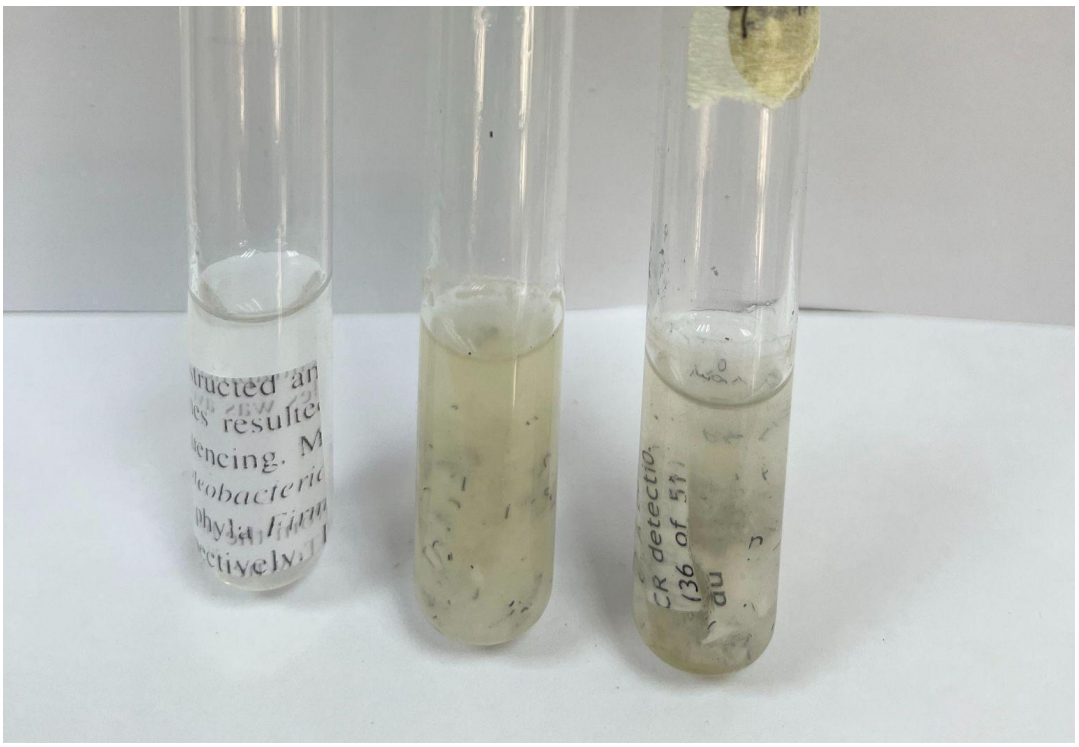

fig 11(a): (from the left- control; isolate SPS-2; isolate SPS-3)

fig: 11(b)

fig: 11(c)

After a small piece of paper with cartridge ink was inserted into inoculated B&H broth, the paper was digested and the ink was decolorized as shown in figures 11(b) and 11(c). Figure 11(a) shows the paper with cartridge ink before and after digestion. Isolates SPS-1, SPS-2, and SPS-3 were found to digest paper and decolorize ink simultaneously.

3.4.2 Screening of Cellulolytic and Agarolytic Bacteria

(i) Cellulolytic Zones

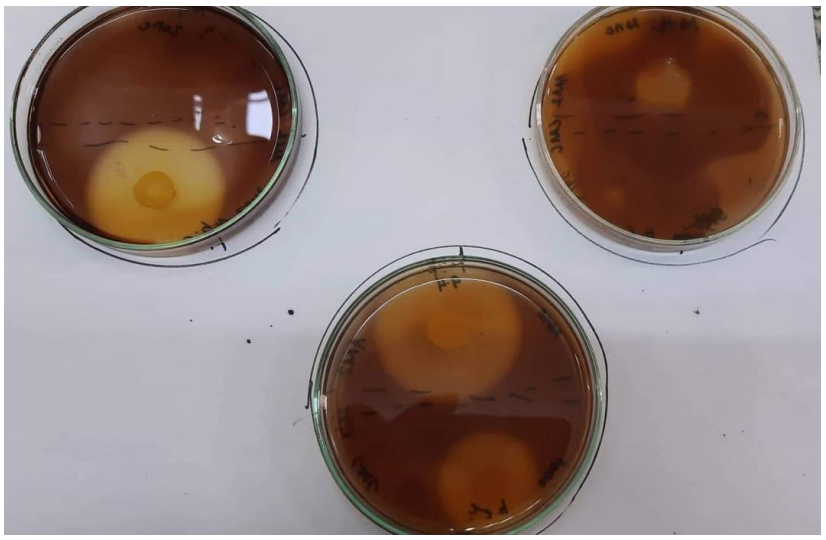

fig: 12

The figure shows cellulolytic zones around isolates SPS-1, SPS-2, and SPS-3.

(ii) Agarolytic Zones

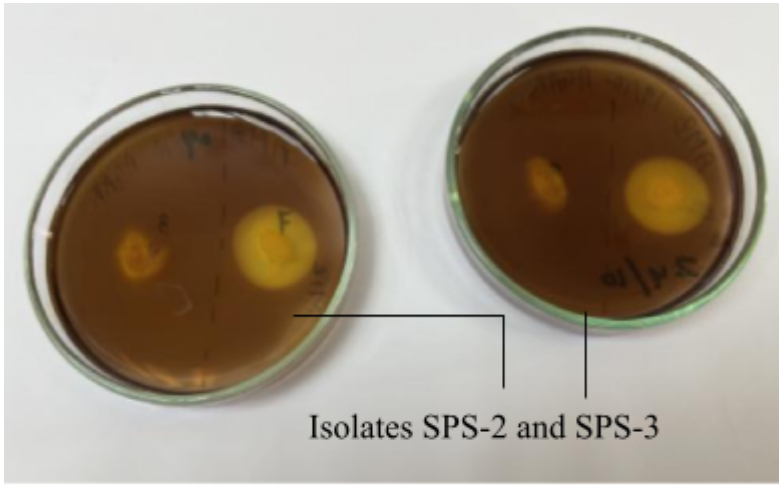

fig: 13

Figure 13 shows isolates SPS-2 and SPS-3, next to positive and negative controls for agarolytic bacteria. Isolate SPS-2 shows zones around agar, proving bacteria to be agarolytic.

(iii) **CMC Broth**

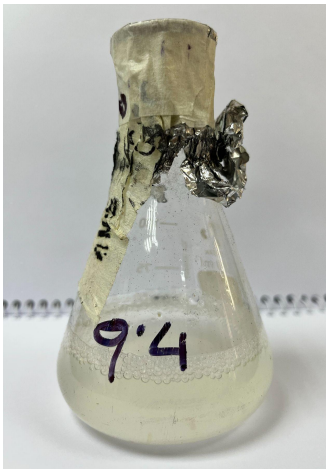

fig 14(a): Isolate SPS-3

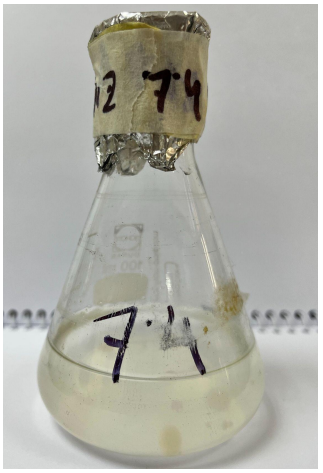

fig 14(b): Isolate SPS-2

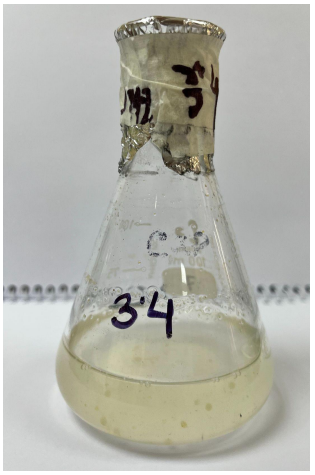

fig 14(c): Isolate SPS-1

The figures 14(a) to (c) show all three isolates SPS-1, SPS-2, and SPS-3 are able to digest cellulose and so are cellulolytic, shown by small masses of bacterial colonies floating as well as gas production in figure 14(a).

3.5 Biochemical Classification

3.5.1 Gram Staining

Table 3: Gram Stains

Isolate	Observation
RGS-1	Gram positive, cocci in clusters
SPS-1	Gram Positive, cocci
RGS-2	Gram Positive cocci
SPS-2	Gram Positive cocci
SPS-3	Gram Negative bacilli

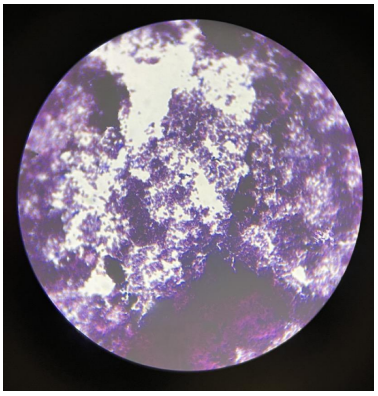

fig 15(a) Isolate RGS-1

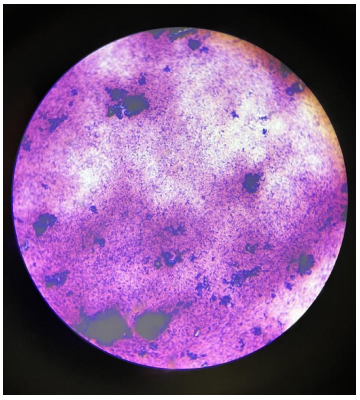

fig 15(b) Isolate SPS-1

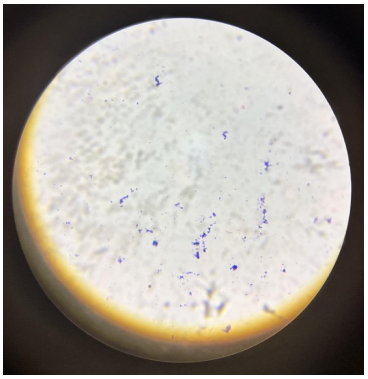

fig 15(c) Isolate SPS-2

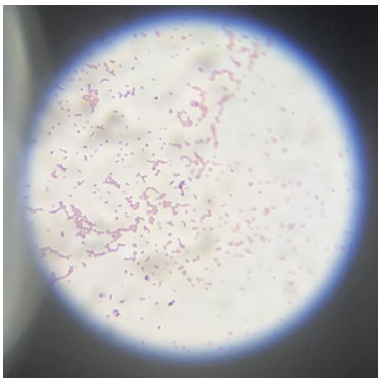

fig 15(d) Isolate SPS-3

3.5.2 Biochemical tests

Table 4: List of biochemical tests in test tubes

Isolate	MR	VP	Indole	Citrate	NRT	Motility	Oxidase	Catalase	Bile Esculin	MIU	TSI
RGS-1	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	M+; U-	No Gas production
SPS-1	-ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	M+; U+	Gas production
RGS-2	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	M+; U-	No Gas production
SPS-2	+ve	+ve	-ve	-ve	+ve (zinc)	+ve	-ve	-ve	+ve	M+; U-	No Gas production
SPS-3	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	M+; U-	Gas production

Table 5: List of biochemical tests in petri dish

Isolate	Starch	SS	MSA	TCBS	Cetrimide	XLD	EMB	Hi-Chrome UTI
RGS-1	No Zone	pink mucoid	no growth	no growth	no growth	no growth	Pink growth	light blue
SPS-1	No Zone	pink mucoid	Yellow glow	Yellow glow	white, opaque	Red center, yellow sides	Purple growth	navy blue
RGS-2	No Zone	bright pink	no growth	no growth	no growth	no growth	Pink growth	light blue
SPS-2	No Zone	bright pink	no growth	no growth	no growth	yellow colonies	Purple growth	blue
SPS-3	No Zone	pink mucoid	Yellow glow	Yellow glow	white, opaque	Red center, yellow sides	Pink growth	navy blue

Table 6: List of phenol red biochemical tests; A = Acid, G = Gas

Isolate	Sucrose	Lactose	Glucose	Maltose
RGS-1	A- ; G-	A- ; G-	A- ; G-	A- ; G-
SPS-1	A+ ; G+	A+ ; G+	A+ ; G+	A+ ; G+
RGS-2	A- ; G-	A- ; G-	A- ; G-	A+ ; G+
SPS-2	A+ ; G+	A+ ; G+	A+ ; G-	A+ ; G+
SPS-3	A+ ; G+	A- ; G+	A+ ; G+	A+ ; G+

The organisms isolates were putatively thought to be as follows according to Microrao:

- RGS-1: *Streptococcus sanguis* I
- SPS-1: *Enterobacter agglomerans*
- RGS-2: *Streptococcus sanguis* I
- SPS-2: *Serratia marcescens*
- SPS-3: *Enterobacter agglomerans*

3.6 Change in pH

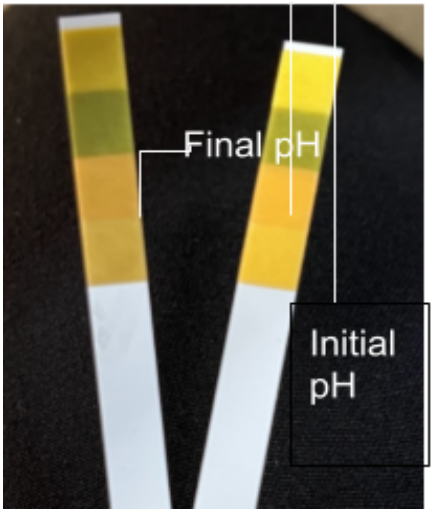

Fig 16: No change in pH after 7 days incubation period

Initially, pH before incubation of B&H was 7 as seen on the litmus paper. After 7 days of inoculation, the decolorized sample also showed a pH of 7 as shown in figure 16. The process was also carried out using a pH meter and the initial reading before incubation was seen to be 6.56 and 6.24 after incubation.

Also, the addition of a pellet of sodium hydroxide brought the pH of the ink from 7 to 11; the addition of 5 ml of 37% hydrochloric acid decreased the pH from 7 to 3. The ink remained unchanged after drastic pH changes.

3.7 Culture Supernatant Precipitation

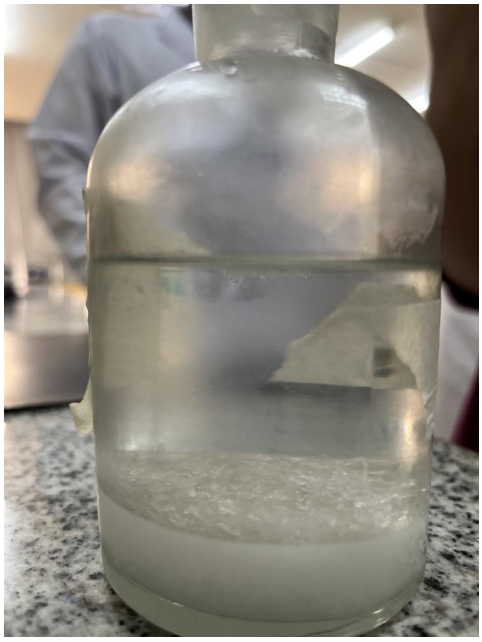

Fig 17: Crystals formed after ammonium sulfate precipitation

The crude culture supernatant amounted to 100 ml, and 100 ml of ammonium sulfate was required for precipitation to occur, hence the cut-off value for saturation was 50%. The crystals of protein precipitated out on the bed of salt as shown in figure 17.

3.8 Snakeskin Dialysis

The snakeskin membrane became turgid after one round of magnetic stirring. After 5 circulations of B&H salt dialysis buffer, 20 ml of concentrated culture supernatant was obtained. Hence, the supernatant had a 5-fold increase in concentration.

3.9 Concentrated Culture Supernatant Activity

Table 7: Percentage decolorization of concentrated, purified culture supernatant

Isolate	Decolorization Percentage (%)
SPS-2	43.50

The table 7 shows an increased decolorization rate for concentrated, purified culture supernatants.

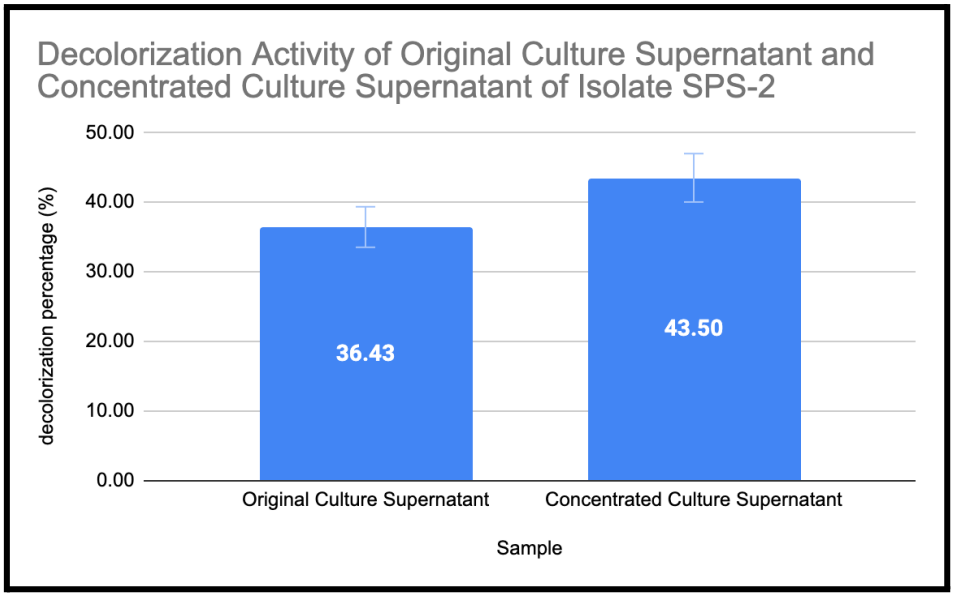

Fig 18: Relationship between the de-inking activity of original and concentrated culture supernatant from SPS-2

Figure 18 compares the decolorization percentage between the original culture supernatant and concentrated culture supernatant from isolate SPS-2. The concentrated culture supernatant demonstrated activity higher than that of the original culture supernatant. The concentrated culture supernatant exhibited a decolorization percentage of 43.50% in 48 hours, while the original culture supernatant showed a decolorization percentage of 36.43% after 7 days of incubation.

3.10 Decolorization Activity of Bacterial Lysate of isolate SPS-2

Table 8: Percentage decolorization of Bacterial Lysate with concentrated, purified culture supernatant from isolate SPS-2

Sample	Decolorization Percentage (%)
Bacterial Lysate	25.25
Bacterial Lysate + Concentrated culture supernatant	70.75

The table 8 shows a low decolorization activity of sonicated bacterial lysate.

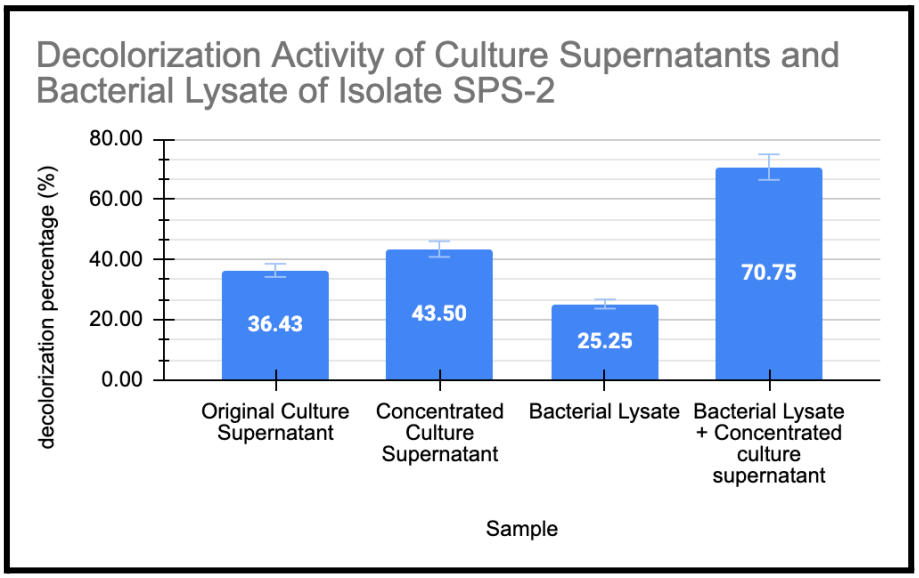

Fig 19: Relationship between the de-inking activity of bacterial lysate and concentrated culture supernatant from Isolate SPS-2

The bacterial lysate gave a decolorization percentage of 25.25%, while the bacterial lysate mixed with concentrated culture supernatant showed a decolorization percentage of 70.75% after 7 days of incubation.

3.11 Process Optimization

Table 9: Percentage of decolorization of isolates SPS-2 and SPS-3 in different conditions

Condition	Isolate SPS-2 Decolorization %	Isolate SPS-3 Decolorization %
pH 5	81.16	74.60
pH 6	23.24	61.53
pH 7	83.27	81.12
pH 8	76.66	73.29
pH 9	80.13	81.35
25 ℃	56.00	63.26
37℃	79.52	77.04
44 ℃	40.02	85.43

According to the results in table 9, isolate SPS-2 showed optimal decolorization activity at pH 7 and 37℃, while isolate SPS-3 showed optimal results at pH 9 and 44℃.

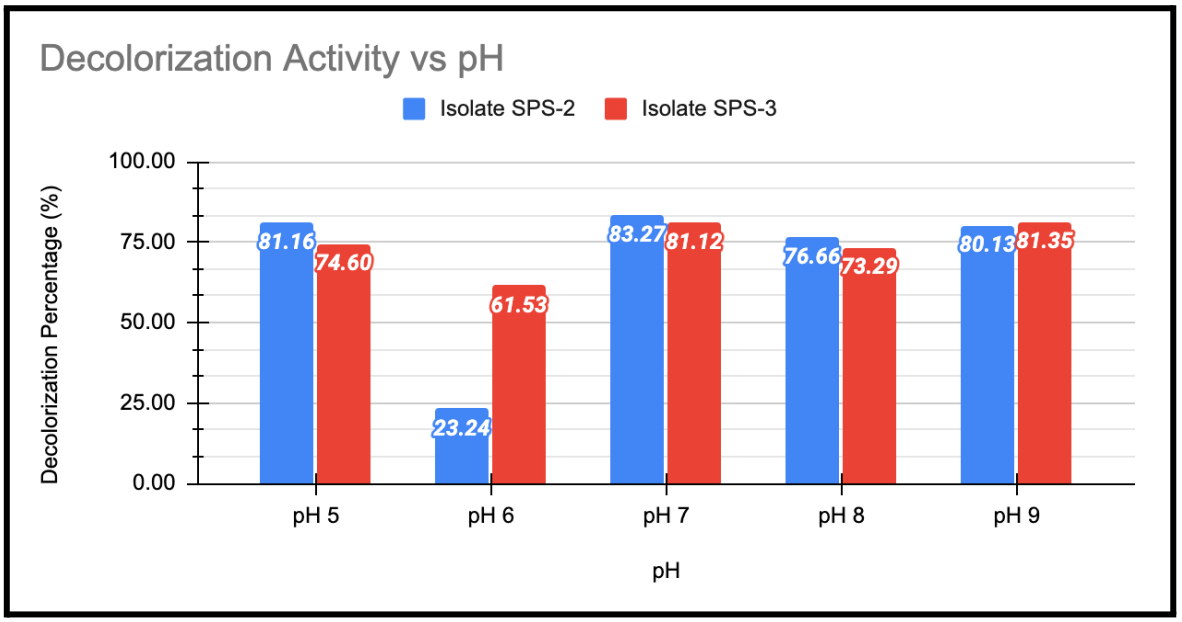

Fig 20: The relationship between de-inking activity and pH

Isolate SPS-2 demonstrated a de-inking activity of 81.16% at pH 5, 23.24% at pH 6, 83.27% at pH 7, 76.66% at pH 8 and 80.13% at pH 9, showing optimal activity at pH 7. Isolate SPS-3 demonstrated a de-inking activity of 74.60% at pH 5,61.53% at pH 6, 81.12% at pH 7, 73.29% at pH 8 and 81.35% at pH 9, showing optimal activity at pH 9.

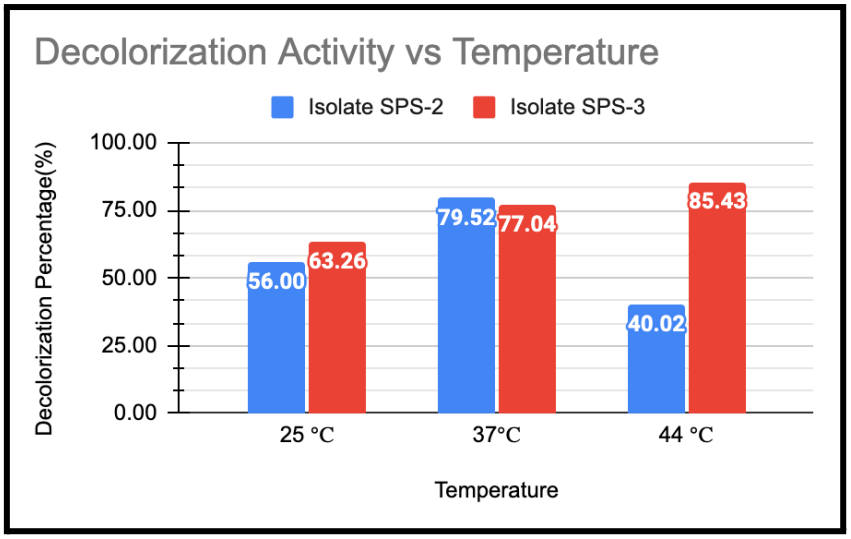

Fig 21: The relationship between de-inking activity and temperature

Isolate SPS-2 demonstrated a decolorization activity of 56% at 25℃, 79.52% at 37℃ and 40.02% at 44℃. Isolate SPS-3 demonstrated decolorization activity of 63.26% at 25℃, 77.04% at 37℃ and 85.43% at 44℃.

Chapter 4
Discussion

4.1 Bacteria Using Ink as a Carbon Source

The bacterial samples were inoculated into 0.9% NaCl to preserve the integrity of the cells (Wachowski et al., 1999). NaCl is isotonic and can therefore prevent cell disruption by lysis. The bacteria were grown on B&H media, which contains all nutrients required for bacterial growth except a carbon source. The media contains magnesium sulfate, calcium chloride, and ferric chloride - which provide trace elements of minerals. Ammonium nitrate is used as the nitrogen source, while monopotassium phosphate and potassium phosphate act as buffers (Bushnell & Haas, 1941; Rogers, Wulf & Hodge, 1963).

Several studies have utilized B&H media with various hydrocarbons as carbon sources to study their breakdown by microorganisms (Hemalatha & Manikandan, 2011; Hanson, Desai & Desai, 1993). In this study, ink was supplied as a carbon source. After the initial inoculation of samples on the agar plates, there were signs of fungal contamination in one sample. Bacterial colonies were circular, raised, and flat, with smooth, opaque surfaces - these were carefully selected for further tests. Fungal colonies had a mold-like appearance and were discarded.

With every subculture, the time required for bacterial growth decreased, and luxuriant growth was observed. This may be because the bacteria became more capable of utilizing the ink as the carbon source for each subsequent subculture. To reduce the number of samples, bacteria possessing extremely similar/identical morphology - form, margin, elevation, size, color, etc. were discarded.

The isolates that showed growth on B&H agar plates were then inoculated into B&H broth with ink; thus, ensuring that agar was excluded and the only source of carbon available for bacterial metabolism was ink. The isolates were originally allowed to grow in the broth for 48 hours at 37°C. Initially, the ink concentration used during incubation was 1.0% (v/v). The isolates were unable to significantly decolorize at this concentration in a 48-hour time frame, hence, the concentration was decreased to 0.3% and later increased incrementally; the time for decolorization was also extended to 7 days. At 0.3% ink after 24 hours, the ink particles could be seen aggregating and depositing at the bottom - these particles were broken down and decolorized after the 7 days period. This suggests that the bacteria were capable of

metabolizing ink as their carbon source, and could effectively break down the ink possibly through initial coagulation. Coagulation is often used for the treatment of colored effluents using a variety of coagulants (Ma & Xia, 2009; Roussy et al., 2005). However, these processes in addition to other chemical de-inking processes require materials that aggregate/coagulate ink that is detrimental to the environment (Borchardt, Miller & Azevedo, 1998). It was also observed that each subsequent subculture of ink at the same concentration resulted in a higher percentage of decolorization in the same time frame. The optical densities of the samples before and after de-inking were measured to find the percentage of decolorization (Varghese & Sebastian, 2015) - the more the ink was digested, the less opaque the broth would become and the lower its optical density would be.

4.2 Analysis of Percentages of Decolorization

4.2.1 Change of Optical Densities

One cuvette was used to measure all the samples in a subculture to increase precision by keeping the light absorption by the cuvette constant. The cuvette was first thoroughly washed with distilled water, wiped, then washed again with the sample broth before each measurement to prevent cross-contamination. As the control broth containing the ink was black in color, it was difficult to decide on the wavelength to be used as the readings were often out of the spectrophotometric optical density range. Hence, ink was diluted by ten, twenty, fifty, and seventy-five folds and the absorbances of this diluted ink were measured. After a series of trials and errors, it was decided that a wavelength of 605 nm (Morais et al., 2020) provided the results of absorbance that were consistently within range, so this wavelength was chosen for the entirety of the study.

For each subculture, the OD of the samples was measured after 7 days, and the respective decolorization percentage (Shah et al., 2021) was calculated. The readings were tabulated and plotted into graphs to find the relationship between ink concentrations and decolorization percentage for each sample.

4.2.2 Decolorization Percentage vs. Ink Concentration

Figure 5 shows the relationship between the de-inking activity of each sample against an increasing ink concentration. The expected result for each sample was that the de-inking activity will decrease with increasing concentration of ink (Habib et al.,

2017) - this was exhibited by Isolate SPS-2 and SPS-3. This is because, within the same time period of 7 days more and more ink particles were present to be decolorized. This should have shown a uniform decreasing pattern for each sample. As for isolates RGS-1 and SPS-1, the decolorization activity decreased to 0.6% (v/v) ink concentration, then increased again. This aligns with a similar study by Habib et al. (2017) where the hydrocarbon-degrading activity of bacteria was compared. Nine bacterial strains were isolated from soil samples and were subjected to diesel of two concentrations: 0.1% (v/v) and 0.5% (v/v). Similar to our study, 4 isolates showed increased diesel utilization activity at 0.1% (v/v) concentration while the activity decreased at 0.5% (v/v) concentration. It was seen that only 2 isolates could utilize diesel at an increased concentration in a similar period.

At each concentration of ink, the isolates SPS-2 and SPS-3 showed the greatest de-inking activity as their decolorization percentage was the highest among the 5 isolates compared. Both isolates showed a smooth trend with each increment of ink concentration. The highest decolorization percentage was of Isolate SPS-3 at 0.3% (v/v) ink concentration, followed closely by Isolate SPS-2 at the same concentration. The isolates SPS-2 and SPS-3 aligned with the expected results of decreasing de-inking activity with increasing ink concentration. Hence, these isolates were chosen to experiment with further.

4.2.3 Change in De-inking Activity at 0.3% (v/v) Ink

According to 2, at an ink concentration of 0.3% (v/v), it is seen that all the samples showed increased de-inking activity in the second subculture compared to the first. The greatest increase was for isolate RGS-1, exhibiting an increase of around 2.5 times that of the first subculture. The highest activity was seen in both isolates SPS-2 and SPS-3, for both subcultures. The increase in activity between subsequent subcultures increased possibly due to the bacteria being able to metabolize ink as a carbon source more readily after each subculture. This may be associated with a higher number of cells present in their log phase (Jain et al., 2020), leading to more exponential growth, and by extension, more metabolization.

4.2.4 Change in De-inking Activity at 0.6% (v/v) Ink

According to figure 6, for ink concentration at 0.6% (v/v), a similar pattern was seen at 0.3% (v/v) ink. The second subculture showed an increase in decolorization percentage than the first subculture. The de-inking activity in all four isolates showed an increase; isolate RGS-1 had a slight decrease in activity. The isolate SPS-2 showed the highest activity in both subcultures at this ink concentration.

4.2.5 Justification of Decolorization through pH Change

The ink may have been acting as an indicator that was causing the decolorization. To rule this out, 37% HCl and NaOH were added to two separate solutions of 1.0% (v/v) ink. No color change was observed; an indicator would have changed colour when exposed to extreme changes in pH. Since this did not occur, it could be concluded that the ink did not behave like an indicator so was not responsible for the decolorization.

Additionally, the pH of the B&H broth before and after bacterial inoculation and incubation was measured. The pH before inoculation was 6.56 and after 48 hours of incubation pH was 6.2 for isolate SPS-2. Since isolate SPS-2 is an acid-producing bacteria, a slight drop in pH is expected. This was also confirmed using litmus paper. Since the pH before and after incubation did not undergo a significant change, it can be concluded that pH is not responsible for the decolorization.

4.3 De-inking Activity of Culture Supernatant

The culture supernatant was collected from isolate SPS-2. The decolorization might have been occurring due to either extracellular or intracellular components of the culture - these could either be enzymes, proteins, or metabolites. The culture supernatant was collected by centrifugation at 10,500 rpm at 4°C for 20 minutes. The low temperature ensured that cells would be preserved and not disrupted due to heat, and 10,500 rpm was used as this was the optimal value of revolutions per minute to ensure cell integrity - higher rpm could disrupt cells (Shankar et al., 2018; Alhelli et al., 2018; Hasan et al., 2013; Sonune & Garode 2018)**.** The culture supernatant was hypothesized to contain the extracellular component/s responsible for de-inking. As this method is employed to obtain extracellular crude enzymes, we can hypothesize that the supernatant included extracellular enzymes (Hasan et al., 2013; Sonune & Garuda 2018). The supernatant was collected through aspiration and incubated

with 1.0% (v/v) ink for 48 hours at 37°C. Since isolates SPS-2 and SPS-3 were concluded to be the most effective at decolorizing ink, the culture supernatant for these samples was collected.

As demonstrated by figure 10, culture supernatant from isolates SPS-2 and SPS-3 showed 40% and 24% decolorization respectively. Isolate SPS-2 shows higher de-inking capacity than isolate SPS-3, which aligns with its de-inking capacities at (v/v) ink concentrations of 0.3%, 0.6%, and 0.8%. It can also be seen that the live culture was able to decolorize 1.0% (v/v) ink to a greater extent than the culture supernatant. This could signify that either the component responsible for de-inking was primarily intracellular, or that the supernatant did not contain an adequately concentrated amount of the component needed for de-inking. Additionally, it was noted that the ink particles aggregated during the process of de-inking. Liquid agglomeration is an alternative to flotation de-inking where a hydrocarbon oil is used to coagulate ink particles and subsequently remove them (Bowen, 1996). Surfactants sometimes aid the coagulation process (Bowen, 1996; Borchardt, Miller & Azevedo, 1998). As both the isolates were capable of aggregating the ink, no additional chemical surfactants or hydrocarbon oils are required which would otherwise be detrimental to the environment.

4.4 De-inking Activity on Paper

Isolates SPS-1, SPS-2, and SPS-3 digested paper printed with cartridge ink and broke the pieces of paper down into smaller segments, in a manner similar to shredding. The isolates also displaced ink from the surface of the paper and significantly digested the ink. Its action mimicked that of surfactants which aid in paper pulping and displacement of ink from paper (van Velzen et al., 2021; Borchardt, Miller & Azevedo, 1998; Biswas et al., 2019). Due to its ability to digest paper, it was hypothesized that the bacteria possess cellulolytic activity. To test this hypothesis, the bacteria were inoculated in CMC agar, where isolates SPS-1, SPS-2, and SPS-3 gave significant zones - alluding that the bacteria might possess cellulase enzymes. Cellulase enzymes can split cellulose fibers, facilitating the loosening and release of ink particles from recycled paper (Hasanin et al., 2020). Cellulase enzymes have been used in enzymatic de-inking (Borchardt, Miller & Azevedo, 1998) - resulting in these bacteria having significant potential for use in the paper industry.

The bacteria were also grown in CMC broth, where they exhibited significant growth. This ensured that the isolates had cellulolytic properties as CMC broth did not contain agar, which is also a potential carbon source. When isolates SPS-1, SPS-2, and SPS-3 were grown on

solely agar, only isolates SPS-2 and SPS-3 showed zones; this suggests that isolates SPS-2 and SPS-3 are both agarolytic and cellulolytic, while isolate SPS-1 is only cellulolytic.

4.5 Precipitation and Purification of Culture Supernatant

Ammonium sulfate precipitation is a frequently used method for protein purification from a salt solution. Proteins form hydrogen bonds with water molecules with their exposed polar and ionic groups. Ammonium sulfate forms small, highly charged ions in the solution. When ammonium sulfate is added to a solution, these ions compete with protein molecules to form bonds with water molecules. This removes water from protein molecules, making them less soluble in solution and causing them to precipitate as crystals (Sakthivel et al., 2010). Factors that affect the concentration at which protein will precipitate include the number and position of polar groups, pH of the solution, molecular weight of protein, and temperature at which the precipitate is formed.

The volume of ammonium sulfate needed is equal to the volume of the sample. The ammonium sulfate is added gradually in small increments (Sango et al., 2021) to the solution while gradually stirred so that the salt concentration at the site of deposition does not exceed desired concentration. After the total volume of ammonium sulfate was added, the beaker was stored at 4°C overnight. In this study, 100 ml of culture supernatant was taken and 100 ml of ammonium sulfate was added, so the cut-off value of saturation was at 50%. The crystallized proteins were then dialyzed through a snakeskin membrane.

Snakeskin dialysis allows the selective diffusion of small molecules from an area of higher concentration to a lower concentration through a semipermeable membrane; large macromolecules such as proteins are retained within the membrane while smaller molecules are dialyzed out. The dialysis buffer was replaced with a new salt solution every 1.5 hours to ensure a new equilibrium is set after the removal of the small molecules that were filtered out. The inner diameter of the pore of the membrane was taken to be 16 mm with a molecular weight cutoff of 10K since the protein was estimated to be of a larger size than the molecular weight cutoff value based on standard studies done previously (Sango et al., 2021). In the study by Sango et al. (2021), the culture supernatant, referred to as the crude enzyme, was first filtered using a membrane of 100K molecular weight cutoff. The filtrate was then further passed through a membrane of 10K molecular weight cutoff and the extracted enzyme was then purified using ammonium sulfate precipitation.

In this study, the culture supernatant was first precipitated using ammonium sulfate and then the protein crystals obtained were purified through snakeskin dialysis. In contrast to the previous study, the filtration was done once using a membrane of 10K molecular weight cutoff in order to minimize the loss of crystals in the process of double filtration and to de-salt the crystals.

After 5 buffer exchanges and the dialysis system kept at 4°C overnight, 20 ml of the concentrated filtrate was retrieved from within the membrane. This was approximately one-fifth of the volume of the culture supernatant originally extracted, and so was hypothesized to have a fivefold increase in decolorization activity.

4.6 De-inking Activity of Concentrated vs Original Culture Supernatant

Figure 18 compares the decolorization percentage between the original culture supernatant and concentrated culture supernatant from isolate SPS-2. While the original culture supernatant showed a decolorization percentage of 36.43% after 7 days of incubation, the concentrated culture supernatant exhibited a decolorization percentage of 43.50%. From this, it can be derived that the concentrated culture supernatant had a much higher de-inking activity compared to the original culture supernatant. This aligns with studies by Abanoz and Kunduhoglu (2018) and Purwanto (2016) that showed an increase in activity via purification and de-salting.

4.7 Characterization of Culture Supernatant

Previous studies have shown that bacteria residing in soil tend to utilize a combination of intracellular and extracellular enzymes for metabolic processes (Nannipieri, Sequi & Fusi, 1996; Burns, 1981). Extracellular or free enzymes are released into bacterial surroundings that are able to digest and break down surrounding nutrients; while intracellular enzymes may be released when a bacterial cell enters the death phase (Burns, 1981). This could play a crucial role in the mechanism of bacterial metabolism in soil. In another study by Burns (1976), it was found that some bacteria have intracellular enzymes that may be able to continue working inside a cell after cell death.

Isolate SPS-2 was lysed using a sonicator (Feliu & Villaverde, 1994;Burden & Whitney, 1995) to release intracellular components. The lysate was collected after centrifugation, while cell debris that precipitated at the bottom was discarded. When the culture supernatant

hypothesized to contain extracellular components was incubated with intracellular lysate, the decolorization percentage was at its highest at 70.75% with 1% (v/v) ink. However, when 1% (v/v) ink was incubated with only bacterial lysate, the decolorization percentage was only 20.25%. This supports that extracellular and intracellular components of bacteria from soil work in tandem, as previous studies suggest.

4.8 Process Optimisation

To find the optimal conditions at which the samples work best, B&H broth containing Isolates SPS-2 and SPS-3 was put under a range of temperatures and pH and incubated for 48 hours. The incubation time was kept the same as other subcultures to ensure uniform results and increase the reliability of the data.

According to figure 19, the decolorization activity of both Isolates SPS-2 and SPS-3 was checked at different pH, ranging from 5 to 9. It was found that both the isolates showed the lowest activity at pH 6, while worked best at pH 7. The highest activity was shown by Isolate SPS-2 at pH 7 at 83.27% and the lowest was also exhibited by Isolate SPS-2 at pH 6 which was 23.24%. From this data set, it can be derived that decolorization by both the isolates occurs best at pH 7. Besides pH 7, both the isolates showed high decolorization activity at pH 9 with Isolate SPS-2 showing 80.13% decolorization and Isolate SPS-3 showing 81.35%. As seen in the previous study by Sango et al.(2021), the de-inking enzymes cellulase and xylanase worked best at pH 9, while in another study by Nathan & Rani(2020), the enzyme lipase which is also employed during the de-inking process worked best at neutral conditions and sometimes up to pH 9.5. These data aligned perfectly with our obtained results where the bacteria exhibited the highest decolorization within the range of pH 7-9.

According to figure 20, the de-inking activity of both Isolates SPS-2 and SPS-3 was checked at 3 different temperatures, 25℃, 37℃, and 44℃. It was found that while Isolate SPS-2 showed the lowest activity at 44 ℃, Isolate SPS-3 had the most decolorization at this temperature. Except for 37℃, Isolate SPS-3 showed slightly better activity than Isolate SPS-2. The highest activity was shown by Isolate SPS-3 at 44℃ which is 85.43% and the lowest was also exhibited by Isolate SPS-2 at the same temperature which was 40.02%. As both the isolates gave similar results at 37 ℃, it can be derived from this data set that decolorization by both the isolates occurs optimally at 37℃.

4.9 Further Scope

The metabolite, protein, or enzyme responsible for de-inking activity has to be characterized and identified in future studies. These could be later optimized, purified, concentrated, and adopted for commercial use in the paper recycling industry. As some of the bacteria alluded to potential cellulolytic activity, these bacteria could be further investigated for use in the paper recycling industry and could provide a cheaper, eco-friendly, and more efficient alternative to chemicals used for de-inking. Additionally, these bacteria could be used in decolorizing ink with a similar composition to the ink decolorized by these bacteria. Also, the paper recovered after de-inking could not be tested for its physical properties such as tensile strength, opacity, brightness, etcetera. Therefore, its physical parameters could not be compared to paper treated with other enzymes used in the industry. If the parameters could be tested, the enzyme could be optimized to give recovered paper with improved physical characteristics. The study should also be repeated on various types, colors of paper, and ink to increase the versatility of the research. The bacteria could not be properly identified by biochemical tests- 16s rRNA sequencing has to be done to identify the exact species of bacteria so further research could be conducted on the bacteria.

Conclusion

Paper and ink pollution is a prevalent issue in Bangladesh that remains largely unaddressed. Although paper recycling is necessary to tackle this problem, de-inking processes in paper recycling utilize harmful chemicals that are expensive to process and detrimental to the environment itself. Therefore, it is imperative to find a suitable, efficient, and sustainable alternative.

In this study, bacteria isolated from soil and water samples in Sylhet, Bangladesh were found capable of degrading ink and exhibiting de-inking activity on paper. The original culture supernatant and concentrated culture supernatant of the bacteria could decolorise ink. However, more significant de-inking activity was demonstrated by the combination of extracellular and intracellular components of the culture.

Further research needs to be conducted to identify the components responsible for the de-inking activity as well as the exact identification of the bacteria by 16s rRNA sequencing. These components can then be further purified and optimized and used in the paper industry. This research has demonstrated bacterial species isolated in Bangladesh that could provide a possible sustainable method of recycling paper, therefore alleviating some of the harmful effects of pollution.

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