

AUTOMATED DETECTION AND MEASUREMENT OF BIOFILM AREA IN MICROSCOPY IMAGES

Submitted in partial fulfillment of the requirements for the award of Bachelor of

Technology degree in Biotechnology

Interdisciplinary Project

By

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SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Rahul Harish S (41230044)**, **Karumanchi Koushal (41230027)**, and **Rohith S (41230048)** who carried out the Interdisciplinary Project entitled "**AUTOMATED DETECTION AND MEASUREMENT OF BIOFILM AREA IN MICROSCOPY IMAGES**" under our supervision from January 2024 to April 2024.

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DECLARATION

We, **Rahul Harish S (41230044)**, **Karumanchi Koushal (41230027)** and **Rohith S (41230048)** hereby declare that the Project Report entitled “**AUTOMATED DETECTION AND MEASUREMENT OF BIOFILM AREA IN MICROSCOPY IMAGES**” done by us under the guidance of **Dr. Prabhakar Singh, Ph.D.** is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 6.5.2024

PLACE: CHENNAI

A handwritten signature in black ink, appearing to read 'Rahul', with a long horizontal stroke extending to the right.

SIGNATURE OF THE CANDIDATE

AKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

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ABSTRACT

Biofilms, complex microbial communities adhering to surfaces, pose significant challenges in various fields including medicine and industry due to their resilience and ability to cause infections and material degradation. Microscopy remains a primary tool for biofilm visualization, but manual analysis of biofilm area is time-consuming and subjective. This study presents an automated approach for detecting and quantifying biofilm area in microscopy images. The proposed method employs image processing techniques including thresholding, morphological operations, and feature extraction to segment biofilm regions accurately. Machine learning algorithms, such as convolutional neural networks, are utilized to enhance detection accuracy and robustness across diverse biofilm structures. Experimental validation demonstrates the efficacy of the proposed approach in accurately quantifying biofilm area, offering a valuable tool for accelerating research in biofilm-related studies and facilitating advancements in biofilm management strategies.

Keywords: Convolution Neutral Networking, Morphological operations, Thresholding, Morphological operations.

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CHAPTER 1

INTRODUCTION

Biofilms are complex communities of microorganisms encased in an extracellular matrix that forms on its own. They are present in many different ecosystems and industries and have a significant impact on environmental science, biotechnology, biomedical engineering, and microbiology. Comprehending their complex dynamics requires advanced analytical techniques, but there are many obstacles to overcome. Difficulties in image processing include morphological variance, noise reduction, and exact segmentation requirements. Contextual information integration with biofilm data adds even more complexity. Notwithstanding these challenges, new developments in biofilm analysis, from conventional image processing methods to cutting-edge machine learning approaches, present encouraging research directions (Zhao, Fei, *et al.*, 2020).

The identification and quantification of biofilm regions in microscope pictures are essential for comprehending the composition, growth, and effectiveness of biofilm defenses. Biofilm research has greatly benefited from the use of microscopy, particularly scanning electron microscopy (SEM), which produces precise pictures that show the shape and spatial organization of biofilms.

Driven by the need to go beyond current constraints, this project sets out to provide an integrated image analysis tool that is specific to the complex details of biofilm characterization. Specifically, the research aims to solve biofilm difficulties as its main purpose. These include developing strong biofilm detection and segmentation algorithms, quantifying important biofilm properties, utilizing machine learning to improve analysis, and integrating data analysis and visualization tools with ease (Miguel, *et al.*, 2022).

More recently, developments in image processing and analysis have improved our capacity to measure biofilm coverage and structure from microscopy pictures. The goal of this research is to create and verify a dependable technique for measuring and identifying biofilm regions from microscope pictures. Our aim is to offer a quantitative

evaluation of biofilm formation through the use of cutting-edge image processing methods and machine learning algorithms, which can facilitate the creation of novel antibiofilm therapies and preventive measures (Sowmya, *et al.*, 2020)

This report describes an automated method for identifying and measuring the biofilm area in microscope pictures in this setting. Our approach intends to precisely delineate biofilm regions by combining cutting-edge image processing techniques with machine learning algorithms. This will enable precise measurement of biofilm area across a range of microbial populations and environmental circumstances. This introduction describes the importance of automated biofilm analysis, gives a summary of current issues, and establishes the framework for the parts that follow, which go into detail about our methodology, experimental validation, and possible implications. (Felix, *et al.*, 2021).

CHAPTER 2

LITERATURE SURVEY

2.1. BIOFILM VIABILITY CHECKER: AN OPEN-SOURCE TOOL FOR AUTOMATED BIOFILM VIABILITY ANALYSIS FROM CONFOCAL MICROSCOPY IMAGES

The study focuses on developing an automated image analysis technique for quantifying biofilm formation from confocal micrographs. Traditional microbiological methods for biofilm analysis are laborious and prone to human error, prompting a need for more direct quantitative analyses. Confocal laser scanning microscopy combined with live/dead staining offers high-resolution 3D visualization of biofilm architecture and viability. Despite confocal laser scanning microscopy's utility, there's little consensus on image analysis methods for quantifying biofilm viability. The study proposes an open-access automated image analysis method using Fiji software, demonstrating its reliability and accuracy across different bacterial species. Validation includes sensitivity and specificity analysis, comparison with traditional microbiological techniques, and application to various biofilm morphologies. The protocol proves effective in analyzing biofilms treated with antimicrobial compounds and provides insights into biofilm architecture. Applications extend to studying the effectiveness of mouthwash on different bacterial biofilms and assessing biofilms on implant surfaces.

2.1.1. Method Developed

This study introduces a user-friendly method for analyzing biofilm micrographs using ImageJ, aimed at researchers in antimicrobial strategies. The method was applied to diverse biofilm types, showcasing its accuracy and efficacy across various bacterial morphologies and ages. The protocol encompasses steps to address uneven fluorescence intensities and eliminate noise, thereby ensuring the reliability of the results. This accessible approach provides a valuable tool for researchers engaged in biofilm studies, facilitating standardized and reproducible analysis for enhanced understanding and development of antimicrobial interventions (Sophie, *et al.*, 2021).

2.2. MICROSCOPY METHODS FOR BIOFILM IMAGING: FOCUS ON SEM AND VPP-SEM PROS AND CONS

The article provides an extensive overview of microscopy techniques, with a focus on scanning electron microscopy, for studying bacterial biofilms. It emphasizes the importance of detailed imaging techniques in biofilm research, considering their complex architecture and role in infectious diseases. Various microscopy methods such as light microscopy, confocal laser scanning microscopy, and atomic force microscopy are discussed, highlighting their strengths and limitations in biofilm analysis. Specifically, the article delves into scanning electron microscope techniques, including conventional scanning electron microscope and variable pressure scanning electron microscope, comparing their advantages and applications in visualizing hydrated biofilms. The OsO₄-RR-TA-IL procedure is proposed as an innovative scanning electron microscope protocol for high-resolution biofilm imaging, offering advantages in preserving biofilm structure and enhancing image quality. Additionally, advanced scanning electron microscope techniques such as cryo- scanning electron microscope, environmental scanning electron microscope, and focused ion beam- scanning electron microscope are explored for their unique capabilities in studying biofilm morphology and subsurface structures. The article also discusses recent advancements in biofilm research, including studies on microbial adhesion forces, nanostructure interactions, and the impact of environmental factors on biofilm formation. Overall, the text underscores the evolving role of scanning electron microscope from a qualitative to a quantitative method, providing valuable insights into biofilm ultrastructure and responses to treatments. (Relucenti, et al., 2021)

2.3. QUANTITATIVE IMAGE ANALYSIS OF MICROBIAL COMMUNITIES WITH BIOFILMQ

The text introduces BiofilmQ, a comprehensive image cytometry software designed for quantification, analysis, and visualization of biofilm properties. It emphasizes the importance of image-based quantitative measurements for understanding biofilm phenotypes and internal properties, particularly in spatially structured microbial

communities exhibiting gradients and spatiotemporal variations. BiofilmQ integrates algorithms for 3D image analysis and offers multiple segmentation options for accurate biofilm segmentation, catering to various microbial communities regardless of size, morphology, or species. The software enables the quantification of internal and global parameters of biofilms, facilitating deeper insights into biofilm biology and enabling analysis of spatiotemporal biofilm development and subpopulation variations.

The article further presents case studies showcasing the application of BiofilmQ in studying gene expression and matrix localization in *Vibrio cholerae* microcolonies, oxygen access in bacterial macro colonies, and phenotypic variations in *Pseudomonas aeruginosa* biofilms. It bridges the gap in spatial and spatiotemporal analysis of 3D microbial communities, aiding data analysis and visualization while enabling scientists without programming expertise to conduct complex analyses. Additionally, the article discusses various image analysis methods, software tools, and methodologies in biofilm research, highlighting contributions from different authors and institutions and providing examples of data visualization outputs for analyzing biofilm parameters over time and space. (Hartmann, *et al.*, 2021)

2.4.A TECHNOLOGICAL UNDETSTANDING OF BIOFILM DETECTION TECHNIQUES

The article comprehensively reviews various techniques for detecting and analyzing biofilms, particularly in the context of bioreactors where biofouling poses significant challenges. It discusses the importance of monitoring and preventing biofilm-related issues in water-based industries and highlights common materials used in bioreactor construction that are prone to biofouling, such as stainless steel and glass.

Numerous detection techniques are examined, including Nuclear Magnetic Resonance Imaging, Ultrasonic Time-Domain Reflectometry, Dry Mass Weighing, Laser-Induced Fluorescence Spectroscopy, Surface-Enhanced Raman Spectroscopy, Microtiter Plate Dye Staining, Light Microscopy, and Confocal Laser Scanning Microscopy, among others. Each technique is evaluated based on its principles, applications, advantages, and limitations.

Additionally, the article explores the influence of surface properties on microbial adhesion and biofilm formation, considering factors like surface charge, hydrophobicity, surface roughness, and topographical configuration. It discusses the use of various microscopy techniques and spectroscopic methods for analyzing biofilm composition, characteristics, diversity, and activity. Furthermore, the text delves into bioreactor design considerations and industrial applications, emphasizing the importance of effective biofilm detection strategies for maintaining operational efficiency and safety across different industries. (Achinas, *et al.*, 2020)

2.5. QUANTITATIVE AND QUALITATIVE ASSESSMENT METHODS FOR BIOFILM GROWTH

The article explores the multifaceted realm of biofilms, emphasizing their significance in various applications while also acknowledging the challenges they pose, such as infections and equipment contamination. Interdisciplinary collaboration is deemed essential for understanding biofilms due to their complex nature.

Biofilm architecture, characterized by live and dead bacterial cells, extracellular substances, and other materials, endows them with resilience against harsh conditions and antimicrobial agents. However, the lack of standardized protocols and training complicates the selection of optimal characterization methods for researchers.

Quantitative methods like colony forming unit counting and adenosine triphosphate bioluminescence are commonly employed for assessing biofilm growth. Software tools like ImageJ and microscopy techniques, including confocal microscopy, facilitate visualization and quantification of biofilm structures and cell counts. (Wilson, *et al.*, 2017)

CHAPTER 3

AIM AND SCOPE

3.1. Aim

- The aim of this project is to develop a comprehensive image analysis tool for the quantitative assessment of biofilm characteristics.
- The goal of this technology is to expedite the biofilm analysis process, which presently depends on researchers manually segmenting biofilms.
- The primary objective is to provide researchers and practitioners with a powerful platform for studying biofilm dynamics, behavior, and response to environmental stimuli.

3.2. Objective

- Researchers can save time, lessen subjectivity, and maybe improve the precision and repeatability of biofilm analysis by automating this operation.
- Automated solutions usually use machine learning and image processing to estimate the area of biofilms, identify their borders, and extract pertinent quantitative data for additional study.
- The scope for automated biofilm area detection and measurement in microscopy images is to equip researchers with efficient, accurate, and quantitative analysis tools.
- This capability enables a deeper understanding of biofilm biology and opens avenues for various applications across different fields.

CHAPTER 4

MATERIALS AND EXPERIMENTAL METHODS

4.1. PROGRAM CODE

Python IDLE was used and the output is verified.

```
import cv2
```

```
import numpy as np
```

```
import os
```

```
def read_image(image_path):
```

```
    image = cv2.imread(image_path)
```

```
    image = cv2.cvtColor(image, cv2.COLOR_BGR2RGB)
```

```
    return image
```

```
def analyze_biofilm(image):
```

```
    resized_image = cv2.resize(image, (500, 500))
```

```
    gray_image = cv2.cvtColor(resized_image, cv2.COLOR_RGB2GRAY) # Convert to  
    grayscale
```

```
    _, binary_image = cv2.threshold(gray_image, 100, 255, cv2.THRESH_BINARY)
```

```
    contours, _ = cv2.findContours(binary_image, cv2.RETR_EXTERNAL,  
    cv2.CHAIN_APPROX_SIMPLE)
```

```
    total_area = 0
```

```

for contour in contours:

    total_area += cv2.contourArea(contour)

return total_area

def analyze_database(database_path):

    total_biofilm_area = 0

    num_images = 0

    for filename in os.listdir(database_path):

        if filename.endswith(".jpg") or filename.endswith(".png"):

            image_path = os.path.join(database_path, filename)

            database_image = read_image(image_path)

            biofilm_area = analyze_biofilm(database_image)

            total_biofilm_area += biofilm_area

            num_images += 1

            print(f'Area of {filename}: {biofilm_area:.2f} square pixels')

    if num_images > 0:

        average_biofilm_area = total_biofilm_area / num_images

        print(f"Average biofilm area: {average_biofilm_area:.2f} square pixels")

    else:

        print("No biofilm images found in the database")

```

```
if __name__ == "__main__":
```

```
    database_path
```

```
=
```

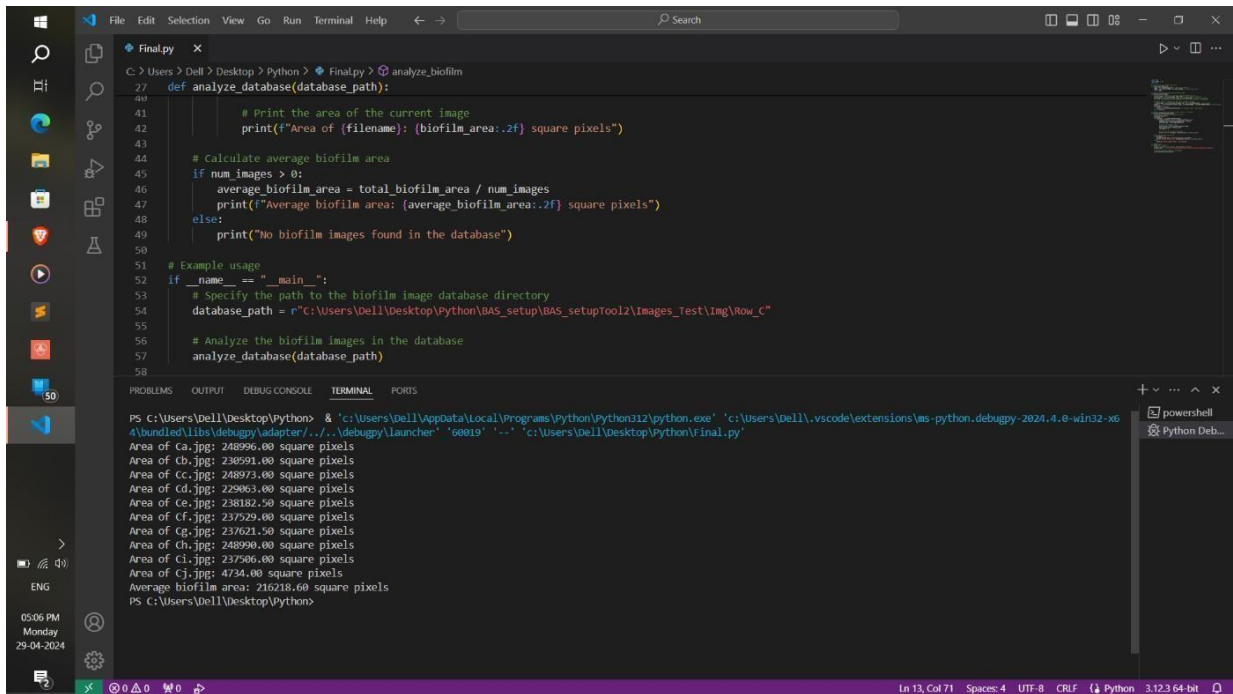
```
r"C:\Users\Dell\Desktop\Python\BAS_setup\BAS_setupTool2\Images_Test\Img\Row_  
C"
```

```
    analyze_database(database_path)
```

CHAPTER 5

RESULT AND DISCUSSION

5.1. PERFORMANCE ANALYSIS



The screenshot shows a VS Code editor with a Python script named `Final.py` and its terminal output. The script defines a function `analyze_biofilm` that calculates the area of biofilm images. The terminal output shows the execution of the script, displaying the area of each image and the average biofilm area.

```
def analyze_biofilm(database_path):
    # Print the area of the current image
    print(f'Area of {filename}: {biofilm_area:.2f} square pixels')

    # Calculate average biofilm area
    if num_images > 0:
        average_biofilm_area = total_biofilm_area / num_images
        print(f'Average biofilm area: {average_biofilm_area:.2f} square pixels')
    else:
        print("No biofilm images found in the database")

# Example usage
if __name__ == "__main__":
    # Specify the path to the biofilm image database directory
    database_path = r"C:\Users\ DELL\Desktop\Python\BAS_setup\BAS_setup\tool2\Images_Test\img_c"

    # Analyze the biofilm images in the database
    analyze_database(database_path)
```

```
PS C:\Users\ DELL\Desktop\Python> & 'c:\Users\ DELL\AppData\Local\Programs\Python\python312\python.exe' 'c:\Users\ DELL\.vscode\extensions\ms-python.debugpy-2024.4.0-win32-x64\bundle\libs\debugpy\adapter\...\.debugpy\launcher' '60819' '--' 'c:\Users\ DELL\Desktop\Python\Final.py'
Area of ca.jpg: 248996.00 square pixels
Area of db.jpg: 230591.00 square pixels
Area of cc.jpg: 248973.00 square pixels
Area of cd.jpg: 229063.00 square pixels
Area of ce.jpg: 238182.50 square pixels
Area of cf.jpg: 237529.00 square pixels
Area of cg.jpg: 237621.50 square pixels
Area of ch.jpg: 248990.00 square pixels
Area of ci.jpg: 237506.00 square pixels
Area of cj.jpg: 4734.00 square pixels
Average biofilm area: 216218.66 square pixels
PS C:\Users\ DELL\Desktop\Python>
```

Fig:5.1: Output for programmed code

5.1.1. Use of square pixels

Standardization of Measurement, by determining the square pixels, you establish a consistent measurement scale for the image. This standardization allows for accurate and reliable quantification of features such as biofilm area, biomass, and cell density across different images and experiments.

Calibration and Comparison, knowing the square pixels enables you to calibrate the image to real-world dimensions. This calibration facilitates the comparison of biofilm properties between different experiments, samples, or imaging modalities. For example, you can compare biofilm growth rates or morphology across images acquired with varying magnifications or resolutions. (Kathirvel, *et al.*, 2023)

5.1.1.1. *Converting square pixels to real world dimensions*

To convert square pixels to real-world dimensions, you need additional information such as the resolution of the image and the aspect ratio if the image is not square. Here is a general approach using the resolution

Find the Resolution: Determine the pixels per inch (PPI) of the device or image. This is often provided in the specifications of the imaging device or can be set in image editing software.

Calculate the Area in Square Inches: Divide the total pixel area by the resolution squared to convert to square inches.

$$\text{Area in square inches} = \frac{\text{Total square pixels}}{(PPI)^2}$$

Convert to Physical Units: If you want the area in square centimeters, for example, you can then convert square inches to square centimeters.

$$\text{Area in square cm} = \text{Area in square inches} \times 6.4516$$

For example, let's take 229063.00 square pixels:

Assuming a common resolution of 300 PPI, the calculation would be:

$$\text{Area in square inches} = \frac{229063.00}{(300)^2}$$

$$\text{Area in square inches} = \frac{229063.00}{90000}$$

$$\text{Area in square inches} = 2.545$$

$$\text{Area in square cm} = 2.545 \times 6.4516$$

$$\text{Area in square cm} = 16.42$$

This means the area would be approximately 16.42 square centimeters at 300 PPI. Remember, this is just an example; you'll need the actual PPI of your image for an accurate conversion. (Karballaei, *et al.*, 2023)

Quantitative Analysis, square pixel information is crucial for performing quantitative analyses of biofilm characteristics. With the correct pixel scale, you can accurately measure biofilm area, volume, thickness, and other morphological parameters. This quantitative analysis provides valuable insights into biofilm development, structure, and behavior.

Interpretation of Results, understanding the scale of the image in real-world units (e.g., micrometers per pixel) aids in the interpretation of analysis results. It allows researchers to relate numerical measurements to physical dimensions, facilitating the interpretation of experimental findings and biological significance. (Qian, *et al.*, 2022)

Visualization and Reporting, square pixel information can be used to annotate or scale biofilm images for visualization purposes. By overlaying scale bars or annotations indicating pixel dimensions, you provide context and scale reference for readers or collaborators. This enhances the clarity and interpretability of your images in presentations, publications, or reports.

Automation and Workflow Efficiency, automating the process of finding square pixels using Python streamlines image analysis workflows, reducing manual effort and potential errors. Once implemented, the pixel scale determination can be integrated into image processing pipelines or analysis scripts for efficient and reproducible data analysis. (Jianhua, *et al.*, 2020)

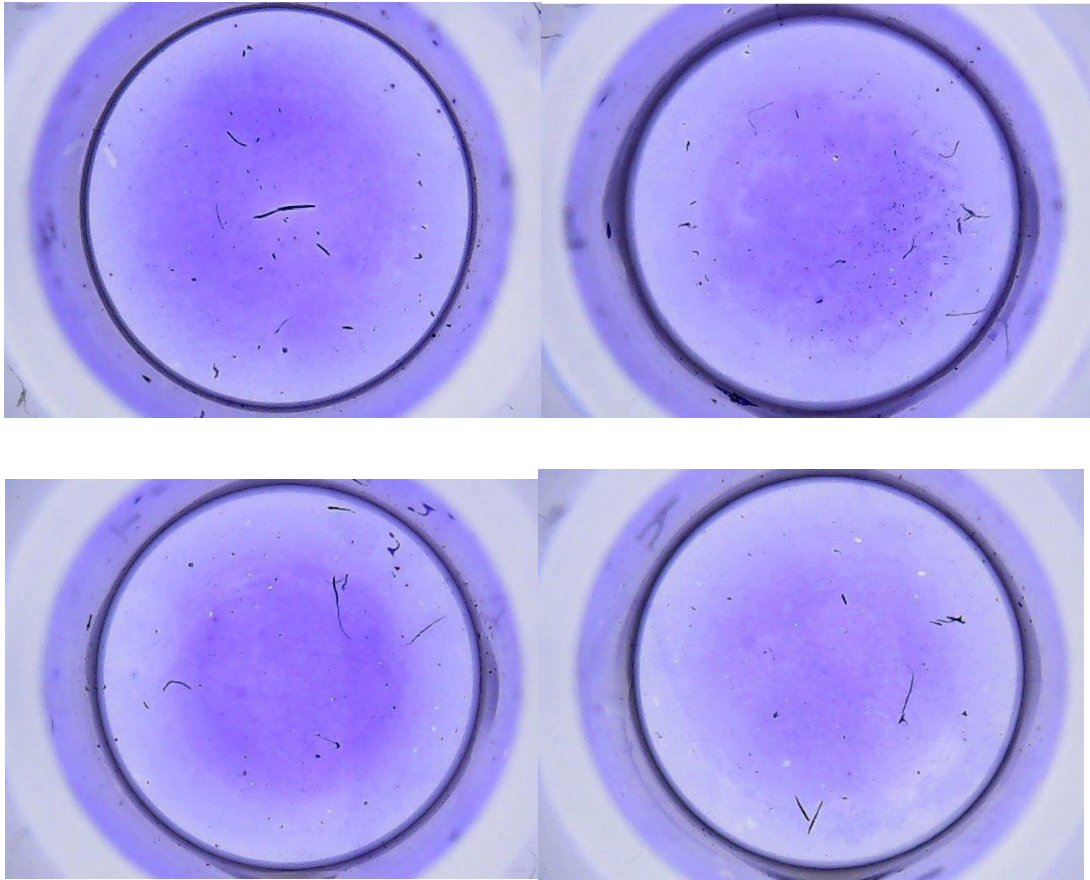


Fig:5.2: Biofilm images used as input

CHAPTER 6

SUMMARY AND CONCLUSIONS

Project Goals Recap, A succinct synopsis of the project's goals, which include using machine learning and image processing tools to biofilm investigation. Important Discoveries, An overview of the key conclusions and outcomes from the biofilm data study. It also highlights significant discoveries and findings that advance our knowledge of the dynamics and behavior of biofilms. Conclusions and Utilization, talk about the project's findings' wider ramifications for a range of disciplines, including biotechnology, environmental science, microbiology, and biomedical engineering.

Prospective Courses of Action, Recommendations for possible topics for additional study and future research directions based on the project's limitations and results. Attainment of Project Goals, talk about how the project's goals were achieved, including the creation of algorithms, the application of machine learning methods, and the incorporation of data analysis tools. Perspectives and Findings, an overview of the most important conclusions and revelations from the biofilm data study and also talks about any surprising findings or outcomes that arose during the study. Contributions to the Domain, Evaluation of the project's contributions to image analysis, biofilm research and identifying topics for more study and investigation in light of the project's findings.

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