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# Ribotyping Analysis of Microbes Producing Lipase from Batik Liquid Waste Samples

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## *Abstracts*

Liquid waste from batik production, when disposed of into the environment, retains high levels of organic compounds as residue from main ingredients such as dyes, wax, starch, soda ash, and more. With its high organic material content, batik liquid waste is a potential habitat for microbial growth. This research aims to isolate and genetically analyze microbes in batik liquid waste, namely by ribotyping analysis of microbes that produce lipase. From the results of microbial screening on selective lipase media, eight types of bacteria were obtained which have the potential to produce hydrolase enzymes. Eight types of lipase-producing bacteria have been genetically identified by analyzing the 16S rRNA gene. Alignment analysis using blastN from NCBI of the 16S rRNA gene nucleotide sequence of the sample suggested that the lipase-producing microbial isolate of the sample had the closest homology to the *Serratia, Burkholderia, Stutzerimonas*, and *Pseudomonas* groups. Four of the eight samples, RR1R, RA1R, RA2R, and ZB16R, have the highest homology <97%. This indicates that the four samples are novel microbial variants distinct from Genbank's microbial data.

**Keywords:** Batik liquid waste, 16S rRNA, hydrolase activity, microbes

## Introduction

Batik is one of Indonesia's cultural heritages, officially approved by the world's UNESCO in 2009. Batik motifs in cloth and other forms are easily found in everyday life, such as on school uniforms, formal shirts, and even shoe motifs. Batik is made by writing dots or patterns on cloth using a canting tool. The patterns made are not arbitrary; each region has different batik patterns, and these patterns have their own meaning.

The process of making batik cloth consists of four main stages, starting with pattern making, pasting, coloring, and “pelorodan”. Pattern making is done by drawing the pattern on paper and then tracing it onto the cloth, or the pattern is drawn directly on the mori cloth. After the pattern is drawn, the pattern is covered using wax to avoid the pattern being colored during the coloring process. The fabric is then dipped in the dye repeatedly until the desired result is obtained. After the fabric has gone through the dyeing process, the wax attached to the fabric is removed or removed. “Pelorodan” can be done by soaking the cloth in boiling water or removing it using a sharp object. The high temperature causes the wax to melt and separate from the fabric.

The batik cloth production process not only produces beautiful batik cloth but also liquid batik waste. This liquid waste can pollute the surrounding aquatic environment due to the chemical content in it. Liquid batik waste consists of various components such as dyes, wax, starch, soda ash, and others. From the composition of this batik liquid waste, there is a potential habitat for microbes. Much research has been carried out regarding the potential for microbes that live in liquid batik waste, including

the identification of microbes from river water contaminated with liquid batik waste by Muchtasjar et al. The eight bacterial genera that were successfully grown were *Mesophilobacter, Methylococcus, Agrobacterium, Neisseria, Xantobacter, Deinococcus, Sporosarcina,* and *Bacillus* [1]. Microbes contained in liquid batik waste also have degradation capabilities. Wijiastuti et al. obtained thermophilic bacterial isolates with the ability to reduce hexavalent chromium from the genus Bacillus, Pseudomonas, and Geobacillus obtained from liquid batik waste [2]. Citrapancayudha et al. obtained isolates of Pseudomonas bacteria from liquid batik waste with the ability to degrade wax [3]. Not only bacteria, Dewi et al. isolated fungi from the genus Aspergillus, which can degrade Indigosol dye [4]. These studies show the various types and activities of microbes contained in liquid batik waste. In this study, we will report the microbes that have hydrolase enzyme activity in liquid batik waste from Tasikmalaya.

## Material and Methods

*Materials*

Materials used in this research are aquabidest, toothpicks, Biologix propylene tips, Biologix microtubes, Himedia M001 nutrient agar media, Himedia M002 nutrient broth media, commercial Rhodamine B dye, commercial olive oil, nuclease-free water, DE buffer solution, isolation reagent DNA Promega WIZARD Genomic DNA purification kit (nuclei lysis solution, RNase solution, protein precipitation solution, rehydration solution), Merck ethanol, Merck isopropanol, GoTaq Promega, a pair of reverse (AAGGAGGTGATCCAGCCGCA) and forward (CAGGCCTAACACATGCAAGTC) primers, Thermo Scientific Top Vision agarose powder, MP Biomedicals Ultra-Pure TRIS base, Merck glacial acetic acid, EDTA, sodium chloride, ethidium bromide, loading dye (bromophenol blue and sucrose). Microbial samples were obtained from river water and batik liquid waste from Tasikmalaya.

*Sampling*

The samples used in this research were taken from liquid batik waste and river water in the environment around one of the batik industries in the Tasikmalaya area, West Java.

*Isolation of microbes by cultivation*

The microbes present in the sample were obtained by culturing the sample on nutrient agar (NA) media. In this study, selective NA media was used by mixing NA media with 0.001% (w/v) Rhodamine B and 2.5% (v/v) olive oil.

*Inoculation of microbes*

After an incubation at 37$^{o}$C for 20 hours, the culture in selective media is observed to determine the presence of growing colonies. Colonies are selected based on their luminescence under UV light. Colonies that glow are marked and transferred for further growth in nutrient broth (NB) media.
Selected colonies from the selective media (NA with olive oil and Rodhamine B) are picked using a sterile toothpick, then placed in NB media and incubated in a shaker incubator at 37°C and 170 rpm for 20 hours.

*Isolation of chromosomal DNA*

The sequence of DNA isolation processes is in accordance with the protocol in the Wizard® Genomic DNA Purification Kit.

*DNA amplification by PCR*

The DNA that had been isolated was then amplified with a polymerase chain reaction (PCR) by using a pair of reverse (AAGGAGGTGATCCAGCCGCA) and forward (CAGGCCTAACACATGCAAGTC) primers). The isolated DNA underwent amplification through a polymerase chain reaction (PCR). The amplification process following this protocol: an initial denaturation at 94°C for 4 minutes, followed by 34 cycles. Each cycle involved denaturation at 94°C for 30 seconds, annealing at 46°C for 30 seconds, elongation at 72°C for 90 seconds, and a final extension lasting 10 minutes

*Agarose gel electrophoresis*

Electrophoresis was carried out using 1% (w/v) agarose in 1x TAE buffer. Electrophoresis was carried out with a voltage of 100 Volts for 30 minutes. The electrophoresis results were visualized using UV light.

*Bioinformatics Analysis*

All the processes of bioinformatics analysis are performed using BioEdit, MEGA XI, and the NCBI site. (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

**Results and Discussion**

***Microbial inoculation on selective media***

Microbes inoculated in NA selective media with rhodamine B and olive oil showed the presence of some fluorescent and some non-fluorescent colonies. Luminescence in microbial colonies indicates the hydrolase activity of the microbes in the colony. The brighter the light, the higher the hydrolase activity. Fluorescent colonies are shown in **Figure 1**. Kouker and Jaeger proposed that the cause of luminescence is due to the interaction between the products of olive oil hydrolysis by lipase and rhodamine B dye [5]. In the hydrolase class, enzymes that can hydrolyze fat are esterase and lipase. Both have similar activities, namely lipolysis, but have different substrate characteristics. Esterase tends to catalyze hydrocarbon fatty compounds that have short chains (<C8), while lipase, on the other hand, tends to catalyze long-chain fats such as triglycerides (>C8) [6]. Substrate characteristics can determine the properties of the enzyme. The substrate of esterase is more hydrophilic than that of lipase, resulting in higher esterase activity on substrates that are easily soluble in water. In contrast, the substrate of lipase typically favors a hydrophobic environment [7].

Olive oil consists of various fats and fatty acids, with the main components being triglycerides and oleic acid [8]. Hydrolase activity assay using olive oil as a substrate is a closest homology to *Pseudomonas, Burkholderia, Stutzerimonas*, and *Serratia*. The positive test for the lipase enzyme [9]. Based on the characteristics of the substrate, the hydrolase activity of the microbes is determined as lipase. Lipase in microbes can be induced using vegetable oil as olive oil, as in this study.

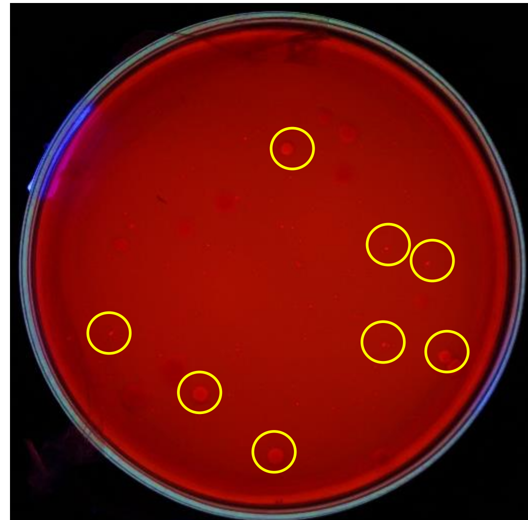


**Figure 1.** Microbial colonies on selective lipase media. The yellow circles show the fluorescent colonies that indicate hydrolase activity.

***16S rRNA gene amplicon of samples***

Amplification of sample DNA using 16S rRNA primers using the PCR technique provides DNA fragments with a size of around 1500 base pairs. The sample 16S rRNA gene amplicon is shown in Figure 2.

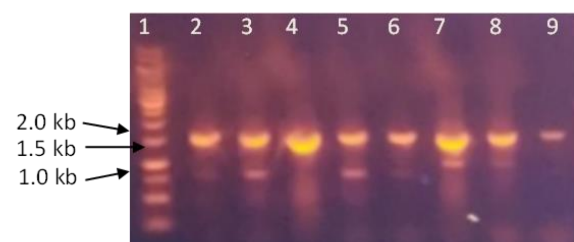



**Figure 2**. 16S rRNA gene amplicons from fluorescent colonies. Lane 1: 1 kb DNA ladder, lane 2-9: 16S rRNA gene amplicons.

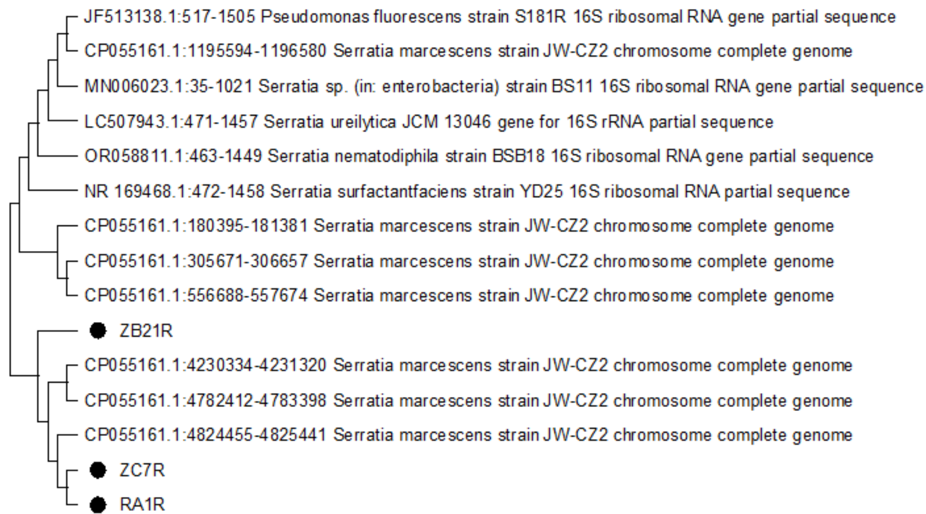
***Sample homology***

The analysis results of the sample's nucleotide sequence aligned with GenBank data at NCBI show that the isolated sample has the closed homology of the samples is shown in Table 1.

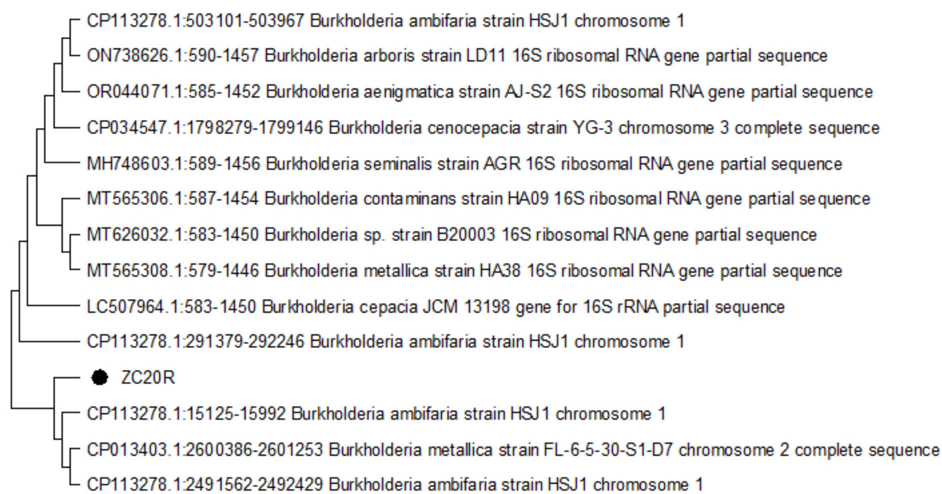
**Table1**. The closed homology of the samples

| No. | Sample name | Closed homology | % Identity |
|---|---|---|---|
| 1 | Z21BR | *Burkholderia sp* | 99.77 |
| | | *Burkholderia metallica* | 99.77 |
| | | *Burkholderia contaminans* | 99.77 |
| | | *Burkholderia cenocepacia* | 99.77 |
| | | *Burkholderia cepacia* | 99.77 |
| 2 | ZC7R | *Pseudomonas fluorescens* | 99.29 |
| | | *Serratia marcescens* | 99.29 |
| | | *Serratia sp* | 99.29 |
| | | *Serratia ureilytica* | 99.29 |
| | | *Serratia nematodiphila* | 99.29 |
| 3 | RS1R | *Pseudomonas aeruginosa* | 99.2 |
| 4 | ZC20R | *Serratia marcescens* | 98.28 |
| | | *Pseudomonas fluorescens* | 98.28 |
| | | *Serratia ureilytica* | 98.18 |
| | | *Serratia nematodiphila* | 98.18 |
| | | *Serratia sp* | 98.18 |
| 5 | RR1R | *Pseudomonas sp* | 85.5 |
| | | *Pseudomonas sihuiensis* | 85.46 |
| | | *Pseudomonas oleovorans* | 85.41 |
| | | *Pseudomonas tianjinensis* | 85.41 |
| | | *Pseudomonas hydrolytica* | 85.41 |
| 6 | ZB16R | *Pseudomonas sp* | 96.25 |
| | | *Stutzerimonas stutzeri* | 96.16 |
| | | *Stutzerimonas frequens* | 96.16 |
| 7 | RA1R | *Serratia marcescens* | 95.23 |
| | | *Serratia sp* | 95.23 |
| | | *Pseudomonas fluorescens* | 95.13 |
| | | *Serratia ureilytica* | 95.13 |
| | | *Serratia nematodiphila* | 95.13 |
| 8 | RA2R | *Pseudomonas guguanensis* | 95.72 |
| | | *Pseudomonas mendocina* | 95.72 |
| | | *Pseudomonas oleovorans* | 95.72 |
| | | *Pseudomonas sihuiensis* | 95.72 |
| | | *Pseudomonas hydrolytica* | 95.72 |

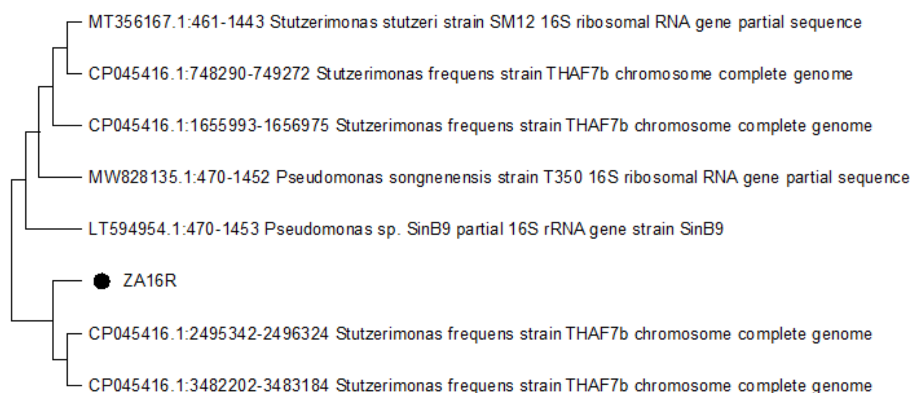
The phylogenetic trees based on the nucleotide sequence of the 16S rRNA gene of the samples are shown in **Figure 3.**




**A**




**B**




**C**

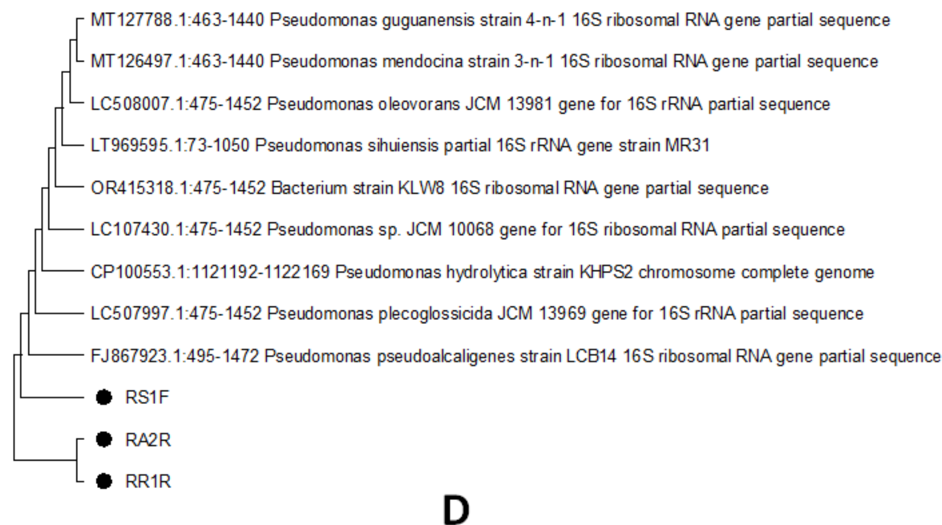



**Figure 3**. Phylogenetic tree of the samples. The ● sign indicates the sample isolates. A: sample group that is close to *Serratia*, B: sample that is close to *Burkholderia*, C: sample that is close to *Stutzerimonas*, and D: sample group that is close to *Pseudomonas*

Table 1 and Figure 3 suggest that microbial samples that have lipase activity can be grouped into four large groups, namely *Serratia, Burkholderia, Stutzerimonas*, and *Pseudomonas*. Based on Table 1, of the eight samples, four samples, RR1R, RA1R, RA2R, and ZB16R, have the highest homology <97%. This suggests that the four samples are new microbial variants, which are different from the microbial data in Genbank. [10]. Currently, sequences of >95% identity of 16S rRNA gene sequences are assumed to represent the same genus, whereas sequences of > 97% identity are assumed to represent the same species. [11]. This suggests that RA1R, RA2R, and ZB16R are different bacterial species, with the same genus. Meanwhile, RR1R is a new bacterium with a different genus level.

## Conclusion

Eight bacteria were effectively isolated from the liquid batik waste of Tasikmalaya. These bacteria exhibit hydrolysis activity, as confirmed through assays employing selective media containing olive oil and rhodamine B. The findings indicate that all eight bacteria demonstrate the ability to produce lipase induced by olive oil. The bacteria can be categorized into four major groups: *Serratia, Burkholderia, Stutzerimonas,* and *Pseudomonas.* Homology analysis showed that four bacteria, RR1R, RA1R, RA2R, and ZB16R were novel bacteria. RA1R, RA2R, and ZB16R are new bacteria with different species in the same genus, while RR1R is a new bacterium with a different genus level from GeneBank microbial data.

## References

[1] B. Muchtasjar, H. Hadiyanto, and M. Izzati, "Microbial degradation of batik wastewater treatment in Indonesia," *IOP Conf. Ser. Earth Environ. Sci.*, vol. 314, no. 1, 2019.
[2] W. Wijiastuti, I. M. Artika, and N. Nurhidayat, "Isolation and Selection of Thermophilic Bacteria as Hexavalent Chromium Reducer from Batik Processing Waste Water," *Curr. Biochem.*, vol. 2, no. 1, pp. 22–31, 2015.
[3] D. R. Citrapancayudha and E. S. Soetarto, "Biodegradasi residu wax dari limbah industri batik oleh bakteri," *Proceeding Biol. Educ. Conf.*, vol. 13, no. 1, pp. 800–806, 2016.
[4] R. S. Dewi, R. S. Kasiamdari, E. Martani, and Y. A. Purwestri, "Decolorization and detoxification of batik dye effluent containing Indigosol Blue-04B using fungi isolated from contaminated dye effluent," *Indones. J. Biotechnol.*, vol. 23, no. 2, pp. 54–60, 2018.
[5] G. Kouker and K. E. Jaeger, "Specific and sensitive plate assay for bacterial lipases," *Appl. Environ. Microbiol.*, vol. 53, no. 1, pp. 211–213, 1987.

[6] L. Ramnath, B. Sithole, and R. Govinden, "Identification of lipolytic enzymes isolated from bacteria indigenous to Eucalyptus wood species for application in the pulping industry," *Biotechnol. Reports*, vol. 15, pp. 114–124, Sep. 2017.

[7] P. Fojan, P. H. Jonson, M. T. N. Petersen, and S. B. Petersen, "What distinguishes an esterase from a lipase: A novel structural approach," *Biochimie*, vol. 82, no. 11, pp. 1033–1041, 2000.

[8] S. Lanka and J. N. L. Latha, "A short review on various screening methods to isolate potential lipase producers: Lipases-the present and future enzymes of biotech industry," *Int. J. Biol. Chem.*, vol. 9, no. 5, pp. 207–219, 2015.

[9] D. Boskou, G. Blekas, and M. Tsimidou, *Olive Oil Composition*, Second Edi. AOCS Press, 2006.

[10] Johnson, J.S., Spakowicz, D.J., Hong, BY. *et al.* Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat Commun* **10**, 5029 (2019). https://doi.org/10.1038/s41467-019-13036-1

[11] Schloss, P. D. & Handelsman, J. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. 71, 1501 (2005). https://doi.org/10.1128/AEM.71.3.1501-1506.2005