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Isolation and Molecular Identification of Direct Red 80 Synthetic Dye Degradation Bacteria from Palembang Indonesia Jumputan Cloth Industrial Waste

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Abstract
Industrial waste from Jumputan cloth production pose a significant risk to environmental safety due to their toxic synthetic dye content. Several studies have shown that the presence of bacteria in these materials plays a very important role in decolorization process of the constituent dye. Therefore, this study aims to isolate bacteria with the ability to decolorize direct red 80 from Jumputan cloth industrial waste. Characterization of isolates was carried out macroscopically, microscopically, and biochemically, followed by molecular identification using the 16S rRNA gene. Decolorization effects of the samples on red dye 80 were then assessed using a spectrophotometer at a maximum wavelength of 528 nm. The results showed that 6 bacteria isolates can degrade dye, with decolorizing power ranging from 26.33±0.94 – 73.67±0.47. The highest potential for decolorizing waste synthetic dye is seen in isolate BD 05. Phylogenetic analysis showed that there were 3 genera of bacteria among the samples obtained, namely *Bacillus*, *Aeromonas*, and *Pseudomonas*. These bacteria were closely related to *Bacillus tropicus*, *Areomonas jandaei*, and *Pseudomonas stutzeri*. *Pseudomonas stutzeri* (BD 05) has the highest potential in handling jumputan industrial waste.

Keywords
Decolorization, Direct Red 80, 16S rRNA Gene

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1. INTRODUCTION

The development of the Jumputan cloth industry has brought significant benefits to people's lives. However, improper management of waste from this industry can increase the risk of environmental damage. The presence of waste containing dye in water interferes with the penetration of sunlight, thereby disrupting the process of photosynthesis. This condition has been reported to have toxic effects on aquatic flora and fauna (Lalnunhlimi and Krishnaswamy, 2016; Panda et al., 2021). A previous study also reported that synthetic dye waste can interfere with the DNA replication process, leading to gene mutations, such as apoptosis or necrosis (Panda et al., 2021). Moreover, these materials have a negative impact on the amount of organic carbon (TOC), biological oxygen demand (BOD), and chemical oxygen demand (COD) (Liu et al., 2022).

Synthetic dye are commonly used in the textile industry because they offer several benefits compared to natural dye, such as color stability, which increases the ability to withstand exposure to light and chemicals (Sałdan et al., 2022). They are also more readily available, cheaper, and come in a wider range of colors (Manna et al., 2017). These materials are also easy to apply, require less energy for production, and have strong covalent bonds in textile fibers with high photolytic structural stability (Brüschweiler and Merlot, 2017).

Direct red is a synthetic dye that is widely used in the Jumputan cloth industry due to its strong binding power, leading to a brighter color. Furthermore, direct red 80, in particular, is composed of formal Sirius red F3B condensation, which produces an organic sodium salt with six equivalents of sodium hydroxide (Chemical, 2022). As a polyazo compound, it does not produce benzidine compounds when degraded, making it safer than traditional direct dye, such as congo red (Dapson et al., 2011).

The treatment of waste containing synthetic dye with physical and chemical methods has proven to be ineffective due to several drawbacks (Saratale et al., 2011; Ngo and Tischler, 2022; Ikram et al., 2022) . Therefore, it is important to develop effective technologies for decolorizing synthetic dye. Several biotechnological approaches have attracted interest in treating these waste by involving bacteria or enzymatic (Saratale et al., 2011). The use of microorganisms or enzymes for decolorization offers various advantages, including being environmentally friendly and cost-effective. It also produces little

precipitate, non-toxic products and requires less water consumption compared to the physicochemical method (Lade et al., 2015).

Several bacteria strains have been identified as biodecolorizing agents, such as *Bacillus sp.* CH12 strain on Reactive Red 239 dye (Guadie et al., 2017). *Aeromonas sp.* has also been reported to have the ability to remove 90% of Novacron Brilliant Blue color (Karim et al., 2018). In addition, previous research showed that *Pseudomonas aeruginosa* and *Staphylococcus aureus* could decolorize congo red by 80% and 96%, respectively (Karnwal, 2019) *Comamonas aquatica* and *Ralstonia mannitolilytica* can also reduce methylene blue by 67.9% and 60.3%, respectively Siregar et al. (2020) and *Bacillus subtilis* WG13 has also been reported to be able to remove 70% of direct red 23 colors (Thiruppathi et al., 2021).

The application of biological methods in waste decolorization involves the use of microorganisms to degrade materials present in wastewater into components that are easily separated with a low pollution effect (Muharni et al., 2018). The effectiveness of these techniques is highly dependent on the adaptability and activity of the selected microorganisms. Therefore, the purpose of this study is to isolate indigenous bacteria from jumputan industrial waste and identify them using the 16S rRNA gene. The use of microorganisms as bioremediation agents has promising prospects because they can break down pollutant molecules through metabolic pathways that are usually used as a source of energy and growth (Marimuthu et al., 2013). This method is also cheap and environmentally friendly, making it an attractive option to explore. Isolation of bacteria from the jumputan industrial waste using direct red 80 has yet to be done.

2. EXPERIMENTAL SECTION

2.1 Method

2.1.1 Sampling Place

The sampling location was the Kertapati area, Tuan Kentang sub-district, Palembang City, South Sumatra Province, Indonesia. This region was a home industry area without a proper waste disposal site. The existing ditches were polluted by dye waste from dyeing jumputan cloth, it is possible to use the bacteria that are native to this area to degrade synthetic dyes since they have adapted to the dye waste. A pictorial illustration of the sampling locations is presented in Figure 1.

Figure 1. Wastewater Ditch in Tuan Kentang, Palembang City, South Sumatra, Indonesia, is the Location for Water/Sludge Sampling for Bacteria Isolation (Geographical Coordinates: 3*01*05*S104*45’23’E). Source: Google Inc, 2022

2.1.2 Isolation and Characterization of Bacteria from Jumputan Cloth Waste

The samples used in this study included water/sludge originating from Jumputan cloth industrial waste. Bacteria isolation was carried out using a pour plate with nutrient agar (NA) media containing 80 ppm of direct red 80 dye (Sigma-Aldrich), followed by incubation at 37°C for 48 hours. The colonies growing with surrounding clear zones were then selected for further characterization. The characterization carried out included colony morphology in the form of shape, color, margin, and elevation, as well as cell morphology through Gram staining. The biochemical tests performed were hydrolysis of starch, catalase, citrate, urea, motility, methyl red, and gelatin. The data obtained were then compared with Bergey’s Manual of Determination Bacteriology (Bergey, 1994).

2.1.3 Decolorizing Bacteria Ability Test

Each bacteria isolate was grown on Nutrient Broth (NB) media containing 80 direct red dye at a concentration of 80 ppm. The isolates were incubated for 120 hours using an incubator shaker at 37°C and with a speed of 120 rpm. Decolorization power was measured after the culture was centrifuged at 10,000 g for 10 minutes. The supernatant was then measured with a UV-Vis spectrophotometer at a wavelength of 524 nm. The initial and final absorbance values were recorded to compute the percent degradation according to Equation (1) given below (Khan et al., 2022).

$$\text{Decolorization Percentage} = (\%) \\ \frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100\% \quad (1)$$

2.1.4 DNA Extraction and 16S rRNA Gene Fragment Amplification

Selected bacteria isolates were grown in a NB at 37°C and harvested after 24 hours. DNA extraction was then performed using the TIANamp Bacteria DNA kit (Tiangen). The amplification was carried out using the primers Bact27_F 5’-AGAGTTT TGATCATGGTCCAG-3’ and Uni1492_R 5’-GGTTACCTT ACGACTT-3’. The PCR reaction consisted of Go Taq green mastermix (Promega), Primer, nuclease-free water, and DNA template. The process was carried out under denaturation at 95°C for 3 minutes, annealing at 55°C for 30 seconds, primary elongation at 72°C for 2 minutes, and final primary extension

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Table 1. Results of Characterization of Colony Morphology, Cell Morphology, and Biochemical Tests of Bacteria Isolates

Characteristics		Isolate					
		BD 01	BD 02	BD 03	BD 05	BD 06	BD 15
Cell Morphology	Form	Bacil	Bacil	Bacil	Bacil	Bacil	Bacil
	Grams	Positive	Negative	Negative	Negative	Negative	Positive
Colony Morphology	Form	Rounds	Rounds	Rounds	Rounds	Rounds	Rounds
	Color	Beige	Yellow	Yellow	Beige	Beige	Beige
	Edge	Lobate	Smooth	Crenate	Wavy	Crenate	Lobate
	Elevation	Flat	Flat	Low convex	Flat	Low convex	Low convex
Biochemistry	Starch Hydrolysis	+	+	+	+	+	+
	Catalase	+	+	+	+	+	+
	Citric	+	-	-	-	+	+
	Hydrolysis of Urea	-	-	+	+	+	-
	Motility	+	+	+	+	+	+
	Methyl Red	+	-	-	-	-	+
	Hydrolysis of Gelatin	+	+	-	-	-	+

Description: (+) Positive Result (-) Negative Result

at 72°C for 7 minutes (Idris et al., 2020). The PCR results were then visualized on a 1% agarose gel stained with Gelred under UV light.

2.1.5 DNA Sequencingn

Sequencing was determined through First Base commercial services, Labs Sdn. Bhd., Selangor Malaysia. The identity of a gene with a known sequence can be assessed by comparing the data available in GenBank through the BLAST program at the national center for biotechnology information or NCBI (`http://www.ncbi.nlm.nih.gov`) (Khaledian et al., 2020).

2.1.6 Phylogenetic Analysis

The data obtained were analyzed using a computer program, including alignment of sample DNA sequences with existing DNA sequences in GenBank, retrieval of DNA sequences from GenBank, editing of DNA sequences by BioEdit, alignment of DNA sequences using the Muscle program and construction of phylogenetic trees with MEGA XI.

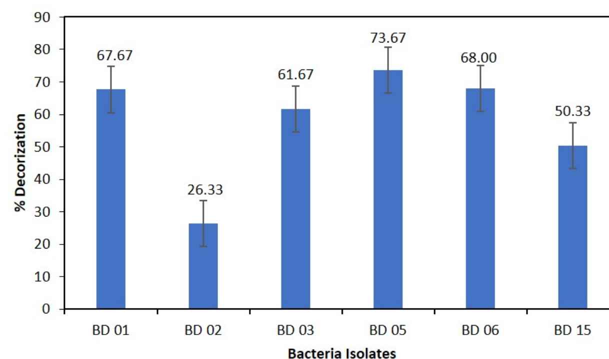

Figure 2. Decolorization Ability of Direct Red 80 Dye by Bacteria Isolates on NB Media, the Concentration of 80 ppm, Temperature 37°C for 120 Hours

3. RESULTS AND DISCUSSION

3.1 Morphological Characteristics of Bacteria Isolates

Isolation of bacteria from Palembang Jumputan cloth industrial waste produced 17 isolates, but only 6 had a clear zone around the colony on nutrient agar media containing 80 ppm of direct red 80 dye. The isolates were coded BD 01, BD 02, BD 03, BD 05, BD 06, and BD 15, which had different morphological characters, as shown in Table 1.

Characterization of colony and cell morphology, as well as several biochemical tests were carried out to support molecular identification. Based on morphological and biochemical characteristics and referring to Bergey's Manual of Determination Bacteriology, it has been shown that the isolates belonging to the dominating features indicate to the genus *Pseudomonas*, *Aeromonas* and *Bacillus*. Bacteria that are abundant in waste containing synthetic dye are the *Pseudomonas*, *Enterobacter*, and *Bacillus* groups, both aerobic and anaerobic (Cong et al., 2022).

3.2 Ability to Decolorize Bacteria Isolates

Biodecolorization was a decrease in dye that can occur through biodegradation and bioabsorption processes. Biodegradation occurred through degradation of enzymes by bacteria. Meanwhile, the mechanism of biosorption was through absorption by bacteria cell wall due to the presence of components, such as peptidoglycan, polysaccharides, and hydroxyl and carboxyl groups. These components can bind to the chemical structure of dye (Sari and Simarani, 2019). The ability to decolorize bacteria isolates ranged from 26.33–73.67%, where the lowest power was found in isolate BD 02 at 26.33±0.94%, while the highest of 73.67±0.47% was obtained in BD 05, as shown in Figure 2.

Each bacteria strain had a different decolorization potential,

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indicating that they were affected by various environmental factors in decolorizing direct red 80 dye. Temperature, pH, and dye concentration were among the environmental parameters. Increasing the dye concentration will affect the decolorization power because it is related to dye's toxic properties. These findings are consistent with Zhuang et al. (2020) that high levels can cause toxicity to bacteria communities. pH was one of the most important environmental factors affecting enzyme activity and bacteria degradation potential. The pH used in this study was 6.8, Shi et al. (2021) stated that the optimal pH range for biodegradation was 6–10. Temperature has also been reported to affect the biodegradation of dye by bacteria, and the optimum range for azo-reactive dye was 30–40°C (Garg and Tripathi, 2017).

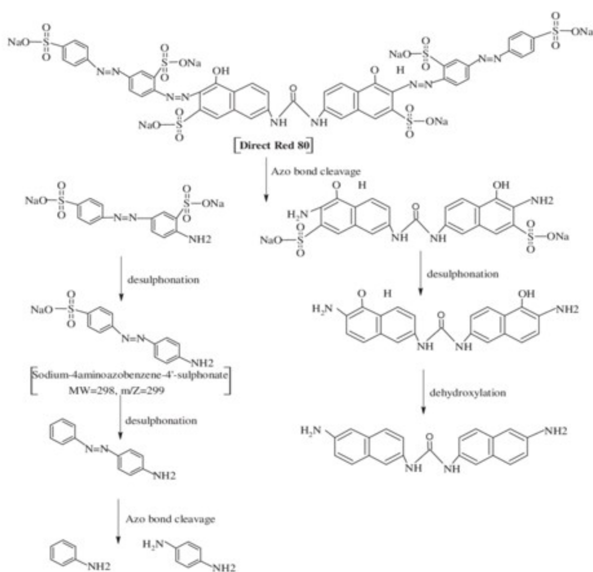

Figure 3. Proposed Mechanism for Breaking the Azo Direct Red 80 Bonds by the Azoreductase Enzyme Miran et al. (2016)

The effectiveness of microbial decolorization can be influenced by various factors, including adaptability, the chemical structure of dye, and various enzyme activities secreted by these microorganisms. The ability of bacteria to degrade dye was closely related to the activity of the enzymes synthesized (Fareed et al., 2022). The biodegradation of azo dye by bacteria occurred enzymatically involving azoreductase and laccase (Sarkar et al., 2020). Azoreductases required reducing agents, such as FADH2, NADPH, and NADH to carry out their functions (Cong et al., 2022). The performance of this enzyme often occurred anaerobically because the presence of oxygen can interfere with the azo bond reduction process. Rasool et al. (2013) stated that degradation of direct red 80 dye produced metabolites in the form of aniline and 1,4-diamino benzene. Figure 3 shows the possible direct red dye degradation pathway 80.

3.3 Molecular Identification

Molecular identification was carried out to verify the six bacteria isolates that can remove direct red dye. The advantage of using molecular techniques is their capacity to identify isolates down to the species level and to reveal family ties among isolates. The 16S rRNA gene of these isolates was successfully PCR amplified, as indicated by the presence of a single DNA band of about 1500 bp in the following agarose gel electrophoresis (Figure 4).

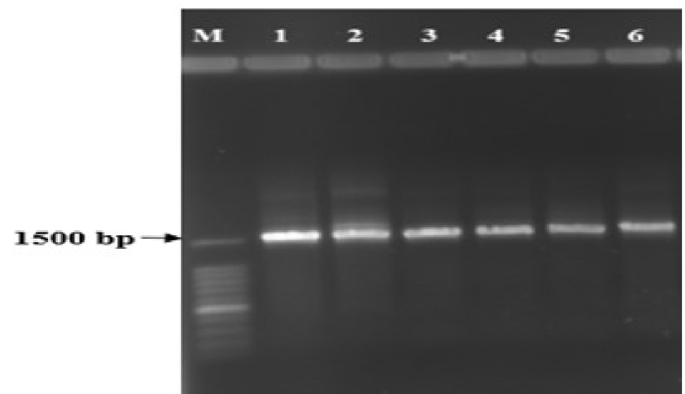

Figure 4. Electrophorogram of Amplified Bacteria Isolates Degrading Direct Red 80 Dye (M=DNA Marker, 1=BD01, 2=BD02, 3=BD03, 4=BD05, 5=BD06 and 6=BD15)

The phylogenetic tree in Figure 5 shows the kinship between bacteria BD 01 and BD 15. The results showed that these two isolates were similar to *Bacillus tropicus* strain MCCC 1A01406 with 97% similarity. BD 01 and BD 15 were related to *Bacillus tropicus* strain MCCC 1A01406, one of the nine new isolates belonging to the *Bacillus cereus* group. Liu et al. (2017) stated that this bacteria was a derivative of *Bacillus cereus* with Gram-positive characteristics. It was also reported to be a facultative anaerobic with motility using perithricous type flagella. This bacteria was isolated from sediment deposits of the South China Sea with a Guanine and Cytosine percentage of 35.2% mol. These bacteria often grew optimally at a pH of 6 with an optimum ambient temperature of 30°C.

Previous reports have shown that *Bacillus tropicus* bacteria has not been utilized as a biodegradable agent for textile industry wastewater. However, *Bacillus cereus* in this same group has been tested for its ability to degrade synthetic dye. It can also decolorize synthetic dye, namely methyl orange by 75% with a concentration of 100 mg/L, at pH 7.2 and temperature 37°C, respectively, for 144 hours (Karnwal, 2019). *Bacillus subtilis* can remove Direct violet color by 68.57% with a concentration of 20 mg/L at pH 7 and 28°C for 72 hours. The metabolites formed during this process were ethanol, 2(2-butyl epoxy), followed by 4-methyl benzoic acid and phenol, 2,4-bis (1,1-dimethyl ethyl) Abd El Rahim et al. (2021) Isolate BD 02 had 97% similarity with the nucleotide base sequence of *Aeromonas jandaei* strain ATTC 49568 bacteria (Figure 6). According to Johnson et al. (2019), sequences with a 97% homology level

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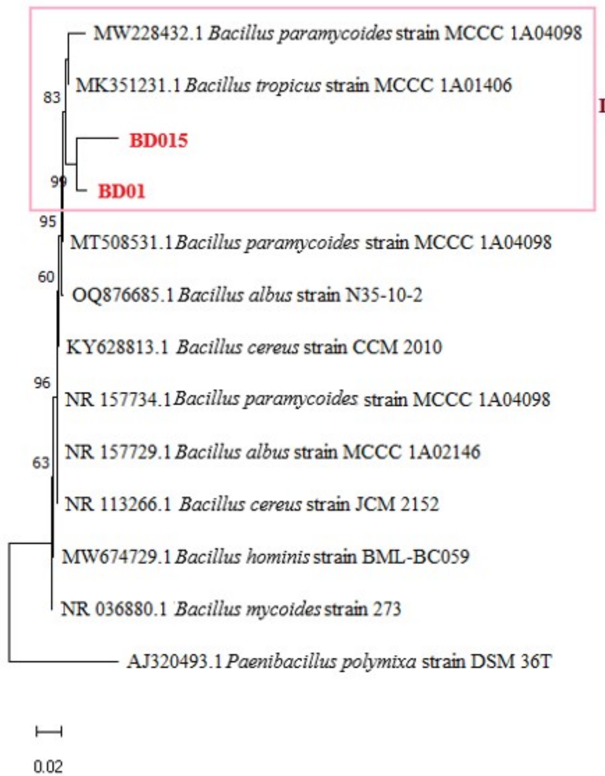

Figure 5. The Phylogenetic Tree of Isolates BD 01 and BD 15 are Similar to *Bacillus tropicus* Strain MCCC 1A01406 Using the Neighbor-joining Method, Bootstrap Values Under 60 are Concealed

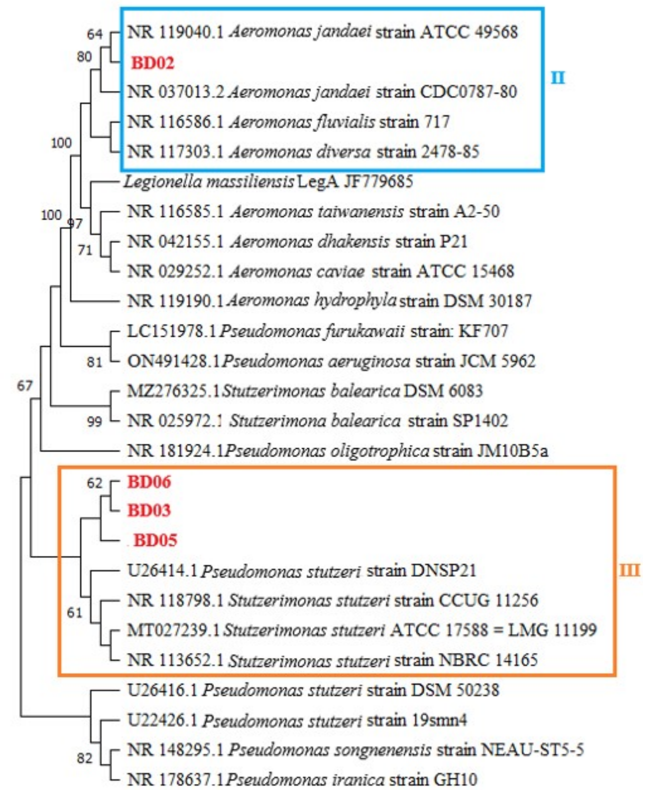

Figure 6. The Phylogenetic Tree of Isolates BD02 are Similar to *Aeromonas jandaei* Strain ATTC 49568 and BD06, BD03, BD05 are Similar to *Pseudomonas stutzeri* Strain DNSP21 using the Neighbor-joining Method, Bootstrap Values Under 60 are Concealed

represented the same species.

The ability of *Aeromonas jandaei* as a degrader of synthetic dye is still unknown, but the *Aeromonas* group has been reported for its decolorization ability. Bharagava et al. (2018) reported that *A. hydrophila* can decolorize crystal violet up to 99% at pH 7 and a temperature of 35°C, in a culture medium consisting of co-substrates, such as yeast extract and sucrose. Several metabolites, such as phenol, 2,6-bis (1,1-dimethyl ethyl), 2ÿ,6ÿ-dihydroxy acetophenone, and benzene were formed during the transformation of crystal violet by *Aeromonas hydrophila*. Decolorization of Novacron Brilliant Blue dye by *Aeromonas sp*. reached 90% at a concentration of 100 mg/L, pH 7, and a temperature of 37°C for 144 hours (Karim et al., 2018).

Phylogenetic analysis showed that BD 03, BD 05 and BD 06 isolates have similar nucleotide sequences with bacteria *Pseudomonas stutzeri* strain DNSP21 (Figure 6), but the similarity index for BD 06, the similarity was below 90%. Isolate BD 06 had less homology with the sequence of *Pseudomonas stutzeri* strain DSM 590, with a sequence similarity percentage of 85%. It also has the potential to be considered a different species from *Pseudomonas stutzeri* strain DSM 590, and a polyphasic taxonomy study was needed for confirmation. Several reports showed that *Pseudomonas stutzeri* has been widely used for processing wastewater containing synthetic dye. This bacteria acted as a biodecolorization agent and detoxifier for dye wastewater. *Pseudomonas stutzeri* showed the highest potential for degradation. However, it was less effective in degrading red dye, especially Red Synozol (Fouda et al., 2016). *Pseudomonas aeruginosa* was reported to have the ability to decolorize reactive red 198 with a concentration of 100 mg/L up by 96% at pH 7.2 and a temperature of 37°C for 144 hours (Karnwal, 2019).

4. CONCLUSION

Bacteria isolation from Jumputan cloth industrial waste yielded 6 bacteria isolates that can degrade synthetic dye direct red 80, with decolorization powers ranging from 26.33±0.94 – 73.67±0.47. The greatest potential for decolorizing waste synthetic dye is seen in isolate BD 05. The phylogenetic analysis showed that there were 3 genera of bacteria namely *Bacillus*, *Aeromonas*, and *Psedomonas*. These bacteria were closely related to *Bacillus tropicus*, *Areomonas jandaei*, and *Pseudomonas stutzeri*. With regard to handling jumputan industrial waste, *Pseudomonas stutzeri* (BD 05) has the greatest potential.

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