Decolorization of Azo Synthetic Textile Dye by Halotolerant Bacteria And Identification Used By 16S rRNA

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Abstract. Textile dye has been used in textile industry to produce carpet, batik, cosmetics as well as pharmaceutical products. Utilizing of textile dye especially azo dye in textile industries is more efficient than natural dye. The high production of textile increases liquid wastes. If the liquid waste is not treated properly and discharged directly to environment, it causes pollution that could harm both organism and the environment itself. Azo textile dye is difficult to be degraded in the naturally environment. The aim of this research is to study the potency of halotolerant bacteria to decolorize azo textile dye. The isolates were isolated from the sea around of Mursala Island, Tapian Nauli village, Central Tapanuli district. In this research, the highest percentage of decolorization was shown by 2 isolates, DH2 and DH3. The quantitative decolorization test was performed in MSM liquid media which were amended by three different pH variations: 6, 7 and 8; Three different azo concentrations which were: 600, 800 dan 1000 ppm and three different salinity of media which were: 0 %, 5%, and 10%. The optimum conditions for isolate to degrade azo dye were shown by isolate DH2 at pH 6, salinity 5% and azo concentration of 600 ppm which was 49.64%. Based on 16S rRNA gene, it was known that DH2 was identified to close to Stenotrophomonas pavanii strain LMG 25348 with homology of 97%.

1. Introduction

Textile dyes have been used in textile industry to produce yarn, batik as well as cosmetics products. Utilizing synthetic dyes for textile industry is more efficient than natural dye because textile dye more tightly banding to fiber material, more practical, efficiency for dyeing and it availability is warrantee. The high usage textile dye for textile industry will influential to increases contaminant substances in liquid wastes which are produced and released to environment [1]. Presence of the dye in aqueous ecosystem diminishes the photosynthetic by impeding the light penetration that will be disruptive for flora and fauna which lives in water. The consequence that emerges from discharge liquid wastes which are not treated properly will be able polluted aqueous ecosystem. Water polluted can be prevented if liquid wastes treated by waste treatment process for decolorization of textile liquid wastes. The conventional treatment methods cannot efficiently to decreases non-biodegradable recalcitrant compound in textile dye. Recalcitrant compound in textile dye could causing health hazards if the liquid wastes appear in environment for a long time because the compound is carsinogen and mutagenic then alternative for treatment process effectiveness is necessary to be found [2]. Common chemical or physicochemical treatment methods are adsorption, chemical transformation, incineration, photocatalyst and ozonation. However, chemical or...
physicochemical methods are generally costly, less efficient and of limited applicability and produce wastes, which are difficult to dispose of.

As a viable alternative, biological process have received increasing interest owing to their cost effectiveness, ability to produce less sludge and environmentally friendly [3,4]. Microorganisms are capable of decolorizing the azo dyes have been reported such as Alcaligens eutrophus, Bacillus subtilis, Klebsiella pneumonia, Pseudomonas stutzeri, and Spingomonas sp. But utilizing halotolerant bacteria for decolorization has rarely been reported. Thus, the aim of this work was to investigate the role of halotolerant bacteria in delorization of textile dye.

2. Material and Method

2.1. Isolation of Halotolerant bacteria from ocean around of Mursala Island
Ocean water from ocean around of Mursala Island was collected randomly and stored in sterilized winkle flask. Then 1 ml of the ocean water added to flask containing Nutrient Broth (NB). Inoculum media incubated in rotary shaker 130 rpm at 30°C for 24 hours. Inoculum media 0.1 ml added to flask containing sterilized water 9.9 ml. Isolate dilute until 10^{-8}. Isolate with dilution 10^{-6}, 10^{-7} and 10^{-8}. Aliquots of 100 µl of each dilution were plated on Zobell media. Plates were incubated for 24 hour. Isolates were purified by plate streaking technique on Nutrient Agar (NA) [5].

2.2. Screening of decolorizers
Ten isolates were cultured to select the effective isolate for decolorization by screening test. Then 45.5 ml of decolorizing medium (MSM) was sterilized in conical flask, inoculated 500 µl bacterial suspension with 0.1 (absorbance measured by spectrophotometer) added to each of the 100 ml conical flask that which containing Minimum Salt Medium (MSM) + Azo dye. The variation culture conditions such as azo dye concentrations, salinity of medium and pH variations. Each variations for screening condition 600 ppm, 5% and pH 6. Cultures were then incubated in rotary shaker 130 rpm at 30°C for 12 days. After 12 days the color intensities were measured in a spectrophotometer at 482 nm. Isolates were then tested for their color removal ability in submerged culture. Two promising isolates were selected.

2.3. Degradation test under different culture conditions
The promising isolate was tested for degradation test. Different decolorization efficiencies were obtained at different concentrations of azo dye (600 ppm, 800 ppm and 1000 ppm), salinity of medium from 40.5 g NaCl (0%, 5%, 10%) and range pH (6, 7, 8). The pH range suitable for growth was deduced. For the pH higher than 6 added NaOH and for lower added HCL. Then 45.5 ml of decolorizing medium (MSM) was sterilized in conical flask, inoculated 500 µl bacterial suspension with 0.1 (absorbance measured by spectrophotometer) added to each of the 100 ml conical flask that which containing Minimum Salt Medium (MSM) + Azo dye. The variation culture conditions such as azo dye concentrations, salinity of medium and pH variations [6]. Each variations for screening condition 600 ppm, 5% and pH 6. Cultures were then incubated and color removal abilities were tested using same procedures.

2.4. Decolorization measured degradation azo dye
To measure decolorization used by spectrophotometer, sampling was done at different time intervals from inoculated decolorizing media. Uninoculated culture media added azo dye were used as control. The decolorization efficiency of different isolates was calculated as

\[
\text{Decolorization} \% = \frac{(A_0 - A)}{A_0} \times 100,
\]

Where \(A_0\) is the initial absorbance and \(A\) is the absorbance of medium after decolorization at the \(\lambda_{\text{max}}\) (nm) of each dye.
2.5. Identification of the isolates
The genomic DNA of the two isolates selected were extracted with conventional methods for DNA extraction. The 16S rRNA were amplified using 63f (5’-CAG GCC TAA CAC ATG CAA GTC-3’) and 1387r (5’-GGG CGG WGT GTA CAA GGG-3’) universal primers (Marchesi et al. 1998). The PCR reaction conditions included initial denaturation at 94°C for 2 min, denaturation 92°C for 30 s, annealing 60°C for 30 s, elongation 72°C for 1 min and post PCR 72°C for 5 min. PCR cycle was run for 35 cycles. The purified PCR products were sequenced in Seoul, South Korea using an automated DNA sequencer (ABI 3730xl DNA Analyzer, Applied Biosystems).

3. Results and Discussions
3.1. Isolation and identification
After isolation and purified halotolerant bacteria were isolated. Primary biochemical and physiological tests were performed to differentiate the isolated. All isolates were assayed to decolorize azo dye. Based on decolorization tests including those performed at higher decolorization in concentration azo dye 600 ppm, 5% and pH 6. Two isolates, namely DH2 and DH3 were selected for further study. 1190 bp of 16S rRNA gene of isolate DH2. the isolates DH2 was 97% similar to Stenotrophomonas pavanii, some characteristics such as Gram negative, nonmotile and catalase positive. Stenotrophomonas sp. In liquid wastes have not be founded. Furthermore, experiments about Stenotrophomonas acidamiphila have been used in consortium bacteria with Bacillus cereus, P. putida and P. fluorescence for bioreactor to decrease synthetic dye liquid wastes concentrations [7]. Moreover, Stenotrophomonas maltophilia is reported to be used as biodegradation agent and decolorization of wastewater textile industry [8].

3.2. Screening tests on Minimum Salt Medium (MSM)
Ten isolates grew on broth of Minimum Salt Medium each supplemented with concentration azo dye 600 ppm, salinity of media 5% and pH 6. Halotolerant bacteria showed various growth abilities in response for the treatment. The best two isolates which ability to decolorize azo dye with higher percentage for decolorization efficiency were DH2 and DH3.

| Isolate code | Decolorization efficiency (%) |
|--------------|-------------------------------|
| DH2          | 19.40                         |
| DH3          | 7.26                          |

The ability of DH2 and DH3 to decolorize azo dye showed that these isolates were capable to used substrate as source of nutrition for grew in harm condition [9]. The ability bacteria for grew can be due such as substrate concentration, medium composition, dyes concentration, pH, oxygen, carbon and nitrogen sources [10,11,12].

3.3. Decolorization efficiency of isolate DH2
The potential isolate from screening test then tested to known isolate ability for decolorization dye substance with various treatments. The reduction in dyestuff on day 12 with the highest decolorization power was, among others, the combination treatment of variation treatment with pH 8, azo concentration of 600 ppm and salinity of 5%; pH 7, 600 ppm, 5% and pH 7, 800 ppm, 5% with decolorization power of 49.64%, 44.48% and 32.48% respectively. Among all treatments the best results were obtained at pH 8, azo concentration of 600 ppm and salinity of 5% with a decrease of 49.64%. The effect of the concentration of dyes on the process of reducing dyes is generally inversely proportional, the higher the concentration of the dyestuff, the ability to decompose the dyes decreases. The reduced ability of bacteria to decolorize dyes is caused by the presence of heavy metal compounds.
contained in textile dyes. So that if the concentration of the dye increases, the toxicity level also increases.

Table 2. Decolorization efficiency through time interval analysis

| Dye concentration (ppm) | Salinity (%) | Mean of absorbance | Decolorization efficiency (%) |
|-------------------------|--------------|--------------------|-------------------------------|
|                         |              | Time interval analysis |                               |
|                         |              | 0     | 3     | 6     | 9     | 12    |
| pH 7                   | 600          | 5     | 1,76  | 1,53  | 1,38  | 1,34  | 0,98  | 44,48 |
|                         | 800          | 5     | 1,89  | 1,62  | 1,47  | 1,32  | 1,27  | 32,48 |
| pH 8                   | 600          | 5     | 2,17  | 1,52  | 1,51  | 1,47  | 1,09  | 49,64 |

This mechanism involves forming metals with proteins in cell membranes, so that heavy metals contained in textile dyes do not accumulate without disrupting bacterial growth. The reduction process occurs because the intracellular electron transport chain to the mediator, which results in color removal. Another possibility is that bacteria interact between intracellular electron transport systems and extracellular dyes through electrons transferring proteins in the outer membrane [13]. However, the higher the concentration of dyes, the higher concentration of metal accumulated. Accumulation of heavy metals from dyes that are too high can inhibit cell growth because the organism's protection system is no longer able to reduce the toxic effects of metals contained in dyes.

3.4. Growth performance of isolate DH2

The measurement of decolorization activity by calculating the number of colonies using the Total Plate Count (TPC) method on day 3 to day 12 of several variations between pH, dye concentration and salinity levels using DH2 isolates shows the growth of isolates at each time interval. The highest growth in the number of bacteria was in the treatment of variations in pH 8, 800 ppm, 5% by 5.74 log cfu with decolorization power of 17.91%. Decolorization power of 17.91% is not the highest. The highest decolorization power was 49.64%, the growth of the number of bacteria was 5.19 log cfu. For the highest growth of the number of bacteria then found at pH 7, 800 ppm, 10% at 5.57 log cfu with decolorization power is quite low 4.17%. At pH 6, 600 ppm, 0% of the amount of bacterial growth is high including 5.31 log cfu with decolorization of 11.69%. The amount of bacterial growth of 5.31 log cfu is also owned by treatment at pH 6, 800 ppm, 10% with decolorization power of 7.26%.

Table 3. Growth performance through time interval analysis

| Dye concentration (ppm) | Salinity (%) | Cell count (log CFU/mL) | Mean Cell count (log CFU/mL) |
|-------------------------|--------------|-------------------------|-----------------------------|
|                         |              | Time interval analysis |                              |
|                         |              | 0     | 3     | 6     | 9     | 12    |
| pH 7                   | 800          | 10    | 3,85  | 5,44  | 7,64  | 5,51  | 4,85  | 5,57  |
| pH 8                   | 800          | 5     | 3,85  | 5,61  | 5,44  | 5,56  | 7,69  | 5,74  |

This is because each bacterium has a generation time interval and the ability to use dyes as a carbon source is different and there are only a few bacteria that can grow with azo compounds as the only carbon source. Microorganism contained in liquid wastes of textile dyes produces azoreductase enzymes so that they can reduce azo dyes [14]. One typical characteristic found in microorganisms
capable of decolorizing textile dyes is the ability of these microorganisms to secrete enzymes such as azoreductase, laccase or peroxidase. Bacteria can produce enzymes constitutively and inductively [15].

3.5. Decolorization efficiency of isolate DH3
The measurement of decolorization activity with variations in pH parameters carried out in the initial conditions, days 3 to 12 of several variations between pH, dye concentration and salinity using DH3 isolates showed a decrease in color in each time interval. The highest reduction in dyestuff on day 12 with decolorization power was among variations in treatment pH 6, 600 ppm, 5% at 23.40% and pH 6, 800 ppm, 10% at 24.89%. Furthermore, in the treatment of variations with pH 7, 800 ppm, 0% was 22.49%.

| Table 4. Decolorization efficiency through time interval analysis |
|---------------------------------------------------------------|
| Dye concentration (ppm) | Salinity (%) | Mean of absorbance | Decolorization efficiency (%) |
|-------------------------|--------------|---------------------|--------------------------------|
| Time interval analysis   | 0 | 3 | 6 | 9 | 12 |                                      |
| pH 7                    | 600 | 5 | 1.88 | 1.77 | 1.76 | 1.67 | 1.44 | 23.40 |
|                         | 800 | 5 | 2.37 | 2.24 | 2.18 | 1.92 | 1.78 | 24.89 |
| pH 8                    | 600 | 5 | 1.88 | 1.78 | 1.63 | 1.56 | 1.46 | 22.49 |

The pH treatment which showed the highest decolorization was found at pH 6 with decolorization power of 24.89% but this pH condition also had the lowest decolorization at 1000 ppm dye concentration with a concentration of 4.83%. The process of decomposing dyes is strongly influenced by pH. Enzymes generally work optimally in the pH range 6-8. In general, microbial groups have different pH characteristics. Although microorganisms grow in a wide pH range and are far from optimum, there are tolerance limits for growth [16].

3.6. Growth performance of isolate DH3
The decrease in dyestuff on day 12 with the highest amount of bacterial growth was found in the treatment of variations in pH 7, 800 ppm, 5% by 6.07 log cfu with decolorization 3.52%. Variation in pH 6, 800 ppm, 10% in the amount of bacterial growth was 5.67 log cfu with decolorization power which was the highest, which was 28.34%. This is in accordance with the greater the number of colonies growth, the higher the power of decoloration. Some of the highest decolorization in DH3 is at pH 6, 600 ppm, 5% at 23.40% and the amount of bacterial growth is 5.21 log cfu. The highest decolorization power of 22.49% was found at pH 7, 800 ppm, 0% with a colony number of 5.24 log cfu.

| Table 5. Growth performance through time interval analysis |
|----------------------------------------------------------|
| Dye concentration (ppm) | Salinity of medium (%) | Cell count (log CFU/mL) | Mean Cell count (log CFU/mL) |
|-------------------------|-------------------------|------------------------|-----------------------------|
| Time interval analysis   | 0 | 3 | 6 | 9 | 12 |                                      |
| pH 7                    | 800 | 10 | 3.85 | 7.63 | 5.63 | 5.42 | 5.26 | 5,67 |
|                         | 800 | 5 | 3.85 | 5.37 | 7.48 | 5.34 | 7.76 | 6,07 |
However, on the 12th day the growth of the number of colonies declined. This is due to the resilience and sustainability of the growth of microorganisms depending on the availability of sufficient nutrients and a good growth environment. In addition, the amount of nutrients present in the initial growth medium has the potential to form final biomass. So that changes in nutrient availability affect the growth of bacteria and biomass products [17]. Nutrients from growth media were no longer available and there was an accumulation of toxic metabolic results which resulted in a drastic decrease in the population of bacteria. In the stationary phase the number of cell populations remains because the number of cells dividing is equal to the number of cells that die [18].

Acknowledgement
The paper is part of undergraduate thesis of first author.

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