KnE Life Sciences
IC-BIOLIS
The 2019 International Conference on Biotechnology and Life Sciences
Volume 2020

Conference Paper

Metabolite Identification from Biodegradation of Congo Red by *Pichia* sp.

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Abstract

Azo dyes are commonly used in textile and paper industries. However, its improper disposal often results in polluting water bodies. Azo dyes can cause adverse health effects because of its carcinogenic properties. Various methods to remove azo dyes from water have been proposed, including biological methods such as biosorption and biodegradation. Biosorption and biodegradation were done by using bacteria, yeast or mold. In general, yeasts have some advantages for azo dyes degradation due to its faster growth compared to mold and better resistance against unfavorable environment compared to bacteria. Previously, we observed that yeast *Pichia* sp. have the ability to degrade Congo red, an azo dye. However, information regarding biodegradation of azo dyes by *Pichia* sp. are still limited. Therefore, in this study, we showed degradation of Congo red by *Pichia* sp. crude enzyme extract obtained from separating *Pichia* cells from medium by centrifugation, followed by identification of its biodegradation products. Biodegradation product was separated from enzyme by ethyl acetate and then Gas Chromatography-Mass Spectroscopy (GC-MS) method was employed to identify biodegradation product. Chromatogram results of GC-MS showed that Congo red were degraded into various products such as biphenyl, naphthalene and smaller molecules with 94 m/z and 51 m/z. These results suggest involvement of azo reductase and laccase-like enzymes which cleaves azo bonds and oxidize the dye molecules to smaller molecules. This study implies the use of *Pichia* sp. as bioremediation agent for the removal of azo dyes.

Keywords: Biodegradation, Congo red, *Pichia* sp., metabolite identification, GC-MS

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Received: 1 February 2020
Accepted: 8 February 2020
Published: 16 February 2020

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the IC-BIOLIS Conference Committee.

1. Introduction

Currently, more than 100,000 types of dyes are used every year and textile industry used 70% of it. Most of them are synthetic dyes which contains aromatic compounds. Textile dyes are grouped into different groups according to their chromophores such as azo, nitro, indigoid, anthraquinone, phthalein, triphenyl methyl, and nitroso[1]. Between those classes, azo dye is one of the most used dyes due to its characteristics: availability in various colors, easier to use in textile production, and resistant to fading due to external

factors. Azo dyes are known to resist degradation by heat, water, light, detergents and perspiration because of its chemical structure [2, 3].

Azo dye stability causes it to remain in water bodies for a long time, causing problems to the ecosystem. Azo dye in concentration as low as 1 ppm is visible, and textile processing wastewater usually contains 10-200 ppm of dye [4]. The dye molecules will block sunlight from photosynthetic organisms in the water, causing photosynthesis to be disrupted and reduces dissolved oxygen in the water. Lack of oxygen will result in the death of organisms and anaerobic degradation of organic materials which yields bad smelling products. There is also danger such as poisoning from acute dye exposure or cancer from chronic exposure due to dyes' mutagenic and carcinogenic properties[5, 6].

There are various textile dye wastewater decolorization methods known and developed, including biological methods. Biological methods include bioaccumulation, biosorption, and biodegradation. Bioaccumulation is the accumulation of dye from the environment by microorganisms into its cells as part of its metabolism. Biosorption is adsorption of dye molecules to biological material such as microbial biomass. Adsorption occurred through the interaction between dye molecules and function groups on cell walls. Biodegradation is the degradation of dye molecules into smaller products by enzymes produced by microorganisms. Well-known enzymes, which participate in the biodegradation process are oxidoreductases such as laccases, manganese peroxidases, lignin peroxidases and azo reductases[6--9].

To date, there are various microbes known to be capable in biological decolorization of textile dye. The microbes reported are classified in the genus *Bacillus*[10], *Enterobacter*[11], *Candida*[12], *Aspergillus*[13], and many others. However, microbes utilized in biological decolorization might be pathogenic[14] and/or produces toxic products, such as aromatic amines[5].

Fermented food is one of the possible sources of non-pathogenic bacteria and fungi because the safety of fermented food is regulated to ensure the safety of its consumer[15]. Yeasts from *Pichia* genus are reported to be found in various fermented foods, including from Indonesian tapai[16, 17]. On the other hand, *Pichia* are known to be able to decolorize various azo dyes such as reactive red[18, 19], reactive orange[20] and reactive blue[21]. However, there is no report yet about enzymatic decolorization of Congo red by yeast from *Pichia* genus and its products. Hence it is important to know the decolorization mechanism and identify its metabolites.

2. Methods

2.1. Materials

Yeast *Pichia* sp. isolate was purified from previous experiment from tapai starter culture. Culture medium used for the experiment were glucose-yeast extract-peptone (GYP) medium composed of 1.0% (w/v) glucose, 0.5% yeast extract and 0.5% peptone. Medium used for decolorization experiment were Congo red GYP medium, which contains GYP medium supplemented by 0.005% (w/v) Congo red. Buffers used in this research was 0.1 M sodium acetate at pH 5. Solvent used were LC-MS grade methanol and ethyl acetate.

2.2. Decolorization of Congo red in liquid medium

Yeast isolate were grown in CRGYP medium for 24 hours at 35 ˚C, and the cell suspension was centrifuged at 8000 rpm to obtain the supernatant. To observe enzymatic decolorization, 250 µl of supernatant was taken and mixed into 4750 µl 0.005% (w/v) Congo red solution and incubated for 1 hour at 35 ˚C, then the changes in absorbance were measured using spectrophotometer. Supernatant were analysed using spectrophotometer (BioDrop DUO, England) to obtain the absorbance spectrum at 300 -- 800 nm.

Decolorization percentage of Congo red was calculated by

$$Decolorization\ (\%) = \frac{A_0 - A_1}{A_0}$$

with A_0 is absorbance at 485 nm before decolorization, and A_1 is absorbance at 485 nm after decolorization.

2.3. Identification of metabolites from decolorization

For identification of metabolites preparation, 5 ml of supernatant of decolorized sample were taken and added with 5 ml of ethyl acetate. Metabolite was extracted by shaking using vortex mixer. The organic phase was separated and dried using a rotary evaporator. The resulting product was re-dissolved in methanol. Remaining water content from the sample was removed using Na_2SO_4.

The metabolites extracted were identified using P2010 SE Standard GC-MS (Shimadzu, Japan) with TraceGold TG -- 5 SiMS GC Column. Ionization voltage was set at 70 eV and helium gas flow was set at 1 ml/minute for 35 minutes of run time. Starting temperature was set at 80 ˚C for two minutes and increased gradually by 10˚C/minute until temperature reached 280 ˚C and held for seven minutes.

3. Result and Discussion

3.1. Decolorization of Congo red in liquid medium

Figure 1 showed Congo red degradation by crude enzyme extract. The result of absorbance spectra from UV-Vis spectrophotometer showed there were two absorbance peaks of Congo red, the slightly lower one at 335 nm and higher one at 485 nm. The observed maximum absorbance wavelength of Congo red was known to be at 497-498 nm, different from the result observed. Since decolorization was tested in sodium acetate buffer, the content of the buffer increases the ionic strength of the solution and causes the peak wavelength of Congo red absorbance to shift toward shorter wavelength[22, 23]. Both peaks at 335 nm and 485 nm existed before and after decolorization by crude enzyme from *Pichia* sp., however there was decrease in overall absorbance. After one hour, there was 51.7% decrease of absorbance at 485 nm which is equivalent to a decrease of Congo red concentration from 0.005% to 0.0026%.

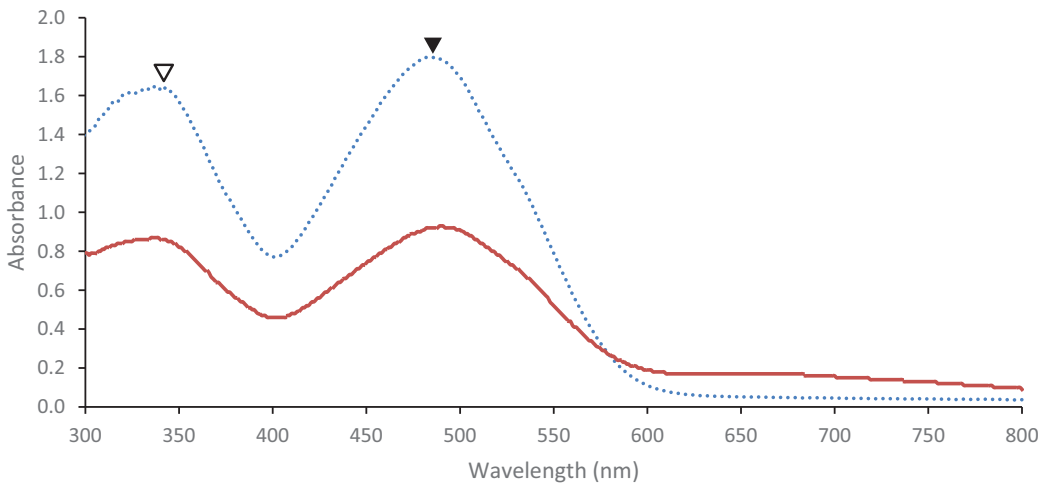

Figure 1: Absorbance spectrum of Congo red degraded by *Pichia* sp. crude enzyme extract.

Empty triangle shows peak at 335 nm and filled triangle shows peak at 485 nm. Dotted lines indicated absorbance before decolorization and dashed line showed absorbance after one hour of decolorization.

3.2. Identification of metabolites from decolorization

GC-MS analysis result in Figure 2 showed there were compounds with lower molecular mass compared to Congo red. It indicated biodegradation of Congo red into various metabolites. Previously, multiple microorganisms were tested to decolorize Congo red with varying decolorization percentage under various incubation time. *Bacillus thuringiensis* could remove 72.84% of Congo red in 12 hours [10], while *Shewanella xiamenensis* BC01 were reported to decolorize 87.5% Congo red after 6 hours[24]. Their results showed fewer compounds in the GC-MS result compared to this study due to shorter incubation time used here and multiple steps involved in enzymatic degradation, there were more intermediates with various molecular weight formed which were not completely degraded to simple products.

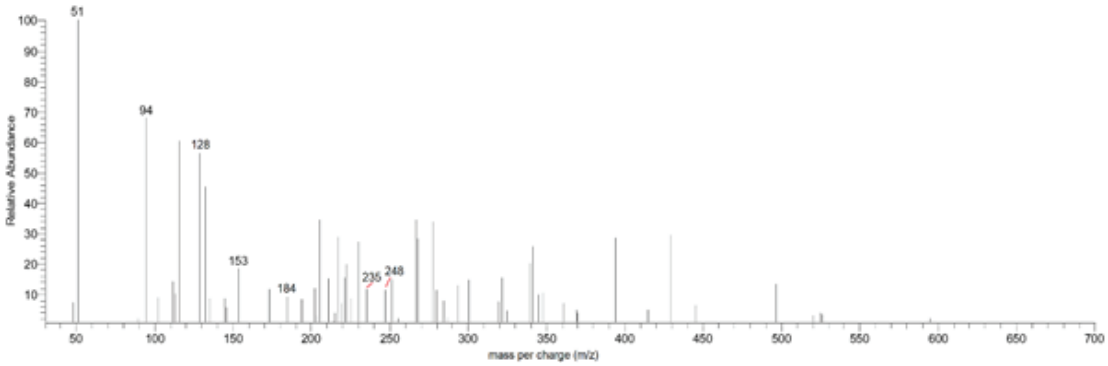

Figure 2: GC-MS result.

In this study we focused to discuss some of the metabolites found were very similar to the suggested decolorization products by azo reductase and laccase [3]. The cleavage of Congo red azo bonds by azo reductase resulted in three smaller molecules. The peak at 184 m/z indicated a molecule with 184 molecular weight (MW = 184), biphenyl-4,4'-diamine and two molecules of sodium 3,4-diaminonaphthalene-1-sulfonate (MW = 260). However, our result shows no peaks around 260 m/z, yet showed one peak at around 235 m/z. It could be explained that the sodium atom from sodium 3,4-diaminonaphthalene-1-sulfonate was removed and resulted in 3,4-diaminonaphthalene-1-sulfonate with approximate MW of 237. Ng *et al.*[24] also suggested a metabolite of MW = 244, which is 1,2'-diaminonaphthalene 4-sulfonic acid. In the present result there was one single peak around 248 m/z which suggested the possibility of Congo red breakdown by *Pichia* sp. resulted in 1,2'-diaminonaphthalene 4-sulfonic acid. Further degradation is suggested to involve laccase and would result in smaller molecules. Biphenyl-4,4'-diamine was broken down into biphenyl, shown by a small peak at 153 m/z. Meanwhile, the 1,2'-diaminonaphthalene 4-sulfonic acid would be deaminated and

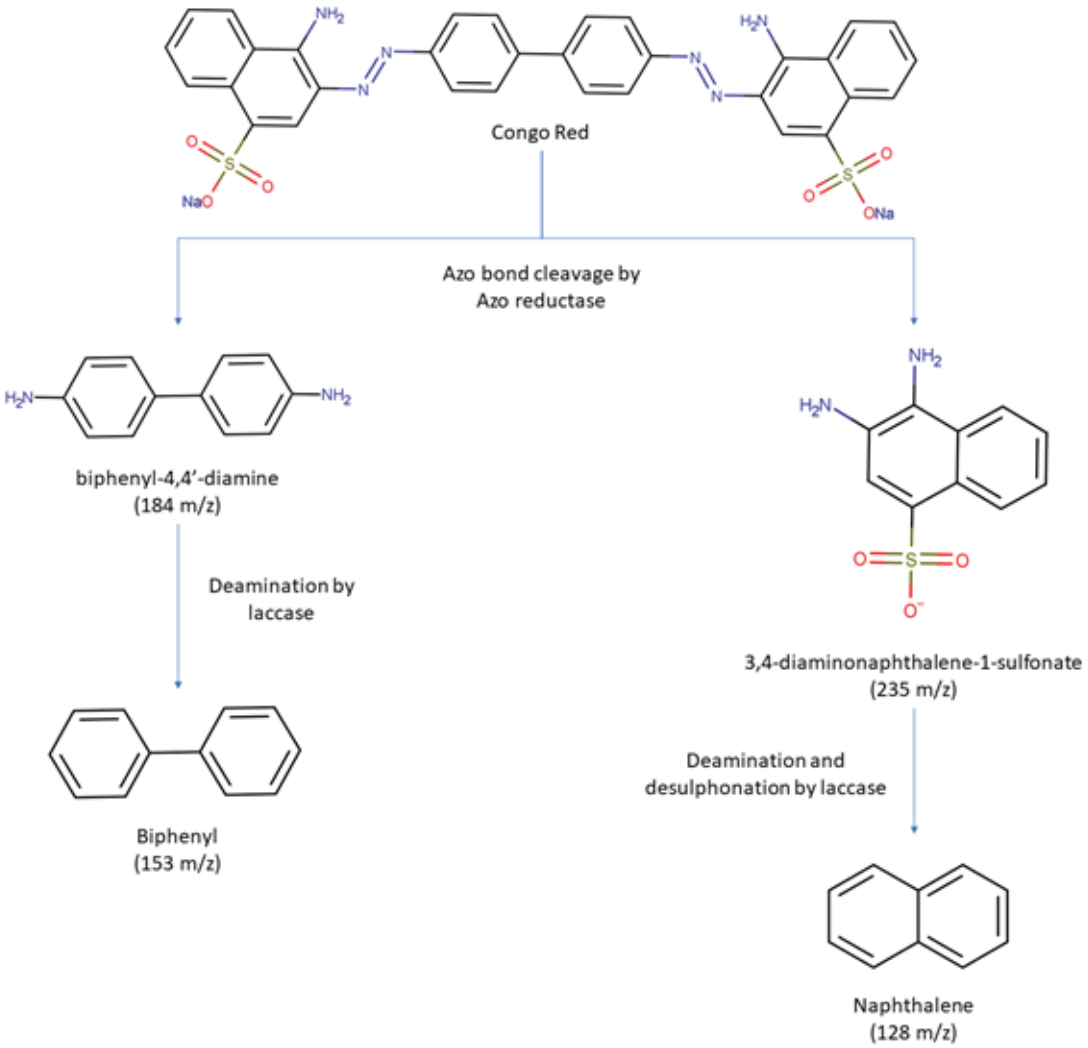

Figure 3: Proposed Congo red biodegradation pathway.

desulphonated into naphthalene shown by a peak at 128 m/z. Therefore, the proposed Congo red degradation pathway from this study result was shown by Figure 3.

Our GC-MS analysis result shows there were two additional products at 94 m/z and 51 m/z. The molecule with 94 m/z indicated an alternative degradation of biphenyl-4,4'-diamine. It is possible that covalent bond between two aromatic rings of biphenyl-4,4'-diamine was broken down into two aniline molecules (MW = 93). The 51 m/z peak showed an unidentified product which probably had 4 carbons. The ability to produce smaller molecules than biphenyl-4,4'-diamine and the 3,4-diaminonaphthalene-1-sulfonate implies there are multiple enzyme with azo reductase-like and laccase-like activity existed in the crude enzyme extract.

4. Conclusion

Yeast *Pichia* sp. isolated from tapai starter culture is capable of decolorizing Congo red enzymatically. Multiple metabolites were formed as the result of decolorization, such as biphenyl, naphthalene and smaller molecules with 94 m/z and 51 m/z, suggesting the involvement of enzymes with azo reductase and laccase-like activity. This study implies the use of *Pichia* sp. as bioremediation agent for the removal of azo dyes.

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