BIODEGRADATION OF DIESEL OIL BY YEAST ISOLATED FROM MANGROVE'S RHIZOSPHERE

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ABSTRACT

Diesel oil-degrading yeast strains isolated from mangrove rhizosphere at Tritih Kulon, Cilacap had been screened with SMSS medium. Four culturable yeast were isolated. Qualitative test was conducted by culturing and incubating the yeasts for one month in a medium added with 1mL diesel oil. By measuring the reduction of diesel oil, two best yeasts were selected. The quantitative test, GC-MS analysis, was conducted to determine the detailed degradation process of diesel oil. *Candida lusitaniae* and *Cryptococcus laurentii* performed the degradative ability. Three highest percent area of hydrocarbon compounds were compared for assessment. The results showed that *C. lusitanie* had better degradative capability than *C. laurentii*, in which hexadecane and methyl hexadecanoate decreased by 90–95%, and 9-octadecenoic acid, methyl ester declined by 30–40%. The increasing pH medium during incubation suggested that fermentation process occurred.

KEY WORDS: yeast, diesel oil-degrading, hydrocarbon compound

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INTRODUCTION

The mangrove forest is a forest located between land and sea. Its ecosystem has a vital role in the ecological and economic cycles since it consists of elements such as nitrogen, accumulated dissolved phosphor, the primary, and secondary productions. The ecosystem services provided include protection of shoreline, prevention of seawater intrusion, regulation of microclimate, germplasm provision of mangrove biota, source of bioenergy, site of animal shelter, site of animal feed and breed (Soedradjad, 2003; Setyawan & Winarno, 2006; Pribadi *et al*., 2009).

The Segara Anakan mangrove, Cilacap covered 24,000 ha area in 1980, then declined to approximately 12.343 ha (Caraka, 2012). The ecosystem is increasingly threatened by sedimentation, industrialization, and overexploitation (Soedradjad, 2003), which was demonstrated by the shrinking reproduction area up to nearly 500 ha, and a shrimp (*Metapenaeus elegans*) production decrease. Both are caused partly by pollution in water bodies in the region (Suradi *et al*., 2005). Oil contaminant is a common problem of pollutant compounds brought by River Donan waters. The river had an oil content of 6.6 ppm (Soedradjad, 2000), while the maximum limit of oil according to the water quality standards based on Kepmen KLH No. 02/1988 is less than 5.0 ppm. Oil pollution in the River Donan comes from the liquid waste of oil refineries (average activities of 167.136 kg/h with the debit of 10.502 m^3/sec) (Soedradjad, 2003).

An effort to reduce the water pollution is by using microbes that are capable of breaking down the oil pollutants to particular molecules (Irianto *et al*., 2003). Yeast is a microbe capable of degrading the oil. Nurhayati *et al*. (2001) reported that yeast could degrade hydrocarbons. They managed to obtain nine isolates from areas contaminated with oil port at Tanjung Perak comprising eight genera consisting of two isolates of Candida and one isolate from each genus of Rhodotorula, Geotrichum, Torulopsis, Trichosporon, Cryptococcus, and Saccharomyces. Miranda *et al*. (2007) isolated yeast from the diesel oil filling station at the Port of Suape Pernambuco, Brazil and cultured two isolates of *Candida ernobii* UFPEDA, i.e., 862 and 845 UFPEDA.

Candida utilizes oil hydrocarbons as its nutrient source for growth by breaking down Carbon to aliphatic and aromatic hydrocarbon. Chandran and Das (2010) isolated *Trichosporon asahii* yeast which became a powerful biosurfactant on mineral salt medium contaminated with diesel fuel in India. *T. asahii* uses the diesel fuel as its carbon source and efficiently degrade (95%) diesel fuel in 10 days.

The ability of yeast to decompose the hydrocarbons, the aliphatic and aromatic compounds, can be enhanced by adding a nitrogen source to the degradation medium. A nitrogen source which has a high nitrogen content at a low price and easily obtained is urea (carbamide). According to Leahy and Colwell (1990), nitrogen fertilizers can stimulate hydrocarbon biodegradation. Therefore, it is necessary to examine the biodegradation of diesel oil by yeast isolates from mangrove Cilacap.

This research was conducted to explore indigenous yeast isolates from the oil-contaminated area. The isolated yeasts were then cultivated on nitrogen enhancing medium and tested for their ability to degrade diesel oil.

METHODS

The yeasts were isolated from Mangrove Forest Area at Tritih Kulon, Cilacap, Central Java. The microbiological analysis was done at the Laboratory of Microbiology, Universitas Jenderal Soedirman. Tests for yeast ability to degrade hydrocarbons was carried out experimentally. Analysis of hydrocarbon compounds using Gas Chromatography-Mass Spektro was conducted at the

Laboratory of Organic Chemistry, Faculty of Mathematics and Natural Sciences Universitas Gadjah Mada.

The experimental design applied to examine the isolated yeast ability to hydrolyze hydrocarbon compound was the factorial pattern with complete randomized design (CRD). The first factor was the two isolated yeast from mangrove rhizosphere either single or mixed isolates, i.e., yeast isolates I (K1), yeast isolates II (K2), and a mixture of isolates I and II (K3). The second factor was the urea as nitrogen sources with a concentration of 0.00 (U0), 0.15% (U1), 0.25% (U2) and 0.35% (U3). Each treatment was repeated three times, and observed at day 0, day 7, day 14 and day 21.

The yeast isolation the mangrove rhizosphere (*Rhizophora* sp. and *Avicennia marina*) was done using SMSs medium containing 0.5 g of $CaCO_3$, 0.25 g NH_4NO_3, 0.1 g $Na_2HPO_4.7H_2O$, 0.05 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$ dissolved in 100 mL water, 2% diesel oil, and 0.05% chloramphenicol antibiotic. The isolates were incubated for seven days at room temperature and shaken using a reciprocal shaker at 120 rpm. Yeast isolates were identified through colony shape, color, edge, and the elevation of the isolates. Identification of yeasts was by rapid identification method API 20 C AUX (Bio-Merieux, France) to determine the species of yeast isolates. Qualitative test was conducted to determine the best isolates by growing isolates on medium SMSs as much as 9 mL + 1 mL of diesel oil (10mL) in a test tube and incubated for 30 days at the shaker incubator. Preparation of the yeast suspension was done by taking 1-2 oses yeast colonies and added to a test tube containing medium SMSs + diesel oil. The isolates were then incubated for two days in a shaker incubator. Ability tests of isolated yeast were done by adding a starter in 100 mL of liquid mineral medium containing 3% (w/v) of diesel oil. The culture medium was incubated at room temperature and shaken in a shaker at 120 rpm. Levels of residual diesel oil and yeast density were calculated at day 7, 14, and 21. The remaining diesel oil in each treatment was extracted and analyzed using gas chromatography. The medium acidity was measured as a supporting parameter.

RESULTS AND DISCUSSION

The qualitative test revealed that Avi2H5 iso2 and Rhi2H1 iso3 had the best degradative ability, and appeared to utilize diesel oil in the medium for their carbon source (Table 2). During 30 days, Avi2H5 iso2 absorbed as much as 0.55 ml (55%), and Rhi2H1 ISO3 for 0.5 ml (50%) of diesel oil. The isolates were identified with rapid identification kit API 20 C AUX which was a precise identification system for almost all yeasts cultured. It consisted of 20 holes containing dehydrated substrates which performed 19 assimilation tests (Bio-Merieux, France). The identification showed Avi2H5 iso2 was *Candida lusitaniae* with a significant value of 92.5% and Rhi2H1 ISO3 was *Cryptococcus laurentii* with a significance value of 99.8%.

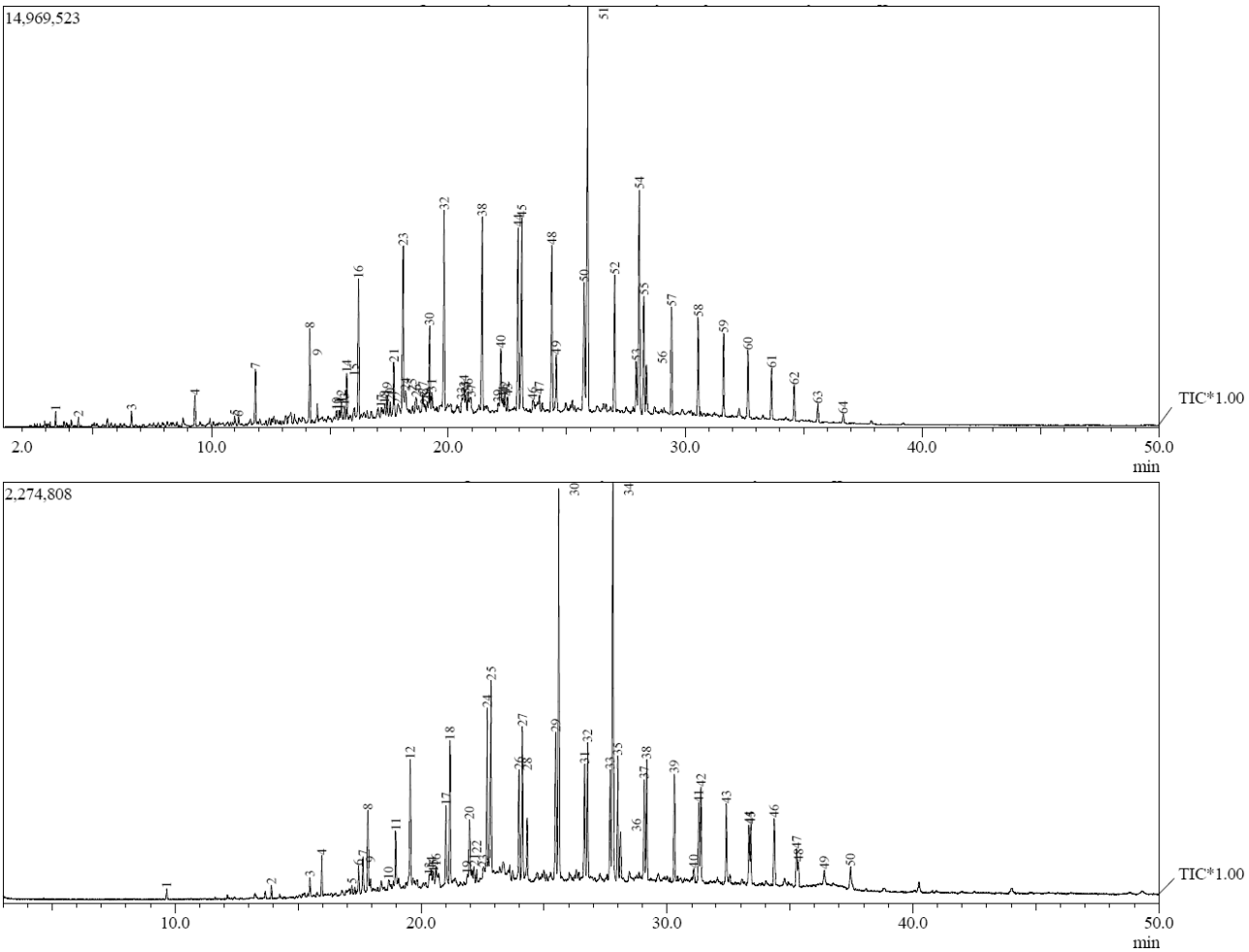

Figure 1. Chromatogram comparison of control K1U0: *Candida lusitaniae* + urea 0% (top), and K1U3: *C. lusitaniae* urea + 0.35% (bottom) after two weeks incubation.

According to Figure 1, gas chromatography on the yeast (K1, K2, and K3) changed on the second week (14 days) period of incubation, producing several hydrocarbon compounds such as methylcyclohexane (C_7H_{14}), octane (C_8H_{18}), and nonane (C_9H_{20}). The decline was demonstrated by the peak of diesel oil components which showed a short retention time of 2–14 minutes absorbed depleted, but the hydrocarbon component with a retention time between 20–30 change their.

Table 1. Characters of the diesel oil-degrading yeasts

Strain	C. Form	Color	C. Edge	Elevation
Rhi2H1 iso3	Round	Cream	Smooth	Flat
Rhi2H1 iso2	Round	Cream	Smooth	Flat
Avi2H5 iso2	Round	White	Smooth	Flat
Avi2H5 iso3	Round	White	Smooth	Flat

Tabel 2. Qualitative test of the yeasts

Strain	Before	After	Degraded
Rhi2H1 iso3	1 ml	0.50 ml	0.50 ml
Rhi2H1 iso2	1 ml	0.80 ml	0.20 ml
Avi2H5 iso2	1 ml	0.45 ml	0.55 ml
Avi2H5 iso3	1 ml	0.80 ml	0.20 ml

The chromatographic analysis after three weeks of incubation showed that in general, all treatments increased the tops of hydrocarbon components of alkanes and aromatics. There were the peaks of a new component that is absent in the chromatogram of diesel oil such as phenanthrene and fluorene in control although the percent area (concentration) were small (Figure 1). According to Sood *et al*. (2010), alkane hydrocarbon degradation process produced monocarboxylic acid intermediates while pyrena polycyclic aromatic hydrocarbons in oil produced an intermediate oxygenated pirenol.

The rise and fall of hydrocarbons compound showing by chromatogram peaks were due to hydrocarbon used by the yeasts for growth. According to Ramadan *et al*. (2012) oil degradation by yeasts was not only reduced, but also an increased the concentration of hydrocarbons contained in the growth medium. For example, it was shown by increases of 2-methyl undecane from 2.06% to 2.71% or naphthalene from 1.72% to 2.77%.

The effect of urea addition to the yeast degradative ability was because the yeast used urea as a nitrogen source. The nitrogen was broken down by urease enzyme into ammonium ions and CO_2. Ammonium was transported to the cell through its membrane then used it for the amination of particular organic compounds to form amino acids to stimulate bacterial growth (Sabarni, 1995).

The dominant compounds based on the results of the retention time of gas chromatography between treatment and control was used as a degradation parameter. The three-highest compound observed was hexadecane (4.85%), methyl hexadecanoate (13.64%), and 9-octadecanoic acid, methyl ester (6.33%).

Percent decrease of hydrocarbons concentration occurred after three weeks. Hexadecane and methyl hexadecanoate reduced up to 90–95%, and the 9-octadecanoic acid declined to 30–40% (Figure 2 and 3). Candida yeasts showed great potential to degrade hydrocarbons, in particular hexadecane that decline to 77.04% and 57.20% (Nurhariyati, 2004), 40 mg eicosane during 10 hours, 72% heneicosane after 8 days at pH 3 (Sood *et al*., 2010), and diesel oil by 98% after 10 days (Chandran & Das, 2010).

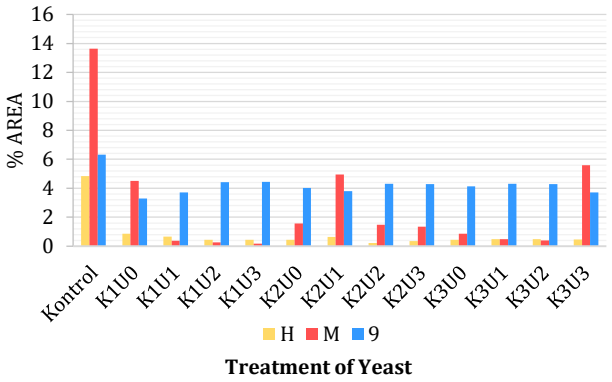

Figure 2. Concentration comparison for hexadecane (H), methyl hexadecanoate (M) and 9-octadecanoic acid, methyl ester (9) after two weeks.

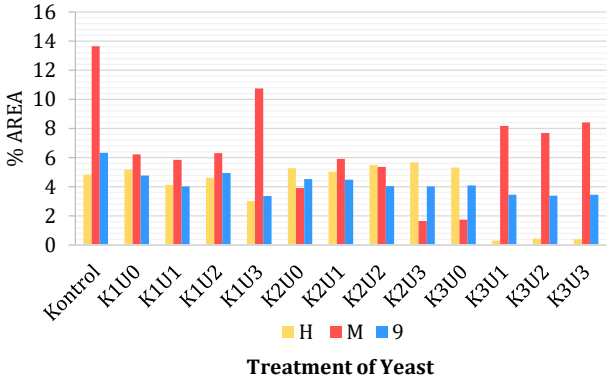

Figure 3. Concentration comparison for hexadecane (H), methyl hexadecanoate (M) and the 9-octadecanoic acid, methyl ester (9) after three weeks.

During the process of diesel-oil degradation, chemical compounds changed during the incubation period. Compound alterations occurred in control were hexadecane ($C_{16}H_{34}$) that changed to pentadecane ($C_{15}H_{34}$), tridecane ($C_{13}H_{28}$) and 1,3,2 dioxaborinane 2.4 6 diethyl 5 methyl propyl ($C_{11}H_{23}O_2$). Based on these changes, the carbon atoms reduced from C_{16} to C_{15}, C_{13}, and C_{11}. The reduction was associated with the use of hydrocarbon of diesel-oil by the yeasts. Additionally, a binding process of two oxygen atoms into two molecules of hydrocarbons 2,4-Diethyl-5-methyl-6-propyl-1,3,2-dioxaborinane occurred because the compounds did not exist in control. This binding process was an activity of the dioxygenase enzyme as a mechanism of hydrocarbon degradation (Haddock, 2010).

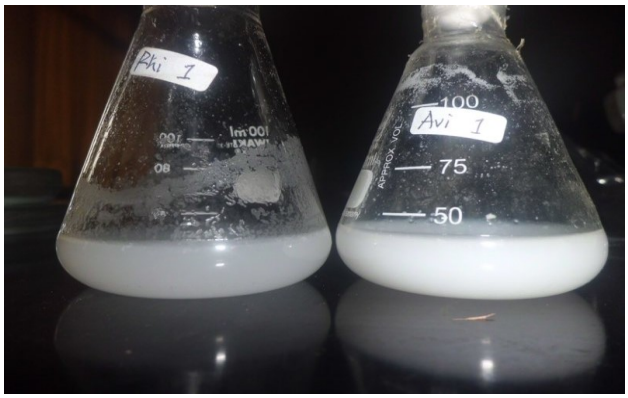

Figure 4. Fermentation results of Rhi2H1 iso3 (left) and Avi2H5 iso2(right) during the first week of observation. The pH was ranged between 6.9 to 7.9. The pH value on week two has increased between 7.1 to 8.5 and week three ranged between 7 to 8.7. The increase in pH was due to the urea decomposition by the urease enzyme into ammonium and CO_2. Higher Ammonium concentrations caused the alkaline medium to increase in pH.

The degradation mechanism of diesel-oil by the yeast occurred because of biosurfactant production during incubation. Biosurfactants were secreted out of the cell causing oil emulsification identified by the discoloration of the fermentation medium. The medium color changed to whitish, and diesel-oil layer transformed into small granules. Biosurfactants are a group of molecules that have surface-active properties, hydrophilic and hydrophobic groups to reduce tension between water and oil (Vater *et al*., 2002). According to Al-Tahhan *et al*. (2000) biosurfactant role in dissolving hydrophobic compounds such as oil to form a micelle structure.

CONCLUSION

There were four yeast isolates from mangrove rhizosphere at Tritih Kulon, Cilacap capable of degrading diesel-oil. The two best isolates were *Candida lusitaniae* and *Cryptococcus laurentii*. *C. lusitanie* had better degradative capability than *C. laurentii*, in which hexadecane and methyl hexadecanoate reduced by 90–95%, and 9-octadecenoic acid, methyl ester decreased by 30–40%.

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