The diversity of active microbial groups in an activated sludge process treating painting process wastewater

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Abstract. Activated sludge process is one of the wastewater treatment method that is applied for many wastewater types including painting process wastewater of automotive industry. This wastewater is well-known to have high heavy metals concentration which could deteriorate water environment if appropriate performance of the wastewater treatment could not be achieved. In this study, we monitored microbial community diversity in a Painting Biological Treatment (PBT) system. We applied a combination of cultivation and genotypic biological methods based on 16S rRNA gene sequence analysis to identify the diversity of active microbial community. The results showed that active microbes that could grow in this activated sludge system were dominated by Gram-negative bacteria. Based on 16S rRNA gene sequencing analysis, it was revealed that their microbial diversity has close association with Bacterium strain E286, *Isosphaera pallida*, *Lycinibacillus fusiformis*, *Microbacterium* sp., *Orchobactrum* sp., *Pseudomonas guariconensis*, *Pseudomonas* sp. strain MR84, *Pseudomonas* sp. MC 54, *Serpens* sp., *Stenotrophomonas acidaminiphila*, and *Xylella fastidiosa* with similarity of 86 – 99%. This findings reflects that microbial community in a Painting Biological Treatment (PBT) system using activated sludge process could adapt with xenobiotics in the wastewater and has a wide range of diversity indicating a complex metabolism mechanism in the treatment process.

1 INTRODUCTION

Every production process carried out by industry produces wastes that require further processing thus they cannot be directly discharged into the environment. These wastes were resulted from the production process as well as waste treatment process. The industrial waste must be treated before being discharged into the environment to prevent environmental pollution, one of which can be due to the presence of heavy metal content. The heavy metal is not biodegradable and tends to accumulate in the environment and cause diseases and other disorders, even though it could be treated by microalgae absorption [1, 2].

One of the wastewater treatment is biological treatment by indigenous bacteria that are environmentally friendly. This treatment configuration will be more effective, inexpensive and sustainable compared to conventional (physico-chemical) methods [3]. In addition, biological methods can also be an attractive choice for conserving water usage through treatment of water produced from wastewater [4].

The activated sludge method is an aerobic biological treatment by taking advantage of a suspended microbial ecosystem. Simphiwe *et al*. (2012) showed that the use of bacteria in the processing of industrial wastewater can be an alternative waste treatment that is more economical and effective in removing dyes, but the efficiency of removing dyes also depends on the type of dye, pH, temperature, and flocculant concentration [5]. This is in line with Mahmood *et al*. (2012) mentioned that the effectiveness of processing using this treatment method depends on three variables, namely: the substrate contained in the waste, the bacterial species, and the environment in which the bacteria live [3].

The object of this study was a biological wastewater treatment system of a metal painting facility owned by a shock-absorber manufacturing industry that generates wastewater consisting paint residues containing heavy metals. This biological wastewater treatment system consists of a Painting Biological Treatment (PBT) unit operating activated sludge treatment process. This PBT unit was constructed not only to treat wastewater, but also to conserve water use by reusing treated wastewater for water curtain system in capturing excess of paint during the painting process.

The PBT unit uses bacteria consortium as biodegraders. This bacteria consortium originated from five seeding tanks. Each of the tanks consists of specific bacteria which has been isolated and cultivated previously [6, 7, 8]. This specific bacteria is augmented to the PBT unit occasionally based on its performance. Another study showed the best composition of that bacteria consortium that needs to be augmented to the PBT unit based on different types of paint used which contains different pigments, binders, extenders, solvents and additives [8].

According to those studies, it could be recognized

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that the degradation of painting process wastewater requires a combination of active microbes, and the change of paint types used influences those active microbes and requires an adjustment of bacteria seed composition to maintain its performance. The measurement of physico-chemical characteristics only is often failed to figure-out the stability of a biological treatment. Monitoring of these varied microbes is very important to maintain the stability of the consortium. However, due to many bacteria that must be routinely monitored, this effort is often constrained by time and implies cost problem. Therefore, instead of evaluating all of the active microbes, a microbial indicator is required for representing those active microbes that can describe the overall condition of the stability and performance of biological degradation accurately and rapidly. Thus, the purpose of this study was to identify the diversity of active microbes in the PBT system that can be used as a basis for further development of a microbial indicator for the performance evaluation of painting process wastewater treatment using activated sludge system, in addition to current conventional physico-chemical characterization.

2 MATERIALS AND METHOD

There were five stages of the research in the study of identification of active microbial diversity in activated sludge system. The diversity of active microbes in the PBT system was revealed by a set of conventional and molecular biological techniques. These methods could figure out the morphological and physiological characteristics of the microbes as well as their genetic properties. This approach was set to isolate and identify the cultivable bacteria which is important to furthermore develop a microbial indicator that could be applied widely.

2.1 Biomass and water samples collection

Samples were taken from the PBT unit and five seeding tanks (Figure 1). Samples were collected during 6 consecutive months using a water sampler at a certain depth and stored properly. Triplicate samples were measured, collected and analyzed to achieve the representative data.

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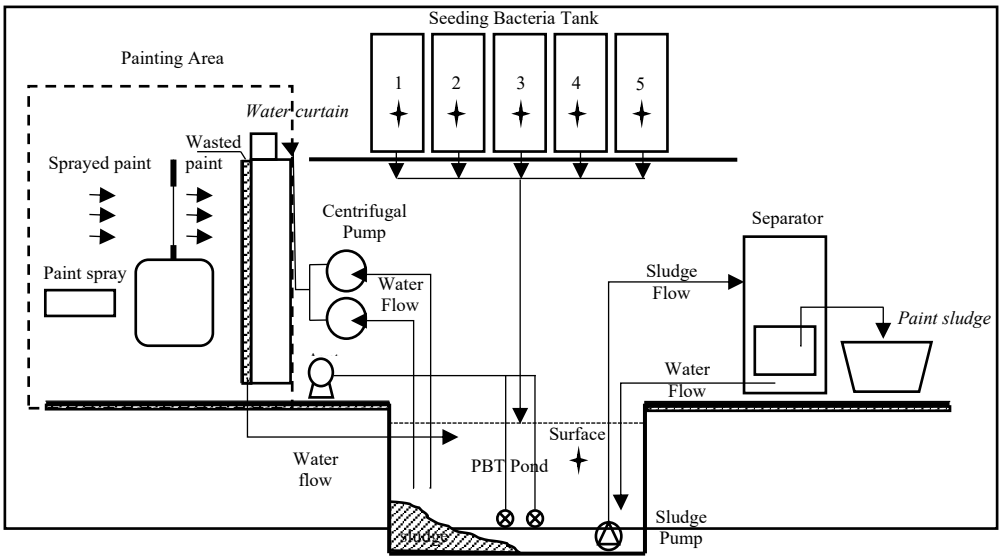

Fig 1. The process flow diagram of PBT Pond/Unit (+ indicates sampling points)

2.2 The physicochemical parameters measurement

The measurements of physicochemical parameters consisted of *in-situ* and *ex-situ* measurements. *In-situ* measurements were conducted for the parameters of temperature, pH, dissolved oxygen (DO), and conductivity. Whereas *ex-situ* measurements were carried out in the laboratory including determination the concentrations of total chemical oxygen demand (COD) and dissolved COD (using the closed reflux method), total-N (using the destruction-distillation-titration method), total-P and orthophosphate (using spectrophotometric method), mixed liquor volatile suspended solid (MLVSS) (using the method of filtration), and Zn and Ni (using the Atomic Absorption Spectroscopy) [9].

2.3 Microbial identification using conventional biological technique

This identification aimed to determine the type of bacteria in general, based on the morphology of the colony using light microscope. In addition, inoculation and isolation of bacteria from the samples were also carried out to obtain pure bacterial cultures prior to their physiology and molecular identification.

The initial stage was the isolation of bacteria from the samples with bacterial culture techniques. Furthermore, the number of colonies was calculated using the method of Total Plate Count (TPC) (cfu/mL) with (1):

$$\text{Tot. colony} = \frac{\text{number of colony x dilution factor}}{\text{volume of the sample}} \dots (1)$$

The purification of isolated bacteria was performed by quadrant streak technique to obtain a single colony. Identification of bacterial morphology was conducted by observing the shape, color, edge and elevation of the colony; microscopic observations of cell wall characteristic by Gram staining; and analysis of the presence of Bacillus bacteria using Luria Bertani (LB) media.

2.4 Identification of microbes using the 16S rRNA gene sequencing analysis

The initial stage of this analysis technique was the extraction of microorganisms' DNA from the seeding tanks and PBT unit. The extract of DNA was then amplified through Polymerase Chain Reaction (PCR) with a universal bacterial primer (Table 1) and a DNAse kit. Subsequently, electrophoresis analysis was carried out, and purified DNA was furthermore sequenced by Macrogen, Korea. The results of DNA sequencing were then compared with the 16S rRNA gene references listed in the GenBank/EMBL/DDBJ database using BLAST search [10]. Sequence arrangements that have a minimum similarity of 95% are grouped in one taxonomic units (OTUs). Phylogenetic analysis was performed using MEGA 7 software, and data alignment was conducted using Bioedit software. The phylogenetic tree was then constructed using the neighbor-joining and maximum-parsimony methods.

Table 1. Primers used in this research

Name	Sequence (5'-3')	Target	16S rRNA target site (*E.coli numbering*)	Reference
B8f	AGRGTTTGATCCTGGCTCAG	SSU rRNA bacteria	8-27	[11]
U1492r	GGTTACCTTGTTACGACTT	SSU rRNA universal	1492-1510	[11]

2.5 Confirmation of the presence of Bacillus with Luria Bertani (LB) Media

Confirmation of the presence of specific bacteria with Luria Bertani (LB) media was to determine the presence of Bacillus bacteria as reported in the previous study [7]. LB media is a common agar medium that can be used for indicating Bacillus group bacterial growth. Bacterial samples from seeding tanks were dissolved in 1x PBS solution and incubated at 4°C for one night. These samples were then incubated for 30 minutes at 80°C and inoculated on LB media with a spread plate technique prior to their incubation at 37°C for two days or 48 hours.

3 RESULTS AND DISCUSSION

3.1 Physicochemical parameters evaluation of PBT Unit

The growth of microbes depends on physicochemical factors in their habitat/environment. Wastewater in PBT unit is originated from metal painting process. There were eight different types of paint used, including Axis Gray M1, Ct/UC Silver, Deep Black Gloss, Met Black, NH 35 Silver, Phantom Silver, Metallic Silver and Solid Black. The use of different paints might differ the physico-chemical characteristics of the wastewater. The result of physico-chemical measurement of the water samples in the PBT unit are shown in Figure 2 and 3.

Based on the data, the temperature was in the range of 27.5±0.71 - 30.3± 0.42 ^{0}C. Whereas, the pH value was in the range of 6.08±0.11 - 6.9±0.57. The results of pH measurement showed that the pH value in the microbial habitat was able to support the microbial growth that microbes can grow and multiply in optimum condition at pH 6.5 - 7.5 [13]. The results of dissolved oxygen (DO) measurements showed values ranging from 1.1±0.14 - 5.94±0.06 mg/L. The wide range of DO value seemed not to be strongly affiliated with temperature or pH value, but likely to be much more associated with lack of proper operation of the PBT unit. During the operation, there were some occurrences of aerator malfunction resulted low DO value. The DO needed by aerobic microorganisms ideally should be more than 2 mg/L, in order to ensure good effluent quality and prevent microbial decay [14]. Meanwhile, the conductivity values were in the range of 51-54 mS/m. In general, those measurements results showed that the values of temperature, pH, DO and conductivity were appropriate for the wastewater treatment using the activated sludge system [13, 14, 15].

The results of the measurement of physico-chemical parameters showed that the value of COD, both dissolved COD and total COD, were relatively high for each sample with an average ratio of 1: 1.56. Total-P and orthophosphate concentration had relatively low values. This circumstance was sufficient for activated sludge system, since high phosphate and orthophosphate values will affect sedimentation rate that could cause a decrease in DO values and subsequently initiate microbial decay.

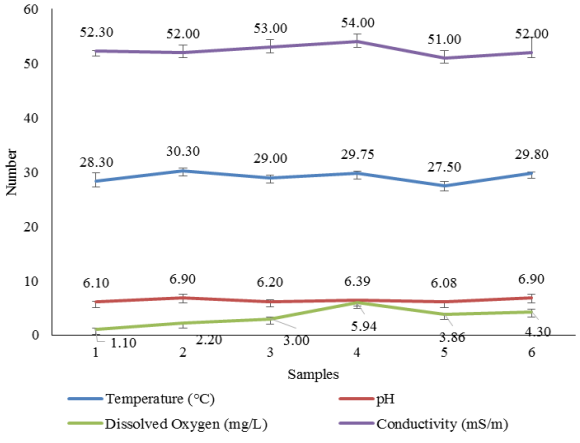

Fig 2. Results of *in situ* measurements of the samples

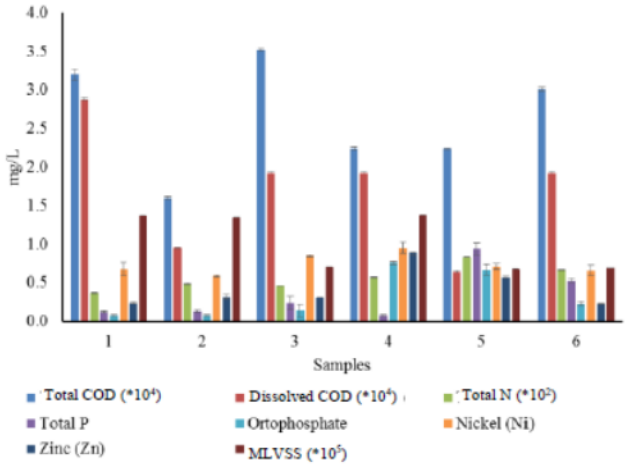

Fig 3. Results of *ex situ* measurements of the samples

Based on previous study [8], the dominant heavy metal contained in the paint used were nickel (Ni) and zinc (Zn). The measurement of heavy metals concentration showed that the concentration of Ni was relatively lower than Zn. These values exceeded the effluent standard of liquid waste for industrial activities, based on the Decree of the Minister of the Environment Republic of Indonesia No. 51/1995, i.e. 1 mg/L. These exceeding values implied that the direct discharge of wastewater could harmful to the environment and appropriate wastewater treatment is indeed required for reducing the content of heavy metals.

It showed that MLVSS for the six samples were in the range of 67.97±42.43 – 137.19±14.14 mg/L. The high value of MLVSS elucidated the large number of microorganisms and certain organic matter in the wastewater that certainly could exist in water environment with high dissolved oxygen concentration [16].

3.2 Identification of active microbes using conventional biological technique

Conventional biological techniques through microbial inoculation using nutrient agar media and dilution techniques were carried out to determine the phenotypic characteristics of active microbes in the seeding tanks and the PBT unit. Inoculation was performed with a spread plate technique, thus bacterial colonies were fragmented and further isolation process could be attempted thoroughly [3]. Dilutions were carried out using 10^{-1} to 10^{-6} dilution factors. After the inoculation was carried out using the spread plate technique, the calculation of the number of bacterial colonies was then carried out to estimate the number of bacteria with the method of Total Plate Count (TPC). Figure 4 shows the microbial abundance of total cultivable microbes in 6 samples based on that TPC method.

It shows that seeding tank no. 4 (ST4) had the highest number of bacteria colonies, i.e. 1.79×10^6 cfu/mL, while the least abundant microbial colonies was found in seeding tank no. 1 (ST1). Surprisingly, the total bacterial colonies in PBT unit was not as high as the total of colonies of ST4. This reflected that there was a competition among seeding bacteria that frequently added to the PBT unit. Meanwhile, the effect of heavy metals in PBT unit also could suppress the microbial growth of seeding bacteria. However, the abundance of microbial consortium in PBT unit suggested that those consortium played a role in wastewater degradation properly.

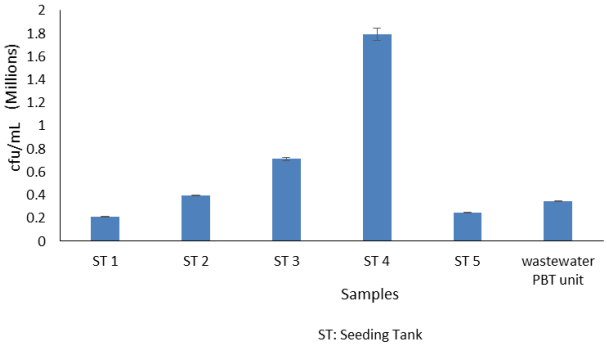

Fig 4. Average number of bacteria colonies in seeding tanks and PBT unit

The identification of morphology of bacterial colonies showed that bacterial colonies generally were round, white and convex. There were 11 types of bacteria that were successfully identified. The Gram-staining results showed that those bacteria was dominated by Gram-negative bacteria (Table 3 and Figure 5).

Many research reported that Gram-negative bacteria constitutes the most genera of bacteria isolated from activated sludge. Sharifi-Yazdi *et al*. (2001) described that the majority (about 22%) of the isolated Gram-negative bacteria belonged to the genus of Pseudomonas [17]. For Gram-positive bacteria, it has been found that this bacteria could cause 'foams non filament' which could generate problems in wastewater treatment performance. However, according to Wagner *et al.* (1994), it has been showed that groups of Gram-positive bacteria are important bacteria to remove phosphate in biological wastewater treatment [18].

Table 3. Gram-staining and morphological analysis results of isolated bacteria

No.	Colony Code	Sample Origin	Gram Stain	Morphology
1	AS 1	PBT Unit	Negative	Rod/Bacilli
2	AS 2	PBT Unit	Negative	Rod/Bacilli
3	AS 3	PBT Unit	Negative	Coccobacilli
4	AS 5	PBT Unit	Negative	Cocci
5	AS 6	PBT Unit	Negative	Diplococci
6	T1.2	ST1	Positive	Coccobacilli
7	T2.1	ST2	Negative	Coccobacilli
8	T2.2	ST2	Negative	Cocci
9	T3.1	ST3	Positive	Rod/Bacilli
10	T4.2	ST4	Negative	Rod/Bacilli
11	T5.1	ST5	Positive	Streptobacilli

Another observation of the conventional biological technique performed was Luria Bertani (LB) media analysis. In this analysis, the growth of the bacterial colony indicates that the bacteria can survive in extreme condition. The following are the results of observations using LB media (Figure 6).

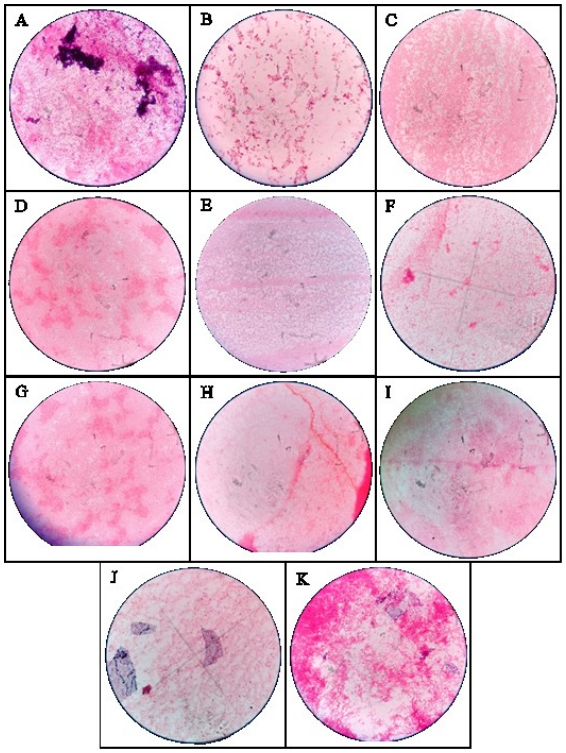

Fig. 5 Gram-staining results (A) AS 1 Colony, (B) AS 2 Colony, (C) AS 3 Colony, (D) AS 5 Colony, (E) AS 6 Colony, (F) T1.2 Colony, (G) T2.1 Colony, (H) T2.2 Colony, (I) T3.1 Colony, (J) T4.2 Colony, (K) T5.1 Colony. Images were taken using 1,000x magnification.

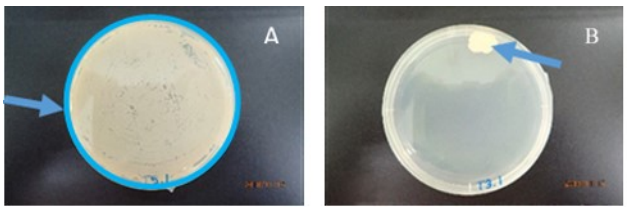

Fig 6. Bacteria colonies that growth in LB media. (A) T5.1 Colony and (B) T3.1 Colony. Arrows indicate colonies growth

One of the bacteria that can grow in LB medium is *Bacillus* sp. Bacillus species are known as spore-forming pathogenic bacteria that are often found in the environment. These bacterial species can deal with a number of disruptive factors that can inhibit growth, including nutrient depletion, temperature fluctuations, pH variation, redox potential, limited water conditions, increased reactive oxygen levels, and osmotic imbalance along with unusual solute concentration.

By applying high temperature on the bacteria grew in LB media, only two bacterial colonies could grow. Munna *et al.* (2015), conducted an experiment on the defense strategy of *Bacillus* spp. when responding to artificial oxidative stress, and they showed that Bacillus could perform sporulation when exposed to aeration speed of 100 rpm and a temperature of 54 °C [19]. In other studies it has been shown that stressosome signaling complexes from *Bacillus* spp. will be active when responding to adverse environmental conditions. This sporulation mechanisms of *Bacillus* spp. support the results of this study. At high temperatures, *Bacillus* spp. could be dormant but they have a possibility to perform sporulation prior to their further normal growth to keep cells alive.

An incubation of the extreme temperature difference of 4 °C to 80 °C was performed. As the result, we could confirm that two bacterial colonies that grew from the previous isolation and cultivation using LB media among 11 bacterial colonies could be indicated as *Bacillus* spp. This finding was in line with previous study [7], described that the dominant bacterium in PBT unit was the Bacillus genus.

3.3 Identification of microbial communities using 16S rRNA gene sequencing analysis

This 16S rRNA gene sequencing analysis technique is a molecular biological technique to determine the genotypic identity of microbes present in seeding tanks and PBT unit. The 16S rRNA gene has a conserved area, so that it is properly to be used in Polymerase Chain Reaction (PCR) and sequences analysis to determine diversity, phylogeny, and taxonomy properties of microbial species. This gene type also has a hypervariable region which is a special characteristic for each microorganism. Eleven isolates that were visually identified in conventional biological technique and Gram-staining were then identified their genotypic properties using this 16S rRNA gene sequencing analysis. Table 4 shows the results of the analysis.

Based on the 16S gene sequences and conducting a blasting method to reveal homologous taxa, it was found that the isolated bacterial entities had similarity of 86 - 99% with closest known taxa. Furthermore, a phylogenetic tree was constructed to identify the affiliation among those bacterial entities (Figure 7).

Table 4. Blasting results of isolated bacteria*

Colony Code	Closest Known Taxa (Accession Number)	Similarity (%)
AS 1	*Pseudomonas guariconensis* strain PCAVU11 16S ribosomal RNA, partial sequence (NR_135703.1)	99
AS 2	*Isosphaera pallida* ATCC 43644, comlpete genome (CP002353.1)	96
AS 3	*Serpens* sp., UA-JF3308 16S ribosomal RNA gene, partial sequence (KC108984.1)	92
AS 5	*Orchobactrum* sp. strain DIBER 16S ribosomal RNA gene, partial sequence (MG021467.1)	88
AS 6	*Pseudomonas* sp., strain MR84 16S ribosomal RNA gene, partial sequence (MG674369.1)	98
T1.2	*Microbacterium* sp. HJX8 16S ribosomal RNA gene, partial sequence (KP979539.1)	95
T2.1	*Pseudomonas* sp. MC 54 partial 16S rRNA gene, isolate MC 54 (LN907782.1)	98
T2.2	*Stenotrophomonas acidaminiphila* strain AMX 19 16S ribosomal RNA, partial sequence (NR_025104.1)	98
T3.1	*Xylella fastidiosa*, strain pistachio8 16S ribosomal RNA gene, partial	86

Colony Code	Closest Known Taxa (Accession Number)	Similarity (%)
	sequence (MG458715.1)	
T4.2	Bacterium strain E286 16S ribosomal RNA gene, partial sequence (MH998446.1)	98
T5.1	*Lysinibacillus fusiformis* strain HBUM07011 16S ribosomal RNA gene, partial sequence (MF662437.1)	98

Notes: *) Blasting was performed online through the website of https://www.blast.ncbi.nih.gov/blast

From these results, seven colonies were contig and constructed in a phylogenetic tree. It can be seen that T4.2 colony was affiliated with *Strenotophomonas malthophila*, while T1.2, T5.1, T2.1, T2.2, AS 1, and AS 5 colony has close affiliation with *Lysinibacillus fusiformis*, *Pseudomonas aeruginosa*, *Bacillus* sp., Bacterium, *Microbacterium schleiferi*, and groups of Pseudomonas and Agromyces, respectively.

Based on the diversity and affiliation of identified microbes in this study, the group of bacteria that commonly found in the activated sludge system was the T4.2 colony that belong to Bacterium group. Besides, T1.2 colony of Actinobacteria group, T5.1 colony of Firmicutes group, T2.1, T2.2, and AS.1 colonies of the Gamma-proteobacteria groups and AS.5 colony of the Alpha-proteobacteria group also have been reported to be subsisted in an activated sludge system of domestic wastewater treatment. These findings suggested that those active microbes in domestic wastewater treatment were also active in painting process wastewater treatment despite their toxic characteristics.

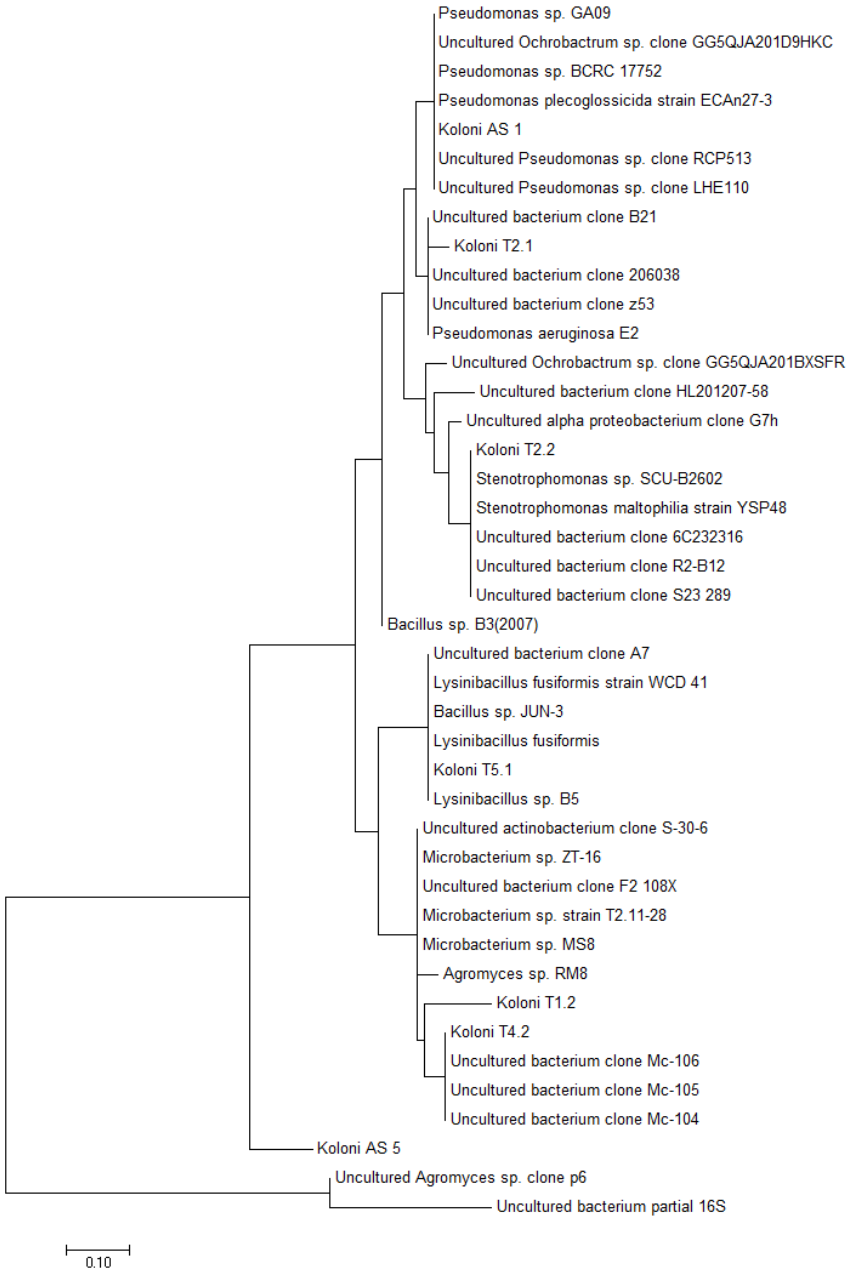

Fig 7. Phylogenetic tree of 16S rRNA gene sequences of selected bacteria

As shown in Table 4 and Figure 7, the presence of Firmicutes and Proteobacteria group have been identified in current study. Another study conducted by Meli *et al.* (2016), a group of Firmicutes bacteria has been found in an activated sludge system treating waste containing zinc oxide nanoparticles (nZnO). This Firmicutes bacteria group was second larger group in the system after the Proteobacteria group that was influenced by the presence of zinc oxide nanoparticles [20].

4 CONCLUSION

We concluded that the microbial community in a Painting Biological Treatment (PBT) system using activated sludge process has a wide range of diversity including Actinobacteria group, Firmicutes group, the Gamma-proteobaceria group and the Alpha-proteobacteria group, indicating complex metabolism pattern in the treatment process. This microbial community seemed to be tolerant and metabolically active in responding toxic substances of painting wastewater.

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