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Identification of *Paracoccus Solventivorans* sp. as Sulphate Oxidizing Bacteria Isolated from Palm Oil Sludge

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Abstract. The emission of hydrogen sulphide (H_2S) from industrial processes causesodour nuisance to the surrounding community. Thus, the ability of sulphur oxidizing bacteria could eliminate H_2S by changing it into non-odorous elemental sulphur or sulphate. In this study, the isolate bacteria of *Paracoccus solventivorans* ATCC 700252 demonstrated pH reduction from pH 8 to about pH 5.27 with sulphate content production of454.0 g/ml. The growth of *Paracoccus* sp. was increased using sulphur as elemental carbon and oxygen as electron acceptor. The optimum temperature and pH of *Paracoccus* sp. were in the range of 30°C to 40°C and pH 7.00 to pH 8.00, respectively. The utilization of *Paracoccus* sp. will create a potential application for H_2S removal and preserve the environment from further odour pollution.

1. Introduction

Sulphur is tasteless, odourless and highly produced. It is a pure element of sulphide and sulphate minerals in nature. Hydrogen sulphide (H_2S), which is a product of sulphur, has very bad odour. One of the challenging factors which limit its use in the gas and petroleum industries is correspond to the hydrogen sulphide composition, which is very corrosive to internal pipelines and other equipment [1]. In addition, excess of H_2S from human exposure limit range may affect health and safety of community [2].

Most of the identified sulphur oxidizing bacteria (SOB) belong to the *Thiobacillus, Thiothrix, Thiomicrospira, Achromatium, Desulfuromonas* and *Paracoccus* [3-4]. However, oxidation of sulphur compounds also occurs in heterotropic bacteria isolated from soil and marine environment. The heterotropic bacteria belong to the *Pseudomonas, Xanthobacter and Escherichia coli* strains [5]. There are mainly found in anaerobic or sulphur rich environment, such as palm oil mill effluent (POME). There are 400 million of POME ponds in Malaysia. POME could be one of the potential supply places for isolation of SOB as it contains elevated concentrations of H_2S, which supports the growth of SOB [6-7]. To date, many studies have reported the utilization of SOB to eliminate high concentration of H_2S in environment. The odorous 'rotten egg' smell is eliminated when H_2S changes into non-odorous elemental sulphur (S°) or sulphate (SO_4^{2-}) [8]. The oxidation reaction depends mainly on metabolic pathway of SOB.

In this study, a set of experiments was designed to study the isolation, identification and prove the abilities of sulphur oxidizing bacteria (SOB) on removal of H_2S from palm oil sludge. The efficiency of isolate was studied in terms of its optimum growth conditions, production of sulphate content and reduction of pH medium.

2. Materials and methods

2.1. Palm Oil Sludge Sample Collection
The sludge sample was collected from United Oil Palm Sdn. Bhd. The top layer of the sludge was removed. Samples were mixed thoroughly and put in the sterile bottle. The samples were brought to the laboratory for further analysis and stored in a refrigerator at 4°C.

2.2. Identification of SOB
One gram of sludge was added with 50 mL of distilled water in 250 mL conical flask. The sample was serially diluted, producing a ten-fold dilution. About 100 µL of each dilution samples were spread on Thiosulphate mineral nutrient (TMN) agar plates. The TMN medium for isolation of SOB contained (per litre) 0.2g $CaCl_2.2H_2O$, 0.4 g NH_4Cl, 0.2 g/L $MgSO_4$, 4.0 g/L KH_2PO_4, 4.0 g/L K_2HPO_4 and 10 g/L $Na_2S_2O_3$ [9]. For agar preparation, 15 g/L of bacteriological agar powder was mixed into TMN medium. The final pH was adjusted to 8 and bromothymol indicator was used as pH indicator [5]. The plates were incubated at 30°C for 14 days.

The purified isolates were cultured and incubated in the 100 mL TMN medium at 30°C for 14 days. Subsequently, the most efficient SOB was selected and observed its morphology and several biochemical characterisations, including colony characteristic, shape and Gram's reaction. It was further identified by 16S rRNA sequences and preserved in stock medium under sterile glycerol (20%) at -80°C.

2.3. Molecular Identification of SOB
Extraction of genomic DNA and amplification of 16S rRNA were carried out by Centre for Chemical Biology (CCB), Penang, Malaysia. GF-1 Bacteria DNA Extraction Kit and Nanodrop 2000 Spectrophotometer were used to obtain pure isolates DNA. The 16s rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers 16S-27F and 16S-1492R. PCR was carried out under the following conditions: 94°C (3 min), 30 cycles of 94°C (30 s), 55°C (30 s) and 72°C (1.4 min) and a cycle of final extension at 72°C (5min). Sequence identification was performed using BLAST analysis provided by NCBI (National Centre for Biotechnology Information, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Sulphur Oxidizing Test

The isolates were tested for pH reduction ability and production of sulphate content. About 10ml of samples were collected using a syringe and filtered using 0.22 µm Nylon syringe filter to remove SOB from broth. The pH of the inoculated broth was determined using a pH2700 Eutech Instrument. Meanwhile, the determination of sulphate ion content was conducted using SulfaVer4 method analysed by UV/visible spectrophotometry (DR 2800 spectrometer) at 450 nm [10].The growth of SOB was monitored by turbidometry using DR 2800 spectrometer at 600 nm wavelength.

2.5. Effect of Temperature and pH Media

The selected SOB was tested for optimal temperature and pH conditions. The starter culture size used was 10% (v/v) in different temperature (20°C, 30°C and 40°C) and pH (pH 5,7, 9) in orbital incubator shaker (Infors HT Ecotron Shaking Incubator) at 150 rpm, 30°C for 14 days. The growth of isolate, sulphate ion content and pH reduction were recorded periodically.

3. Results And Discussion

3.1. Identification of SOB

About 21 different bacterial strains were isolated from the sludge sample. The strains were selected based on their responses towards pH reduction ability and oxidation of sulphate content as summarized in table 1. It can be clearly observed that SOB-18 showed maximum pH reduction and sulphate content.

Table 1. Biooxidation of H_2S in the TMN media by isolated SOB.

Isolates	Colour after 14 days	Initial pH	pH after 14 days	Initial sulphate content (g/ml)	Sulphate content after 14 days (g/ml)
SOB-1	Blue	8.00	8.80	10	370
SOB-2	Blue	8.00	7.65	10	14
SOB-3	Blue	8.00	8.24	10	14
SOB-4	Blue	8.00	8.26	10	25
SOB-5	Green	8.00	6.79	10	33
SOB-6	Blue	8.00	8.17	10	17
SOB-7	Blue	8.00	8.94	10	220
SOB-8	Blue	8.00	7.80	10	20
SOB-9	Blue	8.00	8.80	10	312
SOB-10	Blue	8.00	7.65	10	14
SOB-11	Blue	8.00	8.24	10	40
SOB-12	Blue	8.00	8.26	10	25
SOB-13	Blue	8.00	7.79	10	33
SOB-14	Blue	8.00	8.60	10	247
SOB-15	Blue	8.00	7.85	10	18
SOB-16	Green	8.00	6.89	10	106
SOB-17	Blue	8.00	8.56	10	76
SOB-18	Yellow	8.00	5.40	10	340
SOB-19	Blue	8.00	8.07	10	14
SOB-20	Blue	8.00	7.68	10	56
SOB-21	Blue	8.00	7.89	10	87

SOB-18 was selected as it exhibited better pH reduction ability due to media colour changes indicated by bromothymol from blue to yellow (figure 1). The SOB-18 could reduce pH to 5.40 from the initial pH of 8.00 of TMN medium within 14 days of incubation. The pH reduction in identifying SOB was also reported by Behera *et al.*[5], Veerender *et al.*[11] and Vidyalakshmi and Sridar [12] due to the existence of sulphuric acid during the oxidation process.According to Jin *et al.* [13], since H_2S converted to sulphate and hydrogen ion, the pH of the culture medium changed from alkaline to acidic condition.

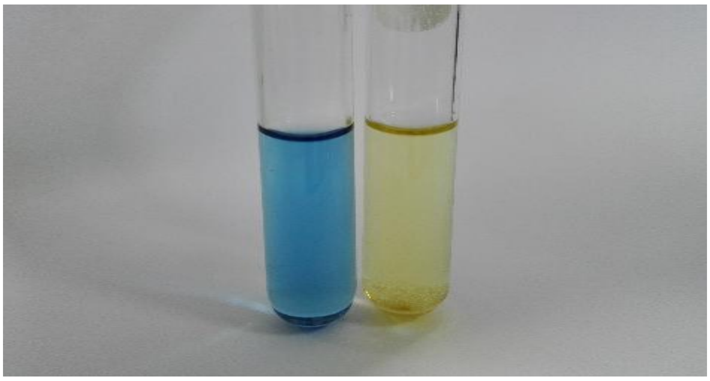


Figure 1. Screening of sulphur oxidising bacteria on TMNmedia supplied with bromothymol indicator (a) control and (b) the growth of SOB-18 after 14 days cultivation time.

SOB-18 was found to be highest sulphur oxidase producer. It oxidized 340 g/ml sulphur from initial sulphate content of 10 g/ml. The sulphate ion revealed that SOB-1818 produced high amount of sulphate ion as sulphur source and took oxygen as the electron accepter. Thus, SOB-18 was considered as the most efficient sulphur reduction bacteria obtained from sludge sample. The molecular identification analysis revealed that the SOB-18 isolates was represented by *Paracoccus solventivorans* ATCC 700252 with DNA similarity of 99%.

3.2. Effect of Temperature on Cell Growth, Sulphate Content and pH Reduction

Figure 2 illustrates the growth of *Paracoccus* sp. (figure 2a), sulphate content (figure 2b) and pH reduction (figure 2c) in the TMN medium at different temperature conditions. For 30°C and 40°C, there was a great difference of each other with the cell growth increased to base on turbidity with OD (Optical density) of 0.252 and 0.245, respectively. The sulphate concentration also increased tremendously to 402.0g/ml (30°C) and 375.0g/ml (40°C). The pH dropped sharply to pH 5.32 (30°C) and pH 5.80 (40°C). The increased growth at 20°C showed a slight increase to OD of 0.196, while the sulphate content produced was 276.5g/ml. The pH value decreased slightly of pH 6.57.For 50°C, the growth reduced vigorously to OD of 0.131. The same trend was recorded for sulphate content (76.5 g/ml) and pH reduction (pH 7.50) almost unchanged.

As mentioned above, *Paracoccus* sp. was grown in a continuous culture at different temperature conditions. The optimum temperature condition was in the range of 30°C to 40°C. In this temperature range, the rate of bacteria multiplication increased constantly. The growth of bacteria continued until H_2S depleted or toxic product accumulated. Beyond the temperature range, *Paracoccus* sp. was not able to sustain itself from high temperature prior to conformation of protein changes and denatured inside cell membrane. However, *Paracoccus* sp. took longer time to grow at low temperature than the temperature range, thus bio-oxidation of sulphur occurred slowly.

a)
b)
c)

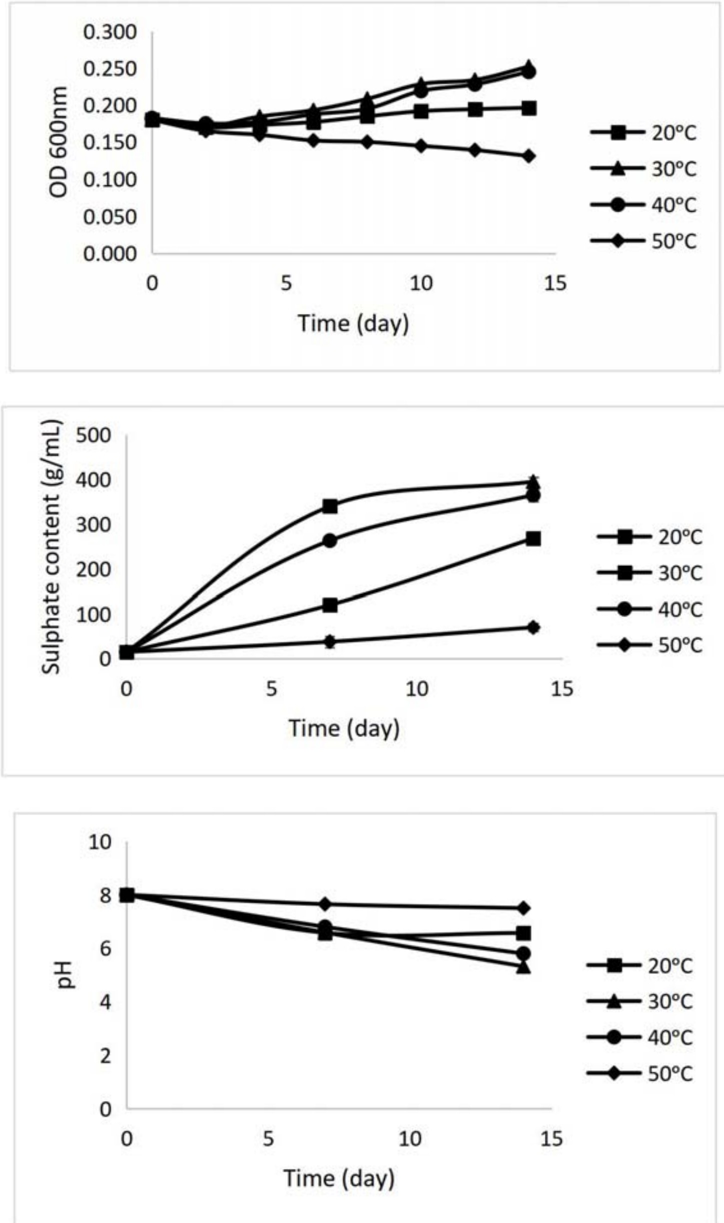
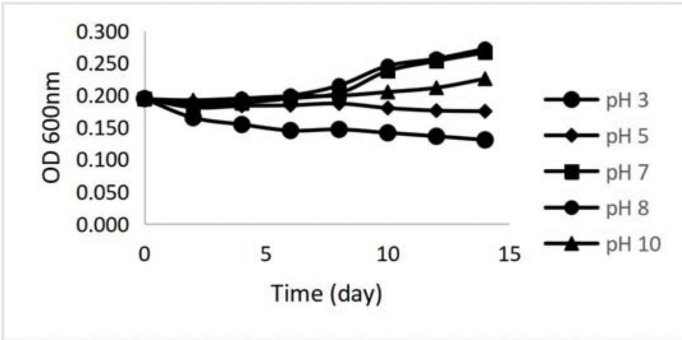


Figure 2. Study of biooxidation sulphur on (a) growth of *Paracoccus* sp. strain, (b) sulphate content production and (c) pH reduction on various temperature conditions for 14 days at pH 8.0 with orbital shaking. The initial culture and sulphate content were standardized at OD of 0.180 and 15 g/ml, respectively.

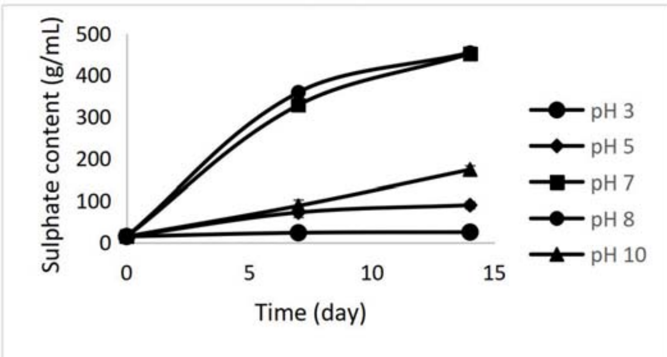
3.3. Effect of pH Media On Cell Growth, Sulphate Content and pH Reduction

Figure 3 shows the growth profile of *Paracoccus* sp. (figure 3a), sulphate content (figure 3b) and pH reduction (figure 3c) in the TMN medium at different pH media.

a)



b)



c)

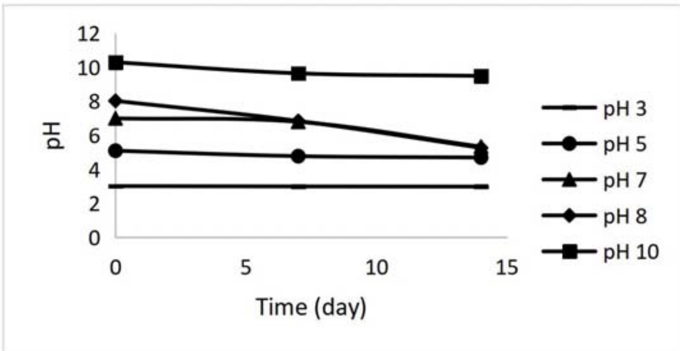


Figure 3. Study of biooxidation sulphate on (a) growth of *Paracoccus* sp. strain, (b) sulphate content production and (c) pH reduction on various pH conditions for 14 days at 30°C with orbital shaking. The initial culture and sulphate content were standardized at OD of 0.195 and 15 g/ml, respectively.

It can be seen at pH 7.0 and 8.0, the sulphate content production obviously increased to 451.5 g/ml and 454.0 g/ml with the increase of OD of 0.267 and 0.272, respectively. The pH reduction decreased sharply from pH 7.0 to pH 5.34 and pH 8.0 to pH 5.27. For the other three, the cell growths were kept steady for sulphate content production and the pH reduction almost remained unchanged. In this study, the optimum pH of *Paracoccus* sp. to grow was in the range of 7.0 to 8.0.

4. Conclusion

This study emphasizes the importance of sulphur oxidizing bacteria in the oxidation of sulphur in palm oil sludge. It was concluded that *Paracoccus solventivorans* ATCC 700252 was able to oxidize sulphur with the reduction of medium pH and efficient production of maximum sulphate ion throughout 14 days cultivation period. The optimum temperature and pH of *Paracoccus* sp. were in the range of 30°C to 40°C and pH 7.0 to pH 8.0, respectively. These *Paracoccus* sp. isolates can be utilized to mitigate the odour nuisance and solve the corrosion problems in the pipeline and equipment of industries. In addition, the pH reduction through the production of sulphuric acid during oxidation of sulphur can be utilized for other application. Sulphur oxidizing bacteria such as *Paracoccus* sp. also has potential to bio filtration of gaseous stream [14].

Acknowledgements

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