CHEMICAL ENGINEERING TRANSACTIONS

VOL. 106, 2023

A publication of AIDIC

The Italian Association of Chemical Engineering
Online at www.cetjournal.it

Guest Editors: Jeng Shiun Lim, Nor Alafiza Yunus, Peck Loo Kiew, Hon Huin Chin

ISBN 979-12-81206-05-2; **ISSN** 2283-9216

DOI: 10.3303/CET23106201

Identification of Facultative Anaerobic Bacteria for Low-Cost Nutrient Removal in Treating Domestic Wastewater

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Facultative Anaerobic Bacteria is the group of bacteria that can grow with or without oxygen whereby they can make Adenosine Triphosphate (ATP) which is the preferred source of useable energy through aerobic respiration if oxygen is available or switch to fermentation if oxygen is not available. Thus, there is an opportunity to explore the bacteria in enhancing nutrient removal and at the same time has potential for energy saving at the Wastewater Treatment Plant (WWTP). This study aimed to screen and isolate the dominant facultative anaerobic bacteria in WWTP where wastewater from four different categories i.e. Extended Aeration (EA), Oxidation Pond (OP), Sequencing Batch Reactor (SBR) and Conventional Activated Sludge (CAS) across the Klang Valley region has been sampled. From the investigation, bacterial strain from the targeted group was successfully isolated i.e Lactobacillus plantarum which found at SBR plant and the suitable low-cost medium for isolated bacterial cultivation was identified. Based on the result, a 5 % of molasses concentration was selected and used for isolated strain performance study. After that, bacterial dosing rate determination was studied using real wastewater with different dosing rates (1 %, 3 % and 5 %). The result indicates at 1 % dosing rate, the percentage of nitrogen removal is higher compared to others whereas Ammoniacal Nitrogen reduced at 97.3 % after 24 h and achieved 98.2 % after 96 h. Meanwhile, Nitrate removal is 63.9 % in 24 hand 39.8 % after 96 h. This finding is useful for the application of L.plantarum as a bioaugmentation product at full scale.

1. Introduction

Malaysia's municipal Wastewater Treatment Plants (WWTP) use an activated sludge technology to treat sewage in order to meet regulatory requirements. This process requires the provision of aeration, which has been recognized as a significant portion of the total amount of energy consumed, which contributes to operational costs. Studies from different countries have shown that between 50 and 70 % of the energy used in a WWTP goes toward biological treatment, and more specifically aeration (Vergara-araya et al., 2021). The facilities that used additional biological treatment for the removal of nutrients required between 30 and 50 percent more energy for aeration, pumping, and the processing of solids as compared to plants that used a normal activated sludge system. It is possible that the use of blowers and aerators accounts for as much as 70 % of the overall energy consumption of the plant (Ramli and Hamid, 2017). As a consequence of this, the approach or solution to minimize energy consumption in operational WWTPs through the study of existing microbial technology is typically favoured since it does not have a negative impact on the environment, and it is cost-effective. Given that microbial are already present in the sewage treatment plant, facultative anaerobic bacteria were discovered to have the potential to be one of the solutions to the problem. In addition, it is thought that boosting the population of this particular species of bacteria will result in the WWTP requiring less aeration and, as a consequence of this, less electricity to run the facility. Past research (How et al., 2018) proved that nitrification is effective under low-Dissolve Oxygen (DO) conditions (0.5 mg/L) for tropical sewage treatment (30 °C). This was accomplished with a 23 % reduction in energy consumption when compared to the conventional nitrification process that uses mechanical aeration (2 mg O_2/L). An organism known as a facultative anaerobic bacterium is one that is able to live in an environment that contains oxygen while also reproducing even more quickly in an

Paper Received: 19 March 2023; Revised: 18 July 2023; Accepted: 30 September 2023
Please cite this article as: Aris A.M., Yuzir A.M., Anuar A.N., Hara H., 2023, Identification of Facultative Anaerobic Bacteria for Low-Cost Nutrient Removal in Treating Domestic Wastewater, Chemical Engineering Transactions, 106, 1201-1206 DOI:10.3303/CET23106201

environment without oxygen. Fermentation or alternative terminal electron acceptors are the methods that allow facultative anaerobes to function in the absence of oxygen. *Lactobacillus* sp. and *Pediococcus* sp. are Gram-positive and facultative anaerobe lactic acid bacteria. According to Gorsuch et al. (2015), isolates heterotrophic bacteria i.e *Lactobacillus* sp. and *Pediococcus* sp. inoculated in the deionized water and wastewater reactor flasks are responsible for the gradual disappearance of ammonia over time. Therefore, this study aimed to investigate microbes' ability in increasing nutrient removal efficiency and as a solution for energy savings based on their capability found in a literature review and the existing abundance of microbes in activated sludge. The significance of facultative anaerobic bacteria was chosen in this study due to the potential of this group of bacteria for simultaneous nitrification and denitrification. The possibility of promoting energy reduction in domestic wastewater treatment plant via the predominance of facultative anaerobic bacteria in activated sludge was taken into consideration due to this organism can survive in an oxygen-containing environment while growing much more productively in an oxygen-free environment. Furthermore, the addition of the potent indigenous microbes into the WWTP through bioaugmentation will accelerate the treatment efficiency since these microbes are continuously exposed to a background mixture of recalcitrant pollutant for an extended period of time in specific ecological niches (Sonkar et al., 2021).

2. Materials and methods

2.1 Determine the target bacterium

In this particular study, numerous categories of WWTPs located in the Klang Valley were sampled in order to look for the facultative anaerobic bacteria, *Pediococcus sp.* or *Lactobacillus sp*. The STP involved include Oxidation Pond (OP) in Subang Jaya, Sequential Batch Reactor (SBR) in Titiwangsa, Extended Aeration (EA) in Petaling Jaya, and Conventional Activated Sludge (CAS) in Pantai Dalam. Only plants with an MLSS that was measured above 1500 mg/L were chosen due to the expectation of plentiful necessary facultative anaerobic bacteria were present in healthy levels. Then, based on the result of the probable bacterial group from preliminary study (Alijah et. al, 2021), the selected colony has been sent to an external laboratory, Apical Scientific Sdn Bhd for colony identification using bacterial species barcoding (16s rRNA). The result from the bacterial identification has been compared to see whether the targeted facultative anaerobic bacteria i.e *Pediococcus sp* and *Lactobacillus sp* preferred to live in aerobic or anaerobic conditions and available in what type of STP whether OP, EA, CAS or SBR. *Pediococcus sp* is the main target bacterium for this study but since it was not identified in the result, the second option i.e *Lactobacillus sp* has proceeded for the next step of the study.

2.2 Study the suitable media

Molasses was used in this study to cultivate the selected facultative anaerobic bacteria and hence determine the suitable molasses concentration through the lab-scale experiment. Molasses has been identified as a cheaper carbon source and proven in increasing nitrogen removal efficiency (Joanna and Katarzyna, 2017). Two molasses concentration has been selected i.e 5 % and 10 % based on a previous study carried out by Farzana et al. (2017). The study has been running triplicate in the 250 mL shake flask and incubator shaker. The maximum bacterial cell yield and the bacterial growth rate has been monitored by the optical density method and measured it using a UV-Vis spectrophotometer

2.3 Study the isolated bacterial performance in lab-scale experiment

The isolated facultative anaerobic bacteria have been further studied for their performance in a 5 L fermenter. For this, real wastewater was placed in the 5 L fermenter, and the experiment has been running under the fixed operating condition (pH 7.5, temperature 30°C and DO 2 mg/L). The bacterial cell was dosed into the real wastewater in the fermenter. A few parameters, including pH, Chemical Oxygen Demand (COD), Ammoniacal Nitrogen (NH_3-N), Total Suspended Solid (TSS), and Nitrate (NO_3), were measured using samples taken before and after the bacteria were dosed. All the measurements were analyzed using the HACH method and Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF, 2012) except for pH which used Eutech pH meter. The experimental activity has been done through two runs in which different doses of bacteria (1 %, 3 % and 5 %) are added to the same volume of wastewater and tested with fixed time (Nasr, 2010).

3. Result and discussions

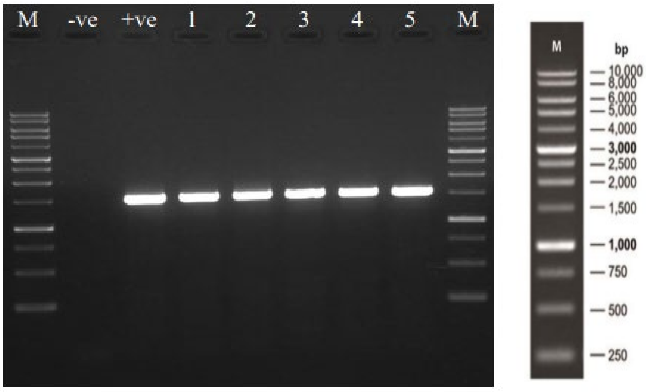
3.1 Bacterial species barcoding (16SrRNA)

By using results of the biochemical test to determine the most likely bacterial group, selected strains have been further isolated by spreading them out on plates and growing them in streaked. Before beginning the process of bacterial species barcoding, the bacterial strain that was grown on the agar was first triplicatively sliced into 0.5

x 0.5 cm squares and then placed in separate 1.5 mL tubes. In accordance with the DNA extraction methodology developed by 1st Base company, bacterial DNA was isolated. Universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') were utilized in order to amplify the whole length of the bacterial 16S rDNA. For each reaction, a total volume of 25 µl reaction mixture containing genomic DNA that had been purified using an in-house extraction method, 0.3 µM of each primer, deoxynucleotides triphosphates (400 mM of each dNTP), 0.5 U Taq DNA polymerase, supplied PCR buffer and water was used. The PCR was carried out in the following manner: 1 cycle at 94 °C for two mins for initial denaturation, followed by 25 cycles at 98 °C for 10 s, 53 °C for 30 s, and 68 °C for 1 min for annealing and extension of the amplified DNA. Utilizing a conventional technique, the PCR products were purified, and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was utilized in order to do direct sequencing using primers 785F and 907R. Table 1 shows the identification result.

Table 1: Identification of the selected bacterial strains by 16s rRNA

Isolates No.	Microbes identified	Descriptions	Max score	Total Score	Query cover	Identification
AE5	*Lactobacillus plantarum*	JCM 1149	2030	2030	100%	100%



-ve: PCR no template control
+ve: Positive control, Bacterial gDNA, 10 ng

Figure 1: Bacteria, 16S rRNA PCR results (~ 1.5 kb full length)

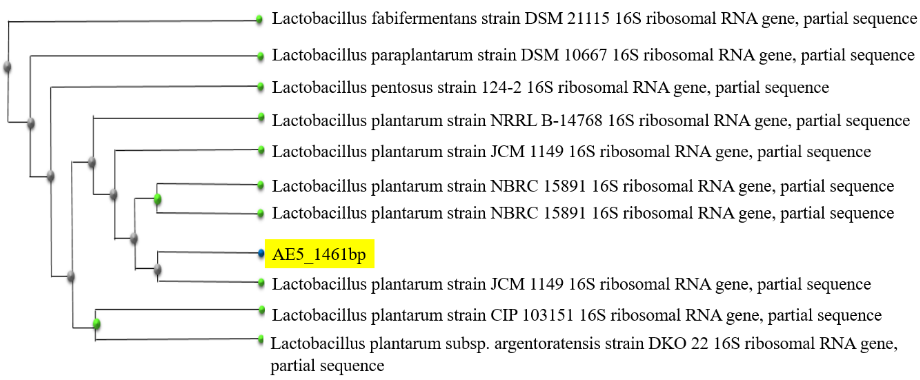


Figure 2: Phylogenetic tree (L. plantarum)

Bacterial species barcoding (16's rRNA) as shown in Figure 1 has been performed and as a result, a bacterial strain from the targeted group was successfully isolated, *L. plantarum* (Figure 2) which found at SBR plant.

3.2 Study the suitable media

Medium Comparison - Two different mediums concentrations were compared for bacterial culture which are 5 % molasses and 10 % molasses. The Optical Density (OD) test revealed that a 5 % molasses medium can be strongly recommended as a suitable medium for *L. plantarum* growth. Besides, the increment trend is quite stable. Moreover, being an economical substance, molasses is a preferable medium.3.3 Study the *L. plantarum* performance in the lab-scale experiment

3.3.1 Study on different microbe dosing volume

Based on the COD data shown in Figure 3, it can be concluded that the addition of *L. plantarum* in molasses media could increase COD in the wastewater if compared to the control (0 %). At a full-scale or actual wastewater treatment plant, this situation is not really affected since the plant itself has a high capability of removing COD and sometimes the addition of carbon sources is required to increase F/M ratio. This is due to most of the wastewater treatment plants receiving low COD or low BOD loading due to the long sewer pipeline that caused sewage dilution. The dilution will be resulting MLSS unable to build up and affect the plant compliance.

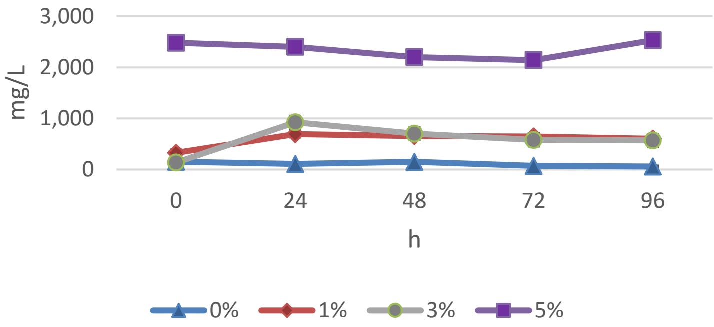


Figure 3: COD concentration in effluent within 96 h at different microbe dosing (1 %, 3 % and 5 %) compared to the control (0% - no microbe dosing)

Meanwhile, NH_3-N removal obviously occurred when the wastewater was dosed with 1 % of *L. plantarum* (as shown in Figure 4). After 24 h of the contact period, NH3-N concentration decreased from the initial 21.9 mg/L to 0.6 mg/L, which indicated 97.26% removal efficiency. The removal continuously occurred to 0.4 mg/L at 96 h and thus proven that 1% was the optimum dosing rate for ammoniacal nitrogen removal.

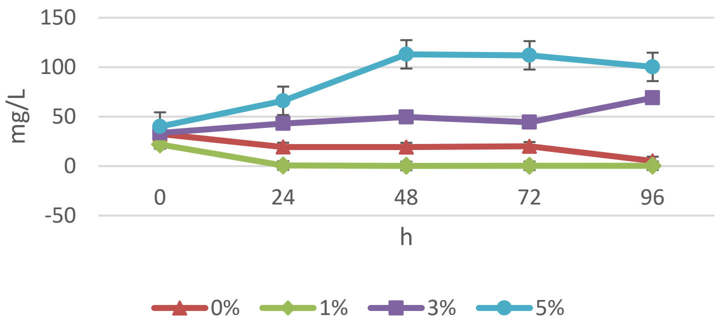


Figure 4: NH_3-N concentration in effluent within 96 h at different microbe dosing (1%, 3% and 5%) compared to the control (0% - no microbe dosing)

Furthermore, after 24 h of the contact period, NO_3 concentration decreased from the initial 10.8 mg/L to 3.9 mg/L, which indicated 63.89% removal efficiency when the wastewater was dosed with 1 % of *L. plantarum* (as shown in Figure 5). However, the reading slightly increased to 6.5mg/L at 96 h. This trend is consistent with the ammoniacal nitrogen result where high ammoniacal nitrogen is converted into nitrite and nitrate finally will cause nitrate result to become slightly higher. Nevertheless, the nitrate results are still acceptable and below the standard A compliance, and this shows that denitrification and nitrification takes place simultaneously in the bioreactor under low Dissolve Oxygen (<2.0 mg/L). Based on the overall study result, 1 % of bacterial dosing showed the best in terms of nitrogen removal performance, especially for ammoniacal nitrogen and nitrate removal.

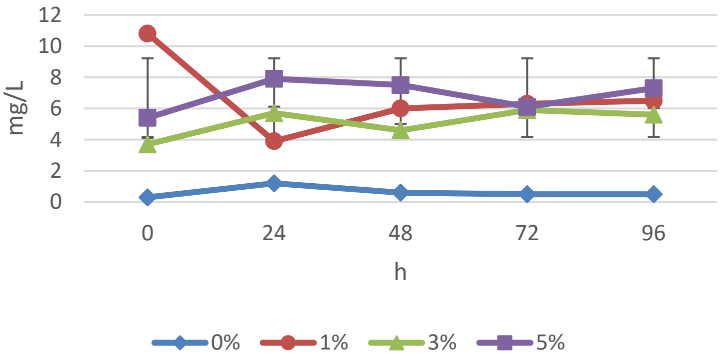


Figure 5: NO_3 concentration in effluent within 96 h at different microbe dosing (1 %, 3 % and 5 %) compared to the control (0% - no microbe dosing)

The result in Figure 6 shows that the pH is still within the range for sewage treatment plant compliance. However, for 1 %, the initial pH is quite higher compared to other dosing rates but slowly become neutral.

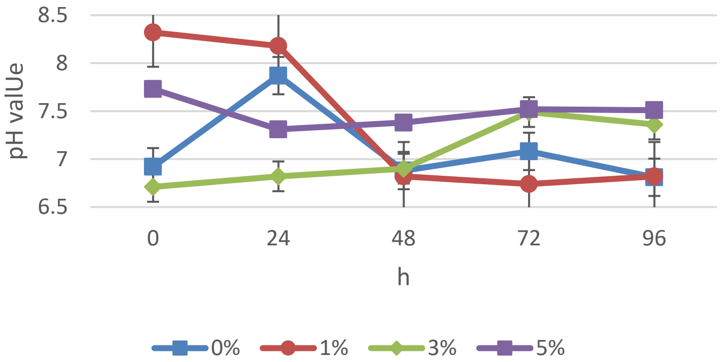


Figure 6: pH in effluent within 96 h at different microbe dosing (1 %, 3 % and 5 %) compared to the control (0% - no microbe dosing)

Overall TSS results as shown in Figure 7 have an increasing trend after 24 h compared to control (0 %). It can conclude that the addition of bacterial products into wastewater will increase sludge production due to additional biomass from the bacterial growth.

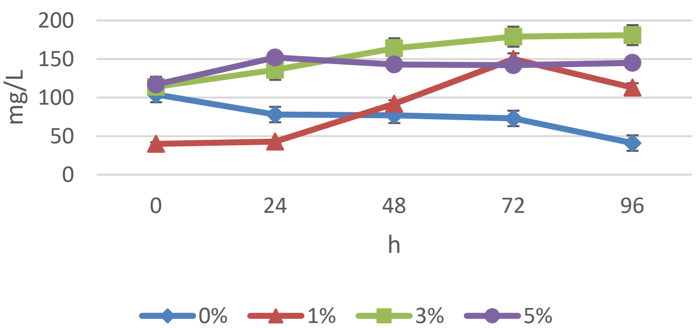


Figure 7: TSS concentration in effluent within 96 h at different microbe dosing (1 %, 3 % and 5 %) compared to the control (0 % - no microbe dosing)

Based on the overall study result, 1 % of bacterial dosing showed the best in terms of nutrient removal performance, especially for ammoniacal nitrogen and nitrate removal.

4. Conclusions

In this study, the targeted facultative anaerobic bacteria have successfully identified from the activated sludge in tropical climate purposely for bioaugmentation, whereas the aim is to improve the biological nitrogen removal in the WWTP and indirectly can pave the way for the energy-saving initiative. The results highlighted that by adding *L. plantarum* into the wastewater, the nitrogen removal was improved as compared to the control reactor (no bacteria dosing). Molasses has been proposed as a low-cost medium for *L. plantarum* cultivation at a commercial scale. A 5 % of molasses concentration was found as an optimum condition for better growth. Finally, a bacterial performance study using real wastewater that has been carried out at different dosing rates (1 %, 3 %, and 5 %) of *L. plantarum,* clearly shown at 1% dosing rate, the percentage of nitrogen removal is higher compared to others where Ammoniacal Nitrogen reduced to 97.3 % after 24 h and achieved 98.2 % after 96 h. Meanwhile, nitrate removal is 63.9 % in 24 h and 39.8 % after 96 h. Thus, the ability of *L. plantarum* isolated from activated sludge in tropical climates as simultaneous heterotrophic nitrifiers and heterotrophic aerobic denitrifies is proven.

Acknowledgements

A sincere acknowledgement to Indah Water Konsortium Sdn. Bhd. (IWK) for supporting this research project in collaboration with Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia (MJIIT, UTM).

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