

Study on Effective of *Bacillus subtilis* for Domestic Wastewater Treatment in the Royal Thai Army Chemical Department Area

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Abstract

Today, wastewater is emitted from various sources into natural water sources, causing the natural water source to be polluted by waste. In this study, the emphasis was placed on using *Bacillus subtilis* to treat wastewater in the pond in front of the Science of Division Royal Thai Army Chemical Department. This pond collects wastewater from the office building and residential areas within the RTA Chemical Department and releases it into the public canal. Therefore, treating the wastewater before releasing it into the canal was necessary. Samples were collected and the bacteria were separated from the soil and water samples within the RTA Chemical Department area. The pure strains of bacteria were then studied for their basic characteristics, with 21 samples tested for their ability to produce enzymes that break down proteins, starch, and fat. It was found that isolates KCT03, KCT04, and KCT05 were bacteria that could produce enzymes from all three of these groups. The test for bacterial resistance was conducted and it was found that all 3 strains were not resistant. The differentiation was carried out using Mass Spectrometry and it was discovered that KCT03 and KCT05 were *Bacillus subtilis*. In order to test their ability to treat bacterial waste in laboratory conditions, The Completely Randomized Design (CRD) experiment consisting of 3 sets of tests with 3 repetitions each was conducted. The quality of the water, including pH, TSS, TDS, COD, BOD and fat oil and grease were measured. It was found that EM3 was the most effective in treating wastewater. It had 5.56% efficiency in treating the organic matter in wastewater, 28.84% efficiency in treating total dissolved substances in wastewater,

19.58% efficiency in treating oil and fat in wastewater, and 16.67% efficiency in treating the organic matter in wastewater.

Keywords: Bacteria, *Bacillus Subtilis*, Wastewater, Wastewater Treatment, Microorganisms.

Introduction

Water is essential for the sustenance of all living beings and is a vital natural resource for agriculture, fishing, and industry. Currently, wastewater is released from various sources into the environment. This causes natural water sources to be contaminated by waste products, such as organic pathogens and various inorganic substances, resulting in the deterioration of water sources (Sura-ut Suphachaturaf, 2545). Initially, nature made the water return clean. However, when there is a large amount of wastewater, the concentration increases as the community grows so that nature cannot keep the water clean in time. Therefore, wastewater must be treated using various methods before being released into natural water sources (Mansin Tanthulavet, B.E. 2542).

Wastewater treatment methods can be divided into physical, chemical, and biological methods. Biological wastewater treatment is the most popular method because it is less costly than other methods and uses microorganisms that exist in nature to utilize organic substances or impurities in wastewater as food. Approximately 95% of organic matter is used as an energy and carbon source and then converted into carbon dioxide, water, and new cells, resulting in wastewater becoming less dirty until it meets the legal standard acceptable for release into natural water sources (Subunnit Nimrat, B.E. 2548). Therefore, this study aimed to investigate the utilization of *Bacillus subtilis* for wastewater treatment and to make the wastewater system stabilize faster.

Research Objective

1. To study the efficiency of *Bacillus subtilis* in wastewater treatment.

Derivation and the significant of problems

According to the National Environment Promotion and Conservation Act 2535, wastewater refers to waste in a liquid state, including contaminated pollutants in that liquid. Pollution sources that affect water quality in water resources can be divided into two main categories. Point sources have a definite origin, such as those from communities and industrial plants. Nonpoint sources, such as agriculture wastewater can be divided into three types.

1. Wastewater from the community, including that generated by the activities of people living there, contains high levels of organic impurities.
2. Industrial wastewater, such as wastewater from industrial processes, from the process of cleaning the raw materials and production process to

factory cleaning, including untreated wastewater or treated wastewater, still needs to meet the industrial effluent standards. The composition of industrial effluent is different and depends on the effluent flow rate, type, and size of the plant.

3. Agricultural wastewater, generated from cultivation activities and animal husbandry farming, contains high levels of nitrogen, phosphorus, potassium, and various toxins, most of which are found in organic matter (Ministry of Natural Resources and Environment, B.E. 2561).

Domestic wastewater refers to the daily activities of people living in the community and activities of some occupations, including cooking and cleaning all dirt within the household and various types of buildings (Thongchai et al.; B.E. 2530). Community wastewater consists of;

1. Organic matter in municipal wastewater may consist of carbohydrates, proteins, and fats, such as rice, noodles, curry paste, and scraps of banana leaves, vegetables, and meat pieces. It can be decomposed by microorganisms that use oxygen, causing the Dissolved Oxygen level to decrease and resulting in putrid conditions. The amount of organic matter in water is commonly measured by the BOD value, which shows that there is much organic matter, and the putrid state will occur easily.

2. Inorganic substances contain minerals that may not cause foul water but may be harmful to live organisms, such as chloride and sulfur. Heavy metals and toxins may be in the form of organic matter or inorganic matter. They can accumulate in the food cycle, causing harm to live organisms such as mercury, chromium, and copper. Industrial wastewater and chemicals are typically used to kill pests contaminated with agricultural wastewater. This pollutant may come from household industries such as Metal Plating shops, Garages from Tanneries, Slaughterhouses, or Canned Food factories in the community area. Many microorganisms use oxygen to live and can reduce the level of dissolved oxygen, causing a putrid state. In addition, some microorganisms, such as microorganisms in hospital wastewater, may harm humans.

3. Nutrients, including nitrogen and phosphorus, when in high amounts, will cause rapid growth and increase algae (algae bloom), causing the oxygen level in the water to drop very low during the night, which also causes aquatic weeds, which is a problem for water traffic.

4. The smell is caused by hydrogen sulfide gas, which is produced by the decomposition of organic matter without oxygen or other odors from various industrial factories such as fishmeal factories and slaughterhouses (Pollution Control Department, B.E. 2563).

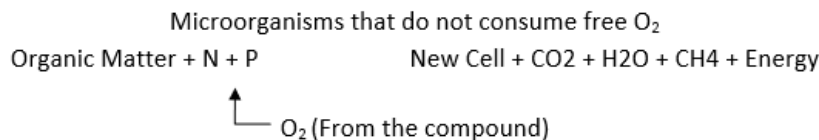
A biological wastewater treatment system is a wastewater treatment process that relies on microorganisms to decompose Biodegradable Organic Compound matter. The microorganisms use organic matter as food and substrate in the life process, growth, and synthesis of New Cells

and yield Carbon Dioxide (CO₂), water (H₂O), and Non-Biodegradable residual (Vera Chiravira, B.E. 2545) with the following treatment processes:

1. Aerobic wastewater Treatment: It is a wastewater treatment process using microorganisms that rely on dissolved or free oxygen for the degradation of organic matter. The organic degradation reactions of Aerobic Bacteria can be classified into two steps:

Step 1: The process of bringing organic matter or nutrients into the cell. The microorganisms send enzymes to decompose organic matter that adheres to the cell wall to change into the form of small molecules that can permeate into the microbial cells.

Step 2: It is a biochemical process within the microbial cell that produces energy for use in various activities and the creation of new cells, which can be written in the form of an overall equation as follows:



When the organic matter in the wastewater is transformed into a new microbial cell, it will gather in a flock (biological flocculation), have more weight, and can be easily separated from the wastewater by sedimentation.

Aerated wastewater treatment processes can be classified into two main types.

1. Treatment systems in which microorganisms are suspended include aerobic ponds, aerated lagoons, and activated sludge systems.
2. Treatment systems where microorganisms adhere to the medium surface or fixed film systems, such as the trickling filter system and rotating biological contactor system.

2. Anaerobic Wastewater Treatment is the process of treating wastewater in an anaerobic state in the absence of Oxygen.

Microorganisms rely on other compounds as electron acceptors instead of Dissolved Oxygen or free Oxygen, which can be divided into four steps, as follows:

Step 1: This is a hydrolysis process based on enzymes sent outside the cell to convert large organic molecules into small molecules.

Step 2: It is an acid formation process (acidogenesis) by acid-producing bacteria. This converts the product obtained from the first hydrolysis reaction to volatile fatty acid (vfa).

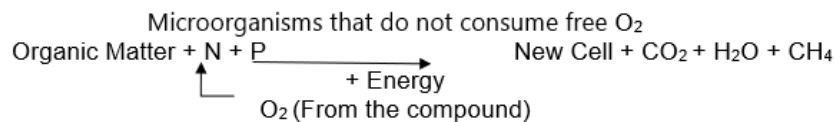
Step 3: Create acetic acid from vfa. (acetogenesis) by Acetogenic Bacteria. It will change vfa. These are essential products in methane production, including acetic acid, formic acid, carbon dioxide, and hydrogen gas.

Step 4: Methane (methanogenesis) is created, in which the product from the acid-forming bacteria in the 3rd step is converted to methane by methane-forming bacteria (methanogenic bacteria).

It can be divided into two types.

1. Bacteria that generate methane from carbon dioxide and hydrogen (hydrogenotrophic bacteria) obtain carbon from carbon dioxide and energy from hydrogen.

2. Methane-Producing Bacteria from acetic acid (acetotrophic bacteria), which use acetate as an electron acceptor and uses hydrogen as an energy source, can be written as an overall equation as follows:



All four steps in the anaerobic treatment process rely on the work of 2 groups of bacteria. Therefore, it is necessary to maintain a suitable environment for continuous interactions between these two groups of bacteria. Therefore, it is necessary to maintain a suitable environment for the continuous interaction of the two groups of bacteria. If the activity of a group of bacteria changes, it affects the work of another group of bacteria and the system's overall efficiency.

For example, suppose the system receives more nutrients or organic matter than normal Acid-Forming Bacteria and has a higher growth rate, an increase in the production of organic acids and various yields results in a group of Methane-Producing Bacteria that are less capable of growing and unable to decompose the increased organic acids in time. There will be an increase in the volume of organic acids accumulated, which, if the system does not have enough buffer power, a decrease in the pH of the system inhibits the growth of Methane-Producing Bacteria until it causes the performance of the system to decrease or the operation of the system eventually fails.

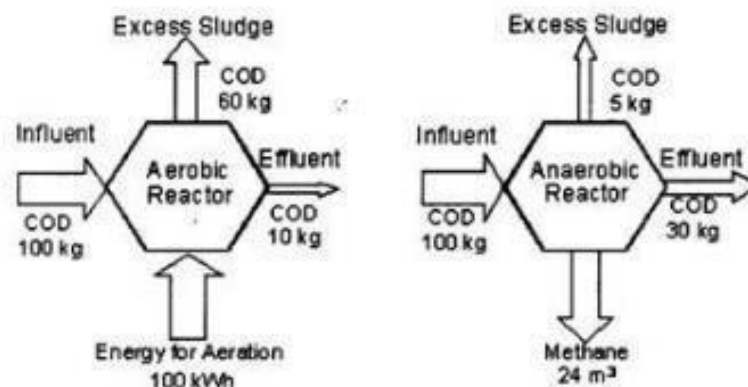
Anaerobic wastewater treatment processes can be classified into two types.

1. Suspended systems include anaerobic ponds, digesters, and anaerobic contacts.

2. Fixed film systems such as anaerobic filters and anaerobic fluidized beds.

The overall differences between aerobic and anaerobic treatment processes can be represented as the COD and energy balance (Figure 1). In other words, aerobic digestion produces better-quality effluent. There are remaining oxygen-demanding substances in the effluent in a small amount (about 10% of the organic matter). Most of the precursor organic matter is converted into Excess Sludge as Bacterial Biomass. Approximately 60% of COD enters the system and requires further treatment. Anaerobic treatment section There will be Residual Solids and substances that require oxygen remaining in the effluent. It produces less excess sludge than conventional aerobic processes (approximately 30% of the organic matter). It is more stable than the aerobic process (representing approximately 5% of the COD that enters the system). In addition, the anaerobic system produces methane as the final product and can be used as the fuel and power source, respectively (Health Science Program Sukhothai Thammathirat University, B.E. 2544).

Figure 1. Comparison of COD and energy balance of anaerobic and anaerobic treatment processes. Source: Journey and Mcniven (1996)



The general characteristics of *Bacillus subtilis* are as follows;

Kingdom: Bacteria, Division: Firmicutes, Class: Bacilli, Family: Enterobacteriaceae, Genus: Bacillus, Species: subtilis.

B. subtilis is a gram-positive Saprophyte bacterium that lives freely by degradation of organic matter to build cells found in water, soil, and air. These bacteria can produce high heat- and drought-tolerant endospores in the environment. It can also produce proteins and various enzymes that contribute to nutrient degradation from plant remains and nutrient

cycling in the environment. However, it was found that most of the lifespan of this bacterium is not in living form but in the form of spores (Alexander, 1978). The wind can spread the spores; however, it has been challenging to determine whether blown *B. subtilis* spores fall into the air, but the environment will thrive in that area (Earl et al., 2008). *B. subtilis* is a harmless bacterium. It does not cause any disease or toxicity to animals and plants (Claus & Berkeley, 1986), although the mechanism of attachment or diffusion in humans has not been reported because they are bacteria that live in a wide range of environments. Therefore, it is expected that bacteria can temporarily reside on the human skin or even the gastrointestinal tract (Edberg, 1991). In addition, Klier et al. (1983) reported that *B. subtilis* could exchange and transmit plasmids; it can also pass the gene to other bacteria in the soil that grow alongside them. It was also found that this type of bacteria can secrete toxins or a mixture of toxins obtained from transfusion and other microorganisms (Edberg, 1991). The toxicity of *B. subtilis* in humans has also been studied. This strain has low toxicity to humans because it does not produce toxins or enzymes that are significantly toxic to humans. Although it has previously been reported that this strain causes disease in humans, when researching in more detail, it was found to be caused by *Bacillus cereus*, a strain closely related to *Bacillus subtilis*. *B. subtilis* is an easily cultivated microorganism that can use carbon sources and complex nitrogen contained in agricultural waste materials such as cottonseed meal and molasses. Minerals are often needed in small amounts, and adding some minerals, such as calcium and manganese, will increase the sporulation rate (Busarakam and Natthima; B.E. 2553). Furthermore, the authors reported that the suitable formula for *B. subtilis* processing was FFS1. It contained a mixture of 10 ml. of fish protein (fermented fish waste or fish manure) mixed with 10 g of soybean meal in 1,000 ml. of distilled water. The ingredients are cheap, easy to buy, and require the shortest time to increase. Bacterial culture under shaking conditions of 150 and 200 rpm was the optimum condition for the endospore formation of *B. subtilis*.

Research Methodology

1. Isolation of microorganisms from the sampling area.
2. The ability of microorganisms to digest organic matter was tested.
3. The efficiency of microbial wastewater treatment was tested in detail as follows:
 1. Well in front of the Science Division Department of Science, Royal Thai Army, coordinates VH2P+2Q, Bangkok (13.8500333,100.5869097) is 29 m wide, 48 m long, and 3 m deep. It can accommodate 4,173 cubic meters of water, and there is a discharge point for wastewater of certain sizes and types of buildings, totaling one sewer pipe. Wells are important collection ponds for wastewater from office buildings and residences

within the Army Science Department. The water in this pond was discharged into a public canal. Therefore, it is necessary to treat wastewater before it is released into public canals.

Figure 2. Show the well in front of the Science Division Department of Science, Royal Thai Army.



■ = Drainage chute from the Royal Thai Army Science School and cafeteria.



= Waste Strainer.



= Water sampling point.

(1. Upstream, 2. Midstream, 3. Downstream)

Soil samples were collected from the soil surface at a depth of ~ 5 cm. and a width of 10 cm. and placed in Polythene Bags of 500 g. Water samples from the wells were collected in a volume of 500 ml. in brown flasks (1,000 ml. The samples were sterilized, stored at 4 °C, and sent to the laboratory within 24 h for bacterial isolation. The wastewater characteristics of the study area were measured according to the standards of the Pollution Control Department, according to the announcement of the Ministry of Natural Resources and Environment. Subject: Control standards for drainage of wastewater from certain types and sizes of buildings, B.E. 2548. The morphological characteristics of the *Bacillus* genus were recorded. The pathogenesis was isolated from two soil samples and two water samples from the well in front of the Science Division and the well in front of the Pyro building.

2. Tests the Degradation Ability of Bacteria, used selective media containing protein, Skim Milk Agar, carbohydrate, Starch Agar and Fat Glycerol Tributyrin Agar, were used as selective media. Microorganisms can decompose proteins, carbohydrates, and fats in the following order.

(1) Isolation of protein-degradable microorganisms using skim milk as a representative by culturing microorganisms in water and soil samples. diluted in Skim Milk Agar medium in a Petri dish. The cells were incubated

at 37 °C for 2 days. Maintaining the germ that grows well, there is a clear area with different colony characteristics around the colony. Stored in the same medium that was slanted into a tube (slant).

(2) Isolation of microorganisms that can degrade carbohydrates using starch as a representative by culturing microorganisms in diluted water and soil samples. with Starch Agar medium on a petri dish and incubated at 37 °C for 2 days. Keep the germs that grow well and have different colonies in the original food that was made tilted in a tube, the colony was then examined for starch degradation by dripping iodine solution on the colony.

(3) Isolation of lipid-degrading microorganisms using tween-80 as representative. The microorganisms in the diluted wastewater samples were cultured with tween-80 Agar on Petri dishes and incubated at 37 °C for 2 days.

(4) Maintaining the germ that grows well, there is a clear area around the colony, and there are different colony characteristics stored in the same medium that was slanted into a tube (slant). The selection of effective microorganisms in organic degradation microorganisms that decompose organic matter into a Clear Zone is formed. Around the microbial colonies on the agar medium, selection of microorganisms that are effective in degrading organic matter from the clear zone width. The width of the clear zone can be obtained from the difference between the diameters of the clear region and the microorganism. This can be calculated from the following equation:

$$\text{Clear Zone Width} = \text{Clear Area Diameter} - \text{Microbial Diameter}$$

Microorganisms with high clear zone width values were selected for storage in wastewater treatment samples (Nongluk, B.E. 2552). The Department of Army Science samples were sent for Mass Spectrometry. testing at the Kamphaeng Saen Animal Disease Research Unit, Faculty of Veterinary Science, Kasetsart University, Kamphaeng Saen Campus. The ability of the bacteria to produce enzymes to digest carbohydrates, proteins, and fats was recorded. The width of the clear zone can be obtained from the difference between the diameters of the clear region and the diameter of the microorganisms.

3. The experimental plan was a completely randomized design (CRD) consisting of four sets of experiments with three repetitions. The Department of Army Science conducted experiments in plastic tanks at the laboratory level using wastewater samples from a well in front of the Science Division. In combination with the microbial inoculum, the best reduction in COD and BOD was obtained from the bacterial degradation ability test (2). The experimental setup was as follows.

Experiment set 1: Control set Experiment set 2: control set with KCT03 inoculum addition Experimental set 3: control set with KCT05 inoculum

addition Experimental set 4: Control set with microbial inoculum KCT03 and KCT05 added. The process as follows:

Preparation of 18-liter plastic water tanks at the laboratory level, totalling 10 wells. Wastewater samples were randomly collected in front of the Science Division, Department of Army Science, and COD and BOD were measured weekly for one month.

Water was replenished using water samples on the day with the highest wastewater value to simulate water entering the well and overflow from the well; water was added daily for four weeks, and approximately the same amount was added to all wells. The COD, BOD, added water, and overflow were measured. Inoculum from the bacterial degradation test (2) was added daily to each batch. By filling in at the same time and in the same amount, it took four weeks to complete the wastewater treatment experiment.

The water quality values before and after microbial inoculum addition were recorded according to standard effluent quality parameters. Water quality measurements were collected daily, and the BOD was checked once a week for four weeks. In each set of experiments, water quality was analyzed using various analytical methods, as shown in Table 1.

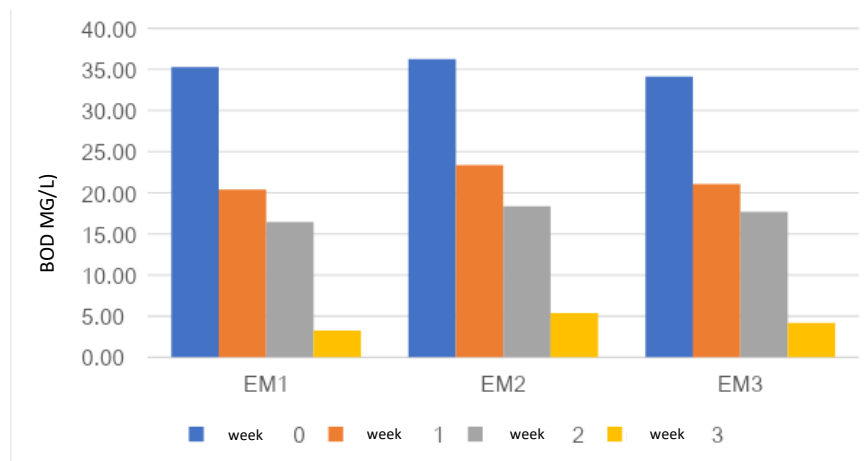
Table 1. Analytical parameters according to the standards controlling the discharge of wastewater from certain types and sizes of buildings (Type A buildings)

Parameter	Unit	Analysis Method
pH value	-	pH Meter
Biochemical Oxygen demand (BOD)	Mg/L	Azide Modification
Suspended Solids	Mg/L	Glass Fibre Filter Disc
Total Dissolved Solid	Mg/L	Dry at 103-105 °C in 1 hour. Titrate
Sulfide	Mg/L	
Nitrogen in TKN form	Mg/L	kjeldahl
Fat, Oil and Grease	Mg/L	Solvent extraction method and weight separation of oils and fats.

Source: Announcement of the Ministry of Natural Resources and Environment on the setting of standards for controlling the discharge of wastewater from certain types and sizes of buildings, B.E. 2548.

Data collected from the analysis of wastewater quality before and after the microbial inoculum addition were analyzed using a statistical program.

Figure 3. BOD measurement results, wastewater samples from water storage ponds in the area of the Department of Science, Royal Thai Army.



Discussion and Conclusion

From the study of the quality of effluent in the water clarifier from the office building and residence within the Army Science Department, the effluent appeared turbid. It had an average pH. of 8.03 and an average Total Suspended Solids of 30.03 milligrams per liter. The total dissolved solids averaged 290 mg/L. The average value of fat oil and grease was 33.64 mg/l, and the average COD was 31.87 mg/l.

The value exceeds the wastewater quality standard type A. The pollution control departments should improve before releasing water into the public canal. The morphological characteristics of the *Bacillus* genus were isolated from two soil samples and two water samples from the wells in front of the Science Division and the wells in front of the Pyro building. All 21 isolates contained smooth, round, white gram-positive, and gram-negative bacteria colonies. The cell walls of gram-positive bacteria are approximately 25-30 mm thick. The cells of isolates are cylindrical or rod-shaped, called *Bacillus* or *Bacilli*, with cell size (width × length) of approximately 2.00 × 5.20 μm. Endospores were produced inside the cell. Isolates KCT03, KCT04, KCT05, KCT07, KCT12, KCT19, and KCT21 can produce protease enzymes; the diameter of the clear zone is between 0.1-2.0 cm. It can grow and decompose the most protein (Casein), which KCT05, KCT03, and KCT04 have mean clear zone diameters of 1.83, 0.52, and 0.37 cm, respectively. Isolates KCT01, KCT02, KCT03, KCT04, KCT05, KCT08, KCT11, and KCT15 were capable of producing amylase. The

diameter of the super clear zone was between 0.1-4.1 cm. The most capable of growing and decomposing carbohydrates (starch) were KCT03, KCT02, and KCT01, with average clear zone diameters of 2.75, 2.35, and 1.65 cm, respectively. All isolates, except KCT06, KCT07, KCT09, and KCT10, could produce lipase. The diameter of the super clear zone was between 1.6-7.5 centimeters. KCT03, KCT02, and KCT01 had average clear-zone diameters of 2.75, 2.35, and 1.65 cm, respectively. Eight pairs of germs exhibited growth inhibition: KCT03 and KCT12, KCT13 and KCT16, KCT05 and KCT13, KCT07 and KCT16, and KCT08 and KCT15. isolate KCT11 versus isolate KCT19 and isolate KCT13 versus isolate KCT15. KTC03 and KTC05 because they can digest protein, starch, and fat well. It can produce proteases, amylases, and clear lipases in all samples. Moreover, there is no hostility of the bacteria on the medium, it can be cultured on a sugar medium well, and sent for Mass Spectrometry and it was discovered that KCT03 and KCT05 were *Bacillus subtilis*. which is suitable for studying the ability to help treat wastewater.

From the test results on day 19, the experimental sets EM1, EM2, and EM3 contributed to the control of effluent quality. (pH, total suspended solids, total dissolved solids, fat oil, and grease), to be in the standard for controlling wastewater discharge from specific types and sizes of buildings, type A, according to the announcement of the Ministry of Science. Technology and Environment Act B.E. 2548 requires a pH between 5-9 batches; the total suspension must be less than 20 mg/L. Total dissolved solids in the effluent must be at most 500 mg/L. The fat and oil contents of the effluent were less than 20 mg/l. The EM3 experimental kit with isolates between KCT03 and KCT05 contributed the most to wastewater treatment, which reduced the total dissolved substances and essential fats and oils.

EM1 had 23.31% total soluble solids treatment efficiency and 11.36% fat and oil effluent treatment efficiency. EM2 had a 26.94% total soluble solids treatment efficiency, 10.76% was effective in treating fats and oils in wastewater, and 5.00% was effective in treating the organic matter in wastewater. While EM3 had the best effluent treatment efficiency, the efficiency in the treatment of the organic matter in the wastewater was 5.56%. The efficiency in treating all dissolved substances in the wastewater was 28.84%, 19.58% effective in treating fats and oils in wastewater, 16.67% effective in treating the organic matter in wastewater, and 10. The BOD trends in the effluents of EM1, EM2, and EM3 were similar and took two weeks to decompose organic matter in the effluent. It passed the quality standard for type A effluent with a BOD value of not more than 20 milligrams per liter of effluent in all test kits, where EM1 was effective in treating organic matter at 90.71%, then EM3 and EM2, having efficiency in treating organic matter at 87.76% and 85.22% respectively. However, the EM2 assay differed in the amount of organic degradation of all subjects each week, which was statistically significant at 0.05.

Recommendations for future research

1. To use *B. subtilis* microorganisms in other areas of wastewater treatment, the Department of Science should study and test for *B. subtilis* microorganisms to ensure better suitability.
2. The use of *B. subtilis* in municipal wastewater treatment affects water quality in terms of sulfide and heavy sediment. TKN covers the other effluent standards.
3. Isolates suitable for water treatment should be clearly identified by the Single 16s ribosomal DNA technique to be studied in other processes in the future.
4. Further studies should investigate the effect of temperature on the wastewater treatment efficiency of *B. subtilis*.
5. *Bacillus subtilis* culture guidelines should be established according to economic guidelines to make them suitable for business use in production.

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