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Influence of Various Factors on Hydrolytic Enzyme Activity and COD Removal Efficiency in Cassava Starch Wastewater by Marine Actinomycete *Streptomyces* sp. A1-3

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Abstract

Wastewater from the production line of cassava starch factories contains a significant amount of organic matter, necessitating treatment before discharging or reusing in the factory. Actinomycetes can produce various hydrolytic enzymes, especially carbohydrate-degrading enzymes, which are important in the biological wastewater treatment process because actinomycetes can utilize several growth substances such as sugar, polysaccharides and protein. In this research, the effects of carbon source, pH, salinity and incubation time on hydrolytic enzyme activity for producing reducing sugar of a marine actinomycete *Streptomyces* sp. A1-3 were evaluated. The maximum hydrolytic enzyme activity was found in the oatmeal yeast extract carboxymethyl cellulose (OYC) medium with a pH value of 4.0-6.2 and a cultivation time of 4 days. Salinities in the range of 0-35 practical salinity units (psu) had no effect on the hydrolytic enzyme activity of strain A1-3. Subsequently, the efficiency of *Streptomyces* sp. A1-3 in reducing the chemical oxygen demand (COD) in cassava starch wastewater was evaluated using a Completely Randomized Design (CRD). The control tank had no *Streptomyces* sp. A1-3, while in the treatment tank, strain A1-3 was added. Aeration was supplied throughout the 16-day experiment. Water samples were collected daily for COD analysis and to measure reducing sugar levels. Within the first 4 days, the efficiency of COD reduction by strain A1-3 was 50%, higher than that in the control tank. During days 7-9, the COD reduction value of strain A1-3 remained almost stable in the range of 58-63%. Adding NaCl

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to adjust the salinity to 17 psu on day 10 increased the COD reduction. Between days 12 and 15 of the experiment, the COD was reduced by 79-81.82%, significantly different from the control tank. However, at the end of the experiment, the COD value in both the control and treatment tanks was higher than the standard of the Ministry of Natural Resources and Environment (Thailand), indicating the need for further treatment of these wastewaters.

Introduction

Thailand has more than fifty cassava starch factories (Thai Tapioca Starch Association, 2023). In general, cassava starch factories are often faced with wastewater management problems from their production processes with high organic concentrations and according to chemical oxygen demand (COD) at 5,000-50,000 mg/L and a biological oxygen demand (BOD) at 3,000-30,000 mg/L (Cai et al., 2019). Moreover, the organic acids, such as lactic acid and acetic acid produced by the activity of starch-decomposing lactic acid bacteria in this wastewater, also contribute to the problem of bad odors (Tosungnoen et al., 2014). These problems can be solved or improved by the right treatment of wastewater, but most technologies such as combination of physical and biological processes (Fettig et al., 2013) and chemical adjustment techniques (Luo et al., 2023) require high expenses for technical equipment as well as for energy. Utilizing microorganisms to reduce organic matter in wastewater can be managed at lower costs. Nevertheless, in certain instances, the efficacy or quantity of microorganisms may prove insufficient for thorough wastewater treatment. Consequently, additional microorganisms must be introduced into the treatment systems to enhance their performance (Wu & Yin, 2020).

Actinomycetes are widely distributed in soil and water, as evidenced by their presence in wastewater treatment plants and bioreactors (Hozzein et al., 2012; Boufercha et al., 2022). They play a crucial role in organic decomposition due to their ability to utilize various growth substrates such as sugars, polysaccharides, proteins, and aromatic compounds (Lemmer & Kroppensted, 1984; Lemmer, 1986), contributing to the degradation and turnover of various materials in the environment. *Streptomycetes*, a significant subgroup of actinomycetes, are economically important organisms and serve as a pivotal source for a wide range of biologically active compounds. They are well-known for their capability to produce various extracellular hydrolytic enzymes (Subramani & Aalbersberg, 2012). These marine actinomycetes offer another option for wastewater treatment as they can produce a variety of hydrolytic enzymes that play a crucial role in digesting organic matter in wastewater. Moreover, they have the ability to remove nutrients such as nitrogen and phosphorus from wastewater (Madkour et al., 2019).

In this study, the suitability of different media (in terms of carbon sources), pH, salinity and cultivation time was investigated for the highest hydrolytic enzyme activity of the marine actinomycete *Streptomyces* sp. A1-3 to produce reducing sugar. *Streptomyces* sp. A1-3, was isolated from mangrove sediment in Chonburi Province, Thailand. *Streptomyces* have the capacity to produce hydrolytic enzymes, such as cellulase and amylase (Sillapachai et al, 2017). Therefore, it can be employed to assess the digestibility of cellulose and starch in wastewater contained at cassava starch factories. The findings from this research will serve as a valuable guideline for implementing actinomycetes in reducing the COD in wastewater from cassava starch factories.

Materials and methods

1. Marine actinomycete source

The marine actinomycete *Streptomyces* sp. A1-3 used in this study was isolated from mangrove sediment in Chonburi Province, Thailand. Its characteristics and the database of the 16S rRNA gene was evaluated by Rattanaporn Siriwibul, a scientist at the Institute of Marine Science, Burapha University. The culture was stored in a nutrient broth medium (NB) containing 20% glycerol at -40ºC.

2. Hydrolytic enzyme production in OYC medium

The culture was streaked on the surface of International *Streptomyces* Project-2 agar (ISP2 agar) (containing 0.4% yeast extract, 1% malt extract, 0.4% dextrose) (Atlas, 2010) with seawater at a salinity of 17 practical salinity units (psu) and incubated for 7 days at 30ºC. Afterward, a piece of agar containing *Streptomyces* sp. A1-3 was extracted using a cork borer with a 5 mm diameter and transferred to four media including cassava starch (with 1% starch), carboxymethyl cellulose (CMC) (containing 0.2% CMC), International *Streptomyces* Project 3 medium (ISP3) (containing 2% oatmeal) and oatmeal yeast extract Carboxymethyl cellulose (OYC) (composed of 1% oatmeal, 0.4% yeast extract and 0.2% carboxymethyl cellulose) (Sillapachai et al., 2017). The medium was prepared using seawater with a salinity of 17 psu, and the pH was adjusted to 6.2±0.5. After that, 100 mL of each medium was transferred into conical flasks and sterilized (model HVE-50, Hirayama). All experimental units were conducted in triplicate and then cultivated on an incubator shaker (model WS-600R, Wiggens) at 30˚C and 110 rpm for 4 days. The crude enzyme was obtained from the culture supernatant through centrifugation (model Sigma2-16KL, Sigma) at 16,215 x g and 4°C for 10 min. Subsequently, the crude enzyme supernatant was utilized to measure reducing sugar and protein content using the dinitrosalicylic acid method (Wood & Bhat, 1988) and the Bradford method (Bradford, 1976), respectively. The ratio of reducing sugar to protein was then calculated (µg/mg) to determine the optimal medium for the strain A1-3 growth and hydrolytic enzyme production.

3. Effects of pH, salinity, and cultivation time on *Streptomyces* sp. A1-3 hydrolytic enzyme activity

3.1 pH on *Streptomyces* sp. A1-3 hydrolytic enzyme activity

Streptomyces sp. A1-3 was cultured in OYC media at 17 psu salinity. The pH of media was varied into 3 different levels including 4.0±0.5, 6.2±0.5 and 10.0±0.5, respectively. They were incubated at 30˚C and shaken at 110 rpm for 4 days. The culture medias were collected to measure reducing sugar and protein to assess enzyme activity.

3.2 Salinity on *Streptomyces* sp. A1-3 hydrolytic enzyme activity

Salinity of OYC media was varied into 3 different levels by adding NaCl, including 0, 17 and 35 psu, pH at 6.2±0.5, then cultivated at 30˚C and shaken at 110 rpm for 4 days. The culture medias were collected to measure reducing sugar and protein.

3.3 Incubation time on culturing *Streptomyces* sp. A1-3 to convert substrate into reducing sugar

Streptomyces sp. A1-3 was cultured in 100 mL OYC medium at salinity 17 psu, pH at 6.2±0.5, temperature at 30˚C and the shaking rate at 110 rpm. The culture media was collected to measure reducing sugar and protein during cultivation for 9 days.

4. The efficiency of COD treatment of wastewater from cassava starch factory by *Streptomyces* sp. A1-3

Streptomyces sp. A1-3 was cultured in OYC medium at 17 psu salinity, pH at 6.2±0.5, temperature at 30˚C and the shaking rate at 110 rpm for 3 days. Therefore, it was used as an inoculum for wastewater treatment. This experiment used original wastewater from a cassava starch factory operated by Eiamburapa Co., Ltd., located in Sa Kaeo Province, Thailand. Wastewater was stored in a freezer (−20°C) until its use. The experimental design used a completely randomized design (CRD) with 2 experimental units. Each unit was performed in triplicate. The wastewater 5,000 mL was used for control experiment in which actinomyces was not added. While in the treatment tank, the wastewater 4,500 mL was filled with 500 mL of actinomycete culture. The control and experimental tanks were continuously aerated at a flow rate of 15 L/hr. After that, NaCl was added into the control tank and the experimental tank on day 10 to adjust the salinity to 17 psu. During the experimental period, COD (American Public Health Association (APHA), 2012), pH and reducing sugar were measured every day.

5. Data analysis

The experimental data were calculated by using mean and standard deviation. Then, variance and comparisons between the groups were analyzed with one-way ANOVA and Tukey's multiple range test at a 95% confidence level by using R version 3.3.1 (Ihaka & Gentleman, 1996).

Results and discussion

1. Hydrolytic enzyme production in OYC medium by *Streptomyces* sp. A1-3

When *Streptomyces* sp. A1-3 cultured in different carbon sources, the results showed that actinomycete gave a reducing sugar to protein ratio of 52.83 mg/mg (Fig. 1). In comparison, cassava starch, CMC and ISP3 media showed reducing sugar to protein ratios of 8.70, 8.30 and 18.51 mg/mg, respectively. This implies that the reducing sugar per protein in OYC medium is higher than that in cassava starch, CMC, or ISP3 media by 6.1, 6.4 and 2.9 times, respectively. Therefore, it can be concluded that the most suitable medium for the strain A1-3 culture is OYC. This may be due to the OYC medium serves as a carbon source for the strain A1-3, comprising glucose from oatmeal and carboxymethyl cellulose. Additionally, it provides a nitrogen source from yeast extract, which stimulates the strain A1-3 to produce hydrolytic enzymes.

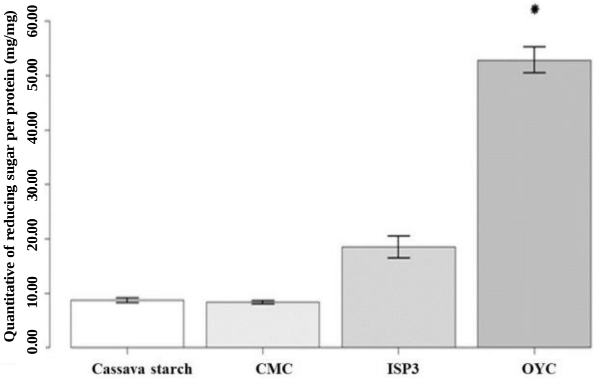

Fig. 1 Activity of hydrolytic enzyme present in the term of reducing sugar production per protein of *Streptomyces* sp. A1-3 grown on various carbon sources

2. The effects of pH, salinity, and time regarding *Streptomyces* sp. A1-3 cultivation on the hydrolytic enzyme activity

Streptomyces sp. A1-3 grown in OYC medium at pH 4.0 and 6.2 exhibited high hydrolytic enzyme activity, with reducing sugars to protein ratios of 7.94 and 6.19 mg/mg, respectively. The results indicate that strain A1-3 grown in OYC medium containing oatmeal and carboxymethyl cellulose as carbon sources was able to produce carbohydrate-degrading enzymes capable of successfully decomposing cellulose and starch present in cassava starch wastewater into reducing sugars. As, the pH range of 4.0-6.2 corresponds to that commonly found in wastewater with high organic loading, such as cassava starch wastewater and olive mill wastewater (Tsigkou et al., 2022; Watthier et al., 2019; Gomes et al., 2016). However, when the pH was increased to 10.0, the enzyme activity decreased significantly (Fig. 2). In this experiment, *Streptomyces* sp. A1-3 was isolated from mangrove sediments with a salinity of 17 psu. When the inoculums were cultured in media with a pH of 6.2 and varying salinity levels, it was found that hydrolytic enzyme activity in media with salinities of 0, 17 and 35 psu was not significantly different. The reducing sugars to protein were 5.37, 6.19 and 6.13 mg/mg, respectively (Fig. 3). This indicates that salinity range of 0-35 psu has no effect on the hydrolytic enzyme activity of the strain A1-3. Actinomycetes grow well at a salinity range of 0-50 psu, but their growth is reduced at higher salinity levels (Omar et al., 1994). Salinity can affect microorganism hydrolytic enzyme activity such as acid phosphatase, alkaline phosphatase, glucosidase, protease and esterase in the submerged fixed-bed bioreactor to treat urban wastewater. The salinity of urban wastewater is in the range of 0.4-1.5 g/L. However, if the salinity is increased to 44.1 g/L, the hydrolytic enzyme activity will decrease (Cortes-Lorenzo et al., 2012). Therefore, *Streptomyces* sp. A1-3 will probably show similar results from other decomposing organic matter with the same salinity conditions. Environmental factors can influence the extracellular hydrolytic enzymes of microorganisms in marine environments (Arnosti et al., 2014). Thus, if the salinity is higher than the environment that the microorganisms used to live in, they will use energy to maintain the cells' osmotic pressure. From the study of substrate converting into reducing sugars at different times, it was found that the reducing sugar to protein level was highest at 6.06 mg/mg on day 4. While reducing sugar was highest at 1.84 mg/mL on day 3 (Fig. 4). It was because the product of carbohydrates digestion in media is a monomolecular reducing sugar. It was concluded that inoculum of *Streptomyces* sp. A1-3 for wastewater treatment was cultured in OYC media and pH at 6.2±0.5 as there were no significant differences observed in the reducing sugar product at different pH values. Of these, OYC media was prepared by using natural seawater with a pH of 6.2±0.5. Moreover, *Streptomyces* sp. A1-3 was cultivated for 3 days because the substrate digestion activity and reducing sugar product was at its highest. Despite the highest hydrolytic enzyme activity observed on day 4, the growth of the strain A1-3 was low, possibly indicating entry into a stationary phase.

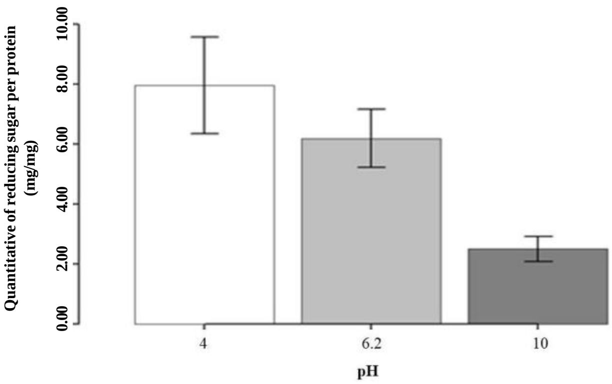

Fig. 2 Effect of pH on the activity of hydrolytic enzyme from *Streptomyces* sp. A1-3

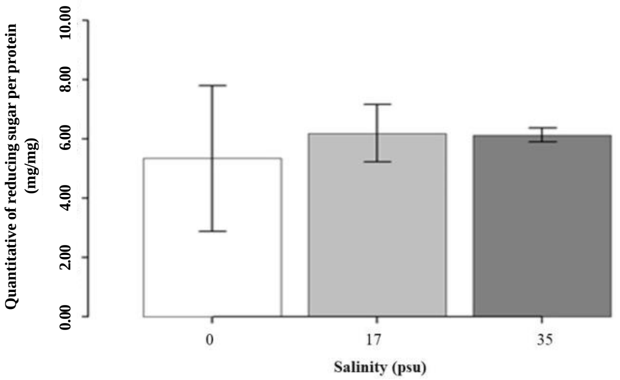

Fig. 3 Effect of salinity on the activity of hydrolytic enzyme from *Streptomyces* sp. A1-3

3. The efficiency of COD treatment of wastewater from cassava starch factory by *Streptomyces* sp. A1-3

The initial COD value in the control tank without strain A1-3 was 23,467 mg/L, whereas in the experimental group with strain A1-3, it was 23,200 mg/L. High COD values are consistently observed in wastewater from cassava starch factories due to the presence of starch and simple sugars such as glucose and fructose (Gomes et al., 2016). The other parameters are approximately the same, e.g. pH is at 4.2 and the reducing sugar level is 31.21 mg/mL. The results of adding *Streptomyces* sp. A1-3 to the wastewater treatment system showed that this can reduce the COD value by 50% within 4 days. Of these, it was faster than the control tank for 4 days. The COD value decreased because microorganisms in the treatment system utilize pollutants, especially starch and cellulose in the wastewater as carbon sources. They convert this organic matter into energy for cellular respiration and protein production (Oljira et al., 2018). Interestingly, even in the control tank without *Streptomyces* sp. A1-3, which suggests that the presence of natural microorganisms are capable of reducing COD as well. The efficiency of COD treatment was stable (58-63%.) during days 7-9. Therefore, on day 10, NaCl was added to both the control and treatment tank at 17 psu. The actinomycetes in this experiment were isolated from marine sediment, leading to the observed result. The result shows that salinity can increase organic treatment by microorganism since the COD value was decreased consequently (Fig. 5). On the last day of the experiment (day 16), both the control and treatment tank with *Streptomyces* sp. A1-3 had a similar COD treatment efficiency at 88.18%. This may be due to the small amount of organic matter in wastewater, which was causing *Streptomyces* sp. A1-3 to grow less. In wastewater with *Streptomyces* sp. A1-3 addition, the pH gradually increased to 7.0, which was faster than the control tank

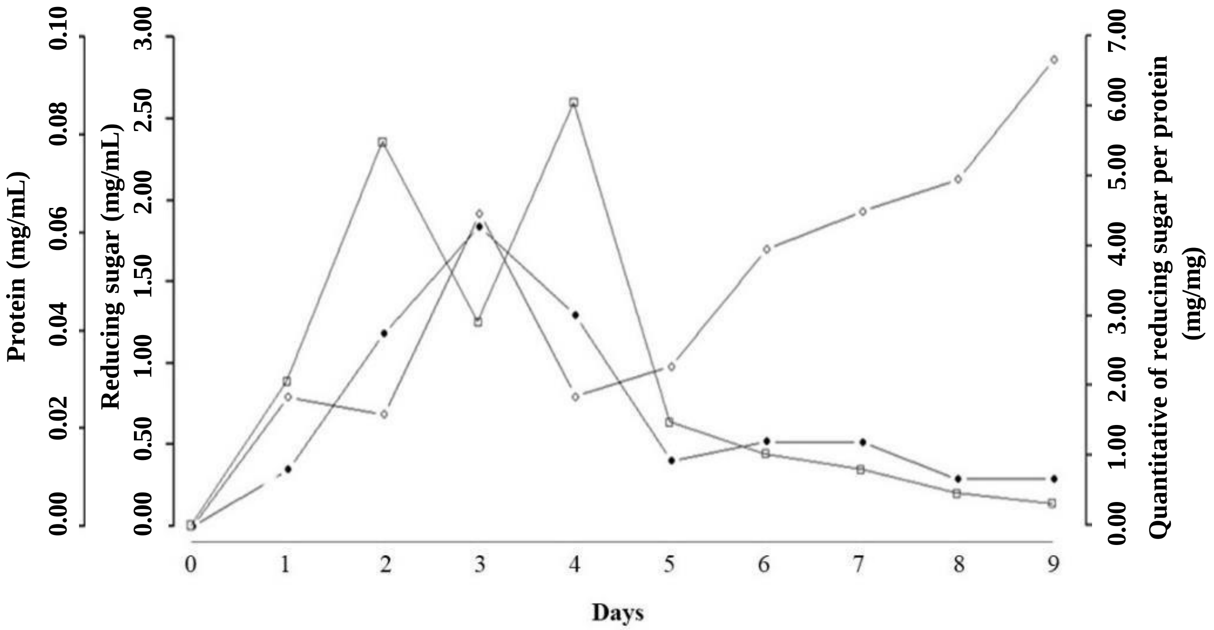

Fig. 4 Changes in protein (✧), reducing sugar (▯) and qualitative of reducing sugar per protein (☐) during 9 days of cultivation of *Streptomyces* sp. A1-3

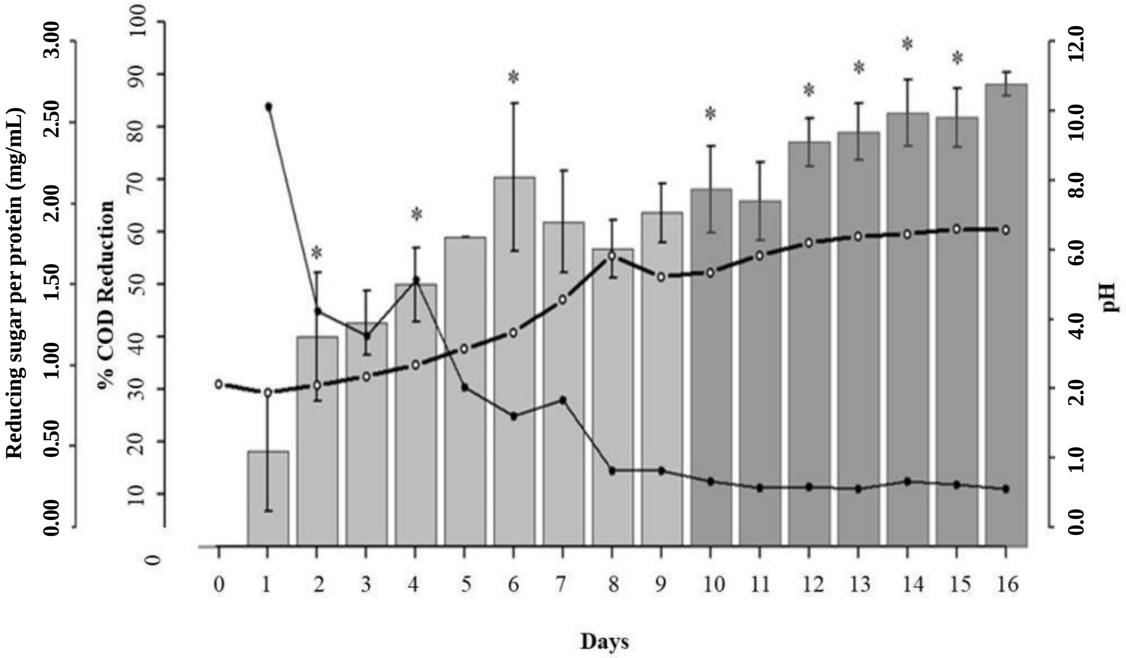

Fig. 5 Changes in % COD reduction showed in bar graph. Line graph represented changes in reducing sugar (●) and pH (○) in a treatment tank (with *Streptomyces* sp. A1-3 addition) during 16 days of the experiment. On days 0-9, the salinity of wastewater was 0 ppt. On day 10, NaCl was added to the treatment tank to adjust the salinity to 17 ppt. * represents the significant difference between % COD reduction in the control tank (without *Streptomyces* sp. A1-3) and the treatment tank (with *Streptomyces* sp. A1-3)

for 4 days. This is because actinomycetes can utilize certain organic acids as an energy source for their growth (Apinya et al., 2015). However, on the end of this experiment, the COD value of both the control and treatment tank was 2,742-2,774 mg/L, which is higher than the standard of the Ministry of Natural Resource and Environment (Thailand), which indicates that the COD value must be less than 120 mg/L before being released into the environment. Therefore, wastewater should be re-treated to reduce the COD value by adding more nutrients especially nitrogen and phosphorus. Cassava contains minimal nitrogen and phosphorus, which might be insufficient for microbial growth (Rojpanit et al., 2017).

Conclusion

Streptomyces sp. A1-3 exhibited high hydrolytic enzyme activity when cultured in OYC medium with a pH range of 4.0-6.2 and a cultivation time of 4 days. Salinity ranging from 0 to 35 psu had no effect on the hydrolytic enzyme activity of strain A1-3. When used to treat wastewater from a cassava starch factory, it demonstrated high efficiency, reducing COD by approximately 70% starting from the 6th day. Subsequently, the COD reduction value of strain A1-3 remained almost stable in the range of 58-63% from day 7 to 9. Despite adjusting the salinity to 17 psu by adding more NaCl, the COD reduction increased by only 10%, reaching a maximum COD reduction value of 88.18% on day 16. However, treated wastewater needs further treatment to reduce the COD value within the standard range. This characteristic suggests that *Streptomyces* sp. A1-3 may find application in the treatment of highly acidic wastewater from factories. Furthermore, *Streptomyces* sp. A1-3, adapted for saline wastewater, holds promise for future applications in various industries such as fermented milk, fishing and the leather industry.

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