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Full Length Article

Isolation and Selection of Bacterial Strains Capable of Degrading Lipid

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Abstract

Fats, oils, and greases are difficult to remove from wastewater treatment systems. This study was conducted to isolate and screen lipid-degrading bacteria from contaminated water samples. The ability of bacteria strains in degrading lipids was tested by growing the bacteria on M1 medium. From three samples of domestic wastewater collected from Ninh Kieu District, Can Tho City, 21 bacterial strains capable of lipolysis were isolated, including 9 strains capable of forming halo rings after 72 h cultured on the M1 medium. The survey resulted on lipase enzyme production showed that the KNT2.2 strain could produce the most effective lipase with an efficiency of 2,670; 2,490 and 6,260 U L^{-1}) after 3, 6 and 9 days of culture, respectively. This study reported that KNT2.2 is a potent strain of bacteria to apply lipase from microorganisms to different industries in the present time.

Keywords: Lipid degradation; Microorganism screening; KNT2.2 strain; Wastewater treatment

Introduction

Lipases (triacylglycerol acyl hydrolases; EC3.1.1.3) are hydrolytic enzymes that catalyze the hydrolysis of saturated triacylglycerols to glycerol, acylglycerols and free fatty acids (Andualema and Gessesse 2012; Geoffry and Achur 2017). Lipases are found in many plants, animals, insects, and microorganisms (Mehta *et al*. 2012; Sarmah *et al*. 2018). Microbial lipases have been widely used in industries due to their selectivity, stability, and specificity with various substrates (Treichel *et al*. 2010; Kumar *et al*. 2012) Many microorganisms are potential lipase producers, including bacteria, filamentous fungi, and yeasts. Studies have shown that many strains of Gram-negative and Gram-positive bacteria are capable of producing lipase enzymes. Many commercial lipases are produced from bacteria of the genus *Bacillus*, such as *Bacillus subtilis* (Suci *et al*. 2018), *Bacillus licheniformis* (Sangeetha *et al*. 2010), *Bacillus pumilus* (Mabizela-Mokoena *et al*. 2017), *Bacillus coagulans* (Gowthami *et al*. 2015), *etc*. Besides, there are several bacterial species capable of producing lipases, such as *Pseudomonas* spp. (Unni *et al*. 2016), *Burkholderia* spp. (Yang *et al*. 2016) and *Staphylococcus* spp. (Daoud *et al*. 2013). The microbial lipases are more valuable than those derived from plants or animals because of the wide range of catalytic activities they can perform, the ease with which they can be genetically modified, the lack of seasonal variations, the regular supply, the increased stability, safety, and convenience, and the extremely high growth rates of microorganisms in economically advantageous media (Mendes *et al*. 2012; Reetz 2013). Research by Rani and Jagtap (2019) suggested that the addition of microbial lipase may significantly reduce the cost of the cheese production by lowering the ripening period by 1 month and maintaining the quality of the final product. In addition, lipase is widely used in the food, dairy, flavoring, detergent, pharmaceutical, biofuel, and cosmetic industries due to its non-toxicity and environmental friendliness (Javed *et al*. 2018).

Can Tho City is the economic and political center of the Mekong Delta. There are a large number of lipids discharge into the environment from daily activities (Loan 2010). Additionally, modern industrial processes require secure, non-toxic raw materials to support sectors including food, pharmaceuticals, and the environment. Therefore, this study was carried out to isolate and select indigenous bacterial strains capable of producing highly potent lipase from wastewater in Can Tho City.

Materials and Methods

Isolation of bacteria capable of degrading lipids

Three domestic wastewater samples were collected in Ninh Kieu district, Can Tho City, Vietnam. One mL of sample was added to 5 mL of density-enriched medium and shake on a shaker at 200 rpm at 30°C. After 72 h of culture, 100

μL of the sample was spread on an M1 medium. The samples were then incubated at 32°C for 48 h. Different discrete colonies were selected for isolation on the M1 medium by the streak plate method. The pure bacterial strains were characterized by colony and cell morphology after 72 h of culture on the M1 medium (Matsumiya *et al*. 2007).

Density-enrichment medium was 10 g/L olive oil; 0.5 g L^{-1} $(NH_4)_2SO_4$; 5 g/L $MgSO_4.7H_2O$ and 1 g L^{-1} KH_2PO_4, pH = 7.5 (Matsumiya *et al*. 2007). M1 medium: 10 g L^{-1} peptone; 5 g L^{-1} NaCl; 20 g L^{-1} agar; 0.1 g L^{-1} $CaCl_2.2H_2O$ and add 10 mL L^{-1} Tween-80, pH = 7.5 (Matsumiya *et al*. 2007).

Investigation of the ability to degrade Tween 80 of isolated bacterial strains

Inoculate a colony of each isolated bacterial strain on M1 medium and incubate at 32°C. After 48 h, the media were examined for the formation of halo rings surrounding the colony, and the diameter of the halo rings was measured.

The relative activity index of lipase was calculated based on Tween-80 degradation halo ring and colony diameters, according to the study of Ventorino *et al*. (2015)

$$\text{Lipase relative activity index (I)} = \frac{\text{Halo ring diameter of Tween degradation (D)}}{\text{Colony diameter (d)}}$$

Substrate degradation and determination of lipase enzyme activity

Substrate degradability: To investigate this bacterial colony was placed in a liquid M1 medium and incubate it at 32°C. After 24 h of growth, the bacterial biomass was centrifuged for 10 min at 10,000 rpm. Bacterial cells were washed, and optical density at 600 nm was adjusted to 0.1 using physiological salt (NaCl 0.9 g L^{-1}). Aspirate 1 mL of the densified bacterial suspension into 50 mL of H_2O, followed by the substrate (7.5 g peanut oil cake). After three days of cultivation at room temperature, lipase enzymes were extracted.

Extraction of bacterial lipase enzymes: After 3, 6 and 9 days of culture, samples were collected and centrifuged at 15,000 rpm for 10 min at 4°C to yield crude lipase (Tišma *et al*. 2019).

Assessment of lipase enzyme activity: Each reaction consisted of 1.25 mL olive oil, 3.75 mL gum Arabic 7%, 2 mL 0.01 M phosphate buffer, and 1 mL crude lipase. The hydrolysis reaction was carried out at 40°C and shaken at 150 rpm. After 30 min, add 15 mL of a mixture of acetone and ethanol (1:1) to stop the reaction. 2–3 drops of indicator phenolphthalein were added to each flask. The sample was titrated with 0.01 *M* NaOH solution. The experiment rechecked the pH value of each treatment with a pH meter (pH=11). Record the volume of 0.01 *M* NaOH solution used to titrate each flask. Lipase activity was calculated according to the formula (Fleuri *et al*. 2014):

$$\text{Lipase activity (UI mL}^{-1}) = (\Delta V_{NaOH} \times CM_{NaOH} \times 1000)/(V_{enzyme} \times t)$$

Where, Lipase activity (UI mL^{-1}) is the amount of lipase required to produce 1 µmol of fatty acids per minute at pH = 7 and temperature 40°C;
ΔV_{NaOH} is the volume difference of 0.01 *M* NaOH used for titration between the control and test sample (mL);
CM_{NaOH} is the molar concentration of NaOH solution;
1000 is the conversion factor;
t is the reaction time (minutes);
V_{enzyme} is the volume of crude enzyme in reaction (mL).

Data processing

The data were entered, stored, and graphed using Microsoft Excel 2013. Minitab 16 software was used for single-factor ANOVA analysis by Fisher's test.

Results

Isolation of bacteria capable of degrading lipids

Twenty-one bacterial strains capable of decomposing lipids were identified from three wastewater samples collected in Can Tho City. There were 16 circular colonies (76%) and 5 non-circular colonies (24%); 14 white strains (66.6%), 4 yellow strains (19%), 1 brown strain (4.8%), 1 red strain (4.8%), and 1 pink strain (4.8%); 18 strains with entire margin (86%) and 3 strains with lobate margin (14%); 14 strains with smooth surface (67%), 7 strains with rough surface (33%); Their dimensions ranged from 1 to 8 mm. The cells of 18 bacterial strains were spherical (86%), with 3 rod-shaped (21%). Cells vary in size from 1 to 2 μm. Figure 1 depicts the colony morphology of some representative bacterial strains capable of digesting lipids on M1 medium after 72 h of culture.

The ability of isolated bacteria to degrade Tween 80

According to the survey results, all identified bacterial strains could form colonies after 72 h of culture on the M1 medium. 9 bacterial strains out of 21 could effectively break down Tween 80 and create halo rings surrounding the territories, with halo ring diameters ranging from 5.3 mm to 35 mm on average. After three days of cultivation, bacterial strain KNT1.2 exhibited the highest halo ring diameter, measuring 35 mm. Table 1 shows the examination findings into Tween '80s deteriorating capacity. Fig. 2 depicts the halo rings of some example bacterial strains capable of digesting lipids on an M1 medium. After 72 h of culture on an M1 medium, all nine strains of these bacteria can produce halo rings, indicating that their potential to degrade substrates should be studied further.

Substrate degradation and determination of lipase enzyme activity

After 3 days, the status of the peanut oil cake was different between the control treatment (without bacterial inoculation) and the treatment with bacterial inoculation. When inoculated with the bacteria, the peanut oil cake became smoother and partially liquefied, which was different when the bacteria were not inoculated (Fig. 3). The result showed an impact from inoculated bacteria on the peanut oil cake after 3 days of culture.

Simultaneously, the lipase activity of bacterial strains was determined by the acid-base titration method. The results of surveying the lipase activity of bacterial strains after 3, 6 and 9 days of culture with the substrate are presented in Table 2. According to the survey results, several bacterial strains displayed lipase activity, with bacterial strain KNT2.2 having the maximum lipase activity, reaching 2.67 U mL^{-1} after 3 days of culture, which was distinct from other treatments. The lipase activity of the bacterial strains was fairly low after 6 days of culture . The bacterial strain KNT2.2 had the highest lipase activity at 2.49 U mL^{-1}, a statistically significant difference from the other treatments. After 9 days of culture, the lipase activity of all bacterial strains rose at 6 days, with KNT2.2 having the greatest lipase activity at 6.26 U mL^{-1}, followed by KNT2.2. The active bacterial strain KNT1.3 achieved 3.59 U mL^{-1}, which was statistically different from the other treatments . After 3, 6, and 9 days of growth, bacterial strain KNT2.2 had the best efficiency, with lipase activity of 2.67, respectively; 2.49 and 6.26 U mL^{-1} (equivalent to 2,670; 2,490 and 6,260 U L^{-1}). It is thought to be a potential bacterial strain for degrading lipids in the medium.

Discussion

Lipids (fats, oils, and greases) constitute the main organic matter in some industrial and municipal effluent and can seriously pollute the environment. Wastewater from the dairy industry, slaughterhouses, wool scouring, and edible oil refineries has a high (> 100 mg/L) lipid concentration (> 100 mg L^{-1}) (Wakelin and Forster 1997; Lefebvre *et al*. 1998). Significant issues in biological wastewater treatment procedures are frequently brought on by high concentrations of these chemicals in wastewater. They naturally form a coating on water surfaces, which reduces the pace at which oxygen enters the aerobic process (Becker *et al*. 1999). Researchers have looked into aerobic and anaerobic methods of bioremediation of lipid-rich wastes (Borja *et al*. 1994; Buhidma *et al*. 2020). Lipases are extracted on an industrial scale from cultures of bacteria, fungi, actinomycetes, and plant and animal cells. Among these, microbial lipases offer an advantage in many industrial processes because they are metabolically adaptable (Akoh *et al*. 1995; Haraldsson *et al*. 1995). Serine hydrolases known as lipases have significant physiological importance and

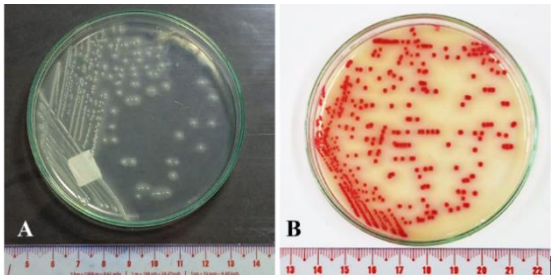

Fig. 1: Colony morphology of some representative bacterial strains capable of degrading lipids on M1 medium after 72 h of incubation**. A:** KNT1.3 strain; **B:** KNT2.2 strain

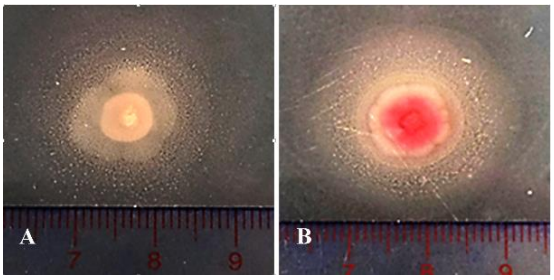

Fig. 2: Halo ring formation of some representative bacterial strains capable of degrading lipids on M1 medium. **A:** KNT1.3 strain; **B:** KNT2.2 strain

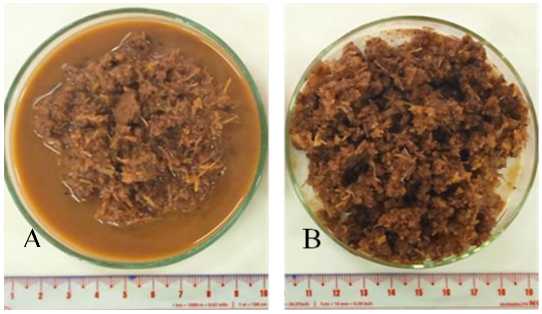

Fig. 3: Substrate sample after incubation with bacteria with lipase activity. **A:** with strains of bacteria; **B:** control (without bacterial inoculation)

industrial potential. They can catalyze a wide range of processes, including hydrolysis, esterification, interesterification, alcoholysis and aminolysis (Pandey *et al*. 1999; Jaeger and Eggert 2002).

A significant biotechnological application and multibillion-dollar global industry is the biological treatment of home and industrial wastewater. Microbial populations in wastewater treatment systems can be increased, and their advantageous functions lead to the effective removal of organic materials, harmful compounds, nutrients, and pathogens (Wang *et al*. 2012). Despite a significance of these processes for the environment and the economy, little is known about how the microbial communities exist in wastewater treatment plants, particularly in developing

Table 1: Degradability of Tween 80 by bacterial strains

Bacterial strains	Halo ring diameter (D, mm)	Colony diameter (d, mm)	Lipase relative activity index (I)
KNT 1.2	35.00 ± 0^{a}	5.33 ± 0.58^{a}	6.61 ± 0.67^{b}
KNT 1.3	28.00 ± 0^{b}	3.00 ± 0^{b}	9.33 ± 0^{a}
KNT 2.2	13.33 ± 1.53^{d}	3.67 ± 0.58^{b}	3.69 ± 0.67^{de}
KNT 2.3	12.33 ± 2.52^{d}	4.00 ± 0^{ab}	3.08 ± 0.63^{de}
KNT 3.1	19.33 ± 1.16^{c}	3.33 ± 0.58^{b}	5.94 ± 1.25^{bc}
KNT 3.2	5.33 ± 0.58^{f}	4.33 ± 0.58^{ab}	1.25 ± 0.25^{f}
KNT 3.6	8.67 ± 0.58^{e}	3.67 ± 0.58^{b}	2.39 ± 0.24^{ef}
KNT 4.3	8.00 ± 0^{ef}	4.00 ± 1^{ab}	2.09 ± 0.54^{ef}
KNT 4.5	20.00 ± 0^{c}	4.33 ± 0.58^{ab}	4.67 ± 0.58^{cd}

Data with different letters in the same column represent statistically significant differences at the 95% confidence level

Table 2: Lipase enzyme activity at 3, 6 and 9 days of culture

Treatment	Lipase activity (U/mL)		
	3 days	6 days	9 days
Control	0 ± 0^{ef}	0 ± 0^{cd}	0 ± 0^{de}
KNT1.2	0.71 ± 0.6^{cd}	0.41 ± 0.26^{bc}	0.48 ± 0.47^{cd}
KNT1.3	1.41 ± 0.47^{b}	0.74 ± 0.36^{bc}	3.59 ± 0.51^{b}
KNT2.2	2.67 ± 0.29^{a}	2.49 ± 0.34^{a}	6.26 ± 0.5^{a}
KNT2.3	0.72 ± 0.16^{bcd}	0 ± 0^{cd}	0.39 ± 0.22^{cd}
KNT3.1	0.56 ± 0.1^{de}	0 ± 0^{cd}	0.39 ± 0.19^{cd}
KNT3.2	0 ± 0^{ef}	0.13 ± 0.23^{cd}	0.38 ± 0.3^{cd}
KNT3.6	1.29 ± 0.57^{bc}	0.64 ± 0.6^{bc}	0.70 ± 0.52^{c}
KNT4.3	0.97 ± 0.5^{bcd}	0.12 ± 0.04^{cd}	0 ± 0^{ef}
KNT4.5	0 ± 0^{ef}	0 ± 0^{cd}	0 ± 0^{ef}

Data with different letters in the same column represent statistically significant differences at the 95% confidence level

nations.

In the screening stage, we isolated 21 strains of bacteria capable of degrading lipids from the wastewater in Can Tho City, Viet Nam. Screening of lipid-degrading bacteria was carried out using media containing 1% of olive oil which acts as a source of carbon and energy for the lipid-degrading bacteria. The oil promotes the growth of lipid-degrading bacteria during the cultivation process. The isolated bacterial strains were observed and selected based on the morphological characteristic. The representative morphology of these obtained isolates in an M1 medium can be seen in Fig. 1. Lipid-degrading bacteria came from different sources. Aryal *et al.* (2015) and Sutrisno *et al* (2016) have isolated bacteria strains from wastewater sites. The percentage of successful isolation of lipid-degrading bacteria varies; Sixty-one bacterial were isolated and only 11 were identified as potential lipid-degrading bacteria collected from wastewater of food processing plant and restaurant in Can Tho City, Viet Nam (Phong *et al.* 2014), whereas Sutrisno *et al.* (2016) had isolated 12 bacterial strains but only 7 strains showed lipolytic activity.

The morphologically different isolated strains were further tested for lipase activity with Tween 80 substrate. According to Kumar *et al.* (2012) when bacterial strains can biosynthesize lipase enzyme, the amount of enzyme produced will hydrolyze Tween 80 substrate, thereby producing a quantity of free fatty acids. The bacterial lipase produces more or less fatty acids depending on the enzyme concentration. When generated in an M1 medium containing calcium ions, these fatty acids will form insoluble particles in the medium and form halo rings. The higher the number of fatty acids, the larger the diameter of the decomposition ring and vice versa. The lipid-degrading bacteria were developed in Tween 80 medium from the precipitation of free fatty acids with calcium (giving a white zone). The halo was used as an indication to detect the bacterial activity for degrading lipids and producing lipase enzymes (Jia *et al.* 2015).

As a result of the free fatty acid precipitation with calcium in the Tween 80 medium, the lipid-degrading bacteria were created (giving a white zone). The halo was employed as a signal to identify bacterial activity for lipid oxidation and lipase enzyme production. This method was used to prove the presence of lipase in the survey environment. The results indicated that 9 bacterial strains showed a significant effect in decomposing Tween 80 with the average diameter of the halo ring ranging from 5.3 mm to 35 mm (Table 1). Notably, bacterial strain KNT1.2 had the largest halo ring diameter, reaching 35 mm after 3 days of culture. Research by Phong *et al.* (2014) includes isolated 102 bacterial strains with 43 samples of wastewater containing vegetable oil from restaurant systems in 5 districts of Can Tho City, Vietnam, on LB medium. There are 61 bacterial strains capable of forming halo rings and only 7 among them are capable of decomposing vegetable oils in wastewater. In this study, 21 bacterial strains were isolated from wastewater in Can Tho, including 9 bacterial strains capable of forming halo rings after 72 h of culturing on an M1 medium. These bacterial strains are the potentials to process lipids in the environment and produce lipase enzymes for industries.

Many studies have demonstrated the ability to produce lipase in isolated bacteria. Matsumiya *et al.* (2007)

determined the lipase-producing ability of *Burkholderia* spp. DW2-1. After 48 h of culture, bacteria DW2-1 produced lipase with an activity of 1,720 U L^{-1} and degraded more than 90% of canola oil after 7 days of culture. Besides, the study of Ilesanmi *et al*. (2020) demonstrated the lipase-producing ability of *Pseudomonas aeruginosa*, with optimized lipase enzyme activity reaching 528.54 U L^{-1} after 12 h of culture. The bacteria showed the best enzyme activity when cultured at pH 11. Olive oil was used as the carbon source, and yeast extract was used as the nitrogen source after 12 h of culture. In this study, bacterial strain KNT2.2 produced lipase with the original substrate of peanut oil cake with lipase enzyme activity of 2,670; 2,490; and 6,260 U L^{-1}, respectively, after 3, 6 and 9 days of culture (Table 2). After three days, there was a difference between the treatment with bacterial inoculation and the control treatment in terms of the peanut oil cake's condition. The peanut oil cake changed when the bacteria were infected; when they weren't, it was rougher and only partially liquefied (Fig. 3). KNT2.2 is a potential bacterial strain that produces lipase for application in the food, pharmaceutical, and environmental industries.

Conclusion

Twenty-one strains of bacteria capable of lipolysis were isolated fromsamples of wastewater contaminated with lipids on the M1 medium. The survey results showed that among 21 bacterial strains, there were 9 strains capable of degrading Tween 80, creating halo rings on the M1 medium after 72 h of culture. The bacterial strain KNT2.2 was one of the strains that achieved the highest efficiency with lipase activity of 2,670; 2,490; and 6,260 U L^{-1}, respectively, after 3, 6, and 9 days when cultured with peanut oil cake.

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Author Contributions

TTM and NHS planned the experiments, HTT and NTDS interpreted the results, VPT, TTM and TTPT did the write up and TTPT statistically analyzed the data and made illustrations.

Conflicts of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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