Screening and Primary Identification of Lipid Degradation Microorganism from Local Bioextracts

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Abstract

Bioextract is a solution of fermented organic matters which mainly are agricultural leftover containing living microorganisms. Studies have shown that bioextract may be applied to agriculture, livestock, gardening and landscaping, composting, and bioremediation. Latterly, bioextract was introduced for wastewater treatment, due to bioextract characteristic and environmentally friendly. From previous studies (Nitsuwat et al., 2013), addition of bioextract was capable to reduce total solid, grease and oil in domestic wastewater. The grease and oil component in wastewater can create problem in sewage system and hard to lose from the wastewater. The aim of this research is to find and identify microorganism contained inside the bioextract that can degrade grease and oil. In the future increasing of these microorganisms in bioextract may improve the ability of bioextract for pretreating domestic wastewater containing grease and oil.

In this research, lipid degradation microorganisms were isolated from the local bioextract sample. The bioextract biodivesity was found contained total aerobic bacteria $1.07x10^4$ CFU.ml^{-1}, actinomycetes $4.53x10^4$ CFU.ml^{-1}, yeast $2.67x10^4$ CFU.ml^{-1}, lactic acid bacteria 3.04×10^3 CFU.ml^{-1}, and mold $1.98x10^3$ CFU.ml^{-1}. The screening methods used media added with tributyrin. Colonies that created clear zone were isolated and tested using tributyrin and vegetable oil. Total of 55 microorganisms were screened for their ability to degrade tributyrin. Ten strains were further selected from the second screening using vegetable oil. Then these ten isolates were primarily identified using morphological and biochemical characteristics. Majority of the selected isolates were in family Bacillaceae.

Keywords: *Bioextract, Lipid, Microorganisms, Identification*

Introduction

Water is the dominant compound on earth. In recent year, worsening water pollution has caused much more serious problem (Zhang *et al*., 2010). One of component pollution in water are fats, oil and greases usually know as FOGs, in wastewater FOGs create problems including the production of foul odours and blockage sewage (Brooksbank, *et al*., 2006).

For the reduction of water pollution level, various treatments are available but the emergence of an amazing technology of a multiculture of anaerobic and aerobic beneficial microorganisms is presently gaining

popularity due to its environmentally friendly nature. This effective microorganism (EM) technology uses naturally occurring microorganisms which are able to purify and revive nature (Zakaria, *et al*., 2010). The concept of EM, also known as bioextracts was developed by Professor Dr. Teruo Higa, University of Ryukyus, Okinawa, Japan in 1980. Bioextracts belongs to the regenerative category whereby they can prevent decomposition in any type of substances and thus maintain the health of both living organisms and the environment (Higa and Parr, 1994).

Now, effective bioextracts has been used as wastewater treatment in several country. In Malaysia, applications of bioextracts using the formula known as effective microorganism activated solution (EMAS) have been experimented in several rivers, with the principal objective of enhancing and improving the water quality (Zakaria, *et al*., 2010).

The main species involved in bioextracts include: Lactic acid bacteria, such as *Lactobacillus plantarum, L. casei, Streptococcus lactis*. Photosynthetic bacteria, such as *Rhodopseudomonas palustrus, Rhodobacter spaeroides*. Yeasts, such as *Saccharomyces cerevisiae, Candida utilis*. Actinomycetes, such as *Streptomyces albus, S. griseus*. Fermenting fungi, such as *Aspergillus oryzae, Mucor hiemalis* (Szymanski and Patterson, 2003). Lipid degraded bacteria such as *Bacillus subtilis, B. licheniformis, B. Amyloliquefaciens, Seratia marsescens, Pseudomonads aeruginosa,* and *Staphylococcus aureus* for using in treatment of wastewater rich in lipid content was formulated as mixed culture, and found to be effective in treatment of lipid-rich wastewater from 25,000 mg/L to 80 mg/L (Prasad and Majunath, 2011).

In previous study by Nitsuwat *et al*. (2013) showed application of bioextracts solution to treat restaurant wastewater, there were significant reduction of total solid at 53.07%, and grease and oil at 69.89% respectively. The reduction of grease and oil level may caused by high percentage of lipid degrading bacteria in bioextracts solution.

In this study, lipid degradation microorganisms were isolated from the local bioextract sample and tested using several tests to identify the isolated strains of the microorganisms. In the future increasing of these microorganisms in bioextract may improve the ability of bioextract for pretreating domestic wastewater containing fat, oil and greases by bioaugmentation technique.

Materials and Methods

Microbial Analysis for Screening Local Bioextracts

The local bioextracts solution provided by NAVA Social Enterprise, Bangkok, Thailand was tested for the presence of lipid degradation microorganisms used enrichment medium. The media used in this research are PCA (Plate Count Agar) to identify total aerobic bacteria , MRS (de Man, Rogosa, and Sharpe) to identify lactic acid bacteria, GYEA (Glycerol-Yeast Extract Agar) to identify actinomycetes, YM (Yeast and Mold) to identify yeast, RBA (Rose Bengal Agar Base) to identify mold. The bioextracts solution was analyzed by serial dilution and spread plate technique. This research done by two step screening using PCA and GYEA

media. First screening using media adding with 1% tributyrin. Isolation process of lipid degradation microorganisms based on the colonies that have clear zone surrounding the lipolytic colonies. For the second screening, isolates was examined using media adding with vegetable oil 1% and tween 80 0.1%. Lipid degradation microorganisms will show opaque zone in the surrounding colonies. Two step of screening is used to test the ability of isolates to degrade the simple lipid in first screening and the complex one in second screening. Then, lipid degradation microorganisms isolates that showing positive result for second screening were primarily identified using morphological and biochemical characteristics.

Morphological and Biochemical Test the Local Bioextracts Isolate

The characteristics of the microorganisms were determined using biochemical test in order to classify them into a more specific classification. There are four types of test, which are catalase test using 3% H_2O_2, sugar fermentation test using phenol red broth (glucose, sucrose, lactose, glycerol, mannitol) and starch hydrolysis test. Isolates also tested the morphological using gram staining and microscope.The result for clasification based on the morphological, biochemichal and phisical characteristic result according to Bergey's Manual of Determinative Bacteriology.

Results and discussion

Table 1. Survey of microbial diversity in local bioextract solution

Description	Colony Forming Unit / mL (mean ± SD)
Total Aerobic Bacteria	$1.07x10^4 \pm 12.91 x10^3$
Actinomycetes	$4.53x10^4 \pm 1.99 x10^4$
Yeast	$2.67x10^4 \pm 3.88 x10^4$
Lactic Acid Bacteria	$3.04 x10^3 \pm 8.47 x10^2$
Mold	$1.98 x10^3 \pm 5.61 x10^2$

Table 2. Total isolates obtained from bioextract from first screening using tributyrin

Media	Number of Isolates
PCA	33
GYEA	22

From analyzing the microorganisms found in bioextracts, mostly contain actinomycetes with colony forming $4.53x10^4 \pm 1.99 x10^4$ CFU/ml. The bioextracts solution also contain aerobic bacteria with colony forming $1.07x10^4 \pm 12.91 x10^3$. The aerobic bacteria was isolated and continued tested using morphological and biochemical test.The bacterial cultures were isolated from bioextract by spread plate and isolated on the slant. The first screening using tributyrin obtained 33 strains from PCA media and 22 strains from GYEA media. Second screening using vegetable obtained 10 strains from PCA with vegetable oil. These strains were collected and tested using several biochemical and morphological tests.

The main biochemical tested was to test the lipid degradation microorganisms in bioextracts using of lipid test. There were two kinds of lipids used in this analysis, tributyrin and vegetable oil. Tributyrin is the simplest triglyceride occurring in natural fats and oils. Short-chain tributyrin (glyceryl-sn-1,2,3-tri-butyric acids) usually use as media to test the lipase activity.

Different with second screening using vegetable oil. Vegetable oil contains palmitic (44.7 g/100 g), oleic (39.8 g/100 g), linoleic (10.2 g/100 g), and

stearic acid (5.4 g/100 g) as major fatty acids, the mono- (MAG) and diacylglycerol from vegetable oil are also composed of several combinations of such long-chain fatty acids (mostly palmitic and oleic acid) (Lee, *et al.* 2007).

The different ability to degrade the two lipids was analyzed in the lipid test. As the result in Table 3, all the ten strains have high efficiency to hydrolysis lipid on the lipid test using tributyrin.

Table 3. Result of Lipid test for PCA isolates from second screening using vegetable oil monitor every 24 hours.

Strain	Lipid Test						
	PCA Tributyrin			PCA Veg. Oil			
	24	48	96	24	48	96	120
PCA D2	++	+++	++++	-	-	+	+
PCA C4X	++	+++	++++	-	-	+	+
PCA A4	++	+++	++++	-	-	+	+
PCA A3	++	++	+++	-	-	+	++
PCA A22	+++	+++	++++	-	-	+	++
PCA A21	+++	+++	++++	-	-	+	+
PCA C311	++	+++	++++	-	-	+	+
PCA D32	++	+++	++++	-	-	+	++
PCA C312	++	+++	++++	-	-	+	+
PCA D21	+++	+++	++++	-	-	+	+

* + on lipid hydrolysis test means it hydrolyzes lipid and - means it does not hydrolyze lipid. + means small size of the clear/opaque zone, ++ =medium, +++=large, ++++=extra large

On the other hand, the result for lipid hydrolysis using vegetable oil showed slower activity. The vegetable oil need more time to show the positive result. In Table 3, show that the strain tested using vegetable oil need minimum of 96 hours to show their lipid hydrolysis activity. This is due to the different character of the tributyrin and vegetable oil. The degradation of lipid begins with bacteria usually secreting extracellular lipase which hydrolyzes the ester bond into glycerol and fatty acids. Next, the fatty acids are degraded via β-oxidation pathway and converted to acetyl-CoA (Ruggieri *et al*. 2008). Therefore, lipid degradation using microbial metabolism takes a long time.

Different biodegradation efficiencies might be due to different chain between tributyrin and vegetable oil. Gray (1980) said, the natural microbial degradation of fat, oil and grease is slow due to their low lipolytic activity. Desbois and Smith (2010) have reported that FFAs with 14, organism than FFAs with 10 or 12 carbons against certain species of bacteria. It mean that, more long chain and complex lipid will be more difficult to breakdown by microorganisms. Furthermore, the biodegradation of lipids often limited by the antimicrobial effect. This is because lipids also encompass molecules such as free fatty acids (FFAs) and their derivatives. The prime target of FFA action is the cell membrane. Here, FFAs disturb the electron transport chain and oxidative phosphorylation. Besides interfering with cellular energy production, FFA action may also result from the inhibition of enzyme activity, impairment of nutrient uptake, generation of toxic peroxidation and auto-oxidation degradation products or direct lysis of bacterial cells (Desbois and Smith, 2010).

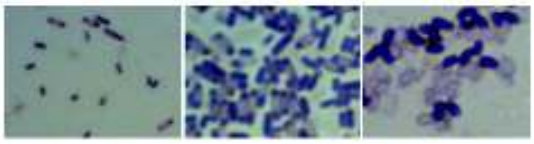
So vegetable oil is harder to degraded than tributyrin, also need longer time to degraded the long-chain fatty acid of vegetable oil. The strain PCA A3, A22 and D32 showed more rapid result for

Table 4. Second screening result tested using Gram's staining.

Strain	Gram Staining
PCA D2	
PCA C4X	
PCA A4	
PCA A3	
PCA A22	
PCA A21	
PCA C311	
PCA D32	
PCA C312	
PCA D21	

biodegradation of vegetable oil. These bacterial isolates may be used to actively degrade the waterwaste containing vegetable oil. According to biochemical, morphological and physiological characteristics in accordance with Bergey's manual of determinative bacteriology, strain namely PCA D2, PCA C4X, PCA A22, PCA A21, PCA C311, PCA D32, and PCA D21 showed characteristic that corresponding to genus *Bacillus*. Ruiz *et al*. (2005) have shown that nearly half of 724 strains isolated from soil rich in organic matter degraded olive oil and tributyrin, and the most active strain belonged to the genus *Bacillus*. For strain PCA C312 belong to genus *Mycobacterium*. Strain A4 belong to genus *Lactobacillus*, and the only one cocci bacteria strain PCA A3 belong to genus *Staphylococcus*.

Even though PCA D2, PCA C4X, PCA A22, PCA A21, PCA C311, PCA D32, and PCA D21 have showed characteristic of the same genus, but it possibly were different species. This is based on the biochemical result and the morphological test using gram staining and microscope. On the microscope result, shown that spore forming and cell size were different. Further test is needed to ensure the classification of the isolates.



Conclusion

Primary analysis showed that bacterial strains namely PCA D2, C4X, A22, A21, C311, D32, and D21 belong to genus *Bacillus*. The strain PCA C312 belong to genus *Mycobacterium*. Strain A4 belong to genus *Lactobacillus*, and

Table 5. Biochemichal and clasification result

Strain	Catalase*	Starch**		Sugar Test***/****					Spore	Gram	Shape	Putative Genus
		24 hrs	48 hrs	Glu	S	L	Gly	M				
PCA D2	+	+	++	-	-	-	-	-	+	+	Rod	*Bacillus*
PCA C4X	+	+	++	-	-	-	-	-	+	+	Rod	*Bacillus*
PCA A4	-	+	++	-	-	-	-	-	-	+	Rod	*Lactobacillus*
PCA A3	+	-	-	A	A	-	-	-	-	+	Cocci	*Staphylococcus*
PCA A22	+	+	++	-	-	-	-	-	+	+	Rod	*Bacillus*
PCA A21	+	-	-	-	-	-	-	-	+	+	Rod	*Bacillus*
PCA C311	+	-	-	-	-	-	-	-	+	+	Rod	*Bacillus*
PCA D32	+	+	++	-	-	-	-	-	+	+	Rod	*Bacillus*
PCA C312	+	-	-	-	-	-	-	-	-	+	Rod	*Mycobacterium*
PCA D21	+	+	++	-	-	-	-	-	+	+	Rod	*Bacillus*

* + on catalase test means it produces catalase enzyme and - means it does not produce catalase enzyme.** + on starch hydrolysis test means it hydrolyzes starch and - means it does not hydrolyze starch. *** A means it produces acid, G means it produces gas, and A/G means it produces acid and gas. **** Glu : glucose, S : Sucrose, L : Lactose, Gly : Glycerol, M : Mannitol

the only one cocci bacteria strain PCA A3 belong to genus *Staphylococcus*. All of this strain bacteria showing positive result for lipid test. These culture may be used to degrade vegetable oil pollutants in the environment. So, increasing of these microorganisms in bioextracts may improve the ability of bioextracts for pretreating domestic wastewater containing fat, oil and greases via bioaugmentation technique.

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