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Isolation and Identification of Lipase-producing Bacteria from Oil-Contaminated Soils

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Abstract: ***In the present study, the indigenous microbes from oil-contaminated soil samples of oil processing mills were isolated based on their lipolytic activities. The isolated bacteria were screened on lipid-enriched media with different substrates and were selected the strains showing lipolytic activity. To be confirmed the lipase activity, the strains were further examined on olive oil and rhodamine B media. And then, the best lipase producer strains were further identified by 16S rDNA sequencing method. Based on the 16S rDNA sequencing results, the strains were close to Acinetobacter species and Stenotrophomonas species.***

Keywords: oil-contaminated soil, lipase, olive oil, 16S rDNA sequencing method

1. Introduction

The large part of the earth's biomass is represented by lipids. Lipids are essential to all living systems. They are the most important source of energy, play structural roles in membranes and are involved in signaling events. To be able to carry out these functions, lipids require lipolytic enzymes during their metabolism. Lipolytic enzymes catalyze the turnover of these water-insoluble compounds [1]. They also breakdown lipids and make them mobile within the cells of individual organisms [2]. Lipolytic enzymes are grouped into 3 main categories, which are esterases, phospholipases and lipases [3].

Lipases are defined basically as fat-splitting enzymes that catalyze the hydrolysis of long-chain triacylglycerols to from glycerols and fatty acids in the presence of excess water. Also, they can catalyze the reverse reaction, synthesis of triacylglycerols, under non-aqueous conditions [4]. Lipases occur widely in nature, but commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Microbial lipases are more widely applied in industries due to their shorter generation time; ease of bulk production which is further enhanced with advancement in fermentation technologies; and ease of manipulation, either genetically or environmentally. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, diaries, soil contaminated with oil, etc [5]. Many microorganisms such as bacteria, yeast and fungi are known to secrete lipases. Of all these, bacterial lipases are more economical and stable [6]. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavor enhancement and lipolysis of butter fat. The industrial demands for new sources of lipases with different enzymatic characterstics that could create novel applications stimulate the isolation and selection of new strains of lipolytic microorganisms. We were interested in this topic because isolation of new lipase-secreting bacteria and study of their enzyme production, purification and characterization could provide new lipase with better quality and wider range of applications.

2. Materials and Methods

A. Chemicals

Tributyrin and *p*-nitrophenyl palmitate (pNPP) were purchased from sigma (USA). The other chemicals were also of analytical grade. Olive oil used for lipase production was commercially available in local market.

B. Methods

1) Collection of sample

Oil-contaminated soil samples from the local oil mills in Myanmar were collected and stored in sterile plastic bags. The samples were transferred to the laboratory under sterile conditions and stored at 4ºC until examination.

2) Isolation of lipolytic bacteria

The isolation was primely processed by serial dilutions of samples and spread plate method on tributyrin agar medium. The composition of tributyrin agar medium is (per liter) 5 g of peptone, 3 g of yeast extract, 10 ml of tributyrin and 15 g of agar. The pH of the medium was adjusted to 7 with 0.1M NaOH. The culture plates were incubated at 35ºC for 48 hrs. This medium was chosen for selectively isolating colonies capable of growing on lipid enriched medium and showing clear zones around them. Colonies showing clear zone diameters were picked up as lipolytic positive strains and streaked onto the nutrient media as pure cultures. Then, these strains were also examined on different enriched media with two different substrates such as egg- yolk and tween 80. On these media, 14 out of 28 strains showed lipolytic activities. These potential lipolytic strains were observed under the microscope and then used for further processes.

3) Screening of lipase producing bacteria

In addition to lipases, esterases are also grouped into hydrolases. Esterases break ester bonds of short-chain fatty acids whereas lipases catalyze the hydrolysis of long-chain fatty acids that are insoluble or poor soluble. Therefore, the lipase must be capable of identifying an insoluble or aggregated substrate. Esterase activity is found to be highest towards more water soluble substrate [7].Olive oil has the

advantage of including high concentration of oleic acid and being more economical. The lipolytic positive strains were further screened for lipase activity by using the modified rhodamine-B agar media consisting of olive oil as substrate at 35ºC. The lipase activity was detected by the presence of pink or orange colored colonies and by fluorescence under UV light.

4) Characetrization and molecular identification of bacteria

The cultures were then characterized morphologically based on Bergey's manual of systemic bacteriology. The identifications were confirmed by using nucleotide sequence analysis of 16S rDNA genes. The genomic DNA was isolated from the bacteria by the DNA extraction method described by Hosek et al. [8]. 16S rRNA gene of isolated strain was amplified from its genomic DNA using a pair of universal bacterial primer, 10F (5'- AGTTTGATCCTGGCTC - 3') as forward primer and 800R (5'- CTACCAGGGTATCTAAT - 3') as reverse primer. The highly purified DNA was then amplified in a thermocycler at conditions: 35 cycles of 95ºC for 30 seconds, 56ºC for 30 seconds and 72ºC for 1 min [9]. PCR products were visualized by agarose gel electrophoresis. DNA sequencing was performed in a highly automated gene sequencer. These sequences had been submitted to the GenBank database (BLASTN) and compared with the other sequences to analyze the bacterial classes and their phylogenies.

3. Results and Discussion

3.1 Isolation of lipolytic strains

Soil samples taken from oil mills in Myanmar were examined for the presence of lipolytic strains. Oil-contaminated soils were chosen as sample sources because they could be good environment for the habitats of lipid degrading bacteria and are rich of lipid. A total of 28 morphologically distinct strains (L1- L28) which showed clear zones around the colonies were isolated from oil-contaminated soils by using tributyrin agar media (Fig.1 (a)).

The egg-yolk suspension allows for the detection of lecithinase and lipase activity. Lipase destroys the fats within the egg yolk, which results in greenish blue colour of the colony surface when flooded with copper II sulphate solution (Fig 1 (b)).

Tweens (fatty acid esters of polyoxyethylene sorbitan) have been the most widely used substrates for the detection of lipase/esterase producing microorganisms in agar media. Screening using tween agar plates shows precipitation around the lipase/esterase producing micro-organisms. The method is based on the precipitation as the calcium salt of the fatty acids released by hydrolysis of tweens. Liberated fatty acids bind with the calcium incorporated into the medium. The calcium complex is visible as insoluble crystals around the inoculation site. On Tween 80 agar plates, formation of precipitation by the isolates also confirmed that this bacterial strains possessed lipolytic activity (Fig 1 (c)).

Totally, 14 strains showed lipolytic activity on these screening media and their microscopic morphology was shown in (Table 1).

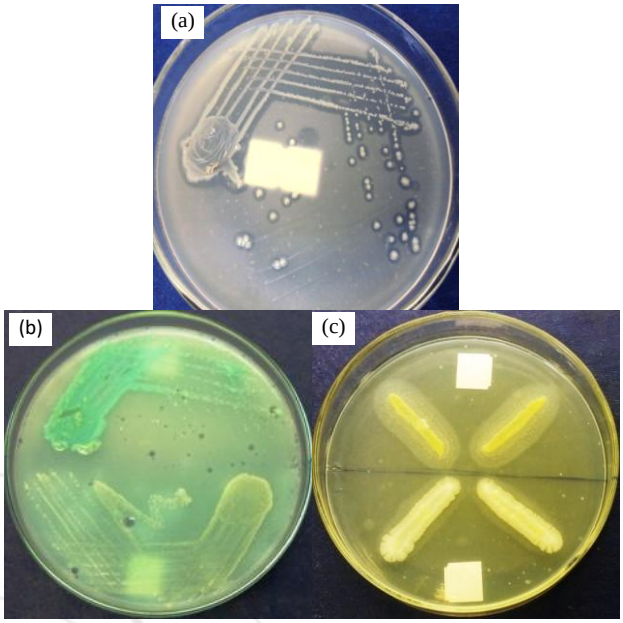

Figure 1: Screening of Lipolytic activity on (a) tributyrin agar medium (b) egg-yolk medium (c) tween 80 medium

Table 1: Cell morphology and physiology of the isolates

Isolate	*Shape*	*Gram's stain*
L1	Rod	Positive
L3	Diplococcoid rod	Negative
L5	Rod	Positive
L8	Cocci	Positive
L10	Cocci	Positive
L11	Rod	Positive
L12	Rod	Positive
L13	Rod	Positive
L14	Diplococcoid rod	Negative
L16	Rod	Negative
L17	Rod	Negative
L19	Rod	Negative
L20	Diplococcoid rod	Negative
L25	Rod	Negative

3.2 Screening of lipase positive bacteria

Lipase producer strains were identified by the formation of orange fluorescent halos around the colonies when olive oil rhodamine B spread plates incubated at 35ºC were exposed to UV light at 350nm. Olive oil is used as lipase substrate and rhodamine B is the indicator of lipase activitiy. This method is not sensitive to pH changes and does not inhibit the growth of bacteria. Seven out of 14 strains were shown the best lipase activity (Fig.2). These rhodamine B-olive oil media can identify lipase-producing bacteria from esterase-producing bacteria. They were selected as the best lipase producer strains and used for further identification. Some biochemical properties of the selected bacteria were also shown in (Table 2).

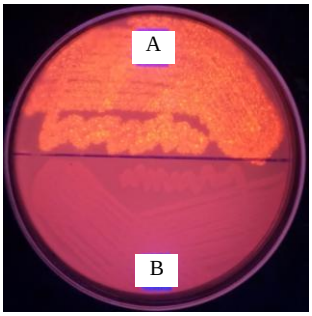

Figure 2: Screening of lipase positive strains under the UV light (A) lipase positive control (B) lipase negative control

Table 2: Some biochemical properties of the isolates

Strain	*Catalase*	*Oxidase*	*Lecithinase*
L3	+	-	+
L14	+	-	+
L16	+	-	+
L17	+	-	+
L19	+	-	+
L20	+	-	+
L25	+	-	+

3.3 Characterization of lipase positive strains

Upon the amplification of 16S rDNA sequence using the primers, the amplified products of 800 bp were obtained (Fig.3) which were then sequenced and compared with the Genbank databases using BLASTN program. It was found to have 95-98% identity with L3 and L14 strains to *Acinetobacter pitti*, L16, L17, L19 and L25 strains to *Stenotrophomonas maltophilia*, L20 strain to *Acinetobacter baumannii* in (Table 3).

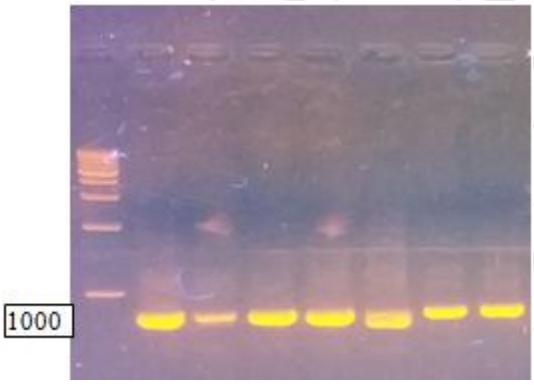

Figure 3: Colony PCR result. Line1: 1kb DNA ladder. Line2: ~800bp PCR products

Table 3: Sequencing result

Sample code	*Primer (10-800bp)*	*Sequence accession description*	*Sequence identity*
L3	10F 800R	*Acinetobacter pittii* PHEA-2 chromosome, complete genome NCBI Reference Sequence: NC_016603.1	97% 98%
L14	10F 800R	*Acinetobacter pittii* PHEA-2 chromosome, complete genome NCBI Reference Sequence: NC_016603.1	97% 98%
L16	10F 800R	*Stenotrophomonas maltophilia* K279a complete genome, strain K279a NCBI Reference Sequence: NC_010943.1	99% 99%
L17	10F 800R	*Stenotrophomonas maltophilia* K279a complete genome, strain K279a NCBI Reference Sequence: NC_010943.1	99% 99%
L19	10F 800R	*Stenotrophomonas maltophilia* K279a complete genome, strain K279a NCBI Reference Sequence: NC_010943.1	99% 99%
L20	10F 800R	*Acinetobacter baumannii* strain AB030, complete genome NCBI Reference Sequence: NZ_CP009257.1	96% 97%
L25	10F 800R	*Stenotrophomonas maltophilia* K279a complete genome, strain K279a NCBI Reference Sequence: NC_010943.1	99% 99%

4. Conclusion

Nowadays, the use of lipases in industries is becoming increasingly important. Extensive and persistent screening for new indigenous microorganisms with improved lipase abilities will lead to faster ways to upgrade the process. With the findings, we obtained three strains of best lipase producers from oil-contaminated soils in oil mills. To obtain maximum production of extracellular lipases, we need to control the best physico-chemical environment that greatly influences the enzyme production. So, the present study requires the optimization of the lipase-production conditions for further industrial applications.

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