Cloning of Lipase Gene from Psychrophilic Microorganism Isolated from Antarctica Fresh Water Sample

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ABSTRACT

Low temperature-active enzymes have recently received increasing attention because of their relevance for both basic and applied research. In biotechnology, novel opportunities might be offered by their catalytic activity at low temperature and, in some cases, unusual specificity.
An obligate psychrophilic microorganism, which hydrolyses lipids at 5°C, was isolated from fresh water sample at Davis Station, Antarctic. The isolate is a rod-shaped with budding, gram-positive bacterium and size around 4.2μm. The isolate grows at 5°C and 15°C for 7 days incubation period. Isolate named PI A was grown on screening plates which contained nutrient agar and lipase substrate such as tributyrin, triolein, palm oil, olive oil and fluorescent assay using Rhodamine B (Kouker & Jagger, 1987) to screen for extracellular lipase. The existence of the halos on the media showed the hydrolytic reaction by lipase activity. Lipase assay using titration was also done to detect the lipase activity. Lipase activity was detected at 1.647U/ml. The genomic DNA of PI A was successfully extracted by using phenol-chloroform extraction with the modification of CTAB (cetyltrimethylammonium bromide) method from plant. 23kb of genomic DNA size was obtained. The extracted genomic DNA from PI A will be used for cloning and expression of lipase gene works. Isolation of lipase gene using PCR method was done using primers designed based on oxyanion hole and active site of selected lipases. The PCR product of these primers was obtained about 350bp. The PCR product was clone using pGEM®-T Vector System and *E. coli* JM109 as host. Plasmids were extracted and were sent for sequencing. The PCR product suspected to be α/β hydrolases. To confirm that this gene fragment was a functional lipase gene, further analysis is being carried out.

INTRODUCTION

Psychrophilic microorganisms have the largest distribution on earth if one considers the extent of area where temperature remains permanently below 10°C including the deep-sea waters, mountains and polar regions. Life in low temperature environments requires physiological and biochemical adaptations of microorganisms. Psychrophilic organisms grow fastest at temperature of 15°C or lower, but cannot survive at temperature above 20°C whereas; psychrotolerants grow well at temperatures close to the freezing point of water, but have fastest growth rates above 20°C up to 40°C (Madigan *et al.*, 2003).

In order for growth to occur in low temperature environments, all cellular components from membranes and transport systems to intracellular solutes, protein and nucleic acids

must adapt to the cold (Cavicchioli *et al.*, 2002). Psychrophilic organisms produce enzymes that can function in cold environment and simply denature at high temperature. The cold-active enzymes have a more flexible structure to undergo the conformational changes necessary for catalysis with a lower energy demand (Arpigny *et al.*, 1997). Cold-active enzymes might offer novel opportunity for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermostability and unusual specificities. These properties are of interest in diverse fields such as detergents, textile and food industry, bioremediation and biocatalysts under low water conditions (Choo *et al.*, 1998). Furthermore, fundamental issues concerning the molecular basis of cold activity and the interplay between flexibility and catalytic efficiency are of importance in the study of structure–function relationships in proteins. In this context, the recent cloning of a few lipases (acylglycerol ester hydrolases, EC 3.1.1.3) active at low temperature is relevant because of their metabolic and industrial role; lipases have been thoroughly investigated by studies encompassing sequence, structure, regulation of expression, activity and specificity (Rashid *et al.*, 2001 and Lee and Rhee, 1992).

Lipases (EC 3.1.1.3) are triacylglycerolester hydrolases, that catalyze the hydrolysis of triglycerides at the interface between the insoluble substrate and water. True lipases are distinguished from esterases by the characteristic interfacial activation they exhibit at an oil–water interface. Lipases are one of the most frequently used biocatalysts for organic reactions performed under mild conditions.

The objectives of this work were to isolate cold-active lipase producing psychrophilic microorganism, to screen the extracellular lipase by using selective agar media for lipase activity and also to clone the lipase gene.

MATERIALS AND METHODS

Organism

Psychrophilic microorganism used in this study was isolated from Antarctic fresh water sample on nutrient agar plates and named as PI A.

Screening for lipase production

Lipase activity of the bacterium was detected through the formation of halos around the colonies in three kinds of lipase specific agar media as follows. The nutrient agar medium containing 5.0g of peptone, 3.0g of beef extract and 12.0g of agar in 1L dH_2O was supplemented with tributyrin medium (1% v/v), or palm oil medium (0.5% v/v) or Rhodomine B medium (1% v/v olive oil and 10ml of Rhodomine B stock 0.01% w/v). The preparation of culture media was done at pH 7.0 and the mixture was well homogenized by a mixer. Cultures were incubated for 7-10 days for tributyrin medium and 2-3 weeks for palm oil and Rhodomine B medium at 5°C. The lipolytic activity was detected through the formation of clear halos around the colonies in three different kinds of agar plates as mentioned above at 5°C. Lipase production on Rhodomine B medium was monitored by fluorescence with UV light at 350nm. Screening for an extracellular

lipase was also carried out by using the supernatant (crude enzyme extract) on tributyrin agar plate. This method was carried out to confirm the lipase produced by PI A strain was an extracellular enzyme. The crude enzyme from the supernatant was pipetted into the holes on tributyrin agar. The agar plates were incubated at 5°C and room temperature (±24°C). The supernatant that contained lipase enzyme would hydrolyse the substrate and produced halo zone surrounding the holes on the agar plates.

Preparation of crude enzyme extract

The cultures from tributyrin, palm oil and olive oil broth medium were used in preparation of crude enzyme extract. PI A cells were harvested by centrifugation at 6,000 rpm for 15 min at 5°C and the supernatant was assayed for extracellular lipase activity.

Lipase Assay

The determination of protein content was carried out by using Bradford method (1976). Three kinds of substrates (tributyrin, palm oil and olive oil) were used in determination of the lipase assay activity. The lipase catalytic activity was measured by titrimetric determination of the rate of free fatty acid release from tributyrin, palm oil and olive oil (contains 70% triolein). Lipase activity was measured by a titrimetric assay with 0.02 M NaOH using emulsified substrates. 1ml portion of crude extract enzyme was added to 5ml of emulsion containing 2% (v/v) olive oil, 1% (v/v) gum arabic, 0.10M Tris and 4ml of 0.2 M phosphate buffer at pH 7. The assay was carried out at 5°C and room temperature (±24°C) during 30 minutes incubation. After this time interval, the reaction was stopped by the addition of 15ml of acetone:ethanol, 1:1 (v/v) and the amount of fatty acids *was then titrated using 1% ethanolic phenolphthalein . One unit of lipase activity was defined as the amount of enzyme required to release 1*μmol of fatty acid per min under these conditions.

Preparation of genomic DNA

Genomic DNA (gDNA) from PI A was isolated directly from the cells grown in nutrient broth using phenol-chloroform extraction with the modification of CTAB (cetyltrimethylammonium bromide) method from plant.

Cloning of lipase gene fragment

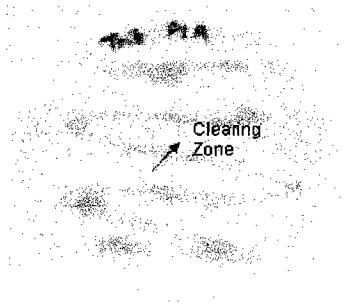
gDNA was successfully extracted as described above. The chromosomal DNA was used as a template for amplification of lipase gene fragments using degenerate consensus primers. The fragment was amplified via PCR with two primers – forward (OXF 1): 5' CCY GTK GTS YTN GTN CAY GG-3'; and reverse (ACR 1): 5'-AGG CCN CCC AKN GAR TGN SC -3'. Reaction was carried out in a total volume of 25μL. The amplification program was as follows: Five thermocycles with (1s 94°C), (1min, 94°C,), (1 s, 37°C), (30s, 37°C), (2min 20s, 72°C), (1min, 72°C). These five cycles were followed by 35-step cycles as follows: (1min, 95°C), (1min, 50°C), (1min, 72°C,). The amplified DNA was

purified using QIAGEN QIAquick PCR purification kits following manufacturer's instructions (QIAGEN, Germany) Purified DNA was ligated in pGEM®-T vector (Promega Corp., USA) overnight at 16°C. The ligated DNA was transformed in *E.coli* JM 109 competent cells. The recombinant clones were identified through blue/white colour selection and the presence of insert in the recombinant clones (white colonies) was confirmed following colony PCR. For sequencing, plasmid DNA was isolated using Wizard ® Plus SV Minipreps DNA Purification System (Promega Corp., USA) and sent for sequencing. A homology search was performed with Genebank database.

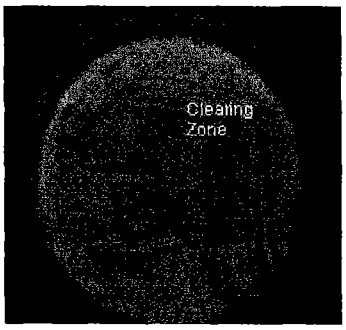
RESULTS

Screening for lipase production

A psychrophilic microorganism which hydrolyses lipids was isolated from fresh water sample from Davis Station, Antarctica. Colonies were able to hydrolyze medium containing tributyrin, palm oil, olive oil and appeared orange fluorescent colour under the UV light when culture on agar plates containing olive oil and rhodamine B.

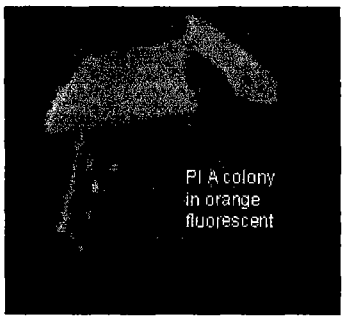


Hydrolisis zone surrounding the PI A colonies on tributyrin agar plate after 7 days of incubation at 5°C.

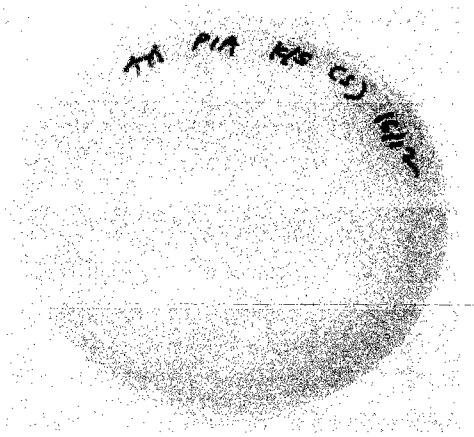


Hydrolisis zone surrounding the PI A colonies on olive oil agar plate after 14 days of incubation at 5°C.

Hydrolisis zone surrounding the PI A colonies on palm oil agar plate after 14 days of incubation at 5°C.



PI A colonies on nutrient agar with rhodomine B showed orange fluorescent colour under the UV light.



Hydrolysis zones were appered surrounding the holes on tributyrin agar that contained the crude enzyme (from palm oil culture medium) at room temperature, ±24°C after 1 day of incubation. Halo zones were appeared surrounding the holes tributyrin agar plates after 1 day of incubation and keep increased with the increasing of incubation

Determination of Lipase Activity

Lipase activity in supernatant cultures at 5°C and room temperature (±24°C) was measured by the titration method and ethanolic phenolphthalein as the titration marker. The volume of NaOH used to neutralized acid was recorded to determine the lipase activity.

PI A has shown to produce significant amounts of extracellular lipase when grown in tributyrin medium with lipase activity of 1.647U/ml at room temperature (±24°C). Whereas the reaction at 5°C only detected 1.253U/ml lipase activity. In palm oil and olive oil medium, the lipase activities for room temperature were 1.547U/ml and 1.523U/ml respectively. While the lipase activity at 5°C in both medium were 1.320U/ml (palm oil) and 1.023U/ml olive oil.

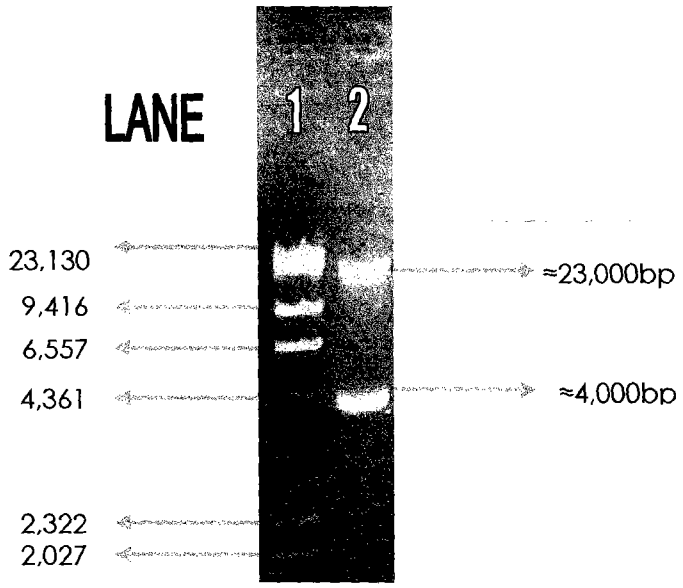
The activity of lipase was higher at the room temperature (±24°C) compared to the activity at lower temperature (5°C) for all types of culture. Enzymes extracted from Antarctica species usually have the specific activities in the range of 0-30°C (Arpigny *et al.*, 1997).

The activity of lipase was higher in the tributyrin medium than those from palm oil and olive oil medium. This was because the tributyrin medium provided more reaction surface for the lipase activity.

Genomic DNA extraction

Genomic DNA (gDNA) from PI A was successfully isolated using phenol-chloroform extraction with the modification of CTAB (cetyltrimethylammonium bromide) method from plant. Figure below shows gDNA of PI A on the agarose gel (0.7%). The exact size

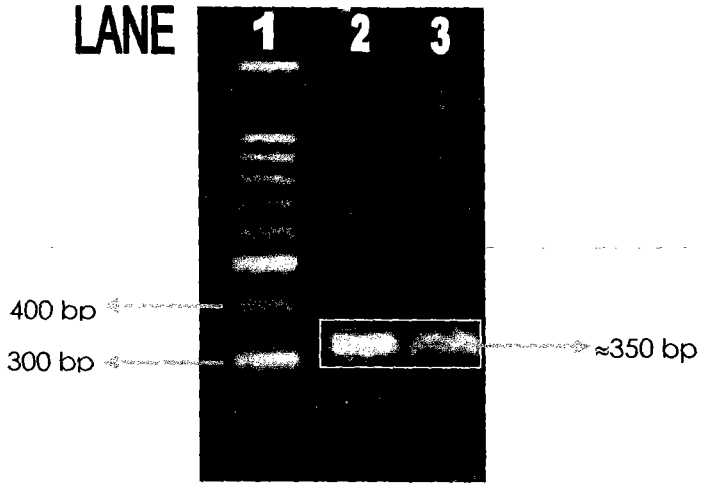
of the genomic was unknown but large bands in the 23kb region were obtained. There was also another band at 4,000bp region which suspected to be the plasmid.



Legends :
Lane 1 : Lambda Hind III / DNA Marker
Lane 2 : DNA of PI A

Cloning of lipase gene fragment

PCR product from degenerate consensus primers was successfully amplified and produced 350 bp size of fragment. This fragment also successfully cloned into pGEM®-T vector (Promega Corp., USA) in *E. coli* JM109 as host. Plasmids were extracted and were sent for sequencing. The result was submitted for BLAST (Basic Local Alignmant Search Tool) at NCBI (National Center for Biotechnological Information). The PCR product suspected to be α/β hydrolases based on sequencing analysis but the sequence does not display similarity to any lipases from other microorganisms. To confirm that this fragment was a functional lipase gene, further analysis is being carried out.



Legends :
Lane 1 : 100 bp Marker
Lane 2&3 : PCR product of PI A

CONCLUSION

In conclusion, a psychrophilic microorganism, namely PI A from Antarctica fresh water sample was isolated after 5 days of incubation at 5°C. PI A was detected to produce an extracellular lipase activity by showing the clear hydrolysis zones on the lipase selective medium. The activity of lipase was also detected in lipase assay. We were also successfully amplified 350 bp fragment using lipase-prospecting primers (P.J. Bell. *et al.*, 2002) from total genomic DNA of PI A via Polimerase Chain Reaction (PCR).

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LIPASE FROM PSYCHROPHILIC MICROORGANISM ISOLATED FROM ANTARCTICA

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Abstract

Low temperature-active enzymes have recently received increasing attention because of their relevance for both basic and applied research. In biotechnology, novel opportunities might be offered by their catalytic activity at low and, in some cases, unusual specificity. An obligately psychrophilic microorganism, which hydrolyses lipids at 5°C was isolated from water at Davis Station, Antarctic. The isolate is a rod-shaped, gram-positive bacteria with budding and size around 4.2µm. Molecular approach to identify the genus of strain by using 16SrRNA gene and Internal Transcribed Spacer (ITS) primer is currently carried out. The isolate grows at 4°C to 15°C for 7 days incubation period. Isolate named PI A was grown on screening plates which contained nutrient agar and lipase substrate such as tributyrin, triolein, palm oil, olive oil and fluorescent assay using Rhodamine B (Kouker & Jagger, 1987) to screen for extracellular lipase. The existence of the halos on the media showed the hydrolytic reaction by lipase activity. Lipase assay using titration was also done to detect the lipase activity. Lipase activity was detected at 1.647 U/ml. The genomic DNA of PI A was successfully extracted by using phenol-chloroform extraction with the modification of CTAB (cetyltrimethylammonium bromide) method from plant. 23 kb of genomic DNA size was obtained. The extracted genomic DNA from PI A will be used for cloning and expression of lipase gene works.

Keywords: *psychrophilic microorganism, 16SrRNA gene, Internal Transcribed Spacer (ITS) primer, Lipase assay*

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