Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 1037 - 1045

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

SCREENING AND IDENTIFICATION OF A NOVEL ALKALINE LIPASE PRODUCING BACTERIUM

FRANCOIS N. NIYONZIMA AND SUNIL S. MORE*

Department of Biochemistry, Center for Post Graduate Studies, Jain University, 18/3, 9th Main Jayanagar 3rd Block, Bangalore-560011, INDIA

ABSTRACT

The lipase producing bacteria were screened from the soil both qualitatively by Tween 80, rhodamine B agar assays and quantitatively by titrimetry with the objective of finding a bacterial strain capable of producing an alkaline lipase. The higher lipolytic activity of 17.43 U/ml was recorded for the isolate L13. Traditional methods viz. morphological, cultural and biochemical characteristics were carried out to identify the most potent bacterial producer of an alkaline lipase. The isolate L13 was identified as *Bacillus flexus*. Based on nucleotides homology and phylogenetic analysis, the isolate displayed 99% sequence similarity to *Bacillus flexus* and was named *Bacillus flexus* strain XJU-1(GenBank Accession Number: DQ837542.1). The screened *Bacillus flexus* from the present study can be exploited for the industrial production of an alkaline lipase that can be used in detergent and leather industries since the isolate is producing higher amounts of enzyme under alkaline conditions.

KEYWORDS: **Olive oil, tween 80, rhodamine B, alkaline lipase, *Bacillus flexus* strain XJU-1**

SUNIL S. MORE
Department of Biochemistry, Center for Post Graduate Studies, Jain University, 18/3, 9th Main Jayanagar 3rd Block, Bangalore-560011, INDIA

INTRODUCTION

Lipases (EC 3.1.1.3) are enzymes that hydrolyze triacylglycerols to fatty acids, diacylglycerols, monoacyl glycerols, and glycerol at the interface between oil and water. However, under non-aqueous conditions, they catalyze reverse reactions such as esterification and trans-esterification. In contrast to esterases which are active with water soluble substrates, lipases are more active with insoluble or poorly soluble substrates. They hydrolyze long chain fatty acid esters of glycerol whereas esterases cleave down ester bonds of short chain fatty acids[1].Lipases constitute the third largest group of enzymes used in industries after proteases and carbohydrases. They have many potential applications in various industries and are selected for each application based on its substrate specificity, position and stereospecificity as well as temperature and pH stability. They are mostly used in the detergent formulations as fat/oil stain removers under the alkaline conditions, in the leather processing to remove residual fats associated with the hides and the skins, in the waste water treatment for degreasing of lipid clogged drains, in the food processing to enhance the flavor, in the pulp and paper industry to remove the pitch, in the organic synthesis to catalyze a wide variety of chemo-, regio-, and stereoselective transformations, in the dairy to aide in the milk fat hydrolysis, in the medical industry as diagnostic tool in blood triacylglycerol assay and in pharmaceutical industries for example in the synthesis of drugs, in the manufacture of agrochemicals and in the textile industry to increase the absorbency of fabrics[1,2].Various methods have been developed to detect lipolytic activity. Tributyrin agar plates are frequently used and the lipase production is indicated by the formation of clear zones around the colonies[3-6]. Tween 80 agar plates are also in use and the lipolytic activity is shown by the occurrence of opaque zones around the colonies[7,8]. Dyes like Victoria blue B, Spirit blue and Nile blue sulfate in combination with Tween 80 can also be used for the lipolytic activity detection. These dyes undergo color change as a result of lipolysis[1,9]. One of the disadvantages of using tributyrin and Tween 80 is that they can be hydrolyzed by the esterases giving false-positive results for lipase presence[10]. In addition, some of these dyes like Victoria blue B can inhibit the growth of some bacteria and are affected by pH changes[11]. Rhodamine B oil agar plates are used to detect true lipase activity which is indicated by the formation of orange fluorescent halos around the colonies under UV light at 350 nm. Unlike tributyrin- and Tween-based methods, this method is not sensitive to the pH changes and does not inhibit the growth of bacteria[9,12,13]. The titrimetric and colorimetric methods are also used to detect the lipase activity. In titrimetry, the fatty acids released during lipolysis are neutralized by NaOH solution of known concentration, whereas the colorimetric method uses synthetic water-soluble substrates like *p*-nitrophenyl palmitate (pNPP)[10,11,14]. Lipases are produced from animals, plants and microorganisms such as fungi and bacteria. However, much attention is paid to lipases from microbes since microbial lipases have vital characteristics such as better stability under alkaline conditions and at higher temperatures, high yields, low production cost, diversity in catalytic activities, easy to manipulate genetic material, stability in organic solvents, broad substrate specificity and ability to grow on cheap media[1,15]. Among the different Gram-positive bacteria, the chief lipolytic producers belong to the genus *Bacillus*[15]. The literature reports different lipase-producing *Bacillus* species (and related species) from diverse environments such as *Brevibacillus borstelensis* AK-P2 isolated from Taptapani hot spring located in Ganjam District (Orissa state, India)[4], *Bacillus* sp. PD-12 isolated from oil industry soil samples[5], *Bacillus megaterium* isolated from mustard oil mills soil[8], *Bacillus pumilus* screened from the soil[14] and *Bacillus subtilis* isolated from soil and tannery wastes[16]. Five different *Bacillus* species viz. *Bacillus flexus*, *Bacillus pseudofirmus*, *Bacillus pumilus*,

Bacillus halodurans and *Bacillus cereus* were also screened from lonal crater[17]. However, the reports on *Bacillus* species that can secrete higher amounts of lipase enzymes with optimum activity under alkaline conditions are scarce. The aim of the present study was therefore to screen and identify a highly potential alkaline lipase-producing bacterium from the soil.

MATERIELS AND METHODS

Primary screening of alkaline lipase-producing bacteria

Soil samples were collected from the Bangalore potato field. All the soils were thoroughly mixed in equal proportions. The lipase-producing bacteria were screened from 1 g of soil sample by serial dilution and spread plating methods on the modified agar medium consisted of Tween 80 (1%, v/v), peptone (1%, w/v), NaCl (0.5%, w/v), $CaCl_2.2H_2O$ (0.01%, w/v) and agar (1.5%, w/v)[18], at pH 10.0. Sodium carbonate solution of 2 M strength was used to adjust the pH of the medium. The medium was treated with niacin before being poured into sterile plates. The plates were incubated at 37 °C for 3 days and checked every day for the growth. The hydrolysis of the Tween opacity medium and/or the presence of white precipitate around the bacterial colonies were an indication of development of lipase producing bacteria.

Secondary screening for the lipase producing bacteria

The colonies with Tween 80 hydrolyzing ability were picked up and streaked on rhodamine B olive oil agar plates containing rhodamine B (0.001%, w/v), olive oil (2%, v/v), sucrose (1%, w/v), KH_2PO_4 (0.1%, w/v), NH_4NO_3 (0.2%, w/v), $MgSO_4$ (0.2%, w/v), $CuSO_4$ (0.006%, w/v) and agar (1.5%, w/v)[19], at pH 10.0. The plates were then incubated at 37 °C for 48 h. The presence of lipase enzyme was confirmed by an orange fluorescence halos around the isolate in rhodamine B olive oil agar plate under UV transilluminator (WEALTEC, India) at 350 nm. A separate agar plate without any bacteria was incubated in the same condition and served as control. The positive strains were streaked on to the freshly prepared sterile nutrient agar slants and preserved at 4 °C in the refrigerator until use.

Screening of the selected isolates for the lipase production under submerged fermentation The inoculum was prepared by transferring one loop of the isolate into 25 ml growth medium contained in 50-ml conical flask. The flask was shaken with an orbital incubator (S150 STUART, India) operating at 100 rpm and at 37 °C for 18 h. 2 ml of the isolate broth was used to carry out the submerged fermentation. The growth medium used for the inoculum preparation and the submerged fermentation was the same as described for the secondary screening excluding agar and rhodamine B. The submerged fermentation was performed by inoculating 2 ml of inoculum into 100 ml of the fermentation medium in 250-ml Erlenmeyer flask. The incubation was carried out at 37 °C with shaking at 100 rpm for 48 h. The broth was centrifuged (REMI C-30 BL cooling centrifuge, India) at 10,000 rpm for 10 min at 4 °C. The supernatant was used as crude enzyme source and was used to quantify alkaline lipase activity. The titrimetric method proposed by Jensen[11] with minor modifications was used to determine the lipase activity and olive oil was used as a substrate. The emulsion was prepared by mixing together with a magnetic stirrer (2MLH REMI, India) 5 ml of olive oil and 5 g gum acacia in 100 ml of 100 mM Tris-HCl buffer (pH 8.5) for 10 min. 10 ml of olive oil emulsion was mixed with 1 ml of crude enzyme and incubated at 37 °C with shaking at 100 rpm for 30 min. The reaction was arrested with the addition of a mixture of 2 ml of acetone and 2 ml of ethanol after the incubation period. 3 drops of phenolphthalein was added as indicator and the mixture was shaken well. 0.05 N NaOH solution was used to titrate the liberated fatty acids to an end point of the pink color at pH 10.0. A blank titration with heat inactivated enzyme at 95 °C for 5 min was also performed. One lipolytic unit (U) of an alkaline lipase was defined as the amount of enzyme which produced 1 µmol of fatty acids from olive

oil per min per ml of the enzyme solution under the assay conditions.

Identification of lipase-producing bacterial isolate

Among the isolates, the isolate that showed a higher lipase activity was subjected to identification. It was first identified by morphological and physiological properties as described in the Bergey's manual of systematic bacteriology[20]. It was further identified based on the 16S rDNA sequence analysis as follow: Genomic DNA was isolated from the selected isolate (L13) using GeNeiUltrapure $_{TM}$ Bacterial Genomic DNA Purification kit (KT162 Cat #.612116200021730). Using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified. The PCR mixture contained 20 ng genomic DNA, 1.0 µl dNTP mix (2.5 mM each), 100 ng forward primer, 100 ng reverse primer, 1 × Taq Buffer A (10X) and 3 U Taq DNA polymerase enzyme. The reaction volume was made up to 50 µl with distilled water. PCR reactions were performed with the following program: 35 cycles consisting of pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1.5 min, followed by a final extension step of 10 min at 72 °C. The amplified PCR products were detected after loading the PCR products on 1.0% agarose gel along with StepUpTM 500bp DNA ladder as the molecular marker. The amplified PCR product was purified and then bi-directionally sequenced using the forward, reverse and an internal primer. The sequence data was aligned using a combination of the National Center for Biotechnology Information (NCBI) GenBank and RDP database and analyzed with nucleotides homology and phylogenetic tree in order to find the closest homologous microbes.

Statistical analysis

Three independent experiments for each isolate were carried out to determine the lipase activity. Each lipase activity reading had thus three replicates. The means of variable and standard deviation were recorded. The data was analyzed using one way analysis of variance (ANOVA) and the possible differences were given by Duncan's multiple range test (DMRT). The statistical analysis was performed at the 5% significance level using the SPSS statistical package software.

RESULTS

Primary and secondary screening of lipase producing microorganisms

In the present investigation, bacteria were screened from the soil collected from potato grown fields around Bangalore (India). 15 colonies (coded as L1 to L15) showed the opaque zones and/or white precipitates around them on agar plates. Out of these, 6 isolates (L2, L4, L7, L8, L9, and L13) were able to form the orange fluorescence halos around them in rhodamine B olive oil agar plates under UV light at 350 nm (Fig 1).

Figure 1

Lipase production by the isolate L13 in an olive oil rhodamine B agar plate. The isolate was incubated at 37 °C for 48 h and then exposed to a UV light at 350 nm.

Screening of the selected isolates for the lipase production under submerged fermentation

The lipase production by the 6 isolates was investigated. The isolate L13 was the highest alkaline lipase producer while L8 was the least (Fig 2).

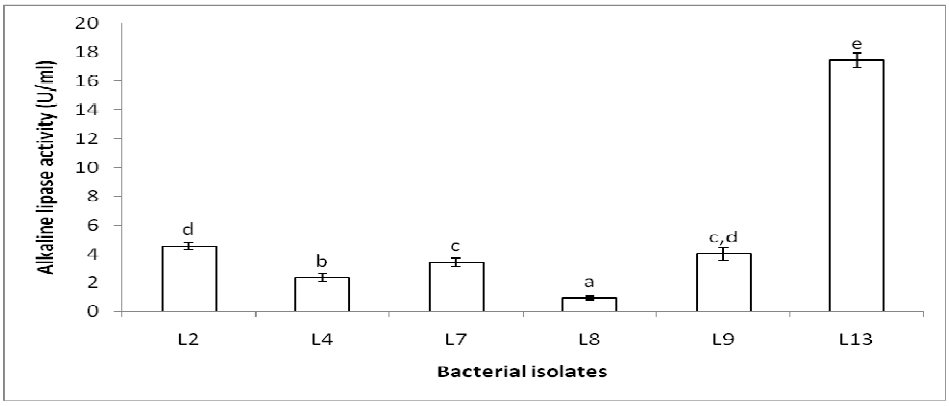

Figure 2

Lipolytic activity of various bacterial isolates. The extracellular lipase activity was assayed by the titrimetric method. The growth medium consisted of olive oil (2%, v/v), sucrose (1%, w/v), KH_2PO_4 (0.1%, w/v), NH_4NO_3 (0.2%, w/v), $MgSO_4$ (0.2%, w/v) and $CuSO_4$ (0.006%, w/v). The different letters on the error bars indicate the significant differences at $P_{0.05}$.

Identification of the isolate L13

The microscopic examination and biochemical tests for the isolate L13 were carried out. It was Gram positive rods, endospore forming and aerobic; had opaque and smooth colonies; growth on nutrient agar medium was abundant, it fermented glucose, mannitol, lactose and sucrose but did not ferment arabinose, mannose, cellobiose and sorbitol; it hydrolyzed gelatin, starch, and casein; it reduced nitrate and was able to produce the indole and the hydrogen sulfide; the Voges-Proskauer test was negative whereas the citrate utilization test was positive; it also showed catalase, urease and oxidase activity. The isolate was identified as *Bacillus flexus* based on these morphological, cultural and biochemical characteristics. It was further subjected to 16S rDNA sequence analysis. Using consensus primers and *Taq* DNA polymerase, the ~1.5 kb 16S rDNA fragment was amplified (Fig 3).

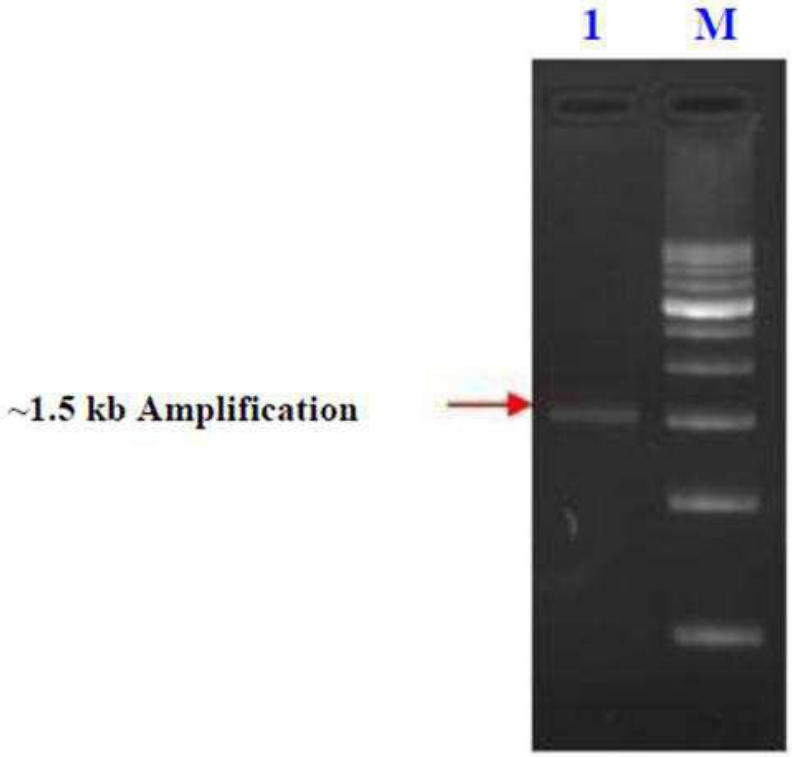

Figure 3

The PCR product of 16S rDNA gene on 1% agarose gel. Lane 1: PCR product of ~1.5 kb from the experimental strain sample, Lane M: DNA ladder (Cat# 612651970501730).

The generated PCR product was sequenced and the sequence data was aligned. After sequence alignment, the sequence was compared to 16S rDNA sequence with other bacteria from GenBank of National Center for Biotechnology Information (NCBI) and analyzed with phylogenetic tree (made using the neighbor joining method) in order to find the closest homologous microbes. Based on nucleotides homology and phylogenetic analysis, the isolate was identified as *Bacillus flexus* strain XJU-1 (GenBank Accession Number: DQ837542.1) (Fig 4).

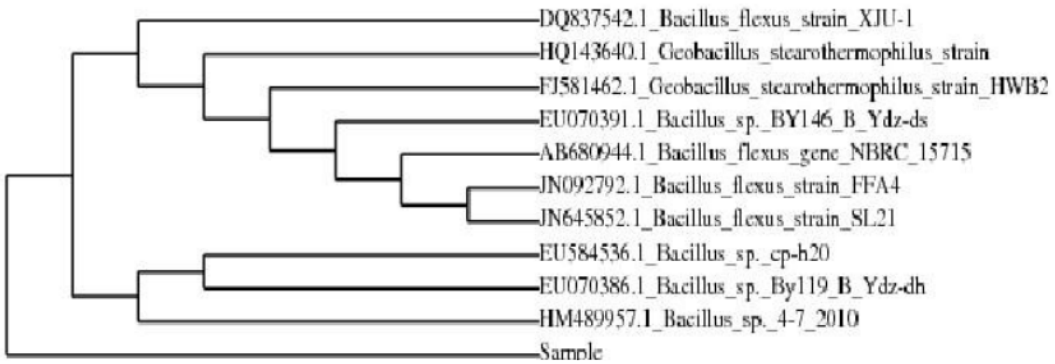

Figure 4

Phylogenetic tree based on the 16S rDNA analysis constructed using neighbor joining method. The closest homologues of the isolate L13 (labeled as sample) with their accession numbers are shown.

DISCUSSION

Primary screening of lipase producing microorganisms

In the present study, lipase-producing bacteria were screened from the soil. The surfactant Tween 80 was used to screen for the lipolytic bacteria. The formation of opaque zones around the colonies was an indication of the lipase producers. These zones occurred due to the hydrolysis of the surfactant by the lipases produced by the isolates. In addition, white crystals were seen around some bacterial isolates. Indeed, the fatty acids released during Tween 80 hydrolysis get complexed with calcium ions present in the growth medium. The calcium-fatty acid complexes are then seen as insoluble white crystals around the colonies[18]. Two roles for Tween 80 were proposed[21], it stimulates the enzyme disassociation from the cell surfaces leading to the complete enzyme release into the culture supernatant (as it is a surfactant) and induces lipase production since it chemically resembles to some natural lipid substrates. Several reports for enhanced lipase production in *Bacillus* species by Tween 80 are available[8,14,16,21].

Secondary screening of the lipase producing microorganisms

15 isolates resulted from screening by Tween 80 were subjected to secondary screening with rhodamine B. Out of these, 6 isolates (labeled as L2, L4, L7, L8, L9, and L13) were found to be true lipase producers. This was confirmed by an orange fluorescence halos around the isolates in rhodamine B olive oil agar plates under UV light at 350 nm. The formation of an orange fluorescence activity is an indication of true lipase activity[12]. The mechanism involved is that fatty acids, monoglycerides and diglycerides resulted from lipolysis are released in to the growth medium and get complexed with the rhodamine B dimers, leading to the formation of fluorescent products seen as orange fluorescent halos around the colonies[13]. The other isolates might be esterase-producing bacteria since they did not show the orange fluorescence; instead they accumulated the rhodamine B as they formed pink colored colonies. Kouker and Jaeger[12] reported the accumulation of rhodamine B by a non-lipase producer, *Escherichia coli*.

Screening of the selected isolates for the lipase production under submerged fermentation

Most of the lipases from bacteria are produced inside the bacterial cells and get released to its external surface or environment[22]. Lipases are thus inducible extracellular enzymes. Olive oil served as a substrate and inducer for the

alkaline lipase production. Similarly olive oil was the best inducer for lipase production by *Bacillus* species[5,14]. The isolates were grown under shaking conditions since mixing and aeration increased in general the biomass and lipase production. This might be attributable to the higher availability of the carbon source and other nutrients to the isolates[5]. The titrimetric and colorimetric methods can be used to find out the lipase activity. Each method has the advantages and disadvantages. The titrimetric method uses simple equipment and the analysis is easy, however, it is time consuming, which may render them unsuitable for the large-scale screening of lipolytic activity[23]. As the colorimetric method is spectrophotometry-based method, it has short reaction time and easy readings. However, these substrates are unsuitable to determine true lipase activity because they are also hydrolyzed by esterases[10]. In the present study, the titrimetric method was used. The gum acacia is known to be a proper tensoactive agent and was used to emulsify olive oil since the lipolytic activity is directly proportional to the degree of emulsification of oil. A stable emulsion is thus necessary for a successful measurement of lipase activity by titration[9,24]. However, the mechanism explaining the effect of emulsifiers in the activity of microbial lipases is not fully understood[10,24].During submerged fermentation, the alkaline lipase activities of the 6 isolates ranged from 0.93 to 17.43 U/ml. This difference in lipase production by the isolates might be due to genetic differences. The variation in lipase production by the isolates was attributed to their ability to tune the growth and metabolic activities with some time lag[6]. The alkaline lipase production was also reported for *Bacillus* species[4,5,14,16,17].

Identification of the lipase-producing isolate L13

Out of the 6 isolates screened with the help of rhodamine B, the isolate (L13) that exhibited appreciable amounts of lipase activity (17.43 U/ml) as compared to others was further identified. Based on morphological, cultural and biochemical characteristics, the isolate was found to be *Bacillus flexus*. It was further detected to be *Bacillus flexus* strain XJU-1 by nucleotides homology and phylogenetic analysis. 16 S rDNA sequence analysis was also used to identify *Bacillus* species[4,17].

CONCLUSION

A highly alkaline lipase-synthesizing bacterium was screened from the soil and identified as *Bacillus flexus* strain XJU-1. The bacterial identification should not only based on microscopic examination and biochemical tests, it should be confirmed by the nucleotide sequence homology and phylogenetic analysis. Higher lipolytic activity at alkaline pH makes this strain a good industrial candidate. The alkaline lipase from this strain is being characterized for use in the detergent and leather industries.

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