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Microbial Potential of Lipase Production from Different Industrial Effluents

Ifrah SHAFQAT[1] Shaheen SHAHZAD[1*] Azra YASMIN[2]
[1] Department of Bioinformatics and Biotechnology, International Islamic University Islamabad, Pakistan
[2]Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi, Pakistan

*Corresponding author:
Email: drshaheen@iiu.edu.pk

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Abstract

Lipases hold significant position in the field of biotechnology due to its ability to catalyze esterification, interesterification, and transesterification reaction in nonaqueous media. In the present study four different lipase producing bacteria were isolated from effluents of leather, marble and textile industry. All the strains (L9, L15, L16 and L20) isolated were gram negative and subjected to both biochemical and molecular characterization. BLAST results for 16S rDNA sequences revealed that L9 (KJ748674) strain had 99% similarity with *Acinetobacter lwoffi*. L15 (KJ748675) and L16 (KJ864927) strains showed 98% and 99% sequence homology with *Acinetobacter junni* and strain L20 (KJ873872) belongs to *Alishewanella agri* sp. For enzyme production studies, pH and temperature were optimized on two different enrichment media. Comparison of two different enrichment media showed that medium containing peptone, yeast extract, NaCl and olive oil gave optimum production for all the strains. *Alishewanella agri* L20 (isolated from textile industry effluent) had growth at all temperatures (30, 37, 42 and 45°C) in medium containing yeast extract, NaCl and olive oil. In comparison to all the reported strains of the present study, *Alishewanella agri* (L20) had maximum enzyme production of 17 U/ml at elevated temperature of 45°C. Partial purification and SDS-PAGE analysis showed that protein bands of lipases were ranged between 35- 66.2 kDa. It is evident that high cost of lipase has generally reduced its exploitation at industrial level. Therefore, screening and identification of these lipolytic strains will act as a significant addition to the database on lipase research and its application at industrial level.

Key Words: Lipase, Industrial effluents, *Acinetobacter junni, Acinetobacter lwoffi, Alishewanella agri*, SDS-PAGE

INTRODUCTION

Life is a well organized form of chemical reactions. In order to sustain life nature has designed catalyst for slowly proceeding reactions. These natural accelerators of chemical reactions referred as "Enzymes" which, facilitates different chemical processes in all forms of life (from bacteria to man). Although nature has designed these enzymes to carry out biochemical reactions at a faster pace but exploitation of their properties at commercial scale is due to retention of their catalytic ability even after extraction from the living organism [1]. Rapidly increasing demand of industrial enzymes, low stability and prolonged biodegradability of synthetic enzymes shifts the focus of researchers towards microbial production of enzymes. Microbial enzymes are generally preferred over animal or plant derived enzyme because of its variety in catalytic activity, stability, high yield, regular supply and involvement of relatively less expensive media [2].

Industrially important enzymes include protease, amylase, lipase, pectinase, cellulase and xylanase. Protease and amylase have dominated the world market because of their hydrolytic potential and industrial applications. However, the explorations of catalytic activity of the most dynamic enzyme lipase have shifted the industrial fronts and research attention towards its immense utilization [1]. In biotechnological applications, lipolytic enzymes constitute the most important group of biocatalyst that facilitates the process of biopolymers, biodiesel, flavoring compounds (for shelf prolongation), agrochemicals (pesticides, insecticides), enantiopure pharmaceuticals (naproxen, ibuprofen) and oleo chemical (oil and fat hydrolysis) industries [3].

On the basis of substrate specificity, lipolytic enzymes are divided into two categories i.e. non specific esterases and lipases [4]. Lipases (triacylglycerol acylhydrolase) catalyze the hydrolysis of carboxylic ester bonds of water insoluble lipids at oil water interface and results in the formation of mono-, and di-acylglyceride, glycerol and free fatty acids [5]. Whereas esterase are specialized for hydrolytic activity of carboxylic ester bonds in water soluble lipid substrate [6].

Advances in lipase-based technologies for the synthesis of novel compounds have rapidly growing the use of lipases in all the technical industries. Modern food industries exploit lipase to improve the shelf life, flavoring and rheological properties of fruit juices, soups, sauces, cheese and baked foods [7]. Detergent industries generally use lipase for removal of fatty stains of oil [2]. In paper industry pulping rate is enhanced by use of lipase, as it removes different hydrophobic and lipid fractions of the wood. In paper recycling it helps to remove lipid stains of paper [8]. Lipase catalyzed polyester synthesis provides best opportunity for conducting environment friendly Green polymer chemistry [9]. All these properties of bacterial lipases are becoming of greater interest as biotechnological catalyst. Lipase biotechnology also opens a new avenue in bioremediation as; it accelerates the remediation process of the lipid containing municipal and industrial waste [10].

Industrial effluents are continuously adding profuse amount of pollutants in our environment but at the same time it provides harsh environment for local inhabitants of microbial community. Therefore, bacteria growing at such contaminated sites could also tolerate high physical and chemical parameters of industrial processing. Considering

the demand of industrial lipase this study is carried out for isolation, characterization and optimization of lipase producing bacteria from different industrial effluents of Pakistan.

MATERIALS AND METHODS

Sampling of Industrial Effluents

Effluent samples from different industries of Islamabad, Sialkot and Faisalabad were collected in May 2012. Marble industry effluent was taken from Iram Marble Industry located in (I-9) industrial area of Islamabad. Effluent of leather industry was taken from Sahab Khan Tannery, Malky, Sialkot. Textile industry effluent was collected from Faisal Fabrics located near Jaranwala road (Faisalabad). All effluent samples were collected directly from the site of their discharge from above mentioned industries.

Isolation and Screening of Lipase Producing Bacteria

Isolation of lipase producing bacteria was done by plating effluent samples on Tween (1%) supplemented nutrient plates. The plates were incubated at 37ºC for 24 hrs and colony forming units (CFU) were counted for each effluent sample. Colonies that showed prominent growth and were also morphologically different were selected for lipolytic character. Qualitative screening of lipase producing bacteria was carried out by the method described by Sierra (1957) [11]. The diameter of lipolytic zone was measured because hydrolysis of lipid substrate results in the appearance of opaque halo zones of calcium oleate.

Biochemical Characterization

Standardized biochemical characterization of isolated lipolytic strains was performed by using API 20 E kit. Both tubes and cupules were filled for CIT (citrate utilization), VP (Voges Proskauer), and GEL (gelatinase) test. Only tubes were filled for other tests. For ADH (arginine dihydrolase), LDC (lysine decarboxylase), ODC (ornithine decarboxylase), H2S production and URE (urease) test anaerobic conditions were created by overlaying tubes with sterile mineral oil. Results were recorded on the basis of color change after 18-24 hrs of incubation at 37°C. For TDA (tryptophan deaminase), VP, nitrate production and nitrate reduction test results were recorded by adding their reagents provided with the kit. Standard protocols were followed for oxidation fermentation, oxidase and MacConkey´s agar test.

Molecular Characterization

Genomic DNA were extracted by CTAB protocol [12]. For all the strains, 16S rRNA gene was amplified by universal primers (27F, 1492R). PCR was performed in a reaction volume of 50 µl consisting of 10X PCR buffer (5.0 µl), $MgCl_2$ (1.5 mM), dNTPs mix (0.2 mM), (0.4 µM) of 27F, (0.4 µM) of 1492R primer, Taq Polymerase (1.5 U), DNA (5 µl). PCR amplification was performed with 30 cycles following denaturation at 94°C for 1 min, annealing at 55ºC for 1 min and extension at 72°C for 2 min [13]. Purified samples were sequenced in ABI 3130 Genetic Analyzer. Sequences were then submitted to Genbank and accession number was assigned for each strain.

Lipase Production and Optimization

Optimization experiments were carried out in two steps. First, inoculum was prepared in tryptic soy broth (TSB). The medium (TSB) was inoculated with one loopful of 24 hrs fresh culture and then incubated at 37°C for 24 hrs. After overnight incubation lipase production medium was supplemented with 1% inoculum. Two different media were used for lipase production experiments. Enrichment medium I comprised of peptone (3.0 gm), yeast extract (1.0 gm), NaCl (0.5 gm) and Olive oil 1% per 100 mL [14]. Enrichment medium II consists of tryptone (6.0 gm), yeast extract (2.0 gm), $CaCl_2$. $2H_2O$ (0.2 gm), $MgSO_4$. $7H_2O$ (0.1 gm), $FeCl_3.6H_2O$ (0.4 mL of 1% stock solution) and 1% olive oil per liter [15]. Crude enzyme was taken for quantitative estimation after every 24 and 48 hrs of incubation.

Physical parameters (pH and temperature) were optimized by changing one variable at a time, while keeping others constant. Two different enrichment media at different pH (6.0, 7.0, and 8.0) and temperature (30, 37, 42, and 45°C) were tested for optimization experiments. Negative control and triplicates of all the experiments were performed for accurate quantitative determination of lipase through lipase assay.

Lipase assay was carried out by standard titrimetric method using olive oil as lipid substrate [16]. The amount of enzyme required to liberate 1 µmol of fatty acids from triglycerides is defined as one standard unit (U) of lipase enzyme [17]. Following formula is applied for estimation of enzyme quantity.

$$\text{Lipase activity} = \frac{(V_S - V_B).\ N.\ 1000}{S}$$

V_S = Volume of 0.05M NaOH solution consumed by enzyme-substrate reaction mixture (ml)

V_B = Volume of 0.05M NaOH solution consumed by control-substrate reaction mixture (ml)

N = Molarity of NaOH used for titration (0.05M)

S = Volume of substrate cocktail solution (5ml)

Partial Purification of Lipase

Lipase precipitation was carried out by ammonium sulphate [$(NH_4)2SO_4$]. Solid [$(NH_4)2SO_4$] was added to crude enzyme solutions at concentration of 20, 40, 50 and 60%. After dissolving salt in crude enzyme precipitates were collected by centrifugation at 10,000 rpm for 15 min. Precipitates were dissolved in 20mM Tris-HCl buffer of pH 7.0. Molecular weight of partially purified enzyme was determined by SDS-PAGE [18].

RESULTS AND DISCUSSION

Modern research exploits extracellular lipase, produced by a wide variety of organisms from different environmental sources [19]. Oil mill effluent, sewage and damp places provide favorable environment to flourish lipase producing bacteria [20]. In the present study lipase producing gram negative bacterium (L9) was isolated from effluent of leather industry and showed lipolytic zone of 11 mm on screening media. Another gram negative cocci (L20) isolated from textile industry effluent when streaked on Tween supplemented agar plate had hydrolytic zone of 16.0 mm. Irrespective of lipid content in microenvironment, few strains have the ability to produce lipase when grown in presence of any lipid substrate. Same is the case of marble industry effluent, it is not a good lipid source but in comparison to the other strains lipase producing L15 and L16 strain isolated from effluent of

marble industry had maximum hydrolytic zone of 21mm on screening media (fig. 1).

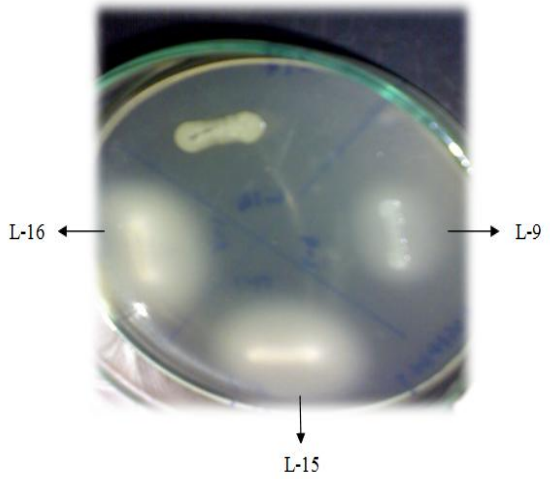

Fig. 1. Hydrolytic zones of lipase producing strains (L9, L15 and L16).

Biochemical and molecular characterization of strains showed that lipase producing strains L9, L15, L16 and L20 have maximum sequence similarity with *Acninetobacter lwoffi*, *Acinetobacter junii* and *Alishewanella agri* respectively (Table 1). *Acinetobacter* lipases have a wide range of applications in the hydrolysis, esterification, and transesterification of triglycerides, and in the synthesis of selective esters [21].

Table 1 Biochemical characterization of strains.

S. No.	Biochemical tests	L9	L15	L16	L20
1	Ortho nitrophenyl-ß galactopyranosidase	-	-	-	-
2	Arginine dihydrolase	-	-	-	-
3	Lysine decarboxylase	-	-	-	-
4	Ornithine decarboxylase	-	-	-	-
5	Citrate utilization	-	-	-	-
6	H_2S production	-	-	-	-
7	Ureaease	-	-	-	+
8	Tryptophan deaminase	-	-	-	-
9	Indole production	-	-	-	-
10	Voges Proskauer	-	-	-	-
11	Gelatinase	-	-	-	+
12	Glucose fermentation	-	-	-	-
13	Mannitol fermentation	-	-	-	-
14	Inositol fermentation	-	-	-	-
15	Sorbitol fermentation	-	-	-	-
16	Rhamnose fermentation	-	-	-	-
17	Saccharose fermentation	-	-	-	-
18	Melibiose fermentation	-	-	-	-
19	Amygdalin fermentation	-	-	-	-
20	Arabinose fermentation	-	-	-	-
21	Nitrate reduction	-	-	-	+
22	Reduction to N2	-	-	-	-
23	MacConkey agar test	+	+	+	+
24	Oxidation fermentation	-	-	-	-
25	Cytochrome oxidase	-	-	-	+

+ = Positive
- = Negative
+A = Growth with acid production

Variation in enzyme production is due to difference in the ability of bacteria to tune the metabolic activities and growth according to physiological conditions [22]. Lipase production of *Acinetobacter lwoffi* (L9) was maximum (11.5±0.5 U/ml) at pH 8 and 6 in enrichment medium (I) containing olive oil and yeast extract. But at pH 6 maximum enzyme was produced at the temperature of 37°C and in alkaline pH (8) maximum enzyme production was at 30°C (Fig. 2). Results of the present study were in consistence with that of the *Bacillus subtilis* isolated from oil contaminated soil that showed maximum enzyme production at 37°C in enrichment medium containing yeast extract and olive oil [23]. Relatively little enzyme was produced by L9 strain at all pH (6.0, 7.0, and 8.0) and temperatures (30°C, 37°C) in enrichment medium (II) containing tryptone, $CaCl_2$, $MgSO_4$, $FeCl_3$ and olive oil.

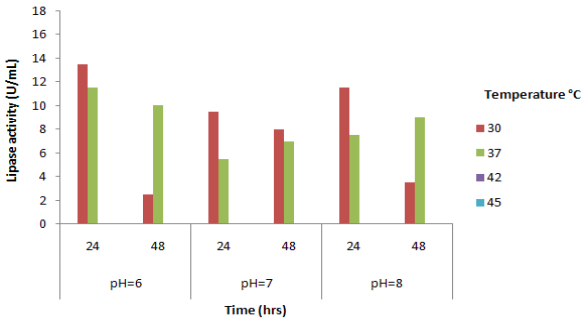

Fig. 2. Optimization of pH and temperature for lipase (U/mL) production of L9 in enrichment medium (I).

Most of the reported data have shown that natural oils are most efficient lipid carbon source to stimulate lipase production [24, 25]. Olive oil, sunflower oil, and soy oil used as carbon source were reported to increase lipase production [26]. Therefore present studies were carried out by using olive oil as lipid substrate in two enrichment media. *Acinetobacter junni* (L15) isolated from marble industry effluent produced maximum lipase (10 U/ml) in enrichment medium (I) at pH 7.0 and 37°C. This strain was unable to grow at elevated temperatures of 42 and 45°C in yeast and olive oil containing medium. Enzyme production was comparatively low at 7.0 and 8.0 pH but still 9 U/ml of lipase produced with standard error of 0.70 at pH 6 and 42°C (Fig. 3). Optimum temperature and pH for enzyme production of *Acinetobacter junni* L16 strain was 30°C and 6.0 respectively as shown in figure 4. In this study *Alishewanella agri* (L20) isolated from textile industry effluent had maximum enzyme production of 15 U/ml at 30°C. Comparison of two production media deduced that yeast extract, olive oil medium and metal salt based medium enhanced the lipase production. Presence of $FeCl_3$ or $CaCl_2$ in enrichment medium (II) might inhibit the enzyme production. In comparison to all the reported strains of the present study, *Alishewanella agri* (L20) had maximum enzyme production of 17 U/ml at elevated temperature of 45°C and pH 6.0 (Fig. 5).

In present study all the four strains had different pattern of enzyme production at different temperatures. Optimum temperature for L9 and L16 was 30°C and for L15 and L20 it was 37°C and 45°C, respectively. pH of the culture medium also plays a critical role in maximizing the enzyme production through maximizing transport of nutrient components across the cell membrane. Maximum lipase activity of *Bacillus* sp. at pH 7 was reported by [27]. Increase of pH from 4 to 8 also resulted in increase of lipase production by *Serratia rubidaea*. Alkaline pH is reported to reduce the enzyme production from 3.83 to 2.71 U/ ml [28]. Acidic pH (6.5-7) is preferred by *Serratia*

marcescens for lipase production [29]. In consistence with the present study strain L9, L15, L16 and L20 also preferred acidic pH and had maximum lipase production at pH 6.0. Acidic lipase plays a significant role in pharmaceutical industry and in the bioremediation of food and industrial wastes [30].

Different salt concentrations (20, 40, 50 and 60%) were used for partial purification of lipase from crude enzyme. SDS-PAGE showed that partial purification at 60% salt concentration purified two protein bands as shown in figure 6. Protein bands of lipases were ranged between 66.2 to 35 kDa. Among different tested saturations 60% saturation was mostly reported to be effective concentration for purification of lipase from *Bacillus pumilus* RK31, *Pseudomonas* sp. and *Thermosyntropha* [31,32].

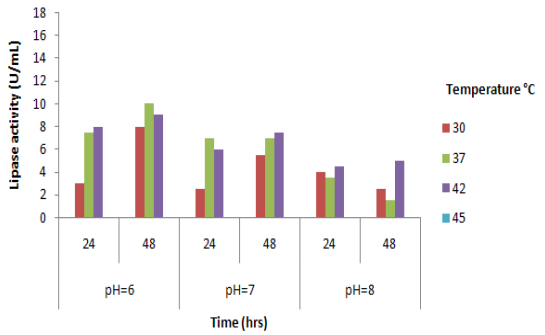

Fig. 3. Optimization of pH and temperature for lipase (U/mL) production of L15 in enrichment medium (II).

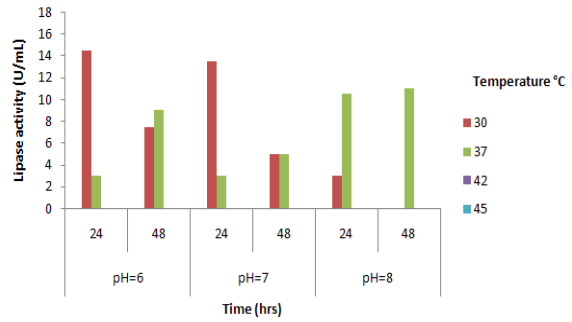

Fig. 4. Optimization of pH and temperature for lipase (U/mL) production of L16 in enrichment medium (I).

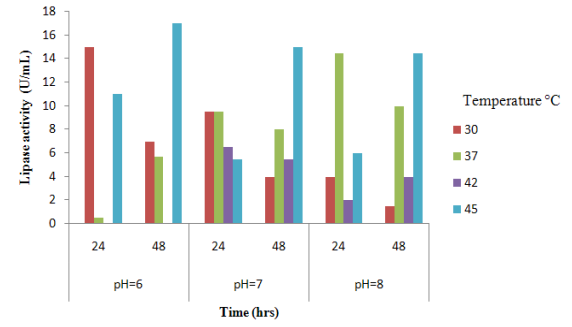

Fig. 5. Optimization of pH and temperature for lipase (U/mL) production of L20 in enrichment medium (I).

CONCLUSION

Isolation of these lipase producing bacteria from effluents of different industries indicate that industrial effluents act as rich source for potential microbes. Characterization of lipase produced by the above mentioned strains will further act as significant addition to lipase technology. Optimization experiments for lipase production showed that all the strains of *Acinetobacter lwoffi*, Acinetobacter junni, and *Alishewanella agri* gave maximum enzyme production at acidic pH (6.0) and acidic lipase always played a significant role in pharmaceutical industry.

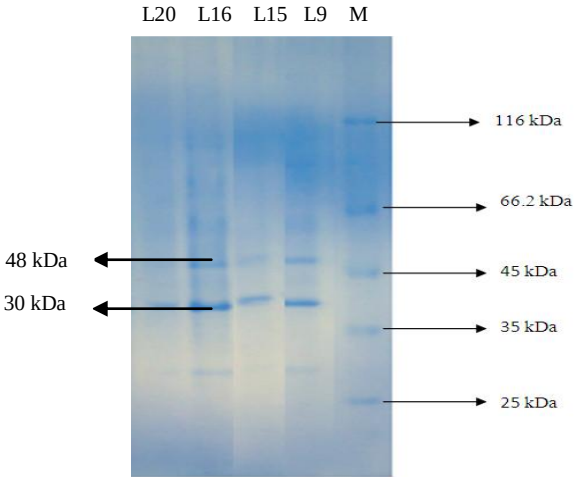

Fig. 6. SDS-PAGE of partially purified lipase from L20, L16, L15 and L9. Last lane (M) comprises of Fermentas protein marker (14.4 kDa to 116 kDa).

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