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Biochemical Characterization of Lipase Produced by *Bacillus* spp. Isolated from Soil and Oil Effluent

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Abstract

The aim of the present work was to isolate *Bacillus* spp. With high lipase activity; to characterize the isolates using both biochemical and molecular methods; to produce lipase using *Bacillus* isolates and to study the biochemical and biophysical characteristics of the produced lipase. Sixty five Bacillus isolates were isolated from soil 20 isolates from guar field soil (G), 15 isolates from Abusabein field soil (B), 15 isolates from sun flower field soil (S) and 15 isolates from oil effluent (O). Lipase producing isolates were screened; a Chromogenic plate's method was used. Enzyme activity was quantitatively assayed. Lipase production under submerged fermentation (SMF) conditions using a production medium that contained metal salts, Tween-20 and olive oil as substrate at different period 24, 48, 72 and 96 h, the optimum pH, temperature for lipase activity was determinated and kinetics as well. The isolates showed the highest lipase activity which was identified as *Bacillus* sp. The optimum pH, temperature, thermostability and kinetic of the produced enzymes were found in three isolates G14, O1 and B10 with the highest enzyme activity and best stability. The isolates G14, O1 and B10 revealed the highest lipase activity of 63.4, 41.2 and 28.3 U/ml, respectively. The results showed optimum pH of the lipase activity from isolates G14, O1 and B10 8.0, 6.0 and 6.0 and the optimum temperature 40, 60 and 75˚C, respectively. Lipase enzymes from isolates O1 and B10 were found to be more thermostable after incubation time for 120 min at 90˚C. The V_{max} and K_m values of lipase for isolates G14, OI and B10 were 17.6, 135 and 24.4 μmole·min^{-1} and 1.3, 1.6 and 0.681 mM, respectively. According to these results *Bacillus* spp. with high lipase activity and thermostability can be used to promote food, pharmaceuticals, paper, detergents agrochemicals industries and pollution control in Sudan.

Keywords

Bacillus, Lipase, Biochemical Characterization, Oil Effluent

1. Introduction

Lipases occur widely in nature, and microbial lipases such as bacterial lipases, fungal lipases and mold lipases are more favorable because of their low cost in production, greater stability and availability than plant and animal lipases. The lipases primarily carried out from fungi, molds or bacteria; and most of lipases formed extracellularly [1]. Lipases are considered to be the third biggest enzymes group following proteases and amylases, based on total sales volume. Because of its extensive range of applications lipase production is a billion dollar business [2]. *Bacillus* lipases have attracted much attention because of their biotechnological potential, which has led to the isolation of several lipolytic enzymes of *Bacillus subtilis* and other species of the genus *Bacillus, Geo-bacillus* and *Paenibacillus* [3]. Many applications of lipases include specialty organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures, and chemical analyses [4].

Lipases (Glycerol ester hydrolases E.C. 3.1.1.3) are much-demanded enzymes with significant commercial applications in industries. Lipases stimulate the hydrolysis of triacylglycerols to glycerol and free fatty acids. A real lipase will cleave emulsified esters of glycerin and lengthy chain fatty acids such as triolen and tripalmitin [5]. Lipases are widely used in the processing of fats and oils, detergents and diaries processing, the synthesis of fine chemicals and pharmaceuticals, paper industries and production of cosmetics [6]. Lipase-producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc. [7]. Microbial lipases detoxify and degrade the oil effluents as one by innovative technologies [8]. The present work aimed at Screening of *Bacillus* spp. producing lipase by using phenol red plate agar assay, identifying the isolates by using both biochemical and molecular methods (RAPD analysis), and to characterize the biochemical properties of the lipase.

2. Materials and Methods

2.1. Sampling

Fifty Soil samples were collected from Khartoum University farm in Shambat (Rhizosphere depth 10 cm of guar, Abusabein and sun flower fields), the soil was alkaline; pH 8.5 and was salty clay. In addition 15 samples were collected from effluent oil at the industrial area Omdurman. Each sample was placed into sterilized plastic package and transferred to laboratory and they kept at room temperature.

2.2. Isolation of *Bacillus* Isolates

Isolation of *Bacillus* was carried out according to [9]. 10 g from each sample were taken and dissolved in 90 ml distilled water. Soil suspension was heated at 80˚C for 15 minutes. The five dilutions of soil and effluent oil were made (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}). And 6 plates of nutrient agar for each sample were inoculated (by the spread plate method) with 0.1 ml of heated dilutions (10^{-3}, 10^{-4},

10^{-5}) and incubated aerobically in 37˚C for 24 hours. Then colonies were purified and prepared for the further work.

2.3. Conventional Identification of *Bacillus* Isolates

Identification of pure culture of each *Bacillus* isolates was carried out according to [10] [11].

2.4. Molecular Characterization of Bacterial Isolates

Molecular RAPD Technique

DNA extraction from *Bacillus* isolates was carried out using a CTAB based protocol described by [12]. Classification of the isolates at the molecular level was carried out using RAPD-PCR technique according to [13]. By three Primer, (1) OPL3 (5-CCA GCA GCT T-3), Primer (2) OPL8 (5-AGC AGG TGG A-3), and Primer (3) OPQ1 (5-GGG ACG ATG G-3).

2.5. Screening for Lipase Activity by Phenol Red with Olive Oil Plate Agar

Typical *Bacillus* isolates was screened for producing lipase enzymes. Using chromogenic plate's assay, which was prepared as followed by [14]. using phenol red (0.1%) along with 1% lipidic substrate olive oil, 10 mM $CaCl_2$ and 2% agar pH is adjusted to 7.3 - 7.4 using 0.1 N NaOH. Then, the plates were incubated with young culture 24 h from the different isolates then incubation for 7 days at 37˚C. After that the plates should be examined for change in color from pink to yellow.

2.6. Lipase Enzyme Productions

The enzyme was produced from the purified isolates that showed a positive reaction on olive oil/phenol red plates test. The tested strains were cultured at 37˚C in 100 ml Erlenmeyer flasks containing 50 ml of, the broth of production media contained peptone 0.2%, $NH_4H_2PO_4$ 0.1%, NaCl 0.25%, $MgSO_4{\cdot}7H_2O$ 0.04%, $CaCl_2{\cdot}2H_2O$ 0.04%, Olive oil 2 ml and Tween-20 2 to 3 drops. One loop full of bacterial culture was inoculated in each flask and incubated at 37˚C. The pH was adjusted at pH 7. Lipase was extracted according to [15], from the production medium after incubation period (24, 48, 72 and 96 h) by centrifugation at 6000 rpm for 10 min.

2.7. Assay of Lipase Enzyme

Lipase activity was assayed according to [16], with slightly modification, using p-nitro phenyl palmitate (p-NPP) as substrate. Enzyme solution (50 µl) was added to 950 µl of the substrate solution consisting of one part solution A (3.0 mM pNPP in 2-propanol) and nine parts solution B (100 mM potassium phosphate buffer pH 7.0, 0.4% Triton X-100 and 0.1% gum arabic), which was freshly prepared before use. The reaction mixture was incubated at 37˚C for 20 min and absorbance was read at 410 nm. Lipase activity and specific activities was calculated as the following formula:

$$\text{Lipase activity}\left(\text{U/ml}\right)=\frac{A*B}{C*D*E} \quad (1)$$

where,

A—μmol of p-Nitro phenol released;

B—Total volume;

C—Volume used in spectrophotometric determination;

D—Volume of enzyme used in assay;

E—Time of incubation.

2.8. Characterization of Lipase Enzyme

2.8.1. pH Optimum

Substrate solution was prepared in 100 mM phosphate buffer at pH 4, 5, 6, 7 and 8 according [7]. The reaction mixture consisted of 50 μl enzyme extract and 950 μl of substrate solution, before incubation the mixture adjusted to desired pH by using HCl or NaOH. The reaction mixture was incubated for 20 min at 37˚C and then the optical density (O.D) was determined at 410 nm.

2.8.2. Temperature Optimum

For determining the optimum temperature [17]; with slightly modification the reaction mixture was prepared as described above. The pH mixture corresponded to determined optimum pH of the respective enzyme. The mixtures were incubated for 20 min at each of the following temperature; 40˚C, 45˚C, 50˚C, 55˚C, 60˚C, 65˚C, 70˚C, 75˚C, 80˚C, 85˚C, or 90˚C, after that lipase activity was determined as described before.

2.8.3. Determination of Thermostability

The thermostability was determined in aqueous solution [17]. With slightly modification, The lipase preparation were pre incubated at 50˚C, 60˚C, 70˚C, 80˚C, or 90˚C for 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, or 120 min after pre incubation periods, the samples were cooled and they were incubated at optimum temperature determined of the respective enzyme and the activities of enzymes were estimated as described before.

2.9. Enzyme Kinetics

Michaelis-Menten constant for lipase substrate pNPP was determined according to [18]. With some modification pNPP instead pNPL (p-nitropheny laurate) and concentration by incubating with concentrations of substrate ranging from 0.05 to 5.0 mmol/l with lipase from three have chosen isolates. The value of constant was calculated according to the Hanes-Woolf plot.

3. Results and Discussion

3.1. Screening for Lipase Activity by Phenol Red with Olive Oil Plate Agar

AII isolate subjected to chromogenic plates assay; isolates were showed a positive

result to the test by convert the color of medium from red to yellow indicate that lipase was produced by different *Bacillus* strains. The free fatty acids liberated by the lipolysis organisms lower the pH resulting in the change of color indicating the formation of lipase [19]. Therefore the isolates were found to be a lipase producer, capable of utilizing the substrate (olive oil) to produce yellow color zone. This result agreed with [20] [21], they proved that the use of phenol red in lipase assay was highly reproducible with sensitivity level as low as 0.5 p-nitrophenyl palmitate (p-NPP) enzyme units within 15 min. Therefore, by using olive oil as the lipidic substrates in phenol red agar, the presence of lipolysis activity could be indicated by the yellow coloration. This assay is based on the principle where free fatty acids were released from the bacterial lipolysis reaction. Lipase from *Bacillus* isolates was produced in liquid production medium. As a more accurate, quantitative method to support chromogenic plates assay for use in screening for *Bacillus* producing lipase. The lipase activities of isolates ranged between 3.4 U/ml (isolate O3) to 63.4 U/ml isolate G14 (**Table 1**). Among the tested isolates, three isolates B10, O1 and G14 showed high potential in Lipase production and had an average lipase activity of 28.3, 41.2 and 63.4 Unit/ml, respectively.

Table 1. Lipase activity and optimum incubation periods of different isolates.

Isolates	Sources	Lipase activity (U/ml)	Optimum time of incubation (hour)
S1	Sun flower Soil	8.8	24
S2	Sun flower Soil	39.9	72
S3	Sun flower Soil	35.2	72
S5	Sun flower Soil	35.2	72
S12	Sun flower Soil	12.6	96
B4	Abusabein Soil	27.0	48
B7	Abusabein Soil	61.2	48
B8	Abusabein Soil	16.4	72
B10	Abusabein Soil	28.3	72
O1	Oil waste	41.2	72
O2	Oil waste	26.9	48
O3	Oil waste	3.4	24
O4	Oil waste	12.8	72
O9	Oil waste	23.6	48
O12	Oil waste	5.7	24
G2	Guar Soil	28.7	72
G4	Guar Soil	52.4	48
G5	Guar Soil	43.6	48
G6	Guar Soil	5.9	96
G7	Guar Soil	17.4	96
G8	Guar Soil	28.2	72
G10	Guar Soil	19.8	48
G11	Guar Soil	31.1	96
G14	Guar Soil	63.4	48
G15	Guar Soil	24.4	72

3.2. Biochemical and Biophysical Characteristics

3.2.1. pH Behavior

The pH optima of lipase from isolates O1, B10 and G14 (Figure 1) were determined by exposing lipases to a range of pH conditions. The pH optima of each lipase preparations from each isolate and the pH behavior of different lipase preparation are shown in Figure 1. The pH optima of lipase from isolates (O1, B10 and G14) are found to be ranged between 4 and 9. The highest activities of lipase for each isolates were measured at pH 6.0 and 8.0. This result is agreement with the previously reports for lipase optimum pH of *Bacillus pumilus RK*31 at pH 6.0 [7], *Bacillus subtilis K-L* at pH 8.0 [22]; However change in pH has a varied effect on enzyme activity, through altering the structure of enzyme and substrate and inhibiting the catalysis of reaction [17].

3.2.2. Optimum Temperature

The optimum temperature of the lipase results were shown in Figure 2. The activities of each lipase from the different isolates after incubation with pNPP solution under different temperature degree for 20 min were mentioned in Figure 2. Isolate **O1** lipases displayed a highest activity at 60˚C while isolate **B10** and

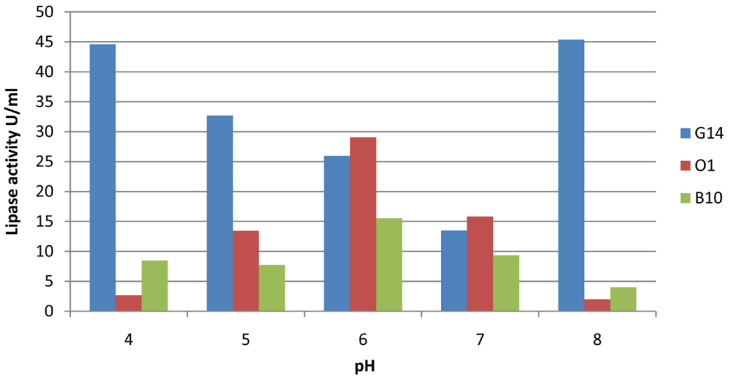

Figure 1. pH behavior of lipase preparations produced from three *Bacillus* isolates. Where, G14 = bacteria isolated from Guar field O1 = bacteria isolated from Oil effluent B10 = bacteria isolated from Abusabein field.

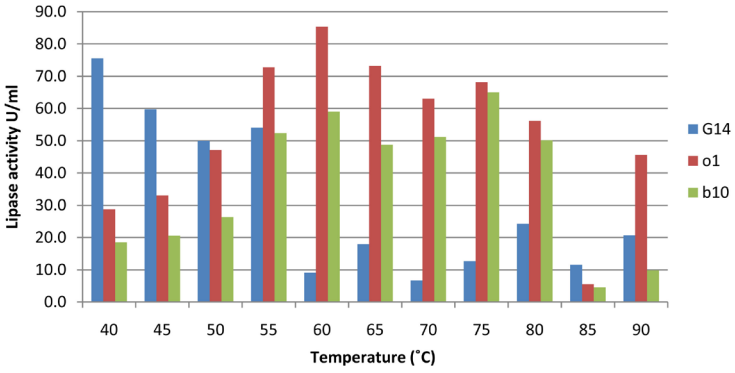

Figure 2. Temperature profile of lipase preparations produced from three *Bacillus* isolates. Where, G14 = bacteria isolated from Guar field O1 = bacteria isolated from Oil effluent B10 = bacteria isolated from Abusabein field.

isolate **G14** released their highest activity at 75˚C and 40˚C respectively. The optimum temperature for a lipase production from *Staphylococcus epidermis* (MTCC 10656) was 40˚C [23], while from *Bacillus coagulans* was 40˚C [24] and from *Bacillus* sp was 60˚C [18] [25], as the reported that the optimum temperature for a lipase production from *Bacillus* sp. H-257 and *Bacillus thermoleovorans* ID-1 was 75˚C by [26] [27]; similarity to the results of the ongoing study are in line with that of previous studies.

3.2.3. Thermostability of Lipases

The thermostability of lipases was measured in aqueous solutions at the optimum temperature of each enzyme; (see **Figures 3-5**) show the lipase activity of the preparation after exposure for different periods at 50˚C, 60˚C, 70˚C, 80˚C and 90˚C. After incubation time for 120 min at 90˚C lipase from isolate **G14** was completely inactivated, while lipase from isolate **O1** lost activity after 90 min but it retain activity within 100 min. also lost activity in 80˚C and retain activity in 90 min. lipase from isolate **B10** lost activity in 80 min at 90˚C and retain activity in 90 min. At all temperature examined three isolate showed high activities. The thermo stability of the lipase at high temperature indicates its suitability for industrial applications and commercialization, because of unique nature of protein and its thermostable nature [17].

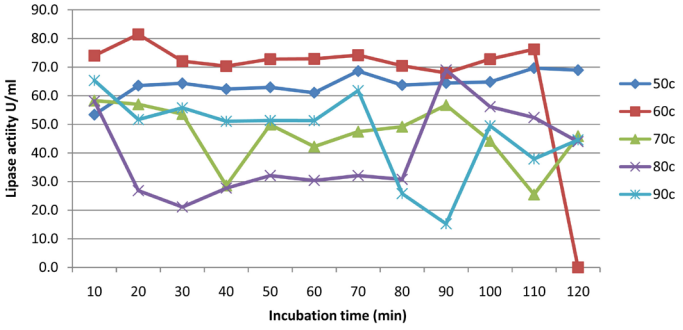

Figure 3. Enzyme activities of lipase preparation from isolate **O1** after exposure for different periods at different temperature. That data are expressed as activity at the optimum temperature for 20 min.

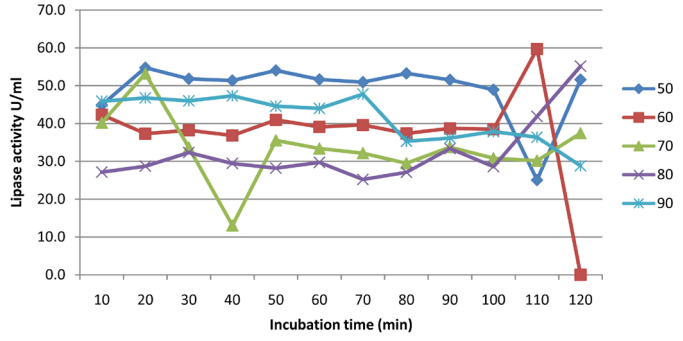

Figure 4. Enzyme activity of lipase preparation from **isolates B10 after** exposure for different periods at different temperature. That data are expressed as activity at the optimum temperature for 20 min.

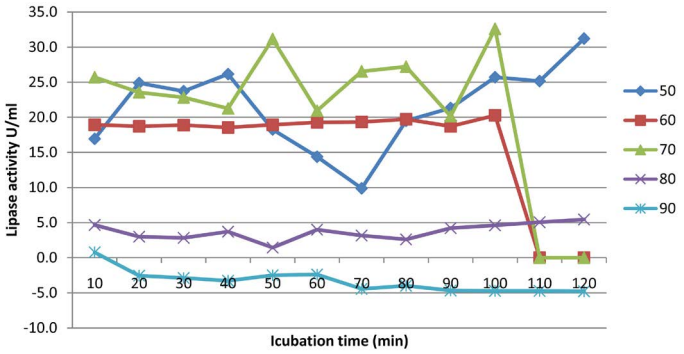

Figure 5. Enzyme activity of lipase preparation from **isolates G14** after exposure for different periods at different temperature. That data are expressed as activity at the optimum temperature for 20 min.

3.3. Lipase Kinetics

The lipase kinetics from *Bacillus* isolates O1, B10 and G14 were measured at the pH and temperature optima of the respective lipase in 0.01 M potassium phosphate buffer and in different pNPP concentration as substrate (0.05, 0.5, 1.0, 2.5 and 5 mM). The V_{max} and K_m values of lipase from isolates G14, OI and B10 were 17.6, 135 and 24.4 μmole/min and 1.3, 1.6 and 0.681 mM, respectively. [28] found that K_m and V_{max} values of the lipase purified from *Bacillus Stearothermophilus* HU1 of 0.235 mM and 161.2 mmol/min/mL, respectively, as the reported by [29] K_m and V_{max} values, were 105.26 mmol and 0.116 mmol·min^{-1}·g^{-1}, respectively from *Bacillus* sp. ITP-001 using olive oil as the substrate.

4. Conclusion

Bacillus is clearly identified using morphological, biochemical and molecular methods (RAPD-PCR). The maximum lipase activity was found to be 63.4 U/ml from isolate G14 (isolate from guar soil), after incubation period of 48 hours. The pH optimum of the isolates O1 and B10 are acidic (pH 6) and G14 is alkaline (pH 8). Isolate B10 is more lipase active at the optimum temperature of 75˚C, this will make it more suitable for producing lipase to use in detergent industry or all that industries depend on higher temperature. Isolate G14 showed the lowest stability after heat treatment in 90˚C. Lipases from isolates G14, OI and B10 have V_{max} values of 17.6, 135 and 24.4 μmole/min and Km values of 1.3, 1.6 and 0.618 mM, respectively.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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