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Molecular Characterization of Thermostable Hydrolytic Enzymes Producing Bacteria Isolated from Hot Spring of Ras Sedr, South Sinai, Egypt.

Maii Elsharayidi[1,*], Ahmed Dewedar[2] , Walid Elkazzaz[2] and Hesham Shafik[1]

[1] Botany Department, Faculty of Science, Port-Said University, Port-Said, Egypt.

[2] Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt

***Corresponding author email**: maiisaleh0@gmail.com

ABSTRACT

Amylases and lipase are among the most important enzymes nowadays in biotechnology. The current study was focused on the partial characterization of extracellular amylase and lipase enzymes produced by two potent isolates which previously isolated from hot spring, Ras-Sedr, south Saini, Egypt. Morphological, biochemical and molecular identification of these potent isolates were also performed. The study revealed that these isolates are belonging to actinobacteria of genus *Thermoactinomyces thalpophilus KCTC 9789* and bacilloid bacteria of genus *Bacillus licheniformis WSE-KSU301*. The effect of physical conditions was also investigated maximum production of the tested enzymes. Crude enzymes were extracted and subjected to different temperature and pHs to measure their effect on the activity and stability of enzymes. The data obtained proved the high stability of these enzymes to high temperature up till 85^{o}C with optimal temperature at 65^{o}C and 55^{o}C, for amylase and lipase, respectively. However, for pHs slightly acidic to neutral displayed the optimal pH for maximum activity for amylase and lipase, respectively. To increase the activity and stability of these enzymes, for industrial large scale production, further studies are in need.

Keywords

Thermophiles, Lipase, Amylase, *Thermoactinomyces thalpophilus KCTC 9789, Bacillus licheniformis WSE-KSU301*.

1. INTRODUCTION

Bioprospecting is defined as biodiversity discovery of unique ecosystems expressing their biodiversity, microorganisms and flora including commercially valuable biological and genetic resources to accomplish financial and environmental goals [8]. Focusing on ecological issues and technological innovations in biotechnology, many chemical processes are now being replaced by biocatalysts, i.e. enzymes, in different industrial applications such as food manufacturing, textiles, cleaning solvents and

printing. Many enzymes have very particular pH and temperature specifications when they're operating. We presently recognize that certain microorganisms, including extremophiles, can produce enzymes which could be stable and operate under harsh conditions [9, 29]. Microbiologists all over the globe are searching for hot springs taking an interest in their physical and chemical properties, thus creating a powerful evolutionary advantage on microorganisms [20]. Due to the extreme and frequently inaccessible habitats from which they emerge, problems in the isolation of thermophiles and consequently the development of these microorganisms under laboratory conditions presented considerable limitations for their study and thereby for relevant biotechnological an industrial applications. In the late seventies and through early nineties, many widely recognized and investigated thermophiles were isolated [28]. It is not surprising, given the usefulness of thermostable enzymes in molecular biological laboratory techniques, that they were also suggested as strong resources for industrial catalysis [30].

Therefore, the current study was focused on characterization of the potent isolates and the optimal conditions required for the target enzymes to obtain the maximum activity. Characterization of targeted enzymes, based on physiological characters, was performed.

2. MATERIALS AND METHODS

1. Source of Microorganisms

Microbial isolates were previously isolated from both sediments and water samples of the hot spring of Ras-Sedr using ATCC as cultural medium for isolation [6, 15]. The pure cultures of these isolates were then subjected to hydrolytic enzyme production screening (amylase and lipase enzymes). The most potent isolates were selected for further studies.

2. Identification techniques of the potent isolates

The most potent isolates for hydrolytic enzyme production were identified using traditional and molecular techniques. The regular identification techniques were performed based on the techniques of Bergey's manual of Determinative Bacteriology (1989). This technique was as follow:

2. a. Morphological characterization

Macromorphological and Micromorphological characterization were performed. For macromorphology, colonial shape, mycelium color (aerial and substrate mycelia) and elevation were recorded. However, for micromorphology microscope examination was carried out after being cultured on oblique slide and stained. Mycelium morphology, arrangement of conidiophores and arthrospore on aerial/substrate mycelium were observed. Meanwhile, shape of spore and their number were also reported using the oil immersion (100X). In parallel, ability for Gram stain and endospore formation was also examined. Gram reaction was carried out according to the method described by [11] and spore formation was performed based on the method of [4].

2. b. Biochemical and physiological characterization

Some biochemical and physiological tests were performed to proceed for potent isolate identification. For biochemical tests including ability to reduce nitrate, melanin pigment production, sugar fermentation, indole acid test, citrate utilization test, Vogus-proskauer test, catalase test and oxidase test, starch and casein hydrolysis were accomplished following the method of [13]. For physiological tests effect of different temperature and pHs were tested.

3. Enzyme production and enzyme extraction techniques:

For extracellular amylase and lipase extraction, the potent isolates, which displayed the greater clearance zones for each enzyme tested, were propagated on type of medium used for each and kept on fridge at 4°C until used. For crude lipase production, the suspension of the isolate was inoculated in nutrient broth supplemented with 1% tributyrin and incubated at 55 ^{o}C for 48hrs. However, for amylase production, starch broth was used in order to obtain OD 0.05 at 550 nm [2]. Subsequent to the incubation period, the broth medium used for each enzyme production was centrifuge at 3000rpm for 20 mins. The supernatant of

the centrifugation was used and the bacterial/actinobacterial cells were discarded. The supernatants, with crude enzymes, were further used for enzymes characterization after being checked for the presence of each enzyme separately.

4. Molecular identification technique

4. a.DNA extraction, PCR amplification

Genomic DNA was isolated from freshly isolates grown in nutrient agar for 24h. The extracted bacterial DNA was followed using standard methods of phenol-chloroform extraction [26], after then ethanol precipitation and TE buffer dissolving were applied. The concentration of DNA was measured spectrophometrically to ensure its purification. For 16S rRNA identification, the universal bacterial primers (**Table 1**) were used.

Table (1): Universal bacterial primer, name and sequence.

Primer name	Sequence (5'-3') :	Reference	Commercial company
B27 F	(5'-AGA GTT TGA TCC TGG CTC AG-3')	[34]	Alpha DNA®
U1492 R	(5'-GGT TAC CTT GTT ACG ACT T-3')		

The PCR reaction condition was carried in which 50 µl of the reaction system, the PCR of 35 cycles, which covers denaturation at 95 °C for 10 s, annealing at 54 °C for 30 s and extends at 72 °C for 1 min. 50 ng/µl of each PCR product was purified and used to prepare the samples which were delivered to MacroGen Company in Korea following their specifications. The sequence similarity search was performed through the http:// blast.ncbi.nlm.nih. gov/Blast.cgi site using BLAST. This was done to measure the level of homology species identified for bacteria in Genebank. In the Basic Local Alignment Search Tool (BLAST) database tracking program on the site the phylogenetic tree was constructed using the 16S-rRNA sequence ratio of other bacteria.

4.b. Molecular identification:

For the nomenclature of the potent isolates to species level the sequence similarity search was performed through the http:// blast.ncbi.nlm.nih.g-ov/Blast.cgi site using BLAS, as mentioned above. In (BLAST) database tracking program on the site the phylogenetic neighbour-joining tree was constructed using the 16S-rRNA sequence of other reference bacteria.

5. Physiological characterization of the extracted enzyme

5. a. Impact of temperature and pH on the activity of Amylase:

The relative activity of amylase at different temperatures and pHs was measured by Bernfeld assay [23]. 1 mL of crude amylase mixed with 2 mL 0.5% starch solution and 1 mL 0.1 M Tris– HCl buffer (pH 6.0) were incubated for 30 min. The reaction was terminated by adding of 2 mL DNS (3, 5- dinitro-salicylic acid), after that the mixture kept in boiling water bath for 5 min. Then, the reaction was diluted by adding 10 mL distilled water. Enzyme activity measurements were carried out spectrophotometrically at 489 nm. One unit of enzymatic activity was described as the quantity of enzyme which created 1 µ mol of sugar reduction under experimental conditions per minute [24]. For optimal temperature which will give the maximum activity will be carried as described above and the range of temperature tested was 35°C to 85°C. However, for optimal pH for enzyme activity, the assessment for the enzyme activity was done by incubating the enzyme for 30 min at the optimal temperature chosen. Enzyme activity was tested at a range of pH between 5.0 to 9.0. For different pHs selected, 50 mM of each of acetate, phosphate, Tris-HCl and glycine buffer was used to obtain pH 5, 6, 7, 8 and 9 respectively. The assessment of enzyme stability under

various degrees of pH was determined by mixing one hundred microliters of crude amylase separately with the previously mentioned buffers for an hour at 65°C.

5.b. Effect of Temperature and pH on the Activity of Lipase:

Lipase activity was measured at different temperature to evaluate the best temperature for maximum activity. The activity was measure for each temperature selected, which ranged from 35 to 75 ^{o}C, using p-nitrophenyl butyrate (PNPB) as substrate that was diluted in 10 mM acetonitrile according to the method of [21]. A final composition of 1:4:95 (v / v / v) of acetonitrile / ethanol / acid, respectively, was then applied to the ethanol and 50 mM potassium phosphate acid (pH 7.5).The crude enzyme (0.3 ml) was added to 0.9 ml of substrate solution, and then the enzyme reaction mixture was incubated at different temperatures. Enzyme activity was assessed after 15 min by measuring the absorbance at 405 nm, which reflects the amount of p-nitrophenyl (PNP) generated and distilled water was used as a blank. One unit of lipase activity is described as the amounts of enzyme that producing 1 µmol of p-nitrophenyl PNP per one min. For lipase activity measurement, a calibration curve of PNP was used. The following equation was used to calculate the enzyme activity:

Lipase activity (U/mL) =

$$\frac{[p-nitrophenol]\ \mu mol\ /\ mL \times 1}{incubation\ time\ (minute) \times dilution\ factor}$$

Relative activity is measured as a percentage of the maximum activity of enzymes under the described assay conditions. A spectrophotometric test was carried out to measure lipase activity and stability at various temperatures and pHs. The quantity of enzyme release is represented as one unit of lipase activity [21].

Lipase activity at different pHs was performed using the same technique followed previously at different temperatures. However, the temperature was fixed at the optimal temperature obtained and the pHs were variable and ranged from pH 5 to pH 9. The optimum pH of lipase enzyme was measured and recorded.

6. Statistical analysis of the results:

The variance assessment was conducted using one-way ANOVA. Using the SPPS software program, significance was calculated as $P \leq 0.05$. For three replicas the error bars reflect standard mean error.

Results and Discussion:

The potent isolates from the previous study [6] were selected for optimization the enzyme production by selecting the suitable conditions for that. The isolate with significant highest diameter for each enzyme production was chosen for the current study. Depending on the highest production activity on specific solid medium, bacteria isolate HRS8 and Actinobacterial isolate HRS17 were selected. In parallel, the study done by [15]**,** the highest enzyme producer-isolates, for lipase and amylase, were selected based on the activity on solid media. Identification of selected potent isolates, based on macro and micromorphology, being referred to belong to actinobacteria where aerial and substrate mycelia were highly distinguished with dispersed spores and the other belongs to Bacilloid form of bacteria (Fig. 1). The macromorphology for actinobacterial isolate showed compacted white aerial hyphae with yellowish white substrate mycelium. No diffusible pigment produced, single spore formed along the aerial hyphae with short stalk or sessile (Fig. 1A). However, for bacterial isolate, vegetative cells are bacilloid in form, G+ve and exist in singly or diploid (Fig 1B).

For the further identification using biochemical characterization, Table 1 showed the ability of the two potent isolates to different biochemical tests. Actinobacteria isolate showed ability for: production of melanin pigment, catalase, ability to ferment different sugar as carbon source, hydrolysis of starch and casein (Table 2). However, the rest of biochemical tests showed negative results. For bacterial isolate (HRS-b), Table 2 showed the different capability for target tests.

For identification of potent isolates based on 16S rRNA, PCR products of genomic DNA amplification of the two isolates, using the universal primers 27F and 1492R, revealed an amplicon band with the size of about 1.5kb. The The alignment sequences, at the data base of NCBI Blast (www.ncbi.nlm.n-

ih.gov/BLAST), was used to compare with those of identified genera. The results showed the highest sequence similarity species is (87.68%) of *Thermoactinomyces thalpophilus* with actinobacterial isolate (HRS-a) and of *Bacillus licheniformis* WSE-KSU301 (90.04%) for bacterial isolate (HRS-b).

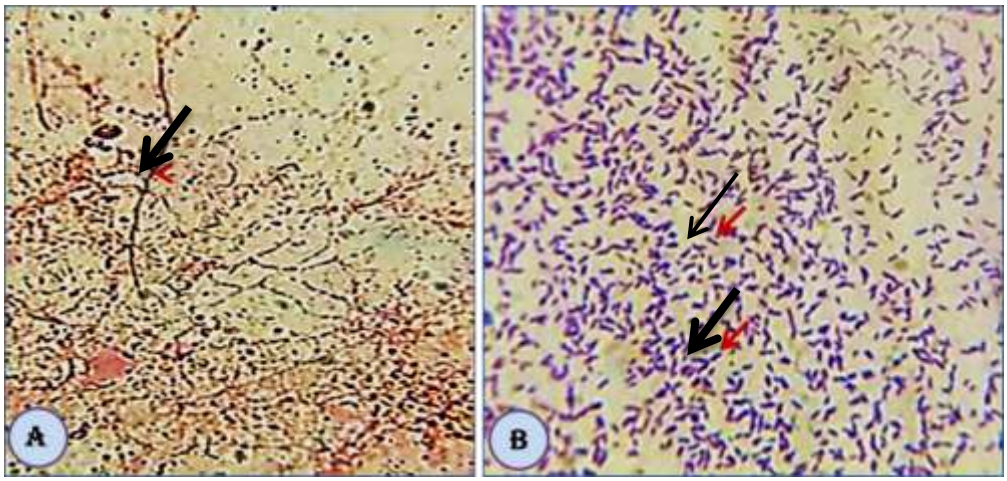

Figure 1: Micromorphology of potent isolates: A, Actinobacterial isolate (HRS-a) showing aerial mycelium (arrowhead) and dispersed spores; B, bacterial isolate (HRS-b) showing G+ve monobacilloid/diplobacilloid form (arrows) with curved tips.

The phylogenetic tree of the potent isolate (Figure 2) showed that HRS-a is closely related to *Thermoactinomyces thalpophilus* KCTC 9789 and HRS-b is closely related to *Bacillus licheniformis* WSE-KSU301. Therefore, it was proposed a name *Thermoactinomyces thalpophilus* and *Bacillus licheniformis* (Figure 2).

Evaluation the temperature influences on the activity of crude α- amylase enzyme was shown in Figure 3. The enzyme displayed a range of temperature (35-85^{o}C) in which activity was recorded. The crude α-amylase activity showed the highest value with 100% at 65°C and relative activity started to decline gradually at temperatures higher than 65°C. At 85°C relative activity reached to 40.92% of that at 65°C. However, the optimal temperature, for the maximum activity, was significantly ($p\leq 0.05$) recorded at 65^{o}C.

Concurrently, the impact of pHs showed a range of significant variation ($p\leq 0.05$) in which pH 6.0 was the optimal for enzyme activity (Fig.4). The crude α-amylase showed the highest activity with100 % at pH 6.0, while the minimum enzyme relative activity was noticed at pH 9.0 with 48.22% of that at pH 6.0. Parallel results were found by some researchers [10, 18] who stated that the highest enzyme activity was recorded at pH ranges of 6.0-7.0.

For lipase enzyme produced by our potent isolate of genus *Bacillus licheniformis*, figures 4 A and B represented the recorded enzyme activity at different temperatures and pHs. The optimum temperature for lipase production corresponds with the growth temperature of the respective microorganism was in match, where optimal temperature recorded at 55^{o}C. The crude lipase maximum activity was obtained at 55°C with 100 %. At 65°C, the enzyme relative activity became 77.23% of that at 55°C and reached to be 62.22% at 85°C. Our data are in confirmation with previous study done by [31]. In their study, they suggested that the preliminary temperature and pH of the growth media and temperature of growth both influence enzymatic activity by controlling cell membrane transport and regulating the enzyme gene expression. In parallel, a study [1] reported the maximum of lipase activity was also reported at 55°C which was in match with the maximum activity of enzyme. Further increases in the incubation temperature beyond 55°C led to a rapid decline in lipase activity (Fig 4A).

†ND, not tested.

Table: 2
Biochemical characterization of the potent enzyme producer-isolates.

Biochemical test	Potent Isolate	
	HRS-a	**HRS-b**
Nitrate reduction	-	+
Melanin pigment production	+	Nd†
Voges–Proskauer	-	+
Catalase	+	+
Oxidase	-	+
Sugar fermentation		
Glucose	+	+
Fructose	+	+
Maltose	+	+
Ribose	+	+
Mannose	+	+
Casein hydrolysis	+	+
Starch Hydrolysis	+	+

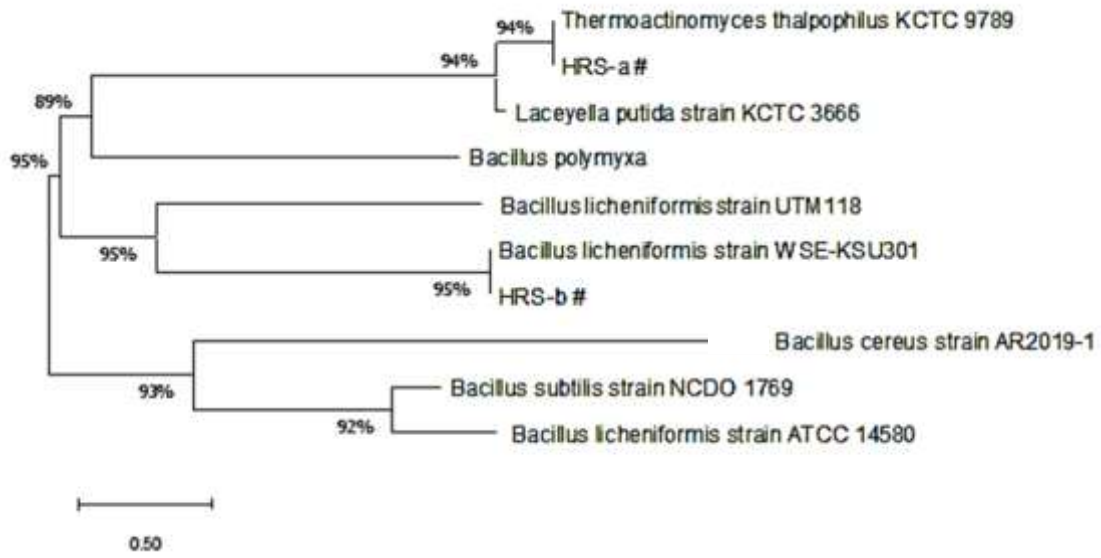

Figure 2: Phylogenetic neighbour-joining tree based on 16S rRNA gene sequences showing the close position of the HRS-and HRS-b to the identified * era with high similarity percent.

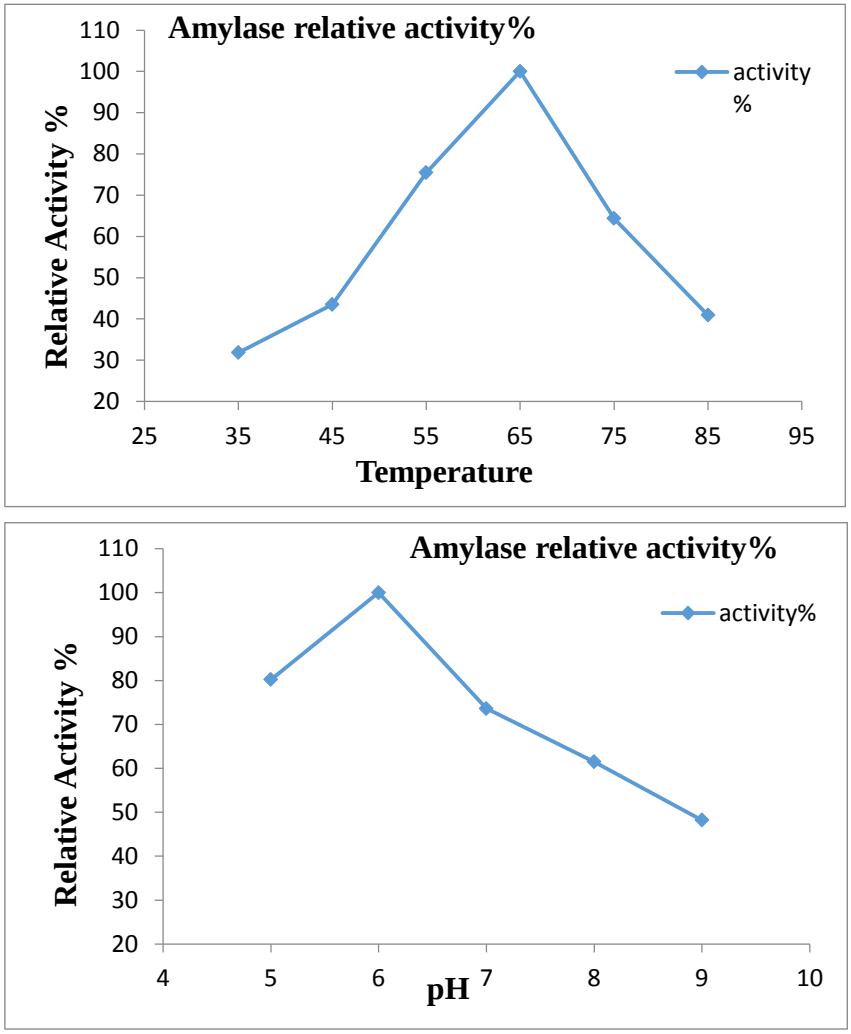

Figure 3: Effect of different temperatures and pHs on the amylase enzyme activity produced by *Thermoactinomyces thalpophilus* KCTC 9789. A, influence of temperature; B, impact of pH.

Lipase enzyme showed its highest activity with 100% at pH 7.0. While the minimum lipase relative activity was observed at alkaline pH 9.0 with 46.54% of that at pH 7.0. (Fig 4B). Although activity recorded between pH 6-7, neutral pH was the optimal for maximum enzyme activity. Reduction of enzyme activity at pH 8 and 9 was also recorded to be 55.74 and 46.52% respectively. Although the activity can be improved by further addition some catalysts like metal ions to be more convenient with industrial purposed on large scale production [22]. The same result also observed by [21] who stated that pH 7 was the optimum for lipase activity.

According to [35] thermophilic microorganisms have application in different industries including the pharmaceutical, chemical, nutrition, textile and leather applications. The most major sources of thermophilic microorganisms are the hot springs. [5] also reported a great number of thermophilic bacilli species belonging to the genus *Bacillus* has been cultured from various geothermal hot springs and identified as thermostable hydrolytic enzymes producers. In this paper, *Thermoactinomyces thalpophilus* KCTC 9789 was obtained and identified on the bases of 16S rRNA gene identification to be members of the genus *Thermoactinomyces* [٣٣]. Researchers reported that members of the genus *Thermoactinomyces* are more closely related to the genus *Bacillus* In a large scale study done by Kawasaki and his coworkers [14], they discussed the existence of *Bacillus* strains in all hot springs researched throughout the world could be

understood by the great extent of bacterial transport all over areas and continents, adapting *Bacillus* spores to severe environmental changes and meeting their basic nutritional growth needs.

3. CONCLUSION

The thermophiles studied through the current research have the ability to produce useful thermostable amylase and lipase enzymes which are greatly worthwhile for industrial applications. These local isolates have been identified which are belonging to thermophilic organisms of genus *Thermoactinomyces thalpophilus KCTC 9789* and *Bacillus licheniformis WSE-KSU301*. Therefore, the source of isolation, Ras-Sedr's hot spring, is considered as a prospective household of economically significant microorganisms which is in need for more studies to explore more useful organisms.

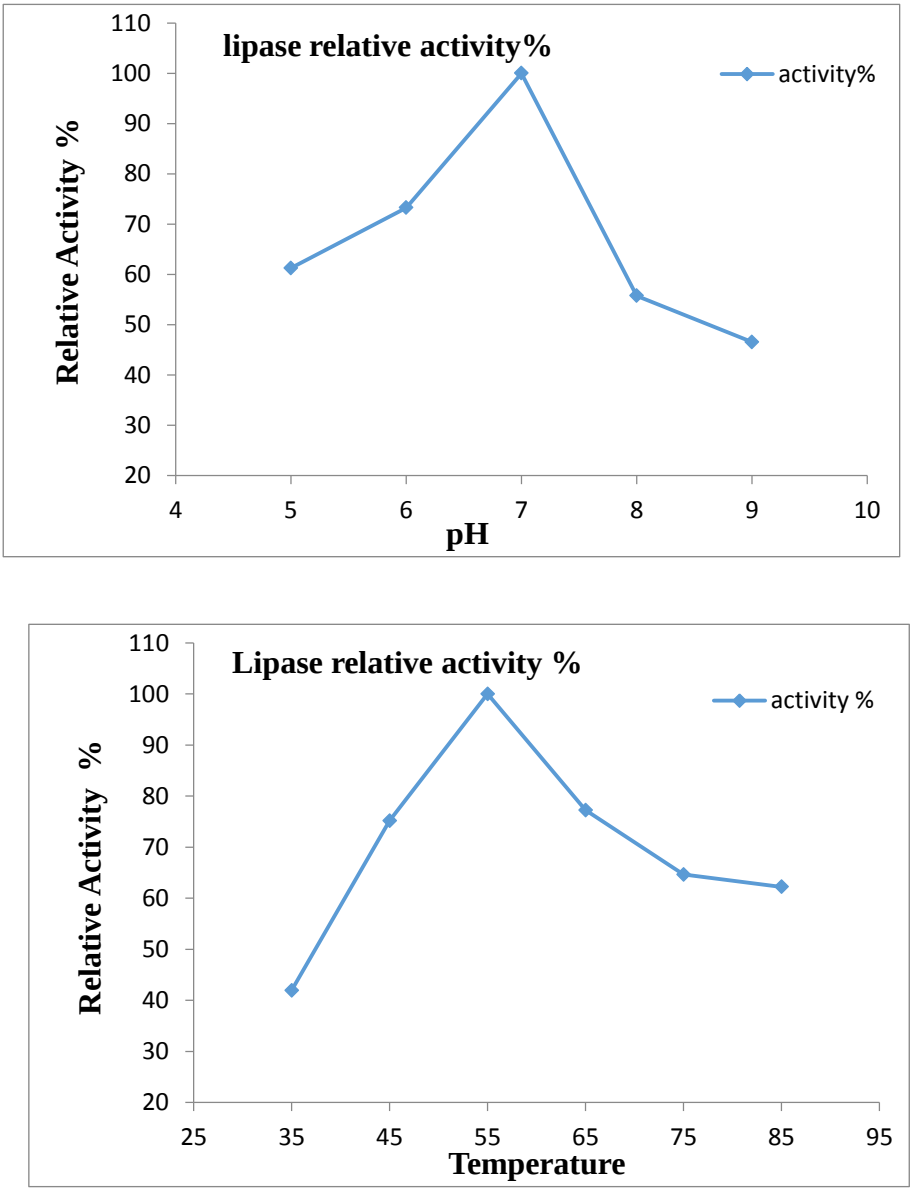

Figure 4: Effect of different temperatures and pHs on lipase enzyme activity produced by *Bacillus licheniformis* WSE-KSU301. A, influence of temperature; B, impact of pH.

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