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Research Article

ISOLATION, PURIFICATION, AND CHARACTERIZATION OF LIPASE FROM *BACILLUS* SP. FROM KITCHEN GREASE

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ABSTRACT

Objective: The objective of this work was to isolation, purification and characterization of solvent tolerant lipase from *Bacillus* sp. The objective of this work was to isolation, purification and characterization of solvent tolerant lipase from Bacillus sp. from kitchen grease for a variety of applications including organic synthetic reactions and preparation of enantiomerically pure pharmaceuticals.

Methods: Lipase producing isolates were screened from kitchen grease on a selective medium rhodamine B olive oil agar, and tributyrin agar was used to screen the lipase and esterase producing an organism, respectively. The isolate identified using 16S rDNA sequencing method and enzyme activity was quantitatively assayed. Lipase production was characterized in different conditions.

Results: The isolate showed highest lipase activity was which later was identified as *Bacillus* sp. using 16S rDNA sequencing method. The lipase was purified using ammonium sulfate precipitation. The isolate showed excellent tolerance to methanol, ethanol, acetonitrile, and moderate tolerance to butanol. The increased biomass concentration, maximum production, and activity were achieved at 37°C in 24 h incubation, then gradual reduction in production was observed. The maximum activity of lipase enzyme was obtained at pH between 6 and 9.

Conclusion: The isolate produce solvent tolerance lipase enzyme and it can be a promising candidate of solvent tolerance lipase enzyme for variety of industrial applications.

Keywords: Lipase, Esterase, *Bacillus* sp., Purification, Biomass.

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INTRODUCTION

Lipolytic enzymes (esterase [carboxyl ester hydrolases E.C. 3.1.1.1] and lipase [triacylglycerol acyl hydrolases E.C. 3.1.1.3]) are the cluster of enzyme used to hydrolyze the ester bond of triglycerides, diglycerides, and monoglycerides into fatty acids and glycerol [1]. Esterase and lipase are distinguished based on their substrates such as tributyrin and triolein, respectively. Carboxyl esterase catalyze the hydrolysis of acylglycerols with short chains of <10 carbon atoms, while lipases catalyze the hydrolyzes of acylglycerols with long chains of ≥10 carbon atoms [2]. Lipolytic enzymes are excellent biocatalyst for various reactions include hydrolysis, esterification, transesterification, acidolysis, alkalosis, and synthesis of peptides [3].

Lipolytic enzymes are produced from several microorganisms including bacteria, yeast, fungi and actinomycetes. These microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oil seeds, compost heaps, coal tips and hot springs. Among these, bacterial lipolytic enzymes are high in demand due to their specificity of the reaction, stereospecificity and less energy consumption than conventional method [5], also they are more economical, stable, easy to produce in large scale easy to manipulate the production either by genetically and environment [6]. Based on the sequence identity and biochemical properties bacterial lipolytic enzymes are classified into eight families (I–VIII), in this true lipase family belongs to Family I, GDSL family belongs to Family II, hormone-sensitive lipase (HSL), and HSL family belongs to Family III and Family IV, respectively, and Families V–VIII [7]. Several lipolytic enzymes from *Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus* sp., and *Chromobacterium* sp. have been isolated and characterized [8].

The bacterial lipolytic enzymes are mostly extracellular, and the production is greatly influenced by various factors such as carbon, nitrogen sources, pH, temperature, aeration, and inoculums size [9]. In biotechnological application and organic chemistry, the most widely used enzyme is lipolytic enzymes, they widely used in dairy industries, detergent industries, processing of fats and oils, fine chemical production, textile industries, food processing, pharmaceuticals, synthesis of surfactants, polymers, paper manufacture, production of cosmetics, leather industries, and wastewater treatment [10-12].

METHODS

Isolation of lipolytic enzyme producing organism

Kitchen grease samples were collected from kitchen to isolate the lipolytic enzyme producing an organism. 1 g of kitchen grease sample was inoculated in 100 ml of sterile distilled water and incubated at 37°C in rotary shaker incubator for 1 h. To isolate the pure culture, 0.5 ml of sample was inoculated on nutrient agar medium and incubated at 37°C for 24 h. The isolated pure cultures were subjected to screen the lipolytic enzyme producing an organism.

Screening of lipolytic enzyme producing organism

The selective medium rhodamine B olive oil agar and tributyin agar were used to screen the lipase and esterase producing organism, respectively, in this the rhodamine B olive oil agar containing: 4 g/l Nutrient broth, 2 g/l NaCl, 10 g/l agar-agar, 5 ml of olive oil, 10 g/l rhodamine B solution, and 150 µl tween 80 were used to screen the lipase producing organism and tributyrin agar containing: 5 g/l Peptic digest of animal tissue, 3 g/l yeast extract, 15 g/l agar-agar, and 10 ml/l tributyrin. The isolated pure cultures were inoculated on both rhodamine B olive oil agar and tributyin agar and incubated at 37°C for 48 h. After incubation, the orange color fluorescence producing organism in rhodamine B olive oil agar under ultraviolet (UV) light and clear zone producing organism in tributyin agar were isolated and identified by 16S rRNA sequencing method.

Identification of organism by 16S rRNA sequencing method

Isolated bacteria were identified by various morphological and biochemical test on the basis of the criteria described in Bergey's Manual of Systematic Bacteriology. Then organism was further confirmed by 16S rRNA sequencing. Genomic DNA is isolated from the bacteria, and the 16s rDNA was amplified, and the products were sequenced with Applied Biosystems 3130xl Genetic Analyzers. Phylogenetic trees were constructed using a Neighbor-Joining method using the MEGA 5.0 program [13].

Estimation of lipase activity

The lipase enzyme activity was determined using *Para*-nitro phenyl palmitate(*p*-NPP) as a substrate by the method of Winkler and Stuckmann [14]. The reaction mixture contain: 2.5 ml of p-nitrophenyl phosphate solution (mixture of solution A [0.001 g pNPA in 1ml isopropanol] and solution B [0.01 g gum arabic, 0.02 g sodium deoxycholate, 50 µl Triton X-100, and 9 ml of 50 mM Tris-HCl buffer, pH 8]) and 2.5 ml 50 mM Tris-HCl buffer (pH 8).The 1ml of cell-free supernatant was added to the reaction mixture incubated for 30 min at 37°C. During this period, the lipase enzyme hydrolyze ester bond and released free para-nitrophenol was estimated using spectrophotometer at 410 nm. One unit of enzyme corresponds to the 1 µmol of released para-nitrophenol per minute under the standard assay conditions.

Partial purification of lipase enzyme by ammonium sulfate

The partial purification of crude lipase enzyme was achieved by ammonium sulfate precipitate method described by Green and Hughen [15]. The culture broth was collected and centrifuged at 10,000 rpm for 15 min in 4°C, and cell-free supernatant containing extracellular enzyme was fractionated with ammonium sulfate to get 60%–80% saturation with continuous stirring for overnight in 4°C. Followed by the fractions were centrifuge at 15,000 rpm for 20 min in 4°C. Then, precipitates were collected and resuspended in 50 Mm Tris-HCL buffer (pH 8) and the enzyme dialyzed there times against buffer for overnight [16].

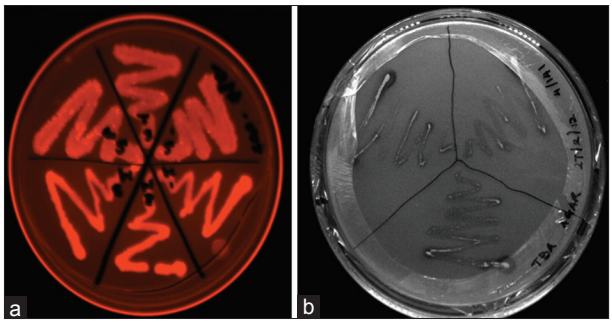

Fig 1: a) Orange fluorescence indicates the Esterase enzyme production in Rhodamine B Olive Oil Agar. b) Zone of clearance indicates the Lipase enzyme production

Optimization of lipase enzyme production

Effect of organic solvent on growth

To determine the organic solvent tolerance of lipase-producing bacteria, the different concentrations (0.5%, 1 %, 2%, and 4%) of methanol, ethanol, butanol, and acetonitrile were supplemented with nutrient broth growth media and to prevent evaporation of the organic solvent all the flask were plugged with a rubber stopper. The culture flask without solvent was taken as control. Then, all flasks were incubated in rotary shaker 37°C for 48 h, followed by growth of solvent tolerance was measured and compared with respect to control.

Effect of optimum temperature and incubation period on enzyme production

To determine the optimum temperature and incubation period on enzyme production, the lipase producing organism was inoculated in nutrient broth growth media and was incubated rotary shaker incubator at various temperatures such as 20°C, 37°C, 50°C, 55°C, and 60°C and every 6 h (6, 18, 24, 30, and 36) the enzyme production was analyzed.

Effect of temperature on growth

The analyze the various temperature (20°C, 37°C, 50°C, 55°C, and 60°C) effect on the lipase producing bacterial growth, organism was inoculated in nutrient broth and incubated at a different temperature in a rotary shaker incubator. After incubation, the biomass concentration was analyzed regularly at every 6 h intervals up to 36 h using spectrophotometer at 600 nm [17]. The carrying out the assay in triplicate and average of the experiment carried out 3 times, and the average values were taken.

Effect of pH on enzyme activity

The lipase producing organisms were subjected to grow in various pH to determine the effect of pH on enzyme activity. The tributyrin agar before the incubation of lipase-producing organism adjusted with the various pH (3, 4, 5, 6, 7, 8, 9, and 10) and incubated at 37°C in rotary shaker incubator for 48 h. Then, the culture broth was collected, centrifuged at 10,000 rpm for 20 min and in the cell-free supernatant, enzyme activity was analyzed. The enzyme activity assay repeated for 3 times and mean value was taken.

Effect of temperature on enzyme activity

To optimize the temperature on enzyme activity, the lipase producing organisms were inoculated in tributyrin agar and incubated under various temperatures (20°C, 40°C, 60°C, and 80°C) in rotary shaker incubator for 48 h. Then, the culture broth was collected, centrifuged at 10,000 rpm for 20 min and in the cell-free supernatant enzyme activity was analyzed. The enzyme activity assay repeated for 3 times and mean value was taken.

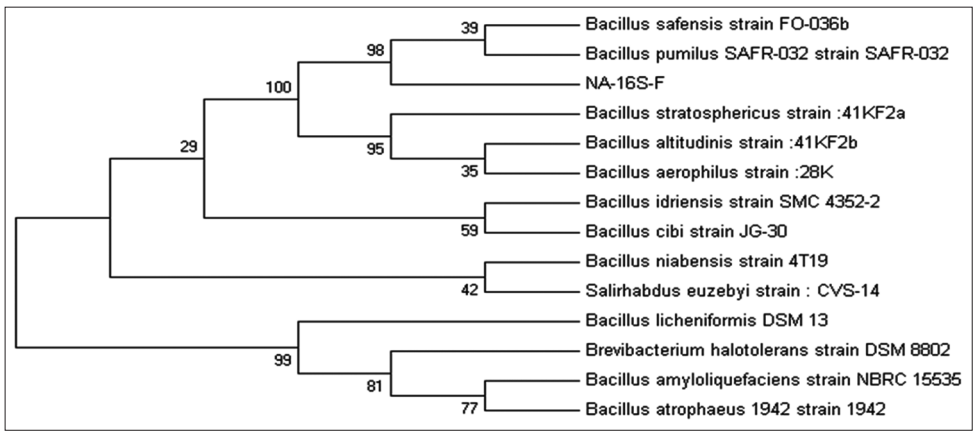

Fig 2: The phylogenetic tree of *Bacillus* MM03 and other close homology microbes

RESULTS AND DISCUSSION

Screening of lipolytic enzyme producing organism

Among the various strain isolated from kitchen grease samples, the lipolytic enzyme producing bacterial isolates were screened using rhodamine B olive oil agar and tributyrin agar. In this, Esterase enzyme producing organism showed the orange color fluorescence in rhodamine B olive oil agar when illuminating under UV light, and lipase enzyme producing organism showed clear zone around the colonies in tributyrin agar [Fig. 1]. Then, the potential lipolytic enzyme producing strain was selected based on the size of zone clearance and intensity of fluorescence and the isolated bacterial strain was identified by 16S rRNA sequencing method. Kanimozhi *et al*., also isolated the lipase producing organism from oil mill waste and oil refinery waste contaminated site, respectively [18].

Identification of organism by 16S rRNA sequencing method

Among the various lipase producing organism, the bacteria which showed maximum lipase production was selected, identified by morphological character, various biochemical characters, and 16S rRNA. Then, the sequence was compared with sequences available in the GenBank using a BLAST search. Their sequence relationships were analyzed using the software MEGA 5.0 to understand the evolutionary distance of sequenced bacteria by constructing the phylogenic tree and identified the bacteria as *Bacillus* MMO3 [Fig. 2].

Ammonium sulfate purification of lipase enzyme

The lipase produced from *Bacillus* sp. purified by ammonium sulfate method, the purification process was summarized in Table 1. Increase of enzyme specific activity observed in 60%–80% of ammonium sulfate precipitation followed by dialysis. The purification process resulted in 2.8-fold purification and a final yield of 80% of the enzyme with a specific activity of 940 U/mg. Abigor *et al*., (2002)[19] also reported that the lipase enzyme produced from *Jatropha curcas* purified with 80% ammonium sulfate precipitation achieved a 2.8-fold increase in the relative activity of the lipase in the supernatant.

Effect of organic solvent on growth

Ogino *et al*. [20,21] reported that most of the organic solvent tolerant organism are producing highly stable lipase enzyme. In this study, the *Bacillus* MM03 shown excellent tolerance to methanol, ethanol, and acetonitrile and moderate tolerance to butanol. Above 75% of growth was observed even in 4% of methanol, ethanol, 0.5% of butanol, and 2% of acetonitrile (Fig. 3). Kumar *et al*. [22] reported that the Gram-positive bacteria originated from the marine environment such as *Rhodococcus, Arthrobacter,* and *Bacillus* showed excellent tolerance to organic solvents.

Effect of optimum temperature and incubation period on enzyme production

The effect of optimum temperature on enzyme production was estimated at every 6 h till 36 h. The maximum production was achieved at 37°C in 24-h incubation, then a gradual reduction in production was observed (Fig. 4). Abdou [23] also reported that the maximum lipase production was found during 30°C–40°C by *Serratia* sp. isolated from raw milk samples, and also our result correlated with the production of lipase by *Micrococcus roseus* [24].

Effect of temperature on growths

To optimize the effect of temperature on growth, organism was inoculated in the growth medium and incubated under 20°C, 37°C, 50°C, 55°C, and 60°C. Among the various temperatures, the increased biomass concentration was observed at 37°C (Fig. 5). Mohan *et al*. [25] also reported that the maximum biomass concentration was obtained at 37°C for the lipase producing *Bacillus* sp.

Effect of pH on enzyme activity

The pH of the medium is most important for the production of enzyme activity. In this study, the maximum activity of lipase enzyme was obtained at pH between 6 and 9 (Fig. 6). This result is correlated with the solid-state fermentation of lipase enzyme activity produced at pH between 5 and 6 by *Rhizopus homothallicus* [26,27]. Similar results were reported, where lipase production from marine actinomycetes

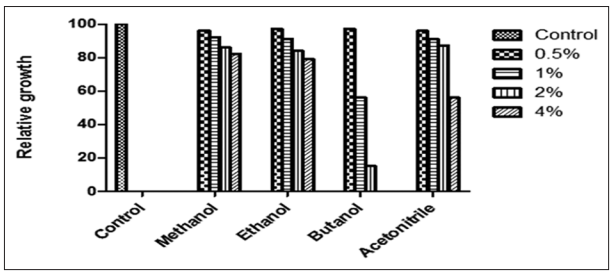

Fig 3: Effect of organic solvent on *Bacillus* MM03 growth

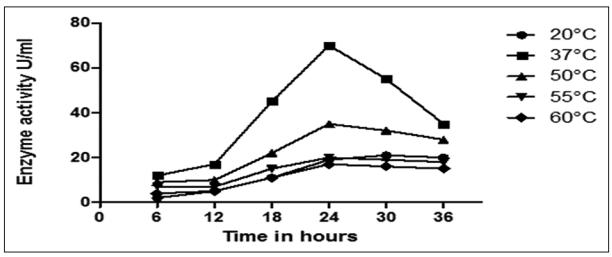

Fig 4: Effect of temperature and incubation period on enzyme production

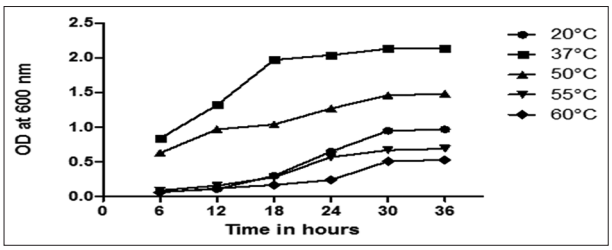

Fig 5: Effect of temperature on growth

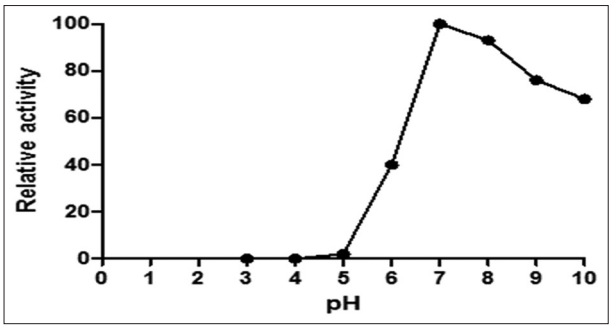

Fig 6: Effect of pH on enzyme activity

Table 1: Purification process of lipase from *Bacillus* MMO3

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	100	8.3	2787	335.8	1	100
Ammonium sulfate	7	2.4	2256	940	2.8	80

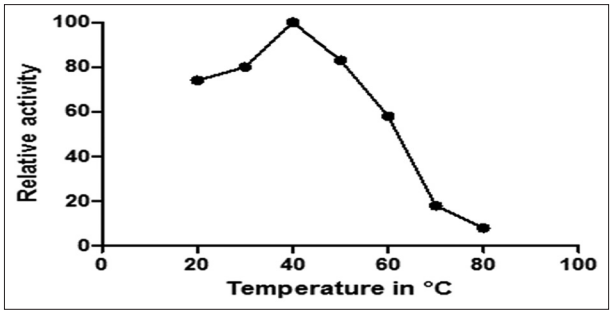

Fig 7: Effect of temperature on enzyme activity

was maximum at pH 6 and the bacteria *Pseudomonas gessardii* has optimum lipase production at pH 7.0 [28,29].

Effect of temperature on enzyme activity

The maximum activity of lipase enzyme at different temperature was analyzed, in this, the maximum activity was achieved at 37°C, and it was slowly reduced, in 80°C the production got ceased [Fig. 7]. Similar result was reported that the maximum lipase production was at 37°C by *Pseudomonas xinjiangensis* [30].

CONCLUSION

In the present study, we isolated solvent tolerance producing bacteria from the kitchen grease. The highest lipase producer in the supernatant was selected, and further characterized by 16S RNA sequencing and was identified as *Bacillus* spp. The results of this study indicate that *Bacillus* sp. is valuable as a source of lipase isolated from kitchen grease. Lipase produced by *Bacillus* sp. shows maximum enzyme production after 24 h and good solvent tolerance. Therefore, *Bacillus* sp. can be a promising candidate of solvent tolerance lipase enzyme for a variety of industrial applications.

AUTHORS CONTRIBUTIONS

JMK and JGR equally contributed to study design, conduct of experiments, data collection manuscript preparation, critical revision, and finalization of the manuscript. Both the authors agree with the content of the manuscript.

CONFLICT OF INTEREST

There is no conflict of interest.

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