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SHORT COMMUNICATION

Production and optimization of lipase from wild and mutant strains of *Bacillus* sp. and *Pseudomonas* sp.

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Abstract

Lipases are ubiquitous enzyme of considerable physiological significance and Industrial potential. Lipolytic bacterial strains were isolated from oil-mill soil samples by enrichment techniques. Totally twenty two isolates were isolated and identified. Among them, two potential lipase producers from oil wastes were optimized by manipulating the cultural environment of the production medium. The isolates were identified by microscopic and biochemical characteristics. The potential isolates were mutated at 260A°. Parent and mutant strai n of *Bacillus* sp. secreted more lipase compared to *Pseudomonas* sp. The wild and mutant strains of the bacterial species were optimized against pH, temperature, carbon and nitrogen sources for lipase production. Optimum biomass and lipase activity was achieved at 37℃, pH 7 whereas, sucrose and tryptone stimulate d lipase production in wild strain of *Bacillus* sp. and mutant strain of *Bacillus* sp.

Keywords: Lipases, oil-mill soil, *Bacillus*, *Pseudomonas*, sucrose, tryptone.

Introduction

Lipases can be naturally and readily discovered from the earth's flora and fauna. Lipases are available from many sources however; the most suitable sources for lipase production are microbes including bacteria, fungi and yeast. These microorganisms can produce high quality lipases in lower cost and shorter time (Trichel *et al.,* 2010). Schmidt *et al*. (1994) and Luisa *et al.* (1997) reported several *Bacillus* sp. as the main sources of lipolytic enzymes. Lipase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairies, industrial wastes, oil seeds and decaying food (Sztajer *et al*., 1988), compost heaps, coal tips and hot springs (Wang *et al*., 1995). Bora and Kalita (2007) and Kanwar *et al.* (2002) reported that microbial lipases are immensely used for biotechnological applications in dairy, detergents and textile industries, production of surfactants and in oil processing industries.

Lipases been widely used in pharmaceutical industries in the production of enantiometrically pure pharmaceuticals; since they have a number of unique characteristics coupled with distinct substrate specificity namely regio-specificity and chiral selectivity (Kim *et al*., 1998). Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation and dissolved oxygen concentration (Gupta *et al.,* 2004). The present study was aimed to isolate and identify bacteria from oil-mill soil samples collected at Rasipuram, Namakkal District. The isolated strains were muted and tested for lipase production by optimizing the cultural conditions of the production medium.

Materials and methods

Sample collection: Five soil samples were collected from different places of oil-mills at Rasipuram, Namakkal District and were subjected to serial dilution. Serially diluted samples from 10^{-1} to 10^{-7} were plated on nutrient agar using spread plate method and kept for incubation at 37℃ for 24-48 h and the plates were observed fo r growth after the incubation time.

Identification of bacterial isolates

The bacteria strains were identified based on the microscopic (Gram staining, Motility test and Spore staining) and biochemical characteristics (Indole, catalase, oxidase and citrate utilization) (Sundarraj, 2005).

Qualitative plate assay for lipase

Lipolytic activity of the isolated organisms was detected on tributyrate agar plates containing 1% tributyrin (pH 7.0) inoculated with 24-48 h old culture of test organisms. A single line streak was made on each plate. The plates were incubated at 37℃ for 24-48 h. Clear zones aro und the isolated colonies were detected which demonstrated lipase production (Sierra, 1957: Akatsuka *et al.,* 2003). Based on the lipase secretion on the tributyrate agar plates, the best enzyme secreting organism was selected for lipase production.

Strain Improvement of the isolates

In order to augment the lipolytic activity, the isolates were mutated by UV radiation (Bornscheuer, 2000). Four test tubes with the bacterial suspension were taken and two of them were kept as control and rest two tubes were treated with UV radiation (260A°) for 5 to 10 min.

UV treated bacterial suspension (0.1 mL) was then inoculated on tributyrate agar plates and incubated at 37℃ for 48 h for zone of clearance.

Preparation of crude enzyme

A loopful of respective 48 h old bacterial cultures of both parent and mutant strain from a nutrient agar slant was inoculated into a 50 mL of production medium (Joseph *et al*., 2006). The initial pH of the medium was adjusted to 7.0 with 1N NaOH/1N HCl. The culture was then incubated for 24 h at 37℃ under shaking condition (150 rpm). The crude enzyme was obtained by centrifugation at 10000 rpm at 4℃ for 10 min and used for further analysis (Vijayaraghavan *et al.,* 2011).

Lipase activity

Lipase activity was assayed quantitatively by using ρ-nitro phenyl palmitate as the substrate (Winkler and Stuckmann, 1979). 10 mL isopropanol containing 30 mg ρ-nitro phenyl palmitate (Sigma) was mixed with 90 mL 0.05 M sodium phosphate buffer (pH 8) containing 207 mg sodiumdeoxycholate and 100 mg gum arabic. A total volume of 2.4 mL freshly prepared substrate solution was prewarmed at 37℃ and mixed with 0.1 ml of crude enzyme solution. After 15 min incubation at 37℃, absorbance at 410 nm was measured against a blank. One enzyme unit was defined as 1µmol of ρ-nitrophenol enzymatically released from the substrate in milliliters per min.

Effect of pH

The isolates of both parent and mutant strains were grown in lipase production medium as described earlier. The effect of pH on lipase production was studied by adjusting the culture media pH to 5.0, 6.0, 7.0, 8.0, and 9.0 respectively by the addition of 1.0 N HCl/NaOH prior to sterilization. One mL of 18 h old cultures were inoculated and incubated at 37℃ for 48 h by shakin g (150 rpm). The cell free extract was used as the crude sample from both parent and mutant strains. The enzyme activity was measured by standard procedure (Winkler and Stuckmann, 1979).

Effect of temperature

Both parent and mutant strains were inoculated in the production medium and incubated at temperatures 25°C, 37℃ and 45℃ for 1 h and lipase activity was measu red after the incubation period.

Effect of nutritional factors

To evaluate the effect of carbon sources on lipase production of both strains of parent and mutant, the isolates were inoculated in the production medium (Peptic digest of animal tissue; 5 g, Yeast extract; 1.5 g, Beef extract; 1.5 g, NaCl; 5 g; Tributrin; 1%, Distilled water; 1L) at 1% (w/v): lactose and sucrose. All the carbon sources were sterilized separately through a 0.22 µm membrane filter (Rankam, NY0213SF) and then added to the reaction mixture.

To determine the effect of nitrogen sources on lipase production, both the strains of parent and mutant were added to the production medium individually at 1.0% (w/v). The nitrogen sources used were yeast extract and tryptone. After incubation, the crude enzyme was collected and the enzyme activity was performed after the incubation period.

Results and discussion

Lipases are versatile enzymes that are widely used and becoming increasingly important in high-value applications in the oleochemical industry and the production of fine chemicals (Kanwar *et al.,* 2002; Bora and Kalita, 2007). In the present study lipolytic bacterial strains were isolated from oil-mill soil samples at Rasipuram. Totally 22 isolates were recovered based on the enzymatic activity and these isolates were purified. Among the 22 isolates, 12 isolates showed same morphology on nutrient agar as Irregular form, undulate margin and other 10 isolates showed same morphology on nutrient agar as large, opaque, irregular colonies and producing green colour pigment (Table 1).

Table 1. Cultural characteristics of the bacterial isolates.

Isolates	Colony morphology
Isolate 1	Irregular form, undulate margin
Isolate 2	Colonies are large, opaque, irregular, producing green colour pigment

Among them two isolates showed highest enzyme activity based on the zone of clearance were selected for further studies. Under microscopic examination, the isolates (isolate 1) were observed as gram-positive rod, motile and spore formers and (isolate 2) gram-negative rod, motile and non spore formers (Table 2). Two bacterial isolates were positive lipase producers and identified based on the morphology, biochemical and cultural characters. Based on the above results and with the help of Bergys manual of systemic bacteriology, the 2 isolates were identified as *Bacillus* sp. and *Pseudomonas* sp. (Table 3). Similar study was done by various researchers (Shah *et al.,* 2006; Kanimozhi *et al*., 2011; Kathiravan *et al*., 2012).

Table 2. Microscopic characteristics of the bacterial isolates.

Isolates	Gram staining	Motility test	Spore staining
Isolate 1	Gram positive rod	Motile	Green colour endospores present with red colour vegetative cells
Isolate 2	Gram negative rod	Motile	Non-spore forming

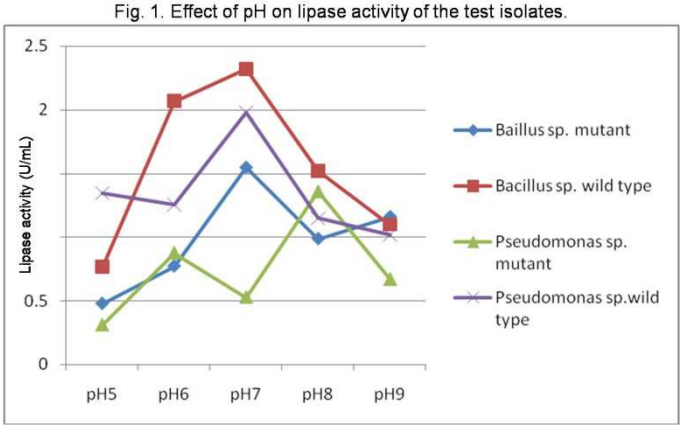
The development of technologies using lipase for synthesis of novel components will result in expansion to new area and increasing number of industrial applications (Bjorling *et al*., 1991).

Table 3. Biochemical characteristics of the bacterial isolates.

Isolates	Catalase test	Oxidase test	Indole test	MR test	VP test	Citrate utilization test	Identified Organism
Isolate 1	+	-	-	-	+	+	*Bacillus* sp.
Isolate 2	+	+	-	-	-	+	*Pseudomonas* sp.

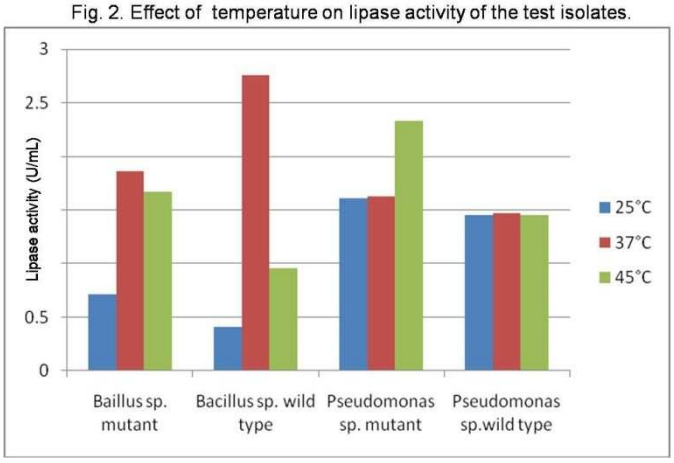
In this study, the mutant and parent strains were evaluated for lipase activity. The effect of pH on lipase production by both wild and mutant strain of *Bacillus* sp. and *Pseudomonas* sp. was evaluated. The lipase production by the isolated strains at different pH showed that the maximum enzyme activity of 1.547 U/mL and 2.066 U/mL by mutant strain and parent strains of *Bacillus* sp. at neutral pH 7 (Fig. 1). *Pseudomonas* sp. showed high production of 1.359 U/mL by mutant strain at pH 8 and 1.979 U/mL by parent strain at pH 7 (Fig. 2). Maximum lipase activity in *Pseudomonas aeruginosa* at pH 7 was reported by Sangeetha *et al.* (2008), Zouaoui and Bouziane (2012).

Fig. 1. Effect of pH on lipase activity of the test isolates.



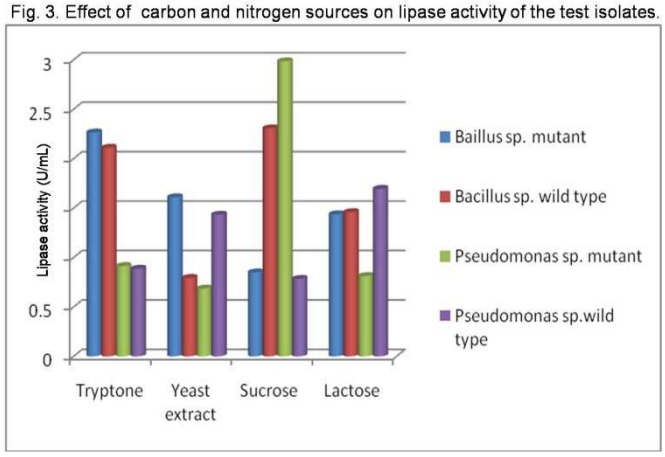
The effect of temperature on lipase production by both wild and mutant strain of *Bacillus* sp. and *Pseudomonas* sp. was evaluated. Mutant *Bacillus* sp. showed maximum activity of 1.859 U/mL and 2.760 U/mL by parent strain at 37℃ (Fig. 3). *Pseudomonas* sp. showed high lipase activity at 45℃, mutant strain recorded 2.328 U/mL and parent strain recorded 1.470 U/mL at 37℃ (Fig. 2). Similar observations were reported by Selva Mohan *et al*. (2008) in his investigation.

Fig. 2. Effect of temperature on lipase activity of the test isolates.



The effect of carbon sources on crude enzyme production by mutant and parent strain of *Bacillus* sp. and *Pseudomonas* sp. in the production medium was studied. In *Bacillus* sp. maximum enzyme production was observed in parent strain (2.307 U/mL) when sucrose is used as the carbon source. In *Pseudomonas* sp. maximum enzyme production was recorded in mutant strain (2.985 U/mL) using sucrose as a carbon source (Fig. 3). Lipase enzyme production was secreted high by mutant strain of *Bacillus* sp. (2.265 U/mL) using tryptone as a nitrogen source. Parent strain of *Pseudomonas* sp. showed maximum enzyme production (1.433 U/mL) when yeast extract is used as a nitrogen source (Fig. 3). Similar observations were reported by Joseph *et al.* (2006) in his study. The rate of activation and inactivation of enzyme, their functional efficiency constituents the kinetic data, determines the way in which the reaction occurs. The reaction rates are often heavily influenced by conditions such as substrate, inhibitor, activator, enzyme concentration, pH and temperature.

Fig. 3. Effect of carbon and nitrogen sources on lipase activity of the test isolates.



Conclusion

Parent and mutant strain of *Bacillus* sp. secreted more lipase compared to *Pseudomonas* sp. The wild and mutant strains of the bacterial species were optimized against pH, temperature, carbon and nitrogen sources for lipase production. Optimum biomass and lipase activity was achieved at 37℃, pH 7 whereas, sucrose and tryptone stimulated lipase production in wild strain of *Bacillus* sp. and mutant strain of *Bacillus* sp. To conclude, the two isolates parent and mutant strains of *Bacillus sp.* is best for the commercial production of extracellular lipase.

Acknowledgements

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