NOTE

Pongamia pinnata seed cake: A promising and inexpensive substrate for production of protease and lipase from Bacillus pumilus SG2 on solid-state fermentation

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The production of a protease and a lipase from Bacillus pumilus SG2 on solid-state fermentation using Pongamia pinnata seed cake as substrate was studied. The seed cake was proved to be a promising substrate for the bacterial growth and the enzyme production. The initial pH, incubation time and moisture content were optimized to achieve maximal enzyme production. Maximum protease production was observed at 72 h and that of the lipase at 96 h of incubation. The production of protease (9840 U/g DM) and lipase (1974 U/g DM) were maximum at pH 7.0 and at 60% moisture content. Triton X-100 (1%) was proved to be an effective extractant for the enzymes and their optimal activity was observed at alkaline pH and at 60°C. The molecular mass of the protease and lipase was 24 and 40 kDa, respectively. Both the enzymes were found to be stable detergent additives. The study demonstrated that inexpensive and easily available Pongamia seed cake could be used for production of industrially important enzymes, such as protease and lipase.

Keywords: Lipase, Protease, Solid state fermentation, Pongamia pinnata seed cake

Proteases (peptide bond hydrolases, E.C 3.4) and lipases (triacyl glycerol ester hydrolases, E.C 3.1.1.3) are commercially important enzymes that find considerable applications in detergent, baking, pharmaceutical and tanning industries. Few industrial processes like soaking, degumming, and cheese making require the presence of both protease and lipase. Soaking is a primary process involved in leather processing which requires the presence of both these enzymes. Biodiesel is a renewable resource of energy and has gained its importance in India due to the soaring oil prices and largely enhanced environmental awareness. Biodiesel and other biofuels are produced from agricultural plants and plant products. Pongamia and Jatropha seeds have topped the biodiesel market in India and Pongamia pinnata has been identified as a superior and more sustainable source for biodiesel.

Pongamia seeds contain 30-40% oil content, which has innumerable uses as lubricant, biopesticide, in alternative medicine and also in detergent and tanning industries. Pongamia seed cake, a by-product of Pongamia oil industry is non-edible and hence is not used as cattle feed, but its use as biofertiliser has been evaluated. Cultivation of Pongamia is easy, as the tree can thrive in any extremes of the weather and on any type of soil and thus the price of Pongamia oil and the cake is usually low. Pongamia is rich in proteins (>30%) as well as carbohydrates (>30%), besides the oil and thus a study aimed at finding further uses for this important material seemed valuable and timely.

In the present study, we report the simultaneous production of two industrially important enzymes, namely a protease and a lipase from Bacillus pumilus by solid state fermentation (SSF) using P. pinnata cake as substrate.
Materials and Methods

Chemicals and reagents
All the chemicals and reagents used were of analytical grade. Acrylamide, agar, ammonium persulphate, gelatin, N,N’-methylene bis-acrylamide, sodium dodecyl sulphate, N,N,N’,N’-tetramethylene-diamine, p-nitrophenol, tributyrin, and Tris base were purchased from Himedia (Mumbai, India). All other chemicals were purchased from Qualigens (Mumbai, India). De-oiled Pongamia cake was purchased from a local market in Chennai, India.

Culture
A bacterial strain which produced both protease and lipase concomitantly was isolated from a food processing industrial effluent and was examined for morphological and biochemical characteristics based on Bergey’s manual of systematic microbiology. It was identified and denoted as Bacillus pumilus SG2.

Solid state fermentation
The ground Pongamia seed cake (10 g) taken in a 250 ml Erlenmeyer flask was moistened with distilled water (5 ml) and after sterilization was inoculated with the overnight culture (0.5 ml), followed by incubation at 37°C. Parameters like pH (6.0, 6.5, 7.0, 7.5 and 8.0), incubation time (48, 60, 72, 84, 96 and 108 h), moisture content (40, 50, 60, 70 and 80%), extracting agents (0.1, 0.5 and 1% Triton X-100, tap water and phosphate buffer) were optimized for maximizing the production of protease and lipase using Pongamia cake by SSF.

Enzyme extraction
A known volume of fermented substrate was mixed thoroughly with 1% Triton-X and the suspension was centrifuged at 5000 rpm for 10 min. The resulting pellet was resuspended in the same extractant and this process was repeated thrice. The supernatants were pooled and centrifuged at 10,000 rpm for 10 min. The supernatant thus obtained was considered as the crude enzyme source. The dry matter (DM) was determined by drying 4 g of the fermented solid matter at 100°C for 16 h.

Enzyme assay
The caseinolytic activity was measured by the photometric method of Rahman et al. One unit (U) of protease activity was equivalent to 0.5 µg of tyrosine liberated by 1.0 ml of enzyme solution under the assay conditions. Lipase activity was also assayed and one unit (U) of lipase activity was equal to 1 micromole of p-nitrophenol liberated per min under the assay conditions.

Partial purification and characterization of the enzymes
The crude enzyme was partially purified by 70% saturated ammonium sulphate precipitation, followed by extensive dialysis against 0.1 M Tris buffer (pH 8.5). The optimum pH of protease and lipase in the partially purified enzyme source was studied over a range of 5.0 to 12.0 by incubating the enzyme mixture with casein and tributyrin as substrate for protease and lipase activity, respectively. The pH stability of the enzymes was also analyzed by pre-incubating the enzyme mixture in different buffers (phosphate, pH 4-7, Tris HCl, pH 8-10 and Glycine-NaOH, pH 11-12) for 1 h at 45°C.

The optimum temperature was studied by incubating the enzyme mixture with their respective substrates at temperatures ranging from 30 to 80°C for 20 min. The stability of the enzymes was also investigated by pre-incubating the enzyme mixture at various temperatures in the range -4 to 90°C for 1 h. The stability of the enzymes in presence of detergents was studied by incubating 5 ml of the enzyme mixture with 10 ml of different commercial detergents (7 mg/ml) at 60°C and the residual activity was studied at regular time intervals up to 2 h.

The purity of the enzyme mixture was determined by polyacrylamide gel electrophoresis (PAGE) using 12.5% polyacrylamide gel according to the method of Laemmli. The enzyme mixture was subjected to activity staining on zymograms for confirmation and to determine their molecular weights. Electrophoresis was performed on 0.1% copolymerized gelatin as the substrate to study the protease activity. Lipase activity staining was also performed.

The ability of the enzyme mixture to decompose gelatin on processed X-ray films was studied by incubating a X-ray film with the enzyme mixture at 40°C. A small portion of a processed X-ray film was cut and immersed in the enzyme mixture. The exposure of plastic or cellulose acetate support of the X-ray film indicated the degradation of gelatin layer.

Results and Discussion
The production of protease by SSF with different agro-industrial wastes like nug meal, pigeon pea waste, green gram husk using Bacillus sp has been reported. Use of SSF for lipase production has also been well studied with the fungi. In this study, we
examined the use of *Pongamia* seed cake as substrate under SSF to produce commercially important protease and lipase using *B. pumilus* SG2.

As no data has been available from literature, the initial attempts were directed towards the optimization of the parameters for maximal production of both protease and lipase. *Pongamia* seed cake was found to be a good substrate for SSF and provided sufficient nutrients and proper anchorage for the bacterial cell growth. The enzyme production could be observed from the 2nd day of fermentation. Maximum protease production was observed at 72 h of incubation (5440 U/g DM), while lipase production was maximal at 96 h of incubation (984 U/g DM). Enzyme production decreased with prolonged incubation time, probably due to the exhaustion of the substrates (Fig. 1a). A similar result is reported for protease production using green gram husk as substrate. However, lipase production by *Bacillus* sp. has been reported at 120 h of incubation.

The initial pH of the media is one of the most significant factors which affects the growth of the organism and enhances extracellular enzyme production. The optimal pH for maximal production of protease (6840 U/g DM) and lipase (1184 U/g DM) was found to be 7.0 (Fig. 1b). The pH of fermented media was observed at regular intervals and was found to vary between 6.5 and 8.0. An initial pH of 6.0 and 7.0 optimum for protease and lipase production, respectively have been reported using *Jatropha* seed cake.

Bacterial growth occurs on the surface of solid particle and at an area of low moisture content in SSF; hence moisture content is a crucial factor in regulating the bacterial growth and hence the enzyme production. The production of both protease and lipase was maximum at 60% moisture content (9840 and 1974 U/g DM, respectively). The enzyme production decreased with increased or decreased moisture content. Increased moisture level in SSF decreases the porosity of agro-substrates by limiting the rate of oxygen transfer, whereas decreased moisture content reduces the swelling of the substrates which decreases enzyme production.

The choice of extractant plays an important role in the recovery of the enzymes in SSF. Three different agents, namely Triton X-100 at three different concentrations, tap water and phosphate buffer (0.1 M) were used as extractants. The enzymes were found to be best extracted when Triton X-100 (at 1% concentration) was used. This could be attributed to the surfactant activity of Triton X-100 which reduces the interface between the enzyme and residual substrates. Phosphate buffer served as a poor extractant of lipase and this might be due to its inability to ease the interface between lipase and the solid support. Use of tap water gave inferior yields of the enzymes (60% and 85% for lipase and protease, respectively).

The activity of both protease and lipase was optimal at pH 10.0 and 11.0 respectively and both the enzymes were stable between pH 7.0 to 12.0. The activity of these enzymes was reduced to more than 50% at acidic pH (Fig. 2a). These enzymes could, therefore, be expected to be effective biocatalysts for the detergent industry, since they require alkaline pH for best activity, similar to the well-known detergent enzyme subtilisin Carlsberg which exhibits its best activity between pH 8.0 and 12.0. The maximum activity of both protease and lipase was detected at 60°C and the optimal activity was observed between temperatures of 40 to 60°C (Fig. 2b). Thus, these enzymes could also be expected to function as effective catalysts in the tannery and detergent industrial processes that require moderate to high temperatures. The enzymes were stable at a wide range of temperatures (between 20 to 80°C) and retained more than 90% of their activity after incubation with commercial detergents (Ariel, Surf Excel Quick Wash and Automatic, Tide, Henko and Pril) (Fig. 2c).

The molecular mass of the protease and lipase produced by *B. pumilus* SG2 on SSF using *Pongamia*
seed cake was found to be 24 and 40 kDa, respectively. The presence of protease and lipase in the enzyme mixture was confirmed using zymogram analysis by the presence of an achromatic band against a blue background and a yellow band against a pink background, respectively (Fig. 3).

The efficiency of the enzyme mixture to degrade gelatin layer of X-ray film was also studied. Decomposition of gelatin layer was initiated after 20 min of incubation at 40°C (Fig. 4). This ability of the enzyme can support the recovery of silver present in the X-ray films. Considering the fact that silver from X-ray films is generally recovered by burning them, the use of enzymes that can facilitate the recovery of silver by degradation of gelatin would be environmentally safe. Earlier, similar results have been reported with a protease produced on a chemical medium from *B. subtilis* ATCC 6633\(^{30}\). The ability of the enzyme mixture to degrade the plastic contained in X-ray films should be evaluated further. The lipase present in the mixture can probably act as an esterase which can degrade the cellulose acetate present in the plastic base of the X-ray film.

**Conclusion**

The study demonstrated *Pongamia* seed cake as a good substrate for the production of protease and lipase from *B. pumilus*. The seed cake was not supplemented with carbon and nitrogen sources and its nutrient content was sufficient to produce promising quantities of lipase and protease. The substrate can be used to investigate the production of other industrially potent enzymes. The protease and lipase produced on SSF using *Pongamia* seed cake have industrial potential and seem suitable for the detergent industry and may serve as bio-catalysts in silver-recovery from processed X-ray films.

**References**

