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RUBBER SEED AS A POTENT SUBSTRATE FOR THE PRODUCTION OF LIPASE FROM *Pseudomonas* SP. STRAIN BUP6 BY SOLID STATE FERMENTATION

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ABSTRACT

Nowadays, increases in demand for cost effective methods to enhance the production of industrially significant biomolecules, especially for microbial enzymes are observed. Solid State Fermentation (SSF) is a much relevant, cheap and ecofriendly fermentation strategy compared to Submerged Fermentation (SmF). Microbial lipases are versatile in nature and they have great applications in industries such as food, diary, paper, pulp, cosmetics, detergent, pharmaceutical, etc. The present study describes the efficacy of de-oiled flours of rubber seed, coconut and cotton seed as solid substrate for the production of lipase by *Pseudomonas* sp. strain BUP6, a novel bacterium isolated from the rumen of Malabari goat. Here, fifty percentage of the flours (w/v) were supplemented with basal salt medium (pH 6.8), and incubated at 37°C for 24 h to 120 h. Lipase production was quantitatively assayed at 24 h intervals. Rubber seed flour supported the maximum lipase production (33.98 U/gds) at 24 h incubation, followed by coconut (9.97 U/gds), while cotton flour showed the lowest production of lipase (5.45 U/gds). Upon this background, the unutilized and cheaply available rubber seed is a potent substrate for the production of lipase which would provide additional profit to rubber farmers.

KEYWORDS: Solid State Fermentation, Lipase, Basal Salt Medium, *Pseudomonas* sp.BUP6, Rubber Seed

The advent of enzymology paved an important breakthrough in biotechnology industry. The worldwide and enormous usage of agro-industrial residues for the production of lipase as well as other value added products would hold a prominent position in future biotechnologies, mainly because of its ecofriendliness and flexibility to both developing and developed countries. Being abundant, cheap and renewable, several agro-residues such as straw, bran and oil cakes attracted many researchers towards biotechnological applications of these natural resources. Lipase is a key enzyme that catalyzes the digestion of fats to form fatty acid and glycerol or other by-products. Compared to esterase, lipases are more vigorous at an oil-water interface. Some of them are specific; although, many lipases are catalyzing a number of useful reactions including hydrolysis, esterification, inter-esterification, acidolysis and alcholysis [Benjamin and Pandey, 1998]. The extensive utilization of lipases has wide range of applications in the synthesis of detergents, biosurfactants, pharmaceuticals and oleo-chemicals, in leather, cosmetic, perfume, dairy and agrochemical industries, in environmental management and in biosensors.

Although lipases are ubiquitous, they are mostly seen in various plants, animals and microorganisms. However, microbial lipases are widely used in industry because of its versatile nature, ease of access, activity under mild conditions, dynamic stability and high substrate specificity [Kempka et. al., 2008]. Microorganisms including bacteria, fungi, yeast and actinomycetes are recognized as preferred sources of extracellular lipases. Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors. The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier. The major lipase producing bacterial sources include *Achromobacter*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas*. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications. Solid State Fermentation (SSF) is a fermentation technique performed on solid substrate that acts as physical support as well as nutrient source. Many microorganisms secrete lipases during growth on organic residues. By dint of low production cost, greater stability, simplicity and wider availability than Submerged Fermentation (SmF), SSF is considered as a novel strategy with higher physiological significance and industrial potentials [Pandey et. al., 1999]. It is more reliable and cost effective.

Rubber tree (*Hevea brasiliensis*) is a perennial tree belonging to the family *Euphorbiaceae* native to the Brazil, South America. India is the sixth largest producer and fourth largest consumer of natural rubber in the world. In India, rubber plantations are mostly concentrated to the state of Kerala (approximately 80%). It is primarily cultivated as industrial crop for the production of milky latex as a source of natural rubber, while the seeds are ancillary. The yield of seeds from the plantations is estimated to be from 150 to 200 kg/ha per year. This study focuses on the exploitation of agro-industrial oil residues for the production of lipase from *Pseudomonas* sp. BUP6

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with an emphasis on solid state fermentation using rubber seed flour.

MATERIALS AND METHODS

Chemicals

Analytical grade chemicals purchased from Hi Media Laboratories (Mumbai, India) and Merck India Ltd. were used for this study. *p*-nitrophenylpalmitate (*p*NPP), substrate for lipase assay was purchased from Sigma Chemical Co., USA.

Culture and Culture Medium

Pseudomonas sp. BUP6 (MTCC No.5925), a new gram negative bacterium described by the Enzyme Technology Laboratory, Department of Botany, University of Calicut, India was used for this study. This was isolated from the rumen of Malabari goat. The bacterium was grown on semi-synthetic basal salt medium (BSM) supplemented with 0.3% of ground nut oil and incubated in temperature controlled incubator (Biolinx; India) at 37°C for 24 h. The mother culture was maintained on BSM plus agar petri-dishes, which was subcultured within 1 month. BSM comprises the following chemicals (g/100 ml): 0.5 g of NH_4NO_3; 0.4 g of $(NH_4)_2SO_4$; 0.3 g of yeast extract; 0.2 g of K_2HPO_4; 0.2 g of NaCl; 0.01 g of $MgSO_4.7H_2O$ and 0.01 g of $CaCl_2$ in double distilled water [Priji et. al., 2015].

Morphological Characterization of *Pseudomonas* sp. BUP6

Well grown *Pseudomonas* sp. BUP6 culture on BSM slants was stained for studying its morphological characteristics. Gram staining was employed for the purpose. The slides were observed under binocular microscope (Magnus MLX). Photographs were taken using Image Analyzer (Nikon Eclipse E400, Towa optical, Japan) fitted with Nikon digital camera (DXM 1200F, Japan).

Colony Forming Unit (cfu) By Serial Dilution Method

The cfu is a rough estimate of the number of viable bacterial cells. After incubation, number of bacteria in 1ml of suspension was counted in cfu using serial dilution method. cfu per mL was calculated using the following equation 1.

$$\frac{\text{Colony count (CFUs) on an agar plate}}{\text{Total dilution of tube (used to make plate for colony count)}} \times \text{volume plated} \quad \text{(Eqn. 1)}$$

Inoculum Preparation

For inoculum preparation one loop full of mother culture was added to the same semi synthetic BSM medium; which was incubated at fully controlled orbital shaker (Scigenics Biotech, India) at 37˚C and 150 rpm for 18 h (in 100 ml Erlenmeyer flasks), so as to reach maximum optical density of 2.5 at 600 nm. The 18 h seed culture was considered as inoculum which was employed for further experiments.

Agro-Waste for Lipase Production

Oil refined agro-industrial oil cakes of rubber seed flour, cotton seed flour and coconut cakes were screened for lipase production by SSF method. The coconut cake and cotton seed flour were purchased from local market, while rubber seeds were collected from a local rubber plantation (University of Calicut; 11.1340° N, 75.8952° E). The collected coconut cake and seeds were ground by using laboratory blender and stored in air tight plastic containers.

SSF Strategy

Fermentation was performed in 100 ml of Erlenmeyer flask. 4g of fine powder of substrate (oil cake) was weighed into a conical flask and then moistened with 50% of BSM medium. All preparations were made in triplicate and autoclaved at 121°C, 15 ψ for 15 min and subsequently inoculated with 0.1 ml of inoculum to sterilized flask under aseptic condition. All flasks were incubated at 37°C for lipase production. Lipase production was quantitatively assayed at each 24 h interval for 5 days.

Extraction of Lipase

After each interval of incubation (24 h), the flasks were withdrawn for lipase assay. Fifty percentage of fermented substrate was taken for the assay and the remaining portion was used for dry weight estimation. Subsequently, the fermented matter was dispersed with 10 ml of 0.1 M tris-HCl buffer (pH 8.0) and stirred for 10 min with magnetic stirrer (Remi; India). Then, the residue was centrifuged at 9400 ×g for 15 min at 4°C and the supernatant was collected for lipase (crude) assay.

Lipase Activity Assay

Lipase activity was determined by the method of Priji et. al., [2014]. High grade *para*-nitro phenyl palmitate (*p*NPP) was used as substrate for lipase assay. The assay test tube containing 1.8 ml of 0.1 M Tris-HCl buffer, 0.15 M NaCl and 0.5% Triton X-100 was pre-incubated with 200 µl of clear supernatant (crude enzyme) at 37°C for 10 min. Subsequently, 20 µl of *p*NPP (18.875 mg/ 1 ml acetonitrile) was added to the reaction tube and incubated at 37°C for 30 min. The quantity of lipase was measured spectrophotometrically at λ_{405}. One unit of lipase corresponds to 1 µmol of *p*-NPP liberated per minute under the standard assay conditions. The lipase activity was estimated using the following formula **(1).**

$$\text{Lipase activity (U/gds)} = \frac{\Delta E \times Vf \times VS}{\Delta t \times \varepsilon \times gds} \quad \ldots (1)$$

Where, ΔE - absorbance at 405 nm; Vf - final volume; VS - volume of lipase used; Δt - time of hydrolysis; ε - extinction co-efficient (0.017); gds - dry weight in grams

Statistics

All studies were performed in triplicate, and an average value calculated and presented with standard deviation. MS Excel was used to illustrate the figures.

RESULTS AND DISCUSSION

Morphological Characterization of *Pseudomonas* sp. BUP6

The clear morphology of the bacteria were observed using binocular microscope (Magnus MLX) and the photographs were taken using Image Analyzer (Nikon Eclipse E400, Towa Optical, Japan) fitted with Nikon digital camera (DXM 1200F, Japan). The pure colonies were smooth, slimy and cream in colour (Figure 1A). *Pseudomonas* sp. strain BUP6 was Gram-negative rods with cell range between 1.0 and 1.5 µM in length and 0.3 and 0.6 µM in diameter (Figure 1B). The viable cells of bacteria (3. 24 $\times 10^9$ cfu/ mL) were determined by serial dilution method.

Effects of Different Substrates on Lipase Activity

Three different de-oiled cakes (rubber seed flour, coconut cake and cotton flour) were employed as solid substrate-cum-inducer for the production of lipase by *Pseudomonas* sp. strain BUP6. Rubber seed flour supported the maximum production of lipase (33.98 U/gds) on 24 h of incubation (Figure 2), subsequently the lipase activity decreased sharply; while coconut cake showed the maximum production of lipase after 72 h of incubation, but in lesser amounts (9.97 U/gds) (Figure 3). Cotton seed flour showed the lowest production of lipase (5.45 U/gds) at 24 h of incubation (Figure 4). When compared to the other two substrates, rubber seed flour showed greater activity, *i.e.*, 33.98 U/gds (Table 1).

Table 1: Maximum lipase activity in different substrates

Substrates	Maximum lipase activity (U/gds)	Incubation (h)
Rubber seed flour	33.98	24
Coconut oil cake	9.97	72
Cotton seed flour	5.45	24

The present study was mainly focused to investigate the optimum production of lipase from agro-industrial wastes by *Pseudomonas* sp BUP6. Large quantities of agricultural residues have been reported to be effective for lipase production and these include brans (wheat, rice, soybean, barley), oil cakes (soya bean, coconut, ground nut, cotton, olive, gingelly, babassu), and bagasse (sugarcane). Oil cakes of various residues obtained after extraction of oils have been utilized for fermentative production of lipases and other industrial enzymes. Most agricultural residues utilized for lipase production contain a mixture of both easily consumable and non-consumable substrates that can support the growth of a wide range of microorganisms. This is because their residual oil contents serve as inducers for lipase production [Singhania et al., 2008]. There are Some reports on the use of different microorganisms such as *Penicillium restrictum, Aspergillus niger, Candida rugosa* and *Yarrowia lipolytica* for lipase production using babassu oil cake, gingelly oil cake, groundnut oil cake and vegetable oil meal respectively [Palma et al., 2000; Kamini et al., 1998; Rekha et al., 2012; Brigida et al., 2014].

Previous studies have revealed that oil cakes are very good solid substrates cum inducers for the production of lipase by SSF [Prakasham et al., 2006]. D'Annibale et al. [2006] reported olive mill waste water as a growth medium for lipase production, which showed the highest lipase activity of 9.23 U/ml. Salihu et al., [2011] used statistical optimization of nutrient components to enhance lipase production by *Candida cylindracea*, and the maximum activity was 20.26 U/ml. Brozzoli et al. [2009] studied lipase production in bench-top reactor using the olive mill waste water medium and obtained the maximum production as 20.4 U/ml. Compared to all these studies,

the present study depicts higher lipase activity (33.98 U/gds) at 24 h incubation. The cultivation period varied with the microorganism, *i.e*., fast growing bacteria were found to secrete lipase within 24 h [Sharma et. al., 2002]. Similarly, in the present study, the highest activity of lipase obtained (33.98 U/gds) was at 24 h of incubation using RSF as substrate. It seems that the residual oil in the cake acted as both inducer and additional nutrient. Microbial lipases are produced mostly by submerged culture but solid state fermentation can also be used.

In the light of the above, the efficiency of *Pseudomonas sp.* strain BUP6 to grow on flours and cakes and extraction of lipase is yet another promising utility of this novel bacterium.

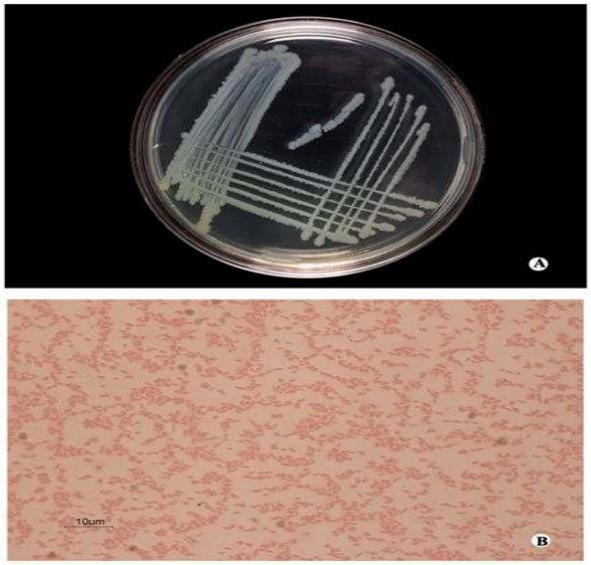

Figure 1: Morphology of *Pseudomonas* sp. BUP6. A) colony morphology of *P.* sp. BUP growing on nutrient agar medium; B) gram staining of *P.* sp. BUP6

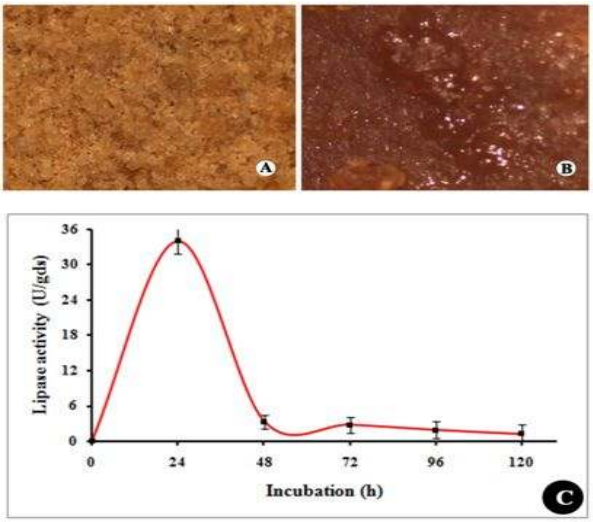

Figure 2: Production of lipase by *Pseudomonas* sp. BUP6 on rubber seed flour. A) rubber seed flour before inoculation. B) rubber seed flour after inoculation. C) lipase activity at different time intervals; maximum lipase activity (33.98 U/gds) was observed at 24 h interval.

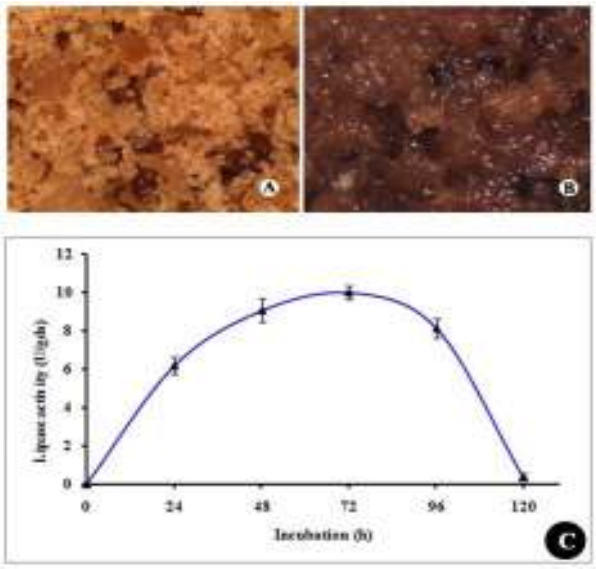

Figure 3: Production of lipase by *Pseudomonas* sp. BUP6 on coconut cake. A) coconut cake before inoculation. B) coconut cake after inoculation. C) lipase activity at different time intervals; maximum lipase activity (9.97 U/gds) was observed at 72 h interval.

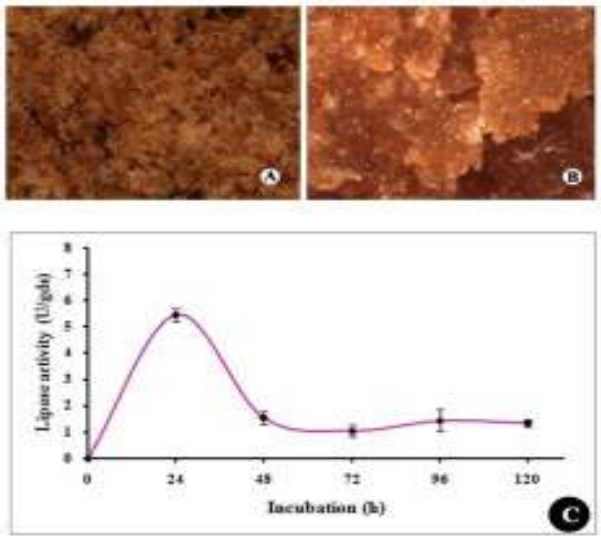

Figure 4: Production of lipase by *Pseudomonas* sp. BUP6 on cotton seed flour. A) cotton seed flour before inoculation. B) cotton seed flour after inoculation. C) lipase activity at different time intervals; maximum lipase activity (5.45 U/gds) was observed at 24 h interval.

CONCLUSION

This study was conducted using a novel strain of bacterium- *Pseudomonas* sp. BUP6 isolated from the rumen of Malabari goat. It showed significant production of lipase when grown in a newly designed basal salt medium supplemented with vegetable oil. In the present study, lipase activity by different natural substrates such as rubber seed flour, coconut oil cake and cotton seed flour was analyzed. Of them, non-edible, unutilized and cheaply available rubber seed flour showed the highest lipase activity of 33.98 U/gds. This would ensure additional income to rubber farmers from their agriculture. Moreover, the ability of *Pseudomonas* sp.strain BUP6 to grow on rubber seed substrate and to secrete extracellular lipase is highly promising. Thus, this study focuses on the use of agro-industrial residues for the production of industrially-significant and human-friendly biomolecules and moreover, its low cost increases the industrial potentials.

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