

Production of Lipase by *Pseudomonas aeruginosa* JCM5962(T) Under Semi-Solid State Fermentation: Potential Use of *Azadirachta indica* (Neem) Oil Cake

Shweta Sachan and Aditi Singh *

Amity Institute of Biotechnology, Amity University Uttar Pradesh,
Lucknow Campus, Malhaur, Gomti Nagar Extension, Lucknow, India.

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A widely employed strategy for increased production is to use crop residues as nutrient source in bioprocess. The plan of present work is to modify growth conditions by using by-products of *Azadirachta indica* (neem) oil seed cake and optimize environmental conditions for maximum production of extracellular lipases by *P. aeruginosa* JCM5962(T), identified for production of extracellular lipases previously. Semi solid state fermentation medium comprising of different concentrations of *Azadirachta indica* oil cake (AIOC) as substrate was studied, in which 4% of AIOC supported maximum lipase production after 96 hours of incubation at 37°C. Optimization of growth condition was done using nitrogen supplements, carbon additives and various oils. 1% Ammonium nitrate as additional nitrogen supplement was found to be ideal parameter for improved production, whereas carbon supplements and oil additives did not have any effect. Mixed substrates of AIOC with six other oil cake residues (1:1) were also studied. Additive effect of mustard and coconut oil cake caused an increase of 29.95% and 38.26% respectively as opposed to AIOC alone. It is the first report demonstrating use of *Azadirachta indica* oil seed cake as fermentation medium for improved production of extracellular lipase from *Pseudomonas aeruginosa* JCM5962(T).

Keywords: *Azadirachta indica*, Lipase, NH_4NO_3 , Oil cakes, *Pseudomonas aeruginosa*.

Oil cakes are by-products, obtained after oil extraction. Composition of oil cakes varies depending on their variety, extraction methods and growing condition. They have a high nutritional value and are used as animal feed because of their rich protein content (15 - 50%). Oil cakes are good in fiber and non-starch polysaccharides (NSP). Their fat content depends on oil extraction method, which is usually less than 3-4% fat¹. Non-edible oil cakes such as Karanja cake,

Castor cake, Neem cake (*A. indica*) are used as nitrogenous fertilizer because of their high N, P, K content. Oilcakes protect the plants from insects, soil nematodes and parasite; there by offer great resistance to infection². *A. indica* manure is by-product obtained after cold pressing of fruits and kernels and the solvent extraction process for *A. indica* oil cake. Its organic manure contains organic nutrients like nitrogen, phosphorous, potassium, calcium, magnesium etc., including sulphur and bitter limonoids. *A. indica* cake organic manure is a proven soil conditioner and it is being used directly or in blends with other manures like, urea, Farmyard manure etc., for its benefits of

* To whom all correspondence should be addressed.
Tel.: 09839009661;
E-mail: asingh3@lko.amity.edu

organic nutrients, pest repellent properties and its control on soil bound nematodes.

Lipases are reported in various microorganisms and plants, apart from animal sources. Over the last decades interest in these enzymes has increased markedly in view of their diverse applications in medicines, food additives, clinical reagents and cleaners and for synthesis of biodiesel and biopolymers. Microbial lipases have special industrial attention because of their selectivity, stability and broad substrate specificity^{3,4}. Microbial enzymes are highly stable in comparison of plant and animal enzymes and their production is also more safe and convenient⁵. Therefore it is important to increase the production by optimizing culture conditions and at the same time reduce the production cost. A widely employed strategy for this is solid state or submerged fermentation modifications for microbial growth. However solid state fermentation (SSF) is most appropriate process due to its various benefits and bioconversion parameters^{6,7}. Crop residue as husk, fruit seeds, bran and bagasse are utilized as a potential organic material because they provide an excellent substratum for growth of organism supplying the required nutrients to them in bioprocesses^{8,9}. Most of the lipase research focuses on production and improvement of extracellular lipase through microorganisms. Technique of semi solid state fermentation (SmSSF) involves the growth of microorganisms on solids the free flowing water.

Previous studies done in our laboratory have identified a novel high lipolytic strain *P. aeruginosa* JCM5962(T), isolated from soil of sugarcane field. The isolate produced an extracellular lipase of 31 kDa with metal cobalt as an enhancer¹⁰. Present work was undertaken to study the potential of AIOC as semi solid state fermentation (SmSSF) medium for improved production of extracellular lipase from JCM5962(T).

MATERIALS AND METHOD

Bacterial Strain

P. aeruginosa JCM5962(T) was used throughout the study. The strain has been isolated from soil and identified by 16S rRNA sequencing (data communicated). It produced an extracellular

lipase of 31 kDa after 24 h of incubation at 37°C. The enzyme was characterized to be stable at 50°C and at pH 8.0. The stock culture was maintained on 1:1 ratio of Nutrient broth (Hi-Media) and glycerol at 20°C. Working culture was prepared on nutrient broth for 24 h at 37°C.

Inoculum Preparation

A loopful of cells of lipolytic isolate *P. aeruginosa* was transferred to a 100 mL nutrient broth. It used as an inoculum after incubation at 37°C for 24 h. Two hundred micro litres of inoculum (approximately 2×10^6 cells) was added to the oil cake media for fermentation.

Semi-Solid State Fermentation

The AIOC substrate used for the production of lipases, it was procured from local market. Dry *A. indica* oil cake was taken with 100 ml of distilled water. The medium were mixed and autoclaved for 15 min at 121°C. The sterilized medium was used as SmSSF throughout the study.

Extraction of Enzyme

Phosphate buffer of pH 8 was added to the medium in 1: 1 ratio after fermentation and enzyme was extracted by centrifugation at 4°C at 10,000 rpm for 10 min. The clear supernatant obtained was used as crude enzyme and stored in sterilized vials for further use.

Lipase Assay

Lipase activity was studied using p-nitrophenyl palmitate (pNPP) as a substrate according to the protocol described previously¹⁰. Substrate solution containing phosphate buffer (pH 8.0) with gum Arabic (Hi Media) and sodium deoxycholate (Sigma Aldrich, USA) along with p-nitrophenylpalmitate (Sigma Aldrich, USA) in isopropanol was pre-incubated with crude enzyme at 37°C. After incubation, the reaction was stopped by adding 0.5 mL of 3M HCl. The reaction mixture was centrifuged at 10000 rpm for 10 minutes at room temperature. The amount of p-nitrophenol released was spectrophotometrically measured at 405 nm.

Optimization of Production Parameters for SmSSF Using Oil Cakes

To obtain maximum lipase production to suit industrial processes, optimization of parameters and manipulation of composition of medium was done. Various parameters were optimized for maximal lipase yield which are incubation time (24, 48, 72 and 96 h), the effect of carbon sources

(1% w/v of glucose, fructose, sucrose, lactose, maltose), lipid sources (0.5% v/v) such as flax, mustard, sesame, soybean, olive oil and nitrogen sources (1% w/v ammonium nitrate, potassium nitrate, peptone, beef extract, yeast extract) in the AIOC SmSSF were also examined.

Mixed Substrate Fermentation

The mixed media used in the study were designated A to F as given in table 1. The mixed substrate was prepared by mixing two different oil cakes in 1:1 ratio and preparing the SmSSF as described before. Best mixed preparation was selected; the above optimized ingredients were also tested for mixed substrate enrichment. The lipase

Table 1. Mixed substrate used in the study

S.No.	Combinations of Oil Cakes	
1	Control	<i>A. indica</i> oil cake (AIOC)
2	Media A	<i>A. indica</i> oil cake (AIOC) + Flax oil cake (FOC)
3	Media B	<i>A. indica</i> oil cake (AIOC) + Mustard oil cake (MOC)
4	Media C	<i>A. indica</i> oil cake (AIOC) + Caster oil cake (CastOC)
5	Media D	<i>A. indica</i> oil cake (AIOC) + Coconut oil cake (COC)
6	Media E	<i>A. indica</i> oil cake (AIOC) + Groundnut oil cake (GOC)
7	Media F	<i>A. indica</i> oil cake (AIOC) + Sesame oil cake (SOC)

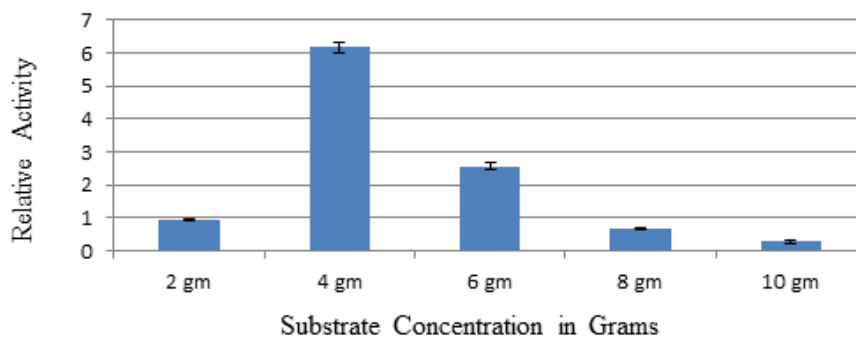


Fig. 1. Effect of different concentrations of *A. indica* oil cake (2%-10%) on lipase activity by *P. aeruginosa* JCM5962(T): strain was incubated at 37°C on different concentrations and lipolytic activity was recorded, 4% AIOC gave the maximum enzyme production whereas 2%, 6%, 8% and 10% substrate gave negligible lipase activity

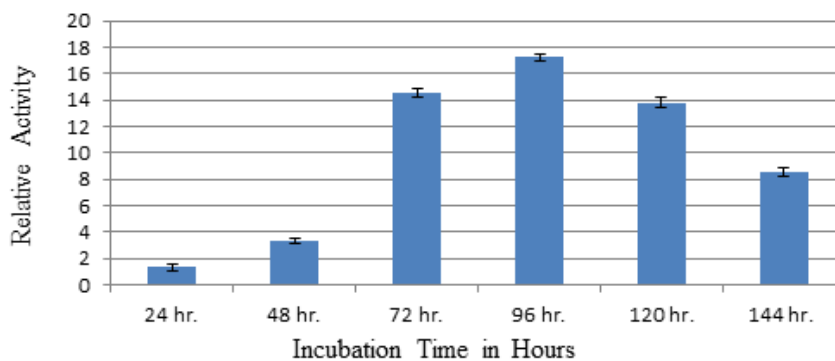


Fig. 2. Effect of different incubation time for *A. indica* oil cake medium (24 – 144 h) on lipase activity by *P. aeruginosa* JCM5962(T): bacterial strain was incubated at 37°C for 24, 48, 72, 96, 120 and 144 h and lipolytic activity was measured which was found to be maximum after 96 hr incubation (17.24 U/gds)

activity was measured for each combination and presented as percent activity.

RESULTS AND DISCUSSION

An important aspect of prime importance in enzyme production is development of an optimum substrate. In the present study, the production of bacterial lipase by *P. aeruginosa* JCM5962(T) was studied in semi-solid state fermentation using AIOC as substrate.

Optimization of AIOC Concentration

Lipase activity in SmSSF was compared with different AIOC concentration (2%, 4%, 6%, 8% and 10%) at 37°C. All media were inoculated with equal number (2×10^6 cells) of microorganisms and incubated at 37°C. The lipase activity was measured in each and relative activity was calculated and presented. As shown in figure 1, the lipase activity was reported maximum at 4% concentration of AIOC which is about 6.16 U/gds.

All further experiments were continued with same concentration of substrate (4%).

Optimization of Incubation Time

To determine incubation time for maximum lipase production, cultures were kept at 37°C for 24, 48, 72, 96, 120 and 144 h. As demonstrated in figure 2, in earlier stages of incubation a low level of lipase activity was obtained, which gradually increased with the passage of time and highest enzyme activity (17.236 U/gds) was obtained after four days (96 h). The gradual decrease in the enzyme activity was observed with increasing incubation time beyond 96 h which could be because of exhaustion of nutrients and production of toxic metabolites suggesting the enzyme being produced in the log phase of the growth^{11,12}. So in further experiments, the lipase production were studied after 96 h culture of *P. aeruginosa* at 37°C. Several reports showed the effect of incubation time on lipase production. *Serratia marcescens* showed a good lipase activity

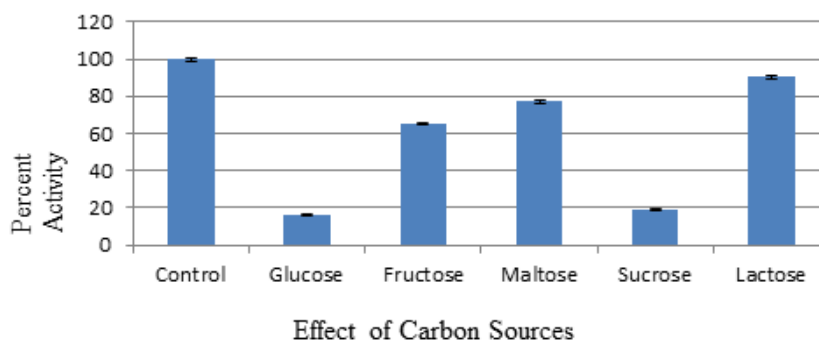


Fig. 3. Effect of different carbon sources on lipase activity by *P. aeruginosa* JCM5962(T): Glucose, fructose, maltose, sucrose and lactose were used as additional nutrient ion, lactose was found to give 90.56% activity in comparison to control. It shows no additional carbon sources required in IAOC medium

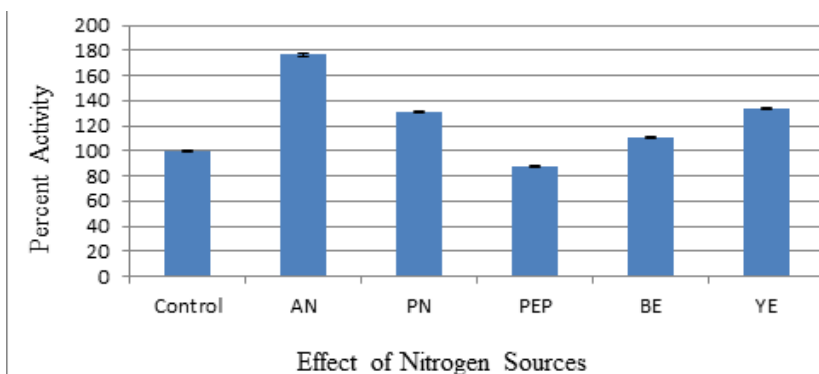


Fig. 4. Effect of different Nitrogen sources on lipase activity by *P. aeruginosa* JCM5962(T): Supplementry nitrogen sources were used as for the AIOC medium, in which AN was found to give 176.49% activity in comparison to control. AN - Ammonium nitrate, PN - Potassium nitrate, PEP - Peptone, BE - Beef extract, YE - Yeast extract

after 6 days of incubation¹³ and *Psychrobacter sp.* Ant300¹⁴, *Pseudoalteromonas sp.* wp27¹⁵ produced cold active lipase after 14 days of incubation.

Effect of Carbon Sources

The impact of additional 1% (w/v) carbon sources was studied (figure 3). Among the tested carbon sources, it was found that lactose induced maximum production of lipase (90%) which was 10% less than control (AIOC without any additional nutrient). However in this study, the enhancing effect in lipase production by supplying different carbon sources was limited, the maximum lipase producing carbon source was found to

be lactose which also did not increase enzyme production. Whereas the results reports of Alkan *et al.*¹⁶ stated that the lipase production by *Bacillus coagulans* under SSF using melon wastes was highly influenced by sucrose. And also maximum lipase production by *Pseudomonas aeruginosa* PseA in sucrose supplied medium¹⁷.

Effect of Nitrogen sources

Nitrogen source is another important factor in any fermentation process for successful production of biomolecules. The effect of organic and inorganic nitrogen sources in SmSSF is shown in figure 4. Ammonium nitrate, potassium nitrate,

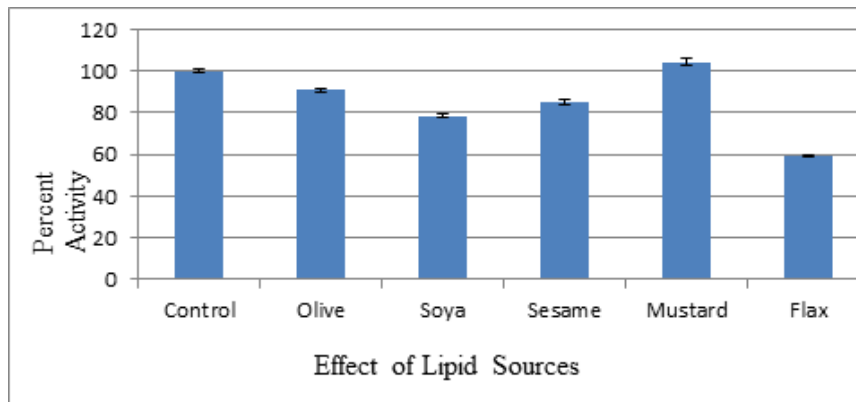


Fig. 5. Effect of different edible oils on lipase activity by *P. aeruginosa* JCM5962(T): Olive oil, soybean oil, sesame oil, mustard oil and flax oil (.5%) were used as additional triglycerides sources for the AIOC medium. Mustard oil was found to give 104.12% activity in comparison to control whereas all others inhibited the lipase activity. It shows AIOC did not need any additional lipid source

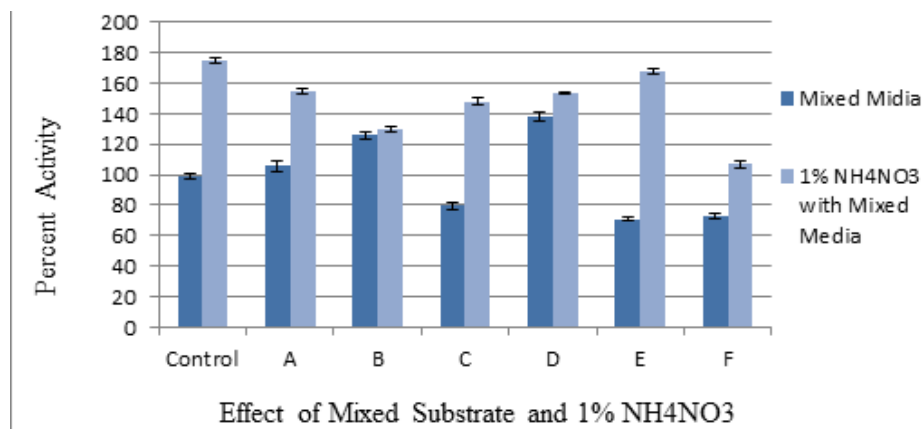


Fig. 6. Effect of mixed media and media with 1% ammonium nitrate on lipase activity by *P. aeruginosa* JCM5962(T): Mixed media, A (AIOC with FOC), B (AIOC with MOC), C (AIOC with castOC), D (AIOC with COC), E (AIOC with GOC) and F (AIOC with SOC) along with control (AIOC) were studied and each combination was also tested with 1% ammonium nitrate as additional nitrogen source. Results presented as percent activity \pm SD, revealed AIOC combined with coconut oil cake in 1:1 ratio gave maximum activity which is 38% more than control (AIOC)

peptone, beef extract and yeast extract (1% w/v) when used as additional sources increased the production of lipase from *P. aeruginosa* upto 179.47%. Ammonium nitrate was found the best additional source as it increased 79% lipase activity in comparison of AIOC without any additive. However, supplementation of peptone was found to have no significant role in increasing the production. An increase in lipase production when ammonium nitrate was added as inorganic nitrogen source to the *Geotrichum candidum* has been shown¹⁸. According to one report lipase production by *Rhizopus homothallicus* under SSF was highly influenced by ammonium nitrate supplied medium¹⁹.

Effect of Lipid sources

The supplementation of different triglycerides, i.e. flax, sesame, mustard, olive, and soybean oil at concentration of 0.5% were added to the AIOC SmSSF medium and lipase activity was detected. The results in the present study revealed that (figure 5) only mustard oil increased lipase activity marginally (4.12% increase) when compared with control, whereas rest of the oil supplements suppressed the lipase production. The reason for this may be presence of required lipids in oil cake itself. According to Eitenmiller et al.²⁰ it may also be due to the fact that higher oil content resulted in the formation of a biphasic system. In the study inhibited production of lipase by *Penicillium roqueforti* was seen by butter oil, corn oil, olive oil. Whereas Lima and coworkers²¹ reported that olive oil induced higher yield of lipase by *Penicillium aurantiogriseum*.

Effect of Mixed Substrate

Further studies were made to evaluate the additive effect of other oil cakes by mixing them with the AIOC in the ratio of 1:1. Preparations of various mixed substrates of AIOC along with sesame, flax, castor, mustard, groundnut and coconut oil cake were made. As represented in figure 6, coconut oil cake was found to be the most suited for mixed substrate with 38% increase in lipase production after 96 h of incubation at 37°C followed by mustard oil cake (25.95%) and flax oil cake (5.6%). However castor, groundnut and sesame oil cakes inhibited the enzyme production. Each combination was also tested with 1% w/v nitrogen source (ammonium nitrate) and though an

increase in production was obtained, it was not so significant with highest yielding mixed substrate (AIOC+COC). Another interesting observation was 29% inhibition in enzyme activity with AIOC and GOC mixed substrate whereas 172% up regulation in lipase production in the same SmSSF medium (AIOC+GOC) supplemented with NH_4NO_3 . In other similar work, increased production of bacterial lipase was found with combination of glucose and ammonium nitrate²². Similarly, a very good lipase production by *Aspergillus niger* by optimizing mixed substrate in ratio of 1: 1 was obtained²³.

This study correlates that lipase production by AIOC and groundnut oil cake under SmSSF was highly influenced by ammonium nitrate supplied medium. Similarly many reports indicate that the lipase production was high in ammonium nitrate added medium²¹.

CONCLUSION

One of the key areas in modern industrial biotechnology is optimum use of by-products of agriculture waste for growth of microorganisms or production of enzymes (e.g. residues from oil seeds). According to present work, the extracellular lipases could be economically produced by a novel high lipolytic *P. aeruginosa* JCM5962(T) by semi-solid state fermentation using easily available, non edible and low cost *A. indica* (neem) oil cake as potent substrate. However, blending of *A. Indica* oil cake along with coconut, mustard and flax oilcake under optimized condition showed better yield of lipase. AIOC along with COC and ammonium nitrate has been found to be a potential substrate for industrial production of extracellular lipase in SmSSF. It is also reported that production of lipase through low cost *A. indica* oil cakes SmSSF might not only reduce the cost of production but also result in many fold increase in the lipase yield when compared with submerged fermentation.

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