Trop J Nat Prod Res, June 2023; 7(6):3240-3245 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Optimization of Culture Conditions for Lipase Production by *Pseudomonas aeruginosa* ECS3

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ARTICLE INFO

Article history:
Received 29 March 2023
Revised 18 May 2023
Accepted 26 May 2023
Published online 01 July 2023

ABSTRACT

Lipases are hydrolytic enzymes with different biotechnological applications in food, detergents, pharmaceuticals, paper, and pulp industries. In lipase-producing bacteria, the encoding genes are responsible for enzyme synthesis. Optimization of medium parameters is an essential factor in the large-scale production of enzymes of biotechnological importance, and this served as the basis for this study. In this study, isolates ECS1, ECS3, ECS11, ECS14, ECS 19, ECS24, and ECS28 were screened for lipase production on tributyrin agar plates. The highest lipase-producing isolate was identified by sequencing the 16S rRNA region following a PCR procedure using a specific primer. The presence of the LipA gene in the isolate was confirmed by polymerase chain reaction (PCR) amplification of their specific primers. In addition, some physical and nutritional parameters were optimized for lipase production. On tributyrin agar plates, isolate ECS3 had the highest zone of hydrolysis (12mm) and was therefore selected. Based on the 16S rRNA sequencing, the highest lipase producer was identified as *Pseudomonas aeruginosa* ECS3. This study also ascertained the presence of the LipA gene in this bacterium with a positive band of 371 bp. Optimum activities were observed at pH (8), temperature (35ºC), and incubation period (24 h). The activity of *Pseudomonas aeruginosa* ECS3 lipase was enhanced by yeast extract (0.5% w/v), lactose (0.5% w/v), agitation speed (120 rpm), and 1% substrate concentration. The study showed the influence of different culture conditions on lipase production by *Pseudomonas aeruginosa* ECS3 with the LipA encoding gene.

Keywords: Identification, Isolation, Lipase, Optimization, *Pseudomonas aeruginosa* ECS3

Introduction

Enzymes have been used in daily human activities since time immemorial, demonstrating the inherent uniqueness of this group of natural catalysts. Lipases, a prominent example, are involved in the catalysis of triacylglycerides to release fatty acids, diacylglycerol, monoacylglycerol, and glycerol [1,2] They are also known to catalyze different bioconversion reactions in living organisms.[3] Over the years, lipases have been produced by microorganisms, plants, and animals. Interestingly, microbial lipases have unique properties such as fast growth, ease of genetic manipulation, specificity, selectivity, high stability, low production cost, and absence of seasonal fluctuations. This has increased their demand in industrial sectors such as textile, detergent, cosmetics, pharmaceuticals, agriculture, food, and paper.[3, 4] It is noteworthy that the global market value for microbial lipase was $349.8 million in 2019 and is estimated to reach $428.6 million by 2025.[3] Major producers of lipases have been identified as gram-negative bacteria, especially *Pseudomonas*.[5] and *Arthrobacter*. Others include *Bacillus*, *Staphylococcus*, *Streptococcus*, *Enterobacter*. Water, soil, and oil-contaminated sites have served as reservoirs for lipase-producing microorganisms.

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Citation: Ogunniran AO, Odeleye BO, Femi-Ola TO. Optimization of Culture Conditions for Lipase Production by *Pseudomonas aeruginosa* ECS3. Trop J Nat Prod Res. 2023; 7(6): 3240-3245 http://www.doi.org/10.26538/tjnpr/v7i6.27

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Approximately 99% of soil microorganisms are yet to be explored in the laboratory, making soil an ideal source for unknown functional organisms.[6] Since enzymes are proteinous, the genes coding for the presence of the proteins are known to influence enzyme function and structure in the cells of living organisms. Therefore, the expression of lipase production by some bacterial species can be attributed to the presence of some lipase-encoding genes. For instance, LipA, which is the gene encoding the lipase activity of some *Pseudomonas* species, has been reported by some authors.[7, 8] In addition, agitation speed, incubation period, pH, temperature, and carbon and nitrogen sources are some fermentation conditions affecting enzyme production. Since the production medium contributes to almost 40 % of the total cost, there is a need to optimize different factors that would enhance lipase production.[9, 10] Although some studies have addressed the optimization of various parameters for lipase production, each bacterial isolate has its specific nutritional and environmental parameters which enhances their growth and enzyme production. In addition, there is a continuous search for bacterial isolates capable of optimally producing the enzyme for various industrial uses. Hence, this research aimed to optimize different culture conditions that would enhance lipase production by the selected isolate.

Materials and Methods

Microorganisms

The bacterial isolates used in this study were previously isolated from engine oil-contaminated soil in Ado-Ekiti, Ekiti State, Nigeria.

Screening for Enzyme Production

The lipolytic activity of the bacterial isolates was tested by slightly modifying the procedure by Sirisha *et al*.[11] The pure bacterial isolates were spot inoculated on the solidified agar, and the plates were

incubated at 37ºC for 24 - 72 h. The colonies with a zone of clearance were considered positive, while an uninoculated plate served as a control.

Identification of the Bacterial isolate
The highest lipase producer following the quantitative screening was identified using morphological and biochemical characterizations such as catalase, gelatin, oxidase, motility, and citrate. For molecular identification, a DNA isolation kit (Promega, USA) was used to isolate and purify the genomic DNA of the bacteria isolates based on the manufacturer's instructions.[12] The 5′ end of the 16S rDNA gene was amplified as outlined by Muhonja *et al*.[13] The PCR products were visualized through electrophoresis on a 2% agarose gel with ethidium bromide added directly. The 1.5kbp products were subjected to Sanger dideoxy sequencing.

PCR Detection of Lipase Gene
Molecular investigation of lipase-coding genes was done by simple PCR on the extracted DNA using LipA primers: Primer1: Sense "5´-ATGGTTCACGGTATTGGAGG-3' Primer2: Antisense 5´-CTGCTGTAAATGGATGTGTA-3' ".[14] Lipase A gene sequence amplified by PCR was approximately 371 bp. A total of 25 µL was used for the amplification, with each reaction mixture including the master mix (10 µL), primer (1 µL), molecular grade (4 µL), and DNA template (3 µL). A negative control (a reaction lacking the template DNA) was included for amplification. The PCR conditions for the amplification were: initial denaturation at 94ºC for 4 min, 30 cycles of 5 sec at 94ºC and 20 sec at 59ºC, and a final extension of 5 min at 72ºC. The gel electrophoresis was done on a 2% agarose gel by slightly modifying the method of Odeyemi *et al*.[15] The gel was electrophoresed at 120V for 45min, visualized by ultraviolet trans-illumination, and photographed. After that, the size of the PCR product was estimated.

Submerged Fermentation of Pseudomonas aeruginosa ECS3
By modifying the method of Mobarak-Qamsari *et al*.[16], a medium of the following composition was used (%, w/v): NaCl (0.25 g), $MgSO_4 \cdot 7H_2O$ (0.04 g), peptone (0.5 g), glucose (0.5 g), K_2HPO_4 (0.09 g), $CaCl_2$ (0.04 g), $(NH_4)_2SO_4$ (0.1 g); olive oil (1.0%), pH 7 was autoclaved at 121ºC for 15 min. The medium was inoculated with the bacterial isolate and was incubated at 37ºC for 24 h. After that, the medium was centrifuged at 4000 rpm for 20 min while the supernatant was used to evaluate the enzyme's activity.
Lipase Activity
The enzyme assay was done following the method outlined by Alami *et al*.[17] with slight modifications. The reaction mixture was incubated at room temperature for 15 min, and absorbance was measured using a spectrophotometer at 410 nm.

Optimization of Culture Conditions
The following parameters were optimized for lipase production, and the experiments were done in triplicates, except pH, which was presented as an average of duplicate readings.

Temperature
By modifying the method of Kamaladevi *et al*.[18], the incubation temperature of the fermentation medium was varied between 25 and 60ºC, at pH 7.0 for 24 h. The lipase activity was determined as earlier stated.

pH
By modifying the method of Devi *et al*.[19], the pH temperature of the fermentation medium was varied between 5 and 10, using different buffer systems. A pH meter was used to adjust the medium before sterilization, and the flasks were incubated at 37ºC for 24 h.

Incubation period
The best incubation period was studied by modifying the method of Kamaladevi *et al*.[18] The fermentation flasks were incubated in an incubator for varying periods (12 to 72 h), pH 7.0 at 37ºC. At a 12-h interval, the flasks were withdrawn, and the enzyme activity was evaluated.[18]

Carbon Source
Different carbon sources (0.5% w/v), such as maltose, galactose, starch, glucose, sucrose, lactose, and fructose, were supplemented into the medium using the modified method of Rawway *et al*.[20] Experimental conditions used were pH 8.0, the temperature of 35ºC. At the same time, the fermentation flasks were incubated for 24 h using an incubator. These were the optimum conditions gotten earlier. After that, their individual effects were determined.[21]

Nitrogen Source
This was estimated by incorporating $NaNO_3$, KNO_3, NH_4Cl, $(NH_4)_2SO_4$, urea, gelatin, peptone, and yeast extract as nitrogen sources into the medium. This was measured by weighing 0.5 g of the individual nitrogen sources into the fermentation medium, pH 8.0. Incubation conditions were at 35ºC for 24 h using an incubator. After that, their individual effects were determined.[21]

Substrate concentration
The effect of substrate concentration, casein, was studied by varying different substrate levels (0.5, 1, 1.5, 2, and 2.5% v/v) in the production medium. This was measured by weighing 0.5 g of the individual nitrogen sources into the fermentation medium, pH 8. Incubation was at 35ºC for 24 h using an incubator.

Agitation speed
By modifying the method of Veerapagu *et al*.[22], the fermentation flasks were incubated on a rotary shaker, with a rate varying between 100 and 200 rpm, pH 8 and 24 h of incubation. Another flask was maintained at static conditions. Afterward, the enzyme activity was determined.

Results and Discussion

The isolates screened, which were ECS1, ECS3, ECS11, ECS14, ECS 19, ECS24, and ECS28, all had a zone of hydrolysis with a diameter of 11, 12, 4, 8, 8, 5, and 6 mm, respectively, on tributyrin agar plates. Isolate ECS3, which had the highest zone of hydrolysis, with a diameter of 12 mm, was selected for further studies. The highest-producing isolate had a smooth surface, a circular colony shape, and was greenish on a nutrient agar plate. The microscopic identification revealed isolated ECS3 as a Gram-negative rod-shaped bacterium. It was positive for catalase, gelatin, oxidase, motility, and citrate. In addition, it utilized monosaccharides, disaccharides, and sugar alcohols (mannitol and sorbitol). On NCBI BLASTn, the 16S rRNA gene sequences of isolate ECS3 were compared to closely related 16S rRNA sequences. Isolate ECS3 had 100% similarity with *Pseudomonas aeruginosa* AT1RP4 accession number LT797517.1.
PCR Amplification of lipase (LipA) gene of *Pseudomonas aeruginosa* is shown in Figure 1. While *Pseudomonas aeruginosa* ECS3 amplified the LipA gene region, positive bands of 371 bp validated that the LipA gene was present in the isolate. Some similar studies revealed that different *Pseudomonas* species isolated from abattoir soil and vegetable oil-polluted soil produced lipase.[23, 24] The lipase gene was confirmed in *Pseudomonas aeruginosa*[7] and *Aspergillus flavus*. [25] The ability of the organism to hydrolyze the substrate clearly shows that the genome harbors the relevant gene.
Parameters influencing enzyme production include temperature, incubation period, pH, etc. It was noticed that lipase activity increased up to 35ºC, which was the optimum, followed by a decline of more than 50%. This indicates that *Pseudomonas aeruginosa* can produce lipase at 35ºC. It appears that the lipase activity slightly increased at 45ºC, possibly due to environmental factors (Figure 2). Similarly, lipase production by *Pseudomonas* sp., *Penicillium fellutanum, Kocuria flava* Y4, and *Providencia stuartii* was stimulated at 35ºC.[26-29] Lipase from *Staphylococcus* sp., *Bacillus cereus*, and *Geobacillus stearothermophilus*, on the other hand, were maximally obtained at 37ºC,[30] 45ºC,[31] and 60ºC,[32] respectively. This shows that the physical properties of the cell membrane, energy metabolism, and translational synthesis of proteins are affected by temperature.[33]

Lipase production by *Pseudomonas aeruginosa* increased from pH 5 to pH 8. At pH 7, more than 50% of the enzyme's activity was retained, while the activity peaked at pH 8. After that, there was a decline in enzyme activity (Figure 3). It was also noticed that enzyme activities were greatly retarded at pH 5 and 10. This shows the alkalophilic nature of the bacteria. Lipases that thrive in alkaline conditions are of great advantage in industrial processes. Some researchers in their investigations also reported a maximum pH of 8 for the lipase activity of *Kocuria flava* Y4,[28] *Staphylococcus* sp.[30], and *Bacillus cereus* NC7401.[34] Meanwhile, pH 7 was reported for *Pseudomonas* sp. and *Providencia stuartii*,[35, 29] while *Bacillus* species produced lipase at an acidic pH of 5.[36] A relationship exists between environmental pH and enzyme production by microorganisms. In addition, the distribution of charges on enzyme molecules and substrates often determines the binding of the substrate and catalysis.[37]

The effect on the incubation period revealed 100% enzyme activity at 24 h, followed by a decline. About 91 and 64% of activity were retained after 36 and 48 h, respectively. Beyond 48 h, lipase activity declined rapidly (Figure 4).

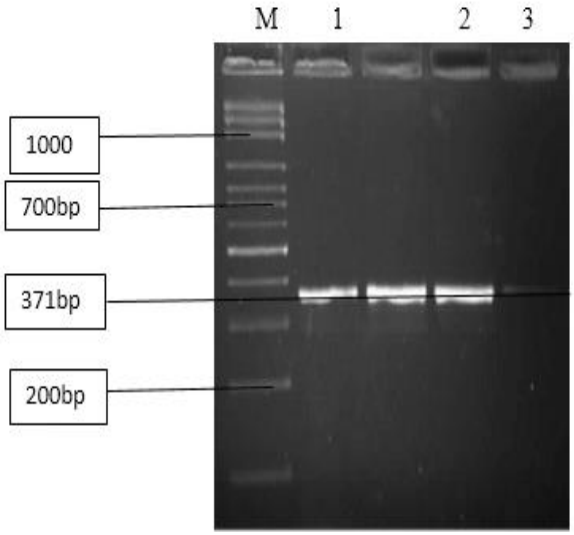

Figure 1: PCR Amplification of lipase (LipA) gene of *Pseudomonas aeruginosa*. Key: M: Molecular marker; 1: Positive control; 2: *Pseudomonas aeruginosa*; 3: Negative control

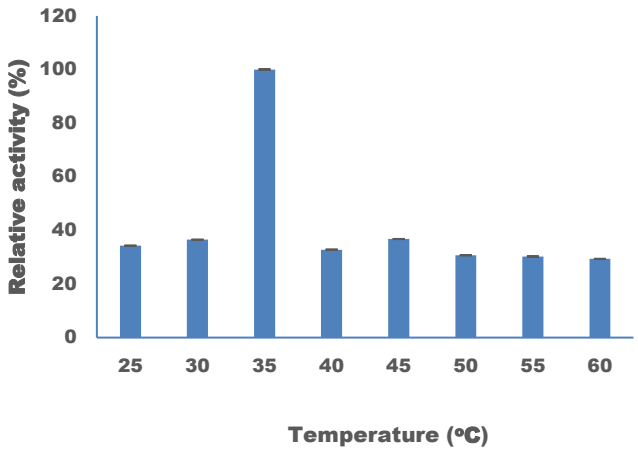

Figure 2: Effect of temperature on lipase activity

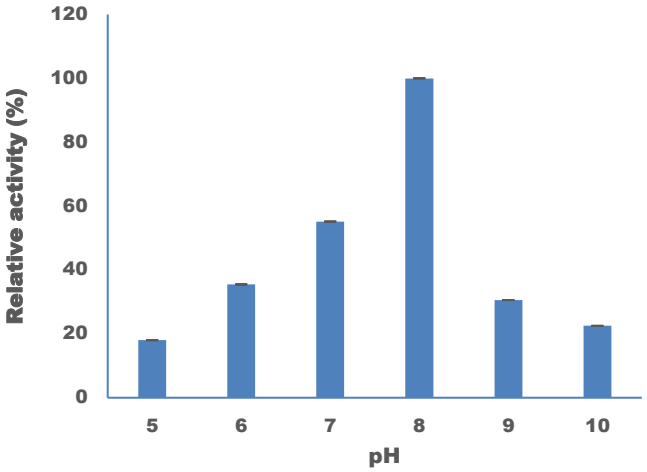

Figure 3: Effect of pH on lipase activity

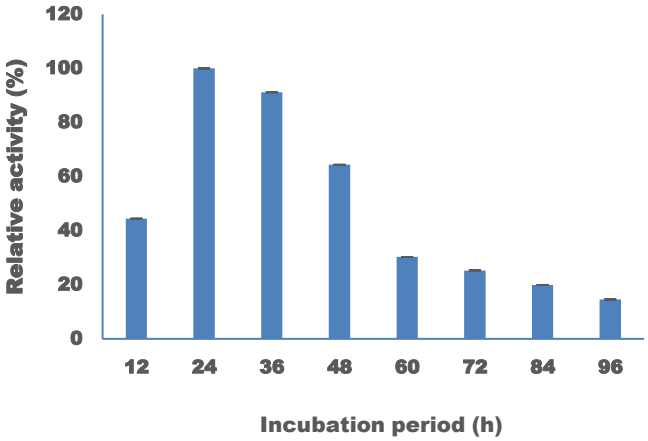

Figure 4: Effect of incubation period on lipase activity

This was confirmed by similar results where the lipase production by *Penicillium fellutanum* and *Bacillus stearothermophilus* was enhanced after 24 h of incubation.[27, 38] Oni *et al*.[39] reported an optimum incubation period of 18 h for lipases produced by *Bacillus subtilis* and *Bacillus cereus*. However, some studies revealed an incubation period of 48h for maximum lipase production by *Pseudomonas aeruginosa* and *Geobacillus stearothermophilus*, respectively.[32, 26] The short incubation period puts this enzyme in an advantageous position because it tends to increase the rate of turnover of this enzyme production. A relationship exists between the incubation period and enzyme production to a certain degree, where enzyme activity tends to reduce beyond the optimum incubation condition. At prolonged incubation periods, low enzyme activity is often noticed. This may be caused by moisture loss, a reduction in nutrients, pH changes, the buildup of toxic by-products, or the fermentation medium containing proteases.[32, 40]

This investigation shows that the addition of fructose as a carbon source inhibited enzyme production, while others enhanced enzyme production. The addition of lactose to the medium enhanced its activity, thus promoting enzyme production (Figure 5). This is consistent with previous studies in which lactose encouraged lipase production.[27, 38] Meanwhile, glucose-enhanced lipase production by *Pseudomonas* sp. and *Kocuria flava* Y4.[35, 28] Contrarily, the lipase of *Pseudomonas aeruginosa* was not enhanced by any carbon source as compared with the control.[26] Since lipases are inducible enzymes, carbon sources often promote their gene expression. Carbon sources, which supply energy for growth and enzyme production, are catabolized by carbon catabolite regulation (CCR).[41] All the nitrogen sources enhanced lipase production except KNO_3, as observed in this study. This result also revealed the preference of the bacteria for organic nitrogen sources as all the organic

nitrogen enhanced the enzyme activity compared to the inorganic nitrogen source. The most effective nitrogen source was yeast extract, followed by peptone (Figure 6). In previous studies, *P. aeruginosa*,[6] *Kocuria flava* Y4,[28] *B. subtilis*, *B. cereus*,[39] and Isolate PKRU-9[42] were reported to utilize yeast extract for lipase production. In addition, KNO_3 did not support lipase production by *P. aeruginosa*.[6] However, some *Pseudomonas* and *Bacillus* species utilized peptone[35] and tryptone[43] as good nitrogen sources for lipase production. There were reports from previous studies where lipase production was greatly enhanced by organic nitrogen sources.[44, 45] Contrarily, the investigation of Demirkan *et al*.[31] showed that inorganic nitrogen sources were more effective in lipase production. Amino acids and growth factors are essential during enzyme production, and these can be readily provided when nitrogen sources are incorporated into the medium.[46] Each microorganism has its preferred nitrogen source.

The stimulatory effect of the concentration of the inducer used in this study was noticed. An increase was detected up to 1% (Olive oil), followed by a gradual decline (Figure 7). Concentrations above 1% reduced lipase activity because microbial growth is inhibited at higher concentrations.[47] In their investigation, Shamim *et al*.[48] reported a positive influence of 1% olive oil on lipase production by *Bacillus glycinifermentans*. Using natural oil, such as olive oil, as the substrate is a significant factor in this study because they generally induce or influence lipase production.[49] They also contain oleic acid, which is characterized by a high level of monounsaturated fatty acids, and this has been shown to aid cell growth.[50]

The lipase production was enhanced at 120 rpm when compared with the activities recorded at static conditions, and this may be because the oxygen in the medium was correctly dispersed (Figure 8).

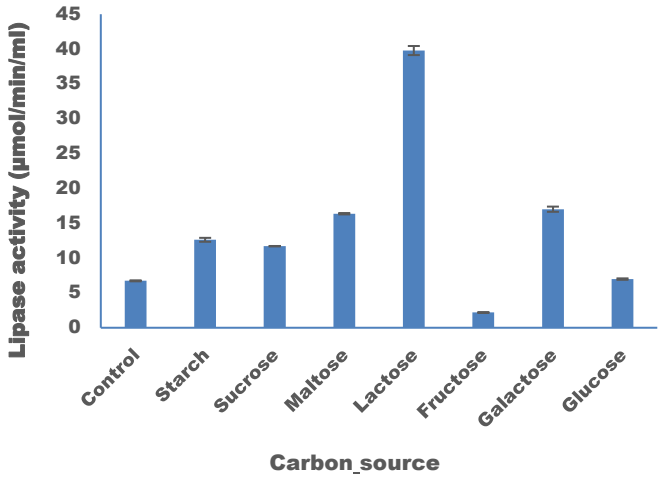

Figure 5: Effect of carbon sources on lipase activity

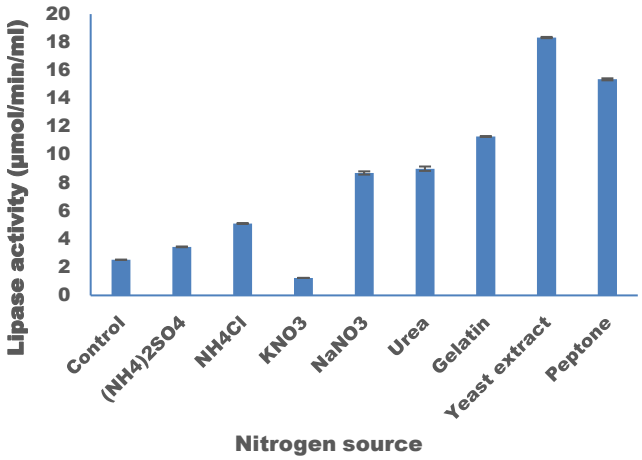

Figure 6: Effect of nitrogen sources on lipase activity

Above 120 rpm, lipase activity was noticed to decrease. Similarly, the lipase enzyme produced by *Bacillus cereus* exhibited its peak activity at 120 rpm.[51] Barik *et al*.[28] also observed an agitation speed of 110 rpm, closely related to our findings. In contrast, Abol-Fotouh *et al*.[32] reported a higher agitation speed of 160 rpm. The decline could be due to the digestion of the enzyme by intracellular enzymes such as protease and esterase during cell disruption, accumulation of hydrogen peroxide, and enzyme denaturation production.[35,52,53]

Conclusion

The lipase activity of *Pseudomonas aeruginosa* ECS3 was modulated by investigating the impacts of physicochemical parameters. A temperature of 35ºC, a pH of 8, and a 24 h incubation time increased lipase activity. The best carbon and nitrogen sources were lactose and yeast extract, respectively. Others include agitation speed (120 rpm) and 1% substrate concentration. The optimum parameters obtained will help in the maximum production of the enzyme.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

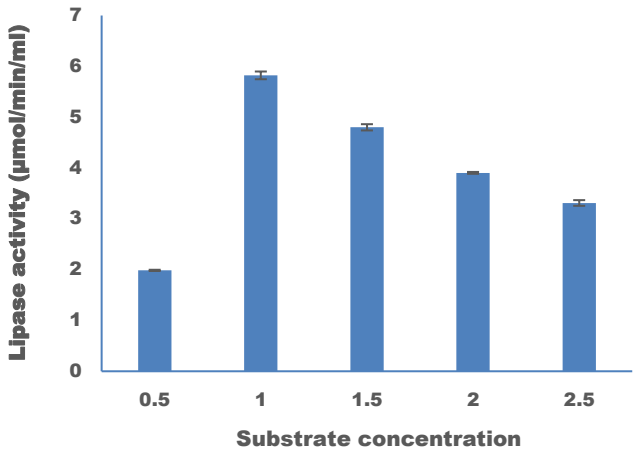

Figure 7: Effect of substrate concentration on lipase activity

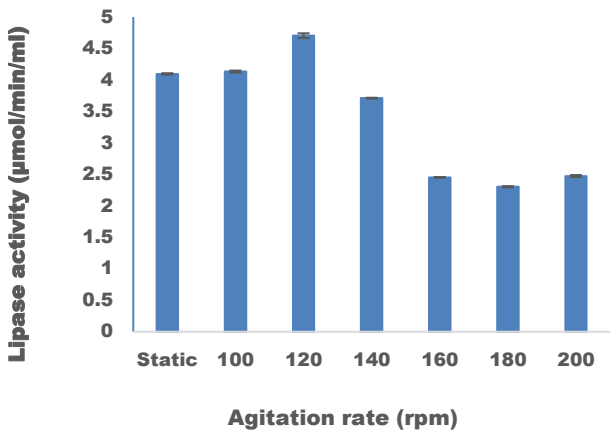

Figure 8: Effect of agitation rates on lipase activity

Acknowledgements

The authors are grateful to the management of Landmark University for providing Laboratory space for this research work.

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