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EVALUATION OF THE LIPASE-PRODUCING POTENTIAL OF *BACILLUS SUBTILIS* AND *PSEUDOMONAS AERUGINOSA* FROM *VITELLARIA PARADOXA* LEAVES

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ABSTRACT
Lipases are industrially important enzymes that are widely used for food product processing, drug processing, biodiesel production, and the bioremediation of contaminants. The main objective of this study was to isolate and identify bacteria that produce lipase from *Vitellaria paradoxa* leaves and to evaluate their enzymatic capabilities. Initial screening was conducted using Rhodamine-olive oil agar plates, where the appearance of orange fluorescent halos under UV light indicated lipase activity. Strains that tested positive underwent additional molecular characterization via 16S rRNA gene sequencing, which confirmed their identities as *Bacillus subtilis* and *Pseudomonas aeruginosa*. Preliminary activity tests revealed that both strains could produce lipase in laboratory conditions; however, each showed distinct enzyme activity patterns when exposed to different pH levels and temperature variations. These initial results suggest that both strains could be refined for targeted industrial applications.

KEYWORDS: Lipase, *Vitellaria paradoxa*, *Bacillus subtilis*, Rhodamine-B, pH, temperature.

INTRODUCTION
Lipases are indeed one of the significant industrially relevant groups of enzymes, especially in the production of biofuels, pharmaceuticals, and food additives. Two of the best-known lipase producers are *Bacillus subtilis* and *Pseudomonas aeruginosa*, hence their attractiveness for biotechnological applications, as described by Abubakar *et al.*[1] Each of these bacteria has shown its ability to produce lipases with different activities and stability under various environmental conditions. *Bacillus Subtilis* has been reported for the production of lipopeptide biosurfactants with immense potential for biotechnological and biopharmaceutical applications, including antimicrobial activities against a wide range of pathogens.[2,3] Similarly, *Pseudomonas aeruginosa* has demonstrated the ability to produce a lipase enzyme, which is very useful in crude oil biodegradation and environmental remediation processes.[4] Interestingly, both species exert synergistic effects when combined, enhancing their overall performance in various applications.[5] Screening for lipase production by *Bacillus subtilis* and *Pseudomonas aeruginosa*, isolated from shea butter leaves, enables the investigation of new sources for such enzymes.

Recent studies have shown the ability of Pseudomonas aeruginosa to harbor extracellular lipases with intense activity at varied physicochemical environments. Unni *et al.*[6] identified that *Pseudomonas aeruginosa* isolated from goats' rumen displayed high lipase activity, with maximum production at pH 8.5 and 37°C. The enzyme possessed high thermal resistance and retained above 80% activity when treated with organic solvents, making it an excellent prospect for non-aqueous catalyzation processes. In addition, Kazeem *et al.*[7] cultured *Pseudomonas sp*. from shea butter mills effluent and reported that shea-nut cake could serve as an effective low-cost substrate for lipase production. Employing response surface methodology, the scientists obtained impressive enzyme yields, indicating that agricultural wastes such as *Vitellaria paradoxa* residues sustain microbial growth and potentially trigger enzyme synthesis due to their intrinsic lipid content.

On the other hand, Gram-positive endospore-forming bacterium *Bacillus subtilis* has proved to have great lipase production capabilities. Its secretion of enzymes directly to the exterior medium and resistance to fluctuating fermentation environments make it of particular industrial fermentation appeal. Kazeem *et al.*[7] built on this by extracting *Bacillus velezensis*—a close relative of *Bacillus subtilis*—from compost and using

shea-nut cake as a growth substrate. Its results demonstrated high lipolytic activity at pH 7.0 and 40°C, and the lipase showed high temperature tolerance, making it useful in thermal-resistant processes. In contrast to *Pseudomonas*, however, which prefers alkaline and weakly saline environments, *Bacillus subtilis* tend to optimally operate at neutral pH with low salinity and so are best suited to some fermentation and biotransformation schemes.

Comparative studies reveal that though both *Pseudomonas aeruginosa* and *Bacillus subtilis* can be effective producers of lipase, both have distinct enzymatic profiles. An example of this is in the research done by Benjamin *et al.*[8] where lipase of *Bacillus* proved to be thermostable but less tolerant to solvent compared to *Pseudomonas* lipase. In addition, Gaur *et al.*[9] noted that *Pseudomonas aeruginosa* lipase hydrolyzed palm oil well at alkaline pH, with 89% substrate hydrolyzed within two hours—the level of efficiency that few of the many *Bacillus* isolates possess. Contrarily, Sharma *et al.*[10] revealed that *Bacillus subtilis* lipase exhibited higher activity remaining following exposure to heat when given enough time, something that ensures higher activity in detergent production in cases where enzymes experience stringent chemicals in processing.

From a biotechnological viewpoint, such co-existence or synergistic application of these bacterial strains would be strategically advantageous. Abubakar *et al.*[11] opined that the combination of lipase-producing strains with different enzymatic profiles can achieve further breakdown of substrate, improved resistance to stress conditions, and expanded substrate specificity. This synergism would be highly useful when it comes to handling complex lipid substrates like derivatives of shea butter that consist of both saturated as well as unsaturated triglycerides. The different metabolic pathways employed by *Bacillus subtilis* and *Pseudomonas aeruginosa* provide varied biocatalytic capabilities, and hence, application of both in combination is an area worthy of further consideration. This work, therefore, aims to study the production of lipases by these bacteria from shea butter leaves and consequently establish new bacterial strains from shea butter leaves as a source of lipases.

MATERIALS AND METHODS

Samples Collection

Shea butter (*Vitellaria paradoxa*) leaves were collected from the Botanical Garden, Akowanjo Area in Ogbomoso. The samples were collected using sterile forceps and stored in sterile glass vials. Following collection, the samples were immediately transferred to the laboratory for examination and subsequent analysis.[12]

Sample Preparation

The leaf samples collected were placed in 10ml of sterile water containing 0.002g of potassium dihydrogen phosphate inside sterile Petri dishes. The Petri dishes containing the leaf samples were then incubated for 3 days at 28^0C. After 3 days, a drop of the solution was aseptically inoculated on a nitrogen-free agar medium for 48 hours at 28^0C.

Culture Examination

Distinct pure colonies were sub cultured in a slant bottle, using Nutrient agar (NA) as the growth medium. The medium was homogenized by autoclaving for 15 minutes at 121°C. The sterilized medium was cooled. 9mls of the medium was poured into the slant bottles and allowed to solidify at room temperature, the isolates were streaked in the slant and incubated at 37°C for 24 hours. The pure cultures were maintained on the slant and stored at 4°C.

Screening Lipase Producing Bacteria

A sensitive and specific plate assay for the detection of lipase-producing bacteria makes use of Rhodamine-olive oil agar (ROA) medium.[13, 14, 15] 300mls of the Rhodamine Olive Oil agar was prepared using 2.4g of nutrient broth, 1.2g of NaCl, 6g of agar agar, 9mls of Olive oil, and 0.5ml Rhodamine B solution. It was then poured into Petri plates under aseptic conditions and allowed to solidify Boonmahome and Mongkoltharuk[16] the bacterial cultures were inoculated into the Petri dishes.

Lipase-producing strains were identified on spread plates incubated for 48 hours at 37°C. Hydrolysis of the substrate resulted in the formation of orange fluorescent halos around bacterial colonies or the appearance of orange fluorescent colonies under UV irradiation, as described by Aknobi *et al.*[17] and Duza and Mastan.[18]

Molecular Characterization of the Isolate

DNA extraction was carried out using a lipase-positive bacterial strain. The target DNA sequence was amplified through PCR utilizing universal primers: forward primer 3′-AGAGTTTGATCCTGGCTCAG-5′ and reverse primer 5′-GGCTGCTGGCACGTAGTTAG-3′. The thermal cycler program began with an initial denaturation step at 94 °C for 2 minutes, followed by 35 cycles that included denaturation at 94 °C for 30 seconds, the annealing of primers was conducted at 55 °C for 30 seconds, followed by an extension phase at 72 °C lasting 1 minute.

A final elongation step at 72 °C for 10 minutes concluded the amplification. The resulting amplicons were analyzed on a 1% agarose gel and compared to a 1 kbp DNA ladder. Quantification of the PCR product was carried out using a Nanodrop spectrophotometer, and the products were purified with the QIA quick purification kit (Qiagen, Germantown, USA). The purified DNA amplicons were then prepared for sequencing, as described by Kozich *et al.*[19]

Lipase Production

A production medium (adjusted to the desired pH values of 7, 9, or 11) was prepared containing 0.75% (w/v) glucose, 0.75% (w/v) peptone, 0.3% (w/v) NaCl, 0.05% (w/v) $MgSO_4$, and 2.0% (v/v) olive oil. The medium was inoculated with 0.75% (v/v) of a 24-hour-old bacterial isolates. Incubation was carried out at different temperatures (37°C, 45°C, 50°C, 55°C, and 65°C) under shaking conditions (150 rpm) for 24 hours to evaluate the effect of these parameters on lipase production. Following incubation, the culture was centrifuged at 10,000 rpm for 10 minutes at 4°C to separate the bacterial cells. The cell-free supernatant was collected and used as the crude enzyme extract for the lipase assay.

Lipase Activity Determination

Lipase activity was determined using the *p*-Nitrophenyl Palmitate (pNPP) assay, following the methodology of Winkler and Stuckmann (1979). A 0.15 M Tris-HCl buffer was prepared and adjusted to pH 7, 9, and 11. The substrate solution was prepared by dissolving *p*-Nitrophenyl Palmitate (pNPP) (10 mM) in isopropanol. For the assay, 2.9 mL of Tris-HCl buffer (pH corresponding to the test condition) was incubated in a water bath at the respective temperature (37°C, 45°C, 50°C, 55°C, or 65°C) for 10 minutes. Following this, 60 µL of pNPP substrate was added and incubated again for 10 minutes. Subsequently, 40 µL of enzyme extract was introduced into the reaction mixture and incubated for another 10 minutes. The reaction was terminated by chilling the solution at -20°C for 1 minute. The amount of *p*-Nitrophenol (pNP) released was measured at 410 nm using a UV/VIS spectrophotometer after bringing the tubes to room temperature. The experiment was performed in triplicate, with appropriate controls, including a blank containing only buffer.

Unit of Enzyme Activity

One unit of lipase activity was defined as the amount of enzyme required to release 1 micromole of *p*-Nitrophenol (pNP) per minute from the substrate *p*-Nitrophenyl Palmitate (pNPP) under the given assay conditions.

RESULT AND DISCUSSION

Leaf samples were submerged in sterile water with potassium dihydrogen phosphate, a technique consistent with current phyllosphere research for extracting leaf-associated microbes which aligns with Bergelson *et al.*[20] Shea butter leaf (*Vitellaria paradoxa*) samples were analyzed to obtain bacterial strains that produce lipase using a screening method used to identify lipase activity as previously reported by Oladipo *et al.*[21] Isolation of lipase-producing bacteria in this study utilized Shea butter leaves (*Vitellaria paradoxa*) as the isolation material, as shown in Figure 1. This was based on the ecological function of Shea butter trees, which are bioactive compound-rich and provide habitat to a rich population of microorganisms. The capacity of plant surfaces to serve as reservoirs of industrially valuable microorganisms renders them significant in biotechnology. This approach aligns with the growing body of research highlighting the potential of the leaf surface as a source of diverse and industrially relevant microbial communities. This is consistent with the findings of Lindow and Brandl,[22] who emphasized the importance of plant surfaces as habitats for diverse microbial populations with potential industrial applications. Isolation on Tributyrin agar was performed as shown in Figure 2, which aligns with the methodology of Mobarak *et al.*[23] who similarly isolated lipolytic bacteria on Tributyrin agar. Tributyrin agar remains an acceptable medium for detecting lipolytic activity because it supports the development of clear zones around lipase-producing colonies. Such bacteria are significant in industries that employ microbial enzymes in the processing of lipids, such as detergent, pharmaceutical, and food industries.

Shea butter (*Vitellaria paradoxa*) leaves

Shea butter leaves soaked in potassium dihydrogen phosphate

Figure 1: Shea butter (Vitellaria paradoxa) leaf samples

Screening for lipase was performed on Rhodamine-B plates containing olive oil, which is a suitable substrate for lipase detection (Figure 2). The strains that produced lipase were identified by colony formation with orange fluorescence under UV light exposure at 350 nm after 48 hours of incubation. Maytham *et al.*[24] also obtained similar findings using spread plates and a 48-hour incubation at 37°C to screen for lipase-producing strains. Hydrolysis of the substrate resulted in the orange fluorescent halo surrounding the bacterial colonies or the formation of orange fluorescent colonies, which were

visible under UV illumination. This is confirmatory of the presence and activity of lipase enzymes and represents an efficient means of screening lipase-producing microbes. Successful utilization of the method finds it effective and suitable in microbial enzyme screening for environmental and industrial uses. The identification of lipase-producing strains using this method ensures the effective selection of potential producers of the enzyme, and this is very important in the optimization of the enzyme for bulk use. Microbial lipases are highly sought after in biotechnological applications, including waste degradation, biofuel production, and synthetic chemistry, thus the identification of new sources would be of great value.

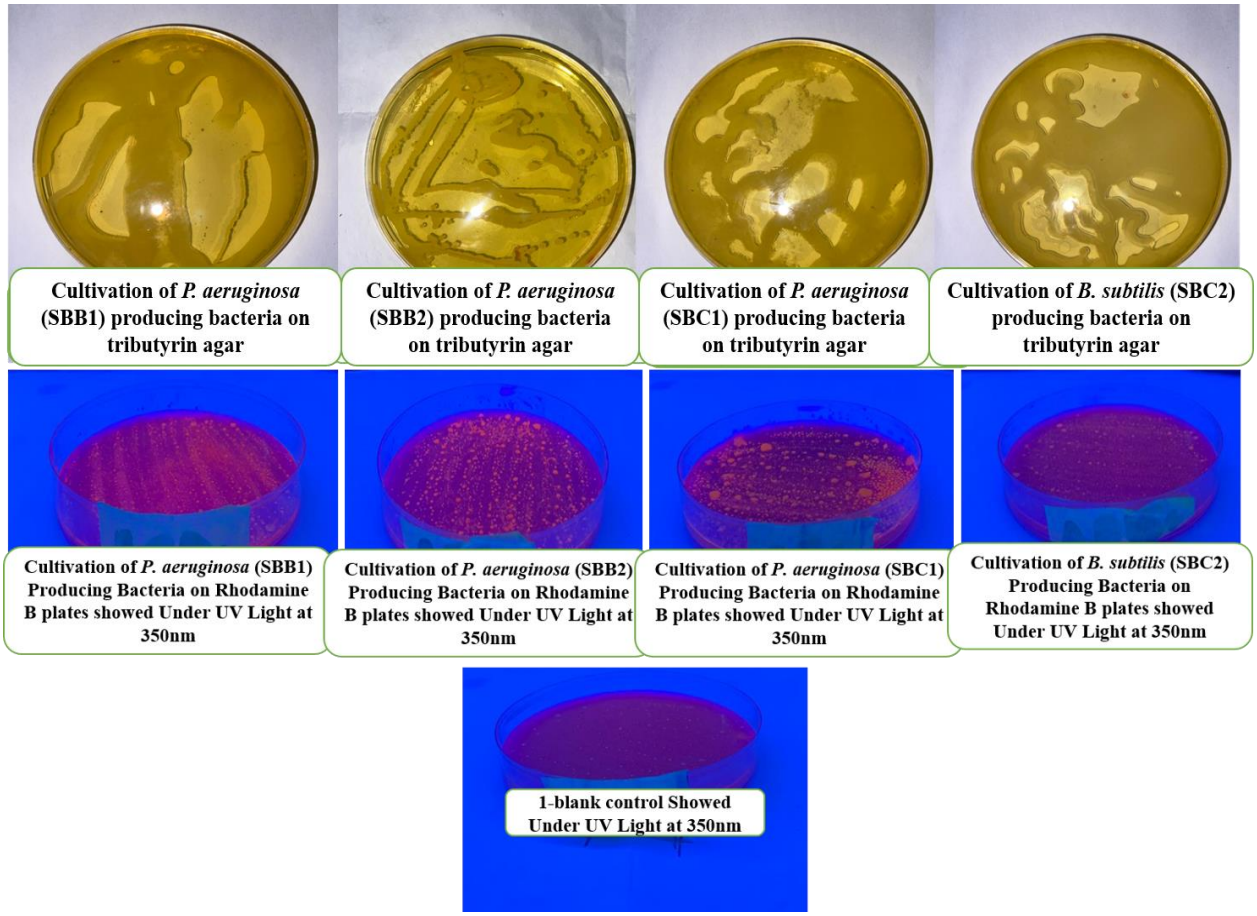

Figure 2: Cultivation of The Lipase Producing Bacteria on both tributyrin agar and Rhodamine B plates (Showed under UV Light at 350nm)

The screening result showed that four Shea butter leaf bacterial isolates were successfully screened as lipase producers. These isolates were further identified through the 16S rRNA gene sequencing technique, a popular bacterial identification method, as shown in Table 1. The method targets a highly conserved ribosomal RNA gene in bacteria and facilitates species-level bacteria identification. This is in agreement with the report of Kim *et al.*[25] who demonstrated the efficacy of 16S rRNA sequencing in identifying bacterial strains with potential lipase production from dairy environments, showing both the utility of this molecular tool and the ecological significance of such studies. The sequencing result revealed the presence of lipase-producing bacteria belonging to two distinct genera: *Pseudomonas aeruginosa* and *Bacillus subtilis*. Three of the isolates were identified as *Pseudomonas aeruginosa*, a well-known lipase producer. Several studies, including Abubakar *et al.*[1] have reported that *Pseudomonas aeruginosa* exhibits greater lipase activity than *Bacillus subtilis*, with the disparity being attributed to genetic diversity and metabolic versatility. Similarly, Mobarak *et al.*[23] confirmed the efficacy of *Pseudomonas aeruginosa* as a lipase producer, isolating a strain from an oil-processing wastewater environment and using 16S rRNA sequencing for effective identification. The identification of these bacterial species is of industrial significance as *Pseudomonas* species are used extensively in commercial enzyme production owing to their capacity for the production of extracellular lipases with broad substrate specificity. *Bacillus subtilis*, on the other hand, is recognized for its stability in enzyme production and capacity to endure a wide range of environmental conditions, and as such, it finds applications in fermentation industries. The presence of these bacterial species on Shea butter leaves suggests that plant-associated bacteria could be exploited as alternative sources of enzyme production to reduce the reliance on costly synthetic enzyme production processes.

Table 1: Molecular Characterization of the selected isolates.

Sample code	Similarity	% identification	Identity
SBB1	*Pseudomonas aeruginosa*	100	*Pseudomonas aeruginosa*
SBB2	*Pseudomonas aeruginosa*	100	*Pseudomonas aeruginosa*
SBC1	*Pseudomonas aeruginosa*	100	*Pseudomonas aeruginosa*
SBC2	*Bacillus subtilis*	100	*Bacillus subtilis*

The lipase production of *Bacillus subtilis* and *Pseudomonas aeruginosa* from *Vitellaria paradoxa* at varying temperatures and incubation times indicates trends consistent with existing research. Lipase production was observed in every condition examined, varying according to the strain of bacteria and the environment.

Several studies Gurung *et al.*[26] have shown that Bacillus subtilis and Pseudomonas aeruginosa produce lipase efficiently in mesophilic to moderate thermophilic temperatures. These findings are in agreement with the results from this study, verifying that the two species can produce lipase efficiently within temperature ranges of 37°C to 65°C (Fig. 3,5, and 7). Also, Jaeger and Eggert.[27] emphasized the duration of incubation as the factor in the biosynthesis of the enzyme, which is in accordance with our findings since lipase production was observed at various times. The same observation from Sarmah *et al.*[28] further supports that enzymatic yield among these bacteria is influenced by the interaction of the strain-specific traits and the external factors.

These results are consistent with recent literature on bacterial lipase production, indicating that *Bacillus subtilis* and *Pseudomonas aeruginosa* of *Vitellaria paradoxa* can serve as stable sources for biosynthesis of lipase if provided with appropriate conditions. The data generated in this study contribute to the growing body of information on microbial lipases with potential biotechnological applications.

The impact of pH on the synthesis of lipase in *Bacillus subtilis* and *Pseudomonas aeruginosa* isolated from *Vitellaria paradoxa* was evident in all the test conditions. The two bacteria produced detectable levels at pH 7, 9, and 11 (Fig. 4, 6, 8), supporting previous findings of Krulwich *et al.*[29] who reported the two species as being capable of producing lipase at neutral to moderately alkaline conditions.

Lipase production was observed at all pH levels, with values being higher at slightly alkaline to neutral conditions. This is consistent with Sarmah *et al.*[28] who indicated that microbial lipase production is stable over a wide range of pH values due to the enzyme's flexibility. The reported production at pH 11, as shown in Figures 4, 6, and 8, is consistent with results by Verma *et al.*[30] where it is indicated that certain bacterial lipases function in extreme environments, yet differences in production could be strain-specific.

The Stability of production under different pH conditions in this study also supports the findings of Yuliani *et al.*[3] who indicated the robustness of bacterial lipases under different physicochemical conditions.

These results validate existing data on the synthesis of bacterial lipases, with both the ability of *Bacillus subtilis* and *Pseudomonas aeruginosa* of *Vitellaria paradoxa* to effectively produce lipases under neutral to alkaline conditions. Such flexibility enhances their viability to be used in industrial processes where enzyme function under different pH conditions is critical.

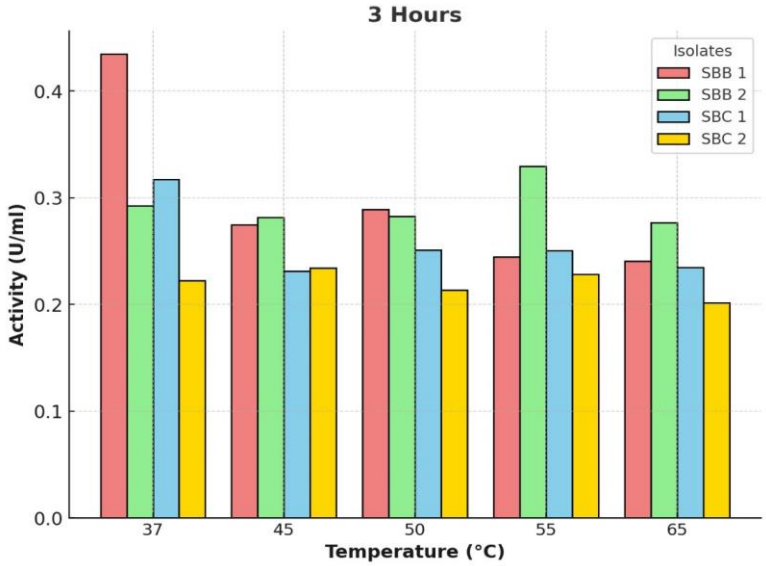

Figure 3: Effect of Temperature on the growth of the isolates at 3 hours

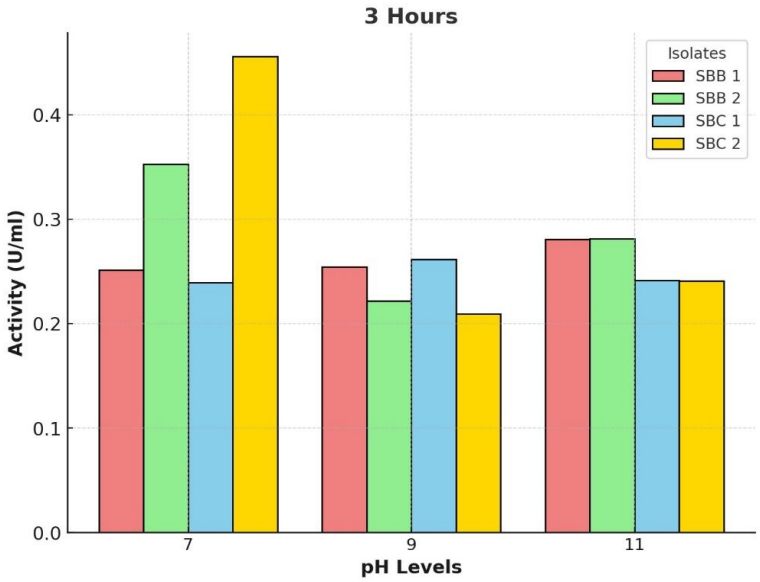

Figure 4: Effect of pH on the growth of the isolates at 3 hours

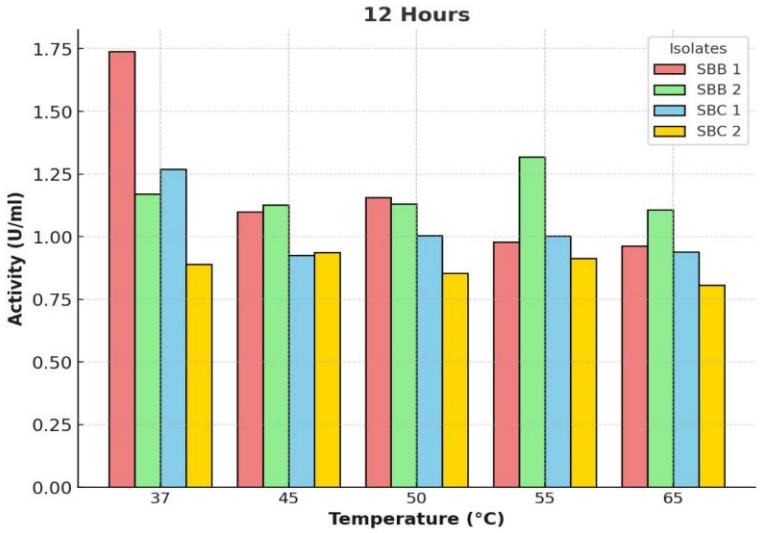

Figure 5: Effect of Temperature on the growth of the isolates at 12 hours

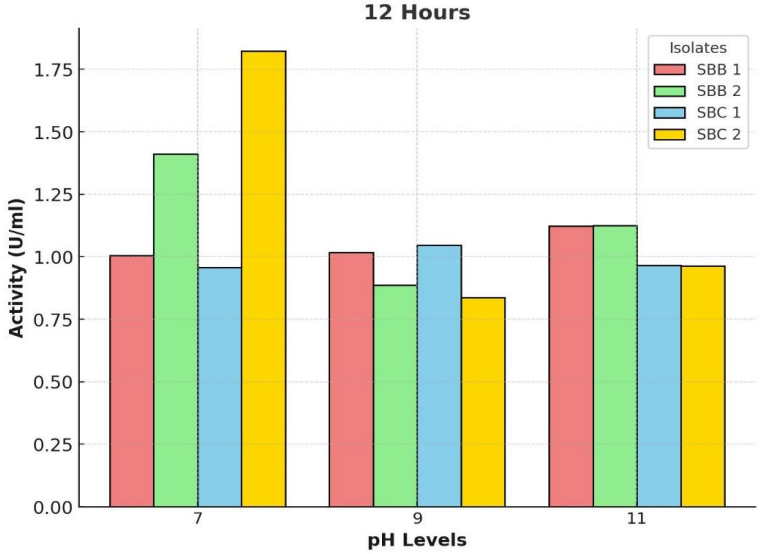

Figure 6: Effect of pH on the growth of the isolates at 12 hours

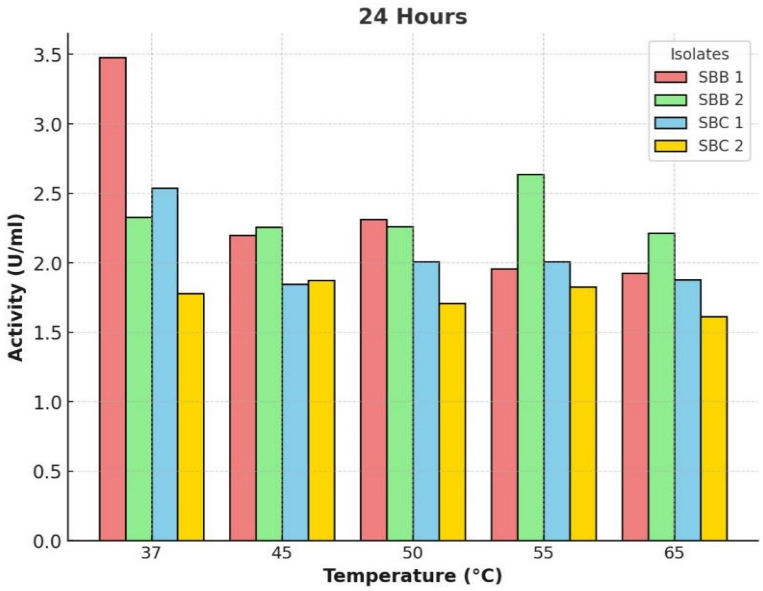

Figure 7: Effect of Temperature on the growth of the isolates at 24 hours

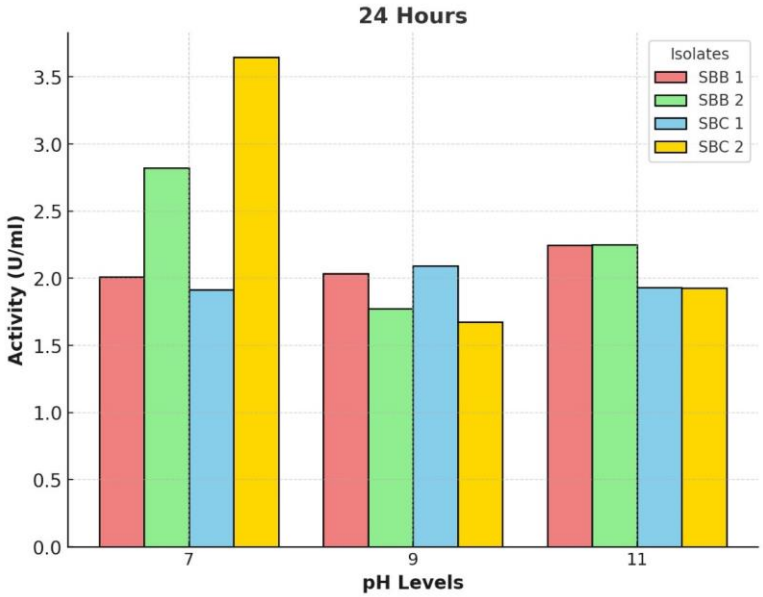

Figure 8: Effect of pH on the growth of the isolates at 24 hours

CONCLUSION

The current study highlights that shea butter leaf samples can be used as a source for the isolation of unique bacterial strains, particularly those with the potential for lipase production. These strains can have significant applications in research laboratories and industrial processes, especially for *Bacillus subtilis* isolated from Shea butter leaves in this study demonstrated lipase-producing potentials.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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