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ENHANCING *BACILLUS* LIPASE PRODUCTION: A COST-EFFECTIVE APPROACH UTILIZING AGRICULTURAL BY-PRODUCTS

ANAM ILYAS, TEHMINA SALEEM KHAN, MADIHA RASHID*, ATIF PREM, USMAN AHMED, NOREEN KAREEM AND MARIA ISHAQ

Department of Botany, Division of Science and Technology, University of Education, College Road, Township Lahore

**Corresponding Author's Email: botanistpk@yahoo.com*

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Abstract

Microbial lipases have significant importance in many industries due to their multidimensional applications. Optimization of cultural conditions can play an important role in their productivity. The present research was based on the isolation of different lipolytic strains of *Bacillus sp.* from oil-contaminated soil, different food products and drainage water. A total of 15 strains were isolated, and 07 showed positive results in Tributyrin Clearing Zone assay. Among them, *Bacillus* sp. UOE-14 (isolated from oil contaminated soil) was quantitatively selected for further studies. Different parameters were studied for enhanced lipase production. Analysis of the data revealed that maximum enzyme activity was observed (27.9 U/mL/min) after 48 h incubation. Effect of pH, agricultural by-products and carbon sources were also studied, and results indicated that pH 8.5, wheat bran, and glucose were the most effective, yielding a 14% lipase increase. $NaNO_3$ as the inorganic nitrogen source was selected because it enhanced lipase production by up to 13% (56.5 U/mL/min). It was also observed that Shan ghee as substrate had great potential to enhance lipase activity (67.4 U/mL/min). The optimal temperature was 30°C for *Bacillus* sp. UOE-14 as at this temperature, lipase activity increased by 8.5% (73.7 U/mL/min). This investigation indicated that different parameters have significant influence on enzyme production and by applying these cultural conditions, lipase production was increased.

Keywords: Agricultural by-products, *Bacillus* sp., Lipase, Oil contaminated soil, Physico-chemical parameters

INTRODUCTION

Lipases (EC 3.1.1.3) are the most significant enzymes for many decades due to their importance in various applications (Cavalcanti *et al*., 2005, Bullo *et al*., 2024). Their main function is to convert diglycerides, monoglycerides, fatty acids and glycerol from triglycerides. Lipases also act on many natural oils, esters of fatty acids and synthetic triglycerides (Kempka *et al*., 2008; Reddy and Pallavi, 2012).

Lipases appeared to be important enzymes due to their multidimensional functions and having vast industrial applications including biomedical sciences, detergents and chemical industry and many other (Vishwe *et al*., 2015; Yasar *et al*., 2020). Furthermore, lipases are very useful in agro-chemical, oleo-chemical, cosmetics, dairy products, nutrition, leather treatment, pesticides, bioremediation processes, surfactants, pollution control, cleaner industries, tannery, wastewater management, meat preservation, fragrance and other organic chemical industries (Liese *et al*., 2000; Hasan *et al*., 2006; Patil *et al*., 2011). Paper and pulp industries also benefit from lipases (Bajpai, 1999; Sharma *et al*., 2014).

Naturally, lipases are produced by plants and animals. A wide range of microbes including bacteria, fungi and actinomycetes also produce high number of lipases in fermentation medium (Reddy and Pallavi, 2012; Lee *et al*., 2015; Amatto *et al*., 2022). The ubiquitous nature of bacteria and fungi helps them in the production of intracellular and extracellular lipases (Gopinath *et al*., 2005). However, bacteria are the best producer of extra-cellular enzymes because they are responsible for the production of half of the industrially important enzymes and are more favorable because they are friendly for environment, non-toxic and no harmful residues (Schallmey *et al*., 2004; Nagarajan, 2012). Many species of the *Bacillus* are good producers of lipases that have useful biotechnological applications (Nthangeni *et al*., 2001; Rahman *et al*., 2003; Ruiz *et al*., 2003). Among these, *Bacillus subtilis* produces a number of lipases whose production may vary under different physical parameters (Eggert *et al*., 2003).

Submerged fermentation (SmF) technique is largely used to produce microbial lipases, preferably from bacteria, because nutrients are easily available to microbes. Solid-state fermentation is also beneficial, however; it is difficult to scale up. The main reason for using SmF is to ease recovery of the end products, disposal of biomass and its purification and it is cost-efficient (Hansen *et al*., 2015; Mazhar *et al.,* 2017).

Optimization of various parameters is the most effective approach for obtaining higher yield of lipases as their production can be enhanced by various physical parameters (pH, temperature and incubation time), nutritional sources (carbon, lipid and nitrogen) and metal ions or salts (Acikel *et al*., 2010; Rajeshkumar *et al*., 2015). It has been illustrated by previous studies that microbial lipases show enhanced production by using agro-waste products like wheat bran, rice husk, banana waste, melon waste and watermelon waste along with optimizing cultural conditions (Dixit and Nigam, 2014; Szymczak *et al*., 2021; Majeed *et al*., 2024).

Pakistan is an agro-based country that produces large quantities of agro-industrial residues which are rich in nutrients like nitrogen, minerals, carbon, and biomass residues. These agricultural wastes can be used as substrates for lipase production. The use of these substrates is economical, as it can help in solving pollution problems. So, the aim of this study was to produce lipases using cost-effective strategies by using agricultural by-products.

Materials and Methods

Sample collection

Samples were obtained from the oil-contaminated soils (OCS), drainage water, decaying foods samples (shawarma, burger and pizza). All samples were collected aseptically in sterile polypropylene containers. To minimize external contamination, sterile collection bottles and spatulas were used during sampling. All samples were transported in an ice box after labeling and processed within 24 h under aseptic conditions.

Isolation of bacteria and cultural maintenance

Samples were serially diluted and inoculated using the streak plate method on sterilized medium containing peptone (0.5%, w/v), yeast extract (0.3%, w/v), Tween-80 (1.0%, v/v), and agar (2.0%, w/v) with pH 7.0. Plates were incubated at 35±1°C for 48 h, and the obtained bacterial colonies were further transferred to get pure cultures. Pure strains were then maintained and sub-cultured on minimal media containing yeast extract (0.3%, w/v), NaCl (0.2%, w/v), peptone (0.5 %, w/v), and agar (2.0%, w/v). The cultures were maintained on slants containing Potato Dextrose Agar (PDA) medium (4.0%) and were stored at 4±1°C in a refrigerator.

Qualitative Characterization and Identification of Isolates

Tributyrin Clearing Zone (TCZ) assay (Carrazco-Palafox *et al*., 2018) was performed for qualitative characterization to screen isolated bacteria for lipolytic ability. Isolates were grown on the minimal media containing tributyrin and were incubated at 30±2ºC for 48 h. Lipolytic bacteria were selected on the basis of appearance of clear zones produced due to hydrolysis of tributyrin. The isolates were tentatively assigned on the basis of their morphology, gram-staining method and microscopic observations. Further preliminary characterization was carried out in the microbiological laboratory of Dr. Ikram-ul-Haq Institute of Industrial Biotechnology (IIIB), GC University Lahore.

Quantitative Screening

Inoculum preparation

Inoculum media was prepared by taking K_2HPO_4 (0.1%, w/v), NaCl (0.2%, w/v), olive oil (0.5%, w/v), and glucose (0.2%, w/v). This medium (25 mL each) was transferred into 250 mL Erlenmeyer conical flasks, these flasks were then cotton plugged and autoclaved. After autoclaving, flasks were allowed to cool at room temperature. Cells of each bacterial strains were transferred aseptically into individual flask using sterilized inoculating needle. The flask was kept in an incubator for maximum growth at 30±2°C for 24 h.

Fermentation technique and Extraction of enzyme

Submerged fermentation technique was used for lipase production. Twenty-five milliliter fermentation media which was prepared using Eggins and Pugh medium (1962) in 1.0 L of water with KH_2PO_4 (1.0g), KCl (0.5g), $(NH_4)_2SO_4$ (0.5g), $MgSO_4.7H_2O$ (0.2g), L-asparaginine (0.5g), yeast extract (0.5g) and $CaCl_2$. $2H_2O$ (0.1g) containing wheat bran (250 mg) with initial pH 5.5 and initial temperature was maintained a 37ºC was added in 250 mL Erlenmeyer conical flask. Flasks were autoclaved and cooled at room temperature, 24 h old freshly grown bacterial cells and were aseptically inoculated and were incubated for 24 h at 30±2°C. Further refinements of cultural conditions were made based on the previously achieved optimal results during this study

After 24 h, fermented broth of each flask was filtered individually by using Whatman filter paper No. 1 to obtain maximum enzyme recovery. The clear filtrate was used in enzyme assay.

Dry Cell Mass

Dry Cell Mass was determined by the modified method of Haq and Daud (1995). The dry weight of the bacterial cell was obtained by harvesting the bacterial cells on the growth medium by filtration through an oven dried (at 50°C for 2 h), and pre-weighed Whatman filter paper No. 1 (9 cm diameter). The cells were washed twice with distilled water to remove traces of medium, and the filter papers together with bacterial cells were dried in oven at 80°C for 24 h, cooled and weighed again using analytical balance. The difference in weight gave the dry weight of the cells.

Enzyme Assay

The filtrate was used for determining the lipase activity (Kundu and Pal, 1970). Without further dilation, the crude enzyme filtrate was used directly. The reaction mixture contained gum acacia 10% (w/v) with 2.0 mL olive oil and 0.6% (w/v) $CaCl_2$ in phosphate buffer (pH 7.0). One milliliter of filtered broth was added to the reaction mixture and then incubated at 30±1ºC for 10 min. After incubation, the reaction was terminated by adding 10 mL acetone: ethanol solution (1:1 ratio). Liberated fatty acids were estimated by titration against 0.05 M NaOH solution using a few drops of phenolphthalein as indicator. One unit (µmole) of lipase activity was defined as "the amount of enzyme which produces one micro mole of fatty acids per minute under defined conditions" (Arima *et al*., 1972). Under identical experimental conditions, a control flask without bacterial inoculum was maintained and served as a blank during titration procedures and enzyme assay.

Lipase activity was calculated using the following formula:

$$\text{Lipase activity (U / mL)} = \frac{(V_s - V_b) \times N \times 1000}{t \times V_e}$$

Where:

V_s = Volume of NaOH used for sample titration (mL)

V_b = Volume of NaOH used for blank titration (mL)

N = Normality of NaOH

t = incubation time (min)

V_e = Volume of enzyme used (mL)

Statistical Analysis

All experiments were performed in triplicate and results were presented as mean ± standard deviation (SD). Treatment effects were compared by the protected Least Significant Difference (LSD) method after Snedecor and Cochran (1980) using software Costat. Bar graphs were made to show the effect of various cultural conditions using Microsoft Excel.

Results

Tributyrin Clearing Zone (TCZ) Assay

Lipase-producing bacteria were isolated from oil-contaminated soils, drainage water, decaying food samples including shawarma, burger, and pizza. Bacterial colonies grown on tributyrin –containing plates were subjected to qualitative screening for lipolytic strains. Several colonies produced a zone of hydrolysis on tributyrin agar. The zone of hydrolysis was produced by 07 strains (UOE-01, UOE-03, UOE-05, UOE-07, UOE-09, UOE-14, and UOE-15) out of 15 (Table 1). The isolates showing zone of hydrolysis were regarded as lipase producers.

Morphological observations

The isolated were appeared to be creamy white, irregular to circular colonies with rough surface under microscope. Further microscopic examination and gram staining method showed gram positive rod shaped structure arranged singly or in chains, which are the characteristic features of *Bacillus* species.

Table 1: Tributyrin Clearing Zone Assay for qualitative characterization of isolates

Strain No.	Source	TCZ Result
UOE-01	OCS	+
UOE-02	Drainage water	-
UOE-03	Shawarma	+
UOE-04	OCS	-
UOE-05	Burger	+
UOE-06	Pizza	-
UOE-07	Pizza	+
UOE-08	Shawarma	-
UOE-09	Drainage water	+
UOE-10	Burger	-
UOE-11	OCS	-
UOE-12	Pizza	-
UOE-13	Burger	-
UOE-14	OCS	+
UOE-15	Shawarma	+

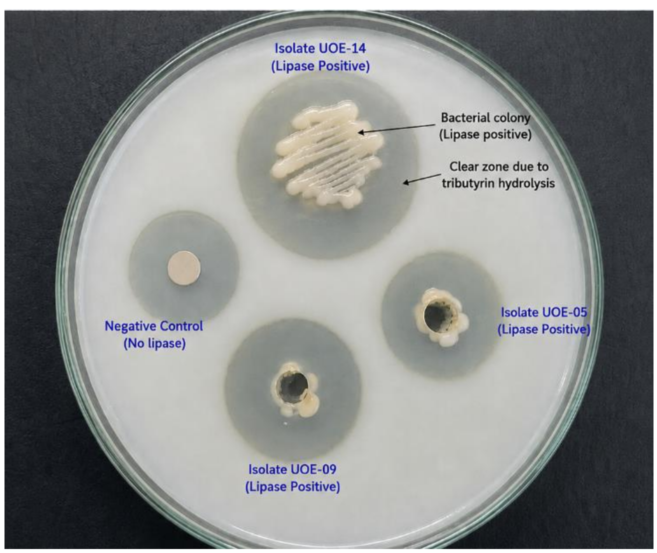

Figure 1: Llipolytic isolates hydrolyze tributyrin assay producing clearing zone around the colonies: UOE-14, UOE-05 and UOE-09 while no clearing zone can be seen in negative control

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Quantitative screening of isolates of *Bacillus* sp.

Bacillus sp.$^{UOE-14}$ strain showed maximum lipase activity of 14.8±0.46 U/mL/min among all tested isolates (Figure 1). In contrast, the minimum activity was observed in the fermented broth of *Bacillus* sp.$^{UOE-03}$ of 3.26±0.60 U/mL/min. Results also showed that *Bacillus* sp.$^{UOE-05}$ and *Bacillus* sp.$^{UOE-09}$ exhibited considerable lipase activity of 9.96±0.51 U/mL/min and 11.8±0.15 U/mL/min, respectively.

Effect of incubation time on the production of lipase

Rate of lipase production by *Bacillus* sp. was checked (0, 24, 48, 72, 96, and 120) hours (Figure 2). It was observed that lipase gave its maximum yield (27.9±0.45 U/mL/min) after 48 h with the dry cell mass (7.34±0.47 mg/mL). But, as the incubation time was further increased, the production rate of lipase was decreased. So, the 48 h was considered as optimized incubation time for *Bacillus* sp. to grow and produce higher amount of lipase.

Effect of initial pH on the production of lipase

In Figure 3, effect of various pH (3.5, 4.5, 5.5, 6.5, 7.5 and 8.5) was investigated for the enhanced production of lipase using submerged fermentation. The lipase activity was high (42.2±0.72 U/mL/min), when the initial pH was 8.5 and at this pH the dry cell mass of *Bacillus* sp. was 6.88±0.81mg/mL. It was also observed that acidic pH below 6.5 was not suitable for lipase production.

Effect of agricultural by-products on the production of lipase

Agricultural by-products were used (1.0 % w/v) as complex carbon sources i.e., wheat straw, rice bran, wheat bran, banola husk and sorghum (Figure 4). Wheat bran was selected as best by maximum yield 42.2±0.25 U/mL/min, that gave the dry cell mass of 7.56±0.96 mg/mL followed by banola husk (4.92±1.36 mg/mL).

Effect of carbon sources on the production of lipase

The effect of different simple carbon source (glucose, sucrose and fructose) on the production of lipase is described by Figure 5. The increased production rate of lipase by *Bacillus* sp. was observed with glucose, but fructose and sucrose affected negatively on production. Hence, glucose (0.1 %; w/v) with dry cell mass 7.22±0.58 mg/mL was selected, as the best carbon source as it gave maximum production of lipase 49.1±0.37 U/mL/min.

Effect of different inorganic nitrogen sources on the production of lipase

To observe the effect of nitrogen source (0.05 %; w/v), $NaNO_3$, $CaNO_3$ and KNO_3 were tested (Figure 6). Among all these, maximum enzyme activity was showed by $NaNO_3$ (56.5±0.41 U/mL/min) with the dry cell mass 8.12±0.2 mg/mL, while $CaNO_3$ minimized the production of lipase. So, $NaNO_3$ was observed to be the rich nitrogen source available to *Bacillus* sp. for the production of lipase.

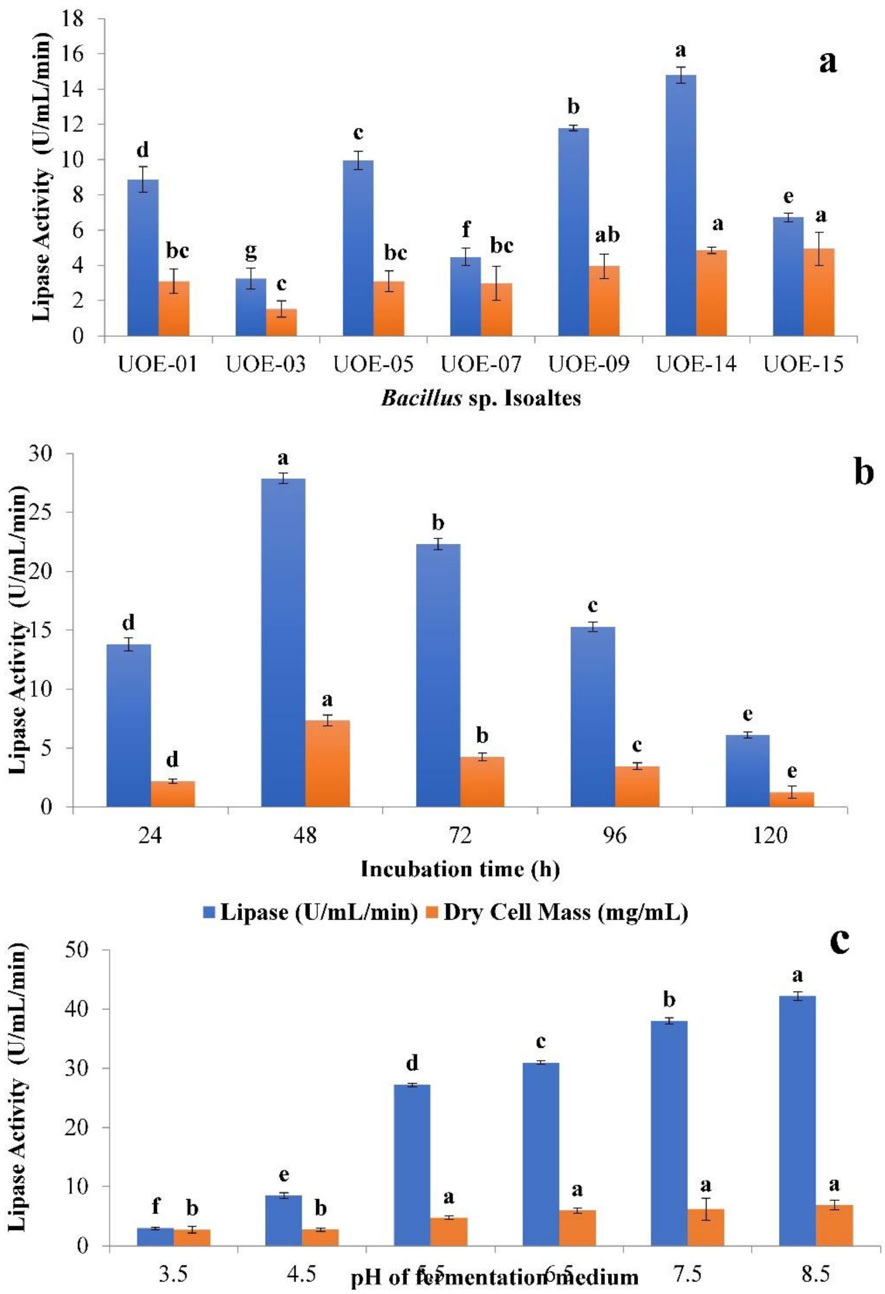

Figure: a) Quantitative screening of different isolates of Bacillus sp. isolates, b) Effect of incubation time on the production of lipase by Bacillus sp. [UOE-14] under submerged fermentation conditions c) Effect of initial pH on the production of lipase by Bacillus sp. [UOE-14] under submerged fermentation conditions. All the experiments were run in triplicates. Error bars show the Standard Deviation among the triplicates. Alphabets in small letters indicate the significance level of treatment effects.

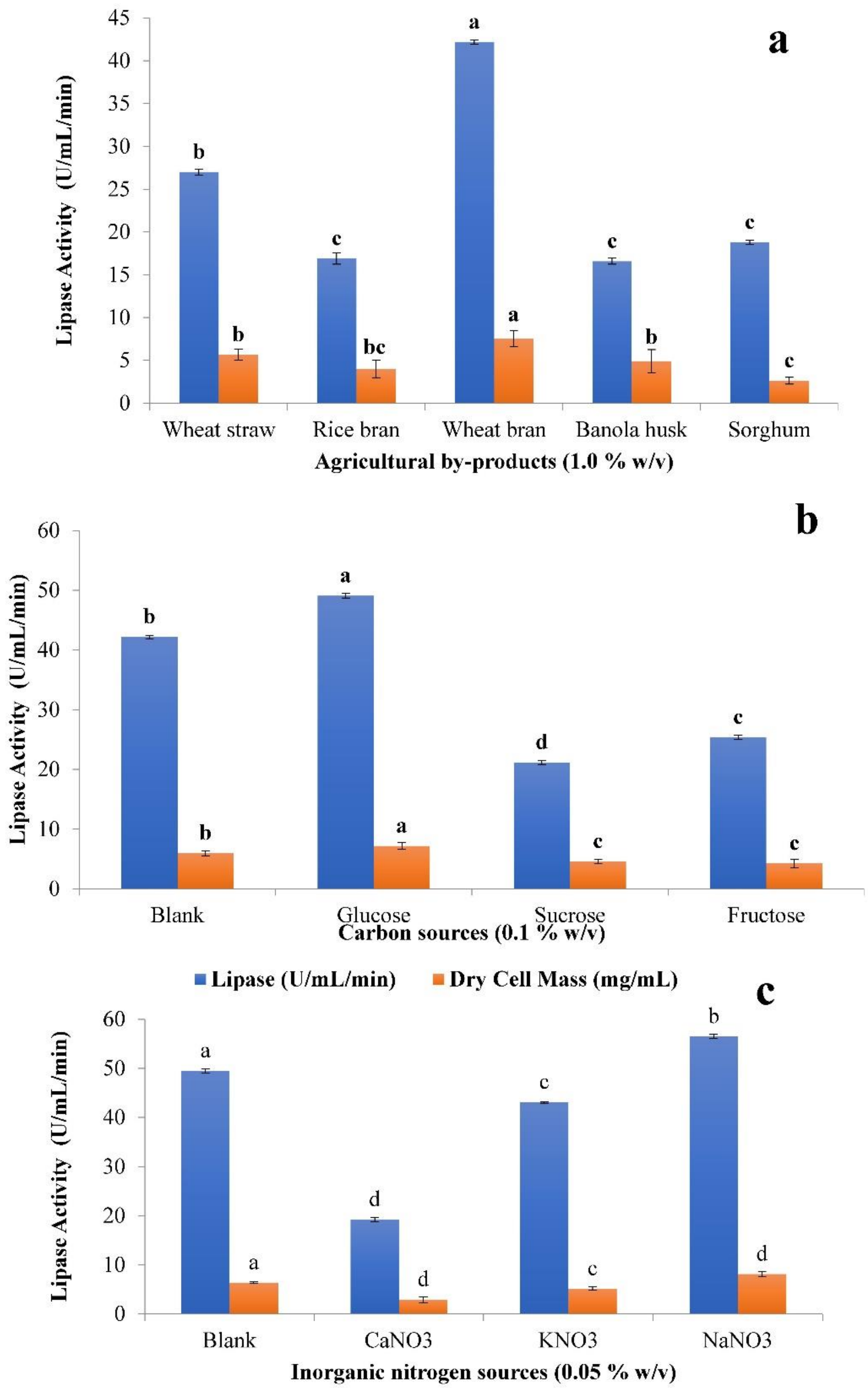

Figure 3: a) Effect of different agricultural by-products (1.0 % w/v) on the production of lipase by Bacillus sp. [UOE-14] under submerged fermentation conditions b) Effect of different carbon sources (0.1 % w/v)on the production of lipase by Bacillus sp. [UOE-14] under submerged fermentation conditions c) Effect of different inorganic nitrogen sources (0.05 % w/v) on the production of lipase by Bacillus sp. [UOE-14] under submerged fermentation conditions

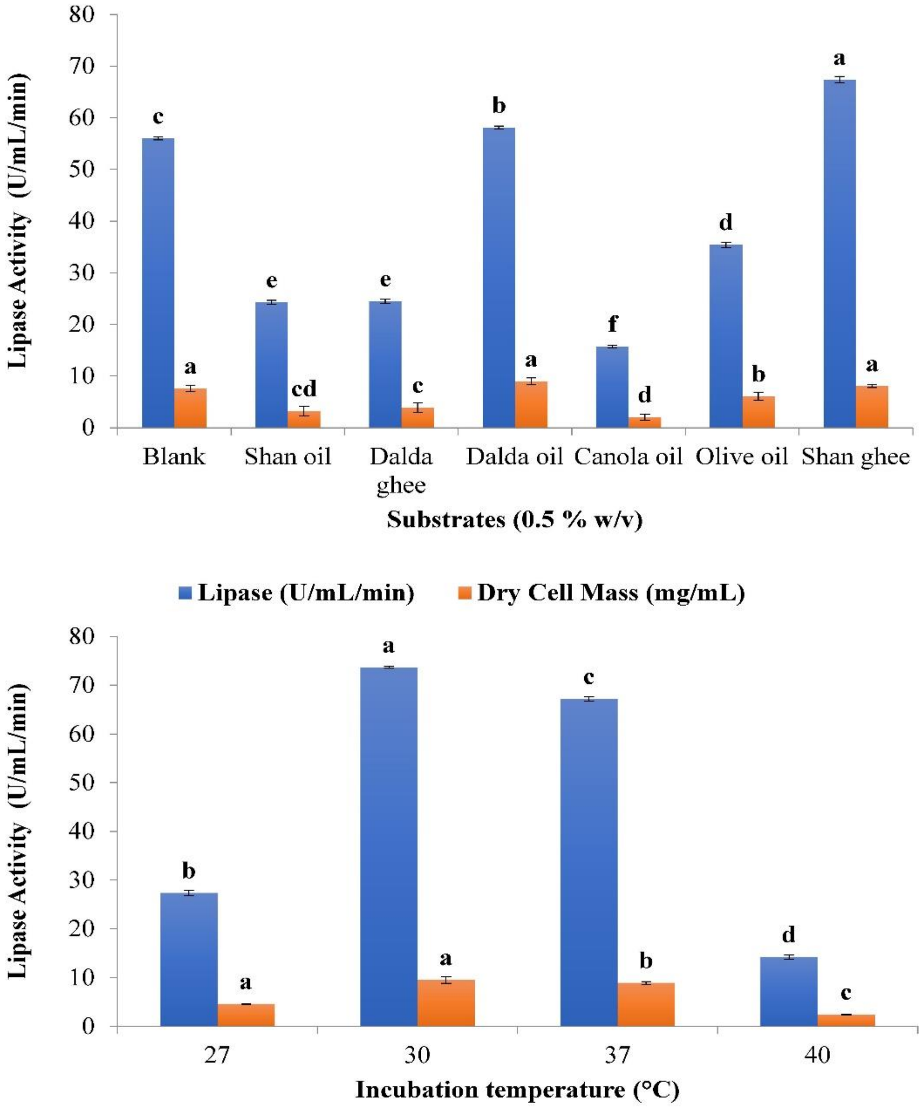

Figure 4 a) Effect of different oil/ghee residues (0.5 % w/v) as substrates on the production of lipase by Bacillus sp. UOE-14 under submerged fermentation conditions b) Effect of different incubation temperatures on the production of lipase by Bacillus sp. UOE-14 under submerged fermentation conditions. All the experiments were run in triplicates. Error bars show the Standard Deviation among the triplicates. Alphabets in small letters indicate the significance level of treatment effects.

Effect of different oil/ghee residues as substrates on the production of lipase

The rate of lipase production was observed, by adding lipid source namely Shan ghee, Shan oil, Dalda ghee, Dalda oil, Canola oil, and Olive oil (Figure 7). Lipase production was obtained maximum

67.4±0.26 U/mL/min with the dry cell mass 8.09±0.60 when Shan ghee (0.5 %; w/v) was added to the fermentation medium followed by Dalda oil 58.1±0.3 U/mL/min. On the other hand, Canola oil, Shan oil, Dalda ghee, Olive oil minimized the production of lipase.

Effect of incubation temperature on the production of lipase

Figure 8 shows range of temperatures i.e., 27, 30, 37 and 40ºC that was applied for the higher yield of lipase produced by *Bacillus* sp. It was found that when the fermented flask was incubated at 30ºC, the maximum yield 73.7±0.2 U/mL/min and the dry cell mass 9.53±0.68 mg/mL was obtained, but further increased in temperature showed the decrease rate in production. Hence, in the present study the optimum temperature of 30ºC was selected for the production of lipase by *Bacillus* sp.

DISCUSSION

Microbial lipases have wide applications and can easily be produced as an important group of commercially valuable enzymes (Yao *et al*., 2021). However, it is a challenging task to isolate a strain which may has potential to produce lipases. Oil or fat-rich habitats are concentrated with bacterial strains having great potential for lipid hydrolysis (Colen *et al*., 2006, Lee *et al* 2015, Vishnoi *et al*., 2020).

Hence, the present study planned to isolate lipase-producing bacteria from various samples including oil contaminated soil, burger, shawarma, pizza. A total of 15 bacterial isolates were obtained from these samples, and 07 isolates showed positive results towards TCZ assay. It revealed that these contaminated samples and foodstuffs are good sources of lipase producing bacteria. However, after quantitative analysis, *Bacillus* sp.$^{UOE-14}$ was found to have greater lipolytic potential among all. So, it was selected for further studies.

Microbial growth is greatly affected by the incubation time. Data analysis showed that *Bacillus* sp.$^{UOE-14}$ gave higher productivity at 48 h of growth period and decreased with further increase in incubation time. It is because after 48 h, the growth phase shifted from the exponential phase. Moreover, bacterial pellets began to form in which nutrients and oxygen supply became growth-limiting. This resulted in the reduction of lipase yield (Chaturvedi *et al*., 2010). Hasan *et al*. (2006), and Pogaku *et al*. (2010) also supported the same findings. Kamzolova *et al*. (2005) investigated that lipase activity was at its maximum during the exponential growth phase and gradually decreased during the late logarithm phase due to the production of citric acid in the medium.

On the other hand, Bokhari *et al*. (2013) reported that lipase activity was observed to be high at 24 h but due to the bacterial growth, only the turbidity of the supernatant is increased at 48 h. pH plays a vital role in enhancing the production of enzymes, and among all microorganisms, bacteria showed great specificity towards pH. In our study, the highest yield was obtained with pH 8.5. The optimum pH from 8.0 to 9.0 has been described already for lipase production from some *Bacillus* species (Nawani *et al*., 2006, Bala *et al*., 2020). But *Lysinibacillus* gave best results at pH 7.0 (Pham *et al*., 2021). This variation was seen due to strain specificity (Qiu *et* al., 2021; Yao *et al*., 2021). However, many data is available that reported *Bacillus* spp. produced the highest lipase yield at pH 8.0 (Hasan *et al*., 2006).

Fibrous bran is associated with most agro-industries (Singhania *et al*., 2008). We obtained be results of lipase (42.2 U/mL/min) production with wheat bran. This might be because wheat bran has high carbohydrate content (Saini *et* al., 2023). The reason might also be that chemical hydrolysis and reduction of particle size by grinding or chopping help increase the accessibility of the nutritional components for microbial uptake and growth (Manpreet *et al*., 2005, Bajor, 2020). Similar results were reported by Gonçalves *et al*. (2020). The culture environment has a dramatic influence on enzyme production, especially carbon sources that play a crucial role in enzyme induction in bacteria. Among all used carbon sources, lipolytic activity was maximum with the glucose. Although our results show contradiction with carbon catabolite repression (CCR) but many results have shown that this is a strain-specific behavior as the glucose presence may enhance the cell biomass in early growth and enzyme expression due the early carbon source for the bacteria (Adetunji and Olaniran, 2021; Lin and Co, 2005). Demirkan *et al*. (2021) and Sangeetha *et al*. (2008) also reported enhanced lipase production in presence of glucose. But our results were in contrast to study of Kumar and Kanwar (2012), and Veerapagu *et al*. (2013). According to them, by adding glucose and other sugars, the lipase production was minimized.

Inorganic nitrogen sources are less expensive than organic nitrogen sources. Sodium nitrate being an inorganic nitrogen source resulted in 16% higher activity. This increase in production was because nitrogen is an essential nutrient to produce enzymes, since these are composed of amino acids, which have nitrogen in their structure (Wu *et al*., 2020). Thakur *et al*. (2014) and Ismail *et al*. (2018) also reported that sodium nitrate enhanced the lipase productivity. Ghee residue is produced in large quantity by industries and most of the ghee residue goes to waste, which can be used as a substrate for large- scale production of lipase enzyme as it contains 32–70% fats. It is obvious that lipids are usually important inducers for lipase production (Wolski *et al*., 2009). We found that 1.0% of shan ghee residue gave 16% higher lipase activity. Hence, it reduced the production cost of lipase, and in this way, it finds applications in a wide range of industries (Sahasrabudhe *et al*., 2012, Wani *et al*., 2022). Bora and Kalita (2008) also observed that biomass of *Bacillus* spp. was increased by increasing oil concentration. Ahmed *et al*. (2019) also reported that the lipase production was more when Shan ghee was used as carbon source.

Optimum temperature for highest production was observed to be 30ºC that showed the maximum lipase activity of 73.7 U/mL/min. It is because microorganisms are very susceptible to temperature changes (Moreira *et al*., 2002). It was also observed that further elevation in temperature results in a decline in production rate because of cell death and might be enzyme had depleted and inhibiting microbial growth and enzyme formation (Palma *et al*., 2000; Bhatti *et al*., 2007; Ghaima *et al*., 2014; Esmaeili *et al*., 2015). Our findings for optimum temperature were like the findings of Fatima *et al*. (2021) and Bharathi and Rajalakshmi (2019). On the other hand, Ghaima *et al*. (2014), and Rajeshkumar *et al*. (2015) investigated that optimal temperature for bacterial growth was 35ºC.

CONCLUSION

Lipase, being a potential enzyme for industrial applications, can be produced by various species of bacteria. Hence, by optimizing all the defined physico-chemical parameters, we obtained 79.9% increase in lipase production. Agricultural residues can add value and minimize the production cost for efficient lipase production. These results are a positive step towards pilot-scale studies, and to further enhance the production rate by strain improvement through mutagenesis. In future, higher lipase productivity can be achieved by optimizing the cultural conditions with other microbial strains including not only bacteria but also fungi. Even though OFAT optimization technique worked well for assessing individual factors, more authenticated optimization techniques such as RSM and molecular identification of the strains as well in future research to gain more knowledge about interaction effects and further enhance lipase production.

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COMPETING INTERESTS

Authors have no conflict of interest

AUTHOR'S CONTRIBUTION

All authors contributed equally.

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