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Production of lipase enzyme using a mixed microbial vaccine technique from two local bacterial isolates, *Kytococcus sedentarius* and *Pseudomonas oleovorans*, utilizing solid medical waste

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Abstract

Objective

The disposal of solid medical waste presents significant environmental challenges, necessitating eco-friendly solutions. Microbial degradation offers a promising approach, particularly when coupled with enzyme production. Among these, lipases hold substantial industrial applications in pharmaceuticals, food processing, and biofuels. This study aims to isolate and identify local bacterial strains capable of degrading medical waste while producing lipase enzymes. Key factors influencing enzyme production, including pH, temperature, and nutrient composition, were optimized to enhance yield. The findings contribute to sustainable waste management and industrial enzyme applications.

Materials and Methods

This study was conducted in the laboratories of the Department of Life Sciences, College of Education for Pure Sciences, University of Anbar. Local bacterial isolates capable of degrading solid medical waste were obtained and identified using standard diagnostic methods. Identification was confirmed using the Vitek system, which classified the isolates as *Kytococcus sedentarius* and *Pseudomonas oleovorans*. Lipase production was carried out using the submerged culture technique, with solid medical waste serving as the primary carbon and energy source. Various factors affecting enzyme production—including pH, temperature, inoculum size, nitrogen source, waste concentration, and incubation duration—were investigated to determine optimal conditions. Enzyme activity was measured, and the effect of nitrogen supplementation on lipase production was assessed.

Results

The bacterial isolates *Kytococcus sedentarius* and *Pseudomonas oleovorans* demonstrated high efficiency in degrading solid medical waste and producing lipase enzymes. Optimal conditions for maximum enzyme yield were determined to be pH 8, a temperature of 45°C, an inoculum volume of 6 mL per 100 mL of medium, and a waste concentration of 2 g per 100 mL of medium. Under these conditions, the highest recorded enzyme activity was 8.5 units/mL. The addition of a nitrogen source led to a decrease in enzyme activity from 8.5 to 8.1 units/mL, suggesting that the solid medical waste medium provided sufficient nitrogen, with additional supplementation acting as an inhibitor of enzyme production.

Conclusions

This study successfully isolated and identified two bacterial strains, *Kytococcus sedentarius* and *Pseudomonas oleovorans*, with high efficiency in degrading solid medical waste and producing lipase enzymes. The optimal conditions for maximum enzyme production were established, highlighting the potential of these bacteria for biotechnological applications. The findings suggest that solid medical waste can serve as a viable substrate for enzyme production, simultaneously reducing waste and generating valuable bioproducts. Furthermore, the observed inhibition of enzyme activity by nitrogen supplementation underscores the importance of optimizing nutrient composition. This research contributes to sustainable waste management and industrial enzyme production, paving the way for further studies on large-scale applications.

Keywords: factors affecting lipase production, *Kytococcus sedentarius*, lipase, mixed microbial vaccines, *Pseudomonas oleovorans*

Paper Type: Research Paper.

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Introduction

Lipase (triacylglycerol hydrolase EC 3.1.1.3) is a hydrolytic enzyme that catalyzes the breakdown of triacylglycerol (TAG) into glycerol and fatty acids. Under specific conditions, it can also catalyze the reverse reaction in non-aqueous media, producing glycerides from glycerol and fatty acids (Jamilu et al. 2022). Lipases facilitate a wide range of biotransformation reactions, including hydrolysis, esterification, and alcoholysis (Abol-Fotouh et al. 2021). These enzymes are ubiquitous in animals, plants, and microorganisms, with microbial lipases being particularly significant due to their higher stability compared to those derived from animal and plant sources (Melani et al. 2020; Veerapagu et al. 2013).

Microbial lipases are preferred for industrial applications due to their ease of production, stability, and adaptability. They can be obtained through liquid-state fermentation (LSF) or solid-state fermentation (SSF). LSF involves cultivating microorganisms in a liquid nutrient medium, whereas SSF entails enzyme production on a solid substrate, where microorganisms metabolize carbon-containing compounds to produce intracellular or extracellular enzymes (Mazhar et al. 2017; Al-Maqtari et al. 2019).

Bacterial lipases are of considerable industrial interest due to their versatility and ease of optimization for enhanced productivity. The growing demand for novel lipases with distinct catalytic properties has driven the isolation and characterization of new bacterial strains. Lipase-producing microorganisms have been sourced from industrial and agricultural wastes, vegetable oil processing plants, dairy factories, oil-contaminated soils, and various natural oils, including olive, coconut, and vegetable oils, as well as petroleum-derived oils (Bharathi et al. 2019). These microorganisms or their enzyme by-products are employed in environmental bioremediation processes, where they degrade or detoxify contaminants, including medical waste. Genetic modifications have further enhanced their enzymatic stability and activity for such applications (Humer et al. 2020; Kumar et al. 2016). Among the key enzymes used in bioremediation are lipases, proteases, and cellulases, which degrade lipids, proteins, and cellulose, respectively. Combining these enzymes may enhance the biodegradation efficiency of various medical waste components (Yadav et al. 2022).

Bioremediation relies on microorganisms to enzymatically degrade pollutants and convert them into non-toxic compounds. However, the success of this process depends on favorable environmental conditions that support microbial growth and enzymatic activity. Key factors influencing microbial efficiency include pH, temperature, nutrient availability, and other physicochemical parameters (Narayanan et al. 2023).

Mixed-culture fermentation, wherein microbial inocula comprise two or more interacting species, is widely employed for enzyme production, protein synthesis, antibiotic development,

and fermentation-based food processing. Compared to monocultures, mixed cultures offer several advantages, such as increased productivity, improved raw material utilization, greater adaptability to environmental fluctuations, and enhanced resistance to contamination (Bajpai 2017). In natural ecosystems, many microorganisms coexist and interact synergistically, with evidence suggesting that essential biochemical processes may be inhibited when microbial fermentations rely solely on a single strain (Shi et al. 2020). Mixed cultures are thus essential for optimizing industrial production processes, as various bacterial, fungal, and yeast strains can enhance each other's metabolic activities. Lipases, which hydrolyze triglycerides into glycerol and fatty acids, are among the most widely utilized industrial enzymes. They have broad applications in the chemical, detergent, pharmaceutical, and food processing industries. Microbial lipases are particularly advantageous due to their stability, selectivity, and substrate specificity (Khabiri et al. 2023). Most lipases used in the food industry originate from non-toxic and non-pathogenic fungal sources classified as safe for consumption (Mohammadabadi et al. 2004; Alavi et al. 2022). The biotechnological production of enzymes is cost-effective, as these biomolecules can be efficiently produced through fermentation and subsequent purification of extracellular enzymes (Shahdadnejad et al. 2016; Amiri Roudbar et al. 2020). The enzyme production process typically involves fermentation, cell separation, concentration, purification, and final product formulation (Mohammadabadi et al. 2011). To further reduce production costs, agricultural waste can serve as a low-cost culture medium, optimizing enzyme yields (Bordbar et al. 2022). The efficiency of biotechnological lipase production depends on various factors, including microbial strain selection, culture medium composition, and fermentation conditions such as pH, aeration rate, and carbon and nitrogen sources (Heidarpour et al. 2011). Therefore, this study aimed to isolate and characterize local bacterial strains capable of degrading medical waste while producing lipase enzymes. Additionally, key factors influencing enzyme production, including pH, temperature, and nutrient composition, were optimized to enhance yield.

Materials and Methods

Isolation of lipase-producing bacteria: Thirty soil samples were collected from different locations in Anbar Governorate to isolate bacteria capable of decomposing solid medical waste, primarily plastic materials, and utilizing them for lipase enzyme production. One gram of each soil sample was subjected to a series of ten-fold dilutions using distilled water in test tubes. Then, 1 mL from the fourth and fifth dilutions of each sample was placed in sterile Petri dishes. Sterile polyvinyl chloride (PVC) agar medium (Shimibio Company, Iran) was then added. This medium consisted of 0.5 g K_2HPO_4, 0.4 g KH_2PO_4, 0.1 g NaCl, 0.02 g $CaCl_2$, 0.2 g $(NH_4)_2SO_4$, 0.02 g

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$MgSO_4$, 0.012 g $FeSO_4$, 0.1 g $MnSO_4$, and 3 g PVC, along with 15 g agar-agar in 1000 mL distilled water. The plates were gently swirled in a circular motion to ensure homogeneous sample distribution and left to solidify. Incubation was carried out at 30°C for six days.

The isolates were purified through sub-culturing on PVC agar to obtain pure single colonies. The purified isolates were then screened (primary and secondary screening) by inoculating them in a circular pattern (1 cm diameter) at the center of the dish. The plates were incubated at 30°C for six days. Growth characteristics were assessed by measuring the colony diameter or the transparent zone formed around the colony.

Testing of selected isolates on solid medical waste agar medium: Following primary and secondary screening, the selected bacterial isolates were cultured on a solid medical waste medium, which had the same composition as PVC agar, except that PVC was replaced with solid medical waste. The selected isolates were inoculated in a 1 cm circular pattern at the center of the dish and incubated at 30°C for six days. The ability of bacteria to degrade solid medical waste was determined by observing growth and measuring the diameter of the colony or the transparent zone around it. Based on these observations, three efficient isolates were selected for further study.

Testing the efficiency of selected isolates in synergistic decomposition of PVC: To evaluate the ability of the selected bacterial isolates to grow and degrade solid PVC medium synergistically, plates containing PVC agar were inoculated with different isolate combinations. Each plate was inoculated with two bacterial isolates, positioned 1 cm apart at opposite ends. The plates were incubated at 30°C for six days, and synergistic growth was analyzed. The two most effective isolates were selected for subsequent experiments.

Identification of bacterial isolates: The selected bacterial isolates were identified based on cultural, microscopic, and biochemical characteristics. Further confirmation was performed using the Vitek 2 Compact system.

Preparation of mixed bacterial vaccines: The two most efficient bacterial isolates were used to prepare a bacterial vaccine for further studies. After growing the isolates on solid nutrient agar, a portion of the colony from each isolate was inoculated into sterile liquid nutrient broth in conical flasks. The flasks were shaken and incubated at 30°C for 24 hours to obtain the bacterial vaccine. This process was repeated at each experimental stage.

Lipase enzyme determination: To estimate lipase production, the selected bacteria were grown as a mixed inoculum in medical waste medium within 250 mL conical flasks containing 100 mL of medium. The pH of the medium was adjusted to 7 before inoculation with 1 mL of each bacterial inoculum. The cultures were incubated at 30°C for 72 hours. After incubation, the bacterial culture was filtered through gauze, and the biomass was separated from the filtrate. The

filtrate was centrifuged at 3000 rpm for 10 minutes, and the supernatant was used to assess lipase activity.

Lipase activity assay: Lipase activity was measured using the titration method described by Watanabe et al. (1977). Olive oil, emulsified with polyvinyl alcohol (PVA), served as the substrate. The enzymatic reaction was stopped by adding an ethanol-acetone mixture (1:1 v/v), followed by titration with 0.02 M NaOH until a stable pink color appeared, using phenolphthalein as an indicator. The amount of NaOH consumed was used to calculate enzyme activity.

Olive oil emulsion was prepared by mixing 25 mL of olive oil with 75 mL of a polyvinyl alcohol (PVA) solution (2 g of PVA dissolved in 100 mL of distilled water). The mixture was homogenized using a mixer for 5 minutes to form a stable emulsion.

Next, 5 mL of the olive oil emulsion was transferred into a 100 mL conical flask, followed by the addition of 4 mL of Tris-HCl buffer (0.2 M, pH 7.0). Subsequently, 1 mL of $CaCl_2$ solution (110 mM) was added, followed by 1 mL of bacterial culture filtrate. A control flask was prepared with the same components, except that 1 mL of boiled culture filtrate was used instead of the natural culture filtrate.

Both flasks were incubated in a shaking water bath at 37 °C and 125 rpm for 1 hour. After incubation, 20 mL of a 1:1 (v/v) ethanol-acetone mixture was added to each flask and mixed thoroughly to terminate the reaction by denaturing the enzyme.

Following this, 2–3 drops of phenolphthalein indicator solution were added to each flask. The contents of each flask were titrated with 0.02 M NaOH at room temperature until the solution turned pink. The volume of NaOH consumed was recorded to determine the extent of the reaction.

Calculation of lipase activity: Lipase activity was calculated using the following equation (Rasmey et al. 2017):

μmol fatty acid/mL subsample = [(mL NaOH for sample − mL NaOH for blank) × N × 1000] / 5 mL

- (mL NaOH for sample − mL NaOH for blank): The difference between the volume of NaOH used for the sample and the volume of NaOH used for the blank solution gives the volume of NaOH consumed by the fatty acids.
- N: Standard value of the (0.02) molar NaOH solution.
- 1000: To convert the unit from moles to millimoles.
- 5 mL: Volume of the sample.

Optimization of lipase production conditions: The production medium contained 0.2 g $NaNO_3$, 0.05 g KH_2PO_4, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.002 g $CaCl_2 \cdot 2H_2O$, 0.002 g $MnSO_4 \cdot H_2O$, 0.002

g $FeSO_4 \cdot 7H_2O$, 0.02 g yeast extract, 0.02 g peptone, and 2 g solid medical waste in 100 mL distilled water. The medium was sterilized by autoclaving and inoculated with the selected bacterial isolates. Cultures were incubated at 30°C for 72 hours, and lipase activity was determined using titration under varying conditions:

1. **Effect of pH**: The medium pH was adjusted to 6, 7, 8, 9, and 10 to determine the optimal pH for lipase production.
2. **Effect of Temperature**: Cultures were incubated at 25, 30, 35, 40, and 45°C to determine the optimal temperature.
3. **Effect of Inoculum Size**: Different inoculum sizes (2, 4, 6, and 8 mL/100 mL) were tested to determine the ideal inoculum concentration.
4. **Effect of Nitrogen Sources**: Urea, ammonium sulfate, and sodium nitrate were compared to identify the most effective nitrogen source.
5. **Effect of Solid Medical Waste Concentration**: Concentrations of 1, 2, 3, 4, and 5 g/100 mL were tested to determine the optimal amount of solid medical waste for lipase production.
6. **Effect of Incubation Time**: Cultures were incubated for 24, 48, 72, 96, and 120 hours to determine the best incubation duration.

Results and discussion

Initial isolation on pure PVC agar medium: Isolation was performed on 30 different soil samples collected from various locations in Anbar Governorate. A total of 72 distinct bacterial isolates were successfully obtained and cultured on sterile PVC agar medium for further analysis. These isolates exhibited varying abilities to degrade PVC, as indicated by differences in colony diameters on the medium, which served as the sole source of carbon and energy. The incubation conditions were maintained at pH 7.0, a temperature of 30°C, and an incubation period of 72 hours.

Following initial isolation, bacterial colonies underwent two rounds of purification by successive streaking on the same medium to obtain single, pure bacterial colonies. These purified isolates were then used in subsequent study steps. The observed differences in PVC degradation may be attributed to variations in the bacterial isolates' metabolic capabilities or their environmental origins.

A study by Fakhri and Aadil Ibrahim (2022) reported a similar approach, where *Kytococcus sedentarius* bacteria were isolated from landfill soil in Anbar Governorate and cultivated in a liquid medium containing nylon bags as the sole carbon and energy source. Under optimal conditions, the study achieved a 43.2% degradation rate of nylon bags.

Primary screening of bacterial isolates producing single-cell protein (SCP): Re-purification of bacterial isolates capable of PVC degradation led to the selection of 35 isolates exhibiting high growth efficiency on PVC agar medium, as determined by colony diameter measurements during secondary screening. The colony diameters varied significantly, ranging from 1 to 5 cm, as presented in Table 1. This variation could be attributed to differences in the isolates' metabolic requirements, environmental sources, or intrinsic characteristics.

A study by Kumar et al. (2017) identified several PVC-degrading bacterial species from plastic-contaminated soil, including *Pseudomonas*, *Klebsiella*, *Staphylococcus*, and *Escherichia coli*. Among these, *Pseudomonas* exhibited the highest degradation rate (40.53%), followed by *Klebsiella* (23.06%), *Staphylococcus* (10.92%), and *E. coli* (5.32%).

Secondary screening of bacterial isolates producing single-cell protein (SCP): Following the primary screening, a secondary screening was conducted to identify the most efficient bacterial isolates for single-cell protein (SCP) production. Thirty-five selected bacterial isolates were inoculated onto PVC medium, each placed in a circular area with a 1 cm diameter at the center of the Petri dish. The objective was to assess their ability to degrade solid PVC medium.

After a six-day incubation period, the degradation efficiency of each isolate was evaluated based on the lysis zone diameter. As shown in Table 2, the four most efficient bacterial isolates were identified based on their ability to degrade the PVC medium, as indicated by the measured lysis diameters. These isolates, labeled A1, L1, N1, and N2, exhibited colony diameters of 3 cm, 5 cm, 3 cm, and 3 cm, respectively.

Shrestha et al. (2019) isolated six different bacterial strains belonging to the genus *Bacillus* spp. from soil, demonstrating their ability to degrade low-density polyethylene (LDPE). Their study identified *Bacillus coagulans*, *Bacillus sporothermodurans*, *Bacillus carboniphilus*, *Bacillus neidei*, *Bacillus smithii*, and *Bacillus megaterium* as effective LDPE degraders, based on visible colony growth on LDPE-containing agar medium.

The four bacterial isolates selected in the previous section were recultured at the center of the plate under identical conditions, except that the PVC material in the culture medium was replaced with solid medical waste. Following incubation, the diameters of the bacterial colonies growing on the medium were measured. The isolates with the largest colony diameters were selected for further analysis. Three bacterial isolates demonstrated efficient decomposition of solid medical waste, with colony diameters ranging as presented in Table 3. These results indicate that the selected bacterial isolates can degrade and utilize solid medical waste as a source of carbon and energy.

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Table 1. Selected Bacterial Isolates from the Initial Screening

	Isolation symbol	Soil type	region	Diameter (cm)
1	A1	Wheat crop soil	Garma	3
2	A2	Wheat crop soil	Garma	2
3	A3	Wheat crop soil	Garma	2
4	A4	Wheat crop soil	Garma	2
5	A5	Wheat crop soil	Garma	2
6	A6	Wheat crop soil	Garma	2
7	B1	Generator soil	AL-khaldia	2
8	C1	Alfalfa crop soil	Eastern Hasiba	2.5
9	C2	Alfalfa crop soil	Eastern Hasiba	2.5
10	C3	Alfalfa crop soil	Eastern Hasiba	2
11	D1	Okra crop soil	Eastern Hasiba	1.5
12	D2	Okra crop soil	Eastern Hasiba	2
13	E1	Vineyard soil	Garma	2
14	E2	Vineyard soil	Garma	2
15	E3	Vineyard soil	Garma	2.5
16	E4	Vineyard soil	Garma	1.5
17	F1	Soil contaminated with household waste	Sofia	2.5
18	G1	Alfalfa crop soil	Garma	2.5
19	G2	Alfalfa crop soil	Garma	1
20	G3	Alfalfa crop soil	Garma	2
21	H1	Soil contaminated with plastic waste	Alsofia	2
22	H2	Soil contaminated with plastic waste	Alsofia	1
23	I1	Generator soil	Alsydkia	2
24	I2	Generator soil	Alsydkia	2.5
25	J1	Swamp soil	The Strait	1.5
26	K1	Apple and orange orchard soil	eastern Hasiba	2
27	K2	Apple and orange orchard soil	eastern Hasiba	2.5
28	L1	Sandy soil	Garma	2
29	L2	Sandy soil	Garma	2
30	L3	Sandy soil	Garma	5
31	M1	Blacksmith soil	Alkafaat	1.5
32	N1	Home garden soil	Fallujah	3
33	N2	Home garden soil	Fallujah	2.5
34	N3	Home garden soil	Fallujah	3
35	N4	Home garden soil	Fallujah	2

Table 2. Selected bacterial isolates from secondary screening

T	isolation	The symbol	Colony diameter (cm)
1	Wheat crop soil - Garma	A1	3
2	Ahbinia Farm Soil - Garma	L1	5
3	Home Garden Soil - Fallujah	N1	3
4	Home Garden Soil - Fallujah	N2	3

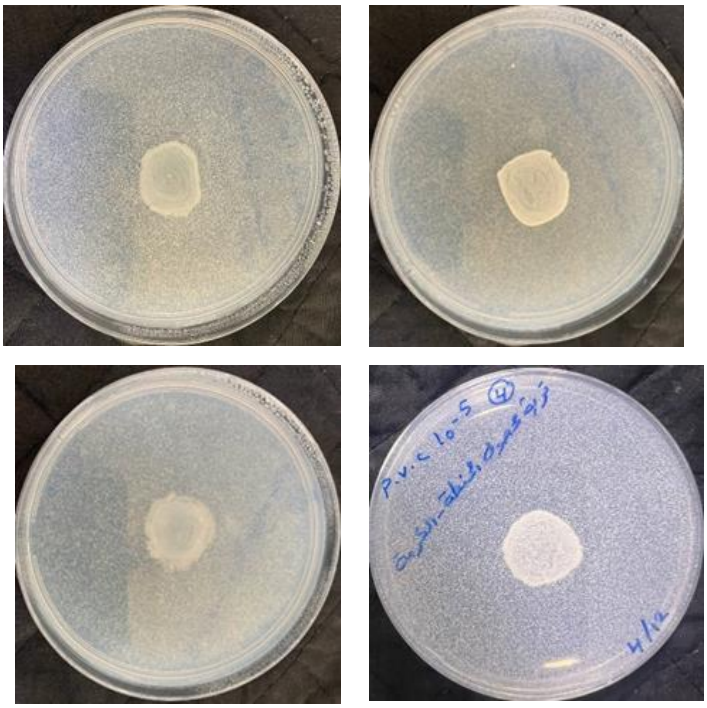

Figure 1. The growth of bacterial isolates selected from secondary screening. Testing the ability of selected bacterial isolates to analyze solid medical waste medium

Table 3. Growth of selected bacterial isolates on solid medical waste medium

T	isolation	The symbol	Colony diameter (cm)
1	Wheat crop soil - Garma	A1	4
2	Home Garden Soil - Fallujah	N1	4
3	Home Garden Soil - Fallujah	N2	3

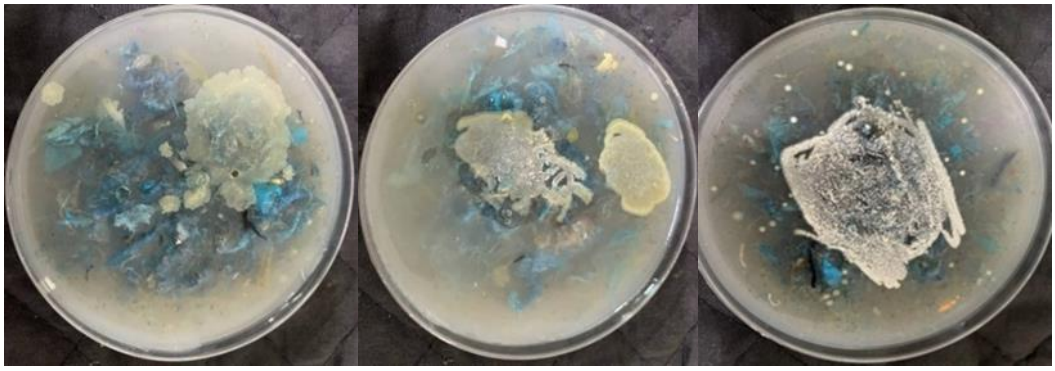

Figure 2. shows the growth of selected isolates on a solid medical waste medium.

Synergistic ability test for selected bacterial isolates on PVC medium

The results of the test evaluating the ability of selected bacterial isolates to grow synergistically demonstrated that the two isolates, designated by local codes N1 and A1, exhibited the highest capacity for synergistic growth and solid medical waste degradation, as shown in Figure 3.

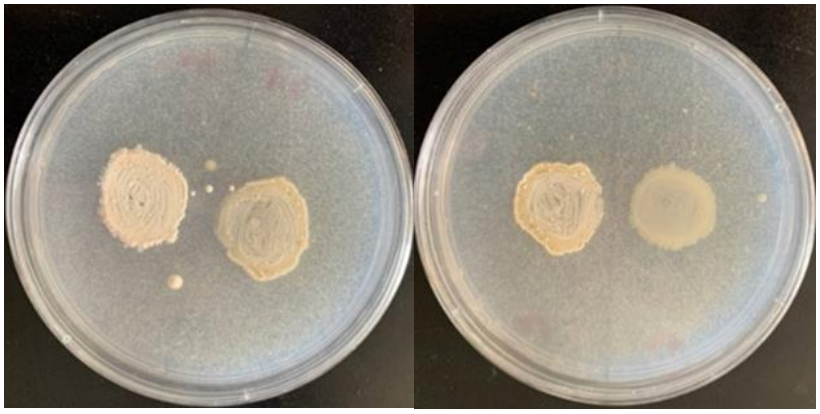

Figure 3. shows the growth of the synergistic bacterial isolates

Diagnosis of the selected isolates: The results of cultural and microscopic examinations, biochemical tests, and the Vitek 2 system for the two selected bacterial isolates (A1 and N1) obtained from soil revealed the following findings:

The first bacterial isolate (A1) was identified as *Kytococcus sedentarius*, a species belonging to the genus *Kytococcus*. This genus was previously classified under *Micrococcus* but was reclassified based on evolutionary and chemical characteristics (Amaraneni et al. 2015; Chan et al. 2012; Chaudhary & Finkle 2010). The identification was confirmed using the Vitek 2 compact system.

The second bacterial isolate (N1) was identified as *Pseudomonas oleovorans*. This identification was also confirmed using the Vitek 2 compact system (Saha et al. 2010). *P.*

oleovorans was first described by Lee and Chandler (1941). Taxonomically, this bacterium belongs to the 16S rRNA group within *Pseudomonas aeruginosa*.

Table 4 presents the cultural and microscopic characteristics of the two selected bacterial isolates, while Table 5 summarizes the biochemical test results.

Table 4. Cultural and microscopic characteristics of the two selected bacterial isolates

T	Diagnostic features	***kytococcus sedentarius* A1**	***Pseudomonas oleovoran* N1**
1	Shape	Circular	Curly round
2	Color	White	white
3	Texture	Solid	dry
4	Transparency	Opaque	dull
5	Edge	Round	round
6	Cell shape	Spherical	spherical
7	Height	High	high
8	Gram stain	Positive	negative
9	Cell aggregation	Pairs or tetrads	single
10	Stain production	not productive	not productive

Table 5. Biochemical tests for the two selected isolates

T	Test type	***Pseudomonas oleovoran* N1**	***kytococcus sedentarius* A1**
1	Gram stain	-	+
2	Oxidase	+	-
3	Catalase	+	+
4	Indole	-	-
5	Methyl red	-	-
6	Voges-Proskauer	-	-
7	Citrate utilization	-	-
8	Motility	+	-
9	Urease	+	+
10	Gelatin hydrolysis	-	-
11	Arginine	-	+

(+)positive test (-) negative test

Factors affecting the production of lipase enzyme-Effect of pH: The results presented in Figure 4 indicate that lipase enzyme production increased with rising pH, reaching its peak at pH

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8.0, where the enzyme activity was recorded at 6.1 U/mL. Beyond this optimal pH, enzyme production declined, with the lowest activity observed at pH 10.0 (4.9 U/mL).

The influence of pH on lipase production and activity is attributed to its impact on the charge distribution of amino acids, which in turn affects the structural conformation of the enzyme's active site (Rehman et al. 2023). Additionally, pH modulates the composition of the bacterial growth medium, particularly influencing nutrient solubility and bioavailability (Hosseini & Sadripour 2017). Variations in pH also alter the ionization state of functional groups, potentially leading to changes in enzyme structure and properties. The enzyme's optimal activity and stability are pH-dependent, with maximum activity occurring at its ideal pH (Maftukhah & Abdullah 2018).

These findings align with previous studies, including Venkatesagowda et al. (2018), who reported that the highest lipase activity from *Lasiodiplodia theobromae* VBE-1 was observed at pH 8.0. Similarly, Abubakar et al. (2024) demonstrated that optimal lipase production from *Bacillus subtilis* and *Pseudomonas aeruginosa* isolates occurred at pH 8.0.

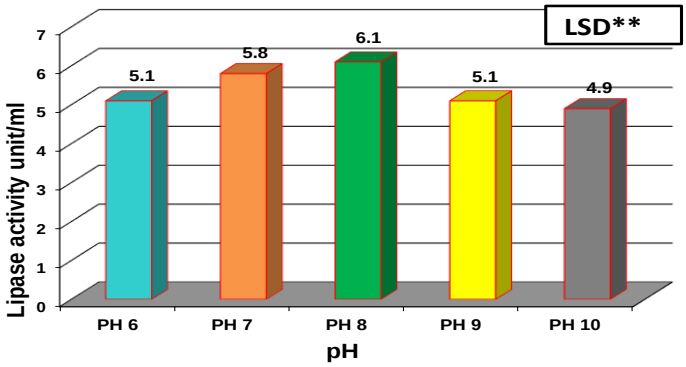

Figure 4. Effect of pH on lipase enzyme production

Effect of temperature: The results presented in Figure 5 indicate that lipase enzyme activity increased with rising temperature, reaching its peak at 45°C with an activity of 8.3 U/mL. In contrast, enzyme activity declined at temperatures below 45°C, with the lowest activity recorded at 5.5 U/mL at 25°C.

Temperature is a crucial factor influencing enzyme productivity, particularly in microbial lipase production. It can alter the physical properties of cell membranes and impact enzyme secretion. The optimum temperature plays a significant role in enzyme release, as low temperatures reduce lipase production, while excessively high temperatures can negatively affect enzyme activity (Kumar et al. 2023). Elevated temperatures typically lead to a reduction in enzyme activity by approximately 50%, primarily due to protein denaturation. This structural alteration disrupts the enzyme's active site, thereby reducing its catalytic efficiency (Sharma et al. 2017). Beyond the optimum temperature, a significant decline in enzyme activity is commonly observed, likely due to the thermal instability of lipase (He et al. 2019). The enzyme reaction rate generally increases with temperature up to a certain threshold, as higher temperatures enhance the kinetic energy of reacting molecules. This increase in energy can, however, lead to the breaking of weak hydrogen and hydrophobic bonds that stabilize the enzyme's structure. Additionally, elevated temperatures may alter substrate properties, increasing resistance to binding at the enzyme's active site. As temperatures exceed the enzyme's stability range, enzymatic efficiency declines due to protein denaturation, which involves structural changes in the enzyme, including disruptions to ionic and hydrogen bonds. These changes ultimately reduce the rate of the catalyzed reaction (Dali Abd-Al Hussien & Fakri Alrawi 2020; Maftukhah & Abdullah 2018).

The present findings align with those of Mazhar et al. (2017), who reported maximum lipase production at 45°C in Bacillus subtilis PCSIRNL-39. Similarly, Demirkan et al. (2021) observed optimal lipase activity at 45°C in the bacterial isolate *Bacillus cereus* ATA179.

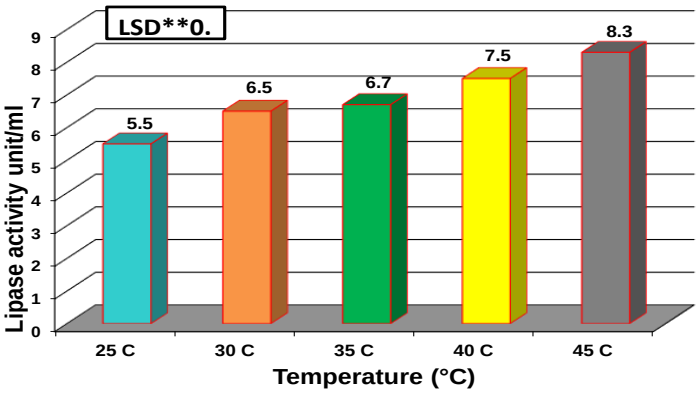

Figure 5. Effect of temperature on lipase enzyme production

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Effect of inoculum size: The results presented in Figure 6 indicate that the optimal mixed inoculum size for enzyme production was 6.0 mL per 100 mL of liquid culture, comprising 3.0 mL of each of the two isolates (*Kytococcus sedentarius* and *Pseudomonas oleovorans*). Under these conditions, enzyme productivity reached a maximum of 8.5 units/mL. In contrast, enzyme productivity declined at other inoculum sizes, with the lowest recorded productivity of 7.5 units/mL observed at an inoculum size of 2.0 mL. Inoculum size plays a critical role in the production process, as it directly influences microbial growth and enzyme synthesis. The optimal inoculum size must be carefully determined based on the type and scale of the production system to maximize efficiency (El-Mansi et al. 2018). Studies have shown that using a small inoculum results in reduced enzyme production due to low cell density, which slows microbial growth rates (Javed & Qazi 2016). Conversely, an excessive inoculum size leads to increased microbial competition for nutrients in the production medium, ultimately reducing growth rates and enzyme productivity (Abood et al. 2021; Salah et al. 2018).

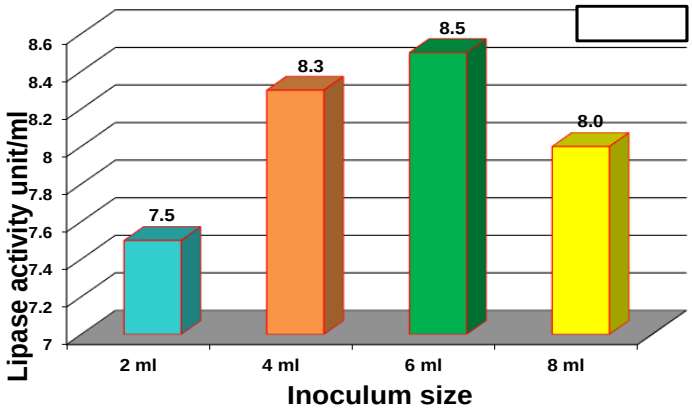

Figure 6. Effect of vaccine volume (milliliter) on lipase enzyme production

Effect of nitrogen sources: The results presented in Figure 7 indicate that the addition of various nitrogen sources led to a reduction in lipase enzyme production. The highest enzymatic activity was observed when sodium nitrate was added to the medium, yielding 8.1 units/mL. In comparison, the optimal enzyme activity recorded in the section discussing the effect of inoculum size was 8.5 units/mL. The decline in enzyme production upon the addition of nitrogen sources may be attributed to the fact that the basal medium already contains sufficient nitrogen to support

optimal enzyme synthesis. Consequently, the introduction of an additional nitrogen source could potentially inhibit bacterial growth and enzyme production. Previous studies have suggested that the type of nitrogen source can significantly influence enzyme synthesis and secretion, as nitrogen compounds provide essential amino acids and growth factors necessary for cellular metabolism and protein biosynthesis (Abu Yazid et al. 2017; Bindal et al. 2022; Hasan et al. 2018). Furthermore, excessive nitrogen concentrations can disrupt normal metabolic pathways due to their high organic matter content, thereby reducing enzyme productivity. Conversely, insufficient nitrogen availability may fail to provide adequate energy for microbial growth and enzyme synthesis. Optimal enzyme production is generally achieved in media containing moderate nitrogen concentrations (Ma et al. 2021).

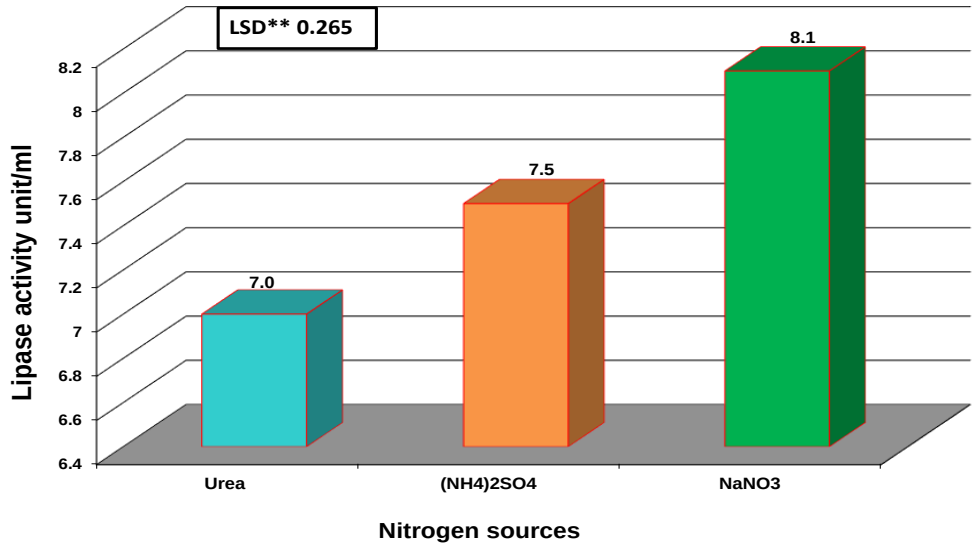

Figure 7. Effect of nitrogen sources on lipase enzyme production

Effect of waste concentration: The results presented in Figure 8 indicate that the highest lipase enzyme production was achieved using solid medical waste at a concentration of 2.0 g/100 mL of medium, with an enzyme activity of 8.5 U/mL. In contrast, enzyme production decreased at other concentrations, with the lowest activity recorded at 7.0 U/mL for all tested concentrations (1, 2, 3, 4, and 5 g/100 mL). A high waste concentration appears to hinder lipase synthesis. This inhibition may be attributed to the excessive presence of proteins and peptides in the waste, which could slow bacterial proliferation, especially due to increased medium viscosity and reduced homogeneity between the microorganism and the production medium. Additionally, excessive waste may lead to a gradual rise in nitrogen content beyond optimal levels, potentially altering lipase metabolism pathways. It is important to note that lipids serve as a primary carbon source

in the medium, and their rapid consumption can limit cell growth and enzyme synthesis when present in low concentrations. Conversely, excessively high lipid concentrations may inhibit lipase production, possibly due to inadequate oxygen transfer within the production medium or the accumulation of excess free fatty acids in the bacterial culture (Abol-Fotouh et al. 2021). A study by Abbas et al. (2017) demonstrated that agricultural waste from rapeseed oil supported optimal lipase production by the bacterial isolate *Bacillus subtilis* PCSIR NL-38. Similarly, Alrumman et al. (2018) reported that *Bacillus sonorensis* (KKUMS14) exhibited the highest lipase production when cultivated with 5% kitchen waste.

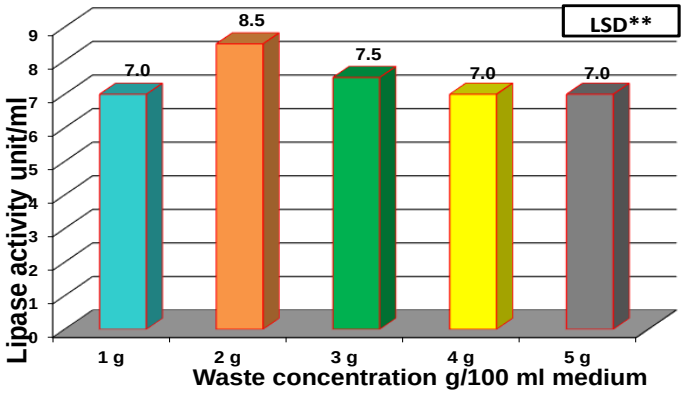

Figure 8. Effect of solid medical waste concentration on lipase enzyme production

Effect of the incubation period: The results presented in Figure 9 indicate that the highest activity of the lipase enzyme was observed after an incubation period of 72 hours, reaching 8.5 units/mL. In contrast, enzyme activity declined with prolonged incubation, with the lowest recorded activity being 6.0 units/mL after 120 hours. Extending the incubation period enhances bacterial activity, leading to increased enzyme secretion outside the bacterial cell, which accelerates the decomposition process. Conversely, a shorter incubation period results in reduced bacterial activity, leading to lower enzyme production and, consequently, affecting the biodegradation process. Additionally, the type and quantity of the carbon source used, along with the specific microorganisms involved, influence the incubation period. Different bacterial strains exhibit varying growth durations, with some requiring a short incubation time while others necessitate a longer period for optimal enzyme production (Arutchelvi et al. 2008; Kempes et al. 2012). Previous studies have reported that the maximum enzyme activity for certain microbes

occurs within 48–72 hours, after which enzyme production declines (Abdullah et al. 2018a; Abdullah et al. 2018b). This reduction is attributed to factors such as nutrient depletion, accumulation of toxic byproducts, low humidity, and pH fluctuations (Amin et al. 2022). After the optimal incubation period, cell autolysis, nutrient exhaustion, and the accumulation of enzyme inhibitors can impair enzyme synthesis by disrupting transport mechanisms across the cell membrane. The initial pH of the medium also plays a crucial role in enzyme production (Alrumman et al. 2018). Mazhar et al. (2023) reported that the maximum activity of the lipase enzyme from *Bacillus amyloliquefaciens* was achieved after 72 hours of incubation.

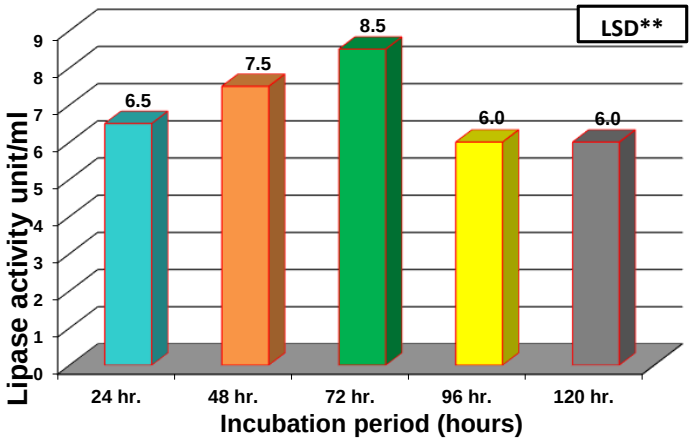

Figure 9. Effect of incubation period on lipase enzyme production

Conclusions: This study successfully isolated and identified two bacterial strains, *Kytococcus sedentarius* and *Pseudomonas oleovorans*, that exhibit high efficiency in degrading solid medical waste and producing lipase enzymes. The optimal conditions for maximum enzyme production were established, emphasizing the potential of these bacteria for biotechnological applications. The results indicate that solid medical waste can serve as a viable substrate for enzyme production, simultaneously reducing waste and generating valuable bioproducts. Furthermore, the observed inhibition of enzyme activity by nitrogen supplementation highlights the necessity of optimizing nutrient composition. This research contributes to sustainable waste management and industrial enzyme production, paving the way for future studies on large-scale applications.

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مجله بیوتکنولوژی کشاورزی
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تولید آنزیم لیپاز با استفاده از تکنیک واکسن میکروبی مخلوط از دو جدایه باکتری محلی Kytococcus sedentarius و Pseudomonas oleovorans با استفاده از مواد زائد جامد پزشکی

کمال سعدون المفرجی

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چکیده

هدف: دفع زباله‌های جامد پزشکی چالش‌های زیست محیطی قابل توجهی را ایجاد می‌کند و راه حل‌های سازگار با محیط زیست را ضروری می‌سازد. تخریب میکروبی یک رویکرد امیدوارکننده ارائه می‌دهد، به ویژه زمانی که با تولید آنزیم همراه باشد. در این میان، لیپازها کاربردهای صنعتی قابل توجهی در داروسازی، فرآوری مواد غذایی و سوخت‌های زیستی دارند. هدف از این مطالعه، جداسازی و شناسایی سویه‌های باکتریایی محلی است که قادر به تجزیه ضایعات پزشکی در حین تولید آنزیم‌های لیپاز هستند. عوامل کلیدی موثر بر تولید آنزیم، از جملهpH، دما و ترکیب مواد مغذی، برای افزایش عملکرد بهینه شدند. این یافته ها به مدیریت پایدار زباله و کاربردهای آنزیم صنعتی کمک می‌کند.

مواد و روش‌ها: این مطالعه در آزمایشگاه‌های گروه علوم زیستی دانشکده آموزش علوم محض دانشگاه انبار انجام شد. جدایه‌های باکتریایی محلی که قادر به تجزیه مواد زائد جامد پزشکی بودند با استفاده از روش‌های تشخیصی استاندارد شناسایی شدند. شناسایی با استفاده از سیستم Vitek تایید شد که جدایه ها را به عنوان Kytococcus sedentarius و Pseudomonas oleovorans طبقه بندی کرد. تولید لیپاز با استفاده از روش کشت غوطه‌ور، با زباله‌های پزشکی جامد به عنوان منبع اصلی کربن و انرژی انجام

شد. عوامل مختلفی که بر تولید آنزیم تأثیر می‌گذارند؛ از جمله pH، دما، اندازه تلقیح، منبع نیتروژن، غلظت ضایعات و مدت زمان هچ برای تعیین شرایط بهینه مورد بررسی قرار گرفتند. فعالیت آنزیم اندازه گیری شد و اثر مکمل نیتروژن بر تولید لیپاز ارزیابی شد.

نتایج: جدایه‌های باکتریایی Kytococcus sedentarius و Pseudomonas oleovorans کارایی بالایی در تخریب مواد زائد جامد پزشکی و تولید آنزیم‌های لیپاز نشان دادند. شرایط بهینه برای حداکثر بازده آنزیم به pH برابر ۸، دمای ۴۵ درجه سانتیگراد، حجم تلقیح ۶ میلی لیتر در هر ۱۰۰ میلی لیتر محیط و غلظت ضایعات ۲ گرم در هر ۱۰۰ میلی لیتر محیط تعیین شد. تحت این شرایط، بیشترین فعالیت آنزیمی ثبت شده ۸/۵ واحد در میلی لیتر بود. افزودن منبع نیتروژن منجر به کاهش فعالیت آنزیم از ۸/۵ به ۸/۱ واحد در میلی لیتر شد، که نشان می‌دهد محیط زباله پزشکی جامد نیتروژن کافی را فراهم می‌کند و مکمل‌های اضافی به عنوان بازدارنده تولید آنزیم عمل می‌کنند.

نتیجه‌گیری: این مطالعه با موفقیت دو سویه باکتری به نام‌های *Kytococcus sedentarius* و *Pseudomonas oleovorans* را با کارایی بالا در تجزیه مواد زائد جامد پزشکی و تولید آنزیم‌های لیپاز جدا و شناسایی کرد. شرایط بهینه برای حداکثر تولید آنزیم ایجاد شد که پتانسیل این باکتری‌ها را برای کاربردهای بیوتکنولوژیکی برجسته می‌کند. یافته‌ها حاکی از آن است که زباله‌های پزشکی جامد می‌توانند به عنوان یک بستر مناسب برای تولید آنزیم عمل کنند و به طور همزمان ضایعات را کاهش دهند و محصولات زیستی ارزشمندی تولید کنند. علاوه بر این، مهار مشاهده شده از فعالیت آنزیم توسط مکمل نیتروژن بر اهمیت بهینه سازی ترکیب مواد مغذی تأکید می‌کند. این تحقیق به مدیریت ضایعات پایدار و تولید آنزیم صنعتی کمک می‌کند و راه را برای مطالعات بیشتر در مورد برنامه‌های کاربردی در مقیاس بزرگ هموار می‌کند.

واژه‌های کلیدی: لیپاز، عوامل موثر بر تولید لیپاز، واکسن های میکروبی مخلوط، *Kytococcus sedentarius*، *Pseudomonas oleovorans*

نوع مقاله: پژوهشی.

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