Optimization of Growth Conditions for Production of Organophosphorous Phosphatase Enzymes in Three Bacteria Species Isolated from Dairy Farms in Kenya

Felix Juma Tom[1], Gibson Kamau Gicharu[1]*, Nzaro Gona Makenzi[1], Lenny Mwagandi Chimbevo[2], Fredrick Mwamburi Mjomba[1], Mzee Juma Amana[2], Adum Atego Norbert[1], Anzazi Lydia Haro[1]

[1]Department of Pure and Applied Sciences, Technical University of Mombasa, P.O. Box 90420-80100, Mombasa, Kenya (felixtom23@yahoo.com, gicharugibsonkamau@tum.ac.ke, clennyson@gmail.com, makenzinzaro@gmail.com, fmjomba@tum.ac.ke, yashoguya2@gmail.com, harolydia@gmail.com); [2]Department of Biochemistry and Biotechnology, School of Pure and Applied Sciences, Pwani University, Kilifi, Kenya (amana_juma@yahoo.com).

*Corresponding author's email: gicharugibsonkamau@tum.ac.ke

Abstract

Chlorfenvinphos is one of the organophosphates largely produced around the world to control mites and ticks in dairy farming. However, it is toxic and has serious effects on the nervous system and other organ systems. It has a long residual effect in the environment posing risks to both aquatic and terrestrial ecosystems. In this study, three species of bacteria isolated from chlorpyrifos contaminated dairy farms in Kenya were screened for their ability to produce chlorfenvinphos degrading organophosphorus phosphatase enzymes. The isolates, *Advenella kashmirensis, Micrococcus luteus,* and *Lysinibacillus sphaericus* grew at different rates when both temperature and pH were varied. The optimal growth temperature for the three isolates was 35°C while the optimal pH of *M. luteus* and *L. sphaericus* was 7 and 6 for *A. kashmirensis*. At these optimal conditions, *A. kashmirensis* had the highest growth (OD 0.175) followed by *L. sphaericus* (OD 0.132) while *M. luteus* had the lowest (OD 0.106). Crude protein concentration was highest for *A. kashmirensis* (465.4 ± 31.8 µg/mL) and was significantly different from the concentrations of the other isolates ($p < 0.05$). This suggests its suitability for applications requiring high concentrations of total proteins. On purification of crude protein using ammonium sulfate precipitation and gel filtration, *A. kashmirensis* exhibited the highest enzyme activity (19.8 ± 0.8 µMol/Min and was significantly different from *M. luteus* (13.8 ± 0.98 µMol/Min and *L. sphaericus* (11.8 ± 0.2 µMol/Min. These results suggest *A. kashmirensis* is superior in bioremediating chlorfenvinphos polluted environments and can be used to clean up polluted dairy farms. However, more studies are required to explore how the isolates interact with each other in their natural environments with a view of coming up with more efficient degradation process to restore polluted dairy farms and reduce the risk of the acaricide entering aquatic and terrestrial ecosystems from where it can easily enter into food chains affecting a wide variety of organisms.

Key Words: Chlorfenvinphos; Organophosphates; Bioremediation; Organophosphorus Phosphatase Enzymes

Introduction

Organophosphate (OPs) pesticides are used extensively in agriculture due to their ability to control pests and increase crop and livestock production and reduce vector-borne disease incidences (Tudi et al.,2021). These compounds inhibit acetylcholinesterase, an essential enzyme for nerve function, leading to the pest's death (Atego, 2021). Examples of OPs include chlorpyrifos, diazinon, and chlorfenvinphos (CFVP) (Kadiru et al., 2022). However, the widely use of these organophosphorus pesticides cause environmental pollution and harm to non-target species through food chains

Published December 2024

(Kadiru et al., 2022). Therefore, it is crucial to adopt control measures to minimize their accumulation in the ecosystem. Chlorfenvinphos (CFVP) is a widely used OP acaricides in Kenya (Atego, 2021) and in various regions globally, like India (Dar et al., 2023) and Pakistan (Ambreen & Yasmin, 2020), for controlling domestic, agricultural, and veterinary pests. The OP act by inhibiting the activityof acetyl cholinesterase (AchE) enzyme in insect pests and other parasites leading to the accumulation of acetylcholine (Ach) and nerve toxicity (Atego, 2021). However, the widespread use of CFVP has resulted in substantial ecological contamination and considerable health hazards to humans, such as neurotoxicity and potential carcinogenic effects (Ambreen & Yasmin, 2020). Traditional methods for removing OPs from the environment include physical and chemical approaches, like adsorption, incineration, and chemical degradation (Singh et al,. 2008). Despite their application, these methods often prove ineffective due to high costs, incomplete removal, and the creation of secondary pollution (Pehkonen, 2002). In contrast, microbial degradation has emerged as a more promising solution for OP detoxification (Saravanan et al., 2021). This method utilizes the natural ability of microorganisms to break down these compounds, offering an environmentally friendly, cost-effective, and efficient alternative (Stadlmair, 2018). However, the process of microbial degradation can be slow, especially in contaminated soils (Singh & Walker, 2006). Recent research has focused on isolating indigenous organisms from a variety ofsources that can eliminate organophosphorus from the environment (Chapalamadugu & Chaudhry, 1992). This approach has several advantages, including being friendly to the environment, effective, and cost-efficient for *in situ* OPs remediation (Ossai et al., 2020). Studies have failed to isolate microorganisms capable of utilizing specific OP as a carbon source (Singh & Walker, 2006). This results from the improper combination and selection of media in man-made settings, the need for specific growth factors by certain strains, and the significant impact of microorganisms that cannot be cultured (Roberts et al., 1993). Imitating natural environments can enable the cultivation of bacteria that were previously uncultivable under laboratory conditions hence can aid in isolation and characterization of new OP degrading bacteria (Kaeberlein et al., 2002). Several studies have successfully isolated and characterized bacterial species which are able to degrade OPs (Atego, 2021). Studies have also shown that individual components of microorganisms, such as isolated enzymes, are capable of hydrolyzing OPs when purified (Karpouzas et al., 2006). However, biodegradation of OPs is dependent on the ecological, physiological, molecular, and biochemical characteristics of bacterial isolates, as well as the presence of a suitable system of metabolizing enzymes (Singh & Walker, 2006; Ambreen & Yasmin, 2020). Therefore, the isolation and characterization of OP degrading bacteria are essential for understanding the biodegradation mechanism and developing effective bioremediation strategies.

Enzymatic degradation is a promising method for removal of OPs from the environment (Xu et al., 2021). Enzymes catalyze specific chemical reactions, and have been identified to break down OPs compounds (Fenner et al., 2013). Enzymatic degradation has several advantages over other methods of organophosphate removal, including specificity and efficiency (Xu et al., 2021). Enzymes are highly specific for their target compounds, and can often degrade them completely without producing harmful by-products. Additionally, enzymes can work at relatively low concentrations and under mild conditions, making them a promising tool for environmental remediation (Fenner et al., 2013). However, enzyme-based methods also have some limitations, including the potential for enzyme deactivation and the need for careful management of enzyme production and delivery (DiCosimo et al., 2013). Phosphotriesterases (PTEs), organophosphorus hydrolases (OPHs) and paraoxonase (PON) are key enzymes that degrade OPs (Ghanem & Raushel, 2005). Bacterial isolates capable of using OPs like chlorpyrifos, chlorfenvinphos, and diazinon as their only supply of carbon for growth are reported (Atego, 2021).

These findings suggest the potential for using microbial degradation as an effective

Published December 2024

method for OPs bioremediation by understanding the molecular and biochemical characteristics of bacterial isolates and their associated enzymes, more efficient and targeted bioremediation strategies can be developed (Atego, 2021; Asamba et al.,2022). The OPH produced by many bacteria and other microorganisms degrade OPs compounds by cleaving the phosphate ester bond, leading to formation of a phosphate group and an alcohol or thiol group (Singh & Walker 2006). The PON can hydrolyze specific OPs compounds, while PTEs can break down more complex OPs such as nerve agents (Singh & Walker 2006).

These strains utilized chlorfenvinphos as the only source of carbon for growth, indicating the potential for decontamination of polluted areas (Atego, 2021; Asamba et al., 2022). Temperature and pH are critical factors that profoundly affect microbial survival and degradation of OPs, such as chlorpyrifos, chlorfenvinphos, and diazinon (Sidhu et al., 2019).Therefore, optimizing the levels of pH and temperature for bacterial strains able to degrade chlorfenvinphos is essential to evaluate their potential to tolerate local environmental conditions.

This study therefore, aimed at optimizing temperature and pH for chlorpyrifos degrading bacteria isolated from OP treated environment (water, dip wash, spray race residues and soils)collected from dairy farms in Kenya for their ability to produce chlorfenvinphos hydrolyzing enzymes and screening for chlorfenvinphos degrading organophosphorus phosphatase enzymes.

Materials and Methods

Viability Assessment of Isolates

The bacterial isolates used in this study had previously been isolated from organophosphate polluted dairy farms soils in Kenya and stored at the Technical University of Mombasa (TUM) laboratory (Atego, 2021; Asamba et al. , 2022). To revive the isolates, they underwent culture in nutrient broth (NB) by dissolving 0.4 g/50 ml of distilled water and adjusting pH to 7.0 then autoclaved at 121˚C. This was followed by incubation at 30°C on a rotor shaker operating at 150 rpm for 24 hours. Subsequently, the viability and growth curve of the bacterial isolates were evaluated using minimal salt medium (MSM) constituted in g/L as follows: $MgSO_4$ $7H_2O$, 0.2; K_2HPO_4, 4.8; Fe_2 $(SO_4)_3$, 0.001; NH_4NO_3, 1.0; KH_2PO_4, 1.2 and $Ca(NO_3)_2.4H_20$, 0.4, (Omolo *et al.*, 2012) supplemented with chlorfenvinphos (98% purity obtained from Sigma Andrich) at a concentration of 25 ppm and autoclaved at 121˚C. Each culture was inoculated with 1 ml of revived bacterial broth having an optical density of 0.6 at 600 nm (24-hour-old), and then incubated at 30°C in a shaker operating at 150 rpm for 5 days. MSM supplemented with CFPV but lacking bacterial inoculation served as a control to monitor non-bacterial-related changes in optical density over time. Growth of bacteria was tracked through the assessment of optical density, utilizing a UV-Vis spectrophotometer (Shimadzu, Japan), at 24-hour intervals. MSM supplemented with CFVP but lacking bacterial inoculation served as a control to monitor non-bacterial-related changes in optical density over time.

Optimization of Growth Conditions

In order to assess how pH affects bacterial development, bacterial cultures were inoculated with mineral salts medium (MSM) supplemented with 25 ppm of chlorfenvinphos at different pH values (ranging from 5, 6, 7, 8, and 9) and then incubated at 30°C for 72 hours while being shaken. Similar to this, temperature optimization was investigated by cultivating bacterial isolates in MSM for 72 hours at a range of temperatures (25°C, 30°C, 35°C, and 40°C). MSM supplemented with CFPV but lacking bacterial inoculation served as a control to monitor non-bacterial-related changes in optical density over time. Over the course of three days, the optical density at 600 nm was taken every 24 hours utilizing a UV-Vis spectrophotometer to monitor the proliferation of bacteria.

Enzyme Extraction

To produce crude enzymes, viable isolates were added to 100 mL of freshly generated MSM containing 25 ppm of chlorfenvinphos (CFVP).

Published December 2024

Afterwards, the 250 mL flasks of Erlenmeyer were placed in incubator set at 30°C for 72 hrs while being rotated at 150 rpm on a rotary shaker. After that, 50 mL of the culture broth was centrifuged for 30 min at 4°C at 8000g.This was followed by washing twice using cold 0.5 M phosphate buffer (pH 7). After reconstituting the bacterial pellets in 5 mL of 0.5 M phosphate buffer (pH 7), the pellets were broken up using an ultrasonic cell disruption machine (Scientz, China). The suspension underwent centrifugation at 12000g for 10 min at 4°C. The culture was then centrifuged for 20 min at 15000g, and supernatant used as crude enzyme source for purification (Liang et al. 2014).

Enzyme Purification

Each stage of the purifying procedure was carried out at 4°C with a few adjustments. Purification was done using methodology outlined by Liang et al., (2014). Ammonium sulfate was added to the supernatant obtained at 15000g, dissolved to 20% saturation, and then agitated for 30 min. The resultant suspension was centrifuged at 15000g for 20 min after being dissolved in ammonium sulfate to 80% saturation once more. The resultant pellet was filtered using a 0.22 µm Nylon Membrane Filter (Tisch Scientific), diluted in 5 mL of 0.5 M phosphate buffer (pH 7), dialyzed against 0.5 M phosphate buffer (pH 7), and concentrated using 80% ammonium sulfate. A Sephadex G-100 column (2.5 x 100 cm) pre-equilibrated with 0.5 M phosphate buffer (pH 7) was loaded with the concentrated enzyme solution. The column was with 200 mL of the same buffer at a flow rate of 0.25 mL/min, and 5-mL fractions were collected at intervals. Fractions that exhibited high activity were concentrated. The purified enzyme fractions were then evaluated for activity.

Determination of Total Protein Concentration

Determination of protein concentration was done following the protocol described by Kejriwal et al., (2014) with adjustments, using bovine serum albumin as the standard. Absorbance at 660 nm was measured using a spectrophotometer (Shimadzu, Japan).

Enzyme Screening

To screen for OPP enzymes, 3 mL of purified enzyme extract was added to 1 mL of the buffer-substrate solution containing p-nitrophenyl phosphate (p-NPP). The resultant solution was then incubated at 30°C for 20 min. To terminate the reaction, 1 mL of 1 Normality (N) sodium hydroxide was added to increase the pH of the mixture to 11. Under alkaline conditions, the colorless p-NPP substrate converted to yellow p-nitrophenol (p-Np).

Enzyme Activity Assay

To determine enzyme activity, UV-VIS spectrophotometer was used at 405 nm. One unit (U) of OPP activity was defined as the amount of enzyme needed to release one nanomol of p-nitrophenol per min at 30°C (Ambreen & Yasmin, 2020). A standard curve of OPP concentration was used to calculate the enzyme activity of crude enzyme, precipitate produced by ammonium sulfate saturation, and the five fractions extracted by gel filtration chromatography (Ambreen & Yasmin, 2020).

Data Analysis and Statistical Test

Data obtained was coded in MS Excel, presented in tables and figures and analyzedusing Fischer Least Variance using Gen Stat discovery V. 14 (Pyne *et al*, 2011). Significant differences in multiple means were analysed using 2-way ANOVA and Duncan's test, and $P < 0.05$ was considered significant.

Results and Discussions

Viability of Bacterial Isolates

After the growth of the isolates on mineral salt medium (MSM) enhanced with chlorfenvinphos at a concentration of 25 ppm for 5 days, different growth rates were observed when optical density (OD) at 600 nm was taken after 24 hrs. *A. kashmirensis* exhibited an increase in OD from 0.0637 at the start of the experiment reaching a peak of 0.181 in day 3 then gradually decreased to 0.104 in day 5. This growth was different from that of *M. luteus* whose peak was observed in day 4 at 0.146 and *L. sphaericus* at 0.144 in day 2 (Figure 1). *A. kashmirensis* exhibited the highest overall growth (OD 0.181) followed by *M. luteus*

Published December 2024

(OD 0.146) with *L. sphaericus* showing slowest growth (OD 0.144).

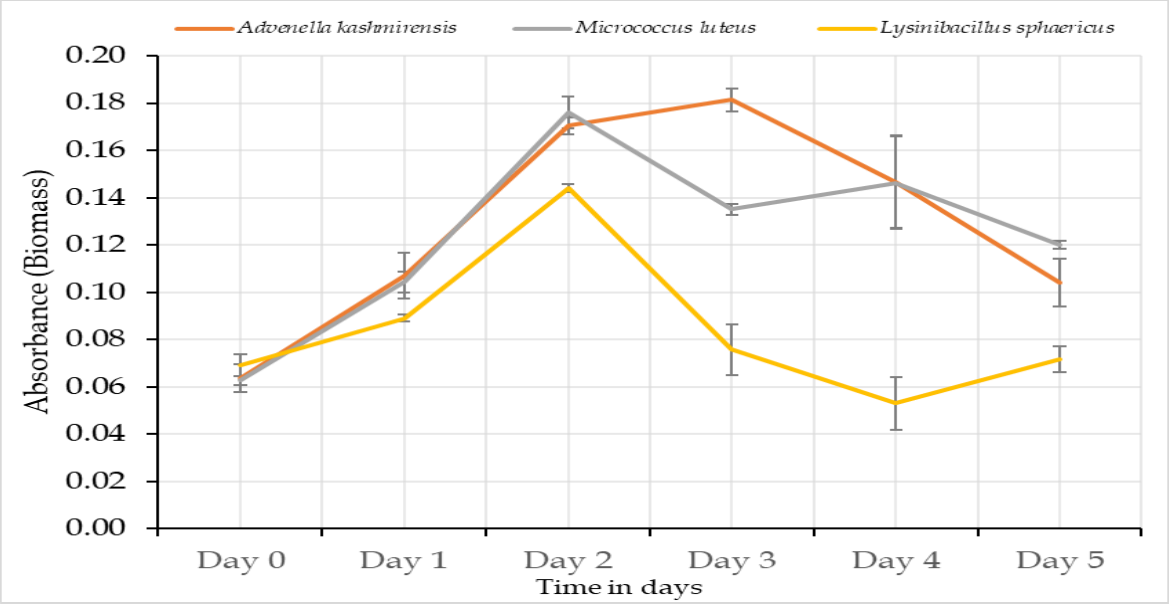

Figure 1. Growth and growth peaks of stored chlorpyrifos degrading bacterial isolates grown on MSM supplemented with chlorfenvinphos at 30 ˚C and pH 7.0.

The variation in growth patterns observed in *A. kashmirensis, M. luteus* and *L. sphaericus* indicated potential differences in their competitive abilities and succession dynamics. *A. kashmirensis* reached the highest peak (OD 0.181) followed by *M. luteus* (OD 0.146 while *L. sphaericus* exhibited the lowest (OD 0.144). The differences in optimal growth days may be due to differences in metabolic rates and nutrient utilization. Therefore, *A. kashmirensis* can be used in environments requiring faster bioremediation compared to other isolates. *L. sphaericus* reached its growth peak earlier on day 2 compared to other isolates and this confer a competitive advantage in succession dynamics by allowing *L. sphaericus* to quickly utilize available resources, potentially outcompeting slower-growing species. Bacteria with faster growth rates efficiently utilize organophosphates, leading to earlier growth peak, while slower-growing species may require more time to reach their maximum population density.

Optimization of pH and Temperature

The isolates were grown in MSM supplemented with CFVP at various pH levels, the three bacterial isolates showed different optimal pH and temperature conditions for growth. L. sphaericus showed optimal growth at pH 7 with an optical density (OD) of 0.132, M. luteus optimal growth was at pH 7 with an OD of 0.106 while A. kashmirensis displayed optimal growth at pH 6 with an OD of 0.175. All isolates reached their highest growth at an optimal temperature of 35°C. These conditions corresponded to the highest OD values, indicating the most favorable environment for the proliferation of these bacteria. The highest growth was observed in A. kashmirensis with an OD of 0.175 at pH 6, whereas the lowest growth was noted in M. luteus with an OD of 0.106 at pH 7. These findings are consistent with prior research, which established varying optimal pH values for different bacteria. For example, pH 5.8-7.0 for Alcaligenes faecalis, pH 6.5 for *Bacillus toyonensis* (Jiménez *et al.*, 2013), pH 7.5 with *Pseudomonas sp.*, pH 7.0 with Bacillus sp. and *Brevundimonas*

Published December 2024

diminuta CB21 (Dossounon *et al.,* 2019), and pH 5.4-7.0 with *Bacillus weihenstephanensis* (Guérin *et al.,* 2017).

The findings are consistent with that of optimum chlorfenvinphos breakdown that requires a pH of neutral to slightly acidic, since microbial strains in contaminated settings prefer a pH range of 5 to 8 (Vidali, 2001). Optimization of *B. diminuta* CB21 exhibited maximum biomass over all pH ranges during optimization, in all studied bacteria, suggesting its potential for bioremediation in chlorfenvinphos contaminated environments (Bhatia & Malik, 2013). This indicated that such bacteria hold promise for mitigative effects of chlorfenvinphos in the ecosystem.

These findings are consistent with Farhan et al., (2021), highlighting significance of bacterial species able to grow under different pH levels, which enhances their likelihood of success in biodegradation efforts amid dynamic ecosystems. Adaptability of *B. diminuta* CB21 emerges as a pivotal factor in the success of contaminant degradation, underscoring its significance in strain selection for bioremediation initiatives (Gaonkar et al., 2019; Farhan et al., 2021; Foong et al., 2020). Understanding significance of bacterial adaptation helps in designing decontamination methods for organophosphates, addressing environmental contamination, and saving the lives of living organisms.

All isolates exhibited optimal chlorfenvinphos degradation when incubated at a temperature of 35°C. These findings contrast with research showing that *A. faecalis* grows best at 37.5°C (Kumar et al., 2013), while *B. diminuta* CB21 and *Pseudomonas* spp. thrive best at 37°C for *B. diminuta* CB21 and *Pseudomonas* spp. (Meng et al., 2017), as well as 30°C for *B. weihenstephanensis* (Trunet et al., 2015), and are in line with a study on *Bacillus toyonensis* which showed an optimum temperature at 35°C (Dossounon et al., 2019). The observed variations might be attributed to the different growth environments, like the use of MSM in experiments. Temperature affects biological processes in a big way, affecting pesticide bioremediation as well as bacterial development (Farhan et al., 2021; Ali et al., 2014).

Screening for production of OPP enzymes

All the 3 isolates (*A. kashmirensis, M. luteus*, and *L. sphaericus*) demonstrated a yellow colour indicating the presence of OPP. This observation aligns with prior research, indicating that alkaline phosphatases, including those isolated from *Rhizobium* and *Pyrococcus abyssi*, typically exhibit their highest catalytic activity at pH levels of 8 or greater, with optimal ranges spanning from 6.8 to 11.8 (Meng et al., 2019; Ganjewala et al., 2008).

Quantification of Total Protein Content

Bovine serum albumin was used as the reference protein and modified Lowry method described by Omolo et al., (2014) was used to determine protein concentration. This process resulted in five distinct purified fractions, each demonstrating separation of proteins according to their sizes. Total protein concentration was determined by use of the standard curves (Table 1).

Published December 2024

Table 1. Total protein concentration (µg/mL) in the crude extract and after precipitation using ammonium sulphate and gel filtration, respectively

Bacterial isolate	Crude enzyme	(NH4)2SO4 precipitation
Advenella kashmirensis	465.407a±31.820	290.222c±1.283
Micrococcus luteus	292.074c±8.373	249.481b±47.965
Lysinibacillus sphaericus	303.556b±20.368	233.556a±41.609

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row and column are significantly different ($p < 0.05$).

The results reveal varying total protein concentrations among bacterial isolates, with *A. kashmirensis* exhibiting the highest concentrations in both crude enzyme and after $(NH_4)_2SO_4$ precipitation, while *M. luteus* and *L. sphaericus* display different degrees of reduction after precipitation steps.

In the crude enzyme extracted from *A. kashmirensis*, the total protein concentration decreased from 465.4 ± 31.8 µg/mL to 290.2 ± 1.3 µg/mL after ammonium sulphate precipitation and was significantly different ($p < 0.05$) from the other isolates (Table 1) implying varying levels of impurities among the crude extracts produced by isolates. The significant decrease in total protein concentration after ammonium sulfate precipitation suggests effective removal of impurities indicating successful purification of the crude enzyme extracts than other isolates. *A. kashmirensis* exhibited the highest total protein concentration after precipitation and was significantly different at $p < 0.05$ from the other isolates. Therefore, *A. kashmirensis* is suitable for applications requiring high concentrations of total proteins compared to the other two isolates.

From gel filtration fractions, the data reveal that *A. kashmirensis* consistently exhibits the highest total protein content in Fraction I, implying its potential application in bioremediation requiring high protein content. Fraction I consistently indicated higher protein content across all isolates, underscoring its significance in bioremediation applications compared to other fractions (Table 2).

Table 2. Shows variations in total protein concentrations among five enzyme fractions obtained after gel filtration at 20-min intervals, with *A. kashmirensis* exhibiting the highest concentrations, and Fraction I consistently displaying the highest concentration across all isolates followed by *M. luteus* and lastly *L. sphaericus*

Bacterial isolate	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V
Advenella kashmirensis	85.037a±10.028	73.926c±4.900	69.111d±1.697	73.185c±4.173	57.259b±11.410
Micrococcus luteus	76.148c±3.920	48.741d±9.945	65.040b±8.2489	60.222e±3.572	51.704a±5.342
Lysinibacillus sphaericus	64.296b±9.392	54.667a±6.939	53.926a±5.456	53.556a±6.420	53.185a± 4.174

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row and column are significantly different ($P < 0.05$).

Published December 2024

Determination of Enzyme Concentration

Enzyme activity, representing an enzyme's capacity to convert a specific substrate into product per unit time and was determined in µmol/mL/min. Enzyme concentrations were calculated for crude enzyme, ammonium sulfate precipitation, and the five fractions obtained through gel filtration chromatography using a standard curve using serial dilutions of p-nitrophenol (p-NP) ranging from 1 to 10 µg/mL, as depicted in Table 3 based on p-Nitrophenol assays (Figure 2).

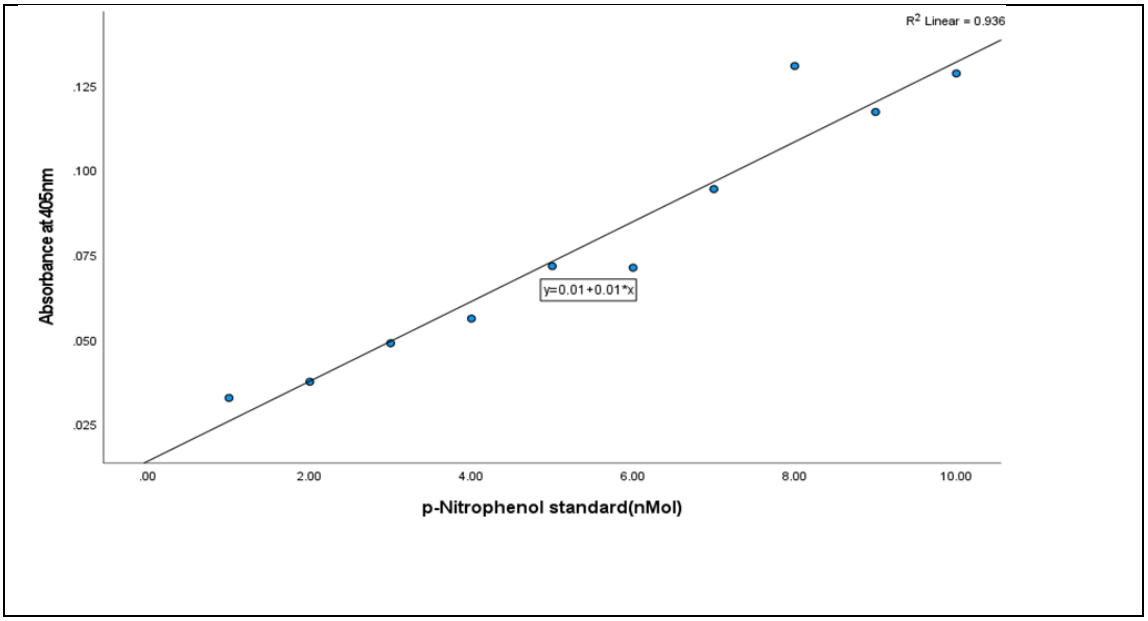

Figure 2. Standard curve of p Nitrophenol

Published December 2024

Table 3. Comparing enzyme concentrations at different purification stages for bacterial isolates (*A. kashmirensis, M. luteus*, and *L. sphaericus*), with *A. kashmirensis* consistently exhibiting the highest concentrations across all fractions, demonstrating a trend of increasing enzymatic activity during the purification process followed by *M. luteus*, and lastly *L. sphaericus*.

Bacterial isolate	Crude enzyme	(NH4)2SO4	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V
A. kashmirensis	21.939c±2.077	28.620d±2.275	54.980g±1.442	14.104a±3.662	34.253e±1.552	17.832f±1.747	40.022b±0.369
M. luteus	12.379a±1.748	19.451b±2.724	38.316c±0.490	14.596a±1.7639	16.004d±1.203	15.370d±1.194	15.734d±1.194
L. sphaericus	7.334b±0.833	15.949d±0.505	32.853e±0.361	11.312c±1.405	12.485c±3.890	14.355a±1.026	6.530f±1.026

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row and column are significantly different (P < 0.05).

Table 4. Enzyme activity (uMol/mL/Min) in crude enzymes, ammonium sulphate precipitation, and purification using gel filtration with *A. kashmirensis* consistently exhibits the highest enzyme activity across all fractions, reaching its maximum in Fraction I (19.761a±0.818), followed by *M. luteus* with intermediate enzyme activity, peaking in Fraction I (13.772b±0.979). *L. sphaericus* demonstrates the lowest enzyme activity, observed in Fraction I (11.808c±0.181)

Bacterial isolate	Crude enzyme	(NH4)2SO4	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V
L. sphaericus	2.638b±0.963	5.732d±0.300	11.808c±0.181	4.066a±0.130	4.487a±0.505	5.160d±1.398	2.347b±0.369
M. luteus	4.449a±0.327	6.991d±0.628	13.772b±0.979	5.246c±0.176	5.752c±0.634	5.524c±0.432	5.655c±0.429
A. kashmirensis	2.638b±0.963	10.287e±0.746	19.761a±0.818	5.069c±0.518	12.311d±1.316	6.409f±0.558	14.385g±2.425

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row and column are significantly different (P < 0.05).

In the crude enzyme preparation, the highest ability for the organophosphate phosphatase synthesis was observed in *M. luteus* (4.449±0.327 µMol/mL/Min) whereas in the crude enzyme extract of *A. kashmirensis* and *L. sphaericus*, the enzyme activity was at 2.638±0.963 µMol/mL/Min. *M. luteus* produced the highest activity of crude extract of 4.449±0.327 µMol/mL/Min and was significantly different at $P < 0.05$. This suggests it had a higher effectiveness for pesticide degradation in bioremediation applications compared to *A. kashmirensis* and *L. sphaericus*. A significant increase in enzyme activity ($p < 0.05$) was observed after each of the purification steps namely, ammonium sulphate precipitation and gel filtration of the crude enzyme (Table 4). This can be due to increase in concentration, thus concentration favored formation of multimer and increases activity. Although the enzyme isolated from the three bacterial isolates showed a substantial increase in enzyme activity after further purification by ammonium sulphate precipitation and gel filtration, the highest enzyme activity observed in the gel filtration step (Table 5). After ammonium sulphate precipitation, the enzyme activity increased with the highest observed in *A. kashmirensis* (10.287±0.746 µMol/mL/Min) followed by *M.luteus* (6.991±0.628 µMol/mL/Min) and finally *L. sphaericus* (5.732±0.300 µMol/mL/Min). *A. kashmirensis* produced the highest enzyme activity (10.287±0.746 µMol/mL/Min) and was significantly different at $P < 0.05$. Ammonium sulfate precipitation increased enzyme activity, with *A. kashmirensis* exhibiting the highest activity, suggests its superior degradation capabilities compared to M. luteus and L. sphaericus, highlighting its potential as the most effective for pesticide remediation in practical applications.

In this study, gel filtration chromatography was employed to separate enzyme components, yielding five distinct fractions collected at 20-minute intervals. After gel filtration, the enzyme activity increased significantly ($p < 0.05$) with fraction I having the highest enzyme activity in all the bacterial isolates that were studied. In fraction I, the highest enzyme activity was observed in *A. kashmirensis* (19.761±0.818 µMol/Min) followed by *M. luteus* (13.772±0.979 µMol/mL/Min) and finally, *L. sphaericus* (11.808±0.181 µMol/mL/Min. *A. kashmirensis* had the highest enzyme activity (19.761±0.818 µMol/Min) and was significantly different at $P < 0.05$. This suggests that *A. kashmirensis*, with elevated enzyme activity in Fraction I, holds promise for applications in bioremediation scenarios that demand high enzyme concentrations. Further, it was observed that *A. kashmirensis* produce organophosphate phosphatase enzyme with the highest activity in all the five fractions, *L. sphaericus* produced organophosphate phosphatase with lowest activity .Therefore, based on the enzyme activity data, *A. kashmirensis* is more effective in degrading chlorfenvinphos compared to *L. sphaericus* and *M. luteus*, as it consistently displays higher enzymatic activity across various fractions. This corroborates with other studies where gel filtration purification a more refined technique separated proteins based on their size (Scopes, 1994; Doonan, 1996; Tripathi, 2021). From this study it was concluded that *A. kashmirensis*, was the best for rapid bioremediation, while *L. sphaericus*, had a competitive advantage by peaking earlier and quickly utilizing resources. These variations in growth patterns reflected differences in metabolic rates and nutrient utilization, influencing their competitive abilities and succession dynamics.

Acknowledgments

This research was supported by a grant (TUM/PRI/IRP/20-21/Vol.3/25) from the Technical University of Mombasa's Office of Registrar, Partnership, Research, and Innovation (PRI).

References

Ali, M., Kazmi, A. A., & Ahmed, N. (2014). Study on effects of temperature, moisture and pH in degradation and degradation kinetics of aldrin, endosulfan, lindane pesticides during full-scale continuous

rotary drum composting. *Chemosphere*, *102*, 68-75.

Ambreen, S., & Yasmin, A. (2020). Isolation, characterization and identification of organophosphate pesticide degrading bacterial isolates and optimization of their potential to degrade chlorpyrifos. *International Journal of Agriculture and Biology*, *24*(4), 699-706.

Asamba, M. N., Mugendi, E. N., Oshule, P. S., Essuman, S., Chimbevo, L. M., & Atego, N. A. (2022). Molecular characterization of chlorpyrifos degrading bacteria isolated from contaminated dairy farm soils in Nakuru County, Kenya. *Heliyon*, *8*(3).

Atego, N. A 2021. Isolation and Characterization of Native Organophosphate Degrading Bacteria and their Degradation Products in Milk and Milk Products in Kenya (Unpublished thesis) Technical University of Mombasa.

Bhatia, D., & Malik, D. K. (2013). Isolation and characterization of chlorpyrifos degrading soil bacteria of environmental and agronomic significance. *Journal of Environmental Science & Engineering*, *55*(2), 227-238.

Chapalamadugu, S., & Chaudhry, G. R. (1992). Microbiological and biotechnological aspects of metabolism of carbamates andorganophosphates. *Critical reviews in biotechnology*, *12*(5-6), 357-389.

Dar, M. A., Kaushik, G., & Chiu, J. F. V. (2020). Pollution status and biodegradation of organophosphate pesticides in the environment. In *Abatement of environmental pollutants* (pp. 25-66). Elsevier.

Dash, D. M., & Osborne, W. J. (2022). A systematic review on the implementation of advanced and evolutionary biotechnological tools for efficient bioremediation of organophosphorus pesticides. *Chemosphere*,137506.

DiCosimo, R., McAuliffe, J., Poulose, A. J., & Bohlmann,G. (2013). Industrial use of immobilized enzymes. *Chemical Society Reviews*, *42*(15),6437-6474.

Doonan, S. (1996). *General Strategies* (pp. 1-16). Humana Press. Tripathi, A. D., Paul, V., Agarwal, A., Sharma, R., Hashempour-Baltork, F., Rashidi, L., & Darani, K. K. (2021). Production of polyhydroxyalkanoates using dairy processing waste–a review. *Bioresource technology*, *326*, 124735.

Dossounon, Y., Blaghen, M., Hager, R., Salam, S., El Magd, N. M., & Almadou, A. (2019). Exopolysaccharide production by Brevundimonas diminuta isolated from Marchica lagoon ecosystem in Morocco. *International Journal of Applied Microbiology and Biotechnology*, *7*, 79-85.

Farhan, M., Ahmad, M., Kanwal, A., Butt, Z. A., Khan, Q. F., Raza, S. A., ... & Wahid, A. (2021). Biodegradation of chlorpyrifos using isolates from contaminated agricultural soil, its kinetic studies. *Scientific reports*, *11*(1), 10320.

Fenner, K., Canonica, S., Wackett, L. P., & Elsner, M. (2013). Evaluating pesticide degradation in the environment: blind spots and emerging opportunities. *science*, *341*(6147), 752-758.

Foong, S. Y., Ma, N. L., Lam, S. S., Peng, W., Low, F., Lee, B. H., ... & Sonne, C. (2020). A recent global review of hazardous chlorpyrifos pesticide in fruit and vegetables: Prevalence, remediation and actions needed. *Journal of hazardous materials*, *400*, 123006.

Ganjewala, M. K. D. (2008). Isolation of Periplasmic Alkaline Phosphatase. *Research Journal of Microbiology*, *3*(3), 157-162.

Gao, Y., Chen, S., Hu, M., Hu, Q., Luo, J., & Li, Y. (2012). Purification and characterization of a novel chlorpyrifos hydrolase from

Cladosporium cladosporioides Hu-01. *PLoS One*, *7*(6), e38137.

Gaonkar, O., Nambi, I. M., & Suresh Kumar, G. (2019). Biodegradation kinetics of dichlorvos and chlorpyrifos by enriched bacterial cultures from an agricultural soil. *Bioremediation Journal*, *23*(4), 259-276.

Ghanem, E., & Raushel, F. M. (2005). Detoxification of organophosphate nerve agents by bacterial phosphotriesterase. *Toxicology and appliedpharmacology*, *207*(2), 459-470.

Guérin, A., Rønning, H. T., Dargaignaratz, C., Clavel, T., Broussolle, V., Mahillon, J., ... & Nguyen-The, C. (2017). Cereulide production by Bacillus weihenstephanensis strains during growth at different pH values and temperatures. *Food microbiology*, *65*, 130-135.

Kadiru, S., Patil, S., & D'Souza, R. (2022). Effect of pesticide toxicity in aquatic environments: A recent review. *Int. J. Fish Aquat. Stud*, *10*(3), 113- 118.

Kaeberlein, T., Lewis, K., & Epstein, S. S. (2002). Isolating" uncultivable" microorganismsin pure culture in a simulated natural environment. *Science*, *296*(5570), 1127-1129.

Karpouzas, D.G., & Singh,B.K.(2006). Microbial degradation oforganophosphorus xenobiotics: metabolic pathways and molecularbasis. *Advances in microbial physiology*, *51*, 119-225.

Kejriwal, S., Bhandary, R., Thomas, B., & Kumari, S. (2014). Estimation of levels of salivary mucin, amylase and total protein in gingivitis and chronic periodontitis patients. *Journal of clinical and diagnostic research: JCDR*, *8*(10), ZC56.

Kumar, A., Bhunia, B., Dasgupta, D., Mandal, T., Dey, A., Datta, S., & Bhattacharya, P. (2013). Optimization of culture condition for growth and phenol degradation by Alcaligenes faecalis JF339228 using Taguchi Methodology. *Desalination and water treatment*, *51*(16-18), 3153-3163.

Meng, D., Jiang, W., Li, J., Huang, L., Zhai, L., Zhang, L., ... & Liao, X. (2019). An alkaline phosphatase from Bacillus amyloliquefaciens YP6 of new application in biodegradation of five broad-spectrum organophosphorus pesticides. *Journal of Environmental Science and Health, Part B*, *54*(4), 336-343.

Meng, L., Zhang, Y., Liu, H., Zhao, S., Wang, J., & Zheng, N. (2017). Characterization of Pseudomonas spp. and associated proteolytic properties in raw milk stored at low temperatures. *Frontiers in microbiology*, *8*, 2158

Omolo, K. M., Magoma, G., Ngamau, K., & Muniru, T. (2012). Characterization of methomyl and carbofuran degrading-bacteria from soils of horticultural farms in Rift Valley and Central Kenya. *African Journal of Environmental Science and Technology*, *6*(2), 104-114.

Ossai, I. C., Ahmed, A., Hassan, A., & Hamid, F. S. (2020). Remediation of soil and water contaminated with petroleum hydrocarbon:A review. *Environmental Technology & Innovation*, *17*, 100526.

Pehkonen, S. O., & Zhang, Q. (2002). The degradation of organophosphorus pesticides in natural waters: a critical review. *Critical reviews in environmental science and technology*, *32*(1), 17-72.

Roberts, S. J., Walker, A., Parekh, N. R., Welch, S. J., & Waddington, M.

J.(1993). Studies on a mixed bacterial culture from soil which degrades the herbicide linuron. *Pesticide science*, *39*(1), 71-78.

Scopes, R. K., & Scopes, R. K. (1994). The protein purification laboratory. *Protein Purification: Principles and Practice*, 1-21.

Sidhu, G. K., Singh, S., Kumar, V., Dhanjal, D. S., Datta, S., & Singh, J. (2019). Toxicity, monitoring and biodegradation of organophosphate pesticides: a review. *Critical reviews in environmental science and technology*, *49*(13), 1135-1187.

Singh, S., Kang, S. H., Mulchandani, A., & Chen, W. (2008).Bioremediation: environmental clean-up through pathway engineering. *Current opinion in biotechnology*, *19*(5), 437-444.

Singh, B.K., &Walker,A.(2006). Microbial degradation of organophosphorus compounds. *FEMS microbiology reviews*, *30*(3), 428-471.

Stadlmair, L. F., Letzel, T., Drewes, J. E., & Grassmann, J. (2018). Enzymes in removal of pharmaceuticals from wastewater: A critical review of challenges, applications and screening methods for their selection. *Chemosphere*, *205*, 649-661.

Tripathi, A. D., Paul, V., Agarwal, A., Sharma, R., Hashempour-Baltork, F., Rashidi, L., & Darani, K. K. (2021). Production of polyhydroxyalkanoates using dairy processing waste–a review. *Bioresource technology*, *326*, 124735.

Trunet, C., Mtimet, N., Mathot, A. G., Postollec, F., Leguerinel, I., Sohier, D., ... & Coroller, L. (2015). Modeling the recovery of heat-treated Bacillus licheniformis Ad978 and Bacillus weihenstephanensis KBAB4 spores at suboptimal temperature and pH using growth limits. *Applied and Environmental Microbiology*, *81*(2), 562-568.

Tudi, M., Daniel Ruan, H., Wang, L., Lyu, J., Sadler, R., Connell, D., ... & Phung, D. T. (2021). Agriculture development, pesticide application and its impact on the environment. *International journal of environmental research and public health*, *18*(3), 1112.

Vidali, M. (2001). Bioremediation. an overview. *Pure and applied chemistry*, *73*(7), 1163-1172.

Xu, W., Zhao, S., Zhang, W., Wu, H., Guang, C., & Mu, W. (2021). Recent advances and future prospective of organophosphorus-degrading enzymes: Identification, modification, and application. *Critical Reviews in Biotechnology*, *41*(7), 1096-1113.