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Production, Partial Purification and Characterization of Free-and Immobilized Urease Isolated from Aspergillus Species Obtained from Long-Horned Beetles (*Cerambycidae Latreille*)

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Abstract

Urease is a vital enzyme with applications in agriculture, environmental biotechnology, and medicine, playing a key role in metabolism and bioremediation. Despite its significance, efficient production and purification methods are still needed to meet industrial demands. This study demonstrated the production, partial purification and physicochemical properties of free-and immobilized urease from *Aspergillus niger* and *Aspergillus ustilago* isolated from the gut of Long-horned beetles. Urease production was achieved through submerged fermentation using urea as the primary substrate. Partial purification was carried out through ammonium sulfate precipitation (70 % saturation). Immobilization of the urease was successfully performed using sodium alginate, which enhanced the enzyme stability and reusability. The activities of the crude and partially purified free-urease were (1.82 and 4.82 U/mL) and (1.78 and 4.23 U/mL) for *A. niger and A. ustilago* respectively. The immobilized enzyme showed optimal activity at pH 7.0 and a temperature of 50 ^{o}C. Metal ions such as Fe^{2+}, Mg^{2+}, and Ca^{2+}, enhanced the activity of the free-urease from *A. ustilago* while Al^{3+}, Cu^{2+} and Co^{2+} were observed to cause inhibitory effects on free-urease from *A. niger*. On the other hand, with immobilized enzyme, Fe^{2+}, Mg^{2+}, Al^{3+} and Ca^{2+} showed a pronounced activation at both concentrations investigated. It was inferred that the immobilized urease from *Aspergillus spp*. was more stable with stronger affinity for all the metal ions tested. This study has provided information on the feasibility of immobilization of urease from *Aspergillus spp*., isolated from the gut of Long-horned beetles with biotechnological implications for industrial and medical applications such as in environmental bioremediation and biosensor development.

1. Introduction

Enzyme are biological macromolecules that catalyze biochemical reactions essential for sustaining life [1-4]. By lowering activation energy, enzymes regulate the rate and specificity of metabolic reactions, ensuring that cellular processes occur efficiently under physiological conditions. Their activity is influenced by several factors, including

temperature, pH, substrate concentration, and the presence of activators or inhibitors [2,5,6]. Urease (urea amidohydrolase; EC 3.5.1.5) is a nickel-dependent enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide [1,7]. This reaction plays a critical role in nitrogen metabolism and contributes significantly to the nitrogen cycle in natural and managed ecosystems [8]. Urea is a major storage and transport form of nitrogen, and its breakdown by urease releases readily available ammonia for plant and microbial uptake. Ammonia serves as a vital building block for essential biological molecules like amino acids and proteins. Through the release of ammonia, urease facilitates nitrogen availability for microbial and plant assimilation. In the absence of effective urease activity, urea may persist in the environment, reducing nitrogen bioavailability and potentially disrupting ecological balance [9]. Urease is commonly found in bacteria, fungi, and plants [1,7,10], and has widespread applications in agriculture, medicine, and environmental biotechnology. In agricultural systems, urease activity governs the efficiency of urea-based fertilizers by influencing the rate of ammonia release, thereby affecting nitrogen use efficiency and fertilizer losses. In medical contexts, urease-producing microorganisms are associated with pathological conditions such as urinary tract infections and gastric disorders [11,12], where urease-producing bacteria, such as *Helicobacter pylori*, contribute to the formation of stones or ulcers in the human body [13-15].

Microbial ureases, particularly those derived from fungi, have attracted attention due to their stability, adaptability, and suitability for industrial processes [1,10]. Fungal enzymes are often preferred because they can be produced extracellularly and remain active under variable environmental conditions [16,17]. Fungi from the genus *Aspergillus* are known to produce urease enzymes with unique properties. *Aspergillus niger* and *Aspergillus ustilago*, isolated from diverse environments, including the guts of insects, have emerged as a promising source of urease for biotechnological applications [18]. For instance, urease from *Aspergillus* species has been used in bio-remediation to neutralize urea in wastewater and agricultural runoff, helping reduce environmental pollution caused by excessive nitrogen [18,19].

Long-horned beetles (*Cerambycidae latreille*) harbor diverse microbial communities that assist in digestion and nutrient acquisition. These microorganisms are adapted to complex substrates, suggesting that enzymes derived from such environments may possess enhanced functional properties. Long-horned beetles have been investigated on their potential for harboring diverse fungi [2,5]. These fungi, residing in the beetle's gut, often produce a range of enzymes, including urease, which help break down plant materials and other organic matter (2,16,20]. This symbiotic relationship presents an opportunity to explore urease production from fungal strains within beetle guts, potentially discovering ureases with enhanced properties. Understanding urease's biochemical characteristics from *Aspergillus spp.* isolated from the Long-horned beetle's gut may open new avenues for environmental and industrial applications [21,22]. Long-horned beetles which feed on plant materials, harbor unique microorganisms within their digestive systems, potentially including urease-producing fungi like *A. niger* and *A. ustilago*.

Despite growing interest in microbial ureases, limited information is available on the production, purification, immobilization, and characterization of urease from *A. ustilago* isolated from insect gut environments. Exploring this source and optimizing urease production could significantly enhance enzyme stability and activity, making it more suitable for industrial applications. Urease is a critical enzyme in biological systems, playing a fundamental role in the catalytic decomposition of urea into ammonia (NH_3) and carbon dioxide (CO_2), and it is responsible for eliminating 80–90 % of nitrogenous waste from the body. Under typical physiological conditions, urea concentrations in blood serum or plasma range between 20–40 mg/dL, and therefore serves as a vital biomarker in clinical diagnostics [15,23].

Urease has been isolated and characterized from several Aspergillus species in previous studies. Although urease is commonly studied in bacteria (e.g., Proteus, Klebsiella) and plants (jack bean urease) [7], multiple reports confirm urease activity and enzyme isolation from members of the genus Aspergillus [1,10,15,24]. However, there are published reports showing that urease is produced and studied in Aspergillus species (as well as isolation of the urease enzyme), although most work focuses on species like *A. niger, A. fumigatus, and A. nidulans* [1,10,15,24], but there are no known published reports demonstrating the immobilization of urease produced specifically by *A. ustilago* derived from Long-horned beetles. Most urease immobilization studies use urease from other microbial or plant sources [1,24]. Urease enzyme has recently found various applications in biotechnology industry, e. g. for the assessment and detoxification of urea in some fluids such as blood, alcoholic beverages, and environmental wastewaters [1,15,16,18,19,25,26]. For industrial purposes, urease is provided from various organisms, including plants, bacteria, fungi, and invertebrates [1,27,28). Due to low price and reusability, the immobilized enzyme has been considered as one of the most significant strategies in industry, medicine, and biotechnology [16,29]. Enzyme immobilization refers to physically entrapment of an enzyme in a certain region to maintain enzyme catalytic activities [30].

Due to the importance of urease, efficient production and purification methods are still needed to meet industrial demands. *A. ustilago* and *A. niger*, isolated from the gut of Long-horned beetles, presents a promising new source of urease with potential for enhanced stability and activity [1,10]. Urease enzymes are critical for catalyzing the hydrolysis of urea into ammonia and carbon dioxide, with significant industrial and environmental

applications [1,10,15]. Isolation and purification of urease had been established in literatures but not from the *Aspergillus* isolated from the gut of Long-horned beetle. Most importantly, scientific reports specifically describing urease production by *A. ustilago* isolated from Long-horned beetles and subsequent immobilization of that urease enzyme are limited based on the available literatures. Therefore, this study sought to produce, partially purify, immobilize, and characterize urease from *Aspergillus* spp. isolated from the gut of Long-horned beetles, with potential applications in environmental, industrial, and medical biotechnology.

Most urease immobilization research involves plant/animal ureases [7], or urease from environmental/soil microbial sources, not insect-derived fungal isolates [18,19]. *A. niger* and *A. ustilago* are filamentous soil fungi capable of producing urease, an enzyme that hydrolyzes urea into ammonia and carbon dioxide [21]. This enzymatic activity lays a crucial role in the natural nitrogen cycle. By breaking down urea from fertilizers, animal waste, and industrial effluents, they enhance nitrogen recycling in soil ecosystems. The released ammonia can be converted into plant-available nitrogen forms, thereby improving soil fertility and supporting sustainable agriculture [18]. Their urease activity also contributes to bioremediation by reducing urea accumulation and minimizing nitrogen-related environmental pollution. Additionally, these fungi can support microbially induced calcium carbonate precipitation, promoting eco-friendly soil stabilization and sustainable construction practices. Therefore, it is highly expedient to explore this niche area by investigating novel ureolytic fungal isolates and immobilized enzyme systems.

2. Materials and Methods

2.1 Source of Organism

Aspergillus niger and *Aspergillus ustilago*, previously isolated from the gut of long-horned beetles (*Cerambycidae* latreille), was obtained from the Department of Biochemistry, Federal University of Technology, Akure.

2.2 Reagents and Equipment

The reagents used included carbon sources (glucose, sucrose, glycerol), nitrogen sources (urea, peptone), mineral salts (magnesium sulfate, potassium phosphate, ammonium sulfate), buffer solutions, phenol red, Nessler's reagent, sodium alginate, and sodium tungstate. All were products of Sigma-Aldrich (St. Louis, MO).

The equipment used for this research were portable pressure steam sterilizer (Model YX-18LM), UV–visible spectrophotometer (Axiom 721 Vis-spectrophotometer UK), pH meter (Philips India), water bath shaker (WHY-2, USA), water bath (HH-W420 thermostatic water cabinet, UK), refrigerated centrifuge (AFI, India), analytical and top loading balance (OHAUS Corporation, US). Other materials include glassware, spatula, glass stirrer, inoculating loop, beaker, measuring cylinder and microliter pipette were of analytical grade, purchased from VWR International, Inc. Clarksburg, MD).

2.3 Procedure for Isolating Aspergillus Species from Long-horned Beetles

Fresh sample of Long-horned beetles was collected from infested wood aseptically into a sterile container, and brought to the Microbiology laboratory Federal University of technology Akure. The sample was surface sterile to reduce the possibility of contamination from external sources by immersing the beetles in 1% hypochlorite for 30 seconds, and then rinsed with sterile water to remove any excess hypochlorite [31]. The beetles were carefully dissected under a laminar flow hood, using sterile tools, to extract internal tissues (e.g., gut, feces or body tissues).

2.3.1 Isolation of Fungal Cultures

Potato dextrose agar (3.9 g) was dissolved in 100 mL of distilled water and sterilized at 121 °C for 15 min. After cooling, the medium was inoculated separately with *A. niger* and *A. ustilago* and incubated at 30 °C with shaking at 150 rpm for 48 h.

2.4 Production of Urease from Aspergillus Spp.

A production medium containing urea (1.3 g), glucose (20 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), calcium chloride (0.3 g), agar (15 g), and phenol red (0.12 g) was prepared in 1,000 mL of distilled water. The pH was adjusted to 7.0 prior to sterilization. The resulting mixture was divided among four conical flasks, autoclaved at 125 °C for 15 minutes, and then allowed to cool. Afterward, 3 milliliters of the seed culture were inoculated into each flask. The flasks were placed on an orbital shaker at 40,000rpm and incubated at 30 °C for 36-48 hours. Following incubation, the culture fluid was sieved with moslin cloth and then centrifuged at 10,000 rpm for 15 minutes using a refrigerated centrifuge. The supernatant, considered the crude enzyme, was collected and stored at 4 °C. Additionally, the volume of the crude enzyme was measured by weight.

2.5 Determination of Urease Activity

Urease activity was determined according to the standard assay method of Rao [23]. Five hundred microliter (500 µL) of substrate solution i. e. 3% urea solution buffered with 500 µL of 0.2M phosphate buffer (pH 7.0) which is the stock solution was pipetted into 3 test tubes. Five hundred microliter (500 µL) of crude enzyme was added in test tube A and B. The reaction mix was incubated at 55 °C for 15 minutes and the reaction was stopped by adding 500µL of H_2SO_4. 500 µL of sodium tungstate solution was added then centrifuged at 40,000 rpm for 5 minutes. Nessler reagent was added to the supernatant and the absorbance was taken at 500 nm. The blank was set up without the enzyme and treated in the same condition with test tube A and B.

2.6 Determination of Protein Concentration

The protein concentration was routinely determined according to the method of Bradford [32] using Bovine serum albumin standard and the absorbance was read against blank at 595 nm with a spectrophotometer.

2.7 Partial Purification of Urease

2.7.1 Ammonium Sulfate Precipitation

The crude enzyme extract was partially purified by ammonium sulfate precipitation at 70% saturation, followed by centrifugation and dialysis against phosphate buffer (pH 7.0) at 4 °C.

2.7.2 Dialysis of Crude Enzyme

The pellet obtained was dialyzed against 0.1 M phosphate buffer (pH 7.0) at 4 ºC using a pre-treated dialysis bag, with three buffer changes. Subsequently, the partially purified enzyme was assayed for both enzyme activity and protein content.

2.8 Immobilization of the Partially Purified Enzyme

Zero point eight gramme of sodium alginate was measured into 20 ml of distilled water and stirred thoroughly to dissolve the sodium alginate, and then 20 ml of the partially purified enzyme was added and mixed evenly. For the control, buffer was used instead of the enzyme. 5.6g of $CaCl_2$ was measured into 200 ml of distilled water. A new syringe was used to draw the sodium alginate and enzyme mixture, which was then added drop by drop into the calcium chloride ($CaCl_2$) solution to form the beads. The mixture was stored in the refrigerator for 2 hours at 4 ºC. After, it was filtered and the beads were washed thoroughly with buffer to remove any unbound enzyme.

2.9 Characterization of the Free-and Immobilized Urease

2.9.1 Effect of pH on the Activity of the Free and Immobilized Urease

The effect of pH on the activity of the immobilized urease was investigated by carrying out urease assay at various pH ranging from 2.0 to 12.0. The buffering system consisted 50 mM glycine-HCl (pH 2.0 and 3.0), citrate buffer (pH 4.0 and 5.0), K_2HPO_4/ KH_2PO_4 buffer (pH 6.0 and 7.0), Tris-HCl (pH 8.0, 9.0 and 10.0) and Tris-NaOH buffer (pH 11.0 and 12.0) were employed. The immobilized urease beads activity at each pH medium was determined using standard assay procedure.

2.9.2 Effects of Temperature on the Activity of the Free and Immobilized Urease

The effect of temperature on the immobilized enzyme was determined by varying the temperature condition of the reacting mixture. The reacting mixture was incubated at different temperature ranging between 30 - 90 °C at an interval of 10 °C. The enzyme activity was carried out according to the standard assay procedure.

2.9.3 Effects of Metal Ions on the Activity of the Free and Immobilized Urease

The effect of metal ions at varying concentrations on the activity of the immobilized enzyme at 5 and 10 mM were determined using Mg^{2+}, Ca^{2+}, Al^{3+}, Cu^{2+}, Fe^{2+}, $Zn^{2+}$and Co^{+}. The substrate was pre-incubated with the above stated metals and enzyme activity was carried out using the standard assay procedure.

J. of Sustainable Materials Processing and Management Vol. 6 No. 1 (2026) p. 29-39

3. Results and Discussion

3.1 Crude Enzyme Activity and Protein Concentration

The results of the crude enzyme activity and protein concentration were found to be (1.819 units/mL and 5.28 mg/mL) for *Aspergillus niger* (4.827 units/mL and 1.36 mg/mL) for *Aspergillus ustilago* respectively. The summary of the partial purification was presented in Table 1. The dialysate obtained after dialysis of precipitated protein using ammonium sulfate showed yield range of 6.54 - 8.24 % with 1.89 – 2.42-fold purification.

Table 1 *Summary of partial purification of urease from Aspergillus spp*

	Step	Vol. (mL)	Activity (U/mL)	Protein Con. (mg/ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification fold
A. niger	Crude	830	1.819	5.28	1509.8	4382.4	0.345	100	1
	$(NH_4)_2SO_4$	70	1.778	2.72	124.52	190.4	0.654	8.24	1.89
A. ustilago	Crude	680	4.827	1.36	3282.4	924.8	3.549	100	1
	$(NH_4)_2SO_4$	50	4.289	0.5	214.5	25	8.58	6.54	2.42

Total Protein (mg) = Protein concentration (mg/mL) x Total volume (mL)
Total Activity (U) = Activity in the fraction (U/mL) x Total volume (mL)
Specific Activity (U/mg) = Total activity (U) / Total protein (mg)
Yield (%) = (Total Activity of Purified step / Total Activity of the crude) x 100
Purification Fold = Specific Activity of Purified Step / Specific Activity of the Crude

The immobilized urease from Aspergillus species was presented in Fig. 1. The enzyme which was cross-linked using calcium chloride in a gel-forming solution of sodium alginate, stored in phosphate buffer at 4 ℃ until use.

Fig. 1 *Immobilized urease from aspergillus spp*

3.2 Effects of pH on the Activity of Partially Purified Free-and Immobilized Urease from Aspergillus Species

The effect of varying pH on the activity of partially purified free and immobilized urease from Aspergillus spp. was presented in Fig. 2a and Fig. 2b for *A. niger* and *A. ustilago* respectively. The free and immobilized urease was found to be active in all the pHs investigated. The relative activity increased from 49.2 – 100 % for free-enzyme and 34.7 – 100% for the immobilized urease from *A. niger*. The optimum pH was observed to be 7.0 for both free- and immobilized urease. However, a gradual decline in activity was observed from pH 8.0 – 12.0 with a relative activity of 25.9 % for free-urease and 14.3% for immobilized urease at pH 12.0. The same observation was applicable to *A. ustilago* urease.

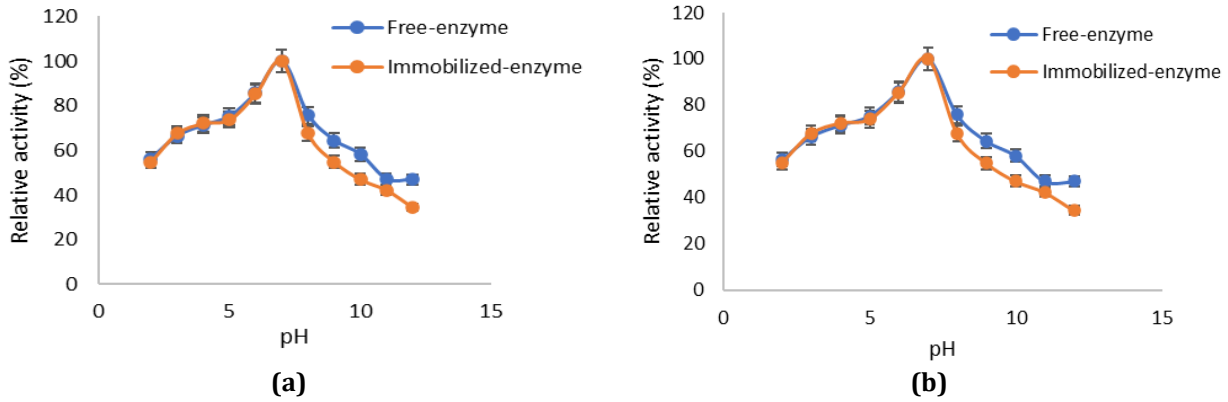

Fig. 2 *Effect of pH on (a) A. niger urease activity; (b) A. ustillago urease activity*

3.3 Effects of Temperature on the Activity of Partially Purified Free-and Immobilized Urease from Aspergillus Species

The effects of temperature on the activity of partially purified free-and immobilized urease produced from Aspergillus spp. was presented in Fig 3a and Fig. 3b. The enzymatic activity of the partially purified free-urease increases with increased temperature. At temperature of 60 °C, free-urease attained an optimum 135.3% relative activity while the immobilized urease was observed with a reduced percentage relative activity of 83.3. However, a minimum relative activity of less than 40 % was obtained for both free-and immobilized urease at temperature above 80 °C.

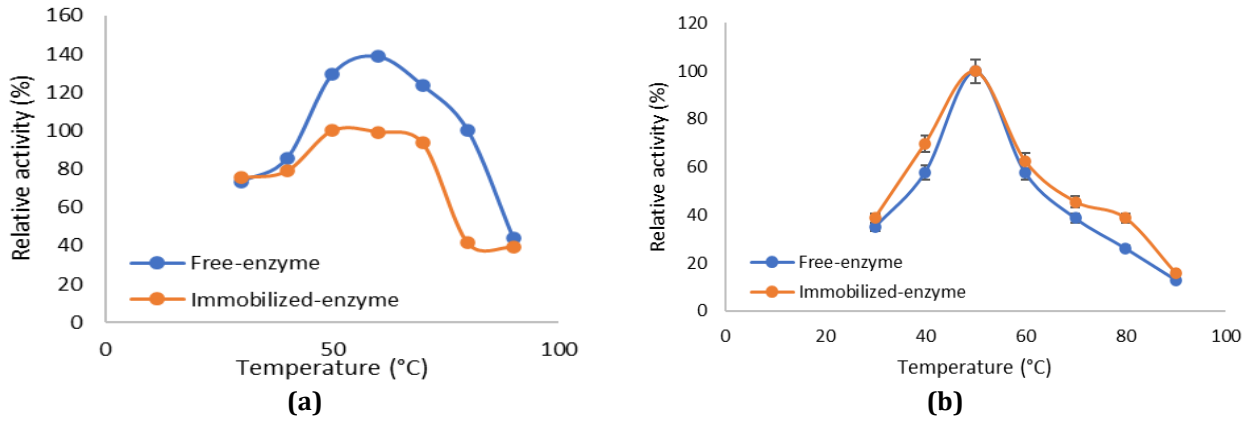

Fig. 3 *Effect of temperature on (a) A. niger urease activity; (b) A. ustillago urease activity*

3.4 Effects of Metal Ions on the Activity of Partially Purified Free-and Immobilized Urease from Aspergillus Species

The effects of various metal ions on the activity of partially purified free-and immobilized urease at 5 and10 mM concentrations was shown in Fig. 4. However, all the investigated metals (Al^{3+}, Fe^{2+}, Ca^{2+}, Co^{+}, Cu^{2+}, Mg^{2+}, and Zn^{2+}) were observed to exert inhibitory effects at 10 mM for free-urease from *A. niger* and immobilized urease from *A. ustilago* while they were observed to stimulate the activity of the immobilized urease from *A. niger* at both concentrations. Moreover, Fe^{2+}, Ca^{2+} and Mg^{2+} were shown to stimulate the activity of free-urease from *A. ustilago*.

J. of Sustainable Materials Processing and Management Vol. 6 No. 1 (2026) p. 29-39

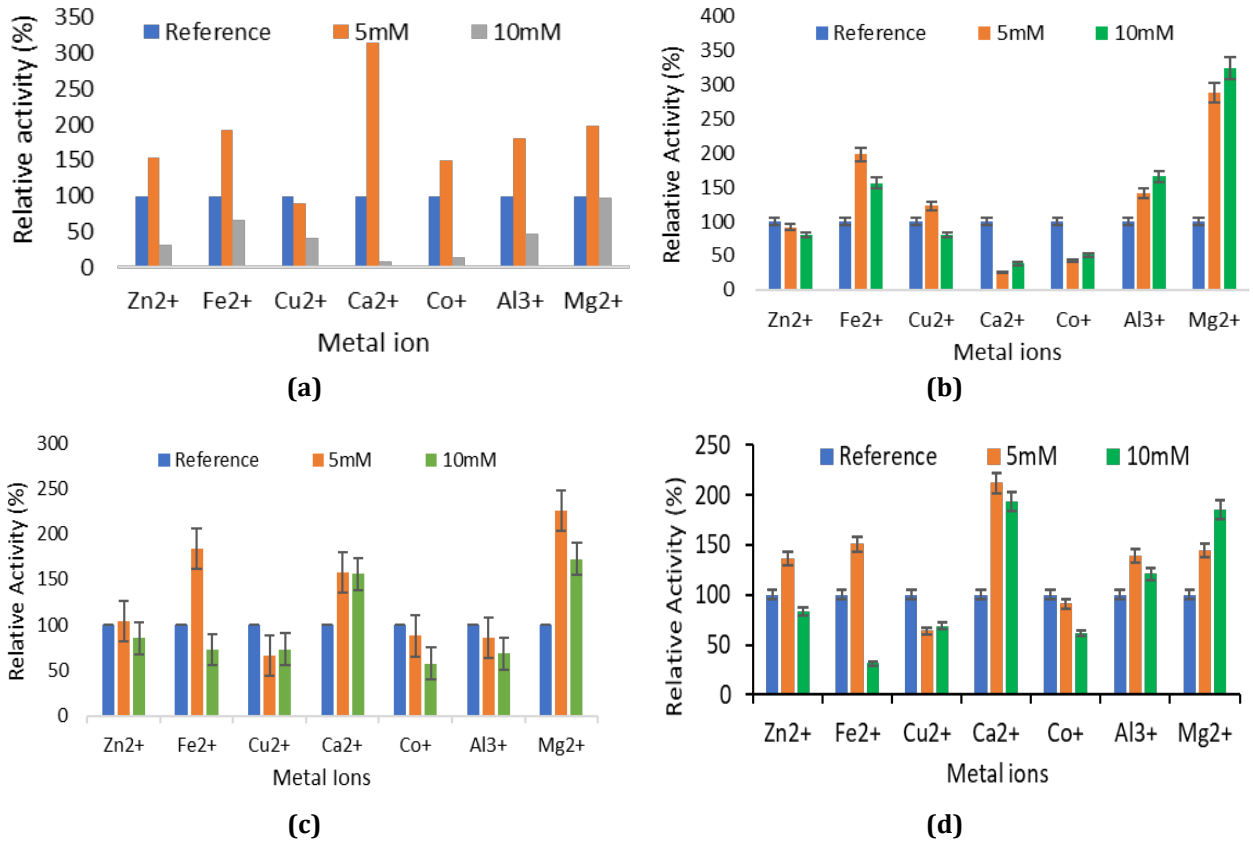

Fig. 4 *Effect of metal ions on (a) Activity of free-urease from A. niger; (b) Immobilized A. niger urease activity; (c) Free-urease of A. ustilago activity; (d) Immobilized urease of A. ustilago activity*

3.5 Discussion

The summary of the partial purification of urease from *A. niger* is presented in Table 1. The dialysate obtained after dialysis of precipitated protein using ammonium sulfate showed yield of 36.4% with 2.0-fold purification while for urease from *A. ustilago*, yield of 40.4% with 14.22-fold purification were recorded. The partial purification of urease from Aspergillus spp. involved two steps, starting with ammonium sulfate precipitation followed by dialysis. This relative moderately high yield indicates that ammonium sulfate precipitation is an effective initial step in urease purification, aligning with its reliability studies [1,33]. Characterization of free-urease enzyme revealed in Fig 2a appears to have an optimal activity at both more acid (pH < 5.0) and more alkaline (pH >8.0) conditions while the immobilized urease shown in Fig. 2b shows a slightly broader optimal range, maintaining higher relative activity at pH (7.0) compared to the free-urease. This result is consistent with many fungal enzymes, which tend to be active in slightly acidic environments [10,34]. This depicts that the immobilization process conferred additional stability to the enzyme structure, allowing it to function under more extreme condition denaturation.

Over the past decades, immobilization technology has been expanded rapidly and has become increasingly conceptualized. The structure of the immobilized urease from *Aspergillus spp*. is presented in Fig. 1. The immobilization of urease from *A. ustilago* using calcium alginate significantly improved its stability and reusability. In order to determine the optimal conditions of the enzymatic reaction, a biochemical study was performed on the free-and immobilized enzymes under different reaction conditions in term of pH, and temperature, effect of metal ions and inhibitors were evaluated [35-38]. The free urease exhibits optimal activity at 60 ^{o}C, as shown in Fig. 3b respectively. In general, following the elevation of temperature, the enzyme activity first increased until it reached the optimum limit and then gradually decreased. The reason for this reduction is that with increasing temperature, there was a gradual reduction in enzyme activities. Thus, in this study, the activity of the free and immobilized enzymes was measured at various temperatures (from 20 to 90 °C). The results showed that the optimum temperature for the aforesaid enzymes was 50 and 60 °C, for A. *ustilago* and *A. niger* respectively. However, there was no significant difference between the activity of the free and immobilized enzymes in terms of optimal reaction temperature. Some previous studies including, Yohanna et al. [1], Sah et al. [39], Taj et al. [40], Tembe et al. [41], and Quiquampoix et al. [42], had shown that stabilization of an enzyme on the matrix results in changes in its optimum pH and temperature by altering the structure and loading of the

enzyme. One of the enzyme immobilization objectives is the production of enzymes with high stability in a wide range of pHs.

The modulation of urease activity by metal ions, where Fe^{2+} was found to inhibit and Zn^{2+} showing an activating effect on urease activity [33,43,44]. The inhibitory effect of Fe^{2+} may stem from its interaction with the enzyme's active site, potentially altering its confirmation and reducing catalytic efficiency [21,44]. Interestingly at 5 mM, Fe^{2+} had a pronounced modulating effect, enhancing urease activity, a trend observed in studies on urease from marine Klebsiella species where divalent ions, including Fe^{2+}, activated the enzyme at low concentrations but inhibited it at higher concentrations levels [21,33]. These results suggest that metal ion concentrations must be carefully controlled to optimize urease activity in industrial processes.

4. Conclusion

This research contributes to the field of enzyme biotechnology by exploring a novel source of urease, *A. ustilago*, isolated from the gut of long-horned beetles *(Cerambycidae latreille).* The study advances the understanding of urease production from non-traditional, fungal-based sources, offering a potentially more sustainable and efficient alternative to conventional methods. By optimizing the production, partial purification, and immobilization processes. It provides insights into improving enzyme stability, reusability, and activity under various conditions. This research could facilitate broader applications of urease in agriculture, medicine, and environmental management, while also paving the way for future research on similar enzyme-producing microorganisms from unique ecological niches.

More importantly, the urease produced by *Aspergillus species* shows significant potential for use in environmental biotechnology, particularly in bioremediation processes. However, future research should focus on optimizing production conditions to enhance its yield and activity. Detailed inhibition kinetics should be conducted on urease with various metal ions, chemical and natural inhibitors, to identify optimal conditions for urease inhibition. This could provide insights into regulating urease activity for specific applications. The exploration of recombinant DNA technology to produce urease variants with enhanced thermal stability and catalytic efficiency would be beneficial for expanding its industrial applicability. Moreover, studies on the role of insect gut microorganisms as reservoirs for novel enzymes should be pursued, particularly focusing on lignocellulosic degradation for biofuel production and sustainable environmental practices.

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Conflict of Interest

The author declares that there is no conflict of interest regarding the publication of the paper.

Author Contribution

The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

Declaration of Generative AI Use

Author hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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