Biophysical properties of thermostable Amidase produced by *Aspergillus fumigatus* in submerged fermentation

ABSTRACT

Amidases, also known as amidohydrolases (EC 3.5.1.4), are enzymes within the nitrilase superfamily or amidase signature family. They hydrolyze amides and nitriles into their corresponding acids while releasing ammonia. These enzymes are widely used in biotechnology and industry. However, their production is limited to a few organisms, which cannot meet industrial demand. Therefore, exploring new amidase sources is crucial. This study focuses on purifying and characterizing amidase from *Aspergillus fumigatus* using cold acetone precipitation and column chromatography with Sephadex G-100. The effects of temperature, pH and metal ions, on the enzyme activity were examined for both crude and purified amidase. The thermal and pH stability of the enzyme alongside the enzyme kinetics were also assessed. The purified amidase exhibited optimal activity of 13.1 U/mL at 70°C, with 65% residual activity after 5 hours at this temperature. The pH studies showed optimal activity at pH 5.0 for the purified enzyme (7.8 U/mL) and at pH 4.0 for the crude enzyme (5.5 U/mL). Metal ion effects indicated that Zn^{2+} , Mg^{2+} , and EDTA enhanced, while Cu^{2+} inhibited, the activities of both crude and purified amidase. Kinetic analysis showed that the enzyme followed Michaelis-Menten kinetics, with K_m and V_{max} values of 0.104 mM and 1.884 U/mL for crude amidase, and 0.017 mM and 1.872 U/mL for purified amidase. These findings suggest that amidase from *Aspergillus fumigatus* holds potential for industrial applications where amidases are essential.

Keywords: Amidase, acetamidase, *Aspergillus fumigatus*, enzyme, purification

INTRODUCTION

Amidases, classified as amidohydrolases (EC 3.5.1.4), belong to either the nitrilase superfamily or the amidase signature family. They catalyze the hydrolysis of amides and nitriles into their respective acids, liberating ammonia during the process.. These amidases usually exist in two forms; the first category consists of aliphatic amidases while the

second category consists of aromatic and mid-chain amidases which hydrolyses acrylamide, aminoamide and hydroxyamide. All these amidases have acyl transferase activity that results in the synthesis of hydroxamic acids (Santoshkumar *et al.,* 2017).

Amidases are important because of their versatility in both industrial and environmental applications where they play important roles in organic synthesis, acting as industrial catalysts to produce an array of molecules that are such as medicines, herbicides, and commodity chemicals such as amino acids, acrylic acids, and hydroxamic acids (Fournand and Arnaud, 2001). The capacity of amidases to generate enantiomerically pure intermediates is critical for the synthesis of these high-value compounds, increasing their efficacy and safety. Furthermore, amidases help to promote environmental sustainability by boosting bioremediation processes as they hydrolyze nitriles and amides, turning potentially hazardous molecules into less toxic substances and assisting in the cleanup of industrial wastes (Silva *et al.,* 2009). Similarly, amidases allow for more ecologically friendly enzymatic processes to produce acrylamide, a crucial chemical utilized in many applications, efficiently in the leather and textile sectors (Sun *et al.,* 2009). Considering their extensive applications, amidases underscore the importance of advancements in both white and green chemistry, as well as their critical role in contemporary biotechnology and industrial operations.

Although several researchers have reported amidase production by various organisms, the supply of these enzymes remains limited and insufficient to meet the growing demand across industries. This shortfall is primarily due to the fact that only a few of these organisms produce amidases with desirable industrial properties, and the production has not been scaled to levels that can satisfy industrial needs. This scarcity underscores the need for enhanced production and supply chain strategies to fully leverage the potential of amidases in industrial processes. In this context, the study was carried out with the aim of purification and characterization of amidase from *Aspergillus fumigatus* using cold acetone precipitation and column chromatography with Sephadex G-100.

METHODS

Microorganism and culture condition

The *Aspergillus fumigatus* used in this study was obtained from the Culture Collection of the Department of Microbiology, University of Ibadan. It was previously isolated from spoilt tomatoes and maintained on acetamide agar slants containing (g/l): K_2HPO_4 (3.5), KH_2PO_4 (2.2), $MgSO_4$ (0.05), Agar (15), Acetamide (20) and 0.5 ml of trace element solution containing (mg/l) $MnSO_4$ (0.3), $CuSO_4.5H_2O$ (0.4), $ZnSO_4.7H_2O$ (0.3), $CaCl_2.2H_2O$ (0.5), $FeSO_4.7H_2O$ (0.6) and 0.01 streptomycin (to prevent bacterial growth)

Inoculum preparation and amidase production

Aspergillus fumigatus was subcultured on amidase agar medium to obtain mycelium and five (5) (5mm) agar discs were inoculated in 50 ml of amidase production containing (g/l): K_2HPO4 (3.5), KH_2PO4 (2.2), $MgSO_4$ (0.05), Phenol red (0.012), Acetamide (20) and 0.5 ml of trace element solution containing (mg/l) $MnSO_4$ (0.3), $CuSO_4.5H_2O$ (0.4), $ZnSO_4.7H_2O$ (0.3), $CaCl_2.2H_2O$ (0.5), $FeSO_4.7H_2O$ (0.6) medium in 250 ml Erlenmeyer flasks using a sterilised cork borer. The flasks were incubated (MEMMERT, 770040) at 37°C on a rotary shaker at 120 rpm for 96. After incubation, the culture was filtered using Whatman filter paper No.1 with the obtained filtrate used as the crude amidase enzyme (Imada *et al.*, 1973).

Amidase assay

Amidase activity in the crude filtrate was investigated according to the method of Imada *et al.* (1973). A reaction mixture containing 0.5 ml of 0.04M L-Asparagine, 0.5 ml of 0.5M buffer (tris- hydroxymethyl) aminomethane. HCl; (Tris HCl) pH 7.2), 0.5 ml of enzyme preparation (obtained filtrate from the suspension of isolates) and distilled water to a total volume of 2 ml in a sterilized bottle were incubated at 37°C for 30 mins. The reaction was stopped by adding 0.5 ml of 1.5M trichloroacetic acid (TCA). Blank test tubes were run by adding the enzyme preparation after the addition of trichloroacetic acid. An aliquot of 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent (an aqueous solution of potassium iodide, mercuric chloride, and potassium hydroxide) were added to 3.7 ml of distilled water. The mixture was incubated at 15-20°C for 20 mins. Amidase activity was determined by measuring the ammonia released (Nesslerization reaction) spectrophotometrically at 450nm (Imada *et al.*, 1973). One International Unit (IU) of amidase is expressed as the amount of enzyme which liberates 1 µmol of ammonia per

minute under the assay conditions.

Amidase Purification

The crude amidases produced by the selected isolates were partially purified using cold acetone method as described by Monica *et al.* (2014) and subsequently fractionated by gel chromatography on Sephadex G-100.

Determination of total protein concentration

Protein concentration was examined by the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard with the protein concentration in the solution was then extrapolated from a standard curve of the different dilutions of the Bovine Serum Albumin.

Characterisation of Amidases

Crude and purified amidases were further analysed and characterised for factors known to affect their activity. The factors analysed include temperature, pH, substrate concentration and effects of metal ions.

Effect of temperature on activity and stability of amidases

The effect of temperature on the activities of both crude and purified amidases was examined by incubating 1.0 mL of enzyme with 1.0 mL of 0.5M Tris HCl buffer (pH 7.2) together with 1.0 mL of 2% acetamide solution as substrate at varying temperatures ranging from 30°C to 80°C for 6hrs. Amidase assay was carried out using the Nesslerization method as described previously (Imada *et al.*, 1973). The thermostability of the crude and purified amidases were examined by incubating a solution of the enzyme (12 mL) over a period of 6 hours at the optimum temperature of activity and thereafter the residual activity was measured.

Effect of pH on activity and stability of amidases

The effect of pH on the activities of both crude and purified amidases was examined by carrying out the reactions in various buffer solution. To 1.0 mL of each enzyme, 1.0 mL of the substrate preparation (2% acetamide) was added at different pHs between 3.0 – 9.0

using 0.1M sodium acetate buffer (pH 3.0 – 5.0), 0.1M potassium phosphate buffer (pH 6.0 – 7.0) and 0.5M tris HCl buffer (pH 8.0 – 9.0) which were incubated at 37°C for 30 minutes. Amidase assay was carried out using the Nesslerization method (Imada *et al.*, 1973). pH stability of free and purified amidases was evaluated by mixing the enzyme solution with their corresponding optimum buffer for 6 h. the residual enzyme yield determined at 30 min regular intervals by the assay method (Imada *et al.*, 1973).

Effect of metal ions on the activity of amidases

The effect of various metal ions on amidase activity was studied by incubating 1 mL of the crude and purified amidases with 1 mL of substrate preparation (2% acetamide) containing 1 mL of the metal ion (Zn^{2+}, Mg^{2+}, Fe^{2+} and Cu^{2+}) at different concentrations at 37°C for 30 minutes. Also, the effect of Ethylene Diamine Tetra Acetic acid (EDTA) on amidase activity was evaluated by incubating 1 mL of the crude and purified amidases with 1 mL EDTA and 2% acetamide at 37°C for 30 min after which the residual activity was assayed (Imada *et al.*, 1973).

Effect of substrate concentration

The kinetic constants V_{max} and K_m were estimated using acetamide (Sigma-Aldrich Chemie GmbH) as the substrate. This was done by measuring rate of reaction at different concentration of citrus pectin (0.1-1mg/mL) and the data from experiments fitted into the Michaelis-Menten model with GraphPad Prism version 9.00 for Windows, GraphPad Software, San Diego, California USA (www.graphpad.com).

Results and Discussion

Enzyme purification is crucial for enhancing their efficiency and stability as the process usually targeted at the removal of impurities such as other enzymes, cellular debris, and salts that can negatively impact enzyme activity and yield. In this study, purification of amidase from *Aspergillus fumigatus*, by cold acetone, achieved a 71.8% yield with a 0.45-fold purification. These results demonstrate a higher yield compared to earlier studies, though with lower specific activity and purification folds (Dange and Peshwe, 2011; Chandrasekhar, 2012). Variations in specific activity and yield are attributed to differences

in purification techniques used across studies, highlighting the influence of methodology on purification outcomes (Mushtaq *et al.,* 2012).

The characterization of crude and purified amidases from *Aspergillus fumigatus* was examined to investigate the effect of pH, temperature, metal ions, and substrate concentration on amidase activity as these factors are reported to plays essential role on the activity and stability of both crude and purified enzymes (Monica *et al.,* 2014; Ahmed *et al.,* 2016; Garuba and Otemoye, 2023).

The effect of temperature on amidase activity was examined ranging from 30°C to 80°C. It was observed that the crude amidase extract had optimum activity of 6.4U/mL while the optimum temperature of 70°C with an enzyme activity of 13.1U/mL was observed for the purified amidases from *Aspergillus fumigatus* (Fig1A). Similar results were reported by Ahmed *et al.* (2016) that purified amidase from *Aspergillus candidus* had maximum activity and stability above 65°C and pH 5.0. The observed trends might be due to the removal of impurities and inhibitory substances present in the crude preparation (by the purification process), leading to an increase in the enzyme's catalytic efficiency (Monica *et al.,* 2014). Additionally, purification might enhance the enzyme's structural stability, enabling it to maintain higher activity levels at elevated temperatures (Sinha *et al.,* 2013). The decline in activity at 80°C for both crude and purified amidase suggests thermal denaturation, though the purified form's activity remains significantly higher, indicating better thermal stability. Similarly, thermal stability studies showed that the purified amidase retained 60% of its residual activity after 6 hours of incubation while the crude amidase had 42% of its relative activity at the same period of incubation (Fig 1B). The high activity of purified amidase by this *Aspergillus fumigatus* at elevated temperatures could be significant for industrial and biotechnological applications as it enhances process efficiency, productivity, and safety by accelerating reaction rates and reducing microbial contamination risk. Furthermore, the amidase can be used longer without denaturation and its utility at high temperatures allows for improved reaction conditions by increasing solubility and reducing viscosity, facilitating better mixing and mass transfer (Hussian and Leong, 2023).

The Effect of pH on the activities of both enzyme preparation was done by the incubation of the crude and purified amidase at different pH and results presented in Fig 2. The results revealed that the purified amidase had optimum activity of 7.8U/mL at pH 5.0 while the crude amidase had 5.2 U/mL at 4.0. pH stability investigation showed that the purified amidase maintained 60% of its residual activity at 6 hours of incubation when incubated at optimum pH of 5.0U/mL while the crude enzyme completely lost its activity at the same incubation time at pH 4.0. This observation is consistent with findings by Rahim *et al.* (2003), reported that *Aspergillus candidus* had optimal amidase activity at pH 5.0, and Sinha *et al*. (2013), indicated that amidase from *Cyberlindnera jadinii* was stable at pH 4.0. Additionally, Manna *et al.* (1995) and Siddalingeshwara and Lingappa (2011) reported optimal amidase activity at pH 9.0 for *Pseudomonas stutzeri MB 405*, *Aspergillus aculeatus*, and *Aspergillus terreus KLS2*. Enzymes are reportedly stable within a specific pH range, with deviations from this range leading to significant decreases in performance, likely due to protonation of ionizable groups on the enzyme's surface, which adversely affects its functionality (Murthy, 2020). The difference in the pH optima of the crude and pure enzyme preparations might be due to a change in the enzyme's microenvironment after purification which ultimately affects its function (Siddalingeshwara and Lingappa, 2011). pH stability studies showed that while the purified amidase retained 60% of its residual activity after incubation for 6 hours at pH 5, the crude enzyme completely lost its activity after incubation for 5 hours at pH of 4.0. The purified amidase retaining more activity at low pH could be because the purification process removes destabilizing impurities. In contrast, the crude enzyme is more susceptible to denaturation at acidic pH due to the presence of impurities and the lack of stabilizers. These factors might have contributed to the complete loss of activity in the crude enzyme under similar conditions.

Effect of different concentration of acetamide on amidase activity was investigated and the results presented in Fig 3. As shown, crude amidase shows a consistent decline in activity from approximately 6.3 U/mL at 0.1 mM to about 3.5 U/mL at 0.5 mM. In contrast, the purified amidase starts with an activity of around 5.22 U/mL at 0.1 mM, increases to a peak of approximately 6.76 U/mL at 0.4 mM, and then decreases to about 5.5 U/mL at

0.5 mM. Higher activity across all tested concentrations exhibited by the purified amidase might likely be due to the removal of inhibitory substances present in the crude preparation which might have inhibited the enzyme or lead to its rapid consumption or denaturation (Rahim *et al.*, 2003). The decreased activity of the amidase at higher substrate concentrations could be attributed to substrate inhibition, where excess substrate impairs the enzyme's activity (Sinha *et al.*, 2013).

Kinetic parameters of the crude and purified amidase as investigated using different concentration of acetamide revealed that the crude amidase had Michaelis Constant (K_m) of 0.710 mM and Maximum Velocity (V_{max}) of 18.84 µM/Min while the purified amidase had K_m of 1.040 mM and V_{max} of 18.72 µM/Min. These results suggests that the crude amidase maintains a superior affinity for acetamide in contrast to the purified amidase. Similarly, the crude amidase demonstrates a marginally higher V_{max} of 18.84 µM/Min compared to the V_{max} of 18.72 µM/Min for the purified amidase. The marginally decreased Km in the crude amidase suggests that purification may have diminished the enzyme's substrate-binding efficiency, potentially due to the removal of stabilizing components present in the crude extract. Nonetheless, the catalytic turnover, as reflected by Vmax, remains nearly unchanged post-purification. These findings highlight the delicate balance between enzyme purification and functional efficiency, suggesting that crude enzymes might offer advantages in applications requiring high substrate affinity, while purified forms may be preferable where purity and catalytic velocity are prioritized.

The results of the effect of metal ions on the activity of the crude and purified amidase is presented in Fig 4. The results showed that Zn^{2+} significantly enhanced both crude and purified amidase activity, consistent with findings from Dange and Peshwe (2011), although other studies have reported varied effects of Zn^{2+} , including inhibition. Fe^{2+} inhibited crude amidases from *Aspergillus fumigatus* but activated the purified amidase from this species, aligning with Dange and Peshwe's (2011) results. Conversely, Mg^{2+} markedly enhanced amidase activity in both crude and purified forms of *Aspergillus fumigatus,* although this effect was not universally observed, as other studies reported inhibition (Manna *et al*. 1995; Dange and Peshwe's 2011; Taylor and Francis, 2015). Cu^{2+} slightly inhibited crude amidase activity but showed stronger inhibition in purified amidase

from *Aspergillus fumigatus*, a finding supported by several studies. EDTA enhanced the activity of both crude and purified amidases from *Aspergillus fumigatus* and showed a greater effect on the purified amidase. These results align with the observations of Manna et al. (1995) and Taylor and Francis (2015), though contrasting with other reports of EDTA-induced inhibition. The observed effects of metal ions and EDTA on amidase activity might be due to their roles as cofactors, inhibitors, or chelating agents, which influence enzyme conformation and function (Pereira et al., 2017, Gulcin and Alwasei, 2022). Zn^{2+} and Mg^{2+} likely enhance amidase activity by stabilizing the enzyme's active site, consistent with their known roles as cofactors, though variability in results suggests the influence of specific experimental conditions. Fe^{2+} exhibited a dual effect, inhibiting the crude amidase potentially due to interactions with other components in the extract, while activating the purified enzyme, possibly by acting as a cofactor in the absence of interfering substances. Cu^{2+}, known for its inhibitory properties, likely reduced activity through active site binding or oxidative stress, with a more pronounced effect on the purified enzyme due to the absence of mitigating factors. EDTA's enhancement of amidase activity is attributed to its chelation of inhibitory metal ions, with the purified enzyme showing greater sensitivity to the removal of such ions, reflecting its reliance on precise metal ion balance for optimal function Overall, the behavior of amidase in the presence of metal ions and EDTA indicates variability, likely due to the enzyme's tetrameric structure and differing interactions with these substances.

Fig 1: Effect of temperature on activity (A) and thermal stability (B) of crude and purified amidase produced by *Aspergillus fumigatus*

Fig 2: Effect of pH on activity (A) and thermal stability (B) of crude and purified amidase

produced by *Aspergillus fumigatus*

Fig 3: Effect of Some selected metal ions on the activity of amidase produced by *Aspergillus fumigatus*

Fig 4: Effect of different concentration of acetamide on the activity of crude and purified amidase produced by *Aspergillus fumigatus*

Conclusion

In conclusion, the study on amidases from *Aspergillus fumigatus* provides valuable insights into the biochemical properties and performance under various conditions of both crude and purified amidases under different conditions of pH, temperature, substrate concentration, and metal ion effects. The purified amidase demonstrated enhanced activity and stability compared to the crude enzyme, with optimal performance observed at higher temperatures and pH levels. Specifically, the purified amidase showed superior thermal stability and maintained activity at its optimum pH, while the crude amidase's performance was more sensitive to these conditions. Additionally, kinetic analysis revealed that although the crude amidase has a higher affinity for acetamide, the purified enzyme's activity profile suggests a more refined catalytic efficiency. The differential effects of metal ions on the amidase activity further underscore the complexities of enzyme regulation, with Zn^{2+} and Mg^{2+} acting as stimulants, whereas Fe^{2+} and Cu^{2+} exhibited inhibitory effects under specific conditions. Overall, these findings highlight the advantages of enzyme purification in enhancing amidase activity and stability, which could have implications for industrial applications and further enzyme engineering studies.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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