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Alpha-Amylase from *Aspergillus niger* XJ42: Isolation, Characterization, and in Silico Analysis

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Abstract

The spoilage of cassava by *Aspergillus niger* strain XJ42 poses significant challenges to food security and industrial biotechnology, necessitating an in-depth understanding of its enzymatic mechanisms. This study aimed to amplify and characterize the α-amylase gene, CDF_Amyl, from *A.* niger strain XJ42 and evaluate its biotechnological potential. The CDF_Amyl gene was successfully amplified and partially sequenced, revealing a protein consisting of 222 amino acids with a molecular weight of 25.13 kDa and an isoelectric point of 4.17. Sequence alignment demonstrated high homology with α-amylase genes from *A.* niger (98%) and *A. oryzae* RIB40 (99%), suggesting evolutionary conservation and potential functional similarities. Kinetic analysis of the enzyme revealed a maximum velocity (Vmax) of 6.90 U/mg protein and a Michaelis constant (Km) of 6.70 mg/ml, indicating its catalytic efficiency. These findings highlight the enzyme's robust activity and potential applications in biotechnology, particularly in starch hydrolysis for industrial, food, and pharmaceutical processes. The unique properties of CDF_Amyl suggest that it may serve as a valuable biocatalyst for enhancing cassava processing and other starch-based industries. Further studies on its structural and functional properties could facilitate its commercial exploitation in biotechnological innovations.

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Keywords: α-amylase, *Aspergillus niger*, Cassava degradation, In silico analysis, Enzyme kinetics, Biotechnology.

1. Introduction

Alpha-amylases (EC 3.2.1.1.) are essential enzymes that catalyze the hydrolysis of starch and related polysaccharides by breaking the internal α-1,4-glycosidic linkages to produce oligosaccharides of varying sizes [1]. These enzymes are integral to various industrial processes, including food, textile, detergent, and pharmaceutical industries, due to their ability to degrade starch efficiently [2]. While amylases can be derived from many organisms, microbial sources, particularly bacteria and fungi, are preferred for industrial applications because of their high enzyme yield, rapid growth, and ease of genetic manipulation [3]. Alpha-amylases belong to family 13 of glycosyl hydrolases, characterized by a three-dimensional (3D) structure that consists of three key domains—A, B, and C. These domains play a vital role in substrate binding and catalytic activity [4]. The catalytic residues are highly conserved within the A-domain, while the B-domain is involved in calcium binding, which is crucial for

enzyme stability [5]. Understanding the structure and function of these enzymes is critical for their manipulation in biotechnological applications, particularly in industrial starch degradation. Bioinformatics has revolutionized enzyme research by providing computational tools to analyze and predict the structure and function of enzyme sequences. In silico approaches such as sequence alignment, molecular weight determination, isoelectric point (pI) prediction, and 3D structural modeling allow researchers to study the functional properties of enzymes without the need for extensive laboratory experiments [6]. These computational tools are especially useful in determining the evolutionary relationships, subcellular localization, and hydrophobicity of proteins, which are important for industrial enzyme applications [7].

Food spoilage is a global challenge, particularly in tropical regions where crops like cassava (*Manihot esculenta*) are highly susceptible to microbial degradation [8]. Cassava tubers are rich in carbohydrates and water, which create an ideal environment for fungi, particularly species of *Aspergillus*, to grow and cause spoilage [9]. These fungi secrete amylases that degrade cassava starch into simpler sugars, thereby accelerating the spoilage process [8]. Identifying and characterizing these enzymes is crucial for understanding microbial spoilage and exploring potential industrial applications.

In this study, we isolated *Aspergillus niger* strain XJ42 from spoiling cassava and performed in silico analysis of its α-amylase gene. The gene was amplified, sequenced, and subjected to various bioinformatics analyses to predict its structure and function. This research aims to enhance our understanding of cassava spoilage mechanisms and explore the potential industrial uses of *Aspergillus niger* α-amylase in biotechnological applications.

2. Materials and Methods

2.1 Cassava Flour Preparation

Fresh cassava tubers (*Manihot esculenta*) were peeled and processed into flour following the method described by Oyewole and Sanni [10]. The prepared cassava flour was used as a primary substrate in subsequent fungal culture experiments.

2.2 YPD Agar Preparation

Yeast extract-peptone dextrose (YPD) agar was prepared as described by Tonukari *et al*. [11]. The medium was sterilized and poured into sterile Petri dishes, allowing it to solidify before use.

2.3 Isolation and Growth of *Aspergillus niger* Strain XJ42

The fungal strain *Aspergillus niger* XJ42, isolated from spoiling cassava, was cultured on YPD agar plates. Identification was carried out using 18S rRNA sequencing, confirming its identity as *A.* niger. The strain was incubated at 28°C in the dark for seven days to promote sporulation. Subcultures were prepared on Cassava Starch Agar (CSA), which included 2% cassava flour as the primary carbon source, 1% $NaNO_2$, and 1.5% agar. Ampicillin (20%, 5 µl) was added to inhibit bacterial contamination during the seven-day incubation period. After incubation, the fungal mycelia were harvested for genomic analysis.

2.4 Genomic DNA Isolation

Genomic DNA was extracted from the *A.* niger XJ42 strain using a modified cetyltrimethylammonium bromide (CTAB) protocol [13], followed by purification via ethanol precipitation. The DNA quality and integrity were verified using agarose gel electrophoresis with a 0.8% agarose gel stained with ethidium bromide. The gel was visualized and imaged using a UV transilluminator (Enduro, USA).

2.5 PCR Amplification of the α-Amylase Gene

The α-amylase gene from *A.* niger strain XJ42 was amplified using polymerase chain reaction (PCR). Specific forward (AmyFP: 5′-CATCTGGATCACCCCCGTTA-3′) and reverse (AmyRP: 5′-AGACTTACGAAGCGAACCGT-3′) primers were used for amplification, as described by Zidani *et al*. [14]. The PCR reaction mixture (25 µl) contained 1.25 µl of each primer (5 mM), 1.25 µl of 2.5 mM dNTPs (Promega), 1.25 µl of 10× NH_4 buffer, 1.0 µl of 50 mM $MgCl_2$, 1.0 µl of Taq DNA polymerase (5 U/µl), 7.25 µl of deionized water, and 7.25 µl of genomic DNA (10 ng/µl). The PCR conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 5 minutes. A final elongation step at 72°C for 5 minutes concluded the reaction. The amplification products were verified through 1.0% agarose gel electrophoresis stained with ethidium bromide and visualized using the Enduro Gel Doc system.

2.6 Sequencing of the α-Amylase Gene

The amplified α-amylase gene fragments were purified and sequenced using an Applied Biosystems 3130xl Genetic Analyzer (ABI Sequencer, USA) at the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria.

2.7 In silico Analysis of the α-Amylase Gene

The nucleotide sequence obtained from the *A.* niger XJ42 α-amylase gene was translated into the corresponding protein sequence using the European Bioinformatics Institute (EBI) EMBOSS translation tool (https://www.ebi.ac.uk/Tools/st/emboss_transeq/).

Identification of conserved domains and similar α-amylase sequences was conducted using NCBI BLASTX and BLASTp tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [15]. Multiple sequence alignments were performed using ClustalW2 at EMBL EBI (https://www.ebi.ac.uk/Tools/msa/clustalw2/) [17], and phylogenetic trees were constructed to analyze evolutionary relationships.

Restriction site mapping of the *A.* niger XJ42 α-amylase gene (AN_Amyl) was carried out using online bioinformatics tools [18]. The molecular weight (Mw) and isoelectric point (pI) of the predicted protein were computed with the ExPASy pI/Mw tool [19]. Protein structural modeling was performed using Phyre2 [20], while subcellular localization predictions were carried out using MultiLoc2 software [21]. Hydrophobicity and transmembrane helix predictions were assessed using bioinformatics tools such as SOSUI and TMHMM-2.0 [22,23].

3 Results

3.1 Nucleotide Sequence Analysis and Characterization of the α-Amylase Gene from *Aspergillus niger* Strain XJ42

The partial nucleotide sequence presented in Figure 1 represents the alpha-amylase gene from *Aspergillus niger* strain XJ42, which was isolated from spoiling cassava. The sequence consists of 926 base pairs (bp), where coding regions (in uppercase) correspond to the protein-coding sequence, and non-coding regions (possibly introns) are denoted in lowercase. The translated amino acid sequence includes key regions such as DAYHG, QDM, YSNLN, and GMMVMVDVVANHM, which are characteristic of alpha-

amylase. The start codon (ATG) is observed at position 1, indicating the beginning of the open reading frame. Several conserved amino acid patterns are seen, particularly repeats of glycine (G), serine (S), and valine (V), which are critical for the enzyme's functionality in starch degradation. This sequence analysis highlights important structural features of the gene involved in the production of alpha-amylase in *Aspergillus niger*.

As shown in Table 1, the BLASTp analysis reveals that the CDF_Amyl protein homologs have high identity scores, with the highest being 99% for Alpha-amylase A type-1/2 from *Aspergillus oryzae* RIB40 (Accession Number: XP_003189619.1). Other notable matches include the orthorhombic crystal structure of *Aspergillus niger* alpha-amylase (98% identity, Accession Number: 2GUY_A) and alpha-amylase from *Aspergillus* flavus NRRL3357 (98% identity, Accession Number: XP_002374124.1). These results indicate a strong conservation of the alpha-amylase gene across different *Aspergillus* species.

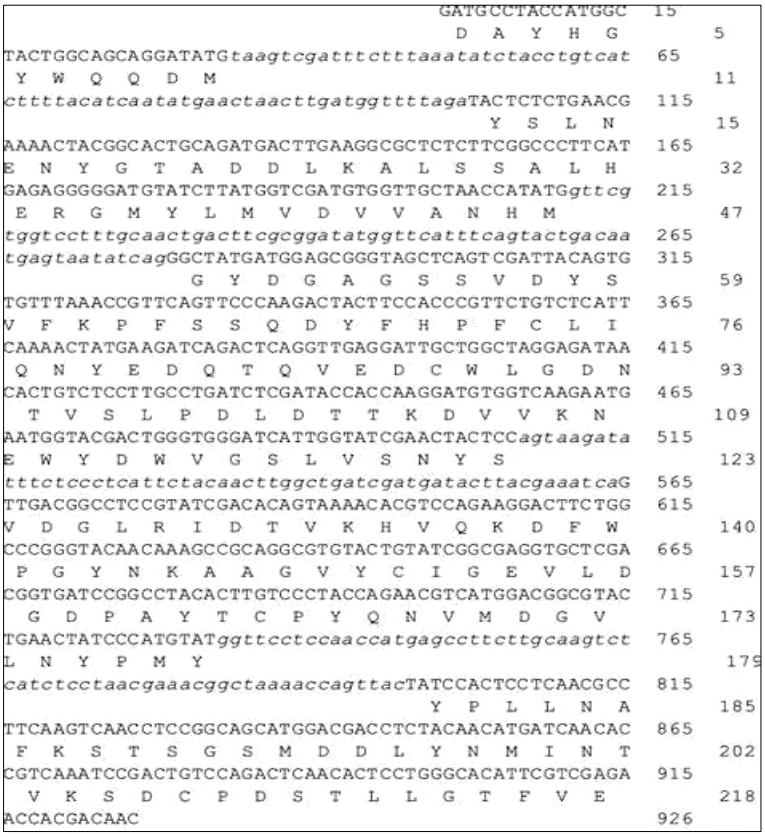

Fig. 1: The partial nucleotide sequence and corresponding amino acid sequence of the amylase gene from cassava-degrading fungi (CDF_Amyl). The gene sequence contains four introns (non-coding regions), indicated by italicized lowercase letters, while the five exons (coding regions) are represented by uppercase letters.

Table 1: BLASTp Analysis of CDF_Amyl Protein Homologs.

Matched Protein/Organism	Max Score	Total Score	Query Cover	E-value	Identity	Accession Number
Alpha-amylase A type-1/2 [*Aspergillus oryzae* RIB40]	462	462	100%	4.00E-159	99%	XP_003189619.1
Chain A, Orthorhombic Crystal Structure Of *Aspergillus niger* Alpha-Amylase	461	461	100%	7.00E-159	98%	2GUY_A
Alpha-amylase, putative [*Aspergillus* flavus NRRL3357]	461	461	100%	1.00E-158	98%	XP_002374124.1
Alpha-amylase [*Aspergillus* kawachii]	461	461	100%	1.00E-158	98%	BAD01051.1
Taka-amylase A (Taa-G1) precursor [*Aspergillus oryzae*]	460	460	100%	3.00E-158	98%	AAA32708.1
Alpha-amylase [*Aspergillus* awamori]	460	460	100%	3.00E-158	98%	BAD06002.1
Alpha amylase Precursor [*Aspergillus* awamori]	459	459	100%	4.00E-158	98%	Q02905.1
Alpha amylase Precursor [*Aspergillus* shirousami]	455	455	100%	3.00E-157	98%	P30292.1
Taka-amylase A precursor [*Aspergillus oryzae*]	450	450	100%	2.00E-154	97%	BAA00336.1
Alpha-amylase [*Aspergillus* sojae]	499	499	100%	7.00E-154	95%	BAM28635.1

3.2 Phylogenetic Analysis of the α-Amylase Gene from *Aspergillus niger* Strain XJ42

The phylogenetic analysis (Fig.2) of the alpha-amylase gene from *Aspergillus niger* strain XJ42, isolated from spoiling cassava, shows its evolutionary relationship with other species. The alpha-amylase gene from *Aspergillus niger* strain XJ42 shows a 98.2% similarity to *Aspergillus flavus* NRRL3357, a 98.65% similarity to *Aspergillus oryzae* RIB40, a 79.28% similarity to *Aspergillus niger* CBS513.88, and a 52.15% similarity to *Fusarium oxysporum*. This data highlights the close genetic relationship between *Aspergillus niger* strain XJ42 and other species, particularly within the *Aspergillus* genus.

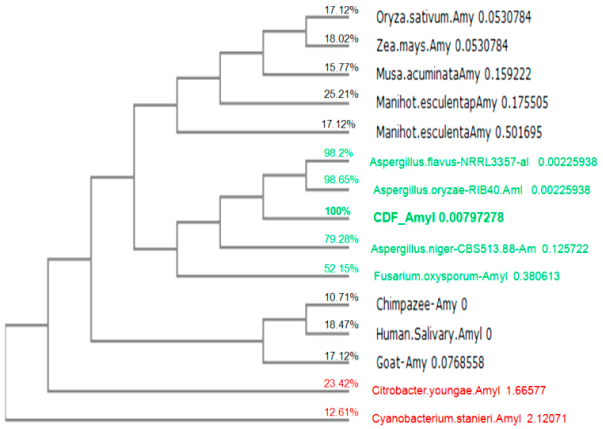

Fig 2. Phylogenetic relationship between the cassava-degrading fungal amylase (CDF_Amyl) and amylases from plants, fungi, bacteria, lower animals, and humans, based on sequences from NCBI GenBank.

3.3 Multiple Sequence Alignment and Conserved Domain Analysis of the Cassava-Degrading Fungal Amylase (CDF_Amyl)

The alpha-amylase gene from *Aspergillus niger* strain XJ42 isolated from spoiling cassava shows a 98.2% similarity to *Aspergillus* flavus NRRL3357, a 98.65% similarity to *Aspergillus oryzae* RIB40, a 79.28% similarity to *Aspergillus niger* CBS513.88, and a 52.15% similarity to *Fusarium oxysporum* (Fig. 3a). This data highlights the close genetic relationship between *Aspergillus niger* strain XJ42 and other species, particularly within the *Aspergillus* genus. Fig. 3b provides a graphical representation of conserved domains within the alpha-amylase gene sequences. The highlighted regions indicate significant conservation across different species. This detailed visualization helps in identifying key areas that may be targeted for further research or biotechnological applications.

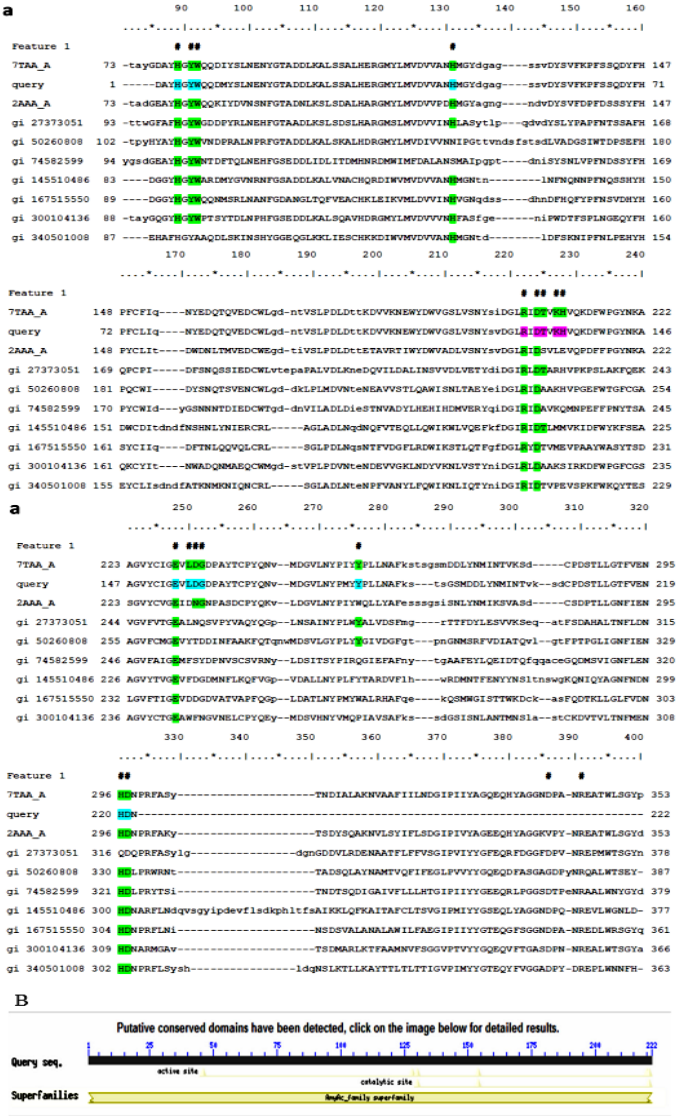

Fig. 3. a. Amino acids within the conserved domains of cassava-degrading fungi amylase (CDF_Amyl) are highlighted. The hash marks above the aligned sequences indicate the locations of conserved residues. The conserved domain of the CDF_Amyl gene includes several amino acid residues, such as 46 His (H), 128 Arg (R), 130 Gln (Q), 154 Glu (E), and 220 His (H), among others. The various amylases compared include 7TAA_A, which denotes Taka Amylase from *Aspergillus oryzae* (GenBank Accession no. 7TAA_A); the query CDF_Amyl gene (from this study); 2AAA_A, representing alpha-amylases from *Aspergillus niger* (Accession: 2AAA_A); gi 27373051, alpha-amylase from *Lipomyces kononenkoae* (GenBank: AAO12212.1); gi 50260808, a hypothetical amylase protein from *Cryptococcus neoformans* var. *neoformans* B-3501A (GenBank: EAL23458.1); gi 74582599, alpha-amylase from *Schizosaccharomyces pombe* 972 h (UniProtKB/Swiss-Prot: O74922.1); gi 145510486, a hypothetical amylase protein from *Paramecium tetraurelia* strain d4-2 (NCBI Reference Sequence: XP_001441176.1); gi 167515550, a hypothetical amylase protein from *Monosiga brevicollis* MX1 (NCBI Reference Sequence: XP_001742116.1); gi 300104136, glycoside hydrolase family 13 protein from *Schizophyllum commune* H4–8 (GenBank: EFI95542.1); and gi 340501008, a hypothetical amylase protein from *Ichthyophthirius multifiliis* (GenBank: EGR27831.1). All the amylases compared for conserved domain amino acids originate from eukaryotic organisms. b. The active and c. catalytic site regions of the cassava-degrading fungi amylase (CDF_Amyl) are illustrated. The CDF_Amyl is classified within the superfamily of amylases, and its catalytic site is located within the active site region.

3.4 Structural Insights from the Alpha-Amylase Gene in *Aspergillus niger* Strain XJ42 and Functional Implications

The alpha-amylase gene from *Aspergillus niger* strain XJ42 isolated from spoiling cassava shows a 98.2% similarity to *Aspergillus* flavus NRRL3357, a 98.65% similarity to *Aspergillus oryzae* RIB40, a 79.28% similarity to *Aspergillus niger* CBS513.88, and a 52.15% similarity to *Fusarium oxysporum* (Fig. 4). This data highlights the close genetic relationship between *Aspergillus niger* strain XJ42 and other species, particularly within the *Aspergillus* genus.

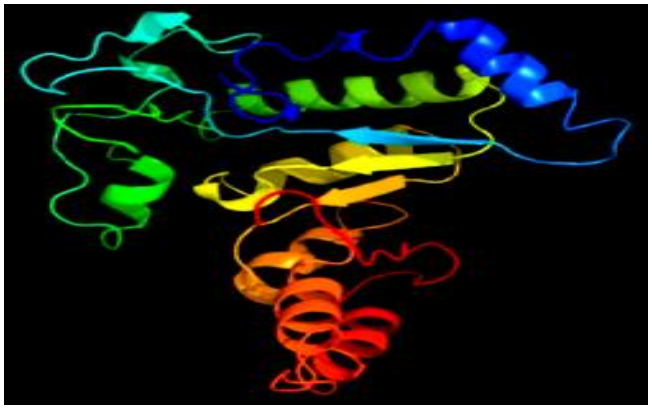

Fig 4: 3D structural representation of *Aspergillus niger* strain XJ42 α-amylase, isolated from spoiling cassava. The structure is visualized with a rainbow color scheme, transitioning from the N-terminus to the C-terminus.

3.5 Restriction Site Analysis and Its Implications for Molecular Investigations

The alpha-amylase gene from *Aspergillus niger* strain XJ42, isolated from spoiling cassava, was analyzed using a restriction map (Fig. 5). The map covers a linear stretch of 1094 bp nucleotide sequence. Sites highlighted in orange represent rare sites that are highly sensitive to methylation and thus not very useful. Sites highlighted in yellow indicate variations between different isolates. Key restriction enzymes and their cut positions include AluI, DraI, and AseI at 135 bp; EcoRI and NlaIV at 324 bp; MluI at 559 bp; TaqI at 679 bp; BsaJI at 748 bp; and BmgBI at 939 bp.

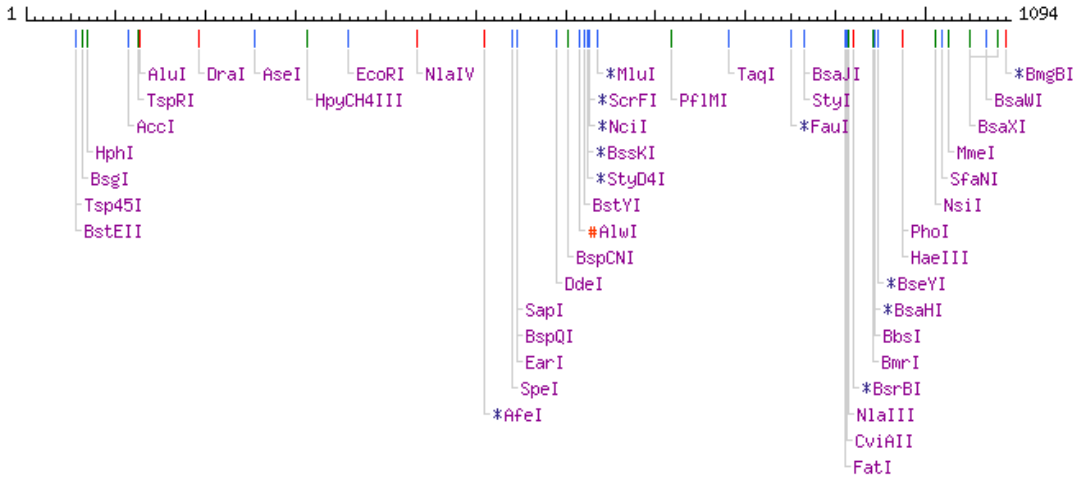

Fig. 5. Restriction enzyme map of the alpha-amylase gene (CDF_Amyl) from *Aspergillus niger* strain XJ42, a cassava-degrading fungus.

3.6 Biochemical Characterization of *Aspergillus niger* XJ42 Alpha-Amylase: Insights into Isoelectric Point, Molecular Weight, and Enzyme Structure

In this study, we investigated the physicochemical properties of the alpha-amylase enzyme (CDF_Amyl) from *Aspergillus niger* strain XJ42, which was isolated from spoiling cassava. Understanding these properties is crucial for potential industrial applications and biotechnological innovations.

Table 2 presents the detailed properties of the enzyme. The enzyme has an isoelectric point (pI) of 4.17, a molecular weight of 25,128.71 Daltons, and consists of 222 amino acid residues.

Table 2: Physicochemical Properties of the Alpha-Amylase Enzyme (CDF_Amyl) from *Aspergillus niger* Strain XJ42

Enzyme	Isoelectric Point (pI)	Molecular Weight (Dalton)	Number of Amino Acid Residues
CDF_Amyl	4.17	25,128.71	222

3.7 Subcellular Localization and Functional Implications of CDF_Amyl in *Aspergillus niger* XJ42

Fig. 6 presents the availability index of alpha-amylase across various subcellular compartments. The secretory pathway shows the highest likelihood with an availability index of 0.9, suggesting predominant localization for secretion. Other compartments, such as the mitochondrial, cytoplasmic, and nuclear pathways, demonstrate significantly lower indices, each below 0.05, indicating minimal presence in these locations.

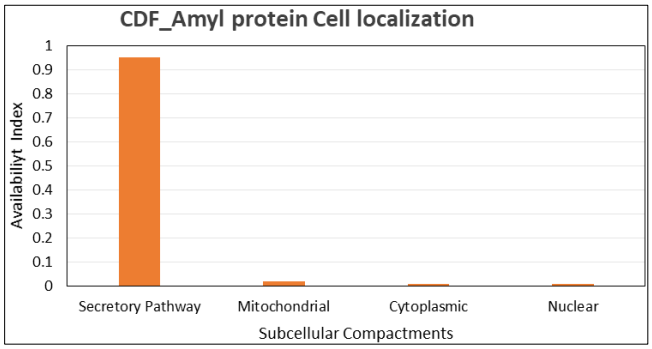

Fig. 6. Localization of CDF_Amyl within the subcellular structures of *Aspergillus sp*. XJ42.

3.8 Analysis of Hydrophobic and Hydrophilic Regions in Protein Sequence Using the Kyte-Doolittle Scale

Fig. 7 illustrates the DAS profile score for predicting transmembrane ™ regions within the alpha-amylase protein sequence from *Aspergillus niger* strain XJ42. Peaks exceeding the loose cutoff of 2.0 suggest potential transmembrane segments, indicating regions within the query sequence that may span the cell membrane. In this analysis, segments around positions 50, 120, and 150 have scores surpassing this threshold, suggesting possible TM regions in these areas. However, none of the segments reach the strict cutoff.

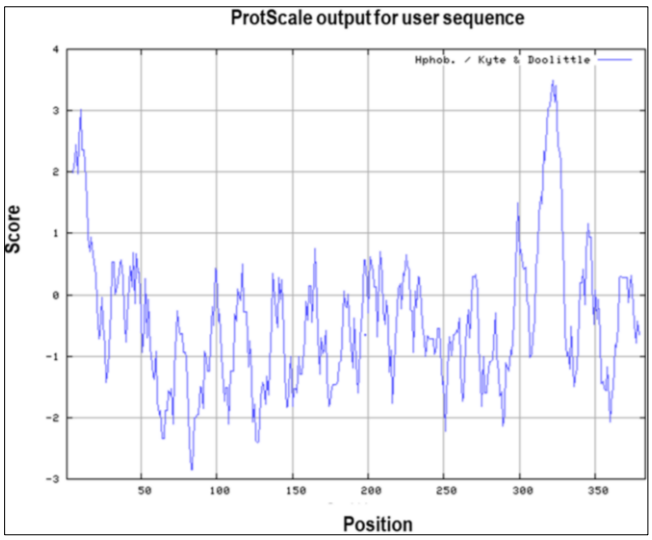

Fig 7

Fig. 7. Kyte-Doolittle hydropathy plot for the alpha-amylase protein from *Aspergillus niger* strain XJ42. The x-axis represents the window position, showing the average hydropathy of the sequence, with the amino acids at the center of each window. Regions above 0 indicate hydrophobic areas within the protein, while regions below 0 represent hydrophilic areas. Peaks exceeding the threshold of 1.8 suggest potential transmembrane regions in the alpha-amylase protein.

3.9 Prediction of transmembrane segments

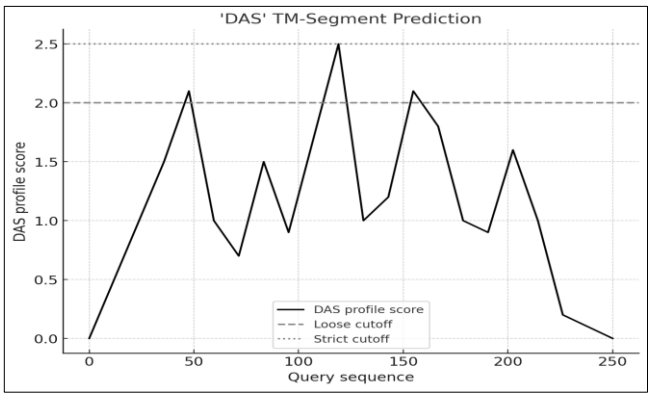

Fig 8: DAS TM-Segment Prediction for Alpha-Amylase Gene from *Aspergillus niger* Strain XJ42.

4 Discussion

Understanding the structure, function, and evolutionary relationships of α-amylases is crucial for applications in biotechnology, food processing, and biofuel production. The present study aimed to investigate the α-amylase enzyme from *Aspergillus niger* strain XJ42, a fungal species commonly associated with cassava spoilage. α-Amylases are enzymes that catalyze the hydrolysis of starch, a complex carbohydrate found in various food sources. We successfully isolated and characterized the α-amylase gene from *Aspergillus niger* strain XJ42, which aligns with previous reports on the conservation of essential domains in α-amylase enzymes across various fungal species. The presence of the catalytic α-amylase domain (PF00128) and the glycosyl hydrolase family 13 (GH13) domain is consistent with earlier findings where similar domains were identified in *Aspergillus* and other fungal α-amylases, underscoring their functional importance in starch degradation [24]. Additionally, we discovered the starch-binding domain (SBD) and calcium-binding sites, which are supported by studies indicating that these regions are essential for the enzyme's substrate specificity and stability under various environmental conditions [25].

Furthermore, the results of our BLASTp analysis (Table 1) provide further evidence of the evolutionary conservation of α-amylase enzymes. Previous research demonstrated a high degree of sequence homology among α-amylase enzymes across different *Aspergillus* species, supporting their functional conservation and evolutionary adaptation to diverse environments [26]. Our identification of homologous proteins from *Aspergillus* species aligns with these findings, suggesting that the α-amylase enzyme from *A.* niger strain XJ42 shares key functional traits with those of other related species. These findings contribute to the growing body of literature on the structure and function of α-amylase enzymes, particularly those isolated from fungal sources. Consistent with previous reports, our study emphasizes the importance of conserved domains for enzymatic activity and stability, providing insights that could have applications in

industrial processes such as starch hydrolysis and bioethanol production [27].

We performed a phylogenetic analysis of the α-amylase gene from *A.* niger strain XJ42 (CDF_Amyl), focusing on the evolutionary relationships with α-amylases from other species. The phylogenetic tree demonstrated that the α-amylase gene from *A.* niger XJ42 exhibits a high degree of similarity with *Aspergillus flavus* (98.2%) and *Aspergillus oryzae* RIB40 (98.65%) [28]. These close similarity values suggest that the α-amylase genes in these *Aspergillus* species likely share a common evolutionary origin, indicative of functional conservation across these strains. The high degree of conservation implies that they may perform similar biological roles in carbohydrate metabolism, particularly in industrial applications such as fermentation and enzyme production [29]. Previous studies highlighted the importance of α-amylases in various biotechnological processes, including starch degradation and bioethanol production [30, 31]. The close phylogenetic relationship between *A.* niger XJ42, *A. flavus*, and *A. oryzae* also points toward possible horizontal gene transfer events or conserved evolutionary pressures in their ecological niches, which favor the retention of α-amylase genes with high efficiency in carbohydrate metabolism. Understanding these evolutionary connections not only enhances knowledge of fungal genomics but also provides potential for utilizing these enzymes in industrial biotechnology, particularly in enzyme optimization and the synthesis of bio-based products.

The multiple sequence alignment of the cassava-degrading fungal amylase (CDF_Amyl) with amylases from various organisms revealed regions of high conservation, particularly around the catalytic sites. The query sequence aligns closely with known amylase sequences from fungi and other species, revealing significant conservation of key residues involved in enzyme activity. Notably, the histidine (H), aspartic acid (D), and glutamic acid (E) residues, typical of α-amylases, were preserved in the query sequence and are crucial for the enzyme's catalytic mechanism, which involves the cleavage of α-1,4-glycosidic bonds in starch [32]. These conserved catalytic domains, part of the glycosyl hydrolase family, are specifically involved in the hydrolysis of starches into simpler sugars. This observation corroborates earlier studies on fungal α-amylases, which reported similar domain structures and active site configurations [33].

The tertiary structure of the α-amylase protein, illustrated in Fig. 4, further supports these findings. As a key enzyme responsible for starch degradation, the arrangement of α-helices (in red and orange) and β-sheets (in green and blue) observed in the structure contributes to the enzyme's stability and activity in starch hydrolysis. Previous studies have shown that conserved regions in the enzyme's tertiary structure play a critical role in binding substrates and catalyzing starch breakdown. In our study, the domains displayed are likely part of the glycosyl hydrolase family, with active sites embedded within the folded structure, facilitating the cleavage of glycosidic bonds. This is consistent with reports from other researchers on fungal α-amylases [33].

Our In silico analysis of the α-amylase gene from *A.* niger strain XJ42 provides further insights into its functional properties. As Huang and Qian [33] noted, conserved catalytic domains are commonly found in fungal amylases, especially within active site configurations that contribute to substrate specificity and enzymatic efficiency. The visualization of these structural features, as depicted in Fig. 4, supports prior research linking enzyme structure with its biochemical functions.

Furthermore, we discovered that the α-amylase from *A.* niger strain XJ42 is highly efficient in breaking down cassava starch. This trait is advantageous for industrial and biotechnological applications, such as food spoilage control and fermentation processes. The structure displayed in Fig. 4 underscores this enzyme's robustness and potential for starch degradation, a characteristic that has been previously noted in studies on microbial amylases used in similar processes [33].

We also identified multiple restriction sites, including EcoRI, TaqI, AluI, BsaI, and BspQI, within the α-amylase gene (Fig. 5). These enzymes recognize specific nucleotide sequences, enabling gene fragmentation into manageable sections for cloning or sequencing. Restriction enzymes such as EcoRI and TaqI are widely used in molecular biology, and their cleavage sites in this gene highlight their potential utility in designing future experiments, such as gene expression studies and activity assays. These findings are consistent with previous studies on fungal α-amylases, such as those from *A. oryzae* and *A.* niger, which have shown similar restriction patterns, further reinforcing the evolutionary conservation of this gene and its role in starch degradation [34].

The low isoelectric point (pI) of 4.17 for *A.* niger XJ42 α-amylase (CDF_Amyl) suggests that the enzyme is acidic, enabling it to function optimally in acidic environments like those found in spoiling cassava. This observation is consistent with recent findings, which report that fungal amylases operate efficiently in low pH conditions, particularly in industrial settings where acidic environments are common [35]. Similar trends have been reported for *A. oryzae* and *A.* niger, where amylases exhibit optimal activity under acidic conditions [36].

The molecular weight of 25,128.71 Daltons for CDF_Amyl falls within the typical range for fungal α-amylases, which often range between 20 and 60 kDa. Recent studies have confirmed this trend, showing molecular weights consistent with *Aspergillus* species, further emphasizing the evolutionary conservation of enzyme size across fungal α-amylases [37]. Additionally, our findings align with research that demonstrated similar molecular weights for α-amylases from *A.* niger, underscoring its conserved function in starch hydrolysis [38].

With 222 amino acid residues, CDF_Amyl presents a compact enzyme structure that is associated with efficient catalytic activity. Recent research on fungal α-amylases has indicated that more compact enzymes tend to exhibit higher efficiency in polysaccharide breakdown, particularly in starch hydrolysis applications. This finding is supported by studies showing that compact fungal amylases are highly effective in industrial starch degradation processes [39].

These biochemical characteristics—including the low pI, molecular weight, and compact structure—are consistent with other well-characterized fungal α-amylases from *Aspergillus* species, reinforcing the evolutionary conservation of these enzymes and their essential role in starch degradation.

The subcellular localization of the *A.* niger XJ42 α-amylase (CDF_Amyl), as shown in Fig. 6, reveals a high availability index in the secretory pathway, with minimal presence in mitochondrial, cytoplasmic, or nuclear compartments. This strongly suggests that the enzyme is secreted extracellularly, a characteristic aligned with its biological role in breaking down cassava starch. The localization of CDF_Amyl to the secretory pathway is consistent with findings from previous studies, which show that α-amylases in *Aspergillus* species, such as *A. oryzae* and *A.* niger, are secreted to facilitate extracellular starch degradation [40]. Additionally, research has demonstrated that secretion of amylases is critical for their application in industrial processes, such as bioethanol

production and food processing, where extracellular activity is crucial for efficient substrate breakdown [41-43].

Moreover, studies have highlighted the evolutionary conservation of secretory mechanisms for fungal amylases, further reinforcing the importance of this localization for starch hydrolysis [44, 45]. Our data on subcellular localization complements the in silico analysis, providing a more comprehensive understanding of how the enzyme functions in degrading cassava during spoilage, a process facilitated by the extracellular activity of secreted amylases.

The hydrophobicity plot based on the Kyte and Doolittle scale provides insights into potential regions of hydrophobicity and hydrophilicity within the protein sequence. Hydrophobicity plays a crucial role in protein folding and function, particularly in membrane interactions. According to Kyte and Doolittle's scale, positive values on the plot correspond to hydrophobic regions, which may be buried within the protein's core or associated with membrane-bound regions [46]. In contrast, negative values indicate hydrophilic regions that are likely surface-exposed and interact with the aqueous environment [47]. The peaks observed in the plot highlight key hydrophobic regions, notably around positions 50, 300, and 350. These areas may be important for structural stability or could indicate transmembrane domains [49], while troughs with negative values suggest hydrophilic regions that contribute to protein solubility and interaction with the environment [50].

The graph in Fig. 8 presents a 'DAS' TM-Segment Prediction, indicating potential transmembrane regions based on the DAS profile score. Peaks exceeding the loose cutoff (2.0) around positions 100 and 180 suggest that these regions may be transmembrane segments. In the context of α-amylase, these transmembrane regions could indicate membrane association, potentially contributing to enzyme secretion or interaction with the starch substrate near membrane structures. Similar findings have been reported in studies on fungal amylases, where membrane association was linked to increased enzymatic efficiency in degrading polysaccharides [51]. Thus, the DAS TM-segment prediction suggests a functional role for transmembrane regions in the α-amylase enzyme from *A. niger* strain XJ42, supporting its role in cassava spoilage by facilitating effective starch breakdown.

5. Conclusion

The findings of this study strongly affirm that the amplified amylase gene from *Aspergillus niger* strain XJ42, isolated from decaying cassava, is a member of the α-amylase superfamily. The unique characteristics of the CDF_Amyl gene and its deduced protein suggest potential applications in the biotechnological, food, and pharmaceutical industries. Cloning and further modification of this gene could be essential for product development and improvement.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Adriel A. Ekozin: Writing – review & editing, Writing – original draft, Conceptualization, Project administration. **Kingsley E. Enerijiofi:** Methodology, Investigation, Validation. **David E. Ekanem:** Software, Resources, Formal analysis.

John M. Obiasi: Visualization, Data curation, Formal analysis. **Taiwo S. Okanlawon:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

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