P-ISSN 2586-9000
E-ISSN 2586-9027

Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia

Vol. 29 No.3 July – September 2024
Page: [224-243]

Original research article

Comparative Analysis of α-Amylase Sequences in Selected *Aspergillus* Species and Analysis of Amylase Activity in Three Locally Isolated *Aspergillus* Species

Karen Jing Yi Hon[1, *], Nurul Arrisya Aimi Mohd Amran[1], Afiq Nasri Abdullah[1],
Ngui Sing Ngieng[2], Hairul Roslan[2]

[1]*Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Sarawak 94300, Malaysia*
[2]*Center For Sago Research, Faculty of Resource Science and Technology,*
Universiti Malaysia Sarawak, Sarawak 94300, Malaysia

Received 2 April 2024; Received in revised form 8 June 2024
Accepted 2 July 2024; Available online 25 September 2024

ABSTRACT

Amylase is an important enzyme in industries where members of the *Aspergillus* genus are significant producers of industrial amylase enzymes; α-amylase is an endoamylase which plays a crucial role in starch hydrolysis. However, knowledge regarding the sequences and evolutionary relationship of α-amylase among sections *Nigri*, *Flavi* and *Fumigati* in the *Aspergillus* genus have remained unknown. The amylase activity of some previously isolated important *Aspergillus* species, namely *A. niger*, *A. fumigatus* and *A. flavus*, also requires further investigation. In this study, α-amylase sequences of species under three sections in the *Aspergillus* genus were retrieved from NCBI for sequence analysis and alignment for phylogenetic tree construction. The solid-based method was then applied to screen for amylase production and the Enzymatic Activity Index (EAI) was evaluated to analyze amylase activity. Similarities in enzyme sequences among the selected species were established through the presence of α-amylase domains and phylogenetic tree branching that rooted to a common ancestor. This study also found that the amylase activity of three selected species differed significantly, with *A. flavus* having the highest EAI of 1.03 ± 0.049, followed by *A. niger* (0.77 ± 0.049) and *A. fumigatus* (0.60 ± 0.103).

Keywords: *α*-amylase; Amylase activity index; *Aspergillus*; Phylogenetic tree; Sequence analysis

---

***Corresponding author:** honjingyi131@gmail.com

1. Introduction

The presence of catalysts within living organisms allows these organisms to carry out reactions necessary for their survival and proliferation [1]. These are biocatalysts known as enzymes which have excellent catalytic efficiency and possess outstanding specificity in both substrates and reactions [2].Nowadays the development of the enzyme market is very promising and growth in global sales of enzymes is estimated to reach approximately 5.5 billion Euros in 2025 [3].

The first enzyme to be studied was 'diastase', dating back to 1833 [3], where the enzyme isolated by Payen and Persoz was in fact a mixture of amylases [1]. Amylases are enzymes that are categorized under the hydrolase subdivision of glycosylases [4] with the classification number of EC 3.2. Amylases are enzymes that facilitate the hydrolysis of starch into simple sugars [5]. Amylase is an important enzyme that accounts for around 30% of global enzyme output and is a crucial enzyme in starch-based industries [6]. Amylase is widely utilized in a range of industries such as baking, detergent, textile, and many more [7].

Alpha-amylase is a type of endoamylase categorized under family 13 of glycoside hydrolase (GH) [6], which is a principal family of α-amylase [8]. Members of the family have similar catalytic machinery, sharing 4 to 7 conserved sequence regions and having a catalytic domain constituting (α/β) 8-barrel fold [9]. Orthologs of α-amylase from the fungi kingdom are more related to one another than they are to the α-amylase orthologs from other kingdoms [10]. *In silico* analysis of enzymes covering conserved domains, protein localization and transmembrane helices were made possible by computational tools [11]. Sequences of α-amylase, for example, could be investigated to reveal conserved regions and evolutionary relationships between subfamilies of the enzyme [12].

Fungi and yeasts play a prominent role in the enzyme industry, responsible for over half of the enzymes that this industry produces. This production is mostly associated with the genera *Aspergillus*, *Trichoderma*, *Rhizopus* and *Penicillium* [7]. Filamentous fungi are favored over other microbes due to their stability, high production potential and simpler purification process [7].

Members of the *Aspergillus* genus are the main source of fungal amylase [13]. By 1926, the *Aspergillus* genus was one of the most recognized and best researched mold groups, as humans recognized the relevance of this species as a causal agent for both spoilage and diseases, as well as the producer of beneficial metabolites [14]. Currently consisting of 339 known species [15], this genus is divided into several sections, with the black aspergilli from section *Nigri* being important in biotechnology, enzyme production and mycotoxin excretion [16], similar to members of the section *Flavi* [17]. On the other hand, the section *Fumigati* has significant medical importance as several of its members are recognized as pathogens to both human and animals [18].

For this work, 49 *Aspergillu*s species from section *Nigri*, *Flavi* and *Fumigati* were selected for sequencing and phylogenetic analysis of their α-amylase gene, while three *Aspergillus* species, *A. niger*, *A. flavus* and *A. fumigatus*, from the three sections were used in the research of amylase activity. The objectives of this study were to investigate the evolutionary link between selected *Aspergillus* α-amylase sequences and to investigate the amylase activities of three *Aspergillus* species.

2. Materials and Methods

2.1 Analysis of *α*-amylase sequences

2.1.1 Retrieval of amino acid sequence data

Aspergillus species categorized under section *Nigri* were identified by Varga et al. [19]; meanwhile Frisvad et al. was referred to for species from section *Flavi* [17], while a study by Frisvad and Larsen was referred to for those belonging to section *Fumigati [*20*]*. The National Centre for Biotechnology Information (NCBI) database was used to access *α*-amylase sequences from the selected species. A total of 49 species were found to have their *α*-amylase sequences in the database, with 23 species from section *Nigri*, 20 from section *Flavi* and 7 from section *Fumigati*. Two *α*-amylase sequences were retrieved from the NCBI database for each species and species with different strains (whenever available), as shown in Table 1. An additional sequence of a citrate synthase (citA) gene was obtained as the outgroup for the phylogenetic tree. Complete information of the selected sequences can be found in Supplementary Table 1.

Table 1. The α-amylase sequences of selected Aspergillus species.

Section *Nigri*

Species	GenPept Accession Number	Genome Accession Number	Num of Amino Acids
A. aculeatinus CBS 121060	RAH74414.1	KZ824935.1	639 aa
	RAH73821.1	KZ824937.1	571 aa
A. brasiliensis	GKZ31594.1	BROR01000101.1	319 aa
	GKZ33145.1	BROR01000174.1	549 aa
A. costaricaensis CBS 115574	RAK85335.1	KZ824566.1	635 aa
	RAK85549.1	KZ824565.1	573 aa
A. ellipticus CBS 707.79	PYH96808.1	KZ825833.1	525 aa
	PYH96683.1	KZ825834.1	532 aa
A. eucalypticola CBS 122712	PWY84188.1	MSFU01000002.1	538 aa
	PWY82341.1	MSFU01000003.1	642 aa
A. fijiensis CBS 313.89	RAK72695.1	KZ824690.1	548 aa
	RAK79041.1	KZ824634.1	571 aa
A. heteromorphus CBS 117.55	PWY92827.1	MSFL01000001.1	486 aa
	PWY90092.1	MSFL01000003.1	529 aa
A. homomorphus CBS 101889	RAL13909.1	KZ824277.1	572 aa
	RAL12205.1	KZ824284.1	588 aa
A. ibericus CBS 121593	RAK97663.1	KZ824460.1	631 aa
	RAK99978.1	KZ824443.1	548 aa
A. indologenus CBS 114.80	PYI36077.1	KZ825466.1	557 aa
	PYI36076.1	KZ825466.1	552 aa
A. luchuensis	GAT24829.1	BCWF01000018.1	550 aa
	GAT20922.1	BCWF01000008.1	555 aa
A. luchuensis IFO 4308	GAA93340.1	DF126574.1	499 aa
	GAA92144.1	DF126489.1	567 aa
A. neoniger CBS 115656	PYH37168.1	KZ821451.1	552 aa
	PYH37276.1	KZ821450.1	634 aa
A. niger	GJP87368.1	BQMC01000001.1	524 aa
	GJP89740.1	BQMC01000002.1	567 aa
A. niger CBS 101883	PYH61062.1	KZ821339.1	555 aa
	PYH57279.1	KZ821347.1	558 aa
A. niger ATCC 13496	RDH24425.1	KZ851902.1	562 aa
	RDH22091.1	KZ851908.1	567 aa
A. niger ATCC 1015	EHA28336.1	ACJE01000001.1	552 aa
	EHA27488.1	ACJE01000003.1	562 aa

A. niger CBS 513.88	XP_001393626.1	XM_001393589.2	555 aa
	XP_001389762.2	XM_001389725.2	557 aa
A. piperis CBS 112811	RAH52383.1	KZ825085.1	548 aa
	RAH53315.1	KZ825077.1	567 aa
A. sclerotiicarbonarius CBS 121057	PYI11254.1	KZ826318.1	630 aa
	PYI04009.1	KZ826374.1	550 aa
A. saccharolyticus JOP 1030-1	PYH48040.1	KZ821222.1	576 aa
	PYH47838.1	KZ821223.1	589 aa
A. sclerotioniger CBS 115572	PWY91510.1	MSFK01000009.1	554 aa
	PWY91509.1	MSFK01000009.1	546 aa
A. tubingensis	GFN12725.1	BLWE01000003.1	567 aa
	GFN13310.1	BLWE01000004.1	524 aa
A. uvarum CBS 121591	PYH84072.1	KZ821686.1	513 aa
	PYH83360.1	KZ821690.1	572 aa
A. vadensis CBS 113365	PYH73420.1	KZ821615.1	493 aa
	PYH69636.1	KZ821623.1	530 aa
A. violaceofuscus CBS 115571	PYI24515.1	KZ825102.1	558 aa
	PYI20221.1	KZ825127.1	558 aa
A. japonicus CBS 114.51	RAH81691.1	KZ824794.1	557 aa
	RAH77919.1	KZ824834.1	499 aa

Section *Flavi*

Species	GenPept Accession Number	Genome Accession Number	Num of Amino Acids
A. alliaceus	KAB8239457.1	ML733060.1	497 aa
	KAE8392003.1	ML735240.1	497 aa
A. arachidicola	KAE8340873.1	ML737144.1	498 aa
	PIG85909.1	NEXV01000296.1	502 aa
A. avenaceus	KAE8146912.1	ML742236.1	530 aa
	KAE8155106.1	ML742024.1	528 aa
A. bertholletius	KAE8380264.1	ML736183.1	498 aa
A. bombycis	OGM45404.1	LYCR01000043.1	551 aa
	OGM43080.1	LYCR01000077.1	498 aa
A. flavus	AAF14264.1	AF139925.1	499 aa
	KAJ1716275.1	JAOAMT010000010.1	533 aa
A. flavus AF70	KOC15411.1	JZDT01000667.1	615 aa
	KOC12109.1	JZDT01000385.1	469 aa
A. hancockii	KAF7592720.1	MBFL02000092.1	552 aa
A. leporis	KAB8070316.1	ML732306.1	537 aa
	KAB8074018.1	ML732217.1	511 aa
A. minisclerotigenes	KAB8273707.1	ML732794.1	498 aa
A. nomiae NRRL 13137	KNG81364.1	JNOM01000457.1	498 aa
	KNG84553.1	JNOM01000201.1	542 aa
A. novoparasiticus IBT 16806	KAB8214608.1	ML733529.1	498 aa
A. sergii	KAE8324966.1	ML741812.1	498 aa
A. sojae	BAM28635.1	AB733130.1	498 aa
A. tamarii	KAE8162449.1	ML738628.1	498 aa
A. transmontanensis	KAE8310350.1	ML738354.1	498 aa
A. oryzae	CAA31220.1	X12727.1	499 aa
	CAA31218.1	X12725.1	499 aa
A. oryzae 100-8	KDE84105.1	AMCJ01000074.1	602 aa
	KDE82914.1	AMCJ01000090.1	566 aa
A. oryzae 3.042	EIT81950.1	AKHY01000091.1	549 aa
	EIT81352.1	AKHY01000106.1	602 aa
A. parasiticus	KAB8200363.1	ML735046.1	498 aa
A. parasiticus SU-1	KJK64034.1	JZEE01000530.1	582 aa

	KJK63736.1	JZEE01000545.1	575 aa
A. pseudocaelatus	KAE8411038.1	ML735896.1	498 aa
A. pseudonomiae	KAB8257239.1	ML734321.1	537 aa
	KAE8402772.1	ML736784.1	537 aa
A. pseudotamarii	KAE8140395.1	ML743561.1	451 aa
	KAE8131762.1	ML743650.1	498 aa
Section *Fumigati*			
Species	**GenPept Accession Number**	**Genome Accession Number**	**Num of Amino Acids**
A. fischeri NRRL 181	EAW18731.1	DS027696.1	612 aa
	EAW25104.1	DS027685.1	422 aa
A. fumigatus	KEY83532.1	JHOI01000358.1	494 aa
	KEY80426.1	JHOI01000543.1	630 aa
A. fumigatus Z5	KMK61686.1	KQ087360.1	487 aa
	KMK58769.1	KQ087364.1	561 aa
A. fumigatus Af293	EAL93641.1	AAHF01000001.1	561 aa
	EAL87170.1	AAHF01000008.1	630 aa
A. fumigatus A1163	EDP53984.1	DS499595.1	568 aa
	EDP54851.1	DS499595.1	471 aa
A. lentulus	GFF88611.1	BLKI01000064.1	554 aa
	GFF93265.1	BLKD01000070.1	604 aa
A. novofumigatus IBT 16806	PKX98967.1	MSZS01000001.1	597 aa
	PKX89274.1	MSZS01000010.1	561 aa
A. pseudoviridinutans	GIJ82803.1	BHVY01000001.1	509 aa
A. udagawae	GFG18418.1	BLKE01000238.1	569 aa
	GFF53486.1	BLKF01000090.1	484 aa
A. viridinutans	GIJ99235.1	BOPL01000001.1	448 aa

Table 2. Identification of Aamy domain, other domains and features with their position in the *α*-amylase protein sequences through SMART tool.

Species	GenPept Accession Number	Domains/Features	Start	End	E-Value
A. aculeatinus CBS 121060	RAH74414.1	Aamy	34	390	2.29e-94
		Low complexity	434	454	N/A
		CBM_2	533	633	2.87e-20
	RAH73821.1	Aamy	38	396	3.37e-74
		Low complexity	451	460	N/A
		Low complexity	523	547	N/A
A. brasiliensis	GKZ31594.1	Low complexity	5	20	N/A
		Aamy	29	319	8.46e-57
	GKZ33145.1	Aamy	41	396	2.85e-72
		Transmembrane region	525	547	N/A
A. costaricaensis CBS 115574	RAK85335.1	Low complexity	8	25	N/A
		Aamy	34	390	2.54e-101
		Low complexity	434	453	N/A
		CBM_2	535	631	7.06e-29
	RAK85549.1	Aamy	40	398	1.47e-74
		Low complexity	524	544	N/A
		Transmembrane region	550	572	N/A
A. ellipticus CBS 707.79	PYH96808.1	Aamy	29	385	1.8e-101
		Low complexity	488	508	N/A
	PYH96683.1	Aamy	41	427	2.33e-36
A. eucalypticola CBS 122712	PWY84188.1	Aamy	41	427	2.27e-48
	PWY82341.1	Aamy	34	390	8.39e-102
		Low complexity	434	453	N/A
		CBM_2	542	638	7.06e-29
A. fijiensis CBS 313.89	RAK72695.1	Aamy	42	435	2.38e-42
	RAK79041.1	Aamy	38	396	5.03e-73
		Low complexity	451	460	N/A

		Low complexity	523	547	N/A
A. heteromorphus CBS 117.55	PWY92827.1	Aamy	1	340	2.2e-55
	PWY90092.1	Aamy	41	421	1.67e-46
		Low complexity	514	528	N/A
A. homomorphus CBS 101889	RAL13909.1	Aamy	38	396	1.73e-71
		Low complexity	451	460	N/A
		Low complexity	523	547	N/A
	RAL12205.1	Aamy	20	439	6.35e-143
A. ibericus CBS 121593	RAK97663.1	Low complexity	3	25	N/A
		Aamy	34	390	1.62e-94
		Low complexity	434	453	N/A
		CBM_2	531	627	1.27e-30
	RAK99978.1	Aamy	39	394	7.07e-68
A. indologenus CBS 114.80	PYI36077.1	Aamy	42	435	4.58e-44
		Low complexity	516	537	N/A
	PYI36076.1	Aamy	38	398	4.84e-75
		Low complexity	511	530	N/A
A. luchuensis	GAT24829.1	Aamy	41	396	7.01e-72
		Transmembrane region	526	548	N/A
	GAT20922.1	Aamy	38	397	1.45e-87
		Low complexity	512	525	N/A
		Low complexity	534	553	N/A
A. luchuensis IFO 4308	GAA93340.1	Low complexity	16	25	N/A
		Aamy	34	390	9.91e-113
	GAA92144.1	Aamy	34	392	7.75e-74
		Low complexity	518	538	N/A
		Transmembrane region	544	566	N/A
A. neoniger CBS 115656	PYH37168.1	Aamy	35	394	6.23e-87
		Low complexity	509	522	N/A
		Low complexity	533	550	N/A
	PYH37276.1	Low complexity	3	14	N/A
		Aamy	34	390	8.99e-102
		Low complexity	434	453	N/A
		CBM_2	534	630	9.99e-29
A. niger	GJP87368.1	Aamy	29	385	5.57e-107
		Transmembrane region	501	523	N/A
	GJP89740.1	Transmembrane region	5	24	N/A
		Aamy	35	393	2.04e-73
		Low complexity	448	457	N/A
		Low complexity	522	541	N/A
		Transmembrane region	544	566	N/A
A. niger CBS 101883	PYH61062.1	Low complexity	14	27	N/A
		Aamy	38	397	2.09e-88
		Low complexity	512	525	N/A
		Low complexity	534	550	N/A
	PYH57279.1	Aamy	41	427	2.91e-46
A. niger ATCC 13496	RDH24425.1	Aamy	20	451	7.43e-126
	RDH22091.1	Transmembrane region	5	24	N/A
		Aamy	35	393	5.4e-73
		Low complexity	522	534	N/A
		Transmembrane region	544	566	N/A
A. niger ATCC 1015	EHA28336.1	Low complexity	11	24	N/A
		Aamy	35	394	2.09e-88
		Low complexity	509	522	N/A
		Low complexity	531	547	N/A
	EHA27488.1	Aamy	20	451	1.14e-126
A. niger CBS 513.88	XP_001393626.1	Low complexity	14	27	N/A
		Aamy	38	397	2.09e-88
		Low complexity	512	525	N/A
		Low complexity	534	550	N/A
	XP_001389762.2	Aamy	41	427	5.14e-47
A. piperis CBS 112811	RAH52383.1	Aamy	41	427	9.67e-43
	RAH53315.1	Aamy	34	392	6.64e-73

		Low complexity	518	538	N/A
		Transmembrane region	544	566	N/A
A. sclerotiicarbonarius CBS 121057	PYI11254.1	Low complexity	3	11	N/A
		Aamy	34	390	1.76e-95
		Low complexity	434	453	N/A
		CBM_2	530	626	7.06e-29
	PYI04009.1	Aamy	41	396	1.66e-64
		Low complexity	539	548	N/A
A. saccharolyticus JOP 1030-1	PYH48040.1	Aamy	37	395	6.75e-74
		Low complexity	450	459	N/A
		Low complexity	528	549	N/A
	PYH47838.1	Aamy	20	439	2.81e-144
A. sclerotioniger CBS 115572	PWY91510.1	Aamy	34	393	7.97e-85
		Low complexity	508	525	N/A
		Transmembrane region	531	553	N/A
	PWY91509.1	Low complexity	2	13	N/A
		Aamy	42	429	1.1e-37
A. tubingensis	GFN12725.1	Aamy	34	392	1.78e-73
		Low complexity	518	538	N/A
		Transmembrane region	544	566	N/A
	GFN13310.1	Low complexity	2	18	N/A
		Aamy	29	385	2.3e-108
		Transmembrane region	501	523	N/A
A. uvarum CBS 121591	PYH84072.1	Aamy	24	388	3.21e-89
	PYH83360.1	Aamy	38	396	1.5e-71
		Low complexity	451	460	N/A
		Low complexity	524	548	N/A
A. vadensis CBS 113365	PYH73420.1	Low complexity	2	18	N/A
		Aamy	29	385	5.28e-108
	PYH69636.1	Aamy	41	427	1.16e-45
A. violaceofuscus CBS 115571	PYI24515.1	Aamy	38	398	6.39e-75
		Low complexity	511	529	N/A
	PYI20221.1	Aamy	47	402	1.03e-69
		Low complexity	522	531	N/A
		Low complexity	541	558	N/A
A. japonicus CBS 114.51	RAH81691.1	Aamy	42	435	1.5e-39
	RAH77919.1	Aamy	34	390	3.62e-65

Section *Flavi*

Species	**GenPept Accession Number**	**Domains/Features**	**Start**	**End**	**E-Value**
A. alliaceus	KAB8239457.1	Low complexity	14	21	N/A
		Aamy	33	389	1.52e-108
	KAE8392003.1	Low complexity	14	21	N/A
		Aamy	33	389	6.25e-110
A. arachidicola	KAE8340873.1	Low complexity	15	24	N/A
		Aamy	33	389	8.17e-114
	PIG85909.1	Transmembrane region	7	29	N/A
		Aamy	37	393	8.17e-114
A. avenaceus	KAE8146912.1	Aamy	41	420	2.23e-33
	KAE8155106.1	Aamy	40	425	1.95e-38
A. bertholletius	KAE8380264.1	Low complexity	7	24	N/A
		Aamy	33	389	1.17e-114
A. bombycis	OGM45404.1	Aamy	38	397	4.68e-91
		Low complexity	518	531	N/A
		Low complexity	537	551	N/A
	OGM43080.1	Aamy	33	389	1.77e-109
A. flavus	AAF14264.1	Low complexity	16	25	N/A
		Aamy	34	390	2.48e-113
	KAJ1716275.1	Aamy	44	430	7.51e-40
A. flavus AF70	KOC15411.1	Aamy	119	505	6.02e-30
	KOC12109.1	Aamy	1	360	1.39e-11
A. hancockii	KAF7592720.1	Aamy	37	396	6.19e-82
A. leporis	KAB8070316.1	Low complexity	11	22	N/A

		Aamy	42	427	1.17e-41
	KAB8074018.1	Aamy	39	398	6.5e-67
A. minisclerotigenes	KAB8273707.1	Low complexity	15	24	N/A
		Aamy	33	389	1.31e-112
A. nomiae NRRL 13137	KNG81364.1	Low complexity	14	24	N/A
		Aamy	33	389	4.06e-109
	KNG84553.1	Aamy	39	388	1.06e-80
		Low complexity	507	542	N/A
A. novoparasiticus IBT 16806	KAB8214608.1	Low complexity	15	24	N/A
		Aamy	33	389	1.22e-112
A. sergii	KAE8324966.1	Low complexity	15	24	N/A
		Aamy	33	389	3.76e-113
A. sojae	BAM28635.1	Low complexity	15	24	N/A
		Aamy	33	389	1.22e-112
A. tamarii	KAE8162449.1	Low complexity	14	24	N/A
		Aamy	33	389	9.61e-111
A. transmontanensis	KAE8310350.1	Low complexity	15	24	N/A
		Aamy	33	389	1.22e-112
A. oryzae	CAA31220.1	Low complexity	16	25	N/A
		Aamy	34	390	2.48e-113
	CAA31218.1	Low complexity	16	25	N/A
		Aamy	34	390	9.91e-113
A. oryzae 100-8	KDE84105.1	Aamy	24	443	9.9e-154
	KDE82914.1	Aamy	18	436	6.21e-105
A. oryzae 3.042	EIT81950.1	Aamy	36	395	4.94e-90
		Low complexity	529	549	N/A
	EIT81352.1	Aamy	24	443	9.9e-154
A. parasiticus	KAB8200363.1	Low complexity	15	24	N/A
		Aamy	33	389	1.22e-112
A. parasiticus SU-1	KJK64034.1	Low complexity	3	27	N/A
		Aamy	34	452	1.24e-104
	KJK63736.1	Aamy	18	441	6.41e-139
		Low complexity	447	459	N/A
A. pseudocaelatus	KAE8411038.1	Low complexity	15	24	N/A
		Aamy	33	389	8.5e-112
A. pseudonomiae	KAB8257239.1	Aamy	41	427	1.32e-35
	KAE8402772.1	Aamy	41	427	1.32e-35
A. pseudotamarii	KAE8140395.1	Aamy	11	342	2.4e-88
		Low complexity	386	402	N/A
	KAE8131762.1	Low complexity	15	24	N/A
		Aamy	33	389	2.62e-112

Section *Fumigati*

Species	**GenPept Accession Number**	**Domains/Features**	**Start**	**End**	**E-Value**
A. fischeri NRRL 181	EAW18731.1	Aamy	42	469	6.09e-122
	EAW25104.1	Aamy	1	315	1.92e-78
		Low complexity	361	374	N/A
A. fumigatus	KEY83532.1	Aamy	32	387	3.61e-115
		Low complexity	433	446	N/A
	KEY80426.1	Aamy	36	392	2.52e-105
		Low complexity	437	453	N/A
		CBM_2	528	624	4.8e-31
A. fumigatus Z5	KMK61686.1	Aamy	16	351	1.85e-62
		Low complexity	434	447	N/A
	KMK58769.1	Low complexity	14	26	N/A
		Aamy	38	400	2.24e-79
		Transmembrane region	537	559	N/A
A. fumigatus Af293	EAL93641.1	Low complexity	13	25	N/A
		Aamy	35	394	1.75e-90
		Low complexity	508	521	N/A
		Low complexity	525	538	N/A
	EAL87170.1	Aamy	36	392	2.52e-105
		Low complexity	437	453	N/A

		CBM_2	528	624	4.8e-31
A. fumigatus A1163	EDP53984.1	Aamy	38	417	2.12e-80
		Low complexity	555	568	N/A
	EDP54851.1	Low complexity	12	25	N/A
		Aamy	35	394	7.44e-85
A. lentulus	GFF88611.1	Aamy	54	479	1.14e-121
	GFF93265.1	Aamy	54	479	1.98e-121
A. novofumigatus IBT 16806	PKX98967.1	Aamy	42	454	1.48e-111
	PKX89274.1	Low complexity	14	26	N/A
		Aamy	38	400	8.69e-77
		Transmembrane region	537	559	N/A
A. pseudoviridinutans	GIJ82803.1	Aamy	1	357	1.11e-83
A. udagawae	GFG18418.1	Low complexity	15	27	N/A
		Aamy	38	417	5.26e-85
	GFF53486.1	Aamy	1	332	1.36e-46
A. viridinutans	GIJ99235.1	Aamy	1	311	2.8e-48

2.1.2 Analysis of protein sequences

The α-amylase sequences were analyzed using Simple Modular Architecture Research Tool (SMART) for identification of the protein domain [21]. SignalP was used to predict the presence of signal peptides [22] and to determine the exact length of the signal peptides [23]. Another prediction tool, DeepLoc was used to determine the subcellular localization of the protein as the combination of SignalP and DeepLoc was reported to be more effective [24]. DeepLoc was also used to determine the predicted sorting signals of proteins other than signal peptides, as it is a multi-label subcellular localization prediction tool [23].

2.1.3 Multiple sequence alignment and evolutionary tree

Amino acid sequences were aligned using the multiple sequence alignment tool, Clustal Omega [25]. The aligned sequences were manually checked by identifying the regions that aligned reasonably well with one another, reflecting similarity in structure and function of the proteins [26]. The phylogenetic tree of the genes was constructed using the Molecular Evolutionary Genetic Analysis (MEGA) software that contained tools for analyzing raw sequence data [27]. The query sequence was the α-amylase gene from *Aspergillus niger* CBS 513.88 with the locus tag XP_001393626.1 while the outlier was the citrate synthase citA sequence of *Aspergillus niger* ATCC 1015 with locus tag EHA28609.1. The evolutionary relationship of the genes was investigated using a maximum likelihood phylogenetic tree.

2.2 Analysis of three *Aspergillus* species

Three *Aspergillus* species, namely *A. niger* from section *Nigri*, *A. flavus* from section *Flavi* and *A. fumigatus* from section *Fumigati*, were selected for further study of their amylase activity. The fungal stocks of the selected species were previously isolated and stored in the form of fungal plugs in 25% glycerol in the Genetic Engineering Lab (GEL), Faculty of Resource Science and Technology (FRST), Universiti Malaysia Sarawak (UNIMAS). The selected species were revived by obtaining a fungal plug from the glycerol stock and were then cultured on PDA for 5 days at room temperature (27°C).

2.2.1 Screening for amylase production

A. niger, *A. flavus* and *A. fumigatus* were assayed for their ability to produce amylase. The solid-based method was applied for screening, where it was performed on nutrient agar [28]. The fungi were cultured on 2% starch agar [29], by inoculating a loopful of spores scraped off from 5-day-old stock cultures. Starch agar was prepared by adding starch powder to PDA. The fungi were allowed to grow for 5 days at 27°C. Staining for amylase production monitoring was performed by dropping an adequate amount of 1% iodine solution onto plates then allowing the plates to settle for 10 minutes [30]. Excess iodine solution was removed, and clear zones were detected, which is an indicator of amylase production [30]. Screening was not performed in replicates as there was no measurement of halo zone diameter.

2.2.2 Amylase activity of selected Aspergillus species

The selected *Aspergillus* species were studied to determine which had the highest amylase activity. Spores from stock cultures of each species were inoculated on 2% starch agar, where the spores were obtained with an inoculation loop and transferred to the starch agar. Inoculation was performed in triplicate for each species. The fungi were allowed to grow for 5 days at 27°C and after which an adequate amount of 1% iodine solution was poured onto each plate. The plates were left to settle for 10 minutes, and then excess iodine solution was discarded. The diameter of the fungus colony and the halo zone formed after staining were taken horizontally and vertically. Enzyme activity index (EAI) was determined as the ratio between fungus colony diameter and halo zone diameter [31]. Eq. (2.1) was used to calculate EAI.

$$\text{EAI} = \text{Halo zone diameter} / \text{Colony diameter}. \quad (2.1)$$

3. Results and Discussion

3.1 Analysis for conserved domain

Identification and annotation of the protein sequences showed various domain locations especially for α-amylase, where it was detected in all sequences as summarized in Table 1. The complete results of SMART analysis are shown in Supplementary Tables 2-3. The α-amylase domain was detected in 109 amino acid sequences out of 110 sequences at various positions through Normal SMART, while the α-amylase domain in *A. niger* CBS 513.88 (XP_001389762.2) could only be detected through Genomic SMART. Expected value (E-value) indicates the probabilities for sequences to be identical where a smaller E-value indicates a higher probability that the sequences are evolutionarily related (Supplementary Table 1).

Conserved domains in α-amylase protein sequences were identified using SMART. Fungal extracellular α-amylases are classified under subfamily 1 of family GH13 [12]. The presence of an α-amylase domain therefore justified the close evolutionary relationship among the fungal species as this domain is the catalytic domain which possesses the $(\beta/\alpha)_8$-barrel active site. The catalytic barrels are present in members of the α-amylase family and are related [32].

Apart from the catalytic domain, Domain B and Domain C are also part of the composition of α-amylase [8]. However, no Domain B and Domain C were observed in the results of protein sequences analysis. SMART is a type of sequence-based domain database that identifies domains which belong to the same family based on sequence alignment, but reliability of sequence comparisons will decrease rapidly in the twilight zone [33]. In the development of the sequence-based method, there is the assumption that family members of the domain share similar sequence attributes. High prediction accuracy could be achieved when close templates are present, but accuracy will drop significantly when homologous templates are absent. This characteristic might explain the inability to detect the presence of both Domain B and C, as they differ among species.

Low complexity regions were also detected in the protein sequences (Table 2). These are regions that are identified by the presence of homo-polymeric repeats of one amino acid or by hetero-polymeric repeats of amino acid residues, or by irregular patterns of a few amino acids [34], and are often functional [35]. A carbohydrate binding module domain could be found in several α-amylase protein sequences. Its function is to hold the starch molecules properly in place for the catalysis to occur [36]. This additional terminal domain is present in approximately 10% of bacterial and fungal amylases, and exhibits an open, distorted *β*-barrel [37]. Additional domains, particularly the domains that enable the enzymes to bind and degrade thermally untreated starch, are present in numerous fungal *α*-amylase sequences [12].

Transmembrane regions were detected in the alpha-amylase sequences of several species, such as *A. brasiliensis* (Table 2). The presence of this feature suggested that the synthesized amylase is a transmembrane protein, as this helix is the most observed secondary structure in transmembrane proteins within their transmembrane segments [38]. Therefore, transmembrane domains are a marker for integral membrane proteins [39]. This domain has been reported to play a role in the secretory pathways of integral membrane proteins as it contains factors that control protein sorting in the pathways, and this type of sorting is suggested to be more important than the cytosolic sorting signals [40].

3.2 Analysis of signal peptide cleavage site

Prediction results from SignalP showed the presence of a signal peptide cleavage site in more than two thirds of the amino acid sequences. Position of the cleavage site and localization of the protein is shown in Table 1. The cleavage site is identified by observing the vertical green dotted line. Graphical results of signal peptide cleavage site prediction, as well as predicted signals for sorting signals other than signal peptides are shown in Supplementary Table 1. Sec signal peptide (Sec/SPI) is predicted to be present in all sequences with signal peptides, except in *A. ibericus* CBS 121593 (RAK99978.1) where lipoprotein signal peptide (Sec/SPII) is also detected.

Signal peptides are short amino acid sequences located at the N-terminal [41]. Regions of the signal peptides are also predicted by the model, shown in the plot with labeling of -n, -h, and -c. Regions with -n labeling are the N-terminal region of the signal peptide, while H-region represents the center hydrophobic region and -c labeling indicates the C-terminal region. The C-terminal region is typically composed of 3 to 7 polar residues [22] which begins with a helix-breaking residue and the signal peptidase recognition site is found in this region [42]. In eukaryotes, signal peptide will be cleaved by the enzyme signal peptidase complex during translocation [22] where conformational changes will be experienced by the C-terminal to expose its cleavage site for the action of signal peptidase [43].

Signal peptides help in targeting the protein to the secretory pathway in eukaryotes [41], while the secretion machinery functions in translocation of proteins across the endoplasmic reticular membranes [42]. Signal peptides also assist in maintaining the transportable folding state of the protein precursor [44]. Presence of a signal peptide does not indicate that the protein is secreted to the extracellular domain, as it could be retained in compartments of the secretory pathway such as the Golgi apparatus, or the protein may be anchored to the cytoplasmic membrane on the outer surface in eukaryotes [22]. An example could be observed in the case of *A. violaceofuscus* CBS 115571. Signal peptides are not necessarily present in all secreted proteins [45]; this could possibly explain why there is an absence in signal peptide prediction in species such as *A. pseudotamarii* (KAE8140395.1) although it is predicted that the localization of amylase is extracellular. Signal peptides are also absent in proteins secreted through nonclassical pathways [46].

The usage of DeepLoc tool had validated the results of SignalP prediction on most α-amylase sequences, where in most cases, sequences with a signal peptide have their α-amylase secreted extracellularly. The tool had also predicted protein localization apart from extracellular, where the cytoplasm is the most predicted destination of the protein, followed by lysosome, vacuole, cell membrane, nucleus and peroxisome. In some species, α-amylase is predicted to be directed to more than one cellular compartment, suggesting that the protein has different functions in the cell. Cellular localization helps to determine the function of a protein, as it provides the physiological environment in which the protein functions [47].

Proteins having a transmembrane helix are proposed to be static, as they are probably functioning in the cellular membrane [46], yet it is observed that sequences of proteins which possess transmembrane regions identified through SMART are being secreted into the extracellular domain. Transmembrane region prediction in SMART is done by the TMHMM 2.0 program. However, after validating the results of the transmembrane region with another prediction tool for this region, DeepTMHMM (Table 1), it was found that this feature is absent. DeepTMHMM tool is shown currently to be the most comprehensive software for transmembrane protein prediction [48].

3.3 Phylogenetic tree analysis

The phylogenetic tree in Fig. 1 showed similarity of the sequences and indicated that the sequences possessed similar catalytic function and monophyletic origin. Mutual similarity of the *Aspergillus* species can be concluded since all species are rooted to a common ancestor. However, it was found that they are not rooted based on the sections they belong to. The phylogenetic tree also did not comply with the subgenus classification apart from the *Aspergillus* sections. Section *Nigri* and *Flavi* are both classified under subgenus *Circumdati* while section *Fumigati* is categorized under subgenus *Fumigati* [49], but the evolutionary relationship of section *Fumigati* species is not distinctly far from the other two sections.

Apart from that, species having multiple strains such as *A. niger* and *A. fumigatus* did not have their strains located next to one another, they were very much scattered in the tree. A similar situation was also observed in the case of *A. flavus* and *A. oryzae*, while *A. oryzae* has been recognized as the domesticated form of *A. flavus* [17], it did not always locate next to *A. flavus* in the tree. A study on cyclopiazonic acid gene clusters of these two species suggested a divergence between these two species [50]. However, a genome-side variant site study found that *A. oryzae* originated from *A. flavus*, where an atoxigenic lineage of *A. flavus* is suggested to have evolved into a cell factory of enzymes and metabolites [51].

The evolutionary tree in this study was constructed based on genetic data, so it reflects the evolution of the α-amylase genes and not necessarily the evolution of the organism encoding the α-amylase genes.

3.4 Screening for amylase production

The selected species were successfully revived, where the *A. niger* colony appeared black in color, *A. flavus* a yellowish-green, and A. *fumigatus* a greyish-green. Vesicles covered with phialides were observed at the end of conidiophore stalks. Observations of *A. niger*, *A. flavus* and *A. fumigatus* aligned with the descriptions from Campbell et al. [52].

All three fungi species showed positive results for amylase activity, as halo zones were present after iodine staining, shows in Fig. 2. The screening procedure evaluates whether amylase is present in hydrolyzing starch, and the activity was evaluated by tracking the change in the treated substrate’s iodine color [53].

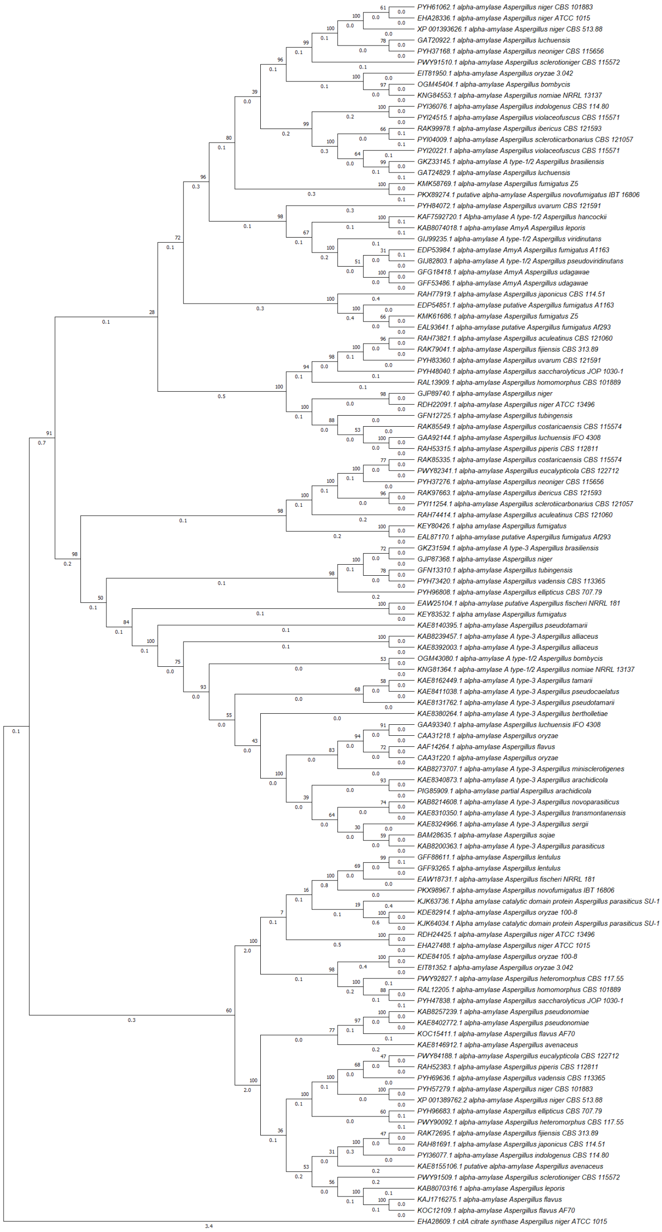

Fig. 1. Phylogenetic tree of α-amylase sequences of *Aspergillus* species under section *Nigri*, *Fumigati* and *Flavi*.

A bluish-black zone was observed on the starch plates indicates that starch is not being hydrolyzed [28]. This happens as amylose in the starch binds with iodine to produce a deep blue complex [54]. As amylose is being hydrolyzed and no longer able to form complexes with iodine to produce the characteristic deep blue color, halo zones around the fungi indicate the occurrence of starch hydrolysis by amylase.

Amylose constitutes less than 35% of the starch composition, which predominantly consists of long linear chains joined together by α-1,4 bonds [55]. All three fungi species produce amylases which act on the α-1,4 bonds such as endoamylase. Thus, formation of halo zones on the three plates after the addition of iodine indicates that *A. niger*, *A. fumigatus* and *A. flavus* are producing amylase. However, the exact identity of amylases produced by these three species could not be identified through this screening process. Therefore, further studies on isolating, purifying and molecular characterization of enzymes produced by these *Aspergillus* species would be beneficial.

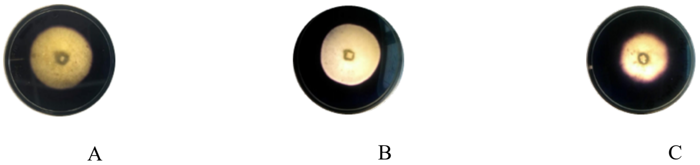

Fig. 2. Results of iodine staining for amylase activity of *A. niger* (A), *A. flavus* (B), and *A. fumigatus* (C) after 5 days of incubation on starch agar.

3.5 Amylase activity of selected *Aspergillus* species

The amylase activity of the selected species was studied with 9 replicates for each species. *A. flavus* showed the highest average EAI of 1.03 ± 0.049, followed by *A. niger* with EAI 0.77 ± 0.049 and finally *A. fumigatus* with 0.60 ± 0.103, as shown in Fig. 3. One Way ANOVA showed that the EAI differed significantly among the three selected species. This indicated that the ability to hydrolyze starch in all three species differs significantly.

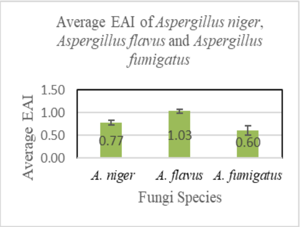

Fig. 3. Average EAI of *A. niger*, *A. flavus* and *A. fumigatus*.

Amylase activity of the selected species was also being reported and compared in several other studies. In the study by Fadahunsi and Garuba, they reported that the halo zone of *A. flavus* formed after staining was 13.0 ± 0.08 mm [56]. While the average *α*-amylase EAI of this species was found to be 1.21 ± 0.10 after culturing the fungus on starch agar for 3 days [57]. Although *A. flavus* had a higher average EAI in this study than the other two species, it cannot be stated that this species is a high amylase producer on solid medium, as some researchers indicated that an EAI of more than 2.5 is needed to classify a microorganism as a good producer of enzymes [58].

Khokhar et al. found an amylase activity index of 1.0 in *A. niger* cultured with 2% starch at 28°C for one week [59]. In contrast, Fifendy et al. isolated *A. niger* and cultured it on rice flour agar at room temperature for two days, resulting in enzymatic indexes of 1.25 and 0.6 for the duplicates, calculated using a different formula than what was used in this study [60]. Meanwhile, for *A. fumigatus*, Tuppad and Shishupala reported an EAI of 1.00 after four days on Czapek Dox agar with starch at 27 ± 2°C [61]. Additionally, Pathak and Sandhu determined an amylase activity index of 1.16 for *A. fumigatus* using the starch hydrolysis test [62].

In the study conducted by Saleem and Ebrahim, the halo zones generated by the selected species were examined in order to compare the amylase activity [63]. They observed that *A. niger* had the highest amylase activity (36 mm), followed by *A. flavus* (33 mm), and *A. fumigatus* (31 mm), which was in contrast to the findings of this study. Aydoğdu et al. investigated amylase activity in *A. flavus*, *A. fumigatus* and *A. niger* under varying pH conditions on Czapek-Dox agar with 2% soluble starch [64]. Regardless of the pH, *A. flavus* consistently displayed the highest amylase activity, followed by *A. fumigatus* and then *A. niger*, as evidenced by their respective degrading indices. Mäkelä et al. reported a similar number of genes responsible for starch degradation in all three species but found no correlation between gene count and polysaccharide degradation efficiency [65].

In a study by Mukunda et al., *A. niger* exhibited higher amylase activity compared to *A. fumigatus* [66]. They found that *A. niger* displayed greater starch hydrolysis in both preliminary and secondary screenings, producing 139 µgs/ml of extracellular proteins, while *A. fumigatus* produced 123 µgs/ml. *A. niger* also had a higher specific amylase activity (4.08 µmol/ml/Min) compared to *A. fumigatus* (3.19 µmol/ml/Min).

Adeniran et al. reported that *A. flavus* showed higher ß-amylase production than *A. fumigatus* when using various agricultural wastes as substrates [67]. *A. flavus* demonstrated superior ß-amylase activity in both static and solid-state cultivation methods. Additionally, Omemu et al. observed a larger hydrolysis zone for *A. flavus* (16.5±5.9 mm) compared to *A. fumigatus* (6.0±1.4 mm) when cultured on starch agar [68].

In this study, similar to Al-Hindi et al., *A. flavus* exhibited higher amylase production than *A. niger*, particularly when fruit peel and PDB were used as substrates [69]. Conversely, Ugoh and Ijigbade reported greater amylase activity in *A. niger* (31.00 ± 4.67 mm halo zone) compared to *A. flavus* (28.67 ± 2.44 mm) [29]. Morya and Yadav found variable amylase production among different isolated strains of *A. flavus*, with three of the ten strains surpassing *A. niger*, reaching 213.2 IU/mL [70]. This suggests that amylase production by fungi may be linked to species origin and strain variability. A comprehensive amylolytic system will determine the amylase activity of a microorganism, where the system contains the entire amylolytic enzymes including, but not limited to, α-amylase, β-amylase, γ-amylase, pullulanase and isoamylase. Amylase activity is also determined by the amino acid residues found within the active site, for example in the case described by Huma et al. [71].

The EAI method employed in our study is a semi-quantitative approach for rapid assessment of microbial enzymatic activity on solid media [58]. However, the observed halo zone size does not necessarily correspond to the actual enzyme quantity produced, as reported in a study by Singh et al [59], wherein they conducted primary screening of fungi for amylase production and found that the S4 strain exhibited a larger hydrolysis zone (3.6 cm) than S2 (2.6 cm) [72]. Nevertheless, in secondary screening via solid-state fermentation, the S2 strain displayed higher amylase activity (143.7 U/mL) compared to S4 strain (113.4 U/mL), that agrees with Khokhar et al. [59]. Therefore, it is essential to employ procedures that directly assess amylase activity.

4. Conclusion

All selected species from the three *Aspergillus* sections demonstrated the ability to produce α-amylase, and similarities were observed in the protein sequence of this enzyme among the species. These similarities were observed via the presence of α-amylase domains and the branching pattern of the phylogenetic tree, indicating a common ancestor. Moreover, the study revealed variations in amylase activity among the three selected species, with *A. flavus* displaying the EAI among the selected species. However, for a more comprehensive understanding, a detailed analysis of α-amylase sequences and enzyme activity, including the quantification of reducing sugar using methods such as the DNS assay, is essential.

Acknowledgements

This work was funded by UNIMAS Tun Openg Chair Research Grant, UNI/F07/TOC/84775/2022. We would like to thank UNIMAS for the use of laboratory facilities and sampling locations to undertake the project.

References

[1] Heckmann CM, Paradisi F. Looking back: A short history of the discovery of enzymes and how they became powerful chemical tools. Chem Cat Chem. 2020;12(24):6082-102.

[2] Bhatia S. Introduction to enzymes and their applications. In: Bhatia S, editor. Introduction to pharmaceutical biotechnology, Volume 2. IOP Publishing; 2018:1-29.

[3] May O. Industrial enzyme applications - Overview and historic perspective. In: Vogel A, May O, editors. Industrial enzyme applications. Chichester, UK: John Wiley & Sons, Ltd; 2019. p. 1-24.

[4] Ivaldo Itabaiana Jr, Rodrigo O. M. A. De Souza. Biocatalysts at room temperature. In: Luque R, Lam FYL, editors. Sustainable catalysis: Energy-efficient reactions and applications. Wiley; 2018. p. 89-134.

[5] Rebello S, Aneesh EM, Sindhu R, et al. Enzyme catalysis: A workforce to productivity of textile industry. In: Saran S, Babu V, Chaubey A, editors. High value fermentation products. Wiley; 2019. p. 49-65.

[6] Balakrishnan D, Kumar SS, Sugathan S. Amylases for food applications—Updated information. In: Parameswaran B, Varjani S, Raveendran S, editors. Energy, environment, and sustainability green bio-processes. Springer Singapore; 2018. p. 199-227.

[7] Sharma A, Gupta G, Ahmad T, et al. Enzyme engineering: Current trends and future perspectives. Food Reviews International. 2021; 37(2): 121-54.

[8] Sidar A, Albuquerque ED, Voshol GP, et al. Carbohydrate binding modules: Diversity of domain architecture in amylases and cellulases from filamentous microorganisms. Frontiers in Bioengineering and Biotechnology. 2020; 8:1-17.

[9] Martinovičová M, Janeček Š. In silico analysis of the α-amylase family GH57: Eventual subfamilies reflecting enzyme specificities. 3 Biotech. 2018; 8: 307.

[10] Janecek S. Sequence similarities and evolutionary relationships of microbial, plant and animal alpha-amylases. European Journal of Biochemistry. 1994; 224(2): 519-24.

[11] Avwioroko OJ, Anigboro AA, Unachukwu NN, et al. Isolation, identification and in silico analysis of alpha-amylase gene of Aspergillus niger strain CSA35 obtained from cassava undergoing spoilage. Biochemistry and Biophysics Reports. 2018; 14: 35-42.

[12] Janíčková Z, Janeček Š. Fungal α-amylases from three GH13 subfamilies: their sequence-structural features and evolutionary relationships. International Journal of Biological Macromolecules. 2020; 159: 763-72.

[13] Liu X, Kokare C. microbial enzymes of use in industry. In: Brahmacharin

G, editor. Biotechnology of microbial enzymes. Elsevier; 2017. p. 267-98.

[14] Arastehfar A, Carvalho A, Houbraken J, et al. Aspergillus fumigatus and aspergillosis: From basics to clinics. Studies in Mycology. 2021; 100(1): 100115.

[15] Samson RA, Visagie CM, Houbraken J, et al. Phylogeny, identification and nomenclature of the genus Aspergillus. Studies in Mycology. 2014; 78(1): 141-73.

[16] Vesth TC, Nybo JL, Theobald S, et al. Investigation of inter- and intraspecies variation through genome sequencing of Aspergillus section Nigri. Nature Genetics. 2018; 50(12): 1688-95.

[17] Frisvad JC, Hubka V, Ezekiel CN, et al. Taxonomy of Aspergillus section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins. Studies in Mycology. 2019; 93(1): 1-63.

[18] Talbot JJ, Barrs VR. One-health pathogens in the Aspergillus viridinutans complex. Medical Mycology. 2018; 56(1): 1-12.

[19] Varga J, Frisvad JC, Kocsubé S, et al. New and revisited species in Aspergillus section Nigri. Studies in Mycology. 2011; 69: 1-17.

[20] Frisvad JC, Larsen TO. Extrolites of Aspergillus fumigatus and other pathogenic species in Aspergillus section Fumigati. Frontiers in Microbiology. 2016; 6: 1-14.

[21] Letunic I, Bork P. 20 years of the SMART protein domain annotation resource. Nucleic Acids Research. 2018; 46: D493-6.

[22] Nielsen H. Predicting secretory proteins with SignalP. In: Kihara D, editor. Protein function prediction. New York: Humana New York; 2017. p. 59-73.

[23] Thumuluri V, Almagro Armenteros JJ, Johansen AR, et al. DeepLoc 2.0: Multi-label subcellular localization prediction using protein language models. Nucleic Acids Research. 2022; 50: W228-34.

[24] Nuanpirom J, Sangket U. Improving identification of animal secretory proteins. In: 7th International Conference on Biochemistry and Molecular Biology. 2021.

[25] Madeira F, Pearce M, Tivey ARN, et al. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Research. 2022; 50: W276-9.

[26] Barton GJ. Sequence alignment for molecular replacement. Acta Crystallographica Section D Biological Crystallography. 2008; 64(1): 25-32.

[27] Kumar S, Stecher G, Li M, et al. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution. 2018; 35(6): 1547-9.

[28] Gopinath SCB, Anbu P, Arshad MKM, et al. Biotechnological processes in microbial amylase production. BioMed Research International. 2017; 2017(1): 1-9.

[29] Ugoh SC, Ijigbade B. Production and characterisation of amylase by fungi isolated from soil samples at Gwagwalada, FCT, Abuja - Nigeria. Report and Opinion. 2013;5(7):44-53.

[30] Das S, Samanta P, Uday USP. Isolation and characterisation of amylase producing fungal strain. Journal of Environment and Sociobiology. 2020; 17(2): 1-7.

[31] Goldbeck R, Andradel CCP, Pereira GAG, et al. Screening and identification of cellulase producing yeast-like microorganisms from Brazilian biomes. African Journal of Biotechnology. 2012;11(53):11595-603.

[32] Janecek S, Svensson B, Henrissat B. Domain evolution in the *α*-amylase family. Journal of Molecular Evolution. 1997; 45(3): 322-31.

[33] Wang Y, Zhang H, Zhong H, et al. Protein domain identification methods and online resources. Computational and Structural Biotechnology Journal. 2021; 19: 1145-53.

[34] Kumari B, Kumar R, Kumar M. Low complexity and disordered regions of proteins have different structural and amino acid preferences. Molecular Biosystems. 2015; 11(2): 585-94.

[35] Mier P, Andrade-Navarro MA. Assessing the low complexity of protein sequences via the low complexity triangle. PLoS One. 2020; 15: e0239154.

[36] Paul JS, Gupta N, Beliya E, et al. Aspects and recent trends in microbial α-amylase: A review. Applied Biochemistry and Biotechnology. 2021; 193(8): 2649-98.

[37] Da Lage J-L. An optional C-terminal domain is ancestral in α-amylases of bilaterian animals. Amylase. 2017; 1: 26-34.

[38] Ren Z, Ren PX, Balusu R, et al. Transmembrane helices tilt, bend, slide, torque, and unwind between functional states of rhodopsin. Scientific Reports. 2016;6(1):34129.

[39] Guna A, Hegde RS. Transmembrane domain recognition during membrane protein biogenesis and quality control. Current Biology. 2018;28:R498-R511.

[40] Singh S, Mittal A. Transmembrane domain lengths serve as signatures of organismal complexity and viral transport mechanisms. Scientific Reports 2016;6(1):22352.

[41] Teufel F, Almagro Armenteros JJ, Johansen AR, et al. SignalP 6.0 predicts all five types of signal peptides using protein language models. Nature Biotechnology. 2022;40(7):1023-5.

[42] Grasso S, Dabene V, Hendriks MMWB, et al. Signal peptide efficiency: From high-throughput data to prediction and explanation. ACS Synthetic Biology. 2023;12(2):390-404.

[43] Pohlschroder M, Pfeiffer F, Schulze S, et al. Archaeal cell surface biogenesis. FEMS Microbiology Reviews. 2018; 42(5): 694-717.

[44] Yao D, Su L, Li N, et al. Enhanced extracellular expression of Bacillus stearothermophilus α-amylase in Bacillus subtilis through signal peptide optimization, chaperone overexpression and α-amylase mutant selection. Microbial Cell Factories. 2019; 18(1): 69.

[45] Rédei GP. Encyclopedia of genetics, genomics, proteomics and

informatics. Dordrecht: Springer Netherlands; 2008.

[46] Dalio RJD, Herlihy J, Oliveira TS, et al. Effector biology in focus: A primer for computational prediction and functional characterization. Molecular Plant-Microbe Interactions. 2018; 31(1):22-33.

[47] Almagro Armenteros JJ, Sønderby CK, Sønderby SK, et al. DeepLoc: Prediction of protein subcellular localization using deep learning. Bioinformatics. 2017;33(21):3387-95.

[48] Hallgren J, Tsirigos KD, Pedersen MD, et al. DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural networks. bioRxiv 2022; 2022.04.08.487609.

[49] Gautier M, Normand A-C, Ranque S. Previously unknown species of Aspergillus. Clinical Microbiology and Infection. 2016; 22(8): 662-9.

[50] Chang PK, Ehrlich K, Fujii I. Cyclopiazonic acid biosynthesis of Aspergillus flavus and Aspergillus oryzae. Toxins (Basel). 2009;1:74-99.

[51] Gibbons JG, Salichos L, Slot JC, et al. The evolutionary imprint of domestication on genome variation and function of the filamentous fungus Aspergillus oryzae. Current Biology. 2012; 22(15): 1403-9.

[52] Campbell CK, Johnson EM, Warnock DW. Identification of pathogenic fungi. Wiley; 2013.

[53] Gupta R, Gigras P, Mohapatra H, et al. Microbial α-amylases: A biotechnological perspective. Process Biochemistry. 2003; 38: 1599-616.

[54] Brust H, Orzechowski S, Fettke J. Starch and glycogen analyses: Methods and techniques. Biomolecules. 2020; 10: 1020.

[55] Seung D. Amylose in starch: Towards an understanding of biosynthesis, structure and function. New Phytologist. 2020; 228: 1490-504.

[56] Fadahunsi IF, Garuba OE. Amylase production by Aspergillus flavus associated with the bio-deterioration of starch-based fermented foods. New York Science Journal. 2012;(1):13-8.

[57] Mythii A, Singh YRB, Priya R, et al. In vitro and comparative study on the extracellular enzyme activity of molds isolated from keratomycosis and soil. International Journal of Ophthalmology. 2014; 7(5): 778-84.

[58] El-Latif Hesham A, Saleh F, Al-Bedak O, et al. Molecular identification of naturally isolated Candida tropicalis AUN-H100 and optimization of their extracellular amylase production. International Journal of Agricultural Sciences and Veterinary Medicine. 2019; 7(4): 28-34.

[59] Khokhar I, Mukhtar I, Mushtaq S. Comparative studies on the amylase and cellulase production of Aspergillus and Penicillium. Journal of Applied Sciences and Environmental Management. 2011; 15: 657-61.

[60] Fifendy M, Indriati G, Osnita S, et al. Isolation and activity of amylase enzyme in isolates of fungi from black rice lemang (Oryza sativa Siarang). Advances in Biological Sciences Research. 2020; 10:46-50.

[61] Tuppad DarshanS, Shishupala S. Endophytic mycobiota of medicinal plant Butea monosperma. International Journal of Current Microbiology and Applied Sciences. 2013; 2(12): 615-27.

[62] Pathak SS, Sandhu SS. Screening of glucoamylase producing fungi from the soils of Jabalpur region. International Journal of Recent Scientific Research. 2019;10:30470-6.

[63] Saleem A, Ebrahim MKH. Production of amylase by fungi isolated from legume seeds collected in Almadinah Almunawwarah, Saudi Arabia. Journal of Taibah University for Science. 2014; 8: 90-7.

[64] Aydoğdu H, Balkan B, Balkan S, et al. Amylolytic activities of fungi species on the screening medium adjusted to different pH. Erzincan University Journal of Science and Technology. 2012; 5: 1-12.

[65] Mäkelä MR, DiFalco M, McDonnell E, et al. Genomic and exoproteomic diversity in plant biomass degradation approaches among Aspergilli. Studies in Mycology. 2018; 91: 79-99.

[66] Mukunda S, Onkarappa R, Prashith Kekuda TR. Isolation and screening of industrially important fungi from the soils of Western Ghats of Agumbe and Koppa, Karnataka, India. Science, Technology and Arts Research Journal. 2012; 1(4): 27-32.

[67] Adeniran HA, Abiose SH, Ogunsua AO. production of fungal β-amylase and amyloglucosidase on some Nigerian agricultural residues. Food and Bioprocess Technology. 2010; 3(5): 693-8.

[68] Omemu A, Bamigbade G, Obadina A, et al. Isolation and screening of amylase from moulds associated with the spoilage of some fermented cereal foods. British Microbiology Research Journal. 2015; 5(4): 359-67.

[69] Al-Hindi RR, Al-Najada AR, Mohamed SA. Isolation and identification of some fruit spoilage fungi: Screening of plant cell wall degrading enzymes. African Journal of Microbiology Research. 2011; 5(4): 443-8.

[70] Morya V, Yadav D. Isolation and screening of different isolates of Aspergillus for amylases production. The Internet Journal of Microbiology. 2008; 7(1): 1-8.

[71] Huma T, Mustafa G, Sethi A, et al. Computational approach to study role of active site amino acids of alpha amylases in thermostability of Pyrococcus furiosus and Bacillus sp. Biochemistry & Molecular Biology Journal. 2021; 7(10): 1-9.

[72] Singh S, Singh S, Bali V, et al. Production of fungal amylases using cheap, readily available agriresidues, for potential application in textile industry. Biomed Research International. 2014; 2014: 1-9.