CHAPTER IV

RESULTS AND DICUSSION

The amylase from endophytic fungi was analyzed using all methods as described in the previous chapter. The results will be shown and discussed in each part of this chapter, respectively.

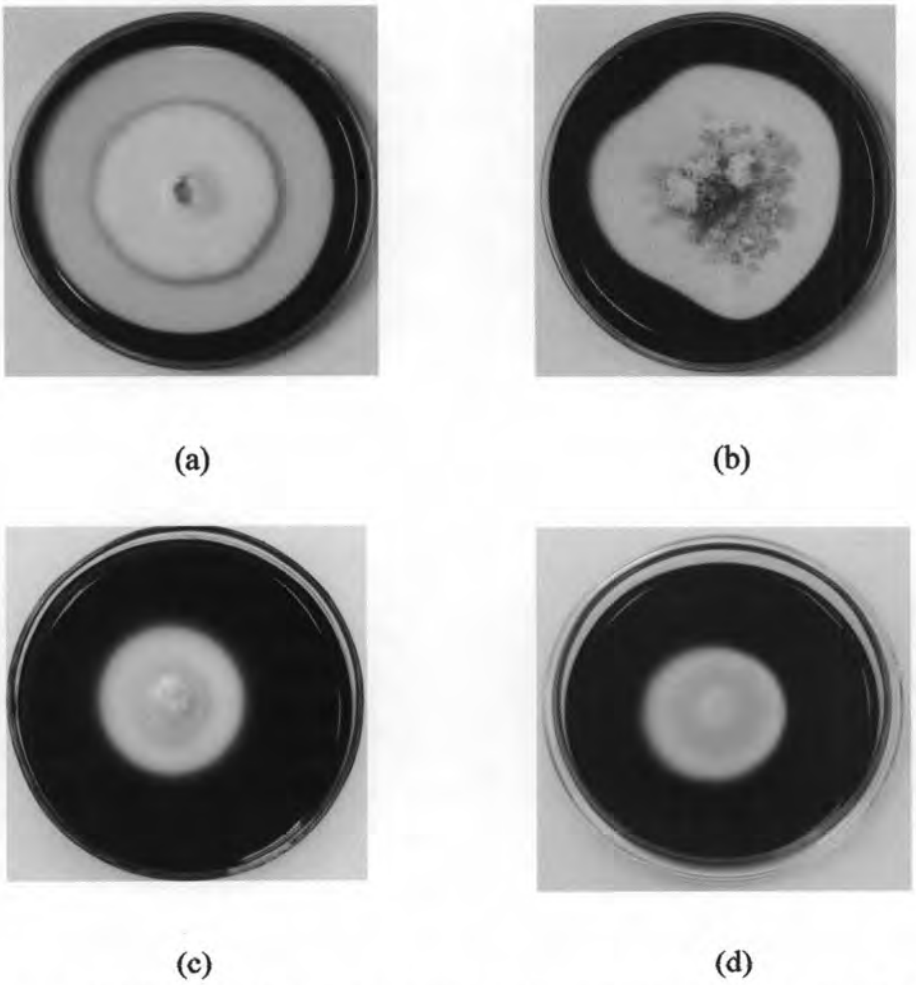
4.1 Screening of Endophytic Fungi for Amylase Activity

Twenty five cultures of endophytic fungi were obtained from Research Centre of Bioorganic Chemistry (RCBC) Chulalongkorn University, Bangkok Thailand. The fungi were tested for amylase activity. Only seven strains gave amylase activity (Table 4.1).

Table 4.1 Amylase activity from 25 isolated of endophytic fungi using soluble starch as substrate.

Isolated strain No.	Diameter (mm)	Isolated strain No.	Diameter (mm)
EF1	3-4	EF14	-
EF2	3-4	EF15	3-4
EF3	-	EF16	7-8
EF4	-	EF17	-
EF5	-	EF18	-
EF6	13-16	EF19	-
EF7	-	EF20	-
EF8	-	EF21	-
EF9	-	EF22	4-7
EF10	-	EF23	-
EF11	-	EF24	-
EF12	13-18	EF25	-
EF13	-		

From Table 4.1, twenty five isolated strains of endophytic fungi were investigated the hydrolyzed starch ability after 8 days of growth on starch agar. When the media flooded with iodine solution, clear zone was observed around the growing colony of the fungi. Petri dish of EF1, EF2, EF15, EF16 and EF22 appeared clear zone, which diameter 3-7 mm. Moreover, isolated EF6 and EF12 appeared clear zone which diameter 13-16 mm and 13-18 mm (Figure 4.1). The clear zone suggested that seven isolated can produce hydrolyzing enzyme to digest soluble starch on starch agar. Isolated EF6 and EF12 showed highest clear zone when compare with other positive strains and is used for further study.



(a) (b)

(c) (d)

Figure 4.1 Colony characteristic of isolates endophytic fungi; (a) EF6 strain, (b) EF12 strain, (c) EF16 strain and (d) EF22 strain.

EF6 and EF12 were inoculated in starch broth. The growth rate was measured by amount of dried weight. The dried weight was determined after drying at 50°C to a constant weight overnight. EF6 have a higher growth rate than EF 12 (Figure 4.2). Consequently EF6 was chosen for further investigation.

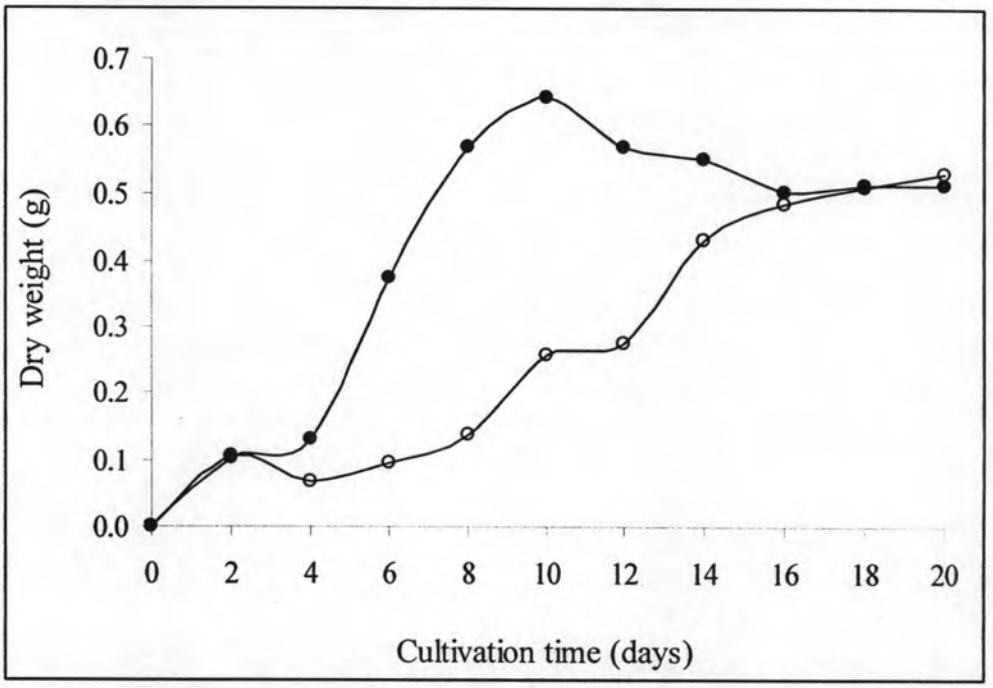
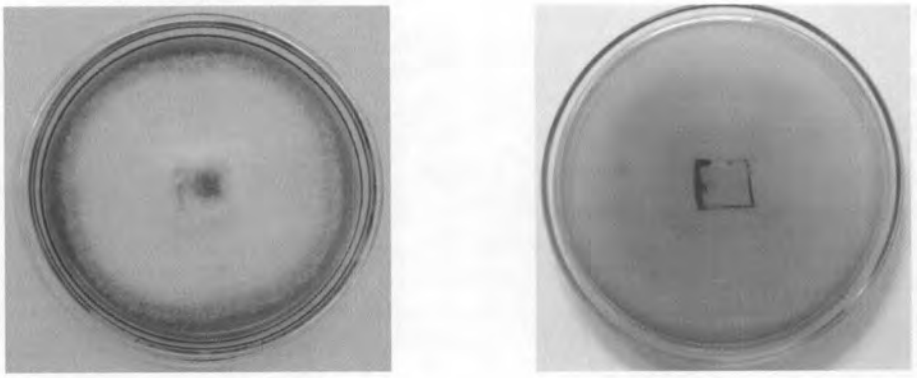


Figure 4.2 Growth curve of endophytic fungi; ● EF6 strain, ○ EF12 strain

4.2 Morphological Characteristic

Endophytic fungi EF6 were isolated from *Sandoricum koetjape* leaves. Isolated EF6 was grown on Potato Dextrose Agar (PDA) at room temperature for 7 day. The fungi mycelium was covered media in Petri dish within 1 week. The characteristic of fungal colony is white mycelium, fluffy and smooth margin. The mycelial color was changed to light yellow after one month old. (Figure 4.3)



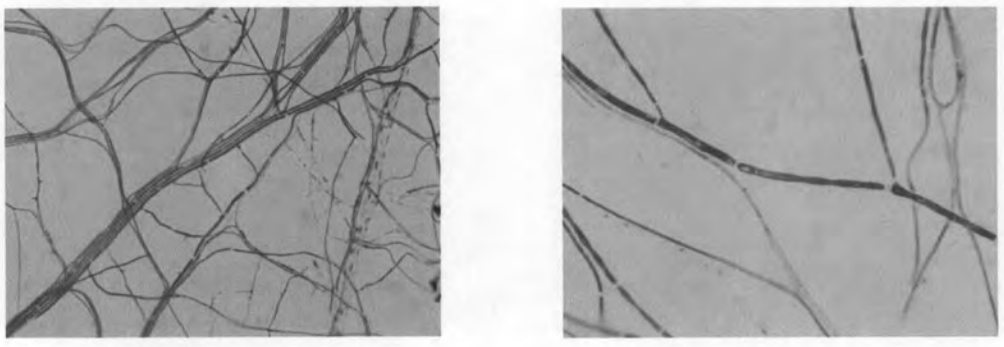
(a) (b)

Figure 4.3 Characteristic of endophytic fungus isolate EF6 grown on PDA for 9 days at room temperature. Top view (a), bottom view (b)

4.3 Identification Endophytic Fungi isolated EF6

4.3.1 Morphological identification

The mycelium of endophytic fungus isolate EF6 was prepared by slide culture technique and observed under light microscope. Isolate EF6 is mycelia sterile because it showed septate hyphae with non spore formation (Figure 4.4) were then identified by molecular technique



(a) (b)

Figure 4.4 Microscopic characteristic of endophytic fungus isolate EF6 was observed under light microscope. magnification x40 (a), magnification x100 (b)

4.3.2 Molecular identification

The ITS sequence of endophytic fungus isolate EF6 was 586 bp., containing a part of the 18S, ITS_1, 5.8 and 28 rDNA, as shown in Figure 4.5. This sequence was compared with reported ITS sequences in the gene bank. The result revealed that ITS region of this fungus had highest sequence similarity of 96% with fungal endophyte sp. J48 from coffee leaves (Accession No. AY601896.1, Score=743 bits), 91% with *Xylaria arbuscula* (Accession No. AY183369.1, Score=708 bits, Accession No.AF5163029, Score=640 bits, Accession No.AF163028, Score=615 bits), 91% with *Xylaria hypoxylon* (Accession No. DQ491487.1, Score=682 bits), 92% with *Xylaria hypoxylon* (Accession No. AF194027, Score=626 bits) and of 91% with *Xylaria mali* (Accession No. AF163040, Score=668 bits).

AAGTCGTAAC AAGGTCTCCG TTGGTGAACC AGCGGAGGGA
TCATTAAAGA GTTTTCGTAA CTCCCAAACC CATGTGAACT
TACCTTCTGT TGCCTCGGCA GGTCGTGTCT ACCCTGTGGT
CGCCTACCCT GTAGGCGACT ACCTGGTAGT CACGGGTTCG
CCTGCCAGTG GCCCGTTAAA ACACTGTTTA TTATATGTTA
TTCTGATTTT ACAACTAAAC AAGTTAAAAC TTTCAACAAC
GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCGAAA
TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC
GAATCTTTGA ACGCACATTG CGCCCATTAG TATTCTAGTG
GGCATGCCTG TTCGAGCGTC ATTTCAACCC TTAAGCCCCT
GTTGCTTAGT GTTGGGAGCC TACAGTCCTC TGTAGCTCCC
TAAATGTAGT GGCGGAGTCG GTTCGCACTC TAGACGTAGT
AGCTTATATC TCGTCAGTGG TATAGGCACG GTCTCTTCCC
GTAAAACCCC CTAATTTTTC AAGGTTGCCC TCGGATCATG
GTAGGAATGC CACGCTCAAA TATAAT

Figure 4.5 Nucleotide sequences of partial 18S region, complete ITS region of endophytic fungus isolate EF6 AB274832, containing a partial of the 18S, ITS1, 5.8S and 28S rDNA.

4.4 Growth measurement of fungal isolated EF6

The growth rate measurement of fungal isolate EF6 and starch hydrolyze enzyme production were performed at room temperature in starch broth as shown in Figure 4.6. By measurement of dry weight, the growth rate EF6 was rapidly during 4-10 days. The maximum growth of EF6 was reached after 10 days of incubation, after that EF 6 was in the stationary phase. The amylase activity was measured every 2 days. The result shown that EF6 can digest starch soluble in broth and then secreted hydrolyze enzyme out of the cell. By log phase during 2-10 days, enzyme activities are increased and the maximum amylase activity was produced on day 8th of incubation which was determined by measurement of the amount of reducing sugar which released from the reaction of amylase. Suitable pH for growth rate and enzyme production was between 5.0 and 6.0 from Figure 4.7, which was measured on 8th.

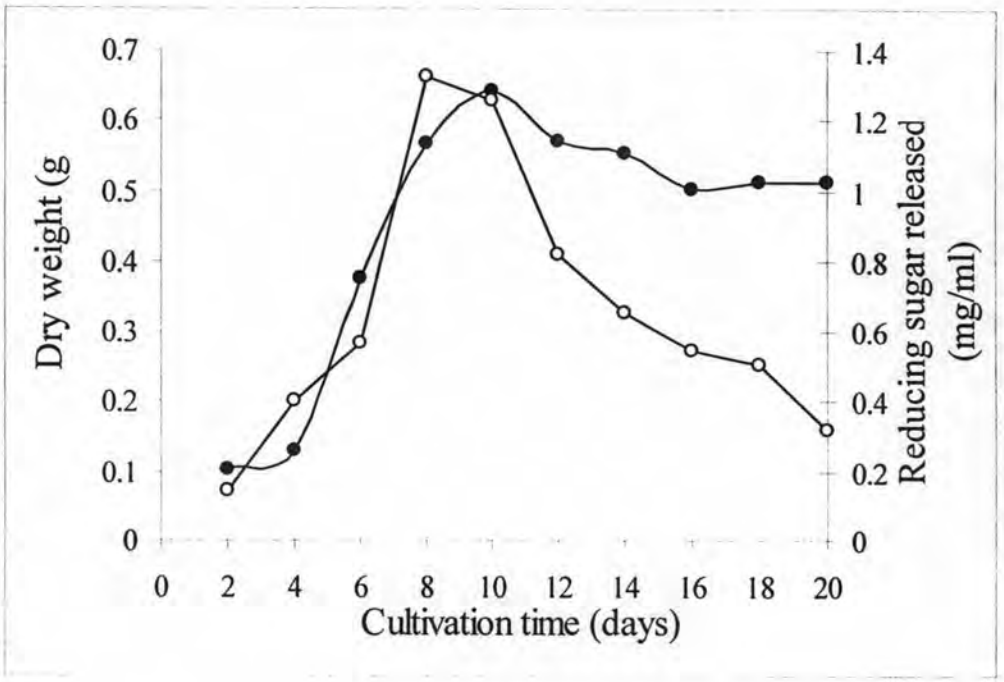


Figure 4.6 Dried weight and amylase activity of crude extract produced by EF6 grown on starch broth. ● Dry weight of mycelia (gram) ; ○ amylase activity

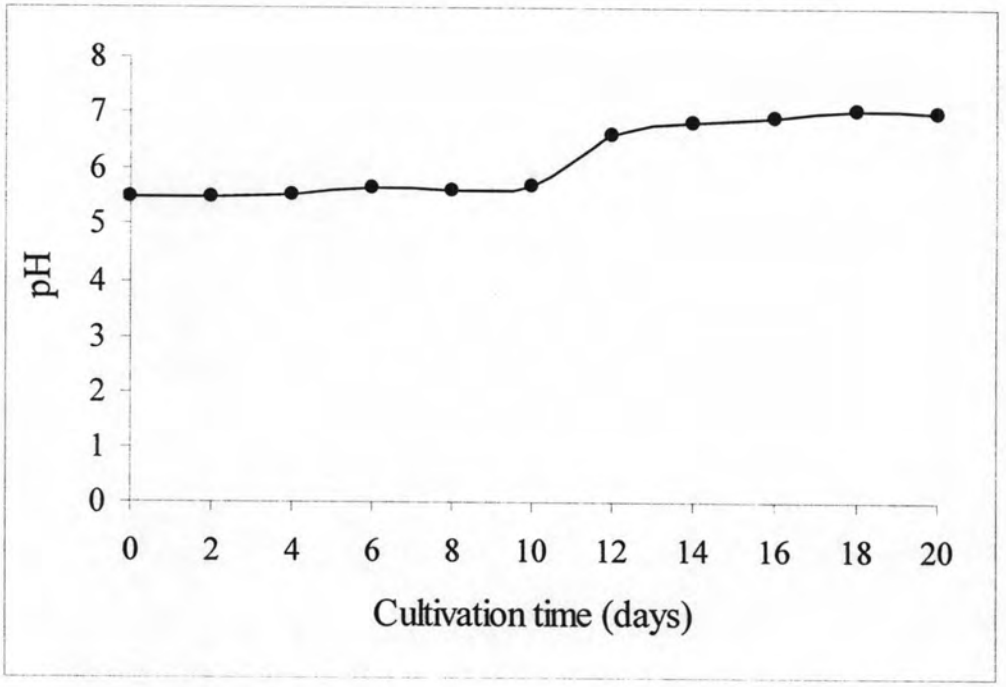


Figure 4.7 pH value of crude extracted from EF6 cultivation

4.5 Protein precipitation with ammonium sulphate

The ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of protein purification. To determine the suitable $(NH_4)_2SO_4$ concentration for precipitation of protein experiment was performed by a stepwise increase at 30% increment from 0-90%. 500 ml of filtrate from broth was used to perform protein precipitation. The results were shown in Table 4.2.

Table 4.2 The amount of protein from precipitate fraction of crude protein

Crude Protein	Amount of protein (mg)
30%	0.24
60%	5.2
90%	19.8

From the result indicated that 90% $(NH_4)_2SO_4$ is yield the highest amount of protein. All fractionation of precipitation protein were performed the amylase activity. The result show as Table 4.3

Table 4.3 Activity of crude protein

Crude Protein	Amylase activity
30%	+
60%	++
90%	+++

Culture filtrated was precipitate with 30% and 60 % $(NH_4)_2SO_4$ were presented the amylase activity less than protein precipitated at 90% $(NH_4)_2SO_4$. Therefore, 90% $(NH_4)_2SO_4$ was chosen for protein precipitation because this fraction showed high amount of proteins and amylase activity.

4.6 Purification of enzyme

The extracellular amylase was purified from crude enzyme obtained from culture filtrate of EF6 after precipitated protein by 90% $(NH_4)_2SO_4$ and dialyzed with distilled water. The amylase was separated by Ion Exchange Chromatography using Q Sepharose column. The chromatogram was shown in Figure 4.8. From the result, there were divided into two parts were bound protein and unbound protein. In the part of bound protein performed two peaks were called A1 and A2. Unbound protein shown two peaks were called A3 and A4. Every fraction was subjected for amylase activity test, only fractions at peak A1 exhibited amylase activity. Active fractions were pooled, dialyzed and concentrated by vacuum freeze dries.

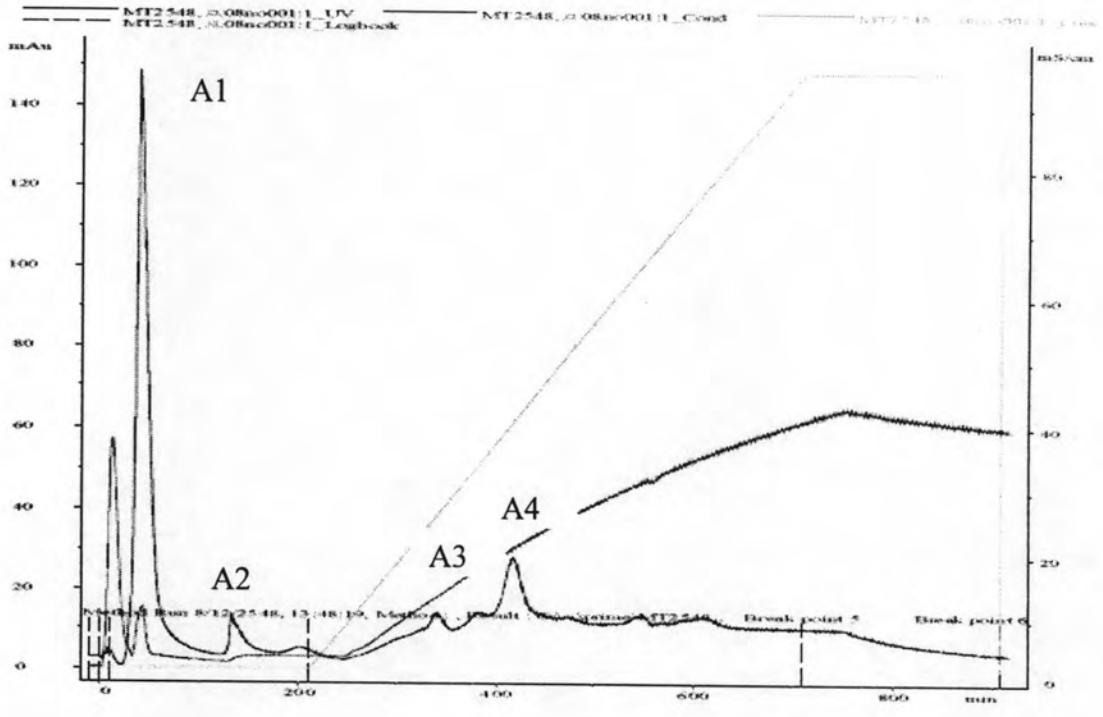


Figure 4.8 Ion Exchange Chromatography of crude protein from EF6 on the Q Sepharose column (1.6x10 cm) equilibrated with 0.02 M piperazine buffer pH 5.5 at a flow rate 1 ml/ min. The bound proteins were eluted with a NaCl salt gradient (0-0.5M) in 0.02 M piperazine buffer pH 5.5.

The fraction A1 was analyzed for purity and protein pattern by SDS-PAGE to compare between original crude protein and purified protein the result show in Figure 4.9

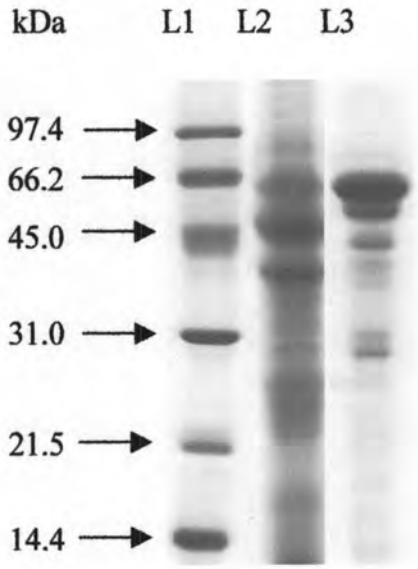


Figure 4.9 Electrophoresis was carried out on 15% SDS-PAGE; L1: standard protein marker, L2: crude protein, L3: A1 from Q sepharose.

From the Figure 4.8, found that the gel from SDS-PAGE when stain with Coomasie Blue, crude enzyme appeared multiple band of protein. Active fraction from Q Sepharose had a less band than crude enzyme. Fraction A1 still being considered impure protein.

The fraction containing amylase activity (fraction 4-6) from Q Sepharose column which impure was concentrated subject for further purification by using gel filtration chromatography. The crude enzyme was applied on to Superdex 75 that separate protein by size between 10^4 and 10^5 Da and eluted with 0.02 M piperazine buffer pH 5.5. The chromatogram was presented in Figure 4.10

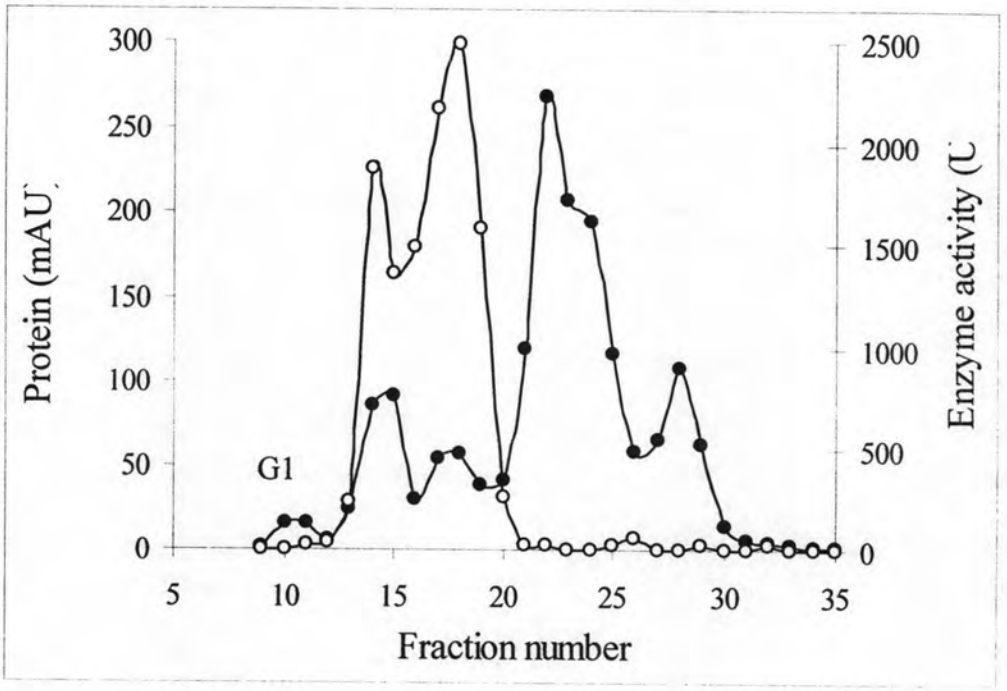


Figure 4.10 Gel filtration of crude proteins on the superdex 75 column (1.6x60 cm) equilibrated and eluted 0.02M piperazine buffer pH5.5, flow rate of 0.5 ml/min. ● absorbance at 280 nm ; ○ Enzyme activity

This chromatogram showed 5 peaks of protein G1, G2, G3, G4 and G5. Every fraction was subjected for amylase activity test, only G2 and G3 showed the activity. G2 and G3 were analyzed for purity and protein pattern by SDS-PAGE. The results show in Figure 4.11

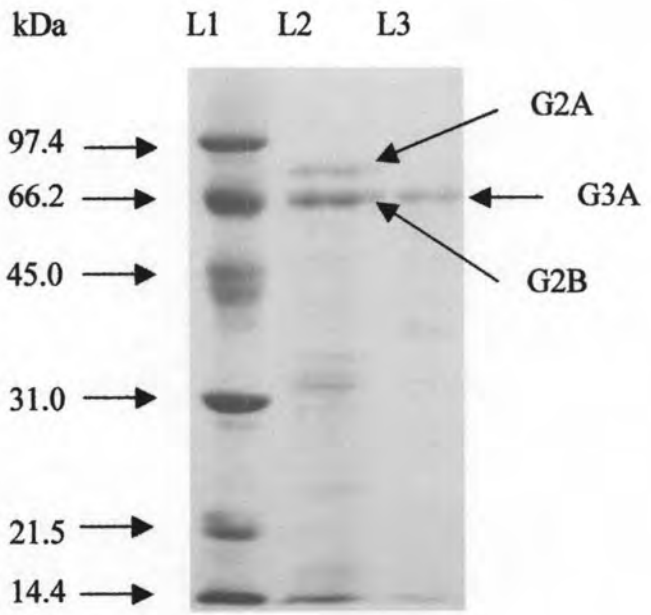


Figure 4.11 Electrophoresis was carried out on 15% SDS-PAGE; L1: standard protein marker, L2: fraction G2 from Superdex 75, L3: fraction G3 from Superdex75.

Figure 4.10 showed that G2 have two bands G2A and G2B. G2A showed molecular weight about 81 kDa and G2B showed molecular weight about 66 kDa. Fraction G3 shows a single band called G3A which also showed molecular weight about 66 kDa.

To confirm amylase activity, the Native-PAGE under non-denaturing condition was used. And then, this gel was incubated in 1% soluble starch for 30 min. After that, the gel was flooded with iodine solution, the result showed in Figure 4.12. From the result, Clear zone appeared around G2B and G3A. G2B and G3A showed the similar of molecular weight and amylase activity which obtained by SDS-PAGE and Native-PAGE. Therefore, both proteins may be the same amylase. The majority of proteins that have amylase activity were eluted in fraction G2 and the minority of amylases was eluted in fraction G3. The purified of G3 was used for next study.

Figure 4.12 Electropharesis was carried out on 15% Native-PAGE; L1: fraction G2 from Superdex 75, L2: fraction G3 from Superdex75.

The result of the purification procedure from 5 liters of culture filtrated were summarized in Table 4.4. One unit of enzyme activity was defined as the amount of enzyme that released 1μmol reducing sugar per minute (U). The specific activity of the amylase was express in term of units per mg protein.

Table 4.4 Summary of the protein purification step for amylase of fungal isolated EF6

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold of purification	Recovery (%)
Crude enzyme	519	27157	52.02	1.0	100
$(NH_4)_2SO_4$	302	22593	74.77	1.44	83.16
Q Sepharose	23	7808	354.91	6.82	28.75
Superdex 75	3.2	2486	776.8	14.93	10.93

4.7 Enzyme Activity

The optimum condition is specific for enzyme activity. For this study is to find optimum temperature, pH substrate and metal ion which were suitable for EF6 amylase activity.

4.7.1 Optimum temperature

Enzyme activity and stability were depended on specific optimum temperature. The optimum temperature of the enzyme was investigated by incubating the reaction mixture at various temperatures as described in section 3.10.4. Purified amylase is the most active in the range from 50-60°C were presented in Figure 4.13. After 30 minutes for incubation, amylase was found to be stable lower than 50°C. Residual activity was retained about 43% at 60°C. The activity decrease rapidly when the enzyme was incubated at temperature higher than 55°C, and the amylase lost most of its activity after 30 min.

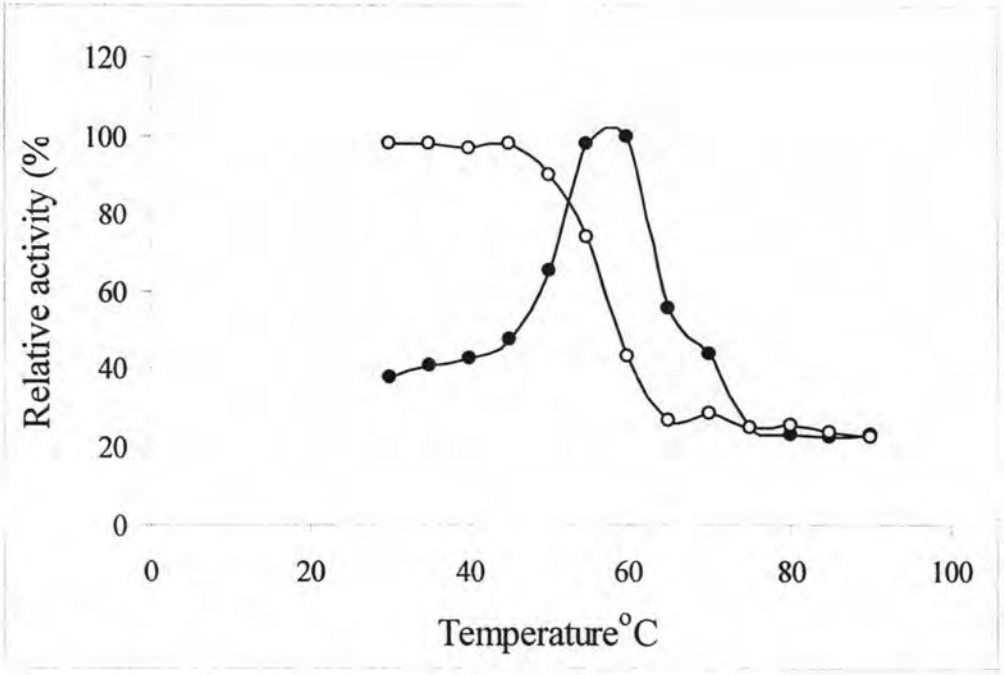


Figure **4.13** Optimum temperature and stability of amylase from EF6. ● optimum temperature ; ○ temperature stability

4.7.2 Optimum pH

The optimum pH of the enzyme was determined as mentioned in section 3.10.3. In this study the 0.02 M sodium acetate pH 3-6, 0.02 M phosphate pH 5-7 and 0.02 M Tris-HCl pH 7-9 were used for as reaction buffer. Activities at the different pH were showed in Figure 4.14. From result, the optimum pH is 6 which give the highest amylase enzyme activity.

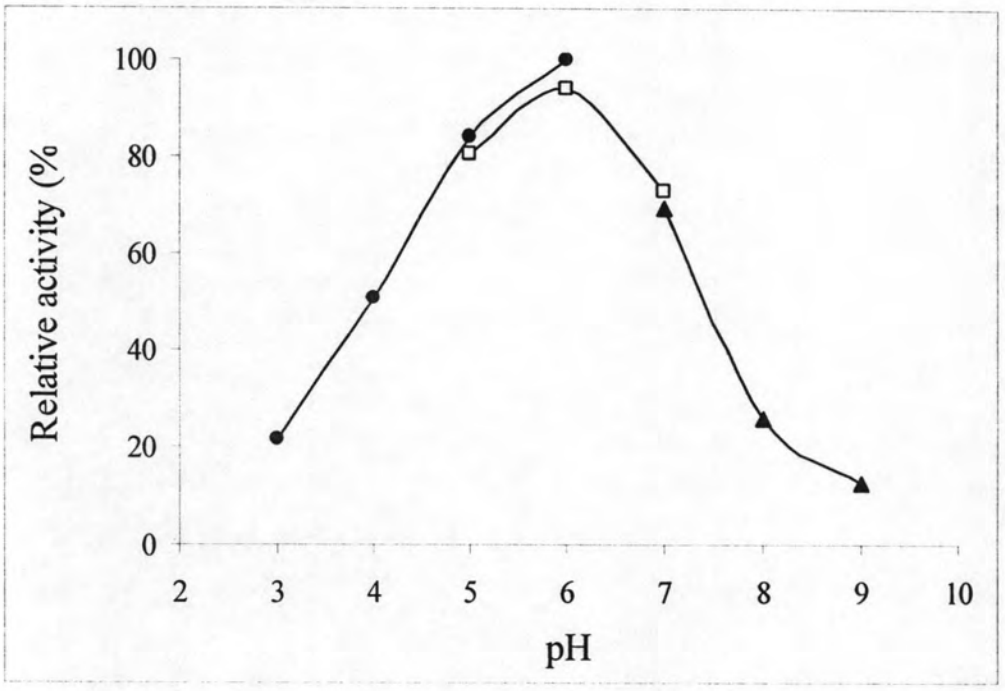


Figure 4.14 Optimum pH on the activity of enzyme. The following buffer system were used 0.02 M sodium acetate pH 3-6 ● ; 0.02 M phosphate pH 5-7 □ and 0.02 M Tris-HCl pH 7-9 ▲

4.7.3 Effect of metal ion

Amylase are usually inhibited or activated by ions. Therefore, the amylase was examined with various metal ions. The method was described in section 3.10.5. The results summarized in Table 4.5. The result suggested that, $FeSO_4$, $HgCl_2$, $MnCl_2$, NaCl, N_2O_6Sr, and $ZnSO_4$ did not significantly influence the enzymatic activity. The amylase activity were increased in the presence of $BaCl_2$, $MgCl_2$, and $CaCl_2$, whereas inhibition effect was occurred with $AgNO_2$, $AlK(SO_4)_2$ and $CuSO_4$.

Table 4.5 Effect of metal ion on the activity of amylase from EF6

Metal ion (10 mM)	Relative activity (%)
None	100
$AgNO_2$	35
$AlK(SO_4)_2$	56
$BaCl_2$	120
$CaCl_2$	107
$CuSO_4$	13
$FeSO_4$	95
$HgCl_2$	81
$MgCl_2$	109
$MnCl_2$	88
NaCl	93
N_2O_6Sr	89
$ZnSO_4$	87

4.7.4 Substrate specificity

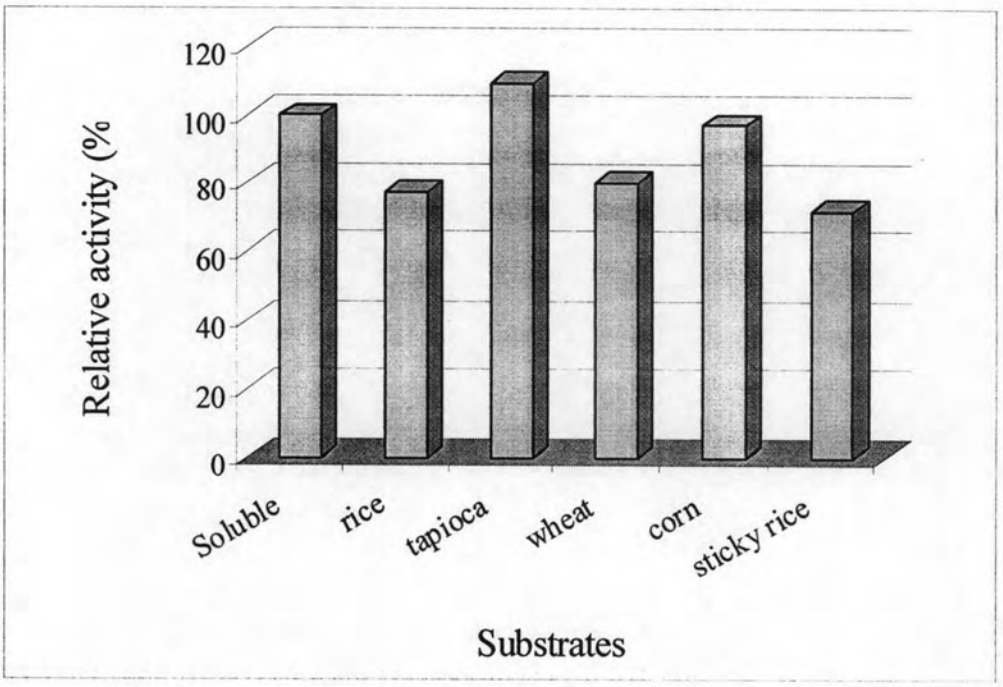


Figure 4.15 Substrate specificity of amylase from EF6 endophytic fungi

The determination of substrate specificity was performed as described in 3.10.6.The ability of hydrolysis on various substrates by amylase was shown in Figure 4.15. From the result suggest that amylase has the highest hydrolyzed activity on tapioca. Soluble starch, corn, wheat, rice and sticky rice flour also show the similar activity but a little bit below.

To confirm the mass of amylase, the mass spectrometry was used. There were two peaks in Figure 4.16. The m/z of 62,031 Da was singlet charged species and m/z of 31,141 Da was doublet charged species of the same protein. From the result, mass spectrum of purified amylase performed mass similar to molecular weight which obtained by SDS-PAGE (approximately 66 kDa). Because mass spectrometry has more accuracy than SDS-PAGE technique, then the molecular weight of this amylase should be 62 kDa.

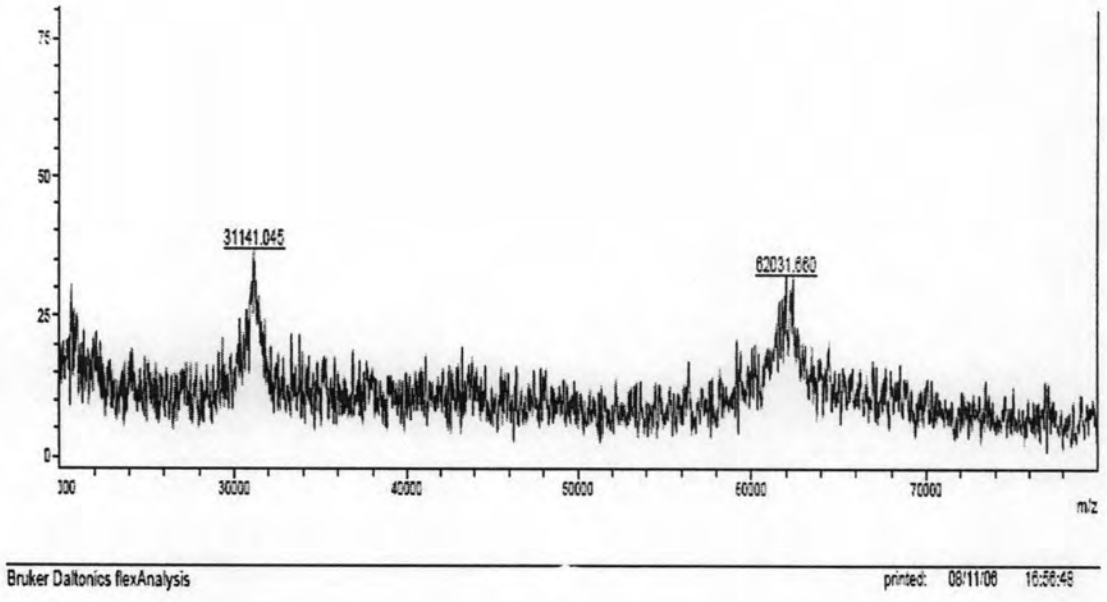


Figure 4.16 MALDI-TOF mass spectrum of G2 fraction from Superdex 75 gel filtration.

4.7.5 Kinetic study

Purified amylase was incubated with 0.2-20 mg soluble starch in 0.02 M piperazine buffer, pH 5.5 at 50°C for 15 min. The hydrolytic products were determined in section 3.7. The typical Lineweaver-Burk plot was shown in Figure 4.17. Kinetic parameters of this enzyme are K_m and V_{max} which calculated from linear equation. The enzyme catalyzed hydrolysis of soluble starch, showed K_m value of 2.63 mg and V_{max} of 1.25 mM/min.

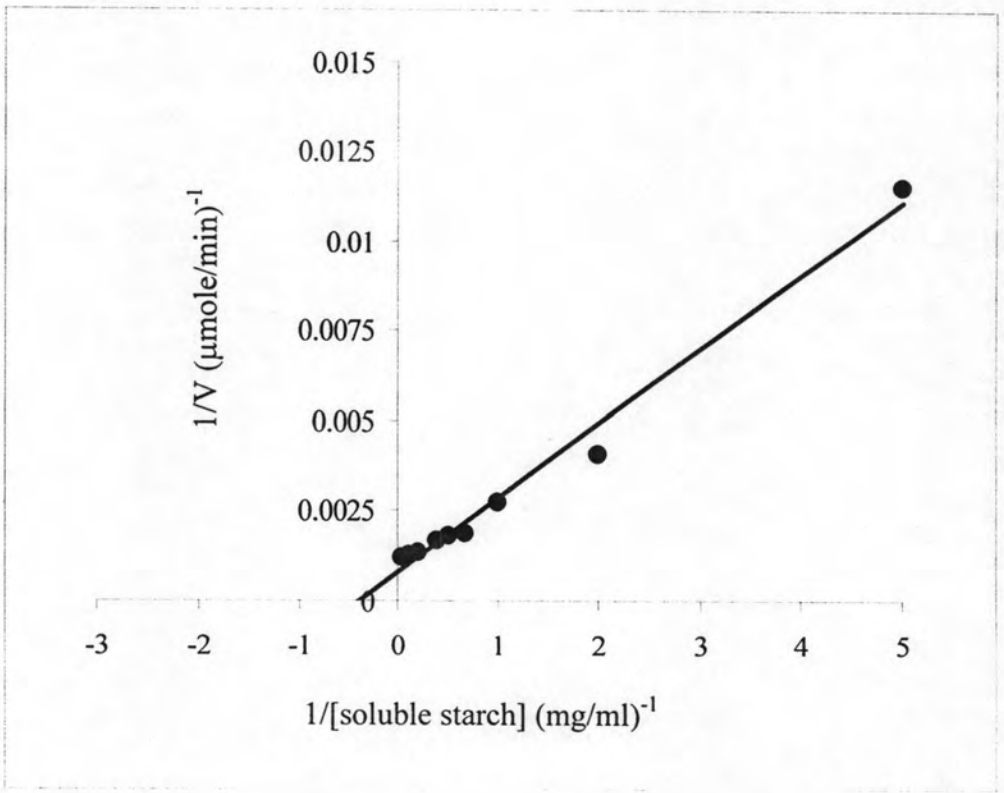


Figure 4.17 Lineweaver-Burk plot of amylase with soluble starch as substrate

4.8 Protein Identification

After proteins were separated by many steps, the G3A from SDS-PAGE was subjected to digestion with trypsin. The peptide sequence of each tryptic fragment was analyzed by ESI-Q-TOF. Product ion spectra of tryptic fragments of purified protein were interpreted by using Peptide Sequencing program on Masslynx software. These sequences were used for protein identification via MS BLAST (http://dove.embl-heidelberg.de/Blast2/msblast.html) (Table 4.6). Search parameters were set, nrdb 95 database and 12 unique peptides. From the result of database searching, partial amino acid sequences of purified protein similar to glucoamylase (3.2.1.3) from *Amorphotheca resinae*, *Neurospora crassa*, *Humicola grisea*, *Aspergillus oryzae*, *Aspergillus kawachii*, *Penicillium chrysogenum*, *Aspergillus awamori* and *Talaromyces emersonii*. So, purified enzyme from EF6 strain may be glucoamylase because HSP (High scoring pair) is higher than the threshold. The results of MS BLAST were presented in Table 4.7-4.16.

Table 4.6 Peptide sequence of precursor ion used in MS BLAST searching

Precursor	Sequence
450.72	-STSLAFFK-
483.58	-HQPLSGGL-
492.26	-VWPLLR-
518.73	-YFYTWTR-
526.24	-FELDATR-
593.32	-GVASGLVLASPSK-
624.83	-TVSNPSGSLSDGK-
635.38	-SLVWPLLRN-
664.34	-SGSLTVT-
666.87	-DNDLFLLRL-
688.83	-QTVSNPSSDGK-
1061.45	-GAPALQPCG-

Table 4.7 The results of MS BLAST of amylase from EF6 compare with glucoamylase P [Precursor] from *Amorphotheca resinae* (Accession number Q03045).

	Comparing	Score
Query:	27 YFYTWTR 33	57
	YFYTW R	
Sbjct:	76 YFYTWSR 82	
Query:	99 QTVSNPS 105	50
	QTVSNPS	
Sbjct:	117 QTVSNPS 123	
Query:	57 TVSNPSG 63	48
	TVSNPSG	
Sbjct:	118 TVSNPSG 124	
Query:	43 GVASGLVLASPSK 55	45
	G G V ASPSK	
Sbjct:	59 GAGAGFVVASPSK 71	
Query:	114 PALQPC 119	38
	P LQPC	
Sbjct:	295 PTLQPC 300	
Query:	73 VWPLLRN 79	34
	+WP++ N	
Sbjct:	184 IWPIIAN 190	
Query:	35 FELDA 39	30
	F++DA	
Sbjct:	286 FDIDA 290	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.8 The results of MS BLAST of amylase from EF6 compare with Glucoamylase precursor from *Neurospora crassa* (Accession number P14804).

	Comparing	Score
Query:	58 VSNPSGSLSDG 68	66
	VSNPSGSLS+G	
Sbjct:	126 VSNPSGSLSNG 136	
Query:	43 GVASGLVLASPSK 55	62
	G ASG V ASPSK	
Sbjct:	65 GAASGVVVASPSK 77	
Query:	27 YFYTWTR 33	51
	Y YTWTR	
Sbjct:	82 YWYTWTR 88	
Query:	71 SLVWPLLRN 79	47
	S++WP+ N	
Sbjct:	188 SIIWPIVKN 196	
Query:	82 GSLTVT 87	38
	GS+TVT	
Sbjct:	374 GSITVT 379	
Query:	99 QTVSNPS 105	37
	Q VSNPS	
Sbjct:	124 QGVSNPS 130	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.9 The results of MS BLAST of amylase from EF6 compare with glucoamylase from *Humicola grisea* (Accession number Q12623).

	Comparing	Score
Query:	43 GVASGLVLASPS 54	54
	G A G V+ASPS	
Sbjct:	61 GAAAGVVIASPS 72	
Query:	58 VSNPSGSLSDG 68	52
	VSNPSG DG	
Sbjct:	108 VSNPSGTFADG 118	
Query:	27 YFYTWT 32	48
	YF+TWT	
Sbjct:	78 YFFTWT 83	
Query:	71 SLVWPLLRN 79	42
	S VWP N	
Sbjct:	170 SVVWPVVKN 178	
Query:	82 GSLTVT 87	38
	GS+TVT	
Sbjct:	360 GSITVT 365	
Query:	99 QTVSNPS 105	38
	Q VSNPS	
Sbjct:	106 QQVSNPS 112	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.10 The results of MS BLAST of amylase from EF6 compare with glucoamylase from *Aspergillus oryzae* (Accession number O59846).

	Comparing	Score
Query:	27 YFYTWTR 33	64
	YFYTWTR	
Sbjct:	75 YFYTWTR 81	
Query:	58 VSNPSGSLSDG 68	64
	VSNPSG LSDG	
Sbjct:	118 VSNPSGGLSDG 128	
Query:	43 GVASGLVLASPSK 55	61
	G A G+V ASPSK	
Sbjct:	58 GAAAGIVVASPSK 70	
Query:	99 QTVSNPS 105	42
	Q VSNPS	
Sbjct:	116 QAVSNPS 122	
Query:	73 VWPLLRN 79	34
	+WP+ N	
Sbjct:	182 IWPIVQN 188	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.11 The results of MS BLAST of amylase from EF6 compare with glucoamylase I precursor from *Aspergillus kawachii* (Accession number P23176).

Comparing		Score
Query:	27 YFYTWTR 33	64
	YFYTWTR	
Sbjct:	72 YFYTWTR 78	
Query:	72 LVWPLLRN 79	54
	+VWPL RN	
Sbjct:	177 IVWPLVRN 184	
Query:	43 GVASGLVLASPS 54	51
	G SG+V ASPS	
Sbjct:	55 GADSGIVVASPS 66	
Query:	58 VSNPSGSLSDG 68	50
	VSNPSG LS G	
Sbjct:	115 VSNPSGDLSSG 125	
Query:	99 QTVSNPS 105	37
	Q VSNPS	
Sbjct:	113 QGVSNPS 119	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.12 The results of MS BLAST of amylase from EF6 compare with glucoamylase from *Penicillium chrysogenum* (Accession number Q76KF7).

	Comparing	Score
Query:	27 YFYTWTR 33	64
	YFYTWTR	
Sbjct:	69 YFYTWTR 75	
Query:	58 VSNPSGSLSDG 68	62
	VSNPSG LSDG	
Sbjct:	112 VSNPSGDLSDG 122	
Query:	47 GLVLASPSK 55	46
	G V ASPSK	
Sbjct:	56 GVVVASPSK 64	
Query:	99 QTVSNPS 105	42
	Q VSNPS	
Sbjct:	110 QAVSNPS 116	
Query:	73 VWPLLRN 79	34
	+WP+ N	
Sbjct:	176 IWPIVQN 182	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.13 The results of MS BLAST of amylase from EF6 compare with glucoamylase precursor from *Aspergillus oryzae* (Accession number P36914).

	Comparing	Score
Query:	27 YFYTWTR 33	64
	YFYTWTR	
Sbjct:	75 YFYTWTR 81	
Query:	43 GVASGLVLASPSK 55	53
	G G V+ASPSK	
Sbjct:	58 GASPGVVIASPSK 70	
Query:	72 LVWPLLRN 79	49
	LVWP RN	
Sbjct:	180 LVWPVVRN 187	
Query:	58 VSNPSGSLSDG 68	49
	+SNPSG LS G	
Sbjct:	118 ISNPSGALSSG 128	
Query:	99 QTVSNPS 105	32
	Q +SNPS	
Sbjct:	116 QGISNPS 122	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.14 The results of MS BLAST of amylase from EF6 compare with glucoamylase precursor from *Aspergillus awamori* (Accession number P69327).

Comparing		Score
Query:	27 YFYTWTR 33	64
	YFYTWTR	
Sbjct:	72 YFYTWTR 78	
Query:	72 LVWPLLRN 79	54
	+VWPL RN	
Sbjct:	178 IVWPLVRN 185	
Query:	43 GVASGLVLASPS 54	51
	G SG+V ASPS	
Sbjct:	55 GADSGIVVASPS 66	
Query:	58 VSNPSGSLSDG 68	45
	+SNPSG LS G	
Sbjct:	115 ISNPSGDLSSG 125	
Query:	99 QTVSNPS 105	32
	Q +SNPS	
Sbjct:	113 QGISNPS 119	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.15 The results of MS BLAST of amylase from EF6 compare with glucoamylase precursor from *Talaromyces emersonii* (Accession number P14804).

Comparing			Score
Query:	27	YFYTWTR 33	57
		YFY WTR	
Sbjct:	77	YFYSWTR 83	
Query:	57	TVSNPSGSLSDG 68	51
		T+SNPSG LS G	
Sbjct:	119	TISNPSGDLSTG 130	
Query:	43	GVASGLVLASPS 54	48
		G G+V ASPS	
Sbjct:	60	GASAGIVVASPS 71	
Query:	99	QTVSNPS 105	45
		QT+SNPS	
Sbjct:	118	QTISNPS 124	
Query:	72	LVWPLLRN 79	39
		++WP+ N	
Sbjct:	182	IIWPIVQN 189	

Note + are indicated the amino acid which differ from retrieved sequence

Table 4.16 Threshold Scores for Statistical Evaluation of MS BLAST Hits of amylase from EF

Number of reported HSP[a]	Threshold Score	Observe Score								
		Q03045	P14804	Q12623	O59846	P23176|	Q76KF7	P36914	P69327	Q9C1V4
1	71	57	66	54	64	64	64	64	64	57
2	119	107	<128[b]	106	<128[b]	118	<126[b]	117	118	108[b]
3	149	<155[b]	<179[b]	<154[b]	<189[b]	<169[b]	<172[b]	<166[b]	<169[b]	<156[b]
4	142	<200[b]	<226[b]	<196[b]	<231[b]	<219[b]	<214[b]	<215[b]	<214[b]	<201[b]
5	197	<238[b]	<264[b]	<234[b]	<265[b]	<256[b]	<248[b]	<247[b]	<246[b]	<240[b]
6	239	<272[b]	<301[b]	<272[b]						
7		<302								
Total		302	301	272	265	256	248	247	246	240

a: HSP (High scoring pair) is a region of high local sequence similarity between the peptide in the query and the protein in a database that was identified by database searching.
b: HSP is higher than the threshold in this ranked, the match is statistically significant.