Supplementary Material

Source	Abbreviation	GH13 subfamily number	Sequence similarity ^a (%)	GenBank or PDB accession number					
Fungi (GH13_1)									
Aspergillus oryzae	AmyA	1	64.4	2GUY_A					
Malbranchea cinnamomea	McAmyA	1	59.0	3VM7_A					
Aspergillus niger	α-amylase	1	55.3	2AAA_A					
Bacteria (GH13_2)									
Bacillus stearothermophilus	Novamyl	2	26.9	1QHO_A					
Thermoanaerobacterium thermosulfurigenes	CGTase	2	23.1	3BMV_A					
Fungi (GH13_5)									
Paecilomyces variotii	alpha-amylase		77.6	XP_028489551.1					
Aspergillus niger	AmyD	5	64.4	XP_001389762.2					
Aspergillus niger	AmyF	5	54.5	XP_001393627.2					
Paracoccidioides brasiliensis	Amy1	5	39.1	ABS11196.1					
Bacteria (GH13_5)									
Bacillus sp. 707	G6-amylase	5	47.5	1WP6_A					
Bacillus licheniformis	alpha-amylase	5	47.0	10B0_A					
Bacillus sp. Ksm-1378	AmyK	5	46.8	2DIE					
Bacillus halmapalus	alpha-amylase	5	46.7	1W9X					
Bacillus amyloliquefaciens	BAA	5	46.4	3BH4_A					
Geobacillus stearothermophilus	AmyS	5	45.3	4UZU_A					
Bacillus sp. strain KSM-K38	AmyK38	5	44.5	1UD2_A					
Alicyclobacillus sp. 18711	AliC	5	44.2	6GXV_A					
Halothermothrix orenii	AmyB	5	31.7	3BC9_A					
Plants (GH13_6)									
Hordeum vulgare	Amy1	6	26.6	2QPS_A					
Hordeum vulgare	Amy2	6	22.6	1AMY_A					
Archaea (GH13_7)									
Pyrococcus woesei	PWA	7	45.0	1MWO_A					
Pyrococcus woesei	alpha-amylase	7	28.2	3QGV_A					
Bacteria (GH13_10)									
Deinococcus radiodurans R1	MTHase	10	33.8	2BHU_A					
Bacteria (GH13_20)									
Thermus sp. IM6501	ThMA	20	24.7	1SMA_A					
Geobacillus stearothermophilus	Neopullulanase	20	20.9	1J0H_A					

Table S1. Amino acid sequences used for phylogenetic tree construction.

Archaea (GH13_20)								
Thermococcus kodakarensis	TUDII	20	27.7	50T1 A				
KOD1	IK-FUL	20	57.7	JU11_A				
Bacteria (GH13_31)								
Erwinia rhapontici	NX-5	31	24.5	4HOW_A				
Listeria monocytogenes EGD-e	Lmo0184	31	23.9	5DO8_A				
Yeast (GH13_40)								
Saccharomyces cerevisiae	Ima1	40	28.7	3AXH_A				
Bacteria (GH13_?)								
Anoxybacillus ayderensis	ASKA	unknown	33.2	5A2A_A				
Geobacillus thermoleovorans	GTA	unknown	32.2	4E2O_A				

^{*a*} The similarities of α-amylases were compared to NFAmy13B, except for those in group GH13_1, which were compared to NFAmy13A.

Substrate	Main linkage/monomor	Glucose equivalents (mM)						
Substrate	Main mikage/monomer	(mean \pm standard deviation)						
Soluble starch	α -(1 \rightarrow 4)- α -(1 \rightarrow 6) glucose	1.23±0.06						
Amylopectin	α -(1 \rightarrow 4)- α -(1 \rightarrow 6) glucose	11.6±0.3						
Amylose	α -(1 \rightarrow 4) glucose	5.73±1.24						
Wheat starch	α -(1 \rightarrow 4)- α -(1 \rightarrow 6) glucose	6.06±1.06						
Potato starch	α -(1 \rightarrow 4)- α -(1 \rightarrow 6) glucose	5.42±0.71						
Corn starch	α -(1 \rightarrow 4)- α -(1 \rightarrow 6) glucose	7.09±0.66						
Xylan	β -(1 \rightarrow 4) xylose	0						
Laminarin	β -(1 \rightarrow 3) and β -(1 \rightarrow 6)-glucose	0						
Dextrin	α -(1 \rightarrow 4) and α -(1 \rightarrow 4)- α -(1 \rightarrow 6) glucose	6.79±0.85						
Glycogen	α -(1 \rightarrow 4)- α (1 \rightarrow 6) glucose	0.225±0.030						
Pullulan	α -(1 \rightarrow 6) glucose	0.0692±0.0948						
Arabinoxylan	β -(1 \rightarrow 4) arabinose and xylose	0						
Cellulose	β -(1 \rightarrow 4) glucose	0						

Table S2. The hydrolysis of NFAmy13B on various substrates.

Each experiment was performed in triplicate.

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Enzyme	Concentration (nM)	Reducing ends (mM)
NFAmy13B	20	6.71±0.46
	900	4.67±0.30
NEA my 13 A	1000	6.63±1.16
NI/Alliy13A	1100	7.70±1.75
	1200	7.18±1.62

Table S3. Degradation capacity of NFAmy13A and NFAmy13B on wheat starch.

Each experiment was performed in triplicate.

Table 54. The invulorities of infamily 15A and infamily 15D on manoungosacchanges (1915)

Cubatrata	Enguina	Product	The yield of reducin	standard deviation)					
Substrate	Elizyille	component	5 min	20 min	60 min				
N/2		M1	1.52±0.11	2.47 ± 0.05	3.29±0.09				
M3	NFAIIY13A	M2	1.96±0.11	2.97 ± 0.05	3.84±0.02				
M4		M1	0.727±0.024	1.34±0.05	2.17±0.08				
	NFAmy13A	M2	7.32±0.07	7.79±0.21	8.53±0.17				
		M3	1.43±0.06	0.657 ± 0.176	0				
		M1	0.0984±0.0394	0.208±0.024	0.335±0.061				
	NFAmy13B	M2	0.269 ± 0.058	1.01 ± 0.06	2.66 ± 0.05				
		M3	0.0867±0.1279	0.483 ± 0.063	0.817 ± 0.195				
		M1	1.02±0.17	2.99±0.04	5.33±0.02				
		M2	8.93±0.39	11.6±0.1	13.5±0.1				
	NFAmy13A	M3	5.59 ± 0.07	3.65±0.15	1.07 ± 0.06				
N/5		M4	0	0	0				
MS		M1	0	0.0951±0.770	0				
	NEA	M2	5.81±0.03	6.94±0.07	7.18±0.04				
	NFAINY13B	M3	7.05 ± 0.22	8.18±0.09	7.99±0.21				
		M4	0	0	0				

Each experiment was performed in triplicate.

Table S5. Comparison of alkali-resisting α -amylases.

Amylase	Source	Optimum pH	pH stability ^b	References
NFAmy13B	Unknown	5.5~6.0	pH 5.5~12.5, 1 h, >90%	This study
G6-amylase	Bacillus sp.	8.8	pH 4.7~10.8, 30 min, >50%	[1]
<i>Td</i> AmyA	Thermomyces dupontii	6.5	pH 4.5~10.0, 30 min, >80%	[2]
Amy1	Bacillus amyloliquefaciens	6.0	pH 7.0~9.0, 1 h, 90%	[3]
α -amylase ^a	Bacillus sp.	5.0	pH 4.5~11.0, 1 h, 80%	[4]
α -amylase ^a	Bacillus sp.	10.5	pH 10.5, 15 h, 55%	[5]
α -amylase ^{<i>a</i>}	Halorubrum xinjiangense	8.5	pH 7~11, 1 h, >45%	[6]
α -amylase ^a	Bacillus sp.	8.0	pH 6~13, 30 min, >67%	[7]
TfAmy48	Tepidimonas fonticaldi	8.0	pH 11, 6 h, 50%	[8]
McAmyA ^a	Malbranchea cinnamomea	6.5	pH 5.0~10.0, 30 min, >90%	[9]

^{*a*} Amylases were produced by the originated organism.

^{*b*} This column indicates the residual activities of α -amylases pre-treated in the range of specified pH with designated time.



Concentration (mM)

Fig. S1 Effect of the concentrations of calcium divalent ion on NFAmy13B thermostability. Enzyme (75 nM) was incubated with different concentrations of CaCl₂ at pH 6.0 and 55 °C for 30 min. The residual activities were determined by incubating 30 nM enzyme with 5 mg/mL potato starch at 54 °C and pH 6.0 for 30 min. Each experiment was performed in triplicate.



Fig. S2 Kinetic studies of the NFAmy13B on the different substrates. 20 nM enzyme with 1.25 mM $CaCl_2$ was incubated with various concentrations substrate between 0.1 and 16 mg/mL at 54 °C for 20 min. Each experiment was performed in quadruplicate.



Fig. S3 Hydrolysis of maltooligosaccharides by NFAmy13A and NFAmy13B. 5 mM M2 and M3 were hydrolyzed by 1 μ M NFAmy13A or NFAmy13B in the presence of 1.25 mM CaCl₂ at pH 6.0 and 54 °C for 2 h. The reaction products were analyzed by HPAEC-PAD. M1, glucose; M2, maltose; M3, maltotriose.



Fig. S4 Phylogenetic analysis of NFAmy13A and NFAmy13B. The amino acid sequences of enzymes from GH13 used for the construction of the phylogenetic tree were selected according to the similarities with NFAmy13A or NFAmy13B. Each enzyme was indicated a number corresponding to

pdb2GUY pdb3VW7 pdb2AAA NFAry13A Ary D Ary F Ary 1 NFAry13B pdb1WP6 pdb1OB0 pdb2DI E	1 1 1 1 1 1 1 1 1 1 1	- M - M - M - M - M - M	√ST SFL FSF ASF KSL	ALF LWC LPC ISS LCC	AAI HPI FK AYI	AAA KKR TRR KYI R	AGS K QRT F	ARI PTI KS	AK RQ RQ RK KQ	- AT AAT - LS AAT LWK SQK KSK KSK	P A I P D I A A S P D I Q I I E I I E I I		S QS S RS T QS A QS A E H A N/ D F N A E H	51 YF 51 YF	UL QVL UL ML QLP QLP QLP	T DR T DR T DR T DR S WD S WH S WH T WD H	F AR F AR F GR F AR - AP - AP HNG - AN HNG	T DG GDG T DN T DN DNT E NT DNQ DNA T NG L NG	STT STT STT LML LMF LMN TMN TLN TMN	AT C AP C AP C QAF QAF QAF /QAF /QY F		ADQI GARI GARI GNEI FAGI HVP HVP HVP YLPI YLPI YMPI HLPI	(Y (Y (Y (Y (Y (Y (Y) (Y) (Y) (Y) (Y) (Y) (Y) (Y) (Y) (Y)	NTAI		GT W GNY GS W GT W GH W S H W S H W K H W N H W QH W O H W	QGI RGL QGI RRL RRL QRL NRL NRL	I DK I SC I DH I NN HQA QAA KSV NSC QNC R DC	(LD) ILD) ILD) ILP ILP ILPS ILPS ILPS ILPS ILPS ILPS I			TAI DS\ TAI TAI (DNI (TSI (TSI (DS) TA\ TA\ TA\	WISSTPPPP WIPPPP WIPPP
pdb2GUY pdb3VM7 pdb2AAA NFAmy13A AmyD AmyF Amy1 NFAmy13B pdb1WP6 pdb10B0 pdb2DIE	64 85 64 80 90 75 76 46 46	PV PI PG PG PA PA PA	TAQ TKQ TEQ CKA CKA CKA CKA CKA VKG VKG	LPC FED WDD MDP MDT MHP ASC TSC	DT DT DT SG SG SG ND AD ND	A DA A	VG NGA DGE DAT NGY NGY VGY VGY	DAY APY AY DI DI DI DI CDI CDI CDI CDI CDI CDI CDI	HGY HGY HGY HGY YDL YDL YDL YDL YDL YDL		DL V DL V GE F GE F GE F GE F GE F		N N N KG9 KG7 KG1 KG1 KG1 KG1	E S - S		GTA GTEA GTA GTA GTA GTA GTA GTA GTA GTA GTA GT	DDL EDL DDL EEL EEL SQL SQL	KAL RAL KSL EDL VSL QAA QGA	SSA ADE SDA ADA VRD VRD VRD VRD VRD VRD VRD VRD VRD VRD	L HE L HA L HA A L HA A A QE A A A C A QE C A C A C C A C A C C A C A C C A C A C	RGI ARGI ARGI DFGI ALG NEI NNGI SRDI	VYLI VFLI VLLI GI VGVI QV QV QV				GYD GYP GYG AGA AGA AGA GGA GGA	GAG GDA GSH DYA DSV DYV DYT DAT DAT	ISSV ASI ISEV ERF ERF EDV EDV	DYS DYS DYS QAV E GV VAV E AV VAV VAV VAV	VFI VFI IFI RVI KVI KVI EVI EVI	(P NP NP DPQE DDDF DPKE DPKE NPNE DPAE	R NN R DI DR R F DR NN R NC	VKI A EDG VVI S ZEVT ZVI S QEI S
pdb2GUY pdb3VM7 pdb2AAA NFAmy13A AmyD AmyF Amy1 NFAmy13B pdb1WP6 pdb10B0 pdb2DIE	140 160 140 167 167 162 163 133 131 133	PA PA PP SP GE GE	EEI QQI QEI EEI YTI VLI YTI	E AV E AV E AV E AV E AV E AV	VGI VTSI VTSI VTSI VTRI VTRI VTRI	F NF F T F F DF F DF F DF F DF F DF	S GF P GF P GF P GF P GF P GF P GF	GN GT GN GK GN GS GN CS CS CS CS CS CS CS CS CS CS CS CS CS	F F S F D F D F D F D F D F D F D F D F D F D	S QE S S S E S S S S S QE S MK S MK S MK S F K DF K NF K	YFF YFF YFF YNF YRS YHV YRS WRV	HPF HPPY HPF KNH AQH SEH AYH AYH AYH				QT Q QT S L T M QE E S R Q L T G L T G I D N S R R S R K S R Q	VE- VE- VE- KC- RN- KN- LNN LNN LQN	GVY AIY GIF AIY RIY RIY KIY	KI C KI L KI L KV V KF R KF C KF R	QG DP S GP C (GP- (GP- QG QG QG QG (GT- (GT (GT))) +4		DC QC DC QC HE DKN NKD SKA GKA GKA	AL GI AL Y AE GI AANI AAQ AARI AARI ADWI ADWI	DNT TGAI DTI DVAI DVG DVS DVS EVD EVD	V V V T-E T-E T-E N-E N-E	NGN NGN NGN NGN NGN F GN		- SL - AL - SL - TL L MF L MF L MF L MY L MY	P DL P DL P DL ADL ADL GDL ADI ADI ADI		KD DPI ET NPC NPE NPE NPE DPI DPI DPI DPI DPI DPI		VEWY 2VHN 1WY FFN DVL DVL DVL DVL DVL DVL DVL NELR NELR
pdb2GUY pdb3VM7 pdb2AAA NFAmy13A Amy1 Amy1 NFAmy13B pdb1WP6 pdb10B0 pdb2DIE	189 208 189 209 250 266 248 248 219 214 219	DW DW DW RW RW RW RW RW RW	VGS AD VAD I KS GKW GEW GEW GVW GTW	L V S L V S L V A L NA I MS I GN I GK Y T N Y T N	NY NY QLI VLI IQLI IELI ITL	S I D S I D					KDF KDF KDF AGF LSF FSF YSF	= WP = FP = QK = QR = QR = LR = TR = LR = LR	GYN GYN DFN DFN DFN DFN DWN DWN		- K - D - K - E RT /RQ /RQ /RQ /RS /RE /RN	AAGG AAGG AAGG AAGG AAGG AAGG AAGG ATF G ATT ATT ATT	 - P D - P D - A G - P H - K N - K P - K P	VYC VYA VYC VFI WFV WFF MFA MFA	I G VG VG I G VA VA VA VA VA	VLD VYH VFN YMK YMK YMK YMK FMK VMS		PAY PAY PASI PAY FKPI RAI VWEI PGEI GAI GAI GAI		Y QN Y QD Y QK Y QE Y L E Y L G Y L Q Y L Q Y L Q Y L N Y L N	V MD V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V L D V D V	G G G YKL YRV HLV HLV HKV HLV HNH	- VL - VL - VL - VL SLF ALF SLF SVF ISVF ISVF	NYF NYF DYF DVF DVF DVF DVF		PLI PLI PLI PLI PLI PLI PLI PLI PLI PLI	NAF DAF DAF STST STST STST STA STA STA STA	KST KSF QTF RVF RTE KSC TQC	S GS S GT S GS /GGN G (R G GGN- GGN- GGG- GGY-
pdb2GUY pdb3VM7 pdb2AAA NFAmy13A AmyD AmyF Amy1 NFAmy13B pdb1WP6 pdb10B0 pdb2DIE	269 288 269 337 354 335 307 302 307	ND NW IS IG - A - A - A - Y - Y - F	DLY SLV DLR DLR DLR DLR DLR DVR DWR	NMI DNI QAI GVL GVL KIF NIF KLL NIL	NT KS TT RG DD NG NG	VKS VFQ VQE TLV TLV SLV TLV TVV SVV	DCF DCS SCk QL - ES - KY - KY - SK - SK - QK -	DS DP DP DP CDS VPI KEI KPC HP HPI	+2 TLL RLL TLL OHS GNA GNA GNA GNA GNA GNA GNA GNA GNA GNA	GTF GNF GNF VTF VTF VTF VTF	VEI SEI VA VT VA VD VD VD		NPF NPF NPF TQF TQF TQF SQF SQF	RFAS RFAS RFAS PGQS PGQS PGQS PGQS PGQS PGQS PGQS PGQ		NDI QDL SDY DDL APV TIV VPI API SFV SFV	ALA SQA ALA TSF EPS AEF ASY QTW QSW	KNV KNV F K K P F K P F K P F K P F K P	AAF LAF LAF LAY LAY LAY LAY LAY LAY	I I L I I L I I L AL I AL I AL I AL I AL	- S D C S D C S D C L L F L L F L L F L T F L T F	GI PI GI PI GI PI GI PI GI PI CI PI		A G Q Q A G Q Q A G Q Q A G Q Q G G Q H I E G Q H I E G Q H I E G Q H I E G Q H I E G Q H I E G Q I I E G Q I I E G Q I I I E G Q I I E G Q I I I E G Q I I I I I I I I I I I I I I I I I I I		Y A G G G G G G G G G G G G G G G G G G	GND DSD GKV DHD DLY DLY DLH DYY DYY DYY	P AN P Y N GL C GT C GI F GI F GI F	REA REA REA DADV DG- CGGC EGV TH- CGD- TH-	T WI T WI T WI K DI C E GI K K I G	SG SG SG PMT PTF LR PLTF - VF RELF	PTE NTE DTS NTE SCF SCF SCC SCC SCC SCC SCC SCC SCC SCC	DSEL DAPL AEL GKL GQL GQL GKL SGKL (HKI (SKI
pdb2GUY pdb3VM7 pdb2AAA NFAmy13A AmyD AmyF Amy1 NFAmy13B pdb1WP6 pdb10B0 pdb2DIE	359 378 359 379 421 437 418 419 387 385 387	YK YK YQ SI PN PH DP DP	LIA: HIA FIK LTR LNR LSL LAL ILE LLQ	S AN ACN KAN AR- AR- AR- AR- AR- AR- AR-	IAI IRI IGI 	R NY R S H R K L R N L K L Y K L Y K L Y K L Y K L Y K Q Y Q K Q Y		KD SNDI SQNI SLQI SEQI SQQI SVQI SKQI SAQI SAQI STQI	T GF DAY S AY P E F R DY R DY R DY R DY R DY H DY H DY	VTY ITT FDK FDK FDK FDK FDK FDF	ANI ANI ANI ANI ANI ANI ANI ANI ANI ANI	API COAF COAF CIG CIG CIG CIG VG	Y K I Y S I Y Q I F V F F V F F V F F V F M T F	DDT DDH DSN DDS QDS QDS QSN QSN QSN QSN QSN QSN QSN QSN QSN QS	FIA FLA FLA NRR NAA OSA NKR NTA OSS OSS	MRK LVK MAK HP- HP- HP- HPN VAN HPN	GT D GA- GG- SG- SG- SG- SG- SG- SG- SG- SG-	GS Q	I VT VI T VVT LAC LAC LAC LAC LAT	VLS VLS VLS VLS VLS VLS VLS VLS VLS LIS LIS	NKC NKC NKC NG NG NG DG DG DG	GAS GAN GSSC GAS GPSI GPSI GPAT SASI AGG PGG	GDS GSS GGD GSS GGD GKR KR KR KR KR KR KR KR KR KR KR KR KR	YTL TTV YTL RTV MYV MYV MFV MFV MYV MYV	SLS TVE TLS GRR GRR GRK GRK GRK GRN GRN GRN GRN GRN	GAG GSG GTG HAK HAG HAG KAG NAG	YTA YAS FEA QTV EKV SVV EHV QVV	GQC GEC GTK GTE TDI TDI TDI TDI TDI TDI SDI HDI RDI	QL T E QV T C L I E L QV L QV Y GT L QV T GN T GN	VI OVL AY OVI CD CR CR NRT NRS NRS	G- CI 5- CI 5- CI 5- CI 5- CI 5- CI 0HP 9 5 0 0HP 9 5 0 0 0HP 9 5 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	T V 1 S I A S V 1 T V 1 S V 1 S V 1 S V 1 S V 1 S V 1 S T V 1 S T V 1	VGS AASD VDS AGD I DA I DA I DR I DR I NA I NS I NA
pdb2GUY pdb3VM7 pdb2AAA NFAry13A Ary D Ary F Ary 1 NFAry13B pdb1WP6 pdb10B0 pdb2DIE	448 463 448 464 500 519 497 498 467 465 467	DGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	NVP RLS DIP YGE YGE YGV YGV YGV WGN WGR	VPN VPL FPV FPV FSV FSV FTV	AG NQ SG SA QS VS NG NG	GL P GL P GL P GL V WR V Y S V GS V GS V GS V	RVI RVI SVI SVV SVV SVV SVV SVV SVV	YP FP VP WN WD WR WR WR WR WR	T E K T D A A S V S S Q S E A E D A E D A A A A K Q	L AG V DS L AK E GR D GR E GR	IS GI S GI S GI DS I DS I DS I DS I	CS CG CA SH GA GCA	SS- N GSC SA- HL EL - - - - - -	GRL (VP) (VY)	YVE AHSI	LST	DDL	LTS	AE L	S DE F HL F DS F DS		YKL / VVV YSS YKH-	4- / GV 5 C -	DV									
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the GH13 subfamily, its abbreviation, and source. Details are provided in Table S1.

◊ Conserved amino acids of fungal α-amylase in group GH13_5

Fig. S5 Amino acid sequences alignment in NFAmy13A/B and high-similarity α -amylases.

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