

Functional Characterization of Recombinant Raw Starch Degrading α -Amylase from *Roseateles terrae* HL11 and Its Application on Cassava Pulp Saccharification

Daran Prongjit ¹, Hataikarn Lekakarn ^{1,*}, Benjarat Bunterngsook ², Katesuda Aiewviriyasakul ², Wipawee Sritusnee ² and Verawat Champreda ²

¹ Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Campus, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand; daranprongjit@gmail.com (D.P.)

² Enzyme Technology Research Team, Biorefinery Technology and Bioproduct Research Group, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Phahonyothin Road, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand; benjarat.bun@biotec.or.th (B.B.); katesuda.aie@biotec.or.th (K.A.); wipawee.sri@ncr.nstda.or.th (W.S.); verawat@biotec.or.th (V.C.)

* Correspondence: hataikarn.lek@sci.tu.ac.th; Tel.: +662-564-4441 (ext. 2452)

Table S1. Summary on characteristics of reported raw starch degrading α -amylases.

Organism	Enzyme	Optimal working condition	Starch binding domain	Family_subfamily	Specific activity (U/mg)	Kinetic parameter values	Reference
Gram-negative bacteria							
<i>Roseateles terrae</i> HL11	HL11Amy	pH 4.0, 50 °C	CBM20	GH13_32	6270 \pm 70	K _m 4.82 mg/ml V _{max} 19,800 U/mg (soluble starch)	In this study
<i>Aeromonas salmonicida</i> ssp	AmyASS	pH 6.0, 40 °C	No SBD	NM	23.5 \pm 1.6 (raw rice starch)	NM	[1]
<i>Bacteroides thetaiotaomicron</i>	SusG	pH 7.0, 37 °C	CBM58	GH13	NM	NM	[2]
<i>Corallococcus</i> sp. Strain EGB	AmyM	pH 7.0, 50 °C	NM	GH13	14,000	K _m 6.61 mg/ml V _{max} 44,301.5 U/mg (soluble starch)	[3]

Organism	Enzyme	Optimal working condition	Starch binding domain	Family_subfamily	Specific activity (U/mg)	Kinetic parameter values	Reference
Gram positive bacteria							
<i>Alicyclobacillus</i> sp. A4	AmyA4	pH 4.2, 75 °C	NM	NM	1,630	NM	[4]
<i>Anoxybacillus flavothermus</i>	AFA	pH 7.0, 40 °C	CBM20	NM	17 (PNP)	NM	[5]
<i>Bacillus amyloliquefaciens</i>	-	pH 5.0, 50 °C	No SBD	NM	72	K _m 3.076 mg V _{max} 4.11 mg/min (soluble starch)	[6]
<i>Bacillus aquimaris</i> MKSC 6.2	BaqA	pH 6.5	No SBD	GH13_new subfamily	NM	NM	[7]
<i>Bacillus megaterium</i> NL3	BmaN2	pH 6.0, 60 °C	No SBD	GH13_36	28.7 ± 1.4	K _m 5.1 ± 0.9 mg/ml V _{max} 141.6 ± 8.1 µg/min k _{cat} 71.5 1/s (soluble starch)	[8]
<i>Bacillus</i> sp. ALSHL3	-	pH 6.0, 60 °C	NM	NM	NM	K _m 4.79 mg/ml V _{max} 0.332 mM/min	[9]
<i>Bacillus subtilis</i> strain AS01a	AmyBS-I	pH 6.0, 70 °C	NM	NM	NM	K _m 2.7 mg/ml V _{max} 454 U/ml	[10]
<i>Streptomyces badius</i> DB-1		pH 6.0, 50 °C			246.9	K _m 1.12 mg/ml V _{max} 454 U/mg	[11]
Marine bacteria	AmyP	pH 7.5, 40 °C	CBM69	GH13_37	453.5 ± 10.7	K _m 2.5 mg/ml k _{cat} 1.53 (1/s) (gel rice) K _m 13.4 (mg/ml) k _{cat} 0.57 (1/s) (raw rice)	[12]
Fungi and yeast							
<i>Geotrichum candidum</i> CMSS06	-	pH 7.0, 40 °C	NM	NM	394.7 mU/ml	NM	[13]

Organism	Enzyme	Optimal working condition	Starch binding domain	Family_subfamily	Specific activity (U/mg)	Kinetic parameter values	Reference
<i>Rhizomucor</i> sp.	RA	pH 4.5, 32 °C	Modified CBM20 from <i>A. niger</i>	NM	NM	NM	[14]
<i>Saccharomycopsis fibuligera</i> KZ	Sfamy KZ	pH 5.1-6.4, 45 °C	No SBD	GH13_1	120–125	NM	[15]

NM= data was not mentioned

Table S2. List of GH13_32 α -amylases for sequence alignment of CBM20.

Organism	Accession no.
<i>Botryobasidium botryosum</i> FD-172 SS1	KDQ06010.1
<i>Jaapia argillacea</i> MUCL 33604	KDQ58058.1
<i>Jaapia argillacea</i> MUCL 33604	KDQ58060.1
<i>Plicaturopsis crispa</i> FD-325 SS-3	KII85613.1
<i>Plicaturopsis crispa</i> FD-325 SS-3	KII90441.1
<i>Sphaerobolus stellatus</i> SS14	KIJ56431.1
<i>Hydnomerulius pinastri</i> MD-312	KIJ69360.1
<i>Gymnopus luxurians</i> FD-317 M1	KIK53845.1
<i>Serendipita vermifera</i> MAFF 305830	KIM25608.1
<i>Phlebiopsis gigantea</i> 11061_1 CR5-6	KIP11288.1
<i>Cylindrobasidium torrendii</i> FP15055 ss-10	KIY62009.1
<i>Cylindrobasidium torrendii</i> FP15055 ss-10	KIY70325.1
<i>Fibularhizoctonia</i> sp. CBS 109695	KZP07860.1
<i>Fibularhizoctonia</i> sp. CBS 109695	KZP22139.1
<i>Neolentinus lepideus</i> HHB14362 ss-1	KZT20455.1
<i>Trametes coccinea</i> BRFM310	OSD07916.1
<i>Amanita thiersii</i> Skay4041	PFH45132.1

Table S3. List of selected animal and animal-like α -amylases for sequence alignment obtained from [16].

Organism	Abbreviation	Accession no.	C-terminus
<i>Drosophila melanogaster</i> (fruit fly)	Drome	CAA28238.1	
<i>Litopenaeus vannamei</i> (white shrimp)	Penva	CAA54524.1	
<i>Homo sapiens</i> (human, saliva)	Homsa	AAA52279.1	
<i>Pseudomonas</i> sp. KFCC10818 (animal-like)	Psesp	AAA86835.1	
<i>Streptomyces limosus</i> (animal-like)	Stmli	AAA88554.1	CBM20
<i>Pseudoalteromonas haloplanktis</i> (animal-like)	Psaha	CAA41481.1	Extra C
<i>Halomonas meridiana</i> (animal-like)	Halme	CAB92963.1	
<i>Microbulbifer degradans</i> (animal-like)	Mibde-A	ZP_00066069.1	CBM20
<i>Thermobifida fusca</i> (animal-like)	Thefu	ZP_00058434	CBM20

Figure

HL11Amy	MPLLPLSSRLTH---ALTAASA--AFALVASPAH--ALNPNATSVQMFEWAWPDIAE	53
Thefu	MGVR---RSLAALLAALLGCATSLVALTVAASPAHAAPSNGNRDVI	56
Drome	--MF----LAKSIVCLALLAVANA--QFD-----TNYASGRSGMVHIFQWRWKSIADE	45
Penva	---M----LRVAPLVVLLAAAAQA--QWD-----PNS-SNGQVIHIFEWKWSIDIAE	43
Homsa	-----MKLFWLL-FTIGFCWA--QYS-----SNTQQGRTSIVHIFEWRWVDIAE	42
Pseesp	MKFN---KSLSA-GLMLFGSLIG--GTQS-----YAQTVPKTTFVHIFEWSWQDIAE	47
Mibde-A	---M----RKL-T-QLFIFAAAL---FSI-----CSHAQPRTAFVHIFEWQWNDIAE	41
Stmli	--MA----RRLATASLAVLAAAAT--ALTAPTAA-AAPPGAKDVTAVIFEWKFASVARA	51
Psaha	MKLN---KIITTAGLS-----LG--LLLP-----SIATATPTTFVHIFEWNWQDVAQE	43
Halme	-----M-----CG--PRLPPRPSGRGFTRVFADTFVHIFEWQWEDVAQE	37

HL11Amy	CTQWLGPKEGGVQISPPGASKN-----ANGWWGVYQPVNYANLTSRMGTPAQLQSMIS	107
Thefu	CRTTLGPHGFAGVQVSPPEHVVLP--AEDYPWWQDYQPVSYKLDQTRRGSRADIFIDMVN	114
Drome	CENFLGPNYAGVQVSPVNENAV---KDSRPWWERYQPVISYKLE-TRSGNEEQFASMVK	100
Penva	CENFLGPRGFAGVQVSPPNNEYVEVYQGDVKRPWWERYQPVSYKLV-TRSGDENAFKDMVT	102
Homsa	CERYLAPKGGVQVSPPNENVAIH--NPFRPWWERYQPVSYKLC-TRSGNEDEFERNMVT	99
Pseesp	CEEFLGPKGFAAVQVSPPKQSV-----NPAAWWSRYQPVSYAFE-GRSGNRAQFADMVQ	100
Mibde-A	CENVLGPKGYAAVQVSPPKQSVS-----SSQWWWSRYQPVSYAIE-GRSGNRNEFASMVS	94
Stmli	CTDSLGPAGYGYVQVSPPEHIQ-----GSQWWTSYQPVSYKIA-GRLGDRAAFKSMVD	104
Psaha	CEQYLGPKGYAAVQVSPPEHIT-----GSQWWTRYQPVSYELQ-SRSGNRAQFIDMVN	96
Halme	CENWLGPKGFKAVQVSPPEHIQ-----GDAWWTRYQPVSYQLE-SRSGSSEAFADMVQ	90

HL11Amy	ACHAAGVRVYADIVNQADGS----GTATDGSTWNAATLTYPFFSASD-FHPNCA----	158
Thefu	TCREAGVKIYVDAVINHMTGTGSAGAGPGSAGSSYSKY--DYPGIYQSQ-DFNDCR----	167
Drome	RCNAVGVRTYVDVFNHMAADG---GTGTGGSTASPSKSYPGVPYSSLDNFPT-----	152
Penva	RCNNVGRIYVDAVINHMSGGW-PMGTGASGGSSFDGAESYPGPVPSAFDFDNGNCHTG	161
Homsa	RCNNVGRIYVDAVINHMCNAVSAGTSSTCGSYFNPGRDFPAVPYSGWDFDNGKCKTG	159
Pseesp	RCKNVGVDIYVDAVINHMAAWN-----R-----NFPEVPYGVNDFNSCT----	139
Mibde-A	RCKNAGVDIYVDAVINHMAAGG-----R-----YYPEVPYYAEHFHTCY----	133
Stmli	TCHAGVKVYVDAVINHMAAGS----GTGTGGSAYQKY--DYPGIWGA-DMDDCR----	153
Psaha	RCSAAGVDIYVDTLINHMAAGS----GTGTAGNSFGNK--SFPIYSPQD-FHESCT----	145
Halme	RCNAGVDVYADAVINHVAHGK----GQGIAGSSYDSEALSYPHYQRDD-FHEPCG----	141

HL11Amy	-INDADYNPAGRSNVQN	CRLGGLPDLATESSYVQGQIVNYLKSLLALGVDGFRIDAAKH	217
Thefu	-RDITNWN---DKWEVQH	CELVGLADLKTSSPYVDRIAYLNELIDLGVAGFRIDAAKH	223
Drome	-CAISNYG---DANEVRN	CELVGLRDLNQNSYVQDKVVEFLDHLIDLGVAGFRIDAAKH	208
Penva	SGNIENY---DANQVRN	CKLVGLNDLNLQGTDYVRGKIREFMNKLISYGVAGFRIDASKH	218
Homsa	SGDIENYN---DATQVRD	CRLSGLLDLALGKDYVRSKIAEYMNHLIDIGVAGFRIDASKH	216
Pseesp	--SDINYSN---RWSVQN	CDLVGLNDLKTSSSEYVRQKIADYMNDAISMVAGFRIDAAKH	194
Mibde-A	--GQIDYGN---RWQVQN	CDLVGLNDLATEQDYVRTKIANYLNDLTNLGVAGFRIDAAKH	188
Stmli	-SEINDYGN---RANVQN	CELVGLADLDTGESYVRDRIAYLNDLLSLGVDGFRIDAAKH	209
Psaha	-INNSDYGN---DRYRVQN	CELVGLADLDTASNYVQNTIAAYINDLQAIGVKGFREDAKH	202
Halme	-IEQSDYAQ--NAESVRQ	CQLVGLPDLNLTSDPTVQSRIADYLDTLAALGVGGIRIDAAKH	198

HL11Amy	MPASAWTSIMSAVKTAYPKT--LQGENIWVT	QSIINDG--EVDPRS	YFPIGTINEFQFTY	273
Thefu	IPEGDLQAILSRKKNVHPAWG---GGKPYIF	QEVIAAD--STISTGS	YTHLGSVTEFQYHR	278
Drome	MWPADLAVIYGRKLNLTDHGFASGSKAYIV	QEVIDMGGEAISKS	EYTGIGAITEFRHSD	268
Penva	MWPGDMKAIFDSLNDLNTDF-FKAGARPFIF	QEVIDLGGGEAISSGE	YVGNRVRTFRYK	277
Homsa	MWPGDIKAILDKLHNLNSNW-FPEGSKPFYI	QEVIDLGGGEPIKSSD	YFGNVRVTEFKYGA	275
Pseesp	MPAEDIAAIAKSKLH-----GTPYIF	QEVIGAENEPVKPAE	EYTYIGDVTEFFAR	243
Mibde-A	IPVGDIAAIAKAKLI-----GNPYIF	QEVIGAPGEPIQPSE	EYTYLADVTEFNFTK	237
Stmli	MPAADLTAIAKAVGN-----GSTYWK	QEAIHGAGEAVQPSE	EYLTGTDVQEFYAR	259
Psaha	VAASDIQSLMAKVN-----GSPVVF	QEVIDQGGGEAVGASE	EYLTGLVTEFKYST	251
Halme	MAPSDIAEILAQVD-----APLYAF	QEVIDLGGGEAISATE	EYQGTADITEFRYGA	247

HL11Amy	ALRDVFRGNNGNTLSSLPGIMGTGWNWGGSWGFIQPQNATV	FVTNWD	TERNGSSSLNINNA	333
Thefu	DISHAFAN---GNIAHL-----TGLG--SGLTPSDKAVF	FVNVHDTQRYE	-----PI-	320
Drome	SIGKVFRG--KDQLQYL-----TNWGTAWGFAASDRSLV	FVDNHDNQR	GHGAGGADV-	318
Penva	YLGEAFRG--NNQLKYL-----NNFGEWGMIDRHDALV	FIDNHDNQR	GHGAGGDMI-	327
Homsa	KLGTVIRKWNGEKMSYL-----KNWGEWGMFMPSDRALV	FVDNHDNQR	GHGAGGASI-	327
Pseesp	TLGPKFKH---GGIKDL-----QGIGSWNGWLSSSDAVT	FVTNHD	DEERHNP	289
Mibde-A	SVSHFFKG--RGPIKEL-----KNIGTWGGWVASADAVT	FVANHDNQR	QDTN---NT-	284

Stmli	DLKRVFQN---ENLAHL-----KNFGEDWGYMASGKSAVFVDNH	TERGG-----DT-	303
Psaha	ELGNTFRN---GSLAWL-----SNFGEGWGFMPSSSAVFVDNH	DNQRGHGGAG-NV-	299
Halme	SLGDIFNN---QALANL-----QQFGESPALLPSEQAIVFTDNH	DNQRGHGAGGSNI-	296
$\beta 8$			
HL11Amy	AGNDAANQRYTLANIFMLAQGYAEALYSSEKFSN-----	TDADRPTTSPYSGGVP	384
Thefu	LTHTD-GARYDLAQKFMALHPYGTpkVMSSYTWSGD-----	DKAGPPMHSdGTT---	368
Drome	LTYKV-PKQYKMASAFMLAHFFGTPrVMSSFSFTD-----	TDQGPPTTDGHNIASP	368
Penva	LTFRV-SKQYKMATAYMLAWPYGYTRVMSSYYWDQWWENGQDKNDWIGPPHDGSGFNIISP		386
Homsa	LTFRV-SKQYKMATAYMLAWPYGYTRVMSSYYWDQWWENGQDKNDWIGPPHDGSGFNIISP		386
Pseps	LSHQDFGNLYFLGNVFTLAYPYGYPKVMSSGYFND-----	FDAGPPATGIHTG---	337
Mibde-A	ITHKDGDNRTMAHVFMLGWPYGYPKVMSSYEWYD-----	HDQGPFAHGASSC---	332
Stmli	LNyKN-GSAYTLAGVFMLAWPYGSPDVHSGYEFTD-----	HDAGPPNGGT-----	347
Psaha	ITFED-GRLYDLANVFMLAYPYGYPKVMSSYDFHGD-----	TDAGGPNVPVHNN---	347
Halme	LTHRD-DQLYRLANFMFLAWPYGYPKVMSSYAFSN-----	SDQGPQAPVYQQ---	343
HL11Amy	QI-----NVVWDFAHRW	TPLANMVGFRNAS-IGQAQQNWTVGDNQVAFSRGNVGFV	436
Thefu	---RPTDCSADRWLCEHR--	AVAGMVGfHNAV-AGQGIGSAV-TDGNRLAFARGSGAGYA	421
Drome	IFNSDNsc-SGGWVCEHRWRQIYNMVAFRNTV-GSDEIQNWW-DNGSNQISFSRGSRGFV		425
Penva	SFNADGSC-GNGWICEHRWRQIYNMVAFRNTV-HGTDMDNWW-DNGSNQIAFCRGNKGFL		443
Homsa	TINPDTTC-GNDWVCEHRWRQIRNMVNFRNVV-DGQPFNTWY-DNGSNQVAFGRGNRGFI		442
Pseps	---NACGFDGGDWVCEHRWRGVANMVAFRNHTAAEWRVTNWW-DDGYNQVAFGRGGLGFV		393
Mibde-A	-----NSGWLCEHRTPAIANMVAFRNFTADEFRVTNYW-DNGNNQIAWGRGGKGFV		382
Stmli	---VNACYSdGWKQCHAWPELSSMVGLRNTA-SGQPVNTWW-DNGGDQIAFGRGDkAYV		401
Psaha	---GNLECFASNWKCEHRWSYIAGGVDFRNTADNwAVTNWW-DNTNNQISFGRGSSGHM		403
Halme	---GEAQc-GEAWVCEHRWPEIANMVAFRQQA-EGAEITHWW-DNGHHQIAFSREAQGGFI		397

Figure S1. The alignment of α -amylases from animal species and animal-like α -amylases from fungal and bacterial species. The sequence alignment has been performed by Clustal W. The residues highlighted with yellow represent conserved residues found in animal and animal-like α -amylases. Green represents catalytic triad and grey represents invariant residues. The information of these sequences are shown in Table S3.

References

- Peng H.; Chen M.; Yi L.; Zhang X.; Wang M.; Xiao Y.; Zhang N. Identification and characterization of a novel raw-starch-degrading α -amylase (AmyASS) from the marine fish pathogen *Aeromonas salmonicida* ssp. *salmonicida*. *J Mol Catal B Enzym* **2015**, *119*, pp. 71-77.
- Koropatkin N.M. and Smith T.J. SusG: A Unique cell-membrane-associated α -amylase from a prominent human gut symbiont targets complex starch molecules. *Structure* **2010**, *18*, pp. 200-215.
- Li Z.; Wu J.; Zhang B.; Wang F.; Ye X.; Huang Y.; Huang Q.; Cui Z.; Liu S.J. AmyM, a novel maltohexaose-forming α -amylase from *Coralloccoccus* sp. Strain EGB. *Appl Environ Microbiol* **2015**, *81*, pp. 1977-1987.
- Bai Y.; Huang H.; Meng K.; Shi P.; Yang P.; Luo H.; Luo C.; Feng Y.; Zhang W.; Yao B. Identification of an acidic α -amylase from *Alicyclobacillus* sp. A4 and assessment of its application in the starch industry. *Food Chem* **2012**, *131*, pp. 1473-1478.
- Tawil G.; Viksø-Nielsen A.; Rolland-Sabaté A.; Colonna P.; Buléon A. Hydrolysis of concentrated raw starch: A new very efficient α -amylase from *Anoxybacillus flavothermus*. *Carbohydr Polym* **2012**, *87*, pp. 46-52.
- Gangadharan D.; Nampoothiri K.M.; Sivaramakrishnan S.; Pandey A. Biochemical characterization of raw-starch-digesting alpha amylase purified from *Bacillus amyloliquefaciens*. *Appl Bioche. Biotechnol.* 653-662.
- Puspasari F.; Radjasa O.K.; Noer A.S.; Nurachman Z.; Syah Y.M.; van der Maarel M.; Dijkhuizen L.; Janeček Š.; Natalia D. Raw starch-degrading α -amylase from *Bacillus aquimaris* MKSC 6.2: isolation and expression of the gene, bioinformatics and biochemical characterization of the recombinant enzyme. *J Appl Microbiol* **2013**, *114*, pp. 108-120.

8. Shofiyah S.S.; Yuliani D.; Widya N.; Sarian F.D.; Puspasari F.; Radjasa O.K.; Ihsanawati; Natalia D. Isolation, expression, and characterization of raw starch degrading α -amylase from a marine lake *Bacillus megaterium* NL3. *Heliyon* **2020**, *6*, pp. e05796.
9. Vidilaseris K.; Hidayat K.; Retnoningrum D.S.; Nurachman Z.; Noer A.S.; Natalia D. Biochemical characterization of a raw starch degrading α -amylase from the Indonesian marine bacterium *Bacillus* sp. ALSHL3. *Biologia* **2009**, *64*, pp. 1047-1052.
10. Roy J.K.; Borah A.; Mahanta C.L.; Mukherjee A.K. Cloning and overexpression of raw starch digesting α -amylase gene from *Bacillus subtilis* strain AS01a in *Escherichia coli* and application of the purified recombinant α -amylase (AmyBS-I) in raw starch digestion and baking industry. *J Mol Catal B Enzym* **2013**, *97*, pp. 118-129.
11. Shivilata L. and Satyanarayana T. Characteristics of raw starch-digesting α -Amylase of *Streptomyces badius* DB-1 with transglycosylation activity and its applications. *Appl Biochem Biotechnol* **2017**, *181*, pp. 1283-1303.
12. Peng H.; Zheng Y.; Chen M.; Wang Y.; Xiao Y.; Gao Y. A starch-binding domain identified in α -amylase (AmyP) represents a new family of carbohydrate-binding modules that contribute to enzymatic hydrolysis of soluble starch. *FEBS Lett* **2014**, *588*, pp. 1161-1167.
13. Attanayaka D.P.S.T.G.; Silva S.N.T.D.; Nirosha S.F.; Aththanayaka A.M.W.S. Isolation of raw starch hydrolysing fungi and purification of α -amylase from *Geotrichum candidum* CMSS06. *Journal of the National Science Foundation of Sri Lanka* **2009**, *37*, pp. 93-98.
14. Tawil G.; Viksø-Nielsen A.; Rolland-Sabaté A.; Colonna P.; Buléon A. In depth study of a new highly efficient raw starch hydrolyzing α -amylase from *Rhizomucor* sp. *Biomacromolecules* **2011**, *12*, pp. 34-42.
15. Hostinová E.; Janeček Š.; Gašperík J. Gene Sequence, Bioinformatics and enzymatic characterization of α -amylase from *Saccharomycopsis fibuligera* KZ. *Protein J* **2010**, *29*, pp. 355-364.
16. Da Lage J.L.; Feller G.; Janecek S. Horizontal gene transfer from Eukarya to bacteria and domain shuffling: the alpha-amylase model. *Cell Mol Life Sci* **2004**, *61*, pp. 97-109.