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Heterologous Expression of *Scytalidium thermophilum* Laccase Gene in *Aspergillus sojae* and Bioinformatic Analysis of Propeptide Cleavage Sites§

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Abstract

Scytalidium thermophilum is a thermophilic fungus with an important role in determining selectivity of compost produced for growing *Agaricus bisporus*. *S. thermophilum* laccase gene was first cloned by Novo Nordisk Bio Tech, Inc. in 1998. This laccase gene (*lccS*) has an open reading frame of 2092 bp. It is composed of five exons punctuated by four small introns. The coding region, excluding intervening sequences is very GC-rich (60.8% G+C) and encodes a preproenzyme of 616 amino acids: a 21 amino acid signal peptide and a 24 amino acid predicted propeptide. *lccS* gene was amplified using specific primers to exclude the signal and pro-peptide coding regions and ligated to expression vector pAN52-4. The recombinant plasmid was used to transform *Aspergillus sojae* ATCC11906 ($pyrG^-$). Heterologuos expression was observed in glucose-containing media, under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and the secretion signal of glucoamylase gene. Heterologous expression of laccase is an important step towards the high level production of this enzyme in a GRAS (Generally Recognized as Safe) eukaryotic host and for further biotransformation and enzyme engineering studies. Also, in this study analysis of N-terminal and C-terminal propeptide cleavage sites of fungal proteins including laccases were studied.

Key words: Laccase, gene cloning, *Scytalidium thermophilum*, propeptide

Scytalidium thermophilum Lakkaz Geninin *Aspergillus sojae'de* Heterolog Ekspresyonu ve Propeptid Kesim Bölgelerinin Biyoinformatik Analizi

Özet

Scytalidium thermophilum sıcaklığa dayanıklı bir küf olup, *Agaricus bisporus* organizmasının büyümesi için üretilen gübrenin seçiciliğinin belirlenmesinde önemli rol oynamaktadır. Bu gen (*lccS*) ilk defa 1998'de Novo Nordisk Bio Tech Inc. tarafından klonlanmıştır. Okuma çerçevesinde 2092 baz çiftinden ve dört kısa intronun böldüğü beş ekzondan oluşmaktadır. Intronların dışında kalan kodlanan bölge sekansı G-C'lerce zengin olup (60.8% G+C), 21 amino asiti signal peptit ve 24 amino asiti propeptit olmak üzere 616 amino asitlik bir preproenzim sentezlemektedir. *lccS* geni, signal peptit ve pro-peptit kodlayan bölgeleri dışarıda bırakacak özgül primerle çoğaltılmış, pAN52- 4 ekspresyon vektörüne takılmış ve *Aspergillus sojae* ATCC11906 ($pyrG^-$) organizmasına klonlanmıştır. Glukoamilaz geninin signal peptit bölgesi altına takılan genin, gliseraldehit 3-fosfat dehidrojenaz promotorı kontrolünde, glukoz içeren ortamda heterolog ekpresyonu gözlenmiştir. Lakkazın heterolog üretimi, enzimin ökaryotik GRAS bir organizmada yüksek düzeyde ekspresyonunu sağlamak ve daha sonraki biotransformasyon ve enzim mühendisliği çalışmalarında kullanılması yönünde atılmış önemli bir adımdır. Bu çalışmada ayrıca lakkazları da içeren küf proteinlerinin N ve C uçlarındaki propeptit kesim bölgeleri incelenmiştir.

Anahtar Kelimeler: Lakkaz, gen klonlama, *Scytalidium thermophilum*, propeptit

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Introduction

Thermophilic fungi are of central importance as a source of thermostable enzymes, which are important for industrial utilization because of the possible economic benefits of being able to degrade plant residues at elevated temperature (Maheshwari et al., 2000). *Scytalidium thermophilum* is a thermophilic Deuteromycete, and a member of the *Torula-Humicola* complex, which are recognized as dominant species in mushroom compost (Wiegant, 1992). *Aspergillus sojae*, which is believed to be a domesticated strain of *Aspergillus parasiticus*, contains all of the aflatoxin biosynthetic genes but is unable to produce aflatoxins and is generally recognized as safe (GRAS) for producing fermented foods (Chang, 2004).

Laccases (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) are multicopper enzymes, which catalyze the oxidation of a variety of organic and inorganic substrates coupled to the reduction of molecular oxygen to water with the one electron oxidation mechanism. Laccase catalyzes the oxidation of a broad range of substrates e.g. polyphenols, substituted phenols, diamines, but also some inorganic compounds (Thurson, 1994). These enzymes are widely distributed in nature. Laccase or laccase-like activity has been demonstrated in higher plants; in some insects and sequence homology analyses suggest that laccases could also occur in bacteria such as *Mycobacterium tuberculosis* (Alexandre and Zhulin, 2000). However, the best-known laccases are of fungal origin and have been reported from ascomycete, basidiomycete and deuteromycete fungi (Bollag and Leonowicz, 1984). Laccase is an important enzyme because of its potential use in several areas such as textile, paper and pulp industries. (Eggert et al., 1997). Laccase can be used in bioremediation (Duran and Esposito, 2000), beverage (wine, fruit juice and beer) processing (Cantarelli et al., 1989), and as biosensor (Couto and Herrera, 2006).

The *Scytalidium thermophilum* laccase gene was first cloned by Novo Nordisk Bio Tech, Inc in 1998 (Patent No: US 5843745 A). This laccase gene (*lccS*) has an open reading frame of 2092 bp. It is composed of five exons punctuated by four small introns. The coding region, excluding intervening sequences is very GC-rich (60.8% G+C) and encodes a preproenzyme of 616 amino acids: a 21 amino acid signal peptide and a 24 amino acid predicted propeptide. In the first part of this study, it was aimed to clone and express the *Scytalidium thermophilum* laccase gene in *Aspergillus sojae* to overexpress the enzyme for future use in biotransformatives and to be able to perform site-directed mutagenesis. In the second part of the study, a bioinformatic approach was used to collect fungal propeptide sequences and to classify them according to their cleavage sites. This information was used to have an overview of the amino acid requirements for cleavage during secretion of fungal proteins. A separate comprise was made between laccase propeptide regions and the overall results were evaluated to draw a number of conclusions that might be of value in further understanding the importance of propeptides in the fungal secretion process.

Materials and Methods

Strains and Media

S. thermophilum (type culture *Humicola insolens*, ATCC No. 16454) kindly provided by ORBA, İstanbul, was inoculated onto YpSs agar plates (Cooney and Emerson, 1964) and incubated at 45 °C for 4-5 days until sporulation was complete. These agar plates can be stored at 20°C for up to 2 months. Spores from these stock cultures were inoculated into a liquid pre-culture media (5 mL) known as YpSs broth, which contains 1% (w/v) glucose instead of starch as a carbon source. After 24 h of incubation at 45°C, the pre-culture (5 mL) was transferred into the main culture (250 mL) supplemented with copper sulfate. Cultures were incubated in a shaker incubator at 45°C and 155 rpm.

Escherichia coli XL1 Blue MRF was supplied from Stratagene (La Jolla, USA) and maintained according to the instructions of the supplier.

Aspergillus sojae strain ATCC11906 was kindly supplied by Dr. P. J. Punt (TNO Nutrition and Food Research, Dept. of Microbiology, Zeist, The Netherlands). Stock cultures of *A. sojae* were grown on complete medium agar (70 mM $NaNO_3$, 7 mM KCl, 11 mM KH_2PO_4, 2 mM Mg_2SO_4, 1% (w/v) glucose, 0.5% (w/v) yeast extract, 0.2% (w/v) casamino acids, 1x trace elements (1.000x stock: 76 mM $ZnSO_4$, 178 mM H_3BO_3, 25 mM $MnCl_2$, 18 mM $FeSO_4$, 7.1 mM $CoCl_2$, 6.4 mM $CuSO_4$, 6.2 mM $NaMoO_4$, 174 mM EDTA), 1x vitamin solution (1.000x stock: 100 mg L^{-1} thiamin, 100 mg L^{-1} riboflavin, 100 mg L^{-1} nicotinamide, 50 mg L^{-1} pyridoxine, 10 mg L^{-1} pantothenic acid, 0.2 mg L^{-1} biotin) and 1.5% (w/v) agar with or without uridine/uracil supplementation (5 mM /5 mM).

PCR Cloning of the lccS Gene

In order to clone *lccS* gene onto vector pAN52-4, a new set of primers were designed as; lacP1(forward)
(5ˈCGTCTCGGCAAGCTTCCTGTCATTCTCCAAGC 3ˈ)
lacP2(reverse)
(5ˈAATCCGCCCAAAGCTTTCACCGATCCCATCGC 3ˈ).

lacP1-lacP2 primer pairs include *Hin*dIII restriction enzyme recognition regions to provide cloning the amplified fragment to vector pAN52-4. The vector pAN52-4 includes glucoamylase gene of *Aspergillus niger* signal peptide and propeptide regions. So the primer lacP1 was designed such that the amplified *lccS* gene lacks the signal peptide and propeptide.

The *lccS* gene was amplified using Taq DNA polymerase (Invitrogene) under the following conditions; *initial* denaturing at 95 °C for 3 min; then 35 cycles at 94°C for 30 s, 57 °C for 60 s, and 72°C for 90 s; and finally a 10 min extension phase at 72°C.

Cloning and Expression in Aspergillus sojae

pAN52-4 (GenBank accession no. Z32699) vector was kindly provided by Dr. P.J. Punt. It is a fungal *expression* and secretion vector. It includes *A. niger* glucoamylase gene (*gla*A) preprosequence. Laccase gene was put under the control of the glyceraldehyde triphosphate dehydrogenase (*gpd*A) promoter of *A. nidulans* using the unique BamHI/HindIII sites of the vector. The plasmid, carrying the laccase gene on vector pAN52-4 was named as pAN52-4LS. The host strain *A. sojae* ATCC11906 (*pyrG*$^-$), which is a uridine auxotroph, was pre-grown in complete medium at 30°C and 155 rpm for 18 h and was transformed with the plasmid pAMDSPYG carrying the marker gene, *pyr*G of *A. niger* and pAN52-4LS together, at a ratio of 1:10, as described by Punt et al (2002). Transformants were selected for uridine prototrophy on complete medium without supplementation. Fungal transformants were cultivated at 30°C and 155 rpm on 1.250 mL modified YpSs broth containing 2% glucose instead of starch.

Laccase Assays

Laccase activity was determined on an aliquot of culture supernatant, by monitoring the oxidation time course of 0.5 mM ABTS at 420 nm, in the presence of 0.1 mM acetate buffer, pH 5.0 at 60 °C, 0.5 ml of culture supernatant and 1 ml of buffer solution. The substrate blank cuvette contained 500 µl buffer solution. One unit of enzyme activity was defined as a change in optical density at 420 nm of 0.01 per minute under the stated assay conditions. The enzyme activity was expressed in units defined as 1 U = 1 µmole of substrate oxidized (or 1 µmole product formed) in one minute by 1 ml of culture supernatant.

Results and Discussion

Multiple Sequence Alignment of Laccase

Laccase gene *(lccS)* has an open reading frame of 2092 bp. It is composed of five exons (243, 91, 70, 1054 and 390 nucleotides) punctuated by four small introns (63, 58, 55 and 65 nucleotides). The coding region encodes a preproenzyme of 616 amino acids: a 21 amino acid signal peptide and a 24 amino acid predicted propeptide (US 5843745 A). Amplification with lacP1-lacP2 primers was yielded a PCR product of an expected size of about 1983 bp without propeptide and signal peptide sites. Comparison with other laccase proteins was performed in order to detect the sequence conservation patterns. For this aim, multiple sequence alignments were performed with the help of software ClustalW and NCBI web sites (Figure 1). The fungal laccases contain several highly conserved ungapped regions, distributed almost throughout the length of the proteins. The copper-ligating residues in the *S. thermophilum* laccase are housed in regions that are conserved across all laccases. A total of 10 histidines and 1 cysteine are completely conserved and they serve as the copper ligands. These residues are housed in the four conserved regions L1-L4. Specifically, the motif HWHG in the region L1 is equivalent to the motif HLHG in the region L3. Similarly, between the region L2 and L4, Gly is the common conserved residue at positions 1 and 16, while His is the common conserved residue at positions 6 and 8 (Kumar et al., 2003). At the C-terminal some laccases from ascomycetes *N. crassa* (Germann et al., 1988), *P. anserine* (Fernandez-Larrea and Stahl, 1996), *M. thermophila* (Bulter et al., 2003) are processed at the processing site Asp-Ser-Gly-Leu, which is the conserved region of these laccases. When we look at the C- terminus of the *S. thermophilum* laccase after the alignment of amino acid sequence with some other fungal sequences, it was observed that it might also require the removal of a C-terminal peptide in addition to the posttranslational removal of a signal sequence and propeptide cleavage at the N-terminus like other ascomycete laccases (Figure 2).

Expression of lccS in Aspergillus sojae

The maximum laccase activity was 70 U L^{-1} at 96 hours (Figure 3). In this study, it was possible to produce *S. thermophilum* laccase gene extracellularly in *A. sojae*. In the previous study *carried* out by Novo Nordisk Bio Tech. activity of *S. thermophilum* laccase gene cloned into *Aspergillus oryzae* was tested by using either syringaldazine or ABTS as substrates and found 2200 or 4200 U L^{-1}, respectively. These values are higher than the activity of the *S. thermophilum* laccase expressed in *A. sojae*. The optimum conditions can be studied to improve the stability and extracellular activity of heterologous laccase enzyme by altering pH, temperature and culture medium contents.

Propeptide Regions of Multicupper Oxidases

S. thermophilum laccase protein possesses a high similarity with *Emericella nidulans* laccase protein preprosequence. The probable signal peptide cleavage site was also found *through* Expasy web site. Both the sequence alignment, and bioinformatic results indicate the same signal peptide cleavage site (Figure 4). *S. thermophilum* laccase protein propeptide cleavage site is not clear however according to sequence alignment it could be at the point of Ser amino acid. (Figure 4).

In conclusion, cloning of *Scytalidium thermophilum* laccase gene would give an opportunity to overexpress the enzyme for future use in biotransformations and to be able to perform site-directed mutagenesis. In this study, PCR cloning studies were performed on the laccase *gene* of *S. thermophilum*. The l*ccS* gene was ligated onto pAN52-4 vector without its signal and propeptide regions. This resulted in a construct where the *lccS* gene is fused to the signal-pro-peptide region of the *A. niger* glucoamylase gene. Furthermore, the gene was put under the control of the constitutive *gdp*A promoter to allow high-level expression in the presence of glucose and absence of an inducer. Accordingly, it was possible to express *S. thermophilum* laccase in *A. sojae* at a level of 70 U L^{-1}, which is within the acceptable limits of laccase production in the literature. In future studies, heterologous expression in *A. sojae* will be optimized and the gene will be used in mutagenesis. This system will also allow the use of recombinant *A. sojae* culture supernatants in biocatalysis.

```
1.    ----MRLSNALVLVAACIS----------------------------------------- 15
2.    ---MSRFHSLLAFVVASLA----------------------------------------- 16
3.    --MK--FLGIAALVAGLLAPSLVLGAPAPGTEGVNLLTPVDKRQDSQAERYGGGGGGCN 56
4.    --MKRFFINSLLLLAGLLN-SGALAAPSTHPR-SNPDILLERDDHSLTSRQ-----GSCH 51
5.    MDVTKSLLCFISFVAFLLFS---------------------------------------- 20
                  :     ::.  :

1.    ----------------------SVVAKTR--TFDFDLVNTRLAPDGFERDTVVIN-GEFP 50
2.    ----------------------AVAHAGIGPVADLTITNAAVSPDGFSRQAVVVN-GGTP 53
3.    SPTNRQCWSPGFNINTDYELGTPNTGKTRRYKLTLTETDNWLGPDGVIKDKVMMVNDNII 116
4.    SPSNRACWCSGFDINTDYETKTPNTGVVRRYTFDITEVDNRPGPDGVIKEKLMLINDKLL 111
5.    ----------------------SVAEANKAHHHEFIIQATKVKRLCETHNSITVN-GMFP 57
                            . .         :             :: : :  .

1.    GTLIQVNKGDSVRIPLHNKLTSPTMRRSVSIHWHGFFQARTSGQDGPSFVNQCPQPPNTT 110
2.    GPLITGNMGDRFQLNVIDNLTDHTMLKSTSIHWHGFFQKGTNWADGPAFINQCPISSGHS 113
3.    GPTIQADWGDYIEITVINKLKS----NGTSIHWHGMHQRNSNIQDGVNGVTECPIPPRGG 172
4.    GPTVFANWGDTIEVTVNNHLRT----NGTSIHWHGLHQKGTNYHDGANGVTECPIPP-GG 166
5.    GPMLVVNNGDTLVVKVINRARY-----NITIHWHGVRQMRTGWADGPEFVTQCPIRPGSS 112
      *. :  : ** . : : :.    :   . :*****. *  :.  **   :.:**  .

1.    FTYEFSVAEQSGTFWYHSHLSTQYCDGLRGAFIVYDPRDPLRHLYDVDDESTVITLAEWY 170
2.    FLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPAADLYDVDNDDTVITLADWY 173
3.    SKVYRWRATQYGTSWYHSHFSAQYGNGIVGPIVING---PASANYDVDLG--PFPLTDYY 227
4.    SRVYSFRARQYGTSWYHSHFSAQYGNGVSGAIQING---PASLPYDIDLG--VLPLQDWY 221
5.    YTYRFTIQGQEGTLWWHAHSSWLRAT-VYGSLLVFPP-AGSSYPFTKPHRNVPLLLGEWW 170
               * ** *:*:* *     .: *.: :  .       :     .  : * :::

1.    HILAPDATNEFFSSGIIPV-QDSGLINGK-GRFNGGPLTPFAVVNVRRGKRYRLRVIAIS 228
2.    HVAAKLGP--AFPLG-----ADATLINGK-GRSPSTTTADLTVISVTPGKRYRFRLVSLS 225
3.    YDTADRLVLLTQHAG-PPP-SNNVLFNGF-AKHPTTGAGQYATVSLTKGKKHRLRLINTS 284
4.    YKSADQLVIETLAKGNAPF-SDNVLINGT-AKHPTTGEGEYAIVKLTPDKRHRLRLINMS 279
5.    DANPVDVLRESIRTGGAPNNSDAYTINGQPGDLYKCSSQDTTVVPINVGETILLRVINSA 230
         .          *  .   :   :**  .          : : :  .:   :*::  :

1.    CRPFFTFSVDNHSLVFMEADGVEHDPVEVQNVDIYAAQRVSVILHANQPIDNYWIRAPMT 288
2.    CDPNHTFSIDGHNMTIIETDSINTAPLVVDSIQIFAAQRYSFVLEANQAVDNYWIRANPS 285
3.    VENHFQLSLVNHSMTIISADLVPVQPYKVDSLLLGIGQRYDVIIDANQAVGNYWFN--VT 342
4.    VENHFQVSLAKHTMTVIAADMVPVNAMTVDSLFMAVGQRYDVTIDASQAVGNYWFN--IT 337
5.    LNQPLFFTVANHKLTVVGADASYLKPFTTNVIVLGPGQTTDVLITGDQPPNRYYMAARAY 290
            .::  *.:..: :*     .  .: : :  .*  .. : ..*. ..*:: :

1.    GGNPDRNPNLNISLTLAILRYHGARHVEPTTVNVP--GHKLLDQE-MHPIR--------- 336
2.    FGNVGFTGGINS----AILRYDGAAAIEPTTTQTT--STEPLNEVNLHPLV--------- 330
3.    FGGNDLCGTSDNKYPAAIFRYQGAPKALPTNKGVAPPDHQCLDLNDLKPVL--------- 393
4.    FGGQQKCGFSHNPAPAAIFRYEGAPDALPTDPGAAPKDHQCLDTLDLSPVV--------- 388
5.    QSAQN--APFGNTTTTAILQYKSAPCCGVGGGSGTKKGNSFKPIMPILPAYNDTNTVTRF 348
       .            . **::*..*          .  . .      : *
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1.  -------------------------------QEG-PGKLGDGPPDKHITLNIAQPN---- 360
2.  -------------------------------ATAVPGSPAAGGVDLAINMAFNFNG---- 355
3.  -------------------------------QRSLNTNSIALNTGNTIPITLDG------ 416
4.  -------------------------------QKNVPVDGFVKEPGNTLPVTLHVDQAAAP 417
5.  SQSFRSLRRAEVPTEIDENLFVTIGLGLNNCPKNFRSRRCQGPNGTRFTASMNNVSFALP 408
                                                .  :   :

1.  --------APFFDINGISYISPTVPVLLQILSGAKRPEDVLP---SEQIFFVPKNSLIEV 409
2.  --------TNFF-INGASFTPPTVPVLLQIISGAQNAQDLLP---SGSVYSLPSNADIEI 403
3.  --------FVWRVNGTAININWNKPVLEYVMTGNTN-YSQSD---NIVQVEGVNQWKYWL 464
4.  H------VFTWKINGSAADVDWDRPVLEYVMNNDLSSIPVKN---NIVRVDGVNEWTYWL 468
5.  SNYSLLQAHHHGIPGVFTTDFPAKPPVKFDYTGNNISRSLYQPDRGTKLYKLKYGSRVQI 468
                  .        * :    ..   .        .             :

1.  NIPG-----EGAHPFHLHGHNFDVVLASNDDTFNFKNPPRRDVY----PINGGN------ 454
2.  SFPATAAAPGAPHPFHLHGHAFAVVRSAGSTVYNYDNPIFRDVVSTGTPAAGDN------ 457
3.  IENDPDGAFSLPHPIHLHGHDFLILGRSPDVTAISQTRYVFDPAVDMARLNGNNPTRRDT 524
4.  VENDPEGRLSLPHPMHLHGHDFFVLGRSPDVSPDSETRFVFDPAVDLPRLRGHNPVRRDV 528
5.  VLQDTGIVTPENHPIHLHGYDFYIIAEGFGNFNPKKDTAKFNLEDPPLRNTVGVPVN--- 525
                **:****: * ::  . .    .     :

1.  --------TTFRFFTDNPGAWFLHCHIDWHLEAGLAIVFAEAPEDNVSGPQSQITPQDWL 506
2.  --------VTIRFRTDNPGPWFLHCHIDFHLEAGFAVVFAEDIPDVAS---ANPVPQAWS 506
3.  AMLPAKGWLLIAFRTDNPGSWLMHCHIAWHVSGGLSNQFLERAQDLRN-SISPADKKAFN 583
4.  TMLPARGWLLLAFRTDNPGAWLFHCHIAWHVSGGLSVDFLERPDELRG-QLTGESKAELE 587
5.  ------GWAVIRFIADNPGVWIMHCHLDAHISWGLAMAFLVE--NGNGVLQTIEQPPHDL 577
              : * :**** *::***:  *:. *::  *      :  .   :

1.              DLCPEYNAIEPEFQ---------------------- 520
2.              DLCPTYDARDPSDQ---------------------- 520
3.              DNCDAWRAYFPDNAPFPKDDSGLRSGVKAREVKMKW 619
4.              RVCREWKDWEAK-SPHGKIDSGLKQ--RRWDA---- 616
5.              PVC--------------------------------- 580
```

Figure 1. Sequence alignment of *Agaricus bisporus* (1) (Genbank AAC18877) *Trametes versicolor* (2) (Genbank CAA77015) *Neurospora crassa* (3) (Genbank AAA33591) *Scytalidium thermophilum* (4), *Arabidopsis thaliana* (5) (Genbank AAM77221) species copper-binding domains in laccase proteins.

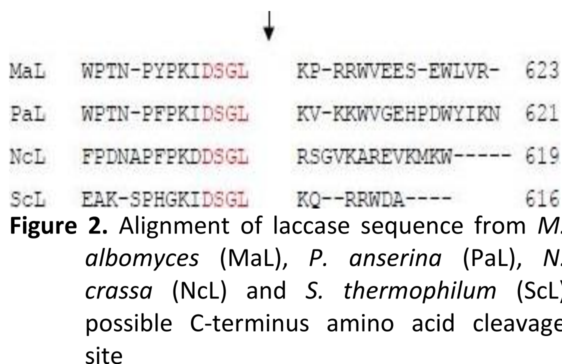

Figure 2. Alignment of laccase sequence from *M. albomyces* (MaL), *P. anserina* (PaL), *N. crassa* (NcL) and *S. thermophilum* (ScL) possible C-terminus amino acid cleavage site

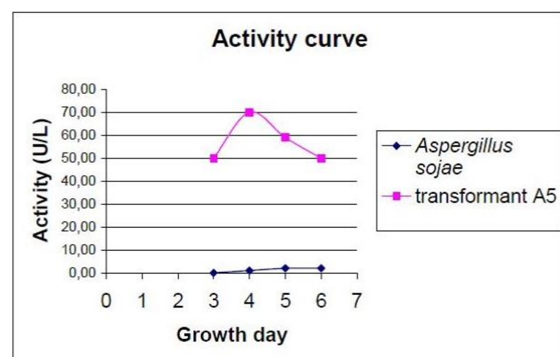

Figure 3. Enzyme activity of transformant A5

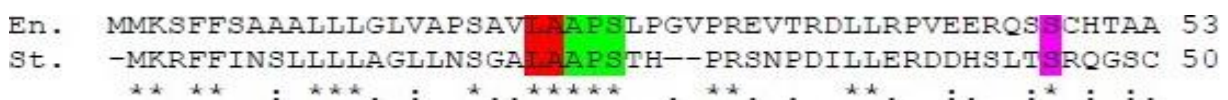

Figure 4. Similarity of signal peptide processing site between laccases of *E. nidulans* (En) and *S. thermophilum* (St).

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