Expression of lignin-degrading enzymes and applications in pretreatment of biomass

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Institute of Biotechnology (IBT)

- IBT is one of the leading national institution in Vietnam with missions in basic research and applications of Biotechnology in agriculture, industry, medicine, environment, ect.

- IBT plays the important role in research, training and technology transfer covering areas involved in human, animal, plant, microorganisms and environment.

Organization of IBT

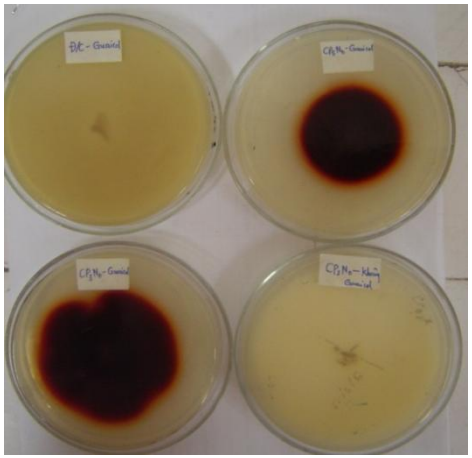
- Established in June, 1993
- Directorate: 1 Director and 2 Deputy Directors
- Scientific Council: Members total 21
- Staff: 288 persons, including 3 Prof., 15 Assoc. Prof., 73 PhD., 68 Masters (*75% under 45, 62% under 35*)
- 25 research laboratories.

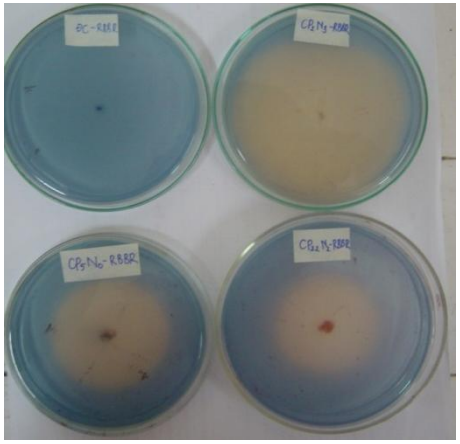
Research fields

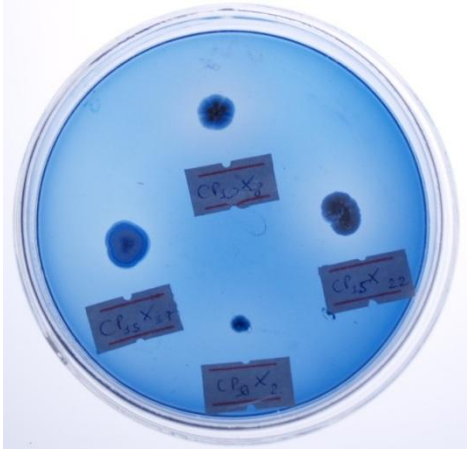
1. Plant Biotechnology
2. Animal Biotechnology
3. Microbiology
4. Molecular Biology & Genetic Engineering
5. Protein & Enzyme Biotechnology
6. Environment protection
7. Marine biotechnology

Screening of microorganisms exhibiting activities of lignin peroxidase (LiP) and laccase (LAC)

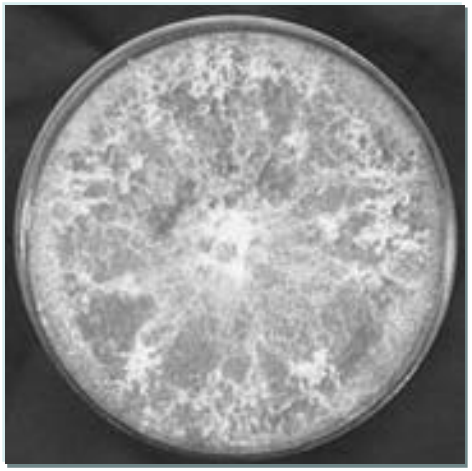
- Isolated 13 bacteria, 208 actinomycetes, 61 fungi/basidiomycetes showing lignin degrading activity.







Catalytic properties of purified LIP isozymes from *Phanerochaete chrysosporium*



Phanerochaete chrysosporium showed the most capability of degrading lignin

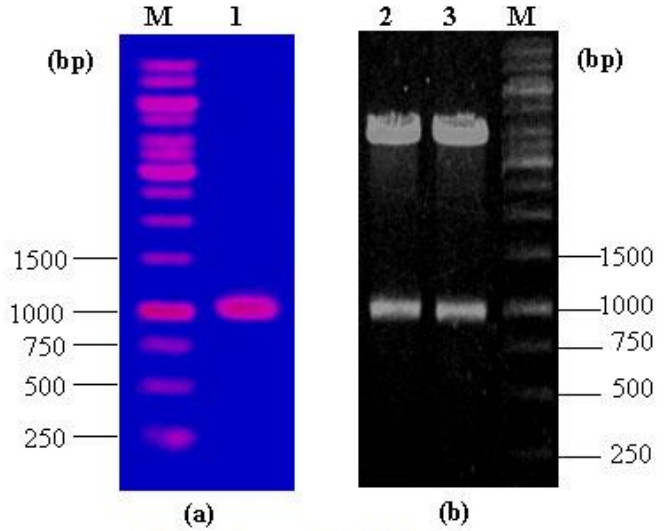
Isozyme	k_{cat} (s^{-1})	K_m – VA (μM)	K_m – H_2O_2 (μM)
H2	17.3 ± 0.66	246.6 ± 29.4	17.7 ± 1.8
H1	22.4 ± 0.43	207.1 ± 12.3	21.4 ± 2.1
H6	18.8 ± 0.54	166.5 ± 20.7	18.8 ± 1.0
Ha	17.7 ± 0.79	169.1 ± 16.1	22.0 ± 3.0
H8	8.1 ± 0.17	165.7 ± 10.3	10.4 ± 2.4
Hb	8.4 ± 0.41	156.2 ± 19.0	13.2 ± 0.8
H10	8.7 ± 0.36	206.3 ± 1.3	9.8 ± 4.8
Hc	11.9 ± 0.40	231.0 ± 7.4	13.3 ± 2.7

Ref: *Appl.Environ.Microbiol.,1997, p. 857–861*

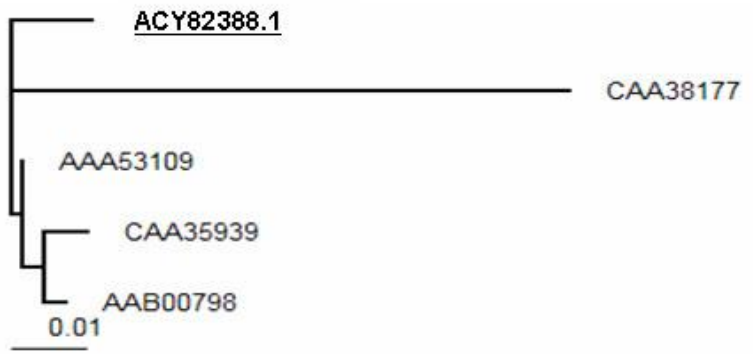
Oxidation of a hierarchical series of methoxy benzenes (~lignin analogs) by WI 71 environment charge mutants

Enzyme	1,2,4 TMB	1,4 DMB	1,2,3 TMB	1,3 DMB
WT LiPH8*	+	+	+	-
[W171F] LiPH8*	-	-	-	-
[D165N] LiPH8*	+	+	+	+
[E168Q] LiPH8*	+	+	+	-
[E250Q] LiPH8*	+	+	-	-
HRP (Kersten *et al.*)	+	-	-	-
Laccase	-	-	-	-
Redox potential	1.37	1.59	1.67	n.d. (>1.74)

Cloning of *lipH8* gene from *P. chrysosporium*



Cloning *lipH8* gene



Phylogenetic tree of LiPH8 sequences

Features Sequence

```
LOCUS       GU119913                  990 bp    DNA     linear
DEFINITION  Phanerochaete chrysosporium strain 36210 lignin peroxidase isozyme
            H8 (lipH8) gene, partial cds.
ACCESSION   GU119913
VERSION     GU119913.1  GI:267850580
KEYWORDS    .
SOURCE      Phanerochaete chrysosporium (anamorph: Sporotrichum pruinosum)
  ORGANISM  Phanerochaete chrysosporium
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Multiple alignment of deduced amino acid sequence of the LiPH8 with the known corralative ones

```
CAA35939        ATCSNGKTVGDASSCAWFDVLDDIQQNLFHGGQCGAEAHESIRLVFHDSIAISPAMEAQG
AAB00798        ATCSNGKTVGDACSCAWFDVLDDIQQNLFHGGQCGAEAHESIRLVFHDSIAISPAMEAQG
AAA53109        ATCSNGKTVGDASCCAWFDVLDDIQQNLFHGGQCGAEAHESIRLVFHDSIAISPAMEAQG
ACY82388.1      ATCSNGKTVGDASCCAWFDVLDDIQQNLFHGGQCGAEAHESIRLVFHDSIAISPAMEAQG
CAA38177        ATCSNGATVGDASCCAWFDVLDDIQQNLFQGGQCGAEAHESIRLVFHDAIAISPAMEAQG
                ****** ******..***************:******************:**********

CAA35939        KFGGGGADGSIMIFDDIETAFHPNIGLDEIVKLQKPFVQKHGCTPGDFIAFAGAVALSNC
AAB00798        KFGGGGADGSIMIFDDIETAFHPNIGLDEIVKLQKPFVQKHGVTPGDFIAFAGAVALSNC
AAA53109        KFGGGGADGSIMIFDDIETAFHPNIGLDEIVKLQKPFVQKHGVTPGDFIAFAGAVALSNC
ACY82388.1      KFGGGGADGSIMIF--------------EIVKLQKPFVQKHGVTPGDFIAFAGAVALSNC
CAA38177        KFGGGGADGSIMIFDDIEPNFHPNIGLDEIINLQKPFVQKHGVTPGAFIAFAGAVALSNC
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CAA35939        PGAPQMNFFTGRAPATQAAPDGLVPEPFHTVDQIINRVNDAGEFDELELVWMLSAHSVAA
AAB00798        PGAPQMNFFTGRAPATQPAPDGLVPEPFHTVDQIINRVNDAGEFDELELVWMLSAHSVAA
AAA53109        PGAPQMNFFTGRAPATQPAPDGLVPEPFHTVDQIINRVNDAGEFDELELVWMLSAHSVAA
ACY82388.1      PGAPQMNFFTGRAPATQPAPDGLVPEPFHTVDQIINRVNDAGEFDELELVWMLSAHSVAA
CAA38177        PGAPQMNFFTGRAPATQPAPDGLVPEPFHTVDQIIARVNDAGEFDELELVWMLSAHSVAA
                *****************.***************** ************************

CAA35939        VNDVDPTVQGLPFDSTPGIFDSQFFVETQLRGTAFPGSGGNQGEVESPLPGEIRIQSDHT
AAB00798        VNDVDPTVQGLPFDSTPGIFDSQFFVETQLRGTAFPGSGGNQGEVESPLPGEIRIQSDHT
AAA53109        VNDVDPTVQGLPFDSTPGIFDSQFFVETQLRGTAFPGSGGNQGEVESPLPGEIRIQSDHT
ACY82388.1      VNDVDPTVQGLPFDSTPGIFDSQFFVETQLRGTAFPGSGGNQGEVESPLPGEIRIQSDHT
CAA38177        VNDVDPTVQGLPFDSTPGIFDSQFFVETQFRGILFPGSGGNQGEVESGMAGEIRIQTDHT
                *******************************:**   ************* :.******:***

CAA35939        IARDSRTACEWQSFVNNQSKLVDDFQFIFLALTQLGQDPNAMTDCSDVIPQSKPIPGNLP
AAB00798        IARDSRTACEWQSFVNNQSKLVDDFQFIFLALTQLGQDPNAMTDCSDVIPQSKPIPGNLP
AAA53109        IARDSRTACEWQSFVNNQSKLVDDFQFIFLALTQLGQDPNAMTDCSDVIPQSKPIPGNLP
ACY82388.1      IARDSRTACEWRSFVNNQSKLVDDFQFIFLALTQLGQDPNAMTDCSDVIPQSKPIPGNLP
CAA38177        LARDSRTACEWQSFVNNQSKLVSDFQFIFLALTQLGQDPNAMTDCSDVIPISKPIPGNLP
                :**********:**********.**************************** *********

CAA35939        FSFFPAGKTIKDVEQACAETPFPTLTTLPGPETSVQRIPPPPGA
AAB00798        FSFFPAGKTIKDVEQACAETPFPTLTTLPGPETSVQRIPPPPGA
AAA53109        FSFFPAGKTIKDVEQACAETPFPTLTTLPGPETSVQRIPPPPGA
ACY82388.1      FSFFPAGKTIKDVEQACAETPFPTLTTPPGPETSVQRIPPPTGC
CAA38177        FSFFPPGKSMKDVEQACAETPFPSLVTLPGPATSVARIPPPPGA
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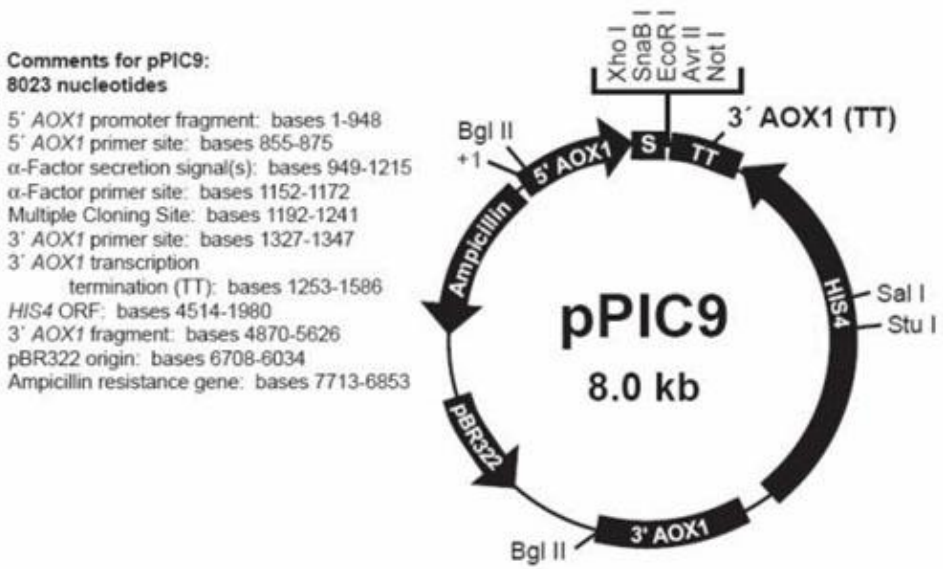
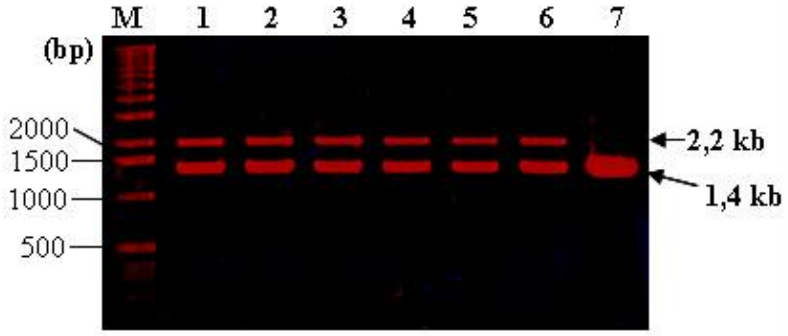
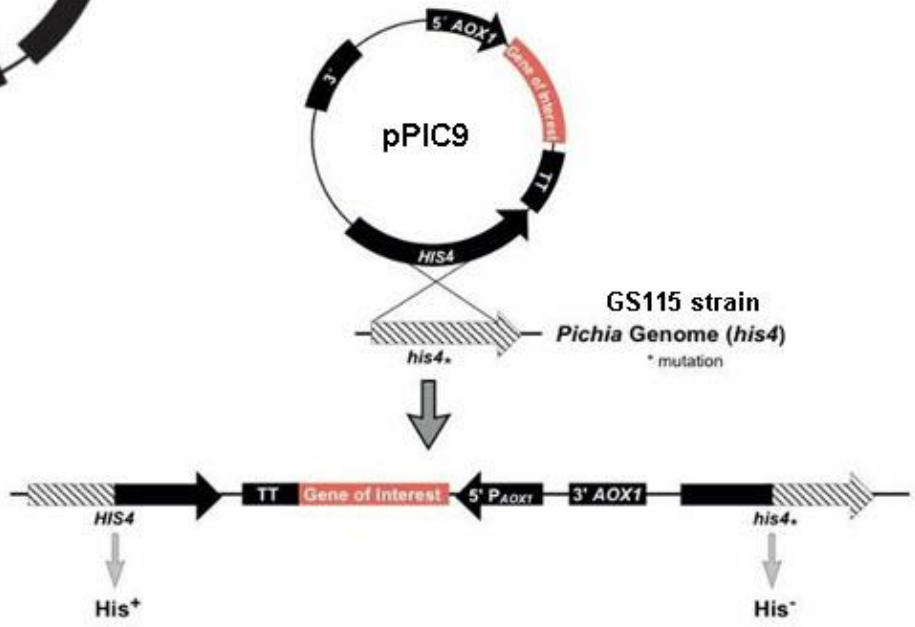


Construction of expression vector for expression of *lipH8* in *Pichia pastoris*



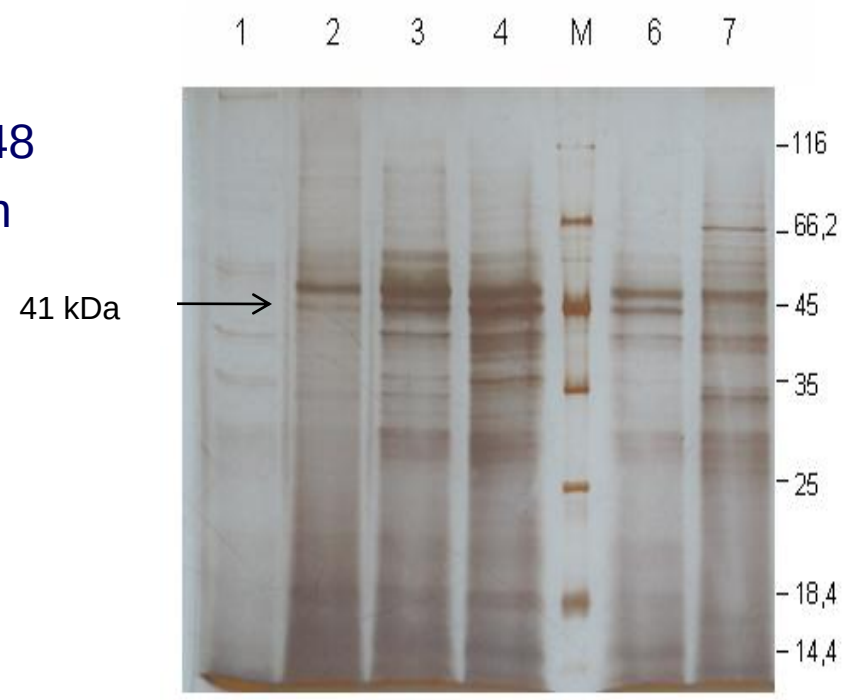
Cheking up the presence of *lipH8* gene on genome of yeast transformants by AOX1 primers



Biosynthesis of recombinant LiPH8 by *P. pastoris*

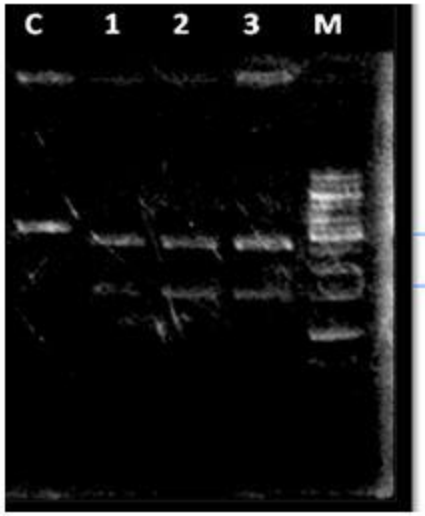
- LiP H8 activity reachs up 11202,48 nkat/l after 66-72 hrs of cultivation

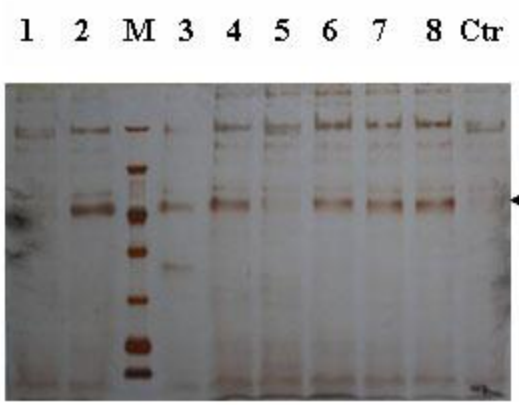
Stt	Medium BMGY-LiP	
1	Yeast extract	2%
2	Pepton	1%
3	K2HPO4 100mM	100 ml
4	YNB	1,34%
5	Biotin	$4x10^{-5}$%
6	Glycerol	1,5%
7	Methanol	2%

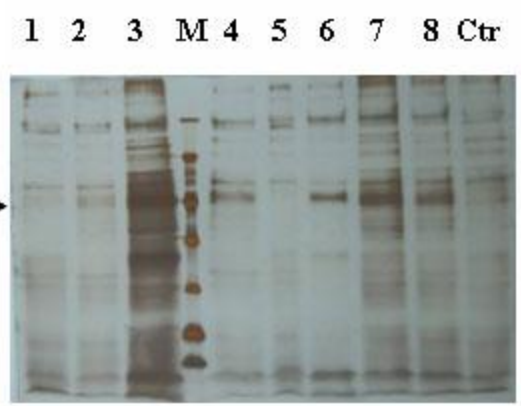


SDS-PAGE analysis of protein produced by *P. pastoris* GS115/pPIC9::*mlipH8* with methanol induction
Lanes 1; 2; 3; 4; 6; 7 showed extracellular proteins after 0; 24; 48; 72; 84 and 96 hrs of induction
Lane M: protein marker (kDa)

Cloning and expression of laccase in transformants of *P. pastoris* GS115



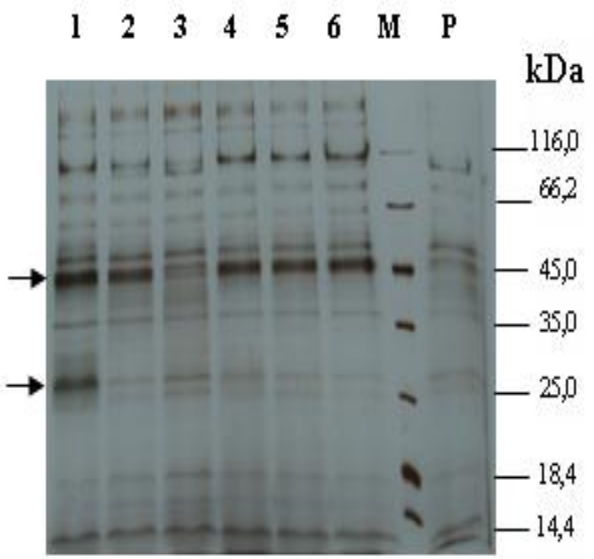




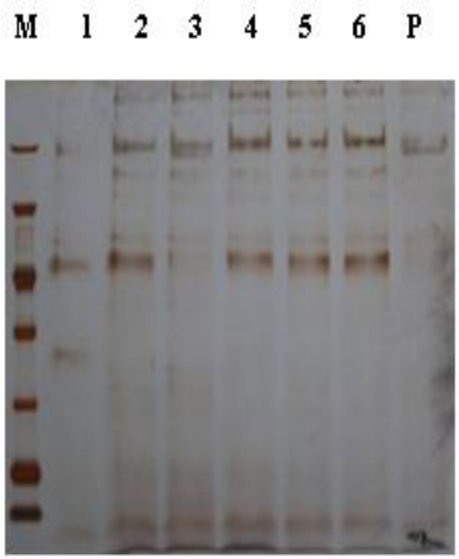
Cloning vector pUC/*lac* cut by restriction enzymes

SDS-PAGE analysis of recombinant laccase expression in broth culture of yeast transformants

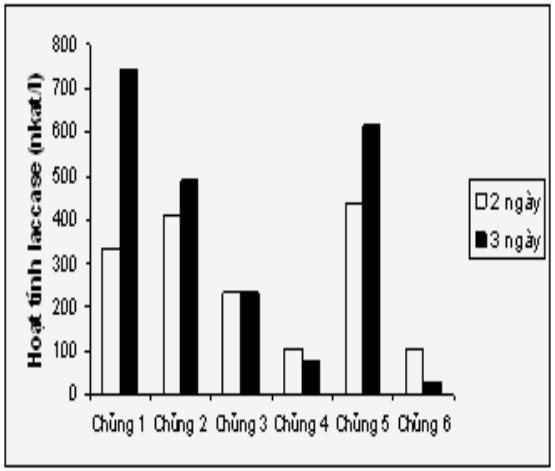
LAC activity of SMD1168/pPIC9::*mlac1* transformants in BMGY medium induced by methanol



After 3 days of induction



After 2 days of induction



Lacase activity of *P. pastoris* transformants

SDS-PAGE analysis of extracelllar proteins synthesized by *P. pastoris* transformants

Lanes 1-6: extracelllar proteins of transformants

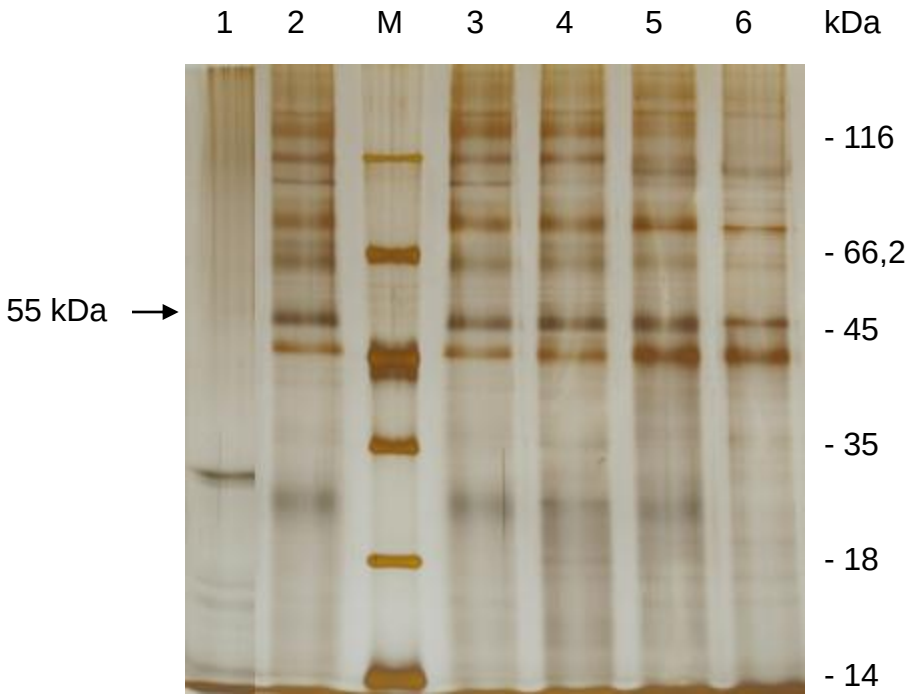
Lane P: *P. pastoris*/pPIC9

Lane M: Protein marker

Biosynthesis of recombinant LAC by *P. pastoris*

- Laccase activity reachs up 1176,02 nkat/l after 72 hrs of cultivation

No	Medium BMGY-LAC	
1	Yeast extract	1,5%
2	Pepton	1,5%
3	100mM phosphat kali	100 ml
4	YNB	1,34%
5	Biotin	$4x10^{-5}$%
6	Glycerol	2%
7	Methanol	1,5%



Extracelular proteins of *P. pastoris* SMD1168/pPIC9::*mlac1*

Lanes 1; 2; 3; 4; 5; 6: after 0; 24; 36; 48; 60 and 78 hrs of induction;
Lane M: Protein marker (kDa).

MAIN CONTENTS

1. Brief introduction to Institute of Biotechnology (IBT), VAST

2. Expression of lignin peroxidase H8 (*lipH8*), laccase (*lac*) in *Pichia pastoris*

3. Preliminary research on hydrolysis of rice straw by combination of chemical pretreatment and degrading enzymes

Lignocellulose from rice straw

- Rice straw contains approximately 80% cellulose and hemicellulose
- These two carbohydrate polymers are tightly bound to lignin and form lignocellulosic complex
- For degradation of lignocellulosic materials, the pretreatment is needed to expand the surface area inside the lignocellulose.

Sugar profile of rice straw hydrolysate

- Sugar concentration in hydrolysate by alkali pretreatment and enzymatic hydrolysis was determined by HPLC: 7.1% glucose; 1.7% celobiose; 4.3% arabinose và 19.4% xylose.
- Finally, total yield of reducing sugar is 32.5% on dry basis sample.

Conditions for preliminary treatment of rice straw for enhancement of sugar content

- The comparison between different chemical pretreatment methods showed that the pretreatment by NaOH (for 21 days at room temperature) combined with subsequent enzyme (lignin peroxidase, xylanase, cellulase) treatment is the most suitable method for conversion of lignocellulose to reducing sugars.
- Up to 36% reducing sugars could be obtained by such approach.

Thank you for your kind attention!