MINISTRY OF EDUCATION AND TRAINING
THAINGUYEN UNIVERSITY

TRINH DINH KHA

PURIFICATION AND STUDIES ON NATURAL CELLULASE PROPERTIES AND PRODUCE RECOMBINANT CELLULASE FROM FUNGUS IN VIETNAM

Specialty/Major: Biochemistry
Code: 62 42 01 16

SUMMARY OF DOCTORAL DISSERTATION OF PHILOSOPHY IN BIOLOGY

THAI NGUYEN - 2015

Works shall be completed at the Faculty of Life Science, Thai Nguyen University of Sciences; Laboratory of Enzyme Biotechnology, Institute of Biotechnology, VAST

Supervisor: 1. Assoc. Prof. Quyen Dinh Thi, Ph.D
2. Assoc. Prof. Nghiem Ngoc Minh, Ph.D

Reviewer 1: .....
Reviewer 2: .....
Reviewer 3: .....

PhD Dissertation will be presented and defended in front of the Council of University Dissertation at the
THAI NGUYEN UNIVERSITY OF SCIENCES
At am/pm date month year 2015

You can find out the PhD Dissertation in National Library Centre for Learning Resource, Thai Nguyen University Library in Thai Nguyen University of Sciences

LIST OF PUBLICATION RELATED TO PhD DISSERTATION

1. Dinh Kha Trinh, Dinh Thi Quyen, Thi Tuyen Do, Thi Thu Huong Nguyen, Ngoc Minh Nghiem (2013), "Optimization of culture conditions and medium components for Carboxymethyl Cellulase (CMCase) production by a novel basidiomycete strain *Peniophora* sp. NDVN01", *Iranian Journal of Biotechnology*, 11(4), pp. 251-259. (SCI-E)
2. Dinh Kha Trinh, Dinh Thi Quyen, Thi Tuyen Do, Ngoc Minh Nghiem (2013), "Purification and characterization of a novel detergent- and organic solvent-resistant endo-beta-1,4-glucanase from a newly isolated basidiomycete *Peniophora* sp. NDVN01", *Turk J Biol*, 37, pp. 377-384. (SCI-E)
3. Trinh Đinh Kha, Quyen Đinh Thi, Nghiem Ngoc Minh (2012), "Cloning and sequencing analysis gene 28S rRNA of strain basidiomycete production cellulase", *Journal of Science and Technology-Thainguyen University*, Volume 96, Issue 8, pp. 115-118.
4. Trinh Dinh Kha, Quyen Dinh Thi, Nghiem Ngoc Minh (2012), "Optimization of carboxymethyl cellulase production by Basidiomycete *Peniophora* sp. NDVN01 under solid state fermentation", *Proceedings The Second Academic conference on Natural Science for Master and PhD Students from Cambodia - Laos - Malaysia – Vietnam*, Publishing House for Science and Technology, pp. 445-450.
5. Trinh Dinh Kha, Quyen Dinh Thi, Nghiem Ngoc Minh (2011), "Optimization of carboxymethyl cellulase production by Basidiomycete *Peniophora* sp. NDVN01 under solid state fermentation", *Vietn J Biotechnol.*, 9(4), pp. 845-852.
6. The gene sequences registered in the international gene banks: Accession number: JF925333

INTRODUCTION

1. Rationale

Cellulases have a broad variety of applications in food, animal feed, brewing, paper pulp, detergent industries, textile industries, fuel, chemical industries, waste management, and pollution treatment.

The cellulase from applications exploiting natural resources face many restrictions due biosynthesis capacity of strains, not proactive, hard intervention changes the kinetic properties of the enzyme, temperature and pH reliability, operability in these conditions high concentrations of detergent and organic solvent.

In the World, there have been many methods are in place to enhance the productivity of cellulase as a selection of strains capable of high cellulase synthesis, optimization of fermentation conditions to obtain large amounts of this enzyme. Especially with the development of technology, a gene encoding the cellulase of microorganisms and plants have been cloned and brought into manifestation great extent in various expression systems (expression in *E. coli*, in yeast, the fungus). In Vietnam, the study mainly cellulase stop isolating a selection of microbial strains producing high enzyme and evaluate some properties of enzymes for applications in biotechnology and environmental remediation. The researchers created recombinant cellulase preparation and application of these products was limited.

From the above reasons we perform the thesis: **"Purification and research of natural cellulase properties and expression cellulase recombinant from fungus in Vietnam".**

2. Objectives of the research

(i) Purification and characterization of natural cellulase from strain fungi selected as the basis for the application and create recombinant cellulase.

(ii) The creation recombinant mature endoglucanase from resources gene were isolated from selected strain fungi in Vietnam.

3. Research Content

3.1. Research screening strains of filamentous fungi capable high production cellulase in collections from various sources;

3.2. Research optimal medium components and fermentation conditions for production natural cellulase of strain fungi selected in conditions laboratory;

3.3. Purification and analysis of physichemical properties of purified cellulase from filamentous fungi strains selected in Vietnam;

3.4. Studies of gene expression mature endoglucanase A from *Aspergillus niger* VTCC-F021 strains in the *Pichia pastoris* GS115 and optimized fermentation broth suitable for the production of recombinant mature endoglucanase A;

3.5. Purification and analysis of physichemical properties of recombinant mature endoglucanase A.

4. New contributions of the thesis

(i) The first time, endoglucanase from strain *Peniophora* sp. NDVN01 selection in Vietnam was purified and had a molecular mass of 32 kDa. Endoglucanase had high stability in the temperature range 30-37°C and pH 4.0 to 7.0. This enzyme resistant to solvents at concentrations of 1-20% acetone; n-butanol and ethanol at a concentration of 1-5%; isopropanol in concentrations of 1-15% and high stability for the detergent Tween 20, Tween 80, Triton X-100 and triton X-114.

(ii) The gene encoding mature endoglucanase A (*meglA*) from *A. niger* VTCC-F021 has been expressed in *P. pastoris* GS115 successfully. Recombinant mature endoglucanase A (rmEglA) was

purified and had a molecular mass of 32 kDa. Optimal enzyme activity at 50°C, pH 3.5, stable at 30-37°C and pH 3.0 to 8.0 reliable. Enzyme had high stability against detergents Tween 20, Tween 80, Triton X-100 and triton X-114.

5. The scientific and practical meanings of thesis

5.1. In Scientifically terms

The research results contribute to elucidate the biochemical properties of endoglucanase is derived from fungi of the genus Peniophora and contribute to clarify the influence of the signal peptide to the nature endoglucanase A of A. niger VTCC-F021 expression in Pichia pastoris.

The resulting recombinant mature endoglucanase further strengthened the scientific basis of the modified activity and properties of recombinant enzymes by cutting off the signal peptide.

The articles published in international technology scientific journals and in the country with 01 gene sequence published in the international gene banks are valuable documents referenced in research and teaching.

5.2. In practical terms

Endoglucanase from strains *Peniophora* sp. NDVN01 and recombinant mature endoglucanase A has properties in line with the production application of supplement in animal feed to metabolize compounds glucan improve feed efficiency and weight gain of animal. The two enzymes can be used in biological conversion of raw materials, agricultural waste into sugar-rich cellulose used in industrial fermentation.

Medium components and optimal fermentation conditions for strain *Peniophora* sp. NDVN01 and recombination strain *P. pastoris* can be used to ferment large, suitable for the production of natural

products endoglucanase and recombination in practical conditions in Vietnam.

*** Structure of thesis:**

The thesis has 126 pages (including references) is divided into chapters and sections: Introduction (04 pages), Chapter 1: Overview of document (27 pages), Chapter 2: Materials and research methods (13 pages); Chapter 3: Results of the study (46 pages); Chapter 4: Discussion of Findings (11 pages); Conclusions and suggestions (02 page); The published works related to the thesis (01 pages); References (22 pages); Appendix (07pages). The thesis has 18 tables, 31 pictures and 193 reference materials.

Chapter 1. DOCUMENT OVERVIEW

Thesis referenced 24 documents in Vietnamese; 165 documents in English and 04 materials from the internet to summarize the relevant content, ncluding: (1) Cellulase; (2) Application cellulase; (3) Study of recombinant cellulase; (4) Fungus *Peniophora* sp., *Aspergillus niger*.

Cellulase are complex enzymes catalyze the hydrolysis of β-1,4-glycosidic linkages in molecules cellulose, oligosaccharide, disaccharide and some of other similar substances (Saranraj *et al*, 2012). Cellulases have a broad variety of applications in food, animal feed, brewing, paper pulp, detergent industries, textile industry, fuel, chemical industries, waste management, pollution treatment and producing bacterial fertilizers (Kuhad *et al*, 2011; Sharada *et al*, 2013).

Until now, the world has had several authors studied expression cellulase gene in various expression systems. Yang *et al* (2010) was cloned and expressed of heat resistance cellulase gene from strain

*Bacillus subtilis*15 in *E. coli* BL21 (DE3). In 2011, Peng *et al* has successfully expressed gene coding for heat resistance cellulase in *E. coli* from *Clostridium thermocellum*. In 2001, Hong *et al* was isolated gene encoding β-glucanase from *A. niger* IF031125 and expressed in yeast. Zhao *et a*l (2010) was synthesized the endo-β-1,4-glucanase (egI) gene from A. niger using optimized codons. In the synthesized endo-β-1,4-glucanase gene syn-egI, 193 nucleotides were changed, and the G+C content was decreased from 54% to 44.2%. The syn-egI gene was inserted into pPIC9K and expressed in *P. pastoris* GS115. Rashid *et al* (2008) was expressed F1-CMCase (24 kDa, 221 aa) from *A. aculeatus* in *A. oryzae* D300. Recombinant enzyme activity reached the highest (18.3 U/ml) after 120 hours of expression in media containing starch source.

In Vietnam, most of the study were only interested in natural glucanase. There is little research about recombinant glucanase. In 2010, Nguyen *et al* cloned genes coding β-glucosidase from *A. niger* PBC and successful expression in *P. pastoris* SMD1168 by vector system is pPIC9. Tran Dinh Man *et al* (2010) based on technical megaprimer has successfully mounted exoglucanase encoding gene fragment (1450 bp) from *Cellulomonas fimi* ATCC484 and promoter (180 bp) from *B. subtilis* and expression in *E. coli* with activity 0.25 U/ml. In 2011, Pham Thi Hoa *et al* were cloned and successfully expressed *eglA* gene encoding endoglucanase A from *A. niger* VTCC-F021 in *P. pastoris* GS115. However, yield expression of recombinant strains of low and inappropriate nature-oriented applications in animal sciences.

Chapter 2. MATERIAL AND METHODS

2.1. Materials, chemicals and research sites

2.1.1. Materials

Collection filamentous fungus (42 strains) provided from Laboratory of Enzyme Biotechnology, Institute of Biotechnology, VAST and Laboratory of Biology, College of Sciences, Thai Nguyen University.

2.1.2. chemicals

Chemicals pure used in experiments provided by the prestigious firm specializing in providing analytical chemistry of USA, Germany, Spain.

2.1.3. Research sites

The experiment was conducted from July, 2009 at the Laboratory of Enzyme Biotechnology and Laboratory of Gene Technology, Institute of Biotechnology, VAST.

Works shall be completed at the Faculty of Life Sciences, College of Sciences, Thai Nguyen University.

2.2. Equipments

The equipment used for experiments are new, modern and high precision from the Laboratory of Enzyme Biotechnology and Laboratory of Gene Technology, Institute of Biotechnology, VAST.

2.3. Research Methodology

2.3.1. Microbial methods

Activation of fungal strains; Culture production enzyme; Culture of *E. coli* and *P. pastoris*; Optimal condition and expression biosynthesis endoglucanase.

2.3.2. Methods of molecular biology

2.3.2.1. Extraction and purified DNA of fungi

2.3.2.2. Extraction and purified DNA of yeast

2.3.2.3. Extraction and purified DNA plasmid by Sambrook and Rusel

2.3.2.4. Plasmid cut by restriction enzyme

2.3.2.5. Purify DNA

2.3.2.6. Cloning of gene by PCR reaction

2.3.2.7. Gene splicing reaction

2.3.2.8. Transformation by sock temperature

2.3.2.9. Transformation by electric

2.3.2.10. Identification and analysis of nucleotide sequence

2.3.3. Biochemical methods

2.3.3.1. Determination of cellulase activity by clean zone

2.3.3.2. Determination of cellulase activity by method of Miller 1959

2.3.3.3. Purified natural cellulase by gel chromatography

2.3.3.4. Purified recombinant protein by affinity chromatography

2.3.3.5. Polyacrylamide gel electrophoresis (SDS-PAGE) by Lemmli 1970

2.3.3.6. Native Polyacrylamide Gel Electrophoresis

2.3.3.7. Determination total protein by Bradford 1976

2.3.3.8. Studied physicochemical properties of natural cellulase and recombinant endoglucanase

Kinetic enzyme

Optimal temperature and optimal pH

Stability temperature and Stability pH

Effect of metal ions, Detergents and organic solvents

2.3.3.9. Determination hydrolases products of substract by TLC

2.4. Analysis methods, data processing

Using Microsoft Excel software, DNAStar software, Blast software, SignalP 4.1 Server software for analysis signal peptide, NetOGlyc 4.0 Server software for analysis O-glycosyl, NetNGlyc 1.0 Server software for analysis N-glycosyl.

Chapter 3. RESULTS AND DISCUSSIONS

3.1. Purification and analysis of physicochemical properties of cellulase from filamentous fungi in Vietnam

3.1.1. Selection and classification of filamentous fungi strain biosynthesis cellulase

The results screening cellulase activity showed that strains NDVN01 produced highest cellulase among surveyed filamentous fungi strains, with an cellulase activity of 1.47 U/ml (Figure 3.1)

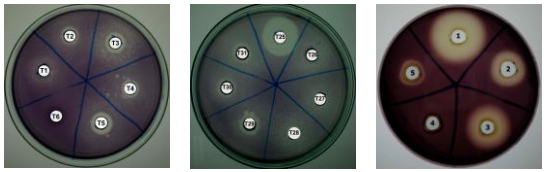


Figure 3.1. Cellulase activity of several fungus

(T1-T31: Trichoderma; 1: Peniophora sp. NDVN01; 2: Pleurotus sajor-caju; 3: Pleurotus ostreatus; 4: Ganoderma lucidum; 5: Flammulina velutipes)

The basidiomycete isolate NDVN01 was identified based on the sequence variation region containing 18S ribosomal RNA gene (partial sequence), internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2 (complete sequence), and 28S ribosomal RNA gene (partial sequence). The ITS sequence consisted of 1255 bp from the basidiomycete isolate NDVN01 (Figure 3.2).

The ITS sequence of the basidiomycete isolate NDVN01 had maximum identity of 93.7 to 99.2 % with those from *Peniophora* strains. Based on the sequence analyzes variations present in internal transcribing spacer (ITS) region, the basidiomycete NDVN01 was identified as *Peniophora* and named as *Peniophora* sp. NDVN01. The sequence was deposited in GenBank with an accession number of JF925333 for *Peniophora* sp. NDVN01.

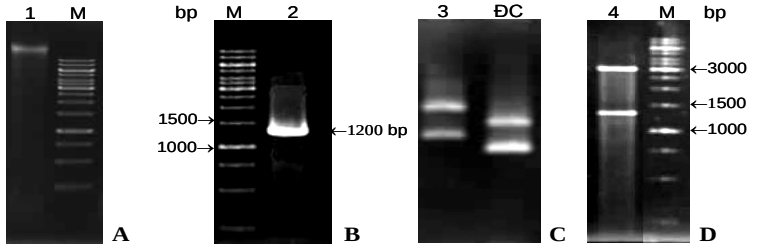
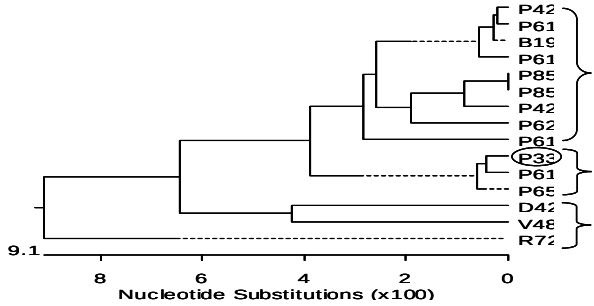


Figure 3.2. The image electrophoresis cloning rRNA gene

*DNA genome (A-1); PCR products (B-2); Recombinant Plasmid (C-3); Products cut recombinant plasmid/Xba*I *and Xho*I *(D-4); Marker 1 kb (M); pJET1.2 (C-ĐC)*

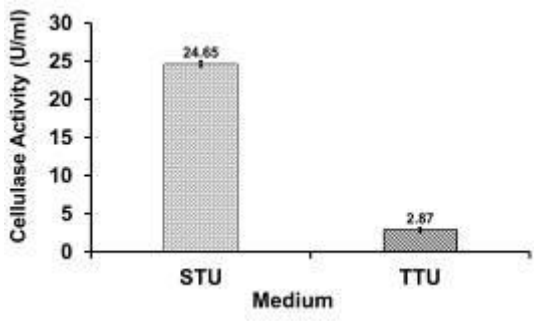


Hình 3.3. The phylogenetic dendrogram for the basidiomycete *Peniophora* sp. NDVN01

P333: *Peniophora* sp. NDVN01 (JF925333); P611: *Peniophora* sp. M104-3B (HM595611); P651: *Peniophora pini* (EU118651)

3.1.2. Optimizing medium conditions for the production of cellulase

The *Peniophora* sp. NDVN01 strain optimized conditions: fermentation time, initial medium pH, culture temperature, the inducer substrate, potato fusion concentration, carbon source, nitrogen source and some mineral source.



Hình 3.9. CMCase production in basal medium and optimal Medium of *Peniophora sp.* NDVN01 strain

STU: basal medium; TTU: optimal Medium

The CMCase production by *Peniophora* sp. NDVN01 in the optimum medium containing 80 % (v/v) of potato in fusion, 0.5 % (w/v) pulp, 0.1 % (w/v) $CaCO_3$ and 0.15 % (w/v) KCl, at 28 °C and initial pH of 7. for 120 hours was 24.65 ± 0.37 (U/ml), that was 8.6 times more than that in the basal medium (2.87 ± 0.37 (U/ml)) (Figure 3.9).

3.1.3. Purification and analysis of physicochemical properties of cellulase from Peniophora sp. NDVN01

3.1.3.1. Purification of cellulase

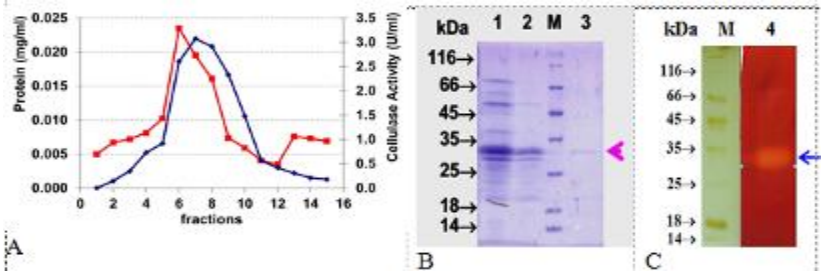


Figure 3.10. Chromatography purification cellulase on Biogel-P100 column (A) SDS-PAGE of the purifed cellulase from (B), Native polyacrylamide gel electrophoresis (C)

lane M: molecular mass marker; lane 1: culture supernatant, lane 2: eluate through Bio-Gel P-100, lane 3: eluate through Sephadex G-75; lane 4: the cellulase activity staining with Congo Red

The cellulase from *Peniophora* sp. NDVN01 was purified to homogeneity through precipitation and gel filtration with Bio-Gel P-100 and Sephadex G-75 with a purification factor of 2.8 and a yield of 3.6%. The purified cellulase exhibited a specific activity of 163.8 U/mg protein and an estimated molecular mass of 32 kDa (Figure 3.10, lane 3).

3.1.3.2. Kinetic of cellulase

The kinetics for barley-β-glucan substrate of cellulase from *Peniophora* sp. NDVN01 strain have lower Km, Kcat and Kcat/km higher than the CMC substrate.Vmax speed of the reaction by cellulase catalytic substrates for CMC reached 1825 U/mg, while for barley-β-glucan substrates reached 9804 U/mg.

3.1.3.3. Substrate specificity

To determine the substrate specificity, the endoglucanase activity towards barley β-glucan, CMC, xylan, LBG, and microcrystalline cellulose (Avicel) was measured. The enzyme displayed the highest activity towards barley β-glucan (5478.8 ± 14.7 U/mg), 4.56 times as high as towards CMC (1202.2 ± 17.3 U/mg). In contrast, no activity towards xylan, LBG, and Avicel was observed.

3.1.3.4. Hydrolysis products

The hydrolysis products of CMC by the purified EG from *Peniophora* sp. NDVN01 were separated and detected with TLC (Figure 3.12). The major product of the CMC hydrolysis was cellobiose (G2) and cellotriose (G3), whereas glucose (G1) and cellotetraose (G4) were obtained in almost equal amounts. In addition, oligomers larger than G4 were also observed.

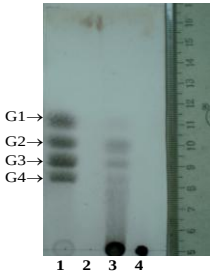


Figure 3.12. TLC analyses of hydrolyzed products

Lane 1: oligosaccharide standards, G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose; lane 2: the purified EG; lane 3: denote hydrolyzed products of CMC after 72 h; lane 4: substrate 1% CMC (w/v).

3.1.3.5. Temperature optima and Temperature stability

The results showed endoglucanase activity increased from 32% at a temperature of 30°C to maximum (100%) at 60°C. Then when the temperature increases, the enzyme activity decreased to 51% at 85 ° C (Figure 3.13A).

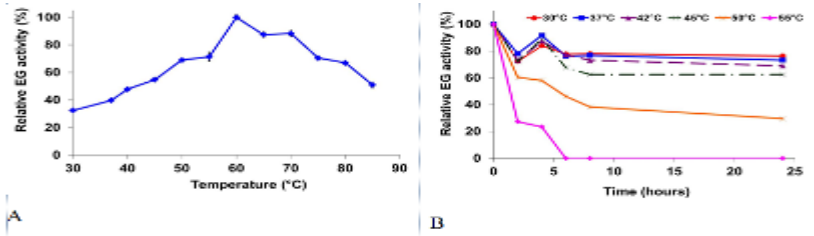


Figure 3.13. The graph influence of temperature reaction (A) and temperature stability (B) of endoglucanase from *Peniophora* sp. NDVN01 strain

Endoglucanase of Peniophora sp. NDVN01 strain remain active at temperatures of 45°C, the relative activity of about 62-76% remaining after 24 hours of treatment at 30-45°C. However, the enzyme activity fell sharply when processed at high temperatures 50-55°C (Figure 3.13B).

3.1.3.6. pHoptima and pH stability

pH phản ứng tối ưu của endoglucanase của chủng *Peniophora* sp. NDVN01 trong khoảng 4,5-5,0. Endoglucanase từ *Peniophora* sp. NDVN01 có độ bền cao trong khoảng pH từ 4,0-5,5 với hoạt tính tương đối còn lại trên 90% sau 24h ủ trong đệm ở nhiệt độ 37°C.

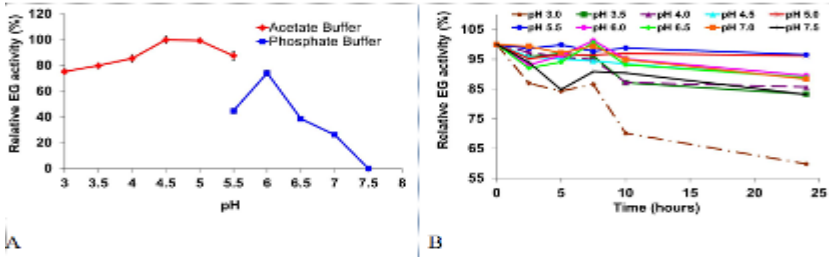


Figure 3.14. The graph influence of pH reaction (A) and pH stability (B) of endoglucanase from *Peniophora* sp. NDVN01 strain

3.1.3.7. Effect of metal on EG activity

Table 3.4. Effect of metal ions and some other reagents on EG activity

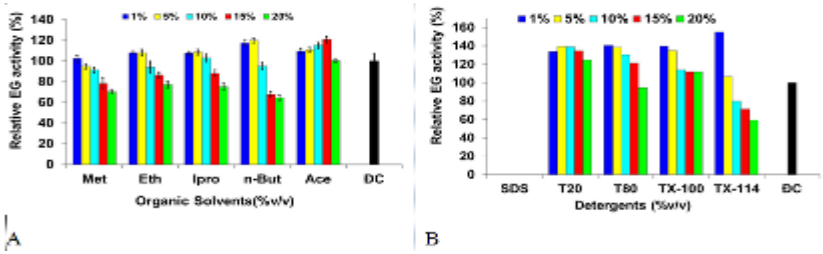
Metal ions and other reagents (mM)	Activity remaining (%)				
	2	4	6	8	10
Ag^{+}	72 ± 2.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
K^{+}	91 ± 3.0	94 ± 2.6	93 ± 3.1	94 ± 3.3	91 ± 3.0
Na^{+}	86 ± 5.8	85 ± 1.8	81 ± 3.5	94 ± 2.8	97 ± 3.0
Ba^{2+}	98 ± 4.8	115 ± 1.8	93 ± 3.5	92 ± 3.2	91 ± 2.1
Ca^{2+}	102 ± 2.3	100 ± 2.9	99 ± 2.0	92 ± 2.4	86 ± 2.8
Cu^{2+}	53 ± 2.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fe^{2+}	70 ± 2.8	77 ± 3.2	92 ± 2.4	93 ± 2.6	101 ± 2.5
Mg^{2+}	79 + 2.8	74 + 3.4	78 + 2.3	80 + 2.5	79 + 3.1
Mn^{2+}	65 ± 3.2	84 ± 2.4	95 ± 2.6	95 ± 3.2	97 ± 3.2
Ni^{2+}	168 ± 1.6	147 ± 1.4	108 ± 3.1	105 ± 3.1	104 ± 2.6
Zn^{2+}	106 ± 2.8	97 ± 2.5	99 ± 2.9	99 ± 1.8	99 ± 1.2
EDTA	61 ± 1.1	65 ± 2.4	66 ± 3.0	71 ± 2.2	67 ± 3.1
2-Mercaptoethanol	115 ± 2.6	108 ± 3.2	103 ± 2.3	100 ± 2.5	94 ± 1.2

The results showed that the enzyme activity was enhanced in the presence of Ni^{2+} in about 2-10 mM, Ca^{2+} at a concentration of 2 mM, Zn^{2+} at a concentration of 2 mM, Ba^{2+} at a concentration 4 mM and mercaptoethanol during 2-6 mM concentration. In particular, Ni^{2+} ions strongly enhance enzyme activity, increase the relative activity up to 168% at concentrations 2 mM. However, cellulase activity was completely inhibited by the addition of Ag^{+} and Cu^{2+} ions at concentrations of 4-10 mM.

3.1.3.8. Effect of organic solvents and detergents

The addition of methanol solvent (1% v/v) ethanol (1-5%), isopropanol (1-10%), n-butanol (1-5%) and acetone (1-15%) intensify of enzyme activity, but when in high concentration methanol solvent (5-20%), ethanol (10-20%), isopropanol (15-20%) and n-butanol (10-20%) inhibited the activity of enzyme. In particular, acetone solvents in concentrations of 15% (v/v) cellulase activity increases sharply with the relative activity reached 121%

compared to non-supplemented control solvent. Solvent n-butanol with a concentration of 10-20% (v/v) strongly inhibited enzyme activity, the relative activity remaining 39-41% compared to control (Figure 3.15A).



Hình 3.15. Effect of organic solvents (A) and detergents (B) on EG activity of *Peniophora* sp. NDVN01

Met: Methanol; Eth: Ethanol; Ipro: Isopropanol; n-But: n-Butanol; Ace: Acetone; T20: Tween 20; T80: Tween 80; TX-100: Triton X-100; TX-114: Triton X-114; ĐC: Control

Additional Triton X-114 at a concentration of 1% (v/v) enhanced activity of the enzyme most strongly, but at a concentration of 10-20% Triton X-114 inhibition of enzyme activity. SDS completely inhibited enzyme activity.

3.2. Cloning and expression *meglA* gene from *Aspergillus niger* VTCC-F021strain in the *Pichia pastoris*

3.2.1. Cloning of gene meglA encoding endoglucanase A

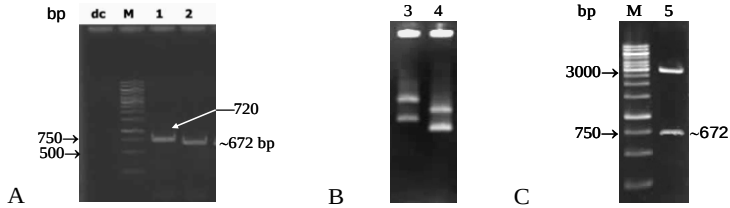


Figure 3.17. The image electrophoresis of PCR products cloning *megl*A gene (A), the recombinant plasmid p*Jmegl*A (B) and the cut p*Jmegl*A by *Eco*RI/*Xba*I (C)

*dc: PCR products native control (not DNA template); 2: PCR products cloning eglA gene (positive control); 3: PCR products cloning meglA gene; 3: plasmid pJmeglA; 4: plasmid pJET1.2 (Control); 5: cut products of pJmeglA by EcoRI/Xba*I

The *megl*A gene sequences were cloned and sequenced with a length of 672 nucleotides.

```
cagacaatgtgctctcagtatgacagtgcctcgagccccccatactcagtgaaccagaac   60
 Q  T  M  C  S  Q  Y  D  S  A  S  S  P  P  Y  S  V  N  Q  N    20
ctctggggcgagtaccaaggcaccggcagccagtgtgcatatgtcgacaaactctccagc  120
 L  W  G  E  Y  Q  G  T  G  S  Q  C  A  Y  V  D  K  L  S  S    40
agtggtgcatcctggcacaccgaatggacctggagcggtggtgagggaacagtgaaaagc  180
 S  G  A  S  W  H  T  E  W  T  W  S  G  G  E  G  T  V  K  S    60
tactctaactctggcgttacatttaacaagaagctcgtgagtgatgtatcaagcatcccc  240
 Y  S  N  S  G  V  T  F  N  K  K  L  V  S  D  V  S  S  I  P    80
acctcggtggaatggaagcaggacaacaccaacgtcaacgccgatgtcgcgtatgatctt  300
 T  S  V  E  W  K  Q  D  N  T  N  V  N  A  D  V  A  Y  D  L   100
ttcaccgcggcgaatgtggaccatgccacttctagcggcgactatgaactgatgatttgg  360
 F  T  A  A  N  V  D  H  A  T  S  S  G  D  Y  E  L  M  I  W   120
cttgcccgctacggcaacatccagcccattggcaagcaaattgccacggccacagtggga  420
 L  A  R  Y  G  N  I  Q  P  I  G  K  Q  I  A  T  A  T  V  G   140
ggcaagtcctgggaggtgtggtatggcagcaccacccaggccggtgcggagcagaggaca  480
 G  K  S  W  E  V  W  Y  G  S  T  T  Q  A  G  A  E  Q  R  T   160
tacagctttgtgtcggaaagccctatcaactcatacagtggggacatcaatgcatttttc  540
 Y  S  F  V  S  E  S  P  I  N  S  Y  S  G  D  I  N  A  F  F   180
agctatctcactcagaaccaaggctttcccgccagctctcagtacttgatcaatctgcag  600
 S  Y  L  T  Q  N  Q  G  F  P  A  S  S  Q  Y  L  I  N  L  Q   200
tttggaactgaggcgttcaccgggggcccggcaaccttcacggttgacaactggaccgcc  660
 F  G  T  E  A  F  T  G  G  P  A  T  F  T  V  D  N  W  T* A   220
agtgtcaactag                                                   672
 S  V  N  -                                                    223
```

Figure 3.18. The *megl*A gene sequence and deduced amino acid sequence of mEglA from *A. niger* VTCC-F021 strain

T*: site Threonine probable glycosylation

Analysis using DNAstar software was showed amino acid sequence deduced of rmEglA had 223 amino acids. Of these, 9 amino acid identifiable strong base (K, R), 19 amino acids brought strong acidic (D, E), 68 amino acids hydrophobic (A, I, L, F, W, V) and 96 amino acids polarity (N, C, Q, S, T, Y). Enzyme rmEglA had molecular mass about 24.24 kDa and pI was 4.24. Compared with rEglA, component amino acid change: down 1 amino acids zo taking three strong (K, R), down 9 amino acids hydrophobic (A, I, L, F, W,

V) and decreased 3 amino acids polarity (N, C, Q, S, T, Y). Enzyme rmEglA volume drop of 1.5 kDa and pI fell 0.129 compared to rEglA. Using NetOGlyc 4.0 Server online software analysis glycosylation point (http://www.cbs.dtu.dk/services/NetOGlyc/) identified on the mrEglA polypeptide chains can occur O-glycosylation at the threonine amino acid position-219 (T *) (Figure 3:18). However, when analyzing by NetNGlyc 1.0 Server online software (http://www.cbs.dtu.dk/services/NetNGlyc/) undetectable probable location N-glycosylation process.

3.2.2. Design vector expression of meglA gene

PJ*megl*A plasmid carrying the gene *megl*A and pPICZαA vector were cut with EcoRI and XbaI. Then, cut products were connected by T4 ligase create recombinant pP*megl*A plasmid. Plasmid insert gene have larger sizes, so is higher than the vector without insert gene (Figure 3.19A).

PP*megl*A recombinant plasmid purification was cut by *Eco*RI and *Xba*I for two band is pPICZαA (~3,6 kb) and *megl*A gene (672 bp) (Figure 3.19B). pP*megl*A vector was sequenced to check the expression structure before transformation and expression in *P. pastoris* GS115. Structure manifestation correct reading frame, *megl*A gen was inserted properly desired location, eligible for expression in the *P. pastoris* GS115.

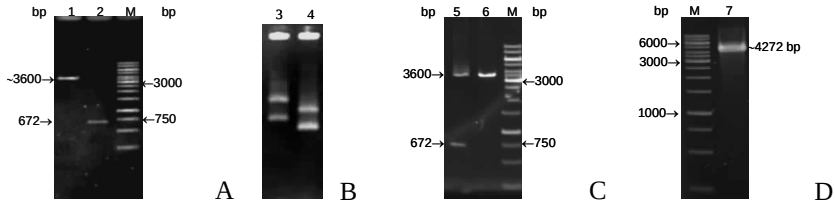
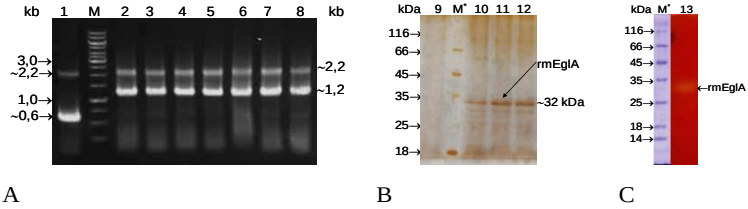


Figure 3.19. The image electrophoresis gel extraction product(A), plasmid pP*megl*A (B), the product cut pP*megl*A by *Eco*RI and *Xba*I (C), the product cut pP*megl*A by *Sac*I (D)

*1: pPICZαA cut by Eco*RI *and Xba*I*; 2: gene meglA; 3: pPmeglA; 4: pPICZαA (Control); 5: pPmeglA cut by Eco*RI *and Xba*I*; 6: pPICZαA cut by Eco*RI *and Xba*I *(control); 7: pPmeglA cut by Sac*I

3.2.3. Expression rmEglA in P. pastoris GS115

3.2.3.1. Construction of expression system P. pastoris GS115/pPmeglA



A B C

Figure 3.21. The image electrophoresis of PCR products with specific primers 3'-5'AOX1 (A); Protein electrophoresis of fermented solution *P. pastoris* GS115 / pP*megl*A strain (B); Native polyacrylamide gel electrophoresis of fermented solution *P. pastoris* GS115/pP*megl*A strains (C)

1: PCR genome P. pastoris GS115/pPicZα (control); 2-8: PCR products genome P. pastoris GS115/pPmeglA; 9: Protein electrophoresis of fermented solution P. pastoris GS115/pPICzαA (đối chứng); 10-12: Protein electrophoresis of fermented solution P. pastoris GS115/pPmeglA; 13: Native polyacrylamide gel electrophoresis of rmEglA

For *megl*A gene expression, recombinant plasmid was cut open loop pP*megl*A by *Sac*I (Figure 3.19D) and was transformed into *P. pastoris* GS115 cells with electric pulses variable. Recombinant strains were cultured in YPG medium additional zeocine overnight, extract DNA, then PCR with primers specific AOX1 for inspection. Some colonies (2-8 wells) (Figure 3.21A) contains foreign DNA fragment corresponding size meglA gene. Thus, we can conclude *P. pastoris* strains containing recombinant gene fragment coding meglA.

3.2.3.2. Selection of clones recombinant P. pastoris GS115/pPEglA

To check the result and expression levels rmEglA, 39 clones recombinant P. pastoris GS115/pPEglA were cultured in expression medium YP additional 1% methanol every 24 hours. The 14 clony has selected with highest expression yield (1.95 U/ml) for subsequent studies.

After 72 hours of expression, extracellular was run electrophoresis on the polyacrylamide gel and silver staining, stained native activity by congo red solution. The results showed that rmEglA was expressed and recombinant protein size of 32 kDa (Figure 3.21B, C).

3.2.4. Optimization medium components and condition ferments for production rmEglA

3.2.4.1. Selection of express medium

3.2.4.2. Effect of concentration yeast extract

3.2.4.3. Effect of concentration peptone

3.2.4.4. Effect of initial pH medium

3.2.4.5. Effect of temperature

3.2.4.6. Effect of concentration methanol

3.2.4.7. Effect of time culture

3.2.4.8. Production rmEglA under optimal medium

The recombinant endoglucanase A production by *P. pastoris* GS115/pPmeglA/14 in the optimum medium containing (1.6% peptone, 1.2% yeast extract; 1.2% methanol induce after 24h, the initial pH 5.0; fermentation temperature 25°C and fermentation time 96h shake 200 cycles/minute) was 17.26 U/ml, that was 8.8 times more than that in the suboptimal medium (1.98 U/ml) (Figure 3.25).

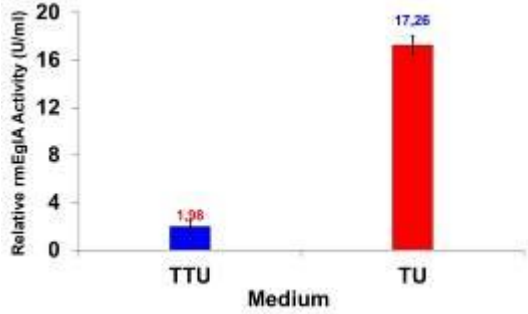


Figure 3.25. rmEglA production in basal medium and optimal medium (B)

TTU: Suboptimal medium; TU: Optimal medium

3.2.5. Purification of rmEglA

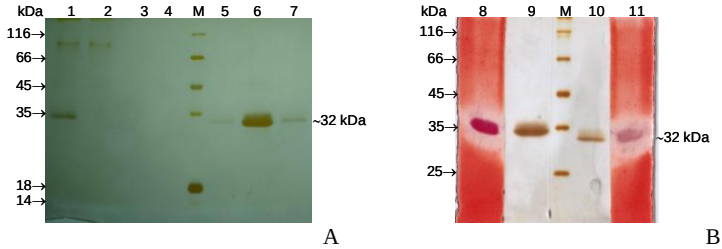


Figure 3.26. The image electrophoresis of purification fractions rmEglA (A), Native polyacrylamide gel electrophoresis (B)

lane 1: culture supernatant, lane 2: eluate column, lane 3-4: Wash; lane 5-7: purification fractions 8: activity staining of rEglA; 9: rEglA purification; 10: rmEglA purification; 11: activity staining rmEglA; lane M: molecular mass marker

The electrophoresis results in Figure 3.26 the showed that rmEglA with high purity and has a mass of about 32 kDa. The mass of rmEglA lowered than the volume of rEglA. Also, using electrophoresis results showed band activity is endoglucanase A purified proteins and has stronger activity than rEglA.

Table 3.6. Purification steps of rmEglA

Sample	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
culture supernatant	1,91	60,75	31,81	1	100
Eluate	0,79	12,53	15,86		20,63
Wash 1	0,72	7,21	10,01		11,87
purified rmEglA	**0,16**	**30,64**	**191,5**	**6,02**	**50,44**

3.2.6. Analysis of physicochemical properties of rmEglA

3.2.6.1. Kinetic enzyme

Kinetics for β-glucan substrates of rmEglA barley hasKm, Kcat and Kcat/Km lower higher than the CMC substrate. This is proves that the affinity of the enzyme barley-β-glucan substrates with higher than the CMC substrate. Vmax of catalytic reactions rmEglA for substrates CMC reached 588.2 U/mg, while for barley-β-glucan substrates reached 666.67 U/mg

3.2.6.2. Substrate specificity

rmEglA have higher specificity for β-glucan barley substrate (relative activity reached 217.6% compared with the metabolic substrates CMC) and CMC (relative activity 100%), the ability to hydrolyze cellulose substrate crystallization (Avicel) very low (1.7%) and inability to hydrolyze xylan substrate, LBG and starch.

3.2.6.3. Hydrolysis products

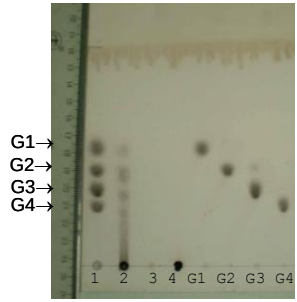


Figure 3.28. TLC analyses of hydrolyzed products

Lane 1: oligosaccharide standards, G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose; lane 2: denote hydrolyzed products of CMC; lane 3: the purified rmEglA; lane 4: substrate 1% CMC (w/v).

The hydrolysis products of CMC by the purified EG from Peniophora sp. NDVN01 were separated and detected with TLC (Figure 3.28). The major product of the CMC hydrolysis was

cellobiose (G2) and cellotriose (G3), whereas glucose (G1) and cellotetraose (G4) were obtained in almost equal amounts. In addition, oligomers larger than G4 were also observed.

3.2.6.4. Optimum and stability temperature of rmEglA

As the temperature rose from 30-50°C reaction, the activity of rmEglA increases and reaches maximum at 50°C. Then, the temperature rises, the activity rmEglA decreased to 45.25% compared to the maximum at 85°C. rmEglA high stability in the temperature range 30-37°C, after 8 hours of treatment relative activity was 88%. However, when high temperatures increase 45-55°C enzyme inactivation rapidly (Figure 3.29).

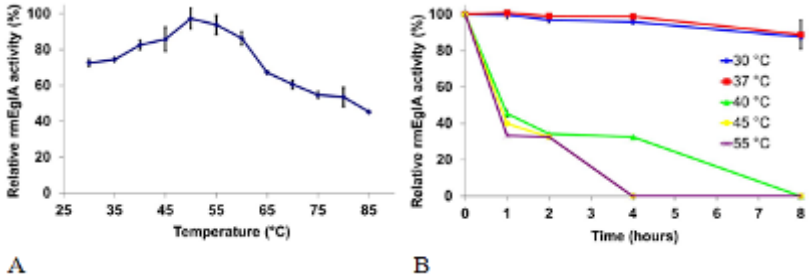


Figure 3.29. The graph influence of temperature reaction (A) and temperature stability (B) of rmEglA

3.2.6.5. Optimum and stability pH of rmEglA

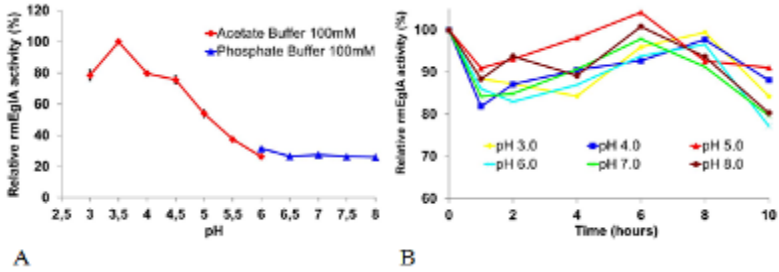


Figure 3.30. Optimum and stability pH of rmEglA

rmEglA have optimal reaction pH 3.5, the pH increased the enzyme activity dropped to 26% of maximum activity at pH 8.0 (Figure 3.30A). Reliability survey the results showed that the pH of the enzyme is very stable pH rmEglA. From 3.0 to 8.0 in wide pH range, after 10 h treatment of enzyme relative activity still 77%. In particular, at 3.0 to 5.0 pH range from relative activity rmEglA still 84-91% (Figure 3.30B).

3.2.6.6. Effect of metal ions on rmEglA activity

Table 3.9. Effect of metal ions on rmEglA activity

Metal ions	Concentration			Metal ions	Concentration		
	5 mM	*10 mM*	*15 mM*		*5 mM*	*10 mM*	*15 mM*
K^+	118,1 ± 1,2	109,0 ± 2,4	104,4 ± 2,1	Ni^{2+}	120,5 ± 2,7	107,3 ± 1,3	103,2 ± 1,8
Ag^+	56,0 ± 1,6	42,5 ± 2,0	41,7 ± 0,7	Mn^{2+}	115,1 ± 2,8	83,7 ± 1,5	73,6 ± 1,7
Ca^{2+}	120,1 ± 3,6	119,5 ± 3,2	112,2 ± 1,5	Cu^{2+}	131,6 ± 3,3	115,2 ± 2,8	100,5 ± 2,4
Ba^{2+}	129,3 ± 2,3	123,5 ± 2,7	123,2 ± 2,1	Co^{2+}	153,0 ± 1,7	158,7 ± 1,9	154,2 ± 2,6
Mg^{2+}	130,1 ± 4,3	67,9 ± 2,9	63,8 ± 2,5	Pb^{2+}	*37,2 ± 0,7*	*0,0*	*0,0*
Zn^{2+}	132,6 ± 1,8	109,6 ± 2,5	97,1 ± 1,7	Al^{3+}	*60,9 ± 0,9*	*0,0*	*0,0*
Fe^{3+}	86,3 ± 1,6	72,5 ± 1,4	69,9 ± 2,2	EDTA	88,6 ± 1,2	86,0 ±1,9	85,3 ± 2,0
Hg^{2+}	*57,0 ± 1,3*	*0,0*	*0,0*	Control		100,0 ± 2,8	

The metal ions (K^+, Ca^{2+}, Ba^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}) at oncentration 5-15 mM were increased activity of rmEglA. The ions (Fe^{2+}, Hg^{2+}, Pb^{2+}, Al^{3+}) powerful inhibitory activity rmEglA. In particular, The Ba^{2+} ion was increased the maximum enzyme activity up 123-129% compared to controls. The Pb^{2+} Ion strongest inhibitor, at a concentration of 5 mM activity was 37.2% relatively compared to the control, while increased to 10 mM, the enzyme completely lost activity. EDTA is the inhibitor features metaloenzyme role inhibiting activity relative rmEglA with 85-88% reduced compared to the control at a concentration of 5-15 mM (Table 3.9).

3.2.6.7. Effect of Organic solvents and Detergents

At concentrations 5% of organic solvent are role increase enzyme activity, isopropanol and acetone which have made the largest

increase with relative activity of 150%. In the survey solvent, methanol does not reduce enzyme activity, n-butanol the strongest inhibitors in 20% complete loss of enzyme activity (Figure 3.31A).

At concentrations survey from 0.5 to 2.0%, detergents (Tween 20, Tween 80, Triton X-100, Triton X-114) were increased from 14.9 to 56.3% enzyme activity compared with control. Of these, Triton X-114 increases maximum enzyme activity. Triton X-114 and Tween 80 influence tends to increase gradually with increasing concentration. SDS completely inhibited enzyme activity (Figure 3.31B).

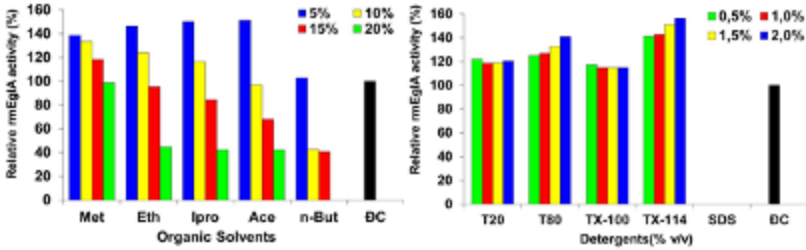


Figure 3.31. Effect of organic solvents (A) and detergents (B) on activity of rmEglA

Met: methanol; Eth: ethanol; Ipro: Isopropanol; Ace: acetone; n-But: n-butanol; T20: Tween 20; T80: Tween 80; TX-100: Triton X-100; TX-114: Triton X-114; SDS: Sodium dodecyl sulphate; ĐC: Control

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

1. The fungi *Peniophora* sp. NDVN01 strain capable high synthesis cellulase were selected from 42 fungus strains in Vietnam. rRNA coding genes isolated from *Peniophora* sp. NDVN01 strain already registered on GenBank sequence with codes JF925333.

2. Medium components of fermentation biosynthesis cellulase suitable in conditions Vietnam has been optimized including 80% (v/v) extracts potatoes, rice straw 0.6%, 0.5% (w/v) pulp as the inducer, 0.2% (w/v) $(NH4)_2HPO_4$, 0.15% KCl and 0.1% $CaCO_3$.

Appropriate fermentation conditions at 28°C, initial pH of 7.0, fermentation time of 120 hours, shaking 200 cycles/minute.

3. Endoglucanase purified from strains *Peniophora* sp. NDVN01 have a molecular mass of about 32 kDa, specific activity reached 169.42 U/mg, purification fold 2.34 times. Optimal activity enzyme at a temperature of 60°C, pH 4.5, stable in pH from 4.0 to 7.0 and 30-45°C. Enzyme was stable for solvents in concentrations of 1-20% acetone; n-butanol and ethanol at a concentration of 1-5%; isopropanol in concentrations of 1-15% and high reliability for the detergents (Tween 20, Tween 80, Triton X-100 and triton X-114).

4. The gene *megl*A has size 672 nucleotides encoding mature endoglucanase A with 223 amino acid from *A. niger* VTCC-F021. It was expressed *P. pastoris* GS115 successfully. Medium components fermentation endoglucanase biosynthesis suitable for recombinant *P. pastoris* GS115/pPmeglA/14 strain include: 1.6% peptone; 1.2% yeast extract; 1.2% methanol induction after 24 hours; initial pH 5.0; fermentation temperature 25°C and fermentation time 96h, shaking 200 cycles/minute.

5. The purified rmEglA has molecular mass about 32 kDa, specific activity reached 191.5 U/mg, purification fold 6.02 times and 50.44% recovery efficiency. Optimal activity enzyme at 50°C, pH 3.5, stable at 30-37°C and very stable at pH 3.0 to 8.0. Enzyme dstable with some detergents (Tween 20, Tween 80, Triton X-100 and triton X-114).

RECOMMENDATIONS

Carry out to ferment the recombinant yeast *P. pastoris* GS115/pP*meglA*/14 for expression of endoglucanase with high yield. Construction of processes for producing recombinant endoglucanase and fermentation test in large scale to create enzyme preparations added to the animal feed and biomass conversion.