β-GLCANASES, ESSENTIAL KEY ENZYMES FOR THE UTILIZATION OF RENEWABLE RAW MATERIALS

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Acknowledgement

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上善若水。

居善地，心善渊，与善仁，言善行，正善治，事善能，动善时。

顺势而为。

故善战者，求之于势，不责于人故能择人而任势。任势者，其战人也，如转木石。木石之性，安则静，危则动，方则止，圆则行。故善战人之势，如转圆石于千仞之山者，势也。

Abstract

During the last decade, the shifting from petroleum based fuel to a greener bio based one was expedited by the increasing concern of global warming. The growing demand oriented firstly to the use of food crops as raw material for producing so called first-generation biofuel. And then converted to the second-generation biofuel, using non-food biomass resources for instance, lignocellulosic raw materials. Although these generations of biofuel offered CO_2 emission benefits and improved domestic energy security, they also caused several environmental impacts. Such as high requirement for pesticide and fertilizer and conversion of agricultural land for food to energy crops (Naik et al. 2010; Chaturvedi and Verma 2013). Consequently, a new raw material, algae, which store high amounts of energy in form of carbohydrates contributed to eliminate these drawbacks. The algal biomass based fuel production was known as the third-generation biofuel (Lee and Lavoie 2013).

In general, algal storage polysaccharides are composed of glucose subunits. Paramylon from *Euglena gracilis*, a linear β-1,3-glucan with very high level of polymerization contributes up to 85% of cell dry weight (Sonck et al. 2010). This glucan is supposed to be a superb potential raw material for sustainable production of bioethanol. The production of bioethanol from algal polysaccharides requires efficient hydrolysis in order to generate fermentable monosaccharides. The hydrolysis is generally carried out by chemical or enzymatic methods. The chemical hydrolysis efficiently yields high concentrations of fermentable sugars, however requires environment harmful chemicals and generates byproducts inhibiting fermentation process. While the enzymatic hydrolysis processes under mild conditions without accumulating inhibitory byproducts (Chen et al. 2013).

Hence, searching for enzymes which could convert paramylon to glucose became the fundamental purpose of this work. As the first part, *E.gracilis* intracellular proteins were extracted for investigating the hydrolyzing ability on paramylon. The cell free extract prepared from late stationary phase rapidly hydrolyzes alkali treated paramylon. After partial purification, one protein potentially belonging to glycoside hydrolase family 22 was found, and followed with trail of obtaining the gene sequence from *E.gracilis* cDNA pool by degenerate PCR.

In the second part of this work, recombinant enzymes of four endo-β-1,3-glucanases (*Tr*GH16, *Tr*GH55, *Tr*GH64, *Tr*GH81), one exo-β-1,3-glucanase (*Tr*GH17) from *Trichoderma reesei* and one exo-β-1,3-glucanase (*Pp*GH5) from *Pichia pastoris* were successfully expressed in *P.pastoris* GS115. Moreover, their activities towards alkali treated paramylon were confirmed by measuring hydrolysis products of reducing groups and glucose, respectively.

In these two parts, alkali was involved for pretreating paramylon, which was not fully compatible with the purpose of direct hydrolysis of paramylon granule by enzymes without chemical pretreatment. However, no enzymatic method to deconstruct the highly crystallized paramylon granule has been reported by now. The newly discovered lytic polysaccharide monooxygenases (LPMOs), which could break glycosidic linkages of recalcitrant polysaccharides by oxidation and introduce new chain ends for hydrolytic enzymes (Forsberg et al. 2016), might become the key role to overcome the barrier. In the last part of this work, two LPMOs, *Tr*AA9 from *T.reesei* and *Ao*AA11 from *Aspergillus oryzae* were expressed in *P.pastoris*. The synergistic action assay between LPMO and glucanase revealed the possibility that *Tr*AA9 from *T.reesei* enhanced oligosaccharide accumulation in enzymatic hydrolysate. The result may open up a new way for depolymerization of recalcitrant paramylon granule in further research.

Abbreviations

CAZy	Carbonhydrate Active Enzyme
GH	Glycoside Hydrolase
AA	Auxiliary Activity
LPMO	Lytic Polysaccharide Monooxygenase
IUBMB	International Union of Biochemistry and Molecular Biology
EC	Enzyme Commission
CDH	Cellobiose Dehydrogenase
pI	Isoelectric Point
SEC	Size Exclusion Chromatography
IEC	Ion Exchange Chromatography
HIC	Hydrophobic Interaction Chromatography
AC	Affinity Chromatography
AOX	Alcohol Oxidase
Mut	Methanol Utilization
*HIS*4	Histidinol Dehydrogenase Gene
TT	Transcription Termination Region
ER	Endoplasmic Reticulum
Kex	Kexin Protease
α-MF	α-Mating Factor
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RNase	Ribonuclease

DNase	Deoxyribonuclease
cDNA	complementary Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
dNTPs	Deoxynucleotide Triphosphates
OD	Optical Density
rpm	Rounds per Minute
kV	Kilo Volt, Electric Potential
µF	Micro Farad, Electrical Capacitance
Ω	Ohm, Electrical Resistance
bp	Basepare
Kb	Kilo base
kDa	Kilo Dalton

1. Introduction

1.1 Euglena gracilis

Euglena was named by Christian G. Ehrenberg in 1830 (Ehrenberg 1830). The word “Euglena” was formed by “eu” and “glene” which mean “good” and “eyeball” in greek, respectively. The “eyeball” was from the clearly visible stigma with optical microscope (Leedale 1967). In 1900, axenically cultivating of *E.gracilis* was performed by Zumstein for the first time (Zumstein 1900). And most of present studies still use Zumstein’s original strain “Z” (Evangelista et al. 2003).

A schematic drawing of *Euglena* is showed in Fig.1. It lacks a cell wall. Instead, it has a pellicle made up of a protein layer supported by a substructure of microtubules, arranging in strips spiraling around the cell. The action of these pellicle strips sliding over one another gives *Euglena* its exceptional flexibility and contractility. The highly flexible cell surface allowes them to change shape from thin cells to spheres with lengths between 20 to 100 µm (Schaechter 2012). The motorial system of *E.gracilis* consistes of two flagella, one emerges from the reservoir and the other one is termed non-emergent flagellum. The stigma, which consists of pigment granules, locates beside the reservoir as part of the visual system. Next to the stigma is the contractile vacuole for regulatory functions concerning the cell fluid. Inside the cell fluid, near the center of the cell, the nucleus can be found. The chloroplasts of *E. gracilis* account for a great percentage of cell organelles and for functions concerning the main energy providing facility. The cornered shape of chloroplast is a characteristic to *E. gracilis*. Due to the presence of chloroplasts, sometimes *E. gracilis* is placed with green algae and can be feed by photosynthesis or heterotrophy. Other

entities such as paramylon granules can be found spread throughout the cell (Marin et al. 2003).

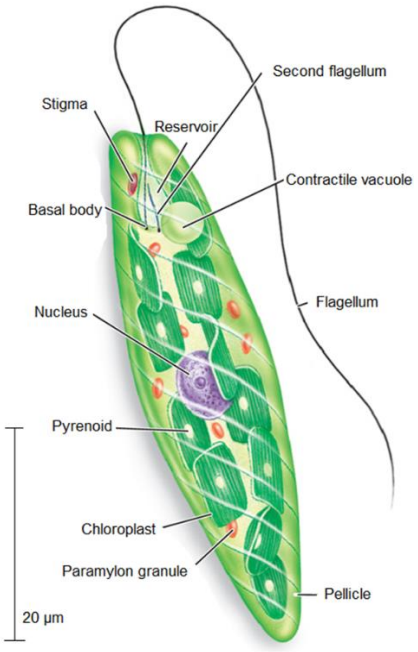

Figure1. A schematic drawing of *Euglena* (Eugene, 2015).

1.2 Paramylon

E. gracilis has been extensively researched for production of vitamins A, C, E (Takeyama et al. 1997), highly nutritious proteins (Schwarz et al. 1995), also as a good source of polyunsaturated fatty acids (Barsanti et al. 2000) and β-glucan (Russo et al. 2016). When grown in the presence of adequate carbon sources under heterotrophic growth conditions, *E. gracilis* can accumulate large amount of insoluble β-1,3-glucan, paramylon, which was firstly discovered by Gottlieb in 1850 (Gottlieb 1850; Bũmer et al. 2001). And in 1960s the β-1,3-glucosidic linkages were identified as the structure basis (Clarke and Stone 1960). The structural investigations by X-ray diffraction and NMR spectrum revealed that it is a linear (unbranched, as showing in Fig.2) β-1,3-glucan type polysaccharide with a very high level of crystallinity about 90% in the native state (Kreger and Meeuse 1952; Kiss et al. 1988).

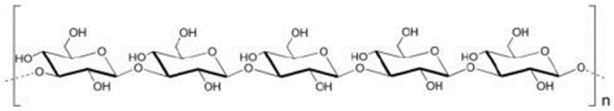

Fig.2 Schematic representation primary structure of paramylon (β-1,3-glucan) (Ramesh and Tharanathan 2003).

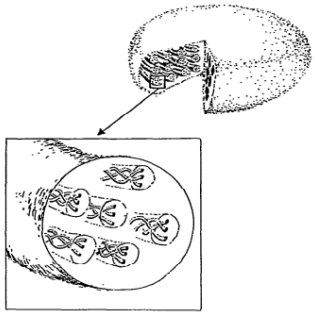

Fig.3 Schematic representation of microfibrils of a paramylon granule (Marchessault and Deslandes 1979).

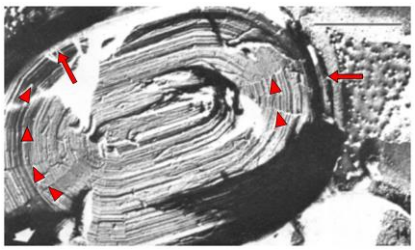

Fig.4 Electron micrograph of a freeze-etched paramylon granule (Holt and Stern 1970).

Paramylon has high molecular mass estimated to be over 500 kDa and a high degree of polymerization about 3000 in form of membrane-bound granule, contributing up to 85% of cell dry weight (Barsanti et al. 2001; Sonck et al. 2010). Microfibrils which are composed of triple helices of β-1,3-glucan chains (as showed in Fig.3) traverse the inner granule and forms the unique high crystallinity (Kiss et al. 1987). The electron micrograph (Fig.4) revealed the concentric layers of the inner structure. In Fig.4, the segmentation of the inner material and outer layer are indicated by the arrows (Holt and Stern 1970).

Paramylon, as well as lentinan and pachyman, belong to a group of naturally occurring bioactive polysaccharides, and possesses antitumor and cytokine-related immunopotentiating activity (Kondo et al. 1992). Sulfated derivatives of

paramylon significantly inhibit the cytopathic effect of human immunodeficiency virus (HIV-1, HIV-2) and the expression of HIV antigen in human peripheral blood mononuclear cells (Koizumi et al. 1993). When incorporated in human or animal diets, paramylon supports cholesterol lowering and moderates the postprandial blood glucose and insulin response (Wang et al. 1997).

1.3 The third-generation bioethanol

For mitigating climate change, the demand of decreasing fossil energy resources and explorations for fossil energy is boosting in worldwide. The strategy of substitute fossil by biomass is the key strategy to achieve this goal, and predominate the next generation biofuels (Aditiya et al. 2016). By now, the production of fuels from biomass is categorized as three generations: the first-generation, which uses good crops as raw material; the second-generation converting also lignocellulosic material into biofuel; and the third-generation, which utilizes algae biomass (Abdallah et al. 2016). In algae, carbohydrates are stored in form of storage sugars and structural material. Beyond varying in the glycosidic bond between monomers, algae energy storage polysaccharides are composed of glucose subunits (Kim 2015). The process of bioethanol production from algae polysaccharides consists of three major steps: biomass pretreatment, enzymatic hydrolysis of algae polysaccharides, and fermentation of sugar monomers to ethanol. The pretreatment step disrupts algal cell and releases intracellular sugars. Subsequently, the pretreated biomass is degraded by lytic enzymes into simple sugars for fermentation. In the last step, simple sugars are used for the microorganism’s growth, while ethanol is produced as a byproduct of the fermentation process (Harun et al. 2014). Studies proved the suitability of algae as feedstock for bioethanol years ago. Microalgae, such as *Chlorococcum infusionum*, *Chlamydomonas reinhardtii*, and *Chlorella vulgaris*, were exploited for the third-generation bioethanol production (Daroch et al. 2013). Mircoalgae were estimated being able to

produce 5000-15,000 gal of ethanol per acre annually, which was more reliable than the first- and second-generation bioethanol feedstock (Nguyen and Vu 2012). Meanwhile, macroalgae (seaweeds) with a world average yield of 73 kg/m^2 per year, were also reported to be one of the best candidates employed as the bioethanol feedstock in 2014 (Noraini et al. 2014). Paramylon, as the storage polysaccharide of *Euglena*, was reported contributing up to 85% of cell dry weight (Sonck et al. 2010), making it an outstanding candidate of renewable resource for the third-generation bioethanol production. However, the Paramylon granule was extremely resistant to physical and chemical attacks. In vitro, hydrolysis of native granules hasn't been performed successfully. The structure of paramylon granule could be destroyed by treatment with alkaline, but without being hydrolyzed. It was believed that the treatment conducts not only a removal of the membrane, but also a restructuring as exposing enzymatic action sites (Monfils et al. 2011). Until now, limited scientific study investigated this type of recalcitrant raw material (Abdallah et al. 2016). And no bioethanol produced from paramylon has been reported to our knowledge.

1.4 Enzymes involved in paramylon degradation

In nature, carbohydrates are degraded through enzymatic cleavage of the glycosidic bonds between monomers. The diversity of polysaccharides and their complex structures results in the existence of huge diversity of enzymes for degradation of these biomolecules. These enzymes are mainly glycoside hydrolases, but can also involve oxidase, such as lytic polysaccharide monooxygenases (LPMOs) (Martinez 2016).

1.4.1 β-1,3-Glucanases

Enzymatic degradation of β-glucans involves a variety of enzymes that hydrolyze glycosidic bonds naming β-glucanases. The enzymatic hydrolysis take place via general acid catalysis requiring a proton donor and a

nucleophile/base. Two mechanisms are involved in the hydrolysis resulting in either a retention or an inversion of the anomeric configuration (See Fig.5) (McCarter and Withers 1994). In retaining mechanism, the glycosidic oxygen is protonated by the acid catalyst (AH) and the base B^- conducts nucleophilic assistance to aglycon departure. Then the glycosyl enzyme interacts with a water molecule, generating a product with the same stereochemistry as the substrate at the anomeric carbon. In the inverting mechanism, protonation of the glycosidic oxygen and aglycon departure are accompanied by a concomitant attack of a water molecule which is activated by the base residue B^-. This reaction results in a product having opposite stereochemistry to the substrate.

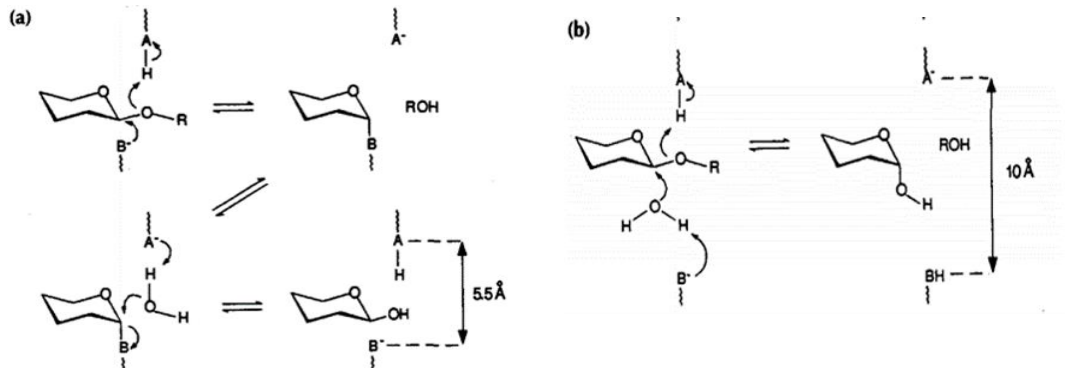

Fig.5 Schematic representation of two enzymatic glycosidic bond hydrolysis mechanisms. (a) The retaining mechanism; (b) The inverting mechanism (McCarter and Withers 1994).

In both mechanisms, positions of the proton donor are identical. But positions of the nucleophile relative to the sugar anomeric carbon are different. In inverting enzymes accommodating a water molecule between the base and the sugar are more distant than in retaining enzymes (Davies and Henrissat 1995).

β-glucanases are systematically classified by the International Union of Biochemistry and Molecular Biology (IUBMB) depending on the selectivity of glucosidic bond and the hydrolytic reaction patterns against specified substrates. The IUBMB sorts these enzymes by Enzyme Commission (EC) number 3.2.1.x. Degradation of β-1,3-glucan, such as paramylon, involves the actions of two groups of glucanases: exo-β-1,3-glucanase (E.C.3.2.1.58) and endo-β-1,3-glucanase (E.C.3.2.1.39). The exo-β-1,3-glucanases are expected to produce glucose by sequential hydrolysis of β-1,3-glucan from the non-reducing end (Yamamoto and Nevins 1983). Endo-β-1,3-glucanases cleave at random sites along the β-1,3-glucan chain, generating oligosaccharides (Pitson et al. 1993). Quite different to IUBMB, the Carbonhydrate Active Enzyme (CAZy) database provides a sequence based family classification of the enzymes which could assemble, modify, or breakdown oligo- and polysaccharides (Lombard et al. 2014). These carbonhydrate active enzymes are classified into different glycoside hydrolase (GH) families by amino acid sequence similarity, members of each family share a common ancestor, conserved catalytic residues and a similar tertiary structure. Each clan in IUBMB contains enzymes from several GH families. For instance, the exo-β-1,3-glucanase (E.C.3.2.1.58) is classified in families 3, 5, 17, and 55, while the endo-β-1,3-glucanase (E.C.3.2.1.39) exists in families 16, 17, 55, 64, 81, and 128 (Sakamoto et al. 2011).

A GH family 5 exo-β-1,3-glucanase from the human pathogen *Candida albicans* naming *Exg* is implicated in cell wall β-glucan remodelling through its glucosyl hydrolase and/or transglucosylase activities. The tertiary structure of *Exg* displays an expected irregular $(\beta/\alpha)_8$-barrel structure and the distinctive “pocket” comprises eight conserved residues including Arg92, His135, Asn191, Glu192, His253, Tyr255, Glu292, Trp363. Structure of the “pocket” characterizes the active sited of GH family 5 glycoside hydrolases. A glucose from the non-reducing end of β-1,3-glucan chain is hydrolyzed through retaining

mechanism (Fig.5-a). The Glu292 acts as the nucleophile, and the Glu192 as the proton donor to conduct the so called double displacement by forming a covalent glycosyl-enzyme intermediate (See Fig.6). (Cutfield et al. 1999; Patrick et al. 2010).

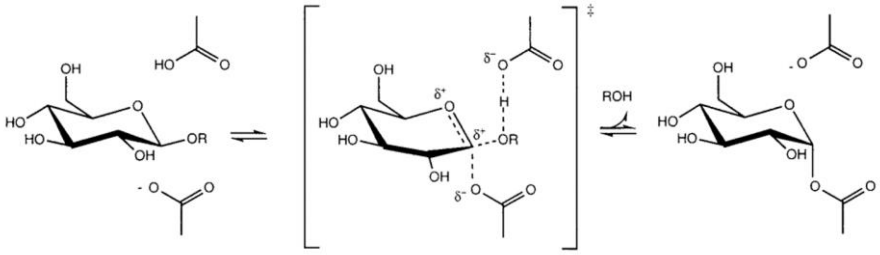

Fig.6 Schematic representation of double displacement reaction.

Formation of the covalent glycosyl-enzyme intermediate is presumed to proceed through an oxo-carbenium ion-like transition state and involve nucleophile Glu292 and proton donor Glu192, which act on the glycosidic bond at the nonreducing end of a β-1,3-glucan chain (Cutfield et al. 1999).

In 2015, a high-resolution crystal structure of an exo-β-1,3-glucanase belonging to GH family 55, namely *SacteLam*55A, from *Streptomyces* sp. was presented (Bianchetti et al. 2015). The enzyme contains two right handed β-helical domains in a single polypeptide chain. These two domains are separated by a long linker region but positioned side by side with a substrate binding cleft on the interface. The enzyme acts in exo-mode with inversion of anomeric configuration and produces glucose from nonreducing end of β-1,3-glucan. The acidic and basic residues activate water as nucleophile to cleave glycosidic bond (As showed in Fig.5-b) (Vuong and Wilson 2010). In the predicted catalytic region, three residues Asp449, Glu480 and Glu502 are strictly conserved among the GH family 55 members. The Glu502 interacting with the anomeric oxygen as the catalytic acid. The residue Glu480 also has

important role in catalysis acting as a general base in the catalytic mechanism by interaction with Gln174, Ser198, and the active site water to be the nucleophile. The conserved residue Asp499 has a primary role in positioning the substrate (Bianchetti et al. 2015).

Although the GH familiy 3 and 17 contain a number of exo-β-1,3-glucanases from different prokaryotes and eukaryotes, no crystallographic data is available for any of these enzymes.

Endo-β-1,3-glucanases are classified into six glycoside hydrolase families. To date, crystallographic studies have been reported for three of these families except GH family 55 and 128. Endo-β-1,3-glucanases in GH family 16 share a β-jelly roll fold and conduct the hydrolysis reaction in a retaining mechanism. At the catalytic region, two glutamate residues are highly conserved acting as nucleophile and proton donor to complete the double displacement action (See Fig.6) (Fibriansah et al. 2007). The crytal structure of a laminarinase (Lam, endo-β-1,3-glucanases) from *Thermotaga maritima* MSB8 was reported in 2011 (Jeng et al. 2011). The enyzme has the classical sandwich-like β-jelly roll fold forming by two antiparallel β-sheets against each other, and forms a concavity for locating the substrates. In the catalytic groove, Glu132 is the base/nucleophile to attack the C1 atom of the sugar ring to promote the cleavage of β-1,3-linkage in the substrate chain. Glu137 acts as the acid to receive the electron and transfer it to adjacent water molecule for attacking the same C1 atom in the β-position, releasing interaction between Glu132 base and the product to complete the retaining catalysis.

Endo-β-1,3-glucanases in GH family 17 possess the $(\beta/\alpha)_8$ barrel fold and two catalytic glutamate residues as proton donor and nucleophile respectively. The hydrolysis of the glycosidic bond in β-1,3-glucan chain is catalyzed by retention of the stereochemistry of anomeric carbon at cleavage point through a double displacement mechanism with covalent glycosyl-enzyme intermediate

(Jenkins et al. 1995). For instance, in the endo-β-1,3-glucanases from *Solanum tuberosum*, Glu118 (proton donor) and Glu259 (nucleophile) are strictly conserved as catalytic residues (Wojtkowiak et al. 2012). Moreover, residues Tyr58, Tyr201, Phe204, Phe305 and Phe322 inside of the catalytic cleft are also strictly conserved among members of GH family 17, which may involve in stacking interactions with the substrate for positioning (Varghese et al. 1994).

Some endo-β-1,3-glucanases are members of GH family 64 cleaving a long chain polysaccharide β-1,3-glucan into specific pentasaccharide oligomer (Nishimura et al. 2001). In 2009, the structure, catalysis mechanism and essential residues of *LPHase* from *Streptomyces matensis* DIC-108 was reported (Wu et al. 2009). The structure of this enzyme consists of a barrel domain and a mixed (α/β) domain, forming a wide-open groove. Within the wide groove, several conserved residues including Glu154 and Asp170 act as acid and base catalysts, respectively, and residues Thr156, Asn158, Trp163 and Thr167 act as substrate binding residues. It cleaves the substrate chain from the reducing end through direct displacement mechanism yielding a pentaose product with the inverted anomeric configuration.

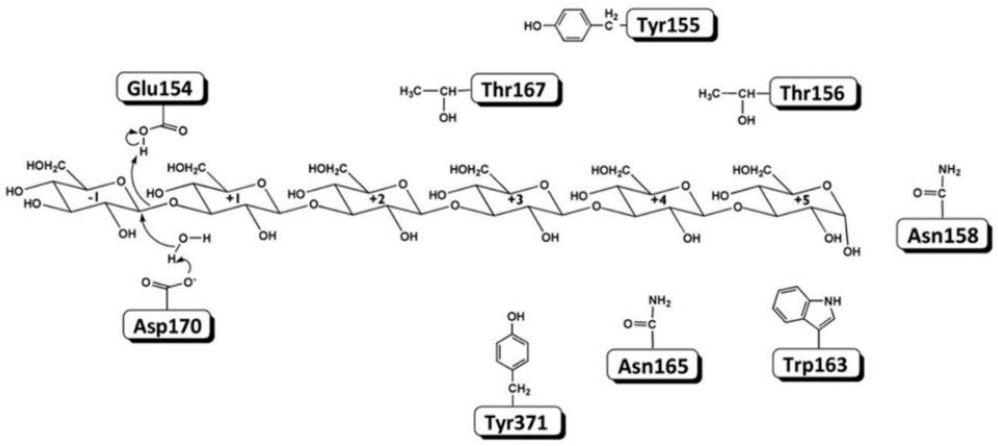

Fig.7 Schematic representation of the proposed catalysis of *LPHase*.

When a long chain polysaccharide β-1,3-glucan diffuses into the wide groove. The substrate binding residues Thr156, Asn158 and Trp163 positioned the

reducing end at the +5 subsite, and then bound by Glu154 and Thr167. Hydrolysis of a β-glucosidic bond by Glu154 acting as a proton donor to the glycosidic oxygen and the Asp170 as a base-assisted nucleophilic from the opposite side of the sugar ring (Wu et al. 2009).

Members of GH family 81 are mainly β-1,3-glucanases from fungi, plant and bacteria. The first crystal structure of this family member was reported in 2013, which is *RmLam*81A from *Rhizomucor miehei* (Zhou et al. 2013). The enzyme has three distinct domains: domain A, B and C. The domain A is comprised of two eight-stranded antiparallel β-sheets, while domain C has a core of $(\alpha/\alpha)_6$-barrel. These two domains compact with each other forming a long catalysis cleft. Domain B exists on the reverse side of the structure to stabilize the whole molecule. Two residues Glu553 and Glu557 are deduced to be a proton donor and a basic catalyst. This agrees with the inversion mechanism of the hydrolytic catalysis.

Enzymatic hydrolyzing paramylon by endo- and exo-1,3-β-glucanase was concerned decades ago. In 1969, Barras and Stone reported that pyramylon was enzymatically degraded by the action of endo- and exo-1,3-β-glucanases in *E.gracilis* cell-extract (Barras and Stone 1969). Vogel and Barber succeeded in characterizing enzymes degrading the treated paramylon in 1986. They found an enzyme act on the substrate yielding glucose as the sole product, and concluded the enzyme an exo-glucanase. This enzyme had optimum conditions of pH 5.0 in 0.5 mol/L acetate buffer and temperature of 60 °C. The highest enzyme activity appeared when the stationary phase *E. gracilis* cells were disruped by French press at about 207 bar. Additionally, an endo-glucanase was also characterized. The optimum pH was at pH 5.2. But in contrast to the exo-glucanase, the endo-glucanase lost its activity by heating to 55 °C. Another difference between the two glucanases was their solubility: the

exo-glucanase was mostly exited in the supernatant after centrifugation. But the endo-glucanase remained in the pellet of percentage about 15-39%. The pellet contained large amounts of insoluble paramylon, indicating that the endo-glucanase was associated with the membrane or the surface of the paramylon granule (Vogel and Barber 1968).

1.4.2 Lytic polysaccharide monooxygenases (LPMOs)

For the derivation of biofuels from non-edible biomass in a more environment friendly transition and breaking the bottleneck of enzymatic conversion, studies are stimulated. Desires on enzymes capable of breaking down recalcitrant polysaccharides, such as paramylon in this work, are boosted. In the traditional model, degradation of such polysaccharides is performed by glycoside hydrolase cocktails, consisting of endo-acting enzymes that cut randomly in the polymer chain and exo-acting enzymes which degrade the substrate from free chain ends. Despite the well acceptance of this model, efficient enzymatic conversion of highly crystalline polysaccharide remains a major bottleneck. Difficulties for the glycoside hydrolases directly deconstruct crystalline substrate into fermentable sugars remains. An unidentified component which may overcome these difficulties was firstly speculated in 1950 (Reese et al. 1950). Researchers proposed that two components were involved in microbial cellulose degradation: One component broke the barrier of polysaccharide crystallinity and the second one corresponded to glycoside hydrolase. Early studies in 1974 firstly reported oxidation as an important enzymatic reaction in cellulose degradation (Eriksson et al. 1974). Decades have passed between the neglect and discovery of lytic polysaccharide monooxygenase (LPMO) activity against recalcitrant polysaccharide such as cellulose (Forsberg et al. 2011) and chitin (Vaaje et al. 2010). The discovery of the LPMOs spured introspection of the classical recalcitrant polysaccharide degradation by hydrolytic enzymes.

LPMOs catalyze the oxidative cleavage of glycosidic linkages of polysaccharides for introducing new chain ends in present of molecular oxygen and an external electron donor, which boosts the activities of hydrolytic enzymes (Forsberg et al. 2016). Chain cleavages are formed by oxidation at either C1 or C4 carbon of a sugar ring (Beeson et al. 2012; Isaksen et al. 2014). Enzymes with mixed C1/C4 activitiy have also been reported (Forsberg et al. 2014a). As showed in Fig.8, oxidation at C1 results in the formation of a lactone, then be further hydrated to a reducing-end aldonic acid, while oxidation at C4 formed a ketoaldose at the non-reducing end (Hemsworth et al. 2015). LPMOs can accept electrons from variety of donors, for instance cellobiose dehydrogenase (CDH) (Courtade et al. 2016), small molecule reducing agents such as ascorbate and gallic acid (Vaaje et al. 2010), and lignin derived redox mediators (Westereng et al. 2015).

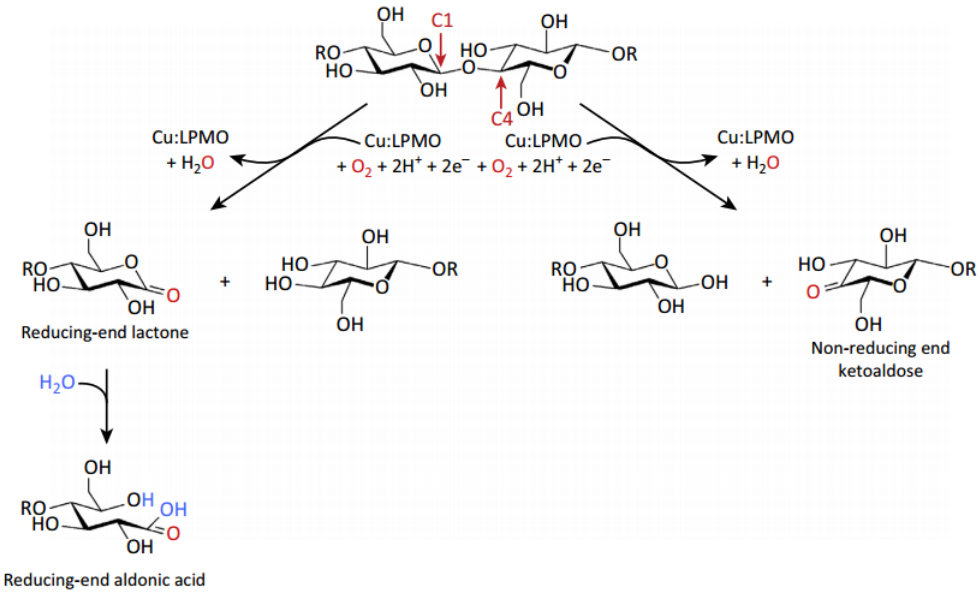

Fig.8 Schematic representation of the LPMO reactions.

LPMOs catalyze oxidation within a polysaccharide chain leading to chain cleavage. Oxidation at C1 results in the formation of a lactone, which is

hydrated to become a reducing-end aldonic acid. C4 oxidation leads to the formation of a ketoaldose at the non-reducing end (Hemsworth et al. 2015).

Originally, LPMOs were classified mistakenly in two families in the carbohydrate active enzymes (CAZy) database: glycoside hydrolase family 61 (GH61), which mainly consisting of fungal enzymes having weak endo-glucanase activity; and carbohydrage binding module family 33 (CBM33) in which were mostly bacterial proteins. In 2010, CBM33s were found deconstructing chitin in an oxidative mechanism (Vaaje et al. 2010), and subsequently GH61s were found able to boost the activity of glycoside hyrolase enzymes on lignocellulose (Harris et al. 2010b). In 2011, CBM33s (Forsberg et al. 2011) and GH61s (Westereng et al. 2011) were then demonstrated as oxidative enzymes and cooper was identified as the correct metal cofactor in the oxidation. These enzymes were first described as “lytic oxidases” (Mba et al. 2012), and then changed to the term “lytic polysaccharide monooxygenase” (LPMO) in 2012 (Horn et al. 2012), reflecting the ability to break and lossen polysaccharide chains from recalcitrant substrates.

Due to the potentially key role of breaking the bottle neck of enzymatic biomass conversion, there was a great interest in unraveling the ability of LPMOs to boost the conversion. Since the discovery of LPMOs, high diversity in sequence, modularity and substrate preference has been explored. Currently, LPMOs are classified in auxiliary activity (AA) families 9, 10, 11, and 13 in CAZy database (Levasseur et al. 2013). The fungal LPMOs belong to families AA9, AA11, and AA13, whereas bacterial LPMOs are sorted in family AA10. In the family of fungal LPMOs, 328 genes related to cellulose cleaving, 66 genes of chitin cleaving, 14 genes of starch cleaving, while 1840 genes of chitin and cellulose cleaving bacterial LPMOs are identified (Martinez 2016). The enzyme has been demonstrated acting on 1,4-glycosidic bonds for deconstructing

cellulose, chitin, starch, and hemicelluloses. In Table 1, some of the characterized LPMOs and their activities and substrate specificities are listed.

Table 1 Characterized LPMOs.

CAZy family	Enzyme	Organism	Substrate	Position	Reference
AA9	Cel61A	*Trichoderma reesei*	cellulose	N/D	(Harris et al. 2010)
	*Ta*AA9	*Thermoascus aurantiacus*	cellulose	C1, C4	(Quinlan et al. 2011)
	*Pc*GH61D	*Phanerochaete chrysosoporium*	cellulose	C1	(Wu et al. 2013)
	*Nc*LPMO9D	*Neurospora crassa*	cellulose	C4	(Beeson et al. 2012)
AA10	*Bl*AA10A	*Bacillus licheniformis*	chitin	C1	(Forsberg et al. 2014b)
	*Cj*LPMO10B	*Cellvibrio japonicus*	cellulose	C1	(Forsberg et al. 2014a)
	*Ef*AA10A	*Enterococcus faecalis*	chitin	C1	(Gudmundsson et al. 2014)
	*Sg*LPMO10F	*Streptomyces gresius*	chitin	C1	(Nakagawa et al. 2015)

	*Tf*AA10B	*Thermobifida fusca*	cellulose	C1	(Forsberg et al. 2014a)
AA11	*Ao*AA11	*Aspergillus oryzae*	chitin	C1	(Forsberg et al. 2014b)
AA13	*An*AA13	*Aspergillus nidulans*	starch	C1	(Leggio et al. 2015)
	*Nc*AA13	*Neurospora crassa*	starch	C1	(Van et al. 2014)

Since 2012, LPMOs have been involved in several commercial cellulose preparations, resulting in efficiently depolymerzation by combined action of LPMOs and glycoside hydrolases (Harris et al. 2014). Considering both the benefit of improving recalcitrant substrate utilization and lowering glycoside hydrolases dosage, the cocktails including LPMOs need to be formulated for every raw material. But by now, no activity has been demonstrated against β-1,3-glucan, such as paramylon. This situation stimulated the trial in this work of searching LPMOs which could act directly on paramylon granule and depolymerize it with synergy of β-1,3-glucanases.

1.5 *Pichia pastoris* expression system

Pichia pastoris is the most cost-effective eukaryotic protein expression system that results in high protein expression yield and achieves a high success rate for a variety of recombinant proteins. As a yeast, *P. pastoris* has many advantages of higher eukaryotic expression systems, such as protein processing, protein folding, and posttranslational modification, straightforward molecular and genetic manipulations and has the added advantage of secreting

the highly expressed protein into the medium (Ahmad et al. 2014). Therefore, the *P.pastoris* was used as recombinant enzyme expression system in this work. Basic knowledges, such as methanol metabolism, gene integration, and protein secretion of this system were introduced in following sections (1.5.1-1.5.10).

1.5.1 A brief history of *P. pastoris*

Methylotroph is the type of cultivation of organism grow on reduced one-carbon (C1) compounds, such as methanol, as sole source of carbon and energy. Methylotrophs are confined to a few prokaryote and eukaryote microorganisms. To prokaryotic methylotrophs, the C1 compound could be methanol, methylamine or methane, whereas in eukaryotic methylotrophs this is limited to methanol (Van et al. 2006).

In yeasts, only a limited number of species belonging to the genera *Pichia*, *Candida*, or *Torulopsis* are able to grow on methanol as sole carbon source, (Gellissen 2000). Among them, *P. pastoris* is well studied. It has unicellular oval shape, 1-5 μm wide and 5-30 μm long (as showing in Fig.9), with a typical eukaryotic cell wall structure consisting of 30-60% polycacharides (mainly β-glucan and mannan sugar polymers), 15-30% proteins, 5-20% lipids, and small amount of chitin (Klis 1994). It has broad pH and temperature range for living between pH of 3-7 (Cregg et al. 2000) and 25-35 °C (Patrick et al. 2005).

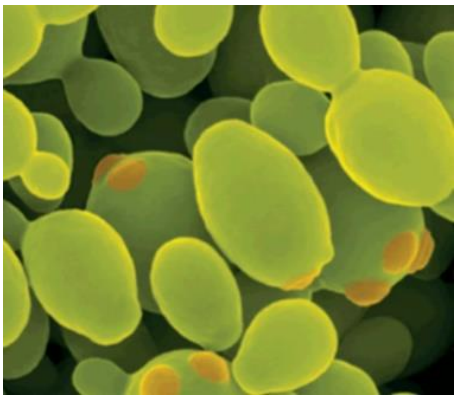

Fig.9 Scanning electron micrograph of *Pichia pastoris* (Schutter et al. 2009).

P. pastoris is taxonomically classified under the kingdom *Fungi*, division *Eumycota*, subdivision *Ascomycotina*, class *Hemoascomycetes*, order *Endomycetales*, family *Saccharomycetaceae*, and subfamily *Saccharomycetoideae*. Since 1940s, Herman Phaff isolated several yeasts from the Yosemite region of California and established a new *Pichia* species, *Pichia pastoris* (Phaff 1986). In 1969, the ability of yeast utilizing methanol as the sole carbon source was firstly described by Koichi Ogata (Koichi Ogata 1969). Immediate attention was attracted in the following decade, because of *P. pastoris*' potential ability of generating yeast biomass and protein for animal feed. In 1970s, mediums and protocols for *P. pastoris* growing on methanol at high cell densities were developed by Phillips Petroleum Company. In early 1980s, Phillips Petroleum contracted with Salk Institute Biotechnology/ Industrial Associate Inc.(SIBIA), developed the *P. pastoris* system as a heterologous protein expression system (Higgins and Cregg 1998). The enzyme alcohol oxidase I (*AOX*1) gene and its promoter were isolated (Roggenkamp et al. 1984; Roa and Blobel 1983), vectors, strains and methods for molecular genetic manipulation were also developed for *P. pastoris* expression system (Cregg et al. 1985). High levels of heterologous protein could be expressed by this new system, due to the combination of the strong and tightly regulated *AOX*1 promoter, and by optimized fermentation methods (Tschopp et al. 1987; Sreekrishna et al. 1988). In 1993, Phillips Petroleum sold the patent to the current holder Research Corporation Technologies (RCT) and licensed Invitrogen Corporation to sell the commercial products of this system which were widely used in molecular biology studies and industries until now (Cereghino and Cregg 2000).

Since 1984, the *P. pastoris* expression system has been selected for cloning and producing hundreds of heterologous proteins due to advantages over other microorganisms. Properties that make the *P. pastoris* an outstanding system were summarized as ability to reach high cell densities in uncomplex growth

medium, easy manipulations in genetic terms, using strong and tightly regulated eukaryotic promoters such as alcohol oxidase (*AOX*) promoter and post translational modification performances as glycosylation, disulfide bond formation, proteolytic processing, secretion of foreign proteins by signal peptides into extracellular medium and therefore simplifying downstream processes (Cregg et al. 2000; Daly and Hearn 2005; Ahmad et al. 2014).

1.5.2 Methanol metabolism in *P. pastoris*

The general mode of methylotrophs to utilize carbon is converting C1 molecules into C3 compounds by a cyclic pathway. In *P. pastoris* and other methylotrophic yeasts, the C1 assimilate uniquely via the xylulose monophosphate cycle. The initial oxidation of methanol takes place in specialized microbodies, peroxisomes, and followed by subsequent metabolic steps in cytoplasm (Sibirny et al. 1990).

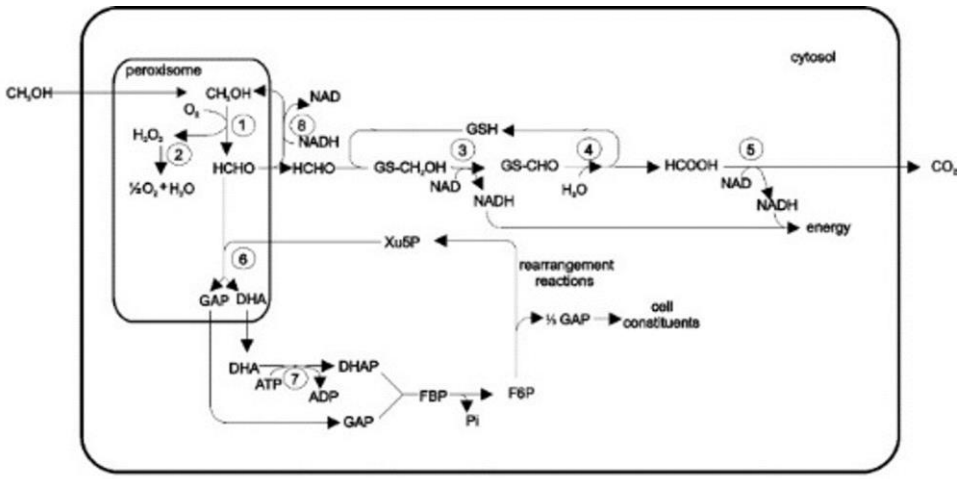

Fig.10 Schematic representation of methanol metabolism in *P.pastoris* (Van et al. 2006).

1: alcohol oxidase (*AOX*), 2: catalase (*CAT*), 3: formaldehyde dehydrogenase (*FLD*), 4: S-formylglutathione hydrolase (*FGH*), 5: formate dehydrogenase (*FDH*), 6: dihydroxyacetone synthase (*DHAS*), 7: dihydroxyacetone kinase, 8: formaldehyde reductase.

In peroxisome, oxidation of the sole carbon source methanol is catalyzed by the alcohol oxidase (*AOX*, EC 1.1.3.13), an enzyme belongs to the family of glucose methanol choline oxidoreductase. The oxidation of methanol by *AOX* results in the generation of hydrogen peroxide and formaldehyde. Enzyme catalase (*CAT*) decomposes hydrogen peroxide into water and oxygen (Ozimek et al. 2005). Formaldehyde is assimilated in the peroxisome or dissimilated in the cytosol, the proper partitioning over these two pathways is fully depending on whether sufficient xylulose-5-phosphate (Xu_5P) is present within the peroxisome (Yurimoto et al. 2005).

Dihydroxyacetone synthase (*DHAS*, EC 2.2.1.3) catalyzes the transketolase reaction between xylulose-5-phosphate (Xu_5P) and formaldehyde yielding dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP) (Sakai et al. 1998). These C3 compounds are diffused to the cytosol. DHA is phosphorylated by dihydroxyacetone kinase forming dihydroxyacetone phosphate (DHAP). In the following aldolase reaction with GAP, it forms fructose-1,6-phosphate (FBP) which is converted to fructose-6-phosphate (F_6P) catalyzed by phosphatase. Xu_5P is regenerated by the subsequent pentose phosphate cycle and transferred into the peroxisome. One third of GAP is exploited for biomass formation via gluconeogenesis (Yurimoto et al. 2011).

Within the cytosolic dissimilation pathway, formaldehyde is trapped by the reduced form of glutathione (GSH) to generate S-hydroxymethyl glutathione ($GS\text{-}CH_2OH$) which is a suitable substrate for formaldehyde dehydrogenase (*FLD*, EC 1.2.1.1) yielding S-formylglutathione (GS-CHO) and NADH. GS-CHO is than hydrolyzed by S-formylglutathione hydrolase (*FGH*, EC 3.1.2.12) to formate and GSH. Formate oxidation is catalyzed by formate dehydrogenase (*FDH*, EC 1.2.1.2) yielding CO_2 and NADH. Dissimilation of formaldehyde results on one hand in the generation of NADH, on the other hand in production of ATP via mitochondrial oxidative phosphorylation. The enhancement of AP

will stimulate the formation of Xu_5P and import it into the peroxisome for assimilatory pathway (Van et al. 2006).

1.5.3 Methanol utilization phenotype in *P. pastoris*

Two alcohol oxidase genes participate the methanol metabolism of *P. pastoris* : *AOX*1 which was isolated in 1985 by Ellis *et al.* (Koshland 1953) for the first time and *AOX*2 which was characterized in 1989 by Cregg *et al.* (Cregg et al. 1989). The major alcohol oxidase activity detected in *P. pastoris* fermentation using methanol as carbon source is contributed by *AOX*1 (responsible for 85% of alcohol oxidase activity), which is more active than *AOX*2 (responsible for 15% of alcohol oxidase activity) (Cereghino and Cregg 2000). This activity difference is caused by the difference in nucleotide sequence located at 5' end of the protein coding portion, although the *AOX*2 is about 97% homologous to *AOX*1. The presence of methanol as the only carbon source in the environment can induce the *AOX*1 promoter, and be repressed when other carbon source exists, such as glucose or glycerol (Paulova et al. 2012).

Because of the presence of two alcohol oxidases in the system, *P. pastoris* strains can be divided into three phenotypes respecting to methanol utilizing abilities (Patrick et al. 2005).

a) Methanol utilization plus (Mut^+) phenotype: Both *AOX* genes are active and the wildtype cells have efficient growth on methanol (Jungo et al. 2006).

b) Methanol utilization slow (Mut^S) phenotype: Only the weak *AOX*2 gene is active, thus cells grow slower using methanol as only carbon source comparing to wild type (Jungo et al. 2006), but can avoid oxygen limitations and difficulties associated with high cell density cultivation (Cos et al. 2006).

c) Methanol utilization minus (Mut^-) phenotype: This type of *P.pastoris* are unable to utilize methanol for growth, as both *AOX*1 and *AOX*2 genes are defective.

The most commonly used expression host of *P. pastoris* is GS115, the wild type with both the *AOX*1 and *AOX*2 genes, and has a phenotype of Mut^+ (Cregg et al. 1985). The frequently used Mut^S strain is KM71, in which the chromosomal *AOX*1 gene is largely replaced with the *S.cerevisiae ARG*4 gene, resulting in slow growth on methanol relying on the weak *AOX*2 gene for the *AOX* activity. The Mut^- type of *P.pastoris* host, for instance MC100-3, has deleted both *AOX* genes and is unable to grow on methanol (Martín-Cuadrado et al. 2008).

1.5.4 Promoter in *P. pastoris*

The promoter *AOX*1 (alcohol oxidase 1, P_{AOX1}) is the most used inducible promoter, which was firstly employed in 1987 for β-galactosidase expression (Tschopp et al. 1987). As reported, the P_{AOX1} is induced only when methanol is the only carbon source, and is strongly repressed by glucose, glycerol or ethanol until these carbon source be depleted (Inan and Meagher 2001). Based on the analysis of the *AOX*1 promoter sequence, a consensus sequence of core motif consisting 5'-CYCCNY-3' was found as specific binding sites for transcription factors (Kranthi et al. 2010). The zinc finger protein *Mxr*1p (methanol expression regulator 1) (Cereghino et al. 2006) and ROP (repressor of phosphoenolpyruvate carboxykinase) (Kumar and Rangarajan 2012) share this DNA binding specificity, but regulate the P_{AOX1} antagonistically in *P.pastoris*. In the promoter of *AOX*1, *Mxr*1p binds at least six regions with the core motif, and deletions of these regions resulting a significant activity reduction of the promoter (Kranthi et al. 2009). Contrarily, deletion of the gene encoding ROP results in increasing activity of P_{AOX1} in a nutrient rich medium (Kumar and Rangarajan 2012).

Although the *P. pastoris* with strong *AOX* promoter is an effective system for protein expression, there are disadvantages due to the usage of methanol. These can be summarized as not suitable for food and therapeutic products manufacturing, as well as the explosive and flammable danger when storing large quantities of methanol (Patrick et al. 2005). Taking these limitations into consideration, alternative promoters for heterologous protein expression in *P. pastoris*, either constitutive or inducible have been developed. For constitutive promoters, promoter *GAP* (Glyceraldehyde-3-P dehydrogenase) (Waterham et al. 1997), promoter *TEF*1 (Translation elongation factor 1) (Adamian et al. 2011), promoter *PGK*1 (3-Phosphoglycerate kinase) (Almeida et al. 2005), and promoter Glycosyl *GCW*14 (Phosphatidyl inositol anchored protein) (Liang et al. 2013) are often used. Beside promoter *AOX*1, the most used and recently established inducible promoters in *P. pastoris* for heterologous expression are: promoter *DAS* (Dihydroxyacetone synthase) (Ellis et al. 1985), promoter *FLD*1 (Formaldehyde dehydrogenase 1) (Shen et al. 1998) and promoter *ADH*1 (Alcohol dehydrogenase), promoter *ENO*1 (Enolase), and promoter *GUT*1 (Glycerol kinase) (Cregg 2012). Among the constitutive promoters, promoter *GAP* (Glyceraldehyde-3-phosphate) is the commonly used for constitutive protein expression (Waterham et al. 1997).

1.5.5 Selection marker in *P. pastoris*

The standard setup of selection marker used in *P. pastoris* are either auxotrophy markers or genes conferring resistance to antibiotics. For the former type, several auxotrophic strains together with vectors having the respective genes have been developed. For instance, *HIS*4 (Histidinol dehydrogenase gene) (Cregg et al. 1985), *ARG*4 (Aregininosuccinate lyase gene), *ADE*1 (PR-aminoimidazole succino carboxamide synthase gene), *URA*3 (Orotidine-5′-phosphate decarboxylase gene) (Cereghino et al. 2001) and *MET*2 (Methyl transferase gene) (Thor et al. 2005). In the latter strategy, zeocin resistance

gene (Drocourt et al. 1990), blasticidin S deaminase gene (Kimura et al. 1994), and kanamycin resisitance gene (G418) (Scorer et al. 1994) are commonly used. In this work, as the *P. pastoris* GS115 is a well studied *HIS*4 auxotrophic mutant, a vector with wild type *HIS*4 gene was used for transformation and protein expression.

1.5.6 Gene integration in *P. pastoris*

Stable gene integration in *P. pastoris* is performed via homologous recombination between linearized vector and genome in homology regions. The integration can be conducted in two ways, gene insertion and gene replacement (Ahmad et al. 2014).

The gene insertion commonly events at the *AOX*1 locus or *HIS*4 locus. Single or multiple gene insertion occurs at both loci. The multiple insertion happens at a low frequency less than 10%. Gene insertion at the *AOX*1 locus conduct via a crossover mechanism at one of these regions in the vector: the *AOX*1 promoter, the *AOX*1 transcription termination region (TT), or 3'-end of *AOX*1 expression cassette. The Fig.11 shows the insertion of linearized recombinant vector at 3'-end *AOX*1 region, gaining an expression cassette of promoter *AOX*1, gene of interest, and *HIS*4.

Gene insertion at the *HIS*4 locus occurs by crossover event between the *HIS*4 locus in the *P. pastoris* chromosome and the *HIS*4 gene in the recombinant plasmid as shown in Fig.12, by linearizing the vector at a restriction enzyme site located in *HIS*4 gene.

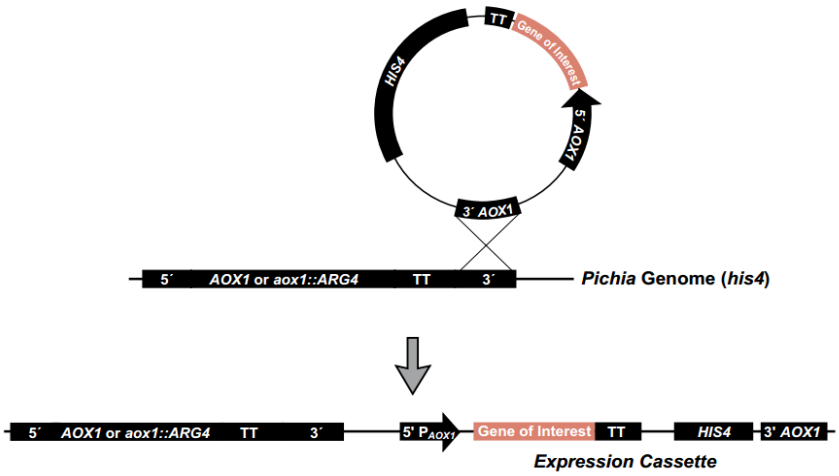

Fig.11 Insertion of linearized plasmid to the *AOX*1 locus (Life technologies 2014).

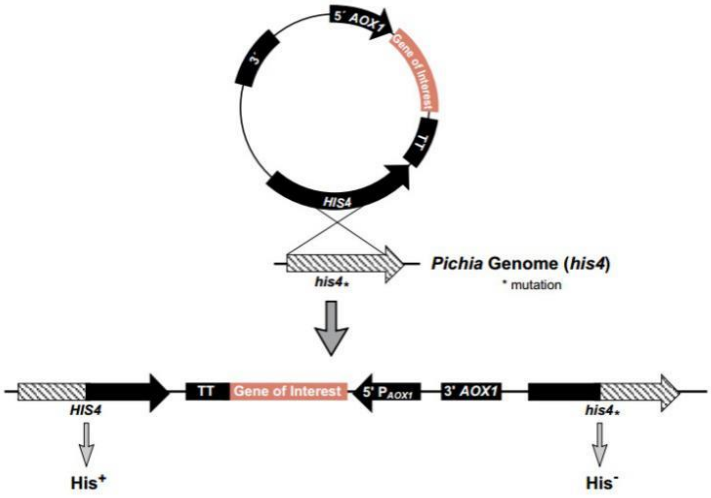

Fig.12 Insertion of linearized plasmid to the *HIS*4 locus (Life technologies 2014).

Gene replacement conducts via double crossover event between the *AOX*1 promoter and 3' *AOX*1 terminal regions between the recombinant vector and *P. pastoris* genome, resulting in *AOX*1 gene deletion and replacement by the expression cassette containing promoter *AOX*1, gene of interest and selection marker. Because of the disruption of the *AOX*1 gene, these recombinant strains

generated are Mut^{S} phenotype and rely weaker enzyme *AOX*2 for growth on methanol. In this work, all the recombinant vectors were integrated into *P. pastoris* strain GS115 by gene replacement at *AOX*1 locus. The Fig.13 below shows the gene replacement at the *AOX*1 locus.

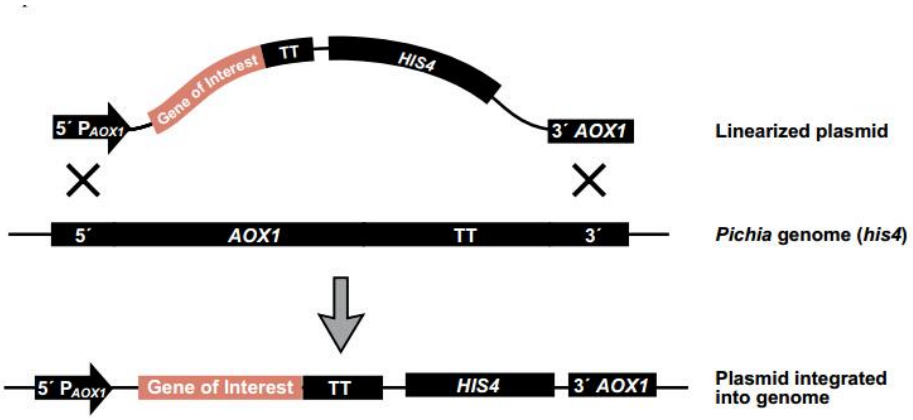

Fig.13 Gene replacement at the *AOX*1 coding region (Life technologies 2014).

1.5.7 Protein secretion in *P. pastoris*

P. pastoris expression system eliminates the costs of expensive downstream purification, as the secreted heterologous protein constitutes the majority of total protein in the medium (Weidner et al. 2010). Protein secretory pathway in *P. pastoris* involves several key steps as showing in Fig.14 (Lambertz et al. 2014). The peptide with an endoplasmic reticulum (ER) secretion signal is synthesized by the ribosome, then targeted to the ER where N-glycosylation, folding and cleavage occurs. The peptide exits the ER and enters the golgi apparatus where their pro-region is removed by dibasic endo-peptidases, such as *Kex*2. Afterward, the recombinant mature protein is packed into a secretory vesicle that will bud from the golgi apparatus and fuse with the plasma membrane. It is then delivered to the periplasm, and transported into the extracellular medium via the cell wall afterwards. For secretion of the

recombinant protein, a secretion signal leader peptide is needed. The commonly used secretion signals in *P. pastoris* are α-mating factor (α-MF), and the invertase signal sequence (*SUC*2) from *S.cerevisiae*, or the *P. pastoris* endogenous acid phosphatase signal sequence (*PHO*1) (Daly and Hearn 2005). In this work, the secretion signal α-MF was involved for protein secretion (See Fig.15).

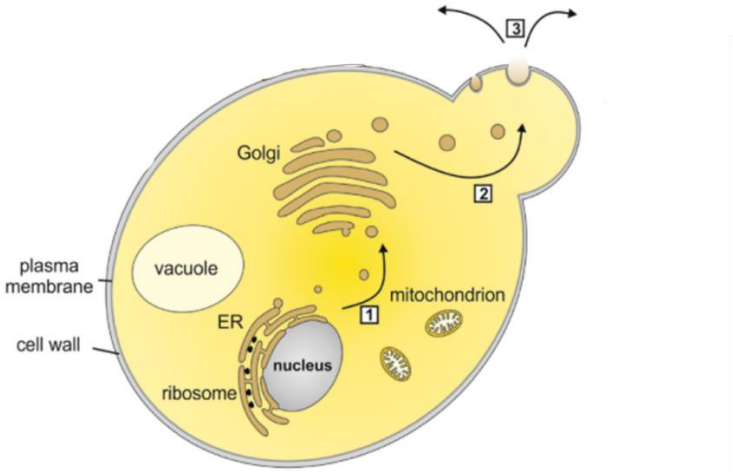

Fig.14 Schematic representation of protein secretion in *P. pastoris* (Lambertz et al. 2014). Arrows indicate the secretory pathway, from the ER (1) the protein is transferred via the golgi apparatus (2) to the medium (3) in secretory vesicles.

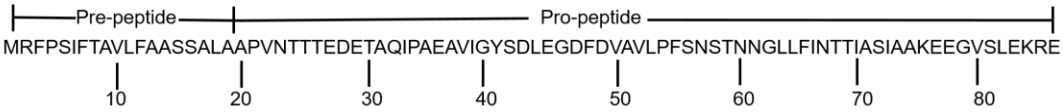

Fig.15 Primary structure of α-MF secretion signal leader.

The α-MF leader consists of two regions, a pre-sequence signal peptide of 19 amino acids and a pro-region of 67 amino acids. The pre-region is responsible for directing the nascent protein post translationally into the ER and is cleaved off subsequently by signal peptidase (Waters et al. 1988). The pro-

region is needed for transferring the protein from the ER to the golgi apparatus compartment and is cleaved at the Lysine-Arginine (K-R) site by the protease *Kex*2 to release the mature protein.

1.5.8 Glycosylation in *P. pastoris*

Glycosylation is the most common post-translational modification to proteins. *P. pastoris* is capable of both *O*-linked and *N*-linked glycosylation of expressed proteins (Pourcq et al. 2010).

Eukaryotic cells assemble *O*-linked saccharide onto the hydroxyl groups of serine or threonine residues in proteins and mainly occur in the golgi apparatus. In yeast such as *P.pastoris*, the *O*-oligosaccharides usually comprise one to five mannose α-1,2 linked residues in arrangement (Duman et al. 1998).

In all eukaryotes, the initial step of *N*-linked glycosylation takes place at the cytoplasmic side of the ER, begins with the sequential addition of *N*-acetylglucosamine (GlcNAc) and mannose (Man) residues, and synthesizes a dolichol-linked glycan precursor. The further elongating occurs after the intermediate $Man_5GlcNAc_2$-PP-Dol structure is flipped to the lumen of the ER until $Glc_3Man_9GlcNAc_2$ is formed. The resultant oligosaccharide is transferred to asparagine residues of the nascent polypeptide chain trimming to $Man_8GlcNAc_2$. At this point, glycosylation pattern of yeast and higher eukaryote differs. In yeast, processing after the glycoconjugate is transported to the golgi apparatus starts with the addition of α-1,6-mannose residues. This branch is elongated by stepwise addition of mannose residues leading to a very complicated hypermannosylated glycoform mixture (Dean 1999).

1.5.9 Conclusion of *P. pastoris* expression system

The use of *P.pastoris* as host for recombinant protein expression has become increasingly popular in recent years. *P.pastoris* is easy to manipulate genetically. The linearized foreign DNA can be inserted in high efficiency via

homologous recombination to generate stable transformats. The benefit of the strong promoters drive promising high-level expression of target proteins. More importantly, *P. pastoris* is an eukaryote provides the potential of producing soluble, correctly folded recombinant proteins with post-translational modifications. A further benefit of *P. pastoris* is that the ability of growing in high cell density enabling large scale fermentations at lower cost than most other eukaryotic systems (Ahmad et al. 2014).

Although many basic elements of *P. pastoris* expression system are well studied and many industrial enzymes have been successfully expressed, further work could still be conducted for optimizing the system. Improving protein secretion by employing different secretion signals is one of the foremost goal in researches (Vadhana et al. 2013). Another general interest is to find effective alternatives to induce protein expression instead of toxic methanol for industrial scale fermentation (Delic et al. 2013). Moreover, by engineering the glycosylation pathways, *P. pastoris* may produce proper post-translational modifications of therapeutic proteins for better pharmacokinetic behaviors (Pourcq et al. 2010).

1.5.10 β-1,3-glucanases expressed in *P. pastoris*

To produce β-1,3-glucanase, the heterologous expression of the enzyme has been tried in various host organisms, including *Escherichia coli* (Kutty et al. 2015), *Bacillus subtilis* (Chuen and Lynch 1998), *Streptomyces lydicus* (Wu et al. 2015), *Saccharomyces cerevisiae* (Giczey et al. 2001), and higher plants (Zhang et al. 2015). The popular yeast *P.pastoris* was also used as expression host. For instance, endo-β-1,3-glucanases from *Lentinula edodes* (Sakamoto et al. 2011), *Aspergillus fumigatus* (Hartl et al. 2011), and exo-β-1,3-glucanases from *Phanerochaete chrysosporium* (Ishida et al. 2009), *Candida albicans* (Patrick et al. 2010) have been successfully expressed in *P. pastoris*. In 2015, the β-1,3-glucananse from thermophilic fungus *Chaetomium thermophilum*

which belonging to GH family 55 was expressed in *P. pastoris* (Papageorgiou and Li 2015). Two exo-β-glucanases of GH family 3 from *Saccharomycopsis fibuligera* and *Trichoderma reesei* were also cloned and expressed in *P. pastoris*. These two enzymes both exhibit significant hydrolysis activity against substrate p-nitrophenyl-beta-d-glucopyranoside, but no evidence of glycosidc bond specificity was reported (Ma et al. 2015). An endo-β-1,3-glucanases of GH family 17 from Ash pollen was produced in *P. pastoris*. Meanwhile, the N-terminal catalytic domain of this enzyme was expressed as independent protein in *P. pastoris*. Although full-length enzyme, showed low enzymatic activity, the recombinant N-terminal catalytic domain had 200-fold higher activity on laminarin as substrate (Torres et al. 2015). As a common enzyme used in biomass conversion, it's believed that more work about β-1,3-glucanase expression in the *P. pastoris* system will be reported, such as optimizations of codon usage, protein structure, medium and cultivation conditions, fermentation engineering and so on.

2. Materials and Methods

2.1 Basic nucleic acid manipulation

2.1.1 Isolation and cleanup of total ribonucleic acid (RNA)

Total ribonucleic acid (RNA) isolation was performed using RNeasy Plant Mini Kit (Qiagen) according to the standard protocol.

RNA purity and integrity are essential for RNA-related experiments. Ribonuclease (RNase) is a highly stable contaminant in laboratory environment which degrades RNA. To prevent prepared samples from RNase degradation, following guidelines should be abided. A set of separate instruments such as pipettes, centrifuge, votexer and disposable nuclease-free plastic-wares are needed exclusively for work with RNA. For glasswares: incubate in 0.1% (v/v) diethylpyrocarbonate (DEPC, Sigma-Aldrich, USA) solution overnight at 37 °C following by autoclaving to ensure complete decomposition of DEPC to CO_2 and ethanol. The work surfaces are cleaned with RNaseZap solution (Thermo Scientific, USA) before use. And wear gloves and change frequently when handling RNA.

The isolated total RNA was cleaned up using deoxyribonuclease I (DNase I, Sigma-Aldrich, USA) and RNeasy MinElute Cleanup Kit (Thermo Scientific, USA). This work was conducted according to the protocol bellow.

1. Add the following reagents into an RNase-free PCR tube:

Table 2 Total RNA clean-up reaction mixture.

Reagent	Volume (µL)
Isolated total RNA	8

10× Reaction buffer	1
DNase I	1

2. Mix gently and incubate at room temperature for 15 minutes;
3. Add 1 µL of stop solution to bind calcium and magnesium ions for inactivating DNase I;
4. Incubate the mixture at 70 °C for 10 minutes to denature both the DNase I and RNA, then chill on ice;
5. Collect RNA according to the concentration protocol provided by the kit;
6. The concentration and purity of RNA isolated was determined by NanoDrop® spectrophotometer (Thermo Scientific, USA). Absorbance readings at 260 nm should be between 0.05 and 1.00. The ratio between the readings at 260 and 280 nm (A260/A280) provides an estimate of the purity of the RNA. Pure RNA preparations have A260/A280 values of 2.0. RNA samples displaying A260/A280 values in the range of 1.8 to 2.0 could be regarded as highly pure and samples with values below 1.0 should not be used;
7. The pure RNA prepared was used for first strand cDNA synthesis immediately.

2.1.2 First strand complementary deoxyribonucleic acid (cDNA) synthesis

The first strand complementary deoxyribonucleic acid (cDNA) synthesis was performed with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) using the total RNA as template. The synthesis reaction was procced on ice according to the following protocol.

1. Add the following reagents into a sterile, nuclease-free PCR tube and add nuclease-free water to the final volume of 12 µL;

Table 3 cDNA synthesis reaction mixture.

Component	Amount
Template RNA	2.5 µg
10 µmol/L Primers	1 µL
Nuclease-free water	To 12 µL

2. Mix gently, centrifuge briefly and incubate at 65 °C for 5 min. Chill on ice, afterwards;
3. Add the following reagents to the PCR tube and incubate for 60 min at 42 °C;

Table 3 Continued

Component	Volume (µL)
5× Reaction buffer	4
20 U/ µL RNase inhibitor	1
10 mM dNTP Mix	2
200 U/µL Reverse transcriptase	1

4. Terminate the reaction by heating at 70 °C for 5 min. The reverse transcription was used for cloning work immediately or stored at -80 °C for further use.

2.1.3 Plasmid isolation

E. coli transformant was picked to inoculate 5 mL LB medium per well with respective antibiotic in 24 square-well plate (HJ-bioanalytik, Germany). The culture was allowed to grow overnight at 37 °C with shaking at 120 rpm, shaking orbital of 50 mm (Kuhner LS-X, Germany). Plasmid isolation was prepared from *E. coli* in small scale using innuPREP Plasmid Mini Kit (Analytikjena, Germany). The concentration and purity of isolated plasmid was determined by NanoDrop® spectrophotometer. The plasmid was used immediately or stored at -20 °C.

2.1.4 Restriction endonuclease digestion

All restriction endonucleases were FastDigest™ enzymes (Thermo Scientific, USA). The reaction components were mixed according to the standard reaction setup provided by the manufacture and incubated for 2 hours in a 37 °C water bath.

2.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate, identify, and purify deoxyribonucleic acid (DNA) fragments. A 10 g/L gel was prepared by mixing agarose in 0.5×TAE buffer. 5 µL Roti ®-Safe Gel Stain (Carl Roth, Germany) was added to every 100 mL gel solution and mix thoroughly before solidifying.

To run electrophoresis, the gel was submerged with 0.5×TAE buffer in a chamber (Mupid-One, Nippon Genetics, Japan). 5-15 µL DNA sample, which was previously mixed with 1/10 volumes of 10 × DNA loading buffer, as well as 8 µL 1 Kb DNA Ladder (Plasmid Factory, Germany) was loading into each well, respectively. The electrophoresis was carried out at 150 volts until dye markers have migrated an appropriate distance depending on the size of the DNA fragment of interest. An BioDocAnalyze Systeme (Analyticjena, Germany) was used for gel visualization.

2.1.6 Purification and ligation of DNA fragments

DNA fragments from reaction mixtures such as restriction endonuclease digestions, PCR *etc*., were purified using Wizard ® SV PCR Clean-up Kit (Promega, USA) following the manufacturer's instructions.

DNA fragment separated by agarose gel electrophoresis was excised from the gel and then extracted by Wizard ® SV Gel Clean-up Kit (Promega, USA) according to the standard procedure.

T4 DNA Ligase LC (Thermo Scientific, USA) was used for DNA ligations. Inserting DNA and linearized vector were mixed in a molar ratio of 3:1. The reaction mixture was prepared following the standard sticky end ligation protocol provided by the manufacturer. And the reaction was performed by incubating in a water bath at 22 °C for 1 hour. Before transformation, the reaction mixture was further incubated at 65 °C for 10 min, then chilled on ice to inactivate T4 DNA ligase.

2.1.7 Isolation of genomic DNA

To prepare the *P. pastoris* genomic DNA, single colony appeared on YPD plate after incubating at 30 °C was selected. After cultivating overnight in 5 mL/well of YPD medium using 24 square-well plate (HJ-bioanalytik, Germany) at 30 °C with constant shaking (180 rpm, shaking orbital 20 mm, IKA-KS 4000ic control, Germany), yeast cells were harvested by centrifugation. Genomic DNA isolation was performed using MasterPure™ Yeast DNA Purification Kit (Epicentre, USA) following the protocol provided.

To isolate genomic DNA from *Asperfillus oryzae*, a glycerol stock was used to prepare a seed culture by inoculating 5 mL mL/well YPD medium in 24 square-well plate and cultivated for 16 h at 30 °C with shaking frequency of 180 rpm, shaking orbital of 20 mm. The entire seed culture was transferred into 50 mL of the same growth medium in 250 mL baffled shake flask and cultivated

for 24 h at the same conditions. A 10 mL portion was centrifuged for harvesting mycelium. The harvested pellet was washed twice with demineralized water before lyophilization. Then the freeze-dried mycelium pellet was pulverized into fine powder using mortar and pestle in the presence of liquid nitrogen. Genomic DNA isolation from *A. oryzae* was then performed using Wizard® Genomic DNA Purification Kit (Promega, USA) according to plant tissue protocol. The genomic DNA was used immediately or stored at -20 °C.

2.1.8 Polymerase chain reaction (PCR)

1. PCR was performed for the specific amplification of DNA, using plasmid, genomic DNA, or cDNA as template. The reaction mixture was set up following the recommendation provided with the Phusion® High-Fidelity DNA Polymerase (New England Biolabs, USA);

Table 4 PCR reaction mixture.

Component	Volume (µL)	Final Concentration
5× Phusion HF Buffer	4.0	1×
10 mM dNTPs	0.4	200 µM each
Forward primer	1.0	0.5 µM
Reverse primer	1.0	0.5 µM
Template DNA	**	< 250 ng
*DMSO (optional)	(0.6 µL)	3% (v/v)
Phusion DNA Polymerase	0.2	0.02 U/µL

H_2O	Add to 20.0	-

*: For amplification of difficult targets, such as GC-rich sequences, or genomic DNA, a final concentration of 3% (v/v) dimethyl sulfoxide (DMSO) was added.

**: Recommend doses of DNA template are showed in the following table.

Table 5 Recommend doses of DNA template.

Template DNA	Amount for 20 µL Reaction
Genomic DNA	20 ng – 100 ng
Plasmid	0.4 pg – 4 ng
cDNA	≤ 2 µL

2. The reaction was performed by Mastercycler® Pro (Eppendorf, Germany) and the thermocycling conditions below were abided.

Table 6 PCR thermocycling conditions.

step	Temperature (°C)	Duration	Cycles
Initial Denaturation	98	30 s	1
Denaturation	98	10 s	35
Annealing	*	20 s	
Extension	72	** 15-40 s/Kb	

Final Extension	72	10 min	1
Hold	4	∞	1

*: The annealing temperature depends on primers used.

**: Extension time is dependent on amplicon length and complexity. The recommend extension durations were showed below.

Table 7 Recommend extension duration.

Amplicon	Extension Duration (s / kb)
Genomic DNA	30
Plasmid	15
cDNA	40

2.1.9 Transformation of *E. coli*

Transformation of *E. coli* was carried out according to the protocol from Chung *et al*. (Chung et al. 1989). And the rapid *E. coli* transformation procedure was optimized as following.

1. Cultivate *E. coli* DH5α overnight at 37 °C with shaking (120 rpm, orbital 50 mm, Kuhner LS-X, Germany) in 50 mL LB medium in 250 mL shake flask;
2. Dilute the overnight culture by the ratio of 1:50 with LB medium and grow at the same condition until the cell density reach OD_{600} = 0.25-0.4;

3. Harvest the cells by centrifugation at 4 °C, and re-suspend the pellet by 1/10 volume ice-cold TSS buffer (Appendices, solutions and mediums) to prepare competent cells;
4. Add 100 pg-10 ng of DNA to 100 µL competent cells. Gently mix the cells and DNA by flicking the tube and incubate on ice for 10 minutes;
5. Transfer the tube to room temperature and incubate for 10 minutes;
6. Re-incubate the tube on ice for another 10 minutes;
7. Add 800 µL LB medium and incubate the cells at 37 °C with shaking (120 rpm, orbital 50 mm, Kuhner LS-X, Germany) for 1 hour;
8. Spread 100 µL transformed *E. coli* on LB agar plate with 100 µL/mL ampicillin, and incubate overnight at 37 °C.

2.1.10 Transformation of *P. pastoris*

The method for *P. pastoris* transformation was developed from the high efficiency protocol reported by Wu and Letchworth (Wu and Letchworth 2004). The transformation was carried out by electroporation using Gene Pulser® and Pulse Controller® (Bio-Rad, USA). Procedure of competent cell preparation and transformation was optimized below.

1. A single colony of *P. pastoris* strain GS115 was grown overnight in 50 mL YPD medium using 250 mL baffled shake flask at 30 °C with shaking (180 rpm, shaking orbital 50 mm, Kuhner LS-X, Germany);
2. 20 µL of the seed culture was used to inoculate 100 mL YPD medium and grew at the same conditions until the cell density reached OD_{600}= 1.0-2.0;
3. The cells were harvested by centrifugation and re-suspend with the same volume of *P. pastoris* transformation pretreat buffer (0.1 mol/L

lithium acetate, 10.0 mmol/L dithiothreitol) at room temperature for 30 minutes;

4. The cells were washed with ice-cold 1.0 mol/L sorbitol solution thrice;
5. The cells were re-suspended with 0.5 mL sorbitol solution (1.0 mol/L) to prepare competent cells;
6. 100 µL competent cells were mixed with 1.5-2.0 µg DNA gently. Transfer to a 0.2 cm gap electroporation cuvette (Bio-Budget, Germany) and incubate on ice for 5 minutes;
7. The electroporating pulse was applied at 1.5 kV, 25 µF, 200 Ω;
8. The cuvette was incubated on ice for 5 minutes, and the electroporated cells were mixed with 800 µL ice-cold 1.0 mol/L sorbitol solution afterwards;
9. 100 µL aliquot was spread on RDB agar plate and incubate at 30 °C for 3-4 days until yeast colonies appear.

2.2 Basic protein manipulation

2.2.1 Protein concentration assay

The protein concentration assay was performed using Protein Assay Kit (Bio-Rad, USA) based on the Bradford method. Two procedures were developed for different ranges of measurement. The standard procedure was for samples with protein concentration in the range of 0.1 g/L to 0.8 g/L. The more sensitive microassay procedure was for samples in the range up to 80 mg/L.

2.2.1.1 Standard procedure for protein concentration assay

1. Dye reagent was prepared by diluting one part dye reagent concentrate with four parts of demineralized water. Then filtered through a Whatman 1 filter (GE Healthcare, USA) to remove particulates;
2. Bovine serum albumin (BSA) protein standard samples were prepared in following concentrates (0 g/L; 0.1 g/L; 0.2 g/L; 0.3 g/L; 0.5 g/L; 0.8 g/L);
3. 10 µL of each standard or sample solution was pipetted into separate wells in a 96 well micro titer plate (BrandTech Scientific, Thermo Fisher Scientific, USA);
4. 200 µL of diluted dye reagent was added to each well. The sample and reagent were mixed thoroughly using multi-channel pipet to dispense the reagent;
5. The mixture was incubated at room temperature for at least 5 minutes. Samples should incubate at room temperature for less than 1 hour;
6. Absorbance at 595 nm was measured by SpectraMax M Microplate Readers (Molecular Devices, USA);
7. Standard curve for protein concentration from 0 to 0.8 g/l could be seen in Fig.16.

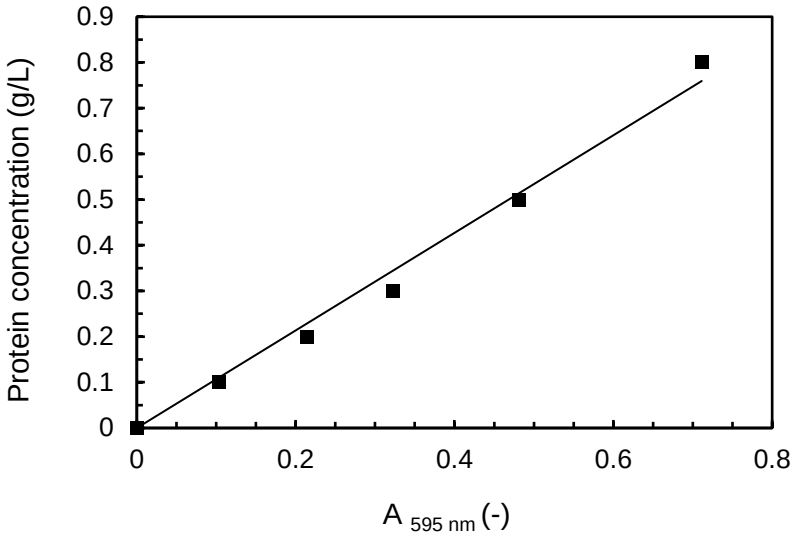

Fig.16 Standard curve for the standard protein concentration assay (0-0.8 g/L).

Fitting formula: $y = 1.068x, R^2 = 0.9887$

2.2.1.2 Micorassay procedure for the more sensitive protein concentration

1. Protein standard samples were prepared in following concentrates (0 mg/L; 10 mg/L; 20 mg/L; 30 mg/L; 50 mg/L; 80 mg/L;
2. 160 µL of each standard or sample solution was pipetted into separate well in 96 well micro titer plate (BrandTech Scientific, Thermo Fisher Scientific, USA);
3. 40 µL of dye reagent concentrate was added to each well. The sample and reagent were mixed thoroughly;
4. The mixture was incubated at room temperature for at least 5 minutes. Samples should incubate at room temperature for less than 1 hour;

5. Absorbance at 595 nm was measured by SpectraMax M Microplate Readers (Molecular Devices, USA);
6. Standard curve for protein concentration from 0 to 80 mg/L could be seen in Fig.17.

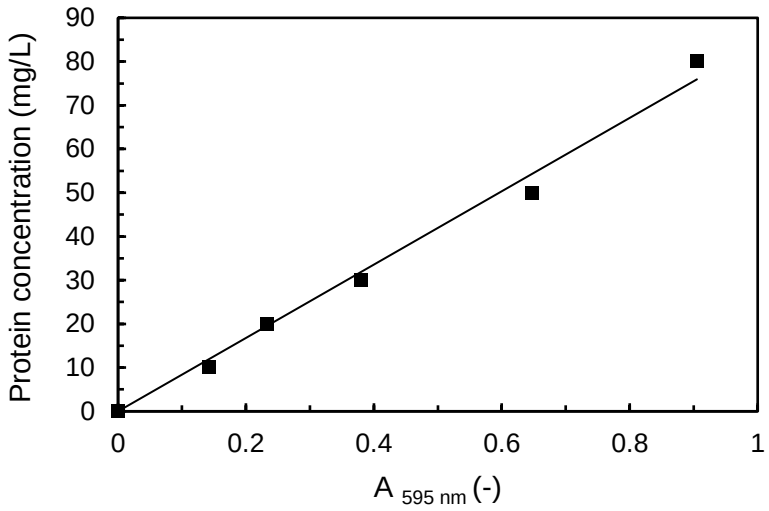

Fig.17 Standard curve for the more sensitive protein concentration assay (0-80 mg/L).

Fitting formula: $y = 83.899x, R^2 = 0.9901$

2.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was applied to separate and analysis proteins, which was carried out using 12.5% (w/w) resolving gel and 5.0% (w/w) stacking gel in tris glycine buffer. The electrophoresis was conducted following to the protocol below.

1. Assemble the glass plates and prepare the 12.5% (w/w) resolving gel solution, add APS and TEMED together at last as described in Table 8 and mix carefully to avoid the formation of bubbles;

Table 8 Resolving gel preparation.

Component	Volume per 15 mL
ddH_2O	5.0 mL
30% (w/w) Acrylamide/bisacrylamide solution	6.25 mL
1.5 mol/L Tris-HCl buffer (pH=8.8)	3.75 mL
50 g/L SDS solution	300 µL
10 g/L APS solution	75 µL
TEMED	10 µL

2. Pour the gel solution between the glass plates, leave free space for the stacking gel. And carefully cover the top of resolving gel with 100 % isopropanol. Discard the isopropanol until the resolving gel was polymerized;
3. Prepare the stacking gel as described in Table 9 and pour into the glass plate sandwich. Insert combs and wait the gel to polymerize;

Table 9 Stacking gel preparation.

Component	Volume per 5 mL
ddH_2O	2.77 mL
30% (w/w) Acrylamide/Bisacrylamide solution	0.83 mL
0.5 mol/L Tris-HCl buffer (pH=6.8)	1.25 mL

50 g/L SDS solution	100 µL
10 g/L APS solution	50 µL
TEMED	6 µL

4. Mix three parts of protein sample with one part of 4× sample buffer (Life Technologies, USA) before incubating in 98 °C for 15 minutes;
5. Remove combs. Put the glass plate sandwich into electrophoresis tank and fill with 1× Tris-glycine-SDS buffer. Load 8 µL PageRuler® Prestained Protein Ladder (Life Technologies, USA) and samples respectively into wells;
6. Set an appropriate voltage and current depending on the number of gels refer to Table 10. Increase power when the dye front reaches the resolving gel;

Table 10 Power management of protein electrophoresis.

Gel	1 gel	2 gels
Stacking gel	13 mA	25 mA
Resolving gel	25 mA	50 A

7. Stop the electrophoresis when protein marker reached appropriate positions and process gel staining.

2.2.3 SDS-PAGE gel staining

2.2.3.1 Coomassie brilliant blue staining

After the electrophoresis, wash the SDS-PAGE gel with water twice to remove running buffer left on the surfaces. Stain the gel with coomassie brilliant blue G-250 staining buffer (appendices, solutions and mediums) with low speed shaking at room temperature overnight. Destain the gel by soaking in water for at least 2 hours.

2.2.3.2 Silver staining

The more sensitive silver staining method was applied for SDS-PAGE gel visualization and detection of protein having low concentration. The staining protocol is shown below, and all manipulations were carried out at room temperature with low speed shaking (appendices, solutions and mediums).

1. Fix the protein by incubate it in fixer solution for 1 hour.
2. Wash the gel twice by soaking in deionized water for 2 minutes;
3. Sensitize by soaking in sensitizing solution for 30 minutes;
4. Wash the gel twice by deionized water for 20 seconds each time;
5. Incubate gel for 20 minutes in silver nitrate solution;
6. Wash the gel twice by deionized water for 1 minutes each time;
7. Develop the gel in visualizing solution until protein bands appear;
8. Terminate the staining by changing to stop solution and incubate for 5 minutes.

2.3 Activity assay and partial purification of *E. gracilis* intracellular proteins

2.3.1 *E. gracilis* cultivation

The *Euglena gracilis* Z wild strain was obtained from the culture collection of Fermentation Technology Research Group, Bielefeld University and cultivated in a basal medium (Table 11), in 300 mL shake flask at 37 °C with shaking (120 rpm, shaking orbital 50 mm, Kuhner LS-X, Germany) under light exclusion. Each 100 mL medium was inoculated with 5 mL culture of exponential phase in the same medium.

Table 11 Medium for *E. gracilis* cultivation.

Component	Concentration (g/L)
$MgSO_4 \times 7\ H_2O$	0.23
$(NH_4)_2HPO_4$	1.00
$CaCl_2 \times 2\ H_2O$	0.03
KH_2PO_4	1.00
$NaC_6H_5O_7 \times 5.5\ H_2O$	0.36
Glucose $\times\ H_2O$	20.00
Trace-element	**Concentration (mg/L)**
$Co(NO_3)_2$	1.30
$MnCl_2 \times 4\ H_2O$	1.80

$CuSO_4 \times 5\ H_2O$	0.02
H_2MoO_4	0.20
$ZnSO_4 \times 7\ H_2O$	0.40
$Fe_2(SO_4)_3 \times 6\ H_2O$	3.00

Vitamine	Concentration (mg/L)
Thiamin × HCl	0.01
Cyanocobalamin	0.05

2.3.2 Intracellular protein extraction preparation

E. gracilis cells were harvested by centrifugation at 3,500 g for 10 min and washed in demineralized water. The pellet was resuspended in 0.1 mol/L sodium acetate buffer (pH=6.0). The cell lysis was prepared by three cycles of sonication at the highest level for 30 s, and cooled in ice bath for 1 min between each cycle. The supernatant obtained by centrifugation at 10,000 g for 20 min was used for the further enzyme activity assay.

2.3.3 Determination of dry biomass and dry paramylon

For determining the dry biomass, a defined volume of culture was centrifuged at 3,500 g for 5 min. The cells were harvested, washed by demineralized water three times, and dried in 37 °C for 72 hours before weighting.

To weight dry paramylon, cells were harvested by centrifugation for 5 min at 3,500 g and the cell pellet was re-suspended in the same volume of demineralized water. The cells were disrupted by three cycles of sonication for 30 s at the highest level. After a further centrifugation at 3,500 g for 5 minutes

the insoluble substances were washed twice with the same volume of 100% ethanol. The insoluble proteins were removed by incubation with 1 g/L trypsin in 0.1 mol/L sodium phosphate buffer (pH 7.6) overnight (37-40 °C) on a shaker (about 20 rpm, shaking orbital 50 mm, Kuhner LS-X, Germany). After the incubation, the pellet was obtained by centrifugation at 3,500 g for 5 min, and followed by washing with demineralized water for twice. The paramylon pellet was dried in 37 °C for 72 hours before weighting.

2.3.4 Paramylon preparation for enzymatic hydrolysis

The native paramylon granule was prepared as the method mentioned in determination of dry paramylon in section 2.3.2.

To prepare 2× lyophilized paramylon, paramylon granule was dissolved in 0.1 mol/L KOH solution (in concentrations of 1.0 g/L, 2.5 g/L, and 5.0 g/L) and transferred to dialysis tubing (MWCO: 8.000 to 10.000, Zellu Trans Roth, Germany), then incubated for at least 24 hours in demineralized water and followed by lyophilization. The procedure was performed twice to produce the so called 2× lyophilized paramylon.

To prepare the alkali treated paramylon, the native granule was mixed with 1.0 mol/L KOH to concentration of 50.0 g/L, the mixture was adjusted to pH 6.0 by 0.1 mol/L HCl forming a paramylon gel. The so-called paramylon gel was diluted with demineralized water in concentrations of 1.0 g/L, 2.0 g/L, 2.5 g/L, and 5.0 g/L. The alkali treated paramylon and 2× lyophilized paramylon solutions were stored for long period at 4 °C and used as substrates for enzyme hydrolysis.

2.3.5 Investigation of intracellular β-1,3-glucanase activity of *E. gracilis* cultures

2.3.5.1 β-1,3-Glucanase activity assay

Glucanases hydrolyze substrate and generate reducing sugars and reducing chain ends with free carbonyl groups. These enzymes' activities are commonly assayed by quantifying the amount of the reducing groups in the product. Typically, by some colorimetric procedures, such as 3,5-dinitrosalicylic acid (DNS) method. The reducing groups react with 3,5-dinitrosalicylic acid (DNS) in alkaline solution forming 3-amino-5-nitro salicylic acid which is orange-red colored and could be detected spectrophotometrically at 540 nm (Fig.18).

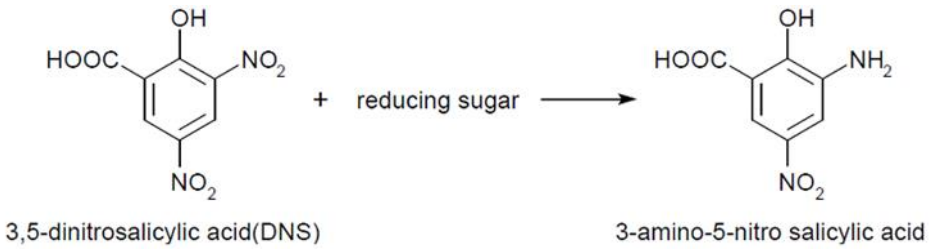

Fig.18 Reaction of DNS with reducing sugar (Kyoto 2011).

The measurement of intracellular β-1,3-glucanase activity of *E.gracilis* cultures were performed according to a standard DNS assay procedure below.

1. Following reagents were prepared: 600 g/L sodium potassium tartrate solution, and 20 g/L 3,5-DNS solution. Prepare DNS work solution freshly by mixing the two solutions mentioned above with equal volume.
2. 180 µL glucose solution of different concentrations or sample was mixed with 60 µL DNS work solution in 96 well micro titer plate (BrandTech Scientific, Thermo Fisher Scientific, USA). Carefully float the plate on 98 °C water bath for 15 min. Measure the absorbance at

540 nm by SpectraMax M Microplate Readers (Molecular Devices, USA) after cooling to room temperature. Absorbance versus concentration of the standards were plotted to construct the standard curve (Fig.19).

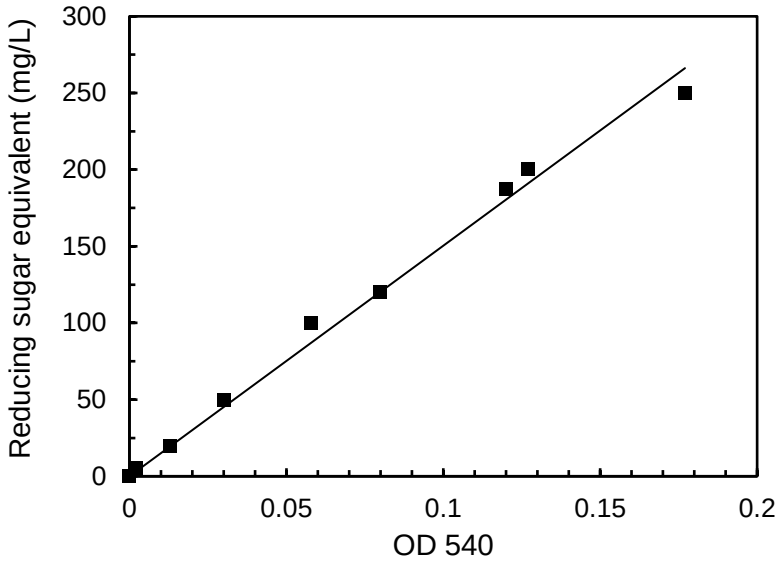

Fig.19 Standard curve of DNS assay.

Fitting formula: $y = 1503.8x,\ \ R^2 = 0.9915$

3. Calculate the reducing sugar equivalent released by glucanase according to the standard sugar curve in term of mmol/L.

$$Reducing\ \ sugar\ \ equivalent = \frac{y}{M} = 8.35x \quad \text{(mmol/L)}$$

Where:

y= reducing sugar equivalent, mg/L;

M = molar mass of glucose, 180.16 g/mol.

2.3.5.2 Influence of substrate

For analyzing the influence of substrates to enzyme activity, native paramylon, 2× lyophilized paramylon or alkali treated paramylon in different concentrations were used as substrates for 100 µL of 10 mg/L laminarinase (endo-β-1,3-glucanase, Sigma-Aldrich, USA), respectively. After 8 hours incubation at 37 °C the enzyme activity was measured by DNS method.

2.3.5.3 Influence of intracellular protein concentration

To study the effect of *E.gracilis* intracellular protein concentration on the degree of conversion towards alkali treated paramylon, concentrated extracts (5-,10-,20-, and 50-fold) from a 7 days culture were prepared. 100 µL extracts were used as crude enzymes and incubated with 1 mL, 1 g/L alkali treated paramylon at 37 °C for 8 hours.

2.3.5.4 Influence of the extraction buffer pH value

pH value of the extraction buffer have influence on hydrolases activity of *E.gracilis* extracts. After cultivation for 7 days, cell free extracts were prepared in 0.1 mol/L sodium acetate buffer at different pH values (6.0, 5.5, 5.0, 4.5) as crude enzymes. 100 µL enzyme samples were incubated with 1 mL of 1 g/L 2× lyophilized paramylon at 37 °C for 8 hours and followed by reducing group measurement.

2.3.5.5 Influence of hydrolysis temperature

1 mL of 1 g/L alkali treated paramylon (pH=5.5) with 100 µL of 50× extract was incubated for 8 hours at 30, 37, 45, 50, and 55 °C, followed by reducing group measurement.

2.3.5.6 Influence of incubation duration

100 µL of 50× extract of the 7-day-old culture was incubated at 37 °C in 1 mL of 1 g/L alkali treated paramylon (pH 5,5) for 16 hours with hourly measurement.

2.3.5.7 Influence of cultivation duration

During cultivation time of 14 days, samples were taken daily and produced a 50× extract. 100 µL of the extraction was incubated in 1 mL of 1 g/L alkali treated paramylon (pH 5,5) for 16 hours at 37 °C and followed by reducing group assay.

2.3.6 Partial purification of intracellular proteins from *E. gracilis*

2.3.6.1 $(NH_4)_2SO_4$ Precipitation

E. gracilis intracellular protein extract was prepared as section 2.3.2. After centrifugation at 22,000 g for 20 min, the supernatant was precipitated to a final $(NH_4)_2SO_4$ concentration of 0-20%, 20-60% and 60-80% saturation respectively. Each precipitate was dialyzed (MWCO: 8.000 to 10.000, Zellu Trans Roth, Germany) against 20 mmol/L Tris-HCl buffer (pH=5.5) at 4 °C for 4 hours. Hydrolytic activity was found in the precipitate of 20-60% $(NH_4)_2SO_4$ saturation.

2.3.6.2 Anion exchange chromatography

The 20–60% saturation precipitate was dialyzed (MWCO: 8.000 to 10.000, Zellu Trans Roth, Germany) against 20 mmol/L Tris-HCl buffer (pH=5.0) at 4 °C for 4 hours. The enzyme extraction was applied to a weak anion exchange chromatography column (Toyopearl DEAE 650, 1.6 cm × 10 cm), using 20 mmol/L Tris-HCl buffer (pH=5.0) as loading buffer at flow rate of 1.0 mL/min. Proteins with glucanase activity against alkali alkali treated paramylon were

found in flow through fraction under pH=5.0 and the flow through peak was collected.

The collected proteins were dialyzed (MWCO: 8.000 to 10.000, Zellu Trans Roth, Germany) for changing buffer to 20 mmol/L Tris-HCl (pH=7.0) at 4 °C for 4 hours and then applied to the same anion exchange chromatography column using 20 mmol/L Tris-HCl buffer (pH=7.0) as loading buffer at flow rate of 1.0 mL/min. Proteins were eluted by stepwise method. The fraction having glucanase activity was eluted by 20% elution buffer (20 mmol/L Tris-HCl, 1 mol/L NaCl, (pH=7.0), flow rate 1.0 mL/min). And this peak was collected for future purification.

2.3.6.3 Hydrophobic chromatography

Enzyme sample obtained from ion exchange chromatography was mixed with the same volume of solution consisting of 40 mmol/L Tris-HCl and 1.8 mol/L ammonium sulfate. Then applied to a hydrophobic chromatography column (Fractogel TSK Butyl 650 (s), 1.6 cm × 6 cm; loading solution: 20 mmol/L Tris-HCl and 0.9 mol/L ammonium sulfate, pH=5.5; eluting solution: 20 mmol/L Tris-HCl, pH=5.5). Proteins were eluted stepwise and the fractions were dialyzed (MWCO: 8.000 to 10.000, Zellu Trans Roth, Germany) against 20 mmol/L Tris-HCl buffer (pH=5.5) at 4 °C for 4 hours followed by hydrolytic assay toward alkali alkali treated paramylon. Paramylon degrading activity was obtained from 45 % to 80% eluting fraction.

2.3.6.4 Size exclusion chromatography

The fraction with glucanase enzyme activity collected from hydrophobic chromatography was concentrated by viva-spin (MWCO 5000, GE Healthcare, USA) to approximately 1.0 mL and then applied to a size exclusion chromatography column (Superdex 200, 1.6 cm × 120 cm) using 20 mmol/L

Tris-HCl buffer (pH=5.5). Fractions were collected by the size of 1.0 mL at a flow rate of 1.0 mL/min for enzyme activity and protein concentration assay.

2.3.7 Trial of gene amplification of paramylon degrading enzyme in *E. gracilis*

2.3.7.1 Total RNA isolation and cDNA synthesis

Total RNA of *E. gracilis* isolation was performed as described in section 2.1.1. 50 mg lyophilized *E. gracilis* cells was used for each mini column according to the standard procedure. After total RNA clean-up, cDNA synthesis was conducted using oligo$(dT)_{16}$ primer with adapter (See Table 12).

2.3.7.2 Design of degenerate primer

According to the same peptide sequence identified by mass spectrum, four degenerate primers (in Table 12) was designed in order to lower the degeneration level.

Table 12 Primers for degenerate PCR.

Primer	Sequence 5'-3'
*EUG*LysoF-a	CGC GGI ATH YTI CAA ATH AAC
*EUG*LysoF-b	CGC GGI ATH YTI CAA ATH AAT
*EUG*LysoF-c	CGC GGI ATH YTI CAG ATH AAC
*EUG*LysoF-d	CGC GGI ATH YTI CAG ATH AAT
Adapter oligo $(dT)_{16}$ primer	GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T

AUAP	GGC CAC GCG TCG ACT AGT AC

I=A+T+G+C, H=A+T+C, Y=C+T.

2.3.7.3 Degenerate PCR

The summary of target protein gene of *E. gracilis* amplification was shown in Fig.20.

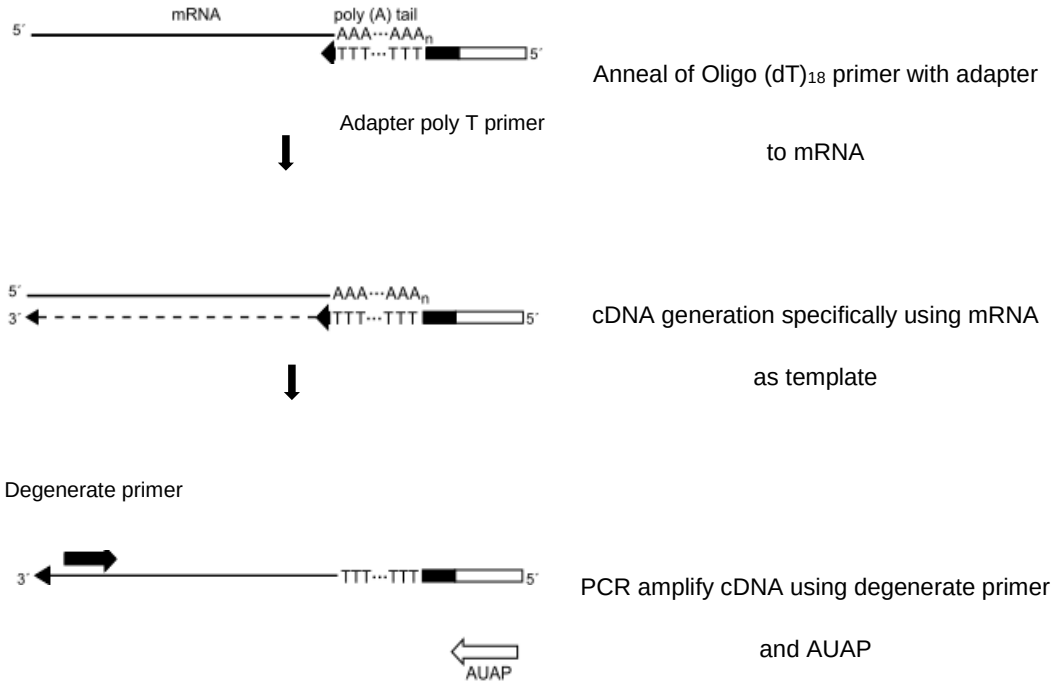

Fig.20 Summary of degenerate amplification.

First strand cDNA synthesis was synthesized from the poly A tail of mRNA using an adapted poly T primer. Amplification of target gene was performed using two primers, the degenerate primer that prospectively annealing to the target region in the cDNA and the other was a primer targeting the adapter of the poly T end generated from cDNA synthesis. The PCR was conducted as followed.

1. Mix the following reagent into a PCR tube as Table 13;

Table 13 Degenerate PCR reaction mixture.

Reagents	Volume (μL)
Template cDNA	1.0
10 μmol/L Oligo $(dT)_{16}$ primer with adapter	1.0
10 μmol/L AUAP primer	1.0
5× Reaction buffer	2.0
10 mmol/L dNTPs	0.2
DNA polymerase	0.1
ddH_2O	to total volume of 10 μL

2. Start the amplification program as described in Table 14;

Table 14 Degenerate PCR thermocycling conditions.

Temperature (°C)	Duration	Cycle
98	30 s	1
98	10 s	45
55	20 s	
72	80 s	
72	10 min	1

4	Hold

3. Analysis of degenerate PCR products by 10.0 g/L agarose gel electrophoresis.

2.4 Cloning and expression of recombinant β-1,3-glucanase in *P. pastoris*

2.4.1 Gene cloning of recombinant β-1,3-glucanase

2.4.1.1 *Trichoderma reesei* cultivation

Spore suspension of *T. reesei* QM 6a (ATCC® 13631™) was routinely maintained from potato dextrose agar plate (PDA) at 28 °C. Suspension containing 1×10^6 spores/mL was washed from the PDA plate with sterile 0.05% (v/v) Tween 80 solution and used to inoculate 100 mL of glucose-yeast extract medium (Table 15, pH=7.0) in a 500 mL baffled shake flask. The spores were grown at 28 °C for 24 h with shaking frequence of 180 rpm, shaking orbital of 50 mm (Kuhner LS-X, Germany).

Table 15 Glucose-yeast extract medium.

Component	Concentration
Glucose	10.0 g/L
Yeast extract	10.0 g/L
$(NH4)_2SO_4$	1.4 g/L
KH_2PO_4	2.0 g/L
$CaCl_2$	0.4 g/L
$MgSO_4$	0.3 g/L

0.5 g/L $FeSO_4$*	1 mL/L
3.7 g/L $CoCl_2$*	1 mL/L
1.6 g/L $MnSO_4$*	1 mL/L
1.4 g/L $ZnSO_4$*	1 mL/L

*: Autoclaved separately and added into medium before use.

The myceliums obtained afterward were harvest by centrifugation and washed twice by sterile demineralized water before changing to inducing medium. For induction, lactose-yeast extract medium (substitute lactose for glucose in glucose-yeast extract medium, pH=7.0) was used and incubated for 48 h under 28 °C on a shaker at 180 rpm (shaking orbital 50 mm, Kuhner LS-X, Germany).

2.4.1.2 Total RNA isolation and cDNA synthesis of *T. reesei*

The myceliums harvested after induction were freeze dried and saved at -80 °C until total RNA isolation. The RNA isolation and cDNA synthesis were carried out as described in section 2.1.1 and 2.1.2. The cDNA produced was kept in -80 °C until use.

2.4.1.3 Gene amplification of β-1,3-glucanase from *T. reesei*

Primers were designed specifically to amplify the β-1,3-glucanase enzyme genes (Table 16). The fragment of *Kex*2 processing site (5´-AAAAGA-3´) for secretion signal α-factor cleavage downstream of *Xho* I site was added into the 5´ end of forward primers. *Sal* I was used instead when amplified gene containing *Xho* I cleavage site. Meanwhile gene of purification tag (Hexa-

histidine) and stop codon followed by restriction cleavage site *Not* Ⅰ were designed at the 3´ end of reverse primers.

β-1,3-glucanase enzyme genes from *T. reesei* were amplified by PCR using cDNA as template. The products were analyzed by agarose gel electrophoresis and fragments with the right size were cut and cleaned up before ligated into the pJET 1.2/blunt Cloning Vector (Thermo Scientific, USA) according to the standard procedure provided, then transformed into *E. coli* DH5α (Procedures see section 2.1). Recombinant vectors were screened by restriction endonuclease digestion and the positive ones containing right size insertion were used for recombinant expression vector construction.

Table 16 Primers for gene amplification from *T. reesei*.

Enzyme	Primer	Sequence 5′-3′
*Tr*GH16 EGR45305.1	P.p45305Forw*SalI*	*GTCGAC* AAAAGATGGGCACCTCCGTCCTATG
	P.p45305Rev	*GCGGCCGC* TTAGTGATGATGATGGTGATGGGTAGAGTACTGGGCAACGTAG
*Tr*GH55 EGR47521.1	P.p47521Forw	TATA *CTCGAG* AAAAGAACGCCCACCCCG
	P.p47521Rev	*GCGGCCGC* CTAATGGTGATGATGATGATGGACGGTATAACGACCGACAT
*Tr*GH64 EGR 45483.1	P.p45483Forw	*CTCGAG* AAAAGAGCCCCCTCTCTCATCACAAGAGTTGC
	P.p45483Rev	*GCGGCCGC* TTAATGATGATGATGATGATGAGGAGGAGGCGCGCCGATG
*Tr*GH81 EGR49603.1	P.p49603Frow	*CTCGAG* AAAAGACTGCCCAATGGCGCAAA
	P.p49603Rev	*GCGGCCGC* CTAGTGGTGATGATGATGATGCGACTGGACAAACTTGAACC
*Tr*GH17 EGR46171.1	P.p46171Forw	TTTG *CTCGAG* AAAAGACAGAACAACTACCTCGGCTTCAAC
	P.p46171Rev	AAA *GCGGCCGC* TTAGTGATGGTGATGGTGATGGGTGGCGGTGGGCTTG

2.4.1.4 Gene amplification of β-1,3-glucanase from *P. pastoris*

For obtaining gene of one exo-β-1,3-glucanase from *P. pastoris* GS115, the intronless gene was cloned by PCR-based strategy using genomic DNA as template (section 2.4.1.7). The specific primer designing (In Table 17), and gene amplification were conducted the same as described in section 2.4.1.3.

Table 17 Primers for exo-β-1,3-glucanase gene amplification from *P. patoris*.

Enzyme	Primer	Sequence 5'-3'
*Pp*GH5 CAY69081.1	P.p69081Forw	CCGG*CTCGAG* AAAAGATACTCCAGT GAAAAAATCATGGG
	P.p69081Rev	ATT*GCGGCCGC*TTAGTGATGGTGATGG TGATGGAACCCACATTGATTTGGATA

2.4.2 Expression vector construction

The secretory expression vector pAaHBgl (collected by our group; Fig.21) was selected for recombinant vector construction. The *Kex*2 processing site for secretion signal α-factor cleavage was added into forward primers upstream of the gene of interest, furthermore the purification tag (Hexa-histidine) and stop codon were added by the end of reverse primers. The gene insertion was performed between restriction cleavage sites *Xho* I (*Sal* I was used when the gene contains *Xho* I site) and *Not* I. Constructed expression vectors were transformed into *E.coli* DH5α and colonies appeared on the selective agar plate were cultivated in order to proof correct clones via plasmid isolation. All related manipulations are described in section 2.1.

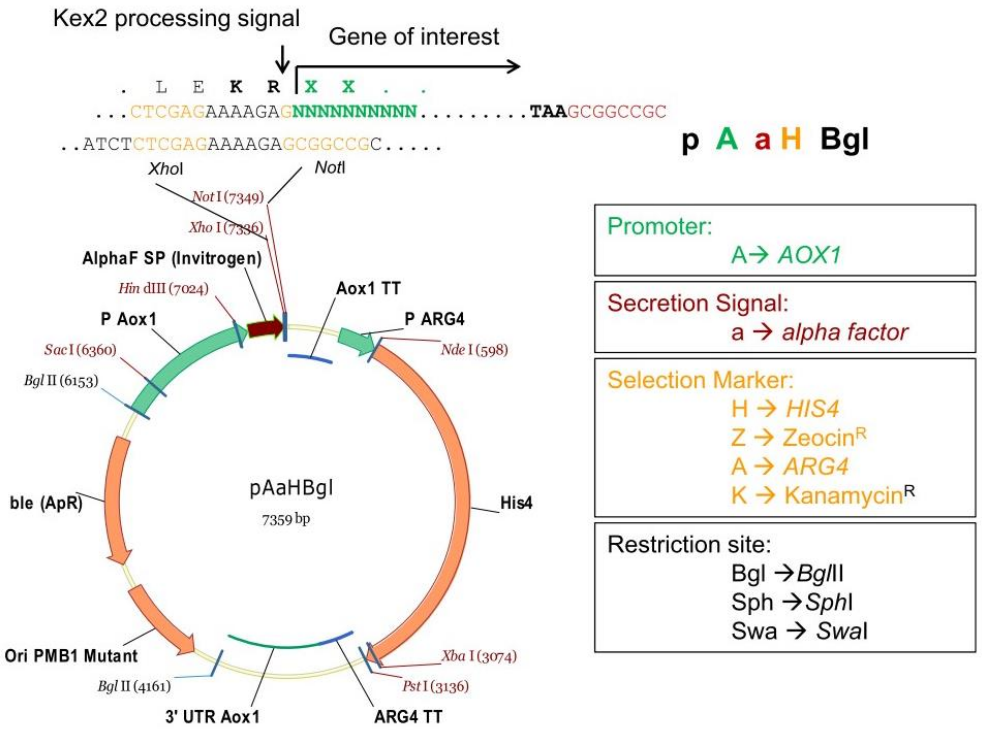

Fig.21 Map of *P. pastoris* expression vector pAaHBgl (Ahmad et al. 2014).

2.4.3 Transformation of recombinant *P. pastoris*

The constructed expression vectors were linearized at two *Bgl* II sites (bases 6153 and 4161 in Fig.21) located upstream of *AOX*1 promoter and downstream of *3'* UTR *AOX*1. The long fragment with a length of about 8 kb was isolated and cleaned up before concentrating with Vacuubrand® vacuum system (Sigma-Aldrich, USA).

Exceptionally, when the gene of interest contains *Bgl* II site, the DNA fragment between two *Bgl* II site (bases 6153 and 4161 in Fig.21) was amplified by PCR using primers given in Table 18. The amplification product was cleaned up directly after PCR reaction.

Table 18 Primers for *P. patoris* transformation.

Primer	Sequence 5′–3′
pAaHBgl-PAOX1-Forw	ATCTAACATCCAAAGACGAAAGGTTGAATGA
pAaHBgl-UTRAOX1-Rev	TCTTGAGATAAATTTCACGTTTAAAATCAGCGTACC

Transformation of *P. pastoris* was carried out by electroporation. The protocol in detail was described in section 2.1.10. Recombinant *P. patoris* were screened for recombinant enzyme expression by SDS-PAGE and enzyme activity assay.

2.4.4 Recombinant protein expression in *P. pastoris*

2.4.4.1 Recombinant expression in 24 square-well plate

For selecting the positive expression recombinant, the pilot expression was performed involving picking colonies from selective plates, cultivation in a small scale culture, methanol inducing, SDS-PAGE and enzyme activity assay. For small scale expression, the 24 square-well plate (HJ-bioanalytik, Germany) was used following the procedure below.

1. A single colony was picked from selective agar plate, and used to inoculate 5 mL YPD medium in one well of the plate. Cultivation was performed for 48 hours at 30 °C, 180 rpm, shaking orbital 20 mm (IKA-KS 4000ic control, Germany);
2. Harvest the cells by centrifuging at 1500g for 5 minutes at 4 °C and dispose the supernatant. Wash the cells with 5 mL BMMY medium twice, then resuspend cell pellet in 3 mL BMMY medium for protein expression;

3. Cultivate under the same conditions and add to each well 150 µL of 10% (v/v) methanol to the final concentration of 0.5% every 24 hours;
4. Stop inducing after 120 hours. The cells and supernatant were separated by centrifugation at 1500g for 5 minutes at 4 °C and stored at -20 °C.
5. Use the supernatant as crude sample for enzyme activity assay and protein electrophoresis.

2.4.4.2 Recombinant expression in shake flask

Positive results from 2.4.4.1 were confirmed by expression in 250 mL baffled flasks following the method below.

1. 100 µL of glycerol stork of positive results from 2.4.4.1 was used to inoculate 5 mL of YPD in 24 square-well plate (HJ-bioanalytik, Germany), and grow at 30 °C in a shaking incubator (180 rpm, shaking orbital 20 mm, IKA-KS 4000ic control, Germany) for 24 hours;
2. The 5 mL seed culture was mixed with 50 mL BMGY medium in a 250 mL baffled flask and grow at 30 °C in a shaking incubator (180 rpm, shaking orbital 50 mm, Kuhner LS-X, Germany) for 24 hours;
3. Harvest the cells by centrifugation at 1,500g for 5 minutes at 4 °C, and decant the supernatant. The cells were washed twice with 50 mL BMMY medium, then resuspended in 50 mL BMMY medium for protein expression;
4. 100% methanol was added to a final concentration of 0.5% (v/v) every 24 hours until the inducting time reaches 120 hours;
5. Samples taken every 24 hours were centrifugated at 1,500g for 5 minutes at 4 °C. The cell pellet and supernatant were stored separately in -20 °C.

2.4.5 β-1,3-glucanase enzyme activity assay

2.4.5.1 Preparation of substrate

Alkali treated paramylon (2.0 g/L, pH=6.5) was prepared as described in section 2.3.4. The alkali treated paramylon could be stored for long period at 4 °C and used as substrate for activity assay.

Laminarin was dissolved in 50 mmol/L sodium acetate buffer (pH=6.5) to a final concentration of 2.0 g/L.

2.4.5.2 Activity assay of recombinant endo-β-1,3-glucanase

Glucanase hydrolyzes glucans to produce reducing sugars and reducing ends with the presence of free carbonyl groups. These enzymes are commonly assayed by quantifying the amount of the reducing groups during the assay. For determining reducing groups in low concentration, a sensitive 3-methyl-2-benzothiazolinone hydrazine (MBTH) assay was used (Anthon and Barrett 2002). This method was developed to measure aldehydes originally (Hauser and Cummins 1964), and had been adapted to determine the quantification of reducing sugars. It had been confirmed for the determination of polygalacturonase activity (Anthon and Barrett 2002) and saccharification assay (Gomez et al. 2010).

In this work, the highly sensitive MBTH method was optimized for standard 96-well microtiter plate with a final volume of 200 µL.

1. Prepare MBTH reagent by freshly mixing equal volumes of 0.43 g/L MBTH and 0.14 g/L DL-dithiothreitol (DTT) before use. The MBTH and DTT stock solutions should be stored at 4 °C;
2. Prepare oxidizing reagent solution containing 5.0 g/L $NH_4Fe(SO_4)_2 \cdot 12H_2O$, 0.5 % (v/v) H_2SO_4 and 0.8 % (v/v) HCl. The oxidizing reagent was stable at room temperature for at least one week;

3. Mix 90 µL enzyme sample with 90 µL substrate solution in 96 well micro titer plate (BrandTech Scientific, Thermo Fisher Scientific, USA). The mixture was incubated at 37 °C for 60 min afterwards;
4. Prepare glucose standard samples with concentration from 0 to 500 µmol/L for standard curve;
5. Pipet 25 µL of standard solution or sample into each well of the microtiter plate;
6. Add 25 µL of 1.0 mol/L NaOH solution followed by 50 µL MBTH reagent in each well and mix thoroughly;
7. Cover the multiwall plate by sealing tape and incubate at 80 °C for 20 min;
8. Mix 100 µL oxidizing reagent to each well. The samples were cooled to room temperature before determining the absorbance at 620 nm by SpectraMax M Microplate Readers (Molecular Devices, USA). Absorbance versus concentration of the standards were plotted to construct the standard curve (Fig.22);

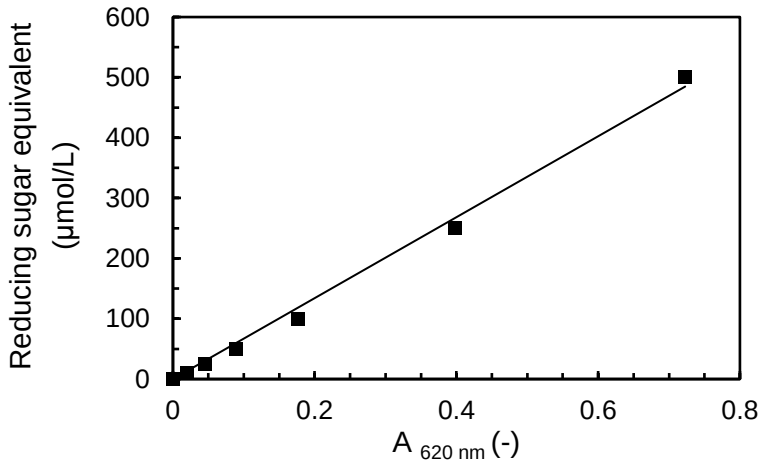

Fig.22 Standard curve of MBTH assay.

Fitting formula: $y = 670.69x,\ R^2 = 0.9952$

9. Calculate the reducing suger equivalents released by glucanase according to the standard sugar curve and convert the enzyme activity in term of IU/L, where one unit of enzyme activity (IU) is defined as 1 µmol of reducing sugar equivalent released in 1 min.

$$Enzyme\ \ activity = \frac{y}{t} \times 8 = 89.43x, \text{ (IU/L)}$$

Where:

y= 670.69x, µmol/L

x= absorbance at 620 nm

t= enzyme reaction duration, 60 min

8= dilution factor.

2.4.5.3 Activity assay of recombinant exo-β-1,3-glucanase

To determine exo-β-1,3-glucanase activity, the hydrolysis product glucose was determined by D-glucose assay kit (R-Biopharm, Germany). D-glucose was phosphorylated to D-glucose-phosphate (G-6-P) in the presence of the enzyme hexokinase and adenpsine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate. In the presence of the enzyme glucose-6-phosphate dehydrogenase, G-6-P was oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The amount of NADPH formed in the rection was stoichiometric to the amount of D-glucose. The increase in NADPH was measured by means of absorbance at 340 nm (Kunst et al. 1984). The experiments were conducted by the following procedure.

In this work, the assay was optimized for 96-well quartz micro titer plate.

1. Mix 100 µL enzyme sample with 100 µL substrate solution and incubate the mixture at 37 °C for 15 min;
2. Terminating the reaction by inactivating the enzyme at 98 °C for 2 min;
3. Determine the produced glucose by D-glucose assay kit;

   The assay reagents triethanolamine buffer (TAE buffer) and standard solution were prepared according to the original procedure. Components of reaction mixture were pipetted into quartz microtiter plate wells as shown in Table 19.

Table 19 Components of glucose assay reaction.

Component	Volume (µL)		
	Blank	Standard	Sample
TAE buffer	80	80	80
Sample solution	-	-	8
Standard solution	-	8	-
Water	160	152	152
Read the absorbance at 340 nm (A_1) approximately 3 min after mixing.			
HK/G6P-DH suspension	1.6	1.6	1.6
Read the absorbance at 340 nm (A_2) approximately 15 min after mixing.			

4. The concentration of glucose in the sample solution was calculated as:

$$c = \frac{V \times \Delta A}{\varepsilon \times d \times v \times 1000} = 0.4794 \times \Delta A, \text{ (mol/L)}$$

Where:

c = glucose concentration in the sample solution, mol/L

v = 0.2416 mL, assay volume

ΔA = (A2-A1) sample-(A2-A1) blank

ε = 6.3 L/(mmol×cm), molecular absorption coefficient of NADPH at 340 nm

d = 0.1 cm, light path from well botton to liquid surface

v = 0.0008 ml, sample volume

1000 $\frac{\text{mol}}{\text{mmol}}$= conversion from mmol to mol

5. Convert exo-β-1,3-glucanase enzyme activity in term of IU/L, where one unit of enzyme activity (IU) was defined as the amount of enzyme required to liberate 1 μmol reducing sugar equivalent in 1 minute under the assay condition;

$$Enzyme\ \ activity = \frac{c \times 10^6}{t} \times 2 = 6.392 \times 10^4 \times \Delta A, \text{ (IU/L)}$$

Where:

c = 0.4794×ΔA, mol/L (see step 4.)

t = 15 min, incubate duration

10^6 $\frac{\mu M}{M}$= conversion from mol/L to μmol/L

2 = dilution factor.

2.5 Cloning and expression of recombinant lytic polysaccharide monooxygenase in *P. pastoris*

2.5.1 Strain and cultivation

2.5.1.1 Strain and cultivation

Trichoderma reesei QM6a and *Aspergillus oryzae* (ATCC® 42149™) were grown on potato dextrose agar (PDA, BD, Germany) at 28 °C to obtain spores. Spore suspensions were diluted to 1×10^6 spores/mL with sterile 0.05% (v/v) Tween 80 solution and used to inoculate 100 mL pre-culivation medium (pH=7.0, Table 20) in a 500 mL baffled shake flask. The pre-cultivation was performed at 28 °C, 180 rpm, shaking orbital of 50 mm (Kuhner LS-X, Germany) for 24 hours.

Biomass obtained from pre-cultivation was harvest by centrifugation at 10,000 g for 15 minutes at 4 °C and washed twice by sterile demineralized water before changing to production medium. In production medium, components were the same as in pre-cultivation medium, except paramylon granule was used at the concentration of 2.5 g/L as the only carbon source instead of glucose.

Table 20 Pre-cultivation medium for *T. reesei* and *A. oryzae*

Component	Concentration
Glucose	10.0 g/L
$(NH_4)_2SO_4$	1.4 g/L

KH_2PO_4	2.0 g/L
$CaCl_2$	0.4 g/L
$MgSO_4$	0.3 g/L
0.5 g/L $FeSO_4$*	1.0 mL/L
3.7 g/L $CoCl_2$*	1.0 mL/L
1.6 g/L $MnSO_4$*	1.0 mL/L
1.4 g/L $ZnSO_4$*	1.0 mL/L

*: Autoclaved separately and added into medium before use.

2.5.1.2 Enzyme activity assay of β-1,3-glucanase induced by paramylon

The supernatant was collected as crude enzyme by centrifugation at 10,000 g, 4 °C for 15 min. The activity assay of β-1,3-glucanase was conducted as described in section 2.3.5.1, using alkali treated paramylon or laminarin as substrate. Enzyme activity was estimated as hydrolysis rate by determining reducing groups by DNS method.

2.5.2 Gene cloning and expression of LPMOs

Lytic polysaccharide mono-oxygenases (LPMOs) are a recently discovered class of enzymes capable of oxidizing recalcitrant polysaccharides. LPMOs from *T. reesei* and *A.oryzae* were selected and the genes were obtained for heteroexpression in *P. pastoris*.

Gene of *Tr*AA9 (GeneBank: EGR52697.1) from *T. reesei* was cloned using cDNA as template. Gene of *Ao*AA11 (GeneBank: BAE61530.1) was obtained by PCR-based strategy using genomic DNA as template. The detailed preparation of cDNA and gnomic DNA are described in section 2.1.

Gene specific primers of LPMOs (See Table 21) were designed and PCR amplification followed the same strategy as described in section 2.4.1.3.

Recombinant expression of LPMOs in the yeast *P. pastoris* were performed following the β-1,3-glucanase expression described in section 2.4.3-2.4.5.

Table 21 Primers for LPMO gene amplification.

Enzyme	Primer	Sequence 5'-3'
*Tr*AA9 EGR52697.1	*P.p*52697Forw	*CTCGAG*AAAAGACATGGACATATTAATGACATTGTCATC
	*P.p*52697Rev	*GCGGCCGC*TTAGTGATGATGATGGTGATGGTTAAGGCACTGGGCGTAG
*Ao*AA11 BAE61530.1	*P.p*61530Forw	TTCC*CTCGAG*AAAAGACACATGATGATGGCGCAGCCC
	*P.p*61530Rev	ATT*GCGGCCGC*TTAGTGGTGATGATGGTGGTGGCAGGCCTGGCTGCCC

2.5.3 Synergistic action assay between LPMOs and β-1,3-glucanase

The copper-dependent lytic polysaccharide monooxygenases (LPMOs) have been shown to play a central role in oxidative degradation of cellulose. But

determination of the activity of LPMO directly remains difficult, therefore the strategy of detecting the soluble product after synergistic action of LMPO and exo- or endo-β-1,3-glucanase was used. The synergistic degrading insoluble paramylon were conducted as described below.

1. As the recombinant enzymes were secreted, the supernatants were used as crude enzyme solution. These colutions were dialyzed against 50 mM sodium acetate (pH 6.5) by dialysis tubular membrane (MWCO 6000-8000, GE Healthcare, USA) for 4 hours at 4 °C followed by filtering sterilization using 0.22 μm filter (Millex-GP, Merck, Germany);
2. Crystalline paramylon granules were autoclaved and dried before use;
3. Enzymes were mixed in the ratio of 1:1 by volume and paramylon was added to the final concentration of 1.0 g/L;
4. The mixtures were incubated at 37 °C with shakeing (30 rpm, shaking orbital 50 mm, Kuhner LS-X, Germany) for 72 hours;
5. The supernatants were centrifuged using Vivaspins (MWCO 5000, Zellu Trans Roth, Germany) at 5000 g for 5 minutes. The filtrate was collected for total carbohydrate assay;
6. Phenol sulfuric acid method was used to assay for total carbohydrate in the product.

Standard procedure of phenol sulfuric acid assay for 96 well mocrotiterplate was performed as described below.

1. Pipet 50 μL sample or glucose standard solution (0-1.5 mmol/L) into 96 well micro titer plate (BrandTech Scientific, Thermo Fisher Scientific, USA). Add 150 μL of 95% (v/v) sulphuric acid into each sample and mix thoroughly;
2. Add 30 μL of 0.5 mol/L phenol into each sample and mix well;

3. Incubate the plate at 90 °C for 5 min and measure the absorbance at 490 nm by SpectraMax M Microplate Readers (Molecular Devices, USA). Absorbance versus concentration of the standards were plotted to construct the standard curve (Fig.23);

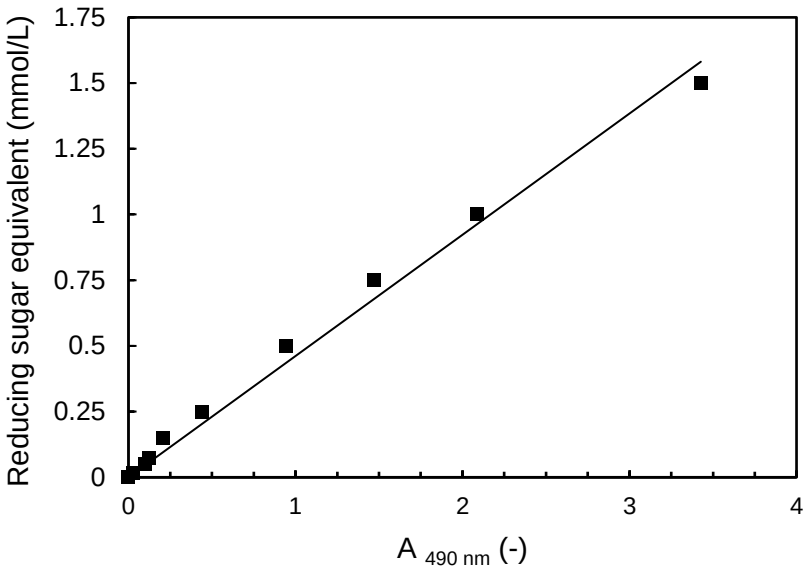

Fig.23 Standard curve of phenol sulfuric acid assay.

Fitting formula: $y = 0.4614x,\ R^2 = 0.9901$

$$Reducing\ \ sugar\ \ equivalent = y \times M = 83.13x \quad \text{(mg/L)}$$

Where:

y = 0.4614x, mmol/L

x = absorbance at 490 nm

M = molar mass of glucose, 180.16 g/mol.

3 Results and Discussion

In this chapter, results from three parts of this work were described and further discussed. The first part (section 3.1) comprised of results from the investigation and partial purification of β-1,3-glucanase from intracellular *E. gracilis* proteins. The results of cloning and expression of recombinant β-1,3-glucanases formed the second part (section 3.2). In this part, four endo-β-1,3-glucanases (*Tr*GH16, *Tr*GH55, *Tr*GH64, *Tr*GH81) and two exo-β-1,3-glucanase (*Tr*GH17, *Pp*GH5) were successfully expressed in *P. pastoris*, and their activities toward alkali treated paramylon were confirmed. In the last part of this work (section 3.3), results from trail of the newly discovered LPMOs deconstructing paramylon granule were presented, including cloning and expression of two LPMOs (*Tr*AA9, *Ao*AA11) in *P. pastoris* and their synergistic actions with β-1,3-glucanase on paramylon granule.

3.1 Investigation of β-1,3-glucanase(s) from intracellular *E. gracilis* proteins

Paramylon acts as storage polysaccharide in *E. gracilis*, therefore, intracellular enzymes should be expressed to deconstruct paramylon for autotrophic nutrition. For this reason, *E. gracilis* intracellular proteins were extracted for investigating the hydrolyzing ability on paramylon in this part. Following the characterizations of enzyme action (section 3.1.1-3.1.6), results from protein purification and gene amplification of paramylon degrading enzyme from intracellular *E. gracilis* proteins were presented in sections 3.1.7 and 3.1.8.

3.1.1 Influence of substrate

The native paramylon granule is extremely resistant to enzymatic attacks. For a proper pretreat which modifies paramylon more accessible, the native granule,

2× lyophilized and alkali treated paramylon were prepared. These three substrates at different concentrations (1.0, 2.5, 5.0 g/L) were hydrolyzed by 10 mg/L laminarinase (1,3-β-D-glucan glucanohydrolase, EC 3.2.1.39; dissolved in 0.1 mol/L sodium acetate buffer, pH=6.0), and the conversions were compared by reducing sugar equivalents as enzymatic hydrolysate.

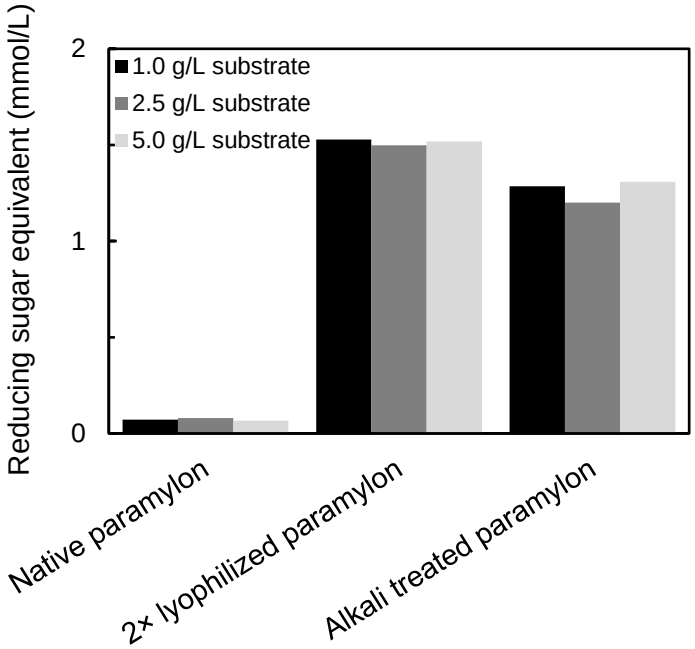

Fig.24 Influence of substrate on hydrolytic activity of laminarinase. 100 µL 10 mg/L laminarinase was incubated with 1 mL substrates respectively at 37 °C for 8 hours. The reducing sugar equivalents were measured by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

Because of the high crystallinity (approaching 90%), the paramylon granules were rather difficult to be hydrolyzed at all concentrations tested, the recalcitrant physical structure blocked the interaction between enzyme and substrate resulting no reducing power of the reaction mixture containing native granule as substrate. By now there is no successful hydrolysis of native granules *in vitro*.

In comparison, enzymatic hydrolysis became possible after pretreatment. Both pretreated substrates showed increasing in conversion. By providing easier access to substrate chain, which were previously hidden within the tightly packed granule, pretreatment became essential for paramylon hydrolyzation. The laminarin activities toward 2× lyophilized and alkali treated paramylon calculated as reducing sugar equivalent were averagely 1.5 mmol/L and 1.3 mmol/L. The 2× lyophilized paramylon was easier for enzymatic hydrolysis than alkali treated paramylon, and it presented in powder form making the determination more accurately. However, 2× lyophilized paramylon was prepared by two cycles of dialysis and freeze drying costing more than 96 hours. Comparing to the time-consuming method, dilution and neutralization of concentrated paramylon-gel dissolved in 0.5 mol/L KOH could be quickly accomplished. When this preparation (alkali treated paramylon) was used as substrate, an obvious high yield hydrolysate was observed. It demonstrated that alkali treated paramylon was also a good substrate for enzyme hydrolysis. Therefore, alkali treated paramylon was used as substrate for further investigating in following sections.

3.1.2 Influence of intracellular protein extract concentration

To study the effect of *E. gracilis* intracellular extract concentration on the degree of conversion towards alkali treated paramylon, concentrated extracts from a 7 days culture in 5, 10, 20 and 50 times were prepared in 0.1 mol/L sodium acetate buffer (pH=6.0). These extracts were used as crude enzymes and incubated with alkali treated paramylon (adjusted to pH=6.0) at 37 °C for 8 hours. Laminarinase (10 mg/L, dissolved in 0.1 mol/L sodium acetate buffer, pH=6.0) and deionized water were used as controls.

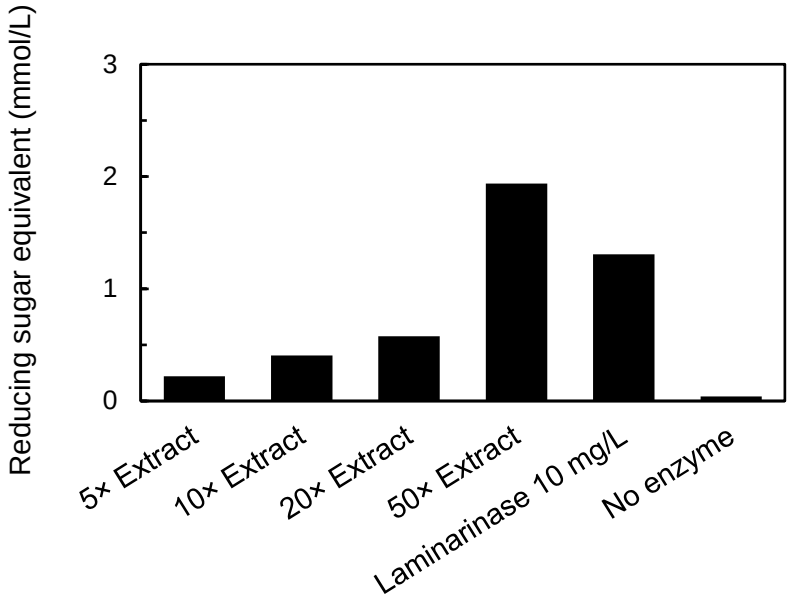

Fig.25 Influence of intracellular extract concentration on hydrolytic activity towards alkali treated paramylon. 100 µL concentrated extracts (5-,10-,20-, and 50-fold) from a 7 days culture were incubated with 1 mL, 1 g/L alkali treated paramylon at 37 °C, pH=6.0, for 8 hours. As controls, 100 µL of 10 mg/L laminarinase and deionized water were used instead of intracellular extracts. The reducing sugar equivalents were measured by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

After incubation, enzymes in 50× extract showed stronger hydrolysis activity comparing to lower concentrated extracts resulting a reducing sugar equivalent of 1.94 mmol/L. And the 50× extract was the only crude enzyme sample generated more reducing sugars than the positive control 10 mg/L laminarinase. Relatively, extract at 5, 10, and 20 folds, contributed lower enzymatic degrading power due to smaller quantity of enzymes. Laminarinase contained only endo-β-1,3-glucanase which catalyzed the hydrolysis of internal β-1,3 linkages in paramylon, different from which, the cell-free extract contained various paramylon degrading enzymes acting synergistically. The presence of active β-

1,3-glucan exo-, endo-hydrolases was reported in Barras and Stone's research (Barras and Stone 1969). Additionally, other enzymes' actions accelerated the degradation of paramylon, for instance, phosphorylases which have been characterized in *E. gracilis* were active toward short oligosaccharides released from paramylon (Kitaoka et al. 2012). Enzymes acted synergistically utilizing paramylon as energy source for cell growth and demonstrated combined hydrolysis power in the extract.

3.1.3 Influence of extraction buffer pH

The influence of pH on activity of the hydrolases in *E. gracilis* was determined with 50 times concentrated cell extract after 7 days cultivation. These extracts and laminarinase solutions were prepared in 0.1 mol/L sodium acetate buffer at different pH values (pH=6.0, 5.5, 5.0, 4.5) as crude enzymes and positive controls. The samples were incubated with 2× lyophilized paramylon at 37 °C for 8 hours and followed by reducing group measurement.

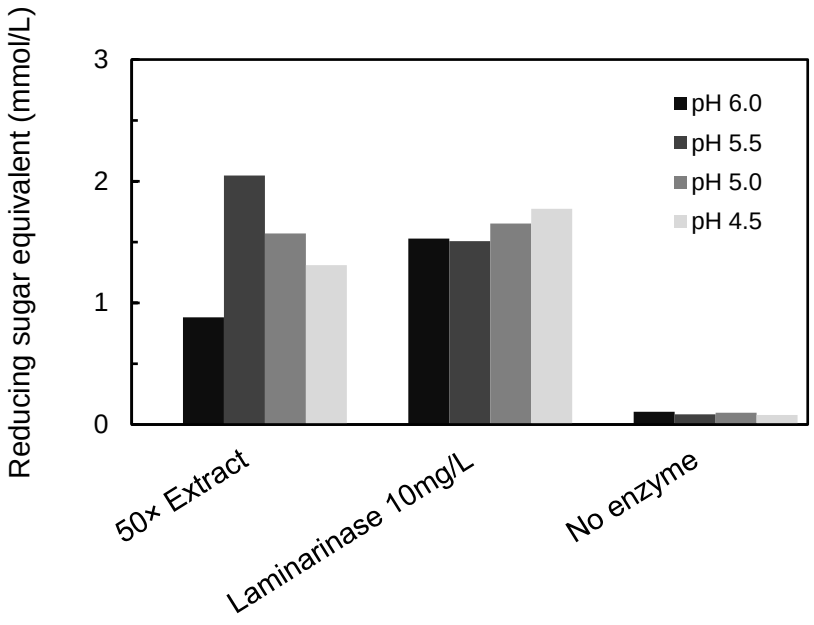

Fig.26 Influence of pH on hydrolytic activity. 100 µL 50-fold concentrated extracts at different pH values (6.0, 5.5, 5.0, 4.5) from a 7 days culture were

incubated with 1 mL of 1 g/L 2× lyophilized paramylon at 37 °C for 8 hours. As controls, 100 µL of 10 mg/L laminarinase and 0.1 mol/L sodium acetate buffer were used instead of intracellular extracts. The reducing sugar equivalents were measured by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

The hydrolases from cell extract had optimal activity at pH 5.5, and contributed a reducing sugar equivalent of 2.05 mmol/L. At pH 4.5, laminarinanse generated 1.77 mmol/L hydrolysate, which indicated the highest activity. It was reported that the intracellular extract contained both endo- and exo-hydrolase, and an endo-β-1,3-glucanase from *E. gracilis* extract rapidly reduced the viscosity of CM-pachyman at pH 5.2 (Barras and Stone 1969). In this work, a significant increasing of reducing sugar equivalent after variation from pH 6.0 to 5.5 and descending of hydrolase activity as the pH values variation from pH 5.5 to 4.5 were oberserved. The highest activity at pH 5.5 suggested that the endo-hydrolase acted on internal glycosidic linkages of paramylon making non-reducing chain ends available for attack by the exo-hydrolase, and might achieve the highest synergy efficiency.

3.1.4 Influence of hydrolysis temperature

In addition to the optimal pH value, a temperature optimum was determined. In comparison of 50 times concentrated extract at different temperature from 30 to 55 °C against alkali treated paramylon, the best result was achieved at 37 °C.

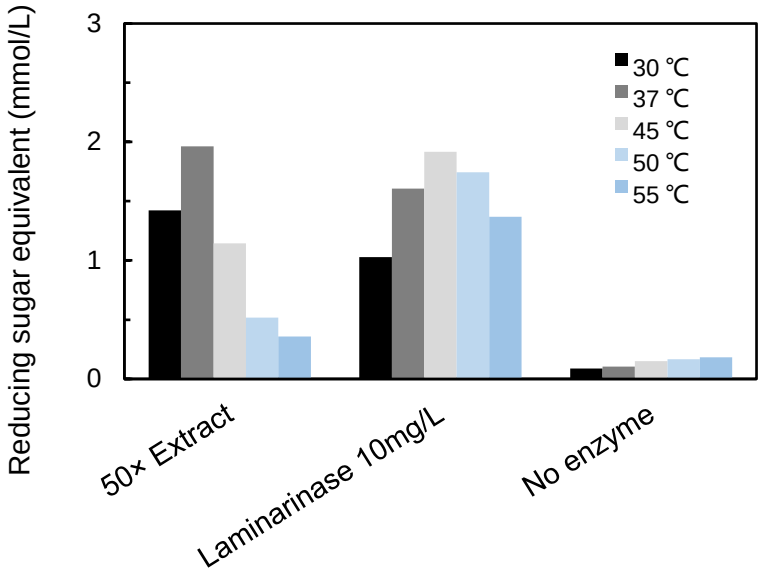

Fig.27 Influence of hydrolysis temperature on hydrolytic activity. 1 mL of 1 g/L alkali treated paramylon (pH=5.5) with 100 µL of 50× extract prepared from a 7-day-old culture in 0.1 mol/L sodium acetate buffer (pH=5.5) was incubated for 8 hours at 30, 37, 45, 50, and 55 °C, followed by reducing group measurement. As controls, 100 µL of 10 mg/L laminarinase and 0.1 mol/L sodium acetate buffer were used instead of intracellular extracts. The reducing sugar equivalents were measured by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

The optimum temperature found in this study was different from reported researches. In works from Vogel and Barber, the optimum temperature of exo-glucanase was 60 °C (Vogel and Barber 1968) and was further confirmed by Barras and Stone that the exo-hydrolase retained most activity at 55 °C (Barras and Stone 1969). The fundamental cause of this difference could lie in the combination of different enzymes in paramylon degradation. The crude enzyme had optimal activity at 37 °C, contributing a reducing sugar equivalent of 1.96 mmol/L, indicated the optimum temperature for paramylon hydrolases

synergistic action in the extract. Results in Fig.27 showed that the laminarinase was active over a broad temperature range and obtained the best at 45 °C. At 45 °C, laminarinanse generated 1.92 mmol/L hydrolysate, which indicated the highest activity.

3.1.5 Influence of incubation duration

To analyze the effect of incubation duration on the degree of paramylon degradation, the 50× extract was incubated with 1.0 g/L alkali treated paramylon as substrate. Over a period of 16 hours, an aliquot was measured every hour concerning the reducing group content.

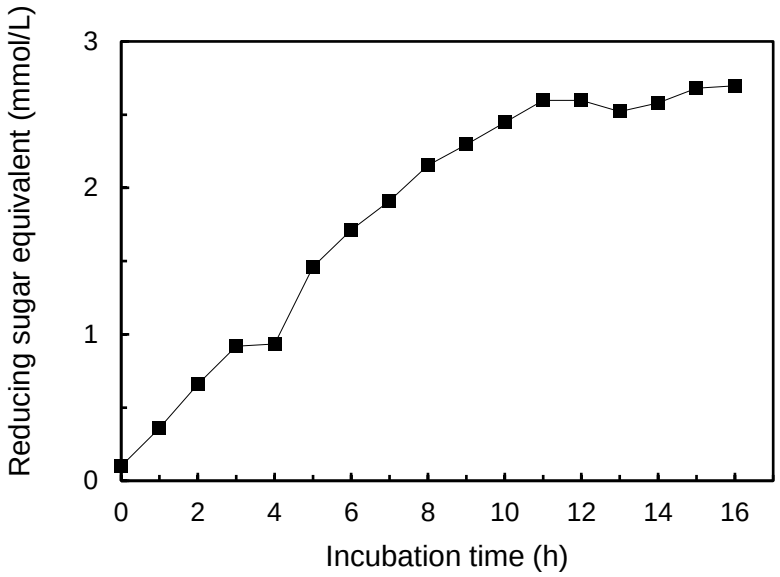

Fig.28 Influence of incubation duration on hydrolytic activity. 1 mL of 1 g/L alkali treated paramylon (pH=5.5) with 100 µL of 50× extract prepared from a 7-day-old culture in 0.1 mol/L sodium acetate buffer (pH=5.5) was incubated for 8 hours at 37 °C for 16 hours with reducing sugar equivalent measurement hourly by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

Under the optimized conditions, the reducing sugar equivalent accumulated continuously during the first 11 hours to 2.59 mmol/L. The amount of degrading product kept in a steady increasing when the incubation time exceeded 11 hours. At 16 h when the experience stopped, the *E. gracilis* intracellular hydrolases contributed a reducing sugar equivalent of 2.70 mmol/L. Similar result was reported by by Barras and Stone that significant amount of reducing sugars were released from untreated laminarin and dispersed paramylon in 12 hours, and continued tardily up to 24 hours (Barras and Stone 1969).

3.1.6 Influence of cultivation duration

The paramylon degrading enzymes were produced to deconstruct it when the cells need to utilize the energy reserve as carbon source. Activity of *E. gracilis* hydrolases from cell-free extract varied with the stage of growth. To invest the hydrolase activities in different growth stage, extract samples were prepared daily and incubated with 1 g/L alkali treated paramylon as substrate at 37 °C for 16 h followed by reducing product assay. Residual glucose and dry cell weight were also showed in the Fig.29.

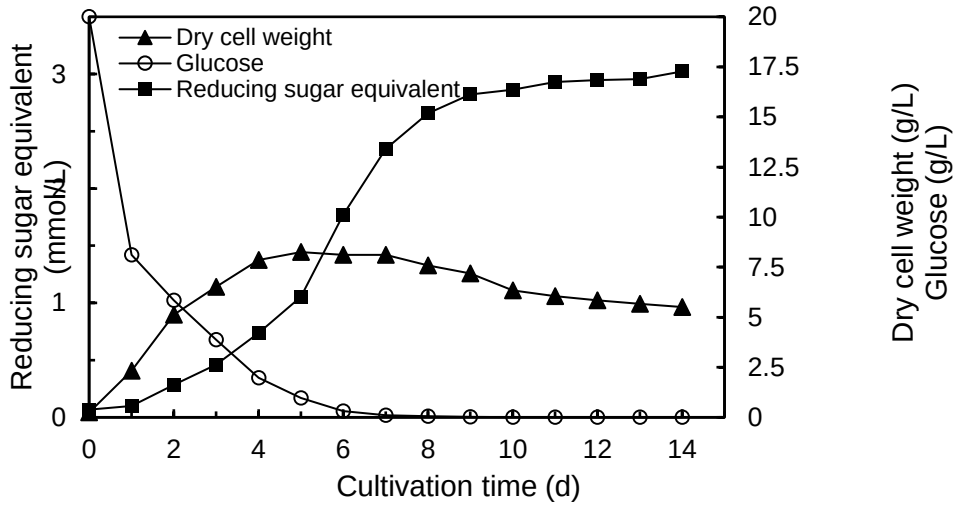

Fig.29 Influence of cultivation duration on hydrolytic activity. During cultivation time of 14 days, samples were taken daily and produced a 50×

extract in 0.1 mol/L sodium acetate buffer (pH=5.5). 100 µL of the extraction was incubated in 1 mL of 1 g/L alkali treated paramylon (pH 5,5) for 16 hours at 37 °C and followed by reducing group assay. The reducing sugar equivalents and residual glucose in the medium were measured by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1). The dry cell weight was measured by method in section 2.3.3.

Paramylon hydrolase activity increased sharply within the first 8 days of cultivation, revealing the initiation of paramylon utilization. As glucose in medium was consumed within 6 days, paramylon played a role as the only energy storage in this carbon-deprived medium. The amount of reducing product revealed that hydrolase activity in cell-free extract kept stable during the last 6 days. Meanwhile, the dry cell weight decreased as paramlon was degraded for metabolism. The similar result was found by Vogel and Barber that the specific glucanase activity in early stationary phase was at least twice as in the logarithmic growth phase whether calculated by protein content or cell number (Vogel and Barber 1968). Indicated by the study of *E. gracilis* cultivation time effect on hydrolase activity, a practical point that crude enzyme extract should be prepared from a culture in late stationary phase after consumption of the carbon source in the medium.

3.1.7 Partial purification of *E. gracilis* intracellular proteins

50× extract of *E. gracilis* was prepared using 12 day-old culture by the method described in section 2.3.2. After centrifugation at 22,000g for 20 min, 50 mL supernatant was precipitated at a final $(NH_4)_2SO_4$ concentration of 0-20, 20–60 and 60–80% saturation, respectively. Each precipitate was dialyzed against 20 mmol/L Tris-HCl buffer (pH=5.5) and adjusted to a volume of 50 mL by the same buffer afterwards. Fraction of 20-60% $(NH_4)_2SO_4$ precipitate showed activity towards alkali treated paramylon. Proteins obtained by $(NH_4)_2SO_4$ precipitation (20-60%) were partially purified by a weak anion exchange chromatography. Fractions collected by stepwise elution were adjust to 50 mL before determination of hydrolytic activity towards alkali treated paramylon. This fraction exhibited high activity towards alkali treated paramylon was mixed with 50 mL of buffer consisting 40 mmol/L Tris-HCl, 1.8 mol/L ammonium sulfate to a total volume of 100 mL, and applied for hydrophobic chromatography. Fractions collected by stepwise elution were dialyzed against 20 mmol/L Tris-HCl buffer (pH=5.5) and fraction eluted by 45-80% elution buffer showed high hydrolytic activity. The results for the partial purification of paramylon degrading enzyme were summarized in Table 22.

Table 22 Results of *E. gracilis* intracellular protein purification.

Step	Protein (g)	Reducing sugar equivalent *	Specific reducing sugar equivalent **	Yield (%)	Purification level
Extraction	11.0	2.47	0.23	100	1
Salt precipitation	2.6	1.85	0.71	74.9	3.1

IEC	1.4	0.77	0.55	31.2	2.4
HIC	0.15	0.14	0.93	5.7	4.0

*: Reducing sugar equivalent measured by DNS method (section 2.3.5.1), mmol/L;

**: Reducing sugar equivalent /Total protein, mmol/(L·g).

As showed in table 21, the reducing sugars generated by unpurified extract from alkali treated paramylon was 2.47 mmol/L. The results of section 3.1.6 suggest that reducing sugars contributed by 50× cell free extract at 12 d was 2.95 mmol/L. For protein purification, 50 mL *E. gracilis* extract was prepared. While in section 3.1.6, the extraction volume was 1 mL using the same ultrasonication. The decreasing in activity was probably caused by an incomplete cell disruption. The first purification step, salt fractionation, led to an increase in purity of 3 folds, and recovered almost 75% of all the target protein in the original extract. After changing buffer by dialysis, the enzyme sample was purified through an ion-exchange column. The purification level increased to 2.4 folds comparing with the original extract, whereas the yield fell to 31.2 %. The followed hydrophobic chromatography resulted in a purification level of 4.0 folds, while recovering 5.7 % target protein form the extract. The SDS-PAGE (Fig.30) showed that the number of bands decreased after each step.

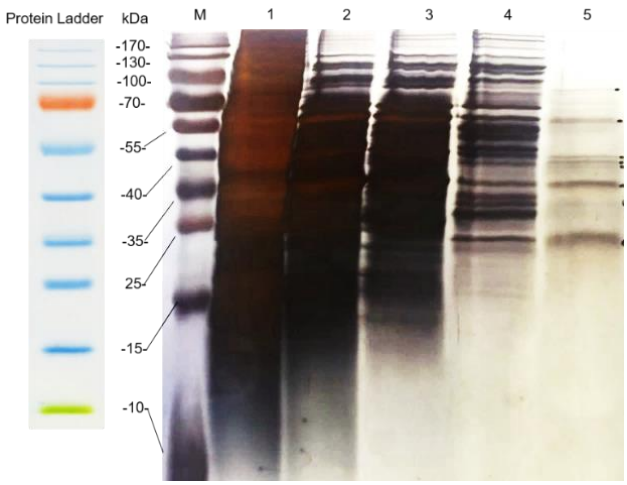

Fig.30 SDS-PAGE analysis of *E.gracilis* intracellular protein purification. Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-5, proteins after partial purification. Lane 1: sell free extraction; lane 2: salt precipitation; lane 3 and 4: IEC elution; lane 5: HIC elution.

The electrophoresis revealed the significant reduction of protein components after each purification step. As showing in Fig.30, Lane 5, elution from hydrophobic chromatography consisted approximately 10 proteins, while generated 0.14 mmol/L reducing product, providing an applicable protein mixture for size exclusion chromatography.

As the last step of purification, the fraction with target activity collected from the hydrophobic chromatography was concentrated by viva-spin before applying a size exclusion chromatography. Fractions were collected by the size of 1.0 mL at the flow rate of 1.0 mL/min of 20 mmol/L Tris-HCl buffer (pH=5.5) for enzyme activity assay. 71 fractions from SEC were collected and their activities responsible for paramylon degradation were analyzed. The results were showed in the following Fig.31.

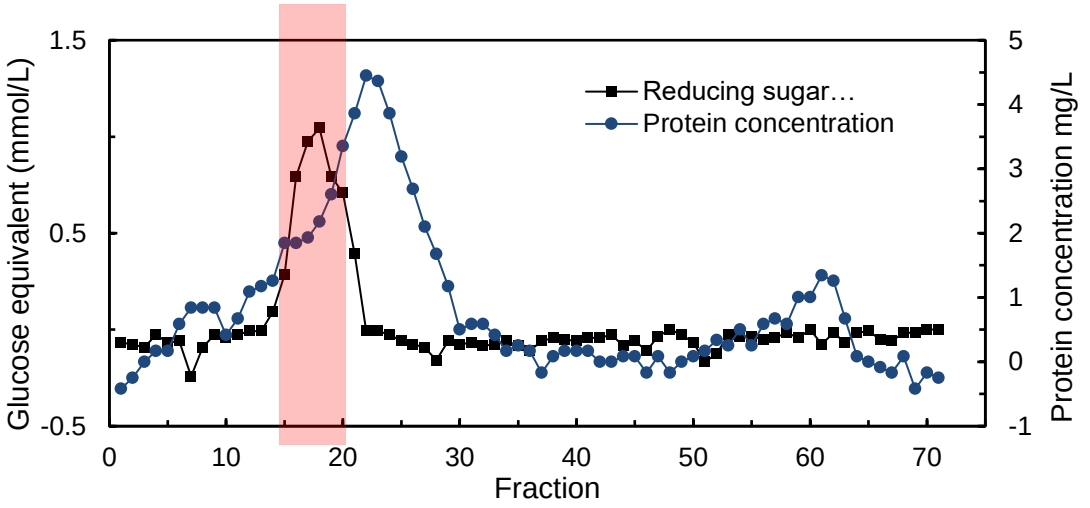

Fig.31 Activity and protein assay of SEC fractions. 100 µL of the collected fraction was incubated in 1 mL of 1 g/L alkali treated paramylon (pH 5,5) for 16 hours at 37 °C and followed by reducing sugar assay by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

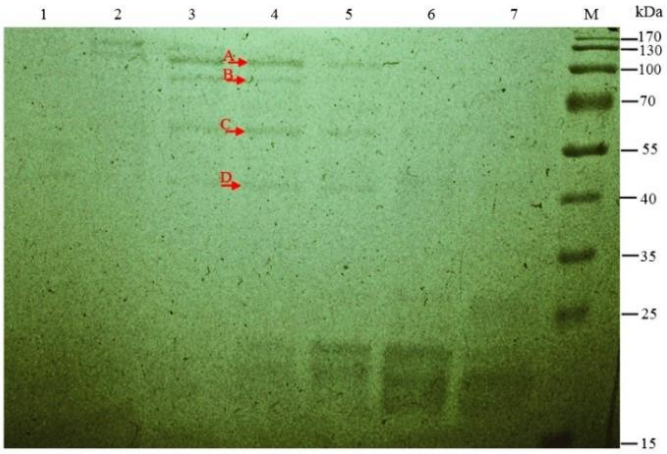

Fig.32 SDS-PAGE analysis of SEC fractions.

Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-7, SEC fractions.

As showed in Fig.31, the pick of reducing sugar equivalent curve indicated obviously that proteins in these fractions had paramylon degrading activities. The seven fractions generating reducing sugar equivalent higher than 0.3 mmol/L were analyzed by SDS-PAGE. After Coomassie brilliant blue staining, different bands were detected in each fraction as showing in Fig.32. Protein bands in the active fraction obtained after separation by SDS-PAGE were sent to central lab for protein analytic of the Ludwig Maxmilians university of Munich and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). For the protein C in Fig.32, a string of 16 amino acids (NTDGSTDYGILQINSR) were found matching 1,4-β-N-acetylmuramidase (EC: 3.2.1.17) with 12% sequence coverage. This GH family 22 enzyme was confirmed having hydrolytic activity against chitin, which was a insoluble long-chain polymer of N-acetylglucosamine with high degree of polymerization (Aam et al. 2010).

Until now, limited enzymes relating with paramylon degradation were indentified. In 1969, an *E.gracilis* intracellular exo-1,3-glucanase was purified and characterized (Barras and Stone 1969). And recently, an endo-β-1,3-glucanase from *E.gracilis* was indentified, which had a molecular weight of about 40 kDa and showed maximum hydrolytic activity towards laminarin at pH 4.0-5.5, 60 °C after 1 h incubation or at 50 °C after 20 h incubation (Takeda et al. 2015). The protein C in this work was purified for the first which might be another enzyme related with paramylon degrading from *E.gracilis*.

3.1.8 Trial of gene amplification of paramylon degrading enzyme purified from intracellular *E. gracilis* proteins

cDNA of *E. gracilis* was synthesized using poly A primer and mRNA as template according to the procedure showed in section 2.3.7. Amplification of the target gene responsible for paramylon degradation protein was performed using degenerate primer initiating from the 3' end of cDNA and the adapter primer from the 5' end. For keeping a low degeneracy, four degenerate primers were designed according to the same protein sequence (-GILQIN-) identified by LC-MS/MS (section 2.3.7.2). However, no clear band was detected by electrophoresis, demonstrating an unsuccessful trial of target gene amplification.

Searching genes in organisms with no available genomic resources could be a low success rate task. Degenerate PCR was one of the methods commonly used for accomplishing this task, either using total genomic DNA or a cDNA library as template. This technique had a low probability but high cost, and its effectiveness depends on various factors. One direct influence might be the quality of cDNA library. In this work, when the total RNA of *E. gracilis* was isolated for cDNA synthesis, abundant polysaccharide, paramylon, remained in the cell seriously impact the quality of isolated total RNA. The commercial RNA isolation kit used in this work was based on an acid guanidinium thiocyanate-phenol-chloroform extraction reagent firstly reported by Chomczynki and Sacchi (Chomczynski and Sacchi 1987). This regent was widely used as lysis buffer before the separation of RNA containing aqueous phase and subsequent RNA precipitation. Polysaccharide in the aqueous phase could physically entrap nucleic acids and entrained into discarded phase during centrifugation. Meanwhile, olligosaccharide precipitate with RNA might interfere with downstream applications (Dellacorte 1994). Both impacts caused by polysaccharide could encumber and fail the subsequent amplification work.

Another direct influence was the degenerate primers used in amplifying. In this work, the limited known of the protein sequence restricted the primer designing. In order to keep a low degeneracy, a pool of four degenerate primers for the same site were designed. However, each of the primer had 288-fold degeneracy, which was still a high one. Moreover, length of the part between the identified peptide coding region and poly T tail in cDNA was unknown. All the uncertainties could completely disrupt the PCR amplification.

Although more exploration need to be conducted before confirming protein C (In Fig.32) be paramylon degrading related enzyme, the purification and identification of protein C for the first time may open a new horizon for paramylon depolymerizer searching for the future work.

3.2 Cloning and expression of recombinant β-1,3-glucanase in *P. pastoris*

In this part, recombinant enzymes including four endo-β-1,3-glucanases (*Tr*GH16, *Tr*GH55, *Tr*GH64, *Tr*GH81), one exo-β-1,3-glucanase (*Tr*GH17) from *T. reesei* and one exo-β-1,3-glucanase (*Pp*GH5) from *P. pastoris* were cloned and expressed in *P. pastoris*, Their activities on laminarin and alkali alkali treated paramylon were confirmed by measuring hydrolysis products of reducing groups and glucose, respectively.

3.2.1 Selection of β-1,3-glucanase

The Carbohydrate Active Enzymes database (CAZy) classified glycoside hydrolases according to significant amino acid sequence similarity, reflecting the functional and structural relationships between classification families (Lombard et al. 2014). This is different to the International Union of Biochemistry and Molecular Biology (IUBMB), by which the glycoside hydrolases are grouped on their substrate specificity and occasionally

molecular mechanism. Consequently, the β-1,3-glucanases could be placed in different groups. For instance, the exo-β-1,3-glucanases (E.C.3.2.1.58) are grouped in glycoside hydrolase (GH) families 3, 5, 17, 55 and 132, while the endo-β-1,3-glucanases (E.C.3.2.1.39) are located in GH families 16, 17, 55, 64 and 81 (http://www.cazy.org/Glycoside-Hydrolases.html).

The filamentous fungus *Trichoderma reesei* attracted considerable attention of the biofuel industry as a workhorse of plant cell wall degrader. The components of plant cell wall polysaccharide were mainly cellulose, hemicellulose and pectin, which were tightly packed and evolved the ability to resist enzymatic attack from microbes (Brink and Vries 2011). *T. reesei* was able to utilize these polysaccharides due to the strong ability of producing wide range of extracellular hydrolytic enzymes. The increasing knowledge of *T. reesei* genome sequence made it a suitable source for β-1,3-glucan degrading enzymes (Martinez et al. 2008).

In yeasts, glucan degrading enzymes were implicated in glucan metabolism and involved in many processes, such as formation and release of ascospores (Larriba et al. 1995), because of their regid cell walls mainly constructed by β-1,3-glucan and other polysaccharides (Jae 1997). Recombinant expression has been conducted for producing β-1,3-glucanases from yeast strains like *Saccharomyces cerevisiae* (Larriba et al. 1995) and *P. pastoris* (Xu et al. 2006). In the following work, one gene coding for exo-β-1,3-glucanase from *P. pastoris* was also selected for cloning and overexpression.

Selected β-1,3-glucanases for recombinant expression in this work were showed in Table 23.

Table 23 Selected β-1,3-glucanases from *T.reesei* and *P. pastpris*.

Organism	GenBank ID	Enzyme	GH family
T. reesei	EGR45305.1	Endo-β-1,3-glucanase, *Tr*GH16	16
T. reesei	EGR47521.1	Endo-β-1,3-glucanase, *Tr*GH55	55
T. reesei	EGR45483.1	Endo-β-1,3-glucanase, *Tr*GH64	64
T. reesei	EGR49603.1	Endo-β-1,3-glucanase, *Tr*GH81	81
T. reesei	EGR46171.1	Exo-β-1,3-glucanase, *Tr*GH17	17
P. pastoris	CAY69081.1	Exo-β-1,3-glucanase, *Pp*GH5	5

3.2.2 Cloning and expression of *Tr*GH16 (EGR45305.1)

Nucleotide and amino acid sequences of *Tr*GH16 are shown in Fig.33. The open reading frame had a size of 864 bp terminated by TAA stop codon, which corresponds to a translation product with 287 amino acids. Predicting by SignalP 3.0, a N-terminal signal peptide existed and the cleavage site lay between residues 19 (a) and 20 (w). No propetide cleavage site was predicted by ProP1.0 Server in downstream of the signal peptide.

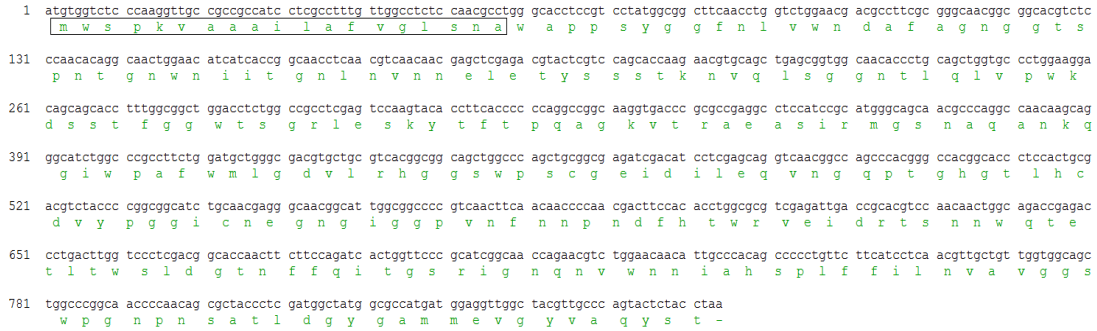

Fig.33 Nucleotide sequence and amino acid sequence of *Tr*GH16.

In amino acid sequence, the putative signal peptide was boxed.

By aligning amino acid sequence of *Tr*GH16 with available protein sequences from the GenBank database, it had high identity of 34% with the endo-β-1,3-glucanase from *Streptomyces sioyaensis* (GenBank: AAF31438.1) (Hong et al. 2002). Additionally, *Tr*GH16 was identical to enzymes belonging to Glycoside Hydrolase Family 16 including the putative catalytic residues (Glu, Asp and Glu) and jelly roll fold related active sites (Mertz et al. 2009) as showing in Fig.34. Within the GH16 family, two conserved glutamates in the pattern E*X*D*X*(*X*)E played the role of the catalytic domain (Viladot et al. 1998), and catalyzed the glycosylic hydrolysis reaction in a retaining mechanism. At active site, the “R1” glutamate residue acted as a nucleophile to attack the C1 atom of the sugar ring to promote the cleavage of β-1,3-linkage in the absence of water molecules.

The “R2” glutamate residue served as the acid to receive the electron and then transfer it back to adjacent water molecule to attack the same C1 atom to complete the hydrolysis (Jeng et al. 2011).

Fig.34 Alignment of the *Tr*GH16 sequence.

EGR45305: *Tr*GH16 from *T. reesei*;

AAF31438: endo-β-1,3-glucanase from *Streptomyces sioyaensis*;

BAE02683: endo-β-1,3-glucanase from *Bursaphelenchus xylophilus*;

ACD93221: endo-β-1,3-glucanase from *Cryptopygus antarcticus*;

BAH84971: endo-β-1,3-glucanase from *Haliotis discus hannai*.

Strictly conserved residues were high-lighted in red, residues predicted as jelly roll fold related active sites were high-lighted with blue background,

conserved catalytic residues Glu (R1), Asp (R2) and Glu (R3) were marked with “#”.

As described in the method (section 2.4.1.3), the enzyme gene was cloned by a PCR-based strategy using cDNA as template. A PCR product of 845 bp was obtained (see Fig.35A, Lane 4) containing 825 nucleotide ORF with TAA as stop codon, which encoding 274 amino acids of mature *Tr*GH16 with a predicted molecular mass of 29.84 kDa. The bright band shown in Fig.35A, Lane 4 was ligated into pJET 1.2 cloning vector for further plasmid construction.

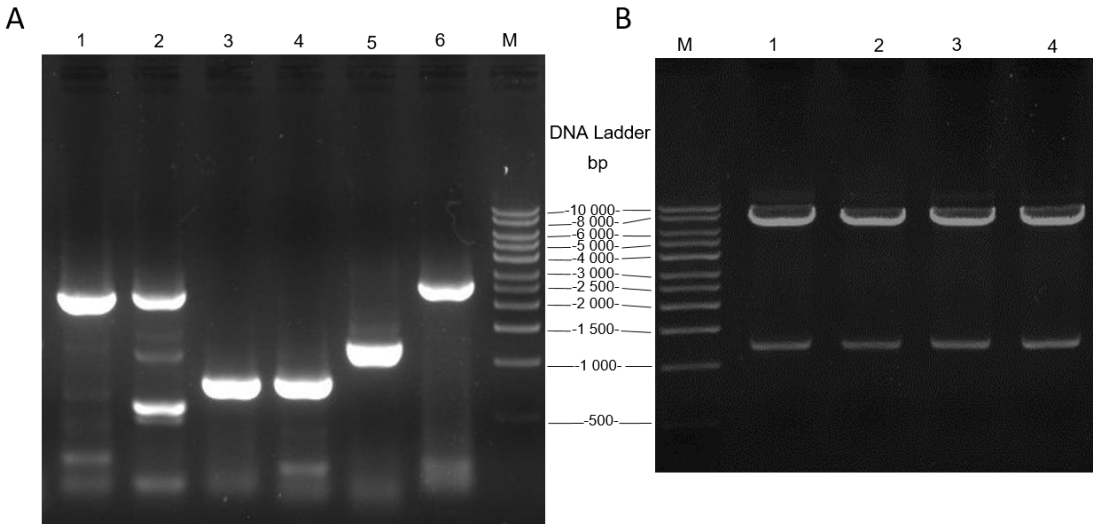

Fig.35 Agarose gel analysis of gene application and plasmid construction.

A: Analysis of PCR products from cDNA of *T. reesei*. Lane 1: Gene of *Tr*GH55a (EGR 47521.1, 2223 bp); lane 2: Gene of *Tr*GH55b (EGR 48699.1, 2259 bp); lane 3: Gene of glucanase (EGR 47490.1, 798 bp, no further study in this work); lane 4: Gene of *Tr*GH16 (EGR 45305.1, 845 bp); lane 5: Gene of *Tr*GH64 (EGR 45483.1, 1316 bp); lane 6: Gene of *Tr*GH81 (EGR 49603.1, 2576 bp); M: 1 kb DNA ladder (Plasmid Factory, Germany).

B: Recombinant vector screening by restriction endonucleases. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1 to 4, results of four recombinant pAaHBgl-*Tr*GH16 vectors digested by Hind III and Not I.

As in the gene sequence of *Tr*GH16 contained the restriction site of *Xho* I (C^TCGAG), the endonuclease of its isocaudomer *Sal* I (G^TCGAC) was introduced instead for constructing. Consequently, after the fragment being inserted into the vector pAaHBgl between sites of *Xho* I and *Not* I, the recombinant vector could not be linearized by either *Sal* I or *Xho* I. Restriction nuclease *Hind* III site existed in the recombinant vector, locating at 312 bp upstream of the replaced *Xho* I. Two fragments from the reconstructed expressing vector pAaHBgl with the sizes of 7047 bp and 1157 bp should be produced after cleavage with *Not* I when the gene of interested was inserted correctly. As shown in Fig.35B, lane 1 to 4, one band about 1200 bp and one band with the size between 8000 and 6000 bp were generated. The faint bands about 10 kb represented uncut plasmid.

The recombinant vector represented by Fig.35B, lane 1 was named as pAaHBgl-*Tr*GH16 and selected as expression vector for transformation into *P. pastoris* GS115. The transformation was performed by the method described in section 2.4.3. Colonies appeared on the RDB agar plate were cultivated in small scale using 24 square-well plate for protein expression. After methanol induction for 120 hours, supernatants were analyzed by SDS-PAGE and MBTH assay toward laminarin as well as alkali treated paramylon respectively. Fig.36 revealed the results of SDS-PAGE analysis of secreted proteins from six recombinant *P. pastoris* GS115 transformants after methanol induction. In lane 5 and 6, the secreted *Tr*GH16 displayed apparent molecular mass about 35 kDa (highlighted by red line), which was higher than the theoretical mass of 29.84 kDa due to glycosylation predicted by NetNGlyc 1.0 Server.

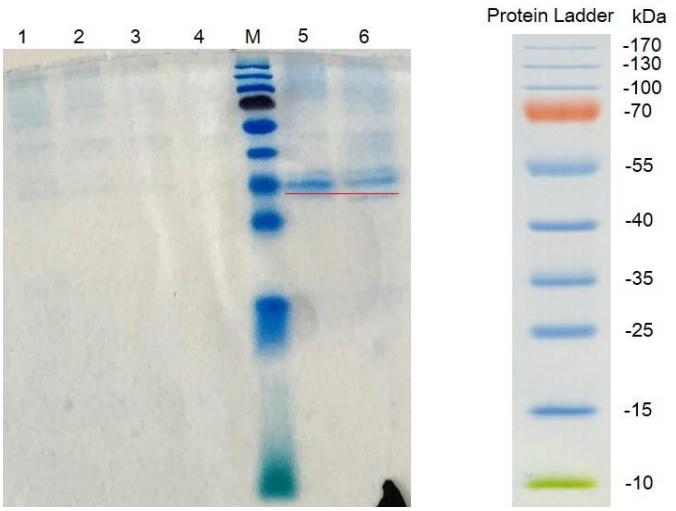

Fig.36 SDS-PAGE analysis secreting expression of recombinant *Tr*GH16.

Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-6, 15 µL culture supernatant of recombinant *P. pastoris* GS115 after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

To investigate the enzyme activity, laminarin and alkali treated paramylon cleavage assays were performed. The reducing groups generated by endo-β-1,3-glucanase *Tr*GH16 were determined using MBTH method (section 2.4.5.2). One unit of enzyme activity (IU) was defined as 1 µmol of reducing sugar equivalent released in 1 min. As shown in Fig.37, enzyme activities of sample 5 and 6 (corresponding to lane 5 and 6 in Fig.36) were obviously higher. When alkali treated paramylon was used as substrate, enzyme activities of sample 5 and 6 were 46.6 and 22.9 IU/L respectively. While acting against laminarin, their activities were 49.4 and 38.3 IU/L. This phenomenon was also observed in other recombinant enzymes in the following work, as the better dissolved laminarin making substrate approaching much easier comparing to the alkali treated paramylon. The other samples had activities lower than 5.0 IU/L toward both substrates.

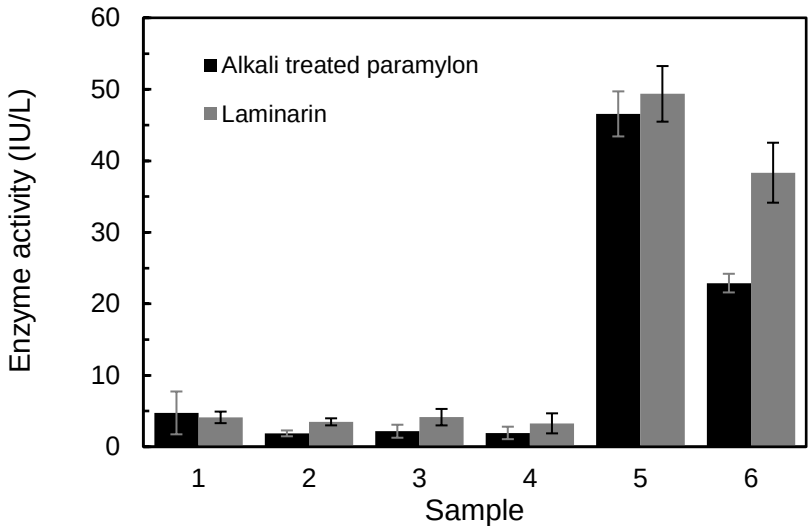

Fig.37 Enzyme activity assay from culture supernatants of selected transformants (*Tr*GH16). Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction in small scale using 24 square-well plate (section 2.4.4.1) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5, indicated by black column) and alkali treated paramylon (2.0 g/L, pH=6.5, indicated by gray column), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

Results of SDS-PAGE and activity assay both indicated that the endo-β-1,3-glucanase *Tr*GH16 was expressed and secreted by transforms NO. 5 and 6. The recombinant strain NO.5 was named as *P. pastoris* GS115-*Tr*GH16 and selected for protein expression. Recombinant *Tr*GH16 expression was conducted in 250 mL baffled flask as described in (section 2.4.4.2). Supernatant collected in every 24 h after changing to BMMY medium was assayed for endo-β-1,3-glucanase activity using laminarin and alkali treated paramylon as substrate, respectively.

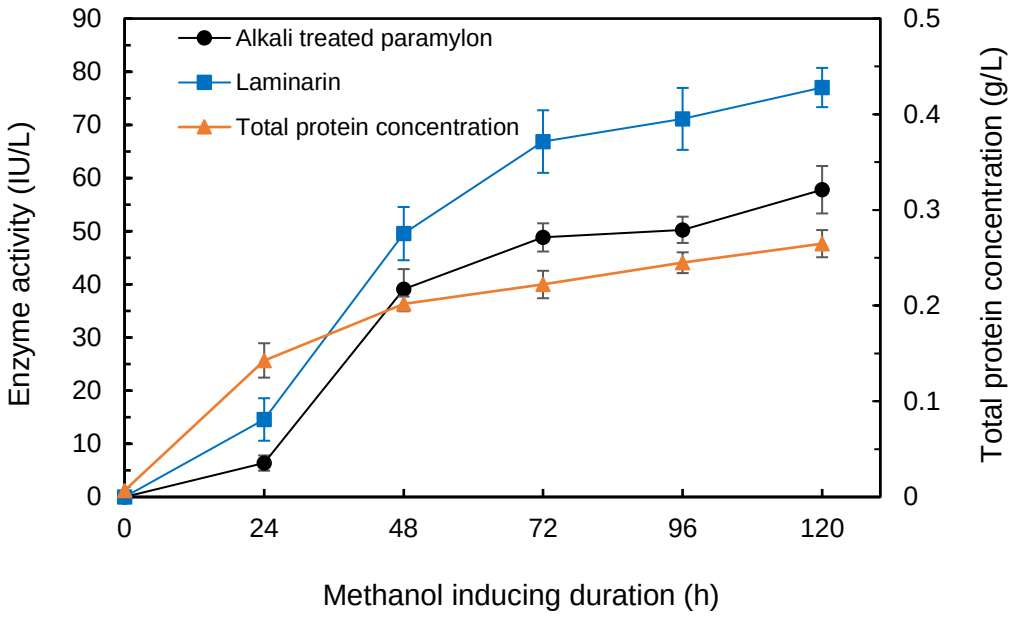

Fig.38 Recombinant *Tr*GH16 expression in *P. pastoris*. Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction using 24 shake flask (section 2.4.4.2) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5) and alkali treated paramylon (2.0 g/L, pH=6.5), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

Cells were harvested and changed to BMMY medium for methanol induction after precultivation in shake flask (methord in section 2.4.4.2). In this way, no activity was detected at the time point of 0 h. The enzyme activity was firstly measured after 24 hours and increased along the inducting time. In general, recombinant *Tr*GH16 maintained higher activity when acting on laminarin, as paramylon existed in the state of colloidal form, providing less free polysaccharide chains for the enzyme. When acting on alkali treated paramylon,

recombinant *Tr*GH16 activity increased rapidly from 6.4 IU/L to 39.1 IU/L, in the second 24 hours of induction, due to the efficient secretion revealed by protein concentration in the supernatant. From time point 72 to 120 h, concentration of total protein kept stable relatively and the increasing of enzyme activity slowed down, achieving the highest activity of 57.8 IU/L after inducing for 120 h. The data suggested that trend of enzyme activity increasement identically when using laminarin as substrate, and represented higher activities. At time point of 120 h, recombinant *Tr*GH16 showed the highest activity of 77.0 IU/L with 0.26 g/L protein in the supernatant.

Revealed by the results above, a putative endo-β-1,3-glucanase *Tr*GH16 was successfully cloned and expressed by recombinant *P. pastoris* using α-factor as leading signal. The glycosylic hydrolase domain of this enzyme contained a conserved E*X*D*X*(*X*)E motif of GH16 members, and the catalytic importance of this conserved motif has been reported by mutational analyses of laminarinase (Krah et al. 1998). Even when the supernatant was used directly as crude enzyme, it efficiently catalysed the hydrolysis of both substrates in this work. In comparison to alkali treated paramylon, larminarin was hydrolysed at a relatively higher rate. Although the genes encoding endo-β-1,3-glucanase in GH 16 have been studied from a variety of hosts for decades, this work expressed *Tr*GH16 (GenBank: EGR45305.1) in *P. pastoris* for the first time and confirmed its activity towards the unique substrate paramylon.

3.2.3 Cloning and expression of TrGH55 (EGR47521.1)

The gene of*Tr*GH55a had an open reading frame of 2277 bp coding for 759 amino acid sequence (Fig.39). Predicting by SignalP 3.0, a N-terminal signal peptide of 17 residues existed and the cleavage site lay between positions 17 (a) and 18 (t). Additionally, the processing site for Kex2 (Lys-Arg) lay at positions 33-34, which would cleave the proprotein after preproprotein entering the secretory pathway leading by the signal peptide.

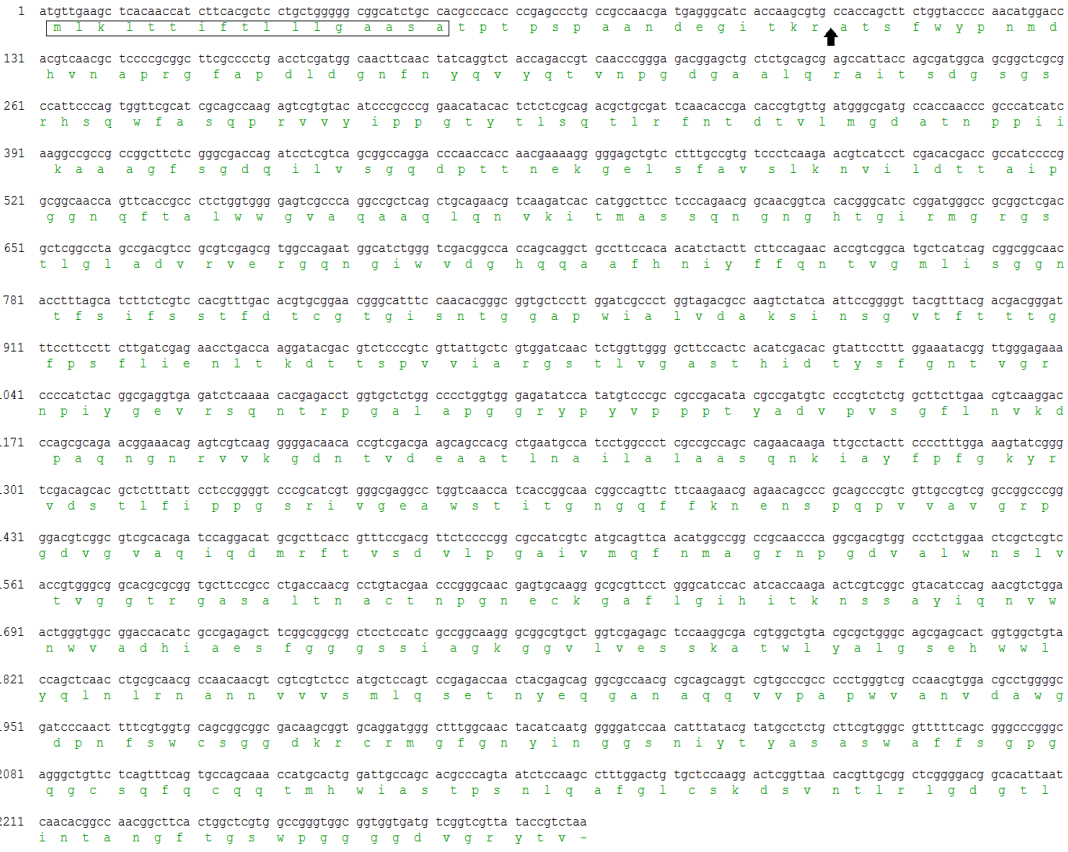

Fig.39 Nucleotide sequence and amino acid sequence of *Tr*GH55.

In the coding region, the putative signal peptide was boxed, and the processing site for Kex2 endoprotease was indicated by arrow.

By aligning amino acid sequence with available protein sequences from the GenBank database, the protein had 80% sequence identity to the characterized

GH 55 enzyme from *Trichoderma harzianum* CECT 2413 naming *Bgn*13.1 (GenBank: ACE81432.1).

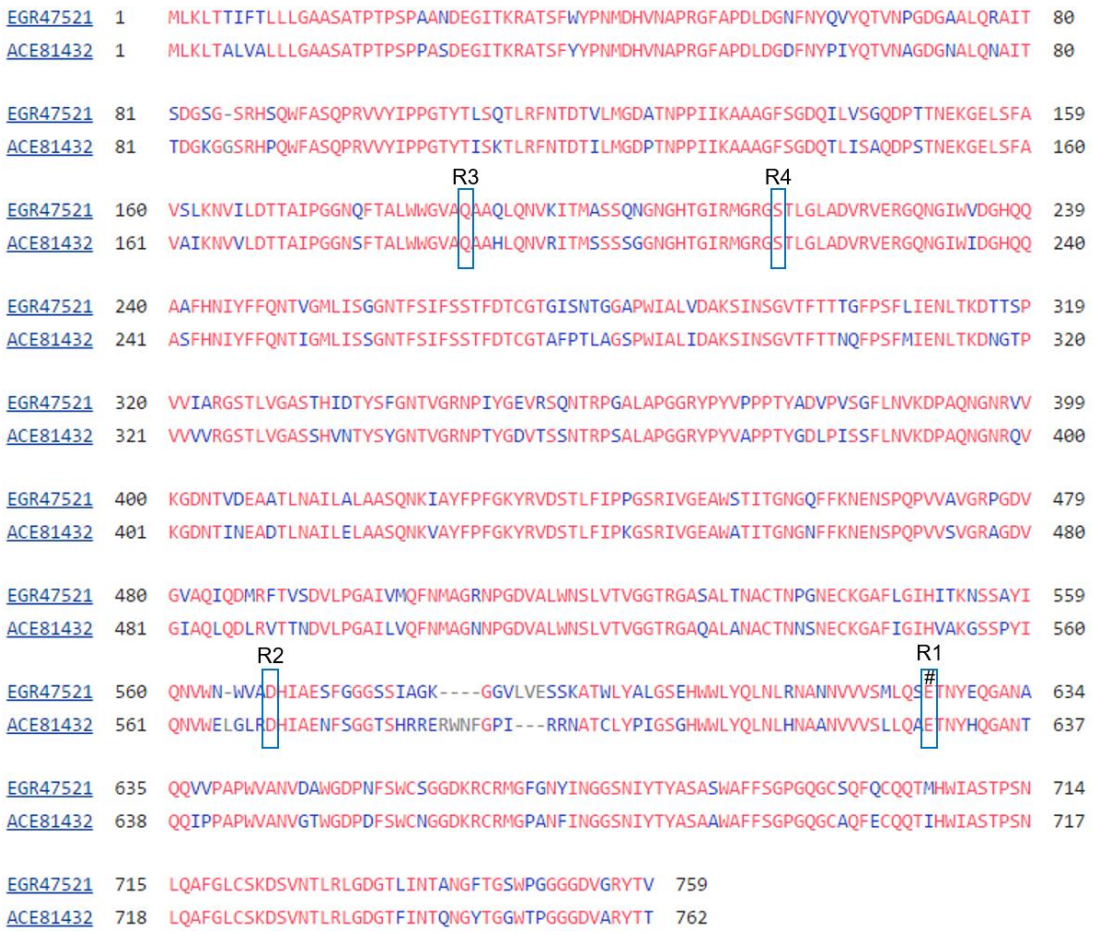

Fig.40 Alignment of the *Tr*GH55 sequence.

EGR47521: *Tr*GH55from *T. reesei*;

ACE81432: *Bgn*13.1 from *T. harzianum* CECT 2413.

Strictly conserved residues were high-lighted in red, conserved catalytic residue R1 (Glu) was marked with "#". Strictly conserved residues related in catalytic reaction R2 (Asp), R3 (Ser) and R4 (Gln) are framed.

The residue R1 (Glu) was concluded as a catalytic site (Ishida et al. 2009). Residue R2 (Asp) had a primary role in positioning the substrate, R3 (Ser) and R4 (Gln) were reported both important, but not essential catalytic related

residues in the latest research (Bianchetti et al. 2015). The Bgn13.1 was confirmed to be an endo-β-1,3-glucanase by experimental evidence for the first time (La-Cruz et al. 1995). It contributed enzymatic activity on periodate oxidized laminarin, which could not be hydrolyzed by exo-β-1,3-glucanase, but showed no ability of hydrolyze laminaribiose. Based on the high identity with Bgn13.1, *Tr*GH55 in this work was predicted as an endolytic mode β-1,3-glucanase.

Gene of putative endo-β-1,3-glucanase *Tr*GH55 was cloned from cDNA of *T. reesei*. A PCR product of 2223 bp was obtained, which contains a 2199 nucleotide ORF terminated by a TAG stop codon, encoding 732 amino acids of mature protein with a predicted molecular mass of 77.94 kDa. In Fig.35A, lane 1, the bright band between 2000 and 2500 bp showed the product of gene amplification. The faint bands in this lane were probably nonspecific PCR products, nevertheless their existence had no influence on isolation of the target band from agarose gel. The purified product was then ligated into pJET 1.2 cloning vector for further plasmid construction.

Gene of *Tr*GH55 was insert to the vector pAaHBgl by ligation between sites *Xho* I and *Not* I, and named as pAaHBgl-*Tr*GH55 in the following work. Two fragments with the sizes of 7395 bp and 2223 bp should be produced by digestion with *Xho* I and *Not* I when the gene of interested was inserted correctly.

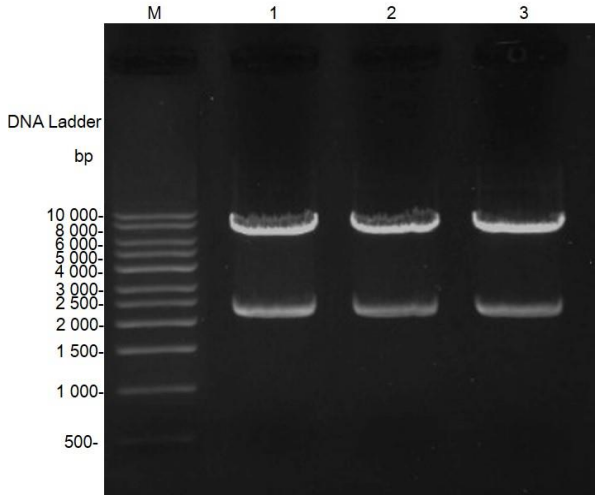

Fig.41 Analysis of pAaHBgl-*Tr*GH55 construction.

M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1 to 3, fragments of recombinant plasmids digested by *Xho* I and *Not* I.

As shown in Fig.41 from lane 1 to 3, two fragments were generated after restrict digestion of endonuclease, with lengths between 2000 to 2500 bp and 8000 to 6000 bp in each lane, respectively. The results revealed by DNA electrophoresis meet the requirements mentioned above, suggesting the recombinant plasmid construction was conducted correctly.

The recombinant pAaHBgl-*Tr*GH55 shown in Fig.41 Lane 1 was selected afterward for transformation into *P. pastoris* strain GS115. Recombinants appeared on the selective plate were picked and cultivated for recombinant protein secretion in small scale cultivation using 24-well plate. After methanol induction for 120 hours, supernatants were used as crude enzyme samples for protein and endo-β-1,3-glucanase activity assay. The Fig.42 below was the SDS-PAGE of secreted protein from the recombinant strains.

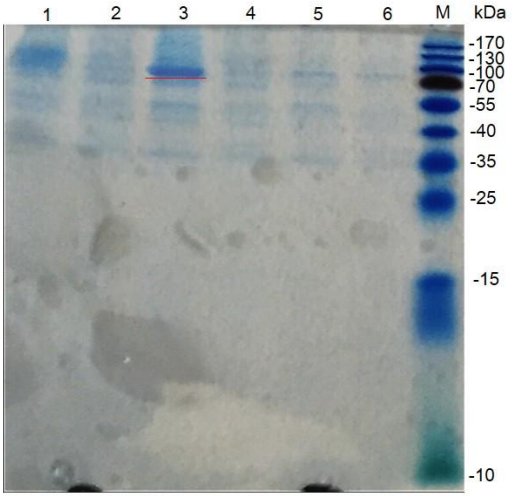

Fig.42 SDS-PAGE analysis secreting expression of recombinant *Tr*GH55.

Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-6, 15 µL culture supernatant of recombinant *P. pastoris* GS115 strains after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

The existence of an evident protein band in lane 3 (highlighted by red line) with a molecular mass between 70 and 100 kDa, which met the theoretical mass of 77.94 kDa, confirmed the secretion of recombinant *Tr*GH55 in *P. pastoris*. Faint protein bands with the same molecular mass in lane 5 and 6 revealed the potential existences of target enzyme. However, it was not difficult to estimate the bands from the gel. For the bands in lane 5 and 6, it was hard to confirm efficient expressions of the potential enzyme from respective recombinants. To investigate the *Tr*GH55 activity in these samples, cleavage assays against laminarin and alkali treated paramylon were performed and enzyme activities were determined by MBTH method (section 2.4.5.2).

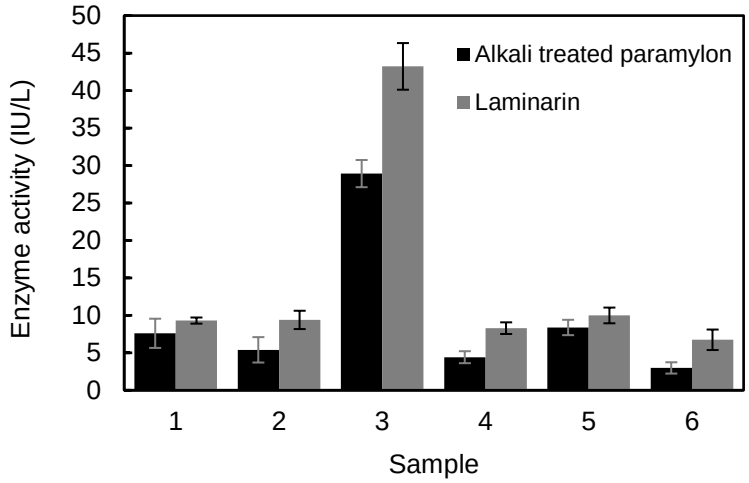

Fig.43 Enzyme activity assay from culture supernatants of selected transformants (*Tr*GH55). Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction in small scale using 24 square-well plate (section 2.4.4.1) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5, indicated by black column) and alkali treated paramylon (2.0 g/L, pH=6.5, indicated by gray column), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

The only sample (Lane.3 in Fig.42) containing an evident protein band with the right molecular mass had an activity of 28.9 IU/L on alkali treated paramylon and 43.2 IU/L on laminarin. The other samples selected all have activity about 10 IU/L on ether alkali treated paramylon or laminarin, indicating the recombinant endo-β-1,3-glucanase *Tr*GH55 was not or inefficiently expressed. Combining the results from SDS-PAGE, the recombinant *P. pastoris* GS115 strain NO.3 successfully secreted *Tr*GH55 as main extracellular protein. And the recombinant enzyme had activity on both laminarin and alkali treated paramylon, providing reducing ends from the substrates. Therefore, this

recombinant strain was selected for further research and named as *P. pastoris* GS115-*Tr*GH55 in this work. Recombinant *Tr*GH55 expression was performed in 250 mL baffled flask. Supernatant collected in every 24 h since methanol inducing starts was assayed for endo-β-1,3-glucanase activity using laminarin and alkali treated paramylon as substrate respectively.

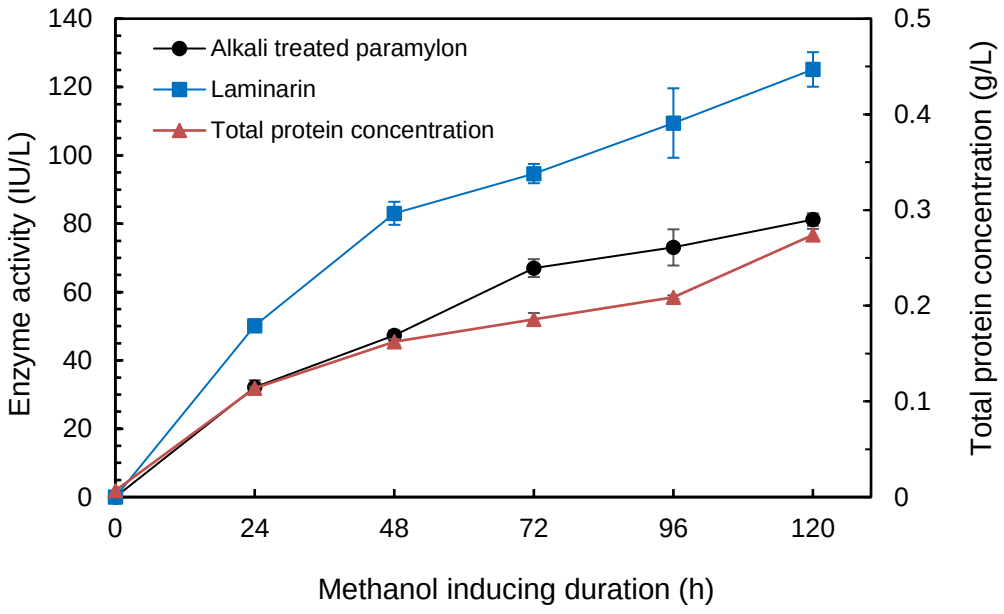

Fig.44 Recombinant *Tr*GH55 expression in *P. pastoris*. Supernatants of selected recombinant P.pastoris GS115 transformants after methanol induction using 24 shake flask (section 2.4.4.2) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5) and alkali treated paramylon (2.0 g/L, pH=6.5), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

During the methanol induction, endo-β-1,3-glucanase activity detected by determining reducing ends generated from laminarin increased from none to 125.1 IU/L in 120 hours. Comparing to 42.2 IU/L detected from 24-well plate

cultivation, the activity of recombinant enzyme achieved a threefold enhancement. A similar trend appeared when acting on alkali treated paramylon as substrate. After 120 hours inducing, the enzyme activity was 81.2 IU/L with a total protein concentration of 0.26 g/L in the supernatant. As discussed before, the recombinant *Tr*GH55 showed higher activity toward laminarin, as the well dissolved laminarin was probably more accessible to enzymes than paramylon.

According to the results of enzyme assay and protein electrophoresis, the conclusion can be made, that a putative endo-β-1,3-glucanase from *T.reesei* namely *Tr*GH55 was successfully cloned and secreted by recombinant *P.pastoris* using α-factor as leading signal. The recombinant *Tr*GH55a is predicted to be a GH55 family enzyme, because of the high amino acid sequence similarity with GH55 family member *Bgn*13.1 from *T. harzianum*. The enzyme *Bgn*13.1 expressed in strawberry was confirmed as an endo-β-1,3-glucanase by hydrolyzing laminaring. The recombinant enzyme had the highest activity of 2.4 mmol/L revealing by glucose equivalent produced after acting on laminarin for 20 hours (Mercado et al. 2015). While in this work, the *Tr*GH55 had the reducing sugar equivalent of 937.5 μmol/L. Although the activity was lower than *Bgn*13.1, the *Tr*GH55 was secreted by *P. pastoris* for the first time since genome sequence of *T. reesei* was published in 2008 (Martinez et al. 2008), and the activity could be enhanced by gene codon and express conditions optimization in further work.

3.2.4 Cloning and expression of *Tr*GH64 (EGR45483.1)

Nucleotide and amino acid sequences of *Tr*GH64 from *T. reesei* are shown in Fig.45. The open reading frame had a size of 1350 bp terminating by stop codon TGA, which corresponds to a translation product of 449 amino acids. Predicting by SignalP 3.0, a N-terminal signal peptide existed and the cleavage site lay between positions 16 (a) and 17 (a). Additionally, basic residues Thr (t) and Arg (r) at positions 23-24 were predicted as propetide cleavage site by ProP1.0 Server, indicating the mature protein started from residue at position 33 (v).

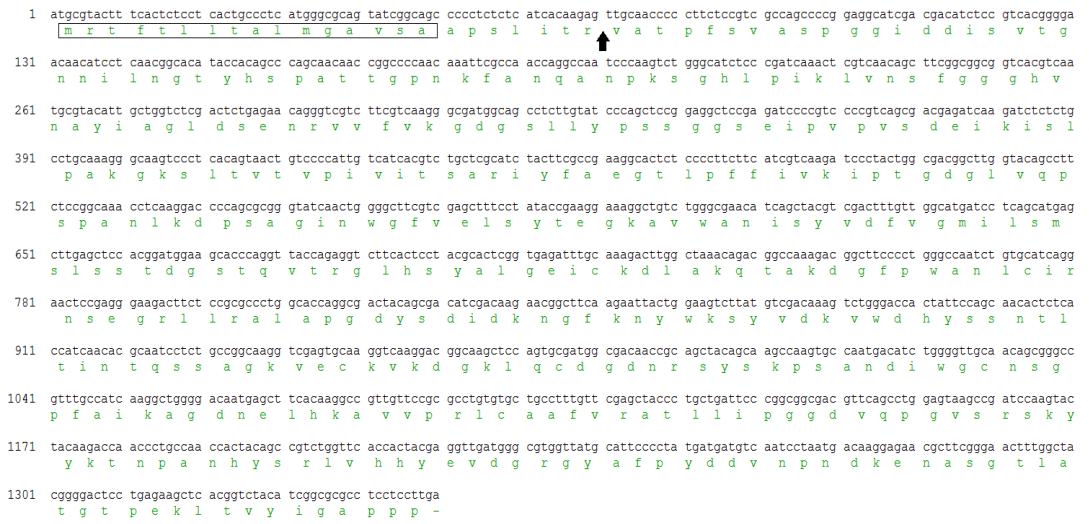

Fig.45 Nucleotide sequence and amino acid sequence of *Tr*GH64.

In the coding region, the putative signal peptide was boxed, and the predicted propeptide cleavage site was indicated by an arrow.

By aligning amino acid sequence with available protein sequences from the GenBank database, the protein showed 25% similarity to laminarinpentaose-producing β-1,3-glucanase naming *LPHase* from *Streptomyces matensis* (GenBank: BAA34349.1) (as showing in Fig.46). The *LPHase* was confirmed to be a GH family 64 member, endolyticly cleaving β-1,3-glucan chain into

specific pentasaccharide oligomers in a direct displacement mechanism. In the catalytic domain, the conserved Glu acted as a porton donor to the glycosidic oxygen, and the conserved Asp facilitated a base assisted nucleophilic attack by a water molecule from the opposite side of the sugar ring, while the side chains of Thr, Asn and Trp acted for polysaccharide chain binding (Wu et al. 2009). When a polysaccharide chain of β-1,3-glucan diffused into the catalytic domain, the reducing end would be positioned bound in the region by Trp(R3), Asn(R4) and Thr(R5). Hydrolysis of the β-1,3-glycosidic bond from the end of the chain was performed by Glu(R1) and Asp(R2), generating pentasaccharide as product.

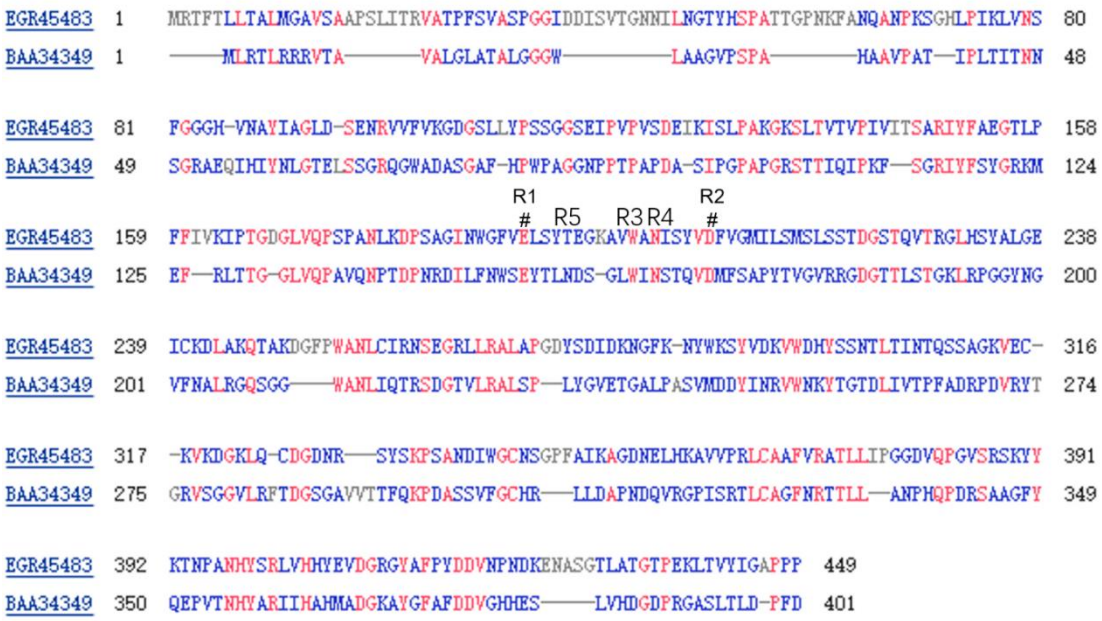

Fig.46 Alignment of the *Tr*GH64 sequence.

EGR45483: *Tr*GH64 from *T. reesei*;

BAA34349: LPHase from *S. matensis*.

The conserved catalytic residues are R1 and R2, substrate binding residues were maked as R3, R4, and R5.

Gene of putative endo-β-1,3-glucanase *Tr*GH64 was cloned by a PCR-based strategy using cDNA as template. A PCR product of 1316 bp was obtained containing a 1299 nucleotide ORF terminated by a TAG stop codon, which encoding 432 amino acids of mature protein with a predicted molecular mass of 46.35 kDa. As shown in Fig.35A, lane 5, the bright band with a size between 1000 and 1500 bp was obtained as the main product. The purified PCR product was then ligated into pJET 1.2 cloning vector for further plasmid construction.

Recombinant plasmid pAaHBgl-*Tr*GH64 construction was performed as described in (section 2.4.2). Gene of *Tr*GH64 was ligated into the vector pAaHBgl between sites *Xho* I and *Not* I. Two fragments with the sizes of 7395 bp and 1316 bp should be produced by endonucleases digestion of *Xho* I and *Not* I when the gene of interested was inserted correctly.

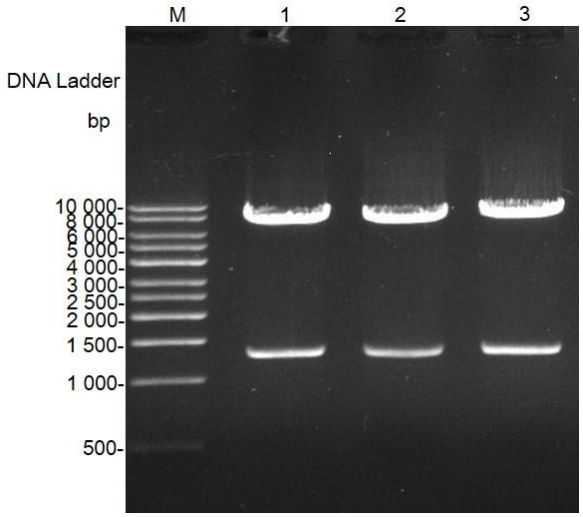

Fig.47 Analysis of pAaHBgl-*Tr*GH64 construction. Recombinant vector pAaHBgl-*Tr*GH64 screening by digestion with restriction endonucleases. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1-3, results of the recombinant plasmids digested by *Xho* I and *Not* I.

As shown in Fig.47 Lane 1 to 3, two evident fragments were obtained by *Xho* I and *Not* I digestion, the band between the 1000 and 1500 bp was the gene

coding *Tr*GH64 and the band with the size between 6000 and 8000 bp was linearized vector pAaHBgl. The recombinant vector shown in lane 1 in Fig.47 was selected as expression vector for further transformation into *P.pastoris* strain GS115. Performed as mentioned in the methods 2.4.4.1, transformants were picked from selective agar plate and induced by methanol for recombinant protein expression in 24-well plates. Supernatants from the small-scale cultivation were used as crude enzyme samples for SDS-PAGE and activity assay. Only one positive transformant was obtained demonstrating by the SDS-PAGE result of secreted proteins. In Fig.48 lane 5, a distinct band (highlighted with red underline) shows a secreted protein with a molecular mass between 40 and 55 kDa, which meets the predicted molecular mass of target enzyme *Tr*GH64 (46.35 kDa). No band with similar molecular mass appeared in other samples.

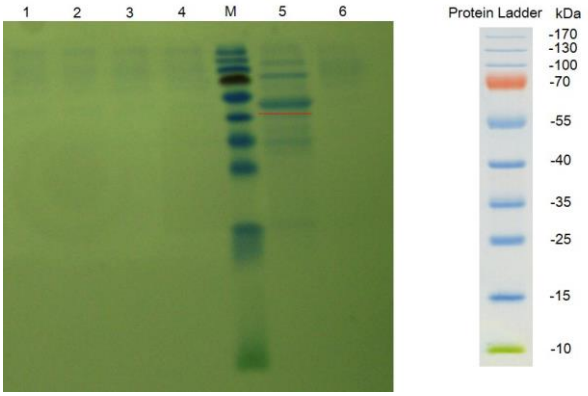

Fig.48 SDS-PAGE analysis secreting expression of recombinant *Tr*GH64. Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-6, 15 µL culture supernatant of six recombinant *P. pastoris* GS115 strains after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

Results in Fig.49 further confirmed the success of cloning and expression of recombinant *Tr*GH64 in *P. pastoris*. To measure the enzyme activity, alkali

treated paramylon and laminarin were used as substrate for recombinant enzyme hydrolyze assay, respectively. Enzyme activity was analyzed by determining reducing ends using MBTH method.

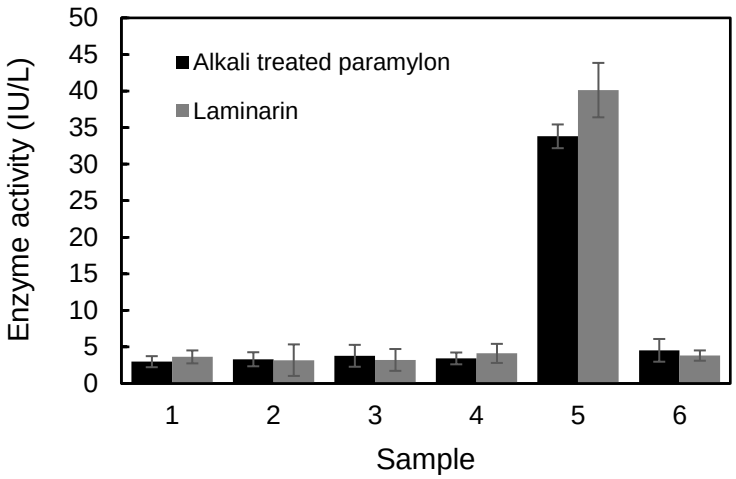

Fig.49 Enzyme activity assay from culture supernatants of selected transformants (*Tr*GH64). Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction in small scale using 24 square-well plate (section 2.4.4.1) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5, indicated by black column) and alkali treated paramylon (2.0 g/L, pH=6.5, indicated by gray column), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

Suggested by hydrolytic activity assay toward these two substrates, only the supernatant of transformant NO.5 (Fig.48 lane 5) had definite higher recombinant *Tr*GH64 activity than the other samples. As shown in Fig.49, recombinant *Tr*GH64 demonstrated activity of 33.8 IU/L towards alkali treated paramylon, and 40.1 IU/L when acting on laminarin. While the other samples

containing only background proteins of *P. pastoris* in SDS-PAGE, these samples all showed activities lower than 5.0 IU/L towards either substrate. Based on these results, it's confirmed that the active recombinant endo-β-1,3-glucanase *Tr*GH64 was secreted. This recombinant strain was named as *P. pastoris* GS115- *Tr*GH64 and cultivated in 250 mL baffled flask for further research.

During methanol induction, supernatant was taken every 24 hours and used as crude enzyme for hydrolytic assay. The total protein concentration and enzyme activity results are shown in Fig.50 below.

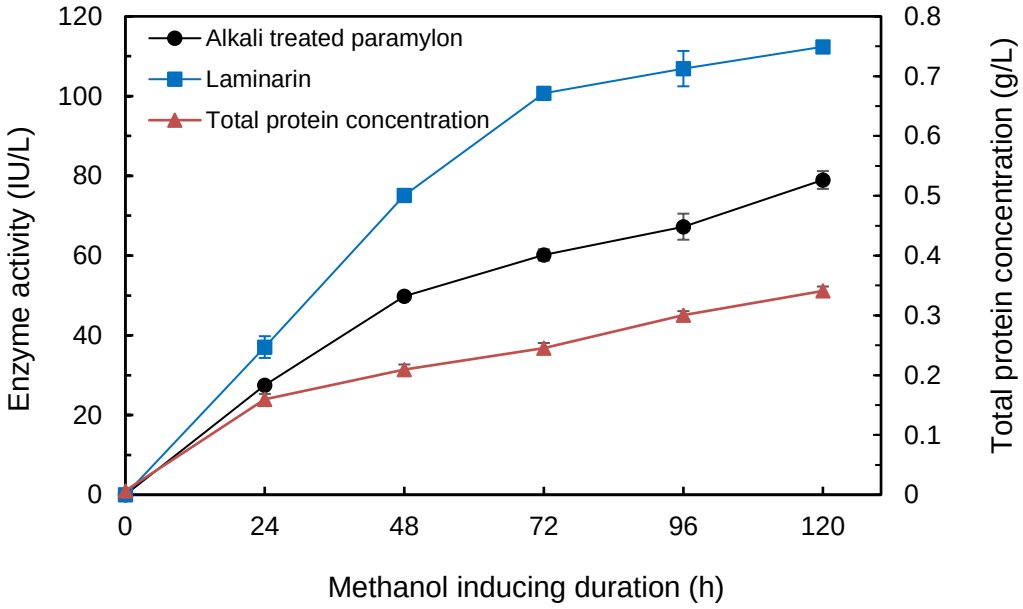

Fig.50 Recombinant *Tr*GH64 expression in *P. pastoris*. Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction using 24 shake flask (section 2.4.4.2) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5) and alkali treated paramylon (2.0 g/L, pH=6.5), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

Since the methanol inducing started, proteins in the supernatant accumulated and achieved a concentration of 0.34 g/L in 120 hours. Consequently, activity of endo-β-1,3-glucanase increased along the protein accumulation. When use laminarin as substrate, the enzyme activity rose rapidly to 100.7 IU/L in the first 72 hours, and increased steadily to 112.4 IU/L at time point 120h. The hydrolysis action of *Tr*GH64 towards β-1,3-glucan started by positioning a free chain of the polysaccharide and breaking the glycosidic bond by interactions of residues including Glu and Asp in the catalytic domain, afterwards. Therefore, enzyme activities toward alkali treated paramylon were relatively lower due to the difficult of accessing between enzyme and substrate. After methanol induction for 120 hours, the recombinant *Tr*GH64 had an activity of 79.0 IU/L on paramylon, which is about 30% lower than acting on laminarin.

Although 296 proteins were predicted relating to glycoside hydrolase family 64 enzymes by now according to sequence similarity (Davies and Sinnott 2008). The GH family β-1,3-glucanase was firstly confirmed to hydrolysis substrate by inverting mechanism in 2001 (Nishimura et al. 2001). The crystal structure was resolved in 2009 for the first time (Wu et al. 2009), and essential residues related to catalytic activity were firstly reported in 2011 (Shrestha et al. 2011). Including the *LPHase* from *S. matensis*, only four GH family enzymes were characterized by now. The other three characterized enzymes, *glu*B from *Lysobacter enzymogenes* Strain N4-7 (Palumbo et al. 2003), *LPHase* from *Streptomyces matensis* DIC-108 (Palumbo et al. 2003), and an unnamed glucanase from *Oerskovia xanthineolytica* (Shen et al. 1991), were all expressed in *E. coli*. Based on the experimental evidences and current knowledges, this work conducted the successful recombinant expression of *Tr*GH64 from *T. reesei* in *P. pastoris* for the first time.

2.3.5 Cloning and expression of *Tr*GH81 (EGR49603.1)

Nucleotide and amino acid sequences of *Tr*GH81 (GenBank: EGR49603.1) are shown in Fig.51. The open reading frame was 2592 bp and terminated by TAA stop codon, corresponding to a translation product of 863 aa. Predicting by SignalP 3.0, a N-terminal signal peptide existed and the cleavage site was between positions 18 (a) and 19 (l). No propetide cleavage site was predicted by ProP1.0 Server downstream of the signal peptide. Therefore, the putative mature enzyme from residue Leu (l) to Ser (s) from position 19 (l) to 863 (s) was cloned.

```
   1  atgctggcct ctctttgtgaa cctcgtcatc gcctcggcgc ttggcgttgc ggctctgccc aatggcgcaa agctcagcaa cattgccgga cgcggtctgt tcgccccgat ctcgacgagc aacccggccg
        m  l  a  s  l  v  n  l  v  i  a  s  a  l  g  v  a  a  l  p  n  g  a  k  l  s  n  i  a  g  r  g  l  f  a  p  i  s  t  s  n  p  a

 131  gcatctcggg cggcaccacg gccaccggcg ccgatggagc gcccgtgagc tcgtttttcg caggcctgaa gcctccgttc ccgaccaact cctggtgggc ctcgtacgcc gccacgcccg gcaacggcac
        g  i  s  g  g  t  t  a  t  g  a  d  g  a  p  v  s  s  f  f  a  g  l  k  p  p  f  p  t  n  s  w  w  a  s  y  a  a  t  p  g  n  g

 261  cgcctcgggc ccctttccct acgagtcgca gctcgacggc ctgggcatca actttggcgt gagcaacagc cgccagttcg acggcacgtc catcaagcag ccgacgcaga acgactggcg cgcgggcttc
        t  a  s  g  p  f  p  y  e  s  q  l  d  g  l  g  i  n  f  g  v  s  n  s  r  q  f  d  g  t  s  i  k  q  p  t  q  n  d  w  r  a  g  f

 391  gccgagcact cgggcgcctt tgccaaccac aaggcgacgg cctttgacac gcactcggtc acggtgcagt acttccaggg cggcgcgagc atgacctcgc cgctgattcc cggatcgccg tacattacgc
        a  e  h  s  g  a  f  a  n  h  k  a  t  a  f  d  t  h  s  v  t  v  q  y  f  q  g  g  a  s  m  t  s  p  l  i  p  g  s  p  y  i  t

 521  tgcagtacca ggctgccacg ccgctgctga cgtcgaggaa cgggggcatt gcgtcgttta acggacagaa tctctcgaat ggacagagtg tcactgctac tggcacctcg ttcaccgtcg tcgacaccac
        l  q  y  q  a  a  t  p  l  l  t  s  r  n  g  g  i  a  s  f  n  g  q  n  l  s  n  g  q  s  v  t  a  t  g  t  s  f  t  v  v  d  t

 651  cggcaccacc tacgtgatct acgccctctc gtccatctcc ctgactgcca cggccaccaa cagcgcccag ggcactatca aggccaccgg cacgtacaac ggcgtgctcc gcctcgtcag gctcacccag
        t  g  t  t  y  v  i  y  a  l  s  s  i  s  l  t  a  t  a  t  n  s  a  q  g  t  i  k  a  t  g  t  y  n  g  v  l  r  l  v  r  l  t  q

 781  gccagccaca aggcgctcct cgaccagcac tacaccgtct accccacggg cgtcggcctc gactacagct tcaccaccac caccggcacg ctcatcttca actacaacac cgtgggcgac ggcagccagc
        a  s  h  k  a  l  l  d  q  h  y  t  v  y  p  t  g  v  g  l  d  y  s  f  t  t  t  g  t  l  i  f  n  y  n  t  v  g  d  g  s  q

 911  tgctgatgct gacctggccg caccaccgtc tgtctctgca gggcgccaac cagccggcca cttcgtccct gggctacctc accaccaagg gatggatgta ccccatcatc ggcaaccagt ggaagctcct
        l  l  m  l  t  w  p  h  h  r  l  s  l  q  g  a  n  q  p  a  t  s  s  l  g  y  l  t  t  k  g  w  m  y  p  i  i  g  n  q  w  k  l

1041  gtaccagctg agcagcatca cctggaaccc tcctcgcgcg ctcgactcct cgtgcagctc ggccgtcatc cagggtctcc agtacgagat tggtctgctc gcgaattcta ctcctcctgt gcccaacgag
        l  y  q  l  s  s  i  t  w  n  p  p  r  a  l  d  s  s  c  s  s  a  v  i  q  g  l  q  y  e  i  g  l  l  a  n  s  t  p  p  v  p  n  e

1171  ttttactact ggggaggcag cctcgccgct caggctcgtc ttgccctcat tgccgaggct gtcggccgca ccgacctcat ccccacggtg acaaactacc tcaagaccag cttccagaac tggttcacgc
        f  y  y  w  g  g  s  l  a  a  q  a  r  l  a  l  i  a  e  a  v  g  r  t  d  l  i  p  t  v  t  n  y  l  k  t  s  f  q  n  w  f  t

1301  ccagcaccgg cgcgtctccc gcgtacgaaa cctcttgggg cggcgtcatc gacaaggccg gcgccaccaa ctcgggcatc gactttggca acggctacta caacgaccac cacttccact acggctactt
        p  s  t  g  a  s  p  a  y  e  t  s  w  g  g  v  i  d  k  a  g  a  t  n  s  g  i  d  f  g  n  g  y  y  n  d  h  h  f  h  y  g  y

1431  tttgtacgtg gccgccgtca ttgccaagta cgacgccaac tggctggcgc agcataagga ctttatcaac tggtttgccc gagacatcat caacccgtcg cccaacgacc cctacttccc cgtcacccgc
        f  l  y  v  a  a  v  i  a  k  y  d  a  n  w  l  a  q  h  k  d  f  i  n  w  f  a  r  d  i  i  n  p  s  p  n  d  p  y  f  p  v  t  r

1561  tgccgcgact ggttcgccgg ccactcgtgg gcctcgggca tcgccaacgg ggccggcagc cgagaccagg agtcgaccgg cgaggccgtc aacggctact acggcgcgct gctctgggcc acggtcgcgc
        c  r  d  w  f  a  g  h  s  w  a  s  g  i  a  n  g  a  g  s  r  d  q  e  s  t  g  e  a  v  n  g  y  y  g  a  l  l  w  a  t  v  a

1691  tgtcgcagga ctacgtcaac tacgcccggc tgctcgtcgc cacgggagcag cagggcgccc aggtctactg gcacctgtac ccgcagcaga gccagacaga ccccaacaac ccgtacccgg agccggcggt
        l  s  q  d  y  v  n  y  a  r  l  l  v  a  t  e  q  q  g  a  q  v  y  w  h  l  y  p  q  q  s  q  t  d  p  n  n  p  y  p  e  p  a

1821  gcgcaacctg gtgaccatgg gcaacgtcga ggactggcag tcgggcgcct ggctgttctg gggcaaccag aagagcgaga ttgcggccat ccagatgctg cccgtgacgc ccatcaacga ggtgctgtac
        v  r  n  l  v  t  m  g  n  v  e  d  w  q  s  g  a  w  l  f  w  g  n  q  k  s  e  i  a  a  i  q  m  l  p  v  t  p  i  n  e  v  l  y

1951  gacgcccagt gggtcaacaa tgtgtggtcg tatgcccaga acgagattgt cgacccgtcg attgccgatg actggcgctc cgtcatgatt gcggcctact ccaacgccaa ccctcagact gctgccgcct
        d  a  q  w  v  n  n  v  w  s  y  a  q  n  e  i  v  d  p  s  i  a  d  d  w  r  s  v  m  i  a  a  y  s  n  a  n  p  q  t  a  a  a

2081  ggagcgccaa cctgaccacc tggggctccg gaaacacctt ctccaacgag ctcttcttca tcggcacccg gcccaacccc agcggccagc ccatctgcgg ctccaacttc ccgcagaacc cgtacggcaa
        w  s  a  n  l  t  t  w  g  s  g  n  t  f  s  n  e  l  f  f  i  g  t  r  p  n  p  s  g  q  p  i  c  g  s  n  f  p  q  n  p  y  g

2211  cttcaagatc cagtcggcca cgacgggcca gtgggtcgtc gcgtcgacgg cgtcgtccaa cctcgtggcc tcgggctcgc aggccagcgc cggcgtcttc atcagctcgt acacgcccaa cgcgggcaac
        n  f  k  i  q  s  a  t  t  g  q  w  v  v  a  s  t  a  s  s  n  l  v  a  s  g  s  q  a  s  a  g  v  f  i  s  s  y  t  p  n  a  g  n

2341  ctcaagctga cgagcaacaa ccagttcgtg acggcggacc agtcgggcaa ctttgcgctg caggcggccc gcgccacggc ctcgtcctgg gaggtgttta ccatccgcca aaaggtcggc gccgcggcgg
        l  k  l  t  s  n  n  q  f  v  t  a  d  q  s  g  n  f  a  l  q  a  a  r  a  t  a  s  s  w  e  v  f  t  i  r  q  k  v  g  a  a  a

2471  gcgtgtacac gatcaaggct gggagcaacg gcaagtgggt gacgctcgcg tcggacgggt cgttgatcaa caatggggcg acggaggctt cggctgctgg gttcaagttt gtccagtcgt aa
        g  v  y  t  i  k  a  g  s  n  g  k  w  v  t  l  a  s  d  g  s  l  i  n  n  g  a  t  e  a  s  a  a  g  f  k  f  v  q  s  -
```

Fig.51 Nucleotide sequence and amino acid sequence of *Tr*GH81.

In the coding region, the putative signal peptide was boxed.

Comparison of the amino acid sequences of *Tr*GH81 with characterized protein *Rm*Lam81A from *Rhizomucor mieher* (GeneBank: AGV00786.1) resulted a similarity of 24%. The enzyme *Rm*Lam81A was an endo-β-1,3-glucannase of GH81 family. For the first time, crystal structure of *Rm*Lam81A was reported consisting three distinct domains: domain B stabilized the structure of the protein, as well as the core cleft formed by domain A and C (Zhou et al. 2013). As shown in Fig 52, strictly conserved residues in the sugar-binding motif were framed in blue. Among them, Glu (R1) and Glu (R3) were predicted catalytic residues. R1 and R3 were likely a proton donor and a basic catalyst respectively, and residues Ser (R2), Tyr (R4) and Try (R5) contributed to position and ionization state of catalytic residues. For instance, residue R2 stabilized the position of R3 by forming a hydrogen bond to the backbone N atom (Zhou et al. 2013). These residues were highly conserved among GH family 81 emzymes.

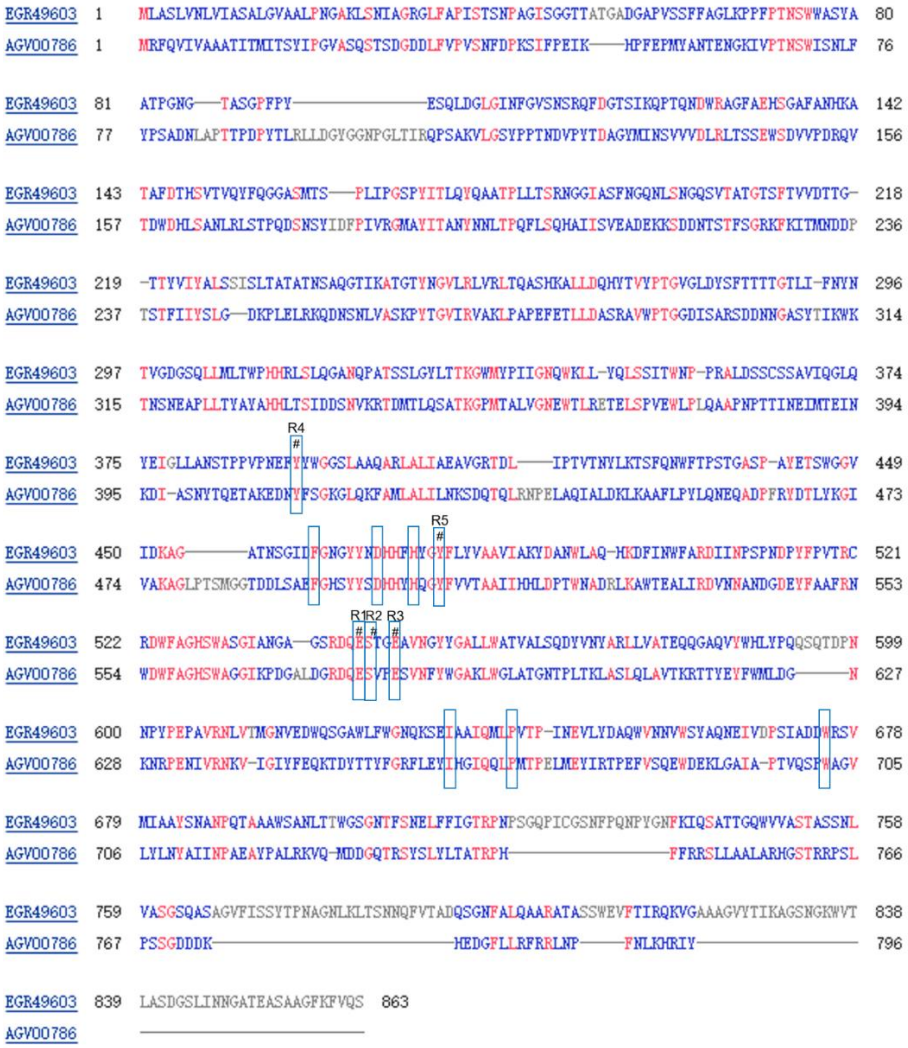

Fig.52 Alignment of the *Tr*GH81 sequence.

EGR49603: *Tr*GH81from *T. reesei*;

AGO00786: *Rm*Lam81A from *R. miehei*;

Strictly conserved residues were highlighted in red; sugar-binding related residues were framed in blue; and catalytic related residues were marked with “#”.

The gene of putative endo-β-1,3-glucannase *Tr*GH81 was cloned by a PCR-based strategy using *T.reesei* cDNA as template. A PCR product of 2576 bp was obtained, containing a mature enzyme (851 aa) nucleotide ORF of 2556

bp. The predicted molecular mass of *Tr*GH81 is 91.24 kDa. As shown in Fig.35A, lane 6, the only bright band with a size around 2.5 kb was obtained, and ligated into pJET 1.2 cloning vector for plasmid construction.

The gene was ligated into the vector pAaHBgl by *Xho* I and *Not* I to construct the recombinant plasmids pAaHBgl-*Tr*GH81. Two fragments with sizes of 7395 bp and 2576 bp should arise after digestion with *Xho* I and *Not* I when plasmid construction was correct.

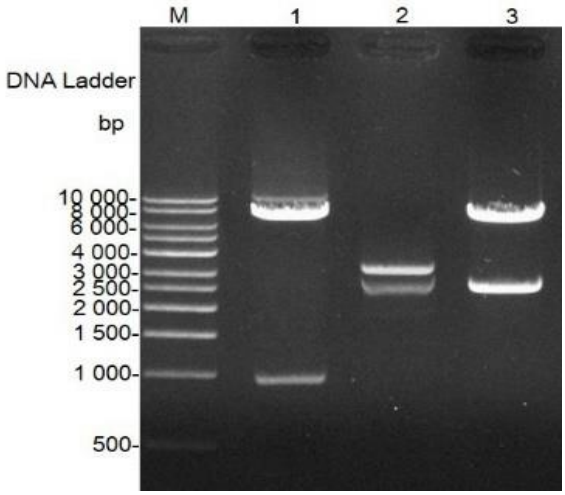

Fig.53 Analysis of pAaHBgl-*Tr*GH81 construction.

Recombinant vector pAaHBgl-*Tr*GH81 digestion with restriction endonucleases. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1-3, results of three recombinant plasmids digested by *Xho* I and *Not* I.

In Fig.53, only lane 3 showed two bands in right size representing two fragments cleaved by *Xho* I and *Not* I from correctly constructed recombinant plasmid. In lane 1, the first faint band about 10 kb was probably uncut original vector pAaHBgl, the second bright band was the longer fragment linearized by restriction endonucleases from pAaHBgl, and the third band with the size about 1,000 bp was the gene inserted between *Xho* I and *Not* I. The uncut original vector existed from the first step of construction and was transformed into *E.coli*

causing a false positive clone. In lane 2, the smaller band was about 2.5 kb, which met the size of target gene (2576 bp), but the other band about 3.0 kb might be a linearized cloning vector pJET 1.2. The bands in lane 2 indicated that circular cloning vector pJET 1.2 with target gene were transformed into *E. coli* strain during the construction procedure. Therefore, the recombinant vector showing in Fig.53 Lane 3 was selected as positive expression vector for transformation into *P. pastoris* strain GS115. Colonies appeared on the selective plates were picked and cultivated in small scale for recombinant protein expression screening.

SDS-PAGE analysis of the supernatants after methanol induction demonstrate that a predominant protein with the molecular mass between 70 and 100 kDa (Fig.54, Lane 2 and 4 (highlighted with red underline)) was expressed, respectively, which meets the predicted molecular mass of 91.24 kDa, indicating a secretion of recombinant *Tr*GH81. Enzyme activities of these two samples were also observed when act on both alkali treated paramylon and laminarin (in Fig.55).

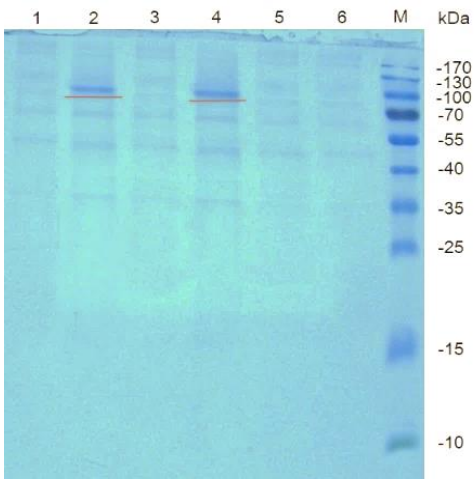

Fig.54 SDS-PAGE analysis secreting expression of recombinant *Tr*GH81. Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-6, 15 µL culture supernatant of six recombinant *P.*

pastoris GS115 strains after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

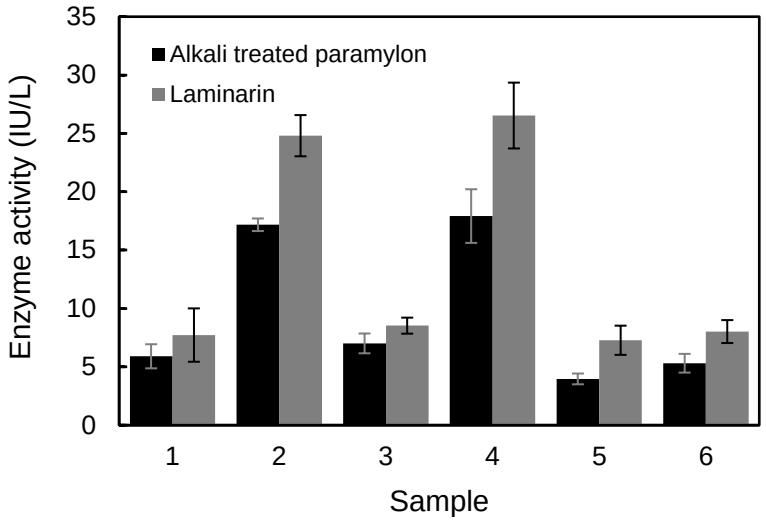

Fig.55 Enzyme activity assay from culture supernatants of selected transformants (*Tr*GH81). Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction in small scale using 24 square-well plate (section 2.4.4.1) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5, indicated by black column) and alkali treated paramylon (2.0 g/L, pH=6.5, indicated by gray column), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

The secreted proteins in Lane 2 and 4 of Fig.54 were active on alkali treated paramylon and laminarin. The crude enzyme sample NO.2 had activity of 17.2 IU/L toward alkali treated paramylon and 24.8 IU/L on laminarin, which were slightly lower comparing with sample NO.4, whose activites on these substrates were 17.9 IU/L and 26.5 IU/L respectively. Activities of sample NO.2 and NO.4 were obviously higher than those of the other four samples selected, which had

no predominant protein as showing in Fig.55. This suggested that the major secreted protein contributed cleavage activites against both substrates. Based on these results, the expression and enzymatic activity of recombinant *Tr*GH81 as an endo-β1,3-glucanase was preliminarily confirmed. And the recombinant strain NO.4 was named as *P. pastoris* GS115-*Tr*GH81 for shake flask cultivation.

During the methanol induction, supernatant was taken at each 24 h and used as crude enzyme for hydrolytic assay. The total protein concentration and enzyme activity results are showing in Fig.56 below.

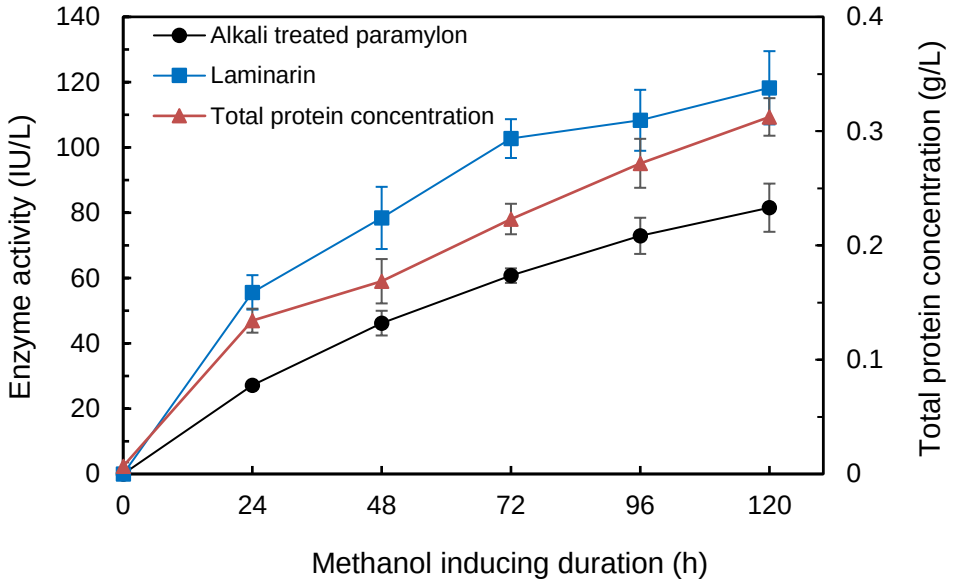

Fig.56 Recombinant *Tr*GH81 expression in *P. pastoris*. Supernatants of selected recombinant *P.pastoris* GS115 transformants after methanol induction using 24 shake flask (section 2.4.4.2) were used as crude enzymes for endo-β-1,3-glucanase activity assay. 90 µL enzyme sample was mixed with 90 µL laminarin (2.0 g/L, pH=6.5) and alkali treated paramylon (2.0 g/L, pH=6.5), respectively, and incubated at 37 °C for 60 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

During the methanol induction, endo-β-1,3-glucanase activity detected by determining reducing ends generated from laminarin increased from none to 118.3 IU/L at 120 h, while the total protein concentration in the supernatant was 0.26 g/L. Comparing to 26.5 IU/L detected from 24-well plate cultivation, the activity of recombinant enzyme achieved a fourfold increasing. As being observed in other recombinant enzyme assay, similar trend appeared also when alkali treated paramylon was used as substrate, but with lower activity. After induction for 120 hours, the enzyme activity of recombinant *Tr*GH81 was 81.5 IU/L in the supernatant. This was a fourfold increasing by comparison with 17.9 IU/L in multi-well plate cultivation.

Hundreds of proteins were classified in glycoside hydrolase family 81, which were widely distributed in fungi, bacteria and plants (Davies and Sinnott 2008). In an inverting hydrolytic mechanism, all GH family enzymes specificly broke β-1,3-glycosidic bond by endolytic mode (McGrath and Wilson 2006). However, limited members of this family have been characterized, such as *Eng*A from *Aspergillus nidulans* FGSC A4 (Mouyna et al. 2002), *Lam*81 from *Thermobifida fusca* (McGrath and Wilson 2006), a β-glucan elicitor binding protein expressed in tobacco suspension cultured cells and in *E. coli* for elicitor activity assay (Umemoto et al. 1997), and *Eng* from *Pneumocystis carinii* expressed in *E. coli* for determining immunoreactivity (Kutty et al. 2015). Nevertheless, as a putative endo-β-1,3-glucanase, *Tr*GH81 was initially secreted in *P. pastoris* and be confirmed by both protein electrophoresis and enzyme assay, especially displayed activity towards alkali treated paramylon.

2.3.6 Cloning and expression of *Tr*GH17 (EGR46171.1)

Nucleotide and amino acid sequences of *Tr*GH17 (GenBank: EGR46171.1) are shown in Fig.57. The open reading frame had a size of 1206 bp, corresponds to a translation product with 401 amino acids. Predicting by SignalP 3.0, a N-terminal signal peptide existed and the cleavage site was between positions 19 (a) and 20 (g). No propetide cleavage site was predicted by ProP1.0 Server at the downstream of signal peptide.

```
   1  atgaagctgt ctacgaccat cgcgactggc gctgcccttg tggccggtgt ttctgctgga cagaacaact acctcggctt caactcgggc aacaccctcc ccgacgagtc cgccaagttc gagaaggact
        m  k  l  s  t  t  i  a  t  g  a  a  l  v  a  g  v  s  a  g  q  n  n  y  l  g  f  n  s  g  n  t  l  p  d  e  s  a  k  f  e  k  d

 131  tccttgccga gttctccacg gcccagaagc tcgtcggcgc ccccggcacc ttcaacgccg tccgtctcta caccaacatc caggcctact ccaaggacac tcctatcgag gccttctccg ccgccatcaa
        f  l  a  e  f  s  t  a  q  k  l  v  g  a  p  g  t  f  n  a  v  r  l  y  t  n  i  q  a  y  s  k  d  t  p  i  e  a  f  s  a  a  i

 261  gaccaagacg tacattctcc tcggtgtctg ggcctcgggc actgataaca tcgacaatga gctggctgcc ctgagcgccg ctgtcaagca gtacggcaag gacctgacgg acctgatcat tggtgtctcc
        k  t  k  t  y  i  l  l  g  v  w  a  s  g  t  d  n  i  d  n  e  l  a  a  l  s  a  a  v  k  q  y  g  k  d  l  t  d  l  i  i  g  v  s

 391  atcggcagcg aggatctgta ccgtgactcg cagactggtc gaaccaacaa ggccggtgtc ggcaacggcc ccaaggaggt cctcggcttc atcaacgact acaagaagac ctttgccaac actgctctgg
          i  g  s  e  d  l  y  r  d  s  q  t  g  r  t  n  k  a  g  v  g  n  g  p  k  e  v  l  g  f  i  n  d  y  k  k  t  f  a  n  t  a  l

 521  ccaatgtccc catcggccac gtcgacacct gggatgcctg ggtcaacggc accaacaagc ccgtcctcga cgccgtcgac tggatcggtg ttgacgagta ccccttctac gagacaggca agggcaacga
        a  n  v  p  i  g  h  v  d  t  w  d  a  w  v  n  g  t  n  k  p  v  l  d  a  v  d  w  i  g  v  d  e  y  p  f  y  e  t  g  k  g  n

 651  cattagcaac gccggcaagc tcttcgacac ggctttcgag accacgctgg gcgctgccaa tggcaagccc gtctgggtga cggagaccgg ctggcccctg accggccccg actgggacga ggccaagccc
        d  i  s  n  a  g  k  l  f  d  t  a  f  e  t  t  l  g  a  a  n  g  k  p  v  w  v  t  e  t  g  w  p  l  t  g  p  d  w  d  e  a  k  p

 781  agcgtcaaga acgcccagaa gtactggcag gacattggct gcaagaggct cttcaacaag taccccacct tctggtacaa cctgcgtgac tccaacccgg ccaaccaggt caagttcggc atcagccaga
          s  v  k  n  a  q  k  y  w  q  d  i  g  c  k  r  l  f  n  k  y  p  t  f  w  y  n  l  r  d  s  n  p  a  n  q  v  k  f  g  i  s  q

 911  gcctctcttc cacccctttcg ttcgacctga cctgccccaa ggaggagacc accacctccg cggccaagcc caccgccacc accctcgtca aggcttctgg aacacccgaa gctgtcaagt ctggctccga
        s  l  s  s  t  p  s  f  d  l  t  c  p  k  e  e  t  t  t  s  a  a  k  p  t  a  t  t  l  v  k  a  s  g  t  p  e  a  v  k  s  g  s

1041  gggcaccgct aacgccggag gctccaagtc cagcaacagc gatgcttcca ccacctcatc aggccctgcc gcggccacta ccaccggcgc cggtgccgtg accaaggtct ccggcgctgc ccttgccggt
        e  g  t  a  n  a  g  g  s  k  s  s  n  s  d  a  s  t  t  s  s  g  p  a  a  a  t  t  t  g  a  g  a  v  t  k  v  s  g  a  a  l  a  g

1171  gtggccatgg ttgccggtct cctggccctg ttctaa
        v  a  m  v  a  g  l  l  a  l  f  -
```

Fig.57 Nucleotide sequence and amino acid sequence of *Tr*GH17.

In the coding region, the putative signal peptide was boxed.

By aligning amino acid sequence with available protein sequences from the GenBank database, *Tr*GH17 showed high similarity to the GH family 17 members, such as 67% indent to exo-β-1,3-glucanase from *Pochonia chlamydosporia* 170 (GenBank: XP_018147378.1) (Wang et al. 2016), and 69 % indent to 1,3-beta-glucosidase from *Colletotrichum incanum* (GenBank: KZL82930.1) (Hacquard et al. 2016). When aligned with the characterized exo-β-1,3-glucanase naming *Bgl*2 from *Saccharomyces cerevisiae* (GenBank: AAA34648.1), as shown in Fig.58, they shared the GH family 17 signature

fragment [LIVMKS]-x-[LIVMFYWA](3)-[STAG]-E-[STACVI]-G-[WY]-P-[STN]-x-[SAGQ] and the two strictly conserved Glu residues (R1 and R2) (Klebl and Tanner 1989).

Fig.58 Alignment of the *Tr*GH17 sequence.

EGR46171: *Tr*GH17 from *T. reesei*, AA1-330;

AAA34648: *Bgl2* from *S. cerevisiae*.

Strictly conserved catalytic Glu residues (R1 and R2) were marked with "#". Glycoside hydrolase family 17 signature fragment were framed.

The gene of putative exo-β-1,3-glucanase *Tr*GH17 was cloned by a PCR-based strategy using cDNA as template. A PCR product of 1190 bp was obtained containing a 1170 nucleotide ORF, which encoding 381 amino acids of mature protein with a predicted molecular mass of 41.06 kD. Fig.59A shows the agarose gel analysis of PCR product from cDNA of *T. reesei* by PCR using *Tr*GH17 gene specific primers. There was one bright band between 1000 and 1500 bp as the main product, which met the right size according to the target gene. Meanwhile, some strain bands existed, which might due to non-specific

amplifications. However, these products had no influence on isolating the target gene from agarose gel for cloning vector construction.

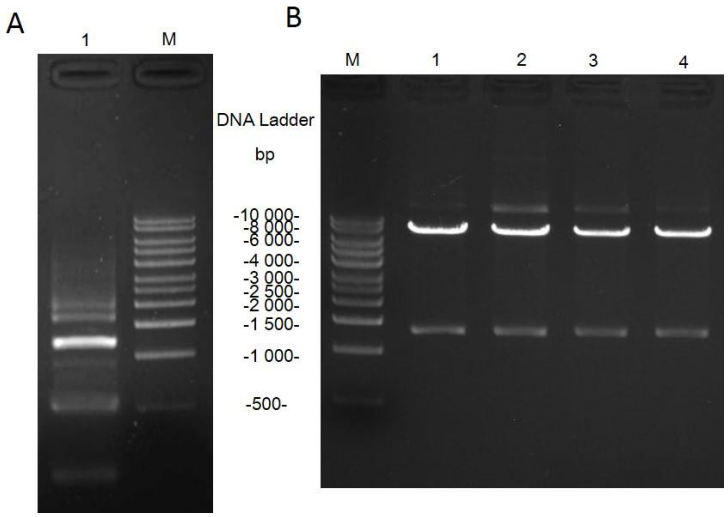

Fig.59 Analysis of *Tr*GH17 gene application and pAaHBgl-*Tr*GH17 construction.

A: Analysis of genes of glucanases obtained from cDNA of *T. reesei* QM6a by PCR using specific primers. M: 1 kb DNA ladder; lane 1: Gene of glucanase *Tr*GH17 (1190 bp);

B: Recombinant vector screening by digestion with restriction endonucleases. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1 to 4, results of four recombinant pAaHBgl-*Tr*GH17 plasmids digested by *Xho* I and *Not* I.

Recombinant plasmid pAaHBgl-*Tr*GH64 construction was performed as described in (section 2.4.2). Gene of *Tr*GH64 was ligated into the vector pAaHBgl between sites *Xho* I and *Not* I. To screen the positive constructions, *Xho* I and *Not* I were used to digest the recombinant plasmids. Two fragments with the sizes of 7395 bp and 1190 bp should be produced when the gene was inserted correctly. The digestion products were visualized by agarose gel

electrophoresis (see Fig.59B). All four plasmids tested had two fragments in right size, and the bands larger than 10 kb were probably un-cut circular vectors.

Afterwards, the recombinant vector shown in Fig.59B, lane 1 was selected as positive expression vector for further transformation into *P. pastoris* strain GS115. Performed as mentioned in section 2.4.4.1, transformants were picked from selective agar plate and induced by methanol for recombinant protein expression in 24 square-well plates. Supernatants from the small-scale cultivation were used as crude enzyme samples for SDS-PAGE and activity assay. Two positive recombinant strains were obtained demonstrating by the SDS-PAGE of secreted proteins. In Fig.60, lane 2 and 4 respectively, both contains a distinct band (highlighted with red underline) indicating a secreted protein with molecular mass about 40 kDa, which meets the predicted molecular mass of *Tr*GH17 (41.06 kDa). No band with similar molecular mass appears in other samples (lane 1,3,5 and 6).

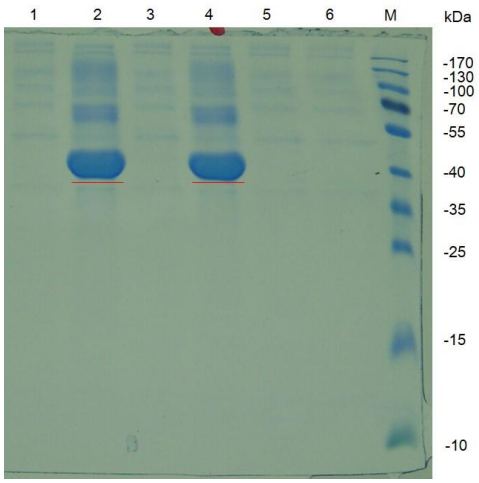

Fig.60 SDS-PAGE analysis secreting expression of recombinant *Tr*GH17. Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-6, 15 µL culture supernatant of six recombinant *P. pastoris* GS115 strains after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

Activities of these crude enzyme samples were measure by D-Glucose assay kit as described in section 2.4.5.3. The exo-β-1,3-glucanase enzyme activity was converted in term of IU/L, where one unit of enzyme activity (IU) was defined as the amount of enzyme required to liberate 1 µmol reducing sugar equivalent in 1 minute under the assay condition. The results in Fig.61 confirmed the distinct proteins showing in Fig.60, lane 2 and 4 were active towards laminarin and alkali treated paramylon and released free glucose from the substrates as hydrolytic product.

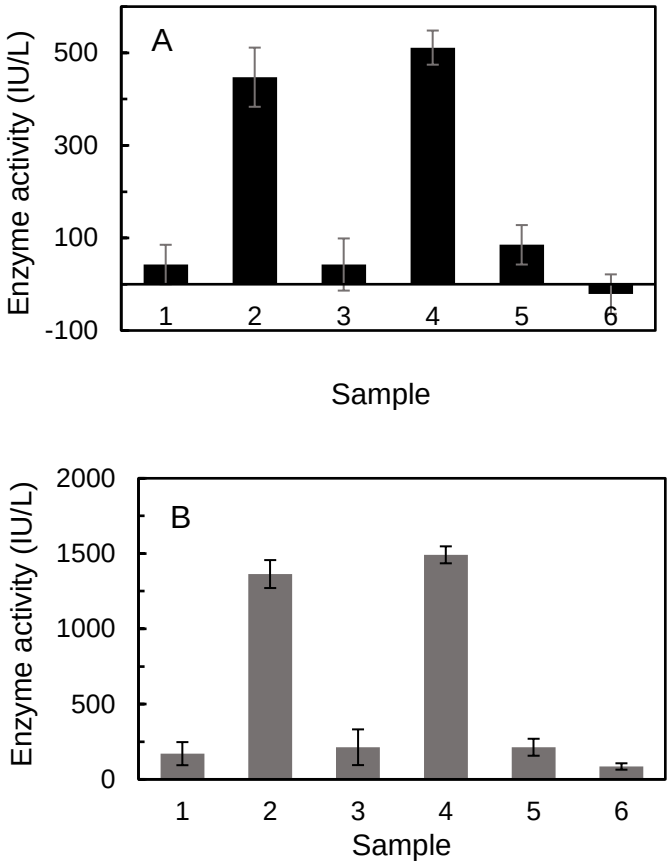

Fig.61 Enzyme activity assay from culture supernatants of selected transformants (*Tr*GH17). Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction in small scale using 24 square-well plate (section 2.4.4.1) were tested for exo-β-1,3-glucanase

activity. 100 µL enzyme sample was mixed with 100 µL using alkali treated paramylon (A, 2.0 g/L, pH=6.5, indicated by black column) and laminarin (B, 2.0 g/L, pH=6.5, indicated by gray column), respectively, and incubated at 37 °C for 15 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

After 120 hours methanol induction in 24-well plate, two of the selected recombinant strains (No.2 and 4) secreted putative exo-β-1,3-glucanase *Tr*GH17 as main product in the supernatant. The data of enzyme activity assay confirmed the conclusion by showing high activity on both substrates. With the total protein concentration of 0.65 g/L, crude enzyme No.2 showed activity of 447.4 IU/L on alkali treated paramylon and 1363.6 IU/L on laminarin, obtained the relative activity of 688.3 IU/g and 2097.8 IU/g, respectively. Moreover, the measured activity of crude enzyme No.4 were 511.4 IU/L on alkali treated paramylon, and 1491.5 IU/L on laminarin with protein concentration 0.7 mg/L corresponding to relative activities of 730.6 IU/g and 2130.7 IU/g, respectively. Based on result of SDS-PAGE and activity assay, the recombinant *P. pastoris* strain No.4 was selected for further work and named as *P. pastoris* GS115-*Tr*GH17.

Similarly, the *P.pastoris* GS115-*Tr*GH17 cultivation was performed in 250 mL shake flask and changed the medium to BMMY for methanol inducing. Supernatants of every 24 h were assaied for total protein concentration and exo-hydrolysis activity (section 2.4.5.3).

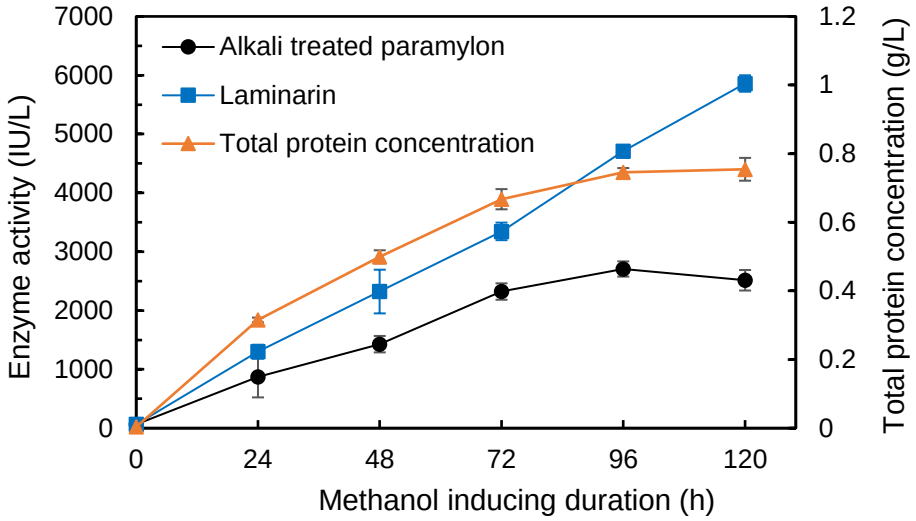

Fig.62 Recombinant *Tr*GH17 expression in *P. pastoris*. Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction using 24 shake flask (section 2.4.4.2) were used as crude enzymes for exo-β-1,3-glucanase activity assay. 100 µL enzyme sample was mixed with 100 µL laminarin (2.0 g/L, pH=6.5) and alkali treated paramylon (2.0 g/L, pH=6.5), respectively, and incubated at 37 °C for 15 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

Along the methanol induction, extracellular proteins accumulated steadily and reached a relatively stable concentration of about 0.75 g/L after 96 h of induction. As confirmed by the SDS-PAGE (Fig.60), recombinant *Tr*GH17 was the main protein in the supernatant. The putative exo-β-1,3-glucanase was supposed to release free glucose from the non-reducing end of the substrate as end product. The quantitative analysis of the enzymatic hydrolysate demonstrated that during the 120 hours of cultivation, enzyme activity increased along the induction time. Generally, the recombinant *Tr*GH17 hydrolyzed laminarin more efficiently and showed high enzyme activities. Along the extracellular protein secretion, hydrolytic activity of *Tr*GH17 towards

laminarin increased steadily, and achieved the highest value of 5859.3 IU/L after 120 hours. When using alkali treated paramylon as enzyme assay substrate, the increasing trend was also observed in general, but with lower activity at each time point. Moreover, sample collected at 96 h obtained the highest 2705.9 IU/L. When the crude enzyme collected at 120 h acted on paramylon, the activity of 2514.2 IU/L was lower than the activity at time point 96 h. Although the hydrolysis was conducted mainly by recombinant *Tr*GH17, exo-β-1,3-glucanases produced by *P. pastoris* for cell growth and metabolism might also participate at the substrate degradation.

Abundant enzymes were classified as GH family 17 members, and 83 enzymes were characterized as β-1,3-glucanases. Among these enzymes, only *Bgl*2 from *S. cerevisiae* (GenBank: AAA34648.1) (Plotnikova et al. 2006) and a β-1,3-glucan transferase from *Candia albicans* were characterized as exo-glucanase (Tsai et al. 2011). In this work, *Tr*GH17 was cloned and expressed in *P. pastoris* for the first time. The activity of the recombinant enzyme on laminarin and alkali treated paramylon were confirmed by determination of glucose as hydrolysis product.

2.3.7 Cloning and expression of *Pp*GH5 (CAY69081.1)

Nucleotide and amino acid sequences of *Pp*GH5 (GenBank: CAY69081.1) are shown in Fig.63. The open reading frame had a size of 1245 bp terminated by TAA stop codon, corresponding to a translation product with 414 amino acids. Predicting by SignalP 3.0, a N-terminal signal peptide existed and the cleavage site lay between positions 16 (s) and 17 (i). And a processing site for Kex2-like endoprotease was found downstream, resulting the mature protein N-terminal from position 23 (d).

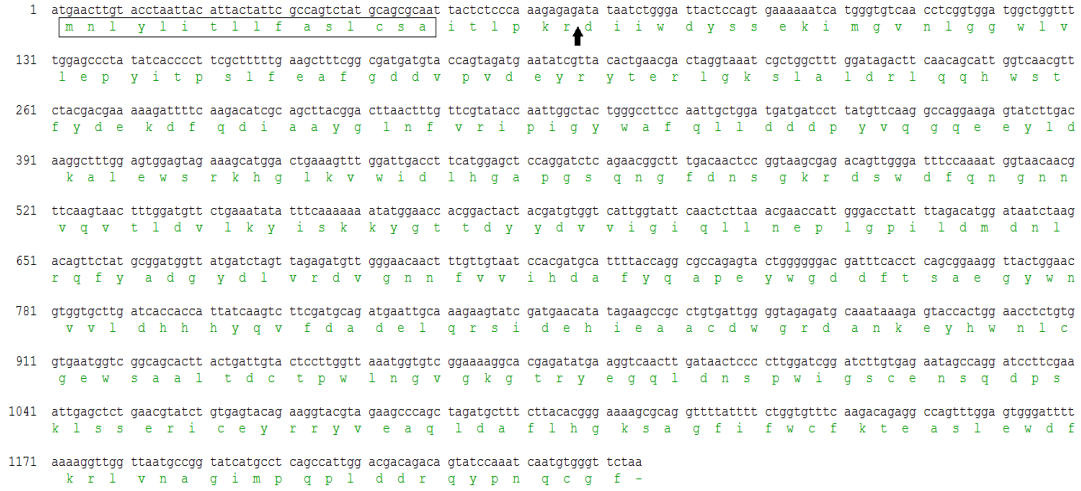

Fig.63 Nucleotide sequence and amino acid sequence of *Pp*GH5.

In the coding region, the putative signal peptide was boxed, and the predicted propeptide cleavage site was indicated by arrow.

A comparison of *Pp*GH5 amino acid sequence with known proteins in GenBank database indicated high sequence identity and similarity to exo-β-1,3-glucanses from glycoside hydrolase family 5 (Fig.64). These enzymes shared conserved the active relating residues R1 (Glu) and R2 (Glu) and several regions including the GH family 5 signature fragment in form of:

-[LIV]-[LIVMFYWGA](2)-[DNEQG]-[LIVMGST]-X-N-E-[PV]-[RHDNSTLIVFY]- (Xu et al. 2006). R1 and R2 served as the proton and nucleophile respectively

cleaving the glycosidic bond in a double-displacement mechanism (Cutfield et al. 1999).

Fig.64 Alignment of the *Pp*GH5 sequence.

CAY69081: *Pp*GH5 from *P. pastoris*;

AAA34599: *EXG*1 from *S. cerevisiae*;

CAA86951: *SoEXG*1 from *Schwanniomyces occidentalis*;

ACP74152: exo-β-1,3-glucanase from *Cyberlindnera saturnus*.

Strictly conserved residues were high-lighted in red, conserved catalytic residues R1 (Glu) and R2 (Glu) were marked with "#". Glycoside hydrolase family 5 signature fragment were boxed and strictly conserved residues in this region were high-lighted with blue background.

Gene of putative exo-β-1,3-glucanase *Pp*GH5 was cloned by a PCR-based strategy using genomic DNA as template. A PCR product of 1224 bp was obtained containing a 1197 nucleotide ORF encoding 398 amino acids of mature protein with a predicted molecular mass of 46.2 kDa. As shown in Fig.65A, lane 1, the only bright band with a size between 1.0 a 1.5 kb was the gene obtained. The bright band shown in Fig.65A, lane 1 was cleaned up and inserted into pJET 1.2 cloning vector for recombinant plasmid construction.

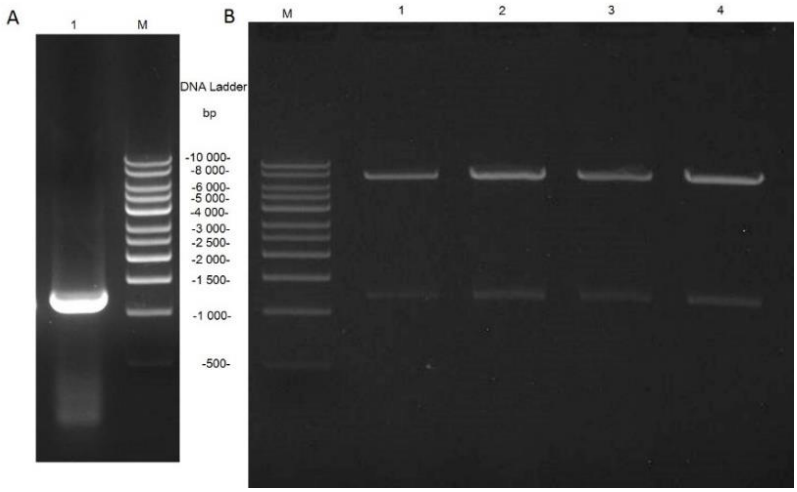

Fig.65 Analysis of *Pp*GH5 gene application and pAaHBgl-*Pp*GH5 construction.

A: Analysis of gene of *Pp*GH5 obtained from genomic DNA of *P. pastoris* GS115 by PCR using specific primers. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1: Gene of *Pp*GH5 (1224 bp);

B: Recombinant vector screening by digestion with restriction endonucleases. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1 to 4, results of four recombinant pAaHBgl-*Pp*GH5 plasmids digested by *Xho* I and *Not* I.

The gene was inserted into the vector pAaHBgl at sites *Xho* I and *Not* I afterward. Two fragments with the sizes about 7.5 kb and 1.2 kb should be

produced when the gene of interested was inserted correctly, when digest by *Xho* I and *Not* I. The screening results were showed in Fig.65B. All plasmids analyzed were rightly constructed, confirmed by the two right length cleavage products. The recombinant plasmid corresponding to Fig.65B, lane 4 was transformed into *P. pastoris* GS115 for protein expression.

Transformants were picked from selective agar plate and induced by methanol for recombinant protein expression in 24-well plates. Supernatants cultivation were used as crude enzyme samples for SDS-PAGE and activity assay. The only distinct protein band showing in Fig.66, lane 4 with a molecular mass about 50 kDa met the predicted *Pp*GH5 molecular weight (46.2 kDa). Results of protein electrophoresis suggested that lane 4 corresponds to recombinant *Pp*GH5 successfully expressed in *P. pastoris*. The activity assay performed afterwards (see Fig.67) confirmed this hypothesis and demonstrate that the recombinant *Pp*GH5 was an active enzyme on both laminarin and alkali treated paramylon.

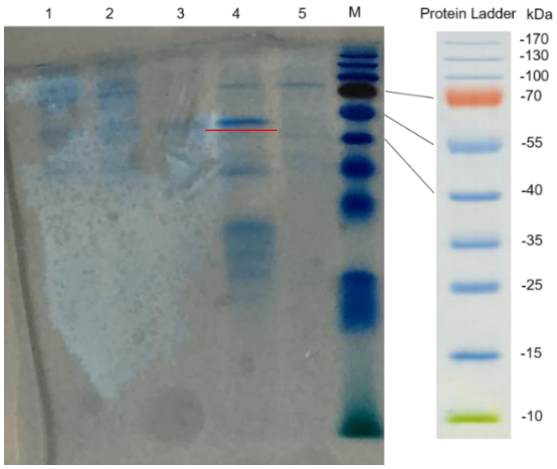

Fig.66 SDS-PAGE analysis secreting expression of recombinant *Pp*GH5. Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); lane 1-5, 10 µL culture supernatant of recombinant *P. pastoris* strains after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

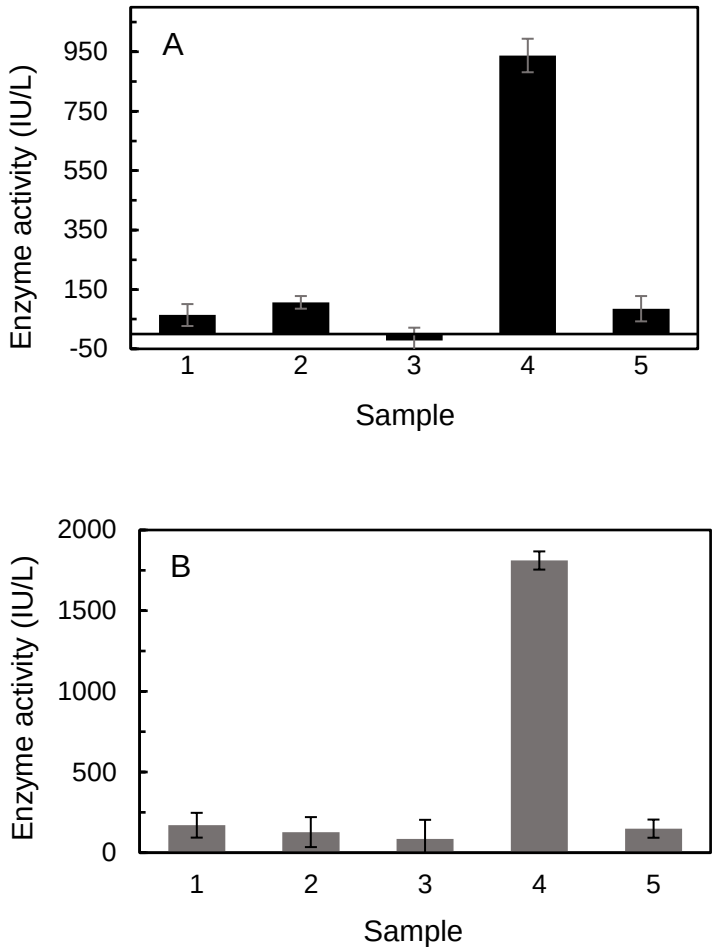

Fig.67 Enzyme activity assay from culture supernatants of selected transformants (*Pp*GH5). Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction in small scale using 24 square-well plate (section 2.4.4.1) were tested for exo-β-1,3-glucanase activity. 100 µL enzyme sample was mixed with 100 µL using alkali treated paramylon (A, 2.0 g/L, pH=6.5, indicated by black column) and laminarin (B, 2.0 g/L, pH=6.5, indicated by gray column), respectively, and incubated at 37 °C for 15 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

The exo-β-1,3-glucanase hydrolysate was measured by D-Glucose assay kit and converted to enzyme activity (section 2.4.5.3). After methanol induction in 24-well plate for 120 hours, *Pp*GH5 was secreted as main protein by recombinant strain No.4 (see Fig.66, lane 4) and generated high activity toward both laminarin and alkali treated paramylon. Although the total protein concentration of the crude enzyme was 0.05 g/L, the activity on alkali treated paramylon was 937.5 IU/L, and 1811.1 IU/L on laminarin. The relative activities were 18.8 IU/mg and 36.2 IU/mg respectively, which were significantly higher than recombinant *Tr*GH17 mentioned in section 2.3.6. The enzyme activity results as well as the SDS-PAGE proved that the exo-β-1,3-glucanase *Pp*GH5 was successfully overexpressed by recombinant *P. pastoris* GS115-*Pp*GH5. The distinctive activity was also showed in the shake flask cultivation.

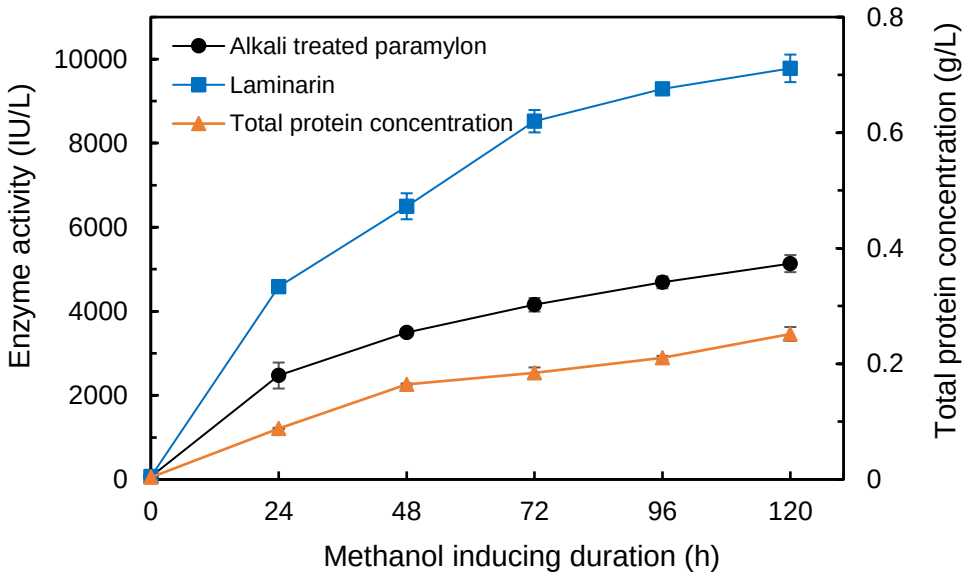

Fig.68 Recombinant *Pp*GH5 expression in *P. pastoris*. Supernatants of selected recombinant *P. pastoris* GS115 transformants after methanol induction using 24 shake flask (section 2.4.4.2) were used as crude enzymes for exo-β-1,3-glucanase activity assay. 100 µL enzyme sample was mixed with 100 µL laminarin (2.0 g/L, pH=6.5) and alkali treated paramylon (2.0 g/L,

pH=6.5), respectively, and incubated at 37 °C for 15 min. Substrate preparation and enzyme activity assay were described in section 2.4.5.

*Pp*GH5 was secreted as the main protein in the supernatant and contributed anomalous effective hydrolysis activity on both substrates. In general, the activity was over 5 times higher comparing to the data from multi-well plate cultivation, due to the more effective protein secretion in shake flask. When laminarin was used as substrate, activity of *Pp*GH5 increased dramatically to 8522.7 IU/L in the first 72 hours. The increasing behaved steady afterwards, and achieved the highest activity of 9779.8 IU/L at the time point of 120. The enzyme hydrolyzed alkali treated paramylon less effectively. After 24 hours inducing, activity on this recalcitrant substrate reached 2471.6 IU/L. Since then, the enhancement of enzyme activity achieved 5134.9 IU/L at 120 h in a steady pace.

However, the overexpression of this exo-β-1,3-glucanase from *P. pastoris* performed in this work was not the first implement. In 2006, the enzyme *Pp*GH5 (EXG1 in the literature) was cloned and expressed in *P. pastoris* X-33 and showed highest activity toward laminarin was 192 IU/mg (Xu et al. 2006). As unpurified enzyme was used in this work, the relative activity was about 39.1 IU/mg which revealed a great potential for improving. Despite the un-initiative expression, this work was the first time confirming *Pp*GH5's activity on alkali treated paramylon, which met the original purpose of searching paramylon degrading enzymes for future biorefinery research.

3.3 Cloning and expression of recombinant lytic polysaccharide monooxygenase in *P. pastoris*

Trail of the newly discovered LPMOs deconstructing paramylon granule were conducted in this part. Two LPMOs, *Tr*AA9 and from *T. reesei* and *Ao*AA11 from *A. oryzae* were cloned and expressed in *P. pastoris*. Moreover, their synergistic actions with β-1,3-glucanase were investigated by determining soluble products generated from paramylon granule.

3.3.1 Extracellular hydrolytic enzyme assay

Biomass of *T. reesei* and *A. oryzae* obtained from pre-cultivation was harvest and transferred to medium with 2.5 g/L paramylon granule as the only carbon source. These experiments were performed aiming at investigation whether these microbes could express paramylon hydrolytic enzymes when the substrate was only available as highly recalcitrant form. The supernatants were used as crude enzymes for activity assay using alkali treated paramylon and laminarin as substrates, respectively. The results were showed in the following figures (Fig.69 and Fig.70).

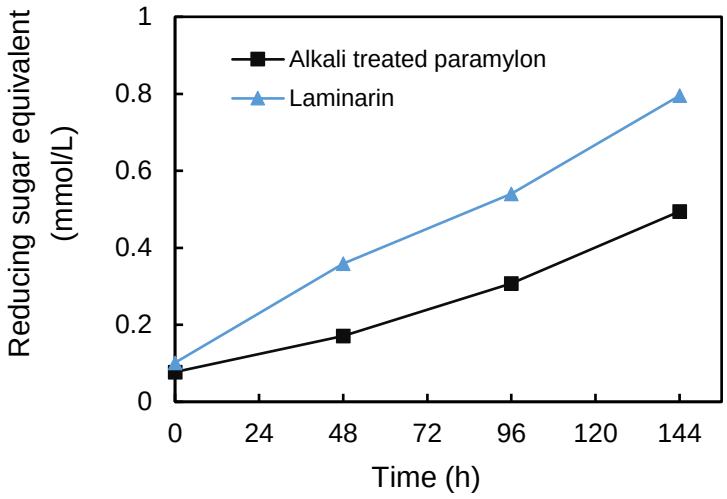

Fig.69 Extracellular hydrolytic enzyme assay of *T. reesei* induced by paramylon. During cultivation time, supernatants were taken as crude

enzyme solutions for activity assay (section 2.5.1). 100 µL enzyme solution was incubated in 1 mL of 1 g/L alkali treated paramylon (pH 6,5) and 1 mL of 1 g/L laminarin (pH 6,5) for 16 hours at 37 °C, respectively. The reducing sugar equivalents were measured by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

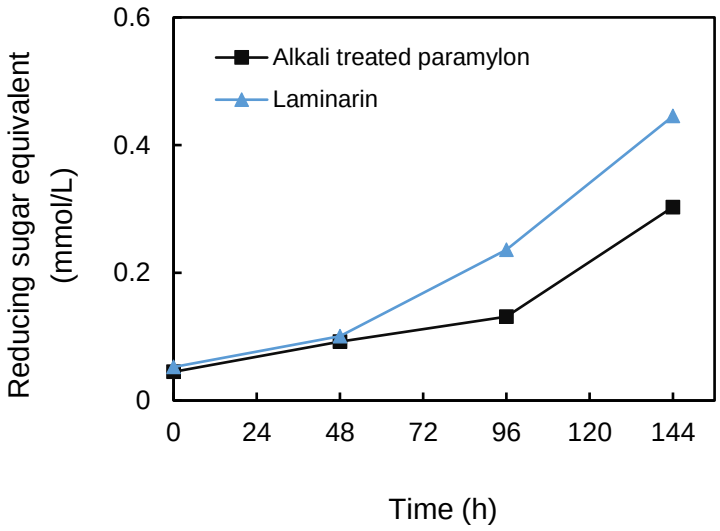

Fig.70 Extracellular hydrolytic enzyme assay of *A. oryzae* induced by paramylon. During cultivation time, supernatants were taken as crude enzyme solutions for activity assay (section 2.5.1). 100 µL enzyme solution was incubated in 1 mL of 1 g/L alkali treated paramylon (pH 6,5) and 1 mL of 1 g/L laminarin (pH 6,5) for 16 hours at 37 °C, respectively. The reducing sugar equivalents were measured by 3,5-dinitrosalicylic acid (DNS) method (section 2.3.5.1).

At the time point of 0 h, crude enzyme samples from *T. reesei* and *A. oryzas* both generated reducing sugars from laminarin and alkali treated paramylon as reducing sugar equivalent of around 0.1 mmol/L. These reducing products were probably glucose residual from the step of medium changing. During 144 hours of cultivation, paramylon granule induced both strains to secret hydrolytic

enzymes which could degrade β-1,3-glucan and generating increasing reducing groups. Enzymes from both *T. reesei* and *A. oryzae* supernatants had stronger activity on laminarin than alkali treated paramylon. After 144 hours cultivation, hydrolytic enzymes secreted by *T. reesei* generated reducing sugar equivalent of 0.8 mmol/L from laminarin in the assay, which was a significantly higher than 0.49 mmol/L from alkali treated paramylon. The results from *A. oryzae* crude enzyme assay showed the same trend. From laminarin and alkali treated paramylon, 0.45 and 0.30 mmol/L of reducing sugar equivalents were hydrolyzed, respectively. The cause of this behavior might be the higher solubility of laminarin than alkali treated paramylon, exposing more accessible hydrolysis sites for glycosidic hydrolyse enzymes. In addition, enzyme mixture produced by *T. reesei* seemed to have better hydrolytic ability on both substrates than enzymes from *A. oryzae*, which may indicate the former strain has better capacity for breaking barrier of recalcitrant substrate.

These results might conform to the hypothesis that some enzymes deconstructed the crystal form of paramylon providing free polysaccharide chain ends for glycoside hydrolyses, when the recalcitrant paramylon was the exclusive carbon source in the medium. Based on current knowledges, the key enzymes should be LPMOs (Vaaje et al. 2010), although no research confirms the existence of LPMO which act on β-1,3-glucan specifically has been reported unti now.

3.3.2 Cloning and expression of *Tr*AA9 (EGR52697.1)

The nucleotide and amino acid sequences of *Tr*AA9 (also names *Cel*61A; GenBank: EGR52697.1) are showing in Fig.22 with a nucleotide sequence of 1035 bp terminated by TAG stop codon, which corresponds to a translation product of 343 amino acids. Analysis of the amino acid sequence of this LPMO candidate showed that a N-terminal signal peptide existed which cleavage site lay between positions 21 (g) and 22 (h) (arrowed in black). The mature protein

contained one catalytic domain (form aa 22 (h) to 256 (n)) belonging to AA9 and one C-terminal carbohydrate binding domain (from aa 308 (a) to 343 (n)) with a linker (from aa 257 (y) to 307 (p), framed in blue) between.

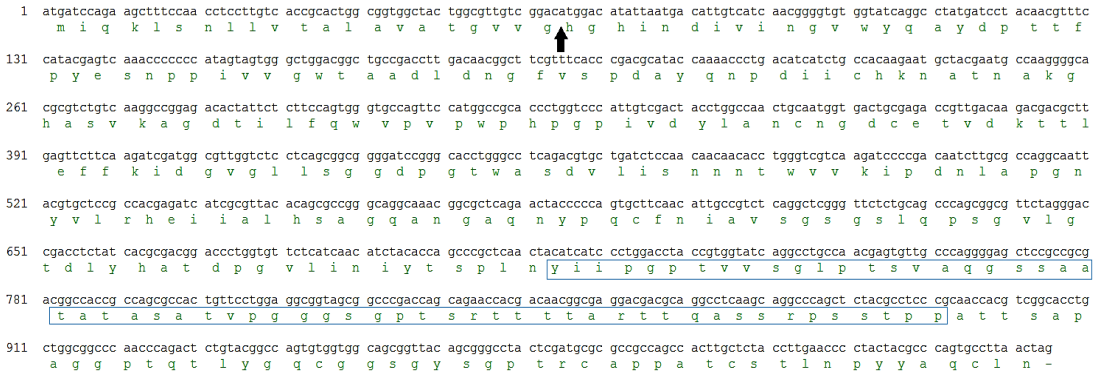

Fig.71 Nucleotide sequence and amino acid sequence of *Tr*AA9.

As described in section 2.5.2, the PCR based strategy was used for cloning this putative LPMO. The mature protein was expressed with a 6×histidine tag on the C-terminus in *P. pastoris*. As one intron exited in encoding region, the cDNA from *T.reesei* was used as PCR template. A PCR product of 1010 bp was obtained containing a 1002 nucleotide ORF terminated by a TAA stop codon, which encoding 333 amino acids of mature protein with a predicted molecular mass of 34.26 kD. As shown in Fig.72A, lane 1, the bright band about 1000 bp was the target product. Some faint bands smaller than 500 bp were unspecific PCR products having no influence to the following gel cutting. The target shown in Fig.72A, lane 1 was than cleaned up and inserted into pJET 1.2 cloning vector for plasmid construction.

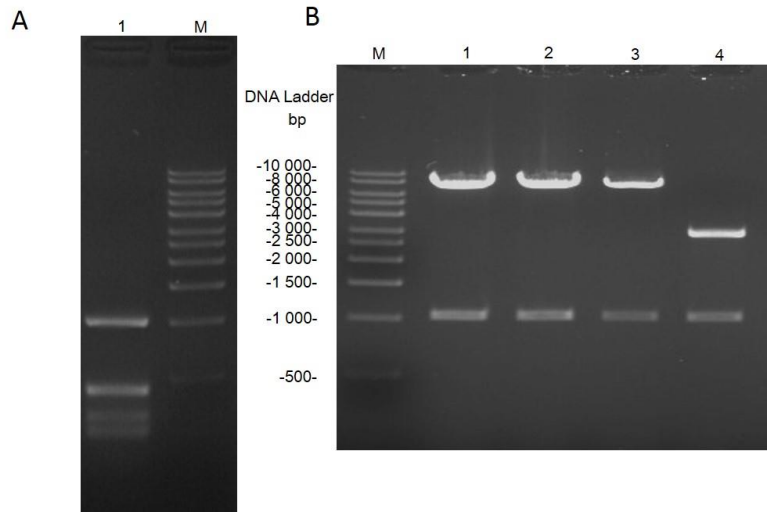

Fig.72 Analysis of *Tr*AA9 gene application and pAaHBgl-*Tr*AA9 construction.

A: Analysis of gene of *Tr*AA9 obtained from cDNA of *T. reesei* by PCR using specific primers. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1: Gene of *Tr*AA9 (EGR52697.1, 1010 bp);

B: Recombinant vector screening by digestion with restriction endonucleases. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1 to 4, results of four recombinant pAaHBgl-*Tr*AA9 plasmids digested by *Xho* I and *Not* I.

The gene was inserted into the vector pAaHBgl between sites *Xho* I and *Not* I. To screen the positive constructions, *Xho* I and *Not* I were used to digest the recombinant plasmids pAaHBgl-*Tr*AA9. Two fragments with the sizes of 7.5 kb and 1.2 kb should be produced when the gene of interest was inserted correctly. The result was showed in Fig.72B. Three plasmids analyzed were all rightly constructed, due to the two right length cleavage products (lane 1-3). The result showing in lane 4 indicated the cloning vector pJET 1.2 with target gene was transformed into *E. coli*. The 3 kb fragment had the size of linearized pJET 1.2 vector, and the shorter fragment shared the same size of target gene as other samples. The recombinant plasmid corresponding to Fig.72B, lane 1 was

selected to transform *P. pastoris* GS115 for protein expression. The selection and small scale cultivation were performed as described in section 2.4.4.1. After methanol induction for 120 hours, proteins in supernatants were analyzed by SDS-PAGE (Fig.73).

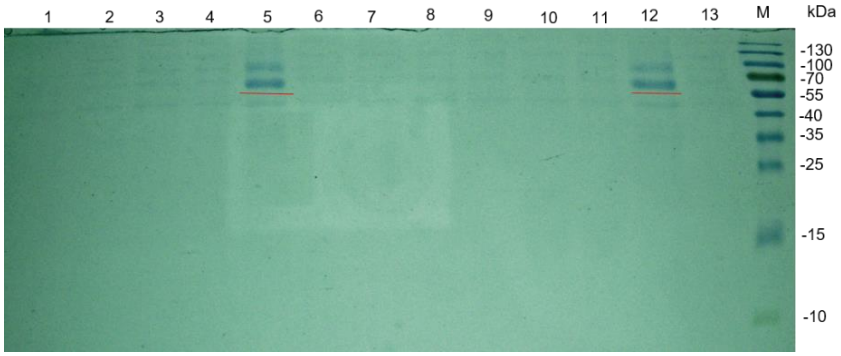

Fig.73 SDS-PAGE analysis secreting expression of recombinant *Tr*AA9.

Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); Lane 1-13, 15 µL culture supernatant of six recombinant *P.pastoris* GS115 strains after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

Lane 5 and 12 revealed positive results out of 13 assayed samples. The recombinant *Tr*AA9 protein was detected at a molecular mass of about 60 kDa, which was higher than its calculated mass of 35 kDa due to over glycosylation. This phenomenon of molecular mass increasing was also reported when the enzyme was expressed in *P. pastoris* system by other researches, and the activity on phosphoric acid swollen cellulose (PASC) was not influenced generating mainly C1-oxidized cello-oligosaccharides, small amount of C4- and double (C1- and C4-) oxidized products (Tanghe et al. 2015).

The result indicated a successful *Tr*AA9 expression in *P. pastoris* using alpha-mating factor from *S. cerevisiae* (α-MF) as secretion signal. The recombinant *P. pastoris* GS115, that expressed enzyme *Tr*AA9 corresponding

to lane 5, was selected for further research. Although the LPMO *Tr*AA9 was confirmed active on carboxymethylcellulose (CMC), barly-glucan, microcrystalline cellulose (Avicel) and phosphoric acid swollen cellulose (PASC) (Karlsson et al. 2001), there was still no clear evidence whether *Tr*AA9 could deconstruct highly crystallized paramylon. The first priority of this part of work is not to preempt heterogenous expression of this enzyme, but to investigate *Tr*AA9's activity on native paramylon by experimental evidence.

3.3.3 Cloning and expression of *Ao*AA11 (BAE61530.1)

The nucleotide and amino acid sequences of *Ao*AA11, which was the only characterized LPMO of the newly discovered AA11 family from *A. oryzae* namely *Ao*AA11 (GenBank: BAE61530.1) (Hemsworth et al. 2014a), were showed in Fig.74. The nucleotide sequence is 1266 bp long with TAA as stop codon, encoding a 421 amino acids translation product. In this work, the *Ao*AA11 mature protein of 220 residues (from aa 20 (h) to 235 (c) in Fig.74, framed in blue) was selected as the target domain for cloning and expression following the method in section 2.4.3 and 2.4.4.

The genomic DNA of *A. oryzae* was isolated as template for obtaining target gene by PCR using gene specific primers. A PCR product of 696 bp was obtained containing a 669 bp nucleotide ORF encoding the mature protein and a 6×histidine tag at the C-terminus. The predicted molecular mass of *Ao*AA11 was 23.88 kD. As shown in Fig.75A, lane 1, the only bright band represented the gene amplified from genomic DNA of *A. oryzae*. The band was cleaned up and inserted into pJET 1.2 cloning vector for expression vector construction.

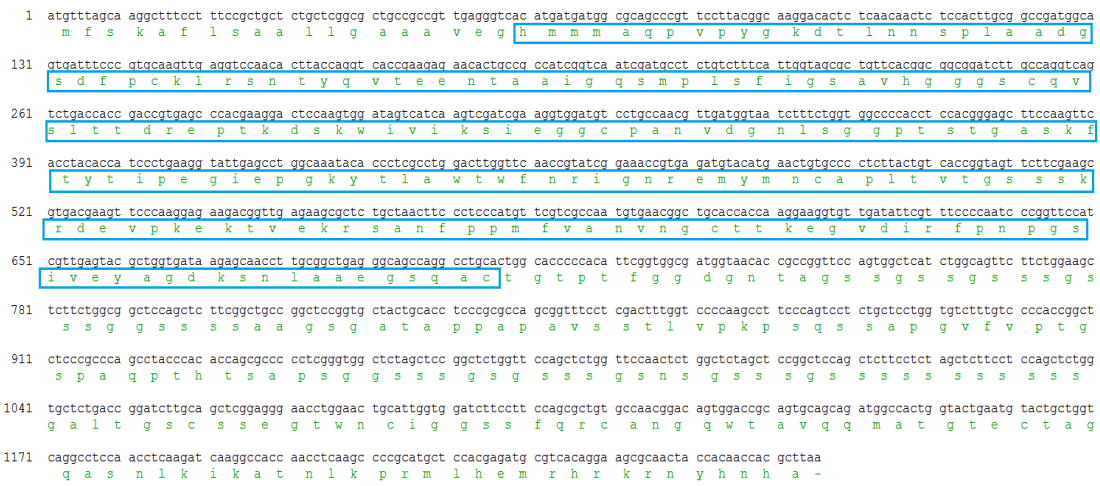

Fig.74 Nucleotide sequence and amino acid sequence of *Ao*AA11.

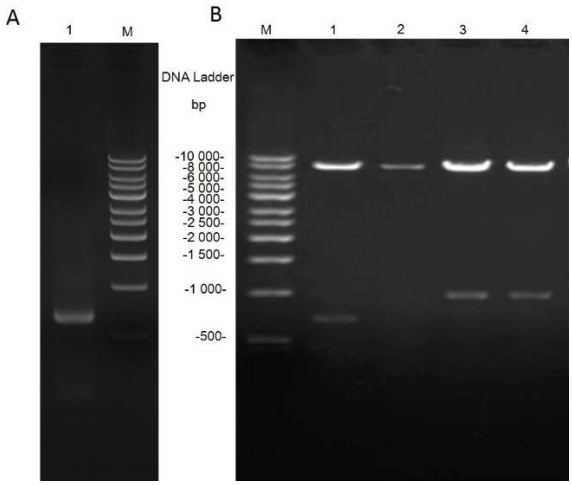

Fig.75 Analysis of *Ao*AA11 gene application and pAaHBgl-*Ao*AA11 construction.

A: Analysis of gene of *Ao*AA11 obtained from genomic DNA of *A. oryzae* by PCR using specific primers. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1: Gene of LPMO *Ao*AA11 (696 bp);

B: Recombinant vector screening by digestion with restriction endonucleases. M: 1 kb DNA ladder (Plasmid Factory, Germany); lane 1: Results of constructed pAaHBgl-*Ao*AA11 plasmids digested by *Xho* I and *Not* I.

In Fig.75B, only one plasmid (lane 1) was constructed correctly, confirmed by the length of cleavage products, which were 7.5 kb and 700 bp. In lane 3 and 4, these two plasmids were vector pAaHBgl. There was only one band in lane 2, which may due to low loading dosage causing an invisible short fragment. Therefore, the recombinant plasmid corresponding to Fig.75B, lane 1 was chosen for *P. pastoris* GS115 transformation. Selection and cultivation of recombinant *P. pastoris* were conducted as mentioned before. After 120 hours methanol induction, proteins in supernatants were analyzed by SDS-PAGE.

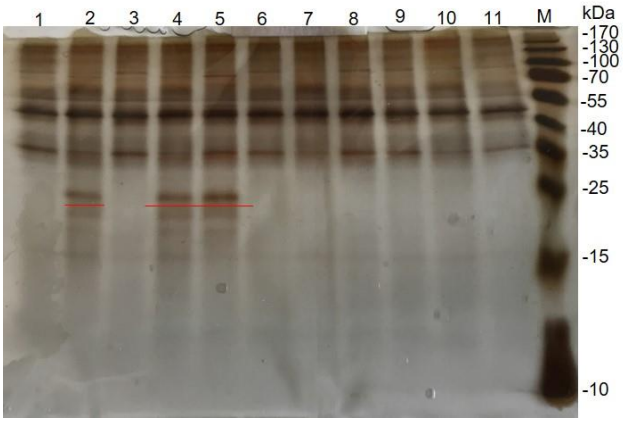

Fig.76 SDS-PAGE analysis secreting expression of recombinant *Ao*AA11.

Lane M, PageRuler Prestained protein ladder (Part No.26616, Thermo scientific, USA); Lane 1-11, 15 µL culture supernatant of six recombinant *P. pastoris* GS115 strains after methanol induction for 120 hours in small scale using 24 square-well plate (section 2.4.4.1).

By comparing proteins in supernatant from 11 transformers, recombinant *Ao*AA11 was successfully over-expressed by three of them (Lane 2, 4 and 5), as a soluble protein. And molecular mass of the target protein was about 24 kDa revealing by the SDS-PAGE, which met the predicted 23.88 kDa. As reported, *Ao*AA11 was cloned and expressed via periplasmic secretion in *E.*

coli (Hemsworth et al. 2014b). The *E. coli* expressed *Ao*AA11 was active on chitin, generating aldonic acid oligosaccharides with even-numbered degrees of polymerization by oxidation at C1, but no activity on other substrates, such as cellulose and starch. The following work further confirmed this conclusion.

3.3.4 Synergistic Action of LPMO and β-1,3-Glucanase

LPMOs had a major significance in biomass conversion, due to the ability to boost the activity of classical glucoside hydrolyses. In nature, the most known renewable material of the second-generation bio-ethanol, cellulose, was degraded by the cooperation of enzymes, including various hydrolytic enzymes in different glycoside hydrolase families: endo-glucanase, which created free chain ends (Ljungdahl 2008); cellubiohydrolase cleaving cellobiose from free chain ends of substrate (Sanchez 2009), and β-glucosidase which produced glucose by hydrolyzing cellobiose (Pothiraj et al. 2006). In addition to these major glycoside hydrolases, the non-hydrolytic auxiliary enzyme, lytic polysaccharide monooxygenase, was confirmed as a significant member to improve the substrate conversion to biorefinery products (Courtade et al. 2016). Mechanism of this boosting effect was assumed as introduction of new chain breaks in crystalline substrate, providing free substrates for hydrolytic enzymes and enhancing biomass degradation (Dimarogona et al. 2013). Meanwhile, this interesting finding offered profound curiosity, whether *Tr*AA9 or *Ao*AA11 could improve recalcitrant paramylon deconstruction when incorporate with β-1,3-glucanases? In this work, trials of synergistic action between LPMO and β-1,3-glucanase were performed. Commercial enzyme (laminarinase), endo- and exo-β-1,3-glucanase expressed in this work were synergistically acted on paramylon granules with *Tr*AA9 or *Ao*AA11 respectively for 72 hours, followed by oligosaccharides analysis.

The qualitative analysis of soluble products generated by LPMOs were commonly performed by two methods. The first method used was matrix

assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) which detects oligosaccharides in different degree of polymerization (Vaaje Kolstad et al. 2010). And the other way was high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) (Forsberg et al. 2011). In this method, the compatibility of electrochemical detection with gradient elution, coupled with the high selectivity of the anion-exchange stationary phases, allowed mixtures of mono-, oligo-and polysaccharides to be separated with high resolution.

In this part of work, a convenient and sensitive colorimetric method, phenol-sulfuric acid method, was applied to determine the oligosaccharides generated by synergistic action of LPMOs and β-1,3-glucanases from insoluble paramylon granule. The concentrated sulfuric acid broke glycosidic bond of polysaccharides, oligosaccharides and disaccharides to monomers, then dehydrated the product to hydroxymethylfurfural (HMF). HMF reacted with phenol generating a yellow-gold colored product which could be measured at 490 nm (DuBois et al. 1956). A microtiter plate format of the method had been developed for pure sugar, oligosaccharide or polysaccharide solutions in a sensitive linear range of substrate (1-150 nmol) (Masuko et al. 2005). However, in enzymatic reaction mixture, the protein concentration may significantly affect the absorption measurements (Agbenorhevi and Kontogiorgos 2010). The various amounts of ammonium sulphate, sulphur and carbondioxide produced by adding sulphuric acid to proteins would interfere with the overall chemistry of the reactions.

Taking this aspect into consideration, dialysis membranes and vivaspins were used for reducing the impact of proteins. Small proteins were removed from the enzyme samples by dialysis using a tubular membrane. Samples taken at incubation time points of 0 and 72 h were centrifuged using ultrafiltration spin, which only allowed potentially generated oligosaccharides

with low polymerize degree to pass the membrane. The collected filtrate was analyzed then by phenol-sulfuric acid method. For confirming the ability of *Tr*AA9 and *Ao*AA11 to improve the activity of β-1,3-glucanase on paramylon granule, laminarinase (100 mg/L) collaborated with *Tr*AA9 and *Ao*AA11 respectively. 100 mg/L BSA was mixed with laminarinase as negative control. Paramylon granule was added to all enzyme combinations to a final concentration of 1.0 g/L. The detailed procedure was described in section 2.5.3.

Revealing by glucose equivalent, the carbohydrate content produced by combinations of laminarinase with BSA and *Tr*AA9 both increased. As a negative control, BSA was incapable of boosting the activity of laminarinase. The unexpected increasing of glucose equivalent by 47.6 % may due to protein denaturation after incubation at 37 °C for 72 hours, causing impurities in the filtrate and affecting the assay. The result of enzyme combination of laminarinase and *Ao*AA11 even showed a decreasing of 19.0 % in carbohydrate assay, which further demonstrated that the measurement could be easily affected and negating *Ao*AA11's oxidative ability on crystalline paramylon.

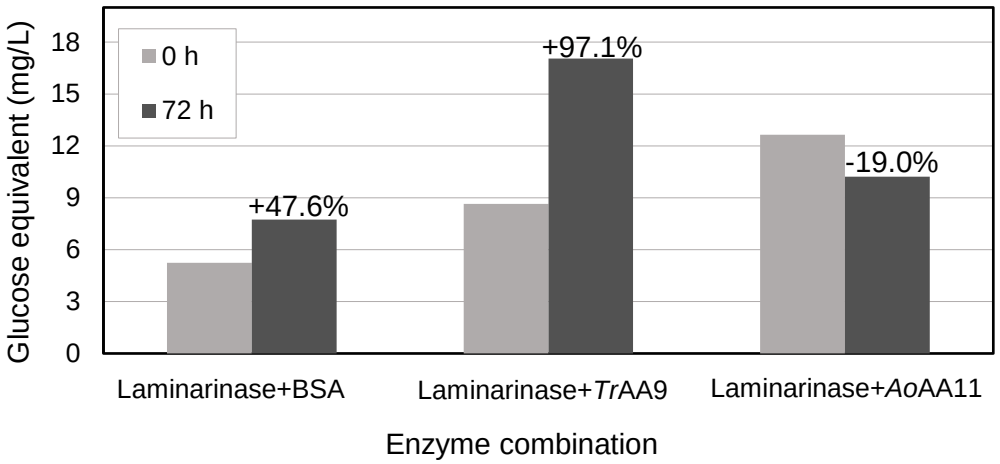

Fig.77 Synergistic action of laminarinase and LPMOs. Experimental conditions were described in section 2.5.3.

Besides that, cooperation of laminarinase and *Tr*AA9 generated a significant carbohydrate accumulation. The ratio of glucose equivalents increased up to 97.1% to 17 mg/L after 72 h synergistic action between LPMO and β-1,3-glucanase, which was most likely a confirming of *Tr*AA9's activity towards paramylon granule. Considering that the measurement of glucose equivalent might be inaccurate, the result from the enzymatic reaction was not sufficient to draw the conclusion, that *Tr*AA9 actively stimulated the hydrolytic reaction of laminarinase. But the trend of sharp increasing could be regarded as a sign to reconsider the substrate specificity of *Tr*AA9.

The facilitating ability of *Tr*AA9 boosting oligosaccharide accumulation from paramylon granule when acting with recombinant endo- and exo-β-1,3-glucanase were also investigated.

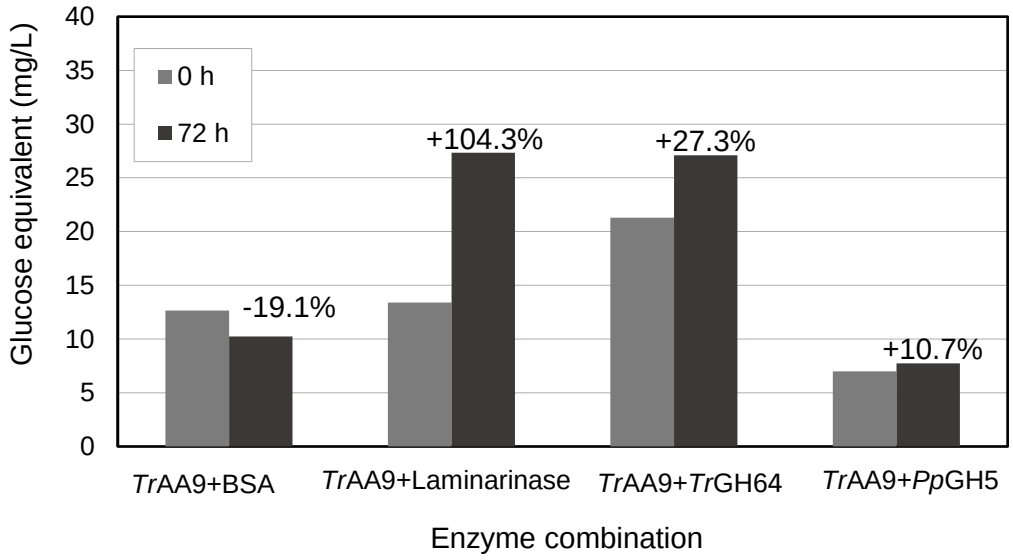

Fig.78 Synergistic action of *Tr*AA9 and recombinant β-1,3-glucanases. Experimental conditions were described in section 2.5.3.

As showing in Fig.78, combination of *Tr*AA9 (LPMO) and *Tr*GH64 (endo-β-1,3-glucanase) generated a 27.3% ratio of glucose equivalent increasement. Meanwhile, combination of *Tr*AA9 (LPMO) and *Pp*GH5 (exo-β-1,3-glucanase)

also slightly enhanced paramylon deconstruction, by additional 10.7 % glucose equivalent after synergistic action for 72 hours. However, it was suspectable that these proportions were approaching the error range. Combination of *Tr*AA9 and BSA acted as negative control, resulting a glucose equivalent decreasing of 19.1%. Nevertheless, as a positive control, combination of *Tr*AA9 and laminarinase generated the highest conversion, resulting a 104.3 % increasement of glucose equivalent. It was consistent with the result in Fig.77, and provoked a keen curiosity over the substrate specificity of LPMOs.

*Tr*AA9 belonged to a cellulose degrading LPMO family AA9, while *Ao*AA11 was a chitin degrading LPMO family AA11 member. The substrate specificity of different families was believed maintaining unity, until researchers discovered that some AA10 members could also degrade cellulose (Forsberg et al. 2011). Actually, the mechanism of LPMOs' substrate specificity remained unconfirmed. Many LPMOs interacted with crystalline substrates at relatively flat surfaces. AA9 enzymes achieved this binding through interactions with aromatic residues in the CBM-like motif (Harris et al. 2010a). For AA10 enzymes, the mechanism of substrate binding was revealed through polar interactions with hydrophilic residues (Aachmann et al. 2012). Since then, researches generally agreed that the specificity of LPMOs were defined by the ability of enzymes to bind substrates. In 2014, structures of cellulose-degrading AA10s were published, revealing a clear configuration difference in active sites, as *Sc*AA10_C and *Sc*AA10_B resembled active site structure of AA10 and AA9, respectively (Forsberg et al. 2014b). Furthermore, the cellulose-active *Sc*AA10 (Book et al. 2014) was found binding chitin without degrading function (Forsberg et al. 2014a). Based on this, it was proposed that enzyme functionality of LPMOs and substrate binding affinity were hardly correlated, but that the substrate specificity depended on active center configuration. More interestingly, EPR spectrum of *Ao*AA11 appeared to resemble a group of cellulose-active LPMOs, but had experimentally measured chitinolytic active

(Hemsworth et al. 2014b). Up to date, the substrate specificity of LPMOs were mainly based on experimental evidences without generally confirmed criteria, leaving a challenge of further structural and mutagenesis studies.

After being neglected for decades, LPMO, the oxidative enzyme that degrade recalcitrant polysaccharides became supreme since its “discovery” in 2010. The abilities of LPMOs to enhance the biomass conversion and lower the cost of deconstruction enzyme cocktails made it a milestone in biorefinery field by deconstructing recalcitrant materials, such as cellulose, hemicellulose, chitin, and lignin without power consuming physical pretreatments, and synergistically acting with hydrolases. The combination of LPMO and cellulase resulted in improvement of glucose yields between two- to eight-fold from cellulose (Langston et al. 2011). The study of LPMOs continued to progress rapidly, and kept going deeper in biology and application. But up to date, there was still no LPMO being confirmed having a substrate specificity for β-1,3-glucans, such as paramylon. Based on the desire of utilize crystalline paramylon as raw material for biorefinery production, the challenge is not whether LPMOs should be used in paramylon conversion, but rather which one can deconstruct the substrate? Although the results from this work were hardly reliable to answer this problem, the basic knowledge and recombinant LPMO expression lay the foundation for further research in this field.

4. Conclusion and Outlook

As the first part of this work, investigation and partial purification of *E. gracilis* extracellular proteins were conducted. Extract prepared from *E. gracilis* cultivation in late stationary phase rapidly hydrolyzes alkali treated or 2× lyophilized paramylon. The 50× concentrated extract showed the highest hydrolase activity when incubated at pH 5.5 and 37 °C for 16 hours. After partial purification for enzymes responsible for paramylon degradation, one protein potentially belonging to glycoside hydrolase family 22 was found. The original mRNA template was isolated for cDNA library synthesis, and roled as template in degenerate PCR. However, the full amino acid or gene sequence of the target protein are yet to obtain. Paramylon as a storage polysaccharide synthesized by *E. gracilis* is an important renewable raw material for bioenergy producing in the future. Although structural changes of paramylon caused by alkali treatment of native granule increased the hydrolyze activity of cell free extract, no reaction product was obtained from untreated paramylon. So far, however, an exo- and one endo-β-1,3-glucanase were reported (Barras and Stone 1969; Takeda et al. 2015), variety and property of enzymes in the cell free extract responsible of paramylon degradation still need further research. Whatsmore, no heterogeneous enzyme has been reported capable of hydrolyzing paramylon yet, thus leaves numerous choices of paramylon depolymerizer from biomass conversion microbes, such as *T. reeei*, *Bacillus subtilis, etc.*.

The bioconversion of paramylon into simple fermentable sugars requires synergistic interaction of at least two types of glycoside hydrolases: endo- and exo-β-1,3-glucanase. Functionally, the endo-β-1,3-glucanase disrupts the substrate at random internal sites in the polysaccharide, generating chain ends for exo-β-1,3-glucanase. In second part of this work, six β-1,3-glucanases

including four endo-β-1,3-glucanases and two exo-β-1,3-glucanases were selected. The endo-β-1,3-glucanases are from GH family 16, 55, 64, and 81, and the exo-β-1,3-glucanases are from GH family 5 and 17. These enzymes were successfully cloned and expressed in *P. pastoris*, and confirmed their activities on laminarin, and more importantly, the alkali alkali treated paramylon. Aside from the exo-β-1,3-glucanase *Pp*GH5, all enzymes were expressed in *P. pastoris* for the first time.

P.pastoris was reported having many advantages over other expression systems, including simple molecular manipulation, promising expression, fast growth, ability for posttranslational modifications, and docility for large scale fermentation (Li et al. 2007). Recombinant protein expression in *P. pastoris* can be improved using various strategies, as protein expression is influenced by multiple parameters, such as divergence of codon usage, cultivation conditions including temperature, pH, composition of the culture medium, inducer concentration, and inducing duration. Codon usage optimization was believed as a main factor affecting the successful expression of foreign proteins in different hosts (Saravana Perumal et al. 2016). Codon optimization process involves retranslating the nucleotide sequence of the wild type gene by replacing codons having low frequencies in the expression host with more frequently used ones. Rare codons decrease mRNA stability and translation rate, while high G+C contents could cause reduced translation rate or failed expression (Sinclair and Choy 2002). In *P. pastoris*, codon optimization technique has been widely used (Akbarzadeh et al. 2014), resulting in significant improvement of enzyme expression in *P. pastoris* (Sun et al. 2016). For further work, strategy of gene condon optimized could be conducted primarily to improve recombinant protein productivity, as in this work all enzymes from *T.reesei* were expressed using wild type genes. Besides this, cultivation parameters could be optimized by designing experiments using response surface methodology. The methodology has been widely used for

studying the interactions of several parameters during biotechnological processes (Muntari et al. 2012). Recombinant enzymes expressed in this work were studied preliminarily using supernatant as crude enzymes. With integrated hexa-histidine tag, purifications could be conducted easily for characterizations. For instance, the effects of temperature, pH, substrate concentration, metal ions and inhibitors on enzyme activity.

In this work, endo- and exo-β-1,3-glucanases were expressed and confirmed their activities on alkali treated paramylon. However, glycoside hydrolases can hardly act on native paramylon granule. As the last part of this work, two lytic polysaccharide monooxygenases from *T. reesei* and *A. oryzae* were expressed in *P. pastoris*. The investigation of synergistic action between LPMOs and glycoside hydrolases toward native paramylon were performed. Firstly, inductions of *T. reesei* and *A. oryzae* were performed using paramylon granule as carbon source. The increasing hydrolytic activities on both laminarin and alkali treated paramylon along the cultivating duration indicated that these two microbes expressed enzymes, which were able to deconstruct paramylon. From both strains, total glucanase activity toward laminarin were significantly higher than acting on alkali treated paramylon due to the difference of substrate solubility. And on both substrates, enzymes secreted by *T. reesei* demonstrated better hydrolytic ability than *A. oryzae*. Laminarin was easier to access, resulting in reducing sugar equivalent of 0.8 mmol/L for *T. reesei* enzymes and 0.45 mmol/L for *A. oryzae* enzymes. While using alkali treated paramylon as enzyme substrate, the result were 0.49 mmol/L for *T. reesei* enzymes and 0.3 mmol/L for *A. oryzae* enzymes. In the secreted enzyme cocktail, LPMO is supposed to be the key role which breaks the barrier of paramylon's highly-crystallization. For further confirming the hypothesis, two LPMOs, *Tr*AA9 and from *T. reesei* and *Ao*AA11 from *A. oryzae* were cloned and expressed in *P. pastoris*. Results from SDS-PAGE revealed that recombinant *Tr*AA9 and *Ao*AA11 were both successfully expressed in *P. pastoris*. In the experiment of

synergistic action of LPMOs and β-1,3-glucanases, *Tr*AA9 acted as improving the activity of laminarinase and recombinant β-1,3-glucanases. Particularly, the combination of *Tr*AA9 and laminarinase yielded an increasing of about 100.0% glucose equivalent after incubation for 72 hours. On the contrary, the result of *Ao*AA11 acting on paramylon granule with laminarinase revealed a decreasing trend. This result confirmed *Ao*AA11's chitin specificity, and on the other hand, indicated the unreliability of phenol sulfuric acid method. In future, accurate analysis method such as MALDI-TOF MS and HPAEC-PAD should be involved for confirming *Tr*AA9's enhancing function to β-1,3-glucanases. Additionally, enzymes are definitely expressed in *E. gracilis* to deconstruct paramylon for autotrophic nutrition. The intracellular proteins could be resources for screening such enzymes. Along with the studied LPMO, for instance *Tr*AA9 in this work, these enzymes need further research of deconstructing abilities on native paramylon granule in order to achieve the goal of low-cost and efficient degradation.

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Appendices

Solutions and mediums

Tris-acetate-EDTA (TAE) stock solution (50×)	
Component	Concentration
Tris base	242.0 g/L
Glacial acetate acid	5.71% (v/v)
EDTA	9.3 g/L

Tris-glycine stock solution (5×)	
Component	Concentration
Tris base	15.1 g/L
Glycine	94.0 g/L
SDS	50.0 g/L

TSS stock solution (2×)	
Component	Concentration
PEG 8000	100 g/L
$MgCl_2 \times 6H_2O$	6 g/L
DMSO	1% (v/v)

Coomassie brilliant blue G-250 staining buffer

Component	Concentration (1L)
Methanol	450 mL
Glacial acetic acid	100 mL
Coomassie brilliant blue G-250	3.0 g
Deionized H_2O	450 mL

Silver staining fixer

Component	Concentration (1L)
Ethanol	400 mL
Acetic acid	100 mL
Deionized H_2O	500 mL

Silver staining sensitizing solution

Component	Concentration (1L)
$Na_2S_2O_3$	0.2 g
Deionized H_2O	1 L

Silver nitrate solution

Component	Concentration (1L)
$AgNO_3$	1 g
Deionized H_2O	1 L
35% Formaldehyde	200 µL add before use

Silver staining visualizing solution

Component	Concentration (1L)
Na_2CO_3	30 g
Deionized H_2O	1 L
35% Formaldehyde	500 µL add before use

Silver staining stop solution

Component	Concentration (1L)
Acetic acid	50 mL
Deionized H_2O	950 mL

LB medium

Component	Concentration (1L)
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Deionized H_2O	Add to 1 L

pH=7.4

YPD medium

Component	Concentration (1L)
Peptone	20 g
Yeast extract	10 g
400 g/L glucose (sterile by 0.22 μm filter)	50 mL
Deionized H_2O	Add to 1 L

pH=6.5

BMGY and BMMY medium

Component	Concentration (1L)
Peptone	20 g
Yeast extract	10 g
1.0 mol/L Potassium phosphate buffer (pH 6.0)	50 mL
10× Yeast nitrogen base (YNB)	100 mL
500× Biotin	2 mL
10× Glycerol	100 mL for BMGY
10× Methanol	100 mL for BMMY
Deionized H_2O	Add to 1 L

pH=6.0

Declaration

I hereby declare that this thesis is a presentation of my original research work. Wherever contributions of other are involved, every effort is made to indicate this clearly, with due reference to the literature. Care has been taken to give credit to all sources that have given information in the form of images, discussions or materials.

Bielefeld, …… ……...

Yingfei Shi