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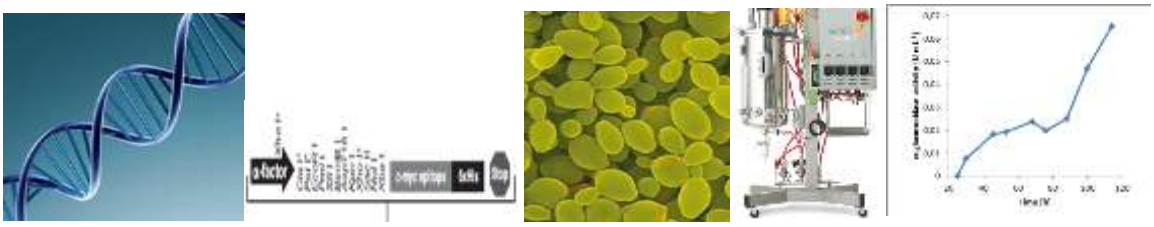
Recombinant *Schizophyllum commune* α-glucuronidase expression and production in the methylotrophic yeast *Pichia pastoris*



Master of Science Thesis

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Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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THESIS FOR THE DEGREE OF MASTER OF SCIENCE in BIOTECNOLOGY

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Cover illustration: Schematic representation of the project from gene optimization, expression cassette construction and fermentation.

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To my family……

‘‘There can be no progress without head-on confrontation’’

(Christopher Hitchens)

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Abstract

The α-glucuronidase of *Schizophyllum commune* was expressed heterologously in *Pichia pastoris*. The α-glucuronidase of the wood degrading fungus *S. commune* belongs to glycoside hydrolase (GH) family 115 and its extracellular activity release D-glucuronic and 4-O-methyl-D-glucuronic acid residues from polymeric xylan backbone and aldouronic acids. The recombinant *P. pastoris* strain was grown in repressive BMGY medium that supported biomass generation and induced in BMMY medium for extracellular secretion of the recombinant α-glucuronidase. The activity of the secreted enzyme was assayed using α-glucuronidase measurement kit on a mixture of tri:tetra:penta aldouronic acid substrates. The glicosidic hydrolysis activity of the enzyme was assayed at pH 6.0 and 37℃ and reached 1.08 nkat mL^{-1}. Also, the enzyme was stable for 18 h at 4℃. The fed-batch fermentation of the recombinant of *P. pastoris* resulted in production of 0.723 mg of the crude recombinant enzyme per liter of the fermentation culture. The engineered *P. pastoris* expression system produced in this project can be used for development and optimization of a cost effective cell line for production of high yield of correctly folded and functional α-glucuronidase.

Key words:
α-glucuronidase, polymeric xylan, glucuronic acid, enzymatic debranching, glycosidic bond, GH115 family, *Agu1* gene, AOX promoter, fermentation.

1. Table of Contents

1. Introduction

Lignocellulosic biomass is of great interest for production of novel materials. Lignocellulosic raw materials possess two prominent properties; renewability and biodegradability that make them desirable over fossil resources, which are not guaranteed resources, for long term utilization. A major portion of fossil resources is used for production of synthetic polymers specially packaging materials. Massive consumption of the plastic packaging materials contributes to waste accumulation in the environment, thus alternative raw materials should be biodegradable as a desired property of novel packaging material. Apparently, renewable lignocellulosic feedstock is a promising alternative resource for production of novel materials with engineered properties and less environmental impacts due to their inherent biodegradability property (Gatenholm, et al., 2004).

The major components of the plant cell wall are cellulose, hemicelluloses, and lignin which altogether form 70% of the biomass (Brink & Vries, 2011)**.** Xylan is the most frequent hemicellulose among hardwood species, and after cellulose it is the most second copious polysaccharide available in nature (Saxenaa, et al., 2011). While cellulose is a homopolysaccharide formed by β (1-4) glycosidic bonds between glucose units, hemicelluloses are categorized into subgroups, such as xylan, xyloglucan, glucuronoxylan, arabinoxylan, mannan and glucomannan, based on the sugars existing in the backbone of the polysaccharide (Brink & Vries, 2011). Process bottlenecks for utilization of xylose (monomer unit of xylan) as a fermentable sugar for biofuel applications (Olsson & Hahn-Hägerdal, 1993) are important factors that make xylan available as a precursor biomaterial.

Previous studies have illustrated that the mechanical properties of xylan are adversely affected by moisture (Dammström, et al., 2005) due to the glucuronic acid substituents present on the backbone of xylan (Viikari, et al., 1994). There is a direct correlation between mechanical properties of the xylan film and the amount of glucuronic acid residues attached to the backbone of xylan that can adversely affect the water transmission rate, which is an important property for many packaging film applications (Saxenaa, et al., 2011). Gatenholm, et al. (2004), demonstrated that oxygen permeability of glucuronoxylan film is reduced by increasing solubility.

The challenge of water absorption, which is caused by glucuronic side groups, could be overcome by removing them from the polymeric xylan backbone. Although glucuronic side groups of xylan could be decreased in the alkaline process in kraft pulping (Viikari, et al., 1994), but during the cooking xylan chains are partly degraded and short chains of xylan form, which is not desirable. The idea of releasing glucuronic acid residues and producing intact xylan chains motivated this project for the development of environmentally benign processes, as well as new products from forest industry. One way to overcome this challenge is through the enzymatic cleavage of the glucuronic acid residues off the polymeric xylan backbone. Tenkanen & Siika-aho (2000) found that α-glucuronidase from *Schizophyllum commune* is capable of debranching glucuronic acid residues of polymeric glucuronoxylan of softwood.

In the present work we want to heterologously express *Agu1* gene, which encodes α-glucuronidase from *S. commune*, in methylotrophic yeast *P. pastoris*. This α-glucuronidase is active on polymeric glucuronoxylan. Initially we will construct the expression vector for the transformation of the *P. pastoris* cells. Then the potential transformants will be screened for the expression levels of active α-glucuronidase. Finally, the fermentation of the selected *P. pastoris* transformant will be scaled up to produce high enzyme titers.

2. Background

2.1 Hemicellulose

Hemicelluloses are branched heteropolysaccharides containing hexoses (D-glucose, D-mannose, D-galactose) and pentoses (D-xylose, and L-arabinose) on their backbone. The backbone of hemicelluloses is usually decorated with D-galactose, D-xylose, L-arabinose and D-glucuronic acid side groups (Brink & Vries, 2011). Hemicelluloses (like cellulose) are called glycans due to O-glycosidic linkages of their monomers. The dominant glycan in the primary cell wall of the higher plants is xyloglucan which consists of covalently β-1,4-linked D-glucose units with D-xylose residues glycosidically bonded to the O-6 position of the glucose monomer. The secondary cell wall, which prevails in the lignocellulosic biomass, differs depending on the origin of plant. The secondary cell wall of hardwood contains glucuronoxylan, whereas arabinoxylan is dominating in cereal plants. Despite hardwood and cereal plants, the major hemicellulosic component in softwood is galactoglucomannans (Buchanan, et al., 2000; De Vries & Visser, 2001). The main monosaccharide in the backbone of the D-xylan is β-D-xylopyranosyl which are covalently (1→4)-linked together. Depending on the source of xylan, some of the monomeric units carry (1→3)-linked α-L-arabinofuranosyl, and (1→2)-linked 4-O-methyl-α-D-glucopyranosyl uranic acid (glucuronic acid) substituents which are glycosidically attached to the backbone of the polymeric xylan. Furthermore, feruloyl, acetyl and p-coumaroyl residues are the other categories of sugar moieties bonded with the D-xylan backbone via ester linkage (Castanare, et al., 1995). The composition of hemicelluloses occurring in the plant cell wall varies significantly between different plant species. For example, a comparison between hard wood and cereal xylans shows that D-glucuronic acid residues are the dominating branches linked to xylan's backbone in hardwood, while in cereal xylans the major side group on the polymer backbone is L-arabinose residue (Brink & Vries, 2011).

2.2 Application of Xylan films

Synthetic plastic materials are, nowadays, manufactured from fossil resources. Among all applications for such materials, the majority (more than 40%) of plastics are consumed for packaging purposes. Short shelf life of the packaging plastics and long post consumption residence of these materials in the environment contribute to drastic growth of landfills and rising of greenhouse gases after burning for the extraction of their thermal content. As mentioned before glucuronoxylan is one of the major components of wood and also available in great amounts in agriculture waste. Statistics revealed that, the annual biosynthesis of

hemicellulose is 60 billion tons. Not only commercial food application of glucuronoxylan are limited (e.g. food emulsifier and sweetening agent) (Gatenholm, et al., 2004), but also its non-food applications are very restricted such as, adhesive and thickener to plastics, pulp and paper industry etc. (Dasilva, et al., 2012). Availability and biodegradability of glucuronoxylan make it an alternative candidate biomaterial for packaging applications (Gatenholm, et al., 2004).

Materials for food packaging applications need the right mechanical and chemical properties so that the quality of the food does not change over the storage period. Strong oxygen barrier properties are obligatory for packaging materials to prohibit oxygen from damaging the food. Another important property these materials should possess is water resistance. Furthermore such materials should not release poisonous components into the food. A desirable property for packaging materials is film forming ability, and glucuronoxylan film could be casted from aqueous solution (Gatenholm, et al., 2004). In addition to the availability of glucuronoxylan, its good mechanical properties also contribute to make it an alternative packaging material. With regards to all the advantages of xylan, it has drawbacks to be argued here. Glucuronoxylan is an amorphous polymer in its natural state, even though its structure can accommodate water, it is not water soluble. Side groups substituted on the backbone of xylan such as glucuronic acid as well as free spaces in the molecular lattice of glucuronoxylan are responsible for water absorption. The consequence of an increased content of water in glucuronoxylan film is higher oxygen permeability (Gatenholm, et al., 2004) which is not a desirable property.

2.3 Structural modification of xylan

The term structural modification refers to removal of the substituted side groups on the backbone of glucuronoxylan. This modification can take place chemically as well as biologically. Debranching of xylan could improve the mechanical film properties by reducing water absorption and oxygen permeability.

2.3.1 Chemical modification

Since xylan has been proposed recently as an alternative precursor biomaterial for food packaging (Gatenholm, et al., 2004; Saxenaa et al., 2010), there was no known industrial process to modify the structure of glucuronoxylan itself for film production. So far, the only possible chemical processes for modification of glucuronoxylan are the kraft pulping and the isolation of hemicellulose from intact wood in which both processes use high concentrations

of alkali. In the kraft pulping process the acetyl side groups are totally cleaved and the amount of glucuronic acids is reduced but the number of arabinose substitutes is not reduced significantly. The reduced number of the side groups on xylan varies after the isolation, depending on the alkaline conditions used. In addition to cleavage of side groups on the backbone of xylan, the above chemical processes reduce the length of xylan polymeric chain (degree of polymerization) that is not desirable (Viikari, et al., 1994)

2.3.2 Enzymatic modification

So far, enzymatic treatment of lignocellulosic biomass has been applied in industry to degrade the polysaccharides into their basic sugars (Brink & Vries, 2011). However, accessory enzymes perform hydrolysis of the side residues on polysaccharides. Accessory enzymes that are active on xylan (fig 2.3.2) are α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase and feruloyl esterase etc (Heneghan, et al., 2007). Among this group of enzymes α-glucuronidase can hydrolyze the bond between glucuronic acid side groups and polymeric xylan (Tenkanen & Siika-aho, 2000). Tenkanen & Siika-aho (2000) showed α-glucuronidase from *S. commune* reacts with polymeric xylan and release glucuronic acid residues. In the following sections, the different families of α-glucuronidase and their mode of actions will be discussed in more details. Also, one-third of the glucuronic acid substituents present on the backbone of xylan are bonded to lignin via ester linkage, thus implying the importance of the cleavage of this residue to release the xylan (hemicellolase) from lignin (Heneghan, et al., 2007).

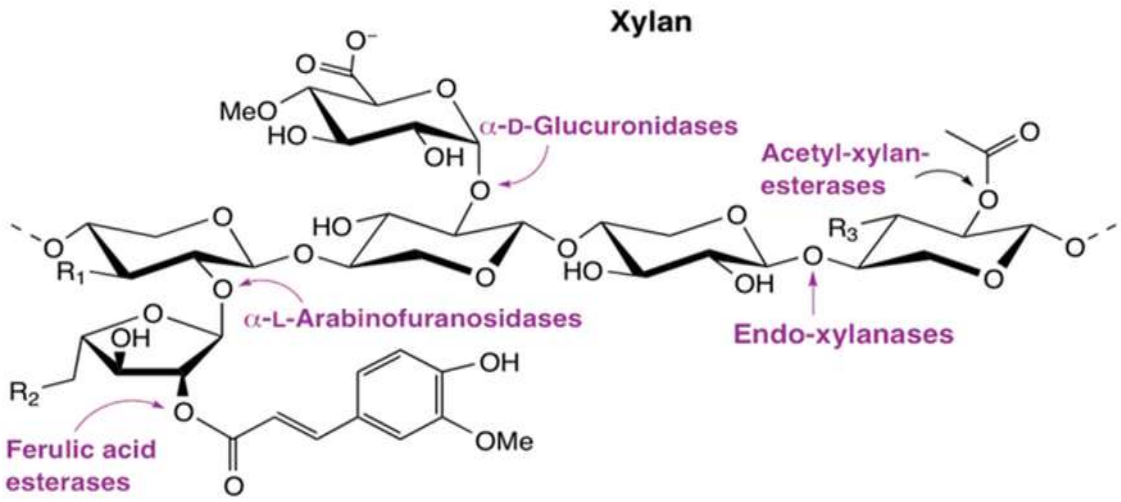

Figure 2.3.2: Schematic illustration of xylan backbone and the reaction sites of the xylan degrading enzymes (http://www.sc.mahidol.ac.th/scmi/phytase.htm).

2.4 Properties of α-Glucuronidase

Alpha-glucuronidase is a glycoside hydrolase. Glycoside hydrolases facilitate the hydrolysis of the glycosidic bonds of the polysaccharides like cellulose and hemicellulose. There are two families of α-glucuronidases, GH67 and GH115. Members of the GH67 family of α-glucuronidases are active on short oligoxylans. These enzymes hydrolyze the α-1,2 glycosidic linkage between 4-O-glucuronic acid residues substituted at the non-reducing end of oligoxylan (Nurizzo, et al., 2002). Alpha-glucuronidases, in which belong to GH115 family, are active on the polymeric xylan (Ryabova , et al., 2009). The recent family requires the two adjacent xylose residues of the substituted residue, to be available for the enzyme binding (Kolenova, et al., 2010). Kolenova and colleagues 2010 illustrated that the GH115 family α-glucuronidases are inverting enzymes that use single displacement mechanism to hydrolyze the substrate, which formerly was shown by Nurizzo and colleagues 2002 & 2003, to be the GH67 family α-glucuronidase mode of the action.

2.4.1 The GH67 family α-glucuronidases

The structure of *Pseudomonas cellulosa* GH67 α-glucuronidase has been determined by (Nurizzo, et al., 2002). They have heterologously expressed the respective gene in *E. coli* and the molecular mass of the recombinant α-glucuronidase was determined by SDS-PAGE at 77kDa, compatible with the expected molecular mass of the protein from the translated sequence of the gene. Nevertheless, gel filtration revealed that the enzyme molecular mass was 150 kDa in solution, indicating that the recombinant α-glucuronidase exists as a dimer.

The recombinant α-glucuronidase from *P. cellulosa* is a dimer, in which monomers composed of three domains. The catalytic center of this enzyme is a $(\beta/\alpha)_8$ barrel located in the central domain. Nurizzo and colleagues also commented on the evolutionary divergence of α-glucuronidase. They found that the catalytic center of α-glucuronidase is conserved among glycoside hydrolases that are involved in the decomposition of di, oligo and polysaccharides. The search for triggering structural similarities of the GH67 α-glucuronidase with other proteins by DALI (distance alignment matrix method) showed that the top seven hits were glycoside hydrolases with the central $(\alpha/\beta)_8$ cavity domain in common but in the evolution these enzymes diversified in terms of the stereochemistry and the substrate specificity.

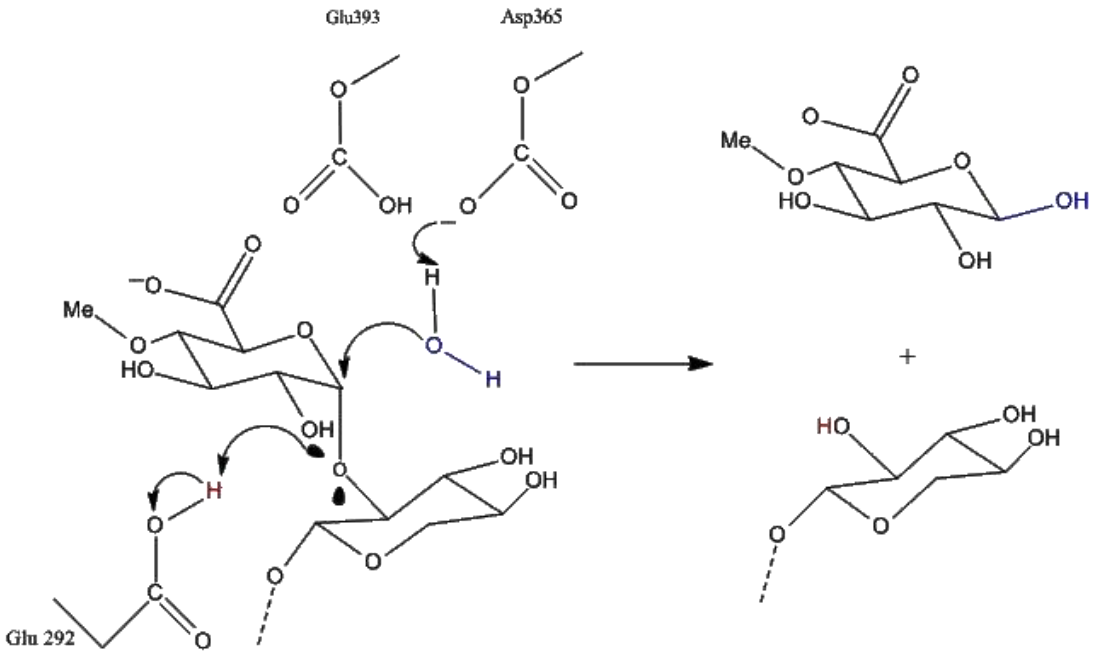

Figure 2.4.1: Single displacement mechanism of hydrolysis of the α1,2-glycosidic bond between uronic acid and xylan moiety by the family GH67 α-glucuronidase (Nurizzo, et al., 2002).

Two catalytic substituents of the enzyme are required for this mechanism: the first is an acid for the protonation of the glycosidic oxygen bridge, which facilitates the departure of the glucuronic acid residue and the second is a base in order to assist the activation of a water molecule prior to the nucleophilic attack of the anomeric carbon C_1 (Nurizzo, et al., 2002). Three carboxylate groups participate in enzymatic cleavage of the glycosidic bridge: Glu297, a proton donor residue which acts as an acid in the catalysis and two residues, Asp365 or Glu393, which are suggested to act as a base contribution to the nucleophilic attack at the α-conformation of the anomeric carbon $C_{1,}$ which results in the inversion of the anomeric carbon to β-conformation (Nurizzo, et al., 2003).

2.4.2 The GH115 family α-glucuronidase

The purified α-glucuronidase from *S. commune* is a member of GH115 family with molecular mass of 125 kDa and the isoelectric point of 3.6, determined by SDS-PAGE and IEF gels respectively (Tenkanen & Siika-aho, 2000). Activity of the *S. commune* GH115 family α-glucuronidase has been investigated on series of aldo-uronic acid substrates by Kalenova and colleagues (2010) (fig 2.4.2a). They illustrated that α-glucuronidases from *S. commune* and the other members of GH115 family are inverting enzymes with single displacement mechanism (explained in the previous section), as it was proposed by Nurizzo, et al., (2002) for α-glucuronidases from GH67 family. They observed the specific activity of the enzyme

increased by length of the backbone xylan, for aldotetraouronic acid, aldopentaouronic acid and aldohexaouronic acid. These results imply that the two adjacent residues of the methylglucuronic acid substituted residues have an important role in recognition and binding of the enzyme to the substrate (fig 2.4.2b). Apart from the catalytic center, which is conserved between glycoside hydrolyzes, topology of the binding site explains the evolutionary gap between them (Nurizzo, et al., 2002). Kolenova and colleagues (2010) assumed the substrate binding site of GH115 α-glucuronidases is a cleft that can accommodate a few neighbor xylose residues in addition to the substituted residue with glucuronic acid. Whereas, situation of the catalytic apparatus of the GH67 α-glucuronidase in the deep pocket topology, demands for substitution of the glucuronic acid residue on the non-reducing end of the substrate. These explain the selection of substrate by both the GH115 and GH67 α-glucuronidases (Kolenova, et al., 2010).

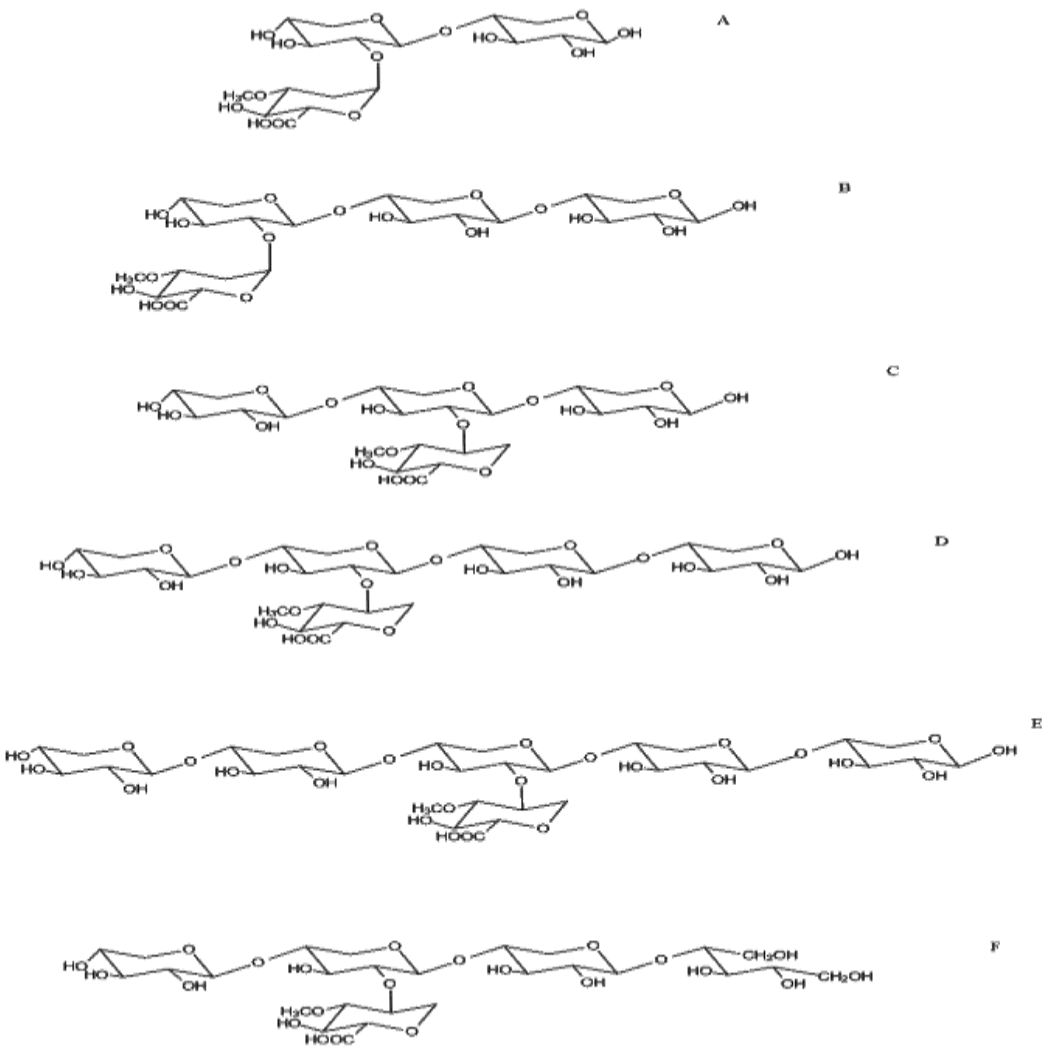

Figure 2.4.2a: Substrates used by Kolenova and colleagues (2010) for determining the activity of the *S. commune* GH115 α-gucurnidase (Kolenova, et al., 2010): A) $MeGlc^{2}AXyl_{2}$, B) $MeGlcA^{3}Xyl_{3}$ C) $MeGlcA^{2}Xyl_{3}$ D) $MeGlcA^{3}Xyl_{4}$ E) $MeGlcA^{3}Xyl_{5}$ F) Reduced $MeGlcA^{3}Xyl_{3}$-xylitol.

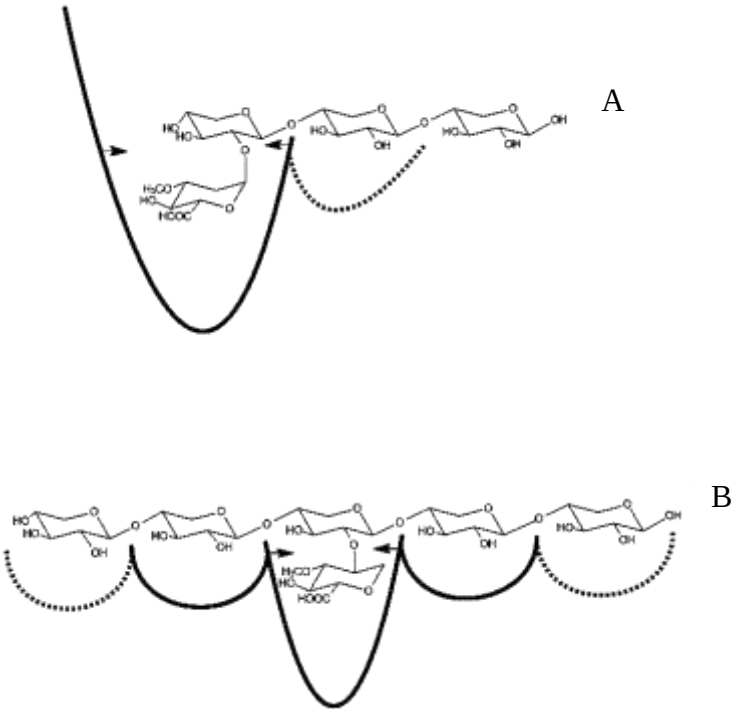

Figure 2.4.2b: Schematic illustration of the substrate binding of both GH67 and GH115 α-glucuronidase families (Kolenova, et al., 2010). A) GH67 α-glucuronidase binding to non-reducing end of xylan chain. B) GH115 α-glucuronidase binding to glucuronic acid residues with two adjacent xylose residues.

2.5 *P. pastoris* expression strains

P. pastoris is a methylotropic yeast which is able to grow on methanol as its sole carbon source (Cereghino & Cregg, 2000). Alcohol oxidase (AOX) is responsible for methanol consumption in *P. pastoris*. *P. pastoris* can also grow on other nutrient media containing carbon sources such as glucose and glycerol, both of which have extremely repressive effect on the activity of the AOX1 (Cregg & Higgins, 1995). Depending on the required application a wide spectrum of *P. pastoris* genotype strains are commercially available.

2.5.1 AOX pathway

The Alcohol oxidase is located in the peroxisome and oxidizes the methanol to formaldehyde by consumption of O_2 and generation of H_2O_2. There are two possibilities for the produced formaldehyde (fig.5). The first is to be further oxidized via the assimilatory pathway in the cytosol. In this case, formaldehyde is converted into dihydroxyacetone through the activity of dihydroxyacetone synthase, which is activated in cytoplasm before the oxidation of formaldehyde. The produced H_2O_2 from the oxidation of methanol to formaldehyde by the activity of AOX is reduced to H_2O and oxygen through the activity of peroxisome catalase. The assimilatory pathway yields the production of biomass (Gellissen, et al., 1992). The second possibility for the formaldehyde is to undergo oxidation in the cytosolic dissimilarly

pathway to generate energy (NADH). Activity of the formaldehyde dehydrogenase (FLD) converts the formaldehyde to formate and yields one NADH in the dissimilatory pathway and finally formate dehydrogenase (FDH) generates CO_2 and another NADH (Cereghino & Cregg, 2000). Other basic alcohols can also be oxidized by AOX, the more the number of carbons in alcohol is, the less the activity of the alcohol oxidase would be. The rate limiting step in the AOX pathway is the formation of formaldehyde from methanol. The methanol consumption rate is regulated via the increase of the intracellular amount of AOX (Couderc & Baratti, 1980).

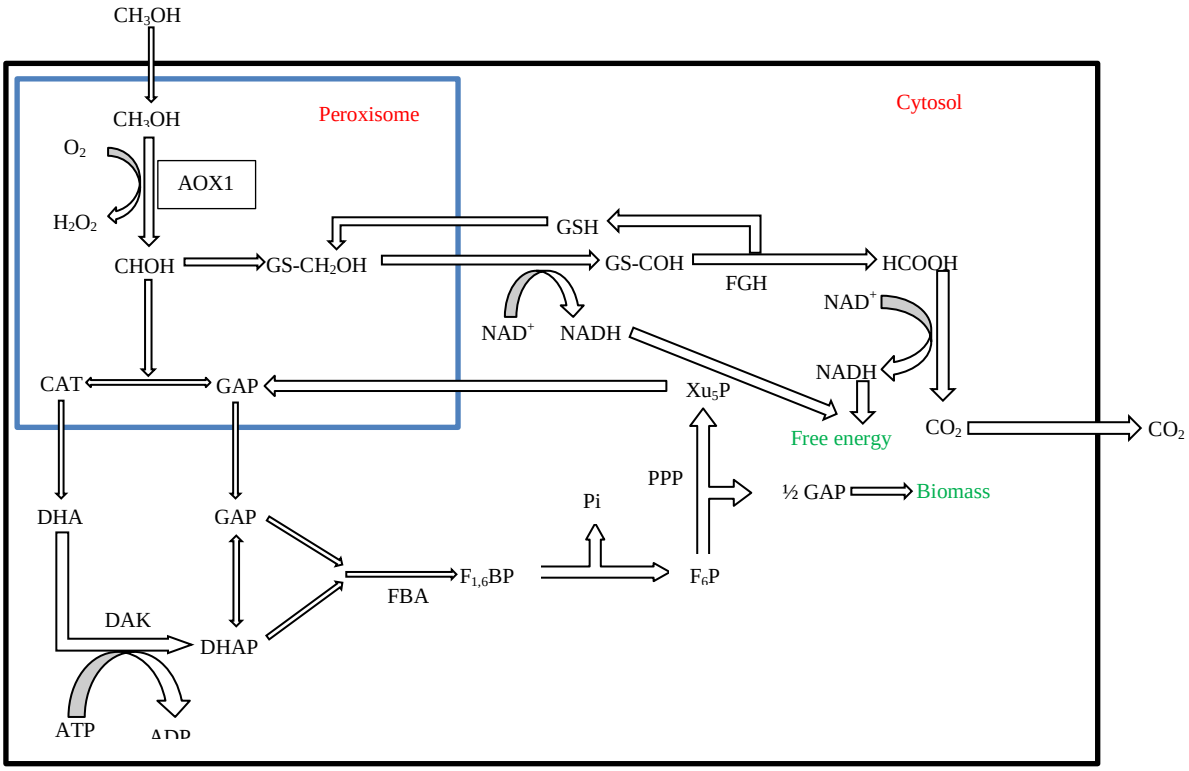

Figure 2.5.1: M ethanol utilization pathway in *P. pastoris* (De Schutter, et al., 2009)**.**

2.5.2 Effects of the Mut⁺ and Mut^S Phenotypes

The phenotype of *P. pastoris* is termed Mut$^+$ when methanol utilization rate is high due to the high level of *aox1* expression. The alcohol oxidase 1 gene is responsible for 85% of the methanol consumption by the alcohol oxidase enzyme. In contrast Mut^s phenotype is termed to methanol utilization slow strains which contain the *aox2* gene that encodes alcohol oxidase enzyme with the same activity as AOX1, but with a lower level of expression. Lower levels of AOX2 expression, which has a weak promoter, limit the methanol consumption rate and the protein production consequently when the inducible AOX promoter is used to initiate the transcription of the recombinant gene. Mut$^+$ phenotype contains functional versions of *aox1* and *aox2* genes, whereas Mut^s relies only on a functional copy of *aox2*. Aeration also

influences the level of recombinant protein production because AOX has a poor affinity for oxygen and cell compensates by expressing a high level of *aox1* which has the same promoter as the recombinant gene (Daly & Hearn, 2005). In order to determine the culture conditions it is essential to determine the Mut^+/Mus^s phenotypes. Typical methanol concentrations in the medium used for Mut^+ are 0.5-1.0% v/v whereas, higher concentrations of methanol than 0.3% v/v potentially results in poisoning of the Mut^s constructs. In large scale industrial fermenters Mut^+ strains require a large methanol supply throughout the induction phase which might be hazardous due to the flammability of methanol. An alternative solution for reducing the explosion risk could be to use Mut^s strains due to their lower methanol demand. Mut^s constructs are able to grow on mannitol and sorbitol, and consume less methanol to keep the induction. The slow growth rate and protein production rate of Mut^s phenotypes can be exploited to produce proteins with slow folding rate (Romanos, et al., 1995).

2.5.3 Expression vector

Among all commercial vectors that are used for expression of recombinant protein in *P. pastoris,* pPICZαC was selected to transfer the *agu1* gene into *P. pastoris*. The pPICZαC expression cassette contains a copy of α-mating factor (α- MF) signal sequence before the multicloning site, which leads to secretion of the recombinant protein. This vector also carries the 5′ AOX1 promoter. The pPICZαC expression vector contains a zeocin resistance gene that allows selection for positive transformants that are resistant to zeocin. The short length of the pPICZαC (3.6kb) allows for efficient transformation and contributes to prepare stable expression strains compared to larger expression vectors developed for transformation into the *P. pastoris* (Daly & Hearn, 2005).

2.5.4 The signal sequence

The alpha–mating factor pre-pro leader sequence (α-MF) consists of two regions, namely the pre and pro sequences. The pre-sequence contains a 19 amino acid signal peptide and the pro-sequence consists of a 60 amino acids. Upon translation, the pro-protein is translocated into the endoplasmic reticulum, while the signal peptidase cleaves the signal peptide sequence off the protein. For further processing the pro-protein is carried to Golgi where kex2 protease removes the pro-sequence prior to secretion of the mature protein into the extracellular media (Brake, et al., 1984).

2.5.5 Culture condition for expression

Before beginning the fermentation, there are some issues regarding the culture conditions of *P. pastoris* to take into account. The first issue is the scale of the culture. Shake flasks are mostly used in the early stage of the cell culture to perform screening and to obtain relevant data for scaling the culture up. Limited aeration and methanol supply are the main restriction integrated with the shaken flask expression system, especially when Mut^+ phenotype is utilized. While using Mut^+ transformants for the culture of *P. pastoris*, the concentration of methanol is an important parameter for optimizing the expression levels of the enzyme. The normal range of methanol concentration, reported for *P. pastoris* cell lines, lies between 0.5-1.0%. The accumulation of methanol can repress the protein production, which is a negative effect. This implies the requirement of the optimal methanol concentration assessment, which tolerates among different family members of *P. pastoris* and the type of the synthesized protein (Romanos, et al., 1995). The pH and ingredients of the medium have a significant effect on the level of the expression of the recombinant protein. The extent of proteolysis of the secreted protein will reduce if the medium is buffered to pH values from 3 to 6 (Sreekrishna, et al., 1997; Cregg, et al., 1993). There are other ways to diminish the proteolysis such as supplementing the medium with peptone and yeast extract, or addition of 1 % casamino acids. Addition of ammonium ions has also been recommended, in the form of ammonium sulfate. Tsujikawa and colleagues (1996), observed 10 fold reduction of proteolysis by supplementing medium IM medium with ammonium ions. Proteolysis is found to amplify over the induction period when the amount of viable cells in the culture is reduced. The way to engineer around this problem is to exchange the culture medium with the fresh medium and recover the product; this method reduces the proteolysis in the lab scale semi continuous cultures. The fermenter can be rebooted as many times as needed with only one start up (Sreekrishna, et al., 1997).

3. Materials and Methods

3.1 Strains and vectors

The codon optimized *Agu1* gene (2925bp) in pUC57 vector was provided by NZYtech (Lda, Lisbon, Portugal). pPICZαC plasmid (carried in *E. coli* TOP10 strain) was used for construction of the expression cassette and was purchased from Invitrogen (Life Technologies, NY, USA). *E.coli* DH5α was used for vector construction and maintenance. The *P. pastoris* SMD1168H strain which was used was for expression was purchased from Invitrogen (Life Technologies, NY, USA).

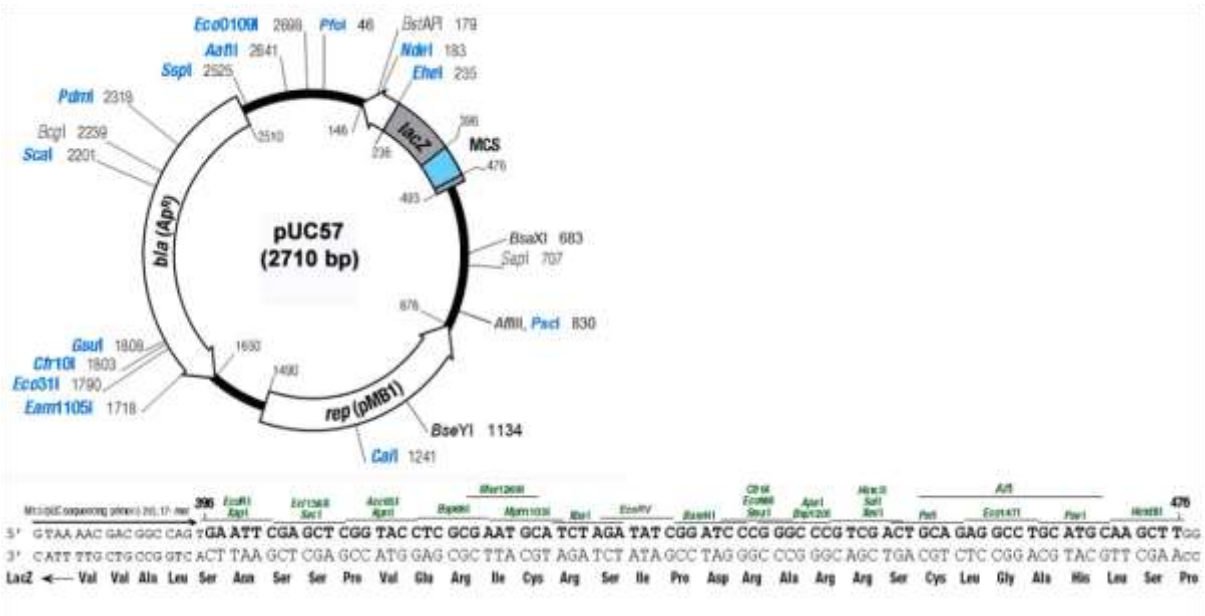

Figure 3.1.a: pUC57 restriction map. pUC57 was used for cloning of *Agu1* into *E. coli.*(http://www.genscript.com).

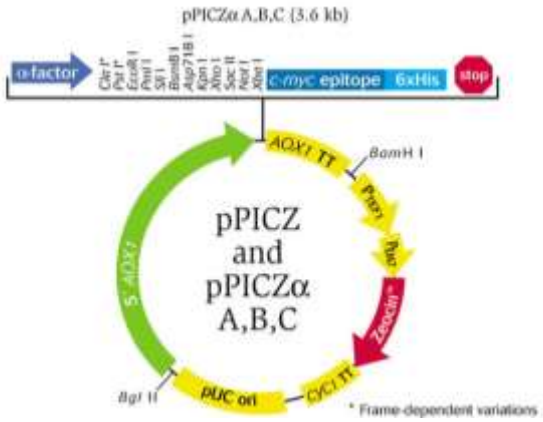

Figure 3.1.b: The commercial pPICZαA,B,C expression vector. *Agu1*was cloned into pPICZαC plasmid for overexpression in *P. pastoris* cells. All three reading frames (A, B, C versions) are provided to facilitate in-frame cloning with the C-terminal peptide (http://www.invitrogen.com).

3.2 Media

All media were prepared according to the Invitrogen manuals (Cat. no. V195-20, Cat. no. K1710-01 and *P. pastoris* Fermentation Process Guidelines) and were sterilized by autoclave on liquid cycle at 15 psi and 121℃ for 30 min, unless otherwise stated.

3.2.1 Lysogeny Broth (LB medium)

The LB medium was prepared according to table 3.2.1. Before addition of the antibiotics to the sterilized medium, it was cooled down in room temperature and then was supplemented with 100 μg mL^{-1} of ampicillin or 25μg mL^{-1} of zeocin under sterile conditions. To prepare LB agar plates, 15 g L^{-1} agar (Merck, Darmstadt, Germany) was added to the above medium prior to autoclave. The LB agar plates containing antibiotic were stored at 4℃.

Table 3.2.1. Composition of LB and low salt LB medium.

Component	Concentration (g L^{-1})	Supplier
Peptone (tryptone)	10	Merck,Darmstadt, Germany
Yeast extract	5	SIGMA ALDRICH, St Louis, USA
NaCl	10 (5 for Low salt LB medium)	Merck, Darmstadt, Germany

3.2.2 Yeast Extract Peptone Dextrose (YPD medium)

YPD had a composition shown in table 3.2.2. Filter sterilized glucose was added to the medium after autoclaving.

Table 3.2.2. Yeast Extract Peptone Dextrose medium (YPD) composition.

Components	Concentration (g L^{-1})	Supplier
Peptone	20	Merck,Darmstadt, Germany
Yeast extract	10	SIGMA ALDRICH, St Louis, USA
Glucose	20	Merck, Darmstadt, Germany

3.2.3 Yeast Extract Pepton Dextrose with Sorbitol (YPDS medium)

YPDS medium was prepared according to table 3.2.3. The medium broth was supplemented with 20 g/L agar before sterilization. The medium was cooled down in room temperature after sterilization. Filter sterilized glucose was added to the medium. The medium was supplemented with 100 µg mL^{-1} of Zeocin from Invitrogen (Life Technologies, NY, USA) when the medium temperature fell below 60℃. The YPDS plates were stored in dark place at 4℃.

Table 3.2.3. Yeast Extract Pepton Dextrose with Sorbitol medium (YPDS).

Components	Concentration (g L^{-1})	Supplier
Peptone	20	BD, Lepont de, France
Yeast extract	10	SIGMA ALDRICH, St Louis, USA
Sorbitol	182.2	SIGMA ALDRICH, St Louis, USA
Agar	20	Merck, Darmstadt, Germany
Dextrose	20	Merck, Darmstadt, Germany

3.2.4 Buffered Glycerol-complex Medium (BMGY medium)

BMGY medium was prepared according to table 3.2.4. The medium containing yeast extract and peptone was sterilized at the first stage. After sterilization, the medium was cooled down to room temperature and was supplemented with filter sterilized 1M potassium phosphate buffer pH 6.0, YNB, biotin and glycerol. Stock solutions for BMGY and BMMY media:

- 10X YNB: 134g yeast nitrogen base with ammonium sulfate and without aminoacids was dissolved in 1 liter water.
- 500X biotin: 20 mg biotin was dissolved in100 water and filter was sterilized. It was store at 4℃.
- 1M Potassium phosphate buffer: 132 mL of 1M K_2HPO_4 and 868 mL of 1M KH_2PO_4 were combined. The pH was adjusted to 6.0 and was filter sterilized. It was stored at 4℃.

3.2.5 Buffered Methanol-complex Medium (BMMY medium)

BMMY medium was prepared according to table 3.2.5. The medium containing yeast extract and peptone was sterilized at the first stage. After sterilization, the medium was cooled down to room temperature and was supplemented with filter sterilized 1M potassium phosphate buffer pH 6.0, YNB, biotin and methanol.

Table 3.2.4. Composition of buffered Glycerol-complex Medium (BMGY).

Components	Concentration (g L^{-1})	Supplier
Yeast extract	10	Merck, Darmstadt, Germany
Peptone	20	SIGMA ALDRICH, St Louis, USA
YNB	13.4	Alfa Aesor, Karlsruhe, Germany
Biotin	4×10^{-4}	SIGMA ALDRICH, St Louis, USA
Glycerol	10	Merck, Hohenbrunn, Germany
Potassium phosphate buffer	100 mL 1M	Merck, Darmstadt, Germany

Table 3.2.5. Composition of Buffered Methanol-complex Medium (BMMY).

Components	Concentration(g L^{-1})	Supplier
Yeast extract	10	Merck, Darmstadt, Germany
Peptone	20	SIGMA ALDRICH, St Louis, USA
YNB	13.4	Alfa Aesor, Karlsruhe, Germany
Biotin	4×10^{-4}	SIGMA ALDRICH, St Louis, USA
Methanol	5	
Potassium phosphate buffer	100 mL1M	Merck, Darmstadt, Germany

3.2.6 Fermentation basal salts medium

Six liters of fermentation basal salt was prepared according to table 3.2.6 and was sterilized in a 30 liters fermenter from infors HT Company (Basel, Switzerland). After the medium cooled down to room temperature, its pH was adjusted with 28% ammonium hydroxide to pH 5.

Table 3.2.6. Composition of fermentation basal salts.

Components	Concentration (g L^{-1})
Phosphoric acid 85%	26.7 (mL L^{-1})
Calcium sulfate	0.93
Potassium sulfate	18.2
Magnesium sulfate-7 H_2O	14.9
Potassium hydroxide	4.13
Glycerol	40.0

3.2.7 PTM_1 trace salts

PTM_1 trace salts were prepared according to table 3.2.7 and were filter sterilized. Trace salts were stored at room temperature before utilization.

Table 3.2.7. Composition of PTM_1 trace salts

Components	Concentration (g L^{-1})
Cupric sulfate-$5H_2O$	6.00
Sodium iodide	0.08
Manganese sulfate-H_2O	3.00
Sodium molybdate-$2H_2O$	0.20
Boric acid	0.02
Cobalt chloride	0.50
Zinc chloride	20.0
Ferrous sulfate-$7H_2O$	65.0
Biotin	0.20
Sulfuric acid	5.00

3.3 Transformation to *E. coli*

500 µL of competent cells of *E.coli* DH5α were supplemented with 1 µl of the vectors. The mixture was incubated for 30 min on ice before thermal heat shock at 42℃ for 90 sec. Immediately, the transformation tube was incubated on ice for additional 10 minutes and 1mL LB medium(table 3.2.1) was added. Cells were incubated at 37℃ and 200 rpm for 1 hour and then streaked on LB agar plates (in the sterile hood) containing 100 µg mL^{-1} ampicillin (table 3.2.1). Plates were incubated at 30℃ overnight and positive transformant cells were selected.

3.4 Plasmid extraction

Cells were harvested by centrifuging at 12000 rpm for 2 min and their plasmids were extracted using the GeneJet Plasmid Miniprep Kit according to Fermentas' (Maryland, USA) instructions (Appx 10.2). The content of the eluted plasmid from the plasmid DNA extraction step was quantified by determining of the absorbance at 260 nm.

3.5 Construction of the expression cassette

3.5.1 Enzymatic restriction

All restriction enzymes were provided by Fermentas (Maryland, USA). Plasmid and DNA fragments restriction were performed according to respective manufacturer's protocols. Digests were purified using Illustra GFX PCR DNA and gel band Purifiation Kit GE Healthcare (Buckinghamshire, UK).

3.5.2 Ligation

Digested DNA fragments were mixed with the linearized vector in the presence of T4 DNA ligase from Fermentas (Maryland, USA). The ligation reaction set up was according the manufacturer's instructions. The ligation mixtures were incubated overnight at room temperature. Ligation products were used for transformation into *E. coli* DH5α cells to test the integrity and amplification of the construct.

3.6 *P. pastoris* transformation

3.6.1 Transformation by electroporation

P.pastoris SMD1168H was grown overnight, in shake flasks, in YPD medium (table 3.2.2) at 30℃ and 170 rpm in a rotary shaker. Cells were harvested for electroporation according to the Invitrogen protocol (Cat. no.V195-20, version F) and resuspended in 1 mL of 1 M ice cold sorbitol. The linearized vectors were mixed with the resuspended cells and then transferred to an electroporation cuvette, which was then incubated on ice for 5 min. The BIO-RAD electroporation device's parameters were adjusted to *P. pastoris* set up. Cells were pulsed and then 1 mL of 1 M ice cold sorbitol was added to the electroporation cuvette. Contents of the cuvette were transferred to a 15 mL Falcon tube and incubated at 30℃ without shaking for 2 hours. Finally the cells were streaked out on YPDS (table 3.2.3) agar plates containing 100 µg mL^{-1} of zeocin and incubated at 30℃ for 72 hours.

3.6.2 Screening for positive transformants

Zeocin resistant colonies were selected and grown overnight, in shake flasks, in YPD medium (table 3.2.2) containing 100 µg mL^{-1} of zeocin at 30 ℃. Cells were harvested at 12000 rpm and their genomic DNA were isolated by phenol chloroform method and amplified by PCR through AOX forward and reverse primers to verify the recombination of the expression cassette into the SMD1168H strains genome. Then the PCR products were ran on gel electrophoresis.

3.6.3 Glycerol stock

P. pastoris strains containing *agu1* gene were grown in YPD medium (table 3.2.2) over night. 500 µL of 80% glycerol was mixed with 500 µl of *P. pastoris* overnight cell culture in a cryovial. The mixture was frozen in liquid nitrogen and stored at -80℃.

3.7 Fermenter preparation

The engineered *P. pastoris* from the frozen glycerol stock was inoculated into BMGY medium in a baffled flask containing a total of 5-10% of the initial fermentation volume. The culture was grown overnight at 30°C and 200 rpm. The fermenter was sterilized with the fermentation Basal salts medium (Table 3.2.6) containing 4% glycerol. After sterilization and cooling, temperature was set to 30°C, agitation and aeration were adjusted to operating conditions, 1000 rpm and 0.1-1.0 vvm air respectively. pH of the fermentation medium was adjusted to 5.0 with 28% ammonium hydroxide (undiluted ammonium hydroxide). The fermentation medium was supplemented aseptically with 4.35 mL of PTM1 trace salts per liter of the fermentation medium.

3.7.1 Glycerol Batch phase

The fermenter was inoculated with the culture generated in the propagation shake flask to 10% of initial fermentation volume. The dissolved oxygen (DO) was kept above 20% by adding oxygen as needed. The batch culture was grown until the glycerol is completely consumed. This was indicated by an increase in the DO to 100%. Sampling was performed at the end of each stage and twice daily. 10 mL samples were taken for each time point. Samples were analyzed for cell growth (OD600 and wet cell weight), protein concentration and α-glucuronidase activity. Cell pellets and supernatants were frozen at -80°C for later analysis.

3.7.2 Glycerol fed-batch phase

A 50% w/v glycerol feed containing 12 ml PTM1 trace salts per liter of glycerol feed was initiated. The feed rate was set to 18.15 mL hr^{-1} L^{-1} of initial fermentation volume. Glycerol feeding was carried out for five hours.

3.7.3 Methanol fed-batch phase

Methanol induction was initiated by starting a 100% methanol feed containing 12 mL PTM1 trace salts per liter of methanol. The feed rate was set to 3.6 mL hr^{-1} per liter initial fermentation volume for the first 2 hours until OD was stabilized. One hour after adaptation

of the culture to methanol utilization, the feed rate is doubled to7.3 $mL^{-1}hr^{-1}$ per liter of initial fermentation volume. After, two hours at the 7.3 mL hr^{-1} per liter of initial fermentation volume, the methanol feed rate was increased to10 mL hr^{-1} per liter of initial fermentation volume. This feed rate was maintained throughout the rest of the fermentation. The entire methanol fed-batch phase lasted 84 hours.

3.8 Biochemical analyses

3.8.1 Bradford assay

Quantification of protein was performed with the Bradford reagent supplied by Bio-Rad (California, USA) in a microplate format according to the manufacturer's instructions. A standard curve of bovine serum albumin in duplicate concentrations from 0.05 µg mL^{-1} to 0.5 µg mL^{-1} was prepared.

3.8.2 α-glucuronidase assay

Alpha-glucuronidase was assayed using the α-D-Glucuronidase assay Kit (K-AGLUA 10/11) from Megazyme International, Co.(Wicklow, Ireland). The assay is a coupled assay where the first step (Figure 3.7.2) consists of the liberation of 4-O-methyl glucuronic acid from aldouronic acids (glucuronic acid-substituted xylose oligomers) by the assayed enzyme. In the second step (Figure 3.7.2) the liberated glucuronic acid is oxidized by uronate dehydrogenase reducing one molecule of NAD^+ per glucuronic acid. This reaction yields one NADH and one glucarate. The total amount of glucuronic acid is stoichiometric with the amount of formed NADH. Measurement of NADH absorbance at 340 nm monitors the amount of available glucuronic acid for the second reaction.

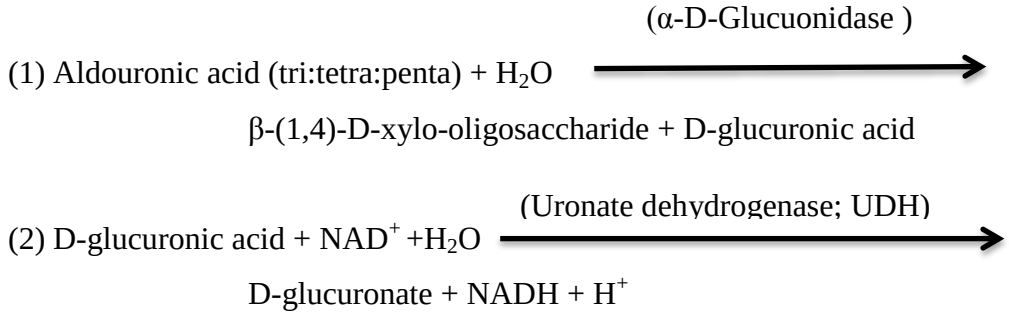

Figure 7.3.2: Principle of the α-D-Glucuronidase Assay Kit. Samples taken over the induction phase were assayed according to the procedure for microplate assay. MES buffer was used as the extraction buffer for this assay and its pH was adjusted to 6.0.

4. Results

The constructed strain was checked in a set of experiments to determine the integration of the expression cassette and to investigate its influences on the production of α-glucuronidase. To investigate the recombination of *Agu1* gene into the expression vector, the constructed vector was analyzed by restriction digestion and DNA sequencing. Also integration of the expression cassette into SMD1168H *P. pastoris* transformants' genome was confirmed by isolation of the genomic DNA and PCR. In order to screen the best α-glucuronidase producing strain, the transformants were grown in BMGY and BMMY media and the activity of the secreted α-glucuronidase was assayed. The candidate strain was further evaluated by cultivation in a shake flask and fermentation in a 30 liter fermenter to investigate the effect of the genetic modifications on the protein production yield and to assay the activity of α-glucuronidase.

4.1 Construction of expression cassette

4.1.1 Propagation of the *Agu1* gene

The synthetic *Agu1* was codon optimized for expression in *P. pastoris.* It was cloned between *Xba*I and *Cla*I restriction sites into EcoRV region of pUC57 donor vector. The vector carries the ampicillin resistant gene that enables the selection of positive transformants on the LB agar plates containing 100 µg mL^{-1} ampicillin (table 3.2.1). Efficient transformation into *E. coli* DH5α was checked by overnight growth of ampicillin resistant colonies in LB medium (table 3.2.1) supplemented with 100 µg mL^{-1} ampicillin. Cells were harvested and their plasmids were isolated using the GeneJet Plasmid Miniprep Kit according to manufacturer's instructions (Appx 10.2). The quantity of the eluted plasmid was 219 ng μL^{-1}, determined by absorbance at 260 nm (table 4.1.2).

4.1.2 Isolation of pPICZαC

pPICZαC was isolated from from *E. coli* TOPO strain grown in LB medium supplemented with 25 µg mL^{-1} of zeocin. Concentration of the purified pPICZαC vector was determined 74 ng μL^{-1} by measurement of absorbance at 260nm (table 4.1.2).

4.1.3 Isolation of *Agu1* from pUC57

Agu1 was isolated from pUC57 by enzymatic digestion with *Xba*I, *Cla*I and *Sca*I, in a two step restriction. Initially, plasmid was digested with *Sca*I and *Cla*I overnight at 37℃ (table 4.1.3a). This digestion resulted in two separate bands, at 3830 bp and 1870 bp. The larger band that contained *Agu1* was purified using illustra GFX PCR DNA and gel band

purification kit GE Healthcare (Buckinghamshire, UK). Concentration of the purified DNA fragment was determined 92 ng μL^{-1} in this step. The second step of the digestion, released the *Agu1* from the attached DNA fragment through the activity of *Xba*I. The reaction was set up according to the table 4.1.3b. Digestion products were separated on an agarose gel and resulted in three DNA fragments (Figure 4.1.3). The larger fragments at 3830 bp is probably undigested DNA, the second fragment at 2925 bp represents *Agu1* and the last fragment at approximately 900 bp the cleaved DNA fragment between *Xba*I and *Sca*I sites. *Agu1* band in the middle was purified using illustra GFX PCR DNA and gel band Purifiation Kit from GE Healthcare (Buckinghamshire, UK). Concentration of the purified gene was determined 24 ng μL^{-1}.

Components	**quantity**
Plasmid	8 µL (1753.6 ng)
***Cla*I**	1 µLl (1 U)
***Sca*I**	1 µL (1 U)
FD buffer	2 µLl
Water	8 µL
Total	20 µL

Table 4.1.3a. The first enzymatic digestion of pUC57 with *Cla*I and *Sca*I. The reaction was incubated overnight in conventional incubator at 37℃. Separation of the reaction products yielded two bands at 3830 and 1870 bp.

Components	**pUC57**
plasmid	12 µL (1099.2 ng)
***Xba* I**	1 µL (1 U)
FD buffer	2 µL
Water	5 µL
Total	20 µL

Table 4.1.3b. The second enzymatic digestion of the DNA fragment (containing *Agu1*) with Xba*I*. The reaction was incubated overnight in conventional incubator at 37℃. Separation of the reaction products yielded two bands at 2925 and 905 bp.

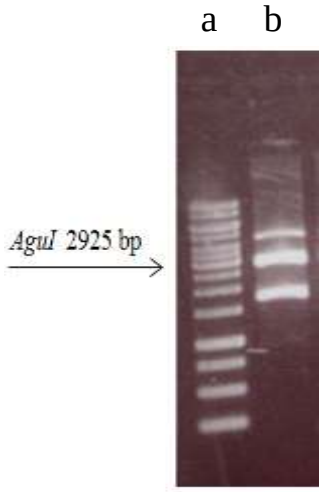

Figure 4.1.3: Electrophoresis analysis of the digested pUC57 with *Cla* I , *Xba* I and *Sca* I . One kb gene ruler (a) and the digestion products (b) were running in the electrophoresis gel for 1 hour at 85V. Eelectrophoresis results illustrate that *Agu1* gene has been isolated from the rest of DNA fragments. The band in the middle at 2925 bp shows *Agu1*.

4.1.4 Cloning of *Agu1* into pPICZαC

Linearization of pPICZαC

Along with the digestion of pUC57 vector, pPICZαC was digested with *Cla*I and *Xba*I in a two steps restriction reaction to provide a linearized vector with sticky ends. The restriction reaction was set up according to tables 4.1.4a and 4.1.4b. Full digestion of pPICZαC was observed with agarose gel electrophoresis of the reaction mixture and revealed a single band at 3600 bp (Figure 4.1.4a). Measurement of the DNA concentration revealed a reduction in concentration of the digested vector after each purification step, from 34 ng μL^{-1} to 13 ng μL^{-1}. The purified gene and the linearized plasmid with complementary sticky ends were used for the construction of the expression vector.

Components	**pPICZαC**
plasmid	12 µL (895.2 ng)
***Cla*I**	1 µL (1 U)
FD buffer	2 µL
Water	5 µL
Total	20 µL

Table 4.1.4a. Enzymatic digestion of pPICZαC with *Cla*I. The reaction was incubated overnight in conventional incubator at 37℃. Separation of the reaction products yielded a single band at 3600 bp.

Components	**pPICZαC**
plasmid	16 µL (545.6 ng)
***Xba*I**	1 µL (1 U)
FD buffer	2 µL
Water	1 µL
Total	20 µL

Table 4.1.4b. Enzymatic digestion of the linearized pPICZαC with *Xba*I. The reaction was incubated overnight in conventional incubator at 37℃. Separation of the reaction products yielded a single band at 3600 bp.

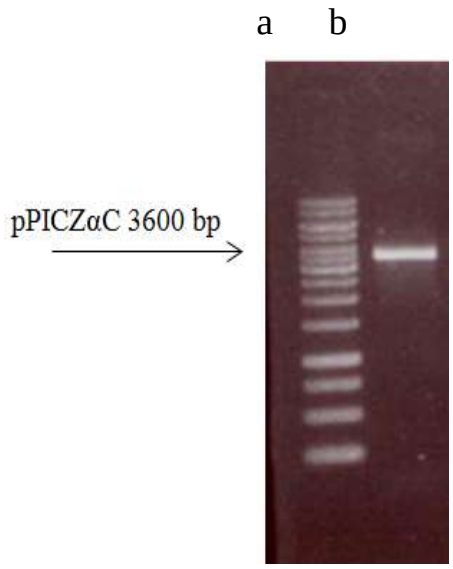

Figure 4.1.4a: Electrophoresis analysis of the digested pPICZ*α*C with *Cla* I and *Xba* I. One kb gene ruler (a) and digestion product (b) were running in the electrophoresis gel for 1 hour at 85V. Electrophoresis results illustrate that pPICZαC has been fully digested and the band at 3600 bp corresponds to pPICZαC**.**

Ligation of Agu1 *into pPICZαC*

The cleaved *Agu1* was combined with the linearized pPICZαC vector in the presence of T4 DNA ligase to clone the gene into the pPICZαC vector by the function of T4 DNA ligase. The amount of the vector and the insert required for 1:1, 1:3 and 1:5 vector: insert ratios was calculated according to the concentrations of the purified vector and insert presented in tables 4.1.3c and 4.1.4c. The ligation reaction was set up (table 4.1.4c) according to the manufacturer's instructions. The reaction was incubated in room temperature overnight and the recombinant expression vectors were transformed into *E.coli* DH5α.

Table 4.1.4c. Ligation of the insert into the expression vector. The reaction was incubated overnight in room temperature.

Components	**Quantity (1:1)**	**Quantity (1:3)**	**Quantity (1:5)**
Digested plasmid	1.5µL (19 ng)	2.0µL (26 ng)	1.0 µL (13 ng)
Insert	2.5 µL (60 ng)	5.5 µL (133 ng)	4.5 µL (108 ng)
T4 ligation buffer 10X	1.0 µL	1.0 µL	1.0 µL
T4 Ligase	0.7 µL (3.5 U)	0.7 µL (3.5 U)	0.7 µL (3.5 U)
MilliQ-Water	Fill to 10 µL	Fill to 10 µL	Fill 10 µL

Evaluation of transformation of the expression vector into E. coli *DH5α*

After the transformation of the ligation products into *E. coli* DH5α, selection of the positive transformants was carried out on low salt LB agar plates containing 25 µg mL^{-1} zeocin. Zeocin resistant colonies were selected and grown overnight in low salt liquid LB medium supplemented with 25 µg mL^{-1} zeocin. Cells were harvested and the plasmids of the cells were extracted. Finally, content of the eluted plasmid from the plasmid DNA extraction step was quantified by measurement of absorbance at 260 nm (table 4.1.4d). Purified DNA plasmids were sent for sequencing to verify the integration of the insert into pPICZαC. Two separate diagnostic restrictions were also performed on the sample plasmids. The first reaction was performed with *Xba*I and *Cla*I with the same set up as the cleavage of the *Agu1* gene from the pUC57 vector. In the second reaction, the recombinant plasmids were digested with *Spe*I, a single cutter enzyme, which cleaves inside the gene region (table 4.1.4e). Diagnostic digestion of the purified plasmid with *Spe*I showed that 9 of the colonies out of 12 gave rise to a single band at 6600 bp (figure 4.1.4b), which implies the insert has been cloned into the vector. In addition, two of the samples, which were diagnosed positive recombination of the insert, were sent for sequencing. The sequencing results also confirmed integration of the recombinant gene in to the pPICZαC plasmid. Eventually one of the expression cassettes, which had full alignment with the sequence of *Agu1*, was used for transformation into *P. pastoris* SMD1168H.

Table 4.1.4d. Concentration of the recombinant expression vector purified from zeocin resistant colony.

	Concentration
Expression cassette	101 ng $µL^{-1}$

Components	**Expression cassette**
Plasmid	8 µl (809 ng)
***Spe*I**	1 µl (1 U)
FD buffer	2 µl
Water	9 µl
Total	20 µl

Table 4.1.4e. Enzymatic digestion of the expression vector with *Spe*I. The reaction was incubated overnight in conventional incubator at 37℃. Separation of the reaction products by electrophoresis yielded a single band at 6600 bp.

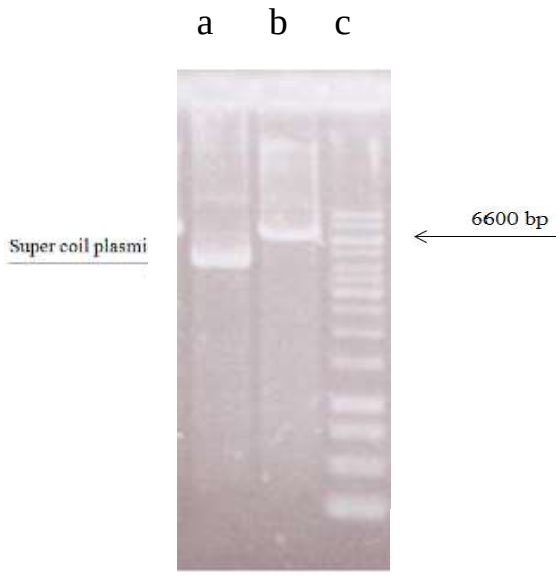

Figure 4.1.4b: Electrophoresis analysis of the digested expression vectors isolated from potential positive DH5α colonies with *Spe*I. The undigested expression vector (a), the digestion product (b) and 1 kb gene ruler were running in the electrophoresis gel for 1 hour at 85V. *Spe* I cuts inside *Agu1* region therefore we expect a single band at 6600 bp. Nine out of twelve colonies contained the expression vector. Undigested cassettes corresponding to each digested cassette have been loaded to their left hand well as control.

4.2 Integration of the expression cassette into *P. pastoris* SMD1168H

4.2.1 Linearization of the pPICZαC construct

pPICZαC plasmid containing α-glucuronidase gene was linearized with *Mss*I (*Pme* I) (table 4.2.1). The linearization reaction took place for 12 hours at 37℃. The concentration of the purified expression vector isolated from *E.coli* DH5α was 100 ng μL^{-1}.

Components	pPICZαC
Plasmid	9.5 µl (950 ng)
***Pme* I**	1 µl (1 U)
FD buffer	2 µl
Water	6.5 µl
Total	20 µl

Table 4.2.1. Enzymatic digestion of the expression vector with *Pme*I. The reaction was incubated overnight in conventional incubator at 37℃. Separation of the reaction products yielded a single band at 6600 bp. The final concentration of the linearized vector after purification reached 0.55 µg μL^{-1}.

4.2.2 Transformation of the linearized pPICZαC construct into *P. pastoris*

P. pastoris SMD1168H cells were grown overnight in YPD medium (table 3.2.2) and harvested at OD_{600} 2.73 by centrifuging at 5000 rpm at 4℃. Harvested cells were resuspended in 1 ml of 1M ice cold sorbitol. Then 5.5 µg of the linearized acceptor vectors were mixed with 80 µl of the resuspended cells and added into an electroporation cuvette. The mixture was incubated on ice for 5 min. The BIO-RAD electroporation device

parameters were adjusted to *P. pastoris* set ups. After pulsing the cells, 1 ml of 1M ice cold sorbitol was added to the electroporation cuvette. Contents of the cuvette were transferred to a 15 ml falcon tube and incubated at 30℃ without shaking for 2 hours. Finally the cells were streaked on YPDS agar plates containing 100 µg mL^{-1} of zeocin until colonies were formed. Transformation yielded 5 positive colonies.

4.2.3 Screening for positive transformants

This section acknowledges the results from the evaluation of the potential transformants grown in BMGY (table 3.2.4) and BMMY (table. 8.2.5) media. This was performed in order to know whether and to which content the cells are able to produce and secrete α-glucuronidase into extracellular media.

In order to screen the positive transformants, they were inoculated into 10 ml of BMGY medium in a 50 ml Falcon tube. Cell cultures were incubated on a shaker at 30℃ and 250 rpm for 28 hours. When the culture concentration reached an OD_{600} =3, cells were harvested by centrifuging at room temperature and 5000 rpm for 5 min. The cell pellet was resuspended in 35 ml of BMMY medium until OD_{600} =1 to induce expression. Cell cultures have been supplemented with 100% methanol, to 0.5% of their final volume every 24 hours, to maintain induction. After 96 hours of induction with methanol, cells were harvested by centrifuging at 8000 rpm for 5 min. Cells and supernatant of each cell culture were stored at -80℃ for further analysis.

Genomic DNA of the zeocin resistant transformants, namely strains GluA, GluB, GluC, GluD, GluE, were isolated and amplified by PCR using AOX1 forward and reverse primers. Analysis of the PCR reaction by electrophoresis (fig 4.2.3) showed two bands, one between 3500 and 3000 bp and the other between 2500 and 2000 bp, in which the fragment between 3500 and 3000 bp confirms the heterologous integration of *Agu1*into *P. pastoris* genome.

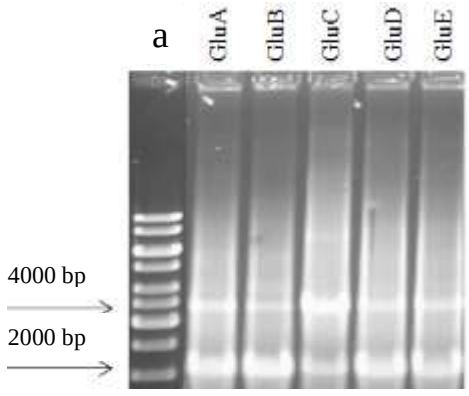

Figure 4.2.3 Electrophoresis analysis of the PCR amplified region of the genomic DNA of the selected transformants by AOX primers. The amplified *Agu1* gene is represented by a band between 3000 and 3500 bp. The second band between 2000 and 2500 bp shows chromosomal AOX1 of *P. pastoris.* Positive transformants from left to right on the gel were named GluA, GluB, GluC, GluD and GluE and for further characterization assayed by α-glucuronidase assay kit.

The analysis of the secreted protein via the α-glucuronidase assay kit resulted in cleavage of D-glucuronic acid residues substituted on (tri:tetra:penta) aldouronic acid substrates through the activity of the produced protein. The supplementation of uronate dehydrogenase (UDH) and NAD^+ in the test samples converted the released D-glucuronic acid into D-glucarate and NAD^+ into NADH. The consumption of D-glucuronic acid in the coupled reaction is stoichiometric with produced NADH that absorbs UV at 340nm. The activity of the enzyme was monitored by the change in absorbance at 340 nm. Since the activity of extracellular enzyme content of the GluC strain was 0.02 U mL^{-1}, slightly higher than the rest of positive transformants (table 4.2.3), it was selected for further investigation.

Mut^+ phenotypes	**C (U mL^{-1})**
STD	0.005
GluA	0.016
GluB	0.018
GluC	0.020
GluD	0.019
GluE	0.017

Table 4.2.3 Analysis of the extracellular α-glucuronidase activity. Positive transformants were induced with methanol for 96 h in BMMY medium. Since the activity of GluC strain was 0.0205, slightly higher than the rest of positive transformants, it was selected for further investigation.

4.3 Small scale cultivation of the recombinant *P. pastoris* strain Gluc

This section acknowledges the results of the cultivation of the screened strain (GluC) in BMGY and BMMY. The experiments were done to monitor the growth of the engineered strain in the repressive BMGY medium and to determine the protein production yield. The engineered SMD1168H was inoculated into 25 ml of BMGY medium in a 250 ml baffled flask. Cell cultures were incubated on a shaker at 30℃ and 250 rpm for 28 hours to grow in the repressive medium before induction. When the culture concentration reached an OD_{600} =2.8 the cells were harvested by centrifuging at room temperature and 5000 rpm for 5 min. The cell pellet was resuspended in 90 ml of BMMY medium until OD_{600} =1 to induce expression. The culture was incubated on a shaker at 175 rpm and 30℃ for 4 days. The growth and induction took place in a 1L baffled flask covered by double layer sterilized gauze to maintain aeration. The cell culture was induced every 24 hour after the start of cultivation with methanol to 0.5% of the total volume of the culture to compensate for methanol loss caused by evaporation from medium and uptake by the cells. Samples were

taken in following time points (hours): 24 h, 48 h, 72 h and 96 h. Samples were centrifuged at 16000 rcf and room temperature for 3 min. The supernatant and cells pellet were separated and frozen in liquid nitrogen and stored at -80℃ for further analysis.

The extracellular α-glucuronidase activity of the samples resulted in an increased absorbance of NADH. The maximum activity of the GluC strain was observed at 96 h and it was found to be 0.02 U mL^{-1}. Compared to the corresponding standard enzyme (supplied by the kit), which had a maximum activity of 0.005 U mL^{-1} (Table 4.2.3), α-glucuronidase produced by *P. pastoris* GluC strain had a 3.7–fold higher activity. The measured activity in the shake flask cultivation is comparable with the previously measured 0.020 U mL^{-1} α-glucuronidase activity of this strain during the screening phase.

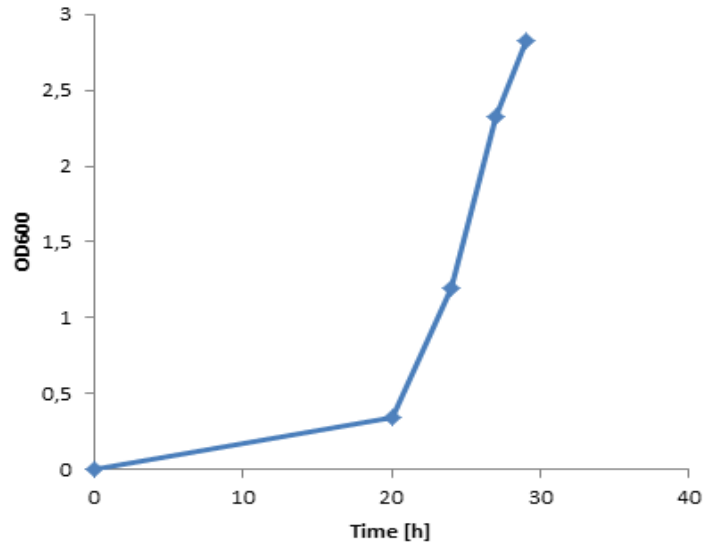

Figure 4.3a: Biomass concentration over time. The cell culture in BMGY medium, which is a glycerol repressive medium, was harvested at OD_{600} 2.8 after 28 hours of the.

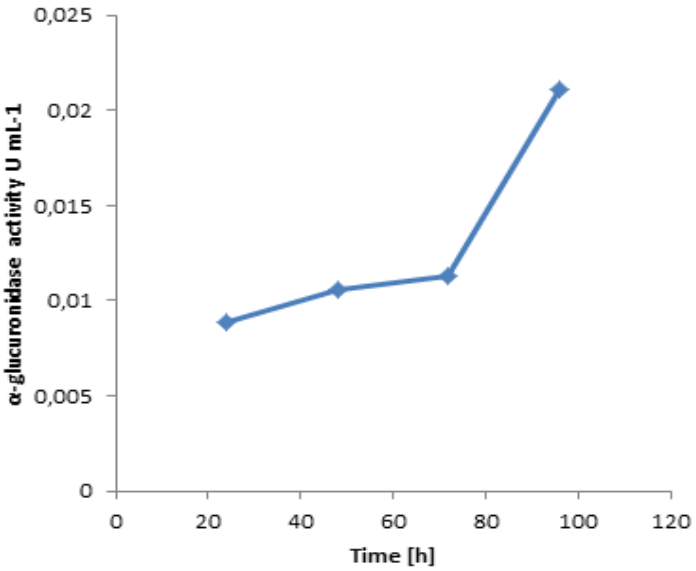

Figure 4.3b. Activity of the enzyme over the induction phase. Samples were taken every 24 hours and the activity of the enzyme in each sample (time point) was assay by α-glucuronidase assay.

4.4 Large-scale production of α-glucuronidase

The main objective of the fermentation of *P. pastoris* GluC was to analyze the performance of the strain under controlled conditions for the large scale production of recombinant α-glucuronidase. The fermentation was performed in three phases; glycerol batch cultivation, glycerol fed batch cultivation and methanol fed-batch cultivation.

4.4.1 Batch phase

The engineered GluC was grown in a 30 liter fermenter on Basal salts, glycerol and ammonium hydroxide (table 3.2.6). The initial volume of the fermentation medium was 6 liters and were inoculated with 600 ml of *P. pastoris* GluC overnight culture grown up to OD_{600} =1.03. The dissolved oxygen was relatively 100% before the culture starts to grow. Then, the cells were grown for 25 hours at 30℃ to generate enough biomass. At the end of glycerol batch phase, cell density reached OD_{600} of 41.8 and wet cell weight 42 g L^{-1} were achieved. Analysis of the sample at 25 hours by the α-glucuronidase and the Bradford assays did not show any detectable trace of extracellular protein secretion. During the batch phase as the cells grow, they will consume oxygen, decreasing dissolved oxygen (DO). The DO was kept above 20% over the glycerol batch phase. After consumption of the available glycerol in the medium, the dissolved oxygen raised to 100% before the initiation of the glycerol feeding.

4.4.2 Glycerol fed-batch phase

In order to increase the biomass concentration, the culture was fed for an additional 5 hours with 50% w/v glycerol containing 12 ml PTM trace salt. The feed rate of glycerol remained constant at 120 mL h^{-1} during this phase. Analysis of the sample at the end of this phase (30 hours after inoculation) by OD_{600} and wet weight measurements showed that a biomass concentration of 90 and 145 g L^{-1} were achieved, respectively (Figures 4.4.3a and 4.4.3b). Also, this sample was analyzed to determine the amount of extracellular protein and possibly the activity of α-glucuronidase (figures 4.4.3c and 4.4.3d) but as expected, the amount of the secreted protein in the repressive medium was negligible. By the end of this phase, the feed from glycerol was switched to methanol.

4.4.3 Methanol induction phase

Induction of α-glucuronidase was initiated by switching the feed from glycerol to 100% methanol feed containing 12 ml PTM_1 trace salts per liter of methanol. The feed rate has been set to 24 mL h^{-1} for two hours, to allow the culture to adapt to methanol and the dissolved

oxygen to stabilize. The dissolved oxygen was fluctuating between 30% and 50% until the end of fermentation. One hour after adaptation of the cells to methanol feed rate doubled to 50 mL h^{-1} for 2 hours. Finally, the feed rate was fixed to 72 mL h^{-1} throughout the remainder of the induction phase (80 hours). Agitation and temperature were kept constant during the fed-batch phases and set to 1000 rpm and 30℃, respectively.

Sampling took place twice a day for analysis of growth (figures 4.4.3a-b) and protein production (figures 4.4.3 c-d). The optical density of the fermentation culture and wet weight exhibited an increasing pattern to the end of fermentation, from OD_{600} 90 and 145 g L^{-1} to OD_{600} 140 and 332 g L^{-1}, respectively.

Samples were also analyzed for extra cellular protein production by Bradford assay and α-glucuronidase assay for α-glucuronidase activity 18 hours after harvesting the cells. Analysis showed that α-glucuronidase was secreted into medium after initiation of the methanol induction phase at 46 h (16 hours after induction). The extracellular protein content of the sample was determined 0.12 mg mL^{-1} by Bradford assay while its α-glucuronidase activity was 0.018 U mL^{-1}. The amount of extracellular protein reached 0.72 mg mL^{-1} (figure 4.4.3d) at the end of fermentation, however α-glucuronidase assay activity increases slowly until 88 h but the activity augments sharply from 0.024 to 0.065 U mL^{-1}, between 88 h to 114 h (figure 4.4.3.c).

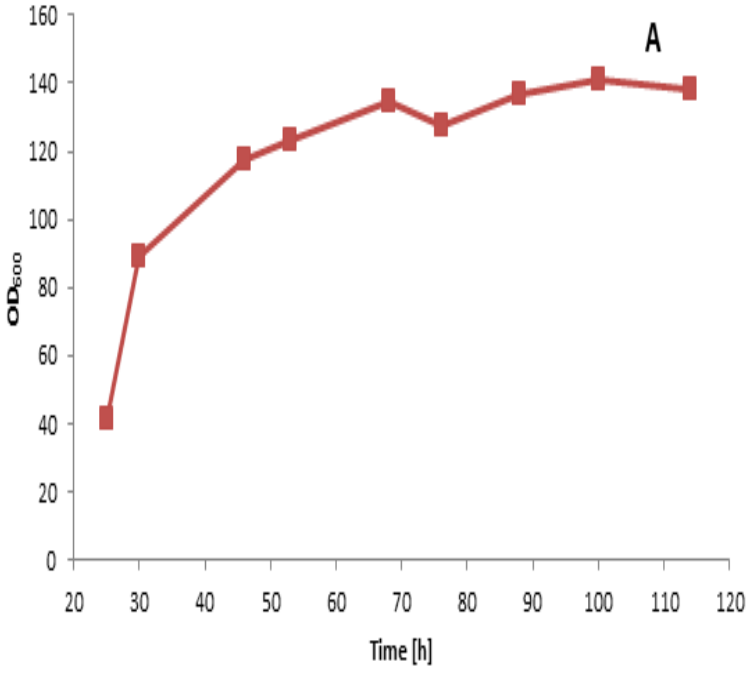

Figure 4.4.3.a Biomass concentration over the fermentation time (OD_{600} absorbance). The first sample was taken at the end of the batch phase.

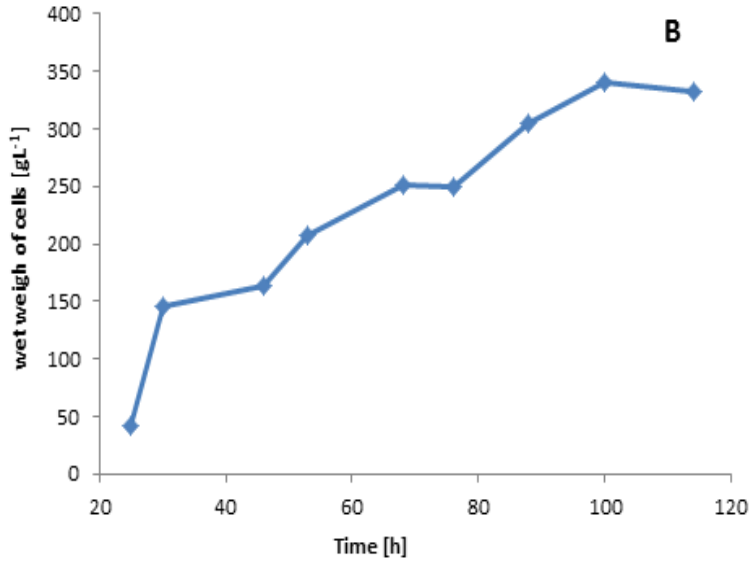

Figure 4.4.3b: Biomass concentration over the fermentation time (cells wet weight (g L^{-1})). The first sample was taken at the end of the batch phase.

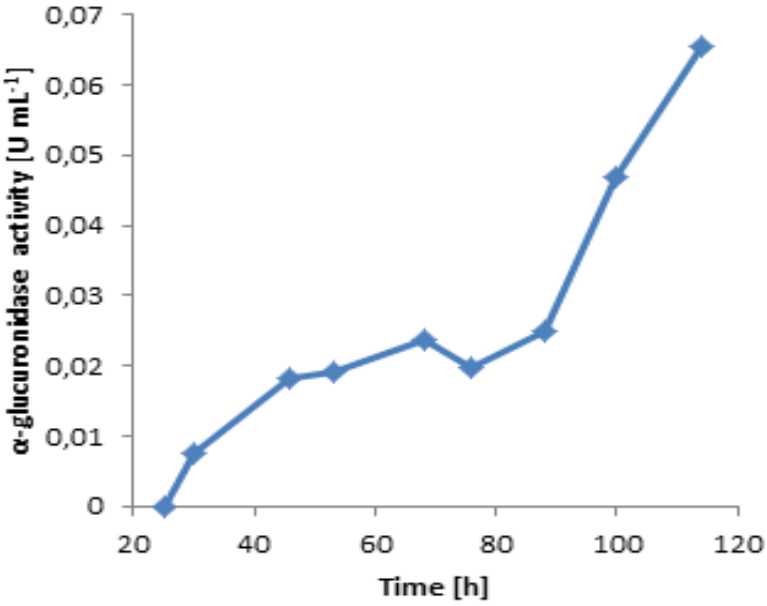

Figure 4.4.3c: α-glucuronidase activity of the engineered *P. pastoris* during the methanol fed-batch phase. During the methanol induction phase *P. pastoris* starts producing α-glucuronidase under activity of AOX promoter. The secretion of α-glucuronidase into extracellular medium increases over the induction phase.

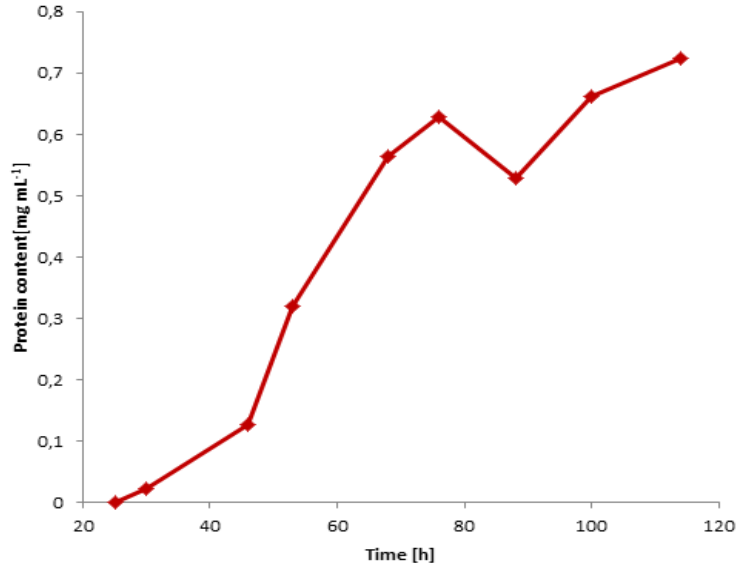

Figure 4.4.3d: Concentration of extracellular protein content by Bradford assay. The concentration of secreted extracellular protein in each sampling time was estimated based on bovine serum albumin.

5. Discussion

In recent years many proteins has been produced recombinantly in *P. pastoris*. Basic features that make methylotrophic *P. pastoris* a good choice for protein production are combination of easy genetic manipulation, high specific growth rates comparable to *E. coli* and an eukaryotic subcellular machinery for performing PTMs (Post Translational Modifications) on proteins (Aoki, et al., 2003). *P. pastoris* SMD1168H was used in this study for the expression of α-glucuronidase enzyme that has wild type methanol utilization phenotype (Mut^+) and benefits from *aox1*. SMD1168H is a protease (carboxypeptidase Y and protease B1) deficient strain (*pep4*$^-$) and it is proven that it reduces the degradation of foreign proteins effectively (Cereghino & Cregg, 2000). Strains such as GS115, SMD1163 and SMD1165 are also Mut^+ phenotypes of *P.pastoris* but KM71phenotype contains *aox2* gene that slows down utilization of methanol while MC100-3 phenotype does not contain even *aox2* gene and cannot grow on methanol and is known as Mut^- phenotype. Except from SMD1168H strain, all of the mentioned strains produce vacuole peptidase A. The amount of vacuole peptidases are considerable in fermenter cultures with high cell density level, due to lysis of a small percentage of cells (Daly & Hearn, 2005).

The amount of secreted protein into extracellular medium in fed-batch fermentation (figure 4.4.3d) of the engineered *P. pastoris* GluC supported our decision to use pPICZαC plasmid for constructing expression vector. AOX1 promoter in the vector derived expression of *Agu1* subcloned in the vector, in transcriptional level. The other important feature of AOX1 promoter is the ability to switching on or off the promoter by changing carbon sources. Bradford and α-glucuronidase assays' results (figures 4.4.3d and 4.4.3e) clearly show that switching between carbon sources from repressive glycerol to methanol at 30 hours after inoculation, started extracellular protein production.

Selection of the signal sequence can be based on the protein's native signal sequence such as α-MF, PHO or SUC2 signal sequences (Li, et al., 2001). It is not possible to predetermine whether using any of the mentioned signal sequences will lead to successful and efficient secretion of the recombinant protein or not (Daly & Hearn, 2005). Many recombinant expression studies has been performed in *P. pastoris* comparing effects of different signal sequences that illustrate α-MF signal sequence has yielded higher secretion of the recombinant protein (Daly & Hearn, 2005). Fermentation of the engineered SMD1168H strain resulted in secretion of large amount of extracellular α-glucuronidase but so far no one

has attempted to overexpress *Agu1*in *P. pastoris* and secrete it by using other signal sequences therefore there is no direct evidence to know the secretion yield of the α-glucuronidase will improve or decrease by using the other signal sequences.

The synthetic *Agu1* originate from *S. commune* which is a non-yeast organism and it should be optimized by altering the codons bias and the GC bases content prior to expression in *P. pastoris*. Codons that are available in *S. commune* may be rare in *P. pastoris*, therefore to avoid limited translation by the amounts of available tRNA in *P. pastoris*, the gene should be codon optimized (biased) for expression in *P. pastoris* (Romanos, et al., 1995). When specific tRNAs are consumed, premature termination of the protein may happen (Leuking, et al., 2000). Also if the gene has high content of AT bases, GC content of the gene should be improved as well (Daly & Hearn, 2005).

We employed triple digestion of the pUC57 vector to isolate *Agu1* with sticky ends. In the first trial *XbaI* and *ClaI* restriction enzymes were used to isolate the gene from pUC57 vector. After running the restriction products on electrophoresis gel instead of two separate bands, one strong intense band between 2500 bp and 3000 bp appeared. The reason why we got a single band was relatively similar molecular weights of the gene and the empty vector 2925 bp and 2710 bp, respectively. We decided to break pUC57 further down with *Sca*I to smaller sizes for precise separation of the gene from the vector. *Sca*I converted empty pUC57 to two separate bands with approximately sizes of 900bp and 1800 bp. By using this strategy a single band at 2900bp corresponding to *Agu1* gene appeared on the electrophoresis gel. Also, pPICZαC vectors were digested with *XbaI* and *ClaI* to prepare an open frame for construction of the expression vector.

During the construction of the expression vector, yield of the ligation reaction significantly improved by increasing the incubation time from 4 hours to 18 hours. The first ligation reaction trial was incubated 4 hours at room temperature. After transformation of the ligation products to *E. coli* DH5α competent cells some ampicillin resistance colonies grown on LB plates. Electrophoresis analysis of the digested plasmids that were isolated from positive transformants revealed that the gene was not inserted in pUC57 vector and resistance of the positive transformants was due to integration of the empty plasmids. The second ligation trial was performed at room temperature and incubated for 18 hours, the results of the diagnostic digestion by *SpeI* and sequencing confirmed cloning of the gene into pPICZαC. *SpeI* is a

single cutter restriction enzyme that just cut inside the gene, precise band at 6600 bp was observed, which is the expected size of the gene and pPICZαC plasmid. As a part of confirmation tests, two of the expression vectors were subjected to sequencing that resulted in full alignment of the sequenced expression vector with the sequence of the gene.

Low efficiency transformation of the expression vector into SMD1168H was improved by increasing the linearized vector concentration. Previously purified vectors from electrophoresis gel resulted in a very low concentration, approximately 0.07 µg μL^{-1}. The required concentration of the linearized vector for electroporation is 1-2 µg μL^{-1}. Concentration of the linearized vectors was improved by direct purification of the restriction products to 0.55 µg μL^{-1}, instead of purification from electrophoresis gel. Another important factor that influences the efficiency of the transformation to *P. pastoris* is the expression vector size. Expression vectors with large size can result in less stable transformats (Daly & Hearn, 2005), so, due to relatively large size of *Agu1*which was 2900 bp, it could have been an additional reason for the low transformation efficiency of electroporation. Alternatively spheroplast transformation method could have been used but the risk of contamination is high and cell lysing enzyme reduces the cell viability (Daly & Hearn, 2005).

We precultured the inoculum in YPD medium at 30℃ and 175 rpm to OD_{600} ≈1.03 for 24 hours before inoculation of the fermentation medium. After 25 hours of glycerol batch culture we reached a wet cell weight of 42 g L^{-1} that was half the expected cellular titer of 90 g L^{-1} wet cell weight suggested by Invitrogen protocol. It could have been due to low cellular concentration of the non-fully grown inoculum, OD_{600} ≈1.03 instead of 3-6. Further feeding of the culture for 5 hour with glycerol resulted in 145 g L^{-1} wet cell weight, which is relatively less than 180 g L^{-1} wet cell weight suggested by Invitrogen protocol. During the methanol induction phase the overall 332 g L^{-1} of wet cellular weight was tittered. In comparison to 350 g L^{-1} wet cell weight in the protocol, the difference between theoretical and experimental titer of wet cell weight in the fermentation was reduced.

Methanol induction phase carried out at 30 ℃ according to Invitrogen's manual for *P. pastoris* Fermentation Process Guidelines, but generally the protein synthesis rate is higher in optimal temperature 25-26℃ (Daly & Hearn, 2005;). Induction of the protein production at 26℃ enhances protein folding pathway and increases cell viability during (Aoki, et al., 2003). Induction in lower temperatures provide more time for the nascent polypeptide chains to be

correctly folded specifically for proteins with the high content of disulfide bridges. In addition lowering the induction temperature may reduce the proteolytic degradation of extracellular enzymes (Li, et al., 2001) over the long induction phase and improve the protein solubility (Aoki, et al., 2003; Chen , et al., 2000).

The time course of the recombinant production of α-glucuronidase by engineered SMD1168H was studied via Bradford and α-glucuronidase assays to determine the yield of the protein production and optimal length of the cultivation. A detectable trace of extra cellular protein was observed at 46 h after inoculation of the fermentation medium by Bradford assay and gradually increased until harvesting the fermentation culture at 114 hour (fig 4.4.3c). Similar increasing pattern was observed by α-glucuronidase assay as can be seen in fig 4.4.3d. The enzyme activity increased drastically after 88 h (58 h after induction) until the end of induction phase. Tenkanen & Siika-aho, (2000) in their attept to produce α-glucuronidase in *S. commune*, produced 4717 mg of extracellular protein after 188 hours of cultivation in final volume of 17 L, whereas, we reached 5784 mg of extracellular protein after 114 hours of the cultivation (86 hours of induction) of the modified *P. pastoris* to the final volume of 12 L. They reported 16 nkat mL^{-1}α-glucuronidase activity of the culture filtrate concentrate (15 time concentrated to the final volume of 265 ml) purified by ion exchange chromatography (DEAE Sepharose FF). The maximum α-glucuronidase activity that we assayed from the culture filtrate was 1.08 nkat mL^{-1}. Lower activity of our enzyme resulted from low concentration of the enzyme in the culture filtrate (8 L filtrate) that can be improved by performing purification of the filtrate with ion exchange chromatography.

Low selectivity of GH115 α-glucuronidase to the aldouronic acids substrate with D-glucuronic acid residues on the non-reducing end of xylan of the oligoxylans contributes to the lower activity of the α-glucuronidase (Kolenova, et al., 2010). Aldouronic acids (tri:tetra:penta) supplied by the kit lacking one adjacent residue due to terminal substitution of glucuronic acid groups on the non-reducing end. Whereas, Tenkanen & Siika-aho, (2000) had investigated the activity of GH115 α-glucuronidase from *S. commune* on a series of polymeric substrates such as glucuronoxylan and acetyl glucuronoxylan from birch wood and arabino-glucuronoxylan from spruce that could be another explanation of the higher activity that they reached.

6. Future works

During the fermentation of the engineered strains identical process parameters suggested by Invitrogen were applied. According to the literature, it is evident that reducing temperature during methanol induction phase increases protein production. Feeding strategy of *P. pastoris* in glycerol and methanol fed batch phases is another process bottleneck to improve. Optimal feed rate increases protein production via increasing biomass generation yield and reducing toxic effect of the accumulated glycerol or methanol. Therefore a set of designed experiments can be suggested to find the optimal fermentation parameters.

The ionic interaction between enzyme and substrate seems to play a key role for the reaction. The role of methyl group of aldouronic acid is not understood so far in the substrate binding mechanism of GH115 α-glucuronidase. To draw a conclusion on the architecture of the substrate binding site, substrate binding and catalysis mechanisms of the enzyme a crystal structure of GH115 family coupled with substrate has to be solved.

7. Conclusion

Modification of SMD1168H *P. pastoris* parental strain for production of GH115 family α-glucuronidase resulted in generation of new strain which is able to extracellular secretion of this enzyme. *Agu1* was codon optimized for overexpression in *P. pastoris* and used for expression cassette construction in pPICZαC vector. All candidate transformants produced extracellular α-glucuronidase and were screened base on the highest α-glucuronidase activity. Batch-fermentation of the screened strain in basal salts and later feeding with glycerol and methanol yielded 732 mg extracellular protein per liter of fermentation filtrate with 0.062 U mL^{-1} α-glucuronidase activity. This enzyme is the only known α-glucuronidase with catalytic activity on polymeric xylan to cleave D-glucuronic acid residues. Development of *P. pastoris*, which is able to grow on a cheap carbon source such as methanol is promising economical process for production of α-glucuronidase in larger scales. On the whole, the results illustrate the capability of *P. pastoris* for heterologous production of biologically active enzymes.

8. Acknowledgment

This thesis is dedicated to the friendship and memory of Kianoosh Asa. He was a chemical engineering master student at Tehran University of Science and Technology who encouraged me over a number of years. He lived his life well, acting upon his spiritual beliefs conscientiously by assisting both friends and strangers in need. Kianooh's strength and faith during the last year of his life gave me a new appreciation for the meaning and importance of friendship. He faced his too early death bravely for freedom and democracy in Iran. Kianoosh shot dead by revolutionary guards in peaceful demonstration after 2009 presidential elections in Iran and his funeral ceremony was held on his thesis defense date. His memories always inspired me and kept me working whenever I wanted to give up.

I would like to express my deepest gratitude to my advisor, Prof. Lisbeth Olsson, for her excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. My special thanks go to Dr. George Anasontzis and Hampus Sunner for guiding my research for the past several months and helping me to develop my background in molecular biology, biochemistry, and fermentation. Undoubtedly without their day and night support this project could have not been successful. The support and encouragement of all friends and colleagues in industrial biotechnology group have been indispensable.

9. References

Aoki, H., Ahsan, M. N. & Watabe, S., 2003. Heterologous expression in *Pichia pastoris* and single-step purification of a cysteine proteinase from northern shrimp. *Protein Expression and Purification,* Volume 31, p. 213–221.

Biely, P., Vries, R. d., Vrsanska, M. & Visser, J., 2000. Inverting charactor of α-glucuronidase A from Aspergillus tubingensis. *Biochimica et Biophysica ,* Volume 1474, pp. 360-364.

Brake, A. J. et al., 1984. Alpha-factor-directed synthesis and secretion of mature foreing proteins in *Saccharomyces cerevisiae*. *Proc Natl Sci USA,* Volume 81(15), pp. 4642-4646.

Brink, J. v. d. & Vries, R. P. d., 2011. Fungal enzyme sets for plant polysaccharide degradation. *App Microbiol Biotechnol,* Volume 91, pp. 1477-1492.

Buchanan, B., Gruissem, W. & Jones, R., 2000. Biochemistry and molecular biology of plants 2 ed.. *American Society of plant Biologists,* Volume 2, pp. 61-75.

Castanare, A., Hay, A. J., Gordon, A. H. & McCrae, S. I., 1995. D-Xylan-degrading enzyme system from the fungus *Phanerochaete Chrysosporium*: isolation and partial characterization of an α-(4-o-methyl)-D-glucuronidase. *Journal of Biotechnology,* Volume 43, pp. 183-194.

Cereghino, J. L. & Cregg, J. M., 2000. Heterologous protein expression in methylotropic yeast *Pichia pastoris*. *FEMS Microbiology reviews,* Volume 24, pp. 45-66.

Chen , Y. et al., 2000. Expression and characterization of glycosylated and catalytically active recombinant human alpha-galactosidase a produced in *Pichia pastoris*. *Protein Expression nad Purification,* Volume 20, pp. 472-484.

Chong, S.-L.et al., 2011. The α-glucuronnidase *Agu1* from *Schizophyllum commune* is a member of a novel glycoside hydrolase family(GH115). *Appl Microbiol Biotechnol,* Volume 90, pp. 1323-1332.

Couderc, R. & Baratti, J., 1980. Oxidation of methanol by the yeast, *Pichia pastoris*. Purification and properties of the Alcohol Oxidase. *Agri. Biol. Chem.,* Volume 44 (10), pp. 2279-2289.

Cregg, J. M. & Higgins, D. R., 1995. Production of foreign proteins in the yeast *Pichia pastoris*. *Canadian Journal of Botany,* Volume 73(S1), pp. 981-897.

Cregg, J. M., Vedvick, T. S. & Raschke, w. C., 1993. Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*. *Nature biotechnology,* Volume 11, pp. 905-910.

Daly, R. & Hearn, M. T., 2005. Expression of heterologous proteins in *Pichia pastoris*: a usefull experimental tool in protein engineering and production. *journal of molecular recognition,* Volume 18, pp. 119-138.

Dammström, S., Salmen, L. & Gatenholm, . P., 2005. The effect of moisture on the dynamical mechanical properties of bacterial. *Polymer,* Volume 46 , p. 10364–10371.

Dasilva, A. E. et al., 2012. Xylan, A Promising Hemicellulose for for Pharmaceutical Use. In: C. J. R. Verbeek, ed. *Products and Applications of Biopolymers.* s.l.:Intech, pp. 61-84.

De Schutter, K. et al., 2009. Genome sequence of the recombinant protein production host *Pichia pastoris*. *Nature Biotechnology,* Volume 27, pp. 561-566.

De Vries, R. P. & Visser, J., 2001. *Aspergillus* Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. *Microbiology and Molecular Biology reviews,* Volume 65, pp. 497-522.

Gatenholm, p., Gröndahl, M. & Eriksson, L., 2004. Material Properties of Plasticized Hardwood Xylans for Potential Application as Oxygen Barrier Films. *Biomacromolecules,* Volume 5, pp. 1528-1535.

Gellissen, G. et al., 1992. Heterologous protein production in yeast. *Antonie van Leeuwenhoek,* Volume 62, pp. 79-93.

Heneghan, M., McLoughlin , L., Murray, P. & Tuohy, M., 2007. Cloning, characterisation and expression analysis of α-glucuronidase. *Enzyme and Microbial Technology,* Volume 41, p. 677–682.

Kolenova, k., Ryabova, O., Vrsanska, m. & Biely, P., 2010. Inverting character of familt Gh115 α-glucuronidases. *FEBS Letters,* Volume 584, pp. 4063-4068.

Leuking, a. et al., 2000. A system for dual protein expression in *Pichia pastoris* and *Escherichia coli*. *Protein Expression and Purification,* Volume 20, pp. 372-378.

Li, P., Gao, X. & Renugopalakrishan, V., 2001. Glycosylated and phosphorylated proteins--expression in yeast and oocytes of Xenopus: prospects and challenges--relevance to expression of thermostable proteins.. *Protein Expression and Purification,* 22(3), pp. 368-380.

Li, Z. et al., 2001. Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. *Protein Expression and Purification,* Volume 21, pp. 438-445.

Nurizzo, D. et al., 2003. the α-Glucuronidase, GlcA67A, of *Cellevibrio japonicus* utilize the carboxylate and methyl groups of aldobiouronic acid as important substrate recognition determinants. *The journal of biological chemistry ,* Volume 278, pp. 20286-20292.

Nurizzo, D., Nagy, T., Harry J., G. & Davies, G. J., 2002. The Structural Basis for Catalysis and specificity of the *Pseudomonas cellulosa* α-glucuronidase, GlcA67A. *Structure,* Volume 10, pp. 547-556.

Olsson, L. & Hahn- Hägerdal, B., 1993. fermentative process of bacteria and yeasts in lignocellulose hydrolysates. *brocess biochem,* Volume 28, pp. 249-257.

Romanos, M., Wellcome, G. & Beckenham, 1995. advances in the use of *Pichia pastoris* for high-level gene expression. *Current Opinion in Biotechnology,* Volume 6, pp. 527-533.

Ryabova , O. et al., 2009. A novel family of hemicellulolytic α-glucuronidase. *FEBS Letters*, Volume 583, p. 1457–1462.

Saxenaa, A., Elderb, . T. J. & Ragauskas, . A. J., 2011. Moisture barrier properties of xylan composite films. *Carbohydrate Polymers*, Volume 84 , p. 1371–1377.

Sreekrishna, K. et al., 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotropic *Pichia pastoris*. *Gene,* Volume 190, pp. 55-62.

Tenkanen, M. & Siika-aho, M., 2000. An α-glucuronidase of Schizophyllum commune acting on polymeric xylan. *Journal of Biotechnology* , Volume 78 , p. 149–161.

Tsujikawa, M., Okabayashi, K., Morita, M. & Tanabe, T., 1996. Secretion of a Variant of Human Single-Chain Urokinase-Type Plasminogen Activator without an N-GlycosylatLon Site in the Methylotrophic Yeast, *Pichia pastoris* and Characterization of the Secreted Product. *Yeast,* Volume 12, pp. 541-553.

Viikari, L., Kantelinen, A., Buchert, J. & Plus, J., 1994. Enzymatic accessibility of xylans in lignocellulosic materials. *Appl Microbiol Biotechnol,* Volume 41, pp. 124-129.

10. Appendix

10.1 Preparing 1 % agarose gel for DNA analysis

Components	Quantity
TAE buffer 1X	125 ml
Agarose powder	1.25 g

Dissolve the solution by incubation in microwave for 3 min. Cast the solution in a mold with appropriate size.Gel forms after 40 min incubation in the room temperature.

10.1.1 Protocol for preparing 50X TAE buffer stock solution

1. Dissolve 242 g Tris base in 750 mL water.
2. Supplement the solution with 100 mL of 0.5 M EDTA pH 8.0 and 57.1 mL of glacial acid.
3. Fill the bottle to the final volume of 1 L.
4. Store the 50X TAE stock solution at room temperature.

10.1.2 Gel electrophoresis protocol

1. Put the agarose gel in the electrophoresis chamber and fill the chamber with 1X TAE buffer to cover the gel completely.
2. Mix appropriate amount of 6X loading dye with 5-50 µl of DNA fragments and load into each well on the gel.
3. Cover the chamber with the lead and apply 60-85 vlot for 30-120 minutes depending on the size of the DNA fragments.
4. Stain the gel in gel red solution for 30 minutes to visualize the bands.
5. Observe the stained gel in UV chamber.

10.2 Extraction of plasmid DNA from *E. coli* DH5α

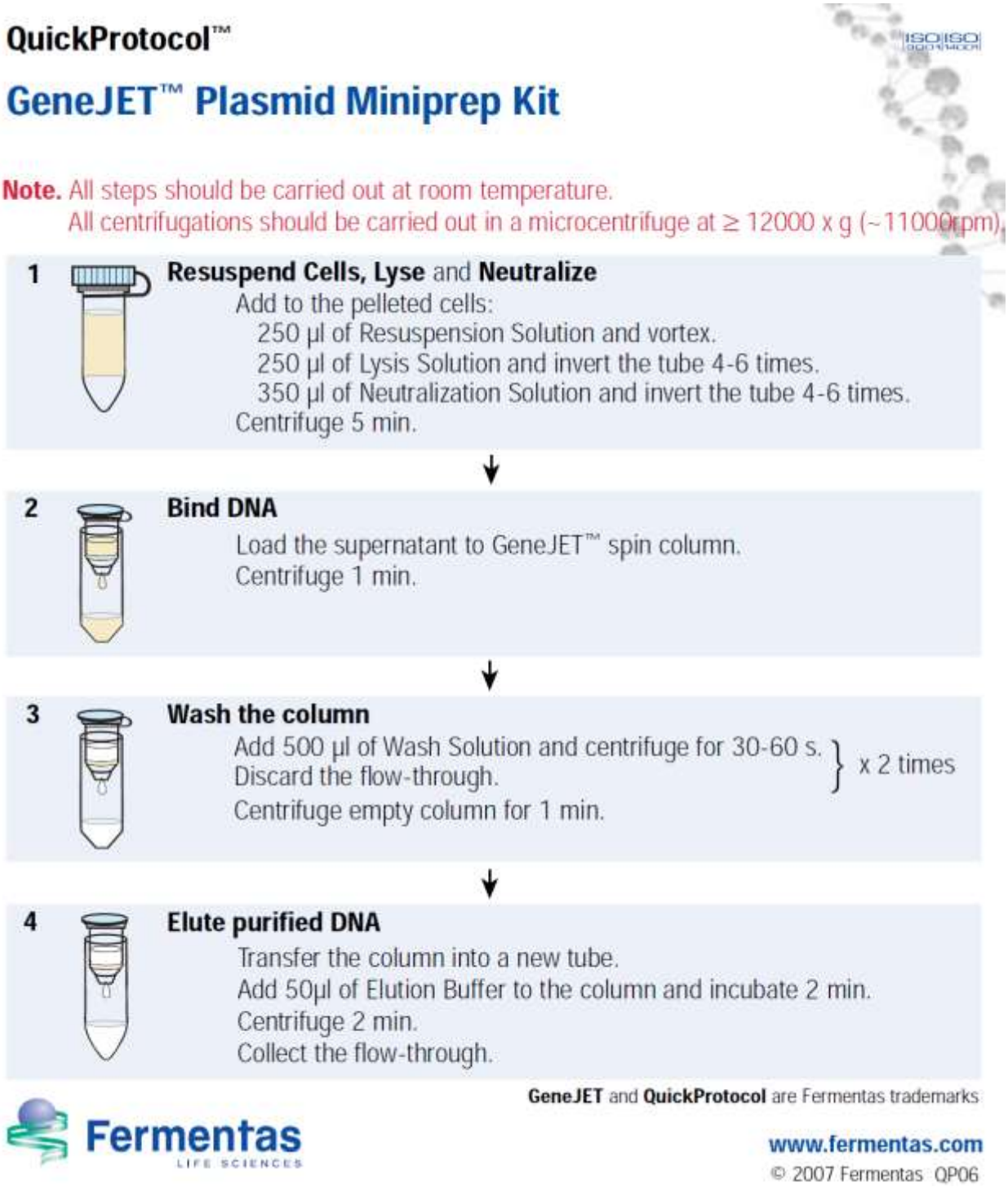

Figure 10.2 Quickprotocol™ – GeneJET™ Plasmid Miniprep Kit: Used for extraction and purification of DNA/plasmids from *E.coli* (http://www.fermentas.com).

10.3 Fermentation of *P. pastoris*

Parameter	Value
Temperature	30℃
Dissolved oxygen	30%
pH	5.0
Agitation	1000
Aeration	0.1-1.0 vvm
Carbon sources	Glycerol- Methanol

Ref:

Invitrogen. *Pichia* Fermentation Process Guidelines. Version B 30 May 2002 [cited 2012,05,01];

Available from: https://tools.invitrogen.com/content/sfs/manuals/pichiaferm_prot.pdf