TAMPERE UNIVERSITY OF TECHNOLOGY
Degree Programme in Environmental and Energy Technology

NACHI ANADA

Towards Extracellular Foreign Protein Expression in *Acinetobacter baylyi*

Master of Science Thesis

Examiners: Professor Matti Karp
and Ph.D. Ville Santala
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Abstract

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Acinetobacter baylyi is a soil bacterium which has been known for unique biological features and metabolic pathways. Particular interest has been paid to *A. baylyi* ADP1 strain to study metabolism due to their high competence with natural transformation. The ability of natural transformation in ADP1 is also found in $B2^T$ wild-type strain; therefore, this study focused on heterologous gene expression in $B2^T$ wild-type strain.

Foreign proteins expressed in this study were *Bacillus stearothermophilus* α-amylase and yellow fluorescent protein Citrine. The putative fusion partner proteins were co-expressed with Citrine for studying extracellular expression in *A. baylyi*. The level of expression was studied by measuring enzymatic activity and fluorescence signal.

α-Amylase was successfully expressed and produced in *A. baylyi*; though its enzymatic activity level was not high due to the weak promoter used in the plasmid construction. The Citrine fusion protein expression resulted in high fluorescence signal detection in intracellular parts, but not in extracellular parts. This result showed that extracellular expression of Citrine using the selected fusion partner proteins was not observed in *A. baylyi*. The high fluorescence signal was remained up to 72 hour incubation when the cells were grown in optimal growth medium; therefore, fluorescent proteins could be used as reporter genes in the fusion gene construction in *A. baylyi*.

Preface

This thesis is based on the study conducted during September 2009 to August 2010 at the Department of Chemistry and Bioengineering, Tampere University of Technology, Finland. The study is a continuation of the project that aims to characterize the strain *Acinetobacter baylyi* as a new model organism for biotechnological applications.

I would like to express my sincere gratitude to my supervisor professor Matti Karp, for his guidance and kind support during the course of the study. I would like to thank my advisors Ph.D. Ville Santala and researcher Suvi Myllyntausta for giving me useful advice and directing the laboratory experiments. This thesis could not have been completed without their support and encouragement.

I would like to thank all of my coworkers in the laboratory and the staff in the Department of Chemistry and Bioengineering for their assistance and advice. Especially I would like to thank researcher Bobin Abraham for conducting the excitation/emission spectra measurement and providing the pAK400c plasmid. Finally I wish to express my greatest thanks to my family and friends for their kind support.

11th August 2010, Tampere

NACHI ANADA

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Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine 5’-triphosphate
DE	Dextrose equivalent
DNA	Deoxyribonucleic acid
DNS	3,5-Dinitrosalicylic acid
dNTP	Deoxyribonucleotide triphosphate
ED	Entner-Doudoroff
EM	Embden-Meyerhof
GFP	Green fluorescent protein
LB	Luria broth
MFP	Membrane fusion protein
OD_{600}	Optical density at 600 nm
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEF	Pulsed electric field
RBS	Ribosome binding site
YFP	Yellow fluorescent protein

1 Introduction

Ever since the utility of restriction endonuclease was discovered by K. Danna and D. Nathans in 1971 (Danna and Nathans 1971), recombinant DNA technology has played a significant role in scientific fields. It is an essential tool in wide range of scientific research areas from studying novel proteins to newly developed biofuel production. Among many other microorganisms, non-pathogenic *Escherichia coli* has served as the most common host organism used in recombinant engineering in laboratories. Enormous amount of data about genomics, proteomics and metabolomics of *E. coli* strains has been collected and elucidated for further interactional and functional mechanisms.

Recently, new model prokaryotic organisms for study of metabolism have been explored in addition to *E. coli*, and a particular interest has been paid to *Acinetobacter baylyi* ADP1 strain (de Berardinis et al. 2009). Its unique biological features and metabolic pathways are worth studying, and on top of that, some of *A. baylyi* strains are known to be highly competent for natural transformation. For this matter, exploration of heterologous protein expression in *A. baylyi* would be an interesting step for utilizing *A. baylyi* as a common host organism in recombinant engineering.

Recombinant protein expression and production are quite common processes in biotechnology. Protein production is often accompanied by additional steps for harvesting, since many host organisms used in protein expression do not secrete the expressed proteins. Therefore, expression of recombinant proteins in extracellular or periplasmic regions has been studied for simpler harvesting process and less damage to the proteins. Extracellular protein production has been successfully achieved by several methods including use of a secretive fusion partner.

In this thesis work, foreign protein expression in *A. baylyi* type strain $B2^T$ wild-type strain was conducted. The proteins expressed were thermostable α-amylase and yellow fluorescent protein (YFP) Citrine. Extracellular expression of these proteins was also investigated by co-expressing possible fusion partner genes from *A. baylyi* $B2^T$ wild-type. Level of expression was studied by α-amylase activity and fluorescence signal measurement. Further discussion and concluding remarks were drawn based on the obtained results.

2 Theoretical background

2.1 Microbiology of Acinetobacter baylyi

2.1.1 Acinetobacter species

The genus *Acinetobacter* is a Gram-negative, non-motile and non-fermentative bacterium which belongs to the class *γ-proteobacteria*. *Acinetobacter* species are widespread in nature and can be obtained from water, soil, and living organisms (Barbe et al. 2004). They are known to mineralize aromatic compounds in soil environments. So far 32 genomic groups have been identified by DNA-DNA hybridization, and 17 species have been given names (Dijkshoorn and Nemec 2008). Little is known about the biology of the *Acinetobacter* species because methods for species identification are not fully developed. However, because of their availability and metabolic versatility, *Acinetobacter* species have been studied for wide varieties of biotechnological applications since the early seventies: production and application of environmentally useful products and processes such as biodegradation and bioremediation of organic pollutants, biopolymer production, bioemulsifier and enzyme production, development of potential applications for biosorption and removal of toxic heavy metals from aquoeous systems, as well as the use of *Acinetobacter* species in the removal of inorganic phosphate from waste water treatment facilities (Gutnick and Bach 2008).

2.1.2 Acinetobacter baylyi

Acinetobacter baylyi was first identified at metagenomic level from the activated sludge (Carr et al. 2003), and later the complete genome sequence of *A. baylyi* strain ADP1 was obtained by the work of Barbe et al. in 2004. A single circular chromosome was about 3.6 Mbp (mega base pairs) and its G+C content was 40 %. The sequence analysis identified that about 35 % of the sequence is devoted to transport, metabolic and enzymatic functions, indicating the large metabolic versatility of this organism (Barbe et al. 2004). Moreover, *A. baylyi* ADP1 has a highly competent transformation system and an ability to be manipulated by homology-facilitated recombination with linear DNA fragments, which is desirable for genetic manipulation and introduction of foreign genes (de Vries and Wackernagel 2002; Gutnick and Bach 2008). The strain used in this study, $B2^T$ wild-type strain, was isolated from the activated sludge in Australia (Carr et al. 2003). The complete genome sequence has not been obtained for $B2^T$; however, 16S rRNA gene sequence analysis demonstrated that $B2^T$ shared the high similarity (98.2 %)

with ADP1. Natural transformation efficiency of ADP1 and $B2^T$ was reported to be of the same order of magnitude, suggesting that these strains share the similarity both at genotypic and phenotypic levels. (Vaneechoutte et al. 2006.)

Genetic investigations of *A. baylyi* ADP1 have revealed its potential to be a model organism for studies of metabolism. ADP1 possesses excellent natural competence, ease of heterologous gene expression and relatively small number of genes (around 3300 genes), which is great for construction of novel metabolic systems (Young et al. 2005; de Berardinis et al. 2008). In addition, ADP1 shares most of the features of *E. coli* as an excellent laboratory host organism. For example, it is non-pathogenic, aerobic, able to grow overnight on both rich and minimal salts media and grows optimally between 30 to 37 °C, and most of the antibiotics used with *E. coli* are effective for ADP1 (Metzgar et al. 2004). Gene functions and the identification of the essential genes of ADP1 were studied and compared with other organisms by constructing single-gene deletion mutants. According to de Berardinis and coworkers, 499 (16 % of total genes) of total 2594 deletion mutants were possibly essential genes in ADP1, of which 88 % was shared with *E. coli* deletion mutant data set and 80 % was consistent with *Pseudomonas aeruginosa* orthologous essential genes. (de Berardinis et al. 2008.) Close relationship with these well-studied organisms suggests that it is possible to directly apply vast knowledge about metabolism and gene function of these organisms to ADP1 system (Metzgar et al. 2004).

Although *A. baylyi* ADP1 and *E. coli* have similarity at the genetic level, novel metabolic pathways have also been elucidated. One example is glucose catabolism in the absence of pyruvate kinase. Pyruvate kinase is involved in the final conversion of phosphoenolpyruvate to pyruvate in glycolysis; therefore, lack of pyruvate kinase gene confirms that ADP1 uses either Entner-Doudoroff (ED) pathway or a modification of ED pathway instead of Embden-Meyerhof (EM) pathway used in many prokaryotes including *E. coli* (Barbe et al. 2004; Young et al. 2005). As shown in Figure 1, the initial uptake of glucose into the cell is facilitated by a periplasmic oxidation through the membrane-bound glucose dehydrogenase. Glucose is then oxidized to gluconate and followed by ED pathway (Barbe et al. 2004). In principle, ED pathway can generate 1 adenosine triphosphate (ATP) per 1 glucose; therefore, it is metabolically less efficient compared to EM pathway that generates 2 ATP per 1 glucose. However, *Acinetobacter* species including ADP1 can utilize variety of other carbon sources such as aromatic compounds using β-ketoadipate pathway (Metzgar et al. 2004; Siehler et al. 2007).

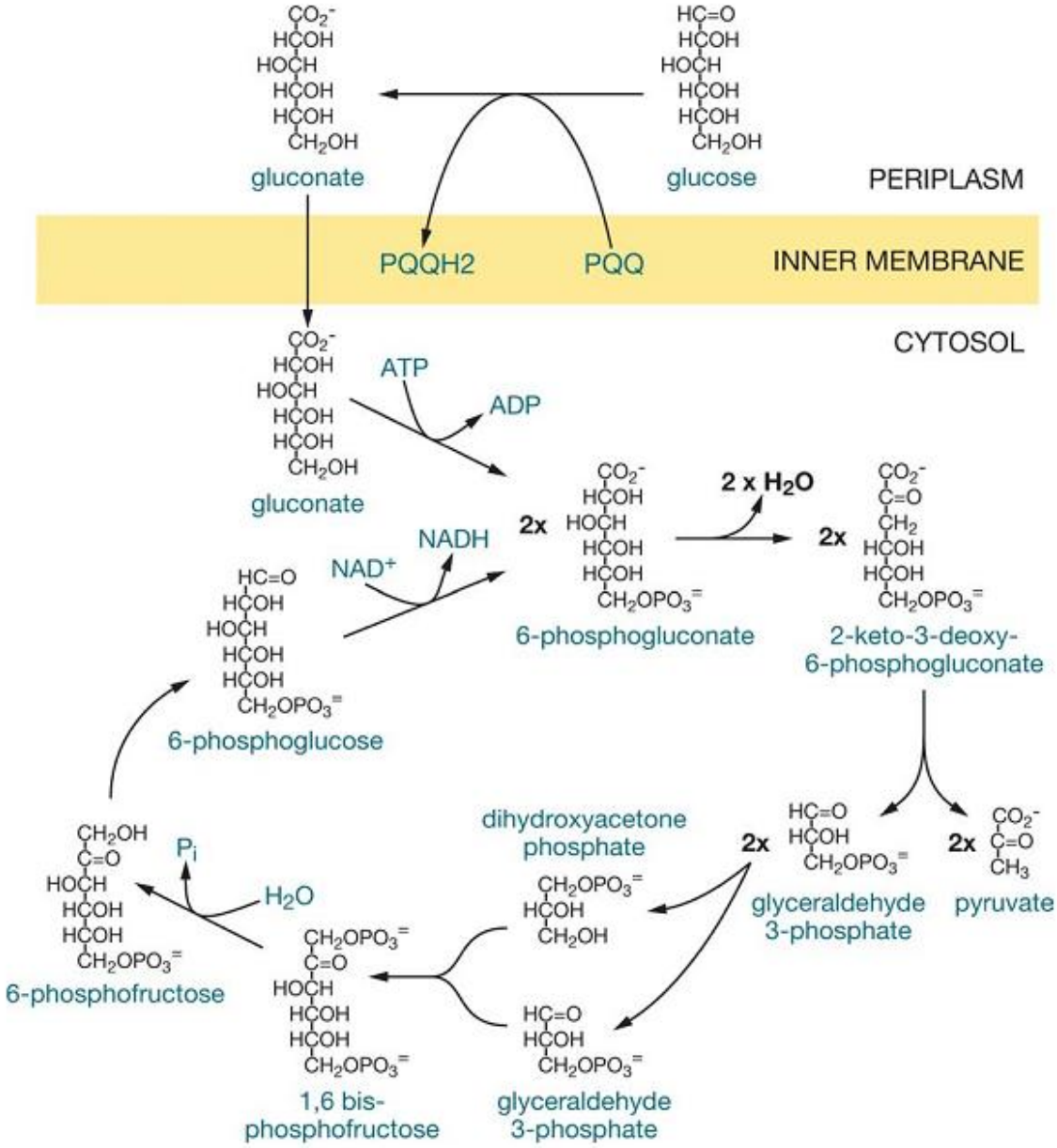


Figure 1. Glucose utilization in the absence of pyruvate kinase presented by Lessie and Phibbs (1984) (Figure taken from Young et al. 2005). PQQ = pyrroloquinoline quinone; PQQH2 = pyrroloquinoline quinol; ADP = adenosine diphosphate; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced form of NAD^+; P_i = phosphate group.

The accumulated 'omics' knowledge allows us to use *A. baylyi* in various applications. Recently, interest has been paid to utilizing *A. baylyi* as a producer of biofuels due to their ability to produce wax ester derivatives (Stöveken et al. 2005; Uthoff et al. 2004). Kalscheuer and coworkers demonstrated biosynthesis of biodiesel-adequate fatty acid ethyl esters (FAEEs) in metabolically engineered *E. coli* with a wax ester synthase gene *atfA* from *A. baylyi* ADP1 and ethanol producing genes from *Zymonomas mobilis* (Kalscheuer et al. 2006). By this approach, 26 % of the cellular dry mass was identified as a FAEE content of the cells. In this case, *A. baylyi* gene was used in recombinant

genes of *E. coli*; however, their study indicates that this gene may be a key element for accumulation of lipids intracellularly, thus promoting biodiesel production. Furthermore, the biosynthesis system similar to the above was combined with using hemicelluloses as carbohydrates for microbial production of biodiesel (Steen et al. 2010). Since microbial production of fatty acid esters saves the costly chemical transesterification process, the biofuel production system presented here would be a promising platform for production of high-energy fuels (Rude and Schirmer 2009).

2.2 Extracellular expression of recombinant proteins

2.2.1 Extracellular expression in E. coli

Non-pathogenic, Gram-negative *E. coli* strains do not usually secrete high amount of proteins (Mergulhão et al. 2005). However, extracellular production of recombinant proteins in *E. coli* has several advantages over intracellular production. The secretory recombinant proteins offer many applications, for example vaccine development, immobilized enzymes and bioremediation, due to simple downstream processing and higher product stability and solubility (Cornelis 2000; Mergulhão et al. 2005). In addition, they can be used for N-terminus authenticity (Makrides 1996). Secretion of proteins to the culture medium may occur either by extracellular production of the proteins or 'leakage' of the proteins located in the periplasmic region of the cells. *E. coli* are known to have five different types (type I-V) of secretory pathways, of which the most common type I and II are explained below.

Type I secretory pathway

Type I secretion mechanism allows the secretion of proteins from the cytoplasm to the extracellular medium in a single step, without a periplasmic intermediate. The secretion is carried out by a translocator consisting of three proteins that span the cell envelope: a specific outer membrane protein (OMP), an ATP-binding cassette (ABC) and a membrane fusion protein (MFP) (Delepelaire 2004). ABC protein is localized in the cytoplasmic membrane while MFP is located in the periplasmic region and OMP is located across the outer membrane (Figure 2). ABC proteins, mostly consisting of two membrane-embedded hydrophobic and two conserved hydrophilic ATP-binding domain, translocate proteins into the extracellular space by directly using the energy generated from ATP hydrolysis (Binet et al. 1997; Omori and Idei 2003). The two accessory proteins, MFP and OMP, are also required for protein secretion in most Gram-negative bacteria, which is why the whole apparatus is referred to as the ABC protein exporter or ABC transporter.

These protein exporters are dedicated to the secretion of proteins belonging to or closely related to the toxin, protease and lipase families (Binet et al. 1997). Even though the ABC protein exporters and the extracellular proteins are usually linked as cluster genes,

exoproteins and recombinant proteins are efficiently secreted by heterologous exporters when co-expressed in *E. coli* (Chung et al. 2009; Eom et al. 2005; Guzzo et al. 1991). As a recent example, Chung and coworkers designed the partial lipase ABC transporter domains based on comparative modeling of *Pseudomonas fluorescens* lipase, attached them genetically to green fluorescent protein (GFP) and epidermal growth factor (EGF), and expressed these fusion proteins in *E. coli* together with ABC transporter of either *P. fluorescens* or *Erwinia chrysanthemi* (Chung et al. 2009). Even though the secretory protein expression in *E. coli* facilitating the ABC transporter of *P. fluorescens* was not superior to the homologous expression in the original *P. fluorescens* (Ahn et al. 2001), their result showed that partial ABC transporter domains could be designed and applied to a common host organism like *E. coli*.

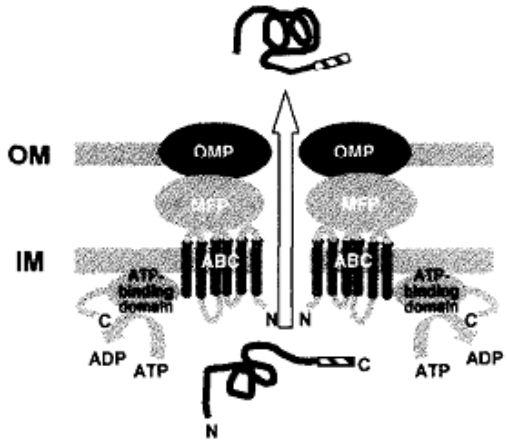


Figure 2. Schematic view of type I secretion pathway in Gram-negative bacteria (figure taken from Omori and Idei 2003). OM = outer membrane; IM = inner membrane; N = amino terminus; C = carboxyl terminus.

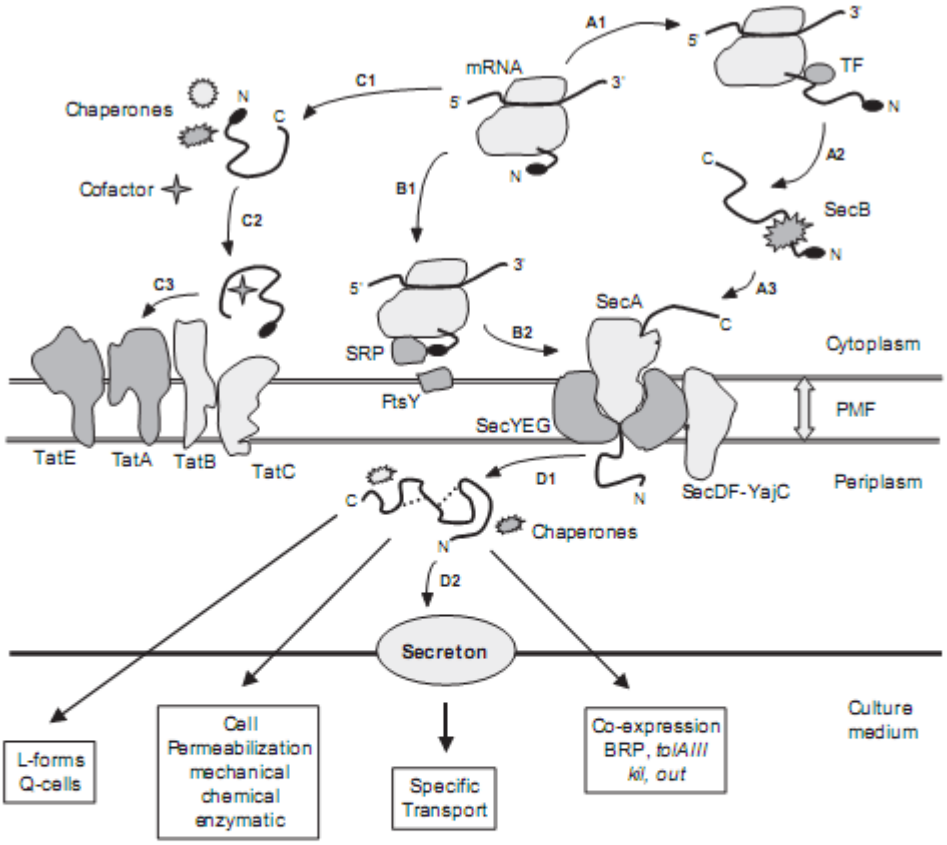
Type II secretory pathway

Type II secretion system is a two-step process called the general secretory pathway (GSP), in which exoproteins are translocated across the inner and outer membranes in two steps. The first step involves translocation across the inner membrane, where the transported protein is synthesized as a precursor with an N-terminal cleavage signal peptide. The translocation across the inner membrane occurs in three ways: the Sec-B dependent pathway, the signal recognition particle (SRP) pathway and twin-arginine translocation (TAT) pathway. These inner membrane translocation pathways are well summarized in Figure 3, which was reviewed by Mergulhão and coworkers (Mergulhão et al. 2005). After cleavage of the signal peptide, the mature proteins are released into the periplasm and folded correctly. The translocation across the outer membrane is further assisted by machinery which involves 12-16 different proteins called "secreton" (Filloux 2004).

Extracellular expression of recombinant protein using type II secretion pathway has been attempted in Gram-negative bacteria including *E. coli*; however, it has shown difficulty presumably due to high specificity to species (Lindeberg et al. 1996). Though

species-specificity of type II secretion machinery, Zhou and coworkers successfully expressed and secreted *Erwinia chrysanthemi* endoglucanase (*CelZ*) in *E. coli* B by co-expressing *E. chrysanthemi out* secretory system (Zhou et al. 1996). By adding the *out* genes, they were able to secrete over a half of active enzyme into the growth medium. The *E. coli* secreton machinery was also studied for extracellular expression of endogenous protein. Francetic and coworkers showed that in *E. coli* K12, transcription of the two operons of the main *gsp* locus, encoding the secreton components, was silenced by the nucleoid-structuring protein H-NS (Francetic et al. 2000). The mutants lacking H-NS successfully secreted endogenous, co-regulated endochitinase *chiA*, promoted by the secreton genes co-expressed.

Strategies for extracellular protein expression in *E. coli* can be divided into three categories: 1) use secretion systems that naturally exist in *E. coli* pathogens; 2) use fusion partner proteins with no known translocation mechanisms; 3) alter outer membrane permeability by using cell envelope mutants or co-expression of a lysis-promoting protein (Ni and Chen 2009). Some examples of extracellular protein expression in *E. coli* strains using secretion systems such as type I and II are presented above. The third strategy aims at secretion of proteins by changing cell structures, rather than engineering extracellular proteins, so that downstream processing would become easier. In this study, the main focus fell into the second strategy, since it was necessary to translocate fusion proteins in both *E. coli* and *A. baylyi*, where no known secretory mechanism is available so far in *A. baylyi* strains. Extracellular expression in *E. coli* using fusion partner was therefore reviewed in the next section and several fusion partner genes were selected for this study.



Recombinant protein secretion by the type II mechanism and strategies for the extracellular release of recombinant proteins from the periplasm. On the SecB-dependent pathway, the protein emerges from the ribosome and binds to trigger factor (TF) (step A1). The protein is then recognised by SecB (step A2), which targets it to the membrane-bound SecA (step A3). At the translocation point, a group of proteins (Sec Y, SecE, and SecG) forms a translocation complex that threads the protein at the expense of ATP hydrolysis. At a later stage, the proton-motive force (PMF) can drive the translocation. On the SRP pathway, the nascent chain is recognised by SRP (step B1). The SRP–ribosome complex interacts with FtsY, thus releasing the nascent chain to the translocation site (step B2). On the TAT pathway, the protein is fully synthesized and folds in the cytoplasm where it can bind specific cofactors (step C1). The signal peptide is then recognised by TatC in the TatBC complex (step C2). Signal peptide binding promotes association of the complex with TatA oligomers at the expense of PMF. Protein translocation occurs through a channel formed by TatA and possibly TatE oligomers (step C3). Within the periplasm, the protein is folded and adopts tertiary or even quaternary structures (step D1). The protein is then transported by a secretion machinery named "secreton" which is composed by 12–16 proteins (step D2). Extracellular release of periplasmic proteins can also be achieved by several strategies like the use of leaky strains, cell membrane permeabilization, or coexpression of release proteins.

Figure 3. Type II secretion system (Mergulhão et al. 2005).

2.2.2 Extracellular expression using fusion partner

The fusion proteins were commonly used for the production of extracellular recombinant protein in *E. coli*, even though the mechanism by which the fusion proteins pass through the outer membrane is not known and factors that influence the transport are not identified (Ni and Chen 2009). Main study of extracellular expression using fusion partner proteins is summarized in Table 1.

Table 1. Fusion partner proteins for extracellular protein expression in *E. coli* strains.

Fusion partner protein	Protein secreted	*E. coli* strain	Characteristics	References
OmpA	Human growth hormone (hGH)	K12	> 98 % purity achieved by reverse-phase column chromatography	Hsiung et al. (1989)
OmpF	β-Endorphin	BL21(DE3)	5.6 g/L fusion protein excreted	Jeong and Lee (2002)
Signal peptide of OmpT	Penicillin amidase (PA)	K5	Translocation efficiency increased	Ignatova et al. (2003)
YebF	α-Amylase	HB101	42.6 % protein secreted	Zhang et al. (2005)
OsmY	α-Amylase, alkaline phosphatase (PhoA), human leptin	BL21(DE3)	250-700 mg/L excretion by high-cell-density cultivation	Qian et al. (2008)

All of *E. coli* strains in Table 1 were non-pathogenic, commonly used strains which normally do not secrete proteins. Therefore, these fusion partner proteins have great potential to be used for extracellular recombinant protein production in larger scale.

Little is known about secretory systems of *A. baylyi* except for secretion mechanism for extracellular protein lipase. Lipase (EC 3.1.1.3: triacylglycerol hydrolase) catalyzes the hydrolysis and synthesis of long-chain acylglycerols with trioleoylglycerol as the standard substrate (Jaeger and Eggert 2002). The extracellular lipase of *A. baylyi* ADP1 is presumably exported through a two-step translocation process: the translocation across the cytoplasmic membrane mediated by the Sec system, whereas the subsequent translocation through the outer membrane is processed by a translocation complex similar to the Xcp system in *P. aeruginosa* (Kok et al. 1995). In addition to these secretion-assisting proteins, a lipase-specific chaperone LipB and disulfide bridge formation by periplasmic protein PdoA are required for the processing of lipase (Kok et al. 1996). Secretory systems derived from toxins are considered to be absent in *A. baylyi* ADP1, even though hemolysin-like proteins are encoded in ADP1 genome (Barbe et al. 2004). The putative secretive proteins are all enzymatic, where the protein expressed in ADP1 strain or similar organism was secreted (Elsemore and Ornston 1995). One of the proteins involved in quinate metabolism, QuiB, may function as a fusion partner protein in *A. baylyi* due to its relatively small molecular weight.

According to Microbial Genome Annotation Platform (MaGe) for ADP1 genome, however, none of the proteins listed in Table 1 are encoded in ADP1 strain genome, except for a putative outer membrane protein precursor which is similar to OmpA (Vallenet et al. 2009). In addition, Qian and coworkers conducted proteome analysis of *E. coli* BL21 (DE3) strain for seeking suitable fusion partner proteins. They were able to pick 22 proteins with molecular weight below 40 kDa, of which 12 overexpressed proteins were detected in the culture medium (Qian et al. 2008). 5 of these proteins were

encoded in ADP1 genome and 4 of them were suitable for cloning; therefore, they were chosen as potential fusion partner proteins for extracellular expression in *A. baylyi*. Table 2 shows main characteristics of the selected fusion partner proteins for this study. Since OmpA-like putative protein may function as membrane-bound precursor, only signal peptide region of the protein was used for cloning. Cloning of signal peptide region was also applied to DsbA protein. These signal peptide regions were predicted using SignalP 3.0 server (Bendtsen et al. 2004).

Table 2. Proteins of *A. baylyi* selected for fusion partners in this study.

Protein name [a]	Accession No. [a]	Protein description [a]	Localization [a]	Alignment score with *E. coli* K12[b]	M_w (kDa)
OmpA	ACIAD0697	Putative outer membrane precursor (OmpA-like)	Membrane	18	38.5
CysP	ACIAD2591	Sulfate transport protein	Periplasmic	45	37.2
DsbA	ACIAD0045	Thiol-disulfide interchange protein, alkali-inducible	Periplasmic	28	23.2
YceI	ACIAD2034	Conserved hypothetical protein; putative signal peptide	Unknown	31	20.8
QuiB	ACIAD1713	Catabolic 3-dehydroquinate dehydratase	Secreted	n/a	30.1

[a]The protein name, accession No., protein description and localization were obtained from MaGe server (https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php?).

[b]Protein sequences of *A. baylyi* ADP1 and *E. coli* K12 strains were aligned using ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.3 Proteins co-expressed with fusion partners in this study

2.3.1 α-Amylase

2.3.1.1 Properties of α-amylases

α-Amylases are one of the oldest yet most important industrial enzymes. α-Amylases (EC 3.2.1.1: endo-α-1,4 glucan-4-glucanohydrolase) belong to enzyme class hydrolase, and they catalyze the endohydrolysis of 1,4-α-glycosidic linkages in starch and other polysaccharides containing 3 or more 1,4-α-linked D-glucose units (Figure 4). Hydrolysis of starch by α-amylase gives maltodextrins, maltose and glucose as byproducts (Gangadharan et al. 2008). As shown in Figure 5, maltose is a molecule in which two D-glucose units are linked together with 1,4-α-bond. Maltodextrin is a saccharide polymer consisting of D-glucose units linked primarily by 1,4-α-bonds and that has a dextrose equivalent (DE) of less than 20. DE is defined as follows (Chaplin and Burke 1990):

$$DE = 100 \times \left(\frac{Number\ of\ glycosidic\ bonds\ cleaved}{Initial\ number\ of\ glycosidic\ bonds\ present}\right) \quad (1)$$

In practice, DE shows how much starch is converted to glucose and also represents sweetness of a saccharide.

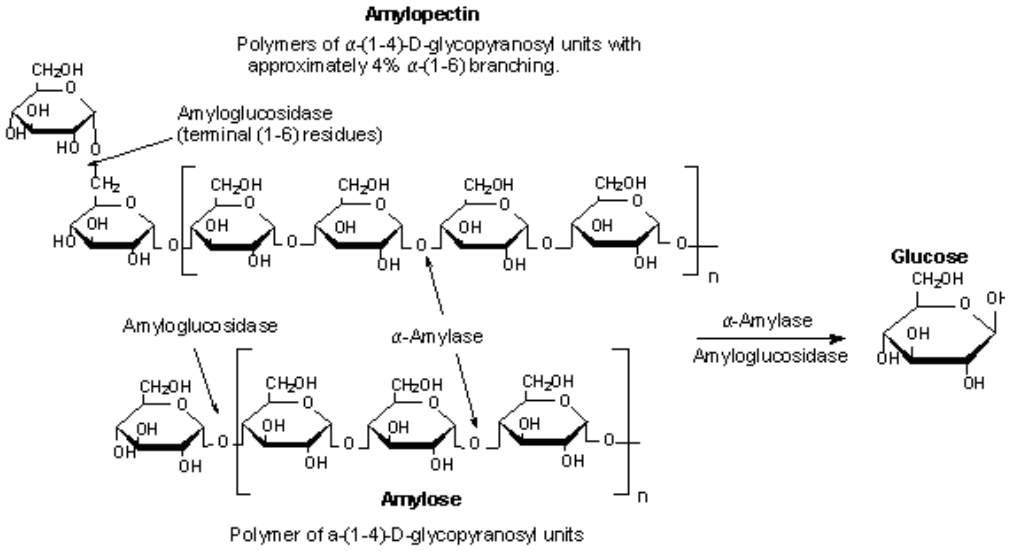


Figure 4. Hydrolysis of starch (figure taken from: http://www.sigmaaldrich.com/life-science/metabolomics/enzymatic-kits.html).

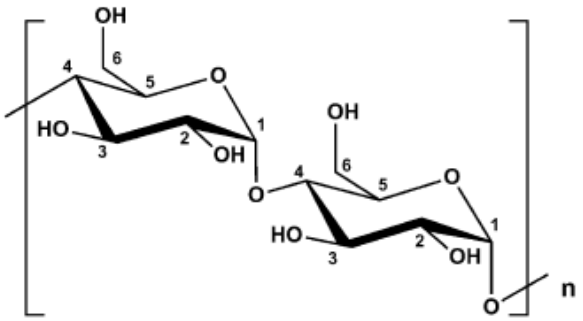


Figure 5. Structure of maltose unit and maltodextrin (figure taken from Wangsakan et al. 2004).

α-Amylases are distributed among a variety of organisms including animal, plant and microorganisms; however, industrial α-amylases are mostly derived from microorganisms because of their desirable characteristics for enzyme production and industrial applications (Gupta et al. 2003). Wide variety of microorganisms produces extracellular α-amylase, but fungal and bacterial α-amylases are mostly dominant in industrial use. Table 3 shows different sources of α-amylase, their optimal conditions for α-amylase activity and industrial usages.

Table 3. Source, optimal conditions and industrial use of some microbial α-amylases.

Source	Optimal pH	Optimal temperature	Industrial use	Reference
Fungal				
Aspergillus oryzae	5.4	50 °C	Used to produce more glucose in baking industry; used as a digestive aid	Yabuki et al. (1977), Wiseman (1975)
A. niger	4.0-4.5	60 °C	Saccharification process in production of glucose syrup	Chaplin and Burke (1990)
Bacterial				
Bacillus subtilis	6.5	50 °C	Starch liquefaction and processing industries; distilling starch glucose and syrups; removal of starch sizes on textiles	Wiseman (1975)
B. amyloliquefaciens	5.0	50 °C	Starch liquefaction process	Chaplin and Burke (1990), Gangadharan et al. (2009)
B. licheniformis	9.0	76 °C	Starch liquefaction process	Saito (1973)
B. stearothermophilus	4.6-5.1	55-70 °C	Starch liquefaction process	Manning (1961)

2.3.1.2 Industrial applications of α-amylases

α-Amylases are widely used in food processing, fermentation processes, paper and pulp industry, and textile industry which require the hydrolysis of starch. Microbial amylases have completely replaced chemical hydrolysis in the starch processing industry, and they can be potentially used in the pharmaceutical and fine chemical industries. (Gupta et al. 2003.) This section deals with short introduction of various applications of α-amylases.

Starch liquefaction and saccharification

The discovery of converting starch to oligosaccharides and dextrose by the use of various types of amylase accelerated the growth of starch-processing industries (Gerhartz 1990). The most important applications of starch processing are high-fructose corn syrup manufacturing and fuel alcohol production. Figure 6 shows the process for the conversion of starch to syrups. The conversion of starch is divided into three stages: 1) gelatinization, 2) liquefaction and 3) saccharification, which involves different degrees of hydrolysis expressed as DE.

As shown in Figure 6, bacterial α-amylases are used in gelatinization and liquefaction processes since these processes require hydrolysis at high temperature to remove lipid-starch complexes. Therefore, thermostable α-amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are used in these processes. The pH of starch slurry is naturally around 4.5; however, it should be adjusted to near 6.0 by adding NaOH as the α-

amylases are not active at a pH below 5.9. After the starch is liquefied with 11-20 DE, either fungal α-amylases or glucoamylases are used for further conversion to glucose and maltose, respectively. *Aspergillus niger* or similar species are the source of enzymes for saccharification. (van der Maarel et al. 2002.)

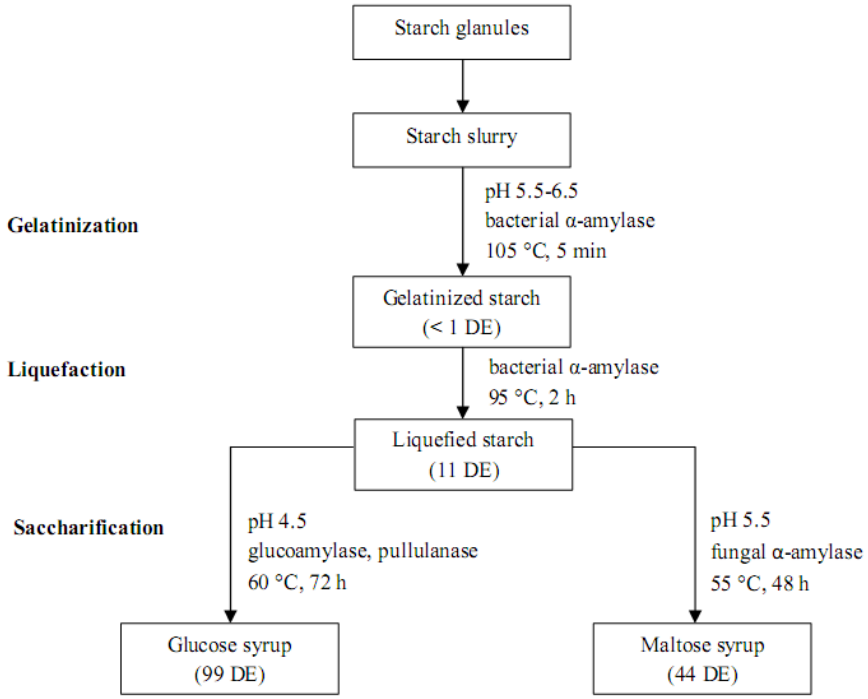


Figure 6. Process for the conversion of starch to glucose and maltose syrups (figure taken from Chaplin and Burke 1990).

Starch liquefaction and saccharification processes are also used in bioethanol production from starchy biomass. Bioethanol can be produced from different processes as shown in Figure 7. The more conventional processes such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) require addition of large amounts of glucoamylase and α-amylase prior to microbial fermentation. These enzymes can be costly thus hamper the low production cost. Recent interest has been paid to consolidated bioprocessing (CBP), since it allows the utilization of whole-cell biocatalyst promoted by secretion of enzymes to the cell surfaces. (Fukuda et al. 2009.)

The yeast *Saccharomyces cerevisiae* is the most commonly used microorganism for bioethanol fermentation, since they can hydrolyze cane sucrose into glucose and fructose which are easily assimilative (Sánchez and Cardona 2008). When starchy biomass is used as feedstock; however, it has to be degraded by amylolytic enzymes prior to fermentation by *S. cerevisiae*. Therefore, integration of hydrolysis and fermentation steps has been developed by strain improvement including surface expression of the amylolytic enzymes in *S. cerevisiae*. This will be discussed further in section 2.3.1.3.

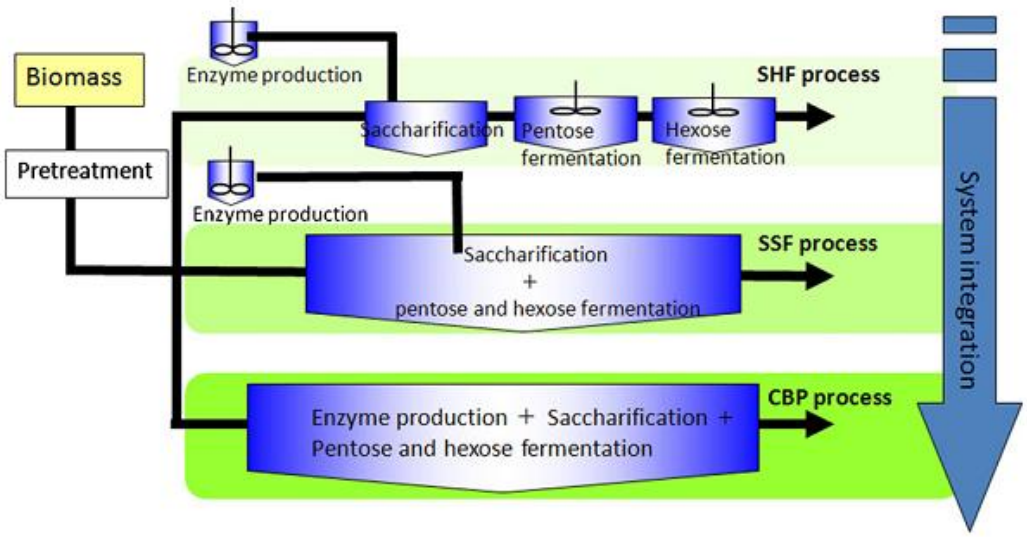


Figure 7. Different bioethanol production methods (figure taken from Fukuda et al. 2009).

For traditional industries

α-Amylases have long been used in the baking industry for making bread with a higher volume, better color and a softer crumb (Gupta et al. 2003). α-Amylase and other enzymes are added in dough making process to help the yeast ferment the available sugars such as dextrins. Fungal or malt α-amylase is commonly used in dough preparation; however, maltogenic α-amylase of *Bacillus stearothermophilus* is also commercially available due to its thermostability and ability to prevent stickiness of bread (van der Maarel et al. 2002).

α-Amylases have been used in powder laundry detergents since 1970s and nowadays, most of commercially available liquid detergents contain α-amylase (Gupta et al. 2003). α-Amylases are effective in removing starch-containing stains from clothes and porcelain with less severe conditions such as mild temperatures. Downsides of using α-amylases in detergents are that they show sensitivity to alkaline and oxidants that are common in washing environments. Therefore, wild-type α-amylases have been improved to be more suited to washing conditions. For example, it is now known that methionine residues in α-amylase are oxidized readily and lead to inactivation of the enzyme (Nielsen and Borchert 2000). Therefore, site-directed mutagenesis was used for optimal substitution of methionine residues and engineering oxidant-resistant α-amylase and protease (Estell et al. 1985; Hagihara et al. 2001).

2.3.1.3 Expression of α-amylase in recombinant organisms

Numerous studies have been conducted for α-amylase expression and production due to its high demand and many applications in the industries. Although strain improvement of α-amylase producing wide-type microorganisms is a common method for high yield of the enzyme, use of recombinant organisms is also studied. Extracellular production of α-amylase is especially interesting for better recovery of the enzyme and for particular purposes such as whole-cell biocatalysts. As mentioned in section 2.3.1.2, surface expression of amylolytic enzymes in the yeast *S. cerevisiae* has also been paid attention to for more efficient bioethanol fermentation. In this section, extracellular and surface expression of α-amylase in recombinant *E. coli* and *S. cerevisiae* is shortly reviewed.

Extracellular expression of α-amylase in recombinant bacteria is difficult to achieve when the host organism is non-pathogenic, Gram-negative species such as *E. coli*. However, several studies have reported extracellular expression of α-amylase in recombinant *E. coli* by overexpression of the protein (Lin and Hsu 1997; Suominen et al. 1987a) and using fusion partner proteins as discussed in section 2.2.2 (Qian et al. 2008; Zhang et al. 2005). Overexpression of α-amylase in *E. coli* often damages the cells and eventually leads to growth defects (Willemot and Cornelis 1983); however, according to the study by Suominen and coworkers, growth defects were mended by controlled induction by inactivation of the temperature-sensitive repressor or addition of Mg^{2+} to the medium (Suominen et al. 1987a). Shiina and coworkers demonstrated the release of recombinant α-amylase from *E. coli* into the medium by using high-voltage pulsed electric field (PEF) during fed-batch cultivation (Shiina et al. 2007). PEF treatment caused disruption of the outer membrane which assisted the release of periplasmic protein (Ohshima et al. 2000; Shiina et al. 2007).

Cell-surface display of amylolytic or cellulolytic enzymes in the recombinant *S. cerevisiae* strains has been a promising approach for simplification of bioethanol production. The outer cell wall layer of *S. cerevisiae* consists of mannoproteins that are covalently coupled to cell wall polysaccharides (Klis et al. 2002). One type of the mannoproteins is anchored to glycosylphosphatidylinositol (GPI) at the C-terminus site, which is suitable for surface expression of the heterologous proteins. One of the GPI-anchored mannoproteins used in surface expression is α-agglutinin, composed of a secretion-signal region, an active region, a support region rich in serine and threonine, and a putative GPI anchor-attachment region (Kondo and Ueda 2004). By using recombinant engineering, the anchoring signal of the α-agglutinin is combined with the signal of the secreted enzymes, so that the target proteins at N-terminal end are transported to the cell surface through the *S. cerevisiae* secretory pathway (Figure 8).

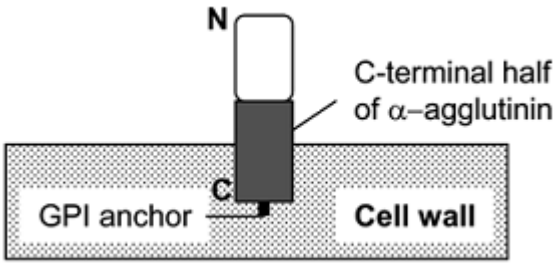


Figure 8. Yeast cell surface display system using α-agglutinin (Kondo and Ueda 2004). C: C-terminus; N: N-terminus of the fusion protein.

The early construction of the starch-utilizing *S. cerevisiae* started with display of a single amylolytic enzyme, either glucoamylase or α-amylase (de Moraes et al. 1995; Murai et al. 1997; Ruohonen et al. 1987), and then later followed co-expression of glucoamylase and α-amylase, which demonstrated improved digestion of insoluble starch (Shigechi et al. 2004). Significant ethanol production was achieved in the fermentation study by Birol and coworkers using the *S. cerevisiae* strains constructed by de Moraes and coworkers (Birol et al. 1998; de Moraes et al. 1995). In their study, the strain secreting *B. subtilis* α-amylase and *Aspergillus awamori* glucoamylase as separate proteins was able to produce 43.8 g l^{-1} ethanol in soluble starch-containing medium (Birol et al. 1998). This value was higher than that from the strain secreting these enzymes as fusion proteins. Shigechi and coworkers demonstrated direct ethanol production from low-temperature-cooked (80 °C, 5 min) corn starch using the yeast strain co-displaying *Rhizopus oryzae* glucoamylase and *B. stearothermophilus* α-amylase, which produced 18 g l^{-1} ethanol after 36 hour incubation (Shigechi et al. 2004). These studies showed that co-display or co-secretion of glucoamylase and α-amylase as separate enzymes could increase ethanol production, probably due to efficiency of the co-operative and sequential reaction of two enzymes.

2.3.2 Citrine

Citrine is a yellow fluorescent protein (YFP) that is highly tolerant to environmental sensitivity such as pH, halides and photobleaching (Griesbeck et al. 2001). YFPs are mutants of the GFP from the jellyfish *Aequorea victoria* (Tsien 1998), of which threonine residue 203 (Thr^{203}) was mutated to aromatic amino acids such as tyrosine (Ormö et al. 1996). The mutation results in a 20 nm shift to longer wavelengths for excitation/emission spectra compared to GFPs (Table 4). Since YFPs showed sensitivity to pH, chloride and photobleaches, or poor expression at 37 °C, an improved mutant of YFP was engineered and named Citrine to indicate its yellow color and acid resistance (Griesbeck et al. 2001). Physical properties of major GFPs and YFPs are shown in Table 4.

Table 4. Physical properties of major fluorescent proteins.

Protein	Excitation peak (nm)	Emission peak (nm)	Brightness[a]	pK_a	Reference
wild-type GFP	395 (470)[b]	509	16	n/a	Chalfie et al. 1994
EGFP	488	507	54	4.3	Heim et al. 1995
EYFP	514	527	51	6.9	Miyawaki et al. 1999
Venus	515	528	53	6.0	Nagai et al. 2002
Citrine	516	529	59	5.7	Griesbeck et al. 2001
YPet	517	530	80	5.6	Nguyen and Daugherty 2005

[a]Product of the molar excitation coefficient and the quantum yield $(mM \times cm)^{-1}$.
[b]Excitation peak maximally at 395 nm with a minor peak at 470 nm.

Use of Citrine is not widespread compared to GFPs since it is not commercially available; however, YFPs have been applied to variety of scientific research such as detection of protein-protein interactions, protein trafficking in the living cells, and observation of intramolecular conformational change (Griesbeck et al. 2001; Nyfeler et al. 2005). Another YFP called Venus was successfully detected in the medium when fused with a neuropeptide Y (Nagai et al. 2002); therefore, the physically similar Citrine could be used in expression of secretive fusion proteins.

Fusion protein expression in *A. baylyi* using Citrine or other YFPs has not been reported so far; however, GFP and luminescent gene *luxCDABE* have been co-expressed with the inducible salicylate operon *salA* in ADP1 for the detection of salicylate (Huang et al. 2005). Their study suggested that a faster turnover of the *lux* gene was suitable for whole-cell luminescence assays of salicylate concentration; whereas the longer maturation and slower turnover of the GFP variant was more suited to whole-cell imaging of the presence of salicylate.

2.4 Other extracellular enzymes of industrial importance

In addition to α-amylase, there are several other extracellular enzymes of industrial importance that are worth describing. Table 4 lists some of the important extracellular enzymes used in industry, source organisms and their applications. Among these enzymes, cellulase and xylanase will be described in detail since enzymatic functions of cellulase and xylanase are similar to those of α-amylase. Although they have been mainly used as industrial enzymes in preparation of beverages, detergents, paper and animal feed, these enzymes have recently gained attentions because of their potential contributions to alternative biofuel production.

Table 4. Extracellular enzymes of industrial importance.

Enzyme	Source organism	Industrial use
α-Amylase (EC 3.2.1.1)	*Aspergillus spp., Bacillus spp.*	Glucose formation in starch industry, baking industry
Protease (EC 3.4)	*B. subtilis*	Protein degradation in detergents
Lactase (EC 3.2.1.108)	*Kluyveromyces fragilis*	Lactose hydrolysis in milk industry
Penicillin amidase (EC 3.5.1.11)	*E. coli*	Antibiotics synthesis
Cellulase (EC 3.2.1.4)	*Trichoderma reesei*	Color brightening in detergents, fruit juice extraction in beverage industry
Xylanase (EC 3.2.1.8)	*Trichoderma fungus*	Fiber solubility in animal feed, biobleaching in paper and pulp industry, dough conditioning in baking industry
Lipase (EC 3.1.1.3)	*Aspergillus spp.*	Fat removal in detergents

2.4.1 Properties of cellulase and xylanase

Most biomass materials present in the world exist in the form of lignocelluloses, which are mainly composed of lignin (a complex polyphenolic structure), hemicellulose (noncellulosic polysaccharides including xylans, mannans, and glucans), and cellulose (insoluble fibers of β-1,4-glucan) (Kumar et al. 2008). Among lignocellulosic components, cellulose accounts for about 40 % of the lignocellulosic biomass; whereas hemicellulose accounts for 25-35 %. Most lignocellulosic residues from agriculture and industries are currently discarded as wastes; therefore, conversion of the lignocellulosic biomass to biofuel such as ethanol has great potential to be an alternative to energy sources with high greenhouse gas emission. Cellulases and xylanases, both of them important catalytic enzymes in various industries, are the main lignocellulosic biomass-degrading enzymes.

Cellulases

Cellulose is the major component of lignocellulosic materials, which is composed of polymers of D-glucose linked by β-1,4-glycosidic bonds. Cellulose is hydrolyzed by a group of enzymes called cellulase. As shown in Figure 9, there are three major cellulase enzymes: endoglucanases (EC 3.2.1.4) randomly cleave internal bonds of the non-crystal cellulose chain; exoglucanases (EC 3.2.1.91: cellobiohydrolase) catalyze the chain ends of the crystal cellulose and release cellobiose; β-glucosidases (EC 3.2.1.21) further break a β-1,4-D-glucosidic linkage from cellobiose and release glucose monomers (Kumar et al. 2008).

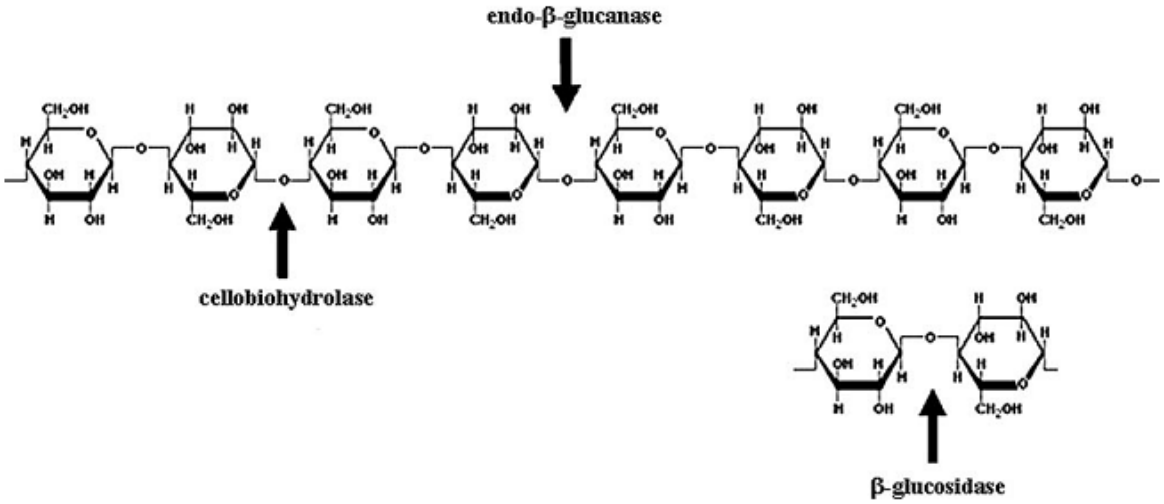


Figure 9. Cellulose hydrolysis by cellulase (figure taken from Kumar et al. 2008).

Xylanases

Xylan, which is mostly present in hardwood, is predominant hemicellulose structure and composed of D-xylopyranosyl units linked by β-1,4-glycosidic bonds. In order to hydrolyze xylans, different kinds of xylanase enzyme are required. Endo-xylanase (EC 3.2.1.8: 1,4-β-D-xylan xylanohydrolase) and β-xylosidase (EC 3.2.1.37: 1,4-β-D-xylan xylohydrolase) hydrolyze the glycosidic bond in the xylan backbone to yield short xyloorigomers. Other accessory enzymes are also involved in xylan degradation. α-D-glucuronidases (EC 3.2.1.139) hydrolyze the α-1,2 bonds between the glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan (Girio et al. 2010). α-L-arabinofuranosidases (EC 3.2.1.55) catalyze the hydrolysis of non-reducing terminal α-L-arabinofuranosidic linkages (Shallom and Shoham 2003). Other carbohydrate esterase enzymes are involved in deacetylation of side-chain substituent, such as acetylxylan esterase (EC 3.1.1.72) and ferulic and *p*-coumaric acid esterase (EC 3.1.1.73).

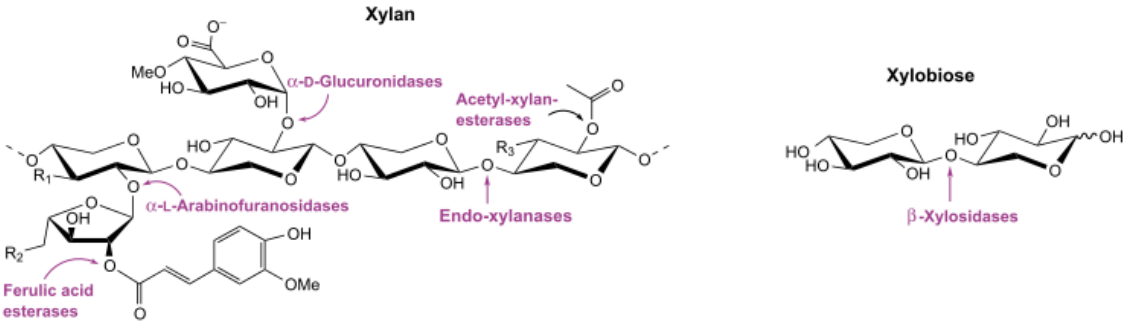


Figure 10. Xylose and xylanase enzymes (figure taken from Shallom and Shoham 2003).

2.4.2 Cellulolytic microorganisms and genetic studies

The filamentous mesophilic fungus *Trichoderma reesei* is known for encoding various types of secretive cellulase and hemicellulase genes with high yields of these enzymes; therefore, they are expected to be suited for production of industrial enzymes. However, *T. reesei* strains and other fungi do not show high thermostability and activity at a broad pH range, which is often required for fermentation processes. Therefore, genetic engineering of *T. reesei* wild-type strain has been conducted for obtaining thermostability and alkali-tolerance. For example, Wang and coworkers used error-prone polymerase chain reaction (PCR) to randomly mutate full-length cDNA sequence of endoglucanase III, followed by screening the mutants at high pH. The site-directed mutagenesis was then conducted on the screened mutants to study the specific amino acid residue in determining the pH activity profile of the enzyme in *T. reesei* (Wang et al. 2005). It was also shown that thermostability increased by introducing a disulfide bridge into the N-terminal region of *T. reesei* endo-1,4-β-xylanase II by substituting threonine residue 2 and 28 with cysteine (Fenel et al. 2004). Expression of glycosylated active endoglucanase II from *T. reesei* in the yeast *S. cerevisiae* resulted in wider pH range of activity and higher thermostability of the enzyme, showing that increased glycosylation extent helped stabilization of the enzyme (Qin et al. 2008). Although the yields of the cellulolytic enzymes are not as much compared to *T. reesei*, there are other organisms that produce thermophilic or alkali-tolerant enzymes, which were expressed heterologously, for example, in *E. coli* (Shendye et al. 1993; Voget et al. 2006). In many cases, however, extracellular expression of cellulolytic enzymes in *E. coli* was not achieved, probably due to lack of secretion system specific to the enzymes and the absence of post-translational modifications in *E. coli* (Kulkarni et al. 1999).

Prokaryotic bacteria also have the ability to degrade cellulose and hemicelluloses into simpler saccharide components. Among them, Gram-positive and strict anaerobic bacteria *Clostridium* strains have been extensively studied due to their efficient cellulose degradation using an extracellular cellulase complex called cellulosome. Cellulosomes are large extracellular complexes that can degrade cellulose, hemicelluloses and pectin, and are produced by anaerobic bacteria such as *Clostridium, Acetivibrio, Bacteroides,* and *Ruminococcus*. The first recognized cellulosome from *Clostridium thermocellum* was found to consist of a non-catalytic scaffolding protein CipA to which a number of cellulolytic and hemicellulolytic enzymes were attached. (Doi et al. 2003.) In the *C. thermocellum* cellulosome, the scaffolding protein contains a single cellulose-binding domain (CBD) and nine repeating domains termed type I cohesins, which interact with the cellulosomal enzymes. Each cellulosomal enzymatic subunit possesses an additional domain, type I dockerin, which binds with the cohesins of the scaffolding protein. (Shoham et al. 1999.) Type II cohesin-dockerin linkage binds the cellulosome with anchoring protein, which is attached to the surface of the cell wall. Figure 11 represents the basic structure of the cellulosome of *C. thermocellum*.

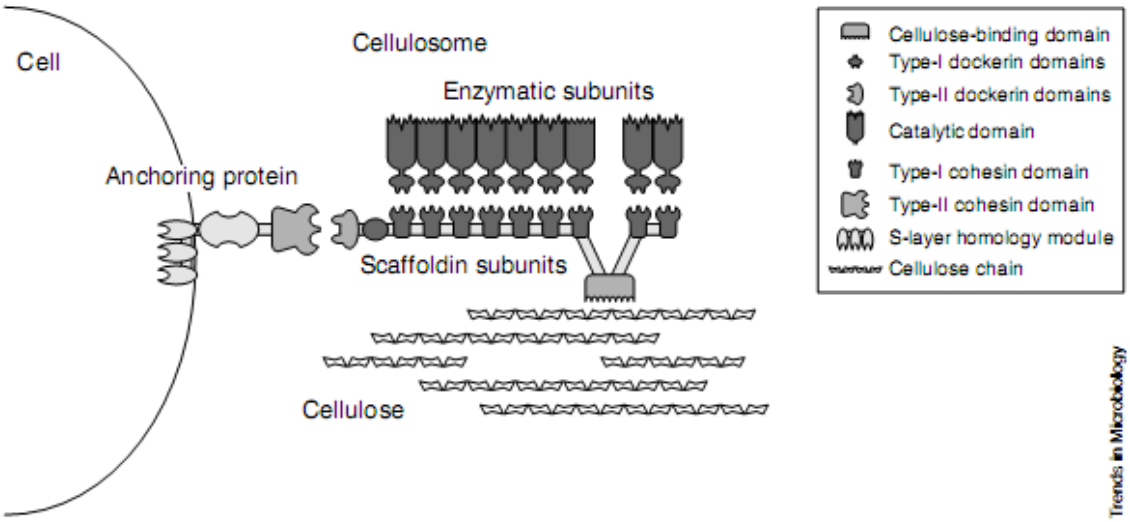


Figure 11. Structure of the C. thermocellum cellulosome (Shoham et al. 1999).

Unlike fungal cellulolytic enzymes, cellulosomes from some anaerobic bacteria like *C. thermocellum* are capable of degrading the insoluble, crystalline cellulose; therefore, many studies have been conducted to study cellulosomal functions and to increase the enzymatic activity level. There are several approaches made for improvement of cellulose/hemicellulose degradation including reconstruction of cellulosome components and cell-surface display of cellulosome subunits in the yeast.

Fierobe and coworkers used complementary protein modules from the cellulosomes of *C. thermocellum* and *C. cellulolyticum* to form a series of chimeric scaffolding proteins, in which selected enzymes were incorporated in specific locations within a multicomponent complex (Fierobe et al. 2001). The *in vitro* assembled bi-functional cellulosome chimeras exhibited enhanced synergy on microcrystalline cellulose (Avicel®). They further constructed a tri-functional chimeric cellulosome using xylanase XynZ from *C. thermocellum* and demonstrated 6-fold more efficient in hydrolysis of complex cellulose (straw) than the bi-functional cellulosome (Fierobe et al. 2005). Similarly the *cipA* gene from the cellulosome of *C. acetobutylicum* was cloned and expressed in *E. coli* (Sabathé and Soucaille 2003). In their study, the constructed cellulosome was assembled *in vivo*, where CipA showed affinity to Avicel® even though the wild-type *C. acetobutylicum* is unable to degrade cellulose. These results show that 'designer' cellulosomes may create efficient synergism among cellulolytic enzymes from different organisms on complex cellulosic substrates.

In order to achieve direct conversion of amorphous or crystalline cellulose into ethanol, cell-surface display of cellulolytic enzymes in the yeast *S. cerevisiae* has been studied. Fujita and coworkers engineered a recombinant yeast *S. cerevisiae* co-displaying *T. reesei* endoglucanase II and cellobiohydrolase II and *Aspergillus aculeatus* β-glucosidase 1 as individual fusion proteins with C-terminal-half region of α-agglutinin

(Fujita et al. 2004). The yeast strain co-displaying three enzymes directly produced ethanol from amorphous cellulose with a yield of 0.45 g/g cellulose, reaching 88.5 % of the theoretical yield. Cellulosome display on the yeast was also reported by several researchers recently, in which they assembled a functional minicellulosome from different organisms on the yeast cell surface (Tsai et al. 2009; Wen et al. 2010). The recombinant strains successfully degraded amorphous cellulose, but degradation of crystalline cellulose has not yet been achieved in the cell-surface display of the yeast strains, where further research may be expected.

3 Materials and Methods

3.1 Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this experiment and their characteristics are listed in Table 5. *E. coli* XL-1 was used for construction of recombinant plasmid and its extraction. *A. baylyi* $B2^T$ wild-type was used as protein expression host. Cells were routinely grown at 30-37 °C in Luria broth (LB) medium or MA/9 medium, with agitation at 300 rpm in the shaker. Antibiotics were used for growth of recombinant bacteria; 25 μg ml^{-1} of chloramphenicol (Cam) for *E. coli* recombinant bacteria and 20-50 μg ml^{-1} of kanamycin (Kan) for *A. baylyi* recombinant bacteria.

Table 5. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
Bacterial strains		
E. coli XL-1	Electrocompetent	Santala, V.
A. baylyi $B2^T$ wild-type		Myllyntausta, S.
Plasmids		
pCSS4	α-Amylase gene	Suominen et al. (1987b)
pAK400c	Citrine gene	Abraham, B.
SM203/pIX	Kan^R, Cam^R, putative lipase promoter	Myllyntausta, S.
SM100/pIX	Kan^R, Cam^R, lac/T5 promotor	Myllyntausta, S.

3.2 Gene construction

α-Amylase gene construction

As shown in Figure 12, the DNA fragment encoding the α-amylase gene derived from *B. stearothermophilus* (GenBank accession number: M57457) was amplified from the plasmid pCSS4 (Suominen et al. 1987b) by a primer pair of vs09_3 and vs09_4 (Table 6). The primer vs09_3 amplified the α-amylase gene containing the endogenous signal peptide region. The start codon of the signal peptide was changed from valine to methionine (Met), since *NdeI* site in the primer vs09_3 contained Met. Native promoter regions of the α-amylase gene were removed. The PCR product was digested with *NdeI*/*XhoI* restriction enzymes and ligated into the corresponding sites of the vector SM203/pIX. SM203/pIX had the following features: chloramphenicol resistance gene (Cam^R), kanamycin resistance gene (Kan^R, located between *XhoI* and *PstI* in Figure 12) and a constitutively expressed lipase promoter (located between *MfeI* and *NdeI* in

Figure 12) which was amplified from ADP1 genome and cloned to the plasmid (Myllyntausta 2010).

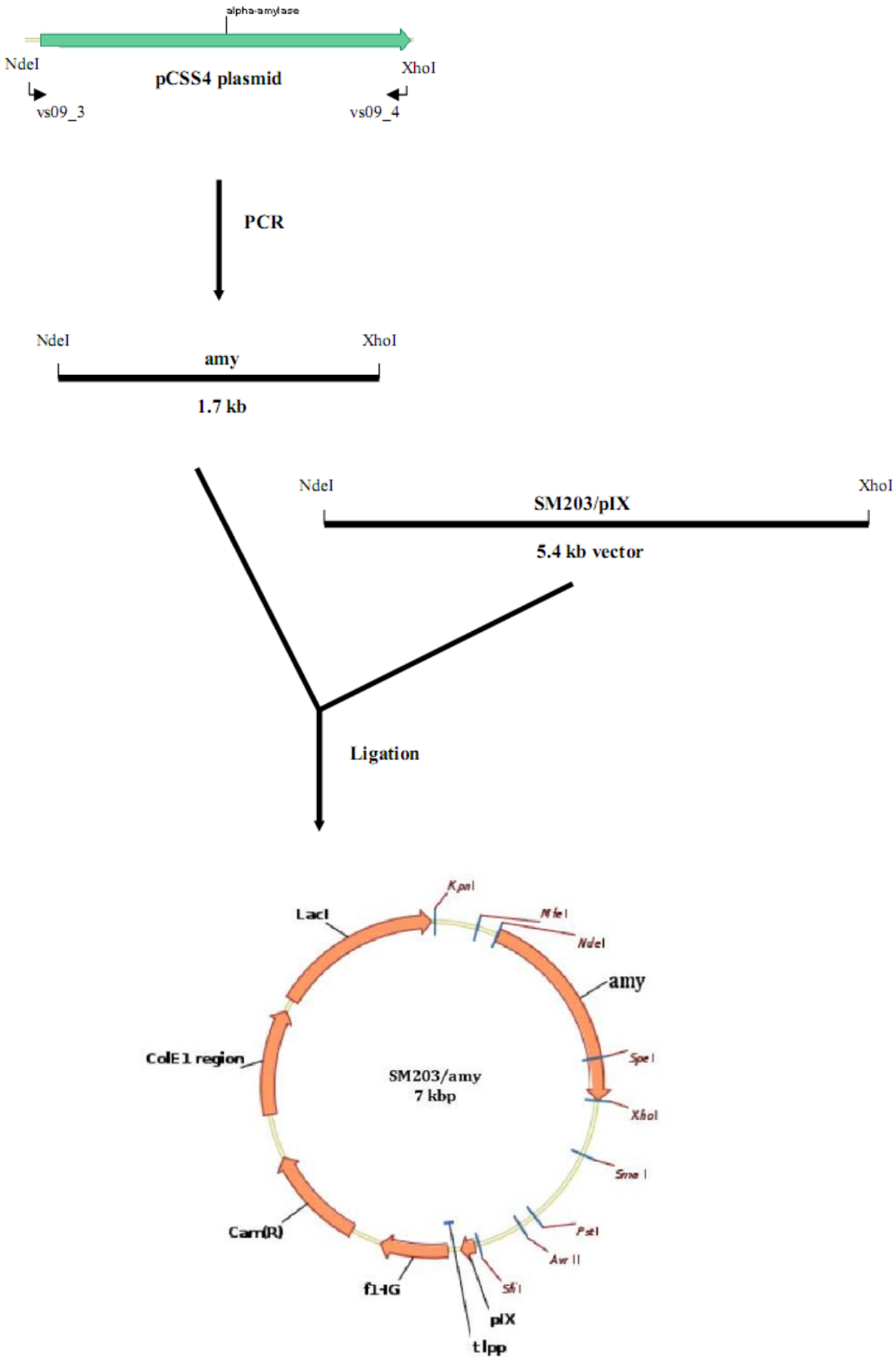


Figure 12. Construction of the SM203/amy plasmid.

Citrine fusion gene construction

Figure 13 shows cloning strategy for Citrine fusion gene construction. The *A. baylyi* genome fragments were amplified by colony PCR from *A. baylyi* $B2^T$ wild-type by the corresponding primer pairs (Table 6). The forward primers contained ribosome binding site (RBS) after *NdeI* site. Stop codon of the fusion partner genes (see section 2.2.2) was removed by the reverse primers. Citrine gene was amplified from the plasmid pAK400c using a primer pair of na10_1 and na10_2. Start codon of Citrine gene was removed by the primer na10_1. After confirming correct amplification of target genes by gel electrophoresis, all PCR products were digested with *SpeI* restriction enzyme. Digestion products were loaded on agarose gel for electrophoresis, followed by gel purification. Each fusion partner gene was then ligated into Citrine gene using T4 ligase to obtain a fusion gene. The fusion genes were further amplified by PCR, followed by digestion with *NdeI*/*XhoI* restriction enzymes. Finally the digested fusion genes were ligated with an *NdeI*/*XhoI* digested SM100/pIX vector. The fusion gene was located at downstream region of lac/T5 promoter. The constructed plasmid was digested with *KpnI*/*SfiI* sites in transformation of *A. baylyi*, so that the integration of the DNA into the *A. baylyi* genome would be efficient. The lac/T5 promoter was always active after integration into *A. baylyi* genome. In addition to the fusion gene constructs, the intracellular Citrine-carrying *A. baylyi* was constructed for control and comparison purposes. For the intracellular Citrine construct, the forward primer did not contain RBS site and the RBS region of the plasmid SM100/pIX was used for initiation of translation.

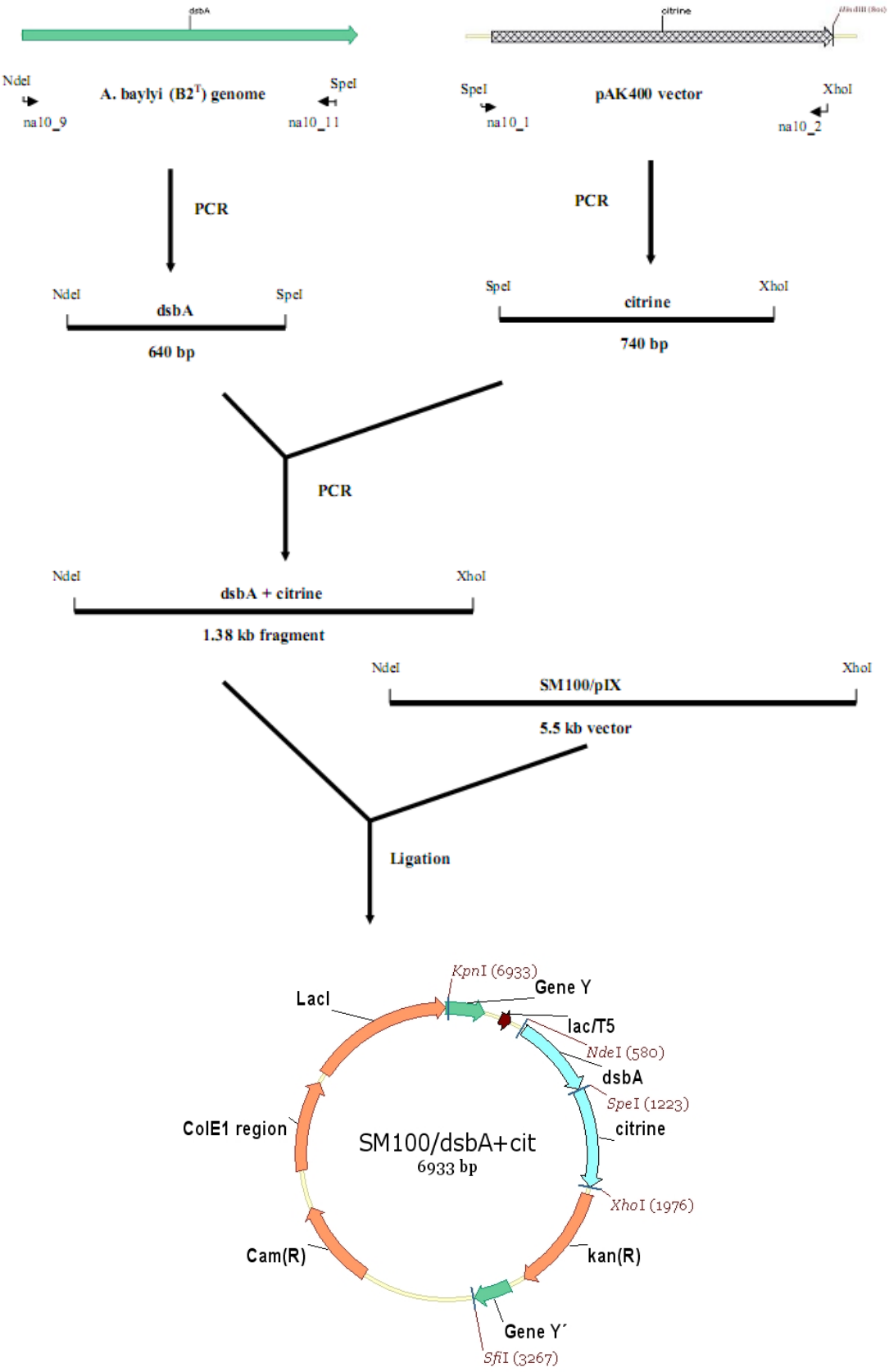


Figure 13. Construction of the *dsbA*+Citrine gene fusion.

Table 6. Oligonucleotide PCR primers used in this study.

Primer name	Gene amplified	Sequence (5'-3')
vs09_3	α-amylase (forward)	atatgctagctagagaaggagatatacatatg**ctaacgtttcaccgcatcattc**
vs09_4	α-amylase (reverse)	atatctcgagtt**aaggccatgccaccaaccgtg**
gfp7	Citrine (forward)	tatacat**atgcatcaccatcaccatcactctggttctgtgagcaagggcgagga**
na10_1	Citrine (forward)	agtacactagtatc**catcaccatcaccatcac**
na10_2	Citrine (reverse)	gaatctcgag**ttacttgtacagctcgtcc**
na10_7	*yceI* (forward)	aatacatatggagaaggagatataaat**atgaatcttaaaacattatctc**
na10_4	*yceI* (reverse)	ggatactagtgta**ctttttggcagcttcagcttc**
na10_8	*cysP* (forward)	aatacatatggagaaggagatataaat**atgcgttttgcacagttaaaaatc**
na10_6	*cysP* (reverse)	ggatactagtgtattttgcgctgttttctttaacgatc
na10_9	*dsbA* SP (forward)	aatacatatggagaaggagatataaat**atgaaaaaatttgtattgggc**
na10_10	*dsbA* SP (reverse)	ggatactagt**agccatggtggtgccc**
na10_11	*dsbA* (reverse)	ggatactagtgta**tttcttggcagtacgttctttc**
na10_12	*ompA* SP (forward)	aatacatatggagaaggagatataaat**atgaaattgagtcgtattg**
na10_13	*ompA* SP (reverse)	ggatactagt**cgcgttagcagc**
na10_14	*quiB* (forward)	aatacatatggagaaggagatataaat**ttgttgatgtcattgcctatattg**
na10_15	*quiB* (reverse)	ggatactagtgta**aggtgtaggttgaactgttttaagg**

*Underlines indicate restriction sites: *NdeI* (catatg), *SpeI* (actagt) and *XhoI* (ctcgag). The sequences in bold are homologous to the respective templates. SP = signal peptide.

3.3 Gene manipulation

PCR amplification

Amplification of the target genes was done using PCR. The following reaction was routinely used for amplification: 100-200 ng of plasmid, 0.25 μmol L^{-1} sense and antisense primers, 1× Optimized DyNAzyme™ Buffer, 0.5 U DyNAzyme™ II PCR polymerase (Finnzymes, Finland), 0.2 mmol L^{-1} deonucleotide triphosphate (dNTP) and sterile Milli-Q water up to 50 μl. The PCR reaction was conducted in T3000 Thermocycler (Biometra, Germany). After the reaction, success of target gene amplification was confirmed by gel electrophoresis of the PCR reaction mixture in 1-2 % (w/v) agarose gel. The PCR program used for gene amplification is shown in Table 7.

Table 7. PCR program and temperature used in target gene amplification.

Program and temperature	Time
1) Initial denaturation 94 °C	2 min
2) Denaturation 94 °C	1 min
3) Annealing 50 °C	1 min
4) Elongation 72 °C	1 min 30 sec
5) Go to 2 for 25 times	
6) Final elongation 72 °C	3 min
7) Final hold 4 °C	

Digestion and ligation reaction

The target genes and plasmids were digested with restriction enzymes and the digested fragments were purified using electrophoresis in 1-2 % (w/v) agarose gel. The gel containing the gene fragments was further purified using GeneJET^{TM} Gel Extraction Kit (Fermentas, Finland). The purified linear vectors and target genes were used for ligation reaction by T4 ligase. All the restriction enzymes used in this study were obtained from Fermentas Finland.

Transformation in E. coli

The electrocompetent cells from *E. coli* XL-1 strain were prepared by the standard procedures (Sambrook et al. 1990). The mixture of 2 µl ligation products and 50 µl aliquot of the electrocompetent cells was transferred to a cooled electroporation cuvette. Transformation was performed with Gene Pulser MXcell electroporation system (Bio-Rad, USA) using Eco-1 program. The transformed cells were spread on pre-made LB agar plate. Control and product plates were incubated at 37 °C overnight or under room temperature until colonies appeared on the plate. A few colonies on the plate were inoculated in LB medium supplemented with 25 µg ml^{-1} of chloramphenicol. The DNA plasmids were extracted from the cell cultures using GeneJET^{TM} Plasmid Miniprep Kit (Fermentas, Finland). The target gene was confirmed by digestion with restriction enzymes and gel electrophoresis of the digested products.

Transformation in A. baylyi

The constructed plasmid (2 µg) was digested with *KpnI* and *SfiI* prior to transformation in 1 ml culture of *A. baylyi*. The overnight-grown *A. baylyi* preculture in 40 µl volume was inoculated in 3 ml of LB medium supplemented with 0.5 % glucose. When the cells reached log-phase after 2-3 hours, the culture was divided into 1 ml aliquot and the digestion reactions were added into the culture tubes. The cultures were further incubated for 2-3 hours. The cultures were transferred in 1.5 ml Eppendorf tubes and centrifuged with Centrifuge 5417R (Eppendorf, Germany) at 5000 rpm for 10 min. The supernatant was removed and 100 µl of re-suspended cells were spread on LB agar + 0.5 % glucose + 50 µg ml^{-1} kanamycin plate. The plate was incubated at 30 °C overnight, and further incubated at room temperature until colonies were formed on the plate.

3.4 Screening

Screening for α-amylase cloning was conducted in two ways: plate-staining assay and colony PCR. For fusion protein cloning, the transformant colony plates were placed under blue light using Safe Imager™ Transilluminator (Invitrogen, USA) and the light-emitting colonies were picked for colony PCR to confirm the fusion genes.

Plate-staining assay

For screening α-amylase activity, transformant colonies were spread on LB agar + 0.25 % glucose + 1 % starch + 50 μg ml^{-1} kanamycin plates. After incubating 4-5 days at room temperature, the plates were stained by pouring 2-3 ml of iodine solution (1 g iodine crystals and 2 g potassium iodine in 300 ml distilled water, followed by 0.2 μm filter filtration). After incubated for 2-3 minutes, halo-forming colony was picked and used for colony PCR.

Colony PCR

Colony PCR was conducted for confirming that the positive transformant carried target genes. Positive colony was transferred to a PCR tube containing 50 μl of Tris-EDTA buffer. The colony was re-suspended in the buffer, followed by boiling at 95 °C for 10 min. After boiling, the cells were centrifuged down and 5 μl of the supernatant was used as PCR template. The following reaction was used routinely for colony PCR: 5 μl of DNA template, 0.25 μmol L^{-1} forward and reverse primers, 1× Phusion® High-Fidelity buffer, 0.5 U Phusion® High-Fidelity DNA Polymerase (Finnzymes, Finland), 0.2 mmol L^{-1} dNTP, 2-3 mmol L^{-1} $MgCl_2$ and sterile Milli-Q water up to 50 μl. Table 8 shows PCR program used for colony PCR. Target insert genes were confirmed by gel electrophoresis.

Table 8. PCR program and temperature used in colony PCR.

Program and temperature	Time
1) Initial denaturation 98 °C	3 min
2) Denaturation 98 °C	10 sec
3) Annealing 72-50 °C (incremental decrease of 0.7 °C every cycle)	10 sec
4) Elongation 72 °C	1 min 30 sec
5) Go to 2 for 35 times	
6) Final elongation 72 °C	3 min
7) Final hold 4 °C	

3.5 Enzyme activity

3.5.1 α-Amylase activity assay

α-Amylase hydrolyzes starch to produce free carboxymethyl maltose units. The free carboxymethyl maltose units react with 3,5-dinitrosalicyclic acid (DNS) reagent to form a colored complex which is detected spectrophotometrically at 540 nm. Soluble starch, DNS reagent and Rochelle salt ($NaKC_4H_4O_6$) were obtained from Sigma-Aldrich, Inc. (USA).

3.5.1.1 Reagents

Substrate solution
Soluble starch (1 g) was dissolved in 100 ml of 20 mmol l^{-1} phosphate buffer (pH 6.8).

DNS reagent
3,5-dinitrosalicylic acid (1 g) was dissolved in 20 ml of 2 mol l^{-1} NaOH and 50 ml of distilled water. $NaKC_4H_4O_6$ (30 g) was added and made up to 100 ml with distilled water.

3.5.1.2 Standard curve

Different concentrations of maltose solution were prepared. To each test tube containing 1 ml of maltose solution 2 ml of DNS reagent was added. The mixture was boiled for 10 minutes, followed by cooling in running tap water. The mixture was diluted with 20 ml of distilled water and absorbance was measured at 540 nm using Shimadzu UV-1601 spectrophotometer. As shown in Figure 14, maltose standard curve was drawn and linear equation of the curve was used for converting absorbance to maltose concentration of the samples.

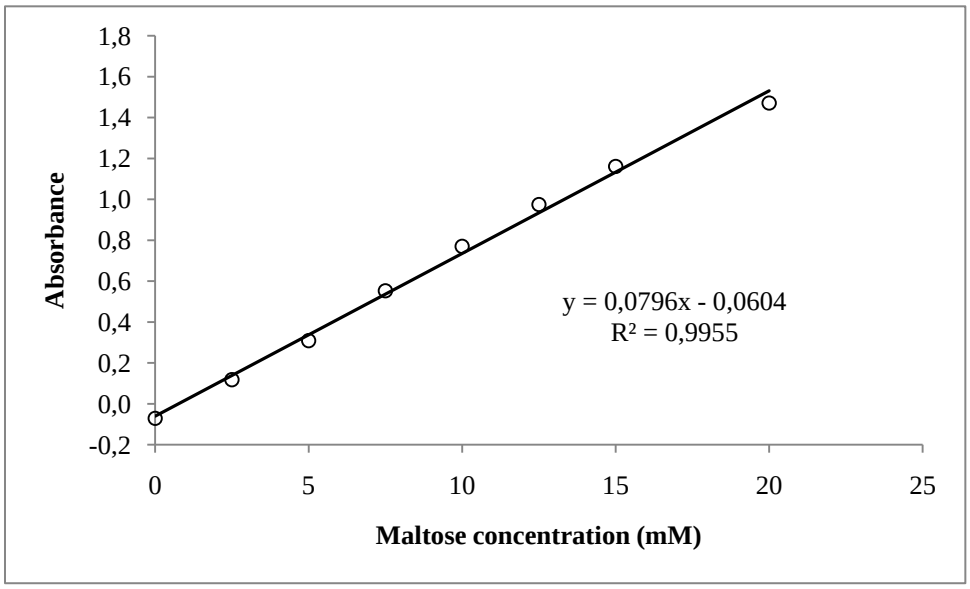


Figure 14. Standard curve of absorbance as a function of maltose concentration.

3.5.1.3 Enzyme assay

Enzyme solution was prepared in the following way. The overnight preculture in 2 ml was inoculated in 50 ml of M9 minimal medium + 0.2 % casamino acids + 0.5 % starch + 20 μg ml^{-1} kanamycin and incubated at 300 rpm shaker at 30 °C. At certain time intervals the cells were transferred to a 1.5 ml Eppendorf tube and boiled at 80 °C for 10 min. The cells were centrifuged down at 7600 rpm for 10 min and 1 ml of supernatant was used as crude enzyme solution. This enzyme solution was incubated at 55 °C for 10 min with 1 ml of the substrate solution containing 1 % starch. The reaction was terminated by adding 2 ml of the DNA reagent and the mixture was boiled for 10 min, followed by cooling. The mixture was diluted with 20 ml of distilled water and absorbance was read at 540 nm. Activity unit (U/μmol) was defined as μmol maltose catalyzed by enzyme in the assay conditions. Table 9 lists the ingredients for 1 L M9 minimal medium.

Table 9. M9 minimal medium composition.

Ingredient	Volume	Note
M9 salts (5 x)	200 ml	64 g/l $Na_2HPO_4 \cdot 7H_2O$; 15 g/l KH_2PO_4; 5 g/l NH_4Cl; 2.5 g/l NaCl
$MgSO_4$ (1 M stock)	2 ml	
$CaCl_2$ (1 M stock)	100 μl	
Sterile H_2O	Up to 1 l	

3.6 Fluorescence measurement

3.6.1 Culture growth

Fluorescent intensity was measured in order to verify extracellular expression of recombinant proteins. The precultures were grown in LB medium supplemented with 0.5 % glucose and 50 µg ml^{-1} of kanamycin for 16-18 hours at 30 °C, 300 rpm. The overnight precultures in 50 µl were inoculated in 5 ml MA/9 or M9 minimal medium supplemented with 0.2 % casamino acids, 2 % of glucose and 20 µg ml^{-1} of kanamycin. The cultures were grown at 30 °C, 300 rpm. The ingredients for 1 L MA/9 medium are shown in Table 10.

Table 10. MA/9 medium composition.

Ingredient	Volume	Note
MA/9 salts (10 x)	100 ml	55.18 g/l $Na_2HPO_4 \cdot 2H_2O$; 34.02 g/l KH_2PO_4; 9.63 g/l NH_4Cl; 0.08 g/l Nitrilotriacetic acid; 10 g/l NaCl
$FeCl_3$ (10 mg/ml stock)	50 µl	
$MgSO_4$ (1 M stock)	2 ml	
$CaCl_2$ (1 M stock)	100 µl	
Basal salts solution	5 ml	Zhang et al. (2000)
Sterile H_2O	Up to 1 l	

3.6.2 Fluorescence measurement

OD_{600} was measured at certain time intervals to obtain the same amount of biomass at each time interval and among the compared cultures. Depending on OD_{600} values, 400-600 µl of the cultures were transferred to a 1.5 ml Eppendorf tube, followed by centrifugation at 10000 rpm for 10 min. The supernatant (extracellular part) and the cell pellets (intracellular part) were separated, and the cell pellets were washed once with 1× phosphate buffered saline (PBS) and then re-suspended in 400 µl of 1× PBS. Both the duplicate supernatants and the re-suspended cell mixtures were placed in a 96-well microplate. Fluorescence was measured at excitation wavelength of 485 nm and emission wavelength of 535 nm using Plate Chameleon Microplate Reader (Hidex, Finland). Fluorescence emission/excitation spectra of the supernatants and the cell cultures were measured using Fluorometer Fluorolog-3-111 (ISA-Jobin Yvon, France). The emission spectra were scanned between 490-700 nm at the excitation wavelength of 480 nm. The spectral bandwidth was set to 2 nm for both the excitation and emission monochromators in all the measurements. The emission spectra were corrected using the correction function provided by the manufacturer after subtracting dark counts of the photomultiplier.

4 Results

4.1 α-Amylase expression

4.1.1 Screening

Plate-staining

Out of 10 transformant colonies screened with iodine staining, one colony formed halo around it, indicating that α-amylase was secreted from the cells to hydrolyze starch. Compared to iodine staining of wild type colony, clear halo was confirmed around transformant colony (Figure 15).

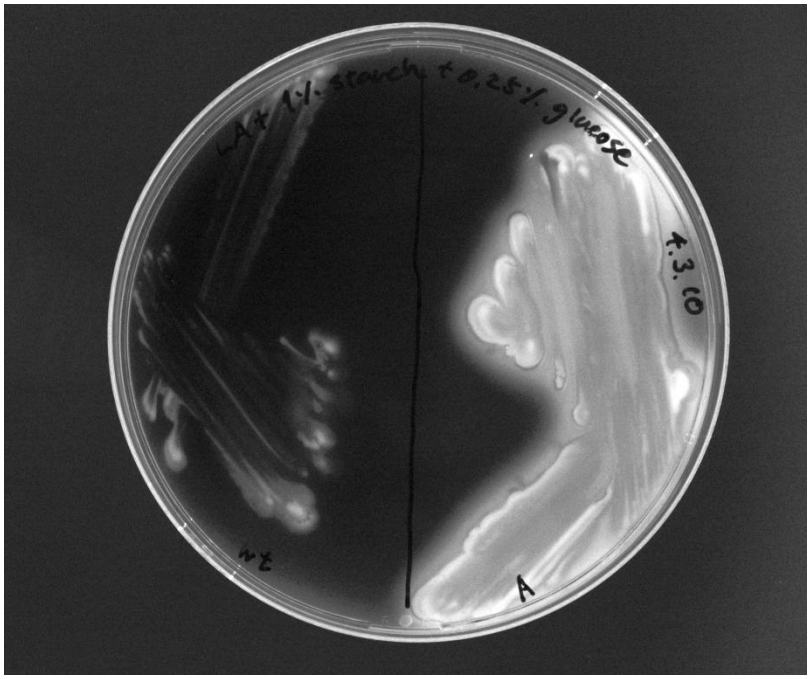


Figure 15. Plate staining of *A. baylyi* wild type (left) and *A. baylyi* carrying *amy* gene (right).

Colony PCR

As shown in Figure 16, 1.7 kb of α-amylase insert gene was confirmed in both *E. coli* and *A. baylyi* transformants.

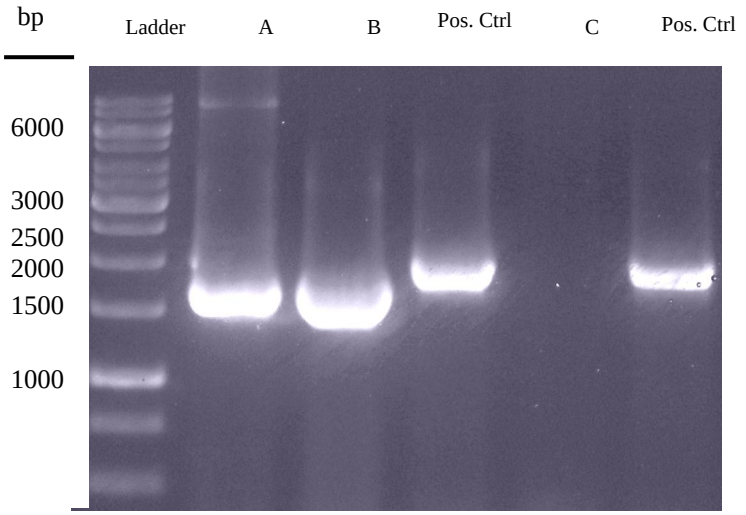


Figure 16. *Amy* insert band check by agarose gel electrophoresis. A: *E. coli* carrying amy gene; B: *A. baylyi* carrying *amy* gene; C: *A. baylyi* wild type (no *amy* gene); Pos. Ctrl: Positive control (2 kb) to check that the template works for colony PCR.

4.1.2 Enzyme activity

Figure 17 shows α-amylase enzyme activity along with cell growth compared between wild-type and the SM203/amy construct. Cell growth was indicated as optical density measured at 600 nm. The cultures of both strains were grown in M9 minimal medium supplemented with 0.2 % casamino acids, 0.5 % starch and 20 μg ml^{-1} of kanamycin for the SM203/amy construct.

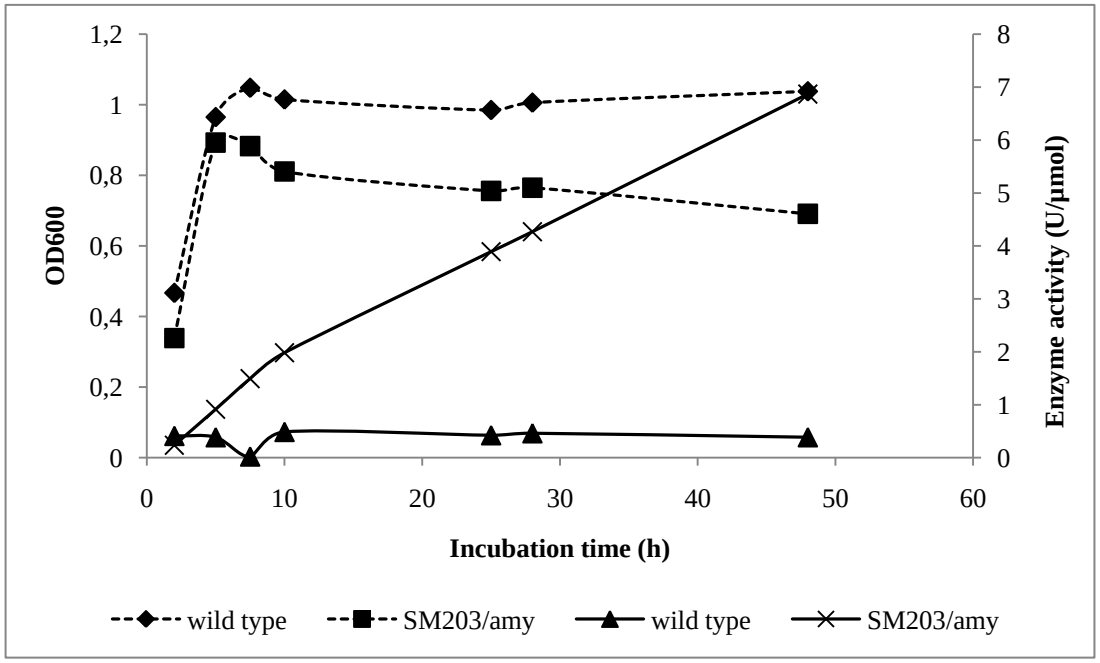


Figure 17. Time profiles of OD_{600} of *A. baylyi* wild type (♦) and SM203/amy (■), and enzyme activity of *A. baylyi* wild type (▲) and SM203/amy (×).

4.2 Citrine fusion protein expression

4.2.1 Construction of fusion genes

Citrine fusion genes were constructed by PCR and ligated into the plasmid SM100/pIX. The RBS site was introduced to the constructed plasmids by the forward primers because upstream of the RBS site of the plasmid SM100/pIX carried histidine-tagged region for protein purification purpose, so that the signal peptides of the fusion partner proteins would probably not work. The constructed plasmids were inserted into *E. coli* XL-1 cells by electroporation, then after incubation at room temperature for 3 days, 4-5 fluorescence-emitting colonies appeared on LB agar plates. There were numerous (more than 500 colonies) non-fluorescence-emitting colonies on the same plate; therefore, the transformation efficiency was very low. After confirming the correct genes, homology-directed transformation of *A. baylyi* wild-type was conducted. This time most of the colonies appearing on the plates emitted the light such as the one shown in Figure 18; however, screening of the correct clones was not easily achieved. Gel electrophoresis of some colony PCR products from fluorescent colonies confirmed the band at around 740 bp, which corresponded to Citrine gene; however, the bands of the fusion genes were not confirmed. Clearly some colonies carried only Citrine gene, which made screening process difficult. In this case, only Citrine gene might have been integrated into *A. baylyi* genome due to unspecific cut of the insert gene fragments; though the reason for this phenomenon remained unclear. As a result of cloning, *yceI*+Citrine fusion gene was not able to confirm after transformation of *E. coli*. Figure 19 shows the result of colony PCR to confirm the fusion genes in transformed *A. baylyi* genome.

Appearance of fluorescent colonies varied among fusion gene constructs. Citrine construct, *cysP*+Citrine, *dsbA* signal peptide+Citrine and *ompA* signal peptide+Citrine constructs emitted bright fluorescent light only after overnight incubation at 30 °C. However, it took 3-4 days until the colonies emitted visually detectable fluorescent light from the *dsbA*+Citrine plate. Visibility of fluorescence was very weak on the *quiB*+Citrine construct plate.

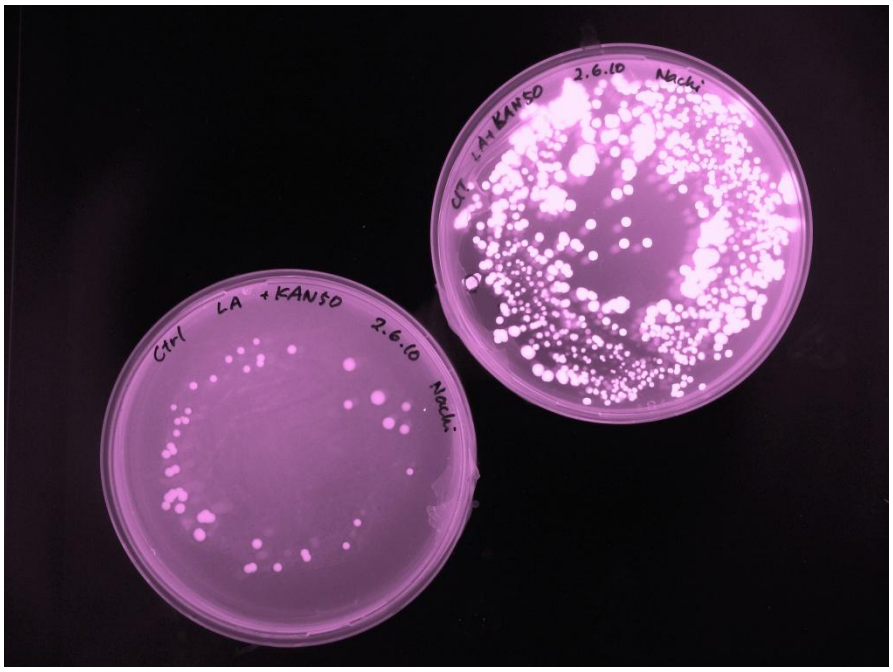


Figure 18. Physiological comparison between Citrine-carrying *A. baylyi* (right) and wild-type *A. baylyi* (left).

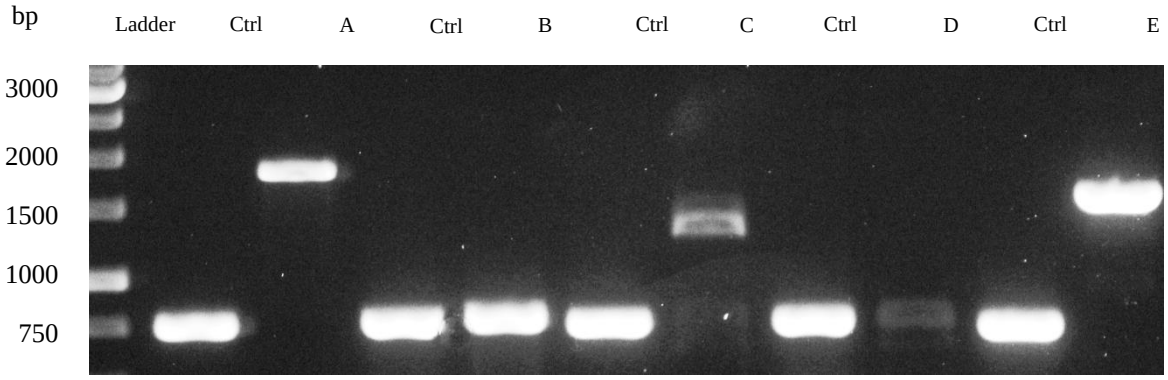


Figure 19. Fugion gene inserts check by gel electrophoresis. Ctrl: positive control (740 bp) which amplifies Citrine gene using the primers na10_1 and na10_2; A: *cysP*+Citrine gene (~1.7 kb); B: *dsbA* SP+Citrine gene (~800 bp); C: *dsbA*+Citrine gene (~1.4 kb); D: *ompA* SP+Citrine gene (~800 bp); E: *quiB*+Citrine gene (~1.6 kb). SP = signal peptide.

4.2.2 Fluorescence measurement

Fluorescence signal was measured for the supernatants (extracellular part) and the re-suspended cells (intracellular part) separately. Figure 20 and 21 show time profiles of optical density measured at 600 nm for the fusion constructs and wide-type grown in M9 minimal medium and MA/9 medium, respectively. There was no growth defects observed in the fusion constructs compared to wild-type when grown in both M9 and MA/9 medium. When grown in M9 minimal medium, the *quiB*+Citrine construct grew slowly compared to the other constructs. Figure 22 and 23 show the relative fluorescence units (RFUs) of the fusion constructs and wild-type strain compared with the same amount of biomass. All the cultures were grown in M9 minimal medium

(Figure 22) or in MA/9 medium (Figure 23) supplemented with 0.2 % casamino acids, 2 % glucose and 20 µg ml^{-1} of kanamycin for the recombinant constructs.

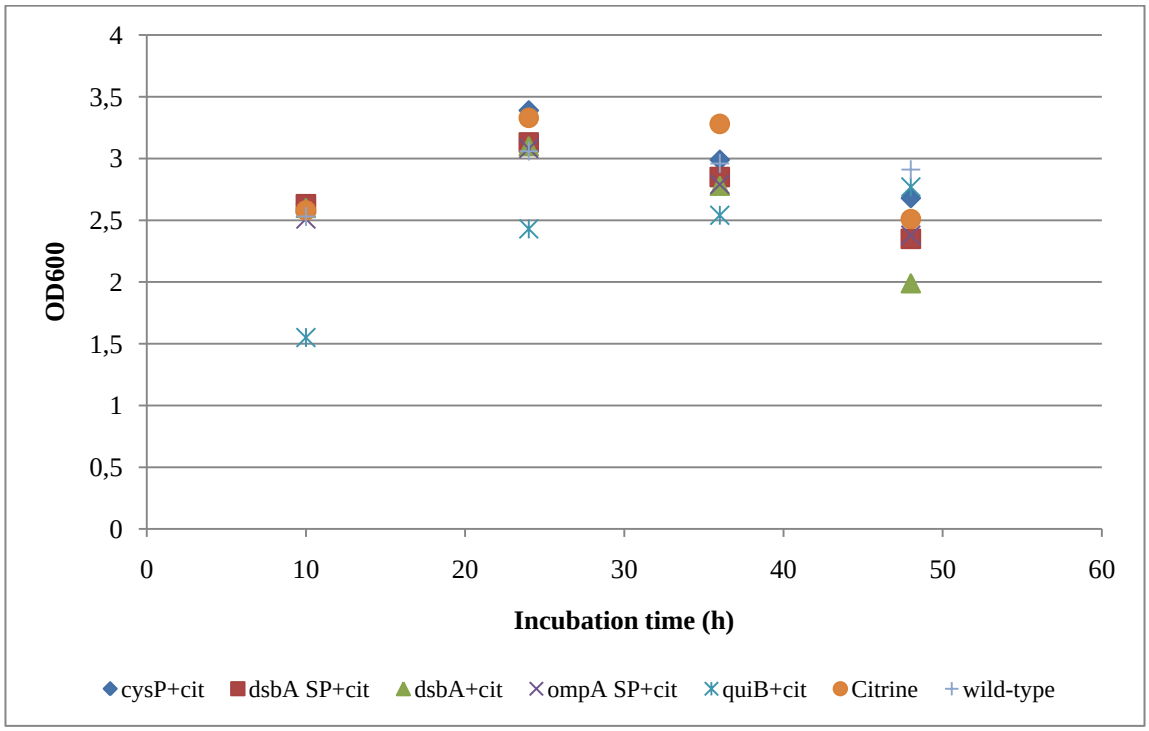


Figure 20. OD_{600} of the fusion construct and wild-type cultures grown in M9 minimal medium.

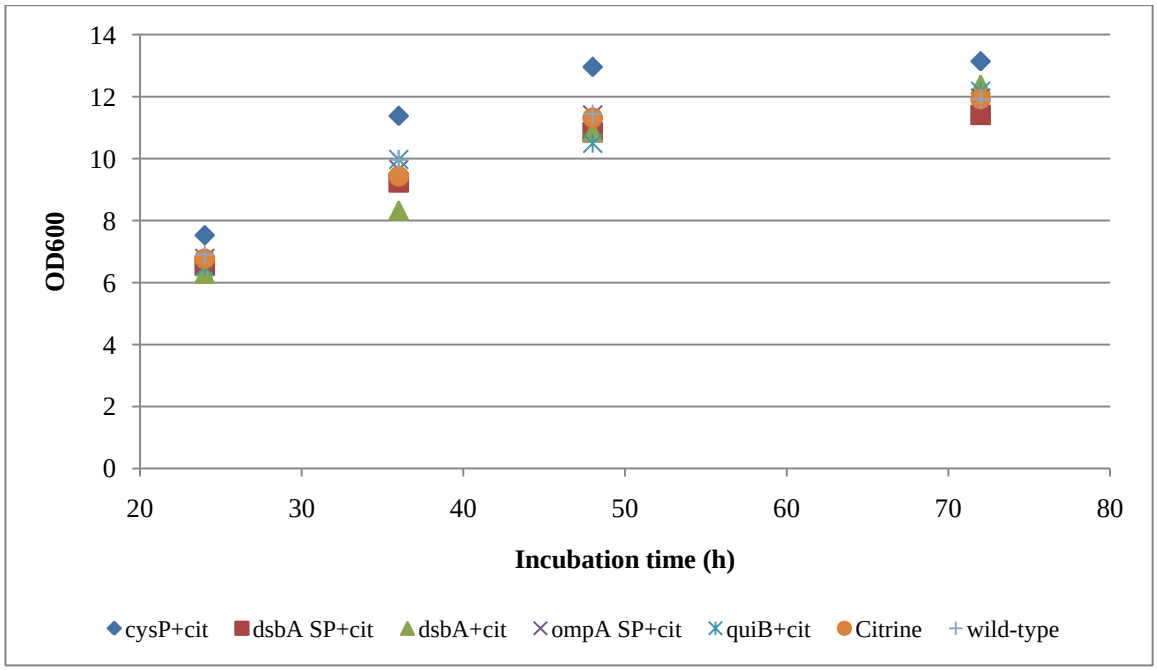


Figure 21. OD_{600} of the fusion construct and wild-type cultures grown in MA/9 medium.

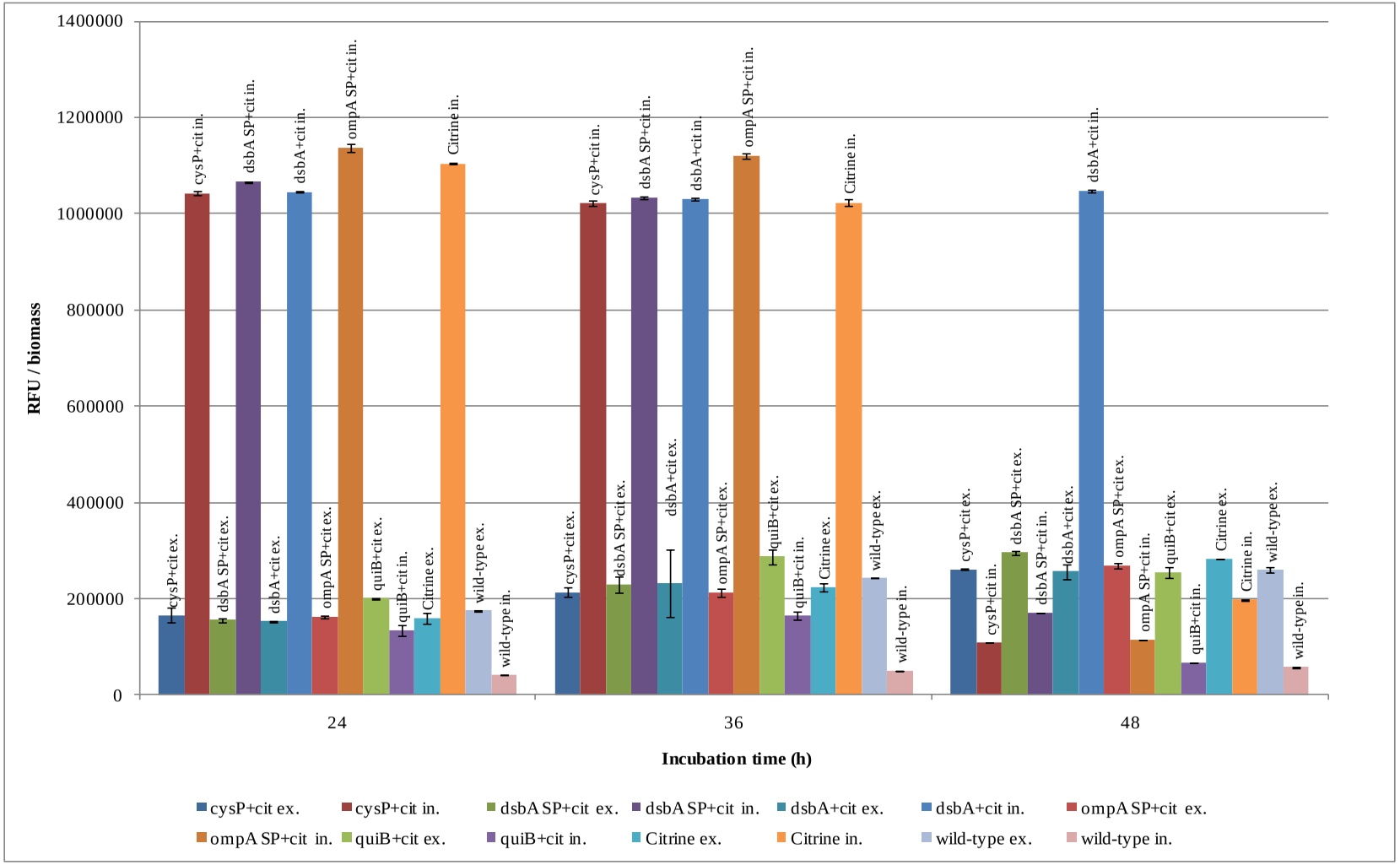


Figure 22. Relative fluorescence unit (RFU) per biomass from the supernatants and the re-suspended cells of the fusion construct cultures grown in M9 minimal medium. ex. = extracellular part; in. = intracellular part; cit = Citrine; SP = signal peptide.

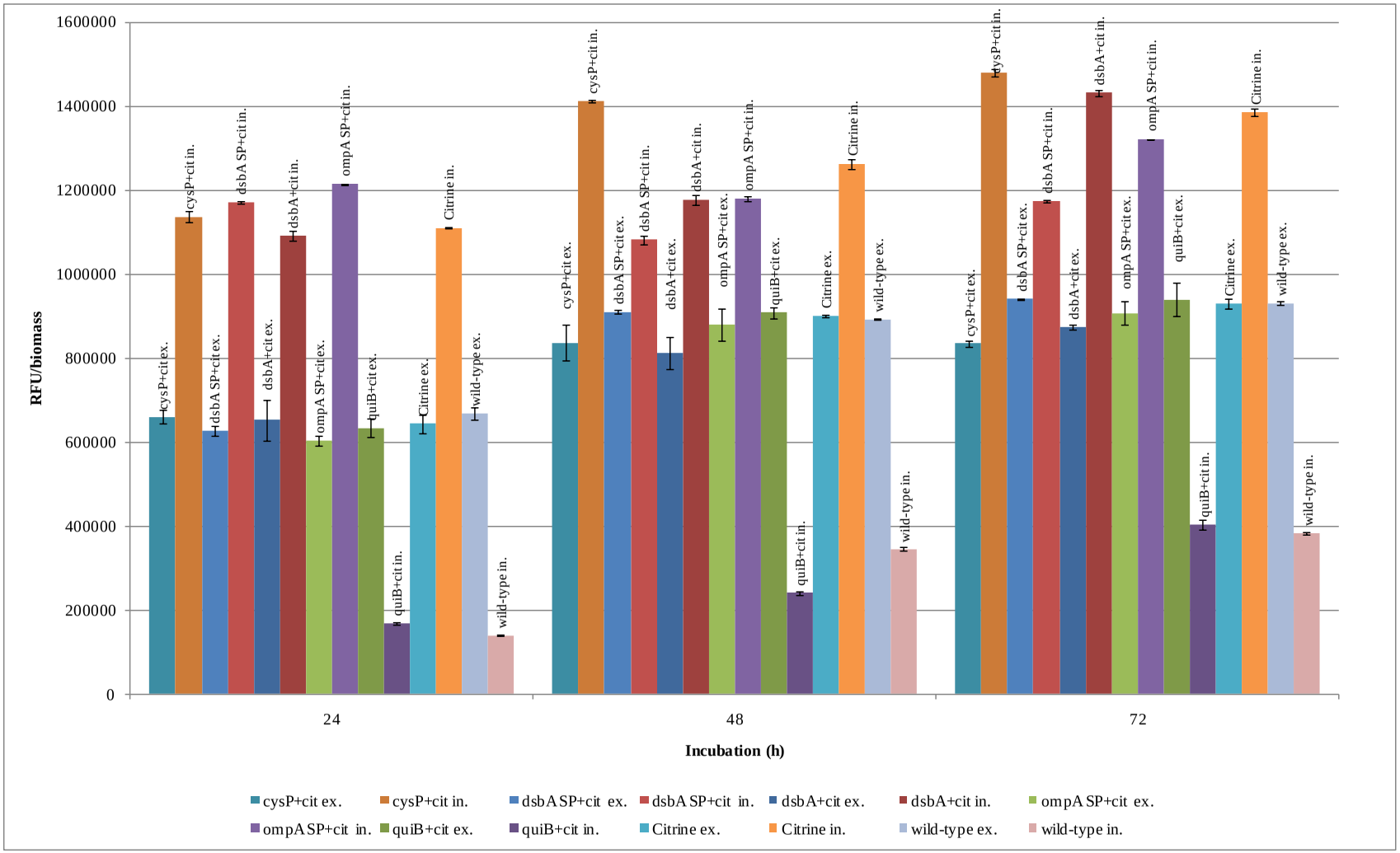


Figure 23. Relative fluorescence unit (RFU) per biomass from the supernatants and the re-suspended cells of the fusion construct cultures grown in MA/9 medium.

Fluorescence excitation and emission spectra of the supernatants and intracellular cultures from wild-type, the Citrine construct, and the fusion construct (*cysP*+Citrine) were also measured in order to confirm the presence of fluorescence. The cultures were grown in MA/9 medium supplemented with 0.2 % casamino acids and 2 % glucose, and incubated for 24 hours. OD_{600} was ~7 for all the cell cultures. Figure 24 and 25 represent emission spectra of all the samples and the supernatants, respectively.

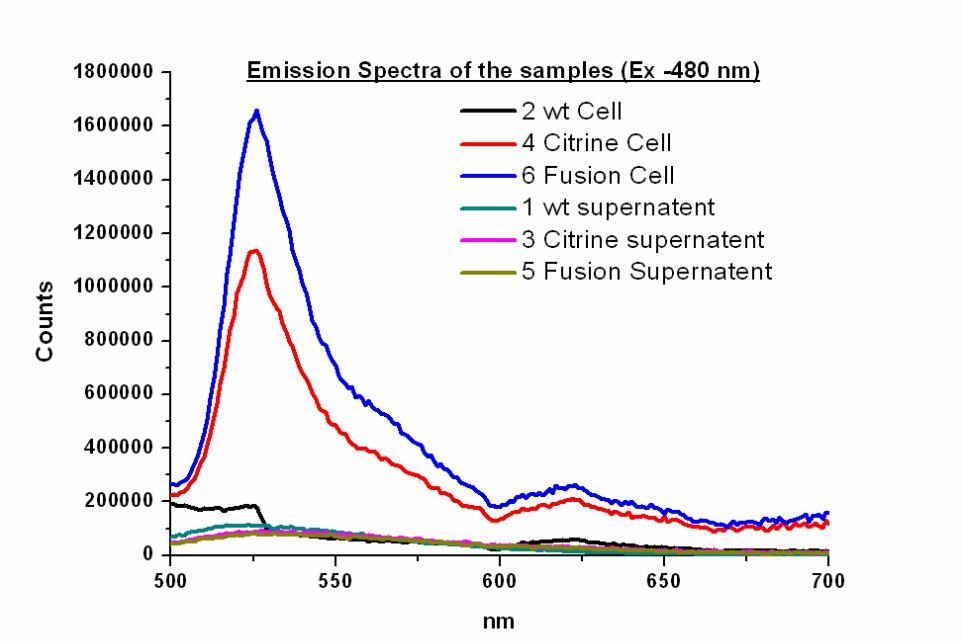


Figure 24. Emission spectra of the samples. wt = wild-type; Citrine = the Citrine construct; Fusion = the *cysP*+Citrine construct; Cell = cell culture; Counts = count per second.

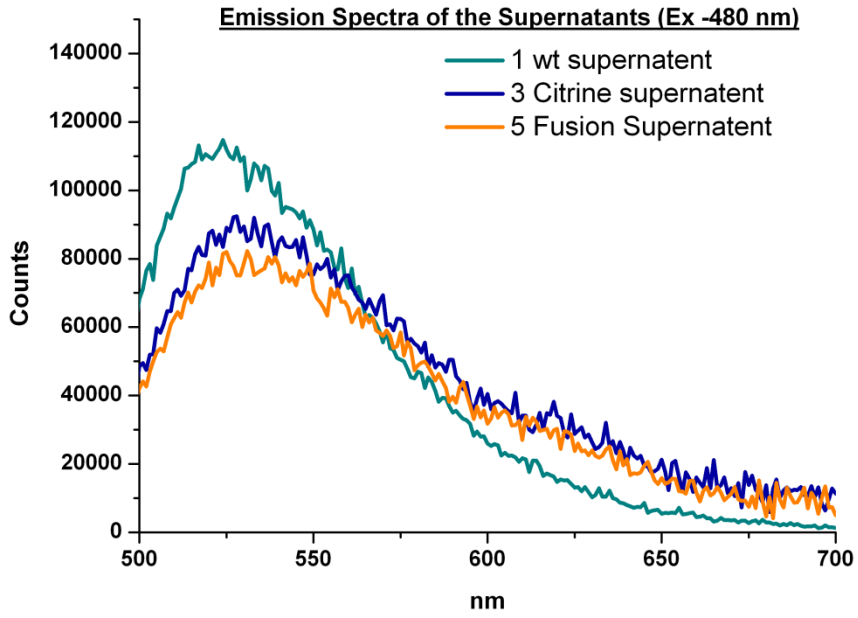


Figure 25. Emission spectra of the supernatants. wt = wild-type; Citrine = the Citrine construct; Fusion = the *cysP*+Citrine construct.

5 Discussion

5.1 α-Amylase enzyme activity

As Figure 17 indicated, α-Amylase activity was quite low for the SM203/amy construct cells, and it was also shown that wild-type strain did not carry naturally ocurring α-amylase gene due to the fact that enzyme activity was almost none during the whole incubation time. Low activity of the construct was considered due to the following reasons: 1) the plasmid carrying α-amylase gene did not carry a strong promoter that could support overexpression of α-amylase enzyme, and 2) the growth medium was not optimal for the construct. As reported in the study by Suominen and coworkers, attachment of the α-amylase native promoters that were removed from the pCSS4 plasmid would not affect the strength of the promoter (Suominen et al. 1987b). Compared to the wild-type, growth of the SM203/amy construct cells was defected faster, which could have been caused by metabolic burden due to expression of foreign gene. For optimization of α-amylase expression, gene construction using a plasmid with strong promoter and optimization of cell growth using different growth media could be further investigated.

Enzymatic activity was also measured using the cultures grown in different growth medium. When grown in LB medium supplimented with 1-2 % glucose, only very weak activity was observed. Addition of 1 % starch into the above medium did not increase the activity at significant level (data not shown). Since the activity in Figure 17 was obtained when the culture was grown in M9 minimal medium supplimented with 0.2 % casamino acids and 0.5 % starch, rich media such as LB medium might have no effect on increasing α-amylase production in *A. baylyi*. Addition of starch in the growth medium led to slight increase of α-amylase activity; however, it was not a strong inducer.

5.2 Fluorescence measurements

Fluorescence measurements were done in order to confirm the presence and localization of Citrine fluorescence. The fluorescence signal was measured for the supernatant (extracellular) cultures and the cell (intracellular) cultures. The excitation and emission spectra were also measured to confirm the presence of any other fluorescent compounds besides Citrine in the supernatants because the high fluorescence signal was detected from the supernatant cultures (Figure 23) when the cultures were grown in MA/9

medium. The following observations from fluorescence measurements are further discussed.

1) High fluorescence signal was obtained from the intracellular parts of *cysP*+Citrine, *dsbA* signal peptide+Citrine, *dsbA*+Citrine and *ompA* signal peptide+Citrine constructs. The high signal was clearly derived from Citrine due to the high emission peaks (~529 nm) detected from the intracellular parts of Citrine and *cysP*+Citrine constructs shown in Figure 24. Compared to the Citrine construct, signals of the fusion constructs were almost at the same level. It can be assumed that expression level of these fusion constructs and the Citrine construct was the same. When grown in M9 minimal medium, fluorescence signal from the *dsbA*+Citrine construct remained high, even after all the other constructs ceased to emit fluorescence after 48 hour incubation. The high signal remained in the *dsbA*+Citrine construct at 72 hour incubation (data not shown). On the other hand, the fluorescence signal from the intracellular part of the *quiB*+Citrine construct remained low during the whole incubation time. This phenomenon corresponded to weak visibility of fluorescence on the LB agar plate. When grown in MA/9 medium, intracellular fluorescence signal remained high after 72 hour incubation in all the constructs except the *quiB*+Citrine construct.

2) As shown in Figure 22, low fluorescence signal was detected in the supernatants from all the fusion constructs. The signals of the fusion constructs were nearly at the same level of those from intracellularly expressed Citrine construct and wild-type strain; therefore, extracellular expression of Citrine using the fusion partners selected in this study was not observed. The same result was confirmed from Figure 24, where no peak from Citrine was observed from the supernatants. This result illustrates that the selected fusion partner proteins that assisted secretion of the co-expressed protein in *E. coli* did not have the same function in *A. baylyi*. The reasons for this phenomenon would not be easy to elucidate since proteins can undergo a variety of changes in a cell; however, it can be assumed that post-translational modification, translocation of these proteins, or secretory system of *A. baylyi* may be different from that of *E. coli*. The result also suggests that another systematic way has to be sought out in order to achieve extracellular expression of recombinant proteins in *A. baylyi*. For instance, other putative fusion partner genes can be selected by conducting proteome-based analysis of *A. baylyi*. So far proteome analysis has been performed on *A. baumannii* strains and *A. radioresistens* S13 strain among *Acinetobacter* species especially for membrane proteins (Pessione et al. 2003; Yun et al. 2008). Pessione and coworkers conducted membrane proteome analysis from the cell cultures grown under different aromatic substrate conditions. According to their study, two-dimensional gel electrophoresis of membrane extracts from benzoate- and phenol-grown cells gave different pattern of spots compared to acetate-grown cultures as control (Pessione et al. 2003). Interestingly, the higher amount of OmpA-like protein, which shows high homology (85 %) to *A. baylyi* OmpA-like protein used in this study, was found in aromatic-grown cultures.

Since these OmpA-like proteins are involved in emulsification of aliphatic and aromatic compounds, overexpression of OmpA-like protein could be induced by growing the cultures with aromatic substrates, which could possibly lead to secretion of the protein. Alternatively, a well-studied secretion system of extracellular protein such as lipase could be used for assisting foreign proteins out of the cells. However, protein-specific chaperone gene might be essential to be co-expressed with heterologous protein gene for correct folding and assisting the protein, like the chaperone protein LipB necessary for production and biofunction of lipase in *A. baylyi* ADP1 (Kok et al. 1995).

Computational- and laboratory-based methods could also be combined to screen membrane- or exo-proteins. Lewenza and coworkers used the computational identification of signal peptides which showed 38 % of the pathogen *P. aeruginosa* PAO1 strain proteome to encode membrane-localized proteins (Lewenza et al. 2005). Screening of the predicted proteins was then conducted by the alkaline phosphatase (PhoA) fusion construction in *E. coli*. By using these methods, 296 out of 310 proteins identified in PhoA screening displayed a predicted export signal and the remaining proteins revealed unique features which were not possible to predict computationally. Computational prediction of target proteins and laboratory-based screening can be complementary to each other, which would be a prominent approach in proteomic studies.

3) Fluorescence signals from the supernatants of all the constructs and wild-type increased along the incubation time, even after the decrease of OD_{600} was observed (Figure 20 and 21). These 'background' signals were significantly high (70-80 % of the intracellular signal) when the cultures were grown in MA/9 medium supplemented with 0.2 % casamino acids and 2 % glucose (Figure 23). It was initially assumed that *A. baylyi* released autofluorescent compounds when the growth condition was optimal and high cell density was achieved. For example, high signal was detected from the supernatants when OD_{600} reached ~6, and the signal continued to increase as OD_{600} increased up to ~12 in this measurement. The putative autofluorescent compounds were removed by using the filters with which compounds of less than 10 kDa (kilo Dalton) molecular weight are filtrated through (data not shown). Emission spectra measurement of the supernatants of wild-type, Citrine construct and *cysP*+Citrine construct showed similar emission peaks at around 525 nm, even though the peaks were considerably small compared to those from Citrine (Figure 25). Further investigation of the putative autofluorescent compounds was beyond the scope of this study; however, one possibility is that certain metabolites excreted from *A. baylyi* cells might be intrinsic fluorescent compounds. For example, riboflavin (vitamin B_2) is a metabolic precursor commonly found in plants and microorganisms and it is known as intrinsic fluorescent compound detected in the 500-600 nm spectral region (Benson et al. 1979). Worst and coworkers studied the iron-regulated riboflavin production from the *Helicobacter pylori* riboflavin synthase genes *ribA* and *ribB*. They found that *E. coli* EB53 carrying a

bifunctional *H. pylori ribBA* gene excreted riboflavin in the culture medium, and the secretion of riboflavin was iron regulated (Worst et al. 1998). Since the high background signals were detected only when grown in MA/9 medium containing $FeCl_3$, riboflavin production in *A. baylyi* might be also regulated by ferric ion.

It is also worth noting that growing the cultures in two different media, MA/9 medium and M9 minimal medium, resulted in considerable differences both in cell growth, Citrine stability and the background signals of the supernatants, but not so much difference in the intracellur signal intensity. When grown in MA/9 medium, the cells grew much faster and continued their stationary phase much longer (Figure 21). The optimal growth of the cells in MA/9 medium led to longer stability of Citrine, as strong fluorescence signal was detected from the intracellular parts of all the fusion constructs except for the *quiB*+Citrine construct up to 72 hour incubation.

6 Conclusions

The following concluding remarks were drawn from this study:

1) α-Amylase was successfully expressed using homology-facilitated transformation of *A. baylyi*. However, its expression level was weak due to weak promoter of the plasmid SM203/pIX and less optimized growth condition.

2) Citrine and Citrine fusion genes were successfully expressed in *A. baylyi*, and strong fluorescence signal was detected over long time of culture incubation. Therefore, Citrine can be used as reporter gene for fusion protein construction in *A. baylyi*.

3) Citrine was not detected with significant level in the culture medium when expressed with the candidates of fusion partner gene. These fusion partner proteins were successfully detected in the culture medium in the expression in *E. coli* K12 strain by other study; therefore, post-translational regulation, translocation, or secretion system of *A. baylyi* may be different from those of *E. coli*.

4) High background of fluorescence signal was detected in wild-type and the fusion gene-carrying *A. baylyi*, and the signal increased significantly along the incubation time. The compounds which caused the high background are not known; however, they were detected into the medium only when the cultures were grown in the optimal growth medium, for example, MA/9 medium supplemented with 0.2 % casamino acids and 2 % glucose, so that the high optical density was obtained.

Expression of foreign proteins in *A. baylyi* was achieved by homology-facilitated transformation; however, secretion of the proteins could not be accomplished in this study due to unknown secretion mechanism of this organism. Proteome analysis could be useful in order to find more suitable fusion partner genes for extracellular expression of heterologous proteins in *A. baylyi*. It is hoped by the author that this study served as a starting point in exploring extracellular expression of the recombinant proteins in *A. baylyi*.

7 References

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