HETEROLOGOUS EXPRESSION OF CELLULASE GENE FROM *BACILLUS AMYLOLIQUEFACIENS* UMAS 1002 IN *ESCHERICHIA COLI* BL21 (DE3)

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Heterologous expression of cellulase gene from *Bacillus amyloliquefaciens* in *Escherichia coli* BL21

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This project is submitted in partial fulfillment of the requirements for the degree Of Bachelor of Science with Honors (Resource Biotechnology)

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<tbody>
<tr>
<td>AGE</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethylcellulase</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-b-d-thiogalactopyronoside</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>K₂PO₄</td>
<td>Potassium acetate</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamphere</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pg</td>
<td>Pictogram</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris-hydrochloric acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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B  Expression vector, pET100 Directional TOPO used for expression of cellulase gene in *E. coli* BL21 (DE3)
Heterologous Expression of Cellulase Gene from *B. amyloliquefaciens* in *E. coli* BL21 (DE3)

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ABSTRACT

Cloning and expression of cellulase gene from *B. amyloliquefaciens* in *E. coli* BL21 (DE3) was attempted to optimize the activity of recombinant cellulase. PCR amplification of cellulase gene from Top10 cell was not successful although different approaches were carried out. Alternative method was taken as time become limiting factor for further works. The pET100 with subcloned cellulase gene was used for expression studies and the work started with extraction of pET100 from Top10 cell and transformation into expression host, *E. coli* BL21 (DE3). Screening of cellulolytic organisms carried by Congo red assay revealed that recombinant CMCase activity on the LB media is higher compared to minimal media. This is based on measurement of diameters of the clear zone produced on CMC plates. Besides the clearing zones on plates, enzymatic and Bradford assay were used to determine the cellulase activity of the selected bacterial isolate in liquid medium. Different parameters including temperature and pH were analyzed to identify cellulase maximal activity. Recombinant cellulase from *E. coli* BL21 (DE3) was shown to have higher activity at temperature 37°C and pH 7. SDS-PAGE analysis showed the presence of two intense bands with estimated molecular weight of about 42kDa and 40kDa. It was suggested that partial degradation of the enzyme took place due to a proteolytic cleavage and this cleavage from the C-terminal ends of the gene could have given rise to the smaller active bands.

Key words: cellulase, *Escherichia coli* BL21 (DE3), Congo red assay, SDS-PAGE analysis

ABSTRAK


*Kata kunci: cellulase, Escherichia coli* BL21 (DE3), assay Congo merah, analisis SDS-PAGE
1.0 INTRODUCTION

*Bacillus amyloliquefaciens* UMAS 1002 is one of the indigenous bacteria that produces cellulase enzyme which able to degrade cellulose efficiently. The cellulase gene has been characterized and expressed successfully in previous studies (Jong, 2002). The recombinant cellulase has been expressed in pET expression system which capable to produce higher amount of enzyme when cloned in *E. coli* BL21 (DE3) expression host.

Cloning and expression of cellulase gene in non-cellulolytic host enable the separation of the specific cellulase from the parental bacterial systems. This allows further investigation of the catalytic properties of the enzyme, nucleotide sequences of the gene and subsequently amino acid sequence of the enzyme it encodes. An understanding of these aspects would enable further genetic manipulations to be carried out.

In recent research, Morimoto *et al.* (2008) in his report states that recombinant protein productivity can be increased by reduction in microbial genome as done in *Bacillus subtilis* strain, MBG874. When compared with wild-type cells, the regulatory network of gene expression of the mutant strain is reorganized after entry into the transition state. To our knowledge, this is the first report demonstrating that genome reduction actually contributes to an in increase in recombinant productivity and lead to creation of bacterial cells with a practical application in industry.

The preferred hosts for protein secretion and expression are *Bacillus* sp., *Streptomyces* sp., *E. coli* and *Saccaromyces cerevisiae* (Jong, 2002). Among the various expression hosts available for heterologous protein production, *E. coli* remain most attractive. In previous works, *E. coli* BL21 (DE3) had been used as expression vector for studies of cellulase gene
and results revealed that *Bacillus subtilis* host strains display much larger and clearer zones around their colonies compared to *E. coli* hosts (Jong, 2002). This implies that *B. subtilis* degrade cellulase more efficiently compared to *E. coli*.

Similar *E. coli* bacterial expression host was used throughout this study but using different expression vector, pET100 which has a strong T7 promoter activity. So, the ultimate aim of this study was to successfully express the isolated cellulase gene from *B. amyloliquefaciens* in *E. coli* besides comparing the recombinant protein produced in *E. coli* with native cellulase in *Bacillus* sp. to determine the activity, efficiency and production rate of recombinant cellulase.

To investigate the expression of cellulase gene in heterologous background, the complete open reading frame of the gene was subcloned into the protein expression plasmid pET100 and the recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) and chemically induced using IPTG. The effects of different parameters including temperature and pH over recombinant protein were tested to optimize the enzyme synthesis. In addition, the enzyme activity and protein concentration also have been analyzed throughout the study by enzymatic and Bradford assay, respectively.

Expression of foreign gene in *E. coli* expression host strain BL21 (DE3) requires three basic steps which includes the insertion of the gene into an expression vector, pET plasmid, introduction of the expression vector into the *E. coli* BL21 (DE3) genome and examination of the foreign gene product (Cereghino and Cregg, 2000).
Heterologous expression of cellulase gene in *E. coli* BL21 (DE3) would include following objectives:

- To transform the isolated cellulase gene from *B. amyloliquefaciens* UMAS1002 in *E. coli*
- To have recombinant protein (cellulase) successfully produced in *E. coli* system
- To compare the production rate of cellulase between *B. amyloliquefaciens* and *E. coli*
- To optimize cellulase enzyme production by manipulating different parameters
2.0 LITERATURE REVIEW

2.1 An overview of cellulose

Cellulose is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere (~100 billion dry tons/year) (Holtzapple, 1993; Jarvis, 2003 and Zhang and Lynd, 2004). Cellulose is a linear essentially insoluble β-1, 4-glucosidically linked homopolymer with a size of about 8,000 to 12,000 glucose units. It mainly used as energy source by numerous and diverse microorganisms including fungi and bacteria which produce functionally complete cellulase enzyme systems (Ekinci et al., 2002).

Each cellulose molecule is a linear glucopyranose polymer of anhydroglucose residues form long chain with various degree of polymerization. Each residue is rotated 180 degrees about the main axis with respect to its neighboring residues. These cellulose chains are held together by hydrogen bond to form insoluble fibrils (Jong, 2002). An important feature of cellulose that is relatively unusual in the polysaccharide world is its crystalline structure.

Cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues) which undergo self-assembly at the site of biosynthesis (Lynd et al., 2002). Depending on the degree of hydrogen bonding within and between cellulose molecules this polysaccharide is found in crystalline or paracrystalline (amorphous) forms. Cellulose exists within a matrix of other polymers primarily hemicellulose and lignin. Hemicellulose is a branched sugar polymer composed of mostly pentose (five-carbon sugars) and some hexose (six-carbon sugars). Lignin is a complex, highly cross-linked aromatic polymer that is covalently linked to hemicellulose thus stabilizing the mature cell wall. These polymers
provide plant cell walls with strength and resistance to degradation which also makes these materials a challenge to use as substrates for biofuel production.

In general, native cellulose is classified into three types. The first type is pure cellulose that has high degree of crystalline such as cotton cellulose and the second type contains crystallized cellulose and a mixture of impurities such as lignin and hemicelluloses. The third type not contains crystalline cellulose but still have some impurities, such as carboxymethylcellulose (Jong, 2002).

2.2 Cellulase enzyme system

In recent years, the molecular biology of bacterial cellulase has received considerable attention. Cellulase are enzymes that hydrolyze the β-1, 4 linkages of cellulose, the most abundant organic compounds on Earth. The enzyme commonly divided into three functional types such as endo-β-1, 4-glucanase (endocellulase), exo-β-1, 4 glucanase (exocellulase) and β-1, 4-glucosidase (Lo et al., 2003).

Cellulase enzymes exist in complex systems in order to efficiently harvest energy from polysaccharides. According to Ohrmund and Susan (n.d), these systems are complex for two reasons. First, many of the enzymes themselves are complex in which they are modular proteins that are comprised of one or more catalytic domains and substrate binding domains. Secondly, the systems are complex and may contain from a few to more than twenty enzymes. All of these enzymes hydrolyze a particular substrate. Researchers have proposed that there are at least two steps involved with hydrolysis. The first step involves a prehydrolytic step
where anhydroglucose chains are swollen and hydrated. The second step involves hydrolytic cleavage of the hydrated polymer by one or many enzymes and steps.

According to Gilbert et al. (1993), only exo-β-1, 4-glucanase has the ability to degrade the crystalline cellulose and other enzymes can degrade cellodextrin and amorphous cellulose. The degradation of cellulose by these cellulases can occur either aerobic or anaerobic. Cellulase (a complex multienzyme system) acts collectively to hydrolyze cellulose from wastes to produce simple glucose unit. These simple glucose units can be further fermented to produce ethanol.

Cellulase enzyme degrades carboxymethyl cellulose (CMC molecular weight 180 000) in an endo-acting manner to yield carboxymethyl cellulo-oligomers (mean molecular weight of 20 000). This cellulase which has a molecular weight of 50 000 had optimum activities at pH 6.8 and 44°C (Ohmiya et al., 1988). Lynd et al. (2002) in his report states that, the great majority of cellulose hydrolysis research to date has focused on the genetics, structure, function, and interaction of components of cellulase enzyme systems.

With the advent of gene libraries, DNA and protein databases, over 400 genes of the cellulase family of enzymes have been cloned and sequenced. Currently, a nomenclature system has been implemented and these enzymes are now classified into 82 families based on amino acid sequence and functional similarity (Ohrmund & Susan, n.d.).
2.3 Cellulose degradation by bacteria

Cellulolytic microorganisms play an important role of recycling the cellulose in the biosphere. Cellulose is used as energy source by diverse type of microorganisms such as fungi and bacteria, which producing cellulose-degrading enzyme systems. Oikawa et al. (1997) characterized a cellulose-producing acetic acid bacterium, *Acetobacter xylinum* strain KU1 which produces high level of extracellular endo-β-glucanase. Other than that, an anaerobic thermophilic and cellulolytic bacterium *Clostridium thermocellum* strain F1 is able to degrade crystalline cellulose efficiently. The ability to degrade crystalline cellulose is due to the action of cellulosome, a high molecular mass, extracellular cellulase complex composed of endoglucanase, xylanase and exoglucanases (Ahsan et al., 1996).
2.4 *Escherichia coli* as expression host

*Escherichia coli* is by far the most widely employed host, provided post-translational modifications of the product are not essential. Its popularity is due to the vast body of knowledge about its genetics, physiology and complete genomic sequence which greatly facilitates gene cloning and cultivation (Carson and Robertson, 2006, p5). The gram-negative bacterium, *E. coli* is commonly used for heterologous protein production due to the availability of an increasingly large number of cloning vectors and mutant host strains. High growth rates combined with the ability to of the strains producing up to 30% of their total protein as the expressed gene product result in high volumetric productivity (Dale, 1989). Strains used for recombinant production have been genetically manipulated so that they are generally regarded as safe for large-scale fermentation.

The ease of its transformability and genetic manipulation has subsequently solidifies the role of *E. coli* as the host of choice for the propagation, manipulation and characterization of recombinant DNA. *E. coli* is one of the most intensively studied living species and widely used hosts for the production of heterologous proteins and its genetics are far better characterized than those of any other microorganism. Glover (1984) in his report state that wild-type *E. coli* has no growth factor requirements and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O₂. Under anaerobic conditions it will grow by means of fermentation producing ethanol as end products.

According to Ekinci (2001) for some genes isolated from rumen bacteria there is evidence that *E. coli* recognizes promoters different from those used in the original host
organism. This has been shown for a cellulase gene from *P. ruminicola* but may also apply to genes from other species.

2.5 **Heterologous protein production in bacterial system**

In general, over expressed recombinant proteins accumulate either in the cytoplasm or periplasmic space. Most frequently, the cytoplasm is the first choice for heterologous protein production because the higher yield seems to be more attractive. Remarkable yields of secreted proteins are well-documented (reviewed in Georgiou and Segatori, 2005). Additionally, purification has been greatly simplified by producing recombinant fusion proteins which can be affinity-purified such as glutathione-S-transferase and maltosebinding fusion proteins.

Currently, strategies to secrete the target proteins by translocation into the periplasmic space or to release the target proteins by linking to existing excretory systems are being developed (Meena and Harish, 2001). The efficiency of expression will also depend on differences of codon utilization by bacteria. So, the original sequence of the heterologous gene has to be modified to reflect the codon usage by the chosen expression system. A disadvantage of recombinant proteins produced in *E. coli* is the accumulation of lipopolysaccharide (LPS), generally referred as endotoxins which are pyrogenic in humans and other mammals. As *E. coli* has toxic cell wall pyrogens hence products need to be tested more extensively before use.

To maximize protein expression in *E. coli* system, the cloned gene must be transcribed and translated at high efficiency. One way of optimizing the transcription is to have increased gene dosage. To obtain high gene dosage, heterologous gene are normally cloned into plasmid
which are present at high copy number, ranging from 15 to several hundreds copies per cell. Another effective and convenient way to maximize the level of transcription is to position the cloned gene downstream from a strong promoter (Kay, 2006). Other than that, expression vector play paramount importance in efficient protein expression. The most popular are the commercially available vector utilizing the bacteriophage T7 promoter. For routine protein expression, *E. coli* BL21 and K12 and their derivatives are most frequently used. In contrast to K12 strains, BL21 derivates are lon (Phillips et al., 1984) and ompT protease deficient.

### 2.6 Quantitative assay

All existing cellulase activity assays can be divided into three types such as the accumulation of products after hydrolysis, the reduction in substrate quantity and the change in the physical properties of substrates. The two basic approaches to measuring cellulase activity are measuring the individual cellulase such as endoglucanases, exoglucanases or β-glucosidases activities and measuring the total cellulase activity. For cellulase activity assays, there is always a gap between initial cellulase activity assays and final hydrolysis measurement (Sheehan and Himmel, 1999). To be most meaningful, individual cellulase component assays must also be based on a reliable estimation of the amount of individual enzyme component present in the assay. This information permits the calculation of specific activity such as bonds broken per milligram enzyme per unit time.

Cellulase activities can be measured based on a reduction in substrate viscosity and an increase in reducing ends determined by a reducing sugar assay. Cellulase activities can also be easily detected on agar plates by staining residual polysaccharides (CMC or cellulose) with various dyes because these dyes are adsorbed only by long chains of polysaccharides (Jang et
al., 2003; Kim et al., 2000; Murashima et al., 2002 and Ten et al., 2004). These methods are semi-quantitative and are well suited to monitoring large numbers of samples. Precision is limited because of the relationship between the cleared zone diameters and the logarithm of enzyme activities. Cellulase activities are detected easily by examination of halo zones on solid agar plates using CMC as the substrate followed by Congo red staining and washing. Higher hydrolysis rates of mutants usually result in larger halos.
3.0 MATERIALS AND METHODS

3.1 Material and Reagent Preparation

a) LB Broth

1.0 g tryptone, 0.5 g yeast extract and 0.5 g NaCl were added into a 250 mL Duran bottle and top up with distilled water to 250 mL. The contents were shaken and autoclaved with loosen cap. After autoclaving, the LB Broth was cooled down at room temperature and stored at 4 °C for future use.

b) 25 mM Tris HCl, pH 8

12.1 g of Tris HCl was dissolved in 80 mL of distilled water, pH adjusted to 8 using 5 M NaCl and 3 M HCl, distilled water top up until reached 100 mL.

c) LB Agar

7.4 g of LB Agar powder was added into 250 mL Duran bottle and top up with distilled water to 200 mL. The contents were shaken and autoclaved with loosen cap. After autoclaving, the agar solution was cooled down to room temperature and agar plate was prepared in laminar plate hood. The plates were wrapped in plastic and stored at 4 °C.
d) **0.2 M NaOH, 1 % SDS**

NaOH/SDS was prepared fresh from 10 M NaOH and 10 % SDS before every use. To make 1 mL NaOH/SDS solution, 100 μL 10 % SDS, 20 μL 10 M NaOH AND 880 μL dH₂O were added and mixed.

e) **3 M KAc, pH 8**

14.72 g potassium acetate was dissolved in 80 mL dH₂O. The pH was adjusted using sodium hydroxide and glacial acetic acid and topped up with dH₂O to 100 mL.

f) **SDS, 10 %**

1 g SDS was dissolved in 10 mL distilled water and shaken to dissolve completely.

g) **10 M NaOH**

8 g NaOH was dissolved 10 mL of distilled water. The pH was adjusted using sodium hydroxide and glacial acetic acid and topped up with dH₂O to 20 mL.

h) **Sodium Potassium Tartrate Solution**

12 g of sodium potassium tartrate tetrahydrate was dissolved in 8 mL of 2 M NaOH. The solution was heated directly on heating plate using constant stirring to dissolve it.

i) **96 mM 3, 5 dinitrosalicylic acid (DNS) solution**

The solution was prepared in deionized water using 3, 5 dinitrosalicylic acid. The solution was heated directly on a stirrer plate using constant stirring to dissolve it.
j) **1 % (w/v) soluble CMC solution**

Soluble CMC solution was prepared by dissolving 1 g soluble CMC in 100 mL distilled water. The solubilization was facilitated by heating CMC solution in a glass beaker directly on heating plate using constant stirring. The solution was brought to boil and the solution was maintained at this temperature for 15 minutes. Then the solution was allowed to cool to room temperature with stirring. CMC solution was returned to original volume by addition of water.

k) **D-glucose standard solution**

0.2 % (w/v) glucose solution prepared by dissolving 0.2 g of D-glucose in 10 mL deionized water.

l) **Bradford stock solution**

Mix 100 mL of 95 % ethanol, 200 mL 88 % phosphoric acid and 35 mg Serva Blue G Coomasie blue. The solution stable indefinitely at room temperature.

m) **Bradford working buffer**

425 mL of distilled water, 15 mL of 95 % ethanol, and 30 mL of 88 % phosphoric acid and 30 mL of Bradford stock solution were added. The solution was filtered through Whatman Number 1 paper and store at room temperature in brown glass bottle or wrapped with aluminum foil.