Heterologous Expression of Xylanase Gene from *Bacillus* sp. in *E.coli* BL21 (DE3)

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List of Abbreviations

AGE	Agarose Gel Electrophoresis
bp	Base pair
BSA	Bovine serum albumin
Da	Dalton
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
EC	Enzyme Commision
EDTA	Ethylene diamine tetra acetic acid
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
KAc	Potassium acetate
Kb	Kilobase pairs
KH_2PO_4	Potassium dihydrogen phosphate
LB	Luria-Bertani
$MgSO_4$	Magnesium Sulfate
mRNA	messenger Ribonucleic acid
Na_2HPO_4	Disodium hydrogen orthophosphate
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NH_4Cl	Ammonium Chloride
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
sp.	Species

TBE	Tris-Borate-EDTA
TE	Tris-EDTA
V	Volt

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ABSTRACT

Xylanase gene of 642 bp with molecular weight 23kDa which was isolated from indigenous *Bacillus* sp. The isolated xylanase gene from indigenous *Bacillus* sp. was cloned into pET expression vector to obtain a high level expression of this recombinant family 11 xylanase in expression host *E.coli* BL21 (DE3). This attempt to clone the gene was initiated with the extraction of xylanase gene previously isolated from pGEM®-T easy cloning vector. The cloning vector was digested with restriction endonuclease and the xylanase insert was cloned into pET41(a) and transformed into *E.coli* BL21(DE3) via heat shock transformation. The expression was attempted to be observed through formation of halos in congo red staining method. Qualitative xylanase screening showed no detectable xylanase activity. This was predicted to be due to reasons like improper framing (frameshift) of cloned xylanase gene to *Lac*Z promoter or incapability of *E.coli* BL21(DE3) to grow optimally in M9 minimal media with corn cob xylan source. It is highly recommended to get the full sequence of recombinant pET41(a)-Xyn to confirm the position of ligation of xylanase gene. The minimal media should also be altered in salt composition for optimized growth of *E.coli* BL21 (DE3).

Key words: Xylanase, *Bacillus* sp., heat shock transformation, frameshift, minimal media

ABSTRAK

Gen xylanase yang mempunyai 642bp dengan jisim molekul 23kDa diasing dari Bacillus sp. yang berhabitat di kawasan tanaman sago. Gen yang diasing diklon dalam vektor ekspresi pET untuk memperolehi xylanase rekombinan keluarga 11 dalam E.coli BL21 (DE3). Cubaan ini dimulakan dengan reaksi enzim EcoR1 untuk asingkan gen xylanase dari vektor pGEM®-T easy. Gen yang diasingkan diklon dalam pET41(a) dan ditranformasikan ke dalam E.coli BL21 (DE3) dengan cara transformasi heat shock. Protein yang diekspress dikesan dengan nyahwarnaan pelumuran Congo red sekitar koloni. Walaubagaimanapun, tiada penyahwarnaan yang diperhatikan. Antara sebab yang diramalkan ialah rangkaan gen xylanase yang tidak selurus dengan pendorong LacZ dan kekurangan dalam pertumbuhan E.coli BL21 (DE3) di media minimal M9 yang ditumbuh dengan sumber xylan dari jagung. Ia dinasihatkan supaya mendapatkan rangkaian nukleotid pET41(a) yang diklon dengan gen xylanase untuk mengetahui posisi gen xylanase. Media minimal juga harus dioptimumkan dari segi kandungan garam supaya pertumbuhan E.coli BL21 (DE3) yang optimum dapat diperolehi.

Kata kunci: Xylanase, Bacillus sp., transformasi heat shock, rangkaan gen, media minimal

1.0 Introduction

Agricultural wastes have been in abundance and sometimes were not given right attention to the usefulness of these wastes. In Malaysia, generation of agricultural wastes has been a fundamental issue which could actually serve for the production of value added commodities and act as a preventive step for environmental pollution (Pang et al., 2006). Xylan is a type of hemicellulose which is copiously found in plant cell wall and so as in agricultural wastes. The degradation of xylan could benefit in fermentation processes during bioconversion.

Indigenous bacterial xylanase have shown promising potential in degradation of xylan. Xylanase is an essential enzyme involved in breaking of the main branch of xylan to xylo-oligosaccharide and xylose. Various researches have been done involving isolation and characterization of xylanase gene from microorganisms like bacteria, fungi and actinomycetes and cloning of these genes into different expression hosts, namely *Escherichia coli* (Subramaniam & Prema, 2002).

The expression of the cloned xylanase gene is either periplasmic or intracellular. There are also secretion been observed but the recombinant protein secreted was found to be non-glycosylated (Kulkarni et al., 1995 & Helianti et al., 2008). It has also been reported that recombinant xylanase has slight differing properties in comparison to the xylanase produced by the native parent *Bacillus* sp. and expression of recombinant xylanase from different genus of *Bacillus* sp. The difference was mainly observed in terms of stability in industrial ph and temperature. The most commonly used host system for recombinant protein expression and engineering is *E.coli* as reported by Ni et al. (2009). Xylanase gene from other organism apart from *Bacillus* sp. which has been studied through heterologous expression has also been published. For example, thermophilic bacteria like

Thermotoga sp. (Saul et al., 1995) and *Caldocellum saccharolyticum* (Luthi et al., 1990) and filamentous fungus like *Cochliobolus carbonum* (Apel-Birkhold et al., 1996).

In general, the application of recombinant technology in the production of xylanase is for the economical benefits towards higher productivity and trouble-free downstream processing in fermentation (Farliahati et al., 2009). In addition to that, it has also been reported by Lee et al. (2008) that xylanase produced through recombinant technology enable us to produce cellulase-free xylanase in case of maintaining the cellulose structure and provides tolerance to various cations.

The objective of this research is to successfully clone the xylanase gene from *Bacillus* sp. into *E.coli* BL21 (DE3) for high quantity, extracellular xylanase production and analyse the presence of xylanase through detecting the formation of visible clearings around microorganisms on solid media with Congo Red solution after an induction by IPTG.

2.0 Literature Review

2.1 Xylanolytic *Bacillus sp.*

The thermophilic, gram positive and endospore forming xylanolytic bacteria which is used in this project was isolated from soil at the vicinity of sago plant. Based on the study done by Chong (2009), the bacteria has been said to show different xylanase thermostability properties compared to the ubiquitous strains. This may be due to the indigenous environmental adaptation of the isolated bacteria.

Numerous literatures have reported on the cloning from various xylanolytic *Bacillus sp.* into different expression hosts. Thermophilic xylanolytic strains are more in research demand due to the stability possessed in term of temperature and pH as industrial applications like biobleaching is known to require enzymes that are thermostable and active at neutral or alkaline pH for improved bleaching even at extreme temperatures (Jiang et al., 2006). Among the bacteria normally used for research purposes, *Bacillaceae* is the most favored for industrial applications because of its ability to express most of the enzymes (Morales et al., 1993).

2.2 Xylanase

β-1,4-Xylans are heterogenous polysaccharide found in cell wall of all land plants and almost in all parts of plants (Wong et al., 1988). This molecule is β-1,4-linked with xylosidic linkage. Degradation of xylans is carried out by a complex of several hydrolytic enzymes with diverse specificity and modes of action. This is due to the heterogeneity and complex constituent and structure of xylan (Beg et al., 2001). Therefore, xylan degrading cells produce a set of enzymes for complete degradation of xylan. The main chain of xylan

is degraded by endoxylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8), β-xylosidase (β-D-xylosidase xylohydrolase; EC 3.2.1.37) and acetyl xylan esterase (EC 3.1.1.72) (Kosugi et al., 2002). The side chains in xylan such as acetyl and arabinofuranose are removed by different enzymes present in xylanolyitc system before xylan is digested by xylanase (EC 3.2.1.8). Then, xylan will be degraded to xylo-oligomers, which will be transported into the cell by permease to further degrade forming xylose by intracellular β-xylosidase (Erlandson et al., 2001). According to Beg et al. (2001), xylanase are usually induced when there is media containing pure xylan or xylan-rich residues.

Xylanases which are produced by various organisms like bacteria, algae, fungi, protozoa, gastropods and anthropods were originally known as pentonases and recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961. There are numerous xylanases that varies according to their specificity, primary sequences and folds. These enzymes are later grouped according to primary structure comparisons of catalytic domain and sequence similarities into families of glycosyl hydrolases(Collins et al., 2005). However, there are also plants like Japanese pear that produce xylanase during the over-ripening period in a fruit life-cycle. It was also found that some higher animals and freshwater mollusc are able to produce xylanase (Subramaniam & Prema, 2002).

Based on the research by Collins et al. (2005), the search from database using the classification number EC 3.2.1.8 showed that enzymes with xylanase activity was found generally in family 5, 7, 8, 10, 11, 16, 26, 43, 52 and 62 of glycosyl hydrolase. However, those family with distinct catalytic domain and demonstrated endo-1,4-β-xylanase activity are enzymes from family 5, 7, 8, 10, 11 and 43. From the reviews of journals on cloning of xylanase gene, those from family 10 and 11 are generally favored for research purpose. For application of xylanase in industry, xylanase from family 11 is more efficient than that of family 10 as family 11 endoxylanase have no cellulose activity, more compact in size and

they have smaller molecular mass. Therefore, they could penetrate easily into cellulose fibers (Helianti et al., 2008).

Concomitant to this phenomenon, activity of fungal xylanase has been shown to contribute to pathogenic invasion of fungal into plant hosts. Xylanase, in association with cellulose degrading enzyme is reported to play role in primary invasion into host cells by softening the region of penetration and causing further effects like eliciting necrosis, leakage of electrolyte and synthesis of pathogenic proteins (Subramanian & Prema, 2002; Apel-Birkhold, et al., 1996). Xylanase has also been proved to be an essential tool in the industrial use of starch conversion or saccharification. It has widely used for an indirect purpose whereby xylanase is applied on wheat starch viscous slurry which is caused by xylan (Crabb et al., 1999). Early studies have shown that, microbial xylanase with the association of cellulase was necessary to show pathogenic property for primary invasion into plants. This process directly acts as to softening of the cell wall of penetration region through partial cell wall degradation of that particular region (Subramaniam & Prema, 2002).

According to Helianti et al. (2008) and Yang et al. (1988), cloning of xylanase gene into an expression host could increase the activity of recombinant xylanase. Therefore, to engineer the enzyme in terms of the molecule and to elucidate the mechanism of enzyme-substrate interaction, subcloning of xylanase gene into mesophilic bacteria should be practiced so that the genes could be overexpressed in an extracellular manner. Yang et al. (1988) has also reported that continuous cloning of xylanase in various host could facilitate in the progress to engineer the enzyme to elucidate desirable properties.

2.3 Xylanase gene

Based on the report published by Helianti et al. (2008), it is showed that *Bacillus sp.* xylanase gene contain an ORF of 642 bp(base pair) encoding 213 amino acids that has a isoelectronic point of 9.96 and molecular mass 23kDa. It also reported that different microbes from the genus *Bacillus* show high homology range of xylanase gene which indicates that the gene of family 11 xylanase were highly conserved among different *Bacillus sp.* Preferably experimented species for gene homology was *Bacillus subtilis, Bacillus circulans, Bacillus halodurans* and *Bacillus firmus.*

Various different size of the ORF which codes for xylanase has also been reported. Based on the study done by Blanco et al. (1999), xylanase gene of 3258bp belonging to the ORF which codes for xylanase with 120kDa was characterized. Apart from that, it was also found that this ORF codes for xylanase with multiple domains which include catalytic domain, cellulose binding domain and thermostabilizing domain.

Different bacterial microflora possesses multiple forms of xylanase activity (Yang et al., 1988). It is understood that genetic constituency and regulation of xylanase gene plays a key role in the multiplicity of xylanase. One possible reason is the existence of separate xylanase genes with distinct DNA sequences. Yang et al. (1988) & Wong et al. (1988) also reported that *B.polymyxa* appeared to contain two xylanase genes, one coding for alkaline xylanase with low molecular weight (16000-22000) and one for slightly acidic with high molecular weight (43000-50000). It is also suspected that one of these forms of xylanase maybe a prexylanase. However, these two forms of xylanase appeared to be conserved in *Bacillus* sp., exceptionally, xylanases from *B.subtilis* shows intermediate molecular weight of 32000 (Wong et al., 1988). There are certain plausible reasons for the multiple occurrence of xylanase in *Bacillus sp.* which are due to the presence of proteolytic

modifications of the parent enzyme, existence of separate xylanase genes with distinct DNA sequences or disparity in the mRNA readouts (Yang et al., 1988).

The xylanase gene cloned in this project was isolated from *Bacillus sp.* by Nikson (2009) which is found in the vicinity of sago plantation soil. Isolated xylanase gene was predicted to code for an enzyme with molecular weight of 23.3kDa and isoelectric point of 9.44 which is similar to the xylanase isolated by Helianti et al. (2008) and Lee et al. (2008) from *B.licheniformis* found in hot spring. However, the thermostability might not be the same due to the environmental adaptation of crude xylanase in this study was not thermophilic and accounts for the slight different in amino acid sequence as compared to the one from *B.licheniformis* from hot spring.

Multiple alignment of ORF 642bp xylanase gene with sequences from closely related *Bacillus* sp. showed 99% homology with *B. subtilis*, *B. pumilus*, *B. cereus* and *B. amyloliquefaciens* while 90% homology to *B. lichenoformis* xylanase. It was envisaged that different source of isolation could be a reason for the low homology with *B. licheniformis*. Based on the phylogenetic analysis based on xylanase amino acid sequence revealed that isolated xylanase for this project showed closer relationship to xylanase gene from *B. cereus, B. amyloliquefaciens* and *B. pumilus*. Similar phylogenetic analysis of family 11 xylanase amino acid sequences by Helianti et al. (2008) showed that the deduced amino acid has highest homology to the xylanase from *Bacillus subtilis* wich is ninety one percent (91%) of identity.

Another isolate of xylanase from the gut of a worm (*Rhynchoporus ferrugineus* larva) was also studied in the study by Nikson (2009). The xylanase gene have shown its highest BLAST match with endo-1,4-beta-xylanase (xylB) gene from *Bacillus subtilis* with 99% identity. In addition, *B. licheniformis* xylanase gene from *R. ferrugineus* gut was also

aligned to xylanase gene from xylanolytic *Bacillus* sp. This alignment showed 98% identity. As mentioned by Helianti et al. (2008), the xylanase gene is found to be ubiquitous enzyme and is widely distributed in a range of microbes inhabiting a particular environment, which in this case, it is the sago plantation soil.

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1   ATGTTTAAGTTTAAAAAGAATTTTTTAGTTGGATTAACGGCAGCTTTAATGAGTATTAGC
61  TTGTTTTCGGCAACCGCCTCTGCAGCTAGTACAGACTACTGGCCAAATTGGACCGATGGG
121 GGCGGAACAGTAAACGCGGTCAATGGGTCTGGCGGGAATTACAGTGTTAATTGGTCTAAT
181 ACCGGAAATTTCGTTGTTGGTAAAGGTTGGACTAAAGGTTCGCCATCTAGAACAATAAAT
241 TATAATGCCGGAGTTTGGGCGCCGAATGGCAATGGATATTTGGCTTTATATGGTTGGACG
301 AGATCACCGCTCATAGAATATTATGTAGTGGATTCATGGGGTACTTATAGACCTACTGGA
361 ACGTATAAGGGTACTGTATACAGTGATGGGGGTACATATGACATATATACAACTAAACGT
421 TATAACGCACCTTCCATTGAGGGCCAACATTCTACTTTTACGCAATATTGGAGTGTTCGC
481 CGGTCTAAGAGACCAACTGGAAACAACGCTAAAATCACTTTCAGCAATCATGTTAAAGCA
541 TGGAAGAGTCATGGAATGAATCTGGGGAGTATTTGGTCTTACCAAGTCTTAGCGACAGAG
601 GGATATCAAAGTAGTGGAAGTTCTAACGTAACAGTGTGGTAA
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Figure 1: Sequence of 642bp xylanase gene isolated by Nikson (2009) from xylanolytic *Bacillus* sp. This gene was cloned into *E.coli* BL21(DE3) to observe the recombinant expression of xylanase.

2.4 Recombinant xylanase

Quite a large number of research publications have analyzed the characteristics of recombinant xylanase under the prospects of varying temperature and pH on the stability of the enzymes produced comparing to the original xylanase. Based on the study by Kulkarni et al. (1995), heterologous expression of xylanase gene from an alkaliphilic and thermophilic *Bacillus* sp. in *E.coli* shows properties of recombinant xylanase which differ markedly from the xylanase from native bacteria. It is also studied that different composition of amino acid occurred in recombinant xylanase which could be due to differential processing of proteins in *E.coli* and *Bacillus* sp. However, according to a recent research done be Yin et al. (2008), similar properties of recombinant and native

xylanase are of greater importance in commercialization. It was also reported that majority of recombinant xylanase was found to be in insoluble fractions which hints that the expression of xylanase gene produces inclusion bodies even after optimization.

Heterologous expression of xylanase from *Bacillus* sp. is studied in greater detail as this project deals with xylanase gene extracted from *Bacillus* sp. from the vicinity of sago plantation. Expression of xylanase gene from *B.licheniformis* in *E.coli* and the characterization of the recombinant xylanase produced were reported by Helianti et al. (2008) & Lee et al. (2008). The recombinant xylanase was detected on solid media through the xylan-Congo red clearance protocol and dinitrosalicylic acid (DNS) which detects the presence of reducing sugar. Recombinant xylanase from reported *Bacillus* sp. has been observed to show optimum activity at pH 5 – 7 and temperature at 40°C to 50°C (Helianti et al., 2008, Lee et al., 2008, Yang et al., 1988 & Kulkarni et al., 1995). Cloning of xylanase gene into heterologous expression host, *E. coli* has shown increased activity comparing to the native enzyme. Multiple recombinant xylanase has also been found to be produced by xylanase gene from *Bacillus* sp in the report published by Kulkarni et al. (1995) and Honda et al. (1985).

2.5 Xylan substrate

Xylan in insoluble form is normally used as substrate for the study of xylanase activity. Xylan for the study of xylanase activity is normally derived from the natural sources. According to research done by Damiano et al. (2003), *B.licheniformis* isolate from decaying wood showed xylanase activity at various rates to substrates like birchwood xylan, corn cob, corn starch, corn straw, eucalyptus brown pulp, eucalyptus sawdust, eucalyptus xylan, glucose, orange bagasse, sucrose and xylose. Xylanase activity was

remarkably high for sources with from birchwood xylan, corn cob, corn straw, eucalyptus xylan and orange bagasse. Xylanolytic activity towards substrate may differ in recombinant xylanase owing to the difference in substrate binding ability in recombinant xylanase (Kulkarni et al., 1995, 1999) in comparison to the native xylanase. However, based on a recent study, corn cob xylan has been reported to be the best substrate for the production of endoxylanase by thermotolerant *B.licheniformis* isolated from decaying wood matter. Maximum endoxylanase activity could be achieved with the optimized particle size of the corn cob powder (Gupta & Kar, 2008).

3.0 Materials and Methods

3.1 Materials and Reagents

a) LB Agar

LB agar was prepared by first disolving 7.4 g of LB agar powder with 200 mL of dH_2O in Scott bottle. The bottle with the content was shaken vigorously and autoclaved. The agar was then poured into sterile plates until quarter of the plate. 20 plates were prepare and stored the freezer especially for uncultured media for future use.

b) LB Broth

The broth was prepared using the same technique as LB agar but LB broth powder was used instead. 7.4 g of LB broth powder was dissolved in 200 mL dH_2O of Scott bottle. It was then shaken vigorously and autoclaved. The Scott bottle with autoclaved LB broth was

stored in the same freezer for uncultured media. Aseptic techniques were used precisely every time the broth was used for culturing to prevent any form of contamination.

c) Ampicillin(25 mg/mL)

Sterilized ampicillin stock was obtained from the postgraduate students belonging to Proteomics lab. An aliquot of ampicilin was prepared by carefully isolating 1.5 mL into a microcentrifuge tube and stored in 4°C.

d) Kanamycin(100mg/ml)

Kanamycin solution was obtained from Genetic Engineering lab. Aliquot was prepared by isolating 1.5 mL into a microcentrifuge tube and stored in 4°C. This was only used for cultivation of bacterial culture harbouring pET plasmid.

e) GTE Solution (50 mM Glucose + 25 mM Tris-HCl, pH 8)

0.495 g of glucose and 0.15 g of Tris HCl was added to 50 mL of dH_2O in a Scott bottle and shaken vigorously. The pH was adjusted using a pH meter with the addition of NaCl or HCl. The solution was then autoclaved and pre-chilled before using.

f) 0.2 N NaOH/ 1% SDS Solution

4 g of NaOH and 0.5 g SDS was dissolved into 50 mL of distilled water in a Scott bottle and shaken. The bottle was then solution was then autoclaved.

g) 3M KAc/10mM EDTA pH 8 Solution

0.186 g of EDTA was added to 50 mL of dH_2O in a Scott bottle. The pH of the solution was then adjusted with a pH meter by adding HCl and NaCl. 14.72 g of KAc was then added to the solution once it has reached pH 8. The solution was autoclaved.

h) 70% Ethanol

70 mL of 100% ethanol was diluted with 30 mL of dH_2O in a Scott bottle.

i) TE Buffer (10mM Tris-HCl, pH 7.5 + 1mM EDTA, pH 8)

0.0605 g of Tris-HCl was added to 50 mL of dH_2O. Subsequently, 0.0186 g of EDTA was added to the mixture. The pH of the solution was adjusted to 8. The bottle was then autoclaved.

j) 3M sodium acetate, pH 5.2

12.30 g of sodium acetate was added to 30 mL of dH_2O. The pH was adjusted with glacial acid and topped up with distilled water to 50 mL.

k) Isopropyl-β-D-1-thiogalactopyranoside (IPTG)

0.12 g of IPTG was dissolved into 5 mL of ultrapure water in a universal bottle and sterilized using microfilter of 0.22 uM filter. The bottle was then stored in -20°C and kept on ice whenever it is used.

l) 1M NaCl solution

5.84 g of NaCl was dissolved in 100 mL dH_2O and autoclaved. The solution was stored at room temperature.

m) Congo red reagent (0.2% w/v)

Congo red reagent was obtained from Mr. Nikson belonging Proteomics lab. The containing bottle was wrapped with foil and stored at room temperature.

n) Corn cob powder

Rough corn cob powder was obtained from Mr. Nikson belonging to Proteomics lab. The powder was sieved to obtain a finer particle of corn cob. The powder was then stored in beaker in a dried area.

o) Bovine Serum Albumin (BSA)

BSA was obtained from the Proteomics lab. Aliquot was prepared in a sterile 0.5 mL microcentrifuge tube and stored at -20°C.

o) 1X M9 minimal media with 1% corn cob xylan

5 g of corn cob powder, 3 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl and 7.5 g of agar was added into a 500 mL bottle and filled with dH_2O. The mixture was shaken vigorously and then autoclaved. The autoclaved solution was cooled down until it

can be hold with bare hands prior to the addition of 0.5 mL $MgSO_4$. Fresh petri dishes were sterilized by exposing to UV radiation for 20 minutes in laminar flow hood. Once the agar has cooled down, it was poured into the plates equally to make 20 plates in laminar flow hood. The agar content was swirled gently frequently to ensure the distribution of insoluble xylan particles is equal. Poured plates were left to cool down before being stored at 4°C.

3.2 Bacterial strain and plasmid

E.coli JM109 in glycerol stock which harbors pGEM-T with xylanase gene and *E.coli* BL21 (DE3) were obtained from Mr. Muhammad Suhaib Mat Hussin from Proteomics Lab. The xylanase gene harboured in the obtained bacterial stock was isolated by Mr.Nikson Chong Fatt Ming for his previous study on the characterization of xylanase from indigenous *Bacillus* sp. pET41(a) expression vector harboured in *E. coli* JM109 was obtained from Genetic Engineering Lab. *Bacillus licheniformis* strain p7 was obtained in glycerol stock from Proteomics lab.

3.3 Isolation of single colony of *E. coli*

The xylanase gene which was previously isolated was cloned into pGEM-T cloning vector and propagated and stored in glycerol for long term storage. pET 41(a) plasmid which will be used for protein expression was also stored in *E. coli* stock. Single colony from this stock was isolated. Serial dilution, spread plate and streak plate were the three milestone methods used for the isolation of single and pure colony. Serial dilution was done based on the method described by Beishir (1991) and Chan et al. (1993) with certain

modifications. Bacterial stock was consecutively diluted using LB broth. According to Schoenborn et al. (2004), liquid dilution has shown more dilution and culturability compared to solid or agar dilution. Based on the research, it has been postulated that liquid dilution provides inferior facilitation in isolation of a pure colony.

Three 1.5 mL tubes were labeled 10^{-1}, 10^{-2} and 10^{-3} respectively. Each tube was filled with 900 µL of LB broth. 100 µL of bacteria from glycerol stock which was thawed initially was transferred into the 10^{-1} tube and shaken gently. 100 µL from 10^{-1} was transferred into 10^{-2}. The procedure was repeated until the third tube. 100 µL of the culture was poured on the plate for the preparation of spread plate. The plates were left overnight under incubation at 37°C.

The isolated colonies from the incubated plates was chosen and subcultured into 3 mL of LB broth with 3 µL of ampicillin(25mg/mL) for culture involving pGEM®-T or kanamycin(100 mg/mL) for cultures with pET expression vector in a universal bottle. The bottle was then incubated overnight at 37°C. The bacterial broth was then used for plasmid extraction. Appropriate aseptic technique was handled throughout these experiments. All the culturing work was done entirely in a laminar flow hood.

3.4 Plasmid extraction through alkaline lysis method

The plasmid was extracted based on the method described by Zyskind and Bernstein (1992) with slight modifications. This method was applied for the extraction of both pGEM®-T easy and pET41(a). 1.5 mL of bacterial culture was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 2 minutes to pellet the cells. The supernatant was removed carefully and the step was repeated for the remaining samples. The supernatant was removed prior to addition of pre-chilled 250 µL GTE solution. The