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Study on cloning of protease gene from *Xanthomonas campestris* into *Escherichia coli*

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Abstract

The aim of the research was to clone the protease gene from Xanthomonas campestris *into* Escherichia coli. *This enzyme is active over a particulary broad pH range. Shotgun cloning was used to compare techniques using four different plasmids and vector. The results indicated that when (a) plasmid pUC19 in* E. coli *DH5a was used, 5 recombinant plasmids with a 0,5 - 7,5 kb* EcoR1 *insert were obtained, (b) plasmid pBluescript in* E. coli *XL-1-Blue was used, 1 recombinants plasmid with a 8 kb* EcoR1 *insert were obtained, (c) plasmid pRK415 in* E. coli *DH5a were used, 3 recombinant plasmids with a 1,5 - 10 kb* EcoR1 *insert were obtained, (d) plasmid pBBR1MCS2 in* E. coli *JM 107 was used, 47 recombinant plasmids with a 1,4 - 8 kb* EcoR1 *insert were obtained. Of these recombinants, two produced a typical clearing zone, when analysed using Skim Milk Agar. However, the typical clearing zone was not clear or stable. When the* E. coli *(pMB8) recombinant was transferred back into a protease negative mutant of* Xanthomonas campestris, *using a triparental method, a typical clearing zone in the Skim Milk Agar media was observed, confirming that the protease gene had succesfully cloned. A recombinant of* E.coli *(pMB.8) was grown in nutrient broth containing glycerol, the protease activity was detected after 20-24 hours. The crude protease showed optimum pH at 8,5 - 10, optimum temperature 60°C and molecular sizes approximately 49 and 45 kD. The enzyme was inhibited by EDTA and also by PMSF indicating a serine protease function.*

Keywords: *cloning -- protease* - Xanthomonas - *recombinant* - *plasmid*

Introduction

Protease plays significant roles in various physiological reaction. It is involved the reaction of the degradation of protein, spore formation, germination, post translation, modulation of gene expression, enzyme modification, the secretion of others protein and enzymes (Ward, 1983). Protease is also important industrially. The sources of most industrial protease is the genus *Bacillus*, with species such as *B. cereus*, *B. pumilus*, *B. subtilis*, *B. licheniformis*, *B. stearothermophilus* and *B. polymixa*. Other bacteria used as protease producers include *Aeromonas*, *Pseudomonas*, *Streptomyces* and *Staphylococcus*. Fungi have also been used as a protease source, such as *Aspergillus*, *Mucor* and *Rhizopus* (Ward, 1983; Mangunwardoyo, 1994).

Xanthomonas campestris (Xc) is a pathogenic bacterial of soybean. This bacterium is able to produce xanthan gum and is thus useful in the food industry. The bacterium also produces a protease with a broad range of pH activity which might also have industrial application. The characteristics of the enzyme from these bacteria has not been throughly explored. Tang *et al.* (1987) reported expression of the protease gene from Xc pv *campestris* in *E. coli* JM 107, but not after transfer to *E. coli* ED8767. Down *et al.* (1990) reported extracellular proteases from

Xc pv *campestris* which were characterized as two proteases, namely, PRT1 and PRT2. PRT1 was found as a serine protease and PRT2 was zincs metallo protease. Liu *et al.* (1990) studied the location of t protease gene from the recombinant pIJ3070. Deletion analysis showed that the gene was located within the 2,2 kb DNA fragment. Sequencing of the protease gene revealed an open reading frame encoding 580 amino acid polypeptide with molecular weight 57 of kD.

Xanthomonas campestris pv *glycine* (Xcg) IFL and YR 64 have been collected from the infected soybean in Indonesia (Mesak *et al.*, 1994; Rukayadi, 1995). Research on the molecular and biochemical characteristics of the protease of the Xcg IFL has been conducted in our Laboratory. The enzyme was stable at pH 6-12, the optimum temperature was 60°C and optimum pH 7 and 12 (Santosa, 1997). Transposon mutagenesis using Tn5 has been reported by Rosana (1997) the result was designed as pLRΩ 6 containing 6.2 kb fragment DNA of *Xanthomonas campestris* 8ra. The plasmid was furtherly used as a probe for Southern hybridization of the isolates of Xcg. IFL, YR64 and YR32, to detect the presence of similar protease gene within the isolat. The study indicated a signal hybridization in the 6,2 kb *Eco*R1 in IFL and YR32, 6,2 and 5,5 kb *Eco*R1 in YR 64 (Rundupadang, 1998).

The aim of this research was to clone the protease gene from *Xanthomonas campestris* into *E. coli* in order to study expression of the recombinants.

Materials and Methods

Materials

Microorganism and Plasmids. The bacterium and plasmids were obtained from the collection of Microbiology and Biochemistry Laboratory, Inter University Center for Biotechnology, Bogor Agricultural Institute. *E. coli* JM 107 and the *X. campestris* (*prt*$^-$) mutant were gifts from Prof. M.J. Daniels (The Sainbury Laboratory, John Innes Institute, UK) (Table 1).

Methods

DNA Chromosome preparation. Chromosome isolation followed the procedure of Leach *et al.* (1994) with some modification. A single colony of Xcg grown for 48 hours on a Luria Agar (LA) plate was grown in Luria Broth (LB) consisting of peptone 1 g, NaCl 1g and yeast extract 0,5 g/100ml of distilled water, at pH 7,2. Of 1,5 ml overnight culture of bacteria were harvested by centrifugation at 10.000 g for 2 minutes. The pellet was suspended in 250 µl of TE buffer containing 12,5µl of lysozyme and incubated at 37°C for 60 minutes. This was followed by addition of 50 µl SDS 10% and 5 µl proteinaseK, before shaking and further incubation at 37°C for 60 minutes. 100 µl 5 M NaCl and 100 µl CTAB/NaCl were than added and the mixture was incubated at 65°C for 20 minutes. The protein debris was extracted using chloroform:isoamylalcohol (24:1), shaken for 30 minutes and centrifuge at 10 000 g for 15 minutes. The supernatant was pipetted into a clean eppendorf and 600 µl isopropanol was added before incubation at 20°C for 10-15 minutes. Stranded DNA floated in the suspension. Of 1000 µl was added ice cold ethanol followed by centrifugation at 10.000 g for 5 minutes. The pellet was then dried using a concentrator for 15 minutes and 10-20 µl sterile water was added to dissolve the DNA. One µl of DNAase free RNAase was then added to eliminate the remaining RNA traces. One µl of the resultant DNA suspension was electrophoresed to check the quality of DNA.

DNA Plasmid Isolation. The DNA plasmid was extracted using a Wizard™ Plus Miniprep DNA Purification System (Promega) according to the manufacture's

Table 1. Bacterial and plasmids used

	Genotype	Sources
Bacteria		
Xanthomonas campestris YR64	wild type	Rukayadi, 1995
Xanthomonas campestris IFL	wild type	Mesak *et al.*, 1994
Xanthomonas campestris	protease mutant	Daniels, 1998
E. coli		
DH5α	*sup*E44*Flacz*U169 (80 lacZΔM15) *recAhsd*R17 *Gyr*A, thi⁻ rel A1	Sambrook *et al.*, 1986
JM 107	*sup*E44*end*A1*hsd*R17*gyr*A96 *rel*A1thi⁻ *lac*-proAB	Sambrook *et al.*, 1986 Daniels, 1998
XL-1-Blue	*sup*E44 *rec* A1*end*A1*hsd*R17*gyr*A46 *rel*A1thi- lac-	Sambrook *et al.*, 1986
Plasmids		
pUC19	Ap^r	Sambrook *et al.*, 1989
pRK415	Tc^r	Keen *et al.*, 1988
pBluescript	Ap^r	Strata Gene, 1991
pBBR1MCS2	Km^r	Kovach *et al.*, 1994
pMB1-43	fragment DNA	This research

instruction.

Partial digestion of chromosome and plasmid: The chromosome preparation was partially digested with *Eco*R1 (Promega). The plasmid preparation was digested with the same enzyme and both were incubated at 37°C for 16 hours. The DNA was electrophoresed in 0.1%(w/v) agarose (Pharmacia) at 40 V for 2 hours. The appropriate part of specific lane containing chromosomal DNA was excised and purified using a Genen Clean Kit Bio 101 Inc, La Jolla. California.

Gene clean analysis (Gene Clean Kit Bio 101) The excised chromosomal DNA was purified using a protocol according to the manufacture's instruction.

Ligation, Competent cell, Transformation and Selection (Sambrook *et al.*, 1986).

Ligation: the ligation process was conducted at 4°C for 16 hours using T4 ligase (Promega) in buffer. The total volume for ligation was 20 µl consisting of 1 µl of DNA plasmid and 10 µl DNA chromosome, 2 µl T4 ligase buffer and 7 µl sterile dH_20. A control ligation was carried out using a digested plasmid without adddition of the DNA chromosome fragment. A control transformation was also prepared using uncut plasmid.

Preparation of competent cell: a single colony was picked from a plate incubated for 16-20 hours at 37°C and transferred to 10 ml of LB medium and incubated for 16 hours at 37°C. Of 1 ml the cell suspension was grown in 10 ml of LB medium for 3 hours to reach the logarithmic phase. Of 1.5 ml cell suspension was centrifuged and 1000 µl of 50mM CaCl2 was added to the pellet. This was incubated for 20 minutes in ice. After centrifugation for 1 minutes, it was resuspended with 200 µl fresh pre-chilled 50 mM $CaCl_2$ before being further incubated for 10 minutes. The competent cells were then ready for the transformation.

Transformation: the fresh competent cells were added to the ligated DNA (50 ng in volume of 10 µl or less to each tube). The tube was mixed by swirling gently and then stored in ice for 30 minutes. The tube was then transferred to a circulating water bath preheated at 42°C. It was left in the water bath for exactly for 2 minutes without shaking. The tube was then rapidly transferred to an ice bath. The cells were chilled for 1-3 minutes. Of 1000 µl of fresh LB medium were added to each resulted transformant mixture and cultured for 60-90 minutes to allow the bacteria to recover and express the antibiotic resistance markers encoded by the plasmid. They were then agitated vigorously at 200 cycle/minute. Controls for transformation and ligation were done at the same time.

Selection of the recombinant: of 50-100 µl the transformed competent cell preparation were transferred into LA+ Ap(50 mg/ml for pUC19/DH5(and pBluescript/X-L-1Blue); Tc(10 µg/ml for pRK415/DH5α); LA+Km (50µg/ml for pBBR1MCS2/JM107) preplated agar. They were surface spread with 4 µl IPTG (200mg/ml) and 40 µl X-gal (20 mg/ml) using a sterile bent glass rod. The plates were left at room temperature until the liquid has been absorbed. They were then incubated at 37°C. Colonies appeared after 12-16 hours incubation. The control transformation and ligation were prepared in the same way except that the competent cells only were spread on the LA medium.

Triparental mating: the donor protease gene used was from *E. coli* DH5α (pMB8), the helper was E.coli HB 101 (pRK2013) and recipient *Xanthomonas campestris* (prt^-). This was carried out following the procedure of Suwanto (1993).

Protease activity: the analysis carried out using casein 2% (w/v) as a substrate. One unit activity is defined as released 1µmol of tyrosin/minute under assay conditions. The procedure was carried out followed Walter (1984).

Protein analysis: analysis was carried out following the procedure of Bradford (1976). Polyacrylamide gel electrophoresis (PAGE): PAGE was carried out by the method of Laemmli (1970), with 10% acrylamide for the resolving gel and 4% polyacrylamide for the stacking gel, using Tris-HCl buffer pH 8.8 and 6.8.

The zymogram: was prepared without SDS and the substrate casein was added in the resolving gel. Proteins were stained using Coomassie Blue R-250.

Results and Discussion

A. Shot gun cloning using plasmid vector pUC19 and *E. coli* DH5α host.

After transfer to the selective medium 8 white colonies were obtained, out only 5 were stable on subculture. No recombinants showed protease acitivy on LA+Ap+2% skim milk medium. Verification showed that 5 recombinants harboured inserts of 0,5; 0,8; 1,9; 4,9 and 7,5 kb (Figure 1).

B. Cloning a 2-8 kb insert using pBluescript vector and *E. coli* XL-1Blue host.

After the bacteria were transferred to the selective medium LA+Ap+IPTG+X-gal, 7 white colonies were obtained. When these transformants were grown on the LA+Ap+

2%skim milk medium, clear zones sorrounding the colonies were not observed. Verification of the recombinants showed that only a single insert was identified, 8 kb in size (Figure 2).

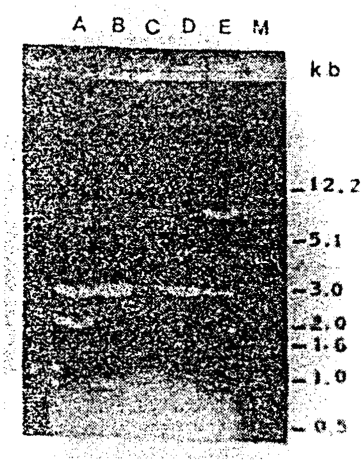

Figure1. Verification of plasmid recombinants resulted from shot gun cloning of *Xanthomonas campestris* IFL, using pUC19 vector and *E.coli* DH5α host (A) (pMB1) 1.9 (B) (pMB2) 0.8 (C) (pMB3) 4.9 (D) (pMB4) 0.5 (E) (pMB5) 7.5 kb and (M) 1 kb DNA Ladder.

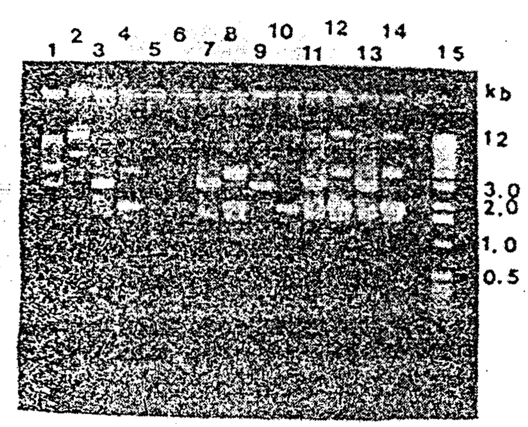

Figure 2. Verification of plasmid recombinants resulting from cloning of a 3-8 kb EcoR1 *Xanthomonas campestris* IFL, using pBluescript vector and *E. coli* XL-1Blue host. Nos 1, 3, 5, 7, 9, 11and 13 *Eco*R1 digested plasmid. Nos 2, 4, 6, 8, 10, 12, and 14 non digested plasmid (15) 1 kb DNA Ladder. Lane 1 shows the 8 kb insert.

C. Cloning a 6,2 kb insert using pRK415 vector and *E. coli* DH5α host.

The protease gene was probed using pLRΩ on the 6,2 kb *Ase*1 fragment constructed by Rosana (1997). Rundupadang (1998) has previously probed the chromosome of Xcg IFL and a positive signal for hybridization was detected on the 6,2 kb *Eco*R1 fragment. The 6,2 kb *Eco*R1 fragment from Xcg IFL was excised from the agarose gel, ligated with pRK415, and transformed. On the selection medium, 23 white colonies were obtained but only 3 were stable on subculture. Verification resulted in inserts of 1,3 and 2,8; 10 and 2,9 kb (Figure 3). The transformants grown on LA+Tc+2% skim milk medium did not give clearing zone on the Skim Milk Agar medium.

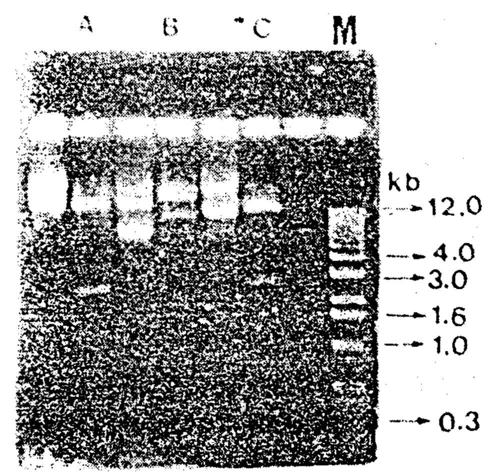

Figure 3. Verification of plasmid recombinants resulting from cloning of a 6,2 kb *EcoR* fragment using pRK415 vector and *E.coli* DH5α host. (A) (pMB6) 2.8 and 1.5 (B) (pMB7)10 dan (C) (pMB8) 2.9 (M) 1 kb DNA Ladder.

D. Cloning a 5,5- 6,2 kb insert using pBBR1MCS2 plasmid and *E. coli* JM 107 host.

Fourty seven white colonies were observed on the selected medium LA+Km+ IPTG+X-gal (Figure 4 A, B and C). Some recombinants seemed to produce concatemers (self ligation of the plasmid), whilst others had 2 inserts and the remainder had only one insert. The insert size varied from 1,4 - 8kb. When transferred to the LA+Km+ 2% skim milk medium, there were no positive results.

Selected retransformation from the

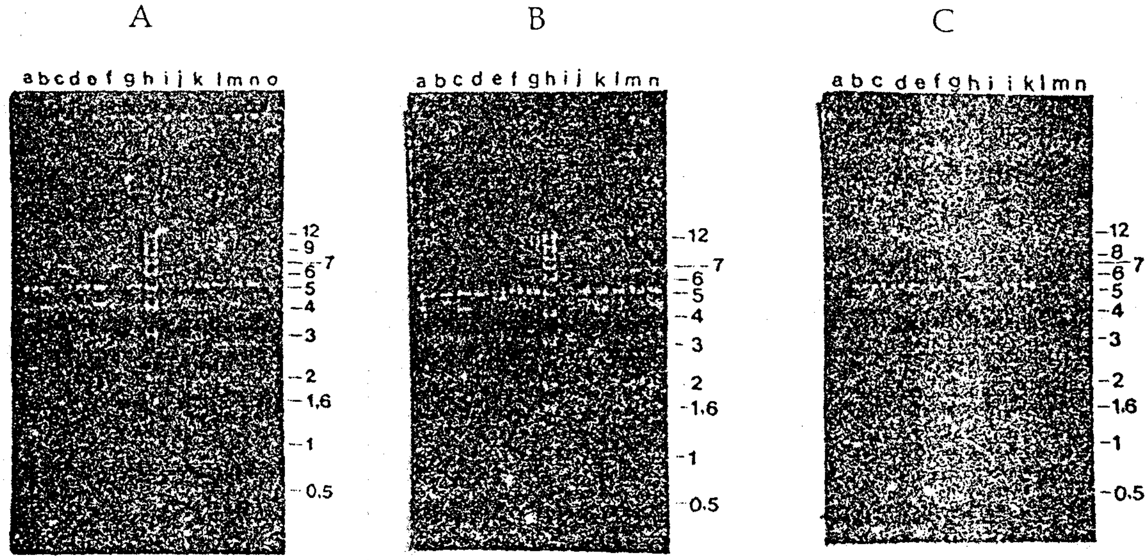

Figure 4.A. Verification of plasmid recombinants resulted from cloning of a 5.5-6.2 kb *Eco*RI fragment using pBBR1MCS2 vector and *E. coli* JM 107 host. (a) (pMB9) 3.7 (b) (pMB10) 4.0 (c) (pMB11) 6.5 (d) (pMB12) 3.1 (e) (pMB13) 4.2 (f) (pMB14) 6.5 (g) (pMB15) 3.1 (h) i kb DNA Ladder (i) intact plasmid (j) (pMB16) 4.5 (k) (pMB17) 9.5 (l) (pMB18) 2.8 (m) (pMB19) 2.8 (n) (pMB20) 2.8 (o) (pMB21) 3.7

Figure 4.B. Verification of plasmid recombinants resulted from cloning of a 5.5-6.2 kb *Eco*RI fragment using pBBR1MCS2 vector and *E. coli* JM 107 host. (a) (pMB22) 4.5 (b) (pMB23) 4.9 (c) (pMB24) 4.3 (d) (pMB25) 2.8 (e) (pMB26) 4.8 (f) concatemer (g) (pMB27) 1.7 and 2.1 (h) i kb DNA Ladder (i) intact plasmid (j) (pMB28) 1.7 and 2.1 (k) (pMB29) 4.3 (l) (pMB30) 1.7 and 2.1 (m) (pMB31) 1.7 and 2.1 (n) (pMB32) 4.5.

Figure 4.C. Verification of plasmid recombinants resulted from cloning of a 5.5-6.2 kb *Eco*RI fragment DNA using pBBR1MCS2 vector and *E. coli* JM 107 host. (a) (pMB33) 7.5 (b) (pMB34) 4.0 (c) (pMB35) 1.7 and 2.1 (d) (pMB36) 1.7 and 2.1 (e) (pMB37) 4.5 (f) (pMB38) 4.0 (g) (pMB39) 4.1 (h) i kb DNA Ladder (i) intact plasmid (j) (pMB40) 1.4 (k) concatemer (l) (pMB41) 8.0 (m) (pMB42) 3.7 and (n) (pMB43) 7.0

transformants with a 2,8-4,8 kb insert was carried out using *E. coli* JM107 from Prof. M.J. Daniels collection. The recombinants were grown NYGA+Km+2% skim milk medium, 2 showed clear zones around the colonies (Figure 5). However, the recombinant plasmids did not exhibit stability after subculturing.

The possibility of instability recombinants in the *E. coli* host was studied using a triparental mating method. *X. campestris* protease negative mutant (prt$^-$) was used as a host. The resultant transconjugants of X. campestris (pMB8) grown on YDC+Tc(10 µg/ml)+Km(25 µg/ml)+Rif(50µg/ml)+ 2% skim milk medium, showed a clear zone around the colonies. The zone was significanly larger than obtained for *X. campestris* (pRK415). Proteolytic indices from the transconjugant *X. campestris* (pMB8) and *X. campestris* (pRK415) were 1,85 and 1,25. This indicated that physiology of the host plays an important role in the expression of the recombinant. While the typical clearing zone sorrounding the transformant colonies was not found when *E. coli* was used as a host. This clearing zone significanly appeared whwn the *X. campestris* (prt$^-$) was used as a host. The instability of the recombinant may be caused by incompatibility between host and vector plasmid.

The attempts at transformation using

several methods and various plasmids or vector described here gave different results. These differences may be caused by differing interspecific characters of the plasmids vector and hosts used. Transformation is a natural phenomenon but can be carried out in the laboratory. The genus *Bacillus* and *Haemophillus* have been found to contain a DNA binding protein whose function appear to be necessary for the initial stage of transformation (Rodriguez and Tait,1983). Transformation is also influenced by the temperature, size/amount of DNA, endonuclease activity, heat shock, ion strength, conformation and concentration of DNA, and host specificity (Rodriguez and Tait, 1983; Hanahan, 1983; Smith, 1990). Factors that can affect the expression of cloned gene include: promotor strength, translational initiation sequences, codon choice, secondary structure of mRNA, transcription termination, plasmid copy number, plasmid stability, host cell physiology, maturation of the enzyme, folding, degradation, and tertiary structure (Friedfelder, 1987; Brown, 1990; Suhartono, 1992; Dale, 1993).

Bacteria also possess restriction and modification systems. The host restriction endonuclease can cleave DNA that has not been modified previously by the same strain. With an insert size between 0,5 - 10 kb, it is possible that the bacteria contained a restriction and modification system which involved the genes *hsd*M, *hsd*S dan *hsd*R. (Rodriguez and Tait, 1983). This may explain the difficulty in obtaining optimum expression of the protease gene in the experiment (using selective solid media).

The plasmid recombinants were not stable. Having maximized the expression of the protease gene, it is important to consider the effect of the recombinant on the host bacterium. The instability of the recombinant plasmid was possibly due to segregational instability causing the loss of plasmids due to defective partitioning. As we observed in our experiment, minimal experession in the selective media was found (Figure 5), eventhough this was lost in further examination. Naturally occuring plasmids are maintained stably because they contain a partitioning function which ensures that they are accurately segregated at each cell division. Structural instability of the recombinant plasmids may arise by deletion, insertion or rearrangement of DNA (Corfield *et al.*, 1988; Old and Primrose, 1985). Several reports describe the instability of *B. subtilis* recombinant plasmids. Even bifunctional (hybrid) vectors that can replicate in both *B. subtilis* and *E. coli* are known to replicate stably in *E. coli*, but suffer effective rearrangement in *B subtilis* (Zaghloul *et al.*, 1994). Eventhough the expression of the recombinant in the selective solid not optimum/instable, we found interesting result, that when grown in the liquid media, the extracellular protease activity of the transformant was indeed detected significantly.

Analysis of the protease activity and biochemical characterization were carried out. The recombinant (pMB8) was grown in on 2% (w/v) peptone, 1%(w/v) yeast extract and 3% (v/v) glycerol, the activity of recombinant gave a significant different compare with *E. coli* (pRK415) control. After 16 hours of fermentation protease activity of recombinant was increased while that of the E.coli (pRK415) control was not detected. (Figure 6).

Further analysis confirmed the present of extracellular protease of the recombinant. SDS-PAGE and Zymogram analysis for determination of molecular weight is shown in Figure 7. The recombinant protease has two proteases with molecular weight of 49 kD and 45 kD. This is in agreement with previous report of two types proteases from *Xanthomonas campestris* pv *campestris* (Dow *et al.*, 1990).

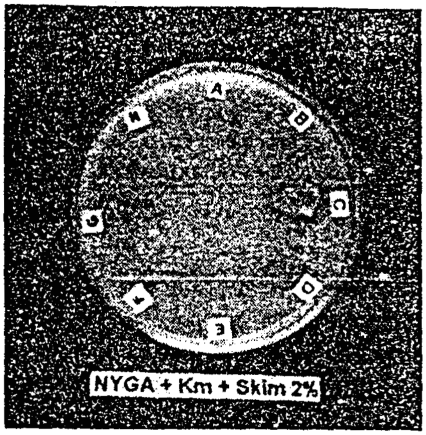

Figure 5. Retransformation of a recombinant using *E. coli* JM107 (Prof. M.J. Daniels collection) on NYGA + Km + 2% skim milk. A-G recombinant *E.coli*. H. *E. coli* (pBBR1MCS2).

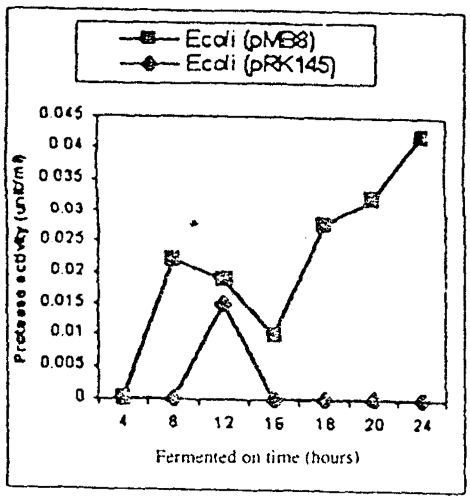

Figure 6. Protease activity of *E. coli* (pMB8) and *E.coli* (pRK415) grown on NYGB medium

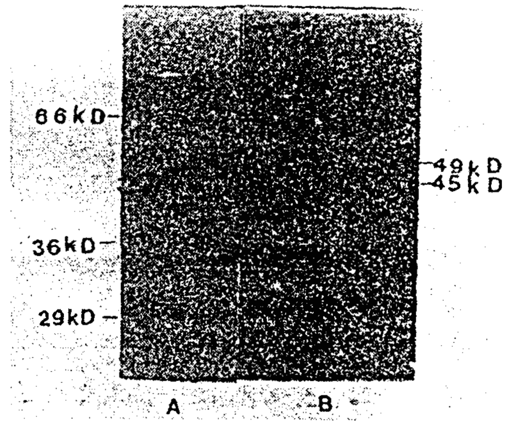

Figure 7. SDS-PAGE and Zymogram staining of recombinant protein
A :Protein marker
B. Recombinant protein

Based on the data from fermentation, the production of the protease was carried out with the *E. coli* DH5α (pMB8) recombinant. The results obtained were compared with the previous research on protease of Xcg IFL donor. Analysis of the temperature optimum, pH optimum, inhibition by PMSF/ EDTA and molecular weight of the *E. coli* (pMB8) recombinant and Xcg IFL as a donor gene is shown in Table 2. The biochemical characterization of the recombinant indicated similarity enzyme product of donor gene. This biochemical characterization data confirmed that the protease gene was indeed cloned into *E. coli* and was expressed more significantly when the transformant is grown in the liquid media.

Table 2. Biochemical characterization of protease recombinants and the donor

	Protease recombinant	Protease donor (Santoso, 1977)
pH optimum	8,5 - 10	7,5 - 10
Temperature optimum	60°C	60°C
PMSF inhibitor (5mM)	remaining activity 26%	remaining activity 56%
EDTA inhibitor (5mM)	remaining activity 34%	remaining activity 25%
Molecular weight	49 and 45 kD	45 kD (YR32)

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