SCREENING AND CLONING OF BACTERIAL β-GLUCOSIDASE GENE THAT CAN DEGRADE SALICIN FROM SOME NIF AND VIRULENT BACTERIA

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β-Glucosidase activity of the most virulent strains of *Agrobacterium tumefaciens* was found to be much higher than that of the weakly virulent strains. It appears that conversion of coniferin to coniferyl alcohol *via* bacterially encoded β-glucosidase affects genes induction and thus virulence. If indeed β-glucosidase is a necessary component of the pathway, this will be the first reported evidence of plant signal compound processing by *Agrobacterium tumefaciens*. The isolation, characterization of β-glucosidase gene from *Agrobacterium tumefaciens* B3/73 and sequencing of *cbgl* were done. The encoded enzyme catalyzes controlled hydrolysis of coniferin but not cellobiose (Morris and Morris, 1990).

The *Agrobacterium tumefaciens* has two β-glucosidase genes *cbgl* and Cbgl, Cbg1 activity hydrolyzes coniferin but not cellobiose. *Agrobacterium tumefaciens* B3/73 beta-glucosidase genes Cbgl was cloned and sequenced, *cbg1* expressed in *Escherichia coli*. The 88-kDa predicted product of *cbg1* β-Glucosidase is highly similar to another bacterial β-Glucosidase and several fungal β-glucosidases (Morris and Morris, 1990).

To confirm that *Agrobacterium tumefaciens* has strongly β-glucosidase activity and slight reaction, clones screened showed that two categories were indeed present DNA from individual clones was digested with *HindIII* and restriction fragment patterns were compared. (Linda *et al*., 1992).

Two type of pattern were obtained, the relative ability of each gene to cleave coniferin was assessed. Clones containing *Agrobacterium tumefaciens* B3/73 DNA rapidly and completely hydrolyzed coniferin to coniferyl alcohol. Over the same period, type 2 clones were completely inactive. The different substrate specificities of clones were also evident from their ability to grow on cellobiose. *Agrobacterium tumefaciens* B3/73 was able to use cellobiose as the sole carbon source. *Escherichia col*i DH5α and type 1 clones were not able to grow on cellobiose. Other clones were able to utilize cellobiose but grew very slowly. (Linda *et al*., 1992).

The 5.7 kb *HindIll* fragment common to all type 1 clones was purified and ligated into pBR322. Clones with

inserts in either orientation were able to cleave X-glucose, indicating that the entire β-glucosidase gene was probably located within this insert. An *Eco*RI, *BamHI*, *BglII*, and *PstI* restriction map of the insert showed that a 3.5 kb *BamHI-PstI* fragment with an internal *PstI* site was found to have the activitty to cleave X-glucose when cloned into pUC19. The sequence surrounding the EcoRI site in the pUC19:3.5 kb BamHI-PstI clone and the sequence were done. (Linda *et al*., 1992).

Woodward and Wiseman (1982) reported that there are two constitutive, β-glucosidase genes in *Agrobacterium tumefaciens* B3/73 were appeared. Those represented by clones able to hydrolyze X-glucose and coniferin but not cellobiose. On the other hand, those represented by other clones having lower activity on X-glucose, non activity on coniferin and able to utilize cellobiose for growth.

β -glucosidase, α-glucosidase, and β-galactosidase activities have been reported to be associated with 45 strains of rhizobia, and some cellulolytic and pectinolytic activities have been detected in *Rhizobium leguminosarum*. (Singh and Singh, 1985).

Several glycosidases have also been detected in *Bradyrhizobium lupini* A β-glucosidase that is particularly active with cellobiose has been purified from *Agrobacterium faecalis*. In addition, a β-galactosidase and a β-glucosidase have been purified from the periplasmic space of *Rhizobium trifolii*, and an endoglucanase gene from *Azorhizobium caulinodans* has been cloned. (Geelen *et al*., 1995).

Listeria spp. transcription of *bvrB* gene was induced by cellobiose and salicin but not by arbutin. Disruption of the *bvr* operon by replacing part of *bvrAB* with an interposon abolished the repression by cellobiose and salicin but not that by arbutin indicating that the *bvr* locus encodes a β-glucoside-specific sensor that mediates virulence gene repression upon detection of cellobiose and salicin. Bvr is the first sensory system found in *Listeria. monocytogenes* that is involved in environmental regulation of virulence genes (Klaus *et al*., 1999).

Caulobacter crescentus ability to utilize lactose was examined to obtain an additional genetic tool for study this model organism, Identified a gene *lacA* that required for growth on lactose as the sole carbon source involved in the catabolism of two glucosides salicin and trehalose., this enzymatic activity is inducible and increased *lac* expression in the presence of lactose and salicin. (Benjamin *et al*., 2010).

MATERIALS AND METHODS

Media used

The media used were carried out according to Ronlald (2004). The bacteria were grown at 28°C for 48 hours. *Rhizobium leguminosarum* bv. trifloii

isolate was maintained on *Rhizobium* agar composed of (g/l)

The composition of the media is agar 15 g., yeast extract 10 g., K_2HPO_4 0.5 g., $MgSO_4.7H_2O$ 0.2 g., NaCl 0.2 g., $FeCl_3$. 6 H_2O 0.002 g., pH 7.2

Azo*rhizobium caulinodans* isolate was grown on Yeast Extract beef (medium YEB) The composition of the media is agar 15 g., yeast extract 1 g., beef extract 5 g., sucrose 5 g., $MgSO_4$ 0.25 g., pH 6.8.

Rhodococcus fascians isolate was grown on the composition of the media is agar 15 g., peptone 5 g., yeast extract 5 g., glucose 20 g., pH 7.

Agrobacterium tumefaciens isolate was maintained on the composition of the media is agar 15 g., peptone 20 g., K_2HPO_4 1 g., glucose 10 g., pH 7.2.

Escherichia coli HC1061 was used as cloning host was grown on LB medium g/l the composition of the media is agar 15 g., bacto tryptone 20 g., bacto yeast 5 g., NaCl 0.2 g., pH 7.5 supplemented with Carbenicillin (100 μg/ml) antibiotic stock.

William *et al*. (1988) the composition of the final concentration 200 μg/ml Carbenicillin is 2500x stock Carbenicillin, 500 mg/ml in water, add 200 μl/500 ml.

SOC media: The composition of the media according to William *et al*. (1988) is agar 15 g., bacto tryptone 2%., bacto yeast extract 0.5 % g., NaCl 10 mM, KCl 2.5 mM, $MgCl_2$ 10 mM, $MgSO_4$ 10 mM, glucose 20mM.

SOB media: The composition of the media according to William *et al*. (1988) is tryptone 2%., yeast extract 0.5 % g., NaCl 10 mM, KCl 2.5 mM.

Screening of β- glucosidase activity

Very sensitive test for detecting colonies of cellulase producing microorganisms was used. For β-glucosidase enzyme assay bacterial colonies or spots of bacterial culture were grown 24 hours on appropriate agar media containing 0.2% Salicin used as a source of carbon Pierre (1983). Salicin containing plates were stained with Congo Red 0.1%, after two to three washes with 1M NaCl, the appearance of a clear halo indicated glucosidase activity.

Bacterial DNA Preparation: chromosomal DNA was isolated from Bacterial isolates as described by Desomer *et al*. (1991).

Gel electrophoresis: 1% Agarose gel electrophoresis was performed as described by *Sharp et al.* (1973).

Construction and screening of genomic library

1-Restriction endonucleases enzymes used:

The DNA was partially digested with restriction enzymes used according to the manufacturer's instructions standard conditions to *EcoRI*, or *SamI*, *HincII*, *SalI* and *HindIII* were used. Total DNA

digestion by the Restriction enzymes the volumes used were 5 µl, DNA, 2 µl, Restriction enzymes, 1 µl, RNase enzyme, 3 µl, buffer, 14 µl, deionized water then incubated for 30 min.

Elution and Purification of DNA fragment from agarose gel

The obtained product by the digestion of genomic DNA was extracted after using *EcoRI*, *SamI* restriction enzyme to get (2-5 kbp) fragment. *Agrobacterium tumefaciens* isolate and *Rizobium leguminosarum* bv. trifloii isolates digestion products were electrophoresed to obtain the desired fragments. The fragments were isolated by electroelution from agarose gel by DNA agarose gel purification kit (Cat. No.1732676, Roche) used according to manufacturer manual.

Digested pUC19 plasmid with Restriction enzymes

pUC19 plasmid DNA was linearized by digested to completion with *EcoRI* and *SamI* by using 5 µl plasmid DNA, 2 µl Restriction enzymes, 1 µl RNase enzyme, 3 µl buffer, 14 µl deionized water then incubated at 25°C for 30 min. and treated with calf intestine alkaline phosphatase.

Ligation 2-5 kbp fragment with pUC19 vector

Genomic DNA was prepared from the bacterial isolates according to Maniatis *et al.* (1982). The DNA was partially digested with *EcoRI*, *SamI*, and the 2 to 5 kb fragments were isolated by electroelution from agarose gel. pUC19 plasmid DNA was digested with *EcoRI*, treated with calf intestine alkaline phosphatase, fragments were used in the ligation mixture 20 µl DNA (insert), 2 µl pUC19, 1 µl T_4 ligase DNA enzyme, 6 µl ligase DNA buffer. The ligation mixture was kept at 16°C overnight.

Preparation and transformation of electrocompetent cells

Ttransformation of *E. coli* bacteria by electroporation was carried out according to William *et al*. (1988).

Transformation by electroporation

Electroporation at high voltage was used by a cell suspention of very low conductivity 10 µl of daylilies ligation mixture, 90 µl of *Escherichia coli* HC1061 were added. The previous volume was put in precool cuvatte without air bubbles, plasmid was introduced into *Escherichia coli* HC1061 by electroporation with Eppendorf multiporater V/240, time/0.8 ms OHMS/200 µF 25. The treated *E. coli* HC1061 cells by electroporation, appropriate dilutions were plated on LB medium- Carbenicillin plates. Colonies were screened for, β-glucosidase activity by replicaplating and exposing the replicated colonies to top agar containing Salicin.

Color selection

To made LB plus cb antibiotic plate add 40µl (20 mg/ml) of a stock solution of X –gal solution was added, 4 µl (200 mg/ml) of a stock solution of (IPTG) and cb antibiotic directly into the medium when you pour the plates, place them at 4°C for several hours to enhance were incubated until developed blue color.

Extraction of plasmids from transformed positive clones

Preparation of plasmids from transformed positive clones for mapping of β-glucosidase gene cleaving site on the pUC19 plasmid was carried out according to Birnboim and Doy (1979).

The polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was conducted to ensure the presence of the gene of interest (2 and 3 kb fragment) in the extracted DNA from recombinant plasmid digested with *EcoRI* restriction enzyme. Forward and reverse primers were used in the reaction and designed to anneal with pUC19. The sequence of both primers was as followed:

Forward primer:
GTTAAACGACGGCCAGTG
Reverse primer:
CAGGAAACAGCTATGACC

The reaction was conducted in total volume of 50 µl containing

Template DNA (50 ng/µl) 1µl, 10 x PCR buffer 5µl, dNTP (2.5 mM) 4µl, $MgCl_2$ (25 mM) 3 µl, Forward Primer (0.1 p mol/ µl each) 5 µl, Reverse Primer (0.1 p mol/ µl each) 5 µl, Taq polymerase (5 unit/µl) 1µl, Dd H_2O up to 50 µl.

Procedure

The PCR program involved an initially step of denaturation at 94°C for 4 min.; followed by 40 cycles of amplification involving denaturation step at 94°C for 1 min., primers annealing to the template realised at 55°C for 1 min., extension step at 72°C for 1 min. and final extension at 72°C for ten min. The PCR DNA products were separated in (1%) agrose gel and the bands were stained by ethidium bromide and detected on UV transilluminator and photographed.

RESULTS AND DISCUSSION

The efficacy of bacterial isolates to produced β-glucosidase enzyme

Four Egyptian isolates of bacterial isolates namely *Rhizobium leguminosarum bv. Trifloii, Azorhizobium caulinodans, Rhodococcus fascins and Agrobacterium tumefaciens* were tested for their ability to use salicin as a sole source carbon. The detection was carried out by appearing a clear halo zone around the colonies which stained with Congo Red. Figure (1) clearly show the capability of *Rhizobium leguminosarum bv. Trifloii, and Agrobacterium tumefaciens* to produce β-glucosidase enzyme as shown in plates (4 and 5). (Saddler *et al*., 1982; Geelen *et al.*, 1995). The screening of β-glucosidase, cellobiohydrolase, endogluconase and polygalacturonase

activities showed the clear formation of haloes around bacterial colonies or spots after overnight incubation on appropriate agar medium, plates stained with Congo Red 0.1% after two or three washes with 1 M NaCl the appearance of a clear halo indicated enzyme activity.

Klaus *et al.* (1999) studied *bvrB* transcription in *Listeria monocytogenes* in the presence of glucose (control) or the β-glucosides cellobiose, salicin, and arbutin. The level of *bvrB* expression was significantly higher in the presence of cellobiose and salicin, The *Listeria monocytogenes bvrB* gene induced by cellobiose and salicin but not by arbutin. The results indicated that the *bvr* locus encodes a β-glucoside-specific sensor that mediates virulence gene repression upon detection of cellobiose and salicin. Bvr is the first sensory system found in *L. monocytogenes* that is involved in environmental regulation of virulence genes. In general, cellulolytic organisms produce cellulases to break down cellulose to cellobiose and glucose, which are used as carbon sources. Because *Azorhizobium caulinodans* cannot grow on glucose or cellobiose, it is very unlikely that the Egl enzyme would serve this purpose. Cellulases might also assist in the release of host plant nutrients, as suggested for *Xanthomonas campestris*, *Erwinia carotovora* (Gough *et al.*, 1988) and *Pseudomonas solanacearum* (Walker *et al.*, 1994).

Gene construction in vitro

Figure (2) showed the digested genomic DNA from *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* bv. Trifloii and size fractionated by agarose gel electrophoresis. *EcoRI* restriction enzyme have a long smear, the 2-5 kb DNA fragments for *Agrobacterium tumefaciens* and *SamI* for *Rhizobium leguminosarum* bv. Trifloii that was eluted and ligated into pUC19 vector. Linda *et al.* (1992) found that *Agrobacterium tumefaciens* the 5.7 kb *HindIlI* fragment common to all type 1 clones purified from a digest of clone 22 and ligated into pBR322. Clones with inserts in either orientation were able to cleave X-glucose, indicating that the entire β-glucosidase gene was probably located within this insert. An *EcoRI, BamHI, BglII,* and *PstI* restriction map of the insert was prepared, and appropriate fragments were subcloned and screened for activity on X-glucose. A 3.5 kb *BamHI-PstI* fragment with an internal PstI site was found to cleave X-glucose when cloned into pUC19.

Plasmid extraction from transformed clones

The new constructs of pUC19 plasmid were introduced into *E. coli HC1061* strain by electroporation. Selection of colonies was done by plate assay. These plates LB media contained IPTG with X-gal substrate and carbanicilin. The blue and white colonies obtained were selected. Transformed colonies were confirmed using *EcoRI* in which the expected band sizes was detected as illustrated in Fig. (3). It was shown that digesting the plasmid from *E. coli*

DH1061 white clones with *EcoRI* restriction enzyme resulted in the production of 2 and 3 kb (insert) fragments. Linda *et al.* (1992) studied the *Agrobacterium tumefaciens* the *EcoRI* site in the pUC19 3.5-kb *BamHI-PstI* clone and the sequence upstream of the *BamHI* site. Sequence data (3,710 nucleotides) were obtained for 176 nucleotides upstream of the *BamHI* site through the downstream *PstI* site. There are at least two constitutive, β-glucosidase genes in *A. tumefaciens* B3/73. Those represented by two types at clones were able to hydrolyze X-glucose and coniferin but not cellobiose. On the other hand, those represented by two types of clones had lower activity on X-glucose and no activity on coniferin and were able to utilize cellobiose for growth.

The white *E. coli* HC1061 recombinant clones, carrying a β-glucosidase gene from *Agrobacterium tumefacien* and *Rhizobium leguminosarum* bv. Trifloii were selected using plate assay grown overnight on LB medium containing Salicin, followed by Congo Red staining. The recombinant clones were characterized by a large halo formation around the white clones *E.coli HC1061*. (Fig. 3). Other white *E. coli* clones but relatively with no activity. Specific fluidity by recombinant plasmid-encoded β-glucosidase, Salicin hydrolysis from *E. coli* carrying recombinant plasmid. The *E. coli* positive clones showed clones on LB medium with Salicin to repeat and confirm the Congo Red stain, the positive *E. coli* clones were again active.

Azorhizobium caulinodans ORS571, cell extracts were tested for the presence of β-glucosidase and cellobiohydrolase activities, using chromophoric substrates. Hydrolysis of the substrates was not observed, suggesting the absence of exoglucanases. Subcloning into pUC19and expression into *E. coli* assigned the Egl activity to a 3 kb DNA fragment. This fragment, which was sufficient to confer CMC degradation, (Geelen *et al.*, 1995)

Preparation of recombinant plasmid DNA from *E. coli HC1061* clones and digestion with the same enzyme showed that insert of about 2-3 kb was present (Fig. 4). Electroelution of the fragments insert (separately) ligated into pUC19 plasmid and transformed into *E. coliHC1061*. Selection derived recombinant clones from each transformation from the white clones from each transformed *E. coliHC1061* and using Congo Red plate assay and recombinant clones expressing β-glucosidase activity by clear halo formation around the clones the result indicated *Rhizobium leguminosarum* have at least two β- glucosidase genes (Fig. 5).

Agrobacterium tumefaciens the *EcoRI* site in the pUC19 3.5-kb *BamHI-PstI* clone and the sequence upstream of the *BamHI* site in the pBR322:5.7-kb *HindIII* plasmid were obtained by double-stranded plasmid sequencing with

synthetic oligonucleotide primers. Sequence data (3,710 nucleotides) were obtained for 176 nucleotides upstream of the *BamHI* site through the downstream *PstI* site. There appear to be at least two constitutive, β-glucosidase genes in *A. tumefaciens* B3/73. Those represented by type 1 clones were able to hydrolyze X-glucose and coniferin but not cellobiose. On the other hand, those represented by type 2 clones had lower activity on X-glucose and no activity on coniferin and were able to utilize cellobiose for growth. (Linda *et al.*, 1992).

6-PCR confirmation

Selected clones were checked for the presence of about 2 and 3 kb and fragment of β-glucosidase gene. PCR confirmed the presence of about 2 and 3 kb fragment β-glucosidase gene in recombinant pUC19 vector. Two PCR primers were used forword and reverse primers annealing to pUC19 based on published data available in the Gene Bank. Using these primers, a PCR product fragment with a MW. of about 2 and 3 kb was obtained. As shown in Fig. (6). The recombinant plasmids showed the expected band size for β- glucosidase gene (2 and 3 kbp) there appear to be at least two β- glucosidase genes in *Rhizobium leguminosarum* bv. Trifloii able to utilize Salicin. Linda *et al.* (1992) found that there is two β-glucosidases in *Agrobacterium tumefaciens* B3/73. There appear to be at least two constitutive, β-glucosidase genes in *A. tumefaciens* B3/73. Those represented by type 1 clones were able to hydrolyze X-glucose and coniferin but not cellobiose. On the other hand, those represented by two types of clones had lower activity on X-glucose and no activity on coniferin and were able to utilize cellobiose for growth.

SUMMARY

Two β-glucosidase genes in *Rizobium leguminosarum* bv. Trifloii able to utilize Salicin. Two and three kbp *SamI* fragments from *Rizobium leguminosarum* bv. Trifloii were ligated into pUC19 plasmid and expressed in *E. coli HC1061*. Transformed clones with β- glucosidase activity were selected by using Congo Red stain plate assay. Restriction enzyme analysis for recombinant plasmid indicated that positive clones contained the 2 and 3 kbp DNA inserts. The *E. coli HC1061* which transformed with 2 or 3 kbp fragment produces extracellular β-glucosidase enzyme can degrade Salicin.

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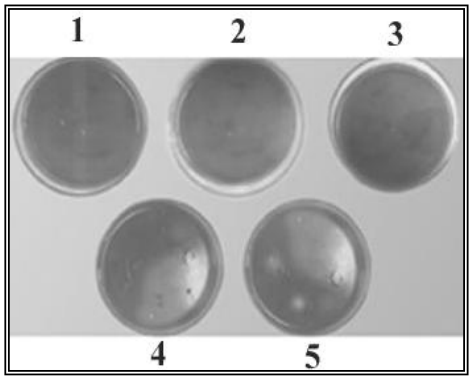

Fig. (1): Glucosidase activity detection assays were performed with 0.2% Salicin as the substrate No (2) Control, No (1, 3, 4 and 5) were *Rhizobium leguminosarum* bv Trifloii, Azo*rhizobium caulinodans*, *Rhodococcus fascins and Agrobacterium tumefaciens*, respectively.

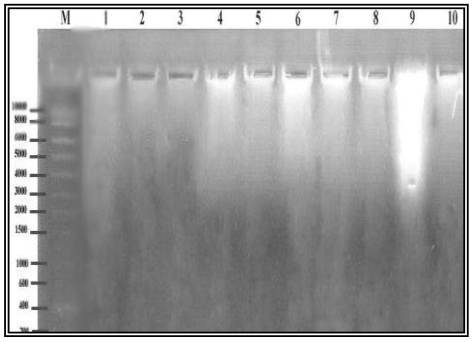

Fig. (2): Digestion of the genomic DNA of *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* bv. Trifloii with different restriction enzymes (*EcoRI,or SamI, HincII, SalI* and *HindIII*) 1, 2, 3, 4 and 5 were *Agrobacterium tumefaciens* 6, 7, 8, 9 and 10 were *Rhizobium leguminosarum* bv. Trifloii, respectively and Smart Ladder as a marker.

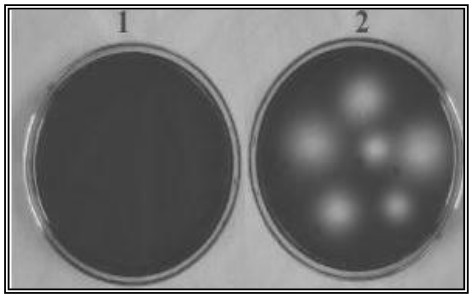

Fig. (3): Screening of recombinant plasmids origin of glucosidase genes activity in the transformed clones, No 1 is *Agrobacterium tumefacien*, No 2 is *Rhizobium leguminosarum* bv Trifloii.

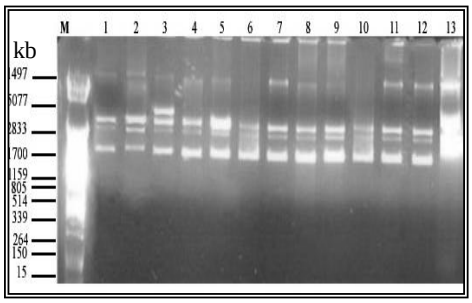

Fig. (4): Fragment length determination by electrophoresis of recombinant plasmid prepared from transformed clones (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13) digested with *EcoRI* restriction enzyme and λ phage *PstI* as a marker.

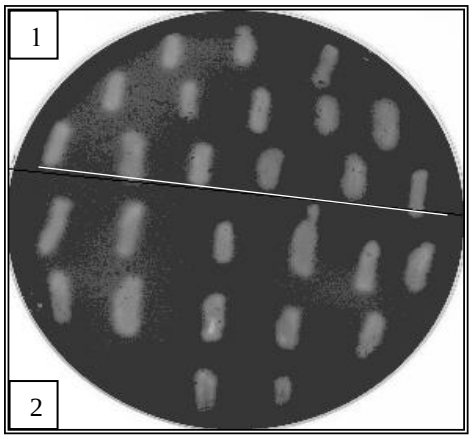

Fig. (5): Verification and Screening of plasmids origin of glucosidase genes activity in the transformed clones, No 1 is 2kbp fragment, No2 is 3kbp fragment of *Rhizobium leguminosarum* bv. Trifloii.

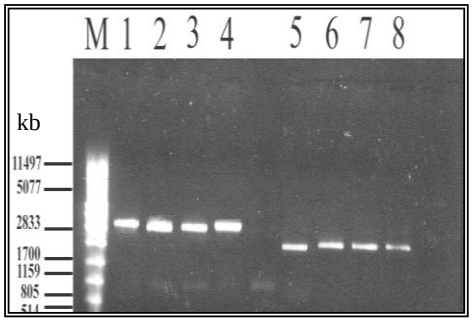

Fig. (6): Charactreization pattern of the PCR product 2 and 3 kb fragment fragment glucosidase genes 1, 2, 3 and 4 glucosidase genes 2 kb 5, 6, 7 and 8 glucosidase genes 3 kb, Lambada *PstI* size marker.