

A Dual Protein Expression System in *Bacillus subtilis*

Running title: Dual expression system

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ABSTRACT

We have developed a dual expression system for the simultaneous overexpression of two proteins in *Bacillus subtilis*. Two candidate genes, xylanase (*xynA*) and glucanase (*bglS*) from *B. subtilis* strain 168, which were engineered with independent Shine-Dalgarno (SD) sequences, were cloned in tandem into a transfer vector, which was then transformed into *B. subtilis* strain 1A304 (ϕ 105MU331). The genes were under the transcriptional control of a strong promoter of a bacteriophage, ϕ 105, where transcription was initiated upon thermal induction. Six constructs were made to compare the factors that affected the yields of the gene products. The expression level of each candidate gene was found to correspond to its position relative to the phage promoter, irrespective of the identity of the insert. The lower expression level of the second insert might have been due to limited resources for protein synthesis, a short half-life of the mRNA, or an early termination of the RNA polymerase. Curiously, gene duplications in tandem did not lead to further production.

INTRODUCTION

Many industrial enzymes are produced by recombinant technology, and usually more than one enzyme is involved in an industrial process. For example, mixtures of protease, lipase, and amylase are sold for detergent applications. We developed a dual expression system based on the *Bacillus subtilis* ϕ 105 MU331 prophage system (1-3). In our system, two proteins were produced simultaneously after heat induction, and the factors that affected their yields were studied. Briefly, two genes were cloned in tandem at a position downstream from the bacteriophage ϕ 105 promoter, with their expression controlled by the ϕ 105 repressor protein. The *cts-52* mutation in the 1A304(ϕ 105MU331) strain renders the ϕ 105 repressor temperature sensitive so that protein expression is induced by temperature shift. Upon thermal induction, the ϕ 105 repressor protein falls off, the promoter is de-repressed, and transcription commences.

To examine the factors that affect the yields of the two proteins, the *xynA* (4) and *bglS* (5) genes from *B. subtilis* 168 were chosen as test models. As these genes are endogenous to the host strain *B. subtilis* 1A304 (ϕ 105MU331), they are better candidates as test models than the other heterologous genes. The expression of the other heterologous genes could be hindered by many factors, such as codon biases, inefficient signal peptide processing, incorrect protein folding, and susceptibility to the host's cellular proteases, etc. The gene product of *xynA*, endo-1,4- β -D-xylanohydrolase (EC

3.2.1.8), hydrolyses xylan, is the major constituent of hemicellulose (6). It has many applications, especially in the paper and pulp industry (6). The gene product of *bglS*, β -(1,3)-(1,4)-glucanase (EC 3.2.1.73), catalyses hydrolysis of β -1,4 bonds in mixed β -glucan. It is particularly useful when it acts in combination with the other hydrolytic enzymes. For instance, both xylanase and glucanase are used as additives to increase the digestibility of animal feed.

MATERIALS AND METHODS

Bacterial strains and culture media

B. subtilis strain 168 served as the template for the glucanase and xylanase genes. *E. coli* Top 10 (Invitrogen, Netherlands) was used as a host strain for plasmid transformation. Luria-Bertani (LB) agar was used for the selection of both *B. subtilis* and *E. coli* strains, and was supplemented with ampicillin (100 mg/L) or chloramphenicol (25 mg/L) when necessary. BHY medium (bovine brain heart infusion, 37 g/L and yeast extract 5 g/L) was used for the *B. subtilis* cultivation.

Construction of expression vectors

The terminator of the α -amylase gene from *B. licheniformis* was cloned into the plasmid pSG1112, which is a derivative of pSG703 (Thornwell et al., 1993), to create the backbone plasmid pSGt (Fig. 1). pSGt contains a promoter and a Shine-Dalgarno (SD) sequence (a ribosome binding site) of the prophage ϕ 105 MU331, followed by a multiple cloning site and a cat gene. The promoter region and the cat gene allow the insertion of heterologous genes into the lysogenic *B. subtilis* 1A304(ϕ 105MU331) chromosome via homologous recombination, as described by Thornwell et al. (7).

To create vectors with dual protein expression capacity, the chromosome DNA

from *B. subtilis* strain 168 was used as a template for glucanase (*bglS*, GeneBank Accession no. Z46268) and xylanase (*xynA*, GeneBank Accession no. Z34519) gene amplification. The primers are listed in Table 1. Restriction sites (VspI, BamHI, and XbaI) were included in the primers to facilitate target gene insertion into cloning vector pSGt. Six plasmid vectors were created: pSGt-X, pSGt-XX, pSGt-XG, pSGt-GX, pSGt-GG and pSGt-G (Fig. 2). All of the constructs contained an upstream ϕ 105 promoter and a downstream terminator of the α -amylase gene from *B. licheniformis*. The plasmids pSGt-X and pSGt-G carried a single gene only, and the other four constructs contained two genes in tandem. Both genes had an upstream SD sequence (GGAGGG) that was located 7 base pairs upstream from the initiation codon ATG.

Generation of recombinant Bacillus subtilis strains

The construction of recombinant plasmids was manipulated in *E. coli* strain Top 10. To generate recombinant *Bacillus subtilis* strains, plasmids were extracted from *E. coli* by a kit (Quantum Prep, BIO-RAD, Hong Kong) before they were transformed into *B. subtilis*. The preparation of *B. subtilis* 1A304(ϕ 105MU331) competent cells and plasmid transformation was accomplished according to the procedure that was described by Osbume et al. (8). Positive transformants were selected on agar plates that were supplemented with chloramphenical (25 ug/ml). The colonies were picked for

further PCR screening using a $\phi 105$ promoter-specific primer (sense) and an insert-specific primer (antisense) (Table 1).

Over-expression of target protein in B.subtilis 1A304($\phi 105$ MU331)

For the production of recombinant proteins, recombinant *B. subtilis* strains were grown in 10 ml of BHY medium with chloramphenicol in a 250 ml flask and shaken at 37 °C at 280 rpm overnight. 1 ml of the overnight culture was then transferred to 15 ml of BHY medium without antibiotics, and the culture was shaken at 37 °C at 280 rpm. After 2 hours, when OD600 reached 3.5 to 4.3, recombinant protein expression was induced by heat shock at 50 °C for 5 minutes. As the enzymes were secreted into the culture medium, 1 ml of cell-free supernatant was collected at various time points from each clone for SDS-PAGE analysis and enzyme assaying. For the OD600 measurement, the cells were diluted 10-fold with culture medium before reading OD.

Enzyme assays

Xylanase and glucanase activities were assayed according to Dygert's Method (9). 0.1% (w/v) oat spelt xylan and 0.5% (w/v) barley- β -glucan were used as substrates for the xylanase and glucanase assays. Culture supernatants were diluted in 50 mM of phosphate buffer (xylanase assay), pH 6.5, or 50 mM of acetate buffer (glucanase

assay), pH 6.0. Both assays were carried out at 50 °C. The amount of reducing sugar that was released was assayed by using xylose and glucose as standards. One unit of activity was defined as the amount of enzyme that produced 1 umol of reducing sugar per minute.

Total RNA extraction and one-step RT-PCR

To confirm the presence of a fusion-transcript, a one-step RT-PCR (QIAGEN OnseStep RT-PCR Kit) was performed. Clones pSGt and GX were cultured as described above. 30 minutes after induction, total RNA was collected using an RNease[®] Mini Kit (QIAGEN). Contaminated DNA was digested and removed by treating the total RNA with components from the DNA-free kit (Ambion). The integrity of the RNA sample was monitored by gel electrophoresis and OD_{260/280} measurement. 50 ng of each RNA sample was added as a template in an individual PCR reaction, for which t1112 was employed as a control sample. A reaction mix without template RNA was included as a negative control. Three sets of one-step RT-PCR with different primer combinations were prepared. In one of these reactions, gene-specific primer C and primer F (Table 1) were added for amplification of the fusion-transcripts *bglSxynA*. The second reaction was aimed at amplifying *bglS* using another pair of gene-specific primers C and D (Table 1). *XynA* was detected using gene-specific primers E and F

(Table 1). Each primer was added at a final concentration of 0.6 μ M. The RNA samples were reverse-transcribed at 50 °C for 30 minutes, followed by initial DNA polymerase activation by incubation at 95 °C for 15 minutes before amplification. This incubation also inactivated the reverse transcriptase. An amplification cycle was carried out by denaturation of cDNA at 94 °C for 40 seconds, annealing at 50°C for 40 seconds, then polymerization at 72 °C for 2 minutes. A final polymerization step was performed at 72 °C for 10 minutes.

RESULTS

Generation of the recombinant Bacillus strains

The PCR products of the xylanase and glucanase genes were 642 and 729 bp respectively. The identities of the six constructs that are shown in Fig. 2 were confirmed by HincII digestion, because different constructs produce different DNA fragments. The genotype of the host *B. subtilis* strain 1A304 (ϕ 105MU331) is *ind-125cts-52 Ω* (*lacZ'*[ClaI]-*ermC-cat'*[NcoI])331 Δ (DI:1t)trpC2metB52xon-1 SPB(S), in which an engineered temperate bacteriophage ϕ 105MU331 has been incorporated into the bacterial genome (7). The pSGt constructs and the *B. subtilis* 1A304(ϕ 105MU331) chromosome DNA contain homologous regions. The target inserts were flanked by a ϕ 105 fragment (approximately 600 bp) and a complete *cat* gene in the plasmid (Fig. 1), whereas an *ermC* gene was flanked by the same ϕ 105 fragment and a truncated *cat'* gene in the *B. subtilis* 1A304(ϕ 105MU331) chromosomal DNA (7). After the plasmid was taken up by the competent *B. subtilis* 1A304(ϕ 105MU331) strain in its late exponential phase, homologous double crossover recombination occurred. Therefore, the transformants should have obtained the phenotype Cm^REr^S. Among the colonies that were picked for PCR screening, 70% of those with the Cm^REr^S phenotype showed positive transformation. The transformants were designated as X, XX, XG, GX, GG,

and G, according to their insert identity. As the plasmid contains no replication origin for the binding of *B. subtilis* DNA polymerase, it cannot autonomously replicate unless stably integrated into the bacterial DNA.

Overproduction of glucanase and xylanase

After thermal induction during the late log phase, all six clones were found to have overexpressed the corresponding gene products, and the target proteins gradually increased from 1 hour to 4 hours after induction. Culture supernatant (15 ul) that was collected at 4 hours after induction was analyzed by SDS-PAGE (Fig. 3). The protein bands of 22 kDa and 25 kDa represent xylanase and glucanase.

Enzyme activity assays

Enzyme assays were carried out using cell free supernatant collected at 4 hours after induction. The results of the activity assays are shown in Fig. 4. It is obvious that the clones GX and XG can produce both glucanase and xylanase, but at different levels. The clone GX produced more glucanase (6.5 U/ml) than xylanase (3.0 U/ml), whereas the clone XG produced more xylanase (9.0 U/ml) than glucanase (4.4 U/ml). In contrast, the double clones XX and GG, which carried two identical genes, only secreted a similar level of enzymes when compared with the clones (X and G) that carried a single

copy gene. This might have been due to limited resources that were available for enzyme production within the host. The elevated expression of these enzymes was not derived from the endogenous *xylA* and *bglS* genes, as the host strain *B. subtilis* 1A304(ϕ 105MU331) produced negligible enzyme activities (data not shown).

Total RNA extraction and one-step RT-PCR

To confirm that the two genes within the same vector were transcribed from the same promoter, a RT-PCR was performed on the clone that carried the pSGt-GX construct. mRNA was first isolated at 30 minutes after heat induction, and various sets of PCR primers were used. As shown in lane 3 of Fig. 5, a PCR product of approximately 1300 bp was amplified by the glucanase sense primer (C) and the xylanase antisense primer (F). The PCR results also indicated that no endogenous glucanase or xylanase mRNAs were transcribed in the host strain that was transformed with the pSGt vector.

DISCUSSION

We have developed a dual expression system in *B. subtilis* that can simultaneously co-express two proteins. However, the expression levels of these two proteins vary. As shown in Fig. 4, the clone GX secreted more glucanase than xylanase, whereas the clone XG secreted more xylanase. This indicates that the expression level of the genes was related to their relative positions in the construct. The closer that the gene was to the prophage promoter, the higher was the expression level. This is unrelated to the identity of the gene or its signal peptide sequence.

After thermal induction, the $\phi 105$ prophage DNA is excised from the *B. subtilis* host chromosome and starts DNA replication for gene expression. Thereafter, a DNAase targeting host DNA is produced. As the DNase accumulates and destroys the host DNA, the host is unable to supply sufficient amino acids and energy for recombinant protein synthesis. As the 5' ends of mRNA molecules are first transcribed, the gene in close proximity to the $\phi 105$ promoter could be translated before the mRNA synthesis is completed, which will result in a higher expression level. When the second gene that is located at the 3' end of the mRNA molecule is available for translation, the preceding gene has already depleted a certain amount of limited resources. Thereafter, the gene that is located at the 3' end of the mRNA shares the depleting resources with the gene that is located at the 5' end. In addition, RNA polymerase has a certain

probability of terminating before the completion of the message, thereby favoring the expression of promoter-proximal genes as compared to those further downstream from the promoter.

The dual expression system may be applied to enhance the level of protein secretion in *Bacillus subtilis*. The high level expression of secretory proteins can saturate the secretion apparatus in *B. subtilis*. For example, in *B. subtilis* strains that overexpress α -amylase, amylase precursors with uncleaved signal peptide were found to accumulate either in the cytoplasm or associate with the cytoplasmic membrane (10, 11). The secretory protein usually carries a signal peptide that is recognized and processed by a signal peptidase on the cell membrane. After the nascent polypeptide is translocated across the cytoplasmic membrane, post-translational folding of the polypeptide is required for the generation of a functional protein. The correct folding of many secreted proteins is not spontaneous, but is dependent on assisting factors such as chaperones or foldases. (12-14). To date, only one extra-cytoplasmic protein in the gram-positive bacterium *B. subtilis*, Prs A, is known to be involved in protein secretion. Prs A is a 33 kDa protein that is anchored on the outer surface of the cytoplasmic membrane. The overexpression of Prs A was shown to enhance the secretion of α -amylase in *B. subtilis* (15) by approximately 5-fold. Therefore, the co-expression of a target protein with PrsA using the dual expression system that is described in this article

may enhance the secretion of the target protein.

B. subtilis has an excellent secretory ability, which is partly due to its abundance in signal peptides. There are six signal peptide genes in the chromosome DNA of *B. subtilis*, including five type I signal peptidases (SPase I) and one type II signal peptidase (SPase II). These two types of signal peptidases are believed to be responsible for the cleavage of the signal peptides of secretory proteins and lipoproteins respectively (16, 17). Even though the five type I signal peptidases, SipS, SipT, SipU, SipV, and Sip W, have various degrees of sequence homology, it is believed that they have different substrate specificities. In addition, their transcription levels are controlled by different mechanisms. While SipU and SipV are constitutively transcribed at a low level, the transcriptions of SipS and SipT are more active in the post-exponential growth phase and exponential growth phase respectively (18). In the future, we intend to study the effects of incorporating these signal peptide genes into the dual expression system to test whether any of them can enhance the secretion of the protein of interest.

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FIGURE LEGENDS

Fig. 1. The plasmid map of pSGt. Heterologous genes were inserted into the multicloning sites (MCS) of the vector. The vector carried a pBR 322 replication origin for *E. coli*, the promoter and the ribosome binding site (SD) of prophage Φ -105 MU331 (Φ -105), the transcription terminator of *B. licheniformis* α -amylase (term), a cat gene for chloramphenicol resistance, and a bla gene for ampicillin resistance.

FIG. 2. Construction map of the six constructs. The thick solid line represents the sequence that was derived from the vector pSGt. The gray box represents the ϕ -105 promoter; the black circle 'SD' represents Shine Dalgarno sequence GGAGGG; and the checkered box represents the terminator of the *B. licheniformis* α -amylase gene, ACGGATTCCTGAAGGAAATCCGT.

FIG. 3. SDS-PAGE of the culture supernatant that was collected 4 hours after induction.

The left-most lane contains the molecular weight markers. Lanes 1 to 6 were collected from clones X, XX, XG, GX, GG, and G. Lane 7 is the culture supernatant from the cells that were transformed with the vector only. 15 μ l samples were loaded into each lane.

FIG. 4. Comparison of the xylanase and glucanase activities that were secreted by

various constructs.

FIG. 5. RT-PCR products from clones pSGt and GX.

Lanes 1 to 3, 4 to 6, and 7 to 9 were amplified by primers C/F, C/D, and E/F respectively.

Lanes 3, 6, and 9 demonstrated the presence of transcripts *bglSxynA*, *BglS*, and *xynA* in the clone GX. No PCR products were shown in lanes 1, 4, and 7 in the absence of template RNA, or in lanes 2, 5, and 8 where mRNA from clone pSGt was used as a template. Molecular weight markers M₁ (1 kb Molecular weight marker, Gibco BRL) and M₂ (GeneRuler 100 bp DNA ladder plus, MBI) were employed.

Table 1: Sequences of oligonucleotides

Primer	Sequence (5' → 3')	R. E. site
A	GAAAC <u>ATTAAT</u> GT TTAAGTTTAAAAAGAATTCTTAG	Vsp I
B	TAGGATCCGCTACCCCTGAT TAAGGATG	BamH I
C	AATCGATTA <u>ATGC</u> CTTATCTGAAACGAGT	Vsp I
D	TAGGATCCGCAGCAGGTTCTTTCACA	BamH I
E	ATGGATCCGGAGGGTAACATAT GT TTAAGTTTAAAAAG	BamH I
F	ACTCTAGATTACCACACTGTTACGTTAGA	Xba I
G	ATGGATCCGGAGGGGCAATAT GC CTTATCTGAAAC	BamH I
H	AGTCTAGAGCAGCAGGTTCTTTCACA	Xba I
Phi105S	CACCGATAAT AAGGTGGGAG	Nil

Restriction sites are underlined. Initiation codon ATG is shown in bold type.

SD sequences (GGAGGG) are shown in italics.

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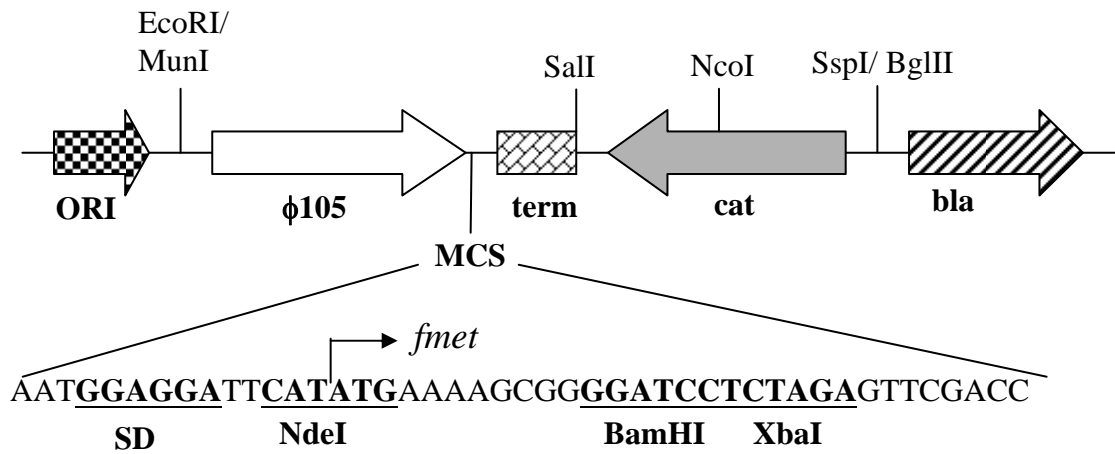
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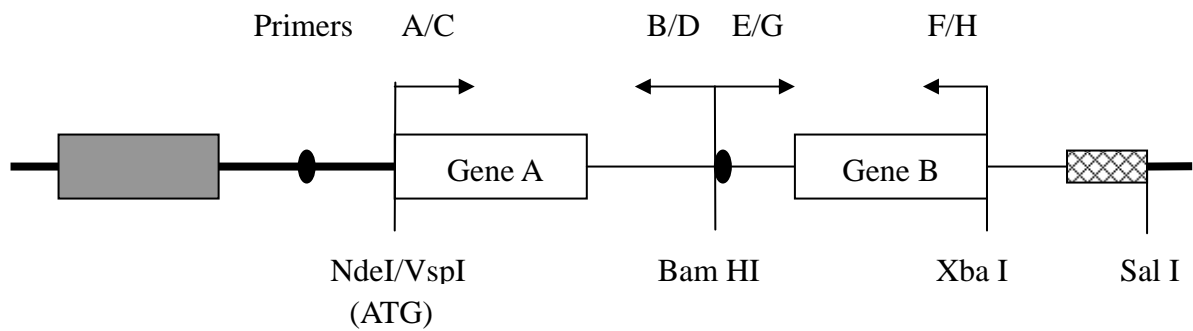
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Fig.1





ϕ 105 promoter



SD sequence



terminator of the α -amylase gene from *Bacillus licheniformis*

Vector	Gene A	Primers	Gene B	Primers
pSGt-X	Xylanase	A/B	N.A.	N.A.
pSGt-XX	Xylanase	A/B	Xylanase	E/F
pSGt-XG	Xylanase	A/B	Glucanase	G/H
pSGt-GX	Glucanase	C/D	Xylanase	E/F
pSGt-GG	Glucanase	C/D	Glucanase	G/H
pSGt-G	Glucanase	C/D	N.A.	N.A.

N.A. Not Applicable.

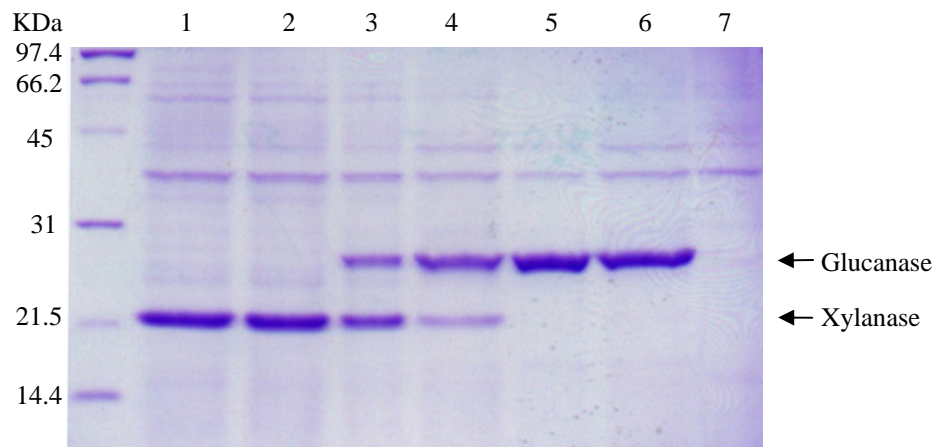
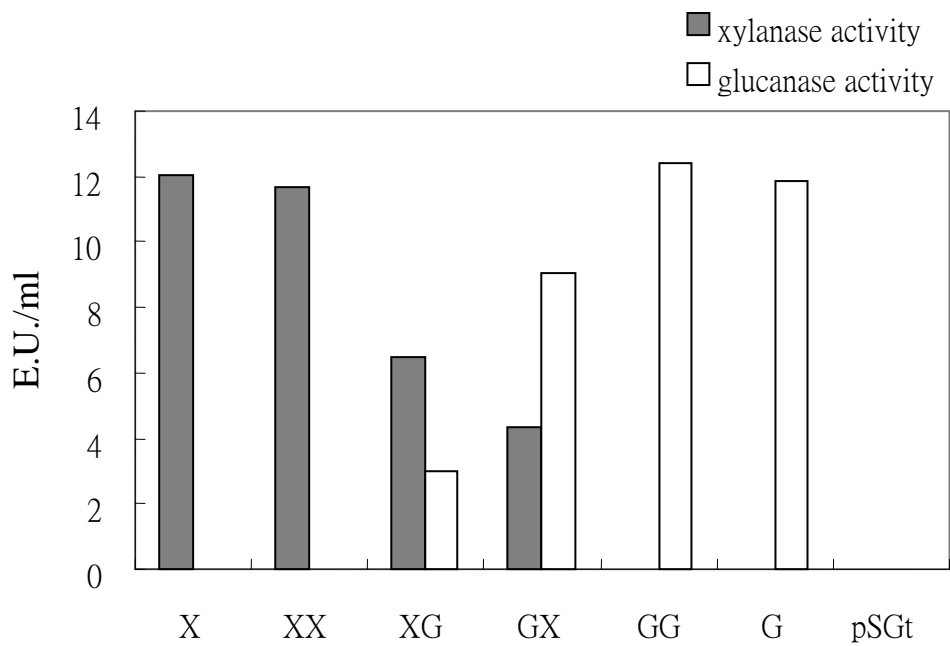


Fig. 3



Clone	Xylanase Activity (E.U./ml)	Fold*	Glucanase activity (E.U./ml)	Fold*
X	12.1	86.1X	0.06	0.8X
XX	11.7	83.6X	0.08	1.0X
XG	6.5	46.1X	3.0	37.3X
GX	4.4	31.1X	9.0	113.1X
GG	0.08	0.6X	12.4	155.1X
G	0.12	0.9X	11.9	148.4X
pSGt	0.14	1.0X	0.08	1.0X

E.U.: Enzyme Unit, defined as the amount of enzyme producing 1 μ mol reducing sugar per minute.

*Number of fold refers to the enzyme activities compared with that of the vector clone (pSGt).

Figure 5.

