

An Alternative Bacterial Expression System Using *Bacillus pumilus* SG2 Chitinase Promoter

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Background: Chitin is an abundant natural polysaccharide found in fungi, algae, and exoskeleton of insects. Several bacterial species are capable of utilizing chitin as their carbon source. These bacteria produce chitinases for degradation of chitin into *N*-acetyl-D-glucosamine. So far, regulation of the chitinase encoding genes has been studied in different bacterial species. Among *Bacillus* species, *B. pumilus* strain SG2 encodes two chitinases, ChiS and ChiL. The promoter region of *chiSL* genes (P_{chiS}) is mainly regulated by the general carbon catabolite repression (CCR) system in *B. subtilis* due to the presence of a catabolite responsive element (*cre*).

Objectives: Use of P_{chiS} in constructing an inducible expression system in *B. subtilis* was investigated.

Materials and Methods: In the first step, complete and shortened versions of P_{chiS} were inserted upstream of the *lacZ* on a pBS72/pUC18 shuttle plasmid. The β -galactosidase activity of *B. subtilis* carrying one of the relevant plasmids was measured in the presence of different carbon sources.

Results: An expression system based on the chitinase promoter of *B. pumilus* SG2 was established. Modification of P_{chiS} and the culture medium resulted in production of β -galactosidase in *B. subtilis* up to 1,800 Miller unit (MU) activity.

Conclusions: The chitinase promoter developed in this study, has potential to be used in an expression vector that could be induced by chitin. In addition, compared to the other inducers like IPTG and lactose, chitin is definitely cheaper and more available as an inducer.

Keywords: Carbon catabolite repression; Chitin; Induction; *N*-acetylglucosamine; Regulation

1. Background

Chitin ($C_8H_{13}O_5N$)_n is the second most abundant natural polysaccharide composed of *N*-acetyl-D-glucosamine (GlcNAc) monomers. Chitin is found in many species including outer skeleton of crustaceans, insects, and as a component of fungi and algae cell walls (1-4). Chitinases (EC.3.2.1.14) are hydrolases able to degrade chitin into GlcNAc. There are endo- and exo-chitinases that degrade chitin as a substrate (2). Different organisms including bacteria, protozoa, fungi, and plants produce chitinases (1, 5, 6). These enzymes have several biological roles in different organisms. For instance, these enzymes are produced to provide carbon and nitrogen sources in bacteria (2, 3, 5, 6).

Generally, heterotrophic bacteria utilize glucose as a preferred carbon source. Meanwhile, in the presence of glucose, transcription of many genes that use other carbon sources are repressed in a mechanism called carbon catabolite repression (CCR) (7, 8). Most of reported chitinase encoding genes are also regulated by CCR. In *Streptomyces plicatus*, characterization of the promoter region of two chitinase genes, namely *chi63* and *chi35*, showed their induction by chitin and repression in the presence of glucose (9, 10). Several other chitinase genes including *chiA* and *chiC* of *Streptomyces lividans* and *chiA*, *chiB*, *chiC*, *chiD* and *chiF* of *Streptomyces coelicolor* A3 (5) are similarly regulated by chitin and glucose. In *Bacillus subtilis*, most of the genes or operons that are under the control

of CCR have a *cis*-acting catabolite responsive element (*cre*) (7, 11-14). This *cre* site (WTGNAANCGN-WNNCW) located within, upstream or downstream of a promoter region and interact with a *trans*-acting complex, carbon catabolite protein A (CcpA) and HPr (S46~P) complex. CcpA is a DNA binding protein that belongs to LacI/GalR transcriptional regulators, which is able to repress or activate the transcription of genes depending on the location of *cre* site at the target promoter (11, 14-16). HPr is a histidine containing protein that forms a complex with CcpA, when it is phosphorylated at its serine residue number 46 (17).

B. pumilus strain SG2 is a halophilic and chitinolytic bacterium that produces two chitinases, ChiS and ChiL (18, 19). The expression of *chiSL* genes is induced by chitin as a sole carbon source, while it is repressed in the presence of glucose. In other words, *B. pumilus* SG2 chitinase encoding genes is controlled by carbon catabolite repression (CCR) (20). In the current study, activity of P_{chiS} from *B. pumilus* SG2 was evaluated on a low copy plasmid in *B. subtilis* in order to

investigate the possibility of construction of an expression system.

2. Materials and Methods

2.1. Strains, Media and Growth Condition

All strains are listed in (Table 1). The original genes and promoter are from *B. pumilus* SG2 strain which was isolated from high salt ecosystem, but cloning and experimental works were carried out in a standard *B. subtilis* called 3NA as explained in (Table 1). *Escherichia coli* JM109 was used for plasmid propagation. LB-agar supplemented with ampicillin (100 µg.mL⁻¹), chloramphenicol (5 µg.mL⁻¹), or spectinomycin (100 µg.mL⁻¹) were used for selection of *E. coli* and *B. subtilis* 3NA transformants. Minimal medium (MI) for transformation of *B. subtilis* was prepared by mixing 97.5 mL of Spizizen's minimal salts containing 2 g.L⁻¹ (NH₄)₂SO₄, 14 g.L⁻¹ K₂HPO₄, 6 g.L⁻¹ KH₂PO₄, 1 g.L⁻¹ Na₃C₆H₅O₇.2H₂O, 0.2 g.L⁻¹ MgSO₄.7H₂O, 5 g.L⁻¹ glucose, supplemented with 0.02% (w/v)

Table 1. Primers, plasmids and strains were used in this study

Name	Description	Reference
Oligonucleotides		
ChiSLF10	GGGCCCGGGTCATCAAGACGCAGATGTC	(20)
ChiSR1J	GGGGCATGCGAGCCCACTCTCTCTTTA	(20)
UP-CRE1	TATGAAAAC TAGAAATGTTGTCTTCAGTGC	This study
UP-CRE2	GCACTGAAGACAACAACATTTCTAGTTTTCATATGC	This study
s5767	AAAGCTAGCTCATCAAGACGCAGATGTC	This study
s5768	AAACTTAAGCCCTTTTCATTAATTTTT	This study
Plasmids		
pDHAFB	<i>bla</i> , <i>amyE</i> ::[<i>cat</i> , <i>lacI</i> , P _{spac}]	(22)
pSUN279.2	<i>ori</i> _{pBS72} , <i>ori</i> _{pUC18} , <i>ter</i> -P _{manR} - <i>manR</i> -P _{manP} - <i>lacZ</i> , <i>spcR</i>	(36)
pUP-Chi2	<i>bla</i> , <i>amyE</i> ::[P _{chiS} - <i>chiS</i> , <i>cat</i>]	(20)
pUP-Chi2Δ <i>cre</i> sig	<i>bla</i> , <i>amyE</i> ::[P _{chiSΔ<i>cre</i>sig} - <i>chiS</i> , <i>cat</i>]	(20)
pUP-Chi2Δ <i>cre</i>	<i>bla</i> , <i>amyE</i> ::[P _{chiSΔ<i>cre</i>} - <i>chiS</i> , <i>cat</i>]	This study
pChi1	<i>ori</i> _{pBS72} , <i>ori</i> _{pUC18} , <i>ter</i> -P _{chiS} - <i>lacZ</i> , <i>spcR</i>	This study
pChi2	<i>ori</i> _{pBS72} , <i>ori</i> _{pUC18} , <i>ter</i> -P _{chiSΔ<i>cre</i>} - <i>lacZ</i> , <i>spcR</i>	This study
pChi3	<i>ori</i> _{pBS72} , <i>ori</i> _{pUC18} , <i>ter</i> -P _{chiSΔ<i>cre</i>sig} - <i>lacZ</i> , <i>spcR</i>	This study
Strains		
<i>E. coli</i>		
JM109	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (r _K ⁻ , m _K ⁺), <i>mcrA</i> , <i>supE44</i> , <i>gyrA96</i> , <i>relA1</i> , λ ⁻ , Δ(<i>lac-proAB</i>), F' (<i>traD36</i> , <i>proAB</i> ⁺ , <i>lacI</i> ^q , (Δ <i>lacZ</i>)M15)	(37)
<i>B. subtilis</i>		
3NA	<i>spo0A3</i>	(38)
Chi7	<i>spo0A3 amyE</i> ::[P _{chiSΔ<i>cre</i>sig} - <i>chiS</i> , <i>cat</i>]	pUP-Chi2Δ <i>cre</i> sig →3NA pChi1
Chi8	<i>spo0A3 amyE</i> ::[P _{chiSΔ<i>cre</i>sig} - <i>chiS</i> , <i>cat</i>]	pUP-Chi2Δ <i>cre</i> sig →3NA pChi2
Chi9	<i>spo0A3 amyE</i> ::[P _{chiSΔ<i>cre</i>sig} - <i>chiS</i> , <i>cat</i>]	pUP-Chi2Δ <i>cre</i> sig →3NA pChi3

casamino acids, and 5 mM MgSO₄ (21). Induction of strain 3NA containing pChi1, pChi2 or pChi3 was carried out using LB medium. LB medium (85 mL) in a 500 mL Erlenmeyer flask was inoculated by 1.7 mL of the overnight culture and incubated at 37°C with 200 rpm shake. Cells were grown to an optical density of 0.4 at 600 nm. Aliquots of 8 mL were divided in 100 mL Erlenmeyer flasks and induced with 0.2% (w/v) inducers, *i.e.* chitin, chitin+glucose, *N*-acetylglucosamine (GlcNAc), GlcNAc+glucose or without any inducer as the negative control. Colloidal chitin was prepared as described (18). Samples were collected 1 h after the addition of carbohydrates.

Minimal medium (96.5 mL of MI medium (without glucose) + 0.02% v/v glycerol) was used to study the β-galactosidase activity of prepared strains, namely 3NA pChi1, 3NA pChi2, 3NA pChi3, Chi7, Chi8 and Chi9. The inducers were 0.2% (w/v) chitin + 0.2% v/v glycerol, 0.2% (w/v) chitin + 0.2% (w/v) glucose, 0.2% (w/v) glucose, and 0.2% (v/v) glycerol. Sample collection was performed at 2 h intervals until 11 h after the induction. All experiments were performed three times and mean values were used for comparison.

2.2. Construction of Expression Plasmids and Strains

Plasmids and oligonucleotide primers are listed in (Table 1). Construction of pUP-Chi2Δcre was carried out using overlapping PCR to fuse the chitinases promoter to the coding region of β-galactosidase gene. PCR₁ was performed using ChiSLF10 and UP-CRE2

primer pairs and plasmid pUPChi2 as a template. PCR₂ was performed using primers (ChiSR1J and UP-CRE1) and plasmid pUPChi2 as a template. PCR₃ was performed using primers (ChiSLF10 and ChiSR1J) and 20 ng of each PCR₁ and PCR₂ amplicons as templates. The final PCR product was inserted into pDHAFB via *Cfr*^{9I} and *Sph*I restriction sites. The final cassette was integrated into the *amyE* locus of *B. subtilis* chromosome to have a constitutive expression of chitinase. Using this method, the α-amylase encoding gene was inactivated and the transformants were selected based on their chloramphenicol resistance (22). In order to create pChi1, pChi2 and pChi3 constructions, promoter of chitinase gene of *B. pumilus* SG2 was amplified in a PCR using s5767 and s5768 oligonucleotides and pUP-Chi2, pUP-Chi2Δcre, and pUP-Chi2Δcresig as the templates. The amplified fragment was inserted into a derivative of the theta replicating pBS72 plasmid (pSUN279.2), which is a low copy number plasmid in *B. subtilis*, upstream of the *lacZ* as the reporter gene using *Nhe*I (NEB) and *Af*III (NEB) restriction enzymes (Figure 1). The pSUN279.2 has also a pUC18 origin of replication for plasmid propagation in *E. coli*.

To integrate the P_{chiSΔcresig}-*chiS* cassette into the chromosome of *B. subtilis* 3NA, plasmid pUP-Chi2Δcresig was used containing an *amyE* integration cassette. In this way, strain 3NA containing pChi1, pChi2 or pChi3 was transformed to create Chi7, Chi8, and Chi9, respectively.

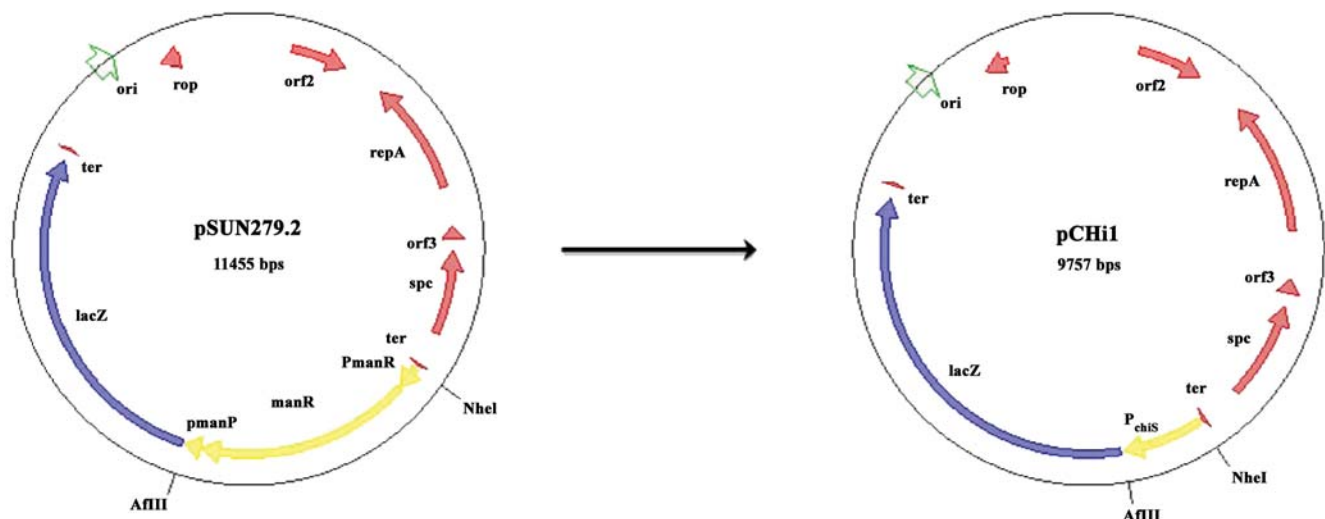


Figure 1. The plasmid maps of pSUN279.2 used as the parental vector and its derivative pChi1. The P_{chis} DNA sequence was inserted into pSUN279.1 via *Nhe*I and *Af*III restriction sites

2.3. Enzyme Activity

Production of α -amylase was detected by the addition of iodine solution (0.5% iodine in 1% potassium iodide solution) in nutrient agar containing 1% (w/v) starch (23), while production of chitinase was detected using MI agar plates containing 0.5% (w/v) chitin and 0.001% (v/v) Congo Red (24). β -galactosidase activity was measured using *o*-nitrophenyl-D-galactopyranoside (ONPG) as substrate according to Miller assay (25) and the result was given as Miller unit (MU). T-test statistical analysis was used to demonstrate the significance and reliability of the quantitative results.

3. Results

3.1. Activity of the Chitinase Promoter on a pBS72 Derivative in *B. subtilis*

Regulation of P_{chiS} and its derivative $P_{chiS\Delta cresig}$ has been already investigated in *B. subtilis* using an integrated P_{chiS} -*lacZ* cassette (20). To develop a new gene expression system for *B. subtilis*, P_{chiS} was inserted upstream of *lacZ*, as a reporter gene, on pSUN279. Plasmid pSUN279 is an *E. coli* (*ori*_{pUC18})-*B. subtilis* (*ori*_{pBS72}) stable low copy number shuttle vector in *B. subtilis*. Transformation of *B. subtilis* 3NA with pChi1 (P_{chiS}) was followed by induction of the transformed mutants in LB in the presence of chitin or GlcNAc with (out) glucose. Measurement of the β -galactosidase activity of the 3NA pChi1 strain showed low

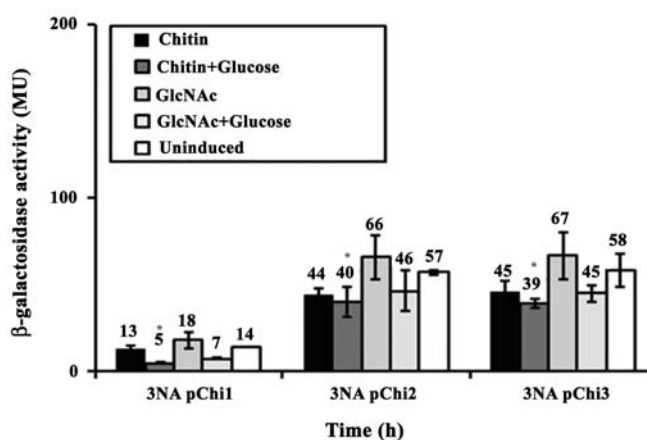


Figure 2. β -galactosidase activity of the *B. subtilis* strains 3NA pChi1 (A), 3NA pChi2 (B) and 3NA pChi3 (C) in LB medium with chitin (0.2%) or GlcNAc (0.2%) alone or together with glucose (0.2%). As the control, no additional carbohydrate was added to the medium (uninduced). The β -galactosidase measurements were carried out 1 h after the addition of carbohydrates. The experiment was performed three times and mean values and standard deviations (error bar) are shown

amount of the β -galactosidase activity up to 18 MU after 1 h of induction in LB. Since there was not a significant difference in the β -galactosidase activity with chitin or GlcNAc as an inducer compared to the uninduced culture, it was concluded that the expression from the P_{chiS} promoter was constitutive (Figure 2). In contrast, the addition of glucose reduced β -galactosidase activity of 3NA pChi1 by 2.6- and 2.5-fold in the presence of chitin and GlcNAc, respectively (Figure 2). In order to investigate the effect of the *cre* site on the activity of P_{chiS} , two shortened versions of P_{chiS} , namely $P_{chiS\Delta cre}$ and $P_{chiS\Delta cresig}$, were inserted upstream of *lacZ*. The $P_{chiS\Delta cre}$ lacked only the *cre* site, whereas $P_{chiS\Delta cresig}$ had an additional deletion upstream of the *cre* site (for the exact promoter sequence see Heravi, Shali (20)). Like 3NA pChi1, the β -galactosidase activities of strains 3NA pChi2 ($P_{chiS\Delta cre}$) and 3NA pChi3 ($P_{chiS\Delta cresig}$) were measured. The results revealed that the deletion of the *cre* site increased the activity in all culture conditions (Figure 2). Unlike 3NA pChi1, in both 3NA pChi2 and 3NA pChi3, P_{chiS} was constitutive. As expected, glucose had no effect on the production of β -galactosidase and the level of enzyme was significantly increased up to 67 MU in 3NA pChi2 and 3NA pChi3. Likewise, no drastic difference was observed between the β -galactosidase activity of 3NA pChi2 and 3NA pChi3 (Figure 2). Altogether, the results indicated that $P_{chiS\Delta cre}$ and $P_{chiS\Delta cresig}$ had higher activities than P_{chiS} . In between, only P_{chiS} was regulated by glucose.

3.2. Strain and Media Optimization for the Activity of P_{chiS} and its Derivatives

Since the β -galactosidase activities of strains 3NA pChi1, 3NA pChi2 and 3NA pChi3 were too low in LB, MI medium containing glycerol was used with the addition of different carbohydrates (glycerol, glucose and chitin). The β -galactosidase activity of each strain was measured with 2 h intervals up to 11 h (Figure 3 A-C). Comparing to LB medium, the results indicated an increase in β -galactosidase activity. Moreover, the β -galactosidase activity of each construct was similar in different conditions, showing that the P_{chiS} and its derivatives were constitutive in minimal medium with the defined composition. The higher β -galactosidase activities were obtained for 3NA pChi2 and 3NA pChi3 after 11 h of induction with about 800-1,100 MU.

Plasmid pUP-Chi2 $\Delta cresig$ with the $P_{chiS\Delta cresig}$ -*chiS* cassette was integrated into the genome of strain 3NA pChi1, 3NA pChi2 and 3NA pChi3 at *amyE* locus. The

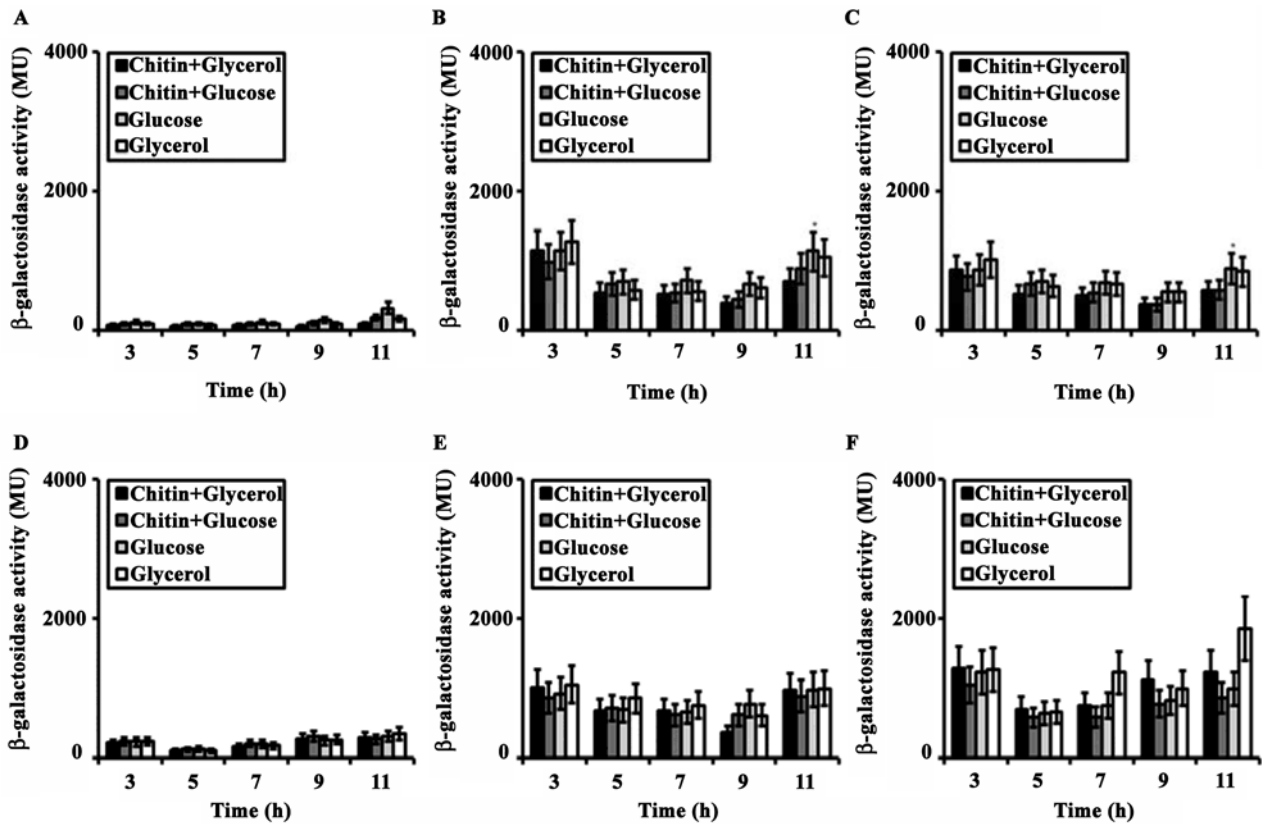


Figure 3. β -galactosidase activity of the strains A: 3NA pChi1, B: 3NA pChi2, C: 3NA pChi3, D: Chi7, E: Chi8 and F: Chi9 in the minimal MI medium containing 0.02% glycerol. Different carbohydrates, *i.e.* chitin, glucose and glycerol (0.2%), were added to the culture media. β -galactosidase activity was measured 3, 5, 7, 9 and 11 h after induction. All the experiments were performed three times and mean values and standard deviations (error bar) are shown

newly constructed strains Chi7, Chi8 and Chi9 were able to degrade chitin and use it as carbon source. The integration of $P_{chiS\Delta cresig}$ -*chiS* into the *amyE* gene was confirmed using starch agar (Figure 4 A-F). As a result, strains 3NA pChi1, 3NA pChi2 and 3NA pChi3 showed a halo around their colonies

degradation (negative controls; Figure 4 A-C), whereas strains Chi7, Chi8 and Chi9 showed no amylase activity and therefore, they showed no halo around the colonies (Figure 4 D-F). Likewise, expression of chitinase was confirmed using chitinase-Congo Red agar. The production of clear halos around the colonies of

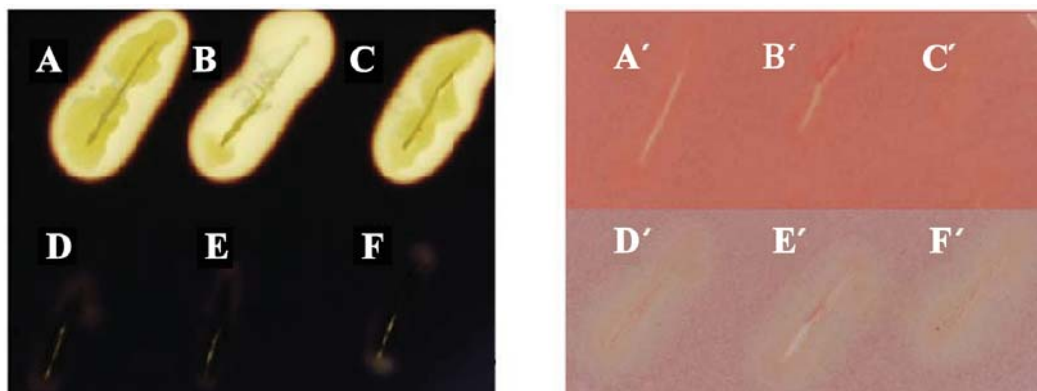


Figure 4. Production of α -amylase was detected on the starch agar (A-F), while chitinase production was tested on the chitin-Congo Red agar (A'-F'). Strains 3NA pChi1 (A), 3NA pChi2 (B), 3NA pChi3 (C), Chi7 (D), Chi8 (E) and Chi9 (F) were streaked on the agar plates. Production of clear halos around the colonies was tested after 48 h of incubation at 37°C.

Chi7, Chi8 and Chi9 showed the chitinase activity compared to strains 3NA pChi1, 3NA pChi2 and 3NA pChi3 (Figure 4 A'-F').

Given that chitinase breaks the chitin branches increasing the amount of GlcNAc as carbon source, the activity of P_{chiS} in strain Chi7 with the addition of different carbohydrates was investigated with 2 h interval up to 11 h (Figure 3D). Strain Chi7 showed identical activity with different carbon sources similar to 3NA pChi1. Overall, the β -galactosidase activity of Chi7 was 2- to 3-fold higher than 3NA pChi1. Additionally, the activities of $P_{chiS\Delta cre}$ and $P_{chiS\Delta cresig}$ from strains Chi8 and Chi9 in the same growth condition were measured. The β -galactosidase activities of both Chi8 and Chi9 in different carbohydrate sources were similar and greater than Chi7 (Figure 3D-4F). Comparison of Chi8 and Chi9 with their parental strains 3NA pChi2 and 3NA indicated that the integration of *chiS* enhanced the β -galactosidase activity in Chi9 (Figure 3 B-F). Furthermore, the β -galactosidase activity of Chi9 was slightly increased in the presence of glycerol and chitin glycerol compared to chitin with (out) glucose reaching up to 1,800 MU (Figure 3F). Overall, the β -galactosidase activity was significantly increased by changing the medium to a defined medium. Moreover, the P_{chiS} and its derivatives showed constitutive activity in the minimal medium.

4. Discussion

Since chitin is an abundant polysaccharide in nature, it can be used as a cheap carbon source in growth media. Nevertheless, the genome of *B. subtilis* 3NA which was used to analyse the promoter in this study, contains no chitinase encoding gene to secret into the surrounding environment and sense the chitin and degrade it, the chitinase gene of *B. pumilus* SG2 plus its upstream promoter was inserted into the genome of this standard *B. subtilis*. promoter. Therefore, Chitinase encoding genes of *B. pumilus* SG2 are expressed by two promoters with a *cre* site in between. The activity of this promoter region is inducible by chitin and repressible by glucose in *B. pumilus* SG2, while the promoter activity is only repressible by glucose in *B. subtilis* (20). In the present study, the activity of chitinase promoter was investigated on a low copy plasmid using *lacZ* (encodes β -galactosidase), as a reporter gene. In addition to the wild type P_{chiS} , two deletions were carried out in the *cis*-regulatory element of the P_{chiS} to test the possible effect of these deletions on the promoter activity.

Firstly, LB medium was used as the basal medium,

which resulted in a weak activity of the wild type promoter in *B. subtilis* (max. 18 MU). Deletion of the *cre* site and its flanking region increased the activity slightly although the level of *lacZ* expression was low and constitutive (approx. 70 MU). This could be due to the shortened untranslated region of the *lacZ* mRNA on pChi2 and pChi3. Similarly, shortening of the untranslated region of *lacZ* mRNA expressed by P_{milA} increased the β -galactosidase activity in the *B. subtilis* host strain (26). Replacing the LB medium with M9 medium led to weak growth of the strains (data not shown). Hence, MI minimal medium containing casamino acids was utilized as the basal media for the chitinases producing strains. In the latter medium, stronger activity of the chitinase promoter was observed. Comparison of the different growth conditions with the negative control showed that the activities of these promoters neither can be regulated nor was strong in *B. subtilis*. Besides, catabolite repression was not observed in the presence of glucose in minimal medium. Integration of the chitinase had increased the β -galactosidase activity in the presence of chitin and chitin + glucose in strain Chi7 compared with Chi1 as well as Chi3 compared with Chi9. *B. subtilis* contains no chitin degradation system (27, 28); however, it has only an *N*-acetylglucosamine utilization system (29). Therefore, the over-produced chitinase could provide further *N*-acetylglucosamine for the growth of the cells. Nevertheless, the increase of the β -galactosidase activity in the absence of chitin remains unclear. In current study, *B. subtilis* selected as a host organism for recombinant protein expression. *B. subtilis* has significant potential for production of industrial enzyme including proteases, α -amylase and lipases (30). Using *B. subtilis* as an expression system has several advantages. The genome of this species has been sequenced. In addition, *B. subtilis* classified as a GRAS organism and without production of any harmful exotoxins or endotoxins. In addition, it could naturally secret recombinant proteins into the extracellular medium, which facilitate purification stage. Despite these benefits, using *B. subtilis* has several drawbacks including lack of suitable expression vectors (31-33). Until now several expression systems have been developed in *B. subtilis*. Among these expression systems are the starch-inducible amylase promoter, xylose-inducible *xylA* promoter, P_{glv} promoter, prophage-derived heat-inducible gene expression systems and *B. subtilis* expression system involving the mannose operon (34). In addition *E. coli lac* repressor-based expression system has been developed for *B. subtilis* that could pro-

duce very high levels of recombinant proteins after induction using IPTG (30, 34, 35).

The chitinase promoter developed in this study, has potential to be used in an expression vector that could be induced by chitin. In addition, compared to the other inducers like IPTG and lactose, chitin is definitely cheaper and more available as an inducer.

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