Cloning and enhanced expression of an extracellular alkaline protease from a soil isolate of *Bacillus clausii* in *Bacillus subtilis*

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Abstract
Alkaline proteases are of industrial importance, mainly in the detergent industry. In this study, the extracellular alkaline serine protease gene, *aprE*, from *Bacillus clausii* was amplified by PCR and further cloned and expressed in *B. subtilis* WB600 using the pWB980 expression vector. Protease activity of the recombinant *B. subtilis* WB600 harboring the plasmid pWB980/aprE reached up to 1020 U/ml, approximately 3-folds higher than the native *B. clausii* strain. Characterization of the recombinant alkaline protease by SDS-PAGE and zymogram analyses indicated a molecular weight of 31 kDa. DNA sequence analysis and the deduced amino acid sequence revealed 98% homology with the extracellular alkaline serine protease from *B. clausii* KSM-K16.
Keywords**:** Alkaline protease; cloning; expression; *Bacillus clausii*; *Bacillus subtilis*

INTRODUCTION

Microbial proteases are among the most important hydrolytic enzymes which dominate the worldwide enzyme market. Among the environmental organisms, *Bacillus* species are prolific producers of extracellular proteases with a wide range of applications, particularly in the detergent, food, pharmaceutical, leather and chemical industries (Horikoshi, 1999; Ward, 1985). In the 1960s, the first detergent containing a bacterial protease (Carlsberg's subtilisin from *Bacillus licheniformis*) appeared on the market. Of the numerous microbial proteases, alkaline proteases are better suited as detergent additives because they digest proteinaceous stains, such as keratin, blood, milk, and gravy on fabrics in highly alkaline detergent solution matrices. Environmental alkalophilic *Bacilli* including *B. clausii* have been characterized and extracellular alkaline serine proteases suitable for industrial-scale production have been reported (Saeki *et al*., 2007; Horikoshi, 2006). However, there are disadvantages in using environmental isolates for large-scale industrial applications such as slow growth, requirements for high pH and complex media. Hence, gene cloning has been used in order to produce high enzyme levels for commercial purposes. A number of studies have undertaken cloning of alkaline proteases in *E. coli* (Sadeghi *et al*., 2009; Sareen *et al*., 2005; Tang *et al*., 2004). In most of these studies, expression of the cloned genes was detected in the *E. coli* hosts but not at high levels. In fact in one study, intracellular accumulation of the alkaline protease was toxic to the host cell (Tang *et al*., 2004). On the other hand, *Bacillus subtilis* has many attractive features as an expression host for foreign proteins. These features include the non-pathogenic nature of *B. subtilis*, well-established safety record, ability to secrete extracellular proteins directly to the culture medium, easy genetic manipulation, non-biased codon usage and fast growth rate. (Doi *et al*., 1986; Priest, 1977). Reports on cloning and over

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expression of alkaline protease genes from different environmental *Bacillus* sp. in *B. subtilis* have shown much higher expression levels which are more suitable for commercial enzyme production (Yang and Wang, 2007; Tang *et al*., 2004). We previously isolated and characterized an alkalophilic soil isolate of *B. clausii* capable of producing an extracellular alkaline protease (Eftekhar *et al*., 2003). This work describes cloning and expression of the extracellular alkaline protease from *B. clausii* into *B. subtilis* in order to characterize the enzyme as well as to enhance its expression.

MATERIALS AND METHODS

Bacterial strains, plasmids and media: The microorganism used as the source of alkaline protease was an indigenous soil isolate previously identified as *B. clausii* (NCBI Accession No. DQ866812). For cloning experiments, *B. subtilis* strain WB600, deficient in six extracellular proteases (Wu *et al*., 1991, NCBI Accession No. NC_006582), and pWB980 expression vector (Wu and Wong, 1999) were kindly provided by Professor Wong (University of Calgary, Canada). Luria-Bertani (LB, Sigma-Aldrich Chemicals, Germany) broth was used for bacterial growth. LB agar containing 25 µg kanamycin/ml (Merck, Germany) was used for screening of recombinant *B. subtilis* clones and screening for protease expression by individual colonies was carried out on LB agar containing 2% (w/v) skim milk. For transformation of *B. subtilis*, we modified Spizizen's medium based on the available reports in order to increase the frequency of transformation in a shorter period of time (Jamer *et al*., 2002; Stewart, 1986; Anagnostopoulos and Spizizen, 1961). The modified medium of this study contained: 15 g K_2HPO_4; 6 g KH_2PO_4; 2 g Na_2SO_4; 1 g sodium citrate; 0.2 g $MgSO_4$; 0.05 g $CaCl_2$; 0.005 g $FeCl_3$; 0.001 g $MnSO_4$, 80 mg of L-tryptophan, 0.5% glucose as the carbon source and 0.2% glutamate as the nitrogen source/l (pH, 7.5).

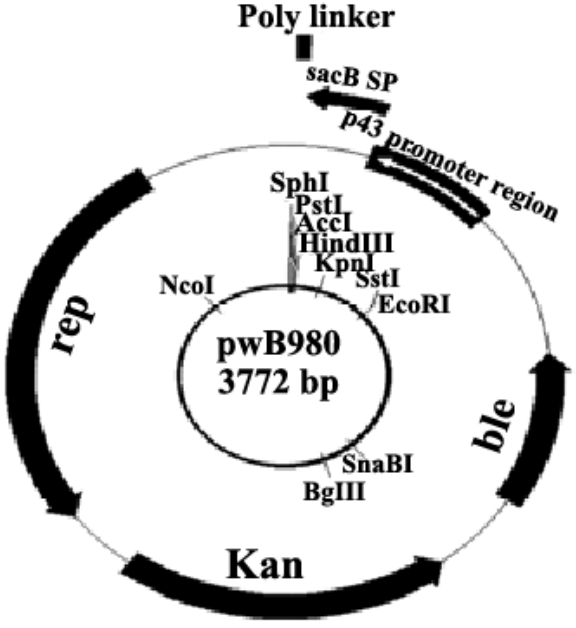

Figure 1. pWB980 Map (Wu and Wong, 1999).

DNA manipulation: *Bacillus clausii* was grown aerobically in LB broth for 18 h at 37ºC prior to chromosomal DNA extraction (Cheng and Jiang, 2006). Plasmid isolation from *Bacillus clausii* was performed according to Voskuil and Chambliss (1993). Other DNA manipulations such as enzyme digestion, ligation and agarose gel electrophoresis were carried out as outlined by Sambrook *et al*. (1989).

Primer synthesis, *PCR and construction of the expression vector*: Primers for amplification of the aprE gene were designed based on the reported sequence of the extracellular alkaline serine protease from *B. clausii* KSM-K16 (NCBI Gene Bank *GM864696.1*) and were: ArpF (5′-GGAAGCTTATGAAGAAACCGTTGG-3′), harboring a signal peptide start codon and a HindIII site, and ArpR (5′-TGTCGACTTAGCGTGTTGCCGCTTC-3′), harboring a stop codon and the SalI recognition site. The oligonucleotides were synthesized by Bioneer (Korea).

The PCR reaction mixture (25 µl) contained 10 ng genomic DNA, 10 pM of each oligonucleotide primer, 10 mM dNTP mix and 2.5 u *Pfu* DNA polymerase in PCR buffer with 20 mM $MgSO_4$ (Fermentas). Amplifications were performed using a Bioer Little Genius thermal cycler with the following thermal cycling profile: an initial denaturation at 95ºC for 4 min followed by 30 cycles of amplification (denaturation at 95ºC for 1 min, annealing at 53ºC for 45 s and extension at 72ºC for 1 min) and a final extension period of 10 min at 72ºC. PCR products were visualized by 1% (w/v) agarose gels. The PCR product was double digested by *Hind*III and *Sal*I and inserted in pWB980 (cut with the same enzymes) downstream of the p43 constitutive promoter regulated by sacB as shown in Figure 1 (Wu and Wong 1999). The recombinant plasmid (pWB980-*aprE*) was then used to transform *B. subtilis* WB600 as described below. DNA sequencing

of the *aprE* gene was carried out by Millegene (France) and homology search was sought using BlastN (http://www.ncib.nlm.nih.gov/blast).

Transformation of *B. subtilis* strain WB600: To generate competent cells, bacteria were grown in the modified Spizizen's medium of this study at 37°C with continuous shaking (200 rpm) until the culture reached an optical density of 0.4-0.6 at 600 nm (approximately 10^7 to 10^8 cfu per ml). The bacteria (0.5 ml) were then transferred into a sterile microtube and 1 µg of plasmid DNA was added. Incubation was carried out for 1 h at 37°C without shaking followed by 1 h shaking at 100 rpm before plating the cells on selective LB-agar containing kanamycin.

Screening recombinant clones for alkaline protease activity: All *B. subtilis* recombinant clones were plated on LB-agar containing 2% skim milk for 24-72 h to screen for protease activity, measured by formation of halos around individual clones due to skim milk degradation.

Quantitative measurement of alkaline protease activity in culture supernatants was carried out by the spectrophotometric assay of Fujiwara *et al*. (1993). Bacterial single colonies were inoculated into 50 ml LB broth and were incubated at 37°C with shaking (180 rpm) for 24 h. Samples were centrifuged at 8000 ×g for 10 min. The supernatants (50 µl) were then added to 450 µl of the reaction solutions containing 1% (w/v) casein in 50 mM glycine/NaOH buffer (pH, 10.0) and were incubated at 35°C for 10 min. The reaction was then stopped by addition of 500 µl TCA solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid) and the mixture was centrifuged at 13000 ×g for 15 min. Protease activity was determined by measuring the absorbance of the acid soluble fragments at 275 nm indicating the amount of tyrosine released. One unit of enzyme activity was defined as the amount of enzyme that released 1 µg tyrosine/min/ml at 35°C.

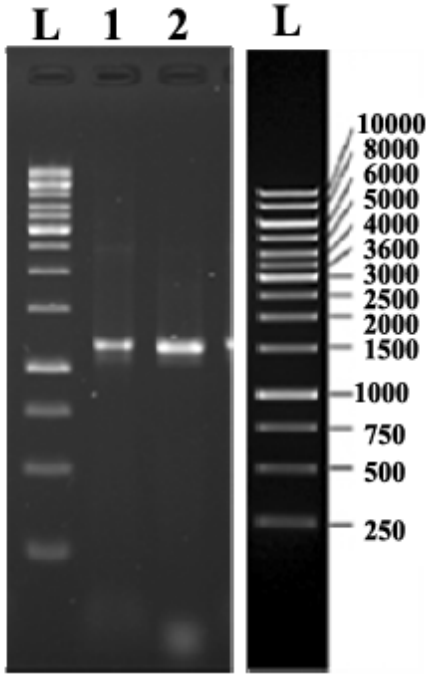

Figure 2. PCR product: lanes 1 and 2 show fragment size of amplified *aprE*, L shows 1Kbp DNA ladder.

SDS-PAGE and Zymogram: In order to characterize the cloned alkaline protease, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% acrylamide gels as described by Laemmli (1970). Zymograms were also performed in 10% (w/v) acrylamide, copolymerized with 0.1% gelatin under nondenaturing conditions and run at 100V for 4 h (Heussen and Dowdle, 1980). Following electrophoresis, the Zymogram gel was washed for 30 min in 50 mM Tris-HCl pH 8.5 containing 2.5% Triton X-100 with gentle agitation in order to remove excess SDS. The gel was then incubated overnight in the same buffer. Both gels were stained with 0.5% Coomassie blue R 250. Proteolytic activity appeared as clear bands in a blue background on the zymogram gel.

RESULTS

Construction of recombinant plasmids and cloning of the *aprE* gene: PCR amplification of the alkaline protease from *B. clausii* revealed an approximately 1100-1200 bp fragment shown in Figure 2. Subsequent cloning of the DNA fragment in *B. subtilis* WB600 resulted in formation of recombinant clones harboring the *aprE* gene. Figure 3 shows the result of plasmid extraction from a recombinant *Bacillus* clone, confirming the presence of the 1100-1200 bp fragment after double digestion by *Hind*III and *Sal*I. The DNA sequence (GenBank Accession No. HM771695) analysis of the cloned *aprE* showed 98% homology with *Bacillus clausii* KSM-K16. We also found that compared to other methods, the use of our modified Spizizen's medium resulted in a higher transformation frequency in a shorter period of time.

Expression of alkaline protease activity in *B. sub-*

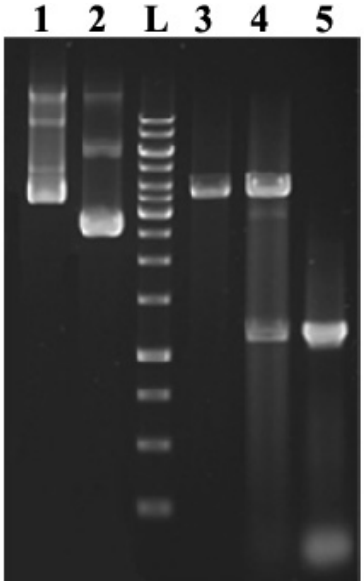

Figure 3. Lane 1, pWB980/*aprE;* 2, pWB980; L, 1Kbp DNA ladder; 3, linear pWB980; 4, pWB980-*aprE Sal*I *Hind*III digest; 5, amplified *aprE*.

***tilis* WB600:** Figure 4 shows the expression of the *aprE* gene in a *B*. *subtilis* WB600 recombinant clone on skim milk agar plates. As observed, the degradation zone around the recombinant clone was much larger than the native *B*. *clausii*. Furthermore, enzyme expression occurred after overnight incubation in *B*. *subtilis* WB600/*aprE* compared to 48-72 h under optimal conditions in the native *B*. *clausii*. Quantitative measurement of the alkaline protease activity showed a value of 1020 EU/ml (specific activity of 204) in *B*. *subtilis* WB600/*aprE* compared to 347 EU/ml (specific activity of 69) in the native *B*. *clausii*. Alkaline protease production in the recombinant clone occurred at

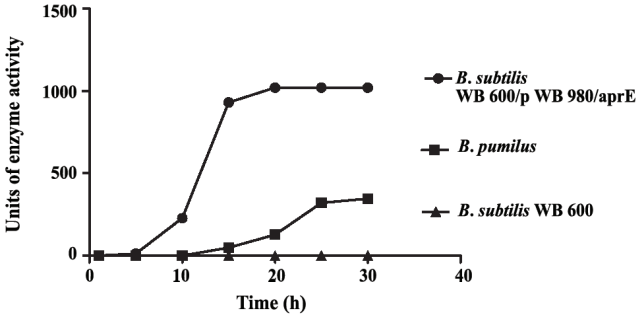

Figure 5. Comparison of alkaline protease production (activity) in culture supernatants of the native *B. pumilus* (squares), *B. subtilis* WB600 (triangles) and *B. subtilis* WB600/pWB980/aprE (circles), grown at 37ºC and shaken at 200 rpm for up to 30 h. The enzyme assays were performed in triplicate and the means ± SD are shown.

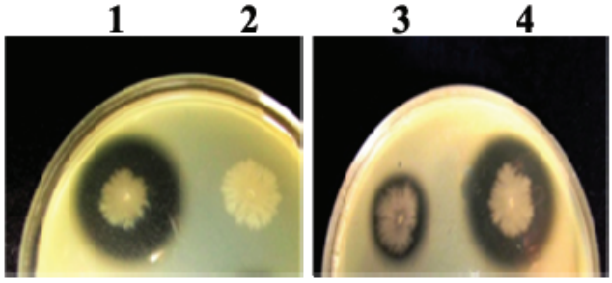

Figure 4. Protease expression on 2% skim milk LB agar at 37ºC. 1 and 4, *B. subtilis* WB600 harboring pWB980/*aprE*; 2, WB600/pWB980; 3, *B. clausii*.

pH, 7.5 compared to the native host where the enzyme was produced within the pH range of 7-11. The temperature for protease production and activity of the recombinant enzyme was identical to that in the native host (37ºC). In addition, under our experimental conditions, enzyme production started after 5 h in the culture supernatants of the recombinant clone compared to the native *B*. *clausii*, where no enzyme activity was shown before 15 h of growth. There was also no enzyme activity in *B*. *subtilis* WB600 culture supernatants (Figure 5). The results not only show the expression of the *aprE* gene in the new host but also an enhancement of almost 3 fold compared to the native host.

Characterization of the cloned *aprE* by SDS-PAGE: SDS-PAGE protein profile of the supernatants of *B*. *clausii*, *B*. *subtilis* WB600 and *B*. *subtilis* WB600/pWB980-*aprE* are shown in Figure 6A. Figure 6B shows the biological activity of the enzyme shown by SDS-PAGE zymogram. As observed, an approximately 31 kDa protein band showed enzyme activity as the product of the cloned gene, corresponding with the prediction made from the gene sequence. The amino acid sequence of the cloned protein showed 98% homology with the extracellular alkaline serine protease from *B*. *clausii* KSM-K16.

DISCUSSION

Environmental isolates of *Bacilllus* sp. are good sources of extracellular hydrolytic enzymes (Priest, 1977). We previously isolated and characterized an alkalophilic soil isolate of *B*. *clausii* capable of producing an extracellular alkaline protease (Eftekhar *et al*., 2003). The organism needed defined alkaline

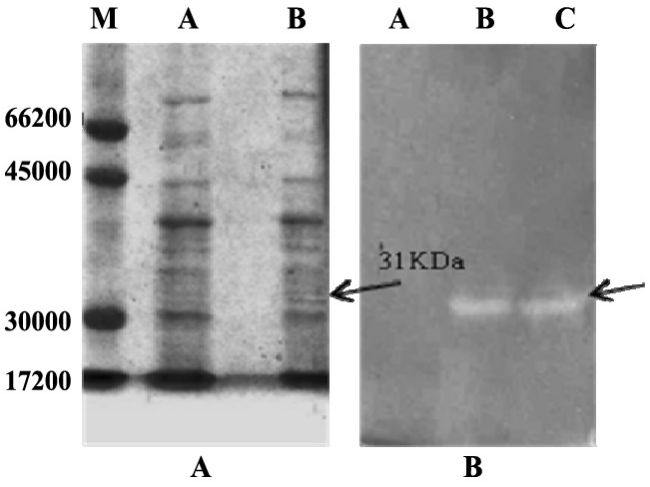

Figure 6. A: SDS-PAGE profile of extracellular proteins from *B. subtilis* and the recombinant clone. M, protein size marker; A, supernatant from *B. subtilis* WB600; B, supernatant from *B. subtilis* WB600/pWB980/aprE; B: SDS-PAGE Zymogram of extracellular proteins from: A, *B. subtilis* WB600; B, *B. subtilis* WB600/pWB980/apr E; C, *B. clausii*.

media in order to produce and excrete high levels of alkaline protease, usually up to 72 h after incubation. *B. subtilis*, another environmental organism, has the advantage of excreting not only high levels of extracellular enzymes but also efficiently express and export foreign proteins directly into the culture medium (Doi *et al*., 1986; Priest, 1977).

Several proteases have been cloned mostly in *E. coli* and a few in *B. subtilis*. Sareen *et al*. (2005), cloned and expressed an alkaline protease from *B. licheniformis* into *E. coli* and predicted a molecular weight of 55 kDa for the mature protein. Sadeghi *et al*. (2009) also cloned an alkaline protease from *B. subtilis* strain 168 in *E. coli* in order to characterize the enzyme but did not measure enzyme expression in the recombinant host. Tang *et al*. (2004) cloned an alkaline protease gene from *B. licheniformis* strain 2709 into *B. subtilis* WB600 and showed 65% enhancement of enzyme expression in the recombinant host compared to the original strain and a molecular weight of 30.5 kDa. Yang and Wong (2007) also cloned an alkaline protease from a *B. pumilus* strain in *B. subtilis* and demonstrated over 6 fold enhanced expression in the recombinant strain. We cloned and expressed the *aprE* gene from *B. clausii* in *B. subtilis* WB600, deficient in 6 proteases using the expression vector pWB980. Our results showed a 3 fold higher expression of the cloned *aprE* gene compared to its natural host before any optimization studies. The molecular weight of the protease recovered from the recombinant strain was 31 kDa, similar to the enzyme obtained from the *B. clausii* parent strain. The expression vector pWB980 allows for better expression of the inserted gene (s) under the constitutively expressed promoter P43, and the *sacB* regulatory gene (Wu and Wong, 1999). On the other hand, protease production in *B. clausii* was inducible and occurred in the presence of casein. This would explain the reason for the rapid detection of protease expression in the recombinant clone compared to the native host (5 h vs. 15 h). Following the same trend, maximum protease production occurred after 18-24 h in *B. subtilis* WB600/aprE compared to 72 h in the native strain. Another advantage of using the expression vector pWB980 is that it carries a *Hind*III site which could be used in combination with other unique restriction sites for subcloning DNA fragments in the desired orientation (Wu and Wong, 1999). Optimization studies will be needed to enhance the alkaline protease expression for future industrial purposes. Despite the number of alkaline proteases which have been introduced, development of recombinant organisms which can over express the enzyme is useful for the expanding industrial needs in different communities.

Acknowledgements

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