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Full Length Research Paper

High-level expression of alkaline protease using recombinant *Bacillus amyloliquefaciens*

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***Bacillus licheniformis* CICIM B5102 was used for the commercial production of alkaline protease. The full-length gene *apr* encoding the alkaline protease was amplified *via* polymerase chain reaction (PCR) using the genomic DNA of *B. licheniformis* CICIM B5102 as a template. The *apr* gene was cloned into plasmid pUB110, resulting in the recombinant plasmid pUB-*apr*, which was then transformed into *Bacillus amyloliquefaciens* CICIM B4803. The protease productivity was significantly improved in the transformants of *B. amyloliquefaciens* CICIM B4803. A transformant with high alkaline protease productivity was selected, and the alkaline protease productivity of the strain increased by 46% when compared with that of wild-type *B. licheniformis* CICIM B5102.**

Key word: Alkaline protease, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*.

INTRODUCTION

Proteases are one of the most important industrial enzyme groups, accounting for approximately 60% of the total enzyme sales (Beg et al., 2003). Alkaline protease accounts for more than 50% of the total protease enzyme sales in various industries, including the detergent, food, leather, diagnostic, waste management, daily cosmetic, photographic and silver recovery industries (Gupta et al., 2002). It is widely present in bacteria, actinomycetes and fungi. However, almost all alkaline proteases used by detergent and leather industries are produced by *Bacillus* strains. *Bacillus licheniformis* CICIM B5102 is used in the commercial production of alkaline protease in China, the products of which are mainly used in the leather industry. Alkaline protease treatment is more environment friendly than the traditional chemical pretreatment of leather.

The gene encoding alkaline protease from *B. licheniformis* has been cloned and successfully expressed in *Escherichia coli* and *Bacillus subtilis* expressed in *Escherichia coli* and *Bacillus subtilis* (Jacobs et al., 1985). In the current study, the gene encoding alkaline protease was cloned from *B. licheniformis* 2709 to improve the productivity of alkaline protease, and recombinant *B. subtilis* was constructed to express alkaline protease (Tang et al., 2004). Although, the productivity of the recombinant *B. subtilis* has improved considerably, this strain has not been used in commercial production because it grows rather slowly in industrial media (data unpublished). In contrast, *B. licheniformis* CICIM B5102 grows quickly in industrial media and its alkaline protease productivity is high. Increasing the gene copy number is an effective method for improving enzyme productivity (Jorgensen et al., 2000). However, the transformation method necessary for this process has not yet been developed for *B. licheniformis* CICIM B5102. *Bacillus amyloliquefaciens* CICIM B4803 is used in the production of α-amylase in China. This strain grows quickly in various media of cheap industrial raw materials, making it an ideal host for heterologous protein expression. The present study aimed to improve alkaline protease production by constructing a recombinant strain

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of *B. amyloliquefaciens* CICIM B4803.

MATERIALS AND METHODS

Bacterial strains and media

B. licheniformis CICIM B5102 and *B. amyloliquefaciens* CICIM B4803 were obtained from the Culture and Information Center of Industrial Microorganisms of China Universities (http://cicim-cu.sytu.edu.cn). In the present study, *B. licheniformis* CICIM B5102 was used as a source of genomic DNA for the amplification of the gene (*apr*) encoding the alkaline protease. *B. amyloliquefaciens* CICIM B4803 was used as the host for the over-expression of alkaline protease. *B. subtilis* WB600 that was deficient in six extracellular proteases was provided by Prof. Wong (Wu et al., 1991). Plasmid pUB110 was provided by the Bacillus Genetic Stock Center (www.bgsc.org). The bacterial strains were routinely cultured at 30°C in lysogeny broth (LB) medium (1% NaCl, 1% tryptone, and 0.5% yeast extract). The transformants were selected in LB medium supplemented with 20 µg/ml kanamycin. The fermentation medium was composed of 0.5% yeast extract, 1% citric acid, 0.33% sodium citrate, 0.26% $CaCl_2$, 3% glucose and 0.26% K_2HPO_4. The casein plate was composed of 1% casein, 0.5% yeast extract, 0.2% NaCl and 1% agar (pH 7.0).

DNA manipulation

DNA manipulation was conducted according to standard protocols (Sambrook et al., 1989). The genomic DNA of *B. licheniformis* CICIM B5102 was extracted and purified according to standard methods described by Dubnau et al. (1982). *B. amyloliquefaciens* CICIM B4803 was electro-transformed according to the method described by Vehmaanpera (1989). The nucleotide sequence was determined using a Bigdye Terminator cycle-sequencing kit for ABI 3200 PRISM (Applied Biosystems).

Construction of recombinant plasmid and strain

Two primers, hwj01: 5'-AATTACCGagatct-GCATCAGGAAAAAGCCGCTG-3' and Phwj02: 5'-AATTACCGagatctAATACGCCTTTCACATGAGCTG-3', were designed based on the nucleotide sequence of the subtilisin protease gene of *B. licheniformis* ATCC 14580 (NCBI accession no. CP000002). An additional restriction site of *Bgl*II (lowercase sequence) was incorporated into either primer to facilitate cloning. Polymerase chain reaction (PCR) was performed in a 50 µL reaction volume containing about 2 µL genomic DNA (20 ng/µL), 1 µL forward primer, 1 µL reverse primer, 21 µL H_2O and 25 µL Premix TaqTM (Ex TaqTM version) (TaKaRa). The thermal cycler was programmed with an initial step of 5 min at 94°C, and the amplification reaction was conducted at 30 cycles of 30 s at 94°C for denaturation, an annealing step of 1 min at 56°C, an extension step of 90 s at 72°C, and finally, a 10 min extension at 72°C. A 1.8 kb product was obtained. This product was digested with *Bgl*II and ligated with *Bam*HI-treated pUB110. The constructed plasmid was designated as pUB-*apr*.

Two primers, Pub01: 5'- ATGAGTTGCTAGTAACATCTG -3' and Pub02: 5'-TTGCTTGCCATATCGTTCG -3', were designed based on the nucleotide sequence around the *Bam*HI site of the pUB110 (NCBI accession no. M37273). The thermal cycler was programmed with an initial step of 10 min at 96°C, and the amplification reaction was conducted at 25 cycles of 30 s at 94°C for denaturation, an annealing step of 1 min at 52°C, an extension step of 90 s at 72°C, and finally, a 10 min extension at 72°C. The plasmid pUB-*apr* was transformed into *B. amyloliquefaciens* CICIM B4803 via electroporation using the method described by Vehmaanpera (1989). The transformants were screened on an LB plate supplemented with 20 µg/ml kanamycin.

Shaker flask cultivation of *B. amyloliquefaciens*

A colony of the *B. amyloliquefaciens* CICIM B4803 transformant was inoculated in 30 mL of the LB medium containing 20 µg/mL kanamycin and grown for 12 h as a seed culture. Subsequently, 5 mL of the seed culture was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of the fermentation medium and then aerobically cultivated at 34°C and 200 rpm for 120 h.

Enzyme activity assay

The fermentation medium was centrifuged at 8,000 rpm for 10 min. The supernatant was used to determine alkaline protease production, and the alkaline protease activity was assayed using the method described by Afify et al. (2009), with casein as the substrate. One unit of protease activity was defined as the amount of enzyme that liberates 1 µg of tyrosine per min under specific reaction conditions. The culture supernatant of *B. subtilis* WB/pUB-*apr* was used as the crude enzyme for characterization of the cloned enzyme. The optimal pH for the recombinant enzyme was determined at 50°C and 50 mM Tris-HCl (pH 6.5 to 11.5). The optimal temperature was determined at pH 9.5 and temperatures ranging from 35 to 70°C. The culture supernatants of *B. subtilis* WB/pUB-*apr* and *B. subtilis* WB600 were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8% polyacrylamide), with PageRuler™ Prestained Protein Ladder (Fermentas) as the standard.

RESULTS AND DISCUSSION

Construction of recombinant plasmid and characterization of the cloned enzyme

The *apr* gene was obtained via PCR using the genomic DNA of *B. licheniformis* CICIM B5102 as a template. A 1.8 kb fragment of the *apr* gene was obtained (Figure 1) and then sequenced on both strands, revealing a 1,800 bp fragment. The sequence of this fragment was deposited in Genbank under accession number JQ285995. Sequence analysis of the 1,800 bp fragment revealed the existence of a 1,140 bp open reading frame (ORF), encoding a polypeptide of 379 amino acids. Approximately 406 bp of the upstream fragment and 254 bp of the downstream fragment were also included in the 1,800 bp fragment. The amplified fragment was compared with other protease encoding genes using the Blast program to search the NCBI database. The sequence of the amplified fragment showed the highest homology with a previously published alkaline protease encoding gene from *B. licheniformis* ATCC14580 (NCBI accession no. CP000002). The 61st base pair upstream from the start codon featured a substitution of A to G when compared with the *apr* gene from *B. licheniformis* ATCC14580. Another substitution of T to C occurred at the 556th base pair, showing a synonymous mutation of codons CTG to

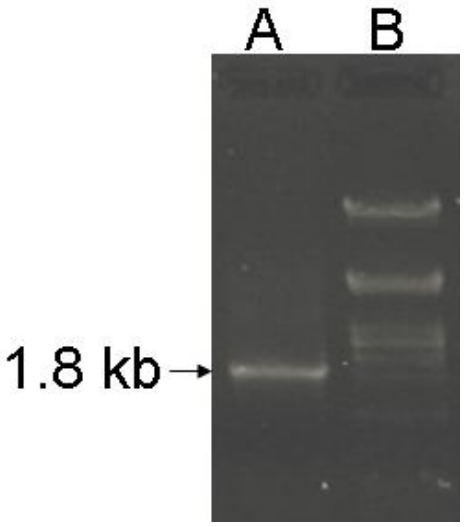

Figure 1. PCR amplification of the *apr* gene encoding alkaline protease from *B. licheniformis* CICIM B5102. A, PCR product of *apr* gene; B, Lamda-DNA/PstI. PCR, Polymerase chain reaction.

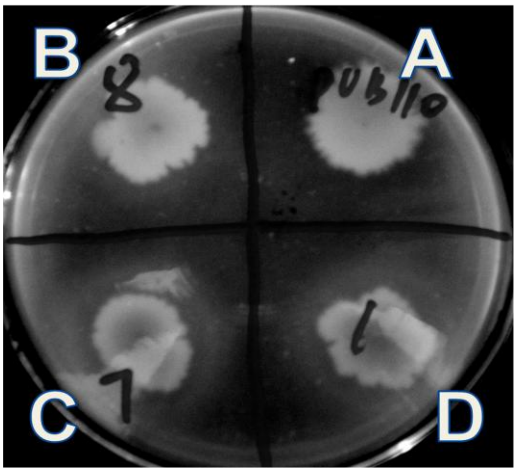

Figure 2. Assay of the *B. subtilis* WB/pUB-*apr* on a casein plate. (A) *B. subtilis* WB600 containing pUB110 plasmids as a control. (B), (C) and (D) show the three colonies of *B. subtilis* WB/pUB-*apr* containing the recombinant plasmid pUB110-*apr*. Colonies of *B. subtilis* WB/pUB-*apr* produced alkaline protease, which degraded the casein on the plate; a clear halo developed around the colonies.

TTG. The fragment was digested with *Bgl*II and then ligated with the *Bam*HI-treated pUB110. The ligation mixture was treated with *Bam*HI prior to transformation to eradicate circular pUB110. The mixture was transformed into *B. subtilis* WB600, a protease-deficient strain. Three transformants were obtained after several transformations. The transformant plasmids were then sequenced

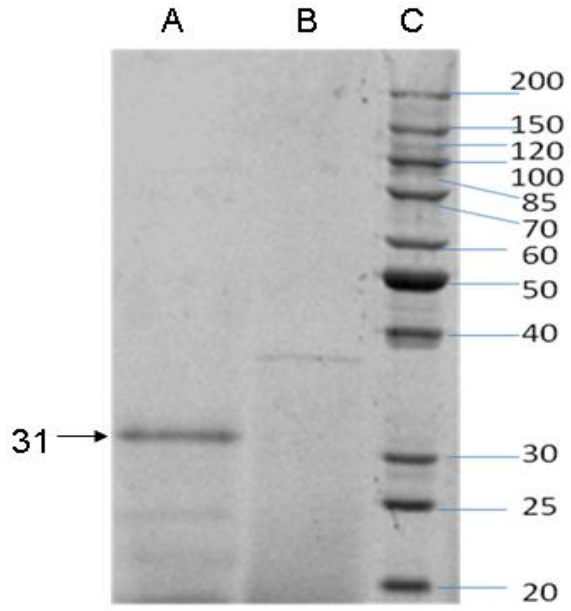

Figure 3. SDS-PAGE analysis of the extracellular protein of recombinant strain *B. subtilis* WB/pUB-*apr*. A, Supernatant of *B. subtilis* WB/pUB-*apr*; B, supernatant of *B. subtilis* WB600; C, protein weight marker (kDa). SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

using Pub01 and Pub02 as primers. Sequence analysis showed that one of the transformants, designated as T02, contained the recombinant plasmid, with the full-length gene *apr* inserted in the *Bam*HI site of pUB110. The recombinant plasmid was designated as pUB-*apr*, and the T02 transformant was designated as *B. subtilis* WB/pUB-*apr*. The *B. subtilis* WB/pUB-*apr* colonies on the casein plate developed a clear halo, while the control protease-deficient strain *B. subtilis* WB600 did not (Figure 2). SDS-PAGE electrophoresis showed that a novel polypeptide with a molecular mass of about 31 kDa was expressed in *B. subtilis* WB/pUB-*apr* (Figure 3). Jacobs et al. (1985) reported that the total protein encoded by *apr* gene of *B. licheniformis* consists of 379 amino acids and includes a 29-residue signal peptide followed by a 76-residue pro-region. The mature peptide of the alkaline protease of *B. licheniformis* comprised of 274 residues with calculated molecular weights of 27.5 kDa. The apparent molecular weight of the recombinant alkaline protease was larger than its calculated molecular weight, which is supported by the findings of previous reports (Wells et al., 1983), indicating that the alkaline proteases from *B. amyloliquefaciens*, *B. subtilis*, and *B. licheniformis* produce similar anomalously slow mobilities on SDS gel.

B. subtilis WB/pUB-*apr* was aerobically cultured in the fermentation media at 30°C. The protease activity was 536 U/ml after 72 h. As a control, the culture supernatant of *B. subtilis* WB600 showed no protease activity. The

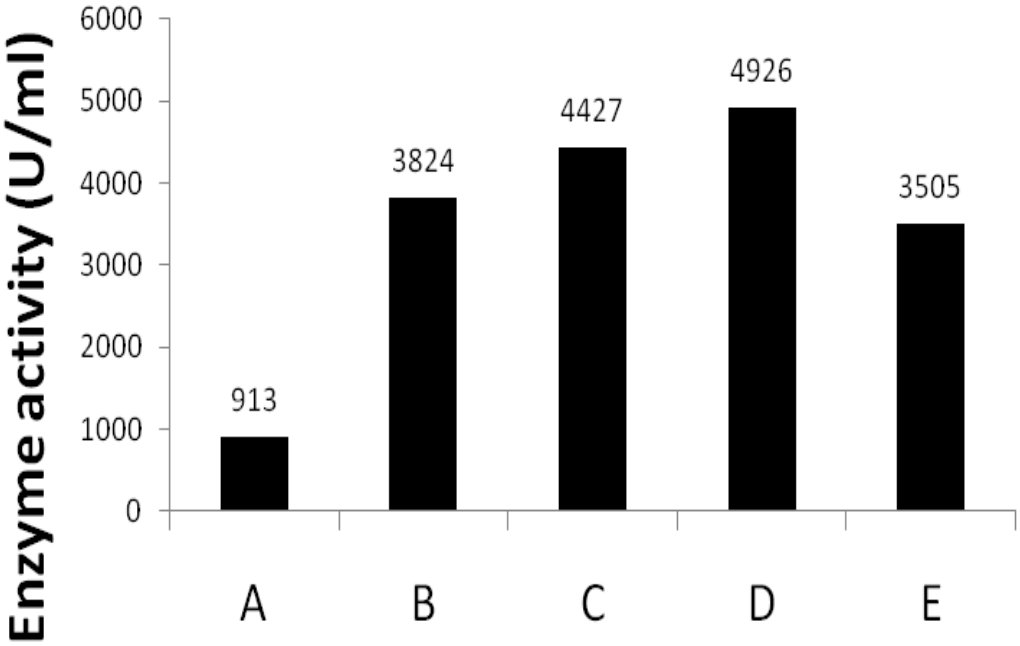

Figure 4. Alkaline protease production in the shaker flask. Column A, *B. amyloliquefaciens* CICIM B4803; Columns B, C and D, different transformants of *B. amyloliquefaciens*/pUB110-*apr*; Column E, *B. licheniformis* CICIM B5102.

maximum protease activity was observed at pH 9.5 and 50°C. The protease exhibited more than 80% of its maximum activity at 40 to 60°C. The thermal stability profile of the protease showed that the enzyme was stable at 40 and 50°C. The enzyme retained more than 90.2 and 88.3% of its initial activity after 2 h of incubation at40 and 50°C, respectively. The observed characteristics of the cloned enzyme were similar to those of the alkaline protease from *B. licheniformis* reported by Sellami-Kamoun et al. (2008) and Hadj-Alia et al. (2007).

Construction of the recombinant *B. amyloliquefacien* and improvement of alkaline protease productivity

The recombinant plasmid pUB110-*apr* was extracted from *B. subtilis* WB/pUB-*apr* and transformed into *B. amyloliquefaciens* CICIM B4803 via electroporation. The transformants were screened on an LB agar plate containing 20 μg/mL kanamycin. Three *B. amyloliquefaciens* transformants, designated as *B. amyloliquefaciens*/pUB-*apr*, were randomly selected, confirmed via PCR, and then further tested using shaker flask fermentation. The protease activities produced by the three recombinants differed from one another, as shown in Figure 4. The experiment was repeated to ensure the credibility of the results. The obtained results confirm differences in the productivities of the transformants. Sequence analysis revealed that the *apr* gene was the same in each transformant. Since the pUB-*apr* is an autonomously replicating plasmid, we deduced that the different levels of protease productivity observed may be attributed to the different damages that occurred during the electrotransformation process. A maximum yield of 4,926 U/ml was obtained from strain D, five-fold higher than that obtained from the original wild-type strain A. The increase in productivity of the alkaline protease by the transformants may be attributed to the donor plasmid harboring the *apr* gene. In this context, previous reports were in support of these findings (Jorgensen et al., 2000; Sabir et al., 2009), indicating that the increase in gene copy number enhances the enzymatic production. The maximum crude enzyme activity of alkaline protease in *B. licheniformis* CICIM B5102 was 3,505 U/ml after 96 h, whereas that of the recombinant *B. amyloliquefaciens* strain D was 4,926 U/ml after 60 h. More transformants were selected for the shaker flask fermentation test to obtain higher protease production. In approximately 200 transformants, one strain, designated as strain 121, exhibited the highest protease production, and thus it was selected for further characterization. The maximum protease activity of strain 121 was 5122 U/ml after 60 h. Under the same conditions, *B. licheniformis* CICIM B5102 had a maximum activity of 3,505 U/ml after 96 h. Therefore, it can be concluded that the enzyme productivity of *B. amyloliquefaciens* transformant strain 121 increased by 46% and its enzyme production time decreased by 37% when compared with those of *B. licheniformis* CICIM

B5102.

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