Iran. J. Biotechnol. October 2024;22(4): e3840 DOI: 10.30498/ijb.2024.427242.3840

Research Article

Simultaneous Expression of Recombinant Cellulase and Protease in An Indigenous *Bacillus Cereus* Strain

Zainab Farhangi [2], Farideh Tabatabaee Yazdi [2], Bagher Yakhchali [1*]**

[1]Institute of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, I. R. Iran.
[2]Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.

[*]*Corresponding author*: Bagher Yakhchali Institute of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, I. R. Iran. Tel: +98-2144787301,Fax:+98-2144787399, E-mail: bahar@nigeb.ac.ir

[**]*Co-Corresponding author*: Farideh Tabatabaee Yazdi, Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran. Tel: +98-5138805764, Fax: +98-5138787430, E-mail: tabatabai@um.ac.ir

Received: 2024/06/06 ; **Accepted:** 2024/10/29

Background: Gene manipulation has a wide array of applications in microorganisms. We can construct multifunctional bacterial strains by gene manipulation and gene editing in order to produce several industrial biomaterials including enzymes at the same time.

Objective: According to the importance of cellulase in various industries, including food industry, the purpose of this study was aimed to produce cellulase in an indigenous *Bacillus cereus* EG296 strain through gene manipulation.

Materials and Methods: The *Bacillus subtilis 168* cellulase gene, located between the regulatory upstream and downstream regions of *Bacillus cereus* protease gene (*aprE)*, was amplified by SOEing PCR and transformed into the *Bacillus cereus* EG296 by natural transformation. After selection of the strains with cellulase activity, the *scoC* gene (Negative transcriptional regulator of *aprE* gene) was also deleted from the genome of the transformant by homologous recombination in order to increase the cellulase and protease activities simultaneously.

Results: The *Bacillus cereus* cells were acquired the cellulase gene into their genome with cellulase activity of about 0.61 $u.mL^{-1}$. By *scoC* gene deletion, the protease activity reached to 363.14 $u.mL^{-1}$ from 230 $u.mL^{-1}$. Meanwhile, the cellulase activity under the control of the protease promoter was also increased to 0.78 $u.mL^{-1}$ from 0.61 $u.mL^{-1}$. The cellulase and protease expressed in *B. cereus* have an instability index of 26.16 and 20.18 respectively, which is much lower than threshold of 40. Accordingly, it can be concluded that both enzymes are considered to be stable.

Conclusion: As a result, we obtained a genetically engineered strain that had the ability to produce and secrete two important industrial extracellular enzymes (cellulase and protease), with easy downstream purification processes.

Keywords: Bacillus cereus, Cellulase, Homologous recombination, Heterologous expression, Metabolic engineering, Protease

1. Background

Enzymes are proteins with high catalytic functions which are responsible for many biochemical reactions in microorganisms, plants, animals and human. Enzymes with broad applicability have a significant market demand and are used as alternatives for chemicals in industrial tasks and prevents the release of approximately 700 million kg/year CO_2 into the atmosphere (1). Microbial industrial enzymes such as protease, amylase, lipase and cellulase have received more attention owing to

their catalytic activity, nontoxicity, eco-friendly nature, stability, cost-effectiveness and easy production (1,2).

Cellulase complex enzymes are the third most important enzymes for industrial uses, which have been commercially available for more than 30 years (3). Cellulases cleave β-1,4-glucosidic bonds in cellulose, the most abundant natural bio-resource (4). Cellulase has many applications in various industries including biofuels, food, animal feed, poultry and aquaculture, pharmaceuticals, agriculture, textiles, paper and waste treatment (3,5). Besides cellulases, proteases are also significant industrial enzymes which have a broad spectrum of applications such as food processing, pharmaceuticals, detergents, leather industry, etc. Most commercial proteases, mainly neutral and alkaline, are produced by microorganisms belonging to the *Bacillus* genus (6).

Bacillus species are a group of gram-positive bacteria that have a recognized history of safe use in foods. Recently, *Bacillus* species have received increased attention for their use in biotechnology as a valuable host for production of heterologous proteins, valuable enzymes, vitamins and antibiotics with respect to nontoxicity, convenience for gene manipulation and high yield of target proteins (7–10).

Strain development based on metabolic engineering is of central importance for biotechnological production processes and identifying the individual Regulatory Factors (RF) that control gene expression has gained reasonable attention (11,12).

In this study, the recombinant cellulase expression along with the production of native protease of the host strain was followed by manipulation of the metabolic pathway of the protease production, through deletion of its negative transcriptional control factor named *scoC* gene, in order to increase the production of both enzymes.

The *scoC* gene, also known as *hpr*, is one of the multiple regulators of *Bacillus* family which directly and indirectly affects the expression of approximately 560 genes in *Bacillus subtilis* (13). The homologue of *scoC* was firstly identified in a study of *Bacillus* mutants and found to be effective in alkaline and neutral protease expression (13). Furthermore, it identifies and binds to specific sequences in the upstream regions of the *aprE* (alkaline protease) and *nprE* (neutral protease) genes and represses them. *Bacillus cereus* has several extracellular protease genes, and the *aprE* is the major protease gene (14). The *aprE* gene is directly repressed by AbrB, scoC, and SinR and is activated by phosphorylated DegU and also indirectly regulated by other proteins, including Spo0A, AbbA, SalA, TnrA, SinI, DegS, DegQ, DegR and RapG (15–17).

2. Objectives

Due to the need of cellulase and protease for application in various industries, the purpose of this study was to produce cellulase and protease simultaneously in an indigenous *Bacillus cereus* strain. In the present study, cellulase gene from *Bacillus subtilis* 168 was introduced into the genome of a protease producing *Bacillus cereus* by natural transformation and recombination. In addition, the *scoC* gene regulator was deleted from the genome of the bacterium by natural transformation and homologous recombination to increase the cellulase and protease production.

3. Materials and Methods

3.1. Strains, Plasmid and Culture Media

Escherichia coli DH5α (Invitrogen) was used as an intermediate host for cloning, *Bacillus subtilis* strain 168 was used as a cellulase gene source and indigenous native *B. cereus* strain EG296 was used as host for production of cellulase and protease (18). The pGEM-5Zf vector (Promega) was utilized as a cloning vector. Luria–Bertani (LB) medium was used to grow *E. coli* at 37 °C and LB agar containing 100 mg. mL^{-1} Ampicillin, 0.5 mM, IPTG and 20 $\mu g.\mu L^{-1}$ X-Gal were used to screen the recombinant bacterial colonies. The LB agar containing 1% skim milk was used for screening colonies with protease activity by detecting clear zone around the colony and LB agar containing 0.2% Carboxymethyl Cellulose (CMC) was used for screening and detecting colonies with cellulolytic activity. The *Bacillus* strains were cultivated in a basal broth medium containing ($g.L^{-1}$) glucose (1), peptone (0.5), KH_2PO_4 (0.1), K_2HPO_4 (0.3), $MgSO_4$ (0.02), yeast extract (0.5) for protease production and a LB medium containing 0.05% CMC for cellulase production.

3.2. Gene Amplification and Cloning

Extraction and purification of *B. cereus* and *Bacillus subtilis* genomic DNA were performed using a DNA purification kit (Thermo Fisher kit). The

upstream and downstream regions of *scoC* gene were amplified by Pfu DNA polymerase using *B. cereus* EG296 genomic DNA as template and scoCF1 (AGCAGTTGCTGGACTAGC), scoCR1 (GATATGCCGTGTTAAGGTC), scoCF2 (GACCTT AACACGGCATATCTTTACTTTTCGCA ATCATGC) and scoCR2 (TGTTAGAAGATTA GAAC AAGC) primers respectively. The purified amplicons were fused together by SOEing PCR and amplified with scoCF1 and scoCR2 primers to produce the *ΔscoC* (*scoC* gene without coding sequence) fragment and confirmed by Electrophoresis on 1% agarose gel (19). The *ΔscoC* pure amplicon was ligated to the *Eco*RV linearized pGEM-5Zf vector and transformed into the *E. coli* competent cells by the heat shock method (20). The transformants were screened by blue and white colonies and recombinant plasmids were confirmed by colony PCR using gene-specific primers (scoCF1 and scoCR2) and DNA sequencing. The resulting plasmid was assigned as Pgem5:ΔscoC and was digested with the *Sca1* restriction enzyme (BioLabs Company) for transformation into *B. cereus*.

The coding region of the cellulase gene, which consisted of 1500 nucleotides encoding a polypeptide with an expected molecular weight of 55 kDa, instability index of 26.6 and aliphatic index of 73.91 was amplified from *B. subtilis* strain 168 using kerCelF (GAAAAGGGAGGAAAAATCTT ATGAAACGGTCAATCTCT) and celKerDwR (GATTAAAATTTCACTTTATTTCCTAATTTGG TTCTGTTCC) primers in 44 °C annealing temperature. The promotor sequence of *aprE* which was located at the upstream region of the cellulase, was specified by the www.softberry.com website. The 282 bp upstream region of the protease gene, consisting of its promoter region was amplified by TOUCH-UP Gradient Amplification Method PCR with annealing temperature of 50 °C in the first 10 cycles and 52 °C in the further 20 cycles using BcKerF (ATCGCTTTTTACAAGCAGAG) *and* KerUpR (AAGATTTTTCCTCCCTTTTG) primers. After the purification of the above mentioned amplicons, they were attached by SOeing PCR using BcKerF and celKerDwR primers to produce a 1782bp DNA fragment of *proUp:cell*. The 183 bp downstream region of the protease was also amplified using KerDwF (GAAATAAAGTGAAATTTTAATCTAAA) and BcKerR (CGATGATTCGTCAACTTGG) primers with annealing temperature of 50 °C. Finally, the *proUp:cell* cassette was ligated to *proDwn* gene through TOUCH-UP SOEing PCR in 47 and 52 °C annealing temperatures and a 1965 bp cassette of *proUp:cell::proDwn* was constructed. All of the amplicons were checked on 1% agarose gel (20).

3.3. Natural Transformation of proUp:Cell:ProDwn Cassette into B. Cereus

The *B. cereus* competent cells were prepared as following with some self-modifications in compare to the reference: 1 mL of overnight culture in LB broth was inoculated into 50 mL of LB broth and incubated at 37 °C and 100 x g for 3h; cells from 30 mL of the culture were harvested by centrifugation at 8000 x g and 4 °C for 5 min. The cells were suspended in 5 mL TF1 (0.1 mL tryptone 0.5%, 1 mL glucose 5%+ $MgSO_4.7H_2O$ 0.2%, 1 mL Spizizen's Salts 10X, 0.1 mL yeast 2% and 8 mL ddH_2O) and incubated at 37 °C and 100 x g for 5 h till the OD600 reached to 2.5. Accordingly, 500 µL of the culture was added to 5 mL TF2 (0.009 mL tryptone 0.5%, 0.9 mL glucose 5%+ $MgSO_4.7H_2O$ 0.2%, 0.9 mL Spizizen's Salts 10X, 0.045 mL yeast 2% and 8 mL ddH_2O) and incubated at 37 °C and 100 x g for 3 h. In this stage, the bacterial cells are naturally susceptible for DNA uptake. 10µL of DNA (about 1 µg with an A 260/280 absorbance of 1.79) was added to 50 µL competent cells and incubated at 37 °C and 180 x g in a shaker incubator for 2 h. After the incubation time, the cells from 10^{-7} and 10^{-8} dilutions were plated on selective LB-agar plates (21).

3.4. Detection of B. Cereus Transformants

The colonies on LB-agar plates were picked up and dotted on CMC agar medium to identify cellulase expressing bacteria. The plates were incubated at 37 °C for 48 h. Thereupon, the plates flooded with 0.1% Congo red for 20 min and then with 1 M NaCl for 15 to 20 min. After incubation, some colonies showed distinct, clear, and prominent zones of clearance indicating cellulase activity. After screening the colonies based on cellulase clear zones, genomic DNA was extracted and the presence of *proUp:cell:proDwn* cassette was confirmed by PCR.

3.5. Deletion of ScoC

10 µL of pure linearized *PGEM5:ΔscoC* (about 1 µg with an A 260/280 absorbance of 1.83) was added to 50

µL of *B. cereus:cellulase* competent cells and incubated at 37 °C and 180 x g in a shaker incubator for 2 h. The cells were diluted to 10^{-7} and 10^{-8} with sterile water and dispensed on LB agar plate and incubated at 37 °C overnight.

Single colonies grown on LB agar plates were transferred to Skim Milk agar plates and incubated at 37 °C for 42 h. Colonies with a larger protease clear zone in comparison to the control were selected for further evaluation. DNA from selected colonies was extracted and PCR reaction was performed by scoCF1 and scoCR2 primers.

3.6. Cellulase Activity

Quantitative analysis of recombinant cellulase activity was performed by DNSA (Dinitro salicylic Acid) assay through measurement of the amount of reducing sugars released during cellulose hydrolysis (22). A standard glucose curve was plotted based on the absorption of different concentrations of glucose at 540 nm. The strain was inoculated in liquid LB medium containing 0.05% CMC and incubated for 37 h at 37 °C and 180 x g. The DNSA assay was carried out as follows: 200 µL of culture supernatant was mixed with 200 µL of 1% CMC in pH 5 Tris–HCl buffer and incubated at 40 °C for 30 min. The reaction was terminated by adding 1200 µL of DNS reagent. The tube was then incubated at 100 °C for 10 min and the optical density (A 540) of the supernatant was measured against a control (native *B. cereus strain* EG296 culture without cellulase activity). According to the equation from the standard glucose curve **(Fig. 1)**, the glucose concentration released in the medium was measured and the cellulase activity was calculated by this formula:

Enzyme Activity (µmoL.min mL^{-1}) or (U. mL^{-1}) = (Concentration of Glucose released) (µmoL. mL^{-1}) ×Total Reaction Volume (mL) / (Reaction time (min)) × (Enzyme volume(mL))

3.7. Protease Activity

To determine the protease activity, 50 µL of culture supernatant was added to 250 µL of 1% casein in pH 7 Tris–HCl buffer and incubated at 35 °C for 10 min. The reaction was stopped by adding 300 µL of 10% Trichloroacetic acid and kept at 35 °C for 10 min. The suspension was centrifuged for 15 min in 10000 x g and the amount of released tyrosine in the supernatant was measured by the Todd method (23). A standard tyrosine curve was used to measure the protease activity **(Fig. 2)**. It was plotted based on the absorption of different concentrations of tyrosine at 275 nm and the protease activity was finally calculated by this formula:

Enzyme Activity (µmoL.min mL^{-1}) or (U. mL^{-1}) = (Concentration of Tyrosine released) (µmoL.mL^{-1}) ×Total Reaction Volume (mL) / (Reaction time (min)) × (Enzyme volume(mL))

3.8. In-Silico Evaluation of Destructive Effect of B. Cereus Protease on Cellulase

The peptide cutter-Expasy online software was used to predict the destructive effect of native *B. cereus* strain EG296 secretory main protease (AprE) on the cellulase. The amino acid sequence of the cellulase

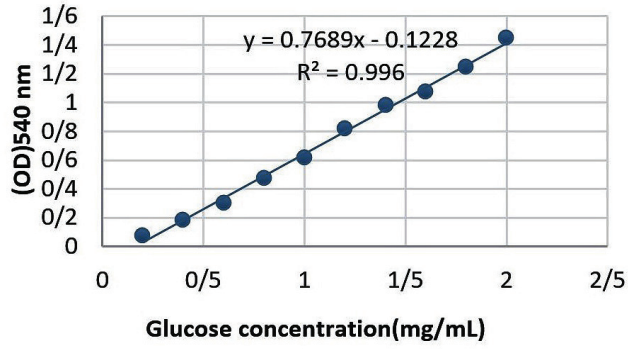

Figure 1. Glucose standard curve

(under study) was obtained from NCBI database and the effect of 30 peptidases in the Expassy database on it was investigated. Alignment of the 30 peptidases and AprE protease of *B. cereus* strain EG296 was carried out using BLAST programs in NCBI and European Molecular Biology Laboratory (EMBL)

3.9. Definition Of the Instability Index and Aliphatic Coefficient

The instability index provides a prediction of the stability of any considered protein. An instability index less than 40 predicts a stable protein, whereas values higher than 40 denote a potentially unstable protein.

The aliphatic coefficient also indicates the relative volume occupied by the side chains of aliphatic amino acids, which is a positive factor to increase the thermal stability of the proteins. These two factors can be calculated at an online https://web.expasy.org/protparam tool by giving the amino acid sequence of a considered protein (24,25).

4. Results

4.1. The Effect of B. Cereus Protease on Cellulase

From 30 peptidases in the Expassy database (Arg-C proteinase, Asp-N endopeptidase, Asp-N endopeptidase + N-terminal Glu, Chymotrypsin-high specificity, Chymotrypsin-low specificity, Clostripain, Glutamyl endopeptidase, LysC, LysN, Pepsin (pH1.3, pH>2), Proline-endopeptidase, Proteinase K, Staphylococcal peptidase, Thermolysin, Tobacco etch virus protease, Trypsin, Caspase10, Caspase2, Caspase3, Caspase4, Caspase5, Caspase6, Caspase7, Caspase8, Caspase9, Enterokinase, Factor Xa, GranzymeB, Thrombin), 13 peptidases (Caspase10, Caspase2, Caspase3, Caspase4, Caspase5, Caspase6, Caspase7, Caspase8, Caspase9, Enterokinase, Factor Xa, GranzymeB, Thrombin) lacked a cleavage site on the amino acid sequence of cellulase. Amino acid sequence of the remaining 17 peptidases (Arg-C proteinase, Asp-N endopeptidase, Asp-N endopeptidase + N-terminal Glu, Chymotrypsin-high specificity, Chymotrypsin-low specificity, Clostripain, Glutamyl endopeptidase, LysC, LysN, Pepsin (pH1.3, pH>2), Proline-endopeptidase, Proteinase K, Staphylococcal peptidase, Thermolysin, Tobacco etch virus protease, Trypsin), which had cleavage sites in the amino acid sequence of cellulase, were aligned with the *Bacillus cereus* EG296 AprE sequence to find their similarity. The BLAST results stated that among the 17 enzymes with cleavage sites, 16 enzymes had no significant similarity (less than 25%) with amino acid sequence of *Bacillus cereus* protease and only proteinase K had about 35 % similarity. So, it may conclude that the *Bacillus cereus* EG296 main protease (AprE) could not cleave the cellulase to be produced in this strain. In the other hand, the amino acid sequence of the 12 peptidases without cutting site in the cellulase have also no significant similarity with the *Bacillus cereus* EG296 AprE.

4.2. Transformation Of Cellulase Gene into the Bacillus Cereus

For production of recombinant cellulase in *Bacillus cereus* a DNA cassette containing coding region of the cellulase gene and regulatory region of the *aprE* gene of *Bacillus cereus* EG 296 was designed **(Fig.**

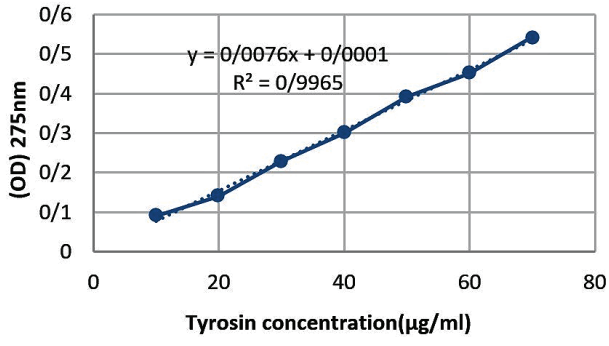

Figure 2. Tyrosin standard curve

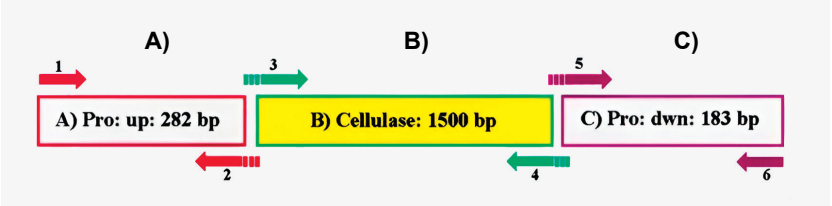

Figure. 3. The *proUp: cell: proDwn* constructed cassette Scheme. The primers used in this stage: 1 (BcKerF), 2 (KerUpR), 3 (kerCelF), 4 (celKerDwR), 5 (KerDwF) and 6 (BcKerR). The upstream region of protease **A)** with the length of 282 bp was amplified by the primers 1 and 2 (*B. cereus* EG296 DNA template*)*. The 1500 bp cellulase gene **B)** was amplified by primers 3 and 4 (*B. subtilis* 168 DNA template) and the downstream region of protease **C)** was amplified by primers 5 and 6 (*B. cereus* EG296 DNA template*)*. Thereupon part (A) and (B) get ligated with the length of 1782 pb by SOEing PCR with primers 1 and 4 and finally, the whole cassette (A+B+C) with the length of 1962 pb was constructed by SOEing PCR and amplified with primers 1 and 6.

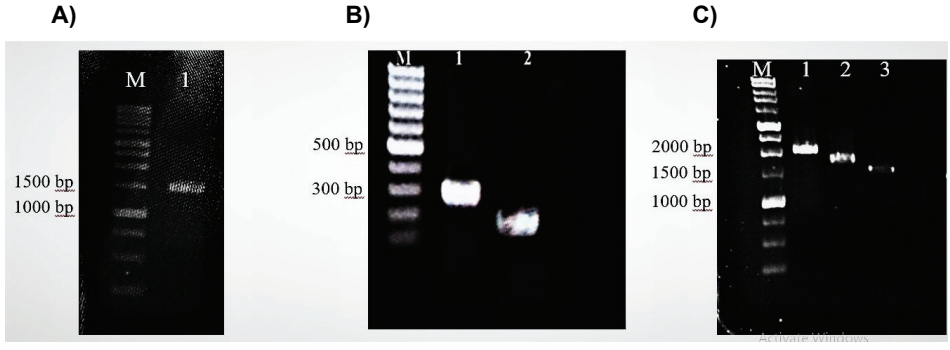

Figure 4. *proUp: cell: proDwn amplicon.* A) M: DNA marker 1 kb, 1: 1500 bp *endoglucanase* amplicon. **B)** M: DNA marker 100 bp, 1: 282 bp *protease gene upstream* and 2:182 bp *protease gene downstream* amplicons. **C)** M: DNA marker 1 kb, 1: 1965 bp *(upstream protease+ endoglucanase+ downstream protease) amplicon*, 2: 1782 bp *(upstream protease+ endoglucanase)* and 3: 1500 bp *endoglucanase* amplicon.

3) and constructed. The coding region of the cellulase gene, which consisted of 1500 nucleotides encoding a polypeptide with an expected molecular weight of 55 kDa, instability index of 26.6 and aliphatic index of 73.91 was amplified from *B. subtilis* strain 168 using kerCelF and celKerDwR primers. The promotor sequence of *aprE* which should be located at the upstream of the cellulase gene coding region, was specified by the www.softberry.com website. The 282 bp upstream region of the protease gene, consisting of its promoter region was amplified using BcKerF and KerUpR primers as mentioned in the Materials and Methods section. After the purification of the above mentioned amplicons, they were attached by SOeing PCR using BcKerF and celKerDwR primers to produce a 1782bp DNA fragment of *proUp:cell*. The 183 bp downstream region of the protease was also amplified using KerDwF and BcKerR primers. Finally, the *proUp:cell* cassette was ligated to *proDwn* gene and a 1965 bp cassette of *proUp::cell::proDwn* was constructed **(Fig. 4)** . All amplicons were checked on 1% agarose gel (20).

proUp:cell:proDwn cassette was transformed into the *B. cereus* competent cells through natural transformation. Recombinant colonies were approved by identifying the growth of bacteria and cellulose hydrolysis on CMC agar plate **(Fig. 5)** and colony PCR. These results highlighted that the manipulated

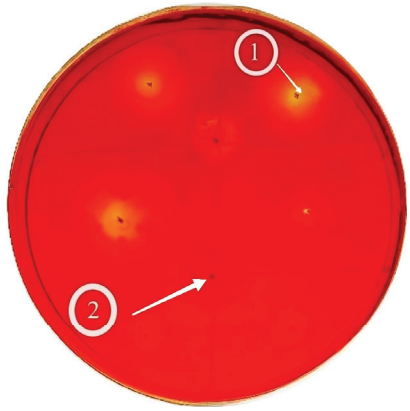

Figure 5. Screening *Bacillus cereus EG302* cellulase activity on CMC Agar plate. 1: *Bacillus cereus* EG302 recombinant colony with cellulase activity. **2:** *Bacillus cereus* EG296 wild type strain without cellulase activity.

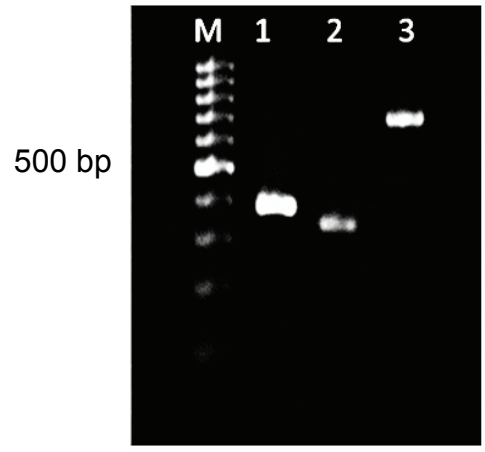

Figure 6. *ΔscoC* construction. M: DNA marker 100 bp, **1:** 390 bp *scoC* upstream PCR product, **2:** 340 bp *scoC* downstream PCR product and 3: 730 bp *ΔscoC PCR product*

strain released extracellular cellulase in the culture medium. Accordingly, 28% of the analyzed *B. cereus* colonies received cellulase gene through natural transformation and recombination. These transformants could express the cellulase under control of the protease promoter. The resulting strain, was assigned as *Bacillus cereus* EG302.

4.3. Deletion of the scoC in B. Cereus Expressing Cellulase

The upstream and downstream regions of *scoC* gene were amplified from *B. cereus* wild type genomic DNA, using scoCF1, scoCR1, scoCF2 and scoCR2 specific primers. The 390 bp upstream and 340 bp downstream amplicons were amplified at an optimum annealing temperature of 50 °C in 30 cycles. The upstream and downstream fragments were attached through SOeing PCR reaction in order to remove the coding region of the *scoC* gene. Thereupon, a 730 bp *ΔscoC* fragment was amplified at an annealing temperature of 52 °C in 35 cycles using *scoCF1* and *scoCR2* primers **(Fig. 6)**. The *ΔscoC* pure amplicon was ligated to the *EcoRV* linearized pGEM-5Zf vector and transformed into the *E. coli* DH5 α competent cells. plasmids were confirmed by colony PCR using gene-specific primers (scoCF1 and scoCR2) and DNA sequencing. The resulting plasmid was assigned as *Pgem5:ΔscoC* and was digested with the *Sca1* for transformation into *B. cereus*.

The linearized PGEM5:ΔscoC was transformed into *B. cereus* EG302 competent cells. The transformants were screened on Skim Milk agar plates and colonies with a larger protease clear zone in comparison to the control were selected. Deletion of the *scoC* gene coding reign was further approved by PCR using scoCF1 and scoCR2 primers. In 25% of the selected colonies, homologous recombination was accomplished and the coding region of the *scoC* gene was successfully deleted. Finally, the cellulase gene of *B. subtilis*168 was heterologously expressed under the control of protease gene promoter of the *Bacillus cereus* and also *scoC* deletion was performed by homologous recombination. By the deletion of *scoC* as one of the most important repressors of protease gene, the protease expression increased. The cellulase expression which was expressed under the control of the protease promoter region was also increased simultaneously. The resulting strain, was assigned as *Bacillus cereus* EG303.

4.4. Cellulase and Protease Activities

According to the previous studies on the genome of the *B. cereus* EG296 and its growth on CMC agar plate, this strain had no cellulase activity. By transforming the *Bacillus subtilis* 168 cellulase

gene under the control of the protease promoter, the cellulase activity of the strain reached to 0.61 u.mL^{-1}. After the *scoC* deletion, the cellulase activity reached to 0.78 u.mL^{-1} (~28% increase). The protease activity of the strain was also measured to be 230 u.mL^{-1}. After the *scoC* deletion, the activity reached to 363.14 u.mL^{-1} (~ 58% increase).

5. Discussion

Many microorganisms have the ability to secrete some proteins into their culture medium at high concentrations. Therefore, remarkable endeavor has been done in order to develop the secretion systems for recombinant protein production (26) to ease downstream purification of secreted heterologous proteins from culture medium (27). Advances in genetic and metabolic engineering strategies have helped to realize the potential of *Bacillus* species as a production host.

Cellulase and protease are the most important commercial enzymes and have widespread uses in industries. The global demand for these enzymes have increased, so their production have a special place in industrial biotechnology (4). Nowadays, using a single microorganism to simultaneous production of multiple enzymes or enzyme cocktails using inexpensive substrates is also attracting more attention. (28).

Simultaneous use of several enzymes can act as a co-catalyst in variety of industries and substitution of expensive raw material with low cost agro-wastes for multi-enzyme production are of interest and beneficial in biotechnology (29,30). Numerous studies have been carried out with respect to the optimization of growth conditions for single enzyme production. However, very few studies have examined the simultaneous production of multiple enzymes in the same host and medium. There is a serious need to investigate simultaneous production of industrially important enzymes using the same media and microorganism due to its economic concern with respect to energy, time and production cost reduction (31).

In most studies, the intrinsic cellulase activity of the bacteria or the cellulase expression in *E. coli* has been investigated. However, in the present study, the expression of the recombinant cellulase via integration of its gene into an indigenous *Bacillus cereus* genome under the control of host protease promoter has been studied.

The *cellulase* gene was introduced into the genome of the *Bacillus cereus* by natural transformation and recombination. The cellulase activity of the recombinant strain expressing cellulase under the control of the protease promoter was 0.61 u.mL^{-1}. After the *scoC* deletion by homologous recombination, the protease and cellulase activities reached to 363.14 and 0.78 u.mL^{-1} respectively.

The endoglucanase gene of *B. subtilis UMC7* (99% similar to the cellulase gene of Bacillus subtilis 168, used in this study) under the control of the T7 promoter had been cloned into *E. coli* BL21. The endoglucanase activity at an optimum temperature of 60 °C and pH 6 from the wild strain and recombinant *E. coli* were 0.12 and 0.51 u.mL^{-1} respectively (32). In another research, the cellulase activity *of B. subtilis* AS3 was 0.07 u.mL^{-1} and after optimizing the bacterial culture medium, this amount reached to 0.49 u.mL^{-1} (33). The manipulated strain in our study compared to other *Bacillus* strains had higher cellulase activity both before and after the *scoC* gene deletion without optimization of the production conditions. Production of higher amounts of cellulase in our system could be carried out using a stronger promoter or other strategies of strain improvement and metabolic engineering.

The *scoC (hpr)* gene is one of the major transcriptional regulators of *Bacillus* genus. The activity of this gene in *Bacillus subtilis* was firstly studied by Higerd *et al*. and was shown that the *scoC* deletion, increases the production of alkaline and neutral proteases (34). In addition, in our previous experiment, the *scoC* deletion increased the protease activity of *B. licheniformis* strain 1.8 times (35). Moreover, the deletion of the *scoC* gene in *Bacillus subtilis* increased the protease activity and decreased the cell motility (36). In our study, indigenous *Bacillus cereus* had significant protease activity (230 u.mL^{-1}). Deletion of the *scoC* gene increased the protease activity to a considerable amount of 363.14 u.mL^{-1} (1.58 times), without optimizing the culture condition. The increase in protease and cellulase gene expression was performed by regulating the expression of the *scoC* repressor. Thus, by deleting the *scoC* gene from the genome of the recombinant *Bacillus cereus: cellulase* strain, its repressive effect on the expression of protease and cellulase was removed resulted in increase in protease and cellulase production.

Competence development is controlled by a com-plex

signal transduction network in *B. subtilis* under specific environmental conditions (37). However, orthologues of most proteins involved in natural DNA uptake in *Bacillus subtilis* could be identified in *B. cereus* (38). The Efficiency of the natural transformation and especially homologous recombination are usually very low (39). Whereas, the *Bacillus cereus* strain used in this study had high potential for DNA uptake and integration into its genome (28%). In addition, PCR product with short homologous upstream and downstream arms of the target gene was introduced and integrated into the genome with high efficiency (about 28%). This property is very important and useful for further genetic manipulation of the strain for improvement the protease and cellulase production and other biological products. According to bioinformatics studies and the results obtained in the present study, the stability index for cellulase and protease, was calculated to be 26.16 and 20.18 respectively, which are less than 40. So, it can be considered as stable proteins. The aliphatic coefficient of cellulase and protease were also 73.91 and 75.92. Due to their high range of index (42.08- 90.68), they could also have good thermal resistance.

6. Conclusion

There are many different techniques that are used in order to manipulate a bacterial strain as a mean to reach desired goals. We used natural transformation, homologous and heterologous recombination to produce and secrete cellulase and protease as two important industrial extracellular enzymes with easy downstream processes for purification and application. In terms of phenotypic characteristics, the *Bacillus cereus* protease does not hydrolyze the secreted cellulase so they can be produced and be active in the culture medium simultaneously.

Acknowledgement

We gratefully acknowledge the partial financial support for Mrs. Farhangi from Ferdowsi University of Mashhad, project no 3/51805, and National Institute of Genetic Engineering and Biotechnology (NIGEB) for full financial support via research project no. 694.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

References

1. Okpara MO. Microbial Enzymes and Their Applications in Food Industry : *A Mini-Rev*. 2022;**23**-47. doi: 10.4236/aer.2022.101002
2. Adrio JL, Demain AL. Microbial enzymes: tools for biotechnological processes. *Biomolecules*. 2014;**4(**1):117-139. doi: 10.3390/biom4010117
3. Kuhad RC, Gupta R, Singh A. Microbial cellulases and their industrial applications. Vol. 2011, *Enzyme Res*. 2011. doi:10.4061/2011/280696
4. Jayasekara S, Ratnayake R. Microbial cellulases: an overview and applications. *Cellulose*. 2019;**22**:92. doi:10.5772/intechopen.84531
5. Ejaz U, Sohail M, Ghanemi A. Cellulases: From bioactivity to a variety of industrial applications. *Biomimetics*. 2021;**6**(3):1-11. doi:10.3390/biomimetics6030044
6. Rao MB, Tanksale AM, Ghatge MS, Deshpande V V. Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev*. 1998;**62**(3):597-635. doi: 10.1128/mmbr.62.3.597-635.1998
7. Cai D, Rao Y, Zhan Y, Wang Q, Chen S. Engineering Bacillus for efficient production of heterologous protein: current progress, challenge and prospect. *J Appl Microbiol*. 2019;**126**(6):1632-1642. doi: 10.1111/jam.14192
8. Schumann W. Production of recombinant proteins in Bacillus subtilis. *Adv Appl Microbiol*. 2007;**62**:137–189. doi:10.1016/S0065-2164(07)62006-1
9. Schallmey M, Singh A, Ward OP. Developments in the use of Bacillus species for industrial production. *Can J Microbiol*. 2004;**50**(1):1-17. doi: 10.1139/w03-076.
10. Liu L, Liu Y, Shin H, Chen RR, Wang NS, Li J, *et al*. Developing Bacillus spp. as a cell factory for production of microbial enzymes and industrially important biochemicals in the context of systems and synthetic biology. *Appl Microbiol Biotechnol*. 2013;**97**:6113-6127. doi: 10.1007/s00253-013-4960-4.
11. Pickens LB, Tang Y, Chooi Y-H. Metabolic engineering for the production of natural products. *Annu Rev Chem Biomol Eng*. 2011;**2**:211-236. doi: 10.1146/annurev-chembioeng - 061010-114209.
12. Bate AR, Bonneau R, Eichenberger P. Bacillus subtilis systems biology: applications of-omics techniques to the study of endospore formation. *Bact Spore from Mol to Syst*. 2016;129-144. doi: 10.1128/microbiolspec.TBS-0019-2013.
13. Caldwell R, Sapolsky R, Weyler W, Maile RR, Causey SC, Ferrari E. Correlation between Bacillus subtilis scoC phenotype and gene expression determined using microarrays for transcriptome analysis. *Am Soc Microbiol*. 2001. doi: 10.1128/JB.183.24.7329-7340.2001.
14. Han L-L, Liu Y-C, Miao C-C, Feng H. Disruption of the pleiotropic gene scoC causes transcriptomic and phenotypical changes in Bacillus pumilus BA06. B*MC Genomics*. 2019;**20**(1):1-11. doi:10.1186/s12864-019-5671-8
15. Pero J, Sloma A. Proteases, p 939–952. Bacillus subtilisand other Gram-positive. *Bact Biochem Physiol Mol Genet Am Soc Microbiol Washington*, DC. 1993; doi:10.1128/9781555818388
16. Abe S, Yasumura A, Tanaka T. Regulation of Bacillus subtilis aprE expression by glnA through inhibition of scoC and σD-dependent degR expression. *J Bacteriol*. 2009;**191**(9):3050-3058. doi: 10.1128/JB.00049-09
17. Ogura M, Shimane K, Asai K, Ogasawara N, Tanaka T. Binding of response regulator DegU to the aprE promoter

is inhibited by RapG, which is counteracted by extracellular PhrG in Bacillus subtilis. *Mol Microbiol*. 2003;**49**(6):1685-1697. doi: 10.1046/j.1365-2958.2003.03665.x
18. Ahmadpour F, Yakhchali B. Development of an asporogenic Bacillus cereus strain to improve keratinase production in exponential phase by switching sigmaH on and sigmaF off. *FEMS Microbiol Lett*. 2017;**364**(24):fnx216. doi:10.1093/femsle/fnx216
19. Karkhane AA, Yakhchali B, Jazii FR, Bambai B, Aminzadeh S, Rahimi F. A nested-splicing by overlap extension PCR improves specificity of this standard method. *Iran J Biotechnol*. 2015;**13**(2):56. doi: 10.15171/ijb.1090
20. Green MR, Sambrook J. *Molecular cloning*. A Lab Man 4th. 2012;
21. Sadaie Y, Kada T. Formation of competent Bacillus subtilis cells. *J Bacteriol*. 1983;**153**(2):813-821. doi: 10.1128/jb.153.2.813-821.1983
22. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem*. 1959;**31**(3):426-428. doi:10.1021/ac60147a030
23. Wang S-L, Hsu W-T, Liang T-W, Yen Y-H, Wang C-L. Purification and characterization of three novel keratinolytic metalloproteases produced by Chryseobacterium indologenes TKU014 in a shrimp shell powder medium. *Bioresour Technol*. 2008;**99**(13):5679-5686. doi:10.1016/j.biortech.2007.10.024
24. Guruprasad K, Reddy BVB, Pandit MW. Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting *in vivo* stability of a protein from its primary sequence. *Protein Eng Des Sel*. 1990;**4**(2):155-161. doi: 10.1093/protein/4.2.155.
25. Ikai A. Thermostability and aliphatic index of globular proteins. *J Biochem.* 1980;**88**(6):1895-1898. doi:10.1093/oxfordjournals.jbchem.a133168
26. Choi JH, Lee S. Secretory and extracellular production of recombinant proteins using Escherichia coli. *Appl Microbiol Biotechnol*. 2004;**64**:625-635. doi:10.1007/s00253-004-1559-9
27. Brockmeier U, Caspers M, Freudl R, Jockwer A, Noll T, Eggert T. Systematic screening of all signal peptides from Bacillus subtilis: a powerful strategy in optimizing heterologous protein secretion in Gram-positive bacteria. *J Mol Biol*. 2006;**362**(3):393-402. doi: 10.1016/j.jmb.2006.07.034
28. Amadi OC, Awodiran IP, Moneke AN, Nwagu TN, Egong JE, Chukwu GC. Concurrent production of cellulase, xylanase, pectinase and immobilization by combined cross-linked enzyme aggregate strategy-advancing tri-enzyme biocatalysis. *Bioresour Technol Rep*. 2022;**18**:101019. doi:10.1016/j.biteb.2022.101019
29. Laothanachareon T, Bunterngsook B, Champreda V. Profiling multi-enzyme activities of Aspergillus niger strains growing on various agro-industrial residues. 3 *Biotech*. 2022;**12**(1):17. doi: 10.1007/s13205-021-03086-y
30. Limkar MB, Pawar S V, Rathod VK. Statistical optimization of xylanase and alkaline protease co-production by Bacillus spp using Box-Behnken Design under submerged fermentation using wheat bran as a substrate. *Biocatal Agric Biotechnol*. 2019;**17**:455-464. doi:10.1016/j.bcab.2018.12.008
31. Shrestha S, Chio C, Khatiwada JR, Kognou ALM, Qin W. Formulation of the agro-waste mixture for multi-enzyme (pectinase, xylanase, and cellulase) production by mixture design method exploiting Streptomyces sp. *Bioresour Technol Rep*. 2022;**19**:101142. doi:10.1016/j.biteb.2022.101142
32. Wei KSC, Teoh TC, Koshy P, Salmah I, Zainudin A. Cloning, expression and characterization of the endoglucanase gene from bacillus subtilis UMC7 isolated from the gut of the indigenous termite macrotermes malaccensis in *Escherichia coli*. *Electronic J Biotechnol*. 2015 Mar;18(2):103-9. doi:10.1016/j.ejbt.2014.12.007
33. Deka D, Bhargavi P, Sharma A, Goyal D, Jawed M, Goyal A. Enhancement of cellulase activity from a new strain of Bacillus subtilis by medium optimization and analysis with various cellulosic substrates. *Enzyme Res*. 2011;2011. doi: 10.4061/2011/151656.
34. Higerd TB, Hoch JA, Spizizen J. Hyperprotease-producing mutants of Bacillus subtilis. *J Bacteriol*. 1972;**112**(2):1026-1028. doi:10.1128/jb.112.2.1026-1028.1972
35. Farazmand A, Yakhchali B, Shariati P, Ofoghi H. Bacillus clausii and Bacillus halodurans lack GlnR but possess two paralogs of glnA. *J Proteomics Bioinform*. 2011;**4**(9). doi:10.4172/jpb.1000187
36. Numberger D, Ganzert L, Zoccarato L, Mühldorfer K, Sauer S, Grossart H-P, *et al*. Characterization of bacterial communities in wastewater with enhanced taxonomic resolution by full-length 16S rRNA sequencing. *Sci Rep*. 2019;**9**(1):1-14. doi:10.1083/s41598-019-46015-z
37. Hamoen LW, Venema G, Kuipers OP. Controlling competence in Bacillus subtilis: shared use of regulators. *Microbiol*. 2003;**149**(1):9-17. doi: 10.1099/mic.0.26003-0
38. Mirończuk AM, Kovács ÁT, Kuipers OP. Induction of natural competence in *Bacillus cereus* ATCC14579. *Microb Biotechnol*. 2008;**1**(3):226-235. doi: 10.1111/j.1751-7915.2008.00023.x.
39. Vojcic L, Despotovic D, Martinez R, Maurer K-H, Schwaneberg U. An efficient transformation method for *Bacillus subtilis* DB104. *Appl Microbiol Biotechnol*. 2012;**94**:487-493. doi: 10.1007/s00253-012-3987-2.