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Development of an asporogenic Bacillus cereus strain to improve keratinase production in exponential phase by switching sigmaH on and sigmaF off

Fathollah Ahmadpour and Bagher Yakhchali*

National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

*Corresponding author: National Institute of Genetic Engineering and Biotechnology, Pajoohesh BLV, 17 KM, Karaj Highway, Tehran, Islamic Republic of Iran. Tel: +98-2144787391; E-mail: bahar@nigeb.ac.ir

One sentence summary: A Bacillus cereus strain was engineered to develop an asporogenic strain producing proteases in the exponential phase by deletion of sigma-F and overexpression of sigma-H genes in the exponential growth phase.

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ABSTRACT

Many bacteria, including the genus Bacillus, are able to produce proteases (keratinase). In Bacillus, proteases are produced in the stationary phase and initial stages of sporulation. Protease production is coordinated with sporulation in which expression of various genes by different sigma factors manages the transition from the exponential to the stationary phase. In the present study the sigma-F gene of an indigenous Bacillus cereus strain, which is involved in transcription of genes maintaining sporulation, was deleted. The sigma-H gene, whose product activates the genes that function in the zero phase of sporulation and inhibits suppression of protease production, and spo0B genes were expressed in the exponential phase under the control of a sucrose-inducible promoter from the Bacillus sacPA operon. For the first time, an asporogenic strain of B. cereus was generated that produced higher keratinase (390 U compared with the 198 U of the wild-type strain) and protease (450 U compared with the 290 U of the wild-type strain) activities in the exponential growth phase by induction with sucrose. The new strain is promising for production of keratinase for degradation of feather waste to produce feather meal for poultry feed and decrease environmental pollution from the poultry industry.

Keywords: keratinase; protease; Bacillus cereus; sporulation; metabolic engineering

INTRODUCTION

Bacillus species, especially engineered strains, have been used extensively for commercial production of protease, amylase and antibiotics (Fleming et al. 1995). Bacillus strains produce a wide variety of products including extracellular enzymes, antibiotics and insecticides synchronous with sporulation. Sporulation is carried out in seven steps in which asymmetric septation in the cell wall divides the bacterial cell into a pre-spore and a mother cell. The pre-spore is covered by spore cortex and covering proteins. At the final step, the mother cell eventually lyses and the spores are released (Wang et al. 2008).

In general, sporulation is induced by adverse environmental conditions such as starvation. Initiation of sporulation is tightly regulated. Sporogenic bacteria use multiple sigma factors during sporulation (Haldenwang 1995). In step 1, a shift occurs in the bacterial sigma factor gene expression and Sigma-H activates transcription of other sporulation genes such as spo0A, Spo0B, Spo0F and kinase genes (KinA, KinB, etc.) (Carter and Moran 1986; Albano, Hahn and Dubnau 1987; Asai et al. 1995; Grossman 1995). When these genes are expressed, KinA triggers the phosphorelay system by adding phosphate to other transcription factors, which ultimately leads to phosphorylation and activation of Spo0A. Spo0A–P is known as the master regulator of the
transition phase that regulates gene transcription at the beginning of sporulation and production of degradative enzymes. The phosphorylated Spo0A suppresses inhibitor genes of protease expression such as sinR, abrB and scoC and activates other genes of sporulation, synthesis of antibiotics, degradative enzymes, competency and biofilm formation resulting in production of protease and other degradative enzymes (Quisel, Burkholder and Grossman 2001; Fujita and Losick 2003; Molle et al. 2003; Fujita, González-Pastor and Losick 2005). A summary of the regulation of sporulation and protease production is shown in Fig. 1.

Sugar promoters are the most common promoters in bacterial systems. The sucrose operon of Bacillus subtilis contains SacA and SacP genes and a sucrose-inducible promoter that is activated in a medium containing sucrose (Debarbouille et al. 1990).

In this work, an indigenous Bacillus cereus strain was developed with null mutation in the sigma-F gene and overexpression of sigma-H in the exponential growth phase from a sucrose-inducible promoter (sacPA operon promoter). Expression of sigma-H induces the expression and activation of Spo0A, which leads to production of protease by negative regulation of sinR, abrB and scoC and trigger the sporulation process, but deletion of the sigma-F gene (the spoIAC gene) impairs the continuation of sporulation. Thus, the asporogenic strain of B. cereus could produce protease in the exponential growth phase by induction with sucrose and remain in the transition phase.

DNA manipulation

Extraction and purification of genomic DNA were performed using a DNA purification kit (Najm DNA purification kit, Iran). PCR primers sigF (5’-CTGCAGATGGACATAGAGGTTAAATGAGGT-3’ and sigR (5’-GGATCCTTATTCGTCTATTCGATCTTTC-3’) (Cinnagen, Iran) were used for amplification of the sigma-F gene. kanF and kanR primers (Cinnagen, Iran) were used for amplification of the DNA fragment containing the kanamycin gene from pWB980 plasmid. PCR reactions were carried out in a DNA thermal cycler (Techne Flexigen, USA).

The sigma-F PCR product was cloned into pGEM-S2f (−) vector linearized with EcoRV enzyme to construct the pGSGF plasmid. The DNA fragment containing the kanamycin gene was cloned in the pGEM-S2f (−) vector resulting in pGKAN. The kanamycin gene was then excised from pGKAN with Ndel and ligated to pGSGF linearized with the same enzyme at position 267 of the sigma-F gene. The new recombinant plasmid containing mutated sigma-F gene was designated as pGSF-Kn.

MATERIALS AND METHODS

Strains, plasmids and media

Indigenous B. cereus strain NYA101, previously isolated and characterized, was used in this study as the wild type strain (Ahmadpour, Yakhchali and Mousavi 2016). Bacillus subtilis 168 was used as the source of the sucrose promoter. Escherichia coli K12 DH5α strain was used as an intermediate host for cloning experiments. The pTZ57R (Fermentas), pGEM-S2f (Promega) and pHY300PLK vectors were used as cloning vectors. Luria–Bertani (LB) medium was used to grow E. coli strains at 37°C and LB agar containing appropriate antibiotics was used for screening of the recombinant clones. The Bacillus strains were cultivated in a basal broth medium containing (g/l) NH4Cl (0.5), NaCl (0.5), KH2PO4 (0.4), K2HPO4 (0.3), MgCl2.6H2O (0.1), yeast extract (0.1) and feather (10) and incubated at room temperature at 150 rpm for keratinase production.
The sigma-H and spo0B genes were amplified by PCR using FsigH (5′-GGGAGAAATAGTTGGAACGGCAGCTCTGAAGAGTGTCTCT-3′) and RsigH (5′-TTTTTCAATTCATGTGGCGCATAGATTTAAGTAGTACTCTC-3′), and FspoB (5′-CATGACAGCACTGAAATGAAAATGGACAAATTAG-3′) and RspoB (5′-GAATTCCTGCGGATCCCATCAGGACCATATTTTCAGCTTC-3′) primers, respectively. The DNA fragment containing the sucrose promoter of the spaC operon of B. subtilis was amplified using Fpro (5′-GAATTCGGCATGATGCGACGATATG-3′) and Rpro (5′-GCCGTGTTCCACCTATTCTCCCGCCTTTGATA-3′) primers. The sigma-H amplicon and the sucrose promoter were fused by the SOEing PCR method (Karkhane et al. 2015) to produce the psac::sigma-H cassette. The psac::sigma-H cassette and the spo0B amplicon were also attached together by SOEing PCR. The transcription of the sucrose operon was located at the 5′-end of the reverse RsigH primer to introduce transcriptional termination in the end of the spo0B gene in order to terminate the transcription of the cassette. The psac::sigma-H::spo0B cassette was cloned into linearized pHY300PLK plasmid to construct pHYSH-SUC. All recombinant plasmids were confirmed by PCR, restriction digestion and sequencing.

For mutation in the sigma-F gene, the pgSF-Kn plasmid was transformed into the B. cereus wild type strain by electroporation. The mutant strains were selected on LB agar medium containing kanamycin (30 μg/ml) and the recombination was confirmed by PCR using Rsigf and Fsigf primers. The pHYSH-SUC plasmid was transformed into the kanamycin resistance mutant strain of B. cereus by electroporation. The transformants were grown on LB agar containing tetracycline. To analyze the transcriptional profile of the engineered strains, RT-PCR was carried out using RsigH and Fsigh primers.

Protease and keratinase activity

Keratinase activity was measured by the modified method of Takiuchi et al. (1982, 1984). The reaction comprised 3 ml of 0.3% keratin in phosphate buffer and 2 ml of culture supernatant. After 1 h of incubation at 30 °C, the reaction was stopped by addition of 1 ml of 10% trichloroacetic acid and centrifuged for 10 min at 13 000 g at 4 °C. The absorbance at 280 nm of the supernatant was measured and compared with control. The control was treated in the same way, except that trichloroacetic acid was added before incubation. One unit (U) of keratinolytic activity was defined as 0.01 increase in absorbance at 280 nm under the defined conditions.

To determine protease activity, 0.2 ml of culture supernatant was added to 1 ml of 1% casein in pH 7 Tris–HCl buffer and kept at 37 °C for 30 min. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid. Then it was centrifuged and the supernatant absorbance was measured by the Todd method using tyrosine as the standard compound. One unit of protease activity was defined as 0.01 increase in the absorbance at 280 nm (Wang et al. 2008).

Spore formation monitoring

A heat treatment assay was applied to evaluate spore formation in mutant and wild type strains (Nicholson et al. 2000). The mutant and wild type strains were cultured in Schaeffer’s sporulation medium (Schaeffer, Millet and Aubert 1965) for 24 h at 37 °C. To reveal the sporogenic bacteria, the cultured media were boiled and cultured on LB agar and incubated for 24 h at 37 °C.
Production of protease and keratinase in asporogenic B. cereus by induction of sigma-H in the exponential phase

To assess the effects of null mutation in the sigma-F gene on protease and keratinase production, the mutant strain was cultivated in basal medium with sucrose as sole carbon and energy source, and growth, protease and keratinase production were measured separately. The results showed that the trends of protease and keratinase production in both native and mutant (sigma F mutant) strains were nearly similar, but the mutant strain was not able to form spores (Figs 4 and 5). In these strains protease and keratinase activity (production) are not adapted to the growth curve. There is a delay in protease and keratinase production at the exponential phase and a sharp increase at the end of the exponential growth phase/initiation of stationary phase. The effects of simultaneous inactivation of the sigma-F and overexpression of sigma-H gene in the exponential growth phase were evaluated by measurement of protease and keratinase production during the growth curve. In this strain the protease and keratinase activity coincided with the growth curve (Fig. 6). The results indicated that protease (450 U) and keratinase (390 U) production were also enhanced in the response to induction with sucrose mostly in the exponential growth phase instead of the stationary phase in the native strain (protease 290 U, keratinase 198 U).

DISCUSSION AND CONCLUSION

In the current study an indigenous B. cereus strain was genetically manipulated to develop an asporogenic strain producing high amounts of proteolytic enzymes using a sucrose-inducible promoter from Bacillus subtilis. An asporogenic B. cereus strain was capable of producing proteolytic enzymes in the exponential growth phase coincident with the growth curve.

Deletion and disruption in the sigma-F gene by insertion of a DNA fragment resulted in asporogenic Bacillus licheniformis, which has been also reported previously (Fleming et al. 1995; Wang, Greenhut and Shih 2005). The sigma-F mutant is asporogenic, which is in accordance with the role of sigma factors in sporulation that has been proven in many studies and was predictable because sigma-F is the first activated gene in the pre-spore, and thus sporulation in the sigma-F null mutant strain stopped at the initiation of the sporulation process (Margolis, Driks and Losick 1991; Driks and Losick 1991; Losick and Stragier 1992; Errington 1993).

The mutation in the sigma-F gene had no significant effect on cell growth and proteolytic enzyme production, as has been reported previously (Wang, Greenhut and Shih 2005). Low amounts of proteolytic enzymes are produced from the early stage of the growth curve with a delay at the exponential growth stage and an increase at the initiation of the stationary phase prior to stage 2 of the sporulation process (Priest 1977) as assayed through the growth curve. Induction of sigma-H and spo0B genes with sucrose in a sigma-F-deficient strain was carried out in this research and it was shown that a sharp increase of protease production occurred in the exponential phase instead of the stationary phase. This is explainable in accordance with the role of the sigma-H factor.

Sigma-H controls expression of several genes such as Vpr, AprE and NprE, which are involved in adaptation to nutrient deficiency and proteases secretion (Sloma et al. 1991; Britton et al. 2002). Previous studies have indicated that sigma-H is typically expressed in the sporulation phase or stationary phase and leads to the expression of spo0A, which suppresses the inhibitors of proteolytic enzymes expression. In this study, sigma-H was overexpressed in the exponential stage with sucrose, so sporulation and protease expression began, but sporulation did
not occur due to destruction of sigma-F, while protease expression continued. This confirms increased production of proteases by the accumulation of sigma-H (Fleming et al. 1995; Wang, Greenhut and Shih 2005).

In summary, the manipulated strain developed in this study lacks spores and can produce proteolytic enzymes in the exponential growth phase, which is very important in large scale production of secondary metabolites. The fermentation process of this strain can be carried out in fed-batch culture, which is suitable for industry, and even in a continuous manner, which means keeping the bacterium in the exponential phase without entering the stationary phase and sporulation process. Protease production in sporogenic strains is mainly limited to the stationary phase in which control and optimization of the process is difficult. Spore forming bacteria can long survive in the environment, which is not suitable for industrial processes using genetically modified organisms (GMOs). Treatment of the industrial waste water containing spores is more difficult and costly than for water without spores. Thus due to easier waste treatment operation and costs, property protection, the fermentation process and environmental considerations, asporogenic strains are desirable. The amount of enzyme produced was greater than that by the wild type strain because of the overexpression of sigma-H (Fleming et al. 1995), which can tightly repress the inhibitors of the production of secondary metabolites (including proteases). The results of this study indicated the coordination and communication between protease production and sporulation in indigenous B. cereus strain NYA101, and the role of sigma-H as a key factor in this harmony was strongly confirmed. This strain has potential for industrial applications because it lacks spores and produces protease and keratinase enzymes simultaneously from the beginning of the fermentation process; this needs more improvement and optimization of the conditions of enzyme production.

The poultry, leather and similar industries generates several million tons of keratinous waste material annually, which is a worrying level of environmental pollution. This pollution could be decreased by the use of keratinase enzyme (Gupta and Ramnani 2006; Brandelli 2008; Cai et al. 2011; Gupta et al. 2013) or keratinase-producing bacteria. Therefore, the capability of the feather and leather degradation of B. cereus strain NYA101 could be used for an environmentally friendly process of recycling poultry and leather industry waste. However, the engineered B. cereus strain NYA101 should be further improved by manipulation of other parts of the metabolic network of sporulation, competency and secondary metabolite production. This strain has also the industrial potential for proteases production, for which its conditions need to be optimized. This approach can also be applied in other wild type strains with the potential for production of proteases or other secondary metabolites.

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Conflict of interest. None declared.

REFERENCES


