Construction of a $\beta$-Glucanase Hyperproducing Bacillus subtilis Using the Cloned $\beta$-Glucanase Gene and a Multi-copy Plasmid

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Received February 23, 1989

We have cloned the $\beta$-glucanase gene from a $\beta$-glucanase producing strain, Bacillus subtilis Y-25, to construct a $\beta$-glucanase hyperproducing strain. The cloned 1.9 Kb EcoRI-HpaI fragment containing the entire $\beta$-glucanase gene was inserted into the EcoRI and PvuII sites of a multi-copy vector plasmid, pUB110. The resulting plasmid, named pLB100, was introduced into B. subtilis Y-25 to construct B. subtilis HL-25. HL-25 produced 347 units/ml of $\beta$-glucanase in a culture supernatant, which was about 19-fold higher than the amount produced by the original strain, Y-25, and corresponded to approximately 2 g of $\beta$-glucanase per l. Furthermore, pLB100 is so stable in HL-25 that the proportion of cells carrying pLB100 was almost 100% after cultivation for 100 generations without a selective antibiotic.

Several Bacillus species produce extracellular enzymes which are of considerably industrial importance. Among these enzymes, $\beta$-glucanase (1,3;1,4-$\beta$-D-glucan 4-glucanohydrolase) has applications within the brewing industry, where it is used to reduce the wort filtration time and haze formation in the finished products.\(^1,2\)

The molecular cloning and DNA sequence of the B. subtilis $\beta$-glucanase gene have already been reported,\(^3,4\) and expression of the gene in brewing yeast has also been attempted by several groups.\(^5,6\) Furthermore, the addition of $\beta$-glucanase can reduce the wort filtration time and give a better extraction yield. Hence, high productivity of the $\beta$-glucanase is industrially interesting. In this paper, we describe the construction of a Bacillus subtilis strain capable of hyperproducing the enzyme.

Materials and Methods

Bacteria, media and transformation. The strains used are listed in Table I. Escherichia coli HB101 was used as the cloning host. B. subtilis UOT0734 was used for subcloning of the $\beta$-glucanase (1,3;1,4-$\beta$-D-glucan 4-glucanohydrolase) gene. $\beta$-Glucanase producing Bacillus strain Y-25 was originally isolated in our laboratory and identified as B. subtilis according to Bergey's manual.\(^7\) The bacteria were grown in L-medium\(^8\) or AH-medium\(^9\) at 37°C, or brewer's spent grain medium (200 g of brewer's spent grain in 1 l of 40 mM phosphate buffer, pH 7.0)\(^10\) at 30°C. Transformation of E. coli HB101 was performed by the method of Mandel and Higa.\(^11\) B. subtilis was transformed according to Chang and Cohen.\(^12\)

Table I. LIST OF BACTERIAL STRAINS USED

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli HB 101</td>
<td>F$^-$, hsdS20, recA13, ara14, proA2, lacY1, galK2, rpsL20, xyl5, mitI, supE44, λ$^+$</td>
</tr>
<tr>
<td>Bacillus subtilis IAM 12118</td>
<td>ATCC 6051, Strain Marburg. Type strain</td>
</tr>
<tr>
<td>IAM 3134</td>
<td>ATCC 6633, Produces Subtilin</td>
</tr>
<tr>
<td>IFO 13722</td>
<td>ATCC 19659</td>
</tr>
<tr>
<td>UOT 0734</td>
<td>hisA1, metB5, lys21, amyE07</td>
</tr>
<tr>
<td>DB 104</td>
<td>hisH, nprE18, nprR2, aprA3</td>
</tr>
<tr>
<td>Y 25</td>
<td>Produces $\beta$-glucanase (this study)</td>
</tr>
</tbody>
</table>

Preparation of chromosomal and plasmid DNAs. Chromosomal DNA from B. subtilis was prepared according to the method of Saito and Miura.\(^13\) Plasmid DNA was prepared by the method of Birnboim and Doly,\(^14\) followed by purification involving ethidium bromide-CsCl density gradient ultracentrifugation.

DNA sequence analysis. EcoRI-digested pLE100 was inserted into the pBS-M13 plasmid vector in two directions, and digested unidirectionally with exonuclease III.\(^15\) After the digestion, the plasmids were selfligated.
with T4 ligase to obtain multiple nested deletion plasmids. These plasmids were used for DNA sequence analysis.\(^{16}\) M13-K07 was used as a helper phage for the recovery of the single strand DNAs.

**β-Glucanase assay.** β-Glucanase was assayed colorimetrically at 540 nm using dinitrosalicylic acid, which reacted with reducing sugar released from lichenan (Sigma) at pH 7.0. One unit of β-glucanase was defined as the amount of enzyme capable of liberating 1 mg of maltose per minute at 40°C.

**Detection of β-glucanase producing transformants.** Bacteria were plated on L-agar plates containing 0.1% (w/v) lichenan. After overnight incubation at 37°C, the plates were stained by flooding with 0.1% (w/v) Congo Red. Colonies of β-glucanase producing transformants had clear halos around them on the red background.

**Enzymes.** The restriction enzymes and T4 ligase were purchased from Takara Shuzo Co., Ltd.

### Results and Discussion

**β-Glucanase productivities of six *B. subtilis* strains**

The β-glucanase productivities of six *B. subtilis* strains were examined using the culture supernatants after incubation in L-medium and AH-medium at 37°C for 3 days (Table II). The highest productivity was observed in the culture supernatant of *B. subtilis* Y-25. We therefore selected Y-25 as both the host and the gene donor to construct a β-glucanase hyperproducing strain.

#### Table II. β-Glucanase Productivities of Six *B. subtilis* Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>L-medium</th>
<th>AH-medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAM 12118</td>
<td>4.4</td>
<td>3.5</td>
</tr>
<tr>
<td>IFO 3134</td>
<td>ND</td>
<td>4.1</td>
</tr>
<tr>
<td>IFO 13722</td>
<td>ND</td>
<td>NT</td>
</tr>
<tr>
<td>UOT 0734</td>
<td>3.5</td>
<td>NT</td>
</tr>
<tr>
<td>DB 104</td>
<td>3.2</td>
<td>NT</td>
</tr>
<tr>
<td>Y 25</td>
<td>6.1</td>
<td>12.6</td>
</tr>
</tbody>
</table>

ND, not detected; NT, not tested.

**Cloning of the *B. subtilis* β-glucanase gene**

Chromosomal DNA prepared from *B. subtilis* Y-25 was digested with EcoRI. The vector plasmid, pBR322, was also digested with the same restriction enzyme and treated with bacterial alkaline phosphatase. The EcoRI-digested *B. subtilis* DNA fragments were then inserted into the EcoRI site of pBR322 using T4 ligase. The ligated plasmids were used to transform *E. coli* HB101. The transformants were grown on L-agar plates supplemented with 50 μg/ml of ampicillin and 0.1% of lichenan. β-Glucanase producing transformants were detected upon staining of the plates with Congo Red after overnight incubation at 37°C. Among 5000 transformants, four had clear halos around their colonies after staining. Each of the plasmids extracted from these four transformants had the same 3.7 kb EcoRI fragment, with its direction in the plasmids in two orientations.

**Fig. 1.** Restriction Maps of the Plasmids Carried by β-Glucanase Positive Transformants.

Closed bars and single lines denote *B. subtilis* Y-25 DNA and pBR322, respectively. Arrows indicate the directions of transcription. Glu, β-glucanase gene. Restriction sites: E, EcoRI; P1, Pvul; H, HindIII; Bg, BglII; S, SacI; B, BamHI; P2, PvuII.
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opposite ways, pLE100 and pLE200 (Fig 1). Further analysis of the 3.7 kb EcoRI fragment showed that the entire β-glucanase gene is contained in the 1.9 kb EcoRI–HpaI fragment. Furthermore, Southern blot analysis of the *B. subtilis* Y-25 chromosomal DNA using the 3.7 kb EcoRI fragment as the probe indicated no deletion or rearrangement in the cloned DNA fragment (data not shown).

DNA sequence of the β-glucanase gene

The DNA sequence of the 1.9 kb EcoRI–HpaI fragment in pLE100 was determined. Figure 2 shows the DNA sequence of the β-

![DNA Sequence](image)

**Fig. 2.** Nucleotide and Deduced Amino Acid Sequences of the *Bacillus subtilis* Y-25 β-Glucanase Gene with Its Flanking Regions.

The putative ribosome binding site (SD) and recognition sequences for σ^{70}RNA polymerase (−35, −10) are underlined. Palindromic sequences in the 3′ flanking region are indicated by horizontal arrows. The putative signal peptide cleavage site is indicated by a vertical arrow.
glucanase gene, including its 5' and 3' flanking regions, and the amino acid sequence deduced from the DNA sequence. There was a single open reading frame of 726 bp, which corresponds to a protein with a molecular weight of 27,338. The deduced N-terminal amino acid sequence has a typical feature of signal peptide that consists of the existence of basic amino acid residues in the N-terminus, a hydrophobic region, and two alanine residues just before the putative cleavage site (Fig. 2). The open reading frame is proceeded by a putative ribosome binding site and a putative recognition sequence for σ^34 RNA polymerase. Including these features, this DNA sequence showed differences in 26 nucleotides compared with that of *B. subtilis* C-120 determined by Murphy *et al.*[^3] The discrepancy between the two β-glucanases resulted in 4 amino acids substitutions in 4 amino acids substitutions (Ser to Ala at 24 and 83, Pro to Leu at 204, and Thr to Arg at 240 see Fig. 2). The substitutions at amino acid positions 24 and 83 might be nonessential and based on the strain specificity, because the same substitutions (Ser to Ala) were observed in the enzymatically functional regions[^19][^20] in the case of *Bacillus α-amylase*.[^21] The significance of the remaining substitutions (at positions 204 and 240) are not clear. Further work is necessary to elucidate the significance of these differences.

Subcloning of the cloned β-glucanase gene into pUB110

In order to construct a β-glucanase hyper-producing *B. subtilis* strain, the cloned 1.9 kb EcoRI–HpaI fragment containing the entire β-glucanase gene was replaced by the EcoRI–PvuII fragment of pUB110 (Fig. 3). The resulting plasmid, designated pLB100, was introduced into *B. subtilis* Y-25 to obtain strain HL-25.

β-Glucanase production by *B. subtilis* HL-25 carrying pLB100

β-Glucanase activities in culture supernatants of *B. subtilis* HL-25 were determined and compared with those of the original strain, Y-25, after 3 days incubation at 37°C in both L-medium and AH-medium (Table III). HL-25 produced 147 units/ml of β-glucanase in AH-medium containing starch as a carbon source and 5 μg/ml of kanamycin, which was about 12-fold higher than the amount of β-glucanase produced by Y-25.

Stability of pLB100 in *B. subtilis* HL-25

The stability of plasmid pLB100 is industrially important. The use of an antibiotic results in high cost and complexity of purification. Hence, the stability of pLB100 in *B. subtilis* HL-25 was examined, as follows. *B. subtilis* HL25 was grown in L-medium without a selective antibiotic (kanamycin) and then plated on L-agar medium to detect viable cells, and then the colonies formed were replicated...
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Fig. 4. Stability of pLB100 in B. subtilis HL-25.

onto L-agar medium containing 5 μg/ml of kanamycin to determine the proportion of cells carrying the plasmid. As shown in Fig. 4, almost 100% of the colonies grown on an L-agar plate were kanamycin resistant, even after cultivation for 100 generations. This result suggested that pLB100 is very stable in HL-25.

β-Glucanase production by B. subtilis HL-25 in a jar fermentor

The above results suggested that B. subtilis HL-25 is industrially useful. We therefore examined β-glucanase production by HL-25 in a 5 l-jar fermentor. The agitation speed and aeration rate were kept at 600 rpm and 1.21 per minute, respectively. HL-25 was precultured overnight at 37°C in L-medium containing 5 μg/ml of kanamycin and then inoculated into brewer’s spent grain medium (with a 1% inoculum), which has been reported to induce β-glucanase production9). After 3 days cultivation at 30°C, HL-25 had produced 347 units/ml of β-glucanase in the culture supernatant (Table III), which is about 19-fold higher than the amount produced in the supernatant by the original strain, Y-25, and corresponded approximately to 2 g of β-glucanase per liter based on the specific activity of the purified enzyme. In this case also, the proportion of cells carrying pLB100 after 3 days cultivation in a jar fermentor has almost 100%. In conclusion, we have succeeded in constructing a β-glucanase hyperproducing B. subtilis strain, HL-25. This strain has two advantages for commercial production of the enzyme:

1. HL-25 stably produces a high amount of β-glucanase without a selective antibiotic.
2. Simple purification may be expected because approximately 70% of the protein in the culture supernatant is β-glucanase (data not shown).

Further work to elucidate the signal peptide processing and the properties of the β-glucanase will be published in a subsequent paper.

Acknowledgment. The authors wish to express their thanks to Dr. Hironori Shimatake (Toho University) for the useful advice and for supporting this experiment in part.

References