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Expression of *Bacillus macerans* Cyclodextrin Glucanotransferase in *Bacillus subtilis*

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Abstract A plasmid vector was constructed for the expression and secretion of *Bacillus macerans* cyclodextrin glucanotransferase (CGTase) in *Bacillus subtilis*. The vector, pUBACGT, was composed of the ribosome-binding sequence, signal sequence, and *cgt* gene from *B. macerans* under the control of *amy*R2, the promoter of *amy*E gene coding for α-amylase from *B. subtilis* var. *natto*. *Bacillus subtilis* LKS88, a mutant strain lacking genes for an amylase and two proteases, was used as a host for the transformation of the plasmid vector. The transformants were selected on kanamycin-containing Luria-Bertani plates. The starch hydrolyzing activity was observed on the starch-containing plates by the iodine method and cyclodextrin-forming activity was detected in the culture medium. A SDS-PAGE analysis showed that most of the expressed CGTase in the recombinant *B. subtilis* was secreted into the medium at a high expression level.

Key words: Cyclodextrin glucanotransferase, *Bacillus macerans*, *Bacillus subtilis*, enzyme secretion, protein expression

Cyclodextrin glucanotransferase (CGTase, E.C.2.4.1.19) is an extracellular enzyme produced by *Bacillus* sp. and *Klebsiella* sp. This enzyme synthesizes cyclodextrins (CDs) by transferring the glucose residue of the reducing end of glucan to the non-reducing end of its chain when it reacts with starch. The CGTase from *Bacillus macerans* mainly produces six glucose-linked CD (α-CD), while CGTase from other *Bacilli*, such as *B. circulans*, produces seven glucose-linked CD (β-CD). α-CD is more soluble than β-CD and can be widely used in food and pharmaceutical areas. Since the CGTase secretion level from *B. macerans* to a culture medium is quite low, an over-expression system is necessary for the biochemical study and industrial use of the enzyme. We have already constructed a CGTase expression system for *E. coli* and studied the influences of environmental factors on CGTase production [9, 10]. However, in an attempt to improve the product yield, CGTase synthesized in *E. coli* was aggregated to form an insoluble inclusion body. The process for recovering active and soluble enzymes from inclusion body is often laborious and cost-dependent. Alternatively, *Bacillus* species have been referred to being a useful microorganism for the expression and secretion of heterologous proteins [1]. Since *Bacillus* sp. can secret proteins in large amounts, many researchers have used this organism as a host for the manufacture of recombinant proteins such as α-amylase [3], β-glucosidase [5], human alpha 2 and beta-interferon [14], human serum albumin [13], and maltotetraose-producing amylase [6]. Secreted proteins are generally much easier and less costly to purify than intracellular proteins. Furthermore, they exist in a soluble and biologically active form. Many *Bacillus* species are also nonpathogenic to humans and animals since they lack pyrogenic lipopolysaccharides in contrast to some *E. coli* strains. In addition, a large-scale cultivation process of various *Bacillus* species is well developed [2].

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Vector Construction And Transformation

An expression vector was constructed as shown in Fig. 1. The *cgt* gene originated from *B. macerans* was obtained from plasmid pTCGT1 [7]. The 2.1 kb DNA fragment containing the ribosome-binding sequence (SD sequence), signal sequence, and *cgt* structural gene from *B. macerans* was ligated with plasmid pUC119, which had also been treated with the same endonucleases and calf intestinal alkaline phosphatase (CIP). Plasmid p8A1 [4] containing the *amy*R2 promoter region was digested with both *Xba*I and *Hin*dIII to isolate a 1 kb DNA fragment containing the *amy*R2 promoter region. In this case, the *amy*R2 promoter was an α-amylase hyperproducing promoter derived from *B. subtilis* var. *natto* [15]. The fragment was further digested by *Hin*fI and then end-filled with Klenow fragment. The *amy*R2 promoter region (0.32 kb) was isolated and combined with pUC-CGT119 which had been treated with *Sma*I endonuclease and CIP to obtain pUC-A-CGT119.

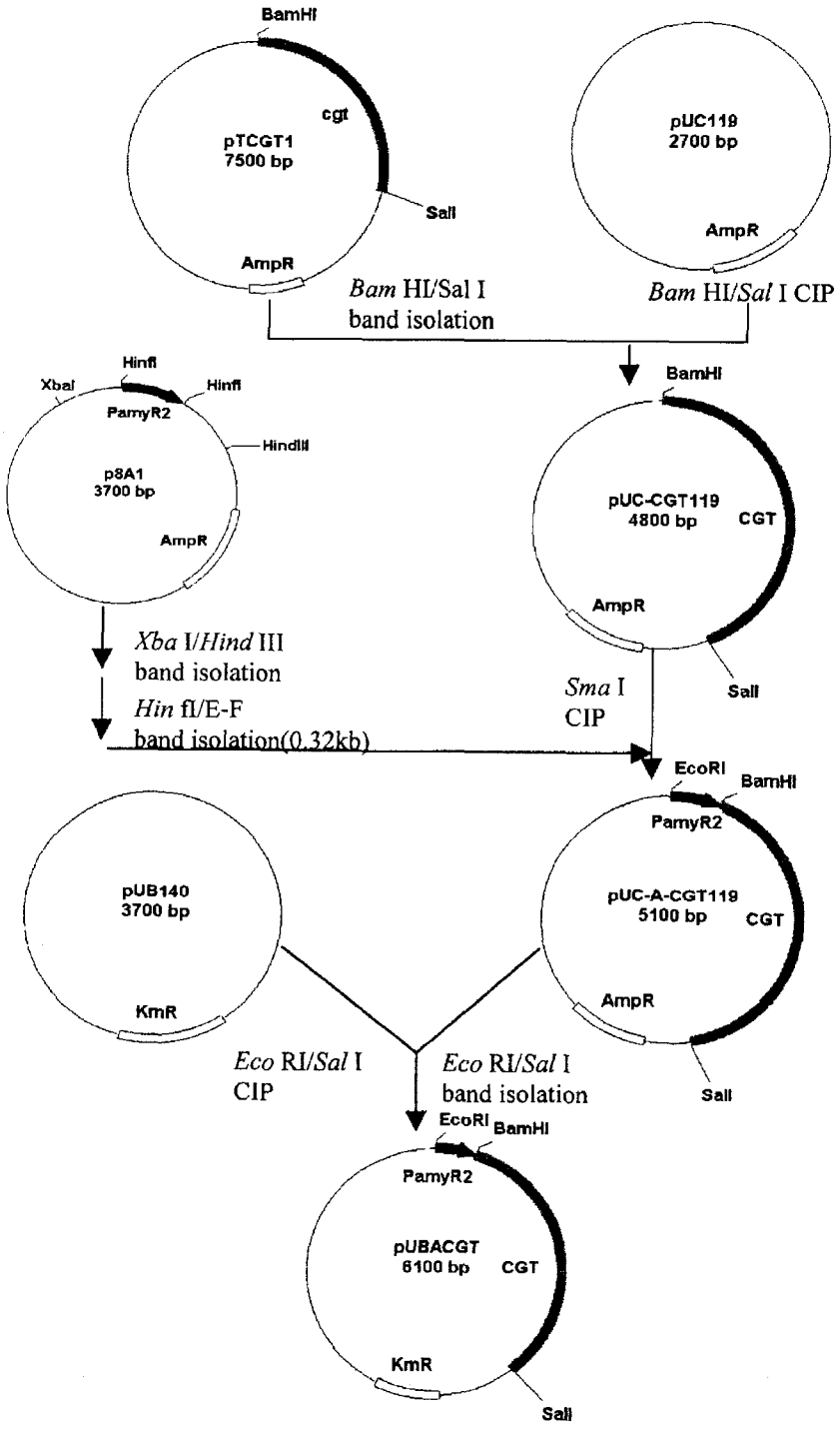

Fig. 1. Construction of pUBACGT, a CGTase expression vector for *B. subtilis* LKS88.
PamyR2, *amy*R2 promoter; CGT, *cgt* gene for CGTase; KmR, kanamycin resistant gene; AmpR, ampicillin resistance gene; CIP, calf intestinal alkaline phosphatase.

The nucleotide and amino acid sequence of the junction region of *B. macerans* CGTase gene and *amy*R2 promoter region of *B. subtilis* var. *natto* was represented in Fig. 2. The DNA sequence analysis of the plasmid confirmed that the *amy*R2 promoter region was inserted in the correct direction. DNA sequencing was carried out by the modified Sanger method [12] according to the procedure of the USB sequenase kit version 2.0. The small DNA fragment (2.5 kb) was finally ligated with plasmid pUB140 containing kanamycin marker after double digestion by *Eco*RI and the

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CCC/ATTCTTCT TTTTATCGTC TGCGGCGGCG TTCTGTTTCT GCTTCGGTAT GTGATTGTGA
SmaI/HinfI-EF
  AGCTGGCTTA CAGAAGAGCG GTAAAAGAAG AAATAAAAAA GAAATCATCT TGAAAAATAG

  ATGGTTTTTT TTTTTGTTTG GAAAGCGAGG GAAACAGTCT CGGGCAGTTT TTTATAGGAC

  CATTGATTTG TATTCACTCT GCCAAGTTGT TTTGATAGAG TGATTGTGAT AATTTAAAAT

  GTAAGCGTAA ACAAAATTCT CCAGTCTTCG CATCAGTTTG AAAGGAGGAA GCGGAAGAAT

  GAAGTAAGAG GGATTTTTGA CTCGGGATC CGCAGAACAT TTGAGAGGAG AGGTAGGACA
                HinfI-EF/SmaI
Met Lys Ser Arg Tyr Lys Arg Leu Thr Ser Leu Ala Leu Ser Leu Ser Met
ATG AAA TCG CGG TAC AAA CGT TTG ACC TCC CTG GCG CTT TCG CTG AGT ATG
Signal Sequence
Ala Leu Gly Ile Ser Leu Pro Ala Trp Ala Ser Pro Asp Thr Ser Val Asp
GCG TTG GGG ATT TCA CTG CCC GCA TGG GCA TCA CCC GAT ACG AGC GTG GAC
                                        CGTase
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Fig. 2. The nucleotide and amino acid sequence of the junction region of *B. macerans* CGTase gene and PamyR2 of *B. subtilis* var. *natto*.

PamyR2 gene is located in between *Sma*I sites. Underlines represent *Sma*I sites constructed after *Hinf* I digestion and Klenow end-filling. The box and arrow indicate the signal sequence and the N-terminus of *B. macerans* CGTase, respectively.

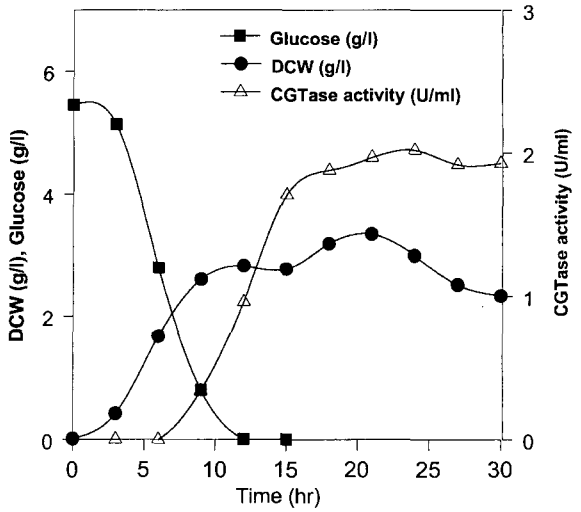

Fig. 3. Fermentation profile of recombinant *B. subtilis* LKS88.

The recombinant *B. subtilis* was cultured in a glucose containing LB medium and growth parameters including dry cell weight (DCW), glucose content, and CGTase activity in the medium were plotted in relation to cultivation time.

*Sal*I endonucleases, and the resulting plasmid was designated as pUBACGT. *B. subtilis* LKS88 [DB104 (*his npr*R2 *npr*E18 (*apr*A3)), WLN7 (*amy*E *aro*I906 *met*B *sac*A321), *trp*C2, *sac*U^{h}32] was used as a host strain after deletion of the α-amylase gene [4]. The transformation of *B. subtilis* LKS88 was carried out using the above ligation mixture according to the method of Sadai *et al.* [11]. For the preparation of *B. subtilis* competent cells, the SPI medium was used and its composition was as follows: 0.5% (w/v) glucose, 0.02% (w/v) casamino acid, 0.05% (w/v) yeast extract, and SP salt [0.2% (w/v) $(NH_4)_2SO_4$; 1.4% (w/v) K_2HPO_4; 0.6% (w/v) KH_2PO_4; 0.1% (w/v) sodium citrate; 0.02% (w/v) $MgSO_4$; pH 7.5]. Luria-Bertani (LB) medium [1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (w/v) NaCl] was used to grow *B. subtilis*. Kanamycin (10 μg/ml) was added to select the transformants.

Secretion of CGTase from Recombinant *B. subtilis* LKS88

The transformants of *B. subtilis* LKS88 selected from the kanamycin medium were tested on starch-containing LB plates by the iodine method in order to detect the hydrolytic activity of the CGTase secreted into the medium. Seven out of nine colonies showed a positive signal, a halo around the colony, indicating starch degradation by the secreted CGTase.

The transformant, *B. subtilis* LKS88 containing pUBACGT, was precultured in 5 ml of an LB medium and the cells were then transferred to a 200 ml medium containing 5 g/l of glucose and incubated at 37°C while shaking at 200 rpm. The cyclodextrin-forming activity of the CGTase was measured according to the absorbance change of the CD conjugates with methyl orange [8]. The dry cell weight, CGTase activity, and glucose content in the medium were plotted along with cultivation time in Fig. 3. The cyclizing activity of the CGTase was detected after 6 h in the medium and it reached a maximum level after 24 h. On the other hand, when cells were harvested by centrifugation and disrupted by sonication during fermentation, the enzyme activity inside the cells was undetectable. A SDS-PAGE analysis showed that a significant amount of CGTase was secreted from the cells at a high expression level (Fig. 4). When the protein concentration of the bands corresponding to the CGTase was analyzed by a densitometer (Bio-Rad GS-700), it was approximately 40% of total proteins secreted from cells and there was a good correlation between the

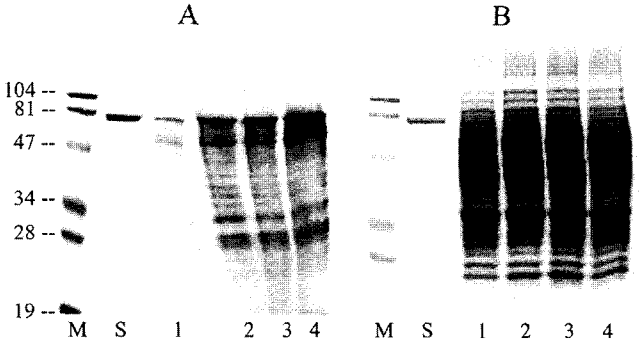

Fig. 4. SDS-PAGE analysis of CGTase expressed in recombinant *B. subtilis* LKS88.

A. Extracellular medium fraction; B. Intracellular fraction; M. Protein size markers (kDa); S. Standard CGTase from *B. macerans*. Numbers 1, 2, 3, and 4 correspond to the post-induction times of 15, 20, 24, and 26 h, respectively.

density profile of CGTase in Fig. 4 and CGTase activity levels in Fig. 3.

In conclusion, *B. subtilis* can be used as an effective and useful host for the expression and secretion of *B. macerans* CGTase.

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