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Cloning of a *Paenibacillus* sp. Endo-β-1,4-Glucanase Gene and Its Coexpression with the *Endomyces fibuliger* β-Glucosidase Gene in *Saccharomyces cerevisiae*

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Abstract A gene, *Egl*, from *Paenibacillus* sp. KCTC 8848P encoding endo-β-1,4-glucanase was cloned and expressed in *Escherichia coli*. It consisted of an open reading frame of 1,191 bp for a protein that consisted of 397 amino acids with a molecular weight of 44,539 Da. The deduced amino acid sequence of the endo-β-1,4-glucanase gene had a 94% similarity to the endo-β-1,4-glucanase of *Bacillus polymyxa*. The *Egl* gene was also expressed in *Saccharomyces cerevisiae* secreting *Endomyces fibuliger* β-glucosidase (*BGL1*) under the control of the alcohol dehydrogenase (*ADC1*) gene promoter. *S. cerevisiae* transformant producing both endo-β-1,4-glucanase and β-glucosidase grew on carboxymethyl cellulose as the sole carbon source.

Key words: *Endomyces fibuliger* β-glucosidase gene, *Paenibacillus* sp. endo-β-1,4-glucanase gene, *Saccharomyces cerevisiae*

The bioconversion of cellulose into glucose requires actions of three types of enzyme (cellulase enzyme complex), including endo-β-1,4-glucanase, cellobiohydrolase, and β-glucosidases [3]. The first two enzymes act synergistically to depolymerize cellulose to cellobiose and cellooligosaccharides, while β-glucosidase (or cellobiase) subsequently hydrolyzes these sugars to glucose. Endo-β-1,4-glucanase (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4) attacks the centers of the cellulose chains at random, breaking into smaller fragments, and progressively generates nonreducing ends on which the cellobiohydrolase can take effect. Numerous studies conducted on endo-β-1,4-glucanase genes from bacteria to fungi have been reported [4]. Members of the industrially important *Bacillus* also produce endo-β-1,4-glucanases, and the genes encoding these enzymes have been cloned and characterized [1, 15, 16, 17, 19]. *Saccharomyces cerevisiae*, in contrast to other cellulolytic bacteria and fungi, is unable to utilize cellulose-rich substrates. In an attempt to supply *S. cerevisiae* with endo-β-1,4-glucanase activity, several heterologous endo-β-1,4-glucanase genes have been cloned and expressed in *S. cerevisiae* [6, 13, 21, 29, 30]. Recently, an endo-β-1,4-glucanase producing bacterium, *Paenibacillus* sp. KCTC 8848P, was isolated from soil [14]. In this paper, the cloning and nucleotide sequence determination of the endo-β-1,4-glucanase gene from this bacterium and its coexpression with the *Endomyces fibuliger* (*Saccharomycopsis fibuligera*) β-glucosidase gene (*BGL1*) in *S. cerevisiae* are described.

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Materials and Methods

Strains and Plasmids

Paenibacillus sp. KCTC 8848P [14] was used as a source of the endo-β-1,4-glucanase gene, and *Endomyces fibuliger* CBS 342.83 (CBS 6310) [18] was used as a source of the β-glucosidase gene. *Escherichia coli* JM83 was used for all bacterial transformations and plasmid preparations. pUC19 was not only used as the cloning vector, but as the subcloning vector for the DNA sequencing as well. *Saccharomyces cerevisiae* W303-1A [10] was used as a host for yeast transformation. pYES2 (Invitrogen, San Diego, U.S.A.) and pAUR123 (TaKaRa, Japan) containing the aureobasidin A (antifungal antibiotic) resistance gene (*AUR1-C*) were used for the construction of yeast recombinant plasmids. All procedures for the plasmid manipulation and preparation, and the transformation of *E. coli* were performed by the methods described by Sambrook and Russell [25].

Media and Culture

For the endo-β-1,4-glucanase production, *Paenibacillus* sp. KCTC 8848P was cultured on a buffered Luria-Bertani

(BLB) medium containing 0.1 M sodium phosphate buffer (pH 6.0), 0.5% Difco yeast extract, 1% Difco tryptone, and 1% NaCl supplemented with 0.5% carboxymethyl cellulose (CMC, Sigma, St. Louis, U.S.A.) at 37°C for 2 days with shaking. The *E. coli* transformants were grown at 37°C for 2 days with shaking in a BLB medium supplemented with 50 μg/ml of ampicillin and 0.5% CMC when required. A YPD medium (1% Difco yeast extract, 2% Difco peptone, and 2% dextrose) was used as a complete medium for the culture of yeast cells. Various concentrations of aureobasidin A (0.1–0.5 μg/ml, TaKaRa, Japan) were added to the YPD plates. The concentration of aureobasidin A in which the yeast colony could not grow was determined [27]. The yeast cells were then transformed according to the lithium acetate/DMSO method of Hill *et al.* [9]. The minimal selective medium (SD) for the yeast transformants contained 0.67% Difco yeast nitrogen base (without amino acid), 2% dextrose, 2% Bacto-agar, and nutritional supplements as required [26]. The yeast transformants grown on the SD agar plates were transferred onto the YPD agar plates containing 20–50 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-glu, Sigma, St. Louis, U.S.A.) (YPDXG) to test the blue color-forming ability as a result of β-glucosidase activity after incubation for 3 days at 30°C. The yeast transformants grown on the YPD plates containing aureobasidin A were transferred onto the YPDC agar plates (YPD containing 0.5% CMC and 2% Bacto-agar) to test the halo-forming ability by staining with 0.2% Congo red solution as a result of endo-β-1,4-glucanase activity after incubation for 3–5 days at 30°C [28]. Buffered YPD (BYPD) medium containing 0.1 M sodium phosphate buffer (pH 6.0) was used to assay the endo-β-1,4-glucanase activity and β-glucosidase activity which were secreted by yeast transformants [10]. BYP medium containing 2% cellobiose was also used to assay the β-glucosidase activity secreted by *End. fibuliger* [18]. Mitotic stability of the *BGL1* gene was determined by the method of Kim and Kim [12].

Preparation of Chromosomal DNA and Construction of Genomic Libraries

The chromosomal DNA of *Paenibacillus* sp. was isolated according to the procedure of Murray and Thompson [20] using cetyl trimethyl ammonium bromide (CTAB, Sigma, St. Louis, U.S.A.). The DNA was partially digested with *Sau*3AI, and ligated to the *Bam*HI site of pUC19. The ligated mixture was then transformed in *E. coli*, and the resulting bacterial transformants were incubated and selected on LB plates supplemented with 50 μg/ml of ampicillin and 0.5% CMC for 2 days at 37°C. The clones showing endo-β-1,4-glucanase activity were detected by the halos around the colonies by Congo red staining. Recombinant plasmid DNA was isolated by using a QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, U.S.A.).

DNA Sequence Analysis

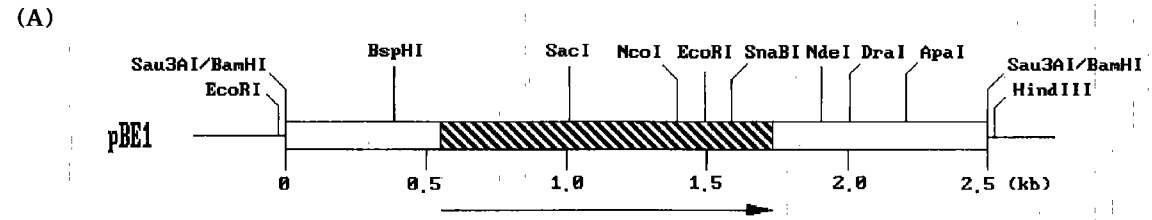
The nucleotide sequence of the endo-β-1,4-glucanase gene was determined by an ABI PRISM 377 DNA sequencer. The PC/GENE and DNASIS software systems were employed to analyze the DNA sequence. The nucleotide sequence and deduced amino acid sequence were analyzed with the databases by using BLAST programs. The nucleotide sequence reported in this paper was deposited in the GenBank database under the accession number of AF345984.

Amplification of the Endo-β-1,4-glucanase Gene and *BGL1* Gene by Polymerase Chain Reaction (PCR)

For the amplification of the endo-β-1,4-glucanase gene from the start codon to the termination codon, two oligo primers (p5' and p3') were designed based on the sequence analysis. The sequence around the start codon and the sequence downstream of the TAG termination codon were used for the design of p5' and p3' oligo primers, respectively. In two oligo primers, a *Bam*HI site (GGATCC) was introduced to facilitate the cloning of the endo-β-1,4-glucanase gene. The primer sequences were as follows: p5' (5'-GGAGGATCCATTATGAAGAAAAAAGGGTTAAAA-3') and p3' (5'-TCCGGATCCTGAATAAACAAAGTGAAGTC-3'). The PCR conditions were: an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 0.5 min, and an extension at 72°C for 1 min. On the other hand, the *BGL1* gene of *End. fibuliger* was amplified with oligonucleotides 5'-TTCGAATTCACCATGTTGATGATAGTACAGCTTTTG-3' and 5'-CCGCTGCAGGAATTCTCAAATAGTAAACAGGACAGACGT-3'. These primers were synthesized from the published nucleotide sequence of the genomic copy of the *BGL1* gene from *Saccharomycopsis fibuligera* HUT7212 producing extracellular β-glucosidases (GenBank accession number M22475) [18]. The PCR condition was known as an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 0.5 min, annealing at 45°C for 0.5 min, and an extension at 72°C for 1 min.

Enzyme Assays

The reaction mixture for measuring the endo-β-1,4-glucanase activity contained 900 μl of 0.1 M sodium phosphate buffer (pH 6.0) with 0.5% CMC and 100 μl of centrifuged culture fluid as a crude enzyme. After 60 min incubation at 55°C, the contents of the reducing sugars were measured by using the DNS method [10]. One unit of endo-β-1,4-glucanase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per ml per min. β-Glucosidase activity was measured, with *p*-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma, St. Louis, U.S.A.) as a substrate, by the method described by Chun *et al.* [2]. One unit of β-glucosidase activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per ml per min.



(B)

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gat ctt cgt caa ccc ctt aac aag ctc acc ctc ttt gcg cac agg cgg ctg tac att ctc gcc cca ttc ctg aag      75
tgt gcg gta agc ggc ttc ctg cac agc ata tac tgt atc att gtt caa acg gtg tgt cag caa gtc aat cgt ttg     150
ctg gtt ttt cca tgc ccc tag ttc att cac cgc agc tag acg cgt ttt cca atc gga ggt gcg gtt tgc agc tac     225
ttt aag ctg ctc ata tac ttc tgt aaa ttc ctg ttg cgt ttc att gtt ttc caa aat aat ctc atc ctt ttc taa     300
aaa tca cta tat atc tgt tca tca cga ttg ccg ttt gtt aaa tat aac ata ctt gca ggt ctt ctt cta aaa aag     375
                                                                    -35
att atc atg aaa tgt gta ggg gaa aac tta acc ttc cgt tgt taa aaa tct aat gag ttc gaa caa aat ctt aaa     450
  -10
aat ata cga att tta tga act tgt ctg atg gaa aga ttt gaa cat aaa atg aaa atg taa tcc ggt tac aaa taa     525
att aat caa gga gga agc aga att ATG AAG AAA AAA GGG TTA AAA AAA ACA TTT TTC GTC ATT GCC TCC CTC GTC     600
            SD                  M   K   K   K   G   L   K   K   T   F   F   V   I   A   S   L   V    (17)
ATG GGC TTT ACG CTG TAT AGC TAC AGT CCC CCT TCA GCA GAT GCA GCC AGT GTG AAA GGA TAT TAC CAC ACT CAA     675
 M   G   F   T   L   Y   S   Y   S   P   P   S   A   D   A   A   S   V   K   G   Y   Y   H   T   Q    (42)
GGA AAC AAG ATT GTA GAT GAA ACC GGG AAA GAA GCT GCA TTT AAC GGT CTG AAC TGG TTC GGT CTG GAA ACC CCT     750
 G   N   K   I   V   D   E   T   G   K   E   A   A   F   N   G   L   N   W   F   G   L   E   T   P    (67)
AAT TAC ACC TTG CAT GGA CTG TGG AGC CGC TCA ATG GAC GAC ATG CTG GAT CAG GTG AAG AAG GAA GGC TAT AAT     825
 N   Y   T   L   H   G   L   W   S   R   S   M   D   D   M   L   D   Q   V   K   K   E   G   Y   N    (92)
CTC ATC CGT CTG CCT TAT AGC AAT CAG CTA TTC GAT TCC AGT TCC CGT GCG GAC AGT ATT GAT TAC TAC AAA AAT     900
 L   I   R   L   P   Y   S   N   Q   L   F   D   S   S   S   R   A   D   S   I   D   Y   Y   K   N   (117)
CCT GAT CTG GTC GGA TTG ACC CCG ATT CAA ATT ATG GAC AAG CTG ATC GAA AAA GCT GGA CAA CGC GGT ATC CAG     975
 P   D   L   V   G   L   T   P   I   Q   I   M   D   K   L   I   E   K   A   G   Q   R   G   I   Q   (142)
ATT ATC CTT GAC CGT CAC CGT CCA GGC TCA GGT GGG CAA TCG GAG CTC TGG TAT ACC TCC CAG TAC CCT GAG TCC    1050
 I   I   L   D   R   H   R   P   G   S   G   G   Q   S   E   L   W   Y   T   S   Q   Y   P   E   S   (167)
CGC TGG ATT AGT GAC TGG AAG ATG TTG GCC GAA CGA TAT AAA AAC AAT CCT ACC GTT ATT GGT GCA GAT TTA CAC    1125
 R   W   I   S   D   W   K   M   L   A   E   R   Y   K   N   N   P   T   V   I   G   A   D   L   H   (192)
AAC GAG CCA CAC GGT CAG GCA AGC TGG GGG ACA GGC GAT GTC TCC ACA GAC TGG CGT CTT GCG GCG CAG CGT GCA    1200
 N   E   P   H   G   Q   A   S   W   G   T   G   D   V   S   T   D   W   R   L   A   A   Q   R   A   (217)
GGG AAT GCC ATT CTG TCC GTG AAT CCG AAT TGG CTG ATT CTC GTA GAA GGT GTG GAT CAC AAT GTC AAA GGC AAC    1275
 G   N   A   I   L   S   V   N   P   N   W   L   I   L   V   E   G   V   D   H   N   V   K   G   N   (242)
AAC AGC CAA TAC TGG TGG GGC GGC AAC TTG ACA GGT GTA GCT AAT TAT CCT GTC GTT CTG GAC GTA CCG AAC CGT    1350
 N   S   Q   Y   W   W   G   G   N   L   T   G   V   A   N   Y   P   V   V   L   D   V   P   N   R   (267)
GTC GTC TAT TCT CCA CAT GAC TAC GGC CCT GGT GTG TCT TCA CAG CCA TGG TTC AAC GAC TCG ACC TTC CCA TCC    1425
 V   V   Y   S   P   H   D   Y   G   P   G   V   S   S   Q   P   W   F   N   D   S   T   F   P   S   (292)
AAC CTG CCA GCG ATC TGG GAT CAA ACC TGG GGC TAC ATC AGC AAG CAA AAC ATT GCC CCA GTG CTG GTC GGT GAA    1500
 N   L   P   A   I   W   D   Q   T   W   G   Y   I   S   K   Q   N   I   A   P   V   L   V   G   E   (317)
TTC GGC GGC CGC AAT GTT GAT TTG TCG TCC CCT GAG GGG AAA TGG CAA AAT GCG CTT GTT GAC TAT ATC GGT GCC    1575
 F   G   G   R   N   V   D   L   S   S   P   E   G   K   W   Q   N   A   L   V   D   Y   I   G   A   (342)
AAC AAC CTG TAC TTT ACG TAT TGG TCC CTC AAT CCG AAT AGC GGC GAC ACA GGC GGT CTA CTG CTG GAT GAC TGG    1650
 N   N   L   Y   F   T   Y   W   S   L   N   P   N   S   G   D   T   G   G   L   L   L   D   D   W   (367)
ACT ACC TGG AAT CGT CCG AAA CAG GAC ATG CTG AGC CGG ATT ATG AAG CCT GTT CAT TTC ATA GCA GAG CAA GCA    1725
 T   T   W   N   R   P   K   Q   D   M   L   S   R   I   M   K   P   V   H   F   I   A   E   Q   A   (392)
AAA GCA GCC GCC GAA TAG gca tag gac ttc act ttg ttt att caa aag ccg att ttc ctc ctt cag gtc aga tta    1800
 K   A   A   A   E   *                                                                               (397)
tgc tga gac tat gaa gag gtg gag aaa cgg ttt ttt tgt tct tcc agt ttt aca cat tgt aaa tcc cac att gaa    1875

tgg ggc att ttg taa act aaa tct ctc ttt taa ccg ata aat aca tat gtt gga ttt gaa att tat ttc gac gca    1950

gga cgc acc atg aac gta aac taa tca gga aag gaa gtt tgc aag tga tta ttt gga agg gtt tcg gta ttt taa    2025
acg tca tta ttc cag caa ttt tat ttt tta ttg tcg gga gtt tgg tat ccg ctc tag ggc ttg act cta           2094
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Fig. 1. Restriction map of the 2.5-kb insert DNA of pBE1 (A) and the complete nucleotide and deduced amino acid sequences of the *Paenibacillus* sp. *Egl* gene (B).

(A) The box represents the coding region of *Egl* and the transcription direction is indicated by an arrow. The thick and thin lines represent the *Paenibacillus* sp. and pUC19 DNAs, respectively. (B) The putative promoter, Shine-Dalgarno (SD) sequence, and 32-amino acid putative signal peptide are underlined. The upward arrow indicates the putative cleavage site. The primers used for the amplification of the endo-β-1,4-glucanase gene are marked by double underlines. The arrows indicate inverted repeat sequences.

RESULTS AND DISCUSSION

Cloning of the *Paenibacillus* sp. Endo-β-1,4-glucanase Gene and Its Sequence Analysis

A genomic library of *Paenibacillus* sp. KCTC 8848P was constructed in *E. coli* by using pUC19, and approximately 6,000 transformants were screened for their endo-β-1,4-glucanase activity. Five endo-β-1,4-glucanase-positive clones were found to produce halos on the LB plates which were supplemented with CMC and ampicillin after staining with Congo red [1]. However, only one clone showed clear halos around its colonies, even after several transfers onto the same plates. When the recombinant plasmid isolated from this clone, designated as pBE1, was used to transform *E. coli*, all the transformants stably produced endo-β-1,4-glucanase. The restriction map of the 2.5-kb insert on pBE1 is shown in Fig. 1A. The nucleotide sequence of the endo-β-1,4-glucanase encoding gene (*Egl*) and its flanking regions in pBE1 is shown in Fig. 1B. The 1,191-bp open reading frame started with the ATG codon at position 550 and terminated with the TAG stop codon at position 1,741, encoding a protein of 397 amino acids with a molecular mass of 44,539 Da. A putative ribosome-binding site (Shine-Dalgarno sequence) was located 11 bp upstream from the initiation codon. GCAGGT and ATTATC sequences located at positions 355 and 376, respectively, upstream from the Shine-Dalgarno sequence corresponded to the putative −35 and −10 regions of the promoter. When the putative signal sequence of a 397-amino acid protein was predicted by the −3, −1 rule of von Heijne [31], its cleavage site appeared to be between Ala (position 32) and Ala (position 33). As shown in Fig. 2, the amino acid sequence alignment analysis of *Egl* from *Paenibacillus* sp. by the family A cellulases [4] showed the following degrees of identity: 94% with *Bacillus polymyxa Egl* (accession No. M33791), followed by 46% with *Xanthomonas campestris Eng*XCA (M32700), and 38% with *Clostridium thermocellum CelB* (X03592), but very little similarity was shown to other endo-β-1,4-glucanases from *Bacillaceae* belonging to family 5 and 8 glycosyl hydrolases [1, 7].

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Paeni    1  ---MKKKGLKKTFFV IASLVMGFTLYSYSP PSADAASVKGYYHTQ GNKIVDETGKEAAFN GLNWFGLETPNYTLH GLWSRSMDDMLDQVK   87
B.pol    1  ---MKKKGLKKTFFV IASLVMGFTLYGYTP VSADAASVKGYYHTQ GNKIVDESGKEAAFN GLNWFGLETPNYTLH GLWSRSMDDMLDQVK   87
X.cam    1  ---------------- MSIFRTASTLALATA LALAAGPAFSYSINN SRQIVDDSGKVVQLK GVNVFGFETGNHVMH GLWARNWKDMIVQMQ   75
C.the    1  MKKFLVLLIALIMIA TLLVVPGVQTSAEGS YADLAEPDDDWLHVE GTNIVDKYGNKVWIT GANWFGFNCRERMLL DSYHSDIIADIELVA   90
                                                                  ***  *       * * **

Paeni   88  KEGYNLIRLPYSNQL FDSSSR----ADSID YYKNPDLVGLTPIQI MDKLIEKAGQRGIQI ILDRHRPGSGGQSE- --LWYTSQYPESRWI  170
B.pol   88  KEGYNLIRLPYSNQL FDSSSR-----PDSID YHKNPDLVGLNPIQI MDKLIEKAGQRGIQI ILDRHRPGSGGQSE- --LWYTSQYPESRWI  170
X.cam   76  GLGFNAVRLPFCPAT LRSDTM-----PASID YSRNADLQGLTSLQI LDKVIAEFNARGMYV LLDHHTPDCAGISE- --LWYTGSYTEAQWL  158
C.the   91  DKGINVVRMPIATDL LYAWSQGIYPPSTDT SYNNPALAGLNSYEL FNFMLENFKRVGIKV ILDVHSPETDNQGHN YPLWYNTTITEEIFK  180
              * *  * *                          * * **              *     ** * *          ***     *

Paeni  171  SDWKMLAERYKNNPT VIGADLHNEPHG--- -------QASWGTGD VSTDWRLAAQRAGNA ILSVNPNWLILVEGV DHN-----------  238
B.pol  171  SDWKMLADRYKNNPT VIGADLHNEPHG--- -------QASWGTGN ASTDWRLAAQRAGNA ILSVNPNWLILVEGV DHN-----------  238
X.cam  159  ADLRFVANRYKNVPY VLGLDLKNEPHG--- -------AATWGTGN AATDWNKAAERGSAA VLAVAPKWLIAVEGI TDNP-----------  227
C.the  181  KAWVWVAERYKNDDT IIGFDLKNEPHTNTG TMKIKAQSAIWDDSN HPNNWKRVAEETALA ILEVHPNVLIFVEGV EMYPKDGIWDDETFD  270
                 * ****      * ** ****             *        *  *     *  * * *  ** ***

Paeni  239  -----VKGNNSQYWW GGNLTGVANYPVVLD VPN-RVVYSPHDYGP GVSSQPWFNDSTFPS N---------LPAIWD QTWGYISKQNIAPVL  314
B.pol  239  -----VQGNNSQYWW GGNLTGVANYPVVLD VPN-RVVYSPHDYGP GVSSQPWFNDPAFPS N---------LPAIWD QTWGYISKQNIAPVL  314
X.cam  228  -----VCSTNGGIFW GGNLQPLACTPLNIP AN--RLLLAPHVYGP DVFVQSYFNDSNFPN N---------MPAIWE RHFGQFAG--THALL  300
C.the  271  TSPWTGNNDYYGNWW GGNLRGVKDYPINLG KYQSQLVYSPHDYGP IVYEQDWFKGDFITA NDEQAKRILYEQCWR DNWAYIMEEGISPLL  360
                       * ****      *             ** ***  *  *  *        *           *                *

Paeni  315  VGEFGGRNVD-----L SSPEGKWQNALVDYI GAN-NLYFTYWSLNP NSGDTGGLLLDDWTT WNRPKQDMLS------ ----------------  379
B.pol  315  VGEFGGRNVD-----L SCPEGKWQNALVHYI GAN-NLYFTYWSLNP NSGDTGGLLLDDWTT WNRPKQDMLG------ ----------------  379
X.cam  301  LGEFGGKYGE-----G DARDKTWQDALVKYL RSKGINQGFYWSWNP NSGDTGGILRDDWTS VRQDKMTLLRTLWGT AGNTTPTPTPTPTPT  386
C.the  361  LGEWGGMTEGGHPLL DLNLKYLRCMRDFIL ENKYKLHHTFWCINI DSADTGGLFTRDEGT PFPGGRDLKWNDNKY DNYLYPVLWKTEDGK  450
              ** **                                  * *   * ****    *

Paeni  380  ---------------- ------RIMKPVHFI AEQAKAAAE------ ---------------- ----------------  397
B.pol  380  ---------------- ------RIMKPVVSV AQQAEAAAE------ ---------------- ----------------  397
X.cam  387  PTPTPTPTPTPTPGT STFSTKVIASPVVGS AARKLPAASRLACHW PASSTG----WRVWV IAAPSVTWKRPHAAR AIERRMRVTRLRRAT  472
C.the  451  FIGLDHKIPLGRNG- ISISQLSNYTPSVTP SPSATPSPTTITAPP TDTVTYGDVNGDGRV NSSDVALLKRYLLGL VENINKEAADVNVSG  539

Paeni  397  ---------------- ---------  397
B.pol  397  ---------------- ---------  397
X.cam  473  RLNRGPLPAPAHTTQ HAPRLR---  493
C.the  540  TVNSTDLAIMKRYVL RSISELPYK   563
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Fig. 2. Comparison of the amino acid sequence of the *Paenibacillus* sp. endo-β-1,4-glucanase gene with those of other endo-β-1,4-glucanases.

The sequence of the *Paenibacillus* sp. endo-β-1,4-glucanase (Paeni) is shown compared with *Bacillus polymyxa Egl* (B. pol), *Xanthmonas campestris Eng*XCA (X. cam), *Clostridium thermocellum CelB* (C. the). The identical amino acids are indicated by asterisks.

Construction of Recombinant Plasmids for the Expression of the *Egl* Gene and *BGL1* Gene in *S. cerevisiae*

Since the *Paenibacillus* sp. gene promoter is not functional in *S. cerevisiae*, the expression of the *Egl* gene was directed by the promoter sequence derived from the yeast alcohol dehydrogenase I (*ADC1*) gene. However, the secretion of endo-β-1,4-glucanase in *S. cerevisiae* was obtained with its own signal sequence, similar to the secretions of *B. amyloliquefaciens* α-amylase and *B. subtilis* endo-β-1,4-glucanase in *S. cerevisiae* by their own signal sequences [6, 24]. A 1.2-kb *Bam*HI amplified DNA fragment of the endo-β-1,4-glucanase coding region containing its own signal sequence (from the start codon to the stop codon) was inserted into the same site between the *ADC1* promoter and *CYC1* terminator of pYES2 containing the *ADC1* promoter [10], thereby generating pYBE1 (Fig. 3A). A 1.2-kb *Spe*I-*Xba*I DNA fragment containing the *Egl* gene was isolated from pYBE1, and inserted into the *Xba*I site between the *ADC1* promoter and *ADC1* terminator of pAUR123, generating pAURBE1 (Fig. 3A). On the other hand, A 2.6-kb amplified DNA fragment of the *BGL1* gene (containing its own signal sequence) from *End. fibuliger* producing extracellular β-glucosidase [18], after being digested with *Eco*RI, was inserted into the same

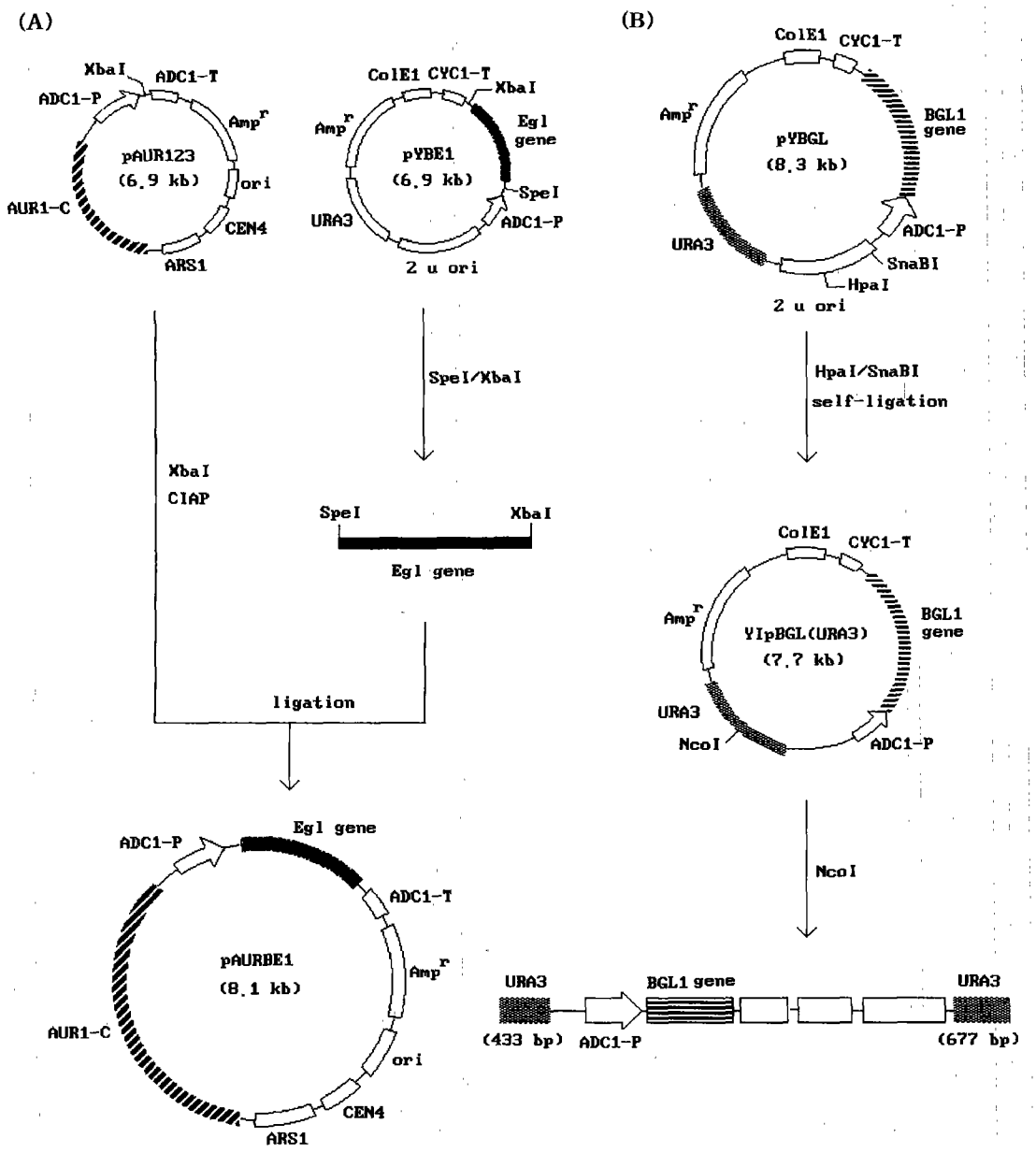

Fig. 3. Construction of recombinant plasmids pAURBE1, containing the *Egl* gene (A), and YIpBGL (*URA3*), containing the *BGL1* gene (B).

site of pYES2 containing the *ADC1* promoter, thereby generating pYBGL (Fig. 3B). For the stable expression of the *BGL1* gene in yeasts, a linearized integrating vector, YIpBGL (*URA3*), was constructed by self-ligation after a 2 micron origin was excised by digesting pYBGL with *Hpa*I and *Sna*BI. YIpBGL (*URA3*) exhibited a unique restriction site for *Nco*I within the *URA3* gene. Therefore, the linearized YIpBGL (*URA3*) digested with *Nco*I could be integrated into a homologous sequence of the *URA3* or *ura3* loci on the chromosome of a recipient yeast cell by initiating homologous recombination [8, 12]. The mitotic stability of YIpBGL (*URA3*) in yeast transformants (grown in nonselective media) was calculated as 100% after 100 generations [22].

Expression and Secretion of Endo-β-1,4-glucanase and β-Glucosidase in *S. cerevisiae*

S. cerevisiae W303-1A was unable to grow at a concentration of 0.4 μg/ml of aureobasidin A. *S. cerevisiae* W303-1A was transformed to Egl^+ AUR1-C (>0.4 μg/ml of aureobasidin A) with pAURBE1, generating W303-1A/pAURBE1. All the transformants secreting endo-β-1,4-glucanase formed halos around their colonies on YPDC agar plates (Fig. 4). *S. cerevisiae* was transformed to Ura^-Bgl^+ with YIpBGL (*URA3*), generating W303-1A/YIpBGL (*URA3*). All the transformants secreting β-glucosidase formed blue colonies on the YPDXG agar plates (data not shown). For coexpression of the *Egl* gene and *BGL1* gene, the *Egl* gene was introduced into a *BGL1*-integrated *S. cerevisiae* transformant [W303-1A/YIpBGL (*URA3*)] by using pAURBE1, thereby generating W303-1A/YIpBGL (*URA3*)/pAURBE1, which secreted both β-glucosidase and endo-β-1,4-glucanase. Endo-β-1,4-glucanase

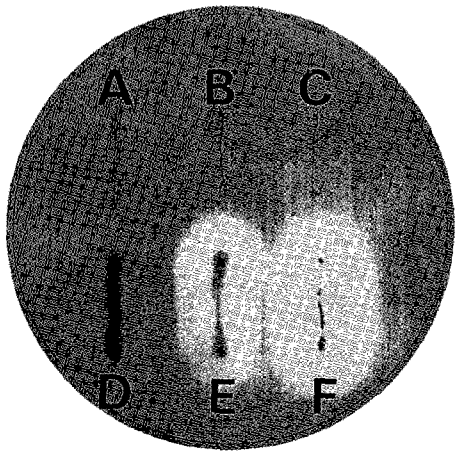

Fig. 4. Secretion of endo-β-1,4-glucanase by *S. cerevisiae* transformants on YPDC plate.
A, *S. cerevisiae* W303-1A; B, W303-1A/pAUR123; C, W303-1A/YIpBGL (*URA3*); D, W303-1A/pYBE1; E, W303-1A/pAURBE1; F, W303-1A/YIpBGL (*URA3*)/pAURBE1. Halos developed around W303-1A/pYBE1, W303-1A/pAURBE1, and W303-1A/YIpBGL (*URA3*)/pAURBE1 secreting endo-β-1,4-glucanase.

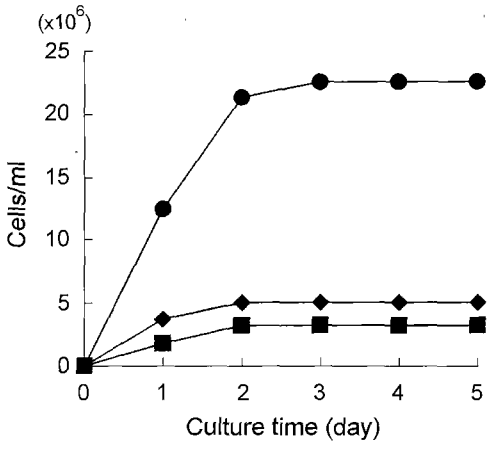

Fig. 5. Growth curve of *S. cerevisiae* transformants on minimal medium containing 0.5% carboxymethyl cellulose as the sole carbon source.
●, W303-1A/YIpBGL (*URA3*)/pAURBE1; ◆, W303-1A/pAURBE1; ■, W303-1A/YIpBGL (*URA3*).

production by *S. cerevisiae* transformants, which harbored pAURBE1 and grew in the glucose-containing media, was caused by the *ADC1* promoter without the regulatory site that was responsible for the repression of *ADC1* gene expression in the presence of glucose [11]. The utilizations of CMC by the different yeast transformants were compared with each other (Fig. 5). As expected, the control strain *S. cerevisiae* W303-1A, producing neither endo-β-1,4-glucanase nor β-glucosidase, consumed almost no CMC. Transformant W303-1A/pAURBE1 secreting only endo-β-1,4-glucanase also could not efficiently utilize CMC. According to the previous report by Raynal and Guerineau [23], *S. cerevisiae* cannot utilize cellobiose or cellotriose produced from CMC by endo-β-1,4-glucanase, because it does not possess a permease to transport cellobiose from the growth medium, and does not produce an extracellular β-glucosidase. Transformant W303-1A/YIpBGL (*URA3*)/pAURBE1, producing both endo-β-1,4-glucanase and β-glucosidase, grew on CMC as the sole carbon source (Fig. 5). This result indicates the degradation of CMC into glucose by the cooperative action of endo-β-1,4-glucanase and β-glucosidase [13]. To quantify the levels of extracellular cellulolytic activity produced by the various yeast transformants, their cell-free culture fluids were assayed for their endo-β-1,4-glucanase and β-glucosidase activities (Table 1). Endo-β-1,4-glucanase activity was present in the culture supernatant of the transformants containing *Egl*, W303-1A/pAURBE1 and W303-1A/YIpBGL (*URA3*)/pAURBE1. The endo-β-1,4-glucanase activity of W303-1A/YIpBGL (*URA3*)/pAURBE1 was 1.8 times higher than that of W303-1A/pAURBE1 when reducing sugar was assayed. It was considered that the β-glucosidase from W303-1A/YIpBGL (*URA3*) was in fact acting cooperatively with the endo-β-1,4-glucanase. The cellobiose released from cellulose hydrolysis has been

Table 1. Endo-β-1,4-glucanase and β-glucosidase activities in cell-free culture supernatants of various yeast strains.

Strains	Endo-β-1,4-glucanase acitivity (U/l)	β-Glucosidase acitivity (U/l)
Paenibacillus sp. KCTC 8848P	40[a]	ND[b]
Endomyces fibuliger CBS 342.83	0	163
Saccharomyces cerevisiae W303-1A	0	0
W303-1A/YIpBGL (*URA3*)	0	158
W303-1A/pAURBE1	41	20
W303-1A/YIpBGL (*URA3*)/pAURBE1	74	191

[a]Values are the means of results from triplicate experiments, and express the endo-β-1,4-glucanase activity and the β-glucosidase activity present in the culture supernatants.
[b]Not determined.

reported to be an inhibitor to endo-β-1,4-glucanase and cellobiohydrolase action [5, 32]. Thus, the removal of cellobiose from the reaction solution by β-glucosidase allows both endo-β-1,4-glucanase and cellobiohydrolase to function more efficiently. For the production of useful industrial products, such as fuel alcohol and protein-rich animal feed from cellulose-rich biomass, further attempts are now being made to develop stable industrial strains of *S. cerevisiae* capable of producing both endo-β-1,4-glucanase and β-glucosidase, and to introduce a cellobiohydrolase gene into the vector that carries both *Egl* and *BGL1* genes for the hydrolysis of crystalline cellulose [13, 29, 30, 33].

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