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REVIEW

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Alkaline cellulases from alkaliphilic *Bacillus*: Enzymatic properties, genetics, and application to detergents

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Abstract We have isolated a number of alkaliphilic *Bacillus* that produce alkaline exoenzymes and found a possible use for alkaline cellulase (carboxymethylcellulase) as an additive for improving the cleaning effect of detergents. Enzymatic properties of some candidate cellulases fulfilled the essential requirements for enzymes to be used practically in laundry detergents. Here I describe the properties and possible catalytic mechanism of the hydrolytic reaction and the gene for the industrial alkaline cellulase produced by one of the isolates, *Bacillus* sp. KSM-635.

Key words Alkaliphile · *Bacillus* · Cellulase · Cloning · Amino acid sequence · Detergent

Introduction

The heavy-duty detergents that are now commercially available already have good wash performance. The cleaning power of detergents seems to have peaked: all detergents contain similar ingredients and are based on the same detergent mechanism. Soil that has been adsorbed onto the surfaces of fibers or has been accumulated in their interstices is removed by surfactants and builders, which lower surface tension at interfaces and enhance the repulsive forces between soil and fabric. Various alkaline proteases have long been incorporated as biobuilders into heavy-duty detergents to hydrolyze and remove proteinaceous materials in soiled clothes (Horikoshi 1971, Hoshino et al. 1995, Kobayashi et al. 1995).

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In 1987, we developed a new compact-type detergent that incorporates an alkaline cellulase produced by our isolate, the alkaliphilic *Bacillus* sp. KSM-635. The alkaline cellulase is effective for the removal of soils from cotton fabrics without degradation of the cotton fibers. In this review, I describe the enzymatic properties and genetics of the alkaline cellulase from *Bacillus* sp. KSM-635. Furthermore, I show briefly that a new detergent mechanism may be associated with the use of the alkaline cellulase.

Alkaline cellulase-producing *Bacillus* strains

The industrial application of cellulases has mainly focused on fungal enzymes in terms of the potential of the enzymes for use in saccharification of cellulosic materials. Several species of bacteria, such as *Clostridium*, *Cellulomonas*, and *Ruminococcus*, have also been studied for their ability to produce cellulolytic enzymes. The members of the genus *Bacillus* are bacteria that produce various extracellular enzymes of industrial importance, including cellulase (Priest 1977). However, there have been no reports on the application of cellulases produced by these bacteria.

Alkaliphilic *Bacillus* strains often produce various alkaline enzymes, including alkaline cellulases (Horikoshi and Akiba 1982, Horikoshi 1996). Horikoshi and his colleagues found that alkaliphilic *Bacillus* strains No. N-4 (Horikoshi et al. 1984) and No.1139 (Fukumori et al. 1985), both of which had first been described in a Japanese patent, produced alkaline cellulases (or carboxymethylcellulose (CMC)-hydrolyzing enzymes, CMCases). Their discovery of the alkaline enzymes from the alkaliphiles led us to the eventual incorporation of cellulase in detergents. According to the screening method of Horikoshi, we isolated from soil samples many alkaliphilic strains, as well as neutrophilic strains, of *Bacillus* that produced alkaline cellulase, namely CMCase (Ito et al. 1989, Shikata et al. 1990, Okoshi et al. 1990, Lusterio et al. 1992). The cellulase- (CMCase) producing strains of *Bacillus* were easily picked up as colonies around which shallow craters or clear zones had been

Table 1. Effects of carbon sources on the production of alkaline cellulase by *Bacillus* sp. KSM-635.

Carbon source	Growth (A_{600}/3 days)	Cellulase produced (units/ml)
No addition	3.7	1.04
Carboxymethylcellulose	3.7	1.66
Cellulose powder	–	1.41
Avicel	–	1.53
Ribose	3.3	1.33
Glucose	5.8	1.22
Sucrose	5.3	2.77
Maltose	4.2	3.64
Cellobiose	3.7	1.91
Mannitol	4.4	3.03
Glycerol	5.5	2.12

The organism was grown at 30°C for 3 days, with shaking, in flasks that contained 50 ml of NY medium (Ito et al. 1989) plus a carbon source at 1% (w/v). The medium consisted (w/v) of 0.8% nutrient broth, 0.1% KH_2PO_4, and 0.5% Na_2CO_3. The carbon source and Na_2CO_3 were autoclaved separately. The amount of growth was measured in terms of absorbancy of the culture broth at 600 nm (A_{600}). Cellulase activities were measured by the amount of reducing sugar released from a 1.0% (w/v) solution of each substrate at pH 9.5 in 0.1 M glycine-NaOH buffer by the dinitrosalicylic acid procedure (Miller 1959). One unit of enzymatic activity was defined as the amount of protein that produced 1.0 µmole of reducing sugar, expressed as glucose, per min.

formed on alkaline CMC-agar plates or on alkaline plates that contained CMC and Trypan blue (or Congo red) dye, respectively. Among the isolates that produced alkaline cellulases potentially suitable for use in detergents, alkaliphilic *Bacillus* sp. KSM-635 was chosen (Ito et al. 1989). The optimum growth and productivity of alkaline cellulase of this isolate were observed in alkaline media, at 30°C–37°C and at pH values higher than 8. Members of the genus *Bacillus* usually require CMC for the production of cellulases (Horikoshi et al. 1984, Fukumori et al. 1985, Au and Chan 1987), but the cellulase of *Bacillus* sp. KSM-635 was produced almost constitutively, in terms of quantity, on various carbohydrates, and no apparent pattern was observed with respect to either the structure or composition of the carbon source used, as shown in Table 1.

One of the most important subsequent advances towards the economical industrial production of alkaline-cellulase-based detergents was the isolation of cellulase-hyperproductive variants by successive mutagenesis or the improvement of enzyme productivity by genetic engineering that involved host-vector systems. For instance, we found that some vancomycin- and risticetin-resistant variants of *Bacillus* sp. KSM-635 produced very high cellulase activity extracellularly (Ito et al. 1991). We improved this strain to increase productivity by single colony isolation, successive mutations, and optimization of culture conditions, and finally succeeded in producing the alkaline cellulase on a noncellulosic carbohydrate in an industrial scale plant. In addition, we also demonstrated the efficient production of the alkaline cellulase by recombinant *B. subtilis* cells carrying a new expression vector that contained the gene for this enzyme (Sumitomo et al. 1992, 1995).

Properties of the alkaline cellulases

The alkaline cellulase was purified to homogeneity from cultures of alkaliphilic *Bacillus* sp. KSM-635 by a two-step column-chromatographic procedure (Yoshimatsu et al. 1990). After gel filtration on Bio-Gel A-0.5 m, with prior chromatography on DEAE-Toyopearl 650S, two peaks of cellulase activity, a major peak (E-H, 130 kDa) and a minor peak (E-L, 103 kDa), were detected, with an activity ratio of approximately 7:3. Specific activities against CMC at pH 9.5 and at 40°C were 34 and 59 units/mg protein for E-H and E-L, respectively. E-H and E-L were alkaline enzymes, both having pH optima of 9.5, and they were very stable to incubation at pH 6–11, as shown in Fig. 1. The optimum temperature for activity at pH 9.5 was 40°C for both.

As shown in Table 2, E-H and E-L hydrolyzed CMC, lichenan (β-1,3;1,4-linkage), and 4-nitrophenyl β-D-cellobioside. Crystalline and amorphous forms of cellulose (Avicel, filter paper, H_2PO_4-swollen cellulose, and NaOH-swollen cellulose), curdlan (β-1,3-linkage), laminarin (β-1,3;1,6-linkage), and 4-nitrophenyl β-D-glucopyranoside were barely hydrolyzed at all. Both enzymes rapidly decreased the viscosity of solutions of CMC. Excess E-H and E-L hydrolyzed cellooligosaccharides (cellotriose through cellohexaose), yielding cellobiose and glucose after the completion of the reactions. Cellobiose was not hydrolyzed by these enzymes. Hence, it is probably more appropriate to refer to these enzymes as endo-1,4-β-glucanases rather than as cellulases (or CMCase). The enzyme activities were both

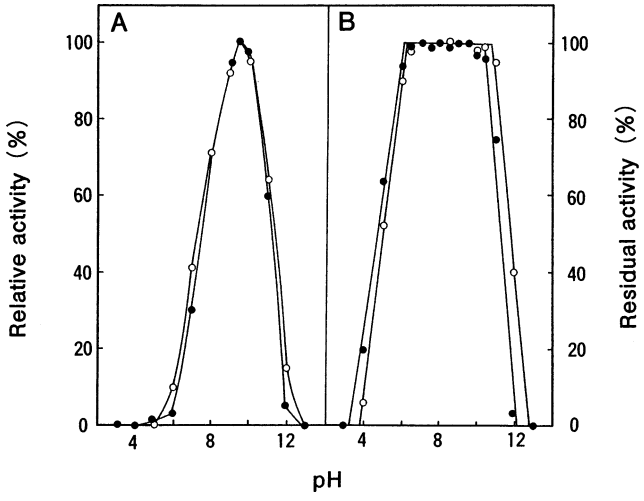

Fig. 1. Optimum pH for the carboxymethylcellulose (CMC)-hydrolyzing reactions of the major and minor peaks (E-H and E-L) of alkaline cellulase purified from the culture of *Bacillus* sp. KSM-635. **A:** The effect of pH on the activities of E-H (*open circles*) and E-L (*closed circles*) was determined with CMC as substrate at 40°C in various buffers ranging from pH 3 to pH 13. Each enzyme activity at pH 9.5 in 0.1 M glycine-NaOH buffer was taken as 100%. **B:** To determine the enzyme stability with changes in pH, E-H (*open circles*) and E-L (*closed circles*) were incubated at 5°C for 3 h in various buffers and then the residual activities were assayed at 40°C and at pH 9.5 in 0.1 M glycine-NaOH buffer. The values are shown as percentages of the respective original activity

Table 2. Substrate specificities of E-H and E-L

Substrate (1.0%, w/v)	E-H (units/mg protein)	E-L
CMC	23.5	43.8
Avicel	<1.1	<1.1
Filter paper	<1.1	<1.1
H_3PO_4-swollen cellulose	<1.1	<1.1
NaOH-swollen cellulose	<1.1	<1.1
Lichenan	19.7	42.5
Curdlan	0	0
Laminarin	0	0
Cellobiose	0	0
4-Nitrophenyl β-D-cellobiose[a]	0.3	0.6
4-Nitrophenyl β-D-glucopyronase[a]	0	0

Assays were done at 30°C in 0.1 M glycine-NaOH buffer (pH 9.5). E-H, E-L, major and minor peaks of alkaline cellulase; CMC, carboxymethylcellulose.
[a] A 4 mM solution of each substrate was used for assays.

inhibited by Hg^{2+} and Cu^{2+} ions, but various sulfhydryl inhibitors had either no effect or a slightly inhibitory effect. The activities were stimulated by Co^{2+} ions rather than Ca^{2+} ions. Both enzymes required Ca^{2+}, Mg^{2+}, Mn^{2+}, or Co^{2+} ions for thermal stability. *N*-Bromosuccinimide at low concentrations abolished the activities of both enzymes, a result indicating that one or more Trp residues is essential for the enzymatic activities. Both enzymes were resistant to various surfactants, such as linear-alkylbenzene sulfonate, alkyl sulfate α-sulfonate, and polyoxyethylene alkyl ether, and to chelating agents such as sodium tripolyphosphate, sodium citrate, zeolite, ethyleneglycoltetraacetic acid (EGTA), and ethylenediaminetetraacetic acid (EDTA). These properties of the enzymes conformed to the essential requirements for enzymes that can be used as effective additives in laundry detergents.

Genes for alkaline cellulases from *Bacillus*

The gene encoding an alkaline cellulase from *Bacillus* sp. KSM-635 was cloned into the *Hin*dIII site of pBR322 and expressed in *Escherichia coli* (Ozaki et al. 1990). In the coding region of the gene sequence cloned (3498 bp), there was only one open reading frame, which started with an ATG initiation codon (nucleotide 1) and ended with a TAA stop codon (nucleotide 2823). The complete open reading frame encoded an enzyme of 941 amino acid residues with a predicted molecular mass of 104 626 Da. The deduced amino acid sequence contained a short, hydrophilic, basic region and an adjacent, long, hydrophobic region. The hydrophilic–hydrophobic sequence is the characteristic of signal peptides of *Bacillus* (Mézes and Lampen 1985), and the residues Ala27-Ser28-Ala29 in the hydrophobic region may be the site required for recognition by a signal peptidase (Perlman and Halvorson 1983). If the signal peptide is cleaved on the carboxy-terminal side of Ala29, the molecular mass of the mature extracellular cellulase can be 101 412 Da, a value close to the 103 kDa determined for E-L. The sequence of amino acids 249–578 in a 2.4-kb fragment, which was proven by subcloning of the gene to be necessary for the cellulase activity, showed high homology to sequences of various alkaline and neutral cellulases. When the homologous sequences were suitably aligned, 90 amino acid residues were conserved in all the cellulases. Among these amino acid residues, 9 and 18 amino acid residues were conserved only in the alkaline cellulases and in the neutral enzymes, respectively. In particular, the locations of Trp residues showed a remarkably high degree of conservation: 10 of 10 or 11 Trp residues in the homologous region of each enzyme were conserved. These conserved amino acid residues, specific for alkaline or neutral cellulases, were concentrated in the carboxy-terminal regions of

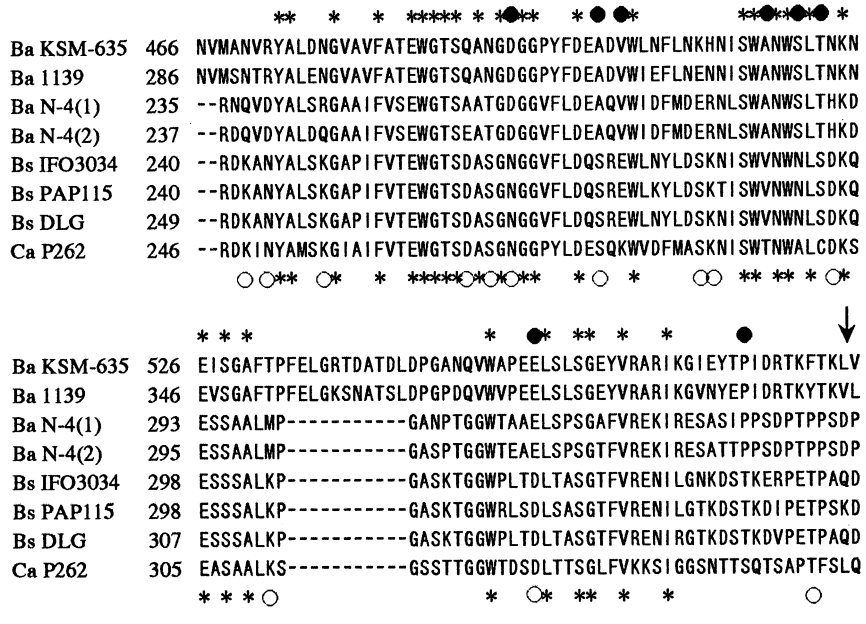

Fig. 2. Comparison of the carboxy-terminal amino acid sequence of alkaline cellulase from *Bacillus* sp. KSM-635 with those of alkaline and neutral enzymes from other bacteria. Amino acid residues conserved in all the cellulases are indicated by *asterisks*. Amino acid residues conserved only in alkaline cellulases or in neutral enzymes are shown by *closed circles* or *open circles*, respectively. The *vertical arrow* indicates the carboxy-terminus (Leu584) of the truncated enzymes encoded by the 2.4-kb fragment. *Ba*, *Bacillus* sp. (alkaliphilic); *Bs*, *B. subtilis* (neutrophilic); *Ca*, *Clostridium acetobutylicum* (neutrophilic)

the homologous amino acid sequences, as shown in Fig. 2. Some of these residues may be involved in catalysis and/or determination of the optimum pH for the cellulases. Nakamura et al. (1991) constructed many chimeric, recombinant cellulases from genes for a neutral enzyme from *Bacillus subtilis* and an alkaline enzyme from *Bacillus* sp. no. 4 and showed that the pH–activity profiles of some chimeric enzymes in the alkaline region depended on the origin of the C-terminal regions.

Mechanisms of the hydrolytic reactions

Enzymatic hydrolysis of glycosidic bonds is often an example of general acid catalysis promoted by Asp and/or Glu residues. The reaction leads to formation of an intermediate carbonium ion, which is stabilized by a negatively charged group of Asp or Glu or by a His residue (Henrissat et al. 1989, Gilkes et al. 1991). Such amino acid residues are highly conserved in homologous regions of the sequences of cellulases in family A, which include the alkaline cellulase from *Bacillus* sp. KSM-635. We purified and characterized an acid cellulase, endo-K, produced by *Bacillus* sp. KSM-330, which is active over an extremely narrow range of pH values, between 4.5 and 6.6, with an optimum pH at 5.2 (Ozaki and Ito 1991). To allow comparisons to be made between endo-K and other alkaline or neutral cellulases, we cloned and sequenced the gene for endo-K (Ozaki et al. 1991). It is noteworthy that the acid cellulase (family D) exhibited no homology to the alkaline cellulase of *Bacillus* sp. KSM-635 or to the other alkaline and neutral enzymes of *Bacillus* reported to date. We identified one Glu, three Asp, and two Trp residues that are important for the action of endo-K from *Bacillus* sp. KSM-330 by site-directed mutagenesis (Ozaki et al. 1994) and stable isotope-aided NMR specroscopy (Kawaminami et al. 1994). We demonstrated that the inactivation by *N*-bromosuccinimide of the alkaline cellulase from *Bacillus* sp. KSM-635 was prevented by CMC, a result that suggests that one or more Trp residues might be involved in the catalysis, in spite of the difference in pH optima. Specific His residues are conserved in, and one of them may be essential for, all cellulases in family A, and His331 may correspond to a residue in the alkaline cellulase from *Bacillus* sp. KSM-635. The possible involvement of this His residue in the mechanism of the hydrolysis is supported by the observation that the activity of this alkaline enzyme was inactivated by diethylpyrocarbonate, and such inactivation was effectively prevented by CMC.

Cellulases and xylanases can be grouped into nine families (A through I) on the basis of amino acid identities in their putative catalytic domains (Béguin 1990), and this grouping is confirmed and extended by hydrophobic cluster analysis (Henrissat et al. 1989). Gebler et al. (1992) showed the stereochemical courses of reactions of enzymes in families A, C, F, G, and H proceed by a double displacement catalytic (retention) mechanism, while those in families B, J, and K proceed by a single displacement catalytic (inversion) mechanism. Using a ^{1}H-NMR spectrophotometer, we

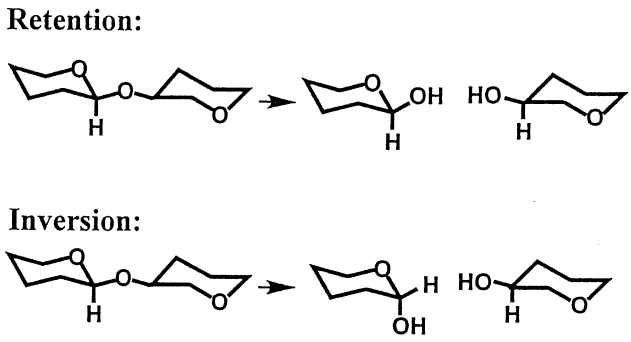

Fig. 3. Stereoselective hydrolysis of β-1,4-glucosidic bond by cellulases. The sugar structure of cellulose (CMC, cello-oligosaccharides, and 4-nitrophenyl β-D-cello-oligosaccharides) is shown only as the cellobiose moiety whose β-1,4 bond is attacked by cellulase. The alkaline cellulase (family A) from *Bacillus* sp. KSM-635 or the acid cellulase (family D) from *Bacillus* sp. KSM-330 hydrolyzes 4-nitrophenyl β-D-cellotriose to generate cellotriose and 4-nitrophenol with a retention or an inversion mechanism, respectively

demonstrated that the hydrolysis of the β-1,4 glucosidic bond of 4-nitrophenyl β-D-cellotriose by the acid cellulase from *Bacillus* sp. KSM-330 proceeds with inversion of the anomeric configuration (Kawaminami et al. 1995). By contrast, the stereochemical course of the reaction by the alkaline cellulase from *Bacillus* sp. KSM-635 proceeds by a retention mechanism, as illustrated in Fig. 3.

Truncation by subcloning and expression of alkaline cellulase

With the ultimate goal of determining the essential region and the tertiary structure of the alkaline cellulase, we truncated the 2.4-kb fragment that encoded the amino-terminal sequence of 584 amino acid residues (65 kDa) by subcloning (Ozaki et al. 1995). The resultant plasmid, pHSP-BC115, was overexpressed in *B. subtilis* cells (Sumitomo et al. 1995). The truncated enzyme consisted of 357 amino acid residues, extending from Ala228 to Leu584 of the wild-type enzyme, plus seven additional amino acid residues derived from a cloning linker, with a predicted molecular mass of 40204 Da. The expressed enzyme [41 kDa, determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)] that we purified was active against CMC, and its specific activity, 94 units/mg protein, was higher than that of E-H (34 units/mg protein) or E-L (59 units/mg protein). The optimum pH and temperature for the activity of the truncated enzyme were pH 9.5 and 45°C, respectively. The degrees of inhibition or stimulation by chemical reagents and inorganic cations of the enzymatic activity were almost identical with those of the native enzymes, E-H and E-L. Other catalytic properties of the enzyme coincided closely with those of the native enzymes, suggesting that the deleted amino- and carboxy-terminal regions have very little influence on the three-dimensional conformation around the active site of this enzyme and are not essential for catalysis.

We have already succeeded in preparing a crystal of the truncated alkaline cellulase and are now in the process of analyzing the enzyme by X-ray crystallography (Yamane and Ito unpublished results). Taking into account the high specific activity of, and length of the gene for, the smaller enzyme, it is expected that the truncated cellulase gene, rather than the entire cellulase gene, may be overexpressed extracellularly by recombinant *B. subtilis* cells.

A new detergent mechanism with the use of alkaline cellulase

It is believed that enzymatic hydrolysis of native cellulose occurs first at its internal glucosidic linkages within an intact glucan chain of amorphous regions by preferential random attack of endo-type cellulase, resulting in exposure of crystalline regions to attack by exo-type cellulase (Wood and McCrae 1972). The alkaline cellulase from *Bacillus* sp. KSM-635 is an endo-type enzyme and compatible with the alkaline ingredients of heavy-duty detergents. Murata and his colleagues demonstrated the cleaning power of an alkaline cellulase-based compact detergent on naturally-soiled cotton undershirts under Japanese washing conditions and confirmed that soiled cotton fibers became clearer as a consequence of the action of the enzyme (Murata et al. 1991, 1993). Since our alkaline cellulases, E-H and E-L, cannot attack the crystalline regions of cotton fibers (Yoshimatsu et al. 1990), clothes are not damaged after repeated wash–wear cycles. In fact, there are no changes in the tensile strength or in the apparent degree of polymerization of cotton cellulose after treatment with alkaline cellulases at high concentrations. It can, therefore, be assumed that the alkaline cellulase contributes indirectly to removal of soil trapped in the amorphous region of cotton fibers by its reaction with the cellulose molecule in the region, but not by its reaction with the soil. Thus, a novel and practically applicable cleaning mechanism has been established, using an endo-type alkaline cellulase.

Conclusions

The use of an alkaline cellulase from a *Bacillus* strain was extended by us to inclusion in compact, heavy-duty detergents for the first time, and the new detergent mechanism of cellulase was proposed for use in enzyme-based detergents. Launching the alkaline cellulase-based compact detergents has had a strong impact on household industries worldwide.

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References

Au K-S, Chan K-Y (1987) Purification and properties of the endo-1,4-β-glucanase from *Bacillus subtilis*. J Gen Microbiol 133:2155–2161

Béguin P (1990) Molecular biology of cellulose degradation. Annu Rev Microbiol 44:219–248

Fukumori F, Sashihara N, Kudo T, Horikoshi K (1985) Purification and properties of a cellulase from alkalophilic *Bacillus* sp. no. 1139. J Gen Microbiol 131:3339–3345

Gebler J, Gilkes NR, Claeyssens M, Willson DB, Béguin P, Wakarchuk WW, Kilburn DG, Miller RC Jr, Warren RAJ, Withers SG (1992) Stereoselective hydrolysis catalyzed by related β-1,4-glucanases and β-1,4-xylanases. J Biol Chem 267:12559–12561

Gilkes NR, Henrissat B, Kilburn DG, Miller RC Jr, Warren RAJ (1991) Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol Rev 55:303–315

Henrissat B, Claeyssens M, Tomme P, Lemesle L, Mornon J-P (1989) Cellulase families revealed by hydrophobic cluster analysis. Gene 81:83–95

Horikoshi K (1971) Production of alkaline enzymes by alkalophilic microorganisms: Part I. Alkaline protease produced by *Bacillus* N. 221. Agric Biol Chem 36:1407–1414

Horikoshi K (1996) Alkaliphiles – from an industrial point of view. FEMS Microbiol Rev 18:259–270

Horikoshi K, Akiba T (1982) Alkalophilic microorganisms: a new microbial world. Springer, Heidelberg

Horikoshi K, Nakano M, Kurono Y, Sashihara N (1984) Cellulases of an alkalophilic *Bacillus* strain isolated from soil. Can J Microbiol 30:774–779

Hoshino E, Murata M, Wada T, Mori K (1995) Hydrolysis of human horny cells by alkaline protease: morphological observation of the process. J Am Oil Chem Soc 72:785–791

Ito S, Shikata S, Ozaki K, Kawai S, Okamoto K, Inoue S, Takei A, Ohta Y, Satoh T (1989) Alkaline cellulase for laundry detergents: production by *Bacillus* sp. KSM-635 and enzymatic properties. Agric Biol Chem 53:1275–1281

Ito S, Ohta Y, Shimooka M, Takaiwa M, Ozaki K, Adachi S, Okamoto K (1991) Enhanced production of extracellular enzymes by mutants of *Bacillus* that have acquired resistance to vancomycin and ristocetin. Agric Biol Chem 55:2387–2391

Kawaminami S, Ozaki K, Sumitomo N, Hayashi Y, Ito S, Shimada I, Arata Y (1994) A stable isotope-aided NMR study of the active site of an endoglucanase from a strain of *Bacillus*. J Biol Chem 269:28752–28756

Kawaminami S, Ozaki K, Ito S (1995) Stereoselective hydrolysis catalyzed by a *Bacillus* endoglucanase in family D. Biochem Biophys Res Commun 212:539–543

Kobayashi T, Hakamada Y, Adachi S, Hitomi J, Yoshimatsu T, Koike K, Kawai S, Ito S (1995) Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. Appl Microbiol Biotechnol 43:473–481

Lusterio DD, Suizo FG, Labunos NM, Valledor MN, Ueda S, Kawai S, Koike K, Shikata S, Yoshimatsu T, Ito S (1992) Alkali-resistant, alkaline endo-1,4-β-glucanase produced by *Bacillus* sp. PKM-5430. Biosci Biotech Biochem 56:1671–1672

Mézes PSF, Lampen JO (1985) Secretion of proteins by bacilli. In: Dubnau DA (ed) The molecular biology of the *bacilli*, vol II, Academic, Orlando, pp 151–183

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426–428

Murata M, Hoshino E, Yokosuka M, Suzuki A (1991) New detergent mechanism with use of novel alkaline cellulase. J Am Oil Chem Soc 68:553–558

Murata M, Hoshino E, Yokosuka M, Suzuki A (1993) New detergent mechanism using cellulase revealed by change in physicochemical properties of cellulose. J Am Oil Chem Soc 70:53–58

Nakamura A, Fukumori F, Horinouchi S, Masaki H, Kodo T, Uozumi T, Horikoshi K, Beppu T (1991) Construction and characterization of the chimeric enzymes between the *Bacillus subtilis* cellulase and an alkalophilic *Bacillus* cellulase. J Biol Chem 266:1579–1583

Okoshi H, Ozaki K, Shikata S, Oshino K, Kawai S, Ito S (1990) Purification and characterization of multiple carboxymethyl cellulase from *Bacillus* sp. KSM-522. Agric Biol Chem 54:83–89

Ozaki K, Ito S (1991) Purification and properties of an acid endo-1,4-β-glucanase from *Bacillus* sp. KSM-330. J Gen Microbiol 137:41–48

Ozaki K, Shikata S, Kawai S, Ito S, Okamoto K (1990) Molecular cloning and nucleotide sequence of a gene for alkaline cellulase from *Bacillus* sp. KSM-635. J Gen Microbiol 136:1327–1334

Ozaki K, Sumitomo N, Ito S (1991) Molecular cloning and nucleotide sequence of the gene encoding an endo-1,4-β-glucanase from *Bacillus* sp. KSM-330. J Gen Microbiol 137:2299–2305

Ozaki K, Sumitomo N, Hayashi Y, Kawai S, Ito S (1994) Site-directed mutagenesis of the putative active site of endoglucanase K from *Bacillus* sp. KSM-330. Biochim Biophys Acta 1207:159–164

Ozaki K, Hayashi Y, Sumitomo N, Kawai S, Ito S (1995) Construction, purification, and properties of a truncated alkaline endoglucanase from *Bacillus* sp. KSM-635. Biosci Biotech Biochem 59:1613–1618

Perlman D, Halvorson HO (1983) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J Mol Biol 167:391–409

Priest FG (1977) Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol Rev 41:711–753

Shikata S, Saeki K, Okoshi H, Yoshimatsu T, Ozaki K, Kawai S, Ito S (1990) Alkaline cellulases laundry detergents: production by alkalophilic strains of *Bacillus* and some properties of the crude enzymes. Agric Biol Chem 54:91–96

Sumitomo N, Ozaki K, Kawai S, Ito S (1992) Nucleotide sequence of the gene for an alkaline endoglucanase from an alkalophilic *Bacillus* and its expression in *Escherichia coli* and *Bacillus subtilis*. Biosci Biotech Biochem 56:872–877

Sumitomo N, Ozaki K, Hitomi J, Kawaminami S, Kobayashi T, Kawai S, Ito S (1995) Application of the upstream region of a *Bacillus* endoglucanase gene to high-level expression of foreign genes in *Bacillus subtilis*. Biosci Biotech Biochem 59:2172

Wood TM, McCrae SI (1972) The purification and properties of the C_1 component of *Trichoderma koningii* cellulae. Biochem J 128:1183–1192

Yoshimatsu T, Ozaki K, Shikata S, Ohta Y, Koike K, Kawai K, Ito S (1990) Purification and characterization of alkaline endo-1,4-β-glucanases from alkalophilic *Bacillis* sp. KSM-635. J Gen Microbiol 136:1973–1979