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ORIGINAL PAPER

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Purification and characterisation of alkaline cellulase produced by a novel isolate, *Bacillus sphaericus* JS1

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Abstract A novel strain of *Bacillus sphaericus* JS1 producing thermostable alkaline carboxymethyl cellulase (CMCase; endo-1,4-β-glucanase, E.C. 3.2.1.4) was isolated from soil using Horikoshi medium at pH 9.5. CMCase was purified 192-fold by $(NH_4)_2SO_4$ precipitation, ion exchange and gel filtration chromatography, with an overall recovery of 23%. The CMCase is a multimeric protein with a molecular weight estimated by native-PAGE of 183 kDa. Using SDS-PAGE a single band is found at 42 kDa. This suggests presence of four homogeneous polypeptides, which would differentiate this enzyme from other known alkaline cellulases. The activity of the enzyme was significantly inhibited by bivalent cations (Fe^{3+} and Hg^{2+}, 1.0 mM each) and activated by Co^{2+}, K^+ and Na^+. The purified enzyme revealed the products of carboxymethyl cellulose (CMC) hydrolysis to be CM glucose, cellobiose and cellotriose. Thermostability, pH stability, good hydrolytic capability, and stability in the presence of detergents, surfactants, chelators and commercial proteases make this enzyme potentially useful in laundry detergents.

Keywords CMCase · Laundry · Alkaline cellulase · Detergents · Stability

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Introduction

Bacillus spp. are known to produce a variety of industrially important alkaline hydrolytic extracellular enzymes, such as amylases, cellulases, pectinases and proteases [12, 13, 16, 18, 19, 31, 37]. The potential of cellulases has been revealed in various industrial processes, including food, textiles and laundry, pulp and paper, and agriculture as well as in research and development [2, 3, 4, 5, 8, 15, 21, 25, 26, 27, 28, 30, 34, 42, 43, 44, 45].

We have purified and characterised an alkaline carboxymethyl cellulase (CMCase) from a novel isolate, *Bacillus sphaericus* JS1. This species is related to *Bacillus circulans* and *Bacillus lentus*, which is known for activity against mosquito larvae. Characterisation of this CMCase is presented in this paper.

Materials and methods

All analytical and media components were purchased from Hi-Media (Bombay, India) and Sigma (St. Louis, Mo.).

Bacterial strains and preparation of enzyme

Soil samples were collected from the premises of paper, cotton and wool industries and screened for alkaliphilic bacteria using Congo Red staining [3]. The isolation medium contained (g/l): CMC (carboxymethyl cellulose) 10 g; peptone 5 g; yeast extract 5 g; NaCl 5 g, KH_2PO_4 1 g; the pH was adjusted to 9.5 with Na_2CO_3 (10%, w/v). Cultures were grown on the same medium [containing 2.0% agar (w/v)] at 40°C in Petri plates. The isolate producing the maximum zone of hydrolysis was selected for further work.

The seed culture was prepared by inoculating a single colony from the maintenance plate (freshly sub-cultured *B. sphaericus* JS1) into the liquid isolation medium and incubating on a rotary shaker (40°C, 150 rev/min) for 12 h. This inoculum (6.0%, v/v) was used for the production of enzyme in 250 ml Erlenmeyer flasks containing 50 ml medium, incubated on a shaker (40°C, 150 rev/min). The culture broth was centrifuged at 15,000 *g* at 4°C for 10 min and the supernatant was used for purification and characterisation.

CMCase assay

Diluted enzyme (250 µl) was mixed with an equal amount of CMC (4.0% w/v in Tris-HCl buffer, pH 8.0). After an incubation of 1 h at 60°C, 750 µl dinitrosalicylic acid reagent solution was added and the mixture was heated in a boiling water bath for 10 min [39]. This was followed by addition of 1,250 µl Tris-HCl buffer and the absorbance was measured at 600 nm. One unit of enzyme activity was defined as the quantity of enzyme required to catalyse the formation of 1.0 µM min^{-1} ml^{-1} reducing sugar (expressed as glucose).

Purification of CMCase

Purification was carried out at 4°C. Enzyme in the cell-free supernatant portion of the culture was precipitated with $(NH_4)_2SO_4$ (30–80% saturation). The precipitate was dialysed against Tris-HCl buffer (50 mM, pH 8.0). The dialysate was applied to a DEAE Sephadex A-50 column (3.6×30 cm) equilibrated with Tris-HCl buffer. The adsorbed material was eluted with a linear gradient of sodium chloride in the range of 0.05–0.5 M in the equilibrating buffer. Fractions (6.0 ml) were collected at a flow rate of 15 ml h^{-1}. The active fractions (20–28), corresponding to the peak of CMCase activity, were combined, dialysed against 0.05 M Tris-HCl buffer and concentrated using a Centrisart filter (Sartorius, Göttingen, Germany), before loading onto a Sephadex G-100 column (2.0×35 cm). Fractions (2 ml; 12–18) corresponding to CMCase activity were collected and the purity was checked on native PAGE. Extracellular protein was measured using the method of Lowry [22] with bovine serum albumin (BSA) as standard. The purified enzyme was subsequently used for characterisation work.

Polyacrylamide gel electrophoresis

Native PAGE was performed as described in the manufacturer's instruction manual (Sigma, Technical Bulletin MKR 137). Urease (272, 542 kDa), BSA (66, 132 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa), α-lactalbumin (14 kDa) were used as molecular mass markers. Electrophoresis was carried out using 4.5–10% gels at 200 V for 10 min following the application of sample/standard, and subsequently at 150 V. The acrylamide gel was overlayed with agarose gel [0.8%; w/v agarose containing 0.5% (w/v) CMC], and incubated at 40°C for 1 h. The agarose gel was stained with Congo Red for zymography and the acrylamide gel with Coomassie Brilliant Blue for detection of JS1 CMCase.

SDS-PAGE was performed on 7.0% polyacrylamide gels as described by Hakamada et al. [10]. The standard molecular weight markers (MW-SDS-200, Sigma) used were: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (97.4 kDa), albumin bovine (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

Effect of pH and temperature on enzyme activity and stability

The enzyme assay was carried out at different temperatures (35–65°C) and at different pH values by using sodium phosphate (7), Tris-HCl (8–9), glycine-NaOH (10) and KCl-NaOH (10.5–11.5) buffers.

Thermal stability studies were carried out by incubating the enzyme at different temperatures (60–70°C). Samples were withdrawn periodically to determine residual enzyme activity. For pH stability, relative activity (%) was determined at different pH values (pH 8.0 and pH 10).

Chromatography of hydrolysed products

Purified CMCase was incubated with CMC (1.0%, w/v) at 60°C for 1 h. An aliquot (0.5 ml) of the enzyme digest was added to 1.0 ml ice-cold acetone to precipitate proteins. The supernatant was obtained by centrifugation at 10, 000 *g* for 10 min, and residual acetone was removed under vacuum. The hydrolysis products were assayed by ascending chromatography of 5.0 µl samples on Silica Gel 60 plates (Merck, Darmstadt, Germany) using a chloroform: acetic acid: water (6:7:1) solvent system. The chromatogram was developed by spraying with the following solution: aniline (1.0%, v/v), diphenylamine (1.0%, w/v), orthophosphoric acid (10%, v/v) in acetone. Movement of products was compared with standard cellobiose, cellotriose and glucose (10 mg/ml).

Effect of effectors (metal ions, surfactants, detergents, chelators and commercial proteases) on CMCase activity

The effect of metal ions [Co^{2+} ($CoCl_2$), K^+ (KCl), Na^+ (NaCl), Ca^{2+} ($CaCl_2$), Mn^{2+} ($MnCl_2$), Fe^{3+} ($FeCl_3$), Mg^{2+} ($MgCl_2$), Pb^{2+} ($PbCl_2$), Zn^{2+} ($ZnSO_4$), Cu^{2+} ($CuCl_2$), Fe^{2+} ($FeCl_2$) and Hg^{2+} ($HgCl_2$)] was determined by adding them to the reaction mixture and incubating it for 30 min (at 60°C) followed by determination of relative activity. Effects of different chelating agents (zeolite, EDTA, sodium citrate and sodium triphosphate; 0.5% w/v each) were determined by preincubating the enzyme solution with each agent for 30 min at 40°C before the addition of substrate (CMC). Relative enzyme activity was measured under standard assay conditions. The effects of different cationic/anionic detergents (citramide/sodium deoxycholate; 0.4% w/v each), non-ionic detergents (Triton X-100, Tween 20, Tween 80; 1.5% w/v each) and commercial proteases [Maxacal (IBSI, Netherlands) and Savinase (Novo, Bagsraerd, Denmark); 0.2% each] were similarly determined.

Results and discussion

Taxonomic characterisation of strain JS1

The isolate designated JS1 is a facultative anaerobe, spore-forming (round, terminal endospore with bulging sporangia), Gram-positive and rod-shaped (0.55–0.65 µm ×1.7–2.1 µm) with peritrichous flagella. It forms irregular, rough, convex and round-shaped colonies. It grew over a pH range of 6.8–11.0, but not at pH 6.0, and the range of temperature for growth was 30–55°C. Strain JS1 was positive for utilisation of salicine, CMC, cellulose, citrate (in Koser's medium), and gelatin liquefaction, and negative for the formation of indol and H_2S, urease, oxidation/fermentation, reduction of nitrate and hydrolysis of casein. Based on the morphological, physiological and biochemical characteristics, isolate JS1 was identified as *Bacillus sphaericus* by the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh (India) and assigned the accession number 3243.

Purification of CMCase and mass determination

The purification and total recovery of CMCase is summarised in Table 1. CMCase was purified to homogeneity, as indicated by native polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue 250. The band of purified protein detected by Coomassie staining coincided fairly well with the clearance zone seen on the agarose gel by zymography

Table 1 Summary of purification steps of alkaline carboxymethyl cellulase (CMCase) from the culture supernatant of *Bacillus sphaericus* JS1

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield
Crude enzyme	2,067	412	0.20	1	100
$(NH_4)_2SO_4$Precipitation (30–80% Saturation)	48	265	5.5	27.5	64
DEAE Sephadex A-50	9.0	180	20	100	44
Sephadex G-100	2.5	96	38.4	192	23

(Fig. 1). The molecular mass of the CMCase was approximately 183 kDa in the native gel.

SDS-PAGE resulted a single band at ~42 kDa (Fig. 2). This finding is similar to the monomeric CMCase (31–94 kDa) reported by many workers [6, 8, 9, 10, 11, 20, 23, 24, 33, 35, 43]. The apparent molecular mass of JS1 CMCase on native PAGE of 183 kDa indicates the possibility of four homogenous subunits in JS1 CMCase.

Effect of pH and temperature on the purified enzyme

Figure 3 shows the effect of pH on the activity of the purified enzyme. JS1 CMCase was active over a broad range of pH (7.0–10.5). Similar observations were also made by others [7, 8, 12, 13, 14, 23, 41, 43, 45].

The activity of JS1 CMCase was maximal at 60°C, which is similar to the optimum temperature reported for such enzymes from other sources [1, 6, 7, 8, 11, 23, 24, 32, 33, 35, 37, 41].

Thermostabilty and pH stability

The half-life of the enzyme at 60°C was 1,080 min in buffer at pH 8.0 and 270 min in buffer at pH 10. At 65°C, it was 120 min and 52 min in buffer at pH 8.0 and 10, respectively, and at 70°C, it was 60 min and 7.0 min. These findings are similar to those reported for the purified enzymes used for thermostability (50–70°C) and pH stability in the alkaline region (8–13) by many workers [6, 8, 10, 11, 17, 23, 36, 37, 38, 41].

Substrate specificity

The relative hydrolytic activities of JS1 CMCase with various polysaccharides were compared by measuring the amount of hydrolysed products. Substrates such as laminarin (β-1,4;1,6 linkages), *p*-NPG *p*-(nitrophenyl β-D-glucopyranoside), curdlan (β-1,3 linkage), Whatman paper, Avicel, cellulose, methyl cellulose, Sigmacell, discs of newspaper and discs of tissue paper, showed the relative activities of 5, 8, 10, 12, 15, 20, 27, 30, 44 and 45%, respectively. These substrates were relatively resistant to JS1 CMCase. CMC and lichenan (β-1,3; 1,4 linkages) were the best substrates, with relative activities of 100 and 232%, respectively. This behaviour is quite similar to that reported for this enzyme from other sources [8, 10, 11, 16, 29, 36, 45].

Chromatography of hydrolysed products

Thin layer chromatography of the CMC hydrolysate revealed the presence of CM glucose, cellobiose,

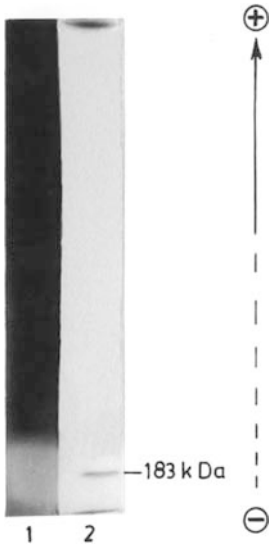

Fig. 1 Native PAGE pattern of purified JS1 carboxymethyl cellulase (CMCase). Lanes: *1* Activity staining of CMCase with Congo Red, *2* Coomassie Brilliant Blue staining of purified enzyme

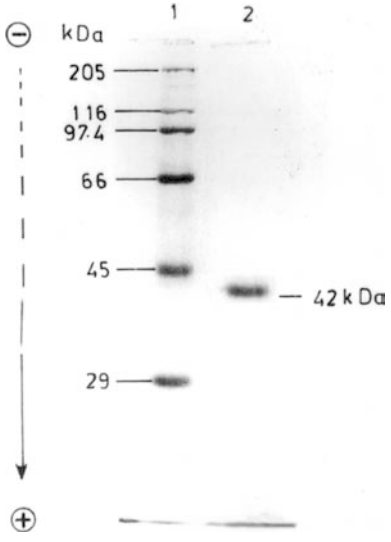

Fig. 2 SDS-PAGE pattern of purified CMCase isolated from *Bacillus sphaericus* JS1. Lanes: *1* Marker proteins, *2* monomeric band of a purified JS1 CMCase

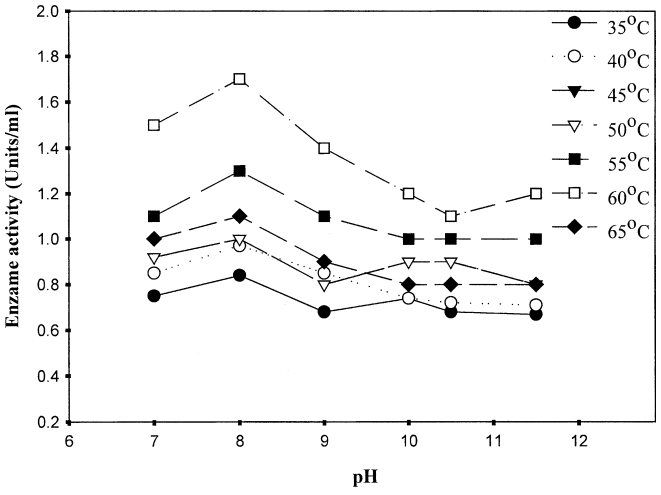

Fig. 3 Effect of pH and temperature on activity of the purified CMCase produced by *Bacillus sphaericus* JS1. The enzyme was diluted in different buffers (50 mM): sodium phosphate (pH 7.0); Tris-HCl (pH 8.0 and 9.0); glycine-NaOH (pH 10) and KCl-NaOH (pH 10.5 and 11), mixed with carboxymethyl cellulose (CMC) (0.4%, w/v in compatible buffers) and incubated at different temperatures (35–65°C). The assay was performed under standard conditions

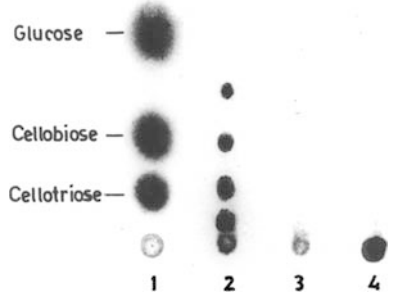

Fig. 4 Thin layer chromatography showing the hydrolysed products of CMC produced by the action of purified JS1 CMCase. Lanes: *1* Standard sugars (10 mg/ml), *2* reaction mixture (purified enzyme + CMC), *3* substrate blank, *4* enzyme blank

cellotriose, etc. (Fig. 4). Normally, the CMC (Fluka; low viscosity, degree of substitution = 0.70–0.85) with the lowest viscosity is substituted to 0.7. Degradation of this substrate produced substituted glucose units (around 120, some with a carboxymethyl group at C2, C3, or C6). After degradation, glucose contained one carboxymethyl group and this gave rise to the difference in chromatographic behaviours of pure glucose and the CMC-degraded product.

Effect of effectors on CMCase activity

Table 2 shows the effect of various metal ions on the activity of the purified enzyme. JS1 CMCase was stimulated by Co^{2+}, Na^{+}, and K^{+} ions [1, 9, 16, 28, 45] and strongly inhibited by Hg^{2+} (88%) [17, 19, 20, 23, 24, 38, 41]. However, it was unaffected by Ca^{2+}, Mg^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3} [23].

The enzyme-based detergent compatibility studies showed the resistance of the enzyme to various anionic surfactants (alkyl ethoxy sulphonate, α-olefin sulphonate, linear alkyl benzene sulphonate; 0.05% w/v each), non-ionic surfactants (polyethylene alkyl ethers), chelating agents (zeolite, EDTA, sodium citrate, sodium triphosphate; 0.05% w/v each), by preserving more than 85% activity. Similar findings have been made by other workers [10, 17, 20, 23, 24, 35]. The non-ionic detergents Triton X-100, Tween 20 and Tween 80 showed no inhibitory effect up to 1.5% (v/v) concentration, with more than 75% activity being retained. More than 65% activity was maintained with cationic (sodium citramide) and anionic (sodium deoxycholate) detergents (0.4% w/v; each). These findings are similar to those obtained with *Bacillus* sp. VG1 [38]. Similarly, proteases and cellulases function to improve the activity of detergents. Cellulases for detergent use should not be hydrolysed by proteases, and should maintain activity in their presence [40]. Alkaline JS1 CMCase is resistant to proteases currently employed in detergents [Maxacal (IBSI, Netherlands) and Savinase (Novo, Denmark)]. *Bacillus* sp. KSM-635 [16], *Bacillus* strain KSM-522 [20], *Bacillus* sp. PKM-5430 [23], *Bacillus* strain ferm bp-3431 [33], and *Bacillus* sp VG1 [38] also showed similar properties. These results indicate that these proteases, which are often included in laundry detergents, are almost completely unable to attack or inactivate the CMCase from the *Bacillus sphaericus* JS1. These properties confirm the essential requirements for use of the enzyme as an effective laundry additive.

The outstanding stability of JS1 CMCase to alkaline pH, high temperature, as well as the absence of inhibition of its activity by inorganic ions, surfactants, deter-

Table 2 Effect of metal ions on the activity of purified JS1 CMCase. The enzyme was preincubated with the defined concentration of metal ions in Tris-HCl buffer pH 8.0 at 60°C for 30 min and the reaction was carried out under standard assay conditions. The relative activities were measured

Metal ion	Concentration (mM)	Percentage relative activity	Metal ion	Concentration (mM)	Percentage relative activity
Control	–	100	Control	–	100
$Co^{2+}(CoCl_2)$	1.0	157	$Mg^{2+}(MgCl_2)$	1.0	103
$K^{+}(KCl)$	50	117	$Pb^{2+}(PbCl_2)$	1.0	99
$Na^{+}(NaCl)$	50	122	$Zn^{2+}(ZnSO_4)$	1.0	96
$Ca^{2+}(CaCl_2)$	1.0	100	$Cu^{2+}(CuCl_2)$	1.0	98
$Mn^{2+}(MnCl_2)$	1.0	92	$Fe^{2+}(FeCl_2)$	1.0	101
$Fe^{3+}(FeCl_3)$	1.0	58	$Hg^{2+}(HgCl_2)$	1.0	12

gents and laundry components, make it a good candidate for use as an effective additive to laundry detergents.

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References

1. Au K-S, Chan K-Y (1987) Purification and properties of the endo-1,4-β-glucanase from *Bacillus subtilis*. J Gen Microbiol 133:2155–2162
2. Bajpai P (1999) Application of enzyme in pulp and paper industry. Biotechnol Progr 15:147–157
3. Beguin P (1983) Detection of cellulase activity in polysaccharide gels using Congo Red-stained agar replicas. Anal Biochem 131:333–336
4. Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383
5. Cavaco-Paulo A (1998) Mechanism of cellulase action in textile process. Carbohydr Polym 37:273–277
6. Christakopoulos P, Hatzinikolaou DG, Fountoukidis G, Kekos D, Claeyssens M, Macris BJ (1999) Purification and mode of action of an alkali-resistant endo-1,4-beta-glucanase from *Bacillus pumilus*. Arch Biochem Biophys 364:61–66
7. Dasilva R, Yim DK, Asquieri ER, Park YK (1993) Production of microbial alkaline cellulase and studies of their characteristics. Microbiol Rev 24:269–274
8. Endo K, Hakamada Y, Takizawa S, Kubota H (2001) A novel alkaline endoglucanase from an alkaliphilic *Bacillus* isolate: enzymatic properties, and nucleotide and deduced amino acid sequences. Appl Microbiol Biotechnol 57:109–116
9. Fukumori F, Kudo T, Horikoshi K (1985) Purification and properties of a cellulase from alkalophilic *Bacillus* sp. No. 1139. J Gen Microbiol 131:3339–3345
10. Hakamada Y, Koike K, Yoshimatsu T, Mori H, Kobayashi T, Ito S (1997) Thermostable alkaline cellulase from an alkalophilic isolate, *Bacillus* sp. KSM-S237. Extremophiles 1:151–156
11. Hakamada Y, Endo K, Takizawa S, Kobayashi T, Shirai T, Yamane T, Ito S (2002) Enzymatic properties, crystallisation, and deduced amino acid sequence of an alkaline endoglucanase from *Bacillus circulans*. Biochem Biophys Acta 1570:174–180
12. Horikoshi K (1997) Alkaliphiles—from an industrial point of view. FEMS Microbiol Rev 18:259–270
13. Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 63:735–750
14. Horikoshi K, Nakao M, Kurono Y, Sashihara N (1984) Cellulase of an alkalophilic *Bacillus* strain isolated from soil. Can J Microbiol 30:774–779
15. Hoshino E, Ito S (1997) Application of alkaline cellulases that contribute to soil removal in detergents. In: van Ee JH, Misset O, Baas EJ (eds) Enzymes in detergency. Dekker, New York, pp 149–174
16. Ito S (1997) Alkaline cellulase from alkaliphilic *Bacillus*: enzymatic properties, genetics, and application to detergents. Extremophiles 1:61–66
17. Ito S, Shikata S, Ozaki K, Kawai S, Okamoto K, Inoue S, Takei A (1989) Alkaline cellulase for laundry detergent: production by *Bacillus* sp. KSM-635 and enzymatic properties. Agric Biol Chem 53:1275–1281
18. Ito S, Shimooka Y, Takaiwa M, Ozaki K, Adachi S, Okamoto K (1991) Enhanced production of extracellular enzymes by mutant of *Bacillus* that have required resistance to vancomycin and ristocetin. Agric Biol Chem 55:2387–2391
19. Ito S, Kobayashi T, Ara K, Ozaki K, Kawai S, Hatada Y (1998) Alkaline detergent enzymes from alkaliphiles: enzymatic properties, genetics, and structures. Extremophiles 2:185–190
20. Kawai S, Okoshi H, Ozaki K, Shikata S, Ara K, Ito S (1988) Neutrophilic *Bacillus* strains KSM-522 that produces an alkaline carboxymethyl cellulase. Agric Biol Chem 52:1425–1431
21. Lenting HB, Warmoeskerken MM (2001) Mechanism of interaction between cellulase action and applied shear force, an hypothesis. J Biotechnol 89:217–226
22. Lowry OH, Rosenbrough W, Farr AL, Rondal RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193:265–273
23. Lusterio DD, Suizo FG, Labunos NM, Valledor MN, Ureda 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 Biotechnol Biochem 56:1671–1672
24. Mawadza C, Hatti-Kaul R, Zvauya R, Mattiasson B (2000) Purification and characterisation of cellulases produced by *Bacillus* strain. J Biotechnol 83:177–187
25. Miettinenen-Oinonen A, Suominen P (2002) Enhanced production of *Trichoderma reesei* endoglucanases and use of new cellulase preparations in producing the stonewashed effect on denim fabrics. Appl Environ Microbiol 68:3956–3964
26. Murata M, Hoshino E, Yokosuka M, Suzuki A (1991) New detergent mechanism with use of novel alkaline cellulase. J Am Oil Chem Soc 68:553–557
27. 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
28. Okoshi H, Ozaki K, Oshino S, Kawai S, Ito S (1990) Purification and characterisation of multiple carboxymethyl cellulases from *Bacillus* sp. KSM-522. Agric Biol Chem 54:83–89
29. Ozaki K, Ito S (1991) Purification and properties of an acid endo β-1,4-glucanase from *Bacillus* sp. KSM-330. J Gen Microbiol 137:41–48
30. Poutanen K (1997) Enzymes: an important tool in the improvement of the quality of cereal food. Trends Food Sci Technol 8:300–306
31. Riisgaard S (1990) The enzyme industry and modern biotechnology. Genet Eng Biotechnol 10:11–14
32. Robson LM, Chambliss GH (1984) Characterisation of the cellulolytic activity of a *Bacillus* isolate. Appl Environ Microbiol 47:1039–1046
33. Saito K, Seko M, Masatsuji E (1994) Detergent comprising isolated cellulase from *Bacillus* ferm bp-3431 or a mutant strain thereof, surfactant, and builder. US Patent 5 314 637
34. Screws GA, Pedersen G (1998) Process for the treatment of cellulosic fibres with cellulases. US Patent 5 707 858
35. Sharma P, Gupta JK, Vadehra DV, Dube DK (1990) Purification and properties of an endoglucanase from a *Bacillus* isolate PDV. Enzyme Microb Technol 12:132–137
36. Shikata S, Saeki K, Okoshi H, Yoshimatsu T, Ozaki K, Kawai S, Ito S (1990) Alkaline cellulase laundry detergents: production by alkalophilic strains of *Bacillus* and some properties of the crude enzymes. Agric Biol Chem 54:91–96
37. Shirai T, Ishida H, Noda J, Yamane T, Ozaki K, Hakamada Y, Ito S (2001) Crystal structure of alkaline cellulase K: insight into the alkaline adaptation of an industrial enzyme. J Mol Biol 310:1079–1087
38. Singh J, Batra N, Sobti RC (2001) A highly thermostable, alkaline CMCase produced by a newly isolated *Bacillus* sp. VG1. World J Microbiol Biotechnol 17: 761–765
39. Summer JB, Somers GF (1954) Dinitrosalicylic acid for glucose. In: Laboratory experiments in Biological Chemistry. Academic Press, New York, pp 34–39
40. Suzuki A, Ito S, Okamoto K, Hoshino E, Yokosyka M, Murarta M (1990) Detergent composition for clothing: containing cellulase enzyme with non-degrading index of 500 or more. US Patent 4 978 470
41. Tian X, Wong X (1998) Purification and properties of alkaline cellulase from alkalophilic *Bacillus* N6–27. Wei Sheng Wu Xue Bao 38:310–312

42. Uhlig H (1998) Industrial enzymes and their applications. Wiley, New York
43. Van Solingen P (1999) Alkaline cellulase and method of producing the same. US Patent 5 856 165
44. Wong KKY, Saddler JN (1993) Applications of hemicellulases in the food, feed and pulp, and pulp industries. In: Coughlan MP, Hazlewood GP (eds) Hemicellulose and hemicellulase. Portland Press, London, pp 127–143
45. Yoshimatsu T, Ozaki K, Shikata S, Ohta Y, Koike K, Kawai S, Ito S (1990) Purification and characterisation of alkaline endo-1,4- β-glucanases from alkalophilic *Bacillus* sp. KSM-635. J Gen Microbiol 136:1973–1979