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Cellulase activity of a haloalkaliphilic anaerobic bacterium, strain Z-7026

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Summary The cellulolytic activity of an alkaliphilic obligate anaerobic bacterium, Z-7026, which was isolated from the microbial community of soda-lake sediments and belongs to the cluster III of Clostridia with low G+C content, was studied. The bacterium was capable of growing in media with cellulose or cellobiose as the sole energy sources. Its maximal growth rate on cellobiose (0.042–0.046 h^{-1}) was observed at an initial pH value of 8.5–9.0, whereas the maximal rate of cellulase synthesis, assayed by using a novel fluorimetric approach, was found to be 0.1 h^{-1} at pH 8–8.5. Secreted proteins revealed high affinity for cellulose and were represented by two major forms of molecular masses of 75 and 84 kDa, whereas the general protein composition of the precipitated and cellulose-bound preparations was similar to cellulosome subunits of *Clostridium thermocellum*. The optimum pH of the partially purified enzyme preparation towards both amorphous and crystalline cellulose was in the range 6–9, with more than 70% and less than 50% of maximal activity being retained at pH 9.2 and 5.0, respectively.

Keywords Cellulose · Cellulases · Cellulosomes · Haloalkaliphile · Anaerobe · Soda lakes

Abbreviations B: Protein band in the electrophoretic separation · BSA: bovine serum albumin · Cbh: Cellobiohydrolase · Cel: Cellulase · CBM: Cellulose-binding module · CPB: Citrate phosphate buffer · GH: Glycosyl hydrolase · MCC: Microcrystalline cellulose · MUF: 4-methylum belliferon · MUFC: 4-methylumbelliferyl-β-D-cellobioside · MUFL: 4-methylumbelliferyl-β-D-lactopyranoside · PASC: Phosphoric acid-swollen cellulose · S: Protein subunit of the *C. thermocellum* cellulosome · SDS-PAGE: Electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate

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Introduction

In spite of extensive studies of cellulolytic enzymes of extremely alkaliphilic bacteria (generally, aerobic bacilli), there are yet no data available on the nature of microorganisms and a set of cellulolytic enzymes capable of completely degrading cellulose in the anaerobic zone of soda lakes (Lynd 2002; Leschine 1995). Although cellulolytic enzymes which are most active at alkaline pH are widely used in detergents and denim wash (Horikoshi 1999), the mechanism of the enzymatic hydrolysis of the β-1,4-glycosidic bond under these alkaline conditions is not yet clear. A protonated carboxyl group is believed to take part in the catalytic event (Schaw et al. 2002), however, protonation of the carboxylate anion at pH 10, where cellulases of alkaliphiles function, seems to be complicated. In addition, the structure of cellulolytic systems of alkaliphilic anaerobes has also not been elucidated to date. Phylogenetically relevant neutrophilic anaerobes (*Clostridium thermocellum*, *C. cellulolyticum*, *Acetivibrio cellulolyticus*, *A. cellulosolvens*, etc.) form special cell-bound protein aggregates for cellulose decomposition (Doi et al. 2003), which were termed cellulosome (Bayer et al. 1985, 1998, 2004; Felix and Ljungdahl 1993; Schwarz 2001). According to Morag et al. (1992) cellulosomes are capable of the most efficient cellulose decomposition (up to 13 μmol of glucose formed in 1 min/1 mg of cellulosome protein). However, the structural integrity of cellulosomes depends on the presence of Ca^{2+} ions (Choi and Ljungdahl 1996; Lytle et al. 2000), the accessibility of

which is limited in soda lakes. Therefore, one might expect a different structure of cellulase systems of anaerobic alkaliphiles as compared with those of taxonomically relevant neutrophilic *Clostridia*. These and other questions regarding cellulose decomposition under alkaline conditions cannot be answered without the isolation of a pure culture of an anaerobic alkaliphilic bacterium.

Studies on alkaliphilic anaerobes constituting microbial communities of soda lakes have been undertaken since the beginning of the 1990s (Zavarzin 1993; Zhilina and Zavarzin 1994), and representatives of major trophic pathways of organic matter decomposition have already been described (Zavarzin et al. 1999). Despite of a first recent publication (Grant et al. 2004), microbial species, representing hydrolytic steps of these degradation pathways and particularly cellulolytic ones, remain unexplored, even though cellulose degradation was shown to proceed very fast at pH 10 and a high-mineral environment (up to 200 g/l of total salt content) (Zhilina and Zavarzin 1994).

Studies on cellulose decomposition by an alkaliphilic anaerobic community isolated at the low-mineral soda lakes Beloye (Buryatia, Russian Federation) and Khadyn (Tuva, Russian Federation) revealed the presence of a variety of rod-shaped bacteria during the first hydrolytic stage. These bacteria were partially associated with cellulose fibers and some of them were forming spores (Kevbrin et al. 1999). Recently, a rod-shaped, spore-forming, strictly anaerobic bacterium, strain Z-7026, was isolated from a cellulose-decomposing consortium isolated at lake Verkhnee Beloye (Russian Federation) at pH 10. This represents the first example of an anaerobic, alkaliphilic, cellulolytic bacterium known so far (Zhilina et al. 2005). Phylogenetically, strain Z-7026 belongs to the cluster III of Gram-positive *Clostridia* with low G + C content, where it forms a new taxon most closely related to *C. thermocellum*. Description of the strain will be presented elsewhere (Zhilina et al. 2005).

This work was undertaken to obtain some primary information on the cellulase system of this novel haloalkaliphilic anaerobic bacterium and to develop an assay for the determination of cellulase activity in the cultures of anaerobes growing at alkaline pH in the presence of cellulose.

Materials and methods

Cultivation

Strain Z-7026 was cultivated in two media which were prepared strictly anaerobically under nitrogen flow and differed in pH and salt content. Medium I was used for isolation and maintenance of the bacterium and contained (g/l): $NaHCO_3$ (10); Na_2CO_3 (6); NaCl (2.6); KH_2PO_4 (0.3); $(NH_4)_2SO_4$ (0.33); NH_4Cl (0.27); $MgSO_4 \times 7H_2O$ (0.1); $CaCl_2 \times 2H_2O$ (0.015); yeast extract (0.2); Sigmacell cellulose type 101 (1); trace elements solution (1 ml); and $Na_2S \times 9H_2O$ (0.5). Trace elements solution contained (mM): $(NH_4)_2SO_4 \times FeSO_4$ (2); $(NH_4)_2SO_4 \times NiSO_4$ (1); $CoCl_2$ (1); $MnCl_2$ (0.5); $ZnSO_4$ (0.5); Na_2MoO_4 (0.1); Na_2WO_4 (0.1); H_3BO_3 (0.1); Na_2SeO_4 (0.05); $CuCl_2$ (0.05). The pH of medium I after sterilization was 9.4. Medium II was designed after optimization of medium I and provided significantly better growth. It contained (g/l): $NaHCO_3$ (7.6); Na_2CO_3 (1); NaCl (10). Other components were the same as in medium I. The pH of medium II after sterilization was pH 8.8. Sigmacell cellulose type 101 (Sigma, USA) or cellobiose were used as the sole carbon sources. Cellobiose was added to the sterilized alkaline medium as a stock sterile solution in order to prevent its caramelization. The strain was grown at 37°C.

Determination of the rate of cellulase synthesis directly in the growing culture

The rate of cellulase synthesis was measured by using a nonspecific fluorogenic substrate, 4-methylumbelliferyl-β-D-lactopyranoside (MUFL, Sigma), of clostridial cellobiohydrolases type CbhA (Melnik et al. 1991). For this purpose, medium II containing cellulose was adjusted to various pH values (6.0–10.5) using 5 M NaOH or HCl. These media (2 ml) were sterilized in test tubes supplied with gas-tight screw caps with rubber stoppers similar to Hungate test tubes, but small enough to be fitted into the cuvette unit of a spectrofluorometer (outer diameter 12 mm, total volume 7 ml, Schott 26-135-11-55). After sterilization, these media were cooled, and their pH value was again determined. Then, an ethanolic stock solution of MUFL was added to each test tube to a final concentration of 0.15 mM by using a Hamilton air-tight microsyringe. Equimolar amounts of 4-methylumbelliferon (MUF, Sigma) were added into the respective control samples having the same pH, in order to evaluate possible quenching of MUF fluorescence by the growing culture and to correct the results for different quantum yields at different pHs. Both the experimental and the control sets were prepared in triplicates and inoculated by adding one drop (0.03 ml) of inoculum cultured on cellulosic medium II. Incubation was performed strictly anaerobically without shaking. The increase in fluorescence at 446 nm (excitation at 368 nm), which resulted from the release of MUF from MUFL by the synthesized cellulases, was measured directly in the test tubes containing the growing anaerobic culture by using a spectrofluorometer (Shimadzu RF-5301PC). By comparison of the fluorescence of experimental and control runs, performed at the same pH and for an equal cultivation time, the concentrations of liberated MUF were calculated for each cultivation period and expressed as the fraction of hydrolyzed MUFL. The specific rates of cellulase synthesis during exponential growth were calculated by linear regression of the logarithm of the yield of MUF versus time.

Isolation of cellulases

One liter of culture fluid (media I or II) containing cellulose was centrifuged aerobically at 10,000*g* and 4°C for 15 min. The supernatant was then used for precipitation of the extracellular cellulase proteins by ammonium sulfate (100% saturation) or 96% ethanol in the ratio 1:4. The protein precipitate was allowed to settle for 12 h at 4°C and was harvested by centrifugation at 20,000*g* and 4°C for 30 min. The precipitate was further fractionated by dissolving it stepwise in minimal volumes of 50 mM Tris–HCl buffer, pH 8.5, and the fractions containing maximal MUF-cellobioside hydrolase activity (ca. 60% saturation by ammonium sulfate) were used as cellulase source.

Attempts to isolate cell-bound or cellulose-bound cellulase proteins were also undertaken. The isolation of cell-bound cellulases included sonication of 1 g wet biomass (intact or pre-incubated at 37°C with 3 mg/ml of hen egg-white lysozyme for 20 min) resuspended in 15 ml of 50 mM Tris-HCl buffer, pH 8.5, for ten times 30 s in an ice-cold water bath with 1-min intervals. The cellular debris and residual cellulose were removed by centrifugation at 10,000*g* for 30 min, and MUF-cellobioside (MUFC) hydrolase activity was measured in the supernatant according to Melnik et al. (1991). Isolation of cellulose-bound proteins was done by incubating the culture pellet with 100% glycerol in a ratio of 1:1.5 (w/v) and in the presence of 4 mM dithiothreitol for 2 h at 20°C, followed by centrifugation at 10,000*g* for 30 min. Cellulase activity and protein content were measured in the supernatant.

Gel filtration on Sepharose 4B

Proteins obtained after ethanolic precipitation of the culture supernatant were dissolved in a minimal volume of 0.1 M CPB, pH 8.0, the solution was then clarified by centrifugation at 10,000*g* for 5 min, and loaded onto a Pharmacia column (15 × 400 mm) filled with Sepharose 4B. The same procedure was performed with the glycerol extract of residual cellulose in the cultural broth. The exclusion volume was determined by using Blue Dextran 2000. The elution of the high-molecular mass cellulosomal protein fraction was monitored by using the Lowry method (Lowry et al. 1951) with BSA as a standard protein.

Electrophoretic determination of cellulase composition

Electrophoresis (SDS-PAGE) was performed in a Helicon II VE-4M vertical system (Helicon, Russia), using 8 or 12% polyacrylamide. MW-SDS-200 (Sigma) and Protein kit H1301 (Helicon, Russia) served as molecular weight markers. Coomassie Brilliant Blue R-250 was used for protein staining. All procedures were performed in compliance with the manufacturers' recommendations. In the case of proteins adsorbed onto microcrystalline (MCC) or phosphoric acid-swollen cellulose (PASC), 2 or 4 mg of cellulose were consecutively washed with 2 ml of 0.1 M CPB, pH 6.5 (twice), and 2 ml of deionized water (once), after which 200 µl of standard electrophoresis buffer was added and the sample was further processed under standard conditions. Following centrifugation as the final step, the supernatant (10–40 µl) was loaded onto the lane.

Cellulase activity

During cultivation of the microorganism cellulase activity was measured by using the fluorogenic substrates MUFL or MUFC according to Melnik et al. (1991).

Cellulase activity towards PASC (Walseth 1952) and MCC (Sigmacell cellulose type 50, Sigma) was assayed using the hydroxybenzoic acid hydrazide (Sigma) reagent with glucose as a calibration standard, as previously described (Lever 1973). Incubation of insoluble substrate (50 mg of MCC or 10 mg of PASC (dry weight)) with different aliquots of enzyme solution was performed at 50° for 30–60 min depending on the enzyme activity at different pH. Xylanase activity towards oat spelt xylan (10 mg/ml, Sigma) was assayed using the same method with xylose as a calibration standard. The results are expressed as mg of glucose (xylose) produced per mg of protein per hour.

Results and discussion

Optimal pH for growth and cellulase production

Studies on the dependence of the growth rate of strain Z-7026 on pH (cellobiose as a substrate) have identified this bacterium as an obligate alkaliphile capable of growing in the pH range 8.0–10.2, with optimal growth from pH 8.5 to 9.0 (Table 1, columns 1 and 2). No growth was observed at pH ≤ 7.7 and ≥ 10.4 in the presence of cellobiose (data not shown).

Soluble growth substrates (e.g. cellobiose) are widely used as substituents of natural substrates in the physiological studies of microorganisms which utilize cellulose or other insoluble biopolymers, because the presence of the latter makes the cultivation medium essentially heterogeneous and complicates correct determination of the growth characteristics. For this reason, investigations of the growth rates and cellulase biosynthesis by anaerobic cellulolytic bacteria in cellulose-based media represent a separate problem (Kennedy et al. 1992; Lynd and Zhang 2002), particularly when taking into account the attachment of some bacteria to the surface of cellulose. In our opinion, the most adequate approach for the evaluation of cell biomass under these conditions was suggested by Solo-

mon and Erickson (1983) and is based on the determination of total DNA. However, this approach is not applicable for investigations of the dynamics of cellulase activity in the developing culture. A priori it cannot be ruled out that cell-free soluble cellulases constitute only a minor fraction of the overall cellulase activity, and that the major fraction is distributed between cellular surfaces and the cellulosic substrate. Partial inactivation of cellulases of anaerobic producers in the presence of oxygen can also not be excluded and further complicates the cellulase assay in aliquots of anaerobic cultures. To overcome these difficulties, Lynd and Zhang (2002) propose immunoassays for the evaluation of cellulase proteins in anaerobic cultures grown in the presence of cellulose. However, this approach requires preliminary isolation of cellulases for immunization and is not applicable for the purposes of screening of new anaerobic producers, which may have low basal level of cellulase biosynthesis.

Table 1 The growth rates on cellobiose and the rates of cellulase biosynthesis on cellulose by the strain Z-7026 at different pHs

Growth on cellobiose		Biosynthesis of cellulase activity on cellulose			
		novel approach		conventional approach	
pH*	μ, h^{-1}	pH	μ, h^{-1}	pH*	μ, h^{-1}
7.72 – 7.33	0	7.38	0.047 ± 0.01	8.15–7.72	0.05 ± 0.006
8.06 – 7.57	0.018	7.72	0.076 ± 0.01	8,3–7.8	0.045 ± 0.005
8.28 – 7.26	0.036	8.06	0.1 ± 0.01	8.6–8.0	0.038 ± 0.005
8.52 – 7.38	0.042	8.28	0.1 ± 0.01	8,85–8.05	0.05 ± 0.001
8.95 – 7.84	0.046	8.52	0.1 ± 0.01	9.3–8.5	0.047 ± 0.003
9.44 – 9.33	0.019	8.95	0.076 ± 0.01		
9.90 – 9.90	0.007				
10.4 – 10.4	0.0002				

*Initial and final pHs of cultivation are given

Here a novel assay for the identification of cellulase activity in anaerobic cultures grown in cellulose-based media at pH > 7 is proposed and termed "a reporter substrate assay". It is based on the cultivation of the cellulolytic anaerobe under strict anaerobic conditions in a test tube in the presence of cellulose and a fluorogenic reporter, namely, the nonspecific substrate of cellobiohydrolase, MUFL. The reporter slowly liberates the free fluorophore MUF when being hydrolyzed by a diverse group of cellulases that are capable of hydrolysis of the more specific substrate MUFC. This group includes cellobiohydrolases CbhA and CelK (subunits S3 and S5 of *C. thermocellum* cellulosome) (Melnik et al. 1991; Zverlov et al. 1999), and, apparently, enzymes comprising subunits S8, S9, and S10 of the *C. thermocellum* cellulosome (Morag et al. 1990; Bhat et al. 2001), as well as noncellulosomal CelI (Gilad et al. 2003) and CelC from *C. thermocellum* (Mel'nik et al. 1999), as well as related enzymes of the same glycosyl hydrolase families isolated from mesophilic clostridia and other bacterial and fungal producers. The advantage of MUFL over MUFC is its lower susceptibility to small amounts of cellulases that are always present in the inoculum, as well as its resistance to β-glucosidases. The liberated MUF shows a strong fluorescence at 446 nm at neutral and alkaline pH when excited at 368 nm. This excitation wavelength allows direct measurements in the glass test tubes during the growth of microorganisms.

The results of cellulase activity monitoring by this new method in cultures growing at different pHs are shown in Fig. 1a. At pH 6.8, 9.44, and 10.4 (curves 1, 8, and 9, respectively) linear liberation of MUF from MUFL was detected during the entire period of obser-

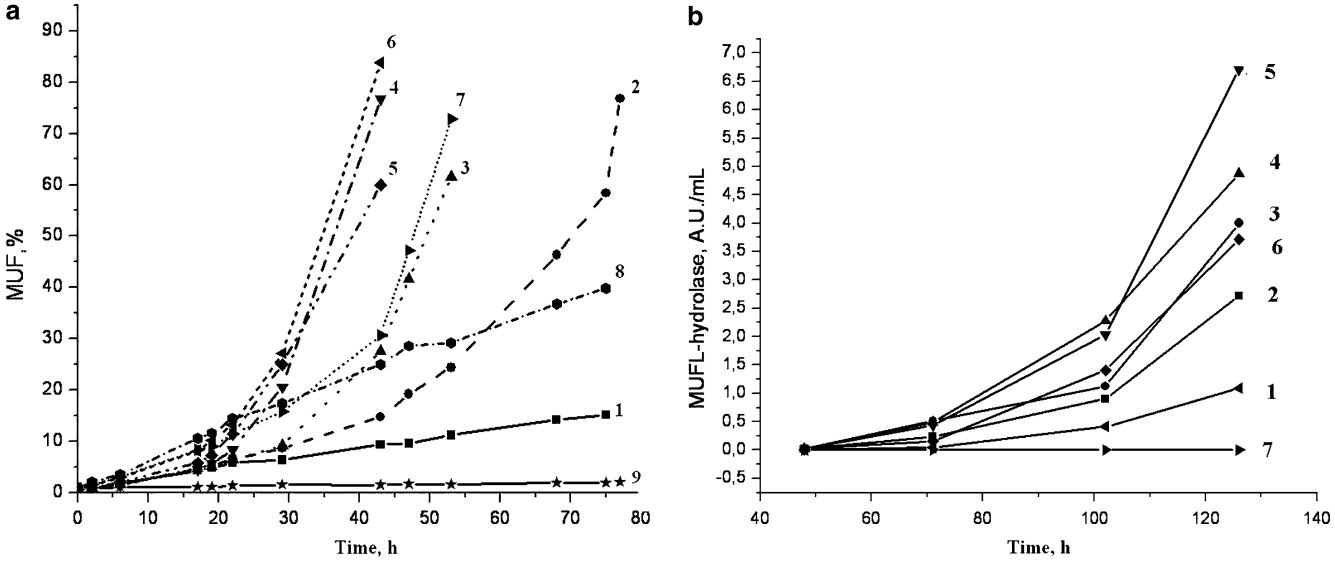

Fig. 1 Dynamics of MUF liberation during MUFL hydrolysis (**a**) and MUFL-hydrolase activity (**b**) by the strain Z-7026 growing in cellulose media. **a** Initial pH values: 6.8, 7.38, 7.72, 8.06, 8.28, 8.52, 8.95, 9.44, and 10.4 (curves 1–9, respectively). **b** Cultivation at pH: 7.9 ± 0.15, 7.95 ± 0.2, 8.05 ± 0.25, 8.3 ± 0.3, 8.45 ± 0.4, 8.9 ± 0.4, 9.5 ± 0.15 (curves 1–7, respectively)

Table 2 Protein composition of the precipitated and cellulose-bound preparations as compared with composition of cellulosome of *C. thermocellum*

Protein		Corresponding *C. thermocellum* [Bayer et al. 1985]		Reference
$(NH_4)_2SO_4$-precipitated	Cellulose-bound	cellulosome subunit (kDa)	Enzyme type	
B1	B1	S1 (210)	CipA	Bayer et al. 1998
B2	B2	S2 (170)	CelJ (Xyn)	Ahsan et al. 1996
B3	B3	S3 (150)	CbhA	Zverlov et al. 1999
B4	B4	S4 (115)		
B5		S5 (98)	CelK	Zverlov et al. 1999
B6		S6 (91)	XynZ?	Beguin and Aubert 1994
B7	B7	S7 (84)	Xylanase?	Morag et al. 1990
B8	B8	S8 (75)	CelS. CelD.CelO. CelF.CelQ?	Arai et al. (1996), Bayer et al. (1998), Beguin and Lemaire (1996), Schwarz (2001)
B9	B9	S9 (67)	XynC	Beguin and Aubert 1994
B10	B10	S10 (66)	CelB. CelG.	Beguin and Aubert 1994
B11	B11	S11 (60)	Endo/Xylanase?	Morag et al. (1990)
B12		S12 (57)	CelA?	Beguin and Aubert (1994)
		S13 (54)	Eng/Xyl	Morag et al. (1990)
B14		S14 (48)		

? – assumption

vation (80 h). The rate of MUF liberation under these conditions, where no growth of the culture was detected, depended on the amount of cellulases coming from the inoculum, as well as on enzyme activity at the given pH. At pH 7.4–8.9, i.e. under conditions appropriate for growth, MUF accumulated exponentially (Fig. 1a, curves 2–7). Autocatalytic liberation of MUF has only one simple explanation, i.e. de novo synthesis of cellulases by growing cultures. Otherwise, a decrease in the enzymatic reaction velocity should be expected because of the decrease of the MUFL concentration below the Michaelis constant.

Differentiation of the obtained curves allows the calculation of an increase in enzymatic activity with time and, therefore, the estimation of the rates of cellulase biosynthesis at different pH (Table 1, columns 3 and 4). The maximal rates calculated from these curves for pH 8–8.5 (0.10 ± 0.01 h^{-1}, Table 1, columns 3 and 4) were twice as high as the maximal growth rate on cellobiose observed for the cultivation in the pH range of 7.84–8.95 (0.046 h^{-1}, Table 1, columns 1 and 2). The comparison of the data given in Table 2 (columns 1–4) also shows a prevalence of the cellulase synthesis rates in cellulose-based media over the growth rates of the culture in cellobiose-containing media and a slight shift of the optimal conditions of enzyme production towards acid pH, although qualitative similarity of the results of these two quite different assays is evident.

The proposed new assay was further verified by a comparison with the conventional one. For this purpose, aliquots of the growing culture were withdrawn at different time periods and their MUFL-hydrolyzing activity was measured aerobically according to Melnik et al. (1991). Figure 1b shows that the maximal rate of cellulase synthesis was also observed in the pH range 8.1–8.5, in agreement with the results of novel assay. However, the maximal synthesis rates corresponded to approx. half of those determined when using the novel assay, and were nearly equal to the values obtained for the culture growth rate in the presence of cellobiose. The reasons of this twofold difference between the two methods of monitoring of cellulase biosynthesis are not yet fully understood. One might assume that cellulase activity in the aliquots withdrawn relates mainly to the water-soluble enzyme fraction that is released after partial cellulose digestion or cell lysis, whereas the novel assay measures the total amount of cellulases including those adsorbed tightly onto cellulose, which may appear first during the course of the cultivation.

Table 1 summarizes the data on the rates of growth and cellulase biosynthesis by the new bacterium obtained using different methods. Taken together, strain Z-7026 is slowly growing anaerobic species, since the obtained rates fall close to the lower limit of corresponding values known for other cellulolytic anaerobes (from 0.05 for *C. cellulolyticum* to 0.51 h^{-1} for *Ruminococcus flavefaciens*) (Lynd et al. 2000).

Proteins secreted by the bacterium grown in cellulose medium

Because of relatively low level of cellulase activity the attempts undertaken for the isolation of appropriate amounts of cell-bound or cellulose-bound cellulases (see Materials and methods) failed. Sufficient activity was obtained only after cultivation of Z-7026 in cellulose-containing medium II. The composition of extracellular proteins precipitated from the culture supernatant is shown in Fig. 2a. Table 2 demonstrates the similarity of

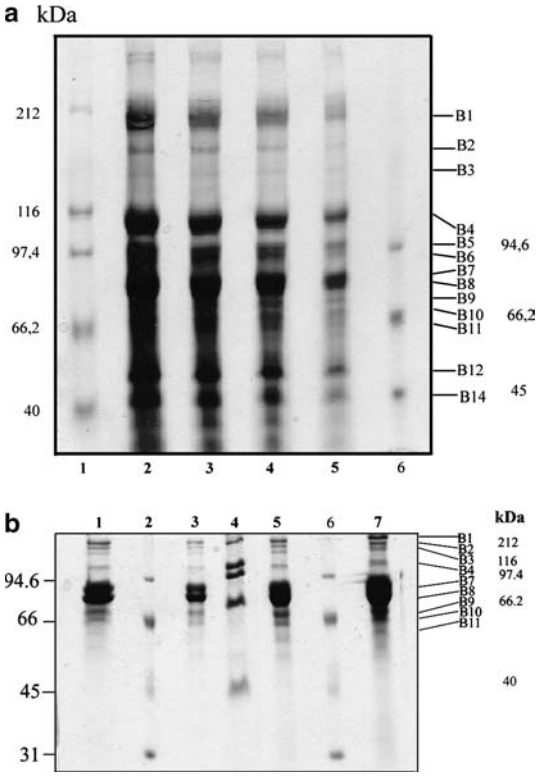

Fig. 2 Composition of extracellular proteins in the $(NH_4)_2SO_4$-precipitated culture supernatant according to SDS-PAGE at different loadings (**a**) and soluble proteins with high affinity for cellulose (**b**). **a**: *1* high-molecular-mass markers, *2–5* precipitated proteins at loadings 50, 30, 20, and 10 µl of sample solution, respectively; *6* low-molecularmass markers (8% gel). **b**: *1,3* proteins adsorbed onto MCC Sigmacell 50 (4 and 2 mg, respectively), *2,6* low-molecularmass markers; *4* high-molecularmass markers; *5,7* proteins adsorbed onto PASC (2 and 4 mg, respectively) (12% gel). The proteins are numbered according to the subunits of *Clostridium thermocellum* cellulosome of similar molecular mass (see Table 2)

an array of obtained protein bands and the composition of the classical cellulosome of *C. thermocellum*. However, the number of proteins showing high affinity for amorphous or crystalline cellulose is significantly lower (Fig. 2b). Among them the proteins with molecular masses of 84 and 75 kDa dominate, which correspond to the subunits S7 and S8 of the clostridial cellulosome. In addition, several other proteins were identified on the cellulose surface that roughly correspond to the subunits S1 (scaffoldin), S2–S4 and S9–S11 of the cellulosome; however, their relative content is remarkably lower than that of the major proteins. Interestingly, according to Morag et al. (1990) subunit S8, the major clostridial cellobiohydrolase bearing a catalytic domain of family GH 48, is not capable of splitting MUFC, the closest analogue of MUFL, whereas according to Bhat et al. (2001) S8 reveals MUFC-hydrolase activity. MUFC activity is well documented for the cellulosome components S3 (CbhA) and S5 (CelK) (Zverlov et al. 1999), as well as for S9–10 (Morag et al. 1990). However, among proteins of Z-7026 that reveal strong affinity for cellulose the corresponding components B3 and B9,10 are the minor forms (Fig. 2b).

Essential identity of the composition of proteins adsorbed on either amorphous or crystalline cellulose at different loadings might be interpreted as binding of the cellulosome as a whole. The fact that the spectrum of proteins of this new strain that shows strong affinity for cellulose is smaller than that of the *C. thermocellum* cellulosome does not, in principle, reject this assumption. It is well established that not every cellulosomal protein is equally important for efficient cellulose decomposition. For example, a high efficiency of *C. thermocellum* subcellulosomes was demonstrated (Kobayashi et al. 1990). Examples of cellulosomes containing only nine constituents are also known (Doi et al. 1998).

However, gel filtration of precipitated proteins of Z-7026 culture fluid on Sepharose 4B did not reveal a high-molecular mass fraction typical for *C. thermocellum* cellulosomes (data not shown). Thus, the proteins B1–B4 and B7–B11 may bear both type-4 cellulose-binding modules (CBM) specific for amorphous cellulose and type-3b CBMs specific for crystalline cellulose, alike, for example, CbhA (S3) of *C. thermocellum* (Zverlov et al. 1999). It is also important to note that cellulolytic anaerobes do not necessarily form cellulosome-like aggregates. For example, *Anaerocellum thermophilum* (Svetlichnyi et al. 1990) and *Halocella cellulolytica* _(Simankova et al. 1993) do not produce cellulosome-like structures. The absence of cellulosomes in cellulolytic haloalkaliphile might be explained by the crucial role of Ca^{2+} ions, which provide structural integrity of dockerins of separate cellulosome subunits (Choi and Ljungdahl 1996; Lytle et al. 2000). The concentration of Ca^{2+} ions in lake Verkhnee Beloye does not exceed 0.5 mM (Gorlenko et al. 1999). This con-

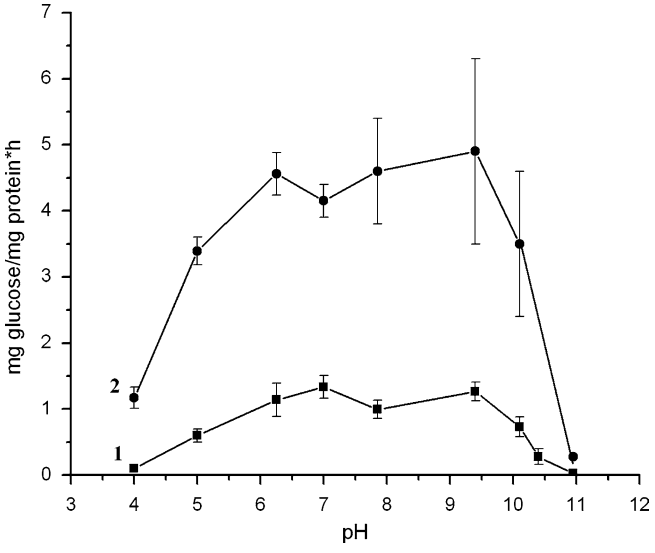

Fig. 3 Dependence of the activity of the precipitated protein preparation from strain Z-7026 towards MCC (*1*) and PASC (*2*) on pH

centration is 20-fold below the optimal Ca^{2+} concentration necessary for supporting optimal activity and stability of cellulosomes. Depletion of Ca^{2+} ions, which play an important role in cellulosome self-assembly, results in the dissociation of the cellulosome with simultaneous splitting of one of two hydrophobic repeats forming subunit dockerins (Choi and Ljungdahl 1996).

To finally resolve the supermolecular structure of the cellulase system of the new bacterium, both detailed electron microscopic studies and investigations of the gene structure of the cellulolytic system are required. These latter studies should investigate the presence of a gene of the cellulosome-integrating protein as well as DNA repeats coding for typical cohesin and dockerin domains.

Cellulase activity of precipitated extracellular proteins

Figure 3 illustrates the pH dependence of cellulase activity of the precipitated proteins from the culture supernatant towards MCC and PASC as substrates (curves 1 and 2, respectively). At pH 4 and 11 cellulase activity towards MCC was negligible. Optimal activity towards both MCC and PASC was observed in the pH range 6–9, the latter substrate being approx. 3.5 times more easily hydrolyzed. However, the specific activity towards PASC at 50° and pH 6–9 did not exceed 0.5 μmol/min per 1 mg protein, i.e. it was significantly lower than the activity of intact *C. thermocellum* cellulosome but comparable to the activity of individual clostridial cellulases (Lynd et al. 2002). In contrast to the majority of fungal cellulases, the new preparation retains more than 50% of cellulase activity towards MCC within the pH range 5–10 and more than 75% of activity towards PASC in the pH range of 5.0–9.4. This opens a number of opportunities for practical applications of cellulases formed by Z-7026, particularly in the areas requiring high pH values, e.g., detergents.

Strain Z-7026 also exhibited xylanase activity when grown in the presence of cellulose. At pH 8 the activity of precipitated proteins towards oat spelt xylan was 60-fold greater than towards PASC (300 mg xylose/mg protein×h). However, the pH optimum of xylanase activity was less alkaline than that of cellulases. At pH 9 and 10 xylanase activity comprised only 50 and 7% of the value obtained at pH 8 (150 and 20 mg xylose/mg protein×h, respectively). The decrease in xylanase activity of the new preparation in the alkaline pH range was even more pronounced than for xylanases of *C. thermocellum* (Morag et al. 1990).

High residual activity of cellulases of alkaliphilic microorganisms at pH 9–10 indicates the existence of a compensation mechanism of glycosidic bond cleavage, diminishing the negative effect of deprotonation of the catalytic residue (usually COOH of a conserved Glu) due to an increase in pH. One such mechanism might be the formation of the catalytic triad Ser-His-Glu that is highly conserved in the family GH 5, which includes, among others, a number of clostridial endoglucanases (Shaw et al. 2002). The function of this triad might be to provide the catalytic Glu under alkaline conditions with a proton for the primary attack on the glycosidic oxygen via a proton-transfer chain from His or Ser. Further studies will show whether this is in fact the case for alkaline cellulases of anaerobic bacteria belonging to different GH families.

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