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Characterization of a metagenome-derived halotolerant cellulase

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Abstract

Metagenomes of uncultured microorganisms represent a sheer unlimited resource for discovery of novel biocatalysts. Here, we report on the biochemical characterisation of a novel, soil metagenome-derived cellulase (endoglucanase), Cel5A. The deduced amino acid sequence of Cel5A was similar to a family 5, single domain cellulase with no distinct cellulose binding domain from *Cellvibrio mixtus*. The 1092 bp ORF encoding Cel5A was overexpressed in *Escherichia coli* and the corresponding 42.1 kDa protein purified using three-step chromatography. The recombinant Cel5A protein was highly active against soluble cellulose substrates containing β-1,4 linkages, such as lichenan and barley β-glucan, and not active against insoluble cellulose. Glucose was not among the initial hydrolysis products, indicating an endo mode of action. Cel5A displayed a wide range of pH activity with a maximum at pH 6.5 and at least 60% activity at pH 5.5 and 9.0. The enzyme was highly stable at 40 °C for up to 11 days, and retained 86–87% activity after incubation with 3 M NaCl, 3 M RbCl or 4 M KCl for 20 h. Cel5A was also active in the presence of diverse divalent cations, detergents and EDTA. This highly stable, salt and pH tolerant cellulase is an ideal candidate for industrial applications.

Keywords: Metagenomic; Cellulase; Halotolerant; pH profile

1. Introduction

Cellulases have many industrial applications from the generation of bioethanol, a realistic long-term energy source, to the finishing of textiles (Ando et al., 2002; Lynd et al., 2002). These industrial processes require cellulolytic activity under a range of pH, temperature and ionic conditions, and they are normally carried out by mixtures of cellulases. Investigation of the diverse range of cellulolytic enzymes involved in the natural degradation of cellulose is necessary for optimisation of these processes.

Three major types of enzymatic activity are involved in the degradation of cellulose in nature: endoglucanases (1,4-β-D-glucan-4-glucanohydrolases; EC 3.2.1.4), exoglucanases including cellodextrinases (1,4-β-D-glucan glucanohydrolases; EC 3.2.1.74) and cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases; EC 3.2.1.91), and (β-glucosidases β-glucoside

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glucohydrolases; EC 3.2.1.21). The most recent nomenclature describes approximately 100 families of glycosyl hydrolases which are organised into 14 clans as listed at the CAZy server (http://afmb.cnrs-mrs.fr/CAZY/).

Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze β-1,4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1,4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism using a proton donor and nucleophile or base. The hydrolysis can either result in the inversion or retention (double replacement mechanism) of the anomeric configuration of carbon-1 at the reducing end (Béguin and Aubert, 1994; Birsan et al., 1998; Hildén and Johansson, 2004).

While there remains much interest in the isolation of cellulases from fungal sources, there has been a recent increase in the isolation of diverse novel cellulases from prokaryotic organisms (Hildén and Johansson, 2004). The two different structural types of cellulase systems found in bacteria are designated non-complexed and complexed. Some anaerobes are known to produce an extracellular multi-enzyme complex called a cellulosome (Schwarz, 2001). The cellulosome comprises cellulases organised on a non-catalytic scaffolding protein which mediates binding to the cellulose (Schwarz, 2001). In contrast, cellulases from the majority of aerobes are not produced as complexes but bind directly to the cellulose (Zhang and Lynd, 2004). These non-complexed cellulases can have a modular structure with non-catalytic carbohydrate binding domains (CBD) linked to catalytic domains by flexible linkers. CBD play a role in binding of the cellulase to insoluble cellulose (Bolam et al., 1998; Carvalho et al., 2004). In addition to enzymes with clearly defined carbohydrate-binding domains a significant number of cellulases have been identified that have no defined CBD and are thus referred to as non-modular cellulases (Lynd et al., 2002). Cellulases lacking CBD show reduced activities against insoluble cellulose while retaining the capacity to depolymerise soluble cellulosic substrates (Bolam et al., 1998; Coutinho et al., 1993; Fontes et al., 1997).

The majority of prokaryotic cellulases have been isolated from cultured microorganisms. Cellulases from specific organisms tend to display activity only in the pH range corresponding to their environment such as the β-1,4-endoglucanase from the larvae gut bacterium *Cellulomonas pachnodae* which had a pH range between pH 4.8 and 6.0 (Cazemier et al., 1999) and the endoglucanase from an alkalophilic *Bacillus* species which had a pH range from 7.0 to 12.0 (Sánchez-Torres et al., 1996). Also, many of the cellulases with industrially relevant characteristics are obtained from extremophiles (Ando et al., 2002; Gomes and Steiner, 2004; van Solingen et al., 2001). Cultivation of microorganisms from these environments is particularly problematic with the majority remaining uncultured. There is a vast biocatalytic potential locked within the uncultured portion of environmental microbial consortia. Metagenomics is a cultivation independent analysis of the metagenome of a habitat and involves direct isolation of DNA from the environment followed by cloning and expression of the metagenome in a heterologous host (Handelsman et al., 1998). This technique had been used to detect a wide range of biocatalysts from uncultured microorganisms (Schloss and Handelsman, 2003; Steele and Streit, 2005; Streit et al., 2004). Although characterisation of cellulases from uncultured microorganisms could reveal much information about cellulolytic diversity and industrial potential there are only two reports to date of metagenome-derived cellulases (Healy et al., 1995; Rees et al., 2003).

To further increase knowledge on cellulases, we isolated and biochemically characterized a soil metagenome-derived cellulase, Cel5A. This enzyme was shown to be an endoglucanase (endo 1,4-β-glucan hydrolase) which was highly active towards soluble forms of cellulose. This novel cellulase was remarkably stable over a wide pH and temperature range, and in the presence of high salt concentrations. This is the first report of such characteristics in a cellulase from a non-extremophile environment.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Escherichia coli was grown at 37 °C on complex Luria–Bertani (LB) medium supplemented with appropriate antibiotics (Sambrook and Russell, 2001). The metagenomic cosmid library was generated from

soil microbial consortia, which had been enriched for agarolytic activity, as previously described (Voget et al., 2003).

2.2. Identification of cellulase-positive clones, subcloning and DNA sequence analysis

Cellulase-positive clones were screened for by using a colorimetric assay on Congo red indicator plates (Teather and Wood, 1982). For this, colonies harbouring metagenomic DNA in the cosmid vector pWE15 (Stratagene, La Jolla, USA) were grown on LB agar supplemented with 0.2% carboxymethylcellulose (CMC). Colonies were stained with 0.2% Congo red for 20 min and destained with 1 M NaCl (Teather and Wood, 1982). Cellulase-expressing colonies were surrounded by a yellow halo against a red background. In total, eight clones were identified which showed reproducible cellulase activity (pSVCosCEL-1 to pSVCosCEL-8). Cellulase-positive cosmid clones were digested with the restriction enzyme *Eco*RI, subcloned in pTZ19R (Cm^R, Göttingen genome laboratory, Germany) and screened again for cellulase activity. This reduced the size of the inserts as well as confirming their genetic diversity. To obtain the nucleotide sequence of the activity possessing gene, a transposon mediated mutagenesis was performed on one of the cellulase-positive subclones, pSVCEL-1 (pTZ19R carrying *celA* on a 9.7 kb *Eco*RI clone derivative of pSVCosCEL-1), using the EZ:TN ⟨Kan-2⟩ Tnp Transposome Kit (Epicentre, Madison, USA). Using the inserted priming sites the corresponding gene was sequenced using automated sequencing technologies (MegaBACE 1000 System, Amersham Bioscience). The sequence was deposited at GenBank under the accession number DQ139834.

2.3. Cloning, expression of celA in E. coli and protein purification

The cellulase encoding gene, *celA*, was amplified from cosmid DNA using PCR in 35 cycles with primer pairs celA-forward 5′-CATATGAAATTTCCGCATTGCTTG-3′ and celA-reverse 5′-GGATCCCTATTTCTTATTTTCACG-3′. Primers were designed to introduce a 3′-*Bam*HI restriction site and a 5′-*Nde*I site into the cloned fragments. To increase cloning efficiency the PCR fragments were first ligated into pBSK+, then excised with *Nde*I and *Bam*HI and ligated into pET19b (Novagen/Merck KGaA, Darmstadt, Germany). The recombinant clones were sequenced and one clone, which was designated pSVpETCEL-1, was chosen for overproduction. *E. coli* BL21 (Novagen/Merck KGaA, Darmstadt, Germany), carrying pSVpETCEL-1 (the *celA* gene in pET19b), was grown to OD 0.6 in LB broth containing ampicillin (100 μg ml^{-1}) at 30 °C and 90 rpm, and expression of the *celA* gene was induced by adding 0.1 mM isopropyl thio-β-D-galactoside (IPTG) followed by further incubation overnight. Cells were harvested and resuspended in 50 mM Tris–HCl pH 8.0 prior to disruption through a French pressure cell at 20,000 psi cell pressure. Although the cloning had produced a N-terminal 6-His fusion protein of Cel5A, the His-tag could not be used for purification. Instead, the cellulase was purified from the supernatant in three steps using an AKTA FPLC system (GE Healthcare) and appropriate columns. The first purification step was done by ion exchange chromatography and a Source 30Q column (1 cm × 10.6 cm) equilibrated with 50 mM Tris–HCl pH 8.0. Linear gradient elution was performed using NaCl (1 M NaCl, 50 mM Tris–HCl pH 8.0). The fractions containing active cellulase protein were identified by assaying enzyme activity and by protein mobility on SDS-PAGE. Collected fractions were dialyzed overnight against 50 mM K_2HPO_4, 1 M NH_4SO_4 pH 6.5 in preparation for the second purification step by hydrophobic interaction chromatography (HIC) using a HIC phenyl sepharose HP column (1.6 cm × 10 cm). The column was equilibrated with 50 mM K_2HPO_4, 1 M NH_4SO_4 pH 6.5 and elution was performed with an linear gradient using 50 mM K_2HPO_4 pH 6.5. The active fractions were pooled, concentrated and applied to a Superdex 200 column (1.6 cm × 60 cm), which was previously equilibrated with 50 mM K_2HPO_4 at pH 6.5. Elution was carried out using the same buffer at 60 ml h^{-1}. The fractions containing active cellulase were pooled and stored at 4 °C. The level of purity of Cel5A, as well as the molecular mass, was determined by SDS-gel electrophoresis.

2.4. Enzyme assays

The purified enzyme was used to examine substrate specificity and to characterize the properties of Cel5A. Cellulase activity was routinely assayed by measuring

the amount of reducing sugar released from carboxymethylcellulose (Sigma) using dinitro-salicylic acid reagent (Miller, 1959). The standard assay mixture contained 2 μg of the enzyme and 1% CMC in a final volume of 0.5 ml of 50 mM K_2HPO_4 at pH 6.5. This mixture was incubated at 40 °C for 15 min. Enzyme assays were carried out in duplicate for each treatment. Units of enzyme activity (U) are expressed as micromoles of reducing sugar released per minute per milligram protein.

To test the temperature range of the enzyme, the standard assay mixture activity was measured at temperatures between 23 and 95 °C. The pH range was determined by measuring standard assay activity between pH 4 and 11.5 using 50 mM of the appropriate buffers. Acetate buffer was used for pH 4–6.0, citrate/phosphate buffer (Macllvaine buffer) was used for pH 6–7.5, Tris–HCl was used for pH 7.5–9.0, and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) was used for pH 9.7–10.5. To test for substrate specificity, CMC was replaced in the standard assay mixture by lichenan, barley β-glucan, laminarin, oat spelt xylan or avicel.

Inhibition or enhancement of cellulase activity was determined for a range of different metal chloride salts, solvents, detergents and EDTA using 1 mM concentrations, unless otherwise stated, and the standard assay mixture.

Thermotolerance and halotolerance was determined by measuring residual activity with the standard assay after preincubation of enzyme samples under the following conditions: temperatures were 40, 50, 55 and 60 °C for up to 11 days, and salt conditions tested included 3 M NaCl or 4 M KCl or 3 M RbCl for up to 20 h.

2.5. Analysis of reaction products by thin-layer chromatography

To determine whether Cel5A has an endo or exo mode of action, cellooligosaccharides (1% from Sigma, Heidelberg, Germany), lichenan (1%, from *Cetraria islandica*, Sigma, Heidelberg, Germany) and CMC (1% from Sigma, Heidelberg, Germany) were digested with Cel5A (2 μg) in 50 mM K_2HPO_4, pH 6.5. Subsequently, aliquots from various incubation times were spotted on a silica 60 TLC plate (Merck KGaA, Darmstadt, Germany). The cellooligosaccharide reaction products were developed in 1-propanol, nitromethane, H_2O (5:3:2, v/v/v) for 2 h. After separation, sugars were visualized by spraying the plates with a freshly prepared mixture of ethanol/concentrated sulphuric acid (9:1, v/v). The lichenan reaction products were developed in ethylacetate, acetic acid, H_2O (2:1:1, v/v/v) for 3 h. After separation, sugars were visualized by spraying the plates with a mixture of 1 ml phosphoric acid and 10 ml stock solution (1 g diphenylamine, 1 ml anilin, 100 ml acetone). The CMC reaction products were developed in 1-propanol, ethylacetate, H_2O (6:1:3, v/v/v) for 2× 3 h, and the sugars visualized using the same mixture as for visualization of lichenan products.

3. Results

3.1. Isolation and partial DNA sequence analysis of pSVCosCEL-1

The soil metagenome library containing 1700 clones yielded eight cellulase-positive clones which were shown to be different based on restriction analysis. One clone, designated pSVCosCEL-1, was selected for more detailed characterisation. Partial DNA sequencing together with restriction analysis revealed an insert size of approximately 22 kb for the pSVCosCEL-1 clone. A 9.7 kb *Eco*RI derivative, pSVCEL-1, was sequenced revealing a total of five complete ORFs (Fig. 1A and Table 1). Additional in vitro transposon mutagenesis verified the location of the ORF responsible for the hydrolytic activities. The putative cellulase gene, which had a length of 1092 bp encoding for 363 amino acids, was designated *celA* (Fig. 1A and Table 1). A putative 23 amino acid long signal peptide was predicted from sequence analysis. The amino acid sequence of Cel5A was similar (86% similarity and 77% identity) to a known cellulase (GenBank accession number AAB61461) from *Cellvibrio mixtus* (Table 1) (Fontes et al., 1997). Sequence analysis of Cel5A indicated that it belongs to glycosyl hydrolase family 5, formerly known as cellulase family A. Interestingly, two of the identified ORFs on pSVCosCEL-1 showed high similarities to proteins involved in polysaccharide hydrolysis. *Orf01* showed 76% similarity and 62% identity to a predicted glycosyl hydrolase of family 81 from *Microbulbifer degradans* and *orf03* was highly

Table 1
Open reading frames (ORF) identified on pSVCEL-1

ORF	AA[a]	Function, closest match	AA[b]	% Identity	Accession number
orf01	1095	Predicted glycosyl hydrolase—*M. degradans*	1238	62	ZP00315839
orf02	310	Conserved hypothetical protein—*Chromobacterium violaceum*	278	33	AAQ60986
orf03	386	Pectate lyase—*Frankia* sp.	365	45	ZP00572934
celA	363	Cellulase A—*C. mixtus*	363	77	AAB61461
orf04	729	Hypothetical protein plu4553—*Photorhabdus luminescens* subsp. laumondii	1257	27	NP931717

[a] The size of putative protein corresponding to ORF.
[b] The size of closest matching protein. Sequences of the analyzed 9.7 kb contig have been deposited at GenBank under accession number DQ139834.

similar (59%) to a predicted pectate lyase from *Frankia* sp.

3.2. Purification and molecular mass of Cel5A

The metagenome-derived *celA* gene was amplified and cloned into an expression vector, pET19b, as described in materials and methods. After overexpression, the recombinant Cel5A protein was purified from the supernatants of *E. coli* strain BL21 cell extracts harbouring the pET19b-*celA* constructs using three-step chromatography. The first purification step was done by ion exchange chromatography and a Source 30Q column followed by a purification step using hydrophobic interaction chromatography (HIC) with a HIC phenyl sepharose HP column. The final purification step using Superdex 200 yielded a 25-fold purified Cel5A protein (Table 2). SDS-PAGE analysis revealed that the protein was homogenous and indicated a molecular mass of approximately 42 kDa for the recombinant Cel5A protein (Fig. 1B). The estimated molecular mass of 42 kDa is in accordance with the theoretical molecular mass of 42.1 kDa.

3.3. Substrate profile of Cel5A

Cel5A was initially identified with carboxymethylcellulose (CMC) as substrate. The purified enzyme

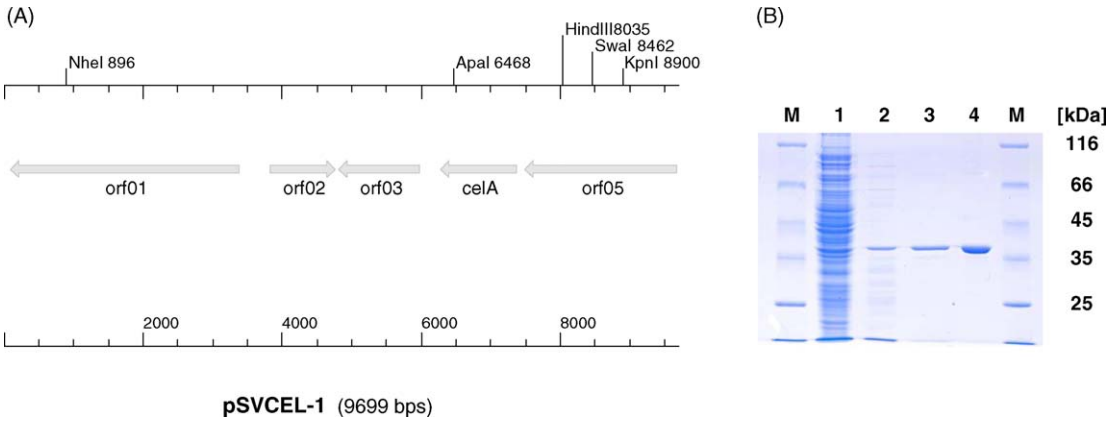

Fig. 1. (A) Physical map of the pSVCEL-1 clone. (B) SDS PAGE of the 42.1 kDa Cel5A protein at each purification step. Lane M is the protein molecular weight marker (Fermentas): lane 1 is the cell-free extract of *E. coli* pSVpETCEL-1; lane 2 is Cel5A after the purification step using a Source Q30 column; lane 3 is Cel5A after HIC Phenyl-Sepharose; lane 4 is Cel5A after Superdex 200.

Table 2
Purification of Cel5A from *E. coli* pSVpETCEL-1 supernatant

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Yield (%)	Purification (fold)
Cell-free extract	21	968	14952	100	1
Source Q30	20	47.8	5580	37	2
Phenylsepharose	22	7.7	770	5	6.6
Superdex 200	4	1.6	624	4	25

showed high activities with β-1,4-linked glucans, such as CMC (68 U mg^{-1}), lichenan (401 U mg^{-1}) and β-glucan from barley (710 U mg^{-1}). No activity was found against crystalline forms of cellulose such as filter-paper or Avicel. Cel5A was also unable to attack the β-1,3-linked glucan laminarin, and displayed no activity with Xylan (β-1,4-linked xylose).

3.4. Mode of action of Cel5A

In order to determine whether Cel5A had an endo or exo mode of action, CMC and lichenan were degraded and the products analyzed by TLC. A time course degradation of CMC showed that at the beginning only high molecular mass products were released suggesting an endowise action of the enzyme (Fig. 2A). This was supported by further tests involving a time course degradation of lichenan by Cel5A where again only high molecular mass products were released at the start. The major product had a degree of polymerization (DP) of three, but minor quantities of smaller and larger oligosaccharides were also produced (Fig. 2B).

To further confirm the endowise action of the enzyme linear cellooligomers were used as substrates and the products were analyzed using thin-layer chromatography (TLC) (Fig. 2C). The smallest cellooligosaccharide used by Cel5A as a substrate is cellotriose. Cellobiose is the product from the cleavage of cellotetraose. The breakdown from cellopentaose produces cellobiose and glucose as end-products.

3.5. Temperature and pH optima of Cel5A

Enzyme activity was measured using the standard assay with CMC as substrate. The maximum activity was detected at 45 °C with the enzyme displaying a broad temperature range of activity functioning at 76% of its maximal activity at 30 °C and 89% at 50 °C (Fig. 3A).

Tests using different buffer systems revealed that the enzyme was stable over a broad pH range (Fig. 3B). The highest activity was observed at pH 6.5 with the enzyme still displaying 60% of its activity at pH 5.5 and 68% at pH 9. Only low activity was recorded below pH 5 and above pH 9.7.

3.6. Stability against solvents, detergents, metal ions and EDTA

The influence of various solvents on the activity of Cel5A was tested using CMC as a substrate (Fig. 4). Cel5A was greatly inhibited by the presence of 10% (v/v) acetonitrile, displaying only 11% residual activity in comparison to the control. The enzyme was much more stable against solvents such as methanol and ethanol, requiring concentrations as high as 30% (v/v) before activity was reduced to 38% or 8% compared with controls, respectively (Fig. 4). Glycerol had very little influence on enzyme activity at the concentrations tested. In contrast, Cel5A was stimulated in the presence of 5% and 15% (v/v) DMSO, although 30% (v/v) inhibited enzyme activity by more than 50%. Up to 5% (v/v) concentration of the detergent triton had little influence upon the enzyme which retained 88% activity whereas 1% (v/v) SDS resulted in an almost complete loss of the residual activity of Cel5A (Fig. 4).

Additional tests were performed to assay the influence of cations and EDTA on Cel5A by measuring residual activity. In these tests the enzyme activity was only slightly reduced by 1 mM concentrations of cations such as Cu^{2+} (88%), Mg^{2+} (92%), Cd^{2+} (92%), Ni^{2+} (96%), Co^{2+} (96%) and Fe^{2+} (97%). In the presence of 1 mM Fe^{3+} the residual enzyme activity was lower at 73%. The same level of inhibition was detected in the presence of Ba^{2+} (79%), Zn^{2+} (78%) and Ca^{2+} (74%). Cel5A was stimulated in the presence of 1 mM Mn^{2+} up to 130% of its original activity. When the concentration was increased to 2.5 mM the enzyme

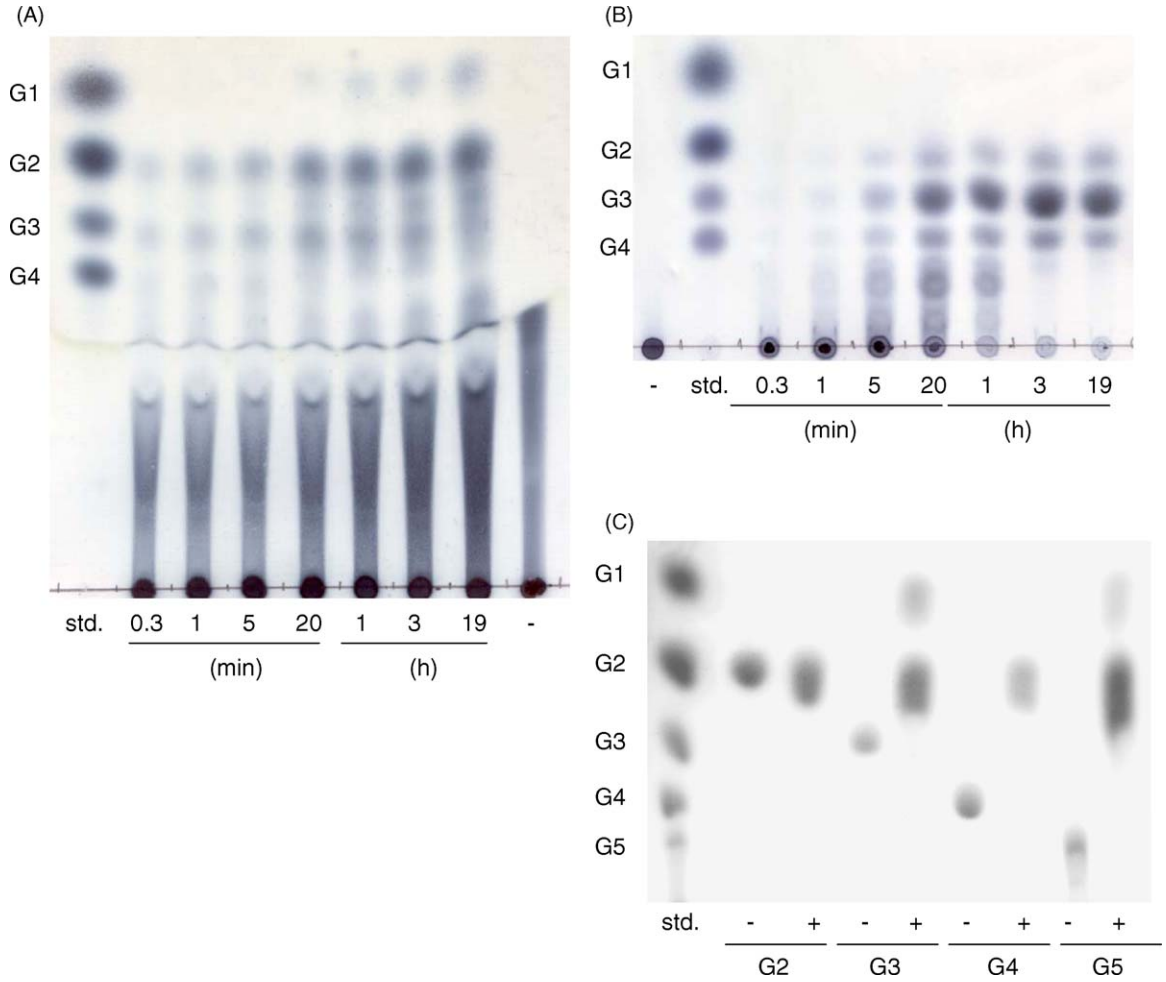

Fig. 2. Thin layer chromatography analysis of degradation products released by Cel5A. Time course degradation of CMC (A) and lichenan (B) sampled at the times indicated. Lane (—) is the control containing substrate without enzyme, and lane (std) is the standard mixture: glucose (G1), cellobiose (G2), cellotriose (G3) and cellotetraose (G4). (C) Hydrolysis of cellooligosaccharides after 24 h incubation at 40 °C. Standards/substrates G1 to G4 as previously. In addition, cellopentaose (G5) was used. (+) indicates enzyme was present and (−) indicates incubation in the absence of enzyme.

was stimulated to 112%. A further increase to 5 mM resulted in a reduction in the activity of the enzyme which still retained 80% residual activity under these conditions (Fig. 4). The enzyme was quite stable in the presence of 50 mM EDTA displaying 86% of the control level of activity.

3.7. Halotolerance and thermotolerance of Cel5A

To test the level of halotolerance of Cel5A it was incubated with either 3 M NaCl, 4 M KCl or 3 M RbCl for up to 20 h and residual activity measured using the standard assay. Cel5A was still active in the presence of very high salt concentrations retaining 87% of its activity after 20 h of preincubation in the presence of 3 M NaCl (Fig. 5A). Preincubation of 20 h in the presence of 3 M RbCl resulted in 77% activity of Cel5A, whereas preincubation of the enzyme for 20 h in the presence of 4 M KCl retained 86% of the original activity. This is a remarkable salt stability for an enzyme derived from a non-halophilic soil microbial community.

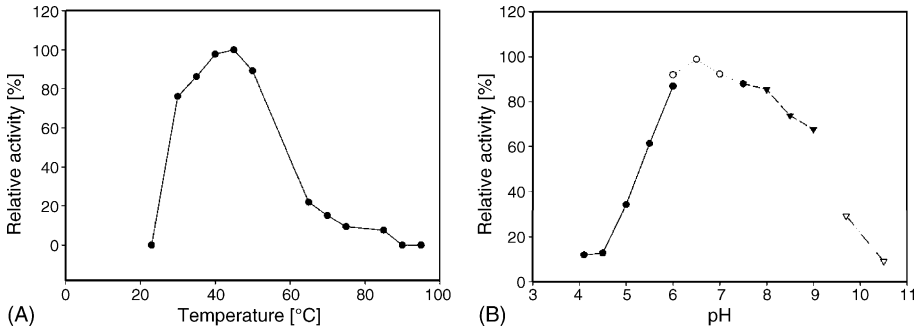

Fig. 3. (A) Influence of temperature on Cel5A activity. Cel5A activity was measured using the standard assay mixture incubated at temperatures between 23 and 95 °C. The maximum activity measured at 45 °C was taken as 100%. (B) Influence of pH on Cel5A activity. Cel5A activity was measured using the standard assay mixture incubated at pHs between pH 4 and 10.5. The maximum activity measured at pH 6.5 was taken as 100%. Buffers used were acetate (●), Macllvaine (◯), Tris–HCl (▼) and CAPS (▽).

In order to test the thermostability of the enzyme, it was incubated at various temperatures and the residual activity measured using the standard assay. Preincubation of the enzyme at 60 °C resulted in immediate and complete inactivation of the enzyme (Fig. 5B). Preincubation for 10 min at 55 °C resulted in only 20% of control activity remaining. In contrast, preincubation at 50 °C for 10 min actually stimulated enzyme activity slightly and after 180 min Cel5A still exhibited 36% of the control activity. Cel5A was remarkably stable at 40 °C. After 4 h incubation at 40 °C the enzyme showed no loss in level of activity. Further tests showed an enzyme activity of 95% after extended incubation at this temperature for 11 days (Fig. 5B).

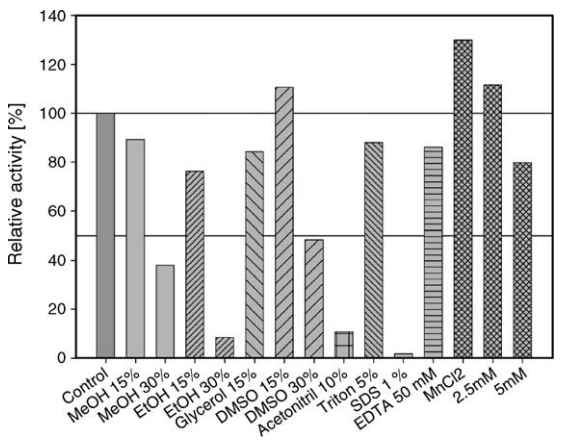

Fig. 4. Influence of solvents, detergents, EDTA and bivalent metals on Cel5A activity. Activity was measured using the standard assay, and control activity was taken as 100%.

4. Discussion

There is catalytic diversity at the habitat level with reactions, such as cellulose degradation, often being carried out by mixtures of synergistically interacting enzymes (Hildén and Johansson, 2004; Väljamäe et al., 1999; Zhang and Lynd, 2004). Metagenomics is an excellent, cultivation-independent strategy for detection and isolation of all enzymes involved in cellulose degradation in a single habitat. Screening of a soil metagenomic library revealed that 8 out of 1700 clones displayed cellulolytic activity. The detection of eight genetically different cellulase-positive clones from a single environment reflects the natural diversity of this type of enzyme. This relatively high number of positive clones may be attributed to the selective enrichment step prior to DNA isolation, a strategy used in metagenomics to increase the likelihood of detecting clones displaying the desired trait (Entcheva et al., 2001; Voget et al., 2003). Previous metagenomic studies targeting cellulolytic activity used either selective preenrichment in the laboratory (Rees et al., 2003) or generated the library from an environment where the microbial population has been naturally enriched for cellulolytic ability (Healy et al., 1995).

Sequencing analysis of pSVCEL-1, a 9.7 kb subclone of cosmid pSVCosCE-1 displaying cellulolytic activity, revealed five ORFS. The identified cellulase, c*elA*, was flanked by two other putative hydrolases suggesting that this cellulase may be part of a gene cluster or operon linked to hydrolytic activities. The amino acid sequence of Cel5A was determined and

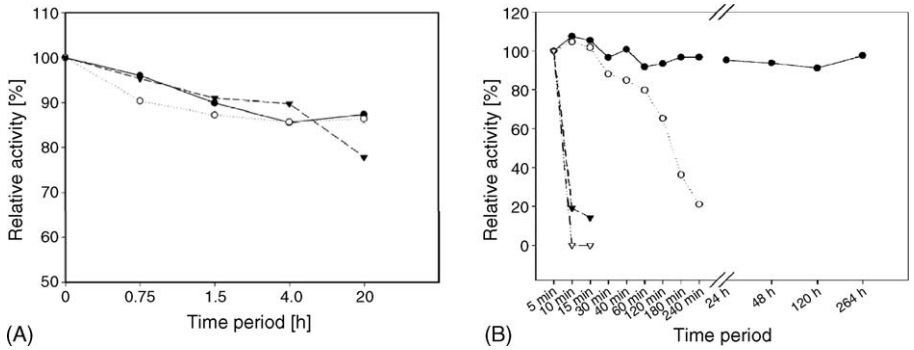

Fig. 5. (A) Halotolerance of Cel5A was tested by assaying residual activity towards carboxymethylcellulose after incubation in 3 M NaCl (●), 4 M KCl (▼) or 3 M RbCl (◯) for up to 20 h. (B) Thermal stability of Cel5A examined over a time period of 240 min by measuring the residual activity after incubation of the standard assay at 40 °C (●), 50 °C (◯), 55 °C (▼) and 60 °C (▽). This was extended for up to 264 h (11 days) for the incubation at 40 °C.

found to be similar to a cellulase isolated from *C. mixtus* (Fontes et al., 1997). Motif searches in the NCBI databases allocated the metagenome-derived cellulase to the same glycoside hydrolase family as the *C. mixtus* cellulase, family 5 (GH5). These cellulases include single domain enzymes lacking the carbohydrate binding domain (CBD). The carbohydrate binding domain is involved in degradation of insoluble cellulose, and cellulases lacking CBD are capable of degrading only the soluble forms of cellulose (Bolam et al., 1998; Coutinho et al., 1993; Fontes et al., 1997).

Cel5A was overproduced in *E. coli* and purified to homogeneity. The experimentally determined protein molecular mass was 42 kDa. As GH5 cellulases are capable of hydrolysing a wide range of soluble cellulose substrates, the substrate range of the metagenome cellulase was tested. Cel5A was unable to hydrolyse Avicel, a crystalline form of cellulose. It was also unable to hydrolyse substrates that lack β-1,4-linkages, such as laminarin (β-1,3/β-1,6), and substrates such as β-1,4 linked xylose. Cel5A displayed typical β-glucanase activity with much higher activity towards barley β-glucan and lichenan, β-1,4/β-1,3-mixed linkage substrates, than towards carboxymethyl cellulose. Hydrolysis of CMC and lichenan produced mainly cellobiose and cellotriose, with no evidence of glucose formation in the early stages. Cellotriose had the minimum length required for hydrolysis. Based on these findings the metagenome cellulase, Cel5A, is most likely an endo 1,4-β-glucan hydrolase. The level of Cel5A activity towards barley β-glucan and lichenan was six to seven times higher than that determined for the *C. mixtus* cellulase, the cellulase most similar to Cel5A (Fontes et al., 1997), although this may be partially explained by the fact that Cel5A was purified to a higher level.

In a series of classical biochemical tests the metagenome cellulase was shown to be highly stable over a wide pH range with activity from acidic pH 5.5 to alkaline pH 9.0. Cel5A displays a broad pH range being more pH stable than any other metagenome-derived cellulase (Healy et al., 1995) and more stable than the cellulase from *C. mixtus* (Fontes et al., 1997). Cel5A displays a similar pH range to that determined for an endocellulase isolated from a *Streptomyces* sp. inhabiting an East African soda lake (van Solingen et al., 2001). As Cel5A is a metagenome-derived cellulase it is not possible to determine which microorganism it is derived from, but the original soil sample was not an extreme environment nor an obvious source for extremophiles. Some extreme environments are more stable and less susceptible to fluctuations in pH and temperature conditions than soils which experience less extreme, but more variable conditions. Therefore, the enzymes isolated from such extreme environments function ideally under a limited set of conditions as set by their environment whereas enzymes from soil have to be more stable to cope with fluctuations.

In addition to the pH stability, Cel5A was also shown to have a broad temperature range of activity, from 30 to 50 °C, and it is thermostable at 40 °C for up to 11 days. Thermostability of a family 5 cellulase from a mesophilic background has been previously reported and this stability is possibly a general feature

of extracellular cellulases which lack cellulose binding modules (Fontes et al., 1997). An interesting feature of Cel5A is its stability in the presence of high concentrations of salts. This is remarkable as all other halophilic enzymes have been derived from microorganisms living in extreme environments. Among them are the cellulolytic enzymes from *Thermotoga maritima* which are stabilised at high temperatures by the addition of up to 5 M NaCl (Bronnenmeier et al., 1995; Liebl et al., 1996).

Further investigation of the stability of Cel5A showed that it is active in the presence of a range of divalent cations, detergents and chelating agents which are commonly used in industrial detergents. Cel5A activity was even enhanced in the presence of low concentrations of manganese, a phenomenon also reported for a cellulase from *Bacillus* (Sánchez-Torres et al., 1996). Cel5A was also shown to be stable at solvent concentrations up to 15%, but inhibited when concentrations reached 30%. These characteristics together with the pH, temperature stability and halotolerance of Cel5A are all properties, which indicate that this novel, metagenome-derived cellulase has the potential to be used in industrial applications.

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