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Purification and Biochemical Characterization of Carboxymethyl Cellulase (CMCase) from a Catabolite Repression Insensitive Mutant of *Bacillus pumilus*

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ABSTRACT

The generation of cellulolytic bacterial candidates free from the catabolite repression system is highly desirable for the production of cellulosic degrading enzymes, which are useful in various industrial applications. Previously, we reported the isolation of a chemical-mediated mutant of *Bacillus pumilus* termed as BpCRI 6 for Catabolite Repression Insensitive no. 6, which was tested for its ability to produce cellulase enzyme. The dimeric purified enzyme has a molecular mass of 80 and 170 kDa by SDS-PAGE and size exclusion chromatography, respectively and has been subsequently characterized in the present study. The enzyme was highly active on carboxymethyl cellulose (CMC) but much reduced or no activity on Avicel, cellobiose and 4-methylumbelliferyl-β-D-cellobiose (MUC) and was suggested to be a CMCase/endoglucanase. The enzyme expression pattern in BpCRI 6 showed apparently stable biochemical characteristics over a long period when stored at –20ºC and was ten times higher than in the wild type in presence of high concentration of glucose (40 mM). How the chemical agent induced the CRI mutation is still under investigation. Nevertheless, we regard the selection of this CRI bacterial mutant as a promising discovery that could be of great biological use.

Key Words: *Bacillus pumilus*; Catabolite repression; Chemical mutation; CMCase; Enzyme expression

INTRODUCTION

The homopolymeric cellulose made of anhydro-D-glucose linked by β-1,4 bonds constitutes the most abundant biopolymer on earth (Coughlan & Mayer, 1992). The degradation of the cellulose to glucose requires a combined or cooperative action of at least three enzymes namely an endo-1,4-β-glucanase (also referred to as carboxymethyl cellulase or CMCase; EC 3.2.1.4), an exo-1,4-β-glucanase (EC 3.2.1.91) and a β-glucosidase (EC 3.2.1.21) (Wood, 1989). Studies have highlighted the need for cellulase and its application in various industries (brewery and wine, textile, detergent, pulp and paper industries), in agriculture and in animal feeds (Bhat & Bhat, 1997; Jang & Chen, 2003). To fully degrade the abundant and available cellulosic material, it is imperative to purify and characterize the various components of the cellulase system (Huang & Monk, 2004). This enzyme can be produced industrially or by microorganisms. The industrial production of cellulase is however very expensive (Solomon *et al.*, 1997), but considerable cost reduction may be possible by exploring ways of cellulose degradation using cellulase-producing organisms. The well-characterized cellulase-producing organisms are the mesophilic fungi and anaerobic thermophilic cellulolytic bacteria (Huang & Monk, 2004). Relatively few aerobic cellulolytic bacteria have been reported (Huang & Monk, 2004). The aerobic bacteria are rather known as producers of extracellular enzymes such as amylases, proteinases, lyases, and polysaccharide hydrolases (Priest, 1977). The synthesis of these enzymes is unfortunately under the control of catabolite repression and very few cellulolytic organisms could still produce cellulases in presence of glucose (Kotchoni & Shonukan, 2002). Therefore, generation of strains with enhanced cellulase production, which can escape the end product inhibition either by chemical mutagenesis or genetic engineering, should receive high priority (Labudova & Farkas, 1983; Kotchoni & Shonukan, 2002).

The demand for constitutive production of enzymes in many industrial applications is crucial (Yoshimatsu *et al.*, 1990; Khasin *et al.*, 1993; Bhushan *et al.*, 1994). In paper making industry for instance, the cellulase- and xylanase-producing organisms free from catabolite repression control are highly expected. To date the available commercial cellulases display optimum activity over a pH range from 4.0 to 6.0. In addition, the discovery of alkaline carboxymethyl cellulase (CMCase) and cellulases has created a new industrial application of cellulase used generally as laundry detergent additives. In this regard, only the *Bacillus* sp. strains (the alkaliphilies) are able to produce active cellulase with an alkaline optimum pH of 10 or even higher (Horikoshi *et al.*, 1984; Fukumori *et al.*, 1985).

However, the synthesis of this enzyme is under the control of catabolite repression. Cultures containing 0.5% (w/v) glucose as carbon source repressed the CMCase production in these organisms (Chen *et al.*, 2004).

We had previously isolated a catabolite repression insensitive (CRI) *Bacillus pumilus* mutant following a chemical-mediated mutagenesis in which ethyl methanesulphonate (EMS) was used as mutagenic agent (Kotchoni & Shonukan, 2002). The bacterial mutant namely BpCRI 6 showed high CMCase activity in presence of glucose (Kotchoni & Shonukan, 2002). The CRI screening procedure consisted of growing the wild type and BpCRI 6 bacterial mutant on broth cultures containing CMC as the sole carbon source supplemented with different concentrations of glucose and monitoring the CMCase protein synthesis via PAGE analysis. In this work, we further report the biochemical characterization of the purified CMCase from the selected BpCRI 6 mutant. We mainly focused on the genus *Bacillus* for the production of cellulolytic enzymes because of their fast growth rate. The properties of the enzyme isolated from *B. pumilus* CRI 6 are discussed in comparison with some well-established cellulase-producing organisms.

MATERIALS AND METHODS

Bacterial strains and chemical mutagenesis. Wild strain of *Bacillus pumilus* used in this work was isolated from soybean fermentation and grown in liquid broth culture as described previously (Kotchoni & Shonukan, 2002). Ethyl methanesulphonate (EMS) was used to induce chemical mutation. Overnight cultures of wild strain *B. pumilus* cells were inoculated into new broth cultures and allowed to grow to an OD (670 nm) of 0.6 corresponding to the exponential phase of the bacterium. The culture was centrifuged (6000 rpm, RT, 5 min), pellet resuspended in 1 mM EMS in saline media and further incubated for 5 h to induce chemical mutation. Different dilutions of the cell cultures were made and streaked on agar plates to obtain independent bacterial clones. The clones were screened for CMCase production together with the wild strain *B. pumilus* in overnight (24 h) broth cultures containing different concentration of glucose in order to select an improved enzyme-producing bacterial candidate as previously described (Kotchoni & Shonukan, 2002).

Enzyme assays. The CMCase activity was estimated by quantifying the reducing sugars liberated during growth according to the Somogyi-Nelson Method (Somogyi, 1944). One unit of the enzyme activity (U) is defined as the amount of enzyme needed to liberate one μmol of glucose equivalent per minute. The mixture for the enzyme assay was made of 1:1 enzyme:substrate [0.2% (w/v)], in 0.1 M phosphate buffer (pH 6.5) and incubated at 45 °C for 1 h. The enzymatic reaction was stopped by adding one volume of a combined copper and arsenomolybdate reagents (CAR) i.e. 1 ml of CAR for 1 ml of enzyme-substrate solution as described by Somogyi (1944). Activities on CMC, Avicel, cellobiose, methylumbelliferyl-β-D-cellobiose (MUC), and maltodisaccharide (MdS) were assayed to assess the specific mode of action of the enzyme, that is whether it cleaves only the β-1,4-glucosidic bonds or the α-1,4-glucosidic bonds or both of them. The enzymatic cleavage ability on 4-methylumbelliferyl-β-D-cellobiose (MUC) was assayed by measuring the 4-methyllumbelliferyl group (MU) released from MUC as described by Schofield and Daniel (1993) and using the excitation (365 nm) and the emission (455 nm) wavelengths of MU in a RF-1501 spectrofluoro-photometer (Shimadzu).

Protein purification and electrophoresis gel analysis. Unless otherwise stated, all the steps were performed at 4ºC. The bacterial culture was centrifuged (10000 rpm, 10 min) and the supernatant was precipitated with ammonium sulphate (80% saturation). The precipitate was resuspended in a small amount of 10 mM phosphate buffer, pH 7.5, which was dialysed in 3 litres of the same buffer for 45 h, with three changes made every 15 h. The dialysed sample was loaded onto an anion-exchange chromatographic separation (DEAE-Sephadex A-50, Bio-Rad Laboratories) eluted with a linear NaCl gradient (0-400 mM) in 10 mM phosphate buffer, pH 7.5. The active fractions were pooled, concentrated by ultrafiltration and dialysed against the same buffer as above. The dialysed sample was then applied to a gel filtration chromatography (sephadex G-100, Amersham Buchler-Braunschweig, Germany) pre-equilibrated with 10 mM phosphate buffer, pH 7.5. Active fractions with specific activity greater than 3500 units/mg were collected and concentrated with YM-10 membrane (Milipore USA). The purified enzyme was separated in 12 % (w/v) SDS-PAGE (Zehr *et al.*, 1989) and stained with Coomassie blue R-250 (Merck D-6100 Darmstadt, Germany). The protein molecular weight standards (MW) used were: phosphorylase (97 kDda), albumin bovine (66 kDa), albumin egg (45 kDa), carbonic anhydrase (29 kDa), α-lactalbumin (14 kDa) from (Sigma, Munich, Germany).

Determination of molecular weight of the active CMCase protein. Molecular weight of the native enzyme was estimated by size exclusion HPLC using a BioSep-SEC-S3000 column (Phenomonex, USA). Gel filtration molecular weight standard (MW) used were β-galactosidase (465 kDa), IgG (150 kDa), Fab fragment IgG (50 kDa), myoglobin (17 kDa). CMCase protein (1 μg) together with MW were applied to the BioSep-SEC-S3000 column, which was pre-equilibrated with 50 mM MOPS buffer, pH 7.5 at a flow rate of 1 ml per min. The molecular weight of active CMCase was estimated from a plot of the log molecular weight of the standard proteins against the elution volume.

Quantification analysis of purified CMCase production under different concentrations of glucose. The quantitative estimation of the purified enzyme was performed after SDS-PAGE analysis via the densitometric analysis of the signal intensity of the corresponding protein bands detected in both wild type and BpCRI 6 bacterial

mutant. The data represent mean values of three replicates, which were analysed by using AIDA Image analyzer version 2.11 (Raytest GmbH, Straubenstadt, Germany).

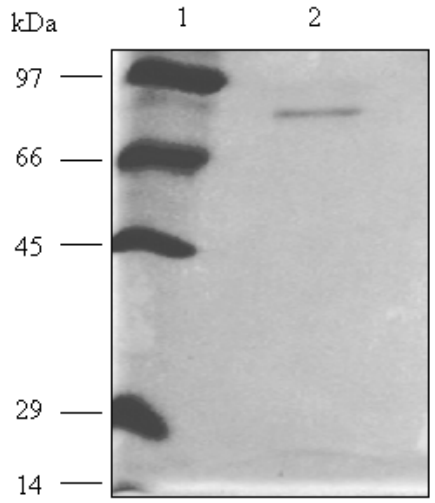
RESULTS

Purification and molecular weight of native CMCase from *Bacillus pumilus* CRI 6. To purify the enzyme, bacterial cells were incubated in broth culture containing 0.5% (w/v) CMC as carbon source to induce the synthesis of the CMCase. The enzyme was purified to approximately 9 fold with a specific activity of 40.5 U mg^{-1} and a yield of 25%. The molecular weight of the purified enzyme analysed by SDS-PAGE and stained with Coomassie Brilliant Blue showed a single band of approximately 80 kDa (Fig. 1) corresponding to the CMCase enzyme based on its high activity on CMC substrate. The estimation of the molecular weight of the active enzyme eluted from the gel filtration column and analysed under non-denatured conditions corresponded to 170 kDa, suggesting that the native CMCase from *B. pumilus* is a dimeric enzyme and 80 kDa protein on SDS-PAGE indicates probably the molecular weight of one of the two subunits of the active enzyme.

Substrate specific analysis and mode of action of the enzyme. The ability of the enzyme to hydrolyse various substrates such as CMC, Avicel, cellobiose, MUC and maltodisaccharide was assayed to investigate the mode of action of the enzyme. The specific activity on CMC was 40.5 U mg^{-1} purified protein, and 3.3, and 1.5 U mg^{-1} purified protein on cellobiose and Avicel respectively. The enzyme was not able to cleave MUC substrate because no fluorescent MU by-product was detectable after the enzymatic reaction. MUC is generally the typical substrate used for the detection of exoglucanase activity, which can hydrolyse the β-1,4-link between the cellobiose and the methylumbelliferyl group (MU) of MUC. The absence of detected MU indicates that the enzyme displayed no exoglucanase activity. In addition, no measurable activity was detected on maltodisaccharide substrate (Table I) suggesting that the enzyme did not cleave α-1,4-glucosidic bonds. These data with the ability of the enzyme to actively degrade CMC, not Avicel suggests that the enzyme is a specific endo-(β-1,4-glucosidic bonds) cleaving enzyme and therefore referred to as CMCase.

CMCase production under conditions of catabolite repression. The enzyme screenings for catabolite repression insensitivity assessment were performed with extracts prepared from WT and BpCRI 6 mutant grown overnight (24 h) at 37°C in broth cultures containing 0.5% (w/v) CMC as carbon source and supplemented with different concentrations of glucose. The crude protein extracted from sonicated bacterial pellets was first precipitated with ammonium sulphate (80% saturation) followed by two-step chromatography procedure (see materials and methods) and then used for the qualitative and quantitative analysis of CMCase protein accumulation profile (Fig. 2a, b). Qualitative CMCase protein accumulation assessed via PAGE showed that the corresponding CMCase band from WT *B. pumilus* was progressively repressed as the concentration of glucose increased in the cultural medium (Fig. 2a) indicating a production pattern strictly under the control of catabolite repression. A complete repression of CMCase synthesis in WT was observed in presence of 40 mM glucose (Fig. 2a). In contrast, BpCRI 6 bacterial mutant was able to display a strong protein bands corresponding to the expected CMCase enzyme even under glucose-inhibitory conditions (40 mM) (Fig. 2a). The CMCase production was also repressed in BpCRI 6 bacterial mutant in presence of 40 mM glucose, but the repression level was much more pronounced in the wild type than in the BpCRI 6 mutant. Some faint secondary bands were visible besides the 80 kDa bands (Fig. 2a) probably due to the source of the

Fig. 1. SDS-PAGE analysis of CMCase purified from *Bacillus pumilus* CRI 6



The standard protein molecular weight markers are shown in lane 1. The gel was loaded with 10 µg of purified BpCRI 6 CMCase protein (lane 2)

Table I. Summary of the mode of action of CMCase on different substrates

Enzymatic source	Substrate specificity-mode of action				
	CMC	Cbs	Avc	MUC	MdS
WT	↗	→	→	→	→
BpCRI 6	↑	→	→	→	→

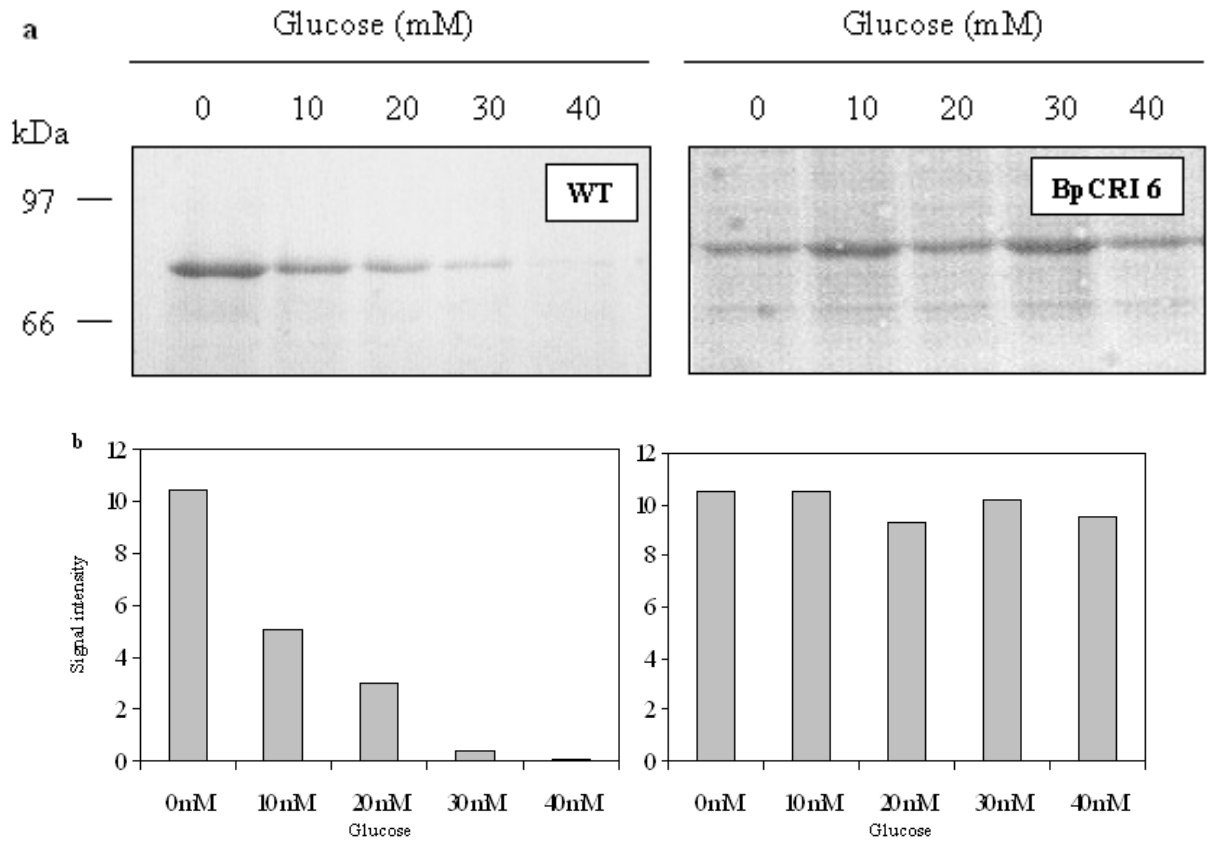
Cbs: cellobiose; Avc: Avicel; Mds: maltodisaccharide

→ No or very low detectable enzymatic activity

↗ Enzymatic activity ten times less than in BpCRI 6

↑ High enzymatic activity

Fig. 2. Effect of end product inhibition on the synthesis of CMCase by the wild type and the CRI bacterial mutant



CMCase expression profile under different concentrations of glucose was assessed by PAGE analysis between the wild type and the mutant (a). Quantitative estimation of the proteins produced by both strains respectively was recorded using AIDA Image Analyser (b)

extracted protein (bacterial pellets), which could not be purified to homogeneity by the two-step chromatography and therefore degraded as impurities in the SDS-PAGE. Densitometric analysis showed that 10 and 30 mM glucose resulted to about 50 and 90% CMCase inhibition in WT respectively, while BpCRI 6 mutant still produced approximately 99 and 90% of CMCase respectively under similar conditions (Fig. 2b).

Biochemical characterization of CMCase production by *Bacillus pumilus* CRI 6. In order to monitor the stability and biochemical properties of BpCRI 6 enzyme, the purified CMCase from *B. pumilus* CRI 6 bacterial mutant was further characterized in the presence of different chemical reagents. The effects of the chemical reagents on the BpCR 6 CMCase activity are presented as the percentage of CMCase activity compared to the control condition. Both Ca^{2+} and Mg^{2+} enhanced the enzyme activity. However, Cu^{2+}, Cd^{2+}, Ni^{2+} and EDTA had a negative effect on the CMCase activity (Table II). The enzyme displayed a broad optimum activity between 40 and 65ºC and beyond this temperature range; the enzyme lost approximately 40% of its activity. When assayed at 60ºC, the optimum pH for maximum CMCase activity (100%) was detected between pH 6.5 and 7.0. At pH 7.5 to 8.5 only 40% of the enzymatic activity was recorded; beyond which, the enzyme was not active. Below pH 6.5 the enzyme displayed 65% of its activity.

DISCUSSION

Regulatory expression of cellulosic degrading enzymes in different bacteria. Industries are generally interested in rapidly growing cellulolytic bacteria that can cope with catabolite repression known also as end product inhibition. The importance of cellulase in biological, chemical, and industrial applications is crucial (Bhat & Bhat, 1997; Jang & Chen, 2003). The end product inhibition is generally the feed-back regulatory repression of inducible enzymes occurring in the presence of glucose or other rapidly metabolised carbon sources used by microorganisms during their growth phase (De Crombrugghe *et al.*, 1984). Most reported cellulase-producing organisms are generally under the control of catabolite repression (Kawamori *et al.*, 1985; Hrmova *et al.*, 1991). For example, *Caldibacillus cellulovorans* and other cellulolytic bacilli cultured in

presence of glucose showed no CMCase production (Hrmova *et al.*, 1991; Huang & Monk, 2004) and *Trichoderma reesei*, a well-known cellulase-producing organism synthesized unfortunately very low amounts of cellulase enzyme after slow addition of glucose or cellobiose in the culture medium (Woodward *et al.*, 1992). The activity of cellulases produced by *Clostridium acetobutylicum*, a solventogenic bacterium, was also very low in cultures grown on glucose compared to any other carbon sources suggesting that a catabolite repression mechanism regulates the expression of glycoside hydrolase-encoding genes in this bacterium (Lopez-contreras *et al.*, 2004). In addition, only few strains (*B. subtilis*) are able to produce cellulase in short time, roughly within 12 h (Chan & Au, 1987; Sharma *et al.*, 1990). Generally, the synthesis of CMCase in various *Bacillus* strains is often achieved only after 2 or 3 days of growth (Robson & Chambliss, 1984; Kawai *et al.*, 1988; Ito *et al.*, 1989). This study represents a contribution towards producing CMCase by a mutant of *B. pumilus* within 12 to 14 h of growth phase representing also the optimum CMCase-producing phase of the bacterium (Kotchoni & Shonukan, 2002). Of particular significance to this study, CMCase produced by BpCRI 6 bacterial mutant has escaped the catabolite repression system unlike the known CMCase-producing bacilli (Woodward *et al.*, 1992). The mutation was stable and the bacterial mutant shows no altered growth pattern compared to the wild strain. This is an interesting discovery with respect to a large-scale synthesis of this enzyme. The EMS chemical mutagenesis approach (Kotchoni *et al.*, 2002) has been recently used to successfully generate catabolite repression resistant mutants (CRRmut) in *Pseudomonas fluorescens* (Bakare *et al.*, 2005a). They showed that cellulase activity in selected *P. fluorescens* mutants namely CRRmut4 and CRRmut24 is higher in presence of 1% (w/v) glucose than in wild-type *P. fluorescens* (Bakare *et al.*, 2005a), indicating therefore the reproducibility of the method. On the other hand, β-xylosidase (EC 3.2.1.37) is one of the component enzymes of hemicellulase complex, which catalyses the hydrolysis of alkyl- and aryl-glycosides as well as xylobiose and xylo-oligosaccharides to xylose (Tsujibo *et al.*, 2001; Rajoka, 2005). Therefore this enzyme as well as other enzymes of the cellulase complex is important in the complete degradation of the naturally available cellulosic material. Recently, γ-irradiation was used by Rajoka (2005) to produce a *Kluyveromyces marxianus* mutant with improved β-xylosidase synthesis when compared to the corresponding wild yeast. The improved yeast mutant termed *Kluyveromyces marxianus* PPY 125 showed a hyper-production of β-xylosidase and addition of glucose to the growth medium did not inhibit the synthesis of the enzyme by the yeast mutant (Rajoka, 2005).

Table II. Effect of chemical reagents on CMCase activity produced by *Bacillus pumilus* CRI 6

Chemical reagents	Relative activity (%)	Reagent effects
Control	100	Reference
Ca^{2+}	120	+ +
Mg^{2+}	118	+ +
Cu^{2+}	88	- -
Cd^{2+}	72	- -
Ni^{2+}	65	- -
EDTA	58	- -

The activity of the enzyme was assayed in the control using 0.5% (w/v) CMC as substrate without any chemical reagents. All reagents were added to the solution at 1.5 mM end concentration. The purified enzyme used was at 7000 U/mg. The assay was carried out in triplicates and estimated in percentage. (+) = increases the enzyme activity; (-) = inhibits the enzyme activity.

The regulation of carbon metabolism in bacteria is generally mediated by a general regulatory protein termed catabolite control protein (Ccp) via a well-known mechanism (Boisset *et al.*, 2000; Miwa *et al.*, 2000; Lopez-Contreras *et al.*, 2004). The Ccp binds to a specific cis-acting element known as CRE (catabolite responsive element), repressing or activating gene expression (Lopez-Contreras *et al.*, 2004). How the BpCRI 6 bacterial mutant managed to cope with catabolite repression is still not known and therefore elucidating the molecular basis leading to the generation of the bacterial mutant is crucial to identifying probable molecular alterations on CMCase-Ccp, CRE, or on the CMCase structural gene and this is one of our future objectives.

CMCase from BpCRI 6 bacterial mutant: properties and stability. The active CMCase enzyme was shown to be a dimeric based on size exclusion chromatography analysis. Recently, Huang and Monk (2004) have isolated a thermophilic CMCase from *Caldibacillus cellulovorans* with a molecular mass of 85.1 kDa revealed by SDS-PAGE analysis and 174 kDa by size exclusion HPLC using a gel filtration column. They also confirmed that the active *C. cellulovorans*-CMCase structure was a dimeric enzyme and the SDS-PAGE analysis has induced the dissociation of the two subunits. The molecular mass of native CMCases from various cellulolytic bacteria is highly variable and organised in different structural subunit arrangements. For example, some CMCases isolated from Caldibacilli are monomeric (Bronnenmeier & Staudenbauer, 1988; Romaniec *et al.*, 1992), while some from alkalophilic bacilli are tetrameric (Yoshimatsu *et al.*, 1990). Bakari *et al.* (2005b) showed that the molecular weight of cellulase proteins from various mutants of *Pseudomonas fluorescens* was between 26 to 36 kDa. To confirm this structural variability, we show an overview of comparative morphological characteristics of various cellulase systems isolated from cellulolytic organisms (Table III). CMCase isolated from BpCRI 6 bacterial mutant is closely related to the thermophilic *Caldibacillus cellulovorans* CMCase described by Huang and Monk (2004) based on the molecular weight and subunit components, although BpCRI 6 CMCase is not thermophilic because it looses more than 50% of its activity when assayed above 70°C. In addition, the enzyme is not an alkaline enzyme because its looses more than 65% of its

Table III. Structural and regulatory expression of cellulase systems produced by different organisms

Enzyme	Source	MW per subunit	Structure	Regulatory expression	References
CMCase	*B. pumilus* CRI 6	80 kDa	Dimer	CRI	This study
CMCase	*C. cellulovarans*	85.1 kDa	Dimer	UCCR	[1]
Endoglucanase	*C. thermocellum*	76 kDa	Monomer	UCCR	[2]
CMCase	*C. stercorarium*	100 kDa	Monomer	UCCR	[3]
Endoglucanase	Rhodothermus marinus	49 kDa	Monomer	UCCR	[4]
Endoglucanase	*Bacillus* sp. KSM-635	150 kDa	Tetramer	UCCR	[5]
CMCase	Sinorhizobium fredii	94 kDa	Monomer	UCCR	[6]
Cellulase	Clostridium acetobutylicum	75 kDa	Monomer	UCCR	[7]
Cellulase	*Aspergillus niger*	100 kDa	Monomer	UCCR	[8]
Cellulase	*P. fluorescens* CRRmt24	36 kDa	NS	CRI	[9]

CRI = catabolite repression insensitive; UCCR = under control of catabolite repression; NS = not specified; [1] Huang & Monk, 2004; [2] Romaniec *et al.*, 1992; [3] Bhushan *et al.*, 1994: [4] Hreggvidsson *et al.*, 1996; [5] Yoshimasu *et al.*, 1990; [6] Chen *et al.*, 2004; [7] Lopez-Contreras *et al.*, 2004; [8] Boisset *et al.*, 2000; [9] Bakare *et al.*, 2005b

activity at pH 8 to 9 unlike the alkalophilic *Bacilli*, which still produced active CMCase at pH 10 or even higher, and therefore used generally as laundry detergent additives (Yoshimatsu *et al.*, 1990; Horikoshi, 1999).

The effect of different culture carbon sources determined in our previous report suggested that glycerol was the best carbon source after CMC to obtain a significant production of the enzyme in BpCRI 6 mutant (Kotchoni & Shonukan, 2002). The activity of the purified enzyme was higher on CMC but very low on Avicel and cellobiose, and the enzyme failed to cleave MUC a typical specific substrate for exoglucanase activity indicating that the enzyme displayed only a CMCase activity and no exoglucanase activity. Based on the mode of action, the end product of the enzymatic reaction on CMC was hypothesized to be probably cellobiose, which could not completely be degraded into glucose because the enzyme showed reduced or no activity on cellobiose. The term CMCases is commonly used to describe endoglucanases, which randomly degrade CMC or amorphous cellulose and generally have very low or no activity on microcrystalline cellulose (Avicel) (Coughlan & Mayer, 1992). The absence of detectable activity on Avicel and MUC and rather high activity on CMC suggests that this enzyme belongs to an endoglucanase (Coughlan & Mayer, 1992). CMCase is one of the cellulase multi-enzymatic system, which by cleaving the endo-1,4 β-D-glucosidic bond, creates open ends for the exo-1,4-β-glucanase of the enzymatic complex to further degrade the substrate residue. During the efficient degradation of cellulose, all type of cellulase systems (endoglucanase, exoglucanase, cellobiase) act synergistically, and several known cellulolytic organisms so far produced at least one of the cellulase enzyme systems, but in most cases two different types (Lynd *et al.*, 2002). In this study, *B. pumilus* was able to display the production of only one type of the cellulase enzyme systems (endoglucanase/CMCase) because the enzyme failed to degrade MUC and showed very low activity on Avicel and cellobiose.

Metal ions have been reported to influence enzyme production by increasing their activity in microorganisms (Rani & Nand, 2000; Rani *et al.*, 2004). Ca^{2+} and Mg^{2+} were found to increase the activity of the enzyme (Table II). These cations might be probably involved in the protection of the enzyme or strengthening of the active site thereby maintaining the conformation of the enzyme in active state, whilst Cu^{2+}, Cd^{2+} and Ni^{2+} were found to inhibit the activity. Cu^{2+}, Cd^{2+} and Ni^{2+} have been reported as heavy metals and generally toxic to some extent to organisms (Sunkar *et al.*, 2003; Rani *et al.*, 2004). The toxic effect of these heavy metals is here hypothesized to negatively influencing the optimum activity of BpCRI 6 CMCase (Table II). EDTA was found to inhibit the activity of the enzyme. EDTA is known as an ionic chelator (Ali & Sayed, 1992) and its inhibition ability indicates that specific ions might be actively involved in the catalytic reaction of the enzyme. This could be the reason of enhanced CMCase activity detected with additional ions such as Ca^{2+} and Mg^{2+}. The purified BpCRI 6 CMCase retains its full activity (100%) over a long period when stored at –20ºC, confirming the stability of this enzyme.

CONCLUSION AND FUTURE PERSPECTIVE

Little is known about successful catabolite repression insensitive cellulase-producing bacilli. Hence, our next step is to elucidate the molecular basis of EMS-mediated CRI-mutation in *B. pumilus* and exploring the cDNA libraries of the mutant compared to the WT to bring insight into whether the mutation has affected the structural gene, the promoter region or the transcription factors of the gene or whether the resulted CRI phenotype was due to a combination of mutations affecting all the transcription by the gene. Although the establishment of the mutation characteristics on the BpCRI 6 genome is currently in progress, this work represents a valuable contribution towards producing bacterial CMCase enzyme within a relatively short growing time, and free from catabolite repression. These findings prove that chemical mutation could be used to improve enzymatic production in bacteria.

REFERENCES

Ali, S. and A. Sayed, 1992. Regulation of cellulase biosynthesis in *Aspergillus terreus*. *World J. Microbiol. Biotechnol.*, 8: 73–5

Bakare, M.K., I.O. Adewale, A. Ajayi, A.I. Okoh and O.O. Shonukan, 2005a. Regulatory mutations affecting the synthesis of cellulase in *Pseudomonas fluorescens*. *African J. Biotechnol.*, 4: 838–43

Bakare, M.K., I.O. Adewale, A. Ajayi and O.O. Shonukan, 2005b. Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*. *African J. Biotechnol.*, 4: 898–904

Bhat, M.K. and S. Bhat, 1997. Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv.*, 15: 583–620.

Bhushan, B., N.S. Dosanjh, K. Kumar and G.S. Hoondal, 1994. Lipase production from an alkaloplilic yeast sp. by solid state fermentation. *Biotechnol. Lett.*, 16: 841–2

Boisset, C., C. Fraschini, M. Schulein, B. Henrissat and H. Chanzy, 2000. Imaging the enzymatic digestion of bacterial cellulose ribbons reveals the endo character of the cellobiohydrolase Cel6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase Cel7A. *Appl. Environ. Microbiol.*, 66: 1444–52

Bronnenmeier, K. and W.L. Staudenbauer, 1988. Resolution of *Clostridium stercorarium* cellulase by fast protein liquid chromatography (FPLC). *Appl. Microbiol. Biotechnol.*, 27: 432–6

Chan, K.Y. and K.S. Au, 1987. Studies on cellulase production by *Bacillus subtilis*. *Antoine van Leeuwenhoek*, 53: 125–36

Chen, P-J., T-C. Wei, Y-T. Chang and L-P. Lin, 2004. Purification and characterization of carboxymethyl cellulase from *Sinorhizobium fredii*. *Bot. Bull. Acad. Sin.*, 45: 111–8

Coughlan, M.P. and F. Mayer, 1992. The cellulose decomposing bacteria and their enzyme systems. In: Balows A, H.G. Truper, M. Dworkin, W. Harder, K.H. and Schleifer (Eds) *The Prokaryotes* Vol. 1. pp 460-516. Springer-Verlag, New York..

DeCrombrugghe, B., S. Busby and H. Buc, 1984. Cyclic AMP receptor protein: role in transcription activation. *Science*, 224: 831–8

Fukumori, F., T. Kudo and K. Horikoshi, 1985. Purification and properties of a cellulase from alkalophilic *Bacillus* sp. no. 1139. *J. Gen. Microbiol.*, 131: 3339–45

Horikoshi, K., 1999. Alkaliphiles: some applications of their products for biotechnology. *Microbiol. Mol. Biol. Rev.*, 69: 735–50

Horikoshi, K., M. Nakao, Y. Kurono and N. Sashihara, 1984. Cellulases of an alkalophilic *Bacillus* strain isolated from soil. *Can. J. Microbiol.*, 30: 774–9

Hreggvidsson, G.O., E. Kaiste, O. Holst, G. Eggerstsson, A. Palsdottir and J.K. Kristjansson, 1996. An extremely thermostable cellulase from the thermophilic eubacteria *Rhodothermus marinus*. *Appl. Environ. Microbiol.*, 62: 3047–9

Hrmova, M., E. Petrakova and P. Biely, 1991. Induction of cellulase- and Xylan-degrading enzyme systems in *Aspergillus terreus* by homo and heterodisaccharides composed of glucose and xylose. *J. Gen. Microbiol.*, 137: 541–7

Huang, X.P. and C. Monk, 2004. Purification and characterization of cellulase (CMCase) from a newly isolated thermophilic aerobic bacterium *Caldibacillus cellulovorans* gen. Nov., sp. nov. *World J. Microbiol. Biotechnol.*, 20: 85–92

Ito, S., S. Shikata, K. Ozaki, S. Okamoto, S. Inque, A. Takel, Y-I. Ohta and T. Satoh, 1989. Alkaline cellulase for laundry detergents: production by *Bacillus* sp. KSM-635 and enzymatic properties. *Agric. Biol. Chem.*, 52: 1275–81

Jang, H–D. and K–S. Chen, 2003. Production and characterization of thermostable cellulases from *Streptomyces* transformant T 3–1. *World J. Microbiol. Biotechnol.*, 19: 263–8

Kawai, S., H. Okoshi, K. Ozaki, S. Shikata, K. Ara and S. Ito, 1988. Neutrophilic *Bacillus* strain, KSM–522, that produces an alkaline carboxymethyl cellulase. *Agric. Biol. Chem.*, 52: 1425–31

Kawamori, M., Y. Orikawa, K. Takayama and S. Takasawa, 1985. Preparation of mutants resistant to catabolite repression of *Trichoderma reesei*. *Agric. Biol. Chem.*, 49: 2875–9

Khasin, A., I. Alchanati and Y. Shoham, 1993. Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. *Appl. Environ. Microbiol.*, 59: 1725–30

Kotchoni, O.S and O.O. Shonukan, 2002. Regulatory mutations affecting the synthesis of cellulase in *B. pumilus*. *World J. Microbiol. Biotechnol.*, 18: 487–91

Labudova, I. And V. Farkas, 1983. Enrichment technique for the selection of catabolite repression–resistant mutants of *T.* as producers of cellulase. *FEMS Microbiol. Lett.*, 20: 211–5

Lopez-Contreras, A.M., K. Gabor, A.A. Martens, B.A.M. Renckens, P.A.M. Claassen, J. van der Oost and W.M. de Vos, 2004. Substrate–induced production and secretion of cellulases by *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.*, 70: 5238–43

Lynd, L.R., P.J. Weiner, W.H. van Zyl and I.S. Pretorius, 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.*, 66: 506–77

Miwa, Y., A. Nakata, A. Ogiwara, M. Yamamoto and Y. Fujita, 2000. Evaluation and characterization of catabolite-responsive elements (cre) of *Bacillus subtilis*. *Nucleic Acids Res.*, 28:1206–10

Priest, F., 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev.*, 41: 711–53

Rajoka, M.I., 2005. Hyper-production of a thermotolerant β–xylosidase by a deoxy-D-glucose and cycloheximide resistant mutant derivative of *Kluyveromyces marxianus* PPY 125. *Electronic J. Biotechnol.*, 8: 177–84

Rani, D.S. and K. Nand, 2000. Production of thermostable cellulase–free xylanase by *Clostridium absonum*. *Process Biochem.*, 36: 355–62

Rani, D.S., S. Thirumade and K. Nand, 2004. Production of cellulase by *Clostridium papyrosolvens* CFR-703. *World J. Microbiol. Biotechnol.*, 20: 629–32

Robson, L.M. and G.H. Chambliss, 1984. Characterization of the cellulolytic activity of a *Bacillus* isolate. *Appl. Environ. Microbiol.*, 47: 1039–46

Romaniec, M.P.M., U. Fauth, T. Kobayashi, N. Huskisson, P.J. Barker and A.L. Demain, 1992. Purification and characterization of a new endoglucanase from *Clostridium thermocellum*. *Biochem. J.*, 283: 69–73

Schofield, L.R. and R.M. Daniel, 1993. Purification and properties of a β-1,4-xylanase from a cellulolytic extreme thermophile expressed in *E. coli*. *Int. J. Biochem.*, 25: 609–17

Sharma, P., J.K. Gupta, D.V. Vadehra and D.K. Dube, 1990. Purification and properties of an endoglucanase from *Bacillus* isolate. *Enz. Microbial. Technol.*, 12: 132–7

Solomon, B.O., B. Amigun, E. Betiku, T.V. Ojumu and S.K. Layokun, 1997. Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR101 grown on bagasse. *J. Nigerian Soc. Chem. Engi.*, 16: 61–8

Somogyi, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.*, 153: 375–80

Sunkar, R., D. Bartels and H-H. Kirch, 2003. Overexpression of a stress-inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. *Plant J.*, 35: 452–64

Tsujibo, H., A. Takada, M, Kosaka, K. Miyamoto and Y. Inamori, 2001. Cloning, sequencing, and expression of the gene encoding an intracellular β-D-xylosidase from Streptomyces thermoviolaceus OPC-520. *Biosc. Biotechnol. & Biochem.*, 65: 1824–31

Wood, T.M., 1989. Mechanisms of cellulose degradation by enzymes from aerobic and anaerobic fungi. In: Coughlan, M.P., (Ed) *Enzyme Systems for Lignocellulose Degradation*.pp: 17–35. Elsevier Applied Science,London.

Woodward, J., K.A. Affholter, K.K. Koles, N.T. Troy and S.F. Gaslightwala, 1992. Does cellobiohydrolase II core protein from *Trichoderma reesei* disperse cellulose microfibrils? *Enz. Microbial. Technol.*, 14: 625–30

Yoshimatsu, T., K. Ozaki, S. Shikata. Y.I. Ohta, K. Koike, S. Kawai and S. Ito, 1990. Purification and characterization of alkaline endo-1,4-β-glucanases from alkalophilic *bacillus* sp. KSM–635. *J. Gen. Microbiol.*, 136: 1973–9

Zehr, B., T. Savin and R. Hall, 1989. A one step low background Coomasie staining procedure for polyacrylamide gels. *Anal. Biochem.*, 182: 157–9

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