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Molecular Cloning and Characterization of CMCase gene (*celC*) from *Salmonella typhimurium* UR

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The sequence coding for carboxymethylcellulase (CMCase, CelC) was isolated from the DNA of *Salmonella typhimurium* UR1. Comparison between the deduced amino acid sequence of CelC (368 amino acid residues, Molecular mass 41 kDa) and that of the previously published CMCase revealed that this enzyme belongs to the cellulase family 8 and D. The protein was overproduced in *Escherichia coli* using T7 expression system, and its activity was confirmed by CMC-SDS-PAGE. When the overexpressed CelC protein was tested on cellulose-type substrates, the recombinant protein is able to degrade cellulose-type substrates, such as CM-cellulose, xylan, avicel, lichenan, and laminarin. Optimal temperature and pH for enzyme activity were found to be 50°C and pH 6.5, respectively.

Key words: *Salmonella typhimurium,* CMCase, *celC,* CMC-SDS-PAGE

Cellulose is the most abundant biomass on earth, and has great potential for future use as an energy source. Therefore, an important application is the conversion of cellulose to glucose or ethanol (Her *et al.,* 1999). Cellulose is degraded to varying extents, by a variety of organisms, depending upon the properties of the cellulosic substrate and the type of glycosidase (Coughlan, 1985). Highly efficient cellulolytic bacteria and fungi generally produce one or more of each of the enzymes of the three classes required to degrade microcrystalline cellulose to glucose (Stephen *et al.,* 1990). These enzymes hydrolyze β-1,4-glycosidic bonds, but can be distinguished by their differing specificities with regard to the precise molecular environment of the bond. Most cellulolytic bacteria synthesize isoenzyme forms of endoglucanase and β-glucosidase (Gilkes *et al.,* 1984; Millet *et al.,* 1985). Nucleotide sequence data indicate that homology is observed between the cellulases from the same organism, but not between the comparable enzymes from different bacteria (Nakai *et al.,* 1998). Several genes involved in cellulose biosynthesis have been identified. Two of these have been shown to represent the structural genes for phosphoglucomutase and UDPG pyrophosphorylase. The genetics of the steps following precursor synthesis are not yet fully understood, but an operon consisting of four genes (*bcsABCD*) is known to be essential for the final steps in cellulose biosynthesis. The biochemical functions of the gene products encoded by the *bcs* operon are also not yet clear, but it has been suggested that the products of the *bcsA* and *bcsB* genes are involved in the polymerization of glucose, while that of *bcsC* is required for the *in vivo* synthesis of β-(1,4)-glucan *in vivo* but not *in vitro*. Absence of the product of that of *bcsD* gene was observed to result in a 40% reduction in the rates of cellulose synthesis (Valla *et al.,* 1989; Ross *et al.,* 1991; Rune *et al.,* 1994; Pear *et al.,* 1996; Park *et al.,* 1997). Subsequently, it was discovered that the *cel* operon allows *Escherichia coli* to utilize cellobiose, as well as arbutin and salicin. Sofia *et al.* (1994) have suggested, based on the analysis of multiple sequence alignment, that *E. coli* carries a set of enzymes involved in cellulase activity and cellulose production, but so far there has been no report of endo-1, 4-D-glucanase activity in *E. coli*. Genes encoding enzymes involved in the hydrolysis of cellobiose have previously been assigned to the *cel* operon in *E. coli*. The bacterium *Salmonella typhimurium* expresses cellulase, but also possesses cellulose synthase genes. In this study, we isolated distinct DNA sequences encoding carboxymethyl-cellulase, indicating that this cellulase is the consequence of multiple cellulose synthase genes. In this report, we also determined the nucleotide sequences in this region, and constructed a characterization of the CMCase.

S. typhimurium (Curtiss *et al.,* 1991) was cultured in LB medium, and used as the source of chromosomal DNA. *E. coli* JM109 cells and BL21 (DE3) were used as a host for the analysis of the gene products of *celC,* which were cultured in LB containing appropriate antibiotics (Ampicillin,

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50 μg/ml). Plasmid pBluescript KS (+) and pUC18 were used as the cloning vector. Small-scale plasmid isolation was performed using a Promega (USA) Wizard Kit. Total DNA from *Salmonella typhimurium*, which was grown in LB broth at 37°C for 24 h, was prepared as described using a Genomic-Tip100/G (Qiagen, USA). Cloning of the cellulase gene was accomplished by the shotgun method (Yoo *et al.*, 1998). The genomic DNA of *S. typhimurium* was partially digested by *Sau*3AI. Three to five kb of these partially digested fragments of genomic DNA, which was found to possess the relevant signal by Southern blotting with a *celA* region probe, were ligated into the *Bam*HI site of the pBluescript KS(+) vector. In order to screen for cellulase activity in the *S. typhimurium* or *E. coli* harboring the cloned cellulase genes, bacterial colonies were grown on LB plates with appropriate antibiotics and 1% carboxymethylcellulose (CMC, low viscosity. Sigma, USA). After 3 days of incubation, the plates were stained with 0.5% Congo red for 30 min, rinsed with water, and then washed twice with 1 M NaCl (Teather and Wood, 1982). After the Congo Red staining, clones which appeared to be surrounded by a yellow halo against a red background were designated as testing positive for extracellular cellulase activity (Sigma, USA) (Barros *et al.*, 1987; Kim *et al.*, 1987; Romaniec *et al.*, 1987; Faure *et al.*, 1988). Genomic Southern hybridization was then performed, in order to identify this cloned gene from *Salmonella* sp., using a ^{32}P-dCTP-labelled probe prepared from the 200 bp PCR fragment from the *celA* of *E. coli* (Southern, 1975). The 200-bp probe was labeled using the Prime-A-Gene system DNA labeling kit (Promega, USA). DNA digested with *Kpn*I were then electrophoresed on an agarose gel, denatured by treatment with 0.2 M NaOH/0.5 M NaCl, transferred to a nylon membrane (Amersham Bioscience, USA) and hybridized with the DNA probe, according to the manufacturer's directions. Other methods followed of the protocols established by Southern (1975). PCR primers were designed to amplify the nucleotide sequence of the gene encoding the CelC protein. Restriction sites were added at the 5' of each primer (*Nco*I at the start of the gene and *Bam*HI at the end). The primers were, respectively, as follows: p1; 5'-TATACCATGGATGATGACTATGCTGCG-3', p2; 5'-AGCCGGATCCTTAGTGTGAATTTGCGC-3'. After the digestion, the PCR-amplified fragment was ligated into *Nco*I-*Bam*HI-digested pET3d vector. The gene fusion was under control of the T7 promoter. The constructed plasmid, pBT3cp, was transferred to *E. coli* BL21 (DE3). The transformed cells were grown overnight at 37°C, with agitation in LB medium (10 ml) supplemented with ampicillin (200 μg/ml). The bacterial suspension was used to inoculate fresh media, and culture was grown to A_{600} of 1-1.5. IPTG (0.1 mM) was added to the culture, and incubation was allowed to continue for 4 h. A CMCase produced by *E. coli* BL21 (DE3) containing plasmid pBT3cp was purified. The cultured cells were harvested by centrifugation at 5,500 rpm for 10 min, and the cells were then removed. The supernatant was used as an extracellular enzyme source. The enzyme was concentrated by freeze-drying, and then ultrafiltrated with an Amicon membrane (MV: 10000) in an Amicon model 4205 (USA). Whole cell extracts from these cultures were prepared by sonication (6 times for 30 s each on ice) of after the cell suspension in 50 mM citrate buffer (pH 5.2). The partially-purified CMCase was further separated through SDS-PAGE on 10% gel. CMCase activity was determined *in vitro*, using CMC as the substrate. Appropriate dilutions of cell-free culture broth (extracellular activity) or cell harvest extractions were assayed at 50°C in 50 mM citrate buffer (pH 5.2). Reactions were terminated by heating to 100°C for 10 min. Reducing sugars were measured using 3, 5-dinitrosalicylic acid reagent, with glucose as a standard (Zhou and Ingram, 1999; Zhou *et al.*, 1999). Endoglucanase activity (CMCase) is expressed as micromoles of reducing sugar per minute. Results given represent an average of two or more determinations. Protein levels were estimated according to the methods of Lowry *et al.*(1951). CMC-SDS-PAGE was performed by the method described by Park *et al.* (Park *et al.*, 1997; Park *et al.*, 2000). *E. coli* cells harboring the *celC* gene were cultured at 37°C for 24 h in LB medium supplemented with 0.1% (w/v) CMC. The cell extracts were mixed with sample buffer (100 mM Tris-HCL pH 6.8, 10% glycerol, 0.025% bromophenol blue, 5% β-mercaptoethanol, and 2% SDS), heated at 98°C for 5 min. The protein samples were electrophoresed. The optimal pH and temperature for endoglucanase activity were determined using the crude enzyme and 1% (w/v) CMC in citrate buffer. The mixture was incubated at the optimal temperature for 60 min. Pro-

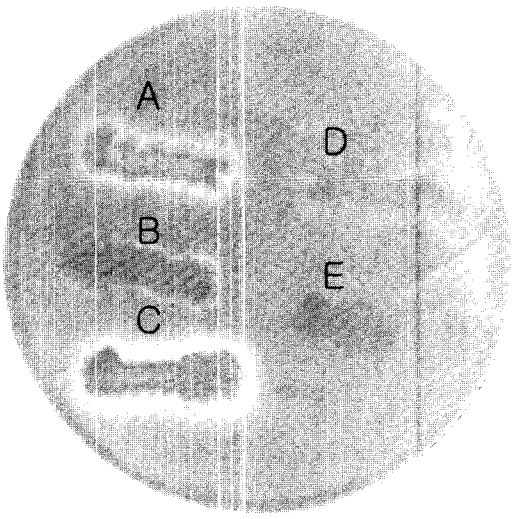

Fig. 1. Detection of cellulase positive clone on the CMC. The cells were incubated for 4 days at 37°C. A, pBT3c/JM109; B, pBT3/JM109; C, pBT3cp/BL21; D, pUC18/JM109; E, pET3d/BL21.

tein concentrations were determined by the methods of Bradford (Bradford, 1976).

Several CM-cellulose positive colonies, designated JM109 (pBT3, pBT312, pBU8) were obtained. pBT3, possessing 5.2 kbp of insertion DNA exhibited the most pronounced halo zone on CMC media (Fig. 1). The plasmid pBT3 DNA was digested with several restriction enzymes. The size of the insert DNA in pBT3 and the orientation of the restriction cleavage sites were detected. In order to ascertain the location of the CMCase gene, various restriction fragments from pBT3 were subcloned into the pUC18 vector. By subsequent subcloning, a 1.2 kb fragment containing *celC* was defined. The recombinant plasmid DNA from the positive clone was designated as pBT3c, harboring the *Sna*BI-*Hinc*II-digested 1.2 kbp fragment of pBT3 (Fig. 2). Its capacity to direct the expression of functional CMCase was then determined, and the fragment was found to be sufficient with respect to cellulase activity, as determined with the CMC indicator medium.

We then used the dideoxy chain-termination method to sequence pBT3. The sequence comparison of translation products with the data obtained from the BLAST program at NCBI revealed that the clone contained three ORFs, which were similar in sequence to those of several endoglucanase and cellulose synthase genes. They contained two complete ORFs and one 5-frame partial ORF. The nucleotide sequences determined for the three ORFs, *celA*, *celB*, and *celC*, exhibited high homology with *E. coli* and other organisms possessing cellulose synthesis ability. The amino acid sequences of CelA and CelB were homologous with cellulose synthase and CelC was homologous with the endoglucanase, cellulose-degrading enzyme. The three genes are organized as an operon for cellulose metabolism, in which two genes are cellulose synthase-like subunit genes, and the other codes for endoglucanase. At the regulatory region, a putative binding site of CRP (cAMP receptor protein) was located upstream of *celA* (data not shown). The *celC* consisting of 1,164 bp (GenBank accession number, AF464897) encodes a protein of 368 amino acid residues, with a predicted molecular mass of the putative Shine-Dalgarno sequence, ATGAG. However, no typical prokaryotic promoter-like sequence was found upstream of *celC*. The

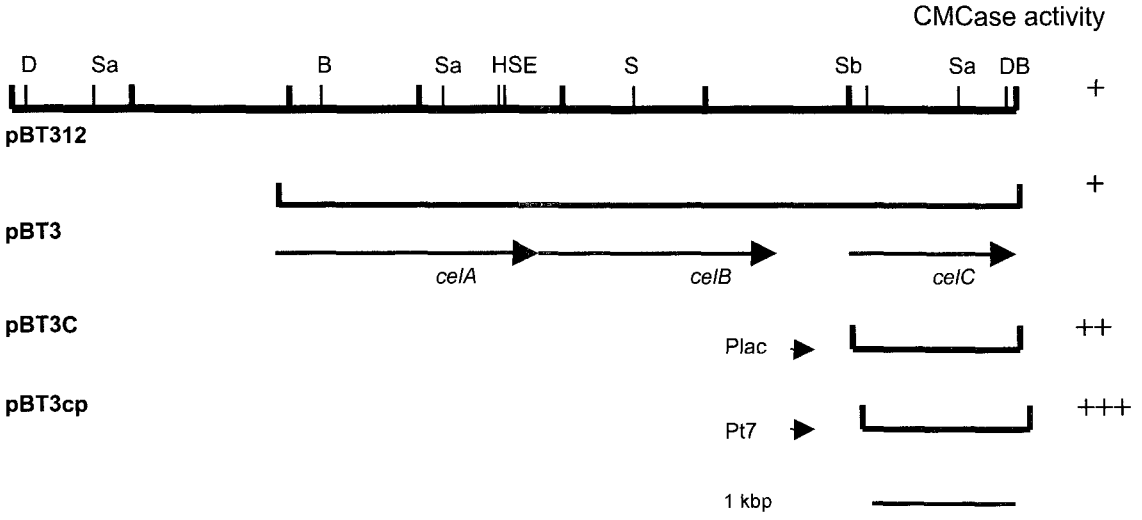

Fig. 2. Restriction map and subcloning of pBT3 derived plasmid harboring CMCase gene. The arrow indicates the direction of gene translation, and the parentheses indicate CMCase activity. B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; S, *Sac*II; Sa, *Sal*I; Sb, *Sna*BI.

Table 1. Comparisons of the *CelC* with various species

Homologous protein	Genbank	Organism	Identity (%)	Positivity (%)
Endo-1, 4D-glucanase	NP462518	*Salmonella typhimurium LT2*	95	95
endoglucase	NC002655	*E. coli*	79	86
CMCase	P37651	*E. coli*	79	86
endoglucase	JT0585	*Erwinia chrysanthemi*	34	56
endoglucase	I40696	*Cellulomonase uda*	32	51
endoglucase	AB010645	*Gluconacetobacter xylius*	30	44
endoglucase	NC003063	*Agrobacterium tumefacience*	28	45
Xylanase Y	AF045480	*Bacillus sp.*	29	48

The amino acid sequences were compared using the NCBI's BLAST search

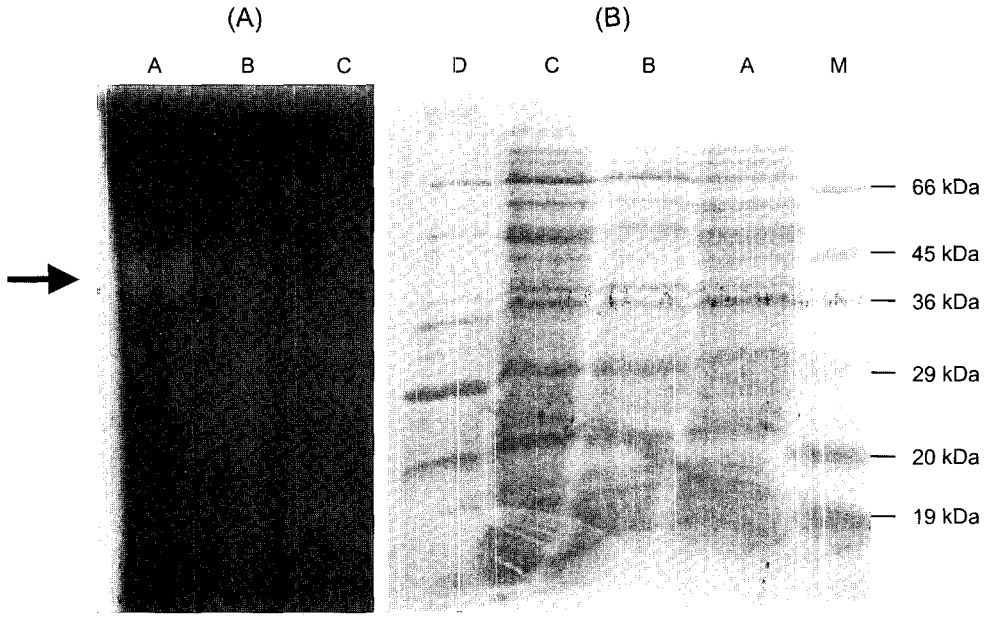

Fig. 3. Active staining of CMC-SDS-PAGE. Detection of CMCase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (B) and CMC-SDS-PAGE (A). lanes, A: pBT3cp/BL21; B: pET3d/BL21, C: pBT3c/JM109; D: pUC18/JM109; M: Molecular weight standards.

amino acid sequence homology studies indicated that CMCase is evolutionarily closely related to the endoglucanases produced by *E. chrysanthemi* and *C. uda* (Nakamura *et al.*, 1986; Guiseppi *et al.*, 1991; Sofia *et al.*, 1994; Umeda *et al.*, 1998; Yoon *et al.*, 1998; Goodner *et al.*, 2001; McClelland *et al.*, 2001; Perna *et al.*, 2001) (Table 1). The CelC, CMCase are members of cellulase family D (Raimbaud *et al.*, 1989; Gilkes *et al.*, 1991). The family D cellulases contain a conserved motif with the consensus sequence A-[ST]-D-[AG]-D-X (2)-[IM]-A-[SA]-[LIVM]-[LIVMG]-A-X (3)-[FW]. This suggests that the *CelC* protein belongs to family D and family 8 of the glycoside hydroases. According to our comparison of the *S. typhimurium CelC* sequence to the database, most of CMCase was secreted extracellularly, and has a signal peptide in its N-terminal. *E. coli* and *Erwinia chrysanthemi* endoglucase BcsC protein carries a typical prokaryotic signal peptide consisting of 21 amino acid residues. No signal peptide was found in its N-terminal region, nevertheless the recombinant protein activity was strong than intracellular crude protein to 120 fold.

The *bgl* and *cel* operon in *E. coli* are both cryptic systems, which allow utilization of β-glycoside sugars. Also, *celC* in *S. typhimurium* was predicted to function in the metabolism of β-glycoside sugars. These operons are located almost opposite each other on the *E. coli* genetic map. An interesting finding was that enzyme CelC of *S. typhimurium* exhibits a striking degree of homology to *A. xylium* and *A. tumefaciens* at both the DNA and protein levels. These two genes are found in the operon containing bacterial cellulose synthase genes.

In order to characterize the CMCase produced in the *E. coli* transformant containing pBT3c, we used a direct staining technique, which allowed for specific detection of CMCase in polyacrylamide gel (Park *et al.*, 1997) (Fig. 3). The predicted *celC* gene product consists of 368 amino acids with an estimated molecular mass of 41kDa, which compared with the size of the enzyme on the CMC-SDS-PAGE. In order to confirm secretion of the recombinant protein was cultured in L-broth containing 0.1% CMC. CMCase activity was assayed in both the extracellular and cytoplasmic fractions. About 24% of total cellulase activity occurred in the cytoplasmic fraction. No activity was detected in corresponding extracts prepared from cells harboring the cloning vector without the insert. This suggests that the cloned *celC* expressed in *E. coli* is, in fact, an extracellular cellulase. The purified enzyme exhibited a 120-fold increase in activity when 1% CMC was used as the substrate (Table 2). Endoglucanase activity was assayed at 30°C on 4 ml substrates using pure (1-4)/(1-3)-β-D-polysaccharides, such as CM-cellulose, Avicel cellulose, lichenan, laminarin,

Table 2. Specific activity of CMCase in *E .coli* carrying the recombinant plasmids[a]

Plasmid	CMCase activity[b] in the following fraction	
	Extracellular fraction	Intracellular fraction
pBT312/JM109	1.406	0.444
pBT3c/JM109	2.644	0.835
pUC18/JM109	ND	0.051
pBT3cp/BL21	12.150	1.120
pET3d/BL21	0.072	0.048

[a]*E. coli* strains were aerobically grown in glucose minus LB broth containing 0.1% CMCase for 48 h at 37°C.
[b]CMCase activity is defined as ng (product)/μg · min (substrate).

Table 3. Substrate specificity for CMCase in *E. coli* carrying the recombinant plasmids[a]

substrate	Substrate specificity	
	Extracellular (unit)	percentage (%)
CMC	5.74	100
Xylan	7.94	138
Avicel	6.26	109
Lichenan	8.6	149
Laminarin	8.8	158

[a]*E. coli* strains were aerobically grown in glucose minus LB broth containing 0.1% CMCase for 48 h at 37°C.
[b]activity is defined as ng (product)/μg · min (substrate).
[c]activity was expressed as percent of the unit per milliliter of the cultured cells.

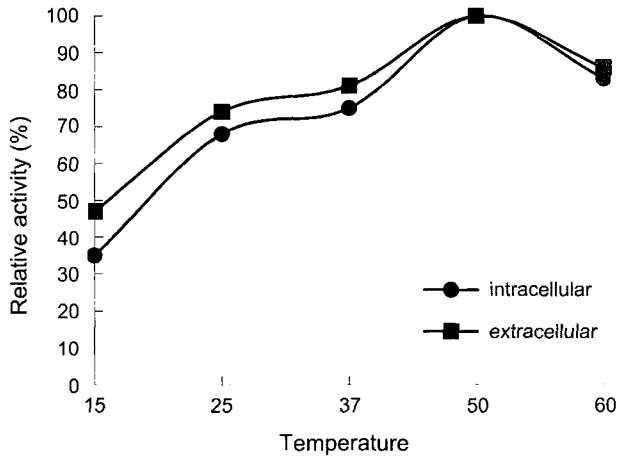

Fig. 4. Effect of temperature on CMCase activity of *celC* enzyme. Enzyme activity was assayed at pH6 for 50 min at the indicated temperature.

or xylane (1% w/v) in 300 mM NaCl/10 mM Tris-HCl buffer at pH 7, mixed with 1 ml of the appropriate enzyme dilution. During incubation, 200 μl samples were collected, and the reducing sugars were detected by the DNS method. The crude extracellular CMCase protein exhibited substrate specificity, as shown in table 3. As seen in table 3, the activity of the enzyme was significantly higher toward laminarin, a polysaccharide with a β-1-3,1-6 linkage, than to CMC. Some activity was also noted on lichenan containing a β-1-3,1-4 linkage, which indicates a reasonable sensitivity of the β-1-3 linkage to the enzyme. Furthermore, the enzyme exhibited significant activity toward crystalline cellulose material. Recombinant CMCase degraded the substrate, suggesting that the recombinant protein possesses multiple degradation ability, specifically with regard to the 1-3 and 1-4 linker of polysaccharides. The effect of pH on the CMCase activity of the CelC enzyme toward CMC was determined at 50°C using various buffers, ranging from pH 3 to 12. Maximal activity was observed at pH 6.5, although CMCase activity was observed in a broad range, as 70% of maximal activity (data not shown). Optimal temperature for enzyme activity was determined by assaying CMCase activity at various temperatures in 50 mM citrate-sodium phosphate buffer (pH 6) with 0.1% CMC. The optimal temperature range for enzyme activity was found to be 40-55°C (Fig. 4). Maximum activity was observed at 50°C.

Here we report on the cloning and characterization of an active form of the *S. typhimurium* UR CelC in *E. coli*. The recombinant protein, a (1-4)-β-D-endoglucanase, is able to degrade cellulose-type substrates, such as CM-cellulose, xylan and avicel, lichenan, and laminarin. The molecular mass of the overexpressed protein is consistent with the 41 kDa value of the native protein activity detected on a CM-cellulose overlay after SDS-PAGE electrophoresis.

The data presented in this paper confirms the presence of a gene in *S. typhimurium*, which codes for an endoglucanase, as an enzyme substrate specific to the 1-4-β-glucosidic bond, 1-3, 1-4, and 1-3 linked sugars. One mM Ni^{+} did not influence the activity, whereas Co^{2+} and Mn^{2+} ions induced a significant increase in residual activity to 262% and 202%, while Fe^{3+} and Mg^{2+} caused a decrease in activity (data not shown). Various ions slightly augmented or diminished CMCase activity. The most typical inhibitors were Mg^{+} and Mn^{2+}, but the recombinant CMCase derived from *S. typhimurium* had a different response to Mn^{2+}.

How the production of cellulose as a substrate for the enzyme CelA, CelB is achieved, if at all. Cellulose synthase activity decreased in the absence of the *celC* gene (data not shown). Further work is needed in order to elucidate the functions and regulation of *celC* in the mechanism underlying the cellulose synthesis qualities of *S. typhimurium*.

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