BACILLUS SUBTILIS SJ01 PRODUCES HEMICELLULOSE DEGRADING MULTI-ENZYME COMPLEXES

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Cellulose and hemicellulose account for a large portion of the world's plant biomass. In nature, these polysaccharides are intertwined, forming complex materials that require multiple enzymes to degrade them. Multi-enzyme complexes (MECs) consist of a number of enzymes working in close proximity and synergistically to degrade complex substrates with higher efficiency than individual enzymes. The aim of this study was to isolate and characterise a (hemi-) cellulolytic MEC from the aerobic bacterium, *Bacillus subtilis* SJ01, using ultrafiltration followed by size-exclusion chromatography on a Sephacryl S-400 column. Two MECs, C1 and C2 of 371 and 267 kDa, respectively, were purified, consisting of 16 and 18 subunits, respectively, five of which degraded birchwood and oat spelt xylan. The MECs degraded xylan substrates (C1: 0.24 U/mg, C2: 0.14 U/mg birchwood xylan) with higher efficiency than amorphous cellulose substrates (C1: 0.002 U/mg, C2: 0.01 U/mg carboxymethyl cellulose - CMC). Low or no binding to insoluble substrates indicated that the MECs lacked some of the features characteristic of cellulosomes. The significance of this study lies in the discovery of MECs that differ structurally from cellulosomes that can hydrolyse substrates with high hemicellulose content.

Keywords: Bacillus subtilis; Cellulosome; Hemicellulases; Lignocellulose; Multi-enzyme complexes; Synergy; Xylanase; Xylanosome

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INTRODUCTION

Cellulose and hemicellulose are among the most abundant sources of carbon on earth. They are found mostly in plants, as structural components, and comprise 75% of the world's biomass (Lynd et al. 1991). Cellulose consists of chains of glucose monomers packed parallel to one another in structures called microfibrils (Beguin and Aubert 1994; Coughlan and Folan 1979; Houtman and Atalla 1995). Hemicellulose is a heterogeneous polymer containing varying compositions of sugars that are naturally found intertwined with cellulose and lignin (Biely 1985; Foyle et al. 2007; Gray et al. 2006; Petersson et al. 2007). Xylan, the most common monomer in hemicellulose, consists of chains of D-xylose molecules (xylan) as a backbone with side chains containing arabinose, acetyl groups, and other sugars (Beguin and Aubert 1994; Gray et al. 2006).

There are several microorganisms capable of degrading (hemi-)cellulose by enzymatic hydrolysis (Beguin and Aubert 1994; Coughlan and Folan 1979). Cellulose is degraded into cellobiose by β-(1,4)-glucanases, which attack the β-D-1,4 glucosidic

linkages; and cellobiose is further converted to glucose monomers by β-glucosidases (Aliyu and Hepher 2000). The xylan backbone in hemicellulose is degraded by endo-β-1,4-xylanases and β-1,4-xylosidase, and the hemicellulose side-chains are degraded by enzymes such as α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, and phenolic acid esterase (Biely 1985; Howard et al. 2003). This enzymatic hydrolysis results in the production of glucose, xylose, arabinose and other sugars, which can be used for nutrition or for fermentation to alcohols. An important application of lignocellulose-degrading enzymes is in the biofuels industry, in which the sugars released from lignocellulose hydrolysis are fermented into butanol or ethanol (Howard et al. 2003; Linde et al. 2008).

In some bacterial (hemi-)cellulase systems the enzyme components are produced as or form aggregates, commonly known as multi-enzyme complexes (MECs) (Gilkes et al. 1991). The most rigorously studied example is the cellulosome of *C. thermocellum*, which consists of a number of cellulolytic enzymes attached to a protein scaffold that holds the enzymes together and binds to the substrate via a cellulose binding domain (CBD) (Bayer et al. 1998). Cellulosomes, xylanosomes and cellulosome-like MECs have been isolated from a variety of bacterial and fungal species. Cellulosomes have been identified in many anaerobic microorganisms such as clostridia (Bayer et al. 1998). Other MECs, often with predominantly xylanase activity, have been reported in *Streptomyces olivaceoviridis*, *Butyrivibrio fibrisolvens*, *Chaetomium* spp., *Bacillus licheniformis, Bacillus circulans*, *Paenibacillus curdlanolyticus*, and *Bacillus megatarium* (Beukes and Pletschke 2006; Jiang et al. 2006; Kim and Kim 1993; Ohtsuki et al. 2005; Pason et al. 2006b; Van Dyk et al. 2009b; Waeonukul et al. 2009). MECs concentrate a variety of enzymes to specific sites on a substrate, which leads to improved hydrolysis, and the arrangement of the enzymes in a complex increases their synergistic action (Bayer et al. 1994; Doi, 2008; Johnson et al. 1982). While cellulosome structure has been extensively elucidated, the structures of other MECs are still under investigation.

Most of the cellulosome-producing organisms are anaerobic, and since these conditions are more difficult and more expensive to maintain, aerobic cellulolytic organisms have been investigated for the presence of MECs (Van Dyk et al. 2009a). Currently, species of *Bacillus* are receiving the most attention, since these aerobic bacteria are abundant in nature and are easy to isolate and culture (Sonenshein et al. 1993). *Bacillus subtilis* has been used as a model system for Gram-positive bacteria in many aspects of biochemistry, genetics and physiology, and detailed genome analyses of *B. subtilis* have been undertaken (Devine 1995; Harwood 1992; Harwood and Wipat 1996; Moszer 1998).

Research into these MECs is important in terms of expanding the current information on MECs. This study is also significant because *Bacillus* MECs have the potential for industrial application since these bacteria can be rapidly isolated and cultured. In this study, bacteria isolated from soil and compost environments were screened for bacilli that produced cellulose and/or hemicellulose degrading MECs. One strain, *B. subtilis* SJ01, was selected based on the presence of a high molecular weight protein complex with high xylanase activity. Growth conditions for the selected strain

were optimised and the MECs produced by this strain were isolated and partially characterised.

MATERIALS AND METHODS

Bacterial Identification

Bacillus subtilis SJ01 was isolated from a soil sample in Grahamstown, South Africa and identified using 16S rDNA sequencing. Chromosomal DNA purification was performed using a Promega Wizard Genomic DNA Purification Kit (Promega Corporation). A PCR reaction using the primers 27F: (GAGTTTGATCCTGGCTCAG) and 1492R: (GGTTACCTTGTTACGACT) for amplification of the 16S rDNA sequence was performed using the following program on a 'PCRSprint' (Hybaid, Ltd.): 95°C, 2 minutes; 98°C, 30 seconds; 61°C, 30 seconds; 68°C, 1 minute (for 25 cycles); 68°C, 5 minutes. The PCR products were sequenced by Inqaba Biotechnology Industries Pty. Ltd., South Africa, and the sequences were used in a nucleotide-nucleotide BLAST search.

Enzyme Assays

The cellulolytic and hemicellulolytic enzyme activities were determined using the dinitrosalicylic acid (DNS) method (Miller 1959), which measures the amount of reducing sugars. The samples were first incubated at 50°C for 18 hours in a solution containing 250 µL 20 mM potassium phosphate buffer, pH 7.0, 50 µL of 2% (w/v) substrate and 100 µL enzyme sample. Appropriate enzyme controls were included. After incubation, 300 µL of the assay preparation was added to 600 µL of DNS reagent and boiled (100°C) for 5 minutes in a dry bath (Labnet, inc.). The reaction was terminated by placing the tubes on ice for 5 minutes, and the absorbance of the resulting coloured samples were read at 540 nm (Bio-Tek Instruments, inc. PowerWave). The µmoles of glucose or xylose produced per hour (U) were calculated using glucose and xylose as standards.

Protein Determination

Protein content was determined using the Bradford's assay (according to the Sigma protocol) and the protein concentration calculated using bovine serum albumin (BSA; Sigma) as a standard (Bradford 1976).

Growth Curve

B. subtilis SJ01 was cultured in an optimised enrichment medium, containing 5 g/L birchwood (bw) xylan, 2 g/L NaCl, 0.5 g/L $MgSO_4.7H_2O$, 2 g/L K_2HPO_4, 2 g/L KH_2PO_4, 5 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$, 0.05% (v/v) vitamin solution (2 mg biotin, 2 mg folic acid, 10 mg pyridoxine hydrochloride, 0.1 mg Vitamin B12, 5 mg *p*-aminobenzoic acid in 100 mL), 1% (v/v) trace element solution (1 g $MnSO_4.2H_2O$, 0.2 g $FeSO_4.7H_2O$, 0.2g $CoCl_2$, 0.2 g $CaCl_2.2H_2O$, 0.2 g $ZnSO_4$, 0.02 g $CuSO_4.5H_2O$, 0.02 g $Al(SO_4)_2$, 0.2 g H_3BO_3, 0.02 g $Na_2MO_4.2H_2O$ in 1 L) and 0.1% (v/v) of 0.035 g/mL phenylmethanesulfonyl fluoride (PMSF) (Fluka) at 37°C and 200 rpm, and samples (3

mL) were taken regularly for a total of three weeks (516 hours). The pH and OD_{600} of each sample were determined and then the samples centrifuged at 3000 *g* for 5 min. The total protein (Bradford's assay), total reducing sugar (DNS assay) and avicelase, CMCase and xylanase activities (DNS assay, after incubating at 50°C for 18 hours) of the pellets and supernatants at each time interval were measured.

Purification of MECs

B. subtilus SJ01 was cultured in the optimised enrichment medium, incubating at 37°C and 200 rpm. After 84 hours of growth (according to the growth curve) the culture was centrifuged at 12 000 *g* for 5 minutes to remove the cells. The supernatant was concentrated by ultrafiltration using an Amicon 8200 ultrafiltration cell with a 50 kDa cut-off membrane (Millipore, South Africa). Proteins retained by the filter were resuspended in Tris-HCl, pH 7.0. Samples of this retentate (1.2 mL) were loaded on a Hi-Prep™ 16/60 Sephacryl S-400 High Resolution size-exclusion column (column dimensions: 60 x 2 cm) (GE Healthcare Bio-Sciences) connected to an ÄKTA™ fast protein liquid chromatography (FPLC) system with Unicorn 5.1 software. The standards; Blue Dextran (2000 kDa), thyroglobulin (670 kDa), ferritin (450 kDa), catalase (250 kDa), γ-globulin (150 kDa), and BSA (66 kDa) were used to construct a calibration curve for the Sephacryl S-400 column. Dextran (5000 to 40 000 kDa) was used to determine the void volume. The fractions in each of the protein peaks on the chromatogram were pooled and concentrated with PEG 20 000.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% resolving gel and 4% stacking gel according to the method described by Laemmli (1970). The boiled protein samples were loaded into the wells, and the gel was electrophoresed at 180 V for about 45 minutes. A peqGOLD protein marker II (peqLab Biotechnologie GmbH) was included in order to determine the molecular weights of the bands. The SDS-PAGE gels were stained with Coomassie brilliant blue R-250.

Zymograms were prepared by placing an SDS-PAGE gel (containing 50 µg protein per well) in 2.5% (v/v) Triton-X 100 prepared in 20 mM potassium phosphate buffer, pH 7.0, for 1 hour to renature the enzymes after electrophoresis. The gel was then incubated in 20 mM potassium phosphate buffer, pH 7.0, at 50°C for 48 hours (for oat spelt (os) xylan) and 24 hours (for bw xylan). The gel was then stained with 0.3% Congo red for 15 minutes, destained with 1 M NaCl and counterstained with 0.5% acetic acid. The gels were photographed using a Uviprochemi geldoc imaging system (Whitehead Scientific).

Substrate Specificity

To determine the range of activities of the MECs from *B. subtilis* SJ01, 2% (w/v) solutions of the substrates i.e. bw xylan, os xylan, CMC, bagasse, Avicel®, and α-cellulose powder (Sigma-Aldrich) were used in a DNS assay, incubating at 50°C for 60 hours.

Substrate Binding Study

Insoluble birchwood xylan was prepared according to Kittur et al. (2003). About 50 mg of Avicel® and insoluble bw xylan were added to microcentrifuge tubes (Eppendorf) containing 1 mL of 20 mM potassium phosphate buffer, pH 7.0. The mixtures were left at room temperature for 1 hour and then centrifuged at 13,000 *g* for 5 minutes to remove the buffer. Concentrated enzyme samples (approximately 0.5 mg/mL protein) were added to the insoluble substrates to a volume of 1 mL each, with 100 µL of 10 mg/mL bovine serum albumin (BSA) to prevent non-specific binding to the tube (Goldstein et al. 1993). The microcentrifuge tubes were incubated on an Intelli-Mixer shaker (Sky Liner, ELMI) at 4°C at 40 rpm for 1 hour and then centrifuged at 13,000 *g* for 1 minute. Controls for the binding study were set up with 1 mL of 20 mM potassium phosphate buffer only; 50 mg insoluble substrate in 1 mL 20 mM potassium phosphate buffer; and 1 mg/mL BSA plus 1 mL MEC. These controls were incubated and centrifuged in the same way as the experimental samples. A Bradford's assay was performed on the supernatants and the % binding calculated using the following equation: % binding = (protein concentration without substrate – protein concentration after binding) / protein concentration without substrate.

RESULTS AND DISCUSSION

Based on the nucleotide BLAST search performed, the organism had the highest sequence homology and score with a number of strains of *B. subtilis* (99%, *E* value 0.0, Score 2545 for strains LZ030, LZ054, LZ045). The sequence of 1380 base pairs was lodged with GenBank with accession number JQ424901.

A growth curve with *B. subtilis* SJ01 was carried out to determine optimal release of extracellular enzymes. As avicelase and CMCase activities were very low in the fractions, only xylanase activity is displayed on the growth curve in Fig. 1. As shown, xylanase enzymes were already produced during the logarithmic phase by *B. subtilis* SJ01 and continued to be produced rapidly for the first 18 hours. Production of xylanase enzymes increased to a peak and reached a plateau between 20 and 60 hours, after which it increased again, with a peak of activity at 84 hours. The overall trend in xylanase activity coincided with bacterial growth (absorbance at 600 nm), where rapid enzyme production coincided with exponential growth (0 to 24 hours). Although xylanase production continued into the late stationary growth phase, it stabilised after 84 hours. Therefore, 84 hours was selected for termination of incubation and purification of extracellular enzymes. During the growth of *B. subtilis* SJ01, the pH of the medium increased from pH 6.5 to pH 7.2 (data not shown). Based on the measurement of total reducing sugars in the supernatant, a sharp increase in reducing sugars was observed, reaching a peak at 12 hours, after which it declined rapidly. This indicated that *B. subtilis* SJ01 was able to utilise the sugars released from the substrate. It should be kept in mind that *Bacillus subtilis* species are able to produce biosurfactants, which may be antimicrobial and cause cell lysis. Although the growth conditions in this case were not optimal for biosurfactant production, it could result in lysis, which would release the

intracellular proteins. These intracellular proteins may have formed part of the enzymes from which the MEC was purified.

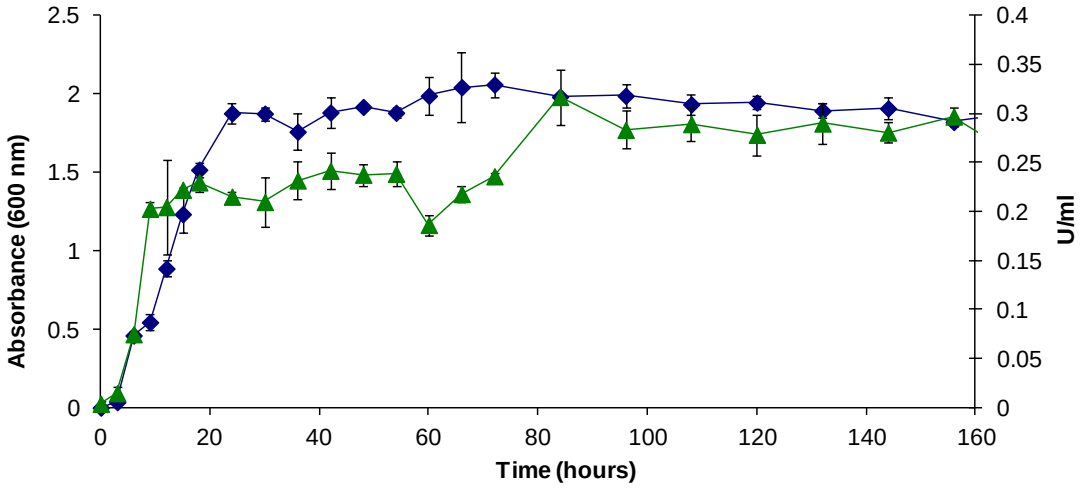

Fig. 1. Cell growth (♦ OD600) and xylanase activity (▲ U/mL) in the culture supernatant of *Bacillus subtilis* SJ01 grown in enrichment media for a total of 160 hours. Values are presented as mean values ±SD (n = 3). U = µmole reducing sugar produced/hour.

According to the literature, extracellular enzymes are released from bacilli in their stationary growth phase (Howard et al. 2003; Robson and Chambliss 1984; Sá-Pereira et al. 2002), which correlated with the data in Fig. 1, where xylanase activity was the highest during the stationary growth phase.

As our main interest was in the purification of MECs, purification was carried out using ultrafiltration with a 50 kDa cutoff to remove smaller proteins. This was followed by size exclusion chromatography using Sephacryl S-400, which has a very high exclusion limit at 2 x 10^6 for dextrans and 8 x 10^6 for globular proteins. This would ensure resolution and separation of large MECs. The size exclusion chromatogram for the purification is displayed in Fig. 2. Protein elution was followed by measurement of absorbance at 280 nm, followed by a Bradford's assay to confirm protein concentration.

Although measurement at 280 nm should detect protein, it is clear from Fig. 2 that absorbance at 280 nm and protein measurement using the Bradford's assay did not correspond. The reason for this was not clear. Based on standards eluted on the same column, it was calculated that peaks S1 and S2 in Fig. 2 were high molecular weight fractions of 2210 kDa and 371 kDa, respectively. Peak S3 was calculated to be 81 kDa and so was unlikely to be an MEC and was therefore discarded. Peaks S4, S5 and S6 were calculated to be smaller than 50 kDa and were also discarded. Peak S2 eluted as a doublet and was subsequently purified as two peaks and termed C1 (calculated to be 371 kDa) and C2 (calculated to be 267 kDa). Peak S1 had such low protein concentration that it was most likely caused by some interference. Therefore only the potential MECs in peak S2 (C1 and C2) were further investigated.

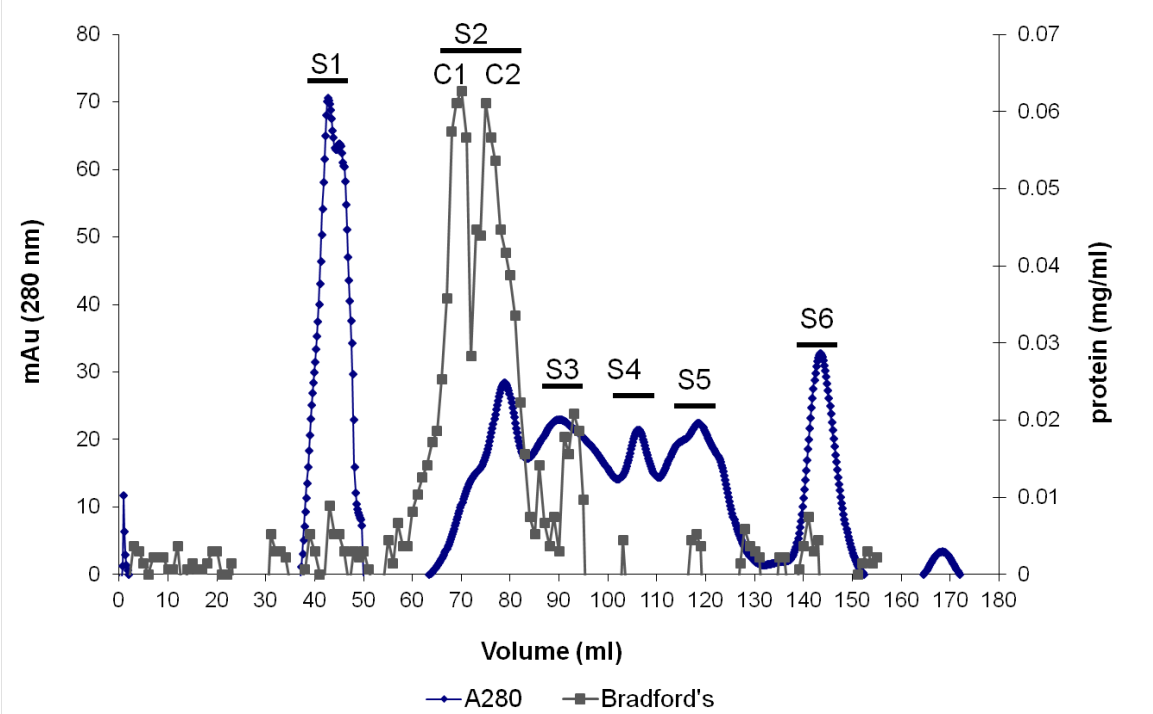

Fig. 2. Sephacryl S-400 chromatogram of ultrafiltration retentrate. Column dimensions: 60 X 2 cm; flow rate: 0.5 mL/min. ♦ A_{280} in mAU; ■ protein concentration in mg/ml. Peaks S1 (2210 kDa), C1 (372 kDa) C2 (267 kDa) and S3 (81 kDa) were pooled and concentrated.

The size of C1 (371 kDa) was similar to that of complexes isolated by Kim and Kim (1993) and Pason et al. (2006a,b) from *B. circulans* and *P. curdlanolyticus*, respectively, which were both 400 kDa in size. Both C1 and C2, however, were smaller than many other MECs and cellulosomes, as reported in the literature, which can reach sizes of 2000 kDa or larger (Bayer and Lamed 1986; Desvaux 2005; Doi et al. 1998; Pason et al. 2006b; Van Dyk et al. 2009a,b). The small sizes of the MECs isolated from *B. subtilis* SJ01 probably indicate that only a limited number of enzymes are present in these complexes.

Table 1 shows the purification table of fractions collected, based on xylanase and CMCase activities recorded in each fraction. The high xylanase and CMCase activities in the ultrafiltration filtrate (Table 1) indicated that there were many free xylanases and CMCases in the crude supernatant that were removed from the sample before loading on the SEC column. The ultrafiltration retentate was loaded on the Sephacryl S-400 column and resulted in the production of peaks S1, S2 and S3. Peaks S1 and S3 had very low protein concentration and xylanase activity compared to S2. Peak S1 also had low CMCase activity and so might be due to interference at A_{280} rather than a protein. Peak S3 had high CMCase activity, but was too small (81 kDa) to be an MEC and, therefore, was probably a non-complexed, free CMCase. Peak S2 had activities of 12.98 U and 0.38 U on xylan and CMC, respectively (Table 1), and was approximately 371 kDa in size (Fig. 2). It was, therefore, possible that this peak represented a (hemi-)cellulolytic MEC.

According to Table 1, the percentage yield for the MEC in peak S2 appeared to be low, with a 10.6% yield of xylanase and 3.1% yield of CMCase. However, it was an objective of the purification to remove the smaller free enzymes, which can explain the reduced yield. Therefore the loss of free enzyme activity was not considered significant. The same applied in the case of the fold purification, which was low due to the loss of CMCase and xylanase activities as a result of the removal of free enzymes.

Table 1. Xylanase and CMCase Purification Tables for the Purification of MECs from *B. subtilus* SJ01 using Ultrafiltration and Size Exclusion Chromatography *

Step	Total Protein (mg)	Total Activity(U)	Specific Activity (U/mg)	Purification Factor	Yield (%)
Xylanase activity					
C	713.50	122.15	0.17	1.00	100.0
CS	339.00	116.40	0.34	2.01	95.3
UR	221.50	29.86	0.13	0.79	24.5
UF	252.80	127.20	0.50	2.94	104.1
S1	2.08	0.03	0.02	0.10	0.0
S2	**50.25**	**12.98**	**0.26**	**1.51**	**10.6**
S3	0.73	0.03	0.05	0.27	0.0
CMCase activity					
C	713.50	12.36	0.02	1.00	100.0
CS	339.15	9.36	0.03	1.59	75.7
UR	221.50	3.34	0.02	0.87	27.1
UF	252.92	14.03	0.06	3.20	113.6
S1	2.09	0.01	0.00	0.19	0.1
S2	**50.35**	**0.38**	**0.01**	**0.44**	**3.1**
S3	0.74	0.70	0.94	54.54	5.6

* C-crude; CS-crude supernatant; UR-ultrafiltration retentate; UF-ultrafiltration filtrate

The purification procedure was repeated several times to generate sufficient protein for preliminary characterization of the MEC in peak S2. In each case, a similar chromatogram to that in Fig. 2 was obtained, and therefore the doublet in peak S2 was separated into MECs C1 and C2. These peaks were pooled separately, concentrated, and used for electrophoresis and zymograms as displayed in Fig. 3, as well as activity assays as displayed in Fig. 4.

Figure 3A displays the protein patterns of C1 and C2 on SDS-PAGE. Each of the MECs had at least 18 protein bands of similar sizes, although the concentrations of the proteins in each MEC varied from each other. The major bands in C1 were 67, 40, 32, 22, and 15 kDa, while the major bands in C2 were 54, 38, 25, 19, and 16 kDa in size. Figure 3B displays the zymogram using birchwood xylan as a substrate, while Fig. 3C displays the zymogram with oat spelt xylan as a substrate.

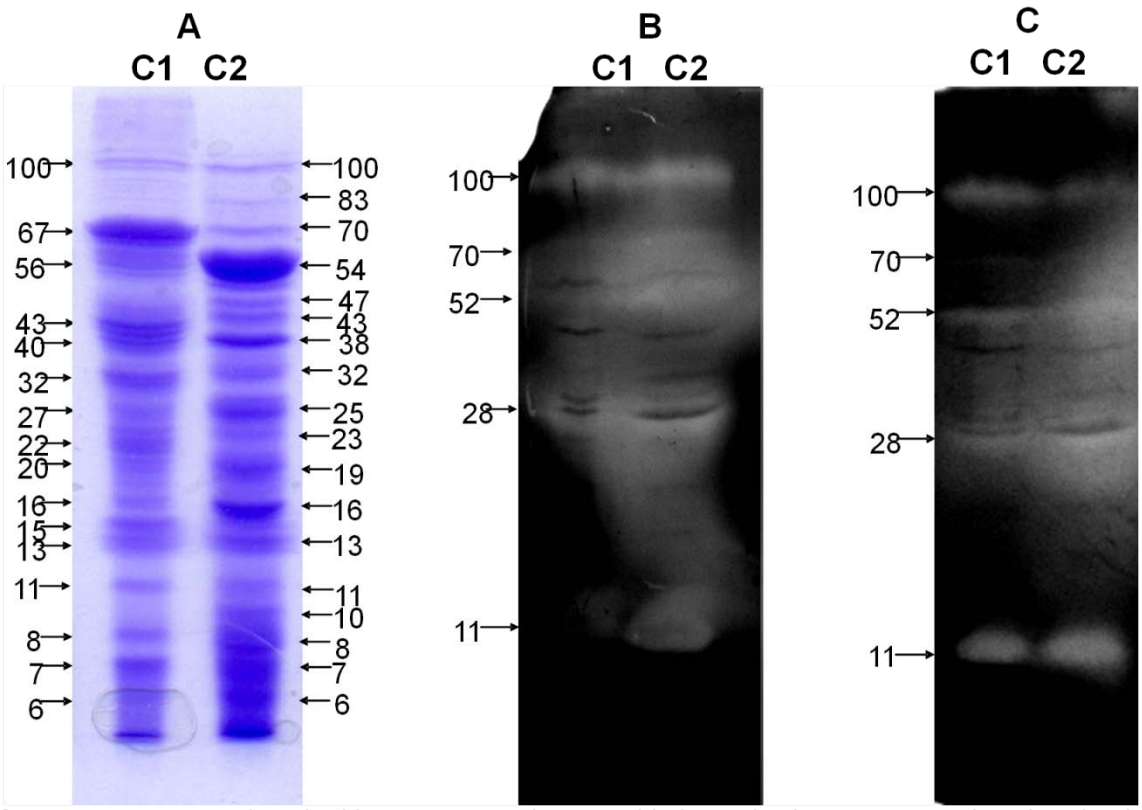

Fig. 3A: SDS-PAGE (10%) with 20 µg C1 and C2; **B:** birchwood xylan zymogram (10%) stained with Coomassie stain and 50 µg C1 and C2; **C:** oatspelt xylan zymogram (10%) with 100 µg C1 and C2. Sizes of bands were calculated based on a peqGold protein marker.

From the zymograms it is apparent that the same enzymes were involved in degradation of birchwood xylan and oat spelt xylan. Activity was lower on oat spelt xylan, as higher concentrations of protein were required to obtain zymogram patterns. Each complex consisted of five xylanases that degraded both birchwood and oat spelt xylan (100 kDa, 70 kDa, 52 kDa, 28 kDa, and 11 kDa). Birchwood xylan is routinely used to identify endo-xylanase activity, as the substrate is composed of 90% polymers of xylose. Oat spelt xylan consists of chains of xylose with arabinose substituents and requires both endo-xylanases and arabinofuranosidases for its degradation. Therefore additional enzymes may be involved in oat spelt xylan, although additional enzymes were not detected on oat spelt xylan. The clearance zones on the zymograms were very broad and accurate determination of the molecular weights was difficult, so the sizes of bands were estimated. Due to the low CMCase activity in these fractions, zymograms on CMC were not successful.

The five clearance zones on zymograms may indicate that five xylanase enzymes were present in the MECs. However, Jiang et al. (2006) found that, although four xylanases were identified through zymograms in a MEC, these were truncated forms of only two enzymes. The presence of four clearance zones on the xylan zymogram was ascribed to protease action which resulted in truncated forms of the same enzymes.

The composition of the MECs purified in this study was compared to similar hemicellulolytic MECs in the literature. The two MECs isolated from *Paenibacillus*

curdlanolyticus contained 7 and 5 xylanases (Pason et al. 2006b), while the MEC from *B. licheniformis* contained seven xylanases (van Dyk et al. 2009a, 2010). The xylanosomes of *Streptomyces olivaceoviridis*, *Butyrivibrio fibrisolvens* and *Chaetomium* spp. contained 4, 11, and 5 xylanases, respectively (Jiang et al. 2006; Lin and Thomson 1991; Ohtsuki et al. 2005). Many of these MECs also contained other enzymes such as endoglucanases, mannanases, and pectinases (Lin and Thomson 1991; Pason et al. 2006b; Van Dyk et al. 2010). Therefore additional characterisation of the MECs in this study can be carried out to identify further enzymes.

No bands were observed on zymograms of C1 and C2 using Avicel® and CMC as substrates due to the low activities on these substrates, and the substrate specificity test in Fig. 4 indicated that C1 and C2 had predominantly xylanase activity, with small amounts of activity on CMC and bagasse. The production of xylanases from species of *Bacillus* is well described in literature (Archana and Satyanarayana 1997; Battan et al. 2007; Dhillon et al. 2000; Dhiman et al. 2008; Kapoor et al. 2008; Pham et al. 1998) and from *B. subtilis* in particular (Jalal et al. 2009; Sá-Pereira et al. 2002, 2004), indicating that xylanases are common in these bacteria. Xylanases are often the predominant enzymes in MECs produced by bacilli (Kim and Kim 1993; Pason et al. 2006b; Van Dyk et al. 2009a,b), and these MECs should probably be termed xylanosomes.

The MECs C1 and C2 were further assayed on a range of substrates to determine their ability to degrade both simple and complex cellulose and hemicellulose. Fig. 4 demonstrates that both C1 and C2 degraded xylan substrates with much higher efficiency than cellulose substrates. The complex C2 displayed slightly lower enzyme activities than C1, but it is interesting to note that C2 displayed a small amount of activity on sugarcane bagasse, which could indicate that additional enzymes were present in this fraction.

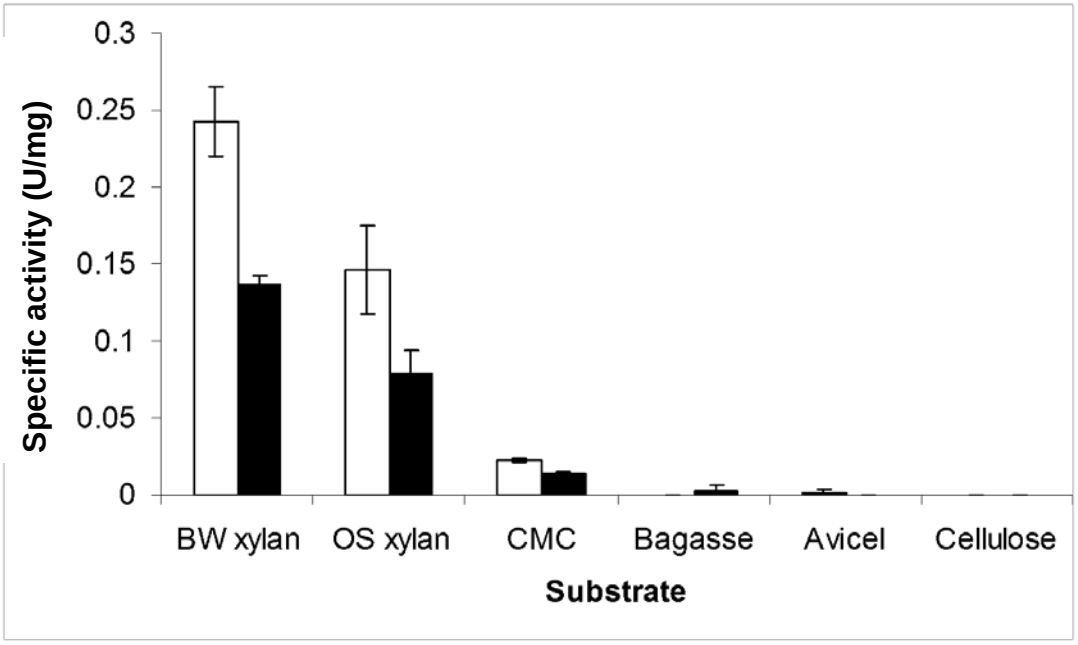

Fig. 4. Specific activity (U/mg protein) of □C1 and ■C2 on various substrates, where 'Bagasse' represents sugarcane bagasse and 'Cellulose' represents α-cellulose powder. Values are presented as mean values ±SD (n = 3). U = µmol reducing sugar produced/hour.

The xylanase activities of C1 and C2 (0.25 µmol/h/mg and 0.14 µmol/h/mg, respectively) were low when compared to xylanase activities of other xylanolytic MECs reported in the literature (3.17 µmol/min/mg for *P. curdlanolyticus*; and 4.37 µmol/h/mg *B. licheniformis*), which may limit the use of these MECs in research and industry. However, further studies should be performed to investigate the structure and function of these MECs, and to determine their usefulness compared to other xylanolytic MECs. For example, further substrate specificity tests could be performed on a wider variety of hemicellulose substrates to identify the presence of β-xylosidases and other enzymes that degrade hemicellulose side-chains.

Cellulosomes typically contain a non-catalytic glycoprotein called a scaffolding protein that binds to the enzyme subunits of the cellulosomes and mediates binding to cellulose substrates (Bhat et al. 1997; Garcia-Campayo and Béguin 1997; Pohlschroder et al. 1994). Scaffolding proteins in cellulosomes characteristically possess binding domains for binding to insoluble substrates such as cellulose (Bayer et al. 1994). Therefore a binding study was carried out to determine whether C1 and C2 were able to bind to insoluble cellulose (Avicel) and insoluble birchwood xylan. The results of the binding study are shown in Table 2. MEC C1 displayed no binding to Avicel and only 16% binding to insoluble xylan, while C2 displayed 11% binding to Avicel and 26% binding to xylan.

Table 2. Binding (%) of Two MECs (C1 and C2) to Insoluble Birchwood Xylan and Avicel® Substrates

	C1	C2
Avicel	0	10.92
Xylan	16.07	25.93

The limited binding to Avicel® and insoluble xylan indicated the absence of substrate binding domains such as found in cellulosomes. In previous binding studies Jiang et al. (2006) reported 50% binding of the *Streptomyces olivaceoviridis* xylanosome to insoluble xylan. Pason et al. (2006a) reported 57% binding of the *P. curdlanolyticus* (hemi-) cellulolytic MEC to insoluble xylan and Ratanakhanokchai et al. (1999) reported 60% binding of an endoxylanase to insoluble xylan. The MECs from *B. subtilis* SJ01 therefore appear to have structures different from many of the known cellulosomes and (hemi-) cellulolytic MECs.

Although the activities of complexes C1 and C2 were predominantly limited to xylanases, these MECs may have a significant application in the paper and pulp industry, since this industry requires cellulase-free xylanases (Balakrishnan et al. 2006). Xylanases are also important for the processing of fabrics (Dhiman et al. 2008), in food processing, poultry feeds, degumming of plant fibres (Battan et al. 2007), and clarification of juices (Dhillon et al. 2000). Xylanolytic MECs may be useful in the degradation of natural xylan substrates such as beechwood xylan, kraft pulp xylan, corncob xylan and wheat arabinoxylan (Jiang et al. 2004) and other natural substrates with high hemicellulose content. Xylanolytic MECs can also be used in conjuction with cellulosomes for the degradation of recalcitrant lignocellulose substrates, and for the construction of designer cellulosomes. There is already a great deal of genetic

information available on *B. subtilis*, making this bacterium an ideal candidate for genetic manipulation to produce MECs containing a desired combination of enzymes (Bayer et al. 1994; Murashima et al. 2003).

CONCLUSIONS

1. This study demonstrated that *B. subtilis* SJ01 produces two MECs of 371 kDa and 267 kDa that displayed predominantly xylanase activity.
2. C1 and C2 consisted of 16 and 18 subunits respectively based on SDS-PAGE analysis.
3. Five proteins in C1 and C2 degraded birchwood xylan and oat spelt xylan.
4. Activity of the MECs, C1 and C2, were determined on birchwood xylan (C1: 0.24 U/mg, C2: 0.14 U/mg) and amorphous cellulose (CMC) (C1: 0.002 U/mg, C2: 0.01 U/mg), indicating that highest activity was found on xylan, corresponding to the zymogram results.
5. C1 and C2 displayed very low binding to insoluble substrates, which is in contrast to what is usually observed with cellulosomes. This probably indicates that C1 and C2 have a different structure than those of cellulosomes.

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