Study on enzyme properties of bacillus subtilis LC-9 multifunction cellulose

Ming Sui1,2, Chunming Li2, Guoying Li1,3, Rongqing Zhou1,3,*

1Key Laboratory of Leather Chemistry and Engineering, Ministry of Education and College of Light Industry, Textile & Food Engineering, Sichuan University, Chengdu 610065, China
2Department of wine and food engineering, Si Chuan Technology and Business College, Si chuan, Du jiang-yan 611800, China
3National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, China

*Corresponding author e-mail: 348237548@qq.com

Abstract. Cellulose is the most abundant renewable resource in nature and can be used for the production of soluble sugars, biofuels and as raw materials for other important chemical products. Therefore, the development and research of multifunction’s enzyme is of great significance for the degradation and utilization of cellulose. In this study, two-step DEAE-Sepharose FF weak anion exchange of fermentation supernatant of Bacillus subtilis LC-9 with different pH buffer as mobile phase was used to obtain the electrophoretically pure protein with a molecular weight of about 32 KDa, 25 KDa. The specific activity of filter paper comprehensively characterized by enzyme was 22.68U/mg, the purification fold was 33.35 and the recovery rate was 20.52%. In this study, the purified multifunction’s enzyme not only has the activity of endoglucanase, xylanase, filter paper enzyme, exoglucanase and other cellulases, but also has the advantages of short enzyme producing time, simple purification method And other advantages, has a good prospect of industrialization.

1. Introduction
In general, cellulose is defined as a system of multifunctional enzymes that hydrolyze cellulose into simple sugars such as glucose and cellobiose, and the two systems work synergistically with one another and can always do this together task. The complex enzyme system is quite complex, so far, people have not fully understand its internal structure and the principle of action, but according to the existing literature, it includes exo-β-1,4-glucan Enzymes and endo-β-1,4-glucanases and β-1,4-glucosidases. The definition of endo-β-1, 4-glucanase means that he is able to exert a random function in the amorphous region inside cellulose, the product of which is to produce a large amount of oligosaccharides and new ends, but exo-β-1, 4-glucanase can act on both the reducing and non-reducing ends described above, and at the same time act as fiber binaural. After the second fiber is produced, the exo-β-1, 4-glucanase converts the second fiber into two molecules of glucose. Cellulases can be extracted from cellulose and this enzyme can have a significant effect on converting cellulose and damaging plant cell walls.
The mechanism by which cellulases degrade natural cellulose is not well understood and until now there have been mainly several hypotheses as follows: (a). C1-Cx Theory: Reese et al. Proposed the C1-Cx theory in 1950 that C1 (endoglucanase) enzyme acts on crystalline cellulose to become non-crystalline cellulose; then we use Cx Glucoses) enzymes hydrolyze non-crystalline cellulose in a random fashion. In this manner, soluble cellulose and glucose [beta]-1, 4-oligomers are obtained; finally, beta-glucan Enzymes hydrolyze cellubiose and disaccharide to glucose molecules. (B). Synergy: The hydrolysis of natural cellulose requires the synergy of three cellulases. In general, the synergistic effect is proportional to the degree of crystallization of the enzymatic substrate. (C). The sequential action hypothesis is that endoglucanases open the gap at the β-1,4 glucosidic bond of cellulose and then the exo-dextran cellobiase cleaves a dimer at the non-reducing end position of the gap, And finally beta-1,4 glucanase hydrolyzes cellubiose to glucose. (D). Oxidative degradation mechanism: This was discovered when brown-rot fungi degraded wood. It has been found through research that it is very likely that there is a very special substance in brown rot fungi that is not an enzyme but an alternative system in which a small molecule substance is present into the cellulose in order to break down cellulose. The Fenton-induced model of cellulose average degree of polymerization of wood is very similar to the brown rot fungus degrading wood. After a large number of studies concluded that: brown rot fungi may be involved in a small molecule, non-enzymatic, involving HO * oxidation mechanism of cellulose degradation.

In nature degradation of plant cell wall has a variety of forms of degradation, mainly contains: free enzyme system, fiber body, cell wall auxiliary enzyme, multifunctional enzyme. There are two main types of enzymes, one is a multi-catalytic domain, and each catalytic domain can only hydrolyze one substrate. The other is a single catalytic domain. One catalytic domain can hydrolyze many substrates. Most of the multifunction cellulose enzymes are found in fungi such as Trichoderma reesei, Aspergillus niger, Penicillum and other fungi. Zverlov et al. screened a multifunctional CelA enzyme from Anaerocellum thermophilum and found that the enzyme hydrolysed CMC, Xylan, and microcrystalline cellulose. Nathan A and so on screened CelAB from Hydrous pediculate’s and found that the enzyme can hydrolyze dextran, CMC, lichenin, and the like. Kim HM (2012) and other multi-functional endoglucanase Cel15B screened from Gloeophyllum trabeum, found that the enzyme can hydrolyze CMC, dextran, filter paper, but rarely found in bacteria, especially in Bacillus Very few, therefore, bacillus subtilis LC-9 multifunction cellulose appears to be particularly important in this article.

2. Experimental method

2.1. Experimental Materials
Species: This experiment is the use of the laboratory pre-screening to produce a multi-functional glycosyl hydrolase can produce bacillus subtilisLC-9, this versatile glycoside hydrolase CMC, pNPG, xylan, microcrystalline Cellulose and other substrates, in industrial applications have broad prospects.
Drug: 2.1.4 medium (Bacillus subtilis LC-9 seed solution 25ml/bottle adjusted to pH5.3 with NaOH), fermentation broth 50ml/bottle, adjusted to pH5.3 with NaOH), SDS-PAGE related solution Glue solution, 12.5% separation gel, 4% concentrated gel, 6 × loading buffer, 10×electrode buffer solution (pH 8.3), fixative solution, decolorizing solution and staining solution) Buffer, 2.5% x-100, 1 M NaCl, Congo red, acetic acid), 3,5 dinitrosalicylic acid.

2.2. Detection of cellulose activity
2.2.1. Glucose standard curve. Using 3, 5-dinitrosalicylic acid colorimetric method (DNS method) determination:
(A). Preparation of glucose standard solution: Weigh accurately 100mg glucose deionized water to a volume of 100 ml made of 1 mg/ml glucose standard solution.
(B). Reagents were placed in a gradient with glucose standards and deionized water.
(C). Add reagent after boiling in boiling water for 5 minutes, cooled and shaken, measured at 540 nm absorbance value.

The fitted regression linear equation is shown in Figure 1.

![Glucose standard curve](image)

\[
y = (3.36758)x + (-0.26356) \quad R = 0.999385
\]

**Figure 1.** Glucose standard curve

After many experiments and thesis research, we found that microcrystalline cellulose decomposes into monosaccharides at high temperature, which has great influence on the experiment. For this reason, afterwards, we experimented on the improvement and the experiment of microcrystalline cellulose Group and blank control group before adding DNS were centrifuged at 8000 r/min, while discarding the bottom sediment, take the same volume of supernatant plus DNS staining boil 5 min at 540 nm with a spectrophotometer to measure the light transmittance.

2.2.2. **Enzyme activity determination**

(A). Endocellulase (CMCase): Take 1% sodium carboxymethyl cellulose (dissolved in 0.2 mol/L citric acid buffer), take 1.0 ml as a substrate, add 0.5 ml of a suitable dilution of the cellulose solution, The reaction was carried out at 45 °C for 30 min in a water bath, and the reducing sugar was determined by the DNS method, and the blank test value was deducted.

(B). Exocellulase (microcrystalline cellulose): Take 1% microcrystalline cellulose, take 2.0 ml as a substrate, add 1 ml appropriate dilution of the cellulose solution, the reaction at 45 °C water bath for 60 min, using the DNS method Determination of reducing sugar, and deduct the blank test measured value. The amount of enzyme required to produce 1 μmol of reducing sugar per minute from the substrate under the above conditions is defined as one unit of activity, expressed as IU/ml.

(C). Xylanase: Take 1% of xylan, take 1.0 ml as substrate, add 0.5 ml of appropriate dilution of cellulose solution, and react in water bath at 45 °C for 30 min, measure the reducing sugar by DNS method, And deduct the blank test measured value. The amount of enzyme required to produce 1 μmol of reducing sugar per minute from the substrate under the above conditions is defined as one unit of activity, expressed as IU/ml.

(D). Filter paper enzyme (FPA): filter paper immersed in 1.0 ml 0.2 mol/L citric acid buffer, PH 6.5, add 0.5 ml of a suitable dilution of the cellulose solution, the reaction at 45°C water bath for 60 min, determination of reducing sugar , And deduct the blank test measured value. The amount of enzyme required to produce 1 μmol of reducing sugar per minute from the substrate under the above conditions was defined as one unit of activity expressed in IU/ml.
2.2.3. **Enzyme activity verification.** Congo red with polysaccharides such as cellulose, a strong color reaction occurred, resulting in red complexes, and reducing sugar does not react. Cellulose is a polysaccharide composed of D-glucopyranose residues linked by β-1,4-glycosidic bonds, which can form red complex with Congo red, while cellulose hydrolyzes carboxymethyl cellulose substrate The main production of reducing sugar, so the hydrolysis zone is not color.

Drill holes in a solid plate with a yellow pipette tip, add a candidate bacterial culture, and a culture medium with no culture as a negative control. 30℃ incubator for about 2 ~ 3 h, with 0.1% congo red solution staining 1 h, then 1 mol/L NaCl solution elution, with a certain cellulose activity will appear clear transparent circle.

2.3. **Determination of protein concentration**

Determination of protein content Bradford method according to the reference, with bovine serum albumin (BSA) as a standard protein Bradfor method for the determination of protein concentration. Draw 0~1mg/ml protein standard curve. Draw as shown in Figure 2:

![Figure 2. Mg/ml protein concentration](image)

After use with a color plate with ethanol, and properly preserved.

2.4. **Fermentation process**

(A). Coating plate 35℃ 36h incubation
(B). Pick bacteria, pay attention to pick a single colony
(C). Inoculation to the seed liquid for 12h, at a temperature of 35℃ shaker culture
(D). 2% inoculated into the fermentation broth at 35℃ constant temperature shaking culture 36h fermentation
(E). the speed of centrifugation at low temperature is 8000r/min and the centrifugation time is 20min. At the end of the collection, the clear liquid above the fermentation broth is collected.

2.5. **B. Subtilis LC-9 Intermediate cellulose validation**

(A). In accordance with the previous method, with a good 12.5% of the separation gel evenly mixed, carefully injected into the plastic glass, the glue surface from the top of the groove about 3 cm stop, the plastic surface gently filled with aqueous solution Seal, place the plastic sheet vertically, the gel between
25℃ ~ 30℃ for about 30 min, to be gelled, pour the water on the glue surface, blot the residual moisture with filter paper.

(B). Press 2.1.5 will be equipped with a good 4% concentrated plastic quickly injected into the upper layer of the separation gel, so that the liquid level flush with the glass plate groove, carefully insert the comb, placed at 25℃ ~ 30℃ 15 ~ 20 min, to be gelled solid After carefully pulling out the comb, rinse the comb hole carefully with the electrode buffer.

(C). Sample Processing: The enzyme solution and 5 × Loading Buffer 3: 1 ratio of mixing, boiled 1min, cooled and centrifuged, using a micro sampler slowly added to the comb hole.

(D). the electrophoresis tank connected electrophoresis apparatus, electrophoresis conditions: 80 V, 30 min; 120 V continue to run to the sample dye migration to the bottom of the glass plate to stop electrophoresis.

(E). Carefully peel the gel from the glass plate and refolding in 2.5% (v/v) Triton-X100 for 1.5 h at 4 C (shaking every 15 min for Triton-X100 at 30 min).

(F). The Triton-X100 gel was washed with ddH2O and placed in a 45°C water bath for reaction with the substrate for 1 h.

(G). The substrate was washed with ddH2O, placed in Congo red staining solution, stained at 45°C 1h.

(H). the gel was soaked in 1 M NaCL solution for decolorization, placed on a decanter shaker repeatedly rinsed the film until the film can be observed obvious bands appear.

(I). If the band is not obvious available 5% acetic acid counterstain.

3. Results and Analysis

3.1. B. subtilis LC-9 cellulose activity verification
According to the test of the activity of cellulose, the fermentation broth of the strain was added to the Congo red plate containing different substrates (sodium carboxymethylcellulose and xylan) for the verification of cellulose activity. As can be seen from FIG. 3, the control fermentation broth did not appear obvious hydrolysis circle, and added to the bacterial liquid whole obvious hydrolysis region

Figure 3. Congo red plates were tested for the viability of cellulose produced by B. subtilis LC9. Panel A showed CMC as substrate and Panel B showed xylan as substrate.
Figure 4. B. subtilis LC9 intermediate cellulose hydrolysis of the filter paper (left a right B)

In addition, we also did a hydrolysis experiment on the filter paper. As clearly shown in Figure 4, the filter paper in bottle B was almost decomposed, while the control a filter paper was still intact.

3.2. B. Subtilis LC-9 Intermediate cellulose validation
The cellulose enzyme solution was electrophoresed on SDS-PAGE, refolded and reacted with two substrates of sodium carboxymethylcellulose and xylan, respectively. From the figure, we can see that xylanase and endoglucanase have the same bands at molecular weight of 18.4KDa, 20KDa and 35KDa, so we conclude that the cellulose is multifunctional.

Figure 5. B. subtilis LC9 activity of cellulose production of active staining

Note: the left picture to xylan as substrate, enzyme activity staining; the right to CMC as a substrate, the activity of the enzyme staining

4. Summary
In this paper, the versatility of cellulose produced by fermentation of B. subtilis LC-9 was verified by Native SDS-PAGE. It is obvious that at the 18.4KDa, 25KDa, 35KDa xylanase and endoglucan Enzymes have the same band. Therefore, B. subtilis LC-9 cellulose has versatility. After two steps of different pH DEAE Sepharose Tm Fast Flow anion exchange column purification method to obtain electrophoretic pure protein, now the protein sequencing work, the protein sequence can be completed after the expression of the enzyme, and ultimately can be used in industrial In production, this is of great practical significance for solving the current energy and environmental problems.
References

[1] Q Xu, You, S-Y Ding, and ME Himmel, Multifunction Enzyme Systems for Plant Cell Wall Degradation [J], Bio resource Technology: 2011, 20 - 21.

[2] Zverlov V, Mahr, S, Riedel K, and, Bronnenmeier K (1998) Properties and gene structure of a bifunctional cellulolytic enzyme (CelA) form the extreme thermophile, Anaerocellum thermophilum, with separate glycosyl hydrolase family 9 and 48 catalytic domains. Microbiology - SGM 144: 457-465.

[3] Nathan A. Ekborg, Wendy Morrill, Adam M. Burgoyne, li li , and Daniel L. Distel CelAB , a Multifunctional Cellulose Encoded by Teredinibacter turnerae T7902T, a Culturable Symbiont Isolated from the Wood-Boring Marine Bivalve Lyrodus pediculate’s [J], AppLED AND EnVIRONMENTAL MICROBIOGY, Dec.2007, P: 7785 - 7788.