

Green Purification Process of *Bacillus Subtilis* (LC-9) Multifunction Cellulase

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Abstract. The thorough degradation of Cellulose requires the interaction of various functions of cellulase. The single function of cellulase degrades cellulose, which is not only inefficient but also costly. In this paper, the fermentation of *Bacillus subtilis* LC-9, a cellulase-producing strain, was screened by the laboratory for hydrolysis experiments and found that it could degrade many substrates such as sodium carboxymethyl cellulose, xylan, filter paper, microcrystalline cellulose and pNPG. The activity of renatured SDS-PAGE gel electrophoresis showed that the 18.4KDa, 20KDa, 35KDa protein bands at the same time with endoglucanase and xylan; enzyme activity, and thus determine the strain may have produced Multiple groups of multifunctional cellulase. Therefore, the development and research of multifunctional cellulase is of great significance for the degradation and utilization of cellulose.

1. Introduction

In biology, cellulose can be hydrolyzed under certain conditions to be decomposed into monosaccharides that can be directly used by people. And all kinds of microorganisms in nature can use the cellulose that has been hydrolyzed into monosaccharides to produce products for daily use, such as the raw materials used in chemical industry, environmental friendly biofuels, biopharmaceuticals, biological feed for aquaculture and our Daily consumption of food and so on, if this technology is mature, you can replace the original fermentation in the field of starch as a raw material for fermentation products, but also can be used instead of direct use of chemical raw materials and chemical products produced, until now , Many institutes both at home and abroad are devoted to the research and development of cellulose. Unfortunately, at present, the utilization rate of cellulose is still only one percent and so low. The most important reason for such low utilization rate is that bioconversion. The cost is too high, and the catalytic efficiency of the enzymes used to degrade cellulose is low and the function of the enzymes that catalyze cellulose is too single. Say garbage is misplaced resources, but so far we humans just use a small part of the cellulose as building materials needed for construction, textiles used in the textile and daily life Of paper, feed, biogas produced by



fermentation and re-utilization of the field, however, to a large extent, most of the cellulose cannot be used to cause the irrational use of resources, which is not only a great waste of resources, In the wrong circumstances, even great pollution to the environment, such as the very serious incineration of straw nowadays, is caused by the problems of air pollution, haze, PM2.5 and so on, which are caused by the incorrect and effective utilization of cellulose resources Nowadays, "Fresh Words" has caused many of us to suffer. However, using biological methods to extract natural cellulase from nature and then increasing its enzyme activity or its yield by gene or purification method will greatly increase the utilization of cellulose Rate, we have the opportunity to break through the bottleneck of today's research, bring more hope to the current energy problems, and solve the environmental pollution problem at the same time. This indeed has great practical significance.

After decades of research scientists have found that there are many microorganisms that degrade and utilize natural cellulose on an existing basis. Such microorganisms exist in many species, such as fungi, actinomycetes, yeast, and higher fungi. However, there are fewer biomaterials in which they can synthesize a complete cellulase system and outwardly secrete cellulase. Although some bacteria, actinomycetes have strong ability to decompose and degrade cellulose, they only degrade under the contact of enzymes and cellulose, and they need the induction of inducers. Therefore, in this case, it is very difficult for cellulase to be industrialized Mass production.

Cellulose is a difficult problem because of its complex structure and how to transform and utilize it effectively. Therefore, it is imperative to explore multifunctional cellulase that can efficiently hydrolyze cellulose. The current production of cellulase producing cellulase is low in production and high in production costs, making cellulase production still in small-scale and low-volume production and cannot be produced by conventional fermentation systems. This topic hopes to get purified cellulase, then achieve heterologous expression, efficient production eventually meet the needs of industrial production. Taking into account the lack of pretreatment methods, this project will be based on the screening of a bacillus subtilis, based on the study of the hydrolysis of cellulase in fermentation broth, and the resulting enzyme on the degradation of straw without pretreatment to explore the use of The feasibility of enzymatic degradation of wood cellulose, and enzymatic degradation of the best reaction conditions were discussed.

2. Method of experimental

2.1. Experimental Materials

Species: This experiment is used in the laboratory pre-screening to a multifunction glycosyl hydrolase enzyme bacillus subtilisLC-9, this versatile glycosyl hydrolase can hydrolyze CMC, pNPG, xylan, microcrystalline cellulose and other end things, in industrial applications have broad prospects.

Drugs: 2.1.4 Medium (Bacillus subtilis LC-9 seed solution 25 ml/bottle, adjusted to pH 5.3 with NaOH), fermentation broth 50 ml/bottle, adjusted to pH 5.3 with NaOH), SDS-PAGE related solution (Gel stock solution, 12.5% separation gel, 4% concentrated gel, 6 × loading buffer, 10 × electrode buffer solution at pH 8.3), fixative solution, decolorizing solution and staining solution) Citrate buffer, 2.5% x-100, 1 M NaCl, Congo red, acetic acid), 3,5 dinitrosalicylic acid.

2.2. Purification of multifunctional cellulose LC-9

2.2.1. *Method of protein purification.* Protein purification technology is the use of proteins that exist between the similarities and differences between the technologies. The first step uses the similarities of the two proteins to remove impurities that are not proteins between the two substances. The second step uses the differences in the proteins to separate the protein of interest.

Method of protein purification

(A). Affinity chromatography affinity chromatography principle: based on the target protein and immobilized ligand specificity and then there was a phenomenon of retention, other unwanted impurities will flow through the column.

(B). Hydrophobic Interaction In high salinity environments, the hydrophobic region of the protein is exposed and bound to the hydrophobic coordinating groups on the surface of the hydrophobic medium.

(C). Electrophoresis Normally, the complexity of a protein sample is monitored by acrylamide gel electrophoresis and the purification effect of the mixed flavor is monitored. It is also possible to detect which concentration gradient ion-exchange column salt eluent the protein of interest is in.

2.2.2. *Separation and purification process.* Isolation and purification process is Figure 1.

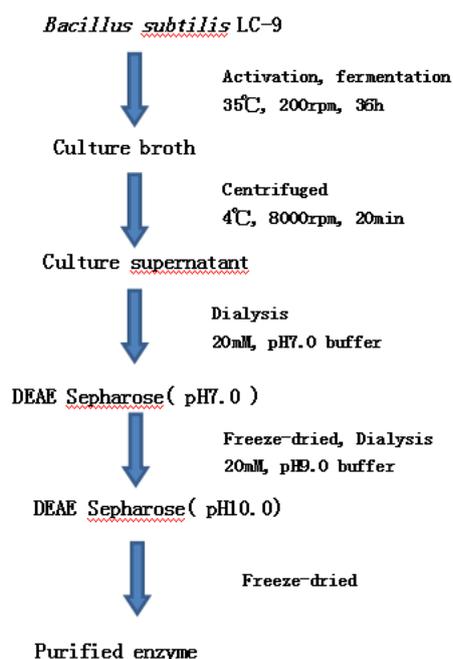


Figure 1. Separation and purification of cellulase LC-9 flow chart

2.2.3. *DEAE Sepharose™ Fast Flow Weak anion exchange chromatography (pH 7.0).* The dialyzed supernatant of the pre-treated fermentation broth was separated by DEAE Sepharose™ FF weak anion exchange chromatography.

Equilibration buffer: Na₂HPO₄-Citrate (20 mmol • L⁻¹, pH 7.0) buffer

Elution buffer: Na₂HPO₄-Citrate (20 mmol • L⁻¹, pH 7.0) buffer + 1 M NaCl

Sample flow rate: 2ml • min⁻¹

Elution flow rate: 2 ml • min⁻¹

Elution method: 100% B directly elution, the penetration peak and the elution peak were collected, the enzyme activity and protein concentration were detected respectively, and samples with enzyme activity were collected for further purification.

2.2.4. *DEAE Sepharose™ Fast Flow Weak anion exchange chromatography (pH 10.0).* The dialyzed supernatant of the fermentation broth was separated by DEAE Sepharose™ FF weak anion exchange chromatography.

Equilibration buffer: Tris-Hcl (20 mmol • L⁻¹, pH 10.0) buffer-phase A.

Elution buffer: Tris-Hcl (20 mmol • L⁻¹, pH 10.0) buffer + 1 M NaCl-B phase

Sample flow rate: 2ml • min⁻¹

Elution flow rate: 2 ml • min⁻¹

Elution method: 100% B directly elution, the penetration peak and the elution peak were collected, the enzyme activity and protein concentration were detected respectively, and samples with enzyme activity were collected for further purification.

Purification results were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as follows:

(a). Will be good with 12.5% of the separation gel evenly mixed, carefully injected into the plastic glass, to the glue surface from the top of the groove about 3 cm stop, the plastic surface gently filled with water seal, vertical plastic placement The plate, gel between 25 °C ~ 30 °C for about 30 min, to be gelled, pour the water on the glue surface, blot the residual moisture with filter paper.

(b). The concentrated 4% concentration gel that has been configured is rapidly injected into the upper part of the separation gel so that the entire liquid level is exactly the same as the groove of the glass plate and then the comb is inserted into it. C ~ 30. C, place 15 to 20 minutes, until the gel solidified, slowly and carefully out of the comb, then rinse the grooming hole with electrophoresis buffer.

(c). Sample Processing: The enzyme solution and 5 × Loading Buffer 3: 1 ratio of mixing, boil 1 min, 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 remove the gel and place in the fixative for about 30 minutes.

(f). Remove the gel, place in staining solution and stain at 45 °C for 15 min.

(g). Soak the gel in the decolorizing solution and place it on a decolorizing shaker for repeated rinsing of the film until the background color of the film is clear.

3. Analysis of the results

The cellulase LC-9 passed through DEAE Sepharose™ Fast Flow ion exchange column, adsorbed more impurity protein under the condition of pH7.0 and collected the penetration peak. The enzyme activity determined that cellulase completely remained in the penetration peak. At pH 10.0, most of the protein is adsorbed on the column, with only a very small amount of protein penetrating. It was found by enzyme activity assay that endo-glucanase activity, xylosease activity, exoglucanase activity and filter paper activity were found in the penetration peak, but most of the endoglucanase activity and xylan; enzyme activity is retained in the elution peak resulting in a decrease in specific enzyme activities of endoglucanase and xylosease activity. Previously, reactive staining contained at least three multifunctional enzymes, so multifunctional cellulase was present in the elution and penetration peaks. When cellulase has exo-glucanase activity, the structure consists of two parts: a catalytic domain and a substrate binding domain. Therefore, the structure of the multifunctional cellulase LC-9 may be opened to form two bands upon SDS-PAGE electrophoresis. Accordingly, Figure 2 shows the electrophoretic pure protein purification conclusion.

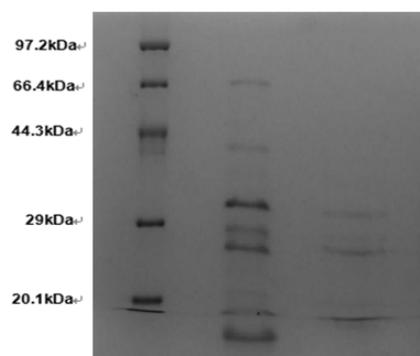


Figure 2. SDS-PAGE of purified endocellulase LC-9

From left to right, followed by M, 1, 2 where M is the standard protein marker, lane 1 is 20 times concentrated 7.0 penetration peak, lane 2 500 times concentrated 10.0 penetration peak

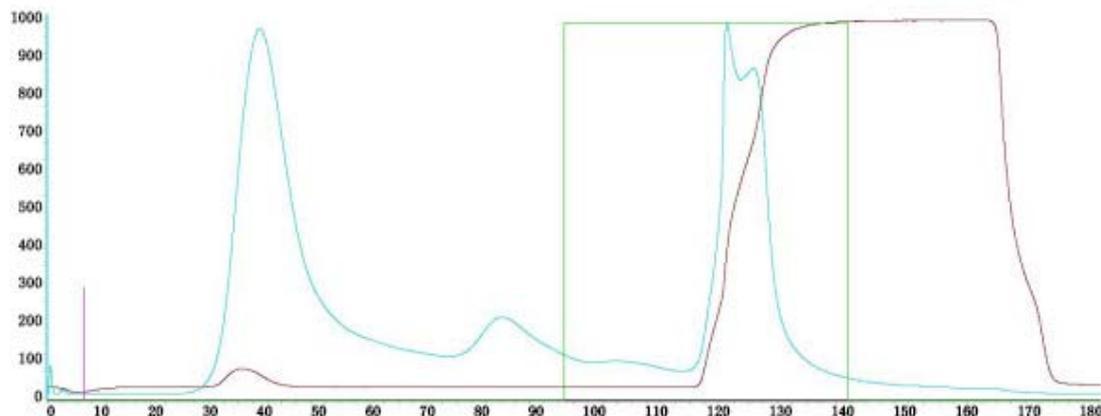


Figure 3. PH 7.0 DEAE Sepharose Tm Fast Flow anion exchange column purification

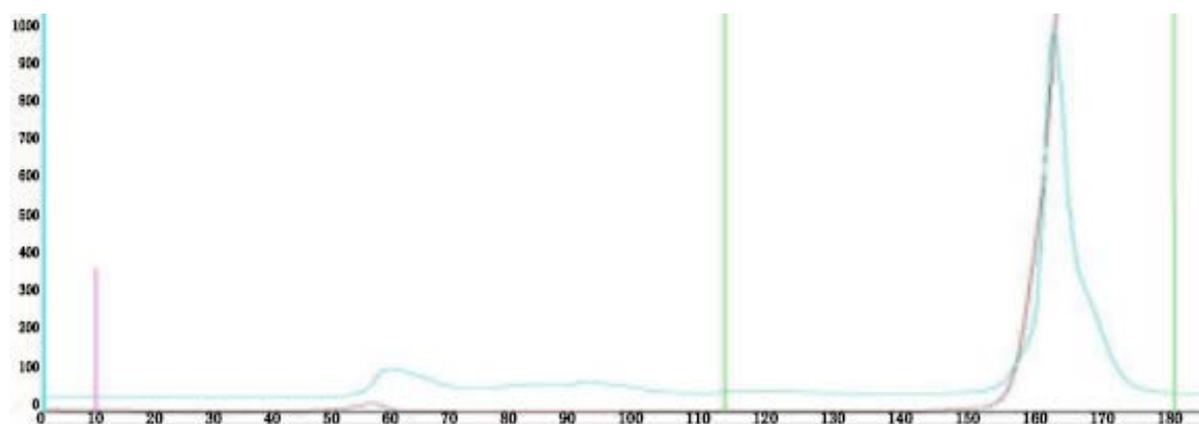


Figure 4. PH10.0 DEAE Sepharose Tm Fast Flow anion exchange column purification map

Table 1. Isolation and purification of cellulase exonuclease activity table

| | Total activity (U) | Total protein (mg) | Specific avicelase activity (U/mg) | Purification folds | Recovery rate (%) |
|-------------------------|--------------------|--------------------|------------------------------------|--------------------|-------------------|
| Culture supernatant | 8.78 | 13.4 | 0.66 | 1 | 1 |
| DEAE Sepharose (pH7.0) | 7.98 | 2.16 | 3.69 | 5.59 | 90.88 |
| DEAE Sepharose (pH10.0) | 2.18 | 0.082 | 26.59 | 40.29 | 24.83 |

Table 2. The filter paper enzyme activity separation and purification coefficient table

| | Total activity (U) | Total protein (mg) | Specific avicelase activity (U/mg) | Purification folds | Recovery rate (%) |
|-------------------------|--------------------|--------------------|------------------------------------|--------------------|-------------------|
| Culture supernatant | 9.06 | 13.4 | 0.68 | 1 | 1 |
| DEAE Sepharose (pH7.0) | 8.28 | 2.16 | 3.83 | 5.63 | 91.39 |
| DEAE Sepharose (pH10.0) | 1.86 | 0.082 | 22.68 | 33.36 | 20.52 |

Table 3. Cellulose endonuclease activity of the separation and purification coefficient table

| | Total activity (U) | Total protein (mg) | Specific avicelase activity (U/mg) | Purification folds | Recovery rate (%) |
|-------------------------|--------------------|--------------------|------------------------------------|--------------------|-------------------|
| Culture supernatant | 355.6 | 13.4 | 26.54 | 1 | 1 |
| DEAE Sepharose (pH7.0) | 311.6 | 2.16 | 144.29 | 5.43 | 87.62 |
| DEAE Sepharose (pH10.0) | 1.308 | 0.082 | 15.96 | 0.60 | 0.3 |

Table 4. Cellulase xylan; enzyme activity of separation and purification coefficient table

| | Total activity (U) | Total protein (mg) | Specific avicelase activity (U/mg) | <i>3.1. Purification folds</i> | Recovery rate (%) |
|-------------------------|--------------------|--------------------|------------------------------------|--------------------------------|-------------------|
| Culture supernatant | 143.2 | 13.4 | 10.68 | <i>3.2. 1</i> | 1 |
| DEAE Sepharose (pH7.0) | 124.4 | 2.16 | 57.6 | <i>3.3. 5.39</i> | 87.62 |
| DEAE Sepharose (pH10.0) | 1.416 | 0.082 | 17.27 | <i>3.4. 1.617</i> | 0.98 |

4. Summary

In this paper, we tried to use two steps of different pH DEAE Sepharose Tm Fast Flow anion exchange column purification method, and finally get electrophoretic pure protein band, the enzyme two subunits relative molecular mass were 32KDa and 25KDa, purified protein The specific activity of filter paper reached 22.68 IU/mg, 33.35 times of purification, and the activity recovery rate was 20.52%. Currently, two protein bands have been excised for LC-MS/MS.

The electrophoretically pure protein was obtained at pH10.0 DEAE Sepharose Tm Fast Flow Penetration peak, but still contains other multifunctional cellulase in the elution peak. However, we still need to purify it in the next phase. Although we obtained electrophoretically pure proteins, the

enzymatic properties and enzyme kinetics of this enzyme have not been studied and will be completed in the following work.

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