Characterization of cellulase from *E. coli* BPPTCC-EGRK2

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Abstract. Cellulase enzymes are widely used in various industries such as detergent industry, bioethanol, animal feed, textile and paper. This research focused on characterization of cellulase produced from Eschericia coli BPPT-CC EgRK2, which is a recombinant that can produce protein enzymes endo- β-1,4-glucanase. Eschericia coli BPPT-CC EgRK2 was cultured in 1 litre liquid medium Luria Bertani. Because the bacteria is intracellular, sonication is needed for cell disruption to get the cellulase enzyme. The enzyme activity was then analyzed by CMC substrate at different concentration. The protein content analysis was carried out using Bradford method; the molecular weight analysis was done using SDS-PAGE; while the enzyme kinetics was plotted using Michaelis-Menten model. Our results showed the highest enzyme activity was 2.403 U/ml and the protein concentration was 5.352 mg/ml. The Michaelis-Menten constant (Km) and maximum velocity (Vmax) for CMC substrate hydrolysis were 0.07 µmol/ml and 2.49 µmol/ml/sec, respectively. The cellulase molecular weight was 58 kDa using SDS-PAGE with 7.5% stacking gel. The results indicated that Eschericia coli BPPT-CC EgRK2 is a promising renewable source for cellulase production for industrial application.

1 Introduction

Enzymes are proteins that function as biocatalysts in a chemical or biological reaction [1]. The enzyme is a protein biomolecule that acts as a catalyst (a compound that speeds the reaction process without reacting) to *an organic chemical reaction. Substrate as the initial molecule will be accelerated its conversion into another molecule called product. The type of product to be produced depends on a condition / substance, called a promoter. The work of enzymes is influenced by several factors, mainly substrate, temperature, acidity, cofactor

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and inhibitor. Each enzyme requires different temperatures and pH (acidity levels) because different enzymes are proteins, which can change shape if temperature and acidity change.

One of the most widely used enzymes in the industry is the cellulase enzyme. Cellulase can convert cellulose, the main polymer in biomass, into glucose which can be further fermented to ethanol. Cellulase is currently the third largest industrial enzyme worldwide [2]. Cellulase is widely used in biotechnology applications, such as fiber modification in the paper and textile industries, but cellulase also has excellent capabilities in the industrial development of ethanol production from lignocellulose. Cellulase can be used for biomass fermentation into bioethanol [3]. A specific enzyme that is used to degrade cellulose is cellulase enzyme. Enzymatic process in hydrolyzing cellulose is a better and more beneficial way because the surrounding condition for enzyme's activity could be controlled [4].

The conventional method to produce cellulase is by using lignocellulosic fungi such as Aspergillus sp., Trichoderma sp., and Neurospora sp. [5-9]. However, cellulase can also be secreted by bacteria, such as Bacillus sp. Bacteria has a faster growth rate and is genetically easier to recombine compared to fungi [10]. This study examined an *E. coli* EgRK2 recombinant strain. The recombinant strain was used in this experiment because the result of the previous research showed the wild type has low activity , the recombinant strain grows faster on inexpensive media, ease of isolation and purification of proteins excreted in comparison with the wild type [11].This study examined the effects the enzyme's hydrolysis performance on Carboxymethyl Cellulose (CMC) as the substrate. The aim of the present research is to describe the biosynthesis, isolation and characterization of a cellulase from *E. coli EgRK2* isolate.

2 Materials and Methods

2.1 Sample Collection

The research was performed with an inoculum of *E. coli* EgRK2 recombinant strain which obtained from BPPT (Agency for the Assessment and Application of Technology Culture Collection) Laptiab, Puspiptek, Serpong,Tangerang, Indonesia.

E. coli EgRK2 is a recombined product of a Bacillus sp. RK2 isolate. The gene that expressed endo-β-1.4-glucanase (gene attB-EgRK2) in Bacillus sp. RK2 was isolated and amplified by the Polymerase Chain Reaction (PCR) method.In this study we used GatewayR (Invitrogen) method and two primers such as egrk2F: 5'- AAA AAG CAG GCT CGA TGA AAC GGT CAA TYT Y -3'; egrk2R: 5'- AGA AAG CTG GGT ACT AAT TKG KTT CTG WTC CC -3' and attB1 adapter: 5'- G GGG ACA AGT TTG TAC AAA AAA GCA GGC T -3'; attB2 adapter: 5'- GGG GAC CAC TTT GTA AA GAA AGC TGG GT -3'[11].

These design primers were used for amplifying endonuclease EgRK2 gen from chromosomal of B amyloliquefaciens BPPTCC RK2. pDONR221 as a donor vector with Kanamycin resistance and pDESTI4 as a destination vector with ampicillin resistance. E. coli DH5α for host cloning and E. coli BL21 Star for expression gen. It was then recombined and expressed in the pEXP-EgRK2 vector, which was transformed to E. coli DH5α [11]. The isolate was inoculated to Luria-Bertani (LB) slants of agar, and incubated at 37°C for 24 h and used as a culture stock.

2.2 Cellulase Enzyme Production

A starter medium was made by inoculating 1 loops of culture stock into sterile 1L LB medium, which was previously autoclaved at 121°C, and 1.2 atm for 2 hours. After autoclaved the LB medium, we put on the rotary shaker at 37°C and 150 rpm for 24 hours. Then we centrifuge the medium at 4°C and 5000 rpm for 25 minutes. After the pellet and supernatant separate, we add tris-HCl buffer to the pellet and mixed until homogen. At the end of the process, the culture medium was sonicated for 15 min to break the cells, and centrifuged at 4,000 rpm for 15 min, to obtain the crude extract that was meant to be used as the enzyme source.

2.3 Enzyme Assay

The assay of enzyme activity was done by the DNS (3.5-Dinitrosalicylic Acid) method. The cellulose existence was identified by determining reducing sugar content that was formed as a result of hydrolysis. The enzyme activity calculated using Eq. (1):

$$\text{Activity (U/ml)} = \frac{mg\ glukosa\ x\ 1000}{Mr\ glukosa\ x\ 30\ \text{min}\ x\ 0.1\ ml} \quad (1)$$

DNS method determined the amount of reducing sugar which was formed (mg) as the sugar content; The molar mass of glucose (180 g/mol) as Mr reducing sugar, the hydrolysis period of cellulose (30 min), and the amount of crude extract analyzed as sample (0.1 mL). Cellulase activity was measured in U/mL. The amount of enzyme required to liberate 1 µmol of reducing sugar per minute under the assay condition is defined as 1 unit.

The protein content was measured using the Bradford protein assay. An enzyme sample of 2 µL was mixed with 795 µL aquades and 0.2 mL Bradford reagent. After that measuring the sample absorbance at 595 nm wavelength.

2.4 Enzyme Characterization

2.4.1 Gel Electrophoresis

The determination of cellulose enzyme molecular weight used sodium deodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) with 7.5% stacking gel at constant current of 20 mA. The following protein calibration kit was used: ovotransferrin (78 kDa), albumin (66.25 kDa), ovalbumin (45 kDa), carbonic anhydhrase (30 kDa). The molecular weight of crude enzyme was determined after gel staining with Comassie Brilliant Blue G-250.

2.4.2 Substrate Specificity and Kinetic Parameters

To investigate the kinetic paramaters of CMC as cellulose enzyme substrate, hydrolysis experiments were conducted. Experiments were carried out on CMC (1; 1.25; 1.5; 1.75; and 2.0% wt). The kinetics of CMC degradation by cellulase was evaluated using the Michaelis–Menten model, with linearization using the Lineweaver-Burk approach shown in Eq. (2).

$$\frac{1}{Vo} = \frac{Km}{\text{Vmaks}}\frac{1}{[S]} + \frac{1}{\text{Vmax}} \quad (2)$$

[S] is the concentration of carbon source (mg/mL), Vmax is the maximum rate of reaction at infinite reactant concentration (µmol/mL/sec), and Km is the Michaelis constant (µmol/mL). The pH condition is 7 and temperature 40°C.

3 Results and Discussion

3.1 Cellulase Enzyme Activity

Our study showed that the highest enzyme activity on CMC hydrolysis is 2.403 U/ml which obtained from 2% CMC concentration as shown in Fig 1.

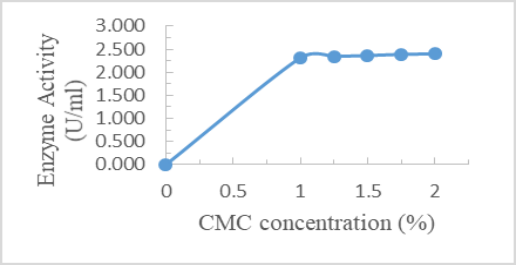

Fig. 1. Cellulase Enzyme activity over different CMC concentration

From this figure we know that reducing sugar concentration gradually increasing during the the substrate is higher. The result of enzyme activity not significantly different from the previous work with CMC as substrate [11] and the optimum condition in this research is 40°C for the temperature and 7 for pH based on the previous work [4] [11]. This result was slightly higher compared to cellulase activity from several studies as shown in figure 2.

Table 1 The variation of microorganisms for cellulase production

Microorganism	Carbon Source	Enzyme activity	Reference
E. coli EgRK2	CMC	Cellulase - 2.49 U/mL	This research
E. coli EgRK2	OPEFB CMC	Cellulase - 2.665 U/mL Celluase - 2.5 U/mL	[11]
*Bacillus sp.*RK2 *Bacillus subtilis*	OPEFB CMC	Cellulase - 2.498 U/mL Cellulase – 0.9 U/mL	[11] [12]
Bacillus pumilus EB3	OPEFB CMC Cellulase Powder	Endoglucanase – 0.076 U/mL Endoglucanase – 0.063 U/mL Endoglucanase – 0.025 U/mL	[13]
Bacillus pumilus	CMC	CMCase – 1.9 U/mL, cellobiase – 1.2 U/mL	[14]
Neurospora crassa	Wheat Straw	FPase – 0.667 U/mL	[15]
Chaetomium globusum	Avicel CMC	FPase – 0.11 U/mL, CMCase – 1.05 U/mL, β-glucosidase – 0.5 U/mL FPase – 0.18 U/mL, CMCase – 2.00 U/mL, β-glucosidase – 1.5 U/mL	[16]
Trichoderma ressei *Phenerocheate*	Cellulose Rice straw	β-glucosidase – 4.54 U/mL FPase – 1.438 U/mL	[17] [18]

chrysosporium			
Trichoderma harzianum	OPEFB	FPase – 5.1 U/g	[7]
Trichoderma ressei	Straw	FPase – 0.041 U/mL	[9]
Trichoderma koningii	Cellulose	FPase – 1.4 U/mL	[8]
Trichoderma ressei	Corn stover	CMCase activity 2596.3 IU/g dry culture	[19]

The cellulase activity values were 2.665 U/mL using *E. coli* EgRK2 and OPEFB as substrate, 2.5 U/mL using CMC as substrate [11]; 0.041 U/mL using *Trichoderma reesei* and straw [9]; 2.00 U/mL using *Chaetomium globusumn* and CMC [16]; 1.9 U/mL using *Bacillus pumilus* and CMC [14]; 0.063 U/mL using *Bacillus pumilus* EB3 using CMC [13]; and 0.9 U/mL using *Bacillus subtilis* and CMC [12].

3.2 Enzyme Characterization

3.2.1 Molecular Weight and Protein Assay

The crude enzyme from the culture broth was analyzed with SDS-PAGE to know the molecular weight of this protein. Because this crude enzyme was not purified yet, there was still more than one protein band. From the SDS-PAGE we know the molecular weight is 58 kDa. After that we analyzed the protein concentration of this broth. The concentration result is 5.352 mg/ml. This is not concentration of cellulose enzyme, because it is no purified, we can't measure the enzyme concentration.

3.2.2 Enzyme Kinetics on CMC Hydrolysis

Cellulase enzyme from *Eschericia coli* EgRK2 was used to hydrolysis of CMC. CMC degradation experiments showed the Vmax values were 2.49 µmol/mL/sec. The Km values for CMC hydrolysis were 0.07 µmol/mL for the recombinant strain. Previous research on Km and Vmax values which used CMC as substrate have been conducted by using same isolate, *Acinetobacter anitratus and Branhamella sp*. as shown in Figure 3 [11][20]. In CMC degradation, cellulase from the *E. coli* EgRK2 had higher Vmax values (2.49 µmol/mL/sec) compared to two other strains (0.48 and 2.56 µmol/mL/sec for *A. anitratus and Branhamella sp*., respectively. But had lower Vmax values compared to the previous research with same EgRK2 isolate (2.543 µmol/mL/sec). Additionally, Vmax values were also higher than cellobiose degradation by using A. anitratus (0.24 µmol/mL/sec) and Branhamella sp. (0.34 µmol/mL/sec). Thus the cellulase produced in this research had a better performance value than that produced in other research [20]. The Km values of cellulase from the *E. coli* EgRK2 recombinant in CMC degradation (0.07 µmol/mL) were smaller than the cellulase from same EgRK2 isolate in the previous research, *A.anitratus* (0.497 µmol/mL) and *Branhamella sp*. (0.790 µmol/mL). Thus in our research the enzyme affinity was better and the carbon source needed to reach its half maximum rate was lower.

Table 2 Values of cellulase Vmax and Km from various carbon source and microorganism

Carbon Source	Microorganism	Maximum enzymatic rate, Vmax (µmol/mL/sec)	Michaelis-Menten Constant, Km	Reference
CMC	*E. coli* EgRK2	2.49	0.07 µmol/mL	This research
CMC	*E. coli* EgRK2	2.543	0.097 µmol/mL	[11]
OPEFB	*E. coli* EgRK2	1.75	0.26 µmol/mL	[20]
CMC	*Acinetobacter anitratus*	0.48	4.97 mg/ml	[20]

	Branhamella sp.	2.56	7.90 mg/ml	
Cellobiose	*Acinetobacter anitratus*	0.24	0.32 mM	[20]
	Branhamella sp.	0.34	2.50 mM	[20]

4 Conclusion

Our research shows that cellulase production by the *E. coli* EgRK2 had Vmax value for CMC degradation were 2.49 µmol/mL/sec, while Km values were 0.07 µmol/mL. The molecular weight was 58 kDa and the protein concentration wa 5.352 mg/ml. This research demonstrated a possible way to develop an efficient hydrolysis system for CMC using *E. coli* EgRK2. The result of this research have known that *E. coli* EgRK2 become a promising renewable source for cellulase enzyme for industrial application.

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