Cellulase Enzyme Production Using *Actinobacillus* sp. on Several Alternative Growth Media

Mohammad Anam Al-Arif[1]*, Mirni Lamid[1], Sunaryo Hadi Warsito[1], Aswin Rafif Khairullah[2], Abdullah Hasib[3]

[1]Division of Animal Husbandry, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia
[2]Research Center for Veterinary Science, National Research and Innovation Agency (BRIN), Jl. Raya Bogor Km. 46 Cibinong, Bogor 16911, West Java, Indonesia
[3]School of Agriculture and Food Sustainability, The University of Queensland, Queensland Australia

*e-mail: alarif_anam@yahoo.com

ABSTRACT

Cellulase enzymes are widely used in the food, beverage, animal feed, textile, and paper industries. The high cost of producing enzymes and low enzyme activity provides opportunities for using chemicals, and efforts are needed to produce cellulase enzymes economically through media optimization. This research aims to find alternative media that make it possible to produce cellulase enzymes with high activity. This research created a control media (M0) using pro-analysis chemicals and CMC as a carbon source. Three types of cellulolytic bacterial growth media were also created, consisting of technical chemicals, rice straw as a substrate, and different protein sources. M1: alternative media using urea as a protein source; M2: alternative media using powdered milk as a protein source; and M3: alternative media using chicken liver as a protein source. A total of 1% of cellulolytic bacteria (*Actinobacillus* sp.) was grown in each media and then incubated at 40°C for 33 hours at a speed of 140 rpm, and the production curve and cellulase enzyme activity were measured at every 3-hour interval. The results showed that the highest cellulase enzyme production was achieved at 24 hours using alternative media with powdered milk (M2) as a protein source. The cellulase enzyme activity produced was 2.9612 µ/ml.

Key Words: Cellulase activity, Cellulase enzymes, Cellulolytic bacteria, Growth media.

INTRODUCTION

Cellulase enzymes are widely used in the textile industry to soften cotton and denim finishing; in the detergent industry for color maintenance, cleaning, and anti-settling; in the food industry to provide a soft texture, soften the fruit, clarify the color of fruit juice, reduce fiber in the dough, hydrolyze roasted coffee, extract tea

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polyphenols and essential oils from olives, and improve the aroma and taste of food; in the pulp and paper industry for deinking and fiber modification; as well as for making beer, alcohol, biofuel, and processing agricultural waste for animal feed and organic fertilizer (Amraini et al., 217; Basak et al., 2021; Korsa et al., 2023).

Although cellulase has been used for more than three decades in the commercial sector, this enzyme remains a topic of interest for both academic and industrial research (*Ejaz et al.,* 2021). Recent trends in the industrial sector tend to favor the use of enzymes as biological molecules (biocatalysts) to replace chemicals that can harm consumers and the environment, causing the demand for enzymes to increase (Robinson, 2015). Its wide application in the textile, animal food, pharmaceutical, detergent, and paper processing industries ranks it second in the global industrial enzymes market by business volume (Patel et al., 2019). The enzyme industry market is around 45% in the food sector (including 11% in starch processing), 34% in detergent, 11% in textiles, 3% in the leather tanning industry, and 1.2% in wood fiber and paper (Demain and Adrio, 2008).

The cellulase enzyme consists of three main components: endoglucanase, exoglucanase, and β-D-glucosidase (Kumar et al., 2019). The endoglucanase enzyme plays a role in breaking the internal bonds of amorphous β-1,4-glycosides in natural cellulose into smaller units to produce cellulose-oligosaccharides (cellulodextrin). Still, endoglucanase is inactive against cellulose crystals (Champreda et al., 2019). Exoglucanase consists of two parts, namely β-1,4-glucan glucanohydrolase, which releases glucose units from the non-reducing pole (β-glucan), and β-1,4-glucan cellobiohydrolase which removes cellobiose units from the non-reducing pole (β-glucan) (Staudenbauer and Schwarz, 2004). The function of exoglucanase is to produce glucose or cellobiose units by attacking the reducing or non-reducing end of the cellulose chain. Exoglucanase is also very active in degrading crystalline cellulose substrates such as avicel or cellooligosaccharides (Chavda et al., 2021).

Cellulolytic bacteria produce cellulase enzymes, which can degrade the substrate in cellulose and convert it into a more straightforward product, namely glucose (Biswas et al., 2020). The synergy between cellulase enzymes is the combined effect of three enzyme elements with endolytic activity (endoglucanase) on the one hand and exoglucanase with exolytic activity on the other hand. The synergistic effect of endo-exo can occur when the endoglucanase enzyme internally initiates the breaking of cellulose chains, producing new reducing and non-reducing ends in cellulose fibers, which are then hydrolyzed by exoglucanase (cellobiohydrolase) to produce cellobiose (Badino et al., 2017). Finally, β-glucosidase hydrolyzes cellobiose to glucose (Chavda et al., 2021).

The availability of cost-effective enzyme substrates is essential so that large-scale production can be carried out. Plant cell walls contain polysaccharides, which can function as a source of energy and carbon that microorganisms can utilize. Therefore, plant cell walls can be a central component in the carbon cycle. Therefore, researchers are interested in the structural characteristics of plant cell wall

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polysaccharides, and the characterization of the enzymes used to degrade them, and the genes encoding them (Jayasekara and Ratnayake, 2019). Cellulose is a polysaccharide consisting of glucose unit monomers linked by β-1,4-glycosidic bonds, and is the most abundant carbohydrate on earth, and the most significant component of plant biomass (Behera et al., 2017; Anggara et al., 2021).

Bacteria require large amounts of nutrient for growth and produce enzymes: there are nitrogen sources (peptides, whey protein, amino acids, casein peptone, soy peptone, tryptone, beef extracts, yeast extracts etc.), carbon sources (glucose, lactose, sucrose, galactose, maltose and soluble starch), minerals and vitamins (Cheng et al., 2019; De Reuse and Skouloubris, 2001; Hayek et al., 2019; Zhang et al., 2020). Among these nutrients, nitrogen sources are the most expensive materials for producing enzymes (Ayivi et al., 2022; Zhang et al., 2022). Nitrogen is required for the synthesis of almost all major cell elements, including amino acids, pyrimidines, purines, and nicotinamide adenine dinucleotide (NAD) (De Reuse and Skouloubris, 2001).

This research aims to produce cellulase enzymes at a low cost. For this reason, an alternative cellulolytic bacterial growth media is needed using readily cheap raw materials in the form of technical chemicals and agricultural waste as a carbon source. The protein source also uses cheap ingredients, like urea, powdered milk, and chicken liver. To determine its effectiveness, a study of cellulase activity is needed by comparing it with pro-analysis (PA) media.

MATERIALS AND METHODS

As much as 300 ml of cellulolytic bacterial growth media was prepared containing CMC as a carbon source. The media consists of $MgSO_4.7H_2O$ 0.05 g/100 ml, $Na_2HPO_4.2H_2O$ 0.5 g/100 ml, NH_2SO_4 0.2 g/100 ml, KH_2PO_4 0.16 g/100 ml, NaCl 0.23 g/100 ml, yeast extract 0.5 g/100 ml, and CMC 2 g/100 ml. The media was added to 300 ml of distilled water, and then fixed in an autoclave at 121^{o}C, 15 psi (pounds per square inch) pressure for 15 minutes. Next, this media was used as the control media (M-0).

Three alternative media were also created with the same volume. The mineral ingredients are made the same, but using technical chemicals. The carbon source (CMC) is replaced with finely ground rice straw and sieved with a 60mesh size, while the nitrogen source (yeast extract) is replaced with urea, powdered milk, and chicken liver. The alternative media is also treated the same as the control media. In total, the research media are as follows:

1. M-0: Control medium, is media that uses pro-analysis materials.
2. M-1: Medium uses technical materials. The nitrogen sources use urea.
3. M-2: Medium using technical materials. The nitrogen sources use powdered milk.
4. M-3: Medium using technical materials. The nitrogen sources use chicken liver.

A total of 1% inoculum of *Actinobacillus* sp. bacteria with a concentration of 1.3 x 10^8 CFU/ml was added to each production

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media. The culture was then incubated for 33 hours at 40°C and shaken at 140 rpm. Cells were harvested by taking 3x1 ml samples every three hours and then centrifuged at 4°C for 15 minutes at a speed of 6000 rpm. The pellet was discarded, while the cellulase enzyme supernatant was taken for use in further analysis.

Cellulase enzyme activity was measured using the DNS (dinitro salicylic acid) method. For this reason, the DNS reagent was prepared by dissolving 1g of DNS in 12.5 ml of 2N NaOH, adding 100 ml of distilled water, and then stirring it using a magnetic stirrer until homogeneous. Then, 100 µl of enzyme was added with 100 µl of 1% CMC substrate (CMC in acetate buffer, pH 7) into an Eppendorf tube, then incubated at 40°C in a water bath for 30 minutes. Next, 600 µl of DNS was added to the solution, soaked in boiling water for 15 minutes, then cooled in an ice bath for 20 minutes.

As a control, 100 µl of enzyme was added with 600 µl of DNS, and then 100 µl of 1% CMC substrate was added. Without an incubation process, the solution was immediately soaked in boiling water for 15 minutes and then cooled in an ice bath for 20 minutes. The absorbance of samples and controls was read using a spectrophotometer at a 540 nm. DNS, or 3,5-dinitrosalicylic acid, is an aromatic compound that reacts with reducing sugar and forms a 3-amino-5-nitrosalicylic acid molecule that absorbs light at a wavelength of 540 nm. The yellow color, which becomes orange-red, indicates the presence of a certain amount of glucose resulting from the activity of the cellulase enzyme. One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol of glucose per minute for each ml of enzyme.

A standard glucose solution was made with 15, 20, 25, 30, 35, 40, 45, and 50 µg/ml concentrations. A total of 1 ml of each standard solution was added to 3 ml of DNS reagent and then homogenized. Next, it was heated in boiling water for 15 minutes until the solution was red-brown, then cooled in an ice bath for 20 minutes. Absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The glucose standard is used to obtain a linear regression equation which can later be used to calculate cellulase enzyme activity. Based on the data obtained, the standard glucose regression equation used is as follows:

$$Y = 0.822\ X - 0.697$$

Cellulase enzyme activity is calculated as follows:

$$Cellulase\ activity = \frac{Y \times P \times 10 \times 1000}{t \times BM\ Glucose}$$

Note: Y = absorbance; P = Dilution; t = Incubation time (30 minutes); BM Glucose = 180

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RESULTS AND DISCUSSION

Optimization of cellulase enzyme production is used to determine the time needed to obtain the optimum amount of enzyme from the production process. Cellulase enzyme production in various growth media is shown in Table 1.

Table 1. Cellulase enzyme production in several growth media

Time (Hour)	Control (M0)	Urea (M1)	Milk (M2)	Liver (M3)
3	0.0131	0.0339	0.6335	0.0209
6	0.0170	0.0228	0.4387	0.0194
9	0.0175	0.0145	0.3427	0.0180
12	0.0194	0.0136	0.3400	0.0075
15	0.0199	0.0143	0.4198	0.0064
18	0.0246	0.0270	0.5288	0.0218
21	0.0468	0.0316	1.8058	0.0414
24	0.0820	0.0514	**1.9318**	0.0596
27	0.1243	0.0690	0.6800	0.0811
30	**0.1410**	**0.1710**	0.5150	**0.0865**
33	0.0736	0.0472	0.2043	0.0594

The cellulase enzyme activity test was carried out using the DNS method, which aims to determine the presence of aldehyde groups in reducing sugars, which are then oxidized to carboxyl. 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions (Valls et al., 2019). The change in the yellow color of 3,5-dinitrosalicylic acid to brownish comes from 3-amino-5-nitrosalicylic acid. The presence of an aldehyde group is indicated by a change in color from yellow to brownish.

Cellulase enzyme activity determines the best media for producing enzymes and the optimum time for making enzymes. The activity of the cellulase enzyme in various growth media is shown in Table 2 where it can be seen that the cellulase enzyme activity produced in the control media, and alternative media using urea and liver in the same period of time had almost the same activity. Urea is source of Nitrogen Non Protein (NPN). Bacteria can hydrolysis urea into ammonia (NH3) and then use it for microbial protein syntesis. Bacteria replicate and produce enzymes (Mahmoudi-Abyane et al., 2020; Hailemariam et al., 2021). Chicken liver is is excellent source of important nutrient for microbial growth, like peptone, protein, and essential amino acid as nitrogen source, vitamins (A, B, B1, B3, B5, and B6), and minerals (Fe, Cu, Mn, and Zn) (Dourou et al., 2021; Nakamura et al., 2021). Bacteria can use a variety of alternative nitrogen sources, as well as a nitrogen source in the form of yeast extract in control media.

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Table 2. Cellulase enzyme activity in several growth media (µ/ml)

Time (Hour)	Control (M0)	Urea (M1)	Milk (M2)	Liver (M3)
3	0.7999	0.8233	1.4987	0.8087
6	0.8043	0.8108	1.2793	0.8069
9	0.8048	0.8014	1.1711	0.8054
12	0.8070	0.8004	1.1681	0.7936
15	0.8075	0.8012	1.2580	0.7923
18	0.8128	0.8155	1.3808	0.8097
21	0.8378	0.8207	2.8192	0.8318
24	0.8775	0.8431	**2.9612**	0.8523
27	0.9251	0.8629	1.5511	0.8764
30	**0.9439**	**0.9777**	1.3652	**0.8826**
33	0.8680	0.8382	1.0152	0.8521

Figure 1 shows the activity of the cellulase enzyme in the control media and indicates that, when using the control media, the highest cellulase activity was achieved at 30 hours of incubation with an activity of 0.9439.

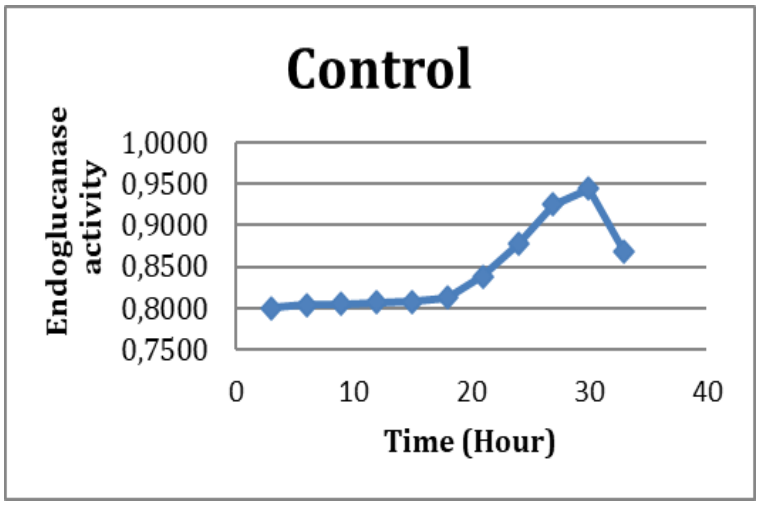

Figure 1. Cellulase enzyme activity curve in control media

Cellulase enzyme activity in media that uses urea as a protein source is shown in Figure 2 below. Figure 2 shows that, when using media containing urea, the highest cellulase activity was achieved at 30 hours of incubation, as was the case when using control media with an activity of 0.9777, which is almost equivalent to control media. The decomposition of urea into NH3 occurs rapidly (Mahmoudi-Abyane et al., 2020). This shows that urea can be used as an alternative protein source to replace yeast extract.

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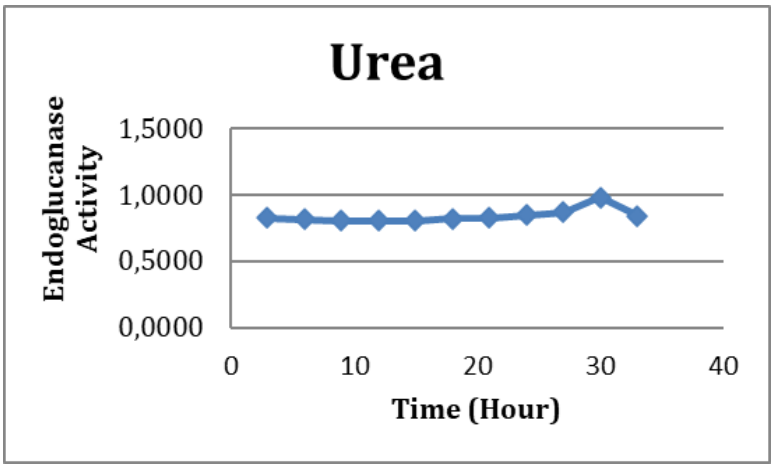

Figure 2. Cellulase enzyme activity curve in urea media

Cellulase enzyme activity in media that uses powdered milk as a protein source is shown in Figure 3 which shows that when medium containing powdered milk is used, the highest cellulase activity is achieved at 24 hours of incubation, with an activity of 2.9612, which is much higher than that of the control medium and requires a shorter time.

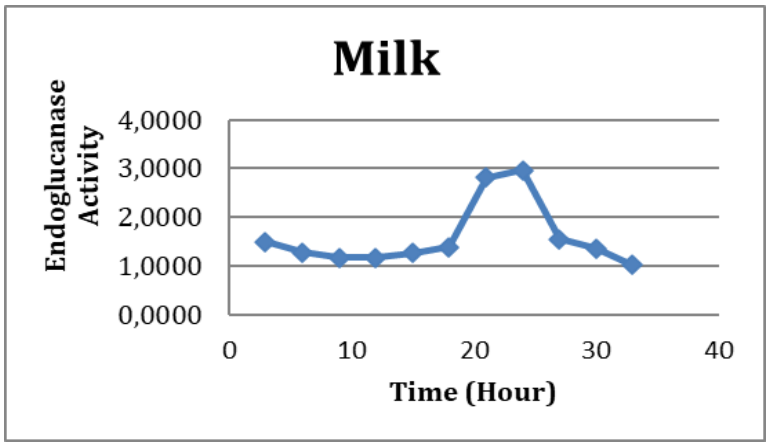

Figure 3. Cellulase enzyme activity curve in powdered milk media

Cellulase enzyme activity in media using chicken liver as a protein source is shown in Figure 4 below indicating that when using media containing chicken liver, the highest cellulase activity was achieved at 30 hours of incubation, as was the case when using control media with an activity of 0.8826, or almost equivalent to control media. Chicken liver contains various nutrients, for example essential amino

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acids, vitamins and minerals that bacteria need to grow and proliferation (Nakamura et al., 2021). Bacterial proliferation will be followed by enzyme production. The more bacteria there are, the more enzymes are produced. This causes bacteria grown on media containing chicken liver to have enzyme activity as good as bacteria grown on control media.

Of the three alternative media for producing cellulase enzymes, the highest activity results were obtained when using powdered milk as a nitrogen source; besides that, the time required was also the shortest, namely 24 hours. When using powdered milk, cellulase activity was high from the start and increased significantly at the 21st hour, peaking at the 24th hour, after which it decreased, although at the 33rd hour, it was still relatively high compared to other media. Powdered milk has a high protein content, around 41% (Dey et al., 2023). Powdered milk also contains lots of vitamins A, B1, B2, B3, C, and minerals (Mourad et al., 2014). These nutrients are needed by bacteria to grow and reproduce.

Media using urea and liver had almost the same cellulase activity as control media. This shows that cellulase enzyme production can be done at a lower cost using alternative media. The use of this alternative media has been proven to produce enzyme activity equivalent to enzyme production using more expensive pro-analysis (PA) media.

Elisashvili et al. (2008) found several fungi that have the potential to produce cellulase, xylanase, laxase, and manganese peroxidase enzymes. The amount of enzyme produced and the ratio between the four enzymes are influenced by the fungal species, lignocellulosic substrate, and cultivation method (Kumla et al., 2020). *Pleurotus ostreatus* grown on media containing wheat straw or leaf substrate will experience an increase in endoglucanase and xylanase enzyme activity if peptone or NH_4NO_3 is added to the media as a protein source (De Mastro et al., 2023).

Misra et al. (2007) stated that among the four *Phanerochaete chrysosporium* strains, there were two strains (NCIM 1073 and MTCC 787) that had the highest cellulase activity, namely endoglucanase (0.36-0.42 IU/ml), exoglucanase (0.55-0.88 IU/ml), and cellobiase (1.07-1.18 IU/ml) after the fermentation process in rice straw for 15 days. *Cellulomonas cellulans* MTCC 23 bacteria showed the highest exoglucanase (0.72 IU/ml) and cellobiase (1.05 IU/ml) activities, while the highest endoglucanase (0.38 IU/ml) came from *Cellulomonas uda.*

Kashem et al. (2004) reported endoglucanase activity (13.78-82.68 µg/ml) in *Cellulomonas cellusea* using several different carbon sources. The highest endoglucanase activity was achieved when using galactose, while the highest exoglucanase and cellobiase were achieved when using lactose as a carbon source.

CONCLUSION

The highest enzyme production was achieved in alternative media using powdered milk (M2) as a protein source. The cellulase enzyme produced by the bacteria *Actinobacillus* sp. using alternative media with powdered milk as a protein source had the highest activity of 2.9612

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μ/ml at the 24th hour of culturing, after which the activity decreased.

ETHICS APPROVAL

This study did not use experimental animals so ethical clearance was not required.

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