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Research article

ACTIVITIES OF CMC-ASE AND β-GLUCOSIDASE IN CELLULOLYTIC BACTERIA FROM BUFFALO RUMEN GROWN ON CARBOXYMETHYL CELLULOSE

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Abstract

Cellulase is an enzyme widely used in the feed and food sectors. This study aims to examine the CMC- ase and β- glucosidase activity of cellulolytic bacteria in degrading cellulose content in CMC substrate and determine the optimum incubation period to produce cellulase enzymes from buffalo rumen bacteria. This study used a factorial Completely Randomized Design (CRD) consisting of 2 factors and 4 replications. The first factor is the type of bacterial isolate consisting of 2 types, namely ST6 and ST8. The second factor is the difference in the incubation period consisting of 5 treatments (T0: 0 days, T1: 3 days, T2: 6 days, T3: 9 days, and T4: 12 days) on the use of 1% CMC (Carboxy Methyl Cellulose) substrate. The data were analyzed using variance (ANOVA) and further tested using Duncan's Multiple Range Test (DMRT). The results of data analysis showed that CMC-ase activity was influenced by the incubation period with the best incubation period at 9 days (T3), and there was a significant interaction between the type of isolate and the incubation period. The activity of the β- glucosidase was influenced by the type of isolate and the incubation period, with the best results at an incubation period of 6 days. The interaction between the isolate type and the incubation period significantly affected the activity of the β- glucosidase.

1. Introduction

Cellulases are key enzymes with widespread applications in various industries, including animal feed, agriculture, paper and pulp, winemaking and brewing, food processing, olive oil extraction, carotenoid extraction, pharmaceuticals, and pollution treatment (Artono et al., 2023; Mrudula et al.,

2011; Retnoningtyas et al., 2013). Their significance extends to the bio-conversion of agricultural waste into valuable products like fermentable sugars and bioethanol (Al-baarri et al., 2018; Damayanti et al., 2020; Paramita et al., 2021). Cellulase enzymes hydrolyze cellulose by breaking the β-1,4 glycosidic bonds in cellulose, cellodextrins, cellobiose, and other cellulose derivatives, converting them into simple sugars or glucose (Mrudula et al., 2011; Wibawanti et al., 2021). Typically, cellulase can be derived from various microorganisms, but bacteria are particularly advantageous due to their rapid growth, which shortens production time (Alam et al., 2004).

Among cellulolytic microorganisms, cellulolytic bacteria hold notable promise for efficient cellulose degradation in fields such as waste reduction, biofuel production, and sustainable agriculture. These bacteria, capable of breaking down cellulose complexes into oligosaccharides and glucose can be sourced from the rumen fluid of ruminant livestock, including buffalo (Gharechahi et al., 2023). Cellulolytic bacteria are more easily isolated from the digestive tract of livestock because the digestive system of animals, such as ruminants, supports cellulose fermentation by microbes in the rumen (Kong et al., 2012). Cellulose is broken down into sugars, which are fermented into short-chain fatty acids (SCFA) as an energy source. In contrast, humans cannot directly digest cellulose, which is only fermented by microbes in the large intestine in smaller amounts, making the isolation of cellulolytic bacteria in humans more difficult (Kamil et al., 2021). Buffalo rumen fluid is preferred due to its higher cellulolytic bacterial content and greater cellulolytic activity than cow rumen fluid, as Pradhan (1994) reported; buffalo rumen fluid contains 6.86 x 10^8 CFU/ml cellulolytic bacteria compared to 2.58 x 10^8 CFU/ml in cow rumen fluid. A cellulose-rich environment, coupled with higher bacterial populations, drives more efficient enzyme production, potentially enhancing the effectiveness of cellulase-based technologies (Borthakur et al., 2024)

The production of cellulase enzymes often employs media containing Carboxymethyl Cellulose (CMC) as a substrate, which serves as a primary carbon and energy source without containing reducing sugars or lignin, thereby enhancing enzymatic activity (Doan et al., 2024). Enzyme activity is also influenced by factors like the length of incubation, where optimal incubation periods can maximize hydrolysis efficiency (Meilany et al., 2020). However, studies exploring the conditions for β-glucosidase activity in cellulolytic bacteria from buffalo rumen are limited (Borthakur et al., 2024b; Murtius et al., 2022; Wonoputri et al., 2018). Consequently, optimizing the incubation period for cellulase activity in these bacteria could reveal new opportunities for enhancing cellulose degradation. By advancing the practical applications of cellulases in biofuel production and sustainable waste management, this study could provide valuable insights for developing more efficient, cellulose-based biotechnologies.

2. Materials and methods

2.1.Tools and materials

The tools used in this study include thermoses, gauze, cloth, CO_2 tubes, test tubes, erlenmeyer flasks, test tube racks, butyl rubber, cotton, petri dishes, pH meters, vortex, magnetic stirrers , analytical scales, spatulas, stirring rods, aluminum foil, plastic wrap , bunsen, ose needles, microtubes, anaerobic jars, centrifuges, water bath, incubator, autoclave, scissors, UV-Vis spectrophotometer, measuring cylinder, micropipette, and measuring pipette.

The materials used in this study included cellulolytic bacterial isolates ST6 and ST8, which were isolated from buffalo rumen fluid in Kudus, Central Java, Indonesia. Selection of bacterial isolates based on the highest CMC-ase activity of the 8 isolates that were successfully isolated. Other materials are aquabidest, aquades, alcohol, Nutrient Broth (NB), Carboxyl Methyl Cellulose (CMC), agar, $NaNO_3$, K_2HPO_4, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$, $CaCl_2.2H_2O$, *2*-Ortho-Nitrophenyl-β-D-glucopyranoside (NPG), 3,5-dinitrosalicylic acid (DNS), glucose, NaOH, DNS reagent, and standard glucose solution.

2.2.Method

The research design used in this study was a factorial, Completely Randomized Design (CRD) consisting of 2 factors and 4 replications. The first factor is the type of isolate, consist of 2 types, namely ST6 and ST8. The second factor is the difference in incubation time consisting of 5 treatments on the use of 1% CMC substrate, namely:

T_0 : zero day incubation
T_1 : three days incubation
T_2 : six days incubation
T_3 : nine days incubation
T_4 : twelve days incubation

2.3.Research Procedures

2.3.1.CMC fermentation with different incubation times

The materials used in the manufacture of liquid treatment media are $NaNO_3$ 0.75 g, K_2HPO_4 0.1875 g, $MgSO_4.7H_2O$ 0.0075 g, $MnSO_4.7H_2O$ 0.0075 g, $FeSO_4.7H_2O$ 0.0075 g, $CaCl_2.2H_2O$ 1.875 g, aquabidest 375 ml, CMC 3.75 g which are divided into 24 test tubes. First, the weighed materials are mixed until homogeneous then measured and adjusted the pH until the pH value becomes neutral. The homogeneous media is then put into a tube as much as 15 ml in each test tube that already contains 0.15 g of CMC. After that, the media is sterilized using an autoclave at a temperature of 121^0 C with a pressure of 1 atm for 20 minutes. The next step is when the media is warm, isolates ST6 and ST8 are inoculated as much as 0.1 ml into a test tube, each inoculated isolate is repeated four times. After that, all the tubes are flowed with CO_2 and then tightly closed so that the conditions in the tube are anaerobic. Furthermore, all the tubes that have been tightly closed are stored in an incubator at a temperature of 39° C for 0, 3, 6, 9 and 12 days. After the incubation process is complete, then harvesting is carried out by cold centrifugation at a speed of 3000 g for 20 - 30 minutes. The filtrate obtained (as a crude enzyme extract) is put into a 1.5 ml microtube and stored in the freezer for enzyme activity testing.

2.3.2.DNS reagent preparation

The DNS reagent was made by weighing 1 gram of 3,5- dinitrosalicylic acid (DNS) which was then dissolved in 20 ml of 2 N NaOH solution with 50 ml of distilled water, then 30 grams of K-Na tartrate were added and homogenized using a magnetic stirrer . Finally, distilled water was added until the final volume of the solution was 100 ml.

2.3.3.Preparation of glucose standard curve

A standard curve was made to determine the concentration of reducing sugar by making a glucose stock solution, namely 1 gram of glucose dissolved in 100 ml of aquabidest, which means that 1 ml of stock solution contains 10 mg of glucose. Then a dilution series was made with concentrations of 0, 50, 100, 150, 200, 250, and 300 ppm by inserting 0.1 ml of 1 mg/ml glucose solution into a test tube and then adding 1.9 ml of aquades as listed in Table 1. Next, 2 ml of DNS solution (3.5- Dinitro salicylic acid) was added and homogenized.

Table 1. Concentration of Glucose Solution in Making Glucose Standards

Final Concentration		Aquadest (ml)	Standard stock solution of glucose added (ml)
ppm	mg/ml		
0	0.00	2.00	0.00
50	0.05	1.90	0.10
100	0.10	1.80	0.20
150	0.15	1.70	0.30
200	0.20	1.60	0.40
250	0.25	1.50	0.50
300	0.30	1.40	0.60

The resulting mixture solution was then incubated at 100° C for 15 minutes. After the incubation process was complete, it was cooled and the absorbance was measured using a spectrophotometer with a wavelength of 540 nm. The standard curve was made 3 times. The absorbance obtained from the measurement results was processed using Microsoft Excel with the absorbance value as the x-axis and the concentration value as the y-axis, until the reaction equation and regression were obtained.

2.3.4.CMC-ase activity test

Cellulase enzyme measurement was carried out by measuring the reducing sugar content of 3 groups of test tubes consisting of samples, controls, and blanks, the method of analysis referred to Yogyaswari (Yogyaswari et al., 2016) with modification. In the sample, 1 ml of crude extract enzyme was added with 1 ml of 1% CMC solution, then homogenized using a vortex and incubated at room temperature for 60 minutes. A CMC level of 1% was used because it is the best for testing CMC-ase activity and is in accordance with the reference.

After that, 2 ml of DNS and incubated in a water bath at 100° C for 10 minutes and cooled. In control, 1 ml of 1% CMC solution was added with 2 ml of DNS and added with 1 ml of crude extract enzyme, then vortexed and incubated in a water bath at a temperature of 100° C for 10 minutes. Control tubes contain substrate and enzyme but are immediately incubated at a temperature of 100° C to stop the enzyme reaction.

In the blank, 1 ml of 1% CMC solution was added with 2 ml of DNS and 1 ml of aquadest, then vortexed and incubated in a water bath at 100° C for 10 minutes. After cooling, the absorbance was measured in the three tubes using a spectrophotometer with a wavelength of 540 nm. The concentration of reducing sugar can be generated using the formula (Sample Absorbance – Blank Absorbance) – (Control Absorbance – Blank Absorbance) (Wijanarka et al ., 2016). The determination of cellulase activity value can be calculated using the following equation:

Cellulase activity (U/ml) = reducing sugar concentration x DF x 1000MW of flucose x t (1)

Information :
DF: Enzyme dilution factor
MW of glucose : Molecular weight of glucose (180.18 g/mol)
t : Incubation time

Table 2. Concentration of NPG Solution in Making Standard NPG Solution Graphs

Solution Concentration		H_2O Sterile (ml)	NPG Standard Stock Solution (ml)
µg/ml	mg/ml		
0	0,000	5	0.00
20	0.02	4.9	0.10
40	0.04	4.8	0.20
60	0.06	4.7	0.30
80	0.08	4.6	0.40
100	0.1	4.5	0.50
120	0.12	4.4	0.60
140	0.14	4.3	0.70
160	0.16	4.2	0.80
180	0.18	4.1	0.90
200	0.20	4.0	1.00

2.3.5.Making standard graph of NPG solution

The standard graph of NPG solution was made using a stock solution of 2-Ortho-Nitrophenyl-β-D-glucopyranoside (NPG). First, a solution with a concentration of 1 mg/ml was made by dissolving 10 mg of NPG into 10 ml of H_2O . Then a dilution series was made with concentrations of 0, 20, 40, 60, 80, 100, 120,

140, 160, 180 and 200 µg/ml by adding 0.1 ml of NPG solution with a concentration of 1 mg/ml and adding 4.9 ml of sterile H_2O as listed in Table 2.

2.3.6.β-Glucosidase enzyme activity test

The materials used in the β-glucosidase enzyme activity test include: 0.1 M acetate buffer solution at pH 4.8 (200 ml of 0.2 M acetic acid solution plus 300 ml of 0.1 M sodium acetate solution, then dissolved to 1000 ml of H_2O), glycine NaOH solution at pH 10.5 (1.675 g of glycine plus 0.91 g of NaOH then dissolved to 500 ml of H_2O), NPG solution (1 mg of 2-ortho-nitrophenyl-β-D-glucopyranoside dissolved with 1 ml of H_2O). All tubes were incubated at 37° C for 60 minutes. Furthermore, the enzymatic reaction was stopped by adding 4 ml of glycine NaOH pH 10.6. Then, a standard graph is made using an ortho-nitrophenol solution with a concentration of 0 - 200 µg/ml in a sample of 2 ml and read on a spectrophotometer with a wavelength of 425 nm. The calculation of enzyme activity (U / ml) can be calculated using the formula:

Enzyme Activity (U/ml) =[C x 10 x DF]T x BM o-nitrofenol (2)

Information :
C: Concentration of *o* -nitrophenol
T : Incubation time
DF : Dilution factor
BM *o* -nitrophenol : 139.11

3. Results and discussions

Tables 4 and 5 present the CMC-ase and β-glucosidase activity tests of ST6 and ST8 isolates isolated from buffalo rumen fluid and grown on CMC substrate.

Table 4. Effect of Incubation Time on CMC- ase Activity Produced by ST 6 and ST 8 Bacterial Isolates (U/ml)

Incubation Period	Isolate Type	
	ST 6	ST 8
T0	0.4644±0.2754 ^{p}	0.7516±0.1075 ^{qr}
T1	0.4849±0.0460 ^{p}	0.8840±0.3169 ^{r}
T2	0.7758±0.0717 ^{qr}	0.5707±0.0714 ^{pq}
T3	1.9153±0.2033 ^{t}	1.6057±0.1376 ^{s}
T4	0.8112±0.0510 ^{qr}	0.7068±0.0485 ^{pqr}

Note: Different superscript ^{pqrst} indicates significant difference ($p<0.05$). T0: 0 days incubation, T1: 3 days incubation, T2: 6 days incubation, T3: 9 days incubation, T4: 12 days incubation.

The results in Table 4 show that the incubation period significantly affects CMC-ase activity for both bacterial isolates, with peak activity observed at 9 days (T3). Specifically, isolate ST6 reached the highest CMC-ase activity at 1.9153 U/ml, and ST8 at 1.6057 U/ml, indicating that 9 days is the optimal incubation period for maximizing enzyme production. This suggests that adjusting incubation time can enhance cellulase yield, which is valuable for industrial applications where enzyme efficiency is crucial. Lower activities at other incubation times highlight the importance of time optimization in achieving effective cellulolytic activity.

Analysis of variance showed no significant effect from different types of isolates. This is thought to be because both isolates produce enzymes with relatively the same ability because they are grown on the same substrate, namely CMC, which has a pure cellulose content. According to Damayanti et al., (2020) and Thielemans et al., (2023) Cellulose hydrolysis can take place very slowly because cellulose has a dense structure in the crystalline part, which is difficult to degrade so both isolates cannot work optimally on the substrate.

The difference in incubation time significantly affects the activity of the CMC-ase, presumably because CMC has fine particles. Torrado et al., (2014) stated that smaller particle sizes will increase glucose from cellulose hydrolysis. According to Yeh et al. (2010), based on kinetic studies, the hydrolysis

of cellulose into glucose runs faster and produces more glucose at smaller particle sizes than larger particle sizes. Hidayat, (2013) also stated that the smaller the particle size, the more efficient the hydrolysis process will be because the enzyme is more easily in contact with the substrate. Differences in incubation time also provide different opportunities for bacteria to grow, produce enzymes and hydrolyze substrates. Increasing enzyme concentration will increase its activity (Murtius et al., 2022).

There is a significant interaction between the type of isolate and the incubation period. The interaction between the type of bacterial isolate and the incubation period is thought to occur because, at the specified incubation time, the two bacterial isolates have different interaction rates with the substrate. Wang et al., (2022) stated that incubation is the time required for the enzyme to bind to the substrate; a short incubation time will result in low enzyme activity because the time to interact is short, which results in the interaction not taking place as a whole so that the resulting product becomes small.

Enzyme activity in ST6 bacterial isolates showed an increasing trend in activity and reached the highest activity on day 9 (T3). The increase in cellulase activity indicates that ST6 bacterial isolates can degrade cellulose contained in the CMC substrate to produce glucose, which is used for cell metabolism along with the length of incubation. In contrast to ST6 isolates, ST8 isolates experienced a decrease in enzyme activity on 6 days (T2) and were the lowest enzyme activity values and increased again on 9 days of incubation. The low activity is thought to be because the enzyme used is a crude extract enzyme, so it is possible that it still contains other components or other proteins that can interfere with the work of the enzyme so that the enzyme cannot work optimally and cause low enzyme activity values (Plaxton, 2019;Hardoko et al., 2019).

The highest enzyme activity in isolates ST6 and ST8 occurred at 9 days of incubation, namely 1.9153 U/ml and 1.6057 U/ml. Then, there was a decrease in cellulase enzyme activity in both isolates on the 12th day (P4). This is thought to be due to the reduction in nutrients available in the fermentation medium, so the rate of bacterial cell division is the same as cell death. According to Laila et al. (2019) and Yudiati et al., (2020), metabolite production, including enzymes, tends to decrease because the source of nutrients for cell growth has begun to decrease.

Table 5. Effect of Incubation Time on the Activity of β- glucosidase Produced by Bacterial Isolates ST 6 and ST 8 (U/ml)

Incubation Period	Isolate Type	
	ST 6	ST 8
T0	0.0279±0.0058 ^{rs}	0.0038±0.0117 ^{pq}
T1	0.0013 ±0.0069 ^{p}	0.0123±0.0073 ^{pq}
T2	0.0429±0.0 094 ^{s}	0.0402±0.0 157 ^{s}
T3	0.0417±0.0119 ^{s}	0.0211±0.0122 $^{q r}$
T4	0.0284±0.0 175 ^{rs}	0.0201±0.0 077 $^{q r}$

Note: Different superscript ^{pqrs} indicate significant differences ($p<0.05$). T0: 0 days incubation, T1: 3 days incubation, T2: 6 days incubation, T3: 9 days incubation, T4: 12 days incubation

In Table 5, the β-glucosidase activity varies with incubation time for both bacterial isolates, with the highest activity observed at 6 days (T2). Specifically, ST6 shows a β-glucosidase activity of 0.0429 U/ml, while ST8 shows 0.0402 U/ml at this incubation period, indicating that 6 days is optimal for maximizing β-glucosidase activity in both isolates. Lower activity at other time points highlights the critical role of incubation period optimization for effective enzyme production. These findings suggest that a carefully controlled incubation period can enhance enzyme output, benefiting industrial processes dependent on efficient cellulose

breakdown.The results of the variance analysis showed that the type of bacterial isolate had a significant effect on the activity of the β-glucosidase. This is thought to be because the types of bacteria found in the ST6 and ST8 bacterial isolates are different. Each type of bacteria has different abilities in degrading cellulose, the cellulase enzyme complex produced by cellulolytic bacteria varies depending on their genes and the substrates used (Ma et al., 2024).The difference in incubation time has a significant effect on the activity of the β-glucosidase, presumably because at the specified incubation time, the cellulase enzyme produced by cellulolytic bacteria has the opportunity to interact with the substrate at different times, thus producing products in different amounts. Metabolite, such as enzyme production, will increase with increasing incubation time because the cellulase enzyme has enough time to interact with the substrate and produce glucose. The small size of the CMC substrate also facilitates the interaction between the enzyme and the substrate. Yeh et al., (2010) found that small particle sizes increase the rate of cellobiose production 11.8 times compared to large particle sizes. Torrado et al. (2014); Yeh et al., (2010) Also explained that the smaller the particle size, up to the optimum limit, the surface area becomes more expansive, and the porosity becomes smaller, making it easier for reactions to occur.The interaction between the type of bacterial isolate and the incubation period also significantly affects the activity of theβ glucosidase. This shows that the difference influences the isolate used in incubation time in enzyme production. The two bacterial isolates are suspected to have different responses in degrading cellobiose into glucose at a predetermined incubation time.β-glucosidase activity showed that both isolates had very low cellulase activity on 0 day (T0) and 3 days 3 (T1) incubation. After that, it increased and reached the highest activity value on the 6th day of fermentation (T2). The increase in cellulase activity was balanced by factors such as the nature of microorganisms to the environment, nutrient content, temperature, pH, incubation time and substrate concentration (Laila et al., 2019). In addition, it was also caused by microorganisms in the sample that could degrade the substrate optimally and use cellulose as their main nutrient.The addition of incubation time to 12 days caused a decrease in the activity of the β- glucosidase. Razie et al., (2011) stated that the decrease in the activity of the β- glucosidase with the addition of incubation time after reaching maximum activity is likely caused by reduced available nutrients, accumulation of harmful substances from products in the fermentation medium and enzyme proteolysis. This is also in line with the opinion of Ikram-ul-Haq et al. (2006) who said that the decrease in enzyme activity with increasing fermentation time after reaching the highest level of activity can be caused by the depletion of micronutrients and macronutrients in the fermentation medium, in line with the ongoing fermentation process, thus suppressing the physiology of bacteria which causes inactivation of enzyme-producing cell organelles.

4. Conclusions

Based on the research that has been conducted, it can be concluded that the CMC ase activity of 2 isolates of buffalo rumen cellulolytic bacteria is influenced by the incubation period and the interaction between the type of isolate and the incubation period with the best incubation period at 9 days, while the β-glucosidase activity is influenced by the type of isolate and the incubation period as well as the interaction between the type of isolate and the incubation period with the best results at an incubation period of 6 days. Bactericellulolytic isolates from buffalo rumen can be utilized in the degradation process of feedstuffs and cellulose foods with an incubation period of 9 days. The potential use of cellulase enzymes in industries reliant on cellulose breakdown highlights their importance for advancing sustainable biotechnological applications.

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