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CMC-ase and β-Glucosidase activity of buffalo rumen cellulolytic bacteria isolates cultivated on a rice straw substrate at different incubation periods

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ABSTRACT

This study aimed to evaluate the activities of CMC-ase and β-glucosidase in buffalo rumen bacterial isolates cultured on a rice straw substrate at different incubation periods. The experiment followed a Completely Randomized Design with a 2 × 5 factorial pattern. The first factor was the bacterial isolate type (ST6 and ST8), while the second factor was the incubation period with five levels: T0 (0 days), T1 (3 days), T2 (6 days), T3 (9 days), and T4 (12 days). Each treatment was replicated four times. The observed variables were CMC-ase and β-glucosidase activities. Data were analyzed using one-way ANOVA, followed by Duncan's Multiple Range Test (DMRT). The results showed that the interaction between isolate type and incubation period had no significant effect on CMC-ase activity ($P > 0.05$). Neither factor independently influenced CMC-ase activity ($P > 0.05$), with an average enzyme activity of 0.78122±0.238 U/mL. Similarly, the interaction effect on β-glucosidase activity was not significant ($P > 0.05$). The type of isolate did not affect beta glucosidase activity, but differences in incubation time affected beta glucosidase activity with the best results at 6 days of incubation (0.030221±0.008 U/mL). In conclusion, the CMC-ase activity of buffalo rumen cellulolytic bacterial isolates cultured on a rice straw substrate is not affected by isolate type or incubation period, whereas β-glucosidase activity is influenced by incubation time.

Introduction

Rice straw is an abundant agricultural waste and presents a global challenge in lignocellulosic biomass conversion, particularly in supporting sustainable agriculture and agricultural waste management. Utilizing rice straw as ruminant feed is one approach to addressing this challenge, as ruminants can digest this material with the aid of cellulase enzymes produced by rumen microbes. These enzymes break down cellulose into glucose, cellobiose, and cello-oligosaccharides. The enzymatic digestion of cellulose by cellulase involves a sequential process with three enzyme types. First, endo-1,4-β-glucanase (CMC-ase) cleaves the internal bonds of the cellulose crystal structure, opening the polysaccharide chain. Second, exo-1,4-β-glucanase hydrolyzes cellulose from the non-reducing end, producing cellobiose. Finally, β-D-glucosidase breaks down cellobiose, generated by exo-1,4-β-glucanase activity, into glucose or monosaccharides (Arifin *et al*., 2019). Together, these three cellulase enzymes facilitate the complete breakdown of cellulose into glucose, the final product of cellulose digestion in ruminants.

Buffalo rumen harbors a larger population of cellulolytic bacteria compared to other ruminants, making buffalo rumen contents a preferred choice for processing fibrous feed before being fed to livestock. Buffalo rumen fluid contains a higher concentration of cellulolytic bacteria (3.3×10^9 CFU/mL) than cow rumen fluid (2.7×10^8 CFU/mL) (Wanapat *et al*., 2013). Studies on the use of buffalo rumen contents for the pre-treatment of fibrous feed have been widely reported (Khejornsart and Wanapat, 2011; Natsir *et al*., 2020; Khejornsart *et al*., 2021). However, most research has focused on feed digestibility rather than elucidating the cellulase mechanism in improving feed digestibility. Understanding the enzymatic activity of cellulase in breaking down fibrous feed is essential for developing effective feed processing strategies before feeding livestock. Therefore, this study aimed to evaluate cellulase activity during the fermentation of rice straw using buffalo rumen fluid.

Materials and methods

Materials

The materials used in this study included buffalo rumen fluid, rice straw, and various chemicals for sample preparation and analysis. Rice straw was selected due to its complex cellulose structure, making it a representative fiber source for ruminant feed. Rumen fluid was collected from adult buffalos immediately after slaughter. The fluid was placed in a pre-prepared container and transported directly to the laboratory. Rumen bacteria are grown on media containing CMC (carboxymethyl cellulose) substrate to ensure that cellulolytic bacteria grow. The bacteria that grow are then regrown using media with a cellulose substrate in the form of rice straw. The rice straw used in this study was sourced from the surrounding district of the research site. Before fermentation, the rice straw was chopped into small pieces (3–5 cm in length) and oven-dried at 55°C for three days. The dried sample was ground using a blender and passed through a 65-mesh sieve (Chasanah *et al*., 2013). The chemicals used in this study included bidistilled water and distilled water, alcohol, spiritus, tissues, wipes, cotton, Nutrient Broth, carboxymethyl cellulose (CMC), agarose, $NaNO_3$, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, glucose, 3,5-dinitrosalicylic acid (DNS), DNS reagent, potassium sodium tartrate, and 2-Ortho-Nitrophenyl-β-D-Glucopyranoside (NPG). These chemicals were used for sample preparation, incubation, and chemical analysis.

Methods

This study was conducted using a Factorial Completely Randomized Design (FCRD) with two factors and four replications. The first factor was the bacterial isolate type (ST6 and ST8). The second factor was the incubation period, consisting of five time points: T0 (0 days), T1 (3 days), T2 (6

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days), T3 (9 days), and T4 (12 days). These incubation periods were selected based on preliminary studies indicating a gradual decline in enzyme activity after 12 days of incubation. The variables observed in this study were CMC-ase and β-glucosidase activities.

Rice straw fermentation

The bacterial isolates obtained from previous isolation were cultured in liquid media according to Jutono *et al.* (1980). All chemicals were dissolved until homogeneous, and the pH was adjusted to 7. Rice straw was portioned into 0.15 g per tube, followed by the addition of 15 mL liquid media. The tubes containing the rice straw substrate and liquid media were sterilized in an autoclave at 121°C for 20 minutes at 1 atm pressure. Starter inoculation was performed while the medium was still warm by adding 0.1 mL of inoculant to each tube. The inoculated tubes were flushed with CO_2 and tightly sealed with butyl rubber stoppers. The tubes were incubated at 39°C for 0, 3, 6, and 9 days, depending on the treatment group. At the designated incubation period, enzyme extraction was conducted by cold centrifugation at 3,000 g for 20–30 minutes. The resulting filtrate was transferred into 1.5 mL microtubes and stored in a freezer for further analysis.

DNS reagent preparation and glucose standard curve

The DNS reagent was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid (DNS) in 20 mL of 2 N NaOH solution and 50 mL of distilled water. Next, 30 g of potassium sodium tartrate (K-Na tartrate) was added, and the solution was stirred using a magnetic stirrer until homogeneous. The final volume was adjusted to 100 mL with distilled water (Azizah, 2017). A glucose standard curve was created to determine the concentration of reduced glucose levels. A glucose stock solution was prepared by dissolving 1 g of glucose in 100 mL of sterile H_2O, resulting in a concentration of 10 mg/mL. Serial dilutions of glucose were prepared at concentrations of 0 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, and 300 ppm. One milliliter of each glucose solution was transferred into separate test tubes, as listed in Table 1 (Murtiyaningsih and Hazmi, 2017). One milliliter of DNS reagent was added to each test tube and mixed until homogeneous. The mixture was then incubated at 100°C for 15 minutes. After cooling, absorbance measurements were taken at a wavelength of 540 nm. The standard curve was created in triplicate. The absorbance values obtained were processed using Microsoft Excel, with absorbance plotted on the x-axis and glucose concentration on the y-axis, generating the regression equation.

Table 1. Concentration of Glucose Solutions.

Concentration		Distilled water (mL)	Glucose Stock Solution (mL)
ppm	mg/mL		
0	0	2	0
50	0.05	1.9	0.1
100	0.1	1.8	0.2
150	0.15	1.7	0.3
200	0.2	1.6	0.4
250	0.25	1.5	0.5
300	0.3	1.4	0.6

CMC-ase activity measurement

Cellulase activity was measured based on the concentration of reducing sugars using three groups of test tubes: samples, controls, and blanks. For the sample group, 1 mL of enzyme solution was added to 1 mL of 1% CMC solution, mixed until homogeneous, and incubated at room temperature for 60 minutes. After incubation, 2 mL of DNS reagent was added, and the mixture was incubated in a 100°C water bath for 10 minutes, followed by cooling. For the control group, 1 mL of 1% CMC solution was mixed with 2 mL of DNS reagent, followed by the addition of 1 mL of enzyme solution. The mixture was homogenized and incubated in a 100°C water bath for 10 minutes. For the blank group, 1 mL of 1% CMC solution was combined with 2 mL of DNS reagent and 1 mL of distilled water. The mixture was homogenized and incubated at 100°C for 10 minutes. After cooling, the absorbance of all three groups was measured at a wavelength of 540 nm. The CMC-ase activity was calculated using the following equation:

Cellulase activity (U/mL) = (µg glucose x DF x1000)/(V x BM glucose x t)

where:

µg glucose: reduced sugar concentration (mg/mL)

DF: dilution factor

BM glucose: molecular weight of glucose (180.18 mg/mL)

1000: conversion from mmol to µmol

V: enzyme volume (mL)

t: Incubation time (minutes)

Standard Curve of 2-Orto-Nitrhophenyl-β-D-Glukopiranoside (NPG) solution

A standard glucose curve was generated using a 2-Ortho-Nitrophenyl-β-D-Glucopyranoside (NPG) stock solution at a concentration of 1 mg/mL. According to Natsir *et al*. (2020), the NPG stock solution was prepared by dissolving 10 mg of NPG in 10 mL of sterile H_2O, followed by a serial dilution at concentrations of 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 µg/mL (Natsir *et al*., 2020). The standard solution composition is presented in Table 2.

Table 2. Composition of NPG Standard Solutions.

Concentration of solution		Distilled water (mL)	NPG Stock Solution (mL)
µg/mL	mg/mL		
0	0	5	0
20	0.00	4.9	0.1
40	0.04	4.8	0.2
60	0.06	4.7	0.3
80	0.08	4.6	0.4
100	0.1	4.5	0.5
120	0.12	4.4	0.6
140	0.14	4.3	0.7
160	0.16	4.2	0.8
180	0.18	4.1	0.9
200	0.2	4	1

β-Glucosidase activity measurement

The activity of the β-glucosidase was analyzed using the substrate 2-Orto-Nitrophenyl-β-D-glukopiranoside (NPG), and the amount of O-nitrophenol released in the reaction with NaOH-glycine was calculated (Halliwell, 1985; Natsir *et al*., 2020). The materials used in the β-Glucosidase activity test included: a 0.1 M acetate buffer solution at pH 4.8 (prepared by mixing 200 mL of a 0.2 M acetic acid solution with 300 mL of a 0.1 M sodium acetate solution, then diluted to 1,000 mL with H_2O); a NaOH-glycine solution at pH 10.5 (prepared by dissolving 1.675 g of glycine and 0.91 g of NaOH in 500 mL of H_2O); and an NPG solution (prepared by dissolving 1 mg of 2-ortho-nitrophenyl-β-D-glucopyranoside in 1 mL of H_2O). All tubes were incubated at 37°C for 60 minutes. To stop the enzymatic reaction, 4 mL of NaOH-glycine (pH 10.6) was added. A standard curve was generated using an ortho-nitrophenol solution with concentrations ranging from 0 to 200 µg/mL, measured in 2 mL samples and analyzed using a spectrophotometer at 425 nm. The enzyme activity

of β-glucosidase was calculated using the following formula:
Enzyme activity (units/mL) = [C x 10 x DF]/T x BM p-nitrophenol
Where:
C: O-nitrophenol concentration
T: Incubation time
DF: Dilution factor
BM o-nitrophenol: Molecular weight of o-nitrophenol (301.25)

Data Analysis

The collected data were analyzed using factorial analysis of variance (ANOVA) based on a completely randomized design (CRD). The first factor consisted of two types of isolates, while the second factor comprised five levels of incubation time. Before analysis, the data were tested for normality to ensure a normal distribution. The Duncan's Multiple Range Test (DMRT) was used to assess the effects of the factors. Statistical analysis was performed using SPSS software (SPSS, 2007).

Results

The results of this study indicate that there was no significant effect (P > 0.05) of either the interaction between isolate type and incubation time or the individual factors on CMC-ase activity (Table 3). Similarly, no significant interaction effect (P > 0.05) was observed for β-glucosidase activity (Table 4). Among the two factors examined, incubation time significantly affected β-glucosidase activity (P < 0.05), whereas the effect of isolate type was not significant (P > 0.05). These findings suggest that among the three cellulases derived from buffalo rumen fluid, incubation time is the primary influencing factor. The type of isolate and length of incubation time had no significant interaction effect on β-glucosidase activity (P > 0.05). However, incubation time alone significantly influenced enzyme activity (P < 0.05) (Table 4).

Table 3. Effect of Incubation Time on CMC-ase Activity in ST6 and ST8 Bacterial Isolates Grown on Rice Straw Substrate (U/mL).

Incubation time[ns]	Types of Isolates[ns]	
	ST 6	ST 8
T0	0.57813±0.08206	0.66391±0.09878
T1	0.75156±0.03412	0.81124±0.19570
T2	0.72172±0.11319	0.6005±0.29773
T3	1.38937±1.27297	0.89889±0.12490
T4	0.80192±0.20150	0.59491±0.15341

Description: U/mL = units per milliliter, ST = Tidar Cellulolytic, T0 = incubation period 0 days, T1 = incubation period 3 days, T2 = incubation period 6 days, T3 = incubation period 9 days, T4 = incubation period 12 days; nsNon-significant.

Table 4. Effect of Incubation Time on β-Glucosidase Activity in ST6 and ST8 Bacterial Isolates Grown on Rice Straw Substrate (U/mL).

Incubation Time[sig]	Types of Isolates[ns]		Average
	ST 6	ST 8	
T0	0.0210±0.006	0.01235±0.006	0.01669±0.007[b]
T1	0.01010±0.009	0.03018±0.009	0.02014±0.009[b]
T2	0.02618±0.002	0.03425±0.011	0.030221±0.008[a]
T3	0.02334±0.004	0.020010±0.016	0.021265±0.010[ab]
T4	0.00000±0.0000	0.00090±0.0008	0.00045±0.0004[c]

ns = Non-significant; sig = Significant; [abc]Different superscripts in the same column indicate significant differences (P < 0.05); U/mL = units per milliliters; ST = Tidar Cellulolytic; T0 = incubation period 0 days; T1 = incubation period 3 days; T2 = incubation period 6 days; T3 = incubation period 9 days; T4 = incubation period 12 days.

Discussion

The average CMC-ase activity recorded in this study was 0.78122±0.238 U/mL, which is higher than the activity of a similar enzyme reported in Nababan *et al*. (2019) and Tingthong *et al*. (2021), namely 0.142±0.008 U/mL. The lack of variation in CMC-ase activity across different isolates and incubation times suggests that ST6 and ST8 isolates produced CMC-ase in consistent quantities throughout the 0-day to-12-day incubation period. However, this value remains within a reasonable range reported by Sakpetch *et al*. (2017). Variations in CMC-ase activity are generally attributed to differences in microbial strains, incubation periods, and substrate types. In this case, the variation is primarily due to differences in substrate types or microbial strains rather than incubation conditions. The impact of microbial strain variations on cellulose degradation has been previously documented by Bahri *et al*. (2023). For instance, Aspergillus niger isolates cultured on rice straw, rice bran, and sugarcane bagasse substrates were reported to produce CMC-ase activity of 0.9818, 0.6035, and 0.0041 U/mL, respectively Pratiwi and Ardiansyah (2022). In contrast, Actinomyces isolates grown on rice straw substrates exhibited CMC-ase activity of 0.142±0.008 U/mL (Tingthong *et al*., 2021). Based on these comparisons, it can be concluded that the CMC-ase activity of ST6 and ST8 isolates derived from buffalo rumen bacteria remains within the reasonable range for rice straw cellulose digestion.

In this study, CMC-ase activity of ST6 and ST8 isolates derived from buffalo rumen microbes showed no significant difference, with an average activity of 0.78122±0.238 U/mL. This finding suggests that the enzyme produced by ST6 and ST8 isolates exhibits the same capability in hydrolyzing cellulose in rice straw. Although the lignin content in rice straw serves as a barrier, enzymes from different isolates can still degrade it into simple sugars with similar activity levels. The dense and rigid structure of lignin in rice straw inhibits cellulase activity, which is responsible for breaking down cellulose into simple sugars Anindyawati (2009). Rice straw consists of 37.71% cellulose, 21.99% hemicellulose, and 16.62% lignin (Pratiwi *et al*., 2016). The lignin content of 16.62% is considered high, as the maximum tolerable lignin level for livestock digestion is 7% (Halili, 2014). This finding has significant implications for the utilization of buffalo rumen fluid cellulase, particularly in the fermentation process during the hydrolysis phase of the amorphous region of the cellulose molecule. CMC-ase plays a crucial role in cleaving internal β-1,4-glucosidic bonds in cellulose (Datta, 2024). Given these results, differentiation between ST6 and ST8 isolates is unnecessary, as both isolates produce cellulase with identical activity in digesting rice straw.

CMC-ase activity in this study was not significantly different throughout the 0 to 12-day incubation period. This may be due to the fact that CMC-ase is part of the cellulase enzyme system, which primarily functions in the slow hydrolysis of cellulose (Lynd *et al*., 2002). The CMC-ase used in this study was derived from Ruminococcus species, which produces complex cellulase enzymes in the form of cellulosomes (Ding *et al*., 2001), functioning in a coordinated manner. CMC-ase hydrolyzes the extracellular region of amorphous cellulose, meaning its activity remains constant depending on the availability of amorphous substrates. The efficiency of cellulase (CMC-ase) activity in hydrolyzing natural cellulose is influenced by several factors, including crystallinity, degree of polymerization, particle size, pore volume, and accessible surface area (Fan *et al*., 1980). Therefore, the assumption that CMC-ase activity remained unchanged throughout the 0–12 days incubation period in this study can be attributed to the limited amorphous substrate, which is produced by other components of the cellulase system.

The highest β-glucosidase activity was observed at 6 days of incubation (0.030221±0.008 U/mL), while the lowest activity was recorded at 12 days of incubation (0.00045±0.0004 U/mL). The range of enzyme activity in this study was higher than the values reported by (Nababan *et al*. 2019) and (Tingthong *et al*., 2021), which recorded activity levels of 0.142±0.008 U/mL.

The type of bacterial isolate did not significantly affect β-glucosidase activity, likely because ST6 and ST8 isolates were derived from the same source, buffalo rumen fluid. As a result, both isolates exhibited similar enzymatic activity. Although Khoirunnisa *et al*. (2020) suggests that different types of cellulolytic bacteria may influence the ability of the cellulase enzyme, this effect was not observed in this study. In contrast, incubation time significantly affected β-glucosidase activity, likely due to differences in enzyme concentration at different time points, which influenced the substrate degradation process. Additionally, the substrate milling process likely enhanced enzyme-substrate interaction, as smaller particles increase surface area, thereby improving digestibility and facilitating enzyme action (Setiana *et al*., 2015).

The increase in β-Glucosidase activity at 6 days of incubation occurred because the bacteria had entered the logarithmic growth phase, characterized by rapid and high cell division. This phase led to an increase in total enzyme production, thereby raising enzyme concentration. According to Khoirunnisa *et al*. (2020), bacterial species and environmental factors influence the rate of bacterial replication. β-glucosidase activity decreased in incubation periods beyond 6 days, possibly due to the presence of other enzymes that interfered with cellulase activity in cellulose degradation. Bacteria are generally capable of synthesizing multiple types of enzymes, including proteases, which can degrade cellulase (Martina *et al*., 2002; Datta 2024). Additionally, the decline in β-glucosidase activity may have resulted from nutrient depletion, as bacterial cell growth and enzyme production cease when available nutrients become insufficient (Baharuddin *et al*., 2014)

Conclusion

The activity of CMC-ase cellulase from buffalo rumen cellulolytic bacteria grown on a rice straw substrate was not significantly affected by either isolate type or incubation time, and no interaction was observed between these two factors. In contrast, β-glucosidase activity was not influenced by isolate type but was significantly affected by incubation duration, with 6 days identified as the optimal incubation period.

Conflict of interest

The authors have no conflict of interest to declare.

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