Characterization of Cellulase from the Rumen Metagenome and Evaluation of its Hydrolytic Potential for Agricultural Wastes

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The cellulase RuCel224, derived from the rumen metagenome, was successfully expressed in *Escherichia coli* BL21(DE3), with a molecular weight of ~43 kDa, as confirmed by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Substrate specificity assays revealed the highest activity against sodium carboxymethyl cellulose (CMC-Na) (0.861 ± 0.011 U/mg), followed by wheat straw xylan (0.150 ± 0.050 U/mg). RuCel224 exhibited optimal activity at pH 5.0 and 35 °C, retaining over 70% activity between pH 5.0 and 7.0 and 80% activity within the temperature range of 20 to 40 °C. Thermal stability tests showed that RuCel224 retained 80% activity after 30 min at 50 °C. Metal ion analysis demonstrated that Mn^{2+} significantly enhanced RuCel224 activity by 68.5% and 83.1% at 1 mM and 5 mM concentrations, respectively. Hydrolysis efficiency on lignocellulosic substrates revealed the highest reducing sugar release from corn stalk (206.21 ± 19.11 μg/mL), followed by wheat bran (106.63 ± 20.08 μg/mL) and rapeseed straw (93.83 ± 3.57 μg/mL). Overall, RuCel224 is a highly versatile cellulase under mild conditions, demonstrating strong adaptability to agricultural residues. Its superior performance on corn stalk highlights its potential for bioethanol production and other biomass valorization processes.

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Keywords: Rumen metagenome; Cellulases; Enzymatic properties; Agricultural wastes

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INTRODUCTION

The growing worldwide need for renewable energy and the pressing necessity to decrease reliance on fossil fuels have highlighted lignocellulosic biomass as a key focus in sustainable resource studies. As Earth's most plentiful renewable organic material, lignocellulose plays a vital role as a feedstock for bioenergy and bioproducts (Isikgor and Becer 2015; Fatma *et al.* 2018). Structurally, it is composed of three primary components: cellulose, hemicellulose, and lignin, which form a complex and recalcitrant matrix in plant cell walls. These structural features, while providing mechanical strength to plants, present significant challenges for efficient biomass conversion (Chen and Chen 2014; Andlar *et al.* 2018). Efficiently unlocking the potential of lignocellulose is pivotal for addressing environmental challenges, such as greenhouse gas emissions, and for advancing the development of bio-based economies. Among the diverse lignocellulosic resources, agricultural residues, particularly agricultural wastes, represent a vast and underutilized

resource (Marriott *et al.* 2016; Seglah *et al.* 2019). These residues, characterized by high cellulose and hemicellulose content, are produced in significant quantities globally. However, their dense crystalline structure and close association with lignin pose significant barriers to enzymatic hydrolysis, limiting their direct utilization as a feedstock for bioenergy and biochemical production (Beig *et al.* 2021). Developing efficient methods to overcome these recalcitrance challenges remains a cornerstone of lignocellulosic biomass research.

Enzymatic hydrolysis has emerged as a highly promising approach to lignocellulose bioconversion (Zhang *et al.* 2012). Cellulases and hemicellulases, as key biocatalysts, play a pivotal role in degrading cellulose and hemicellulose into fermentable sugars under mild reaction conditions (Xiao *et al.* 2024). Cellulase, including endoglucanase, exoglucanase, and β-glucosidase, catalyzes the depolymerization of cellulose chains, while xylanase specifically targets the xylan backbone of hemicellulose (Beg *et al.* 2001; Jayasekara and Ratnayake 2019). Despite their potential, the efficiency of enzymatic hydrolysis in real-world applications is often hindered by enzyme instability, substrate heterogeneity, and inhibitory by-products (Agrawal *et al.* 2021), underscoring the need for robust and highly active enzymes. In nature, the rumen of ruminant animals represents one of the most effective ecosystems for lignocellulose degradation (Gharechahi *et al.* 2023). This anaerobic fermentation chamber is home to a diverse microbial community that produces a plethora of glycoside hydrolases with remarkable efficiency and specificity (Gharechahi *et al.* 2023). The exploration of rumen microbiota as a source of novel lignocellulose-degrading enzymes has attracted growing attention, facilitated by advancements in metagenomic and bioinformatic technologies. By mining metagenomic datasets from the rumen environment, it is possible to uncover novel enzymes with enhanced catalytic properties, tailored for the degradation of recalcitrant biomass.

In this study, the metagenomic data from beef cattle rumen microbiota (NCBI accession number PRJNA806344) was leveraged to identify and characterize a cellulase, RuCel224 (EC 3.2.1.4), with potential applications in lignocellulose bioconversion. Through comprehensive enzymatic assays, its catalytic properties were investigated, and its efficiency in degrading agricultural straw was assessed. These findings provide valuable insights into the enzymatic degradation of lignocellulose and highlight the potential of RuCel224 as a biocatalyst for sustainable biomass utilization.

EXPERIMENTAL

Metagenomic Sequencing of Rumen Samples

The metagenomic sequencing data of rumen samples were derived from prior assessments (Hu and Zhao 2022), with the NCBI accession number PRJNA806344.

Cloning of RuCel224

The RuCel224 gene was identified from rumen metagenomic DNA derived from liquid-phase rumen bacteria. Subsequently, liquid-phase DNA served as a template for the PCR amplification of the RuCel224 gene using the primer pair 5'-CTTTAAGAAGGAGATATACGGATCCATGAAAAAAGAATTAACAGCTC-3' and 5'-AGTGGTGGTGGTGGTGGTGCTCGAGAAGTCCGAATGCTTCGGGGAAT-3'.

The amplified fragment was purified and inserted into the pET-28a expression vector using homologous recombination with the Hieff Clone® Plus Multi One Step

Cloning Kit (Yeasen Biotechnology, Shanghai). The recombinant vector, pET-RuCel224, was then transformed into *Escherichia coli* Top10 competent cells *via* heat shock. The pET-RuCel224 plasmid was extracted, verified by PCR amplification, and sequenced. The DNA sequence data are listed in Table S1 (see Appendix).

Expression and IMAC of RuCel224

The pET-RuCel224 plasmid was introduced into *E. coli* BL21 (DE3) cells *via* heat shock, and transformants were selected on LB agar plates containing kanamycin at 37 °C overnight. Positive colonies were identified by PCR using specific primers. A seed culture was initiated in kanamycin-supplemented LB broth and incubated at 37 ℃ until an OD600 of 0.7, at which point 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce RuCel224 expression. The culture was then subsequently shifted to 20 °C for over 18 h. After cultivation, cells were harvested by centrifugation at 8,000 rpm for 10 min at 4 °C. The cell pellet was resuspended in PBS and lysed ultrasonically, and centrifuged to separate the supernatant. The presence of RuCel224 in the supernatant was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining. The recombinant RuCel224 strain was then cultured and induced with 0.2 mM IPTG to produce sufficient quantities of the enzyme for subsequent experimental procedures. The purification of RuCel224 was performed by affinity chromatography using a 5 mL Bio-Scale Mini Nuvia IMAC Ni-Charged column (BioRad, Hercules, CA, United States) connected to a low-pressure chromatographic system (Biologic LP; Bio-Rad, Hercules, CA, United States).

Determination of Substrate Specificity of RuCel224

The substrate specificity of RuCel224 was assessed using five different substrates: CMC-Na (Sinopharm, China, carboxymethyl cellulose), Avicel (Sigma-Aldrich, St. Louis, MO, microcrystalline cellulose), wheat straw xylan (prepared as described by Faryar (Faryar *et al*. 2015)) and chitosan (Solarbio, Beijing), pachyman (BBI-lifesciences, China). The enzyme was incubated in a 50 mM disodium hydrogen phosphate-citrate buffer (pH 6.0) containing 1% (w/v) substrate and incubated at 40 °C for 30 min. The reducing sugars released were quantified using alkaline 3,5-dinitrosalicylic acid reagent, with xylose or glucose as standards (Ghose 1987). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute under assay conditions.

Characteristics of Recombinant RuCel224

The pH-dependent activity of RuCel224 was assessed over a range of pH values (3.0 to 8.0) using 50 mM disodium hydrogen phosphate-citrate buffers. Reactions were carried out at 40 °C for 30 min with 1% (w/v) CMC-Na as the substrate. To determine pH tolerance, RuCel224 was pre-incubated in the same buffer systems without substrate at 35 °C for 30 min. Residual enzyme activity was measured under optimal reaction conditions. The pH stability of RuCel224 was expressed as the percentage of residual activity relative to an untreated enzyme sample, which was set as 100%.

The optimal temperature for RuCel224 activity was determined by conducting reactions at temperatures ranging from 20 to 80 °C for 30 min in 50 mM disodium hydrogen phosphate-citrate buffer (pH 5.0) with 1% (w/v) CMC-Na. Thermal stability was evaluated by incubating the enzyme at 50, 60, 70, and 80 °C for varying durations (5, 10, 20, and 30 min) under pH 5.0 conditions, followed by measuring residual activity under

optimal conditions. Thermal stability was evaluated by comparing the residual activity to that of the untreated enzyme sample, which was set at 100%.

The effects of metal ions, including K^+, Ca^{2+}, Na^+, Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, and Ni^{2+}, as well as EDTA, SDS, β-mercaptoethanol, and DTT, on enzyme activity were examined at final concentrations of 1 and 5 mM. Additionally, the impact of Tween-20 and Triton X-100 on enzymatic activity was evaluated at final concentrations of 1% (v/v) and 5% (v/v). All reactions were carried out under optimal conditions in a 50 mM disodium hydrogen phosphate-citrate buffer. A reaction mixture without any additives served as the positive control, and its activity was defined as 100%.

Hydrolysis of Agricultural Wastes by RuCel224

To evaluate the lignocellulose-degrading potential of RuCel224, substrates including wheat bran, rice straw, wheat straw, corn stalk, soybean straw, rape straw, and *Camellia oleifera* meal were tested at a final concentration of 1% (w/v). Reactions were performed in 50 mM disodium hydrogen phosphate-citrate buffer (pH 5.0) at 35 °C under optimal enzyme conditions. Control reactions without enzyme were conducted in parallel to establish the baseline hydrolysis of each substrate. Hydrolytic efficiency was assessed by comparing the reducing sugars released from enzyme-treated samples to those of the controls.

Statistical Analyses

Statistical analysis was performed using a one-way ANOVA in IBM SPSS statistics version 27 (IBM, Chicago, IL, United States). Significance was declared at $P \leq 0.05$.

RESULTS AND DISCUSSION

Expression of the RuCel224

The RuCel224 gene was successfully cloned into the pET-28a expression vector and heterologously expressed in *E. coli* BL21(DE3) under IPTG induction conditions.

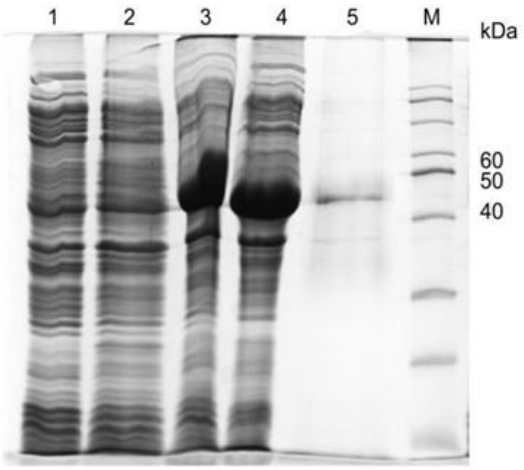

Fig. 1. Analysis of RuCel224 by SDS-PAGE. M, protein marker; 1, non-transformed E. coli BL21 (DE3); 2, Uninduced RuCel224; 3, RuCel224 transformants induced with 0.2 mM IPTG; 4, The supernatant after inducing RuCel224; 5, Purified RuCel224.

SDS-PAGE analysis revealed that the recombinant protein accumulated with an

approximate molecular weight of 43 kDa (Fig. 1), which is consistent with the theoretical molecular weight predicted from the amino acid sequence of RuCel224. This result not only confirms the successful expression of the target protein, but it also suggests proper protein folding and absence of major degradation products, as evidenced by the clear single band on the SDS-PAGE gel.

Substrate Specificity

To evaluate the substrate specificity of RuCel224, its enzymatic activities were measured using five substrates: CMC-Na, wheat straw xylan, Avicel, chitosan, and pachyman. As shown in Table 1, RuCel224 had the highest activity against CMC-Na (0.861 ± 0.011 U/mg), indicating a strong ability to degrade cellulose, particularly amorphous polysaccharides. The enzyme also showed moderate activity on wheat straw xylan (0.150 ± 0.050 U/mg), suggesting some ability to hydrolyze hemicellulose. In contrast, significantly lower activities were observed for Avicel, chitosan, and pachyman. This reduced enzymatic efficiency can be attributed to the substrates' higher structural complexity and recalcitrance. Lower activity was observed with Avicel, chitosan, and pachyman, which was likely due to their higher structural complexity. For instance, Avicel's crystalline cellulose is more resistant to hydrolysis than the amorphous CMC-Na (Gao *et al.* 2014), and pachyman's unique β-(1,3)- and β-(1,6)-glycosidic linkages may limit enzyme access (Gupta and Lee 2009). These results suggest that RuCel224 primarily targets β-(1,4)-linked glucans, consistent with its classification as a cellulase (EC 3.2.1.4). However, the assay conditions (*e.g.*, temperature, reaction time, pH) were not fully optimized, which could affect the results.

Table 1. Substrate Specificity of RuCel143

Substrates	Main Linkage	Enzyme Activity (U/mg)
CMC-Na	1,4-β-(Glucose)	0.861 ± 0.011
Wheat straw xylan	1,4-β-(Xylose)	0.150 ± 0.050
Avicel	1,4-β-(Glucose)	0.025 ± 0.002
Chitosan	1,4-β-(Glucosamine)/1,4-β-(N-acetylglucosamine)	0.019 ± 0.001
Pachyman	1,3-β-(Glucose)	0.047 ± 0.009

Note: Enzyme activity values are expressed as mean ± SD (n = 3). Assays were performed at 40 °C, pH 6.0.

Characterization of RuCel224

This study used CMC-Na as a substrate to investigate the cellulase characteristics of RuCel224. As shown in Fig. 2, the enzyme exhibited peak activity at pH 5.0, maintaining over 70% of its activity in the pH range of 5.0 to 7.0. This result aligns with previous studies on enzymes from the rumen microbiome (Cheng *et al.* 2016; Loaces *et al.* 2016; Lee *et al.* 2018). To assess pH stability, RuCel224 was incubated at various pH levels for 30 min, and residual activity was measured. The enzyme retained over 90% of its activity across a wide pH range (4.0 to 8.0), indicating strong stability. However, exposure to pH 3.0 for 30 min resulted in complete inactivation, suggesting RuCel224's sensitivity to highly acidic conditions. These findings indicate that RuCel224 can tolerate mild acidity but is fully inactivated at pH values below 4.0.

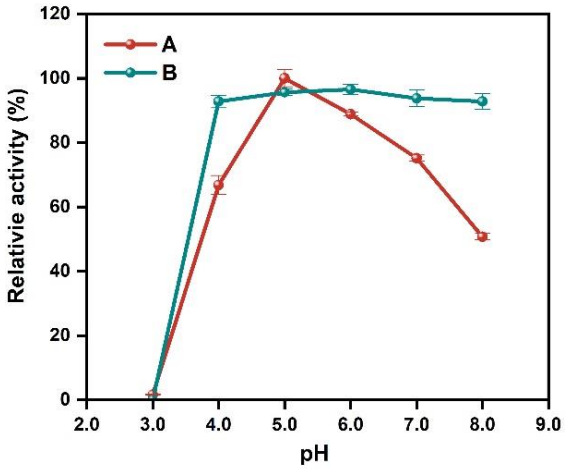

Fig. 2. (A) Optimal pH and (B) pH stability of RuCel224

Temperature dependence and thermal stability were examined using CMC-Na at pH 5.0. As shown in Fig. 3A, RuCel224 exhibited the highest activity at 35 °C, retaining over 80% of its activity within the 20 to 40 °C range. At 50 °C, activity decreased to 43.1%, and at 60 °C, it was nearly undetectable. The optimal temperature of 35 °C corresponds to the typical bovine rumen temperature of 38 to 39 °C (Wanapat 2000), further supporting the adaptability of RuCel224 to function within the rumen's temperature range. Thermal stability tests revealed RuCel224's sensitivity to high temperatures (Fig. 3B). At 50 °C, activity decreased gradually over time, retaining around 80% after 30 min. However, at 60 °C, only 3% of activity remained after 5 min, with the enzyme nearly fully inactivated. No detectable activity was observed at 70 or 80 °C, indicating limited tolerance to elevated temperatures. These results suggest that RuCel224 functions best under moderate temperatures and is prone to thermal denaturation at higher temperatures.

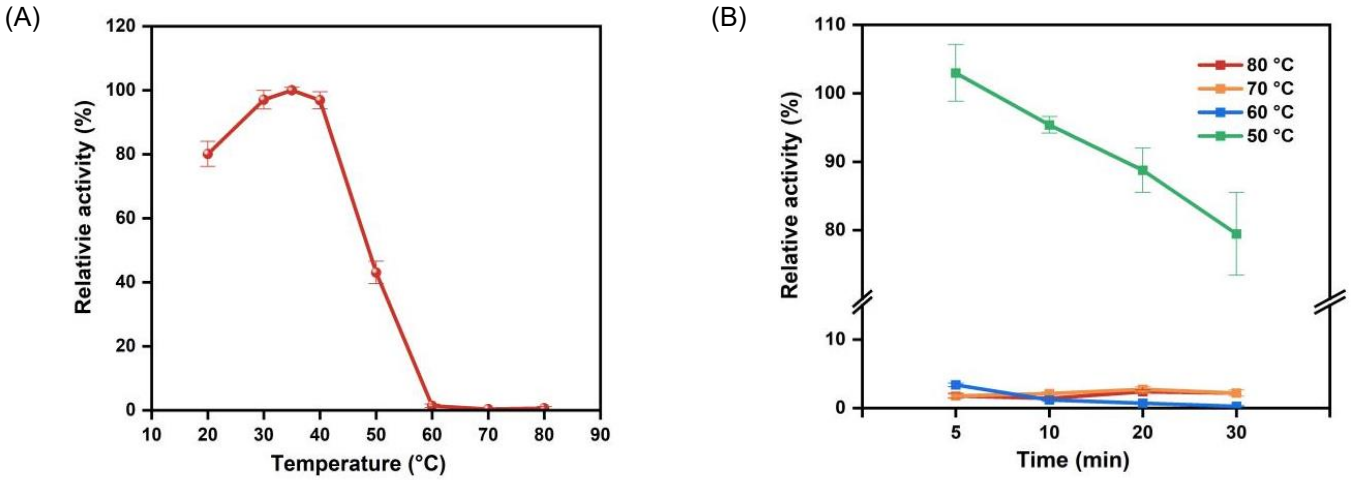

Fig. 3. Optimum temperature (A) and thermal stability of RuCel224 (B)

Mn^{2+} significantly enhanced RuCel224's cellulase activity, with a pronounced stimulatory effect (Fig. 4A). At concentrations of 1 mM and 5 mM, Mn^{2+} increased activity by 68.5% and 83.1%, respectively. This enhancement is consistent with previous reports showing that manganese ions act as cofactors for glycoside hydrolases, stabilizing their

tertiary structures and improving catalytic efficiency. Mn^{2+} likely interacts with negatively charged amino acid residues or coordinates with functional groups in the active site, stabilizing the enzyme-substrate complex and lowering the activation energy for catalysis (Huy *et al.* 2016; Vasconcellos *et al.* 2016). In contrast, RuCel224 activity was significantly inhibited by Cu^{2+} and Ni^{2+}, with Cu^{2+} showing a particularly strong inhibitory effect, reducing activity to 12.3% at 5 mM. This is consistent with the redox activity of Cu^{2+}, which can alternate between +1 and +2 oxidation states. At higher concentrations, this redox cycling generates reactive oxygen species (ROS), leading to oxidative damage to the enzyme (Dudev and Lim 2013). Several reports support these findings, showing Cu^{2+} inhibition of various enzymes (Loaces *et al.* 2016; Tan *et al.* 2017; López-López *et al.* 2023). Other metal ions, such as K^+, Na^+, Ca^{2+}, and Mg^{2+}, had negligible effects on RuCel224 activity, indicating that the enzyme is relatively resilient to ionic variations under standard conditions.

Additionally, RuCel224 showed significant sensitivity to chelating agents (EDTA), surfactants (SDS), and reducing agents (DTT), as shown in Fig. 4B. DTT at 1 mM and 5 mM reduced activity to about 45%, likely by breaking disulfide bonds and compromising the enzyme's structural integrity. EDTA, as a potent chelator, may disrupt metal-dependent stabilization of the enzyme (Auld 1988; Bergan *et al.* 2001), reducing activity to 76.5% and 59.7% at 1 mM and 5 mM, respectively. SDS caused the most dramatic inhibition, reducing activity to 26.8% at 1 mM and almost completely inactivating the enzyme at 5 mM. SDS is known to disrupt non-covalent interactions, such as hydrogen bonding and hydrophobic forces, leading to denaturation (Turner *et al.* 2003). Interestingly, β-mercaptoethanol also exhibited moderate inhibition, further highlighting the role of disulfide bonds in maintaining the enzyme's stability. In contrast, nonionic surfactants, namely Tween-20 and Triton X-100 had minimal impact on RuCel224 activity, suggesting the enzyme's stability in the presence of hydrophilic surface-active agents. This stability points to RuCel224's potential suitability for industrial applications involving detergents or emulsifiers.

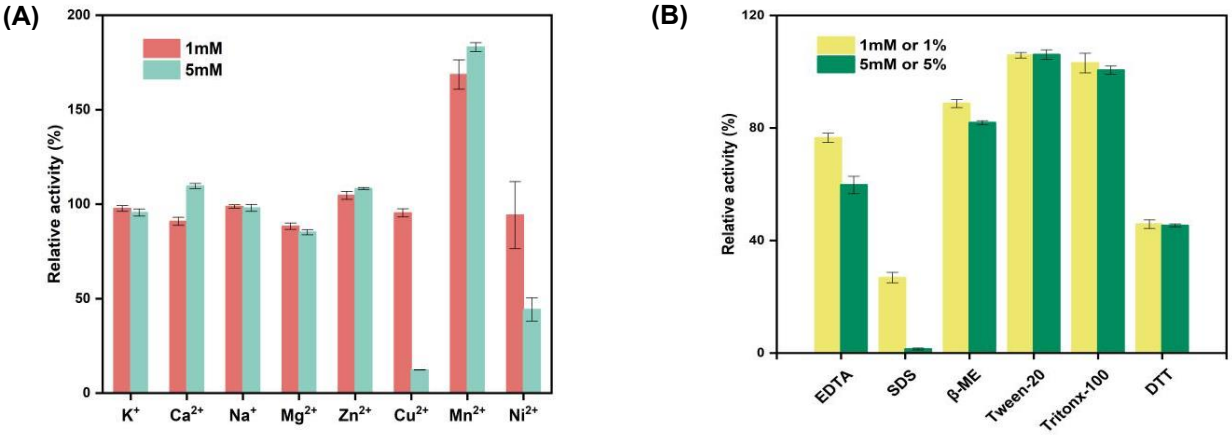

Fig. 4. Effects of metal ions (A), inhibitors and detergents (B)

Hydrolysis of Agricultural Wastes by RuCel224

The hydrolysis efficiency of RuCel224 was evaluated using various lignocellulosic substrates, including wheat bran, rice straw, wheat straw, corn stalk, soybean straw, rape straw, and *Camellia oleifera* meal. The released reducing sugars were quantified, and the results are summarized in Fig. 5. Among the tested substrates, corn stalk exhibited the highest hydrolysis efficiency, with an average reducing sugar concentration of 206.21 μg/mL and a standard deviation of 19.11 μg/mL. This was followed by wheat bran (106.63 μg/mL, SD = 20.08) and rape straw (93.83 μg/mL, SD = 3.57). In contrast, wheat straw showed the lowest hydrolytic efficiency, releasing only 47.89 μg/mL of reducing sugars with minimal variation (SD = 0.62).

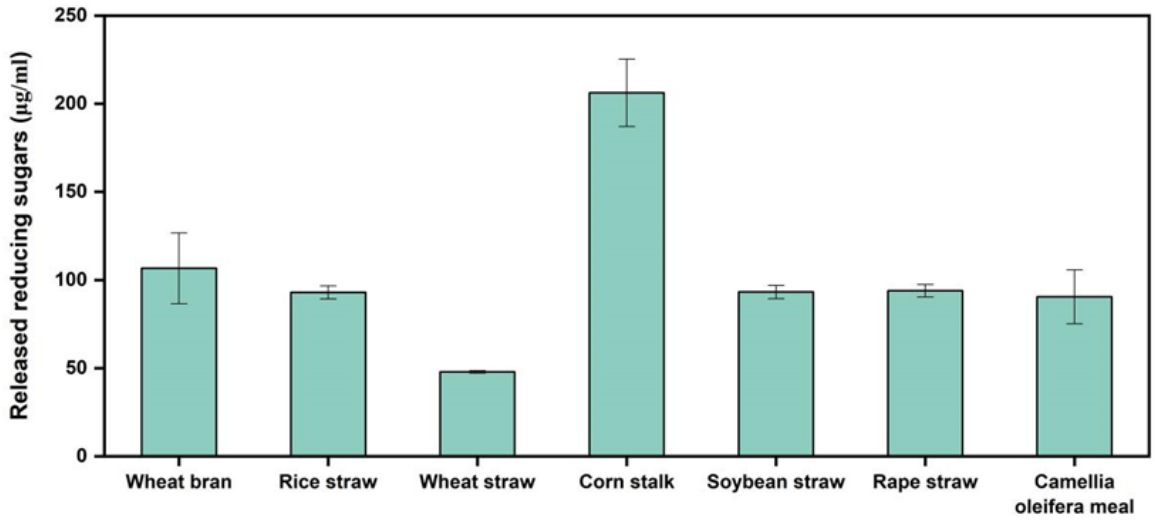

Fig. 5. Hydrolysis of agricultural wastes by RuCel224. The results of the hydrolysis were calculated as the difference between the experimental group (with enzyme treatment) and the control group (without enzyme treatment).

The observed variation in hydrolytic efficiency can be attributed to differences in lignocellulosic composition and structural complexity among substrates (Mansfield *et al.* 1999). Corn stalk, which demonstrated the highest reducing sugar release, is known to contain higher levels of hemicellulose and amorphous cellulose, which are more susceptible to enzymatic hydrolysis (Reddy and Yang 2005). Conversely, wheat straw's dense crystalline cellulose and higher lignin content likely hindered enzymatic access, resulting in lower sugar release (Fan *et al.* 1981). These findings emphasize the potential of RuCel224 in agricultural residue valorization, particularly in the context of bioethanol production, where efficient lignocellulose degradation is crucial. Moreover, the enzyme's effectiveness on substrates such as tea seed cake and rapeseed cake, indicates its potential value in processing by-products from oilseed crops, further broadening its industrial applications. The authors' previous studies align with these results, demonstrating the enzyme's wide substrate range under mild conditions. Notably, wheat bran, a by-product of flour milling, exhibited substantial enzymatic hydrolysis, suggesting its suitability as a feedstock for bioprocessing. Due to its relatively lower lignin content, wheat bran presents a promising substrate for the production of high-value sugars. However, despite RuCel224's significant activity across diverse substrates, its reduced efficiency on lignin-rich materials, such as wheat straw, highlights a key limitation. This limitation underscores the need for optimization strategies to enhance the enzyme's efficacy on such recalcitrant

substrates. Potential solutions could include enzyme engineering or the synergistic use of auxiliary enzymes, such as laccases or peroxidases, which may improve the breakdown of lignin (Shin *et al.* 2019). Furthermore, pretreatment methods like steam explosion or alkali treatment could disrupt lignin-cellulose complexes, thereby enhancing the enzyme's access to the cellulose fraction and improving hydrolysis efficiency (Hendriks and Zeeman 2009). These substrate-specific variations in reducing sugar release reinforce the necessity of tailoring enzymatic processes to the unique characteristics of different feedstocks.

Beyond enzymatic performance, the cost of RuCel224 production presents another significant challenge. The industrial production of enzymes is inherently expensive due to the requirements for fermentation, purification, and stabilization, which can hinder their widespread application, particularly in cost-sensitive sectors like bioethanol production. Strategies to reduce enzyme production costs are therefore critical. Optimizing microbial fermentation processes, improving strain engineering for higher expression yields, and exploring alternative host systems—such as bacterial or yeast-based recombinant platforms—could substantially enhance the cost-effectiveness of RuCel224 production (Adegboye *et al.* 2021).

Additionally, the enzyme's stability and availability under varying storage and operational conditions are crucial for its industrial deployment. Enzymes are susceptible to denaturation and activity loss due to prolonged storage, temperature fluctuations, and interactions with inhibitors or stabilizing agents. To mitigate these challenges, enzyme immobilization or chemical modifications could be employed to enhance RuCel224's structural stability and extend its shelf life, ultimately improving its practicality in continuous industrial processes (Ansari and Husain 2012).

In summary, while RuCel224 holds great promise for lignocellulosic biomass conversion, addressing its limitations—including its narrow operational range, high production costs, and sensitivity to lignin-rich substrates—will be critical for maximizing its commercial potential. Advancements in enzyme optimization, synergistic enzymatic applications, and effective pretreatment strategies will play a pivotal role in overcoming these challenges, ultimately enabling the broader adoption of RuCel224 in bioprocessing industries.

CONCLUSIONS

1. RuCel224 is a rumen-derived cellulase with broad substrate specificity, capable of degrading both cellulose and hemicellulose.
2. RuCel224 exhibits optimal activity at pH 5.0 and 35 °C, with good pH stability, but it is sensitive to high temperatures and highly acidic environments, making it suitable for operation under mild conditions.
3. Mn^{2+} significantly enhances the cellulase activity of RuCel224.
4. RuCel224 demonstrates good degradation capacity on agricultural wastes such as corn stalks, providing a foundation for the further development of RuCel224-based enzymatic systems in biomass conversion and green biorefining applications.

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APPENDIX
Supplementary Information

Table S1. Nucleotide Sequence of RuCel224

Item	Sequence
RuCel224	ATGAAAAAAGAATTAACAGCTCTTGTACCGTTTGCACTCGCCCTGAGTTTGTGCCTGACC GCCTGCGACAACGGCGGCTCCCATAACGCCGTGGCCCCTAAAGAAGATGATTCGACAGC GACGAAGGCTATCGACTATTCCAAGGGTCGCGCCATGAACAAGAGGCTCGGCAAGGGTA TCAACCTTGGCAACTCCTGGGAATCCCAAGGGAGCAGCCTTGACTGCAGCTGGGGTAAC TGCATTGAAGACACCGATTTTGCTGTCGTTAAGGCGGCAGGATTCAATTCTATTCGACTCC CCGTGCGTTGGCAGCAGGATTCCGACTATGGCACCCATACCGTAGACCCTGCCCGTCTC GCCGGCGTGAAAGAAGATATCGAGCTTGCCCTTGCCCAAGGACTCGCTGTCATCGTGAA CTTCCACCACTATGTCGAGCTGAACGAAGCCGGTAACAACTATACGACCGACCCCCAGGC ATACGAAGAGGAAAAGGCTCACTTCTTGAAATTGTGGGAACAGGTCGCCACGGAATTGAA CAAATACCCCGATTCCATGCTCGTGCTTGAAATTTTGAACGAGCCCACCATTTCTAACGCC GAACGCGTAAGCAACCTCATGAATGACGCTTACCAGGTGATCCGTGCAAACGCACCCGG CAAGACAATCATGTTCGAAGCCTACCATGCCGCAAAATTTGCAGACCTTAAGGACTTGAAA CTGCCGGAAGACGGAAACATCATCTATTCCGGGCATTATTACGAACCCTACACCTATAGCC ACCAGGGCCATAGCTACGCCTGCAAGGGCGACGACGCTTACGCCAACACCGCTATTTAC GACATGGCCACCTATTCAAAGATTGCAACGGAGCTCTACCCCGACGTAAATGGCGGACAT GTTCCCATGAACATGGGTGAATTCGGCATTTCGGGCGGCGGCGACTTTGCCAACAGCAG AAGTTGCAACGAAGGCGAATCGCTCCCGTCGGCAAGAATGAAGGCCAAGTGGGCAAAGT TGACAATTCAGGCTGCAGAATCGAACGATATTTCCTGGCACTACTGGGGATTTACGAAAGT AGGCGGATTCGAAGCCTACAGCCGTAATGATGACGCTTGGTACGAAGGATTCCCCGAAG CATTCGGACTT