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Characterization of a Thermophilic Lignocellulose-Degrading Microbial Consortium with High Extracellular Xylanase Activity

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A microbial consortium, TMC7, was enriched for the degradation of natural lignocellulosic materials under high temperature. TMC7 degraded 79.7% of rice straw during 15 days of incubation at 65°C. Extracellular xylanase was effectively secreted and hemicellulose was mainly degraded in the early stage (first 3 days), whereas primary decomposition of cellulose was observed as of day 3. The optimal temperature and initial pH for extracellular xylanase activity and lignocellulose degradation were 65°C and between 7.0 and 9.0, respectively. Extracellular xylanase activity was maintained above 80% and 85% over a wide range of temperature (50–75°C) and pH values (6.0–11.0), respectively. *Clostridium* likely had the largest contribution to lignocellulose conversion in TMC7 initially, and *Geobacillus*, *Aeribacillus*, and *Thermoanaerobacterium* might have also been involved in the later phase. These results demonstrate the potential practical application of TMC7 for lignocellulosic biomass utilization in the biotechnological industry under hot and alkaline conditions.

Keywords: Lignocellulose degradation, extracellular xylanase activity, thermophilic, alkaline condition, microbial consortium

Introduction

Lignocellulosic biomass is considered an attractive alternative to fossil fuel, and can be used for the production of bioethanol, methane, and forage after being broken down into simple sugars [1–3]. As a complex polymeric substance, lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin, which form a strong network [4]. Hemicellulose is the most abundant heteropolymer, accounting for 25–35% of the lignocellulosic material [3]. Because of the strong binding of cellulose with heterogeneous hemicellulose and lignin, lignocellulose hydrolysis is the most significant technological and economical limiting factor [4, 5]. The degradation of hemicellulose could make lignocellulosic materials more susceptible to bio-utilization [6, 7]; thus, hemicellulose degradation and breakdown are very important steps towards the proper utilization of lignocellulosic biomass [3, 8, 9]. Enzymatic hydrolysis is currently considered to be an attractive alternative strategy owing to its moderate and environment-friendly features [3, 10]. Hemicellulose is mainly composed of xylan; therefore, xylanase is considered to be absolutely required for efficient utilization of lignocellulosic materials and has attracted considerable research interest for biotechnological applications [6, 7, 11]. To date, xylanase has been utilized in various industrial fields, including the baking and pulp and paper industries [12, 13], especially extracellular xylanase, which is much easier to extract and more available compared with cell-associated enzymes [14, 15].

The utilization of lignocelluloses by pure-culture microorganisms has been extensively studied. However, most cellulolytic pure-culture isolates can only degrade relatively simple substrates such as artificial xylan and carboxymethyl starch, and they are generally limited to

catabolizing natural lignocellulosic materials [16]. The decomposition of natural lignocelluloses by microbial co-cultures or communities has been proposed as a highly efficient strategy because an efficient microbial reaction requires a complex set of lignocellulolytic enzymes [14, 17]. Considerable research has been devoted to studying the effective degradation capabilities of lignocellulose-degrading microbial communities, and it is difficult to achieve degradation capability equivalent to that of a microbial consortium by using any single-component isolate or a simple mixture of these isolates from a consortium [14, 15, 18].

Our previous study showed that the microbial consortium XDC-2 could degrade natural lignocelluloses and secrete extracellular xylanase at room temperature [14]. However, the xylanase activity decreased sharply and only 3% of the activity was retained when cultured at 45°C. Most of the xylanases secreted by bacteria or fungi show optimal activity at neutral or slightly acidic conditions and at temperatures between approximately 40°C and 55°C. Overall, lignocellulose degradation by a microbial consortium with high extracellular xylanase activity under high temperature (>55°C) has been largely neglected in this field of research. However, the degradation of hemicelluloses is generally favored under hot and alkaline conditions, such as in the paper and pulp industry [19]. To resolve this issue from a practical point of view, it is necessary to establish microbial consortia for thermophilic and alkaliphilic lignocellulose degradation with high extracellular xylanase activity.

In the present study, a stable thermophilic lignocellulolytic microbial consortium (TMC7) that shows high lignocellulosic substrate degradation and extracellular xylanase activities was established under high temperature (65°C). The characteristics of the enzyme activity and degradation capability towards natural lignocelluloses were investigated along with the compositional diversity of TMC7. The results of this study should contribute to further exploration and development of microbial systems for the efficient production of extracellular xylanase, and highlight the potential of TMC7 in biotechnological applications for improving lignocellulose degradation under high-temperature and alkaline conditions.

Materials and Methods

Preparation of Lignocellulosic Materials

Lignocellulosic materials (rice straw, corn stalk, and wheat straw) obtained locally from Wuhan, China were air-dried. The contents of the main lignocellulose components of these materials, namely cellulose, hemicellulose, and lignin, were respectively 39.7%, 21.3%, and 16.5% in rice straw; 37.1%, 24.1%, and 12.1% in corn stalk; and 42.2%, 23.7%, and 18.2% in wheat straw. The alkali-treated lignocellulosic materials were submerged in 0.5% (w/v) sodium hydroxide at room temperature for 24 h to increase the exposure of polysaccharides to the microbes and hydrolytic enzymes, and then washed with tap water to neutral pH. After treatment, the contents of cellulose, hemicellulose, and lignin were, respectively, 65.1%, 14.6%, and 5.9% for rice straw; 62.4%, 17.6%, and 5.7% for corn stalk; and 63.3%, 15.3%, and 6.2% for wheat straw. Finally, the alkali-treated and untreated lignocellulosic materials were oven-dried at 80°C and then cut into 1–2-cm sections for further use.

Enrichment of a Lignocellulose-Degrading Microbial Consortium

A lignocellulose-degrading microbial consortium was enriched from a local thermophilic compost of agricultural wastes composed of straw, sawdust, and animal feces in Wuhan, China. In brief, 20 g of compost was transferred to 350 ml of autoclaved peptone cellulose solution (PCS) medium (pH 7.2, composed of (per liter) 1 g of peptone, 2 g of yeast extract, 2 g of $CaCO_3$, 5 g of NaCl, 0.35 g of $MgSO_4{\cdot}7H_2O$, and 1 g of K_2HPO_4) [20] containing 1% (w/v) alkali-treated rice straw as a carbon source, and then incubated under static conditions in the dark at 65°C in a 500-ml flask with a loose aluminum cap. After 3 days, when the straw in the medium started to degrade, 5% (v/v) of the active culture was transferred to new PCS medium with 1% (w/v) rice straw. The subculture process was repeated four times after appropriate incubation periods (3–6 days). This lignocellulose-degrading microbial consortium is hereafter referred to as TMC7. The degradation efficiency of TMC7 for alkali-treated corn stalk and wheat straw, as well as the untreated lignocellulosic materials (rice straw, corn stalk, or wheat straw), was determined. Alkali-treated rice straw was used throughout the study unless otherwise stated. All experiments were performed in triplicate.

The pH of the TMC7 culture during rice straw degradation was measured using a compact pH meter (B-212; HORIBA, Japan).

Determination of Weight Loss, Components of Residual Cellulosic Substrates, and Enzyme Activities

The cultures were sampled on days 0, 3, 6, 9, 12, and 15 for analysis of weight loss, components of residual cellulosic substrates, and enzyme activities. The weight loss of natural lignocelluloses and lignocellulosic components was assayed with a gravimetrical method after fermentation [14]. Uninoculated medium served as the control.

For crude enzyme preparation, 7 ml of culture samples was centrifuged at 12,000 ×*g* for 10 min at 4°C, and the supernatants were used as extracellular enzyme samples. The pelleted cells were washed in phosphate-buffered saline solution (pH 7.4) and resuspended in 4 ml of 20 mM Tris-HCl buffer (pH 8.0). The mixture was incubated at 37°C for 30 min after the addition of 0.5 ml of 1% (w/v) lysozyme solution. The solutions were then centrifuged at 12,000 ×*g* for 10 min at 4°C, and the supernatants

were used for measurements of cell-associated enzyme activity. Xylanase activities were determined according to the methods of Bailey *et al*. [21], and filter paper activities (FPA) were measured according to the protocol of the International Union of Pure and Applied Chemistry system [22]. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar during a 1-min reaction. All samples were analyzed in triplicate, and mean values were calculated.

Optimization of Cultivation Temperature and pH

To determine the optimal cultivation temperature and pH for TMC7, the consortium was incubated as described above under varying temperatures (50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, and 85°C) or different initial pH values of the medium (3.0–12.0) adjusted with HCl or NaOH. The weight loss and xylanase activity were determined on day 12.

Effects of Temperature and pH on Xylanase Activity, Thermostability, and pH Stability

Crude extracellular enzyme samples were obtained from the cultures after 12 days of incubation to analyze the effects of temperature and pH on the extracellular xylanase hydrolysis reaction and to determine the thermostability and pH stability of the extracellular xylanase. The effect of temperature on the xylanase reaction was determined by incubating the enzyme with 1% birchwood xylan in 50 mM phosphate buffer (pH 7.0) at different temperatures (50°C, 60°C, 65°C, 70°C, 75°C, 80°C, and 85°C) for 30 min. For thermostability analysis, xylanase activity was determined by incubating the enzyme in 50 mM phosphate buffer (pH 7.0) at different temperatures (50°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, and 100°C) for 120 min. Before measurement of the activity, xylanase was cooled on ice for 30 min.

The effect of pH on the enzymatic reaction was evaluated by incubating the xylanase in different buffers (50 mM) (glycine-HCl (pH 2–3), acetate buffer (pH 4–5), phosphate buffer (pH 6–7), Tris-HCl buffer (pH 8–9), glycine-NaOH buffer (pH 10), or Na_2HPO_4-Na_3PO_4 buffer (pH 11–13)) at 65°C for 30 min. The pH stability of the extracellular xylanase was determined by incubation in the various buffers listed above at 65°C for 120 min. The remaining activities of the various temperature- and pH-treated xylanase samples were measured as described above and compared with the activity of the intact enzyme to calculate the relative activity.

Compositional Analysis of the Microbial Consortium by 16S rRNA Gene Sequencing

Genomic DNA of TMC7 was extracted from 4 ml of the culture with the E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. The 16S rRNA gene was sequenced using an Illumina MiSeq PE300 sequencing platform (Illumina, Inc., USA). The universal bacterial primer set of 338F and 806R was used to amplify the V3–V4 hypervariable region of the 16S rRNA gene [23]. The unique sequence set was classified into operational taxonomic units (OTUs) with a threshold of 97% identity using UCLUST. The OTUs were then classified taxonomically at the genus level with RDP Classifier.

Results and Discussion

Degradation Capacity and Enzyme Activities of TMC7

The weight loss of the alkali-treated rice straw, cellulose, and hemicellulose was determined during 15 days of incubation under 65°C (Fig. 1A). TMC7 showed the highest degradation rate of 35% for straw in the first 3 days; 55% of hemicellulose was drastically decomposed and only 15% of cellulose was degraded during this period. However, cellulose was strongly degraded from day 3 to day 9, with degradation rates of 42%, 73%, and 84% after 6, 9, and 12 days, respectively. After 12 days, the weight loss of straw, cellulose, and hemicellulose was not substantial, but reached 78%, 86%, and 90% on day 15. Therefore, hemicellulose was mainly degraded in the first 3 days, whereas the decomposition of cellulose occurred mainly from day 3 to

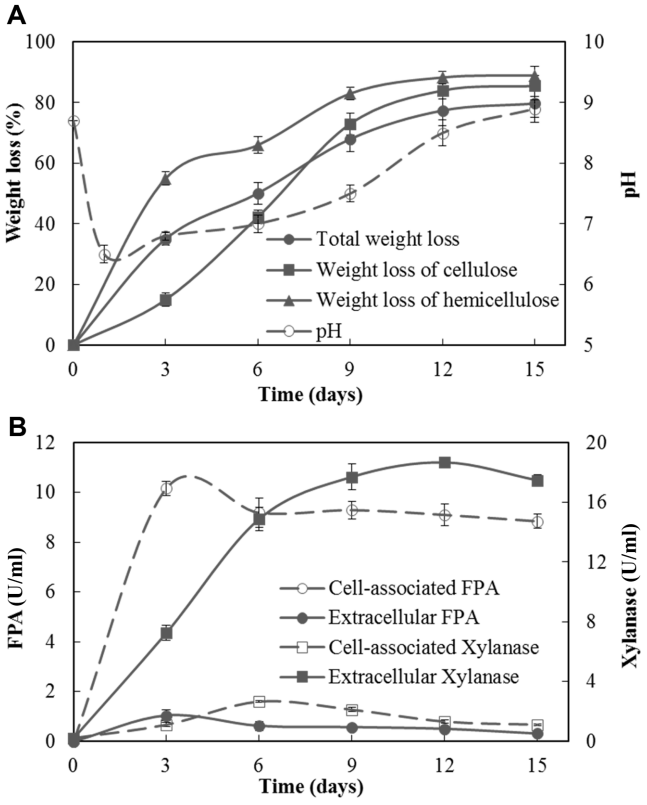

Fig. 1. Changes of pH and weight loss of alkali-treated rice straw, hemicellulose, and cellulose (**A**), and the filter paper activity (FPA) and xylanase activity (**B**) of TMC7 during cultivation.

Vertical bars represent the standard deviation of the mean.

day 9. These results agree with those of previous reports finding that degradation of hemicellulose could improve cellulose bio-utilization [6, 7].

The pH of the culture was also determined during the degradation of rice straw. The pH declined rapidly from an initial value of 8.7 to 6.5 after 1 day of incubation; thereafter, it increased, reaching 8.5 on day 12, and then remained relatively stable, which might be ascribed to the production of acids from saccharide fermentation at the beginning and consumption of these acids afterwards. This pH-changing trend is consistent with previous reports about lignocellulose-degrading microbial consortia [14, 20, 24]. Before 9 days, the pH of the culture was slightly acidic, and the degradation of rice straw was the most vigorous during this period. The rice straw degradation became much weaker and gradually disappeared when the pH became more neutral and then alkaline. The relationship between the pH and rice straw degradation ability of the microbial consortium in this study is also consistent with previous reports [14, 20, 24]. The mechanism underlying this relationship should be further investigated.

The extracellular and cell-associated enzyme activities were determined during the 15-day degradation of the alkali-treated rice straw (Fig. 1B). The extracellular xylanase activity increased dramatically in the first 6 days before it peaked at 18.7 U/ml on day 12. By contrast, the cell-associated xylanase activity peaked on day 6 with an activity of 2.7 U/ml, which is negligible compared with the extracellular xylanase activity. The extracellular xylanase activity was higher than that of previous studies, which specifically demonstrated optimal xylanase activities of 13.2 U/ml at 50°C in pH 8.0 in a microbial consortium [25], 3.75 U/ml at 60°C in pH 6.4 in a microbial consortium [26], and 5.12 U/ml at 70°C in pH 8.0 in a pure culture of *Kluyvera* sp. strain OM3 [3].

Both the extracellular FPA and cell-associated FPA reached their highest levels at approximately 3 days; the cell-associated FPA was 10.2 U/ml, which was more than 9 times higher than the extracellular FPA. These results indicate that extracellular xylanase and cell-associated cellulase serve as the functional enzymes for lignocellulose decomposition during the whole degradation process.

Optimization of the Cultivation Temperature and pH of TMC7

For optimization of cultivation temperature and pH, TMC7 was cultivated at varying temperatures (45–85°C; Fig. 2A) or initial pH (3.0–12.0; Fig. 2B). The optimal temperature for both the rice straw degradation and xylanase activity

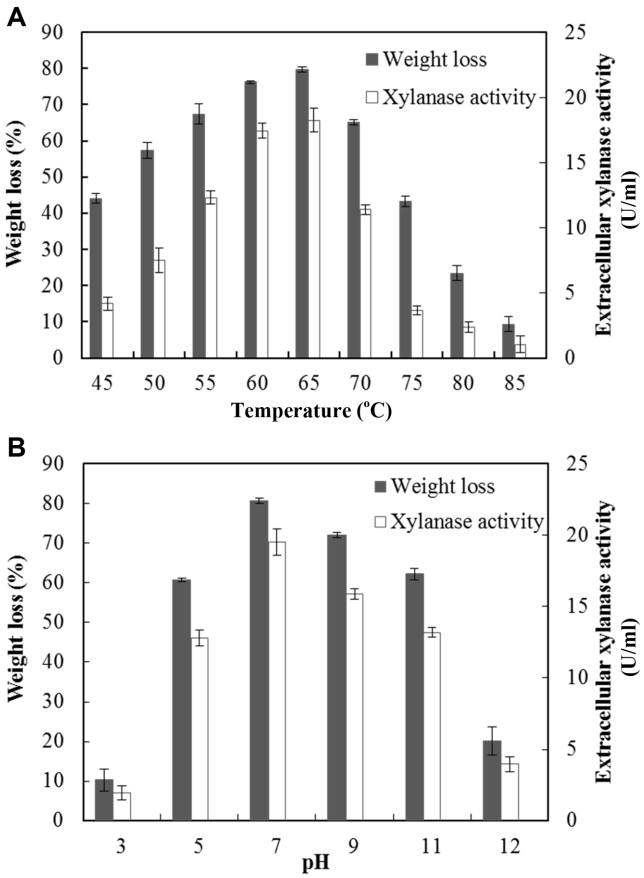

Fig. 2. Optimization of the cultivation temperature (**A**) and initial pH (**B**) of TMC7 for rice straw degradation and xylanase production.
Vertical bars represent the standard deviation of the mean.

was 65°C. The weight loss of rice straw and extracellular xylanase activity peaked at 79.7% and 18.2 U/ml at 65°C, and only decreased slightly at 70°C, with values of 65.1% and 11.4 U/ml, respectively. However, when the cultivation temperature was increased to 75°C or set to lower than 50°C, the degradation capability and xylanase activity decreased substantially. The optimal initial incubation pH of TMC7 was between 7.0 and 9.0; the rice straw degradation and xylanase activity were maintained at 72.1–80.7% and 15.9–19.5 U/ml, respectively. When the initial incubation pH was 11.0, the TMC7 still showed 62.2% weight loss and 13.2 U/ml of extracellular xylanase activity. The lignocellulose-degrading ability and xylanase activity of TMC7 were sharply decreased when the initial pH of the medium was adjusted to 3.3 and 12.0. These results indicate that TMC7 is a thermophilic and alkaliphilic lignocellulose-degrading microbial consortium with a high level of extracellular xylanase.

Degradation of Different Lignocellulosic Materials by TMC7

The degradation efficiencies of TMC7 for alkali-treated rice straw, corn stalk, wheat straw, and the untreated lignocellulosic materials were determined. The dry-mass weight-loss percentages for alkali-treated rice straw, corn stalk, and wheat straw were 50.1%, 44.2%, and 34.4% after 6 days, and 77.4%, 70.2%, and 57.4% after 12 days, respectively (Fig. 3A). The lignocellulosic materials were degraded more strongly during the first 6 days. The degradation ratios of the main lignocellulose components of rice straw, corn stalk, and wheat straw after 6 days were as follows: cellulose, 42.3%, 45.2%, and 35.7%; hemicellulose, 66.2%, 56.8%, and 47.8%, respectively. The degradation of hemicellulose in all samples was higher than that of cellulose, demonstrating that this microbial consortium has a stronger capability for hemicellulose degradation.

TMC7 showed a similar degradation trend for the different untreated lignocellulosic materials (Fig. 3B). Compared with wheat straw and corn stalk, rice straw was degraded more thoroughly, regardless of alkali treatment. It is noteworthy that TMC7 showed strong degradation ability even for the untreated lignocellulosic materials, although these degradation ratios were decreased and the weight loss was 52–55% of that observed with the alkali-treated biomass. After 12 days, the weight-loss percentages of untreated lignocellulosic materials were 42.2% for rice straw, with 60.1% of cellulose lost and 62.2% of hemicellulose lost; 36.7% for corn stalk, with 50.1% of cellulose lost and 60.2% of hemicellulose lost; and 30.1% for wheat straw, with 40.2% of cellulose lost and 51.2% of hemicellulose lost. A recent report on the bioconversion of non-pretreated lignocellulosic biomass by a microbial consortium at 35°C showed a weight loss of 39.0% for rice straw, 25.2% for wheat straw, and 17.6% for corn stalk [27]. Therefore, TMC7 showed better performance for the bioconversion of non-pretreated lignocellulosic materials. Moreover, the extracellular xylanase of TMC7 showed high activity at high temperature. To further characterize the extracellular xylanase of TMC7 and its potential for practical application, the effects of temperature and pH on the xylanase enzyme reaction, and the thermostability and pH stability of the extracellular xylanase were analyzed.

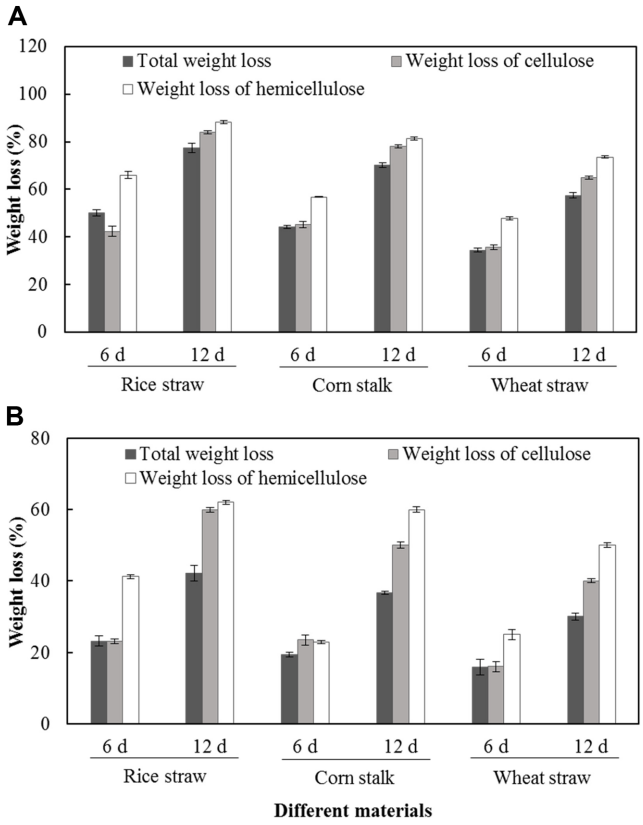

Fig. 3. Weight loss of alkali-treated (**A**) and untreated (**B**) lignocellulosic biomasses (rice straw, corn stalk, and wheat straw) in TMC7.

Vertical bars represent the standard deviation of the mean.

Characteristics of Extracellular Xylanase

To test the effects of temperature and pH on the xylanase enzyme reaction, the extracellular xylanase activity was determined under varying temperatures from 50°C to 85°C at pH 7.0 and across a pH range of 4–11 at a constant temperature of 65°C. The relative activity of xylanase was higher than 80% at reaction temperatures of 55–75°C and higher than 90% at pH 7–10 (Fig. 4A). The optimal xylanase reaction temperature and pH were determined to be 65°C and 9, respectively. These results are in good agreement with the analysis of the optimization of the cultivation temperature of TMC7.

The thermostability and pH stability of extracellular xylanase were also analyzed. The xylanase activity was relatively high after treatment between 50°C and 75°C, with more than 80% activity retained, and more than 95% and 85% activity was retained after 120 min of incubation at 70°C and 75°C, respectively. The xylanase activity decreased sharply when the incubation temperature was increased to 85°C, with residual activities of 36.4%, 12.7%, and 9.1% after incubation at 85°C, 90°C, and 100°C, respectively (Fig. 4B). This indicates that 85°C is the temperature threshold for efficient extracellular xylanase activity of TMC7. Moreover, stable xylanase activity was

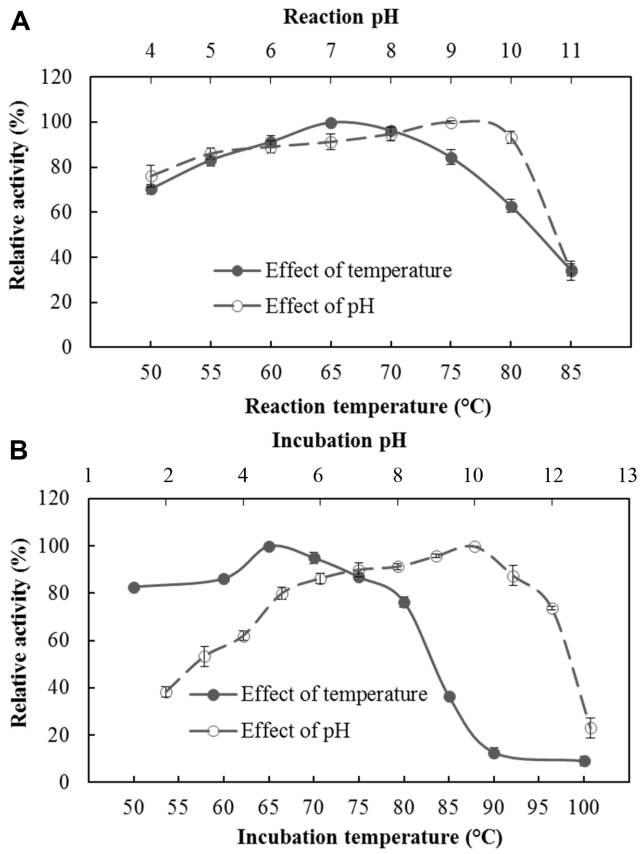

Fig. 4. Effects of temperature and pH on the xylanase enzyme reaction (**A**), and effects of incubation temperature and pH on extracellular xylanase activity (**B**).
In each experiment, the highest xylanase activity was defined as 100%. Vertical bars represent the standard deviation of the mean.

observed over an incubation pH range of 6.0–11.0, and more than 85% of the relative activity was maintained. Even after 120 min of incubation at pH 12, 73.6% of the relative xylanase activity was retained (Fig. 4B). Incubation at a pH of 13 resulted in a substantial decrease of xylanase activity, indicating that the pH tolerability limit of the enzyme system might be pH 13.

Xylanase showed maximum relative activity after treatment under 65°C and pH 10, and the stability was well maintained over a wide range of temperature (50–80°C) and pH values (5–12), indicating its extreme thermal and pH stability, and strong tolerance to elevated temperature and alkaline pH. The stability of TMC7 was much higher than those reported previously for extracellular enzymes [3, 14, 25, 26]. A thermostable xylanase was previously reported in which 71% of the activity was retained after 1 h of incubation at 70°C, and its activity could only be maintained between pH 5 and 9 [3]. Yang *et al*. [26] studied a microbial community for the degradation of switchgrass, which showed optimal cellulase activity at 60°C and pH 6.4; however, xylanase activity was maintained above 80% only within a narrow pH range (5.5–7.5) and temperature range (50–70°C) [26]. An alkaliphilic xylanase showed optimum activity at 50°C and pH 8.0; however, the relative activity declined drastically to below 30% when the pH was higher than 9 or lower than 5 [25]. Moreover, the enzyme only functioned at mesophilic temperatures (between approximately 30°C and 50°C), and lower than 60% activity remained when it was cultured above this range. A mesophilic (30–40°C) lignocellulose-degrading microbial consortium (XDC-2) was previously reported to show high extracellular xylanase activity under acidic or alkaline conditions; however, only 3% of the xylanase activity was retained when the incubation temperature was increased to 45°C [14]. The strong tolerance of the extracellular xylanase of TMC7 to broad temperature and pH conditions could provide a convenient way to prepare effective xylanase for the treatment of lignocellulose under hot and alkaline conditions.

Dynamics of the TMC7 Consortium during Lignocellulose Degradation

Given that TMC7 showed the most effective degradation of rice straw, the dynamics of the composition of the TMC7 microbial consortium during rice straw degradation were determined using high-throughput sequencing at day 0, day 3, day 6, and day 12. The major genera identified in TMC7 on day 0 were *Clostridium* III (31.8%), *Geobacillus* (8.8%), and *Tepidimicrobium* (8.9%). The abundance of *Clostridium* III increased to 51.2% in the first 3 days. The abundance of *Geobacillus*, *Aeribacillus*, and *Thermoanaerobacterium* increased to 25.3%, 3.8%, and 4.3% after 6 days, respectively, and then the abundance of *Geobacillus* and *Thermoanaerobacterium* decreased to 16.2% and 1.7% on day 12, whereas that of *Aeribacillus* increased continuously, reaching 7.9% on day 12. The abundance of *Clostridium* III decreased to 40.9% on day 6 and finally to 32.1% on day 12, although *Clostridium* remained the dominant genus throughout the culture (Fig. 5).

The rapid initial growth of *Clostridium* and its high abundance in TMC7 suggests that *Clostridium* might play the greatest role in the degradation of rice straw in this consortium. The cellulose degradation capability of *Clostridium* has been well established [28–32]. *Clostridium stercorarium* is an anaerobic thermophile bacterium that shows high ethanol production during cellulose and

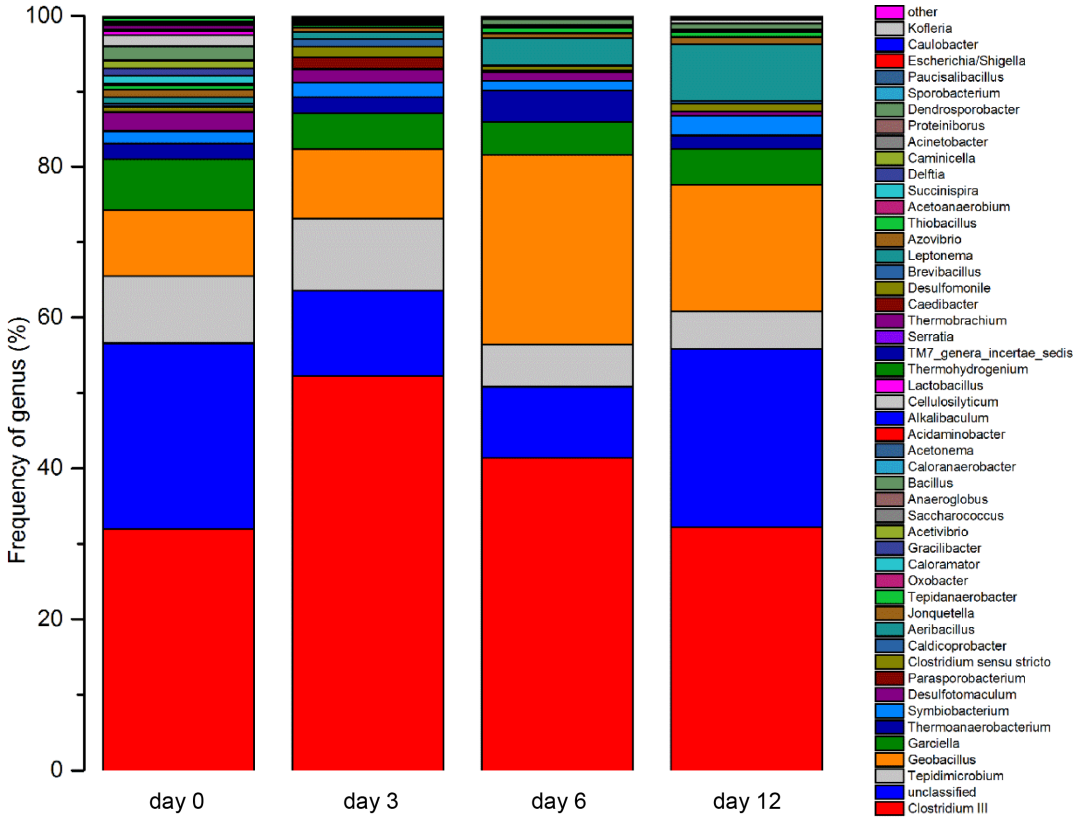

Fig. 5. Diversity dynamics of TMC7 during rice straw degradation.
The culture was sampled on day 0, day 3, day 6, and day 12.

hemicellulose decomposition [29]. *Clostridium caenicola* belongs to cluster III and has been reported as a thermophilic cellulose-digesting bacterium [30]. *Clostridium thermocellum* has attracted research interest owing to its cellulolytic and ethanologenic abilities, as it is capable of producing ethanol by cellulose hydrolysis [28, 31, 32]. Previous studies demonstrated that *Geobacillus* had xylosidase activity during growth on hemicellulose biomass and could produce isobutanol from cellobiose [33, 34]. *Geobacillus*, *Aeribacillus*, and *Thermoanaerobacterium* species in TMC7 might also participate in the lignocellulose conversion process in the later phase.

Overall, this study showed that the microbial community of TMC7 is relatively complex, and the lignocellulosic materials were degraded through multiple stages involving several lignocellulose-degrading bacteria with different extents of contribution at each stage. Further work confirming the mechanisms underlying the synergistic interaction and mutual coordination among these cellulolytic bacteria is required to effectively produce extracellular enzymes by TMC7 and fully utilize this consortium for practical applications under high-temperature and alkaline conditions.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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