RESEARCH ARTICLE

Preparation and fiber degradation characteristics of a fusion subunit RZ1

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Efficient biodegradation of crop straw in saline-alkali lands is a crucial strategy for promoting its sustainable development. This study isolated cellulose-degrading fungi strains from wild elk feces in the coastal wetlands of Yancheng, Jiangsu Province, China and applied cell fusion biotechnology to develop novel microbial resources for optimizing agricultural waste straw utilization in saline-alkali areas. A high-efficiency fiber-degrading fusion strain was engineered using cell fusion technology to enhance the biodegradation rate of plant straw. *Penicillium* and *Mucor* were selected as parent strains and fused under polyethylene glycol (PEG) mediation, resulting in the highly efficient fiber-degrading fusion strain RZ1. The results showed that the enzymatic activity of RZ1 was remarkable with filter paper enzyme activity, endocellulase activity, exocellulase activity, and β-glucosidase activity reaching 121.92 U/mL, 270.74 U/mL, 267.46 U/mL, and 263.09 U/mL, respectively. Following 9 days of anaerobic incubation, the fiber degradation efficiency of strain RZ1 reached 40.78 ± 1.48%. Environmental tolerance studies revealed distinct pH-dependent growth characteristics as acidic conditions of pH 3-5 significantly inhibited RZ1 proliferation, while neutral to alkaline conditions of pH 7-9 remarkably enhanced its growth. The strain had salt tolerance within a 1 - 5% salinity range, indicating excellent adaptability to saline-alkali environments. The co-culture with rice seeds demonstrated that the germination index showed significant improvement across all tested bacterial suspension concentrations after 36 hours of soaking with increases of 11.91, 7.73, 9.58, and 11.78 compared to 14.71 in the control group. These results confirmed that RZ1 exerted no phytotoxic effects on crop growth. The developed fusion strain RZ1 exhibited superior cellulose-degrading capacity and demonstrated promising potential for enhancing straw decomposition in saline-alkali soils. These findings suggested that RZ1 could serve as an effective biological agent for sustainable agricultural waste management in saline-alkali environments.

Keywords: fungi; cell fusion; cellulose degradation; saline-alkali environments; elk.

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Introduction

According to recent statistical data, the global saline-alkali land area spans approximately 1.1×10^9 hectares. China alone accounts for about 36.667 million hectares of utilizable saline-alkali land resources [1]. Despite the increasing cultivation coverage of crops such as rice, wheat, soybeans, and corn in these regions, the degradation and resource utilization of straw from salt-tolerant crops remain under explored. Currently, three primary methods are employed for straw utilization, which are direct field incineration, agricultural utilization, and industrial application [2]. However, direct incineration releases harmful pollutants such as

carcinogenic benzo[a]pyrene, contributing to air pollution and posing significant health risks. Agricultural utilization primarily involves returning straw to the fields, but its natural degradation cycle is prolonged, and the accumulation of undegraded straw can foster harmful bacterial growth, ultimately inhibiting crop growth. Industrial utilization, which involves converting straw into resources like ethanol through microbial or enzymatic processes, presents technical challenges and limitations.

Biodegradation technology offers a sustainable solution by enhancing the degradation rate of straw while enabling the return of organic matter to the soil for crop reuse instead. This approach aligns with contemporary green development principles and has emerged as a key research focus in straw degradation. Due to their unique growth environment, saline-alkali crops accumulate high concentrations of sodium chloride, sodium sulfate, sodium carbonate, and sodium bicarbonate, making them resistant to conventional biodegradation methods. Efficient straw degradation in saline-alkali soils is therefore crucial for promoting sustainable land utilization. In Dafeng, Jiangsu, China, wild elk primarily feed on saline-alkali-tolerant reeds and *Spartina alterniflora* as 96.73% of their diet, suggesting that their gut microbiota possesses exceptional fiber-digesting capabilities [3]. Aro *et al.* used protoplast fusion technology to obtain fusion offspring when studying the genetics of *T. reesei* and found that its carboxymethyl cellulose (CMC) enzyme activity was twice as high as that of the parent strain [4]. Later, the fusion technology was applied to fuse different wood molds, which opened the application of cell fusion technology in improving cellulase activity. Li chose *Penicillium* Q5 that produced cellulase and *Bacillus subtilis* K3 that had stress resistance as parents to obtain fusion strains that could secrete high yield cellulase and had strong stress resistance by using the protoplast fusion technology [5].

This research isolated fiber-degrading microbial strains from wild elk feces in Dafeng, Yancheng, Jiangsu, China and prepared novel microbial partners using cell fusion technology to enhance fiber degradation efficiency. The resulting strains offered promising new microbial resources for the efficient biodegradation of saline-alkali crop straw.

Materials and methods

Sample source

Penicillium and *Mucor* were screened from the feces of wild elk obtained from Dafeng, Yancheng, Jiangsu, China and preserved in the Cell Engineering Laboratory of Yancheng Normal University (Yancheng, Jiangsu, China).

Prepare and screen fusion subunit RZ1

(1) Activate strains and subculture

The mycelium of *Penicillium* and *Mucor* from the inclined surface of the preservation test tube were selected and inoculated into potato culture medium (PDA) (Thermo Fisher Scientific China Co., Ltd, Beijing, China) for activation. After culturing at 28℃ for 3-5 days on a shaker, the bacteria were inoculated into Czapek-Dox medium (Thermo Fisher Scientific China Co., Ltd, Beijing, China) for subculture and continue culturing for 3-5 days.

(2) Prepare protoplasts

Penicillium and *Mucor* cultured in PDA medium for 48 hours were taken as parents and treated with a mixed enzyme solution containing 1% snailase and 2% lysozyme (Shanghai Guchen Biotechnology Co., Ltd, Shanghai, China) [6]. Enzymatic hydrolysis was performed using 0.6 mol/L sucrose solution (Beijing Coastal Hongmeng Standard Material Technology Co., Ltd, Beijing, China) as a stabilizer [7, 8]. The protoplast isolation was conducted by taking 5 mL of parental strain solution in logarithmic growth stage, centrifuging at 7,000 rpm for 20 min. The precipitate was washed twice with a stabilizing agent before suspending in the stabilizing agent. 5 mL of each wall breaking enzyme was added and hydrolyzed at 38℃ for 2 hours before centrifuging at 7,000 rpm for 5

minutes. After removing the supernatant, the precipitate was washed twice with stabilizer, centrifuged at 7,000 rpm for 10 minutes. The protoplast precipitate was collected and suspended in the stabilizer for later use.

(3) Prepare fusion subunit RZ1

The prepared protoplasts of *Penicillium* and *Mucor* were diluted with a stabilizer and mixed in a 1:1 ratio before centrifugation at 7,000 rpm for 10 minutes. The parental precipitate was then collected. 40% polyethylene glycol (PEG) was prepared by using isotonic solution and used immediately. The fresh calcium solution was prepared by mixing equal volumes of 54% KH_2PO_4 and 29.4% $CaCl_2{\cdot}H_2O$ after sterilization. The fusion subunit was prepared by adding 1.8 mL of 40% PEG and 0.2 mL of fresh calcium solution to the parental precipitate. The mixture was incubated in a 38°C water bath for 10 minutes before centrifugation at 7,000 rpm for 5 minutes. The precipitate was diluted in a stabilizer, coated with high osmotic yeast extract peptone dextrose medium (YEPD) (Thermo Fisher Scientific China Co., Ltd, Beijing, China), and incubated at 28°C for 3 days.

(4) Screen fusion subunit RZ1

The initial screen of fusion strain was performed by the observation of the color and morphological characteristics of parental colonies and fusion colonies. The strains with significantly different morphologies from parental colonies on YEPD high osmotic medium were selected, streaked, and cultured on the initial screening medium and incubated at 28℃ for 3 days before the plates were stained with 1 mg/mL Congo Red solution (Shanghai Guchen Biotechnology Co., Ltd, Shanghai, China) for 20 minutes. After removing the staining solution, decolorization was performed three times using 1 M NaCl solution with 15 minutes each time. Strains were then re-screened based on the diameter of the hydrolysis zones. The selected fusion strain, designated as RZ1, was subsequently cultured in liquid medium at 28℃, 180 rpm [9].

Optimize the enzyme production conditions

(1) Orthogonal optimization

Five factors that affected the activities of cellulose degrading enzymes were selected including initial pH (5, 6, 7, 8), temperature (25, 30, 35, 40℃), time (24, 36, 48, 60 h), carbon source (Glucose, Sucrose, carboxymethyl cellulose sodium (CMC-Na), Starch), and nitrogen source of the culture medium (NH_4Cl, Urea, Peptone, $NaNO_3$) with four levels set for each factor [10-14].

(2) Determination of the cellulose degradation rate of RZ1

A nylon bag of 8 cm × 10 cm and 300 mesh was sewed on three sides with fine polyester thread and double-stitched. The straw of rice growing in the coastal mudflat of Yancheng, Jiangsu, China was cut into 1 cm small pieces and dried in a 60℃ oven for 24 hours. After soaked the samples in $Ca(OH)_2$ with a mass fraction of 2% for 24 hours, the samples were filtered, washed with water, and baked in the oven at 60℃ for 24 hours before grounded into powder to obtain the processed samples. 0.5 g of the sample was then added into a nylon bag with the total mass of the sample and the nylon bag being weighed and denoted as m1. The degradation effect of RZ1 was tested by placing each pre-packaged nylon bag into an optimized enzyme producing culture medium before inoculating 1% fusion strain RZ1 and parent strain into the enzyme producing culture medium, respectively, and cultivated at 25℃ and 120 rpm. Three parallel tests were set for each sample. After culturing for 2 and 7 days, the nylon bags were washed with distilled water and dried in a 60 ℃ oven to a constant weight. The total mass of the sample and nylon bag was recorded as m2. The degradation rate was calculated as follows.

$$\text{Degradation rate} = ((m1 - m2)/m1) \times 100\%$$

A Phenom Pharos G2 scanning electron microscopy (SEM) (Funa Scientific Instruments Co., Ltd, Shanghai, China) was applied to observe the physical deformation of rice straw after degrading 2 and 7 days by the fusion strain RZ1.

Determine the cellulase activity

The enzyme activity was calculated by using the following formula.

$$X = (S \times D \times 1{,}000)/(N \times T)$$

where X was the enzyme activity of the strain (U/mL). S was the glucose content corresponding to the absorbance value of the bacterial solution on the standard curve (mg). D was the dilution factor of the crude enzyme solution. 1,000 was the conversion factor between the units of mg and μg. N was the volume of crude enzyme solution involved in the reaction (mL). T was the reaction time (min). The cellulase activities including endocellulase (EG), exocellulase (CBH), and β-glucosidase (BG) were measured using carboxymethyl cellulose, microcrystalline cellulose, and salidroside as substrates [15-17]. Among them, the filter paper activity (FPA) that reflected the total cellulase activity of the comprehensive effect of fiber degradation was determined using a modified DNS method. After mixing 0.5 mL of crude enzyme extract with 1.5 mL of 0.1 M sodium acetate buffer (pH 4.8), a strip of Whatman No. 1 filter paper (1 × 6 cm) was added as substrate. The reaction mixture was incubated at 50℃ for 60 min in a water bath, then immediately quenched by adding 3 mL of 3,5-dinitrosalicylic acid (DNS). After 10 min of color development in a boiling water bath, the reaction was terminated by rapid cooling down under running cold water and brought to a final volume of 25 mL with distilled water. For the blank control, 0.5 mL of distilled water replaced the enzyme solution while maintaining all other conditions. Absorbance was measured at 530 nm using a N6000 plus spectrophotometer (Beijing Yongguangming Medical Instrument Co., Ltd, Beijing, China). FPA activity (U/mL) was calculated by comparing the optical density against a glucose standard curve, where one unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmoL of reducing sugars per minute under the assay conditions. The endocellulase activity was measured using 1% (w/v) CMC-Na, while exocellulase was determined using 1% (w/v) microcrystalline cellulose and β-glucosidase was measured with 1% (w/v) salicylic acid.

The environmental tolerance of RZ1

(1) The acid-base tolerance of RZ1

The pH gradients of 3, 5, 8, and 9 were tested in the re-screening medium with three replicates for each treatment. After cultivation in a 30℃ constant temperature shaking chamber, the samples were collected at different time points of 12, 24, 36, 48 hours. The growth characteristics of RZ1 at different pH values were analyzed.

(2) The salt tolerance of RZ1

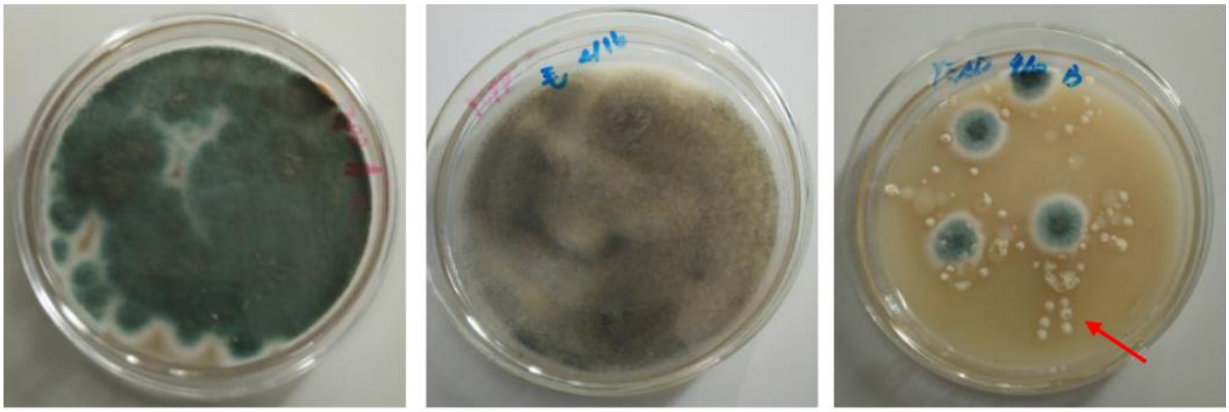
The salt concentrations (NaCl) in the medium were set to 1%, 3%, 5%, 7%, and 8% with three replicates for each treatment. After shaking incubation at 30℃, the samples were collected at different time points of 12, 24, 36, 48, 60 hours to analyze the growth characteristics of RZ1 at different salt concentrations.

(3) The oxygen tolerance of RZ1

The growth characteristics and fiber degradation rate under different oxygen environments of fusion strain RZ1 were measured. Briefly, the activated RZ1 fusion strain was inoculated into 100 mL of liquid enzyme production medium and were sealed with sterile kraft paper secured with rubber bands. The culture was incubated at 30°C, 150 rpm for aerobic cultivation. The anaerobic culture was conducted by transferring the activated RZ1 inoculum to an oxygen-scavenging system that was established by placing 0.2 g gallic acid (Thermo Fisher Scientific China Co., Ltd, Beijing, China) between two 90 mm diameter sterile Whatman No. 1 filter papers that were saturated by 0.5 mL of 10% NaOH (w/v) solution. This assembly was immediately positioned at the opening of cultural container, sealed with two layers of parafilm, secured with kraft paper, and incubated under identical temperature and agitation conditions as the aerobic treatment.

Evaluation of RZ1 biosafety

One hundred rice and cabbage seeds were soaked in four dilution gradients of bacterial



1-1 Penicillium 1-2 Mucor 1-3 Fusion strain RZ1

Figure 1. Comparison of colony morphology between fungal fusion strain RZ1 and its parent strains.

solutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}) and sterile water, respectively. After soaking the seeds under sterile conditions for different time periods of 24, 36, and 48 h, 30 rice seeds and 30 cabbage seeds were selected at each concentration and transferred to a petri dish covered with moist filter paper and cultivated at room temperature with natural light with three repeats. The moisture content of the seeds was observed and recorded at fixed intervals to maintain mass conservation throughout the cultivation process. The effects of strain RZ1 on seed germination and seedling growth were analyzed. After seed germination, the germination number was recorded daily for 7 consecutive days, and the relevant indicators of seed germination were analyzed. The germination potential was measured on the 4^{th} day of seed germination, and the germination rate, germination index, and vitality index were measured on the 7^{th} day of germination [18]. The germination rate and index were calculated as below.

Germination rate (%) = (number of normally germinated seeds on the 7^{th} day/total number of seeds in each dish) × 100%

Germination index = $\sum Gt/Dt$

where Gt was the number of germination days on day t. Dt was the relative number of germination days. After 7 days of seed germination, 60 early seedlings from the experimental group were randomly collected. 15 seedlings were harvested and frozen for preparation of crude enzyme solutions to detect each biochemical indicator. The superoxide dismutase (SOD) and peroxidase (POD) indicators were determined by using a reagent kit (Shanghai Guchen Biotechnology Co. Ltd, Shanghai, China) following manufacturer's instructions.

Statistical analysis

Microsoft Excel (Microsoft, Redmond, WA, USA) was used to organize and process data [19]. The data was expressed as mean ± standard deviation. SPSS (IBM, Armonk, NY, USA) was used for multiple comparisons. Path analysis was performed to analyze the impact of three variables of endocellulase, exocellulase, β-glucosidase, and filter paper activity on the dependent variable fiber degradation rate.

Results and discussion

Screening of fusion subunit RZ1

The initial color of the *Penicillium* colony was dark green, and eventually a green moss appeared (Figure 1-1). The mold initially appeared as white velvet and covered the entire plate after 3 days (Figure 1-2). The mycelium eventually turned

dark brown in color. After the two parent strains were fused, a strain with a completely different colony morphology from the parent strain grew on the YEPD medium. The initial colony color was white, and after 3 days, wrinkles appeared on the colony surface and bulged toward the middle (Figure 1-3). It was preliminarily determined that this strain was the fusion strain RZ1. The strains in the initial screening medium were stained with Congo Red dye and then decolorized with sodium chloride solution, forming clear transparent circles around the colonies (Figure 2). The target strain RZ1 was selected based on the size of the transparent bacteria.

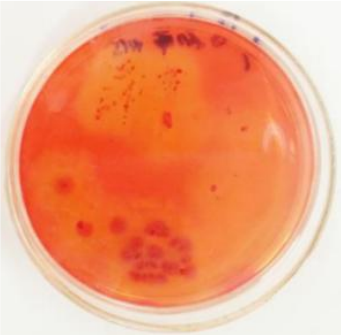


Figure 2. Results of strain re-screening.

Analysis of the cellulase characteristic of fungal fusion subunit RZ1

The characteristics of cellulose degradation rate of fusion subunit RZ1 were analyzed [20]. Under the optimized enzyme production conditions, the filter paper enzyme activities of endocellulase, exocellulase, and β-glucosidase in fusion strain RZ1 were all higher than those in the parent strains. The cellulase activity of the fusion strain RZ1 was significantly different from that of the parent *Mucor* ($P < 0.05$), and the endocellulase activity was extremely significantly different from that of the parent *Penicillium* ($P < 0.01$) (Figure 3).

The plant cellulose degradation rate of RZ1

After 2 days of degradation, the cellulose degradation rate of fusion strain RZ1 was 10.93%, which was 138.4% higher than that of *Penicillium* and 211.4% higher than that of *Mucor*. After 7 days of degradation, the cellulose degradation rate of fusion strain RZ1 was 19.08% with an increase of 106.2% compared to *Penicillium* and 112.4% compared to *Mucor* (Figure 4).

Growth status of RZ1 under different pH and salt concentrations

The results of salt concentrations and pH tolerance analysis of the fusion strain showed that, regarding environmental tolerance, pH significantly influenced RZ1 growth. The growth was inhibited at pH 3 - 5 but enhanced at pH 7 - 9. RZ1 exhibited growth even at a 7% salt concentration, surpassing the typical 6% threshold known to inhibit most fungal growth [20, 21] (Figure 5). This exceptional salt tolerance might stem from the parental strains of RZ1, which were isolated from the feces of wild elk locally. These elk primarily consumed plants from saline-alkali environments, potentially contributing to RZ1's adaptive traits under alkaline conditions.

RZ1 cellulose degradation rate under different oxygen environments

The cellulose degradation rate of strain RZ1 under anaerobic conditions was higher than that under aerobic conditions. The cellulose degradation rate reached its peak on the 9^{th} day at 32.19 ± 7.06% and 40.78 ± 1.48%, respectively (Figure 6).

Path analysis of fiber degradation rate and cellulase activity of strains

Among the four enzyme activities of FPA (X_1), EG (X_2), CBH (X_3), and BG (X_4), FPA had the highest direct determination coefficient on cellulose degradation rate in the parental strains *Penicillium* and *Mucor*. CBH had the greatest impact on fiber degradation rate in the fusion strain RZ1 with corresponding coefficients of 0.50, 0.67, 0.89, and -1.71, respectively. The order of decision coefficients was $R^2(3) > R^2(2) > R^2(1) > R^2(4)$, where $R^2(4)$ was less than 0. Therefore, X_3 was the main decision variable with the smallest direct determining effect, which meant that it was necessary to increase the activity of external cellulase (X_3), maintain

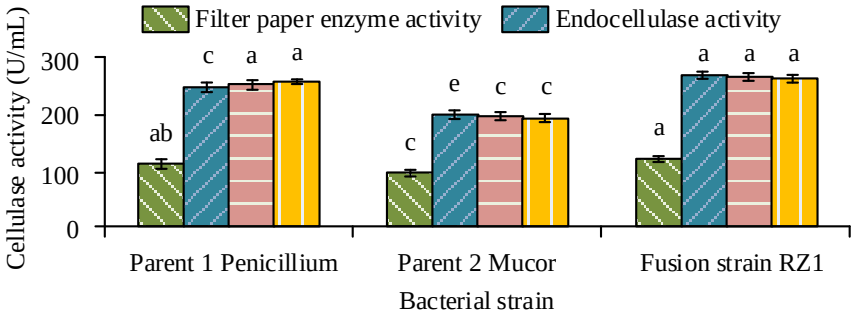


Figure 3. Characteristics analysis of cellulose degradation rate of fusion subunit RZ1.

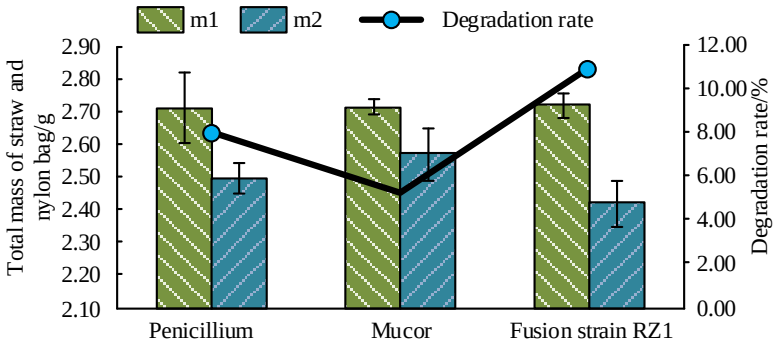


Figure 4. Comparison of cellulose degradation rates between fusion strain RZ1 and its parents.

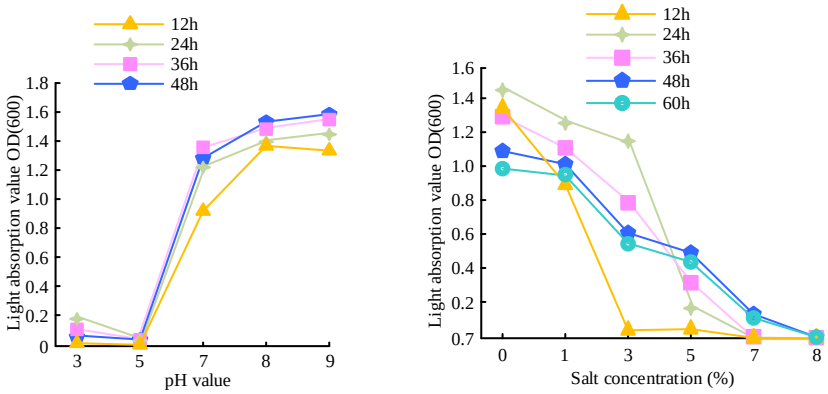


Figure 5. Analysis of pH and salt concentration tolerance of fusion strain RZ1.

the activity of internal cellulase (X_2) or filter paper enzyme (X_1) and limit the activity of β-glucosidase (X_4) to improve the cellulose degradation rate of fusion strain RZ1 (Figure 7).

Scanning electron microscopy results of straw degradation

After 2 days of degradation by the fusion strain RZ1, the surface of the straw produced varying degrees of depression, and the fiber bundles became loose, revealing a neatly arranged fiber bundle skeleton (Figure 8A). After 7 days of degradation, the surface roughness of the straw was more obvious, and the fiber bundles underwent varying degrees of breakage (Figure 8B).

Bio-safety analysis of fusion strain RZ1

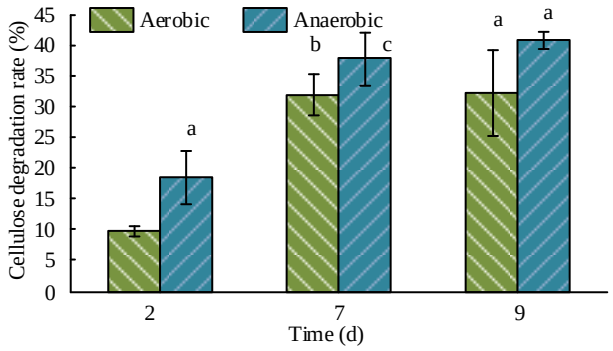


Figure 6. Results of cellulose degradation rate of fusion subunit RZ1 under different oxygen environments.

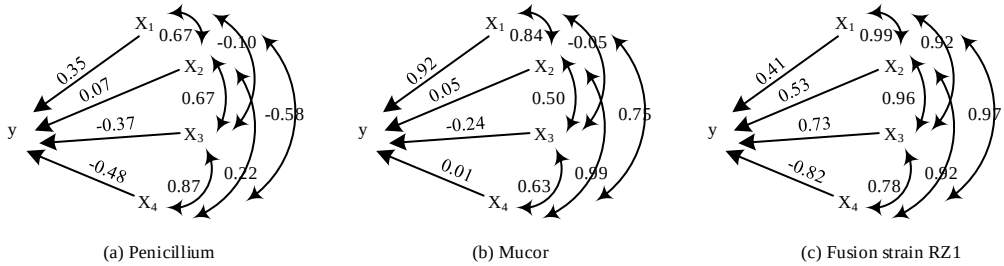


Figure 7. Path analysis results of degradation rates and four cellulase activities of different bacterial strains. **Note:** y: degradation rate. X_1: filter paper enzyme activity. X_2: endocellulase activity. X_3: external cellulase activity. X_4: β-glucosidase activity.

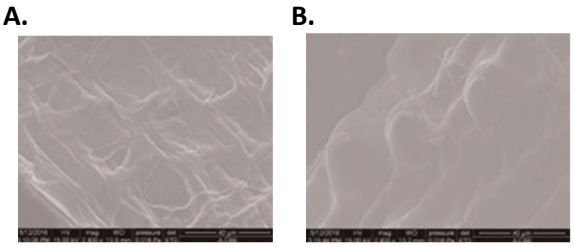


Figure 8. Scanning electron microscopy of straw biodegradation by fusion strain RZ1 for 2 days (A) and 7 days (B).

This study found that RZ1 had no significant effect on the germination rate of rice and vegetable seeds ($P > 0.05$), but the germination index was significantly increased. Among them, taking rice seeds as an example, under different bacterial solution concentrations, the germination index of seeds soaked for 36 hours increased by 11.91, 7.73, 9.58, and 11.78, respectively, compared with the blank group (14.71). The co-treatment of RZ1 increased the POD and SOD enzyme activities in rice and green vegetables. The POD enzyme activity in green vegetable seedlings soaked for 24 and 36 hours showed different forms of increase. After soaking for 48 hours, the POD enzyme activity of rice seedlings reached the highest value of 786 U/mL, which increased the yield and stress resistance of rice and vegetables and had certain biological safety [22].

Acknowledgements

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