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Isolation of Xylanase Producing Strains, Optimization of Fermentation Conditions and Research on Enzymatic Properties

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Abstract

In this study, we successfully isolated a strain of *Aspergillus oryzae* TR08, which produced xylanase secreted to the outside of the cell productively. The enzyme activity and specific activity in the fermentation broth of this strain reached peak values of 451 IU/mL and 1963 IU/mg after 156 h of fermentation. A single factor experiment was designed, and it was found that the strain was adjusted to the initial pH of the fermentation broth to 7.5 in a shaker at 180 rpm and 32 °C. After 156 h of fermentation, the enzyme activity reached a maximum of 1264 IU/mL. The optimal reaction temperature and pH value of the xylanase were 55 °C and 7.5, respectively, and it had excellent acid and alkali resistance and a wide pH activity range. The xylanase was increased the catalytic activity by 15% in 0.25 mM Fe^{3+}, and the biological activity of the enzyme was not affected in the sodium dodecyl sulfate environment.

Keywords: *Aspergillus oryzae*, xylanase, fermentation, enzymatic properties

1. Introduction

1.1 Xylan

Xylan, the second most abundant heteropolysaccharide in nature, was the main component of hemicellulose (Schell *et al.*, 2016). The xylan from lignocellulosic biomass was connected by *β*-1,4-xylosidic bonds to form a backbone, and was further covered by 4-O-methyl-D-glucuronic acid groups, acetyl groups, and α-arabinofuranose side chain group modification (Sha *et al.*, 2020; Khaire *et al.*, 2021). At present, xylan was mainly used to degrade to produce xylose, which was then chemically or biologically converted into biofuels, furfural, xylitol, etc. Secondly, because xylan also contained a small amount of arabinose, mannose, galactose, galacturonic acid, glucose and glucuronic acid, etc., it could be used as a raw material to produce a variety of high-value chemicals.

1.2 Enzyme-Catalyzed Production of Xylo-Oligosaccharides

In recent years, with the widespread application of xylo-oligosaccharides (XOS) in the feed industry (49.6%), health and medical products (25.4%), food and beverages (23.2%) and other fields (1.8%), XOS obtained through enzymatic hydrolysis or acid hydrolysis and other methods had become a more promising application (Mathew *et al.*, 2017; Li *et al.*, 2019). Generally, the production of XOS based on lignocellulose was carried out using direct autohydrolysis or pretreatment combined enzymatic methods. Direct autohydrolysis, using high-temperature and high-pressure water vapor or liquid water to directly depolymerize the semi-fibers, during the process would produce by-products such as formic acid, acetic acid, furfural, pentahydroxymethyl furfural (Ashraf and Schmidt, 2018), but this method was environmentally friendly, no or a small amount of corrosive compounds were involved. Pretreatment combined with enzyme method, the extracted xylan was pretreated by acid, alkali or hydrothermal method, and then xylanase was used to catalyze the production of XOS with the target degree of polymerization (Qian *et al.*, 2020). This method produced the usual xylose content in XOS that was high, the cost of enzymes was high, but the degree of polymerization was more consistent. In order to solve the problem of xylanase, it was important to find a strain that produces xylanase.

1.3 Xylanase Secreting Strain

The current research on xylanase was mainly focused on the screening of enzyme-producing strains and the construction of genetically engineered bacteria. A variety of microorganisms could produce xylanase, such as bacteria, yeast, fungi, protozoa, gastropods and arthropods (Collins *et al.*, 2005). Among them, xylanase was mainly produced by fungi and bacteria in extracellular secretion, because they secreted the enzyme into the culture medium through exocrine secretion, thereby avoiding the process of disrupting cells. Quite a few studies had reported strains that produce xylanase, such as *Bacillus pumilus* (Poorna and Prema, 2007), *Cellulomonas uda* (Rapp and Wagner, 1986), *Bacillus mojavensis* (Kallel *et al.*, 2015) , *Aspergillus niger* (Robl *et al.*, 2015), *Paecilomyces thermophile* (Yan *et al.*, 2008) and *Pichia stipitis* (Den Haan and Van Zyl, 2003) etc. A large amount of xylanase was produced on an industrial scale mainly from the fermentation broth of fungi belonging to the genus *Trichoderma, Aspergillus* and *Penicillium*. Therefore, commercially available xylanases were mainly derived from fungi (Zhuo *et al.*, 2018).

1.4 Research Objectives

The purpose of this study was to screen a fungus that could secrete xylanase extracellularly. Sequencing identification was performed by modern molecular biology methods to determine the species of the strain. In view of the current problems of long fermentation period and poor activity of xylanase, the fermentation conditions were optimized through single-factor experiments. In order to find the most suitable application conditions, the enzyme solution obtained under the optimal fermentation conditions was used to study the properties of xylanase in the solution, which also provided experimental and theoretical basis for the production and application of xylanase.

2. Method

2.1 Soil Sample and Chemical

Soil samples were collected near the roots of the plants in the Yellow Sea of Tongzhou Bay, Nantong, Jiangsu, China. Collect the soil sample in a sterile plastic bag, and sterilize the surface of the soil sample in a clean bench. The experimental reagents were purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd.

2.2 Culture Medium

Enrichment medium: yeast powder 1 g/L, peptone 2 g/L, commercial xylan 2 g/L, natural pH. Sterilize at 121 °C for 20 min.

Plate screening medium: commercial xylan 10 g/L, yeast powder 1.0 g/L, KNO_3 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.3 g/L, NaCl 0.5 g/L, K_2HPO_4 0.5 g/L, agar 20 g/L, pH 6.5. Sterilize at 121 °C for 20 min.

Incline preservation medium (PDA medium): potato 200 g/L, glucose 20 g/L, agar 20 g/L, natural pH. Sterilize at 115 °C for 30 min.

Seed medium: sucrose 10 g/L, yeast powder 5 g/L, NaCl 5 g/L, pH 6.5. Sterilize at 121 °C for 20 min.

Basic enzyme production medium: 20 g/L corncob (through 45 mesh sieve), 1 g/L commercial xylan, 5 g/L yeast powder, 0.5 g/L NaCl, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, K_2HPO_4 2 g/L, pH 5.5. Sterilize at 121 ^oC for 20 min.

2.3 Strain Screening

2.3.1 Initial Isolation of Strains

Take 10 g soil sample and mix it into 90 mL sterile distilled water, then add appropriate amount of glass beads, shake in a 200 rpm shaker for 30 minutes, and absorb 1 mL of the suspension in the enrichment medium at 30 ^oC, 180 rpm enriched and cultivated for 7 days. Take out the enriched bacterial solution by centrifugation, and then dilute it into 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} bacterial solution. Take 200 μL of the diluted bacterial solution and spread it on the plate screening medium. Incubate in a constant temperature incubator at 28 ^oC for 72 h. Pick a single colony with a transparent circle and fungal morphology on the plate, transfer it to a fresh plate screening medium for repeated purification, and cooperate with microscopy for morphological observation. The isolated and purified strains were transferred to PDA slant medium for storage at 4 ^oC.

2.3.2 Strain Isolation and Purification

The selected strains were inserted into 100 mL seed culture medium, 3 replicates in each group, and cultured at 30 ^oC and 180 rpm for 24 h. The strains in the seed solution were inoculated into the basic enzyme-producing medium with an inoculum of 5%, 3 replicates in each group, and cultured at 30 ^oC and 180 rpm for 3 days. The fermentation broth was frozen and centrifuged for 20 min to take the supernatant, and the enzyme activity was determined,

and the strain with higher enzyme activity was selected.

2.3.3 18S rDNA Detection

The TR08 strain was sent to Shenggong Bioengineering (Shanghai) Co., Ltd. for 18S rDNA sequencing analysis. The measured sequence was submitted to the National Center for Biotechnology Information (NCBI), and the basic search tool for partial sequence alignment (BLAST) was used for homology sequence comparison analysis in the gene bank (Khusro *et al.*, 2016).

2.4 Xylanase Activity Determination

The xylanase activity was determined according to the method in the literature (Pennacchio *et al.*, 2018). Definition of xylanase enzyme activity unit (IU/mL): the amount of xylanase enzyme required to hydrolyze xylan to produce 1 μmol of xylose and its homologues per minute under the conditions of 50 °C and pH 5.0. The formula for calculating xylanase activity is as follows:

$$\text{Xylanase activity (IU/mL)}=S\times D\times 1000/(V\times T\times 150)\times 1.7\ \text{(IU/mL)} \quad (1)$$

Note. S: the amount of xylose (mg) corresponding to the measured absorbance on the standard curve; D: the dilution factor of the enzyme solution; 1000: the conversion factor between mg and μg; V: the amount of enzyme solution taken (mL); T: Reaction time (min); 150: conversion factor between g and mol; 1.7 method conversion factor.

2.5 Determination of the Enzyme Production Process of the Strain

After the strain was inoculated, take 2 mL of fermentation broth every 12 h, and measure the enzyme activity and protein content of the fermentation broth respectively, until the enzyme activity and protein content of the fermentation broth no longer changed significantly, and each level was repeated three times. The amount of protein was determined according to the Coomassie Brilliant Blue method (Grintzalis *et al.*, 2015).

2.6 Optimization of Fermentation Conditions

Other fermentation conditions remained unchanged, a single-factor experiment was designed, and the initial fermentation pH and fermentation temperature of the fermentation broth were successively changed. Set the initial pH of the fermentation broth to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, take the fermentation broth after 156 h of fermentation to measure the enzyme activity, and compare the enzyme activities to determine the optimal initial pH of the fermentation broth. Set the fermentation temperature to 28, 30, 32, 35, 37, and 40 °C under the optimum initial pH of the fermentation broth. Take the fermentation broth after 156 h of fermentation to measure the enzyme activity, and compare the enzyme activities to determine the optimum fermentation temperature.

2.7 Enzymatic Properties Research

2.7.1 Optimum Reaction Temperature of Enzyme

Take the enzyme solution after dialysis and desalination, and set the reaction temperature to

40, 45, 50, 55, 60, 65, and 70 °C in a buffer with pH 7, and determine the xylanase activity at different reaction temperatures. The untreated xylanase activity was defined as 100%, and each level was repeated 3 times.

2.7.2 Optimal Reaction pH of Enzyme

First configure buffers with different pHs: CA-Na_2HPO_4 buffer (pH 3~4), HAC-NaAC buffer (pH 5), K_2HPO_4-KH_2PO_4 buffer (pH 6~8), Gly-NaOH buffer (pH 9~10) and Na_2HPO_4-NaOH buffer (pH 11). The enzyme solution after dialysis desalination was dissolved in different pH buffers, and the enzyme activity under different pH conditions was measured. The untreated xylanase activity was defined as 100%, and each level was repeated 3 times.

2.7.3 Enzyme Temperature Stability

Take the enzyme solution after dialysis and desalination, and keep it in a buffer with pH 7 and a water bath at 40, 45, 50, 55, 60, 65, 70 °C for 0.5, 1, 2, 4 h, and measure the enzyme activity at different temperatures and different times. The untreated xylanase activity was defined as 100%, and each level was repeated 3 times.

2.7.4 pH Stability of Enzymes

Take the enzyme solution after dialysis and desalination, and keep it at 0 °C and pH 3~11 buffer solution for 0.5, 1, 2, 4 h, and measure the enzyme activity at different pH and different time. The untreated xylanase activity was defined as 100%, and each level was repeated 3 times.

2.7.5 The Effect of Metal Ions on Enzyme Activity

Under optimal conditions, add 0.05 mM various metal ions to the diluted crude enzyme solution. The xylanase enzyme activity after adding metal ions was determined, and the enzyme activity of the crude enzyme solution without metal ions was defined as 100%, and each level was repeated 3 times.

2.7.6 The Effect of Surfactants on Enzyme Activity

Under the optimal conditions, 0.05 mM surfactant (Sodium dodecyl sulfate, Tween 60-80, hexadecyltrimethylammonium bromide, Ethylene diamine tetraacetic acid, Polyethylene1glycol 1000-6000) was added to the diluted crude enzyme solution, and the xylanase enzyme activity after adding the surfactant was measured. Define the enzyme activity of the crude enzyme solution without added surfactant as 100%, and each level was repeated 3 times.

3. Results

3.1 Screening Results of Xylanase-Producing Strains

The strains with obvious clear circles during the initial screening were inoculated on selective medium for repeated separation and purification, and the enzyme activity of 22 strains with larger clear circles was determined. As shown in Table 1, the TR08 strain had the highest

xylanase enzyme activity and was a potential strain with high xylanase production. At the same time, it was found that the strains with transparent circles on the surface of the selective medium may not have the ability to produce xylanase, and it may be that the commercial xylan itself contains a small amount of impurities such as cellulose and sucrose.

Table 1. Enzyme activity of strains after 72 hours of culture

Serial number	Enzyme activity, IU/mL	Serial number	Enzyme activity, IU/mL
TR01	11.34	TR12	0.19
TR02	2.73	TR13	1.58
TR03	2.33	TR14	5.26
TR04	0.78	TR15	4.24
TR05	8.95	TR16	0.17
TR06	3.18	TR17	3.24
TR07	3.69	TR18	7.66
TR08	28.67	TR19	2.04
TR09	0.89	TR20	0.45
TR10	1.27	TR21	0.68
TR11	4.61	TR22	5.24

3.2 Identification of TR08 Strain

As shown in Figure 1, the TR08 strain was cultured on a screening medium plate at 28 °C for 72 hours. The colony texture was loose, white and light yellow at first, and then yellowish brown. The conidiophore was divided into upper and lower layers, with apical sacs, and the conidia head was radial. The conidia were pear-shaped or ovoid when they were young, and most of them became spherical or sub-spherical after senescence.

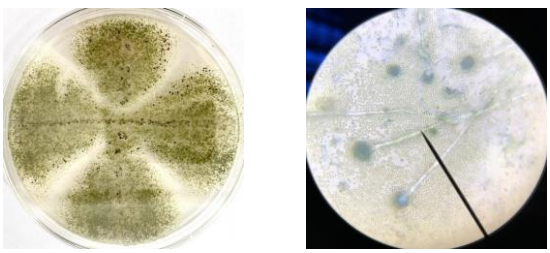


Figure 1. TR08 colony and strain morphology

As shown in Figure 2, PCR(Polymerase chain reaction) amplification of genomic DNA yielded a band of about 1321 bp. TR08 strain and *Aspergillus oryzae* RIB40 the similarity of the 18S rDNA of was 99.70%, which was the closest to its genetic relationship, so the TR08 strain was classified as *Aspergillus oryzae*. As shown in Figure 3, use MEGA-X software to construct its phylogenetic tree.

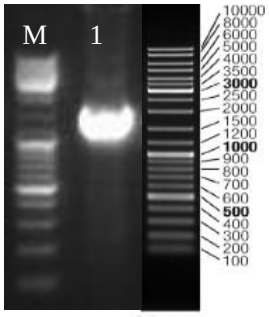


Figure 2. Electrophoresis of PCR products

Note. M, Marker; 1, 18S rDNA of TR08 strain

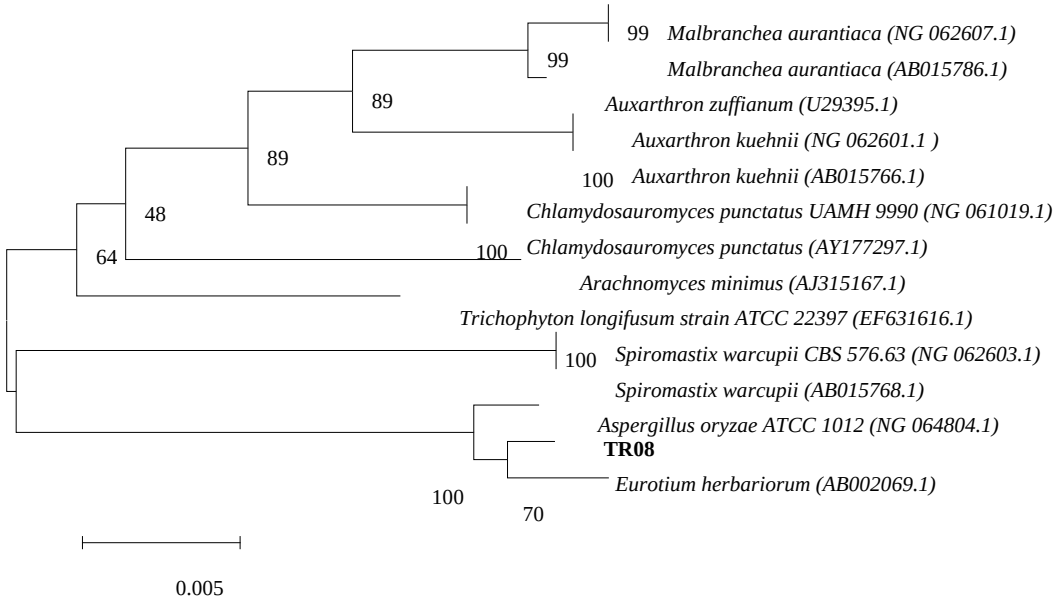


Figure 3. Phylogenetic tree of TR08 strain

3.3 Enzyme Production Process Determination

As shown in Figure 4, after 156 h of fermentation of TR08 strain, the enzyme activity and specific activity in the fermentation broth reached peak values of 451 IU/mL and 1963 IU/mg, respectively. The strains in the pre-fermentation stage needed to complete material accumulation to meet their own growth and metabolism. Therefore, the fermentation before 96 h did not secrete or secretes a very small amount of xylanase, and produced a small amount of protein necessary for growth. When the strain entered the logarithmic growth phase, the amount of enzyme produced with the exponential increase in the biomass of the strain also increased, and the enzyme activity in the fermentation broth also increased accordingly. When the growth of the strain entered the stable period, the enzyme activity and

specific activity in the fermentation broth reached the highest at this time. Therefore, in the optimization of fermentation conditions and the study of enzymatic properties, the fermentation time was selected to be 156 h.

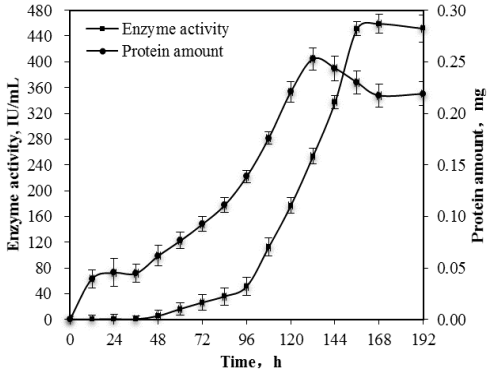


Figure 4. Determination of the enzyme production process of TR08 strain

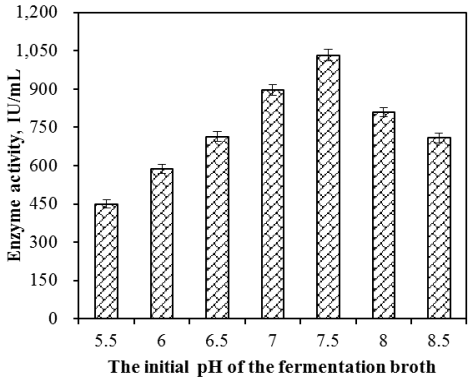
3.4 Ancillary Analyses

As shown in Figure 5, the initial pH of the fermentation broth had a great influence on the enzyme activity in the fermentation broth. As the pH increased from 5.5 to 7.5, the enzyme activity of the strain increased rapidly after 156 hours of fermentation, reaching a maximum of 1033 IU/mL at the initial pH of 7.5. The proliferation of strains required the participation of various enzymes, and the enzyme activity must be at a certain pH and temperature to play a role. The increasing initial pH of the fermentation broth created pH conditions for the accumulation of essential nutrients in the initial growth of the strain, and also improved the permeability of the strain's cell membrane and growth and metabolism to a certain extent (Potocnik *et al.*, 2019), greatly shortening the strain's sluggishness period, promoted the proliferation of strains. The enzyme activity decreased rapidly after the pH continued to increase from 7.5 to 8.5, also due to the initial pH environment that affected the growth of the strain. Therefore, the initial pH of the fermentation broth was selected to be 7.5 for the subsequent optimization of the fermentation temperature.

It can be seen from Figures 6 that temperature had an important effect on the activity of xylanase. When the temperature was between 28 °C and 32 °C, as the temperature increases, the enzyme activity of the strain increased after 156 h of fermentation, reaching a maximum of 1264 IU/mL at a fermentation temperature of 32 °C. At this time, the increase in temperature significantly increased the enzyme activity of the fermentation broth, because a large amount of heat was required for frequent growth and metabolism activities at each stage of the growth of the strain. At the same time, the various enzymes required for the growth and development of the strains needed suitable temperature conditions for efficient catalysis, including the synthesis of xylanase-related substances in the strains. When the fermentation temperature increased from 32 °C to 40 °C, the enzyme activity of the fermentation broth gradually decreased. At too high temperature, the strain not only needed to resist the energy consumption caused by the too high temperature to maintain its own growth and metabolism,

but also a small part of the enzyme protein might lose activity at the high temperature.

In summary, it was determined that the initial pH of the fermentation broth was adjusted to 7.5 in a shaker at 180 rpm and 32 °C. At this time, the fermentation conditions were optimal. After 156 h of fermentation, the maximum enzyme activity was 1264 IU/mL.



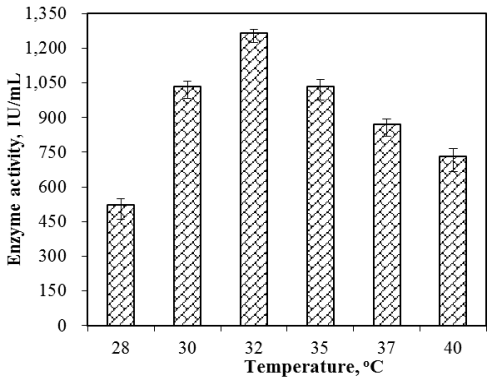


Figure 5. Effect of initial pH of TR08 fermentation broth on enzyme activity

Figure 6. Effect of TR08 Fermentation Temperature on Enzyme Activity

3.5 Enzymatic Properties Research

3.5.1 Optimum Reaction Temperature of Enzyme

As shown in Figure 7, the optimal reaction temperature of the enzyme was 55 °C. When the reaction temperature was increased from 40 °C to 55 °C, the relative enzyme activity increased rapidly, because the temperature rise at this time would provide more energy for the enzyme's catalytic reaction, while maintaining the invariance of the enzyme protein. When the reaction temperature continued to rise from 55 °C to 70 °C, the relative enzyme activity dropped rapidly. At this time, too high temperature would denature part of the enzyme protein with loose conformation. However, the enzyme still retained 61.4% of the relative enzyme activity when reacting at 70 °C. According to Figure 9, it is found that this is due to the catalytic effect produced by the incomplete denaturation of the enzyme protein in the early stage of the reaction, not the heat resistance of the enzyme protein itself.

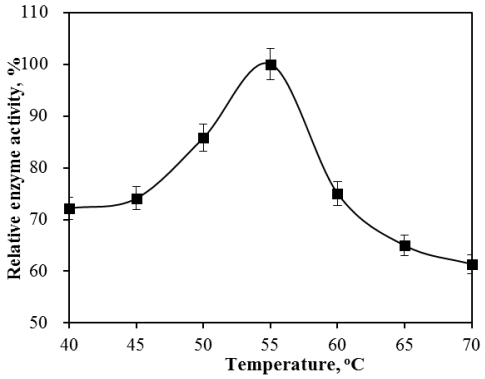


Figure 7. Optimum reaction temperature of xylanase

3.5.2 Optimal Reaction pH of Enzyme

As shown in Figure 8, the optimal reaction pH of the enzyme was 5. When the reaction pH was between 3 and 5, the relative activity of the enzyme rose rapidly as the pH value rose, and when the pH continued to rise from 5 to 11, the relative activity of the enzyme began to drop rapidly to 4.1%, and finally remained basically unchanged. On the one hand, the change in pH affected the charged state of the substrate and the enzyme protein, thereby affecting the binding of the substrate and the enzyme. The closer the enzyme was to the optimal reaction pH, the more suitable the dissociation state of the active group of the enzyme was for the specificity binding of the substrate; on the other hand, under strong acid and strong alkaline conditions, the highly regular structure of the protein became a disorderly loose stretched structure, especially the secondary and tertiary structure was destroyed.

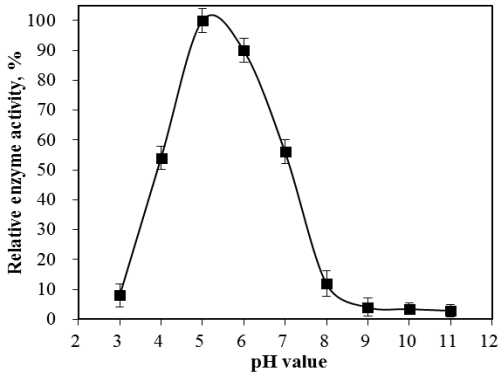


Figure 8. Optimum reaction pH of xylanase

3.5.3 Enzyme Temperature Stability

As shown in Figure 9, the relative enzyme activity of the xylanase remained 75.8% after being kept at 40~50 °C for 1 h, and after 4 h, the relative enzyme activity remained more than 61.3%, almost no longer Decrease; under the condition of 60~70 °C, after keeping for 1 h, the enzyme activity dropped rapidly to below 33%, and after keeping for 4 h, the enzyme activity was very low or almost undetectable. It was concluded that the enzyme had a certain degree of heat resistance, but could not withstand high temperatures. Interestingly, the enzyme retained only 43.0% of its activity after 4 hours of incubation at the optimal reaction temperature of the enzyme. This further showed that the optimal catalytic conditions of the enzyme were not suitable for the preservation of the enzyme. This brought the difficulties to

industrialization of the enzyme.

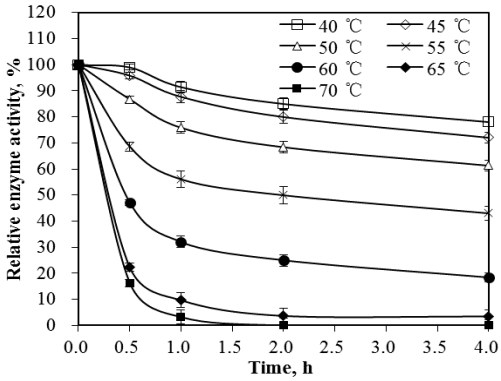


Figure 9. Temperature stability of xylanase

3.5.4 pH Stability of Enzymes

As shown in Figure 10, the xylanase still retained more than 87.3% relative enzyme activity after being kept in a buffer solution at 0 °C and pH 5~6 for 4 h; while at 0 °C pH 3~4 and pH 8~11 after being kept in the buffer solution for 4 h, the enzyme retained at least 50.0% of the enzyme activity. This result seemed to be inconsistent with the results under strong acid and strong alkali when investigating the optimal reaction pH determination. Compared with the reaction conditions, it was found that the two reactions had a high temperature difference, obviously due to the combined effect of temperature and pH, the enzyme protein was basically lost Biological activity. According to the results of pH stability measurement, it could be determined that the enzyme had acid and alkali resistance at low temperatures, which made the preservation of the enzyme very easy.

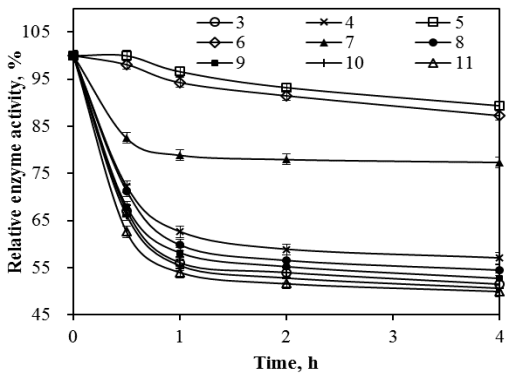


Figure 10. pH stability of xylanase

3.5.5 The Effect of metal Ions on Enzyme Activity

As shown in Figure 11, Fe^{3+} alone increased the catalytic activity of the enzyme by 8.3% compared to the blank. Al^{3+}, Fe^{2+}, and Co^{2+} plasmas had almost no effect on the enzyme activity, while Zn^{2+}, Mn^{2+}, and Ca^{2+} had no effect on the enzyme activity. The inhibition of enzyme activity was the most obvious, but these three ions only reduced the biocatalytic activity of the enzyme by less than 20%. In addition, the effect of Fe^{3+} concentration (0-3 mM) on enzyme activity was studied in a buffer solution at 55 °C and pH 5.0. As shown in

Figure 12, it was found that the catalytic activity of low concentration Fe^{3+} enzyme had a promoting effect, but after the Fe^{3+} concentration reached 0.5 mM, the high concentration Fe^{3+} began to inhibit the catalytic activity. When the Fe^{3+} concentration was 0.25 mM, the xylanase activity produced by this strain reached the highest value.

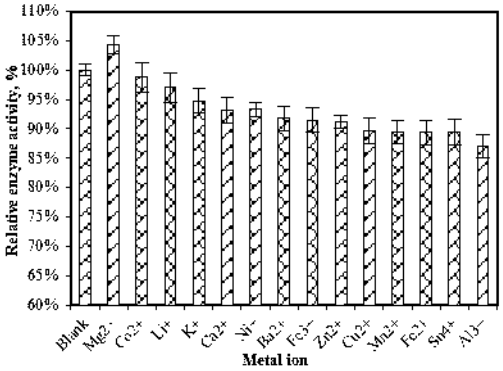


Figure 11. The influence of different metal ions on xylanase

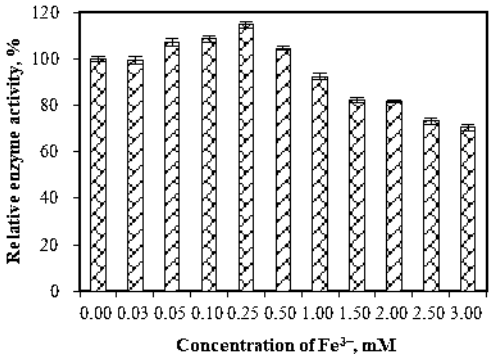


Figure 12. The effect of Fe^{3+} ion concentration on xylanase

3.5.6 The Effect of Surfactants on Enzyme Activity

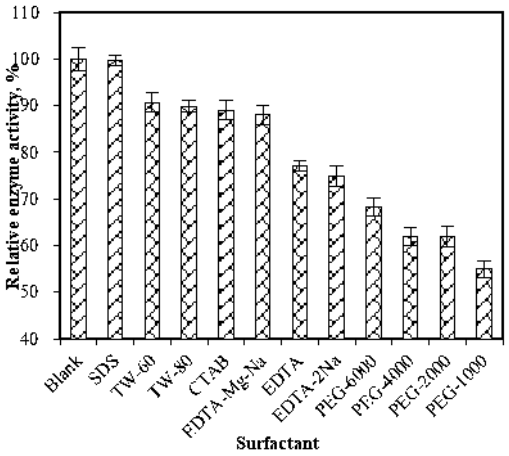


Figure 13. The effect of surfactants on the activity of xylanase

As shown in Figure 13, compared with the blank, only SDS did not affect the catalytic activity of the xylanase. TW60, TW80, CTAB, and EDTA-Mg-Na inhibited the catalytic

activity of the xylanase lower, but they could also Maintained above 89%, and PEG4000, PEG2000, PEG1000 inhibited the enzyme catalytic activity most significantly, reducing the biocatalytic activity of the enzyme to less than 60%.

4. Discussion

In this study, an Aspergillus oryzae strain TR08 (*spergillus sp.*) that secretes xylanase from the soil was successfully isolated. Through the determination of the enzyme production process and the optimization of fermentation conditions, the optimal fermentation conditions for TR08 enzyme production were determined. TR08 was in a shaker at 180 rpm and 32 °C. The initial pH of the fermentation broth was 7.5. After 156 hours of fermentation, the enzyme activity of the fermentation broth reached a maximum of 1264 IU/mL. In addition, the optimal reaction temperature and pH value of xylanase produced by TR08 are 55 °C and 5.0, respectively, and the pH stability is high. 0.25 mM Fe^{3+} can increase the catalytic activity of the enzyme by 15%. The biological activity of the enzyme is not inhibited in the environment of 0.05 mM SDS. These features help reduce the preservation cost of the enzyme and expand its application range. The isolation of the TR08 strain provides a low-cost, high-activity xylanase for the subsequent enzymatic catalysis of lignocellulose to produce xylanase, and also provides an experimental and theoretical basis for the study of xylanase produced by Aspergillus oryzae.

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