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Communication

Solid-State Fermentation of Hyperactive Pectinase by the Novel Strain *Aspergillus* sp. CM96

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Abstract: Pectinase, a kind of hydrolase, mainly contains polygalacturonase, pectinase, and pectin lyase, which can hydrolyze pectin to generate galacturonide and is widely used in industry. At present, pectinase's activity is still relatively low. Hyperactive pectinase was produced with solid-phase fermentation and a tray bioreactor using the novel strain *Aspergillus* sp. CM96 in this study. This pectinase's activity can reach 17,000 U·g^{-1} after fermentation with a tray bioreactor, an increase of 86% compared to that obtained using flask liquid fermentation. The pectinase was purified and its characteristics were explored. Additionally, during pectinase fermentation, the activities of protease, glucanase, and cellulase were also determined to reach 7000, 8000, and 3000 U·g^{-1}. The enzyme mixture was used to improve substrate digestion efficiency in 144 Soviet white pigs after adding a 0.05% cocktail enzyme for 38 days. The results showed that the average daily gain (ADG) increased by 139.41 ± 1.04 g·day^{-1}, while the average daily feed intake (ADFI) and the feed conversion rate (FCR) decreased by 19.82 ± 1.64 g·day^{-1} and 0.07 ± 0.01 in 38 days, which indicated that the addition of hyperactive pectinase from the strain CM96 can increase nutrient digestibility and improve feed efficiency.

Keywords: *Aspergillus* sp.; hyperactive pectinase; solid-state fermentation; nutrient digestibility; enzyme cocktail

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1. Introduction

Pectin is an acidic heteropolysaccharide found in the cell walls and the primary and middle lamella of land plants [1–3]. Pectin is generally exploited as a gelling and stabilizing agent in the food and cosmetic industries. Pectin also is a common anti-nutritional factor in plant feed, which affects the effective absorption of feed nutrients by animals [3–5]. Pectinase is a kind of hydrolase and contains polygalacturonase, pectinase, and pectin lyase, which can hydrolyze pectin to generate galacturonide with a lower molecular weight and are mainly used for fruit processing, food, pharmaceuticals, and many other industrial fields [1,3–7]. Pectinase plays an important role in the field of biotechnology catalysis and accounts for 1/4 of the biological enzyme preparation market. Pectinase usually comes from fungi, bacterium, and yeast fermentations, and now filamentous fungi, such as *Aspergillus* sp., are often selected for pectinase production [1,3,7–9]. Therefore, microorganisms are currently the main means of the industrial production of pectinase [3,9,10]. However, the most critical factor is determining whether product quality and yield tally with the standard for the microbial strains used in the industrial enzyme fermentation process [9,10].

Plant-based feeds contain a high amount of non-starch polysaccharides (NSPs), which are indigestible for livestock and poultry [11,12]. Exogenous enzymes can improve the digestibility of nutrients and the feed efficiency of livestock and poultry [13–15]. Exogenous enzymes can hydrolyze some specific chemical bonds present in the undigestible components of feed ingredients in livestock and poultry [13–15], such as plant materials.

Therefore, feed-substrate-specific enzymes are economically sustainable, and the most widely used feed enzymes are pectinase, protease, glucanase, pectinase, and cellulase. An exogenous enzyme cocktail, such as carbohydrase, can break the specific chemical bond in plant-based diets [12,13,15,16].

In this study, the novel strain was employed for pectinase production by solid-state fermentation. In order to reduce the cost, we utilized crop waste soybean meal to reduce the production cost of hyperactive pectinase, and a mixture of enzymes containing pectinase, protease, glucanase, and cellulase was produced by solid-state fermentation. Compared with liquid fermentation, solid fermentation saves costs regarding equipment, production, and operation, and bacterial pollution is less likely to occur during operation [17]. The pectinase's characteristics show that the pectinase has a good pH and the temperature stability of strain CM96 shows favorable application prospects in feed biotechnology.

2. Materials and Methods

2.1. Microorganism and Chemicals

A wild type of *Aspergillus* sp. CM96 with increased pectinase activity and yield was isolated from soils located at the south of Changzhou, Jiangsu Province, China [13,18,19], and stored in the Lab of Applied Microbiology, Changzhou University, and China General Microbiological Culture Collection Center (CGMCC No. 40340).

The DNA and protein markers, 2 × Loading Buffer, SDS-PAGE gel preparation kit, TRIzol Lysate, M-MuLV First-Strand cDNA Synthesis Kit, and 2 × SG Fast qPCR Master Mix used in the experiment were purchased from Sangon Biotech (Shanghai, China); glucose, peptone, ammonium sulfate, and other chemical reagents are analytical reagents and were purchased from Sinopharm Chemical Reagent (Shanghai, China).

2.2. Optimization of Fermentation Substrate Composition and Condition

The main sources of carbon and nitrogen in this experiment were wheat bran, soybean meal, soybean cake, soybean meal, and cottonseed meal. Then, the total moisture content of the substrate was controlled at 60%. Fermentation optimization took into account substrate composition, additional carbon and nitrogen sources and their concentrations, different metal ions and their concentrations, and the water content of pectinase fermentation. The spores of *Aspergillus* sp. CM96 were inoculated into the fermentation medium and cultured at 28 °C for 120 h. The concentration of the spore suspension is 10^7–10^8 spores/mL.

2.3. Tray Bioreactor Design and Configuration

The bioreactor was designed as shown in Figure 1 for the fermentation of pectinase. The air was filtered and pumped into the bioreactor from the bottom, and escaped from the top. The depth of the fermentation bed was approximately 1.5~2.0 cm to avoid temperature gradients in the bed [13,19]. The temperature and moisture were set at 28 °C and approximately 75%, respectively, which could minimize water loss in the fermentation medium because of evaporation. Each tray was filled with 300–400 g of dry fermentation medium, and samples were taken every 24 h to measure pectinase activity and cell growth. The fermentation process lasted 96–120 h.

2.4. Enzyme Fermentation and Purification

Pectinase was extracted using 0.1 M phosphate-buffered saline, pH 7.2, in the following steps: 30 mL of phosphate buffer was added to a flask of 5 g of fermentation medium for 72 h and placed in a shaker at 28 °C, 200 rpm, for 30 min. Then, the mixture was centrifuged at 5000 rpm, 4 °C, for 10 min. The supernatant was a crude pectinase solution and stored in a 4 °C freezer for further analysis and purification. Pectinase was purified as previously specified [19–21].

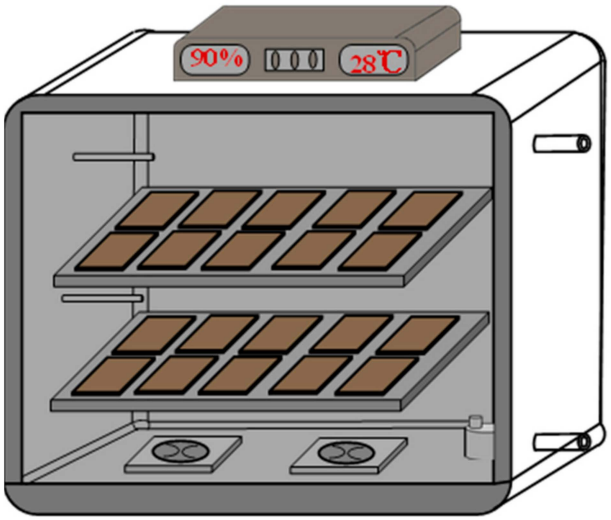


Figure 1. Schematic diagram of a benchtop plate bioreactor.

The enzyme activities were determined at different temperatures (45–75 °C) in the Na_2HPO_4–citric acid buffer (pH 3.5) and pH values (3.0–8.0) in order to study the optimal temperature and pH for pectinase. After incubation at temperatures of 45, 50, 55, 60, 65, 70, and 75 °C and different pH values (3.0–8.0) for 0, 30, 60, 90, and 120 min, respectively, residual pectinase activity was determined to investigate its thermal and pH stability. Different metal ions (Na^+, Mg^{2+}, K^+, Fe^{2+}, and Ca^{2+}) were added to the purified enzyme at final concentrations of 1 mmol/L, 5 mmol/L, and 10 mmol/L to test their effects on pectinase activity. The combination was preincubated for up to an hour at 4 °C. Under optimal conditions, the enzyme's residual activity was measured [20–22].

2.5. Analytical Methods

The method used for the determination of pectinase activity refers to Lipase preparations (GB/T 23535-2009). The unit of pectinase activity was defined as the hydrolysis of 1 μmol of pectin ($U \cdot g^{-1}$) in one minute at a certain temperature and pH. Bovine serum albumin (BSA) was used as the standard protein, and the protein content was determined by the Bradford method [13].

Enzyme activity was measured by adding 5 mL pectin solution into colorimetric tubes A and B and preheating them in a constant-temperature bath at 50 °C ± 0.2 °C for 8 min. A total of 5 mL citric acid–sodium citrate buffer was added to the nail tube (blank); 1 mL diluent enzyme solution and 4 mL citrate–sodium citrate buffer were added into tube B (sample), shaken well immediately, timed, and accurately reacted for 30 min before being immediately removed. Then, another tube was taken, 1 mL reaction solution was measured, and 3 mL 3,5-Dinitrosalicylic acid (DNS) reagent was added, heated, and boiled for 5 min, before terminating the reaction, cooling the mixture, filling the tube with distilled water to 25 mL, and shaking it well. The absorbance value at 540 nm was measured, with the nail tube used as the control. Parallel sample measurements were taken at the same time.

The enzyme recovery rate calculation formula was as follows: enzyme recovery rate = (total activity of enzyme preparation/total activity of crude enzyme solution) × 100% [17].

3. Results and Discussion

3.1. Effect of Medium Composition on Pectinase Activity

Wheat bran, soybean meal, soybean cake, and soybean powder were selected as the main carbon and nitrogen sources for pectinase fermentation. The results in Figure 2A show that pectinase activity reaches its maximum when soybean meal or a mixture of wheat bran and soybean meal was used as the solid fermentation substrate. The mixture of wheat bran and soybean meal is fluffy, which can promote the growth of hypha and spores

of the strain CM96. The mixture of bran and soybean meal was selected as a fermentation substrate for pectinase production based on the lower economic cost of raw bran materials.

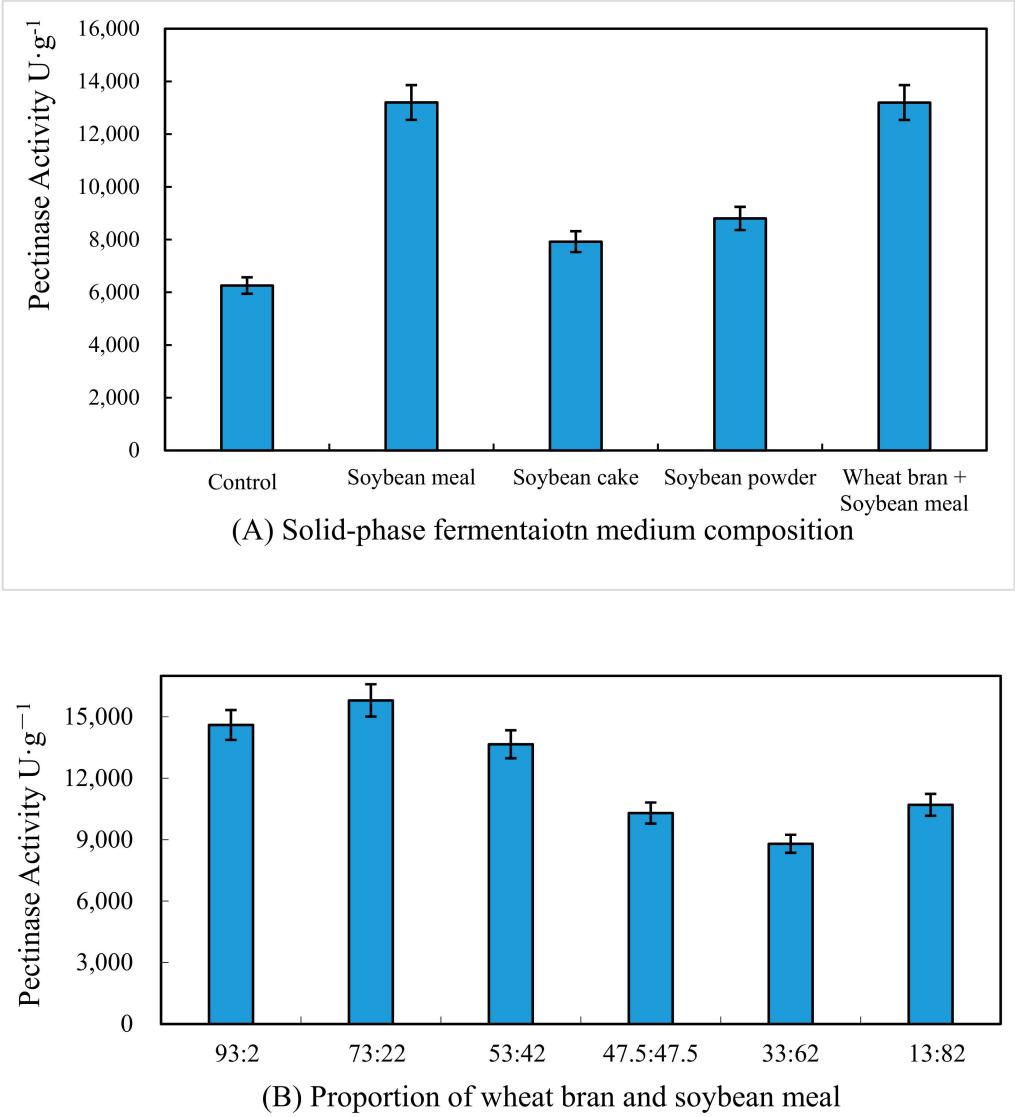


Figure 2. Effect of solid substrates (**A**) and their proportion (**B**) on pectinase fermentation by *Aspergillus* sp. CM96.

The C/N ratio is a key parameter for the growth of fungal cell and enzyme activity. Wu et al. reported that the ratio of bran and soybean was 53/42 (m/m), and the enzyme activity of *Aspergillus* sp. SH312-26-19 reaches its maximum, 2518.69 U·mL^{-1} [23]. The effect of the proportion of bran and soybean meal (*w*/*w*, 93/2, 73/22, 53/42, 47.5/47.5, 33/62, 13/82) on pectinase activity and yield was investigated in this study, and the results are shown in Figure 2B. The pectinase activity reached its maximum (15,127 U·g^{-1}) at a proportion of 73/22 (m/m).

3.2. Effect of Water Content and Inoculant on Pectinase Activity

The effect of water content (45–65%, *w*/*w*) and inoculant (0.5–4.5%, *w*/*w*) on the enzyme production by strain CM96 were investigated. The results are shown in Figure 3A,B. Pectinase activity reached its maximum at 60% water content. A reduction in water content reduces the growth rate of microbial cells, and the addition of excessive water is not conducive to the ventilation and heat dissipation of solid media, resulting in reduced enzyme activity [9,13,18]. Pectinase activity with 3.5% (*w*/*w*) inoculant was

15,200 U·g^{-1} and achieved the maximum enzymatic activity. The use of less inoculant could prolong the fermentation process, and the use of more inoculant could also affect enzyme production [13,16,24].

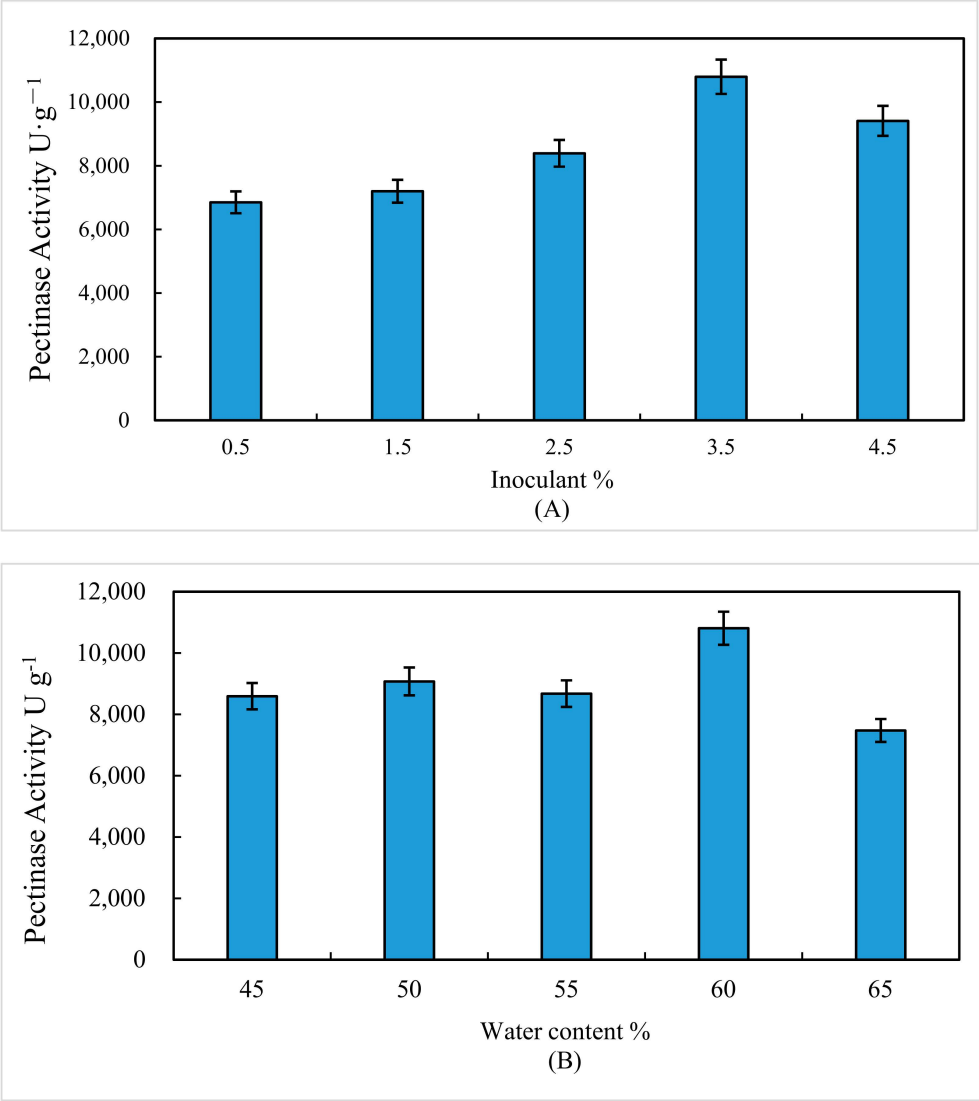


Figure 3. Effect of inoculant (**A**) and substrate moisture (**B**) on pectinase activity.

3.3. Effect of Additional Metal Ion on Pectinase Activity

Additional K^+, Na^+, Mg^{2+}, Ca^{2+}, and Fe^{2+} were added to the fermentation medium, and the effect of metal ion concentration (0.4‰, 0.6‰, 0.8‰, 1.0‰, 1.2‰ *w*/*w*) on pectinase activity was investigated. The effects of different concentrations of ions on pectinase fermentation are different, as shown in Figure 4A,B. The mycelium of strain CM96 is smaller, and seems a little yellow with a small number of spores, after the addition of K^+ and Ca^{2+} to the fermentation medium. The results show that K^+ and Ca^{2+} can inhibit strain CM96's growth and pectinase activity. While Na^+, Mg^{2+}, and Fe^{2+} can promote strain CM96's growth and increase pectinase activity, the mycelium is longer and thicker, and the spores are numerous and black. When Mg^{2+} is used as the metal ion, pectinase activity has the maximum value, and Mg^{2+} is essential to pectinase's structure. The results are consistent with the previous literature [9,13,18]. In Figure 4B, the addition of 0.6‰ of Mg^{2+} could greatly enhance the enzyme-producing ability.

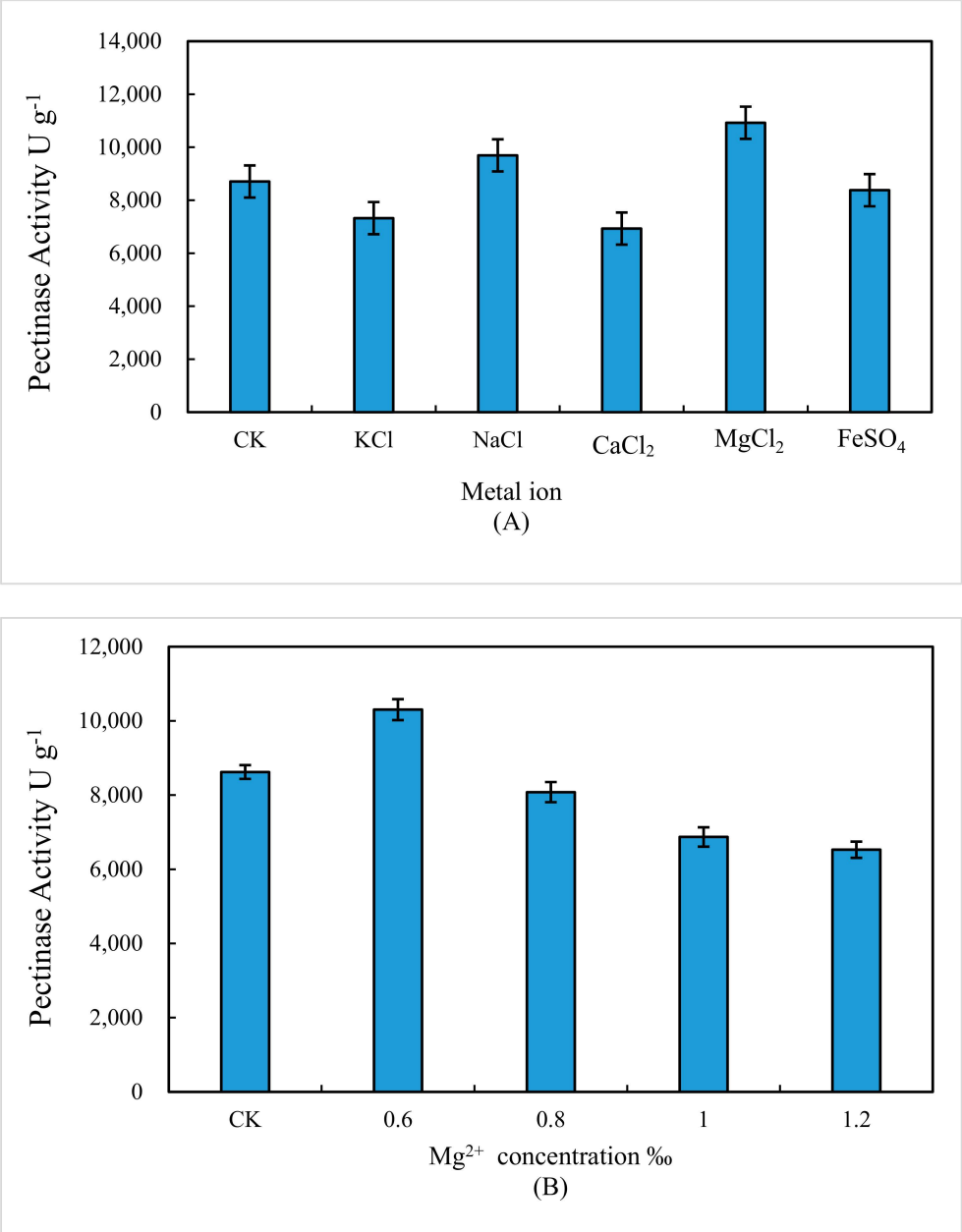
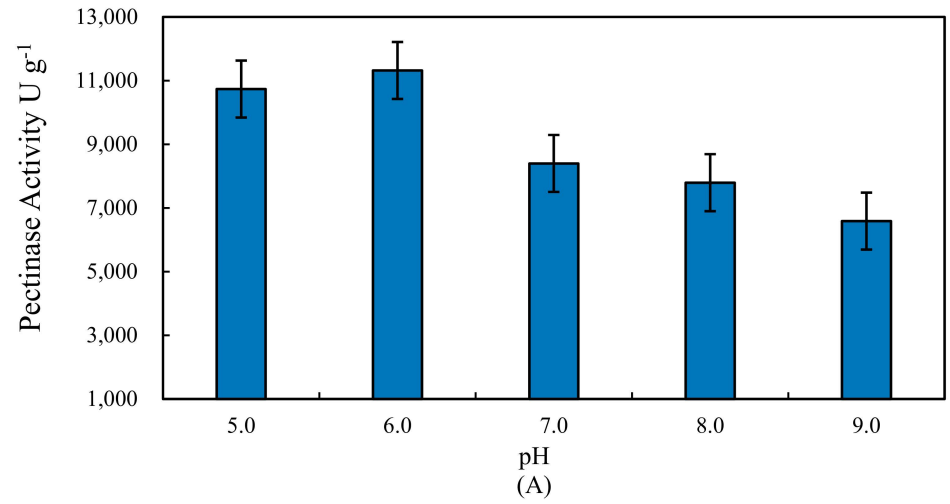


Figure 4. (**A**) Effect of metal ions on enzyme production by strains. (**B**) Effect of Mg^{2+} concentration on enzyme production by strains. Note: CK stands for control group.

3.4. Effect of Temperature and Initial pH Value on Pectinase Activity

Microbial fermentation is sensitive to environmental temperature, and the optimal growth temperature varies depending on microbial species. Strain CM96 belongs to Aspergillus sp. and can be grown at around 28~30 °C. The effect of a temperature ranging from 22 to 34 °C on pectinase fermentation and activity was investigated. As shown in Figure 5B, temperature has great impact on cell growth and pectinase synthesis, and significantly affects the number of hyphae and spores. When the fermentation temperature is set at 31 °C, pectinase activity reaches its maximum. Similarly, the initial pH has a great influence on the growth of bacteria, the spore number, and pectinase activity as shown in Figure 5A. When the initial pH is 6.0, it is most conducive to pectinase synthesis, and a higher or lower pH value can inhibit CM96 growth and pectinase fermentation. When the pH gradually increases, the number of hyphae and spores also decreases, indicating

that strain CM96 is not suitable for growing in a medium-alkaline environment. Pectinase activity reaches the maximum value under the slightly acidic conditions of pH 6.0.



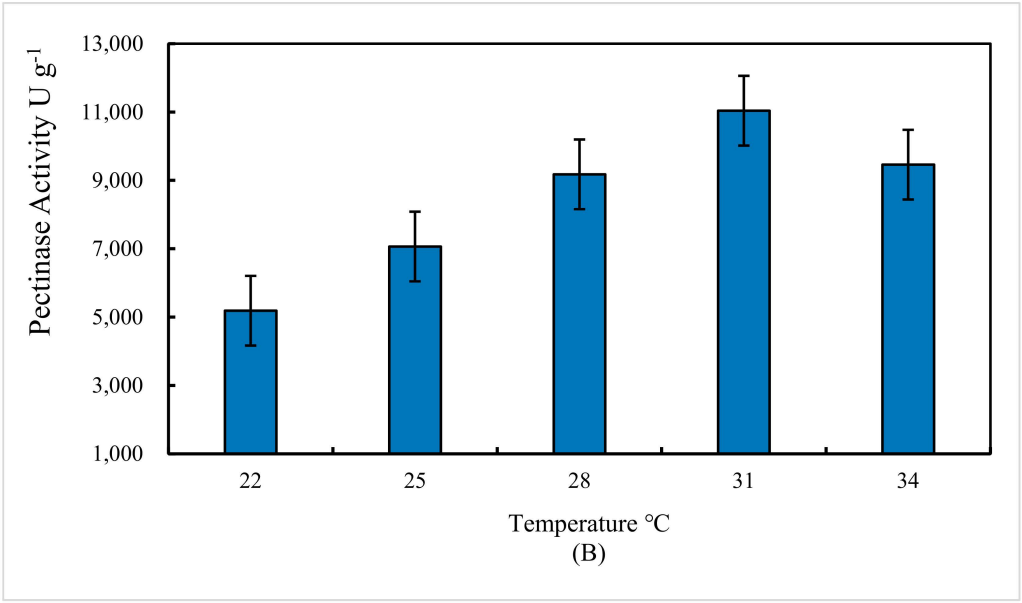


Figure 5. Effect of pH value and temperature on enzyme production ((**A**) pH; (**B**) temperature).

3.5. Pectinase Fermentation and Purification

Based on the above optimization results, a tray bioreactor (Figure 6) was designed for the production of pectinase. During the pectinase fermentation process, other enzymes including proteinase and glucanase were also determined, and their activity is shown in Table 1. The results showed that pectinase, protease, and glucanase were produced simultaneously during the solid fermentation process, and the pectinase activity reached the maximum value at 144 h of fermentation, while the maximum value of protease and dextranase reached the maximum value at 72 h of fermentation. The yields of pectinase, protease, and glucanase in the ray bioreactor were significantly, at 72.3%, 36%, and 41.5%, respectively, higher than those in the flasks. The results of this study were consistent with those previously reported for filamentous fungi, with a better enzyme production performance being obtained in the static bed [13,19].

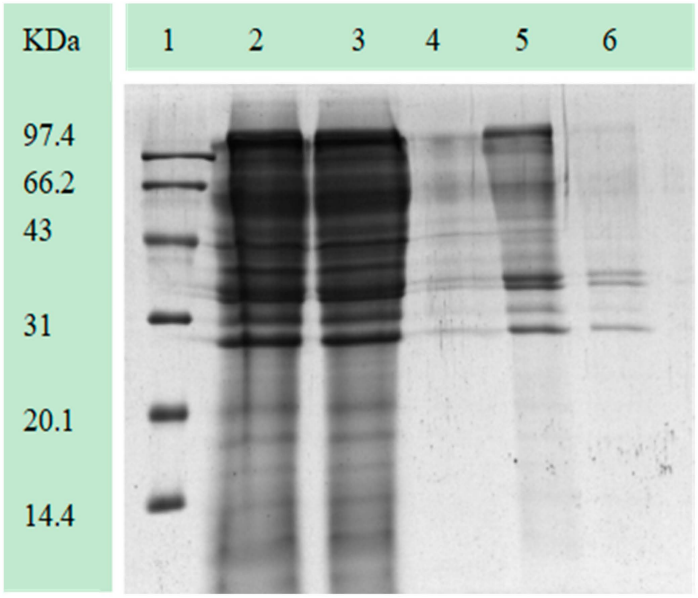


Figure 6. SDS-PAGE electrophoresis of solid fermentation cocktail enzymes with purified pectinase (1. marker; 2 and 3. enzyme cocktail; 4. 50% of ammonium sulfate; 5. 80% of ammonium sulfate; 6. purified lipase).

Table 1. Cocktail enzyme activity.

Kind of Enzyme	Activity ($U \cdot g^{-1}$)
Glucanase	7000 ± 400
Protease	8000 ± 600
Pectinase	15,000 ± 700
Cellulose	3000 ± 200

Pectinase was purified 50.33 times through ammonium sulfate precipitation and chromatography. The recovery rate was 33.67%, and the specific activity was 65,366.67 U/mg. The molecular weight of purified pectinase is 30 kDa.

3.6. Characteristics of Purified Pectinase

Temperature and pH are the most important factors when evaluating enzymatic activity. Based on relative activity, the activity of pectinase at different pH and temperatures was reported [4,25]. In this study, the maximum activity under the optimal conditions was considered to be 100% and was compared with other conditions [4,26]. In addition, the thermal stability and pH during the process were evaluated based on residual activity, and the initial activity of the pectinases was 100% at different temperatures and pH [27].

Temperature plays a very important role. As shown in Figure 7A, the activity of free pectinase showed a trend of first increasing and then decreasing with the gradual increase in temperature, and the maximum activity was obtained at 50 °C. When temperature exceeded 50 °C, pectinase activity decreased rapidly; pectinase activity was only less than 20% when reacting at 50 °C for 30 min. This result indicated that certain conformation changes occurred at 50 °C, leading to a significant decrease in activity, but the enzyme was not completely denatured. The effects of pH on pectinase activity are measured in the pH range of 2.5–6.5. The result is shown in Figure 7B. Pectinase showed a trend of first increasing and then decreasing with the increase in pH, and reached the optimum pH at pH 4.5. This may be attributed to the good adaptability to acidity; the pectinase produced by the strain CM96 is an acidic enzyme. When pectinase was placed in an acidic environment with pH 4.5 for 150 min, it was found that pectinase activity could remain above 69.4%, which indicated that the pectinase used in this study has good acidic stability.

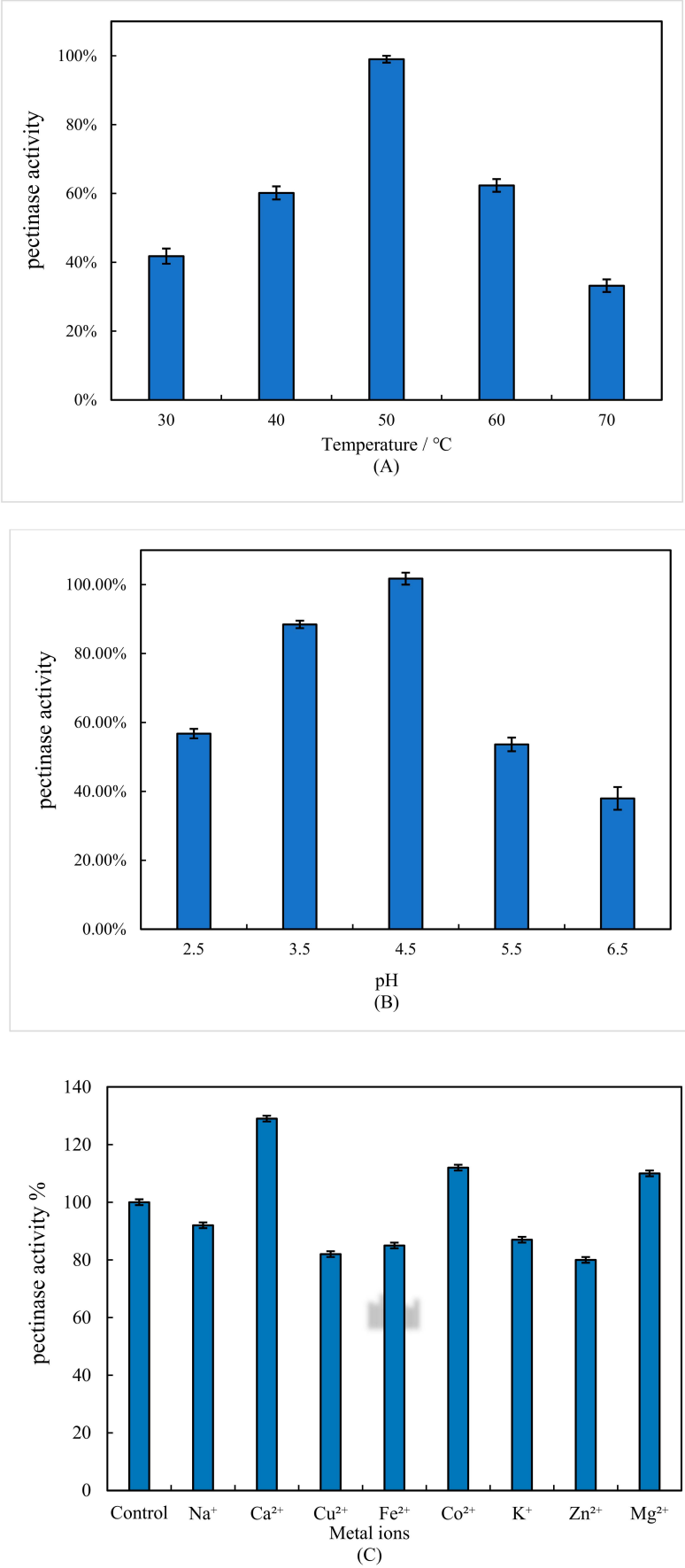


Figure 7. Effect of temperature (**A**), pH (**B**), and metal ions (**C**) on activity of purified pectinase.

Metal ions are also important for pectinase. Different metal ions have different effects on the enzymatic activity of pectinase, as shown in Figure 7C. Different metal ions (Ca^{2+}, Co^{2+}, Mg^{2+}, Na^{+}, Cu^{2+}, Fe^{2+}, K^{+}, Mn^{2+}, and Zn^{2+}) were added during the enzymatic hydrolysis reaction of pectinase. Ca^{2+}, Co^{2+} and Mg^{2+} have a promoting effect on pectinase, while Na^{+}, Cu^{2+}, Fe^{2+}, K^{+}, Mn^{2+}, and Zn^{2+} inhibit the enzymatic activity of pectinase. Ca^{2+} has the strongest promoting effect on pectinase, while Mn^{2+} almost completely inhibited the activity of pectinase.

3.7. Characterization of Enzyme Cocktail and Its Application

The activity and properties of the enzyme mixture were studied. The results showed that the enzyme cocktail contains glucanase, acidic protease, pectinase, and cellulase, and their enzymatic activities units were 7000 ± 400 U·g^{-1}, 8000 ± 600 U·g^{-1}, 15,000 ± 700 U·g^{-1}, and 3000 ± 200 U·g^{-1}, respectively. This enzyme cocktail includes relatively high glucanase, protease, and pectinase activity, and relatively low cellulase activities.

The addition of exogenous enzymes to animal diets had been considered as a strategy to improve nutrient digestibility and feed efficiency. A total of 0.1% of exogenous enzyme cocktail (*w*/*w*) is usually added to chicken and pig feed as an additive). The Association recommended excessive enzyme mixture loading to improve the efficiency of substrate digestibility. In order to understand the effect of enzymatic hydrolysis, 144 Soviet white pigs were selected in this experiment, and 0.05% mixed enzyme was added at 38 days of the feeding cycle to verify the digestion efficiency of the substrate. A total of 144 Soviet white pigs were divided into a control group and a treatment group. As shown in Table 2, the average daily gain (ADG, g·day^{-1}), average daily feed intake (ADFI, g·day^{-1}), and feed conversion rate (FCR) were monitored and analyzed.

Table 2. ADG, ADFI and FCR of 144 Soviet white pigs before and after adding the exogenous cocktail enzyme (feed period 1–38 d).

Items	Control	Treatment	*t*	*p*
Initial average weight (kg)	5.74 ± 0.02	5.87 ± 0.01	−60.360	<0.01
Final average weight (kg)	16.15 ± 0.56	18.04 ± 0.39	−58.063	<0.01
ADG (g/d)	252.20 ± 1.10	391.61 ± 0.06	−15	<0.01
ADFI (g/d)	522.13 ± 0.01	502.31 ± 1.65	143.051	<0.01
FCR	1.43 ± 0.00	1.36 ± 0.01	158.995	<0.01

As shown in Table 2, ADG was 252.20 ± 1.10 g·day^{-1}, and 391.61 ± 0.06 g·day^{-1} before and after adding the cocktail enzyme over 1–38 days, and the ADG increase was 139.41 ± 1.04 g·day^{-1} over 38 d. ADFI and FCR decreased by 19.82 ± 1.64 g·d^{-1} and 0.07 ± 0.01, respectively, in 38 days. The results showed that the addition of this enzyme mixture to pig feed could significantly promote animal growth and improve feed efficiency. The independent-samples *t* test was carried out on the results, and the statistical results showed significant differences ($p < 0.01$). The multi-enzyme complexes used in the experiment have four different enzymes and a combination of glucanase, acid protease, pectinase, and cellulase. In order to determine the effect of enzyme addition in growth–fattening feeds, further studies on the addition of enzymes, either alone or in combination, are needed [13,15,28–31]. The cocktail enzyme containing pectinase used in this study is a multi-enzyme complex addition that also depends on the content of the raw material, and is the most sustainable way to improve the growth and efficiency of the feed.

4. Conclusions

Aspergillus sp. CM96 was employed for pectinase fermentation and production in this study. The results showed that the pectinase activity of *Aspergillus* sp. CM96 reached 18,000 U·g^{-1} through solid-phase fermentation for 5 d in the tray bioreactor. Pectinase was purified through ammonium sulfate precipitation, anion chromatography, and concentration. The recovery rate was 33.67%, and the specific activity and molecular weight

of purified pectinase were 65,366.67 U·mg^{-1} and 30 kDa. During pectinase fermentation, the activities of protease, dextranase, and cellulase were determined, and the substrate digestion efficiency was improved after the addition of a 0.05% cocktail enzyme over a 38 d of feed period for 144 Soviet white pigs. The results showed an ADG increase of 109.41 g· day^{-1}. Therefore, the *Aspergillus* sp. CM96 strain can be used as an important source of pectinase, which contains glucanase, acid protease, and cellulase, and its mixed enzyme can be used as a feed additive to improve feed efficiency.

Author Contributions: Conceptualization: H.C., M.W. and Y.L.; methodology: H.C., G.Y. and Z.C.; data analysis: H.C. and G.Y.; software: H.C.; writing—original manuscript: H.C.; reviewing and revising the manuscript: G.Y. and Z.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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