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PURIFICATION AND CHARACTERIZATION OF ACIDIC PROTEASE FROM *ASPERGILLUS ORYZAE* BCRC 30118

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Key words: purification, acidic protease, *Aspergillus oryzae.*

ABSTRACT

The acidic protease was purified from 4-day cultivation of *Aspergillus oryzae* BCRC 30118 by DEAE Sephacel and Sephacryl S-200 HR chromatographs. The specific activity, yield and purification fold were 117.62 kU/mg, 15.1% and 6.6, respectively. The molecular weight (*M*) was 41.0 kDa, while the optimal pH and temperature were 3.0 and 60°C, respectively. It was stable at pH 3.0-6.0 and 4-35°C. However, it was inhibited by Fe^{2+}, Hg^{2+}, Fe^{3+} and pepstatin A, and slightly by leupeptin and TPCK. According to substrate specificity and inhibitor study, it was a cysteine protease with activation energy of 37.5 kcal/mol. Its K_m, V_{max}, K_{cat} and K_{cat}/K_m for the hydrolysis of hemoglobin were 0.12 mM, 14.29 μmol/min, 14.55 sec^{-1} and 125.80 (sec^{-1} mM^{-1}), respectively.

I. INTRODUCTION

Enzymes, extracting from the viscera of animal, had been used in food industries during the early years. Nowadays, since bacteria could quickly grow and be induced by environmental changes to obtain the desirable enzymes during incubation, production of microbial enzymes has extensively studied during the past decade by many scientists and industries. Furthermore, the secreted enzymes are easily isolated from the broth of bacteria. They have long been directly or indirectly used in food fermentation, baking and alcohol making industries [30]. Enzymatic reaction can decrease the reaction energy and undergo highly efficiently [28]. They have high specificity to attack the specific site of substrate to produce high purity products and subsequently decrease undesirable by-products formation during fermentation. Accordingly, they are recognized to have high application potentials in food and cosmetic industries, base on the economic, health and safety point of view [30]. Among these enzymes, accounted about 65% of global enzymes market is proteases which are frequently used in detergent, leather, pharmaceuticals, and food industries [2, 25]. Acid proteases are frequently used in the production of seasoning materials, protein hydrolysate, soy sauce, or used as digestive aids [25]. They are also widely used to improve the texture of flour paste, to tender the fibril muscle, and to clear beer and fruit juice [48].

Filamentous fungi are also exploited for the production of industrial enzymes due to their ability to grow on solid substrate or broths and produce a wide range of extracellular enzymes. Among the advantages of production of enzymes by fungi, low cost, high productivity, fast production and the ease with which the enzymes can be modified are most concerned. Several studies have recently performed on the isolation of acid proteases from different fungi ~ *Aspergillus saitoi* [11], *Aspergillus oryzae* [35-39], *Aspergillus niger* [29, 41, 49], *Mucor pusillus* [24], *Rhizopus hangchow* [10], *Monascus pilosus* [9, 18, 33] and *Penicillium duponti* [8]. However, many reported activity levels were still poor. Therefore, new species for the production of acid proteases are still searching [3, 31, 32, 43]. The present work reports purification and characterization of an acid protease produced extracellularly from *Aspergillus oryzae* BCRC 30118.

II. MATERIALS AND METHODS

1. Materials

Aspergillus oryzae BCRC 30118 was obtained from the Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Institute, Hsinchu, Taiwan. It was activated in malt extract broth (containing malt extract 20 g/L, glucose 20 g/L, peptone 1.0 g/L) at 25°C for 4 days. To potato dextrose agar (PDA), 0.1 mL of *Asp. oryzae* broth was inoculated and incubated at 25°C for another

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4 days. The spores, washed from PDA using 5 mL of 0.05% Tween 20, were used as starter. They were inoculated into malt extract broth and incubated at 25°C with 150 rpm shaking for 4 days and then centrifuged at 6,500 g for 20 min. The supernatant was passed through a 0.22 μm membrane to remove the cells and used as crude protease solution.

DEAE-Sepharose and Sephacryl S-200 were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, bis-acrylamide and coomassie brilliant blue G-250 were obtained from Merck (Darmstadt, Germany), while the malt extract, peptone, PDA were the products of Difco. Hemoglobin, bovine serum albumin (BSA), glucose, sodium dodecyl sulfate (SDS), N, N, N', N'-tetramethyl-ethylenediamine (TEMED), 1-(L-trans-epoxysuccinylleucylamido-4-guanidinobutane) (E-64), phenylmethyl-sulfonyl fluoride (PMSF), iodoacetic acid (IAA), N-ethylmaleimide (NEM), leupeptin, pepstatin A, toyllysine chloromethyl ketone (TLCK), tosylphenylalanine chloromethyl ketone (TPCK) were obtained from Sigma (USA). Protein-dye binding reagent was obtained from Bio-Rad (USA). $FeCl_3$, $FeCl_2$, $MgCl_2$, $MnCl_2$, NH_4Cl, urea, ethylenediamine tetraacetic acid (EDTA) and trichloroacetic acid (TCA) were the products of Hayashi Pure Chemical Industries (Osaka, Japan).

2. Purification of Acidic Protease

Preparation of the crude enzyme: The acidic protease was purified by a series of chromatographic steps. All purification procedures were performed at 4°C. The 4-day cultivated broth was centrifuged at 6000 × g for 10 min to precipitate pellets and then passed through a 0.22 μm membrane to remove the cells. The filtrate was used as crude enzyme.

Ion exchange chromatograph: The DEAE-Sephacel Fast Flow column (2.6 × 40 cm) was employed to purify the acidic protease. It was equilibrated with 50 mM citrate buffer, pH 6.0 and washed with 1.0 L of the same buffer after the crude enzyme was loaded. The acidic protease was then eluted with a linear gradient of 0.0-1.0 M NaCl in 50 mM citrate buffer, pH 6.0. Fractions with acidic protease activity were collected and concentrated to a minimal volume by Amicon Ultrafiltration (cutoff: 10,000).

Gel filtration: The Sephacryl S-200 was used to further purify the acidic protease. It was equilibrated with 50 mM citrate buffer (pH 6.0). Acidic protease collected on DEAE-Sephacel Fast Flow column was concentrated. The resulted sample was loaded on Sephacryl S-200 which was equilibrated with 50 mM citrate buffer (pH 6.0) and then eluted with the same buffer. Fractions with acidic protease activity were collected and subjected to the following assays. All purification procedures were performed at 4°C.

3. Determination of Enzyme Activity

The activity of acidic protease was measured mainly as described by Narahara *et al*. [20]. To 1.0 mL of 2% hemoglobin in 50 mM citrate buffer (pH 3.0), 1.0 mL of appropriate concentration of enzyme was added. After 30 min reaction at 37°C in a water bath with shaking, equal volume of 10% TCA (2.0 mL) was added to stop the reaction. The resulted samples were centrifuged for 10 min at 6000 × g to remove the precipitate. Absorbance at 280 nm (A_{280}) of the supernatant was determined by using a spectrophotometer (Hitachi U-2001, Hitachi, Japan). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze hemoglobin and release equivalent to 1.0 μg of tyrosine within 1 min reaction at 37°C.

4. Protein Concentration

Protein concentration was determined according to protein-dye binding method [1] using bovine serum albumin (Sigma Chemical, St. Louis, MO) as standard.

5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purified enzyme in 62.5 mM Tris-HCl buffer containing 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol (β-Me) and 0.002% bromophenol blue was boiled at 100°C for 5 min and then subjected to SDS-PAGE according to Laemmli [14]. Gels were stained according to Neuhoff *et al*. [22]. Protein Ladder (consisting of 12 bands from 10 to 120 kDa in 10 kDa increments, which are produced by partial cleavage of a 120 kDa protein, plus a 200 kDa band) were used as standards (Life Technologies, Gaithersburg, MD).

6. Optimal pH and pH Stability

The activity of acidic protease in 50 mM buffer with various pHs (citrate buffer, pH 2-6; Tris-HCl, pH 7-8; carbonate buffer, pH 9) was measured. To determine the pH stability, the acidic protease in 50 mM buffer with various pHs was incubated at 37°C for 30 min and then determined the activity as described by Narahara *et al*. [20].

7. Optimal Temperature and Thermal Stability

The activity of acidic protease in 50 mM citrate buffer (pH 6.0) was measured at various temperatures (4-80°C). To measure the thermal stability, the acidic protease in 50 mM citrate buffer (pH 6.0) was incubated at various temperatures (4-90°C) for 30 min. At definite time intervals, the enzyme solution was cooled immediately in ice water for 5 min. The activity was measured as described by Narahara *et al*. [20].

8. Effect of Metal Ions

Various metals (1.0-10 mM) suspended in 50 mM citrate buffer (pH 6.0) were added to the purified acidic protease. The resulted samples were incubated at 37°C for 30 min. The residual activity was measured.

9. Effect of Inhibitors

Various inhibitors (0.5-2.0 mM) suspended in 50 mM citrate buffer (pH 6.0) were added to the purified acidic protease. The resulted samples were incubated at 37°C for 30 min. The residual activity was measured.

10. Activation Energy (Ea)

The purified acidic protease in 50 mM citrate buffer (pH 6.0) was incubated at 25, 30, 35, 40, 50, 60°C. During incubation, samples were removed at a 10-min interval, cooled and measured the activity [20]. The residual activity of each sample during incubation were used to calculate the inactivation rate constant of the purified acid protease and then to construct the Arrhenius plot.

11. Determination of Enzyme Kinetics

Acidic protease in 50 mM citrate buffer (pH 6.0) with various concentrations of substrate was equilibrated at 25°C for 5 min. The reaction rate under different concentrations of substrate was measured. The V_{max} and K_m were then calculated from Lineweaver-Burk double reciprocal plot [16].

III. RESULTS AND DISCUSSION

1. Growth, pH and Protease Activity of *Aspergillus oryzae* during Incubation

One percent of starter was inoculated into 100 mL of malt extract broth using a 250 mL flask and incubated at 25°C with 150 rpm shaking for 4 days. At a 24 h interval, the pH, mycelial dry weight, and protease activity were determined. According to Fukamoto *et al.* [6], during the early stage of *Asp. oryzae* fermentation, proteases were produced inside the myceles and then secreted gradually to the broth. The highest production of proteases occurred at the stage during spore formation. The mycelial dry weight and protease activity increased with the incubation time and reached a plateau after 4 days incubation, while the acidic protease activity were about 370 units/mL (data not shown). According to Fumiyosi *et al.* [7], the types and production of proteases produced by *Aspergillus* were highly related to the ratio of carbon to nitrogen source (C/N ratio). At low C/N ratio, the dominant proteases would be neutral and alkaline protease. However, the dominant proteases were acidic protease type at high C/N ratio [7]. In our preliminary experiments, during 7 days cultivation in malt extract broth at 25°C with 150 rpm shaking, pH decreased from 5.23 to 3.13, while acidic protease activity increased to 370 units/mL after 4 days incubation. No further decline in pH and increase in activity was observed during the prolonged incubation. However, the mycelial dry weight increased with the duration up to 5 days cultivation (data not shown).

2. Purification

The acidic protease was purified to electrophoretical homogeneity after DEAE-Sephacel Fast Flow and Sephacryl S-200 chromatographs (Fig. 1). The recovery and purifycation were 25.8% and 3.8 fold, respectively, after the 1st Sephacryl S-200. Although approximately 50% activity was lost after the 1st Sephacryl S-200 chromatography, more than 50% of protein was eliminated. About 6.6 fold of purification and 15.1% recovery were achieved after the 2nd Sephacryl S-200 chromatography. It was summarized in Table 1. According to SDS-PAGE analysis, the purified acidic protease was a monomer with *M* of 41.0 kDa (Fig. 1).

Table 1. Summary of the purification of acidic protease from *Aspergillus oryzae*.

Procedures	Total protein (mg)	Total activity (kU)	Specific activity (kU/mg)	Recovery (%)	Purification (fold)
Crude enzyme	44.1	781.52	17.81	100.0	1.0
DEAE Sephacel	6.8	359.37	52.85	46.0	3.0
1st Sephacryl S-200 HR	3.0	201.61	67.20	25.8	3.8
2nd Sephacryl S-200 HR	1.0	117.62	117.62	15.1	6.6

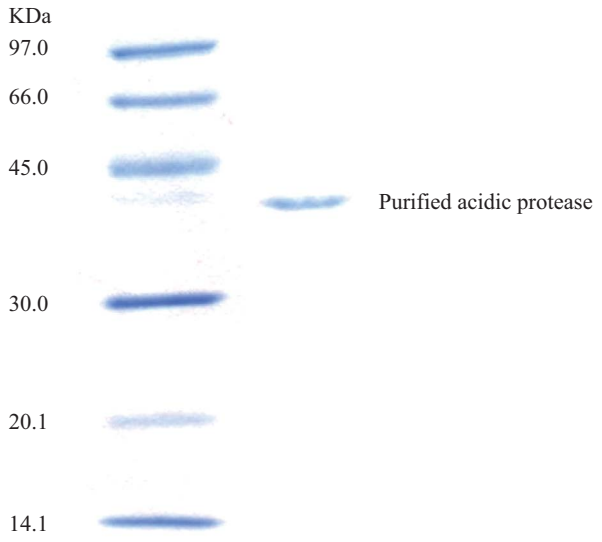

Fig. 1. Electrophoretic analysis of the purified acidic protease from *Aspergillus oryzae*.

3. Effects of pH and Temperature

The purified protease was very stable at pH 3.0-6.0 with an optimum pH at 3.0 (hemoglobin as substrate) (Fig. 2). As shown in Fig. 2, there was still 80% activity left even after 30 min incubation at < 35°C. This result was similar to those reported by Numokawa [23], Li [17], Wang *et al.* [46], Sawada [26], Vicente *et al.* [42], Wu and Hang [47], and Eneyslaya *et al.* [4]. According to Tsujita and Endo's study [34], the optimal pH of acidic protease A_1 and A_2 purified from commercial *Asp. oryzae* (Takadiastase) were 3 (casein as substrate) and 5.8 (hemoglobin as substrate), respectively. The optimal pH of all acidic proteases, E_1, E_{1a}, E_{1b} and E_2, extracted from the broth of *Asp. oryzae* were 4.2 (hemoglobin as substrate) [37]. The optimal temperature of purified acidic

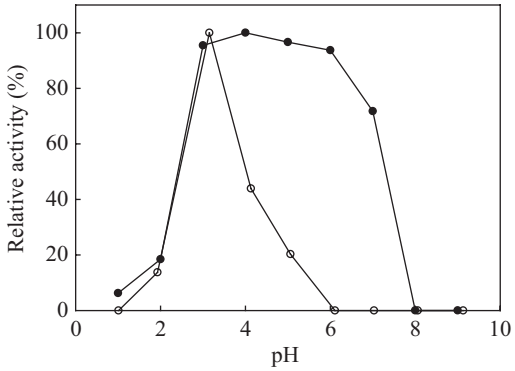

Fig. 2. Effect of pH on the activity of purified acidic protease from *Aspergillus oryzae*. —●—: pH stability; —○—: optimal pH.

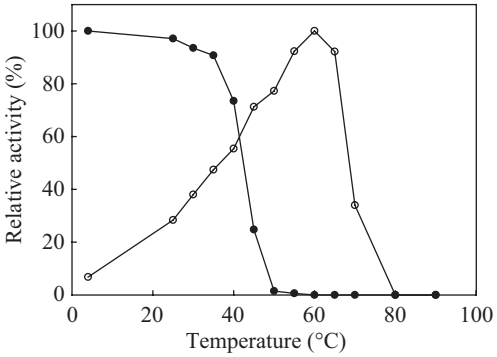

Fig. 3. Effect of temperature on purified acidic protease from *Aspergillus oryzae*. —●—: thermal stability; —○—: optimal temperature.

protease was 60°C, however, its activity was quickly disappeared at 70°C (Fig. 3). This phenomenon was similar to the aspartic protease purified from *Phycomyces blakesleeanus* [42]. In this study, the purified acidic protease was very stable at temperature ≤ 40°C, but unstable at temperature ≥ 50°C (Fig. 3). *Aspergillus oryzae* can produce various proteases with optimal pH at 2.5-5.0 and optimal temperatures at 50-70°C [13, 15, 23, 37]. They were very stable at pH 2.5-6.5 [6, 27]. However, their thermostability highly varied with the species. *Rhizopus oryzae* and *Asp. oryzae* MTCC 5341 were at 30-45°C and 40-57°C, respectively [13, 44], while *Rhizopus oryzae* was at 25-40°C [45]. *Asp. niger* I1 was around 30-40°C [27]; *Monascus pilosus* was at 25-55°C [15]. The acidic proteases A_1 and A_2 extracted from commercial *Asp. oryzae* (Takadiastase) were very stable even after 10 min incubation at 55° and 40°C, respectively, using hemoglobin as substrate [34]. According to Fukumoto *et al*. [6], about 90% activity of the acidic protease from *Rhizopus chinensis* was left after 15 min heating at 60°C. The acidic protease from *Rhizopus oryzae* was stable after 20 min incubation at 25-40°C, however, about 50% activity lost after 20 min incubation at 50°C and almost completely lost after 20 min incubation at 60°C [46]. According to the previous studies, the acidic proteases seemed to be heat unstable.

Table 2. Effect of metal ions on the acidic protease activity (relative activity, %).

Metal ions	Relative activity (%)		
	Concentration (mM)		
	1.0	5.0	10.0
None	100.0	100.0	100.0
Ag^{+}	102.2	102.0	101.4
K^{+}	103.1	102.6	102.1
Li^{+}	104.8	103.9	103.0
Na^{+}	99.5	98.9	97.5
NH_4^{+}	96.2	98.7	104.6
Ba^{2+}	95.3	98.8	99.2
Ca^{2+}	97.7	91.2	100.3
Cd^{2+}	100.5	91.5	97.0
Co^{2+}	100.7	103.0	104.2
Cu^{2+}	101.9	97.4	99.4
Fe^{2+}	91.8	37.8	37.1
Hg^{2+}	81.5	76.3	64.3
Mg^{2+}	100.6	104.1	99.2
Mn^{2+}	101.1	95.4	95.1
Ni^{2+}	98.9	98.8	108.3
Zn^{2+}	102.1	101.6	111.8
Fe^{3+}	99.8	0.0	0.0

* The counter ion of all metals was chloride.

4. Effect of Metal Ion

The purified enzyme was not affected by any metals at a concentration of 1.0 mM. However, it was moderately inhibited by Fe^{3+}, strongly inhibited by Fe^{2+} and Hg^{2+} at 5.0 mM (Table 2). It is considered that the active site contained –SH group. Metal effect on the activity of purified acidic protease was similar to that reported by Narahara *et al*. [21].

5. Effect of Inhibitor

The purified acidic protease was completely inhibited by pepstatin and partially inhibited by leupeptin and TPCK (42.37 and 35.93% activity left, respectively). No significant loss of purified acidic protease activity was observed on samples with EDTA, IAA, NEM, PMSF, and urea added (Table 3). This phenomenon was similar to that from *Phycomyces blakesleeanus* [42]. Pepstatin is a potent inhibitor of aspartyl proteases and nearly all acid proteases [40], while leupeptin inhibits serine, cysteine and threonine proteases, but not inhibit α-chymotrypsin or thrombin [19]. TPCK is the irreversible inhibitor of chymotrypsin. It also inhibits some cysteine proteases [5]. According to the data obtained, it is considered to be a cysteine protease.

6. Activation Energy (Ea)

According to the Arrhenius's Plot, the slope and Ea were -8.12 and 37.5 kcal/mol (25-60°C) (Fig. 4). According to Vicente *et al.* [42], the Ea of aspartic protease from *Phycomyces blakesleeanus* was 29.7 kJ/mol (about 7.10 kcal/mol)

Table 3. Effect of inhibitors on the purified acidic protease activity.

Inhibitors	Relative activity (%)		
	Concentration (mM)		
	0.5	1.0	2.0
None	100.0	100.0	100.0
IAA	95.20	91.02	89.52
NEM	91.13	89.58	88.32
EDTA	91.13	88.02	88.17
Urea	90.84	89.52	86.39
Leupeptin	68.31	55.84	42.37
E-64	89.68	89.62	89.97
pepstatin A	0.00	0.00	0.00
TLCK	99.21	97.32	96.17
TPCK	88.08	77.99	35.93
PMSF	91.28	92.22	94.46

[a] IAA, iodoacetic acid; NEM, N-ethylmaleinide; EDTA, ethylenediamine tetraacetic acid; E-64, 1-(L-trans-epoxysuccinyl-leucylamido-4-guanidine-butane); TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride.

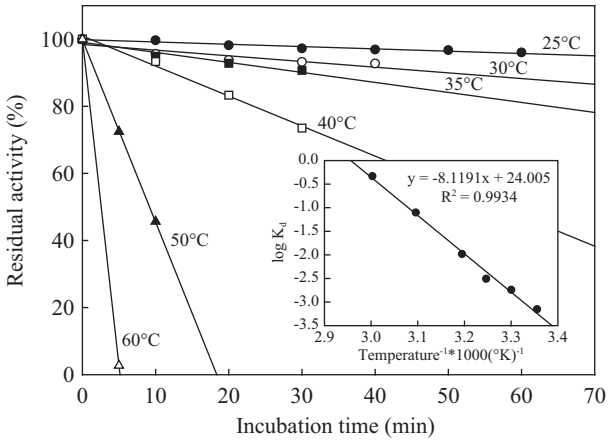

Fig. 4. Inactivation constant (K_d) of purified acidic protease incubated at various temperatures and its correspondent Arrhenius Plot.

(20-70°C) by using the reaction rate (log V) via T^{-1} as Arrhenius's plot. The Ea of the purified acidic protease, a cysteine protease, is higher than the aspartic protease from *Phycomyces blakesleeanus* [42].

7. Enzyme Kinetics

As shown in Lineweaver-Burk plot of *Aspergillus oryza* acidic protease with substrate of hemoglobin (Fig. 5), the apparent K_m and V_{max} were estimated to be 0.12 mM and 14.29 μmol/min. The K_{cat} and K_{cat}/K_m were 14.55 sec^{-1} and 125.80 (sec^{-1} mM^{-1}), respectively (Table 4). According to Jeng [12], the apparent K_m and V_{max} of acidic protease from *Aspergillus* sp. FC-10 using casein as substrate were estimated to be 0.14% and 2000 μg/min, respectively. The K_m

Table 4. Kinetic parameters of purified protease for hydrolysis of hemoglobin.

Substrate	K_m (mM)	V_{max} (μmol min^{-1})	K_{cat} (sec^{-1})	K_{cat}/K_m ($sec^{-1}mM^{-1}$)
Hemoglobin	0.12	14.29	14.55	125.80

$[E]_0$: Enzyme mole concentration (0.9815 μmol).

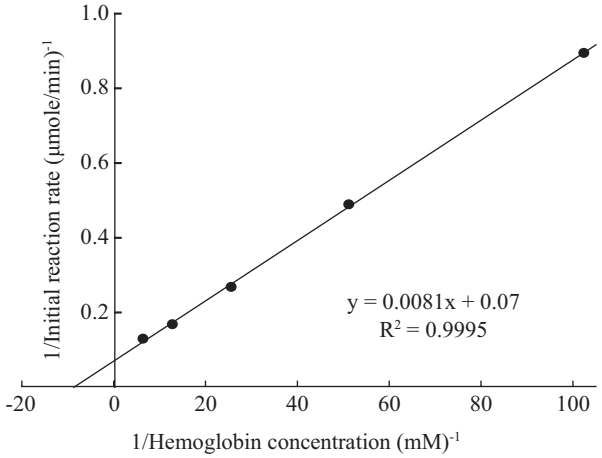

Fig. 5. A double-reciprocal of hemoglobin concentration against the activity of purified acidic protease.

and V_{max} for the caseinolytic activity of acidic protease from *Aspergillus niger* I1 were estimated to be 1.02 mM and 2.2 μmol/min, respectively [27]. The purified acidic protease from *Aspergillus oryza* had lower K_m and higher V_{max} than those from *Asp. niger* I1 and *Asp.* sp. FC-10, suggesting its higher affinity to the substrate and reaction velocity, which consequently has higher industrial application than others from *Aspergilli*.

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