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Purification and Characterization of a Unique Chitinase from a *Malbranchea chrysosporioidea* Isolated from Chitin Rich Soil

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Chitinases have received substantial attention owing to their potential application in environmental protection for clearing the waste generated in shrimp and crab processing industry. We report a chitinase producing fungus isolated from soil samples of Andhra Pradesh, India, where shrimp shells were dumped regularly. The isolated fungus was identified as a *Malbranchea chrysosporioidea* and an extracellular chitinase was purified from the culture filtrate of *Malbranchea chrysosporioidea*. The purified chitinase has a molecular weight of 100 kDa as estimated by SDS-PAGE further being confirmed by activity staining using glycol chitin in the gel. The isoelectric point of purified enzyme was 8.45. The enzyme exhibited an optimum pH of 9.0 and a temperature of 37^{0}C, with swollen chitin as the substrate. The enzyme was unusually activated by Cd^{2+} and inhibited by Zn^{2+}, Co^{2+} and As^{3+}. Results of RP-HPLC analysis of hydrolytic products revealed a unique nature of purified chitinase, exhibiting exochitinase and β-N-acetyl hexosaminidase activities releasing chitobiose and N-acetylglucosamine respectively as end products. To our knowledge, this is the first report on purification and characterization of a chitinase from *Malbranchea chrysosporioidea* isolated from soil of Andhra Pradesh, India.

Key words: *Malbranchea chrysosporioidea,* Chitinase purification, Exochitinase and β-N-acetyl hexosaminidase activities.

Chitin, a linear biopolymer, is the second most common and abundant organic molecule of biological origin found on our planet, after cellulose with an annual production of 100 billion tons. It is widely distributed in nature and forms a major constituent of the shells of crustaceans such as crabs, shrimps, and exoskeleton of insects, and as a component of variety of fungi and algae[1]. The catabolism of chitin is catalysed by a chitinolytic system classified into: endo-chitinases (EC 3.2.1.1.4), exo-chitinases (EC 3.2.1.14), chitobiase (EC 3.2.1.30) and β-N-acetyl hexosaminidases (EC 3.2.1.52). Endochitinases cleave randomly along the internal chain of chitin, producing chitotetraose and chitotriose, eventually giving diacetylchitobiose as predominant reaction products. However, exochitinases release diacetylchitobiose without production of N-acetyl glucosamine or oligomers. β-N-acetyl hexosaminidases split diacetylchitobiose as well as chitotriose and chitotetraose into N-acetyl glucosamine monomers, since β-N-acetyl hexosaminidases also acts on di-acetylchitobiose it is also called chitobiase[2] . Physiological role of chitinases is different among various organisms. Microorganisms produce the enzyme 'chitinase' to digest the chitinous nutrient or to partially hydrolyze the chitinous cell wall for cell proliferation[3]. In insects and crustaceans, chitinase

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acts by degrading the exoskeletal chitin in the cuticle or shell for ecdysis[4]. In plants, chitinases are used for defense against plant pathogens and pests[5]. The seaweed chitinases also play a vital role in defense mechanism like in plants[6]. Chitinase presence has been demonstrated even in mammals and recently, human chitinases and their nature of combating with human pathogens have been reported[7].

There is considerable interest in chitinases derived from various sources due to their wide range of applications such as isolation of protoplasts from fungi and yeast, preparation of pharmaceutically important chitooligosaccharides and N-acetyl-D-glucosamine, control of pathogenic fungi, treatment of chitinous waste, and the control of malaria transmission[8]. Hence, chitinolytic enzymes have been purified from many microorganisms, and their enzymatic properties have been investigated. In our study, while exploring for efficient chitin-degrading organism from soil, we found a fungus, which grew well on a chitin-agar medium. We identified the isolated fungus as *Malbranchea chrysosporioidea* using molecular methods. Species of *Mabranchea* living in soil are known to serve in the breakdown of keratin containing waste such as hair, fur and feathers with a role in the digestion of soil debris[9]. In the current study, we demonstrated that *Malbranchea chrysosporioidea* showed an impressive chitinolytic activity, which may be helpful in environmental protection for clearing the waste generated in shrimp and crab processing industry. So far, no description about chitinase from *Malbranchea chrysosporioidea* had been reported in the literature. We described the purification and characterization of a unique chitinase produced by *Malbranchea chrysosporioidea*. To our knowledge, this is the first report to demonstrate the purification and characterization of chitinase from *Malbranchea chrysosporioidea* isolated from soil of Andhra Pradesh, India.

MATERIALS AND METHODS

Materials

All chemicals were procured from Sigma or Himedia. Swollen chitin and glycol chitin were prepared using the methods described earlier[10,11].

Growth conditions

The fungus was isolated from soil where shrimp waste was regularly dumped. The cultures were grown in modified Sabouraud's medium containing (g/L): swollen chitin, 15; peptone, 5; and yeast extract, 3 with shaking (120 rpm) at 28^0C for 8 days.

Purification of chitinase

All steps of purification were performed at 4^0C until unless mentioned.

Enzyme Assay

Chitinase was assayed using 200 µl of reaction mixture containing 1% swollen chitin, 20µg enzyme in 50 mM borate buffer pH. 9.0(assay buffer) at 37^0C with shaking for 6 h and was analyzed for N-acetyl glucosamine spectrophotometrically according to the method of Reissig *et al*.[12]. Specific activity is expressed as µmols of N-acetylglucosamine released per mg protein per min. One unit of chitinase activity is defined as the amount of enzyme required to release 1 µmol of N-acetylglucosamine per min under the assay conditions mentioned above.

Ammonium sulfate precipitation

The culture fluid was subjected to 80% ammonium sulfate saturation and the precipitate dissolved in assay buffer, dialyzed and lyophilized. Protein content of enzyme preparation at different stages of purification was estimated using BSA as the standard.

Adsorption of chitinase on chitin

Chitinase was further purified by Roberts and Cabib method[13]. The lyophilized enzyme was dissolved in assay buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Swollen chitin (10 mg/ml) was mixed with the chitinase and kept on ice for 1 h with shaking and washed with excess buffer and finally re-suspended in 2 ml of the same buffer. The suspension was incubated for 3 h at 37^0C with shaking (50 rpm) for the release of the enzyme from chitin. The enzyme suspension was dialyzed extensively against the assay buffer to eliminate the soluble chitin degradation products.

Sephadex G-100 gel filtration chromatography

Chitinase from the above step was concentrated and applied to a Sephadex G-100 column previously equilibrated with assay buffer. 2 ml fractions were collected and the fractions with enzyme activity were pooled and once again

concentrated by lyophilization.

SDS-PAGE and Activity staining of chitinase

SDS - PAGE was used to check the protein purity and molecular size. The activity staining of the gels was according to the procedure of Marek *et al*[14]. Purified chitinase was subjected to SDS-PAGE (10% gels) containing 0.01% glycol chitin. β-Mercaptoethanol was omitted from sample buffer. Gels were immersed in assay buffer containing 1% Triton X-100 for 1 h to remove SDS. Further the gel was washed to remove Triton X-100, and incubated at 37^{0}C overnight with the same buffer.

Determination of the isoelectric point (pI) of chitinase

The pI was determined by the isoelectric focusing (IEF) technique of O'Farrell[15] with ampholines of pH 3-9. The enzyme sample (2 µg) was dissolved in the buffer (9.5 M urea containing 2% igepal, 2% ampholines and 5% β-Mercaptoethanol) and electrophoresed for 4 h at a constant voltage of 450 V along with standard IEF markers [Lentil lectins of 8.65, 8.45 and 8.15; Horse myoglobulins of 7.35 and 6.85; Human carbonic anhydrase (6.55); Bovine carbonic anhydrase (5.85); Lactoglobulin (5.20); Soybean trypsin inhibitor (4.55)].

Determination of optimum pH for activity and stability of the chitinase

The optimum pH was determined by varying the pH between pH values 3 and 12, at an increment of 1.0 unit. The buffers used were citrate for pH 3.0, acetate for pH 4.0 and 5.0, phosphate for pH 6.0 and 7.0, Tris-HCl for pH 8.0, borate for pH 9.0, carbonate for pH 10.0, and glycine-NaOH buffer for 11.0 and 12.0. The reaction mixture contained enzyme plus chitin and buffer of required pH to a final concentration of 50 µM. The activity of the chitinase was then assayed under standard conditions. Effect of pH on stability of chitinase was investigated by pre-incubating 20 µg of the enzyme in 200 µl of 50 µM of the buffer described for 1 h at 37^{0}C. After 1 h, 1% chitin was added and residual activity was then assayed under standard conditions.

Determination of optimum temperature for activity and stability of the chitinase

The optimal temperature was determined as described above except that chitinase activity was assayed at different temperatures between 20 and 70^{0}C with an incubation period of 6 h. Temperature stability was examined by pre incubating 20 µg of enzyme in assay buffer for 15 min at corresponding temperature. After 15 min, 1% swollen chitin was added and the residual activity was assayed as above.

Effect of Metal ions on chitinase activity

The effect of several metal ions on the activity of the enzyme was investigated by incubation of chitinase in swollen chitin plus the metal ion under test at a final concentration of 10mM. Control assay (without metal ion) was taken as 100% activity.

Analysis of reaction products of the chitinase by Reverse phase-high pressure liquid chromatography (RP-HPLC)

The reaction mixture (500 µl) containing 1% swollen chitin, 50 µg of purified chitinase in buffer (pH 9.0) was incubated for 6 h. Reaction products of swollen chitin by chitinase action were separated by RP-HPLC using a Whatman partisil ODS column (4.6 X 250 mm). The mobile phase was 70% acetonitrile and 30% water. The flow rate was 1.2 ml/min. Hydrolysis products were detected on the basis of their absorbance at 210 nm with a Shimadzu LC-6A and identified by comparison to (N- acetylglucosamine)$_n$ standards from n=1-6[16].

RESULTS AND DISCUSSION

In search of identifying chitin degrading microorganisms from soil, we isolated *Malbranchea chrysosporioidea* from soil of Andhra Pradesh, India. The application of molecular identification determined that the isolate shows resemblance with *Malbranchea chrysosporioidea* (data not shown). Sanjana Kaul and Geeta Sumbali reported *Malbranchea* as a keratinophilic fungi from feathers of Indian poultry birds, exhibiting keratinophilic activity and demonstrated that *Malbranchea chrysosporioidea* is a new addition to Indian mycoflora[9, 17]. Other species like *Malbranchea gypsea* keratinolytic capabilities were studied by Chandra Jeet Singh[18]. This is the first report of *Malbranchea chrysosporioidea* isolated from soil showing chitinolytic activity and likely to contribute significantly in breaking down and recycling chitin and keratin wastes, thereby controlling pollution

hazards and regulating the essential ecological processes in the ecosystem.

Purification of chitinase

The crude enzyme obtained from culture filtrate of *Malbranchea chrysosporioidea* was subjected to 80% ammonium-sulfate fractionation, increasing the specific activity from 0.1 to 0.16, the yield being 66.6%. In the next step it was adsorbed on to swollen chitin, enhancing the specific activity to 0.85, yield being only 25.5%. This fraction was further purified by Sephadex G-100gel filtration chromatography and the chromatography pattern is shown in Fig. 1a. The gel filtration resulted in an increase of the specific activity from 0.85 to 1.05. Although the purification procedure resulted in only a 10.5-fold purification with a yield of 12% (Table 1), the preparation appeared to be highly purified, manifesting a single band following silver staining of the protein after SDS-PAGE (Fig. 1b). Activity staining revealed only one chitin clearance band matching with the corresponding protein band of lane A4 (Fig. 1b). Similarly isoelectric focusing gave a single band with a pI of 8.45 (Fig. 2). The presence of a single band in isoelectric focusing provided further evidence of the high purity of the enzyme preparation. A number of chitinolytic enzymes have been purified and well characterized biochemically from various sources. However, no reports are available with regard to the purification of an extracelluar chitinase from *Malbranchea chrysosporioidea*. This is the first report on purification and characterization of chitinase from *Malbranchea chrysosporioidea*. One of the purification steps involving adsorption on chitin was very effective and most of the proteins other than chitinases were eliminated at this stage. The overall recovery of enzyme obtained from this step was 25.5%, comparable to the recovery (27.4%) of chitinase from *Serratia marcescens* by this method reported earlier[13].

Table 1. Purification of a 100 kDa chitinase from *Malbranchea chrysosporioidea*. Chitinase activity is expressed in terms of specific activity, defined as of N-acetyl glucosamine/mg protein/min not mmols of N-acetyl glucosamine/mg protein/min

Purification step	Total protein (mg)	Total activity (units)	Specific activity (µmole of GlcNAc /mg protein/min)	Purification fold	Yield %
Culture filtrate	840	84	0.1	1	100
Ammonium sulfate precipitation	350	56	0.16	1.6	66.6
Chitin adsorption	25.2	21.5	0.85	8.5	25.5
Gel filtration	9.6	10.08	1.05	10.5	12

Table 2. Effect of various metal ions on the activity of purified chitinase: Each metal ion was added to a final concentration of 10 µM to the reaction mixture containing 20 µg of pure enzyme in 200 µl of borate buffer and 1% swollen chitin. Activity of control (without metal ion) was taken as 100%

S. No	Metal ion (20 µM)	Relative activity (%)
1	None	100
2	Cd^{2+}	132.4
3	Mn^{2+}	55.2
4	Mg^{2+}	63
5	Mo^{2+}	31
6	As^{3+}	20
7	K^{+}	50
8	Co^{2+}	18.7
9	Ca^{2+}	95
10	Fe^{2+}	78
11	Cu^{2+}	26
12	Zn^{2+}	16.6

Biochemical properties of chitinase

The purified chitinase migrated as a single band on SDS-PAGE with estimated molecular weight of 100 kDa (Fig.1b) and in agreement with the 100 kDa chitinase of *Bacillus* sp which showed chitinolytic and glyco-transferring activities[19]. A wide range of molecular sizes from 30 to 120 kDa was observed in bacteria and fungi. The isoelectric point (pI) of chitinase from *Malbranchea chrysosporioidea* isolated was

found to be 8.45(Fig.2). Chitinases have the following wide range of pI values: 3.5-8.8 in microorganisms; 3.0-10.0 in higher plants and algae; and 4.7-9.3 in insects, crustaceans, molluscs and fishes[20].

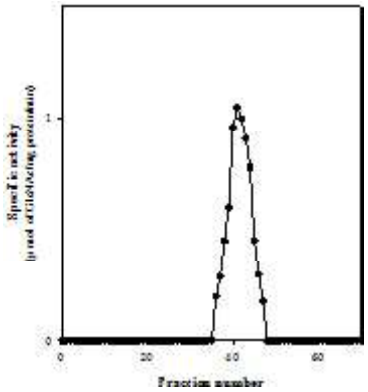

Fig.1a. Elution profile of the chitinase on Sephadex G-100: Chitinase obtained after adsorption on swollen chitin was applied to a Sephadex G-100 column. Chitinase fractions collected were pooled and rechromatographed on the same column. Specific activity was expressed as µmols of N-acetylglucosamine released /mg protein/min

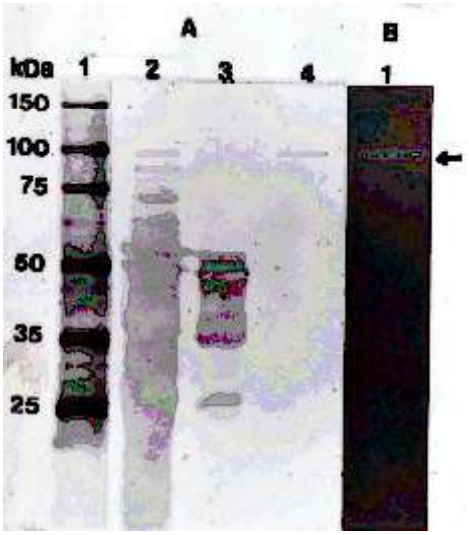

Fig.1b. SDS-PAGE (A) and Activity staining (B) of *Malbranchea chrysosporoidea* chitinase: Total proteins were resolved on a 10% SDS-PAGE and the chitinolytic activity was detected on the resolving gel contained 0.01% glycol chitin by silver stain. Lane A 1. Sigma SDS-PAGE recombinant protein markers. Lane A 2. Proteins precipitated with 80% saturation of ammonium sulfate (5 µg). Lane A 3. Proteins after chitin adsorption. Lane A 4. 500 ng of purified enzyme. Lane B 1. Activity staining of purified chitinase

The effect of pH for the purified enzyme against swollen chitin was observed to increase as the pH increased from 3 to 9 almost in a linear fashion. The enzyme exhibited a pH optimum of 9.0 that is identical to that of *Bombyx mori* chitinase, with a pH optimum between 9.5 and 10.0[21]. The stability curve of the enzyme is more or less parallel to pH curve and is more stable between pH 7-10 (Fig.3a). The enzyme is stable over a wide range of pH, retaining around 90% of its original activity between pH 7.0 to 10.0. In this regard it is similar to many plant chitinases which are stable between pH 6 and 11 and different from most fungal chitinolytic enzymes. Generally, fungal chitinolytic enzymes exhibited a pH optimum between 4.0 and 7.0 with some exceptions. One of such example is the cytosolic chitinolytic enzymes of *Saccharomyces cerevisiae* with pH optimum of 2.5[22].The optimum temperature was 37^{0}C and the enzyme was stable upto 40^{0}C and retained only

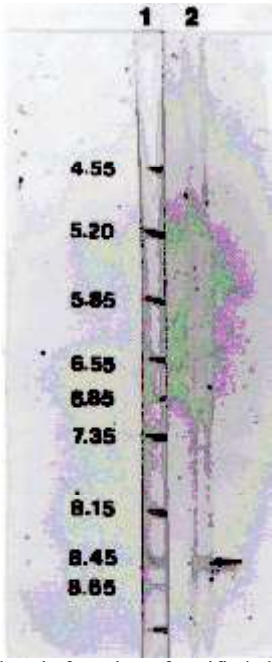

Fig.2. Isoelectric focusing of purified chitinase from a *Malbranchea chrysosporoidea* : The enzyme was subjected to isoelectric focusing on a (3-9 pH gradient) precast gel using Pharmacia LKB phast gel system. Lane 1. Standard marker- Lentil lectin (8.65); Lentil lectin (8.45); Lentil lectin (8.15); Horse myoglobulin (7.35); Horse myoglobulin (6.85); Human lactoglobulin (5.20); Soybean trypsin inhibitor (4.55). Lane 2. Purified chitinase, indicated by arrow

28% of its activity at 50^{0}C. It was completely inactivated at 60^{0}C (Fig. 3b). In this regard it is similar to insect chitinases such as the silkworm, which are also stable up to 40^{0}C[23]. We found that above 40^{0}C the enzyme was very sensitive, rapidly losing its activity.

Effect of metal ions on chitinase activity

Metal ions are sometimes necessary for enzyme activity as an additional co-factor. The enzyme was assayed in the presence of various metal ions at concentrations of 10 mM. The results obtained are shown in Table 2. Our results showed Cd^{2+}, a toxic metal enhanced the activity of the enzyme by 32% at a concentration of 10 mM and this behaviour of enzyme requires further investigation in detail to elucidate its significance. The other metals like Zn^{2+} (83.4%), Co^{2+} (81.3%), As^{3+} (80%), etc. inhibited the enzyme activity. Several chitinolytic enzymes are inhibited by the metal ions like Fe^{2+}, Hg^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} and Mg^{2+}. In our study Zn^{2+}, Co^{2+} and As^{3+} were found to be strong inhibitors of this enzyme.

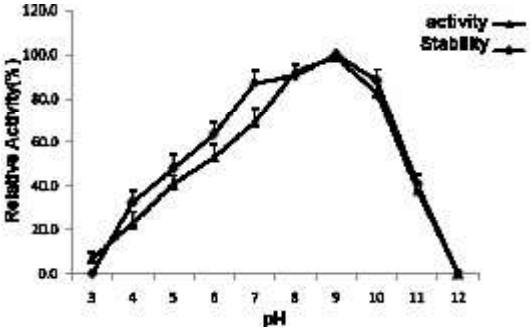

Fig.3a. Effect of pH on activity and stability of chitinase: The reaction mixture containing 20 µg of enzyme and 1% swollen chitin was maintained at the above indicated pH, using different buffers (50 mM) in the range of 3-12. For pH stability the enzyme was pre incubated at indicated pH for 1 h and the residual activity was assayed at 37^{0}C. The maximum activity was taken as 100%

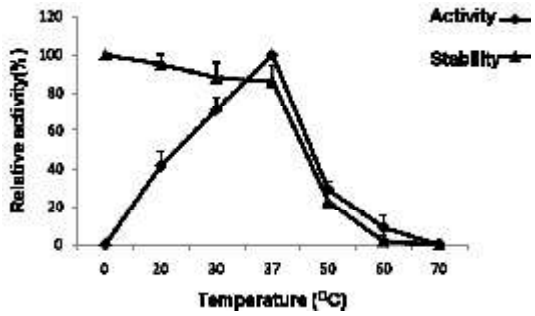

Fig.3b. Effect of temperature on activity and stability of chitinase: The reaction mixture containing 20 µg of enzyme in 50 mM borate buffer (pH 9.0) and 1% swollen chitin was incubated at the above indicated temperatures for 6 h. For thermal stability, the enzyme was pre incubated at indicated temperatures for 15 min. After adding 1% chitin the enzyme was further incubated at 37^{0}C for 6 h to measure the residual activity. The maximum activity was taken as 100%

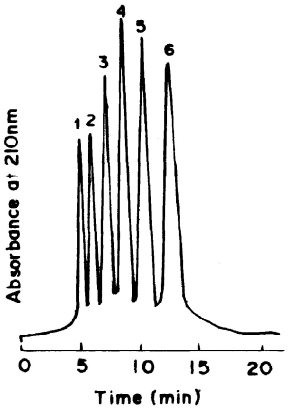

Fig.4a. RP-HPLC analysis of reaction products of *Malbranchea chrysosporoidea* chitinase: a. RP-HPLC profile of authentic standards ranging from GlcNAc 1 to GlcNAc 6 peak 1, GlcNAc1; peak 2, GlcNAc2; peak 3, GlcNAc3; peak 4 GlcNAc4; peak 5, GlcNAc5; peak 6, GlcNAc6

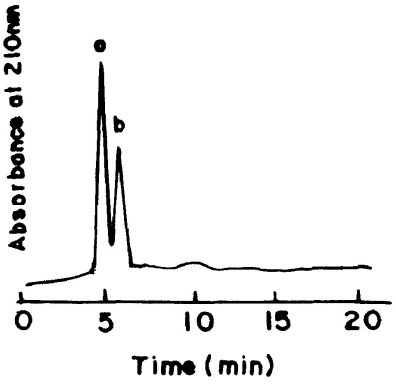

Fig.4b. RP-HPLC profile of hydrolysis products of swollen chitin by purified chitinase. 50 µg of purified chitinase in 500 µl of borate buffer pH 9.0 and 1% swollen chitin was incubated for 6 h and boiled for 3 min to stop the reaction followed by centrifugation and lyophilization of the supernatant. The hydrolysis products of swollen chitin were separated on RP-HPLC

Products of the chitinase action

To know the specificity of chitinase action, the products derived from the hydrolysis of swollen chitin by chitinase were analyzed by RP-HPLC. RP-HPLC analysis showed two peaks corresponding to N-acetylglucosamine (peak a) and chitobiose (peak b) as shown in Fig.4. These results indicate that purified chitinase of *Malbranchea chrysosporioidea* degraded swollen chitin to GlcNAc and $GlcNAc_2$ as minimum units, exhibiting both exochitinase and β-N-acetyl hexosaminidase activities. Chitinolytic organisms convert chitin to N-acetylglucosamine by a cooperative interaction of two enzymes, chitinase and N- acetylglucosaminidase and this process is a key transformation step in the biological carbon and nitrogen cycles in nature. Interestingly, in our study we detected both the monomer N-acetylglucosamine and the dimer chitobiose indicating both β-N-acetyl hexosaminidase and exochitinase activities respectively. Our results are in accordance with that of a novel chitinase from *Metarhizium anisopliae*, which showed two types of chitinase action[24]. This type of chitinase action was also observed with the chitinases of a plant, parsley, belonging to a rare class of plant chitinases. Nearly all plant chitinases isolated to date are endo-chitinases[25]. Chitinase purified from *Malbranchea chrysosporioidea* exhibiting both exochitinase and β-N-acetyl hexosaminidase activities with broad pH activity and stability are few promising properties for its biotechnological applications.

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