International Journal of Biochemistry and Biotechnology ISSN: 2167-0404 Vol. 2 (3), pp. 298-301, March 2013. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Identification of *aspergillus species* using morphological characteristic and the effect of temperature on the protease activity

Indratiningsih[1], Endang Wahyuni[1*], Ambar Pertiwiningrum[1], and Shanti Asri Suwarti[1]

[1]Dept. of Animal Products Technology, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Accepted 21 February, 2013

Aspergillus sp. is a fungus that can produce protease enzyme. Aproteolytic enzyme of Aspergillussp is enzymes group that attack the peptide bonds of proteins. **In this study, we emphasize on morphological methods including macroscopic and microscopic characteristics for identification of Aspergillus sp. Aspergillus sp was isolated from waste of milk industry. Protease was produced by *Aspergillus sp.* and the temperature effect was evaluated on its activity, at 37, 40, 45, and 50°C with casein as a substrate. The results obtained show that isolates selected have the green color colony (Fig.1. A,B,C),grown evenly on PDA medium were incubated at 37°C for 4 days, round-shaped vesicles(Fig.1.1), conidiophores somewhat yellowish green nodes, and conidiaspore round and light green. The data of protein contents is shown on Table 1. The average protein contents of the crude enzyme protease extract were 0.95 mg/ml of isolates a, 0.98 mg/ml of isolates B, and 1.03 mg/ml of isolates. Isolate C was selected to produce enzyme and analyze enzyme activities. The enzyme activities of isolate C were 85.85 U/ml at 37°C, 101.87 U/ml at 40°C, 179.00 U/ml at 45°C, and 136.81 U/ml at 50°C. The optimum activity was 179.00 IU/ml and it was reached at 45°C.**

Key words: *Aspergillus,* identification, temperature, protease activity.

INTRODUCTION

Aspergillus sp. is a fungus that can produce protease. In some Asian countries, some fungus are widely applied to producing traditional fermented food (Fogarty, 1983). Proteases from fungi have more profit than the protease from bacteria in the separation of the enzyme because the mycelium can be removed simply by filtration. Proteases produced by *A. sp.* is more important because the ability of the resulting higher protease. Solid-state fermentation (SSF) has many advantages, including large-scale productivity; substrate used is not expensive, simple process, low energy requirements and wastes little (Malathi and Chakraborty, 1990).

Generally identification of the Aspergillus species is based on the morphological characteristics of the colony and microscopic examinations (McClenny, 2005). Although molecular methods continue to improve and become more rapidly available, micros-copy and culture remain commonly used and essential tools for identification of *Aspergillus sp*.

The use of microorganisms for enzyme production has several advantages, including easy producing on a large scale; production time is relatively short and is produced simultaneously with a relatively low cost. Protease-producing microorganisms can be bacteria, fungi, and yeast (Such as, *B. licheniformis, B. subtilis, A. niger, A. sp., A. oryzae* etc). The enzymes produced by microorganisms were separated by centrifugation to separate the cells and subsequent purification is done by precipitation, gel filtration and ion exchange chromatography (Smith, l990). The most common simple method is precipitation in cold temperatures with a high concentration of ammonium salt specific (different) or the use of organic solvent, the precipitate was dissolved in buffer (Deutcher, 1990).

Proteases are enzymes that catalyse hydrolytic reactions in which protein molecules are degraded to peptides and amino acids (Sumantha et al., 2006). They constitute one of the most important groups of

*Correspondingauthor.E-mail: endang.wahyuni@gmail.com

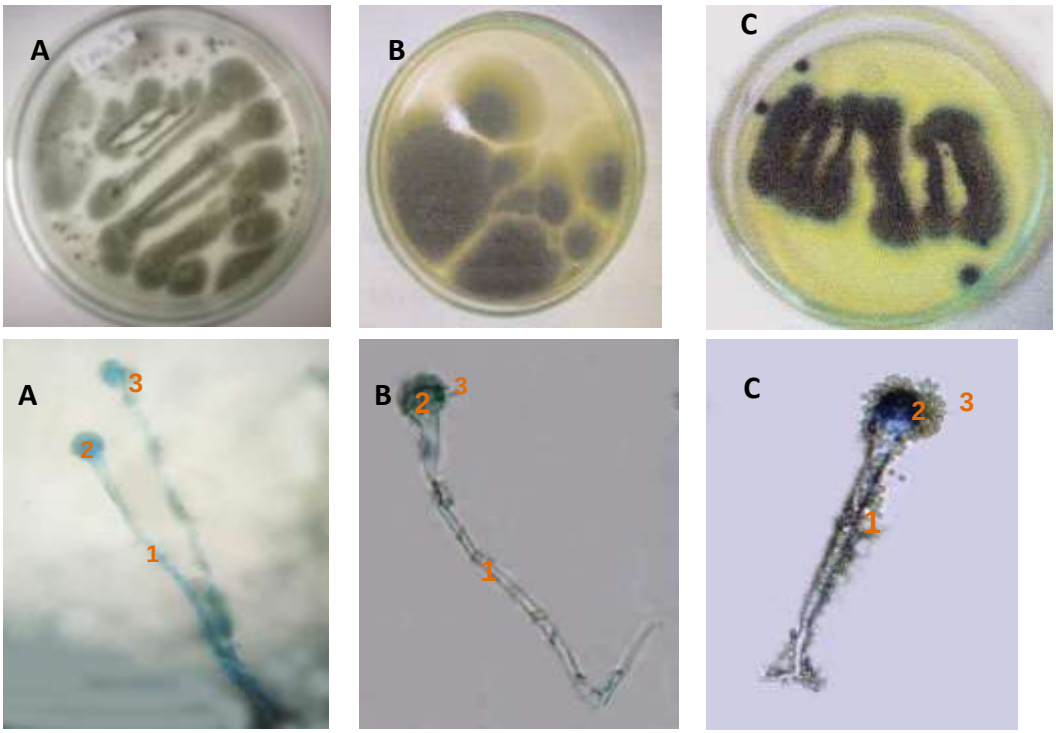

Figure 1. Photographs of the colonies and cells morphology of Aspergillus in PDA medium after 7 days showed similar morphologic. Isolates Aspergillus (A, B, C) are from dairy waste with a magnification of 40 times. 1: vesicles, 2: conidiophores, 3: conidia

industrial enzymes and have applications in different industries such as detergent, food, feed, pharmaceutical, leather and waste processing. Proteolytic enzymes are important in the industry, about 60% of the total enzyme traded in the world (Woods et al., 2001). In Indonesia, the needs of protease enzymes also increased but the need is still dependent on imported production. One way to anticipate dependence on imports is necessary to attempt to produce protease enzymes (Thomas, 1984; Suhartono, 1989).

An enzymatic reaction is the conversion of one molecule into another; a chemical reaction catalyzed at the reactive sites on the enzyme. Considering the complex nature of the enzyme itself, it is not reasonable to expect that many parameters will affect the rate of this catalytic activity. Enzyme activity can be influenced by steric hindrance, pH, temperature, and substrate concentration (Michaelis-Menten Kinetics). Temperature, a measure of the intensity of heat, is an important factor in the activity of enzymes. The velocity of an enzymatic reaction is influenced by temperature. This is because substrates with active sites frequently in the presence of rapidly moving molecules. In addition, although these molecules do move rapidly the speed of the reaction drops sharply. In short, thermal agitation causes protein molecules (enzymes) to denature (breakdown of protein structures). All enzymes have an optimal temperature at which reaction rates go fastest without denaturing the enzyme (Campbell and Reece, 2002)

This study deals to obtain protease-producing Aspergillus in solid-state fermentation conditions (SSF) using rice bran and find the optimum temperature effect on protease activity was determined.

MATERIALS AND METHODS

Sample preparation

Samples were collected from one of Indonesian milk cooperative in Yogyakarta. 1 ml of waste milk was diluted in sterile distilled water until volume reached 100 ml, and then the samples incubation were at 50°C for 45 min in a water bath shaker (Nadem et al., 2007).

Culture and identification

0.1 ml of Dairy wastes samples was inoculated onto PDA medium enriched with 1% casein, and then incubated at 37°C for 7 days (Hanjhani and Setyaningsih, 2006). Isolates obtained were observed on colony and cell morphology. Aspergillus was identified according to its specific colony

Table 1. Protein content of waste milk isolates (mg/ml)

Name of isolate	Protein content (mg/ml)
Isolate A	0.92[c]
Isolate B	0.98[b]
Isolate C	1.03[a]
Average	0.98

[a, b, c] Different superscripts in the same column indicate differences (P<0.05)

Table 2. Effect of incubation temperature on protease activity (U/ml)

Incubation temperature (°C)	Enzyme activity (U/ml)
37	85,85[a]
40	101,87[ab]
45	179,00[c]
50	136,81[b]
Average	125,88

[a, b, c] Different superscripts in the same column indicate differences (P<0.01)

characteristics, slides were also prepared for identification of mycelium and hyphial arrangement with lactophenol blue staining method (Darise, 1987). Isolate produced highest protein content were selected for protease enzyme production.

Protease Production

Isolates were grown in the medium as much as 10 g of rice bran plus 15 ml of saline solution (consisting of 2 g NaNO3, 0.5 g MgSO4.7H2O, 0.5 g KCl, 2 g KH2PO4, and 1 mg F2SO4.7H2O and ZnSO4.7H2O). Medium was sterilized at 121°C for 20 min. Incubation was carried out at room temperature for 7 days (Malathi and Chakraborty, 1990). Prepared substrate plus 200 ml phosphate buffer pH 7, and then shaked until homogeneous and filtered. The filtrate obtained is a source of crude protease enzymes.

Protein Determination

Protein was estimated according to the method of Lowry *et al*. (1951). So, 1 ml enzyme solution plus 5 ml Lowry B, then shaked and left for 10 minutes. The solution was added with 0.5 ml of Lowry A, then shaked and left for 20 minutes. The solution was read with a spectrophotometer at 600 nm absorbance.

Protease activity assays at different temperatures

It was determined using casein as a substrate according to the method reported by Pagare *et al.* (2009). 0.2 ml of crude Enzyme extract plus 2 ml casein 0.2% and 1.8 ml phosphate buffer pH 7, then incubated at 37, 40, 45, 50°C for 10 min. At the mixture we added 4 ml of 5 % TCA and allowed to stand at room temperature for 20 minutes. The mixture was filtered with Whatman paper No. 1, which was read by spectrophotometer at 280 nm absorbance (Pagare et al., 2009).

RESULTS AND DISCUSSION

Isolates selected have the green color colony (Figure.1. A, B, C), grown evenly on PDA medium were incubated at 37°C for 4 days, round-shaped vesicles (Fig.1. 1), conidiophores somewhat yellowish green nodes (Fig.1. 2), and conidiaspore round and light green (Fig.1. 3). Based on the results of cell morphology observers, these isolates belong to the group of *Aspergillus sp*. that have the same characteristics, namely globular vesicles, conidiophores shaped translucent yellowish green, semi conidiaspore round to round-shaped light green to brownish green (Yunasfi, 2008).
Coniodiaspore colors caused by lipid compounds red, brown, yellow on the walls of spores, while the dark

Spores caused by walls containing melanin (Schenck, 1982).

The average protein contents of the crude enzyme protease extract were 0.95 mg/ml of isolates A, 0.98 mg/ml of isolates B, and 1.03 mg/ml of isolates C (Table 1). According to Harlis (2008), different types of mold provide different levels of protein. The differences in levels of a protein produced in each mold was due to different populations of fungi that get each activity in the enzyme protease is also different.

On the other hand, the results obtained shows that the temperature affect significantly the activity of proteolytic enzymes. The data of effect of temperatures on protease activity of Aspergillus sp.is shown on Table 2.The difference temperatures can affect the protease activity of *Aspergillus sp.* with a range between 200 U/ml and 1,000 U/ml with 3% casein as a substrate. (Table 2). So, the highest enzyme activity, i.e., 179 U/ml was obtained at an incubation temperature of 45°C ($P<0.01$). Similar results were reported by Hossain et al. (2006); Morimura *et al.* (1994).

According to Chaplin and Christopher (1990), the use of enzymes at high temperatures was better because it improved the response and able to protect against microbial contamination. However, enzymes are proteins that can undergo denaturation at temperatures above the action of the enzyme and usually are influenced by the environment.

CONCLUSION

Isolates selected are Aspergillus sp that have the green color colony, round-shaped vesicles, yellowish green nodes conidiophores, and round and light green conidiaspore. The highest protease enzyme activity of Aspergillus sp. isolated from waste milk (179 U / ml) was achieved at 45°C.

REFERENCES

Campbell NA, Reece JB (2002). Biology, 6th ed. Don O'Neal ed. Benjamin Cummings Publishing, Menlo Park, California. Pp.300-340

Chaplin M, Christopher B (1990). Enzyme Technology. Cambridge University Press. Cambridge.

Darise HL (1987). Medically important Fungi. A guide to identification. Pp.14-15.

Deutscher MP (1990). Guide to Protein Purification, Vol. 182. Academic Press, San Diego, CA.

Fogarty WM (1983). Microbial enzymes and biotechnology, Applied Science Publishers, London, 317p, 1983.

Hanjhani NS, Setyaningsih R (2006). Identifikasi Jamur dan Deteksi Aflatoksin B1 Terhadap Petis Udang Komersial. Biodiversitas Vol. 7(3): 212-215

Harlis (2008). Pengaruh Konsentrasi Aspergillus oryzae (Ahlburg) Cohn dan *Rhizopus oligosporus* Saito dalam Fermentasi Kedelai pada Pembuatan Kecap. ISSN 0854-8986 Vol.91. Fakultas Keguruan dan Ilmu Pendidikan. Universitas Jambi. Jambi.

Hossain TMD, Das F, Maran LW, Shafiqur Rahman MD, Anwar MN (2006). Some Properties of Protease of Fungal Strain *Aspergillus flavus*. Int. J. Agr.and Biol. Chittagong. Bangladesh.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275

Malathi S, Chakraborty R (1990). Production of Alkaline Protease by A New *Aspergillus flavus* Isolate under Solid-Substrate Fermentation Conditions for Use as a Depilation Agent. Dept. of Biochem. Central Leather Research Institute, Madras, India.

McClenny N (2005). Laboratory detection and identification of Aspergillus species by microscopic observation and clture. J. Md. Mycol.1:125-128

Morimura S, Kida, Sonada Y (1994). Production of protease using waste water from the manufacture of Smith GR (1990).RecBCD enzymein Nucleic acids and molecular biology, eds Eckstein, F., Lilley, D.M.J. Springer-Verlag, Berlin. Pp.78–98.

Shochu. J.Ferment.Bioeng. 77:183-187.

Nadem M, Qazi JI, Baig S, Syed Q (2007). Studies on Commercially Important Alkaline Protease from *Bacillus lichniformis* N-2 Isolated from Decaying Organic Soil. Turkish J. Biochem Vol. 32(4):171-177.

Pagare RS, Ramdasi AM, Khandelwal SR, Lokhande MO, Aglave BA (2009). Production and Enzyme Activity of An Extracellular Protease from *Aspergillus niger* and *Bacillus subtilis*. Int. J. Biotechnol. Biochem. India.

Schenck NC (1982). Methods and Principles of Mycorrhizal Research. The American Phytopathological Society.

Suhartono MT (1989). Enzim dan Bioteknologi. Departemen Pendidikan dan Kebudayaan Direktorat Jenderal Pendidikan Tinggi Antar Universitas Bioteknologi Institut Pertanian Bogor. Bogor.

Sumantha A, Larroche Ch, Pandey A (2006). Microbiology and industrial biotechnology of food-grade proteases: a perspective. Food-Grade Proteases, Food Technol. Biotechnol. 44(2):211-220.

Thomas DB (1984). A Textbook of Industrial Microbiology. Sinaver Associates Sunderland, USA.

Woods YL, Cohen P, Becker W, Jakes R, Goedert M, Wang X, Proud CG (2001). The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bepsilon at Ser539 and the microtubule-associated protein tau at Thr212: potential role for

DYRK as a glycogen synthase kinase 3-priming kinase. Biochem J. 355(Pt 3):609-615.
Yunasfi (2008). Fungi at *Eucalyptus urophylla* S.T. Blake in Log Yard (TPK) PT. Toba Lestari, Tbk. Kabupaten Toba Samosir, North Sumatera. J. Hutan dan Masyarakat Vol 3 (1).