Screening of Cellulase- and Amylase- Producing Fungi to Fermenting the Agro-industrial Waste for Using as Feedstuffs

การคัดเลือกเชื้อราที่มีคุณสมบัติผลิตเอนไซม์เซลลูเลสและอะไมเลสเพื่อใช้ในกระบวนการหมักเศษเหลือทิ้งอุตสาหกรรมเกษตรเพื่อใช้เป็นวัตถุดิบอาหารสัตว์

Usaneeporn Soipeth (อุษณีย์ภรณ์ สร้อยเพ็ชร์)* Dr.Saowanit Tongpim (ดร.เสาวนิต ทองพิมพ์)**

Dr.Vichai Leelavatcharamas (ดร.วิชัย ลีลาวัชรมาศ)*** Terdsak Khammeng (เทอดศักดิ์ คำเหมิ่ง)****

ABSTRACT

Agro-industrial wastes can be used as feedstuffs, however its nutritive value should be improved. This study focus on selected fungi which produced cellulase and amylase enzyme and utilized agro-industrial wastes as substrate. Four fungal isolates, *Aspergillus niger* TISTR 3056, 3245, 3254 and 3281 were examined for cellulase and amylase production with Gram's Iodine on Carboxyl methyl cellulose (CMC) agar and PDA with 1 % soluble starch (w/v), measuring enzyme activity by Dinitrosalicylic acid (DNS) method. *A. niger* 3056 showed highest amylase activity (127.17 U/ml) and cellulase activity (184.13 U/ml) compared with other isolates. Solid state fermentation of DDGS to increase protein levels by *A.niger* 3056 was 20.22 % of crude protein at 30 ^{o}C for five day. So, *A. niger* could be developed agro-industrial wastes to utilizable raw materials for various beneficial uses such as feedstuffs

บทคัดย่อ

เศษเหลือทิ้งอุตสาหกรรมเกษตรสามารถใช้เป็นวัตถุดิบอาหารสัตว์ได้ด้วยการปรับปรุงคุณภาพทางโภชนาการ การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อคัดเลือกเชื้อราที่มีคุณสมบัติผลิตเอนไซม์เซลลูเลสและอะไมเลสเพื่อใช้ในกระบวนการหมักเศษเหลือทิ้งจากอุตสาหกรรมเกษตร เชื้อรา *Aspergillus niger* จำนวน 4 สายพันธุ์ ได้แก่ *A. niger* TISTR 3056, 3281, 3245 และ 3254 ถูกนำมาทดสอบประสิทธิภาพการผลิตเอนไซม์เซลลูเลสและอะไมเลส โดยการวัดเส้นผ่าศูนย์กลางของบริเวณใสที่เกิดจากการทดสอบด้วย Gram's iodine บนอาหารจำเพาะ CMC และ PDA ที่เติมสารละลายแป้ง 1 % w/v และวัดค่ากิจกรรมของเอนไซม์โดยวิธี Dinitrosalicylic acid (DNS) พบว่า *A. niger* TISTR 3056 มีกิจกรรมของเอนไซม์อะไมเลสสูงสุดเท่ากับ 127.17 Unit/ml และเซลลูเลสสูงสุดเท่ากับ 184.13 Unit/ml เมื่อเทียบกับไอโซเลทอื่น และการใช้เชื้อรา *A. niger* TISTR 3056 ในกระบวนการหมักกากเอทานอล สามารถเพิ่มระดับโปรตีนเป็น 20.22 % ที่อุณหภูมิ 30 องศาเซลเซียส ระยะเวลา 5 วัน ดังนั้นเศษเหลือทิ้งที่หมักด้วยเชื้อรา *A. niger* ในสภาวะที่เหมาะสม สามารถพัฒนานำสู่การใช้เป็นวัตถุดิบอาหารสัตว์ได้อย่างมีประสิทธิภาพ

Key Words: *Aspergillus niger*, Agro-industrial waste, Feedstuffs

คำสำคัญ: เชื้อรา *Aspergillus niger* เศษเหลือทิ้งอุตสาหกรรมเกษตร วัตถุดิบอาหารสัตว์

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* *Ph.D. student of Animal Science, Department of Animal Science, Faculty of Agriculture, Khon Kaen University*

** *Assistant Professor, Microbiology Department, Faculty of Science, Khon Kaen University*

*** *Assistant Professor, Biotechnology Department, Faculty of Technology, Khon Kaen University*

**** *Associate Professor, Animal Science Department, Faculty of Agriculture, Khon Kaen University*

Introduction

Fermentation technology is the importance technology for utilize the cheap wastes as the raw material for microorganism to growth and producing the enzyme or specific essential nutrients by the optimize environment such as temperature, pH, moisture, source of nitrogen and energy. Utilization of agro-industrial wastes as feedstuffs by fermentation need microorganisms which high digestible enzyme as cellulase and amylase enzyme. These enzyme produced by microorganisms as bacteria and fungi (Klyosov, 1990) and varieties of enzyme from fungi are importance in the biotechnology industry, several important researches are including the study of single cell protein, eukaryotic protein secretion in general and mechanism involved in control of fungal morphology such as *Aspergillus niger*, *Trichoderma* sp. etc. (Amir *et al*., 2011 and Dashtban *et al.,* 2009.)

The increasing demand for raw materials for industrial production, the non-renewable resources are dwindling day-by-day, wet products are the main pollution because no area to dry and smell not good for health. Production of cassava powder industry is 200-2,000 tons/day, by-products is 40-50 tons/day as wet by-products 23.39 % (Pirapot *et al.,* 2009) The amount of citric acid by-product from citric acid industry is 73-74 % of day, include crude protein 1-3 %, crude fiber 4-15 %, NFE 66-81 % and pH 4 (Pirapot *et al.,* 2009 and Terdsak *et al*., 2006.) This acid raw material is optimizing for fermentation by *A. niger* (Usaneeporn, 2007). The DDGS from ethanol industry production is 30-40 tons/day (from 150,000 liters/day ethanol), that include 10 % crude protein and 10 % fiber. These agro-industrial wastes can be used as feedstuffs because these wastes have enough carbon sources such as carbohydrate fiber and cellulose for fermentation and produce protein or essential nutrients by microorganisms. (Ministry of Energy, 2006)

A. niger is a filamentous ascomycete fungus, which is ubiquitous in the environment. *A. niger* is a common member of the microbial communities found, that plays a significant role in the global carbon cycle. This organism is a producer of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocelluloses. *A. niger* is economically importance as a fermentation organism used for production of citric acid, as a model fungal fermentation process (Scott, 2006). And Jayant *et al.* (2011) was produced cellulase enzyme by *A. niger*, the maximum enzyme activity was 3.56 unit/ml. (Olufunke *et al*., 2010) using cassava peels as raw material in submerge fermentation by *Trichoderma viride* ATCC 36361 combined with enzyme, the product was 37.63 % crude protein. And David (2010) was used solid state fermentation by *A. niger* TC-4 to increase protein in *Terminalia catappa* fruit meal (FTCM) from 47.2 % CP to 74.3% CP. The gain by-products fermented with *Candida tropicalis* (ccum 8223) was 8.69-9.19 % crude protein.

Objectives of the study

This study focus on the selection of fungi to have cellulase- amylase enzyme production using agro-industrial waste as substrate and efforts are to be made for controlling pollution arising out of the disposal of wastes by conversion of these unwanted wastes into utilizable raw materials for various beneficial uses such as feedstuffs.

Methodology

Substrate selection

Agro-industrial waste of citric acid by-product and DDGS (Distiller's Dried Grains with Soluble) collected from local industry of Kalasin Province, Thailand and Ethanol Public Limited Company, Khon Kaen, Thailand. The substrates were dried in oven at 60 ^{o}C and grinded mechanically with electric grinder to make in particle form to 4 meshes, sterilized at 121 ^{o}C for 15 minutes.

Microorganism selection

The strain TISTR 3056, 3281, 3245, 3254 were obtained from stock cultures of TISTR, Bangkok, Thailand.

Maintenance of *Aspergillus niger*

Strains of *A. niger* maintained on potato dextrose agar (PDA) medium slants under sterilized at 121 ^{o}C for 15 minutes in autoclave, incubated at 30 ^{o}C for 72 hrs and subsequently stored at 4 ^{o}C for inoculums preparations (Saowapa, 2010). The spores of cultured *A. niger* on PDA medium were isolated aseptically using sterilized water with 0.1 % Tween 80 was used for inoculation.

Spore suspension

The inoculums were counted on a Haemacytometer slide bridge before used as spore suspension. The inoculums were diluted to $1x10^{5}$ spore/ml. (Saowapa, 2010) for initiate growth on cellulose activity and amylase activity and diluted to $1x10^{8}$ spore/ml for fermentation (Usaneeporn, 2007).

Assessment of amylase activity in starch agar medium

The starch agar medium contained 1 % of soluble starch in PDA medium, pH of medium was adjusted to 7 with 1M HCl/ 1M NaOH and was sterilized at 110 ^{o}C for 28 minutes. The starch agar plates were prepared and were inoculated with *A. niger*. The plates were incubated at 28 ^{o}C for 3 days. In order to detect amylase activity, plates were flooded with gram's iodine solution for 2 minutes which indicated the amylase producing ability of the strains.

Assessment of cellulase activity in CMC agar medium

The CMC agar medium contained $CaCl_2$ (0.1 g/l), $(NH_4)_2SO_4$ (0.5 g/l), K_2HPO_4 (1.0 g/l), $MgSO_4$ (0.2 g/l), KCl (0.5 g/l), yeast extract (0.5 g/l), Carboxymethylcellulose (10.0 g/l), agar powder (17.0 g/l), pH of medium was adjusted to 5 with 1M HCl/ 1M NaOH and was sterilized at 110 ^{o}C for 28 minutes. The CMC agar plates were prepared and were inoculated with *A. niger*. The plates were incubated at 30 ^{o}C for 3 days. In order to detect cellulsae activity, plates were flooded with gram's iodine solution for 2 minutes. (Positive control was cellulase enzyme from *A. niger* (sigma) 10 mg/ml (2.41 Unit/ml) and negative control was distilled water).

Analytical Procedure

The spore suspension (1 x 10^{5} spore/ml) 5 ml was used to initiate growth in 250 ml Erlenmeyer flask supplemented with 50 ml CMC medium ; $CaCl_2$ (0.1 g/l), $(NH_4)_2SO_4$ (0.5 g/l), K_2HPO_4 (1.0 g/l), $MgSO_4$ (0.2 g/l), KCl (0.5 g/l), yeast extract (0.5 g/l), Carboxymethylcellulose (10.0 g/l). Then inoculated flasks were placed in shaker incubator at 28 ^{o}C and 150 rpm for 72 hrs and pH was adjusted at 7. The shaked flasks were filtered by Whatman filter paper No.1 and centrifuged at 10,000 rpm and 4 ^{o}C for 10 minutes to eliminate impurities and insoluble

materials. The supernatants were carefully collected with the help of auto-pipette.

Amylase activity

The determination of enzyme activity was done by using Dinitrosalicylic acid (DNS) method. Taking 0.25 ml of starch soluble in Tris-HCl buffer, incubated at 90 ^{o}C for 15 minutes, added 0.25 ml of crude enzyme and incubated at 90 ^{o}C for 15 minutes then enzyme sample was broiled with 0.5 ml DNS 3, 5-Dinitrosalicylic acid for 10 minutes, following cooling and added 5 ml distilled water, absorbance of sample was taken at 520 nm. (Mandel *et al.,* 1976). The absorbance was translated by plotting against regression equation to get µg/ml/min of glucose by inserting into the following formula to calculate units of enzyme activity.

Cellulase activity

Cellulase activity was done by using Dinitrosalicylic acid (DNS) method. Taking 0.25 ml of CMC soluble in citrate buffer, incubated at 40 ^{o}C for 15 minutes, added 0.25 ml of crude enzyme and incubated at 40 ^{o}C for 15 minutes then enzyme sample was broiled with 0.5 ml DNS 3, 5-Dinitrosalicylic acid for 10 minutes, following cooling and added 5 ml distilled water, absorbance of sample was taken at 520 nm. (Mandel *et al.,* 1976 ; Tabao and Monsalud, 2010). The absorbance was translated by plotting against regression equation to get µg/ml/min of glucose by inserting into the following formula to calculate units of enzyme activity.

Enzyme activity (µg/ml/min) = (Absorbance of enz. x Regression equation)/ Time of incubation

(One unit of enzyme activity was defined as the amount of glucose (µg) released per ml of enzyme solution per minute)

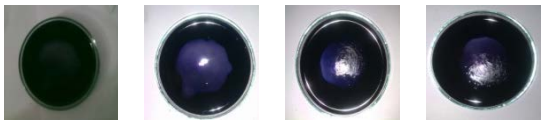
Solid state fermentation

Sterilized citric acid by-product and DDGS 100 g which added in each tray then added 10 ml spore suspension (1x10^{8} spore/ml) and 20 ml culture media. Spread for 1 cm high and cover the tray by moisture cloth at room temperature. Collected sample at 0, 24, 48, 72, 96, 120, 144, 168 hrs for proximate analysis (AOAC, 1984).

Results and Discussion

Screening of *Aspergillus niger* for amylase production

A. niger 4 strains were inoculated on 1 % starch agar plate and incubated at 28 ^{o}C for 3 days, plates were flooded with gram's iodine solution for 2 minutes. The clear zone after pouring surface media with gram's iodine solution indicated starch degradation activity of *A. niger* on PDA consisted of 1 % starch soluble (w/v) showed in Fig. 1



3245	3056	3254	3281
(3 ± 0.1 cm)	(5 ± 0.1 cm)	(2.5 ± 0.1 cm)	(3 ± 0.1 cm)

Figure 1 Clear zone of amylase producing of *A. niger*

The average diameter of the clear zone that *A. niger* 3056 had larger clear zone (5 ± 0.1 cm) than the other (*A. niger* 3245, 3254 and 3281 were 3 ± 0.1 cm, 3 ± 0.1 cm, and 2.5 ± 0.1 cm respectively), and was marked. The diameter of clear zone was not confirmed that *A. niger* 3056 was better than the others after determinate of reducing sugar by Dinitrosalicylic acid (DNS) method in PDA broth consisted of 1 % starch soluble (w/v). (Table 1)

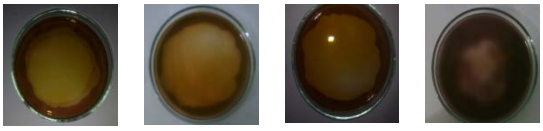
Table 1 Amylase activity of *A. niger*

A. niger	Clear zone (cm)	OD 520 nm	enzyme activity (U/ml)
TISTR 3056	5 ± 0.1	0.60	127.17
TISTR 3245	3 ± 0.1	0.10	21.195
TISTR 3254	3 ± 0.1	0.09	19.075
TISTR 3281	2.5 ± 0.1	0.34	72.063

The starch degradation efficiency was considered from maximum average diameter of the clear zone. Results showed that *A. niger* 3056 had larger clear zone (5 ± 0.1 cm) than the other and had higher enzyme activity (127.17 Unit/ml), however, *A. niger* 3281 had smallest clear zone (2.5 ± 0.1 cm) but had high activity (72.063 Unit/ml)

Screening of *A. niger* for cellulase production

The clear zone formation around the colony which indicated the cellulase production from *A. niger* (Fig. 2) Cellulase ability of *A.niger* 3056 was approximated to the others (*A.niger* 3056 (8.0 ± 0.1 cm) *A. niger* 3245 (7.5 ± 0.1 cm) *A. niger* 3254 (8 ± 0.1 cm) and *A. niger* 3281 (7 ± 0.1 cm), respectively.



3245 (7.5 ± 0.1 cm) | 3056 (8 ± 0.1 cm) | 3254 (8 ± 0.1 cm) | 3281 (7 ± 0.1 cm)

Figure 2 Clear zone of cellulase producing of *A. niger*

All of fungal isolates were examined for cellulase production by stain with Gram's Iodine on Carboxyl methyl cellulose (CMC) agar and then determined of reducing sugar by Dinitrosalicylic acid (DNS) method in CMC broth. (Table 2)

Table 2 Cellulase activity of *A. niger*

A. niger	Clear zone (cm)	OD 520 nm	enzyme activity (U/ml)
TISTR 3056	8 ± 0.1	0.87	184.13
TISTR 3245	7.5 ± 0.1	0.55	116.40
TISTR 3254	8 ± 0.1	0.30	63.49
TISTR 3281	7 ± 0.1	0.61	129.10

The *A. niger* was high degradation efficiency of cellulase, *A. niger* 3056 was best activity (184.13 Unit/ml) than others, and the same as amylase production, *A. niger* 3281 had smallest clear zone (7 ± 0.1 cm) but was second activity (129.10 Unit/ml) followed by *A. niger* 3245 and (116.40 and 63.49 Unit/ml, respectively).

Cellulase and amylase, these enzyme produced by microorganisms as bacteria and fungi (Klyosov, 1990). Jayant *et al.* (2011) used wastes from paper, cotton waste and bagasse as substrate in solid state fermentation to produce cellulase enzyme by *A. niger*, the maximum enzyme activity at 8 days was 3.56 unit/ml. Suganthi *et al.* (2011) inoculation and incubation for 6 days at room temperature with pH 7, the specific activity was recorded as 86 U/mg for amylase enzyme produced by *A.niger* BAN 3E.

However, the ability of *A.niger* had been screened, *A. niger* 3056 gave higher enzyme activity and optimization of growth conditions were important for best growth of fungi. The fungal enzymes produced cannot explain if the enzyme activity was proportional to the width of the clear zone. Therefore, necessary to analyze the activity of enzymes produced by fungi to confirm amount of each fungal isolate enzyme.

Solid state fermentation

The composition of citric acid by-product and DDGS by *A.niger* under solid state fermentation was represented (Table 3)

Table 3 Compositions of citric acid by-product and DDGS fermentation products

Nutrients (%)	F-cassava[3/]	Citric acid	DDGS	F-citric acid	F-DDGS
Dry matter[1/]	90.17	-	91.65	92.00	90.50
Crude protein[2/]	11.25	7.58	9.64	16.57	20.22
Ether extract[2/]	2.64	1.19	1.49	1.05	0.65
Crude fiber[2/]	4.30	-	26.78	6.62	10.11
Ash[2/]	8.62	19.72	12.71	8.45	6.67
NFE[2/]	63.36	-	41.03	59.31	52.85
GE (kcal/kg)[2/]	4,290	3,470	-	3,500	3,530

[1/] air dry basis (%), [2/] dry matter basis (%), NFE (nitrogen free extract)=100-(% H_2O +% CP + % EE + % CF + % Ash), [3/]Usaneeporn (2007)

The fermentation of citric acid by-product and DDGS by *A.niger* had optimum condition at 25-30 degree Celsius for 5 days (120 hrs), pH 6 and 60 % of initial moist. The fermented citric acid by-product was 16.57 % CP from 7.58 % CP and fermented DDGS was 20.22 % CP from 9.64 % CP, both of the waste had efficiency to use as protein source in animal feedstuffs and the low content of crude fiber was caused by the fungi used as carbon source.

Similarly, Odunsei et al. (2002) the result of *A. niger* gave the improvement of protein that high enough as much as 52.04% and the decreasing crude fiber around 42.03 % of the waste and same as Abalaka and Safiya (2010) *Candida tropicalis* using fiber from cereal wastes to enriched protein.

This experiment used citric acid by-product and DDGS as the main carbon source for fungal. The fungi had grown as well and sporulation in the 1-5 days and the growth rate decreased with increasing fermentation time. The optimum temperature was 25-30 degrees Celsius at pH 6 with 60 % of initial moisture and spores suspension from $1x10^8$ spores/ml.

A. niger 3056 and 3281 produced the amylase and cellulase enzyme higher than the other isolates. As a result, the growth rate in the fermentation process as well. When analyzing the chemical composition of the material showed that fermented citric acid by-product had crude protein 16.57 % from 7.58 % and the fermented DDGS had crude protein 20.22 % from 9.64 % by *A. niger* 3056, which can be used as protein source as feedstuffs. However, the further experiment needed to confirm and measuring in feeding trials.

Conclusions

Selection of fungi that has the ability of amylase- and cellulase-production to increase protein levels in raw materials by solid state fermentation, *A. niger* TISTR 3056 has efficiency of amylase and cellulase production confirmed by diameter of clear zone and enzyme activity. Increasing protein levels of fermented DDGS from 9.64 to 20.22 % CP as protein source.

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References

Abalaka, M. E. and Safiya, Y. D. 2010. Assessment of performance of chicks fed with cereal wastes enriched with single cell protein – *Candida tropicalis*. Au. J. T. 13(4): 261-264.

Amir, I., Zahid, A., Yusuf, Z., Iqbal, H., Aish, M., Muhammad, I. and Sajid, M. 2011. Optimization of cellulase enzyme production from corn cobs using *Alternaria alternate* by solid state fermentation. J. Cell and Mole.Biol. 9(2): 51-56.

AOAC (Association of official Analytical Chemists). 1984. Official meyhods of Analysis. 14th edn. A.O.A.C. Arlington. VA.

Dashtban, M., Schraft, H. and Qin, W. 2009. Fungal Bioconversion of lignocellulosic residues; opportunities & perspective. Int. J. Biol. Sci. 5: 578-595.

David, F. A. 2010. Effect of *Terminalia catappa* fruit meal fermented by *Aspergillus niger* as replacement of maize on growth performance, nutrient digestibility and serum biochemical profile of broiler chickens. Biotech. Res. Int. vol. 2011.

Jayant, M., Rashmi, J., Shailendra, M. and Deepesh, Y. 2011. Production of cellulase by different co-culture of *Aspergillus niger* and *Penicillium chrysogenum* from waste paper, cotton waste and baggase. J. Yeast and Fungal Res. 2(2): 24-27.

Klyosov, A. A. 1990. Trends in biochemistry and enzymology of cellulase degradation. Biochem. 29:10577-10585.

Mandel, M., Andreotti, R. and Roche, C. 1976. Measurement of saccharifying cellulase. Biotech. Bioeng Symp. 6: 21-23.

Ministry of Energy. 2006. Value added to ethanol waste product. Report of Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Bangkok, Thailand.

Odunsei, A. A., Akande, T. O., Yusup, A. S. and Salam, R. J. 2002. Comparative utilization high inclusion rate of four agro industrial by products in the diet of egg type chickens. Arch. Zootech. 51: 465-468.

Olufunke, O. E., Ogugua, C. A., Hans, P. B. and Thaddeus, C. E. 2010. Protein enrichment of cassava peel by submerged fermentation with *Trichoderma viride* (AT36316). Afr. J. Biotech. 9(2): 187-194.

Pirapot, N., Takehiro, N., Rumpai, C., Virote, P. and Kritapol, S. 2009. Metabolizable energy evaluation of Pangola grass hay, cassava chip, cassava plup and brewery waste in Thai Native cattle. Proceedings of the Agricultural Conference, 26-27 January 2009, Khon Kaen University, Khon Kaen, Thailand.

Saowapa, S., Prasan, S., Winyou, P., Dueantem, T., Kanjana, R. and Wira, S. 2010. Isolation of cellulase producing fungi in plant genetics conservation project under the Royal Institute of Her Royal Highness Princess Maha Chakri Sirindhorn Rambhai Barni Rajabhat University. Proceedings of the 5th Thai resources Conference, 3-5 November 2011, Rajamangala University of Technology Isan, Nakhon Ratchasima, Thailand.

Scott, E. B. 2006. *Aspergillus niger* genomics: Past, present and into the future. Med.Micro. 44: 17-21.

Suganthi, R., Benazir, J. F., Santhi, R., Ramesh, K. V., Anjana, H., Nitya, M., Nidhiya, K. A., Kavitha, G., Lakshmi, R. 2011. Amylase production by *Aspergillus niger* under solid state fermentation using agro industrial wastes. Int. J. Eng. Sci. Tech. 3(2): 1756-1763.

Tabao, N. S. C. and Monsalud, R. G. 2010. Screening and optimization of cellulase production of Bacillus strains isolated from Philippine mangroves. Philippines J. Syst. biol. 4: 79-87.

Terdsak, K., Chainarong, N., Phairuch, L., Tanapat, S., Usaneeporn, S., Yupawan, S. and Boonmee, D. 2006. Utilization of Waste Obtained from Cassava Starch and Citric Acid Manufacturing as Feeds in Muscovy Ducks Rations. Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand.

Usaneeporn, S. 2007. Effects of Protein-Enriched and Phytase Cassava from *Aspergillus niger* on Commercial Duck Rations. Master of Science Thesis in Animal Science, Graduate School, Khon Kaen University, Khon Kaen, Thailand.