Production of Fungal-Xylanase using Agricultural Waste by Solid State Fermentation

Ancharida Svarachorn

In comparative studies on using rice straw, corn cob, rice husk, penut hull, sugar cane bagasse, rice bran, as a substrate for xylanase production by *Aspergillus fumigatus* strain 4-45-1F, rice straw was found to be the best substrate. Maximum xylanase production (16.8× 10^4 units/g rice straw) was obtained when 1.2×10^5 spores/g rice straw were grown at 40°C for 6 days on a substrate containing 5 mesh ground rice straw, supplemented with 0.8% (w/w) NH_4NO_3 and 82 g rice straw/100 ml of water, at initial pH of 4.5. The previous induction of xylanase production by using xylan as a substrate for cultivation of spore inoculum was not necessary. In addition to xylanase, a substantial amount of cellulase was also detected. Hydrolysis of various cellulosic wastes by the crude xylanase prepared showed that the highest amount of reducing sugar (0.26 g/g) was obtained when sugar cane bagasse was used as a substrate.

Key words: xylanase, xylan, *Aspergillus*, agricultural waste, solid state fermentation.

Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

การผลิตเอนไซม์ไซลาเนสจากเชื้อราโดยใช้วัสดุเหลือทิ้งทางเกษตรกรรมโดยกระบวนการหมักแบบแข็ง

อัญชริดา สวารชร (2542)

วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **24 (1)**

ผลการเปรียบเทียบการใช้ฟางข้าว ซังข้าวโพด แกลบ เปลือกถั่ว ชานอ้อยและรำข้าว เป็นสารตั้งต้นของการผลิตเอนไซม์ไซลาเนส (xylanase) โดย *Aspergillus fumigatus* รหัส 4-45-1F พบว่าสารตั้งต้นที่ให้ผลดีที่สุดคือฟางข้าว ปริมาณเอนไซม์ไซลาเนสสูงสุดที่ได้คือ 1,120 หน่วยเอนไซม์/กรัมของฟางข้าว เมื่อฟางข้าวที่ใช้มีขนาด 5 เมช เติม NH_4NO_3 0.8% (น้ำหนัก/น้ำหนัก) 82 กรัมของฟางข้าว/ 100 มิลลิลิตรน้ำ เชื้อเริ่มต้น 1.2×10^5 สปอร์/กรัมของฟางข้าว pH เริ่มต้น 4.5 บ่มเชื้อที่อุณหภูมิ 40° ซ. เป็นเวลา 6 วัน เชื้อเริ่มต้นไม่จำเป็นต้องเพาะเลี้ยงไว้ในอาหารเลี้ยงเชื้อที่มีไซแลนเป็นแหล่งคาร์บอนก่อน *A. fumigatus* รหัส 4-45-1F เมื่อเจริญบนฟางข้าว นอกจากจะผลิตเอนไซม์ไซลาเนสแล้วยังผลิตเอนไซม์เซลลูเลสในปริมาณมากด้วย โดย crude enzyme ที่ผลิตได้สามารถย่อยสลายชานอ้อยไปเป็นน้ำตาลรีดิวซ์ได้มากกว่าเมื่อใช้วัสดุเหลือทิ้งทางเกษตรกรรมชนิดอื่นเป็นสารตั้งต้น ปริมาณน้ำตาลรีดิวซ์สูงสุดที่ได้คือ 0.26 กรัม/กรัมของชานอ้อย

คำสำคัญ ไซลาเนส, ไซแลน *Aspergillus,* วัสดุเหลือทิ้งทางเกษตรกรรม, กระบวนการหมักแบบแข็ง

INTRODUCTION

Cellulosic waste is composed of cellulose, hemicellulose and lignin in the approximate ratio of 4:3:3.[1] Therefore, the efficient usage of cellulosic waste, not only cellulose but also hemicellulose and lignin, should be considered.

Xylan, the polymer of xylose,[2] is the main component of hemicellulose.[3] Acid or enzymatic hydrolysis of xylan results in xylose which is a substrate for the production of several useful compounds, for example, single cell proteins, ethanol, butanol, and xylitol.[4,5,6,7] Enzymatic hydrolysis has an advantage over acid hydrolysis in that the xylose obtained has a higher purity, and it does not cause a chemical pollution problem.

Various kinds of microorganisms including bacteria, fungi, and actinomycetes have been reported as xylanase producers [8,9,10,11] by using xylan as the best inducer for the enzyme production.[2] *Aspergillus fumigatus* strain 4-45-1F, a thermostable hyper-xylanase producing *Aspergillus*, was isolated from decomposed corn cob in Thailand[12] by an enrichment culture at 45°C. The highest xylanase, obtained after 5 days of incubation at 40°C in the medium containing 1.5% purified larchwood xylan, 0.2% NH_4NO_3, 0.02% yeast extract, and an initial pH of 4.5, was 8.5×10^3 units/ml, and the xylanase was produced only in the presence of xylan or xylose. Since agricultural waste contains a high concentration of xylan (15-30%),[13] and solid state fermentation, which is low in cost and technology, always faces the problem of heat accumulation during incubation period, therefore, the possibility to produce xylanase at a low cost using thermostable hyper-xylanase producing fungi isolated from agricultural waste by solid state fermentation was studied.

MATERIALS AND METHODS

Microorganism and cultivation

Aspergillus fumigatus strain 4-45-1F was used throughout the study. Spores of the culture grown on xylan agar slant containing 1% purified larchwood xylan (Sigma, USA), 0.2% NH_4NO_3, 0.2% KH_2PO_4, 0.02% $MgSO_4\cdot 7H_2O$, 0.02% yeast extract, 1.5% Bacto-agar, and initial pH 4.5, incubated at 40°C for 5 days, were suspended in sterile distilled water. The inoculum size was 1.2×10^5 spores/g substrate.

Selection of agricultural waste for maximum xylanase production

Spore suspension was inoculated into 50 ml liquid medium in a 250 ml-flask containing 5% of 20 mesh ground rice straw, corn cob, rice husk, peanut hull, sugar cane bagasse, or rice bran; 0.2% NH_4NO_3; 0.02% yeast extract; and initial pH 4.5. Cultivations were performed on a gyrotary shaker at the speed of 200 rpm, and at 40°C for 6 days. Culture filtrate was used as the crude enzyme.

Optimal conditions for xylanase production by solid state fermentation

Spore suspension was inoculated into 100×15 mm petri-dishes containing 2.5 g of 5 mesh ground rice straw, 0.01 g yeast extract, 80 g rice straw/100 ml of water, at initial pH 4.5, and incubated at 40°C. After 6 days or the time indicated, the incubation mixture was suspended in 25 ml of 0.02 M citrate phosphate buffer pH 5.8, and incubated on a gyrotary shaker at the speed of 200 rpm for 30 min. Culture filtrate separated from rice straw and spores by filtration through white cotton mesh cloth and then centrifugation at 7,000 rpm for 15 min was used as the crude enzyme.

Enzyme activity assay

In 0.02 M citrate phosphate buffer of pH 5.8, 1% purified larchwood xylan (Sigma, USA) and 1% carboxymethyl cellulose were used as substrates for xylanase and cellulase activity assay, respectively.[14] A 0.5 ml solution each of crude enzyme and substrate was incubated at 50°C for 10 min. The reaction mixture was heated in a boiling water bath for 10 min and was analysed for reducing sugar by the method of Somogyi and Nelson.[15] One unit of enzyme activity was defined as the amount of enzyme that released 1 μg of xylose or glucose per min in reaction conditions tested.

Selection of agricultural waste to use as substrate for maximum fermentable sugar production

A 2 g sample of 5 mesh ground agricultural wastes suspended in 80 ml of crude enzyme was incubated on a 200 rpm gyrotary shaker at 50°C for 3 h. The reaction was stopped by boiling in a boiling water bath for 10 min. The supernatant obtained from centrifugation of the filtrate through a white cotton mesh cloth was analysed for reducing sugar by the Somogyi-Nelson method, and concentrated by boiling in a boiling water bath for 1 hr. Concentrated agricultural waste hydrolyzate (10 μl) was analysed for hydrolysis products by ascending paper chromatography,[12] using acetonitril and 0.1 M ammonium acetate in the ratio 4:1 as the eluent, and colour was developed by diphenylamine-aniline-phosphoric acid. Glucose and xylose at 1% (W/V) were used as authetic sugars.

RESULTS AND DISCUSSION

Selection of agricultural waste for maximum xylanase production

Effects of various kinds of agricultural wastes such as rice straw, corn cob, rice husk, peanut hull, sugar cane bagasse and rice bran on xylanase production were compared in suspension culture. Maximum xylanase production was obtained from the rice straw medium (Fig. 1). Rice straw might contain a growth factor that supports better growth of *A. fumigatus* strain 4-45-1F than other agricultural wastes tested. *A. fumigatus* strain 4-45-1F produced xylanase at the stationary growth phase. Maximum xylanase was obtained at the death phase when a mold pellet dispersion and lost in cell dry weight was observed.[12] Svarachorn et al.[12] suggested that the maximum xylanase obtained from *A. fumigatus* strain 4-45-1F might be the sum of intracellular and extracellular xylanases. Purification of xylanase from *A. fumigatus* strain 4-45-1F showed two types of xylanase.[14] Extension of the lag phase might occur in a medium supplemented with other agricultural wastes tested, and cells then could not reach the death phase within 6 days of incubation. Fadel and Fouda (1993) reported that among alkali-treated agricultural wastes tested (sugar cane bagasse, rice straw and wheat straw), rice straw was the best substrate of *Penicillium funiculosum* for xylanase production by suspension culture. Xylanase was not detectable in a sugar cane bagasse medium. The high concentration of glucose in sugar cane bagasse might be the reason for no xylanase production, since *A. fumigatus* strain 4-45-1F can more efficiently use glucose than xylan for growth. Therefore, in the following experiments on studying the optimal conditions for xylanase production by solid-state fermentation, rice straw was used as carbon source.

Fig. 1 Effect of carbon source on xylanase production. The spore suspension was inoculated to a liquid medium containing 5% (20 meshes) of various kinds of agricultural wastes as carbon sources, 0.2% NH_4NO_3, 0.02% yeast extract, and initial pH 4.5. Cultures were incubated at 40°C for 6 days. Culture filtrate was used as crude enzyme for xylanase activity assay.

Optimal conditions for xylanase production by solid state fermentation

The optimal concentration of NH_4NO_3 was 0.8% (w/w) (Fig 2.). Among the inorganic nitrogen sources tested, NH_4NO_3, $(NH_4)_2 SO_4$, and $(NH_4)_2 HPO_4$, NH_4Cl, NH_4NO_3 was found to be the best for xylanase production by *A. fumigatus* strain 4-45-1F.[12] Xylanase production remained unchanged either with or without yeast extract (data not shown).

The optimal water content was 82 g rice straw/100 ml of water (Fig. 3). Scott reported that low water content in solid-state fermentation leads to a longer lag phase for spore germination.[17] At a higher water content than 82 g rice straw/ 100 ml of water, rice straw became clumpy which reduced aeration, and poor growth occurred, followed by a low amount of xylanase production.

Fig. 2 Effect of NH_4NO_3 concentration on xylanase production. The spore suspension was inoculated to a medium in a 100×15 mm petri-dish containing 2.5 g of 5 meshes rice straw, 0.01 g of yeast extract, various concentrations of NH_4NO_3 in % (g/g rice straw), 80 g rice straw/100 ml of water, initial pH 4.5. Culture was incubated at 40°C for 6 days, and then suspended in 25 ml of 0.02 M citrate phosphate buffer pH 5.8 on gyrotary shaker at 200 rpm for 30 min. The supernatant separated by filtration through cotton mesh cloth and centrifugation at 7,000 rpm for 15 min was used as crude enzyme for xylanase activity assay.

A comparative study on using spore inoculum prepared from medium containing purified larchwood xylan or glucose (Czapek's agar) as a carbon source showed no effect (Fig. 4). This result indicated that, although this fungus did not produce any xylanase in a medium containing glucose.[12] The use of xylan to prepare spore inoculum was not necessary.

The suitable particle size of ground rice straw for xylanase production was 5 meshes (Fig. 5). This might be the result of the lower growth surface area of 4 mm-long chopped rice straw while for the particle size of 20 mesh ground rice straw, clumps lead to low aeration, resulting in poor growth and then a low amount of xylanase produced.

Fig. 3 Effect of water content on xylanase production. Medium, incubation conditions, and crude enzyme preparation were the same as shown in Fig. 2. Various water contents in g rice straw/100 ml of water and 0.8% (w/w) NH_4NO_3 without addition of yeast extract were used.

There was no effect of a spore inoculum size of 1.2×10^5, or 1.2×10^6, or 1.2×10^7 spores/g rice straw (data not shown). To decrease the

time of spore inoculum preparation, the spore inoculum size of 1.2×10^5 spores/g rice straw was selected.

From the above results, the optimal conditions for xylanase production in solid cultivation were used and the maximum xylanase obtained was 16.8×10^4 units/g rice straw after 6 days of incubation. Not only xylanase but also cellulase at 3.4×10^3 units/g rice straw was found(Fig. 6). Therefore, crude enzyme produced by this fungus was suitable for converting hydrolyse agricultural wastes containing both cellulose and xylan to fermentable sugar. Xylanase production using agricultural wastes by solid state fermentation was reported by Jain;[18] *Molanocarpus albomyces* could produce xylanase from untreated wheat straw or sugar cane bagasse. This fungus could use rice straw or rice husk as a substrate for xylanase production only after alkali or acid chlorite treatment.

Fig. 4 Effect of inoculum medium on xylanase production. Symbols used: xylan agar medium(●), Czapek's agar medium (▲), Medium, incubation conditions, and crude enzyme preparation were the same as shown in Fig. 2. The 82 g rice straw/100 ml of water, and 0.8% (w/w) NH_4NO_3 without addition of yeast extract were used.

Selection of agricultural waste for maximum fermentable sugar production

When 2 g of each 5 mesh ground agricultural waste was suspended in 80 ml of crude xylanase preparation at 50°C with shaking for 3 h, the maximum reducing sugar was obtained from sugar cane bagasse at the concentration of 0.26 g/g sugar cane bagasse (Fig. 7). The analysis of hydrolysis products from all of the agricultural wastes tested by paper chromatography showed that they were mostly xylose and glucose (Fig. 8). As shown in Fig. 8, sugar cane bagasse itself contained a high concentration of glucose, and in the hydrolysis product, there was a higher concentration of xylose obtained from sugar cane bagasse than that from other agricultural wastes tested. The structure of xylan in sugar cane bagasse might be less complex and more susceptible to xylanase of *A. fumigatus* strain 4-45-1F.

Fig. 5 Effect of particle size of substrate on xylanase production. Symbols used: chopped rice straw, 4 mm long (■); ground rice straw, 5 meshes (●); ground rice straw, 20 meshes (▲). Medium, incubation conditions, and crude enzyme preparation were the same as shown in Fig. 2. The 82 g rice straw/100 ml of water and 0.8% (w/w) NH_4NO_3 without addition of yeast extract were used.

Sugar cane bagasse was more susceptible to xylanase of *A. fumigatus* strain 4-45-1F than that of rice straw, but sugar cane bagasse contained a high concentration of glucose

which inhibited xylanase production by this fungus.[12] Therefore, production and hydrolysis processes of this enzyme should be separated.

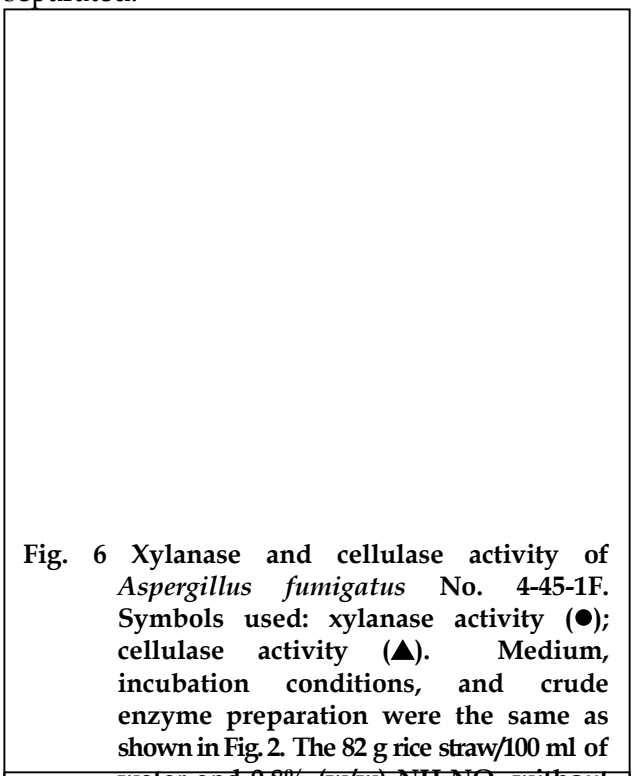

Fig. 6 Xylanase and cellulase activity of *Aspergillus fumigatus* No. 4-45-1F. Symbols used: xylanase activity (●); cellulase activity (▲). Medium, incubation conditions, and crude enzyme preparation were the same as shown in Fig. 2. The 82 g rice straw/100 ml of water and 0.8% (w/w) NH_4NO_3 without addition of yeast extract, 5 meshes ground rice straw and 1.2×10^5 spores/g rice straw were used.

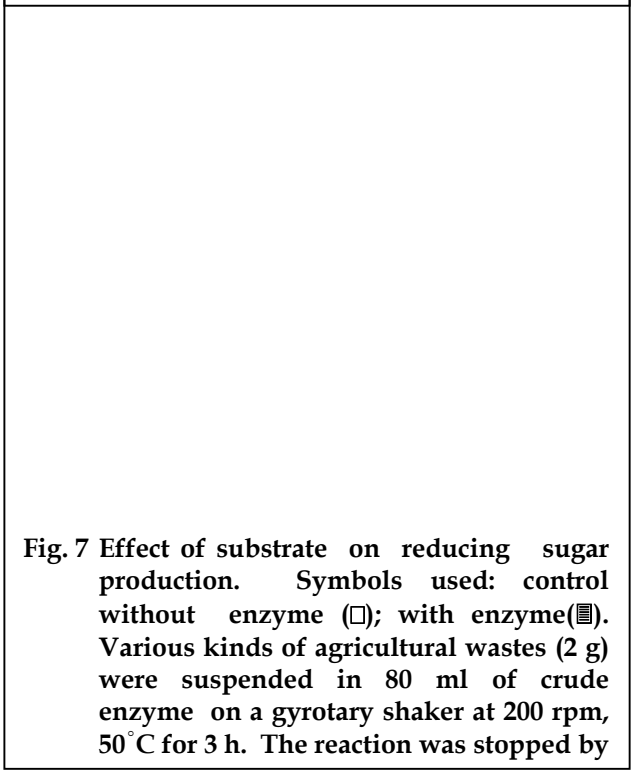

Fig. 7 Effect of substrate on reducing sugar production. Symbols used: control without enzyme (□); with enzyme(▤). Various kinds of agricultural wastes (2 g) were suspended in 80 ml of crude enzyme on a gyrotary shaker at 200 rpm, 50°C for 3 h. The reaction was stopped by boiling in boiling water for 10 min. Supernatant obtained after separation of agricultural waste substrate was analysed by the Somogyi-Nelson method.

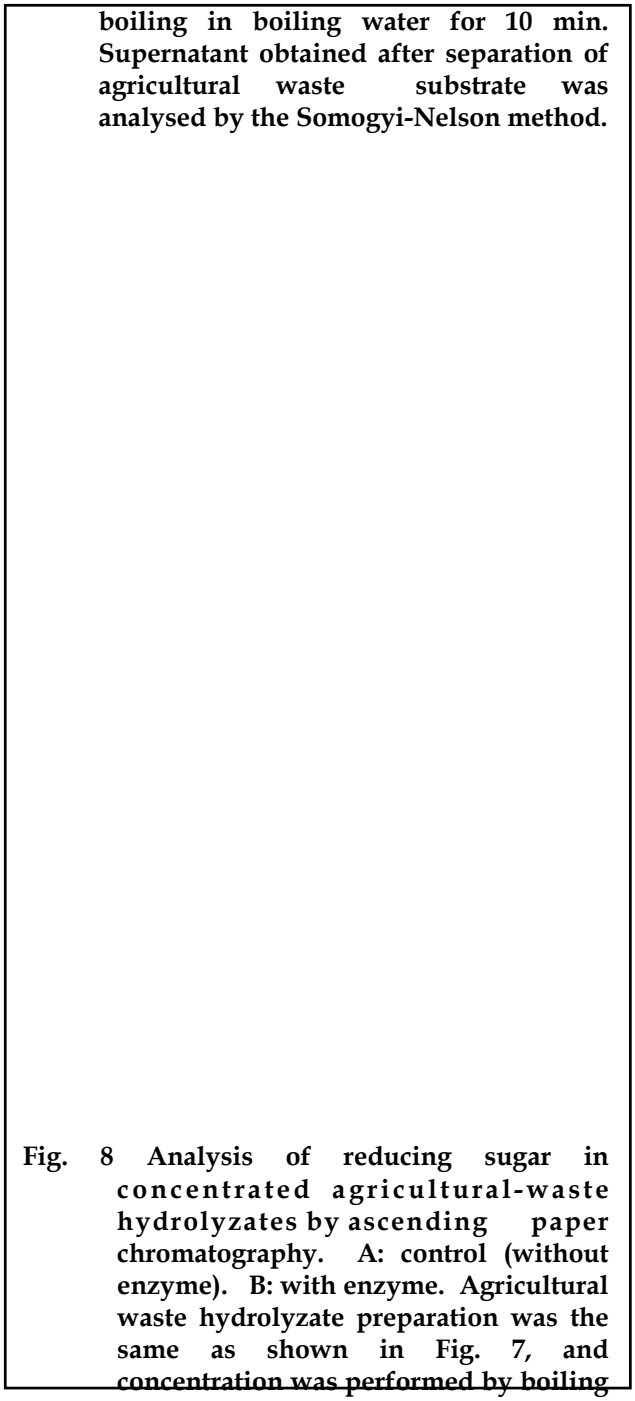

Fig. 8 Analysis of reducing sugar in concentrated agricultural-waste hydrolyzates by ascending paper chromatography. A: control (without enzyme). B: with enzyme. Agricultural waste hydrolyzate preparation was the same as shown in Fig. 7, and concentration was performed by boiling in a boiling water bath for 1 hr. The method of ascending chromatography was decribed in the text.

REFERENCES

1. Tsao, G. T. (1978) Cellulosic Material as a Renewable Resource. *Proc. Biochem.* 10 (13): 12- 14.

2. Hampot, H. A., Hawarth, W.N. and Hirst, E. L. (1992) Polysaccharides Part I V. The Constitution of xylan. *J. Chem. Soc.* 1739 - 1753.
3. Estebam, R.,Villanueva J.R. and Villa, T.G. (1982) β-D Xylanase of *Bacillus circulans* WL-12 *Can. J. Microbiol.* 28: 733 - 739.
4. Biely, P (1985) Microbial Xylanolytic Systems. *Trends Biotechol* 3 (11): 286 - 290.
5. Magee, R, J., and Kosaric, N. (1985) Bioconversion of Hemicellulosic. *Adv. Biochem. Bioeng*. 32: 64-93.
6. Desphande, V., Lachke, A.,Mishra, C., Kesher, S. and Roat, M (1985) Mode of Action and Properties of xylanase and β-xylosidase from *Neurospora crassa. Bio-technol. Bioeng*. 28: 1832-1837.
7. Gilbert, H. J. , and Hazlewod, G.P.(1993) Bacterial Cellulases and Xylanases. *J. Gen Microbial*. 139: 187-194.
8. Khasin, A., Alichanati I., and Shoham,Y. (1993) Purification and Characterization of a Thermostable Xylanase from *Bacillus stearothermophilus* T-6. *Appl. Env. Microbiol.,* 59 (6): 1752-1730.
9. Kitpreechavanich, V., Hayashi, M., and Nagai, S. (1984) Production of Xylan Degrading Enzymes by Thermophilic Fungi, *Aspergillus fumigatus* and *Humicola Canuginosa. J. Ferment Technol*. 62(1): 63-69.
10. Rhyum, S. B., Kang, M. K., Maeng, P. J., Park, H. M., and Rhee Y. M. (1993). Purifcation and Charactarization of Xylanase from Alkalophilic *Streptomyces* sp. S-510. *Kor. J. Microbiol.* 31(5): 436-444.
11. Patel. B. N., and Ray, R. M. (1994) Production and Characterization of a Xylanase from *Streptomyces* sp. Grown on Agricultural Waste. *World. J. Microbiol. and Biotechnol*. 10(5): 594-599.
12. Svarachorn, A., Kitpreechavanich, V., and N. Lotong (1987) Selection of Thermophilic Xylanase Producing Mold. *J. Kasetsart (Sci.),* 22: 294-302.
13. Whistler, R.L. and Smart, C.L.(1953) Polysaccharide Chemistry. New York: Academic Press.
14. Svarachorn, A., Kinoshita, S.,Pichyangkura, S., and Lotong, N. (1984) Purification and Characterization of Xylanase from *Aspergillus fumigatus* strain 4-45-1F. *Proceedings of the 22th Kasetsart University Conference*. p. 66-76.
15. Nelson, N. (1944) A Photometric Adaptation of the Somogyi Method for the Determination of Glucose. *J. Biol. Chem*. 153: 375-380.
16. Fadel, M., Fouda, M.S. (1993) Physiological studies on Xylanase Production by *Penicillium fumiculosum* on some Agricultural Wastes. *Zentralbl. Microbiol*. 148: 304-312.
17. Scott, W.J. (1957) *Adv. Food Res*. 7:83 in Nishio, N. and Nagai, S. (1980) Thermostable Cellulase Production by *Taralomyces* sp. in Solid-state Cultivation. *Proceeding of JSPS-NRCT Seminar on Agro-Industry Including Microbial Technology "Microbial Utilization of Renewable Resources"*. 1: 54-65.
18. Jain, A (1995) Production of Xylanase by Thermophilic *Melanocarpus albomyces* IIS-68. *Process. Biochem*. 30: 705-709.

Received: August 3, 1998
Accepted: April 19, 1999

……..……Ancharida Svarachorn