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The Production of Fungal Mannanase, Cellulase and Xylanase Using Palm Kernel Meal as a Substrate

Nisa SAE-LEE

Department of Biotechnology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80161, Thailand

ABSTRACT

Extracellular enzymes including mannanase, cellulase and xylanase from *Aspergillus wentii* TISTR 3075, *Aspergillus niger*, *Aspergillus oryzae, Trichoderma reesei* TISTR 3080 and *Penicillium* sp. were investigated. The enzymes were produced in solid-state fermentation using palm kernel meal (PKM) as a substrate. All fungal strains produced mainly mannanase. A maximum activity of 24.9 U/g koji was observed in *A. wentii* TISTR 3075 with a specific activity of 1.5 U/mg protein. During PKM fermentation, there was also found low concomitantly of cellulase and xylanase activities with high mannanase activity in all strains. The degradation of non-starch polysaccharides (NSPs) in PKM by these fungal strains was indicated by the increased mannanase, cellulase and xylanase activities which correlated with the increase in reducing sugar content and pH profiles during PKM fermentation. PKM was shown to be more suitable for production of mannanase than cellulase and xylanase for all strains because of the high content of mannan as an inducer in PKM. Increases in enzyme yield might be obtained by optimizing the culture conditions.

Keywords: Mannanases, cellulases, xylanases, palm kernel meal, solid-state fermentation, *Aspergillus* spp., *Trichoderma* sp., *Penicillium* sp.

INTRODUCTION

Solid state fermentation (SSF) has usually been exploited for the production of value-added agro-industrial residues such as soybean meal, canola meal, wheat bran, cellulosic pulp, corncobs. Palm kernel meal (PKM) is the main by-product of the palm oil industry. PKM is used as a low cost source of protein, energy and fibre in animal feed [1,2]. However, the use of PKM in monogastric animal feed, particularly poultry is limited (up to 20 % by weight) [1] as it contains high levels of non-starch polysaccharides (NSPs) (60 % NSPs). These NSPs, are present mainly in the form of mannan (78 %) which is found in the cell wall, and impair the digestibility and utilization of nutrients either by direct encapsulation of the nutrients or by increasing the viscosity of the intestinal contents. This causes a reduction in the rate of hydrolysis and the absorption of nutrients in the diet.

The use of enzymes to digest agricultural wastes is known to release digestible sugars which can then be fully absorbed and metabolized by monogastric animals. The enzyme supplements in broiler diets of monogastric animals also increase weight gain and feed intake [3,4], improve overall crude protein digestibility [5], and prevent increase in viscosity and subsequent reduction in bacterial overgrowth in the intestinal contents [6].

From the composition of carbohydrates in PKM, it appears that at least three main cellulolytic and hemicellulolytic enzymes are needed to improve the nutritive value of PKM, namely: mannanases, cellulases and xylanases. These enzymes are used to digest the internal glucosidic bonds of mannan, cellulose and xylan resulting in a conversion to mannose, glucose and xylose, respectively.

A wide variety of microorganisms are known to produce mannanases in the form of endo-β-mannanase (EC 3.2.1.78) and exo-mannanase (β-manosidase, EC 3.2.1.25). These forms of mannanases have been found in *Aspergillus niger* [7,8], *A. awamori* [9], *A. fumigatus* [10], *Sclerotium rolfsii* [11], *Bacillus subtilis* [12], *Trichoderma reesei* [13,14], and *Pichia pastoris* [15]. These enzymes are capable of hydrolyzing β-1,4 glycosidic linkages in mannan, galactomannan, glucomannan and galactoglucomannan. However, there are some constraints in producing exo-mannanase commercially due to the instability of this enzyme in acidity conditions [16]. Fungi are the main group of organisms that produce cellulase such as *Aspergillus* spp. [17,18], *Trichoderma* spp. [19-22] and *Penicillium* sp. [23]. Several microorganisms including *Trichodrema* spp. [20,24], *Penicillium* spp. [23,25], *Aspergillus* spp. [26,27], *Bacillus s*pp. [28-30] and *Thermomonospora* sp. [31] are used in the production of xylanolytic enzymes such as xylanases which have applications in the pulp and paper industry.

This work focuses on mannanase, cellulase and xylanase production by fungal strains of *A. wentii* TISTR 3075, *A. niger*, *A. oryzae*, *T. reesei* TISTR 3080 and *Penicillium* sp. using solid state fermentation of PKM as a substrate. This study attempts to develop a method to improve the quality of PKM making it more suitable as an effective feed additive for animal feed.

MATERIALS AND METHODS

Fungal strains and inoculum preparation

A. wentii TISTR 3075 and *T. reesei* TISTR 3080 were purchased from Thailand Institute of Scientific and Technological Research (TISTR). *A. niger*, *A. oryzae*, and *Penicillium* sp. were obtained from a source Laboratory of Walailak University, Thailand. The cultures were maintained on potato dextrose agar (PDA) slants at 4 °C. The spores of these microorganisms were obtained by culturing the strains at 30 °C on PDA agar plates for 5 days. The spores were collected from the culture surfaces using sterile distilled water. The concentration of the spores was adjusted to 1×10^7 spores/ml with the same diluents and used as inoculums.

Screening of mannanase, cellulase and xylanase producing fungi

The potent strains of fungi were tested for NSPs (mannan, cellulose and xylan) hydrolytic activity using the modified agar diffusion method. Ten µl of spore suspension was dropped onto 6 mm diameter of autoclaved paper disc (Whatman No. 1). Inoculated paper discs were dried at room temperature and put onto the center of medium plates. Exo-mannanase was tested by using a mannan-agar medium containing 0.5 % phyta gel (Sigma) and 0.5 % locust bean gum (Sigma) [32,33]. Cellulase assay was used as a cellulose-agar medium containing 0.5 % carboxymethylcellulose (Sigma) [34] and xylan-agar medium added 0.5 % brichwood xylan (Sigma) for xylanase activity measurement [35]. After 48 h incubation at 30 °C, the plates were stained with 0.4 % congo red for 10 min and then destained with 1 M NaCl. The hydrolysis zones were observed.

Culture conditions

This study used PKM as a test substrate. The PKM was obtained from a palm oil factory. SSF was carried out in 1 liter Erlenmeyer flasks containing 80 g of PKM as a main substrate. Distilled water was added to the final

substrate to adjust the moisture content to 30 %. The substrates were sterilized by autoclaving, cooled to room temperature and inoculated with 5 % (w/w) inoculum of 10^7 spores/ml and incubated at 30 °C for 8 days. Cultures were sampled daily at day 0, 1, 2, 3, 4, 5, 6, 7 and 8 for mannanase, cellulase and xylanase analyzes. All culture assays were repeated 3 times. The pH was determined using a pH meter and the soluble reducing sugar was assayed by the dinitrosalicylic acid (DNS) method using glucose as a standard.

Determination of enzyme activities

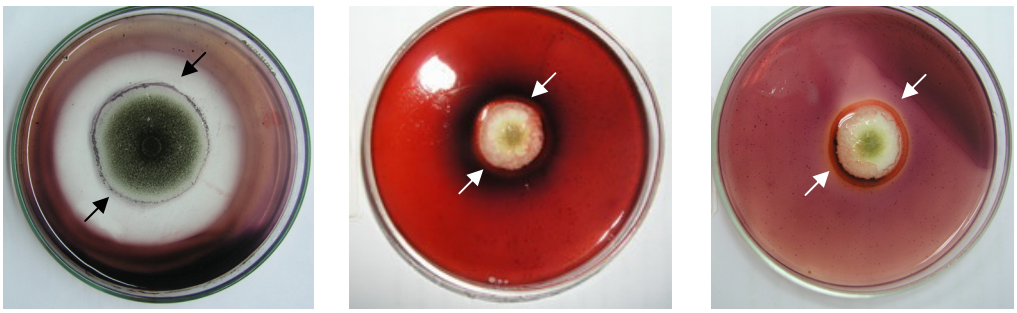
Ten grams of koji was extracted with 100 ml of sterile distilled water. The extractions were performed on a rotary shaker at 200 rpm, 20 °C for 30 min, were then squeezed through 200 mesh cloths, and the volume was noted. The enzyme extracts were filtered through Whatman No.1 filter paper and the clear supernatants were kept at –20 °C and used as the crude enzyme sources for analysis of the enzyme activities, pH valve and residual sugar. If necessary, the enzyme was concentrated using 0 - 100 % saturated ammonium sulfate and dialysis overnight using a 10 kDa cut-off membrane (Biorad) before doing the activity assay.

Mannanase activity was assayed by mixing 50 µl of an appropriately diluted enzyme solution with 450 µl of 0.5 % locust bean gum in a 50 mM sodium acetate buffer, pH 5.0 [15]. Determination of cellulase activity was adapted from the method of Baldrian and Gabriel [36]. The reaction mixture contained 0.5 ml of the enzyme, 0.5 ml of 0.75 % carboxymethylcellulose in a 50 mM sodium acetate buffer, pH 5.0. Xylanase activity was assayed using the method of Christakopoulos *et al* [37]. The reaction mixture contained 2 ml of 0.8 % w/v brichwood xylan solution in a 0.1 M sodium acetate buffer, pH 5.0 and 2 ml of a suitable diluted enzyme solution in the same buffer. The reaction mixtures were incubated at 40 °C for 30 min. Mannanase, cellulase and xylanase activities were determined by quantifying the release of reducing sugar by the dinitrosalicylic acid (DNS) method using mannose, glucose and xylose as standards, respectively.

One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 µmol of product per minute under the assay conditions. Enzymatic activities in the extracts recovered from the solid-state cultures were determined in U/ml. Total units per culture were obtained by multiplying U/ml by the volumes of the extracts. Enzyme production was expressed as U/g koji. Each test was repeated 3 times. The average enzymatic activities from triplicate cultures are reported with standard deviations as error bars.

RESULTS AND DISCUSSION

The five strains of fungi used in this study showed the ability to produce mannanase, cellulase and xylanase enzymes. **Figures 1a-c**, shows an example of the hydrolysis zones. The colonies with mannanase activity resulted in a discoloration of the mannan-agar plate to a clear zone with red background. The yellow zone on the xylan-agar plates resulted from xylanase activity. The yellow halo zone with dark or red background on the cellulose-agar plate resulted from cellulase secretion. In all tested species strong activities were observed on mannan-agar plates but little activity was observed on cellulose-agar and xylan-agar plates. Only *T. reesei* TISTR 3080 and *Penicillium* sp. showed only little activity on all agar plates. These results indicated that these fungi had the ability to secrete mannanase, cellulase and xylanase and they were selected for PKM fermentation as a potential feed additive for monogastric animals.



(a) (b) (c)

Figure 1 Plate assays showing hydrolysis zone (arrow) by (a) mannanase, (b) cellulase and (c) xylanase.

Enzyme activities, soluble reducing sugar content and pH change were observed for eight days in SSF of PKM as a main substrate by different strains of fungi (**Figures 2 - 6**). During fungal growth on PKM, mannanase was the major enzyme secreted with the highest activity of 24.9 U/g koji (2.5 U/ml) at the 6th day by *A. wentii* TISTR 3075. The maximum activities obtained from *A. niger*, *A. oryzae*, *T. reesei* TISTR 3080 and *Penicillium* sp. were 20.2, 12.3, 7.4 and 8.2 U/g koji, at 6 days, 4 days and at the end of fermentations, respectively (**Figure 2**).

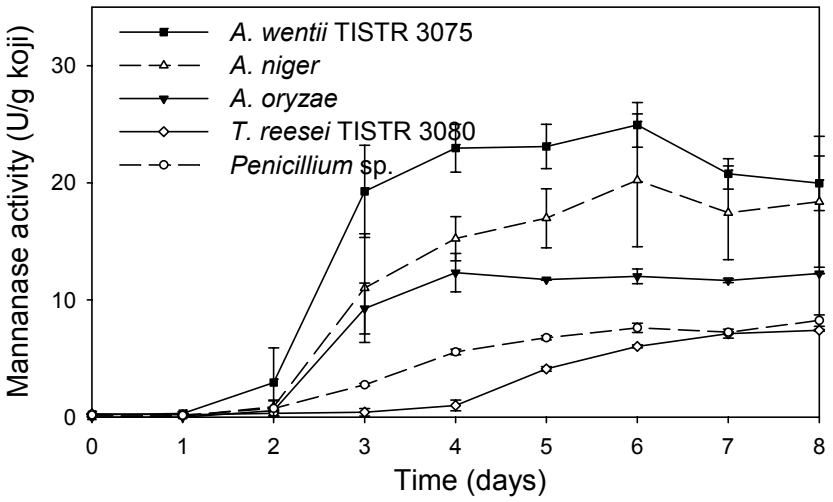


Figure 2 Time courses of mannanase production by *Aspergillus wentii* TISTR 3075, *Aspergillus niger*, *Aspergillus oryzae, Trichoderma reesei* TISTR 3080 and *Penicillium* sp. during solid state fermentation using PKM as a substrate.

Among these fungal strains, the ability of extra-mannanase production during growing on PKM was highest in *A. wentii* TISTR 3075. This might result from its ability as an xerophile which exhibits strong growth in both sugar and salt environments [38]. This indicates that *A. wentii* TISTR 3075 grew better on PKM than the other strains. The reduction of mannanase yield after the maximum period might result from the depletion of nutrients available to the fungi. Since PKM contains 46.8 % mannan together with 7.2 % cellulose and 3.6 % xylan [2], this provides an appropriate substrate for *A. wentii* TISTR 3075 and its allied fungi to produce mannanase.

In this study, low amounts of extracellular xylanase and cellulase activities were detected during the mannanase production on PKM as shown in **Figures 3** and **4**. The maximum yield of cellulase and xylanase were mostly

achieved after 8 days from the beginning. Within the five strains used in PKM fermentation, the highest cellulase and xylanase activities were achieved by *A. niger* at 1.3 and 3.2 U/g koji, respectively. The highest activities obtained from *A. wentii* TISTR 3075, *A. oryzae*, *T. reesei* TISTR 3080 and *Penicillium* sp. were 1.2, 1.0, 0.85 and 1.1 U/g koji and 1.9, 1.0, 1.3 and 3.0 U/g koji of cellulase and xylanase activities, respectively. However very low amounts of the enzymes were detected and this might be due to the low substrate concentration in PKM and xylan as reported by Abdel-Sater and El-Said [39]. They reported that when mannose and glucose or a mixture of glucose and xylan were used, the xylanase activity disappeared.

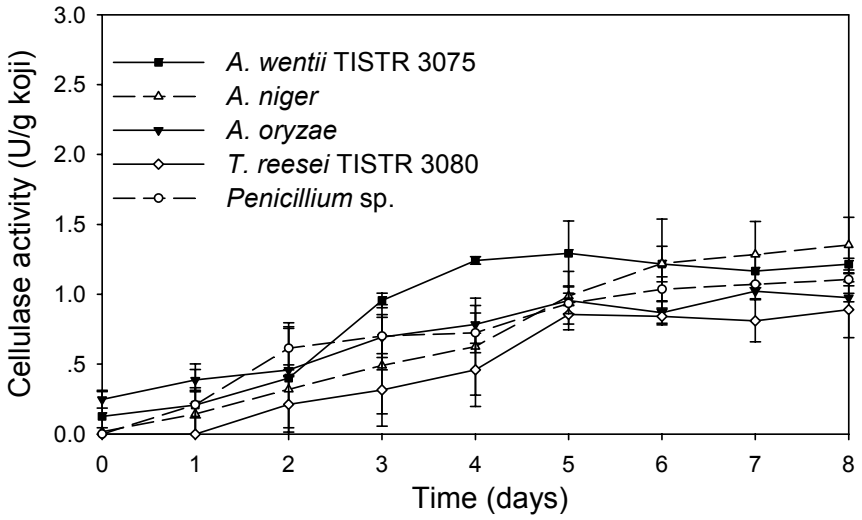


Figure 3 Time courses of cellulase production by *Aspergillus wentii* TISTR 3075, *Aspergillus niger*, *Aspergillus oryzae, Trichoderma reesei* TISTR 3080 and *Penicillium* sp. during solid state fermentation using PKM as a substrate.

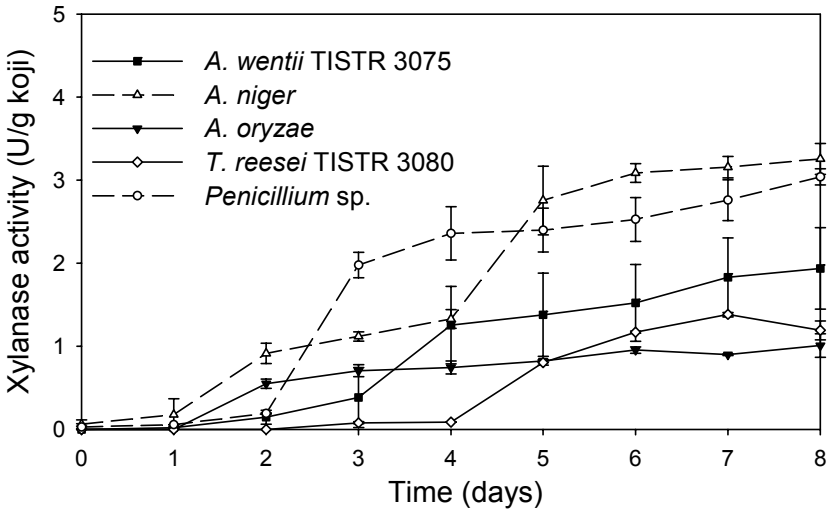


Figure 4 Time courses of xylanase production by *Aspergillus wentii* TISTR 3075, *Aspergillus niger*, *Aspergillus oryzae, Trichoderma reesei* TISTR 3080 and *Penicillium* sp. during solid state fermentation using PKM as a substrate.

Compared to other studies, mannanase activity production using PKM by *A. wentii* TISTR 3075, *A. niger*, *A. oryzae*, *T. reesei* TISTR 3080 and *Penicillium* sp were lower than that by *A. niger* and *B. licheniformis* NK-27 in which the maximum activity of mannanase obtained was 28 U/ml after three days and 212 U/ml using defatted copra and konjac powder as a substrate, respectively [8,40].

The maximum xylanase activity found in this experiment was 3.2 U/g koji (0.3 U/ml). This activity was very low compared to those reported in *A. fumigatus*, *B. pumilus*, *B. megaterium* and *A. sulphureus* in which the maximum xylanase production was 10,000 U/g rice straw, 328 U/ml, 8 U/g of oven dried pulp and >1000 IU/g dry koji when grown on rice straw, brichwood and wheat bran as a substrate, respectively [2,26,27,41].

Cellulase activities of 1.0 - 1.3 U/g koji (0.1 - 0.13 U/ml) obtained from this study were far lower than that of *T. reesei* QM 9414, *Trichoderma* sp. and *A. wentii* LOCK 0459 which had the highest activities of 16 U/g cellulose, 3 - 4 U/ml and 1.14 U/ml when grown on sugarcane bagasse, corn fibre and cellulose, respectively [17,19,20].

In this study, the low concomitantly produced cellulase and xylanase at high mannanase activity occurred during growth on PKM. This might result from inducers (mannan, cellulose and xylan) contained in PKM. This result agreed with the study by Jiang *et al* [12] when growing *B. subtilis* WY34 on

konjac powder. The co-production of mannanase, cellulase, xylanases and other cellulolytic and hemicellulolytic enzyme systems were found in many studies when growing microorganisms using agricultural residues as a carbon source, the enhancement of which is strictly dependent on suitable inducers and/or catabolic repression [10,31,42,43]. Many reports in the literature indicate that most xylanases exhibit significant cellulolytic activity which makes them suitable for many applications [17,20,23,27,28,30,44]. Williams *et al* [14], Guzinska *et al* [17] and Subramaniyan and Prema [30] indicated that the ability of *Trichoderma* spp. in secreting both cellulase and xylanase lies in its ability to switch strategies based on the environmental conditions. The trace cellulolytic activity may be due to the release of xylose from the cellulose substrates and some of the microbial xylanases contained in cellulose binding domains.

In this study, high enzyme yield production was limited and this might result from the limited amount of nitrogen present. The effect of the nitrogen source on enzyme production was also reported by Bakir *et al* [18]. They indicated that the production of xylanase by *R. oryzae*, when omitting the nitrogen sources, increased the enzyme yield and the fermentation period. This indicates that the conditions of fermentation must be optimized if PKM is used as a substrate to obtain a high enzyme yield.

The pH profile during SSF of PKM, in general, shows that the pH valves initially decrease slightly from 5.0 to 4.7 after 2 - 3 days of fermentation and then slightly increase to around 5.4 - 6.1 for all fungal strains as shown in **Figure 5**. Initial pHs were similar to that of the crude extract of *A. awamori* K4 (pH 5 - 6) grown on coffee waste and wheat bran [9]. The decrease in the pH during early fermentation might be due to organic acid accumulation during cultivation. The subsequent increase in the pH correlates with the increase in the mannanase activity and soluble reducing sugars profiles during fermentation. This is because fungi grew faster on PKM as a carbon source and produced more nitrogenous waste, which lead to an increase in the pH [8].

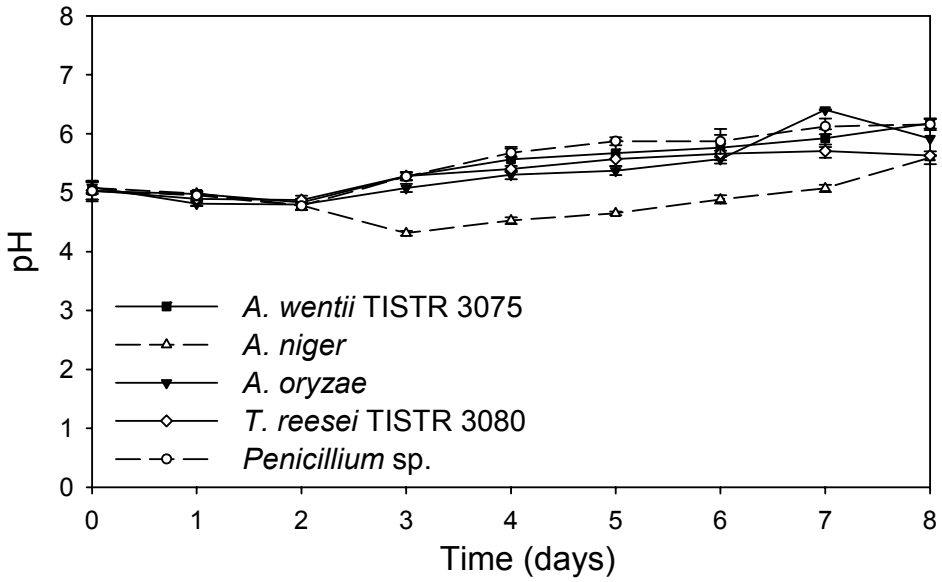


Figure 5 Profiles of pH during solid state fermentation of PKM cultivated with *Aspergillus wentii* TISTR 3075, *Aspergillus niger*, *Aspergillus oryzae, Trichoderma reesei* TISTR 3080 and *Penicillium* sp.

During fermentation of PKM by these fungi, soluble reducing sugar contents rapidly increased from initial concentrations of 7.3 - 7.8 % to 11.6 - 14.5 % on day 3 - 4, then decreased to 7.3 - 8.9 % at the end of fermentation (**Figure 6**). This resulted from mannose, glucose and xylose production in the system. These sugars were then used as a carbon source and as energy for growth. This change correlated with the change in the amount of mannanase, cellulase and xylanase activities during fermentation. This indicates that cellulose and hemicellulose-degrading enzymes are necessary to derive carbon and energy sources from PKM substrate for the growth of fungi. In conditions where NSPs were limited, the reducing sugar would be completely utilized [8].

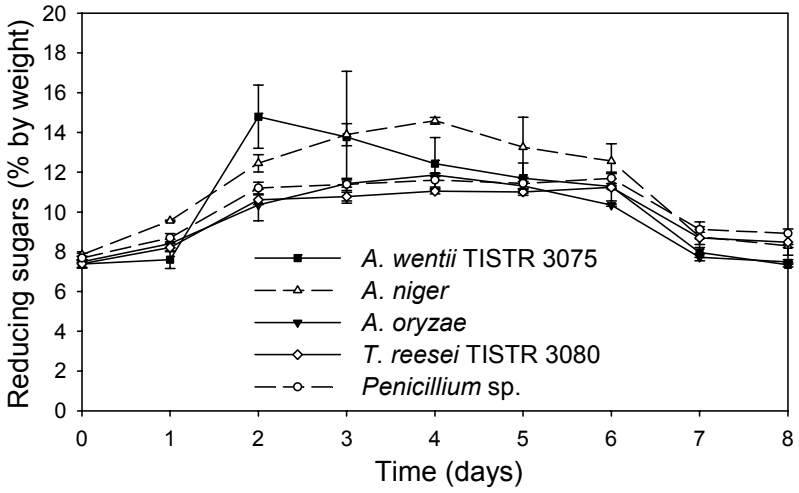


Figure 6 Reducing sugar contents during solid state fermentation of PKM cultivated with *Aspergillus wentii* TISTR 3075, *Aspergillus niger*, *Aspergillus oryzae, Trichoderma reesei* TISTR 3080 and *Penicillium* sp.

CONCLUSIONS

This study clearly indicated that these fungal strains were capable of producing mannanase, cellulase and xylanase during solid-state fermentation of PKM as a main substrate. A significant amount of mannanase was found in *A. wentii* TISTR 3070, *A. niger* and *A. oryzae* whereas *T. reesei* TISTR 3080 and *Penicillium* sp. had lower activities. They had the ability to degrade NSPs in the form of mannan as shown by an increase in the amount of reducing sugar at the beginning of fermentation before slowly depleting. Interestingly, PKM is chemically heterogeneous, with mannan as a main component, which is a good carbon source for fungal production of mannanase. In addition to mannan, it also contains a minor component of cellulose and xylan. Thus breakdown of this component may need the interaction of several cellulolytic and hemicellulolytic enzymes. Hence, a combination of mannanase, cellulase and xylanase may be used when developing an appropriate method to digest PKM. Fungi are not only capable of increasing soluble sugars and reducing the complex NSPs in PKM but also of increasing the protein in these wastes. The tested strains also showed the potential to produce NSPs-saccharifying enzymes for hydrolysis of PKM substrate. The hydrolysis property may be applied in the feed industry in order to reduce viscosity of the NSPs. However, optimizing conditions for mannanase production may help to increase the exo-mannanase production using PKM as a carbon source.

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บทคัดย่อ

นิสา แซ่หลี

การผลิตเอนไซม์แมนนาเนส เอนไซม์เซลลูเลส และเอนไซม์ไซลาเนส โดยเชื้อรา จากกากเนื้อในเมล็ดปาล์มน้ำมันโดยกระบวนการหมักแบบแข็ง

เอนไซม์แมนนาเนส เอนไซม์เซลลูเลส และเอนไซม์ไซลาเนส โดยเชื้อรา *Aspergillus wentii* TISTR 3075, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei* TISTR 3080 และ *Penicillium* sp. ด้วยกระบวนการหมักแบบแข็งโดยใช้กากเนื้อในเมล็ดปาล์มน้ำมันเป็นสารตั้งต้น การทดลองพบว่าเชื้อที่ทดสอบทุกสายพันธุ์ผลิตเอนไซม์แมนนาเนสเป็นหลัก โดยปริมาณเอนไซม์แมนนาเนสสูงสุดที่ได้จากเชื้อ *A. wentii* TISTR 3075 คือ 24.9 ยูนิตต่อกรัมโคจิ และมีค่ากิจกรรมจำเพาะสูงสุดของเอนไซม์ 1.5 ยูนิตต่อมิลลิกรัมโปรตีน เชื้อราทุกสายพันธุ์ที่ทดสอบนอกจากผลิตเอนไซม์แมนนาเนสปริมาณสูงแล้วยังพบการผลิตเอนไซม์เซลลูเลสและเอนไซม์ไซลาเนสปริมาณเล็กน้อยในระหว่างการหมักกากเนื้อในเมล็ดปาล์มน้ำมัน แสดงว่าเชื้อราดังกล่าวมีการย่อยสลายสารประกอบพอลิแซ็กคาไรด์ที่ไม่ใช่แป้งที่เป็นส่วนประกอบหลักในกากเนื้อในเมล็ดปาล์มน้ำมัน ซึ่งแสดงให้เห็นจากการเพิ่มขึ้นของปริมาณเอนไซม์แมนนาเนส เอนไซม์เซลลูเลส และเอนไซม์ไซลาเนส ที่เพิ่มขึ้นสัมพันธ์กับการเพิ่มขึ้นของปริมาณน้ำตาลรีดิวซ์และการเปลี่ยนแปลงค่าพีเอชระหว่างการหมักกากเนื้อในเมล็ดปาล์มน้ำมันดังกล่าว กากเนื้อในเมล็ดปาล์มน้ำมันมีความเหมาะสมสำหรับเชื้อราทุกสายพันธุ์ที่ทดสอบในการผลิตเอนไซม์แมนนาเนส เอนไซม์เซลลูเลสและเอนไซม์ไซลาเนส เนื่องจากมีส่วนประกอบของแมนแนนซึ่งเป็นสารเหนี่ยวนำการผลิตเอนไซม์แมนนาเนสปริมาณสูง ดังนั้นการเพิ่มผลได้ของเอนไซม์สามารถทำได้โดยการปรับสภาวะการหมักให้เหมาะสม

สาขาวิชาเทคโนโลยีชีวภาพ สำนักวิชาเทคโนโลยีการเกษตร มหาวิทยาลัยวลัยลักษณ์ อำเภอท่าศาลา จังหวัดนครศรีธรรมราช 80161