INTRODUCTION

Feed is the major operational cost for most animal farms and, depending on the farming intensity, accounts for approximately 60–80% of the variable cost (Arana, 2007). The rising cost of feed is driving some farmers to utilize alternative feedstuffs. In Thailand, over 1 million t each of palm kernel meal (PKM), cassava pulp (CP) and rough rice bran (RRB) are generated annually (Sriroth et al., 1999; Chutmanop et al., 2008; Teoh and Mashitah, 2010). They contain high levels of non starch polysaccharides (NSPs) such as cellulose and hemicellulose (Khempaka et al., 2009). RRB is a byproduct of the rice milling industry and contains 28–36% cellulose and 34–45% other crude fibers, including hemicelluloses and lignin, which have low digestibility (Kodali and Pogaku, 2007). PKM, a byproduct from palm oil manufacturing, contains 20–30% cellulose, 6%...
hemicellulose and 2% lignin (Bono et al., 2009). CP is the fibrous residual material generated by the starch industry, consisting of 20% cellulose, 19% hemicellulose and 2–7% lignin (Howard et al., 2003; Saha, 2003; Kosugi et al., 2009; Khempaka et al., 2009; Wanrosli et al., 2011). These NSPs can be used as feed and carbon sources for microbial cultivation. However, the protective lignin coat limits both cellulose and hemicellulose hydrolysis (Choct, 1997). Therefore, pretreatment technology is required to obtain valuable energy from these fiber sources.

The use of pretreated materials, such as RRB, PKM and CP, as alternative substrates to enhance cellulosytic enzyme production is attractive. Recently, a few reports have been published relating to pretreatments of these materials for cellulosytic enzyme production, mostly concerned with ethanol production (Eyini et al., 2004; Yan et al., 2009; Akaracharanya et al., 2011). An alkaline pretreatment has been used on RRB, with Sridevi (2009) reporting that cellulase production from alkaline pretreated rice bran (0.1% NaOH, 121 °C at 1 hr) by A. niger improved the activities of filter paper degrading enzyme, carboxymethylcellulase (CMCase) and β-glucosidase (BG) by 53, 59 and 58%, respectively. The most successful CP pretreatment was in an acid bath at high temperature. Kosugi et al. (2009) reported that diluted acid (0.1% H₂SO₄, at 140 °C for 1 hr) used to pretreat CP provided a maximal glucose yield of 0.744 g per gram dry pulp. When the pretreated CP was used as a carbon source for Rhizopus oryzae, 60 units.g⁻¹ glucoamylase were produced. Several pretreatment techniques, including high pressure and the application of chemicals, were proposed for PKM (Yan et al., 2009; Abdeshahian et al., 2011). Teoh and Mashitah (2010) reported that the pretreatment of palm oil residues at 121 °C for 15 min could enhance cellulase activities. Activities of 50.11 units.mL⁻¹ CMCase, 29.01 units.mL⁻¹ FPase and 5.58 units.mL⁻¹ BG for Pycnoporus sanguineus were observed.

The demand for cellulases has increased tremendously in the animal feed industry, where the cellulase system consists of three classes of endoglucanase (EC 3.2.1.4), exoglucanase or celllobiohydrolase (EC3.2.1.91) and β-glucosidase (EC 3.2.1.21) synergistically converting complex carbohydrates that are present in the lignocellulosic biomass into glucose (Aro et al., 2005). The cellulolytic enzyme system of A. niger is known to produce several enzymes for lignocellulosic material degradation, particularly, the degradation of cellulose. A major concern in a solid state fermentation (SSF) system is spore production by fungi, which could lead to some health problems (Bennett and Klich, 2003; Blumenthal, 2004). Aspergillus niger 386017M1, isolated by Alltech Inc. (USA), is one of the most effective strains in producing fiber digestion enzymes for the animal feed industry. This strain does not produce spores during the 10 d of the SSF system cycle (unpublished data). In addition, this strain contains seven powerful and complex enzymes: cellulase, xylanase, pectinase, β-glucanase, phytase, protease and α-amylase are essential for fiber degradation (Min et al., 2009; Hooge et al., 2010; Mahai et al., 2010). The current study aimed to investigate the effect of suitable pretreatments of RRB, CP and PKM on the growth of Aspergillus niger 386017M1, its cellulase production and the chemical composition of the fiber degraded.

**MATERIALS AND METHODS**

**Agricultural waste by products**

Three agricultural wastes—cassava pulp (CP), palm kernel meal (PKM) and rough rice bran (RRB)—were obtained from the Suppaluek farm in Rachaburi province, Thailand. The samples of both RRB and CP were 0.5 mm in diameter and had 12% moisture content. PKM was 0.2 mm in diameter and had 14% moisture content.

**Fungal strain and its cultivation**

A. niger 386017M1 was obtained from
Alltech Inc. (USA) and maintained in its freeze-dried form. In preparation for experiments, $1 \times 10^8$ spores.mL$^{-1}$ were collected from a 21 d culture on potato dextrose agar medium and subcultured into 200 mL cultivation liquid medium (in g per 100 milliliters: corn starch, 1.4; glucose, 1.0; bactopeptone, 1.8; KCl, 0.05; MgSO$_4$$\cdot$7H$_2$O, 0.15; KH$_2$PO$_4$, 0.1 and wheat bran, 2.0) by submerged fermentation at 30 °C with shaking at 250 rpm for 2 d. The cultured solution was then used as a starter for SSF.

**SSF system**

The SSF system used a modified method from Anupama and Ravinda (2001). The fermentation process was performed in a 500 mL Erlenmeyer flask that contained 3 mL of the starter culture and 10 g of the substrate. This mixture was adjusted to a final moisture content of 50% and cultivated at 30 °C. The culture samples were dried in a freeze drier (Lyph-Lock6; LABCONCO; Kansas City, MO, USA) at -40 °C for 4–5 d and then kept at -20 °C until use.

**Pretreatment of substrates**

Three pretreatments—acid, alkaline and steam—were studied. The acid pretreatment was carried out according to a modified method from Schell et al. (1999). The substrate was soaked in 0.35% H$_2$SO$_4$ at a 1:2 ratio and autoclaved at 121 °C for 1 hr. The alkaline pretreatment followed a modified method from Tatsumoto et al. (1988). The mixture of 5% substrates and 4% NaOH (w/v) was autoclaved at 121 °C for 1 hr. The steam pretreatment followed a modified method from Brownell and Saddler (1987). The substrates were soaked in distilled water at a ratio of 1:2 and autoclaved at 140 °C for 2 hr. The three hydrolysate materials were washed with tap water and dried at 40 °C overnight to obtain 15% moisture content. Then, the samples were kept at -20 °C until use.

**Indirect biomass estimation**

The glucosamine content was estimated using the method of Chumanop et al. (2008). In brief, 2 mL of 60% sulfuric acid was added to 0.5 g of the SSF sample and kept at 25 °C for 24 hr. The reaction was then diluted with deionized water to a concentration of approximately 1 N and autoclaved at 121 °C for 1 hr. After cooling, the mixture was neutralized with 3 N NaOH and diluted to a final volume of 100 mL. The mixture containing 2 mL of sample and 2 mL of acetylacetone solution was incubated at 96 °C for 20 min. After cooling, 20 mL of 96% ethanol and 2 mL of Ehrlich reagent were added and left for 1 hr at room temperature. The amount of N-acetyl glucosamine was measured by the absorbance at 530 nm against a standard curve.

**Determination of cellulolytic enzyme activity**

The crude cellulase was extracted by mixing 1 g of dry SSF and 50 mL distilled water using a shaker at 200 rpm for 1 hr and then centrifuging for 15 min. The supernatant was used as crude enzyme. The CMCase activity was assayed according to the method of Nitisinprasert and Temmes (1991) using carboxymethyl cellulose as the substrate. The amount of reducing sugar was determined according to Somogyi and Nelson (1992). One unit of CMCase activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per minute. The activity of CBH was determined according to the method of Laymon et al. (1996) using 4–methyumbelliferylcellobioside as substrate. One unit of CBH activity was defined as the amount of enzyme required to liberate 1 microequivalent of 4–methylumbelliferone per minute. The activity of BG was assayed according to the method of Macris (1984) using ρ-nitrophenyl-β-D-glucopyranoside(ρ-NPG) as substrate. One unit of β-glucosidase activity was defined as the amount of enzyme required to liberate 1 μmol of ρ-nitrophenol per minute.
Analysis of cellulose, hemicellulose and lignin contents

The cellulose and hemicellulose contents were analyzed by the modified method of Linskens et al. (1999). One gram of sample was suspended in ethanol (four times the volume) and incubated at 40 °C overnight. Then, 30 mL of 1% diastase was added and incubated at 25 °C for 30 min to remove all residual starch. The residual enzymes were removed by distilled water and dried at 40 °C. Half of the sample was removed to determine its dry weight (A), and the rest was treated with 25 mL of 24% KOH at 25 °C for 4 hr. After removing the KOH with distilled water, the sample was dried at 80 °C to obtain the dry weight (B). The B fraction was further treated with 25 mL of 72% H$_2$SO$_4$ for 3 hr and refluxed with 25 mL of 5% H$_2$SO$_4$ for 2 hr to hydrolyze all of the cellulose. The residual H$_2$SO$_4$ was removed by distilled water. The sample was dried at 80 °C to obtain the dry weight, (fraction C). The percentages of cellulose, hemicellulose and lignin were calculated from the following equations: %Cellulose = (B-C) × 100/A, %Hemicellulose = (A-B) × 100/A and %Lignin = C × 100/A.

Analysis of monosaccharide content

The saccharides were analyzed using a modified method from Hoebler et al. (1989). A 50 mg sample was first hydrolyzed with 1 mL of 72% H$_2$SO$_4$ (weight per weight) for 30 min. After hydrolysis, the sample was diluted to 2 N H$_2$SO$_4$ and further heated in a boiling water bath for 2 hr. Its supernatant was collected by filtration with Whatman filter paper and neutralized to pH 5–6 with calcium carbonate. To determine the contents of arabinose, xylose, glucose, galactose, mannose and cellobiose, 10 mL aliquots were filtered through a 0.2 μm filter membrane and analyzed by high-pressure liquid chromatography (HPLC) using an Aminex HPX-87P carbohydrate column (300 × 7.8 mm) at 85 °C with deionized water as the mobile phase and the flow rate of 0.6 mL.min$^{-1}$. The data obtained were analyzed using a chromatographic data management system (HPLC LC–20A; Shimadzu Corporation; Kyoto, Japan).

Scanning electron microscopy (SEM)

The material was completely dried by freeze drying. The dry material was mounted on a specimen, fixed by electrically conductive, double sided, adhesive tape, and then coated with gold using a vacuum sputter coater (JSFC 1,200 series high resolution sputter coater; JEOL Ltd.; Tokyo, Japan) to improve the conductivity of the sample. Analysis was performed with a scanning electron microscope (JSM5410 series; JEOL Ltd.; Tokyo, Japan) operated at 20 kV.

RESULTS AND DISCUSSION

Suitable pretreatment of rough rice bran, palm kernel meal and cassava pulp for enzyme production

Three pretreatments—acid, alkaline and steam—were performed. The qualities of the pretreated substrates of RRB, PKM and CP were assessed by the activities of endoglucanase (CMCase), cellobiohydrolase and β-glucosidase obtained by SSF (Table 1). The SSF extracts from the pretreated RRB contained low activities of CMCase that were not significantly different ($P > 0.5$) from the untreated, except for the alkaline pretreatment which exhibited a much lower activity than the control ($P < 0.5$). Gossett et al. (1982) reported that alkaline pretreatment causes solubilization, redistribution and repolymerization of lignin and then is modified into crystalline cellulose that prevents enzyme hydrolysis which would provide lower carbon sources and subsequently delay both growth and cellulase production. However, compared with the untreated values, the SSF from the acid and steam pretreated RRB exhibited increases in the CBH and BG activity of 2.9–3.7 and 1.4–1.6 fold, respectively. Nevertheless, the activities of CBH and BG from the SSF for acid- and steam-pretreated RRB were
higher with values of 6,244–7,809 and 28,485–33,072 units.g⁻¹, respectively. Compared with the untreated samples the acid- and steam-pretreated PKM samples exhibited increased CBH and BG activities of 55–135 and 3.5–3.7 fold, respectively. These findings indicated that when these materials are steamed, the cell wall structure of PKM could be more effectively degraded than that of RRB.

After SSF for the three pretreatments of CP, the activities of both CMCase and BG were not significantly different (P > 0.5) from those of the untreated sample. The extract from acid-pretreated CP after SSF had the maximum CBH activity, which was 521 fold higher than that of the untreated sample. The positive effects from dilute-acid pretreatment (0.3% sulfuric acid) for CP were similar to previous studies; Kosugi et al. (2009) and Srinorakutara et al. (2006) reported that the optimum diluted-acid pretreatment (0.1–0.6% H₂SO₄, at 121 °C, 0.5–1 hr) was able to maximize the glucose yield and enzymatic hydrolysis using cassava waste for ethanol production. This pretreatment displayed better results in partial dissolution, the deacetylation of hemicelluloses and increased accessible surface area for cellulase hydrolysis without the inhibitor effect of furfural and hydroxymethylfurfural (Taherzadeh and Karimi, 2008). The current findings revealed that the suitable pretreatments as a means of producing hydrolysis power are necessary for these agricultural wastes, particularly, CP and PKM. However when the activities of these enzymes produced by a different pretreatment were taken into account, both the acid- and steam-pretreatment of RRB provided the highest activities of CMCase, CBH and BG. This result might have been due to the presence of excess nutrient sources for fungal growth.

SSF of pretreated cassava pulp, palm kernel meal and rough rice bran

According to previous results, the suitable pretreatment process for RRB, PKM and CP, is steam, steam and acid, respectively; these
process were therefore selected for further study. The chemical composition of both pretreated and native materials were analyzed, (Table 2). Compared to the native material, the amounts of cellulose from pretreated RRB, PKM and CP increased 2.0, 3.2 and 1.6 fold, respectively; hemicellulose and lignin decreased 1.3–1.5 fold compared to the native material. Acid pretreatment and elevated temperature by steam pretreatment could significantly decrease the β-0-4 linkage in the lignin and break down the ester bond of the lignin-hemicelluloses linkage which would cause a decrease of lignin and dissolution of hemicelluloses as well as produce the desirable result of an increase in cellulose (Dien et al., 2006; Sreenath et al., 1999). It was presumed that the higher lignin removal from the pretreated substrates would provide an accessible structure of the cellulose for enzyme digestion and produce suitable carbon sources for fermentation, thereby increasing the CBH activity. As observed by scanning electron microscopy, the structure of the pretreated materials changed to cracked and rough cell wall surfaces, (Figure 1). This result confirmed that the pretreatment used had an effect on the outer surface of the materials.

Both pretreated and native materials were further subjected to SSF by A. niger to determine the maximum cellulolytic enzyme production during cultivation. The results are shown in Figure 2. The glucosamine per gram of dry solids as growth of A. niger in both of native and pretreated materials tended to its maximum value over 3–5 d of cultivation. The pH tended to be stable or a bit lower during SSF due to the acetic acid, ferulic acid and glucoronic acid released from the hemicellulose during enzyme hydrolysis (Taherzadeh and Karimi, 2008). Moreover, the carbon to nitrogen ratio of substrate utilization was the other effect on pH change. These fungal species can balance pH, for example, by using NO3− for an alkaline pH and NH4+ for an acidic pH (Kitpreekhanich et al., 1992). The pretreatment of these three materials resulted in significantly (P < 0.05) enhanced CBH production compared to the native material. However, the pretreated materials did not significantly affect the yields of CMCase and β–glucosidase at 3, 5 and 7 d. The CBH activities of 1,610 and 5,895 units.g−1 were observed after 3 d of SSF of steam-pretreated PKM and acid-pretreated CP, respectively; these activities were stable over the 7 d cultivation, and the native material exhibited low activities of 8–13 units.g−1. The activities of CBH from the SSF of steam-pretreated PKM and acid-pretreated CP were 1,610–5,157 and 5,697–7,683 units.g−1, respectively, which significantly increased by 135–146 and 434–698 fold, respectively, compared to the control from days 3–7. These findings showed that A. niger could ferment only pretreated samples to produce high yields of CBH from SSF. The CBH activity of SSF for steam-pretreated RRB was 6,246 units.g−1 at 3 d and remained stable over the 7 d of cultivation.

Table 2  Chemical composition of the native and pretreated materials.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native RRB</td>
<td>28±8</td>
<td>26±5</td>
<td>26±3</td>
<td>20±4</td>
</tr>
<tr>
<td>Steam-pretreated RRB</td>
<td>57±6</td>
<td>19±6</td>
<td>17±3</td>
<td>7±1</td>
</tr>
<tr>
<td>Native PKM</td>
<td>14±4</td>
<td>38±9</td>
<td>28±4</td>
<td>20±7</td>
</tr>
<tr>
<td>Steam-pretreated PKM</td>
<td>45±7</td>
<td>27±6</td>
<td>20±9</td>
<td>8±4</td>
</tr>
<tr>
<td>Native CP</td>
<td>31±5</td>
<td>37±4</td>
<td>21±4</td>
<td>11±3</td>
</tr>
<tr>
<td>Acid-pretreated CP</td>
<td>50±6</td>
<td>27±4</td>
<td>16±8</td>
<td>7±3</td>
</tr>
</tbody>
</table>

RRB = Rough rice bran; PKM = Palm kernel meal; CP = Cassava pulp.
Values are presented as mean ± SD
However, high CBH activity for native RRB (2,127 units g⁻¹) was observed at 5 d. This increase in activity caused the CBH activities from SSF for steam-pretreated RRB to increase 568 and 3.4 fold and were observed at 3 and 5 d of cultivation, respectively, compared to the native material. Thus, CBH can be produced from SSF of both native and pretreated RRB, but the production rate of native RRB fermentation is lower. The reducing sugars remaining during fermentation, as shown

Figure 1  Photographs of native and pretreated samples of rough rice bran (RRB), palm kernel meal (PKM) and cassava pulp (CP) by scanning electron microscopy: (A) Native RRB (200×); (B) Steam-pretreated RRB (200×); (C) Native PKM (750×); (D) Steam-pretreated PKM (750×); (E) Native CP (350×); (F) Acid pretreated CP (350×).
in Table 3, were lower in the steam-pretreated RRB and steam-pretreated PKM cultivations. The higher reducing sugar concentrations remaining in the native RBB and PKM cultures might be the rate-limiting step of CBH production. However, a high glucose concentration of 376 mg·g⁻¹ in the acid-pretreated CP culture was 1.4 fold higher than the native CP concentration of 271 mg·g⁻¹. These findings indicate that the glucose concentration remaining did not affect the catabolic repression of CBH production, as proposed by other works (Nakari–Setala and Penttila, 1995; Atif et al., 2004; Gautam et al., 2011). The current study results were also supported by the findings of McKelvey and Murphy (2010), who reported that the addition of 1, 5 and 10% glucose in the wheat bran fermentation of A. niger BFJS did not increase the CMCase activity. Interestingly, a high galactose concentration of 58 mg·g⁻¹ was detected from the SSF for acid-pretreated CP, while a high galactose concentration was not observed for native CP. Karafta et al. (2006) reported that the

Figure 2  Solid state fermentation (SSF) of native and pretreated materials with Aspergillus niger 386017M1: (A) SSF for native rough rice bran; (B) SSF for steam-pretreated RRB; (C) SSF for native palm kernel meal; (D), SSF for steam-pretreated PKM; (E), SSF for native cassava pulp; (F), SSF for acid-pretreated CP. Growth of A. niger 386017M1 measured for glucosamine concentration (●); reducing sugar (▲); pH (●); cellobiohydrolase (CBH) activity (□); carboxymethylcellulase (CMCase) activity (●); and β-glucosidase (BG) activity (△). Vertical bars represent the standard deviation from the mean.
addition of D-galactose may be an inducer and may not be related to carbon catabolic repression in Hypocrea jecorina. In addition, D-galactose induced cellulobiohydrolase 2 gene transcription and led to the accumulation in a synthetic medium of endoglucanase, Cel 7A and Cel 6A proteins, which are regulated under the same promoter region in A. niger. (Karafa et al., 2006). This observation suggests that the galactose detected from the acid-pretreated CP culture is involved in enhancing CBH production.

The formation of spores in the SSF of steam-pretreated PKM was not observed over the 7 d of cultivation or 3 wk after cultivation, whereas spores could be found in steam-pretreated RBB and acid-pretreated CP after 20 d.

Cost estimation

The market prices of RRB, PKM and CP were low over the research period, with values of only 3.0–4.5, 7–10 and 3.5–4 baht.kg⁻¹, respectively. The CBH production cost based only on the price of RRB, PKM and CP was only approximately 0.34–0.5, 1.34–1.94 and 0.45–0.52 baht per million units, respectively. Therefore, both RRB and CP would be a good choice for commercial production in the future. However, the cost of the material is dependent on the yield of the crop. Therefore, the decision regarding which material to use in commercial production should involve a consideration of the volume supply on the industrial scale as well.

CONCLUSION

Three pretreatments of plant based materials—RRB, PKM and CP—containing lignocellulose were studied to improve cellulase production. These findings revealed that increasing CBH production by A. niger 386017M1 was successfully accomplished by feedstocks of steam-pretreated RBB and PKM and diluted-acid-pretreated CP. The lignin content after pretreatment of steam-pretreated RRB, steam-pretreated PKM and acid-pretreated CP was reduced 1.5, 1.4 and 1.3 fold, respectively, while the cellulose content increased 2, 3.2, and 1.6 fold, respectively. The CBH activities obtained from steam-pretreated RRB, steam-pretreated PKM and acid-pretreated CP increased 3.4–568, 135–146, and 434–698 fold, respectively, compared to the native controls. Spore formation was not observed over 20 d of cultivation. Both RRB and CP would be a good choice for commercial production in the future due to the low cost of their substrates, being approximately 0.34–0.5 and 0.45–0.52 baht per million units, respectively.

ACKNOWLEDGEMENTS

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<table>
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<th>Table 3</th>
<th>Residual sugar content after 5 d solid state fermentation for both native and pretreated materials.</th>
<th>(milligrams per gram on a dry basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Cellubiose</td>
<td>Glucose</td>
</tr>
<tr>
<td>Native RRB</td>
<td>ND</td>
<td>245±17</td>
</tr>
<tr>
<td>Steam-pretreated RRB</td>
<td>ND</td>
<td>78±8</td>
</tr>
<tr>
<td>Native PKM</td>
<td>108±12</td>
<td>254±39</td>
</tr>
<tr>
<td>Steam-pretreated PKM</td>
<td>ND</td>
<td>120±24</td>
</tr>
<tr>
<td>Native CP</td>
<td>ND</td>
<td>271±31</td>
</tr>
<tr>
<td>Acid-pretreated CP</td>
<td>ND</td>
<td>376±28</td>
</tr>
</tbody>
</table>

RRB = Rough rice bran; PKM = Palm kernel meal; CP = Cassava pulp. Values are presented as mean ± SD.

LITERATURE CITED


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