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Original Article

Cellulase and xylanase production from *Candida easanensis* using agricultural wastes as a substrate

Jantaporn Thongekkaew*, Wanlee Patangtasa, and Apichat Jansri

Department of Biological Science, Faculty of Science,
Ubon Ratchathani University, Warin Chamrap, Ubon Ratchathani, 34190 Thailand.

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Abstract

The production of cellulase and xylanase from *Candida easanensis* strain JK-8 was investigated. Different fermentation conditions were standardized for the growth and enzyme activity, the optimum being 72–96 hrs growth at initial pH 4.0, and cultivation temperature at 35°C. Of the different carbon sources on cellulase production, carboxymethyl cellulose gave the maximum production of 0.23 UmL^{-1}. Among the carbon sources on xylanase production, the maximum enzyme activity was achieved in the medium containing Birchwood xylan (1.14 UmL^{-1}). Amongst different agricultural waste samples (such as rice straw, corn husk, and sugarcane bagasse), corn husk gave the highest yields of cellulase and xylanase and the activities were 0.089 and 0.82 UmL^{-1}, respectively. This study suggests that corn husk could be utilized as a carbon source for economical production of cellulase and xylanase by *C. easanensis* JK-8. This may in turn reduce the cost of enzyme production leading to efficient use of ligno-cellulosic materials to produce value-added products.

Keywords: cellulase; xylanase; *Candida easanensis*; agricultural waste

1. Introduction

In recent years, increasing concern over preserving resources and environment has initiated a growing interest in producing microbial enzymes. Cellulases and xylanases from microorganisms have attracted a great deal of attention in the last decade because of their biotechnological potential in various industrial processes such as waste water treatment, food, feed, and paper-pulp industries (Enari, 1983; Bedford and Classen, 1992; Kapoor *et al*., 2001; Bocchini *et al*., 2003; Damiano *et al*., 2003; Suchita and Ramesh, 2006), as well as biofuel production from cellulosic biomass (Ali and Saad, 2008).

Cellulases and xylanases are produced by both prokaryotes and eucaryotes. A large number of bacteria and fungi are known to produce these enzymes (Kulkarni *et al*., 1999; Subramaniyan and Prema, 2002). Cellulases can convert the world's most abundant biopolymer, 'cellulose', into reducing sugars. Bacterial and fungal cellulases are traditionally separated into three classes, endoglucanases (EGs) (EC 3.2.1.4), exoglucanases (EC 3.2.1.91, EC 3.2.1.176), and β-glucosidases (EC 3.2.1.21). The endoglucanases are responsible for the scission of the inner bonds in the cellulose chains yielding glucose and celloligosaccharides. Exoglucanases (cellobiohydrolases) cleave the non-reducing end of the cellulose with cellobiose as the main structure, whereas β-glucosidases hydrolyse cellobiose to glucose (Whitakers, 1971). Xylanase (1, 4-β-D-glucan xylanohydrolase, EC 3.2.1.8) is a key enzyme for the degradation of β-(1,4)-xylan, the major plant cell-wall polysaccharide of hemicelluloses, into xylose (Shah and Madamwar, 2005).

The cost of an enzyme is one of the main factors determining the economics of a process. The high costs of cellulase and xylanase enzyme production hinders the application of these enzymes for bioethanol production (Himmel, *et al*., 1999; Wooley *et al*., 1999). This can be partially achieved by optimizing fermentation medium and using agricultural wastes for the cultivation of the producer micro-

* Corresponding author.
Email address: jantaporn_25@yahoo.com

organisms, which has been suggested as an alternative to reduce the production costs (Rajaram and Varma, 1990; Dhillon *et al*., 2000).

Thermotolerant and/or thermophilic microorganisms are quite useful for certain industrial processes. The production of biological materials at high temperatures rather than room temperature makes it possible to reduce the risk of contamination and the cost of maintaining low growth temperatures in large-scale systems. It also increases the productivity rate. Moreover, the organisms are valuable sources of thermostable enzymes that are often stable also in solvents and detergents used in many biotechnological and industrial applications (Lasa and Berenguer, 1993; Haki and Rakshit, 2003). For these reasons, a new strain of thermotolerant yeast, *Candida easanensis* strain JK-8, growing at temperatures up to 40ºC, isolated here in Thailand, and showing an extracellular cellulase and xylanase producing ability was investigated and used in the optimization of cellulolytic and xylanolytic enzymes production.

2. Materials and Methods

2.1 Preculture of microorganism selection

Candida easanensis strain JK-8 was grown in sterile YM broth medium (3 gL^{-1} yeast extract, 3 gL^{-1} malt extract, 5 gL^{-1} peptone and 10 gL^{-1} glucose) for 16 hrs at 35ºC was used as the preculture.

2.2 Optimization of cellulase and xylanase production

Cellulase and xylanase production by *Candida easanensis* strain JK-8 was optimized following one factor at a time (OFAT) approach. The effect of initial pH of medium (3.5-6.0) and different carbon sources was tested. A medium containing 1.0% different carbon source (CM-cellulose, carboxymethyl cellulose (CMC), cellobiose and glucose) in 1.0% yeast extract and 0.5% peptone was used to detect cellulase while Birchwood xylan, glucose and xylose were used as a carbon source to detect xylanase. Each medium was inoculated with overnight preculture with initial OD_{660} at 0.1. The cultures were grown under shaking with 130 rpm at 35ºC. The cell free supernatant was measured for cellulase and xylanase activity at 24 hr-intervals for seven days by measuring the releasing of reducing sugar with the Somogyi-Nelson method (Somogyi, 1952). The effect of different concentrations of carboxymethyl cellulose and xylan (0.5-1.5%) were also examined.

2.3 Utilization of agricultural waste as substrate for enzyme production

Agricultural waste, samples of rice straw, corn husk and sugarcane bagasse, were collected from local industries of Ubon Ratchathani Province, Thailand. The substrates were dried in an oven at 70ºC and chopped into smaller pieces. The material was sieved (200 mesh size) and the finer particles were used at 1.0% concentration as a carbon source (Rani and Nand, 2000).

2.4 Determination of xylanase and cellulase production

Cellulase activity was determined by measuring the amount of glucose released from carboxymethyl cellulose by the Somogyi-Nelson method with glucose as the standard (Somogyi, 1952). Reaction mixtures contained 0.45 mL of 1.0% carboxymethyl cellulose sodium salt in 50 mM citrate buffer, pH 5.0 and 0.05 mL of each enzyme fraction. Control lacked the enzyme fraction. After incubation at 50ºC for 15 min, the reaction was terminated by adding 0.5 mL of Somogyi reagent. The mixture was mixed, placed in a boiling-water bath for 10 min, and cooled to room temperature. A 0.5 mL of Nelson reagent was added. After being vortexed, the mixture was centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 660 nm. One international unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 mmol of reducing sugars in glucose equivalents per min.

Xylanase activity was determined by measuring the release of reducing sugars from Birchwood xylan using the Somogyi–Nelson method (Somogyi, 1952). Reaction mixtures contained 0.45 mL of 0.5% Birchwood xylan in 50 mM citrate buffer, pH 5.0 and 0.05 mL of each enzyme fraction. Control lacked the enzyme fraction. After incubation at 50ºC for 15 min, the reaction was terminated by adding 0.5 mL of Somogyi reagent. The mixture was vortexed, placed in a boiling-water bath for 10 min, and cooled to room temperature. A 0.5 mL of Nelson reagent was added. After being vortexed, the mixture was centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 660 nm. One international unit (IU) of enzyme activity was defined as the amount of enzyme required to release 1 mmol of xylose from Birchwood xylan in 1 minute under the assay condition.

3. Results and Discussion

3.1 Effect of initial pH of medium on cellulase and xylanase production

The initial pH of medium is one of the most critical environmental parameter affecting enzyme production. The cellulase and xylanase production by *C. easanensis* JK-8 were tested at different pH ranging from 3.5 to 6.0. This yeast was observed to produce maximum cellulase and xylanase at initial pH of 4.0 at day 4 of cultivation (Figure 1). At higher acidic pH (pH 6), less cellulase and xylanase production was noticed. This result suggests that the enzyme production depended on the pH of the growth medium. Good growth and higher enzyme activities were always noticed in the pH adjusted culture medium as compared with growth without pH adjusted. The pH optimum for cellulase and xylanase production by *C. easanensis* JK-8 was similar to these

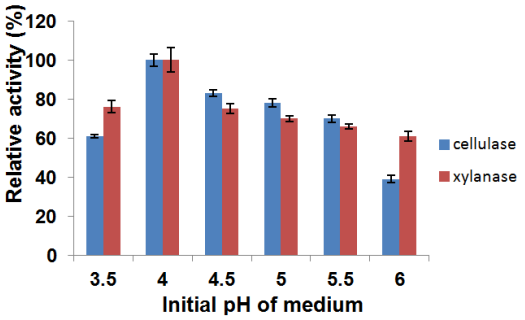

Figure 1. Effect of initial pH of medium on cellulase and xylanase production. The culture media containing yeast extract, peptone and cellulose/xylan at different initial pH from 3.5 to 6.0 were inoculated with yeast cells and cultivated under shaking (130 rpm) at 35°C. The cell free supernatant was measured cellulase and xylanase production after 96 hrs of cultivation under the method of Somgyi-Nelson. Each experiment was measured in triplicate.

enzymes production from *Cryptococcus sp.* S-2 (Iefuji *et al.*, 1996; Thongekkaew *et al.*, 2008).

3.2 Effect of different carbon sources on cellulase production

Various cellulosic substrates (cellobiose, carboxymethyl cellulose (CMC) and CM-cellulose) and glucose were tested as a carbon source on cellulase production. The maximum growth was exhibited in the medium containing cellobiose, followed by glucose, CMC and least with CM-cellulose. However, the highest cellulase was produced in the medium containing CMC and the enzyme activity was 0.23 UmL^{-1} at day 5 of cultivation whereas cellobiose and CM-cellulose resulted in enzyme activity of 0.15 and 0.09 UmL^{-1}, respectively. However, the lowest enzyme production was found in the medium containing glucose (0.05 UmL^{-1}) (Figure 2). This result can be correlated with previous report that mentioned about an increase in endoglucanases activity when *Trichoderma* sp., was grown in the presence of CMC (Aslam *et al.*, 2010). Some previous studies also reported that CMC is the substrate of choice for endoglucanase production (Shambe, 1987; Gautam, 2010). Moreover, it was found that the cellulase production was repressed when *C. easanensis* JK-8 was grown in the medium containing glucose, suggesting that cellulase production was under catabolite-repression as has been reported for *Alternaria solani* earlier by Sands and Lukens (1974), where a decreased production of cellulases was observed in cellulose-deficient medium.

3.3 Effect of substrate concentration on cellulase production

As the maximum enzyme activity was obtained with CMC, the influence of its varying concentrations on cellulase production was examined. We found that culture medium containing 1.0% CMC is the most effective induction of the enzyme production (Figure 3); this value is quite close to the results for cellulase production from *Alternaria solani* (Sands and Lukens, 1974), *Pseudomonas* sp. (Gautam *et al.*, 2010) and *Alternaria sp.* MS28 (Sohail *et al.*, 2011).

3.4 Effect of different carbon sources on xylanase production

To investigate the optimization of xylanase production by *C. easanensis* JK-8, the effect of different carbon sources (xylose, glucose, and Birchwood xylan) on the growth and enzyme production was studied. The maximum growth was exhibited in the medium containing xylose, followed by glucose and least with Birchwood xylan. However, the highest xylanase activity was obtained with Birchwood xylan (1.14 UmL^{-1}) at day 4 of cultivation, followed by glucose and least with xylose (Figure 4). Birchwood xylan was found to

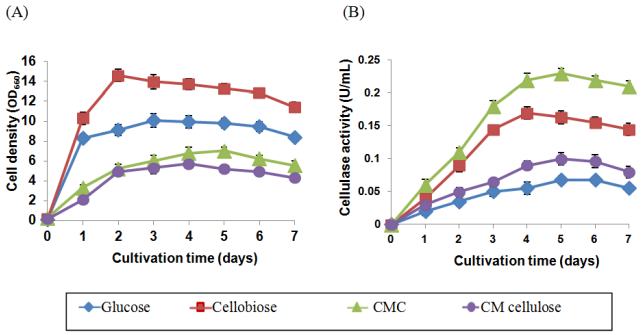

Figure 2. Effect of different carbon sources on cellulase production. The culture media (pH 4.0) contain various carbon sources (glucose, cellobiose, CMC, and CM cellulose) at concentration of 1% (w/v). Growth (A) and enzyme production (B) were measured at 24 hr-intervals for 7 days. Each experiment was done in duplicate and measured in triplicate.

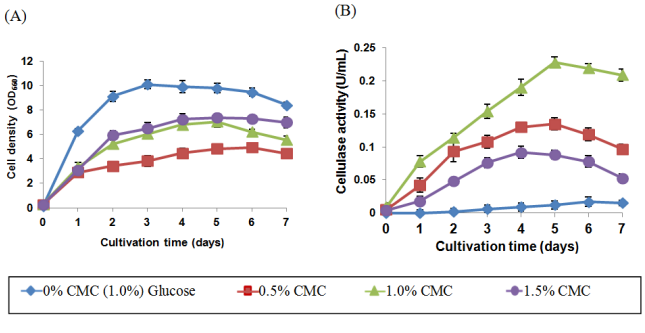

Figure 3. Effect of carboxymethyl cellulose (CMC) concentration on cellulase production. The culture media (pH 4.0) contain CMC at concentration from 0 to 1.5% (w/v). Growth (A) and enzyme production (B) were measured at 24 hr-intervals for 7 days. Each experiment was done in duplicate and measured in triplicate.

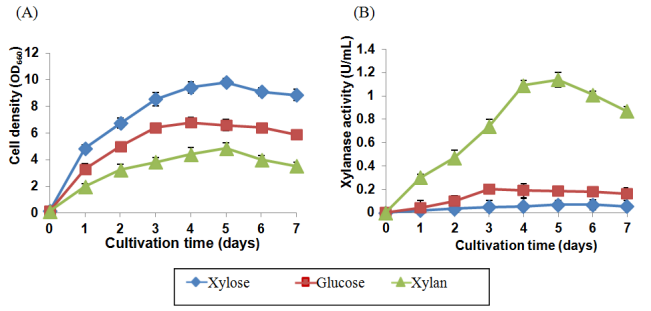

Figure 4. Effect of different carbon sources on xylanase production. The cultures medium (pH 4.0) contain various carbon sources (xylose, glucose and xylan) at concentration of 1% (w/v). Growth (A) and enzyme production (B) were measured at 24 h-intervals for 7 days. Each experiment was done in duplicate and measured in triplicate.

be the best substrate for xylanase production. This result correlates well with previous reports of xylanase production by *Thermoascus aurantiacus* (Yu, *et al*., 1987), *A. sydowii* MG49 (Ghosh and Nanda, 1994), thermophilic *Bacillus* sp. (Pertulla *et al*., 1993), and *B. circulans* (Ratto *et al*., 1992), respectively. Glucose and xylose (1.0% (w/v)) proved to be ineffective as inducers for xylanase production which was similar to xylanase production from *Fusarium oxysporum* as reported by Christakopoulos *et al*. (1996). Smith and Wood (1991) also reported very low activity of xylanases in the glucose medium. Moreover, Gupta and Kar (2008) stated that xylanase activity was not observed until the glucose was depleted from culture medium during the growth of *Streptomyces syaneus* SN32 in the medium containing both xylan and glucose. A significant decrease in production was observed from the sixth day onwards, indicating that xylanases are usually expressed at the end of the exponential phase and harvesting time as reported by Kulkarni *et al*. (1999). The phenomenon of sudden increase and subsequent decrease in enzyme activities during the cultivation period has also been noted in xylanase produced by *Aspergillus sydowii* MG-49 (Ghosh *et al*., 1993) and *Streptomyces* sp. CH-M-035 (Flores *et al*., 1996).

3.5 Effect of substrate concentration on xylanase production

In order to further increase the yield of xylanase, the influence of Birchwood xylan varying concentrations on xylanase production was examined. The result showed that no significant difference in enzyme production was observed in the medium containing 0.5 to 1.5% Birchwood xylan, suggesting that 0.5% Birchwood xylan is sufficient for xylanase production by this yeast strain (Figure 5). A similar result was obtained for enzyme production with *Cellulosimicrobium* sp. MTCC 10645 (Kamble and Jadhav, 2012) whereas in *Gracilibacillus* sp. TSCPVG xylanase activity was maximum in 0.75% Birchwood xylan (Giridhar and Chandra, 2010).

3.6 Effect of agricultural wastes on cellulase and xylanase production

Carboxymethyl cellulose or pure xylan due to its high cost are not affordable for large scale industrial production of cellulase or xylanase, therefore, low cost agricultural residues such as wheat bran, rice straw, corn husk, sugarcane bagasse, and others have been explored as cheap substrates for cost-effective production of enzymes (Gupta and Kar, 2008; Bajaj and Singh, 2010; Geetha and Gunasekaran, 2010; Bajaj *et al*., 2012). In the present study, cellulase and xylanase were produced by *C. easanensis* JK-8 in the medium with different agricultural waste (rice straw, corn husk, and sugarcane bagasse) as compared to the values obtained with CMC and pure xylan (Figure 6 and 7). The maximum growth was observed in the medium with corn husk followed by sugarcane bagasse and least with rice straw. The highest yield of cellulase and xylanase were produced in the medium containing corn husk as a sole carbon source and activities of these enzymes were 0.089 and 0.82 UmL^{-1}, respectively. Our results

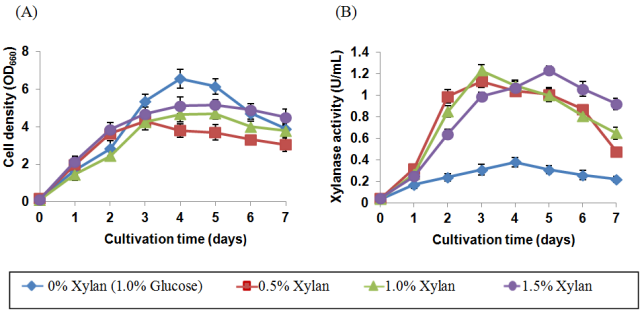

Figure 5. Effect of xylan concentration on xylanase production. The cultures medium (pH 4.0) contain xylan at concentration from 0 to 1.5% (w/v). Growth (A) and enzyme production (B) were measured at 24 hr-intervals for 7 days. Each experiment was done in duplicate and measured in triplicate.

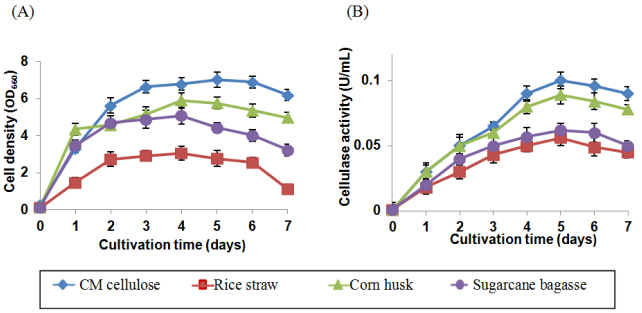

Figure 6. Effect of agricultural wastes on cellulase production. The culture media (pH 4.0) contain different agricultural wastes (rice straw, corn husk, and sugarcane bagasse) at concentration of 1% (w/v) with CM cellulose as control. Growth (A) and enzyme production (B) were measured at 24 hr-interval for 7 days. Each experiment was done in duplicate and measured in triplicate.

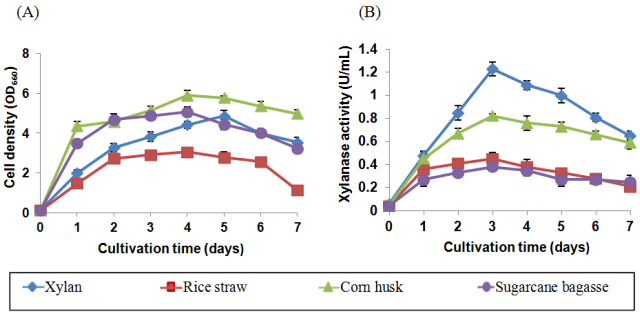

Figure 7. Effect of agricultural wastes on xylanase production. The culture media (pH 4.0) contain different agricultural wastes (rice straw, corn husk, and sugarcane bagasse) at concentration of 1% (w/v) with xylan as control. Growth (A) and enzyme production (B) were measured at 24 hr-interval for 7 days. Each experiment was done in duplicate and measured in triplicate.

indicate that corn husk was a relatively good inducer of cellulase and xylanase. However, Jaafaru and Fagade (2010) studied cellulase synthesis by *Aspergillus niger* and reported that addition of sugarcane bagasse as sole carbon source. Some previous studies (Rani and Nand 2000; Kang *et al*., 2004; Haq *et al*., 2006; Immanuel *et al*., 2006; Oliveira *et al*., 2006; Bansal *et al*., 2012) reported that corn cobs and wheat bran produced maximum cellulase and xylanase enzyme synthesis.

The difference in cellulase and xylanase activities on agricultural waste utilization may be due to variation in the amounts of utilizable amorphous cellulose present in each sample, which could be attributed to the accessibility of microorganisms towards the cellulose and hemicellulose in the polymer matrix as has been reported earlier by Adsul *et al*., 2004, Gupta and Kar (2008), Geetha and Gunasekaran (2010), and Bajaj *et al*., 2012.

4. Conclusion

Our results show that thermotolerant yeast, *Candida easanensis* JK-8 has the potential for cellulase and xylanase production. This yeast produces cellulase and xylanase which is induced by the addition of 1.0% carboxymethyl cellulose and 0.5% Birchwood xylan in the culture medium, respectively. Moreover, our study can conclude that the use of corn husk is an efficient alternative to reduce the costs of cellulase and xylanase production by *C. easanensis* JK-8 in submerged fermentation, since this material is often available in tropical countries like Thailand, as an inexpensive source of components that propitiate the enzyme production.

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