Songklanakarin J. Sci. Technol.
35 (1), 17-22, Jan. - Feb. 2013

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
http://www.sjst.psu.ac.th

Original Article

Optimization of extracellular mannanase production from *Penicillium oxalicum* KUB-SN2-1 and application for hydrolysis property

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Received 21 May 2012; Accepted 4 December 2012

Abstract

Effects of media composition, and physical properties on the production of crude mannanase by *Penicillium oxalicum* KUB-SN2-1 were investigated. *P.oxalicum* KUB-SN2-1 was propagated in a shaking incubator at 30°C with rotation speed of 200 rpm of 7 days. The specific activity obtained during growth on robusta coffee residues (RCR) of 16.21 U/mg protein was much higher than other carbon sources tested. For nitrogen sources, yeast extract (0.11 U/mg protein) and ammonium nitrate (0.09 U/mg protein) showed maximum specific activity. Hence, guar gum was the best inducer for producing mannanase (14 U/mg protein). For evaluating the optimal concentration, the result showed that 1% guar gum, 0.5% yeast extract, 0.25% ammonium nitrate, and 0.25% RCR were the suitable sources of inducer, organic nitrogen, inorganic nitrogen, and carbon, respectively. Modified medium with initial culture pH of 5.0 at 30°C was optimum for mannanase production (53.77 U/ml for 3 day). Reducing sugars were analyzed by dinitrosalicylic acid methods. The highest reducing sugar of 7517.82 µg/ml was obtained from copra meal hydrolysate after 30 h.

Keywords: *Penicillium oxalicum*, mannanase, copra meal, oligosaccharide production

1. Introduction

Lignocellulose is a major component of plant cell walls and is mainly composed of lignin, cellulose and hemicellulose. Mannans and heteromannans are a part of the hemicellulose fraction in plant cell walls. The structure of hemicellulose is a key component of many types of sugar such as xylans, mannans, heteromannans, galactans, and arabinans (Zyl *et al*., 2010). Mannans and heteromannans are widely distributed in hardwoods and softwoods, seeds of leguminous plants, and bean (Dhawan and Kaur, 2007). They can be divided into 4 types: (1) unsubstituted (1,4)-linked β-D-mannans, (2) galactomannans, (3) glucomannans, and (4) galactoglucomannans (Kote *et al*., 2009). Mannans, which have main component as D-mannose, are important for several industries, including food, feed, and feed stocks. They can be broken down into simple sugars or oligosaccharides by a synergistic action of endo-mannanases (EC number 3.2.1.78, mannan endo-1,4-β-mannosidase) and exo-acting β-mannosidase (EC number 3.2.1.25).

Various microorganisms can produce mannanases such as *Aspergillus awamori* (Kurakake and Komaki, 2001), *Trichoderma harzianum* (Ferreira and Filho, 2004), *Rhodothermus marinus* (Politiz *et al*., 2000), *Bacillus subtilis* (Jiang *et al*., 2006), and *Streptomyces ipomoea* (Monitel *et al*., 1999). Many mannan-based carbon sources including locust bean

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gum (LBG), guar gum, and copra meal have been used to cultivate filamentous fungi (Ademark *et al*., 1998; McCutchen *et al*., 1996; Oda *et al*., 1993). Moreover, various inducers were used for improving mannanase production.

Chaiongkarn *et al*. (2009) isolated microorganisms from soil using copra meal as a carbon source for their growth. The results showed that the *Penicillium oxalicum* KUB-SN2-1 secreted extracellular enzyme with mannanase activities of 19.31 Unit/ml. Therefore, this study aimed to search for cheaper carbon, nitrogen, and inducer sources for mannanases and oligosaccharides production by *P.oxalicum* KUB-SN2-1.

2. Materials and Methods

2.1 Materials

Locust bean gum (LBG) and guar gum were purchased from Sigma Chemical, USA. All other chemicals used were of analytical grade.

2.2 Preparation of agricultural wastes

Seven kinds of agricultural wastes (AWs) including arabica coffee residues (ACR), robusta coffee residues (RCR), soy bean meal, copra meal, potato peel, baggasse, and corn cob were used as carbon sources, inducers, and substrates for mannanase production. AWs were dried at 60°C for 48 h, blended, milled by a hammer mill (IKA Labortechnik; Janke & Kunkel, Germany), and then sieved to obtain a product with an average particle size of 30 mesh. All samples were kept in a desiccator until used.

2.3 Microorganism and cultivation

Penicillium oxalicum KUB-SN2-1 was obtained from the laboratory stock culture at the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand. *P. oxalicum* KUB-SN2-1 was maintained on potato dextose agar (PDA) plate. After 7 days of the cultivation at 30°C, spore concentration was adjusted to 10^6 spore/ml with sterile 0.2% (v/v) tween 80 and used as a starting inoculum.

2.4 Enzyme production

Basic liquid medium (control medium) for growth and enzyme production had the following composition (%w/v): 0.4 bacto-peptone, 0.3 KH_2PO_4, 0.2 K_2HPO_4, 0.05 $MgSO_4.7H_2O$, 0.0002 $CoCl_2.6H_2O$, 0.0005 $FeSO_4.7H_2O$, 0.0002 $MnSO_4.H_2O$, and 0.0016 $ZnCl_2$. The 10% (v/v) of inoculum was transferred to 500 ml of control medium and incubated at 30°C with shaking at 200 rpm for 7 days. Samples were collected at day 1 to day 7 by centrifugation at 11,000Xg, 4°C for 15 min. Supernatant was collected and used as crude extracellular mannanase. All the experiments were investigated in triplicate, and results represent mean values of the activities.

2.5 Determination of mannanase activity and protein concentration

Mannanase activity was measured at 60°C for 15 min using a reaction mixture containing 0.5 ml of crude enzyme samples and 0.5 ml of 0.1M citrate buffer pH 4.0 with 1% (w/v) LBG. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme producing 1 micromole of mannose per minute under experimental conditions. Protein concentration was determined employing the method of Lowry *et al.* (1951) with bovine serum albumin as a standard protein.

2.6 Effect of temperature and initial pH

The crude extracellular mannanase was preincubated at different temperatures ranging from 25 to 45°C for different time intervals and then assayed for enzyme activity. The effect of initial pH of enzyme production medium were studied between pH 4 and 7 using 0.5% (w/v) of tartaric acid. All the experiments were carried out in triplicate.

2.7 Determination of medium composition

Effect of 1% (w/v) supplementation of additional carbons, and nitrogen sources, and inducers to control medium were investigated. The AWs as carbon sources such as ACR, RCR, potato peel, and corn cob for the mannanase production were studied. Nitrogen sources, such as organic nitrogen as yeast extract and meat extract and inorganic nitrogen as ammonium nitrate (NH_4NO_3), ammonium sulfate [$(NH_4)_2SO_4$], and urea [$CO(NH_2)_2$], were tested. The effect of inducers as LBG, guar gum, and copra meal were examined. In addition, carbon and nitrogen sources and inducers were also evaluated for optimal concentrations in the range of 0 to 2% (w/v).

2.8 Time course of mannanase production

Time course of mannanase production was determined before and after media formulation at the same conditions as above. After inoculation, mannanase activities in the supernatant were followed from the first to seventh day.

2.9 Hydrolysis study

Crude extracellular mannanase of 50 U/ml were performed in the reaction mixture of 15 ml of crude extracellular enzyme sample and 15ml of 1% (w/v) AWs in 100 mM citrate-phosphate buffer pH4.0 and carried out at 60°C for 48h. The reaction after 3, 6, 9, 24, 27, 30, and 48 h of incubation period were stopped by placing the samples in boiling water for 10 min. The amount of reducing sugar of hydrolysis products were analyzed by DNS methods. Hence, the hydrolysis product patterns were determined by modified thin-

layer chromatography (TLC) (Apiraksakorn *et al*., 2008).

3. Results and Discussion

3.1 Effect of enzyme production medium

Nutrient compositions in the medium influenced the production of mannanase by *P. oxalicum* KUB-SN2-1 as shown in Table 1. The results showed that the maximum production of mannanase were 16.21, 0.11, 0.09, and 14 U/mg protein when the medium was supplemented with RCR, yeast extract, ammonium nitrate, and guar gum, respectively. On the contary, various simple sugar or sugar alcohol as glucose, mannose, maltose, and sorbitol inhibited mannanase production (data not shown). The monosugars could cause a catabolite repression, as reported for *Aspergillus niger* and *A. flavus* (Kote *et al*., 2009). Hence, *A. nidulans* was also observed to sensitive to carbon catabolite repression (Loera and Cordova, 2003).

A comparison between nitrogen and carbon sources revealed that mannanase specific activity from the medium supplemented nitrogen sources was lower, since nitrogen source are usually used for protein synthesis substances more than growth (Stanbury and Whitaker, 1984). In addition, among nitrogen sources, organic nitrogen enhanced mannanase production more than the inorganic sources (Table 1). This is similar to the results of Kote *et al*. (2009).

Occasionally, combined effects on the mannanase production were observed for carbon sources and inducers tested. Because both carbon sources and inducers usually contain high content of polysaccharides which are substrate-like or substrates of the enzyme. As shown in Table 1, LBG, guar gum, and copra meal are galactomannans with different mannan contents in theirs molecules. In LBG, the ratio of mannose to galactose is 4:1, it is 2:1 in guar gum and 14:1 in copra meal (Regaldo *et al*., 2000). Although RCR, which is composed of 20-30% galactomannan, has more branches than ACR, RCR appeared to be a more efficient carbon source for *P. oxalicum* KUB-SN2-1 mannanase. This might be due to its higher water solubility.

Thus, RCR, yeast extract, ammonium nitrate, and guar gum were selected for further evaluating the optimal concentrations in the range of 0 to 2% (w/v). The highest specific mannanase activity of 15.13 U/mg protein on day 6 of the cultivation with 1% guar gum supplemented medium was achieved (Figure 1D), whereas, 0.5% yeast extract, 0.25% ammonium nitrate, and 0.25%RCR, which were the best sources of organic nitrogen, inorganic nitrogen, and carbon, respectively, gave specific enzyme activities of 0.16, 0.12, and 3.67 U/mg protein on day 5 of the cultivation as shown in Figures 1A-1C, respectively. The results suggest that guar gum can be used as an inducer for mannanase production by *P. oxalicum* KUB-SN2-1. Guar gum consists of a β-1,4 linked D-mannose backbone and α-1,6 linked D-galactose side chains, in which the D-galactose and D-mannose contents are 38% and 62%, respectively (Kurakake *et al*., 2006). Similarly, Mohamad *et al*. (2010) showed that the highest mannanase production was obtained when *A. niger* was cultivated with guar gum compared to cultivation with LBG, α-cellulose, glucose, and carboxymethylcellulose.

In addition, the modified medium consisting of control medium supplemented with 1% (w/v) guar gum, 0.5% (w/v) yeast extract, 0.25% (w/v) ammonium nitrate, and 0.25% (w/v) RCR was evaluated for mannanase production comparing to control medium. Figure 2 shows that *P. oxalicum* KUB-SN2-1 had enhanced mannanase production in the modified medium. Additionally, the enzyme production reached a maximum activity after 3 days compared to 5 days using each

Table 1. Effects of different carbon and nitrogen sources and inducers on mannanase production

Source	Mannanase productivity (maximum activity/day)	Specific mannanase activity (U/mg protein)
Control	6	0.11±0.02
Carbon sources		
Arabica coffee residues, ACR	5	2.21±0.1
Robusta coffee residues, RCR	5	16.21±2
Potato peel	5	0.22±0.01
Corn cob	5	0.62±0.02
Nitrogen sources		
Yeast extract	4	0.11±0.003
Meat extract	3	0.04±0.002
Ammonium nitrate	5	0.09±0.006
Ammonium sulfate	2	0.03±0.004
Urea	2	0.02±0.002
Inducer sources		
Locust bean gum	5	10.12±0.92
Guar gum	5	14±1.10
Copra meal	5	0.50±0.02

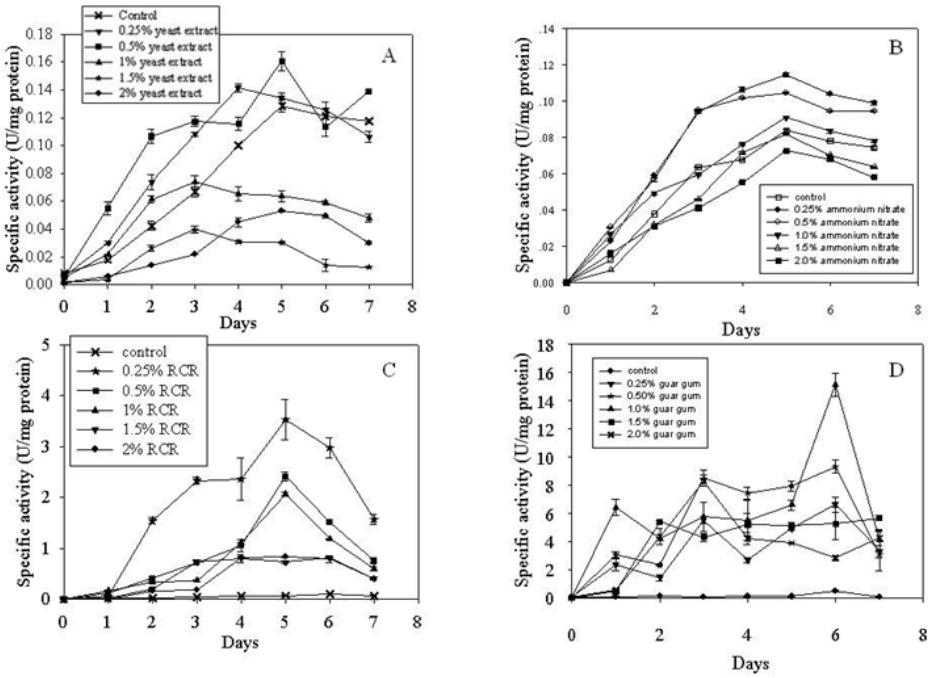

Figure 1. Effects of concentrations on various supplements in the range of 0 to 2% (w/v) of: (A) yeast extract; (B) ammonium nitrate; (C) RCR; and (D) guar gum

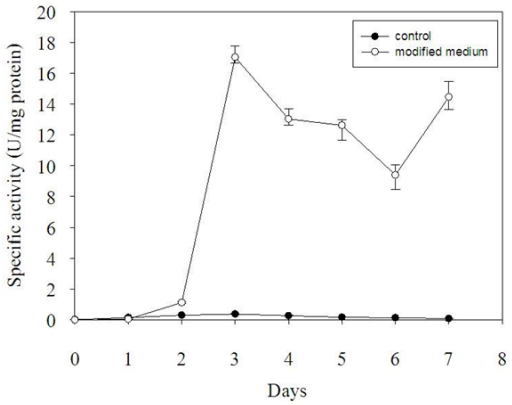

Figure 2. Mannanase production with modified medium comparing to that of control medium

source alone (Table 1). The highest production of mannanase was 17.89 U/mg protein on day 3 of the cultivation time.

3.2 Effect of initial pH and temperature

The influence of initial pH of the culture medium on mannanase production was investigated in the range of 4.0-7.0. Maximum mannanase production was achieved at pH 5.0 (Figure 3A). Similarly, Kote *et al*. 2009 reported maximum production of mannanase from *Aspergillus flavus* grown in a fermentation medium having an initial pH at 5.0.

The effect of growth temperature on mannanase production in the range of 25-45°C was studied. Optimum enzyme activities were obtained at 30°C (Figure 3B). Moreover, the results show that the initial pH of 5.0 for *P. oxalicum* KUB-SN2-1 was suitable for mannanase production at 30°C and yielded the maximum mannanase activity of 53.77 U/ml. Thus, the optimum conditions for mannanase production were produced by modified medium at the initial pH of 5.0 and carried out at 30°C for 72h.

3.3 Hydrolysis property

AWs are alternative economical raw materials for oligosaccharides production. This study aimed to change locally available AWs into value-added products. Various AWs including copra meal, potato peel, soy bean meal, RCR, and baggasse were hydrolyzed by crude extracellular mannanase from *P. oxalicum* KUB-SN2-1. The maximum amount of reducing sugar released are shown in Table 2. The

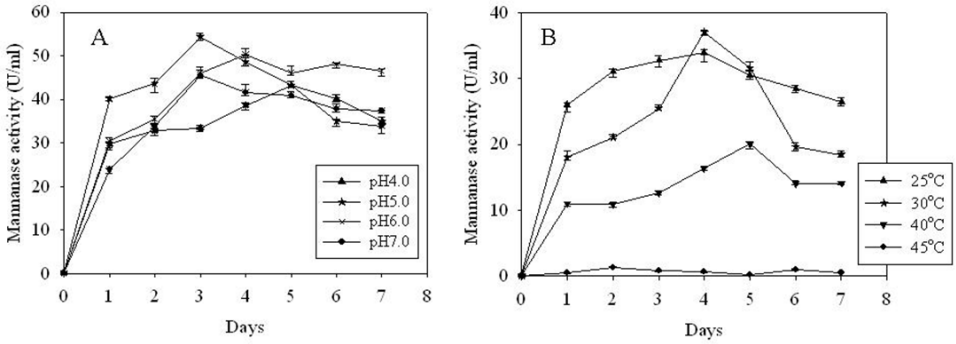

Figure 3. Mannanase production at different initial pH (A), and temperature (B)

Table 2. Amount of reducing sugar against various AWs

Type of agricultural waste	Amount of reducing sugar (μg/ml)	Relative hydrolysis (%)
Copra meal	7,517.82 ± 436.436	100
Potato peel	3,309.49 ±176.174	44.02
Soy bean meal	2,325 ± 109.109	30.93
RCR	3,355.91 ±549.814	44.64
Bagasse	3,827.64 ±214.286	50.92

highest reducing sugar content achieved was from copra meal (7,517.82 μg/ml) followed by baggasse (3,827.64 μg/ml), RCR (3,355.91 μg/ml), potato peel (3,309.49 μg/ml), and soy bean meal (2,325 μg/ml), respectively. Additionally, various sizes of oligosaccharide detected by TLC were obtained from the hydrolysis of copra meal (Figure 4).

As shown in Figure 4, mannotriose and mannobiose were produced after 3h. Similarly, mannanases from *Bacillus* sp.KK01 and *B. subtilis* WY34 hydrolyzed copra mannan to mainly mannotriose and mannobiose (Hossain *et al*., 1996; Jiang *et al*., 2006). Hence, some fungal mannanases hydrolyse mannotetraose to mannotriose and mannobiose through transglycosylation reaction (Puchart *et al*., 2004). Moreover, the medium supplemented with guar gum showed maximum mannanase activity (50U/ml), similar to that reported by Kurakake *et al*. (2006). The authors revealed that *P.oxalicum* SO mannanase efficiently hydrolyzed guar gum to manno-oligosaccharide. Thus, the mannanase is suitable to produce oligosaccharides such as manno-oligosaccharides, which are the products from the enzyme hydrolysis. However, the degradation products were determined by TLC method. Therefore, to confirm the type and concentration of the hydrolysate products, further analyzes will be done using high performance liquid chromatography (HPLC) method.

4. Conclusion

This study focuses on the optimization of culture parameters for the maximal production of crude extracellular mannanase from *P. oxalicum* KUB-SN2-1. The modified medium with initial medium pH of 5.0 at 30°C gave the highest mannanase activity of 53.77 U/ml on day 3. There were increases of up to about 50 times and faster production rate comparing of control medium. Finally, hydrolysis products of the enzyme produced were mannooligosaccharides, which may be used in food and feed industries.

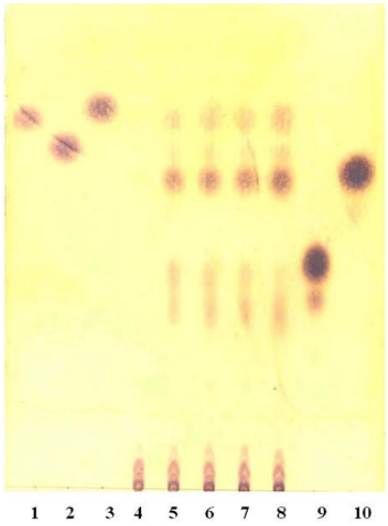

Figure 4. Copra meal degradation by crude extracellular mannanase at various times of 0, 3, 6, 9 and 24h. Lane 1: Glucose, Lane 2: galactose, Lane 3: mannose, Lane 4: 0h, Lane 5: 3h, Lane 6: 6h, Lane 7: 9h, Lane 8: 24h, Lane 9: mannotriose, Lane 10: mannobiose.

Acknowledgements

This work was supported by TU research fund.

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