Productions of xylanase and cellulase from lignocellulosic wastes by

Paenibacillus curdlanolyticus B6

การผลิตไซลาเนสและเซลลูเลสจากวัสดุเหลือทิ้งทางการเกษตรโดย

Paenibacillus curdlanolyticus B6

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Weera Piyatheerawong (วีระ ปิยธีระวัฒน์)***

ABSTRACT

To determine the effects of carbon sources on the productions of xylanase and cellulase by Paenibacillus curdlanolyticus B6, the bacterium was cultured in mineral medium containing various lignocellulosic materials (corn husk, sugarcane bagasses and rice straw). The result showed that corn husk was the best carbon source for xylanase (2.7 Uml⁻¹) and cellulase (0.12 Uml⁻¹) productions. Moreover, factor affecting enzyme productions such as inoculum size, substrate concentration, temperature and initial pH were further investigated. The results indicated that combinations among 1% (vv⁻¹) of inoculum size, 1.5% (wv⁻¹) of substrate concentration, 37°C of temperature and 6.3 of initial pH value were the optimum condition. According to the optimum condition, the highest xylanase (3.01 Uml⁻¹) and cellulase (0.21 Uml⁻¹) activities were detected. In addition, the crude enzymes were partial purification by precipitating with 30-60% (wv⁻¹) of ammonium sulphate. The activities of xylanase and cellulase were increase to be 5.5 and 0.95 Uml⁻¹, respectively.

Key words: Xylanase, Cellulase, Lignocellulosic wastes, Paenibacillus curdlanolyticus B6

บทคัดย่อ

เพื่อศึกษาอิทธิพลของแหล่งคาร์บอนต่อการผลิตไซลาเนสและเซลลูเลสโดย Paenibacillus curdlanolyticus B6 จึงน้าเทคนิคการเพาะขี้ตาไก์ในอาหารที่เติมแหล่งคาร์บอนต่างๆ คือ (เปลือกข้าวโพด ชานอ้อย และ ฟางข้าว) ผลการศึกษาพบว่า เปลือกข้าวโพดเป็นแหล่งคาร์บอนที่สูดสำหรับการผลิตไซลานเซส (2.7 Uml⁻¹) และเซลลูเลส (0.12 Uml⁻¹) หลังจากนั้น ปัจจัยอื่นที่มีผลต่อการผลิตไซลานเซส ได้แก่ ปริมาณเชื้อเริ่มต้น ความเข้มข้นแหล่งคาร์บอน อุณหภูมิ และค่าพีเอชเริ่มต้น ได้ถูกศึกษาเพิ่มเติม ผลการศึกษาทั้งหมดนี้ ทำให้พบว่า ที่ปริมาณเชื้อเริ่มต้น ร้อยละ 1 (vv⁻¹) ความเข้มข้นแหล่งคาร์บอน ร้อยละ 1.5 (wv⁻¹) อุณหภูมิ 37 องศาเซลเซียส และค่าพีเอชเริ่มต้น 6.3 เป็นสภาวะที่เหมาะสมในการผลิตไซลานเซส จากสภาวะทนสมั้นนี้ พบกิจกรรมของไซลานเซส (3.01 Uml⁻¹) และเซลลูเลส (0.21 Uml⁻¹) สูงสุด นอกจากนี้ เมื่อน้าแอมโมเนียมสำเร็จสำเร็จส่วนใหญ่การคัดแบรนเด้ยและولاتในน้ำมันชีพเพลิง ร้อยละ 30-60 พบว่า ปัจจัยประกอบการใช้ข้อมูลของเซลลูเลสที่ขึ้นเป็น 5.5 และ 0.95 Uml⁻¹ ตามลำดับ

Key words: Xylanase, Cellulase, Lignocellulosic wastes, Paenibacillus curdlanolyticus B6

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Introduction

The ability of some microorganisms to metabolize cellulose or hemicelluloses makes them potentially important to take advantage of vegetable residues. Hemicelluloses and cellulose represent more than 50% of dry weight of agricultural residues (Ferreira, 2004). They can be converted into soluble sugars either by acid or enzymatic hydrolysis. Its complete breakdown requires the action of several hydrolytic enzymes with different specificities and mechanisms of hydrolysis (Sunna and Autranikian, 1997 and Beguin et al., 1994). Xylanase and cellulase have widely used for potential biotechnological applications such as a food additive for poultry to increase feed efficiency (Classen, 1996), juice clarification (Kapoor et al., 2001) and hydrolysis of lignocellulosic wastess to valuable products (Demain et al., 2005). The industrial enzyme production is often limited by the cost of substrates for cultivation the producer microorganisms. The use of low cost substrates, such agricultural wastes has been suggested as an alternative to reduce the cost of enzyme production (Hinman, 1994).

*Paenibacillus curdlanolyticus* B6 was reported to be the producer of xylanolytic and cellulolytic enzymes. *P. curdlanolyticus* B6, when grown on mineral salt medium containing 0.5% commercial oat spelt xylan, the xylanolytic-cellulolytic enzyme system produced by this strain is capable of hydrolyzing not only pure insoluble polysaccharides, but also xylan and cellulose present in lignocellulosic substances such as corn hulls (Pason et al., 2006).

In this paper we study the use of lignocellulosic wastes for xylanase and cellulase productions by *P. curdlanolyticus* B6. Furthermore, factors affecting on the enzyme productions such as inoculum size, substrate concentration, temperature and initial culture pH were investigated. In addition, partial purification of enzyme in the culture filtrate was determined.

Materials and methods

Bacterial strain and growth conditions

*Paenibacillus curdlanolyticus* B6 isolated from an anaerobic digester fed with pineapple wastess received from Assoc. Prof. Kanok Ratanakhanokchai, King Mongkut’s University of Technology Thonburi, Bangkok. The bacterium was grown on Berg’s miner salt medium (1972) containing 0.2% NaNO₃, 0.05% K₂HPO₄, 0.02% MgSO₄·7H₂O, 0.002% MnSO₄·2H₂O, 0.002% FeSO₄·7H₂O, 0.002% CaCl₂·H₂O and 0.5% Birch wood xylan or varied percentage of carbon sources. The inoculum was prepared in basal medium containing 1% glucose. The inoculum culture was grown on the same medium for 48 h. and incubated at 37°C and 200 rpm. (Rattithumkul, 1998)

Enzymatic assay

Xylanase and cellulase activities were assayed by measuring the formation of reducing sugar. The enzyme activities were assayed at 50 °C. In case of xylanase activity, the reaction mixture was containing 0.1 M, pH 7.0, Tris-HCl buffer, 0.5 ml of 1% (wv⁻¹) xylan and crude enzyme 0.1 ml to give final reaction volume 0.6 ml for 15 min. One unit of xylanase activity is defined as the amount of enzyme that
released 1 µmol reducing sugar equivalent to xylose per min under the above assay conditions. In case of cellulase activity, it was determined as above but 0.05M, pH 5.0, sodium acetate buffer 0.5 ml of 1% (wv⁻¹) carboxymethyl cellulose was incubated with crude enzyme 0.1 ml for 30 min. One unit of cellulase activity is defined as the amount of enzyme that released 1 µmol reducing sugar equivalent to glucose per min under the above assay conditions.

Chemical analysis

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Enzyme activity (Uml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylanase</td>
</tr>
<tr>
<td>Rice straw</td>
<td>0.1 a</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>1.3 b</td>
</tr>
<tr>
<td>Corn husk</td>
<td>2.7 c</td>
</tr>
</tbody>
</table>

Reducing sugar was qualified by the Somogyi-Nelson method (Nelson, 1944), using xylose or glucose as a standard. Protein concentration was determined by Lowry method (Lowry, 1951).

Substrate preparation

Agricultural wastes; corn husk, sugarcane bagasse and rice straw were chopped into small pieces, dried and ground in a hammer mill. This ground material was then separated by sieves into particles of different sizes.

Results

Xylanase and cellulase productions with different lignocellulosic wastes

P. curdlanolyticus B6 was cultured in medium supplemented with 1.0 (wv⁻¹) of various lignocellulosic wastes to determine the effects of these lignocellulosic wastes (carbon sources) on xylanase and cellulase productions. The results showed that corn husk gave the highest xylanase (2.7 Uml⁻¹) and cellulase (0.12 Uml⁻¹) activities significantly (p<0.05) compared to sugarcane bagasse and rice straw. On the other hand, rice straw gave the lowest enzyme activities (Table 1).

Table 1 Xylanase and cellulase activity obtained from different substrates after 3 days of cultivation of P. curdlanolyticus B6

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Enzyme activity (Uml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylanase</td>
</tr>
<tr>
<td>Rice straw</td>
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<td>1.3 b</td>
</tr>
<tr>
<td>Corn husk</td>
<td>2.7 c</td>
</tr>
</tbody>
</table>

a, b, c means with different letters within each column are significantly different (p ≤ 0.05)

The result suggested that corn husk was the best carbon source for xylanase and cellulase productions. Once, the suitable carbon source was detected. Factors affecting enzyme productions such as inoculum size, substrate concentration, culture temperature and initial culture pH were further investigated.

The effect of inoculum size and substrate concentration on xylanase and cellulase productions

The effect of inoculum size and substrate concentration on the enzyme productions was
determined by three-level full factorial experiments. The levels of substrate concentration were 0.5%, 1.0% and 1.5% (wv⁻¹) and also, three inoculum size levels were 1.0%, 2.0% and 5.0% (vv⁻¹). The result indicated that the highest xylanase activity was 3.01 Uml⁻¹ when supplemented medium with 1.5% (wv⁻¹) of corn husk (Figure 1). Statistical analysis suggested that substrate concentration had significant influence to the enzyme activity (p<0.05) as shown in the Table 2. However, in the case of cellulase activity, the highest enzyme activity was 0.27 Uml⁻¹ when 1% (vv⁻¹) inoculum size and 1% (wv⁻¹) of corn husk were applied (Figure 2).

Effect of temperature on xylanase and cellulase productions

P. curdlanolyticus B6 was grown on mineral medium supplemented with 1.5% (wv⁻¹) corn husk as a carbon source. The bacterium was grown in various temperatures ranging from 30 to 45 °C. The result showed that xylanase and cellulase activities were highly detected at 37 °C of cultured condition. However, the enzyme activities were very low at 45 °C of cultured condition (Figure 2).

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**Figure 1** The highest productions of xylanase (□) and cellulase (■) from *P. curdlanolyticus* B6 by supplemented medium with different amount of corn husk 0.5% (wv⁻¹), 1.0% (wv⁻¹), 1.5% (wv⁻¹) and 1.0% (vv⁻¹) inoculum size.

**Figure 2** The highest productions of xylanase (□) and cellulase (■) from *P. curdlanolyticus* B6 in various temperature culture.

**Figure 3** The highest productions of xylanase (□) and cellulase (■) from *P. curdlanolyticus* B6 in various initial pH value.
Table 2 Analysis of variance for the production of xylanase from *P. Curdlanolyticus* B6

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>F-value in statistic table.</th>
</tr>
</thead>
<tbody>
<tr>
<td>inoculum size</td>
<td>0.105</td>
<td>2</td>
<td>0.052</td>
<td>1.185</td>
<td>4.26</td>
</tr>
<tr>
<td>substrate concentration</td>
<td>1.201</td>
<td>2</td>
<td>0.600</td>
<td>13.608*</td>
<td>4.26</td>
</tr>
<tr>
<td>interaction</td>
<td>0.499</td>
<td>4</td>
<td>0.125</td>
<td>2.826</td>
<td>3.63</td>
</tr>
<tr>
<td>error</td>
<td>0.397</td>
<td>9</td>
<td>0.044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>2.202</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant at 95% of confidence level

Table 3 Analysis of variance for the production of cellulase from *P. curdlanolyticus* B6

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>F-value in statistic table.</th>
</tr>
</thead>
<tbody>
<tr>
<td>inoculum size</td>
<td>0.001</td>
<td>2</td>
<td>0.000</td>
<td>0.924</td>
<td>4.26</td>
</tr>
<tr>
<td>substrate concentration</td>
<td>0.043</td>
<td>2</td>
<td>0.022</td>
<td>59.178*</td>
<td>4.26</td>
</tr>
<tr>
<td>interaction</td>
<td>0.010</td>
<td>4</td>
<td>0.002</td>
<td>6.546*</td>
<td>3.63</td>
</tr>
<tr>
<td>error</td>
<td>0.003</td>
<td>9</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>0.057</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant at 95% of confidence level

Effect of ammonium sulphate concentration on partial purification of xylanase and cellulase

<table>
<thead>
<tr>
<th>Percentage of ammonium sulphate (%)</th>
<th>Enzyme activity (Uml⁻¹)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Xylanase</td>
</tr>
<tr>
<td>0-30</td>
<td>3.49</td>
<td>0.69</td>
</tr>
<tr>
<td>30-60</td>
<td>5.54</td>
<td>0.90</td>
</tr>
<tr>
<td>60-80</td>
<td>2.78</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Ammonium sulphate was added into the culture filtrate to precipitate and concentrate xylanase and cellulase. The percentage of ammonium sulphate tasted was 0-30, 30-60 and 60-80. The result showed that xylanase and cellulase activities were highly detected by precipitating with ammonium sulphate between 30-60% (Table 4).

Table 4 Partial purification of xylanase and cellulase from culture filtrate
Discussion and conclusion

Among the various agricultural residues tested (Table 1), corn husk was the best inducer of xylanase and cellulase activities in *P. curdlanolyticus* B6. The highest induction capacity of corn husk was probably due to the composition of substrate. It was reported that when *Clostridium cellulovorans* was grown on different carbon sources (such, avicel, pectin, xylan or a mixture of all three), the subunit composition of cellulosomal subpopulations and their enzymatic activities varied significantly (Lucina et al., 2006). Previously researches have been shown that 44.5% hemicellulose was the most partly of chemical structure in corn husk (Welch, 1983). The hemicellulose polysaccharides of sugarcane bagasse and rice straw were in the range 20-30% and about 10.2%, respectively (Wadmark, 1984 and Zhu et. al, 2005). As shown in Table 1, the mean of xylanase or cellulase activities was significantly different on various carbon sources and enzyme activities on media supplemented with corn husk were the highest compared to sugarcane bagasse and rice straw (p<0.05). The result suggested that corn husk was the suitable carbon source for xylanase and cellulase productions.

According to the above result, corn husk was applied to study the affecting factor on xylanase and cellulase productions by *P. curdlanolyticus* B6 such as inoculum size, substrate concentration, temperature and initial pH.

The result showed substrate concentration had significantly effect on xylanase or cellulase productions by *P. curdlanolyticus* B6 (Figure 1). The xylanase activity in media supplemented with 1.5% (wv⁻¹) of corn husk was the highest when comparison to 1.0% and 0.5% (wv⁻¹) (Figure 1). The combination of inoculum size and substrate concentration had significantly effect on cellulase production by *P. curdlanolyticus* B6 (p<0.05). The highest cellulase activity was found when 1% (vv⁻¹) inoculum size combined with 1.0% (wv⁻¹) was applied (Figure 1). The result suggested that an increasing of substrate concentration significantly enlarged the production of enzyme production. This result was corresponding to the report of Haltrich et. al (1955), which was found that an increasing of avicel from 1.2 to 3.0 mgml⁻¹ led to an almost 5 fold increasing in xylanase activity. However, larger amount of carbon sources may reduce dissolved oxygen in the media and it would be affect the growth. As shown in figure 1, 1% (vv⁻¹) inoculum size and 1.5% (wv⁻¹) of corn husk was suitable condition for xylanase and cellulase productions from *P. curdlanolyticus* B6.

Once substrate concentration and inoculum size was designed, the bacterium was grown in various temperatures ranging from 30 °C to 45 °C. The highest xylanase and cellulase activities were achieved at 37 °C (figure 2). In contrast, the xylanase and cellulase activities at 45 °C were significantly lower than that at 30 and 37 °C. The optimum temperature for xylanase and cellulase productions was therefore 37 °C.

Finally, initial pH had significantly effect on xylanase and cellulase productions (p<0.05). The highest xylanase and cellulase activities were observed when the bacterium was grown on the initial culture pH of 6.3 (Figure 3). The result was analogous to the reported of Chipeta et. al (1955) which was found that *Aspergillus oryzae* gave the maximum xylanase activity when it was grown on
xylan as a carbon source and initial pH was 6.5. The result was also similar to the reported of Koomnok, 2005 which was found that *Aspergillus* sp. LA1 and *Aspergillus* sp. SA5 gave the maximum cellulase activity when they were grown on cellulose broth at initial pH 6.5, while the optimum pH of *Sporotrichum* sp. was 6.0-6.5.

The maximum xylanase and cellulase activities were highly detected by precipitating with ammonium sulphate between 30-60% (Table 4). Bailey et al (1993) also reported that xylanase and cellulase activities were stabilized by precipitating with ammonium sulphate between 25 and 50%, with full recovery of the xylanase activity of the liquid concentrate.

In conclusion, the results of our report suggested that the feasibility of using the agricultural wastes as an alternative to save costs on the enzyme production.

**Acknowledgement**

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