การหาสภาวะที่เหมาะสมต่อการผลิต Cellulase-free Xylanase จาก alkaliphilic *Bacillus firmus* K-1

ชนิดา เลิศศลารักษ์ [1] กนก รัตนะกนกชัย [2] และ คิน เลย์ คู [3]

มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี บางมด ทุ่งครุ กรุงเทพฯ 10140

บทคัดย่อ

Alkaliphilic *Bacillus firmus* K-1 ผลิต extracellular cellulase-free xylanase ซึ่งมีศักยภาพที่จะนำไปใช้ในการช่วยฟอกสีเยื่อกระดาษเมื่อเพาะเลี้ยงในสูตรอาหารที่มีไซแลนเป็นแหล่งคาร์บอนในสภาวะเป็นด่าง แต่เนื่องจากไซแลนมีราคาแพง ดังนั้นเพื่อลดราคาการผลิตไซลาเนสงานวิจัยนี้จึงทำการศึกษาเพื่อนำวัสดุเหลือทิ้งทางการเกษตรต่างๆ มาใช้เป็นแหล่งคาร์บอนทดแทนไซแลน และศึกษาถึงแหล่งไนโตรเจนที่เหมาะสมต่อการผลิตไซลาเนส โดยทำการเพาะเลี้ยงเชื้อในเครื่องเขย่า จากการทดลองเปรียบเทียบวัสดุเหลือทิ้งทางการเกษตรต่างๆ ได้แก่ ฟางข้าว ซังข้าวโพด ชานอ้อย และรำข้าว พบว่าเปลือกข้าวโพดร้อยละ 1.0 เป็นแหล่งคาร์บอนที่เหมาะสมที่สุดต่อการผลิตไซลาเนส และยูเรียร้อยละ 0.4 เป็นแหล่งไนโตรเจนที่เหมาะสมที่สุดเมื่อเปรียบเทียบกับแอมโมเนียมไนเตรท แอมโมเนียมซัลเฟต โซเดียมไนเตรท และโปตัสเซียมไนเตรท จากสูตรอาหารเหมาะสมที่ได้ให้ค่า specific xylanase activity เท่ากับ 3.0 ยูนิตต่อมิลลิกรัมโปรตีน และค่ากิจกรรมของไซลาเนสเพิ่มขึ้นร้อยละ 62.0 เมื่อเปรียบเทียบกับสูตรอาหารเดิมที่ใช้ไซแลนร้อยละ 0.5 และโซเดียมไนเตรทร้อยละ 0.2 ส่วนเกลือแร่ชนิด $CaCl_2$ $FeSO_4$ และ $MgSO_4$ ไม่มีผลต่อการผลิตไซลาเนส ขณะที่ $CoSO_4$ $ZnCl_2$ และ $CdSO_4$ ยับยั้งการผลิตไซลาเนส

คำสำคัญ : Extracellular Cellulase-free Xylanase / Alkaliphilic *Bacillus firmus* K-1 / สูตรอาหารที่เหมาะสม / การฟอกสีเยื่อกระดาษ / เปลือกข้าวโพด

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[1] *นักศึกษาปริญญาโท สายวิชาเทคโนโลยีชีวภาพ คณะทรัพยากรชีวภาพและเทคโนโลยี*

[2] *รองศาสตราจารย์ สายวิชาเทคโนโลยีชีวเคมี คณะทรัพยากรชีวภาพและเทคโนโลยี*

[3] *ผู้เชี่ยวชาญต่างประเทศ คณะทรัพยากรชีวภาพและเทคโนโลยี*

Optimization of Extracellular Cellulase-free Xylanase Production by Alkaliphilic *Bacillus firmus* K-1

Chanida Leartslarus [1] **Khanok Ratanakhanokchai** [2] **and Khin Lay Kyu** [3]
King Mongkut's University of Technology Thonburi, Bangmod, Toongkru, Bangkok 10140

Abstract

When an alkaliphilic bacterium *Bacillus firmus* K-1 was grown in alkaline xylan medium, pH 10.5, it produced extracellular cellulase-free xylanase that has a potential to use in pulp prebleaching process. Due to the high price of xylan, optimization of media for the extracellular cellulase-free xylanase production by the *Bacillus firmus* K-1 using various agricultural wastes as carbon sources instead of xylan and various nitrogen sources in shaking flask was studied. It was found that 1.0% corn hull was the best carbon source for production of the xylanase when compared with the other agricultural wastes such as rice straw, corn cob, sugarcane bagasse and rice bran. For the nitrogen source, 0.4% urea was found to be the best when compared with ammonium nitrate, ammonium sulfate, sodium nitrate and potassium nitrate. The optimized alkaline medium gave the specific xylanase activity of 3.0 U/mg protein, resulting 62.0% increase of the activity when compared to that obtained in the alkaline mineral salt medium with 0.5% xylan and 0.2% sodium nitrate. The mineral salts in the form of $CaCl_2$, $FeSO_4$ and $MgSO_4$ had no effect on xylanase production whereas $CoSO_4$, $ZnCl_2$ and $CdSO_4$ inhibited the production of xylanase.

Keywords : Extracellular Cellulase-free Xylanase / Alkaliphilic *Bacillus firmus* K-1 / Optimization of Medium / Pulp Prebleaching / Corn Hull

[1] *Graduate Student, Division of Biotechnology, School of Bioresources and Technology.*
[2] *Associate Professor, Division of Biochemical Technology, School of Bioresources and Technology.*
[3] *Expert, School of Bioresources and Technology.*

1. Introduction

Recently, interest has been shown in the production of cellulase-free xylanase for application in the pulp and paper industry. The conventional bleaching of pulps with chlorine causes environmental pollution due to the formation of chlorinated organic compounds in the effluents. The use of xylanase in a prebleaching treatment reduced the amount of chlorine needed to attain the target brightness. After enzyme treatment on kraft pulps, lignin-residual may then be trapped and extracted out more easily due to the decreased degree of polymerization of the matrix formed by xylans. Therefore, a significantly lower amount of chlorine was expected to be needed in bleaching process. Used of xylanolytic enzymes in bleaching process have been reported to be able to decrease the consumption of chlorine, AOX (adsorbable organic halogen), COD (chemical oxygen demand) and improve waste water. Furthermore, after prebleaching pulp with xylanolytic enzymes, the bleaching time with chemical was reduced and paper brightness and strength were increased [1]. In Thailand, sugarcane bagasse and eucalyptus are being used in manufacturing of kraft pulp in the pulp and paper industry. Therefore, it is of interest to use the enzymes in prebleaching of kraft pulp in Thailand.

In our previous study, it has been reported that the alkaliphilic *Bacillus firmus* K-1 (formerly known as *Bacillus* sp. K-1), isolated from the wastewater treatment plant of pulp and paper industry in Ayutthaya province, has a potential to be used in the pulp prebleaching process [2]. The bacterium produced extracellular cellulase free xylanolytic enzymes that were two types of xylanases, β-xylosidase, arabinofuranosidase and acetyl esterase, when grown in an alkaline xylan medium. The enzyme was active and stable in alkaline pHs up to 12. Two types of xylanases were difference in molecular weights (23 and 45 kDa) and the ability in binding to insoluble xylan. The low molecular weight, 23 kDa, xylanase had a strong affinity to insoluble xylan. The crude enzyme could hydrolyze xylan in corn hull, sugarcane bagasse and eucalyptus wood directly, without any pretreatment of those agriculture products and it hydrolyzed xylan in pulps especially sugarcane bagasse and eucalyptus pulps [3][4]. Eventhough the crude enzyme can be used without purification in prebleaching of kraft pulps, the production cost of the enzyme was high because xylan, a sole source of carbon in the production of the enzyme, is expensive (4,494 bahts/100g when 1$ = 45 baht). Agricultural wastes which are inexpensive and abundant, are more attractive to be used as carbon sources for the production of enzyme. As Thailand is an agricultural country, there are more than 45 million tons/year of agricultural wastes (Statistic of agricultural wastes in Thailand in the agricultural year of 1996/1997 and FAO Agricultural Services Bulletin, 1982). This paper describes the optimization of media with some agricultural wastes are used for the production of the extracellular cellulase-free xylanase by *Bacillus firmus* K-1.

2. Materials and methods

2.1 Microorganism

Alkaliphilic *Bacillus firmus* K-1 was isolated from the wastewater treatment plant of pulp and paper industry in Ayutthaya province, Thailand [2].

2.2 Culture medium

The bacterium was grown on Berg's mineral salts medium [5] containing 0.200% $NaNO_3$, 0.050% K_2HPO_4, 0.020% $MgSO_4.7H_2O$, 0.002% $MnSO_4.H_2O$, 0.002% $FeSO_4.7H_2O$ and 0.002% $CaCl_2.2H_2O$ with agricultural wastes or xylan, as carbon sources. After sterilization, pH of the media were initially adjusted to 10.5 with 1% Na_2CO_3.

2.3 Preparation of carbon sources

Agricultural wastes such as sugarcane bagasse, corn hull, corn cob, rice straw and rice bran were ground to 40 mesh and washed with hot water for several times to remove the residual sugars in those substances before dried in an oven.

2.4 Xylanase production

The inoculated cells of $1.6x10^8$ CFU/ml was cultivated in Berg's mineral salt medium supplemented with 0.5% xylan. The culture was incubated in the rotary shaking incubator at 200 rpm and 37 ^oC. The samples were taken at every 12 h. After 72 h, all samples were centrifuged at 4 ^oC and crude enzyme was assayed for xylanase activity.

2.5 Carbon source

For the effect of different carbon sources on the enzyme production, 0.5% xylan was replaced by 1.0% each of the agricultural wastes, corn hull, corn cob, sugarcane bagasse, rice straw and rice bran.

2.6 Nitrogen source

The effect of nitrogen sources was assessed by replacing sodium nitrate with various nitrogen sources such as potassium nitrate, urea, ammonium nitrate and ammonium sulphate.

2.7 Mineral salt

In the culture medium, various trace elements and mineral salts were also used. Various mineral salts, $CaCl_2$, $CdSO_4$, $CoSO_4$, $FeSO_4$, $MgSO_4$ and $ZnCl_2$, were tested for the production of the enzyme.

2.8 Xylanase assay

Assay mixture consisted of 0.15 ml of enzyme solution and 0.15 ml of 0.5% oat spelt xylan in 0.1 M Tris-HCl buffer, pH 9.0. After incubation for 10 min at 50 °C, reducing sugars-released were determined by the dinitrosalicylic acid method [6] using xylose as a standard.

One unit of the enzyme (U) was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min.

2.9 Determination of protein

Protein concentration was determined followed the method of Lowry *et al.*, [7].

3. Results and Discussion

3.1 Effect of carbon source

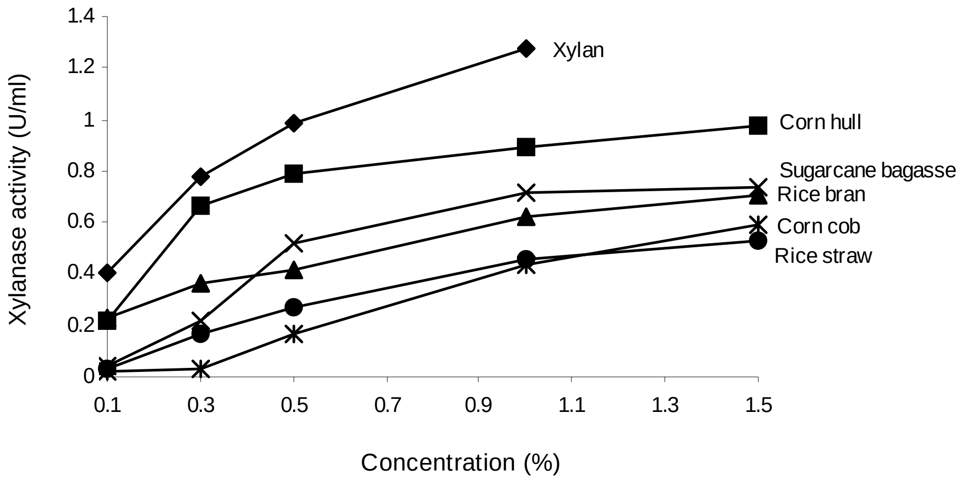

Fig. 1 Production of xylanase by *B. firmus* K-1 using various carbon sources in the different concentrations. (All datae were the maximum activities of each concentration.)

As shown in Fig. 1, *Bacillus firmus* K-1 could grow on the agricultural wastes which were corn hull, sugarcane bagasse, rice bran, corn cob and rice straw and produced xylanase within 72 h. Singh *et al.*, [8] found that 14.9, 11.3, 22.8 and 15.0 IU/ml of xylanase were produced by a hyperxylanolytic mutant of *Fusarium oxysorum* in 120 h when agricultural wastes, rice straw, sugarcane bagasse, wheat bran and wheat straw, respectively, were used and Biswas *et al.*, [9] also reported that when *Aspergillus ochraceus* that was grown on wheat bran, wheat straw and sugarcane bagasse as carbon sources by solid-state culture, produced 28.1, 24.4 and 18.6 U/ml of xylanase and 8.1, 5.1 and 5.1 U/ml of β-xylosidase, respectively. Xylanase production of *B. firmus* K-1 increased when all carbon sources increased due to the increasing of cell growth (datae not shown) (Fig. 1). That was similar to the result of Haltrich *et al.*, [10] which was found that when 2.5-fold increasing in the concentration of avicel (from 1.2 to 3.0 mg/ml) led to an almost 5-fold increasing in xylanase activity. However, xylanase activity increased slightly from 1.0% to 1.5% of all carbon sources. Larger amount of carbon sources may reduce dissolved oxygen in the medium and it would affect the growth. Singh *et al.*, [8] also reported that when *Fusarium oxysporum* NTG-19 was grown on wheat bran, xylanase and β-xylosidase production were increased from 1.0 to 3.0%. However, those two enzymes did not increased when wheat bran concentration was increased to more than 3.0%.

Corn hull was found to be the best carbon source when compared with other tested agricultural wastes. It may be due to the hemicellulose (xylan) content and structural differences of xylan between the agricultural wastes [11]-[13]. In our previous study, 0.5% xylan was normally used as the carbon source and when compared with the agricultural wastes [2][3]. Fig. 1 shows that 0.9 U/ml xylanase was produced in medium contained 1.0% corn hull which was comparable to that in medium contained 0.5% xylan (0.99 U/ml).

Time course of the enzyme production by *Bacillus firmus* K-1 using various agricultural wastes as carbon sources were shown in Fig. 2. Rapid production of the xylanase was found in 12 h when cultivated on corn hull and some could be detected in the medium with sugarcane bagasse but slower production of xylanase appeared in 24 h when grown on 0.5% xylan and rice bran. At 24 h, the enzyme production in 1% corn hull was approximately 1.7 times higher than that in 0.5% xylan, although the production in the former one was slightly lower in 36 h.

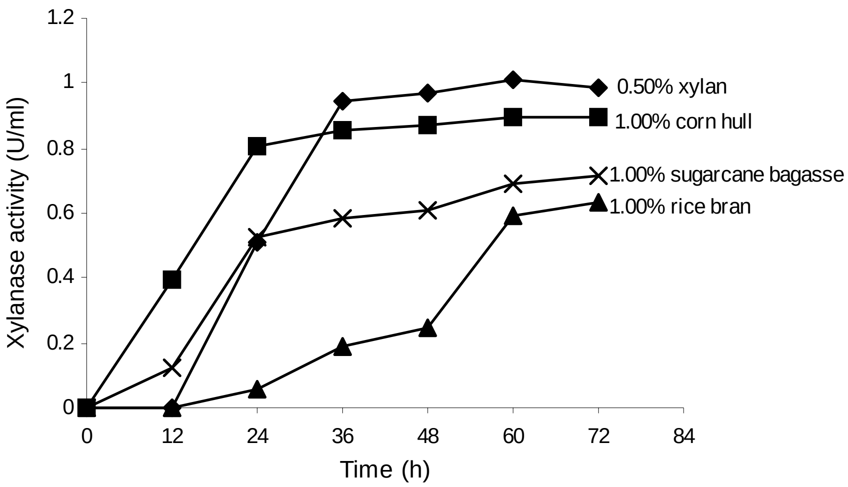

Fig. 2 Time course of xylanase production by *B. firmus* K-1 using various carbon sources.

3.2 Effect of nitrogen source

To study the effect of nitrogen source on the production of xylanase using corn hull as the carbon source, various nitrogen sources such as ammonium nitrate, ammonium sulphate, urea, sodium nitrate and potassium nitrate were tested.

Fig. 3 shows that the best concentrations of each nitrogen source for the production of xylanase were 0.4% urea, 0.2% ammonium nitrate, 0.1% ammonium sulfate, 0.4% sodium nitrate and 0.6% potassium nitrate and the xylanase activities were detected at 1.60, 1.44, 1.31, 0.89 and 0.40 U/ml, respectively. However, the use of 0.4% urea and 0.2% ammonium nitrate was found to give higher xylanase than that of other nitrogen sources in the bacterial culture with corn hull as the carbon source.

Liu *et al.*, [14] found that ammonium sulfate was the best nitrogen source for the production of xylanase in *Trichosporon cutaneum* SL409. As shown in Fig. 3, when the concentration of ammonium nitrate and ammonium sulfate were up to 0.4%, very low xylanase production was found. It may be due to the accumulation of some chemical that may toxic to cells. Leejeerajumnean *et al.*, [15] reported that 0.5 M ammonia inhibited growth of *Bacillus pasteurii* and *Bacillus pumilus*. As shown in Table 1, the production and the specific activity of xylanase with urea was found to be the highest among the other nitrogen sources so that 0.4% urea was the best nitrogen source for the production of xylanase.

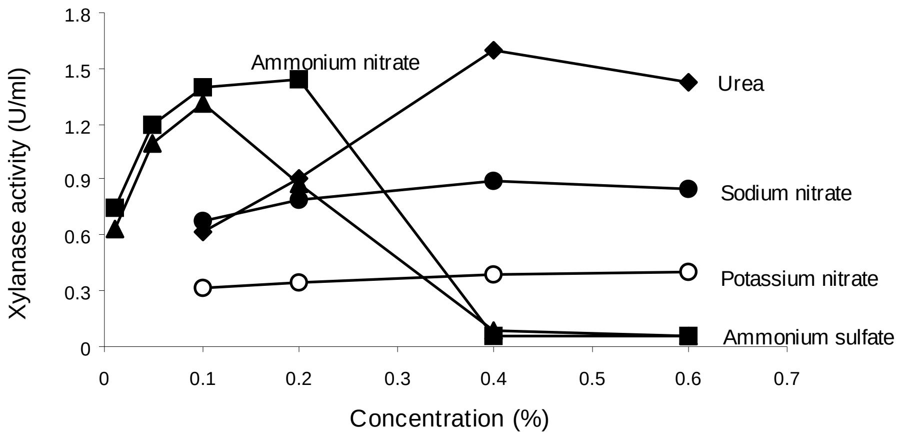

Fig. 3 Effect of nitrogen sources on the production of xylanase from *B. firmus* K-1, using corn hull as the carbon source. (All datae were the maximum activities of each concentration.)

Table 1 Effect of nitrogen sources on growth and xylanase production by *B. firmus* K-1 grown on 1.0 % corn hull.

Nitrogen source	Protein (mg/ml)	Xylanase (U/ml)	Specific activity (U/mg)
1. Urea (0.4%)	0.54	1.60	2.96
2. Ammonium nitrate (0.2%)	0.55	1.44	2.62
3. Ammonium sulphate (0.1%)	0.56	1.31	2.34
4. Sodium nitrate (0.4%)	0.43	0.89	2.07
5. Potassium nitrate (0.6%)	0.25	0.40	1.60

The studies on the effect of carbon and nitrogen sources on production of the xylanase revealed that the optimized alkaline mineral salt medium contained 1.0% corn hull and 0.4% urea was the best one for the xylanase production. The enzyme produced in such medium was 1.60 U/ml (3.0 U/mg protein) which was 62.0% increased when compared with the medium containing 0.5% xylan and 0.2% sodium nitrate which was 0.99 U/ml.

The cost of xylanase production that is important for application in an industry, could be 225 times reduced by using the corn hull (xylan = 4,494 bahts/100 g, corn hull = 20 bahts/100 g).

3.3 Effect of mineral salts

In our previous studies, it was found that Fe^{2+}, Ca^{2+} and Mg^{2+} enhanced the xylanase activity [4] and Co^{2+}, Zn^{2+} and Cd^{2+} increased the adsorption of xylan-binding xylanase on insoluble xylan [16]. Therefore, the effect of those metal ions on the production of xylanase were examined and the results have been shown in Table 2.

Table 2 Effect of mineral salts on the production of the xylanase.

Mineral salt	Relative rate of production of xylanase (%)
Control	100
$CaCl_2$ (0.01%)	100
$FeSO_4$ (0.01%)	100
$MgSO_4$ (0.10%)	91
$CoSO_4$ (0.01%)	15
$ZnCl_2$ (0.01%)	12
$CdSO_4$ (0.01%)	0

Control = Berg's mineral salt medium [5]

The addition of 0.01% $CoSO_4$, $ZnCl_2$ and $CdSO_4$ inhibited the production of xylanase while $MgSO_4$ showed slightly inhibitory effect, too. Calcium chloride ($CaCl_2$) and $FeSO_4$ did not effect on the production. This result was similar to the result of Haltrich *et al.*, [17] which was reported that changing of concentrations of mineral salt, $MgSO_4$ has no effect on the production of xylanase by *Sclerotium rolfsii*.

4. Conclusion

The production of xylanase by the alkaliphilic *Bacillus firmus* K-1 was conducted using agricultural wastes as carbon sources in shaking flask. The agricultural wastes, corn hull, corn cob, sugarcane bagasse, rice straw and rice bran, were used for the enzyme production. The results showed that among the others, 1.0% corn hull was the best to be used as carbon source. For the source of nitrogen, 0.4% urea was better than the others. The bacterium cultured with 1.0% corn hull and 0.4% urea produced xylanase with high specific activity of 3.0 U/mg protein. The use of corn hull and urea resulted in 62.0% increase in xylanase activity over the value obtained with 0.5% xylan and 0.2% sodium nitrate. Mineral salts, $CaCl_2$, and $FeSO_4$ had no effect on xylanase production but $CoSO_4$, $ZnCl_2$ and $CdSO_4$ inhibited the production of xylanase and $MgSO_4$ showed slightly inhibition, too. This work indicated that corn hull which is available as an agricultural waste in large quantity, can be used as a carbon source under alkaline condition for xylanase production and considered to reduce the production cost. Production of xylanase by *B. firmus* K-1 in fermentor with this optimized medium will be further studied.

5. Acknowledgement

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