Optimization of Culture Medium and Conditions for α-L-Arabinofuranosidase Production by *Streptomyces* sp. PC22

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Low-cost carbon and nitrogen sources for the production of α-L-arabinofuranosidase (AFase), one of the accessory enzymes required for complete degradation of xylan, by *Streptomyces* sp. PC22 was investigated. Maximum AFase activity of 0.85 U ml^{-1} was obtained when cultivated for 2 days at 45°C in the presence of 3%(w/v) wheat bran and $(NH_4)_2SO_4$ equivalent to 0.1% (w/v) nitrogen content at an initial medium pH 10 whereas a maximum activity of only about 0.2 U ml^{-1} was obtained when grown in 1% (w/v) commercial oat-spelt xylan and polypeptone equivalent to 0.05% (w/v) nitrogen content at an initial pH 9 under the same cultivation conditions. The crude AFase displayed optimal activity at 60°C and pH 6. It was stable at temperatures up to 60°C and to a broad pH ranges from 5-9.

Key words: α-L-Arabinofuranosidase, Enzyme production and *Streptomyces*

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องค์ประกอบอาหารเลี้ยงเชื้อและภาวะเหมาะสมในการผลิตแอลฟา-แอล-อะราบิโนฟิวราโนสิเดสโดย *Streptomyces* sp. PC22

วิชุตา เหล่าเรืองธนา และ ไพเราะ ปิ่นพานิชการ (2549)
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ผลการหาแหล่งคาร์บอนและไนโตรเจนราคาถูกและภาวะที่เหมาะสม สำหรับ *Streptomyces* sp. PC22 ในการผลิตแอลฟา-แอล-อะราบิโนฟิวราโนสิเดส (AFase) ซึ่งเป็นหนึ่งในกลุ่มเอนไซม์จำเป็นต่อการย่อยสลายไซแลนอย่างสมบูรณ์ พบว่าเชื้อนี้สามารถผลิต AFase ได้สูงสุด 0.85 หน่วยต่อมิลลิลิตร เมื่อเลี้ยงเป็นเวลา 2 วัน ที่ 45 องศาเซลเซียส โดยมีรำข้าวสาลี ร้อยละ 3 (น้ำหนักต่อปริมาตร) และ $(NH_4)_2SO_4$ ที่ความเข้มข้นเทียบเท่ากับไนโตรเจน ร้อยละ 0.1 (น้ำหนักต่อปริมาตร) ที่ pH เริ่มต้นของอาหารเลี้ยงเชื้อเท่ากับ 10 ขณะที่สามารถผลิตเอนไซม์นี้ได้สูงสุดเพียงประมาณ 0.2 หน่วยต่อมิลลิลิตร เมื่อเลี้ยงโดยใช้ 1% (น้ำหนักต่อปริมาตร) ไซแลน ร้อยละ 1 (น้ำหนักต่อปริมาตร) จากเปลือกข้าวโอ๊ตที่ได้จากทางการค้าและพอลิเพบโทนที่ความเข้มข้นเทียบเท่ากับไนโตรเจน0.05% (น้ำหนักต่อปริมาตร) ที่ pH เริ่มต้นของอาหารเลี้ยงเชื้อเท่ากับ 9 ภายใต้ภาวะเดียวกัน ผลการศึกษาสมบัติเบื้องต้นของเอนไซม์หยาบ พบว่าให้แอคติวิตีสูงสุดที่ 60 องศาเซลเซียส pH 6 เอนไซม์มีความเสถียรต่ออุณหภูมิได้สูงถึง 60 องศาเซลเซียส และเสถียรต่อ pH ในช่วงกว้างตั้งแต่ 5-9

คำสำคัญ แอลฟา-แอล-อะราบิโนฟิวราโนสิเดส การผลิตเอนไซม์ *Streptomyces*

INTRODUCTION

Xylan is the major constituent of hemicellulose which is the second most abundant renewable polysaccharide in nature after cellulose. It consists of a backbone of β-1,4-linked xylopyranose and side chains of α,L-arabinofuranoside, an acetyl group, and/or 4-O-methyl glucuronic acid at the C-2 and C-3 positions of the xylose units.(1) Due to the complexity of xylan structure, its complete degradation requires the cooperative action of several enzymes which include xylanase and β-xylosidase, the backbone hydrolyzing enzymes, and debranching enzymes such as α,L-arabinofuranosidase (AFase), acetyl esterase (AE) and α-glucuronidase.(2-4)

AFase catalyzes the hydrolysis of α-1,2 and α-1,3-L-arabinofuranosidic bonds in hemicellulose like arabinoxylan commomly found in softwood,(5) arabinan and other L-arabinose containing polysaccharides. Recently, AFases have received much attention because of their practical applications in various biotechnological processes, such as the efficient conversion of hemicellulosic biomass to simple fermentable sugars for subsequent production of fuels and chemicals, delignification of pulp, efficient utilization of plant materials for animal feeds, and hydrolysis of grape monoterpenyl glycoside during wine fermentation.(6)

We previously reported xylan degrading enzymes from *Streptomyces* spp. isolated from soil in Thailand, these included xylanase from *Streptomyces sp*. PC22(7) and β - xylosidase from *Streptomyces sp*. CH7.(8-9) Later study found that *Streptomyces sp*. PC22, capable of growing at pH 9 and 45°C, produced at least 2 xylanases which were active at high temperature and stable to a broad range of pH.(10,11) It is therefore worth studying AFase, one of the accessory enzymes for xylan degradation, from this organism. In the present work, we describe optimization for AFase production by *Streptomyces sp*. PC22 using low-cost carbon and nitrogen sources including preliminary characterization of the crude enzyme properties.

MATERIALS AND METHODS

Microorganism and culture conditions

Streptomyces sp. PC22 was isolated from soil collected from a sugar cane field in Thailand using humic acid-vitamin agar medium adjusted to pH 9 and an incubation temperature of 45°C as isolation conditions as previously reported.(7) It was maintained as spore suspension prepared according to Kieser *et al*.(12) at -20°C.

Cultivation was by preinoculating 100 μl of spore suspension (approx. 2×10^8 spores ml^{-1}) into 30 ml Tryptic Soy Broth (TSB) medium adjusted to pH 9 and incubating at 45°C with shaking at 200 rpm until mid-log phase was reached at about 9 hr. It was used as a seed culture. For enzyme production, 10% (v/v) of the seed culture was inoculated into 30 ml of complex medium consisting of (w/v) 0.5% polypeptone equivalent to 0.05% nitrogen content as determined by Kjeldahl method using Buchi 425 Digester and Buchi 315 Distillation Unit, 0.1% yeast extract, 0.4% K_2HPO_4, 0.02% KCl, 0.1% $MgSO_4.7H_2O$ and 0.002% $FeSO_4.7H_2O$ with carbon source as indicated in the results and adjusting the pH of the medium to 9 or as otherwise indicated in the results. Incubation was at 45ºC with 200 rpm-shaking for 2 days or otherwise indicated. Culture supernatant obtained after removing the mycelium by centrifugation at 10,000 rpm for 10 min was used for enzyme assays. For studying the effects of temperature and pH on activity and stability of the enzyme, the crude enzyme was prepared by precipitating the culture supernatant with ammonium sulfate at 80% saturation and dialyzed twice against 100 mM phosphate buffer, pH 6.0 and finally against 25% glycerol in the same buffer.

Carbon sources

Xylan from oat spelts, birchwood, beechwood and arabinose were from Sigma Chemical Co. (USA). Agricultural by-products including rice straw, rice bran, wheat bran, corn cob, corn hulls and cottonseed hulls were obtained locally. Cottonseed hulls were prepared by soaking 10 g of cottonseed hulls in 100 ml of 1% NaOH solution at room temperature for 1 hr to remove lignin then washed thoroughly with water, dried and ground. Rice straw, corn cob and corn hulls were dried at 60°C for 48 hr milled and passed through a 60-mesh screen.

Inorganic nitrogen sources

The effects of inorganic nitrogen sources on the enzyme production were assessed by replacing polypeptone in the complex medium with various inorganic nitrogen salts; NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and KNO_3 at the concentration equivalent to 0.05% (w/v) nitrogen content or as otherwise indicated in the results.

Enzyme assays

AFase was assayed at 65°C as described by Manin *et al.*[13] using 1 mM *p*-nitrophenyl α-L-arabinofuranoside as a substrate in a 100 mM sodium phosphate buffer at pH 6. Incubation time was for 10 min and the released *p*-nitrophenol was quantitatively determined from the absorbance at 405 nm using *p*-nitrophenol as a standard. One unit of the enzyme was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per min under the assay conditions.

Effects of temperature and pH on enzyme activity

AFase was determined under standard conditions except at different temperatures as indicated in the results. Enzyme activities were then determined at different pHs from 4 to 9 at the optimal temperature.

Effects of temperature and pH on enzyme stability

The enzyme solutions were preincubated in the absence of substrate at different temperatures or pHs for 30 min, thereafter the residual activities were determined under the optimal conditions.

Data presentation

All results presented are the average of three replicates.

Table 1. Effect of carbon source on AFase production by *Streptomyces* sp. PC22.

Carbon source (1%, w/v)	AFase (U ml^{-1})
xylan, oat spelt	0.20
xylan, birchwood	0.12
xylan, beechwood	0.01
arabinose	0.30
rice straw	0.02
rice bran	0.01
wheat bran	0.13
corn cob	0.07
corn hulls	0.09
cottonseed hulls	0.02

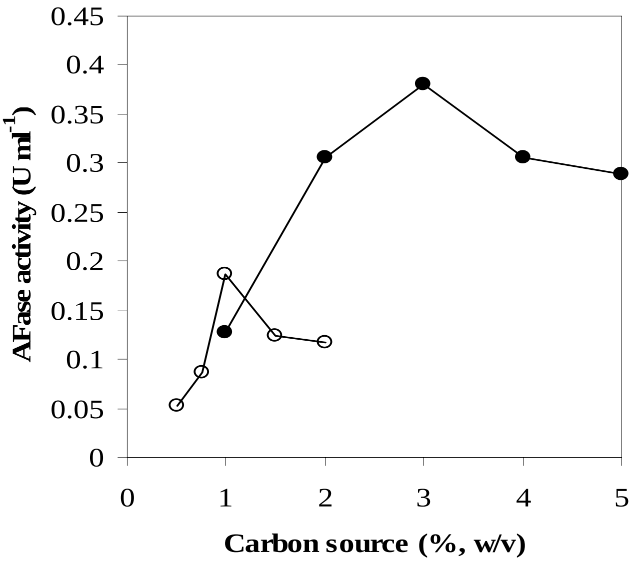

Figure 1. Effect of concentrations of oat-spelt xylan (○) or wheat bran (●) on AFase production from 2 day-culture.

RESULTS AND DISCUSSION

Effect of various carbon sources on AFase production

Time courses of AFase production by *Streptomyces* sp. PC22 were preliminarily determined using 1% (w/v) oat-spelt xylan as a carbon source. The AFase activity reached the maximum level on day 2 of cultivation (data not shown). To determine the effect of various carbon sources on the production of this enzyme, cultivation time of 2 days was therefore used. As shown in Table 1, arabinose, a simple sugar molecule, could induce fairly high level of AFase production which was similar to those observed for AFase production by *Aureobasidium pullulans*[14] and by *Fusarium oxysporum*.[15] However, with xylan as a carbon source, oat-spelt xylan which contains α,L-arabinofuranosyl residue at O-3 position of xylose backbone at every 8-10 xylose molecules[16] was the best inducer. Among the various agricultural by-products wheat bran having high arabinoxylan content,[17] was the best carbon source.

In order to use wheat bran, which is cheap and abundantly available, as a carbon source to replace expensive commercial xylan and arabinose for the enzyme production, the effects of carbon source - concentrations were then comparatively studied between wheat bran and xylan. At the optimal substrate concentrations, AFase production with wheat bran was about 2 fold higher than with oat-spelt xylan (Figure 1).

Time courses of the enzyme production when *Streptomyces* sp. PC22 was grown on xylan or wheat bran at their optimal concentrations were also determined. With either substrate, the maximum enzyme production was reached at the same cultivation period as shown in Figure 2.

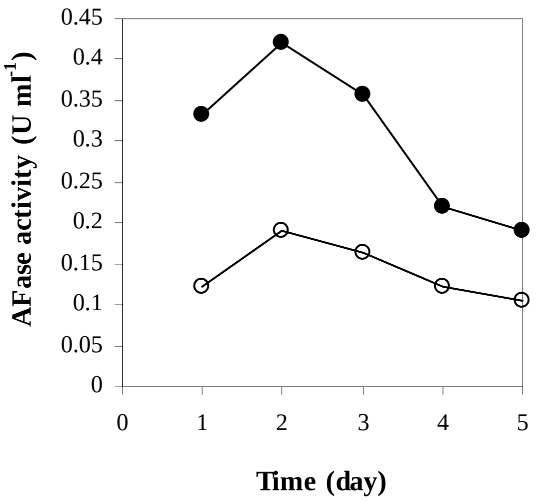

Figure 2. Time courses of AFase production with 1% oat-spelt xylan (○) or 3% wheat bran (●) as a carbon source.

Table 2. Effect of inorganic nitrogen salts compared with polypeptone at the final concentration equivalent to 0.05% (w/v) nitrogen content on AFase production by *Streptomyces* sp. PC22 with oat-spelt xylan or wheat bran as a carbon source cultivated for 2 days.

Nitrogen source	AFase (U ml^{-1})	
	1% oat-spelt xylan	3% wheat bran
polypeptone	0.20	0.40
NH_4Cl	0.09	0.29
NH_4NO_3	0.23	0.26
$(NH_4)_2SO_4$	0.11	0.41
$(NH_4)_2HPO_4$	0.15	0.35
KNO_3	0.03	0.23

Effect of various inorganic nitrogen sources

To further reduce the cost of enzyme production, replacing the expensive commercial polypeptone in the culture medium with cheaper and commonly available compounds such as various inorganic nitrogen salts was attempted. From the results shown in Table 2, NH_4NO_3 and $(NH_4)_2SO_4$ were selected for AFase production with oat-spelt xylan and wheat bran as a carbon source, respectively. The results in Figure 3 indicated that using wheat bran as a carbon source produced substantially higher activity of the enzyme as compared with that of xylan in which about 2.5 fold higher activity was observed at the optimal concentrations of inorganic nitrogen. These findings suggested that agricultural by-products and inorganic nitrogen salts could replace expensive commercial xylan and polypeptone for the efficient production of AFase. This medium was then chosen for further optimization.

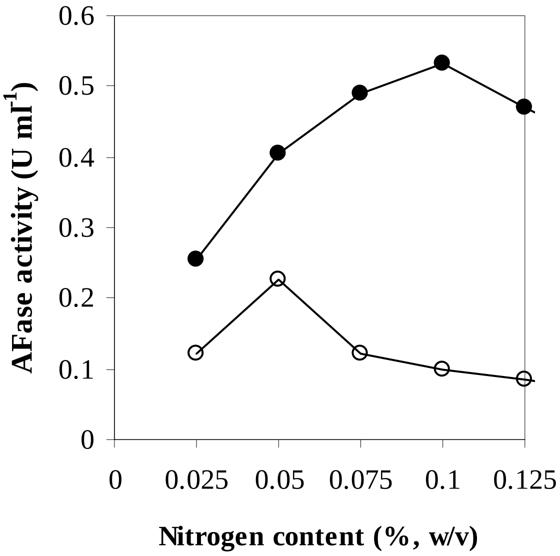

Figure 3. Effect of concentrations equivalent to nitrogen content of NH_4NO_3 with oat-spelt xylan as carbon source (○) or $(NH_4)_2SO_4$ with wheat bran as carbon source (●) on AFase production.

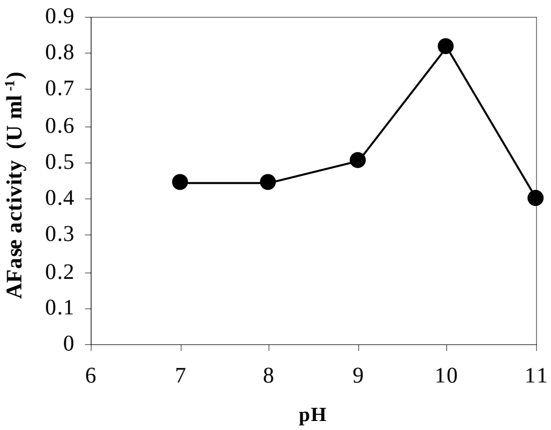

Figure 4. Effect of initial medium pH on AFase production with wheat bran and $(NH_4)_2SO_4$.

Effect of initial medium pH

The initial pH of the medium for the production of AFase by *Streptomyces* sp. PC22 growing on wheat bran in the presence of $(NH_4)_2SO_4$ was optimized. As shown in Figure 4, the optimal initial pH was 10. This might be due to the fact that at higher pH and at a high cultivation temperature of 45°C, the wheat bran was partially hydrolyzed and liberated arabinose which was shown to be the best inducer for AFase production (Table 1).

The results presented here demonstrated that *Streptomyces* sp. PC22 produced AFase at higher levels of about 0.85 U ml^{-1} when grown on wheat bran and $(NH_4)_2SO_4$ under the optimal conditions compared with those reported by others, such as 0.067 U ml^{-1} from *Penicillium chrysogenum* grown on mixed carbon sources of glucose and sugar beet pulp using both peptone and NH_4NO_3 as nitrogen sources(18) and 0.117 U ml^{-1} from *Streptomyces chartreusis* GS901 grown on arabinan and $(NH_4)_2HPO_4$.(19)

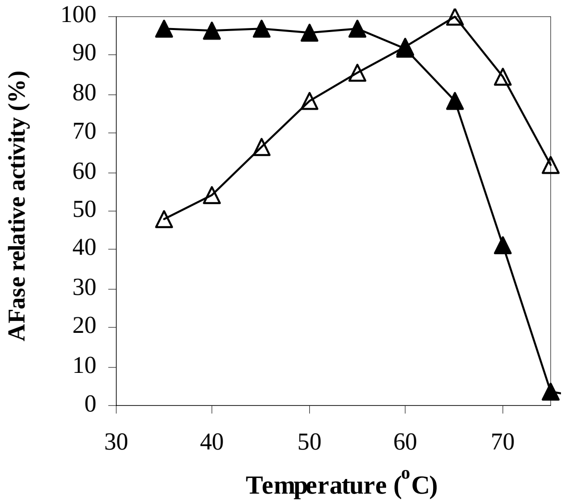

Figure 5. Effect of temperature on activity (△) and stability (▲) of AFase.

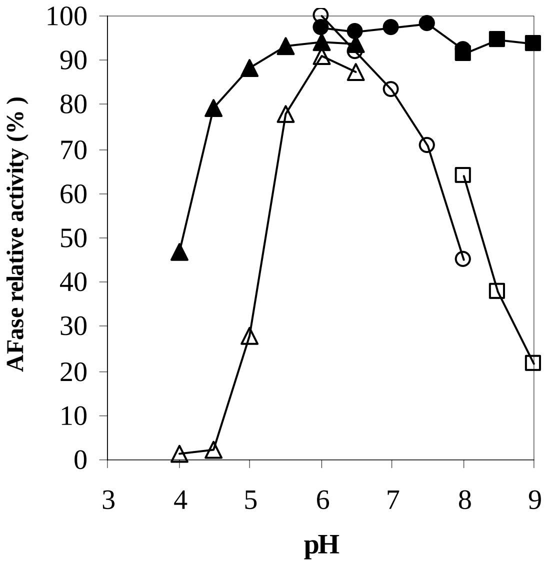

Figure 6. Effect of pH on activity (△, ○ and □) and stability (▲, ● and ■) of AFase. Sodium acetate buffer (△ and ▲); sodium phosphate buffer (○ and ●); and tris-HCl buffer (□ and ■).

Effects of temperature and pH on activity and stability of the crude enzymes

The crude preparation of AFase displayed optimal activity at a temperature of 65°C and optimal stability at a temperature of 60°C (Figure 5). It had a higher optimal temperature than AFase I (55°C) and AFase II (50°C) from *Streptomyces chartreusis* GS901 and was also more thermal stable than both enzymes which were reported to be stable up to 45°C for AFase I and 40°C for AFase II.[19] The optimal pH for AFase activity was 6.0 and it was stable to a broad pH range between 5 and 9 (Figure 6). However, at pH lower than 5, its activity decreased sharply whereas slow decrease in activity was found at higher pH. This observation was in agreement with the moderate alkaliphillic property of this organism.[7]

CONCLUSIONS

The present work demonstrated that high levels of AFase activity could be produced by *Streptomyces* sp. PC22 grown on low cost agricultural by-products and inorganic nitrogen sources. The enzyme was active at considerably high temperature and it had both high thermal and broad range of pH stability. The findings suggested that this enzyme will be suitable for industrial applications in food and feed processing including saccharification of hemicellulosic residues which are renewable resources to fermentable sugars for the production of fuel ethanol as well as other useful fermentation products.

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