Biosci. Biotechnol. Biochem., **63** (1), 226–228, 1999

Note

Citric Acid Production from Xylan and Xylan Hydrolysate by Semi-Solid Culture of *Aspergillus niger*

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Received July 29, 1998; Accepted October 5, 1998

Citric acid production from xylan and xylan hydrolysate was done by *Aspergillus niger* Yang no. 2 cultivated in a semi-solid culture using bagasse as a carrier. Yang no. 2 produced 72.4 g/*l* and 52.6 g/*l* of citric acid in 5 d from 140 g/*l* of xylose and arabinose, respectively. Yang no. 2 produced 51.6 g/*l* of citric acid in 3 d from a concentrated xylan hydrolysate prepared by cellulase treatment, containing 100 g/*l* of reducing sugars. Moreover, Yang no. 2 directly produced 39.6 g/*l* of citric acid maximally in 3 d from 140 g/*l* of xylan.

Key words: *Aspergillus niger*; citric acid; hemicellulose; xylan; xylose

Today, citric acid is mainly produced from molasses or starch hydrolysates by a fermentation process with the filamentous fungus *Aspergillus niger*.[1–4] Although the demand for citric acid used in the world exceeds 500,000 tons/yr, it is provided at a low price as a commodity chemical. Therefore, it is important to establish a method for the production from inexpensive and readily available raw materials in industrial processes.[1,2,5,6]

From this view-point, we are studying the possibility of citric acid production from what is called plant biomass; this contains large amounts of cellulosic and starchy materials but is mainly scrapped without use as agricultural waste, especially in Southeast Asian countries. At present, direct production of citric acid from cellulose has not yet succeeded, since *A. niger* usually cannot degrade nor assimilate crystalline cellulose as a sole carbon source. However, we confirmed that citric acid production from cellobiose[6] and cellulose hydrolysate[7] is possible, especially the production from cellulose hydrolysate gave a high conversion yield of 68.2% in semi-solid culture.[7]

Usually, lignocellulosic biomass contains approximately 15–35% hemicellulose.[8] Although no attempt has been made so far to produce citric acid from xylan nor xylan hydrolysate, we considered that such studies would be necessary to help in understanding the potentiality of *A. niger* for the citric acid production from plant biomass. On the other hand, since xylan itself is insoluble in water, a semi-solid cultivation method seems to be suitable for the production. In this note, we report citric acid production from xylan and xylan hydrolysate in a semi-solid culture of *A. niger*.

For citric acid production in semi-solid culture, *A. niger* Yang no. 2,[1] a hyperproducer of citric acid in semi-solid culture, was used. A basal synthetic medium solution[9] was used, containing (per liter of distilled water): NH_4NO_3, 2 g; KH_2PO_4, 10 g; $MgSO_4 \cdot 7H_2O$, 250 mg; $FeCl_3 \cdot 6H_2O$, 20 mg; $MnSO_4$, 14 mg. As a carbon source, 140 g/*l* of xylose, arabinose, xylooligosaccharides (2.9% xylose, 67.6% xylobiose, and 29.1% xylotriose; Wako Pure Chemical Ind., Osaka), or xylan (Sigma, St. Louis, MO, U.S.A.) from oat spelts, was used. Since the solubility of xylan in water is low, xylan was suspended in the basal medium solution and used. Xylan hydrolysate was obtained by enzymatic treatment of xylan 50 g/*l* in 50 mM sodium citrate buffer (pH 4.5) at 45°C containing a commercial cellulase, Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd., Tokyo) produced by *Trichoderma viride*, 5 g/*l* for 72 h, since this commercial enzyme contains a large amount of xylanase. After centrifugation for removal of unhydrolyzed xylan, the supernatant was used as a carbon source of medium for citric acid production. When the xylan hydrolysate was used as a carbon source, other components such as supplementary mineral nutrients of a basal synthetic medium solution were dissolved in the xylan hydrolysate to give each concentration. The pH of all the media was initially adjusted to 4.25.

Citric acid production in semi-solid culture was done basically as described previously.[6,9] Conidia of Yang no.2 were suspended in the medium solution at a concentration of 1×10^6/ml. Cultivation was started by adding 1 ml of the conidial suspension to the semi-solid medium, consisting of 15 ml of the basal synthetic medium solution with each carbon source and 3.9 g sugar cane bagasse as a carrier, in a Petri dish (9 cm in diameter) after sterilization in an autoclave and cooling. All cultivation was done at 30°C. After an appropriate cultivation time, the cultivated samples in the Petri dishes were taken out and broken into pieces, and 200 ml distilled water at 40°C was added. The mixture was vigorously stirred for 15 min, and filtered through Whatman GF/A with suction. The filtrate was then used as the extract of the semi-solid culture for measurement of the amounts of citric acid, reducing sugars, etc., and the activity of xylanase.

The amount of citric acid in the culture filtrate was roughly measured by titration with 0.1 M NaOH against

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phenolphthalein, used as an indicator. After this titration, analysis of the organic acids and measurement of citric acid were done by an HPLC system (Shimadzu Scientific Instruments Ltd., Kyoto) as described previously.[10] When xylan hydrolysate was used as a carbon source, the media initially contained citric acid derived from the citrate buffer. Therefore, the initial concentration of citric acid was subtracted from the total amount of citric acid after the cultivation, and the value calculated is shown as the amount of citric acid produced. The amounts of xylose, xylooligosaccharides, arabinose, and glucose were also measured by an HPLC system as described previously.[6] The amounts of xylooligosaccharides were calculated as the sum total of the amounts of xylobiose and xylotriose. Total amounts of reducing sugars were measured by a colorimetric method at 540 nm using dinitrosalicylic acid reagent.[11] In this study, reducing sugars in xylan hydrolysate were measured using xylose as a standard.

Xylanase activity was measured by the method of Morosoli *et al.*[12] with some modifications. The reaction mixture (1 ml) containing 0.9 ml of a 1% (w/v) suspension of xylan in 30 mM acetate buffer (pH 4.5) and 0.1 ml of appropriately diluted culture filtrate was incubated at 40°C for 30 min. The reaction was stopped by immersing the tube in a boiling water bath for 5 min. The amounts of reducing sugars released in the reaction mixture were measured by the Somogyi-Nelson method with some modifications[13] using xylose as a standard. The blank was prepared using the culture filtrate after inactivation of enzyme by boiling. The units of xylanase activity were defined as the amount (μmol) of reducing sugars released per minute.

First, to examine abilities of *A. niger* Yang no. 2 to produce citric acid from pentose, production tests from xylose and arabinose, the main constituents of xylan, were done as a prerequisite to production of citric acid from xylan. As shown in Fig. 1, Yang no. 2 produced 72.4 g/*l* and 52.6 g/*l* of citric acid in 5 d from 140 g/*l* of xylose or arabinose as sole carbon sources, respectively, with yields of 51.7% and 37.6%, respectively, based on sugars supplied. Both the amount of sugar consumption and the productivity of citric acid for arabinose were lower than that for xylose. As shown in Fig. 1(A), the productivities from these saccharides were lower than that from glucose, and higher than that from cellobiose. The cultivation period of 5 d gave the highest production for xylose and arabinose, and this was 3 d for glucose and cellobiose. The differences of such fermentation periods for each saccharide might be explained by the difference of metabolic pathways: arabinose and xylose as pentoses are metabolized through the HMP pathway, but glucose and the cellobiose component (β-glucose), being hexose, go through the EMP pathway.

When 140 g/*l* of xylooligosaccharides were used as sole carbon sources, xylanase activity (8.11 unit/ml at 3 d) was detected and 72.0 g/*l* of citric acid was produced in 5 d (details not shown). Xylanase activity for cultivation with xylooligosaccharides was two times as high as that with xylose, and the yield of citric acid from xylooligosaccharides was on the same level as that from

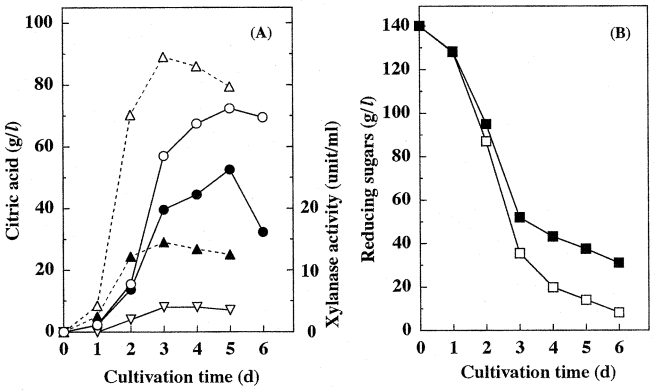

Fig. 1. Citric Acid Production and Sugar Consumption from Xylose and Arabinose.

The amounts of citric acid (A) produced from xylose (○) and arabinose (●), and the amounts of reducing sugars (B) for xylose (□) and arabinose (■) are shown. The amounts of citric acid (A) produced from glucose (△) and cellobiose (▲) are also shown with broken lines. Xylanase activity which detected for xylose are also shown (▽).

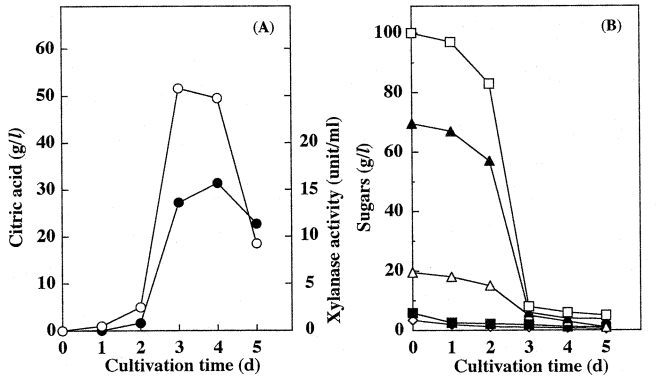

Fig. 2. Citric Acid Production and Sugar Consumption from Xylan Hydrolysate.

Xylan hydrolysate contained 100 g/*l* of reducing sugars. The amounts of citric acid (○) and xylanase activity (●) are shown (A), and the amounts of reducing sugars (□) and each sugar in the hydrolysate: xylose (▲); glucose (△); arabinose (■); and xylooligosaccharides (◇), are shown (B).

xylose. These results indicate that xylooligosaccharides, but not xylose, induced the xylanase of *A. niger* Yang no. 2 is semi-solid culture.

Therefore, we tried citric acid production from the xylan hydrolysate prepared by enzymatic treatment. Approximately 95% of xylan was hydrolyzed mainly to xylose (33.8 g/*l*), with a little xylooligosaccharides (1.67 g/*l*), glucose (9.42 g/*l*), and arabinose (2.84 g/*l*); the concentration of reducing sugars was 48.3 g/*l*.

When the hydrolysate containing 48.3 g/*l* of reducing sugars was used as a carbon source, the amount of citric acid produced maximally at 3 d reached only 15.0 g/*l*. Since usually such a high concentration of sugars is used for efficient production of citric acid,[1-6] we concentrated the hydrolysate on a rotary evaporator to make 100 g/*l* of reducing sugars and used this for the production. The concentrated hydrolysate contained 69.6 g/*l* of xy-

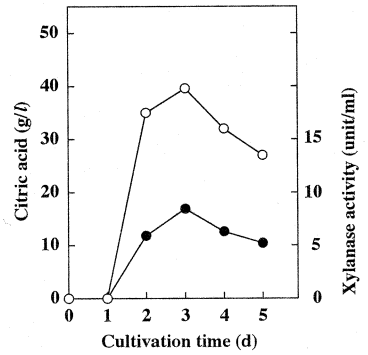

Fig. 3. Direct Production of Citric Acid from Xylan.
The amounts of citric acid (○) and xylanase activity (●) are shown.

lose, 3.46 g/*l* of xylooligosaccharides, 19.5 g/*l* of glucose, and 5.87 g/*l* of arabinose. As shown in Fig. 2, Yang no. 2 produced 51.6 g/*l* of citric acid for 3 d from 100 g/*l* of reducing sugars supplied, and a high xylanase activity, maximally 15.7 unit/ml at 4 d, was detected. The yield of citric acid produced from the concentrated hydrolysate based on reducing sugars supplied was at the same level as that from xylose. Reducing sugars such as xylose, xylooligosaccharides, glucose, and arabinose were comsumed for 3 d, and citric acid productivity reached a maximum at 3 d.

Since Yang no. 2 was confirmed to produce xylanase, direct production of citric acid from 140 g/*l* of xylan was tried. The courses of citric acid production and xylanase activity are shown in Fig. 3. Xylanase activity was detected after 2 d and reached a maximum level of 8.45 unit/ml at 3 d, and maximally 39.6 g/*l* of citric acid was produced at 3 d. Citric acid productivity from xylan was increased with xylanase activity.

Although there have been some reports on citric acid production from cellulose hydrolysate,[7,14,15] there have been no reports on citric acid production from hemicellulose nor xylan hydrolysate. To our knowledge, this is the first paper showing such hyper-production of citric acid from xylan and xylan hydrolysate. In this study, we used oat spelts xylan as a model of hemicellulose. And, from the practical view-point, the cost for enzymatic hydrolysis of xylan and for concentration of the hydrolysate must be lowered. However, we consider that xylan is useful for citric acid production as a carbon source together with the cellulose fraction of lignocellulosic materials, and that the results shown in this note would be useful for development of a new system to produce citric acid from the cellulosic materials of plant biomass.

Maddox *et al.*[16] reported that *A. niger* produced only 22 g/*l* and 6 g/*l* of citric acid from 100 g/*l* of xylose and arabinose, respectively, after long cultivation periods of 14 d in flask culture. However, in this study, the yields from xylose and arabinose reached 51.7% and 37.6%, respectively, in 5 d, and Yang no. 2 used all sugars in the medium containing xylan hydrolysate. These characteristics of semi-solid culture with Yang no. 2 are suitable for citric acid production from xylan and xylan hydrolysate.

Furthermore, considering that the productivity of citric acid from xylooligosaccharides and xylan hydrolysate was higher than that from xylan, it must be possible to increase the yield of citric acid directly produced from xylan through more efficient saccharification of xylan and an increase of xylanase production by the citric-acid producing *A. niger* itself or expression of some heterologus xylanase-encoding gene in the *A. niger* strain.

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