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Yam bean starch: a novel substrate for citric acid production by the protease-negative mutant strain of *Aspergillus niger*

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Abstract

Selection of protease-negative mutant strains of *Aspergillus niger* in semi-solid culture was carried out in order to enhance citric acid production from yam bean. The protease-negative mutants were obtained by UV-irradiation of the parental strain Yang no. 2. Using a halo-selection medium, a number of mutants with decreased extracellular protease activity were selected. Citric acid productivity by the selected mutant strains was tested on a modified starch–methyl red agar plate. The best mutant strain YW-112 was obtained and produced 106 g/l of citric acid, whereas the parental strain Yang no. 2 produced 58 g/l, from 140 g/l of soluble starch in semi-solid culture at 5 days of cultivation time. During the whole period of cultivation with soluble starch, the extracellular glucoamylase activity of YW-112 was higher than that of the parental strain Yang no. 2. In addition, when 17 g/plate of rod-shaped yam bean was used instead of soluble starch, the protease-negative mutant strain YW-112 produced 490 g/l of citric acid, which is approximately 1.5 times as much citric acid as Yang no. 2 produced and showed enhanced extracellular glucoamylase production.

Keywords: Protease-negative mutant; *Aspergillus niger*; Semi-solid culture; Yam bean; UV-irradiation; Extracellular glucoamylase production

1. Introduction

Citric acid is widely used in the food, pharmaceutical, cosmetic and beverage industries. The entire world-wide demand for citric acid is met by fermentative production, mainly by methods involving the filamentous fungus *Aspergillus niger* (Usami and Fukutomi, 1977; Usami, 1978; Crueger and Crueger, 1984; Steinboch et al., 1991). Since citric acid is a commodity chemical, it is necessary to use inexpensive and readily available raw materials in industrial production processes (Taketomi and Usami, 1960; Usami, 1978). For the production of citric acid from starch, Ozaki et al. (1957) and Kawahara and Matsukubo (1956) reported that the preliminary hydrolysis of raw starch was necessary due to the low saccharification ability of citric acid-producing strains of *A. niger*. Similar results were also reported by Taketomi et al. (1959) and Taketomi and Usami (1960) who investigated the optimal conditions for the preliminary saccharification for citric acid production. In practice, starch hydrolysate generally gives a low yield of citric acid (Usami and Fukutomi, 1977; Usami, 1978; Steinboch et al., 1991). However, if direct production of citric acid from starchy material were possible, pretreatment processes such as liquefaction and saccharification could be omitted, and as a consequence the energy requirements and cost of citric acid production would be reduced.

Yam bean contains large amounts of amylopectin, a branched and water-insoluble polysaccharide as compared with starch prepared from other plant sources such as maize (corn), wheat and rice. Therefore, we considered that yam bean may be one of the suitable substrates for the production of citric acid in Thailand since it is cheap and renewable especially in the Northeastern part of Thailand. In the previous data, we found that 2-deoxy-D-glucose (DG)-resistant mutant strain of *A. niger* showed enhanced extracellular glucoamylase activity and also exhibited enhanced citric acid production from starch (Suzuki et al., 1996). Moreover, we also previously reported the effect of protease activity on extracellular glucoamylase level in *A. niger* (Sarangbin et al., 1994). The addition of protease inhibitor, pepstatin, decreased protease activity and thereby indirectly increased glucoamylase activity, by reducing its degradation (Sarangbin et al., 1994). In addition, glucoamylase activity from the

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filamentous fungus *Aspergillus awamori* var. *kawachi* was improved by the induction of protease- and glycosidase-negative mutant strains (Fukuda et al., 1992).

Here, we report on the citric acid production from yam bean by the protease-negative mutant strains in semi-solid culture. The percentage yields of citric acid from yam bean reached 58–74%.

2. Materials and methods

2.1. Microorganisms

A. niger Yang no. 2 (Kirimura et al., 1992; Sarangbin et al., 1993; Suzuki et al., 1996), a hyper-producer of citric acid in semi-solid culture, was used as the parental strain. The medium used was potato dextrose agar (PDA).

2.2. Media

For citric acid production, a synthetic (SS) medium (Sarangbin et al., 1993) 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, was used. The SS media containing either glucose or soluble starch at 140 g/l as a sole carbon source were sterilized at 120°C for 20 min. The pH was initially adjusted to 4.25. All cultivations were carried out at 30°C. The selection medium consisted of Czapek's medium containing 30 g/l of soluble starch as a sole carbon source and methyl red, being solidified with 2% (w/v) agar. The pH was initially adjusted to 6.0. As a pH indicator, 1% (w/v) methanol solution of methyl red was added to a part of the sterile medium to a final concentration of 20 ppm.

2.3. Induction and isolation of mutants

A 9-day conidial cultivation of *A. niger* Yang no. 2 on PDA medium was subjected to treatment with UV-light as described previously (Sarangbin et al., 1993). After UV-irradiation, 10 ml of conidial suspension was inoculated into 50 ml of Czapek's medium containing 30 g/l of casein (Wako Pure Chemicals, Japan) as a sole carbon source in a 500-ml Sakaguchi flask, and cultivated aerobically with shaking. After 24 h, the broth was filtered on sintered glass filter with a pore size of 20–30 μm to remove germlings, which are supposed to be high protease-producing strains. The conidial suspension, after passing through the sintered glass filter, was suspended in distilled water at an appropriate concentration, and then spread on the glucose–agar medium, which consisted of SS medium containing 140 g/l of glucose, solidified with 2% (w/v) agar, pH 4.25. The colonies appearing during 36-h cultivation were picked up, purified by a single-colony isolation method, and maintained on PDA agar slants. Primary screening of protease-negative mutant strains was carried out by plating the mutants individually onto casein medium (casein, 5 g; casamino acid, 0.05 g; Na_2HPO_4, 1.07 g; KH_2PO_4, 0.36 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; $ZnCl_2$, 0.14 g; $CaCl_2$, 2 mg; $FeSO_4 \cdot 7H_2O$, 2 mg; agar, 20 g; and distilled water, 1000 ml). Mutants with significantly smaller halos (clear zones) than those of the parental strain were selected and cultured on PDA medium that contained 0.1% (w/v) yeast extract and 0.1% (w/v) casamino acid. Spores of the selected mutants were inoculated and maintained on PDA agar slants. Consequently, these mutants were used for selection of high citric acid-producing strains.

2.4. Selection of citric acid-hyperproducing mutants from starch

Selection was carried out according to the method of Rugsaseel et al. (1993) with some modifications. The selection criteria were divided into two sections as follows.

First, selection of strains showing higher amylolytic activity was done on the modified starch–methyl red agar plate. Conidia from each strain were inoculated onto the centre of agar plate. Triplicate plates were prepared for each strain and incubated at 30°C. Amylolytic activity of each strain on the agar plate was determined by measuring the ratio of clear zone diameter, formed after flooding with 0.1% (w/v) iodine solution, to that of the colony.

Second, selection of strains showing higher citric acid productivity was also done on the same modified starch–methyl red agar plate. In parallel experiments, the acid productivity of each strain on the agar plate was determined by measuring the ratio of acid zone diameter, indicated by methyl red, to that of the colony.

Strains showing significant increases in both amylolytic activity and acid production were selected and subjected to tests for citric acid production in semi-solid culture.

2.5. Preparation of yam bean for citric acid production

Yam bean (*Pachyrrhizus erosus* (L.) Urban) was obtained from the local market. The washed yam bean was peeled, cut into rod shape (0.3 × 4.5 cm), cubic shape (0.5 × 0.5 cm) and dried at 60°C for 5 h.

2.6. Citric acid production

Citric acid production in semi-solid culture was essentially done as described previously (Kirimura et al., 1992). When glucose and soluble starch were used, SS medium containing these saccharides was prepared. Conidia of each strain were suspended in the medium solution at a concentration of 1×10^6/ml. Cultivation was initiated by adding 1 ml of the conidial suspension to the sterile semi-solid medium with 3.9 g sugar cane bagasse as a carrier, in a Petri dish (9 cm in diameter). When yam bean was used, the following modifications were made. For the semi-solid

culture, yam bean at 14, 17 and 20 g/plate of both rod and cubic shape were mixed with 15 ml basal SS solution (pH 4.25) and autoclaved. After cooling, 1 ml of conidial suspension was added to the semi-solid medium, and cultivation was initiated. After appropriate cultivation time, the samples in cultivated plates were broken into pieces, and 200 ml distilled water at 60°C was added. For enzyme assay, only distilled water at 30°C was added to prevent enzyme inactivation. The mixture was then vigorously stirred for 15 min, and filtered through Whatman GF/A with suction. The filtrate was finally used as the semi-solid culture extract for measurement of citric acid and residual sugar as well as glucoamylase and α-amylase activities.

2.7. Analysis of organic acids

Citric acid in the culture filtrate was measured enzymatically using a test kit (Boehringer Mannheim, Germany). To determine whether or not the other by-product organic acids such as oxalic acid were produced, the culture filtrate was analyzed with an HPLC system (Shimadzu, Kyoto) equipped with an LC-6A pump, an RID-6A refractive index detector, and a SCR-101N column (7.9 × 300 mm), using pure water as a mobile phase with the flow rate of 0.6 ml/min at 40°C. The mycelial dry weight was not measured since it was impossible to separate the mycelia from the bagasse.

2.8. Assay of glucoamylase

The glucoamylase activity was measured according to the method of Abe et al. (1988) with some modifications. The reaction mixture (1 ml) containing 0.8 ml of 2% (w/v) soluble starch in 50 mM acetate buffer (pH 5.5) and 0.2 ml of appropriately diluted culture filtrate was incubated at 45°C for 15 min. The reaction was stopped by immersing the tube in a boiling water bath for 5 min. The amount of released glucose in the reaction mixture was measured by a glucose C-test kit (Wako Pure Chemicals), using glucose solution as a standard. The blank was prepared using the culture filtrate after inactivation of the enzyme by boiling. One unit (U) of glucoamylase activity was defined as the amount of enzyme liberating 1 μmol of glucose/min under the assay conditions.

2.9. Assay of acid protease

Acid protease activity was measured according to the method of Lovrien et al. (1985) with modifications. The reaction mixture (4 ml) containing 3 ml of 1% (w/v) azocasein (Sigma, USA) in 20 mM citrate–phosphate buffer (pH 5.5) and 1 ml of appropriately diluted culture filtrate was incubated at 37°C for 30 min. The reaction was stopped by addition of 12% (w/v) trichloroacetic acid, and the precipitate was removed by filtration on Whatman no. 1 filter paper. The absorbance of the reaction mixture was measured at 366 nm. One unit of acid protease activity was defined as the amount of enzyme causing an increase of 0.01 A_{366}/min under the assay conditions.

2.10. Assay of α-amylase

α-Amylase activity was measured using the mono-test [a] α-amylase test kit according to the instructions of the manufactures (Boehringer Mannheim).

2.11. Measurement of reducing sugar

For measurement of the total reducing sugars in the culture filtrate, the colorimetric method using dinitrosalicylic acid reagent (DNS) was employed at 540 nm (Miller, 1959).

3. Results

3.1. Selection of protease-negative mutant strains with enhanced glucoamylase activity and acid production

A comparison of amylolytic enzyme, α-amylase and glucoamylase, activities between the mutant strains and parental strain was performed. The results indicated that both the mutant strains and the parental strain Yang no. 2, showed only negligible α-amylase activity, therefore, we evaluated

Table 1
The variability (in %) of amylase and acid production levels for the mutant strains of *A. niger* in comparison with those for the parental strain on a modified starch–methyl red agar plate

Characterization[a]	Relative (%)			No. of mutant strains (total)
	$R < 1$	$R = 1$	$R > 1$	
Amylase[b]	31.0	17.0	52.0	299
Acid[c]	21.6	12.0	66.4	128
Both amylase and acid[d]			28.4	85

[a]When r is defined as the zone diameter ratio of amylase or acid to that of the solid medium, the R value is the ratio (r for the mutant strain)/(r for the parental strain). The actual values for r are shown in Table 2.
[b]A total of 299 mutant strains selected as described in the text was examined as to amylase productivity.
[c]A total of 128 mutant strains showing $R > 1$ for amylase production was examined as to acid productivity.
[d]The relative percentage to a total of the initial 299 mutant strains is shown.

Table 2
Amylase and acid production by the mutant strains of *A. niger* on the modified starch–methyl red agar plates and those in semi-solid culture[a]

Strain	On the plate[b]		In semi-solid culture[c]	
	Zone ratio of amylase	Zone ratio of acid	Glucoamylase (U/ml)	Citric acid (g/l)
Parent				
Yang no. 2	1.2 (0.3)	2.9 (0.6)	12 (1.1)	58 (5.7)
Mutant				
YW-60	2.1 (0.5)	3.8 (0.3)	22 (3.4)	102 (8.4)
YW-79	2.0 (0.6)	3.7 (0.7)	18 (3.1)	97 (7.2)
YW-99	2.0 (0.5)	3.9 (1.1)	20 (3.7)	99 (5.9)
YW-112	2.1 (0.4)	4.1 (0.9)	28 (3.6)	106 (7.7)
YW-125	2.0 (0.4)	3.6 (1.2)	20 (2.9)	99 (10.2)

[a]Means and standard deviations ($n = 3$, in parentheses) are given.
[b]On the modified starch–methyl red agar plates, the diameters (ϕ) in mm were measured for zones of amylase and acid. Zone diameter ratio of amylase is shown as the value of (ϕ amylase zone)/(ϕ colony) and that of acid as the value of (ϕ acid zone)/(ϕ colony). All measurements were done on the peak day of each zone ratio during 3–5 days.
[c]Cultivation was performed for 5 days in semi-solid culture.

the amylolytic activity as the glucoamylase activity (data not shown).

Mutants with reduced levels of extracellular protease were isolated by screening for loss of halo production on a casein medium. A total of 299 protease-negative mutant strains were tested for their abilities to produce glucoamylase and acid on modified starch–methyl red agar plates in comparison with the parental strain Yang no. 2. During 3–5 days cultivation, we recorded the maximum zone ratios of glucoamylase and acid for all the strains tested. The mutant strains were classified according to the relative values (R) of their zone diameter ratios (As shown in Table 1). Since the R value of the zone diameter ratio of the parental strain was approximately 1.2 and 2.9 for glucoamylase activity and acid production, respectively, therefore, the mutants with the R value higher than that of the parental strain were supposed to be important from the viewpoint of glucoamylase activity and acid production from soluble starch. Based on this criterion, the results in Table 1 show that among the total of 299 mutant strains only 128 (52%) mutant strains showed increased glucoamylase activity ($R > 1$). Further analysis indicated that 85 (64%) mutant strains out of the 128 mutant strains showed increased acid production. Therefore, only 85 (28.4%) of the total mutant strains showed the increase in both glucoamylase activity and acid production.

3.2. Glucoamylase and citric acid productivities of the selected mutants in semi-solid culture

For the production test in semi-solid culture, further selection of mutant strains were carried out. Among the 85 mutants, only the mutants whose zone diameter ratios of glucoamylase and acid were beyond 2.0 and 4.0, compared to the parental strain Yang no. 2 of 1.2 and 2.9, respectively, were selected. As shown in Table 2, the production levels of glucoamylase and citric acid in semi-solid culture with soluble starch showed a correlation with the zone diameter ratios of glucoamylase and acid on plates. Therefore, as expected, all of the selected mutant strains showed significant increased glucoamylase and citric acid productivity in semi-solid culture. The best protease-negative mutant strain, YW-112, produced 106 g/l citric acid, whereas Yang no. 2 produced only 58 g/l from 140 g/l of soluble starch after 5 days of cultivation. In addition, no by-product organic acids besides citric acid were detected in the culture filtrate by HPLC analysis. Furthermore, very low level of protease activity was detected in the culture filtrate.

3.3. Citric acid production from yam bean by the mutant strain YW-112

As shown in Table 2, strain YW-112 showed glucoamylase activity and citric acid productivity from soluble starch higher than that of the parental strain Yang no. 2. Our preliminary experiments confirmed that the culture filtrates of Yang no. 2 and the mutant strain YW-112 cultivated with soluble starch in semi-solid culture efficiently liquefied and saccharified yam bean (data not shown). Therefore, the direct citric acid production from yam bean might give a sufficient yield.

Table 3
Citric acid production from rod and cubic shapes at various concentrations of yam bean as the sole carbon source by *A. niger* Yang no. 2[a]

Shapes of yam bean	Amount (g/plate)	Citric acid[b] (g/l)
(1) Rod	14	292 (9.7)
	17	331 (10.5)
	20	316 (9.3)
(2) Cubic	14	166 (9.9)
	17	199 (8.7)
	20	238 (9.8)

[a]Means and standard deviations ($n = 3$, in parentheses) are given.
[b]Amounts of citric acid are shown as the values per milliliter of the medium supplied.

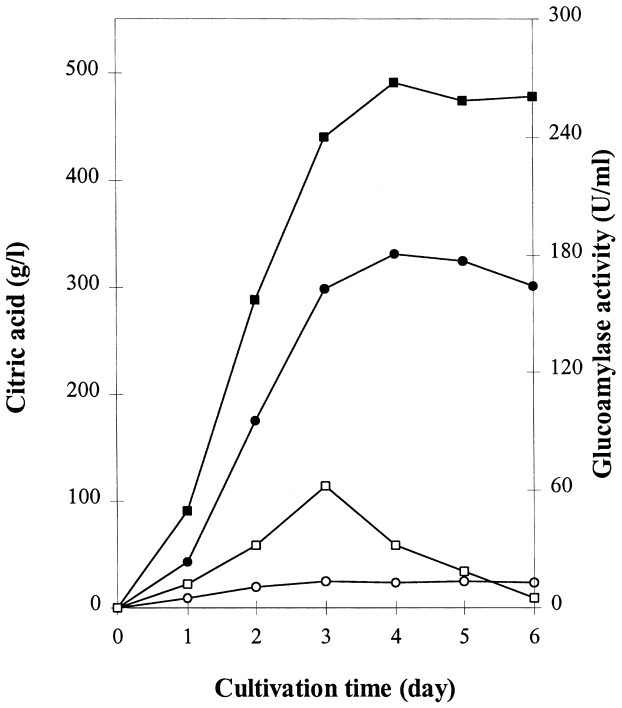

Fig. 1. Time courses of citric acid production by the protease-negative mutant strains of *A. niger*. Amount of citric acid (filled) and extracellular glucoamylase activity (unfilled) are shown. Symbols: (○) Yang no. 2; (□) YW-112.

The maximum citric acid production from rod and cubic shapes at different concentrations of yam bean by *A. niger* Yang no. 2 was studied. The results indicated that rod-shaped yam bean at 17 g/plate gave the maximum citric acid production (Table 3). The results of time courses of citric acid and glucoamylase production by Yang no. 2 and YW-112 with SS medium containing 17 g/plate of yam bean as the sole carbon source are shown in Fig. 1. The maximum amounts of citric acid produced by YW-112 and Yang no. 2 were 490 and 331 g/l, respectively, after 5 days of cultivation. The amount produced by YW-112 was thus approximately 1.5 times higher than that of Yang no. 2. Moreover, based on the supplied carbohydrate, the yield obtained from YW-112 reached 73.7%, whereas that of Yang no. 2 was only 42.8%. Interestingly, the mutant strain YW-112 in semi-solid culture exhibited extracellular glucoamylase activity only after 1 day of cultivation, reaching the maximum of 62 U/ml at 3 days, and thereafter decreased. On the other hand, the parental strain Yang no. 2 showed the maximum extracellular glucoamylase activity after 3 days of cultivation and thereafter did not decrease.

4. Discussion

The protease-negative mutant strains in filamentous fungi are shown to enhance ability to digest raw starch due to the increased glucoamylase activity (Fukuda et al., 1992). From our previous study, addition of protease inhibitor, pepstatin, to the SS medium showed a decrease in protease activity leading to indirectly increased glucoamylase activity (Sarangbin et al., 1994). Therefore, it is interesting to induce the protease-negative mutant of *A. niger* for citric acid production from starchy materials. In this study, we have isolated mutants which are defective in the extracellular protease production. Based on the zone diameter ratios of glucoamylase and acid on the methyl red agar plates, the best protease-negative mutant strain YW-112 was obtained and shown to have enhanced glucoamylase activity as compared to the parental strain Yang no. 2. This may be due to the decrease in glucoamylase degradation by extracellular protease. As a consequence, it is of interest that citric acid production from 140 g/l of soluble starch in semi-solid culture produced 106 g/l citric acid, whereas the parental strain Yang no. 2 produced only 58 g/l (Table 2). This result confirmed that the strain YW-112 provided the maximum glucoamylase activity and citric acid production.

In general, sugar cane bagasse is used as a carrier for citric acid production in semi-solid culture by *A. niger* (Taketomi et al., 1959; Taketomi and Usami, 1960; Usami and Fukutomi, 1977; Fukuda et al., 1992; Sarangbin et al., 1993; Suzuki et al., 1996). In this experiment, when yam bean was used as the sole carbon source, there was no need to add sugar cane bagasse as a carrier in the culture medium (Table 2). This result may imply that yam bean can act as both a carrier and a carbon source. In addition, rod-shaped yam bean was shown to increase citric acid production up to the maximum level of 331 g/l, whereas cubic-shaped yam bean provided only 238 g/l (Table 3). Therefore, the rod-shaped yam bean may act as a better carrier than the cubic one. It is very interesting that citric acid production from 17 g/plate of rod-shaped yam bean by the strain YW-112 was increased up to 490 g/l, which is approximately 1.5 times higher than that of the parental strain Yang no. 2.

In conclusion, we have shown that yam bean is an efficient carbon source as well as a carrier in the culture medium for citric acid production. Since *A. niger* Yang no. 2 and the mutant strains can directly use yam bean, pretreatments such as liquefaction and saccharification therefore are not needed. Moreover, *A. niger* Yang no. 2 was found to produce citric acid from 17 g/plate rod-shaped yam bean more than that of the cubic shape. In addition, the protease-negative mutant strain, YW-112 is a more powerful strain for citric acid production from rod-shaped yam bean as compared to the parental strain Yang no. 2. Glucoamylase activity in YW-112 was found to reach a maximum level after 3 days of cultivation, and thereafter reduced compared to the Yang no. 2, which showed constant activity after 3 days of cultivation. This may indicate that, in YW-112, due to the increase in acidity of the culture filtrate, this resulted in the loss of enzyme activity after 3 days. Another possibility is that glucoamylase in YW-112 may have different forms which are not the same as those of the parental strain Yang no. 2. Therefore, further studies including

characterization of glucoamylase from strain YW-112 should be performed since there may be different types of the enzyme, as has been reported by Fukuda et al. (1992) and Takahashi et al. (1985). Genetic and molecular analysis will be carried out in the near future in order to improve the yield of citric acid production from yam bean for large scale production.

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