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Citric Acid Production by *Aspergillus niger* on Corn Distillers' Grains with Solubles*

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Abstract: Selected strains of the fungus *Aspergillus niger* were screened for their ability to produce citric acid on corn distillers' dried grains with solubles. It was determined that citric acid production by *A. niger* ATCC 11414 or ATCC 201122 after 240 h was higher on untreated grains than on autoclaved grains or acid-hydrolyzed grains. Methanol supplementation to the grains was only capable of stimulating citric acid production by *A. niger* strains ATCC 26550 and ATCC 11414. Biomass production by the selected strains after 240 h on the untreated and treated grains varied according to the strain being studied. The highest yield of citric acid was obtained for ATCC 11414 after it was supplemented with phosphate.

Key words: Citric acid, biomass, *Aspergillus niger*, solid-state fermentation, methanol, phosphate, corn distillers' grains with solubles

INTRODUCTION

Citric acid is a commercially important specialty chemical with global production estimated to be over 900,000 tons (Karaffa *et al*., 2001). Its industrial applications include uses in foods, beverages and pharmaceuticals (Tran *et al*., 1998). Citric acid can serve as a flavor enhancer, a pH regulator, a preservative, a chelating agent, a stabilizer and an antioxidant (Karaffa *et al*., 2001). The production of citric acid by strains of the fungus *Aspergillus niger* from brewery wastes has been investigated (Hang *et al*., 1975, 1977; Roukas and Kotzekidou, 1986). Citric acid can also be produced following growth on pineapple waste (Tran *et al*., 1998), figs (Roukas, 2000), sweet potato (Leangon *et al*., 2000) or cassava bagasse (Vandenberghe *et al*., 2004) using solid-state fermentation. Beet molasses, carobod syrup or corn starch served as other substrates for citric acid fermentation (Clement, 1952; Macris, 1975; Nguyen *et al*., 1992). It has also been shown that the addition of phosphate or methanol can stimulate citric acid production by the fungus (Shu and Johnson, 1948; Moyer, 1953a). It was of interest to learn whether coproducts resulting from ethanol fermentation could be utilized as a substrate for citric acid production by *A. niger* strains. The major coproducts produced during ethanol production from corn are corn distillers' grains and condensed corn solubles. About 18 pounds of 90% corn distillers' grains with solubles are produced from each bushel of corn processed at ethanol plants. Currently, corn distillers' grains with solubles is used as a protein supplement in animal feeds (Ham *et al*., 1994). With more than a million tons of grains being produced from ethanol production per year, the low value grains could be more fully utilized to produce specialty chemicals such as citric acid. Corn distillers' grains with solubles contains fermentable sugars and starch that could be utilized as a carbon source by microorganisms (Moyer, 1953b; Nguyen *et al*., 1992). In this study, selected strains of *A. niger* were screened for their ability to produce citric acid from untreated and treated corn

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distillers' grains with solubles by solid-state fermentation. The effect on citric acid production by the fungal strains following phosphate and methanol supplementation to the untreated corn distillers' grains with solubles was also investigated.

MATERIALS AND METHODS

Strains and Growth Conditions

Three known citric acid-producing strains, namely *Aspergillus niger* ATCC 11414, ATCC 201122 and ATCC 26550 (Perlman *et al*., 1946; Wold and Suzuki, 1976; Gradisnik-Grapulin and Legisa, 1996), were used in this study. When sterilized by autoclaving, the corn distillers' grains with solubles was subjected to 121°C at 17 pounds/square inch of pressure for 20 min. Acid-treated grains were treated with 0.5 to 1.5% H_2SO_4 and autoclaved for 20 min (Dunning and Lathrop, 1945). Following autoclaving, the pH of the acid-hydrolyzed grains was adjusted to 6.0. A loopful of fungal mycelium of the *A. niger* strain was inoculated into potato dextrose broth (20 mL) and the culture was grown for 72 h at 25°C. The 5% inoculum (10^3 conidia/mL) was added to 5 g corn distillers grains (82% moisture) in a sterile 125 mL Erlenmeyer flask and the fungus was grown for 240 h at 25°C. When 0.4% phosphate or 3% methanol was added to each culture, the fungus was also grown for 240 h at 25°C.

Processing of Grains

After 240 h at 25°C, the citric acid present in each solid-state fermentation culture was collected using the following procedure. To each culture, sterile water (25 mL) was added. After shaking each culture for 60 min at 25°C, the grains were filtered through a Whatman No. 1 filter. The fungal biomass in each culture was washed with sterile water (10 mL) and also filtered through a Whatman No. 1 filter. The filtrates from each culture were combined. To precipitate any protein present in each culture filtrate, ice-cold 0.5 N $HClO_4$ (0.5 mL) was added and the filtrate was stirred. Any protein precipitate present was removed. The filtrate was subsequently neutralized to pH 7.0 with 1 N NaOH. The volume of each culture filtrate was recorded.

Citric Acid Assay

The neutralized filtrate was assayed for its citric acid content using a coupled enzyme spectrophotometric assay (Moellering and Gruber, 1966; Henniger and Mascaro, 1985). The modified assay mix (1 mL) contained 0.1 M glycylglycine buffer pH 7.8, 0.2 mM NADH, 0.6 mM $ZnCl_2$, 5 units citrate lyase, 6 units malate dehydrogenase, 3 units lactate dehydrogenase and sample. Citric acid standards were also assayed. The reaction was monitored at 340 nm by following the decrease in absorbance that is proportional to the concentration of citric acid present in the sample. Citric acid levels are expressed as g citric acid/kg grains with solubles. All values represent the mean of three independent determinations involving three separate cultures. The Student's t-test was used during statistical analysis.

Biomass and Reducing Sugar Determinations

To determine biomass production, wet fungal biomass collected after 240 h of growth was placed in a preweighed beaker and dried at 105°C to constant weight. The beaker containing the dry fungal biomass was reweighed to derive the weights for each culture. The weight of the inoculum added to each culture was determined by collection on preweighed filters, drying to constant weight at 105°C and subtracting these weights from the biomass levels determined after 240 h. Reducing sugar levels were determined using a previously described assay where glucose served as the standard

(Dygert *et al*., 1965). Biomass levels are expressed as g cell weight/g grains with solubles while % citric acid yield is given as g citric acid/g reducing sugar consumed×100%. All values represent the mean of three independent determinations involving three separate cultures. The Student's t-test was used during statistical analysis.

RESULTS AND DISCUSSION

It was shown earlier that strains of *Aspergillus niger* excrete citric acid after growth on corn substrates such as ground corn and corn starch (Moyer, 1953b; Nguyen *et al*., 1992). In this study, the ability of *A. niger* ATCC 11414, ATCC 26550 or ATCC 201122 to utilize untreated or treated corn distillers' grains with solubles for citric acid production was investigated. Each of the strains was grown on untreated corn distillers' grains with solubles, autoclaved grains or acid-hydrolyzed grains for 240 h at 25°C. All three strains were capable of producing citric acid on the untreated or treated grains (Table 1). With respect to citric acid production by ATCC 11414 on the untreated grains, the fungus produced 2-fold lower citric acid levels on the autoclaved grains ($p<0.01$) while fungal citric acid production decreased ($p<0.01$) as the acid concentration increased. Citric acid production by ATCC 26550 was slightly lower on the autoclaved grains compared to the untreated grains. Citric acid production by ATCC 26550 increased when acid was used to hydrolyze the grains relative to the citric acid levels produced by the fungus on the untreated grains (Table 1). Compared to the untreated grains, a 2.5-fold or 1.9-fold increase in citric acid production by ATCC 26550 on 0.5% or 1.0% acid-hydrolyzed grains, respectively, was observed (Table 1) where the differences in citric acid production were statistically significant ($p<0.01$). Citric acid production by ATCC 201122 was 1.4-fold higher on the untreated grains compared to the autoclaved grains. As the acid concentration used to hydrolyze the grains was increased (Table 1), citric acid production by ATCC 201122 decreased compared to the untreated grains ($p<0.01$). Since it was reported earlier that low concentrations of phosphate or methanol stimulate citric acid production by *A. niger* (Shu and Johnson, 1948; Moyer, 1953a), either was added to the untreated grains to learn whether citric acid production by the three strains could be stimulated. For ATCC 11414 or ATCC 26550 grown on untreated grains, phosphate addition decreased citric acid production while methanol treatment elevated citric acid production. Citric acid production by ATCC 26550 more than doubled ($p<0.01$) after methanol addition compared to the untreated grains (Table 1). In contrast, phosphate or methanol supplementation reduced citric acid production by ATCC 201122 by at least 1.8-fold ($p<0.01$) compared to the untreated grains (Table 1). It appeared that stimulation of *A. niger* citric acid production by phosphate or methanol addition was strain dependent.

Prior studies have examined citric acid production by *A. niger* using solid-state fermentation (Tran *et al*., 1998; Roukas, 2000; Leangon *et al*., 2000; Vandenberghe *et al*., 2004). It was shown that *A. niger* ATCC 9142 and ATCC 12846 were able to produce citric acid on autoclaved pineapple waste with ATCC 9142 producing the highest citric acid level. The presence of methanol slightly increased citric acid production by the strains (Tran *et al*., 1998). The Yang No. 2 strain of the fungus was shown to produce citric acid on sweet potatoes after 4 days (Leangon *et al*., 2000). Using figs as a substrate, *A. niger* ATCC 10577 produced citric acid after 15 days at 30°C. Methanol supplementation stimulated citric acid production by about 1.5-fold (Roukas, 2000). The solid-state fermentation of cassava bagasse by *A. niger* ATCC 9142 produced citric acid after 144 h with 7-8 g citric acid produced/kg dry bagasse (Vandenberghe *et al*., 2004). The concentration of citric acid produced by this strain on cassava bagasse was similar to the levels produced by the strains tested in this study on corn distillers' grains with solubles. Although the citric acid levels produced by the fungal strains on the grains were relatively low compared to substrates with high sugar content, the quantity of grains being produced per year would allow a substantial amount of citric acid to be fermented.

Table 1: Citric acid production by *Aspergillus niger* strains on untreated or treated corn distillers' grains with solubles as a substrate following 240 h of growth

	Citric acid concentration		
Treatment	ATCC 11414	ATCC 26550	ATCC 201122
None	4.81 (0.38)	1.73 (0.03)	4.56 (0.48)
Autoclaved	1.71 (0.43)	1.64 (0.05)	3.17 (0.35)
0.5% H_2SO_4	4.06 (0.73)	4.36 (0.24)	2.02 (0.41)
1.0% H_2SO_4	3.40 (0.64)	3.23 (0.69)	2.02 (0.39)
1.5% H_2SO_4	3.15 (0.41)	2.07 (0.40)	1.59 (0.11)
3% Methanol	5.40 (0.99)	4.06 (0.81)	2.52 (0.14)
0.4% Phosphate	3.20 (0.39)	2.00 (0.50)	2.39 (0.11)

The results are expressed as g citric acid/kg grains with solubles and represents the mean of three separate trials (standard deviation)

Table 2: Biomass production by *Aspergillus niger* strains on untreated or treated corn distillers' grains with solubles as a substrate following 240 h of growth

	Biomass level		
Treatment	ATCC 11414	ATCC 26550	ATCC 201122
None	0.17 (0.02)	0.28 (0.03)	0.16 (0.01)
Autoclaved	0.24 (0.01)	0.29 (0.01)	0.17 (0.01)
0.5% H_2SO_4	0.27 (0.02)	0.32 (0.01)	0.16 (0.01)
1.0% H_2SO_4	0.26 (0.01)	0.34 (0.05)	0.17 (0.01)
1.5% H_2SO_4	0.26 (0.02)	0.30 (0.02)	0.14 (0.01)
3% Methanol	0.25 (0.02)	0.23 (0.02)	0.16 (0.01)
0.4% Phosphate	0.16 (0.00)	0.19 (0.02)	0.13 (0.02)

The results are given as g cell weight/g grains with solubles and indicates the mean of three separate trials (standard deviation)

Table 3: Citric acid yield by *Aspergillus niger* strains on untreated or treated corn distillers'grains with solubles as a substrate following 240 h of growth

	Yield (%)		
Treatment	ATCC 11414	ATCC 26550	ATCC 201122
None	19.27 (4.19)	23.08 (7.27)	20.97 (7.37)
Autoclaved	3.96 (1.07)	12.40 (2.44)	7.95 (1.13)
0.5% H_2SO_4	5.40 (1.57)	7.09 (0.63)	2.68 (0.87)
1.0% H_2SO_4	2.13 (0.55)	1.86 (0.44)	1.12 (0.12)
1.5% H_2SO_4	1.29 (0.16)	0.90 (0.13)	0.75 (0.09)
3% Methanol	12.27 (2.19)	11.06 (6.20)	6.98 (1.32)
0.4% Phosphate	33.30 (9.49)	18.41 (3.08)	10.03 (3.42)

The results are expressed as g citric acid/g reducing sugar consumed ×100% and represents the mean of three separate determinations (standard deviation)

Biomass production by the three *A. niger* strains after growth on the untreated and treated grains was also studied (Table 2). After 240 h of growth, biomass production by ATCC 11414 and ATCC 26550 was higher for the autoclaved grains or acid-hydrolyzed grains than the untreated grains. Biomass production by ATCC 201122 was slightly increased after growth on the autoclaved, 0.5% or 1.0% acid-treated grains but diminished when the fungus was grown on grains treated with 1.5% acid (Table 2). The addition of phosphate to the untreated grains decreased biomass production by the strains. The effect of methanol supplementation on biomass production was strain dependent. Biomass production by ATCC 11414 was increased by 1.4-fold after methanol addition compared to the biomass levels detected on the untreated grains. Also following methanol supplementation, biomass production decreased for ATCC 26550 but remained unchanged for ATCC 201122 relative to the untreated grains (Table 2). Overall, the grains treated with low acid concentrations supported higher biomass production by the strains compared to the untreated grains.

The highest specific productivity for ATCC 11414 of 0.12±0.01 g citric acid/kg grains/h (mean of 3 trials±standard deviation) or for ATCC 201122 of 0.12±0.01 g citric acid/kg grains/h was observed

after growth on the untreated grains. The highest specific productivity for ATCC 26550 of 0.06±0.01 g citric acid/kg grains/h was observed after growth on the 0.5% acid-treated grains or the methanol-treated grains. Relative to citric acid yields, growth on the untreated grains produced the highest yields for ATCC 26550 or ATCC 201122 (Table 3). Interestingly, the highest citric acid yield observed for ATCC 11414 was noted following phosphate addition (Table 3). Only for ATCC 201122 did its growth on the untreated grains produce the highest specific productivity and citric acid yields.

In conclusion, citric acid was produced by the selected strains of *A. niger* whether they were grown on untreated or treated corn distillers' grains, although higher levels of citric acid were detected on the untreated grains than the acid-treated grains. Phosphate supplementation was found to slightly stimulate citric acid production by only *A. niger* ATCC 26550. Stimulation of citric acid production after methanol addition was found to be strain dependent with the greatest increase in citric acid production being observed for ATCC 26550.

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