Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 1 (6): 540-545, 2006
ISSN 1816-4935
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Comparison of Citric Acid Production by *Aspergillus niger* ATCC 9029 and ATCC 12846 on Corn Distillers' Grains with Solubles

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Abstract: Two 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 9029 after 240 h was higher on untreated grains than on autoclaved grains or acid-hydrolyzed grains. In contrast, citric acid production by *A. niger* ATCC 12846 after 240 h was higher on autoclaved grains than on untreated grains or acid-hydrolyzed grains. Methanol or phosphate supplementation to the grains failed to stimulate citric acid production by either *A. niger* strain. Biomass production by both strains after 240 h was higher on the treated grains than the untreated grains. The highest citric acid yields were observed following growth of both strains on the untreated grains.

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

Introduction

The global production of the specialty chemical citric acid is estimated to be over 900,000 tons (Karaffa *et al*., 2001). It is used as a specialty chemical in industrial applications involving foods, beverages and pharmaceuticals (Tran *et al*., 1998). Due to its properties, citric acid can be used as a preservative, flavor enhancer, a chelating agent, a pH regulator and an antioxidant (Karaffa *et al*., 2001). Prior studies have demonstrated that the fungus *Aspergillus niger* can utilize brewery wastes to produce citric acid (Hang *et al*., 1975, 1977; Roukas and Kotzekidou, 1986). In addition, pineapple waste (Tran *et al*., 1998), figs (Roukas, 2000) or cassava bagasse (Vandenberghe *et al*., 2004) can be used by *A. niger* to produce citric acid utilizing solid-state fermentation. The supplementation of phosphate or methanol to the fungal cultures has been shown to stimulate citric acid production (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 distillers' solubles. From each bushel of corn processed at ethanol plants, approximately 18 pounds of corn distillers' grains with solubles are produced. At present, corn distillers' grains with solubles is used in animal feeds as a protein supplement (Ham *et al*., 1994). Considering that more than a million tons of grains are produced from ethanol production per year, the low-value grains could increase in value if utilized to produce specialty chemicals such as citric acid. It is known that fermentable sugars and starch present in corn distillers' grains with solubles could be used by microorganisms as a source of carbon (Moyer, 1953b; Nguyen *et al*., 1992). In this study, the known citric acid-producing *A. niger* strains ATCC 9029 and ATCC 12846 were compared for their

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ability to produce citric acid from untreated and treated corn distillers' grains with solubles by solid-state fermentation. The influence of phosphate and methanol supplementation of the untreated corn distillers' grains with solubles on citric acid production by the fungal strains was also studied.

Materials and Methods

Strains and Growth Conditions

The citric acid-producing strains *Aspergillus niger* ATCC 9029 and ATCC 12846 (Moyer, 1953a, 1953b; Somkuti and Bencivengo, 1981), were used in this study. The corn distillers' grains with solubles was subjected to 121°C at 17 lb $inch^{-2}$ of pressure for 20 min during autoclaving. During acid treatment, the grains were treated with 0.5 to 2.5% H_2SO_4 and autoclaved for 20 min (Dunning and Lathrop, 1945). Subsequently, 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^{-1}) was added to 5 g corn distillers grains (82% moisture) in a sterile 125 mL Erlenmeyer flask and the cultures were grown for 240 h at 25°C. The fungal cultures were grown for 240 or 480 h at 25°C when 0.4% phosphate or 3% methanol, respectively, was supplemented to each culture.

Processing of Grains

After 240 or 480 h at 25°C, the citric acid present in each solid-state fermentation culture was collected using the following protocol. Sterile water (25 mL) was added to each culture and each culture was shaken for 60 min at 25°C. Subsequently, the grains were filtered through a Whatman No. 1 filter. After washing the fungal biomass in each culture with sterile water (10 mL), the liquid was also filtered through a Whatman No. 1 filter. The filtrates from each culture were combined. By adding ice-cold 0.5 N $HClO_4$ (0.5 mL) to each culture filtrate, any protein present in each filtrate was precipitated and 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 citric acid content of the neutralized filtrate was assayed spectrophotometrically using a coupled enzyme assay (Moellering and Gruber, 1966; Henniger and Mascaro, 1985). The composition of the modified assay mix (1 mL) was 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. Standard concentrations of citric acid were also assayed. The reaction was followed at 340 nm by monitoring 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 separate determinations involving three independent cultures. The Student's t-test was utilized during statistical analysis.

Biomass and Reducing Sugar Determinations

Biomass production was measured using wet fungal biomass collected 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 concentrations were measured using a previously published calorimetric method 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 x 100%. All values represent the mean of three separate determinations involving three independent cultures. The Student's t-test was utilized during statistical analysis.

Results and Discussion

Prior studies have shown that strains of *Aspergillus niger* are capable of utilizing ground corn or corn starch to excrete citric acid (Moyer, 1953b; Nguyen *et al*., 1992). In this investigation, the ability of *A. niger* ATCC 9029 or ATCC 12846 to use untreated or treated corn distillers' grains with solubles for citric acid production was examined. The fungal strains were grown on untreated corn distillers' grains with solubles, autoclaved grains or acid-hydrolyzed grains for 240 h at 25°C. Both strains were capable of producing citric acid on the untreated or treated grains (Table 1). ATCC 9029 produced a 1.8 fold higher level of citric acid on the untreated grains than did ATCC 12846 with the difference being statistically significant ($p<0.01$) (Table 1). In contrast, ATCC 12846 produced the highest level of citric acid on the autoclaved grains (Table 1). Citric acid production by ATCC 9029 was slightly less when the grains were autoclaved compared to the untreated grains (Table 1). Citric acid production by ATCC 12846 on the autoclaved grains increased by 1.5 fold relative to the untreated grains with the difference in production being significant ($p<0.01$) (Table 1). It appeared that citric acid production by ATCC 9029 improved as the acid concentration used to hydrolyze the grains increased. ATCC 12846 produced 1.4 fold higher citric acid levels on the 0.5 and 1.5% acid-treated grains than it did on the untreated grains. The 2.0 and 2.5% acid treated grains supported low citric acid production by ATCC 12846 (Table 1). Generally, grains treated with higher acid levels supported greater citric acid production by ATCC 9029 than did ATCC 12846. With previous studies indicating that low concentrations of phosphate or methanol stimulate citric acid production by *A. niger* (Shu and Johnson, 1948; Moyer, 1953a), the possibility of elevating citric acid production by the strains on the supplemented grains was explored. The supplementation of 3% methanol or 0.4% phosphate failed to stimulate citric acid production by ATCC 9029 or ATCC 12846 (Table 1). With respect to both strains, methanol or phosphate supplementation decreased citric acid production (Table 1). Methanol addition to the grains significantly reduced citric acid production by both strains ($p<0.01$).

A number of investigations have studied citric acid production by *A. niger* using solid-state fermentation (Tran *et al*., 1998; Roukas, 2000; Vandenberghe *et al*., 2004). One of the strains used in this study, namely *A. niger* ATCC 12846 and ATCC 9142 were shown to produce citric acid on autoclaved pineapple (Tran *et al*., 1998). During this study, the highest citric acid level was produced by ATCC 9142 on the pineapple waste (Tran *et al*., 1998). The presence of methanol slightly

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

	Citric acid concentration	
Treatment	ATCC 9029	ATCC 12846
None	4.68 (0.38)	2.68 (0.46)
Autoclaved	3.63 (0.40)	4.04 (0.33)
0.5% H_2SO_4	1.51 (0.70)	3.76 (0.46)
1.0% H_2SO_4	2.24 (0.78)	2.48 (0.41)
1.5% H_2SO_4	2.65 (0.39)	3.85 (0.50)
2.0% H_2SO_4	2.97 (0.05)	1.16 (0.39)
2.5% H_2SO_4	3.66 (0.73)	1.65 (0.43)
3% Methanol	2.29 (0.53)	1.69 (0.11)
0.4% Phosphate	3.46 (0.54)	1.74 (0.06)

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

	Biomass level	
Treatment	ATCC 9029	ATCC 12846
None	0.22 (0.02)	0.18 (0.01)
Autoclaved	0.27 (0.02)	0.20 (0.01)
0.5% H_2SO_4	0.30 (0.02)	0.21 (0.01)
1.0% H_2SO_4	0.31 (0.00)	0.35 (0.11)
1.5% H_2SO_4	0.34 (0.03)	0.47 (0.17)
2.0% H_2SO_4	0.32 (0.03)	0.51 (0.04)
2.5% H_2SO_4	0.37 (0.01)	0.62 (0.20)
3% Methanol	0.26 (0.03)	0.18 (0.01)
0.4% Phosphate	0.25 (0.03)	0.16 (0.01)

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

increased citric acid production by ATCC 9142 and ATCC 12846 (Tran *et al*., 1998). When figs served as the substrate, *A. niger* ATCC 10577 produced citric acid after 15 days at 30°C and it was also shown that methanol supplementation stimulated citric acid production by about 1.5 fold (Roukas, 2000). Using cassava bagasse as a substrate for solid-state fermentation, *A. niger* ATCC 9142 produced 7-8 g citric acid/kg dry bagasse after 144 h (Vandenberghe *et al*., 2004). This level of citric acid produced by ATCC 9142 on cassava bagasse was comparable to the concentrations produced by the strains utilized in this study on corn distillers' grains with solubles.

Biomass production by *A. niger* ATCC 9029 and ATCC 12846 after growth on the untreated and treated grains was also explored (Table 2). After 240 h of growth, biomass production by ATCC 9029 and ATCC 12846 was higher for the autoclaved grains or acid-hydrolyzed grains than the untreated grains (Table 2). Biomass production by ATCC 12846 was highest after growth on the 2.5% acid-treated grains and was 1.7 fold higher than biomass production by ATCC 9029 on the 2.5% acid-treated grains (Table 2). The difference in biomass production by the strains was statistically significant ($p<0.01$). On the other hand, biomass production by ATCC 9029 was 1.4 fold higher after growth on the autoclaved grains compared to ATCC 12846 with the difference being statistically significant ($p<0.01$) (Table 2). Methanol supplementation of the grains slightly increased biomass production for ATCC 9029 while it had no effect on ATCC 12846. The addition of phosphate to the grains slightly increased biomass production by ATCC 9029 but decreased biomass production by ATCC 12846 (Table 2). The difference in biomass production by ATCC 12846 on the phosphate-supplemented grains was significantly lower ($p<0.01$) relative to the untreated grains. Overall, biomass production by both strains was increased when higher acid concentrations were used to treat the grains.

The highest specific productivity for ATCC 9029 or ATCC 12846 of 0.09±0.01 g citric acid/kg grains/h (mean of 3 trials±standard deviation) was found after growth on the untreated grains or autoclaved grains, respectively. Relative to citric acid yields, growth on the untreated or autoclaved grains produced the highest yields for ATCC 9029 or ATCC 12846 (Table 3). The citric acid yields observed for ATCC 9029 and ATCC 12846 were found to be significantly lower ($p<0.01$) on the acid-treated grains and the methanol-supplemented grains compared to the yields of the strains on the untreated grains (Table 3). The phosphate-supplemented grains supported less citric acid production by both strains relative to their production on the untreated grains with the decrease in citric acid production being statistically significant ($p<0.01$) for ATCC 9029 (Table 3).

In conclusion, citric acid was produced by *A. niger* ATCC 9029 and ATCC 12846 when the strains were grown on untreated or treated corn distillers' grains. Methanol or phosphate supplementation failed to stimulate citric acid production by either strain. It appeared that either the untreated or autoclaved grains supported the highest citric acid production by the strains. Although the citric acid levels produced by the fungal strains on the grains were low compared to the citric acid

Table 3: Citric acid yield by *Aspergillus niger* strains on untreated or treated corn distiller's grains with solubles as a substrate

	Yield (%)	
Treatment	ATCC 9029	ATCC 12846
None	10.03 (0.30)	20.63 (7.01)
Autoclaved	9.50 (0.58)	17.11 (4.06)
0.5% H_2SO_4	1.91 (0.80)	4.33 (0.77)
1.0% H_2SO_4	1.24 (0.39)	1.53 (0.33)
1.5% H_2SO_4	1.11 (0.20)	1.71 (0.30)
2.0% H_2SO_4	1.05 (0.04)	0.41 (0.13)
2.5% H_2SO_4	1.22 (0.24)	0.54 (0.13)
3% Methanol	4.85 (1.09)	4.51 (1.47)
0.4% Phosphate	6.93 (1.03)	11.80 (5.83)

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

concentrations produced by *A. niger* strains grown on high sugar substrates, the quantity of grains being produced from ethanol fermentation per year would allow a significant amount of this specialty chemical to be fermented.

Acknowledgments

This study was supported by the South Dakota AES (Paper 3569) and South Dakota Corn Utilization Council. This study reports results of research only and the mention of brand or firm names does not constitute an endorsement by the South Dakota AES over those mentioned but of a similar nature.

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