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Citric Acid Production by *Aspergillus niger* on the Ethanol Dry Milling Coproduct Thin Stillage

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Abstract: Seven strains of the fungus *Aspergillus niger* were screened for their ability to produce citric acid on the ethanol dry milling coproduct thin stillage. Citric acid and biomass production by the fungal strains grown on the thin stillage were analyzed using an enzyme assay and a gravimetric method, respectively. Citric acid production by *A. niger* ATCC 9029, ATCC 9142, ATCC 10577, ATCC 12846, ATCC 26550 and ATCC 201122 was similar after 144 h of growth on thin stillage. These strains also exhibited high citric acid specific productivities and yields. Only the citric acid production, specific productivity and yield by *A. niger* ATCC 11414 were lower after growth on thin stillage. Biomass production by ATCC 9029 on thin stillage was the highest of the strains studied. For all strains, at least 88% of the reducing sugar concentration in the thin stillage was consumed after 144 h.

Key words: Citric acid, biomass, yield, thin stillage, surface fermentation, *Aspergillus niger*

INTRODUCTION

Citric acid is a commercially important specialty chemical that has a number of industrial applications for use in beverages, foods and pharmaceuticals (Tran *et al*., 1998). Some applications of citric acid include utilization as a preservative, flavor enhancer, a chelating agent, pH regulator, antioxidant and stabilizer. Global production of citric acid is estimated to be over 1.4 million tons (Soccol *et al*., 2006). Strains of the fungus *Aspergillus niger* have been shown to excrete citric acid (Legisa and Mattey, 2007) and also have been tested for their ability to produce citric acid from brewery wastes (Hang *et al*., 1975, 1977; Roukas and Kotzekidou, 1986). Other substrates that have been utilized for citric acid fermentation by *A. niger* were beet molasses, carobod syrup or corn starch (Clement, 1952; Macris, 1975; Nguyen *et al*., 1992). It was of interest to learn whether the coproduct thin stillage resulting from the ethanol dry milling process could be utilized as a substrate for citric acid production by *A. niger* strains. The major coproducts produced during ethanol production from corn using dry milling are corn distillers grains and thin stillage. Previous studies have shown that citric acid can be produced by *A. niger* strains grown on corn distillers grains (Xie and West, 2006a-c). The thin stillage is recovered from the whole stillage after centrifugation. The thin stillage is mixed with the wet corn distillers grains and dried to produce corn distillers grains with solubles. About 18 pounds of 90% corn distillers grains with solubles are produced from each bushel of corn processed at dry milling 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 the ethanol dry milling process per year and the wet distillers grains also serving as an animal feed, the low value thin stillage could be more fully utilized to produce a specialty chemical such as citric acid. In this study, seven strains of *A. niger* were screened for their ability to produce citric acid from thin stillage by surface fermentation.

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MATERIALS AND METHODS

Strains and Growth Conditions

Seven citric acid-producing strains, namely *Aspergillus niger* ATCC 9029 (Somkuti and Bencivengo, 1981), ATCC 9142 (Doelger and Prescott, 1934), ATCC 10577 (Clement, 1952), ATCC 11414 (Perlman *et al*., 1946), ATCC 12846 (Moyer, 1953), ATCC 26550 (Martin and Waters, 1952) and ATCC 201122 (Gradisnik-Grapulin and Legisa, 1996) were used in this study. The seven strains were tested for their ability to produce citric acid from the thin stillage using surface fermentation. The experiments were conducted (Brookings, SD, USA) in August 2005 and the source of the thin stillage was Dakota Ethanol LLC (Wentworth, SD, USA). The thin stillage was prepared as a substrate by initially adjusting its pH to 6.0 and filtering it through a Whatman No. 1 filter to remove insoluble material. The clarified stillage was sterilized at 117.2 kPa of pressure for 20 min at 121°C. A volume of the processed thin stillage was added to sterile 250 mL Erlenmeyer flasks. To prepare the inoculum, 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. A 5% inoculum (2×10^3 conidia mL^{-1}) of each strain was added to each flask to initiate the surface fermentation of the thin stillage. The cultures were placed in a rotary shaker and shaken at 200 rpm at 25°C for a period of 144 h.

Processing of Grains

Following the incubation period of 144 h, the cultures were processed to allow the assaying of citric acid and biomass levels. Each culture was 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 fungal biomass was removed and utilized during the subsequent biomass determination. To precipitate any protein present in each culture filtrate, ice cold 0.5 M $HClO_4$ (0.5 mL) was added and the filtrate was stirred. After any protein precipitate present was removed by filtration, the filtrate was subsequently neutralized. 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 L^{-1} thin stillage. 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

To determine biomass production, wet fungal biomass collected after 144 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 144 h. Reducing sugar levels were determined using a previously described assay where glucose served as the standard (Dygert *et al*., 1965). 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 has previously been shown that strains of *Aspergillus niger* excrete citric acid after growth on corn substrates such as ground corn and corn starch (Moyer, 1953; Nguyen *et al*., 1992). It has also been shown that citric acid can be produced by *Aspergillus niger* strains grown on brewery wastes (Hang *et al*., 1975, 1977; Roukas and Kotzekidou, 1986). In this study, it was shown that seven citric acid-producing strains of *A. niger* were able to utilize stillage for citric acid production after 144 h at 25°C (Table 1). Six of the seven strains, namely ATCC 9029, ATCC 9142, ATCC 10577, ATCC 12846, ATCC 26550 and ATCC 201122, were capable of producing similar citric acid levels after 144 h of growth on thin stillage (Table 1). No statistically significant difference in the citric acid levels produced by these six strains was observed. There was a statistically significant ($p<0.01$) difference in the levels of citric acid produced by these strains compared to ATCC 11414. ATCC 11414 produced the lowest level of citric acid following its growth on thin stillage for 144 h (Table 1). With respect to biomass production, ATCC 9029 produced the highest biomass level on the thin stillage after 144 h of growth (Table 1). The difference in biomass production by this strain was statistically significant compared to biomass production by ATCC 9142 ($p<0.05$), ATCC 10577 ($p<0.05$), ATCC 11414 ($p<0.05$), ATCC 12846 ($p<0.01$), ATCC 26550 ($p<0.01$) and ATCC 201122 ($p<0.01$). The specific productivities of ATCC 9029, ATCC 9142, ATCC 10577, ATCC 12846, ATCC 26550 and ATCC 201122 were comparable with the lowest specific productivity being noted for ATCC 11414 (Table 2). A statistically significant difference between their specific productivities was not observed. The citric acid yields produced by ATCC 9142, ATCC 10577, ATCC 11414, ATCC 12846, ATCC 26550 and ATCC 201122 were similar (Table 2). The citric acid yield produced by ATCC 11414 was found to be statistically lower ($p<0.01$) than the other strains studied (Table 2). Relative to reducing sugar consumption by the strains, it can be noted that a high reducing sugar concentration is utilized by all seven strains (Table 3). This is clearly evident because nearly 90% of the available reducing sugar concentration of the thin stillage is consumed by all the strains tested (Table 3).

Table 1: Citric acid and biomass production by *Aspergillus niger* strains grown on thin stillage as a substrate for 144 h at 25°C

Strain	Citric acid level	Biomass level
ATCC 9029	5.73 (0.77)	21.6 (1.9)
ATCC 9142	5.28 (0.04)	17.5 (2.2)
ATCC 10577	5.37 (0.22)	18.1 (0.8)
ATCC 11414	3.64 (0.12)	16.9 (2.2)
ATCC 12846	4.28 (1.27)	15.2 (1.1)
ATCC 26550	5.84 (1.16)	16.4 (1.8)
ATCC 201122	6.01 (1.04)	16.1 (1.0)

The citric acid levels are expressed as g citric acid L^{-1} thin stillage while the biomass levels are given as g cell weight L^{-1} thin stillage. All values represent the mean of three separate trials (standard deviation)

Table 2: Citric acid specific productivity and yield by *Aspergillus niger* strains grown on thin stillage as a substrate

Strain	Specific productivity	Yield (%)
ATCC 9029	1.86 (0.40)	69.89 (11.60)
ATCC 9142	2.12 (0.28)	61.87 (1.37)
ATCC 10577	2.06 (0.11)	64.76 (2.11)
ATCC 11414	1.52 (0.22)	43.39 (1.66)
ATCC 12846	1.99 (0.75)	52.05 (16.59)
ATCC 26550	2.47 (0.32)	69.85 (13.76)
ATCC 201122	2.62 (0.63)	71.63 (12.39)

Specific productivity is expressed as g citric acid kg^{-1} biomass h^{-1} while % yield is given as g citric acid g^{-1} reducing sugar. All values represent the mean of three separate trials (standard deviation)

Table 3: Reducing sugars consumed by *Aspergillus niger* strains following growth on thin stillage as a substrate for 144 h at 25°C

	Reducing sugar consumed	
Strain	g reducing sugar L^{-1}	Consumed (%)
ATCC 9029	8.34 (0.27)	88.57 (2.87)
ATCC 9142	8.54 (0.21)	90.67 (2.27)
ATCC 10577	8.29 (0.10)	88.07 (1.05)
ATCC 11414	8.38 (0.10)	88.99 (1.05)
ATCC 12846	8.26 (0.17)	87.73 (1.76)
ATCC 26550	8.36 (0.21)	88.70 (2.18)
ATCC 201122	8.40 (0.04)	89.12 (0.38)

The results indicate the mean of three separate trials (standard deviation)

Although limited in number, prior studies have examined citric acid production by *A. niger* using surface fermentation of brewery wastes (Hang *et al*., 1975, 1977; Roukas and Kotzekidou, 1986). Using lager tank sediment as a substrate, *A. niger* ATCC 10577 produced about 7-8 g L^{-1} citric acid on spent grain liquor after 192 h of growth at 30°C (Roukas and Kotzekidou, 1986). An initial study found that *Aspergillus foetidus* ATCC 10254 produced 8 g L^{-1} citric acid and 12 g L^{-1} dry mycelial weight of growth on spent grain liquor after 96 h at 30°C (Hang *et al*., 1975). A more comprehensive study showed that *A. foetidus* ATCC 10254 produced an average of 7.6 g L^{-1} citric acid with an average citric acid yield of 49.9% when it was grown on spent grain liquor for 96 h at 30°C although some brewery waste samples only supported 3.5 g L^{-1} citric acid (Hang *et al*., 1977). The reducing sugar concentration of the spent grain liquor consumed by the fungus averaged about 86% (Hang *et al*., 1977). The mycelial dry weight ranged from 6-11 g L^{-1} after growth for 96 h at 30°C (Hang *et al*., 1977). Brewery wastes were reported to be excellent substrates for biomass production by yeast and fungal strains (Shannon and Stevenson, 1975). Similar to this investigation, some of the same strains used in this work were utilized in the previous studies and all the previous reports employed surface fermentation (Hang *et al*., 1975, 1977; Roukas and Kotzekidou, 1986). This study was also similar to prior reports in that the fermentation wastes used contained comparable initial reducing sugar concentrations (Hang *et al*., 1975, 1977). There was only one study that differed from this study in that the fermentation waste contained a higher initial level of reducing sugars (Roukas and Kotzekidou, 1986). A comparison of the findings of this investigation with the earlier studies indicated that similar levels of citric acid could be produced by the majority of the *A. niger* strains tested on the fermentation wastes (Hang *et al*., 1975, 1977). Biomass production by ATCC 9142 and ATCC 10577 was higher after growth on thin stillage compared to their biomass production on other fermentation wastes (Hang *et al*., 1975, 1977; Roukas and Kotzekidou, 1986) which is likely due to the higher protein content in the thin stillage supporting better fungal growth. Further, the citric acid yield observed for the majority of strains used in this study was higher than 60% and this compared well with the data from prior investigations (Hang *et al*., 1975, 1977). It is clear that thin stillage could be used by all of the *A. niger* strains screened as a substrate for the production of citric acid and supported high levels of biomass production. The percentage of reducing sugar consumed by the *A. niger* strains tested in this study was nearly equivalent to the percentage observed when *A. foetidus* was grown on spent grain liquor (Hang *et al*., 1977).

CONCLUSIONS

Citric acid was produced by all the *A. niger* strains grown on thin stillage at comparable levels for six of the seven strains tested. Moreover, the citric acid specific productivities and yields were

highest for these six strains. Biomass production on thin stillage was highest for *A. niger* ATCC 9029 but the thin stillage supported excellent growth by all the strains tested. Compared to other brewery wastes, thin stillage appeared to be as effective a substrate for fungal citric acid production.

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REFERENCES

Clement, M.T., 1952. Citric acid fermentation of beet molasses by *Aspergillus niger* in submerged cultures. Can. J. Technol., 30: 82-88.

Doelger, W.P. and S.C. Prescott, 1934. Citric acid fermentation. Ind. Eng. Chem., 26: 1142-1149.

Dygert, S., L.H. Li, D. Florida and J.A. Thoma, 1965. Determination of reducing sugar with increased precision. Anal. Biochem., 13: 367-374.

Gradisnik-Grapulin, M. and M. Legisa, 1996. Comparison of specific metabolic characteristics playing a role in citric acid excretion between some strains of the genus *Aspergillus*. J. Biotechnol., 45: 265-270.

Ham, G.A., R.A. Stock, T.J. Klopfenstein, E.M. Larson, D.H. Shain and R.P. Huffman, 1994. Wet distillers byproducts compared with dried distillers grains with solubles as a source of protein and energy for ruminants. J. Anim. Sci., 72: 3246-3257.

Hang, Y.D., D.F. Splittstoesser and E.E. Woodams, 1975. Utilization of brewery spent grain liquor by *Aspergillus niger*. Applied Microbiol., 30: 879-880.

Hang, Y.D., D.F. Splittstoesser, E.E. Woodams and R.M. Sherman, 1977. Citric acid fermentation of brewery waste. J. Food Sci., 42: 383-384.

Henniger, G. and L. Mascaro, Jr., 1985. Enzymatic-ultraviolet determination of L-citric acid in wine: Collaborative study. J. Assoc. Off. Anal. Chem., 68: 1024-1027.

Legisa, M. and M. Mattey, 2007. Changes in primary metabolism leading to citric acid overflow in *Aspergillus niger*. Biotechnol. Lett., 29: 181-190.

Martin, S.M. and W.R. Waters, 1952. Production of citric acid by submerged fermentation. Ind. Eng. Chem., 44: 2229-2233.

Moellering, H. and W. Gruber, 1966. Determination of citrate with citrate lyase. Anal. Biochem., 17: 369-376.

Moyer, A.J., 1953. Effect of alcohols on the mycological production of citric acid in surface and submerged culture. II. Fermentation of crude carbohydrates. Applied Microbiol., 1: 7-13.

Nguyen, T.K., L. Martinkova, L. Seichert and F. Machek, 1992. Citric acid production by *Aspergillus niger* using media containing low concentrations of glucose or corn starch. Folia Microbiol., 37: 433-441.

Perlman, D., D.A. Kita and W.H. Peterson, 1946. Production of citric acid from cane molasses. Arch. Biochem., 11: 123-129.

Roukas, T. and P. Kotzekidou, 1986. Production of citric acid from brewery wastes by surface fermentation using *Aspergillus niger*. J. Food Sci., 51: 225-228.

Shannon, L.J. and K.E. Stevenson, 1975. Growth of fungi and BOD reduction in selected brewery wastes. J. Food Sci., 40: 826-829.

Soccol, C.R., L.P.S. Vandenberghe, C. Rodrigues and A. Pandey, 2006. New perspectives for citric acid production and application. Food Technol. Biotechnol., 44: 141-149.

Somkuti, G.A. and M.M. Bencivengo, 1981. Citric acid fermentation in whey permeate. Dev. Ind. Microbiol., 22: 557-563.

Tran, C.T., L.I. Sly and D.A. Mitchell, 1998. Selection of a strain of *Aspergillus* for the production of citric acid from pineapple waste in solid state fermentation. World J. Microbiol. Biotechnol., 14: 399-404.

Xie, G. and T.P. West, 2006a. Comparison of citric acid production by *Aspergillus niger* ATCC 9029 and ATCC 12846 on corn distillers' grains with solubles. Res. J. Microbiol., 1: 540-545.

Xie, G. and T.P. West, 2006b. Citric acid production by *Aspergillus niger* on wet corn distillers grains. Lett. Appl. Microbiol., 43: 269-273.

Xie, G. and T.P. West, 2006c. Citric acid production by *Aspergillus niger* on corn distillers' grains with solubles. Res. J. Microbiol., 1: 228-233.