Biotechnol Lett (2011) 33:2463–2467
DOI 10.1007/s10529-011-0720-7

ORIGINAL RESEARCH PAPER

Malic acid production from thin stillage by *Aspergillus* species

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Received: 14 June 2011 / Accepted: 26 July 2011 / Published online: 9 August 2011

Abstract The ability of *Aspergillus* strains to utilize thin stillage to produce malic acid was compared. The highest malic acid was produced by *Aspergillus niger* ATCC 9142 at 17 g l^{-1}. Biomass production from thin stillage was similar with all strains but ATCC 10577 was the highest at 19 g l^{-1}. The highest malic acid yield (0.8 g g^{-1}) was with *A. niger* ATCC 9142 and ATCC 10577 on the stillage. Thus, thin stillage has the potential to act as a substrate for the commercial production of food-grade malic acid by the *A. niger* strains.

Keywords *Aspergillus* · Biomass · Ethanol fermentation · Glycerol · Malic acid · Thin stillage

Introduction

Malic acid is a specialty chemical that has applications in the food and beverage industries as well as in metal cleaning, pharmaceuticals and in the production of plastics (Goldberg et al. 2006). Although originally extracted from apple juice (Peleg et al. 1988), malic acid is currently produced by chemical synthesis and the world production is about 40,000 tons annually (Goldberg et al. 2006). Malic acid can be produced by species of the fungus *Aspergillus* when grown on fermentable substrates (Peleg et al. 1988, 1989; Battat et al. 1991; Goldberg et al. 2006). Genetic engineering has been used to improve malic acid production by *Saccharomyces cerevisiae* and *Escherichia coli* (Zelle et al. 2008; Zhang et al. 2011).

During the dry milling of corn to produce ethanol, one of the primary coproducts is thin stillage. During grain-based ethanol production, thin stillage is recovered from the whole stillage following centrifugation of the yeast cells. Thin stillage is a substrate that contains a high percentage of glycerol and about 1% nitrogen (Kim et al. 2008, 2010). It is usually mixed with wet distillers' grains and dried to produce dried distillers' grains with solubles (Kim et al. 2008). To reduce electrical costs associated with the drying process, many ethanol plants are marketing wet distillers' grains (Kim et al. 2008). As a result, thin stillage is becoming a low value co-product although it could serve as a substrate for the production of alcohols or organic acids. While it has been shown that the glycerol present in thin stillage can be utilized by microorganisms to produce ethanol or butanol (Gonzalez et al. 2010; Ahn et al. 2011), the microbial production of an organic acid, such as malic acid, from thin stillage has not been studied.

Species of *Aspergillus*, including *Aspergillus niger* ATCC 9029, *Aspergillus niger* ATCC 9142, *Aspergillus niger* ATCC 10577 and *Aspergillus flavus*

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ATCC 13697, produce malic acid from glucose as a carbon source (Peleg et al. 1988, 1989; Battat et al. 1991; Goldberg et al. 2006). The objective of this study was to determine the ability of selected *A. niger* strains and *A. flavus* ATCC 13697 to utilize the ethanol fermentation coproduct thin stillage for malic acid production.

Materials and methods

Microorganisms and inoculum

The known malic acid-producing strains of *A. niger* (ATCC 9029, ATCC 9142 and ATCC 10577) and *A. flavus* ATCC 13697 were used. Each was inoculated into potato/dextrose broth (10 ml) and grown for 48 h at 25°C.

Batch culture fermentation and collection of mycelia

Initially, the thin stillage was prepared as a substrate for the medium by filtering it through a Whatman No. 1 filter for the removal of insoluble material. To the clarified thin stillage, 9% (w/v) K_2CO_3 was added as a neutralizing agent (Andersson et al. 2009) and its pH was adjusted to 6.0. The fungal inoculum (17% v/v) was added to 10 ml of sterilized thin stillage (pH 6.0) containing 9% (w/v) K_2CO_3 in a sterile 125 ml Erlenmeyer flask and grown for 192 h at 25°C (200 rpm). Thin stillage (3.4 g glucose l^{-1}; 17.1 g glycerol l^{-1}; 15.8 g lactic acid l^{-1}) was from a local ethanol plant and its glucose, glycerol or lactic acid composition was similar to what has previously been found (Kim et al. 2010). After 192 h at 25°C, each of the three liquid cultures was processed using the following procedure. Each culture was filtered through a Whatman No. 1 filter and the fungal mycelium in each culture was collected and washed. The resultant supernatant for each culture was collected for subsequent malic acid determination while the wet fungal mycelium was saved for biomass determinations.

Malic acid, biomass and substrate determinations

Malic acid in each culture supernatant solution was determined spectrophotometrically using a malate dehydrogenase assay (Elkins and Heuser 1994). The assay (in 1 ml) contained 798 mM glycine buffer pH 9.0, 13.3 mM disodium EDTA, 2 mM 3-acetylpyridine/ADP and 42 units malate dehydrogenase and sample. Malic acid standards were also run using the assay. The reaction was monitored at 365 nm by following the increase in absorbance that is proportional to the concentration of malic acid present in the sample. Fungal biomasses after 192 h and in the inoculum were determined by drying 105°C. Glucose, glycerol and oxalic acid in the thin stillage were assayed by previously described methods (Dygert et al. 1965; Bashar and Townsend 1966; Domínguez et al. 2010). All values represent the mean of three independent determinations involving three separate cultures. The Student's *t*-test was used during statistical analysis.

Results

Although all the *Aspergillus* strains investigated can produce malic acid from thin stillage, *A. niger* ATCC 9142 produced the highest malic acid level after 192 h (Fig. 1). A statistically significant ($P < 0.01$) difference was observed in malic acid production by ATCC 9142 compared to ATCC 9029 but not compared to ATCC 10577. In addition, ATCC 9142 produced a higher malic acid level than did *A. flavus* ATCC 13697 after 192 h of growth on the thin stillage (Fig. 1) with the difference in production being statistically significant ($P < 0.01$). Malic acid production by *A. niger* ATCC 10577 on the thin stillage was also significantly ($P < 0.05$) higher than *A. flavus* ATCC 13697 (Fig. 1). Malic acid production by *A. niger* ATCC 9029 was significantly ($P < 0.01$) lower after growth on thin stillage than *A. niger* ATCC 9142, *A. niger* ATCC 10577 and *A. flavus* ATCC 13697 (Fig. 1). In addition to malic acid, ATCC 9029, ATCC 9142, ATCC 10577 and ATCC 13697 produced oxalic acid at 1, 0.3, 1 and 0.5 g l^{-1}, respectively.

Biomass productions by the Aspergillus strains following growth on the thin stillage are also shown in Fig. 1. Highest values were for *A. niger* ATCC 10577 which was significantly higher than *A. flavus* ATCC 13697 ($P < 0.01$). The differences in biomass production between *A. niger* ATCC 10577 and ATCC 9029 or ATCC 9142 were not statistically significant.

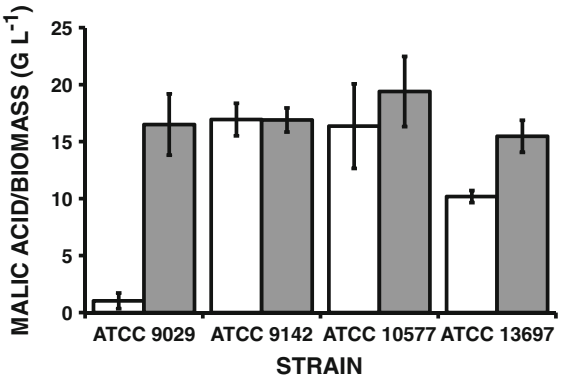

Fig. 1 Malic acid (*open square*) and biomass (*filled square*) production (g l^{-1}) by *Aspergillus* strains grown at 25°C for 192 h in thin stillage (pH 6) with 9% (w/v) K_2CO_3. The data represent the mean of three separate trials ± standard deviation (*error bars*)

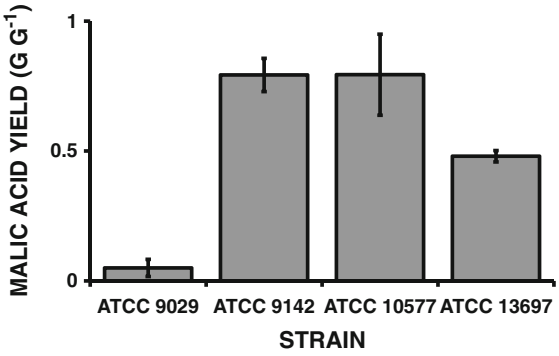

Fig. 2 Malic acid yield (g g^{-1}) relative to glycerol and glucose consumed by *Aspergillus* strains grown in thin stillage (pH 6) with 9% (w/v) K_2CO_3 for 192 h at 25°C. The data represent the mean of three separate trials ± standard deviation (*error bars*)

The lowest biomass production after growth on thin stillage was observed for *A. flavus* ATCC 13697 (Fig. 1).

The highest malic acid yields were for *A. niger* ATCC 9142 and ATCC 10577 (Fig. 2) and were significantly ($P < 0.01$) higher than the malic acid yields measured for *A. niger* ATCC 9029 or *A. flavus* ATCC 13697 (Fig. 2). There was no significant difference in the yields observed for *A. niger* ATCC 9142 and 10577 (Fig. 2). The malic acid yield of ATCC 9029 was significantly lower than the yields for the other *Aspergillus* strains (Fig. 2). As can be seen in Table 1, the amount of glycerol consumed by *A. niger* ATCC 9029, *A. niger* ATCC 9142, *A. niger* ATCC 10577 and *A. flavus* ATCC 13697 was higher than the amount of glucose consumed from the thin stillage.

Table 1 Utilization of glycerol and glucose in thin stillage by *Aspergillus* strains after 192 h of growth

Strain	Glycerol utilized (%)	Glucose utilized (%)
ATCC 9029	99.3 ± 1.2	51.5 ± 0.3
ATCC 9142	100 ± 0	62.7 ± 0.7
ATCC 10577	94.5 ± 1.6	62.2 ± 5.4
ATCC 13697	100 ± 0	60.2 ± 3.5

Glycerol and glucose were determined as indicated in the text at 0 h for the thin stillage and after 192 h of growth in the thin stillage for each strain. The values represent the mean of three independent determinations ± standard deviation. Initial values were glucose at 3.4 g l^{-1} and glycerol at 17.1 g l^{-1}

Discussion

Previous studies have investigated malic acid production by *Aspergillus* species with several reports examining malic acid synthesis by *A. flavus* ATCC 13697. When grown for 192 h at 25°C, ATCC 13697 produced 36.4 g malic acid l^{-1} from 10% (w/v) glucose as the carbon source and calcium carbonate as a neutralizing agent (Peleg et al. 1988). Also, the presence of the protein synthesis inhibitor, cycloheximide, in the medium blocked *A. flavus* ATCC 13697 malic acid production by preventing synthesis of the major isoenzyme of malate dehydrogenase (Peleg et al. 1988, 1989). In *A. flavus* ATCC 13697, malic acid was synthesized from pyruvate with oxaloacetate serving as an intermediate (Peleg et al. 1989). Pyruvate carboxylase was responsible for malic acid production in *Aspergillus* species (Bercovitz et al. 1990). The enzyme was located only in the cytosol fraction of *A. flavus* ATCC 13697 but was located in both the mitochondrial and cytosol fractions of *A. niger* ATCC 9029, ATCC 9142 and ATCC 10577 (Bercovitz et al. 1990). It was also *A. flavus* ATCC 13697 produced several-fold higher malic acid concentrations than *A. niger* ATCC 9029, ATCC 9142 and ATCC 10577 after 135 h at 30°C in a 10% (w/v) glucose-containing production medium (Bercovitz et al. 1990). Moreover, *A. flavus* ATCC 13697 produced more than double the level of malic acid produced by *A. niger* ATCC 9142 when grown in a malic acid production medium containing glucose for

160 h (Goldberg et al. 2006). When malic acid production was studied in a 16 l stirred fermentor, *A. flavus* ATCC 13697 produced 113 g malic acid l^{-1} using 12% (w/v) glucose as a carbon source after 190 h at 32°C (Battat et al. 1991).

The significance of the findings from this latter study (Battat et al. 1991) is that large scale production of malic acid by *A. flavus* ATCC 13697 is possible for non-food uses but the findings do not preclude using *A. niger* strains to produce food-grade malic acid from thin stillage. In this study, the concentration of malic acid produced by *A. niger* ATCC 9142 or ATCC 10577 on thin stillage was about 1.6-fold higher than that for *A. flavus* ATCC 13697. Only *A. niger* ATCC 9029 produced a lower malic acid level than did ATCC 13697. Possibly ATCC 9142 or ATCC 10577 can use the glycerol present in the thin stillage more effectively than the other strains to produce a higher malic acid level. The malic acid yield produced by ATCC 9142 or ATCC 10577 was higher than that produced by ATCC 13697. Prior work has used glucose as the carbon source but, under conditions where high glucose concentrations are present, *A. niger* strains produce higher citric acid levels than malic acid levels (Bercovitz et al. 1990). The accumulation of malic acid rather than citric acid by the *A. niger* strains used in this study is likely due to the glycerol, nitrogen and carbonate levels in the thin stillage medium. The utilization of glycerol by *A. flavus* ATCC 13697 may be slower than its utilization by the *A. niger* strains resulting in less malic acid being produced. The likely pathway of glycerol utilization by the *A. niger* strains is for its conversion to glycerol/3-phosphate and subsequently dihydroxyacetone phosphate (Salazar et al. 2009). Dihydroxyacetone phosphate is likely converted to pyruvic acid which either is converted by pyruvate carboxylase to malic acid or enters the citric acid cycle to form malic acid. The presence of 9% (w/v) carbonate in the thin stillage likely increases malic acid formation using pyruvate carboxylase. Both *A. niger* ATCC 9142 and ATCC 10577 had a higher NAD^+-dependent malate dehydrogenase activity in its cytosolic and mitochondrial fractions than did *A. flavus* ATCC 13697 or *A. niger* ATCC 9029 (Bercovitz et al. 1990). The higher dehydrogenase activity in ATCC 9142 or ATCC 10577 may be responsible for the increased malic acid production on glycerol compared to *A. flavus* ATCC 13697. Also unlike *A. flavus*, *A. niger* grown on glucose produces a high gluconic acid concentration which it cannot produce on glycerol and increased malic acid production may result (Bercovitz et al. 1990). The theoretical yield from glycerol (1 mol malic acid/mol glycerol) is expected to be lower than from glucose (2 mol malic acid/mol glucose). Therefore, glucose would be the preferred substrate. Although the theoretical yield from glycerol is lower than from glucose, *A. niger* strains have not been reported to produce aflatoxins as has been shown for *A. flavus* ATCC 13697 (Mislivec et al. 1968).

Overall, malic acid was produced from thin stillage by the *Aspergillus* strains used in this study. Malic acid production on the thin stillage was noted to be highest for *A. niger* ATCC 9142 while the malic acid yield from the glycerol in the thin stillage was observed to be highest for *A. niger* ATCC 10577. Considering that ethanol plants produce large volumes of thin stillage each year, a process to use the available sugar and glycerol in thin stillage to produce malic acid using the *A. niger* strains has potential for the production of food grade malic acid.

Acknowledgment Financial support of this project by the South Dakota Agricultural Experiment Station Grant SD00H198-07 was greatly appreciated.

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