TECHNICAL NOTE

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Inhibition of Ethanol Production by *Saccharomyces cerevisiae* in Human Blood by Sodium Fluoride

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ABSTRACT: Production of ethanol in antemortem blood samples inoculated with an efficient ethanol-producing microorganism and incubated at various temperatures is discussed. Whole blood samples inoculated with *Saccharomyces cerevisiae* were incubated in gray stoppered Venoject® tubes (approximate draw volume 7 mL) containing sodium fluoride (17.5 mg) and potassium oxalate (14.0 mg) at 4°C, 25°C, and 37°C for 0, 24, 96, 192, and 408 h. No volatile substances (such as ethanol, methanol, isopropanol, acetone, or acetaldehyde) (<0.010 g/dL) were produced in any of the samples at 4 or 25°C. At 24 h incubation a trace amount (<0.018 g/dL) of ethanol was detected at 37°C.

KEYWORDS: forensic science, forensic toxicology, ethanol, driving under the influence, in vitro fermentation, antemortem

The production of ethanol from contaminating microorganisms in antemortem blood samples is of significant importance to laboratories performing forensic blood ethanol determinations submitted in driving under the influence (DUI) arrests. Many manuscripts have been published on postmortem ethanol production (1–11), whereas, little data is available on antemortem ethanol production (e.g., 1,10,11). The contamination and concomitant production of ethanol in postmortem blood samples can be elucidated via analysis of vitreous samples, if available, in death investigation cases (3–5); however, the contamination and production of ethanol in antemortem samples is not easily elucidated. Many elaborate scenarios for contamination of antemortem samples have been proposed to defend against blood ethanol results in court, but there are few legitimate scenarios. Prior contamination of the collection tube (non-sterile), contamination during collection, or contamination during analysis are three possible scenarios by which samples could become adulterated.

The present study seeks to determine if ethanol can be produced in chemically preserved antemortem samples inoculated with *Saccharomyces cerevisiae* (Brewer's yeast). Of forensic interest are the conditions under which significant levels of ethanol and related volatiles can be produced.

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Materials and Methods

Materials

Blood samples were from the local Red Cross (containing citrate, phosphate, dextrose, and adenine). The blood glucose diagnostic kit was from Sigma. Tertiary-butanol (*t*-butanol) was from Fisher Scientific. Aqueous ethanol standards were from The College of American Pathologists (CAP). Acetone and methanol were from J.T. Baker. Trypticase soy broth, trypticase soy agar, and sterile Venoject tubes were from Baxter. Isopropanol and acetaldehyde were from EM Science. *Saccharomyces cerevisiae* was a gift from Dr. Duane Yoch, Department of Biological Sciences, University of South Carolina, Columbia, South Carolina.

Methods

Blood Glucose Determination—Blood glucose levels were determined by a quantitative, enzymatic (glucose oxidase) assay from Sigma Diagnostics. To 0.2 mL of blood was added 1.8 mL of water, and 1.0 mL of 0.3 N barium hydroxide solution. After vortexing, 1.0 mL of 0.3 N zinc sulfate was added. The tubes were capped, vortexed, and centrifuged at 1000 × g for 10 min at room temperature. Aliquots of the supernatant (0.5 mL) were removed and added to 5.0 mL of enzyme color reagent. After vortexing, the tubes were incubated at 37°C for 30 min. Absorbance measurements at 431 nm were recorded on a Beckman Du-7 spectrophotometer. Glucose concentrations (mg/dL) were determined by dividing absorbance (at 431 nm) of the sample by absorbance of the standard and multiplying by 100. The standard deviation and coefficient of variation for the assay were 3.2 mg/dL and 3.4%, respectively (12).

Inoculation—Blood samples were inoculated with 0.1 mL of a suspension of *Saccharomyces cerevisiae* (75–80 by 10^7 colony forming units (CFUs)) and placed in gray stoppered Venoject tubes (approximate draw volume 7 mL) which contained sodium fluoride (17.5 mg) and potassium oxalate (14 mg). Control samples containing no microorganisms were placed in gray stoppered Venoject tubes. Each sample was incubated at 4°C, 25°C, and 37°C. Aliquots (0.1 mL) were removed and assayed for the presence of ethanol, methanol, isopropanol, acetone, and acetaldehyde after 0, 24, 96, 192, and 408 h. Volatiles were analyzed in duplicate at each time given.

Blood Alcohol Determinations—Blood samples (0.1 mL) with 0.1 mL of deionized water were added to 2.0 mL of internal standard (0.2% w/v *t*-butanol) in a glass headspace vial and sealed with a butyl septum. Analysis of volatiles was performed by a Perkin-Elmer HS-100 gas chromatograph with a 6 ft by 1/8 in. stainless steel 5% Carbowax® 20M on a 60/80 mesh Carbopack B column and a flame ionization detector (at 250°C). Injection, transfer line, and GC-oven temperatures were set at 90°C. The sample temperature was 60°C for 25 min.

Microorganism Growth—Saccharomyces cerevisiae was inoculated in trypticase soy broth and trypticase soy agar, incubated for 48 h at 37°C, aliquoted, and stored at −70°C. Growth was monitored by inoculation of tryticase soy agar plates and incubation at 37° C for 72 h.

Results

Background Measurement of Blood Volatiles, Microorganism Growth, and Glucose—Blood samples were assayed before the time course for the presence of volatiles, glucose, and microorganisms. All samples were negative for the presence of ethanol, methanol, isopropanol, acetone, or acetaldehyde (<0.005 g/dL). Whole blood samples contained 114 mg/dL glucose. Tryticase soy broth and tryticase soy agar inoculated with blood samples before inoculation with *Saccharomyces cerevisiae* exhibited no growth after 72 h at 37°C.

In Vitro Production of Ethanol—There was no production of ethanol, methanol, isopropanol, acetone, or acetaldehyde at 4°C, 25°C, or 37°C in any of the control samples (containing no *Saccharomyces cerevisiae* and incubated in gray stoppered Venoject tubes) (line 1, Table 1). Moreover, no microorganism growth was detected in controls (after inoculation of tryticase soy agar and incubation for 72 h) at the 0 or 408 h time points. Whole blood samples inoculated with *Saccharomyces cerevisiae* and incubated in gray stoppered Venoject tubes at 4°C and 25°C produced no ethanol (●-● Fig. 1 and line 2, Table 1), methanol, isopropanol, acetone, or acetaldehyde (not shown) for up to 408 h. At 24 h incubation, trace (<0.018 g/dL) amounts of ethanol were detected in samples inoculated with *Saccharomyces cerervisiae* and incubated at 37°C (○-○, Fig. 1 and line 2, Table 1). Positive control (with *Saccharomyces cerevisiae*) red stoppered tubes (Venoject containing no sodium fluoride or potassium oxalate) did produce ethanol (line 3, Table 1) at 25° and 37°C. Negative control (with no *Saccharomyces cerevisiae*) red stoppered tubes (Venoject) did not produce ethanol (line 4, Table 1), methanol, isopropanol, or acetone (not shown) for up to 408 h.

TABLE 1—*Experimental design and results.*

Tube*	Ethanol Production 4°C	25°C	37°C
Gray stoppered (control)	−	−	−
Gray stoppered with *Saccharomyces cerevisiae* (experimental group)	−	−	trace
Red stoppered with *Saccharomyces cerevisiae* (positive control)	−	+	+
Red stoppered (negative control)	−	−	−

*Gray stoppered tubes (Venoject, 7 mL) contain 17.5 mg sodium fluoride and 14 mg potassium oxalate. Red stoppered tubes (Venoject, 7 mL) contain no additive.

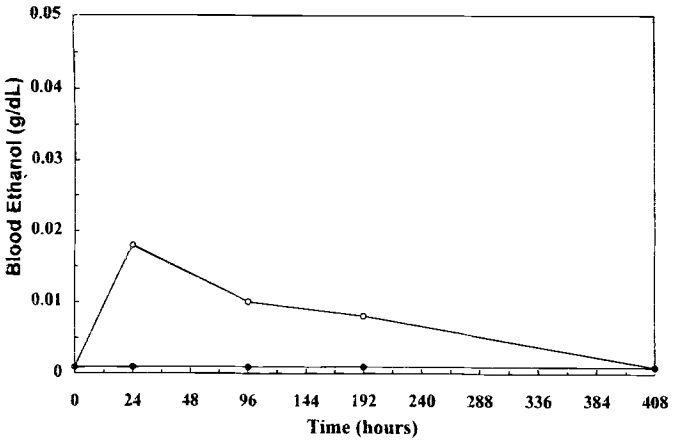

FIG. 1—*In vitro production of ethanol in human whole blood by* Saccharomyces cerevisiae. *Blood samples were inoculated with* Saccharomyces cerervisiae *and incubated for the times indicated. Aliquots (0.1 mL) were removed and assayed for ethanol at 4°C (●-●), 25°C (●-●), and 37°C (○-○) as described under "Experimental Procedures."*

Discussion

Controlled studies were conducted to determine if ethanol production could be inhibited in antemortem blood samples inoculated with an efficient ethanol-producing microorganism following incubation at various temperatures. One aspect of these experimental situations pairs growth conditions (at 37°C) of a proficient ethanol-producing organism (*Saccharomyces cerevisiae*) with a previously recommended condition for collection and storage of forensic blood samples for DUI cases (in tubes containing sodium fluoride (approximately 1%)) (4,15,16). The concentration of sodium fluoride in these experiments was approximately 0.25% (17.5 mg sodium fluoride in approximately 7 mL volume). Even under these experimental conditions, the results in this manuscript demonstrate the inhibition of ethanol production from *Saccharomyces cerevisiae* in human blood.

Although the possibility of contamination of blood samples collected for DUI cases is negligible, there are plausible scenarios. Three possible scenarios include prior contamination of collection tubes, contamination during collection, or contamination during analysis. The possibility of prior contamination of collection tubes is unlikely according to the certificates of sterility available from the manufacturer. Secondly, if aseptic technique is used, microorganism contamination during collection is unlikely. Contamination by skin cleansing volatiles (i.e., ethanol, isopropanol, etc.) may also be of concern during collection. However, in many states, recommendations for the collection of forensic blood samples are published (e.g., ref. 13) to eliminate this type of contamination. Finally, during analysis of over 200 negative control (red stoppered Venoject tubes) samples in this study, ethanol production did not occur (line 4, Table 1). If contamination of the sample during analysis or from the manufacturer is in question, then microorganism growth and production of ethanol could have occurred in these negative control samples. In addition, inoculation of tryticase soy agar plates with the negative control blood samples at the completion of the time courses (408 h) revealed no microorganism growth.

Nevertheless, if contamination occurred during the collection and/or testing of a forensic blood sample, or by any other means, the results in this manuscript show that when the sample was collected in a tube containing sodium fluoride and stored at 4 or 25°C, ethanol production by *Saccharomyces cerevisiae*, would be inhibited.

The results in Fig. 1 illustrate an initial rapid production of ethanol followed by a gradual decrease in ethanol concentration. This is most likely due to substrate (glucose) depletion and concomitant breakdown of ethanol to acetate and, subsequently, to carbon dioxide. Kochova-Kratochvilova has described *Saccharomyces cerevisiae* exhibiting diauxic growth that involves consumption of available sugar, followed by utilization of ethanol for energy production (14). The blood samples were inoculated with 0.1 mL of a suspension containing 75–85 by 10^7 CFUs of *Saccharomyces cerevisiae*, consequently producing an immediate maximal concentration of ethanol as measured under these experimental conditions, followed by a decrease. When blood samples were inocuated with a lesser amount of *Saccharomyces cerevisiae*, there was a lag time before the production of ethanol and a lower maximal concentration (flatter curve) (not shown). A larger inoculation dose may increase the maximal ethanol concentration slightly but would also deplete the substrate quicker resulting in a sharper curve. Nevertheless, under these experimental conditions (37°C with sodium fluoride), the maximal concentration achieved was a trace amount (<0.018 g/dL) at most, and ethanol production in tubes inoculated and incubated at 4 or 25°C was completely inhibited by sodium fluoride.

Sulkowski et al. recently described the in vitro production of ethanol in urine in order to examine the possibility of contamination by possible urinary tract pathogens. They concluded that freezing or storage near 0°C, or addition of sodium fluoride, could inhibit the in vitro production of ethanol in urine by certain species of bacteria and yeast (15).

O'Neal and Poklis have recently described numerous situations and experimental conditions whereby postmortem ethanol (and other volatile substances) production occurs (16). In addition, they provided practical suggestions to assist in the interpretation of results in postmortem cases. These include analysis of multiple specimens (heart blood, peripheral blood, vitreous humor, bile, and urine) in order to rule out postmortem production of ethanol by contaminating microorganisms (16). Although urine and blood are obtainable in DUI cases, the other specimens mentioned are obviously unobtainable.

Saccharomyces cerevisiae was chosen in this study because it is an ubiquitous, proficient producer of ethanol and a very likely source of contamination. Our results demonstrate the inhibition of the production of ethanol from this microorganism under specific conditions. The data supports the recommendation previously given, that samples should contain sodium fluoride for preservation (4,15,16).

Conclusions

We conclude that ethanol production by *Saccharomyces cerevisiae* in blood samples collected for forensic purposes can be completely inhibited by gray stoppered Venoject tubes containing sodium fluoride and potassium oxalate at 4 or 25°C for at least 408 h.

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