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In-Vitro Production of Ethanol in Urine by Fermentation

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ABSTRACT: Driving while under the influence of alcohol (DUI) can lead to serious injuries to the intoxicated driver and surrounding individuals, in addition to revocation or suspension of driving privileges. The accuracy and interpretation of the testing procedures may be compromised if an individual's urine contains sugar, and either bacteria or yeast. Under these conditions, ethanol can be produced in vitro, producing a result that may be erroneously indicative of DUI. In this study three yeast species and six bacterial species were added to a blank urine sample devoid of any alcohol or sugar. Samples were incubated at 0, 25, and 35°C for 24, 48, and 144 hours in the presence of one of four different sugars. Ethanol concentrations were assayed using an enzymatic alcohol dehydrogenase assay. Results showed that when glucose was used as a substrate, all yeast species (*Candida albicans, Candida parapsilosis,* and *Candida sp.* not *albicans*) and three bacterial species (*Klebsiella pneumoniae, Escherichia coli,* and *Proteus mirabilis*) were capable of producing ethanol while the other three (*Enterococcus sp., Staphylococcus sp.* not *aureus,* and *Pseudomonas aeruginosa*) were not. The rate of ethanol production is temperature dependent and can be inhibited by storage of samples at 0°C or the use of approximately 1% sodium fluoride as an antimicrobial agent. Many of these species were also able to use other substrates (sucrose, fructose, and galactose) to produce ethanol by fermentation.

KEYWORDS: forensic science, toxicology, ethanol, DUI, in vitro, fermentation, antimicrobial

In the presence of a suitable substrate, the production of endogenous ethanol by microorganisms through the fermentation of carbohydrates in urine is a potential problem for driving-under-the-influence drug testing and postmortem evaluations. Positive results obtained in this manner can lead to false accusations of ethanol use. Several investigators have shown that ethanol is produced by *Candida albicans* when this yeast species is present in serum [*1*], urine [*2*], and peritoneal fluid [*3*]. The rate of ethanol production is dependent on sample storage temperatures [*2*]. The use of approximately 1% sodium fluoride as a preservative in urine has been shown to be effective in inhibiting ethanol production [*4*]. However, other microorganisms and substrates such as sucrose, fructose, and galactose also have potential for fermentation and have not been examined in a controlled study.

The in-vitro production of ethanol by fermentation was studied on six different bacterial species using one of four different substrates (glucose, sucrose, fructose, and galactose). The rate of ethanol production was studied at three different incubation temperatures over six days. The effectiveness of sodium fluoride was also tested as a preservative. Fluoride acts by inhibiting the activity of phosphoglucomutase, which prevents cell polysaccharide synthesis [*4*].

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Materials and Methods

Organism Culture and Identification

The organisms used in this study were fresh clinical isolates isolated in the Hartford Hospital microbiology laboratory from urine samples (clean catch or catheterized) submitted from patients suspected of having a urinary tract infection. The microorganisms selected represent common causes of nosocomial and community-acquired urethritis and cystitis. Specimens were initially processed on trypticase soy agar containing 5% sheep red blood cells (Becton Dickinson Microbiology Systems, Cockeysville, MD) and MacConkey agar (Becton Dickinson) and incubated at 35°C in ambient air for 24 hours. Both bacterial and yeast isolates were identified by standard methods. Isolates were maintained on trypticase soy agar with 5% sheep red blood cells prior to testing.

Calibration Curves of Bacteria and Yeasts

To estimate the amount of bacteria or yeast added to the urine samples, standard curves were constructed by plotting the log concentrations of colony forming units/mL vs. absorbance. Absorbance measurements were made at 660 nm on a spectrophotometer (Cary 219, Varian Instruments, Walnut Creek, CA) using a 1-cm pathlength quartz cuvette and saline as a reference. A linear curve was produced, which was unique to that particular organism. From the regression equation of the line, unknown concentrations were estimated by extrapolation.

Experimental Procedures

Blank urine used for the spiking studies were provided by one subject. Both the control and experimental samples were made from this urine stock, which was refrigerated at 2°C. The control urine contained 4 mL of clean urine diluted with 1 mL of saline. Spiked urine samples contained 4 mL of clean urine, between 4–8 mg of sugar, and 1 mL of bacteria/yeast suspension. The substrates studied were glucose, fructose, galactose, and sucrose (approximate final concentration was 1.0 mg/mL of each). The theoretical maximum ethanol concentrations of these sugars are between 400–800 mg/dL for glucose, galactose, and fructose, and 216–432 mg/dL for sucrose [*4*]. These sugars were selected because they can be found in human urine. In selected samples, 44.4 mg of sodium

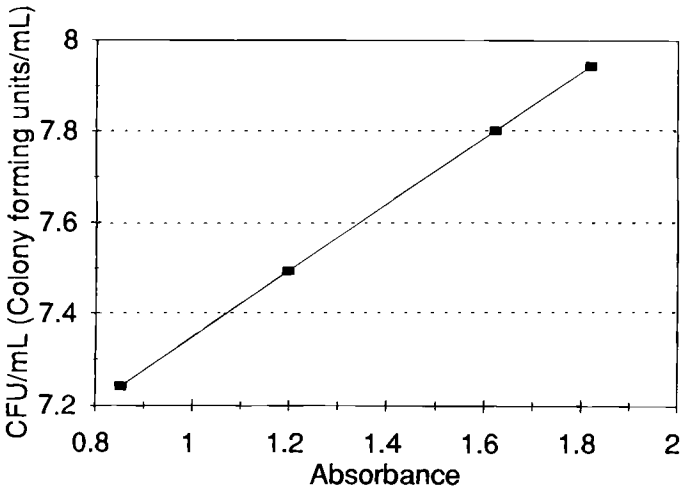

FIG. 1—*The calibration curve for the* C. albicans. *The equation for the line is* y = *0.726x* + *6.6,* r^2 = *0.996.*

fluoride was also added (final concentration 0.89%). Only glucose and sucrose were tested as substrates in the sodium fluoride inhibition study. Samples were aliquoted and incubated at 0, 25, and 35°C. At 24, 48, and 144 hours, a portion of each sample was assayed for ethanol.

Analysis of Ethanol

Urine samples were analyzed for ethanol using a centrifugal analyzer (Cobas Fara II, Roche Diagnostics, Nutley, NJ) using the alcohol dehydrogenase assay [5]. This assay uses reagents available in kit form (No. 330-1, Sigma Chemical Co., St. Louis, MO). The NAD^+ is converted to NADH and the absorbance is measured at 340 nm. The reaction is carried out at 37°C in a glycine buffer, pH 9.0. A competitive inhibitor, pyrazole (Sigma), is added to the buffer to reduce the affinity of ADH for ethanol. This modification allows measurements of ethanol in serum directly without dilution [6]. The assay is calibrated using a one point aqueous standard (100 mg/dL). The assay has a sensitivity of 10 mg/dL. Culture tubes producing ethanol concentrations that were less than 10 mg/dL were assumed to be negative. Based on information provided by the manufacturer, this assay will also react with n-butanol (18% relative to that of ethanol), isopropanol (1.3%), methanol (1.0%), and ethylene glycol (3.0%).

An in-house liquid alcohol control was used for all determinations. This control contained ethanol and methanol at concentrations of 118 and 15.8 mg/dL, respectively. The day-to-day precision (n = 100) for this control was: mean 117.2 mg/dL, standard deviation of 2.7 mg/dL, and a coefficient of variance of 2.3%. A negative control was also used for all analyses.

Results

Figure 1 is a typical calibration curve (*C. albicans*) used for determining the concentration of bacterial and yeast suspensions used in the study. For most species, the calibration curves were linear ($r > 0.90$). However, due to unwanted turbidity, the curves for the *Proteus* and *Pseudomonas* species were less than optimal (r = 0.78 and 0.86, respectively). The approximate range of colonies which made up the various suspensions were as follows: *Staphlococcus sp* 3–9 × 10^7, *K. pneumoniae* 1.2–460 × 10^6, *E. coli* 3.6 × 10^6 – 5.5 × 10^{11}, *Enterococcus sp.* 1.8–100 × 10^8, *P. mirabilis* 1.1–61 × 10^9, *P. aeruginosa* 3.5 × 10^6 – 5.9 × 10^{12}, *C. albicans* 12–77 × 10^6, *C. paraposilosis* 6.3–51.1 × 10^7, *Candida sp.* 2.9–9.3 × 10^5. These colony count values are an approximation of the amount of live bacteria or yeast that is present in the matrix and other components such as dead colonies, gram negative rods, and other debris.

The ethanol production from glucose at 35°C for all nine organisms is illustrated in Fig. 2. All of the yeast isolates studied produced significant amounts of ethanol. The maximum concentration (~52 mg/dL) of ethanol produced was from *C. albicans*. However, the other yeast species studied also produced significant amounts of alcohol at either 24 or 48 hours. At 144 hours, the ethanol concentration in the tubes declined significantly. Figure 2 shows that three of the six bacterial isolates (*E. coli, K. pneumoniae, and P. mirabilis*) were also capable of fermentation. The concentrations at 24 and 48 hours were lower than any of the yeast species, but remained at a constant level at 144 hours. For the other three bacterial species tested (*Enterococcus sp., Staphylococcus sp.* not *aureus,* and *P. aeruginosa*) none were able to ferment glucose to ethanol, as concentrations were less than 10 mg/dL for all time points (Fig. 2). Table 1 lists the ethanol concentrations obtained from glucose by fermentation.

The effect of incubation temperature on ethanol production is illustrated on Fig. 3 for *C. albicans*. As expected, the rate of ethanol production increases with the incubation temperature. Results show that storage at 0°C inhibits production of ethanol for all of the species studied. Of the three incubation times tested, the maximum ethanol concentration was produced after 24 hours at 35°C (~80 mg/dL), while at 25°C, an intermediate ethanol concentration was observed (32 mg/dL). Similar results were observed for each of the other yeast, and bacterial species that were capable of producing ethanol (results not shown).

The effect of sodium fluoride as an antimicrobial agent is illustrated in Fig. 4 (only glucose and sucrose were tested). The optimum temperature conditions were used in order to test the inhibiting property of sodium fluoride. *C. albicans* showed no ethanol production at 35°C for either glucose or sucrose in the presence of this inhibitor.

The results of ethanol production using sucrose, fructose, and galactose as a substrates are summarized in Table 1. As described above, six of the nine microorganisms were able to ferment glucose at 35°C, including *P. mirabilis* but excluding *P. aeruginosa*. Six species of microorganisms were also able to ferment galactose and

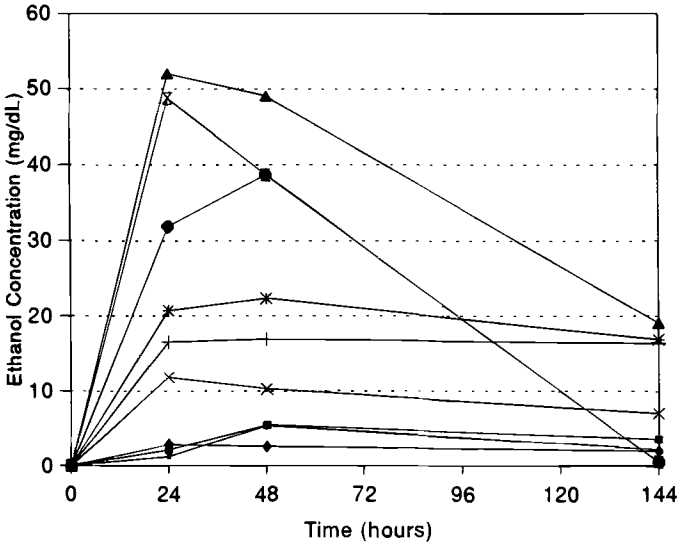

FIG. 2—*Ethanol production of all nine organisms at 35°C using glucose as the substrate.* -▼-C. albicans, -X-C.paraposilosis, -●-Candida sp. *(not albicans)*, -*-E.coli, -+-K. pneumoniae, -×-P. mirabilis, -◆-Enterococcus sp.,-■-P. aeruginosa, -▪-Staphylococcus sp. *not* aereus.

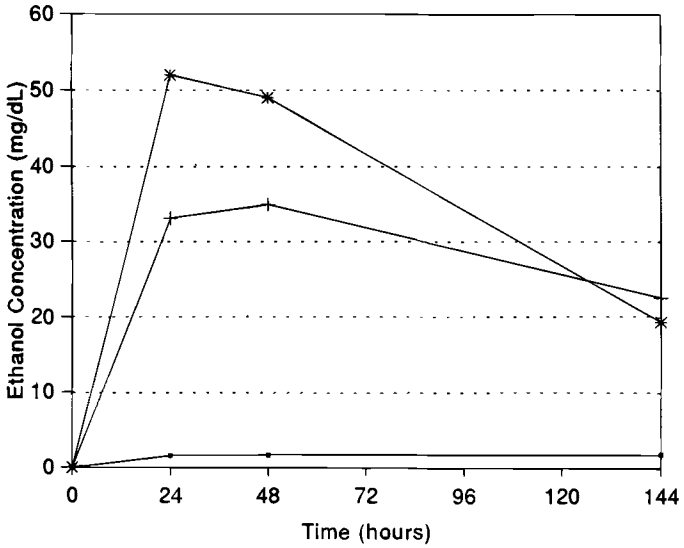

FIG. 3—*The effect of temperature on the production of ethanol. The organism was* C. albicans *and the sugar was glucose,* -▪- at 0°C, -+-at 25°C, -*-at 35°C.

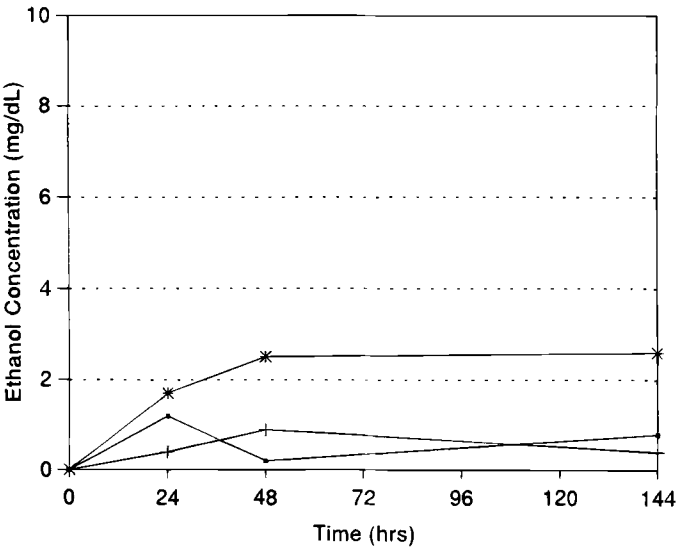

FIG. 4—*The effect sodium fluoride on the ethanol production of* C. albicans *at 35°C.-▪-glucose, -+-sucrose, -*-blank.*

fructose, however, *P. aeruginosa* is included on this list instead of *P. mirabilis.* (*Staphlococcus sp.* and *K. pneumoniae* were not tested for these substrates). In the case of sucrose, neither *P. mirabilis,* nor *P. aeruginosa* were able to use this substrate. Moreover, *C. albicans* was unable to ferment sucrose. This was consistent with Kratochvilova findings [*7*].

Discussion

These studies were conducted to determine the conditions by which urinary tract pathogens might contribute to in vitro production of alcohol. The organisms selected for this study represent common gram positive and negative organisms and yeast species associated with urinary colonization and/or infection. Although bladder urine is normally sterile, microorganisms may enter urine as contaminants in collection containers, from periurethral tissue, from the urethra itself, from gross fecal or vaginal contamination, or from organisms actually multiplying in the urine. Colonization of the periurethral area in both males and females can contaminate urine with staphylococci, streptococci, and enteric gram negative bacilli. The latter of which are the most common organisms associated with urinary tract infections. The presence of yeast in urine can result from skin or vaginal contamination. Urinary tract infections

TABLE 1—*Peak ethanol production by fermentation using different substrates.*[a]

Microorganism	Glucose			Sucrose			Fructose			Galactose		
	24h	48h	144h	24h	48h	144h	24h	48h	144h	24h	48h	144h
Staphylococcus sp.	1.4	5.4	2.2	NA[b]	NA	NA	NA	NA	NA	NA	NA	NA
K. pneumoniae	16.5	16.9	16.4	19.5	22.2	19.2	30.9	34.7	29.5	12.7	18.2	14.2
E. coli	20.7	22.3	16.9	0.8	1.5	18.6	15.7	15.4	13.4	18.0	20.1	10.2
Enterococcus sp.	2.8	2.6	2.0	NA	NA	NA	NA	NA	NA	NA	NA	NA
P. mirabilis	15.7	16.6	15.2	0.3	0.3	0.4	0.7	0.8	0.5	0.1	0.4	0.4
P. aeruginosa	2.1	5.5	3.6	0.7	1.8	1.8	11.1	12.1	15.5	0.0	14.4	17.8
C. albicans	52.0	49.0	19.1	3.4	3.9	2.7	44.8	44.9	22.1	32.7	37.1	26.7
C. paraposilosis	48.7	38.6	0.5	49.5	23.7	1.8	49.5	36.4	0.3	31.7	19.8	1.9
Candida sp.	47.0	27.5	2.1	54.5	33.8	1.7	49.2	32.1	0.9	39.0	25.0	1.5

[a]All results at 35°C.
[b]Not available.

caused by yeast, particularly *C. albicans*, can be seen in patients with diabetes mellitus or previous urinary instrumentation [*8,9*].

Although urine is known to be inhibitory to some microorganisms, it serves as a good culture medium for the replication of most non-fastidious organisms. This is especially true when carbohydrates such as glucose are present in urine.

Previous studies of the in vitro fermentation of ethanol have largely focused on the potential of *C. albicans* to produce ethanol in the presence of glucose. Glucose is excreted into the urine whenever the plasma concentration exceeds the renal threshold concentration, typically 180 mg/dL. In addition to diabetes mellitus, glucosuria is also seen in patients with alimentary glycosuria, Cushing's syndrome, pancreatic diseases, hyperthyroidism, infection, asphyxia, myocardial infarction, gastrectomy, general anesthesia, brain tumors, cerebral hemorrhage, obesity, and glycogen storage diseases. In renal glycosuria, lowering of the reabsorptive threshold of tubules causes urinary glucose excretion. Glycosuria of pregnancy is thought to originate from a lowering of the renal threshold [*10*].

This study has shown that other carbohydrates can serve as substrates for fermentation. For *C. albicans*, the data is consistent with Kratochvilova, who demonstrated production of ethanol from glucose, galactose, and maltose, while no fermentation was seen for the sugars sucrose, lactose, and raffinose [*7*]. Galactosuria is the most significant in infants with congenital metabolic defects. Although not examined in this study, other sugars can be present in urine such as lactose, maltose, mannose, and pentose. Lactosuria may occur in the urine of nursing women, premature infants, and also during late pregnancy. Fructose may appear in the urine after eating honey, syrups and fruits. Pentoses may occur in the urine after eating such fruits as plums, prunes, and cherries, or as a harmless congenital anomaly. Based on the result of this study, many of these other sugars might be suitable substrates for the microorganisms examined.

The time vs. ethanol plot for any yeast showed that the production of ethanol was higher at 24 than at 48 h. After 48 h, the ethanol levels begin to drop off dramatically. An explanation for this is that once the yeast consumes all of the available sugar the organisms begins to digest the ethanol, thereby lowering ethanol concentrations. Kratochvilova states that "Baker's yeast has long been known to exhibit diauxic growth that reflects the utilization of added carbon source, usually saccharide, followed after its exhaustion by utilization of the produced alcohol" [*7*]. The cells oxidize ethanol to acetate and then to carbon dioxide. Most species have the ability to utilize ethanol for energy production.

The amount of ethanol produced by each microorganism was up to approximately 10% of the theoretical maximum amount of ethanol that can be produced from the substrates. For *C. albicans*, this data is in contrast to that of Lough et al. who reported values up to 88% of the theoretical maximum [*4*]. Our results suggest that substrate concentration might not be the only limiting factor for ethanol production. Alcohols are frequently used as a disinfectant against bacterial growth [*11*]. Ethanol levels exceeding some threshold concentration may inhibit further growth of the microorganism.

Conclusion

We conclude that yeast and certain species of bacteria can produce significant amounts of ethanol over a 24 and 48 hours time period, which could lead to possible false positive results. This problem can be remedied by either freezing or storing the sample as close to 0°C as possible, or by adding a small amount of sodium fluoride (approximately 1 mg NaF to every 1 mL of urine). By practicing either one of these methods, the in-vitro production of ethanol by fermentation can be eliminated.

Acknowledgment

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