J Forensic Sci, May 2006, Vol. 51, No. 3
doi:10.1111/j.1556-4029.2006.00112.x
Available online at: www.blackwell-synergy.com

TECHNICAL NOTE

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Enhanced Elution of Sperm from Cotton Swabs Via Enzymatic Digestion for Rape Kit Analysis*

ABSTRACT: This report describes development of a method for enhanced cell elution from cotton swabs. The method exploits an enzyme mixture for digestion of the cotton to remove intact cells, and can be utilized in conjunction with or to circumvent conventional differential extraction (DE). Samples digested with *Aspergillus niger* cellulase yielded sperm cell recoveries (18 ± 3.5%) similar to conventional DE buffer (23 ± 7.8%) while providing intact epithelial cells. Storage time affected the concentration of enzyme required for optimal sperm cell recovery, with longer times requiring increased cellulase concentrations. Cellulase from *A. niger* yielded a twofold enhancement in sperm cell elution over buffer alone, and preliminary testing of higher activity cellulases from *Trichoderma reesei* and *Trichoderma viride* showed even greater enhancement. These results indicate that cellulose-digesting enzymes enhance the release of sperm and epithelial cells from a cotton swab over buffer alone, providing for efficient DNA analysis.

KEYWORDS: forensic science, cellulase, cell elution, differential extraction

Since its inception, forensic DNA analysis has proven to be a considerable factor in crime investigations, providing reliable evidence for suspect identification. The utility of forensic DNA evidence has resulted in an increased demand for DNA analysis services, which has produced a substantial forensic casework sample backlog. A recent Attorney General report estimated that 542,700 criminal cases with biological evidence are awaiting DNA testing; among them, 52,000 homicide cases, 169,000 sexual assault cases, and 264,000 property crime cases (1). Delays in processing evidence, costs associated with the procedure, and lengthy analysis times are all factors that contribute to the existing casework surplus (2). These factors are particularly evident in sexual assault and rape cases, where genetic analysis of perpetrator and victim DNA from vaginal cotton swabs is a well-established forensic technique for investigating and prosecuting sex crimes (3–5). Analytical results suitable for prosecution rely on separation of DNA from the perpetrator and victim to obtain individual DNA profiles. The current protocol for recovery of biological materials from a cotton matrix involves differential extraction (DE), a method that utilizes proteinase K and an anionic detergent to selectively lyse vaginal epithelial cells while eluting sperm cells intact (4). Sperm cells are pelleted by centrifugation, and the supernatant containing the epithelial cell DNA is removed. The sperm cells are then resuspended in a buffer containing dithiothreitol (DTT), reducing the disulfide bond network in the sperm cell head, allowing the nuclear membranes to be lysed. The DE process allows independent recovery of male and female DNA from a swab, but utilizes multiple sample handling steps and is time-consuming, sometimes requiring an overnight incubation for optimal recovery of DNA (6).

Efforts have recently been directed toward improving the DE process. Tereba et al. (7) introduced a modified DE method that incorporates a combination of centrifugal extraction and phase separation to obtain separate sperm and epithelial fractions. This procedure eliminates some wash steps from the traditional DE method; however, it still involves a great deal of sample handling. In addition, the success of the method is dependent on the sample quality, as degraded sperm could contaminate the epithelial fraction. Greenspoon et al. (8) reported improvements in sample processing efficiency and throughput using robotic automation of DE methods. However, implementation of automated DE may be impractical for forensic laboratories handling low-throughput quantities of samples where the high costs associated with the instrumentation cannot be justified.

An alternative to DE involves separation of sperm and vaginal epithelial cells before DNA extraction. Elliott et al. (9) demonstrated selective capture and isolation of sperm cells using laser capture microdissection. Although this method has high specificity and has proven effective in a forensic investigation (10), it is time-consuming and labor-intensive to identify the cells by visual inspection, and not easily amenable to high-throughput applications. Recently, Horsman et al. (11) demonstrated successful microdevice-based sorting of sperm cells from a mixture of sperm and vaginal epithelial cells. The process exploited the differential physical properties of the cells, resulting in sedimentation and adsorption of epithelial cells to the bottom of an inlet reservoir on the glass microdevice. Subsequent buffer flow through the system caused sperm cells to migrate toward the outlet reservoir while epithelial cells remained in the inlet reservoir, resulting in effective separation of the two cell types.

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*This work was presented in part at AAFS, 2005.

Received 1 May 2005; and in revised form 27 Sept. 2005; accepted 29 Oct. 2005; published 5 April 2006.

The microdevice cell separation technique is advantageous because it could be integrated with downstream sample preparation steps on a microfluidic device (12–15) in the form of a micro-total analysis system (μ-TAS) (12,16–19). Integrated systems that combine several analytical techniques on a single microfluidic device reduce analysis times, sample handling, and the sample size required for analysis, making it ideal for samples encountered in forensic laboratories. Effective utilization of the microdevice cell sorting technique developed by Horsman et al. (11), in a μ-TAS or simply as a replacement for traditional DE, relies on efficient recovery of intact cells from a swab sample in a timely manner and in a small sample volume.

Whether associated with the conventional DE or an anticipated μ-TAS platform, enhanced elution of sperm cells from cotton swabs would be beneficial. The work presented here focuses on the effects of a cellulase-based enzyme mixture obtained from *Aspergillus niger* on the recovery of intact sperm and epithelial cells from a cotton swab matrix. The concentration of *A. niger* cellulase for optimal cell recovery was investigated for swabs that were dried then stored for various periods of time. Additionally, fungal sources of cellulase with higher enzyme activities (*Trichoderma reesei*, and *Trichoderma viride)* were evaluated for improved elution enhancement.

Methods and Materials

Preparation of Mock Casework Samples

To mimic vaginal epithelial cells expected on casework sample swabs, buccal epithelial cells were collected onto sterile cotton swabs (Fisher Scientific, Pittsburgh, PA) and allowed to dry for a minimum of 3 days at room temperature. Swabs were then cut into pieces of consistent mass (1.0 ± 0.1 mg, approximately one fifth of a swab), and a 0.5 μL volume of semen (Donor #M99F31U) was applied to each sample. Samples were dried then stored at room temperature for specified amounts of time, as indicated in the results section. Samples prepared in a similar manner were sent for scanning electron microscopy examination. All buccal swabs and semen samples were obtained by voluntary donation from healthy females and males. Semen samples were stored at room temperature for 1 h after collection before frozen storage to reduce the viscosity of the solutions (20).

Procedure for Cell Elution from Cotton Swabs

A solution of 10 mM citrate buffer (pH 5.4) was prepared by dissolving 1.05 g of citric acid monohydrate (Sigma, St. Louis, MO) in 500 mL of Nanopure water (Barnstead/Thermolyne, Dubuque, IA). Cellulase solutions were prepared at the appropriate concentration in citrate buffer using cellulase from *A. niger* (Sigma), *T. reseei* (Sigma), or *T. viride* (Sigma). Swab samples were placed in polypropylene PCR tubes (Fisher Scientific) containing either 100 μL of citrate buffer or cellulase solution, vortexed briefly, and incubated for 1–4 h. Incubations were performed at 37°C, the optimal temperature for *A. niger* cellulase, as recommended by the manufacturer, unless otherwise stated. After incubation, the samples were vortexed briefly, and small holes were created in the bottom of the PCR tubes by inverting the tube and inserting a 21-gauge needle through the center of the bottom of the tube. The PCR tubes were then inserted into 1.5 mL microcentrifuge tubes and centrifuged at 6000 r.p.m for 4 min. The released cells in solution were collected in the microcentrifuge tube while the cotton swab fragment remained in the PCR tube. The collected solution was vortexed, and a 10.0 μL portion was isolated for cell counting. For comparison with the traditional DE method, swabs were incubated for 2 h at 37°C in DE buffer containing 0.01 M Tris-HCl, 0.01 M EDTA, 0.1 M NaCl, 1% (w/v) Sarkosyl, and 20 μg/mL proteinase K (all reagents from Sigma), pH 8.0 (4).

Determination of Cell Recovery

Sperm and epithelial cells eluted from each sample were visualized via light microscopy and counted using a hemacytometer (Fisher Scientific). A fixed volume was applied to the hemacytometer grid, and the sperm and epithelial cells in each of the 81 squares were counted to determine the number of sperm and epithelial cells recovered. The average sperm cell count for the semen donor (#M99F31U) was determined to be 44,150 cells/μL ($\pm$ 3.5%). This value was used to calculate the percent of sperm cells recovered for each set of samples. The results for epithelial cells are reported as the number of cells recovered.

Results and Discussion

The method chosen for elution of dried cells from a cotton swab should be dependent on the nature of the interaction of the cells with the cotton matrix. The idea of sperm cells "entangled" in the cotton fiber matrix construes an image of a sperm cell interacting modestly (based on sperm cell surface area) with several fibers. To evaluate the sperm/cotton interaction more accurately, SEM examination of a semen-imbued swab was performed. The SEM images of a cotton swab in Fig. 1 provide a visual representation of sperm cells captured on cotton swab fibers. It is clear in the figure that the average size of a cotton fiber dwarfs the dimensions

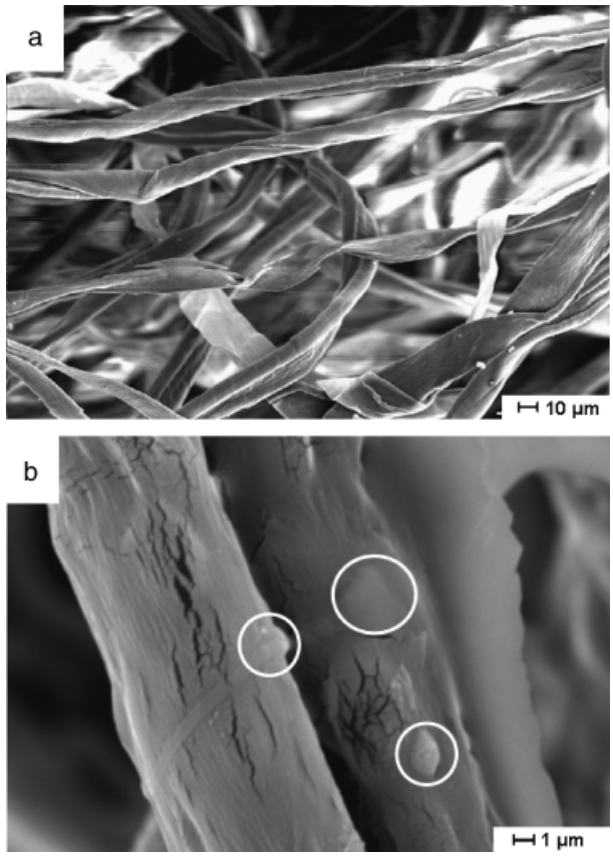

FIG. 1—*SEM images of sperm cells affixed to a cotton fiber surface. (a) Cotton fiber surface at* × *400 magnification. (b) Cotton fiber surface at* × *5000 magnification.*

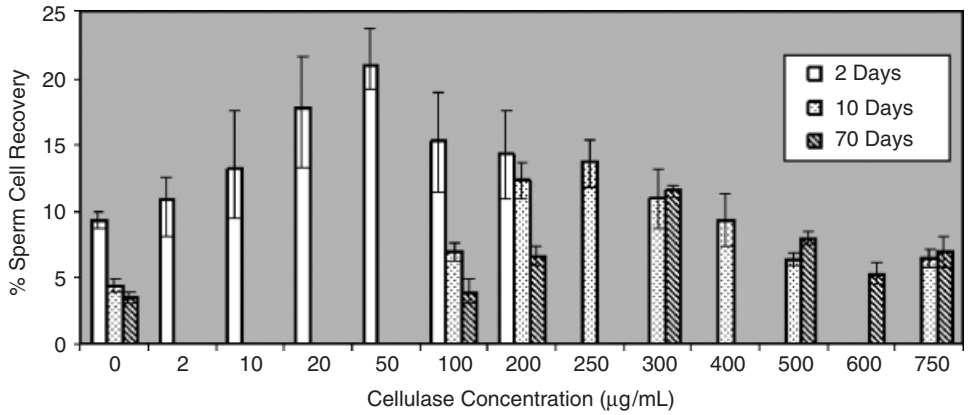

FIG. 2—*Effect of cellulase concentration on the release of sperm cells from samples dried for 2, 10, and 70 days. Samples were incubated for 4 h at 37°C.*

of a sperm cell and that the interaction of sperm cells with cotton fibers is better represented as an adsorption process rather than entanglement. The use of enzymes to remove surface-bound particles from cotton fibers has been described previously (21,22) with digestion of the cotton microfibrils allowing material bound to the surface to be released intact into solution. It was, therefore, hypothesized that enzymatic treatment of cotton swab samples could provide an efficient method for recovery of cellular material from cotton swabs.

Cellulase, the enzyme preparation utilized in the previous studies (21,22), should release intact cells from cotton matrices by digesting the cellulose polymers to which the cells are adsorbed. Cellulose molecules, unbranched polymers consisting of thousands of β(1,4) linked D-glucose units, interact with other cellulose molecules through hydrogen bonds to form fibers, and cotton is one of the purest sources of native crystalline cellulose. Digestion of cellulose by microorganisms such as *T. reesei* and *A. niger* has been extensively studied and reported in the literature (23–25). These microorganisms produce mixtures of enzymes (collectively referred to as "cellulase") with different but synergistic modes of action on the cellulosic substrate. The cooperative behavior between the cellulase components provides a widely accepted mechanism for enzymatic hydrolysis of cellulose (26–28). Hydrolysis of the cellulose chains is initiated at internal glucosidic bonds by the attack of an endoglucanase [1,4-(1,3; 1,4)-β-D-glucan-4-glucanhydrolase], which creates a new nonreducing chain terminus susceptible to attack by an exoglucanase [1,4-β-D-glucan cellobiohydrolase]. The result is cellobiose disaccharide units, which are then hydrolyzed by the β-D-glucosidase component of cellulase to produce glucose monomers. For release of cells, complete hydrolysis of the cotton to glucose is not necessarily required; sufficient digestion would only be needed to break the attachments of the cells to the fiber mass.

Dependence of Cell Elution on the Concentration of A. niger *Cellulase*

Preliminary cell elution experiments indicated that overloading the cotton swab fragments with semen dramatically affected recovery; thus, a saturation curve was generated. Increasing volumes (0.2–3.0 µL) of semen were applied to cotton swab fragments, which were then dried for two days and treated with buffer for cell elution. The results (data not shown) indicated a clear overloading of the swabs above 2.0 µL (*c.* 90,000 sperm cells) of semen added. Smaller semen volumes were more suitable for small cotton swab fragments; therefore, all additional experiments utilized 0.5 µL of semen (*c.* 22,000 sperm cells) to prevent overloading the swab samples. Using these samples, initial investigations focused on optimization of the *A. niger* cellulase concentration for enhancement of cell elution and recovery. Mock casework samples were prepared as described and dried for 2 days. Cells were eluted from the swab in citrate buffer (pH 5.4) containing *A. niger* cellulase ranging from 2 to 200 µg/mL, and compared with samples eluted in citrate buffer in the absence of enzyme. The results, as provided in Fig. 2, showed a substantial variation in sperm cell recovery for this range of enzyme concentrations. An optimal concentration of 50 µg/mL of *A. niger* cellulase was observed for samples dried for 2 days, showing a greater than twofold increase over samples eluted in citrate buffer alone: an average sperm cell recovery of 21 ± 2.7% for 50 µg/mL enzyme vs. 9.4 ± 0.64% for 0 µg/mL enzyme. Lower sperm cell recoveries at lower enzyme concentrations suggest that the cotton matrix was not sufficiently digested for release of adsorbed cells. At concentrations higher than 50 µg/mL of enzyme, sperm cell recovery decreased to 10 ± 2.2%. This could be explained by overcrowding of substrate sites, which would limit the synergistic effects of the endoglucanase, exoglucanase, and β-D-glucosidase components of cellulase (26).

Samples that had been dried for only 2 days do not accurately represent the age range of samples found in forensic casework; therefore, studies were performed on samples that had been dried and stored for longer periods of time. Mock casework samples were prepared and dried for 2, 3, 4, 5, or 30 days. For samples incubated in citrate buffer with or without *A. niger* cellulase (50 µg/mL), the results shown in Fig. 3 indicate that sperm cell recovery dramatically decreased as samples were dried for extended periods of time. The average sperm cell recovery from samples dried for four days before elution decreased to

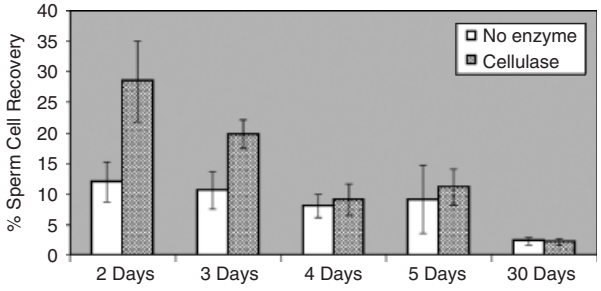

FIG. 3—*Effect of drying time on optimal enzyme concentration. Samples were incubated for 4 h at 37°C.*

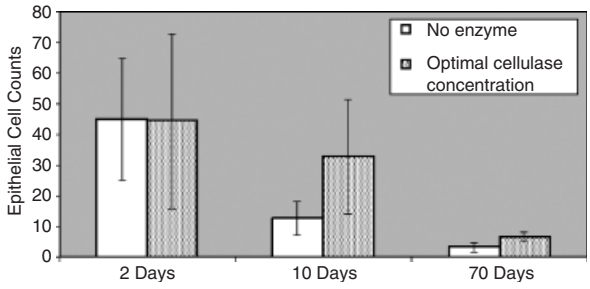

FIG. 4—*Comparison of epithelial cell counts from samples eluted using* Apergillus niger *cellulase and elutions without cellulase present.*

9.1 ± 2.6% in enzyme solution, and was similar to that of samples incubated without enzyme (8.0 ± 1.9%). After 30 days of drying, average sperm cell recoveries from samples incubated with and without cellulase were less than 2%. Based on these results, it was inferred that the concentration of enzyme required for optimal sperm cell recovery was likely to be dependent on the drying time of the sample.

To ascertain the enzyme concentration for optimal cell elution in aged samples, mock casework samples, dried for 10 days, were eluted in citrate buffer containing *A. niger* cellulase concentrations between 100 and 750 μg/mL. Cell recoveries from these samples were compared with those of samples eluted in citrate buffer without enzyme. This study was repeated for samples dried for 70 days. The results of these studies, also shown in Fig. 2, indicated an optimum enzyme concentration of 250 μg/mL for 10-day-old samples, although this did not differ considerably from cell recoveries using 200 and 300 μg/mL concentrations of cellulase. An overall decline in sperm cell recovery was observed for samples dried for 70 days, even at the optimal enzyme concentration (300 μg/mL). However, the results show an improvement in sperm cell recovery with incorporation of cellulase into the elution buffer. These data support the conclusion that samples that have aged longer require higher enzyme concentrations for effective enhancement of cell elution.

Epithelial cell counts were also recorded for samples dried for 2, 10, and 70 days. Crime laboratories are usually concerned with maximum sperm cell recovery to obtain the DNA profile of a perpetrator, while victim profiles are sought only as confirmation of the correct evidence sample. Recovery of epithelial cells from samples is therefore not as critical as that of sperm cells, but the number or intact epithelial cells eluted may be of interest, particularly if these samples are utilized with a cell separation step. Figure 4 shows the cell counts obtained from cellulase-digested (at the optimal concentration for each drying time) and buffer-eluted samples; because of the heterogeneous nature of epithelial cell adsorption with buccal swab collection, percent recoveries could not be calculated. Although the number decreased over time, there were similar or greater numbers of epithelial cells eluted from enzyme-treated samples compared with buffer-eluted samples. As the nuclear membrane of epithelial cells is relatively fragile in comparison with sperm cells, and easily lysed over time, it is not surprising that less epithelial cells were recovered in aged samples. This does, however, indicate that the use of cellulase for cell elution does not cause epithelial cells to lyse.

TABLE 1—*Enzyme activities of commercially produced cellulase systems.*

Cellulase System	Activity (Units Glucose Produced/mg Enzyme)
Aspergillus niger	>0.3
Trichoderma reesei	>1.0
Trichoderma viride	3–10

Comparison of Cellulase with DE Buffer for Cell Elution

The primary buffer used in the conventional DE process utilizes proteinase K and Sarkosyl in the absence of DTT to remove intact sperm cells from swabs while lysing vaginal epithelial cells. It was therefore necessary to compare cellulase digestion and DE buffer for the purpose of sperm cell elution. Samples were prepared, dried for 12 days, and incubated in DE buffer, or in citrate buffer with or without *A. niger* cellulase (250 μg/mL). Figure 5 shows that the average recovery with enzyme-mediated sperm cell desorption was similar to that seen with the conventional DE method. The average sperm cell recoveries for samples treated with DE buffer (23 ± 7.8%) and cellulase (18 ± 3.5%) were higher than samples eluted in citrate buffer alone (5.0 ± 1.3%). Proteinase K has been found to lyse epithelial cells, although this effect is absent with sperm cells (5). Therefore, it was not surprising that epithelial cells were not recovered from the samples incubated in the DE buffer (Fig. 5).

Comparison of Cellulases Derived from Different Fungal Sources

Commercially prepared cellulases contain multiple enzyme activities, but are standardized on the basis of activity on specific cellulosic substrates. The activities listed in Table 1 for various cellulases were measured using an assay described by Worthington (29), based on the ability of the enzymes to hydrolyze Sigmacell 20 (particle size 20 μm), a microcrystalline form of cellulose. It was speculated that use of cellulase enzymes with higher activity would be more effective in the hydrolysis of cotton than the *A. niger* enzyme, which had the lowest activity. Samples treated with *T. viride* cellulase were, therefore, expected to produce higher cell recoveries.

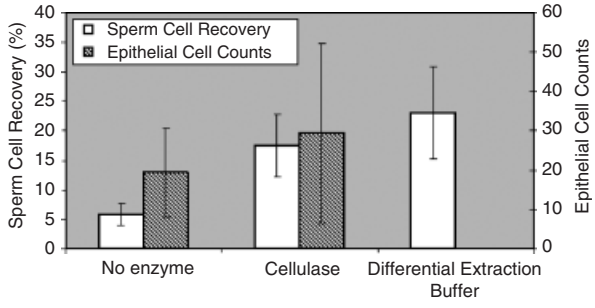

FIG. 5—*Comparison of sperm cell recoveries for differential extraction buffer studies. Samples were incubated for 2 h at 37°C.*

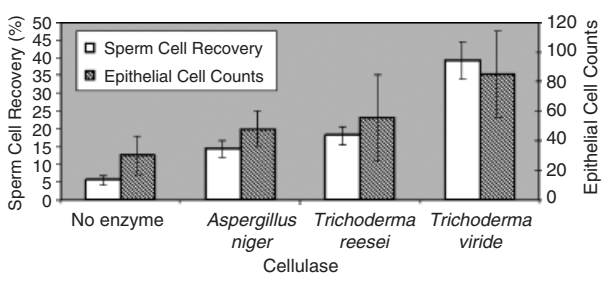

FIG. 6—*Comparison of sperm cell elutions from cotton swabs using commercially prepared cellulases from different fungal sources. Samples were incubated in 50 μg/mL of specified cellulase for 1 h at 37°C.*

Mock casework samples dried for 2 days were incubated in citrate buffer with or without 50 µg/mL cellulase from *A. niger*, *T. reesei*, or *T. viride*. As expected, samples treated with *T. viride* cellulase yielded a higher average sperm cell recovery and epithelial cell counts (Fig. 6) than those treated with identical concentrations of cellulase possessing lower activity. The addition of *T. viride* cellulase to citrate buffer produced a nearly eightfold increase in the average sperm cell recovery compared with no enzyme, and over a twofold increase in recovery compared with samples treated with *A. niger* cellulase. This suggests that the *T. viride* cellulase may provide better sperm cell recovery than the traditional DE method. Future studies will focus on optimizing the concentration of *T. viride* cellulase for enhancement of sperm cell elution and recovery, as well as decreasing the time required for the elution procedure.

Conclusions

This study investigated the hypothesis that enzymatic digestion of cotton swabs using cellulase would increase the number of sperm cells eluted from mock sexual assault samples over buffer elution alone. The results clearly support this hypothesis; however, as drying time for the samples increased, the concentration of *A. niger* cellulase required also increased. Cell elution using *A. niger* cellulase digestion was also compared with a conventional DE elution method. These results demonstrated that enzyme-mediated cell elution provided equivalent removal of sperm cells, with the added benefit of intact epithelial cell removal. Investigation of cellulase preparations from other fungal sources indicated that cellulase from *T. viride* provided an enhanced benefit over the *A. niger* preparation; thus, further investigation of this enzyme preparation for cell elution from cotton swabs is warranted for achieving an even better recovery.

These results illustrate the potential of the enzymatic digestion method as an alternative to the conventional DE procedure in forensic DNA analysis. Optimization of this method to increase cell recovery from cotton swabs would be ideal for low-copy-number samples to increase the efficiency of obtaining accurate DNA profiles. The recovery of intact cells, which this method provides, makes it compatible with conventional DE processing, but also allows for novel processing methods for recovery of male- and female-specific DNA from sexual assault evidence. The proposed cell elution method therefore has the potential to revolutionize forensic DNA analysis through integration with new methods for rapid analysis of forensic sexual assault samples, which will result in a significant reduction of the existing DNA casework backlog. Moreover, the potential exists for application of this method to forensic samples not involved in sexual assault cases. The concept could easily be extrapolated to enhance recovery of microorganisms from a variety of substrates or even the extraction of relevant biomolecules from surfaces, where the choice of enzyme would be guided by the nature of the substrate.

Acknowledgments

Research initially funded by the Federal Bureau of Investigation (Contract #A3N301582). The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed, or implied, of the U.S. government.

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