Robust and effective methodologies for cryopreservation and DNA extraction from anaerobic gut fungi

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Abstract: Cell storage and DNA isolation are essential to developing an expanded suite of microorganisms for biotechnology. However, many features of non-model microbes, such as an anaerobic lifestyle and rigid cell wall, present formidable challenges to creating strain repositories and extracting high quality genomic DNA. Here, we establish accessible, high efficiency, and robust techniques to store lignocellulolytic anaerobic gut fungi long term without specialized equipment. Using glycerol as a cryoprotectant, gut fungal isolates were preserved for a minimum of 23 months at -80°C. Unlike previously reported approaches, this improved protocol is non-toxic and rapid, with samples surviving twice as long with negligible growth impact. Genomic DNA extraction for these isolates was optimized to yield samples compatible with next generation sequencing platforms (e.g. Illumina, PacBio). Popular DNA isolation kits and precipitation protocols yielded preps that were unsuitable for sequencing due to carbohydrate contaminants from the chitin-rich cell wall and extensive energy reserves of gut fungi. To address this, we identified a proprietary method optimized for hardy plant samples that rapidly yielded DNA fragments in excess of 10 kb with minimal RNA, protein or carbohydrate contamination. Collectively, these techniques serve as fundamental tools to manipulate powerful biomass-degrading gut fungi and improve their accessibility among researchers.

Introduction

The growing demands of industrial biotechnology have renewed interest in non-model microbes with unusual properties, such as those that degrade biomass or make novel natural products [1–4]. One promising group of underexplored organisms are Neocallimastigomycota, or anaerobic gut fungi, which efficiently degrade cellulose, hemicellulose and pectin in crude plant biomass into their component sugars [5,6]. Anaerobic gut fungi are the most primitive free living fungi

that persist to this day [7], and they exist ubiquitously in the digestive tracts of large herbivores [5]. Here, they are major contributors to the degradation of ingested plant biomass through their invasive, rhizoidal growth and secretion of an array of powerful enzymes that efficiently degrade the biomass. However, the same attributes that make them attractive for biotechnology hinder their application as industrial strains; gut fungi are exceedingly difficult to lyse, genetically manipulate [8], and to preserve in traditional culture collections.

The rigid cell walls that enable gut fungi to effectively penetrate fibrous plant structures pose a formidable challenge to the preservation and manipulation of fungal cell strains. In particular, cell wall rigidity makes gut fungal cells more susceptible to damage from the expansion of ice crystals formed in cryopreservation processes, leading to poor viability of cultures stored beyond a few months [9,10]. This has led to a number of complex cryopreservation methods requiring hazardous cryoprotectants, numerous reagents, and multi-stage protocols, in some cases, taking a full day to complete successfully [11,12]. Further complicating these procedures is the exceptionally low tolerance to oxygen of gut fungi, which make even the most basic laboratory manipulations non-trivial. Despite best efforts, current methods to preserve isolated strains of gut fungi are not robust and as few as 40% of all stocks retain viability after less than one year [11]. Due to this unreliability in long-term storage, gut fungal strains are maintained with continual passaging in batch culture. Thus, only a handful of researchers who are equipped to isolate and routinely sub-culture gut fungal specimens have been able to explore the superior lignocellulose degradation and innovative biosynthetic potential of these organisms [5].

The rigid cell wall of the fungus also prevents efficient cell lysis, and acts as a tough barrier against the recovery of the cell's genomic contents [13]. Moreover, the chitin-rich composition of their cell wall and abundance of intracellular polysaccharides leads to co-purification of carbohydrate contaminants that render genomic DNA unsuitable for next generation sequencing platforms [13–16]. Molecular characterization is also hindered by low natural abundance of DNA content by cell weight [13], a consequence of the elaborate invasive growth of a single fungal thallus, and the inherent fragility of their A-T rich genomes [17]. As a result, genomic characterization of the Neocallimastigomycota has been limited to only 1 published genome to date [18].

Current state of the art techniques for working with gut fungi require the repeated passage of liquid cultures whose phenotype, and genome, likely adapt and drift over time. Thus, development of simple, rapid, and reliable methods to both “preserve” and “break through” cellular integrity would enable future efforts to develop promising anaerobic fungal strains for biotechnology. Using four unique specimens isolated by our group, one each of *Piromyces* and *Anaeromyces*, and two of *Neocallimastix* [6], we developed, modified, and assessed simple, new methods for genomic DNA isolation and cryopreservation of gut fungi. We evaluated the feasibility of using glycerol as a gut fungal cryoprotectant over a range of concentrations for extended storage at -80°C, and assessed the impact of cryopreservation on fungal viability. These cryopreserved stocks were also examined for their ability to retain their biomass degrading capability after long-term storage, benchmarked against original and passaged cultures. Concurrently, several popular protocols and commercial kits were tested for their ability to provide high-quality genomic DNA suitable for genome sequencing and assembly. These

methods spanned a range of common cell lysis and DNA isolation techniques, which were all assessed for DNA yield, quality and integrity. For one approach, parameters of cell lysis were further explored to better understand their limitations and guide in method optimization.

Methods

Strains Used

Gut fungal cultures used in this study were previously isolated [6] and are listed in Table 1.

Table 1. Anaerobic gut fungal isolates used

Gut Fungal Isolates	IF Number ⁺	Isolated from	Source
<i>Anaeromyces robustus</i>	IF551676	Sheep	Santa Barbara Zoo, Santa Barbara, CA
<i>Neocallimastix californiae</i>	IF551675	Goat	Santa Barbara Zoo, Santa Barbara, CA
<i>Neocallimastix</i> sp. S1	--	Sheep	Santa Barbara Zoo, Santa Barbara, CA
<i>Piromyces finnis</i>	IF551677	Horse	Verrill Farm, Concord, MA

⁺IF = Index Fungorum nomenclature and fungal classification database

Culture maintenance

Gut fungal cultures were continually passaged anaerobically in Medium C containing 15% bovine rumen fluid (Bar Diamond, Parma, ID) and supplemented with up to 0.5% of a soluble or insoluble carbon source under a 100% CO₂ headspace [9]. Fresh rumen fluid was centrifuged to remove particulates, and frozen at -20 °C in single use 75 mL aliquots until media preparation. Prepared media was dispensed in 10 mL volumes, autoclaved, and stored at 4 °C until use. Cultures (10 mL) were grown for 3-5 days in 15 mL Hungate tubes at 39 °C before passaging. During passaging, 1 mL culture was transferred to fresh medium using a sterile needle and disposable syringe. Insoluble substrates were dried and ground (1 mm dry mesh screen) prior to inclusion in Medium C.

Growth quantification

To quantify growth, periodic head space pressure measurements were taken with a pressure transducer [5]. The accumulated pressure was then used as a proxy to generate the fungal growth curve, as done previously [20]. The effective specific growth rates were calculated as the slope of the linear regime of the log-linear plot of accumulated pressure vs. culture time in hours, i.e. during exponential growth.

Fungal biomass estimation

Fungal biomass was estimated using the correlations between culture gas volume at atmospheric pressure and biomass production established by Theoorou et al [21]. Culture gas volumes at atmospheric pressure were estimated from the measured culture pressure and headspace volume (5 mL) using Boyle's Law.

DNA Isolation Methods

Piromyces, *Anaeromyces*, and *Neocallimastix* cultures were grown for 3-4 days in Medium C supplemented with 0.5% glucose before the cultures were harvested by centrifugation. The resulting cell pellets were then processed to isolate the genomic DNA.

Silica slurry adsorbed samples were prepared using the FastDNA™ SPIN KIT for Soil (MP Biomedicals, Santa Ana, CA) according to manufacturer instructions except where an alternative lysis method is noted. Y-PER™ lysed cells were resuspended in 1 mL Y-PER™ Yeast Protein Extraction Reagent (Thermo Scientific, formerly Pierce Biotechnology, Rockford, IL) and incubated at 65 °C for 10 min before being pelleted. The pellet was then resuspended in 400 µL of the FastDNA™ MT buffer and the manufacturer's protocol continued from Step 5. Cells

treated with lyticase (Lyticase from *Arthrobacter luteus* L4025, Sigma-Aldrich, St. Louis, MO) were resuspended in 978 µL of the provided sodium phosphate, 122 µL MT buffer and digested with 200 U lyticase for 10 minutes at 30 °C. Depending on treatment, the included beads (Lysing Matrix E) were added and the manufacturer's protocol continued from Step 4 (with beads) or Step 5 (without beads).

Isopropanol precipitated and silica column adsorbed samples were prepared with Omniprep™ for Fungi (G-Biosciences, St. Louis, MO) and the PowerPlant® Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), respectively, according to manufacturer instructions. PEG precipitation was completed as described by Brownlee [13] with polyethylene glycol (PEG) 8000 (Sigma-Aldrich, St. Louis, MO). CTAB – RNase A was performed as described by Brookman and Nicholson [22]. CTAB + RNase A used a modified protocol where cells were resuspended and lysed in a CTAB DNA isolation buffer supplemented with 0.2% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 0.1 mg/ml proteinase K (New England Biolabs, Ipswich, MA) at 60 °C for 1 h. The lysate was treated with 0.8 ml phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, St. Louis, MO) for 2 minutes before being centrifuged. The aqueous layer was removed and then treated with 1 µL of 300 U/ml RNase A (G Biosciences, St Louis, MO) for 30 minutes at 37 °C before being precipitated in isopropanol (Sigma-Aldrich, St. Louis, MO) and 70% ethanol (Fisher Scientific, Pittsburgh, PA)[23]. DNA was resolubilized in TE buffer. The Sarkosyl/CTAB protocol was provided by Prof. Mostafa Elshahed at Oklahoma State (personal communication). Briefly, fungal biomass was ground under liquid nitrogen before being resuspended in 10 mL TE buffer (100 mM Tris-HCl, pH 8.0; 100 mM EDTA; Sigma-Aldrich, St. Louis, MO) supplemented with 250 mM NaCl (Sigma-Aldrich, St. Louis, MO) and 0.45

mg/ml Proteinase K (G-Biosciences, St. Louis, MO). To this, 1 mL of 10% sodium lauroylsarcosine (Sarkosyl – MP Biomedicals, Santa Ana, CA) was added before overnight incubation at 50 °C with gentle agitation. The lysate was then incubated with 2 ml of 5M NaCl and 1.6 ml 10% CTAB (hexadecyltrimethylammonium bromide – Sigma Aldrich, St. Louis, MO) in 0.7M NaCl at 65 °C. The samples were then deproteinized with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifugation for 10 minutes at 9.7×10^3 g, 4 °C. The aqueous layer was then precipitated in isopropanol and then anhydrous molecular biology grade ethanol (Fisher Scientific, Pittsburgh, PA) before being resuspended in 500 µL 10 mM Tris-HCl, pH 8.5 buffer. This prep was then treated with 0.1 mg RNase A (G-Biosciences, St. Louis, MO) at 37 °C for 1 h before being precipitated in ethanol, washed and resolubilized in 10 mM Tris-HCl, pH 8.5 buffer (Thermo Scientific, Grand Island, NY).

Assessing DNA Yield, Quality, and Integrity

DNA yield and quality were evaluated spectrophotmetrically using a NanoDrop 2000 (Thermo Scientific, Waltham, MA). Intact double stranded DNA was assessed using the Quant-iTTMPicoGreen dsDNA Assay Kit (Invitrogen, formerly Molecular Probes Inc, Eugene, OR). DNA integrity was evaluated with gel electrophoresis using 0.7 % (w/v) agarose at 90V, 60 min [23]. DNA fragment size was quantified using a Bio-Rad CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA) using a 1% SeaKem Agarose (Lonza, Basel, Switzerland) gel in 0.5X TBE (45 mM Tris-HCl, 45 mM borate, 1.0 mM EDTA, pH 8.3) with electrophoresis parameters set at 6 V/cm², an initial switch time of 1 s, a final switch time of 7s, included angle of 120° and run time of 17 h. DNA preps were compared against either the 1 kb ladder (NEB, Ipswich, MA) or the 5 kb Ladder (Bio-Rad Laboratories, Hercules, CA).

Cryopreservation Stock Creation and Revival

Gut fungi were grown in media Hungate tubes as described above, supplemented with 0.1 grams of reed canary grass (graciously provided by Paul Weimer, US Department of Agriculture) for 2 days prior to preservation. Cultures were stored in a preservation medium of sterile, anaerobic Medium C supplemented with 15% rumen fluid [9] and glycerol (Fisher Scientific, Pittsburgh, PA) at 10, 15 or 25% (v/v). After two days of growth, the culture supernatant was removed anaerobically with a syringe needle, leaving only insoluble substrate and colonizing fungal growth. Preservation medium was then added anaerobically and mixed by inversion to disperse the colonized reed canary grass. These tubes were then uncapped under a stream of 100% CO₂ gas (Praxair, Oxnard, CA) and the contents transferred into a 2 mL polypropylene cryovial (Corning Part # 430488, Corning, NY) using a wide bore pipette, also under a stream of CO₂. These vials were capped and rapidly frozen in liquid nitrogen before being stored at -80°C. To revive preserved cultures, cryovials were removed from -80°C and placed into an incubator at 39°C for 15 minutes to quickly thaw the vials and minimize ice crystal damage. Once thawed, the cryovials were opened under a stream of CO₂, the glycerol containing media was removed, and the fungal biomass and particles of reed canary grass resuspended in sterile growth medium. This material was transferred to a fresh 10 mL culture supplemented with reed canary grass and allowed to grow at 39°C. Successfully revived cultures produced fermentation gases within 2 days and displayed visible indicators of fungal growth such as the appearance of rising bubbles and the formation of a buoyant plug of plant material and fungal biomass [5].

Results and Discussion

Robust and reliable long-term storage with cryopreservation

A common consequence of culturing gut fungi is their repeated transfer to fresh medium at regular 2-5 day intervals [5]. Failure to passage results in loss of culture viability. Thus, the use of cryopreservation is essential for the long-term storage of these strains. Cryopreservation not only safeguards culture viability but also prevents genetic drift due to selection during repeated sub-culture. To address these issues, we sought to develop a simple, robust protocol that did not require specialized equipment, and used the inexpensive and non-hazardous cryoprotectant glycerol. Unlike previous methods that (a) preserve fungi grown on soluble substrates (b) use centrifugation to pellet fungal biomass (c) cool fungal stocks in stages over several hours, and (d) demonstrate fungal viability within a range of 3 months to one year [10–12], our protocol avoids the use of pelleted biomass and preserves fungi ‘*in situ*’ on their preferred particulate growth substrates. In essence, our protocol differs from previous methods in that it requires fewer steps, thereby reducing the risk of oxygen exposure during the preservation process. After two days growth on reed canary grass, we use anaerobic procedures to simply replace the liquid growth medium with glycerol-containing cryopreservation medium. Glycerol-incubated strains are quickly aliquoted into cryovials under a stream of CO₂, which are flash frozen in liquid nitrogen, and stored at -80°C.

We tested three different glycerol concentrations (10%, 15%, and 25%) and four different fungal isolates to identify the optimal composition of cryoprotectant to promote cellular viability (Table 2). The four isolates represented three genera of anaerobic gut fungi: *Neocallimastix*, *Anaeromyces*, and *Piromyces* isolated from sheep, goats, and horses fed a fiber-rich diet. For

each concentration, 12 cryovials in total were prepared and revived to quickly verify the feasibility of any cryopreservation. Cryovials were stored for 1-2 weeks at -80°C, thawed, inoculated in fresh media, and assessed for viability via the generation of fungal fermentation gases on reed canary grass. Of these concentrations, 10% and 15% glycerol had slightly higher success rates in short term storage (1-2 weeks) with 8 out of the 12 vials (67% of cryostocks) retaining fermentative viability (Table 2). In contrast, only 7 of the 12 vials tested (58% of cryostocks) were preserved in 25% glycerol (Table 2). Consequently, subsequent studies were conducted with 15% glycerol medium as a cryopreservant.

Table 2. Viability of cryopreserved gut fungi as a function of cryopreservation medium.

Fraction of cryostocks successfully revived for various monocentric and polycentric fungi after 2 weeks at -80°C. Cryovials were stored in one of three different glycerol concentrations. X/Y = #revived strains/#frozen strains

Fungal Isolate	10% Glycerol	15% Glycerol	25% Glycerol
<i>Neocallimastix sp. S1</i>	4/4	3/4	4/4
<i>Anaeromyces robustus</i>	3/3	3/3	3/3
<i>Neocallimastix californiae</i>	1/2	2/2	0/2

Of the fungal isolates tested, *Anaeromyces* was most robust as it retained viability in all the glycerol concentrations tested (Table 2). Similarly, both *Neocallimastix* strains were successfully revived after storage in 10% and 15% glycerol. However, one *Neocallimastix* isolate was non-viable after storage in 25% glycerol while the *Piromyces* isolate was not successfully revived at any concentration of glycerol tested. These differences in storage stability may arise from inherent variations in the morphology and physiology of the various gut fungal genera. The preservation of the *Neocallimastix* and *Anaeromyces* isolates, but not *Piromyces*, may suggest

that these two genera are more capable of forming a resistant survival structure with thicker cell walls that allows them to maintain viability in adverse conditions. Such a structure has been suggested to play a role in long term survival of fungus in liquid culture [24] and may also be important for successful cryopreservation, though further investigation is necessary to verify. Other contributing factors to cryopreservation survival may include their sensitivity to the microaerobic conditions formed during aliquoting, and the ability of these fungi to quickly exchange the water in their cytoplasm with cryoprotecting glycerol before freezing.

Given the robustness of *Anaeromyces* to cryopreservation, we employed this isolate as a model species to determine the stability of gut fungal cryostocks over the course of multiple years in storage. A culture bank of 100 cryovials were created and stored at -80°C. At periodic intervals, 5 cryovials were revived and tested for viability (Figure 1A). In all intervals tested, from 1 month to 23 months of storage, all cryovials led to a vibrant culture that could be repeatedly passaged. That is, this simple and safe storage protocol with 15% glycerol as a cryoprotectant proved to be robust with 100% survival of *Anaeromyces* cultures revived between 1-23 months.

Figure 1. Cryopreservation of anaerobic fungal cultures promotes long-term culture viability. A)

Protocol to test long-term viability of *Anaeromyces* cryostocks. From a repository of identical cryostocks, samples were periodically thawed at the indicated intervals, inoculated, and assessed for growth. Growth curves of initial, cryopreserved and continually passed cultures were generated for species **B)**

Anaeromyces robustus and **C) Neocallimastix californiae**

In parallel with long-term storage at -80°C, the fungal isolate was serially passaged in liquid culture and used as a baseline to benchmark the performance of revived cryostocks. Samples of the polycentric fungus *Anaeromyces* that were cryopreserved for 23 months were used to seed new liquid cultures whose growth patterns were characterized and directly compared to the continually passed cultures (Figure 1B). Comparisons were made using pressure accumulation from fungal fermentations gases [5] to calculate an effective specific growth rate. These studies revealed no significant difference in the growth rates between the initially isolated strains, the cryopreserved strains, and cultures continually passed every 4-5 days for 23 months (Figure 1B). We also examined the health of cryostocks of a monocentric fungus, *Neocallimastix californiae* after 17 months of storage at -80°C to demonstrate the broad applicability of this

method to successfully store monocentric gut fungi long term. These cultures also demonstrated no difference in specific growth rate between the cryopreserved strains, and cultures continually passaged every 4-5 days (Figure 1C). Using inexpensive and safe glycerol, our method robustly stored anaerobic gut fungi at -80°C for up to 23 months, the longest period currently reported [10–12], with a 100% survival rate for several isolates. Additionally, we have demonstrated that this method has no deleterious impact on the rate of growth of cryopreserved cultures on crude biomass when compared to cultures that were continually passaged over the same time period (Figure 1).

Preparation of high-quality, high molecular weight genomic DNA

Since their initial isolation in the 1970s [25], a number of nucleic extraction methods for gut fungi have emerged to allow for basic molecular characterization (e.g. PCR, molecular cloning, taxonomic classification) [6,13,17,18,26]. However, unlike RNA that can be readily isolated from a cellular lysate with minimal degradation or contamination using commercial kits [6,18] due to its small, unmodified nature, DNA must be unpacked from chromatin, and separated from the protein/carbohydrate modifiers that mediate its activity, while not being sheared and degraded during the lysis of the tough cell wall and removal of cellular debris. As a result, DNA extractions typically rely on slow overnight precipitations, and toxic reagents that produce impure samples unsuitable for modern next generation sequencing pipelines [16]. To address this, we assessed a number of common kits and protocols for their ability to produce high molecular weight DNA with sufficient purity and integrity for next generation sequencing platforms; i.e. DNA with minimal degradation to <10 kb fragments, negligible protein

contaminants (A_{260}/A_{280} range of 1.7 – 2.0), and low carbohydrate contamination (A_{260}/A_{230} range of ≥ 1.0).

One commonly used approach to isolate genomic DNA from gut fungal isolates is the FastDNA™ SPIN KIT for Soil [22], which relies on adsorption to a silica slurry (Glassmilk®) for DNA isolation and purification. Following manufacturer instructions, cells are first lysed with detergents (sodium dodecyl sulfate – SDS) and mechanical bead disruption to release the DNA for purification. Under exponential growth with a glucose substrate, gut fungal genomic DNA yields were typically 200 ng DNA/mg fungal biomass as measured by absorbance at 260 nm (Table 3). This DNA displayed minimal degradation to <10 kb fragments (Figure 2) and had an average fragment size of 15-20 kb as revealed by pulsed field gel electrophoresis (Figure 2B). Samples prepared in this manner contained minimal protein contamination ($A_{260}/A_{280} = 1.8$, Table 3) and were suitable for routine PCR amplification[6]. However, due to the high degree of co-purifying carbohydrates ($A_{260}/A_{230} = 0.1$, Table 3) [13] these samples did not meet the purity standards for next generation sequencing platforms

Figure 2. Integrity and size distribution of fungal DNA isolated from a monocentric fungus with the silica slurry adsorption method. A) DNA as separated on native agarose gel electrophoresis at 90 V for 60 min with 1X Tris-Acetate-EDTA B)DNA via pulsed field gel electrophoresis on 0.5X Tris-Borate-EDTA. See Methods for detailed conditions. High MW DNA = High molecular weight (>10 kb) DNA.

Table 3| Overview of DNA isolation methods tested

Method	Silica Slurry Adsorption ^a	Isopropanol Precipitation ^b	PEG Precipitation[13]	CTAB Extraction[22]	Sarkosyl/CTAB Extraction	Silica Column Adsorption ^c
Cell Lysis	Bead beating → SDS	LN2 Grinding → bead beating → Proteinase K	LN2 Grinding → Proteinase K	LN2 Grinding → CTAB	LN2 Grinding → Sarkosyl → CTAB	Bead beating → SDS + proprietary polymer (Phenolics Separation Solution™)
Protein Removal	Acetic Acid Precipitation	Chloroform	Phenol:Cholorform :Isoamyl Alcohol (25:24:1)	Phenol:Cholorform :Isoamyl Alcohol (25:24:1)	Phenol:Cholorform :Isoamyl Alcohol (25:24:1)	Acetic Acid Precipitation
RNA Removal	N/A	RNase A	N/A	+/- RNase A	RNase A	RNase A
DNA Isolation	Glassmilk/Silica adsorption	Isopropanol precipitation	Methoxyethanol + PEG 8000 precipitation	Isopropanol precipitation	Isopropanol precipitation	Silica adsorption
DNA Cleanup	Ethanol Wash	Ethanol Wash	Ethanol Wash	Ethanol Wash	Ethanol Wash	Ethanol Wash
Typical Yields (ng DNA/mg biomass)	200	200	34	200	150	75
Typical A₂₆₀/A₂₈₀	1.8	1.7	2.1	1.6	1.9	1.7
Typical A₂₆₀/A₂₃₀	0.1	0.5	0.1	1.0	1.3	1.1

Typical DNA yields and quality are from the unmodified protocol. ^aFastDNA™ SPIN Kit for Soil; ^bOmniPrep™ for Fungi; ^cPowerPlant® Pro

DNA Isolation Kit. A₂₆₀/A₂₈₀ estimates protein contamination. Target values are 1.7-2.0. A₂₆₀/A₂₃₀ estimates carbohydrate contamination, target range ≥ 1.0

We hypothesized that inefficient cell lysis is a critical barrier to achieving high quantities of pure genomic DNA. With aggressive cell lysis may improve DNA yield and remove co-purifying contaminants, it likely increases DNA shearing and degradation. Conversely, inefficient lysis could reduce yield and DNA purity while improving DNA quality. Thus, we assessed the effect of cell lysis choice on DNA yield and purity (Figure 3). While the lysis methods tested had no significant effect on the resulting DNA purity, there was a marked impact on the amount of DNA recovered (Table 3). Membrane solubilization with Y-PER™ greatly reduced DNA yields, likely due to an inability of the propriety detergent formulation to effectively break the tough chitin-rich cell wall of the gut fungi. Conversely, enzymatic digestion of the cell wall with lyticase, or mechanical disruption with beads was able to produce yields that were more than 2.5-fold greater than Y-PER™ lysis alone. However, combining enzymatic digestion and mechanical disruption did not greatly improve DNA yield suggesting that the standard protocol of mechanical disruption was able to recover the majority of the fungal DNA (Figure 3). Moreover, the elevated temperatures and buffers required for enzymatic digestion (30 °C) and Y-PER™ (65 °C) increased DNA damage with Y-PER™ producing ≤ 100 bp fragments and lyticase producing ~3kb fragments (data not shown). Thus, the recommended cell lysis protocol of cell lysis with bead beating and SDS solubilization was optimal for DNA yield and quality.

Figure 3. Cell lysis technique effects genomic DNA yield and quality with silica slurry adsorption.

DNA yield calculated from absorbance at 260 nm and normalized by estimated fungal mass. DNA purity estimates protein contamination (A_{260}/A_{280} , 1.7 – 2.0 is target range) and carbohydrate contamination (A_{260}/A_{230} , ≥ 1.0 is target range) levels from spectrophotometric absorbance.

To improve the sample purity and reduce carbohydrate contamination, we attempted post-extraction DNA cleanup by precipitating with polyethylene glycol (PEG 8000) or ethanol. PEG precipitation was particularly attractive as it is able to selectively precipitate DNA fragments of high molecular weight (>10 kb) [27] and remove contaminating coprecipitants [13]. However, we were able to recover less than 5% of the initial DNA when precipitated with two-thirds volume of 30% PEG 8000 and 1.5 M sodium chloride. Ethanol precipitation of samples with 0.3 M sodium acetate was marginally more effective with little more than 10% of the DNA recovered and carbohydrate contamination being reduced by an order of magnitude (A_{260}/A_{230} increased to 0.7 from 0.06). To account for this loss, we assayed our samples with a DNA

specific dye to reveal that approximately only 25% of our starting samples were intact double stranded DNA. That is, spectrophotometric quantification greatly overestimated the abundance of quality DNA due to the presence of co-purifying contaminants [28]. More importantly, these contaminants were inhibiting the ability of the DNA to precipitate with high efficiency, potentially causing damage to individual DNA strands.

In addition to the silica slurry adsorption protocol, we tested and evaluated a number of alternate commercial kits and gut fungal genomic DNA isolation protocols for their ability to produce high quality DNA with minimal contaminants (Figure 4). All protocols tested, with the exception of the PEG precipitation which yielded marginal amounts of DNA, produced DNA with comparable yields and purity (Table 3). However, DNA quality varied tremendously. The isopropanol precipitation kit and CTAB extractions appeared spectrophotometrically to produce samples with high yields and superior purity to that of the silica slurry adsorption. However, these samples manifested as a high molecular weight band that was unable to even enter an agarose gel in a standard electrophoresis experiment (Figure 4, isopropanol precipitation not shown). This band was resistant to shearing with a vortexer and digestion with EcoRI suggesting that it was contaminant rich and not high molecular weight chromosomal DNA. In contrast, the Sarkosyl/CTAB protocol was able to produce DNA at high yields and purity that was able to effectively enter the gel. The dramatic improvement in DNA performance on the gel electrophoresis between the CTAB and Sarkosyl/CTAB protocols supports the earlier finding that the CTAB protocol product was primarily contamination as Sarkosyl was needed in the lysis steps to release usable DNA. Nonetheless, all CTAB-based protocols were slow, relied on toxic

deproteinizing reagents such as chloroform, and produced significant amounts of RNA contamination which were not adequately removed with RNase A (Figure 4).

Figure 4. Yield and Integrity of gut fungal DNA varies as a function of genome preparation

approach. Each lane loaded with 2 μ L of anaerobic fungal genomic prep run on a 0.7% (w/v) agarose gel at 90 V, 60 min. Overview of each preparation method provided in Table 2. High MW DNA = High molecular weight (>10 kb) DNA.

All extraction methods applied to gut fungi in the literature were able to produce genomic DNA of varying quality. However, these samples contained persistent carbohydrate impurities, likely arising from the abundance of storage polysaccharide energy reserves and the chitin-rich cell wall of gut fungi [14,15]. Thus, we evaluated a silica spin column based method, PowerPlant®

Pro DNA Isolation Kit, which was designed to remove these contaminants from tough, hardy samples such as seeds and pine needles. Using proprietary buffers, this kit was able to readily produce DNA of moderate yield at 75 ng/mg biomass with minimal carbohydrate and protein contamination (Table 3). More importantly, this genomic DNA was mostly intact double stranded DNA with minimal degradation products (Figure 4) for all isolates tested (*Neocallimastix californiae*, *Anaeromyces robustus*, *Piromyces finnis*). Similarly, the high degree of purity allowed these extracts to readily precipitate in ethanol with minimal loss of sample. This material could be amplified, concentrated and ultimately made into DNA fragment libraries for genomic sequencing with next generation Illumina and PacBio platforms. The high quality of these preps have culminated in the most intact gut fungal genomes sequenced to date, 232 scaffolds vs. the published 32, 574 [18], from organisms with genomes of at least 50 Mb and GC content as low as 17% [29].

Conclusions

In these studies, we developed rapid, robust and inexpensive methods of cryopreserving gut fungal cultures and extracting high quality genomic DNA. We established methods to cryopreserve gut fungi long term at -80°C with safe and inexpensive glycerol. In contrast to previous methods that are complex, slow, and unreliable, our methods are robust, do not require specialized equipment, and generate cryostocks with a two-step protocol that can be completed in less than 2 minutes per vial. More importantly, cultures were viable for at least 2 years, the longest reported for any gut fungi, with no obvious impact on fungal viability. Simultaneously, we identified a commercial kit, PowerPlant® Pro DNA Isolation Kit, which was able to quickly isolate genomic DNA without contaminants common in traditional preparations. This DNA

could be produced at moderate yields with minimal degradation, and of high enough purity for sequencing with next generation platforms. The resulting preps have yielded the most intact gut fungal genomes sequenced to date and will enable a wealth of new molecular level studies.

Taken together, these improved techniques catalyze future opportunities for research and development of gut fungi in biotechnology. Our ability to reliably cryopreserve gut fungal cultures long term facilitates the development of strain repositories that foster scientific collaboration between groups, and enables the development of industrial processes that can meet stringent quality control requirements. Similarly, the isolation of contaminant-free genomes ushers in a new age of research for gut fungi that can leverage the latest advances in sequencing technology to reveal new enzymes and chemistries for biochemical production. More importantly, however, we anticipate that the simplicity and ease of implementation of these techniques will increase the accessibility of gut fungi and lead to their development as interesting new model organisms for biotechnology.

Author Contributions

K.V.S, J.K.H, M.K.T, and M.A.O designed the experiments. K.V.S. carried out the genomic isolation studies, and J.K.H. and M.K.T. executed the cryo-preservation studies. K.V.S, J.K.H., M.K.T., and M.A.O. wrote the manuscript.

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Competing Interest Statement

The authors have no competing financial interests associated with the research described in this manuscript.

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