SUBMISSION: **MCAN**
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Consolidation Request: PC-21-0060

I. Introduction

CinderBio has developed a microbial protein expression platform to produce recombinant proteins in hyper-thermoacidic Archaea

II. Intended Use

The resulting microorganisms are used to produce hyper-thermoacidic enzymes from several enzyme classes, currently including proteases, lipases, carbohydratases, cellulases, and xylanases. The strains are grown in custom bioreactors in our facilities to isolate the target enzymes for use in laboratory biochemical reactions and as non-chemical alternatives for cleaning and sanitation in the food and beverage industries.

III. Taxonomy and Characterization

Sulfolobus species have been isolated from solfataric springs in many different locations around the world. All members of the *Sulfolobus* genus live in nearly boiling acidic waters in areas of active volcanism all around the globe. *Sulfolobus* isolates have been collected from volcanic springs in Yellowstone National Park, Mount St. Helens, Iceland, Italy, Japan, and Russia among many other areas. *Sulfolobus* is located almost wherever there is volcanic activity. They thrive in environments where the temperature is about 80°C with a pH about 3 and sulfur is present.

CinderBio host organisms (*Sulfolobus*) are members of the Archaeal Domain. Such hyper-extremophilic organisms were discovered in the 1970's (Brock, Brock, Belly, & Weiss, 1972). These fascinating organisms were found to be so fundamentally different from previously understood forms of life that a new hierarchy of taxonomy was proposed by Carl Woese in the 1990's (Woese, Kandler, & Wheelis, 1990). Because these new organisms had distinct membrane lipids and diverged in other basic biological characteristics Woese proposed a system of taxonomy that separated all known life into three "Domains" as shown in **Figure 1**; Bacteria, Eukarya, and the new domain including these new extreme life forms, the Archaea. This taxonomical

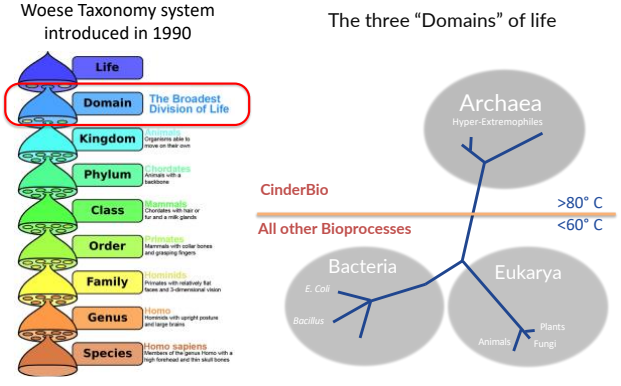


Figure 1. The Woese "three domain" taxonomical system.

system has been widely adopted and ensuing studies strongly validate the legitimacy of this new division and hierarchy in taxonomy (**Figure 1**).

IV. CB Strain History

The original isolate of *Sulfolobus* that our strain was derived from was isolated from a solfataric hot spring in Italy by Karl Stetter and Wolfram Zillig in 1980 (**Figure 2**). The organisms described here are derived from *Sulfolobus solfataricus P1,* renamed recently to *Saccharolobus solfataricus* (Sakai & Kurosawa, 2018), herein referred to as *Sulfolobus solfataricus P1*.

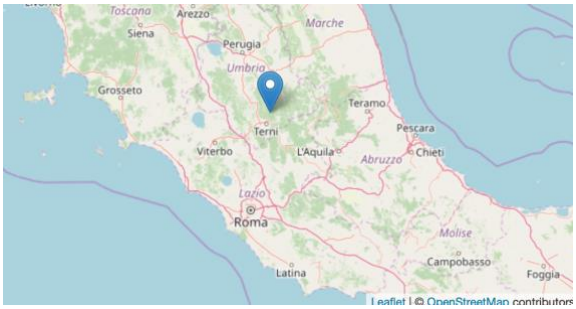


Figure 2. Original source of *Sulfolobus solfataricus* P1. Specifically, in Campi flegrei, Pisciarelli near Agnano, a volcanically active region in central Italy.

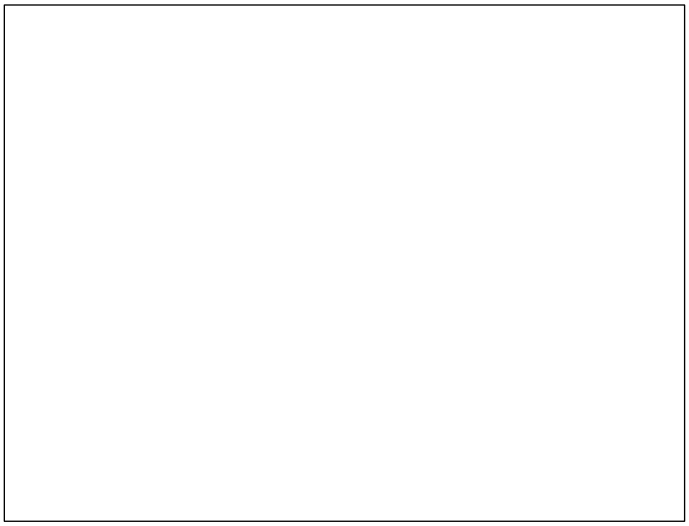
Morphological & Physiological Features: The CinderBio microbial strains under consideration here are morphologically and phenotypically indistinguishable from the wild-type parental strain *Sulfolobus solfataricus* P1 (accession # LT549890.1),

Furthermore, we have prepared genomic DNA from our parental strain

We have amplified, cloned, and sequenced well over 100 separate genes from genomic DNA isolated from the

the genome of *Sulfolobus solfataricus* P2 was completed in 2001 (She et al., 2001) with a follow-on study, providing genetic and genomic search and annotation tools for this genome (Charlebois, Gaasterland, Ragan, Doolittle, & Sensen, 1996). *Sulfolobus solfataricus* Strain 98/2 and evolved derivative genomes were published in 2015 (McCarthy et al., 2015). Currently GenBank holds nearly 100 genome assemblies for the genus *Sulfolobus*.

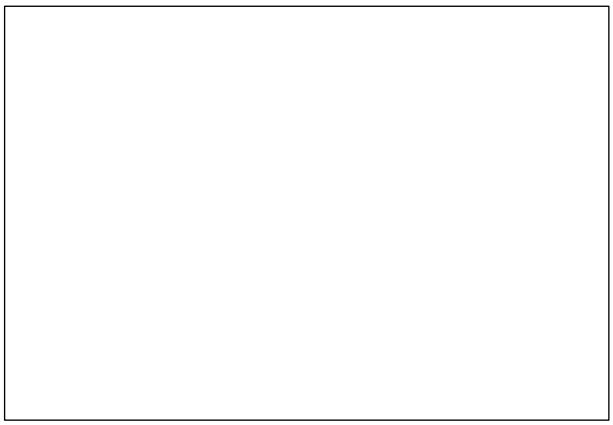
Importantly, all members of this genus are classified as Biosafety Level 1 (BSL1) organisms and listed as such in the American Type Culture Collection (ATCC) where available, reflecting the sum of knowledge about this genus and the very low risks to human and environment health associated with *Sulfolobus* species.



V. Genetic Divergence from Wild Type *Sulfolobus solfataricus* P1

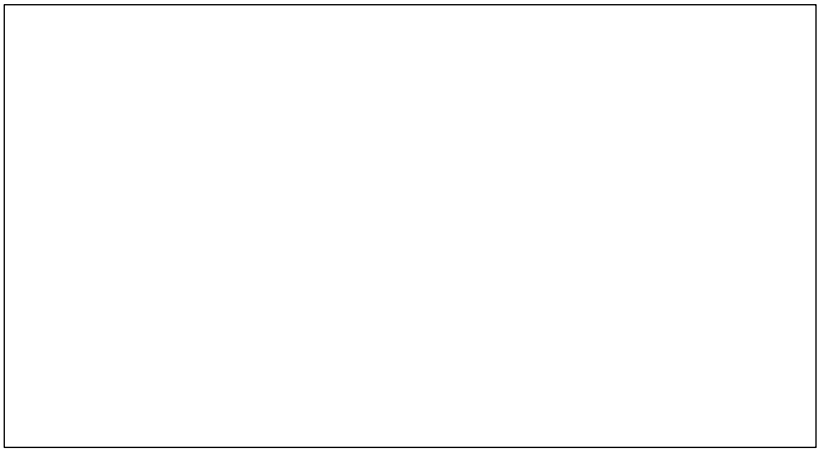
Figure 4.

VI.



The CcdB protein is a potent poison of gyrase and *E. coli* host cells require a *gyrA*462 mutation to survive in the presence of the CcdB protein (Bernard, Gabant, Bahassi, & Couturier, 1994). In addition to the CcdB selection in *E. coli*, we have also added a kanamycin resistance selectable marker on the same cassette. The selection cassette illustrated in **Figure 5** was obtained from PCR reactions that fused

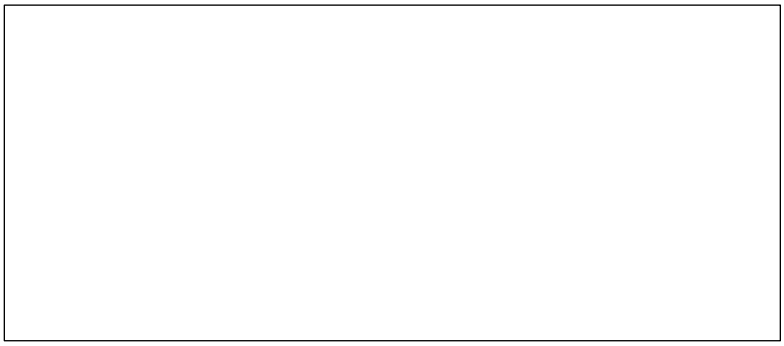
These components permit standard cloning approaches in *E. coli* to move genes of interest into our vector and ultimately into our *Sulfolobus* host.



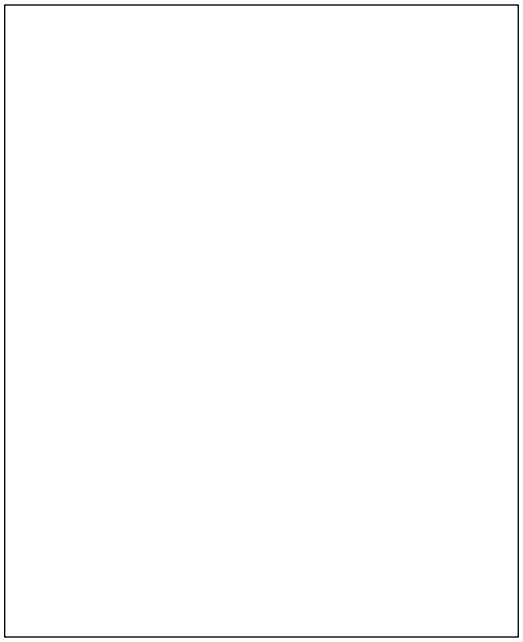
VII.

The likelihood of posing a pathogenic risk to plants, animals, fish or mesophilic microbes is extraordinarily low due to its extreme genetic peculiarity, its single-genus host specificity (*Sulfolobus*), and only having been observed genetically or functionally in hyper-thermoacidic environmental niches ahy & Van Regenmortel, 2008).

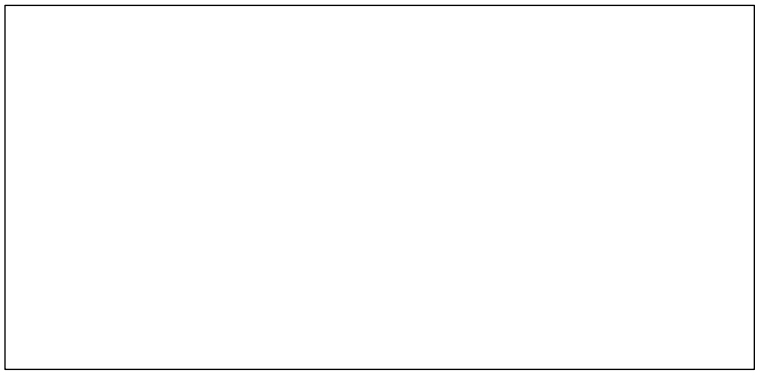
VIII.

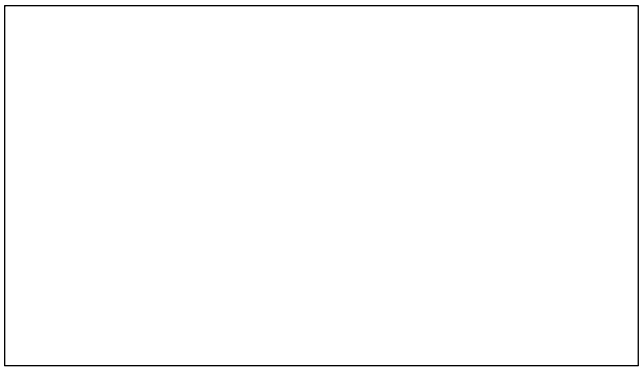


IX.



X. Molecular Process to Generate CB Strains





XI. Six Genes of Interest Defining Six Strains

Proposed Strain Naming Convention

Therefore, the six strains under consideration here are specifically named: *CinderBio-14057, CinderBio-14624, CinderBio-23726, CinderBio-23117, CinderBio-13366, and CinderBio-13184.*

XII. Vector Transformations

For vector propagation in *E.coli*, standard chemically competent cells are procured and used according to manufacturer's instructions. The amplified vector DNA is isolated using standard plasmid preparation techniques and commercially available kits. The resultant vectors are electroporated into our host,

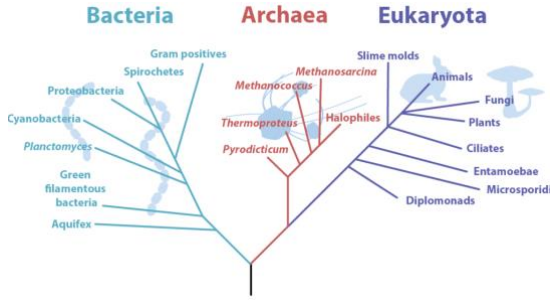


Figure 11. Early Archaeal divergence from other Domains of life.

XIII. Integrated DNA Verification

Importantly, PCR screens use primers that bridge the junction of our and the specific gene of interest, sequence that is unique to each strain and a reliable differentiator between all CinderBio strains.

XIV. Likelihood of Genetic Transfer

(**Figure 11**). Moreover, because the habitat requirements are at such extreme temperatures and pH, it is highly unlikely that the *Sulfolobus* genus has interacted with animals, plants, and fish in any significant way throughout evolutionary time. Importantly, hyper-thermoacidophiles and their viruses are so profoundly divergent from all other known life forms that there is little to no homologous DNA sequence in GenBank for the majority of their genes (Iverson & Stedman, 2012). Moreover, there is no genetic evidence we are aware of for lateral gene transfer between hyper-thermoacidic genomes and mesophilic organisms. This genetic isolation is likely a function of the habitat isolation (**Figure 12**). Notably, proteins evolved in hyper-thermoacidic environments don't function at mesophilic temperatures and pH, and visa-versa. Therefore, these strains and viruses pose little to no risk of genetic transfer to life forms other than *Sulfolobus* or other hyper-thermoacidophiles. Examining the lack of overlap between hyper-

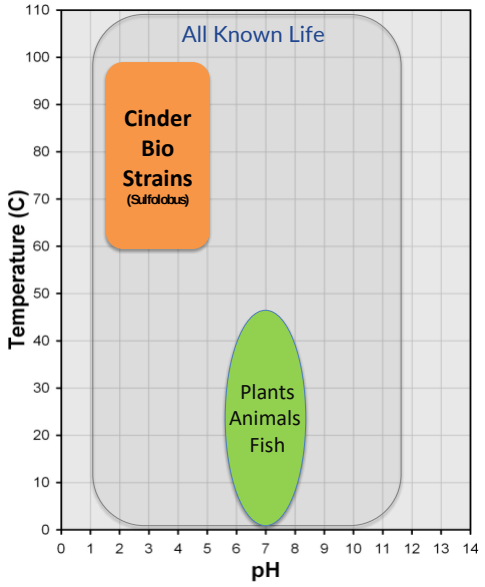
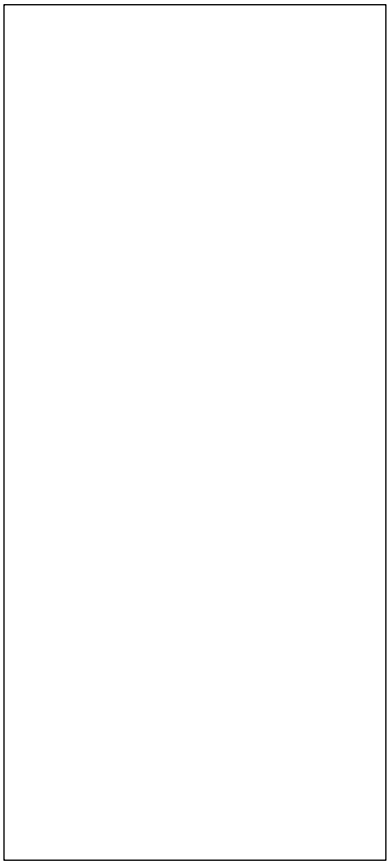


Figure 12. Graphic representation of non-overlapping habitat conditions for survival and propagation of *Sulfolobus* and plant and animal species.

thermoacidic habitats and those inhabited by plants, animals, and fish it is clear that preclude *Sulfolobus* cannot cohabitate or pose a pathogenic risk to people, animals, plants, or fish (**Figure 12**).

This series of unlikely events suggests that hyper-thermoacidic organisms have many inherent properties that render CinderBio processes safer for plants, animals, bacteria, and the environment relative to the thousands of ongoing microbial industrial processes using bacteria or eukaryotic organisms that thrive in moderate conditions (mesophiles). In our assessment, these sets of properties render the relative risk of biological threats posed to people and the environment to be far less with CinderBio strains and CinderBio processes than bioprocesses using mesophilic organisms.

XV.



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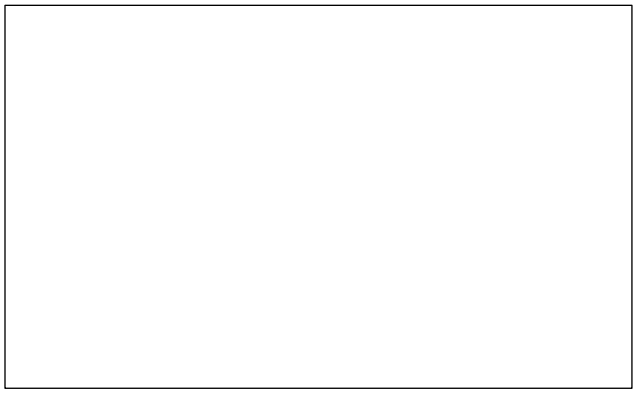
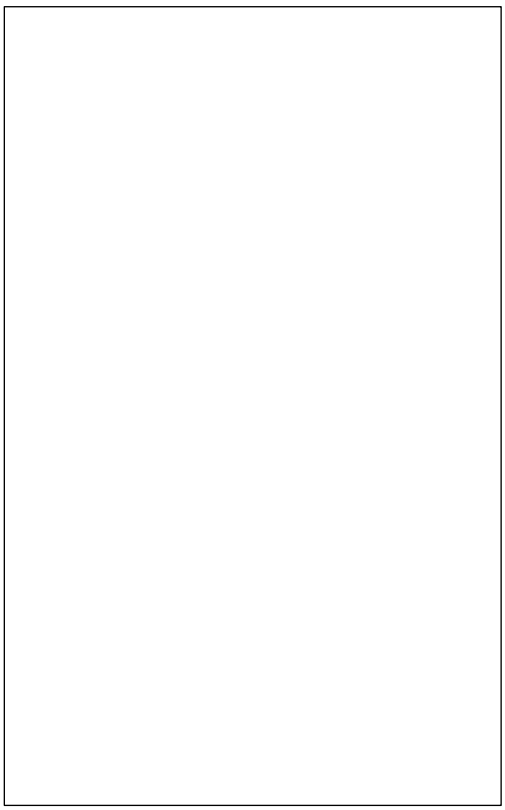
XVI. Recombinant Enzyme Production



We have built an integrated fermentation and downstream processing unit where production will take place. Our process involves microbial fermentation followed by

These steps are followed by chromatography to remove contaminating proteins and residual small molecules. The isolated enzymes are then subjected to a buffer-exchange process which leaves the pure enzyme of interest in a defined buffer (**Figure 14**).

XVII. Production Volume



These studies revealed that cell viability in active log-phase cultures was approximately 100% and an OD_{600} of 1.0 corresponds to **673×10^9** viable cells/liter (or CFUs). Therefore, a 50-liter batch of culture at OD_{600} = 1.0 would have 33.65×10^{12} viable cells.

While a large number, this number of cells is roughly equivalent to that generated from producing the same volume of beer or wine.

infinitesimally small fraction of microbial biomass produced annually to produce beer and wine alone.

All waste products from the CinderBio production process are aqueous biomolecules, none of which are known to cause any threat to human or environmental health.

XVIII. By-Products

Only the cell biomass resulting from production is expected. This material is neutralized with then biologically deactivated with bleach treatments and disposed. No living or dead cells nor DNA are included in our final enzyme products.

XIX. Potential Toxicity or Pathogenicity

Hyper-thermoacidic archaeal organisms are not known to produce endotoxins as they entirely lack the bacterial lipopolysaccharide (LPS) associated with septic shock caused by gram-negative bacteria. Likewise, septic shock caused by gram-negative bacteria is also thought to be caused by membrane components that are entirely different in archaea. Membrane lipids of Archaea are unique and distinct from those found in Eukarya and Bacteria. The polar lipids making up archaeal membranes consist of isoprenoid chains, 20–40 carbons long. Interestingly, a principle means to identify an archaeal microbe is detection of isoprenoid lipids. However, one study (the only such toxicity study on *Sulfolobus*) reports that very high concentrations of *Sulfolobus* membrane fractions can induce local hypersensitivity and pyrogenic activity when directly injected into sensitized animals (Galdiero et al., 1993). Importantly, these observed responses were, 1) only observed in highly sensitized animals, and 2) required injections of 2 milligrams (2×10^{-3}g) of *Sulfolobus* material to elicit a lesser response than 50 micrograms (50×10^{-6}g) of *E. coli* membrane fractions (20-40x). In other words, a response to

Sulfolobus membranes was 20 to 40 times less toxic than *E.coli* and only observed in sensitized animals with direct injection (Galdiero et al., 1993). Notably, the authors of this toxicity study also point out the near impossibility of *Sulfolobus* pathogenicity.

"The physiological parameters of S. solfataricus (optimal growth temperature 88 T, minimum growth temperature 70, optimal pH 3-0, maximum pH 5-0) prevent this bacterium from being even an occasional parasite of higher organisms." -(Galdiero et al., 1993).

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XX. Personal Protective Equipment (PPE) and Engineering Controls

Irrespective of the perceived low-risk associated with fermentation of the CinderBio strains , standard laboratory PPE are employed in all laboratory and production operations. These include nitrile gloves, eye protection and laboratory coats. We also employ engineering interventions for safety, including secondary containment of all liquid containing and handling equipment and dedicated exhaust ventilation on the reactor and downstream processing (DSP) stations (see **Figure 14**).

There are four laboratory/production functions that involve exposure to CinderBio strains in viable forms and in crude biological material originating from CinderBio strains (**Table 1**). In all cases personal protective equipment is used. The exposures are minimal and primarily involve handling liquids in a series of sealed systems. With routine cell culture the cultures are in sealed flasks or bottles and accessed to take manual readings by removing 1 milliliter of culture into a cuvette and measuring the optical density in a spectrophotometer. Production-scale fermentation is also almost entirely free of manual liquid handling.

All equipment including the reactor are equipped with secondary containment and dedicated ventilation.

WORKER ACTIVITY	Protective Equipment /Engineering controls	# of Workers Exposed	Maximum Duration (hrs/day)	Maximum Duration (days/yr)
Routine cell culture (< 2liters)	Lab PPE, secondary containment, ventilation	3	2	100
Production Scale Fermentation	Lab PPE, secondary containment, ventilation	3	2	100
Routine Molecular Biology	Lab PPE	5	2	100
Biochemistry	Lab PPE	5	5	200

Table 1. Worker activities and PPE and engineering controls to reduce risks.

XXI. Environmental Release and Disposal

In our current production method, the target enzyme is the only product, all other 'byproducts' are currently considered 'waste'. All waste streams from this process are liquid and first treated with sodium bicarbonate (baking soda; $NaHCO_3$) to neutralize the acidic media, to bring the pH to near neutral (pH 7-8) and to heavily stress any viable cells. The resulting waste liquid is then rendered biologically inactive by incubation with sodium hypochlorite (NaClO; bleach) for one hour at ambient temperatures. The neutralized and sanitized material is then discharged directly to municipal waste followed with 5-10x of municipal tap water. Spills and absorbent materials contaminated with CinderBio strain cells are put through the same neutralization/sanitation process when liquid and autoclaved if on absorbent material. Given the fragility of *Sulfolobus* in ambient conditions, our secondary containment, and our sanitation protocols, we expect undetectable numbers of viable cells to be released to the environment as we have failed to detect viable cells on work surfaces know to be contaminated with active CinderBio strain cultures. We list 'releases' in **Table 2** and note all waste is released to municipal waste (POTW) after treatment and sanitation. The risk of exposure to the public is extremely low.

Release Number	Amount of new substance released (CFU/day) To environment | To control Tech.	Media of release	Control Tech.	Efficiency
1	0 CFU 0 CFU	Municipal waste	$NaHCO_3$ NaClO	~100%

Table 2.

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