Characterization of residuals from novel anaerobic digestion of organic municipal solid waste for application as liquid fertilizer

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ABSTRACT

Management of organic municipal solid waste presents numerous challenges in India. Anaerobic digestion is one technology that can be used to address this problem, by transforming organic waste into methane via microbial activity. This process generates energy (in the form of methane) and a residual byproduct that can be used as fertilizer. Bottling and transportation of methane, however, presents numerous challenges. A novel anaerobic process is in development which would circumvent many of these challenges by instead producing a liquid biofuel. The purpose of this study is to determine if the residual byproduct from the novel process could still be used as fertilizer. A growth test failed to demonstrate the efficacy of the anaerobic residuals as a fertilizer, most likely because the residual byproduct was not dosed correctly. A follow-up growth test was able to show that the residual byproduct is not toxic to plant growth and if dosed in a lower concentration, could have potential as a fertilizer. The carbon to nitrogen ratio of the anaerobic residuals was favorable for plant growth. Further tests are needed to validate the use of the anaerobic residuals as fertilizer.

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1. Introduction

This report will address one promising avenue for waste management, anaerobic digestion. The focus of this thesis is to explore the potential for a novel anaerobic digestion process in India, and specifically how its residuals could be used as a liquid fertilizer.

1.1. Waste Management Overview

1.1.1. Municipal Solid Waste Challenges

Waste management is a significant concern worldwide, particularly in countries with a high population density like India (Sharholy et al., 2007). Municipal solid waste (MSW) management focuses only on waste generated by households, and not industrial, hazardous, and agricultural wastes. Increasingly, MSW management practices are receiving enhanced scrutiny by local governments and scientific researchers alike. As of 2000, around half of all MSW in the world is landfilled, and 30 percent of that waste is the organic fraction (Mata-Alvarez and Llabres, 2000).

In more highly developed areas, many waste management plans aim for 'zero-waste;' instead of disposal, and waste management planners try to find a myriad of ways to reuse and repurpose both synthetic materials and organics to avoid landfilling (Ngoc and Schnitzer, 2009). In 2011, the United States generated 250 million tons of waste, which included materials that could be recycled and composted (EPA, 2013). Less than 12 percent was used for energy recovery.

Landfilling waste is highly undesirable due to space considerations, especially in nations with a high population density. Incineration, or burning waste, is also frequently employed in waste management. But incineration can be toxic to the environment. The content of MSW oftentimes contains chlorinated compounds,

which can form hydrogen chloride and byproducts during incineration (Hartmann and Ahring, 2006).

Moving towards more sustainable waste management practices, experts envision a hierarchy of waste treatment methods: first minimization and reuse, then recycling and composting, followed by energy recovery, and treatment and disposal only as a last resort (EPA, 2013). More sustainable approaches, however, oftentimes require advanced planning as well as investment and experience in technology that can make such waste management less attractive in developing nations. As such, source reduction and reuse are infrequently target programs in these areas.

In less economically developed countries, the challenge is to implement a model that focuses on resource recovery, oftentimes in the absence of existing waste management. MSW can be rich in resources, and also highly varied in composition, generally consisting of food waste, paper, plastic, metal and glass; hazardous household items, such as electric light bulbs, batteries, and discarded medicines, are also common (Ngoc and Schnitzer, 2009). Resource recovery is both an opportunity and a challenge in waste management.

Wilson (2007) identified six broad drivers for improving waste management practices: public health, environmental protection, resource recovery, institutional responsibility and public awareness. Additionally, he noted that the resource value of waste provides a livelihood for the urban poor (known as 'waste picking'). Given the high removal rate of valuable recyclables, via waste pickers, and the high fraction of MSW that is food waste, the bulk of the remaining waste is organic.

Resource recovery, particularly of organics, presents an opportunity to close the energy and nutrient loop. As Hansen *et al.* (2006) noted, "Waste treatment options allowing recycling of the content of organic matter and nutrients to agricultural land might be a method for closing the cycle between city and agriculture and simultaneously reduce the production and use of commercial fertilizers."

Additionally, improved waste management practices can lower global warming emissions. Eriksson et al. (2005) observed "landfilling of all waste contributes most to the global warming potential of the studied scenarios," and also

noted that recycling of nutrients and materials led to lower global warming emissions than incineration.

1.1.2. Background on India

India is home to more than 1.2 billion people, and with a rapidly growing economy is finding a foothold in the developed world. Economic growth averaged seven percent per year between 1997 and 2011 (CIA, 2014). As a result, economic growth and urbanization have created lifestyle changes that can be correlated to an increase in per capita waste generation of about 1.3 percent per year (Ambade et al., 2013). Like many developing countries, India faces swelling waste management problems and plenty of challenges in addressing them (Annepu, 2013). With a rapidly developing economy, India also has enormous opportunities in waste management.

In particular, cities in India face serious problems in the management of MSW (Kumar et al., 2009). Waste generation generally rises proportionally to population increase and urbanization, and these demands require more land for the ultimate disposal of this MSW (Idris et al. 2004). The annual load of solid waste in Indian cities rose from 6 million tons in 1947 to 48 million tons in 1997. At the current growth rate of 4.25 percent per year, annual waste should reach 300 million tons by 2047 (Sharholy et al., 2007). Additionally, Kumar et al. (2009) observed that in most Indian urban centers, waste is merely disposed of into low-lying areas and unregulated landfilling is common in most cities.

Furthermore, countries with a greater population in rural areas generally produce more organic waste and fewer recyclable items (Idris et al., 2004). As a heavily agricultural nation, India creates high volumes of organic waste. Rural and urban areas of India face distinct and pervasive waste management problems, and both are ripe for improved management strategies.

The impact of reduced waste on quality of life could be significant. The portion of organic waste in MSW generally increases as socio-economic status decreases, so the poorest have the most to contribute, in terms of volume, and the

most to gain, in terms of public health (Sharholy et al., 2008). Improper disposal can have an enormous effect on human health and the environment, as disposal sites tend to be located in poorer districts. Organic waste can contain pathogenic bacteria, such as *Salmonella*, *Escherichia coli*, and heavy metals such as copper, cadmium, lead, mercury, and arsenic (Shyamala and Belagali, 2010).

Waste management problems in India are endemic and characteristic of poverty in developing nations. Perhaps optimistically, these problems also appear to have interesting solutions that would not only improve public health and the environment, but also empower the local community and present opportunities for small-scale entrepreneurship.

1.2. Introduction to Anaerobic Digestion

Anaerobic digestion is the process of transforming organic material into methane (also known as biogas and biomethane) in the absence of oxygen, via microbial activity. The traditional anaerobic digestion process was developed within the past few decades in order to develop useful energy from MSW (Sans et al., 1993). Biogas is commonly produced at an industrial scale, particularly in Europe, China, and India.

Anaerobic digestion is an intriguing approach to waste management, and is oftentimes cited as the most economical and energy-efficient biological treatment method (Mata-Alavarez and Llabres, 2000). In simple terms, anaerobic digestion allows microbes to decompose organic matter in the absence of oxygen. Anaerobic digestion can produce methane, carbon dioxide, and low-molecular weight intermediates such as organic acids and alcohols.

In addition to production of methane, anaerobic digestion also creates a nutrient-rich residual byproduct that is frequently used as fertilizer. The application of residual biomass as liquid fertilizer is an opportunity to reduce demand for chemical fertilizers and lessen the volume of MSW that is landfilled. Moving waste management away from landfills and incineration is a far more effective way to

recycle nutrients in the context of sustainable waste treatment (Hartmann and Ahring, 2006).

1.2.1. Scope of Project

Though biogas is useful waste management technology, it does have some drawbacks, notably the infrastructure and energy needed to capture, bottle and store methane gas. Researchers have begun to consider ways to alter the traditional method in order to overcome this drawback. Transitioning to a liquid fuel process would be useful in this regard.

Dr. Greg Stephanopoulos' chemical engineering laboratory at MIT currently has a modified anaerobic pilot study ongoing (http://bamel.scripts.mit.edu/gns/research/biofuels/). The key products of the process are lipids that can be sold as a fuel additive, but the digestion also produces a byproduct of residual sludge. The goal of this project is to characterize and propose applicability for residuals from the novel anaerobic process.

It is important to have well-thought-out plans for residuals. The goal is to have a waste-reduction technology that does not create a secondary waste stream as a byproduct. Finding a useful destination for the residuals is important to establish bioliquid production as a good waste management practice.

1.2.2. Municipal Solid Waste to Biofuels Process

The traditional anaerobic digestion method for MSW has four stages with different types of microbes: hydrolytic, fermentative, acetogenic, and methanogenic (Parawira et al. 2004). In the process studied here, methanogens are inhibited by the low pH to produce volatile fatty acids instead of methane.

A number of factors can inhibit methanogens during anaerobic digestion, but the key parameter in this study is pH. For example, Parawira et al. (2004) showed that acidic conditions in a reactor during anaerobic digestion inhibited methane generation and resulted in very low gas production. In a mathematical model of

volatile fatty acids production in a plug-flow reactor, pH was found to be the single most critical parameter for production (Negri et al., 1993). The Stephanopoulos laboratory controls pH with sodium hydroxide to inhibit methane production. Instead of producing methane biogas, the anaerobic stage generates a liquid product rich with volatile fatty acids.

Current methodology in the laboratory for developing liquid biofuels is as follows: food waste, collected from the MIT campus, is mechanically disrupted in an industrial blender. The food waste is found to consistently have a solids composition around 20 percent by mass. The influent is then placed into an approximately six-gallon anaerobic digester at about ⅔ full. Activated sludge from the Deer Island Waste Water treatment plant is added to promote microbial activity. Sodium hydroxide is added to maintain pH at 5.5, which inhibits production of methane. The reactor temperature is held constant at 37 degrees Celsius. The waste is left in the reactor for five days, then removed and separated by centrifuge into liquid (volatile fatty acids) and solids (residual biomass). The liquid is then moved to an aerobic reactor for an additional five days for transformation into 16 to 18 carbon chain triglycerides. The schematic is shown in Figure 1 (Courtesy of Devin Currie).

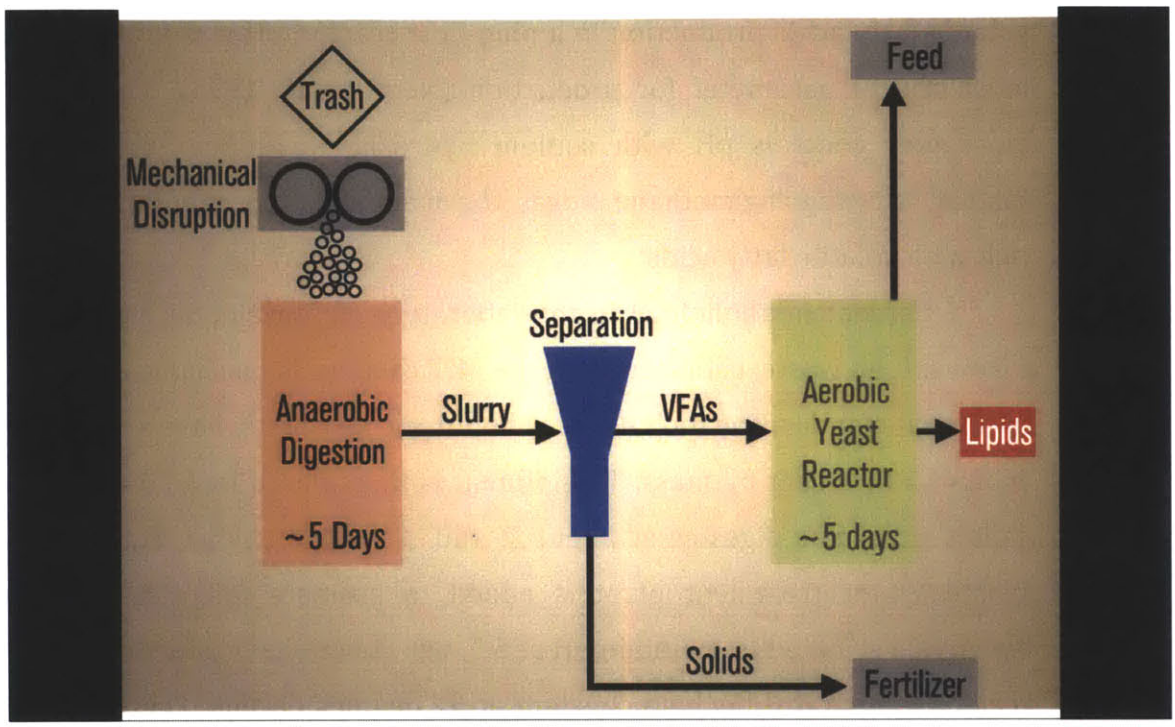

Figure 1: Schematic of liquid biofuel process.

2. Literature Review

2.1. Biogas in India

Biogas is a very versatile energy source. It can replace fossil fuels in power heat production and vehicles (Weiland, 2010). Given biogas' adaptability, it is no surprise that its reputation as a promising fuel source continues to grow. Anaerobic digestion on the industrial scale is viewed as a very promising source of energy for future generations. Compared to hydro, solar, and wind power, anaerobic digestion of biomass requires less capital investment and has a lower per unit production cost (Rao et al., 2010).

With 70 percent of India's population in rural areas, there is great incentive to develop alternative energy supplies that are accessible far from dense urban centers (Bhat et al., 2001). Biogas is currently very popular in India, though disseminated unevenly throughout the country. For example, a study of the town of Sirsi revealed that 85 percent of homes with small-scale biodigesters were able to meet their energy needs simply with the generated biogas (Bhat et al., 2001). Almost half of homes in the town were already in possession of the equipment. Popularity of the process continues to grow.

For a broader perspective, expansion of the biogas industry could be a piece of India's long-term energy security. Today around 80 percent of the world's energy comes from burning fossil fuels (Rao et al., 2010). Given the finite amount of petrol and political instability of oil-rich nations, there is great demand for effective methods of generating alternative fuels.

2.2. Inputs and Process

Justification for anaerobic digestion is well established in the literature. Food waste has a high content of moisture and organic matter, and is viewed as an ideal substrate for anaerobic digestion (Jiang et al., 2013).

Important considerations in the startup of an anaerobic digester are the organic load and food-to-microorganism ratio (Meng et al., 2014). Additionally, the quality of the biogas is dependent on the content of the waste. A few key components are carbohydrates, lipids, proteins, as well as the presence and species of cellulose, hemicellulose and lignin fractions (Hartmann and Ahring, 2006).

A significant challenge in the digester startup process is the portion of solids of the influent stream. Anaerobic degradation of MSW in a reactor with influent of a high-solids fraction has been found to be more difficult in both startup and system control than for a waste stream with low-solids influent (Nopharatana et al., 1998).

Generally, the influent waste is pretreated to make the digestion process more efficient. In particular, the hydrolyzing of starch, grease and lignocellulose expedites the digestion phase (Meng et al., 2014). Co-digestion of sewage sludge along with MSW has been shown to increase the anaerobic digestion potential (Gunaseelan, 1997). Various types of reaction processes have been studied, and D'Addario et al. (1993) found that a batch system performs best.

2.3. Application as Liquid Fertilizer

Scientific literature contains numerous examples of anaerobically digested waste products being used as liquid fertilizer and evaluations of the efficacy of that fertilizer. A few key examples are discussed here:

Mantovi et al. (2005) found that an application of biosolids from sewage sludge on agricultural land for 12 years improved soil fertility. The biosolids gave a crop yield similar to chemical fertilizer, but excessive nitrogen was problematic. Haraldsen et al. (2011) found that applying liquid fertilizer, digested from household waste, gave significantly higher yields of barley grain and straw than unfertilized control crops. They found anaerobically digested household waste to be an excellent source of nitrogen.

Ryu et al. (2010) used methane fermentation digested sludge on paddy fields, and found that it could be successful as liquid fertilizer if the crops and field were

well managed and maintained. Additionally, taste tests were performed with rice in two different years: the first year yielded no taste difference from a control group, and the second year experience a five percent taste difference.

Leidl et al. (2006) suggested that effluent from anaerobically digested poultry waste showed promise as an alternative fertilizer. The success of the fertilizer, however, was largely crop dependent: among test crops, corn yield improved and tomato yield dropped.

Baba et al. (2013) found that grass yield from plots fertilized with anaerobically digested crude glycerol was 1.2 times higher than that of non-fertilized plots.

In another study, the authors examined the use of anaerobically digested wastewater residuals as fertilizer for corn. Application of effluents increased the pH of the soil (Ribas et al., 2010). They found that a high dose of residuals did not negatively affect maize development, and also concluded that residuals application promoted nitrogen adsorption. The authors suggested the anaerobic residuals could have potential as a fertilizer.

A similar field study examined the effect of anaerobic digestion residuals as a fertilizer on potato crop yields (Garfi et al., 2011). The residuals were applied every 10 days, with varying doses according to nutrient needs at each stage of plant growth. Fertilizer doses were calculated based on farmer interest, crop field availability, and volume of residuals produced in small, household digesters. Compared with the control group, the potato yield increased 27 percent with the residuals application, and 15 percent with compost (Garfi et al., 2011). The authors concluded that the residuals could be applied successfully in a range of doses.

Alburquerque et al. (2012) examined an anaerobic residuals derived from an industrial co-digestion plant. The residuals was composed of pig slurry, sludge from a wastewater treatment plant, and biodiesel wastewater. The C/N ratio of the residuals was 1.2. It was tested as a fertilizer on watermelon and cauliflower, compared with mineral fertilizers, manure, and unamended soil. With regards to the cauliflower, all three of the organic treatments performed poorly, and mineral fertilizers provided a much better yield. The watermelon yield gave significantly

better results for mineral fertilizer and residuals than manure and no-additive. The authors also found that the residuals provided a high amount of ammonia nitrogen, which volatilized rapidly and was only available to plants short-term. They suggested the differences between yields in cauliflower and watermelon may be seasonal, as cauliflower is a winter vegetable and has a longer growing cycle that may require more nitrogen.

Lemke et al. (2012) compared anaerobically digested swine manure to undigested manure and traditional chemical fertilizers as soil amendment for barley in a 3-year study. They found that all three treatments produced significantly greater yields than no treatment at all, but also found, on average, slightly better performance for the anaerobic residuals as a fertilizer (Lemke et al., 2012). The authors also concluded that the residuals volatilized less ammonia and was less of a contributor to greenhouse gas emissions than undigested manure.

In addition to growth tests present in the literature, the chemical composition of anaerobic residuals has also been thoroughly evaluated. Nutrient content is a crucial parameter in the efficacy of residuals as fertilizer, and a few critical examples are discussed here:

Tani et al. (2004) found that the combination of livestock waste and industrial waste was nutritionally complementary. They completed grass-growth experiments to evaluate anaerobically-digested dairy slurry as fertilizer, and found that residuals dissolved with four percent chemical fertilizer produced the greatest crop yield. Their results suggested that dairy slurry, in combination with chemicals, can be used on agricultural fields as a replacement for traditional chemical fertilizers by themselves (Tani et al., 2004).

Compared with manure, anaerobically digested residuals have a higher ammonium concentration, higher total nitrogen content, lower organic matter content, decreased biological oxygen demands, a higher pH, and a lower carbon to nitrogen (C/N) ratio (Möller and Müller, 2012). Additionally, the authors compared untreated manure to anaerobically digested manure in a field experiment. Their results suggest that a significant difference in crop yield can only be found when the manures were incorporated directly into the soil. This suggests that a significant

portion of the total nitrogen in the residuals was volatilized as ammonia (Möller and Müller, 2012). Furthermore, they found that the anaerobic digestion process had no effect on phosphorus availability. The authors suggested, in field application, appropriate dilutions are between 1:4 and 1:8 ratios of anaerobic residuals to water.

Tambone et al. (2010) studied anaerobically digested sludge from municipal waste water treatment, suggesting that the sludge could be useful as a fertilizer because of its well-documented high nitrogen and phosphorus content and low carbon to nitrogen ratio. The authors found that the anaerobic digestion residuals studied had a high biological stability, similar to that of composts (Tambone et al., 2010). Their work suggested the soil amendment properties of digested sludge were inferior to those of compost.

Rigby and Smith (2014) compared the physiochemical characteristics of various biowastes, including anaerobically digested food waste, and found nutrient contents to be as follows: total nitrogen ranging from 5.46 to 7.21 kg/m^3 and total phosphorus ranging between 178 to 362 g/m^3. Mineral nitrogen ranged from 39.3 to 71.8 percent of total nitrogen, with the percent of mineral nitrogen increasing in samples that had been left in storage (Rigby and Smith, 2014). Additionally, the samples were field-tested in a growth experiment using ryegrass. They found a significant increase in crop yield in all treatments that contained high levels of nitrogen, including the anaerobic residuals (Rigby and Smith, 2014). The yield was comparable to other treatments with similar nitrogen content.

Nkoa (2014) suggested that normal biochemical values for anaerobic residuals are as follows: percent total nitrogen – 3.1-14.0; percent total carbon – 36.0-45.0; C/N ratio – 2.0–24.8; percent total phosphorus 0.04-0.25. Nkoa also found that the nitrogen content of residuals from plant biomass would generally be 2-3 kg/m^3, a C/N ratio between 5 and 8, and low biodegrability (Nkoa, 2014). The author suggested there is high variability within the residuals 'group' of organic materials, and that their efficacy as a fertilizer is not as good as mineral fertilizers, but better than animal manure.

Moreover, researchers found a few important considerations in the application of waste-derived liquid fertilizer: separation of nutrients allows for

better nutrient management than straight land application of undigested or composted animal residuals, as phosphorus remained in the solid fraction and nitrogen portioned into the liquid fraction (Liedel et al., 2006). This phase separation would allow farmers to deliver more targeted nutrients. Liedel et al. (2006) also noted that the yield from application of liquid fertilizer is highly crop dependent.

Generally, heavy metals were not detected in residuals from anaerobically digested crude glycerol and it was safe to use on land (Baba et al., 2013). Additionally, physical and biological properties of anaerobic residuals should be considered.

Weiland (2010) demonstrated another advantage of liquid fertilizer, improved flow properties. The residuals were able to penetrate faster into the soil than a dry (solid) fertilizer, reducing the risk of nitrogen losses through ammonia emissions. Properly optimizing the viscosity could turn the residuals into a desirable product.

Finally, Müller et al. (2013) examined the hygienic effects of using anaerobic residuals as fertilizer. The authors compared a traditional fertilizer with anaerobic residuals from combined municipal waste and liquid manure. The treatments were administered for two years, and microbial analyses were performed, measuring for total bacteria, *E. coli*, *Bacillus* spores, clostridial spores, molds and yeasts. They found that the pH was higher than expected for of the residuals examined (Müller et al. 2013). They also found, generally, that bacteria counts for crops treated with anaerobically digested residuals were similar to those treated with animal manure. These bacteria counts were deemed safe by national standards (Müller et al., 2013).

In summary, use of anaerobically digested residuals as fertilizer is well documented and justified by the literature. Though we have no direct comparison with regards to pure MSW, a variety of residuals are examined above, with varied results, and validate the need to explore further.

3. Experimental Methods

The following subsections describe the materials and experimental methods used in this study.

3.1. Anaerobic Digestion Process

Residual sludge was produced via a novel anaerobic digestion process performed in the Stephanopoulos laboratory. Full experimental methods are given in Appendix K. Inputs were mixed food waste from MIT's Stata Center.

Initial food waste was 10 percent solids (reactor run four) and 15 percent solids (reactor run five) by mass. Residuals from reactor runs four and five were mixed thoroughly. Five mL of residual sludge was placed into a 50 mL conical tube, and lyophilized (freeze-dried) to remove all liquid. The combined residuals were determined to have a solids content of 19.4 percent.

3.2. Plant Growth Experiment

3.2.1. Background on use of Arabidopsis in plant growth experiments

In order to test the efficacy of the laboratory residual sludge as a liquid fertilizer, a plant growth experiment was set up in the laboratory. Arabidopsis, a small flowering plant, is well suited as a laboratory test plant due to its short life cycle (Sijomons et al., 1991). A manual on Arabidopsis growth can be found in Appendix G.

3.2.2. Development of treatment plan and dose

Four different treatments were applied to the plants: diluted laboratory residual sludge, digestate from a traditional anaerobic digestion process, fertilizer, and compost. As two different anaerobically digested residuals are described, for

clarification, the laboratory-generated residual will be known as 'laboratory residual sludge' or 'laboratory residuals' and the residuals from the Crapo Hill landfill will be known as 'digestate.' Additionally, a control group of pots received no amendments.

A mixture of 50 percent sand and 50 percent loams (by volume) was used, to mimic natural growing conditions. The sand and loams were purchased from a nursery in Massachusetts.

Each pot contained 300 mL of the loam/sand mixture. Each pot was labeled according to treatment, then the soil was wetted and lightly patted down. Two Arabidopsis seeds were placed with tweezers into each pot onto the soil's surface. Seeds were planted on March 19, 2015.

The pots were then placed into a cold room at 4 degrees Celsius for three days to encourage germination (see Appendix G). After 72 hours, they were taken back into the laboratory and placed under an indoor plant growth light (constant light) at 22 degrees Celsius. Treatments were applied immediately after the pots were moved from the cold room to bench top.

Seven days after seeding, plant growth became visible and the light was switch off for eight hours at night by automatic timer to mimic exposure to outdoor light. The pots were watered nearly daily (see Appendix E). Prior to sprouting, each pot was misted with a spray bottle to moisten to soil. The intent was to avoid excess water that might move or disrupt seeds, in lieu of watering. Once the sprouts appeared, the pots were watered until the water just began to seep out the bottom slits of the pot, to thoroughly soak the plant roots.

Pots were randomly arranged in the trays and rotated every 48 hours as described in Appendices D and E.

3.2.3. Dosage Determination

Two academic studies were referenced for determining dosage of anaerobic residuals. Ribas *et al.* (2010) calculated residual dose based on nitrogen content in

the anaerobic residuals, and applied an amount of residual with comparable nitrogen content to fertilizer. The amount of nitrogen 'required' was based on soil mass. No information was provided on root or soil depth, and that plants were cut off at the soil level for evaluation. Garfi *et al.* (2011) dosed approximately 50 kg N/ha of total organic nitrogen and ammonia nitrogen. Since this calculation relied on surface area, rather than soil mass, it was more straightforward to scale to our laboratory study, as we do not know how the root depth required for maize compares with Arabidopsis. Additionally, Garfi et al. (2011) calculated fertilizer doses based on the amount of anaerobic residuals produced in household digesters, a direction we hope this research takes.

Anaerobic residuals doses ranged from 200,000 L/ha to 75,000 L/ha for a field potato trial in the Garfi study. The midpoint of the range was converted into mL/pot for an eight cm by eight cm pot, resulting in a dose of 88 mL/pot. The same dose was used for both the Crapo Hill digestate and laboratory sludge. Additionally 88 mL of the Miracle-Gro and 88 mL of water for the compost and control pots were all dosed at the same time. This would ensure that any differences in growth could not be accounted for by soil saturation of liquid at the time of dosing, but rather by the different chemical composition of each treatment.

All amendments were administered 72 hours after seeding, when the pots were removed from the cold room. The laboratory residual sludge and Crapo Hill digestate formed a solid layer on the top of the soil when added. Because this solid layer could prevent plants from sprouting, these treatments were administered only once. To keep all treatments as comparable as possible, the Miracle-Gro was also only administered once.

3.2.3.1. Control

For the control pots, no was added treatment, and the pots were watered and rotated as described in Appendices D and E.

3.2.3.2. Fertilizer (Miracle Gro)

Upon removal from the cold room, 88 mL of Miracle Gro dilution (0.5 teaspoon/1 gallon water, per directions on box) was administered onto the pots. This treatment was applied only once, to provide a more direct comparison with digestate and the laboratory residual treatments.

3.2.3.3. Worm castings compost

Organic Materials Review Institute (OMRI) Listed 'Wiggle Worm' soil builder earthworm castings was mixed in with soil/sand at 1:3 ratio per instructions on bag. As with the other treatments, 300 mL of soil combination was placed in each pot.

3.2.3.4. Traditional anaerobic digestate

Digestate was collected from the Crapo Hill landfill in New Bedford, MA. The CommonWealth Resource Management Corporation owns this anaerobic digester and landfill gas-to-energy facility. According to the facility operator, inputs are slurried food waste from food processing industry and leachate from compost operations. The inputs are typically four to 12 percent solids by mass and greater than 95 percent organic material. The pH ranges from four to six. The retention time in the digester is approximately 30 days, with a continuously mixed, mesophilic anaerobic digestion. Temperature is maintained at 98 degrees Fahrenheit. The digestate was lyophilized and found to be 1.95 percent solids by mass. Compared with the laboratory residual sludge, this digestate had $1/10^{th}$ of the solids content. Therefore, it was applied to the soil without any modifications.

Upon removal from the cold room, pots were treated with 88 mL of Crapo Hill digestate. The digestate was only dosed once during the duration of the experiment.

3.2.3.5. Laboratory residual sludge

Laboratory residuals were dosed upon removal from the cold room, 88 mL/pot (see methodology for dosing below). This dose contained laboratory residual sludge that had been diluted 1:10 in water so that its solids content was approximately two percent (see traditional anaerobic digestate above). Two hundred fifty mLs of the laboratory residual sludge was mixed with 2,250 mL of water to produce 2.5L. No further modifications were made to the laboratory residual sludge. It was applied only once.

3.2.4. Development of randomized growth grid and growing experiment

A randomized growth grid and system for rotation was developed to ensure equal treatment of all plants, as described in Appendices D and E.

3.2.5. Methodology for data collection

Forty days after seeding, the plants were harvested for measurement. Per common Arabidopsis growth methodology, the plants were removed from soil, rinsed with distilled water, lyophilized (to remove liquid) and weighed (Talon et al., 1990; Huynh et al., 2005; Creelman et al., 1990).

3.2.5.1. Photographs of plants

Each plant was photographed. A complete set of photographs was provided to the MIT Tata Center, sponsor of this research. The images are categorized into subfolders labeled by treatment.

3.2.5.2. Weighing plants

Each plant was placed onto weigh paper and weighed with precision to a tenth of a milligram. All plant weight data is listed in Appendix J.

3.2.6. Follow-Up Germination Test

Small sprouts began to appear after about seven days in pots with fertilizer, compost, and no treatment. In pots dosed with laboratory residual sludge or Crapo Hill digestate, however, the solids separated at the surface of the soil and appeared to prevent sprouts from breaking through. In order to determine the best methods for dosing in future work, three new treatments were devised and administered: (1) laboratory residual sludge diluted to 1:100 in water and 88 mL/pot dosed immediately after seeding; (2) laboratory residual sludge diluted 1:100 in water and 88 mL/pot dosed 72 hours after seeding; and (3) 2 mL of laboratory residual sludge directly mixed into 300 mL of soil per pot. Two pots were setup per treatment, with the methods for seeding exactly as in the primary experiment. Unfortunately, due to space constraints, these six pots could not be placed under the artificial light, and were instead placed on a windowsill after removal from the cold room. They were watered daily.

3.3. Chemical Composition

Samples of dry residuals, from both Crapo Hill and the laboratory experiment, were sent out to a soils analysis laboratory at the University of Massachusetts-Amherst for total nitrogen and total carbon. Methods for sample preparation are presented in Appendix F. The chemical report is in Appendix C.

4. Results

4.1. Chemical Analysis

Chemical analysis reports of residuals were obtained from two biogas facilities in India during January of 2015: Green Elephant and Mailhem, both in Pune, Maharashtra. Reports provided by the facility staff can be found in Appendices A and B.

Mailhem is anaerobically digesting organic MSW. Green Elephant is anaerobically digesting organics obtained from the food processing industry (see Appendix H). As such, the influent from Mailhem bears more similarity with the laboratory residual sludge, and this is reflected in the chemical analysis report of the anaerobic residuals.

Table 1: Chemical Analysis of anaerobic residuals

	%Carbon	%Nitrogen	C/N Ratio
Laboratory	41.2	3.4	12.12
Crapo Hill	32	4.2	7.62
Mailhem			11.61
Green Elephant			2

4.2. Plant Growth

4.2.1. Germination Success

Table 2: Plant counts per pot (note: two seeds planted per pot)

	Compost	Fertilizer	Control	Lab Sludge	Crapo Hill
Pot 1	0	1	2	1	0
Pot 2	1	0	2	0	0
Pot 3	2	1	0	0	0
Pot 4	0	0	1	0	0
Pot 5	2	1	2	0	0
Pot 6	1	2	1	0	0
Pot 7	2	1	1	0	0
Pot 8	1	1	1	2	0
Pot 9	0	1	2	0	0
Pot 10	0	1	2	0	0
Pot 11	2	2	0	1	0
Pot 12	0	0	1	0	0
Pot 13	0	1	0	0	0
Pot 14	2	2	1	0	0
Total	**13**	**14**	**16**	**4**	**0**
Average	**0.93**	**1.00**	**1.14**	**0.29**	**0.00**
Std dev	**0.92**	**0.68**	**0.77**	**0.61**	**0**
Fraction	**0.46**	**0.50**	**0.57**	**0.14**	**0.00**

Table 2 lists plant counts 40 days after seeding. Two seeds were planted per pot; fraction refers to total fraction of seeds that produced a plant for each treatment; average denotes the average plants per pot. Standard deviation was computed via the Microsoft Excel function.

Table 3 lists the results of the follow-up germination test. Thirteen days after seeding, the plants were counted and are listed below:

Table 3: Plant count for 1:100 dilution germination test (note: two seeds planted per pot)

	Dose at seeding	Dose at 72 hours	Residuals mixed in
Pot 1	1	0	1
Pot 2	1	2	2
Total	**2**	**2**	**3**
Fraction:	**0.5**	**0.5**	**0.75**

4.2.2. Plant Mass Data

Table 4 lists plant mass by treatment method:

Table 4: Plant mass, by treatment method

Treatment:	Average Plant Mass (mg):	Standard deviation:	Total mass (mg)
Traditional Sludge	0	0	0.00
Lab Sludge	0.58	0.46	2.30
Compost	6.61	4.68	85.90
Fertilizer	4.35	4.66	60.90
Control	3.84	3.06	61.50

Full results can be found in Appendix J. Average refers the average plants size within a given treatment. Standard deviation was calculated via Microsoft Excel function.

For illustrative purposes, a few photos are included. The largest plant was 16.9 mg, from the fertilizer treatment, and the smallest was 0.1 mg, from the laboratory residual treatment. The average size plant was 4.4 mg.

Figure 2: Average plant size, 4.4 mg

Figure 3: Largest plant, 16.9 mg

Figure 4: Smallest plant, 0.1 mg

5. Discussion

5.1. Implications drawn from growth experiment

The potential of this laboratory residual sludge as an effective fertilizer is still unknown. Though the follow-up germination test ruled out toxicity of the laboratory residuals to plants, the solids from the residuals formed a physical barrier that slowed down growth. This made it impossible to directly compare plants grown under no treatment, fertilizer, and compost to the plants dosed with laboratory residuals, because the plants dosed with laboratory residuals were much further behind in their life cycle.

Still, the follow-up experiment to test germination showed potential avenues for future research. Plants dosed with a 1:100 dilution sprouted in approximately the same 7-10 day window as the three non-residual treatments. This suggests that nothing about the chemical content of the laboratory residual would be counterproductive to germination or plant growth.

In terms of enhancing plant growth, the compost performed the best, followed by the fertilizer. Both showed an improvement, in terms of plant mass, compared with no treatment. This validates the use of Arabidopsis as a test plant in this experiment, as it responded appropriately to nutrient amendments. Perhaps the improvement in growth from compost could serve as a benchmark or goal in further testing the laboratory residuals.

The chemical composition of the laboratory residuals suggests this product can be used effectively as fertilizer. As with compost, a carbon to nitrogen ratio less than 24 is required for plant growth (USDA, 2011). Excess carbon, beyond that ratio, would encourage microbial growth in the soil, and those microbes would consume nitrogen needed by the plant, hindering plant growth. The laboratory residual sludge is below the C/N range where a surplus or carbon would inhibit plant growth, and thus ought to be explored further as a potential fertilizer.

5.1.1. Limitations

This study, though able to demonstrate that the laboratory residual sludge holds potential as a fertilizer, has numerous limitations. The laboratory residuals were only tested on a single plant in a laboratory setting. Arabidopsis is not a target crop for agricultural in India, where primary crops are wheat, corn and rice (Leff et al., 2004). Additionally, Indian climate and geography can be highly variable, particularly with yearly monsoon seasons, and this would affect the efficacy of any fertilizer product.

Furthermore, only the nitrogen and carbon content of the laboratory residuals were examined, when other nutrients can factor into productivity as a soil amendment. Potassium, for example, is a common nutrient in fertilizer and can be very valuable in growing rice (Rengal and Damon, 2008). The residuals were also not evaluated for other chemicals, such as zinc, copper, iron and manganese, which can be vital micronutrients to plant growth (Mortvedt, 1994).

5.1.2. Potential as value added technology

The goal of this project was to determine if the laboratory residuals could serve as a 'value-added' for the new anaerobic digestion progress. If current biogas facilities are collecting income from selling the residuals, then adopting a modified process should not involve loss of that income. At the very least, this residual sludge should be able to serve in the same role as in the previous process, where it can be sold as a fertilizer and not need to be disposed of as a waste stream.

Perhaps with further research, the potential as a fertilizer can be better characterized. A more thorough investigation of nutrient components and dosage parameters would go a long way to determining the value of this product.

5.2. Future Work

Examining how this process could be folded into a life cycle assessment and used to compare the liquid fuel to biogas production, on a larger, scale, would be useful in future work.

In terms of the plant growth study, this experiment was not replicated due to time constraints. If this research is continued, the following subsections provide suggestions and guidance for future work:

5.2.1. Suggested Improvements to experimental methods for future plant experiments

Determining the dose volume and dilution was the biggest challenge encountered in the growth experiment. Both the Crapo Hill digestate and laboratory residual sludge were originally dosed at a volume percent of 1.95 percent solids. The volume dosed was 88 mL, and this provided too much solid content. In the follow-up germination experiment, the laboratory residual sludge was dosed at a volume percent of 0.195 percent solids, again 88 mL, and this solids content did not prevent plants from sprouting.

In future experiments, the dilutions of laboratory residuals should be kept to around 1:100 solids in water; perhaps iterations could be created, of different dilution rates, until the highest possible solids content that does not prohibit plant growth is identified.

A suggested setup would be to compare 1:100 dilution of laboratory residual sludge and a 1:10 dilution of Crapo Hill digestate to compost treatment and no treatment plants. The compost, as producing the best growth data, could serve as a comparative goal for plant growth.

Additionally, it may be worth running a growth test in which the treatments are applied after plant sprouts have taken root. Perhaps early dosing caused seeds to shift and take longer to germinate, and allowing the plants to grow longer before applying treatments would be a more effective experimental method.

A more distant goal would be to compare the laboratory residual sludge as a fertilizer in a field test – perhaps using wheat or corn, which are commonly grown in both the United States and in India.

5.2.2. Suggested alterations to laboratory residuals

The most critical parameter evaluated in this report was the C/N ratio of the laboratory residual sludge. Investigating how changes with the influent – such as variations in the organic waste collected – alter the C/N ratio of the residuals could be useful data in a larger-scale implementation.

Additionally, the value of the laboratory residuals as fertilizer could increase greatly by adding a few micronutrients, such a zinc and copper, which could be targeted towards a specific crop.

6. Conclusions

This study evaluated the residual byproduct of a novel anaerobic digestion process for application as fertilizer. The purpose of this study was to determine if the byproduct can serve as a 'value-added' to a new type of biotechnology that seeks to reduce organic MSW. Anaerobic digestion has potential in both improving waste management practices and increasing energy security. Further, this technology makes for better waste management practices if the process can be a 'closed loop' system in terms of energy and nutrients: with the residual byproduct being repurposed rather than treated as waste.

The experiment could be called a successful failure. The growth test failed to demonstrate the efficacy of the laboratory residuals, but the cause of failed growth could largely be attributed to less than optimal dosage of the residuals. The follow-up germination growth test clearly showed that the laboratory residuals are not toxic or detrimental to plant growth. Further studies, with varied residual dilutions and dosage volumes, are suggested.

The growth tests, however, did suggest that Arabidopsis serves as a good test plant for the purposes of testing soil amendment growth properties. The plants responded well to both fertilizer and compost, with an increase in average plant size beyond no treatment. The compost treated plants, in particular, could serve as a benchmark goal for future growth tests.

Finally, the carbon to nitrogen ratio demonstrated that this residual has potential as a fertilizer, as the values were favorable for plant growth. Although this study did not succeed in validating the use of the laboratory residuals as fertilizer, it did provide a pathway for future research to further test the efficacy of the residuals, and also suggested that this byproduct has potential as an added-value to the new anaerobic digestion process.

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Appendix A: Mailhem Residual Sludge Report

Accurate Analytical Laboratory
Prof. Mrs. S.M.Nadkarni M.Sc. (Lond)
Tel.: 25652744 / Fax 25652744
E-mail :- shalini_nadkarni2006@yahoo.co.in

967/3/1, Bhagyadeep,
Near Ratna Memorial Hospital,
Off Senapati Bapat Road,
Pune - 411 053

TEST REPORT

REPORT No : ATM /L – 728 14.2.2015.

To,
Mailhem Ikos Environment Pvt. Ltd.,
14, Vishrambaug Society,
Senapati Bapat Road. Pune :- 411 016

Kind Attn : Mr. Sameer Rege.

Your Ref No :- Your Letter MIPL / R&D/03 Dated : 30.01.2015 Received On :- 30.01.2015.

Samples :- Three Liquid samples marked as,
1) Inlet Slurry Feed. For : COD, BOD, pH, TS, TSS, TDS, VS @550°C, TOC, N, C:N ratio.
2) Secondary Sludge For : COD, BOD, pH, TS, TSS,TDS,VS@550°C,TOC, NPK, C:N ratio, Humic Acid.
3) Overflow. For :- COD, BOD, pH, TS, TSS, VS @550°C.

Sample collected from: Aundh Plant. Pune.
Collected on: 30th January 2015, Time: 1: 00 P.M
Sent for Analysis on: 30th January 2015, Time: 1: 30 P.M
Sample collected by (MEPL Representative): Mrs. Srideepika S.

Test Conducted :To determine COD, BOD, pH, TS, TSS,VS @550°C, TOC, N P, K, C:N ratio, Humic Acid.

TEST FINDINGS :

Sr.No.	Tests	Unit	Inlet Slurry Feed.	Secondary Sludge	Overflow
1)	COD	mg/L	160169 . 49	106355.93	33541.66
2)	BOD (27°C, 3 days)	mg/L	59000 . 00	6000 . 00	10500 . 00
3)	pH	---	4 . 26	7 . 50	6 . 92
4)	Total Solids	mg/L	67292 . 00	53944 . 00	17376 . 00
5)	Total Dissolved Solids	mg/L	33540 . 00	6147 . 00	5665 . 00
6)	Total Suspended Solids	mg/L	33752 . 00	47797 . 00	11711 . 00
7)	Total Volatile Solids @550°C	mg/L	58488 . 00	39748 . 00	12072 . 00
8)	Total Organic Carbon	mg/L	146690 . 00	52109 . 00	13598 . 00
9)	Total Kjeldahl Notrogen	mg/L	4330 . 00	4486 . 00	----
10)	C : N ratio	---	33 . 8775	11 . 6159	----
11)	Total Phosphorus (P)	mg/L	----	3469 . 00	----
12)	Total Potassium (K)	mg/L	----	1100 . 00	----
13)	Humic Acid.	mg/L	----	2551 . 00	----

For Accurate Analytical Laboratory

M.P.G. / M. M. Mrs. S.M. Nadkarni.

Disclaimer:
- Sample submitted by the Customer in their own container.
- Above analysis result is valid only for the specific sample as stated above, submitted for testing, without any bias to its source.
- We claim no responsibility for changes made in the report after dispatch e.g use of whitener or eraser.
- Above Test Report cannot be produced as legal evidence without our prior written permission.
- Sample stored for one month and Test Report for one year from the date received.
- Duplicate copies of Report or Invoice will be charged extra.

Appendix B: Green Elephant Residual Sludge Report

Parameter	value	Unit
germinable seeds	0	units
Salmonelas	0	units
Foreign substances bigger than 2 mm	0.5	% of total solids
sand/stones	5	% of total solids
Acids as Acetic acid equivalent	1500	mg/L
Dry matter	3.5	% of fresh matter
Ignition loss	50	% of dry matter
C/N ratio	2	
Density	1050	mg/L
pH	8	
Salinity content	18	g/L
Nutrient content total		
Nitrogen	16.5	% of dry matter
Nitrogen	0.5	% of fresh matter
Phosphate (P2O5)	5.5	% of dry matter
Phosphate (P2O5)	0.18	% of fresh matter
Potassium (K2O)	6.4	% of dry matter
Potassium (K2O)	0.2	% of fresh matter
Magnesium (MgO)	0.32	% of dry matter
Magnesium (MgO)	0.01	% of fresh matter
Sulphur (S)	0.8	% of dry matter
Sulphur (S)	0.03	% of fresh matter
Lime (CaO)	6.87	% of dry matter
Soluble nutrients		
Ammonical nitrogen (NH4-N)	3500	mg/L
Nitrate (NO3-N)	1	mg/L
Heavy metals		
Lead (Pb)	3.5	mg/ kg Dry Matter
Cadmium (Cd)	0.35	mg/ kg Dry Matter
Chrom (Cr)	7.5	mg/ kg Dry Matter
Copper (Cu)	5	mg/ kg Dry Matter
Nickel (Ni)	15	mg/ kg Dry Matter
Mercury	0.1	mg/ kg Dry Matter
Zinc (Zn)	20	mg/ kg Dry Matter

Appendix C: Laboratory residual sludge and Crapo Hill digestate chemical analysis report

Soil and Plant Tissue Testing Laboratory
203 Paige Laboratory
161 Holdsworth Way
University of Massachusetts
Amherst, MA 01003
Phone: (413) 545-2311
e-mail: soiltest@umass.edu
website: http://www.soiltest.umass.edu/

April 30, 2015

Julie Karceski
MIT
77 Massachusetts Ave
Cambridge, MA 02139
karceski@mit.edu

Re: Total Carbon and Nitrogen

Sample ID	% Carbon	% Nitrogen
Chill	32.0%	4.2%
Lab	41.2%	3.4%

Thank you for choosing the UMass Soil Testing Lab!

Appendix D: Growth Grid and Randomization Methodology

A9	E14	D13	E3	C5	A14	Empty	D9	D6	A13	A3	E5
C6	A5	C1	A1	E6	B8	C11	Empty	B14	B12	D14	E8
B11	D11	D5	B1	C8	D10	E2	A7	B7	B4	D8	C2
A12	C4	C3	A8	E9	C10	C9	D2	B6	E1	E13	B2
A4	E11	D1	E7	B10	C7	E12	C13	D12	A2	D3	A10
D7	C12	C14	E10	B9	B5	D4	B13	B3	A11	A6	E4
Tray 1			Tray 2			Tray 3			Tray 4		

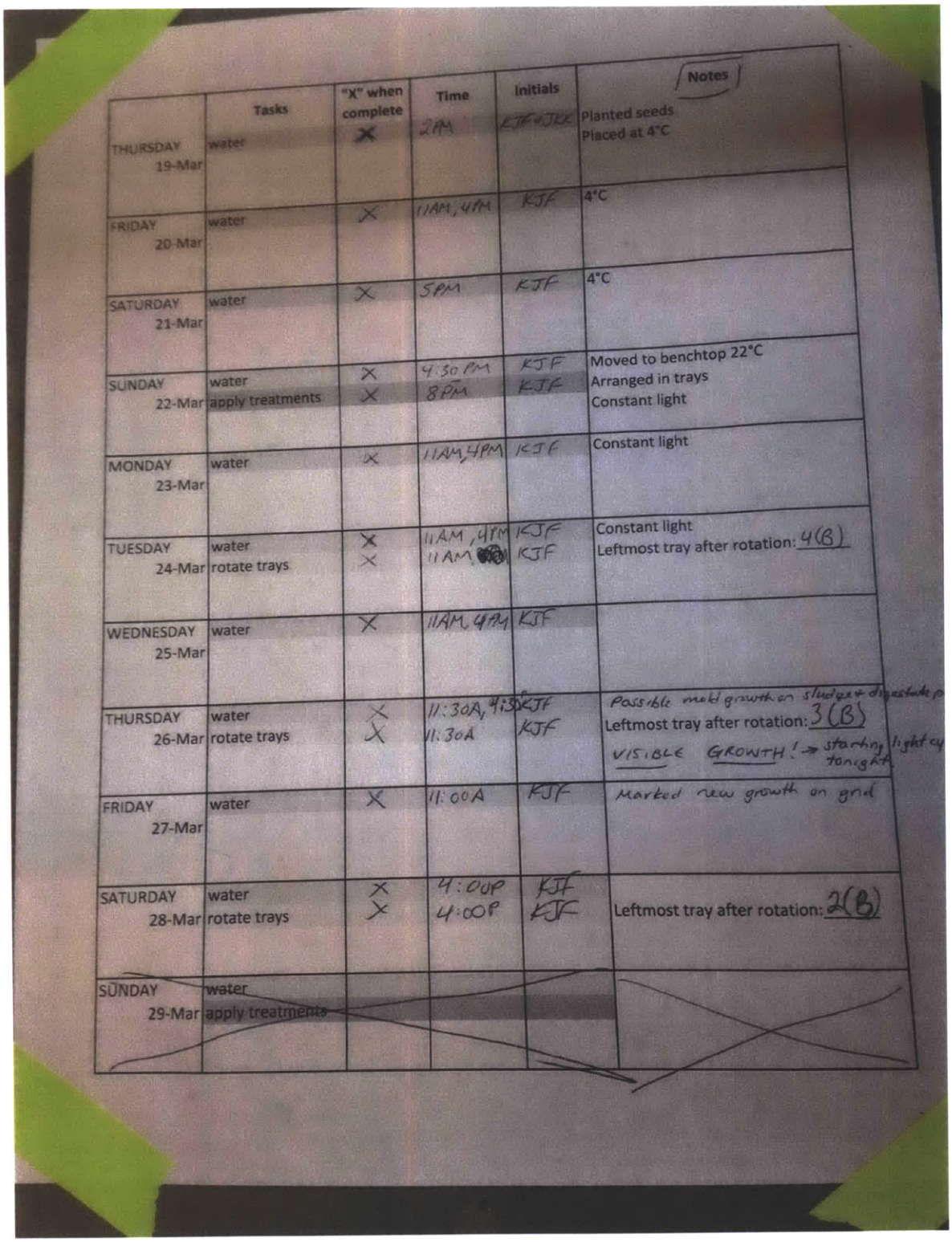
Rotation Routine:

- Every 48 hours, the tray in position 4 is rotated 180 degrees and placed into the position of tray 1
- The trays in places 1,2 and 3 are moved down one space

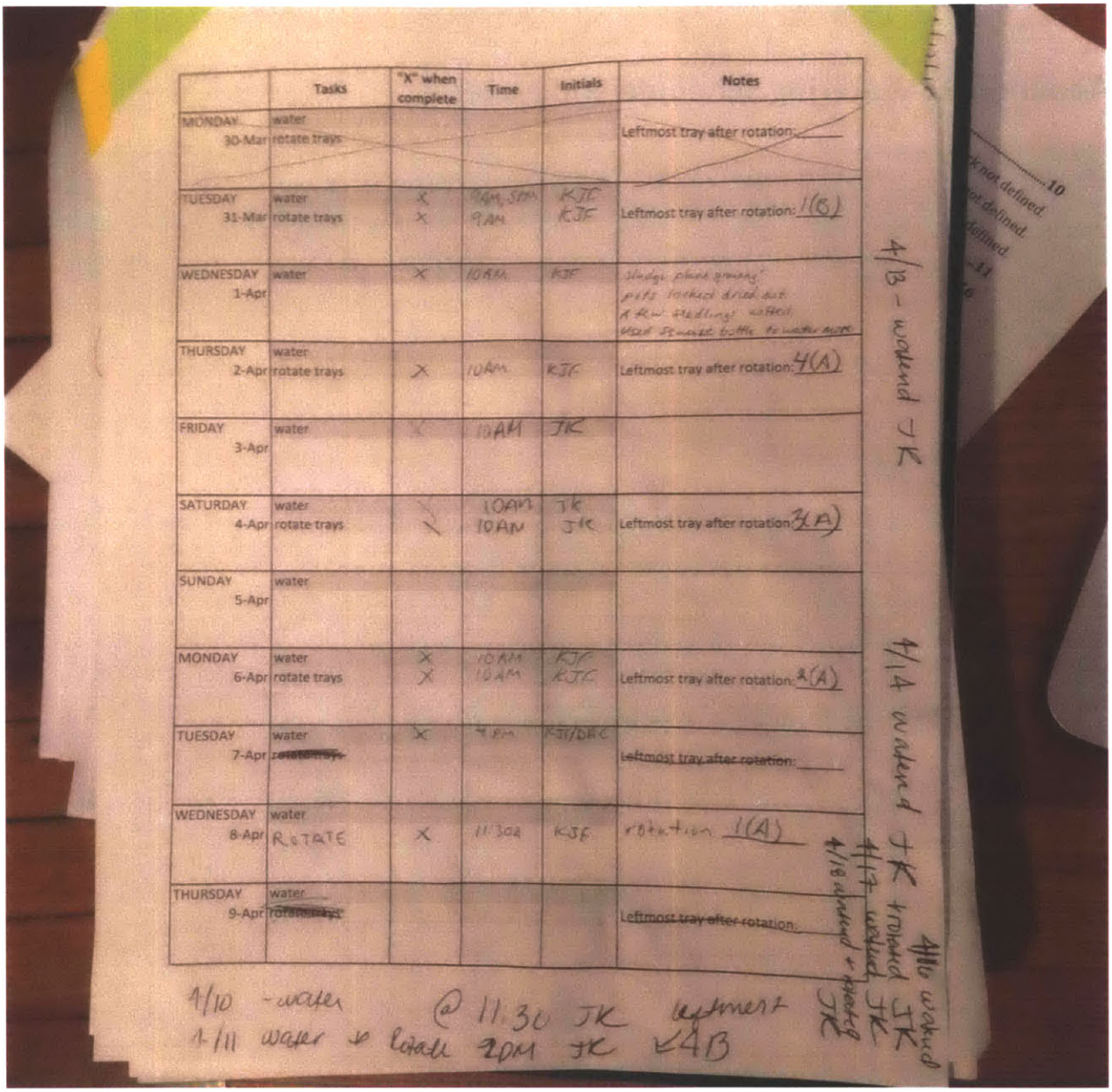
Generation randomized growth grid:

1) The tab 'grid' contains a 6X12 grid that represents the growing space available in the lab.
2) Each numbered cell represents a pot, and each tray represents a 3X6 tray that holds 18 pots.
3) Cells were assigned a number in order to randomly generate the scheme of potted plants.
4) Letters were each assigned a designation, ie, control, compost, fertilizer, etc., in order distinguish treatment method between plants.
5) Each letter was also assigned a number 1-14 to give each potted plant a unique ID. All A's have the same treatment; all B's have the same treatment, etc.
6) A1-14, B1-14, C1-14, D1-14, E1-14 were organized sequentially into one column.
7) Random.org was used to generate a list of random numbers, input 1 as minimum and 72 as maximum.
8) The randomly generated numbers were copied and pasted as a column next to the column of A-E grid designations.
9) Excel 'sort' function was used to sort the numbered column sequentially, giving the A-E grid designations a random order.
10) The A-E grid designations were then transposed onto the corresponding cell, ie, A9 went to position 1, A1 went to position 16, because that is how they were assigned by the random number generator.
11) 48 hours after removal from the cold room, the tray in position 4 was rotated 180 degrees, and moved to position 1, where trays in positions 1,2 and 3 were slid down one spot.
12) This rotation was repeated every 48 hours during the growing experiment.

Appendix E: Watering Schedule and Notes



	Tasks	"X" when complete	Time	Initials	Notes
THURSDAY 19-Mar	water	X	2PM	KJF + JLK	Planted seeds Placed at 4°C
FRIDAY 20-Mar	water	X	11AM, 4PM	KJF	4°C
SATURDAY 21-Mar	water	X	5PM	KJF	4°C
SUNDAY 22-Mar	water	X	4:30 PM	KJF	Moved to benchtop 22°C
	apply treatments	X	8PM	KJF	Arranged in trays Constant light
MONDAY 23-Mar	water	X	11AM, 4PM	KJF	Constant light
TUESDAY 24-Mar	water	X	11AM, 4PM	KJF	Constant light
	rotate trays	X	11AM	KJF	Leftmost tray after rotation: 4(B)
WEDNESDAY 25-Mar	water	X	11AM, 4PM	KJF	
THURSDAY 26-Mar	water	X	11:30A, 4:30P	KJF	Possible mold growth on sludge + digestate p
	rotate trays	X	11:30A	KJF	Leftmost tray after rotation: 3(B) VISIBLE GROWTH! → starting light cy tonight
FRIDAY 27-Mar	water	X	11:00A	KJF	Marked new growth on grid
SATURDAY 28-Mar	water	X	4:00P	KJF	
	rotate trays	X	4:00P	KJF	Leftmost tray after rotation: 2(B)
SUNDAY 29-Mar	water				
	apply treatments				



	Tasks	"X" when complete	Time	Initials	Notes
MONDAY 30-Mar	water rotate trays				Leftmost tray after rotation: ____
TUESDAY 31-Mar	water rotate trays	X X	9AM, 5PM 9AM	KJF KJF	Leftmost tray after rotation: 1(B)
WEDNESDAY 1-Apr	water	X	10AM	KJF	Sludge plant growing' pots looked dried out A few seedlings wilted Used 3 water bottles to water all
THURSDAY 2-Apr	water rotate trays	X	10AM	KJF	Leftmost tray after rotation: 4(A)
FRIDAY 3-Apr	water	X	10AM	JK	
SATURDAY 4-Apr	water rotate trays	X X	10AM 10AM	JK JK	Leftmost tray after rotation: 3(A)
SUNDAY 5-Apr	water				
MONDAY 6-Apr	water rotate trays	X X	10AM 10AM	KJF KJF	Leftmost tray after rotation: 2(A)
TUESDAY 7-Apr	water ~~rotate trays~~	X	4PM	KJF/DRC	~~Leftmost tray after rotation: ____~~
WEDNESDAY 8-Apr	water ROTATE	X	11:30a	KJF	rotation 1(A)
THURSDAY 9-Apr	water ~~rotate trays~~				~~Leftmost tray after rotation: ____~~

4/13 - weekend JK

4/14 weekend JK

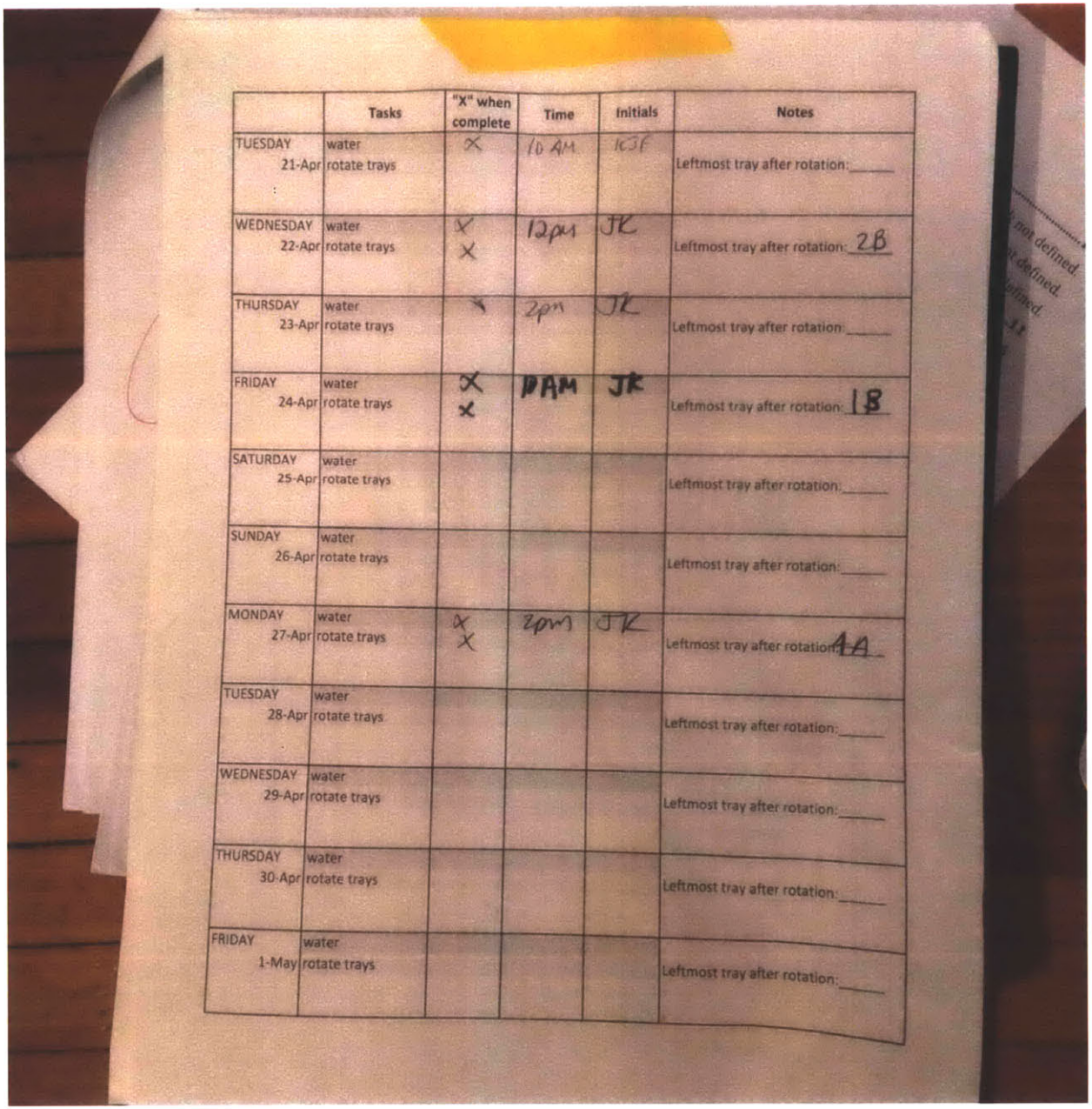
4/16 weekend JK

4/17 weekend JK

4/18 weekend + watering JK

4/10 - water @ 11:30 JK leftmost

4/11 water + rotate 2PM JK KAB



	Tasks	"X" when complete	Time	Initials	Notes
TUESDAY 21-Apr	water rotate trays	X	10 AM	ICJF	Leftmost tray after rotation:_____
WEDNESDAY 22-Apr	water rotate trays	X X	12pm	JK	Leftmost tray after rotation: 2B
THURSDAY 23-Apr	water rotate trays	X	2pm	JK	Leftmost tray after rotation:_____
FRIDAY 24-Apr	water rotate trays	X X	9AM	JK	Leftmost tray after rotation: 1B
SATURDAY 25-Apr	water rotate trays				Leftmost tray after rotation:_____
SUNDAY 26-Apr	water rotate trays				Leftmost tray after rotation:_____
MONDAY 27-Apr	water rotate trays	X X	2pm	JK	Leftmost tray after rotation: 4A
TUESDAY 28-Apr	water rotate trays				Leftmost tray after rotation:_____
WEDNESDAY 29-Apr	water rotate trays				Leftmost tray after rotation:_____
THURSDAY 30-Apr	water rotate trays				Leftmost tray after rotation:_____
FRIDAY 1-May	water rotate trays				Leftmost tray after rotation:_____

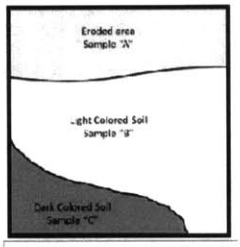
Appendix F: Soil sample instructions

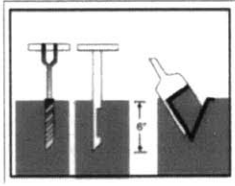
Soil and Plant Tissue Testing Laboratory
203 Paige Laboratory
161 Holdsworth Way
University of Massachusetts
Amherst, MA 01003

Phone: (413) 545-2311
email: soiltest@umass.edu
website: http://soiltest.umass.edu/

Soil Sampling Instructions

The most critical step in soil testing is collecting the sample. It is important that you take the necessary steps to obtain a representative sample; a poor sample could result in erroneous recommendations.





The first step is to determine the area that will be represented by the sample. Soil physical appearance, texture, color, slope, drainage, and past management should be similar throughout the area. It may be helpful to draw a map of the property and identify areas where you will collect samples. Using a clean bucket and a spade, auger, or sampling tube collect 12 or more subsamples to a depth of six to eight inches (four to six inches for turf) from random spots within the defined area. Avoid sampling field or plot edges and other non-representative areas. Avoid sampling when the soil is very wet or within six to eight weeks after a lime or fertilizer application.

Next, break up any lumps or clods of soil, remove stones, roots, and debris, and thoroughly mix subsamples in the bucket. Once the sample is thoroughly mixed, scoop out approximately **one cup** of soil and spread on a clean sheet of paper to air-dry. A fan set on low will help speed the drying; do not apply heat. **Do not submit wet soil samples to the lab.**

Place approximately **one cup** of your dry sample in a plastic zip-lock bag. Label each zip-lock bag with your sample ID (you create this: limit of 5 characters) and complete the submission form.

Complete all information on the sample submission form (found on our website). Provide your contact information, including a phone number and email address, under "Main contact." If you would like a copy of your results sent to anyone else, include their contact information under "Send copy to." Enter your Sample ID using the same five characters you labeled your samples with. Please include the approximate area represented by each sample. This information is useful to the lab and will be reported with your results. A rough approximation is adequate (i.e., +/- 1000 square feet for turf or +/- one acre for row crops).

Be sure to specify a Crop Code for each sample; without a Crop Code, the lab cannot provide lime and nutrient recommendations. Crop codes are listed on the second page of the submission forms. Finally select any optional tests you would like in addition to routine soil analysis. A brief description of these is proved below.

Send your sample(s), completed submission form and payment to the address listed on the front. Enclose check payable to UMass with your order. Please include $2 for postage and handling if you would like your results sent by US Mail.

Soil Test Descriptions & Fees

Ref. No. SPTTL_1

Routine Soil Analysis

Standard fertility test: $1

Appendix G: Arabidopsis Instructions

Handling Arabidopsis plants and seeds

Methods used by the Arabidopsis Biological Resource Center (ABRC)

https://abrc.osu.edu/seed-handling

Arabidopsis Biological Resource Center
The Ohio State University
Rightmire Hall
1060 Carmack Road
Columbus, OH 43210 USA

email: abrc@osu.edu
phone: 614-292-9371
fax: 614-292-0603

1. Growth of plants

1.1. Growth of plants in sterile conditions on solid media

1.2. Growth of plants in sterile conditions in liquid media

1.3. Planting seeds on soil

2. Growth conditions

2.1. Maintenance of plants in greenhouses

2.2. Maintenance of plants in growth chambers and growth rooms

2.3. Monitoring the environmental growth conditions

3. Genetic crosses

4. Seed handling

4.1. Plant isolation, harvesting and preparation for storage

4.2. Seed storage and preservation

4.3. Seed quality control

The methods used by the ABRC for handling Arabidopsis thaliana (Arabidopsis) plants and seeds are outlined below. These procedures are designed to generate healthy plants that give maximum set of pure seeds and to preserve these in the safest and most convenient manner. The plant and seed management protocols are given in the chronological order in which they would normally be utilized. Many other approaches may be equally as good, especially in specific experimental situations.

1. Growth of plants

Proper handling and maintenance of Arabidopsis plants is a prerequisite for accurate and reproducible research, it also enables a high rate of seed production. Arabidopsis can be grown in a variety of locations, growth media and environmental conditions. Environmental settings include growth rooms, growth chambers, greenhouses, lighted shelves and outdoors. Peat moss-based mixes, commercial greenhouse mixes, relatively inert media watered with nutrient solutions, and defined agar media can all be employed as plant substrates. Our focus will be on growth of plants on agar and soil in growth chambers and greenhouses.

1.1. Growth of plants in sterile conditions on solid media

It is necessary to grow Arabidopsis in sterile conditions on solid media for specific experimental settings such as selection of drug resistant and transformed plants, examination of early root and shoot phenotypes, identification of homozygous lethal mutants, etc. Liquid bleach sterilization, described here, is a practical method to sterilize a few seed lines at a time. Larger numbers of lines can be sterilized easily and with less manipulation using chlorine gas. Chlorine gas can also be utilized for seeds infested with powdery mildew or other fungal diseases. Various containers such as sterile plastic Petri plates (10 cm or 15 cm diameter), Magenta® boxes, or culture tubes are used, depending on the purpose of the experiment. Below we describe the use of the most commonly employed medium for sterile growth conditions in Petri plates (1x MS agar media). Adaptation to other sterile formats is straightforward, and most experimental additives can be easily incorporated in the preparation.

1. Add 4.31 g of Murashige and Skoog (MS) basal salt mixture and 0.5 g of 2-(N-Morpholino) ethanesulfonic acid (MES) to a beaker containing 0.8 L of distilled water and stir to dissolve. Add distilled water to final volume of 1 L. Check and adjust pH to 5.7 using 1M KOH.

2. Divide the media into two 1 L glass bottles, 500 mL in each. Add 5 g of agar granulated per bottle. Keep the lid loose.
3. Autoclave for 20 min at 121°C, 15 psi with a magnetic stirring device in the bottle.
4. Place the bottles on a stir plate at low speed, and allow the agar medium to cool to 45-50°C (until the container can be held with bare hands).
5. Starting from this step, perform all the steps in sterile conditions in a laminar flow hood. Add (optional) 1-2% sucrose and 1 mL Gamborg's Vitamin Solution, stirring to evenly dissolve. Optional sucrose and vitamins should be added after autoclaving and only after the agar media cools, because vitamins are thermo-labile and 15-25% of the sucrose may be hydrolyzed to glucose and fructose at elevated temperatures. Plants grow more vigorously and quickly on media containing 1-2% of sucrose, however, fungal and bacterial contamination must be rigorously avoided by seed sterilization. Note that germination of some mutants might be delayed on sucrose-containing media.
6. Label the bottom of Petri plates with identification number or name, including the date.
7. Pour enough media into plates to cover approximately half of the depth of the plate.
8. Allow the plates to cool at room temperature for about an hour to allow the agar to solidify. If the plates are not to be used immediately, wrap them in plastic and store at 4°C (refrigerator temperature). Covered plates, boxes, or tubes with solidified agar can be stored or several weeks at 4°C in a container that prevents desiccation.
9. Surface sterilize seeds in microcentrifuge tubes by soaking for 20 min in 50% household bleach with the addition of 0.05% Tween® 20 detergent.
10. Remove all bleach residue by rinsing 5-7 times with sterile distilled water.
11. For planting of individual seeds at low density, adhere one seed to the tip of a pipette using suction, then release seed onto the agar in desired location. For planting seeds at higher densities, mix seeds in sterile distilled water (or 0.1% cooled top agar), pour onto plate, and immediately swirl to achieve even distribution. Use a sterile pipet tip to adjust the distribution and remove excess water. Allow the water or top agar to dry slightly before placing lid onto plate.
12. Seal with Micropore paper tape to prevent desiccation, while allowing slight aeration.
13. Place the plates at 4°C for 3 days. This cold treatment, also called stratification, will improve the rate and synchrony of germination. The use of an extended cold treatment of approximately 7 days is especially important for freshly harvested seeds, which have more pronounced dormancy. An extended cold treatment is also necessary for certain natural accessions (e.g., Dobra-1, Don-0, Altai-5, Anz-0, Cen-0, WestKar-4). Cold treatment of dry seeds is usually not effective in breaking dormancy. Instead of stratification on plates, seeds suspended in sterile water can also be stratified prior to planting on agar or soil surface.
14. Transfer the plates to the growth environment. Illumination of 120-150 .mol/m2sec continuous light and a temperature of 22-23°C are suitable growth conditions.

1.2. Growth of plants in sterile conditions in liquid media

Seedlings of Arabidopsis can also be grown in liquid growth media. This method provides large amounts of plant tissue suitable for proteomics and metabolomics, or any study that requires a larger amount of starting material. Liquid culture growth is also widely used for

high-throughput genomic studies. In this case, growth protocols are adapted to 96 deep-well plates (or other formats) with the MS media supplemented by gibberelic acid.

1. Prepare MS media, as described in subheading 1.1. Do not add agar.
2. After the media has been autoclaved and cooled to room temperature, distribute 75-100 mL MS media into previously sterilized 250 mL Erlenmeyer flasks in a laminar flow hood.
3. Add bleach- or chlorine gas-sterilized seeds to the media (add up to 10 .l of seeds to each flask, which corresponds to approximately 250 seeds).
4. Grow seedlings under continuous light (120-150 µmol/m2 sec) with gentle rotation in an orbital shaker at 120 rpm for up to two weeks.
5. Remove seedlings from the flask. Growth of more than 200-250 seedlings for more than two weeks may result in difficulty removing plant material from the flask.
6. Remove excess media from the seedlings using filter paper. Plant material is now ready for downstream applications.

1.3. Planting seeds on soil

Diverse mixtures and media can be utilized for growing Arabidopsis. The term "soil" will be used here for any mix or media utilized for non-sterile growth of plants in pots or similar containers. Commercial potting mixes are popular due to their convenience and reliability. Potting media often employ peat moss for moisture retention and perlite for aeration. Soil mixes such as Sunshine® LC1 support healthy Arabidopsis growth and include a starter nutrient charge, so that fertilization is not necessary in early growth phases. Soil can be autoclaved to eliminate pests, but this is usually not necessary. Seeds can be planted by various methods: square pots with a diameter of approximately 5.5 cm can be used to grow one plant, 11 cm diameter pots are suitable for growing up to 60 plants, rectangular flats that are 26 cm x 53 cm can accommodate as many as 200-600 plants grown to maturity. Another option especially suitable for genomic studies is 96-well insets. Higher densities, approximately 3000 plants per 30 cm2, can be used if plants are harvested at early stages. Preparation of soil for planting in pots can be accomplished as follows:

1. Place soil in a clean container. Add fertilizer in slow release pellets, e.g., Osmocote® 14-14-14 (14% nitrogen, 14% phosphate, 14% potassium), feeding up to 3 months from planting. Apply in amounts according to the label. Alternatively, nutrient solution can be used to wet the soil. Wet thoroughly with tap water and mix well with trowel, large spoon or hands.
2. Label plastic pots with holes in the bottom or plastic flats/trays with the stock number or name and date of planting. Always use clean growth supplies, especially new pots and trays to avoid pest contamination.
3. Place soil loosely in pots or other containers using a trowel or large spoon and level, without compressing, to generate a uniform and soft bed. Pots are then ready for planting. Prepared pots can be stored in covered trays at 4°C for several days before planting, although pot preparation and planting should be conducted on the same day if possible.

4. When planting many seeds in a pot, scatter them carefully from a folded piece of 70 mm filter or other paper; distribute them evenly onto the surface of the soil. When planting individual seeds, adhere one seed to the tip of a pipette using suction, then release onto the soil. Various methods can be employed to plant seeds. The density of plants varies with genetic circumstances and purpose of the planting. High yields are achieved with 10-20 plants per 11cm diameter pot. Generally, low densities increase the yield/plant and are suitable for pure lines. High densities reduce the yield/plant, but are useful when it is necessary to maintain the genetic representation in segregating populations. Planted seeds should not be covered with additional soil, since Arabidopsis seeds require light for germination.
5. Place pot(s) in a tray, flat or other container.
6. Cover with a plastic dome or with clear plastic wrap taped to the container. The plastic wrap should not be allowed to contact the soil surface and should be perforated to provide aeration. If clear plastic domes are used, they should not be tightly sealed.
7. Place pots at 4°C for 3 days. Most widely used lines have moderate dormancy, and cold treatment, also called stratification, may not be required for germination when planting older seeds of these lines. However, a cold treatment at 4°C for 3 days will improve the rate and synchrony of germination. The use of an extended cold treatment of approximately 7 days is especially important for freshly harvested seeds, which have more pronounced dormancy. An extended cold treatment is also necessary for certain natural accessions (e.g., Dobra-1, Don-0, Altai-5, Anz-0, Cen-0, WestKar-4). Cold treatment of dry seeds is usually not effective in breaking dormancy.
8. Transfer pots into the growth area.
9. Remove plastic dome or wrap for growth in the greenhouse, but leave them on until germinated seedlings are visible for plants grown in a growth chamber.

2. Growth conditions

In general, the growth and development of Arabidopsis, including flowering time, is influenced by a number of environmental conditions in addition to the genetic background. Seeds of most lines germinate 3 to 5 days after planting under continuous light, 23°C, adequate watering and good nutrition. Plants produce their first flowers within 4-5 weeks, and seeds can be harvested 8-10 weeks after planting. High quality seeds can be produced if light, temperature and watering are carefully controlled.

You should be aware however, that most of the commonly used growth environmental conditions, particularly in greenhouses, may not be similar to the ones in the native habitats of some natural accessions. This is especially important for interpreting phenotypic differences of traits that are known to be strongly influenced by the natural habitat. Therefore, the protocols described here should be taken only as a guide for the experimental setup and design.

Light

For vigorous plant growth, the optimum light intensity is 120-150 .mol/m2sec. Higher intensities may result in death of some seedlings, but are tolerated by older plants; purpling of leaves is the first symptom of high light stress. Very low light intensities may result in weak and chlorotic plants. Arabidopsis is a facultative long-day plant. Plants flower rapidly under continuous light or long day (>12 h) photoperiods, while under short days (<12 h) flowering is delayed, favoring vegetative growth. Plants grow well under a cycle of 16 h light/8 h dark, or under continuous light. Various light sources can be used for optimal plant growth, such as cool-white fluorescent bulbs, incandescent bulbs, very high output (VHO) lamps, high-intensity discharge (HID) lamps and shaded sun light. Cool-white fluorescent bulbs, supplemented by incandescent lighting are recommended in growth chambers or growth rooms. HID lamps of 400-1000 watts are conventional in greenhouses in temperate climates to supplement the sunlight or prolong the natural photoperiod.

Temperature

The optimum temperature is 22-23°C. The temperature range for Arabidopsis growth is 16-25°C. Lower temperatures are permissible, but higher temperatures are not recommended, especially for germination through early rosette development. Temperatures above 28ºC are better tolerated by more mature plants (past early rosette stage). In general, high temperatures result in a reduced number of leaves, flowers and seeds. At lower temperatures, growth is slow, favoring the vegetative phase, and flowering is delayed. Some winter-annual natural accessions require a period of cold to initiate flowering, a process known as vernalization (e.g., Galdo-1, Monte-1, Cit-0, Dog-4, Istisu-1, Valsi-1, Mir-0, Tamm-2). Young rosettes (2-4 weeks old) of late flowering accessions should be placed at 4°C for 4-7 weeks to accelerate flowering.

Water

Water requirement is strongly influenced by relative humidity. Plants tolerate low (20-30%) relative humidity well, but depletion of soil moisture may occur in these conditions. Plant sterility may result from very high (>90%) relative humidity. Mild humidity (50-60%) is considered optimal for plant growth, however low humidity (<50%) is recommended for silique maturation.

The following growth practices are useful for handling plants in any growth context (greenhouse, growth chamber or growth room).

1. Add water to trays containing pots with perforated bottoms.
2. Maintain approximately 2 cm of water around base of pots during germination, to avoid any soil drying before the first true leaves begin expanding.
3. Reduce the watering frequency to as low as once or twice per week as needed after plants have developed true leaves and until the plants flower, to avoid water stress but

allow proper drainage of the soil. Plants should not be over-watered to avoid development of algae, fungi, fungus gnat larvae and other pests who thrive on overly wet soil. Algae can be manually scraped off and the soil allowed to dry.
4. Water daily during silique filling stage for good seed production. The water requirement of plants increases dramatically during this stage.
5. Keep plants spaced apart with good air circulation to prevent the incidence of powdery mildew.
6. Place several yellow or blue sticky cards (e.g., Pest Trap™) in the growth area to monitor insect populations. An important aspect of insect control is detection and identification before populations multiply. These traps are vital in this regard for catching winged insects. Inspect cards and plants daily for pests. Always identify a pest before embarking on treatment. Change cards periodically to better judge the pest populations, and especially after a pesticide application.
7. Prevent the introduction and spread of pests, which can be transported to the growth area via the soil, seeds, plants, or by humans. Wear a lab coat especially assigned to the growth area, since insects and pathogens can readily be transported on clothing. Plan to have plants of similar age in the growth area, since mature plants are more susceptible to pests than very young plants. Any person who has been in infested growth areas should subsequently abstain from entering non-infested areas; when entering multiple areas, entries should be from the cleanest to the more infested. Keep the area clean and regularly sweep the floors and/or shelves to eliminate or reduce potential sources of pest outbreaks. Mature and dry plants should be harvested and old soil and non-viable dry plant debris should be discarded immediately.
8. Avoid infestation of pests like thrips, aphids, fungus gnats, white flies and spider mites by spraying plants with a preventive mixture of Enstar® II and Conserve® SC. Insecticide mixture is prepared by adding 1.2 mL of each to 12 L of water. This mix can be sprayed lightly on rosettes prior to bolting stage, before placement of any isolation devices (see subheading 4.1.). Marathon® 1G, a granular systemic insecticide, can also be applied as directed by the label to control aphids, fungus gnat larvae, white flies, psyllids and thrips. Marathon® 1G is effective as a preventive or as treatment following infestation. It can be applied to the soil surface or included in sub-irrigation watering regime, which reduces damage to the plants. Preventive application of pesticides, if local regulations allow this, can be efficient to avert heavy use of chemicals after infestations have developed. Rotate pesticides, if possible.
9. Note that the use of brand names does not constitute an endorsement of product nor does it imply that other approaches may be necessarily inferior. The chemicals mentioned are for information only. Also, when these or any other pesticides are employed, check the label instructions of the manufacturer before purchase or application for registered usages of the product and recommended application rates and frequencies. Label instructions of pesticides must be strictly followed, and the product applied only by individuals with currently valid licenses. All applications of pesticides should be made in evening hours, and greenhouse rooms flushed with fresh air before the next morning to minimize exposure to workers. Notice of application should always be posted.
10. Use biological pest control, as an alternative to the application of pesticides, introducing parasitic wasps, predatory insects or mites, nematods, etc. These living animals have limited lives, they cannot be stocked on shop shelves, they have to be ordered and released

periodically. Follow carefully the supplier's instructions regarding release and subsequent care of the predators or parasites. The pest must be present, otherwise there is nothing for the predators to feed on or the parasites and nematodes to infest. Predators and parasites should therefore be introduced into a growth area in an early infestation, before a heavy and damaging invasion has developed. These biological control agents cannot give an instant reduction in pest numbers; they need some time to get established. Biological controls are an alternative to pesticides, rather than an additional treatment, they are vulnerable to most insecticides. It relies on predation and parasitism, but typically also involves an active human management role and generally it needs a degree of tolerance to low-level pest populations.

2.1. Maintenance of plants in greenhouses

Greenhouses with satisfactory cooling, heating and supplemental light are suitable for large-scale growth of lines that do not require strict control of environmental conditions, which include most natural accessions (e.g., Col, Ler, Cvi, Ws, Est, Kas, Sha, Kondara, C24) as well as species related to Arabidopsis thaliana. However, conditions are often too hot in temperate climates for Arabidopsis growth in greenhouses during the summer. Successful plant growth should start with an empty room, cleaned and maintained as follows. Mature or diseased plants, plant debris, used soil, pots and other materials, can shelter pathogen spores or insects from former plantings. After removal of the pest and host materials and the sterilization of the growth area, it is very improbable that any pest or pathogen will survive.

1. Remove and properly discard all plants and other materials in the room.
2. Sweep and hose down the entire room interior (benches, floors, window ledges and windows).
3. Increase the temperature in the room to 40°C for 3-5 days. The temperature setting may be higher, depending on the outside environmental conditions and equipment specification. Lights, fans and cooling pads should be turned off and vents closed during this period.
4. Do not place diseased or older plants in the clean room after the high temperature treatment.
5. Provide supplemental evening and morning light during the winter, since the plants generally require a long photoperiod (at least 12 h) for flowering. In the greenhouse, 16 h photoperiods are typically employed.
6. Use shade cloth during the summer, which helps reduce light intensity and regulate temperature.
7. The recommended growth temperature in the greenhouse is 21-23ºC. Night temperatures should be maintained 2-4°C lower than the day temperature.

2.2. Maintenance of plants in growth chambers and growth rooms

Most of the commercial growth chambers precisely control light intensity, photoperiod, temperature (typically ±1°C), and often humidity. Custom plant growth rooms provide

environmental control similar to that of reach-in chambers. Standard architectural rooms, equipped with supplemental lighting and air conditioning, are popular for reproducing Arabidopsis economically. Such rooms must be designed with sufficient light, cooling and ventilation, but typically afford less rigorous control of growth conditions than custom chambers.

Such facilities usually allow better control of temperature and light than is offered by a greenhouse, hence, their popularity among Arabidopsis researchers. Growth rooms can be maintained within 2-3°C of a set point, while greenhouse temperatures may spike to higher deviations with rapid changes in sunlight, unexpected hot days, etc. As is the case for greenhouses, it is imperative to start a new planting in a growth facility that has been previously emptied and properly cleaned. Hence, the use of chemicals to control pests and loss of plants due to pest infestation is minimized.

1. Remove and discard all plant residues and related materials. Mature or diseased plants, plant debris, used soil, pots and other materials, can shelter pathogen spores or insects from former plantings.
2. Sweep and wipe down the interior with wet paper towel.
3. Make sure the intake and exhaust vents are closed.
4. Apply a sterilizing agent, such as Spor-Klenz® Ready To Use, to kill fungal spores if heavy infestation of powdery mildew was present, using a fogger tank (e.g., Tornado™/Flex cold fog ULV mist sprayer) through an external access port of the chamber. Read and follow precautionary measures as suggested by the manufacturer of the cold sterilant SporKlenz®.
5. Leave chamber undisturbed overnight, and wipe down the inside of the growth chamber with a wet paper towel the next day.
6. Increase the temperature to 40-45°C for a period of 3-5 days to eradicate/minimize pests.
7. Do not place diseased or older plants in the cleaned chamber.
8. Use continuous light or a long day photoperiod if you wish to accelerate the reproductive cycle. Short days (less than 12 h) favor growth of vegetative tissue and delay flowering.

2.3. Monitoring the environmental growth conditions

The environmental control systems currently offered for greenhouses, growth chambers and growth rooms allow for remote monitoring, control adjustment and alarm notification via internet connections. These features represent a vital tool for avoiding loss of data during plant production and maintaining control of environmental experiments. Installation of remote sensing is recommended for new growth facilities of all types.

In addition to the control and logging systems in place at the growth facilities, environmental growth conditions can be monitored by placing portable data loggers (e.g., the HOBO® U14) in growth areas. They can act as a complementary, backup or sole resource for recording environmental data. They can be used to display and record temperature and relative humidity conditions in greenhouses, growth chambers, growth

rooms, cold rooms, dry rooms and laboratories. These data loggers offer reliability, accuracy, convenient monitoring and documentation of specific environmental conditions. They can be connected to a computer to quickly display and analyze data.

3. Genetic crosses

Some species of Arabidopsis, particularly A. thaliana, are mostly self-pollinating, especially in a growth chamber or greenhouse setting where insect populations are minimized. It should be noted that the pollen of Arabidopsis does not disperse through the air. Therefore, crossing Arabidopsis is mainly conducted through manual emasculation of flowers just prior to flower opening, followed by hand transfer of pollen from the desired male parent to the stigma of the emasculated flower. Although labor-intensive, the manual method remains a reliable technique for achieving cross-pollination.

Species, such as A. halleri and A. lyrata, have natural self-incompatibility mechanisms, which prevent the plant from self-pollinating and result in obligate outcrossing. For such species, simple maintenance of a genetic stock, cannot easily be accomplished from a single plant, and it is most convenient to start with a small population of founders and perform cross-pollination. The manual techniques for performing genetic crosses of A. thaliana can be generalized to the related species. The use of a magnifying visor (e.g., optical glass binocular magnifier) or dissecting microscope is recommended to visualize floral parts and avoid damage to the pistil. Genetic crosses can be performed as follows:

1. Select the appropriate parent plants. Choose young plants at early stages of flowering. Avoid using the first flowers in the inflorescence, which are usually less fertile and the smaller flowers produced by mature plants. Crosses may be performed throughout the duration of the flowering time, however, the crosses will have a higher rate of success during the earlier stages of flowering.
2. Prepare the female parent:
a. Select a stem with 2-3 flower buds, in which the tips of the petals are barely visible and before the anthers begin to deposit pollen on the stigma. Using unopened flowers for the female parent is important in order to avoid self-pollination. Shortly after this stage, stamen/pistil length ratio, as well as the timing of anther dehiscence, favor self-pollination and open flowers have most likely been self-pollinated. All flower candidates for female crossing should be examined for presence of released pollen prior to their use in crossing.
b. Remove siliques, leaves, and any open flowers above and below the selected buds on the chosen stem with a small pair of scissors; avoid damaging the stem.
c. Remove the sepals, petals and all six stamens from the selected flower buds using a precision clamping tweezers, leaving the pistil intact. If the pistil is damaged, it is highly unlikely that the cross will be successful and the flower should not be used.

3. Prepare the male parent: select a newly opened flower with anthers that are dehiscent. These flowers will contain fresh pollen that will contribute to the success of the cross. Remove the flower by squeezing near the pedicel with tweezers.
4. Pollinate the female parent by taking the fully open flower from the male parent and brushing the anthers over the bare stigma of the female parent. Visually confirm that pollen has been deposited on the stigma.
5. Label the crosses, placing a colored tape on the stem of the female plant, noting the male and female parent and the date of the cross.
6. Inspect developing siliques over the next several days. Successful crosses are visible after 3 days when the siliques start elongating. Siliques are ready for harvest once they turn brown, but before they shatter. Siliques should be ready to harvest in about 2-3 weeks after the cross. If siliques are brown, use care, as it is easy to lose all seeds at this stage.
7. Harvest siliques by cutting them with scissors and placing them into a 1.5 mL microcentrifuge tube or a small paper envelope.
8. Air-dry seeds at room temperature, preferably at 20-30% relative humidity, for 1-3 weeks. Thresh seeds if necessary.

4. Seed handling

4.1. Plant isolation, harvesting and preparation for storage

Prevention of cross-contamination among adjacent pots and avoiding the loss of seeds due to shattering are equally important. Plants must be isolated from their neighbors without compromising seed quality. Various methods and devices exist to accomplish these objectives, including Aracons™, plastic floral sleeves, plastic bags and isolation by space on the open bench. Details of each method are described below.

1. Aracons™: Place Aracons™ over single plants soon after bolting.
2. Plastic transparent floral sleeves (e.g., straight sleeve BOPP 60x40x15 cm for 11 cm diameter pots): Cut four equally spaced holes at the point where the sleeve meets the top of the pot. This will increase aeration and reduce water condensation that may encourage mold growth. Place the sleeve on the pot near the time of bolting, so that all plant inflorescences are maintained within the sleeve. Plastic sleeves fit snugly around a pot, extend upward, and are wider at the top allowing for expansion of the developing inflorescences. Sleeves made of biaxially-oriented polypropylene (BOPP) are very clear, maintain upright stiffness, and tear easily for harvesting. Fold down the tops of the sleeves about 2 cm to ensure they stay open and stable. If plants grow out above the sleeves and are at high plant density, train the top of the plants back down into the sleeve to avoid contamination. This method is very effective for achieving high densities while maintaining productivity and purity of single lines of different genetic backgrounds.
3. Plastic bags: If plastic bags are used, train inflorescences of non-erecta lines into a 4-8 L transparent plastic bag before siliques begin to brown. Bags should be kept open to avoid the accumulation of moisture resulting from transpiration.
4. Open bench growth: Plants can be maintained on the open bench for bulk seed production, keeping all lines separated by adequate space. Avoid disturbance of maturing

inflorescences. This method is appropriate when growing natural accessions that are late flowering and develop large and dense canopies (e.g., Sij-1, Monte-1, Amel-1, Anholt-1, Appt-1, Bik-1, Bl-1, Do-0).

The simplest procedure is to wait until the entire inflorescence has browned before harvesting. However, some siliques may shatter naturally and seed will be lost. Harvest seeds only after the soil in pots or flats has been allowed to dry. It should be noted that delays in harvesting following physiological maturation of the plant result in seed deterioration, especially under non-optimal environmental conditions. Seeds from individual siliques can be harvested after the fruits have turned completely yellow, if rapid turnover is required. However, such seeds have high levels of germination inhibitors. Since formation and maturation of siliques occur over time, early siliques can be harvested before later ones mature. Harvest for each of the four isolation methods is as follows:

1. Aracons™: Slide the plastic cylinder off, and then cut off the dry inflorescence above the cone device in a threshing sieve.
2. Plastic floral sleeves: While holding the pot, cut away and discard plastic sleeve. Cut the dry inflorescences and place them in a threshing sieve.
3. Plastic bags: Cut the entire plant off at its base. Shake the seeds into the bag; inflorescences can be gently hand-pressed from the outside, and the seeds will fall to the bottom of the bag. Most of the dry inflorescences can be removed from the bag by hand before seeds are sieved to separate them from chaff.
4. Open-bench: Cut off the entire inflorescence at its base, and carefully place into a 4-8 L or larger transparent plastic bag, depending on the size of the bulk of plants.

The major factors influencing seed longevity are: 1) genotype, 2) environmental conditions during seed maturation, harvesting and seed handling and 3) seed storage conditions. Harvested seeds should be processed promptly (including threshing, cleaning, drying and packaging) and placed into storage.

Seeds should be threshed when the seed moisture content is approximately 10%, to minimize seed damage during threshing. This seed moisture content will be reached when all plant material appears to be dry. Hand, rather than machine threshing, is recommended mainly because threshing machines need rigorous cleaning between lines to avoid sample cross-contamination, require very careful adjustment and do not accommodate the variable size of Arabidopsis seeds well. The hand method is performed as follows:

1. Set a large, clean, white paper on a bench or table for collection of the threshed seeds.
2. Place a clean threshing hand sieve (e.g., U.S. Standard Stainless Steel Test Sieve No 40) on top of the paper.
3. Place dry plants directly onto the sieve. If plants are larger than the sieve, they can be cut into pieces that fit the screen.
4. Crush plants using hands to remove all the seeds from siliques. Discard plant material.

5. Sieve seeds through the mesh repeatedly until they are clean and free of chaff. After sieving, the seeds are still likely to be mixed with soil and plant residue. A combination of additional sieving, gentle blowing and visual inspection can be employed to clean the seeds completely.
6. Clean small samples by hand with the aid of a pointed tool on an opaque glass plate illuminated from below, if needed.
7. Place cleaned seed samples in small labeled manila envelopes (e.g., 6 cm x 9 cm) or small open glass jars (125 mL) or other containers to allow seeds to air dry. Do not use plastic due to static effects.

The ideal moisture content of seeds for storage is 5-6%. Higher moisture content can cause seed deterioration. There are many methods available for drying seeds. The recommended method is to air-dry the seeds at room temperature and approximately 20% relative humidity for 1-3 weeks. The moisture content of Arabidopsis seeds stored in open containers corresponds to the room humidity. Arabidopsis seeds behave in a similar way to crop seeds with similar chemical composition. Low relative humidity (20-30%) is necessary for seeds to reach the desired moisture content. Seed moisture content can be determined by several methods and it is calculated as the loss in weight as a percentage of the original weight of seeds. Seed packaging for storage can be accomplished as follows:

1. Use polypropylene cryovials with threaded lids and gaskets (e.g., 2 mL screw cap micro tubes) for convenient, safe and permanent seed storage. They hold large numbers of seeds, seal tightly, are moisture-proof and can be resealed many times.
2. Label each vial with pertinent information including date of storage, use printed labels or permanent marker.
3. Determine stored seed quantities (approximately 50 .l = 25 mg = 1,250 seeds).

4.2. Seed storage and preservation

The general conditions for preserving optimal viability of seeds have been well defined. Seed storage principles for Arabidopsis are similar to those for other plants, with the caveat that the small seeds rehydrate very rapidly if exposed to high humidity. When seeds deteriorate, they lose vigor and eventually the ability to germinate. The rate of this 'aging' is determined by interactions of the temperature and moisture content at which seeds are stored, and unknown cellular factors that affect the propensity for damage reactions.

Rapid deterioration of seeds has not been observed for the diverse collections currently maintained at ABRC. However, experience regarding the effect of genotype is limited. A large number of genes involved in embryogenesis, reserve accumulation and seed maturation have been identified. Conspicuously, seeds of the abscisic acid-insensitive mutants fail to degrade chlorophyll during maturation and show no dormancy, leading to low desiccation tolerance and poor longevity. Arabidopsis seeds should retain high viability for long storage periods, under proper conditions. With the increase of storage temperature and seed moisture content, the lifespan of the seeds decreases. Seeds left at

room temperature and ambient relative humidity lose viability within approximately two years. Seed stored dry at 4ºC or -20ºC should last decades. Below are three storage options for safe seed preservation.

1. For active collections which are accessed often, store seeds at 4°C and 20-30% relative humidity. Control of humidity is typically achieved by a dehumidification system in the cold room. Note that the control of relative humidity provides a safety factor in case seed containers are not sealed properly.
2. For long-term or archival storage, the recommended temperature is sub-zero, preferably -20°C, and also preferably 20% relative humidity.
3. For open containers such as envelopes, seeds can be stored at 15-16°C, with a relative humidity maintained very carefully at 15%. Under this controlled environment, seeds will maintain suitable low moisture content. Storing seeds at relative humidity < 15% will not increase shelf-life and may actually accelerate deterioration.

When vials are removed from cold storage, condensation of moisture on the seeds and subsequent damage may occur. For vials stored at 4°C, sealed vials must always be warmed to room temperature before opening. For vials stored at -20°C rapid re-warming (placing the sealed vial in a 37°C water bath for 10 min) is a recognized method to minimize frost damage. If possible, working with seed stocks should take place at low (20-30%) relative humidity. If accumulation of condensation is suspected, vials should be left open in the dry room until seeds have equilibrated before returning the vials to cold storage.

4.3. Seed quality control

The purity and physical integrity of seeds and the presence of pests and seed-borne diseases (especially some fungal diseases) can be detected by visual examination with the naked eye, magnifying lenses or using a dissecting microscope. For a rigorous assessment, spread the seeds on white paper under a well-lit microscope. Generally, gray or white coloration on the seed surface indicates fungal contamination. Discard seeds if possible; otherwise sterilize seeds with fungicides before planting. Do not discard shriveled, small, irregular shaped and other colored seeds that might correspond to specific mutations, assuming that the seeds were produced under optimal conditions.

Seed viability should be monitored at regular intervals by conducting germination tests under a standard set of conditions. It is recommended that seeds in long-term storage under the optimal preservation standards should be monitored at least every ten years. Seeds in short-term storage should be monitored at least every five years.

A germination test for Arabidopsis can be conducted in 3-7 days to determine the proportion of seeds in a sample that will produce normal seedlings. Tests should be carriedout before seeds are stored, so that poor quality samples can be recognized. Arabidopsis seeds may fail to germinate because they are dormant or because they are defective or non-viable. Dormant seeds can be distinguished because they remain firm and

in good condition, while non-viable seeds soften and are attacked by fungi. Most widely used lines have moderate dormancy, and cold treatment, also called stratification, may not be required for germination when planting older seeds of these lines. However, a cold treatment at 4°C for 3 days will improve the rate and synchrony of germination. The use of an extended cold treatment of approximately 7 days is especially important for freshly harvested seeds, which have more pronounced dormancy. An extended cold treatment is also necessary for certain natural accessions. Cold treatment of dry seeds is usually not effective in breaking dormancy.

Initial germination rate should exceed 80%, but may be lower for some lines. Mutations in a significant number of genes, mostly involved in biosynthesis and signaling pathways of certain hormones, affect seed germination and/or dormancy. A germination test can be performed as follows:

1. Label the bottom of a 10 cm diameter plastic Petri plate with name and date.
2. Place two layers of absorbent paper (e.g., filter paper 10 cm diameter) in the bottom of the plate and moisten with distilled water. Remove excess water.
3. Distribute 100 seeds evenly on the surface of the paper. Seal the plate with Parafilm or clear tape, to prevent drying.
4. Stratify seeds by placing the plates at 4°C for 3 days.
5. Move the plates to an illuminated shelf or to a growth chamber under standard light and temperature conditions. Environmental conditions for seed germination tests are the same as for growing plants. Two replicates of 100 seeds each provide reliable germination estimates. Cases in which observed germination is < 80% may warrant follow-up testing.
6. Record germination percentage after 3 to 7 days by dividing the number of seedlings by the total number of seeds, and multiplying by 100.

Germination tests can also be performed on solid media, such as MS, described in subheading
1.1. These methods are used by the ABRC for handling plants and seeds. If you have any questions concerning the above procedures, feel free to contact us at abrc@arabidopsis.org

Arabidopsis Biological Resource Center
Ohio State University
1060 Carmack Road
Columbus OH 43210
Phone: 614-292-9371
Fax: 614-292-0603
Email: abrc@arabidopsis.org

Appendix H: IAP 2015 Notes

IAP 2015 Notes:

Shri Ram College of Engineering, Muzzafarnagar
Visit 1/13/15

Prof. Ashwani Kumar meeting:

-studies use product (sludge) as biofertilizer
1:1000 ratio (water) - spray on crop as bioinsecticide
insecticide - same ratio
diluted 4000 times for use as air freshener
diluted 5000 times for use as plant hormone
gets stronger as it ages
ratio: 1 oil cane: 3 organic waste: 10 water
oil cane locally made from sugar cane
kitchen waste - only delicate material
no dust, no plastic, no glass, etc.
leafy material best
biogas established in India in 1980s by gov't subsididies
removed ~2000 ? (very large area) for biogas plant

compact biogas plant - fabric, plastic material
use dung and food waste
slurry byproduct - used a compost
put into pit for ~3 months for dehydrating
used by farmers
sale direct to farmers
end product is a solid

1/19/15 - Green Elephant Visit

-small biogas facility (outside Pune)
5 metric tons/day
80-90% processed
sources not residential
no segregation
manual sorting
organics from commercial sites
crushers, pulverizer to less than 5 mm
ratio 1:1 waste to water
about 10% solids when loading
loading rate about 2.5 kg/ ?
hydrolysis phase - about 10 days
total retention for whole process - ~30 days
liquid fertilizer - no one wants it

liquid try to recycle
pH - liquid fertilizer ~7.0
~1000-3000 rupees/ton
depends on how its packaged

big plant:
pressure - gas - 8 bar
CO2 scrubbing
facility digesting waste
spent wash from sugar industry
pH - 3-4 incoming (liquid)
COD 110,00 mg/L
VFAs cause acidity ~600 cubic meter/day
spend wash - pumped into 1 settling tank
yeast settles ~2 days
capacity ~ days worth of influent
runs 24/7 (non-monsoon season)
split feed - each is 300 cubic m / day
feet rate 12.3 m3/hr - to each
reactors follow - CSTR
agitators inside - 1 in middle, 3 on sides
60 kw power for mixer
no neutralization of feed stock
generate over 25000 m3/day per tank, 50000 m3/day
hydrogen sulfide scrubber
after scrubbing, less than 350 ppm
byproduct of pure sulfur
make 1 ton S/day
areas of overlap: small scale on skids biogas
sold them to VW and Tata
see VW tomorrow? (we did)
small scale reactor

Sitara -
they own land, site
could set up small operation on site to look at process
also have Bangalore MSW facility 50 tons/day
600,000 L/day of sludge
reduced organic load, still a lot of carbon
65% lower in carbon content
goes into dehydrating pond
spray slurry onto presma (pulp)
makes fertilizer
in windrows
pipe slurry out and it transfers ownership
add micronutrients to slurry
digester mesophilic
whole process takes ~27 days

Mailhelm Visit 1/20/15

pyrolysis - plastic to fuel -
mine scrubbing (?)
cost conscious internally

recycle sludge -
Segregation is biggest issue
2nd issue - awareness
solids are retained - allows separation of solids
liquid, gas - > liquid recycled
HRT - solids retention time - MSW is 26-28 days
(mesophilic range)
thermophilic bacteria more sensitive
heat recovery important - drives process
don't need to take out sludge everyday
7-10% sludge - remains at bottom
system to remove H_2S and CO_2
want to sell gas in India, gas value higher in India
2 ways of selling gas
0.4 kg - 1 m3 gas
goes through flow meter - calorific value low
clean gas through scrubber - remove CO_2
90-92% pure - 200 bar compressed, bottled here
2-3% loss converting from biogas to bio C&G

80-90% segregated waste
mostly MSW
needs fresh water for shredder
pumped to primary digester
solids separate overflow
gas collected into raw gas balloon
pressurized and passed to scrubber
solid says in longer than usual to get more biogas
sludge put into liquid manure tank
dewatered by pump
used for public gardens
coming in 80-90% segregated
5 tons/day influent
retention time is 25-28 days
75% recycle, 25% drained
digested sludge - red bucket
pH is neutral
4-5% solids- liquid format
used on public gardens
nothing added - can add micronutrients
80 m3 capacity biogas balloon

value ~3 rupees/kg of sludge ~market value
50K inside, 10K outside
power generated runs street lights
can power 90 street lights
email: mithila.s@mailhelm.com

VW visit - 1/21/15
Green Elephant - 50-80 m3 gas daily
450 - 475 kg/day

Appendix I: August 2014 Trip Report

Tata August 2014 - Trip Report
CEE M.Eng Tata Fellows - Ellen Huang, Julie Karceski, Ching-ching Liu

Muzzafarnagar
Wednesday, August 13
Morning: Paper Mill
Afternoon: Group Meeting @ Paper Mill Plant

The paper mill plant produces kraft paper (brown paper bag) and duplex board (similar to cereal box cardboard).

Afternoon: Group Meeting 2 @ Paper Mill Plant
Met with: Pankaj Aggarwal (mayor), Akshay Tyagi (facility staff)

Currently the plant is burning pet coke. There is far less ash produced with this material. However, depending on price, the plant might change back to RHA (Rice Husk Ash).Scheduling was discussed for brick-making, ash disposal field, steel recycling plant, A2Z plant, and household interviews.

Afternoon: Visit to Secondary Waste Collection Site
There are 25 secondary collection sites across Muzaffarnagar. There are about 100 waste pickers who segregate plastic and sell the recyclables.

Secondary waste collection site has no formal design. There are cows and pigs browsing through the trash for food. About 30-40 A2Z tricycles carry waste from household to a single secondary collection site. Waste pickers segregate and get about 70-100kg of plastic and 50-60kg of paper/day per site. Interestingly, waste pickers have a mutual agreement where no waste picker infringes upon others' "sites". Later in the day, a truck from A2Z will pick up the trash for processing.

Afternoon: Visit to Another Secondary Waste Collection Site located next to Grand Plaza Mall
This one will be merged with the previous one to be moved so that less of the road is taken up by trash. There's a lot more yard waste and thus might feed from higher income families. They can get 4 rupees/kg of plastic and 3 rupees/kg of cardboard.

Afternoon: Visit to Third Secondary Waste Collection Site located next to Medical Facilities

There is water present at the site since it's by a drain, which can cause issues with flooding. Also people will throw their trash into the drains. In addition to the medical waste from nearby hospitals and clinics, there is also a lot of landscaping material (i.e. leaves and grass). There are 4-6 waste pickers for this site. All shops are rented spaces alongside the road.

Thursday, August 14
Morning: Visit to Fertilizer Plant

The 25 year old plant make a constituent in fertilizer, a micronutrient.

Fertilizer is heavily subsidized by government including micronutrients. The zinc sulfate demand is based entirely on consumer, whichever fertilizer ends up to be cheaper. Tata is one of their biggest clients. About 15% of India doesn't use fertilizer; instead they use compost and organic matter. Unfortunately organic fertilizer is more expensive, usually double in price. It has been shown to be better than chemical.

The organic fertilizer market is growing at about 10% per year.

Thursday, August 14
Afternoon: A2Z Visit
Met with: Shahid Raja (representative), Pankaj Aggarwal (mayor), Ahsan Saifi (plant operations & compost/RDF making, saifi.ahsan@gmail.com), and Ravendra Sing (garbage collection, ravendras40@gmail.com)

Julie arrives in Muzz.

At A2Z, to make the compost they use sieves that filter the material and gradually the sieve size gets smaller. Between the large filter sizes and smaller filter sizes, the compost is piled in windrows (windrow composting) for ~28 days.

The net amount of revenue they collect is 682 rupees/metric ton for garbage collection, paid by municipal government. Only 30% of households give the 30 rupees/month.

Friday, August 15
Morning: Independence Day Celebrations
Libby McDonald arrives in Muzz.

Visit to Muzz town hall in the morning for Independence Day ceremony and march, and local public girl's school for celebrations

Afternoon: More tours

Repeat visits to A2Z and the pyrolysis plant.

Evening: Independence Day Celebrations at Solitaire Hotel
Participants: Pankaj and Councilmembers

Saturday, August 16
Morning: Interviews conducted at high income communities with households and with local kabadiwallas
Afternoon: Drive back to Delhi
Evening: Flight to Mumbai (Ching-Ching, Ellen, Julie, Kate)

<u>Mumbai</u>
Sunday, August 17
All Day: Free day in Mumbai
Evening: Informal dinner with Tata Fellows, postdocs, affiliates, and professors.

Monday, August 18

All Day: Orientation for new Tata Fellows. Discussion of Tata Center/Fellowship purpose and outline for the rest of the week. Introductions. Discussions on assigned readings.
Afternoon: Dharavi Slum tour

Tuesday, August 19
All Day: Orientation for new Tata Fellows. Discussion of Dharavi Slum tour and results of individual Development Index estimates.
Afternoon: Medical hospital tours: King Edward Memorial Public Hospital (Ching-Ching), Tata Cancer Memorial (Julie), B J Wadia Hospital for Children (Ellen).

Wednesday, August 20
All day: Introductions to successful social enterprises:

- Ziqitza (privitized ambulance service)
- Build a Swasth India (low cost small sized health clinics)
- Naandis Midday Meal Program/Manna Trust (centralized kitchen model to provide lunch to public schools)
- SWADHAR (generate employment for visually challenged rural youth while recycling old/torn saris)
- Society for Innovation and Entrepreneurship @ IITB (incubator for startups)

Thursday, August 21
All Day: General orientation for all Tata and IITB fellows. Introductions to Tata Fellow project sectors (Energy, Healthcare, Housing, Agriculture, Water, and Waste) and highlights of projects. Networking followed by break up into individual rooms for sector specific discussions.

Friday, August 22
All day: Introductions to Tata Center Trust and their projects. Introduction to IITB fellows and their projects.

Saturday, August 23
All day: Explore Mumbai
Evening: Flight back to USA

Appendix J: Plant Weight – Full Data

Lab Sludge: Plant Mass (mg)			**Fertilizer: Plant mass (mg)**		
Pot:	Plant 1	Plant 2	Pot:	Plant 1	Plant 2
LS1	1.2		F1	10.4	
LS2			F2		
LS3			F3	1.7	
LS4			F4		
LS5			F5	0.8	
LS6			F6	1.4	2.3
LS7			F7	5.9	
LS8	0.6	0.4	F8	2.9	
LS9			F9	8.2	
LS10			F10	0.9	
LS11	0.1		F11	4.3	3.3
LS12			F12		
LS13			F13	16.9	
LS14			F14	0.6	1.3
Average plant mass:		*0.58*	*Average plant mass:*		*4.35*
Standard deviation:		*0.46*	*Standard deviation:*		*4.66*
Total plant mass:		*2.3*	*Total plant mass:*		*60.9*
Compost: Plant mass (mg)			**Control: Plant mass (mg)**		
Pot:	Plant 1	Plant 2	Pot:	Plant 1	Plant 2
CT1			NT1	4	3.7
CT2	4.4		NT2	4.8	3.4
CT3	3.9	2.9	NT3		
CT4			NT4	5.2	
CT5	12.9	14.3	NT5	0.3	0.3
CT6	1.1		NT6	0.6	
CT7	1.8	0.4	NT7	9.4	
CT8	11.7		NT8	11.7	
CT9			NT9	3.4	2.2
CT10			NT10	4.3	2.1
CT11	7.3	10.8	NT11		
CT12			NT12	2.1	
CT13			NT13		
CT14	7.9	6.5	NT14	4	
Average plant mass:		*6.61*	*Average plant mass:*		*3.84*
Standard deviation:		*4.68*	*Standard deviation:*		*3.06*
Total plant mass:		*85.9*	*Total plant mass:*		*61.5*

Appendix K: Anaerobic Digestion Materials and Methods

General Purpose

The goal of the technology described herein is to biologically produce the commodity chemical lactic acid from organic waste. This is done without the need for sterilization or enzyme addition through a controlled microbial community, which differentiates this technology from the current state of the art. The product is crude lactic acid in the range of 60-100 grams per liter which can be concentrated via a variety of methods known to the field. Organic waste, particularly food waste, is an excellent feedstock due to its low cost (in some cases as low as -$80/ton).

This technology will help fulfill the increasing global demand for lactic acid for use in a variety of industries including the food, cleaning, and polymer sectors. Polylactic acid or PLA is a biodegradable thermoplastic which is seeing increasing use as a result of its superior characteristics and green production method. We envision the primary use of the lactic acid produce via the method described herein will allow for even more cost effective production of lactic acid and as a result other products such as PLA.

Feedstock

The feedstock for this process can be most forms of organic waste, ideally those with higher concentrations of sugar, starch, and protein (i.e. food waste). Other organic feedstock such as agricultural waste can be used, although solid retention time in the reactor will necessarily increase in order to account for the recalcitrance of the material.

Prior to fermentation, the waste is mechanically disrupted (e.g. shredded or ground) to a particle size of approximately 1 mm or smaller. The solid content is adjusted to 8-15% and the pH is adjusted as needed.

Sterility and Process

Despite thousands of years of experience with microbes, with the most notable example being the employment of *Saccharomyces cerevisiae* in beer and wine production [1], process engineering challenges still exist for large scale fermentations [2-6]. For most industries employing microbes or other biocatalysts, sterility remains a significant concern [7]. Either the feedstock or the bioreactor are potential sources of contamination and are generally sterilized and monitored to avoid growth of contaminating phage [8-11], bacteria and fungi [12-15]. To maintain sterility, various methods have been developed that require skilled personnel employing specialized equipment and strict standard operating procedures. These include best practices, clean/steam in place sterilization for equipment, a pH range and medium design that favors the intended organism or community, and the addition of antibiotics [9, 11-13, 16-18]. Unfortunately, all of these will inherently increase production overhead. In general these costs are unavoidable. This is because contaminating microbes can decrease yield and/or titer of a final product, or produce compounds which result in the loss of a batch [19]. These precautions are unnecessary for this process. A general process overview can be seen in Figure 1.

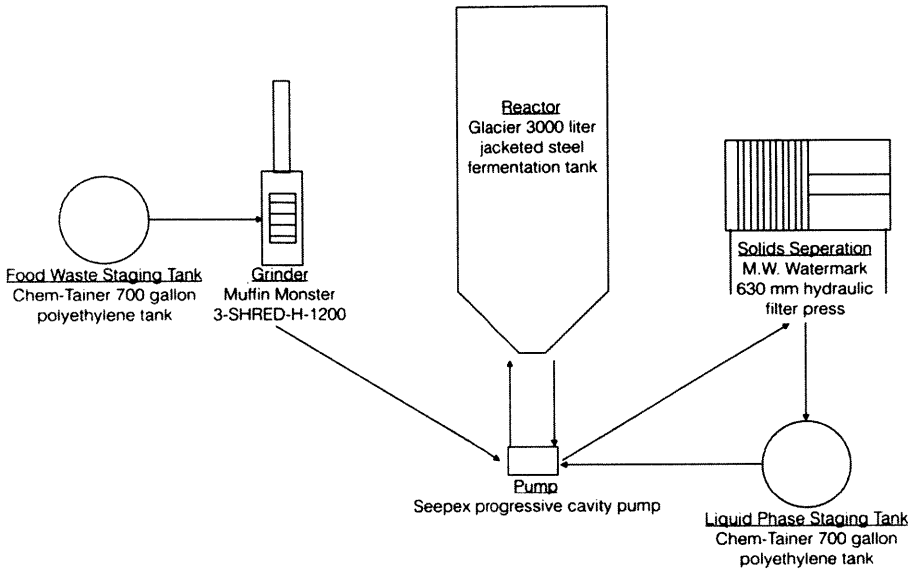


Figure 1. A small-scale process overview of a system to handle 0.5 tons of food waste per day for conversion to crude lactic acid.

Microbial Community

The communities responsible for anaerobic digestion are complex and have been extensively explored [20-23]. Important bacterial community members are from the Firmicutes, Bacteroides and Spirochetes, with the archaeal population largely made up of hydrogenotrophic and acetoclastic methanogens [24]. Anaerobic digestion of MSW to produce methane via short-chain fatty acid intermediates is similar to the process in which we produce lactic acid, although the community is shifted towards lactic acid producers and the methanogens are inhibited.

Commercial applications

The U.S. is the largest consumer of lactic acid in the world, accounting for 45% of the total $22 billion per year market [25]. This high value chemical has applications in the food industry as an acidity regulator, preservative and flavoring agent, and in other industries such as pharmaceuticals, consumer goods, and recently in biodegradable plastics using its polymeric form – Poly Lactic Acid. With this wide array of uses, the lactic acid market is estimated to grow substantially, with a CAGR of 20% until at least 2019 [26].

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