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Identification and Characterization of Microbial Contaminants and Associated Bacterial Viruses in Bioethanol Production Facilities to Suggest a Potential Alternative to Antibiotic Treatment

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IDENTIFICATION AND CHARACTERIZATION OF MICROBIAL CONTAMINANTS AND ASSOCIATED BACTERIAL VIRUSES IN BIOETHANOL PRODUCTION FACILITIES TO SUGGEST A POTENTIAL ALTERNATIVE TO ANTIBIOTIC TREATMENT

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ABSTRACT

In recent years, bioethanol has received worldwide interest as a bioenergy source. This interest has stimulated the production of substantial quantities of ethanol annually. However, the inability to produce bioethanol under sterile conditions plagues the industry, resulting in frequent microbial contamination. Bacterial contamination is one of the more challenging problems facing the bioethanol industry because contaminants drastically lower ethanol yield. Conventional methods of antibiotic application to eradicate bacterial contaminants are expensive and prohibitive. A more sustainable approach to control bacterial contamination of industrial ethanol fermentation systems is to use bacteriophages (phage). The goal of this research was to create a cocktail of phages capable of infecting and eliminating bacterial contaminants that hinder the production of bioethanol. I isolated, purified, and characterized the common bacterial contaminants in an industrial bioethanol fermenter and beerwell and demonstrated that bacteriophage could be induced from some of these cultures. Further research is needed to determine if virulent mutants of these phages can be generated.

Keywords: Bacteriophages, Antibiotic Alternatives, Bacterial Identification, Bioethanol

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PUBLICATIONS

1. Hatfull, G. F., et al (2012) Complete genome sequences of 138 mycobacteriophages. J. Virol. 86: 2382-2384.

2. M. Kearney, C. Kline, E. Anderson, L. Smith, C. Coomer, J. Lifson, J. Mellors, J. Coffin, and Z. Ambrose. Plasma Virus Populations in RT-SHIV-Infected Macaques Are Derived from Multiple Tissues. In prep for Journal of Virology.

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FIELDS OF STUDY

Major Field 1: Biology

Major Field 2: Chemistry

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CHAPTER 1

INTRODUCTION

Fossil fuels are limited in supply, expensive to extract, and contribute to pollution and climate change. The United States is heavily dependent on foreign oil, importing more than 50% of the oil it consumes.[1] In an effort to decrease the United States' dependence on foreign oil and the pollution associated with its use, many politicians and environmental scientists have promoted the production of ethanol fuel as a viable and sustainable alternative to gasoline.[2] Ethanol burns much more cleanly, and it is cheaper to produce. Cellulosic ethanol production reduces emissions by 95% compared to gasoline production.[1] Furthermore, cellulosic ethanol is one of the most promising options available to reduce transportation greenhouse gas emissions.

In recent years, the fuel-ethanol industry has experienced rapid growth, with 10.6 billion gallons of ethanol produced in 2009. It is estimated that 60 billion gallons/year will be needed by 2030 in the United States alone.[3] A major problem plaguing the industry, however, is the inability to produce fuel-ethanol under closed conditions and the substrates utilized for fermentation are not sterile.[4] As a result, it is not feasible to aseptically produce bioethanol. Because of this limitation, chronic and acute microbial contaminations are common and expected. Bacterial contaminations in bioethanol production may reduce ethanol yield by 27%.[5-6] Eradicating bacterial contaminants in fermentation systems often requires production shutdowns for extensive cleaning or

expensive prophylactic antibiotic treatments.[5,7] Developing methods for long-term suppression of microbial contamination is a major challenge in fuel-ethanol production.

There are numerous reasons why bacterial contaminants are detrimental to bioethanol production. The majority of the chronic contaminants are believed to compete for sugars that are utilized by yeast during fermentation.[5-8] Additionally, these microbes reduce the amount of essential micronutrients within the fermenting environment.[6-7] Furthermore, many bacterial contaminants often produce inhibitory byproducts, such as lactic acid and acetic acid, which inevitably leads to the inhibition of yeast growth and "stuck" fermentations—a condition where the yeast become dormant before fermentation has completed.[5-7, 9] These acids suppress yeast growth by lowering the pH below the optimal range for the conversion of sugars to ethanol. [9]

Among the bacteria that contaminate the mash (the organic fermentable material), the fermenters, and the beerwells, lactic acid bacteria are considered to be the most prevalent due to their rapid growth.[9-12] *Pediococcus* species are the second most prevalent. Specifically, it has been reported that the *Lactobacillus* species *L. fermentum* is the most abundant microbial isolate from commercial bioethanol systems.[9-11] Lactobacilli flourish in the commercial fermentation environments because they are well-adapted for survival and rapid growth under high-ethanol, low-pH, and low-oxygen conditions. For an extensive list of common microbial contaminants in fuel-ethanol production, see Table 1.

Table 1 Bacterial and yeast contaminants found in Fuel Ethanol Fermentations

Organism	Reference
Bacteroides forsythus	Skinner and Leathers (2004)
Bifidobacterium sp., *Bif. adolescentis*, *Bif. angulatum*, unidentified *Bifidobacterium* sp.	Skinner and Leathers (2004)
Clostridium sp., *Cl. aerotolerans*, *Cl. clostridiiforme*	Skinner and Leathers (2004)
Eubacterium biforme	Skinner and Leathers (2004)
Fusobacterium nucleatum	Skinner and Leathers (2004)
Lactobacillus sp., *Lact. acidophilus*, *Lact. amylovorus*, *Lact. brevis*, *Lact. buchneri*, *Lact. casei*, *Lact. crispatus*, *Lact. delbrueckii* subsp. *delbrueckii*, *Lact. delbrueckii* subsp. *lactis*, *Lact. diolivorans*-like, *Lact. ferintoshensis*, *Lact. fermentum*, *Lact. gasseri*, *Lact. helveticus*, *Lact. hilgardii*, *Lact. lindneri*, *Lact. manihotivorans*, *Lact. mucosae*, *Lact. nagelii*, *Lact. paracasei* subsp. *paracasei*, *Lact. pentosus*, *Lact. plantarum*, *Lact. reuteri*, *Lact. rhamnosus*, *Lact. salivarius*, *Lact. vini*, unidentified *Lactobacillus* sp.	Skinner and Leathers (2004); Lucena *et al.* (2010); Chang *et al.* (1995)
Lactococcus sp., *L. lactis* subsp. *lactis*, *L. raffinolactis*	Skinner and Leathers (2004)
Leuconostoc sp., *Leuc. carnosum*, *Leuc. citreum*, *Leuc. lactis* subsp. *lactis*, *Leuc. mesenteroides* subsp. *cremoris*	Skinner and Leathers (2004)
Oenoccocuss kitaharae-like	Lucena *et al.* (2010)
Pediococcus sp., *Ped. acidilactici*, *Ped. damnosus*, *Ped. parvulus*, *Ped. pentosaceus*, unidentified *Pediococcus* sp.	Skinner and Leathers (2004)
Propionibacterium granulosum	Skinner and Leathers (2004)
Weisella sp., *W. confusa*, *W. paramesenteroides*, *W. viridescens*	Skinner and Leathers (2004); Lucena *et al.* (2010)
Candida sp., *C. intermedia*, *C. lusitaniae*, *C. pararugosa*, *C. parapsilosis*, *C. tropicalis*, *C. xylopsoci*	Basilio *et al.* (2008)
Dekkera bruxellensis	Basilio *et al.* (2008)
Exophiala dermatitides	Basilio *et al.* (2008)
Hanseniaspora guilliermondii	Basilio *et al.* (2008)
Issatchenkia orientalis	Basilio *et al.* (2008)
Pichia anomala, *P. caribbica*, *P. fabianii*, *P. galeiformis*, *P. guilliermondii*, *P. ohmeri*	Basilio *et al.* (2008)
Pseudozyma hubeiensis	Basilio *et al.* (2008)
Saccharomycodes ludwigii	Basilio *et al.* (2008)
Williopsis sp.	da Silva-Filho *et al.* (2005)
Zygoascus hellenicus	Basilio *et al.* (2008)
Zygosaccharomyces fermentati	Basilio *et al.* (2008)

Table 1.1: Bacterial and yeast contaminants found in fuel-ethanol fermentations. Source: Beckner, M., Ivey, M.L. and Phister, T.G. (2011), Microbial contamination of fuel ethanol fermentations. Letters in Applied Microbiology, 53: 387–394.

To prevent the reduction of biofuel yields by bacterial contaminants, various approaches have been utilized to control their growth. Compounds such as 3,4,4'-trichlorocarbanilide, hydrogen peroxide (H_2O_2), and potassium metabisulfite are commonly used for this purpose. These agents can disrupt the structural integrity of the bacterial cell membrane (3,4,4'-trichlorocarbanilide), produce cytotoxic oxygen radicals that inactivate critical proteins (H_2O_2), or mutate DNA (H_2O_2 and potassium metabisulfite). However, bacterial contaminants are most often controlled by using commercially available antibiotics. [7,11,12] The most common antibiotics utilized by

fermenter facilities are virginiamycin, penicillin, and erythromycin.[7,11,12] Both virginiamycin and erythromycin inhibit protein production in bacterial cells by binding to the bacterial 50S ribosomal subunit.[7,11,12] Antibiotic binding to this subunit inhibits peptidyl transferase activity and interferes with the translocation of the ribosome during translation.[7,11,12] Penicillin is effective against Gram-positive bacteria and works by weakening the peptidoglycan cell wall to the point where osmotic pressure causes cytolysis.[7,11,12] Treatment with antibiotics often requires repeated addition at each cycle of fermentation. However, it has been reported that several *Lactobacillus* species isolated from dry-grind ethanol plants have become resistant to virginiamycin.[12] The emergence of multidrug resistant bacterial contaminants has also been documented.[7,12]

The potential for residual antibiotics in distillers' grains is a significant concern. These byproducts are generated by the fermentation process and are often recycled—in the form of forage or fertilizer.[9,11,12] Any remaining antibiotics in this waste may lead to the emergence, multiplication, and spread of resistant organisms, capable of threatening the safety of human health.[9,11,12] Therefore, it is crucial to develop a method to control lactic acid bacteria and other prevalent contaminants during bioethanol production without the relying on antibiotics.

The long-term goal of this research is to develop a new, less expensive, and sustainable approach to control bacterial contaminations within commercial ethanol fermentation systems. Bacteriophages (phages) are viruses that infect bacteria and are the most numerous biological entities on Earth. The activity of these natural parasites may be harnessed as an environmentally-friendly, inexpensive alternative to antibiotics. Very few studies have been performed to determine if bacteriophages can be used to

eradicate contaminant bacteria in bioethanol production. The use of phages to specifically eradicate lactic-acid bacteria in fermentation systems has been particularly under studied.[8]

There are several advantages of using bacteriophages to control bacterial populations. Bacteriophages are species-specific and do not directly infect human cells. In contrast to antibiotics, phages are self-replicating in environments where a suitable host exists. Specifically, when a phage infects a host bacterium, it uses the host cell machinery to reproduce. Once new phage particles have been assembled, the host cell is lysed, and the phages are released and go on to repeat the infection cycle. Additionally, the use of phage to remove or control bacterial contamination does not exacerbate the problem of antibiotic resistance.

To prevent or treat an active bacterial infection in a fermentation reaction, a cocktail of phages that attack different contaminants could be added at the source of the infection, such as the fermentable grains or the continuous/batch reactor. Ideally, the cocktail would be added in sufficient amounts to infect and lyse the contaminating bacteria. Reducing the number of contaminants should prevent the formation of the acidic environment responsible for inhibiting yeast growth.

To investigate the potential of utilizing bacteriophages as an alternative to antibiotics in the bioethanol industry, a series of experiments were conducted using samples obtained from an ethanol production facility located in Hopkinsville, Kentucky (Figure 1.1). To identify bacteriophages that may be used to eradicate the prevailing contaminants in the bioethanol fermenters, the bacterial hosts had to be identified. This

identification was required because bacteriophages are species-specific and have a narrow host range. Therefore, I collected raw sample specimens from a fermenter and beerwell from a bioethanol reactor and isolated the predominate bacterial species. These isolates were initially characterized by gram staining and light microscopy. This was followed by whole colony polymerase chain reactions (PCR) to precisely identify the contaminants at the genus and species level.

Figure 1.1: Commonwealth Agri-Energy fuel ethanol plant. Source: Brame, David. "Integrated Agriculture." *Commonwealth Agri-Energy*. Inter-Quest. Web. 9 Jan 2014. <http://www.commonwealthagrienergy.com/>.

Transmission electron microscopy (TEM) was used to screen the raw samples for the presence of bacteriophages. Lytic phages are the most desirable since they destroy their host at the end of the infection cycle. Lysogenic phages are less desirable because they can integrate into the host chromosome and enter a dormant state. When this occurs, the host continues to multiply. However, lysogenic phages can be genetically manipulated to adopt the lytic cycle, or lytic mutants could be selected. Alternatively, it

may be possible to use lytic phages that have been characterized by other laboratories. I identified one lysogenic bacteriophage and three prevalent contaminants from the Commonwealth Agri-Energy fuel ethanol plant located in Hopkinsville, KY.

CHAPTER 2

BACTERIOPHAGE BIOLOGY: AN OVERVIEW

Bacteriophages are viruses that infect bacteria and are so named because they destroy their bacterial hosts. The term "bacteriophage"—literally "bacteria-eater"—is derived from the Greek word "phagein," meaning "to eat." These bacterial viruses were independently discovered by British scientist Frederick Twort in 1915 and Felix d'Herelle in 1917, and were termed "filterable infectious agents" and "invisible antagonists" of bacterial cells.

Bacteriophages are genetically diverse and are the most prevalent biological entities on the planet.[13] It is estimated that there is a global population of at least 10^{31} bacteriophages, and less than one percent of the phages observed by electron microscopy, over 5400 to date, have been grown in culture. Scientists currently recognize 1 order, 13 families, and 31 genera of bacteriophages.[13]

Bacteriophages have a variety of structures, but there are four basic bacteriophage shapes: binary, icosahedral, helical, and pleomorphic.[13] All bacteriophages contain a capsid (head) structure, which houses the genetic material, and may vary considerably in size and shape from one bacteriophage to the next. Additionally, a significant portion of bacteriophages have tail structures that vary widely in length and diameter. The tail structure is a hollow tube, where the genome of the phage enters the host cell during the

beginning stages of infection. Figure 2.1 depicts examples of the diversity of bacteriophages morphology.

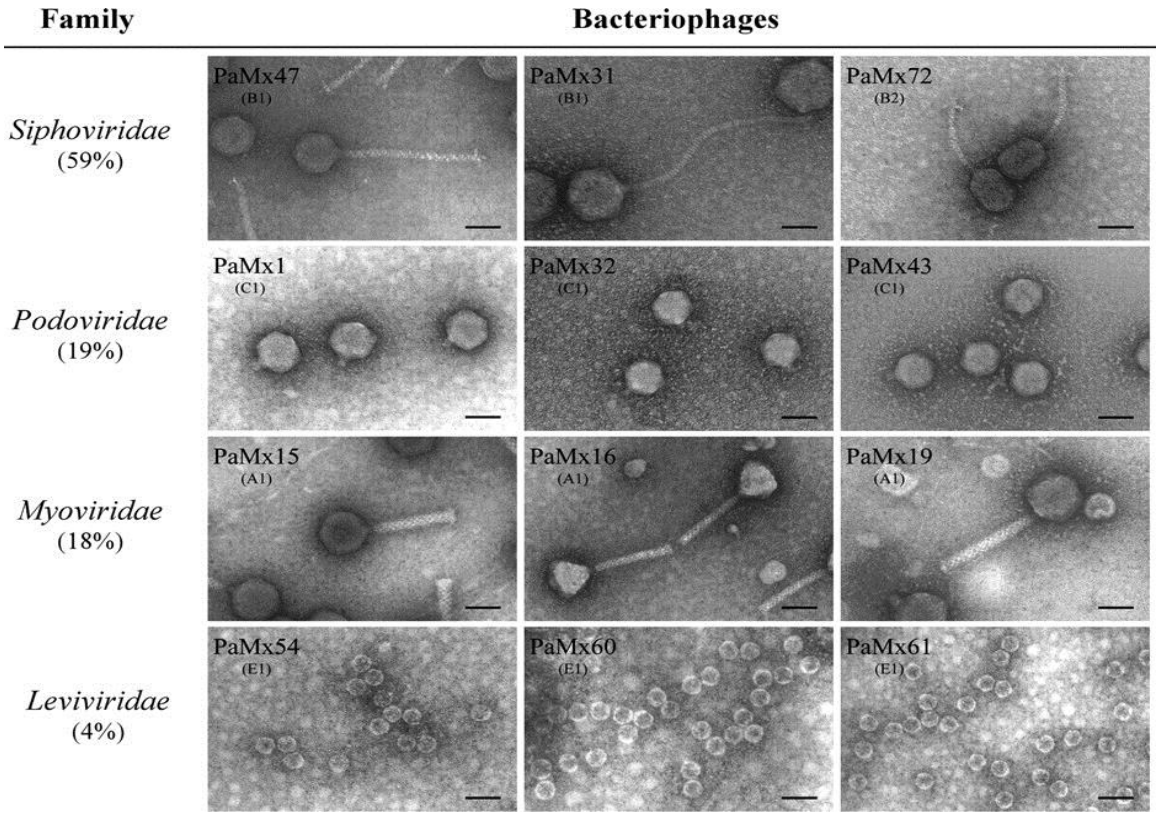


Figure 2.1: Electron micrographs of various bacteriophage morphologies. Electron microscope images of selected phages. Each row shows three examples of the morphological family indicated in the left margin. Each percentage corresponds to the proportion of phages in the collection belonging to each family. The upper left corners are marked with the name and morphotype of the phage in each picture. Source: Ashfield *et al*. "Applied and Environmental Microbiology." *High Diversity and Novel Species of Pseudomonas aeruginosa Bacteriophages.* 79.12 (2012): 4510-4515. Print.

The genome of most phages is comprised of double-stranded DNA and ranges in size from 20 to 500 kilobases. Generally, the length of the phage's genome is reflected in the diameter of the capsid. Bacteriophages genomes tend to be compact, and like most

viruses, they rely on the host cell to provide the metabolic pathways, replication, translation, and transcriptional machinery needed for propagation.

Many bacteriophages possess two alternative lifestyles: the lytic cycle or the lysogenic cycle. Bacterial viruses that undergo the lytic cycle are named “lytic” phages and phages that undergo the lysogenic cycle are termed “temperate” or “lysogenic” phages. Figure 2.2 provides a depiction of the two lifecycles and their differences.

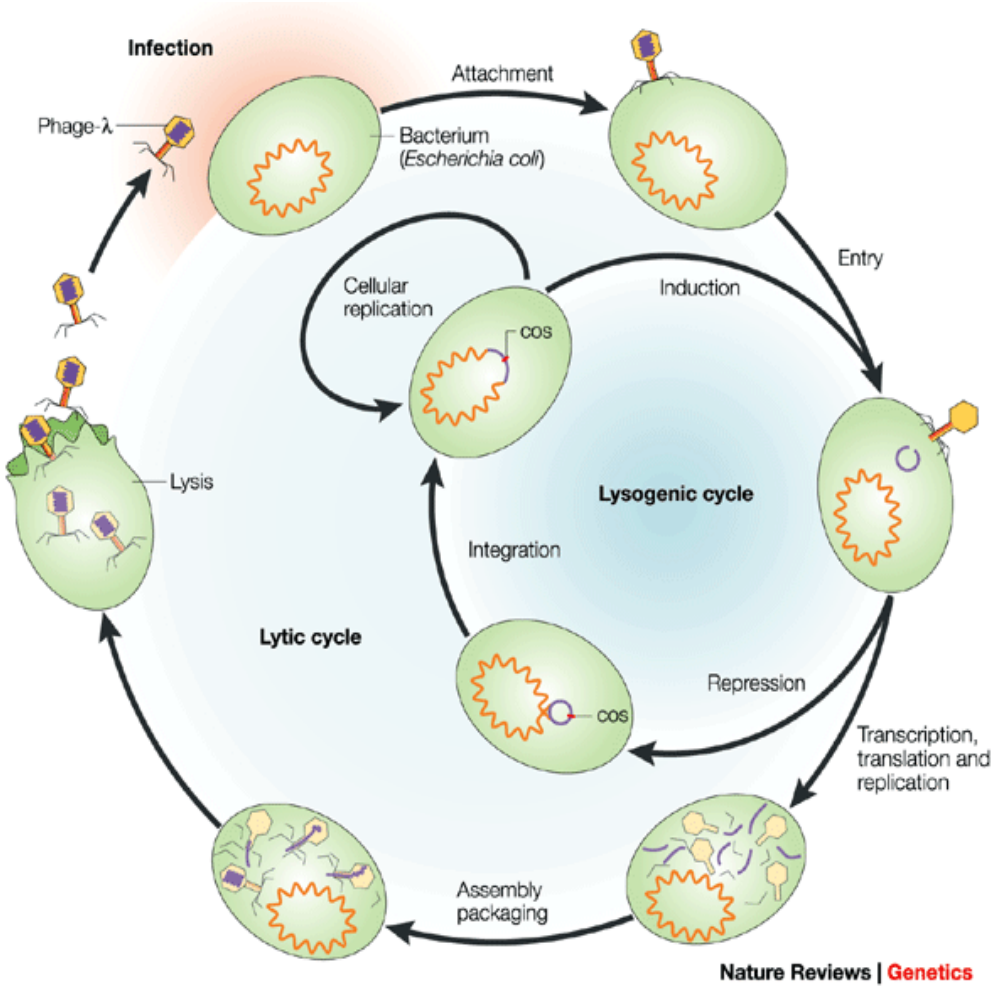


Figure 2.2: Bacteriophage lifecycles. Source: Campbell, Allan. "Nature Reviews Genetics." *Future of bacteriophage biology*. 4.1 (2003): 471-477. Print.

In both cycles, a bacteriophage adsorbs to a receptor, usually a protein, on the surface of the host cell. Receptors include pili, glycoproteins, flagella, lipopolysaccharides, or oligosaccharides.[13] Some bacteriophages may bind to multiple receptors. This attachment, often termed "adsorption," is mediated by specialized structures on the bacteriophage, typically located on the tail fibers.[13] The successful adsorption of a bacteriophage to the host surface receptor often results in a conformational change of the bacteriophage tail fibers and the bacterial surface receptor. In some phages, this conformational change results in the contraction of the bacteriophage tail and subsequent penetration of the host cell wall and cell membrane. After adsorption, the bacteriophage genome is injected into the host cell through the tail sheath. Bacteriophages that lack a tail—and a substantial proportion with non-contractile tails—often utilize cell wall and cell membrane degrading enzymes, such as lysozyme that can attack the cell wall. The genomic material of the bacterial virus then enters the host cell. The mechanisms for genome entry are largely unknown.

Once the genomic DNA of the bacteriophage is inside the host cell, the genome circularizes rapidly, or alternatively, it modifies its ends to protect them from host bacterial nucleases. Furthermore, as the infecting bacteriophages' genome enters the cell, host RNA polymerases immediately recognize the viral DNA promoters and begins to transcribe what are known as "early genes."[13] Because the host bacterial cells lack a nuclear membrane, transcription of these early genes is coupled with translation. Examples of genes that are expressed at the beginning of bacteriophage infection are repair enzymes that repair in the bacterial cell wall and proteins that deactivate host nucleases that could destroy the infectious viral DNA.[13]

After the expression of early genes, the bacteriophage genome is copied multiple times, along with the transcription and translation of phage genes that will comprise the capsid, tail fibers, and other necessary components of the bacteriophage's structure—these components are encoded by the phage "late genes."[13] Additionally, genes that may be involved in lysing the bacterial cell may be transcribed and translated. As soon as all bacteriophage structural proteins have been produced, progeny bacteriophages assemble, and a copy of the phage genome is packaged into the bacterial virus. Bacteriophages encode enzymes, such as endolysins, muramidases, and virolysins, which hydrolyze specific bonds in the cell wall of the bacterial host. These enzymes weaken the cell wall by hydrolyzing critical stabilizing bonds. The cells eventually lyse thus allowing the progeny virus to escape and initiate a new infection. Figure 2.3 depicts the steps of a detailed lytic infection of bacteriophage T4, a prototypical lytic phage.

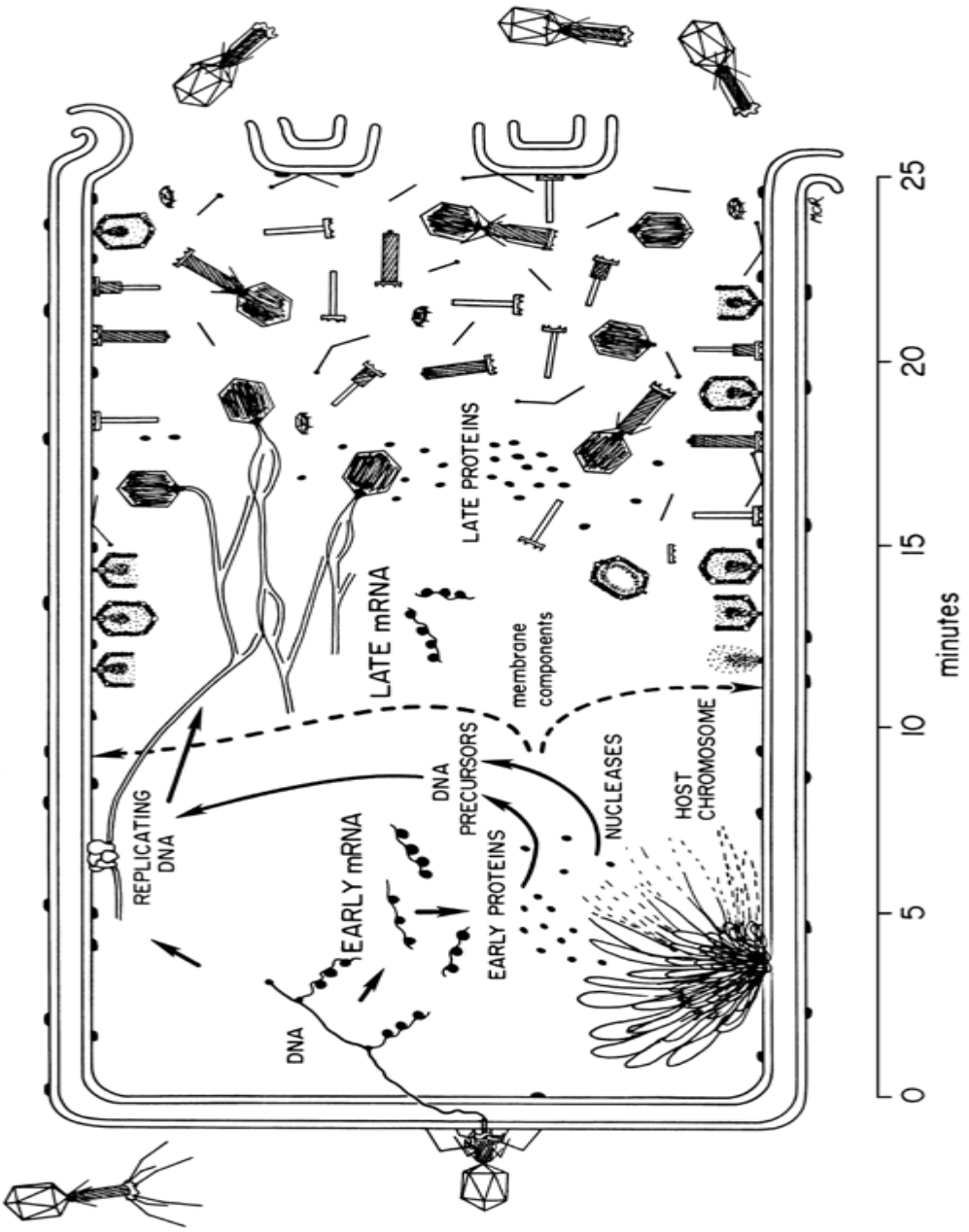


Figure 2.3 The lytic cycle of bacteriophage T4. Source: Abedon, S.T. and Calendar, R.L. *The Bacteriophages,* Second Edition. Oxford University Press, 2005.

Lysogenic, or temperate bacteriophages, undergo a drastically different lifecycle when compared to lytic viruses. Similar to lytic phages, lysogenic bacteriophages follow the same initial steps during infection. However, instead of killing the host directly via

lysis, the bacteriophage becomes dormant. Most lysogenic bacteriophages, such as phage λ, exist in their dormant state by integrating into a specific region of the host chromosome. This recombination event occurs at the attachment sites (*att*B (the bacterial attachment site) and *att*P (the phage attachment site)). Alternatively, some phage circularize their genome and exists as a plasmid, such as phage P1.[13] In addition, some phage integrate randomly via a transposase-mediated mechanism.[13] This mechanism occurs in transposable phages, such as bacteriophages Mu and D3112, specific to the *Pseudomonas* bacterial genus.[14-16] The integrated form of the bacteriophage is termed a "prophage." Specifically, soon after penetration, the phage genome integrates into the host chromosome, and because of this integration, it is replicated along with the cell each time the cell copies its chromosomal DNA. In many bacteriophages, such as phage λ and P22, virtually all viral genes are repressed in the prophage state. Some exceptions include the gene that encodes the phage repressor and some prophage encoded toxin genes (i.e. diphtheria toxin).

If the repressor is inactivated, the bacteriophage DNA is excised from the bacterial chromosome and the lytic cycle ensues. New virus particles are produced that are released upon lysis of the host cell. Derepression of the prophage can occur at a low spontaneous rate. However, derepression often occurs in response to genomic or cellular damage.[13] Bacterial cells undergo an SOS response to repair the damaged DNA. This SOS response leads to the inactivation of the bacteriophage repressor protein, and entry into the lytic cycle. This response allows the phage to escape its dying host. Between 20 and 200 new virus particles can be produced by each infectious cycle of most bacteriophages that have double stranded DNA genomes.

The destructive nature of bacteriophages towards bacteria is a property that has great potential for human health. For example, one of the discoverers of bacteriophages, Felix d'Herelle, actively researched and promoted the idea that phages could be utilized to fight bacterial infectious diseases.[13] Specifically, in his research, he hypothesized that the viral-induced lysis of bacteria could be used to cure patients suffering from many diseases, specifically typhoid, bubonic plague, and dysentery.[13] Using phages, he conducted trials to control an epidemic of chicken typhoid and buffalo pasteurelosis.[13] The overall results suggested that bacteriophage-treated chicken and buffalo populations suffered fewer deaths and shorter epidemics than untreated populations. These successful studies motivated d'Herelle to conduct human trials, using prepared bacteriophage lysates to treat humans suffering from dysentery and bubonic plague with all patients recovering rapidly with bacteriophage treatment.[13] Although promising, bacteriophage therapy was overshadowed by the discovery of antibiotics. However, the development of antibiotic resistance has revived interest in utilizing bacteriophages to treat bacterial infections. Figure 2.4 provides an example of the efficacy of topical bacteriophage cocktails in fighting an external multidrug-resistant *Staphylococcus aureus* infection.

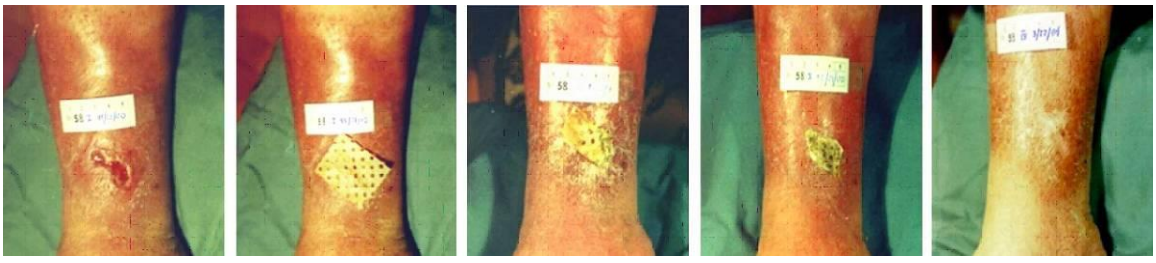


Figure 2.4 Bacteriophages in PhagoBioDerm™ help clear a wound from multidrug-resistant *S. aureus*. Source: Markoishvili, K., *et al* (2002), A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. International Journal of Dermatology, 41: 453–458.

CHAPTER 3

AN INTRODUCTION TO BIOETHANOL FERMENTATION

Bioethanol (fermentation ethanol) is produced from biomass feedstocks, such as corn, and accounts for more than 90% of all ethanol production in the United States.[17] In addition to its principal use in fuel, byproducts from this process are used in the beverage, fodder, forage, and the dry-ice industry.[17] In fact, to meet government-mandated fuel requirements to assist in the reduction of greenhouse gases, 7.3 billion gallons of bioethanol were added to gasoline in the United States in 2009.[17] Fuel-ethanol today is produced by utilizing a process almost indistinguishable from beer brewing. In this process, a significant quantity of starch-producing crops is converted to sugars that are fermented by yeasts into ethanol via glycolysis (Figure 3.1). Upon completion of this cycle, the fermented ethanol is distilled into its final form: biofuel.

At the biochemical level (Figure 3.1), ethanol is formed from pyruvate, an end product of glycolysis. Each step of glycolysis and ethanol fermentation is catalyzed by enzymes. The first step of ethanolic fermentation is the decarboxylation of pyruvate to form acetaldehyde. This reaction is catalyzed by pyruvate decarboxylase, which utilizes thiamine pyrophosphate, a coenzyme derived from the vitamin thiamine (B_1).[19] The second step of ethanolic fermentation is the reduction of acetaldehyde to ethanol by NADH. This reaction is catalyzed by alcohol dehydrogenase, regenerating NAD^+. The

regeneration of NAD^+ completes this sequence of metabolic reactions in a cyclic process. The net result of this anaerobic process is shown by the following chemical equation, using glucose as the carbon source:

$$\text{Glucose} + 2\ P_i + 2\ ADP + 2\ H^+ + 2NAD^+ \rightarrow 2\ \text{ethanol} + 2\ CO_2 + 2\ ATP + 2\ H_2O + 2NADH$$

The NADH generated by the oxidation of glyceraldehyde-3-phosphate in glycolysis, in preparation for its conversion into pyruvate, is consumed in the conversion of glucose into ethanol.[19]

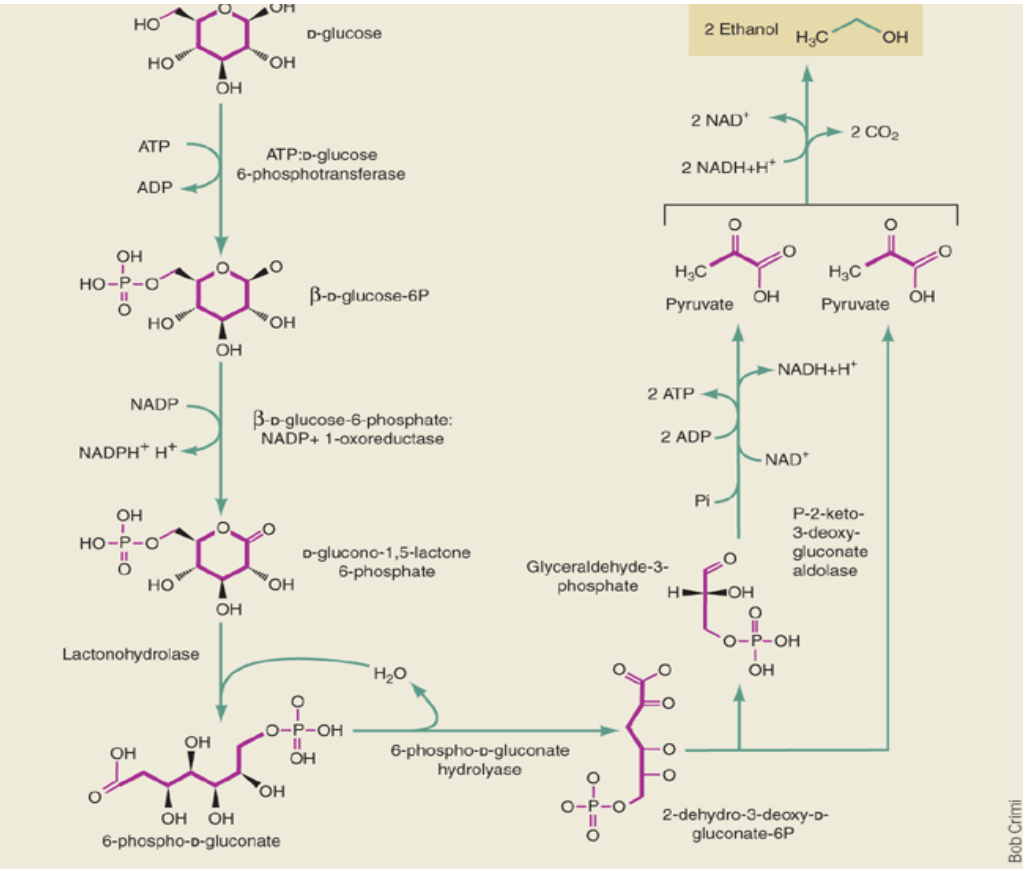


Figure 3.1 Biochemical pathway of glycolysis and ethanol Fermentation. The glycolytic pathway begins with D-glucose and involves a series of reactions catalyzed by enzymes to yield two molecules of pyruvate. Fermentation follows, beginning with the pyruvate molecules, to yield two molecules of ethanol. Source: Jeffries, Thomas. "Nature Biotechnology." *Ethanol fermentation on the move*. 23.1 (2005): 40-41. Print.

In addition to starch-producing crops, bioethanol may also be produced from cellulosic biomass (grasses, trees, and agricultural residues). Biofuel from this source is produced by first utilizing pre-treatment and hydrolysis procedures to extract sugars from the biomass in their monosaccharide form.[17] After this treatment, the monosaccharide forms of the extracted sugars are available for fermentation by yeast. It should be noted that producing bioethanol from cellulosic biomass is significantly more expensive than producing bioethanol from starch crops.[17] However, the United States government recently spearheaded a biofuels initiative motivated by the goal of reducing the cost of cellulosic bioethanol production.[17] Currently, scientists and environmentalists are working on ways to improve the efficiency and economics of the cellulosic bioethanol production process.

Bioethanol is most often produced on the industrial scale in a batch-style progression—using either a wet-mill or dry-mill process. In dry milling, corn is collected from surrounding agricultural areas. The kernels and additional starchy grains are mashed and ground into fine flour, often referred to "meal."[18] The meal is further processed without separating the mixed components of the grains, becoming liquefied upon the addition of water to form a murky "mash."[18] Ammonia is used to adjust the pH and also serves as a nutrient for the yeast. In addition, enzymes are added to convert the grains and corn starch into glucose, which is readily fermented by yeast cells.[18]

After this initial treatment, the mash is subjected to a high-temperature, high-pressure oven in order to reduce microbial levels that may hinder the fermentation of sugars into bioethanol by yeasts. Next, the mash is partially cooled and transferred to

large fermenters, where yeast culture is added to convert sugars to ethanol and carbon dioxide (CO_2). This process generally takes 40 to 50 hours, depending on the amount of yeast used to initiate fermentation as well as the mash-sugar concentration. While this process occurs, the mash is continually agitated and cooled to aid the activity of the yeast.[18] After fermentation, the resulting impure ethanol is transferred to columns where it is distilled—a process in which most of the water is separated by taking advantage of boiling point differences. The remaining stillage is removed by centrifugation, after which the ethanol is approximately 190 proof, and is subsequently dehydrated to approximately 200 proof utilizing a molecular sieve system.[18] Figure 3.2 on the following page depicts the dry-mill process.

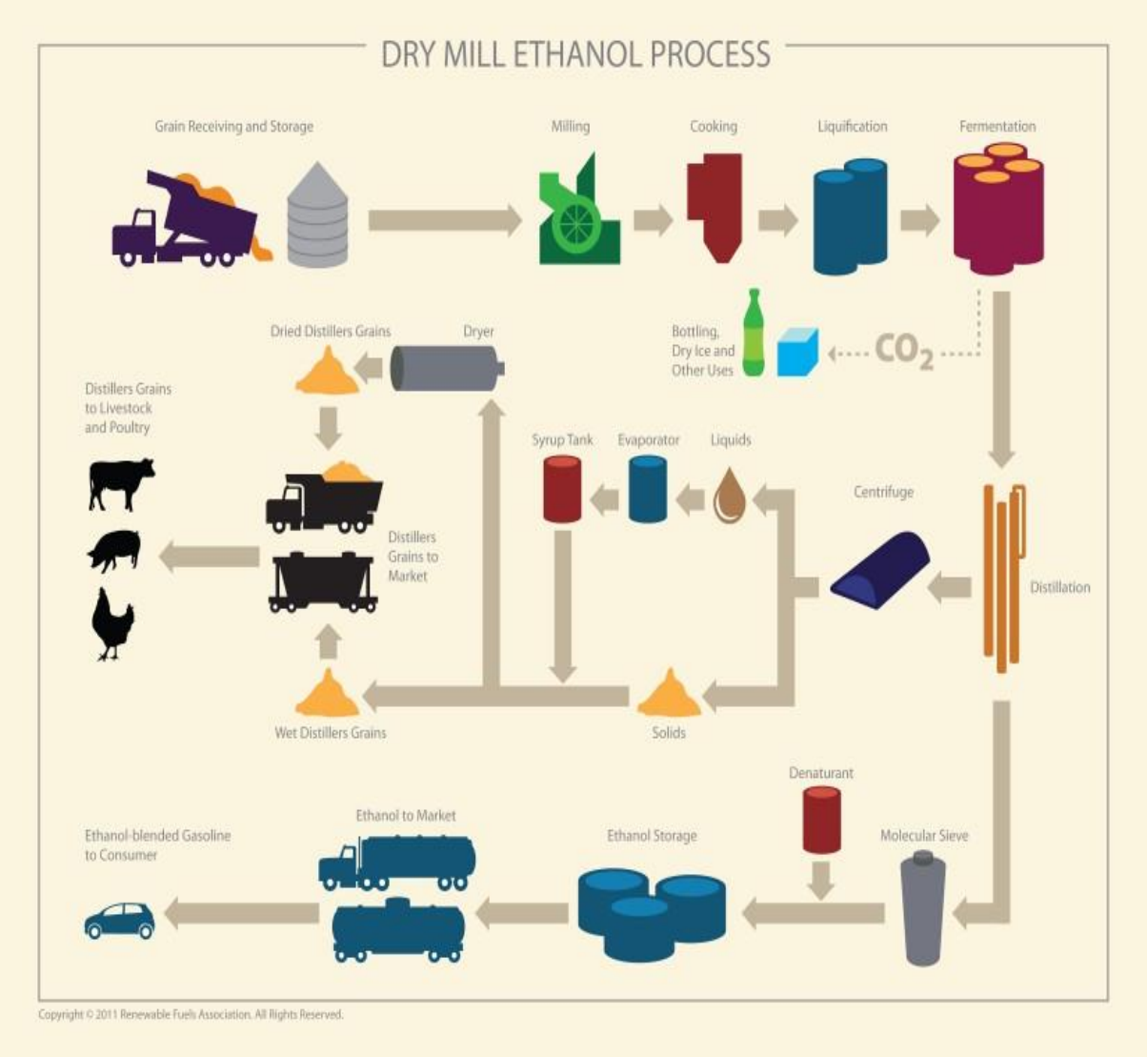


Figure 3.2 The ethanol production process—dry milling. Grains are ground into meal, mixed with water to form a mash, and treated to make simple sugars. The mash is then processed in a cooker, cooled, and fermented. The solution is distilled to separate ethanol and then dehydrated in a sieve system to produce bioethanol. CO_2 is collected after fermentation, and after distillation, the solution is treated to make feed. Source: Dinneen, Bob. N.P. Web. 8 Jan 2014. <http://www.ethanolrfa.org/pages/how-ethanol-is-made>.

Upon completion of fermentation (for both the wet and dry mill process), many recyclable co-products are produced. After distillation, the remaining silage is placed in a centrifuge to separate the coarse grain from the solubles. The solubles are then concentrated by evaporation, which creates condensed distillers solubles (CDS), most commonly known as "syrup." The CDS is further processed to produce nutritious livestock feed. Additionally, the carbon dioxide released during ethanolic fermentation of the sugars is captured, solidified, and sold for use in carbonating soft drinks and manufacturing dry ice. The fuel-ethanol industry supplies approximately 40 percent of the carbon dioxide for the North American merchant market.[18]

In the wet mill process, the grain is soaked, or steeped, in dilute sulfurous acid for two days to catalyze the separation of the grains into its component parts: starch, fiber, germ, and protein.[18] This slurried corn is then developed by passing it through a series of grinders to isolate the corn germ, upon which the corn oil may be extracted.[18] The remaining corn starch and corn fibers, such as cellulose, are separated further using separation techniques such as centrifugation.

The steeping ethanolic mixture is then evaporated in order to concentrate it, and contaminants such as gluten, protein, and fibers are separated by filtration and dried to produce a variety of co-products. The starch and excess water from the wet-mill mash is fermented into ethanol, as described above.

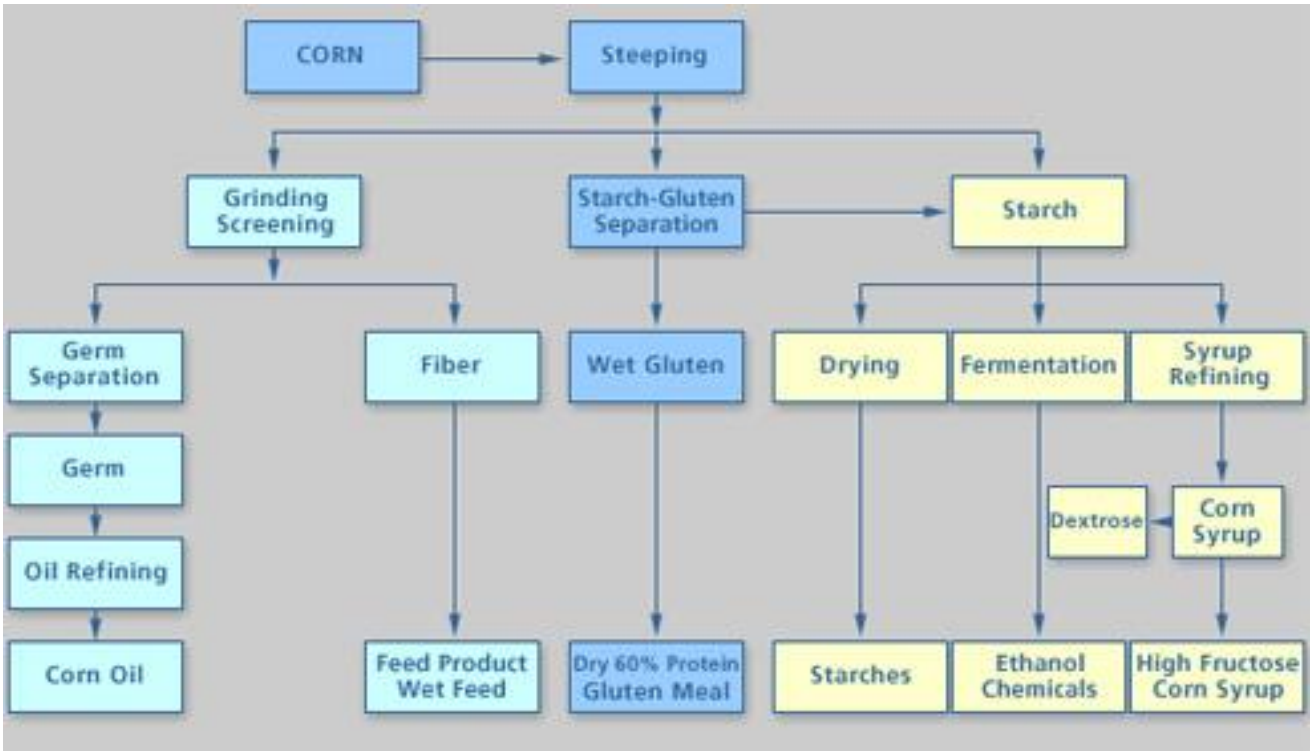


Figure 3.3 The ethanol production process—wet milling. Grain is initially steeped in dilute sulfurous acid to separate the grain into its many components. The resulting slurry is then ground to separate the corn germ and fiber to obtain corn oil or feed product. The gluten and starch are segregated using centrifugal methods to create meal, bioethanol, or corn syrup. Source: Dinneen, Bob. N.P. Web. 8 Jan 2014. <http://www.ethanolrfa.org/pages/how-ethanol-is-made>.

CHAPTER 4

MATERIALS AND METHODS

4.1 Sample Processing for Bacteriophage and Contaminant Isolation

Fermenter and a beerwell samples were obtained from a dry-mill bioethanol production facility in Hopkinsville, KY. The samples were collected into sterile 500mL bottles (Fisher Cat. No. 02-897-10), and were immediately placed on ice in a cooler. These samples, shown in Figure 5.1, contained large particles of debris and residue originating from the various fermentation process steps. Because of the substantial amount of debris, which interfered with downstream protocols, both samples were filtered using sterilized cheese-cloth to remove macroscopic particles. Approximately 8mL of the filtered samples were collected in 10mL conical tubes (Fisher Cat. No. 05-539-5)—two tubes each for both the fermenter and the beerwell samples.

A filtered sample from each source was centrifuged at 4 °C for 20 minutes at 157xg. This low speed centrifugation was performed to remove additional large debris and to facilitate the recovery of bacterial contaminants. The other two samples were centrifuged at 4°C for 15 minutes at maximum-speed, 3030xg. These samples were spun at maximum-speed to not only remove remaining macroscopic particles, but also to separate bacteriophage, if present, from any contaminating bacteria in hopes of being able to visualize bacteriophage by electron microscopy.

Immediately following centrifugation, approximately 8mL of the cleared supernatant from each sample was collected. Two milliliters of glycerol were added to the cleared supernatant from each sample prior to freezing at -80 °C.

4.2 Recovery of Bacterial Contaminants from fermentation samples

To isolate bacterial contaminants, aliquots of the samples from the low speed centrifugation were spread plated on de Man, Regosa, Sharpe agar (Thermo Scientific Cat. No. R01585) plates supplemented with 10 mg/mL cycloheximide (Sigma-Aldrich Cat. No. C1988-1G) (MRS/CHX). Because the concentration of contaminants in each source was unknown, aliquots ranging from 10 to 30 µL in increments of 10 µL were plated on individual plates. Because the aliquots of sample used were too small to be spread evenly on agar plates, each aliquot of sample was mixed with 5mL of MRS top agar and poured onto MRS/CHX plates. MRS agar is selective for *Lactobacillus*, *Lactococcus*, and *Pediococcus* growth—the most commonly cited contaminants of industrial fermentation systems as stated previously. Additionally, cycloheximide was included to prevent the growth of yeast.

All plates were placed in an anaerobic BioBag® chamber (Becton-Dickinson Cat. No. 261215) and were allowed to incubate overnight at 37°C. Independent colonies with different morphologies were picked and streaked for purification by transferring them to separate MRS/CHX plates via an inoculating loop. These plates were also incubated in BioBag® anaerobic chambers for 24 hours at 37°C.

Isolates were purified three times by streaking on the surface of MRS agar plates (standard streak plate method). After the third purification, the isolates were inoculated

into 10mL MRS broth (Thermo Scientific Cat. No. R061428) and allowed to grow overnight at 37 °C with shaking. A subculture was made from each purified strain growing in the MRS broth to allow long-term maintenance of each cell line. Subcultures were prepared by transferring 50 µL overnight culture to 10mL of sterile MRS broth incubating overnight at 37 °C with shaking. Each subculture was centrifuged for 10 minutes at maximum-speed, 3030xg. The supernatant was removed, and the bacterial pellet was re-suspended in 5mL of 10 mM $MgSO_4$. This allowed for short term storage of cells at 4°C for a maximum of two weeks.

4.3 Identification of Bacterial Contaminants by PCR

Polymerase chain reaction (PCR), shown in Figure 4.2, is a method used to amplify DNA. Specificity is achieved by complementary base pairing between the target sequence and the oligonucleotide primer. The bacterial contaminants in this study were identified at the species level using whole colony polymerase chain reactions (PCR). Primers specific for *Lactobacillus, Pediococcus,* and *Lactococcus* (ordered from Integrated DNA Technologies) were chosen because these bacteria are among the most common contaminants of bioethanol production facilities. Tables 4.1-4.3 show the primer pairs used for species identification.

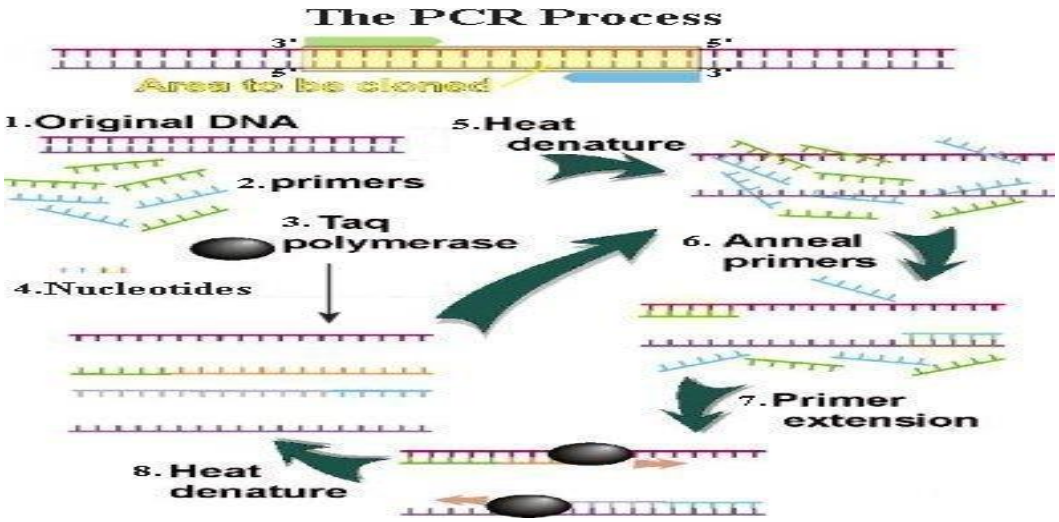


Figure 4.2 The polymerase chain reaction. Template is added to a solution of dNTPs, polymerase, and primers, which define the region to be amplified. The solution is subjected to cyclic temperature changes. By heating to roughly 95°C, the template DNA denatures. Upon cooling, the primers anneal to their complimentary regions. Polymerase recognizes the primers and synthesizes a new strand, yielding two new molecules of DNA. This is repeated multiple times for amplification of the target. Source: N.p. Web. 15 Jan 2014. <http://universe-review.ca/R11-16-DNAsequencing.htm>.

The identification of the bacterial contaminants at the species level was possible because the primers were complementary to unique regions of the 16S, 16S-23S rRNA intergeneic spacer region (ISR), and 23S genes.[20-22] In addition to highly conserved sequences, the 16S rRNA, 16S-23S ISR, and 23S rRNA genes contain hypervariable regions that can provide species-specific signatures that are useful for bacterial identification. Because of the high degree of specificity and accuracy of PCR, traditional phenotypic methods for microbial identification that rely heavily on differences in morphology, enzymatic activities, and metabolic capabilities were not used. The 16S rRNA genes were amplified in the *Lactococcus* isolates, and the 23S rRNA genes and

16S-23S ISR were amplified in the *Pediococcus* and *Lactobacillus* isolates. The 16S rRNA gene is sufficient to differentiate species of *Lactococcus*.[20] However, in the case of closely related species of *Lactobacillus* and *Pediococcus*, 16S rRNA probes or primers have not been used due to little variation of the 16S rRNA sequence.[21] The sequence of the 16S-23S rRNA ISR and 23S rRNA genes exhibits greater variation than that of the 16 rRNA structural gene in either species and hence is more suitable for designing species-specific probes to identify closely related species.[21,22]

Primer designation	Target organism(s)	Target region*	Length†	Sequence (5′–3′)
1RL	All species used in this study	37–61	19 (8)	TTTGAGAGTTTGATCCTGG
2RR	All species used in this study	1642–1666 RC	19 (9)	TCTACGCATTTCACCGCTA
LacreR	*Lactococcus lactis*	779–815 RC	19 (8)	GGGATCATCTTTGAGTGAT
LacF	*L. lactis* subsp. *lactis* (including *L. lactis* subsp. *lactis* biovar diacetylactis), *L. lactis* subsp. *hordniae*	217–342	19 (9)	GTACTTGTACCGACTGGAT
CreF	*L. lactis* subsp. *cremoris*	217–341	19 (9)	GTGCTTGCACCGATTTGAA
LgR	*L. garvieae*	1203–1239 RC	21 (6)	AAGTAATTTTCCACTCTACTT
PiplraR	*L. plantarum*, *L. piscium*, *L. raffinolactis*	1963–2015 RC	18 (11)	CGTCACTGAGGGCTGGAT

*Sequence position numbering from Ribosomal Database Project (RDP) prokaryotic small subunit rRNA sequence alignment database (Maidak *et al.* 2000). Data used in preparing this table were derived from the RDP accessed at Michigan State University (East Lansing, MI, USA) via ftp.cme.msu.edu on 9 November 2000. Numbering indicates the target region, but note that it does not indicate the size of the target.
†Primer length (nucleotides), with total of G and C bases indicated in parenthesis.
RC, 'Reverse' primer, complementary to relevant rRNA sequence.

Table 4.1: Oligonucleotide primers used to identify *Lactococcus* contaminants. Source: Pu, Z.Y. Dobos, M., Limsowtin, G.K.Y. and Powell, I.B. (2002), Integrated polymerase chain reaction-based procedures for the detection and identification of species and subspecies of the Gram-positive bacterial genus *Lactococcus*. Journal of Applied Microbiology, 93: 353–361.

Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)	Reference
16	GCTGGATCACCTCCTTTC	[9]
23-10C	CCTTTCCCTCACGGTACTG	[10]
Ldel-7	ACAGATGGATGGAGAGCAGA	present study
LU-1′	ATTGTAGAGCGACCGAGAAG	present study
LU-3′	AAACCGAGAACACCGCGTT	present study
LU-5	CTAGCGGGTGCGACTTTGTT	present study
Lac-2	CCTCTTCGCTCGCCGCTACT	present study
Laci-1	TGCAAAGTGGTAGCGTAAGC	present study
Ljen-3	AAGAAGGCACTGAGTACGGA	present study
Lcri-3	AGGATATGGAGAGCAGGAAT	present study
Lcri-2	CAACTATCTCTTACACTGCC	present study
Lgas-3	AGCGACCGAGAAGAGAGAGA	present study
Lgas-2	TGCTATCGCTTCAAGTGCTT	present study
Lfer-3	ACTAACTTGACTGATCTACGA	present study
Lfer-4	TTCACTGCTCAAGTAATCATC	present study
Lpla-3	ATTCATAGTCTAGTTGGAGGT	present study
Lpla-2	CCTGAACTGAGAGAATTTGA	present study
Lreu-1	CAGACAATCTTTGATTGTTTAG	present study
Lreu-4	GCTTGTTGGTTTGGGCTCTTC	present study
Lsal-1	AATCGCTAAACTCATAACCT	present study
Lsal-2	CACTCTCTTTGGCTAATCTT	present study
Lpar-4	GGCCAGCTATGTATTCACTGA	present study
RhaII	GCGATGCGAATTTCTATTATT	[19]

Table 4.2: Oligonucleotide primers used to identify *Lactobacillus* contaminants. Source: Song, Y.-L., Kato, N., Liu, C.-X., Matsumiya, Y., Kato, H. and Watanabe, K. (2000), Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S–23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiology Letters, 187: 167–173.

Primer	Target	Sequence	Length [bp]	GC [%]	T_m [°C]
Primers for genus identification					
Pedio23S_F	Typical	GAACTCGTGTACGTTGAAAAGTGCTGA	27	44.4	64.6
Pedio23S_R	pediococci	GCGTCCCTCCATTGTTCAAACAAG	24	50.0	64.5
PDE23S_F	*P. dextrinicus*	CAAGGCGTAGTCGATGGCAAG	21	57.1	64.5
PDE23S_R		GGGCTTCAATTCGTACCTTTGGGT	24	50.0	64.5
Multiplex PCR primers					
PDA23S_F	*P. damnosus*	GTTACCGCCACGTGAATACATAA	23	43.4	60.9
PST23S_F	*P. stilesii*	GTTCTTGAAACCATGTGCCTACAAA	25	40.0	61.3
PPE23S_F	*P. pentosaceus*	CCAGGTTGAAGGTGCAGTAAAAT	23	43.4	60.9
PPA23S_F	*P. parvulus*	TTAGGGCTAGCCTCGGATTA	20	50.0	60.4
PCE23S_F	*P. cellicola*	AACAAGTCTGGTGGAGAGTG	20	50.0	60.4
PIN23S_F	*P. inopinatus*	GAGGAGAGTATCCTAAGGTGT	21	47.6	60.6
PCL23S_F	*P. claussenii*	AGGTCAGCCGCAGTGAAG	18	61.1	62.1
PAC23S_F	*P. acidilactici*	GTTTCGGAGGAGGCGCAA	18	61.1	62.1
P23S_R	Different LAB	CTGTCTCGCAGTCAAGCTC	19	57.8	62.3

Table 4.3: Oligonucleotide primers used to identify *Pediococcus* contaminants. Source: Pfannebecker, J., Frohlich J. (2008) Use of a species-specific multiplex PCR for the identification of pediococci. International Journal of Food Microbiology, 128: 288-296.

Whole colony PCR is a type of polymerase chain reaction that does not require purified template DNA. Instead, this type of PCR uses the bacterial cells directly. The intense heat of the denaturation step destroys the bacterial cell wall and plasma membrane and enables the primers to access the targeted DNA for amplification.

Each PCR performed was carried out in a 0.2mL microcentrifuge tube, containing an aliquot of a master-mix, consisting of the ingredients listed below. Master Mix, per reaction:

- 16.5 µL of PCR mix (a solution containing 330µL of 10X PCR buffer B (Fisher Cat. No. FB 600050), 6µL of each dNTP (CTP-Fisher Cat. No. BP 2592250; ATP-Fisher

Cat. No. BP 2590250; GTP-Fisher Cat. No. BP 2591250; TTP-Fisher Cat. No. BP2593250), (100mM final concentration), 330µL of 25mM $MgCl_2$, 316µL of npH_2O)

- 30.0 µL of npH_2O water
- 0.5 µL of Taq Polymerase (5000units/mL; Fisher Cat. No. FB-6000-15)
- 1 µL of forward and reverse primer (100pmol/µL)

The final volume of this mixture was 49 µL. One microliter of $MgSO_4$ suspended bacterial cells, from step 4.2, was added and placed into a PCR machine. Each primer pair required different cycle conditions, which are shown in Tables 4.4 and 4.5.

After each PCR, the reaction products were separated by gel electrophoresis (see Section 4.4) and stained with 0.5 µg/mL ethidium bromide (Sigma-Aldrich Cat. No. E7637-1G). Ethidium bromide is a chemical that fluoresces when bound to DNA and exposed to UV light. By examining size of the amplicon, the identity of the unknown bacterial isolate could be determined. A positive result was obtained when a PCR product corresponding to the predicted size was detected. Predicted product sizes for each set of primers used are shown in Tables 4.6-4.8.

Genera	Denaturation	Annealing	Extension	Number of Cycles	Final Extension
Lactobacillus*	95°C for 20s	55°C for 2min	(Included in Annealing step)	35 Cycles	N/A
Pediococcus	Initial: 95°C for 15min				72°C for 10min
	94°C for 30s	69 °C decreasing by 0.3°C/0.15s for 60s	72°C for 60s	Conditions for: first 10 cycles	
	94°C for 30s	66°C for 60s	72° for 60s	Conditions for: next 22 cycles	
Lactococcus	94°C for 30s	45°C for 30s	72°C for 30s	35 Cycles	N/A

Table 4.4: PCR cycle conditions for tested genera.

Lactobacilli can be broadly grouped based on phylogenetic relatedness.[21] In this study, the unknowns were initially sorted into phylogenetically related groups using the primers listed in Table 4.6. If the appropriate sized PCR product was produced, a second PCR reaction was run to unequivocally identify the species. Reactions utilized to categorize unknown contaminants into groups required different cycle conditions than the reactions used for identification at the species level. This approach reduced the number of PCR reactions needed to identify *Lactobacillus* contaminants. The cycle conditions used for species identification are listed in Table 4.5, where cycle conditions listed in Table 4.4 were used for initial grouping.

PCR Group	Denaturation	Annealing/Extension	Number of Cycles	Final Extension
I	95^{o}C for 20s	68^{o}C for 2min	35 Cycles	74^{o}C for 5min
II	95^{o}C for 20s	65^{o}C for 2min	35 Cycles	74^{o}C for 5min
III	95^{o}C for 20s	62^{o}C for 2min	35 Cycles	74^{o}C for 5min
IV	95^{o}C for 20s	60^{o}C for 2min	35 Cycles	74^{o}C for 5min

Table 4.5: Species-specific PCR Cycle Conditions for *Lactobacillus*.

Group Primer Pairs (Forward/Reverse)	Expected Product Size (bp)	Species-Specifc Primer Pairs (Forward/Reverse)	Expected Product Size (bp)
Ldel-7/Lac-2 (Group 1)	450bp	Laci-1/23-10C	210
		Ljen-3/23-10C	700
LU-5/Lac-2 (Group 2)	400bp	Lcri-1/Lcri-2	522
		Lgas-1/Lgas-2	360
LU-3/Lac-2 (Group 3)	350bp	LU-5/Lpar-4	312
		LU-5/RhaII	113
LU-1 /Lac-2 (Group 4)	300bp	Lsal-1/Lsal-2	411
		Lreu-1/Lreu-4	303
		Lpla-3/Lpla-2	248
		Lfer-3/Lfer-4	192

Table 4.6: Predicted sizes of amplicons produced by each set of primers used in whole-colony PCR reactions. Each 3 letter abbreviation for the species-specific primer pairs corresponds to the species name in the *Lactobacillus* genus (i.e. "Lfer" corresponds to ***L**actobacillus **fer**mentum*). Source: Song, Y.-L., Kato, N., Liu, C.-X., Matsumiya, Y., Kato, H. and Watanabe, K. (2000), Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S–23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiology Letters, 187: 167–173.

Species-Specific Primer Pairs (Forward/Reverse)	Expected Product Size (bp)
1RL/LacreR	238
1RL/LgR	482
1RL/PiplarR	860
LacF/LacreR	163
CreF/LacreR	165

Table 4.7: Predicted sizes of amplicons produced by *Lactococcus* specific primer pairs sets used in whole-colony PCRs reactions. To determine the species that correspond to each primer pair, please consult Table 4.1. Source: Pu, Z.Y. Dobos, M., Limsowtin, G.K.Y. and Powell, I.B. (2002), Integrated polymerase chain reaction-based procedures for the detection and identification of species and subspecies of the Gram-positive bacterial genus *Lactococcus*. Journal of Applied Microbiology, 93: 353–361.

Species-Specific Primer Pairs (Forward/Reverse)	Expected Product Size (bp)
PDA23S_F/ P23S_R	2244
PST23S_F/ P23S_R	1840
PPE23S_F/ P23S_R	1647
PPA23S_F/ P23S_R	1517
PCE23S_F/ P23S_R	866
PIN23S_F/P23S_R	711
PCL23S_F/ P23S_R	620
PAC23S_F/ P23S_R	213

Table 4.8: Predicted sizes of amplicons produced by *Pediococcus* specific primer pairs used in whole-colony PCR reactions. Each 3 letter abbreviation for the species-specific primer pairs corresponds to the genus and species name (i.e. "PPE" corresponds to ***P****ediococcus* ***pe****ntosaceus*). Source: Pu, Z.Y. Dobos, M., Limsowtin, G.K.Y. and Powell, I.B. (2002), Integrated polymerase chain reaction-based procedures for the detection and identification of species and subspecies of the Gram-positive bacterial genus *Lactococcus*. Journal of Applied Microbiology, 93: 353–361.

4.4 Agarose Gel-Electrophoresis

A 2.0% or 2.5% (mass/volume) gel was created by mixing agarose (Fisher Cat. No. BP160-100) with 1X tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer [diluted from a 50X stock made of 242 g of Tris Base (Fisher Cat. No. BP1521) 57.1 mL of glacial acetic acid (Fisher Cat. No. BP2401C), 100mL of 0.5 M EDTA (Fisher Cat. No. BP2482) and 37.2 g of $Na_2EDTA{\cdot}2H_2O$ (Fisher Cat. No. BP120500), pH adjusted to 7.8 with acetic acid]. This mixture was heated until the agarose completely dissolved. The molten agarose was allowed to cool to 55 °C before pouring. After cooling, 25 mL of the molten agar was poured into a mold containing a 10 well comb and was allowed to solidify. The agarose gel and mold were immersed in a 1X TAE in an appropriate gel

electrophoresis apparatus (Fisher Cat. No. FB-SB-710 or FB-SB R-1316). The DNA ladders used included a 100 bp ladder (Axygen Cat.No. M-DNA-100BP), exACTGene 100bp DNA Ladder (Fisher Cat. No. BP2571100), and/or the low range ladder (Fisher Cat. No. BP2578100).

After the DNA was loaded into the wells, an electrical current was applied (10V/cm) for up to 1.25 hours. Because DNA is a negatively charged molecule, it migrates to the cathode. Smaller DNA molecules move more quickly their larger counterparts. DNA fragments were stained with a 0.5 μg/mL solution of ethidium bromide for 10 minutes, and visualized by exposing the gel to UV light. Species identification was verified when products that correspond to the predicted amplicon size were observed.

4.5 Gram-Staining of Bacterial Isolates

Purified bacterial isolates were Gram-stained using commercial reagents and the manufacturer's protocol. The Gram stain is a differential stain because of the fundamental differences in the cell architecture of gram-positive and gram-negative bacteria. Gram-positive bacteria have a thick, multilayered peptidoglycan cell wall, and lack an outer membrane. These cells appear purple when gram-stained. Gram-negative bacteria have a thin, single-layered peptidoglycan cell wall and possess both an inner and outer membrane. These cells appear red when gram-stained. Knowledge of an unknown's gram reaction provided useful information. However, this technique was abandoned in favor of whole colony PCR, which can rapidly identify bacterial species.

The gram stain procedure is as follows:

• *Escherichia coli* and *Micrococcus luteus* cells were smeared onto a glass slide and served as gram-negative and gram-positive controls, respectively.

• Unknown cells were smeared onto a different area of the slide.

• The cells were heat-fixed onto the slide by gently heating with a Bunsen burner until the slide was warm to the touch.

• Flood slide with crystal violet solution for one minute.

• Rinse with distilled water.

• Flood slide with Gram's iodine solution for one minute.

• Rinse with distilled water.

• Decolorize with concentrated ethanol for one to five seconds.

• Rinse off with distilled water.

• Flood slide with safranin for 30 seconds.

• Rinsewith distilled water.

• Blot dry using bibulous paper

• Immerse in oil and view via light microscopy.

4.6 Induction of Resident Prophages

Two different protocols were used to detect phages. After collecting the raw samples from the beerwell and fermenter, they were filtered and purified as described earlier (see Section 4.1). A 5 mL aliquot of filtered sample was centrifuged to remove additional debris and bacterial cells, and 2 mL of supernatant were transferred to 5 mL syringes that were attached to 0.22 µm filters (Fisher Cat. No. 09 -719G). After filtration, the samples were centrifuged at 14.8 rpms for one hour at 4˚C. After centrifugation, the supernatant was placed in 100 µL of phage buffer, and was examined for the presence of phage by electron microscopy (discussed below).

To determine if lysogenic bacteriophage were present, the purified bacterial isolates were grown in the presence of mitomycin C (Fisher Cat. No. BP2531-2). This drug induces the bacterial SOS response which often leads to the derepression of resident prophage.

For mitomycin C induction, 5 mL of overnight cells were centrifuged and suspended in 5 mL of 10mM $MgSO_4$. This suspension was used to inoculate 5mL of MRS broth and this subculture was incubated overnight at 37 °C with shaking. The following day, the cells were subcultured again by diluting 50 µL of the overnight culture into 5 mL of new MRS broth. Two controls were also created. One contained uninoculated media, the other contained 50 µL of the overnight culture. These cultures were incubated for 1.5 hours at 37˚C, with shaking, in a screw-capped tube. Following this, 5µL of 2 mg/mL stock of mitomycin C was added to the subculture. The controls were incubated with the drug-treated culture and were used to compare the relative cell density throughout the incubation period. All cultures were incubated for 5 hours at 37 °C then examined for signs of cell lysis. Two 1.5 ml aliquots were removed from the drug-treated culture and transferred to separate microcentrifuge tubes. One tube was treated with 100 microliters of chloroform the other was untreated. After vortexing for 30 seconds to ensure thorough mixing, the samples were centrifuged for 5 minutes at 13,400 rpms at 4 °C. The supernatant (lysate) was transferred to fresh tubes and examined by electron microscopy.

Electron Microscopy:

To prepare lysates for electron microscopy examination, 10µL of lysate (from the mitomycin C induction or from extended centrifugation of the filtered raw fermenter and

beerwell samples, see Section 4.1) was placed onto a Formvar-coated EM grid and allowed to incubate for two minutes. After incubation, the sample-side of the grid was washed twice by pipetting 10μL of sterile water onto the grid, allowing the grid to incubate for two minutes, and wicking off the excess water using filter paper. The grid was then stained using the following method, wicking off the material between each step: phage buffer (10 seconds), deionized water (10 seconds), 1% uranyl acetate (1 minute), 1% uranyl acetate (1 minute). The grid was allowed to dry and was then loaded onto the TEM and visualized.

CHAPTER 5

RESULTS

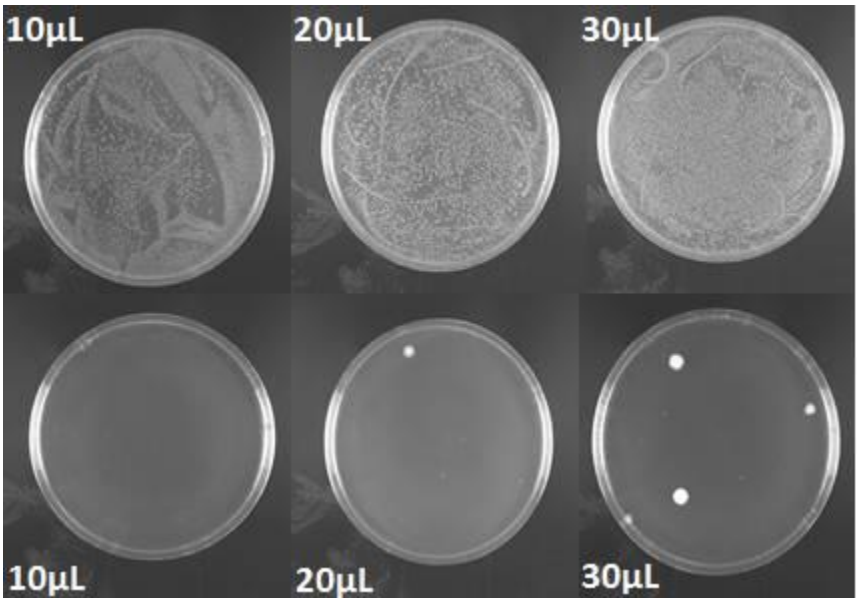
5.1 Sample Collection and Processing.

Industrial production of ethanol is adversely impacted by the presence of contaminating bacteria that compete with the yeast for fermentable sugars and vital micronutrients. An essential first step in this research was to identify the common contaminants present in an industrial fermentation system. To accomplish this, we obtained fermenter and beerwell samples from an ethanol production facility in Hopkinsville, KY. Because the raw samples (depicted in Figure 5.1) contained large particles of debris, they were filtered through sterilized cheese-cloth. These filtered samples were subsequently centrifuged at different speeds to remove any remaining macroscopic particles and to separate bacteriophage, if present, from any contaminating bacteria. Different amounts of processed specimen were plated on MRS agar plates (described in Section 4.2) to select for common contaminants such as *Lactobacillus fermentum* and *Lactococcus lactis*. Plates were incubated overnight at 37°C in anaerobic BioBag ® chambers.

Five different colony morphologies were detected after overnight growth. Figure 5.2 shows the growth on MRS plates. The increasing numbers of contaminant bacteria on the

MRS plates with increasing volume of sample plated is evident. Individual isolates were purified by the streak plate technique (Section 5.2).

Figure 5.1 Raw fermenter and beerwell samples. These raw samples were collected from Commonwealth Agri-Energy**,** Located in Hopkinsville, Kentucky, Samples were immediately stored on ice upon retrieval, and were processed using the methods described in Section 4.1.



Figures 5.2: Aliquots from processed fermenter sample (Top) and processed beerwell sample (Bottom) were plated on MRS agar supplemented with cyclohexamide and incubated overnight at 37 °C.

5.2 Purification of Bacterial Isolates

Seven colonies with different morphologies were purified three times by the streak-plate method, as described in Section 4.2. During the streaking process, some candidates that initially appeared unique were found to be more similar to other isolates. The reason for this change is not known, but it is likely that high density of bacteria on the primary isolation plates could have influenced the observed phenotype. After purification, only five unique bacterial morphotypes remained. Examples of the streak plate purification are shown in Figure 5.3.

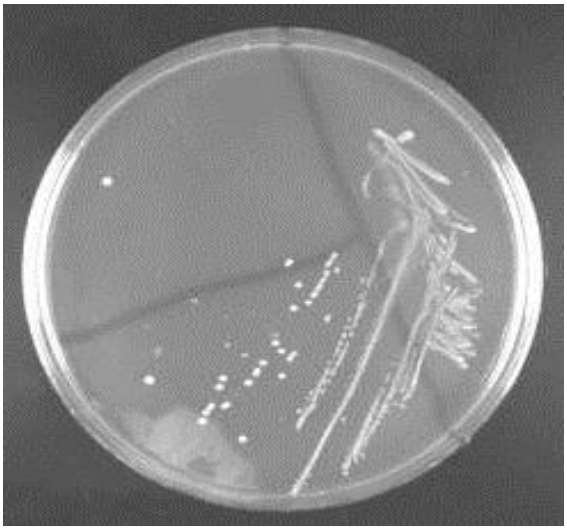
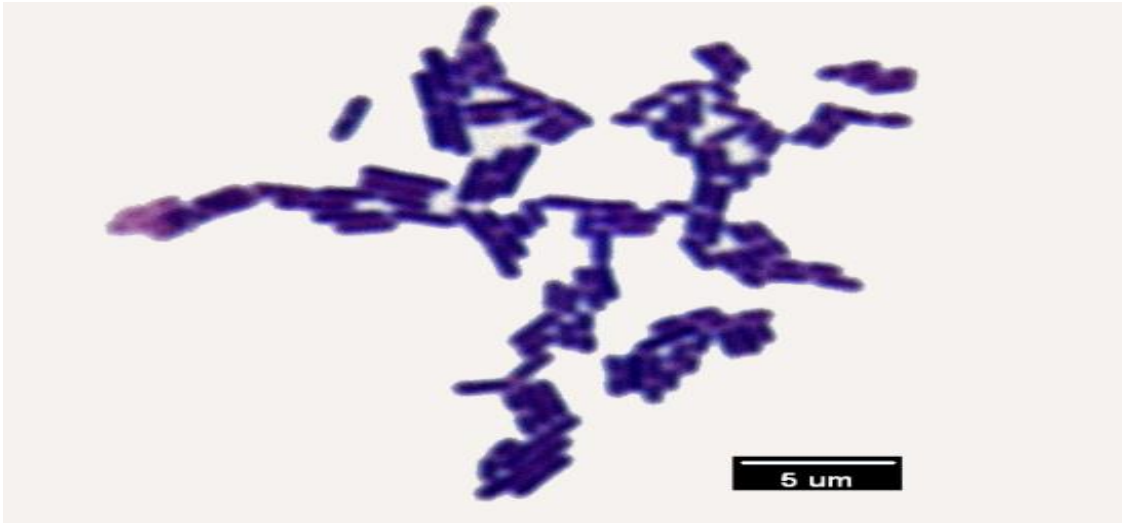


Figure 5.3: Purification of bacterial isolates by the streak plating method.

5.3 Gram-Staining

Gram-stains, described in Section 4.5, were performed for preliminary characterization of the newly isolated unknowns. Gram-stains were not performed on all bacterial isolates because this characterization method was replaced by whole-colony PCR for rapid species identification. Nevertheless, two gram-stains were performed, and

these two isolates were determined to be a gram-positive bacillus (Figures 5.3) and a gram-positive coccus (Figure 5.4).



Figures 5.4: Gram-stain of *Lactobacillus paracasei* cells

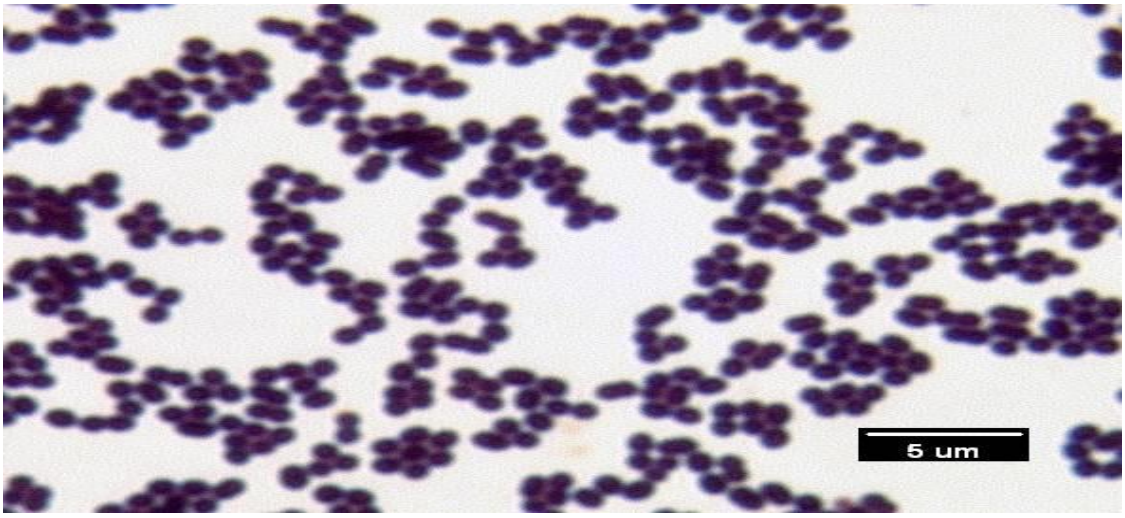


Figure 5.5: Gram-Stain of *Pediococcus pentosceus* cells

5.4 Bacterial Identification by PCR

Three different sets of oligonucleotide primers were used to identify the five microbial contaminants isolated from the fermenter and beerwell samples from the fuel-ethanol facility. These published primer-sets were designed to identify all species of *Lactococcus*, *Lactobacillus*, and *Pediococcus*, by amplifying species-specific 16S rRNA or 23S rRNA gene sequences (see Section 4.3). A unique PCR product of expected size was generated if the primer pair matched the sequence of the unknown bacterial isolate. The predicted sizes of the amplicons produced by each set of primers are listed in Tables 4.6, 4.7 and 4.8.

All PCR products were analyzed by standard agarose gel electrophoresis, using either 2.0% or 2.5% gels. Of the five microbial species isolated from the ethanol production facility, three were identified at the species level: *Lactobacillus fermentum*, *Lactobacillus paracasei*, and *Pediococcus pentasaceus*. These bacterial species, when amplified with primer sets LU-5/Lpar-4, PPE23S_F/P23S_R, and Lfer-3/Lfer-4 created amplicons of the expected size (312bp, 1647bp, and 192bp, shown in Figures 5.6-5.8, respectively). The identity of the two other isolates could not be determined with any of the primer pairs used in this study. One of these unknowns produced small white colonies and was collected from the beerwell. The other produced characteristic small grey colonies, was unable to grow in MRS broth, and was isolated from fermenter samples. These isolates may be an *Enterococcus* species, which are also common contaminants in fuel-ethanol production (see Table 1.1).

Negative results were obtained when no product was generated or if the product was the incorrect size Figures 5.6-5.8 are gel images showing the positive identification of *Lactobacillus fermentum*, *Lactobacillus paracasei*, and *Pediococcus pentasaceus.*

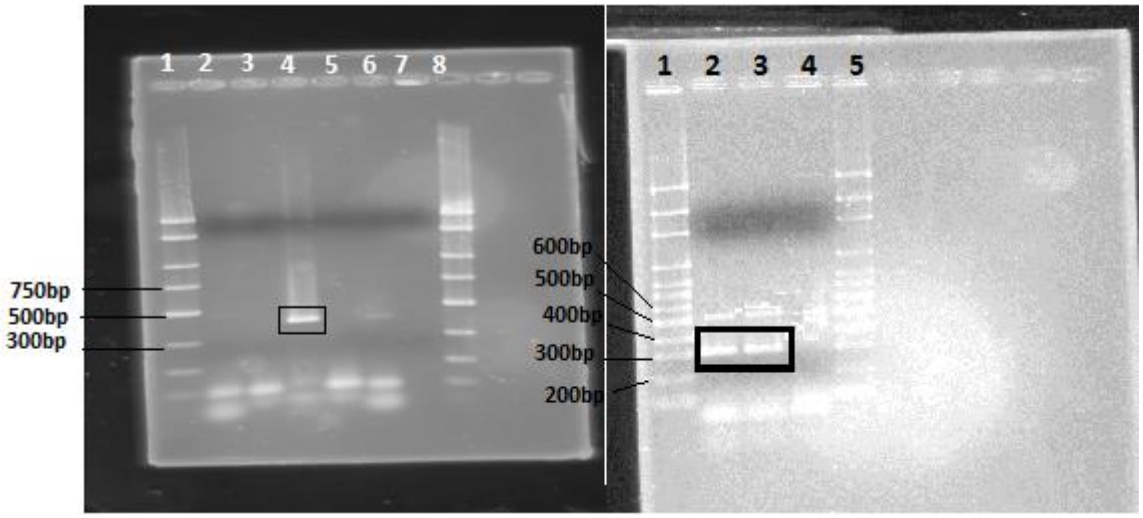


Figure 5.6: PCR amplification products using group primers (Left) and species-specific primers (Right) to identify *Lactobacillus paracasei.* Gel 1 (Left): Lane 1—Low Ladder Fischer (50bp-2kb), Lane 2—Amplification products using LU-3/Lac-2 (expected product size: 350bp), Lane 3—Amplification products using LU-1/Lac-2 (expected product size: 300bp), Lane 4—Amplification products using LU-5/Lac-2 (expected product size: 400bp), Lane 5—Amplification products using Ldel-7/Lac-2 (expected product size: 450bp), Lane 6—Amplification products using LU-3/LU-5/LU-1/Lac-2 (expected product size: 400bp), Lane 7—Blank, Lane 8—Low Ladder Fischer (50bp-2kb). Gel 2: (Right), Lane 1—Axygen 100bp Ladder DNA marker (100bp-3kb), Lane 2—Amplification products using Lpar-4/LU-5 (expected product size: 312bp), Lane 3—Amplification products using Lpar4/LU-5 (expected product size: 312bp), Lane 4—RhaII/LU-5 (expected product size: 113bp), Lane 5—Axygen Ladder DNA marker (100bp-3kb). Boxed bands are amplified products that match expected sizes.

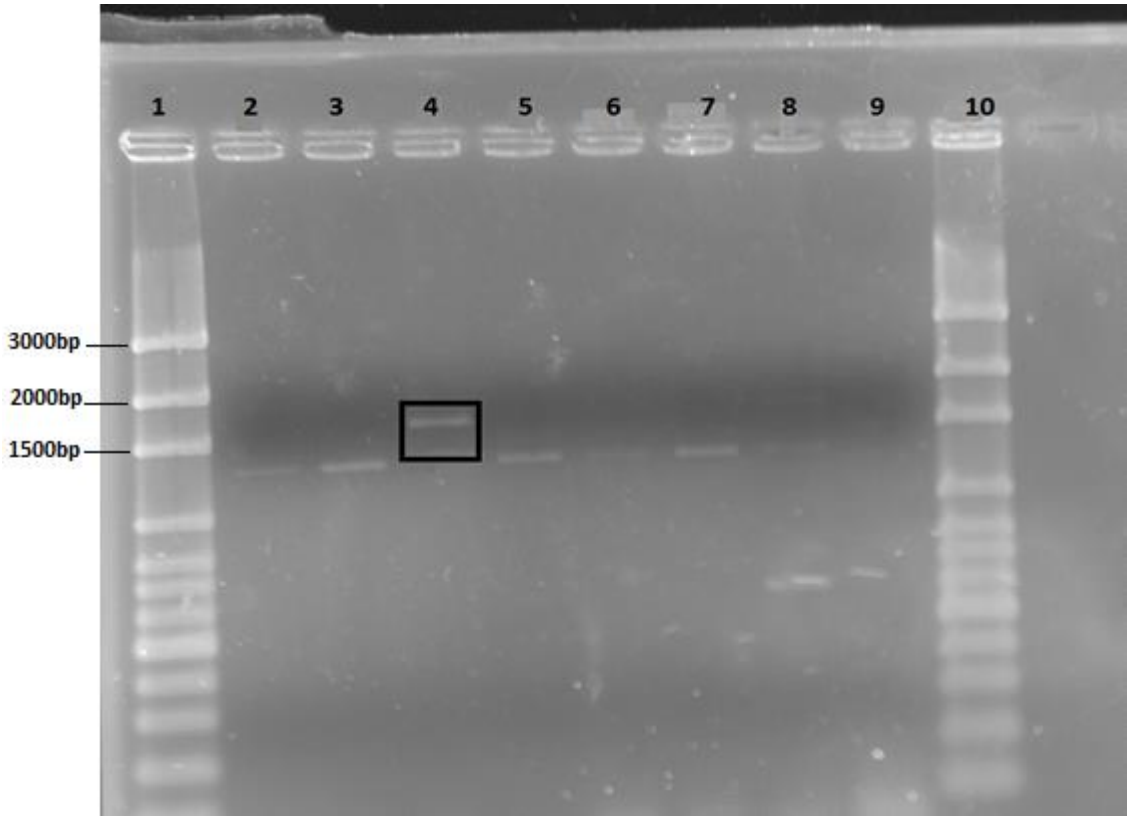


Figure 5.7: PCR amplification products using species-specific primers to identify *Pediococcus pentosaceus.* Lane 1—Axygen 100bp Ladder DNA marker (100bp-3kb), Lane 2—Amplification products using PDA23S_F/ P23S_R 2 (expected product size: 2244bp), Lane 3—Amplification products using PST23S_F/ P23S_R 2 (expected product size: 1840bp), Lane 4—Amplification products using PPE23S_F/ P23S_R 2 (expected product size: 1647bp), Lane 5—Amplification products using PPA23S_F/ P23S_R 2 (expected product size: 1517bp), Lane 6—Amplification products using PCE23S_F/P23S_R 2 (expected product size: 866bp), Lane 7—Amplification products using PAC23S_F/ P23S_R 2 (expected product size: 213bp), Lane 8—Amplification products using PIN23S_F/P23S_R 2 (expected product size: 711bp), Lane 9—Blank 2 (expected product size: N/A), Lane 10—Axygen 100bp Ladder DNA marker (100bp-3kb). The boxed band is the amplified product that matches its expected size.

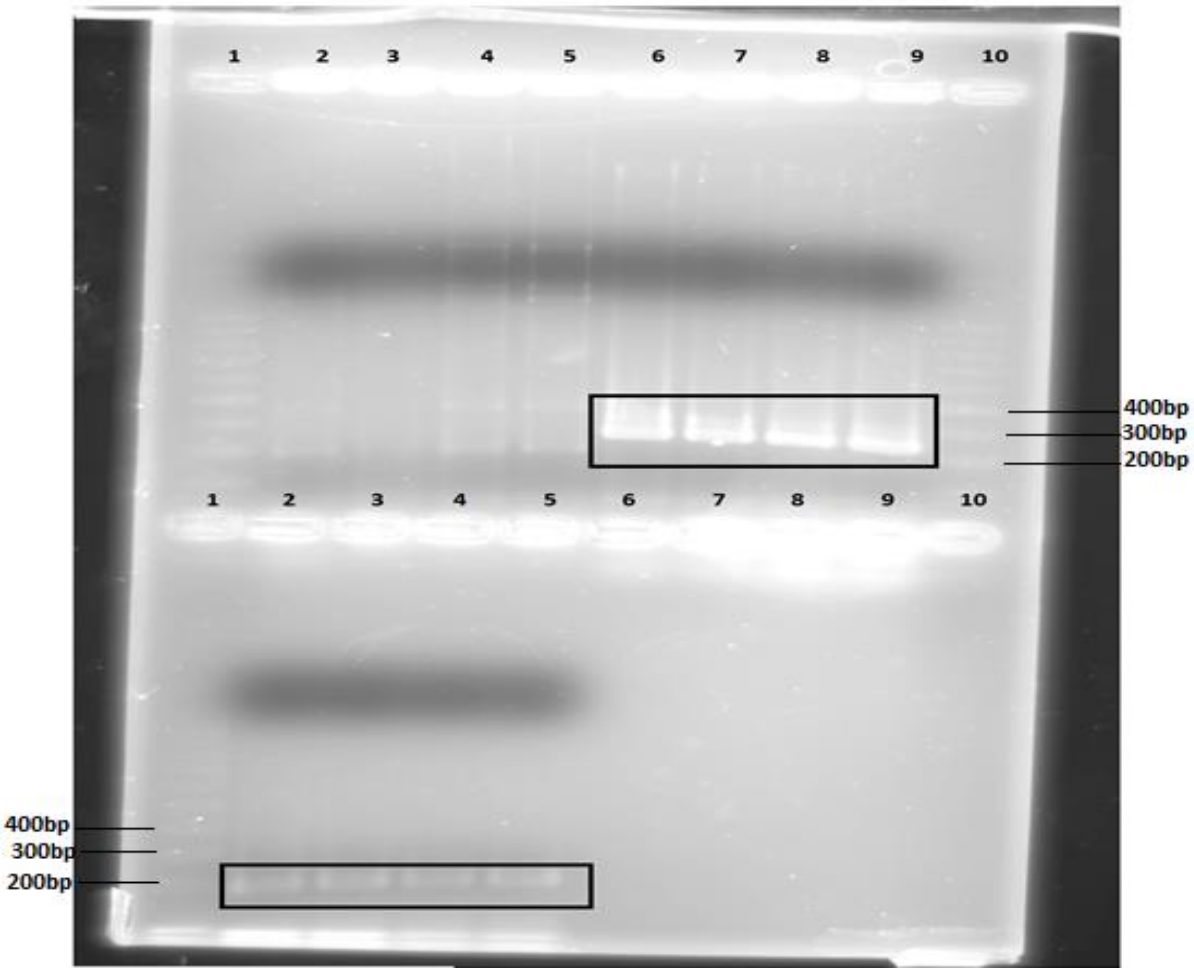


Figure 5.8: PCR amplification products using group primers (Top) and species-specific primers (Bottom) to identify *Lactobacillus fermentum*. Note: the concentration of the gel and the high (100V), may have affected the migration of the amplification products causing the discrepancy in their apparent size. Top: Lane 1—exACTGene 100bp DNA Ladder (25bp-1kb), Lanes 2-5—Amplification products using LU-3/Lac-2 (expected product size: 350bp), Lanes 6-9—Amplification products using LU-3/Lac-2 LU-1/Lac-2 (expected product size: 300bp), Lane 10—exACTGene 100bp DNA Ladder (25bp-1kb). Bottom: Lane 1—exACTGene 100bp DNA Ladder (25bp-1kb), Lanes 2-5—Amplification products using LU-3/Lac-2 Lfer-3/Lfer-4 (expected product size: 192bp) Boxed bands are amplified products that match expected sizes.

5.5 Detecting the Presence of Bacteriophages

My results show that the Hopkinsville ethanol production facility contains bacterial contaminants that are known to impact ethanol production efficiency. The next step was to determine if I could find bacteriophages that would specifically attack these common hosts. Determining whether or not bacteriophages are present in the fermenting environment is a critical step before cocktails for eliminating the contaminants can be formulated. Phages may be present extracellularly or they may exist as a prophage, integrated into the bacterial host's chromosome. The presence of phage particles in the fermenter samples suggest that the contaminating bacteria may be lysogens which periodically release phages either spontaneously or through induction events. The presence of lysogenic phage may lower the efficacy of a phage cocktail due to the phenomenon of immunity. Resident prophages synthesize repressor proteins that effectively block secondary infections by the same phage. Therefore, lysogenic cells are said to be immune to infection by any phage that possesses the same type of repressor and thus the same a repressor-binding sites.

Because temperate phages can adopt two different lifestyles, it was necessary to search for phages located extracellulary and those that have integrated into the chromosome of their host. To look for free phages, the clarified supernatants from the fermenter and beerwell samples (Section 4.1), were examined using a transmission electron microscope (TEM). For each sample, 10µL of clarified supernatant was placed on an EM grid and stained with uranyl acetate. No particles were observed when viewed by TEM, although the entire grid was searched. These results suggest that either the

samples do not contain free phages or that the concentration of released phage may have been far too low to be detected by this approach.

5.6 Induction of Resident Prophage

To determine if the bacteria isolated from the fermenter and beerwell samples contain prophages, I attempted to induce their production by treating the cells with mitomycin C. Mitomycin C is a potent DNA cross-linker, which blocks bacterial DNA replication and leads to cell death. The cells sense this damage and respond by turning on the expression of the SOS regulon. In many cases, this also results in the derepression of resident prophages by inactivating the phage repressor protein. These viruses enter the lytic cycle, and eventually lyse the cell to release the progeny virions.

Only one mitomycin C treated isolate yielded bacteriophage. The culture of *Lactobacillus paracasei*, appeared turbid during the first few hours of incubation but then cleared after 5 hours of incubation. The drastic reduction in turbidity is a characteristic sign of cell lysis. The cell debris was removed by centrifugation and the supernatant was transferred to a sterile tube. An EM grid was prepared using the clarified lysate and examined under the TEM. Phage particles were easily identified in this sample. Although capsid morphology, and tail length were similar, we do not know if the phage population is homogeneous because many phages have similar morphologies. These results suggest that the *Lactobacillus paracasei* contaminant harbors lysogenic bacterial virus(es). The electron micrograph of the bacteriophages is shown in Figure 5.9.

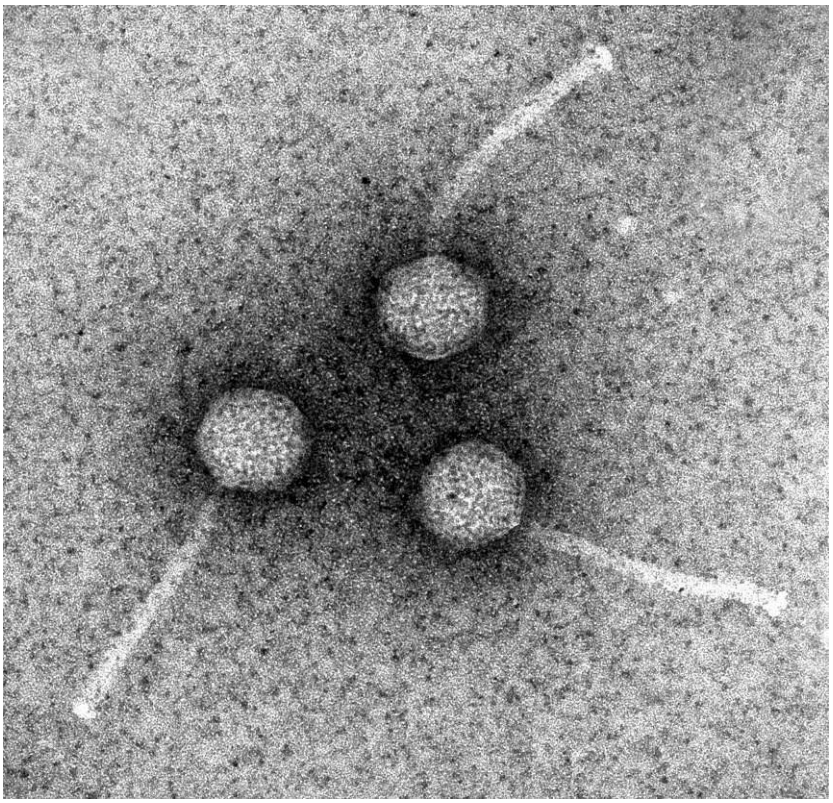


Figure 5.9: Bacteriophages induced from *Lactobacillus paracasei.*

CHAPTER 6

DISCUSSION

Bacterial contamination continues to have a negative impact on the bioethanol industry. Conventional methods for treating such contamination and its associated complications are not sustainable due to inhibitory costs and the development of resistence. The goal of this research was to identify and develop a sustainable alternative to antibiotics and chemical biocides that are currently used to control microbial contamination in biofuel facilities. Bacteriophages are strong candidates as alternatives to antibiotics in the control of bacterial contaminations in fuel-ethanol fermentations. Bacteriophages have several advantages over antibiotics. Specifically, bacteriophages are capable of self-replicating where a suitable host exists due to their lifecycle. Each productive infection results in substantial amplification of the lytic agent and hundreds of progeny phage are produced. This amplification drastically increases the concentration of phage in the vicinity of the host contaminant bacteria. Additionally, bacteriophages do not harm human or animal populations due to their selective toxicity against their small bacterial host range. Furthermore, the selective pressure of an added phage population does not increase the probability of developing resistance to antibiotics.

Bacteria can readily develop resistance to antibiotics but they also can develop resistance against bacteriophage. For example, a mutation in in the bacteriophage receptor could prevent adsorption and entry. A common approach to overcome this type

of phage resistance is to use a collection of bacteriophage that target different receptors on the target host. Thus, if a contaminant acquires a mutation in a receptor protein rendering it resistant to infection by a particular phage, other phages in the cocktail that utilize different receptors may still be able to infect it. Due to the relatively low mutation rate of bacteria, it is unlikely that a bacterial host will acquire multiple mutations to become resistant to all phages in a cocktail simultaneously.

An essential first step in using bacteriophages to combat bacterial contaminants in bioethanol production is to identify the culprits. Only after this information is known can an appropriate cocktail of bacteriophages be formulated. I isolated and cultured five different bacterial species from fermenter and beerwell samples obtained from a bioethanol facility. Of these five different bacterial species, three were positively identified using whole-colony PCR and gel electrophoresis. The identity of two other isolates remains undetermined: one unknown produced small white colonies and was collected from the beerwell and the other produced characteristic small grey colonies, was unable to grow in MRS broth, and was isolated from fermenter samples. Both contaminants were minor constituents of the observed contaminant population. These isolates may belong to the *Enterococcus* genus, which are also common contaminants in fuel-ethanol production. Primer sets which amplify the species-specific 16S rRNA region of each species belonging to the *Enterococcus* genus may be designed and tested with these unknown isolates to determine if these contaminants belong to this genus.

Contaminants identified at the species level included members of the genera *Lactobacillus* and *Pediococcus*. Further research must be conducted to identify additional bacterial contaminants. Only two samples were analyzed and this small sample size may

introduce significant sampling error. In addition, samples should be collected at different times during contaminant blooms in order to determine which populations persist in fermenters and beerwells. Moreover, our protocol selected for the growth of *only Lactococcus*, *Pediococcus*, and *Lactobacillus* populations. This selective medium prevented the growth, and therefore, characterization of other microbial contaminants which may be prevalent in fermenters. Different culture conditions supplemented with different nutrients may result in the growth of new communities of bacteria. Therefore, it may be beneficial to plate future samples initially on nutrient rich media supplemented with CHX to evaluate the diversity of microbiota that are prevalent in bioethanol facilities. This information will help to identify phage that target the predominant contaminating species.

The fermenter contained significantly greater numbers of contaminating bacteria than the beerwell (Figure 5.2). This suggests that the application of bacteriophage in the fermenting vats of the Hopkinsville bioethanol facility would be more effective than applying a phage cocktail to the beerwell due to the potential for a more significant reduction of contaminants. Nevertheless, the addition of phage to the beerwell may still be beneficial since bacteria were also recovered from this location. The addition of bacteriophage early in the bioethanol production cycle may be particularly valuable. Reducing or preventing the growth of bacteria that naturally contaminate the grains, will reduce their negative impact on yeast growth.

Phages that target the majority of the contaminating bacterial species must be used in sufficient amounts to ensure efficient host cell killing. Although naturally lytic bacteriophages are ideal as biocontrol agents, it may be possible to genetically engineer

the temperate bacteriophages to exclusively undergo the lytic cycle. Bacteriophages may also be obtained from research laboratories that have shown effective lytic activity against similar bacterial species. Collecting a variety of phages will broaden the scope of target receptors that can be harnessed for host cell infection, ultimately reducing the chance of the development of phage resistance of target contaminants.

Alternatively, instead of using bacteriophages that strictly lyse infected bacteria and release progeny phage to scout for new hosts, where bacteria quickly evolve to becoming resistant to infection, genetically modified viruses that weaken their hosts to become more susceptible to antibiotics may be used.[23] Lu and Collins genetically engineered a phage called M13, which does not lyse infected cells, to produce a bacterial protein called lexA3. This protein impairs a bacterium's ability to repair damaged DNA by blocking the induction if SOS functions in the cell.[23] When the modified M13 phage infects its host, *Escherichia coli*, it produces lexA3, which renders the bacterium more vulnerable to DNA-damaging drugs.[23] Collectively, it was discovered that the phage increased the ability of the antibiotic ofloxacin to kill *E. coli* grown in culture, even when the bacteria were resistant to the antibiotic.[23] The findings suggest that this type of phage therapy could rejuvenate antibiotics that have been deemed no longer effective, and this research may be extended to cases to eliminate prevalent antibiotic resistant bacteria in bioethanol facilities.

Another alternative approach for using bacteriophages to kill bacterial contaminants in bioethanol production was recently published in the Journal of Biotechnology and Biofuels.[24] Instead of using whole bacteriophages, the investigators used the cell-lysing capabilities of bacteriophage encoded enzymes. This study was conducted by the United

States Department of Agriculture, and successfully demonstrated that phage endolysins are effective in significantly reducing the number of *Lactobacillus*—a common contaminant in the bioethanol industry.[24] Endolysins are hydrolytic enzymes produced by bacterial viruses during the late stages of infection. The endolysins were isolated from streptococcal phages and lactobacillus phages. Specifically, the Lambda Sa2 lysin was isolated from a streptococcal phage, and the lysins LysA, LysA2, and LysgaY were isolated from a variety of lactobacillus phages.[24] These enzymes are responsible for degrading peptidoglycan—a critical component of the bacterial cell wall. Peptidoglycan is composed of an intricate structure comprised of a sugar backbone with two components: alternating units of N-acetyl glucosamine and N-acetyl muramic acid. Forming the cell wall, peptidoglycan maintains the structural integrity of the cell, reinforces the plasma membrane, and counteracts the osmotic pressure of the cytoplasm. Endolysins create a myriad of holes in the peptidoglycan wall in order to lyse the cell. These holes weaken the integrity of the cell wall and the intracellular osmotic pressure causes the wall to rupture. This type of cell death is called osmolysis. A single lysin enzyme is sufficient to cleave an adequate number of bonds to destabilize the bacterial cell wall.[24]

Although these lytic enzymes are normally produced inside the phage-infected cell, and therefore degrade the cell wall internally, many studies have shown that treating bacterial cells externally with lysins is just as lethal.[25-28] Consequently, many bacteriophage lysins are being utilized to control bacterial populations. Externally applied lysins have proven to be highly effective when applied to gram-positive cells because

they lack an outer membrane, the presence of which prevents access of the lysins to the peptidoglycan wall of gram negative bacteria.

The investigators from the Department of Agriculture isolated, purified, and screened four different phage lysins for their ability to lyse *Lactobacillus* strains collected from fuel ethanol fermenters.[24] The endolysins demonstrated strong lytic activity towards the majority of strains of *Lactobacillus* tested, and maintained optimal activity under fermentation conditions (pH 5.5 and in the presence of 5% ethanol), reducing contaminant numbers by many orders of magnitude. Therefore, these lytic enzymes have potential to control unwanted lactobacilli contaminations in fermentation systems and merit further testing in fuel ethanol fermenters as either additives or expressed in genetically modified, fermenting yeast.

Although phage-encoded lysins are capable of significantly reducing contaminants in bioethanol facilities, the expense associated with these proteolytic enzymes may be greater than that of a phage cocktail. This is because exploiting the activities of these enzymes requires a substantial initial investment that encompasses cloning, expressing, and purifying these proteins for their use. However, growing and maintaining phage stocks entails fewer steps and less initial investment. Determining which methods are work best and are the most cost effective will require additional study.

The long-term goal of this research is to develop a new, less expensive, and more sustainable approach to control bacterial contaminations of industrial ethanol fermentation systems. Knowing where the application of phage is most effective will help ethanol fuel industries eradicate the greatest number of bacterial contaminants and avoid

unnecessary or ineffective applications of a phage cocktail. This study has shown that for Hopkinsville's Agri-Energy® Bioethanol production facility, contaminations in the fermenter are more prevalent in the fermenter than the beerwell. Three common contaminants were identified in the Hopkinsville bioethanol facility—*Lactobacillus fermentum, Lactobacillus paracasei, and Pediococcus pentosaceus*. Two other bacterial contaminants remain unidentified. In addition, I found that bacteriophage are already present in the fermenting environment. Because bacteriophages are natural parasites, scientists continue to investigate the variety of contexts where bacteriophages are applicable to harness their capabilities to revitalize their use as antimicrobials in industrial and clinical settings. This preliminary study suggests that bacteriophages may be utilized as efficient alternatives to antibiotics in eradicating bacterial contaminants in fermenters, and this diverse group of microorganisms remains a viable biocontrol agent in the bioethanol industry, and may have other useful applications where microbial contaminants are a problem.

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