AN ABSTRACT OF THE THESIS OF

Amy N. Smoker for the degree of Master of Science in Microbiology presented on April 28, 1998. Title: Screening of *Sporolactobacillus* and *Bacillus* Strains for Use in Silage Inoculation.

Redacted for Privacy

Abstract approved: _____

William E. Sandine

Preservation of silage is commonly promoted by the addition of bacterial inoculates responsible for the production of lactic acid. These inoculates are typically mixed cultures of bacteria in the vegetative cell form, being maintained in low temperature (frozen liquid) or low moisture (lyophilized powder) forms. The cultures typically consist of lactic acid producing bacteria capable of converting naturally occurring or supplemented sugars found in the silage into lactic acid. When produced rapidly enough and to a high percentage, the lactic acid reduces the pH to around 4.0 which renders many common spoilage bacteria unable to grow. This environment of low pH also serves to slow plant enzyme activity, further preserving the silage product. Research performed in this thesis examined sporolactobacilli and bacilli strains for use as silage inoculates. It was found that the test strains were capable of producing high spore numbers; for example *Sporolactobacillus inulinus* when incubated at 37°C for 4 days, produced 1.5 x 10^{10} spores/ml of culture. When grown in MRS broth at 37°C for 5 days, 6.4 x 10^{8} spores/ml of culture were seen. Spray drying of spore preparations showed spore numbers of 2.0 x 10^{7} spores/g of collected powder. For shelf stability confirmation, total plate counts of the powder were run at 6 weeks and 5.0 x 10^{7} cfu/g of powder were present.

Results validated the test strains to behave similarly to the commercially available inoculates in the amount of organic acids monitored by high pressure liquid chromatography. *Sporolactobacillus inulinus* produced 50.47 ug/10ul, compared to the

Quest "LPE" commercial strain which produced 69.08 ug/10ul when inoculated into silage and incubated at 37°C for 72hrs. Comparable results were also noted in pH reduction of silage when inoculated by both test and commercial strains. Pre fermented silage pH was reduced to an average of 4.15 by *S. inulinus* and 4.24 by the Quest "LPE" commercial strain.

The conclusion has been made that the test strains behave like commercially available inoculates and are suitable candidates for use as industrial silage inoculates. Further experiments will need to examine commercial spray drying time and temperature settings for various solids percentages of spore preparations.

Screening of *Sporolactobacillus* and *Bacillus* Strains for Use in Silage Inoculation

by

Amy N. Smoker

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented April 28, 1998
Commencement June 1999

Master of Science thesis of Amy N. Smoker presented on April 28, 1998

APPROVED:

Redacted for Privacy

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Amy N. Smoker, Author

ACKNOWLEDGMENT

I would like to thank Dr. William Sandine for providing this opportunity to me in lending me both financial support and unyielding confidence in my abilities. I truly could not have done this without you.

I would also like to thank Dr. James Dewey who fully supported my academic endeavors while under his supervision at Oregon Freeze Dry, Inc.

Thank you to my husband Jason, my partner in everything.

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Screening of Sporolactobacillus and Bacillus Strains for Use in Silage Inoculation

INTRODUCTION

Virtually any crop can be ensiled as cattle feed (Bolsen, 1985). Ensiling is the process of anaerobically fermenting crop forage into product able to be stored. Once ensiled, anaerobic fermentation by primarily epiphytic lactic acid bacteria occurs converting water soluble carbohydrates into lactic acid and various other by products (Weinberg, 1993). This conversion of sugars into acid also takes place via the many fermentation pathways of the various naturally occurring organisms and can be classified as homofermentative and heterofermentative. Fermentation leads to a reduction in pH as lactic acid accumulates to promote preservation of the forage (Weinberg, 1993).

This preservation is due to pH reduction and can be explained as both a prevention of spoilage bacteria from growing and also as a reduction in plant enzyme activities. Certain plant enzymes such as cellulases can decrease the percent dry matter to an undesirable level. Common spoilage organisms such as Clostridium species, yeasts and coliform bacteria can ruin silage by leaving unwanted metabolic products behind. Examples of unwanted fermentation by-products would be excess butyric acid produced by *C. butyricum* or propionic acid by Propionibacterium species, or by simply molding the surface of the silage (Dellaglio, 1984). Excess butyric acid in silage is referred to as clostridial fermentation. By rapidly lowering the pH to below 4.0, via the production of lactic acid, many of the common spoilage organisms are unable to survive. Application of concentrated bacterial inoculates capable of producing lactic acid can encourage this rapid drop in pH, regardless of the epiphytic microbial composition. Many studies have illustrated these advantages (Carpintero et al., 1979; Lindgren et al., 1983; Seale, 1986; Weinberg et al., 1988). Barnett, at the University of Aberdeen, author of Silage

Fermentation outlined the following as problems associated with ensilage: (1) control of losses due to cell respiration during the earliest phases of ensiling where plant respiration is most active, (2) proper stimulation of lactic acid production to prevent a later attack by butyric acid-producing organisms (Barnett, 1954).

Research Topic

The research documented in this thesis has centered around proving that the chosen candidate bacterial strains performed similarly to marketed or "approved" commercial silage inocula, first in a lab setting and finally on a commercial level. Standards were given defining the characteristics which qualify a strain as "successful" based on what is known of currently marketed inocula as components of commercial silage additives. Considering that the more lactic acid formed the more efficient the fermentation process, commercial inocula rely heavily on use of lactic acid bacteria (for example, *L. plantarum*).

Lori Scarlett, former graduate student of Dr. William Sandine performed several characterization assays of *Lactobacillus sporogenes* (aka. *Sporolactobacillus inulinus*) in March, May and June of 1989. A brief summary of her discoveries is applicable at this point as the present thesis work stems from a discussion she and Dr. Sandine held on the possibilities of using of this bacterium as a silage inoculate. No work was performed at that time towards this idea. In March, an experiment to determine its optimal growth temperature was performed using MRS, MRS+V8 and milk tubes with incubation temperatures of 30°C, 37°C and 45°C. Growth and pH observations were monitored at 24 hours and 3 days when it was concluded that the organism was thermophilic with a wide range of growth conditions. There was little growth in milk (no clot), consistent growth in MRS at all three temperatures and the best growth in MRS+V8 at 45°C. pH ranges were 6.08 at 30°C, 5.62 at 37°C and 4.27 at 45°C measured at day 3.

Also in March, a plasmid profile was determined which indicated *L. sporogenes* to be plasmid free. In May, a mini-tek was used to examine carbohydrate fermentation patterns. Nine of twenty carbohydrates were positive: amygdalin, arabinose, cellobiose, esculin, glucose, maltose, mannose, melezitose and salicin. A paper by Holzapfel and Botha (1988) was used for comparison in which positives found were inulin, maltose, mannitol, raffinose, sorbitol, sucrose and trehalose, with no growth occurring at 15°C or 45°C. Lastly in June, she ran a test tube carbohydrate assay comparing *L. sporogenes* cultures from two different culture collections and found that the culture sent to her from Korean researcher Dr. M. Y. Pack, as mentioned in her notes, fermented 14 of 18 carbohydrates, while the culture from ATCC fermented 2 of 18 with 6 other possible slight positives. Good growth in both salicin and meleztiose was noted.

In the present study, *Sporolactobacillus* and *Bacillus* sp. were screened for use as potential silage inocula. *Sporolactobacillus inulinus* was the target candidate with all other strains serving as controls and/or organisms of potential. These two genera had not previously been screened as potential silage inocula and they differ from the typical lactic acid bacteria (LAB) being used commercially by their ability to produce true heat-resistant endospores (Doores, 1981; Palop, 1996). By using sporeformers, the production costs would be decreased dramatically by elimination of the lyophilization process and using instead the common spray dryer. Also, once in the spore state, the inoculate has the potential for surviving a variety of environmental conditions to the point of application and would have a higher recovery rate, as germination can approach 100%. This is in contrast to current inocula being used, which are both costly to produce as well as store and ship, and are also variable products due to the use of bacteria in the vegetative, non-spore state.

Sporolactobacillus is a genus of gram positive bacteria, its name conveying their ability to form endospores, and, like LAB metabolize hexoses into predominantly lactic acid (Kitahara and Lai, 1967). Kitahara and Suzuki (1963) first isolated the organism from chicken feed , naming it *Sporolactobacillus inulinus* due to its ability to ferment inulin (Kitahara and Suzuki, 1963). Similar to *Lactobacillus* , this organism is catalase negative, microaerophilic, gram positive and rod-shaped; however it is different in its

peritrichous flagellar motility, endospore formation and exclusive homo-fermentation of glucose into D(-)-lactic acid. The organism also shares similarities with *Bacillus* bacteria. Seven strains isolated by Nakayama and Yanoshi (1967) from the rhizosphere of wild plants were found similar to the earlier *Sporolactobacillus* isolates with the exception of their ability to form DL-lactic acid. These strains were also placed in this new genus and under the generic rank *Bacillaceae*. Nakayama also isolated other suspect *Sporolactobacillus* strains which he designated as *S. laevus* and *S. racemicus*, differing also in the type of lactic acid produced (Uchida and Mogi, 1973). These latter two strains have not been listed under the "Approved List of Bacterial Names" (Skerman , 1980).

Little available information on *S. inulinus* and essentially none on *S. cellulosolvens* (other than date of isolation) exists to date. In 1987, Holland defined the genus simply as "an endosporeforming anaerobe which is fermentative, not obligately anaerobic, and resembling *Bacillus* and *Lactobacillus* ". Spore structure is the same as that of aerobic bacilli, although they contain less dipicolinic acid, (average of 51.6 ug/mg spore dry weight), previously described by Janssen in 1958 and have lower heat resistance, dying off at temperatures below 100°C. Vegetative cells contain no dipicolinic acid (Kitahara, 1967). Mature spores are 0.8 x 1.0 u in size and occur at the terminal position in swollen sporangia (Kitahara, 1963). Nutrient requirements are not as extensive as those of *Lactobacillus* but are closer to the *Bacillus.* The most important characteristics for differentiation of *Sporolactobacillus* from other sporeforming organisms was summarized by Hozapfel and Botha: *Sporolactobacillus* and *Lactobacillus* are negative for catalase and nitrate reactions for which *Bacillus* are positive; *Sporolactobacillus* and *Bacillus* are positive for endospore formation and are motile, which differ from *Lactobacillus* (1988). No growth occurs without fermentable carbohydrates, hexoses are fermented predominantly to D(-)-lactic acid with < 1% ethanol or volatile fatty acids, and pentoses are not fermented (Buchanan and Gibbons, 1967). The organism is listed in Bergey's Manual as having no catalase or cytochrome systems and with an optimal growth temperature of 35°C (Holzapfel, 1988). Some strains isolated from soil have the ability to produce DL(-) or L(+) lactic acid (Nakayama and Yanoshi, 1967).

Doores (1983) has done substantial work on *S. inulinus* to define its carbohydrate utilization. Together with D.C. Westoff, experiments were performed to identify the most suitable growth medium for both *Sporolactobacillus* and *Bacillus* species. *Lactobacillus* MRS broth was rated as "excellent" for growth for both organisms and was chosen as the primary lab medium for maintaining cultures of these bacteria. The MRS medium was modified to enrich for the *Sporolactobacillus* by the addition of α-methyl glucoside (1.0% w/v) and potassium sorbate (0.1% w/v) (Doores and Westoff, 1983). Their work also has provided the only information regarding heat resistance of *S. inulinus* endospores. Cells were grown on 24 media with varying carbohydrate sources and concentrations. *Sporolactobacillus* agar with 1 % α- methyl glucoside yielded highest heat resistant total counts while *Sporolactobacillus* agar with 2 % α-methyl glucoside yielded the most heat resistant spores. D-values (decimal reduction time) and z values were determined for spore crops. Average D -values were as follows: 53.2 min at 72°C, 19.5 min. at 80°C, 6.8 min. at 85°C and 5.1 min. at 90°C with a z-value of 13.0° C (Doores and Westoff, 1981).

The four strains of *Bacillus coagulans* which were screened along with the sporolactobacilli, also had the ability to form spores under appropriate conditions and were capable of homofermentative lactic acid production. *Bacillus coagulans* was first identified by Hammer in 1915 (Nakayama et al., 1967). Gordon has defined the genus *Bacillus* as "rodshaped bacteria capable of aerobically forming refractile endospores that are more resistant to heat, drying, and other destructive agencies" (1973). *Bacillus coagulans* forms spores which are ellipsoidal or cylindrical, generally subterminal or terminal in nature. The four ATCC strains used in the present study were 7050, 11369, 31284, and 10545. These gram positive rods range in size from 0.6 u to 1.0 u by 2.5 u to 5.0 u, making some strains easily recognizable and distinct from the others. Despite the variance in the morphological characteristics of the four strains, they behaved similarly for proteolysis, carbohydrate utilization and spore formation. (Gordon, 1973).

Research Approach

Suitability of the sporolactobacilli and bacilli chosen was determined by the following assays. Strains were first subjected to spore counts. These counts confirmed that these organisms did in fact form endospores under "appropriate" conditions. It was also important to determine the ease with which sporulation could be induced, and whether or not the numbers of spores produced would prove practical for commercial use. Next, carbohydrate utilization assays were performed to determine the range of sugars used and which might occur in the process of ensiling. Acquiring this information possibly would allow use of an alternative sugar such as molasses to insure lactic acid production at a rapid rate.

Next, model systems of ensiling were set up in the laboratory to run fermentation assays on grass silage using candidate strains and commercial controls. Glucose Yeast Peptone medium fermentation assays were run to confirm the conversion of glucose into lactic acid by all candidates prior to the silage fermentations. Post silage fermentation samples were analyzed for various organic and volatile fatty acids using high pressure liquid chromatography. These data allowed the various actively fermenting organism to be recognized by their metabolic by-products.

Finally, inoculants were prepared such as would be done in a commercial setting. Inoculants were bulked up and spray dried (versus lyophilization common for non-sporeformers) and then spore counts made to confirm survival and germination rates of strains.

The research approach took the strains through a screening process, into the fermentation setting and concluded with commercial mock-ups of the organisms as inoculants.

Objectives

The problem which has been the focus of this research project has been to prove the worthiness of several never-before-used bacterial strains for use as the silage inoculum component of an industrial silage additive. Information on currently marketed silage inoculants served as a basis for developing standards which the new candidate organisms should parallel. In this way, sufficient qualities of an acceptable inoculate able to meet industry needs for use in silage will have been achieved. The main objective underlying this research was to locate *at least* one new. The benefits of spore-forming bacteria should be evident from an inoculate processing and storage point of view, as well as the resiliance of spores over the more vulnerable vegetative inocula currently marketed. Sufficient data were obtained on the spore forming ability and the number of spores contained in the mock work-up of commercially prepared inocula in the lab setting.

LITERATURE REVIEW

Silage Fermentation

A typical silage fermentation study will usually entail ensilage of a particular forage crop such as rye-grass, alfalfa or corn, inoculation with a commercially available inoculate-containing additive, anaerobic incubation and post-fermentation assays to determine the success or failure of the chosen bacterial inoculates. These post-fermentation assays may include examination of predominant or surviving microflora, percent dry matter (% DM) remaining, pH decline patterns and determination of the organic acids formed in the silage. In one silage fermentation study, Moon and Sudweeks (1981), prepared silages from wheat (early bloom), alfalfa (second cutting 20 - 40 % bloom), and corn (early dent). Fresh forage (55 kg) was inoculated with 5 g/kg commercial silage additive (Sila-bac) containing *L. acidophilus* and clostridial species. Anaerobic conditions were met by packing silage into 6 mm polyethylene bags which were sealed in air tight drums and examined at day 0, 1, 2, 4, 6, 8, 16, 24 and 32. Microbiological analyses found only variable success of inoculates and led to the conclusion that the beneficial effect of the addition of their chosen inoculates could not be established. It was suggested that this could be due to inadequate selection of suitable isolates for use as inoculates. Since then, the silage industry has "fine tuned" which strains will consistently be more suitable. For example, *L. plantarum* is the leader of commercial strains being put to use and was chosen as a control for this research project.

Weinberg et al. (1988) inoculated vetch, wheat and alfalfa silages with a commercial inoculate comprised of *L. plantarum, S. faecium,* and *Pediococcus sp.* at a rate of 5.6×10^4 cfu/g. After anaerobically incubating the silage in sealed jars, analyses of % DM loss, pH decline and lactic acid accumulation were performed at days 1, 2, 5, 7 and 30. They also analyzed volatile fatty acid accumulation, which would indicate efficiency of fermentation patterns by looking at ethanol, acetic and butyric acids. Gas

chromatography was chosen for examination of volatile fatty acids. Microflora assays included those for lactic acid bacteria, yeasts, clostridial spores and enterobacteria. Findings of the study showed less % DM loss in nearly all inoculated silage samples over the control. Rates of pH decline were more significant in inoculated samples over controls which correlated with a faster and higher lactic acid build-up in the inoculated samples. Water soluble carbohydrates decreased faster in control samples. Ethanol and acetic acids were at higher levels in controls, and butyric acid was found only in controls. Exceptions to the success of the commercial inoculum was found in the wheat or cereal crops (Weinberg, 1988). Weinberg had performed a study a year previous to this which gave similar results when applying *L. plantarum* to wheat crop, a high sugar, low protein crop whose fermentation patterns were unreliably spontaneous in nature (Weinberg, 1987). The findings of Weinberg, et al. (1988) support previous work of Carpintero et al. (1979) who used an inoculum containing *L. plantarum, L. mesenteroides* and *S. faecalis* in a laboratory rye grass-clover silage experiment . They found that LAB inoculum improved fermentation patterns in silage, helped reduce DM losses and repressed undesirable microorganisms such as clostridia and enterobacteria .

In order to expand research efforts dealing with silage fermentation, Tanaka and Ohmomo designed a "Repeatable Model System for Silage Fermentation" (1994). The system was designed to supply simple, rapid and repeatable data on silage fermentations carried out in culture tubes, in a laboratory setting. When first reported to the scientific community, they used milled alfalfa hay cubes (AHC), mixed with water and glucose as their silage medium of choice. The forage was inoculated with LAB either alone or in combination with butyric acid bacteria (BAB) and coliform bacteria (CFB) and incubated anaerobically. They followed the principle that butyric acid produced by BAB during ensiling when the pH is near neutral led to poor quality silage . One objective of their research was to determine what size LAB inoculum would protect the silage from this butyric fermentation. Criteria for the fermentation system was as follows: 1) starting plant material should be storable for repeat assays, 2) anaerobic ensiling should be met, and 3) initial microflora and incubation temperatures should be controllable. Bacterial inocula chosen were *L. planatarum*, *C. butyricum* (2 strains) and the 2 coliforms,

Klebsiella pneumoniae and *Escherichia coli.* Alfalfa hay cube medium (5 g) was inoculated in culture tubes (16.5 mm diameter x 165 mm length) and sealed in anaerobic jars for incubation for 72 hours at 20°, 30° and 37° C. Post-fermentation silage supernatant was used for chemical testing, and high pressure liquid chromatography was used to assay the supernatant for organic acids and soluble sugars. Enumeration of LAB, CFB and BAB spores was performed. Results indicated that the pure culture LAB inoculum led to the production of lactic acid within 24 hours of incubation. Mixed culture of LAB, CFB and BAB promoted butyric acid fermentation. Butyric acid was produced under conditions of temperatures greater than 30°C, DM content <20% and a prevention of pH reduction in medium. A low soluble sugar content of the medium (0.35%) or an inoculum containing LAB in numbers less than 10^6 but with CFB at 10^7 were effective in the necessary reduction in pH, and prevention of the butyric acid fermentation (Tanaka and Ohmomo, 1994).

A subsequent experiment, in which Tanaka was involved, utilized the model system of silage fermentation for screening lactic acid bacteria for use as silage inocula, and served as the basis for much of the experimental research that followed. Tanaka et al. (1994) chose 16 strains of LAB which had proven high lactic acid production characteristics in liquid medium and compared their lactic acid production in silage using the model system. They followed the principle that silage pH should be lowered to less than 4.2 by lactic acid in order to prevent multiplication of BAB and CFB. It can not be assumed that if lactic acid production is "good" in liquid medium, it will likewise be so in silage and thus the model system proved a successful tool for which to measure this (2,3,6). First they examined the production of lactic acid in Glucose Yeast Peptone (GPY) broth, an assay which is described in detail in Materials and Methods of this thesis. Next, the model system was used with milled alfalfa hay cube medium (AHC) mixed with water and glucose. AHC (5 g) was inoculated with *L. planatarum, C. butyricum* and *K. pneumoniae* either alone or as a mixed BAB, CFB culture. Anaerobic conditions were established by use of sealed air-ejected plastic pouches (17 cm x 4 cm). Chemical, microbiological and organic acid analyses were performed as described in the

previous experiment (Tanaka and Ohmomo, 1994). pH decline, temperature, lactic and butyric acid production were also followed. Results identified three classes of LAB strains: 1) those with high lactic acid production in GPY and silage, 2) those with lower lactic acid production in GPY but the same in silage as group 1, and 3) those with the same lactic acid production in GPY as group 1, but lower in the silage. This suggested the need for successful lactic acid production in both liquid culture as well as silage when choosing strains for use as silage inocula. The researchers thus documented the varying growth rates of LAB and found that *L. casei* was proven a successful bacterium due to its acid tolerant characteristic at pH levels less than 4.2 (Tanaka et al., 1994).

Sporulation experiments carried out during the present research project were formulated following correspondence with Dr. Raso, known for studies on sporeforming *B. subtilis* and *B. coagulans* (Palop and Raso et al., 1996). Dr. Raso verified modifications made to his method of performing the spore counts, using Roux bottles of nutrient agar which contained 500 mg/l of Bacto dextrose and 3 mg/l manganese sulfate. The importance of manganese sulfate to spore formation of some *Bacillus* species had been noted in previous work of Charney et. al.(1951). Roux bottles were inoculated with young cultures (24 hour, 35°C) of the bacilli in nutrient broth and incubated for 5 days. Glass beads were used to spread a pipetted 1-ml aliquot of inoculate across the surface of the Roux bottle agar. Following incubation, spores were harvested by flooding the agar surface with sterile citrate-phosphate buffer, pH 7.0. Spores were washed 5 times by centrifugation and resuspended in the buffer. Spore counts were performed using phase contrast microscopy (Palop, 1996).

Silage Preservation

Successful lactic acid fermentation of silage requires:

- sufficient plant sugars.
- lactic acid bacteria on forage.

- anaerobic conditions.
- proper DM content (30%-50%).

(Pitt, 1987)

Silage "additives" contain the silage bacterial inoculum as well as various desired vitamins, minerals and organic acids. Silage inocula primarily consisting of LAB have been used for many years to speed up the natural fermentation of the ensiled crop. LAB are chosen due to their ability to rapidly reduce the pH to a level unfavorable for spoilage organisms. Lactobacilli, streptococci, pediococci and enterococci are some of the leaders in bacterial inocula (Moon, 1981). The inocula usually are dominated by homofermentative LAB which convert glucose into lactic acid, with a stoichiometric ratio of 1:2. Heterofermentative LAB convert 1 glucose into 1 lactic acid, 1 ethanol and 1 carbon dioxide, and thus are less efficient for use as inocula (McCullough, 1984). Inocula are commonly a mixed culture of organisms (for example: *Pediococcus* and *Lactobacillus*) and are currently available as either lyophilized or frozen cultures. For example Hansen Laboratories Inc. of Milwaukee, WI are well known as a company which supplies inoculum preparations for use in silage preparations. The goal of an inoculum is to minimize dry matter loss of the forage, to maintain nutritional value, to preserve by lactic acid production, to enhance digestibility and to remain cost effective through processing and sale (Bolson, 1985). Typical of any scientific system, there are many external factors affecting the success of the inoculum such as harvesting the cells, application and silo conditions (Bolson, 1985).

Perhaps of even greater importance than sufficient lactic acid production is the exclusion of oxygen from the silo (Pitt, 1987). Plant enzymes are a means of providing substrate to active microorganisms, as many of them break down complex carbohydrates into simple sugars which can be readily utilized during the fermentation. An example of this would be cellulases breaking down cellulose into glucose, which can be used by LAB to produce lactic acid. Other examples include hemicellulases which breakdown hemicelluloses into 5-carbon sugars, amylases which breakdown starch into sugars (legumes contain starch, grass does not), pectinases which break down pectin into sugars

and proteases which break down plant proteins into soluble non-protein forms. Too much protein solubilization is undesirable in silage fermentation, however and control of this protease activity through the rapid production of lactic acid is accomplished by addition of the LAB inoculum. Proper amounts of forage breakdown serve to ease the digestion process by cattle and allow the needed vitamins and minerals to be absorbed more readily. Prolonged proteolytic activity however can result in loss of nutritive quality and consequently profit loss on many levels.

This loss can be prevented via the exclusion of oxygen from the silo. Oxygen allows plant respiration which leads to the break down of plant sugars to gaseous carbon dioxide, water, and heat release. Early investigations by Amos and Williams in 1922 into temperatures of silage indicated that this heat or energy release was substantial (Barnett, 1954). The following equation defines this reaction, which leads to an overall loss of nutritive energy:

$C_6H_{12}O_6$	+	$6O_2$	$\rightarrow$	$6CO_2$	+	$6H_2O$	+	1.74 Mcal/lb,
(sugar)		(oxygen)		(carbon dioxide)		(water)		(heat)

Plant enzymes active early in fermentation are responsible for this plant protein solubilization. Oxygen extends the respiration resulting in heating and browning, bacterial-induced spoilage, and DM loss (McDonald, 1981).

Percent DM of the ensiled crop contributes significantly to the success of the inoculum. For example if the silage is too wet when it is ensiled, this will cause seepage and encourage the naturally occurring clostridia to flourish. Less than 28% DM is considered too low (McCullough, 1984). This ultimately results in an uncontrolled increase in fermentation and loss of storable material, as well as a reduction in silage consumption and thus poor performance of cattle. Conversely, if the silage is too dry, greater than 36% according to McCullough, when it is ensiled there may be problems associated with improper elimination of air (poor compaction), which can result in

reduced fermentation and ultimately aerobic deterioration with high enough pH levels to support yeasts and molds (Bolsen, 1985). The ideal % DM for ensiling has been found to fall between 28 and 36% (McCullough, 1984).

Leibensperger and Pitt, (1987) noted that the typical sugar content at harvest, expressed as % of DM, is 10%-20% for grass. The minimum required initial sugar content for grass harvested at a DM content of 30% would be 10% of DM in order to assure a proper lactic acid fermentation. Grass harvested at a higher % DM would have a lower minimum required initial sugar content; for example 50% of DM would require a sugar content of 2%. Grass harvested at a lower % DM would require a greater initial sugar content. There must be a large enough supply of fermentable carbohydrates available over an appropriate time span in order for the inoculum to succeed. Many silage additives will include energy and nitrogen containing ingredients such as corn by-products which the LAB can use in the conversion of sucrose into lactic acid . Sometimes however it is necessary to apply a carbohydrate source such as the commonly used molasses (which contains glucose, fructose and sucrose) to the forage as an energy source for the LAB. In a study done by Leibensperger and Pitt (1987), it was noted that molasses applied at the point of ensiling did have an effect on the final pH, depending primarily on the DM content of harvested grass. For example, grass ensiled at 17% DM content, with molasses added at 24 % of DM, had a final pH of 3.8, versus a pH of 4.0 with no molasses added. Many cases have been reported where silage investigators have advocated nutrient additions in combination with bacterial inocula for "guaranteed" success (Moon, 1981).

MATERIALS AND METHODS

Characterization Assays

Candidate organisms listed in Literature Review were subjected to spore counts, carbohydrate utilization testing and a casein hydrolysis assay to determine their suitability for use as silage inoculants. The following table lists the six candidate strains by ATCC (American Type Culture Collection) or NCIMB (National Collection of Industrial and Marine Bacteria) number, in the case of strain 2 only. They will be referred to as strains 1,2,3,4,5 and 6 throughout the Results section to follow.

Strain Identification

Table 1. Strain Identification

Strain Number	**Candidate Strain**	**ATCC/NCIMB Number**
1	*Sporolactobacillus inulinus*	13358
2	*Sporolactobacillus cellulosolvens*	12173 (NCIMB)
3	*Bacillus coagulans*	7050
4	*Bacillus coagulans*	31284
5	*Bacillus coagulans*	10545
6	*Bacillus coagulans*	11369

D-value Determinations

D-values were determined for vegetative cells of *S. inulinus* and *S. cellulosolvens*. For the vegetative cells, Tryptic Soy Broth was inoculated (1%) from an 18-hour culture of the appropriate culture and incubated 24hrous at 37°C. The resulting cells were examined by phase contrast microscopy to insure that essentially only vegetative cells were present. The 24 hour culture was placed in an 80°C water bath with 5 minutes allowed for temperature equilibration. Samples were removed every minute for 10 minutes and plated using Plate Count agar. Plates were incubated for 48 hours at 37°C and the colonies counted. Counts obtained were plotted semi-logarithmically.

For spores, logarithmic die-off was observed but D-values were unable to be reached under the following conditions: the initial culture was grown 4 days at 37°C and then examined by phase contrast microscopy to insure that only spore forms could be seen. This culture was plated immediately to determine whether or not the spores needed to be heat-shocked for activation and germination then the culture was temperature equilibrated to 80°C and samples taken at 5 minute intervals for an hour and plated on PCA medium. Plates were incubated as before and colonies enumerated. Data was plotted semi-logarithmically.

Materials:

Overnight cultures of *S. inulinus* and *S. cellulosolvens* were grown in Tryptic Soy Broth. Tryptic Soy Agar was prepared and stored in 250-ml bottles and then melted ans tempered to 55°C at time of use for making pour plates.

Method:

Water bath was held at 80°C. Serial dilutions of 10^{-2} and 10^{-5} were made of overnight cultures in Tryptic Soy Broth (10ml) and a pour plate of the 10^{-5} dilution was plated to

represent the pre-heat shock culture. A rack containing 1 more 10^{-5} dilution and 10 tubes of the 10^{-2} dilution was placed into the water bath. The 10^{-5} dilution was removed at 30 seconds, with a 10^{-2} tube removed at minute 1, 2, 3 and so on up to 10 minutes.

Spore Counts

Spore counts were performed on all six strains (see Literature Review). Two methods of counting were performed, i) Large Petri Plates and ii) Strain Specific Broth. Results were generated by both plate enumeration of post heat shock cells as well as with the phase contrast microscope.

Large Petri Plate Method : This method was a modified version of the one used by Palop et al. (1996). Instead of using Roux bottles and glass inoculating beads, large petri plates and glass hockey sticks were substituted.

Materials:

Large petri plate nutrient agar: One liter of nutrient agar (Difco(®), 0.5g dextrose and 0.003g manganese sulfate were combined and dispensed (80 ml) into each petri plate.

Sodium citrate-dibasic potassium phosphate buffer, pH 7.0, 0.1 M: Combined were 1.0ml of 1.0M sodium citrate buffer and 1.0ml of 1.0M dibasic potassium phosphate buffer. The volume was brought up to 900ml mark on a 1L-beaker with addition of deionized water. The pH was adjusted to 7.0 using HC1 and/or NaOH, then brought up to the 1L mark with deionized water.

A Nova, Model 926 (VWR®) was used for phase contrast microscopy.

Basic fuschin dye (0.5%) was prepared for spore stains by adding 0.5g of basic fuschin powder (VWR®) into a beaker, followed by 100ml deionized water.

Method:

Overnight cultures (0.5ml) of all six strains (see Introduction) grown in MRS broth were streaked onto the surface of large petri plates, in duplicate. Plates were incubated 4-5 days aerobically or anaerobically depending on strain. Sodium citrate dibasic potassium phospate buffer (5.0ml) were added to plate surface and allowed to sit for 5 minutes before rubbing colonies off with a glass hockey stick and resuspending the cells in 10.0ml of the same buffer. MRS broth (10ml) was used to obtain a final dilution of 10^{-7}. MRS soft agar was steamed to melt and tempered to 50°C before doing a pour plate of the 10^{-7} dilution. The 10^{-7} tubes were then heat shocked in an 80°C water bath for 10 minutes before using MRS soft agar for a post heat shock pour plate. Before and after heat shock, plates were incubated for 24 to 48 hours depending on the strain and spore count results were calculated. Prior to heat shocking, phase contrast microscopy was used in some assays for comparative spore count calculations. To do this, dilutions were heat fixed to microslides, rinsed with methanol (100%) and stained with basic fuschin dye (0.5%) by steaming the dye and rinsing. Phase contrast microscopy (100x, oil) was used to enumerate spores in several fields.

Strain Specific Broth Method: Specific broth preparations were chosen from literature references (Doores, 1983) and the ATCC Catalogue which supplied recommendations for optimal growth parameters for each strain. β-glycerophosphate was added (19g/L) to all broths as a buffer against acid formation during the extended incubation period (Terzaghi and Sandine, 1975).

Materials:

Nutrient Broth (Difco®), (10 ml) was dispensed into 20ml glass test tubes. The NB composition per liter of deionized water was Bacto Beef Extract, 3g; Bacto Peptone, 5g; b-glycerophosphate, 19g/L and calcium carbonate added in excess as a broth supplement.

Nutrient agar: This was the same composition as NB but with the addition of 7.5g of Bacto agar per liter. No additional supplements were added. The agar (20 ml) was dispensed into disposable petri plates.

Nutrient broth transfer tubes: This medium was of the same composition as NB but without additional supplements.

Tryptic Soy Broth (Difco, ®): This medium (10ml) was dispensed into 20ml glass test tubes. The composition per liter of deionized water was: Bacto Tryptone, 17g; Bacto Soytone, 3g; Bacto Dextrose, 2.5g; sodium chloride, 5g and dipotassium phosphate, 2.5g. β-glycerophosphate (19g/L) and calcium carbonate in excess were added as supplements to this broth.

Elliker Broth (Difco®): This medium (10ml) was dispensed into 20ml tubes. The composition per liter of deionized water was: Bacto Tryptone, 20g; Bacto Yeast Extract, 5g; Bacto Gelatin, 2.5g; Bacto Dextrose, 5g; Bacto Lactose, 5g; Bacto Saccharose, 5g; sodium chloride, 4g; sodium acetate, 1.5g and ascorbic acid, 0.5g. β-glycerophosphate (19g/L) was added as a supplement to this broth

Method:

For each of the six strains (see Literature Review), overnight colonies grown on NB agar were selected and used to inoculate each of its specific broth as listed alone in medium preparations. All strains were inoculated into NB and TSB as prepared above, with *S. inulinus* and *S. cellulosolvens* additionally inoculated into Elliker Broth. Broth tubes were incubated for up to 8 days with spore counts performed at 4 and 8 days. NB 10mL transfer tubes were used to perform serial dilutions up to 10^{-7}. NB agar was used to plate out each tube at 10^{-7} before and after heat shocking at various temperatures and times. *Sporolactobacillus inulinus* and *Sporolactobacillus cellulosolvens* were heat shocked at 80°C for 2 min. and all others at 80°C for 10 min. Pour plates were incubated an additional 24 to 48hrs, depending on the strain, and spore count results calculated.

Phase contrast microscopy was used for confirmation as noted in large petri plate method above.

Casein Hydrolysis

This simple milk plate agar assay indicates proteolytic activity by a clearing in the milky agar.

Materials:

Nonfat milk powder broth (11%) was prepared by combining 11g of nonfat milk powder and 89 ml of deionized water to make a 11% nonfat milk powder solution.
Double strength nutrient agar was prepared by combining 6.0g of Beef extract, 10.0g of Peptone, 15g of Agar and 1L of deionized water and then adjusting the pH to 6.8 using HC1. This was autoclaved for 13 minutes, at 121°C to sterilize.

Method:

Two plates of milk agar per strain were streaked, using fresh overnight cultures grown in MRS broth. Incubation was for up to 14 days for monitoring proteolysis of casein in the milk agar. Proteolysis was noted as clear zones in the agar. Plates were incubated at 37°C, either aerobically or anaerobically, depending on strain.

Carbohydrate Utilization

Rapid method carbohydrate testing was performed to determine the preferred sugar usage of each strain during fermentation cycles as could occur in the silo environment. API® test kits were chosen for this assay.

Materials:

Medium was purchased as part of the API® kit, Biomerieux Inc. (Hazelwood, Missouri). The suspension medium was demineralized water.

API ® 50 CH1 Medium: This consisted of Polypeptone, 10g; yeast extract, 5g; Tween 80, lml; dipotassium phosphate, 2g; sodium acetate$\times 3H_2O$, 5g; diammonium citrate, 2g; magnesium sulphate$\times 7H_20$, 20g; manganese sulphate$\times 4H_20$, 0.05g; bromcresol purple, 0.17g and deionized water to make 1L.

Method:

Colony Selection: The purity of strains was determined by subculturing selected colonies twice on MRS agar and checking for gram and catalase reactions as well as sporeforming ability and positive MRS growth. Pure cultures also were verified in a wet mount.

Preparation of Inoculum: From overnight growth on MRS plates, all colonies were subcultured into 2mL of suspension medium. All colonies selected appeared identical morphologically. Sterile swabs were used to make heavy suspensions labeled as (S1). An amount of drops (n) of this suspension was transferred into 5ml of suspension medium to reach the same turbidity as a #2 McFarland Standard. API(®) 50 CH1 Medium was inoculated by adding twice or 2 x (n) of the original heavy suspension (S1).

Inoculation of Strip: Trays containing API(®) carbohydrate strips were inoculated using (S1) suspension medium, and overlayed with sterile minerol oil. Sterile pipets were used for inoculation. The trays were covered with a lid were incubated at 37° C. Results were

observed at 24 and 48 hours. Positive results were noted as a color change in inoculated cupules from purple to yellow.

Fermentation Assays

Having been characterized as sound silage inoculant candidates, the chosen strains were next put through a series of fermentation assays. Silage to be used as test samples in the assays were first characterized microbially, by pH and with a measure of percent dry matter (% DM). Then, all strains, along with chosen control strains, were utilized in two fermentation assays: GPY and Silage Fermentation.

Grass silage was obtained at the time of ensiling from the Oregon State University Dairy Center and immediately stored frozen (-20°C) until use. During growth, the grass for silage had been fertilized with cow manure-based fertilizers but had no commercial microbial inoculant added. Sample preparation for all characterization tests were as follows: 11 g of raw silage was added to 89ml of 0.1% Peptone diluent and homogenized using a Stomacher(®) bag and Stomacher(E3) lab blender for 2 minutes. The following tests were chosen to characterize the normally occuring flora of the raw silage: total plate count, yeast and mold count, coliform count and lactobacilli count. A total of four sample bags were filled at the time of ensiling from diverse areas within the silage truck. The pH of the Raw Silage was also taken to be compared with post fermentation samples.

Microbial Analysis of Raw Silage

Total plate count: Each time assay was performed in triplicate for each of four samples taken at the point of ensiling. Assays were performed at two different times, and the "average count for the raw silage" was obtained from all numbers accrued over the

two test periods [i.e. 2 (twice ran) x 4 (4 bags total) x 3 (each bag run in triplicate) = 24 counts averaged together].

Materials:

Plate Count Agar (PCA or Standard Methods Agar, Difco®); 23.5g was resuspended in 1L of deionized water. Agar was autoclaved 10 minutes, 121°C, 15 psi) in 100ml flasks for use to prepare pour plates. PCA agar consists of Bacto Tryptone, 5g; Bacto Yeast Extract, 2.5g; Bacto Dextrose (Glucose), lg and Bacto Agar, l5g per liter of deionized water.

Method:

Homogenate 0.1mL or 0.01mL was pipetted into a petri dish, followed by 20ml of tempered (46°C) PCA agar. Plates were incubated for 24 to 48 hours and results calculated based on colony counts and dilution factors.

Yeast and mold count: Each time assay was performed in duplicate for each of four samples taken at the point of ensiling. Assays were performed at two different times, and the "average count for the raw silage" was obtained from all numbers accrued over the two test periods [i.e. 2 (twice ran) x 4 (4 bags total) x 2 (each bag ran in duplicate) = 16 counts averaged together].

Materials:

Rose-Bengal Chloramphenicol Agar Base (OXOID®); (32g) was resuspended in 1L of deionized water, brought to a boil and to it was added 10.0ml chloramphenicol solution (0.05g/10ml). Agar was autoclaved for 10 minutes at 121 °C (15 psi) and stored in 100ml flasks until use.

Chloramphenicol Solution: Chloramphenicol (0.05g) was mixed into 10 ml of deionized water and filter sterilized using Nalgene ® filtration unit.

Method:

Homogenate (0.1ml or 0.01ml) was pipetted into a petri dish, followed by 20ml of RBC agar. Plates were incubated for 24 to 48 hours and the results calculated based on colony counts and dilution factors.

Total coliform count: Each time assays were performed in duplicate for each of four samples taken at the point of ensiling. Assays were performed at two different times, and the "average count for the raw silage" was obtained from all numbers accrued over the two test periods [i.e. 2 (twice ran) x 4 (4 bags total) x 2 (each bag ran in duplicate) = 16 counts averaged together].

Materials:

Violet Red Bile Agar (Difco®); (20.75g) was resuspended in 500ml of deionized water and brought to a boil while stirring. Agar was autoclaved for 15 minutes at 121°C (15psi) and stored in 100mL flasks until use.

Method:

Homogenate (0.1ml or 0.01ml) was pipetted into a petri dish, followed by 15ml of VRB agar. After the agar solidified, approximately 4ml of VRB agar was poured onto each dish as an overlay. Plates were incubated for 24 to 48 hours and results calculated based on colony counts and dilution factors.

Lactobacilli count: Each time assay was performed in triplicate for each of four samples taken at the point of ensiling. Assays were performed at two different times, and the "average count for the raw silage" was obtained from all numbers accrued over the two test periods [i.e. 2 (twice ran) x 4 (4 bags total) x 3 (each bag ran in triplicate) = 24 counts averaged together].

Materials:

Lactobacillus Selection Agar (LBS) was prepared according to specifications found in the "Handbook for Microbiological Media for the Examination of Food", Ronald M. Atlas, p. 147. The following were added to 998.7ml of deionized water: sodium acetate$\times 3H_20$, 25g; Glucose, 20g; Agar, 15g; Pancreatic digest of casein, 10g; KH_2PO_4, 6g; yeast extract, 5g; ammonium citrate, 2g; polysorbate 80, lg, $MgSO_4$, 0.575g; $FeSO_4$, 0.034g and $MnSO_4$, 0.12g. This mixture was brought to a boil and to it 1.32ml of glacial acetic acid was added and the mixture held between 90 to 100°C for 2 to 3 minutes.

Method:

Homogenate (0.1ml or 0.01ml) was pipetted into a petri dish, followed by 20ml of LBS, agar. Plates were incubated for 24 to 48 hours and results calculated based on colony counts and dilution factors.

pH of Raw Silage

Materials:

10 grams of frozen raw silage were weighed into a stomacher bag.

Method:

Samples were diluted 3 to 6 times with deionized water and homogenized using a Stomacher lab blender for approximately 2 minutes. The pH meter (Corning Model 340) was calibrated to a pH of 7.02 using VWR® pH 7 buffer. The meter was used to measure the pH of each of the four samples taken from the silage truck at the point of ensiling.

%DM of Raw Silage

%DM was determined on raw silage to be compared to post-fermentation silage . Starting material should be within a proper range of %DM in order to assure a good fermentation. Final %DM can be used as a measure of successful amounts of final or storageable product remaining, and what state of accesibility its nutritive by-products may be in.

Materials:

5g of raw silage samples were weighed using a Mettler P162 electric scale balance, into 4 in. plastic trays.

Method:

Samples were dried using at 62°C for 48h using a Blue M Stabil-Therm Gravity Oven. Samples were then reweighed and the difference between predried and dried samples represented the %DM of the original raw silage samples.

GPY Fermentation Assay

Glucose peptone yeast extract (GPY) assay was a method of establishing which strains are homofermentatively producing lactic acid by metabolizing glucose provided in the GPY medium. Measurements of glucose and lactic acid were done using HPLC before and after fermentation had occured. The stoichiometric conversion of 1 glucose into 2 lactic acid molecules is considered in % calculations.

Materials:

HPLC Specifications: The equipment was a Beckman 163 Variable Wavelength Detector, HP 3394 Integrator, Beckman 340 Organizer, Beckman Altex 210A Valve - injector, Beckman 110B Solvent Delivery Module, Beckman 110A Solvent Delivery Module and a Beckmen 420 Microprocessor controller.

GPY Broth: Tubes (10ml) were prepared by adding peptone, 2g; yeast extract, 1g; glucose, 4g; sodium acetate, 1g and manganese chloride, 0.05g to 1L of deionized water. The pH was adjusted to 5.3 with the use of acetic acid and tap water.

Lactic Acid Standards in GPY Broth: These were prepared as follows:

0.5% = 0.05ml lactic acid in 10mL GPY.

0.4% = 0.04ml lactic acid in 10mL GPY.

0.3% = 0.03ml lactic acid in 10mL GPY.

0.2% = 0.02ml lactic acid in 10mL GPY.

0.1% = 0.01ml lactic acid in 10mL GPY.

Solutions were filtered sterilized using 0.45uM Millipore HVLP membranes, a sidearm flask and vacuum generator. Each standard (2ml) was added to a cryovial and frozen until ready for HPLC analysis.

*Glucose Standards in GPY Broth:*Standards were prepared in the range of 0.5% to 0.1% glucose in GPY broth following the above lactic acid standards in GPY broth.

Acetonitrile - Water Mobile Phase for Glucose extraction: HPLC Grade Acetonitrile (ACN) and HPLC Grade Sterile Water were used as an isocratic system with ACN starting at 80% and fading to 20%; water concentrations ran in the inverse.

Mobile Phase for lactic acid extraction (0.0075N) H_2SO_4: H_2SO_4 (0.3678g) was added to 1L of deionized water. (1997 Supelco HPLC Catalog, A65.)

Method:

Sample treatments: Control and Candidate Strains were incubated for 24h at 37_C.

1. Control = GPY Broth, known glucose concentration of 4%.
2. Strain 1 = *S. inulinus*
3. Strain 2 = *S. cellulosolvens*
4. Strain 3 = *B. coagulans*, ATCC 7050
5. Strain 4 = *B. coagulans* ATCC 31284
6. Strain 5 = *B. coagulans* ATCC 10545
7. Strain 6 = *B. coagulans* ATCC 11369

GPY broth was inoculated with overnight cultures of each strain.

8. through 12. Lactic Acid Standards = GPY Broth with known lactic acid concentrations spanning the expected range of recovery.

13. through 17. Glucose Standards = GPY Broth with known glucose concentrations spanning the expected range of recovery.

GPY tubes were removed from incubator and HPLC samples of control, strains and standards were prepared by centrifugation for 3 minutes at 15,000 rpm. Supernate was removed and filtered using 0.45um HVLP Millipore Filter Papers, vacuum generator and sidearm flask. HPLC samples (2ml) of each were frozen until ready to run on HPLC.

HPLC on Glucose Standards and Strains:

1. HPLC setup: UV abs = 230, A = 0.02, R_t= 30 minutes, chart speed = 1 cm/min, flow = 1ml/min.
2. Isocratic Mobile Phase = Acetonitrile - Water : 80 - 20.

3. Five chromatograms were run for each glucose standard to establish a standard curve for known glucose concentrations.

4. Five chromatograms were run for each strain and % glucose calculated in post fermentation in the GPY Broth. From this % glucose utilized during fermentation was calculated.

HPLC on Lactic Acid Standards and Strains:

1. HPLC setup: UV abs = 210nm, A = 0.002, R_t = 30 minutes, chart speed = 0.2 cm/min, flow = 0.7 ml/min.

2. Mobile Phase = 0.0075N H_2SO_4.

3. Five chromatograms were run on each lactic acid standard to establish a standard curve for known lactic acid concentrations.

4. Five chromatograms were run for each strain and % lactic acid in post fermentation GPY broth calculated. From this % lactic acid produced during fermentation was calculated.

Silage Fermentation Assay

The method of measuring various organic acids produced during the fermentation of grass ensiled with candidate strains and controls against organic acid standards was following the model system for ensilage described by Tanaka and Ohmomo (1994). Microbial analysis and pH also were measured following ensilage to obtain comparative data to pre-ensilage results to determine the effect various strains may have on the silage.

Materials:

HPLC Specifications: Beckman 163 Variable Wavelength Detector, HP 3394 Integrator, Beckman 340 Organizer, Beckman Altex 210A Valve - injector, Beckman 110B Solvent

Delivery Module, Beckman 110A Solvent Delivery Module, Beckman 420 Microprocessor controller.

Grass Silage: Grass silage was collected at the point of cutting from the Oregon State University Dairy Station in June, 1997 and stored frozen (-20_C) until ready to use. Grass (31.25g) was weighed out and mixed with added inoculant to produce various sample treatments, then added to 100ml volume glass screwtop bottles.

Lactic Acid Standard: For 1337ug/ml lactic acid, 0.00157ml of 85% lactic acid was added to a beaker and the volume was brought up to 1ml with deionized water.

Pyruvic Acid Standard: For 38.3ug/ml pyruvic acid, 0.000045ml of 85% pyruvic acid was added to a beaker and the volume brought up to 1ml with deionized water.

Propionic Acid Standard: For 421ug/ml propionic acid, 0.421ul of 100% propionic acid was added to a beaker and the volume brought up to 1ml with deionized water.

Acetic Acid Standard: For 213.2ug/ml acetic acid, 0.2132ul of 100% acetic acid was added to a beaker and the volume brought up to 1ml with deionized water.

n-Butyric Acid Standard: For 553ug/l n-butyric acid, 0.553ul of 100% n-butyric acid was added to a beaker and the volume brought up to 1ml with deionized water.

Formic Acid Internal Standard (ISTD): For 107.6ug/ml formic acid, 0.1076 ul of 100% formic acid was added to a beaker and the volume brought up to 1ml with deionized water.

Combined Organic Acid Standard: Lactic acid (0.00157ml), pyruvic acid (0.045ul), propionic acid (0.421ul), acetic acid (0.2132ul), n-butyric acid (0.553ul) and formic acid (0.1076ul) was added to a beaker and the volume brought up to 1ml with deionized water.

Mobile Phase (0.0075N) H_2SO_4: H_2SO_4 (0.3678g) was added to 1L of deionized water. (1997 Supelco HPLC Catalog, A65.)

Method:

Sample Treatments: Each bottle was inoculated with 1.0ml of a cell suspension at 4 x 10^6 cfu/ml which was equal to approximately 1.45 x 10^5 cfu/g silage. For all treatments

four bottles were inoculated and incubated to represent Sets I, II, III and IV or 37_C for 24h, 37_C for 36h, 42_C for 24h and 42_C for 36h using anaerobic jars and gaspaks (,). The following samples were prepared:

1. Control = Uninoculated Raw Silage
2. Strain 1 = *S. inulinus*
3. Strain 2 = *S. cellulosolvens*
4. Strain 3 = *B. coagulans*, ATCC 7050
5. Strain 4 = *B. coagulans* ATCC 11369
6. Strain 5 = *B. coagulans* ATCC 31284
7. Strain 6 = *B. coagulans* ATCC 10545
8. Biosile = *L. planatarum, P. cerevisiae*
9. Quest = *Pediococcus : Lactobacillus* 1:1 Ratio
10. Quest = *Pediococcus : Lactobacillus* 1:1 Ratio with *Enterococcus* 10%
11. Pioneer Hybrid = *L. planatarum, S. faecium* (*Enterococcus*)

Bottles were removed and HPLC samples prepared by extracting 1ml of supernate from the homogenized silage. Solid Phase Extraction (Supelco, 1997) was used to filter samples which were frozen until ready for HPLC analyses.

Remaining supernate was removed and the final fermentation pH measured.

Silage (10g) was removed for final microbial analyses, following the procedure given in Materials and Methods of Fermentation Assays, section A as well as Characterization Assays and Microbial Analysis described previously in this chapter.

HPLC on Lactic Acid Standards and Strains: The following conditions and procedures were used:

1. HPLC setup: UV abs = 210nm, A = 0.002, R_t = 30 15 minutes, chart speed = 0.2 cm/min, flow = 0.7 ml/min.
2. Mobile Phase = 0.0075N H_2SO_4.
3. Five chromatograms were run on each lactic acid standard to establish a standard curve for known lactic acid concentrations.

4. Five chromatograms were run for each strain to calculate % lactic acid in post fermentation GPY Broth. From this the percent lactic acid produced during the fermentation was calculated.

Microbial Analysis, pH and %DM of Fermented Silage

These assays were performed exactly as found in the section titled Characterization of Raw Silage described previously in this chapter.

Commercial Inoculum Preparation

Pre Dried Spore Counts

These were performed on the pre-spraydried inoculates.

Materials:

Tryptic Soy Broth: Tryptic Soy Broth (10ml-TSB, Difco,) was prepared by adding 30g of TSB powder to 1 L deionized water. The mixture was stirred until homogeneous and 10ml were distributed into 20 ml glass tubes with metal caps. Tubes were autoclaved at 121°C (15psi) for 15 minutes.

Tryptic Soy Broth with b-glycerophosphate: TSB (1L) was prepared by adding 30g of TSB powder to 1L of deionized water. β-glycerophosphate (19g/L) was added and the mixture was stirred, while applying low heat until homogeneous and then 500ml distributed into 1L glass screw top bottles. Bottles were autoclaved at 121°C (15 psi) for 15 minutes.

Nutrient Agar: Nutrient Agar (500mL-NA) was prepared by adding 30g of NA powder to 1L of deionized water. The mixture was stirred until homogeneous mixture and 250ml distributed into 500ml glass screw top bottles and autoclaved at 121°C (15psi) for 15 minutes.

Method:

TSB-bG (1L) was inoculated with overnight cultures which had been grown in 10ml of TSB. These were incubated for 4 to 8 days (usually 5) aerobically or anaerbically depending on strain (see Introduction) with Gas-Pak, in anaerobic jar, at 35°C. Cultures were removed and serial dilutioned using 10ml TSB tubes, reaching final dilution of 10^{-2} and 10^{-3}. Pour plates were made for each dilution using NA tempered to 55°C. Heat shocking of the final dilutions were carried in a water bath at 80°C for 1 to 10 minutes, the time used depending on the strain. A second set of pour plates was prepared for comparative reasons. All plates were incubated for 48h and the percent spores calculated by taking the difference between pre and post heat shock counts.

Spray Dry Experiment

Materials:

The following were used:

1. Spore preparations (1L) of *Sporolactobacillus inulinus*, in Tryptic Soy Broth.
2. Nonfat Milk Powder, standard grade.
3. Yamato Pulvis Mini Spray, Model GA-31, College of Pharmacy, Oregon State University, courtesy of Dr. J. W. Ayres.

Method:

The following stages were carried out or described on p15, Spray Dry Manual (Yamato, 1984).

1. Turned on short circuit breaker at rear of controller.
2. Turned on power and aspirator switches.
3. Began airflow into chamber by rotating aspirator to approximately 7 on dial. Note that the measuring meter is inoperable, but not significant to our work.
4. Turned on heater switch, setting the heater/inlet temperature dial to 100°C.
5. Pushed pump switch *forward* which sent sample to the spray nozzle. Turned the dial to 2 in order to prime the sample movement, then backed it down to approximately 1.
6. During cycle, the brass nozzle clearing button located on top was pushed every few minutes to prevent nozzle from clogging with product. Product/solvent level in beaker was maintained at sufficient level to prevent air from being drawn into chamber.

Note: Droplet size was regulated by adjusting the atomizing airflow (kgf/cm^2); an increase of airflow resulted in decrease in droplet size.

Clean-up:

1. Immediately sent water to nozzle with removal of solvent/sample.
2. Reduced flow rate and turned heater off.
3. Pushed aspirator adjusting dial, then turned aspirator off.
4. Removed clamp holding collection vessel, and collected accumulated powder.
5. Turned power off and disassembled equipment following instructions in the Spray-Dry Manual. All parts which were in contact with solvent/sample were cleaned using warm tap water. Note: Spray-dry nozzle needed to be washed using an ultrasonic water bath.

Post Dried Spore Counts

These were performed on post spray-dried powder.

Materials:

Powder was collected from the spray-dry experiment for enumeration of spores of *S.inulinus*.

The water bath was set at 80°C.

Pour plate agar, Tryptic Soy Agar were used.

Methods:

The following steps were carried out:

1. Rehydrated powder with lactose broth, 10^{-1} dilution.
2. Performed serial dilutions and pour plated 10^{-5}, 10^{-7} dilutions.
3. Prepared pre-heat shock pour plate of dilutions.
4. Placed tubes into water bath for 1 minute and repeated pour plates, adding 10^{-2} dilution as well.
5. Prepared post-heat shock pour plates.
6. Calculated spore counts using pre and post heat shock samples.

% Moisture

Materials:

4.2921g of spray-dried poweder was collected.

A Max 2000 Moisture Analyzer, Computrac, was used.

Method:

1. Weighed out 4.2921g powder onto metal tray.
2. Placed into automatic moisture analyzer.
3. Set settings at 100°C as recommended by Food Technologist, Steve Vinson, Oregon Freeze Dry, Inc.

Shelf Stability Counts

Materials:

One gram of powdered inoculum was used.

10ml of Tryptic Soy Broth.

Method:

1. Rehydrated powdered inoculum, 10^{-1} dilution.
2. Performed serial dilutions of 10^{-5}, and 10^{-7}.
3. Prepared pour plates of dilutions and incubated for 24h at 35°C.
4. Repeat at 6 weeks for shelf stability assessment.

RESULTS

Characterization Assays

Table 1 found in the Material and Methods section lists the six candidate strains by ATCC (American Type Culture Collection) or NCIMB (National Collection of Industrial and Marine Bacteria) number. They will be referred to as strains 1,2,3,4,5 and 6 throughout the Results section corresponding to Table 1.

D-value Determinations

Figures 1 and 2 show the semi-logarithmic plots of D-value determinations for *S. inulinus* and *S. cellulosolvens* when 18 hour cultures incubated at 37°C where heated for 10 minutes at 80°C and plated each minute. It may be seen that at 80°C, the count decreased one log (90%) in about 1 minute for both Sporolactobacilli strains.

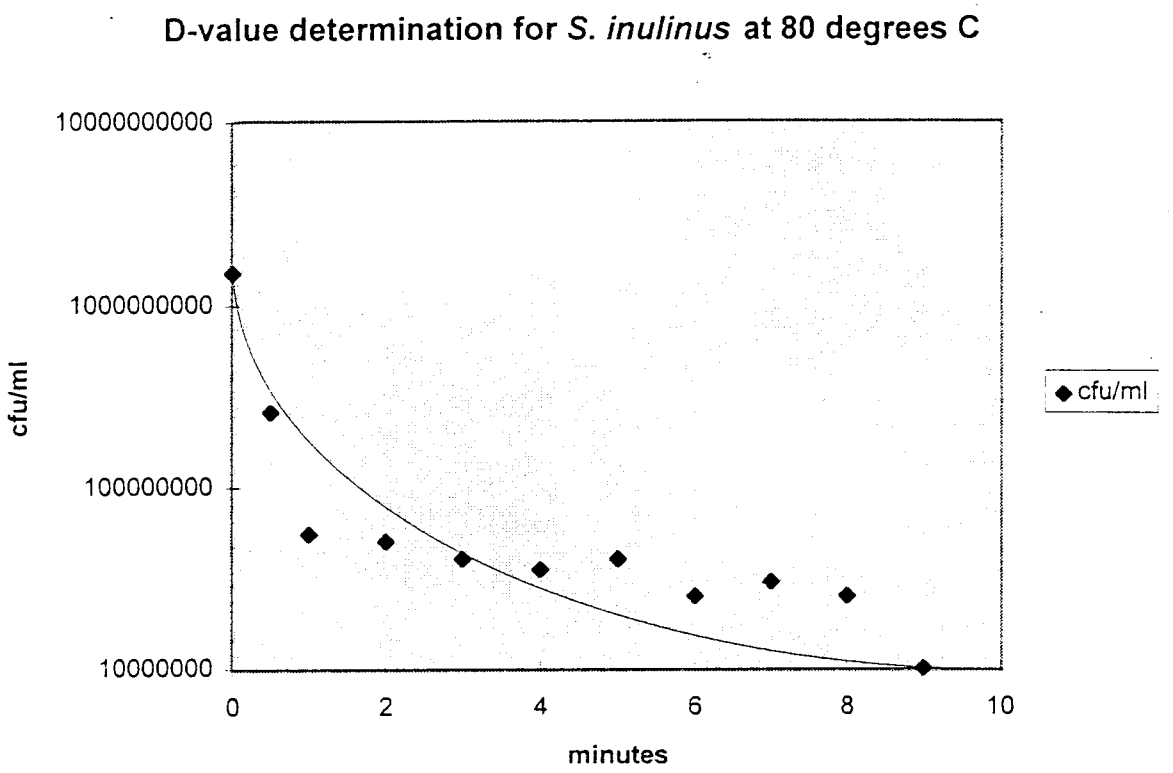

Figure 1. D-value determination for vegetative cells of *S. inulinus*.

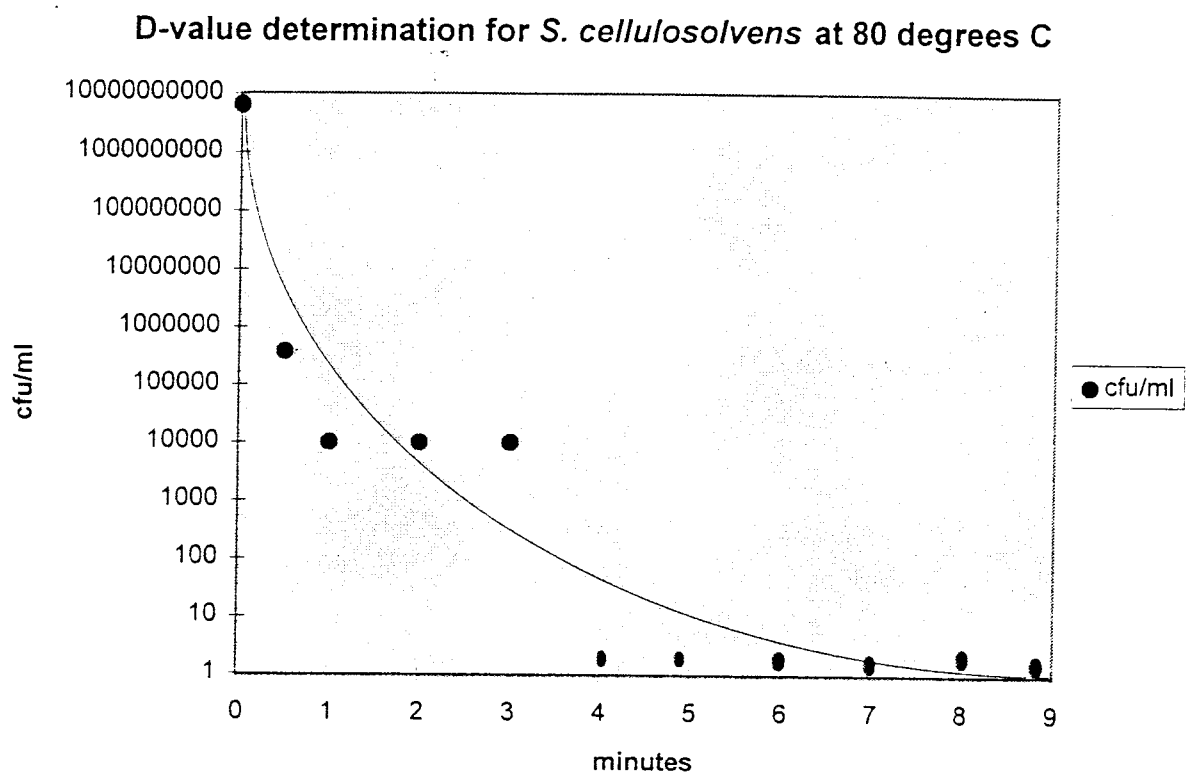

Figure 2. D-value determination for vegetative cells of *S. cellulosolvens*.

Figures 3 and 4 show the semi-logarithmic plots of die-off seen for spores of *S. inulinus* and *S. cellulosolvens* when 96 hour cultures incubated at 37°C where heated for 60 minutes at 80°C and plated every 5 minutes. It may be seen for both strains that while

there was a decrease in spore numbers by about one log within 10 minutes, fairly high numbers of spores where able to survive the entire hour.

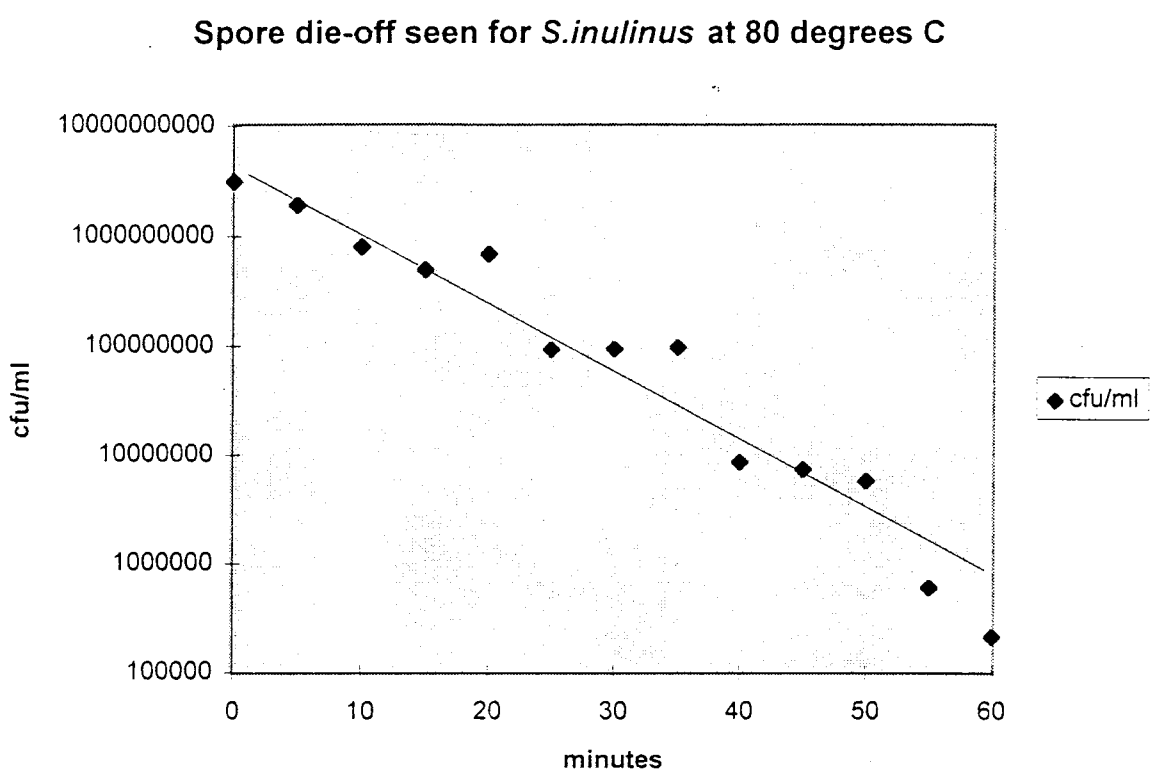

Figure 3. Semi-logarithmic plot of die-off seen for spores of *S. inulinus*.

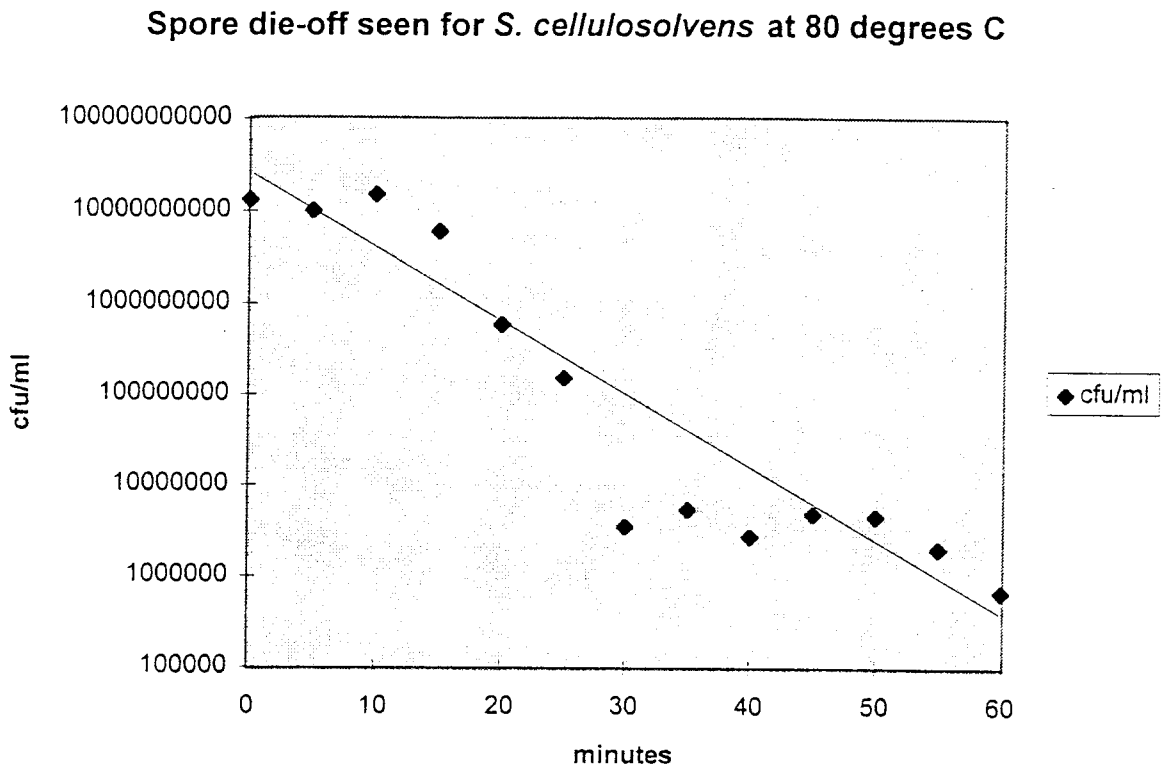

Figure 4. Semi-logarithmic plot of die-off seen for spores of *S. cellulosolvens*.

Spore Counts

Large Petri Plate Method: It may be seen from Tables 2 and 3 that agar served as a suitable medium for generation of high spore numbers by all test strains. Spore percentages exceeding 100% represent high numbers of spores germinating out once vegetative cell death had occurred.

Table2. Pre- and post-heat shock counts at 80°C for 1 to 10 minutes (strain dependent) after incubation for 4 days at 37°C for the various strains tested.

ATCC Strain	pre heat shock counts	post heat shock counts	% spores
15538	$8.2x10^{11}$	$1.5x10^{11}$	18.29
12173	$7.9x10^{12}$	$1.1x10^{9}$	0.01
7050	$6.8x10^{12}$	$6.4x10^{11}$	9.41
31284	$1.1x10^{12}$	$7.3x10^{10}$	6.64
10545	$8.0x10^{11}$	$6.8x10^{10}$	8.50
11369	$1.2x10^{12}$	$2.3x10^{11}$	19.17

Table 3. Pre- and post-heat shock counts at 80°C for 1 to 10 minutes (strain dependent) after incubation for 10 days at 37°C for the various strains tested.

ATCC Strain	pre heat shock counts	post heat shock counts	% spores
15538	$7.0x10^{10}$	$1.4x10^{11}$	200.00
12173	$9.3x10^{11}$	$1.5x10^{9}$	0.16
7050	$1.2x10^{11}$	$7.4x10^{10}$	61.67
31284	$7.0x10^{10}$	$3.3x10^{11}$	471.43
10545	$9.6x10^{10}$	$1.9x10^{11}$	197.92
11369	$1.3x10^{11}$	$2.4x10^{11}$	184.62

Strain Specific Broth Method: From Tables 4, 5 and 6 it may be seen that while the strains did not form as many spores in the broth as they did in the agar medium, there was still high spore numbers generated by day 10.

Table 4. Pre- and post-heat shock counts at 80°C after incubation for 5 days at 37°C for the various strains tested when grown in various media.

Strain/Broth	pre heat shock counts	post heat shock counts	% spores
1N	$1.0x10^{9}$	$6.4x10^{8}$	33.68
1E	$4.0x10^{9}$	$1.9x10^{8}$	4.75
1T	$8.0x10^{9}$	$8.0x10^{8}$	10.00
2N	$5.0x10^{8}$	0	0
2E	$3.2x10^{8}$	0	0
2T	$4.0x10^{9}$	0	0
3N	$5.1x10^{8}$	0	0
3E	$1.6x10^{9}$	$7.4x10^{5}$	0.05
3T	$2.6x10^{9}$	$5.4x10^{5}$	0.02
4N	$3.8x10^{8}$	$6.0x10^{4}$	0.02
4E	$1.6x10^{8}$	0	0
4T	$1.3x10^{9}$	0	0
5N	$2.6x10^{8}$	$3.5x10^{6}$	1.35
5E	$1.6x10^{9}$	$6.1x10^{5}$	0.04
5T	$6.0x10^{9}$	$1.0x10^{6}$	0.02
6N	$1.0x10^{10}$	$1.0x10^{9}$	10.00
6E	$8.0x10^{9}$	$1.6x10^{8}$	2.00
6T	$1.1x10^{10}$	$1.0x10^{10}$	90.91

*Broth: N = Nutrient, E = Elliker, T = Tryptic Soy
See Table 1 for Strain Identification.

Table 5. Pre- and post-heat shock counts at 80°C after incubation for 6 days at 37°C for the various strains tested when grown in various media.

Strain/Broth	pre heat shock counts	post heat shock counts	% spores
1N	$2.3x10^{8}$	$2.9x10^{9}$	1260.87
1E	$1.0x10^{9}$	$4.0x10^{8}$	40.00
1T	$3.0x10^{9}$	$1.8x10^{9}$	60.00
2N	$1.6x10^{10}$	0	0
2E	$1.1x10^{10}$	$1.0x10^{7}$	0.09
2T	$1.4x10^{11}$	$1.0x10^{7}$	0.01
3N	$1.2x10^{8}$	0	0
3E	$8.6x10^{9}$	0	0
3T	$2.5x10^{10}$	$1.0x10^{7}$	0.04
4N	$1.6x10^{8}$	0	0
4E	$1.0x10^{7}$	0	0
4T	$5.1x10^{9}$	0	0
5N	$2.7x10^{8}$	$1.0x10^{7}$	3.70
5E	$3.0x10^{8}$	0	0
5T	$2.6x10^{10}$	0	0
6N	$1.0x10^{11}$	$1.0x10^{11}$	100.00
6E	$7.4x10^{9}$	$1.0x10^{9}$	13.51
6T	$1.4x10^{10}$	$1.4x10^{9}$	10.00

*Broth: N = Nutrient, E = Elliker, T = Tryptic Soy
See Table 1 for Strain Identification.

Table 6. Pre- and post-heat shock counts at 80°C after incubation for 10 days at 37°C for the various strains tested when grown in various media.

Strain/Broth	pre heat shock counts	post heat shock counts	% spores
1N	$4.8x10^{9}$	$2.5x10^{9}$	52.08
1E	$1.9x10^{10}$	$2.0x10^{9}$	10.53
1T	$2.0x10^{9}$	$3.4x10^{10}$	1700.00
2N	$5.6x10^{9}$	$1.0x10^{8}$	1.79
2E	$7.6x10^{7}$	$4.0x10^{8}$	526.32
2T	$1.6x10^{10}$	$1.1x10^{8}$	0.69
3N	$5.2x10^{7}$	0	0
3E	$4.1x10^{7}$	0	0
3T	$3.9x10^{7}$	$4.0x10^{8}$	1025.64
4N	$5.5x10^{9}$	$5.0x10^{8}$	9.09
4E	$1.8x10^{7}$	0	0
4T	$1.3x10^{10}$	$1.0x10^{8}$	0.77
5N	$2.8x10^{7}$	0	0
5E	$1.0x10^{10}$	0	0
5T	$2.9x10^{7}$	$1.0x10^{8}$	344.83
6N	$4.8x10^{9}$	$4.0x10^{11}$	8333.33
6E	$3.0x10^{12}$	$3.2x10^{12}$	106.67
6T	$1.0x10^{11}$	$1.0x10^{12}$	1000.00

*Broth: N = Nutrient, E = Elliker, T = Tryptic Soy
See Table 1 for Strain Identification.

Casein Hydrolysis

After inoculation of each strain onto milk agar plates, presence or absence of growth and proteolytic activity were monitored at three dates following inoculation. This experiment was run twice to confirm first data set and to obtain data for Strain 15538 which failed to grow on first run. Table 7 shows the results. It was found that Strain 7050 achieved approximately 50% proteolysis of milk agar by Day 14. Strains 10545 and 11369 exhibited only slight proteolytic activity by Day 14, visible only when held to a light source. This experiment was repeated and the data appear in Table 7.

Table 7. Growth observations for various strains on milk agar plate incubated at 37°C for 3, 8 and 14 days.

Strain ATCC No.	**Day 3**	**Day 8**	**Day 14**
15538	no growth	no growth	no growth
12173	growth only	growth only	growth only
7050	growth	proteolysis	proteolysis
31284	growth only	growth only	growth only
10545	growth only	growth only	proteolysis
11369	growth only	growth only	proteolysis

Table 8. Growth observations for various strains on milk agar plate incubated at 37°C for 4, 10 and 15 days.

Strain ATCC No.	**Day 4**	**Day10**	**Day 15**
15538	proteolytic	proteolytic	proteolytic
12173	growth only	growth only	growth only
7050	proteolytic	proteolytic	proteolytic
31284	growth only	growth only	growth only
10545	growth only	proteolytic	proteolytic
11369	proteolytic	proteolytic	proteolytic

In this experiment it was found that Strain 15538 exhibited major proteolytic activity by a complete clearing of milk agar from white to clear as early as Day 3. This was later confirmed by additional proteolysis testing of Strain 15538 which can be seen in the photos in Figures 5, 6 and 7. Strains 7050 exhibited a milder form of proteolysis with approximately 50% of the white agar being cleared by Day 14. Strains 10545 and 11369 exhibited very mild proteolytic activity with only slight clearing at Day 14.

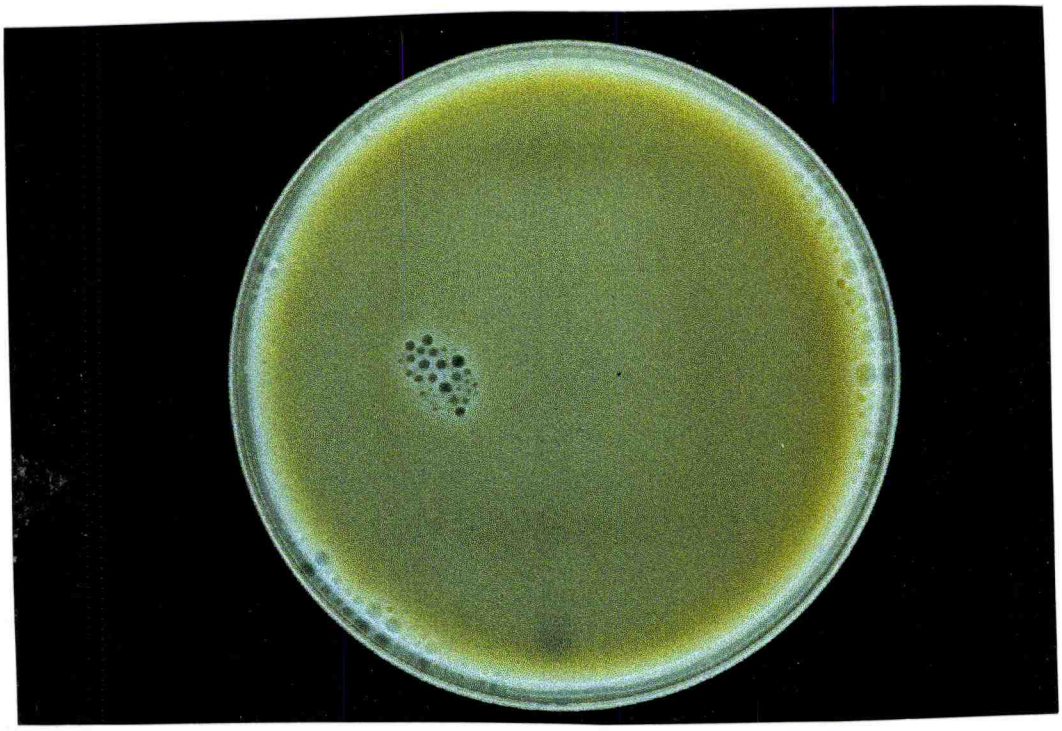

Figure 5. Photograph showing milk agar control plate.

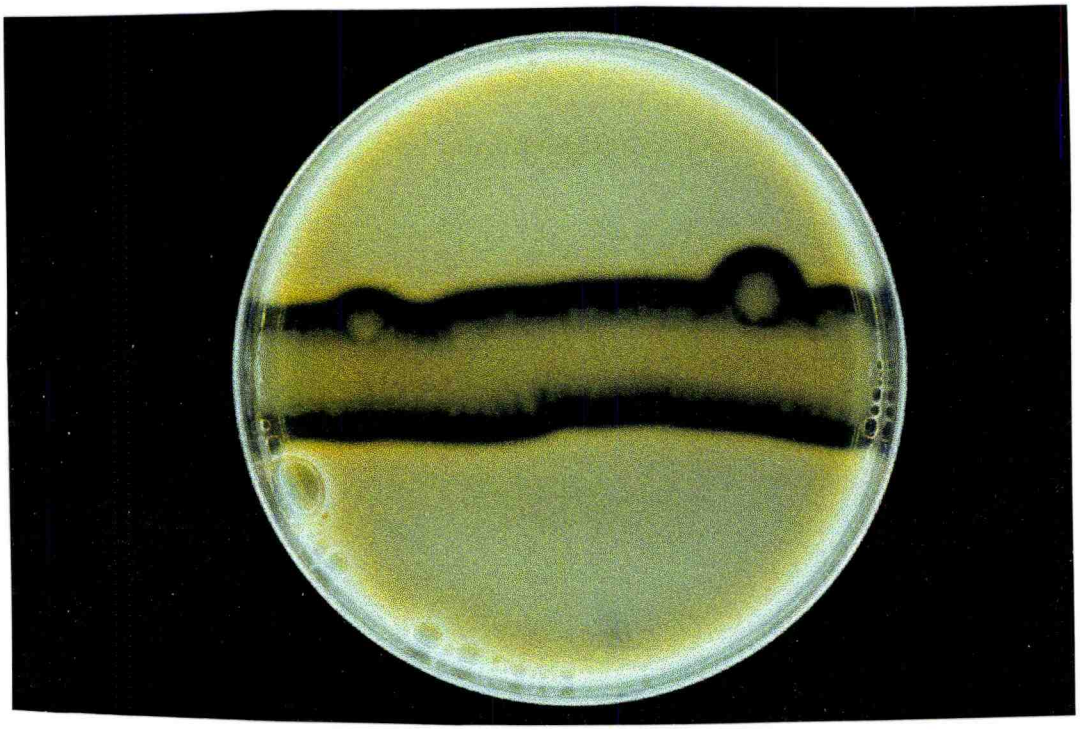

Figure 6. Photograph showing proteolytic activity of *S. inulinus* on milk agar plate when incubated for 24hrs at 37°C.

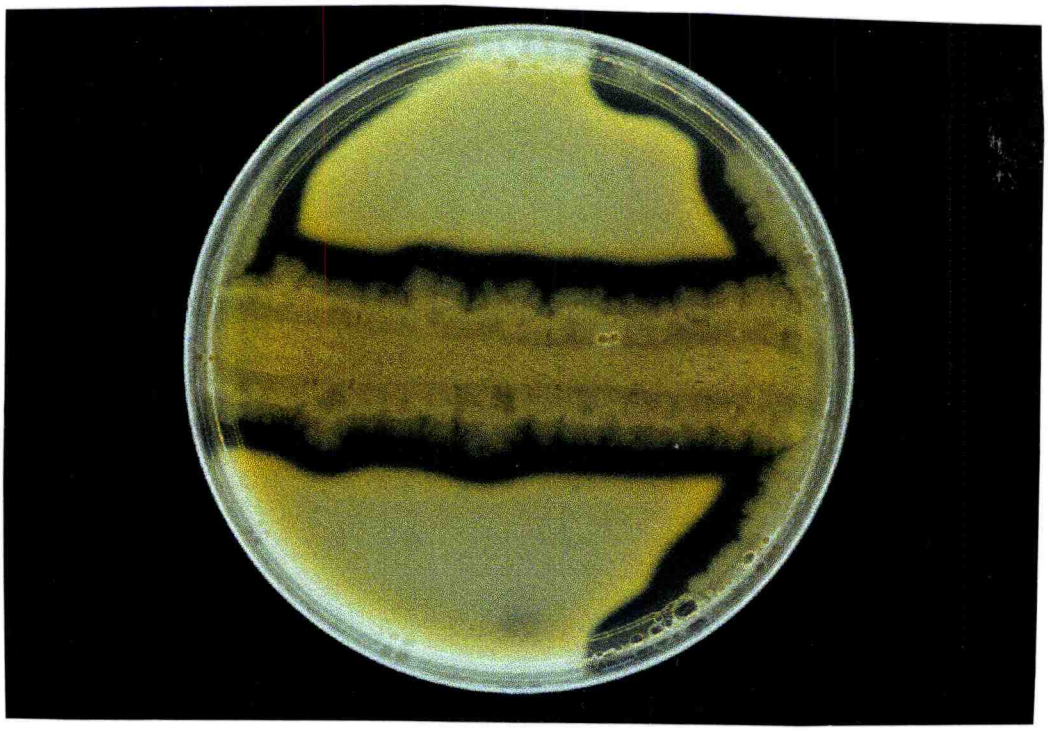

Figure 7. Photograph showing proteolytic activity of *S. inulinus* on milk agar plate when incubated for 48hrs at 37°C.

Carbohydrate Utilization

Table 9 shows the strength of the positive fermentation reactions for carbohydrates inoculated by various strains and incubated at 37°C for 48hrs. It was seen that *S.inulinus* fermented the least amount of carbohydrates available. *S. cellulosolvens* and bacilli strains behaved similarly to the control strain of *B. coagulans*.

Table 9. Positive carbohydrate fermentation reactions of various strains when incubated for 48hrs at 37°C.

		Sporolactobacilli Strains		*Bacilli Strains*			
	Control B. coagulans	***Strain 15538***	***Strain 12173***	***Strain 7050***	***Strain 31284***	***Strain 10545***	***Strain 11369***
		48hr	***48hr***	***48hr***	***48hr***	***48hr***	***48hr***
CTRL							
GLY	+						
LARA						++	
RIB			+++		+++	++	
DXYL					+++		
GAL	+		+++	+++	++	++	+++
GLU	+	+	+++	+++	+++	+++	+++
FRU	+		+++	+++	+++	+++	+++
MNE	+		+++	+++	+++	+++	+++
RHA			+		+	++	
MAN			+++		+++		
SOR						+++	
MDG	+		+		+++	+++	
NAG	+	+	+++	+++	+++	+++	+++
AMY	+				+++		
ARB	+		++		+++	+++	
ESC	+	++	++		+++	++	
SAL	+		+		+++	+++	
CEL	+		+++		+++	+	
MAL	+	+		+++		+	+++

Table 9 (Continued)

LAC	+		+++		+		
MEL	+			+++	+++	+++	+++
SUC	+	+	+++				+++
TRE	+	+	+++	+++	+++	++	
MLZ			+++				
RAF	+					+	
STCH	+			+		+	
GLYG	+						
GEN			+		+		
TUR	+		+++			+++	
TAG			+++				
GNT	+						

*GLY = glycerol, LARA = L-arabinose, RIB = ribose, DXYL = D-xylose, GAL = galactose, GLU = glucose, FRU = fructose, MNE = mannose, RHA = rhamnose, MAN = mannitol, SOR = sorbitol, MDG = α-methyl-D-glucoside, NAG = N-acetyl-glucosamine, AMY = amygdalin, ARB - arbutin, ESC = esculin, SAL = salicin, CEL = cellobiose, MAL = maltose, LAC = lactose, MEL = meliobiose, SUC = sucrose, TRE = trehalose, MLZ = melezitose, RAF = raffinose, STCH = starch, GLYG = glycogen, GEN = gentiobiose, TUR = D-turanose, TAG = D-tagatose, GNT = gluconate.
+ = slight positive at 24 and 48hrs, ++ = slight positive at 24hrs, stronger at 48hrs, +++ = strong positive at 24 and 48hrs.

Figures 8 and 9 show the API carbohydrate tray before and after the dehydrated sugars have been fermented by *Bacillus coagulans*, represented by a yellow color change.

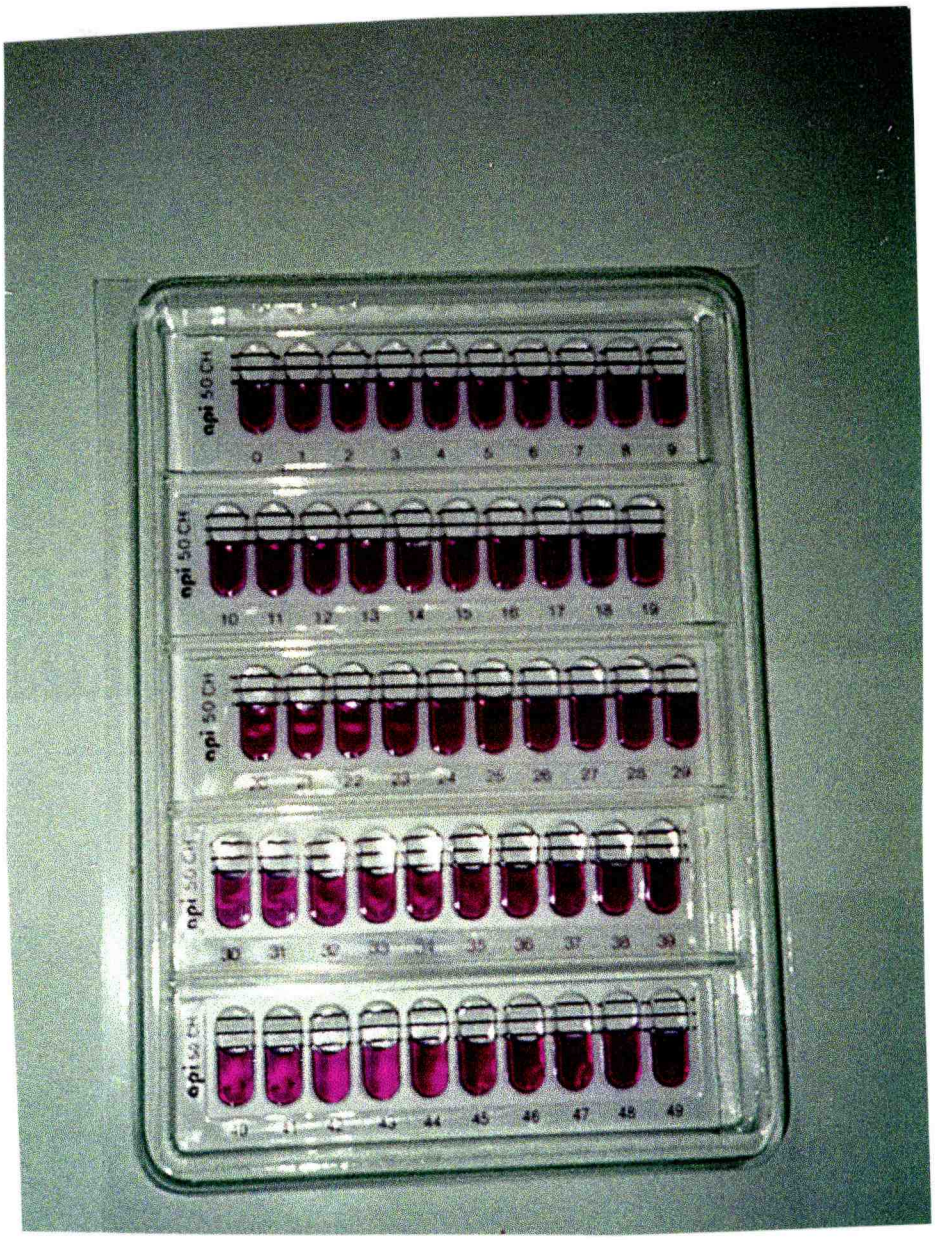

Figure 8. Photograph showing API® control tray of uninoculated carbohydrate cupules.

Figure 9. Photograph showing positive fermentation reactions of carbohydrates, represented by yellow color change, for various strains incubated at 37°C for 48hrs.

Fermentation Assays

The following results of microbial analysis, pH and %DM were used in the characterization of the grass samples as a fermentable product for ensilage. GPY and Silage Test Tube fermentation results will be used to access the strains suitability to perform as inoculants.

Microbial Analysis of Raw Silage

Table 10 shows the total plate count for triplicate runs of each sample bag. It may be seen that an average of $3.0x10^6$ cfu/ml was found.

Table 10. Total plate count cfu/ml of each sample bag when incubated at 37°C for 48hrs.

Sample bag #	**First assay: cfu/ml**	**Second assay: cfu/ml**
1	1.6×10^6 1.2×10^6 9×10^5	2×10^7 1.4×10^7 1.8×10^7
2	4×10^5 5×10^5 4×10^5	7×10^5 $3.4x\ 10^5$ 6.6×10^5
3	1×10^6 5×10^5 1.2×10^6	9.7×10^5 1×10^6 1×10^6
4	2×10^6 2.5×10^6 2×10^6	6×10^5 1.5×10^6 7.8×10^5

Table11 shows the yeast and mold count for duplicate runs of each sample bag. It may be seen that an average of $2.0x10^4$ cfu/ml was found.

Table 11. Yeast and mold count of each sample bag when incubated at 37°C for 48hrs.

Sample bag #	**First assay: cfu/ml**	**Second assay: cfu/ml**
1	2.3×10^4 2×10^4	5×10^3 2×10^3
2	1×10^3 1×10^3	3.3×10^4 5.3×10^4
3	1×10^4 1.7×10^4	5.3×10^4 2.8×10^4
4	1.6×10^4 2×10^4	2.2×10^4 2.4×10^4

Table12 shows the coliform count for duplicate runs of each sample bag. It may be seen that an average of $2.9x10^6$ cfu/ml was found, which is comparable to the total plate count cfu/ml average count.

Table12. Coliform count of each sample bag when incubated at 37°C for 24hrs.

SAMPLE BAG #	FIRST ASSAY: CFU/ML	SECOND ASSAY: CFU/ML
1	1.6×10^5 1.7×10^5	2.7×10^6 1×10^6
2	5×10^6 5×10^6	6.2×10^5 2.4×10^5
3	5×10^6 5×10^6	1.4×10^6 3.4×10^6
4	5×10^6 5×10^6	5.2×10^6 1.6×10^6

Table 13 shows the lactobacilli count for triplicate runs of each sample bag. It may be seen that an average of 5.9×10^4 cfu/ml was found.

Table13. Lactobacilli count of each sample bag when incubated at 37°C for 48hrs.

Sample bag #	First assay: cfu/ml	Second assay: cfu/ml
1	4×10^2 5×10^2 1×10^2	2.5×10^5 3.2×10^5 2.5×10^5
2	2.3×10^4 3×10^4 2.2×10^4	0 0 1×10^3
3	5.6×10^3 6.6×10^3 6.7×10^3	0 0 2×10^3
4	1.7×10^5 1.5×10^5 1.7×10^5	1×10^3 2×10^3 4×10^3

Figures 10-13 illustrate the varying morphology of colonies of naturally occuring microflora in the raw silage samples. While the petri plates shown are heavily colonated, serial dilutions were carried out for enumeration of the microbial populations shown.

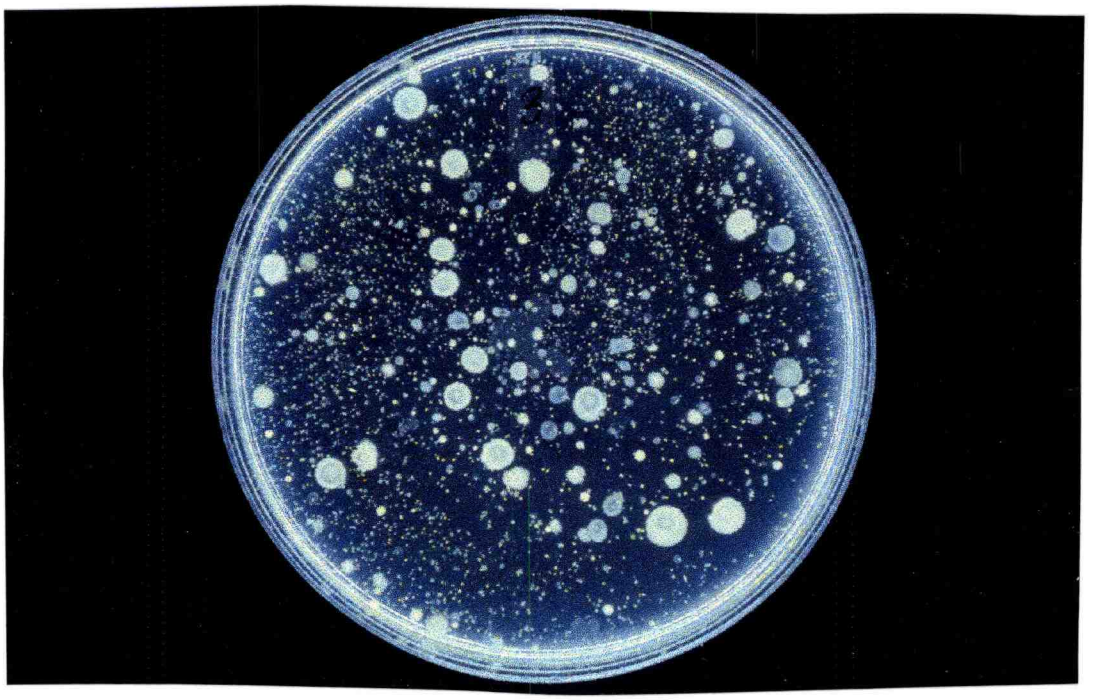

Figure 10. Total plate count colonies from grass silage grown on Plate Count Agar, for 48hrs at 37°C.

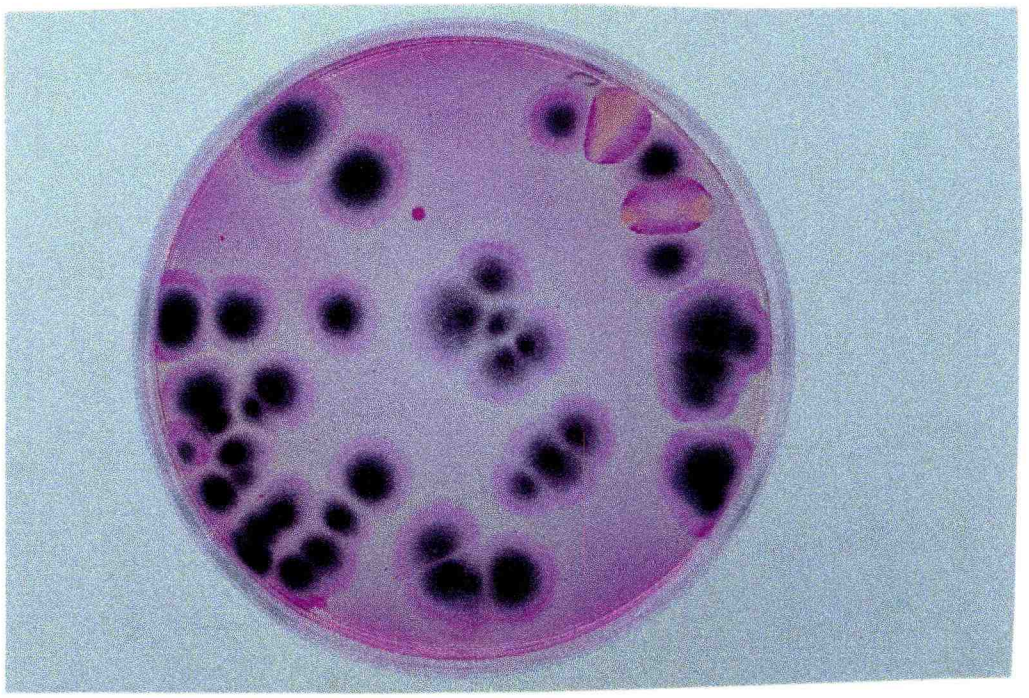

Figure 11. Yeast and Mold colonies from grass silage grown on Rose-Bengal Chloramphenicol Agar for 48hrs at 25°C.

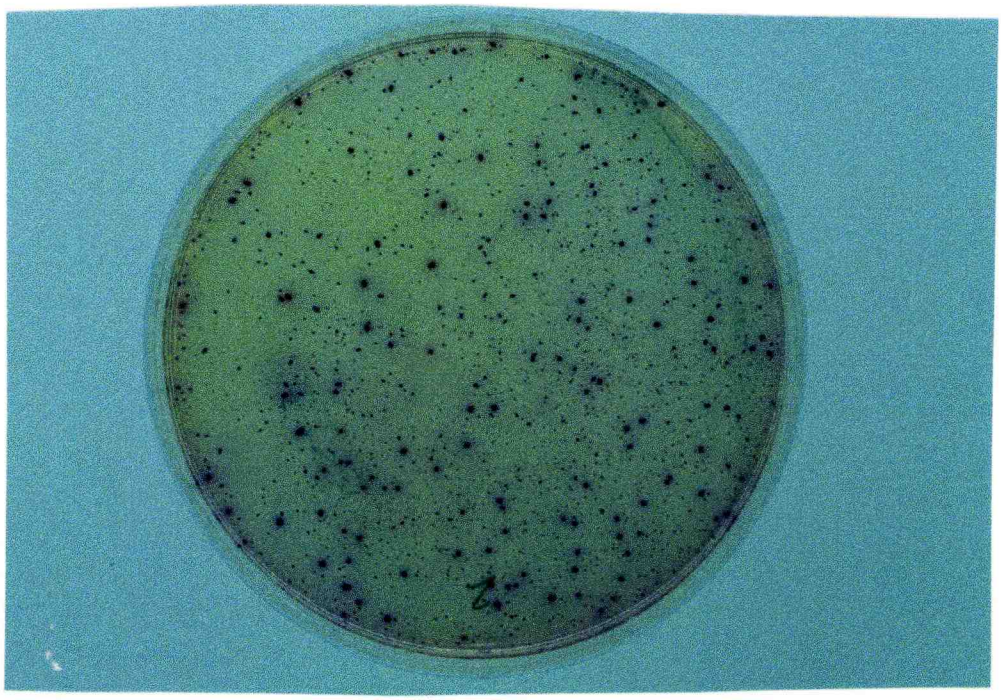

Figure 12. Coliform colonies from grass silage grown on Violet Red Bile Agar for 24hrs at 37°C.

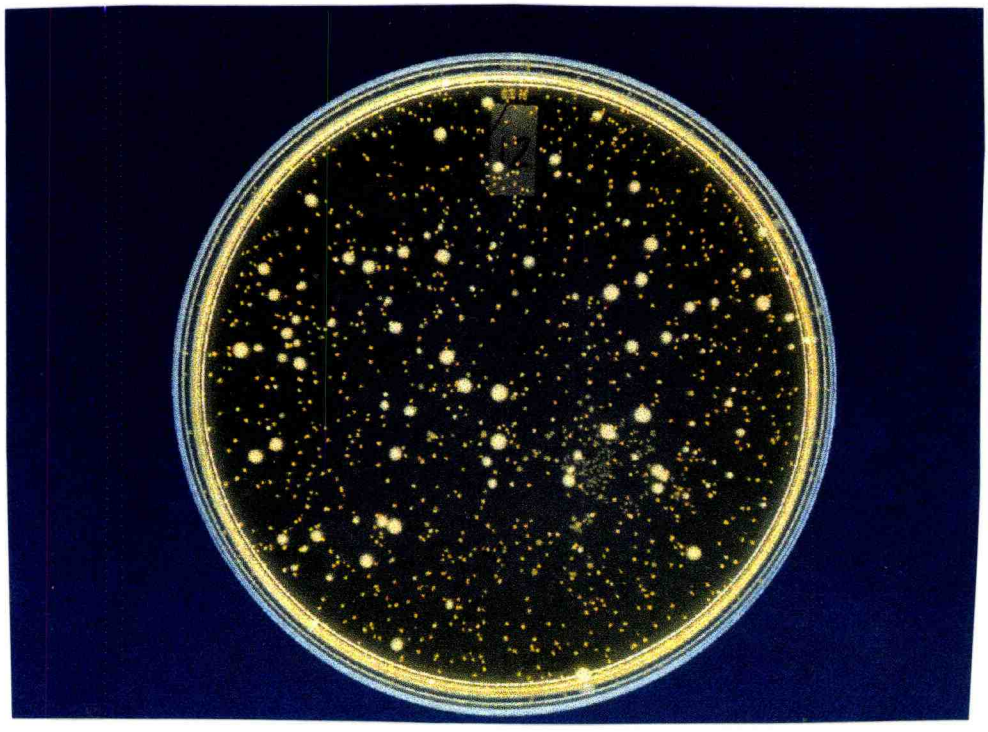

Figure 13. Lactobacilli colonies from grass silage grown on Lactobacillus Selection Agar for 48hrs at 37°C.

pH of Raw Silage

Table 14 shows the pH of each sample bag, taken prior to incubation at various time/temperature settings. It can be seen that the average pH of sample bags was found to be 5.89.

Table 14. pH of each sample bag before incubation.

Sample bag	**pH**
1	5.88
2	5.81
3	5.83
4	5.99

% DM of Raw Silage

Table 15 shows the %DM for each sample bag calculate prior to incubation at various time/temperature settings. It can be seen that the average %DM for sample bags was found to be 30.48.

Table 15. %DM of each sample bag when dried at 62°C for 48hrs.

Sample #	**Pre-dried Weight**	**Post-dried Weight**	**%DM**
1	5.06g	1.49	29.4
2	5.05g	1.53	30.3
3	5.00g	1.40	28.0
4	5.01g	1.56	31.0
5	5.02g	1.69	33.7

GPY Fermentation Assay

Glucose Standard Curve: A standard curve was developed for glucose using the areas under representative peaks generated with HPLC analysis. It was later determined that more than one run would be used for determining an "Average Area" under the peak for other standards, although the above results proved consistent for developing a typical standard curve.

Table 16. HPLC peak areas generated for known glucose percentages.

% Glucose in GPY Broth	**Area Under the Peak**
0	0
0.1	685488
0.2	1370975
0.3	2056463
0.4	2741950
0.5	3427438

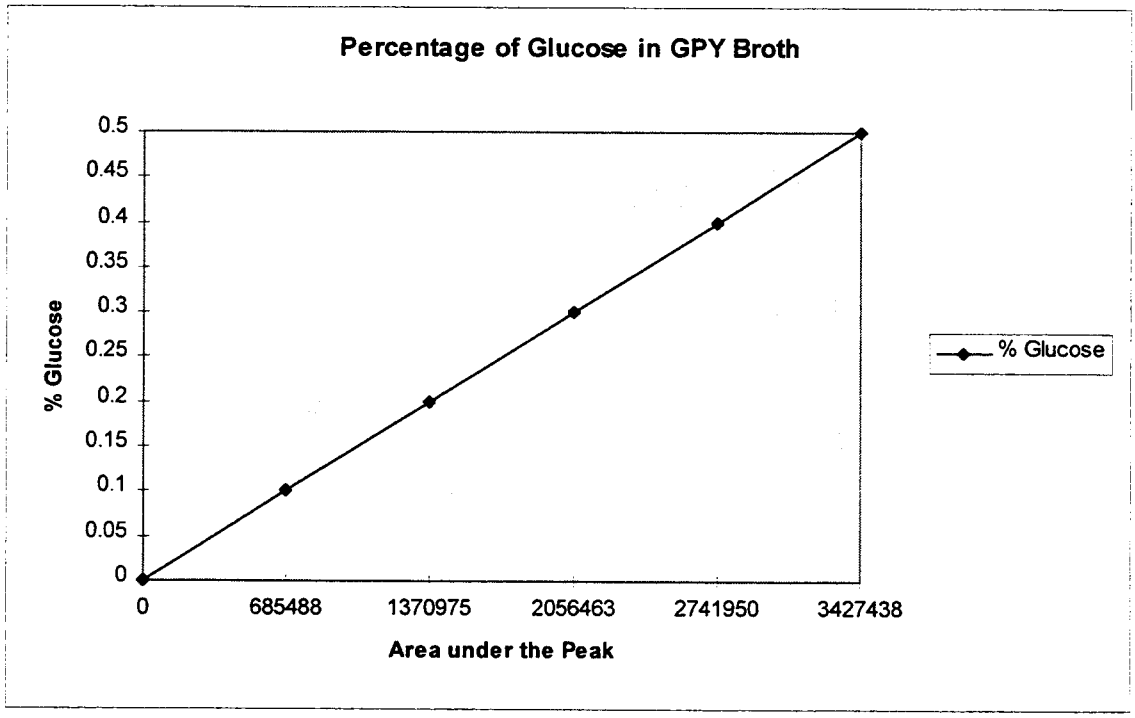

Figure 14. Standard curve for percent glucose in GPY broth.

Lactic Acid Standard Curve: A standard curve was developed for lactic acid using the areas under representative peaks generated with HPLC analysis. Lactic acid, 0.4% had only 3 runs in calculating the Average Area.

Table 17. HPLC peak areas generated for known lactic acid percentages.

% Lactic Acid in GPY Broth	Run 1 Area	Run 2 Area	Run 3 Area	Run 4 Area	Run 5 Area	Average Area Under the Peak
0	0	0	0	0	0	0
0.1	391670	362270	373880	488420	352440	393736
0.2*						464079
0.3	539000	558370	584240	538970	451530	534422
0.4	689270	571150	806270			688896.7
0.5	787300	688960	764340	829560	578700	729772

*Lactic acid, 0.2% Average Area was calculated by averaging 0.1% and 0.3% average areas.

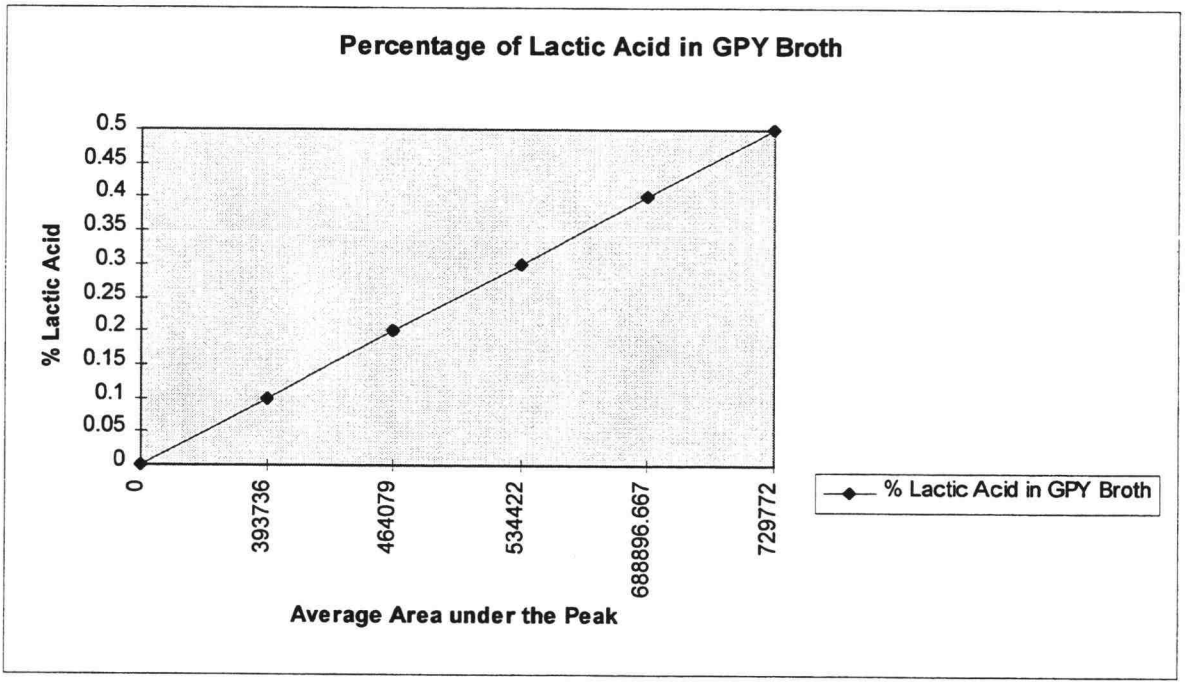

Figure 15. Standard curve for percent glucose in GPY broth.

Glucose Remaining in GPY at 24hrs: % Glucose in GPY was calculated from glucose standard curve using the formula: Area under the Peak x 0.3% glucose/2056463 area = % Glucose in GPY. % of Original 0.4% glucose utilized was calculated from the formula: ((0.4% - % glucose remaining in GPY)/0.4)x100. Table 18 shows that all strains utilized about 95% of the original 0.4% glucose in the GPY broth. The absence of error bars results from each column representing a single chromatographic peak and thus having no standard deviation. There are no error bars for Table 18 because each column represents a single data point (peak) and thus no standard deviation.

Table 18. Percent of glucose utilized from original 0.4% provided in GPY broth by 6 test strains.

Test Sample	**Area under the Peak**	**% Glucose Remaining in GPY**	**% of Orig 0.4% Glucose Utilized**
1. control	628570	0.0917	97.71
2. Strain 1	1471500	0.2147	94.63
3. Strain 2	1210900	0.1766	95.58
4. Strain 3	869110	0.1268	96.83
5. Strain 4	986000	0.1438	96.40
6. Strain 5	1558500	0.2274	94.32
7. Strain 6	966030	0.1409	96.48

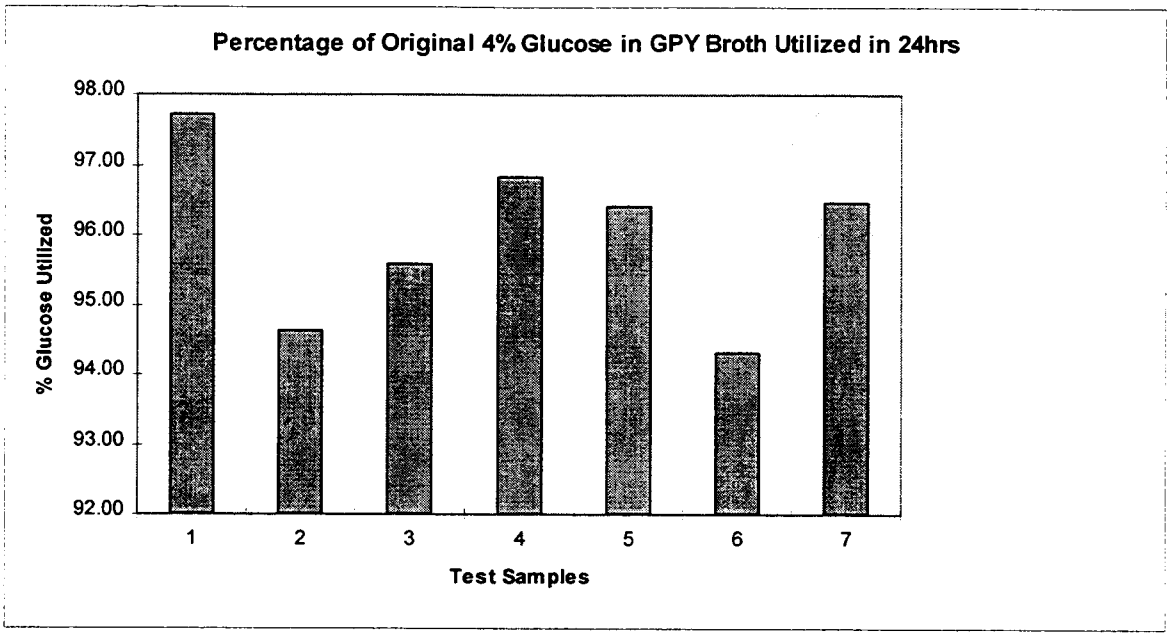

Figure 16. Comparison of % glucose utilized in GPY broth by 6 test strains and various commercial inoculates.

Lactic Acid Produced in GPY at 24hrs: % Lactic acid produced was calculated by using a standard curve for a lactic acid value as follows: (0.2% lactic acid/460079 area) x average area lactic acid in GPY = % lactic acid produced. Strains 1 and 2 only had three runs for calculating the average area. Strain 6 only had four runs for calculating the average area. The adjusted area was calculated by taking the average area of the control from the average area of each strain to account for lactic acid produced in GPY naturally at 24hrs. Table 19 shows a varied glucose/lactic acid percent conversion among the 6 test strains and various commercial inocula ranging from 36.25% to 75.75%.

Table 19. Percent of lactic acid produced in GPY broth by 6 test strains and various commercial preparations.

Test Sample	Run 1 Area	Run 2 Area	Run 3 Area	Run 4 Area	Run 5 Area	Average Area	Adjusted Average Area	% Lactic Acid Prod.*	Gluc / Lactic Acid (%)
Control	39370	93402	72042	27662	26377	51770.6	NA	0.023	5.75
Strain 1	803020	794900	623150	NA	NA	740356.7	688586.1	0.299	74.75
Strain 2	757590	741530	750230	NA	NA	749783.3	698012.7	0.303	75.75
Strain 3	322390	434700	583170	451630	348940	428166.0	376395.4	0.164	41.00
Strain 4	374730	475390	397440	462420	403640	422724.0	370953.4	0.161	40.25
Strain 5	492520	399280	427340	446330	495420	452178.0	400407.4	0.174	43.50
Strain 6	392670	418080	333290	398680	NA	385680	333909.4	0.145	36.25

*%lactic acid determined from the standard curve shown in Figure 13.

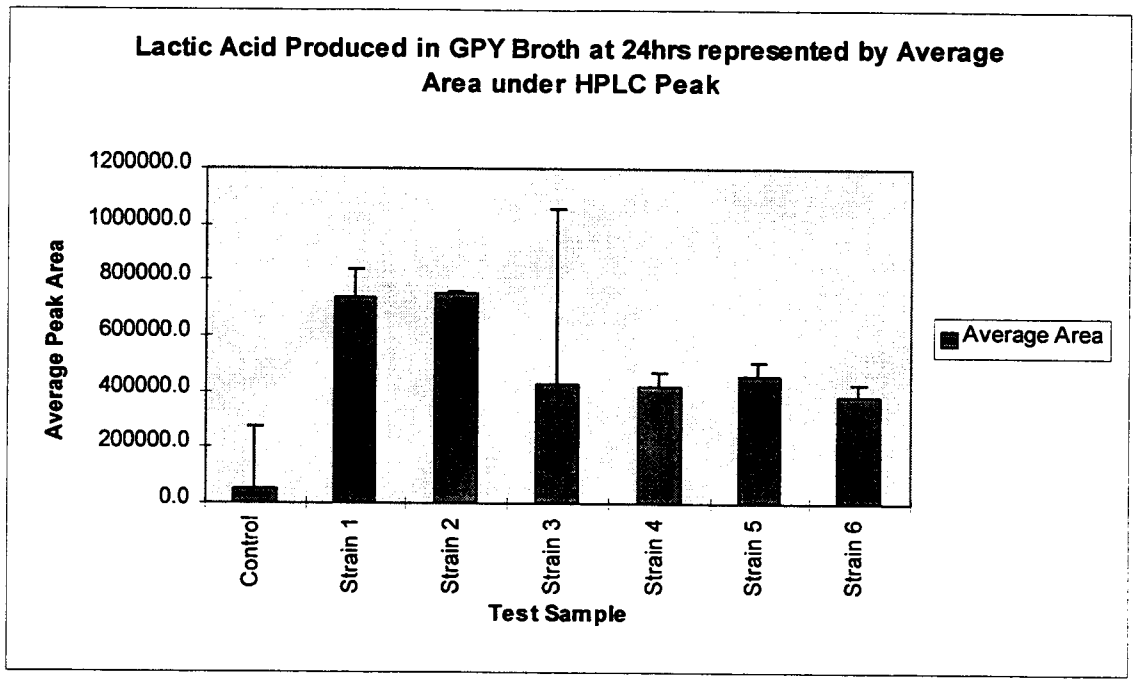

Figure 17. Comparison of % lactic acid produced in GPY broth by 6 test strains and various commericial preparations. Bars represent the standard deviation of 5 peaks generated by HPLC for each sample.

Silage Fermentation Assay

Formula derivitization for calculating ug organic acid/10ul injection: Lactic acid standard of 1337ug/1ml or 13.37ug/10ul injection was run 4 times; Acetic acid standard of 213.2ug/1ml or 2.132ug/10ul injection was run 4 times and Propionic acid standard of 421ug/1ml or 4.21ug/10ul injection was run 5 times. Table 20 shows the averages of these runs which were used to calculate ug of organic acid produced by various strains as seen below. The following formula was derived for calculation of ug organic acid/10ul injecton: (known ug organic acid in 10ul injection/Average area organic acid calculated above)x Area of organic acid at 24hrs for test strain.

Table 20. Average area of lactic, acetic and propionic acids generated by HPLC.

Lactic Acid areas **4 Runs**	**Acetic Acid areas** **4 Runs**	**Propionic Acid areas** **5 Runs**
268640	96335	867560
281880	96191	589880
232460	120550	578300
235720	82816	698220
NA	NA	485980
Average area = 254675	Average area = 98973	Average area = 643988

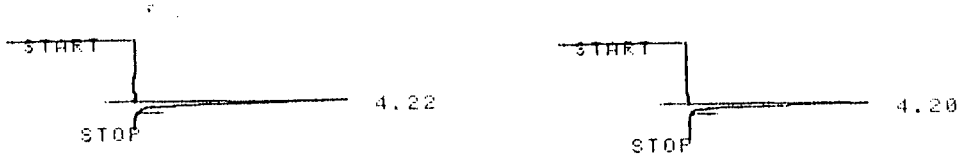

Figure 18. Two chromatograms of lactic acid peaks (13.37ug/10ul injection), with peak areas of 232460 and 281880; and retention times of 4.20 and 4.22.

Analysis of lactic, acetic and propionic acid for each strain, at Time/Temp Sets I, II, III and IV: Organic acid analysis of non-autoclaved silage is seen in Tables 21-26. Analysis revealed that silage made with the 6 test strains was similar to the commercial inoculum preparations for accumulated organic acids. Figures 19, 20 and 22 show a

comparison organic acids (ug/10ul injection) produced for test strains and commercial inoculates.

Set I = Non autoclaved silage, incubation at 37°C for 72hrs.

Set II = Non autoclaved silage, incubation at 20°C for 120hrs.

Set III = Autoclaved silage, incubation at 37°C for 72hrs.

Set IV = Autoclaved silage, incubation at 20°C for 120hrs.

Table 21. HPLC peak areas for lactic acid produced by 6 test strains and various commercial preparations generated.

Test Sample	**Set I**	**Set II**	**Set III**	**Set IV**
Strain 1	961460	1229500	1097100	316960
Strain 2	1331400	1443600	1741400	1166400
Strain 3	1359900	515380	1445600	409390
Strain 4	947470	799870	2116300	223850
Strain 5	624020	965010	1936500	150050
Strain 6	351960	1022500	1727300	449060
Control	725090	779280	355760	582300
Biosile	636140	527080	1284300	176100
Pioneer	1119400	566250	1531200	500310
Quest "LP"	1208300	620640	1431700	289340
Quest "LPE"	1315800	783760	1637100	246620

Table 22. ug of lactic acid produced by 6 test strains and various commercial inoculates.

Test Sample	Set I	Set II	Set III	Set IV
Strain 1	50.47	64.55	57.60	16.64
Strain 2	69.90	75.79	91.42	61.23
Strain 3	71.39	27.06	75.89	21.49
Strain 4	49.74	41.99	111.10	11.75
Strain 5	32.76	50.66	101.66	7.88
Strain 6	18.48	53.68	90.68	23.57
Control	38.07	40.91	18.68	30.57
Biosile	33.40	27.67	67.42	9.24
Pioneer	58.77	29.73	80.39	26.27
Quest "LP"	63.43	32.58	75.16	15.19
Quest "LPE"	69.08	41.15	85.94	12.95

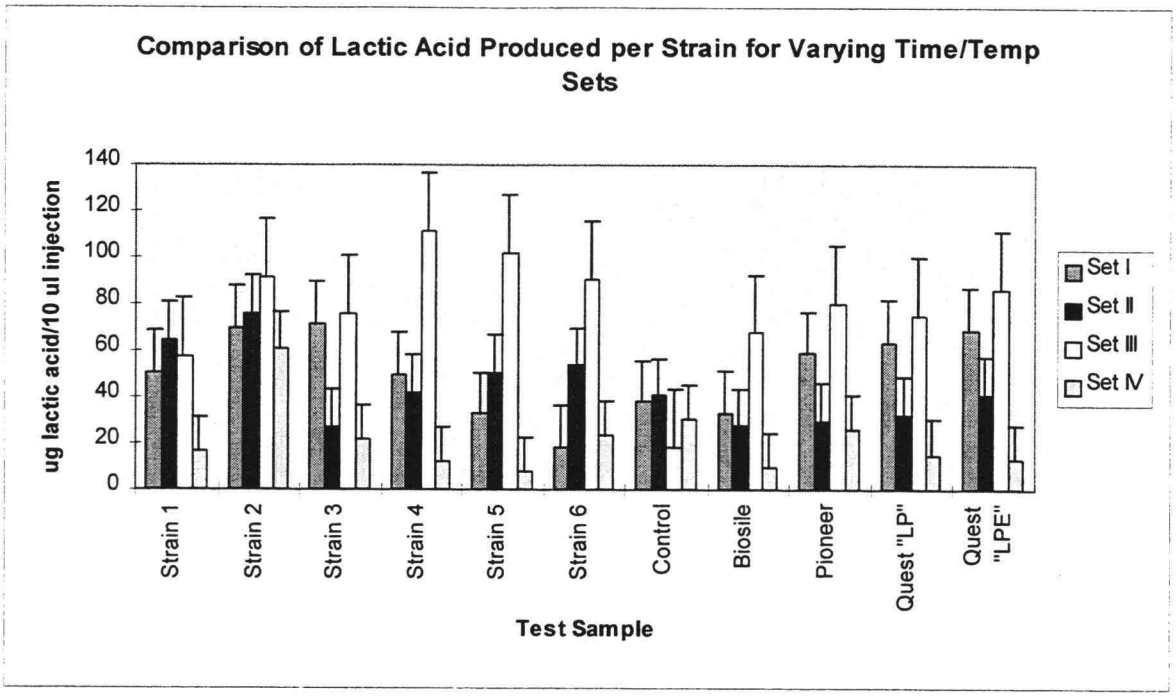

Figure 19. Comparison of lactic acid produced by 6 test strains and various commerical inoculates.

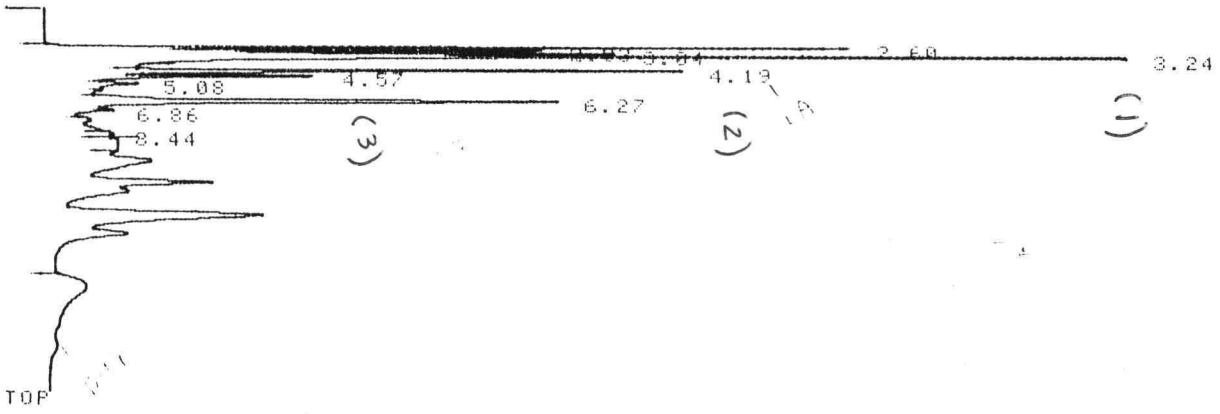

Figure 20. Separation of organic acids from silage supernate by reverse-phase liquid chromatography on an ultrasphere octyl column. Ordinate: u.v. absorbance. Peak numbers represent: (1) formic acid, (2) lactic acid, (3) acetic acid. Lactic acid showed an area of 624020 which calculated into 32.76ug/10ul injection.

Table 23. HPLC peak areas for acetic acid production by 6 test strains and various commercial preparations generated.

Test Sample	Set I	Set II	Set III	Set IV
Strain 1	135490	324440	117840	139220
Strain 2	0	152890	0	0
Strain 3	0	96736	0	308710
Strain 4	107370	80084	0	304500
Strain 5	220830	154830	0	227880
Strain 6	251210	120540	0	346650
Control	163270	115890	322710	208290
Biosile	155940	262100	0	388570
Pioneer	107190	102300	0	285910
Quest "LP"	101770	187810	0	288250
Quest "LPE"	60511	258370	0	233330

Table 24. ug of acetic acid produced by 6 test strains and various commercial preparations.

Test Sample	Set I	Set II	Set III	Set IV
Strain 1	2.92	6.99	2.54	3.00
Strain 2	0.00	3.29	0.00	0.00
Strain 3	0.00	2.08	0.00	6.65
Strain 4	2.31	1.73	0.00	6.56
Strain 5	4.76	3.34	0.00	4.91
Strain 6	5.41	2.60	0.00	7.47
Control	3.52	2.50	6.95	4.49
Biosile	3.36	5.65	0.00	8.37

Pioneer	2.31	2.20	0.00	6.16
Quest "LP"	2.19	4.05	0.00	6.21
Quest "LPE"	1.30	5.57	0.00	5.03

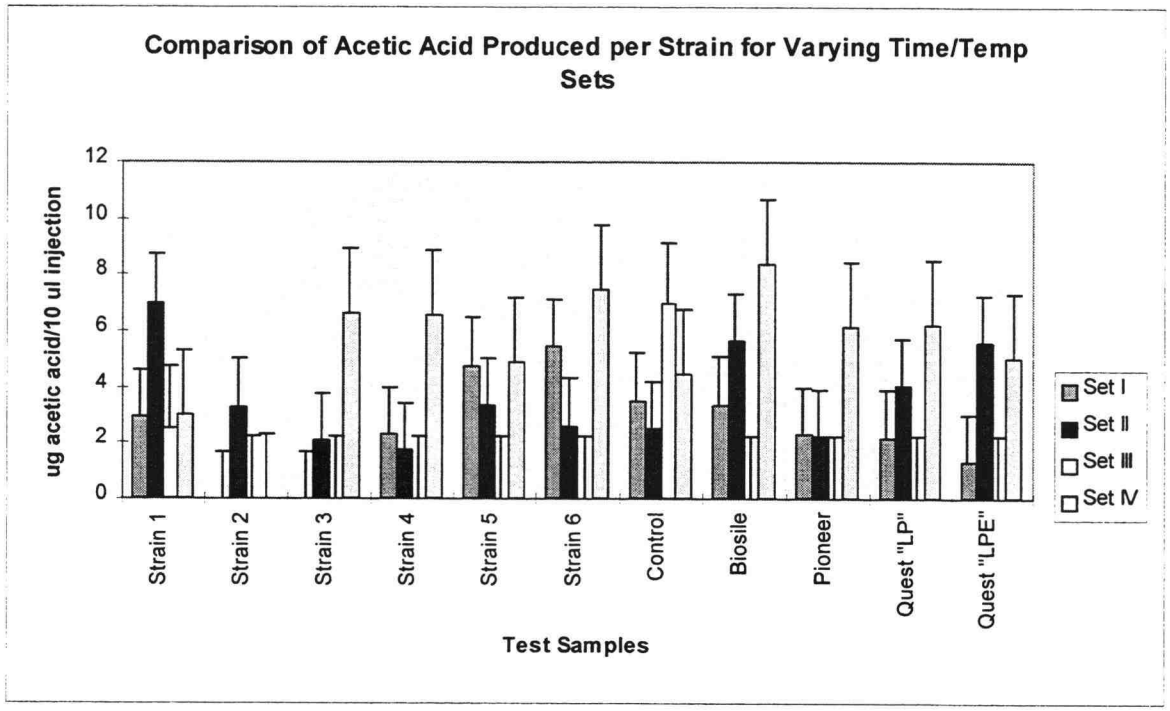

Figure 21. Comparison of acetic acid produced by 6 test strains and various commerical preparations.

Table 25. HPLC peak areas for propionic acid production by 6 test strains and various commercial preparations.

Test Sample	**Set I**	**Set II**	**Set III**	**Set IV**
1	0	0	0	0
2	0	0	0	0
3	57234	0	0	0
4	111330	0	0	0

5	17600	0	0	0
6	0	0	0	0
Control	25686	0	0	54952
Biosile	0	0	0	0
Pioneer	78638	0	0	0
Quest "LP"	0	0	132840	0
Quest "LPE"	0	0	0	0

Table 26. ug of propionic acid produced by 6 test strains and various commercial preparations.

Test Sample	**Set I**	**Set II**	**Set III**	**Set IV**
1	0	0	0	0
2	0	0	0	0
3	0.37	0	0	0
4	0.73	0	0	0
5	0.12	0	0	0
6	0	0	0	0
Control	0.17	0	0	0.36
Biosile	0	0	0	0
Pioneer	0.51	0	0	0
Quest "LP"	0	0	0.87	0
Quest "LPE"	0	0	0	0

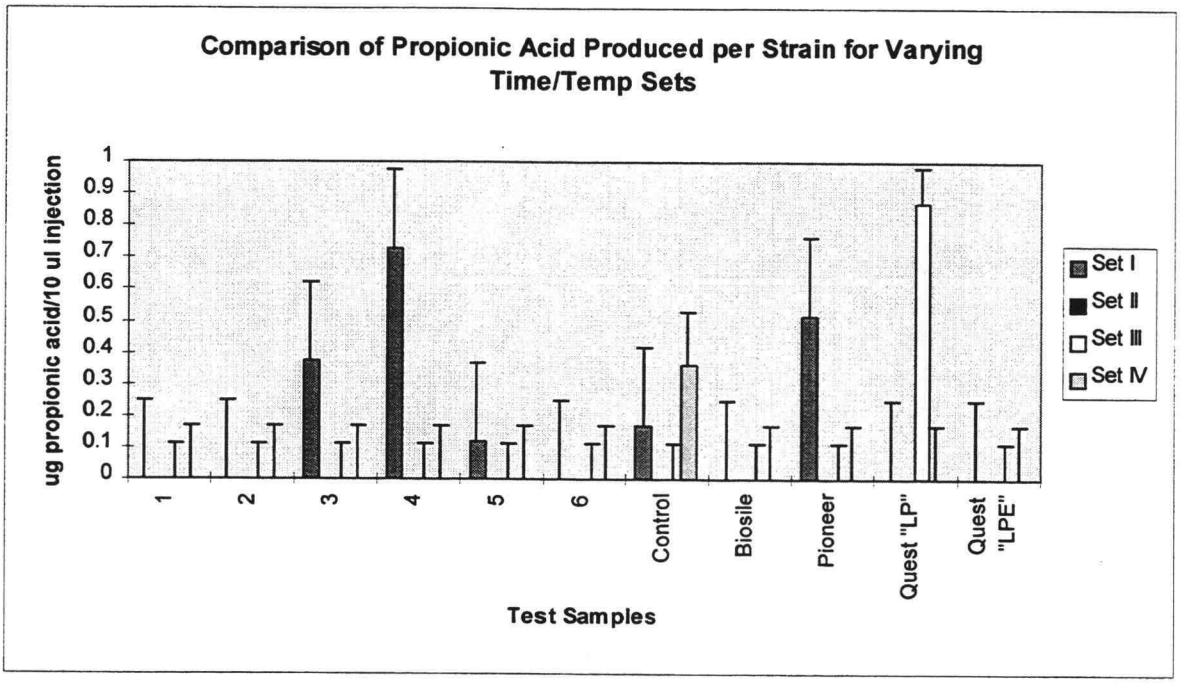

Figure 22. Comparison of propionic acid produced by 6 test strains and various commerical preparations.

Microbial Analysis of Fermented Silage

Microbial analysis of non-autoclaved silage is seen in Table 27 and 28. Analysis revealed that silage made with the 6 test strains was similar to the commercial inoculate preparations.

Table 27. Microbial analysis of non-autoclaved silage incubated at 37°C for 72hrs.

Test Sample	**PCA cfu/ml**	**LBSA cfu/ml**	**VRBA cfu/ml**	**RBCA cfu/ml**
1	$2.0x10^4$	$2.0x10^7$	$2.0x10^4$	$3.0x10^4$
2	$1.5x10^7$	$3.0x10^7$	$3.0x10^4$	$7.6x10^3$

3	$1.0x10^7$	$2.5x10^7$	$3.0x10^4$	$3.7x10^4$
4	$2.9x10^7$	$2.5 x10^7$	$1.0 x10^7$	$1.8x10^6$
5	$2.9 x10^7$	$2.5 x10^7$	$3.0 x10^7$	$1.8 x10^6$
6	$2.9 x10^7$	$3.0 x10^7$	$4.0 x10^7$	$9.0x10^5$
Control	$5.9 x10^7$	$3.0 x10^7$	$1.0x10^8$	$9.0x10^5$
Biosile	$1.5 x10^7$	$3.0 x10^7$	$2.0 x10^7$	$1.8 x10^6$
Pioneer	$2.9 x10^7$	$3.0 x10^7$	$1.0 x10^6$	$1.8 x10^6$
Quest "LP"	$2.9 x10^7$	$3.0 x10^7$	$3.7 x10^7$	$1.8 x10^6$
Quest "LPE"	$1.5 x10^7$	$3.0 x10^7$	$1.0x10^8$	$6.0x10^4$

Table 28. Microbial analysis of non-autoclaved silage incubated at 20°C for 120hrs.

Test Sample	**PCA cfu/ml**	**LBSA cfu/ml**	**VRBA cfu/ml**	**RBCA cfu/ml**
1	$1.0x10^8$	$1.0 x10^9$	0	$9.0x10^5$
2	$1.0 x10^8$	$2.0 x10^8$	0	$1.0x10^4$
3	$2.0 x10^8$	$2.0 x10^9$	$9.0 x10^8$	$9.0x10^6$
4	$1.0x10^9$	$1.0 x10^8$	4.00E+06	$9.0 x10^6$
5	$1.0 x10^8$	$1.0 x10^9$	0	$1.0x10^6$
6	$1.0 x10^8$	$1.0 x10^9$	$1.0x10^4$	$4.0x10^4$
Control	$5.0x10^7$	$2.0 x10^9$	$1.0x10^6$	$1.0 x10^6$
Biosile	$1.0x10^8$	$1.0 x10^9$	$1.0 x10^6$	$3.0 x10^6$
Pioneer	$2.0 x10^8$	$2.0 x10^9$	$2.0 x10^6$	$4.0 x10^6$
Quest "LP"	$2.0x10^9$	$1.0x10^{10}$	$5.0x10^7$	$8.0 x10^6$
Quest "LPE"	$3.0 x10^8$	$1.0 x10^9$	0	0

Table 29. Microbial analysis of autoclaved silage incubated at 37°C for 72hrs.

Test Sample	PCA cfu/ml	LBSA cfu/ml	VRBA cfu/ml	RBCA cfu/ml
1	$1.5x10^{8}$	$2.0 x10^{5}$	0	0
2	$6.0 x10^{7}$	$2.0 x10^{6}$	$7.0 x10^{4}$	$6.0 x10^{5}$
3	$1.0 x10^{8}$	$1.0 x10^{6}$	0	0
4	$2.0 x10^{8}$	$8.0 x10^{6}$	$3.0 x10^{6}$	$2.0 x10^{6}$
5	$6.0 x10^{7}$	$1.0 x10^{5}$	0	$1.0 x10^{4}$
6	$2.0 x10^{8}$	$7.0 x10^{6}$	$6.0 x10^{4}$	$3.0 x10^{7}$
Control	$1.0 x10^{8}$	$2.0 x10^{6}$	$6.0 x10^{5}$	$1.0 x10^{5}$
Biosile	$1.0x10^{9}$	$1.0 x10^{7}$	$4.0 x10^{7}$	$1.0 x10^{6}$
Pioneer	$2.0 x10^{9}$	$3.0 x10^{7}$	$5.0 x10^{7}$	$1.5 x10^{6}$
Quest "LP"	$2.0 x10^{9}$	$1.0 x10^{7}$	$5.0 x10^{6}$	$8.0 x10^{5}$
Quest "LPE"	$2.0 x10^{8}$	$2.0 x10^{5}$	0	$1.0 x10^{5}$

Table 30. Microbial analysis of autoclaved silage incubated at 20°C for 120hrs.

Test Sample	PCA cfu/ml	LBSA cfu/ml	VRBA cfu/ml	RBCA cfu/ml
1	$6.0x10^{7}$	$1.0 x10^{7}$	$2.0 x10^{4}$	$1.0 x10^{4}$
2	$2.0 x10^{7}$	$1.0 x10^{7}$	0	0
3	$2.0 x10^{7}$	$5.0 x10^{7}$	$1.0 x10^{7}$	$9.0 x10^{6}$
4	$2.0 x10^{8}$	$2.0 x10^{7}$	$1.0 x10^{6}$	$1.0 x10^{4}$
5	$1.0 x10^{7}$	$2.0 x10^{8}$	$1.0 x10^{4}$	0
6	$2.0 x10^{8}$	$3.0 x10^{7}$	$2.0 x10^{7}$	$2.0 x10^{7}$
Control	$4.0 x10^{8}$	$6.0 x10^{8}$	0	$1.0 x10^{4}$

Biosile	1.0×10^{7}	1.0×10^{7}	4.0×10^{5}	3.0×10^{5}
Pioneer	1.0×10^{8}	2.0×10^{7}	0	0
Quest "LP"	9.0×10^{7}	2.0×10^{8}	9.0×10^{6}	2.0×10^{5}
Quest "LPE"	2.0×10^{8}	2.0×10^{7}	1.0×10^{7}	1.0×10^{7}

pH of Fermented Silage

pH readings at 72hrs showed simarly reduction by both test strains and commercial inoculates, as illustrated in Table 31.

Table 31. pH of silage in Sets I - IV at 72hrs for 6 test strains and various commercial preparations.

Test Sample	**Set I**	**Set II**	**Set III**	**Set IV**
1	NA	4.31	4.06	4.08
2	3.85	3.99	4.20	3.84
3	4.05	5.50	4.11	6.07
4	4.80	4.44	4.08	6.88
5	5.10	4.08	4.20	4.66
6	4.88	4.10	4.15	6.40
Control	5.00	4.10	5.99	4.48
Biosile	4.40	4.24	4.95	4.50
Pioneer	4.20	5.97	4.48	3.95
Quest "LP"	4.24	3.99	4.70	4.32
Quest "LPE"	3.90	3.90	3.92	5.25

Commercial Inoculum Preparation

Pre Spray Dried Spore Counts

Table 32. Pre spray-drying spore counts of *S. inulinus* and nonfat milk powder.

Sample	**cfu/ml pre heat shock**	**cfu/ml post heat shock**	**% spores**
S. inulinus	$1.0x10^{9}$	$6.7x10^{8}$	37.22
Nonfat Milk Powder	$1.0x10^{2}$	0	0

Grams of Powder Collected

Spray drying of approximately 1 liter of spore preparations, bulked only 4%, yielded approximately 10 grams of powdered inoculant.

% Moisture

Moisture analysis of 4.29g collected powder yielded 4.7% moisture.

Post Dried Spore Counts

Total plate count performed 6 weeks after collection of powder indicates that the spores were stable in this low moisture environment.

Table 33. Viability counts of spray dried powder.

Date/length of storage	**cfu/ml pre heat shock**	**cfu/ml post heat shock**	**% spores**
1 week	$5.0x10^7$	$2.0x10^7$	40
6 weeks	$5.0x10^7$	NA	NA

DISCUSSION

Characterization Assays

D-value Determinations

Early attempts at characterization of the selected sporolactobacilli strains through various spore count assays provided inconsistent and uninterpretable results. Various broth and agar media, as well as incubation time and temperature combinations were explored. While microscopic examination revealed high spore numbers, the post heat shock spore counts were repetitively low or non-existent. The second problem was the unreliable spore counts in broth media. Doores et al. (1981), grew *Sporolactobacillus inulinus* on 24 media varying in carbohydrate source and concentration. Their results suggested an estimated D-value of 5 minutes at 80°C. The highest total counts were obtained when grown on a medium containing 2% α-methylglucoside (carbohydrate source). Highest spore count results were obtained with growth on 1% α-methyl-D-glucoside. Average spore D-values were: 53.2 minutes at 75°C, 19.5 minutes at 80°C, 6.8 minutes at 85.5°C and 5.1 minutes at 90°C. Earlier work by Kitahara and Suzuki (1963) suggested a D-value for *Sporolactobacillus* of 1 minute at 90°C when grown in a glucose-yeast extract-peptone media.

D-value determination assays were run for vegetative cells of the sporolactobacilli strains when grown in Tryptic Soy Broth for 24hrs at 35°C (Figures 1 and 2). Results indicated a total count D-value for each of the strains to be approximately 1 minute at 80°C. The reduced time, in comparison to earlier studies could be attributed to the specific components in the media. For example, Doores et al. obtained best results when using the α-methylglucoside carbohydrate source versus Kitahara's medium which contained glucose similar to the dextrose in the Tryptic Soy Broth. For further heat

shocking of the Sporolactobacilli strains, 80°C at 1 minute was used. *Sporolactobacillus cellulosolvens* was treated with the same parameters as the *S. inulinus* since there is little research information available to date on this strain. From the data seen in Figures 3 and 4, it may be seen that the spores of the two Sporolactobacilli strains did not need heat activation for germination. As noted in the results section, D-value determinations were not determined for spores of *S. inulinus* or *S. cellulosolvens*, but semi-logarithmic plots were made indicating the D-value of the spores for each strain to exceed 60 minutes at 80°C.

Spore Counts

A large petri plate method of performing spore counts was derived from that of Palop and Raso,et. al (1996) in which Roux® bottles of nutrient agar were used. Consultation with Dr. Raso led to confirmation that use of large petri plates containing 80ml of nutrient agar and glass hockey sticks would be a suitable substitute for the Roux® bottles and glass beads used in their experiments. Similar to Palop et. al, who attained 80-90% sporulation after 5 days of incubation at 35°C, high spore counts were obtained at day ten (Table 3). Spore counts >100% could possibly be attributed to a higher ratio of spore to vegetative cells which did not germinate (lower pre shock count) until the cell suspension had been heat shocked (thus higher post shock count). At day four, the % spores appear low in comparison to day 10, however it must be taken into consideration that even at 18.29% spores, the spore count was $1.5x10^{11}$ (Table 2). These results indicate agar to be a suitable environment for production of high spore numbers and the process of scraping, resuspending and preserving as an alternative harvesting technique when preparing inoculates could be a consideration if broth proved a difficult sporulation medium. *Sporolactobacillus inulinus* and *Bacillus coagulans* (ATCC 11369) both appear to be able spore formers.

Several broth preparations were chosen for use in further spore count assays. These were selected based on successful growth (turbidity) for 24hrs at 35°C. Tryptic Soy Broth, Elliker Broth and Nutrient Broth were selected and supplemented with β-glycerophosphate, which served as a buffer against acid build-up during the extended incubations necessary for spore formation to occur. The broths not chosen were MRS and Reinforced Clostridium, which produced inconsistent growth amongst the strains. Sufficient growth in all media was noted for all strains, however sporulation was not as consistent (Table 4). Extended incubation time appeared to be a factor contributing to higher spore counts as well as a higher total number of the strains producing spores at day ten (Table 6). The strains that were predominant early on (day 5 and 6), such as *Sporolactobacillus inulinus* and *Bacillus coagulans* (ATCC 11369) remained the leaders in highest spore counts throughout. Medium also appear to be a factor as it was noted that at days five, six and ten, highest total counts were seen consistently in the Tryptic Soy Broth. Further examination of media components and possible supplements could be explored later to induce better sporulation within the other bacilli strains, although the preshock counts indicate the environment to support good growth. Results from the assay support the previous large petri plate conclusions for *Sporolactobacillus inulinus* and *Bacillus coagulans* (ATCC 11369) tendency towards spore formation.

Casein Hydrolysis

Measurement of the proteolytic nature of the selected strains was accomplished by growth on Double Strength Nutrient Skim Milk agar plates, according to Methods for General and Molecular Bacteriology (Gerhardt, 1994). The assay measured the breakdown of casein, provided by the milk, caused by the prescence of proteinase, which was provided by the various organisms (Table 7 and 8). Results indicated *Sporolactobacillus inulinus* to be a proteolytic strain, illustrated by complete clearing in the milk agar by day three (Figures 7-9). *Bacillus coagulans* (ATCC 7050) exhibited

milder proteolytic activity, with approximately 50% clearing of milk agar by day fourteen. Bacillus coagulans (ATCC 10545 and 11369) showed very slight proteolytic activity at day fourteen, with *Bacillus coagulans* (ATCC 31284) showing no proteolysis. It is important to note this activity, since bitter peptides may be produced in the silage upon the breakdown of proteins into amino acids, then into ammonia compounds, bitter peptides and amines. This is known as Strecker Degradation and results from microorganisms breaking down protein when carbohydrate sources are limited, due to factors such as other competitive microflora or their own fastidious nature. The end result of ammonia by-products in the silage may lead to a bitter flavor or odor which is not palatable for the cattle (Bolden, 1985).

Carbohydrate Utilization

Various carbohydrate sources will occur in the silo environment which may or may not suit the needs of the organisms present. Rapid method carbohydrate testing was performed next to study which sugars each strain could readily ferment. The API® test kits enabled the fermentation of 49 carbohydrate sources to be studied by tabulating various color changes indicative of positive fermentation patterns which were correlated to a *Bacillus coagulans* control strip (Figures 10 and 11). The results indicated *Sporolactobacillus inulinus* to be the most fastidious of the test strains, fermenting only 6 of the 49 carbohydrates. Those which is did ferment were only slightly positive, with the exception of esculin. Surprisingly this did not include inulin or ∝-methyl-D-glucoside. *Sporolactobacillus cellulosolvens* was much less fastidious fermenting 19 of the 49, with the bacilli strains fermenting from 8 to 20 of the 49 carbohydrates, all with strong positive readings (Table 9). Table 9 provides a summary of the carbohydrate utilization findings and has been narrowed to include only those carbohydrates which at least one strain positively fermented. Eighteen additional carbohydrates were included in the API® test strips which no strain fermented.

Knowing the fastidious nature of the organism can provide insight into whether its necessary sugar sources will be found in a typical silo environment. If they are not, then the organism may turn to breaking down protein or possibly not survive long enough to produce enough acid for preservation of the silage. Although *Sporolactobacillus inulinus* proved to be the most fastidious of the representative organisms, analysis of the sugars which it did ferment such as glucose, maltose and sucrose, suggests that it will still be able to utilize natural sugars in various silage products. Another option which is employed quite often today is the addition of sugars at the time of inoculation, such as mollases. Sugar crops such as sweet corn will provide an abundance of carbohydrates.

Fermentation Assays

Characterization of Raw Silage: Microbial analyses, pH and %DM were taken for the pre-ensiled grass obtained from Oregon State University Dairy Farm. These results can help predict the suitability of the grass as a typical silage and how the chosen strains may react in this environment.

Microbial Analysis of Raw Silage

Total plate count, yeast and mold, total coliform and lactobacilli counts were performed on the raw silage which had been stored frozen from day of cutting until assay (approximately 1 month) (Figures 12-15). There may have been a reduction in microbial numbers due to the storage for organisms such as coliform bacteria which are more sensitive to temperature abuse. As expected, total plate count results were the highest at $3.0x10^6$ cfu/g of forage with total coliform results very comparable at $2.9x10^6$ cfu/g of forage (Tables 10 and 12). One would expect to see high coliform levels as the grass had been fertilized with cow manure. The yeast and mold results were $2.1x10^4$cfu/ml which will predictively decrease as fermentation in the silo occurs and the organisms are

inhibited by reduction in pH (Table 11). These organisms serve as one of the more common spoilers in silage and addition of a lactic acid bacteria inoculum should enhance the reduction in their numbers beyond what would be seen in a non-inoculated control sample. Repetitive opening of the silo for feeding allows air to enter promoting aerobic deterioration of the silage to occur by the yeasts and molds. This detereoration is a result of the breakdown of lactic and acetic acids (preservatives) and water soluble carbohydrates (food sources for preservative microorganisms) (Seale, 1986). Lactobacilli counts were at $5.9x10^4$cfu/ml which show that these organisms would have opportunity to naturally ferment the sugars found in the silage, if the conditions are suitable (Table 13). Suitable conditions would include low enough numbers of competitive microorganisms such as *Clostridium* species which are commonly found in silage, a proper starting %DM and pH, as well as maintenance of an anaerobic environment. Lactic acid bacterial inoculants guarantee the overabundance of the microorganisms needed to produce enough lactic acid for quick preservation of the silage product without much loss of %DM.

pH and %DM of Raw Silage

Because of the inhibitory effect a reduction in pH can have on both clostridial and coliform species (<4.0pH), it is important to see a rapid decrease in pH upon ensiling. For this reason, the lower the starting pH of the forage, the better. The average pH of the raw silage was 5.9 and was compared to ensiled grass (Table 14). Lactic and acetic acids produced by the lactic acid bacteria upon fermentation of the water soluble carbohydrates usually provides this needed pH decline in timely enough manner for the *Clostridium* species not to dominate. If low pH levels are not reached soon enough, coliform species can compete with the lactics for water soluble carbohydrates. Moist conditions however favor the *Clostridium* and even at low pH's they can begin a secondary fermentation in which lactate-fermenting clostridia can turn WSC and/or lactic acid into butyric acid. This in turn can raise the pH again and allow proteolytic *Clostridium* to begin breaking

down proteins in the silage, which can lead to bitter flavour compounds and a lowered nutritional value (Seale, 1986).

Table 15 shows that the raw silage average %DM = 30.48 which indicates no need for the addition of a sugar source (Bolden, 1985). %DM has an important effect on the success of the fermentation as seen above in regards to the Clostridium species. DM is controlled in a process called pre-wilting. Wilting the crop increases %DM, which in turn sends less water to the silo and animal consumer. This practice also benefits the fermentation process because the lactics have a higher tolerance for a low moisture environment than many of the major competitors.

GPY Fermentation Assay

In preparation for the model test fermentations to be performed on the silage, each strain was examined for lactic acid productivity. Following Tanaka et al. (1994), the strains were grown in glucose-peptone-yeast broth and then glucose and lactic acid levels were measured by HPLC. First a glucose standard curve was established to cover an expected range of remaining glucose in the post fermented GPY broth (Figure 16). The range ran from 0 to 0.5 % glucose which would represent a maximum of 100% used or a minimum of 0% glucose used with a buffer of an additional 25% increase during fermentation (Table 16).

A lactic acid standard curve was also developed to cover an expected range of lactic acid produced from the known 0.4% glucose provided, with the intention that this is the primary source for lactic acid production but taking into consideration that a percentage of the glucose may be converted into other by-products if the fermentation behaves heterofermentatively (Table 17). The range ran from 0 to 0.5% which would represent a minimum of 0% lactic acid produced or a maximum of 100% lactic acid produced with an additional 25% buffer (Figure 17).

Table 18 illustrates that nearly 100% of the glucose was utilized or broken down into other by-products during incubation. Surprisingly the control showed 97.71 % glucose

utililization, which slightly exceeded all other strains by 0.5-1.0% (Figure 18). The results of the GPY experiment indicated the two sporolactobacilli strains to have converted approximately 75% of the glucose provide into lactic acid (Table 19, Figure 19). From this it was concluded that these two strains were behaving homofermentatively, converting the glucose molecules into 2 lactate molecules with small amounts of other by products. This is done by the phosphorylation of glucose, at the expense of ATP to yield glucose-6-P which is then converted into pyruvic acid, a key player in the fermentation metabolism of all carbohydrates within the many fermentation pathways of various microorganisms. Aldolase is the enzyme responsible for cleaving the hexose diphosphate molecule into two pyruvates which lead to two lactate molecules.

The bacilli strains on the other hand averaged only a 40% conversion of the glucose to lactic acid which may be due to a more heterofermentative behaviour. In this instance about half of the glucose can be converted into other products such as CO_2, alcohol, formic or acetic acid. The major difference in the two fermentations is that the pentose phosphate pathway is employed during heterofermentative conversion instead of the EMP or glycolytic pathway. The control sample showed a 5.75% conversion of the glucose to lactate which may be due to simple breakdown.

Silage Fermenation Assay

When choosing which organic acids to monitor at post fermentation of grass, lactic acid and acetic acid were chosen to measure the efficiency of the strains to produce enough lactic acid for preservation and to examine the homo- or heterofermentative behaviour of each. Propionic acid was also chosen to serve as an indicator of the successful competive behaviour of the lactics against certain clostridial spoilage species. Pyruvic and n-butyric acids were other prospects but the HPLC set-up being used invariably yielded inconsistent results with multiple peaks and low resolution. Standards of each organic assay to be analyzed were developed according to HPLC experiments of

organic acid analysis and by the process of trial and error (Fernandez-Garcia, 1994). Multiple chromatograms were obtained for each known organic standard which were used to calculate the ratios of organic acids found at post fermentation (Table 20). Figure 20 shows a chromatogram run for the lactic acid standard of 0.1337ug/10ul injection, where an area of 232460 was generated with a retention time of 4.20.

Results of lactic acid analysis indicate Set III, which was incubated at 37°C for 72 hrs to be the optimal conditions of ensilage for the production of lactic acid (Tables 21 and 22). Figure 18 shows a chromatogram with a peak area for lactic acid of 624020 or 32.76ug/10ul injection and a retention time of 4.19, which was generated by the *Bacillus coagulans* ATCC 10545 strain in set III. This applied to the test strains as well as commercial inocula. The control sample which had no inoculum, other than normally occuring microflora predictively produced the least amount of lactic acid under any setting with the exception of Set IV. Set IV proved the worst overall conditions for lactic acid production for most all test samples (Figure 21). At 37°C the lactic acid results were fairly consistent regardless of incubation times. These results indicate the test strains behave similarly to the commercial preparations in the amount of lactic acid produced. The sporolactobacilli strains were the most consistent in their overall lactic acid production in all four sets.

Analysis of acetic acid produced were not as consistent, however Set IV appeared to be the most optimal condition for the heterofermentative microorganisms under which to produce acetic acid (Tables 23 and 24). The control sample indicates the prescence of a fair amount of these organisms as acetic acid levels seen in the control were similar in all four Sets. *Sporolactobacillus cellulosolvens* illustrated its potential to quickly produce lactic acid, thereby preventing opportunity for competitors to utilize it for the production of acetic acid (Figure 23). Once again the test strains behaved similarly to the commercial preparations. Overall, there was much less acetic acid produced (approx. 10%) than lactic acid for all test samples throughout the four sets.

Not surprisingly with the levels of lactic acid produced as early as 24 hours, the propionic acid levels produced were only in trace amounts (Tables 25 and 26). Overall,

Set I which was incubated at 37°C for 24h could be considered the most optimal conditions for production of propionic acid although all test samples showed very low levels of propionic acid production (Figure 24). The assumption could be made that lactic acid was produced quickly enough for the pH level to drop, creating a poor growth environment for Propionibacterium.

Microbial Analysis and pH of Fermented Silage

Analysis of the post fermentation microbial results focuses on Sets I and II, the non autoclaved silage, because these best represent the conditions likely to occur in a silo. Temperatures chosen for the sets were representative of various seasonal changes occuring in a silo. Sets III and IV will provide information on the amount of lactic acid produced in the autoclaved silage which should have allowed the inocula access to a higher percentage of nutrients with fewer starting competitive microflora. Those microorganisms which survived the autoclaving did so due to frozen clumps in the test samples where heat did not fully penetrate the grass, but the microbial load should still have been significantly reduced.

Sets I and III showed the highest lactic acid production which could be contributed to the active fermenters preference for the higher temperature (37°C) (Tables 27 and 29). Surprisingly, the greatest amount of lactic acid,as seen in set III, was the set with the lowest overall lactobacilli cfu/g of silage recorded. It was seen that pH of Sets I-IV was comparable and that all decreased from the pre-ensiled pH average of 5.86 to an average of about 4.5, with some falling below 4.0. It was seen that the *Sporolactobacillus cellulosolvens* test strain and Quest "LPE" commercial preparation dropped the pH below 4.0 in 3 of the 4 sets (Table 31).

Coliform counts which began at 2.9×10^6cfu/ml in the pre-ensiled grass varied somewhat in the post fermentation samples. Total inhibition was noted in Set II for both sporolactobacilli strains as well as the Quest "LPE" commercial culture. Set I showed

some increase in Coliform counts but these appeared to decrease by Set II, which could be due to the time or temperature variable difference (Tables 27 and 28). It could also be caused by increased lactic acid productivity, which correlates to the lactobacilli count increase.

Yeast and Mold counts appeared to increase in all four sets from the pre-ensiled counts, except for certain test samples in Sets III or IV where the autoclaving appears to have killed them (Tables 29 and 30). High yeast and mold is most likely due to the extremely low compaction of the grass samples in the test bottles which allowed entry of high amounts of air and thus aerobic deterioration to occur.

Overall the total plate counts rose from an average $3.0x10^6$cfu/ml to an average $1.0x10^8$cfu/ml, which was expected with the addition of an inoculum into a medium incubated at suitable growth temperatures. Results indicate the test strains behaved similarly to the commercial strains in the cfu/ml reached in all sets as measured by total plate and lactobacilli counts. The test strains also parallel the commercial strains in their inhibition towards both coliforms, yeasts and molds. The Quest "LPE" commercial preparations and sporolactobacilli cultures behaved most like a successful inoculum as seen in the Set II results.

Surprisingly, the control behaved simarily to the test and commercial preparations. This could have been due to a high number of naturally occuring microflora, which the pre-ensiled counts reflected. The time and temperatures chosen for the assay also appeared to compliment the growth conditions of the control which was seen earlier in the ug/10ul lactic acid production results observed by the HPLC experiments.

Commercial Inoculum Preparation

Tables 32 and 33 show the spore count results of pre and post spray-drying of test strain broth preparations. It can be seen that the pre spray-dry spore counts of $6.7x10^8$cfu/ml remained sufficiently high at $2.0x10^7$cfu/ml after being spray-dried into a

powder with a moisture content of 4.3%. A second set of total plate counts were performed on the collected powder following 6 weeks of storage at ambient temperature, and $5.0x10^7$cfu/ml was seen. *S. inulinus* demonstrated its ability to germinate into the vegetative form when inoculated and incubated at suitable conditions without the need for heat shock induction as seen in the 6 week counts. This indicates how this microorganism would respond to application on silage upon rehydration. Approximately 10% yield was obtained from 1L of broth culture, when bulked 4% with nonfat milk powder. Further experiments examining time/temperature settings for various percentages of solids in broth preparations is needed for industrial application of the test strains.

CONCLUSION

Successful demonstration of the ability of the chosen candidate strains to behave similar to commercial silage inocula was achieved. In the laboratory setting, the sporolactobacilli test strains were able to ferment various carbohydrates which should occur in a natural silo setting into predominantly lactic acid. The bacilli test strains also were able to produce lactic acid to a lesser degree but in amounts comparable to commercial strains. Model silage fermentation experiments showed similar behavior between test and commercial strains in acid production, microbial inhibition of spoilage organisms, pH reduction and %DM activity.

Commercial broth preparations of the test strain, *Sporolactobacillus inulinus* was spray-dried in a small scale laboratory dryer, bulked with non-fat dry milk and produced a shelf stable powder product comparable to commercial inocula in spore numbers. While further testing should be done to determine appropriate time and temperature settings for industrial size commercial broth preparations, the research indicates the chosen test strains to be good candidates for use in silage additives.

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