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Title: SEMISOLID FERMENTATION OF ALKALINE-TREATED RYEGRASS STRAW FOR ANIMAL FEED

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Annual ryegrass (Lolium multiform Lam) was treated with 2-10% NaOH (w/w) and fermented with a mixed culture of Cellulomonas sp. and Alcaligenes faecalis to increase its feed value. NaOH-treated straw was neutralized and 1% N as $(NH_4)_2SO_4$ was added. Fermentation was carried out aerobically for 2-3 days on a semi-solid substrate (moisture content of about 70%). NaOH-treated, Cellulomonas-fermented straw showed a more than two-fold increase in crude protein and crude fat, and a 70% increase in in vitro rumen digestibility. An alternative process is to treat the substrate with 2-10% NH_4OH (w/w) for four weeks at 40% moisture. When the NH_4OH-treated straw was exposed to air for two days the pH became neutral. The NH_4OH treatment increased the nitrogen content in the straw by 120%. Fermentation was carried out as in the NaOH-treated straw process. NH_4OH-treated, Cellulomonas-fermented straw showed a three-fold increase in crude protein and 60% increase in

in vitro rumen digestibility. Acetic acid was the main volatile fatty acid in the fermented straw. Semisolid fermentation was used in favor of ordinary liquid submerged fermentation because no expensive extraction was needed and the entire product could be used as an animal feed.

Semisolid Fermentation of Alkaline-treated Ryegrass Straw for Animal Feed

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This thesis is dedicated to

my mother

Mrs. Leung Wan-Hoi

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INTRODUCTION

Over 200 million tons of cellulosic agricultural wastes are produced each year in the United States. These high fiber materials create a tremendous waste disposal problem. In the State of Oregon, over a million tons of grass straw are produced annually and disposed of by burning. Public objection to the resulting air pollution eventually led to legislation to prohibit open burning. Thus, considerable efforts are being made to find alternative means of disposal or utilization.

Approximately two-thirds of grass straw consists of cellulose and hemicellulose and the rest is comprised of lignin, soluble cellular matters and ash. The high cellulose content of the straw makes it a potentially attractive source of energy for the ruminants. The major shortcomings of the straw as animal feed, however, are its low digestibility and low protein content.

Many efforts have been made to increase the nutritional value of straw. Alkaline treatment using NaOH or NH_3 has been the most commonly used to improve the digestibility of straw. Microbial fermentation was used to increase the protein content and digestibility of lignocellulosic materials (Han, 1975; Thayer et al., 1975). By

cultivating cellulolytic microorganisms single cell protein was produced from cellulosic wastes (Bellamy, 1975; Han et al., 1971). The submerged fermentation process they used required complex controls, and the fermentation products had to be harvested through a costly centrifugation. Thus, economic factors impeded the usefulness of their process. A semisolid fermentation process used by Han and Anderson (1975) simplified the fermentation process. This process involved acid hydrolysis of straw followed by cultivation of yeast on a semisolid state substrate. We have combined these two processes by growing a pair of symbiotic cellulolytic organisms on alkali treated straw by a semisolid fermentation process (Yu et al., 1976). This research describes the fermentation pattern and the characteristics of fermented straw obtained by the process.

LITERATURE REVIEW

Out of the 35 billion acres of total land area on the world's surface 1, 200 million acres are devoted to cereal production and ten billion acres are for the growing of wood, a lignocellulosic material (Tarkow and Feist, 1969). Over 200 million tons of cellulosic agricultural wastes are produced each year in the United States (Han and Anderson, 1975).

Research in the field of cellulosic wastes utilization has increased markedly during the past years. Impending shortages of conventional foodstuffs for both man and his domestic livestock force us to contemplate the vast food-energy potentially locked up in the millions of tons of currently unused lignocellulosic residues.

In areas where cereal or seed crops are grown, farmers have a problem of disposing of the remainder of the plant after the harvest. The traditional practice has been to burn the straw in the fields.

Historically, field burning was a cultural practice started in the mid-1940's to control blind seed disease then infecting some 90% of the 50, 000 acres of perennial ryegrass in Western Oregon. The incineration practice proved to be an effective control measure. Nearly one million tons of cellulosic fibers were burned in 1970 (Alexander, 1971). However, open field burning is a principal source of atmospheric pollution; in Oregon field burning will be prohibited

by law after January 1, 1977. Alternate means of disposing of this material are currently under investigation.

Straws of various types have been used for many centuries and were once one of the original sources of fiber for the manufacture of paper. It is still used widely for making paper in those countries having a shortage of wood. For many years it was the source of pulp for paper in the United States, but since the end of World War II straw mills in this country have been closed or converted to pulp mills. Reasons for this change include the limited season of harvest, biodegradability of straw in storage, and certain difficulties in manufacturing paper from pulp. All are related to economics, and most mills have converted to wood primarily because it is cheaper to manufacture pulp from wood than from straw. However, the yield of pulp from straw is about equal to that from wood fiber and in addition the pulping time and bleaching requirements are reduced with straw. A major disadvantage of straw is the shortness of its fibers which reduces the tear strength of the paper produced. Pulping cubed straw has proven very satisfactory in preliminary tests, with indications that straw cubes were superior to wood chips in terms of bulk density (Bublitz, 1974). Several wood products and resin companies have examined the feasibility of using straw in the production of interior binding panels but the economics of straw utilization are not yet competitive with wood (Alexander, 1971).

More than half of the dry matter of straw consists of cellulose and hemicellulose. The rest is comprised of lignin, nitrogenous compounds, and ash--mostly silica (Han and Anderson, 1974). Because of the nature of its constituents, straw may be used in animal feed. It cannot be used as the only source of nutrients for ruminants, but must be processed or supplemented. The main shortcomings of straw as animal feed are its (a) low digestibility, (b) low protein content, (c) poor palatability, and (d) bulkiness. By chemical and microbial fermentation of straw, a nutritious animal feed can be produced from straw which may help solve the disposal of straw while improving the rural economy by beginning a new feed industry in Oregon.

Alkaline-treatment of lignocellulosic materials has been widely used in upgrading the nutritive value of forage and forest residues for ruminants (Millett, 1970; Guggolz, 1971). Archibald (1924) in his review of the literature on alkaline-treatment of cellulosic material reported that as early as 1890, Henneberg and Lehmann had carried out feeding experiments with crude fiber prepared from NaOH treated rye straw. Most of the earlier processes applied heating with or without pressure.

In 1919, Beckmann patented an alkaline treatment process which was used extensively in Europe during both World Wars I and II, and still finds occasional use in Norway. His process involved the

hydrolysis of the cellulosic material with eight times its weight of 1.5% NaOH in open vats for 24 hours. The excess liquid was then drained off and the residue was washed with water until the product no longer turned red litmus blue. The treatment was carried out at ambient temperature and the treated cellulosic material was comparable to previous alkaline-treatment with or without pressure for longer periods of time. The digestibility of treated straw increased two-fold (Beckmann, 1921). One disadvantage of the Beckmann process is that considerable hemicellulose is solubilized, resulting in a loss of about 20% of the straw dry matter during washing operations. These washings are both an economic decrement and a disposal problem. Godden (1942) estimated that about 4,000-6,000 gallons of water were required for the treatment of one ton of straw.

To alleviate the problems related to the washing operations in the Beckmann process, Wilson and Pigden (1964) developed a 'dry' process in which straw was treated with NaOH solution at a moisture level of less than 30%. The NaOH-treated straw was then mixed with corn silage or ground alfalfa hay and neutralized with acetic acid before feeding to test animals. Alkali concentration up to 9% caused marked increase in <u>in vitro</u> digestibility but no further increases were obtained above this level. Feeding trials showed that up to 2% residual NaOH was readily accepted by sheep. No detrimental effects were noted when lambs consumed unwashed NaOH-soaked straw

(Anderson, 1972). Singh and Jackson (1971) also reported that cattle fed for six months with unwashed, NaOH-treated wheat straw showed no adverse effects. The NaOH spraying method also reduced the cost to half of that of the Beckmann process. Odell and Miles (1974) devised a dry caustic treatment method, in which chopped ryegrass straw was mixed with 10% volume of 20-40% NaOH solution to give a final treatment level of 2-4% NaOH by dry weight. The treated straw was then cubed as such and no binding agent was needed.

Alkaline treatment is widely used to improve nutritive value of various cellulosic materials. Ferguson (1942) pointed out that alkaline treatment showed marked increase in the digestibility of all constituents of straw except lignin. Feeding of alkaline-treated cereal straw was shown to be beneficial for the growth of young animals (Kehar, 1954). Alkaline-treated straw in digestion tirals with sheep using urea as a nitrogen source is comparable to hay as an energy source for protein synthesis (Lampila, 1963). Alkaline-treatment significantly improved the quality of crop residues such as corn cobs, stalk, stover and husklage (Krause et al., 1968; Klopfenstein et al., 1970). Javed and Donefer (1970) treated straw with a NaOH solution in a horizontal feed mixer. After the treated material was neutralized with acetic acid, it was mixed with molasses, protein and mineral supplements and compared to dehydrated alfalfa meal. The increased nutritive value of the chemically treated and

supplemented straw, due to improved digestibility and voluntary intake, yielded growth results approaching those obtained with the alfalfa ration. Saxena et al. (1971) studied the growth and nitrogen metabolism of lambs fed with alkali-treated oat straw supplemented with either soybean meal, urea, or diammonium phosphate. Animals fed with NaOH-treated straw had lower levels of rumen ammonia and blood urea, indicating that straw treatment was effective in bringing about the release of energy to stimulate bacterial growth. Other lignocellulosic materials other than straw were also studied. Millett et al. (1970) examined 24 species and subspecies of wood for their change in digestibilities by dilute NaOH. Hardwoods such as aspen, ash and maple were digested to a larger extent than softwoods and the results indicated that the response to alkaline treatment is species dependent. Mellenberger et al. (1971) treated aspen sawdust with 0.5% NaOH at 10:1 liquid-to-solid ratio for two hours at room temperature. After draining, washing once with water, and air drying, the product was incorporated into pelleted rations at levels of 0 to 60%. Dry matter digestibilities of about 41% for untreated aspen and 52% for the alkali-treated aspen were obtained.

Sodium hydroxide is an effective delignifying agent. Chandra and Jackson (1971) reported a 26% reduction in lignin content of ground maize cobs treated with 10% NaOH. They also reported a 100% increase in digestibility. Stone et al. (1965) reported that

alkali treatment causes swelling of the cellulose fiber and consequently causes the breaking of the lignin physical structure. This change in structure rendered the cellulose-lignin complex more susceptible to enzyme and ruminant digestion. Tarkow and Feist (1969) postulated that the effect of alkali treatment was essentially the saponification of intermolecular ester bonds, thus promoting the swelling of wood beyond water-swollen dimensions and favoring increased enzymatic and microbiological penetration into the cell-wall fine structure.

Millett et al. (1970) estimated the amount of NaOH necessary for maximum effect on in vitro digestion of wood. Results indicated that from 5 to 6 g of NaOH per 100 g of wood was necessary for maximum increase of digestion. This level of alkali is essentially equivalent to the combined acetyl and carboxyl contents of the woods (Feist et al., 1970).

Alkali treatment can also significantly increase microbial growth as recently described by Han and Callihan (1974). Carbohydrate utilization of rice straw and sugarcane bagasse by Cellulomonas bacteria advanced from an initial 29% up to 73% through a 4% sodium hydroxide treatment at 100 C. Hogan and Weston (1971) studied the bacterial protein synthesis from alkali-treated and urea-supplemented wheat straw. Treated straw served as an effective energy source for promoting nitrogen incorporation into bacterial

cells. Studies with other alkali besides NaOH were also performed. Guggolz et al. (1971) tested the improved digestibilities of various agricultural residues such as straw, sugarcane bagasse, pineapple leaf waste and cotton gin trash. Their results indicated that KOH is roughly equivalent to NaOH on a molar basis in improving the digestibility of rice straw. CaOH might be used to establish a desired cation balance and it is cheaper than NaOH but less effective to increase digestibility and has a lower solubility than NaOH.

Tarkow and Feist (1969) and Feist et al. (1970) studied the mechanism of ammonia on lignocellulosic materials. Ammonia exerts a strong swelling action on wood and cellulose by ammonlysis of esters of 4-O-methylglucuronic acid attached to the xylan chains. The fiber saturation point of hardwoods is doubled following treatment with 1% liquid ammonia. Wang et al. (1964) reported that the amide groups formed in the ammonia-treated wood were derived from the ester groups in the original 4-O-methylglucuronoxylan. Some of the uronic acid groups in the native wood presumably existed as ester crosslinks.

Millett et al. (1970) tested the in vitro digestibility changes of a number of woods after treating with both gaseous and liquid forms of ammonia. The results indicated that digestibility changes were species dependent with hardwoods generally more responsive to treatment than softwoods. The increased nitrogen content of the

ammoniated product was through the formation of amides and ammonium salts by reacting with the acetyl and uronic acid ester group of the wood. Waiss et al. (1972) treated rice straw with 5% NH_3 and 30% water at ambient temperature for 30 days. In vitro digestibility of product was about 62% and it contained about 1.3% nitrogen, an increase of non-protein-nitrogen of 133%. Production of toxic 4-methylimidazole observed in the ammoniation of molasses and other agricultural commodities was not detected at a test level of 10 ppm. Similar results were obtained by Han and Callihan (1974). After treatment of rice straw with 5.2% NH_3, its utilization by Cellulomonas and Alcaligenes sp. was increased from 29% to 57%. Itoh et al. (1974) treated rice straw and hull with 10% by weight of NH_3 and 30% by weight of water for 12 months at ambient temperatures. They reported a three-fold increase in crude protein content. The lignin and cellulose fractions were not affected by the treatment. In vitro dry matter digestibility of ammoniated rice straw was equivalent to that of the orchard grass hay.

Studies of ammonia on the prevention of spoilage molds in corn indicated that ammonia, in concentrations as low as 0.5% of the dry weight of the corn, is an excellent fungicide. Corn treated with 2% NH_3 at 26% moisture eliminated mold and yeast growth (Bothast et al., 1973). This appears to be a beneficial effect of straw treatment with ammonia when long periods of storage time are necessary.

Composting has also proven a useful method of disposing of agricultural wastes. This is a process in which organic matter is degraded to humus by microbial action. Until the 1920's, composting was practiced by individual farmers and gardeners as a means of disposing of agricultural wastes and restoring valuable humus and plant nutrients to the soil. During the 1920's, attention was focused on larger scale composting (Updegraff, 1972). Aerobic thermophilic decomposition of urban refuse which consists of about 50% cellulosic materials (paper, wood, vegetables, etc.) is found to be an ideal method of disposal of solid wastes without creating health hazards or water, land or air pollution. The process is based on the biodegradation of organic solids and liquids into a relatively stable end product which makes an excellent soil conditioner (Hortenstine and Rothwell, 1969; Wiley, 1967). Composting is one possible way of disposing of the large tonnages of straw. However, commercial inorganic fertilizer can be applied to the soil far more cheaply and results are almost as good as that obtained from compost. There is a small market in the United States for compost in specialty gardening at home and in nurseries, but only a limited market in large-scale commercial farming. Thus the utilization of straw through composting does not appear economical under current world marketing conditions.

Hydrolysis of cellulose by cellulolytic organisms depends on

the production of cellulose decomposing enzymes. Reese (1956) suggested the following steps during cellulose degradation. Native cellulose $\xrightarrow{C_1}$ Linear Cellulose $\xrightarrow{C_x}$ Cellobiose $\xrightarrow{\beta\text{-glucosidase}}$ Glucose. A true cellulolytic organism is one which is capable of attacking native cellulose and produces two or more cellulases. The first (C_1) attacks native anhydro-cellulose to break down the aggregates into linear chains of hydrated glucose polymers. These chains are then attacked by a second enzyme (C_x) which hydrolyses them to cellobiose. Cellobiose may then be assimilated directly into the cell, or may be converted to glucose by β-glucosidase before assimilation.

The potential importance of microorganisms as a source of protein for livestock feeding and in human nutrition has been frequently discussed (Mateles and Tannenbaum, 1968). Candida utilis (torula yeast) grows on sulphite liquor residue derived from the manufacture of paper. The sulphite waste liquor contains lignin and various sugars. Lekprayoon (1972) anf Frey (1973) studied the propagation of Candida utilis on ryegrass straw hydrolyzate. The hydrolysis step was done in an autoclave with 3% sulfuric acid for 30-45 minutes. The predominate sugars were xylose, glucose and mannose. Small amounts of galactose and arabinose were also recovered. A wide variety of other substrates have also been proposed, such as the use of sunlight and carbon dioxide for the growth of algae

like Spirulina or Chlorella (Clement, 1968, Lipinsky and Litchfield, 1970); the use of hydrocarbons for yeast production (Champagnat et al., 1963) or methane for bacteria (Wheeler, 1966); and the more traditional carbohydrates such as molasses for Candida utilis (Peppler, 1965), cellulose for bacteria like Cellumonas (Dunlap, 1975), or starch for filamentous fungi (Gray, 1970).

Using a symbiotic pair of microorganisms, Cellulomonas sp. and Alcaligenes sp., Louisiana State University developed a process to produce protein from cellulosic wastes. The substrate (sugarcane bagasse) was ground and treated with 2-4% NaOH before being subjected to microbial fermentation. Treated substrate was fortified with nitrogen and other minerals, and aerobic fermentation was carried out 3-7 days in a fermentor (Han et al., 1971). At the U. S. D. A. Western Regional Research Laboratory, this process was adapted for rice-straw fermentation (Han, 1975). In a typical fermentation, 75% of initial substrate (rice straw) was used, and a net protein yield of 20% (protein/substrate consumed) was obtained. The final product, microbial cells, contained about 50% protein and an amino acid profile equivalent to that of soybeans. The fermentation residue (undigested substrate), contained 12% protein and 40% crude fiber, which is also suitable as an animal feed. In order for the process to be economically feasible, the production cost should be competitive with that of soy protein (Han and Anderson, 1974).

Many efforts have been made to utilize cellulosic substrates by submerged microbial fermentation. However, these methods have disadvantages either because they are too expensive or because they do not yield products of acceptable food value and digestibilility, or both. On the other hand, in the traditional way of preparing food in the Orient, soy sauce, miso, sake, tempeh etc. are prepared by solid state fermentation. In the United States the solid state fermentation technique has been adapted for mycotoxin production. Hesseltine (1972) reported a more effective production of secondary metabolites (ochratoxin and aflatoxin) from Aspergillus and Penicillum species using rice, corn and wheat as substrates on solid state fermentations. He uses the term 'solid state fermentations' for any fermentation process in which the moisture level used was about 28%. The advantages of solid state fermentations were (a) it takes less space, less water and more concentrated substrate, (b) the growth condition is more like the natural habitat, (c) the yield is just as reproducible as conventional-type fermentations, and (d) the final product can be easily extracted. Lindenfelser and Ciegler (1975) utilized a similar concept in the production of ochratoxin A from wheat or rice with the highest yield reported of up to 30-31%. The fermentor they designed was a baffled, air supplied, rotating drum which maintained a homogeneity of the product throughout the fermentation period and also increased the contact between the substrate and the inoculum.

Bothast et al. (1975) cultivated a high pH resistant fungus (Scopulariopsis brevicaulis) on NH_3-treated corn. Results indicated that 28% of the total solids and 25% of the nitrogen in the ammoniated corn infusion broth (pH 10) were converted for mold mycelium. Hartley et al. (1974) reported the modification of beech and poplar sawdusts and barley straw by physical, chemical and fungal treatments. Fungal treatment with Fomes lividus, a white rot fungus which attacks both lignin and cellulose, followed by NaOH treatment was most successful. In Japan, solid-substrate fermentation is being conducted commercially in automated equipment for the production of fermented foods and enzymes which are used in the manufacture of soy foods (Terui, 1966).

Han and Anderson (1975) and Han et al. (1976) have developed a method to ferment grass straw to produce a feed that is sufficiently nutritious and palatable for ruminants. This process involved the hydrolysis of straw with 0.5 N H_2SO_4 (water:substrate ratio of 3:1) for 30 min at 121 C and then adjusted to pH 4.5 to 5.0 with 5 N NH_4OH. The pretreatment yields a material of up to 30% sugar and 2.3% nitrogen. The fermentation was carried on semisolid substrate (moisture level of 75%) for 2-3 days. The acid-hydrolyzed and fermented by Candida utilis straw has a final crude protein content of 12.4% and an in vitro rumen digestibility of 46.7%. A basic advantage of this process is that the adsorptive properties of the

straw provides a substrate in optimum condition for carrying out the fermentation. Aeration equipment such as the spargers and pumps required in submerged liquid fermentations is not needed. Also, various conditions of pH, temperature and the like need not be rigorously controlled. Foaming problems frequently encountered in the submerged liquid fermentations do not occur in this process. Another advantage is that the process yields a product which is useful in its entirety as an animal feed. Feeding trials will be initiated soon at Oregon State University to determine in vivo the value of the fermented straw as feed.

MATERIALS AND METHODS

Microorganisms

A species of Cellulomonas (NRRL B-37323) and Alcaligenes faecalis (NRRL B-3731) were used. The Cellulomonas species is a catalase positive, cellulolytic gram negative rod. The morphological and cultural characteristics have been reported by Han and Srinivasan (1968). The Alcaligenes sp. is a cellobiose utilizing organism.

Culture Media and Growth Conditions

The organisms were grown and maintained on nutrient broth containing 0.8% Bacto-nutrient broth and 0.5% Bacto-yeast extract in distilled water. When test for cellulolytic activity, the composition of the growth medium was as follows: $(NH_4)_2SO_4$ (6.0 g); KH_2PO_4 (1.0 g); K_2HPO_4 (1.0 g); $MgSO_4$ (0.1 g); $CaCl_2$ (0.1 g); yeast extract (0.5 g); $FeCl_3 \cdot 6H_2O$ (16.7 mg); $ZnSO_4 \cdot 7H_2O$ (0.18 mg); $CuSO_4 \cdot 5H_2O$ (0.16 mg); $CoCl_2$ (0.18 mg); EDTA (20.1 mg); and 10-50 g of cellulosic substrate per liter of distilled water.

Substrate

Annual ryegrass (Lolium multiform Lam) straw grown in the Willamette Valley, sun-dried and ground to pass a 20-mesh screen,

was used as the substrate. The straw was supplied by Straw Utilization Center, Corvallis, Oregon.

Digestibility Test

In vitro rumen digestibility (IVRD) of fermented straw was determined by the modified method of Mellenberger et al. (1970). The method was as follows: The straw as first dried overnight at 105 C and 0.5 g was placed in a 50 ml screw-capped tube (Pyrex 9826 culture tube with screw cap 25 x 200 mm). Rumen fluid was obtained from a fistulated Holstein steer and mixed with mineral-and-buffer mixture as described by McDougall (1948) at a ratio of 1:1. The tubes were sealed tight and incubated 2-3 days at 39 C with occasional shaking. The content of each tube was then filtered through sintered-glass crucible (Pyrex C porosity) and dried overnight at 105 C. The weight loss was reported as the percent IVRD.

Substrate Pretreatment

Straw was treated with 4% NaOH (w/w) with substrate to liquid ratio of 1:2 and neutralized with HCl, H_2SO_4, HNO_3, H_3PO_4, CH_3COOH, and HCOOH. Using HNO_3 to neutralize, the moisture content of the straw was adjusted from 11 to 85.8% by adding 10, 30, 50, 70, 90, 120, and 170 g of water to 30 g dried straw. Using a moisture level of 63%, one percent of nitrogen calculated in terms

of urea, ammonium sulfate, ammonium phosphate, ammonium nitrate, ammonium bicarbonate, ammonium bisulfate, ammonium citrate, ammonium chloride, ammonium persulfate, and ammonium acetate, each of which was dissolved in 30 ml distilled water before adding to the straw, was added as a nitrogen source.

Protein Determination

Total-N was determined by the micro-Kjeldahl method of Perrin (1953) and NH_3-N according to the method described by Jackson (1960). Crude protein was calculated by multiplying 6. 25 times the total-N minus the NH_3-N.

Moisture Content

Moisture was determined by drying to a constant weight at 105 C. The weight loss is calculated as the percent of water content in the original sample.

pH Measurement

The pH of the semisolid samples was measured on a sample blended (1:10) in distilled water.

Microbial Counts

Microbial counts were made from 1 g sample blended for 30

seconds in 99 ml sterile water and serially diluted and plated on nutrient broth agar.

Fiber Analysis

Cellulose, hemicellulose, lignin, and ash were determined by the method of Goering and Van Soest (1970). Unless otherwise specified, all other chemical analyses were carried out by the methods of the Association of Official Agricultural Chemists (1970).

Extraction of Volatile Fatty Acids

The extraction procedure employed here was for the short chain (C_2-C_5) and water soluble fatty acids. Ten grams of wet fermented straw was mixed with 20 ml of distilled water in a stopped glass bottle and stored at 4 C for six days. The slurry was centrifuged at 5000 rpm for five minutes. Concentrated HCl (0.2 ml) was then added per 10 ml of supernatant to give a pH of approximately 2. The precipitate formed was removed by recentrifugation at 5000 rpm for five minutes. One microliter of sample was then injected onto a column for gas chromatographic analysis (Rogosa and Love, 1968).

Preparation of Column

Column packing material was obtained from Supelco, Inc., Bellefonte, Pa. The liquid phase was 10% SP-1200/1% H_3PO_4 on a

solid support of acid washed Chromosorb W, 80/100 mesh. About 9 ml of this powder was packed, with vibration into a 6 ft. x 1/8 in. o. d. stainless steel column. Both ends of the column were plugged with silanized glass wool and the column was conditioned overnight at 200 C by purging with helium (40 ml/min). Before using the column, several microliters of water were injected to clear it of extraneous material. This is seen as a peak shortly after the injection. The water conditioning step was carried out at 150 C.

Gas-liquid Chromatography

Volatile fatty acid analysis of fermented and unfermented substrates were made with a F and M Scientific Corporation Gas Chromatograph, Model 402 (F and M Scientific Corporation, Avondale, Pa.), equipped with dual columns hydrogen flame ionization detection systems and a Honeywell Electronik 16 chart recorder (Honeywell, Philadelphia, Pa.). Chromatographic peak area measurements were made with an electronic integrator, model 3370 A, manufactured by Hewlett-Packard, Avondale, Pennsylvania. The column was operated isothermally at 125 C with an injection port and detector temperature of 150 C. The gas flow rates were as follows: helium 30 ml/min., hydrogen 25 ml/min., and air 150 ml/min. (Ottenstein and Bartley, 1971).

Fermentation

Fermentations were carried out at ambient temperature in Mason jars half filled with 30 grams of straw and held in a horizontal position on a rotating wooden plate (Figure 1). This provided a continuous tumbling motion to the substrate and kept the fermentation mixture well aerated. Ten percent inoculum of actively growing Cellulomonas sp. and Alcaligenes faecalis was used. Samples were withdrawn periodically for microbial plate count and other chemical analyses.

RESULTS AND DISCUSSION

Unfermented ryegrass straw contained cellulose, hemicellulose, lignin, crude protein, fat and ash at the level of 35.1, 21.7, 5.9, 3.1, 0.4 and 1.1%, respectively (Table 1). Moisture content of the straw was 7%. When a mixed culture of Cellulomonas sp. and Alcaligenes faecalis were grown on straw substrate, the crude protein content increased to 6.8% for the NaOH treated straw, and to 9.5% for the NH_3 treated straw. The crude fat content of both samples doubled, whereas the hemicellulose content decreased slightly. The levels of cellulose, lignin and ash showed a moderate gain. These results are consistent with the hypothesis that microorganisms preferentially utilize the hemicellulose portion of straw for synthesis of cellular materials.

It is quite likely that crude protein measurements on NH_3-treated straw overestimate the true protein content. NH_3 may bind to the straw forming such compounds as ammonium acetate, pyrazine and imidazole (Tarkow and Feist, 1969), thereby introducing a sizeable error in calculated levels of nitrogen incorporated into crude protein.

The digestibility of untreated straw was 32.7% (IVRD), whereas the digestibility of fermented straw pretreated with either NaOH or NH_3 increased to 55.9% and 51.5% respectively. NH_3 treatment is

Table 1. Chemical Composition and Digestibility of Straw Fermented with Cellulomonas sp. and Alcaligenes faecalis.

Straw component (% dry matter)	Ryegrass straw	Fermented ryegrass straw	
	untreated control	4% NaOH treated	5% NH_3 treated
Cellulose	35. 1	41. 2	48. 0
Hemicellulose	21. 7	18. 8	16. 7
Lignin	5. 9	8. 3	9. 3
Crude protein	3. 1	6. 8	9. 5
Crude fat	0. 4	0. 8	0. 8
Ash	1. 1	1. 8	2. 7
IVRD	32. 7±5. 1	55. 9±0. 5	51. 5±2. 6

especially beneficial in not only increasing the digestibility of the straw, but also provides nitrogen to the straw during alkaline pretreatment prior to fermentation. Consequently, NH_3-treated straw requires no further nitrogen supplementation prior to fermentation.

Figure 1 shows the fermentation rotating wheel set up. The wheel is rotated at 2 rpm to provide a constant and slow tumbling motion to the substrate. As many as 12 samples could be accommodated at one time. Samples could be taken out for microbial and chemical analyses readily.

Figure 2 shows the effect of moisture level on the growth of microorganisms. The microorganisms grew well when the moisture content of the substrate was 63% or above; whereas the organisms did not grow when the moisture content was less than 50%. Therefore, for semisolid fermentation, the straw should contain at least 63% moisture. More water is not only unnecessary, but it would also add to the cost of the process due to drying. The number of organisms increased about 1000 times during the first 26 hours of fermentation. After that period no significant increase was noted. A differential count indicated that the predominate microorganism in the fermented straw was Cellulomonas. Little loss of moisture was found during the course of fermentation.

The length of NaOH and NH_3 treatment time varied before reaching the optimum level of digestibility. NH_3 treatment takes

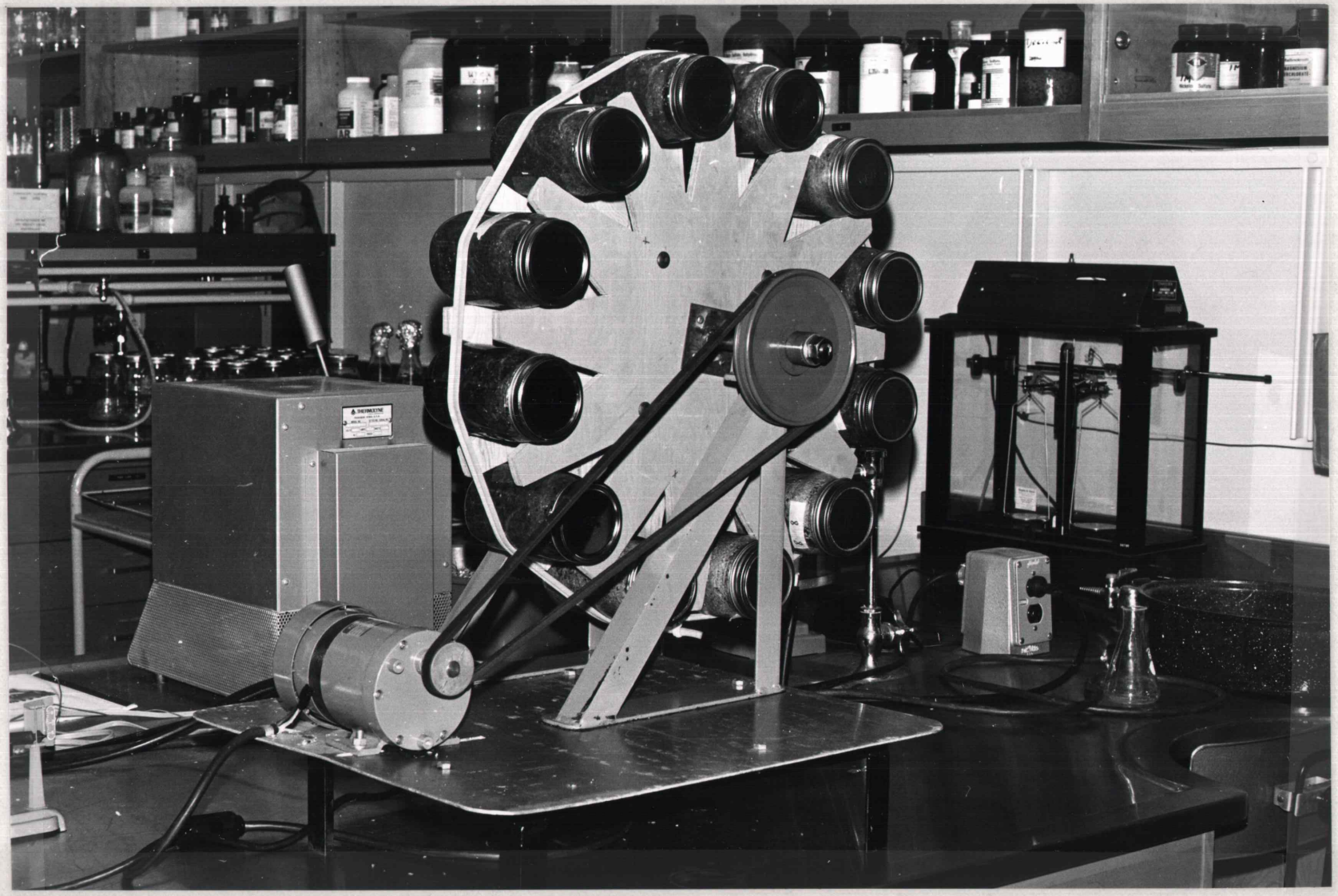

Figure 1. Semisolid Fermentation Set Up

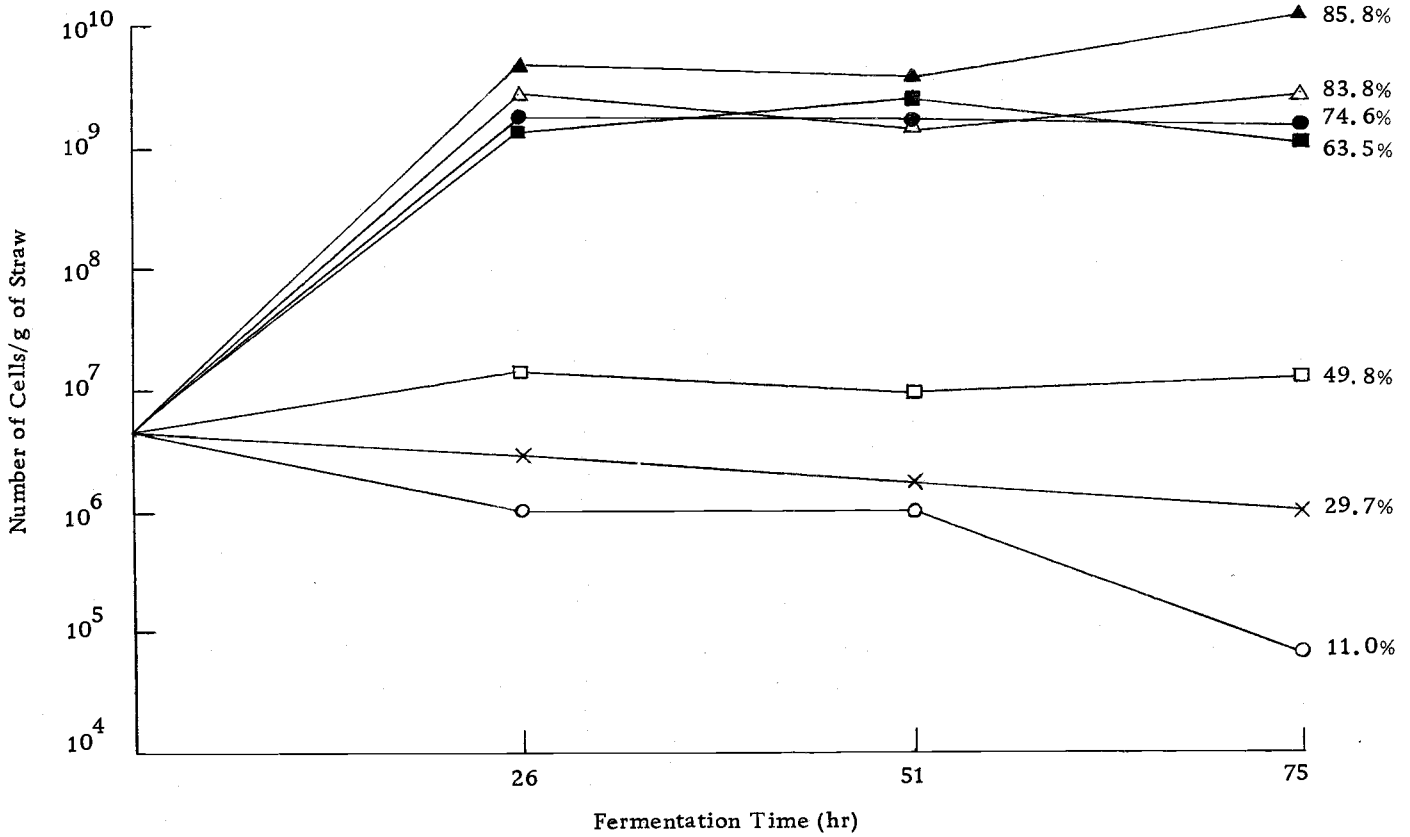

Figure 2. Growth of a Mixed Culture of Cellulomonas sp. and Alcaligenes faecalis on NaOH-Treated Straw at Different Moisture Levels

four weeks while NaOH treatment takes only two hours at ambient temperatures. Table 2 shows the effect of NaOH concentration on the digestibility (IVRD) of unfermented straw. Straw treated for 1 hr, 2 hr, and 24 hr showed no significant differences ($P < .05$) in IVRD at constant NaOH concentrations, but a sizeable increase in IVRD was seen upon increasing the concentrations of NaOH from 2% to 4 %. Samples treated at 4%, 6% and 8% showed no significant differences ($P < .05$) in IVRD. Straw treated for 2 hr at 4% NaOH showed an IVRD increase of 113% over the control (plain, untreated straw). This level of NaOH treatment and time was used in later experiments.

Table 3 shows the characteristics of straw fermented with different levels of nitrogen. When no nitrogen was added the protein content of the fermented straw was 3.9%, suggesting little or no increase in protein above that of untreated straw. When 3.7% $(NH_4)_2SO_4$ was added, the crude protein content of straw increased to 6.6%. The IVRD of the straw was also affected by the addition of nitrogen. Without adding nitrogen the digestibility of the fermented straw was 40.6%, whereas the addition of 3.7% $(NH_4)_2SO_4$ increased the digestibility to 53.2%. Further additions of nitrogen did not increase the crude protein content nor the digestibility. Therefore, increase in crude protein and digestibility of the fermented straw is mainly due to the microbial action, and not to the mere addition of inorganic nitrogen.

Table 2. Effect of NaOH Concentration and Treatment Time on IRVD.

NaOH level (w/w)	Treatment time		
	1 hour	2 hours	24 hours
2%	51.3±0.2[b]	52.7±2.2[b]	51.8±0.4[b]
4%	66.6±3.3[c]	69.1±0.8[c]	64.0±2.7[c]
6%	72.7±2.3[c]	73.2±2.1[c]	76.5±1.1[d]
8%	76.0±0.4[c]	76.3±0.6[c]	77.7±1.7[d]
control[a]	32.3±0.8[e]		

[a]plain, untreated straw

b, c, d, e Means with standard deviations. Means at vertical columns not bearing a common superscript letter are different ($P < .05$)

Table 3. Characteristics of Straw Fermented with Cellulomonas sp. and Alcaligenes faecalis at Different Levels of Nitrogen.

Sample no.	$(NH_4)_2SO_4$ added (%)	Crude protein (%)	IVRD (%)
1	0	3.9	40.6±0.9
2	3.7	6.6	53.2±0.6[a, b]
3	7.9	6.8	52.9±1.0[a, b]
4	12.2	7.1	53.1±0.9[a, b]
5	16.4	6.6	52.6±0.4[a, b]
6	21.1	6.0	55.1±1.1[a, c]

[a]pretreated with 4% NaOH

[b, c]Means with standard deviations. Means not bearing a common superscript letter are different ($P < .10$)

Different acids were used for neutralization in order to determine their effect on microbial growth of NaOH treated straw. The following acids made up to 1.0 N were used: HCl, H_2SO_4, HNO_3, H_3PO_4, CH_3COOH and HCOOH. The characteristics of the fermented straw are shown in Table 4. Different salts formed upon neutralization might either inhibit or promote microbial protein production. Crude protein varied from 4.0% (H_3PO_4 neutralized) to 7.0% (HNO_3 neutralized). All samples increased in pH from 6.8 to 8.5-8.7 with the exception of that neutralized with H_3PO_4, which attained a pH of 6.5-7.0. This is probably due to the buffering action of the phosphate. Ammonium sulphate (4.8% added) was the only nitrogen source. There was no significant difference ($P < .05$) in IVRD among the fermented samples.

Table 5 shows the final crude protein content and IVRD of straw fermented with different nitrogen sources. The nitrogen sources used included an organic nitrogen (urea) and nine inorganic ammonium compounds (ammonium acetate, bicarbonate, bisulfate, chloride, citrate, nitrate, persulfate, phosphate, and sulfate). All samples were pretreated with 4% NaOH and adjusted to pH 6.7 with 1.5 N HCl. One percent of nitrogen was dissolved in 30 ml of distilled water being added to the sample. Crude protein in fermented samples ranged from 3.8% with ammonium persulfate to 7.1% with ammonium nitrate. There was a corresponding decrease in IVRD of

Table 4. Fermentation Characteristics of NaOH-treated Straw[a] Neutralized with Different Acids.

Sample no.	Acid used	Crude protein (%)	IVRD (%)
Control[b]		4.2	41.7±0.7[c]
1	HCl	5.6	58.2±1.4[d]
2	H_2SO_4	5.6	57.5±2.2[d]
3	HNO_3	7.0	59.1±1.0[d]
4	H_3PO_4	4.0	56.6±0.4[d]
5	CH_3COOH	6.0	61.9±2.9[d]
6	HCOOH	5.9	61.3±1.4[d]

[a] all samples except control were treated with 4% NaOH and 4.8% of $(NH_4)_2SO_4$ was added as nitrogen source.

[b] untreated straw but fermented

[c, d] Means with standard deviations. Means not bearing a common superscript letter are different ($P < .05$)

Table 5. Characteristics of Straw Fermented[a] with Different Nitrogen Sources.

Sample no.	Nitrogen source[b]	Crude protein (%)	IVRD (%)
Control[c]		3.1	32.7±5.1
1	urea	4.7	61.8±1.1
2	ammonium sulfate	6.2	58.7±2.1
3	ammonium phosphate	5.9	57.2±0.8
4	ammonium nitrate	7.1	63.0±0.2
5	ammonium bicarbonate	5.3	62.5±0.9
6	ammonium acetate	5.9	58.6±1.6
7	ammonium bisulfate	4.5	44.8±0.4
8	ammonium citrate	5.7	59.0±1.4
9	ammonium chloride	4.4	53.8±2.0
10	ammonium persulfate	3.8	38.9±0.3

[a] pretreated with 4% NaOH

[b] 1% N calculated of each nitrogen source was added

[c] plain, untreated straw

those samples which showed a lower protein content except in the case of urea. A lack of adequate urease to break down urea for microbial protein synthesis may account for the lower protein content in urea treated straw. The presence of high protein concentrations of the following cations: HSO_4^-, Cl^-, and $S_2O_8^=$ could be an inhibitory to microbial growth and may account for low protein yields in samples containing these ions.

Table 6 shows the fiber content following an eight week treatment at different concentrations of NH_3 and H_2O. The samples were analyzed for cell soluble matter (CSM), hemicellulose, cellulose, lignin and ash. There was a decrease in the level of hemicellulose as the concentration of NH_3 increased and an apparent increase of cellulose, lignin, CSM and ash content. Itoh et al. (1975) reported a similar decrease of hemicellulose with ammonia treated rice straw and hulls.

The presence of water may increase the swelling capacity of the straw and consequently improve the penetrating ability of NH_3 into the straw fibers. The results of Table 6 indicate that an increase in H_2O content further increases the cell soluble matter (CSM). These increases might possibly be due to the better dispersion and reaction of NH_3 with the straw at higher moisture levels. Furthermore, the amount of water present could affect the dissociation of ions.

IVRD of straw subjected to different concentrations of NH_3

Table 6. Fiber Analysis Following Eight Weeks in NH_3 Treated Sample.

	Treatment		Composition (% of Total)				
	% NH_3	% H_2O	CSM	Hemicellulose	Cellulose	Lignin	Ash
Control[a]	--	--	27.2	24.9	38.8	7.1	1.8
Group 1	2.1	15.1	24.1	24.8	41.9	7.2	2.3
	4.3	20.4	27.5	18.3	44.5	7.6	1.8
	6.5	25.0	30.9	13.1	45.6	7.7	2.0
	8.7	29.2	32.2	13.9	44.5	7.0	2.1
	10.9	33.0	36.8	11.4	42.9	6.6	2.1
Group 2	2.1	27.4	19.1	25.7	44.8	8.0	2.2
	4.3	31.3	30.7	15.2	43.9	7.9	2.1
	6.5	34.8	38.3	9.3	43.1	8.4	2.1
	8.7	38.0	37.8	9.1	42.6	8.5	2.1
	10.9	40.9	38.1	8.6	43.7	7.6	2.0
Group 3	2.1	36.6	26.6	19.3	43.4	7.6	2.4
	4.3	39.6	29.8	13.6	46.8	7.4	2.2
	6.5	42.3	32.9	9.6	46.4	8.3	2.4
	8.7	44.8	38.2	9.6	42.8	7.2	2.2
	10.9	47.1	38.8	7.1	43.5	8.1	2.1

[a]plain, untreated straw

(2-8%) and varying periods of hydrolysis (2-8 weeks) is shown in Table 7. There was no significant increase ($P < .05$) in IVRD for straws treated at a constant level of NH_3 after two weeks treatment. The IVRD of straw pretreated with 2% NH_3 remained constant beyond two weeks of hydrolysis. At 3-8% NH_3 treatment levels, there were significant increases ($P < .05$) in digestibility when comparing between two and four weeks of hydrolysis. After the fourth week of hydrolysis all samples exhibited identical IVRD values ($P < .05$).

Table 8 shows the nitrogen content in NH_3-treated straw fermented before and after fermentation with Cellulomonas sp. and Alcaligenes faecalis. In untreated straw the crude protein was 3.6%. In NH_3 pretreated straw crude protein was increased by about two-fold over the control. The difference between untreated and NH_3 treated in crude protein analyses is due to the organically bound non-protein nitrogen. Microbial fermentation of the NH_3 treated straw only increased slightly the protein content of straw. The accessibility of the bounded nonprotein nitrogen to the microorganisms and any presence of toxic ammoniacal products might inhibit the growth of the cells. The lack of adequate nutrients also could result in low microbial protein production.

An analysis for volatile fatty acid content of NaOH-treated straw fermented with Cellulomonas sp. and Alcaligenes faecalis is shown in Table 9. Acetic acid was the major volatile acid released and it

Table 7. Treatment Duration and the Effect of the Level of NH_3 on the *in vitro* Rumen Digestibility.

NH_3 level	Treatment duration			
	2 weeks	4 weeks	6 weeks	8 weeks
Control[a]	26.7±1.4[b]			
2%	39.9±3.5[c]	39.3±0.7[c]	36.3±0.9[c]	37.5±1.0[c]
3%	36.6±0.8[c]	43.0±2.4[d]	40.9±0.1[d]	41.5±1.3[d]
4%	37.6±0.3[c]	42.4±2.8[d]	41.2±2.0[d]	40.8±0.5[d]
5%	35.8±0.6[c]	41.7±0.6[d]	40.5±0.5[d]	41.3±1.3[d]
6%	38.7±3.4[c]	41.8±0.4[d]	41.5±1.5[d]	41.7±0.5[d]
8%	37.9±1.0[c]	42.7±1.4[d]	41.6±0.9[d]	42.5±1.5[d]

[a]plain, untreated straw

[b, c, d]Means with standard deviations. Means not bearing a common superscript letter are different ($P < .05$)

Table 8. Nitrogen Content of NH_3 Treated Straw Before and After Fermentation with Cellulomonas sp. and Alcaligenes faecalis.

NH_3[a] treatment level	Before fermentation % Nitrogen			Crude protein[c]	After fermentation % Nitrogen			Crude protein
	Total	NH_3	Organic[b]	(%)	Total	NH_3	Organic	(%)
Control[d]	0.63	0.05	0.58	3.6	0.69	0.01	0.68	4.2
2%	1.62	0.61	1.01	6.3	1.57	0.49	1.08	6.8
3%	2.04	0.82	1.22	7.6	1.90	2.68	1.22	7.6
4%	2.12	0.85	1.27	7.9	2.25	0.96	1.29	8.0
5%	2.18	0.91	1.27	7.9	2.27	0.82	1.45	9.0
6%	2.12	0.89	1.23	7.7	2.27	0.80	1.47	9.2
8%	2.15	0.91	1.24	7.7	2.31	0.79	1.52	9.5
10%	2.15	0.87	1.28	8.0	2.30	0.68	1.62	10.1

[a] two week treatment duration

[b] total N - NH_3-N

[c] organic N x 6.25

[d] plain, untreated straw

Table 9. Volatile Fatty Acid Analysis of NaOH Treated and Fermented with Cellulomonas sp. and Alcaligenes faecalis.

	VFA (mg/g dry sample)					
	Acetic acid C_2	propionic acid C_3	iso-butyric acid i-C_4	N-butyric acid N-C_4	iso-valeric acid i-C_5	N-valeric acid N-C_5
Control I[a]	2.75	--	0.48	--	--	--
Control II[b]	19.60	0.91	--	--	--	--
HCl neutralized	1.76	--	--	--	--	--
H_2SO_4 neutralized	1.98	--	--	--	--	--
HNO_3 neutralized	1.09	--	--	--	--	--
H_3PO_4 neutralized	2.63	--	--	--	--	--
CH_3COOH neutralized	1.96	--	0.12	--	--	--
HCOOH neutralized	4.89	0.36	0.16	--	--	0.17

[a] plain, untreated, unfermented straw

[b] 4% NaOH treated, unfermented straw

appeared to be used during fermentation by the organisms as a carbon and energy source. Only small amounts of propionic acid, iso-butyric acid and n-valeric acid were detected in the fermented samples.

Figure 3 shows a schematic diagram of a proposed semisolid fermentation process for ryegrass straw. The straw is first chopped to 1/4-1 inch length using a hammer mill or knife grinder and conveyed to the NaOH treatment tank to give an alkaline treatment level of 4% by weight of NaOH. The alkaline treated straw is then adjusted to pH 6.5-7.0. The straw is tumbled by a paddle mixer during inorganic nitrogen addition, then inoculated with Cellulomonas sp. and Alcaligenes faecalis. The fermentor provides a constant tumbling motion to the straw and permits free exchange of air. For continuous fermentation, the chamber should be large enough to provide at least 36 hr of residence time for passage of the substrate through the chamber by conveyer. For batch processes, the chamber size is not critical. It is important however, to tumble the substrate slowly (.25-2 rpm) during batch processing to insure proper aeration and mixing of the fermenting substrate. At the end of fermentation, the fermented product is dried with hot air. Owing to the low pH, limited level of nutrients, and the large inoculum size, the process can be operated without strict adherence to aseptic technique.

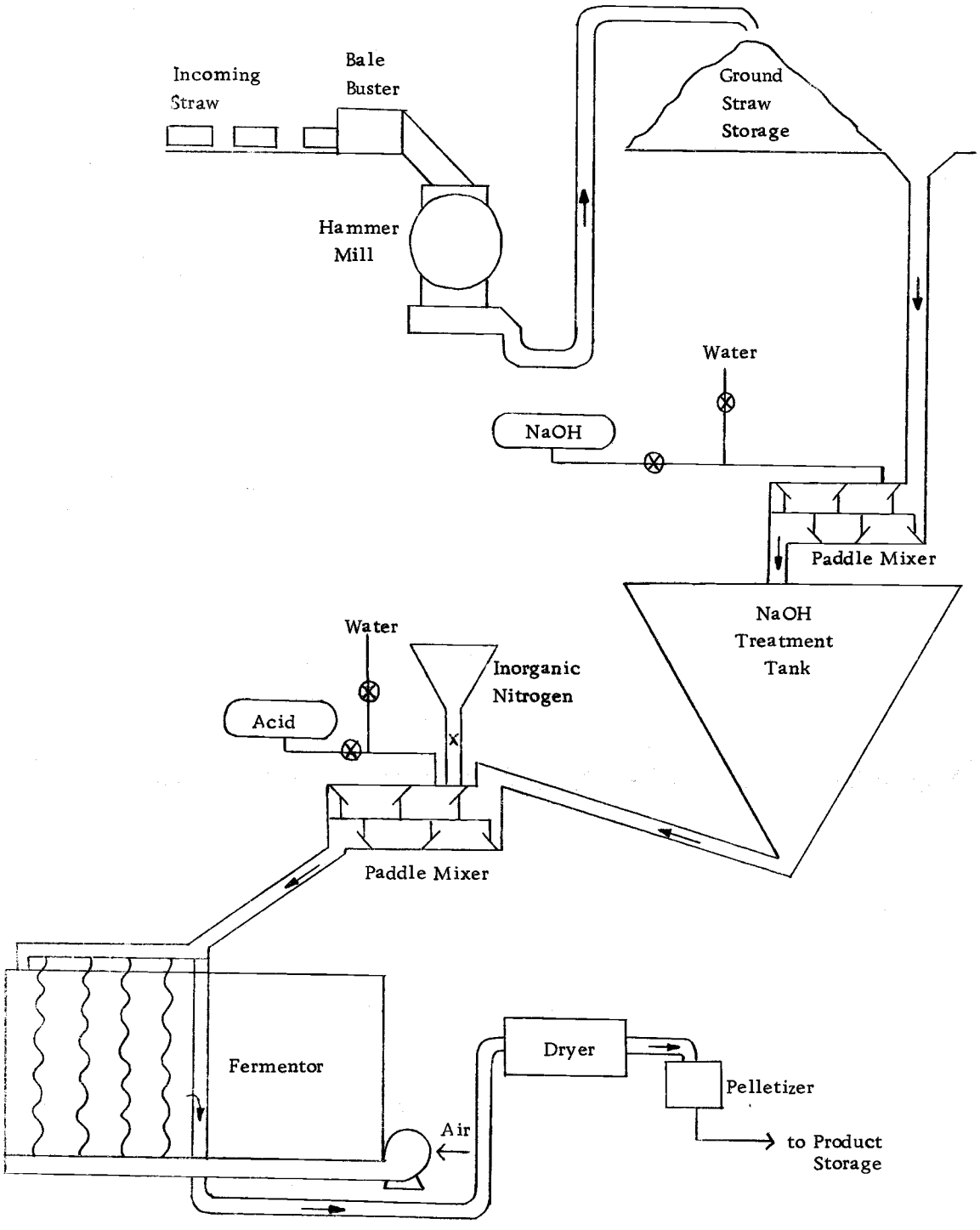

Figure 3. Process Flow Diagram for the NaOH Treatment, Cellulolytic Organism Process

SUMMARY

Annual ryegrass straw was treated with 4% NaOH or 5% NH_3, and fermented with a mixed culture of Cellulomonas sp. and Alcaligenes faecalis. Fermentation was carried out on a semi-solid substrate having a moisture content of 11-85.8%. NaOH treated and fermented straw showed an increase of more than two-fold in crude protein, two-fold in crude fat, and a 70% increase in in vitro rumen digestibility. NH_3 treated and fermented straw showed a three-fold increase in crude protein, two-fold increase in crude fat, and 60% increase in in vitro rumen digestibility. NH_3 treatment alone increased the organic nitrogen content of straw by 120%. Acetic acid was the main volatile fatty acid in the fermented straw. A minimum of 63% moisture was necessary for proper fermentation of straw.

Semisolid fermentation of NaOH or NH_3 treated straw is a promising way of producing nutritious animal feed from waste straw. The low cost and simplicity of the semisolid fermentation process makes it an improvement over other processes using submerged fermentation of cellulosic wastes. However, much of the methodology of semisolid fermentation must still be developed. A better understanding of the kinetics of this process is lacking and would greatly advance this area of study.

A horizontal fermentor design seems to be more appropriate than the traditional vertical type fermentor. Sampling, mixing,

aeration, and pH control could also pose various problems in the new fermentor. Protein determination, IVRD assay and fiber analysis are methods still not yet refined and the experimental errors may be large.

The interaction of microbes with substrate at low moisture levels is also not well understood. Although it is clear that the hemicellulose fraction breaks down into smaller units upon chemical hydrolysis, little is known regarding the decomposition of cellulose and lignocellulose. The latter components account for greater than 50% of the material. Modification of the substrate by chemical means prior to microbial fermentation may not be the most ideal procedure if a large fraction of the substrate is converted to a form which can no longer be degraded by the microorganisms.

The nutritional quality, toxicity and acceptability of the final product by the animal remains to be tested. In addition, the economic feasibility of this process must be determined in relation to other products and methods.

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