PROTEIN GENERATION AND DELIGNIFICATION OF ALDER SAWDUST BY THERMOPHILIC MICROORGANISMS

by

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ABSTRACT

It has been indicated through a review of the literature that wood by-products have a potential as a dietary source of energy for the ruminant animal. However, lignin constitutes a barrier to the proper utilization of cellulose. Generally, any treatment to remove or alter lignin makes the cellulose within lignocellulose materials more susceptible to the activity of the cellulolytic enzymes. Therefore, an efficient biological treatment would require a system to solubilize or to remove lignin from the lignin-carbohydrate complex. Cellulose within ruminant feeds forms an effective substrate for eventual conversion to body protein.

There are several methods available for delignification. This study was carried out using the thermophilic aerobic oxidation method for which swine manure was used both as the source inocula and initial culture media. In the process of degradation, a part of the energy produced was utilized by the bacteria for cell function and multiplication. The remainder of the available energy was released as heat energy. In this method the heat necessary to maintain the temperature in the thermophilic range was derived from both mechanical and from microbial activity. Thermophilic activity is considered to reduce the time required for organic waste digestion over that experienced by mesophilic digestion. The rate of the destruction of pathogenic bacteria, virus and other organisms is increased as a result of the high temperatures fermentation.

Batch studies conducted to delignify alder sawdust by the use of the aerobic thermophilic oxidation method demonstrated that the lignin content of sawdust can be reduced by as much as 74%, and crude bacterial protein was generated by approximately 17%.

Constant supply of small amounts of swine manure ensures high temperature maintenance. Periodical addition of 2 kg sawdust within thermophilic temperature range results in better delignification.

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I. INTRODUCTION

Lignin present in natural fibers provides both a physical and a chemical barrier to enzymes that are able to attack isolated fibers of cellulose. Physically, penetration by enzyme molecules is suppressed; chemically, lignin-carbohydrate complexes form metabolic blocks that inhibit polysaccharide hydrolysis (21). Therefore, an efficient biological treatment may require a system to solubilize or remove lignin thus dissociating the lignin-carbohydrate complex. In general, any treatment to depolymerize and solubilize lignin makes the cellulose in lignocellulosics more susceptible to the activity by cellulolytic enzymes.

The results presented by Kitts et al. (55) and several others have indicated that wood and wood by-products are potential sources of dietary energy for the ruminant animal. However, change in the chemical composition of plants owing to maturity and species as well as the presence of natural inhibitors to cellulases a preliminary physical, chemical and biological method to alter the structure of plant materials appears to be necessary before cellulolytic enzymes can be effective.

Cellulose is a major constituent of all vegetation accounting for from one-fourth to one-half of the plant's dry material. Cellulose also constitutes the world's most plentiful renewable energy resource. Use of cellulose as ruminant feed affords an effective means by which the production of high quality protein can result (97). Incorporation of cellulosic material as the energy source in ruminant rations will permit a greater use of cereal grains for man and non-ruminant animals.

The thermophilic aerobic oxidation is a procedure leading to the degradation, stabilization, pasteurization and deodorization of waste arising from many sources such as animal and plant production. The bacteria that occur naturally in agricultural wastes (eg. swine manure) under aerobic conditions will oxidize the usable substrate converting it to carbon dioxide and water. In order for bacteria to grow and reproduce, a source of nutrients is required. Such nutrients, present in the waste, include nitrogen, phosphorus, sulfur and minor trace elements all of which are essential for bacterial cell production. Bacterial degradation of waste releases a part of the energy for cell function and the remainder of the energy in the form of heat energy, producing an increase in substrate temperature. This increase in temperature has the effect of accelerating the oxidation process by increasing the rate of biochemical reaction (29).

Not only does the thermophilic aerobic process greatly reduce the time over that experienced by mesophilic digestion, but it also leads to the destruction of pathogenic bacteria, viruses, and other organisms as a result of the attainment of high temperature (24).

The study reported here was designed to investigate possible means of reducing the cost of animal feed by generating the cheapest source of energy and possibly crude protein. The object of this project was three-fold:

1. to examine the apparent capacity of aerobic thermophilic bacteria to delignify alder sawdust (wood).
2. to subject fermentation samples collected at different periods of fermentation and levels of temperatures to *in vitro* assays designed to evaluate the digestibility of residual carbohydrates, and
3. to study the potentiality of this biological method in single cell protein production.

II. LITERATURE REVIEW

1. Basic Biological Processes

A microbiological process may be defined as a sequence of biochemical events under the control and direction of microscopically visible living cells, in particular bacteria, fungi, and algae. The important culture parameters to consider in engineering design in order to maximize the desired biochemical events are oxygen requirements, and whether or not the organism is to be held in suspension within medium or fixed to a surface of supporting structure (113).

The term aerobic processes refers to a condition in which dissolved oxygen is present within the medium. The oxidation of organic matter using molecular oxygen as the ultimate electron acceptor serves as the primary pathway yielding useful energy to the microorganisms. Microbes that use oxygen as the ultimate electron acceptor are termed "aerobic micro-organisms" (67).

The term suspended growth refers to a culture condition in which the organism is suspended within the medium. The microorganisms are under some culture conditions able to aggregate into masses containing a large number of cells. Agitation of the liquid medium keeps the microbial "masses" in suspension thereby maintaining a continuous contact with the substrate and removal of waste products (110).

A) Biochemical Reactions

Efficient utilization of the substrate for microorganisms involves a maximum conversion of energy and carbon into an increase in cell numbers. Thus the organism is concerned primarily in cell duplication (110). The organisms also can use previously accumulated internal or endogenous food supplies for respiration purposes and do so for a limited time in the absence of external or exogenous food sources. Synthesis and endogenous respiration occur simultaneously in biological systems with "synthesis" predominating when there is an excess of exogenous food and endogenous respiration dominating when the exogenous food supply is small or nonexistent (67). The general reactions that occur as illustrated by Loehr (67 are as follows:

Energy containing metabolizable substrate + microorganisms
$\longrightarrow$ end products + additional microorganisms.

In explaining the equation, Loehr (67) stated that it represents reactions in which substrates such as biodegradable wastes are metabolized for energy and for the synthesis of new cells. The energy utilized in the equation is obtained during the metabolism of the wastes. Synthesis or growth is affected by the ability of the microorganisms to metabolize and assimilate the food. In addition, the presence of toxic materials, the temperature, the availability of adequate dissolved oxygen (DO), the pH of the system, and also the presence of adequate accessory nutrients and trace elements all determine or limit the extent of microbial activity.

Whatever the nature of the substrate, it must contain a sufficient amount of the major elements such as carbon, nitrogen, phosphorus and minerals to meet the nutritional requirements of the microorganism. Most of the research investigations dealing with substrates such as natural occurring organic wastes indicate that the contained nutrients are available to biodegradation, however, the biological reactions may be constrained by environmental factors such as temperature, pH, dissolved oxygen and inhibitory compounds.

In respect to batch systems, Loehr (67) mentioned that in the biological waste treatment system in which the culture medium and the products of microbial metabolism are contained within an enclosed vessel, carbon may become a limiting factor as the carbonaceous material is metabolized and lost from the systems as carbon dioxide.

When growth becomes limited, or increases in cell mass many of the microorganisms may die and lyse thereby releasing the nutrients of their protoplasm which may be utilized by the remaining or scavenging cells.

Further in his explanation pertaining to biochemical reactions Loehr (67) stated that in the presence of waste material and congenial living conditions, microbial metabolism will occur allowing new cells to be formed; energy and the microbial solids will increase. In the absence of food, endogenous respiration will predominate and a reduction of the net microbial solids will result. The depleted cell residue will not be reduced to a zero net weight even with a long endogenous respiration period. Supporting this statement, Kountz, et al. (61) and Washington, et al. (119) reported that a residue of about 20 to 25% of synthesized microbial mass will remain.

When organic matter is metabolized and resynthesized into microbial cells, the converted waste also being organic matter is partially stabilized. As indicated earlier, the microbial cells are capable of further degradation. Only when the biochemical oxygen demand of the waste degradation has stopped does a stabilized effluent result (67).

B) The Energy Relationships in the System

All cells, whether animal or plant, use similar fundamental mechanisms for their energy transforming activities (108).

Loehr (67) indicated that knowledge of the energy relationships of microbial cells permits an understanding of energy available for synthesis and respiration, of production of microbial cells in biological waste treatment units. This also includes an explanation of the nature of the expected end products occurring under certain conditions.

The energy of the food material utilized by the living cell is in part conserved chemically in the compound known as adenosine triphosphate (ATP). ATP is the carrier of chemical energy from the oxidation of foods to those processes of the cells which do not occur spontaneously and can proceed only if chemical energy is supplied. These processes are involved in the performance of osmotic, mechanical or chemical work (67). In the context of this study, the food for the cells could be from animal wastes (swine) and sawdust (alder).

Adenosine triphosphate is formed from adenosine diphosphate (ADP) during oxidation in the cells. Adenosine triphosphate is the high energy form of the energy transporting system and ADP is the lower energy form. A portion of the energy of the oxidation is conserved as the energy of the ATP. This process operates in a continuous dynamic cycle, receiving energy during the oxidation of foods and releasing energy during the performance of cellular work. A molecule of inorganic phosphate (Pi) is released when ADP is formed and incorporated in ATP when ATP is formed. Although ATP is not the only energy carrying compound in every cellular reaction, it is the common intermediate in the energy transformation in the cells (67).

The ultimate electron acceptor is oxygen in aerobic organisms. However, electrons are not transferred directly from fuel molecules and their breakdown product to oxygen. Instead, these substrates transfer electrons to special carriers, such as pyridine nucleotides or flavins. The reduced forms of these carriers then transfer their high-potential electrons to oxygen via an electron-transport chain located in the inner membrane of mitochondria. It was stated earlier that ATP is formed from ADP and Pi as a result of this flow of electrons. This process called oxidative phosphorylation, is the major source of ATP in aerobic organisms (108).

In a comparison of aerobic and anaerobic organisms Loehr (67) commented that, for a given organic loading, aerobic conditions will produce a more oxidized end product or effluent than will anaerobic conditions and will permit synthesis of a greater quantity of microbial cells. These additional cells are an asset because it is thus possible to have a larger amount of active microbial solids to increase the removal of organic wastes. Aerobic organisms in a complete oxidation can conserve for themselves a greater portion of the available energy from metabolism of organic matter than can anaerobic organisms. Thus, anaerobic organisms must process a greater quantity of food to obtain the same amount of energy. In further comment, Loehr stated that the energy recovery per unit of food is small for anaerobic organisms which indicates that the number of microbial cells synthesized per unit of food metabolized will be significantly less than for aerobic organisms.

C) Biochemical Transformations

Several studies described in literatures (67) (109) (111) and (61) have ascertained the fact that to achieve satisfactory biological breakdown of wastes, the material must contain sufficient carbon, nitrogen, phosphorus and other necessary elements to sustain optimum rates of microbial synthesis. A problem due to lack of these elements in most biological wastes has not been indicated, since there usually is more than enough nitrogen, phosphorus, and trace minerals with respect to the carbon used in cell synthesis. Loehr (67) briefly described these elements as fundamental transformations in a variety of treatment systems. Loehr also describes the signficant role that temperature, oxygen and pH play in respect to aerobic biological systems.

a) Carbon

The oxidation of organic carbon-containing compounds represents the mechanism by which heterotrophic organisms obtain the energy for synthesis. In aerobic treatment systems organic carbon is transformed, via many steps, to synthesized microbial protoplasm, $C_5H_7O_2N$, and CO_2.

$$\text{Organic Carbon} + O_2 \longrightarrow C_5H_7O_2N + CO_2$$

The uptake of oxygen and formation of carbon dioxide represent the effects of respiration.

b) Nitrogen

Nitrogen is an important nutrient in biological systems and is present at a concentration of about 12% in bacterial protoplasm. In waste matter, nitrogen will be present as organic and ammonia nitrogen, the proportion of each depending upon the degradation of organic matter that has occurred. In biological systems, organic nitrogen compounds can be transformed to ammonium nitrogen and oxidized to nitrite and nitrate nitrogen.

$$\text{Organic N} \longrightarrow \text{ammonium N} \longrightarrow \text{nitrite N} \longrightarrow \text{nitrate N.}$$

The oxidation of ammonia to nitrite and nitrate is termed nitrification and occurs under aerobic conditions. A more basic definition of nitrification is the biological conversion of inorganic or organic nitrogen compounds from a reduced to a more oxidized state. In waste treatment the term usually is used to refer to the oxidation of ammonia. According to Loehr's statement, a residual dissolved oxygen concentration of about 2 mg/l has been found necessary to have optimum nitrification. Autotrophic bacteria, such as _Nitrosomonas_, which obtain energy from the oxidation of ammonia to nitrite, and _Nitrobacter_, which obtain energy from the oxidation of nitrite to nitrate, are organisms that in combination can accomplish the complete oxidation of nitrogen. It has been pointed out by Loehr that the release of ammonia nitrogen to aerobic treatment units creates an added oxygen demand to these systems. The oxidation of 1 kg. of ammonia nitrogen to nitrate nitrogen will require 4.57 kg. of oxygen.

c) Nitrification

Nitrification can be defined basically as the biological conversion of nitrogen in inorganic or organic compounds from a reduced to a more oxidized state (67).

Loehr (67) referred to Pasteur's work (1862) which suggested that the oxidation of ammonia was due to microbial activity. This suggestion was verified in sewage and soil studies which showed that oxygen was essential and that alkaline conditions favored nitrification.

Several genera of nitrifying organisms have been reported (14). _Nitrosomonas_, _Nitrosospira_, _Nitrosococcus_ and _Nitrosocystis_ oxidized ammonia to nitrite. _Nitrosogloea_, _Nitrobacter_ and _Nitrocystis_ oxidized nitrite to nitrate. Of these genera, only _Nitrosomonas_ and _Nitrobacter_ are generally encountered in aquatic and soil ecosystems and are the nitrifying autotrophs of importance. Two new genera of obligate autotrophic nitrite-oxydizing bacteria, Nitrospira and Nitrococcus species have been reported (120). Factors like dissolved oxygen, temperature and pH play an important role in the nitrification process.

(i) Dissolved Oxygen: Since the nitrifying organisms are aerobic, adequate dissolved oxygen (DO) is necessary to support nitrification assuming other environmental conditions are satisfactory (67).

Loehr (67) reported that low dissolved oxygen concentration suppressed measurably the growth of Nitrosomonas and Nitrobacter was also affected to an even greater degree. Further in this comment, Loehr (67) stated that sensitivity of the Nitrobacter to low dissolved oxygen concentrations is one of the reasons complete nitrification is difficult to accomplish in heavily loaded systems where the oxygen demand is significant and where adequate oxygen does not exist in the microbial floc.

A critical dissolved oxygen concentration exists below which nitrification does not occur. The critical dissolved oxygen concentration has not been precisely determined, but it appears to be around 0.5 mg/l (123). The actual limit is more dependant on the rate of oxygen diffusion to the microorganisms than on the oxygen concentration in the mixed liquor. To assure that the dissolved oxygen concentration in a treatment unit is not limiting nitrification, the dissolved oxygen in a treatment unit generally is kept above 1.0 mg/l. Nitrification proceeds at a rate independent of the dissolved oxygen above the critical concentration (67).

(ii) Temperature: Nitrification is affected by the temperature of the media. Pure culture studies indicated that the optimum growth of nitrifiers occurred between 30^o and 36^oC (1). One of the early studies (39) indicated that an exposure for 10 min. at 53^oC - 55^oC and at 56^o - 58^oC killed Nitrosomonas and Nitrobacter, respectively.

Laboratory activated sludge studies indicated that the rate of nitrification increased throughout the range of 5^o - 35^oC (123). Information on nitrification at low temperatures indicates conflicting data. Different studies have indicated that nitrification did not develop below 10^oC (96), that it was possible to maintain nitrification at 8^oC (82), that little nitrification was achieved at temperatures below 6^oC (32) and that nitrification was achieved at temperatures below 1^oC (43).

(iii) pH: The optimum pH for the growth of the nitrifiers is not sharply defined, but in pure cultures it has been shown to be generally on the alkaline side (67). In a detailed study of nitrification, optimum pH was found to be 8.4 Ninety percent of the maximum rate occurred in the range of 7.8 - 8.9 and outside the ranges of 7.0 to 9.8 less than 50% of the optimum rate occurred (123).

According to Hang's and McCarty's (43) report nitrifying organisms can adapt to low pH levels and achieve adequate nitrification. When the pH was adjusted to a range of 5.5 to 6.0 in a submerged aerobic filter, the nitrifiers adapted to the lower pH levels and the rate of ammonia oxidation reached that comparable to what had been achieved at a pH of 7.0.

d) Denitrification

Microbial denitrification takes place under anoxic conditions where nitrites and nitrates are used as terminal electron acceptors in place of molecular oxygen (67).

The nitrates and nitrites are reduced to gaseous nitrogen resulting in a reduced nitrogen content of the treatment unit as the gas escapes from the unit. The composition of the gas produced is a function of environment conditions. Nitrogen gas (N_2) is the primary gaseous end product. Other gases produced include nitrous oxide (N_2O), nitric acid (NO), CO_2 and H_2 (67).

Denitrification is brought about by facultative heterotrophic bacteria. Most of the active denitrifying organisms belong to the genera of _Pseudomonas_, _Achromobacter_, _Bacillus_ and _Micrococcus_ (67).

e) Phosphorus

Phosphorus is an important nutrient in biological processes. The phosphorus content of bacterial cells is about two percent (1). The sources of phosphorus in waste processes include organic matter, phosphates originating in cleaning compounds used for cleaning up, and the urine of animals. The organic phosphorus is transformed to inorganic phosphorus during biological treatment (67). Aerobic biological treatment will convert condensed phosphates to orthophosphates.

2. Thermophilic Microorganisms

The word thermophilic, derived from Greek, means thermo = heat, phile = loving: heat loving microorganisms. In a simple schematic classification of microorganisms, thermophiles fall under bacteria.

A) Origin and Distribution

The origin of thermophiles still remains open for debate among authorities in the field. Opinions of various people who conducted a study at measurable depth are given below briefly.

Imsenecki *et al*. (49) speculated that variants of well-known strains of mesophilic bacteria, progressively more completely adapted to higher temperatures up to the final obligate stage; whereas MacFadyen *et al*. (71) considered that they had become adapted even more gradually and more reversibly than connoted by the term variant. Gaughran (38) - in his thermophilic microorganisms review indicated several possibilities - that spontaneous adaptation or mutation, tropics as the central part of the evolution, propelled by the radiation pressures of the sun from Venus to Earth. Nevertheless, most of the recent authorities in the microbiological area seem to accept Imsenecki's *et al*. (49) assumption. Along the same line Loginova and Tsaplina (70) assumed that most of the thermophilic organisms developed from mesophilic species close to them in such a way that the enzymes and other cell components of the thermophiles acquired heat resistance.

a) Division of thermophiles

According to Smith *et al*. (104), thermophilic bacteria are classified into two well defined species:

1. *Bacillus coagulans* - the majority of these strains grow between 33^{o} and $60^{o}C$, none above $60^{o}C$, and
2. *Bacillus stearothermophilus* - growing between 37^{o} and $65^{o}C$, with many growing above $70^{o}C$.

Definite nomenclature is not yet given to thermophiles with sufficient data to make possible a comparison among organisms. The most recent designations reviewing the early descriptive terms of

Fischer (36) and the animal physiologists, are those of Imsenecki and Solnzeva (49).

True thermophilic bacteria are those species whose optimal range of growth lies between 55^{o}C and 60^{o}C. They may be divided into two groups:

a) Stenothermal thermophiles - develop at 60^{o}C but show no growth after many days at 28-30^{o}C.

b) Eurithermal thermophiles - develop at 60^{o}C, and show slight to abundant growth at 28-30^{o}C.

Thermophiles constitute a very heterogenous group, if all organisms are included with an optimum temperature for growth above 50^{o}C. Their morphology and staining reactions are varied. Fundamental differences appear in their nutritional requirments and metabolic activities (38).

Egorova (35) found that the growth of the thermophilic bacteria was considerably poorer when the medium was based on tap water rather than some solid material which in his case was 20% potato broth. The optimum growth temperature for thermophilic bacteria is usually somewhat lower in liquid medium than on an agarized medium. This has been confirmed by Loginova (68). Based on this fact, Egorova (35) assumed that thermophilic bacteria are no exception in this respect.

According to Egorova's studies, no differences in cell morphology were observed during the period of active growth (the first 2-5 h), when the cultures were incubated in the shaker and in the freezer apparatus. However, the same author stated that the extremely thermophilic bacterium, Thermus flavus, exhibited marked morphological differences during different stages of its growth. This microorganism is distinguished from obligative thermophilic sporogenous bacteria by a lower growth rate.

Thermophilic bacteria growing in an aerobic environment, reproduce at a maximum rate (in the exponential phase of growth) for a period of 2-3 hours. In the linear phase of growth, thermophiles metabolize the substrate (glucose) by using both aerobic and anaerobic pathways, which is evident by the increasing values of the respiratory quotient and the volatile acids liberated into the external medium (91). Pozmogova (93), in his earlier work has also indicated that an organism may experience a limited oxygen supply during the stage of rapid growth. As a result of this deprivation, there is a sharp drop in the maximum rate of growth; with a result that there is a changeover to anaerobic metabolism. This observation has been supported by Miller et al. (79).

The ability of thermophiles to survive at higher temperatures still remains unanswered. Among possibilities that are being envisaged by various investigators

1. Suitable media may increase heat resistance by providing favorable conditions for active metabolism so that the organism is better able to repair damage done by heat or they may aid growth at high temperatures because they contain substances which protect proteins from thermal denaturation (2).

2. The effect of divalent ions i.e. Ca or Mg ions is believed to play an important role on thermal resistance aspect, particularly when considered together with the finding that higher calcium content is one of the few chemical differences which have been found between bacterial spores, which are the most thermoresistant living objects known, and vegetable cells (27).

Regarding the growth limitation of thermophilic bacteria Allen (3) reported that it is due to exhaustion of the dissolved oxygen availability at elevated temperatures. This statement has been backed by results of Tanner et al. (111), but neither has mentioned nutrient exhaustion effect.

b) Growth at high temperature

Tanner and Wallace (111) were the first to apply the quantitative growth-curve method to the thermophilic bacteria; this is according to Gaughran's (38) report. At 55°C, Tanner and Wallace observed most rapid increase in cell numbers and, after the period of active growth, a rapid death; and since cultures often became sterile, it may be inferred that these thermophiles did not sporulate at this temperature (38). Casman and Rettger (20) assume that the absence of spores may have been the result of low oxygen tension in a liquid medium at an elevated temperature, in accord with the observed relationship of oxygen tension and the capacity to sporulate among the aerobes, as well as facultative and strict anaerobes.

In Gaughran's (38) report, it was stated that the generation time of thermophiles isolated at 55°C was 16 minutes. In the same investigation, it was mentioned that calcium carbonate was added in order to neutralize acid production. The maximum viable cell yield (6×10^8 per ml) was obtained at 42°C, rather than at 55°C; with a second optimum cell yield at 20°C.

A study of the rate of biochemical activity of a number of thermophiles indicated that the high reproductive rate is inadequate in explaining the intense biochemical activities of the thermophilic bacteria. In some thermophilic cultures the progress of proteolysis may, of course, be related to reproduction rate, death and autolysis, and a resultant accumulation of proteolytic enzymes in the medium. Thus Imsenecki was led to the conclusion that a possible explanation of the high biochemical activity of thermophilic bacteria may be found in the very intense metabolic activity of these organisms, and not merely in their rapid proliferation (38).

It has been suggested that rapidly metabolizing bacterial vegetative cells growing in a depleted medium with an accumulation of waste products of metabolism would limit bacterial survival unless they were capable of existing into a dormant or resistant stage. The ability to form spores has been suggested as a means by which the organism can overcome unfavorable culture conditions. Thermophiles with a narrow temperature range, however, do produce spores in their optimum growth range, but experience a depression in this activity as the maximum growth temperature limit is reached (38).

c) Growth at low temperature

Thermophilic microorganisms, arbitrarily characterized by an optimum temperature above 45 - 50^{o}C exhibit considerable latitude in their overall temperature range for growth. A large number of thermophilic bacteria have been found to grow at 37^{o}C, and even at 20^{o}C (38).

Evidence has been presented (81) to show that the environment (culture medium) exerts an important influence on the temperature limits for growth. Morrison and Tanner (81) have suggested that during the growth of thermophilic bacteria at lower temperatures, the time of observation is of greatest significance.

Foter and Rahn (37) in their analysis of the limiting minimum temperature of thermophilic growth, stated that the most common explanation of cessation of growth at low temperatures is the assumption that the numberous interlinked reactions of the cell are influenced differently by a change in temperature, with the result that the growth mechanism is upset. In their further comment, they mentioned that the accumulation of toxic metabolites within the cell is not considered as a possible cause for a disturbance of the growth mechanism in the case of bacteria or other cells with large surface area. Contrary to their comment, Surucu (110) reported that the growth of microbial populations is normally limited either by the exhaustion of available nutrients or by the accumulation of toxic metabolic products. It should be noted that other environmental changes such as dissolved oxygen level, temperature and pH are also important, since these changes in the environment may be produced by the microorganisms themselves, thus limiting the development of the microbial population.

Brock and Darland (15), in their study pertaining to effect of temperature and pH on microbial existence, concluded that bacteria have the ability to grow at either high temperature or high acidity, but not at both high temperature and high acidity.

B) Potential of Thermophilic Aerobic Digestion of Organic Solids

Thermophilic aerobic digestion is a process which appears to have a great potential for the degradation of organic solids (51). One of the major factors to be considered in the use of thermophilic process is the heat energy required to maintain thermophilic temperatures. However, heat can be produced by microbial thermogenesis as evidenced by the autothermal nature of composting processes which usually operate in the thermophile range (51).

Simulation studies have shown that sufficient heat can be generated in the aerobic digestion of organic solids to make the process self-sustaining in the thermophilic range (45 - 65^{o}C).

Kambhu and Andrews (51) listed the possible advantages of thermophilic aerobic digestion over the corresponding mesophilic process:

a) Increased reaction rate. According to the classic thermodynamic rule of Van't Hoff-Arrhenius, the biochemical reaction rates will double with each 10^{o}C temperature increase (109).

b) Increased efficiency.

c) Improved solids - liquid separation.

d) Increased destruction of pathogenic organisms.

e) Biomass harvested from thermophilic aerobic digestion process may have relatively high protein and vitamin content (109).

f) The single cell protein produced by the thermophilic aerobic digestion process in some cases has been found to be composed of a better balanced amino acids composition for nutritional purposes (73).

Although many thermophiles had been isolated from canned food early in the history of the canning industry, the canned food spoilage effect did not receive sufficient attention until Barlow's (7) work was published. The significance of these bacteria lies in their ability to ferment lactose, or less commonly decompose proteins, and cause undesirable flavours or odours (38).

Apart from their undesirable activities,thermophiles do have some beneficial aspects as well. They are considered as potential agents:

a) in controlled fermentation of cellulose to useful products (38);

b) in the recovery of vegetable oils and fats (8);

c) in the degumming of silk (52);

d) in enzyme application, i.e. amylase (38) and degummase (48) as well as in pharmaceutical products. Certain enzymes of thermophilic microorganism are being tested as drug preparation.

A determination of the proteolytic activity in different species of the isolated spore-forming bacteria showed that the largest zones of casein hydrolysis are characteristic of B. Stearothermophilus, B. substitis B. brevis and B. megaterium (69). The authors (Loginova, et al.) also refer to the role not only of the above listed organisms but also to B. cereus in the decomposition of organic substance.

C) Nutritional Requirements

Early workers indicated that thermophilic bacteria required organic nitrogen (3). Such statements have arisen from the fact that from the limited studies to date, very few thermophilic sporeformers can develop in a simple medium with ammonia nitrogen and a single carbon source (3).

Cleverdon et al. (22) studied the minimal nutritional requirements of 12 strains of B. stearothermophilus and they responded well with the addition of thiamine, nicotinic acid and biotin to the growth medium. However, they did not determine whether these vitamins were absolute requirements for all strains used in their study. Similar experiments have been carried to a greater depth by Cambell and Williams (18) which were based on essential amino acids and vitamins as a function of temperature ($55^{o}C$). In general, their findings indicate that the complexity of nutritional requirements increase with temperature, even though this does not always hold true. Baker et al. (5) stated that the increased complexity of the nutritional requirements of the thermophilic bacteria may reflect the inability of cell to synthesize these vital components ahead of their destruction. Furthermore, Baker et al. (6), after studying strains of B. coagulans and 68 strains of B. stearothermophilus, reported that these organisms fell into four main nutritional patterns, i.e.:

a) methionine;

b) methionine plus other metabolites;

c) methionine but clearly stimulated by other factors and,

d) no methionine.

These results are also in agreement with Cambell and Williams (18), and Bhat and Billimoria (11).

Several investigators have indicated that the three vitamins: biotin, niacin and thiamin are essential for thermophiles growth (3). Similarly, the same authors (3) reported that glucose, lactate and malate were the best carbon sources for growth at temperatures of 20 - $37^{o}C$. However, addition of 0.3% monosodium glutamate to the simple glucose medium permitted growth at $55^{o}C$ for some thermophiles. The addition of 0.1% casein acid or enzyme hydrolysateto the simple medium described above stimulated growth.

As Baker (5) indicated, thermophiles would appear to require high concentrations of nutrients for optimal growth. Such culture media exerts a rather high osmotic pressure on the cells, which in turn suggests that thermophiles may be halophilic (salt loving). Contrary to this conclusion, Allen (3) in his review stated that recent data would indicate that the growth factor requirements of the thermophilic bacilli are in reality quite simple and that they do not need extensive vitamin and amino acid supplement - and that past culture difficulty may be traced to the use of an inadequate mineral base and, possibly, to the use of unsuitable carbon source or substrate concentration.

Thermophily was considered to depend not only on the presence of unusually thermostable enzymes but also on the functioning of greatly expanded biosynthetic systems. The relatively high permeability and high osmotic tolerances of thermophiles permit the efficient provision of catalysts, fuel and building blocks to these systems (5).

Based on their findings, Baker et al. (5) concluded that "high temperature" strains utilize a wider range of substrates, have a higher osmotic tolerance and also a heightened permeability than do "low temperature" strains.

Various thermophilic organisms are unable to utilize the simple mono and disaccharides (121). Prickett (94) indicated that some of the thermophiles are also unable to utilize starch and cellulose.

It has been pointed out that, although some thermophiles are proteolytic (8) and several are non-cellulolytic, the activity of these organisms in symbiotic relationships in nature would suggest that they are, at most, feebly capable of attacking native proteins (38). This has been found to be true with pure cultures of food spoilage organisms, such as the hydrogen sulfide or sulfur producing stinker organisms in canned corn (122) and the thermophiles found in milk (34). Hydrogen sulfide and indole production were the only end products of organic nitrogen metabolism studied (38). It was also pointed out by the same author that both indole and hydrogen sulfide were produced only by a limited number of organisms. Gaughran (38) in his review, cited types of thermophilic microorganisms according to their specific activities, - i.e. nitrogen fixing, nitrosifying, denitrifying, sulfate reducing, etc.

Suitable growth of a number of thermophiles has been achieved by using basal medium which contains several carbon sources, a richer complement of mineral nutrients, biotin and riboflavin. Excellent growth also resulted, surprisingly, if instead of the vitamins a large quantity of calcium ions (12 mg%) was added to the culture solution (3).

D) The Effect of Temperature on the Nutritional Requirement

Cleverdon *et al*. (22) studied the vitamin requirements of *Bacillus coagulans* at 37°C and 55°C. Employing a vitamin-free casein hydrolyzate medium they found that niacin, thiamin and biotin were required for growth at both temperatures, growth and sporulation being most abundant at lower temperature of incubation. In a study of the vitamin requirements of twelve obligate thermophilic strains of *Bacillus stearothermophilus*, these same workers (22) noted that biotin, niacin and thiamin were the only vitamins found to be essential for continued growth of the cultures at 55°C and 65°C in the casein digest medium employed.

These workers (22) followed the classification proposed by Cameron and Esty (19) in order to avoid confusion. Their classification is:

Facultative thermophiles - growth at 37° and 55°C

Obligate thermophiles - growth at 55° but not at 37°C.

Campbell *et al*. (18) in their study dealing with the effect of temperature on nutritional requirements commented, in agreement with the majority of the investigators, that at higher temperature the enzymes responsible for the synthesis of a particular metabolite required, for example, compound X. This compound may undergo thermal inactivation and thus the organism would require an exogenous source before growth can take place.

In an attempt to explain his data, Cambell et al. (18) considered several possibilities and concluded that the genetic material was responsible for the potential capacity of an organism to produce enzymes, which, in turn, synthesizes the metabolites necessary for growth. Thus, the complete inactivation of a gene responsible for the synthesis of an essential metabolite imposes upon the organism a requirement of an external source of the substance which it can no longer produce. One of the bacterial strains Cambell (18) used in his studies failed to synthesize histidine at culture incubation temperatures of 20 - 37 - and 55^{o}C. Histidine therefore was required for growth. In interpreting this particular finding, Cambell (18) assumed that the gene necessary for the production of the enzyme responsible for the synthesis of histidine, if present, has been inactivated; thus this strain can no longer make histidine and now requires an external source of this metabolite before growth can occur.

E) Energy Metabolism

During growth, thermophilic organisms lose a large quantity of energy in the form of heat (70). The maximum weight of the cell biomass at first increases with increasing concentration of the carbon source; however, after a maximum is reached, the weight of the biomass remains constant, limited not on the increase in the concentration of the carbon source, but only on the concentration of oxygen dissolved in the medium (70).

Regarding the effect of dissolved oxygen in this respect, Loginova (70) stated that the concentration of dissolved oxygen is lower at higher temperatures even though the oxygen demand by the organism to meet the metabolism is increased. A portion of the energy requirement of certain thermophilic organisms for the production of supplementary energy of dissimilation (catabolism) may be nitrate reduction.

In a comparison of the amino acid composition of some enzymes, it was shown by O'Brian _et al_. (70) that a correlation is observed between the thermal stability and the increase in the content of isoleucine, arginine and tryptophan, and a decrease in the content of phenylalanine and aspartic acid. The authors suggest that there are stronger hydrophobic bonds between the subunits in the enzyme molecules of the thermophiles, which is responsible for their thermal stability. It has been reported by Heinen (70) that the enzyme amylase activity of the extreme thermophilic bacterium (70^{o}C optimum for growth), plays an important role in thermal stability whereas Ca and Mg ions are essential for ensuring the thermal stability of amylase and its high activity.

F) Oxidative Metabolism

It has been reported by Allen (3) that the difficulty of growing thermophilic organisms in chemically defined media with a single carbon source has inhibited research progress. Knowledge of the types of compounds which can be oxidized by these sporeformers and the pathways of oxidation of those compounds to be utilized remain completely unknown.

However, Allen (3) managed to extract some general conclusions on the oxidative abilities of the thermophiles from the literature and from his personal observations. In his general conclusion he stated that:

a) Sugars and polyhydric alcohols are usually readily oxidized, as are salts of lactic acid and the common C_4 dicarboxylic acids;

b) Some thermophiles do utilize citrate;

c) Utilization of fatty acid salts in pure cultures has been noted, although available information is limited;

d) Amino acids are excellent substrates for oxidation.

Evidence has been reported by Pozmogova and his associates (93) that thermophilic sporeforming bacteria possess both aerobic and anaerobic metabolic pathways. During the growth of the bacilli in a well aerated media the organisms displayed a predominantly aerobic type of metabolism during the first 1 - 3 hours, after which followed an increasingly active anaerobic pathway. Further in their report, they mentioned that the reason for such a change in substrate metabolism was due to the presence in the thermophiles of more active anaerobic dehydrogenases.

The data of Pozmogova _et al_. (93) showed that development at submaximal temperature leads to the activation of enzymes in pathways for the cleavage of glucose (glucose 6-phosphate dehydrogenase),for the anaerobic transformation (alcohol dehydrogenase), and for the reduction in the rate of amination of a - ketoglutarate (a fall in activity of glutamate dehydrogenase).

G) Transformation of Nitrogenous Compounds

The most conspicuous conversions of nitrogenous compounds effected by thermophilic aerobic sporeforming bacteria are proteolysis, ammonification and denitrification (3).

Oxidation of ammonia to nitrite by cultures of thermophilic sporeformers has been reported by Cambell (17).

Allen in his review (3), stated that the nitrogen fixation by thermophilic bacteria in crude culture may proceed at a rate of three to six mg nitrogen per gram of sugar utilized; however, the mechanism is not well understood.

"Nitrification" and "nitrogen fixation" by cultures of thermophilic organisms, although reported by few investigators (78, 72), have not been extensively studied.

H) Decomposition of Natural High Polymeric Materials

It has been noted as early as 1894 (71) that the thermophilic aerobic sporeforming bacteria have the ability to decompose natural high polymeric substances found in plants. The production of important enzymes such as amylase, proteinases, and lipase as by-products was recorded by many early researchers. Several investigators have obtained thermostable hydrolytic enzyme preparations from the culture filtrates of thermophilic sporeformers; however, the properties of these enzymes have not been studies in detail.

Viljoen et al. (117) have isolated the organisms responsible for the primary attack on cellulose and have reached the conclusion that the thermophilic bacteria which actually decompose cellulose are obligate anaerobes. Tetrault (112) has reported that the aerobic and facultatively anaerobic sporeformers associated with the anaerobes play a secondary role. McBee (75) in his study using pure cultures also obtained a similar result. Claims have also been made, however, for cellulose decomposition by aerobic thermophiles. The first of these claims came from Kellerman and McBeth (53). Allen (3) in his review reported that results of several studies have evidence for aerobic thermophilic bacteria's ability to decompose cellulose.

Under natural conditions, anaerobic cellulose breakdown may be considered to be more prevalent (107) (3). Hydrogen, carbon dioxide, ethyl alcohol, acetic acid and occasionally butyric acid have been found as products of high temperature cellulose breakdown (100).

Murray (85) reported on the aerobic growth of thermophilic cellulose bacteria in atmospheres where the humidity was maintained at 98 to 100 percent. He obtained little or no growth when the humidity fell to 90 percent or less. He concluded that the many failures to obtain pure cultures of thermophilic aerobic cellulose decomposers may not be due to the anaerobic nature of the cellulolytic organisms, but due to insufficient water vapor in the air. Still less is known regarding the decomposition of other constituents of plant material by thermophilic bacteria. There is almost no literature on the breakdown by thermophiles of such materials as hemicellulose, pectin, lignin and chitin.

3. Wood and Its Potential in Ruminant Diet

Wood residues, of 70 to 80% carbohydrate, are a potential source of dietary energy for ruminants. However, only a minor percentage of this carbohydrate can be utilized by rumen microflora without some form of pretreatment (99). The high lignin and the low cell contents are believed to be the controlling factors (114, 115).

The purpose of preliminary physical and/or chemical treatment of wood and wood by-products as explained by Kitts *et al*. (55), is to remove factors which contribute a negative impact toward nutritive value. These are due to changes in the chemical composition of the plants owing to age and species and the presence of natural inhibitors of celluloses. It was suggested by Kitts _et al_. (55) that before a decision is made to use wood in practical feeding to ruminants, the following factors must be closely observed:

a) Economic consideration;

b) Nature of treatment applied should allow more nutrients to become available to the rumen microorganisms without causing excessive depolymerization of polysaccharides or loss of micronutrients, and

c) Effect of treatment should not leave any residue which will be toxic to the microorganisms or the host while removing the natural inhibitors of polysaccharides, if any.

The ruminant is adapted physiologically and anatomically to convert fibrous material containing large amounts of cellulose into food that is acceptable to man. Since wood and wood by-products contain mainly free cellulose, cellulose chemically associated with lignin and nitrogen, and pentosans often referred to as "hemicelluloses," it is reasonable to speculate that the rumen microflora and fauna could utilize these substances and in turn have the end-products of this fermentation transformed into human food (55).

The value of forages for meeting the energy needs of ruminant animals is mainly a function of the amount of feed consumed and the digestibility of the feed's energy. These factors are inter-related and depend to a large extent on the chemical composition of the herbage. The relative amounts of solubles, cellulose, hemicellulose and lignin are all aspects of composition that influence digestibility. Intake is related to these composition factors as well as to the rate of digestion and rate of passage of the fiber mass (118).

Smith et al. (105) reported that there is a significant correlation between the rate constant and percent lignin in the plant tissue of several grass and legume forages. Lignin cellulose ratio and soluble dry matter were also correlated with the rate constant.

It has been pointed out by Lechtenberg (64) and his associates that it is difficult to study the effect of plant composition parameters such as intake, digestibility, or digestion rate. Because of the fact that plant materials that differ only in the component of interest, i.e. differences in lignin percentages are generally confounded with maturity, or differences in the content of other fibrous components. They (64) also indicated that occasionally genetic mutants offer an opportunity to obtain samples of plant tissue which differ appreciably in only one chemical component. In order to substantiate their statement, they referred to Muller's et al. (83,84) work which verified the fact that plant material which has less lignin content has greater cell wall digestibility and intake.

The results of Riquelme et al. in lamb fattening rations (97) indicated that the cellulose fibers (bleached hardwood kraft) supported animal weight gain essentially equal to the control ration (70% wheat + 24% alfalfa hay + 4% molasses + 2% premix) and did so efficiently. Furthermore, the carcasses of the fiber fed lambs exhibited a conformation and quality comparable to the control group, and because of the reduction in backfat thickness, kidney and pelvic fat, the calculated yield grade was improved, indicating more salable meat cuts per carcass.

A. Lignin Distribution in Wood Tissues

Ultraviolet microscopy has been used by Goring *et al*. (40) to measure the distribution of lignin in spruce and birch wood. The spruce xylem was considered to consist predominantly of three distinct morphological regions:

1. Secondary wall (S) of the fiber (tracheid);
2. The compound middle lamella (ML) between fibers
3. Middle lamella (ML_{cc}) at the cell corners.

Table 1. The distribution of lignin in black spruce earlywood

Tissue type	Tissue volume (%)	Lignin % total	Lignin concn.,g/g
S	87	72	0.22
ML	9	16	0.50
ML_{cc}	4	12	0.85

As shown in Table 1 most of the lignin in spruce earlywood exists in the secondary wall of the fiber even though the concentration of lignin in the middle lamella is high. It was also mentioned in their report that similar results have been obtained in latewood studies. The distribution of lignin found in these three areas supports the prediction made by Berlyn and Mark (10).

Goring *et al.* (40) concern in this particular study was to remove lignin and record the rate of removal from various regions in the wood tissue during chemical pulping (Kraft and sulfite pulping). According to their results the secondary wall loses lignin fairly rapidly in the early stages of the work while the middle lamella shows strong ultra-violet (UV) absorption until late in the cook. They also reported that similar trends were found in acid sulfite pulping.

Both in kraft and sulfite pulping, it has been noted (40) that low molecular weight lignins are first extracted with higher molecular weight material being made soluble later in the cook. This extraction sequence of spruce lignins and the microscopic pattern of lignin removal have been interpreted in terms of a theory of delignification based on the degradation of a polymer gel.

B) Lignin

Lignin is located in the woody parts of plants, such as bobs, hulls and the fibrous portions of roots, stems and leaves. Its chemical structure remains uncertain. Due to this uncertainty, it has been suggested (74) that the term must be considered to designate a group of substances having a common basic structure but differing as regards attached units. The substances contain carbon, hydrogen, and oxygen, but the proportion of carbon is much higher than in carbohydrates. Nitrogen is also present, ranging from 1 to 5 percent in different products isolated. Methoxy groups have been reported to occur in percentages ranging from 5 to 15 or more. The percentage increases as the plant matures. The nucleus is a polyhydrate aromatic compound. Thus, lignin cannot be classed as a carbohydrate, but it is discussed along with this group of compounds because it occurs in intimate association with cellulose and is included with the carbohydrates in the conventional methods of feed analysis. Its recognition as a separate entity is important because of its dominant influence on the degree of digestibility of many feeds (74).

Lignin occurs in plants chiefly as lignocellulose. There is support to the belief that substances of the glucosanxylan series are the forerunners of lignin, but neither its exact chemical structure nor the manner in which it is combined with cellulose are fully understood. Its behavior in nutrition is likewise unsettled, different feeding tests yielding conflicting results. Proof for or against its utilization by the animal is difficult to establish, for until its molecular structure is known, no criterion of the accuracy of a quantitative test for lignin is possible (26). Crampton and Maynard (26) have shown in their studies that lignin is not appreciably metabolized by animals.

The work of Woodman et al. (125) has shown that lignified plant tissues apparently are not attacked by alimentary bacteria. According to their assumption, this might be due to a certain degree of antiseptic action of the lignin resulting from its phenolic nucleus.

C) Delignification

In one of their studies, Kitts et al. (58) reported that unprocessed sawdust in ruminant diet has been able to provide energy as high as 244 kcal/kg digestible energy, and 22.7% of digestible protein. In a further statement, it was mentioned that because the actual utilization of the untreated sawdust by rumen microorganisms per se is poor, it has not been particularly beneficial to utilize wood wastes without further processing to remove or lessen the lignin content.

Untreated sawdust has been included in production rations at low level, and it would appear that it serves mainly as non-nutritive bulk in rations containing otherwise high levels of energy feeds (4,55). One liquid by-product, a hemicellulose extract prepared from hardboard manufacture, has proved commercially successful as a feedstuff of high nutritive value (89).

Sodium hydroxide has been one of the more popular agents for delignification and has been applied to a variety of materials to improve their digestibility for use as a ruminant feed.

Sodium hydroxide would appear to be the most effective and economical, particularly under mild conditions of atmospheric temperature and pressure. Optimum treatment levels of sodium hydroxide would appear to be less than 8 g of chemical/100 g of substance, an indication that mild heat can be used to reduce chemical levels and thus minimize physiological stress on animals consuming large quantities of treated feedstuffs (31).

Smith et al. (103) compared a variety of agents for improving digestibility of ruminant fecal material. Sodium hydroxide and sodium peroxide substantially improved the availability of carbohydrate in the material. The increase resulted from the improvement in the availability of insoluble residual cell wall as well as the formation of soluble matter.

Brownell (16) found that when 20 mesh wood was subjected to various chemical pretreatments, the amount of lignin obtainable after subsequent ball milling was greatly increased above that obtained without milling. The most striking increase occurred after treatments with sodium hydroxide or ammonium hydroxide.

Pew and Weyna (88) in their study of enzymatic liberation of lignin, observed a large increase in the extent of enzymatic digestion following mild treatment with a base. Similar to Brownell (16), Bland

and Menshum (12) reported that the yield of milled wood lignin was increased by a mild alkali treatment before milling. This suggested that lignin was attached to the carbohydrate by alkali sensitive bonds. Further in their report (12) they mentioned that prolonged standing of milled wood solutions of sodium or ammonium hydroxide at poor temperature failed to increase the yield of lignin. The yield, however, did increase with increasing temperature of alkali treatments between 48^{0} and 86^{0}C. Finally, they (12) concluded that the lignin carbohydrate bond, if such bond does exist, is stable to alkali treatment below approximately 100^{0}C and that the breakup of the complex is due to alkali peeling of the hemicellulose. Above 100^{0}C hydrolysis of lignin begins and complicates further measurements of lignin recovery. It would appear,however, that the lignin-carbohydrate bond cannot be more alkali-labile than bonds within the lignin.

The objective of treating lignocellulosic materials is to remove or disrupt ligninso that their energy potential as a ruminant feedstuff is enhanced severalfold compared with their untreated state.

As it is mentioned in several references, cellulosic materials contain a vast store of energy. Residues from the wood industry, animal wastes, municipal organic wastes, etc. are being investigated for cheap energy sources for livestock feed.

The energy contained in cellulosic waste can be converted to food use by means of three general systems (116);

1. Chemical or enzymatic hydrolysis to produce sugar;
2. Microbial fermentation with added nitrogen source to produce single-cell protein;
3. Feeding to ruminants to produce meat and milk.

Earlier, it was mentioned that ruminants have the ability to utilize cellulosic materials through the rumen fermentation. The nature of fermentation is anaerobic. The anaerobic microorganisms convert carbohydrate to microbial proteins, various volatile fatty acids, carbon dioxide and methane (46).

Microbial proteins, microbial soluble matter and volatile fatty acids are utilized by the ruminant, while methane is a caloric loss. For adequate fermentation the nitrogen requirement of the microbes must be met. Since cellulosic wastes are usually very low in nitrogen, these feedstuffs must be supplemented with nitrogen. Digestion of cellulose is a function of the time the material resides in the rumen. Thus, feeding cellulosic materials in rations containing high levels of concentrates may decrease cellulose utilization because of the faster rates of passage associated with high concentrate rations. Fine grinding of the cellulosic material might obtain a similar result. It has also been postulated that high levels of soluble carbohydrate might reduce the digestion of cellulosic materials (46).

Kitts and Underkrofler (59) investigated the mechanism of breakdown of cellulose to develop methods that would increase the availability of cellulose in low quality roughages dealing with cellulolytic enzymes from mixed rumen microorganisms by grinding the bacterial cells from strained rumen fluid with alumina. They could not detect cellulolytic activity in the centrifuged and filtered rumen fluid, suggesting that the cellulolytic enzymes were not present as in the rumen fluid but were associated with the bacterial cells. Being aware of the fact that glucose was the only major product of hydrolysis of carboxymethyl cellulose (CMC) they hypothesized that the cellulose degrading enzyme of the rumen microorganisms was a "celloglucosidase."

The rumen microorganisms are limited by the composition of the cellulosic matter. They are unable to degrade lignin, which exerts a protective action on the cellulose with which it is combined. This imposes a limit of digestibility on the structural matter of all plant materials fed to ruminants, digestibility being negatively related to lignin content (117). Therefore, removing lignin by the cheapest means possible is a vital concern for ruminant nutritionists. Various methods for delignification are being used by concerned investigators. Some are listed below:

a) Sodium hydroxide (30, 60, 124).
b) Methods similar to those used in the pulping of wood for paper (88).
c) The use of white rot fungi that can aerobically ferment lignin (54) and cellulose.
d) Chlorite treatment (98).
e) Sodium peroxide treatment (103).
f) Fermentation (54).
g) Steaming under different combinations of time and pressure [temperature] (9).
h) Irradiation treatment (55).
i) Ball milling (28).
j) Acid hydrolysis (45), etc.

Kirk and Moore (54) have examined nine white-rot fungi for their ability to remove lignin from bigtooth aspen (Populus grandidentata Michx) and yellow birch (Betula allghaniensis). During decay most of the fungi removed a larger percentage of the lignin than the polysaccharides. Lignin removal was always accompanied by removal of polysaccharides, but lignin removal did not corrolate with removal of any particular component of the polysaccharides. During decay lignin was usually more selectively removed in the first few percentages of weight loss than were the polysaccharides. The decayed wood with less lignin were more digestible by a mixture of polysaccharidases and by rumen fluid than were the control samples. It was known from this type of study that in general the more lignin removed from wood, the more digestible is the residual material.

Although some white-rot fungi are effective in removing lignin faster than polysaccharides from wood, it was mentioned by the authors that the process was relatively slow. For practical use of white-rot fungi it would be desirable to speed up the process. Therefore, they suggested meeting the following criteria which are believed to be optimizing factors:

1. Aeration, moisture, temperature and source and amount of nutrient nitrogen should enhance the rate of decay.
2. To find conditions that would improve the selectivity of lignin removal.
3. To find better fungus species or obtain desirable mutants, as is common in industrial microbiology.

III. MATERIALS AND METHODS

1. Materials

Treatment tank: A fiber glass fermentation tank (Figure 1) with a total capacity of 1690 litres (operating volume of 1362 litres) one inch thick styrofoam blanket bonded to the side for insulation purposes and the entire outer surface covered with a protective coat of canvas and epoxy resin (25) was utilized in the study.

Aerator: Aeration was accomplished with a commercial compressor. A four inch diameter rotor driven by a 1/2 H.P. electric motor at 1725 RPM delivered approximately 1400 gms of oxygen per day. Aeration and subsequent dissolved oxygen in the substrate was a critical factor in thermophilic waste treatment processes (25).

Mixer: Active agitation of the substrate was considered to be essential and was achieved by a 1 HP 1725 RPM motor directly connected to 7.62 cm - 3 blade propellor mounted on a 20.32 cm. diameter stainless steel draft tube (Figure 1). The mixer provided a small amount of aeration to the substrate (supplying approximately 0.54 kg. of oxygen per day). In combination with the aerator, a previous trial showed that the two units supplied a total of over 1.81 kg per day of oxygen to the substrate (25).

Pumps: a) Charge Pump - A "Moyno" charge pump with a 10.16 cm. diameter intake and 3.81 cm. discharge was employed. The pump was capable of delivering 68.10 litres per minute.

b) Transfer Pump - The transfer pump is a smaller reversible action "Myno" screw pump. This unit allowed transfer of the sludge from the clarifier unit to the digester or from the digester to the screening unit.

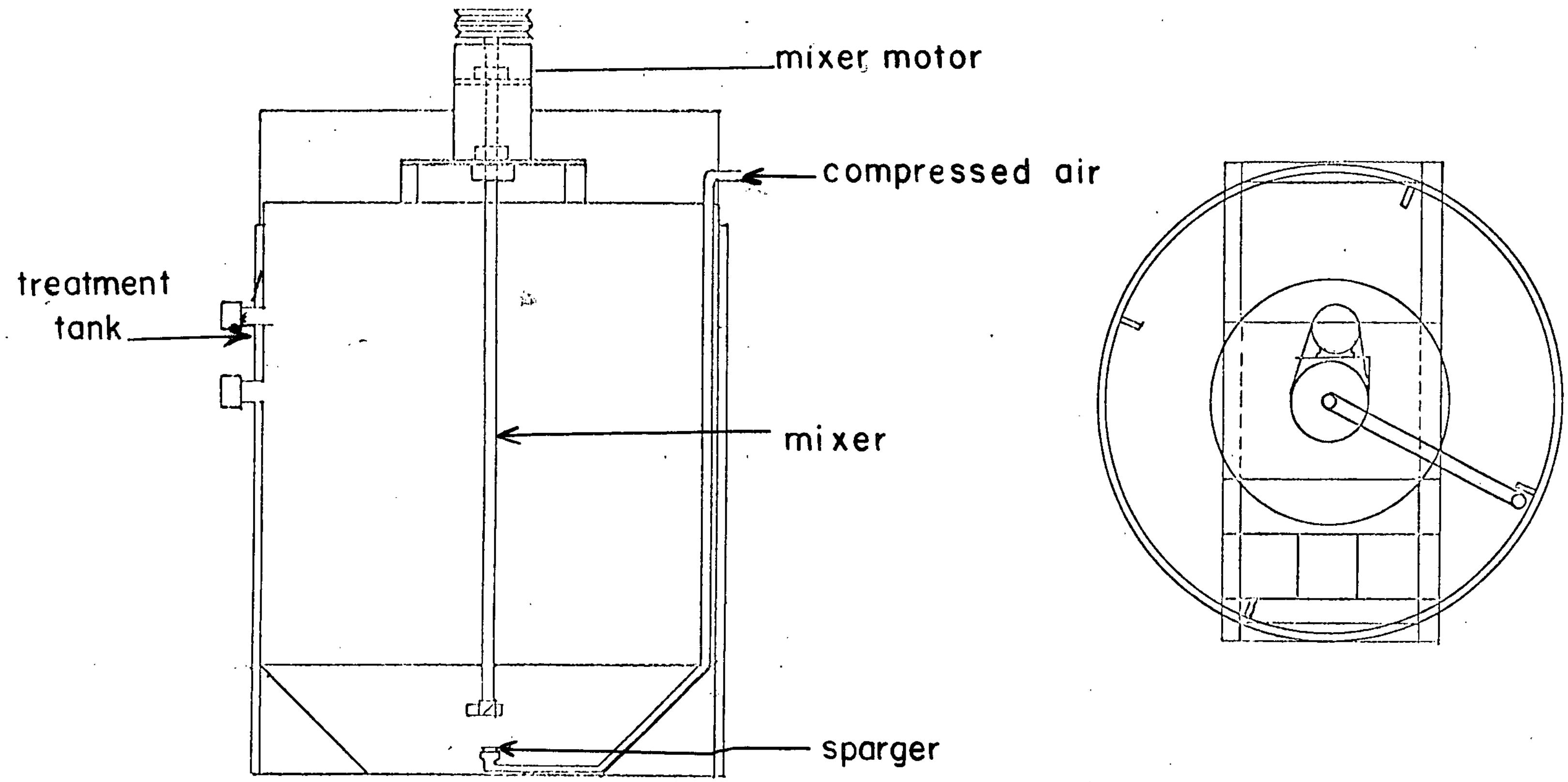


FIGURE:1 SCHEMATIC OF FERMENTER
SHEPHERD, 1977.

39.

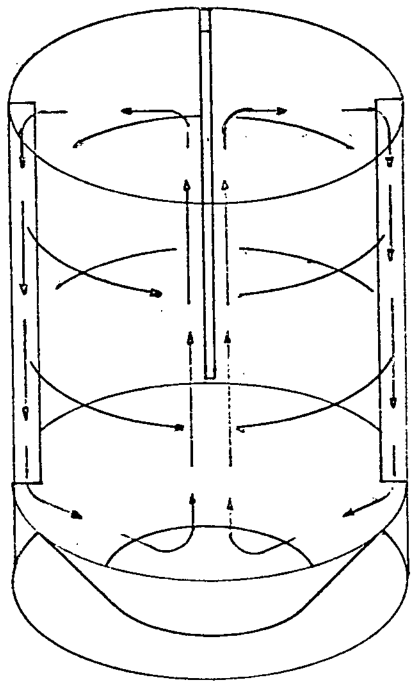


FIGURE: 2 MIXING ACTION

SHEPHERD, 1977.

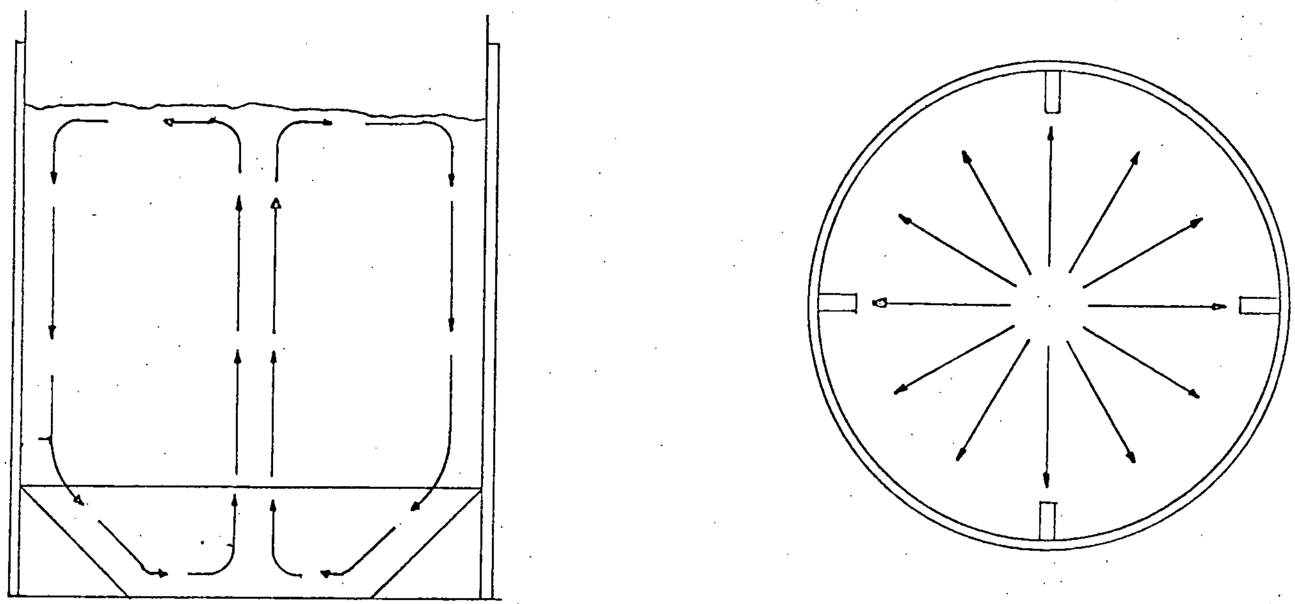


FIGURE: 3 MIXING ACTION
SHEPHERD, 1977.

Transfer lines: All transfer lines were of 5.08 cm. diameter (minimum) rubber tubing, equipped with quick coupling fittings.

Screening: Processed mixed liquor and suspended solids (MLSS) were screened into three major fractions by a "Sweco" vibrating screen which was driven by a 1/4 HP electric motor.

a) The coarse sized material was retained on a 30-mesh sieve (30 holes/2.54 sq. cm.).

b) The finer sized material was collected on a 150-mesh sieve (150 holes/2.54 sq. cm.).

c) The liquid filtrate was collected and subjected to a "basket-type" centrifugation for further solids recovery(56).

Drying: Process MLSS was subjected to drying using two different sources of energy depending on the weather condition.

a) Solar energy was utilized when weather permitted. A thin plastic sheet was spread over a dry ground surface on which the processed sawdust was evenly spread for drying.

b) An electric heater blanket covering a surface area of 5.95 square meters was used as the heating surface to supply drying conditions when the weather did not permit sun drying.

2. Methods of Culturing the Microorganisms

A) Initial Stage Procedure for Substrate Preparation

a) The digester was washed, cleaned, and filled with tap-water to a height at which the mixer propeller was just submerged.

b) Mixer and aerator were turned on. Assuming a regulator flow of air is of vital importance for a successful aerobic fermentation, a constant flow of fine air bubbles at the overhead of the sparger (Figure 1) was maintained.

c) One hundred and thirty to one hundred and forty liters of fresh and partially decomposed (from swine waste sump) swine manure (50:50 v/v) was added to the digester.

d) Additional water was added in order to adjust the working volume to three-fourths of the digester capacity. The remaining one-fourth of the fermentation volumes provided space for foam insulation and for sawdust addition.

e) Two to four kilograms of sawdust were added to the digester in amounts after the temperature of the substrate within the digester reached 55^{o}C.

f) Imhoff cone (volume of settleable solids) and initial temperature (^{o}C) measurements were taken immediately after completion of the filling of the digester with substrate. Coulthard (25) stated that the desirable quantity in the slump for thermophilic treatment process is when settleablesolids or particles of the substrate are between 50 and 75%.

B) Culturing Parameters

a) Temperature - temperature is one of the most important environmental parameters affecting the growth, activity and evolution of organisms (3). Surucu (110) stated that growth rate of microorganisms increases with temperature up to certain points and after that point, it begins to decrease. This would indicate that temperature can be expressed as the resultant of two opposing activities on microorganisms which are:

i) rate of enzymatic activity is low at low temperatures and accelerates as the temperatures rise, and

ii) denaturation of cell proteins (or enzymes) is not significant until moderate temperatures are attained. Protein inactivation increases rapidly with further increase in temperature.

b) pH - the [H] ion concentration effect is one of the factors which has a very pronounced effect on enzyme reactions (77). A change in pH of the substrate or medium frequently occurs as the result of microbial growth (66).

One of the conditions which limits microbial growth is the hydrogen ion concentration of the medium. At low pH denaturation of the key enzyme proteins will occur. With the exception of a few bacterial species, such as the sulfate oxydizers, most bacteria are not active below pH 4.0. The same is true at pH values greater than neutrality. As pH rises over 9.5, the hydroxyl-ion begins to exert a toxic effect. Few, if any, microorganisms can survive above pH 11.0. Control of pH at either a high or low range can be used to prevent or retard bacterial decomposition of potentially degradable materials (77).

c) Dissolved oxygen - aeration in biological processes serves two important functions:

i) provides adequate oxygen to meet the demand of the microorganisms and

ii) provides adequate means to ensure mixing in order to prevent settling of the solids (23).

Aeration and subsequent dissolved oxygen in the substrate is a critical factor in the thermophilic type of waste digestion (25). A low level of dissolved oxygen in the medium tends to favour low thermal energy release, a low operating temperature and the development of undesirable microflora (24). The aerobic thermophilic process demands a high oxygen infusion due to the high rate of biological oxidative activity (24).

d) Imhoff cone measurement (Amount of Settleable Solids)- the settleable solids most frequently encountered in waste treatment practice are of such particle sizes that they tend to settle through water at constant rates depending on the effective sizes and relative densities of the individual particles (47).

While a certain ratio of degradable organic wastes to water are necessary for the normal growth of microorganisms, an excessively high organic matter to water content of the medium can result in a depressed fermentation (77). Coulthard (25) indicated that 50 to 75% settleable solids is a desirable quantity in the slurry for the thermophilic treatment process. Large volumes of water to solids ratio reduces the level of heat energy derived from oxidation while an excessively high solid content reduces the efficiency of mixing and aeration.

C) <u>Delignification</u>

As stated above, the digestor was filled to three-fourths of its capacity. Aeration and mixing (25) continuously creates a foam blanket on the surface; thus, insulating the medium and preventing the heat being produced by the reaction from dissipating to the atmosphere.

With a suitable physical and chemical environment the thermophilic bacteria multiply and a portion of the energy in the carbonaceous material is released to heat the medium to temperatures of 55^{o} to 65^{o} (25).

A portion of 2-4 kg. of alder (<u>Alnus</u> <u>rubra</u>) was added to the digestor after the temperature of the substrate had reached $55^{o}C$. The

Imhoff cone percentage was used as a guide in order to decide when to add the sawdust, i.e. the lower the percent, the greater the amount of material that has been degraded and vice versa.

For several days the temperature remained at the optimum range, resulting in a rapid delignification of the sawdust. The declining phase of the microbial cell growth was accompanied by a gradual decline in temperature of the mixed liquor and suspended sludge (M.L.S.S.), a low biological oxygen demand value (B.O.D.) and a stabilized sludge.

A sample was collected from the vat before adding new unprocessed sawdust. Excessive foam formation and low Imhoff cone percentage were the basic guides for sample collection. Collected samples were stored in a deep-freezer until analyzed. Unprocessed raw sawdust was used as a control for analysis.

The collected samples and unprocessed (control) sawdust were analyzed or tested for:

a) acid detergent fiber

b) cellulose

c) lignin

d) crude protein and

e) digestibility (*in vitro* digestion)

D) Harvesting

Once the microbial growth ceased, the M.L.S.S. was pumped by a transfer pump to a screening unit. The harvested products were collected in three major fractions as described above.

E) Drying

The objectives of drying are to remove moisture, eliminate flies and odours, and to facilitate the storage and handling of the processed product. Regarding the odour and fly problem, the processed product had a negligible effect once it has reached this dry stage.

A moisture content in the 10-15% range will inhibit mold and microbial growth and minimize the dust problem (106).

Solar energy (with one exception) was the only type of energy used for drying purposes throughout the experimental period.

F) Nutritional Value

The processed sawdust as well as unprocessed sawdust (control) was subjected to the following analysis in order to determine the nutritive value of the product.

1. Dry matter
2. Crude protein
3. Bomb calorimetry - gross energy
4. Ash

G) Analyses

Frozen processed sawdust (supernatant) samples were thawed and dried in an oven at 65°C. Similar drying treatment was carried out on unprocessed sawdust (control). Dried samples were ground to pass a 40-mesh screen and analyzed for:

a) dry matter
b) acid detergent fiber
c) cellulose
d) lignin
e) crude protein
f) gross energy and
g) ash

In vitro digestion was also conducted to determine the effect of such microbial treatment towards dry matter digestibility.

Procedures as described in the Association Officials of Agricultural Chemists (A.O.A.C.), 1960, 9th edition were used for all the analyses with the exception of crude protein and gross energy. Crude protein was determined according to the Triebold Modification of A.O.A.C. Gross energy determinations were made by the use of the Parr Bomb.

The procedure for in vitro digestion was a development of the rumen liquor and pepsin-acid procedures from Larsen and Jones (63) with slight modification. The source of inoculum was from a rumen of fistulated steer which was fed a diet of alfalfa hay with premix mineral salt-free choice, and water available at all times.

IV. RESULTS AND DISCUSSION

1. Environmental Parameter

Studies were conducted in order to examine the possibility of alder sawdust delignification by aerobic thermophilic fermentation. The purpose of the studies using the batch technique was to obtain information relating to the environmental variables thought necessary to ensure the growth of microorganisms and to their effectiveness in delignificantion. The variables examined in the environmental parameter study included temperature, dissolved oxygen and pH.

The relevant changes in temperature, pH and dissolved oxygen including ambient temperature of Batch I, II and III are shown in Figures 4, 5, and 6 respectively. In all three batches the pH increased as the fermentation period progressed. In most cases, in 7 to 10 days of processing the pH range was between 7.5 and 8.3. McKinney (77) assumed that the increase of pH in organic fermentation may be due to some ammonia production and also possibly some increase in amino acid content although analyses to support this assumption have not yet been reported. The literature on the growth of thermophilic organisms in pure cultures contains sufficient information to indicate that many thermophilic organisms grow best when the pH of the medium is between 6.5 to 7.5 (67). Regarding mixed liquor and suspended solids, Bragg, et al. (13) reported that for the best result the aerobic thermophilic processing alkalinity should be in the range of 7.5 to 8.5 pH value. Brock and Darland (15) have shown in their work that the bacteria have the ability to grow at either temperature or high pH, but not at both high temperature and high pH. These workers were not specific as to the genus or species of

FIG. 4.
RELEVANT CHANGE IN ENVIRONMENTAL VARIABLES DURING TREATMENT. BATCH I.

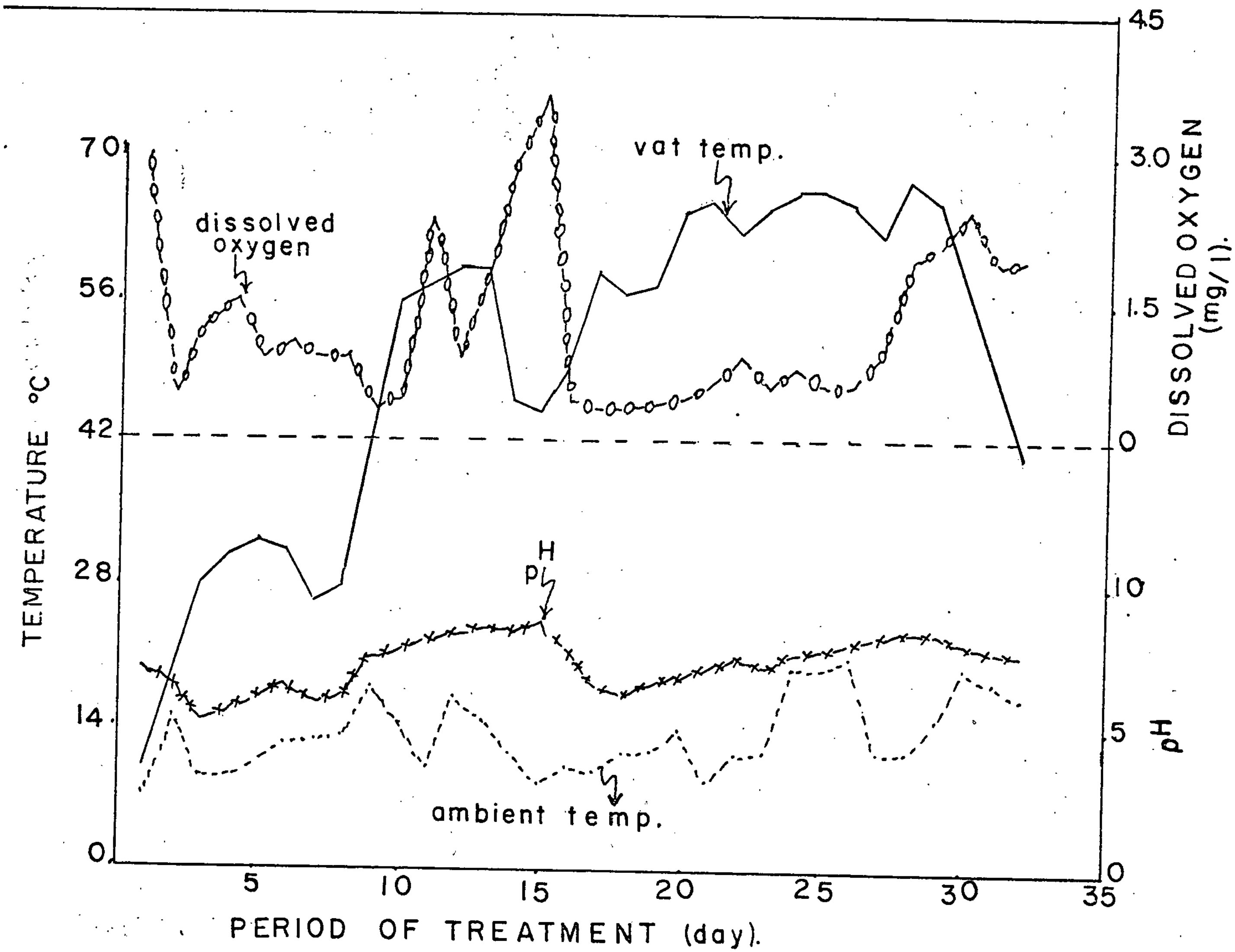


FIG. 5.
RELEVANT CHANGE IN ENVIRONMENTAL VARIABLES DURING TREATMENT

BATCH II

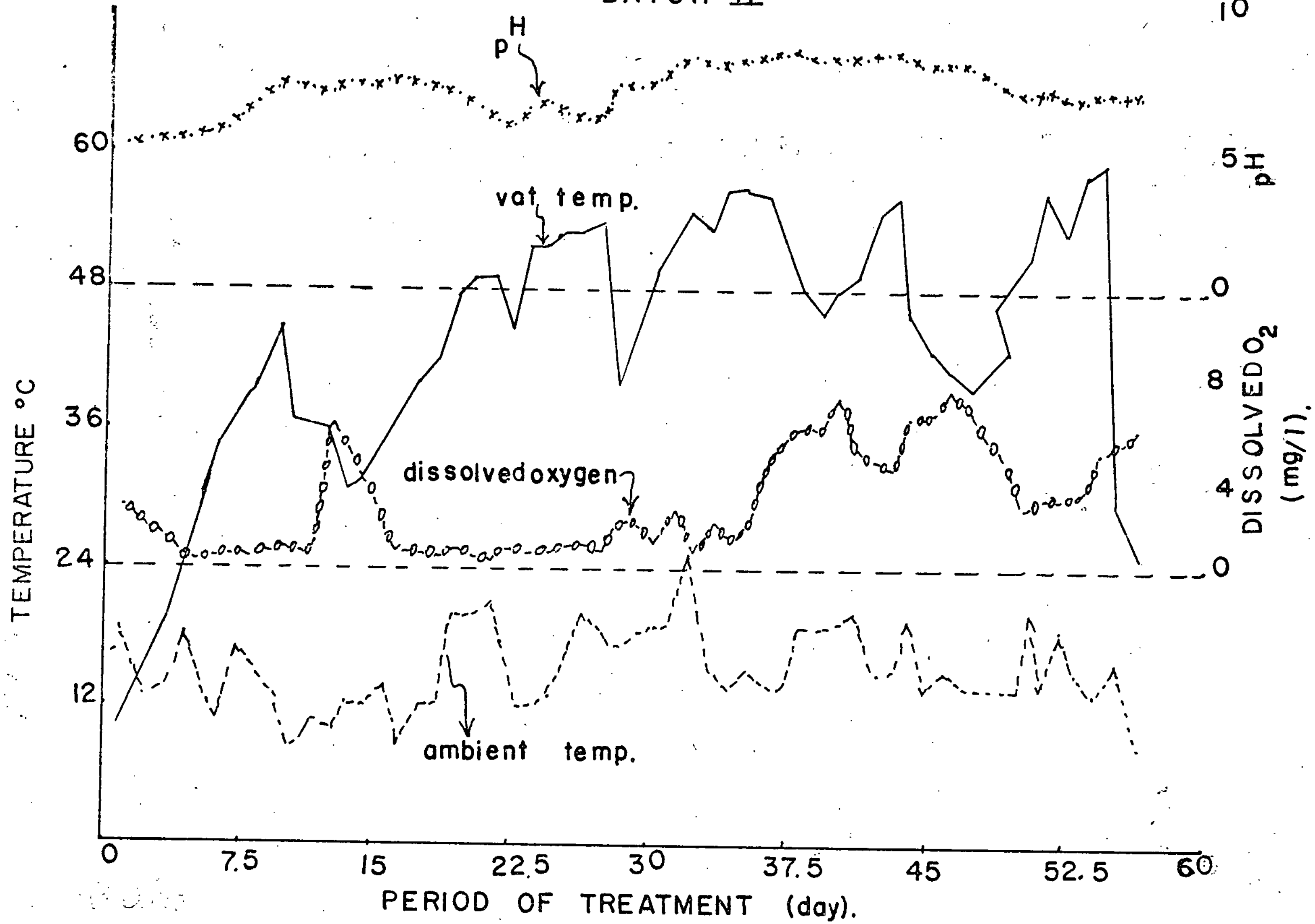
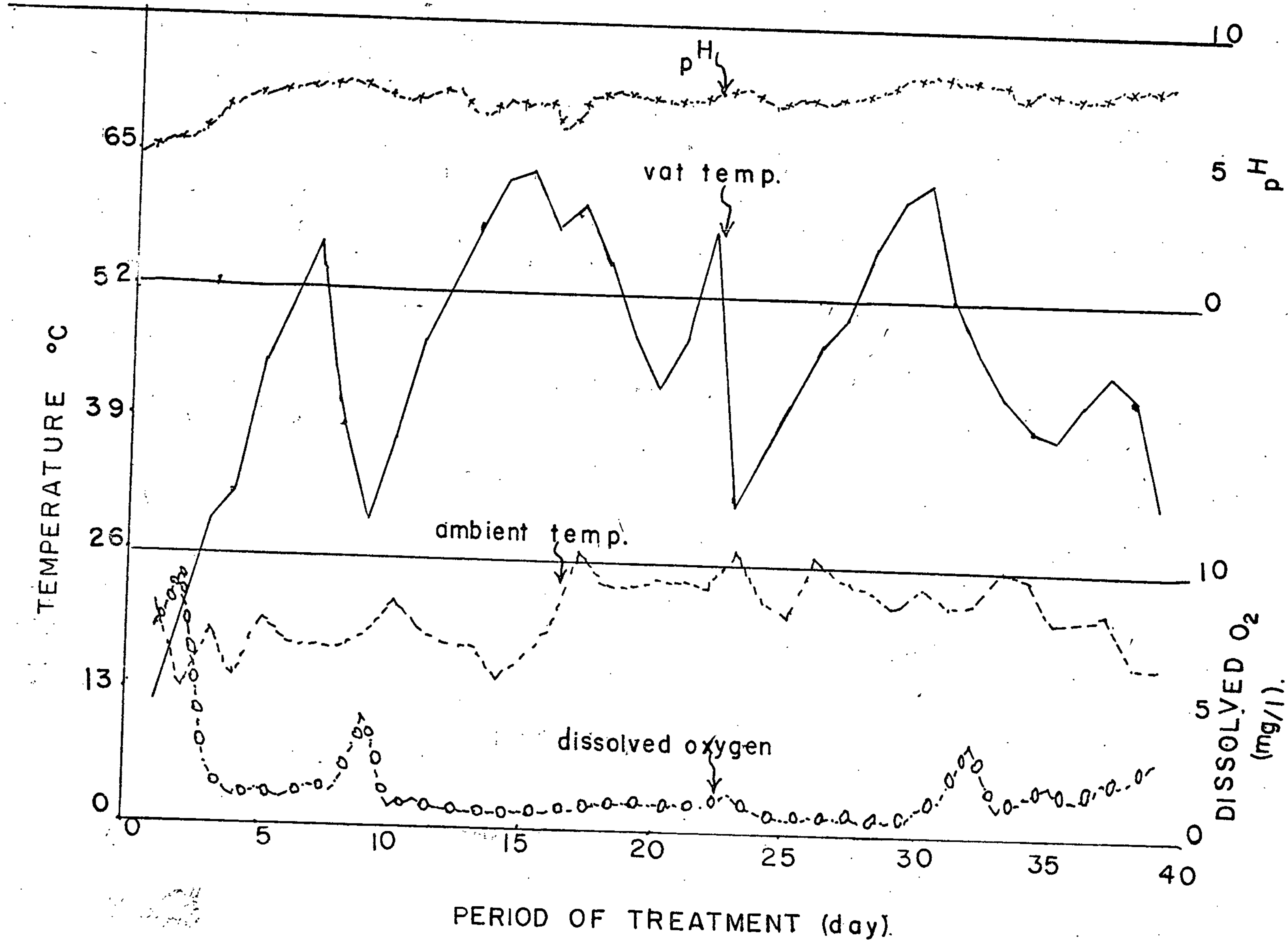


FIG. 6.
RELEVANT CHANGE IN ENVIRONMENTAL VARIABLES DURING TREATMENT

BATCH III



51.

bacteria studied. Nevertheless, these results suggest that there are physico-chemical limitations of the environment beyond which life is impossible. However, Surucu (110) indicated that there is little difference in the pH requirements between thermophilic groups. In case the pH becomes a problem, he suggested the use of a phosphate buffer to maintain the pH of the medium at approximately neutrality.

Temperature changes in Batch I, II, and III are shown in Figures 4, 5, and 6 respectively. These results show a relationship between high rate of delignification and high temperature (Tables 2, 3, and 4). Surucu (110) reported that 55^{o}C is the temperature which the highest observed net cell mass production occurred while the maximum growth and substrate utilization rates were noted at 58^{o}C. However, these results did not differ significantly between 55 to 58^{o}C (110). Samples collected within this range of temperature are low in lignin and acid detergent fiber (Tables 2, 3, and 4). Once the vat temperature reached the optimum range (55^{o} - 60^{o}) it remained isothermal for 4 to 8 days resulting in high delignification. The foam produced as a result of the biochemical activity of the vat, aids to conserve the released heat. The temperature generated in all the "batch" studies was followed by a decrease in temperature. The addition of about 14 to 23 liters of fresh swine manure caused the temperature to return to the optimum range within 24 to 48 hours' time. These results indicate that swine manure in small quantities provides adequate nutrient supply to provide optimum bacterial activity. Regarding the effect of temperature towards the growth of microorganisms, Cambell and Williams (18) commented that in case the incubation temperature is increased there is an increase in the growth requirements of the particular organism. Further in their comment, they stated that at a higher temperature the enzyme(s) responsible for the synthesis of a particular metabolite(s) required, undergoes thermal inactivation and thus the organism requires an exogenous source of compound before growth can be restored.

In aerobic thermophilic type of processing, high temperature is achieved as a result of oxidizing the organic portion of the substrate by microorganisms. A portion of the energy of catabolism is utilized by microorganisms and the surplus energy is released to the substrate in the form of heat and consequently an elevated temperature. The increase of temperature accelerates the biochemical reaction proceeding to a biological stabilization of the waste material (24). The result of Popel's (90) work has indicated that 3.55 kcal of heat is produced per gram of organic solids. Observations of these studies and several other "batch" process trials showed that the more heat produced, the greater the degradation of organic solids by the aerobic bacteria. Therefore, the maintenance of high temperature during processing is of vital importance for increasing the rate of biodegradation. To achieve this, the following factors must be closely observed:

a) Increasing oxygen transfer efficiency (51),

b) Supplying high nutrient content of organic matter, and

c) Insulation of the reactor.

Aeration of a biological system serves two separate physical functions; oxygen transfer and mixing. These two functions are of primary importance in waste treatment systems. Oxygen is essential for proper functioning of the aerobic organisms and mixing is required for transfer of oxygen into the liquid phase (111), for uniform contact of microorganisms with waste material and also for preventing settlementation of sludge in the aeration vat.

In these studies, dissolved oxygen was monitored by using a YSI Model-dissolved oxygen meter. The results of all three batches showed the decrease in dissolved oxygen (Tables 6, 7, and 8); i.e. it was inversely related to temperature.

Harrison (42) showed in his experiment that maximum growth can be obtained with an oxygen concentration of 1.4 to 4.6 mg/liter. Harrison claimed that an increase in oxygen concentration beyond this range retarded the growth of the thermophiles. In a "batch" experiment performed in this study, the dissolved oxygen concentration of the medium was carried out between 2 and 5 mg/liter in order to provide a condition in which oxygen was not limiting.

It has been pointed out by Coulthard (24) that the oxygen transfer coefficient is affected by the physical and chemical characteristics of the system under aeration. Along the same point Eckenfelder (7) lists the variable characteristics as temperature, turbulent mixing, liquid depth and waste composition. This observation can be explained by the fact that the concentration and the biological activity of mixed liquor suspended solids decreased with increasing treatment period. This has been demonstrated also by Surucu (110) that at higher solid retention time values, autoxidation of biological solids becomes very important and increases the apparent amount of oxygen utilized per quantity of organic matter removed. As another possibility, the increase in oxygen utilization may be the result of an increase in rate of endogenous respiration. An increase in air requirement per unit organic matter removed with longer aeration periods is well documented in the literature. For example, air requirements for a conventional biological process are about 14 to 26 cu. meters/kg. of B.O.D. removed and it increases to 34 to 51 cu.m./kg. of B.O.D. removed for the extended aeration process, where solid retention time is longer (78).

The oxygen input and output were expressed as functions of the air flow rate and oxygen transfer efficiency since these are of importance in the heat balance. A study was conducted by Kambhu, et al. (51) to determine the quantity of oxygen leaving the reactor as dissolved oxygen in the medium; the result they found was negligible. Therefore, according to these studies reported in this thesis, the results (Tables 6, 7, and 8) showing the decrease of dissolved oxygen as the temperature rises should not be concluded as an increase of oxygen dissipation to the open atmosphere.

2. Effect of Aerobic Thermophilic Bacteria on Lignin Content of Alder

Alder (*Alnus rubra*) sawdust[1] was obtained from the lower Fraser Valley, British Columbia and was classified as matured wood. The size of the sawdust particle was approximately 2.5 x 3 x 10.15 millimeters.

The chemical composition and digestibility of the untreated alder sawdust (control) the fresh swine manure and the processed samples of all batches are given in Tables 2, 3, and 4.

In the case of "batch" I and II, sawdust was added each time the percent of Imhoff read low which was the indication of a high percentage degradation or digestion, and when the vat temperature was within the optimum growth range (55^{o} - 60^{o}C) of aerobic thermophilic bacteria. Batch III

[1]Purchased from Spruce Specialties Ltd.

was designed to investigate:

a) Whether delignification was taking place before vat temperature reaches $55^{o}C$ or not;

b) Whether the stage of processing would yield maximum delignification. However, the major system of processing was not different from Batch I and II except that 22 kg. of sawdust was added along with fresh swine manure at the initial stage of processing.

Batch I has shown about 41% of delignification with a 24 hour period (Table 2). This was with a limited sawdust load of 22 kg. On the other hand, 59% of delignification was achieved within 22 days of processing having a total load of 99 kg. of sawdust (Table 2).

The result of Batch II showed slightly over 56% of delignification within 13-17 days of processing with 44 kg of sawdust. There was an indication of poor delignification as the vat temperature drops (Table 3). This can be explained by the possibility that aerobic thermophilic bacteria and temperature play an important role in delignification.

With Batch III, the highest delignification rate (74%) was achieved within 15 days of processing (Table 4). As it was mentioned earlier, this batch was treated only with 22 kg of sawdust throughout the processing period. Again, as the vat temperature dropped there was a lower delignification rate (Table 4).

In most cases the extended rate of delignification was not as efficient as it was during the first 24 hours (Batches I and II). Presumably, this was mainly because of low load at the beginning and at this particular stage, the microorganisms are within their optimum growth range, accounting for the high chemical activity and measurable degree of degradation. As the period of processing progressed, there was an increase of sawdust load periodically and exhaustion of the nutrients from the medium resulting in poorer delignification. Under conditions described in these studies 10 to 15 days of processing appears to be sufficient time to delignify 20 to 25 kg of alder sawdust.

Relationship between lignin removal and removal of cellulose

Due to the partial utilization of cellulose by microorganisms, it was difficult to correlate the rate of delignification with the amount of cellulose release. As the amount of raw sawdust increased within the fermenter, the amount of free cellulose in the medium and rate of delignification decreased (Figures 7 and 8). Low level of total cellulose corresponded with high vat temperature (Table 2). Since the higher vat temperature promoted a general increase in chemical activity and growth, these data might indicate that the released cellulose was being utilized by microorganisms as a nutrient source. In most experiments, low analytical values for cellulose was related to high vat temperature. However, this low cellulose-high processing temperature relationship was connected to the fermentation period of 3 to 7 days from the start of processing. During the first few days after sawdust addition, percent of cellulose remained relatively high (Table 2). The high content of cellulose at an early stage of processing may be due to readily available nutrients from swine manure which is preferred to the cellulose by microorganisms. However, as the period of processing advanced, this would be followed by exhaustion of preferred nutrients, and thus cellulose would become the predominate medium. Consequently, more cellulose utilization took place resulting in reduced cellulose content.

Most workers who attempted to isolate the organisms responsible for the primary attack on cellulose have reached the conclusion that the thermophilic bacteria which actually decompose cellulose to carbon dioxide and short chain organic compounds are obligate anaerobes (38). Claims have also been made, however, for cellulose decomposition by aerobic thermophilic bacteria. The first of these claims came from Kellerman and McBeth (53).

Cellulose content decreased as processing time increased. This decrease was accompanied by an increase in lignin especially when the vat temperature was high (approximately 66^{o}C) as shown in Figure 2. A relatively high percentage of ADF depletion had also been exhibited at this temperature (Table 2).

Under conditions described in these studies 10 to 15 days of processing seems to be a reasonable period of time to remove 50-70% of lignin from a 20 to 25 kg load of alder sawdust.

Percent of ash was taken to represent the inorganic (mineral) constituents of the samples. It has been indicated by Macdonald, et al. (76) that the ash may, however, contain material of organic origin such as sulfur and phosphorus from proteins and some loss of volatile material in the form of sodium, chloride, potassium, phosphorus, and sulfur will take place during ignition. The ash content is thus not truly representative of the inorganic material in a given sample quantitatively or qualitatively.

Results of percent of ash of Batches I, II, and III are given in Tables 2, 3, and 4 respectively. In most cases the results indicate that as the period of treatment progressed the percent of ash increased. Processed products have shown higher mineral content than supernatant samples. Negative relationship between mineral concentration and gross energy was observed (Tables 2, 3, and 4); i.e. the higher the mineral content the lower the gross energy and vice versa.

Since the major constituent of the substrate (swine manure and alder sawdust) was predominantly carbohydrate in nature, results of supernatant as well as processed products showed little variation in gross energy content (Tables 2, 3, and 4).

The value of gross energy is not consistently a good indicator of the quality of the feed product in this regard. A substantial quantity of heat can be contributed from lignin by complete oxidation of unit weight of a sample and does not occur (complete oxidation of lignin) in the animal's body.

The yield of processed product obviously depends on the volume of sawdust added and to a certain extent to the amount of swine waste used in the process.

3. In Vitro Digestibility Test

It has been documented in a number of reports that wood residues of 70 to 80% carbohydrate, are potential sources of dietary energy for ruminants. However, the intimate association of carbohydrates with the lignin renders most wood practically indigestible. It is known from this and other types of study that in general the more lignin removed from wood, the more digestible is the residual material by domestic animals.

The chemical analyses and *in vitro* dry matter digestibility results for alder sawdust are given in Tables 2, 3, and 4 for Batch I, II, and III, respectively. Values for digestibility are averages of duplicates.

As the volume of sawdust added to the treatment vat increased, the percent of digestibility increased to a certain point and declined

Table 2. Chemical composition and in vitro digestibility of supernatant and processed product of alder sawdust[a] - - Batch I.

	Amount of sawdust added[b] (kg.)	Treatment time (day)	Acid det. fiber (%)	Lignin[c] content (%)	Cellulose content (%)	Gross energy (cal/g)	Ash (%)	In vitro dry matter digestibility (%)	Vat temperature (^oC)	Yield of processed product[d] (kg)
Raw sawdust (control)	-	-	66	34	52	6298	1	19	-	-
Fresh swine manure	-	-	52	19 (44)	44	5989	12	41	-	-
Supernatant:	22	1	49	20 (41)	43	5970	9	43	59	
	-	4	46	20 (41)	26	6161	10	45	49	
	44 (66)	9	45	19 (44)	14	6309	8	47	60	
	-	11	49	17 (50)	14	6044	10	53	66	
	22 (88)	14	49	16 (53)	22	6096	10	50	67	
	11 (99)	22	44	14 (59)	16	4338	10	55	41	
Processed products:										
30 mesh		22	48	20 (41)	13	6214	6	35	-	144.0
150 mesh		22	42	33 (3)	27	5584	13	29	-	17.0
150 mesh filtrate		22	39	28 (18)	20	5538	18	24	-	2.0
Solka (purified cellulose)								75	-	-

(a) Dry basis
(b) Accumulated amount of sawdust (kg.)
(c) Figures in parentheses represent percent of delignification
(d) Air dry basis

Table 3. Chemical composition and in vitro digestibility of supernatant and processed product of alder sawdust[a] - - Batch II.

	Amount of sawdust Added[b] (kg.)	Treatment time (day)	Acid det. fiber (%)	Lignin[c] content (%)	Cellulose content (%)	Gross energy (cal/g)	Ash (%)	In vitro dry matter digestibility (%)	Vat temper-ature (^{o}C)	Yield of[d] processed product (kg.)
Raw sawdust (control)	-	-	71	32	48	6129	1	19		
Fresh swine manure	-	-	60	22 (31)	40	6285	8	48	-	
Supernatant:	44	13	49	14 (56)	31	5872	12	61	55	
	-	17	53	14 (56)	34	5790	12	63	56	
	11 (55)	26	55	17 (47)	35	5852	11	60	43	
	11 (55)	37	55	19 (41)	32	5919	13	58	25	
Processed products:										
30 mesh		37	60	17 (47)	38	5883	8	37	-	49.0
150 mesh		37	52	26 (19)	30	5531	17	28	-	11.0
150 mesh filtrate		37	53	30 (6)	25	5505	23	24	-	2.0
Solka (purified cellulose)								71	-	-

(a) Dry basis
(b) Accumulated amount of sawdust (kg.)
(c) Figures in parentheses represent percent of delignification
(d) Air dry basis

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Table 4. Chemical composition and in vitro digestibility of supernatant and processed product of alder sawdust[a] - - Batch III.

	Amoung of sawdust Added[b] (kg.)	Treatment time (day)	Acid det. fiber (%)	Lignin[c] content (%)	Cellulose content (%)	Gross energy (cal/g)	Ash (%)	In vitro dry matter digestibility (%)	Vat temperature (^oC)	Yield of[d] processed product (kg.)
Raw sawdust (control)	-	-	73	35	53	6278	3	16		
Fresh swine manure	-	-	52	23	43	6023	8	50		
Supernatant:	20	3	51	21 (40)	30	5617	17	41	46	
	-	9	47	17 (51)	33	5798	16	48	44	
	-	12	50	11 (69)	34	5497	15	50	43	
	-	15	45	9 (74)	35	5639	17	55	57	
	-	21	43	10 (71)	24	5292	19	54	47	
Processed products:										
30 mesh		21	-	-	-	-	-	-	-	
150 mesh		21	45	25 (29)	28	5235	17	30		43.0
150 mesh filtrate		21	37	28 (20)	23	4767	30	25		5.0
Solka (purified cellulose)								73		

(a) Dry basis
(b) Accumulated amount of sawdust (kg.)
(c) Figures in parentheses represent percent of delignification
(d) Air dry basis

FIG. 7

RESULTS OF DELIGNIFICATION TO CELLULOSE CONTENT AND in vitro DIGESTIBILITY IN TERMS OF TREATMENT DURATION.

BATCH I.

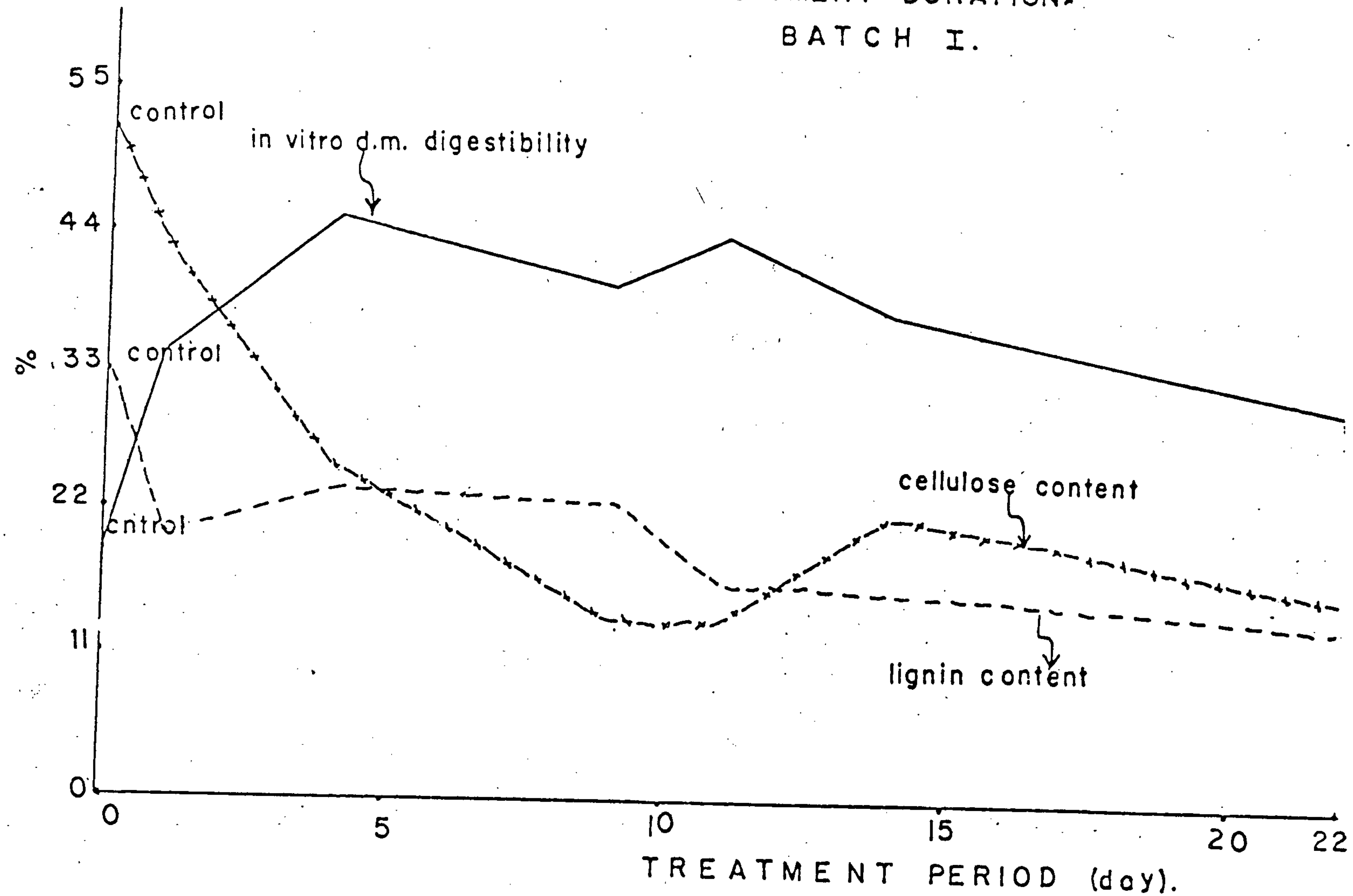


FIG. 8

R E S U L T S OF DELIGNIFICATION TO CELLULOSE CONTENT in vitro DIGESTIBILITY IN TERMS OF TREATMENT.

BATCH II.

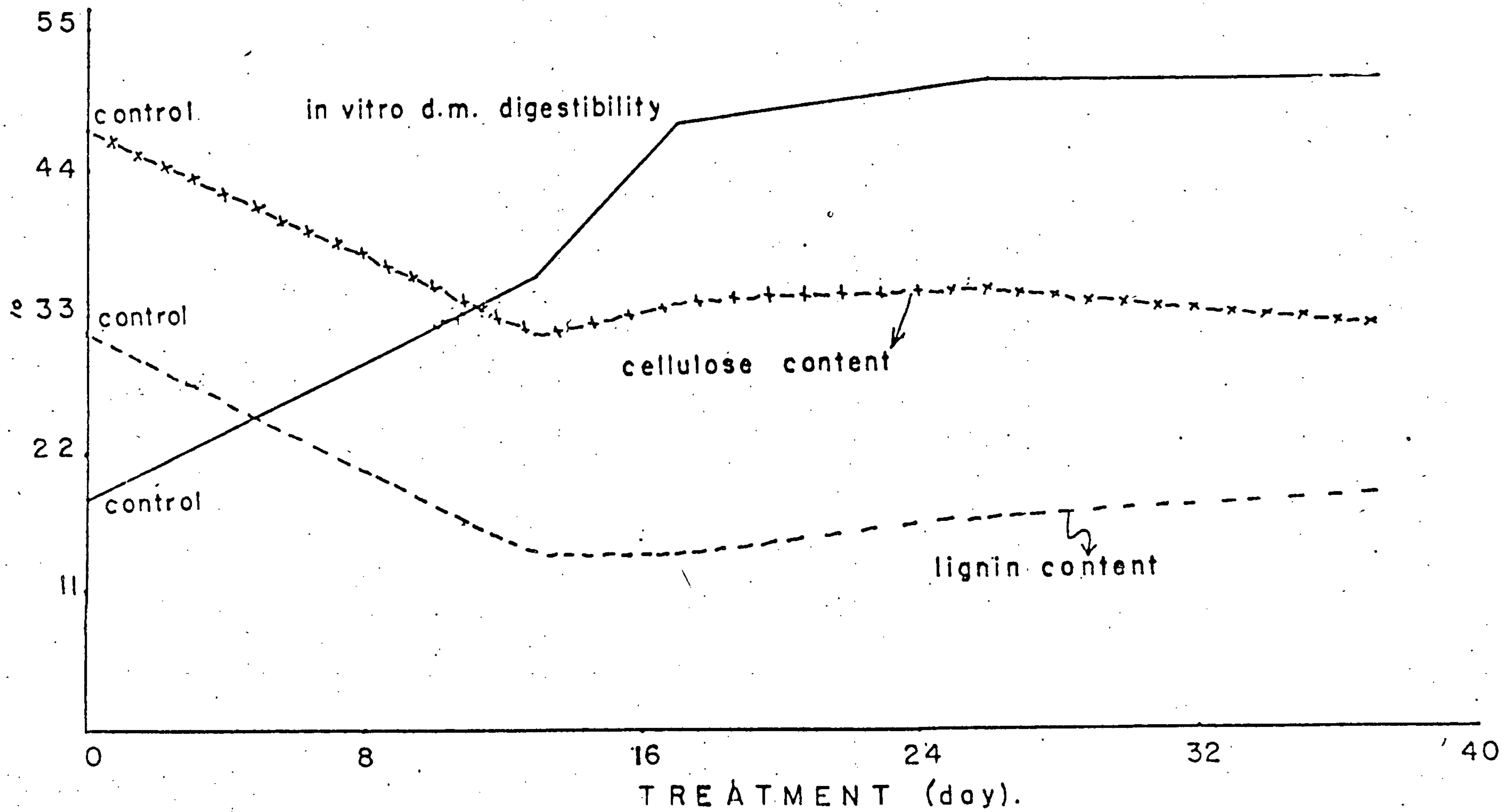
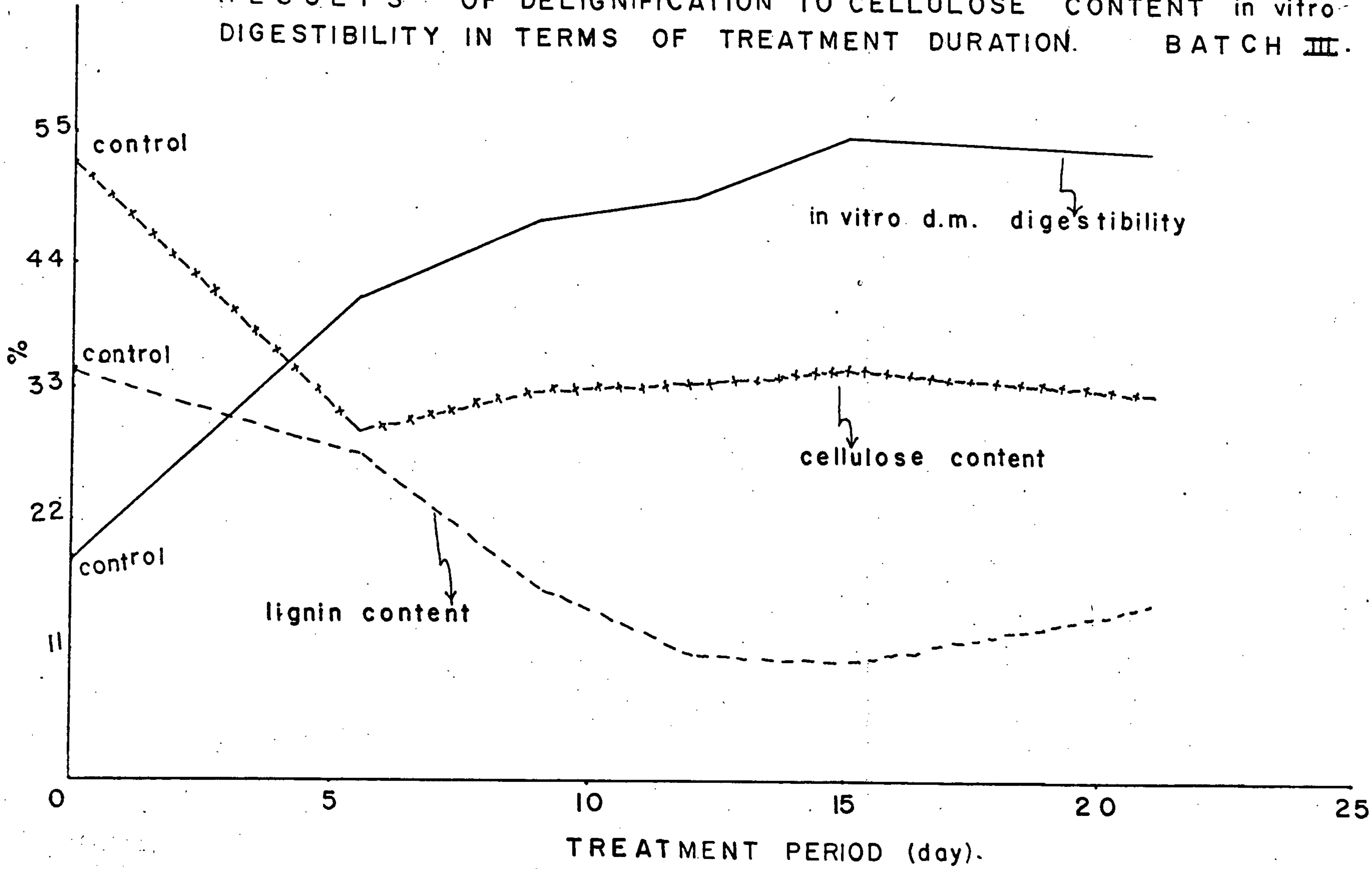


FIG. 9.
RESULTS OF DELIGNIFICATION TO CELLULOSE CONTENT in vitro DIGESTIBILITY IN TERMS OF TREATMENT DURATION. BATCH III.



as the load of sawdust further increased (Figures 7 and 8). A parallel relationship was detected between high delignification and high digestibility. However, low rate of delignification and in vitro digestibility was experienced along with low vat temperature after the sawdust volume reached 88 kg within two weeks of treatment (Figure 7).

In Batch II, the periodical addition of small amounts of sawdust accounted for an improvement in digestibility as well as delignification (Figure 8).

The result obtained from Batch III of which only 22 kg of sawdust was added at the initial stage of treatment, clearly indicate the effectiveness of microorganisms in delignification. Progressive in vitro digestion corresponding with the improvement of delignification was observed (Figure 9).

The first 10 to 15 days of processing appears to have the major influence on digestibility. High digestibility (%) was attained beyond which additional processing is of no value mostly in terms of delignification. In vitro digestibility test showed that processed sawdust is 55% digestible which is equivalent to high quality alfalfa hay in terms of animal feeding value. As a result of aerobic thermophilic bacteria treatment almost threefold increase of alder sawdust dry matter digestibility has been achieved compared to the control (19%). Some of the samples which showed low lignin content failed to show high digestibility (Tabes 2, 3, and 4). It seems reasonable to expect that the rate constant for digestion of a forage is related to the structural composition of the material; however, factors other than lignin content were involved to lower the digestibility value.

Although the experimental design did not allow the interaction between samples and other environmental variables (in addition to swine manure dosage and volume of sawdust added] to be statistically analyzed, there was an impressive trend which indicates that aerobic thermophilic bacteria treatment was more effective in increasing the _in vitro_ dry matter digestibility of alder sawdust.

Particle size in all batch studies reduced [150 mesh, 150 mesh filtrate (centrifuged product)] the _in vitro_ digestibility value as well as the gross energy value, but the ash value increased. These findings indicate high mineral and less organic matter concentration in these particular samples.

Based on these _in vitro_ digestibility observations, it can be concluded that aerobic thermophilic treatment appears to be very effective in making the nutrients in alder sawdust available to rumen microorganisms.

4. Crude Protein

Results of crude protein value of Batch I, II and III are shown in Tables 6, 7, and 8, respectively. The crude protein value of the processed sawdust was improved from 0% (control) to 17% (Table 8). The protein values for Batch I was low (Table 6) compared to Batch II and III. Before Batch II was conducted, it was assumed that some loss of nitrogen might have taken place during the drying process. To confirm this, samples were freeze dried and analyzed for crude protein value. However, no difference was observed. Therefore, Batch II was designed to determine whether long term (37 days) was a major contributing factor for the improvement of the crude protein percent or not. The result of long processing (Table 7) has shown a substantial increase in the crude protein value compared to Batch I; thus length of processing has an important role to play in regards to nitrogen value.

Figure 12 was plotted to show the level of crude protein as it may be affected by environmental variables. In most cases, high crude protein value parallels the sharp temperature rise and the high bacterial activity at the same time.

Comparatively, 150 mesh solid material and 150 mesh-filtrate have shown high crude protein value from supernatant samples. The crude protein value of Batch III was relatively high (Table 8) compared to other batches; this was mainly due to the addition of about 160 liters of partially pre-processed substrate (after the effect of 22 kg of raw sawdust towards delignification study was completed).

The percent of crude protein content (see Tables 6, 7, and 8) of the 150 mesh filtrate is higher than that of the 30 mesh and 150 mesh solids. This indicates that most of the crude protein is in the fines not captured by the 30 and 150 mesh screens. Thus, it is obvious that to recover more protein the 150 mesh filtrate is the fraction requiring further attention, since it contains the highest percentage of crude protein. In most cases, supernatant samples had higher crude protein value than 30 and 150 meshes.

An additional study was also carried out to determine the amount of solid material that remains in the supernatant after centrifugation. This was found to be only 0.7%. However, on a large scale basis this could become a substantial loss in crude protein.

The data of Batch I shows the crude protein value of the sludge reached a maximum of 5% during the 22-day period with 99 kg sawdust load (Table 6). In the case of Batch III a maximum of 17% crude protein was achieved during the 21-day period with only 20 kg sawdust load which was added at the initial stage of processing (Table 8). As it was mentioned earlier, the main contributing factor for high crude protein value in this particular batch was the addition of pre-processed sludge. Batch II required a 37 day period to reach the highest crude protein value which was 12% with 66 kg sawdust load (Table 7). Each time a given amount of sawdust was added there was a drop of 5-10^{o}C in vat temperature; and in some cases a total loss of foam which results in a 15-20^{o}C drop in temperature.

Similar batch treatment studies were conducted by Coulthard and Townsley (24) using identical materials and culturing media (swine manure). They reported on 8% crude protein as a maximum value after a 7 to 9 day period. The data of these studies indicated that the addition of sawdust in a larger volume will increase dilution rates apart from creating a disfavourable environment to the microorganisms. Thus, less cell yield and consequently longer periods of time for a given percent of crude protein (102); i.e. the percent protein of the biomass decrease within increasing dilution rate.

Regarding the quality of the single cell (S.C.) protein, Mateles, et al. (73) reported that the amino acid composition of thermophilic organisms protein is of relatively high quality. Surucu (110) commented that the nutritional value of S.C. protein is directly proportional to the protein content and inversely proportional to the nucleic acids content of the biomass. Since the percentage of protein in the biomass was higher and the nucleic acids were lower at the lower dilution rate (44), it would seem desirable to operate the system accordingly. Therefore, operation of the system at a lower dilution rate is preferable from the standpoint of the protein and nucleic acid content in the biomass, i.e. tradeoffs of dilution rates against protein should be made if maximum protein production is to be achieved.

The most common sources of nitrogen for microorganisms are organic nitrogen, nitrate, nitrite, and ammonium compounds. There are, however, some species which can utilize atmospheric nitrogen; viz. some bacteria and blue-green algae.

Table 5. Nitrogen composition of the waste and/or wood substrate at initial and final stage of processing

Batch	Total Nitrogen Composition (%)*		
	Substrate at initial stage	Processed product H	Processed product L
I	1.28	1.12(47)	0.16(11)**
II	1.12	2.08(65)	1.44(56)
III	1.28	3.36(72)	2.08(62)

*Dry matter basis

**Figures in parentheses implies total nitrogen in percent

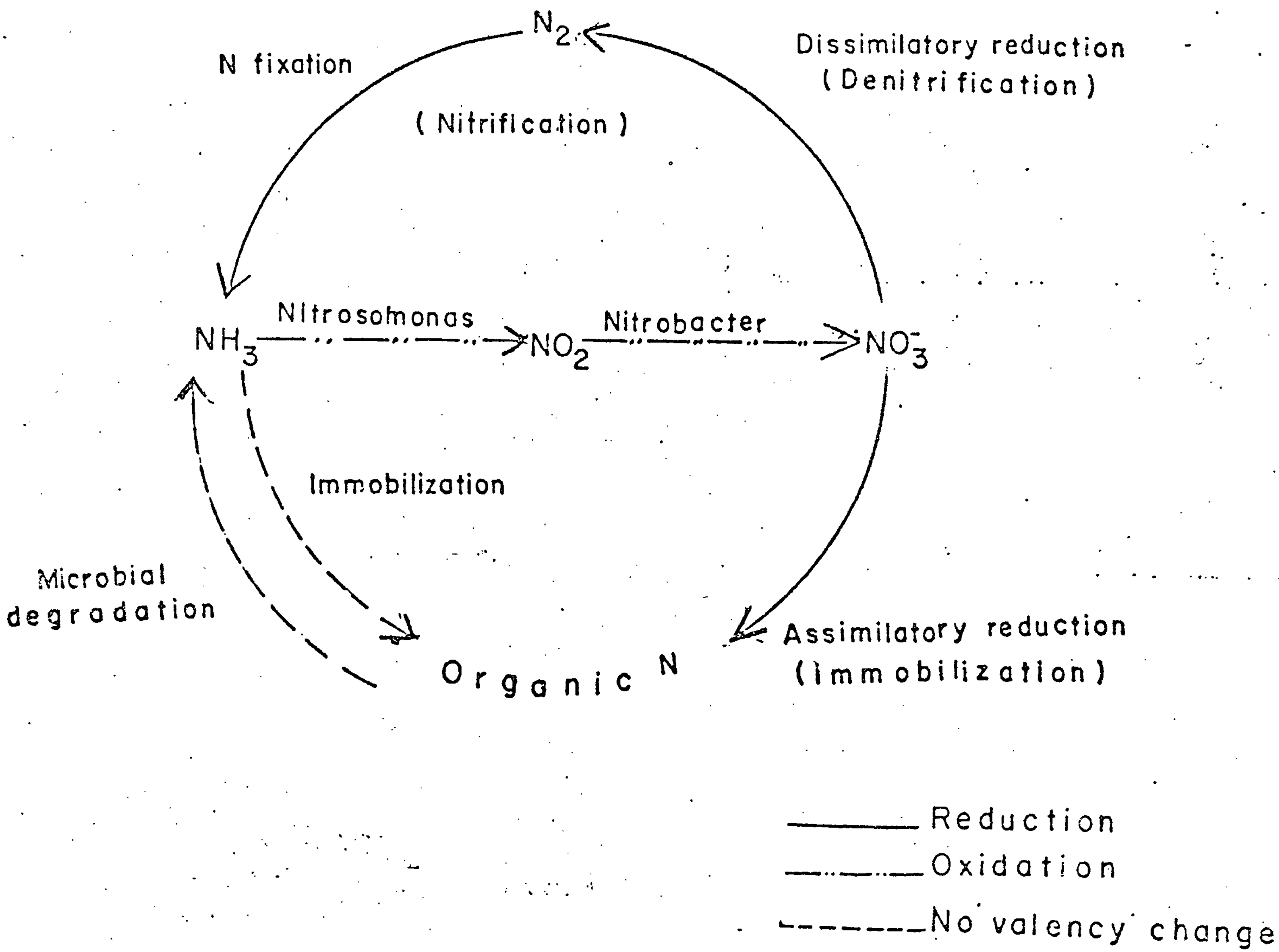
H = Highest
L = Lowest

Results of Batches II and III indicated an increase of 65% to 72% (Table 5) respectively of total nitrogen in the final processed product. The organic nitrogen contribution from the swine waste was only 35% for Batch II and 28% for Batch III (Table 5). But in the case of Batch I, the results showed a 6% reduction in total nitrogen content (Table 5) from the initial nitrogen content of the substrate. Thus, denitrifying organisms might have been involved in the fermentation process compared to nitrifying organisms. Since the crude protein percent value of the sawdust was nil (Tables 6, 7, and 8) the supply of organic nitrogen from this source was negligible.

The data of this study (Batch II and III) indicate that microorganisms particularly those capable of fixing atmospheric nitrogen have contributed a substantial amount of nitrogen to the total nitrogen content of the processed product. Linday (66) stated that atmospheric nitrogen fixation by microorganisms together with electrical discharge in the atmosphere, by which molecular nitrogen can under normal circumstances, be made available for the majority of living things.

The general mechanism of the nitrogen cycle is shown in Figure 10. Nitrate is the most common inorganic source of nitrogen in the aerobic environment. This is due to the rapid oxidation of ammonia to nitrate by bacteria (lithotrophic) Levy, et al. (65). Nitrogen fixation is accomplished both by aerobes and anaerobes. Levy et al. (65) stated that a key nitrogenous compound in microbial nutrition is inorganic ammonium, for it combines with organic acids (d-keto acids) to form amino acids. The principle product of this event is glutamic acid. Further in their statement regarding the mechanism of nitrification, they also mentioned that many bacteria possess this ability and have the additional enzymes to form all of their nitrogen-containing cellular constituents from glutamic acid. Microorganisms, therefore, can use inorganic ammonium salts as their sole source of nitrogen. The utilization of nitrate by microorganisms as a source of nitrogen for cellular synthesis entails the reduction of nitrate to the oxidation level of ammonia; at which it can be incorporated into amino acids and other nitrogen-

Fig. 10. THE NITROGEN CYCLE _ LEVY *et al.* 1973.



containing cell constituents.

As shown in the nitrogen cycle (Figure 10), nitrogen fixation is a reduction process and a supply of potential reducing agents is required. Levy (65) stated that the nitrogen-fixing aerobes (the hetertrophic Azotobacter species, the symbiotic Rhizobia, and the photosynthetic blue-green algae) carry out the reaction in an essentially anaerobic microenvironment. The nitrogenase enzyme system that performs the reduction is in all cases oxygen sensitive. It seems likely that the nitrogenase is protected from oxygen by a very high rate of respiration in Azotobacter, which limits the access of oxygen to the site of nitrogen fixation.

As it was mentioned earlier, the fixed nitrogen finally becomes ammonia, and is then combined to form amino acids, and thence to protein. Degradation of nitrogenous material, for example, dead cells, follows the reverse pathway down to ammonia; in which form it may be released or rebound into protein again. Organic nitrogen compounds yield ammonia when they decompose. This is primarily a hydrolytic reaction involving no valency (65).

Mischustin and Shilnicova (80) have reported that the nitrogen fixing bacteria (Azotobacter chrooccum) is effective within pH range (6.2 to 8.8) which in the case of all batches of this study the pH was within the reported range.

There are also present denitrifying organisms which reverse this process, releasing atmospheric nitrogen. Thus some organisms fix nitrogen for themselves, and this becomes available for others after cell death and breakdown. Whilst others directly convert it to a form more available for other organisms (66). Regarding denitrification, Levy (65) stated that denitrification reaction characteristically carried out by aerobes that find themselves in an anaerobic environment. If there are oxidizable carbon compounds present these will be oxidized and nitrate is utilized as the terminal electron acceptor instead of oxygen. There is a possibility that such a reaction took place in Batch I; because the final processed product has shown a decrease of 13% to 88% in total nitrogen content from the initial stage (Table 5).

Table 6. Results of crude protein value and environmental variables in terms of sawdust volume and treatment time - - Batch I

	Amount of sawdust added (kg.)[a]	Treatment time (day)	Dry matter[b] (%)	Crude protein[c] (%)	Vat temperature (^oC)	pH	Dissolved oxygen (mg/1)
Raw sawdust (control)	-	-	86	0	-	-	-
Fresh swine manure	-	-	97	8	-	-	-
Supernatant:	22	1	97	3	59	8.2	2.4
	-	4	96	4	49	8.4	3.0
	44 (66)	9	97	3	60	6.8	0.4
	-	11	96	4	66	7.2	0.6
	22 (88)	14	96	4	67	7.7	0.8
	11 (99)	22	94	5	41	7.8	2.0
Processed products:							
30 mesh	-	22	93	1			
150 mesh	-	22	89	2			
150 mesh filtrate	-	22	93	7			

(a) Accumulated amount of sawdust (kg.)
(b) Average of triplicate sample
(c) Dry matter basis and average of triplicate sample

Table 7. Results of crude protein value and environmental variables in terms of sawdust volume and treatment time - - Batch II

	Amount of sawdust added(a) (kg.)	Treatment time (day)	Dry matter(b) (%)	Crude(c) protein (%)	Vat temperature (^oC)	pH	Dissolved oxygen (mg/l)
Raw sawdust (control)	-	-	86	0			
Fresh swine manure	-	-	95	7			
Supernatant:	44	13	93	9	55	8.34	0.7
	-	17	93	9	56	8.47	3.9
	11 (55)	26	92	11	43	8.29	5.5
	11 (66)	37	93	12	25	7.36	5.0
Processed products:							
30 mesh	-	37	93	9			
150 mesh	-	37	93	11			
150 mesh filtrate	-	37	93	13			

(a) Accumulated amount of sawdust (kg.)
(b) Average of triplicate sample
(c) Dry matter basis and average of triplicate sample

Table 8. Results of crude protein value and environmental variables in terms of sawdust value and treatment time - - Batch III

	Amout of sawdust added (kg.)	Treatment time (day)	Dry matter (%)[a]	Crude protein (%)[b]	Vat temperature (^oC)	pH	Dissolved oxygen (mg/l)
Raw sawdust (control)	-	-	88	0			
Fresh swine manure	-	-	97	8		6.9	
Supernatant:	22	3	96	15	46	7.8	1.0
	-	9	93	15	44	8.17	2.5
	-	12	96	13	43	8.75	4.4
	-	15	94	15	57	8.20	2.2
	-	21	94	17	47	6.51	3.3
Processed products:							
30 mesh	-	21	-	-			
150 mesh	-	21	94	13			
150 mesh filtrate	-	21	92	21			

(a) Average of triplicate sample
(b) Dry matter basis and average of triplicate sample

FIG. 11

THE RATE OF DELIGNIFICATION AND ENVIRONMENTAL VARIABLES.
(THREE BATCHES COMBINED)

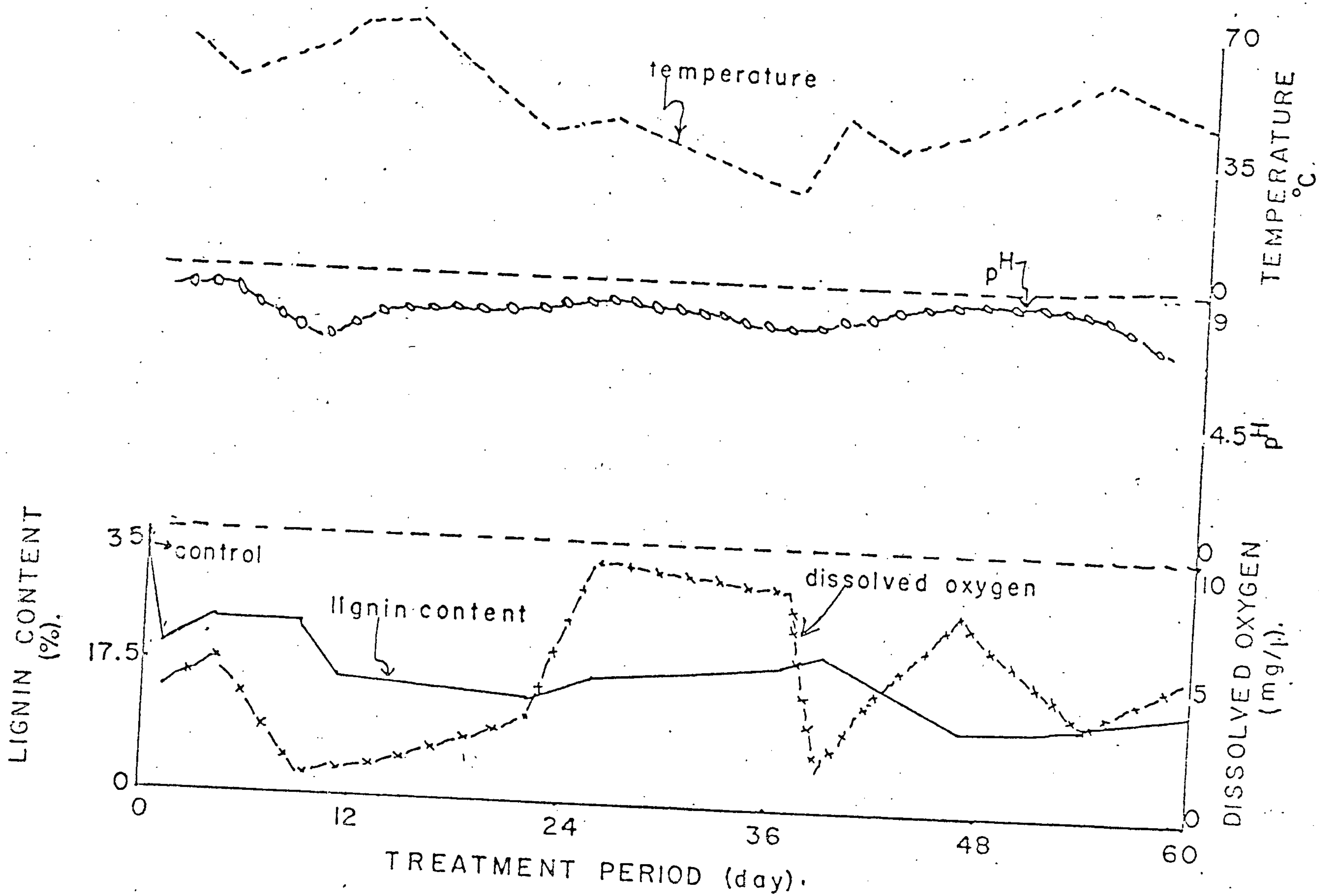
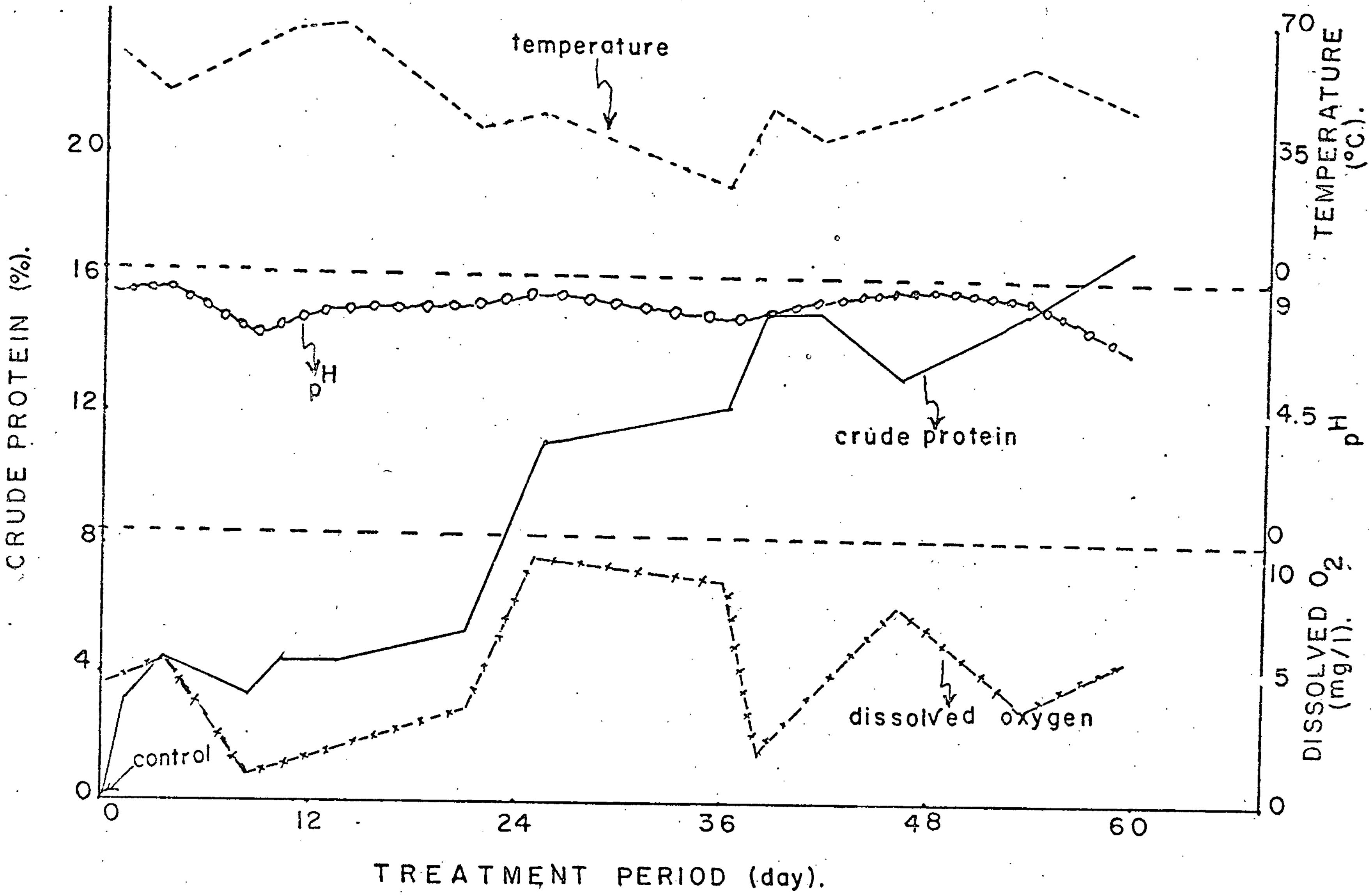


FIG. 12.
RESULTS OF CRUDE PROTEIN VALUE AND ENVIRONMENTAL VARIABLES IN TERMS OF TREATMENT OF THE THREE BATCHES COMBINED.



V. SUMMARY AND CONCLUSION

Aerobic thermophilic bacterial treatment of sawdust material was studied employing a batch system. Swine manure was used both as a source of inoculum and culture media.

The high temperature which was produced solely from microbial action promoted the sawdust delignification rate to a satisfactory degree. The decreases in lignin content of the sawdust was associated with a substantial increase in digestibility.

Studies concerning the effect of longer periods of fermentation on percent protein in the biomass showed that the percentage of protein in the biomass increased with the longer period of treatment and with the decreasing dilution (less addition of sawdust) rate.

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