TWO POTENTIAL APPLICATIONS OF BACTERIAL CELLULOSE PRODUCED BY A. XYLINUM

by

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ABSTRACT

The focal point of this research project was the development of two applications of cellulose produced from bacterial cultures of Acetobacter xylinum. The first part of this study relates to the culture method. Selection of influential factors for maximum cellulose production in defined medium was carried out by using Taguchis' L_{27} (3^{13}) Fractional Factorial Design. Sucrose and peptone concentrations and pH were found to be significant sources of variation on cellulose yields. Stability of three strains of A. xylinum were assessed over serial transfers of static and swirled cultures. It was concluded that all the strains tested were unstable under swirled conditions, ATCC 14851 being the most stable of the three when grown under static conditions.

Growth curves of the three strains were studied and cellulose yields, pH values, sugar utilization, sugar conversion and nitrogen content in dry cellulose were determined over a 40 day incubation period. Comparison of the growth curves of A. xylinum strains showed that this organism varied widely in its efficiency of converting sugar to cellulose. The degree of polymerization (D.P.) values of the cellulose synthesized by two of the strains were followed over an incubation period of 32 days. The D.P. values of both strains appeared to decrease with incubation time.

The second aspect of this study was to develop a process that integrates cellulose fibrils into a cotton-like fibre. Since A. xylinum naturally produces cellulose in the form of highly fibrillar ribbons, a process was devised to mechanically direct bacterial cells to spin these ribbons into oriented parallel filaments by cultivating the organisms in a straight-line flow path. Aeration rates in the system appeared to influence growth and cellulose yields. Flow mode (intermittent and continuous) and A. xylinum strain were shown to be highly significant sources of variation on the tensile strength of fibres. Optimum conditions for the mercerization treatment of fibres for achieving maximum tensile strength were determined by using a mapping super simplex optimization technique. Tensile properties of mercerized and unmercerized fibres were studied by conducting load-elongation tests to specimen failure. Light and electron microscopy were employed to observe the fine fibre structure.

Finally, the third aspect of this study consisted of the development of a process for the purification and hydrolysis of A. xylinum cellulose for the manufacture of microcrystalline cellulose (MCC). Chemical composition and physical properties of the spray dried MCC were determined. The flow behaviour of A. xylinum MCC dispersions was examined and compared to commercial Avicel PH-101 MCC. Rheograms of both MCC dispersions displayed non-Newtonian pseudoplastic behaviour. A. xylinum MCC dispersions were found to have rheological behaviour of the thixotropic type, that is, there was a reversible change in viscosity with time at a constant rate of shear.

Results of this study indicated that it is technically feasible to produce synthetic cotton-like fibres by stimulating A. xylinum cells to extrude their cellulose ribbons in a parallel fashion. A. xylinum cellulose appeared to be a good source of raw material in the manufacture of microcrystalline cellulose because it is a highly purified form of cellulose which can be made available all year around at any location.

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This thesis is dedicated to my husband for his support and enthusiasm throughout this study.

INTRODUCTION

The cellulose formed by A. xylinum is a well known standard type, similar to the highly crystalline homopolymer group which has been represented classically by the alpha cellulose or cellulose I of cotton. The cellulose is also free of lignin, hemicellulose, pectin or other encrusting substances. The cellulose microfibrils accumulate exclusively in the extracellular phase where they form a hydrophilic membranous pellicle. The gas-liquid interface, as the site of pellicle formation, permits the possibility of large scale cellulose membrane production. The cellulose can be readily freed from bacterial cells thereby separating product from substrate.

A wide variety of substrates can be utilized for cellulose synthesis increasing the potential flexibility of utilization of agricultural wastes, such as beet molasses, blackstrap molasses, coconut milk, pineapple pulp trimmings, and sulfite waste liquor. The simplicity of cellulose production suggests that large scale microbial cellulose production plants could be located near sources of convenient and inexpensive substrates.

Over the last two decades research into cellulose biosynthesis by A. xylinum has increased, since it was through this organism that cellulose assembly in living systems was first observed and monitored. Utilization of A. xylinum cellulose for industrial purposes however has been scarcely explored, in spite of its great potential for large-scale production.

In the last two years increased attention has been given to industrial applications of bacterial cellulose. In 1982, Mynnat patented a process for fiber production from continuous cultivation of microorganisms, pertaining to the production of cellulose fibres for use in the manufacture of paper.

Brown (1983) patented a process for the production of a cellulose-synthetic polymer composite fibre. The invention took advantage of the cellulose produced by Acetobacter xylinum in that it was possible to produce cellulose on the surface of a polyester fibre, which conferred to the fibre many of the physical properties of cotton. The composite polymer produced according to that procedure provided a whole new approach to the manufacture of "cotton-like" goods.

The purpose of this thesis was to develop two applications of A. xylinum cellulose. One entailed the production of a "cotton-like" fibrous strand. This was successfully achieved by mechanically directing A. xylinum organisms to spin cellulose ribbons into parallel oriented strands.

The second application developed was in the use of A. xylinum cellulose as the raw material for the production of microcrystalline cellulose (MCC). The multiple uses of MCC in the food and pharmaceutical industries, among others, indicate the potential for utilizing a highly purified form of A. xylinum cellulose as a raw material.

LITERATURE REVIEW

A. *A. Xylinum* Cellulose Production

As long ago as 1886 Brown described a bacterium which produced a solid membrane when growing on a carbohydrate rich medium. It was found that these membranes were soluble in ammoniacal copper hydroxide and yielded reducing sugars when hydrolyzed with sulfuric acid. Since it was known that cotton produced these reactions, the organism was called *Acterium xylinum* (xy' li•num. Gr. adj. xylinus of cotton; L. neut. n. xylinum cotton). The similarity between *A. xylinum* cellulose and cotton cellulose was confirmed by the production of well oriented x-ray diffractograms of stretched bacterial membranes which were identical to those of cotton. Electron micrographs showed that these bacterial membranes consist of a thick network of fibres and ribbons (Muhlethaler, 1949) similar to that of cellulose from the cell walls of numerous plants.

1. Description of Acetobacter xylinum

The genus *Acetobacter* refers to a group of bacteria that have the ability to oxidize ethanol to acetic acid. In particular, the bacterium *Acetobacter xylinum*, noted for its ability to produce vinegar, is usually found in wine vats as " ... a sort of moist skin, swollen gelatinous and slippery ..." It has been referred to in the past as "mother of vinegar" and was the basis of the early vinegar industry (Shramm and Hestrin, 1954). The gelatinous skin is actually a polysaccharide matrix within which the bacterial cells are enmeshed, and is more commonly

known as a pellicle. Because A. xylinum is an obligate aerobe requiring a constant supply of oxygen, it can be speculated that the function of the polysaccharide pellicle is to provide a buoyant environment at the air-liquid interface (Schramm and Hestrin, 1954).

A. xylinum is a rod-shaped non-motile, gram negative bacterium which occurs singly and in chains. The individual cells, which are enveloped by a gelatinous material, measure 2 μm long and 0.1 μm wide. The polysaccharide pellicle forms on all liquid media in which growth occurs. The nature of the medium influences the thickness of the film which may vary from 2 to 250 mm. The pellicle becomes cartilagenous and falls to the bottom if disturbed. The optimum growth temperature of A. xylinum is 28°C (Breed et al., 1957). This organism forms acid from glucose, ethanol, propanol and glycol; but it does not produce acid from arabinose, fructose, galactose, maltose, lactose, raffinose, dextrin, starch, methanol, isopropanol, butanol, isobutanol, pentanol, mannitol or acetaldehyde. It produces catalase (positive); litmus milk is not changed; indole is not formed, gelatin is not liquified; and it oxidizes acetic acid to carbon dioxide and water (Lapuz et al., 1967).

The morphological and physiological characteristics show that this organism belongs to the genus Acetobacter, which according to Breed et al. (1957), was classified as Genus II of the family Pseudomonadacea. The species of Acetobacter may be differentiated from all other Pseudomonadacea by their unique ability to oxidize significant quantities of ethanol under the extremely acidic conditions imposed by the presence of from about 2 to more than 11 percent acetic acid. Acetobacter species

are aerobic, strongly catalase positive, and oxidative in their physiology. Within the genus Acetobacter, two groups of species were recognized. The first group consists of species capable of oxidizing acetic acid to carbon dioxide and water while the second group does not have this ability. A. xylinum belongs to the first group under which five species have been recognized. The distinction between the A. aceti and the other acetic acid oxidizing species is based mainly on growth in Hoyer's medium, where ethanol is the sole carbon source. The inability of A. xylinum to utilize ammonium salts as a sole source of nitrogen (no growth in Hoyer's medium) is due only to the lack of adequate nutrients.

A. xylinum will utilize ammonium salts if glucose is supplied. Finally, a distinctive character within the classification is the production of a thick, leathery, zoogleal, cellulosic membrane on the surface of liquids.

Buchanan and Gibbons (1975) reclassified the genus Acetobacter under the "Genera of Uncertain Affiliation".

2. Acetobacter xylinum cultures

The range of utilizable substrates for cellulose production by resting cells includes hexoses, their anhydrides and compounds which presumably yield hexoses as a result of bacterial action. When the carbon compound used is not a free hexose, nor one apparently capable of conversion into a hexose, as in the case of pentoses such as arabinose and xylose, glycols, polyglycols and erythritol, no membrane formation

takes place. On the other hand mannitol, which is known to undergo oxidation with the formation of fructose under the experimental conditions, gives rise to high yields of polysaccharide. Glycerol behaves similarly, presumably due to a primary oxidation to dihydroxyacetone and conversion of this to fructose. Galactose is much less reactive than glucose, while mannose appears to be relatively inactive. The highest yield of the polysaccharide was obtained from fructose, a result presumably connected with the recognized fact that this bacterium forms little or no acid from this sugar (Tarr and Hibbert, 1931).

Work of this nature was continued by Kaushal and Walker (1951) who studied three other species of Acetobacter. A strain of A. acetigenum, isolated from East African vinegar, formed cellulose from all of the eighteen carbohydrates submitted to its action. By its ability to convert to cellulose α- and β-methyl-D-glucosides, three pentoses, erythritol and ethylene glycol, respectively, the organism showed itself to be enzymatically more active than A. xylinum which proved unable to utilize these substances for this purpose (Tarr and Hibbert, 1931).

As almost all earlier work was done with growing cultures, it did not distinguish between a possible effect of substrate on cell growth from that on cellulose production. This source of ambiguity was eliminated by using washed non-proliferating cells (Gromet et al., 1957). Hexoses (glucose and fructose), three carbon compounds (glycerol and dihydroxyacetone) and hexonates (gluconate, 2- and 5-oxogluconate) have been converted into cellulose by the action of washed cells of A. xylinum. Acetate, pyruvate and citrate-cycle intermediates were oxidized

without attendant conversion into cellulose. Phosphate esters (including glucose 6-phosphate, α-glucose 1-phosphate, β-glucose 1-phosphate and uridine diphosphoglucose) were presumably not taken up by the cells. Exogenous addition of such esters did not result in the formation of cellulose. The range of substrates for both cellulose and CO_2 formation depended on the cell history and on the conditions of the assay.

The work of Dudman (1959b) paid closer attention to the influence of growth conditions on cellulose yield. Acetate, citrate and succinate were highly effective in stimulating cellulose synthesis by A. acetigenum. Ethanol stimulated growth but did not increase cellulose yield. It was found that appropriate concentrations of succinate could cause a large increase in cellulose production. Thus it was postulated that succinate increased cellulose yield by specifically stimulating cellulose synthesis rather than growth in general. It was proposed that succinate was involved in a sparing mechanism where it is preferentially oxidized in place of some sugar substrates, which are then freed for cellulose synthesis. The hypothesis was supported by previous observations of Schramm et al. (1957) in which acetate, citrate, succinate and other intermediates of the tricarboxylic acid-cycle were all readily oxidized to CO_2 by washed suspensions of A. xylinum. It was also noted that the buffering effects from the addition of succinate may have been a factor in the increased cellulose yield.

An adaptive effect of carbohydrate composition of the growth medium on the substrate range was illustrated by the synthesis of

cellulose from pyruvate by succinate grown cells of A. xylinum (Benziman and Burger-Rachaminou, 1962). Their observations indicated that the anhydroglucose carbon chain of cellulose arises from pyruvate in A. xylinum via a condensation involving two molecules of a three carbon compound.

Studies by Weinhouse and Benziman (1974) revealed that A. xylinum cells readily oxidized succinate, pyruvate and acetate to CO_2, and synthesized cellulose from succinate and pyruvate when they were at relatively high concentrations. Furthermore, any of these tricarboxylic acid-cycle intermediates promoted cellulose synthesis from fructose and gluconate, although retarding their oxidation to CO_2.

Weinhouse and Benziman (1976) further showed that glycerol and dihydroxyacetone can be utilized by A. xylinum as a source of carbon and energy for growth and cellulose synthesis. Glycerol utilization by cells of A. xylinum was accompanied by the formation of dihydroxyacetone, cellulose, CO_2 and small amounts of acetate.

Dudman (1959a) published the first study concerning the conditions which gave maximum cellulose yields with inexpensive and readily available substrates, with a view to possible large-scale production of this polysaccharide. Different carbohydrate substrates were compared for cellulose production by A. acetigenum and six strains of A. xylinum. Hydrolyzed molasses was found to give the largest yields. Cellulose yields varied over a wide range, equivalent to conversions of 1.9 to 23.5%. Cellulose synthesis stopped when growth stopped, even when the sugar in the medium was not exhausted, indicating that cellulose was synthesized only by the actively growing organism.

In conjunction with the observations previously described on cellulose production by A. acetigenum in complex media, a study was conducted on growth and cellulose production in defined media (Dudman, 1959b). Growth and cellulose production by A. acetigenum was limited by nitrogen concentrations below 0.01% (W/V) in glucose defined medium. Ammonium sulfate and asparagine + glutamic acid (50% of each) were equivalent as nitrogen sources when compared on a nitrogen weight basis over the range 0.001 - 0.1% N. At higher concentrations, ammonium sulfate decreased growth and cellulose synthesis, while asparagine + glutamic acid became stimulatory. There was no direct evidence to suggest the mechanism by which the amino acid mixture became stimulatory, but it was proposed that it may have been the result of deamination, by the organism, of some of the asparagine and glutamic acid to oxalacetic and ∝-ketoglutaric acids, respectively. Because these are intermediates in the tricarboxylic acid cycle, they would probably act in the same way as acetic, citric and succinic acids. Indirect evidence of this was the decreased recovery, in the amino acid cultures, of utilized nitrogen as cell-N with the increased concentration of nitrogen in the medium.

With the goal of utilizing coconut milk, which is wasted in places where copra is produced, Lapuz et al. (1967) found that supplemented coconut milk is an excellent growth medium for A. xylinum. Cultural requirements included: a nutrient medium, preferably one containing an ingredient with growth promoting factors such as those present in coconut water, temperature, about 28°C; pH 5.0 to 5.5; nitrogen source,

ammonium salts preferably monobasic ammonium phosphate ($NH_4H_2PO_4$); carbon source, glucose and sucrose.

Among other substances that stimulate cellulose formation, one of the more interesting ones is the cellulosic optical brightener Calcofluor White. The use of Calcofluor in the study of Acetobacter species can be traced to Maeda and Ishida (1967), who first reported the specificity of its binding with cellulose. It was because of this specificity that Haigler et al. (1980) originally utilized Calcofluor for observing microfibril assembly in A. xylinum.

Haigler et al. (1980) reported that Calcofluor disrupted the assembly of crystalline cellulose I microfibrils and their association into composite ribbons. This disruption was traced to alterations of hydrogen bonding between glucan chains by the Calcofluor which produced a non-crystalline type of cellulose. Because this non-crystalline cellulose could easily be made crystalline by washing out the Calcofluor and drying the product, the use of Calcofluor demonstrated that it was possible to separate the processes of polymerization and crystallization in the assembly of cellulose microfibrils. Benziman et al. (1980) found that the addition of Calcofluor to growth medium increased the rate of glucose polymerization into cellulose. Since there were not significant increases in general bacterial activity, it was believed that energy which normally was used by cell-directed crystallization was available for increased glucose polymerization.

Schramm and Hestrin (1954) examined the behaviour of A. xylinum in shaken cultures, and reported that although the formation of the

pellicle was prevented, growth occurred instead in the form of stellate bodies. Cellulose production was less than half that in the static cultures grown under the same conditions. It was also reported that agitated conditions favoured the growth of mutants unable to synthesize cellulose and that prolonged culture or repeated subculture on the shaker led to complete failure in the production of cellulose.

Dudman (1960) reported that the normally slow rates of growth and cellulose synthesis by certain strains of A. xylinum in static cultures were accelerated only when growth was carried out under conditions that prevented the formation of pellicles. The dependence of the increased growth rate on obtaining growth in a finely divided state, in which the effective surface area of the solid mass in contact with the medium was greatly increased, suggested that the slow growth under static conditions was caused by the slow rate of penetration of oxygen and nutrients into the pellicle. As cellulose synthesis in static cultures takes place mainly in the upper part of the pellicle, it was likely that the limiting process in static cultures was the rate of diffusion of oxygen into the pellicle. Accelerated growth was obtained, however, at the expense of decreased cellulose yields.

Yields of cellulose in stirred, aerated fermenters decreased with increasing air flow rates, although the growth level remained constant (Dudman, 1960). Results suggested that increased aeration decreased the yield of cellulose by causing decreased synthesis of cellulose per unit cell weight. The inverse relationship between aeration and cellulose yields may be interpreted to mean that increased aeration leads to

a shift in the metabolism of the organisms away from cellulose synthesis towards increased oxidation of the sugar substrate. It is well established, however, that aerated cultural conditions favour the selection of celluloseless mutants of cellulose-producing strains (Schramm and Hestrin, 1954). Therefore, both mechanisms were suggested to be involved depending on the strain and cultural conditions.

The specialists who cultivate A. xylinum under laboratory conditions have wondered for a long time whether the production of cellulose in a tough and leathery pellicle on a suitable liquid medium serves a useful purpose for the organism when it grows in nature. Generally two different views have been offered. The first is that the web of cellulose microfibrils affords some support or advantage for the enmeshed cells in liquid media. The second is that production of cellulose by this bacterium is essentially an accident of evolution without benefit or detriment to the organism.

Cook and Colvin (1980) supported the first view, and reported that cultures of cellulose deficient cells of A. xylinum which were isolated from solid medium revert from the cellulose-deficient condition to the normal cellulose producing form after five transfers in liquid medium. In addition, serial cultures of initial mixtures of cellulose-deficient cells and normal cells in the ratio of 9 to 1 showed a rapid decrease in the proportion of deficient cells, so that after seven transfers in liquid medium, there were less than 1% of cellulose-deficient cells. These results demonstrated that in liquid medium, cells which were normal in cellulose production overgrew those which were deficient in

this capacity. This suggests that cellulose production in liquid medium helped this obligate aerobe to obtain a limited supply of oxygen by floating the cells close to the surface.

3. Cellulose biosynthesis

a. Intracellular processes

The bacterium A. xylinum has served as a model organism for studying cellulose biogenesis, namely, the biosynthesis of linear β 1,4-glucan chains and their crystallization into cellulose fibrils. The polymeric product, formed in abundance (up to 45% of all added glucose), is deposited extracellularly and can be readily visualized (Hestrin and Schramm, 1954; Brown et al., 1976). Synthesis proceeds without a lag period, at a rate that it is linear with respect to cell concentration, and is essentially irreversible (Hestrin and Schramm, 1954; Ohad and Hestrin, 1962). Cellulose formation is not indispensable to the metabolic utilization of carbohydrates, either for energy or for carbon. Net protein synthesis is not required for cellulose synthesis in resting cells, since the process can proceed normally in the absence of a nitrogen source (Hestrin and Schramm, 1954) and is not affected by the presence of protein synthesis inhibitors (Webb and Colvin, 1967). Cellulose production is conditional on concurrent oxidation processes, and utilizable substrates for cellulose synthesis are all metabolically associated with the two amphibolic pathways operative in A. xylinum: the pentose cycle for the oxidation of carbohydrates and the citrate cycle for the oxidation of organic acids and related compounds (Gromet et al., 1957).

Two different approaches have been offered towards the understanding of biosynthesis of cellulose in A. xylinum. Firstly, an indirect in vivo approach using whole cells included studies on the patterns of isotopic distribution and label retention in the cellulose formed by resting cells from substrates specifically labelled with carbon 14 (Schramm et al., 1957; Weinhouse and Benziman, 1976; Cooper and Manley, 1975a; Swissa et al., 1980). A second approach has been to effect the separation of the synthesizing enzyme system from the structure of the cell (Glasser, 1958; Ben-Hayyim and Ohad, 1965; Swissa et al., 1976; Swissa, 1978; Cooper and Manley, 1975b).

The two approaches to the study of cellulose biosynthesis indicated that the initial steps of the process involved the following sequence:

$$\alpha\text{-D-glucose} + \text{ATP} \rightleftarrows \alpha\text{-D-glucose-6-phosphate} + \text{ADP}$$

$$\alpha\text{-D-glucose-6-phosphate} \rightleftarrows \alpha\text{-D-glucose-1-phosphate}$$

$$\alpha\text{-D-glucose-1-phosphate} + \text{UTP} \rightleftarrows \text{uridine diphosphoglucose} + \text{inorganic pyrophosphate}$$

The probable existence of intermediary stages beyond UDP-glucose on the pathway to cellulose has also been indicated. The synthesis, in vivo, of fibrillar cellulose and in vitro of β 1,4-glucan is accompanied by formation of glycolipids that appear to contain in their carbohydrate portion either glucose or β 1,4-linked glucose moieties (Aloni and Benziman, 1982).

Until a few years ago, it was generally believed that the synthesis of crystalline cellulose microfibrils by A. xylinum was an

extracellular process (Colvin, 1971, 1972). According to these ideas, cellulose fibrils were randomly deposited in the surrounding medium and were not appendages of the bacterial cell (Ben-Hayyim and Ohad, 1965).

However, several reports (Brown et al., 1976; Forge and Preston, 1977; Zaar, 1977, 1979) strongly support the concept that each cell synthesizes a single ribbon of cellulose composed of crystalline microfibrils which elongate continuously, in association with multiple synthesizing sites organized in a row along the longitudinal axis of the cell that, in turn, are associated with corresponding extrusion sites in the lipopolysaccharide (LPS) layer of the bacteria. Accordingly, the glucan chains comprising the microfibrils are synthesized in inmediate contact with the cell envelope and must be physically attached to the bacterial surface (Colvin and Leppard, 1977; Colvin et al., 1977). Moreover, it is at this end of the growing glucan chain, which is probably the reducing end, where the incorporation of the new glucosyl residues occurs (Brown, 1979).

Compatible with this concept is the idea that peripheral hydrophobic proteins are the anchoring sites for the growing glugan chains. The formation of β 1,4-polyglucan chains would thus require the cooperative action of distinct enzyme systems which promote the generation of UDP-glucose, transport of active glucosyl units to phospholipid carriers, transfer of glucosyl or cellodextrin to hydrophobic glucoproteins, and transfer of cellodextrins to "anchor protein" in the outer cell wall (Aloni and Benziman, 1982).

It has been proposed by Brown and Willison (1977) that the enzyme systems which catalyze the multiple sequence of reactions leading to the

synthesis of cellulose are arranged as a multienzyme complex, traversing the plasma membrane, the periplasmic space, and the outer LPS layer. Since a large number of glucan chains constitute the microfibril, and each chain is supposed to have an independent terminus in the bacterial envelope, a multisubunit enzyme complex would be expected to participate in the simultaneous synthesis of a number of glucan chains. The glucose polymerizing capacity within each subunit will, in turn, depend on the availability of substrates and on the physical integrity and proper spatial arrangement of its diverse enzymatic and carrier components. The cooperative action of the multitude of synthesizing units thus constitutes the overall cellulose-synthesizing capacity of the cell.

b. Extracellular processes

In the foregoing, the intracellular transformation of glucose from a free molecule to a part of a polyglucosan has been described. In the last decade, it has also become clear how the polymer is transported to outside the cell. Colvin and Leppard (1977) indicated how the assembly of polymers may form the microfibril. In A. xylinum, crystalline microfibrils approximately 3.5 nm in diameter are assembled in association with rows of particles (presumed to be groups of multienzyme complexes) that are situated below extrusion pores in the LPS layer of the bacterium (Brown et al., 1976; Colvin and Leppard, 1977; Zaar, 1979). These microfibrils associate, by hydrogen bonding, into bundles that in turn form a twisted ribbon. Dark-field light microscopy has shown that the bacterial cell turns on its axis as it is propelled

forward by the elongating ribbon. Ribbons from many bacteria interwine into a tough pellicle of cellulose on the surface of the culture medium. The composite ribbon is analogous to microfibrils in walls of other cellulose synthesizing organisms (Brown et al., 1976).

Ribbon extrusion was filmed by using frame by frame analysis, and an extrusion rate of $1x10^8$ glucose residues/h was calculated. In addition, it was observed that the bacteria rotate about the longitudinal axis (Brown, 1981). The important conclusion that emerged from these studies was that microfibrils which comprise the ribbon were synthesized in a row parallel to the longitudinal axis of the bacterial cell (not from the poles of the bacterial cell as previously reported by others).

Haigler et al. (1980) showed that direct dyes and fluorescent brightening agents alter the in vivo assembly of cellulose ribbons. A. xylinum normally produces a highly crystalline, extracellular ribbon of cellulose. A variety of compounds used as industrial dyes and brighteners for cellulosic products and as biological stains for cellulose bind to the subunits of the ribbon as they are synthesized in vivo, prevent their normal aggregation, and radically alter the morphology and crystallinity of cellulose. Most of the dyes and brighteners found so far to alter cellulose assembly are planar derivatives of trans-stilbene with substituents capable of hydrogen bonding with the hydroxyl groups groups of linear β (1,4) polysaccharides. Similarly, carboxy methylcellulose (CMC) of varying degrees of polymerization and substitution have been shown to alter cellulose assembly by A. xylinum but at a higher

level of organization than the dyes and brighteners. Experimental use of these compounds which interrupt cellulose assembly at different levels, had allowed the direct investigation of the relationship between glucan chain polymerization, microfibril crystallization, and fibril assembly in the biogenesis of cellulose I. From these studies, Haigler et. al. (1980) have proposed that polymerization and crystallization are coupled processes which can be experimentally separated in vivo, and that biogenesis of cellulose I fibrils occurred by a cell directed, self assembly process in A. xylinum.

The possibility that native cellulose can be altered provides a mechanism to control crystallite size. Crystallites obtained on drying of Calcofluor-induced cellulose vary in size depending on the initial concentration of Calcofluor used to induce the alteration, a normal 69 A° crystallite resulted when 0.025% Calcofluor is used and a 28 °A crystallites resulted when 0.1-0.5% Calcofluor was used (Haigler et al., 1980; Benziman et al., 1980).

Brown (1981) reviewed the stages of cellulose synthesis, using the basic model previously presented by Haigler et al. (1980). The process in general was summarized in the following stages:

a. Polymerization to form β-1-4 glucans.

b. Parallel glucan chains produce non-dissociable ordered aggregates (12 to 15 glucan chains).

c. Adjacent glucan ordered aggregates crystallize into microfibrils (3.5 nm crystalline elementary microfibril).

d. Microfibrils from discontinuous segments of extrusion channels aggregate into bundles (6.5 to 7.5 nm).

e. Bundles of microfibrils aggregate to form the single twisting ribbon.

f. Direction of ribbon synthesis is polarized but can be reversed (the ribbon always forms parallel to the longitudinal axis; however, the polarity of its synthesis can suddenly be reversed by physical strain during cell division.

g. Cell-directed crystallization is rate limiting.

A diagram of the proposed model of cellulose assembly is presented in Figure 1.

B. Industrial Applications of Bacterial Cellulose

The cellulose pellicles formed within a sugar-rich media by A. xylinum are used in the Philippines as a food product (Jesus et al., 1971; Lapuz et al., 1967). A delicacy among Philippinos, popularly known as "nata", the cellulose pellicles are well liked for dessert. Lapuz et al. (1967) determined the ideal conditions under which "nata" could be produced in sugared, coconut-milk medium. This study opened a way of mass-producing "nata" for preservation in heavy syrup and sale in the marketplace. The popularity of the product has not be confined to the local community. "Nata" is gradually being introduced into foreign markets. It is available in Canada at certain Chinese retail stores.

Jesus et al. (1971) reported the isolation and selection of high-yielding A. xylinum strains from Philippine microflora. A. xylinum was

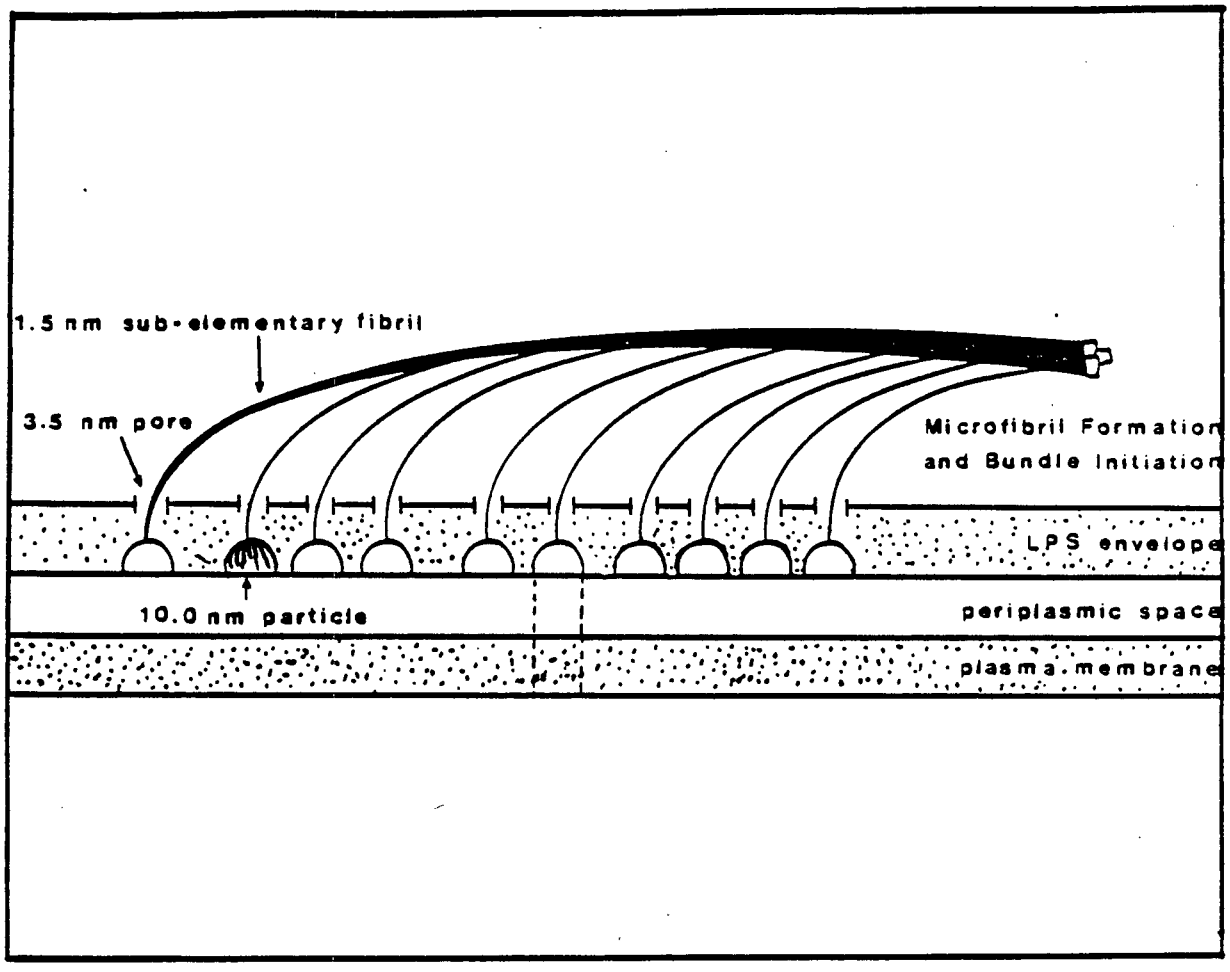

Figure 1. Proposed model of normal cellulose assembly. Glucan chain aggregates from organized multienzyme complexes and extrusion pores crystallize into microfibrils, which then assemble into bundles and the normal ribbon at the cell surface (Brown, 1982).

also reported to form "nata" in tomato juice, citrus juice, coconut milk-sucrose medium and crushed pineapple (Alaban, 1962).

Yamanaka et al. (1979) patented a gel-type dessert which was very similar to the "nata" already produced by the Philippinos.

Bacterial cellulose produced by A. xylinum has also been utilized by fields other than the food industry. Correns et al. (1972a) patented the production of cellulose membranes suitable for filters from intact pellicles produced by cultivating A. xylinum in a nutrient solution. A second patent (Correns et al., 1972b) involved the production of membranes and sheets from bacterial cellulose based on conventional papermaking methods.

Mynatt (1982) patented an apparatus and process for the production of polysaccharide (cellulose) fibres for use in paper manufacturing. In "Fiber production from continuous cultivation of micro-organisms", a method was described in which fibres were produced by harvesting the liberated products of continuous micro-organism cultivation. A suitable microorganism such as Sphaerotilus natans was grown on a pitted metallic plate supplied with a flowing nutrient substrate. With substantial pellicle growth, the nutrient flow was halted temporarily while a blade passed over the plate harvesting the pellicle growth and depositing the harvest products onto a sluice conveyor. The blade was retracted and the nutrient flow restored until the pellicle growth again became abundant. The harvested products were further processed to remove undesirable non-cellulosic materials depending upon the particular

microorganisms used. This process and apparatus thus produces minute cellulosic fibres suitable for paper making, thereby eliminating the processing and digesting of wood pulp. As an alternative, the apparatus might be modified for the growth of Acetobacter.

Brown (1983) patented a method for the production of a cellulose-synthetic polymer composite fibre. Hydrophilic characteristics were imparted to hydrophobic synthetic substrates, such as polyester fibres, by incubating a culture medium with A. xylinum in the presence of the substrate. Many synthetic fibres, while having exceedingly useful properties such as durability, permanent press, etc., still lack some of the physical properties desired in a cotton fabric. One of the most notable properties is the hydrophilicity of a cotton fibre. Brown's invention takes advantage of the cellulose produced by Acetobacter in that it is possible to produce this cellulose on the surface of polyester fibre, thereby conferring to that fibre many of the physical properties of cotton fibre. The advantages of the cellulose-synthetic polymer composite were: a) greater hydrophilicity and subsequent greater comfort in wearing; b) greater absorbancy which may be a useful property for disposal bandages, dressing, and the like; c) a natural biosynthetic reaction being coupled onto the surface of a synthetic polymer as a substrate; and d) the altered surface properties of the cellulose synthetic polymer composite might be advantageous for the adsorption of dyes and several other agents to the surface. The invention also provided a method of enhancing the hydrophilic characteristics of hydrophilic materials (eg., cotton or paper) by incubating A.

xylinum in the presence of a natural material whereby cellulose microfibrils are produced on and attached to its surface. Suitable materials included cotton (e.g., to increase the hydrophilic nature) and paper (e.g., to increase its strength). The composite polymer produced according to Brown's invention with its unique physical properties provided a whole new approach to the manufacture of "cotton-like" goods.

C. Microcrystalline Cellulose

Microcrystalline cellulose (MCC) is one of the few new ingredients available to formulators in the last two decades. The first commercial quantities were produced in 1962; however, MCC did not become a factor in food stabilization until very late in the 1960's. Although the original MCC product was designed for use as a bulking agent in low calorie foods, these foods have never reached their expected potential due to the difficulty in obtaining additional non-caloric ingredients acceptable for use in foods. By far, the major uses of edible MCC are in pharmaceutical tableting and in the stabilization of foods (FMC Corporation, Bulletin G-34).

Mycrocrystalline cellulose is a purified, naturally occurring α-cellulose. It is a linear polymer formed by β-1,4 linked glucose units, is completely insoluble in water but disperses in water to form colloidal solutions and white opaque gels. MCC is not a chemical derivative, but it is produced by converting fibrous cellulose to crystalline cellulose or a redispersible gel. This is accomplished by a simple acid hydrolysis, drying and/or co-processing with various hydrophilic

dispersions. The raw material for MCC is α-cellulose. Wood contains about 40-50% α-cellulose, but the purest natural source of cellulose is cotton fibers or linters, which on a dry basis consist of about 98% α-cellulose. To date, these two have been the most important commercial sources for raw material cellulose (Battista, 1975).

Part of the research of this thesis points towards the potential application of A. xylinum cellulose as a raw material in the production of MCC. Bacterial cellulose has been shown to be native crystalline (type I) cellulose, like the α-cellulose of cotton. Furthermore, analytical recoveries of D-glucose from hydrolysates of A. xylinum cellulose and cotton are within the same range (nearly 90% of theoretic value) indicating the high degree of purity of both materials (Whistler, 1963). Although cotton is the purest form of naturally occurring cellulose, it contains several impurities such as wax, pectins, and colouring matter. Any method of purification must aim at removing these impurities under conditions which will not bring about any fundamental change in the cellulose structure. If the conditions of purification are not rigorously controlled, considerable degradation of the cellulose occurs as indicated by reduced viscosity and α-cellulose content. This is particularly true in the case of wood cellulose, which must be subjected to a delignification treatment. Degradation of the cellulose always occurs during delignification, and it seems impossible to isolate a wood cellulose with the same degree of polymerization as that of the native wood or plant (Whistler, 1963).

The degree of polymerization (DP) of the raw material is extremely important since it is the initial DP which will determine the final

level-off DP (or LODP) and the crystalline residue upon hydrolysis. The LODP value and the crystalline residue are higher when the intial DP value is higher (Magister et al., 1975).

The commercially available microcrystalline cellulose comes as a white, fine powder which is low in ash, metals, and soluble organic materials. It is insoluble in water, dilute acid, common organic solvents, and oils. It is partially soluble, with some swelling in dilute alkali. One of the most important of these products is a microcrystalline cellulose sold under the trade name of Avicel. It is prepared by acid treatment of ∝-cellulose (from wood) under special processing conditions, as disclosed by the patent of Battista and Smith (1961). By controlled hydrolysis with hydrochloric acid, ∝-cellulose is converted to two components - an acid-soluble fraction and an acid-insoluble fraction. The acid-insoluble crystalline residue is washed and separated. It is called cellulose crystalline material or MCC. Essentially, the amorphous regions of the polymer are hydrolyzed completely, leaving the crystalline regions as isolated microcrystallites which are defined as the level-off degree of polymerization or LODP cellulose (Battista, 1950). In other words, if the hydrolysis reaction were continued, the degree of polymerization would not change, indicating that the level period or limit of reactivity, has been reached. The reported level-off DP consists of 15-375 anhydroglucose units depending on the initial DP of the raw material; the constituent chains of each aggregate being separate from those of neighboring

aggregates. These aggregates are characterized by sharp X-ray diffraction patterns indicative of a substantially crystalline structure (Battista and Smith, 1962, 1965).

Acid hydrolysis is followed by mechanical agitation in a water slurry to free a fraction of the unhinged crystals. The slurry is subsequently filtered and neutralized. The resulting filter cake is reslurried and the homogeneous aqueous slurry is spray dried to give a white, free-flowing powder consisting of completely unhinged or disconnected but aggregated microcrystals. A dry MCC which is more conveniently redispersed to a stable thixotropic gel can be produced by blending the filtered wet cake (40% solids) with a hydrophilic barrier such as CMC. This is done in order to homogenize the hydrophilic gum throughout the system so that the free microcrystals are coated with a film of the water-dispersible barrier. The homogenized mix is then either drum-dried and granulated in a hammer mill or spray dried. These commercial grades of MCC are readily dispersible in water (Battista, 1975).

Multiple uses of different grades of MCC have been listed by Battista (1975). In their nonfibrous and gel forms, MCC's have opened up major new applications for pure celluloses never before available for commercial use. The various grades of MCC have been engineered to contribute unique and useful properties to a wide spectrum of commercial products. For example, the regular pharmaceutical grade serves as a pharmaceutical tablet binder. Other grades go into frozen desserts to control ice crystal growth. Colloidal microcrystalline powders are

incorporated as rheology control agents in foods where they may be used as temperature-insensitive thickeners for salad dressings, hollandaise sauces, etc. One of the first major commercial uses for MCC was as a non-caloric ingredient for controlling the caloric content of foods, especially of fat-loaded foods. Industrial uses of cellulose microcrystals as rheology-control agents are also being developed, although they have not yet received as much commercialization as well-proven food and pharmaceutical uses.

The food grade of microcrystalline cellulose is "generally recognized as safe" in the Food and Drug Act (FDA) (1958) of the United States and the ADI for man is "no limit" (WHO, 1974).

MATERIALS AND METHODS

A. Test Organisms

The cultures in this study were obtained in the freeze dried state from the American Type Culture Collection and the National Institute of Science and Technology, Manila, Philippines. Acetobacter xylinum ATCC 10821, ATCC 14851 and the Philippine strain were resuspended in the following medium (% w/v): 2% glucose, 0.1% potassium phosphate, 0.05% magnesium sulfate, 0.15% sodium chloride, 0.5% peptone and 0.25% yeast extract, adjusted to pH 5. The cultures were grown in an incubator at a temperature of 28 to 30°C.

B. Cultivation Method

1. Culture propagation and conditions

Erlenmeyer flasks (250 mL) each containing 100 mL of the appropriate medium and equipped with cotton plugs were inoculated with 10% (v/v) inoculum. Cultures were left undistrubed on the bench at 28 to 30°C and subcultured every two weeks. To ensure the maintenance of a pure culture, the following procedure was used (Cook and Colvin, 1980). Cultures were diluted by a factor of 10^4; 0.1 mL of the diluted culture was spread uniformly on agar surfaces in 10 cm polystyrene Petri plates (1.5 g agar dissolved in 100 mL of the medium previously described). After incubation of the Petri plates for 4 to 7 days at 28 to 30°C, two replicas of each plate were made using the same agar medium and incubated for 4-7 days at 28 to 30°C. The incubated plates were sprayed

with a solution of Calcofluor White M2R New disodium salt of 4,4'-bis [4-anilino-6-bis(2-hydroxyethyl)amino-s-triazin-2-ylamino]-2-2'-stilbene disulfonic acid which fluoresces in the presence of cellulose. A stock solution was prepared with 0.1 g of Calcofluor in 5 mL of water with 5 mL of 0.2 M Na_2HPO_4 added. For spraying, 0.1 mL of the stock solution was diluted into 10 mL of Na_2HPO_4. Colonies that produced cellulose in normal quantity fluoresced brightly when examined with ultraviolet light, wavelength 366 nm, while those colonies which produced less cellulose were markedly darker. Cellulose producing colonies were picked off the extra undisturbed replica, streaked on agar surfaces to ensure that pure cultures were isolated, and then transferred to agar slopes. Cultures once grown were either stored under refrigeration (stock cultures) or in the incubator, but in either case they were subcultured every 1 to 2 months.

2. Selection of influencial factors for maximum cellulose production in defined medium

Erlermeyer flasks (250 mL) each containing 100 mL of nutrient media and provided with cotton plugs were inoculated with 10% (v/v) of adapted culture (Philippine strain). To ensure adaptation to each nutrient medium mixture the organism was grown through two serial subcultures in each mixture before the appropriate flasks were inoculated. Duplicate cultures were incubated at 28-30°C for 1 week. The Fractional Factorial design L_{27} (3^{13}) of Taguchi (1957) was used to select the factors which may significantly affect cellulose production, namely, sucrose and peptone concentrations and pH, as well as possible

interactions. The nutrient media composition (Lapuz et al., 1967) was as follows: sucrose (at 3 levels, 4, 6 and 10%), peptone (at 3 levels, 0.30, 0.50 and 1.23%), 0.1% potassium phosphate, 0.05% magnesium sulfate, 0.15% sodium chloride, and 0.25% yeast extract. The pH was adjusted with acetic acid at 3 different levels, 4.0, 4.5 and 5.5. After one week of incubation, the culture fluid was decanted and the pellicles were left in the flasks. Pellicles were thoroughly washed with repeated changes of distilled water over an 8 h period. Mercuric chloride solution (1 mL, 10% w/v) was added to the first changes of water to prevent the growth of contaminants (Dudman, 1959a). At this stage it was assumed that the pellicles contained only cellulose plus organisms. Next, 100 mL of 8% (w/v) NaOH were added to the flasks and shaken overnight to ensure complete dissolution of the cellular material from the pellicle. To determine cellulose content, the alkali-extracted pellicles were washed first with dilute acetic acid (1% v/v) until they remained acid for several hours, as indicated by the addition of methyl red, and then with repeated changes of distilled water to remove the acid. The cellulose pellicles were hung on glass rods, allowed to drain for 8 h, and then dried to constant weight at 105°C in tared crucibles (Dudman, 1959a). Data collected from the 27 nutrient media were analyzed using a Factorial Analysis of Variance Taguchi's L_{27} $(3)^{13}$ program written for a Monroe calculator (Model 1880, Monroe, Orange, NJ).

C. Culture Stability

Cellulose producing colonies of ATCC 14851, ATCC 10821 and the Philippine strain were propagated in the following defined medium: 10% sucrose, 0.5% peptone, 0.1% potassium phosphate, 0.05% magnesium sulfate, 0.15% sodium chloride, 0.5% peptone and 0.25% yeast extract, adjusted to pH 4.5.

Static and swirled cultures were incubated at 28-30°C. Swirled cultures in 250 mL Erlenmeyer flasks were incubated in a New Brunswick Psychrotherm incubator at 150 rpm. After 48 h incubation, cultures were transferred to fresh media at a 10% v/v inoculum level. This transfer was designated transfer 1. Subsequent transfers were done every 48 h. At each transfer, the cultures were diluted and spread on agar surfaces in 10 cm Petri plates (1.5 g agar dissolved in 100 mL of the medium described above). After 6-7 days growth, they were sprayed with Calcofluor and the numbers of cellulose deficient (mutants) and normal (wild) colonies were determined. The percentage of the colonies found to be deficient was calculated.

Colonies were observed with respect to colour, opacity, form, elevation and margin under a Steromicroscope (Model Wild M3, Wild Heerbrugg, Switzerland). Cellulose deficient colonies were isolated from replicas and maintained on slopes. Cellulose deficient strains as well as the wild type were transferred to liquid medium and allowed to grow for 2 weeks at 28-30°C under static conditions. Cultures of cellulose deficient colonies were tested for pellicle formation by visual comparison with cultures of the wild type.

D. Growth Curves

Cultures were grown in 100 mL of defined medium in 150 mL flasks, under static conditions at 28-30°C. Duplicates were removed from the incubator and analyzed at growth intervals of 0, 4, 8, 12, 16, 20, 30 and 40 days. At the time of analysis, the culture fluid was decanted for pH measurement and residual sugar determination. The pellicles were left in the flasks and washed with repeated changes of distilled water. Mercuric chloride solution (1 mL, 10% w/v) was added to prevent the growth of contaminants.

1. pH measurement

The pH of the culture fluid was determined immediately after sampling using a pH meter (Accumet Model 230, Fisher Scientific Co., Pittsburgh, PA).

2. Nitrogen determination (cell-nitrogen)

Washed pellicles were weighed in the flasks and an equal volume of 2N NaOH was added. The flasks were stoppered and shaken overnight. Nitrogen determinations were carried out on 1 mL samples of the alkaline extracts; from these results, the nitrogen content of the pellicles was calculated (Dudman, 1959a). Nitrogen was determined in duplicate according to the rapid micro-Kjeldahl method of Concon and Soltess (1973). The amount of nitrogen in each digested sample was determined using an Auto Analyser II (Technicon Instruments Corp., Tarrytown, NY). All nitrogen analyses were calculated on a dry basis.

3. Cellulose determination

To determine cellulose content, the alkali-extracted pellicles were washed first with dilute acetic acid (1% v/v) until they remained acid as demonstrated by the addition of methyl red indicator, and then with repeated changes of distilled water to remove the acid. The cellulose pellicles were allowed to drain for 8 h and then dried to constant weight at 105°C (Dudman, 1959a).

4. Sugar utilization and conversion

At the time the culture fluid was decanted for residual sugar determination for the different growth intervals, samples were filter-sterilized with a Millipore membrane (0.22 μm) to remove organisms. Total carbohydrate analyses of the organism-free culture fluids were determined by the phenol-sulfuric acid method of Dubois et al. (1956). Absorbance was determined at 490 nm on a Beckman Spectrophotometer (Model DB, Beckman Instruments Inc., Fullerton, CA.). Triplicate analyses were carried out on duplicate cultures. Total carbohydrate content was estimated from a standard curve for sucrose. The sugar utilized by the cultures was calculated by difference between the residual sugar found and at initially present in the medium (day 0). Percent total sugar conversion to cellulose ("conversion of total sugar") and percent of the sugar utilized by the culture ("conversion of utilized sugar") to cellulose was calculated by comparing the actual, corrected cellulose yield to the number of grams of total or utilized sugar in 100 mL sample and expressing this as a percentage.

E. Average Degree of Polymerization (DP) vs. Incubation Time

Stock cultures of ATCC 14851 and the Philippine strain were propagated in defined medium for 48 h at 28-30°C. After 48 h of incubation, cultures were transferred to fresh media at a 10% (v/v) inoculum level, and left undisturbed. Duplicate cultures were removed at growth intervals of 5, 8, 11, 13, 15, 20, and 32 days. At the time of harvest, the culture fluid was decanted, the pellicles were washed with distilled water and shredded in a Waring Blendor regulated with a Variable Autotransformer (600 rpm). The shredded fibres were passed through several layers of cheesecloth, the fraction on the cloth was scraped off, resuspended in water, and collected by centrifugation (9000 x g for 15 min). This process was repeated several times until centrifugates were solute free. Then, the fibres were immersed in 2% (v/v) NaOH solution for 3 h to remove alkali-soluble components and neutralized with 1% (v/v) acetic acid (Takai et al., 1975). Finally, they were washed exhaustively with large volumes of distilled water and freeze dried. The degree of polymerization (DP) for the purified cellulose was measured by the Cupriethylenediamine disperse viscosity pipet method (Cannon-Fenske Viscometer) for pulp (Tappi, 1966).

The Cupriethylendiamine disperse viscosity method made use of two cupriethylenediamine solutions: 0.167 M in copper and 1.0 M in copper. An amount of freeze dried sample equivalent to 0.125 g of moisture-free cellulose was weighed and placed in the dissolving bottle (this was calculated by determining the moisture on a separate portion). The bottles containing the two $Cu(En)_2$ solutions were connected to 25 mL

side-arm Mohr burettes. The burettes were filled by maintaining a 2 psi nitrogen pressure on the bottles. 15 mL of 0.167 M solution were added to the dissolving bottle, taking care that the cellulose was thoroughly wetted. Then, 10 mL of 1.0 M solution were added, the bottle was flushed with a stream of nitrogen for at least 15 s, and quickly capped. The bottle was shaken for 15 min to disperse the cellulose completely. At the end of the shaking, the solution was poured into the viscosity tube and placed in a water bath maintained at 25°C. The efflux time was determined in duplicates. Viscosity (in mPa•s) was calculated with the formula: V=Ctd, in which C is the viscometer constant (C_{200}=0.1 and C_{300}=0.25), t is the time in seconds and d is the density of the cellulose solution (d=1.052 g/mL). Once the viscosity measurement was obtained, the approximate DP was deduced from nomogram tables (Rydholm, 1965).

F. Cellulosic Fibre Production and Characterization

1. Description of the Fibre Production Apparatus

Acetobacter xylinum cultures were directed to grow within a confined unidirectional flow of sterile medium. This was accomplished in preliminary experiments by growing the culture in the apparatus depicted in Figure 2. As shown in this figure, the apparatus included two 2 L Erlenmeyer flasks stoppered with cotton plugs connected with a sloping glass tube (29.5 cm long and 4.5 cm inside diameter) provided with ground glass 24/40 joints at both ends. The sloping glass tube provided a growing surface along a straight line path where the

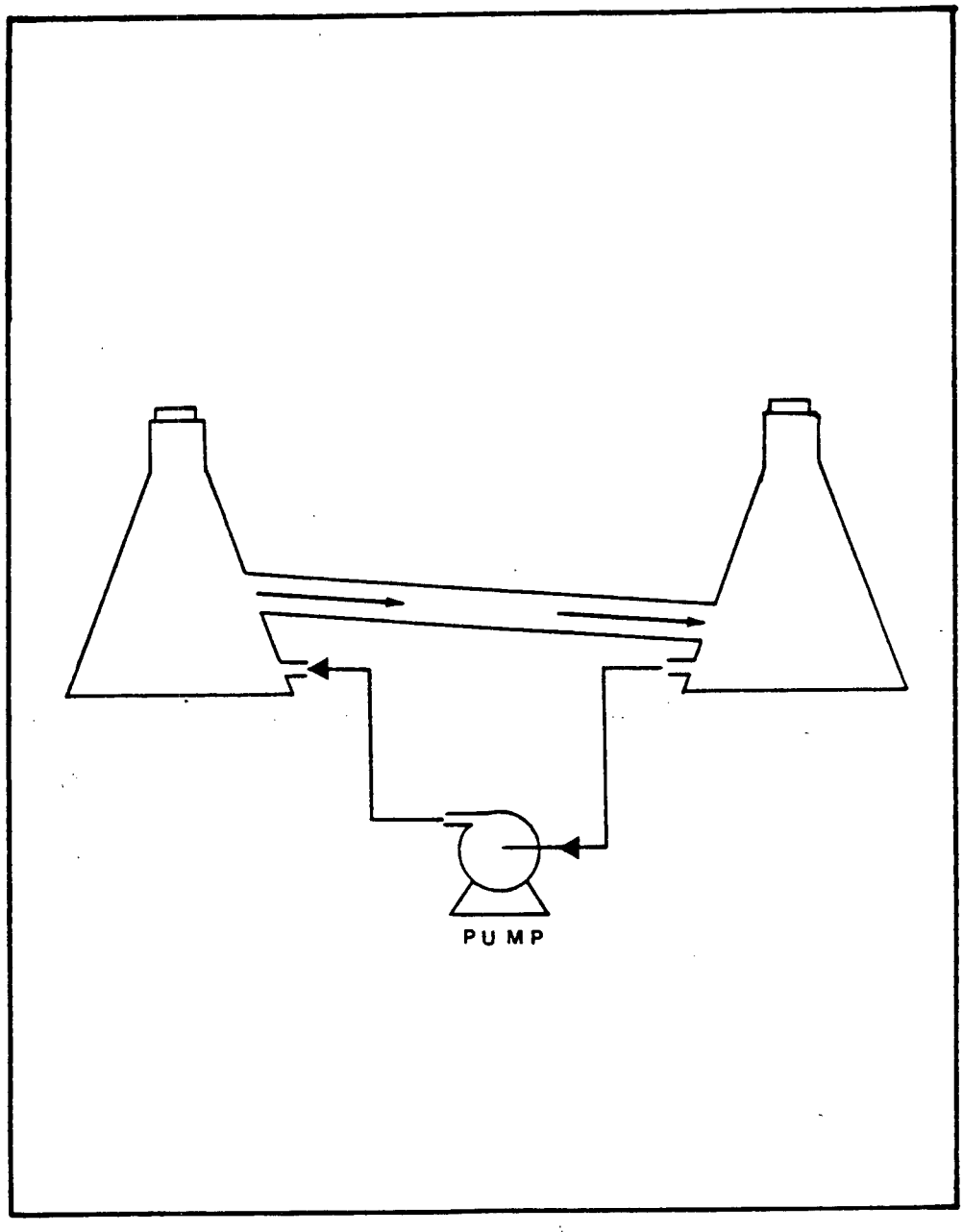

Figure 2. Fibre production apparatus (preliminary design).

cellulose fibres were deposited. The flowing inoculated medium was circulated with a flow rate of 10 L/min by a March Orbital Magnetic Drive pump (Model MDX-3, March Mfg. Inc., IL) regulated with a variable autotransformer. The fluid in the Erlenmeyer flasks was stirred with magnetic stirring bars to prevent pellicle formation in the flasks. The apparatus was placed in an incubator room at 28-30°C.

Based on the same principle of unidirectional flow (Townsley, 1981; personal communication), a second apparatus was designed which provided a larger growing surface along a straight-line path. A side view of the apparatus diagram is shown in Figure 3. In this apparatus, the sterile chamber was provided by a tank (58 cm long, 9.5 cm high and 35 cm wide) constructed of polycarbonate sheet (12.7, 9.2 and 6.3 mm thick for bottom, side walls and top, respectively). The bottom part of the tank was provided with a rubber gasket fitted inside a groove carved along the edges of the side walls, which allowed a tight seal for the cover. The growing surface was a ramp (50 cm long and 35 cm wide) provided with a number of V-shaped grooves or channels with sides approximately 3 mm long (Figures 4 and 5). The ramp was inclined slightly (7°) so that growing medium placed at the top of the ramp adjacent the grooves flowed slowly down (flow rate of 12.2 L/min) the grooves to the bottom. The growing medium was then collected and recirculated to the top of the ramp by a March Orbital Magnetic Drive Pump (Model MDX-3, March Mfg. Inc., IL) regulated with a variable autotransformer. The pH of the growing medium was controlled at 4.5 by a pH control system Speedomax H (Model 300, Leed and Northrup Co., North Wales, PA) provided with a pH electrode which was placed in a glass jar through which

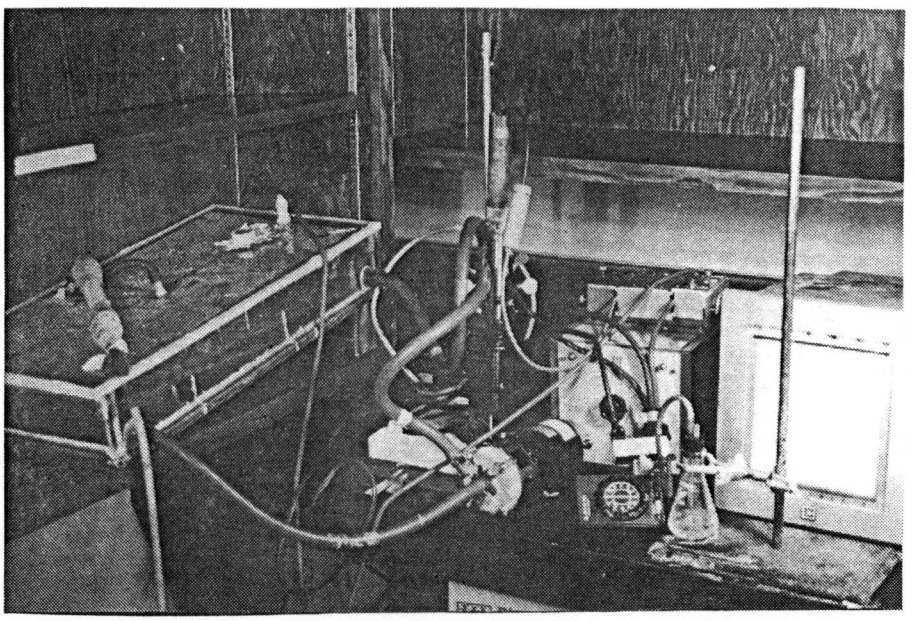

Figure 3. Fibre production apparatus (side view).

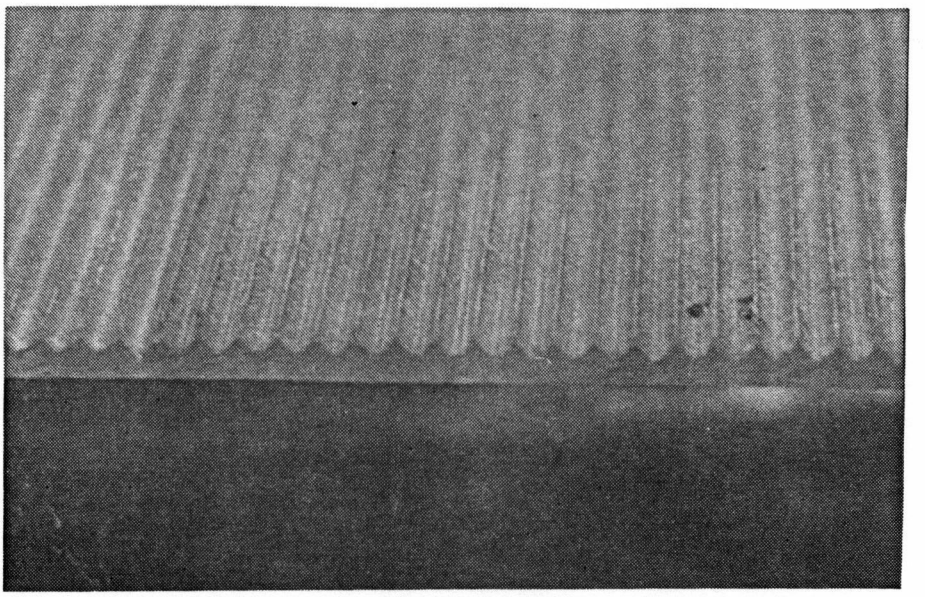

Figure 4. Grooves or channels in the fibre production apparatus.

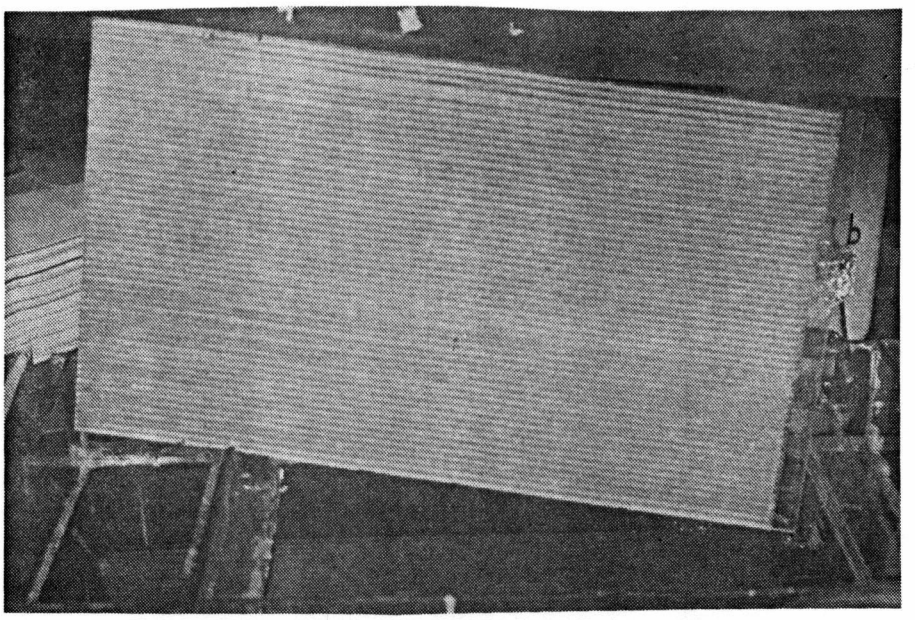

Figure 5. Ramp with carved grooves or channels providing a straight line flow path.

growing medium circulated in and out (Figure 6). 2N NaOH was fed into the tank by a peristaltic pump (Model 1201, Harvard Apparatus Co. Inc., Millis, MA) at an average flow rate of 0.42 mL/min (Figure 7). Intermittent flow was accomplished by means of a Paragon timer (15 min setting capability up to 48 h on/off, AMF Paragon Electric Co., Guelph, ON). In addition, filter sterilized air was sparged through the tank at a rate of 0.04 - 0.28 L air/L medium/min (Figure 8). Air was supplied by an air compressor (Webster Co., London, ON) connected to a flow meter (model No. 3, Gilmont Instruments Inc., Great Neck, NY). The flow meter was calibrated by Gilmont and a graph was supplied to obtain the proper readings by interpolation. The apparatus was located in an incubator room maintained at 28-30°C. The polycarbonate apparatus with its tubing connections and air filter were sterilized in an autoclave at 120°C and 15 psi g for 45 min. The pH electrode was sterilized by flushing it with Cry-Oxide ethylene oxide (11% active ingredient, AMSCO) overnight. It was then aseptically attached to the tubing connected to the polycarbonate apparatus.

2. Process for the production of fibres

Sterilized defined medium (4.5 L) inoculated with 10% (v/v) inoculum were dispensed into the sterile polycarbonate tank. The growing medium was circulated through the ramp for 4 d with a flow rate of 12.2 L/min either intermittently or continuously. In preliminary experiments, a flat wooden ramp was used as a growing surface for the production of fibres by two strains of A. xylinum ATCC 14851 and the Philippine strain. Both strains were tested for their ability to

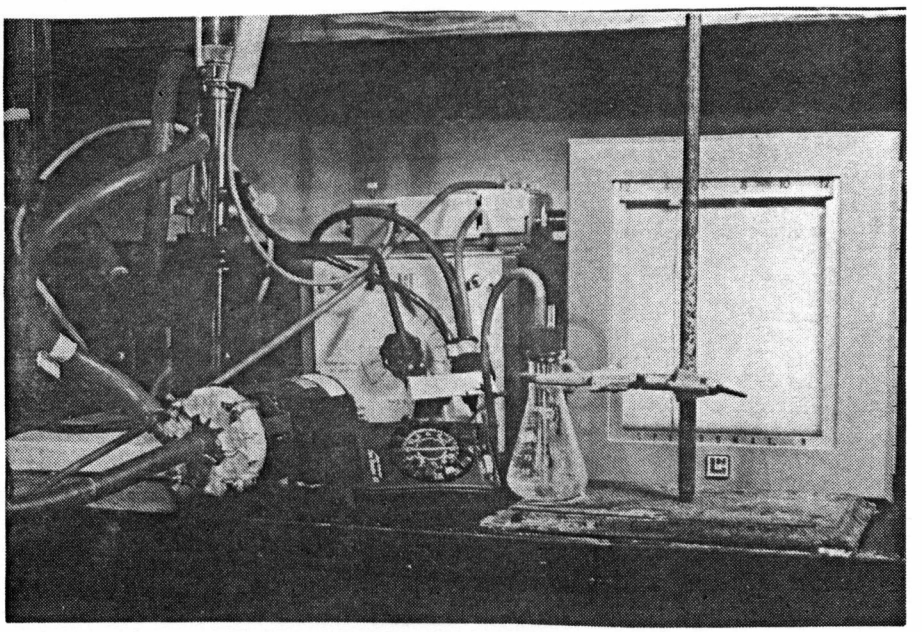

Figure 6. pH controller Speedomax provided with a pH electrode.

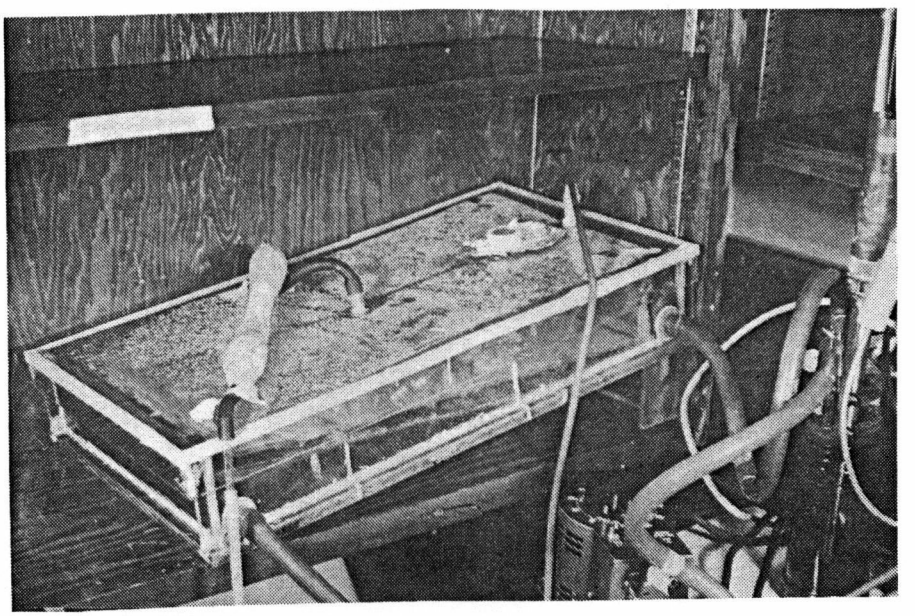

Figure 7. Polycarbonate chamber provided with air filter, cotton plug (air vent), and NaOH feeder.

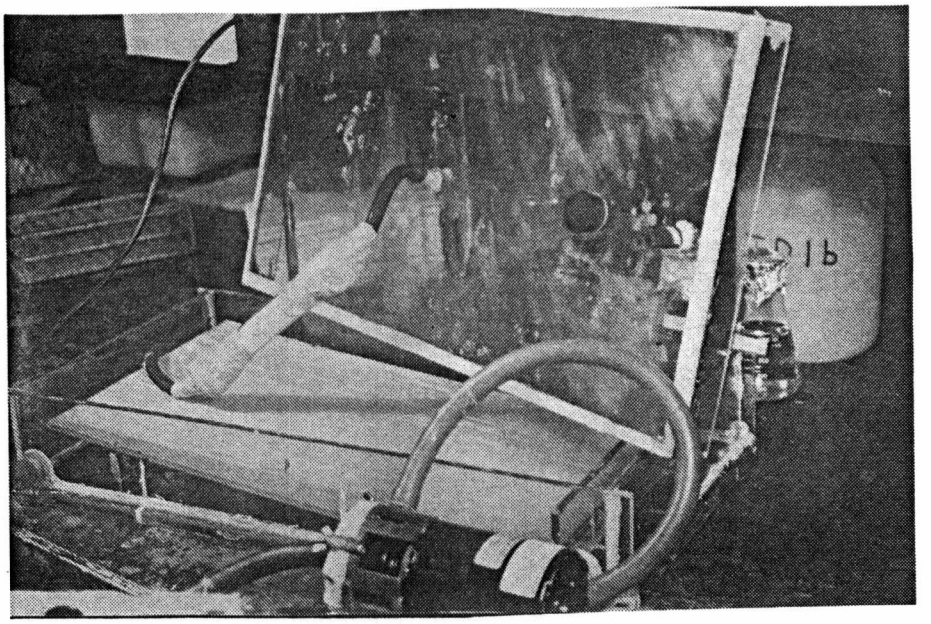

Figure 8. Polycarbonate cover with air filter attached.

produce cellulose under these particular conditions. Cellulosic fibres were observed for uniformity of growth over the wooden ramp for the two organisms. Since it was observed that continuous or intermittent flow determined the rigidity of the fibres at harvest, particularly for one of the strains (Philippine strain), it was necessary to test the tensile strength of the fibres produced under the following conditions: continuous flow and intermittent flow (1 h on/1 h off). Four trials were required for this test, incubation of ATCC 14851 and the Philippine strain with continuous flow and with one hour flow at one hour intervals. Air was sparged through the apparatus at a rate of 0.04 L air/L medium/min. The cultures were incubated for four days at 28-30°C for each trial. Cellulose was harvested, washed with several changes of water to remove remaining culture fluid, and dipped into a 10% (v/v) glycerol solution for 1 h. This procedure was adopted in attempts to eliminate brittleness upon drying of the cellulose fibres. Cellulose was cut into bone shaped pieces 5 cm long, 3 cm wide (widest parts) and 2 cm wide (narrowest center part) and dried in a vacuum oven at 60°C for 2.5 h (40% moisture wet basis). Triplicate samples from each treatment were subjected to tensile strength determination.

Following this experiment, intermittent flow (1 h on/1 h off) was adopted for further trials. In addition, ATCC 14851 strain was selected for further studies. The effect of rate of aeration on growth and cellulose yields was examined with cultures grown as described above. Cultures were all grown under the same conditions with the exception that they were aerated at different rates (0.040, 0.102 and 0.282 L

air/L media/min). Sugar utilization, sugar conversion, cellulose and pH were determined following the methods previously described for the static cultures. Samples (50 mL) were aseptically removed daily for sugar analyses and pH determination. At the end of the incubation period, 25 mL samples were placed into a vessel cup used to measure the dissolved oxygen. This vessel cup was threaded so that it could be screwed onto an oxygen probe (YSI model 57 oxygen meter and YSI model 5739 oxygen probe, Yellow Springs Instruments Co., Inc., Yellow Springs, OH) excluding the air. A small teflon magnetic bar was used to achieve circulation. Cellulose and nitrogen in the pellicle were determined only at the end of the incubation period. In addition to these 3 trials at different aeration rates, a fourth trial was performed following the same parameters but this time the growth medium was pH controlled at 4.5 and air was sparged at a rate of 0.04 L air/L medium/min; all trials were carried out in duplicate. At this stage, a final set of conditions was adopted for further fibre production. pH controlled defined medium (pH 4.5) flowed down a ramp at a rate of 12.2 L/min. Flow was circulated for 1 h at 1 h intervals. The apparatus was sparged with air at a flow rate of 0.04 L air/L medium/min and incubated for 4 days at 28-30°C.

3. Tensile properties determination

The standard load-elongation test conducted to specimen failure, was performed using an Instron Universal Testing Instrument (Model 1122, Instron Corp., Canton, MA) equipped with a tensile/compressive load cell with a full scale range of 10 g to 500 kg and a strip chart recorder.

Tensile strength, modulus of elasticity and percent elongation were calculated for each sample from its load-elongation curve. Tensile strength or stress at failure was defined as the ratio between the force applied and the cross sectional area. The rupture force was determined from the peak height in the load-elongation curve and converted into units of force (Newtons). The cross sectional area, expressed in cm^2, was determined for each individual sample. Just prior to the test, measurements of the samples were taken with the aid of a micrometer and a ruler. Cross sectional area at the narrowest part of the "bone-shaped" strips and strands were calculated by multiplying width by thickness. The strips were wider at the ends to be gripped by the pneumatic action clamps so that the specimens would not fail at the clamping sites. In the case of threads, the diameter was measured and the cross sectional area was calculated.

Modulus of elasticity is defined as the resistance of the fibre extension to the applied force, or a measure of the force required to produce an extension. It was estimated from the slope of the initial part of the curve or the tangent of the angle between the initial part of the curve and the horizontal axis, expressed in units of N/cm.

Finally, percent elongation or breaking extension is expressed by the ratio between the extension at the breaking point and the initial length (gauge length) x 100. The extension at the breaking point was estimated from the distance (on the horizontal axis) between the point of first tension and the breaking point divided by the magnification ratio 10 (chart speed 50 mm/min/crosshead speed 5 mm/min).

Pretest setup procedures were required in order to establish some factors affecting the tensile properties of the fibres. To ensure uniform testing, the same conditions were used for all similar tests. Factors considered were the following: test specimen length (gauge length), testing speed (cross head speed), moisture in the specimen and shape of the test specimen. The gauge length was 2 cm for all tests, this test specimen length was found to minimize the effect known as the "weak link" effect (Booth, 1964). A suitable crosshead speed for all the tests was 5 mm/min and a chart speed of 50 mm/min. Thus, the magnification ratio (Chart speed/Cross head speed) was 10.

If cellulose samples were produced in sheets, strips 5 cm long and 3 cm wide were cut and shaped into a bone form (2 cm wide at the center). At the time of testing, the samples were mounted centrally, securely gripped along the full width to prevent slipping, and the jaws were aligned in a parallel fashion so that the load was applied uniformly across the full specimen center width. Cellulose fibres produced in the channels or grooves were already uniform strands (0.2 cm wide and 50 cm long) which were tested with their original shape unless otherwise specified.

The effect of moisture content on the tensile strength of fibres was determined by equilibrating the samples under different relative humidities. Six bone-shaped samples were placed in each of the desiccators containing the following saturated solutions: KOH (9% ERH), potassium acetate (22% ERH), $NaNO_2$ (65% ERH), NaCl (76% ERH), and K_2SO_4 (97% ERH). Samples were allowed to equilibrate for 48 h; half of the samples were subjected to tensile strength testing and the other half of the

samples were analyzed for moisture content. Previous to equilibration at the different relative humidities, the samples were dried in a vacuum oven for 15 h at 60°C. Thus, dried samples would equilibrate by water adsorption. Preliminary experiments showed that desorption took from 7 to 10 days due to the high water content of the samples in the swollen state. At the time of tensile testing, sample thickness was measured by means of a micrometer.

The Instron was operated with a crosshead speed of 5 mm/min, a chart speed of 50 mm/min, a 5 kg load and a 2 cm gauge length. Tensile strength was plotted against moisture content.

Tensile testing of fibres produced by two different strains under different flow modes (intermittent and continuous) was carried out using the same set of conditions as above.

4. Mapping Super Simplex Optimization of mercerization treatment

Mercerization is perhaps the most commonly employed chemical treatment of cotton fibres and yarns. In this process the swelling and modifying action of sodium hydroxide upon the cellulose fibres of cotton hairs is utilized to advantage to impart certain desirable properties such as luster, improved strength and increased dyeability. A great deal of work has been done on various aspects of mercerization, and studies have been carried out to determine the optimum conditions under which cellulose should be mercerized in order to impart the desired improved properties. As a result, information is available on the extent of changes in the structural and morphological properties of cotton after swelling in NaOH under various conditions of treatment

(Warwicker et al., 1966; Peters, 1967; Warwicker and Hallam, 1975; Rajagopalan et al., 1975). Since it was shown that the effect of any treatment intended to modify the properties of the fibres was greatly influenced by the original characteristics of the fibres, it was necessary to find the optimum conditions under which A. xylinum cellulose fibres would achieve maximum tensile strength.

Mapping Super Simplex Optimization (MSO) was carried out to optimize the treatment conditions using a modified super-simplex optimization program (MSS) (Nakai, 1982), written for a Monroe 1880-88 programmable calculator, a grouping and matching program (Nakai et al., 1984) written for the UBC Amdahl 470 V/8 computer, and a simultaneous factor shift program (Nakai et al., 1984) written for a Monroe 1880-88 programmable calculator. Three factors were optimized for maximum tensile strength, namely, temperature, NaOH concentration and time. Based on preliminary experiments, the boundary values of each factor were established. Thus, sodium hydroxide concentrations ranged between 6 and 22% (w/v); temperature ranged between 2 and 80°C and time ranged between 10 and 60 min. Triplicate cellulose strands were treated under various conditions, neutralized with 1% (v/v) acetic acid, washed several times with distilled water and dipped into a 10% (v/v) glycerol solution for 1 h. During treatment, strands were securely gripped with two clamps to prevent entanglement.

The treated strands were dried under vacuum at 60°C for 2.5 h to reach a final moisture content of 40% (wet basis) and immediately subjected to tensile testing. Individual strands were securely gripped along the full width with the upper grip and twisted by rotation of a

clamp attached to the free end to introduce 10 turns per cm, a total of 20 turns in the 2 cm gauge length for all tested samples. Twist was introduced to the fibres in order to develop strength due to cohesive forces, as is the common practice in textiles (Booth, 1964). Diameter of the threads was measured with a micrometer once they were secure. The standard load-elongation test conducted to specimen failure was carried out with a 5 mm/min crosshead speed, a 50 mm/min chart speed, a 2 kg full scale load range and a 2 cm gauge length. The mean tensile strength calculated with each treatment represented the response value entered into the program.

5. Statistical analyses

a) Effect of A. xylinum strain and flow mode on tensile strength

The purpose of the analysis was to determine if the strain and the flow mode had a significant effect on tensile strength. Data collected were coded for "strain" (2 levels) and "flow mode" (2 levels) and analyzed using a two-way analysis of variance (ANOVA) program package (*MFAV) (Le, 1980) available for use on the UBC computer (Amdahl 470 V/8) system.

b) Effect of batch on tensile properties

Tensile strength, modulus of elasticity and percent elongation or breaking extension were tested in cellulose fibres produced from two different batches. The standard load-elongation tests conducted to specimen failure were carried out with the Instron operated with a 5 mm/min crosshead speed, a 50 mm/min chart speed, a 2 kg full scale load

range and a 2 cm gauge length. Eight replicates were evaluated from each batch. A one-way analysis of variance (ANOVA) with "Batch" as the single factor (2 levels) was performed using the *MFAV program.

c) Effect of Mercerization on tensile properties

Tensile strength, modulus of elasticity and percent elongation or breaking extension were tested in non-treated and mercerized fibres. The load-elongation tests were conducted with the Instron operated with the same conditions previously stated. Eight replicates were evaluated from each treatment. A one-way analysis of variance with "treatment" as the single factor (2 levels) was performed using the *MFAV program.

6. Fibre microstructure

a) Light microscopy

Cellulosic fibres were observed under a Wild M20 Microcope (Wild, Heerbrugg, Switzerland) equipped with a Pentax ME 35 mm camera. Cellulosic strips were dialyzed with distilled water immediately after harvesting and stained with a 1% aqueous solution of Chlorazol Black E (Paszner, 1982; personal communication). Phase contrast was used and photographic images were recorded on Kodak Ektachrome colour slide film. Stained fibres were also observed using polarized light. Phase polarized light was obtained by inserting a Nicol prism in the microscope substage.

b) Scanning electron microscopy

Structural examination of cellulosic fibres produced either within a confined unidirectional flowing medium or under static conditions, non-treated and mercerized samples, was accomplished using scanning electron microscopy techniques. Samples, approximately 3x3x1 mm cubes, were fixed with 4% v/v glutaraldehyde in 0.066 M phosphate pH 7.0 for 15 h at 4°C. Three 5 min rinses in 0.066 M phosphate buffer were followed by secondary fixation in 1% osmium tetroxide in phosphate buffer for 60 min. Rinses of phosphate buffer (3x5 min) preceeded alcohol dehydration through a series of increasing ethanol concentrations (5 min each in 50, 60, 70, 80; 2x10 min in 90; and 3x20 min in 100%). Replacement of ethanol with amyl acetate was accomplished through 10 min changes of 25, 50, and 75% amyl acetate in absolute ethanol, followed by 1 h in 100% amyl acetate. The samples were then dried in a Parr 5770 Critical Point Drying Bomb (Parr Instrument Co., Moline, IL) using carbon dioxide as the transitional fluid (critical temperature and pressure: 30°C; 7468 kPa). The dried samples were mounted with aluminum paste on aluminum stubs and coated with gold by vacuum evaporation. A Cambridge Steroscan 250 Scanning Electron Microscope (Cambridge Co., Cambridge, England) equipped with a Polaroid 545 land film camera was used to observe the structural detail of the samples and photographic images were recorded on positive/negative film (type 55). An operating voltage of 20 kV was employed.

7. Crystallinity index, crystal size and degree of polymerization determination

Crystallinity index and crystal size of mercerized fibres produced under confined unidirectional flowing media were determined in duplicate using X-ray diffraction methods (detailed description of the method is given in section G_2). The degree of polymerization was determined by the Cupriethylenediamine disperse viscosity method (TAPPI, 1966; detailed description of the method was given in section E) in duplicate.

G. Microcrystalline Cellulose (MCC) Production

1. Cellulose production

Cellulose production started with the incubation of static cultures. Defined medium was inoculated with 10% inoculum (Philippine strain) and left undisturbed for two weeks at 28-30°C. Cellulose pellicles were harvested after two weeks incubation and dialyzed overnight with tap water. The dialyzed cellulose was then shredded into small pieces with a Waring Blendor regulated with a variable autotransformer (600 rpm). The shredded cellulose was drained through several layers of cheesecloth to remove excess water and subjected to NaOH extraction.

2. Cellulose purification (NaOH extraction)

An extraction treatment which resulted in a highly purified cellulose ($\geq$ 98% cellulose) without any structural damage was investigated. Cellulose, in the swollen state (98% moisture wb) and freeze dried (2%

moisture, wb) were treated with increasing NaOH concentrations 1, 3, 6 and 8% (w/v). Weighed, swollen samples were mixed with NaOH solutions in a ratio of 1 to 2; thus the final NaOH concentration was approximately 2/3 of the intial concentration assuming the samples were 100% water. Mixtures were shaken for 3 h at 25°C, neutralized with 1% (v/v) acetic acid and rinsed with distilled water to remove the acid. Treated samples were freeze dried and ground with a Wiley mill equipped with a 40 mesh screen. Duplicate samples were subjected to nitrogen determination according to the method of Concon and Soltess (1973) and the digested samples were analyzed using an Auto-Analyser III (Technicon Instruments Co., Tarrytown, NY). The degree of polymerization (DP) was measured by the Cupriethylenediamine disperse viscosity pipette method (TAPPI, 1966) in duplicate.

Crystallinity and crystal size were determined by using a X-ray diffraction method. Approximately 1 g of ground sample was shaped into a thin disk by compression with a hydraulic press (750 psig for 1 min). The samples were carefully placed in position in a Dutch Philips 1009 X-ray Diffractometer (Philips Electronic, Mahwah, NJ) and scanned between 9° to 30° (2θ) angle. The x-rays were generated from a water-cooled copper target at 30 kV and 15 mA current, monochromatized by a nickel filter and passed through a 0.5° divergence slit. The goniometer consisted of a proportional counter through a 0.1° receiving and 0.5° scatter slit, amplified by the preamplifier circuit and the resulting signals were fed into a recorder. The three maxima (peaks) obtained corresponded to 002, 101 and $10\bar{1}$ planes of the cellulose crystal lattice. Percent crystallinity was derived by comparing the intensity

of x-rays diffracted by the crystalline portion with that diffracted by the non-crystalline (amorphous) portion in the sample (Browning, 1967). The method uses the intensity maxima of the 002 peak (I_{002}) and intensity minima at 18° 2Θ (I_{am}). The crystallinity index was calculated with the following formula:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

The crystallite sizes of cellulose were obtained from the line breath of 002 peak. The usual Sherrer formula was used,

$$D = \frac{0.9\ \lambda}{\beta \cos \Theta}$$

where: D is the crystallite size in A°; λ is the wavelength in A° of the x-ray used (λCu = 1.54 °A); Θ is the Bragg angle in degrees (22.6°); and β is the half breadths of the peaks, in rad.

Data collected for all the measured variables were coded for "condition" (2 levels, swollen and freeze dried) and "treatment" (5 levels; 1, 3, 6 and 8% NaOH extraction); an analysis of variance (ANOVA) program package (*MFAV) available for use on the UBC computer was used to analyze the data. Duplicate values were available for all variables. Data were subjected to further statistical analysis using Duncan's multiple range test available with the MFAV program. Duncan's multiple range test (Li, 1964) is used to compare the means of the main effects.

3. Bleaching

Freeze dried NaOH-extracted cellulose samples still contain some colouring matter and have a yellowish colouration. Thus, a mild

bleaching process was necessary to obtain a good white colour. The treatment was carried out with sodium chlorite as the bleaching agent. The method represents further refinements on the Jayme and Wise chlorite cellulose technique (Browning, 1967) in that it was adjusted to suit this type of cellulosic material, since the severity of the treatment required depends on the amount of non-cellulosic material present in the sample. Water (200 mL) was added to a 500 mL Erlenmeyer flask, heated to 70°C, and added to 5 g of sodium chlorite, $NaOCl_2$. The equivalent of 5 g (dry) of unbleached samples were added and shaken to mix. Glacial acetic acid (2 mL) was added and mixed again. The mixture was held in a water bath at 70°C for 3 h. The digestion was stopped and the mixture was washed with 1% acetic acid. Finally, the cellulose was rinsed very thoroughly with water, drained through cheesecloth, and freeze dried.

4. Hydrolysis

A study was carried out on the effect of time on the weight loss and the degree of polymerization of purified cellulose samples using drastic conditions of hydrolysis, i.e. 2.5 N hydrochloric acid at boiling temperatures (Battista, 1950). Cellulose samples of known moisture content were weighed and added to boiling 2.5 N HCl in a ratio 1 (g): 150 (mL). The hydrochloric acid solution was heated by means of a Glass-col mantle connected with a powerstat. A steady stream of nitrogen gas (100 cc per min) was administered through one opening of a three neck flask for the purpose of keeping the temperature of the acid uniform, eliminating bumping, and excluding oxygen from the hydrolyzing

medium. The temperature was kept at 105 ± 0.5°C. The samples were left in the boiling acid for precisely the times specified, 15, 30, 60 and 120 min. The apparatus was dismantled and the contents were rapidly transferred to a tared fritted glass filter of M porosity. The sample was then washed repeatedly with distilled water, dilute ammonium hydroxide (5%), and more distilled water until acid-free, after which it was dried in a vacuum oven to constant weight at 105°C. Changes in the degree of polymerization with hydrolysis time were recorded and plotted. Cellulose yields resulting from acid hydrolysis (based on weight of residue) were determined at each time interval; DP and yields were determined in duplicate.

The aim of this study was to find the time interval needed to reach the "level-off degree of polymerization" (LODP) or when a relatively constant degree of polymerization was reached. Once the hydrolyzing conditions were established, they were adopted for further work.

5. Spray Drying

The resulting neutralized, filtered cellulose crystals were reslurried to about 3% solids and subjected to the action of a Waring Blendor for 30 min to free a fraction of the unhinged crystals. The homogeneous aqueous slurry was spray dried with a Niro Atomizer (Copenhagen, Denmark) at a 96-100°C inlet temperature and 36° outlet temperature. The slurry was fed into the Atomizer with a flow rate of

20 mL/min. After spray drying, the product was stored in bottles placed in a desiccator until used for the various tests.

H. Microcrystalline Cellulose (MCC) Characterization

1. Physical property tests

a) Crystallinity and crystall size

Estimation of crystallinity and crystal size was performed on triplicate samples of the MCC prepared in this study, as well as on commercial MCC Avicel PH-101 by methods previously described.

b) Average degree of polymerization and particle size

The degree of polymerization was determined in triplicate by the Cupriethylenediamine disperse viscosity method as previously described (TAPPI, 1966).

Average particle size was determined by measuring 20 particles in each sample dispersion using a Wild M40 Microscope (Wild Heerbrugg, Switzerland) provided with an ocular and stage micrometer. Both tests were performed on commercial MCC Avicel PH-101 and prepared MCC.

c) Moisture adsorption

Triplicate MCC samples were allowed to equilibrate for 48 h at 25°C with the following saturated solutions: potassium acetate (22 % ERH), sodium chloride (76% ERH) and potassium sulfate (97% ERH).

Water activities were determined by placing the samples in a Rotronic Hygroskop DT DMS 100 water activity measuring station (Rotronic

Ag, Zurich, Switzerland), and allowed to equilibrate for 1 h. The Hygroskop was calibrated with a saturated barium chloride solution. Moisture content was determined by drying in a vacuum oven at 105 kPa vacuum and 80°C for 15 h. Moisture contents were calculated as a percentage (dry basis) and results presented as the average of triplicate analyses.

d) Zeta potential

MCC dispersions (0.1%) were prepared in .01 M phosphate buffer (pH=6.95) by using a Polytron high speed blender/sonicator. Zeta potentials of duplicate dispersions were determined with a Laser Zee^{TM} (Model 500, Pen Kem, Inc., Bedford Hills, NY) and the values were corrected for temperature.

e) Colour determination

Colour of the MCC samples was measured with a Hunterlab Colour Difference Meter (Model D 25 D, Hunter Associates Laboratory, Inc., Fairfax, VA) with the L scale and the standard No. W 272.

2. Chemical composition

a) Cellulose determination

Triplicate MCC samples were subjected to a primary and a secondary hydrolysis as described by Whistler (1963). Total carbohydrate content of the digested samples was determined by the phenol-sulphuric acid method of Dubois et al. (1956) using a glucose standard curve.

Primary hydrolysis:

Cellulose (0.3 g) in 3.0 mL H_2SO_4 (72%) was maintained at 30°C for 1 h. The samples were then diluted with 84 mL of distilled water in a tared 250 mL Erlenmeyer flask.

Secondary Hydrolysis:

The flasks were covered with an inverted beaker and placed in an autoclave for 1 h (15 psig; 120°C).

Neutralization:

The hydrolysates were cooled and neutralized to pH 5.5 by addition of a saturated solution of barium hydroxide during vigorous agitation with a mechanical stirrer. Solutions were diluted to the weight of 261.7 g exactly equivalent to 250 mL of solution plus suspended $BaSO_4$ (11.7 g). The neutralized hydrolysates were centrifuged and aliquots (200 mL) were taken from the clear supernatant. Aliquots were stored under refrigeration until total carbohydrate analyses were performed.

b) Nitrogen determination

Samples (60 mg) of spray dried MCC, experimental and commercial, were placed in a 30 mL micro-Kjeldahl flask and digested using the method of Concon and Soltess (1973). Digested samples were analyzed for nitrogen content by means of a Technicon Autoanalyzer (Technicon Instruments, Co., Tarrytown, NY).

Samples were also analyzed for ammonia nitrogen using a Kjeldahl Distilling Unit, a saturated boric solution as the trapping agent and

NaOH-$Na_2S_2O_3$ as the releasing agent. Distillates were titrated with 0.02 N HCl (AOAC, 1980). Analyses were carried out in triplicate.

c) Ash determination

Samples (1 g) were heated at 550 ±50°C until completely charred in a muffle furnace, then ignited at 800 ±25°C until free from carbon, cooled in a desiccator and weighed (FCC III, 1981). Analyses were carried out in triplicate.

3. Rheological properties

Samples of MCC, prepared from A. xylinum cellulose and commercial Avicel PH-101 were dispersed in distilled deionized water to make duplicate dispersions of 6, 5, 4 and 3% (w/v). Solids were dispersed by using a Polytron high speed blender/sonicator for 2 min. Duplicate samples were then subjected to viscometric evaluations using a coaxial cylinder Fann Viscometer (Model 35, Fann Instrument Co., Houston, TX). Tests were carried out over a range of temperatures (25, 35 and 45°C), shear rates (3-600 rpm) and pH (4.0, 5.5 and 7.0). The effect of 0.4% (w/v) NaCl on the flow behaviour of 3% dispersions was also studied.

Each sample was loaded, allowed to equilibrate and sheared over a shear rate range of 0 to 600 rpm at 10 s intervals followed by a decrease in shear rate to 0, again at 10 s intervals. The sample was then sheared with the same increase/decrease cycle. The dial readings (θ) were recorded from the meter for each speed (rpm). Using a

rheological analysis program (Computer-Aided Rheological Analysis of Drilling Fluids; Speers, 1982), available through an Apple II microcomputer, data from all experiments were analyzed. By using linear regression techniques, this program calculates Bingham, Power law and Casson model parameters. The Bingham model was expressed by the equation $\eta = \eta_{pl} + \frac{\sigma_y}{\dot{\gamma}}$, where η_{pl} was the plastic viscosity, σ_y was the yield point, $\dot{\gamma}$ was the shear rate and η was the apparent viscosity. The Casson model was represented by the equation $\sqrt{\eta} = k_1 + k_0\frac{1}{\sqrt{\dot{\gamma}}}$, where k_0 was $\sigma_y^{1/2}$ and k_1 was $\eta_\infty^{1/2}$. The Power Law model was expressed by the equation $\eta = m\dot{\gamma}^{n-1}$ where m was the consistency coefficient and n was the flow behaviour index. These techniques also indicate the degree to which the model fits the data. The program also provides a graphical portrait of σ versus $\dot{\gamma}$ (or transforms), and η versus $\dot{\gamma}$. In this program the Θ (dial readings) values obtained at several rpms were treated assuming that Newtonian shear rates occur in the annular gap. The shear rate and stress values were calculated from the following two equations:

$$\dot{\gamma} = \frac{4\Pi rpm}{60}\ \frac{r_c^2}{(r_c^2 - r_b^2)} \qquad \text{and} \qquad \sigma = \frac{S_c\Theta}{2\Pi r_b^2 h_e}$$

where r_b was the radius of the bob (1.725 cm), r_c was the radius of the cup (1.842 cm), S_c was the spring constant of the viscometer (387 dyne/cm degree), and h_e was the effective height of bob (corrected for end effect, 4.05 cm). These two equations for shear rate and shear stress were modified by substitution of the values to equations $\dot{\gamma}$ =1.703 rpm in s^{-1} and σ=0.511 Θ in Pa. Therefore, to calculate the Power law index n and consistency coefficients m from data, η was calculated using

regression of log σ on log $\dot{\gamma}$ according to the above equations. Once the n was calculated, a true m was determined by using a corrected shear rate ($\dot{\gamma}_c$) given by the expression $\dot{\gamma}_c = \frac{4\Pi rpm}{60} \frac{r_c^{2/n}}{r_c^{2/n} - r_b^{2/n}}$. The corrected shear rate values were then used in further computations.

Dispersions were also tested for time dependence. Plots of the upcurve and downcurve at 25°C at each concentration were subjected to a line comparison program titled "NLIN", available on the UBC Food Science Department computing ID, to test the presence of a significant difference between two regression lines. This program compares pairs of lines to test for homogeneity of residual variances, slopes and levels. The significance of these comparisons was based on a F-value generated in each case. In this comparison of Power Law rheograms the η and $\dot{\gamma}$ data were transformed to logarithms to provide a linear function so that a difference in slopes would indicate that n values differed, and a difference in levels would indicate that m values differed.

To test if the dispersions were thixotropic, that is, if the fluid possesses a reversible decrease in viscosity to some equilibrium value with time at a constant shear rate; gel strengths (10 sec and 10 min) were recorded at 3 rpm by shearing the dispersion at 600 rpm for 10 sec, then the shear rate was brought to zero for a rest period of 10 sec or 10 min (Gray and Darley, 1981). In addition a test was carried out to check if the two MCC samples tested, commercial Avicel PH-101 and MCC prepared from A. xylinum cellulose, presented significantly different viscosities over a range of concentrations (3, 4, 5 and 6%) and shear rates (3-600 rpm) at 25°C. Plots of the equilibrium curves of both MCC

at 25°C at each concentration were subjected to the line comparison program. In this comparison of Power law rheograms, a difference in slopes would indicate that (n-1) values differed, and a difference in levels would indicate that m values differed.

Using the Power law models apparent viscosities were calculated at 100 s^{-1}. These apparent values were then used to examine viscosity-concentration relationship. The viscosity-concentration model used was a Power-type relationship expressed by the equation $\eta = ac^b$ or $\log \eta = \log a + b \log C$, where a and b were constant parameters (Harper and EL Sahrigi, 1965). The effect of temperature on apparent viscosities was studied by using an Arrhenius-type relationship expressed by the equation

$$\eta = Ae^{\Delta E/RT} \quad \text{or} \quad \log \eta = \log A + \frac{\Delta E}{2.303R} \frac{1}{T}$$

where A is the frequency factor, ΔE is the activation energy, T is the absolute temperature, R is the universal gas constant and e is natural logarithm base (Harper and EL Sahrigi, 1965).

RESULTS AND DISCUSSION

A. Selection of Influential Factors in Defined Medium

Data collected for the 27 nutrient media tested, following a Fractional Factorial Experimental Design $L_{27}(3)^{13}$ of Taguchi (1957) were analyzed by analysis of variance to determine the significance of pH, sucrose and peptone concentration and possible interactions on cellulose production. The results are presented in Table 1. The factors of pH and sucrose concentration were computed to be highly significant sources of variation ($P < 0.01$). Peptone concentration was also found to be significant ($P < 0.05$). The effect curve in Figure 9a illustrates a remarkable increase in cellulose production towards acidic pH, as it is expected for bacteria of the genus Acetobacter, reaching an optimum at pH 4.5. These results agreed very closely with those reported by Lapuz et al. (1967), who indicated that A. xylinum is distinctly acid-tolerant, thriving even as low as at pH 3.5, and that thicker pellicle formation was always observed in nutrient media with an initial pH of 4.5 to 6.0. In coconut water medium, pellicle formation was observed to be optimum at pH 5.0 to 5.5. This study agreed with their findings even though their method of assessing cellulose production was based on volume displacement by the gelatinous material (cellulose pellicle), whereas in this study, dry cellulose weights were determined.

In preliminary experiments several sugar substrates were tested, namely, glucose, fructose, sucrose and supplemented coconut water. All of them sustained good cellulose production, however, the aim in this

Table 1. Analysis of variance (Taguchi's L_{27} 3^{13}) for cellulose yields obtained from 27 nutrient media.

Source of variation	DF	Mean square	F-ratio
pH	2	159.28×10^3	38.90**
Sucrose concentration	2	139.32×10^3	34.03**
Peptone concentration	2	22.64×10^3	5.53*
pH x sucrose concentration	4	37.30×10^3	5.93*
pH x peptone concentration	4	37.08×10^3	9.05**
Peptone concentration x sucrose concentration	4	2.02×10^3	0.49
Error	8	4.09×10^3	
Total	26		

*significant at $p < 0.05$
**significant at $p < 0.01$

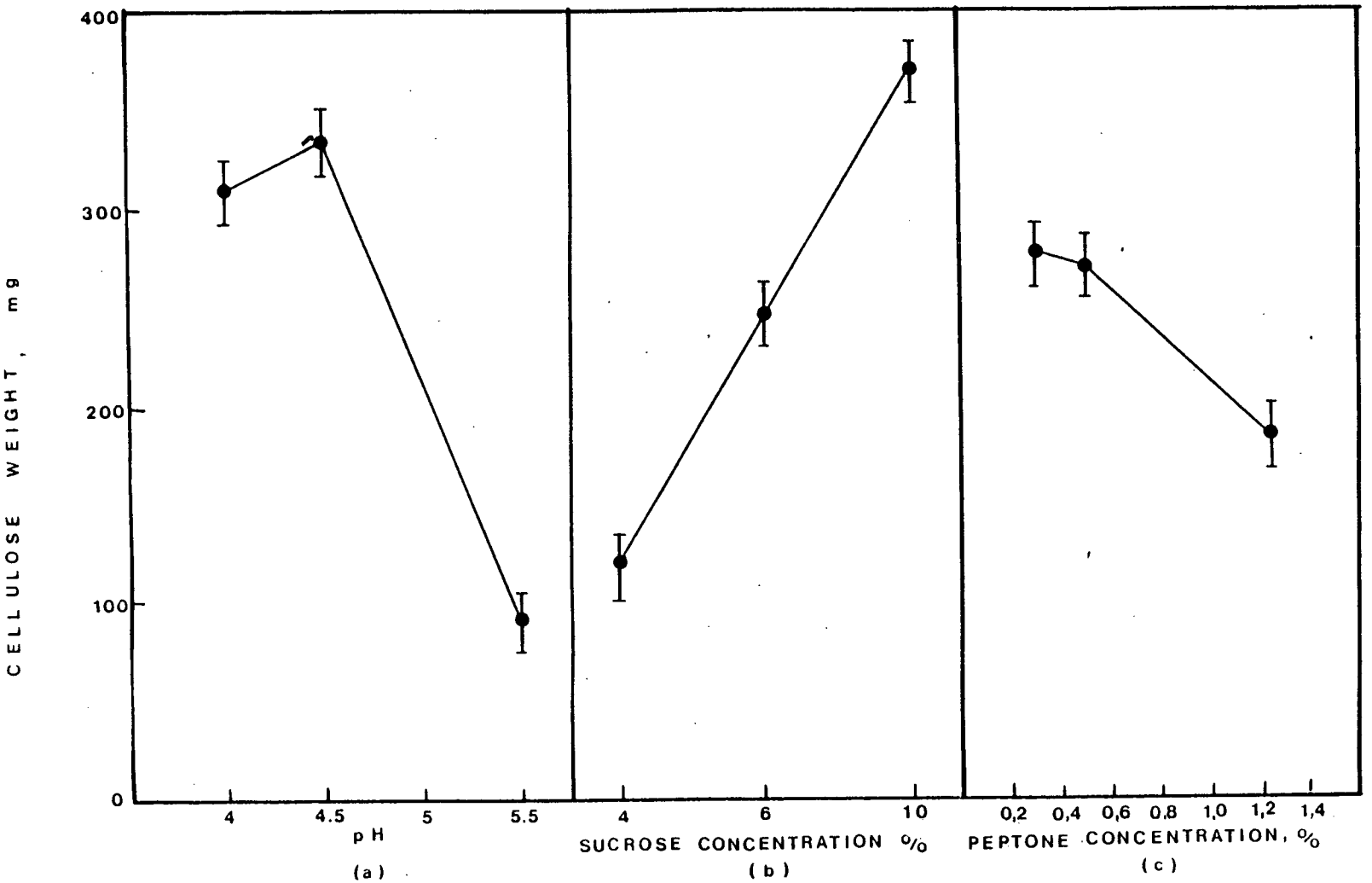

Figure 9. Effect Curves resulting from the selection of influential factors in defined media.

study was to find a suitable defined medium for the strains under investigation that would allow large yields of cellulose from an inexpensive and readily available substrate. Therefore, sucrose was chosen as the sugar substrate for this experiment and for further work.

The effect of sucrose concentration on cellulose production revealed that if the amount of sucrose is limited, the amount of cell material and thus the cellulose would also be reduced. The effect of low nutrient concentrations on growth rate was suggested by Brock (1979). It was proposed that at low nutrient concentrations, the nutrient cannot be transported into the cell at sufficiently rapid rates to satisfy all the metabolic demands for the nutrient. As the nutrient concentration is increased, a concentration will be reached that is no longer limiting, and further increases then no longer lead to increases in total crop.

As it is shown in Figure 9b, cellulose yields were greatly increased as sucrose concentration increased; however, the optimum concentration was not determined since the effect curve did not show a maximum or a plateau. In determining the optimum sucrose concentration further experimentation would be required, searching that region beyond 10%. Factorial designs present this limitation, where the results are highly dependent upon the choice of levels tested (Nakai, 1982). These results agreed well with those reported by Lapuz et al. (1967), who reported maximum cellulose production with 10% glucose or sucrose when 2, 6 and 10% sugar concentrations were tested. Cellulose yields however, as reported by Dudman (1960), were reduced when sugar

concentrations in hydrolyzed molasses were above 3.7% for one of their strains, and were unaffected by concentrations of up to 10.7% with a different strain. These disagreements may have arisen, firstly, from using different strains from those used in this study. Secondly, their cultures were shaken thus increasing aeration which may have lead to a shift in the metabolism of the organism away from cellulose towards increased oxidation of the sugar substrate (Dudman, 1960).

Dudman (1959b) reported that the influence of glucose concentration in defined medium on cellulose production was relatively slight unless succinate was present as an adjunct. The addition of succinate to the medium caused the cellulose yields to show a marked response to glucose concentration reaching a maximum at 5% concentration. Again, this variation in reponse to sugar concentration may be attributed to using different strains.

Peptone proved to be inhibitory at the maximum level tested (1.23%) which is equivalent to 0.2% nitrogen. The effect curve (Figure 9c) shows a slight plateau at low peptone concentrations, 0.3 and 0.5% equivalent to .05 and .08% nitrogen concentrations, respectively.

The inhibitory effect of peptone at 1.23% (0.2%N) agrees well with figures reported by Dudman (1959b). It was reported that cultures containing nitrogen concentrations ranging from 0.001 up to 0.1% as ammonium sulphate or as asparagine + glutamic acid gave similar results when cellulose yields were determined, but a difference was found when nitrogen concentrations were increased. Ammonium sulfate at concentrations equivalent to 0.2% N caused decreased growth and cellulose yields,

while the equivalent concentration of asparagine + glutamic acid gave rise to increased growth and cellulose yields. There was no direct evidence reported to suggest the mechanism by which the amino acid mixture became stimulatory.

Gaudy and Wolfe (1961) reported that peptone concentrations higher than 0.25% significantly inhibited cellulose formation in cultures of Sphacretilus natans. The ratio of cellulose formation to total growth decreased with increasing peptone concentration, although total weight continued to increase. It was suggested that the disturbance in the metabolism due to high concentrations of peptone may have been, as observed by others, due to growth inhibition by tryptophan, cystine, and methionine in concentrations as low as 0.2 mg/L. However, other researchers found no inhibition with tryptophan at 100 mg/L; tyrosine and cystine retarded growth at this concentration. Others reported delayed growth at concentrations of 0.1% of leucine, threonine, cystine and tyrosine.

A possible explanation for the inhibitory effect of high peptone concentrations on cellulose formation by A. xylinum, might be that increased oxidation of the substrate occurred rather than cellulose formation; as suggested by the lower final pH and cellulose yield recorded for those cultures containing 1.23% peptone (0.2% nitrogen) at pH 4.0, whereas this behaviour was not observed at higher pH values. This hypothesis is clearly supported by the highly significant interaction showed between pH and peptone concentration ($p < 0.01$).

A significant interaction was also observed between pH and sucrose concentration ($p < 0.05$). This interaction indicated a different

response to increasing sugar concentration at the different pH tested.

The aim of this study was to develop a carefully defined formulation for determining the specific requirements of cellulose production which would be adopted for further work. Defined medium was preferred since it permits the determination of those specific requirements for growth and cellulose formation, and simultaneous variation of the two most essential nutritional factors to microbial activity 1) a source of energy for cell metabolic processes, a carbon source, and 2) a source of materials from which cellular matter can by synthesized, a nitrogen source. It appears that by simultaneously varying nutrient concentrations and medium pH, much more valuable information can be obtained than when only one factor is studied at a time as was the case in all available reports on nutrient requirements of A. xylinum which totally ignore interactions among factors.

Other advantages of defined medium include its reproducibility, translucency, and the relative ease of cellulose recovery and purification. However, this phase should be followed by a transition to a natural medium in order to scale-up the formulation to a commercially viable process. Natural medium might, for example, be beet molasses which is a by-product of the refining of table sugar from the sugar beet; blackstrap molasses which is the mother liquor remaining after the crystallization of brown sugar from the crude sugar juice extracted from sugar cane; refiner's cane molasses which is the residue remaining after white sugar has been recrystallized from brown sugar. In general, beet and cane molasses have comparable amounts of fermentable sugar (48-58 total sugar), potassium, trace minerals, niacin, pyridoxine and

inositol. Beet molasses can have a five-fold higher organic nitrogen content, but half of it is betaine which is not assimilable by some species. Cane molasses is substantially richer in biotin, pantothenic acid, thiamin, magnesium and calcium.

Molasses appears to be an especially useful medium component, since it is a good source of nitrogen, inorganic constituents, and vitamins as well as carbohydrates. These nutritional characteristics combined with its economy make molasses one of the most widely utilized raw materials in industrial fermentation media (Zabriskie, 1980). In fact, experiments had been described determining the influence of some nutritional factors on cellulose production in media based on blackstrap molasses. Different carbohydrate sources were compared and were found to be in the following order: hydrolyzed molasses>molasses>glucose>-sucrose (Dudman, 1959b).

An agricultural waste material which is an excellent growth medium for A. xylinum may be supplemented coconut milk (Lapuz et al., 1967). The accelerated growth of this organism when coconut milk is added to a basal medium is due to the presence of growth promoting factors which were reported to be present in it by Shantz and Steward (1952; 1955). Another source of carbohydrate is found as a waste material from the sulfite paper-pulping industry. Sulfite waste liquor is the spent fluid remaining after wood for paper manufacturing is digested to form a cellulose fiber pulp using calcium bisulfite. The liquor contains lignin, sulfite, and hexoses derived from the hydrolysis of hemicelluloses. Treated sulfite waste liquor has been used in the production of

single cell protein (SCP) and ethanol (Zabriskie, 1980). The primary objective of those processes had been to upgrade this waste stream into a useful product.

Pineapple pulp derived from trimmings and cores in the pineapple-canning industry is another waste material high in carbohydrates able to support growth of A. xylinum (Alaban, 1962; Villanueva, 1937). Therefore, a great variety of substrates might be utilized for cellulose synthesis increasing the potential flexibility of utilization of agricultural wastes.

B. Culture Stability

There is ample evidence in the literature of the high mutability of cells within species of the acetic acid bacteria (Shimwell and Carr, 1958; Schramm and Hestrin, 1954; Cook and Colvin, 1980). Therefore, the stability of the three strains available in this study was assessed before any of them was chosen for further work. Cultures were grown under static and swirled conditions, since both situations were of interest for the applications intended.

Table 2 shows the percentage of cellulose-deficient colonies from static and swirled liquid cultures on agar surfaces as a function of the number of transfers. There was a rapid increase in the proportion (and therefore of total numbers) of cellulose deficient colonies in the swirled cultures; 100% of the colonies were cellulose deficient between the fifth (10 days) and sixth (12 days) serial transfer for the three strains. The swirled cultures did not develop the characteristic pellicle formed in static cultures, instead, cellulose in the swirled

Table 2. Mean values of percentage of cellulose "deficient" CFU in liquid cultures, swirled and static, and a function of number of transfers (n=3).

No. of Transfers	Cellulose-deficient CFU (%)					
	ATCC 14851		ATCC 10821		Philippine strain	
	Swirled	Static	Swirled	Static	Swirled	Static
1	0	0	0	0	0	0
2	35.4 (1.2)[1]	0	8.1 (7.0)	9.1 (3.1)	1.0 (0.2)	0
3	82.6 (5.7)	0	32.6 (0.6)	9.0 (0.7)	28.4 (3.0)	0
4	96.1 (0.9)	0	50.0 (5.0)	10.7 (4.1)	97.0 (2.1)	13.8 (6.0)
5	97.6 (0.7)	0	100.0 (0.0)	16.0 (3.1)	97.0 (3.2)	25.0 (2.1)
6	100.0 (0.0)	0	100.0 (0.0)	20.0 (3.0)	100.0 (0.0)	75.9 (10.4)
7	100.0 (0.0)	0	100.0 (0.0)	20.7 (2.6)	100.0 (0.0)	85.5 (1.0)
8	100.0 (0.0)	0	100.0 (0.0)	14.6 (0.6)	100.0 (0.0)	87.0 (11.2)
9	100.0 (0.0)	9.2 (9.1)	100.0 (0.0)	18.1 (3.4)	100.0 (0.0)	100.0 (0.0)
10	100.0 (0.0)	10.3 (2.8)	100.0 (0.0)	18.8 (1.9)	100.0 (0.0)	100.0 (0.0)

[1]Standard deviation.

cultures occurred in the form of spherical bodies. It appears that the cells growing in swirled medium gradually lost the ability to form cellulose and assumed a diffuse growth habitat. On the contrary, cultures grown under static conditions were remarkably more stable, as shown by the low percentage of cellulose-deficient colonies. In addition, all the strains developed the characteristic leathery cellulose pellicle at the surface of the liquid medium.

These results seem to agree well with the hypothesis of Schramm and Hestrin (1954). They proposed that in static liquid medium a floating pellicle insures an abundant oxygen supply to the cells, and in swirled liquid medium, on the other hand, the cells need not be floated by the pellicle in order to obtain oxygen and actively proliferating forms which are deficient in cellulose forming ability might thus gain ascendancy. Observations concerning the slow proliferation of cellulose deficient colonies of the Philippine strain in static cultures in serial transfers do not agree with results reported by Cook and Colvin (1980). These researchers demonstrated that in liquid medium, cells which are normal in cellulose production overgrow those which are deficient in this capacity. The reason for this negative selection in static liquid medium was speculative, but it was proposed that cells which produce less cellulose are less able to form a pellicle that tends to keep them on the surface of the medium. Because, A. xylinum is an obligate aerobe, cellulose-deficient cells would thus be less able to obtain the oxygen which they need and therefore would be at a definite disadvantge to normal cells under those conditions.

One interesting feature of static cultures of the Philippine strain which was completely different from that of the other two strains was pellicle thickness and porosity. The former produced highly hydrated porous pellicles approximately 1 to 2 cm thick, whereas the other two form very compact tough pellicles 0.3 to 0.5 mm thick. It appears that cultures with thick porous pellicles would have many more enmeshed cells within the pellicle matrix which do not need to produce cellulose to be able to obtain the oxygen necessary for survival. With the other two thin pellicle producing strains, however, cells have to struggle to reach the surface to obtain oxygen.

On the basis of the types of colonies grown on agar two distinct types were observed. Cellulose producing colonies were round with umbonate elevation, undulate margin, tough, opaque and cream in pigmentation, while cellulose deficient colonies were round, convex, entire margin, soft and translucent for strains ATCC 14851 and 10821. Colony morphology of the Philippine strain was slightly different. Cellulose producing colonies were round, of pulvinate elevation, entire margin, tough, opaque and cream in pigmentation whereas the cellulose deficient colonies had the same features except that they were flatter and extremely mucoid. When cellulose deficient colonies as well as the wild type (isolated from replicas) were transferred to liquid static cultures, all of them formed the characteristic zoogleal film at various rates of production. As expected, the wild type formed the characteristic tough cellulose pellicle in 3 to 4 days whereas the cellulose deficient colonies formed fragile pellicles after 1 to 2 weeks incubation.

Therefore, in the present investigation, it was concluded that all the strains tested were unstable under swirled conditions, while ATCC 14851 was the most stable of the three when grown under static conditions.

C. Growth Curves

Cellulose yields after 40 days of incubation varied from 6500 mg (64% conversion of total sugar) with the Philippine strain, to 600 mg (6.05% conversion of total sugar) produced by ATCC 10821, which may be considered a low-yielding strain. ATCC 14851 produced much less cellulose than the Philippine strain, however it yielded almost as much cellulose as ATCC 10821 (Figure 10). The nitrogen content in dry cellulose after 40 days of incubation, which is an indication of cell growth, varied between narrower limits (1.81-0.47%) than the cellulose yields, showing that the degree of growth was relatively similar between the three strains at the end of incubation (Figure 11).

Thus, it appears that the Philippine strain is a high cellulose-yielding strain, whereas the other two strains appear to shift their metabolism away from cellulose synthesis towards other metabolic pathways. The pH drop (to approximately 3.0) for the two low-yielding strains during the exponential phase, suggests that an oxidative mechanism predominated in these organisms. The pH for the Philippine strain remained constant during the exponential phase and started to decrease after 14 days of incubation (Figure 10). The two low-yielding strains were only able to utilize approximately 25 to 30% of the sugar,

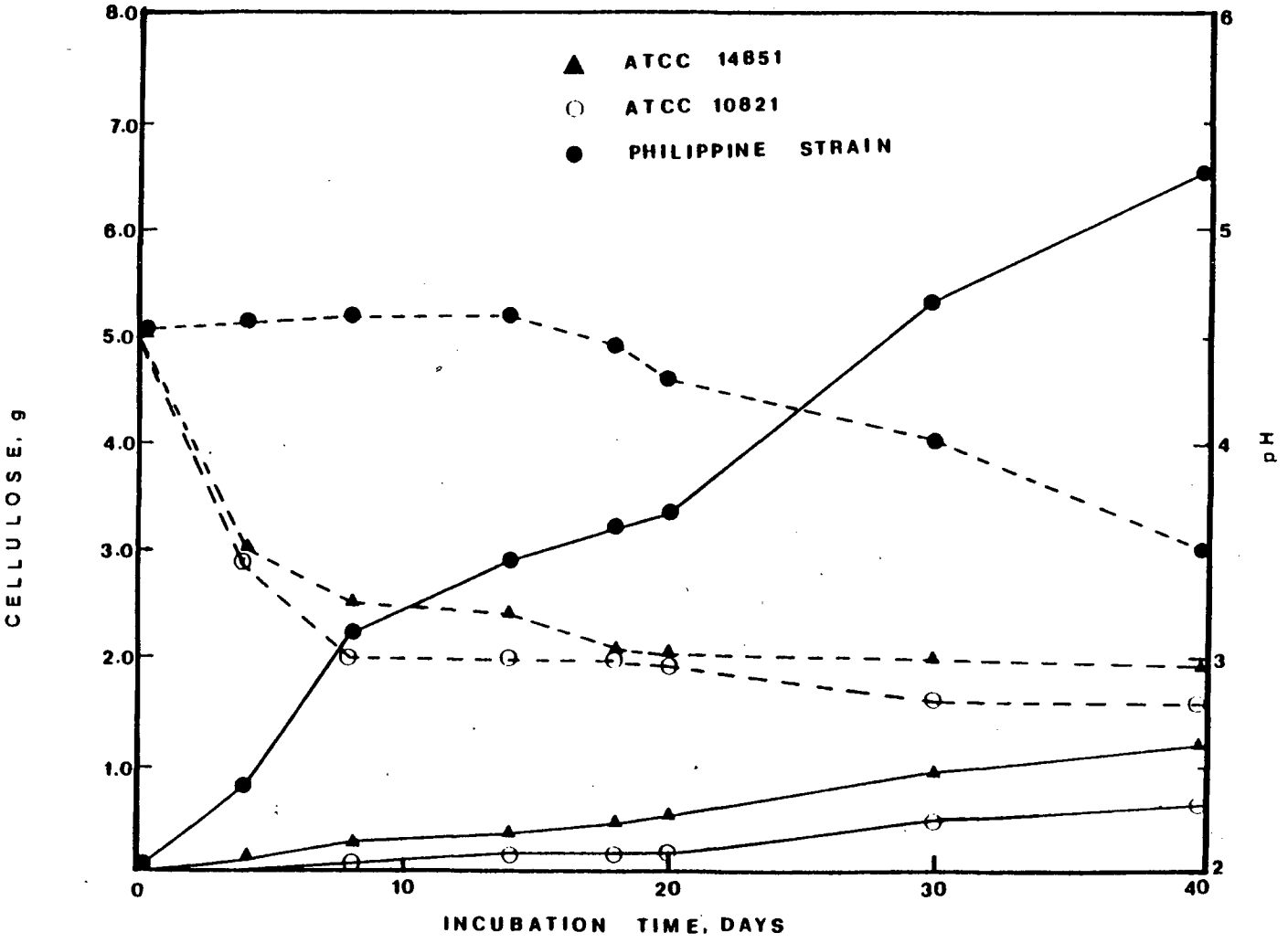

Figure 10. Cellulose production (——) and pH changes (---) during growth of three A. xylinum strains in 100 mL of defined medium.

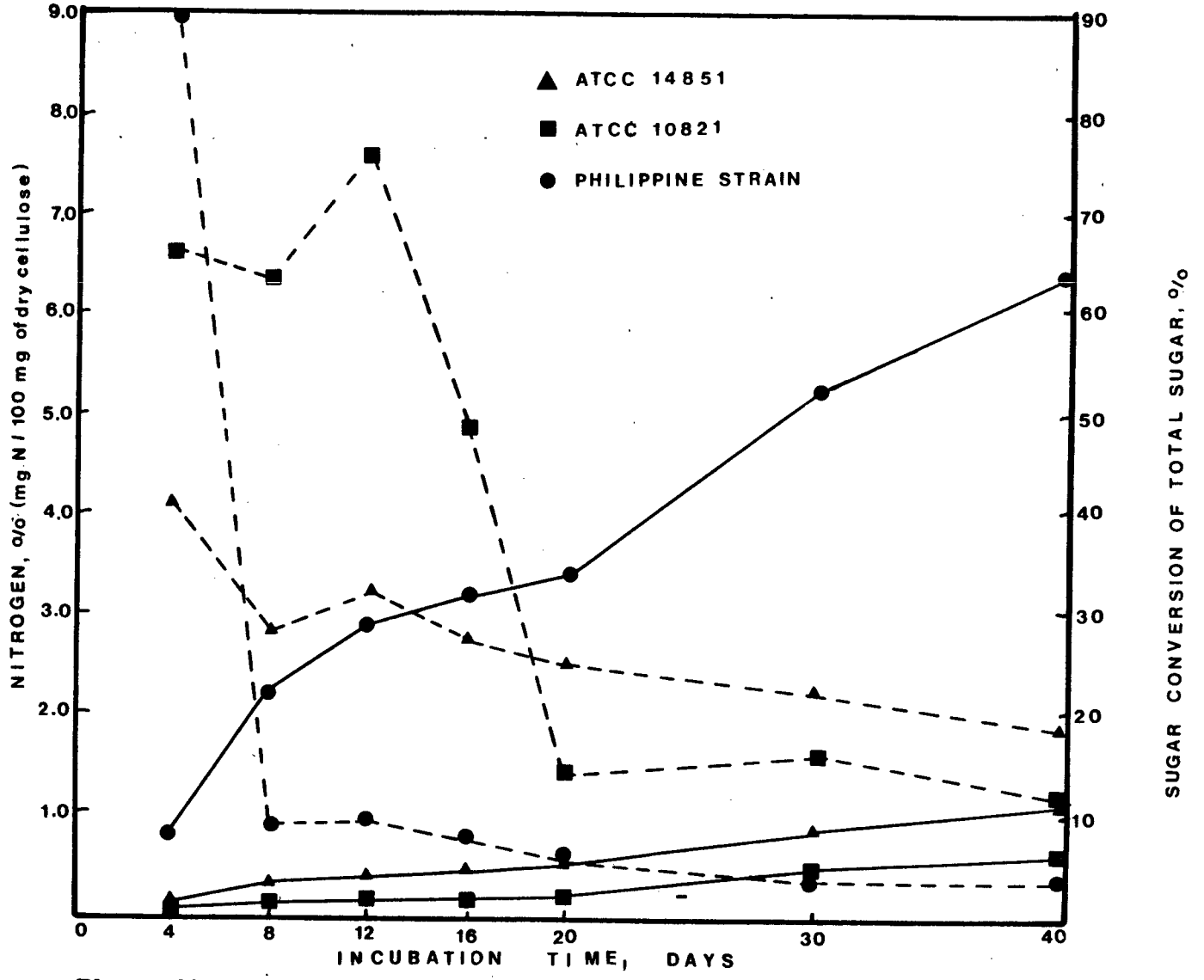

Figure 11. Sugar conversion of total sugar (——) and nitrogen content per 100 g of cellulose (---) of three A. xylinum strains in 100 mL of defined medium.

from which only about 45% was converted to cellulose by ATCC 14851 and about 20% by ATCC 10821. On the contrary, the Philippine strain was able to utilize up to 73%, of which 87% was converted to cellulose (Figure 12). The conversion of total sugar in the medium reached a maximum of 64% with the Philippine strain (Figure 11). Contrary to these results, Dudman (1959a) reported a maximum of 23.5% conversion of total sugar, when A. xylinum strain Hestrin was grown on hydrolyzed molasses for 30 days eventhough 94% of the sugar was utilized.

Comparison of the growth curves of the three A. xylinum strains suggested that this organism varies widely in its efficiency to convert sugar to cellulose. Thus, it appears that the Philippine strain is the only one whose industrial application may be economically feasible. However, it should be emphasized that no attempt was made to further improve sugar conversion by the use of additional adjuncts (Dudman, 1959b).

D. Degree of Polymerization vs. Incubation Time

As shown in Table 3, the degree of polymerization (DP) of the Philippine strain slightly decreases with incubation time to a mimimum of 2000 DP units after 20 days of incubation. The same behaviour appears evident for ATCC 14851 as indicated by the decreasing viscosity after 5 days of incubation (Table 4).

Takai et al. (1975) reported a maximum DP of 3500 attained after 250 h incubation of strain AHV-1595 of A. xylinum which then also decreased. This group of researchers interpreted the decreasing DP

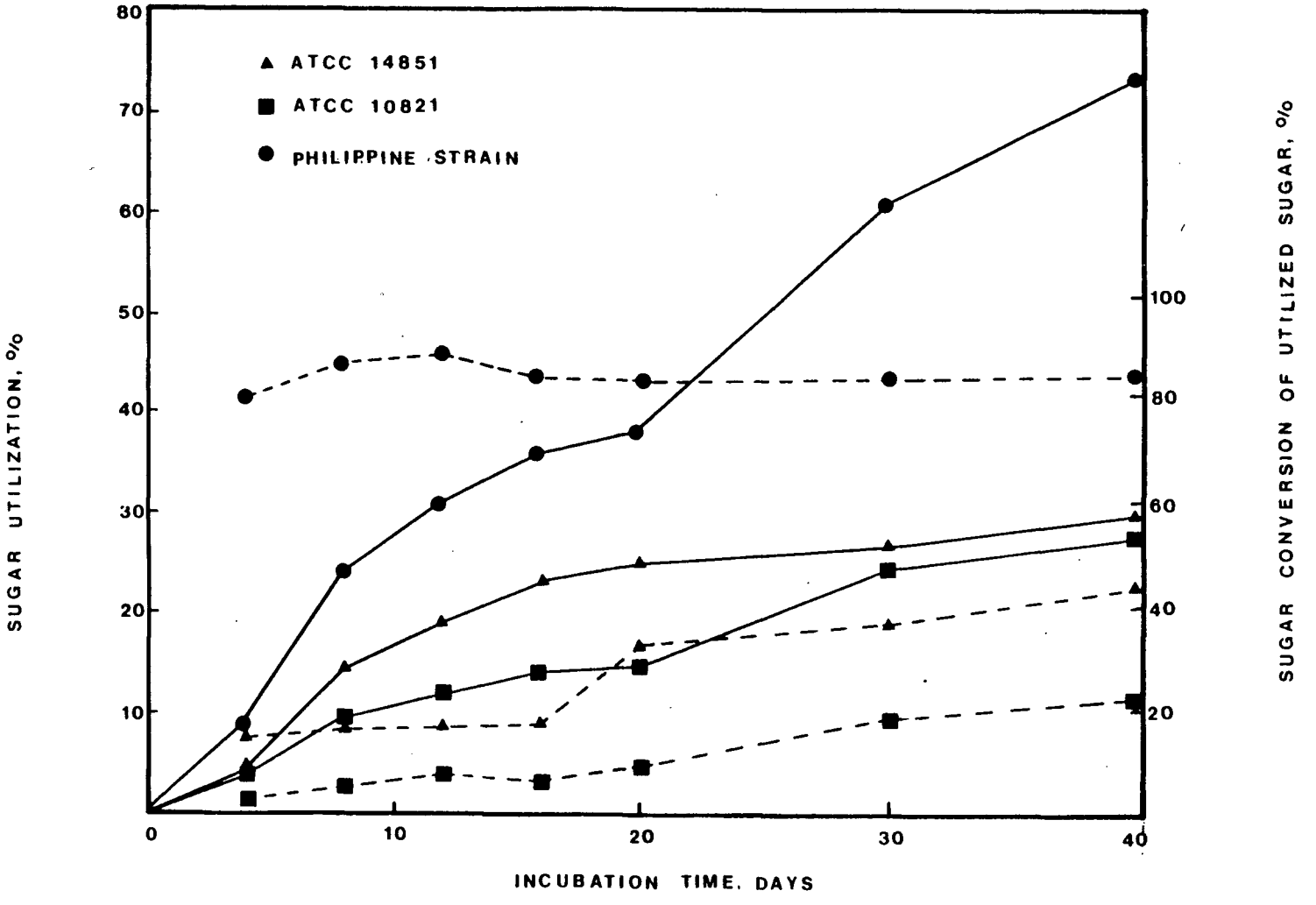

Figure 12. Sugar utilization (——) and sugar conversion (---) of three A. xylinum strains in 100 mL of defined medium.

Table 3. The viscometric average degree of polymerization of bacterial cellulose produced by incubation of the Philippine strain of A. xylinum.

Incubation time (days)	Intrinsic viscosity[a] (mPa•s)	D.P.
5	96.30 ± 1.13	2300
8	84.98 ± 0.67	2200
11	86.59 ± 1.62	2200
13	70.99 ± 0.24	2050
15	73.49 ± 1.23	2075
20	66.60 ± 0.60	2000
32	63.14 ± 0.70	2000

[a]Mean intrinsic viscosity ± S.D. (n=4).

Table 4. The average degree of polymerization of bacterial cellulose produced by incubation of ATCC 14851 of A. xylinum (n=4).

Incubation time (days)	Intrinsic viscosity[a] (mPa•s)	D.P.
5	251.02 ± 11.05	>2350
8	126.24 ± 20.0	>2350
11	131.02 ± 12.3	>2350
13	144.02 ± 18.68	>2350
15	109.50 ± 2.12	>2350
20	109.00 ± 5.01	>2350
32	105.00 ± 2.03	>2350

[a]Mean intrinsic viscosity ± S.D.

to be the result of the action of cellulase released into the medium at the same time as cellulose microfibrils were being formed. The action of a cellulase in cultures of A. xylinum, however, could not be confirmed by other authors. A more precise interpretation of this DP drop was given by Marx-Figini (1982) considering the polymerization mechanism and taking into account a certain synchronization of the bacteria. The relationship between molecular weight, reaction rate, time of synthesis, and generation time of the bacteria in bacterial polymerization processes has been theoretically derived assuming a Poisson like polymerization mechanism and taking into account an exponential increase of chain initiation and a linear chain growth. The theoretical approach (Figini, 1974) confirmed the assumption of a Poisson like mechanism and revealed furthermore that the experimentally observed decrease of DP is a consequence of a certain synchronization of the bacteria and the nonstatistical polymerization mechanism.

A decreasing DP could also be interpreted to be the result of the formation of mutants which possess different degrees of cellulose deficiency, but still able to produce shorter fibrils. This would result in higher polydispersity of the molecular weight, and consequently, a lower average DP would be determined.

E. Cellulosic Fibres Production

1. Fibre production apparatus

The preliminary design of this apparatus resulted in the successful production of cellulose fibres which became interwined to form a

fibrous strand oriented parallel to the medium flow. Unidirectional orientation of the fibres was first confirmed by observing the stained fibres by light microscopy. Photographs showing well defined areas of birefrigence are shown and discussed in the fibre microstructure section.

This fibre production apparatus provided a completely sterile system which could be aseptically inoculated with cultures of A. xylinum.

A second apparatus was designed based on the same principle of unidirectional flow (Townsley, 1981; personal communication) of sterile medium, which would provide a larger growing surface. Further improvements were the inclusion of an air filter through which air was sparged into the chamber, a growing surface provided with channels where individual strands were synthesized, the adaption of a pH control system which maintained an optimum pH and a timer which provided the means to intermittently control the flow.

2. Process for the production of fibres

Two variables, the A. xylinum strain and the flow mode (continuous or intermittent, which appeared to determine the tensile strength of fibres were considered. Flow rate was fixed at 12.2 L/min since it was the lowest flow rate which still provided a smooth evenly distributed flowing media.

a) Effect of A. xylinum strain and flow mode on tensile strength

Values for tensile strength obtained through load-elongation tests of cellulose fibres produced by incubation of two strains of A. xylinum under two flow modes are presented in Table 5. Results of a two-way

Table 5. Tensile strength of cellulose fibres produced by two strains of A. xylinum cultivated under continuous or intermittent flow (Mean ± S.D.).

Tensile strength, N/cm^2 (n=3)			
ATCC 14851		Philippine strain	
Intermittent	Continuous	Intermittent	Continuous
8340 ± 1200	5760 ± 920	4000 ± 420	N.D.[1]

[1]Not detected.

analysis of variance are reported in Table 6. The factors of strain of A. xylinum and flow mode were computed to be highly significant sources of variation ($P<.01$).

Tensile strength of high polymers had been reported to be influenced by the total amount and orientation of crystalline material in a preferred direction (Battista, 1958). Several researchers had reported on the relation between crystalline orientation and rupture properties such as tensile strength and extension at break of cotton fibres; these observations indicated that the structural alignment of cellulose had a major influence on the tensile properties of the fibres (Joshi, et al., 1967; Shelat et al., 1960; Egle and Grant, 1970). This internal fibre arrangement is determined by extensive hydrogen bonding; molecules of cellulose associate through OH bonding, intra and inter-chain (Nissan, 1977). Thus, the significance of water in the formation of bacterial cellulose microfibrils is obvious. Wet cellulose is surrounded by layers of water molecules; one of the effects of drying is the removal of these multiple layers, permitting a close association of the chains and the irreversible formation of hydrogen bonds between closely opposed hydroxyl groups. Sometimes these associations are extensive and strong enough to form the crystallites (Colvin, 1977).

The mechanism by which fibres were produced by intermittent flow in this study, involved the combination of these aggregated and desiccated stages as explained by Colvin (1977). Intermittent flow allowed desiccation to take place with the concurrent closer association of cellulose chains. Strong associations resulted in the formation of oriented crystallites consequently increasing the tensile strength of

Table 6. Analysis of variance of mean tensile strength for cellulose fibres produced by two strains of A. xylinum cultivated under continuous or intermittent flow.

Source of variation	DF	Mean square	F-ratio
Strain of A. xylinum	1	0.7774×10^8	138.82**
Flow mode	1	0.3178×10^8	56.75**
Strain x flow mode	1	0.0136×10^8	2.42
Error	8	0.0056×10^8	
Total	11		

**Significant at $p < 0.01$.

the fibres. On the contrary, cellulose chains of fibres produced under continuous flow were constantly surrounded by water molecules which were competing with adjacent chains for those OH groups involved in hydrogen bonding, consequently weakening associations among cellulose chains.

Significant differences in the tensile strength of fibres produced by two different strains also suggest differences in crystalline orientation. Visual comparison of cellulose fibres produced by the two strains indicated that the Philippine strain produced a fibre network much more highly hydrated than that produced by ATCC 14851. Again, close aggregation of cellulose chains appeared to play an important role in tensile strength.

The interaction between strain and flow mode was computed to be a nonsignificant source of variation ($P > 0.05$). Following this experiment, intermittent flow (1 h on/1 h off) and strain ATCC 14851 were adopted for further trials.

b) Influence of aeration rate on growth and cellulose yield

The influence of aeration rate on growth and cellulose yields was examined in cultures grown in defined medium. Cultures were all grown under the same conditions, but aerated at different rates (0.04, 0.102, and 0.282 L air/L medium/min). The rate of sugar utilization reached a maximum of 15% at the end of incubation when air flow was sparged at a rate of 0.10 L air/L medium/min, whereas lower or higher air flow rates resulted in approximately 6.8 and 7.5% sugar utilization (Figure 13). In all trials, pH fell to about 3.2 to 3.6. Consequently, it appears

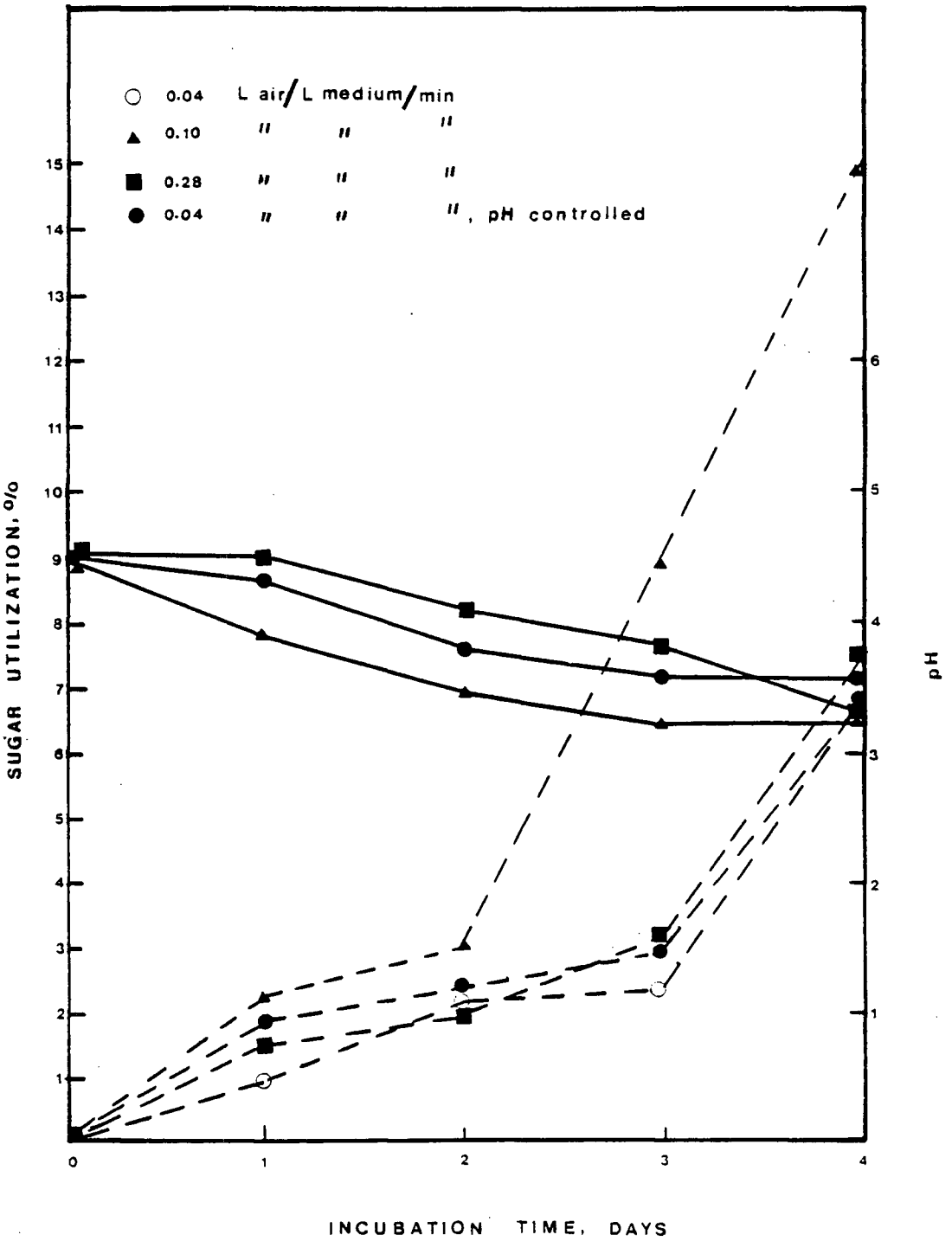

Figure 13. Influence of air flow rate on sugar utilization (---) and pH (——) of A. xylinum ATCC 14851 grown in defined medium.

appears that cellulose yields could be increased if the cultures were neutralized to keep the pH in a more favorable range. Thus, a fourth trial was examined following the same parameters as for the other three trials, except that the pH was controlled at 4.5 and air was sparged at .04 L air/L medium/min. Sugar utilization in the pH controlled trial followed the same trend as that in the non controlled trial, reaching a final sugar utilization of 6.8%. However, cellulose yield in the pH controlled trial represented an increase of approximately 23% over the non controlled trial. Furthermore, the most favourable conversion of utilized sugar to cellulose of 10.24% was in the pH controlled trial (Figure 14). Cellulose yields and sugar conversion were affected by the air flow rates used; cellulose yields reached a maximum of 3100 mg with an air flow rate of 0.10 L air/L medium/min), with lower yields observed at lower or higher flow rates (Figure 14). However, the most favourable conversion of utilized sugar to cellulose of 8.34% was with the lowest air flow rate (0.04 L air/L medium/min).

Increased aeration decreased the cellulose to nitrogen ratio C/N from 74.6 (0.04 L air/L medium/min) to 15.6 and 14.7. Thus, it appears that cellulose synthesis/unit of growth was reduced by increased aeration. This inverse relationship may be interpreted to mean that increased aeration lead to a shift in the metabolism of the organisms away from cellulose towards increased oxidation of the sugar substrate (Dudman, 1960). The C/N for the pH controlled trial was within the highest values, 60.6, as it would be expected for a process with the highest sugar conversion (10.24%).

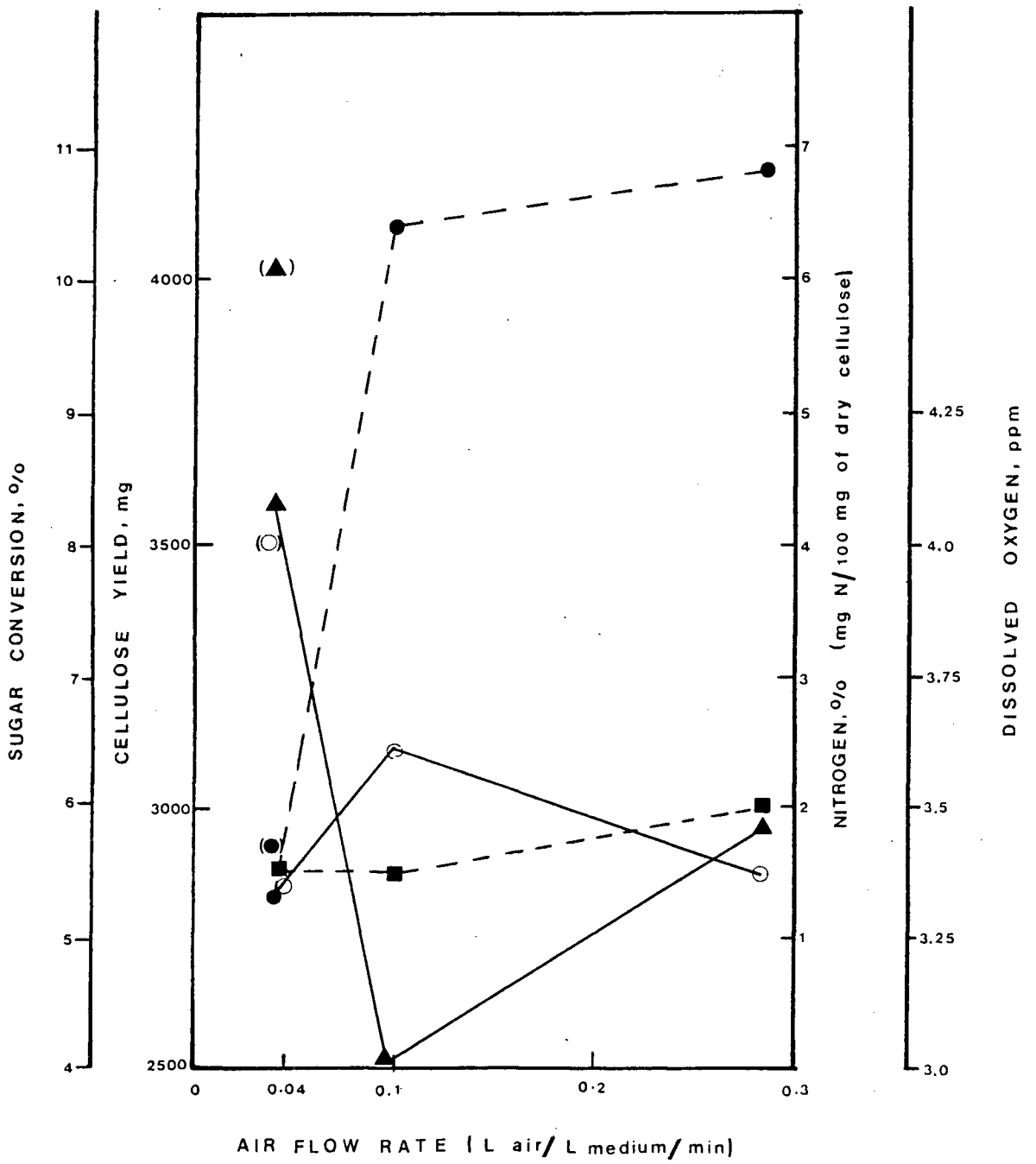

Figure 14. Influence of air flow rate on cellulose yield (○ ___ ○), sugar conversion (▲ ___ ▲), nitrogen content (● --- ●) and dissolved oxygen (■ --- ■) of A. xylinum ATCC 14851 grown in defined medium. Symbols in brackets represent pH controlled trials.

Dissolved oxygen (DO) was measured at the end of the trials to ensure anaerobic conditions did not occur, particularly with lower air flow rates. DO values ranged from 3.3 to 3.5 ppm (Figure 14). Thus, it appears that eventhough A. xylinum is a highly aerobic organism, a limited supply of oxygen is all that was required for maximum sugar conversion to cellulose, since accelerated growth occurred with increased aeration at the expense of decreased cellulose yields.

The following cultivation conditions were adopted for further fibre production: pH controlled defined medium was used with intermittent flow at a rate of 12.2 L medium/min; air was sparged into the system at a rate of 0.04 L air/L medium/min, and the inoculated apparatus was incubated for 4 days at 28-30°C.

F. Cellulosic Fibre Characterization

1. Tensile properties

a) Effect of moisture content on tensile strength

The effect of moisture content on tensile strength of cellulosic fibres is depicted in Figure 15. Each bar represents the mean of triplicate analyses of samples which were equilibrated over different saturated salt solutions.

The mechanical behaviour of the fibre structure appeared to be greatly influenced by the moisture content in the specimen. It has long been recognized that the moisture relationships of various fibre types differ and the degree to which the fibre properties are modified will vary. The load-elongation curve for a hydrophobic material when tested

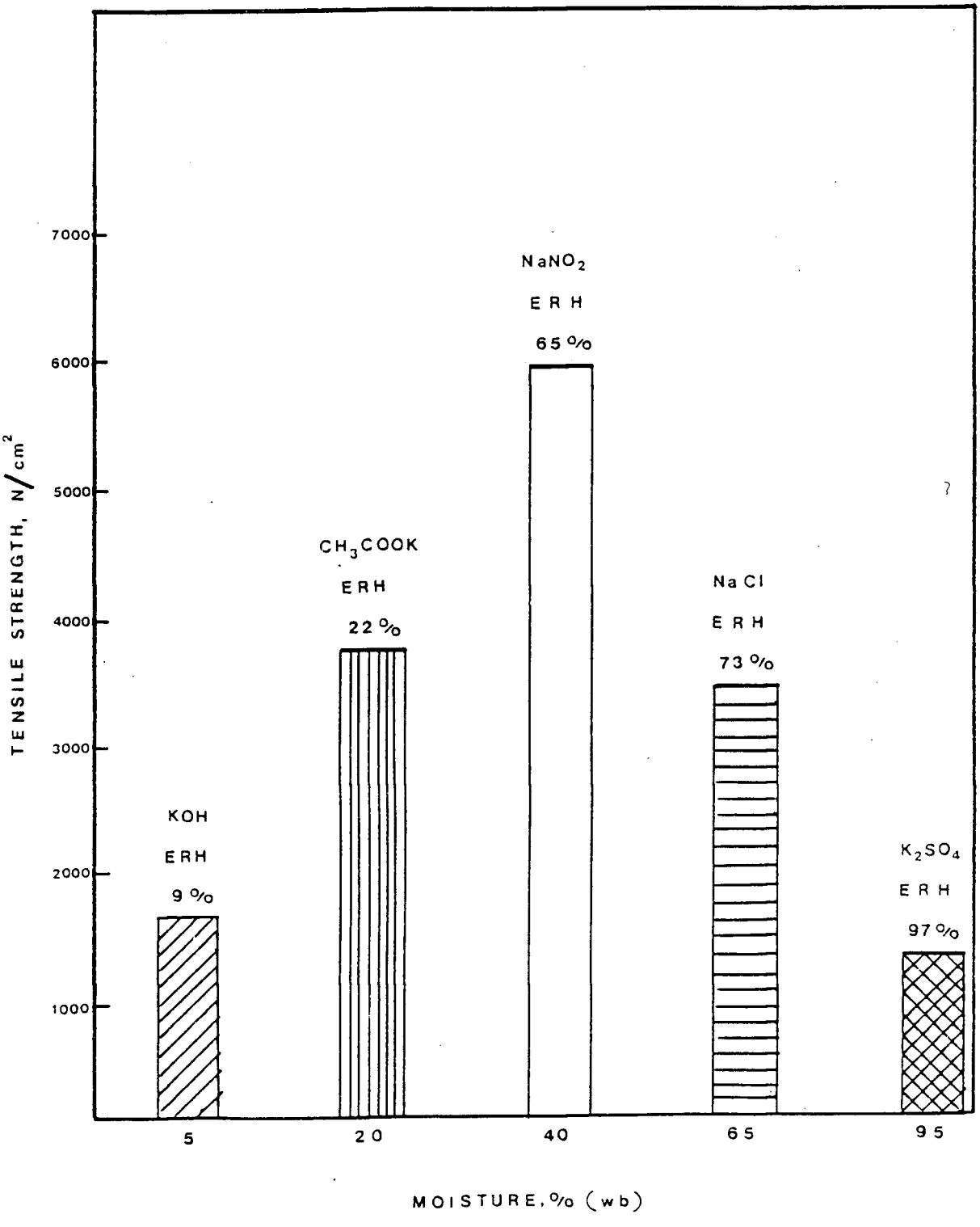

Figure 15. Mean tensile strength of A. xylinum cellulose fibres equilibrated over five saturated salt solutions (n=3).

in the dry state will be similar to the curve obtained from a wet state; on the other hand, the curves obtained when testing hydrophilic materials dry and wet will exhibit significant differences (Booth, 1964). Since cellulose fibres are highly hydrophilic, tensile strength was expected to be highly influenced by the moisture content in the sample. The mechanical behavior of the fibres in this study could be explained in terms of chain mobility in the cellulose fibre structure. Maximum tensile strength in a polymeric structure is attained when the load is applied unformly to all chain segments, i.e., a perfectly oriented structure. With imperfect orientation, strength can be improved if more chains are utilized in resisting the load. Orientation is possible, however, only if there is chain segment mobility under the test conditions. Chain mobility is useful in delaying failure of brittle materials because strain energy can be dissipated in viscous flow and/or transferred away from a region of localized stress concentration, e.g., the tip of a propagating crack (Warburton, 1970). Cellulose is composed principally of stiff, rigid cellulose chains. The greater part of each molecule is constrained to a crystalline lattice. There is little opportunity for chain mobility in such a structure; however, the little that exists is of considerable utility. It appears that approximately 40% moisture provided the cellulose structure with the required chain mobility needed to achieve maximum orientation when subjected to strain, consequently, maximum tensile strength was attained. As moisture content increased beyond 40% tensile strength decreased. This result may be interpreted in terms of excessive hydrogen bonding with water

rather than between cellulose chains, thus when a load was applied, cellulose chains possessed excessive mobility contributing to disorientation. On the other hand, as the moisture content approximated 0% the material became extremely brittle and tensile strength decreased.

For subsequent testing, therefore, it was essential that a standard moisture content in the sample was maintained.

b) Mapping super simplex Optimization (MSO) of mercerization treatment

The objective of this study was to find the conditions under which the tensile strength of A. xylinum cellulose fibres was maximized. After 18 experiments using a modified super simplex optimization (MSS) (Nakai, 1982) the iteration was stopped since the last three vertices to replace the worst (vertices 15, 16 and 18) had approximately the recommended 10% difference in the response size (Nakai et al., 1984). After carrying out eighteen experiments, the response values were plotted against the factor levels on separate graphs for each factor. The large and small limits of each factor were determined and entered into the grouping-matching program to obtain a series of matched data. The matched data points were joined in each graph and the most probable lines were drawn. Figures 16a, b, and c show the result of Mapping with the 18 data from MSS; the conditions for each treatment are summarized in Table 7. From these results, a target value for each factor was set as follows (the present best value is shown in parentheses): NaOH 8.31

(8.31)%, temperature 70 (59.09)°C, and time 20 (24.62) min. After two experiments using simultaneous shift, the search was terminated since the response value became worse. The MSO showed the presence of an optimum at 8.3% NaOH, 59°C and 24 min, with a maximum tensile strength of 83,300 ±1800 N/cm^2 which was an increase over the untreated fibres of 281%. The mean tensile strength of the untreated fibres was 29,600 ±4200 N/cm^2. The results in this study showed the usefulness of mapping super simplex optimization in finding the best conditions for the mercerization treatment of A. xylinum cellulose. Furthermore, MSO could be extremely helpful in textile research since numerous studies have been carried out to determine the optimum conditions under which cellulosic materials, i.e., cotton should be mercerized (Warwicker and Hallam, 1975; Peters, 1963). However, their results were far from being the true optimum; the treatments varied only one factor at a time, thus eliminating the strong interactions among the factors which take place during swelling of cellulose by the action of sodium hydroxide. On the contrary, super simplex optimization allowed the simultaneous variation of the three factors involved in mercerization. Furthermore, after 18 experiments, the mapping procedure provided a useful overview of the response surface around the optimum.

The effects of the mercerization treatments on the mechanical properties of cellulosic fibres were explained by Warwicker et al. (1966) on the basis of changes in fibre orientation and improvements in the uniformity of the strength along the fibre. This was attributed to

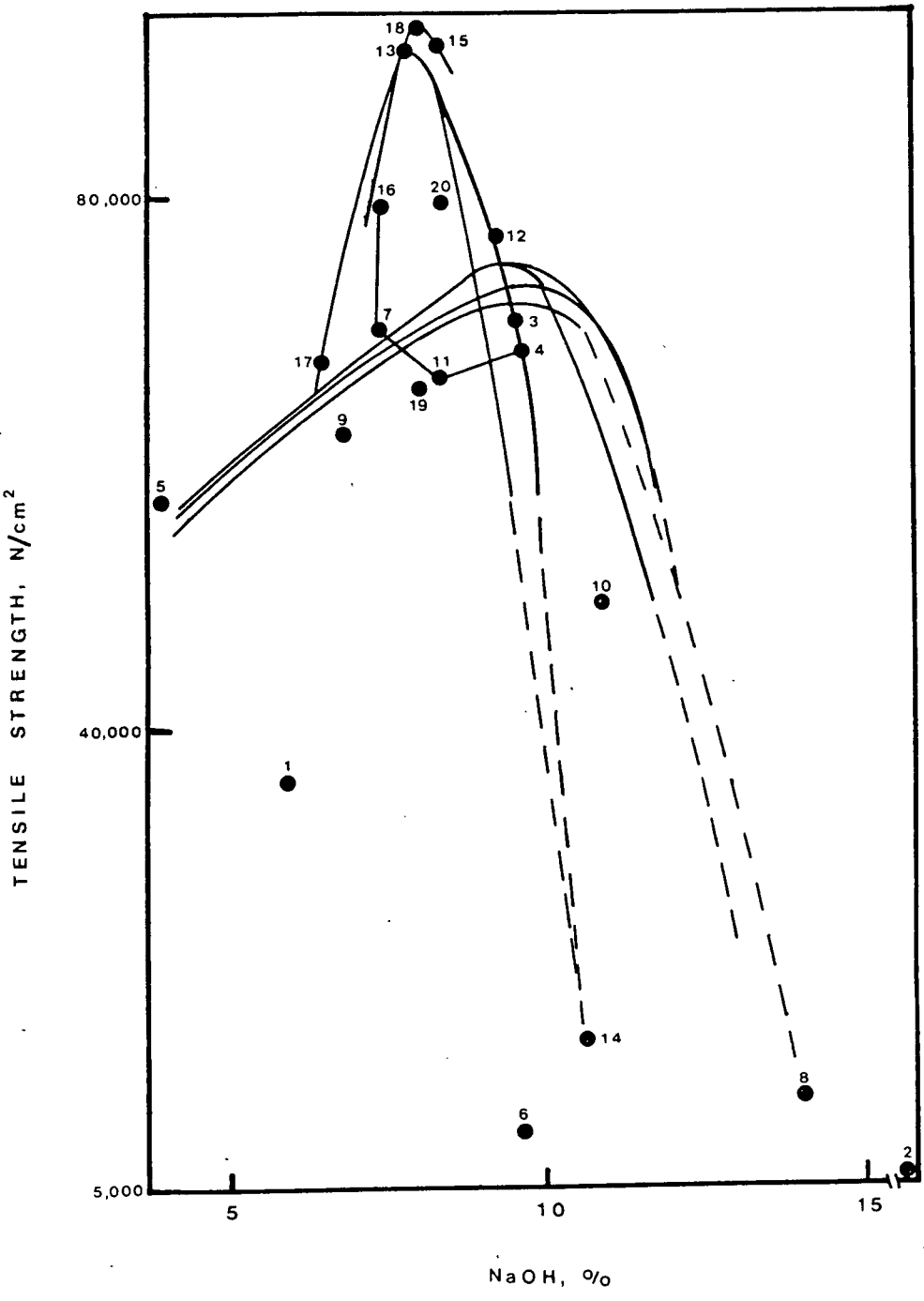

Figure 16a. Mapping responses of experiments to determine conditions to maximize tensile strength of A. xylinum cellulose fibres. Response value plotted against NaOH, %.

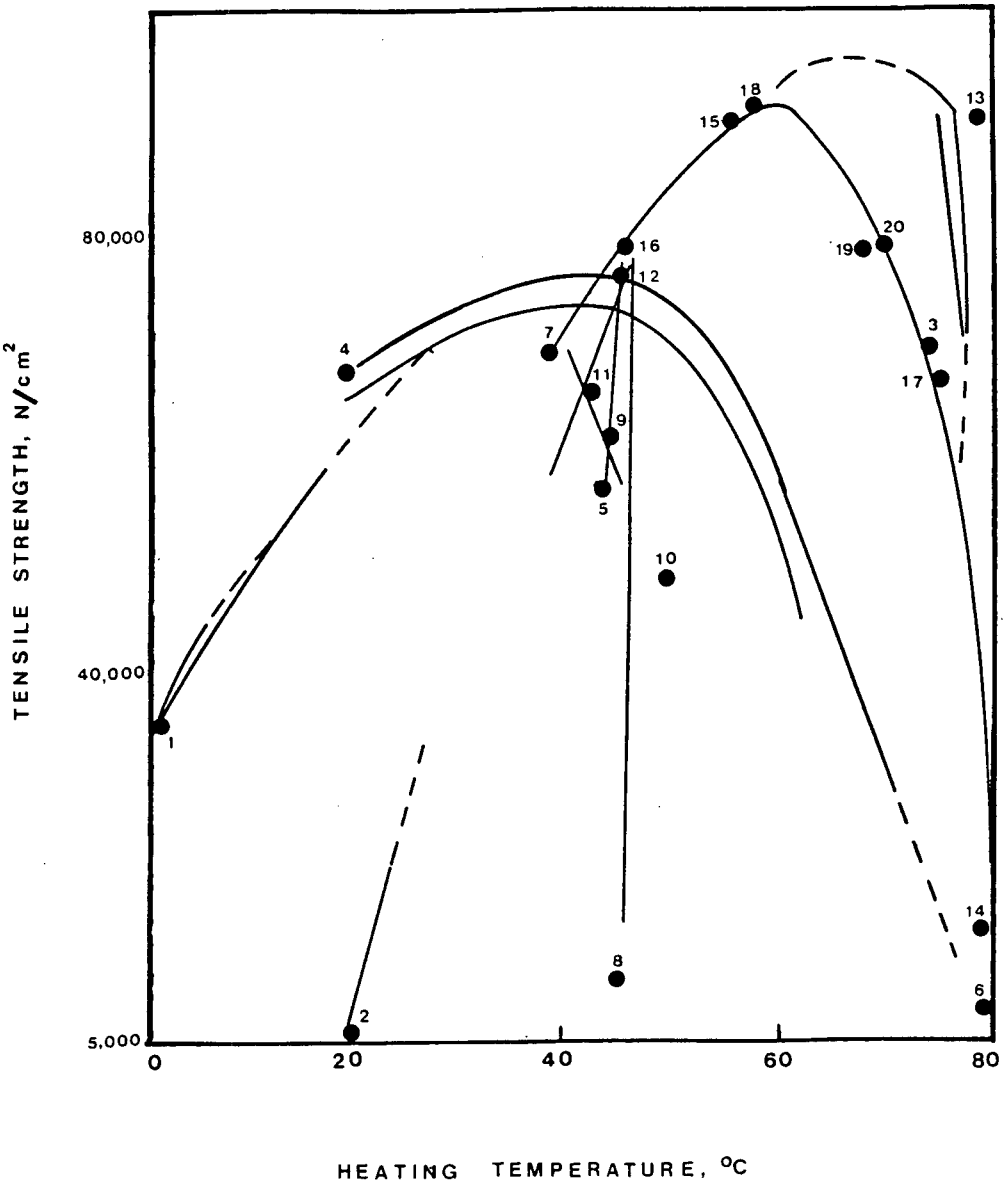

Figure 16b. Response values plotted against heating temperature, °C.

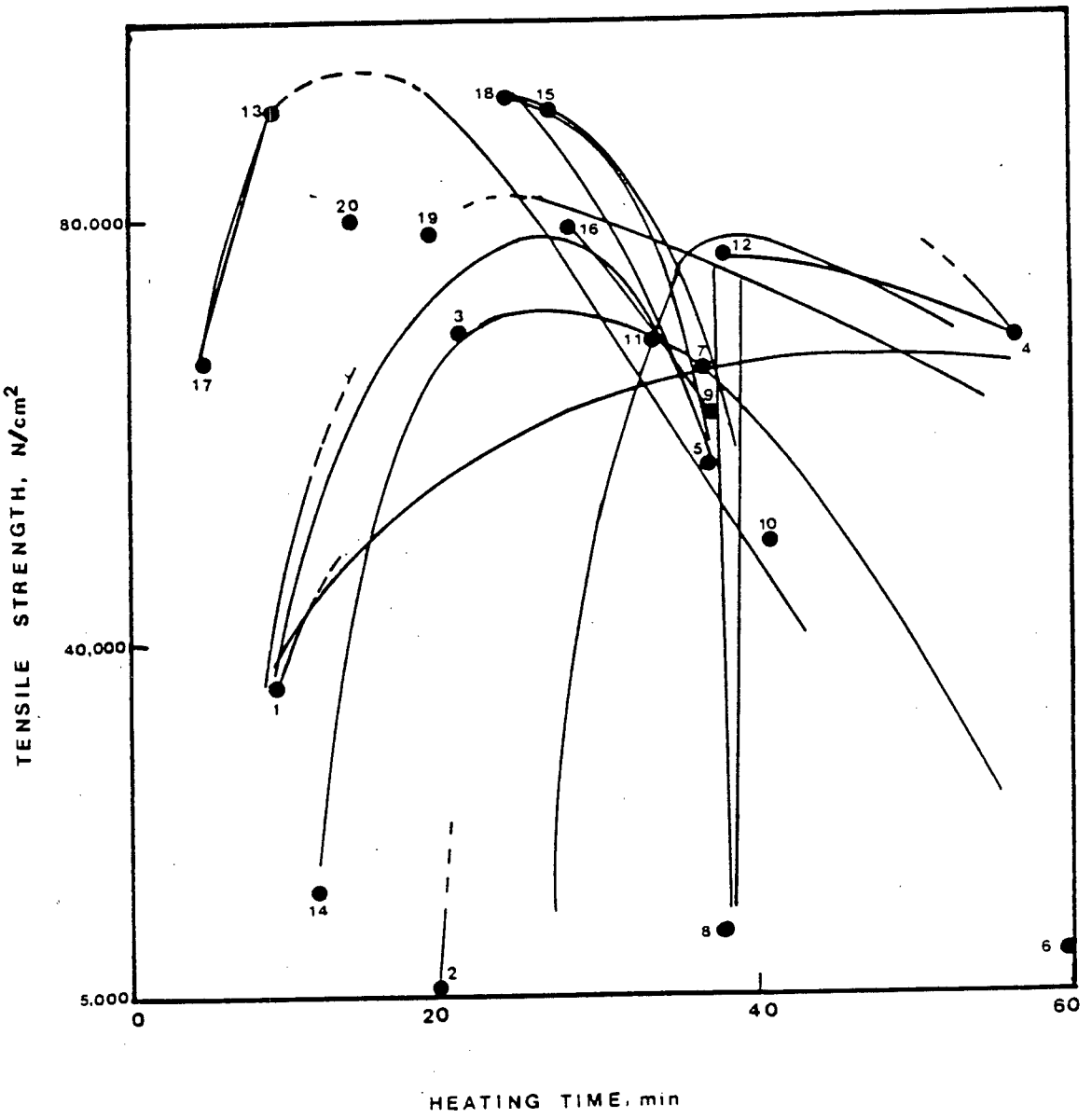

Figure 16c. Response values plotted against heating time, min.

Table 7. Mercerization treatments and mean tensile strength values obtained with Mapping Super Simplex Optimization (n=3).

Vertex No.	NaOH (%)	Temperature (°C)	Time (min)	Tensile Strength (N/cm^2)
1	6.00	2.0	10.0	32,500 ± 4200[a]
2	21.00	20.3	21.7	5,000 ± 400
3	9.77	75.5	21.7	63,200 ± 2800
4	9.77	20.3	57.1	61,600 ± 3200
5	4.00	44.8	37.5	51,600 ± 2500
6	9.69	80.0	60.0	8,300 ± 400
7	7.53	39.8	34.2	63,000 ± 1300
8	14.06	45.6	37.9	11,100 ± 900
9	7.39	45.1	37.6	56,200 ± 800
10	11.06	50.6	41.1	44,800 ± 2500
11	8.55	43.9	36.9	60,100 ± 2200
12	9.51	46.5	38.5	69,400 ± 1300
13	8.11	80.0	10.0	82,000 ± 1900
14	10.71	80.0	12.6	15,100 ± 900
15	8.54	57.2	27.4	82,400 ± 1800
16	7.66	46.9	28.8	71,900 ± 1500
17	6.70	76.2	5.6	61,100 ± 3200
18	8.31	59.0	24.6	83,300 ± 1800
19	8.31	70.0	20.0	72,400 ± 1300
20	8.50	72.0	15.0	79,700 ± 1500
untreated sample				29,600 ± 4200

[a]Standard deviation.

an increase in strength at the weakest points in the fibre. Thus, it was suggested that mercerization increased the tensile strength of fibres by eliminating the weak points; however, very little is known about the factors influencing uniformity and these deserve further investigation.

Modification of the cellulose structure takes place due to the high affinity of NaOH. The alkali is able to penetrate not only the amorphous regions but also the crystalline ones. During this process, the interchain forces are weakened and the strength of the material decreases, but is recovered when the fibre is deswollen and dried. The process of mercerization is irreversible because of the distortion of the polymer network and of the changes in crystalline structure (Peters, 1967). Several authors reported other structural changes taking place; i.e. the transformation of the original cellulose I to cellulose II when the fibre is completely mercerized as revealed by X-ray examination of native and mercerized cotton fibres (Peters, 1963), and the reduction of the amount of crystalline material and effects on the crystallite size as indicated by the leveling off degree of polymerization dependent on alkali concentration (Warwicker et al., 1966).

Although several theories of how swelling occurs have been proposed, it appears that no theory accounts for all the known facts. The most popular theory (Peters, 1963) postulated that the hydroxyl groups of the glucose units behave as weak acids which dissociate

independently of each other. In an alkaline medium, these will become dissociated to an extent dependent on caustic soda concentration, and the presence of ions in the cellulose gives rise to an osmotic pressure which will cause water to enter until counter balanced by the elastic forces of the swollen polymer. When the alkaline solution is replaced by water, undissociated hydroxyl groups are reformed; the osmotic pressure therefore falls, leaving the cellulose in its original chemical state. If the osmotic pressure becomes high enough, however, the polymer will be permanently distorted. Thus it appears that the response values (tensile strength) obtained in this study, when the three interacting factors were varied, were directly related to the degree of swelling taking place in the cellulose structure. The extent of swelling is determined by the osmotic pressure created between the cellulose and the external solution. As it can be seen in Figure 16, excessive swelling appeared to occur with increasing alkali concentration as shown by vertices 2 (21% NaOH, 20.38°C, 21.78 min) and 8 (14.06% NaOH, 45.6°C, 37.9 min), or by increasing temperature and/or time as shown by vertices 6 (9.69% NaOH, 80.0°C, 60 min) and 14 (10.71% NaOH, 80°C, 12.6 min). Extensive swelling resulted in mechanically damaged fibres, as indicated by the lower tensile strength values of these treated fibres than those of untreated fibres. Mean tensile strength values for vertices 2, 8, 6, 14 and for untreated fibres were 5000 ±400, 11,100 ±900, 8300 ±400, 15,100 ±900, and 29,600 ±4200 N/cm^2, respectively.

c) Effect of mercerization on tensile properties

A typical load-elongation curve conducted to specimen failure is depicted in Figure 17. This curve details features such as the elastic and flow regions, the extensibility of the material and the ultimate tensile strength and rupture characteristics. The shape of the curve is one typically obtained when fibres are subjected to an external force; it is balanced by internal forces developed in the molecular structure of the material. In the early stages of stretching the material, the elongation is mainly concerned with the deformation of the amorphous regions in which bonds are stretched and sheared. If the stress were removed at this stage most of the extension would be recovered and the material would exhibit elastic properties. By increasing the stress further, the curve bends slightly and large extensions are produced by small increases in stress. A sort of plastic "flow" of the material occurs. The long-chain molecules rearrange themselves with further breaking of secondary bonds; the curve begins to bend towards the force axis until the breaking point is reached. Such typical behaviour was described by Booth (1964) for different fibres; showing slightly different shapes for fibres with different molecular structure. Since physical and chemical treatments affect the load-elongation properties of fibres (Booth, 1964), the effect of mercerization on the tensile properties of A. xylinum cellulose fibres was studied. Values for tensile strength, modulus of elasticity and percent elongation obtained through load-elongation analysis of mercerized and unmercerized fibres are presented in Table 8. Results of a one-way analysis of variance with treatment as the single factor are reported in Tables 9, 10 and 11.

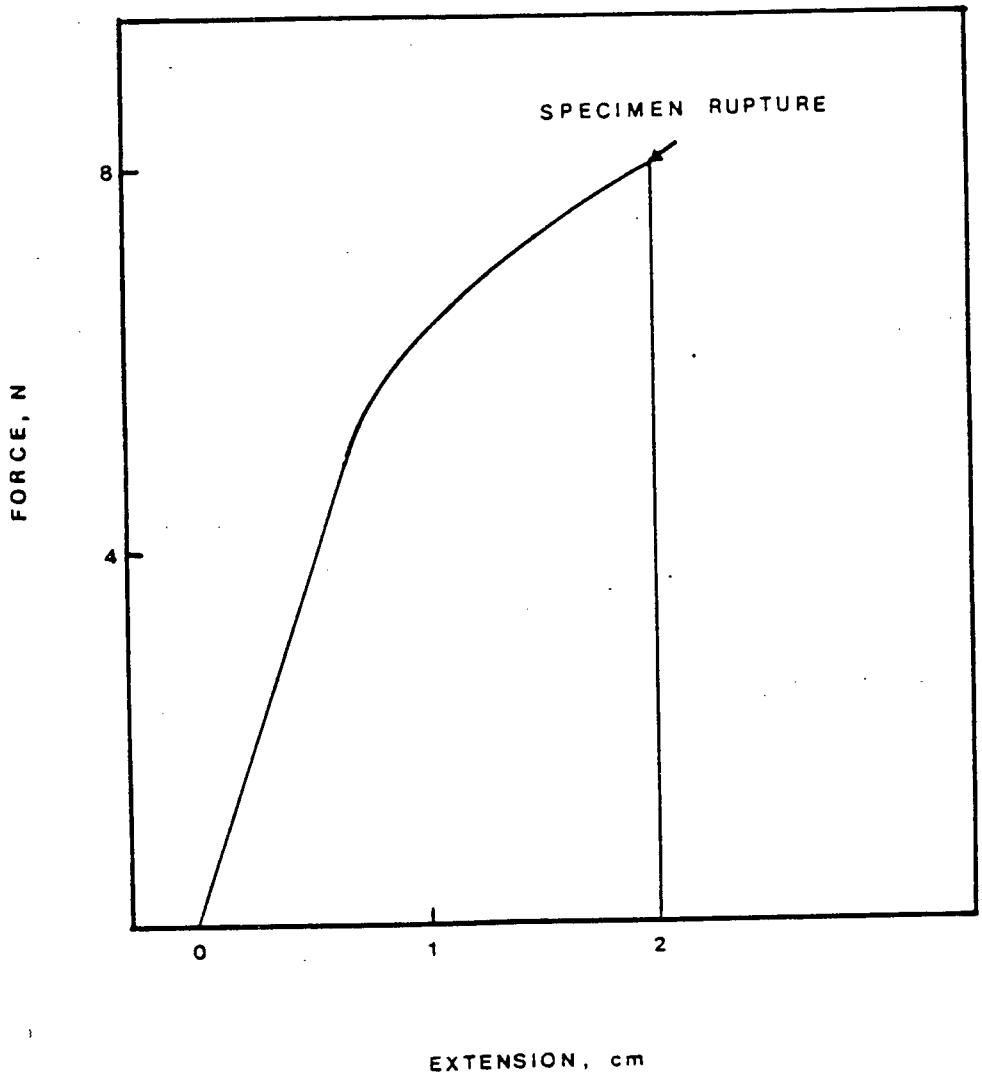

Figure 17. Typical load-elongation curve of A. xylinum cellulose fibres conducted to specimen failure.

Table 8. Mean values of tensile properties of mercerized and unmercerized fibres (n=8).

Treatment	Tensile Strength (N/cm^2)	Elongation (%)	Modulus of Elasticity (N/cm)
Mercerization	22,100 ± 3900[a]	6.56 ± 2.29	22.18 ± 9.74
Control	4,700 ± 400	10.21 ± 3.98	4.07 ± 0.83

[a]Standard deviation.

Table 9. Analysis of variance for tensile strength of mercerized and unmercerized fibres.

Source of variation	DF	Mean square	F-ratio
Treatment	1	0.1217×10^{10}	158.49**
Error	14	0.7680×10^{7}	
Total	15		

**Significant at $p < 0.01$.

Table 10. Analysis of variance for elongation of mercerized and unmercerized fibres.

Source of variation	DF	Mean square	F-ratio
Treatment	1	53.29	5.06
Error	14	10.52	
Total	15		

Table 11. Analysis of variance for modulus of elasticity of mercerized and unmercerized fibres.

Source of variation	DF	Mean square	F-ratio
Treatment	1	1311.40	27.40**
Error	14	47.85	
Total	15		

**Significant at $p < 0.01$.

Tensile strength (N/cm^2) and modulus of elasticity (N/cm) were significantly greater ($p < 0.01$) for the mercerized fibres, whereas elongation (%) was not significantly different for the mercerized and unmercerized fibres. The increased tensile strength of mercerized cellulosic fibres has already been explained in terms of fibre orientation and improved uniformity along the fibre (Warwicker et al., 1966). The modulus of elasticity (N/cm) gives a measure of the force required to produce an extension or reflects the stiffness of the fibres; a higher modulus for mercerized fibres indicates inextensivility, whereas a lower modulus indicates greater extensibility (Booth, 1964). It appears that such behaviour could be attributed to increased orientation of the fibres during mercerization; therefore, the typical chain rearrangement taking place when fibres are subjected to an external force is constrained.

d) Effect of batch on tensile properties

Values for tensile strength, modulus of elasticity and percent elongation obtained through load-elongation analyses of fibres produced from two batches are presented in Table 12. Results of a one-way analysis of variance with batch as the single factor are reported in Tables 13, 14 and 15.

Tensile properties of fibres in the two batches were not significantly different. These analyses support the conclusion that there was no variation of fibre structure, and consequently tensile properties when fibres were produced in two trials. However, this was true

Table 12. Mean values of tensile properties of cellulose fibres produced from two batches (n=8).

Batch	Tensile Strength (N/cm^2)	Elongation (%)	Modulus of Elasticity (N/cm)
1	4700 ± 400 [a]	10.21 ± 3.98	4.07 ± 0.83
2	5300 ± 1100	9.43 ± 6.03	5.81 ± 1.92

[a]Standard deviation.

Table 13. Analysis of variance for tensile strength of fibres produced in two different batches.

Source of variation	DF	Mean square	F-ratio
Batch	1	0.14652×10^7	2.21
Error	14	0.66208×10^6	
Total	15		

Table 14. Analysis of variance for elongation of fibres produced in two different batches.

Source of variation	DF	Mean square	F-ratio
Batch	1	2.48	0.095
Error	14	26.11	
Total	15		

Table 15. Analysis of variance for modulus of elasticity of fibres produced in two different batches.

Source of variation	DF	Mean square	F-ratio
Batch	1	12.02	5.48
Error	14	2.19	
Total	15		

provided that the inoculum was a pure culture of a cellulose producting strain. Therefore, periodic isolation of cellulose producing organisms was necessary in maintaining a pure culture which would provide uniformity throughout the trials.

2. Fibre Microstructure

a) Light microscopy

Micrographs depicting stained cellulose fibres matrix embedded with bacteria are presented in Figure 18 and 19. Observation of Figure 18 clearly reveals bacteria which are generally unidirectionally oriented. Such pattern is the result of cellulose fibres being produced by incubating A. xylinum within a confined unidirectional flow of medium. Initially, some organisms are deposited on the growing surface as medium is circulated over it; thereafter, more of them become attached to the cellulose ribbons already synthesized and attached to the growing surface. Consequently, ribbons from many bacteria associate with each other in a parallel fashion. Cellulose ribbon assembly by A. xylinum has already been observed by several workers (Brown et al., 1976; Colvin and Leppard, 1977; Zaar, 1979); however, in all of these studies A. xylinum was grown in static cultures where cellulose ribbons were interwined in a disorganized fashion. Therefore, this study was the first report on the characterization of the structure of cellulose ribbons which were produced in an organized parallel fashion (see Figure 19).

A micrograph depicting the cellulose fibres under polarized light is presented in Figure 20. Well defined unidirectional luminous areas

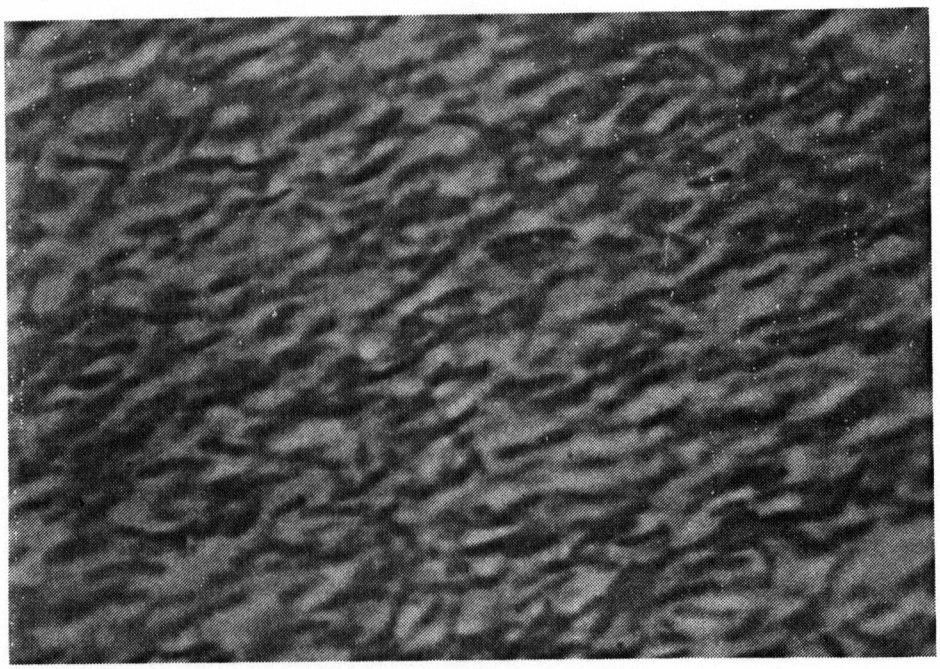

Figure 18. Light micrograph of stained cellulose fibre matrix embedded with bacteria (pw=130µm), pw = photo width.

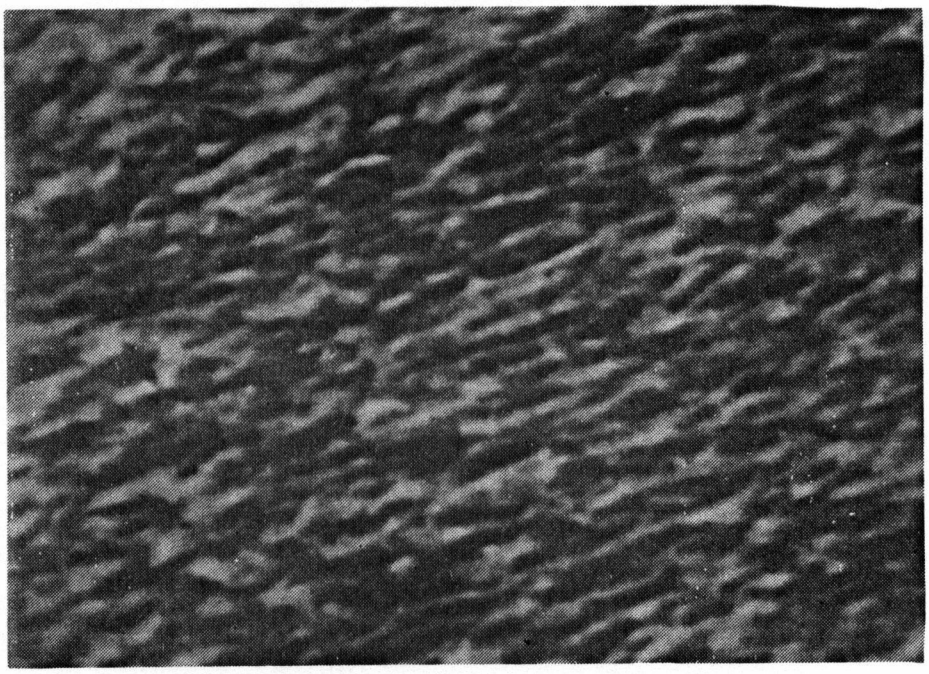

Figure 19. Light micrograph of cellulose fibres in an organized parallel fashion (pw=130µm).

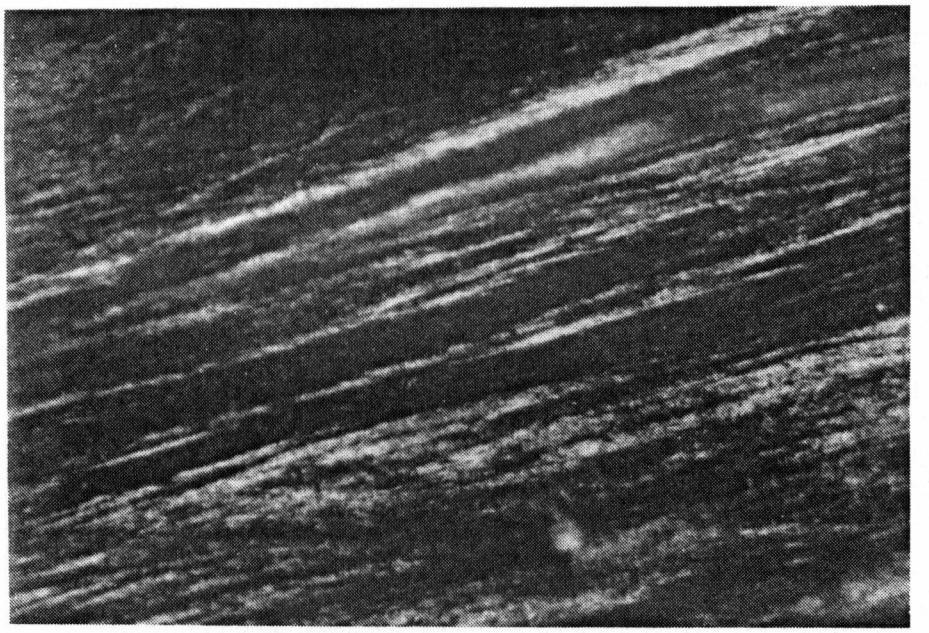

Figure 20. Unidirectionally oriented cellulose fibres under polarized light (pw=130µm).

can be observed which indicate a high degree of unidirectional orientation of this anisotropic material. Optically anisotropic or birefringent materials rotate the plane of polarized light and appear luminous, whereas optically isotropic materials cannot rotate the plane of polarized light and remain dark on rotation between crossed polars (Whistler, 1963). In general, birefrigence is used as a relative measure of orientation and is defined as the difference of refractive indexes in two principal directions; most natural and synthetic fibres are anisotropic or birefringent and can easily be observed through the polarizing microscope (Happey, 1978).

b) Scanning electron microscopy

Scanning electron micrographs of cellulose fibres produced under unidirectional flow are presented in Figures 21, 22 and 23. Fibres in Figures 22 and 23 were subjected to mercerization, whereas fibres in Figure 21 were unmercerized. Examination of the mercerized samples (Figures 22 and 23) revealed that a high degree of swelling occurred when cellulose fibres were exposed to NaOH; individual fibres appeared glued to each other adopting a very compact structure. On the contrary, a finer and looser structure was observed in unmercerized fibres (Figure 21). Width of individual cellulose ribbons ranged between 0.1-0.2 μm. A common feature observed in Figures 21, 22 and 23 was a high degree of parallel orientation of the fibres.

Scanning electron micrographs of cellulose fibres produced under static conditions are presented in Figures 24 to 31. Observation of

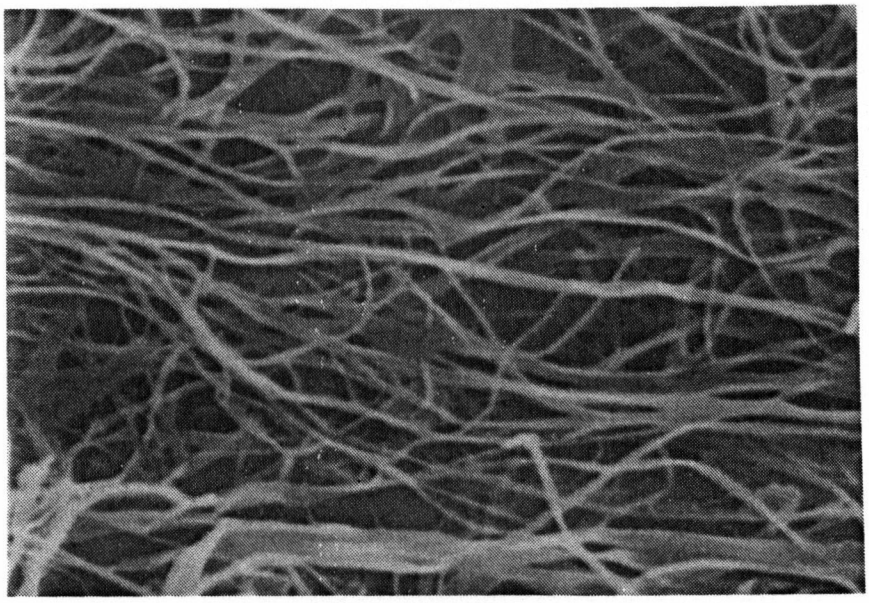

Figure 21. Scanning electron micrograph of unmercerized cellulose fibres produced under unidirectional flow showing a loose fibre structure (pw=10μm).

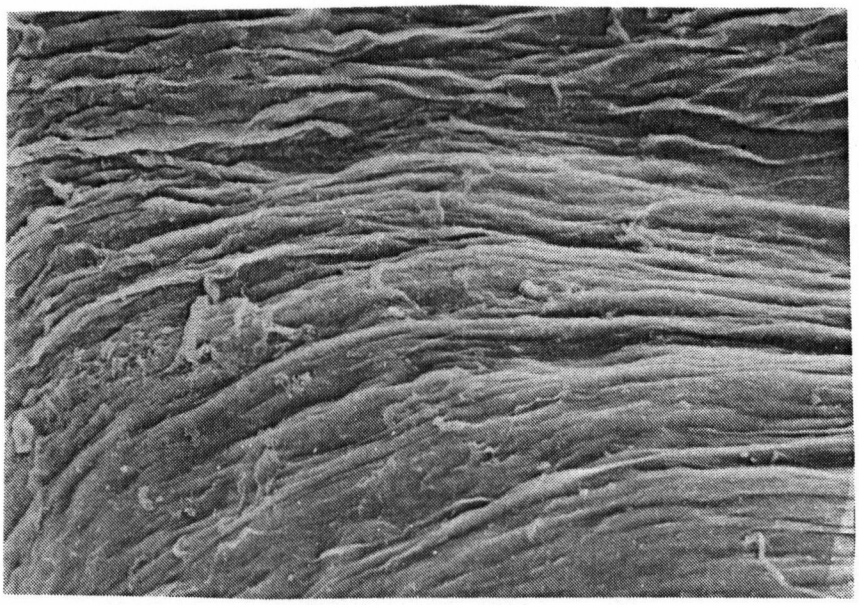

Figure 22. Scanning electron micrograph of mercerized cellulose fibres produced under unidirectional flow (pw=247μm).

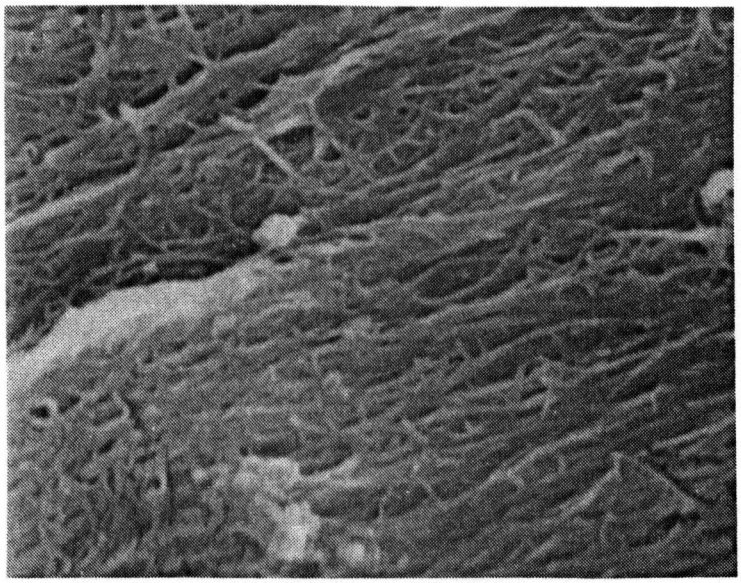

Figure 23. Scanning electron micrograph of mercerized cellulose fibres produced under unidirectional flow showing a compact fibre structure (pw=11 μm).

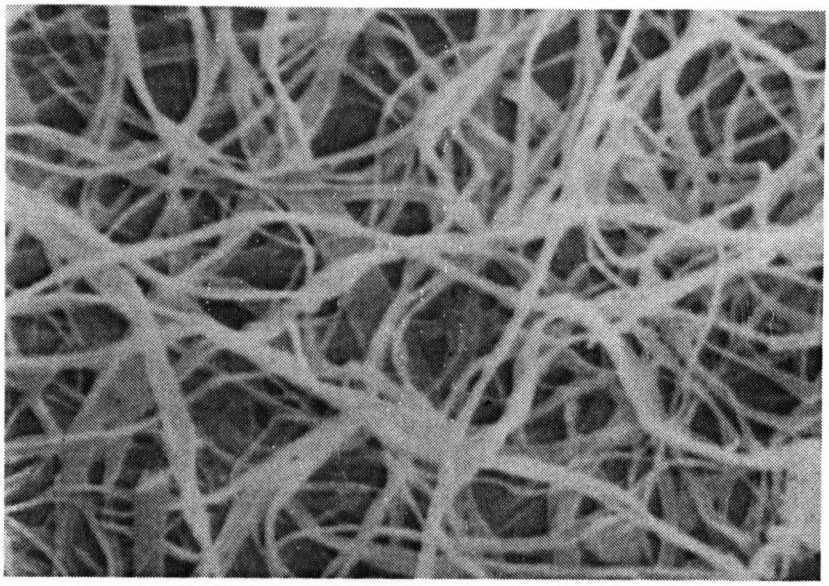

Figure 24. Scanning electron micrograph of unmercerized cellulose fibres produced under static conditions showing a network of entangled loose disoriented cellulose fibres (pw=11µm).

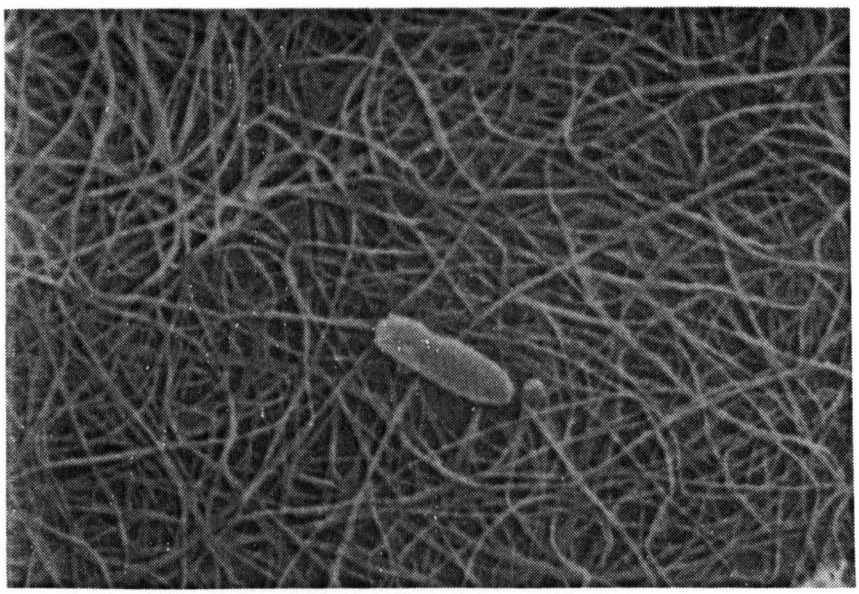

Figure 25. Scanning electron micrograph of unmercerized cellulose fibres produced under static conditions showing a bacterium entrapped within the matrix (pw=12µm).

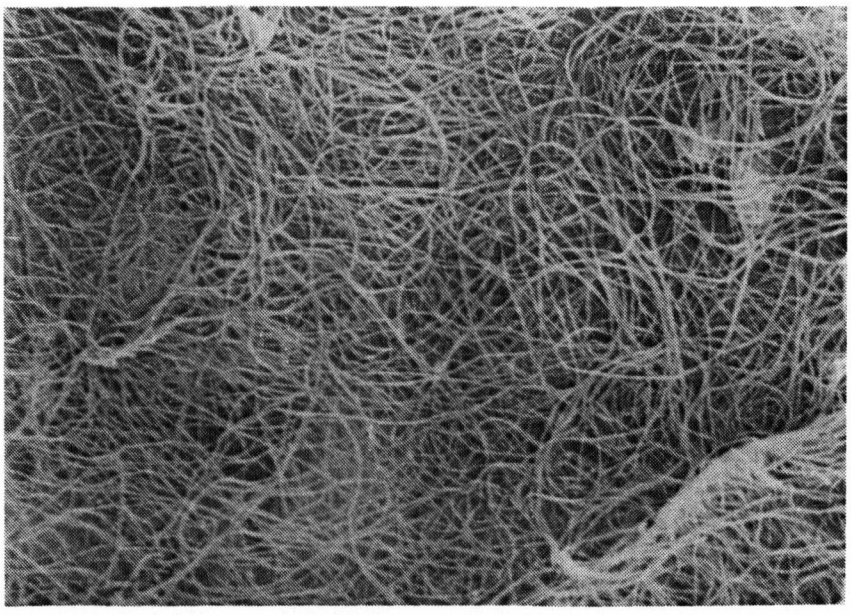

Figure 26. Scanning electron micrograph of unmercerized cellulose fibres produced under static conditions (pw=28μm).

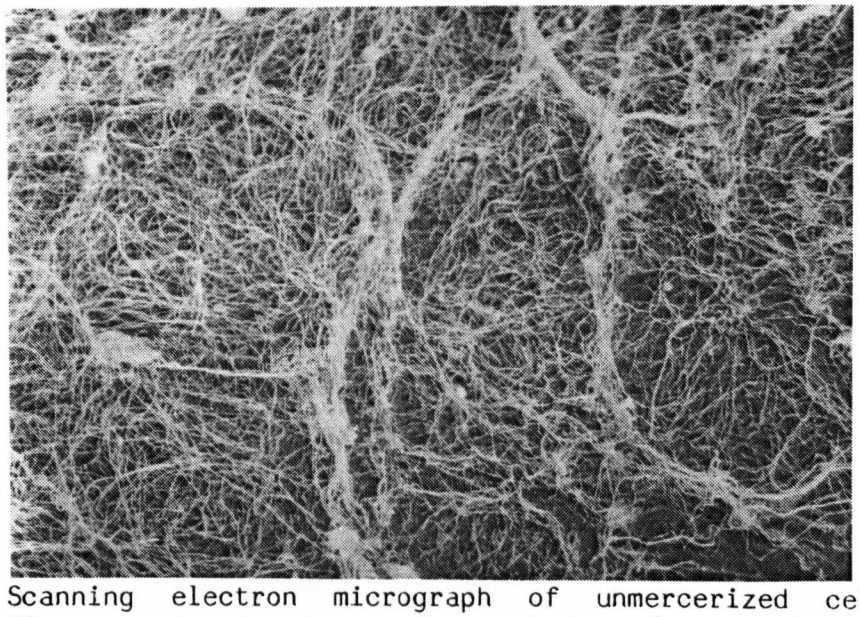

Figure 27. Scanning electron micrograph of unmercerized cellulose fibres produced under static conditions (pw=208μm).

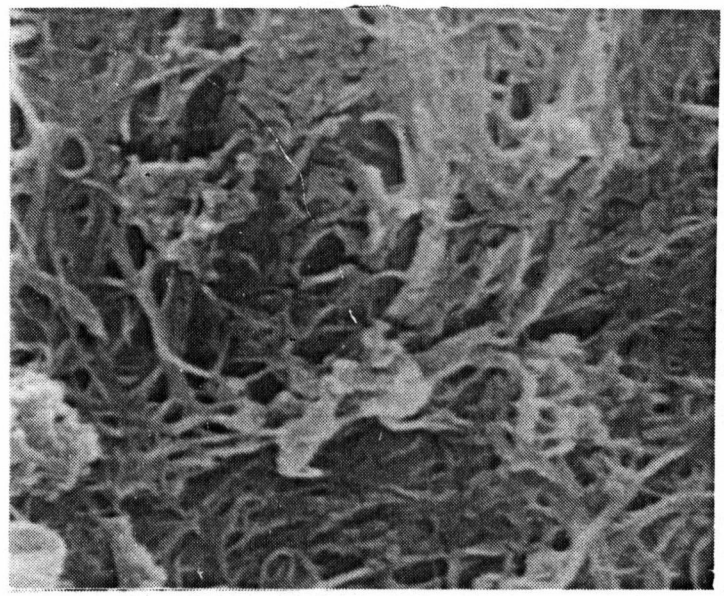

Figure 28. Scanning electron micrograph of mercerized cellulose fibres produced under static conditions (pw=11μm).

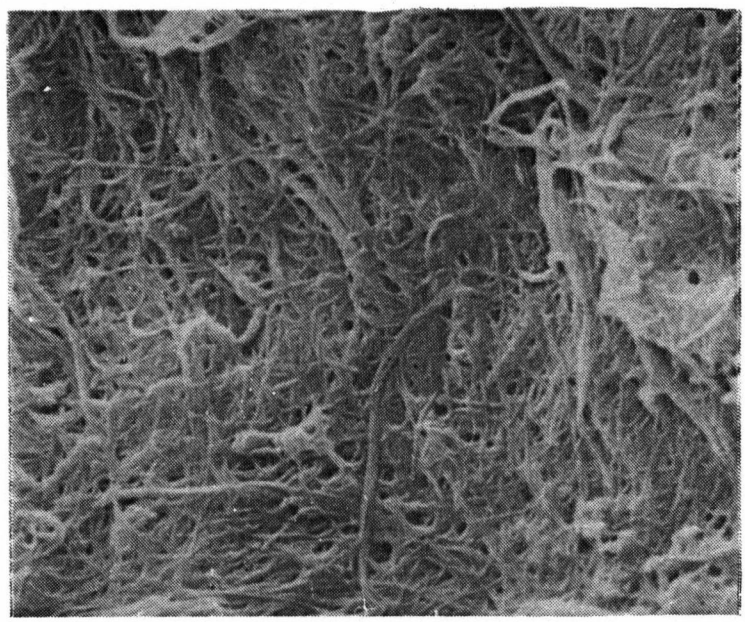

Figure 29. Scanning electron micrograph of mercerized cellulose fibres produced under static conditions showing a compact structure (pw=22μm).

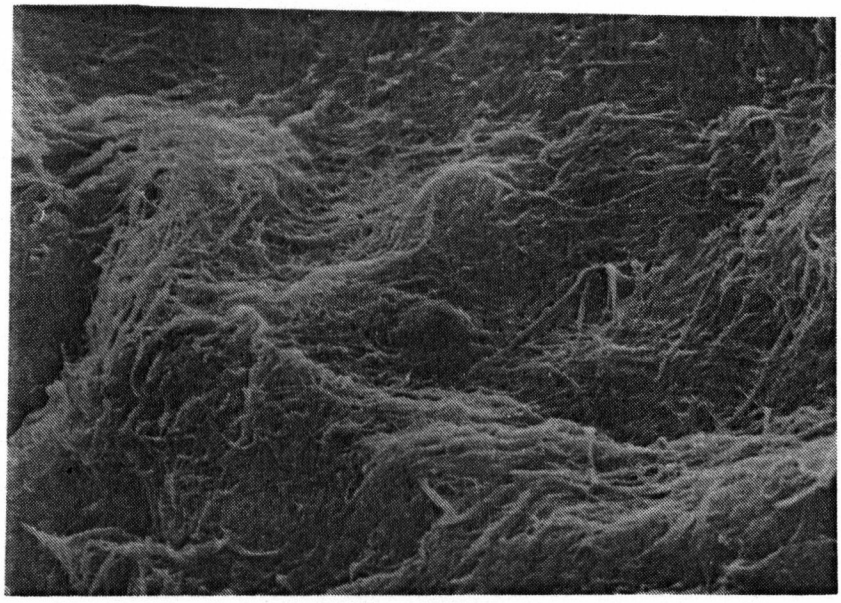

Figure 30. Scanning electron micrograph of mercerized cellulose fibres produced under static conditions (pw=57μm).

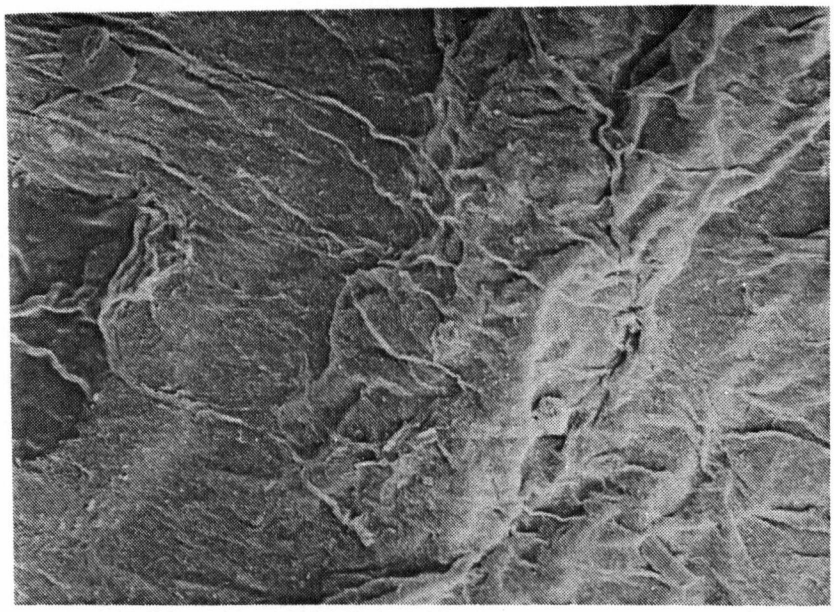

Figure 31. Scanning electron micrograph of mercerized cellulose fibres produced under static conditions (pw=254μm).

these figures clearly revealed a network of entangled disoriented cellulose fibres. Figure 25 shows a bacterium entrapped within the cellulose matrix. It is approximately 2 μm long and 0.6 μm wide, which is in close agreement with dimensions reported previously (Breed, et al., 1957). Again, unmercerized fibres (Figures 24 to 27) show a fine and loose structure; whereas mercerized fibres show a swollen and compact structure (Figures 28 to 31). Such compact structures also appeared in the electron micrographs of Peters (1967). It was described that the well oriented fibres of cotton became comparatively disoriented, and assumed a crimped appearance after mercerization.

3. Crystallinity index, crystal size and degree of polymerization

Mercerized strands presented the typical X-ray diffractogram of cellulose I (Figure 36). According to Peters (1967), cellulose I is transformed to cellulose II when fibres are completely mercerized. Therefore, it appears that the mercerization treatment given to the fibres in this study, did not cause extensive swelling which would bring about a change in the diffraction pattern of cellulose. Crystallinity index and crystal size calculated for the mercerized strands were 95.0 ±1.41% and 61.60 ±0.70°A, respectively. The degree of polymerization of the mercerized strands was 2000 ±200.

G. Microcrystalline Cellulose (MCC) Production

1. Cellulose production and purification

Cellulose was produced and purified according to the procedure outlined in Figure 32. Production started with the incubation of A.

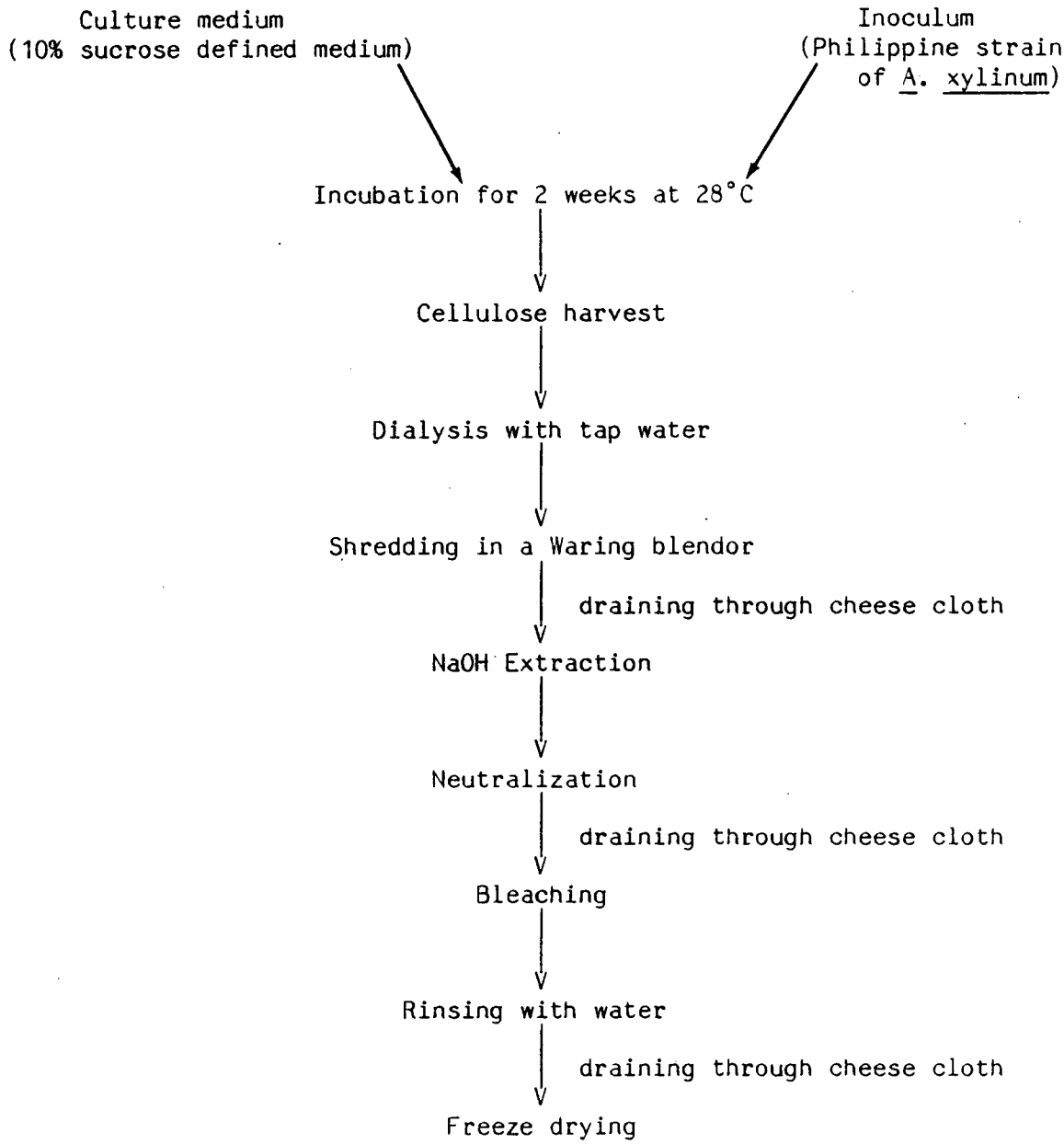

Figure 32. Cellulose production and purification.

xylinum in 10% sucrose defined medium, where celulose pellicles were grown adjacent to the surface of static growing medium (Figure 33). After 2 weeks of incubation, cellulose pellicles were harvested by decanting the culture fluid and removing cellulose (Figure 34). Intact pellicles were dialyzed overnight with tap water to remove remaining growing medium and then shredded to facilitate NaOH extraction (Figure 35). The use of NaOH solutions to remove bacterial cells from cellulose pellicles has been well documented in the literature (Schramm and Hestrin, 1954; Dudman, 1959a; Kaushal and Walker, 1954). However, due to the nature of their work, no attention was given to the possible structural damage taking place in the cellulose matrix. Studies by various researchers (Warwicker, et al., 1966; Ranby, 1952; Peters, 1967) had shown the detrimental action of welling agents, such as NaOH, on the physical structure of various celluloses, namely, decreased crystallinity and degree of polymerization. Furthermore, in preliminary experiments of this study, it was observed that cellulose pellicles which were soaked in 8% (w/v) NaOH for 3 to 4 days at room temperature had a promounced decrease in the degree of polymerization (approx. 700 DP units). Consequently, a very low yield was recovered after acid hydrolysis (30-40%).

Taking these facts into consideration, cellulose was subjected to various extraction treatments to investigate whether structural damage was taking place under the conditions tested. Cellulose, whether in the swollen wet state or freeze dried state, was subjected to increasing NaOH concentrations ranging from 1 to 8% (w/v) for 3 hours. Degree of

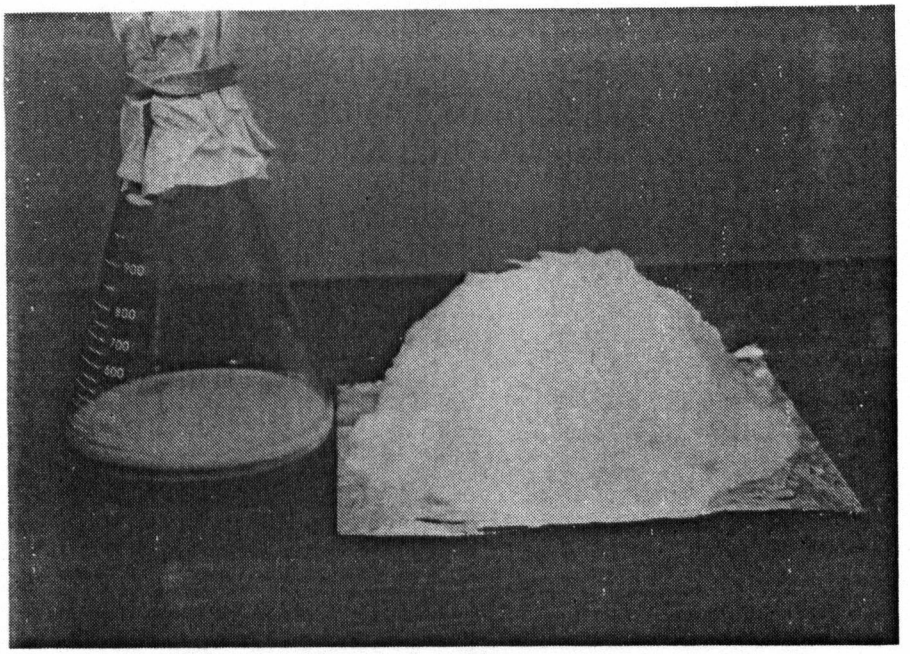

Figure 33. A. xylinum culture with cellulose pellicle at the surface.

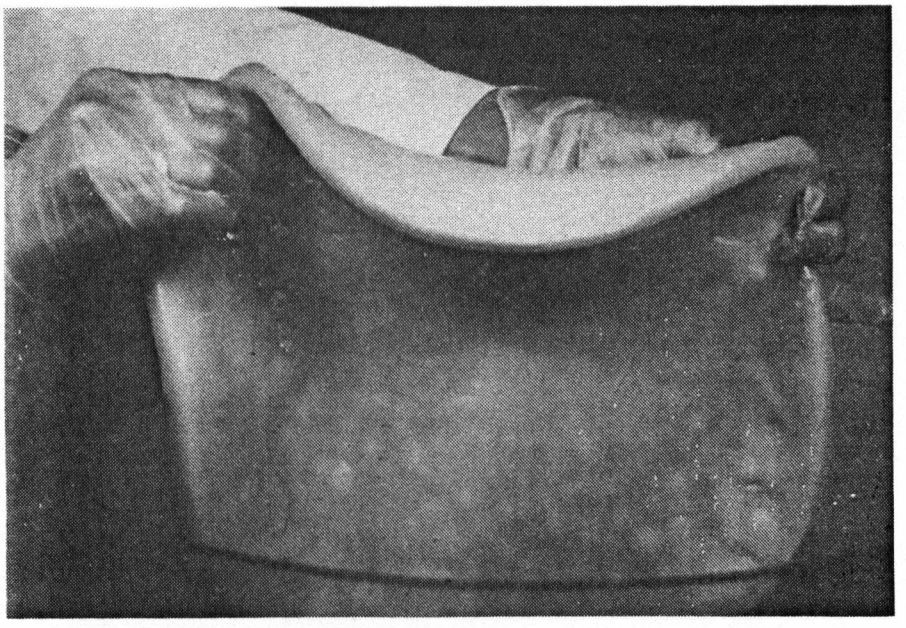

Figure 34. Cellulose pellicle being harvested after two weeks of incubation.

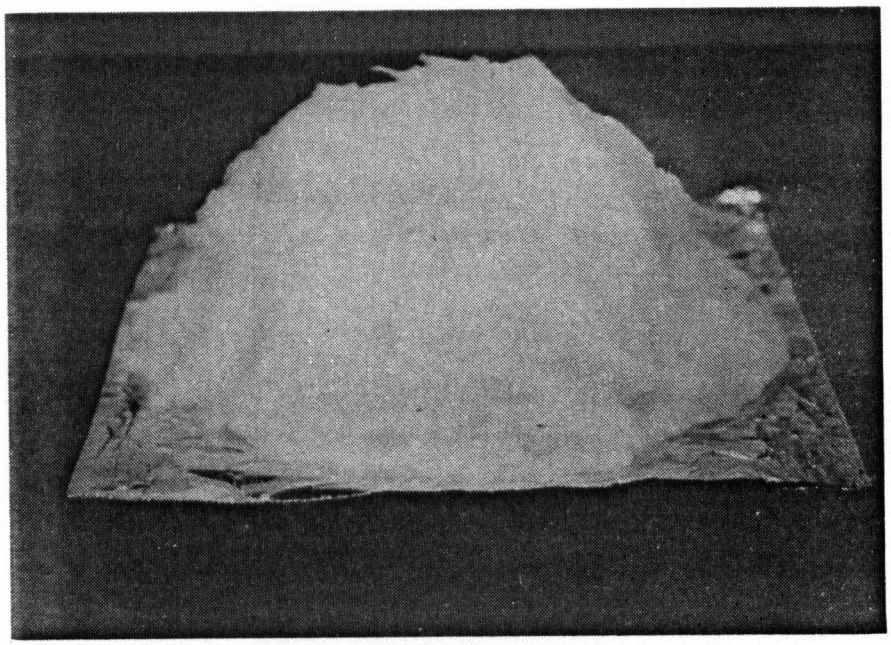

Figure 35. Shredded A. xylinum cellulose prior to NaOH extraction.

polymerization, crystallinity index, crystal size and nitrogen content of the various samples are reported in Table 16.

A typical diffractogram for A. xylinum cellulose is presented in Figure 36, showing the main diffraction maxima of the structure corresponding to the 101, 10T and 002 planes from which the crystallinity index and crystal size were derived.

Results of a two way analysis of variance for each of the four variables tested are presented in Tables 17, 18, 19 and 20. The factor "treatment" was computed to be a highly significant source of variation ($p < 0.01$) for all determined variables, except for crystal size. The factor "condition" was found to be a highly significant source of variation ($p < 0.01$) for all variables tested except for the degree of polymerization. The results of a Duncan's multiple range test on nitrogen content revealed that each treatment was significantly different ($p < 0.05$) from the others, except for 6 and 8% NaOH concentration. Increasing NaOH concentration significantly ($p < 0.05$) reduced the nitrogen content of the cellulose matrix, until it appeared to approach equilibrium at concentrations between 6 and 8%.

Sodium hydroxide extraction was effective in removing entrapped bacteria. Extraction of nitrogenous impurities was significantly ($p < 0.01$) more efficient in freeze dried treated samples than in the swollen samples. These results were somewhat expected, since the swollen samples had a dilution factor of approximately 2/3. However, it is possible that not only the final NaOH concentration during extraction accounted for the decreased nitrogen in the freeze dried samples, but

Table 16. Effect of NaOH extraction of swollen and freeze dried cellulose on Degree of Polymerization (DP), Crystallinity Index (CrI), crystal size and nitrogen content (Mean values ± SD)[1].

Condition of cellulose	NaOH (%)	D.P.	CrI (%)	Crystal size (°A)	Nitrogen (%)
Swollen	0	300 ± 70.7	86.45 ± 0.14	66.35 ± 1.62	0.90 ± 0.14
	1	1200 ± 141.4	94.85 ± 1.69	61.60 ± 0.70	0.71 ± 0.07
	3	1400 ± 35.3	95.00 ± 1.14	61.60 ± 0.70	0.53 ± 0.02
	6	2050 ± 162.6	95.00 ± 1.41	61.60 ± 1.13	0.31 ± 0.01
	8	1900 ± 106.0	94.80 ± 0.70	61.60 ± 4.24	0.15 ± 0.02
Freeze-dried	0	300 ± 70.7	86.40 ± 0.14	66.35 ± 1.62	0.90 ± 0.14
	1	1200 ± 141.4	89.20 ± 2.12	66.35 ± 1.69	0.48 ± 0.02
	3	1600 ± 106.0	87.50 ± 0.28	66.30 ± 2.54	0.21 ± 0.01
	6	1800 ± 141.4	89.60 ± 1.41	66.30 ± 0.7	0.09 ± 0.01
	8	1700 ± 70.7	92.20 ± 1.69	66.30 ± 3.53	0.10 ± 0.02

[1] n=2.

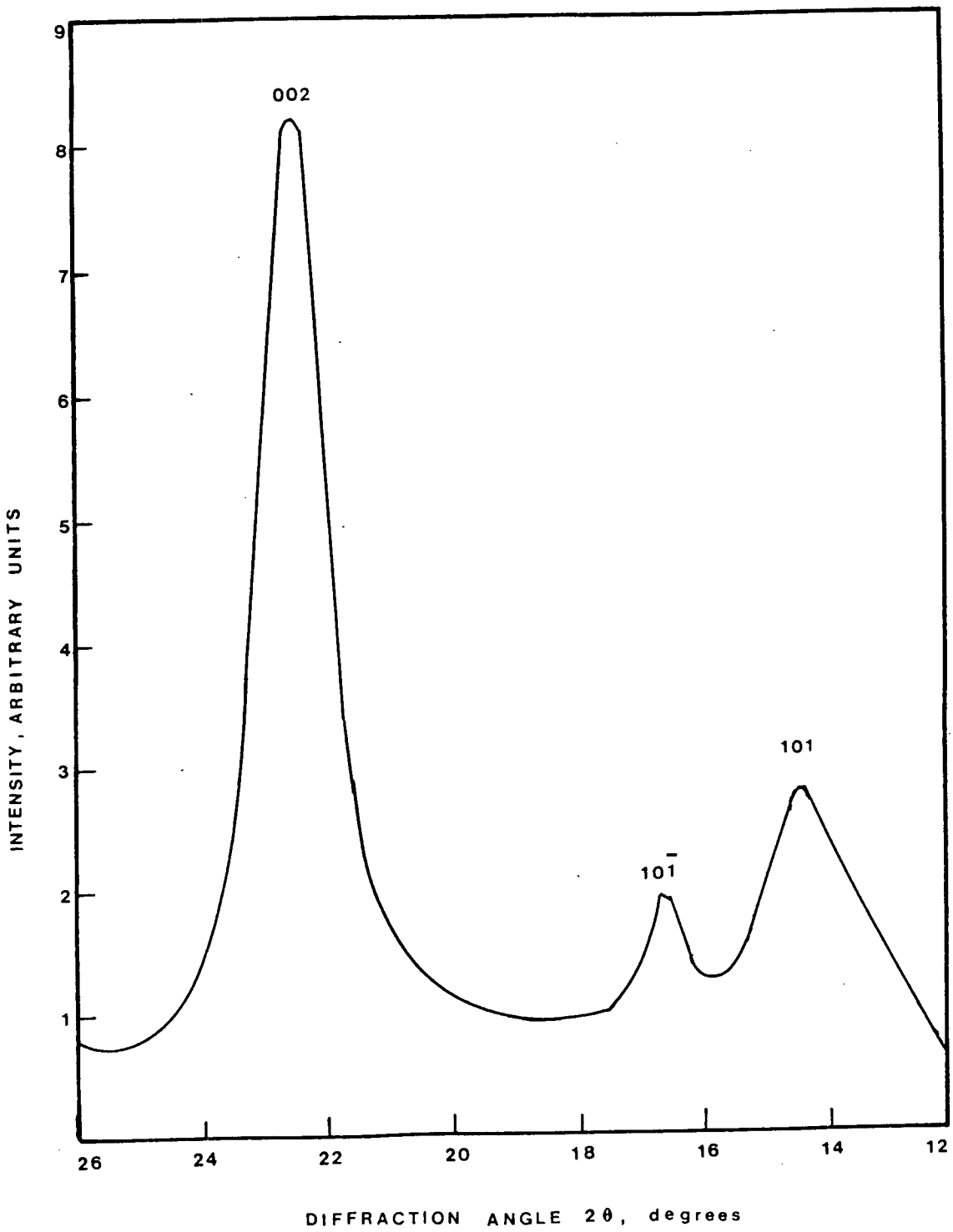

Figure 36. A typical diffratogram of A. xylinum cellulose.

Table 17. Analysis of variance of nitrogen content of NaOH-extracted cellulose samples[a].

Source of variation	DF	Mean square	F-ratio
Condition	1	0.134	33.52**
Treatment	4	0.397	99.32**
C x T	4	0.017	4.42 n.s.
Error	10	0.004	

[a]These data are shown in Table 16.
**Significant at $p < 0.01$.

Table 18. Analysis of variance of crystallinity index of NaOH-extracted cellulose samples[a].

Source of variation	DF	Mean square	F-ratio
Condition	1	90.40	82.18**
Treatment	4	30.40	27.63**
C x T	4	8.00	7.27 n.s.
Error	10	1.1	

[a]These data are shown in Table 16.
**Significant at $p < 0.01$.

Table 19. Analysis of variance of degree of polymerization of NaOH-extracted samples[a].

Source of variation	DF	Mean square	F-ratio
Conditions	1	0.0125×10^6	1.08 n.s.
Treatment	4	1.6805×10^6	146.13**
C x T	4	0.0325×10^6	2.82 n.s.
Error	10	0.0115×10^6	

[a]These data are shown in Table 16.
**Significant at $p < 0.01$.

Table 20. Analysis of variance of crystal size of NaOH-extracted samples[a].

Source of variation	DF	Mean square	F-ratio
Conditions	1	71.10	16.15**
Treatment	4	4.50	1.02 n.s.
C x T	4	4.40	1.00 n.s.
Error	10	4.40	

[a]These data are shown in Table 16.
**Significant at $p < 0.01$.

also an increased NaOH adsorption rate determined by changes in the fibre morphology. Significantly ($p < 0.01$) lower crystallinity indexes in the freeze dried samples supports the assumption of an increased NaOH adsorption rate. Magister et al. (1975) reported a similar phenomenon when cellulose was hydrolyzed; higher reaction rates for dried cellulose were reported (air dried, freeze dried, air dried and wetted again) than for moist celluloses. It was suggested that one of the essential factors was the development of mechanical stresses, contractions during the primary shrinkage stage of the drying process, resulting in microtears which then assisted in the hydrolytic degradation.

The crystallinity indexes of treated samples were significantly ($p < 0.05$) higher than those of untreated samples. These results do not indicate that crystallinity per se was increased by the treatment, but rather that a substantial amount of impurities and amorphous cellulose extracted by NaOH accounted for the percentage of amorphous material in the untreated samples thus, decreasing the ratio $I_{002}-I_{am}/I_{002}$.

A crystallinity index of approximately 95% calculated in this study agrees well with a Cr.I. of 96% reported by Correns and Purz (1975).

It appears that some decrystallization took place in the freeze dried samples, and these results agree well with those reported by Patil et al. (1965). It was reported that sodium hydroxide acts as a decrystallizing agent on cotton cellulose; NaOH concentrations ranging from 0 to 50% resulted in Cr.I. ranging from 70 to 45%.

The degree of polymerization was significantly ($p < 0.05$) higher for the treated samples than for the untreated ones, and the D.P. of 6 and 8% treated samples were not significantly ($p > 0.05$) different.

Since the D.P. is not an absolute measurement, but only an average, impurities and short fibrils in the untreated samples may have caused low D.P. averages. Treated samples freed from impurities result in true estimation of cellulose chain sizes. Increasing NaOH concentration significantly ($p < 0.05$) reduced impurities as indicated by the nitrogen content. Consequently, the average D.P. also increased until it appeared to level off at 6 and 8% NaOH. In conclusion, an extraction treatment with a NaOH concentration ranging between 6 and 8% on swollen cellulose efficiently removed embedded bacteria without any detrimental effect on fibre structure.

The yield of purified cellulose after treatment with 6% NaOH was 43 ±1.44%. NaOH extraction of swollen cellulose not only eliminated degradation, but also eliminated the freeze drying step. Thus, the process of cellulose purification could be continuous.

Since at this stage, cellulose was highly purified (approximately 98% cellulose), a very mild bleaching treatment was necessary to obtain a good white colour. This mild treatment involved only one sequence of chlorite and acid addition. On the contrary, several sequences are usually involved during delignification of wood pulp by the chlorite method resulting in considerable depolymerization (Browning, 1967).

Bleaching resulted in 88% yield thus, a 12% loss in cellulose and colouring matter. The DP value of the bleached sample was 1500,

therefore, some depolymerization had taken place during chlorite treatment. Modifications of the chlorite method are concerned with the time and sequence of the chlorite and acid additions, the control of pH during the chlorite treatments, and the temperature at which the treatment is carried out. For example, Browning (1967) reported that chlorite markedly reduced the DP of cotton at pH 2 (70°C); but at pH 5 (70°C) the drop in pH was negligible, although at 90°C the DP markedly decreased. Sodium chlorite at pH 2.9 and 20°C oxidizes reducing groups to carboxyl groups, but side reactions occur and additional oxidation reactions take place in cotton which leads to lower molecular weight.

Thus, it is suggested that if further work were to be done to avoid depolymerization of A. xylinum cellulose during bleaching, control of pH during the chlorite treatment should be considered.

2. Effect of acid hydrolysis on weight loss and degree of polymerization

Changes in the degree of polymerization and yield with the time of hydrolysis by boiling with 2.5 N HCl are shown in Figures 37 and 38 for 6% NaOH-extracted bleached samples. A highly degraded cellulose (extracted with 8% NaOH for 3-4 days) is also included to illustrate the dependence of the degree of polymerization and the final yield on the extent of preswelling of cellulose samples by sodium hydroxide solutions. Battista (1950) had reported the hydrolysis of several celluloses (purified cotton, bleached cotton and wood pulp) by using 2.5 and 5N HCl at 5, 18, 40 and 105°C. In this work, the use of boiling 2.5 N

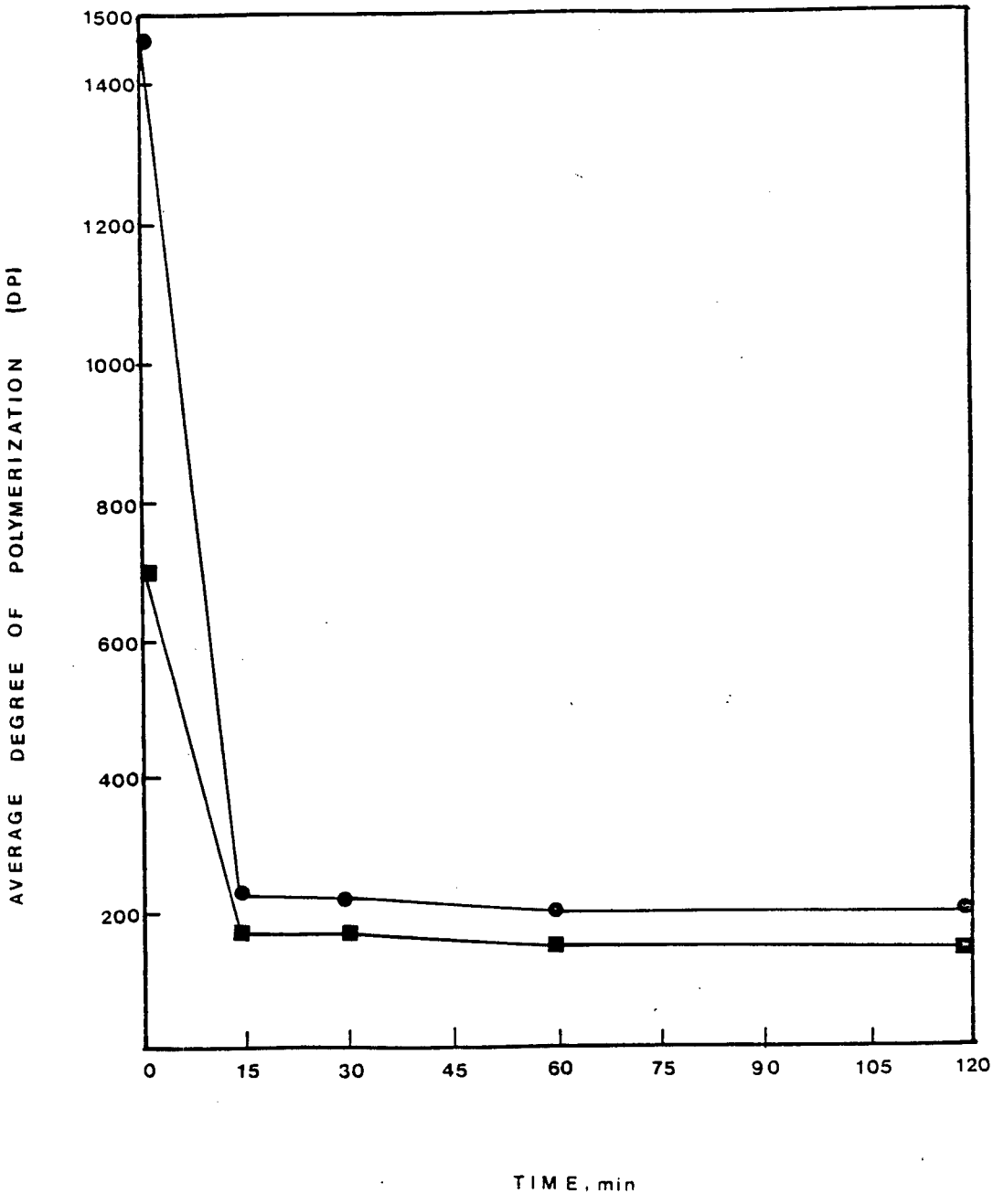

Figure 37. Hydrolyses of 6% NaOH-extracted cellulose (●) and degraded cellulose (■).

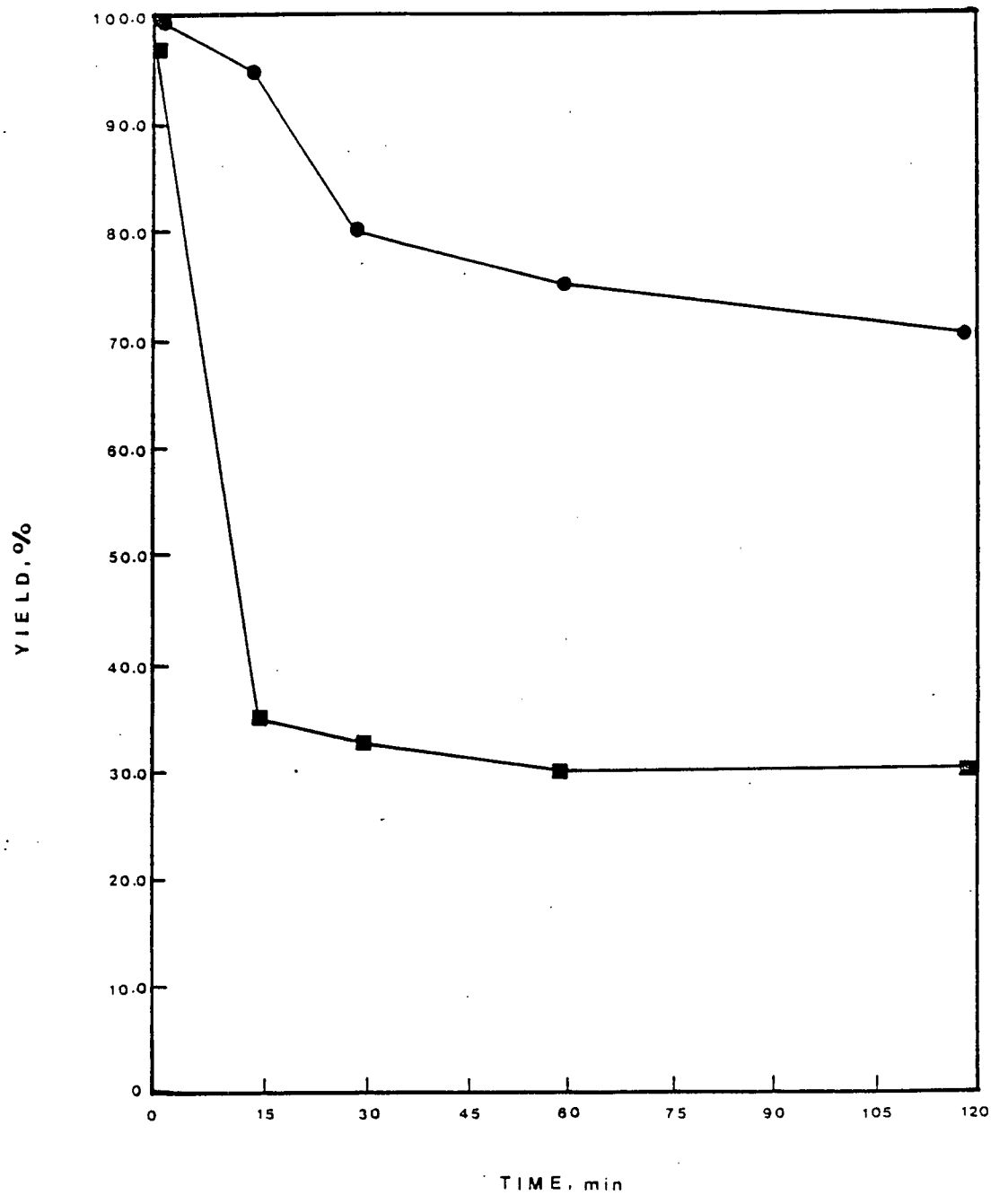

Figure 38. Comparison of yields observed in 2.5 N HCl at 105°C of 6% NaOH-extracted cellulose (●) and degraded cellulose (■).

HCl was recommended, since 5N HCl gave rise to the formation of humic substances and at the lower temperatures the degree of polymerization tended to level-off at higher values. Therefore these conditions were adopted in this study.

In Figure 37, it is shown how quickly the basic degree of polymerization reaches what appears to be a fairly constant value and that this leveling off is higher for the 6% NaOH-extracted (D.P. 200-220) than for a degraded cellulose (D.P. 150-175). Furthermore, the 6% NaOH-extracted cellulose lost weight with time of hydrolysis at a much slower rate than did degraded cellulose (see Figure 38). These same trends were observed by Battista (1950) when purified cotton (native cellulose) and regenerated cellulose (viscose tire yarn) were subjected to acid hydrolysis with boiling 2.5 N HCl for 15 min. Purified cotton (D.P.=3200) yielded a LODP of 242, whereas regenerated cellulose (D.P.=470) yielded a LODP of 43; yields upon hydrolysis were 91 and 70%, respectively.

On the basis of the data shown in Figures 37 and 38, a time of 15 minutes in boiling 2.5 N HCl was adopted as being the optimum point at which the LODP (220) was reached with minimum weight losses (5%).

At this stage of the process, cellulose was been converted to microcrystalline cellulose (MCC) since the amorphous material was completely removed. The crystalline material was then removed by filtration or centrifugation, neutralized with 5% NH_4OH, and washed with several changes of distilled water (see Figure 39). The resulting neutralized filtered cellulose crystals were reslurried to 3% solids and subjected to mechanical disintegration to free the unhinged crystals and to form a homogeneous aqueous slurry which could be easily spray dried.

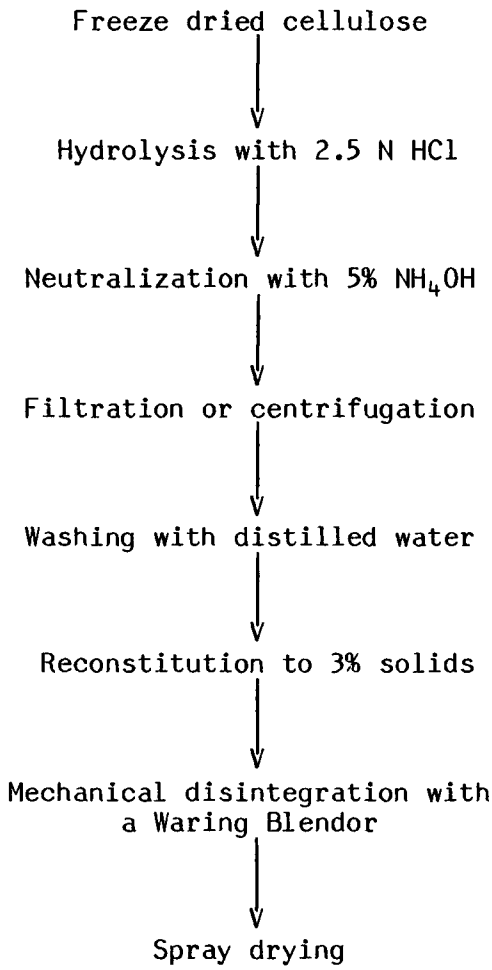

Figure 39. Microcrystalline cellulose production.

MCC production was terminated by spray drying the homogeneous slurry at a temperature of 96 to 100°C (inlet temperature). MCC in the powder form was stored in bottles placed in a desiccator until required for the characterization tests.

H. Microcrystalline Cellulose Characterization

1. Chemical composition, cellulose, nitrogen and ash content

Cellulose determination was carried out by first subjecting the cellulose to a total hydrolysis. The procedure required a primary hydrolysis with strong mineral acid followed by a secondary hydrolysis in dilute acid. The primary hydrolysis resulted in the formation of a mixture of oligosaccharides. It was the function of the secondary hydrolysis to complete the conversion to monomeric sugars.

The initial weights of cellulose samples were corrected dry weights (moisture content of spray dried sample was 4.0 ±0.7% dry basis). The theoretical glucose yield is equal to the cellulose weight multiplied by the factor (1.111); this factor is the ratio 180/162, 162 is the anhydroglucose M.W. and 180 is the monohydrate M.W.

Recovered glucose was determined by the phenol sulfuric acid method of Dubois *et al.* (1951). Cellulose content in the sample was calculated by the ratio recovered glucose/theoretical glucose yield X 100. The results are shown in Table 21.

Cellulose content indicated that *A. xylinum* MCC was purified to 98.5%, within the requirements of MCC as is listed in the FCC III (1981) which states that it should not be less than 97.0% and not more than the

Table 21. Chemical composition of microcrystalline celluloses (Mean ± S.D.).

Sample (MCC)	Cellulose[a] (% d.b.)	Moisture[a] (% w.b.)	Nitrogen[a] (% d.b.)		Ash[a] (% d.b.)
			Total	Ammonia	
A. xylinum	98.5 ± 1.4	4.05 ± 0.7	1.1 ± 0.4	1.01 ± 0.1	1.15 ± 0.1
Avicel PH-101	99.2 ± 0.3	2.64 ± 0.2	N.D.[b]	N.D.	0.85 ± 0.1

[a] n=3.
[b] Not detected.

equivalent of 102.0% of carbohydrate as cellulose, calculated on a dried basis.

Values for total nitrogen content were relatively high for A. xylinum MCC. It appears that most of it was in the ammonium form as indicated by the nitrogen determined as ammonia. These results reflect the need for exhaustive washing with water after neutralization with NH_4OH to eliminate the NH_4Cl being formed.

An ash content of 1.15% for A. xylinum MCC might be the result of using technical grade reagent for the chlorite treatment; sodium chlorite which was 80% $NaOCl_2$ was used for bleaching.

2. Physical properties

a) Crystallinity, crystal size, average degree of polymerization, particle size determination

Values for A. xylinum MCC and Avicel PH-101 are summarized in Table 22. Crystallinity index, crystal size and D.P. were significantly ($p < 0.01$) larger for A. xylinum MCC than for commercial MCC (Avicel PH -101) as indicated by a t-test. These results clearly indicate that the preparation of MCC from non-degraded high D.P. A. xylinum cellulose resulted in a product which was highly crystalline with a high LODP value. Commercial MCC Avicel PH-101, however, is prepared from highly degraded cellulose from wood pulp. Degradation of cellulose always occurs during delignification and it seems impossible to isolate a wood cellulose with the same D.P. as that in the original plant.

Consequently, crystallinity is partly destroyed and the LODP was significantly lower ($p < 0.01$).

Table 22. Mean values of crystallinity index, crystal size, average degree of polymerization and particle size for A. xylinum MCC and Avicel PH-101 MCC.

Sample (MCC)	Crystallinity Index[a] (%)	Crystal Size[a] (°A)	a D.P.	Particle[b] size (μm)
A. xylinum	98.15[x,1] (0.91)[c]	57.25[x] (1.76)	220.0[x] (14.1)	1.05[x] X 0.70[x] (0.60) (0.06)
Avicel PH=101	84.90[y] (1.41)	45.47[y] (3.43)	100.0[y] (0.0)	3.50[y] X 1.75[y] (0.30) (0.20)

[a] n=3.
[b] n=20.
[c] Standard deviation.
[1] Values sharing a common superscript within a column are not significantly different ($p > 0.05$).

The crystallinity index calculated in this study for Avicel PH-101, agrees well with a crystallinity index of 82% reported by Paquot (1982).

Mean particle size values reported in Table 22 were obtained after dispersing 0.1% MCC solutions by a Polytron high speed blender/sonicator. Large aggregates, approximately ten times the particle size were observed before dispersing with a Polytron. Thus, it is possible that the values presented in Table 22 represent dimensions of aggregates rather than individual particles. Values reported in the literature (Battista, 1975) for mechanically disintegrated MCC are within the range of 150Å-5μm. The size and the shape of the microcrystals depend on the history of the precursor cellulose fibres (Battista, 1975).

b) Moisture adsorption

Moisture adsorption isotherms of A. xylinum MCC and Avicel PH-101 MCC are depicted in Figure 40. It appears that Avicel PH-101 retained less moisture than A. xylinum MCC. The reason for this behaviour could be due to the fact that Avicel PH-101 was more highly purified than A. xylinum MCC, as it can be observed from their respective chemical compositions (see Table 21).

Another important factor which may determine this sorption behaviour, however, is the total surface area. Since A. xylinum MCC had a significantly ($p < 0.05$) smaller particle size than Avicel PH-101 (Table 22), the total surface area available for water adsorption in A. xylinum

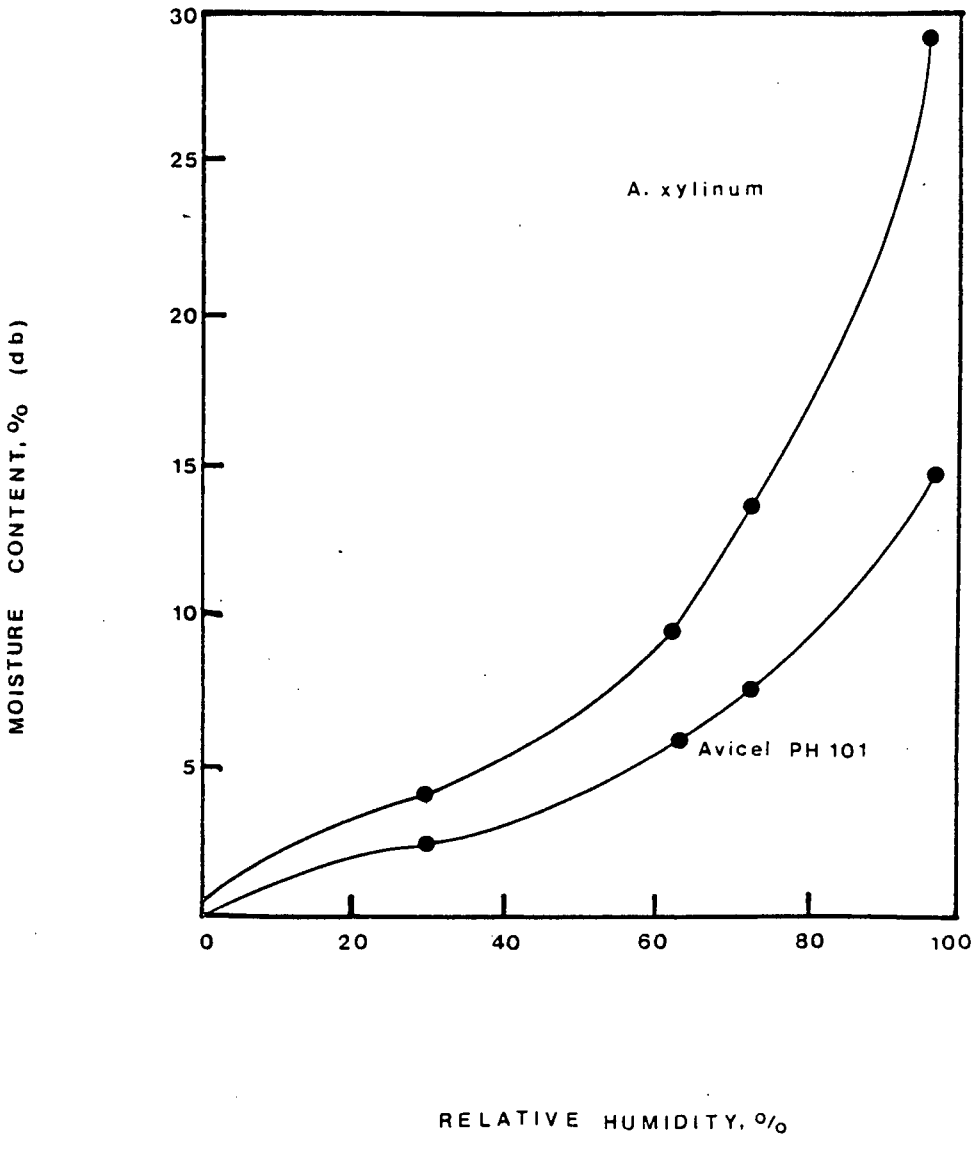

Figure 40. Sorption isotherms of A. xylinum MCC and Avicel PH-101 MCC at 25°C

MCC was greater. Water adsorption of Avicel PH-101 determined in this study is in close agreement with those results reported by Paquot (1982). A moisture content of 16% (d.b) at 93% RH (20°C) was recorded for Avicel PH-101.

Browning (1967) has pointed out that as a first approximation, there is a direct relationship between the capacity of a fiber for water adsorption and the quantity of amorphous material in the fibre. Sorption occurs mainly, if not entirely, in the noncrystalline regions, and depends on the availability of free hydroxyl groups. The surfaces of the crystallites also participate in the sorption.

A. xylinum MCC was significantly ($p < 0.01$) more crystalline than Avicel PH-101, that is, it had less amorphous regions than Avicel PH-101 (Table 22). Consequently, it would be expected to retain less water than Avicel PH-101; however, the opposite occurred. Therefore, it appears that a combination of factors determines the sorption behaviour of microcrystalline cellulose.

c) Zeta potential determination

The mean corrected zeta potential values for 0.1% dispersions of A. xylinum MCC and Avicel PH-101 in 0.01 M phosphate buffer (PH=6.95) were -3.2 ±0.45 mV and -6.05 ±2.19 mV.

The magnitude of the electrostatic charge (zeta potential) of small solid particles dispersed in water or colloids determines the stability of the dispersion. A stable colloid is one in which the colloid particles remain separate and distinct (dispersed). An unstable

colloid is characterized by particles that gradually agglomerate. If the charge of the particles is high, the particles repel one other and the colloid is stable. If the charge is near zero, the random motion (Brownian motion) of the particles cause them to collide and to become attached to one another (Sennett and Olivier, 1965). Thus, it appears that strong agglomeration or incipient instability is expected for dispersions of A. xylinum MCC and Avicel PH-101.

The zeta potential obtained for Avicel PH-101 in this study, totally disagrees with results reported in the literature. Paquot (1982) reported zeta potential values ranging from -35 to -40 mV in a pH range from 5 to 9 for Avicel PH-101.

d) Colour determination

A. xylinum MCC was slightly less white than Avicel PH-101 as reflected in its lower "L" value (Table 23). Avicel was more yellowish than A. xylinum MCC as indicated by its higher "b" value; however, A. xylinum MCC appeared more green as indicated by the "a" value. It is suggested that the greenish shade is the result of neutralization with NH_4OH during MCC preparation (Paszner, 1982; personal communication).

3. Rheological properties

Data from all experiments were analysed by using the rheological analysis program for the purpose of selecting the best model that fit the data; coefficients of determination (r^2) and plots of σ versus $\dot{\gamma}$ (or

Table 23. Colour of A. xylinum MCC and Avicel PH-101 MCC.

Sample	Hunterlab scale		
	L[1]	a[2]	b[3]
Standard No. W272	93.10	-0.60	0.40
A. xylinum MCC	90.23	-0.18	1.41
Avicel PH-101	91.91	0.02	3.69

[1] L white

[2] a=0 grey
a<0 green
a>0 red

[3] b=0 grey
b>0 yellow
b<0 blue

transforms) were examined. The Power Law accurately described the flow behaviour at the concentrations and temperatures tested for both MCC, A. xylinum and Avicel PH-101.

Consistency coefficients, flow behaviour indices, coefficients of determination (r^2) and the number of points used in each regression for the upcurves and downcurves of A. xylinum or Avicel PH-101 MCC are shown in Tables 24 and 25. Statistical analyses of the slopes (n) and levels (m) of A. xylinum MCC curves revealed significant differences ($p < 0.05$); therefore, it appears that the flow properties of A. xylinum MCC are time dependent. Statistical analyses of the slopes (n) and levels (m) of Avicel PH-101 MCC curves, however, revealed no significant difference ($p < 0.05$); therefore it appears that the flow properties of Avicel PH-101 MCC were not time dependent. Rheograms of the upcurves and downcurves for 6% dispersions of A. xylinum and Avicel PH-101 MCC are depicted in Figure 41. For a time dependent fluid, the increasing and decreasing rheograms will not coincide, that is, they will show hysteresis (Holdsworth, 1971). Thus, the well defined hysteresis observed for A. xylinum MCC further confirms its time dependence. The concavity of the lines identifies these curves as characteristic of time dependent thinning.

Gel strength ratios of A. xylinum MCC and Avicel PH-101 are shown in Tables 26 and 27. The gel strength ratio G_0/G_{10} reflects the progressive nature of structural buildup in the dispersions at rest, if the gel strength is measured immediately after shearing and after increasingly longer periods of rest, 10 minutes in this study. The values obtained are found to increase at a decreasing rate until a

Table 24. Consistency coefficients and flow behaviour indices for the upcurve and downcurve of A. xylinum MCC at 25°C and various concentrations.

Concentration (%)	Curve direction	m (Pas^n)	n	r^2	N
6	upward	1.037^{a1}	0.395^{x}	0.983	12
	downward	0.379^{b}	0.540^{y}	0.983	12
5	upward	1.067^{a}	0.347^{x}	0.970	12
	downward	0.257^{b}	0.542^{y}	0.995	12
4	upward	0.679^{a}	0.335^{x}	0.941	12
	downward	0.308^{b}	0.447^{x}	0.964	12
3	upward	0.309^{a}	0.417^{x}	0.972	12
	downward	0.235^{b}	0.422^{x}	0.060	12

[1] m and n values sharing a common superscript letter within a column for each upcurve and downcurve are not significantly different ($p > 0.05$).

Table 25. Consistency coefficients and flow behaviour indices for the upcurve and downcurve of Avicel PH-101 MCC at 25°C and various concentrations.

Concentration (%)	Curve direction	m (Pas^n)	n	r^2	N
6	upward	0.465^{a1}	0.407^x	0.971	12
	downward	0.344^a	0.437^x	0.975	12
5	upward	0.245^a	0.422^x	0.987	12
	downward	0.256^a	0.399^x	0.982	12
4	upward	0.275^a	0.375^x	0.992	12
	downward	0.282^a	0.339^x	0.955	12
3	upward	0.309^a	0.308^x	0.975	12
	downward	0.296^a	0.256^x	0.817	12

[1]m and n values sharing a common superscript letter within a column for each upcurve and downcurve are not significantly different ($p > 0.05$).

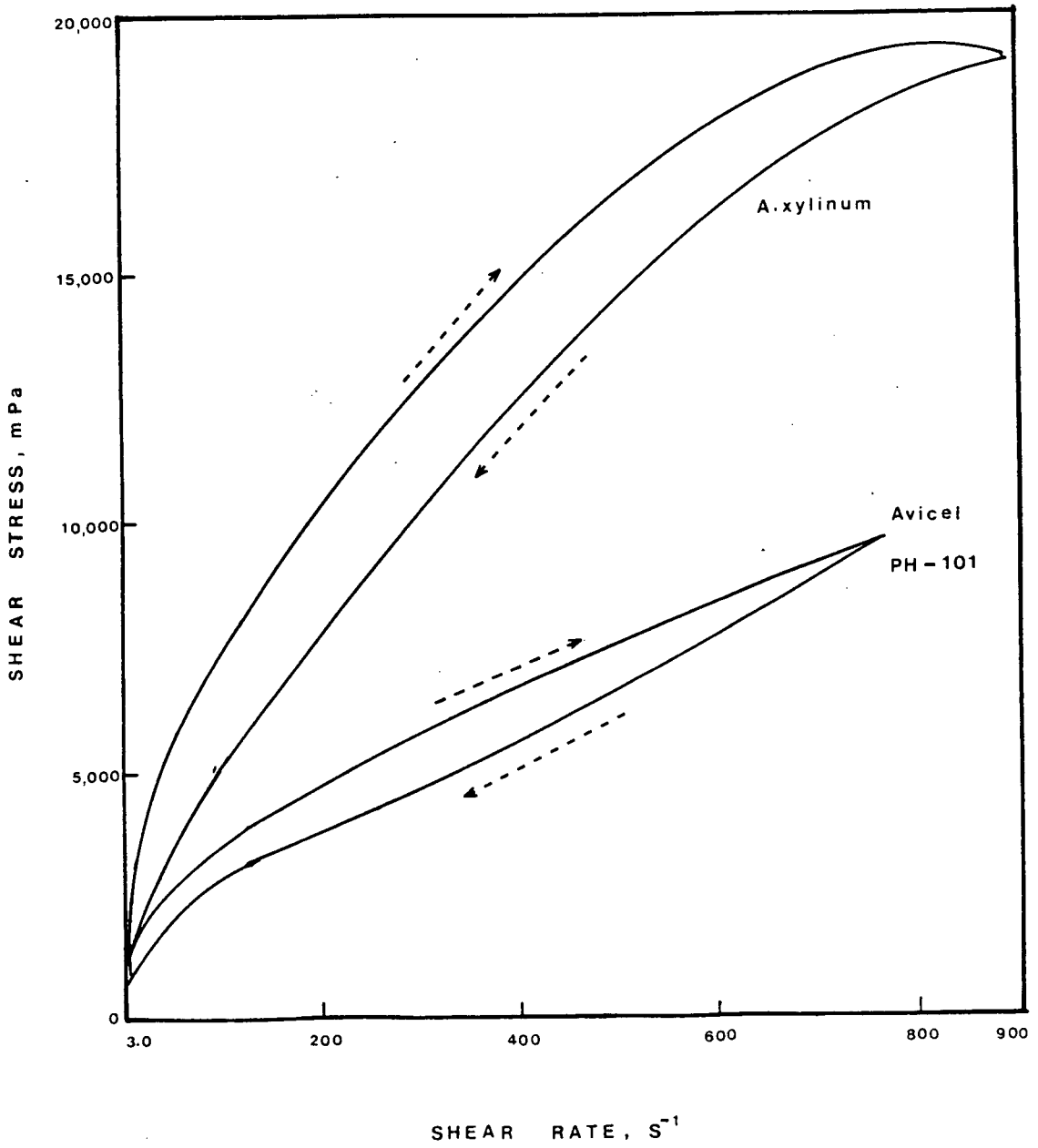

Figure 41. Upcurve and downcurve rheograms showing time dependent behaviour of 6% dispersions of A. xylinum MCC and Avicel PH-101 MCC at 25°C.

Table 26. Gel strength ratio (G_o/G_{10}) of 6, 5, 4 and 3 % dispersions of A. xylinum MCC at different temperatures.

Concentration (%)	Temperature (°C)	Gel strength[1] (G_o/G_{10})
6	25	1/4
	35	2/4
	45	1/2
5	25	2/3
	35	1/2
	45	1/2
4	25	1/1
	35	1/1
	45	1/1
3	25	1/1
	35	1/1
	45	1/1

[1] n=2.

Table 27. Gel strength ratio (G_0/G_{10}) of 6, 5, 4 and 3 % dispersions of Avicel PH-101 MCC at different temperatures.

Concentration (%)	Temperature (°C)	Gel strength[1] (G_0/G_{10})
6	25	1.5/3.0
	35	1.5/3.0
	45	1.5/3.0
5	25	1.0/2.0
	35	1.0/2.0
	45	1.0/1.0
4	25	1.0/1.0
	35	1.0/1.0
	45	1.0/1.0
3	25	1.0/1.0
	35	1.0/1.0
	45	1.0/1.0

[1] n=2.

maximum value is reached, then this behaviour is a manifestation of the phenomenon of thixotropy (Gray and Darley, 1981). Thus, it appears that 6 and 5% dispersions of both MCC present such behaviour, whereas no gel strength was detected ($G_0/G_{10}=1$) at lower concentrations. This may be due to the fact that the gel strength increased very rapidly immediately after cessation of shearing, so that the initial gel strength was very sensitive to time.

In conclusion, it appears that both MCC presented time dependent rheological behaviour of the thixotropic type, that is, there is a reversible isothermal change in viscosity with time at a constant rate of shear.

Microcrystalline cellulose aqueous gels had been reported to present thixotropic behaviour; the flow properties of these gels were interpreted in terms of a particle network model which is reversibly disrupted by the application of an outside mechanical force (Hermans, 1963).

A second approach to evaluating time effects on viscosity of MCC dispersions is suggested for future work, by recording the shear stress decay curve at a constant shear rate by means of a strip-chart at pre-determined time intervals.

Consistency coefficients, flow behaviour indices, coefficients of detemination (r^2) and the number of points used in each regression for the equilibrium curves of A. xylinum and Avicel MCC are shown in Tables 28 and 29.

Since the n values are < 1, that is, viscosity decreases with shear rate the dispersions were non-Newtonian pseudoplastic. Suspensions

Table 28. Consistency coefficients and flow behaviour indices of A. xylinum MCC dispersions at various temperatures and concentrations.

Concentration (%)	Temperature (°C)	m (Pas^n)	n	r^2	N
6	25	0.150	0.634	0.981	12
	35	0.150	0.610	0.989	12
	45	0.160	0.590	0.988	12
5	25	0.173	0.575	0.987	12
	35	0.160	0.570	0.983	12
	45	0.160	0.570	0.989	12
4	25	0.200	0.502	0.979	12
	35	0.170	0.510	0.942	12
	45	0.190	0.470	0.954	12
3	25	0.243	0.406	0.981	12
	35	0.210	0.410	0.966	12
	45	0.210	0.410	0.966	12

Table 29. Consistency coefficients and flow behaviour indices of Avicel PH-101 MCC dispersions at various temperatures and concentrations.

Concentration (%)	Temperature (°C)	m (Pas^n)	n	r^2	N
6	25	0.336	0.437	0.987	12
	35	0.300	0.410	0.965	12
	45	0.310	0.390	0.974	12
5	25	0.260	0.373	0.981	12
	35	0.230	0.380	0.985	12
	45	0.220	0.400	0.980	12
4	25	0.294	0.325	0.991	12
	35	0.240	0.330	0.934	12
	45	0.260	0.310	0.952	12
3	25	0.342	0.268	0.998	12
	35	0.290	0.250	0.928	12
	45	0.310	0.180	0.694	12

of long-chain polymers are known to be typical pseudoplastics. At rest, the chains are randomly entangled, but they do not set up a structure because the electrostatic forces are predominantly repulsive. When the fluid is in motion, the chains tend to align themselves parallel to the direction of flow; this tendency increases with increase in shear rate, so that the effective viscosity decreases (Gray and Darley, 1981).

At the concentrations and temperatures tested, no typical trends were observed for the consistency coefficients and flow behaviour indices. Rheograms for different concentrations of A. xylinum and Avicel PH-101 MCC are depicted in Figures 42 and 43, respectively. These figures demonstrate this decrease in viscosity with increasing shear rates.

Table 30 summarizes the Power Law parameters for different concentrations of A. xylinum and Avicel PH-101 MCC dispersions at 25°C. Statistical analyses of the slopes (n-1) and levels (m) at each concentration, revealed significant differences ($p < 0.05$); therefore, it appears that apparent viscosities for the two types of MCC were significantly different over the entire range of shear rates. By examining their rheograms depicted in Figure 44, it appears that Avicel PH-101 is more pseudoplastic, that is it presents more shear thinning than A. xylinum MCC. Consequently, apparent viscosities at high shear rates were greater for A. xylinum MCC than for Avicel PH-101, while apparent viscosities at low shear rates ($\dot{\gamma}=1s^{-1}$) were greater for Avicel PH-101 than for A. xylinum MCC.

Greater pesudoplasticity of Avicel PH-101 MCC may be explained in terms of interactions between particles. Avicel PH-101 appears to be

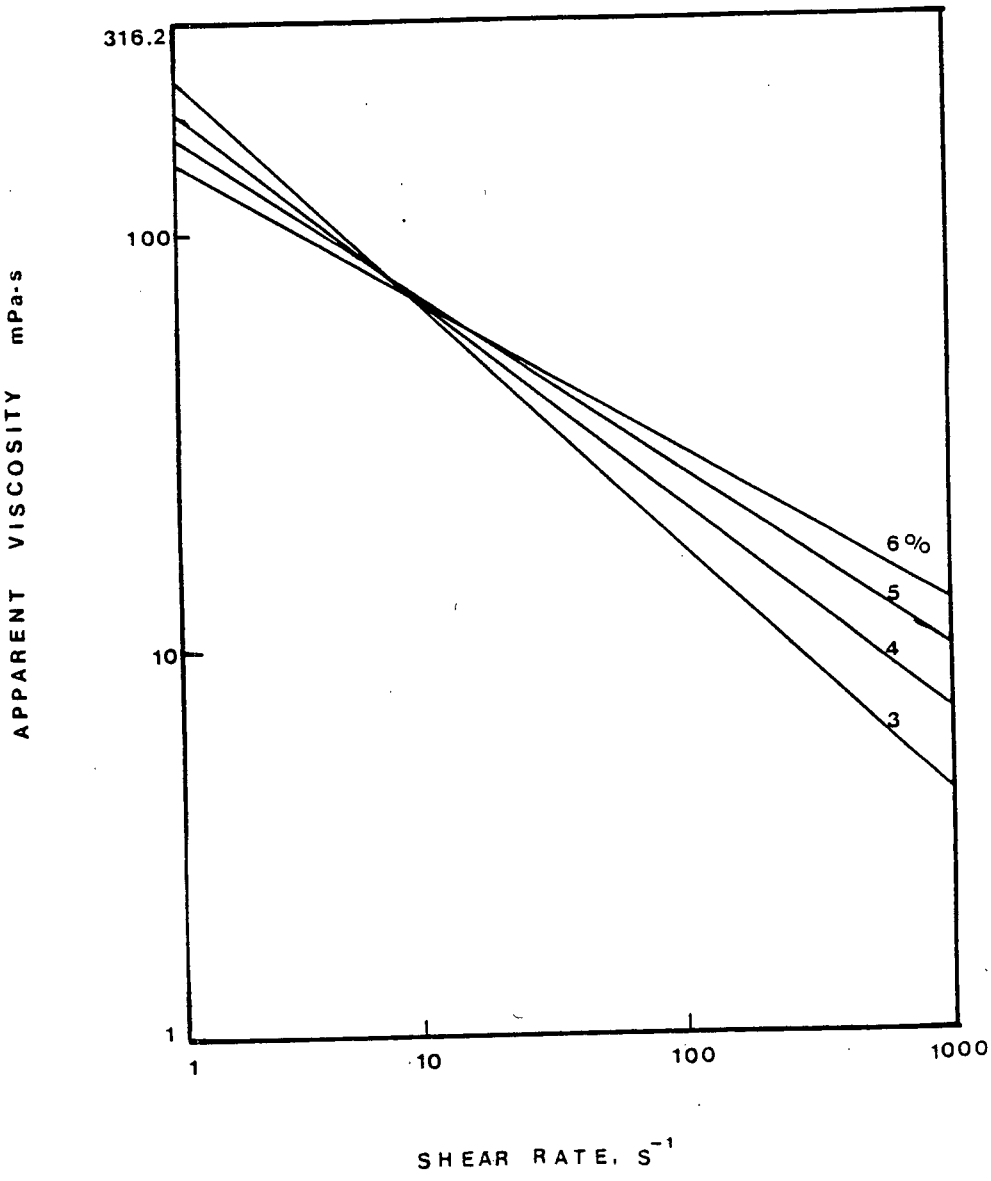

Figure 42. Rheogram for 6, 5 4 and 3 % A. xylinum MCC dispersions at 25°C according to the Power law flow model.

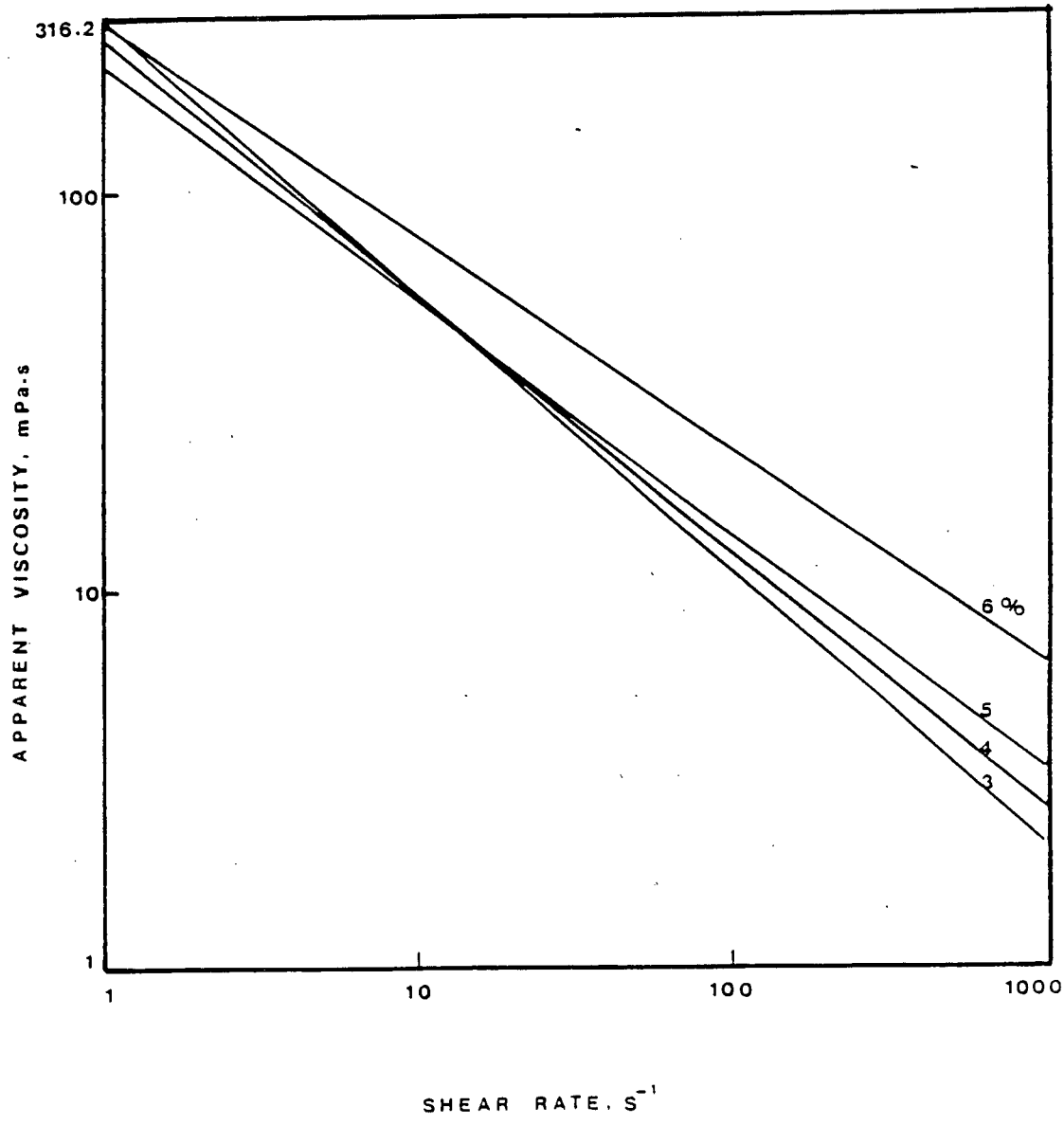

Figure 43. Rheogram for 6, 5, 4 and 3 % Avicel PH-101 MCC dispersions at 25°C acording to the Power Law flow model.

Table 30. Consistency coefficients and flow behaviour indices for bacterial and Avicel PH-101 MCC dispersions at 25°C and various concentrations.

Concentration (%)	MCC	m (Pas^n)	n	r^2	N
6	A. xylinum	0.150^{a1}	0.634^{x}	0.981	12
	Avicel	0.336^{a}	0.437^{y}	0.937	12
5	A. xylinum	0.173^{a}	0.575^{x}	0.987	12
	Avicel	0.260^{b}	0.373^{y}	0.981	12
4	A. xylinum	0.200^{a}	0.502^{x}	0.979	12
	Avicel	0.294^{b}	0.325^{y}	0.991	12
3	A. xylinum	0.243^{a}	0.406^{x}	0.981	12
	Avicel	0.342^{b}	0.268^{y}	0.998	12

[1]m and n values sharing a common superscript letter within a column for A. xylinum and Avicel MCC at each concentration are not significantly different ($p < 0.05$).

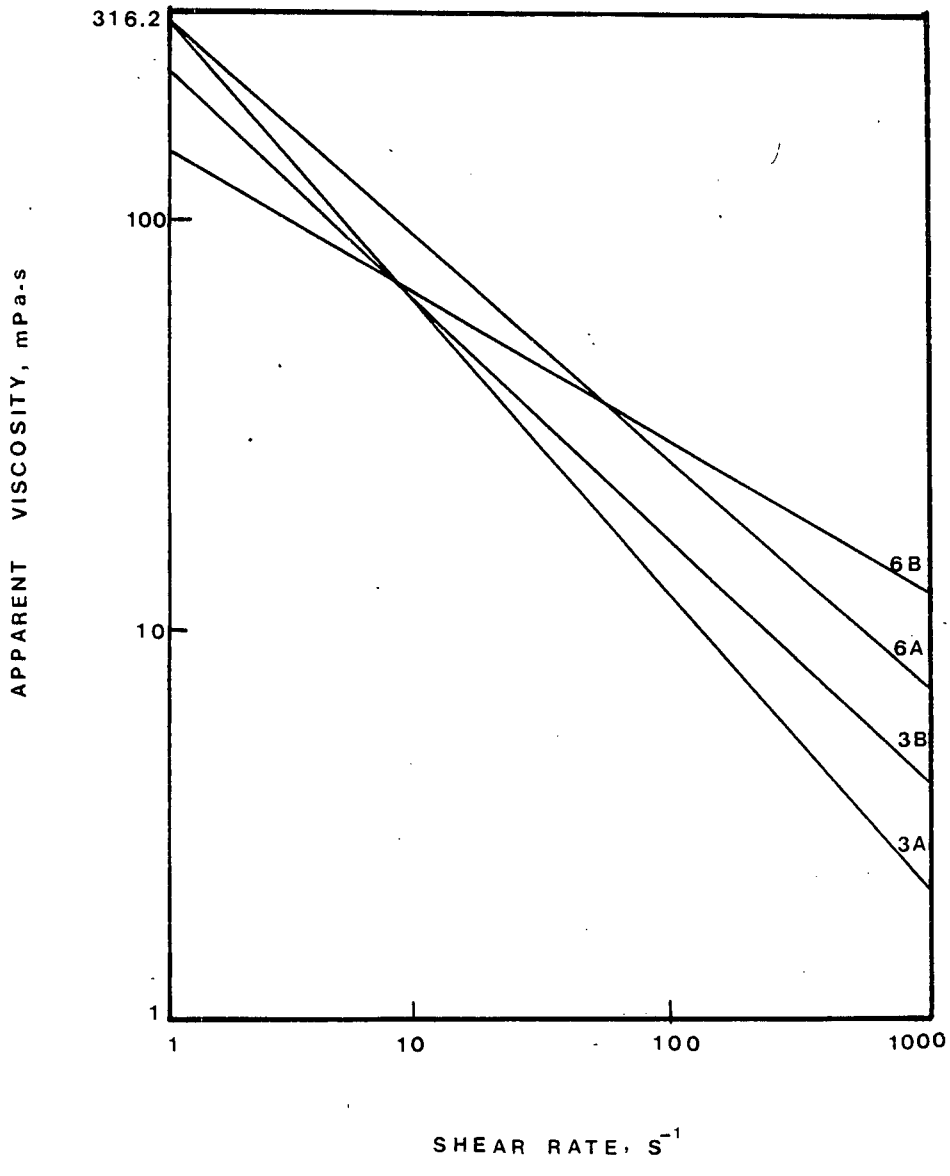

Figure 44. Rheogram for 6 and 3 % Avicel PH-101 MCC (A) and A. xylinum MCC (B) dispersions at 25°C according to the Power law flow model.

more aggregated than A. xylinum MCC as indicated by the significantly larger average particle size of Avicel PH-101 (Table 22). Consequently, there was more breakdown or rearrangement of the structure resulting in a fluid which was more shear sensitive. Shear dependency reveals the nature of structures existing in the system. Rha (1978) attributed the presence of pseudoplastic behaviour to the presence of several factors: 1) high-molecular weight compounds or large particles at sufficient concentration, and 2) high interaction between the particles, causing aggregation, or association by secondary bonding.

Thus, it appears that the differences in viscosity of Avicel PH-101 and A. xylinum MCC were determined by mechanical forces; that is, solid-solid, solid-liquid and liquid-liquid interactions. However, the influence of electrochemical forces should not be ignored, eventhough zeta potential values for Avicel PH-101 and A. xylinum MCC (-6.05 ±2.19 and -3.2 ±0.45 mV, respectively) indicated very small electrostatic charge.

Using the Power law parameters shown in Tables 28 and 29, apparent viscosities were calculated at 100 s^{-1}. These apparent viscosities (at $\dot{\gamma}$=100 s^{-1}) were used to examine viscosity-concentration relationships.

The viscosity-concentration model fit the data accurately over the concentration range tested as is indicated by the r^2 in Tables 31 and 32. Rheograms of these data are depicted in Figures 45 and 46, where the logarithm of apparent viscosity vs the logarithm of concentration were regressed.

Table 31. Apparent viscosity (at $\dot{\gamma}$-100 s^{-1})-concentration relationship for A. xylinum MCC dispersions from 25 to 45°C.

Temperature (°C)	a (m Pa s)	b	r^2 (of log $\eta_{\dot{\gamma}=100\ s^{-1}}$ to log C)
25	6.397	0.825	0.999
35	5.420	0.858	0.998
45	5.3662	0.849	0.986

Table 32. Apparent viscosity (at $\dot{\gamma}$=100 s^{-1})-concentration relationship for Avicel PH-101 MCC dispersions from 25 to 45°C.

Temperature (°C)	a (m Pa s)	b	r^2 (of log $\eta_{\dot{\gamma}=100\ s^{-1}}$ to log C)
25	3.630	0.983	0.870
35	2.704	1.053	0.953
45	1.591	1.366	0.998

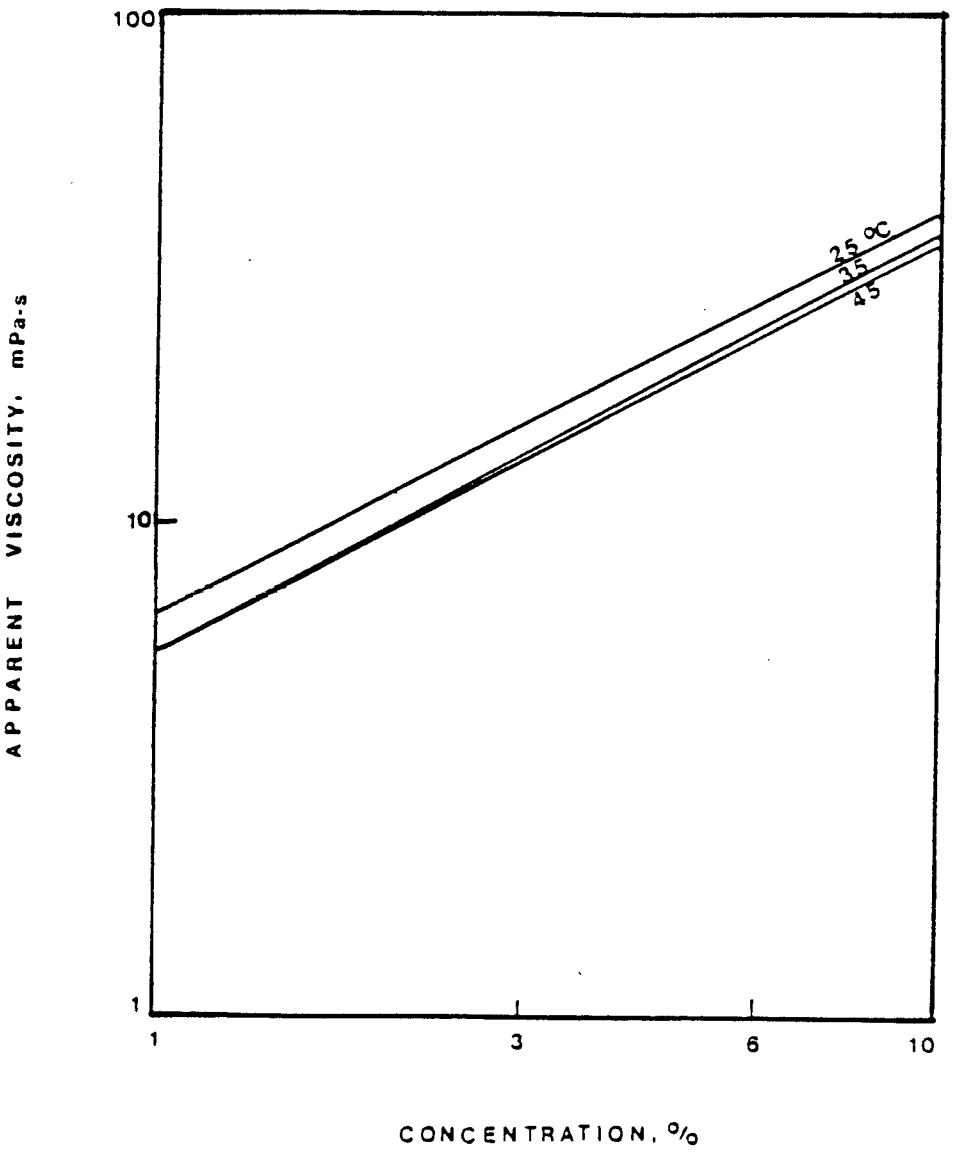

Figure 45. Effect of concentration on apparent viscosity ($\dot{\gamma}$=100 s^{-1}) of A. xylinum MCC dispersions at 25, 35 and 45°C.

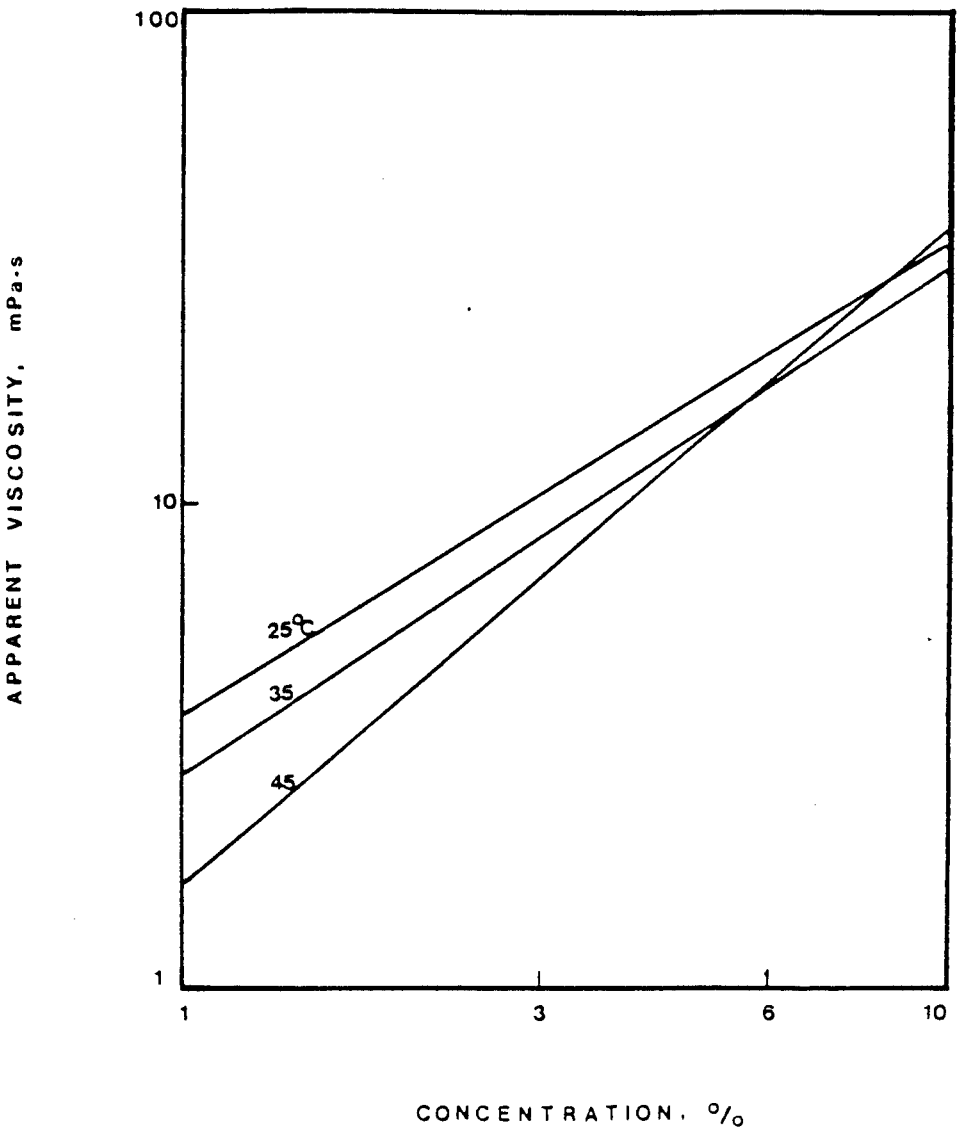

Figure 46. Effect of concentration on apparent viscosity ($\dot{\gamma}$=100 s^{-1}) of Avicel PH-101 MCC dispersions at 25, 35 and 45°C.

Apparent viscosities (at $\dot{\gamma}$=100 s^{-1}) were also analysed with respect to the reciprocal of absolute temperature. An Arrhenius type model was used to fit the data. This model fit the data accurately, except for the 5% dispersion of Avicel PH-101 as indicated by their coefficients of determination (r^2) shown in Tables 33 and 34. The activation energies and frequency factors were also included in these tables. The data were plotted on semilogarthmic coordinates with viscosity on the logarithmic ordinate and the reciprocal of the absolute temperature on the arithmetic abscissa. These rheograms are depicted in Figures 47 and 48. It appears that very small changes in apparent viscosity took place at the temperatures tested, with a general trend towards greater changes at lower concentrations.

Tables 35 and 36 show the consistency coefficients and flow behaviour indices of 3% dispersions of Avicel PH-101 and A. xylinum MCC at different pH values. These data were also portrayed in a graphic form in Figures 49 and 50. A. xylinum MCC did not appear to be greatly affected by pH, whereas Avicel PH-101 showed greater viscosities at lower pH. Avicel PH-101 response to pH was somehwat expected since the system acquired a flocculated appearance as the pH was adjusted to 4.0. The term flocculation is limited to the loose association of particles which form flocs or gel structures (Gray and Darley, 1981). Such behaviour was not as clearly observed with A. xylinum MCC, since its electrostatic charge, as represented by its zeta potential value, was lower than for Avicel PH-101. Flocculation is known to be the result of compression of the double layers when an electrolyte is added (Gray and

Table 33. Apparent viscosity (at shear rate=100 s^{-1})-temperature relationship for A. xylinum MCC dispersions at different concentrations.

Temperature (°C)	ΔE (k cal mole)	A (m Pa s)	r^2 (of log $\eta_{\dot{\gamma}=100\ s^{-1}}$ to $1000/T$)
6	1.304	3.025	0.950
5	0.960	4.745	0.875
4	1.871	0.845	0.991
3	1.211	1.990	0.875

Table 34. Apparent viscosity (at shear rate=100 s^{-1})-temperature relationship for Avicel PH-101 MCC dispersions at different concentrations.

Temperature (°C)	ΔE (k cal mole)	A (m Pa s)	r^2 (of log $\eta_{\dot{\gamma}=100\ s^{-1}}$ to $1000/T$)
6	2.824	0.206	0.950
5	0.4602	6.55	0.582
4	1.8352	0.575	0.901
3	4.7469	0.003	0.999

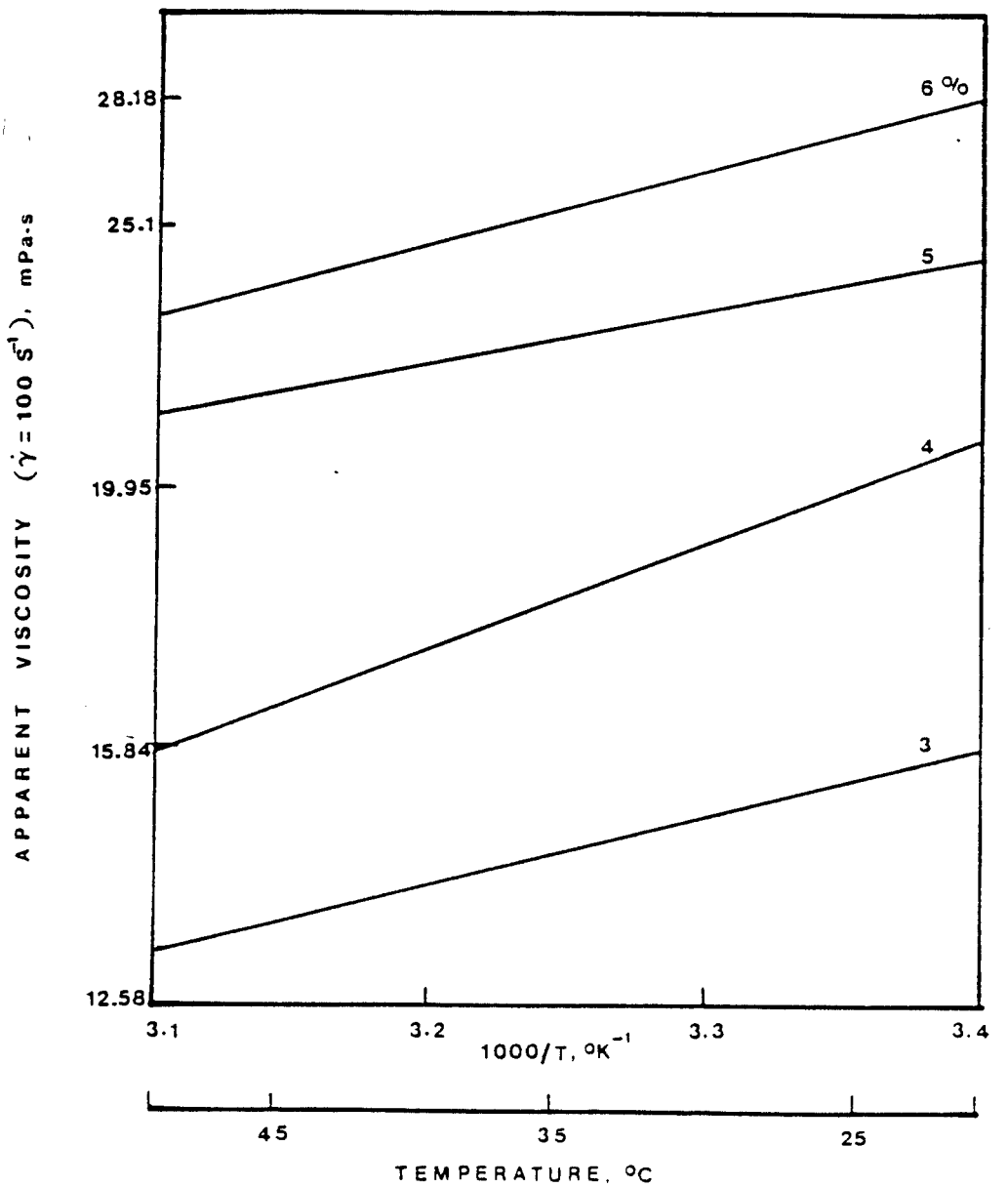

Figure 47. Effect of temperature on apparent viscosity (shear rate=100 s^{-1}) for 6.0 to 3.0 % A. xylinum MCC dispersions.

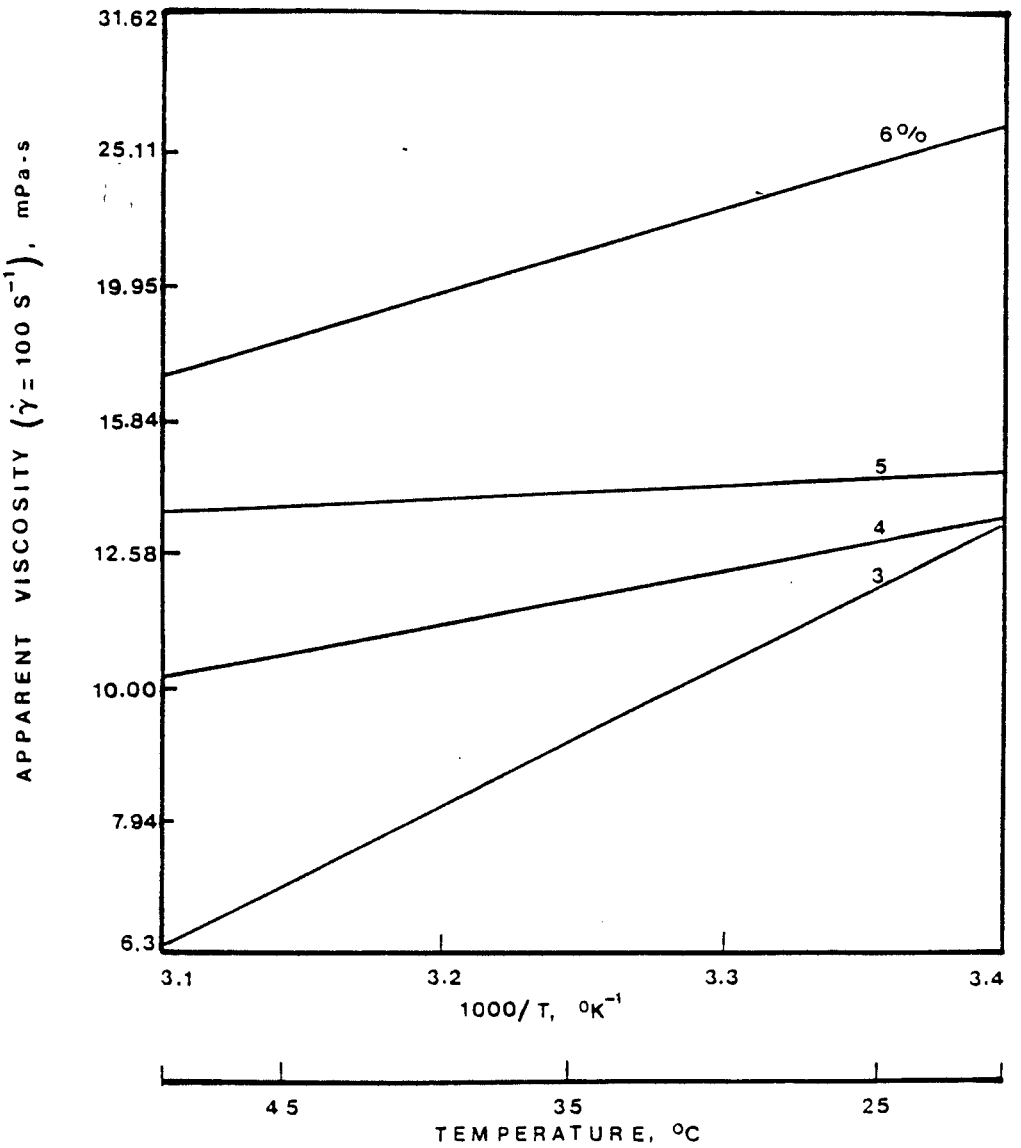

Figure 48. Effect of temperature on apparent viscosity (shear rate=100 s^{-1}) for 6.0 to 3.0% Avicel PH-101 MCC dispersions.

Table 35. Consistency coefficients and flow behaviour indices for a 3% dispersion of A. xylinum MCC at 25°C and at different pH.

pH	m ($Pa \cdot s^n$)	n	r^2
4.0	0.270	0.459	0.984
5.5	0.241	0.473	0.990
7.0	0.304	0.411	0.991

Table 36. Consistency coefficients and flow behaviour indices for a 3% dispersion of Avicel PH-101 MCC at 25°C and at different pH.

pH	m ($Pa \cdot s^n$)	n	r^2
4.0	0.427	0.405	0.991
5.5	0.387	0.269	0.991
7.0	0.376	0.288	0.988

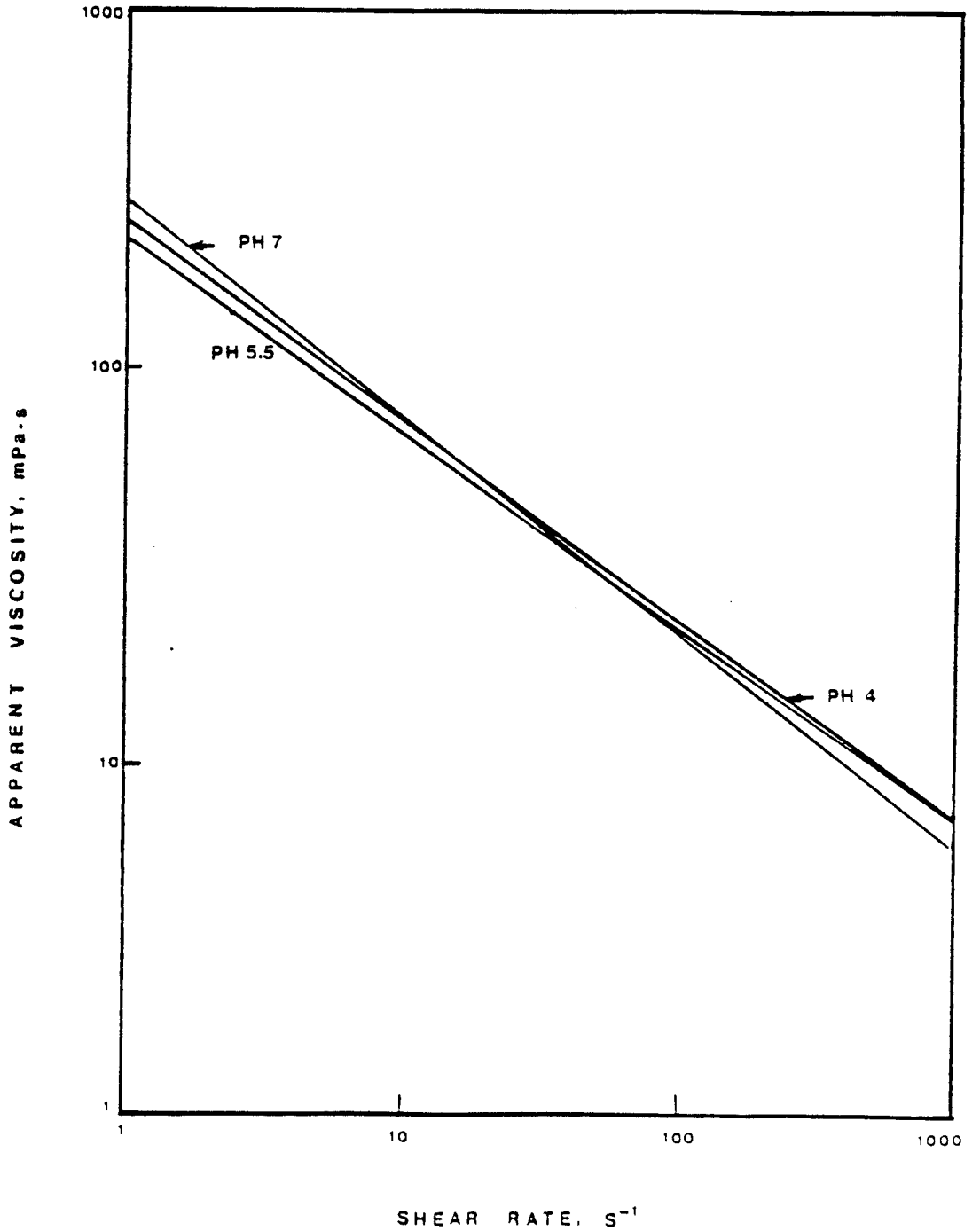

Figure 49. Effect of pH on apparent viscosity of 3% A. xylinum MCC dispersions at 25°C.

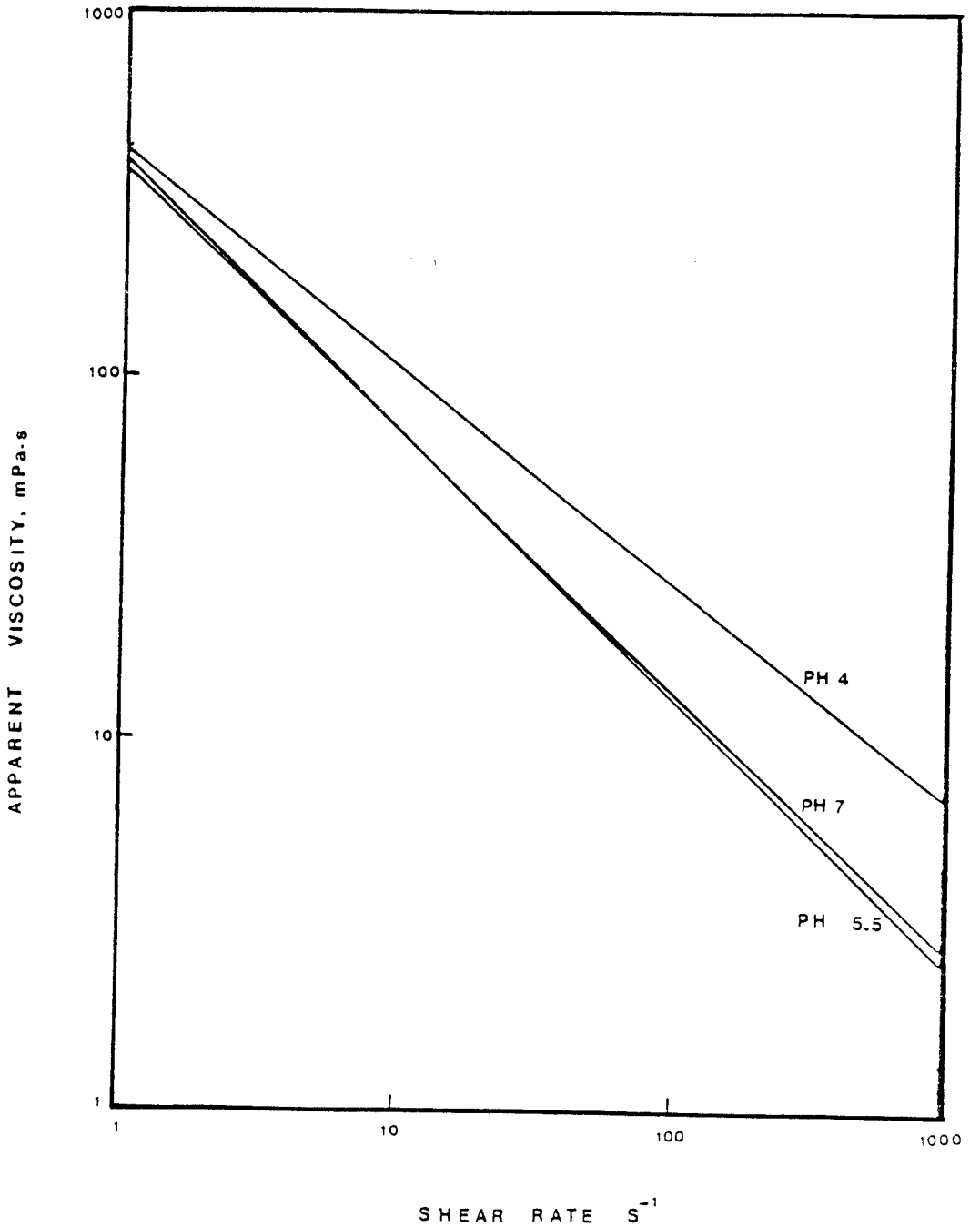

Figure 50. Effect of pH on apparent viscosity at 25°C for 3% Avicel PH-101 MCC dispersions.

Darley, 1981), and so the higher the electrostatic charge, the higher the sensitivity to electrolytes and the greater the chances for flocculation.

Table 37 presents the Power law parameters for 3% dispersions of either Avicel PH-101 or A. xylinum MCC and either of the two dispersions + 0.4% NaCl. These data are also shown in Figure 51. Either of the 3% dispersions appeared flocculated when prepared with 0.4% NaCl. This flocculation was more clearly observed at high shear rates where apparent viscosities were higher for the dispersions prepared with 0.4% NaCl than for either of the 3% MCC dispersions. A study was made by Edelson and Hermans (1963) on the effect of adding electrolyte to microcrystalline cellulose gels; it was reported that provided the gel and the electrolyte solution were properly mixed, only a small increase in the shear stress at any shear rate was measured. It was concluded that the negative charges present on the cellulose microcrystals do not appreciably contribute to the interparticle forces responsible for the gel properties.

Table 37. Consistency coefficients and flow behaviour indices for a 3% dispersion of Avicel PH-101 MCC or A. xylinum MCC and Avicel or A. xylinum MCC + 0.4% NaCl at 25°C.

Sample	m ($Pa \cdot s^n$)	n	r^2	N
A. xylinum MCC	0.243	0.406	0.981	12
Avicel MCC	0.342	0.268	0.998	12
A. xylinum MCC or Avicel MCC + 0.4% NaCl	0.286	0.413	0.972	12

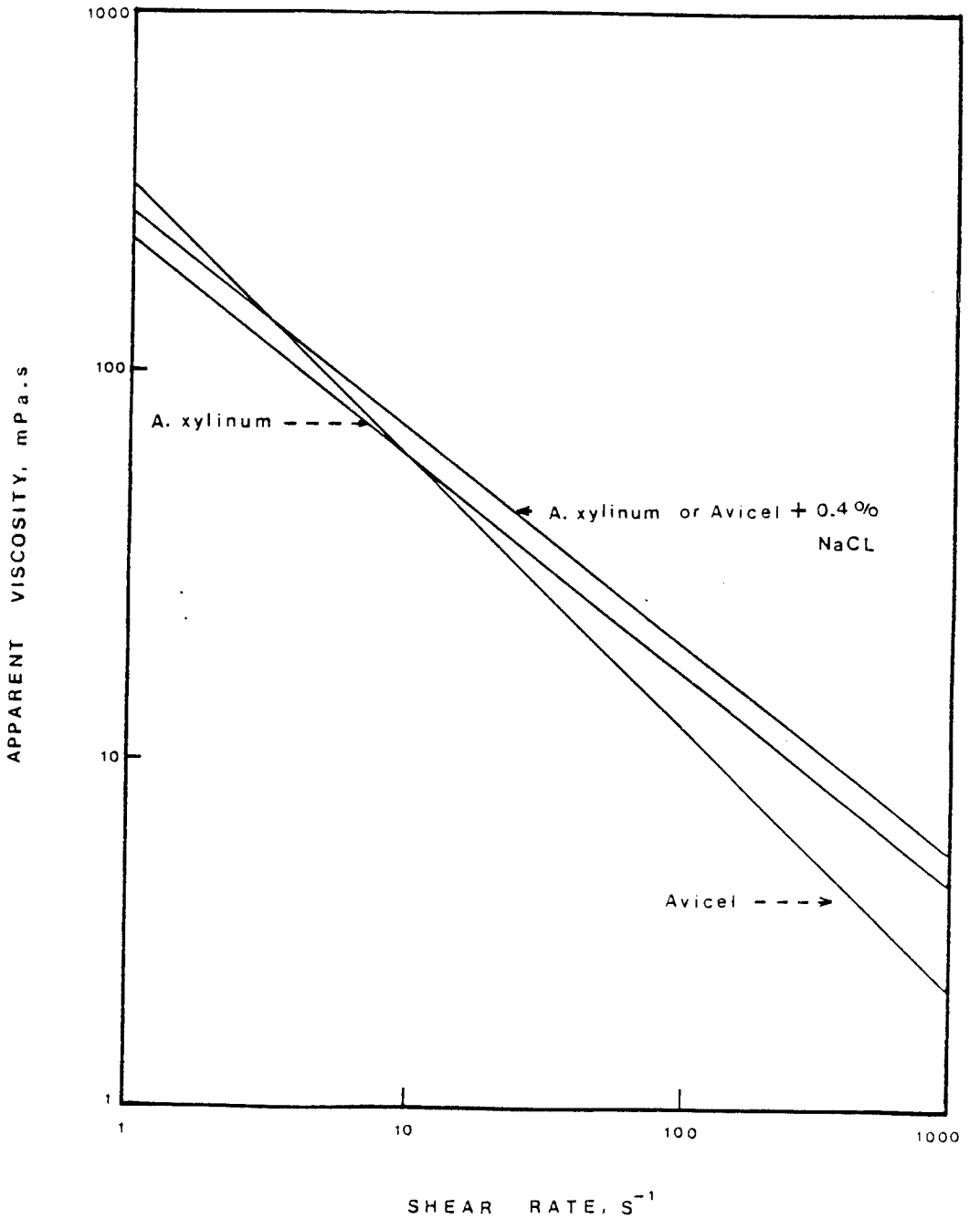

Figure 51. Effect of 0.4% NaCl on apparent viscosity of 3% A. xylinum and Avicel PH-101 MCC dispersions at 25°C.

CONCLUSIONS

Industrial applications of A. xylinum cellulose have been scarcely explored, in spite of its great potential for large scale production. This study was conducted to develop two applications of A. xylinum cellulose.

The first part of the study was aimed at investigating nutrient requirements of Acetobacter xylinum and to compare three strains in terms of cellulose production and culture stability. Previous reports on carbohydrate and nitrogen requirements and pH of the cultivation media of A. xylinum (Lapuz et al., 1967; Dudman, 1959) were confirmed. A defined medium which consisted of 10% sucrose and 0.5% peptone among other ingredients at pH 4.5, was formulated. This formulation was adopted for further work in this study; however, it is suggested that future work should follow this phase by a transition to natural media or agricultural waste in order to scale-up the formulation to a commercially viable process.

High mutability of cells within species of the acetic acid bacteria was also confirmed (Shimwell and Carr, 1958; Schramm and Hestrin, 1954; Cook and Colvin, 1980). The three strains tested were unstable under swirled conditions, 100% of colonies were cellulose-deficient after the fifth and sixth serial transfers (10 to 12 days). On the contrary, all the strains were much more stable under static conditions; ATCC 14851 being the most stable with only 9 to 10% cellulose deficient colonies after the ninth to tenth serial transfer (18 to 20 days).

Cellulose yields after 40 days of incubation varied from 6.5 g/100 mL media (64% conversion of total sugar) with the Philippine strain to 0.6 g/100 mL media (6.05% conversion of total sugar) produced by ATCC 10821. The high-yielding strain was able to utilize up to 73% of the sugar, from which up to 87% was converted to cellulose. On the other hand, the two low-yielding strains were only able to utilize 25 to 30% of the sugar, from which only 45% was converted to cellulose. Thus it appears that the Philippine strain is a high cellulose yielding strain whereas the other two strains appear to shift their metabolism away from cellulose synthesis towards other metabolic pathways. Therefore, it was concluded that these organisms vary widely in their efficiency to convert sugar to cellulose.

The second part of this study involved the continuation of the development of a process which integrated cellulose I fibrils into a "synthetic" fibre originally developed in this laboratory (Townsley, 1974; unpublished results). A method was devised to force A. xylinum cells to spin cellulose ribbons into parallel stable filaments by cultivation of the organisms in a straight-line flow path. Electron micrographs clearly revealed parallel orientation in the fine fibre structure. This investigation confirmed an electron microscopic study carried out in this laboratory (Townsley and Tung, 1974; unpublished results).

Brown (1982) suggested the integration of cellulose I fibrils produced by A. xylinum into a synthetic fibre. According to him, a novel system requires that cellulose I structures be retained throughout

the later stages of synthetic filament formation. Thereby, an integrated process for developing a cellulose fibril would incorporate a natural polymer in the early stages of its aggregation, thus allowing a greater amount of synthetic engineering. Therefore, it appears that the synthetic fibre developed in this study will encourage more integrated research in this field which may benefit industry.

Tensile strength of the fibres was improved by using the mercerization treatment commonly used for cotton. Optimum conditions for the treatment as determined by using Mapping Super Simplex Optimization were 8.3% NaOH, 59°C and 24 minutes. A tensile strength of 83,300 ±1800 N/cm^2 was achieved, an increase over the untreated fibres of 281%. The results of this study thus indicated that the well known mercerization treatment used in textiles could successfully be applied to cellulosic fibres produced by bacteria only if the treatment conditions were optimized to this particular cellulose fibre structure.

Finally, the third part of this study consisted of the development of a process for the purification and hydrolysis of A. xylinum cellulose for the manufacture of mycrocrystalline cellulose (MCC).

MCC from A. xylinum cellulose was produced by a simple procedure. The method consisted of only one extraction procedure with 6% NaOH for 3 hours, one bleaching sequence with $NaOCl_2$ for 2 hours and hydrolysis with 2.5 N HCl for 15 minutes. Nevertheless, a highly purified (98.5 ±1.4% cellulose) and highly crystalline (98.15 ±0.91% crystallinity index) microcrystalline cellulose was obtained without causing any detrimental effect on the fibre structure. On the contrary, commercial

MCC is prepared from highly degraded cellulose from wood pulp as revealed by a low crystallinity index of 84.90 ±1.41%. Degradation of the cellulose always occurs during the severe purification treatments to which wood pulp is subjected to.

The flow behaviour of A. xylinum MCC dispersions was studied and compared to commercial Avicel PH-101. Both MCC displayed non-Newtonian pseudoplastic behaviour. A. xylinum MCC dispersions were found to be thixotropic.

The multiple uses of MCC in the food and pharmaceutical industries indicate the great potential that A. xylinum cellulose may have as a raw material.

Results of this study indicated that the two applications of A. xylinum cellulose developed are technically feasible. What remains is the need to determine economic feasibility.

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