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CHARACTERIZATION OF EXOPOLYSACCHARIDE PRODUCED BY A *CORYNEBACTERIUM* IN CHEESE WHEY SUBSTRATE

A Dissertation

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Degree

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ABSTRACT

A Corynebacterium designated #98 was found to produce an orange pigmented exopolysaccharide in high yield when grown at optimum conditions in a cheese whey substrate.

Sweet whey was reconstituted and deproteinized by lowering the pH to 5.0 and autoclaving. The whey solution was enriched with $MgSO_4$, $FeSO_4$, lactose, its pH was adjusted to 7.0 with 1 N NaOH, and the solution was autoclaved. The Corynebacterium #98 was cultivated in Tryptic Soy Broth (TSB) for 24 hours and then inoculated into 1.5% whey substrate. This was incubated at 30°C in a shaker bath for 7 days. The viscous culture medium was inoculated later at a rate of 15% into a 2-liter fermentor with agitation of 200 rpm, air flow of 1090 ml/min., and temperature of 30°C.

After 15 days, the viscous material was precipitated by a mixture of acetone, phosphate buffer, and saturated KCl. The gum was freeze-dried and stored.

The rheology of the gum indicated that it is a shear thickening (dilatant) polysaccharide. The viscosity increased with increasing gum concentration and pH, and decreased with increasing salt (NaCl) concentration. The emulsifying capacity was 17.02 ml of vegetable oil/g of gum.

Analysis indicated that the freeze-dried gum consisted of 5.93% moisture, 1.11% ether extract, 4.06% protein, 29.67% ash, and 59.23% nitrogen free extract. Mineral determination showed high levels of

potassium and sodium, some chloride and phosphate, and low levels of calcium, magnesium, iron, and silica.

The monosaccharide constituents of the gum as determined by gas chromatography were rhamnose, xylose, mannose, glucose, galactose, glucuronic acid, and mannuronic acid in a ratio of 2:1:6:6:7:16:11.

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CHAPTER I

INTRODUCTION

The polysaccharide hydrocolloids, traditionally from plants, are used for many purposes in food products. In recent years, the term has been extended to include microbial gums which are rapidly emerging as important new sources of polymeric materials. These biopolymers have unique properties and applications in food, pharmaceutical, and other products. These microbial gums are produced by different microorganisms and unlike plant gums can be produced commercially with economic feasibility.

A coryneform bacterium designated as number #98 which was isolated in 1979 by Daeschel from sorghum molasses was found to produce a slimy material in sorghum juice. Further investigation by Rodriguez (1980) indicated that this microorganism can produce an orange pigmented exopolysaccharide by using glucose, lactose, maltose, galactose, and sucrose as carbon sources in the presence of $MgSO_4$ and $FeSO_4$. The organism was also able to produce the polysaccharide in a modified whey substrate.

Whey, a byproduct of the cheese industry, is produced in large quantities. Although some of this product has been used for a variety of purposes, the disposal of the remainder has caused problems to both the dairy industry and city sewage systems. To lessen the magnitude of the problem, research on the effective utilization of whey has been encouraged.

Whey contains several nutritional components. Presently, many investigators are involved in the recovery of protein and lactose and the resulting products are used in many foods and pharmaceuticals. Through investigation, it also has been reported that whey and some of its components may be used as nutrient sources by some microorganisms to produce microbial gums on a commercial scale.

Therefore, the main purpose of this investigation was to optimize the production and recovery of the gum produced by Corynebacterium #98 grown in a sweet whey substrate, and to characterize the gum's rheology and its composition.

CHAPTER II

REVIEW OF LITERATURE

I. IMPORTANCE OF WHEY UTILIZATION

Whey utilization in various areas of food technology has become increasingly important as environmental pollution problems increase and as new techniques of production are developed. In making cheese, for every ten pounds of milk, nine pounds of whey are obtained and one pound of cheese is produced (Groves, 1973).

In 1974, the United States produced 32.5 billion pounds of whey, containing 1.6 billion pounds of lactose, over half of which was disposed, causing environmental problems. However, the remaining whey was used for a variety of nutrient materials (O'Leary and Woychick, 1976; O'Leary et al., 1977). In 1976, between 11 and 15 billion pounds of whey, containing one billion pounds of lactose, was also wasted (Elias, 1979).

Cerbulis et al. (1972) found that the average composition of commercial dried whey included 9.7% protein, 71.7% carbohydrate, 1.28% lipid, and 8.2% ash. Since strict regulations were established for disposal of whey, new interests developed in reclaiming the discarded whey nutrients. For example, utilization of whey (liquid or dried) has been achieved in the production of baked goods, beverages, dairy products, confections, animal feeds and other edible products (Groves, 1973; Holsinger et al., 1974). In addition to its use in

these common food products, whey has been used recently as a substrate for laboratory microbial gum production (Harrison, 1976; Rao, 1977).

II. NATURE AND OCCURRENCE OF MICROBIAL GUM

Gums or hydrophilic polysaccharides are amorphous substances of high molecular weight which occur widely in the plant kingdom. In recent years, the term has been extended to include microbial gums which have been produced by different species of microorganisms. The new interest in developing microbial gums is based on several different reasons, including: a) distinctive properties of microbial gums related to successful performance in food; b) human digestibility of the gums without adverse effect; c) the role of gums as supplementary sources for plant gums; 4) the ability to control the synthesizing process by varying one or more factors which affect the quality and quantity of the yield; e) easy recovery of microbial gums; f) the possibility of using a low cost substrate for production of gums; and g) various advantages of domestic production over foreign importation (Jeanes, 1974; Rao, 1977; Rodriguez, 1980).

Polysaccharides which are produced by bacteria usually belong to one of the following three categories: 1) extracellular polysaccharides which occur as capsules around the cells or as slimes which accumulate outside the cells (most common); 2) cellular structures; and 3) intracellular storage polysaccharides. Polysaccharides usually are classified as homopolysaccharides which contain one kind of sugar such as glucose or as heteropolysaccharides

which contain two or more types of sugar. The former is produced by a single or simple enzyme system and the latter is produced by utilization of a carbon source by a complex enzyme system (Wilkinson, 1958).

There are many factors which influence the ability of microorganisms to produce polysaccharides. Most of these microorganisms are aerobes or facultative anaerobes, and depending upon the type and location of microbes in nature, are capable of converting inexpensive waste carbohydrate (such as lactose in whey) to polysaccharides. The source of nutrients in the fermentation process affects the composition of the homopolysaccharides. However, it does not affect sugar composition of heteropolysaccharides (Sanford and Conrad, 1966). The culture medium usually used for polysaccharide production contains the following sources unless the microorganism is photosynthetic or a nitrogen fixing organism: a) carbon source such as glucose; b) a source of nitrogen, usually inorganic salts of NH_4^+ and NO_3^- ions; 3) cations such as iron, magnesium, manganese, potassium and sodium. The other optimum conditions for the production of polysaccharides include oxygen, pH, temperature, proportion of air, presence or absence of agitation and the size of the inoculum (Harrison, 1976; Rao, 1977; Rodriguez, 1980).

Molecular weight (MW) is one of the important characteristics of an exopolysaccharide which influences the rheology of the gum. This can be controlled by physical alteration in the growth environment. Therefore, the growth environment can affect microbial gum quantity as well as its quality (Collins, 1964).

III. RHEOLOGY AND CHARACTERISTIC PROPERTIES OF SOME COMMERCIAL MICROBIAL GUMS

There has been a continuous effort to select and produce microbial gums which are important to the food industry, pharmaceutical usage, and many other commercial applications. The Northern Regional Research Laboratory has been active in developing these new sources of gums for the food industry. Also, much research has been oriented toward producing high quality microbial gum using waste products, such as whey, as a substrate.

Previously, many microorganisms were found which produced slimy or extracellular materials; some were produced commercially. The following are some of the important microbial gums which are produced in high quantity and quality.

Dextran

Dextran is a polyglucan which is produced by a wide range of microorganisms. Leuconostoc mesenteroides NRRL 512 F strain is usually used for commercial production of dextran, using sucrose as a substrate (Jeanes, 1974). In making the dextran, dextransucranase is the extracellular enzyme responsible for dextran formation. Production of dextran can be achieved by adding the enzyme as a cell-free extract to a sucrose solution, or by adding the microorganism Leuconostoc mesenteroides directly to the sucrose-containing medium (Margaritis and Zajic, 1978).

The dextran structure is made of α - D - glucopyranoside residues that contain about 95% of α - D - (1 $\rightarrow$ 6) - and 5% of

α - D - (1 → 3) - linkages and has a M.W. of 100,000 to 40 - 50 x 10^6. Jeanes (1974) reported that "native" dextran has a M.W. of 30-90 million.

Dextran is dissolved readily in water and is stable to heat, freezing, and thawing. The dextran solution shows no viscosity increase until a concentration of 3 to 5% is reached (Jeanes, 1974).

Dextran is used as a blood plasma extender to improve the flow in capillaries (Jeanes, 1974). The crosslinked dextran produces "Sephadex" which is used widely in the purification and isolation of biochemical materials (Margaritis and Zajic, 1978). Dextran has been used also in the food industry since it prevents crystallization, improves moisture retention, improves body, and maintains flavor and appearance in candies and jellies. Dextran is used also as a stabilizer in ice cream and beverages and as an anti-staling agent in bread products (Glicksman, 1969).

It has been reported that the breakdown of dextran in the intestinal tract is dependent upon the availability of dextranase and the structure of dextran, not molecular size. Dextranases occur in intestinal mucousa of humans and other mammals (Jeanes, 1974).

Xanthan Gum

Xanthan gum is a polysaccharide produced by Xanthomonas spp. The gum synthesized by Xanthomonas campestris NRRL B-1459 is commercially produced by the Kelco Company under the trade name of Keltrol (food grade) and Kelzan (industrial grade) (Jeanes and Pittsley, 1973). The optimum conditions for gum production by these

microorganisms are dependent upon the species of the Xanthomonas. The optimum industrial conditions when B-1459 is used include: 48 hrs incubation at 28 to 31°C with good O_2 transfer at pH of 6.0 - 7.0. This bacterium is killed by pasteurization and the gum is precipitated with 2-propanol or methanol (Margaritis and Zajic, 1978; Moraine and Rogovin, 1973).

Souw and Demain (1979) reported that 40% sucrose or glucose medium yielded the highest amount of xanthan gum. However, Moraine and Rogovin (1973) believe that the growth was not nutrient limited, and, as the cell concentration increased, the product formation rate also increased.

Addition of certain organic acids such as succinnic stimulates xanthan gum production. Concentration of the nitrogen sources higher than the optimal level inhibited gum production while stimulating growth. It is believed that the gum is produced as a stagnant slime layer which builds up around the cells, and, as its viscosity increases, the transport of nitrogen to the cells decreases (Moraine and Rogovin, 1973). It was found that productivity increased when NH_4OH was used to control pH and as a source of nitrogen (Moraine and Rogovin 1973). Xanthan production was also sensitive to high concentrations of inorganic phosphate (Moraine and Rogovin, 1973; Souw and Demain, 1979). HOdag KP-2 was found to be the best antifoam for Xanthamonous spp. (Lilly et al., 1958).

Xanthan gum structure is composed of D-glucose, D-mannose, and D-glucuronate in a ratio of 3:3:1, respectively (Sloneker and Jeanes,

1962). The D-glucuronic glycosidic linkages in the main linear chain are (1 → 4) and all appear to have the β-configuration. Mannose, a single unit side chain, is attached to the main chain by (1 → 2) or (1 → 3) glycosidic linkages. The polymer has a molecular weight of more than one million, sometimes reaching 5-10 million (Glicksman, 1969; Sloneker and Orentos, 1962). Rocks (1971) reported that xanthan gum exists in the form of the potassium salt and has relatively few carboxyl groups. Since the polymer contains short side chains, it does not lend itself to cross-linking.

Xanthan gum rheology has made the gum favorable to various uses, especially in the food industry. Xanthan gum has a high viscosity at low concentrations. The viscosity of a 1% solution is about 800-1000 cps at 60 RPM on Brookfield viscometer at 25°C. Its viscosity increases as the gum concentration increases. It is soluble in hot or cold water and the viscosity decreases with vigorous agitation (pseudoplastic) which gives good flow properties (Jeanes, Pittsley, and Senti, 1961; Tsai, 1976).

The viscosity of the xanthan gum (B-1459) solution is independent of pH between 6-9. Lowering the pH from 5 to 4 increases the viscosity; however, below pH 4, solutions buffer strongly and show a linear decrease in viscosity with pH. Adjustment of pH between 9 to 11 increases the viscosity which follows a slower decrease (Jeanes, 1974; Jeanes, Pittsley, and Senti, 1961).

Xanthan gum can be affected differently at differing concentrations by various salts. Polysaccharide solutions of less than

0.2% KCl showed a decrease in viscosity and then leveled off, but salt concentrations of more than 0.4% increased viscosity and then leveled off. At salt concentrations between 0.2 - 0.4%, the viscosity increased, then decreased and was followed by an increase (Jeanes, Pittsley, and Senti, 1961). Souw and Demain (1979) found that xanthan gum had a lower viscosity with salt concentrations of less than 0.3%. An increase in viscosity was observed at higher salt concentrations, but was not significant (Souw and Demain, 1979). It was also reported that some salts, such as quaternary ammonium salts, precipitated xanthan gum (Stauffer and Zeeder, 1978).

The viscosity of xanthan gum solution (B-1459) was influenced inversely by temperature in the presence of salt. However, this was different in regard to source of microorganisms (Jeanes, Pittsley, and Senti, 1961). This gum showed little sensitivity to temperatures as high as 80°C. The presence of KCl can protect the gum against high temperature (Harada and Yoshimura, 1965). However, Cadmus et al. (1963) reported that the viscosity of polysaccharide B-1973, a different gum, increased as temperature increased. Rocks (1971) reported that xanthan gum was resistant to degradation by heat and had good pump workability.

Xanthan gum has been accepted as a major food additive and placed on the GRAS list by the FDA (Jeanes, 1974). Smiley (1966) reported that use of xanthan gum at 7.5 - 15% and 2% levels in rat and dog rations, respectively, caused diarrheatic conditions among the animals. However, at 1% level it was considered to be safe. Xanthan

gum has been used as suspending agent in beverages at 0.001 to 0.015% level. Its excellent stabilizing and emulsion properties led to its use in canned food. It was also reported that xanthan gum has good freeze thaw stability which makes it useful for pie fillings and puddings (Kovacs, 1973; Rocks, 1971). Xanthan gum also is used in non-food products such as textiles, paints and dyes; it is used in oil well drilling, and flooding (Jeanes, 1974).

Alcaligenes viscolactis

Alcaligenes viscolactis is a non-pathogenic microorganism that causes ropiness in milk. Harrison (1976) used a whey substrate for producing the slimy material (extracellular). She found the yield of gum was about 0.625 g per liter of culture fluid. The viscosity of the gum solution increased as gum concentration increased. Salt concentration had a significant negative effect on the viscosity of the aqueous gum solution. The effect of pH was not significant in altering the viscosity, while heat had a highly significant negative effect, causing viscosity to decrease.

Hansenula holstii

Hansenula holstii is a non-pathogenic yeast isolated from pine trees; it produces polymers using glucose mainly as a carbon source. The polymers produced are composed of mannose, phosphate, and potassium in a ratio of 5:1:1, respectively, when isolated as the potassium salt (Slodki and Cadmus, 1978; Jeanes, 1961). The viscosity of this gum solution increases linearly to a maximum of 1.5%, then at a slower rate to 3%. However, between 3 and 5%, the viscosity increased

nonlinearly. Rao (1976) used a hydrolyzed whey substrate for production of phosphomannan gum by Hansanula holstii and reported that yield was dependent upon glucose concentration. He concluded that yield of polymer was usually 30% of glucose available in the medium. This phosphomannan gum can be used as a thickening and stabilizing agent.

There are many other microorganisms which are capable of producing exopolysaccharides. However, many have not been studied extensively for their properties, structure, rheology, commercial production and safety usage level. Such expolysaccharide producing microorganisms are: Streptomyces strain (HRM P1185) (Miyazaki et al., 1976); Zoogloea romigera (NRRL 13-182) (Parsons and Dugan, 1971); Cryptococcus laurentii (NRRL Y-1401) (Jeanes et al., 1964); Corynebacterium and Brevibacterium (Kanamaru and Yamatodani, 1969); Corynebacterium hydrocarboclastus (Knetting and Zajic, 1972; Zajic and Knetting, 1971); Rhodococcus erthropotis (Rapp, et al., 1979); Corynebacterium autotrophicum strain (7C) (Tunail and Schlegel, 1974).

IV. CHARACTERISTICS OF THE CORYNEBACTERIA

Coryneform bacteria are widely distributed in nature and primarily found in soil, plants, sea water, on fish, in dairy products, and as a part of the normal flora of humans and animals. Certain species of these bacteria are particularly important since they may cause disease in crops and animals (Jensen, 1966).

The coryneform bacterium under current investigation was isolated from raw sorghum juice by Daeschel (1979) who found that his

microorganism produced yellow-orange slimy material. The cells were Gram variable, non-motile, non-acid fast, non-sporulating, aerobic and coccoid (0.54 x 0.75 μ) with a tendency to form small curved rods with time. This Corynebacterium could not be classified under any species of the coryneform group of bacteria, according to Bergey's manual (Buchanan and Gibbons, 1974). There is no clearly established identification of bacteria in this particular group, which includes Corynebacterium, Arthrobacter, Cellulomonas, Microbacterium and Kurthia. However, the cell wall composition at all levels of complexity have made a major contribution to the classification of coryneform bacteria (Rodriguez, 1980).

The Corynebacterium species data related to cell wall composition collected from different investigators have been divided into three categories: 1) human and animal pathogens; 2) plant pathogens; and 3) non-pathogenic coryneform bacteria. The cell wall composition of the coryneform bacterium studied by Rodriguez (1980) contained lysine as diamino acid and large amounts of glycine with galactose and glucose. According to this content combination, the coryneform bacteria could not be classified in the coryneform species. However, the cell wall characteristics of this microorganism are very similar to those of Corynebacterium and Arthrobacter species. Further investigation is warranted for final determination of a distinct classification for this isolate.

Rodriguez (1980) studied the coryneform bacterium and determined the optimum conditions for its synthesis of gum in synthetic medium as well as a whey substrate for producing the orange pigmented gum. The

balance of this section is a description of her findings, the starting point for this investigation. After a series of experiments, she found that tryptic soy broth (TSB) was the best medium for growth of this microorganism. The optimum temperature found was between 30 and 32°C with a pH of 6.0 to 8.0. Since these bacteria are aerobic, aeration of the medium by shaking the flasks accelerated the growth. Surface area also influenced the cell growth and polysaccharide (PSC) biomass. Increased surface area of medium in relation to volume increased the cell growth, while the pH dropped at a slower rate, indicating less sugar fermentation. Both aeration, using a shaker, and large surface area increased availability of nutrients to the bacteria.

The addition of a salt solution affected the growth and gum production remarkably. Among various salt solutions, $FeSO_4$ was found to be very effective. However, $MgSO_4$ or K_2HPO_4 alone suppressed growth. This research also indicated that the coryneform bacteria utilized galactose, lactose, glucose, sucrose, and maltose, respectively, as sources of carbon tryptic soy agar.

Many factors were identified which influenced the polysaccharide production in whey substrate. However, the factors were similar to those which affected the synthetic media in the preliminary examination.

One important factor which influenced all growth and polysaccharide production was the role of crude nitrogen and the ratio of surface area to volume. It was found that 1.5% whey (w/v) in water was a suitable source of nitrogen which gave the highest yield, since nitrogen was a limiting factor for this microorganism. As the ratio of surface area to volume (sa/v) increased, the rate of oxygen

diffusion through the culture increased, and as a result a high yield of polysaccharide was observed. Therefore, oxygen was also a limiting factor for gum production using the coryneform bacterium.

Other important factors were the effect of rotary agitation and addition of salt. During the fermentation, the slimy material produced a decrease in oxygen transfer to the cell wall of the aerobic bacteria. As a result, agitation played an important role in providing oxygen to the cells.

Salts also affected the cell growth and yield of polysaccharide in the whey substrate. It was found that the combination of $MgSO_4$ and $FeSO_4$ yielded maximum cell growth and polysaccharide formation as shown in Figure I. However, an enhancement in cell growth was not associated with polysaccharide production. Therefore, the results were improved and the yield of gum increased when agitation as well as salts were used in an aerated environment.

Optimum initial pH was important for PSC production. It was believed that the initial pH of the medium played a critical role in exopolysaccharide formation. The initial pH was 6.5 and the pH was maintained rather constant through the fermentation; this might be attributed to the polymer properties.

The optimum level of carbon source was important to the bacteria as well as its source. It was found that 3% lactose (in whey substrate) resulted in the highest viscosity.

Rodriguez (1980) investigated the relationship between time and PSC production. She reported that polysaccharide production usually began after the bacteria had reached the stationary phase and

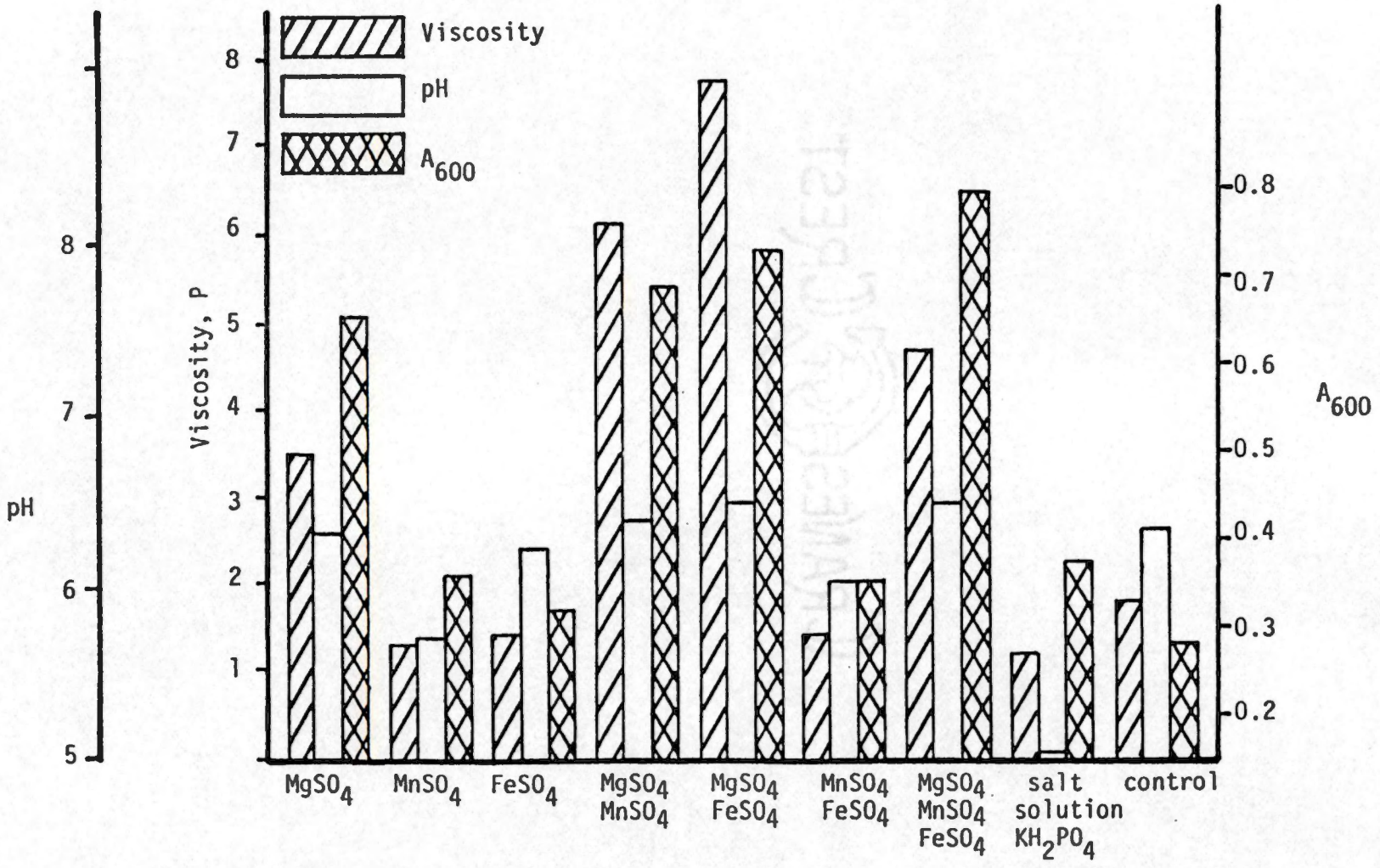


Figure I. The effect of salts on polysaccharide production, growth and pH in 1.5% whey medium (Rodriguez, 1980).

continued during the time of the experiment up to 22 days. She also found that increasing the rate of O_2 from 714 ml/min to 1090 ml/min after 18 days increased the PSC biomass formation as shown in Figure II.

The effect of inoculum on fermentation time was investigated, and it was found that inoculation of the fermentor with an active gum producing culture reduced the time considerably. The reduction in fermentation time may have been attributed to two factors, continuation of polysaccharide production by the cells in a similar substrate, and the inoculum of the adapted culture contained a higher cell concentration than had been previously reached.

After the fermentation process, the gum was precipitated by addition of KCl (0.02/l v/v) K_2HPO_4 (0.02/l v/v) and acetone (4:1 v/v). The gum separation from the cells was difficult, possibly as a result of protein association or the adherence of polymers to the cells (Brown and Lester, 1980). However, mild sonification resulted in complete clarification of medium. The sonification procedure destroyed weak interactions with cell or protein or broke the polymer molecule into smaller fragments.

Rodriguez finally investigated the sugar composition of the gum using paper chromatography. She found that rhamnose, mannose, and galactose were the major sugars in the polymer. However, there were other sugars present which were not identified.

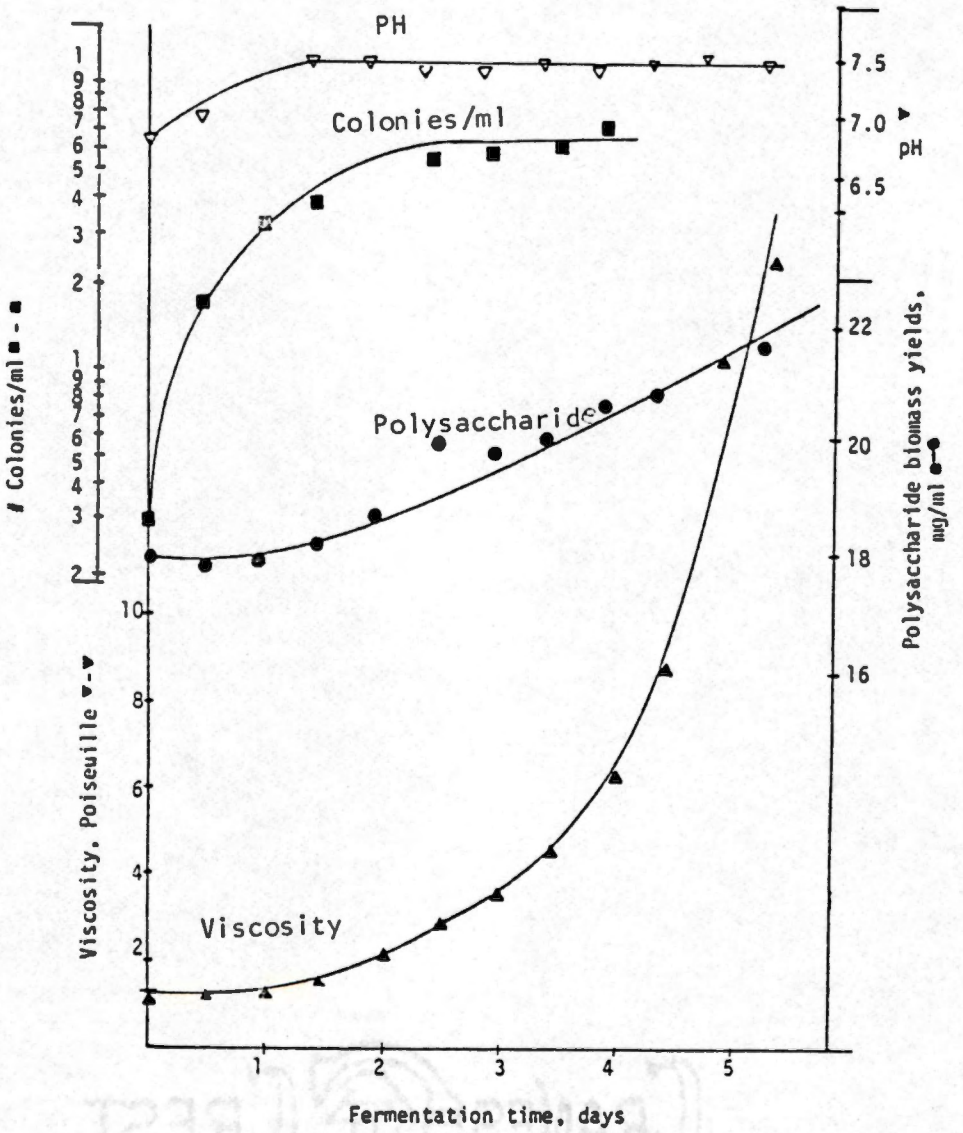


Figure II. Time course of exopolysaccharide production in a 2-liter fermentor inoculated with a three-day viscous whey culture (Rodriguez, 1980).

CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF MATERIALS

The culture used in this study was obtained from Dr. J. O. Mundt of the Microbiology Department of The University of Tennessee, Knoxville, and was isolated from sorghum juice by Daeschel in 1978. Rodriguez (1980) investigated this bacteria primarily for its polysaccharide production and designated it Corynebacterium #98. The culture was preserved in the frozen state as stock culture, while the working cultures were maintained on Brain Heart Infusion Agar (BHIA) (Pfizer) at 4°C (Jeanes, 1968).

One lot of dried cheese whey was obtained from Kraft, Inc., Greeneville, Tennessee, to insure uniformity of the medium throughout the work.

II. CULTURE MAINTENANCE

Culture #98 was maintained in the frozen state by transferring 0.5 ml of a broth culture of cells in the late stationary phase to 3 ml of tryptic soy broth (TSB) containing 16% glycerol (Tuggle et al., 1958). BHIA was found to be the most suitable medium for working cultures which were transferred monthly.

III. PREPARATION OF WHEY SUBSTRATE

A 6% whey solution (w/v) was prepared by rehydrating dried whey in distilled water. After adjusting the pH to 5.0, the mixture was autoclaved for 15 minutes at 121°C causing precipitation of part of the whey protein. The precipitate was removed by filtration through two layers of Whatman no. 1 paper and the volume was adjusted to the original. The solution was then diluted fourfold with distilled water and 0.5% salt solution containing 0.2% $FeSO_4$ and 4% $MgSO_4$ was added. The culture medium also was enriched with 2% lactose raising the content of lactose to about 3%. The pH was later adjusted to 7.0 with 1 N NaOH and the media was autoclaved (121°C, 15 min.).

IV. FERMENTATION

A 50 ml aliquot of autoclaved TSB was inoculated with two loops of culture #98 and incubated in a Blue M shaker-incubator at 30°C for 24 hours (shaker rate - 80 rpm). Five 50 ml aliquots of the whey substrate in 250 ml Erylenmeyer flasks were then inoculated with the 24 hr culture in TSB at a rate of 10% per flask. The flasks were incubated in the same shaker incubator with identical temperature and agitation. The incubation period usually lasted 7 days or until the whey substrate appeared viscous.

The last phase of fermentation was carried out in a Multigen fermentor (F-2000, New Brunswick Sci. Co., Addison, NJ). A 15% inoculum was added to 1.5 liters of sterilized whey substrate. The three impellors in the multigen were operated at 200 rpm and

temperature was held at 30°C while air was sparged in at a rate of 1090 ml/min. Silicon antifoam, manufactured by Dow Corning Company, was added at a level of 0.5 ml per 1000 ml of culture medium to suppress foaming. The pH of the culture medium was adjusted to 7.0 using 1 N NaOH before sterilization. Samples were taken from the fermentor as the fermentation progressed for pH, colony count, and relative viscosity determinations.

All measurement of pH was performed with a Fisher Accumet Model 230 A pH/Ion meter. A Brookfield viscometer, model LVF with spindle number 18, was used to measure relative viscosity. Standard plate count technique was employed in determination of colony counts (A.P.H.A., 1972).

V. ISOLATION OF THE GUM

Gum was precipitated in 1000 ml plastic centrifuge bottles which had a minimum adherence for the precipitated polymers. To 200 ml of the viscous liquid from the fermentor, 5 ml of 2 M phosphate buffer at pH 7.6, 5 ml saturated KCl, and 800 ml of acetone were added. This solution was hand mixed for 30 seconds then refrigerated overnight for complete precipitation. The precipitate was collected on Whatman no. 1 filter paper, and then homogenized with the addition of two to three times hot distilled water in a Vitrus homogenizer. The viscous gum solution later was placed in glass petri dishes and dried in a Virtis (Gardiner, N-Y) freeze-drier for 72 hours.

The freeze-dried gum was placed in a plastic bottle with a few marbles and then put into a Twin Shell Dry Blender at 27 rpm for 30 minutes. Then the composite powdered gum was kept refrigerated at 4°C until further analysis.

VI. MICROBIOLOGICAL PROPERTIES OF THE DRIED GUM

Standard plate counts were performed on the dried gum sample employing the spread plate technique on Standard Methods Agar (Difco) with 1% dextrose added (A.P.H.A., 1972). Dilutions up to 10^{-7} of the sample were made in phosphate buffer, and 0.1 ml of the dilutions were spread on the surface of the agar plates. The plates were incubated at 32°C for 48 hours and then counted. The coryneform organisms were differentiated from contaminants by the orange color of the coryneform colonies.

VII. RHEOLOGICAL PROPERTIES OF THE GUM

The effect of gum concentration, salt (NaCl) concentration, pH, and temperature on viscosity were studied. The levels of these factors were as follows:

1. Gum concentration: 0.5, 1.5, 2.5% (w/v)
2. Salt concentration: 0.5, 1.0, 1.5% (w/v)
3. pH: 5, 6, 7
4. Temperature: 15, 25, 35, 45°C

The experiment was conducted as a 3^3 factorial in randomized blocks of 9 combinations for a total of 27 experimental units. Each

experiment was performed at four different temperatures which made a total of 108 observations.

The aqueous solution of the gum for rheological determination was made prior to testing to let the gum rehydrate sufficiently. The viscosity in centipose (CPS) was measured with a Brookfield viscometer model LVF with spindle no. 18 at 60 rpm. The required temperatures were adjusted and controlled with a Haake instrument, model E 52.

VIII. STATISTICAL ANALYSIS

Data on viscosity were analyzed by analysis of variance (Sokal and Rohlf, 1969). Since there was no replication in the experiment, the four way interactions were used as random error for F-value determination. However, since the three way interactions were not significantly different, they were eliminated and pooled into error. Two-way interaction levels which were significantly different were subjected to multiple regression analysis to determine the interaction trends (Barr and Goodnight, 1979).

Means separation procedures, Duncan's Multiple Range Test, also was used for main factors in the model at levels of 5 and 1% to determine the significant difference among the mean values.

IX. PROXIMATE ANALYSIS OF THE GUM

Moisture content of the gum was determined by the vacuum drying method (A.O.A.C., 1975). Approximately 1.5 g of the gum were dried in triplicate in a vacuum oven at 60°C for 20 hours at a vacuum of 380 torr to obtain a constant weight.

The dried gum samples used in the moisture determination were ashed at 550°C for 6 hours (A.O.A.C., 1975). The samples were cooled to room temperature in a desiccator and then weighed. The results were calculated as ash on the freeze-dried basis.

Total nitrogen was determined by the Kjeldahl method (A.O.A.C., 1975) using 1 g gum samples. The test was performed in triplicate. The percentage of protein was calculated by multiplying the percentage of nitrogen by 6.25. The results were expressed on the freeze-dried basis.

The percentage of fat was determined by the petroleum ether extract method (A.O.A.C., 1975). Approximately 1.0 g gum samples in triplicate were extracted with 60 ml petroleum ether on the Goldfinch extractor for 16 hours. The results were reported on the freeze-dried basis.

The nitrogen free extract was determined by subtracting the percentage of moisture, ash, protein, and fat from 100%.

X. EMULSIFYING CAPACITY

Emulsifying capacity was determined by the method of Webb et al. (1970). A 1.5 g sample of gum was added to 100 ml of 1% salt (NaCl) solution and was shaken vigorously to dissolve the gum. Twenty-five g of the solution with 10 g vegetable oil were placed into a 250 ml blender jar. The blender contained 2 copper electrodes which were immersed in the solution. Vegetable oil was added slowly (1 ml/min.) from a burette to the blender operating at a speed of 12,000 rpm.

Emulsion break point was measured by a volt-ohmmeter attached to the copper electrodes. The results were calculated for three replicate samples and recorded as emulsifying capacity (ml oil/g gum).

XI. MINERAL COMPOSITION

The ash samples from the gum were analyzed for calcium, potassium, magnesium, sodium, iron, phosphate, silica, chloride, and sulfate content. An atomic absorption spectrophotometer (Instrument Laboratory, model 551, Wilmington, Maine) with a standard air-acetylene burner and a single element hollow cathode lamp was used for calcium, potassium, magnesium and iron determination. Atomic emission with an air-acetylene flame was used for sodium detection.

Approximately 1.5 g samples in duplicate were ashed in a muffle furnace at 550°C for 6 hours. The ash was dissolved with 20 drops of 6 N HCl and diluted to 25 ml with deionized water. Further dilution was necessary to bring the concentration of the element into the range of the instrument. A 1% lanthanum oxide solution was also used to overcome interferences. The dilution factors for calcium, magnesium, potassium, iron and sodium were 2,500, 6,250, 625,000, 25, and 250,000, respectively. To calculate the percent of elements present in the ash, the following equation was used:

$$\text{Elements } \% = \frac{(\text{absorbance}) \times (\text{dilution factor}/1000)}{\text{ash wt (mg)}} \times 100$$

The concentration of phosphate was determined by a colorimetric method using molybdovannado phosphate (A.S.T.M., 1974). Three samples of gum, approximately 1.5 g each were ashed at 550°C and

treated with 1 ml 6 N HCl. The dissolved ash was diluted to 500 ml in a volumetric flask. Fifty ml of each sample were placed in a 250 ml Erlenmeyer flask with 25 ml of ammonium molybate reagent and allowed to stand for 2 minutes before measuring the absorbance at 400 nm on a Shimadzu double-beam spectrophotometer UV-190.

Standards were prepared by dissolving 0.2197 g predried potassium dihydrogen phosphate (KH_2PO_4) in 1000 ml distilled water (stock solution). Fifty ml of stock solution were further diluted to 100 ml (1.0 ml of solution contained 0.025 mg phosphorous). Standards were prepared starting from 1 mg/l to 14 mg/l in 1 mg/l intervals. The amount of phosphate was calculated using the equation:

$$y = a + b\,x$$

$$\text{Absorbance} = (\text{intercept}) + (\text{slope})\ (\text{mg P/L})$$

$$\text{mg P in ash} = \frac{\text{mg P/L}}{1000} \times \text{vol. of total ash solution (ml)}$$

$$\text{P\% in ash} = \frac{\text{mg P in ash}}{\text{ash wt (mg)}} \times 100$$

$$PO_4\% \text{ in ash} = \frac{\text{P\% in ash} \times 3.066}{\text{ash wt (mg)}}$$

The chloride ion concentration was determined by a potentiometric method in which a silver-silver chloride reference electrode and a silver indicating electrode (Fisher Scientific Co.) were used (A.P.H.A., 1975). The electrodes were attached to a Fisher Accumet pH/Ion meter 230A. Three replicate samples of 1.5 g ashed gum were dissolved with 1 ml 50% HNO_3 and diluted to 500 ml. One hundred ml of each sample was placed in a 250 ml beaker with 2.5 ml

concentrated nitric acid. Then the electrode was immersed in the beaker with a magnetic stirrer. Silver nitrate reagent (0.012 N) was added slowly to the beaker, so that the voltage end point could be observed on the millivolt scale. A blank sample was also measured using distilled water. The calculations used to measure the chloride ion concentration in the ashed samples were as follows:

$$Cl^-\text{mg/ml} = \frac{(\text{ml } AgNO_3 - \text{ml blank}) \times \text{normality of titrant} \times 35.45}{\text{ml of used sample}}$$

$$\text{mg } Cl^- \text{ in ash} = \text{mg/ml } Cl^- \times \text{vol. of total ash solution (ml)}$$

$$Cl^-\% = \frac{\text{mg } Cl^- \text{ in ash}}{\text{ash wt (mg)}} \times 100$$

Silica determination was performed by the molybdosilicate method (A.P.H.A., 1975) for three samples of 1.5 g gum ashed at 550°C for 6 hours. The ashed samples were dissolved with 1 ml 6 N HCl and diluted to 500 ml. To 50 ml of each sample solution, 1.0 ml of 50% HCl and 2.0 ml ammonium molybate reagent were added. The solution was mixed and allowed to stand for 5 to 10 minutes. Then 1.5 ml oxalic acid solution was added to the solution and mixed thoroughly. After 2 minutes color development time, the absorbance was read at 510 nm on a Shimadzu double-beam spectrophotometer UV-190.

The standards were prepared by dissolving 4.73 g sodium metasilicate, $Na_2SiO_3 \cdot 9H_2O$, in 1000 ml distilled water (stock solution). One ml of stock solution was diluted to 1000 ml (1 mg SiO_2/L). Five different concentrations (1 through 5 mg/L) of standards were prepared. The silica concentration, SiO_2 mg/L, was measured by the following calculation:

$$Y = a + b\,x$$

$$\text{Absorbance} = (\text{intercept}) + (\text{slope})\ (\text{mg } SiO_2/L)$$

$$\text{mg } SiO_2 \text{ in sample} = \frac{\text{mg/L } SiO_2}{1000} \times \text{vol. of ash solution}$$

$$\%\ SiO_2 \text{ in ash} = \frac{\text{mg } SiO_2 \text{ in sample}}{\text{ash wt (mg)}} \times 100$$

The sulfate determination was performed by the gravimetric method (A.P.H.A., 1975). The ash samples of approximately 1.5 g gum were dissolved in 1 ml 6 N HCl, and diluted to 500 ml. One hundred ml of sample was adjusted to pH 5.0 with 1 N HCl, then an additional 2 ml of the acid was added. The solution was heated to boiling and while stirring gently, warm BaCl solution was added until precipitation appeared to be complete. The solution was digested at 90°C for 2 hours and then filtered through porcelain Gooch crucibles using vacuum. Crucibles and their contents were ignited in a muffle furnace at 800°C for one hour and cooled. The differences in weights before and after ignition showed $BaSO_4$ which was calculated as follows:

$$\text{mg/L } SO_4 = \frac{\text{mg } BaSO_4 \times 411.5}{\text{ml sample}}$$

$$\text{mg } SO_4 \text{ in sample} = \frac{\text{mg/L } SO_4}{1000} \times \text{vol. of ash solution}$$

$$\%\ SO_4 \text{ in ash} = \frac{\text{mg } SO_4 \text{ in sample}}{\text{ash wt (mg)}} \times 100$$

XII. GAS CHROMATOGRAPH ANALYSIS

The compositional analysis of the gum was done using gas chromatography (Crawford, 1980; Mason and Slover, 1971; Birch, 1973). The instrument was a Shimadzu Mini-2 fitted with a 1.4 m x 5 mm (O.D.) glass column packed with 1% SE 30 on Chromosorb Q, 100/120 mesh. The column was conditioned overnight at 275°C then conditioned with Silyl-8 (Pierce) until a stable baseline was established. The carrier gas was N_2 at 30 cc/min., H_2 at 30 cc/min. and air at 300 cc/min. Oven temperature was maintained at 180°C throughout the analysis. The inlet and flame ionization detector were set at 210°C. The injection volume was 1 microliter. Peak areas were measured with a Shimadzu Chromator Pack E-1A electronic integrator. Tri-Sil "Z" (Pierce) was used to derivatize (1 ml/10-15 mg sample) the dried samples before injection.

XIII. STRUCTURAL UNITS IN THE GUM

Three samples were used to determine the structural units in the polysaccharide (Adams, 1965). For determination of alpha-linked subunits, each sample was mixed with 200 ml of water and 20 ml of hydrochloric acid (Sp. gr. 1.125). The mixture was placed in a 500 ml round bottom flask with a condenser attached and refluxed for 2.5 hours. The flasks were cooled, and the solutions were neutralized with 1 N NaOH. The solution was transferred to a 250 ml volumetric flask and made to volume with distilled water. The solution was then filtered by gravity through Whatman filter paper

no. 4, placed in 2 or 3 ml quantities in septum vials, and freeze-dried.

Sugar analyses were done by injecting 1 ml Tri-Sil "Z" reagent into each vial and, after one hour derivatization, the samples were injected into a Shimadzu Gas Chromatograph (GC) Mini-2 for sugar determination. The instrument and conditions were identical as stated previously.

A procedure designed to hydrolyze beta-glycosidal linkages was performed using three 1.5 g samples of dried gum (Adams, 1965). The samples were weighed into 30 ml beakers with 5 ml of 72% sulfuric acid solution which had been cooled to 15°C. The mixture was then stirred thoroughly. The beakers were immersed in a water bath at 30°C for 45 min. and the mixture was stirred at 5 min. intervals. The mixture was then washed into an Erlenmeyer flask with 140 ml water and autoclaved for one hour at 121°C. The samples were cooled and diluted to 250 ml, after neutralizing with 1 N NaOH, and filtered through Whatman no. 4 filter paper. The filtered samples were placed in septum vials in 3 ml quantities and freeze-dried. The dried sample in each vial was derivatized with Tri-Sil "Z" reagent for one hour and then injected into the GC for analysis of glycosidal residues. The instrument and conditions were the same as noted above.

XIV. QUALITATIVE SUGAR ANALYSIS OF THE GUM

The qualitative determination of the sugars in the gum was done by comparison to known sugars. Ten mg samples of several known sugars were derivatized individually with 1 ml Tri-Sil "Z" reagent

for one hour, and then 1 microliter of the sample was injected into the GC. This was done in duplicate for each of the sugars used. The retention times of the different sugars were compared to the retention times of the sugars from the gum sample. Identical retention times indicated correspondence to knowns. Two sugar compounds out of seven could not be identified since the available standard sugars did not match the retention times of the peaks in the sample.

From reviewing the literature (Aspinal, 1970; Bell, 1948; Brown and Lester, 1980; Jeanes et al., 1965; Jeanes, Pittsley, and Senti, 1961) the two peaks were believed to be glucuronic and mannuronic acids. D-Glucuronic acid lactone and mannuronic acid lactone (Sigma), the two closest available compounds, were purchased. Ten mg of each were derivatized with Tri-Sil "Z" and injected into the GC. Since the resulting peaks were not identical to the unknown peaks in the gum samples, salts of glucuronic and mannuronic acids were synthesized. They later were derivatized and injected into the GC for positive identification.

XV. SYNTHESIS AND IDENTIFICATION OF GLUCURONIC ACID

Glucuronic acid was prepared in the form of a salt for qualitative determination using the GC. Glucose and sodium gluconate solutions were made at 6 and 1% concentration, respectively. Twenty-five ml of each solution were mixed. An ion exchange column was packed with anion base resin, 1-x 8 Dowex, and washed with 200 ml distilled water. Fifty ml of the glucose and sodium gluconate

solution was then passed through the column and the column later was washed with 0.1 N HCl. The acidic solution was collected as indicated by pH paper. The acidic solution was neutralized with 1 N NaOH, then 5 ml quantities were added to septum vials and vacuum dried at 60°C. After drying the sample, 1 ml of Tri-Sil "Z" was added to each vial and derivatization was allowed to proceed for 1 hour after which the sample was injected into GC for glucuronic acid identification.

XVI. SYNTHESIS AND IDENTIFICATION OF MANNURONIC ACID

Mannuronic acid was synthesized by means of chloramine-T (Hinton and Macara, 1927) and identified qualitatively with the GC. Twenty-five ml of a 5% mannose solution was made and placed in a 250 ml Erlenmeyer flask with a ground glass stopper. A drop of 1 N NaOH was added to the solution to adjust the pH to 9.0. Then 10 ml of 1% potassium iodine (KI) and 50 ml 0.1 N chloramine-T solutions was added to the Erlenmeyer flask containing the mannose solution. The solution was mixed well and set in a dark place for 110 minutes to oxidize completely.

After oxidation, the solution was passed through an anion resin column packed with 1-x 8 Dowex resin (anion base) which was washed previously with distilled water. The column then was washed with 0.1 N HCl and the acidic solution was collected and neutralized with 1 N NaOH. The neutralized solution was placed in septum vials (5 ml per vial) and they were placed in a vacuum oven. After the samples were close to dryness, they were taken out and dried with nitrogen

gas. The dry sample in each vial was derivatized with 1 ml Tri-Sil "Z" for 1 hour and then injected into the GC for qualitative analysis.

XVII. QUANTITATIVE SUGAR ANALYSIS OF THE GUM

Quantitative sugar analysis of the gum sample was conducted with the same Gas Chromatograph and identical conditions which have been stated previously. Sorbitol was selected as an internal standard with a retention time between the glucuronic and mannuronic acid peaks.

Machine response factors (K) were determined primarily for rhamnose, xylose, mannose, glucose, and galactose with sorbitol as the internal standard. Four different procedures were used to determine the response factors which are outlined below: (1) Rhamnose, xylose, and mannose were weighed and combined in ratios of 2:2:2, 3:3:3, 4:4:4 mg in three separate vials with 3 mg sorbital as the internal standard in each vial. Glucose and galactose also were mixed together (2:2, 3:3, 4:4 mg) in two separate vials each containing 3 mg sorbitol as the internal standard. The mixed sugars were derivatized with Tri-Sil "Z" reagent and injected into the GC in triplicate; (2) Ten mg of rhamnose, xylose, mannose, glucose and galactose with sorbitol as internal standard derivatized separately with 1 ml of Tri-Sil "Z" reagent. Three sample sizes of 200, 300, and 400 ml of each sugar were placed in three separate vials in the presence of 20 ml internal standard (sorbitol). After derivatization, they were injected into the GC three times; (3) this method also was conducted as method 2 with the only difference being the use of 100 mg internal standard (sorbitol) rather than 20 ml; (4) the fourth method was also the same

as method 2, except that the larger sample sizes for each sugar 250, 500, and 750 ml were prepared with 500 ml of sorbitol as the internal standard.

For each sugar combination analyzed, the response factor (K) was calculated by:

$$K = \frac{\text{area sugar x wt. std.}}{\text{area std. x wt. sugar}}$$

The overall K value for the individual sugars was established by averaging the K values found for each sugar concentration in the four methods. The response ratios for rhamnose, xylose, mannose, galactose and glucose were 0.96, 0.88, 0.88, 0.76, 0.87, respectively. The response ratio for glucuronic and mannuronic acids was taken as unity.

The quantification of each sugar in the gum was performed with 2 mg sorbitol as the internal standard using the following calculation (Sawardecker et al., 1965):

$$\text{Sugar wt. (mg)} = \frac{\text{area sugar x wt. std. (mg) x response factor}}{\text{area std.}}$$

The ratio of sugar constituents in the polymer was determined using three freeze-dried gum samples resulting from three separate hydrolyses with 2 mg sorbitol as the internal standard. One microliter of each sample was injected into the GC in duplicate. The results were subjected to analysis of variance including replicate, hydrolyses, and sugars as main variables. Then, the sugar with the lowest mean value was taken as unity and the ratio of other sugars were estimated accordingly.

CHAPTER IV

RESULTS AND DISCUSSION

I. PRODUCTION AND RECOVERY OF THE GUM

Polysaccharide production with the Corynebacterium #98 in a whey substrate was found to be practical at optimum conditions. Since whey disposal is a problem for the dairy industry, it would be ideal to use whey as a nutrient source for the microbial gum production. However, Rodriguez (1980) concluded that whey was not the best medium for polysaccharide (PSC) production using the Corynebacterium #98. On the other hand, the coryneform bacterium in whey substrate fortified with necessary ingredients was found to produce the slimy material in high yield.

With establishment of whey as a suitable medium for the Corynebacterium #98, the optimum conditions were investigated and modified for typifying commercial production of the gum. In production of the gum, the whey culture medium first was made and adjusted for optimum levels of carbon, nitrogen, salt, and pH as indicated in the Materials and Methods Chapter. The formation of the coryneform gum, as indicated in the Review of Literature, is influenced by many environmental and nutritional factors. However, some of these internal and external factors were changed at high levels of production due to modification of the procedures. The following is a discussion of some of the important factors which affected the gum yield at the high level of production.

After 24 hours of growth in tryptic soy broth (TSB), cells were inoculated into 1.5% prepared whey media at a level of 10%. The level of inoculation affected the cell growth and polysaccharide (PSC) production. This phenomenon was due to the crude nitrogen concentration in the whey substrate and also the ratio of surface area to the volume. Despite the report that 15% inoculation was the best level (Rodriguez, 1980) to use, it was observed that at the 10% level of inoculation the bacteria could better utilize the crude nitrogen and oxygen available in the medium. Reports from the literature and preliminary experiments indicated that this coryneform bacterium was very sensitive to nitrogen and oxygen levels in the media. As a result, these two factors, nitrogen and oxygen levels, could limit the production of polysaccharide and cell growth.

Shaking the whey culture medium also facilitated the polysaccharide formation and cell growth since air was made more available to the bacteria throughout the viscous material. Visual comparison between the inoculated whey medium in the incubator with and without shaking indicated that shaking had a direct effect on increasing the viscosity of the medium. The viscosity increased to a great extent when a high ratio of surface area to volume existed.

Inoculation of the prepared whey into the fermentor with active gum producing culture at a rate of 15% affected the final PSC recovery. Preliminary work showed that the inoculation of the fermentation media with viscous whey material reduced the fermentation time. High viscosity at shorter fermentation times was due to

adaptation of the culture to the media. Also high cell concentration reached the stationary phase sooner and started earlier polysaccharide formation, resulting in higher viscosity.

The fermentor provided regular and stable conditions for the cells which convert the regular whey media to a viscous and slimy material. This period of fermentation usually lasted approximately 15 days, after which the whey media became a yellow-orange, viscous, foamy material. The viscosity of the media in the fermentor usually dropped after high PSC productions, and this was influenced by interruption of oxygen into the cells. This theory was supported by increasing the oxygen level at the end of the fermentation time, which resulted in an increase in both the cells and PSC-biomass yield.

The orange pigmented gum production from the Corynebacterium #98 lasted about 25 days with an average yield of 33 g of gum from each batch of 1,500 ml of whey.

II. MICROBIOLOGICAL ASSAY OF THE GUM

It was found that culture contamination was a basic problem during the fermentation period, resulting from air contact. The contaminating microorganism was believed to be Bacillus spp. Carefully performed experimental procedures inhibited contamination which could be controlled by covering the top of the fermentor with sterilized aluminum foil. However, in commercial production, this problem is not of importance since aseptic conditions may be employed. Microbial tests also indicated that the coryneform culture was

usually destroyed with acetone at the time of precipitation, while the contaminants (spore formers) survived through the precipitation. Therefore, aseptic production of the gum would lead to high PSC yield free from contamination.

III. RHEOLOGICAL PROPERTIES OF THE GUM

The effects of gum concentration, salt (NaCl) concentration, pH, and heat on the viscosity of the aqueous gum solutions were determined. The primary analysis of data was performed by analysis of variance (Sokal and Rohlf, 1969) as shown in Table I.

Concentration of the gum gave a highly significant positive effect, with viscosity increasing as the gum concentration increased as shown in Table II. The gum was soluble in hot water; however, it could be dissolved in cold water with high speed agitation for a period of time. Since the gum viscosity increased as the shear rate increased, it is believed that it is non-Newtonian in nature and has shear-thickening or dilatant properties (Glicksman, 1969). In reference to the literature, there are few microbial gums that possess shear-thickening properties (Jeanes, Pittsley, and Senti, 1961; Lilly et al., 1958). The viscosity from 1.5% gum concentration was about 29 centipose at a shear rate of 60 using a Brookfield spindle no. 18. This viscosity might not be as high as expected for other microbial gums (Jeanes, Pittsley, and Senti, 1961; Smiley, 1966); however, such expected viscosity may be obtained at a higher concentration.

The effect of pH was also significant in positively altering viscosity at the specific pH levels used as shown in Table II. This

Table I. Analysis of variance of effects of gum concentration, salt concentration (NaCl), pH, and temperature on viscosity of the gum polymer.

Source	DF	SS	MS	F
C (concentration)	2	4441.18	2220.59	7779.16**
pH	2	2.92	1.46	5.12**
C * pH	4	5.03	1.26	4.40**
S (salt)	2	11.92	5.96	20.88**
C * S	4	10.19	2.55	8.92**
pH * S	4	6.34	1.58	5.55**
T (temperature)	3	1107.71	369.24	1293.51**
C * T	6	1031.12	171.85	602.04**
pH * T	6	0.69	0.12	0.40^{NS}
S * T	6	1.57	0.26	0.92^{NS}
Residual error	68	19.41	0.28	

**Significant at the 0.01 level.

^{NS}Not significant at the 0.05 level.

Table II. Mean values for the coryneform gum viscosity as affected by gum concentration, pH, salt (NaCl), and temperature.

Source	Level	Viscosity[1] (CPS)
Concentration	0.5%	2.9930[a]
"	1.5%	5.7708[b]
"	2.5%	17.7708[c]
pH	5	8.7083[a]
"	6	8.7500[a]
"	7	9.0764[b]
Salt (NaCl)	0.5%	9.2639[a]
"	1.0%	8.8194[b]
"	1.5%	8.4514[c]
Temperature	15°C	13.6111[a]
"	25°C	9.6296[b]
"	35°C	7.1389[c]
"	45°C	5.0000[c]

[1]Means within each variable followed by the same letter are not significantly different at the 0.01 level. For concentration, pH and salt n = 36; for temperature n = 27.

effect was not similar to many microbial gums (Jeanes et al., 1961, 1965) since those gums studied were not affected at the pH levels used. However, the use of a broader pH range might result in greater variation. Therefore, it is important to be concerned with the type of food system in which the gum is to be used.

Salt concentration had a significant, though rather small, negative effect on the viscosity of the aqueous gum solution. The effect of salt on the viscosity of other microbial gum solutions (Jeanes et al., 1961, 1965; Margaritis and Zajic 1978) showed some variation, depending on the particular salt used, the ratio of gum, and salt concentration. Therefore, since sodium chloride was used in this experiment, it can be concluded that the gum might give different rheological reactions with other salts which could affect the viscosity differently.

The effect of temperature upon viscosity also was negative and highly significant. This characteristic seems to be typical for many other microbial gums (Jeanes et al., 1961, 1965).

In determination of rheological properties of the gum, the linear and quadratic trends for each main effect also were considered since food rheology includes the combined physical and/or chemical interactions. The interactions of the main effects were investigated and as shown in Table I, all two-way interactions were significantly different at a significance level of 0.01 and 0.05, except for pH*T and S*T. Furthermore, the significant two-way interactions were subjected to the Multiple Regression Range Test (Barr and Goodnight, 1977) and their trends are shown in Figures III, IV, V, and VI.

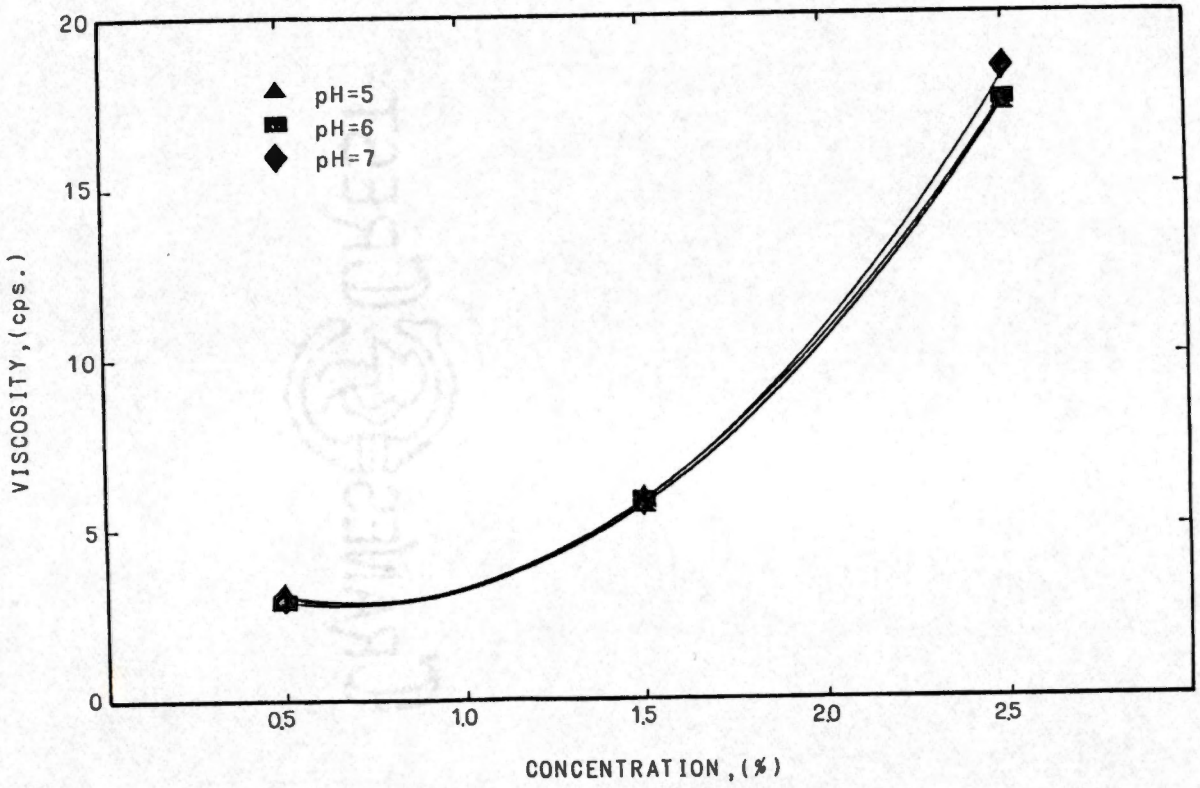


Figure III. The interaction trends between gum concentration and pH levels.

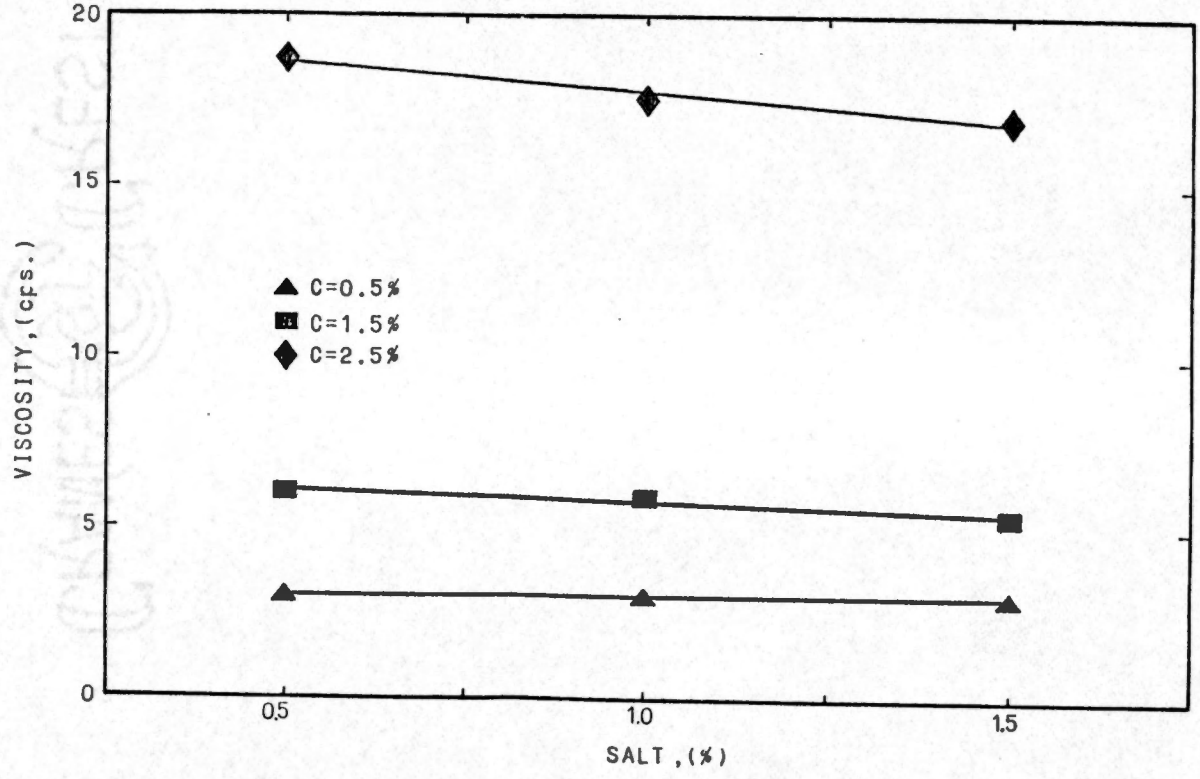


Figure IV. The interaction trends between gum concentration and salt levels.

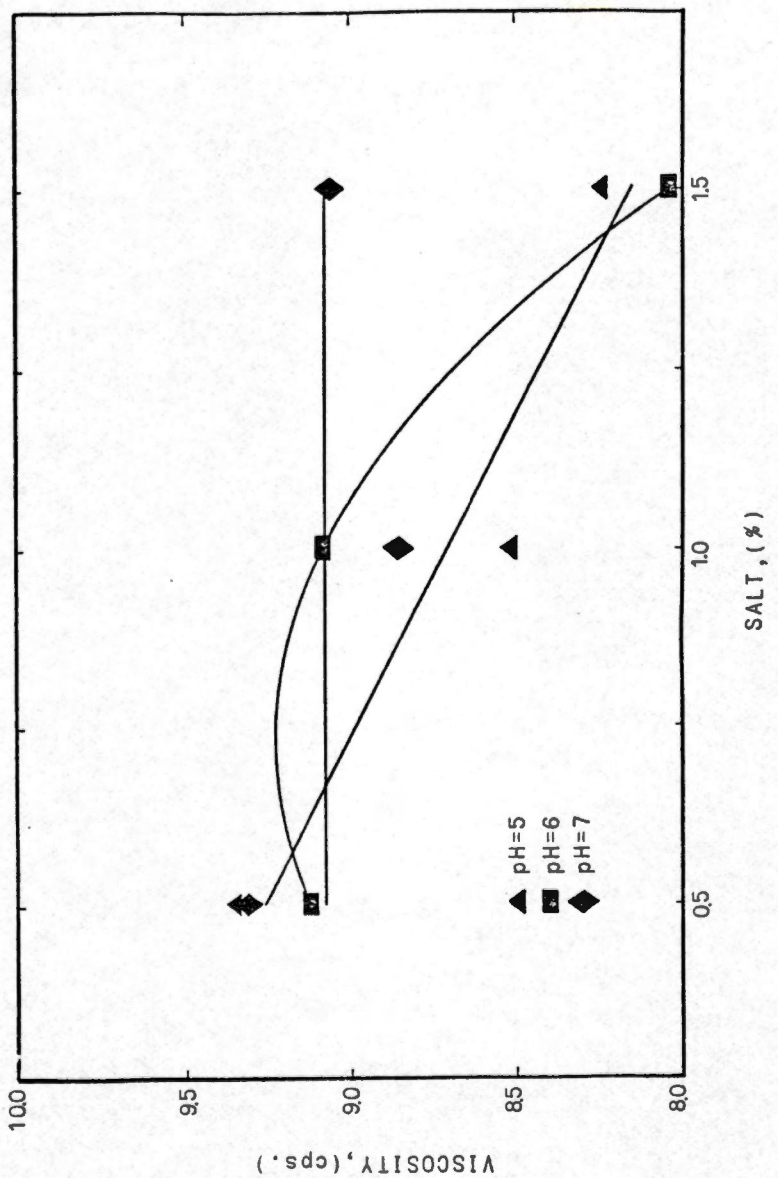


Figure V. The interaction trends between salt levels and pH levels.

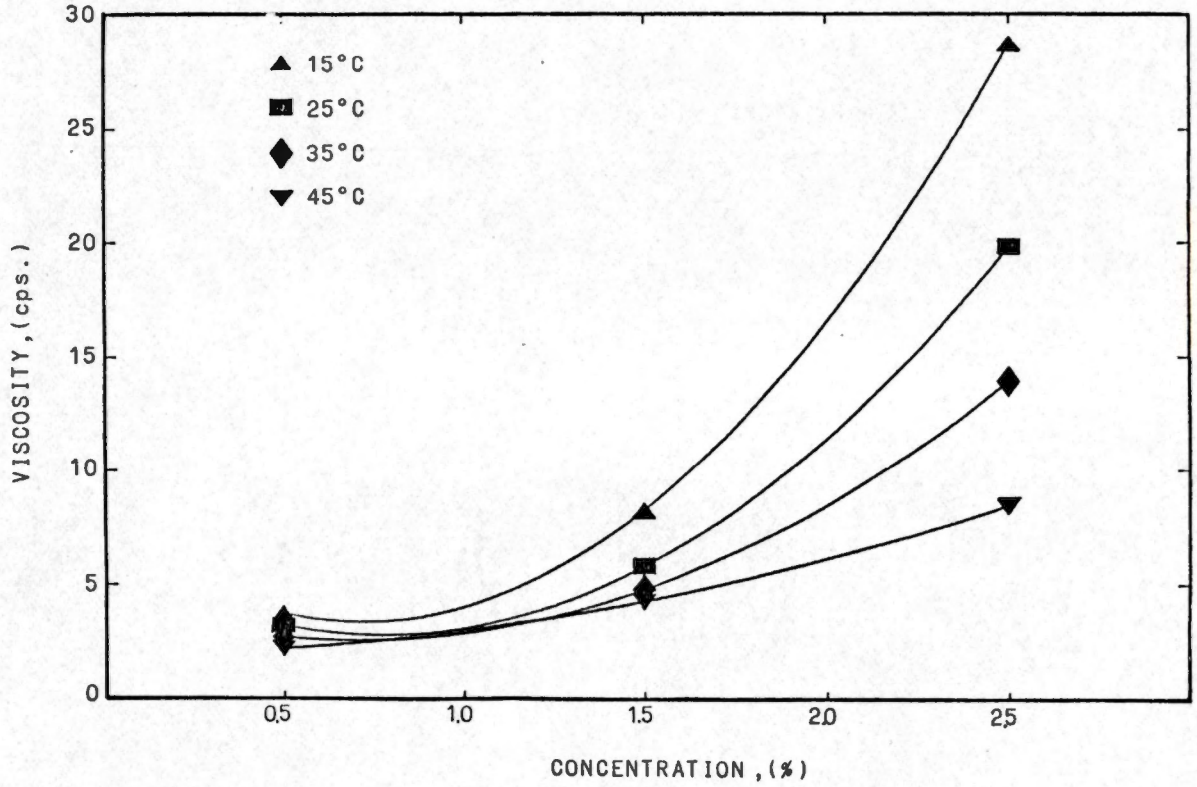


Figure VI. The interaction trends between gum concentration and temperature levels.

Figure III represents the interaction between gum concentration and pH, relative to viscosity. Regardless of the pH, viscosity increased as the gum concentration increased with a linear-quadratic trend.

Figure IV shows the effect of the interaction between gum concentration and salt upon viscosity. It was observed that at low gum concentration there was no change in viscosity as the salt level was increased. However, at a higher gum concentration (1.5 and 2.5%) there was a linear decrease in viscosity as salt level increased.

The interaction effect of pH and salt upon viscosity is illustrated in Figure V, which shows that viscosity decreased linearly with the addition of salt at pH 5. This trend became quadradic as pH increased to 6, while at pH 7 there was no effect on viscosity with the addition of salt.

Figure VI represents the interaction trends of gum concentration and temperature. There was a general linear-quadradic trend at all temperature levels for viscosity, which increased with increasing gum concentrations. However, the viscosity increased to a greater extent as temperature levels decreased.

It can be concluded that since there were significant interactions between main effects (gum concentration, pH, salt and temperature), it is important that these interactions be considered when utilizing the gum in a food system.

IV. PROXIMATE ANALYSIS

Proximate analysis of the gum was performed on the same batch of gum (composite sample) which was used previously for determination of rheological properties. Proximate composition of the gum is presented in Table III. It was found that the gum contained 5.93% moisture, 1.11% fat, 4.06% protein, 29.67% ash, and 59.23% nitrogen free extract.

The fat and protein present in the gum basically was attributed to culture cells existing in the gum, since the cells could not be separated due to their adherence to the gum (Brown and Lester, 1980). Mild sonification could separate part of the polymer from bacteria, but the breakdown of polymer is inevitable (Rodriguez, 1980; Brown and Lester, 1980). Therefore, the commercial production of the gum, including the cells, could be feasible since the culture is killed at the time of precipitation. The literature also did not indicate the significance of the cells in the gum especially when they were not pathogenic and could be killed during the course of preparation (Margarits and Zajic, 1978; Rocks, 1971; Smiley, 1960).

The percentage (29.67) of ash in the gum was due to high mineral content which is common for other microbial gums (Jeanes et al., 1961, 1965). The mineral components were analyzed and will be discussed later.

The major component of the gum is carbohydrate as shown in Table III. The sugar composition of the gum also was subjected to more detailed investigation.

Table III. Proximate compositicn of the gum produced by the Corynebacterium #98.

Components	%[a,b]
Moisture	5.93
Carbohydrate	59.23
Ash	29.67
Protein	4.06
Fat	1.11

[a]Means of three observations.

[b]Values calculated on a freeze-dried basis.

V. MINERAL COMPOSITION OF THE GUM

The high ash content of the gum was investigated through different procedures which established the actual level of each mineral component. The level of each element and procedures used are shown in Table IV.

The high concentration of potassium, 51.52%, indicated that the gum most likely was precipitated in the form of a potassium salt. The high level of potassium was due to saturated potassium chloride which was used to precipitate the gum. The chloride ion concentration at a level of 2.54% also might have resulted from gum contamination with potassium chloride. This supports the above theory of gum precipitation as a potassium salt.

The phosphate concentration in the ash was also high at 3.72% which is common in most microbial gums (Jeanes et al., 1961). This high level of phosphate is believed to be related to the addition of phosphate buffer to the gum sample during the course of gum precipitation.

The sodium content at a level of 5.71% was considered to be chiefly attributed to the whey ingredients and possibly to the addition of an antifoam agent.

The concentration level for other detected elements such as calcium, magnesium, iron, silica, and sulfate were 0.80, 0.51, 0.02, 0.01 and 0.00% of ash, respectively. This indicates that the gum is relatively free from high levels of heavy mineral salts. It can be

Table IV. Mineral components of the coryneform gum with the method of determination.

Component	Method of Measurement	%[a]
Calcium	Atomic Absorption	0.80
Magnesium	Atomic Absorption	0.51
Potassium	Atomic Absorption	51.52
Iron	Atomic Absorption	0.02
Sodium	Flame Emission	5.71
Phosphate	Molybdovanado Phosphate	3.72
Silica	Molybdosilicate	0.01
Sulfate	Gravimetric	0.00
Chloride ion	Potentiometric	2.54

[a]Mean value of triplicate determinations for each item.

concluded that the high levels of potassium, phosphate, sodium, and chloride ion pose no threat since most foods commonly contain higher levels of these materials.

VI. EMULSIFYING CAPACITY

Sharma (1981) reported that the gum polymers are usually responsible for their functionality in oil-water emulsion system. Although gum polymers are not true emulsifiers, their viscous properties generally minimize the dispersed phase coalescence and stabilize the emulsion (Bennett et al., 1968).

The emulsifying capacity for the coryneform gum was established. The average mean value for three observation indicated a value of 17.02 ml oil/g gum. The use of this coryneform gum in any food system is based on the continuous and dispersed phases which require different emulsion stabilizers.

VII. QUALITATIVE AND QUANTITATIVE SUGAR ANALYSIS OF THE GUM

To determine sugar components of the gum qualitatively the gum was hydrolyzed with heat and hydrochloric acid. Two kinds of hydrolysis, alpha and beta linked D-hexoglycan were performed to determine the kind of bonds existing in the gum's structure as well as breakdown of the gum constituents.

The gum hydrolyzed for beta-linked D-hexoglycan was injected into the GC and no peaks or signals were detected. This indicated that there was no beta-linkage between sugar components of the gum.

Conversely, the derivatized samples resulting from alpha-linked D-hexoglycan analysis produced seven peaks with good resolution on the GC, as shown in Figure VII.

To identify the peaks, different standard sugars were derivatized and injected into the GC after injection of the gum sample. The identical retention times enabled positive identification of sugars. Five peaks out of seven were identified using available standard sugars. The identified monosaccharide constituents of the gum were rhamnose, xylose, mannose, galactose and glucose with retention times of 108, 142, 239, 282, and 317 seconds, respectively.

From the literature reviewed (Brown and Lester, 1980; Hendrick 1956; Jeanes et al., 1961, 1965; Sloneker et al., 1964; Sloneker and Jeanes, 1962; Sloneker and Orentos, 1962), it was noted that microbial gum components frequently included glucuronic and mannuronic acids. A gum isolated from Crystococcus laurentii var. flavescens (Jeanes, 1974) contained xylose and mannuronic acid which distinguished this gum from others. Since the gum in the present study also contained xylose, it was believed that mannuronic acid also could be present in the gum. Therefore, the possibility that glucuronic and mannuronic acids produced the two unidentified peaks was investigated.

The two sugar acids, glucuronic and mannuronic acids, are not available in their free form in nature. However, their salts can be synthesized from glucose and mannose, respectively. Glucuronic acid lactone and mannuronic acid lactone, the two closest available compounds, did not produce any peaks with GC analogous to the unknown

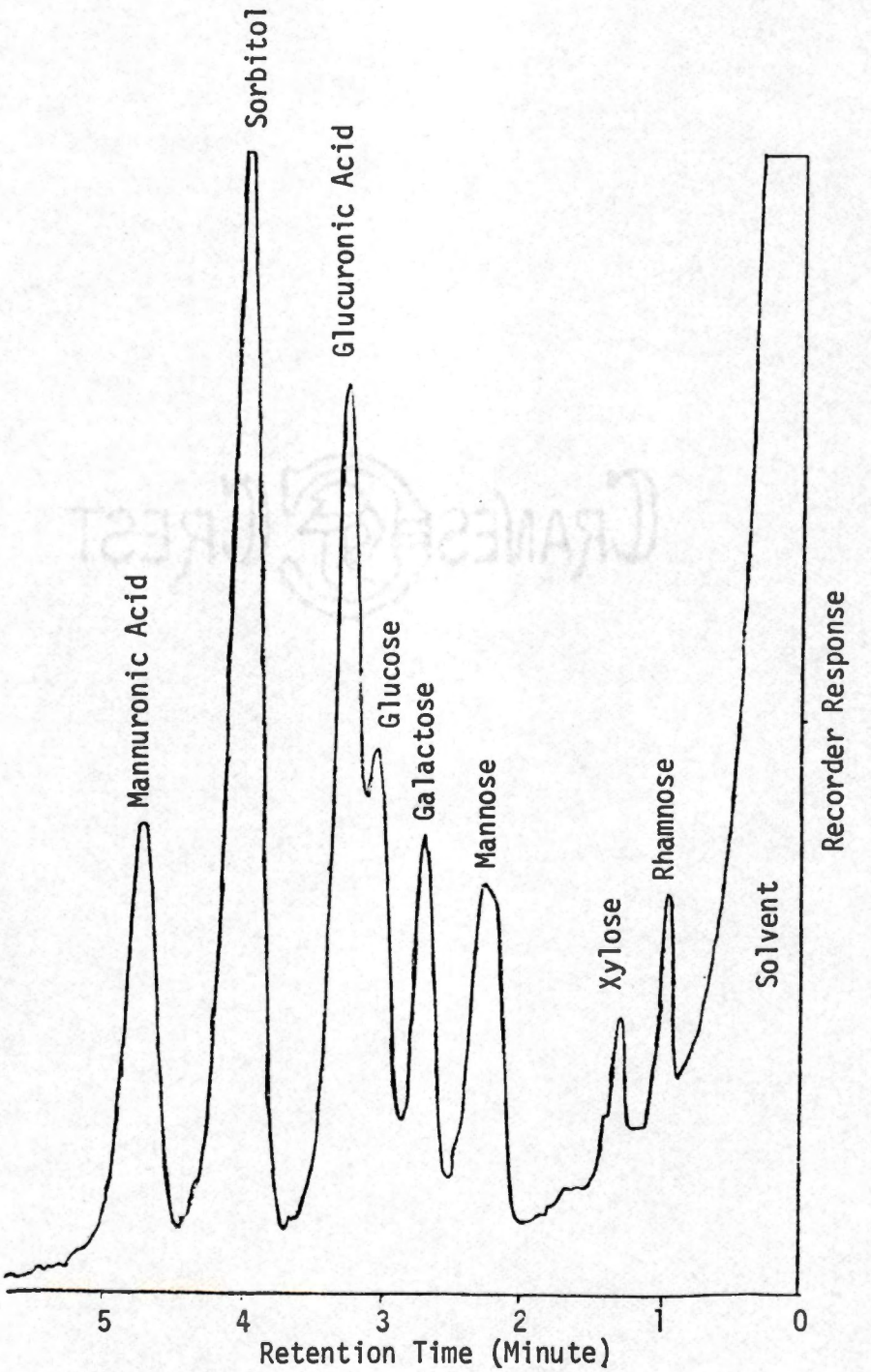


Figure VII. Chromatogram of monosaccharide constituents of the coryneform polymer.

peaks in the gum sample. Therefore, these two sugar acids, glucuronic and mannuronic, were synthesized during the course of experimentation for positive identification.

The sodium salt of glucuronic acid was synthesized from a glucose and sodium gluconate solution which was passed through a resin column (anion base). It is postulated that the carbon in the 6th position in sodium gluconate was oxidized to a carboxyl group, forming sodium glucuronate (Aspinal, 1970; Bell, 1958). This product was derivatized with Tri-Sil "Z" reagent and injected into the GC resulting in a peak identical to that of one of the unknown peaks in the gum sample with a retention time of 339 seconds.

Potassium mannuronate was also synthesized by oxidizing mannose with chloramine T (oxidizing reagent) and potassium iodide. This solution was passed through a resin column (anion base) and potassium mannuronate was collected. The principle of mannuronic acid synthesis is similar to that of glucuronic acid in which the primary alcohol group on the carbon in position 6 of mannose is oxidized to a carboxyl group (Aspinal, 1970; Bell, 1948). The injection of the derivatized potassium mannuronate into the GC resulted in an identical peak with the same retention time as that of the unknown peak in the gum sample. The existence of glucuronic and mannuronic acids in this polysaccharide supports the theory of Jeanes et al. (1961, 1968, 1974) who documented the presence of these sugar acids in many microbial gums.

Although there are other methods (Stacey and Wilson, 1944; Stacy, 1947) for synthesizing glucuronic and mannuronic acids, it is

believed that the established present method is easier and more rapid. It is also considered that the GC can qualitate and quantitate glucuronic and mannuronic acid in gum polymers with greater sensitivity than with thin layer chromatography (TLC) which has been utilized by many researchers in identification of these sugar acids.

The sugar constituents of the polymer also were quantified with sorbitol as an internal standard (Delente and Ladenburg, 1972). The response ratio for rhamnose, xylose, mannose, glucose, and galactose had to be determined by averaging the resulting values from four different procedures, since the machine responses varied from one method to another. The reasons for variable response factors might be attributed to: the properties of the samples which may have caused silicon deposits on the flame ionization detector (FID); reaction of Tri-Sil "Z" reagent with sample (King, 1978); and/or other experimental errors which could not be controlled. The response ratio for glucuronic and mannuronic acids were taken as unity since the established method of synthesis could affect the resulting values.

The amount (mg) of each sugar in the gum samples with 2 mg sorbitol as an internal standard was calculated. The results from three hydrolyzed freeze-dried gum samples (two injections per sample) were subjected to analysis of variance as shown in Table V. Experimental error and isomerization of the sugars (King, 1978) during the investigation contributed to significant differences among the replicates, hydrolyses, and sugar variables. However, the observed peaks from the GC indicated a similar value of each sugar in the replicates and hydrolyses of the samples.

Table V. Analysis of variance of sugars and hydrolyses of the gum samples after injection into the GC.

Source	DF	SS	MS	F
Sugars (S)	6	5.4060	0.9010	5975.01**
Hydrolyses (H)	2	0.0073	0.0036	243.05**
Replicate (R)	1	0.0044	0.0044	29.19**
R * H	2	0.0025	0.0013	8.26**
R * S	6	0.0109	0.0018	12.10**
H * S	12	0.1056	0.0088	58.36**
Residual error	12	0.0018	0.0002	

**Significant at the 0.01 level.

The mean value for each sugar also was calculated and the sugar with the lowest mean value, xylose, was taken as unity and the ratios of other sugars were extrapolated accordingly. It was found that the polymer was composed of rhamnose, xylose, mannose, galactose, glucose, glucuronic acid, and mannuronic acid in the ratio of 2:1:6:6:7:16:11. The results indicated that the sugar constituents in this gum are the same type of sugars found in other microbial gums (Hendrick, 1956). However, the large number of sugars found in this gum may have resulted from the GC which detected with high sensitivity.

CHAPTER V

SUMMARY

The objective of this investigation was to optimize the production and recovery of the gum produced by Corynebacterium #98 grown in sweet whey substrate, and to characterize the gum's rheology and its composition.

The disposal of whey from cheese plants continues to be a problem in the cheese industry, and utilization of whey constituents, such as lactose, by selected microorganisms would be beneficial in the reduction of this disposal problem.

The Corynebacterium #98 was found to produce an orange pigmented exopolysaccharide in high yield when grown at optimum conditions in a cheese whey substrate. Sweet whey was reconstituted and deproteinized by lowering the pH to 5.0 and autoclaving (121°C/15 min.). The whey solution was enriched with $MgSO_4$ (0.2%), $FeSO_4$ (4.0%), lactose (3.0%), and pH was adjusted to 7.0. The Corynebacterium #98 was cultivated in tryptic soy broth (TSB) for 24 hours and then inoculated into 1.5% whey substrate. This was incubated at 30°C in a shaker water bath for 7 days. The viscous culture medium was inoculated later at a rate of 15% into a 2 liter multigen fermentor (New Brunswick Scientific) with agitation of 200 rpm, air flow of 1090 ml/min., and temperature of 30°C.

After 15 days the orange viscous material was precipitated by a mixture of acetone (4 ml/ml), phosphate buffer (pH 7.0, 0.02 ml/ml),

and saturated KCl (0.02 ml/ml). The gum was precipitated, freeze-dried, and stored.

Rheological properties of the gum were evaluated by observing the effects of gum concentration (0.5, 1.5, 2.5%), temperature (15, 25, 35, 45°C), pH (5.0, 6.0, 7.0), and ion strength of NaCl (0.5, 1.0, 1.5%) upon viscosity. The rheology of the gum indicated that it is a shear thickening (dilatant) polysaccharide. The viscosity of 1.5% gum concentration was about 29 (cps) at a shear rate of 60 using a Brookfield viscometer with spindle no. 18. The viscosity increased with increasing gum concentration and pH, and decreased with increasing salt (NaCl) concentration and temperature. The emulsifying capacity for Corynebacterium #98 was established and 1 g of gum emulsified 17.02 ml of vegetable oil.

The proximate composition and mineral components of the gum were investigated. The proximate analysis indicated that the freeze-dried gum consisted of 5.93% moisture, 1.11% ether extract, 4.06% protein, 29.67% ash, and 59.23% nitrogen free extract. Mineral determination of the ash showed high potassium and sodium levels of 51.52%, and 5.71%, respectively. Lower levels of calcium (0.8%), magnesium (0.51%) iron (0.02%), phosphate (3.72%), silica (0.01%), and chloride ion (2.54%) also were detected.

The gum sample was hydrolyzed with HCl under reflux and freeze-dried. Sugar composition was determined by injecting the hydrolyzed sample, derivatized with Tri-Sil "Z" (Pierce), into a gas chromatograph (Shimadzu mini-2) containing a 1.3 meter glass column

packed with 1% SE 30. The monosaccharide constituents of the gum as determined by gas chromatography were rhamnose, xylose, mannose, glucose, galactose, glucuronic acid, and mannuronic acid in the ratio of 2:1:6:6:7:16:11, respectively.

The overall evaluation of the gum indicates that the gum can be produced commercially in high yield from whey substrate. Therefore, conversion of whey substrate into potentially useful products, such as microbial gum, may provide one solution to the whey disposal problem.

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APPENDICES

APPENDIX A

Tryptic Soy Broth
(Fisher)

Formula (g./Liter)

Tryptone	17.0
Soy peptone bacteriological	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Glucose	2.5
pH	7.3

Preparation:

Suspend 30 grams in 1000 ml of distilled water. Mix thoroughly and heat to boiling to dissolve completely. Dispense as desired and sterilize in the autoclave at 121°C for 15 minutes.

Brain Heart Infusion Agar
(Pfizer)

Formula (g./Liter)

Calf brain and beef heart infusion	8.75
Pfizer peptone "M"	15.0
Yeast autolysate	3.75
Sodium chloride	5.0
Dextrose	2.0
Disodium phosphate	2.5
Pfizer agar	15.0
pH	7.4

Preparation:

Suspend 52.0 grams in 1000 ml of distilled water. Heat to boiling with frequent stirring to dissolve medium completely. Dispense as desired and sterilize at 121°C (15 psi) for 15 minutes.

APPENDIX B

Table VI. Biochemical reactions of Corynebacterium #98.

Test	Reaction
Indole	-
Methyl Red test	-
Voges-Proskauer test	-
Citrate	-
H_2S production	-
Nitrate reduction	v
Hemolysis	-
Bile salts tolerance	+
TTC tolerance	+
Gelatin liquefaction	-
Esculin hydrolyzed	+
Starch hydrolyzed	-
Urease	-
Phenylalanine	-
Oxidase	-
Catalase	-

Note: + = positive reaction; - = negative reaction; v = variable (Rodriguez, 1980).

Table VII. Utilization of acids as sole carbon source by Corynebacterium #98 in nutrient broth.

Carbon Source	Result
Acetate	-
Benzoate	0
Citrate	0
Gluconate	+
Lactate	+
Malonate	0
Propionate	+
Succinate	+
Tartrate	+

Note: + = utilization of the acid; - = growth only; 0 = no growth (Rodriguez, 1980).

Table VIII. Utilization of carbohydrates by Corynebacterium #98 in nutrient broth.

Carbon Source	Result
Adonitol	-
Aesculin	+
Arabinose	v
Cellobiose	v
Dulcitol	-
Erythritol	-
Fructose	A
Galactose	-
Glucose	A
Glycerol	-
Inositol	-
Inulin	-
Lactose	-
Maltose	v
Mannitol	A
Mannose	A
Melezitose	v
Melibiose	-
Me-D-glucoside	-
Raffinose	v
Ribose	-
Saliscin	-
Sorbitol	-
Sucrose	A
Trehalose	-
Xylose	v

Note: A = acid production; + = growth only; - = no growth; v = variable reaction (Rodriguez, 1980).

Table IX. pH tolerance of Corynebacterium #98.

pH	Result
4.5	-
5.0	+/-
5.5	+
6.0	+
6.5	+
7.0	+
8.0	+
8.5	+
9.0	-

Note: + = growth; - = no growth; +/- = poor growth (Rodriguez, 1980).

Table X. Salt tolerance of Corynebacterium #98.

% NaCl	Result
0	+
3	+
5	-
7	-
9	-

Note: + = growth; - = no growth (Rodriguez, 1980).

VITA

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