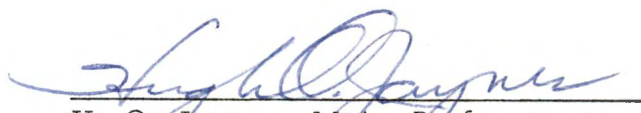
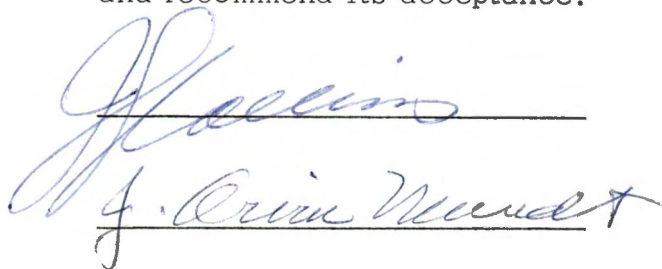


To the Graduate Council:

I am submitting herewith a thesis written by Lakshma O. Rao entitled "Production of Phosphomannan from Cheddar Cheese Whey by Hansenula holstii." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.


H. O. Jaynes, Major Professor

We have read this thesis
and recommend its acceptance:


J. Crim Maudet

Accepted for the Council:


Vice Chancellor
Graduate Studies and Research

Thesis

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PRODUCTION OF PHOSPHOMANNAN FROM CHEDDAR
CHEESE WHEY BY HANSENULA HOLSTII

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Lakshma O. Rao

August 1977

1228577

ACKNOWLEDGMENTS

The author wishes to express sincere gratitude and appreciation to his major professor, Dr. H. O. Jaynes for his valuable advice, guidance, and encouragement throughout the graduate program and for his assistance in planning, conducting, and reporting the present study. Sincere appreciation and thanks are extended to Dr. J. L. Collins and Dr. J. O. Mundt for their advice and assistance in serving as members of the Graduate Committee and for their helpful suggestions.

The author wishes to thank the faculty and staff of the Department of Food Technology and Science, especially to Dr. J. T. Miles, Department Head and Dr. W. W. Overcast for their help and advice during the study. Also thanks to Dr. William Sanders for his assistance in statistical analysis and Mrs. Ann Helms for her assistance and help throughout the study and to Jo M. Hodge for typing the thesis.

A special thanks to his wife Aruna for her endurance, encouragement, support and love and son Srinadh for his patience and understanding during the author's graduate study.

Sincere appreciation is extended to his uncle Mr. Muralidhar Rao for his inspiration, encouragement and guidance which created a positive influence on the author's life, to his brother Dr. Prabhaker Rao for his sacrifice, help and assistance, and to his special friend Dr. V. R. Charya for his assistance and help for the author's education in the United States.

Finally the author wishes to express his gratitude and appreciation to his deceased parents Mr. and Mrs. Narasimha Rao for their sacrifice, love, guidance and support throughout his life and dedicates this graduate thesis to them.

ABSTRACT

The objectives of this study were to produce and isolate phosphomannan gum in whey using the yeast Hansenula holstii NRRL Y-2448 and to study its rheological properties.

A 5 percent inoculum of Hansenula holstii was grown in hydrolyzed whey for 96 hours at 23°C. under continuous agitation. The gum was extracted by ethanol precipitation from cell free medium followed by air drying and lyophilization.

The glucose concentration and protein concentration of whey were determined. Alterations in whey substrate were made in regard to their salt content. Polymer from different batches of whey and control medium was examined for its yield and rheological properties. It was observed that the salts of magnesium, manganese and iron did not have any influence on either the yield or the viscosity but the protein concentration influenced solubility and viscosity of the gum. It was observed that the hydrolyzed whey was a suitable medium to support the growth of yeast Hansenula holstii and its production of polymer phosphomannan.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	3
	The Role of Microorganisms in Foods	3
	Nature and Occurrence of Microbial Gums	4
	Some Gum Producing Organisms and Their Products	6
	Characteristics of <u>Hansenula holstii</u>	9
	Phosphomannan Gum	11
	Whey Utilization	13
III.	MATERIALS AND METHODS	16
	Materials	16
	Culture Preparation	16
	Whey Substrate	17
	Glucose Determination of Whey Substrate	17
	Protein Assay	20
	Control Medium	20
	Inoculation and Fermentation	20
	Recovery of the Gum	21
	Examination of the Gum	21
	Analysis of Data	22
IV.	RESULTS AND DISCUSSION	23
	Composition of Whey	23
	Recovery of Phosphomannan.	23
	Yield of the Gum	27
	Rheological Properties of the Gum	30
V.	SUMMARY	35
	REFERENCES	37
	VITA	43

LIST OF TABLES

TABLE		PAGE
I.	Composition of Dried Cheese Whey	15
II.	Substrate Variations	18
III.	Glucose Content of Whey Substrates	24
IV.	Protein Content of Whey Substrates	25
V.	Phosphomannan Yield from Nine Media	28
VI.	Analysis of Variance on Phosphomannan Yields from Nine Media	29
VII.	Phosphomannan Yield from Nine Media Based on Available Glucose	31
VIII.	Apparent Viscosity Measurements on Gum Solutions . . .	32

CHAPTER I

INTRODUCTION

The polysaccharide hydrocolloids traditionally used for many purposes are obtained from plants. A newly developing source for these hydrocolloids is based on the biosynthetic capability of non-pathogenic microorganisms. The polysaccharide hydrocolloids produced by microorganisms are comparable to plant gums in bringing about physical changes such as free dispersion and increase in viscosity. Unlike plant gums, production of microbial gums do not require extensive labor; thus large amounts of microbial gums can be produced at reasonably low cost and can be successfully introduced in place of plant gums.

Hansenula holstii, which was identified in 1960 by Wickerham (50), is capable of producing a polymer using glucose as a carbon source in addition to phosphate. It is a non-pathogenic yeast usually found on pine trees. The polymer producing strains of Hansenula holstii were specially developed by the Northern Regional Research Laboratories by crossing two of the originally isolated strains.

Whey, which is a by-product in the cheese industry, is usually disposed of through sewage plants. It is the liquid part after separating the cheese and its nutrient content is high. However, its utilization has not been achieved completely. Presently, many investigators are involved in the recovery of protein from whey but not much work has been reported on the utilization of its carbohydrate part which is lactose. Lactose is not easily digestible disaccharide. Even though some

microorganisms do hydrolyze lactose, very few of them polymerize any of its monosaccharide constituents.

The objective of this study was to achieve an enzymatic hydrolysis of lactose into its monosaccharide constituents, glucose and galactose, and polymerization of glucose by using the yeast Hansenula holstii NRRL Y - 2448.

CHAPTER II

REVIEW OF LITERATURE

I. THE ROLE OF MICROORGANISMS IN FOODS

Microorganisms have an important role in foods and food constituents; this could be either in improving the quality of food or in the complete destruction of a food product. One of the important phenomenon that the microorganisms carry out that has a significant impact on food products is fermentation. The use of microbial fermentation in foods dates as far back as prehistoric man. He used this technique without even knowing what he was doing, or without the knowledge of the existence of microorganisms. He thought that it was magic. Some of the examples of the microbial fermentation early man used were the aging of meat and the manufacture of alcohol. In China and Japan man made soy sauce long ago by fermenting soy beans. For centuries the Balkan people in Europe used fermented milk or yogurt. In central Asia nomadic tribesmen made an alcoholic beverage called "Kumiss " from fermented mare's or camel's milk. In some form or other bread was made almost every where in the world (19).

Perhaps the first man to suggest the role of microorganisms in spoilage of foods was Kircher (22). In 1658 he examined the decaying bodies of meat, milk and other products and indicated the presence of organisms which he called "worms," which were invisible to the naked eye. However, some detailed information about the presence and the role of microorganisms was obtained from Pasteur's work in 1873 (22).

Continued investigation has extended man's knowledge of microorganisms which have a great effect in our day to day life. With the identification of microbial polysaccharides, a new avenue was opened whereby microorganisms can serve in the food industry.

II. NATURE AND OCCURRENCE OF MICROBIAL GUMS

Gums are amorphous substances of high molecular weight occurring widely in the plant kingdom. They are mostly hydrophilic polysaccharides or their derivatives and are characterized by dispersability in cold or hot water leading to the production of viscous dispersions or solutions and in some cases gel formation (11, 31). In recent years the term gums has been extended to include materials of microbial origin and synthetic polymers produced in the laboratory (2). The newly developing sources of polysaccharides are based on the biosynthetic capability of non-pathogenic microorganisms (27). These exocellular secretions of the microorganisms have an important function. It is considered that these secretions inhibit the action of bacteriophages in destroying the cells (7). Based on origin the microbial polysaccharides are of two types: (a) capsules, that form the integral part of the cell wall, and (b) slimes, that accumulate outside the cell wall (35).

A major factor in determining the occurrence of the extracellular microbial polysaccharides is the availability of carbon-containing material such as carbohydrates or hydrocarbons under conditions that permit microbial growth. The structure of the microbial gum is determined by the nature of the substrate material. Based on the structure and the

components the microbial polysaccharides have been divided into two groups (19, 25). These include homopolymers which contain one kind of polymeric units such as dextran and heteropolymers which contain several kinds of polymeric units such as phosphomannan.

In the laboratory production of microbial gums the culture medium and the conditions follow the same general pattern. The medium always contains materials from the following sources (25).

Commercial grade glucose or crude enzymatic hydrolyzates of such cereal grain as corn, wheat, and sorghum serve as carbohydrate sources. In some cases starch or sucrose may serve instead of glucose.

Organic nitrogen and growth factors are provided by yeast products such as hydrolyzates or autolyzates, by-product materials such as corn steep liquor, casein hydrolyzates, and distillers dried solubles (from production of alcoholic beverages). These grain hydrolyzates serve as protein sources and provide growth factors.

Inorganic materials come from substances such as phosphates and potassium salts, which serve as buffering agents. Metal ions such as magnesium, manganese and iron may also be needed in the growth medium.

 Other factors include the culture medium, pH, temperature, amount of oxygen, relative volume of inoculum, type and degree of agitation and length of fermentation (27).

Several important factors are directing the attention of the food industry towards the microbial polysaccharides. These include:

a. Successful performance of several of these microbial products based on their distinctive properties.

b. General suitability for ingestion without adverse effects because constituent natural residues and the products of hydrolysis of glycosidic linkages permit either digestion and metabolism or inertness and non-caloric effect.

c. Economic feasibility resulting from extracellular occurrence of these biopolymers and their production in good yield from low cost substrates by fermentation procedures.

d. Need to supplement supplies of natural plant gums.

e. Various advantages of domestic products over foreign imports (27).

III. SOME GUM PRODUCING ORGANISMS AND THEIR PRODUCTS

The Northern Regional Research Laboratory has been engaged in the development and production of extracellular microbial gums as part of a continuing program to increase the industrial uses of cereal grains (40). Dextran is one of the most extensively studied polymers. One of the main reasons for its intensive study was its use as a blood volume extender in World War II (43). Dextran was first recognized as a nuisance material blocking the pipelines in the sugar cane industry in the sugar refineries (19). It is a glucose polymer.

Dextran is synthesized from sucrose by the non-pathogenic bacteria Leuconostoc mesenteroides and Leuconostoc dextranicum. Certain species of Streptococcus and Lactobacillus also produce dextran using glucose as a carbon source. In many strains the enzyme system for dextran is found in cell-free extracts. This has an added advantage in its

successful production (24). The dextran now produced in United States, Canada, Sweden and elsewhere is synthesized from sucrose by the extracellular enzyme dextran sucrose obtained from cell-free culture filtrates of Leuconostoc mesenteroides, strain NRRL B-512(F) (27). Partial depolymerization of this "native" dextran which has a molecular weight of 30 - 90 million resulted in the formation of lower molecular weight substances which are of pharmaceutical and industrial importance (24).

Dextrans of average molecular weight below 100,000 were included among GRAS products, but were deleted in 1973 because of disuse (12, 16). Dextran B-512(F) dissolved readily in water to give a clear solution which was stable to heat, freezing, and thawing. Viscosity increased with concentration in a linear fashion. A 15 percent dextran solution showed viscous characteristics similar to that of 1 percent locust bean gum (37). These similarities were also evident in the mouthfeel of these two gums (45). A 5 percent dextran solution showed Newtonian flow (37).

Xanthan is another microbial gum and is produced by Xanthomonas campestris NRRL B-1459. It is a polyanionic heteropolysaccharide which contains two moles of glucuronic acid and 0.6 moles of pyruvic acid per mole of anionic polymer with a molecular weight of 5 to 10 million (27). In the neutral state its constituents include D-glucose, D-mannose, and D-glucuronic acid in a 3:3:1 ratio (19). The United States Food and Drug Administration approved xanthan gum as a food additive, where its use is not precluded by standards of identity (13). It is used as an

optional emulsifying and stabilizing ingredient in French dressing (14) and in certain cheese and cheese products (15).

Fundamental characteristics of xanthan gum provide the basis for its numerous applications (32). Its homogenous aqueous dispersions have high viscosities at low concentrations, while at high concentrations its property of molecular self interaction and weak gelatinization is manifested. In dilute solutions its viscosity is much less affected by salts, pH and heat than that of plant polyelectrolytes. Its dispersions are pseudoplastic with a high yield value and thin readily with shear (27). Xanthan dispersions have excellent flow properties, mouthfeel and suspending ability (44, 45). Its formation of cohesive flexible films would contribute favorably to its role in emulsification and foam stabilization (23, 26) and possible utilization in coating and encapsulation (6, 32). The concentrations required for xanthan proved to be less and the performance to be superior than the substances used before in bread and French type dressing (3).

Arthrobacter viscosus NRRL B-1973 is another bacterium capable of producing extracellular microbial polymers using glucose as the carbon source (10). It produces a heteropolymer composed of D-glucose, D-galactose and D-manuronic acid (as the potassium salt) in a 1:1:1 ratio (33, 41). Also present is 25 percent of O-acetyl groups, but unlike xanthan gum, it does not contain pyruvic acid (42). The presence of D-manuronic acid is unique in an extracellular polysaccharide from a non-pathogenic bacterium. However, it is a component of the secretions from pathogenic bacteria (28). The polymer is gelatinous, fibrous and somewhat cohesive when first precipitated

from crude culture liquor. The lyophilized material is a white and sponge-like substance. Aqueous solutions of the polysaccharide were found to be very viscous; a 1 percent solution gave a viscosity of 10,000 centipoise (17). The curve of shear rate versus torque measurement indicated that the dispersions of the native polysaccharide had plastic rheological characteristics. After the yield value was exceeded, resistance to increasing rate of shear leveled off rapidly. Potassium (0.4 percent) and calcium (0.1 percent) chloride increased the viscosity of a 1 percent polysaccharide B-1973 solution by 80 percent. The viscosity usually remained constant over a pH range of 4 - 11 and the polymer withstood temperatures up to 98°C (28).

IV. CHARACTERISTICS OF Hansenula holstii

Hansenula holstii was first reported by Wickerham in 1960 to be very significant in the early evolution of heterothallic species of the genus Hansenula (50). Hansenula holstii is a heterothallic yeast which produces hyphae, while its homothallic counterpart Hansenula capsulata does not produce hyphae. Each of the two species gave rise to two phylogenetic lines in which the dominant evolutionary characteristic was increasing tendency of each succeeding species to live as a mixture of haploid and diploid cells. Hansenula holstii is commonly found in frass of a number of genera of coniferous trees (50). The trees that are successfully attacked are generally old, unhealthy, or felled; the sap flow in healthy young trees is sufficient to overcome infestation. The gum produced by the yeast causes adherence of the yeast cells to bark beetles that transport the yeast from one tree to another (51). Because

this yeast is heterothallic and almost exclusively haploid, ascospores are never produced by isolates picked from a single colony. Hansenula holstii, like other frass yeasts, produces a small percentage of vegetative cells which usually bear one long, narrow tapering appendage. The appendage may be many times longer than the cell. However it is not observed in all cultures. It is believed that the appendage serves as a conjugation tube, but this theory lacks any supportive evidence (50, 51).

The colonies of Hansenula holstii are usually mucoid, more watery than viscous, often with a fringe of hyphae that develops in the aged colonies. Pseudohyphae and true hyphae up to 2.5 mm are produced and well differentiated blastospores are formed singly or in pairs or as short chains on the hyphae. The blastospores are usually ellipsoidal, short, cylindrical or occasionally spherical or ovoidal and many have long, tapering ends. Sexually active strains are seldom found on pinus, whereas these are very common on prunus hosts. Bisexual diploid strains have been isolated only from laboratory cultures. These are not found in nature. Ascospores of conjugated haploid strains are hat-shaped, 2 to 2.5 μ in diameter and the maximum number is two per ascus (50).

Hansenula holstii causes gaseous fermentation in glucose. It seldom ferments galactose, maltose, sucrose, lactose and raffinose. Carbon compounds assimilated by all strains are glucose, galactose, maltose, sucrose, cellobiose, ethylalcohol, glycerol, pyruvate, succinate and citrate. Lactose, melibiose, inulin, inositol are not assimilated. Nitrate is not assimilated, and starch is not synthesized.

An exogenous supply of vitamins is required for growth. The sexually potent strain NRRL Y-2155 is designated as the type strain (50).

Hansenula holstii NRRL Y-2448 was developed by mating Hansenula holstii NRRL Y-2154, which is a constantly mucoid and haploid strain, with Hansenula holstii NRRL Y-2155 which is a butyrous strain exhibiting mucoid tendencies (1). The resultant hybrid strain NRRL Y-2448 is a diploid, forming mucoid colonies like Y-2154 while producing larger cells than the parent strains. The reason Hansenula holstii was studied intensively was because of its capability of producing an exocellular polymer phosphomannan (1).

V. PHOSPHOMANNAN GUM

Phosphomannan is a white granular material composed of D-mannose, phosphate and potassium in a ratio of 5:1:1 when isolated as a potassium salt (29). The average mannose to phosphorus molar ratio is 5:7 (40). Phosphomannan is possibly the first polysaccharide isolated to contain a significant amount of phosphate produced by a non-pathogenic yeast (29). The degree of phosphorylation is directly attributed to the primitiveness of the yeast. The mannose-phosphate molar ratio of Hansenula holstii Y-2448 is indicated to contain higher amounts of phosphate than other species of Hansenula and some advanced Saccharomyces species. Hence it was considered that Hansenula holstii was the most primitive yeast. As the species evolved, it has undergone various morphological and biochemical changes, by which it formed large diploid colonies, attained the capability to ferment various sugars and to synthesize vitamins. The organisms

independent of bark beetles in the propagation of the species (51). An inverse relationship was found between the evolutionary status of the organism and the degree of phosphorylation of its phosphomannan (40). In most every instance 6 percent glucose was used in laboratory synthesis of phosphomannan as a carbon source (1, 40). The polymer yield was 50 percent of the glucose supplied when glucose content was 5 - 6 percent (43).

The periodate oxidation of phosphomannan showed that 20 percent of its mannose units were linked to carbon 1 of the main chain, 33 percent were linked to C - 1 and C - 2 and 47 percent were linked to C - 1 and C - 3 of the main chain (30). Guanosine diphosphate was considered to be the precursor for phosphomannan (9). It has five moles of alpha-D-mannopyranose units per mole of monopotassium orthrophosphate (29).

Phosphomannan, which is an anionic polysaccharide, hydrated rapidly in water at ordinary temperatures to form greatly swollen masses, which dispersed into clear solutions. The length of time required to reach the maximum viscosity was influenced by the density of the solid particles and the extent of agitation, but this could be achieved in an hour (25).

Phosphomannan formed a brilliantly clear solution in water; in a concentration of 0.25 percent it showed a soft gel-like aspect (29). These solutions, even though biodegradable, were exceptionally resistant to spontaneous microbial action (27). The polysaccharide dissolved in 10 percent ethanol formamide, ethanolamine, dimethyl sulfoxide and 10 percent aqueous solution of tetra ethyl ammonium hydroxide (25).

Viscosity increased linearly to a maximum at 1.5 percent, then decreased to a minimum at 3 percent. Between 3 and 5 percent, viscosity increased nonlinearly (29).

Although phosphomannan is still in the developmental stage, it has been suggested for use in food processing and related industries as a thickening, stabilizing, dispersing, and suspending agent (20). In low concentration it has a profound beneficial effect as a stabilizer in beer and other carbonated malt beverages (39). Phosphomannan contributes to a pleasing mouthfeel in liquid products (45).

VI. WHEY UTILIZATION

Whey is the serum or watery part of milk that separates from the curd in the process of cheesemaking. It is the largest by-product of the U. S. dairy industry. It is now creating an impact on economic and environmental affairs. The magnitude of the problem to the dairy industry caused by whey is indicated by the statistics. About 29 billion pounds of whey was produced in 1972. The projections for 1980 are somewhere between 36 billion and 41 billion pounds (21).

Since the problem has a multifold effect on the environment, the federal government became involved. The Water Quality Act of 1965 provided for state water quality standards, and it established the Federal Water Pollution Control Administration, which is now called the Federal Water Quality Control Administration (FWQCA) (18). The FWQCA established research and development programs for control of water pollution by the dairy industry (34). The diversified concern over whey-related problems resulted in the Organization of Whey Utilization

Conferences in 1970, 1972 and 1974. Several aspects of whey utilization were recommended in those conferences. In 1969 Weisberg and Goldsmith proposed the solution to the whey pollution control through the beneficial utilization of whey (49).

Whey has a reasonably high nutrient value, with an energy yield of 350 calories per 100 grams. The composition of dried whey is given in Table I (47).

Considerable effort has been made by food processors to develop and perfect processes to fractionate the protein, mineral and lactose components in order to produce an undenatured whey protein concentrate (WPC) suitable for use in a variety of food applications (36).

Whey can make a valuable contribution in food processing. Lactose, the major component, is responsible for most of the flavor intensification observed when whey or lactose is added to many food formulas. The high lactose content seems to enhance food flavors in the same manner as monosodium glutamate (48). It also contributes to the improvement of mouthfeel in liquid products such as skimmilk (45). Whey acts as a tenderizer, producing tender crispiness in pie and pizza crust. Purified lactose is used in many pharmaceuticals (48).

The whey utilization conferences intensified efforts in the utilization of whey. The integrated efforts of FWQCA and EPA may certainly contribute to the development of new products and new processes in whey utilization.

TABLE I
COMPOSITION OF DRIED CHEESE WHEY (47)

<u>Component</u>	<u>Percent</u>
Protein	12.9
Fat	1.1
Ash	8.0
Water	4.5
Lactose	73.5

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

One lot of dried cheddar cheese (sweet) whey was obtained from Kraftco Corporation, Chicago, Illinois, to insure uniformity in the medium.

Cultures of Hansenula holstii NRRL Y-2448 were obtained from the Fermentation Laboratory, Northern Regional Research Center, Peoria, Illinois.

Maxilact F. G. Lactase enzyme was obtained from Enzyme Development Corporation, Keyport, New Jersey.

The enzyme for glucose analysis, fermacozyme 952 DM was obtained from Fermco Biochemics Inc., Elk Grove Village, Illinois, and 4-aminophenazone 124001 was manufactured by Boehringer Mannheim, Gmbh, West Germany.

II. CULTURE PREPARATION

Cultures of Hansenula holstii Y-2448 were maintained on yeast malt agar slants at 4°C. They were transferred onto fresh slants at an interval of four weeks. Prior to inoculation into whey substrates they were grown in Trypticase Soy Broth for 24 hours at 23°C.

III. WHEY SUBSTRATE

A 6 percent (w/v) whey solution was prepared by rehydrating dried whey, and 0.212 g./l. Maxilact F. G. Lactase enzyme was added to hydrolyze lactose present in whey into its monosaccharide constituents glucose and galactose (4). The pH of the solution was adjusted to 7.0 with 1 N KOH by means of a Corning pH Meter, Model 10. The solution was then incubated at 23°C on a Fisher Thermix Magnetic Mixer and mixed continuously by means of a magnetic stirring bar. At the end of a 14 hour incubation period, the pH of the solution was adjusted to 5.0 with 1 N HCl by means of a pH meter.

The whey solution was autoclaved for 15 minutes at 121°C to precipitate the proteins. The whey substrate was cooled and filtered through Whatman No. 12 filter paper to remove proteins and other precipitates. Aliquots of 100 ml whey substrate were distributed in 500 ml Erlenmyer flasks. To each flask salts were added singly or in combinations as described in Table II, and the flasks were autoclaved 5 minutes at 121°C.

IV. GLUCOSE DETERMINATION OF WHEY SUBSTRATE

Prior to inoculation samples of whey substrate were tested for their glucose content by the method of Trinder (46).

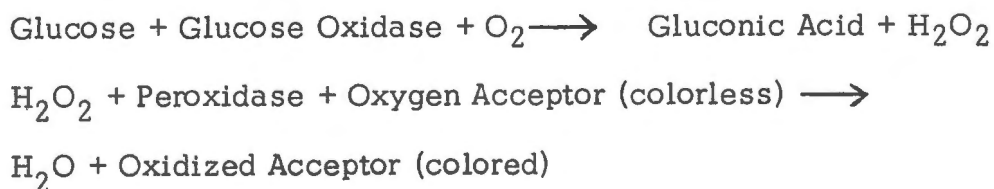


TABLE II
SUBSTRATE VARIATIONS

Treatment Number	Components Added ¹
1	Control Medium of Anderson et al.
2	None
3	0.5% Stock Salt Solution ²
4	0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
5	0.001% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$
6	0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
7	0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ + 0.001% 0.001% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$
8	0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ + 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
9	0.001% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ + 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

¹In treatments number 2 through 9, 6 percent whey solution was the basal medium.

²Anderson et al. (1).

The protein precipitant was made by dissolving 10g $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$; 10g Na_2HPO_4 and 9 g NaCl in 800 ml water. The pH was adjusted to 3.0 by adding about 125 ml of 1 N HCl. Then the volume was made up to a liter after adding 1 g of phenol.

The color reagent was prepared by adding 75 ml of 4 percent Na_2HPO_4 , 5 ml Fermacozyme 952 DM (enzyme complex) and 100 mg 4-aminophenazone in 220 ml of water and stored at 4°C.

Glucose standards were prepared by dissolving appropriate amounts of glucose to obtain (w/v) solutions of 0.05 percent, 0.1 percent, 0.15 percent, 0.2 percent and 0.25 percent.

The whey substrate for glucose analysis was prepared by making a 10 percent dilution from the original medium used for gum production.

One-tenth ml of whey substrate was added to 2.9 ml of protein precipitant and filtered through Whatman No. 4 filter paper. One ml filtrate was taken into a test tube and mixed with 3 ml of color reagent. Then the reactants were mixed and placed in a 37°C water bath for 10 minutes with periodic agitation. At the end of the reaction period the colorless color reagent turned into a pink to strawberry color depending upon the glucose content. The absorbances of the solutions were measured in Bausch and Lomb Spectronic 20 Spectrophotometer at 515 nm. A standard curve of glucose concentration versus absorbance was plotted. The glucose concentration of unknowns (whey substrates) was calculated by means of a linear equation obtained from the standard curve, which had an r^2 value of 0.989:

$$x = 0.3608A - 0.00303$$

Where x = glucose concentration (percent) and A = absorbance.

V. PROTEIN ASSAY

Protein content of each batch of whey substrate was determined by the Kjeldahl method (5), using a factor of $6.38 \times \% N$.

VI. CONTROL MEDIUM

The control medium was prepared according to the procedure described by Anderson et al. (1). It contained 6 percent glucose, 0.5 percent KH_2PO_4 , 0.2 percent yeast extract and 0.5 percent salt solution. The stock salt solution contained 4 percent $MgSO_4 \cdot 7H_2O$ and 0.2 percent each of $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$ and $NaCl$. The pH of the medium was adjusted to 5.0 by means of a Corning pH Meter, Model 10.

VII. INOCULATION AND FERMENTATION

The whey substrates and the control medium were inoculated with 5 percent by volume of Hansenula holstii inoculum grown for 24 hours in Trypticase Soy Broth. Then the flasks were incubated at $23^\circ C$ on a Burrell Wrist Action Shaker allowing continuous shaking at the rate of 130 strokes per minute with an arc length of 2.5 cm for 96 hours. Five replications were carried out.

VIII. RECOVERY OF THE GUM

The recovery procedure followed the method of Anderson et al. (1). The culture fluids were diluted 1:1 with water and centrifuged in an International Equipment Model K Centrifuge at 800 x g for 15 minutes to sediment the cells. The supernatant was decanted into another centrifuge bottle and two volumes of 95 percent ethanol were added to one volume of supernatant and mixed thoroughly. Then 1.0 ml of saturated KCl solution was added to insure complete precipitation of polymer. The precipitate was then separated by centrifuging at 800 x g for 15 minutes. After decanting, the pellet was air dried for five minutes to evaporate ethanol and dissolved in 10 ml of water. After thorough mixing, the gum solution, which was very viscous at this time, was poured into tared petri dishes and freeze dried.

The percent polymer yield from different sources was measured based on the volume of the crude culture.

$$\% \text{ Polymer} = \frac{\text{g of polymer}}{\text{ml crude culture}} \times 100$$

IX. EXAMINATION OF THE GUM

The polymer from different media was examined by apparent viscosity measurement. Dried gum from each medium was dissolved in water to give 0.3, 0.6 and 0.9 percent concentration (w/v). The gum solutions were mixed and set at 4°C for 24 hours for complete hydration. Apparent viscosity measurements were taken on a Brookfield Viscometer at 23°C using a small sample adapter and No. 18 spindle at 30 rpm.

X. ANALYSIS OF DATA

Data on yield of gum from various media were analyzed by analysis of variance. Means from significant effect was partitioned by Duncan's Multiple Range Test.

Apparent viscosity was measured on gums from three replicates. The slopes of the family of plots were subjected to Duncan's Multiple Range Test.

CHAPTER IV

RESULTS AND DISCUSSION

I. COMPOSITION OF WHEY

Whey substrate used in this study was analyzed for its glucose and protein content. The glucose content is shown in Table III along with the standard deviation. The mean glucose content was 1.62 percent, which is considerably lower than the glucose content of the control medium which had 6 percent glucose. This factor may have had a significant influence on the yield of the gum. Since the hydrolysis of lactose was achieved by means of the enzyme lactase (Maxilact), it is probable that the enzyme was not as quite effective as it is claimed to be by the manufacturer (4).

The protein content of whey is shown in Table IV along with the standard deviation. The mean value was 0.42 percent compared to 0.8 percent which is normally found in sweet rennet whey (52). It is evident that only 49.9 percent of protein was precipitated during the process of deproteinization. On the other hand, the analysis of protein in the control medium indicated it to be 0.11 percent which is also considerably lower from that of whey substrate.

II. RECOVERY OF PHOSPHOMANNAN

The gum that was produced by Hansenula holstii precipitated readily after the addition of ethanol to the cell-free crude culture.

TABLE III
GLUCOSE CONTENT OF WHEY SUBSTRATES

Replicate Number	Percent Glucose ¹
1	1.69
2	1.40
3	1.65
4	1.56
5	1.76
Mean	1.62
Standard Deviation	0.157

¹ Means of two determinations.

TABLE IV
PROTEIN CONTENT OF WHEY SUBSTRATES

Replicate Number	Percent Protein ¹
1	0.41
2	0.43
3	0.44
4	0.44
5	0.39
Mean	0.42
Standard Deviation	0.023

¹ Means of two determinations.

The precipitation process was completed after the addition of saturated KCl. The solubility of the gum was quite evident when dissolved in water. However, there was a difference in the solubility of gum from different sources. The gum from control medium dissolved more readily than the gum from whey substrates. No difference in solubility was observed among the gums from various whey substrates. In other words, variations in salt contents did not influence the solubility of the polymer. The difference in solubility among the gums from control medium and whey substrate may be attributed to the difference in the protein content of both media. As was indicated by Avaigad (8), when the growth medium is rich in protein, the microbial gum may adhere to the protein and this may interfere in the solubility of the gum. It was also stated by Anderson et al. (1) that for product purification the fermented culture liquor should have a minimal amount of soluble protein. Based on these two observations it can be concluded that the protein content of the substrate had a role in the solubility of the gum. As indicated in Table IV the whey substrate had a mean protein content of 0.42 percent, about four times more than that of the control medium which had 0.11 percent. This may have made the control gum more soluble than the gum from the whey substrate. Even though extraction was performed by addition of 95 percent ethanol (1, 38), alternate procedures such as using methanol (39) were attempted. The gum from methanol extraction was spongy in appearance and was not soluble in water.

III. YIELD OF THE GUM

The percent yield of phosphomannan gum per milliliter of crude culture is listed in Table V. The mean yield from the control medium was 1.7690 percent, while the yield from whey media ranged from 0.6763 percent in treatment number nine to 0.7927 in treatment number three. Analysis of variance was performed on these data and is listed in Table VI. It indicated that there was a significant difference in the treatments at the 99 percent confidence level. To locate differences in the treatments, Duncan's Multiple Range Test was performed on the data, and the result is listed in Table V along with the yield. This test indicated that the yield from the control medium was significantly different from the yield from the whey substrates. However, the differences in the yields from different whey substrates was not significant.

There is another aspect to consider, that is the yield of gum per gram of glucose available in the medium. As cited in the literature (43), the theoretical yield of the gum is about 50 percent of the glucose available in the medium. But in practicality researchers using a similar control medium (1) and a similar strain obtained yield of about 36 - 40 percent (1, 38, 40). In this study a yield about 30 percent was obtained from the glucose in the control medium. Some modifications in the control medium did improve yield (38, 40), but such modifications were not made in this research. As far as the yield from whey substrate is concerned, an average of 0.737 g. which ranged from 0.676 to 0.793 g of gum was obtained from an average of 1.62 g of glucose available in

TABLE V
PHOSPHOMANNAN YIELD FROM NINE MEDIA

Treatment Number	Yield ^{1,2} g./100 ml.	Range g./100 ml.
9	0.6763a	0.6122 - 0.7833
8	0.7101a	0.6030 - 0.8963
2	0.7129a	0.5930 - 0.9158
7	0.7246a	0.6410 - 0.8629
4	0.7457a	0.6530 - 0.9158
5	0.7652a	0.6610 - 0.8865
6	0.7678a	0.6930 - 0.8207
3	0.7927a	0.7280 - 0.9833
1	1.7690b	1.5439 - 2.0300

¹ Means of five replicates.

² Means tested by Duncan's Multiple Range Test at 5 percent level of significance. Same letters indicate no significant difference.

TABLE VI
ANALYSIS OF VARIANCE ON PHOSPHOMANNAN
YIELDS FROM NINE MEDIA

Sources	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Treatments	8	4.79	0.5987	19.96*
Replicates	4	0.16	0.0400	1.33
Residual	32	0.96	0.0300	
Total	44	5.91		

*Significant at 5 percent.

the medium, which is about 45.5 percent. This was a higher yield than from the control medium. However, more research needs to be done to improve the yield as well as solubility. Table VII indicates the yield of gum in grams per gram of glucose in the media. Duncan's Multiple Range Test was performed on the data. It indicated that yield from control medium, as was expected, was significantly higher than the whey substrates, but the yield from various whey substrate did not show much difference, or their difference was not significant. Here again it is evident that the type of salts does not influence the polymer yield. Similar yield was achieved in sterilized hydrolyzed whey without adding any salts. This factor is an added advantage of whey utilization for gum production. The cost and the addition of salt could become an economic factor in mass operation (38). Besides glucose content, protein content, and type of salts, there are several other factors such as pH, aeration rate, fermentation flask size and culture volume that could have an influence on the polymer yield (1, 38). It is to be noted that since this study was only an exploratory investigation no attempts were made to maximize yield other than substrate variations.

IV. RHEOLOGICAL PROPERTIES OF THE GUM

The apparent viscosity measurements of gum from control medium and whey media showed a difference, which is indicated in Table VIII. The gum solution from the control medium had mean apparent viscosities of 18.82, 31.65 and 49.37 centipoise at concentrations of 0.3, 0.6 and 0.9 percent, respectively, while gum solutions from the whey substrates

TABLE VII
PHOSPHOMANNAN YIELD FROM NINE MEDIA
BASED ON AVAILABLE GLUCOSE

Treatment Number	Yield ^{1,2} g./g. glucose	Range g./g. glucose
1	0.3004b	0.2573 - 0.3383
9	0.4198a	0.3763 - 0.4964
8	0.4388a	0.3655 - 0.4979
2	0.4414a	0.3594 - 0.5180
7	0.4496a	0.3885 - 0.5252
4	0.4624a	0.3876 - 0.5133
5	0.4736a	0.4006 - 0.5295
6	0.4774a	0.4200 - 0.5789
3	0.4956a	0.4308 - 0.5634

¹ Means of five replicates.

² Means tested by Duncan's Multiple Test at 5 percent level of significance. Same letters indicate no significant difference.

TABLE VIII

APPARENT VISCOSITY MEASUREMENTS ON GUM SOLUTIONS

Treatment Number	Apparent Viscosity (Centipoise) ¹		Regression Equation ²
	0.003 g./ml.	0.006 g./ml. 0.009 g./ml.	
4	5.77	9.37 13.70	y = 13.22x + 1.68 (a)
2	4.33	8.83 12.87	y = 14.22x + 0.15 (a)
8	4.25	8.90 13.35	y = 15.17x - 0.33 (a)
7	5.28	11.20 15.00	y = 16.17x + 0.79 (a)
9	5.87	10.55 15.90	y = 16.72x + 0.74 (a)
5	5.47	10.00 16.33	y = 17.61x + 0.03 (a)
6	6.42	12.42 17.07	y = 17.72x + 1.35 (a)
3	5.50	10.17 17.33	y = 19.72x - 0.83 (a)
1	18.82	31.65 49.37	y = 50.89x + 2.75 (b)

¹ Means of three replicates.² Slopes tested by Duncan's Multiple Range Test at 5 percent level of significance. Same letters indicate no significant difference.

had mean apparent viscosities of 5.36, 10.15 and 15.16 centipoise at the same concentrations. The difference in apparent viscosity was probably due to the solubility. As indicated earlier, the protein content may be a factor in solubility and apparent viscosity. Other components in the whey substrate which might be interfering with the solubility is a question which needs further investigation for a suitable answer. The apparent viscosity of gum from control medium was also not as high and the solutions were not as clear as was reported in the literature (27, 29, 45). A solubility problem was indicated here, also.

Analysis of variance was performed on apparent viscosity data from each treatment for significance of differences among whey substrates and no significant difference was evident. That of the control gum was significantly higher. The apparent viscosities followed the general pattern indicated in the literature which showed that the increase in apparent viscosity was linear below a concentration of 1.5 percent (27, 29, 38, 45). To obtain a straight line pattern in apparent viscosity curves, concentrations of 0.3, 0.6 and 0.9 percent were considered in this study for apparent viscosity measurements. A model system was designed to compare the family of apparent viscosity plots obtained from each treatment based on their slopes. As indicated in Table VIII, the apparent viscosity plot of the gum from control medium had a totally different slope from the slopes of apparent viscosity plots obtained from whey substrates. The slopes of gum solutions from whey substrates ranged from 13.22 to 19.72 centipoise. To study the relations among slopes, Duncan's Multiple Range Test was performed and the results are listed in Column 6 of Table VIII. The test indicated that the difference in

the slopes of apparent viscosity regression equations of gum obtained from different whey substrates was not significant. The gum obtained from whey substrate where no salts were added had similar rheological properties to that of the gum obtained from whey substrate with added salts. It can be concluded that the sulfates of Mg, Mn, and Fe did not influence either viscosity or the yield of the phosphomannan gum produced in whey. This also indicated that whey not only supports the growth of polymer producing yeasts but was also sufficient for the production of polymer of uniform character without adding any additional substances. However, further research is needed to produce phosphomannan of high apparent viscosity in good yield in whey by using the yeast Hansenula holstii.

The phosphomannan gum has been recognized as a useful ingredient as a thickening agent (20) and as a stabilizer (39). Economic production may intensify its use and offer new ways for its utilization in the food industry. Several aspects of its industrial production have been studied. A 92 percent pure phosphomannan can be produced at a cost of 93 cents per pound using anhydrous dextrose as a carbon source (38). The same product might be produced at still a cheaper price using hydrolyzed lactose from whey as a carbon source. Such an effort would not only help solve some of the pollution problems caused by whey but also produce gums at a cheaper price for the ever increasing demands for gums in the food industry.

CHAPTER V

SUMMARY

The purpose of this investigation was to produce and isolate phosphomannan gum in hydrolyzed whey using the yeast Hansenula holstii, and to study its yield and rheological properties pertaining to food systems.

The whey that was used as a growth medium was prepared by rehydrating dried sweet whey, and was enzymatically treated to hydrolyze lactose into glucose and galactose. It was deproteinated by heat. A control medium and several whey media having different combinations of salts were used as growth media.

A 5 percent inoculum of Hansenula holstii NRRL Y-2448 was grown in the growth media for 96 hours at 23°C under continuous agitation. The gum was precipitated by ethanol and saturated KCl from cell-free culture media. The gum was air dried and lyophilized after dissolving in small quantity of water.

The dried gum from each source was examined for percent yield and rheological properties. The percent yield from each source based on crude culture and available glucose were compared. For apparent viscosity measurements the gum in appropriate quantities was dissolved in 10 ml water to obtain solutions of 0.3, 0.6, and 0.9 percent concentrations. The apparent viscosities of gum from each source in replicates was compared based on the slope of apparent viscosity plots.

The data on yield and apparent viscosities were subjected to analysis of variance and Duncan's Multiple Range Test. It was observed

that the gums from various whey substrates was statistically similar to one another both in yield and apparent viscosity, but were different from the gum obtained from control medium. It was concluded that salts of Mg, Mn, and Fe did not have any influence on the yield and apparent viscosity of the gum produced in whey. While the yield was dependent on glucose concentration of the medium and the viscosity and solubility were dependent on protein content, the hydrolyzed whey was a suitable medium for growth and phosphomannan production from Hansenula holstii.

Further research is needed to study other factors that influence viscosity of the gum and to produce phosphomannan of high apparent viscosity in whey.

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