JOURNAL OF CLINICAL MICROBIOLOGY, June 1975, p. 495-499.

Vol. 1, No. 6
Printed in U.S.A.

Growth Potential of Cottonseed Culture Media for Various Clinically Significant Aerobic Bacteria

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Received for publication 21 February 1975

Enzymatic hydrolysates of various cottonseed flours were prepared with the proteolytic enzymes bromelain, HT-200, Pronase, and trypsin. The growth of various aerobic bacteria of clinical significance in these hydrolysates was compared to that obtained with a standard casein-soybean peptone culture medium, Trypticase soy. The generation times of the majority of bacteria grown in the bromelain cottonseed flour hydrolysate were shorter than that obtained with the standard control broth. A bromelain cottonseed flour hydrolysate agar preparation supported the growth of the bacteria comparably to that of the casein-soybean agar substrate. All the bacterial colonies were larger on the bromelain cottonseed flour hydrolysate blood agar medium than those grown on the control agar. The peptones derived from the enzymatic hydrolysis of cottonseed flour are sufficient to promote the rapid and luxuriant growth of a wide spectrum of aerobic bacteria without the addition of peptone from other sources. It is suggested that cottonseed flour peptones be utilized as a nutrient source in general-purpose media for the clinical microbiology laboratory.

Cottonseed has been recognized for many years as a potential high-protein food source (17). Extraction of the proteins from cottonseed may be accomplished by single-step procedures with aqueous alkaline or salt solutions (1, 9, 16, 17). A two-step extraction procedure employing water or dilute salt followed by dilute alkali for the isolation of cottonseed proteins was shown to yield a product higher in nitrogen than that obtained by a single-step procedure (3). Of the various protein extraction methods, enzymatic solubilization of cottonseed protein has proven to be the most efficient means of extraction (2). The amount of protein extracted varies according to the procedures employed, but none of these procedures completely extracts the protein (3).

The microbiological applications of cottonseed flour are primarily associated with many industrial fermentations including antibiotic (12, 31) production. Earlier reports have described cottonseed flour as a constituent in a culture medium for the growth of various arthropods (5).

The present investigation concerns the growth potential of enzymatic hydrolysates of cottonseed flours for various aerobic bacteria of clinical importance. The results are compared to that obtained with a standard soybean-casein hydrolysate culture medium, Trypticase soy broth (TSB) and agar (TSA).

MATERIALS AND METHODS

Bacteria. The cultures of bacteria were obtained from clinical specimens and stock cultures. All organisms were maintained at room temperature in stabs of motility agar and transferred every 4 months until required for experimentation.

Standard media. TSB (BBL) was prepared as a 3% solution as suggested by the manufacturer. TSA (BBL) was prepared with and without the addition of sterile defibrinated sheep blood.

Cottonseed flour. The four types of cottonseed flour utilized in these investigations included: Pharmamedia and Proflo (Traders Protein Division, Fort Worth, Tex.), Liquid Cyclone Process (10, 11, 33), and a flour derived from a glandless variety of cotton (13, 19, 32). The latter two flours were obtained from Dwain Mulsow, Texas A & M University. Liquid Cyclone Process flour was also obtained from Homer Gardner, Southern Regional Research Laboratories, New Orleans.

Enzymes. Pronase (Type VI protease) (25, 27, 30) and trypsin were obtained from the Sigma Chemical Co. The enzymes HT Proteolytic-200 and bromelain (23) (Bromelain concentrate 1200) were obtained from Marschall, Division of Miles Laboratories, Inc.

Enzymatic hydrolysis of cottonseed flour. Fifty grams of the respective cottonseed flour, 0.5 g of enzyme, and 500 ml of appropriate buffer were placed in a 1,000-ml flask with a magnetic stirrer. Pronase was reacted with the cottonseed flour substrate at pH 7.0 in tris(hydroxymethyl)aminomethane buffer which was 0.25 M in $CaCl_2$ (27). HT-200 and trypsin were used with tris(hydroxymethyl)aminomethane buffer at pH 6.9 and 7.0, respectively. Bromelain was

used to hydrolyze cottonseed flour at pH 4.6 with citric acid-phosphate buffer. During incubation, samples were removed each hour for 7 h to determine pH alterations. Accordingly, appropriate adjustments were made to correct the pH values for optimal enzymatic activity.

At the end of the 7-h incubation period, the hydrolysate was centrifuged at 2000 × *g* in a refrigerated centrifuge (model PR-2, International Equipment Co., Needham Heights, Mass.) for 10 min at 8 C. The supernate was autoclaved for 1 to 2 min to inactivate the enzymes and precipitate phosphates. The hydrolysates were then passed while hot through various filters (Millipore Corp., Bedford, Mass.) with pore sizes beginning with 1.2 μm and ending with 0.45 μm, to clarify them. The hydrolysates were adjusted to pH 7.2 with 10 N NaOH and sterilized by autoclaving at 121 C and 15 pounds of pressure for 15 min. The hydrolysates were filtered while hot through 0.45-μm filter units (Nalge, Cybron Corp., Rochester, N.Y.) to remove the precipitate that had formed due to the addition of the NaOH. This procedure eliminated any further precipitation with additional heating or autoclaving of the hydrolysates. The broths were then dispensed into glass prescription bottles. The fluid cottonseed hydrolysates (bromelain [BCH], HT-200, Pronase, and trypsin) were stored at 8 C.

The hydrolysate was also diluted to 3% with tris-(hydroxymethyl)aminomethane buffer (pH 7.2). This preparation was prepared as a solid medium with the incorporation of 1.5% agar and 0.85% NaCl. In some instances 5% sterile defibrinated sheep blood was added to the BCH agar medium.

Dehydration of the hydrolysates. Unautoclaved hydrolysates were dried with an evaporator (Buchi Rotovapor-R). The dried flakes were powdered with a spatula and stored at room temperature in plastic screw-capped glass bottles. After rehydration with distilled water, the preparations were sterilized by autoclaving in the usual manner.

Cultivation. The bacterial broth cultures were grown overnight in the same medium in which they were to be examined. The rapidly growing bacteria, however, were incubated for only several hours. Screw-capped tubes (18 by 150 mm) or 50-ml Nephelo culture flasks (Bellco Glass, Inc., Vineland, N.J.) containing 10 ml of medium were inoculated with the bacterial cultures to an optical density between 0.05 and 0.10. The optical density was read at 650 nm in a spectrophotometer (Spectronic 100, Bausch and Lomb). Each bacterium was inoculated in triplicate into a cottonseed flour hydrolysate broth and control TSB medium. The tube cultures were incubated in a stationary manner, and the Nephelo flask cultures were incubated on a Rotory Orbital Shaker (Lab-Line Instruments, Inc.) adjusted to 125 rpm for the desired period of time. All cultures were incubated at 35 C and read at half-hour intervals until their stationary phase was observed. The mean effective generation time was calculated (6), and the lag time was estimated from the intercept of the exponential growth slope with the initial inoculation level (20, 28).

Other analytical methods. Protein was determined by the method of Lowry et al. (14) with crystallized bovine albumin as the standard. The glucose composition of a BCH and TSB preparation was determined by the hexokinase-glucose-6-phosphate dehydrogenase method (35). A BCH preparation was assayed for its calcium (39), iron (4), and magnesium (29) content.

RESULTS

A series of experiments was first performed to establish the potential of cottonseed flour hydrolysates prepared with four enzymes to support the growth of *Serratia marcescens*. The rate of growth of this organism was significantly greater in BCH than in other enzymatic cottonseed hydrolysates (Table 1).

The generation times of *S. marcescens* grown in bromelain-hydrolyzed glandless, deglanded (Liquid Cyclone Process), Pharmamedia, or Proflo preparations of cottonseed flour were essentially similar. In all instances the rate of growth of the organism in each of the cottonseed hydrolysates was greater than in TSB (Tables 1 and 2). A BCH preparation contained 4.5 mg of calcium, 48 mg of iron, and 48 mg of magnesium per 100 ml. Glucose analysis of this cottonseed hydrolysate revealed a concentration of 35 in contrast to 210 mg/100 ml of glucose for TSB. The bromelain hydrolysate of Pharmamedia was used for the remainder of the investigation as the cottonseed culture medium.

BCH was next examined for its growth potential with 15 other bacteria of clinical signifi-

TABLE 1. *Growth rates of Serratia marcescens in cottonseed flour[a] hydrolysates derived by various enzymes and the control Trypticase soy broth*

Enzyme	Mean effective generation time[b] (min)
Bromelain	22.2
HT-200	91.2
Pronase[c]	72.0
Trypsin	40.0
Trypticase soy broth control	41.1

[a] Pharmamedia cottonseed flour.
[b] Based on growth in stationary tubes.
[c] Type VI protease, Sigma Chemical Co.

TABLE 2. *Growth rates of Serratia marcescens in bromelain hydrolysates of various cottonseed flours and control TSB*

Cottonseed flour	Mean effective generation time[a] (min)
Deglanded	21.8
Liquid cyclone process	22.2
Pharmamedia	23.4
Proflo	22.8
TSB control	48.7

[a] Based on growth in stationary tubes.

cance. The results showed that the generation times of the majority of these bacteria were shorter in the cottonseed flour hydrolysate than in TSB. In particular, the exceedingly short generation time of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Streptococcus pneumoniae* in BCH was in contrast to that obtained with TSB (Table 3). The lag periods of *Aeromonas hydrophila*, *S. aureus* and *Yersenia enterocolitica* appeared shorter in BCH than in the TSB control.

The indole test (Ehrlich's reagent) was positive with 48-h BCH cultures of *Escherichia coli* and *Flavobacterium meningosepticum*.

Agar-solidifed BCH was examined for its ability to support bacterial growth. A 3% basal medium was prepared with 1.5% agar and 0.85% NaCl. The BCH agar medium supported the growth of most organisms similarly to that of TSA. The colonies of *Serratia liquefaciens* and *Klebsiella pneumoniae*, however, were distinctly larger and more mucoid on the BCH agar than that on the control TSA. Pigment production by *S. marcescens*, *S. aureus*, and *P. aeruginosa* was similar to that obtained with TSA.

A dehydrated BCH preparation was rehydrated with phosphate buffer (pH 7.2) to a 3% concentration. Agar was added, and the medium was autoclaved. Bacterial growth on the BCH agar medium was similar to that on the unevaporated 3% BCH agar medium.

After a 18-h period of incubation, bacterial colonies were larger in size on BCH blood agar than those grown on TSA blood agar. The hemolytic zones of the *S. aureus*, *S. pneumoniae*, and beta-hemolytic streptococci were as clear as those seen on TSA blood agar.

DISCUSSION

This is the first report that establishes enzymatic hydrolysates of cottonseed flour as an excellent nutrient for the growth of many clinically associated bacteria.

Cottonseed has long been considered an important source of edible protein (17, 31). Numerous procedures were developed to concentrate (16, 17) the proteins and to isolate (3) by extracting the protein of cottonseed. Enzymatic treatment of cottonseed was previously demonstrated to be the most efficient procedure in yielding a soluble extract of protein (2). Arzu and his associates reported that among 10 enzymes investigated, the enzymes bromelain (2) and HT-200, a protease derived from *Bacillus subtilis*, appeared the most active in extracting cottonseed protein. Biological parameters in the present investigation have shown that the growth potential of the Bromelain 1200-derived cottonseed extract was greater than that of the HT-200 extract. Possibly the nature of the proteins and growth factors released in the Bromelain 1200 extract is more growth promoting than that of the HT-200 extract.

This is the first report of the use of Pronase in the protein solubilization of cottonseed flour. This enzyme has a very broad specificty (22, 24, 25); however, it did not yield a hydrolysate of greater growth potential than bromelain hydrolysate. The present investigation has demon-

TABLE 3. *Growth rates of various bacteria in BCH and TSB*

Bacteria	Mean effective generation time (min)			
	BCH		TSB	
	Range	Mean	Range	Mean
Aeromonas hydrophila	32-36	33.6	39-49	44
Arizona hinshawii	39-43	40.7	40-44	41.3
Citrobacter diversus	40-52	46.0	50-64	58.0
Escherichia coli	35-46	40.3	49-54	51.6
Flavobacterium meningosepticum	30-32	30.6	37-45	41
Klebsiella pneumoniae	17-18	17.6	38-39	38.3
Providencia stuartii[a]	55-64	58.3	55-59	56.6
Pseudomonas aeruginosa	33-34	33.3	74-80	76.6
Salmonella typhi	43-50	46.6	48-49	48.3
Serratia liquefaciens	33-41	37.0	39-47	42.6
Serratia marcescens[a]	25	25.0	42-43	42.6
Shigella sonnei	35-37	36.3	30-31	30.6
Staphylococcus aureus	32-34	33	54-61	56.6
Streptococcus pneumoniae	58-59	58.6	429-456	444.3
Streptococcus pyogenes	41-47	44.3	74-78	75.6
Yersinia entercolitica	35-39	37.3	41-43	41.6

[a] Based on growth in Nephelo flasks.

strated that of the enzymatic hydrolysates investigated, the bromelain hydrolysate, without added nutrients, appears to yield the nutritive factors required to stimulate the rapid growth of a wide variety of bacteria associated with clinical specimens.

Tryptophan was reported to be an important amino acid for streptococci (18). It appears to be present in BCH in a concentration that permits a positive indole test with appropriate bacteria and in sufficient concentration to permit growth of the beta-hemolytic streptococci used in this investigation. The addition of magnesium and iron to media was reported as beneficial to growth of certain bacteria (15, 26, 34). Since chemical analysis had disclosed the presence of these electrolytes in the BCH medium their additions with appropriate salts were not made. Furthermore, if these electrolytes were required by any of the bacteria, it would appear that their concentrations in the basal cottonseed medium are at least sufficient for the excellent growth response obtained by the majority of the bacteria examined.

There are, no doubt, various bacterial strain differences in respect to generation times. Although single bacterial strains were utilized for each of the species in this investigation, the trend of results distinctly indicates that rapid growth of bacteria will often occur in BCH broth. Furthermore, the rate of growth of many bacteria in this medium was greater than that obtained with TSB. The glucose content of BCH was significantly lower than that of TSB. Thus, it would appear that the excellent growth-promoting potential of BCH is not related to its glucose content.

As a blood agar base, the basal medium in combination with only defibrinated sheep blood appears to contain the necessary growth-promoting factors to support the growth of all the bacteria utilized in this investigation. The hemolytic zones were as clear and the colony sizes of many of the bacteria, including the streptococci, were larger than those observed on TSA made with defibrinated sheep blood.

The commonly applied general purpose media, and many other bacteriological media consist, in the main, of peptones from several sources. Casein and soybean hydrolysates and in some instances peptones derived from muscle (Myosate, BBL) (8, 21) are incorporated in various combinations in culture media. Their nutritive value is related, in part, to their respective peptide, vitamin, and amino acid concentrations (7). The peptones derived from the enzymatic hydrolysis of cottonseed flour, however, are sufficient to promote the rapid and luxuriant growth of a wide spectrum of aerobic bacteria without the addition of other peptones or other nutritive factors.

Cottonseed flour (Pharmamedia, Proflo) is less expensive than both the soybean meal and casein utilized in the preparation of TSB. Thus it is possible to prepare a hydrolysate from cottonseed flour less expensively than that for TSB or other similar peptone media.

The clinical microbiologist is continually searching for growth media that will permit the early recognition of bacteria from clinical specimens. The cottonseed flour hydrolysate described above represents an excellent general purpose medium. The simplicity of the formulation of the basal cottonseed medium, its ease of preparation, and the relatively low cost of the reagents and cottonseed flour should encourage further interest in its application in the clinical microbiology laboratory.

ACKNOWLEDGMENTS

We thank Isobel Lamb for the secretarial assistance in the preparation of the manuscript and R. J. Hartsock for his helpful comments on the manuscript.

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