STUDIES ON THE NUTRITIONAL REQUIREMENTS OF BACILLUS COAGULANS

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# STUDIES ON THE NUTRITIONAL REQUIREMENTS OF BACILLUS COAGULANS

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## ABSTRACT

A study was made of 177 spore-forming bacteria isolated from the wash waters of Canadian tomato juice packing plants. Only about 30 percent of these were able to produce flat-sour spoilage in tomato juice both aerobically and anaerobically. The remaining 70 percent grew only aerobically, generally producing thick cheesy pellicles and alkaline reactions. Since all organisms grew well on thermoacidurans agar (Difco) (DTA), it was concluded that routine bacteria counts on this medium do not provide a true index of spoilage possibilities. Results indicated that the addition of 0.004 percent bromcresol green indicator to DTA, and increasing its dextrose concentration to 1.0 percent, rendered it more valuable as a plating medium. The significant acid-producing types were readily distinguished in this modified medium.

Taxonomic studies showed that Bacillus coagulans predominated among those organisms producing acid whereas those producing a more alkaline reaction in tomato juice resembled Bacillus subtilis.

The vitamin and amino acid requirements of 15 of the canning plant isolates identified as B. coagulans and 8 other authentic strains of this organism were studied at 37°C. In a medium comprising vitamin-free acid hydrolyzed casein, yeast extract, glucose, and mineral salts, growth occurred earlier

at pH 5.5 than at 7.5. Substitution of malate, succinate, fumarate or citrate for glucose in this medium indicated that citrate was not utilized and response to the other three was feeble. Sporadic results obtained in this medium when vitamins were substituted for yeast extract did not result from a deficiency of cysteine or tryptophan. The variation was eliminated by substituting enzymic casein hydrolysate for the acid hydrolysate. Biotin and thiamin were required by all strains in this semi-synthetic medium. Niacin, also, was required by one strain, and folic acid by another. An additional requirement for folic acid (or PABA) was noted for most strains in a synthetic medium containing 12 amino acids. This was the most important difference between results obtained using the semi-synthetic medium and the synthetic.

The amino acid requirements were generally non-specific at 37°C. However, glutamic acid appeared essential for a few strains and stimulated others.

The enhanced growth generally afforded by the enzymic casein hydrolysate was largely a reflection of buffering action resulting from its high acetate content. Phosphate buffer appeared more favorable for B. coagulans since one strain proved acetate sensitive.

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# STUDIES ON THE NUTRITIONAL REQUIREMENTS OF BACILLUS COAGULANS

## INTRODUCTION

Recently, considerable effort has been made to determine the vitamin and amino acid requirements of *Bacillus coagulans* (synonym: *Bacillus thermoacidurans*), the organism believed to cause flat-sour spoilage of tomato juice. Most reports on the nutritional requirements of this organism have shown a lack of agreement among results for which there has been no concrete explanation. The overall picture is confusing in that different workers tested different strains using various methods and incubation temperatures.

In general, most investigations have involved too few strains to provide sufficient information on variation within the species. Recent, more comprehensive studies indicate that variation within the species, at least with respect to vitamin requirements, may be somewhat less than indicated by earlier, less complete reports.

The present investigation was undertaken in the hope that the examination of a variety of strains under similar conditions would provide a more complete picture of the species and perhaps explain some of the controversy. Strains of *B. coagulans* isolated from

canning plants and some examined previously by other workers were included in this study.

# REVIEW OF LITERATURE

## General

*Bacillus coagulans* was first isolated and described by Hammer (1915) as the cause of an outbreak of coagulation in evaporated milk. Hussong and Hammer (1928) described another organism, *Bacillus calidolacticus*, which coagulated milk by producing lactic acid and small amounts of volatile acid. Later, Sarles and Hammer (1932) noted that *B. coagulans* produced large quantities of lactic, acetic, and propionic acids from carbohydrate. Although the organism showed no visible evidence of proteolysis, it increased greatly both the soluble and amino nitrogen in skim and evaporated milks.

Berry (1933) described an apparently new species, *Bacillus thermoacidurans*, as the cause of off-flavor in commercially packed tomato juice. Still another new species, *Bacillus dextrolacticus*, was described by Andersen and Werkman (1940) which was related to *B. coagulans* but differed from it by producing considerably more lactic acid, forming acid from arabinose and sorbitol, and reducing nitrate to nitrite.

Reliable methods for classifying the aerobic, mesophilic, sporeforming bacteria were published by Smith *et al.* (1946). A description of *B. coagulans* was included but was emended in a later report by Gordon

and Smith (1949). Knight and Proom (1950) identified 15 new isolates as B. coagulans in substantiating the methods of Smith et al. (1946). They further noted that these isolates were urease and lecithinase negative and would grow in 4 percent sodium chloride broth.

In comparing B. thermoacidurans Berry with B. coagulans Hammer (1915), Becker and Pederson (1950) concluded that they were essentially alike and that the latter name had priority. A second publication by Smith et al. (1952) which superseded the first (1946), included the emended description of B. coagulans by Gordon and Smith (1949). These workers reported that B. calidolacticus Hussong and Hammer, B. thermoacidurans Berry, and B. dextrolacticus Andersen and Werkman, were synonymous with B. coagulans Hammer. Therefore, all cultures designated by these names will be considered to be B. coagulans in this study. The standard description given by these workers is as follows:

"Vegetative rods - 0.6μ to 1.0μ by 2.5μ to 5.0μ; not in chains; uniformly stained; motile; Gram positive.

Spores - 0.9μ to 1.0μ by 1.2μ to 1.5μ; oval; thin-walled; sub-terminal to terminal.

Sporangia - definitely swollen in some cases; not swollen in others.

Colonies - not distinctive.

Nutrient agar slants - growth variable.

NaCl broth - no growth in 5 percent concentration of NaCl.

Glucose-nutrient agar slants - growth soft; moist; more abundant than on nutrient agar.

Utilization of citrate - negative.

Potato - growth erratic.

Soybean agar slants - growth usually heavier than on nutrient agar.

Proteose-peptone acid agar slants - good growth.

Stock-culture agar slants - growth as good as or better than on nutrient agar.

Glucose-asparagine agar slants - growth scant if any.

Tomato-yeast milk - curdled in 1 to 3 days at 45°C.

Hydrolysis of starch - positive.

Production of acetylmethylcarbinol - usually positive.

pH of glucose broth - 5.0 or less at 7 days; acid without gas from glucose.

Hydrolysis of gelatin - negative or weak.

Hydrolysis of casein - negative or weak.

Reduction of nitrate to nitrite - usually negative.

Anaerobic production of gas from nitrate - negative.

Anaerobic growth in glucose broth - positive; pH below 5.2.

Temperature for growth - good growth from 33° to 45°C.; maximum temperature for majority of strains 55° to 60°C.; no growth at 65°C.; poor growth if any at 28°C."

Ford et al. (1952) studied 43 strains of spore-forming bacteria associated with flat-sour spoilage of tomato juice. Although variable with respect to gelatin hydrolysis, growth in 5 percent sodium chloride, and acetylmethylcarbinol production, the majority of the strains constituted a homogeneous group.

The above review shows that a reliable description of B. coagulans is of comparatively recent origin. Minor discrepancies noted probably reflect strain variation.

A special proteose-peptone acid agar medium was recommended for the detection and cultivation of B. thermoacidurans by Stern et al. (1942). Sporulation on this medium was apparently greater than on nutrient agar. Also, the medium is of value in differentiating between B. coagulans and Bacillus stearothermophilus (Smith et al., 1952). Cameron and Bohrer (1948) reported that proteose-peptone acid agar "promotes the growth of the flat sour types of bacteria while inhibiting the growth of types not capable of spoilage of tomato juice. The count on this medium represents the number of Bacillus thermoacidurans and other acid

tolerant types present and is an index of spoilage possibilities". This medium is commercially available in dehydrated form from Difco Laboratories, Detroit, Michigan, under the trade name thermoacidurans agar.

## Nutrition

Reports on the nutritional requirements of B. coagulans are somewhat controversial and few data are available on its nutritional requirements at 37°C. This temperature was suggested by Berry (1933) to be the optimum temperature for flat-sour spoilage of tomato juice. Some reports (Campbell and Williams, 1953a,b; Bhat and Bilimoria, 1955) indicate that growth temperatures may influence the nutritional requirements of this organism. The absence of numerical data in most reports makes it difficult to assess the quality of the substrates employed and impossible to determine the quantitative effects of various modifications on growth.

Andersen and Werkman (1940) found that Bacillus dextrolacticus required either thiamin or riboflavin and an unidentified component found in an ether soluble fraction of acidified yeast extract and in hydrolyzed casein. Requirement for this latter factor was satisfied by 3 amino acids; viz., glutamic acid, cystine, and either of arginine or threonine. A single

strain of B. coagulans (N.R.S. No. 27) studied by Cleverdon et al. (1949) grew at 37° and 55°C. in a basal medium containing L-cystine, DL-tryptophan, thiamin, biotin, and niacin. Growth and sporulation were better at the lower temperature. In contrast to these reports, Allen (1953) found that 3 strains of B. coagulans grew in a simple basal medium containing glucose and monosodium glutamate. The addition of casein hydrolysate permitted an additional 2 strains to grow, whereas another 2 strains preferred a mixture of carbon sources and a richer mineral complement. Allen reported that a large excess of calcium ions replaced the biotin and riboflavin requirements for a number of thermophiles.

In a more comprehensive study, Knight and Proom (1950) investigated 15 new isolates of B. coagulans along with a reference strain (A.T.C.C. 7050) of this organism. They used an incubation temperature of 45°C. The nitrogen requirements were satisfied by casein digest but not by ammonia. Although thiamin and biotin were usually required, some strains, or selected variants thereof, became self-sufficient with respect to one or the other of these vitamins upon subculture. In further studies, Proom and Knight (1955) found this group to be nutritionally homogeneous with respect to their requirements for

biotin and thiamin in a 14-amino acid medium. The amino acid demands were considered complex in the light of the absence of growth in a 7-amino acid medium. The authors could not explain differences in their results from those of Cleverdon et al. (1949) and of Allen (1953).

Baker et al. (1953) mentioned that a strain of B. coagulans grew in a basal medium supplemented with either glycerol or carbohydrate. Later, Baker et al. (1955) suggested employing a strain of B. coagulans for folic acid assay at 55°C. in an unsterilized substrate.

Campbell and Williams (1953a) noted that the vitamin and amino acid requirements of B. coagulans (N.C.A. strains 2, 12, 32, 1039) may vary with the temperature of incubation. Folic acid was required by all strains at 45° and 55°C but not necessarily at 36°. In another publication (1953b), they reported on variation with respect to the biotin requirement of 6 strains of this organism (N.C.A. strains 16, 18, 32, 43P, 831, and 1039). Whereas, oxybiotin fulfilled the biotin requirements of all strains at different temperatures of incubation, desthiobiotin, pimelic acid, aspartic acid, oleic acid, and aspartic plus oleic acid varied in this respect. None of the tricarboxylic acid cycle intermediates replaced biotin

at 36°, 45°, or 55°C.

Bhat and Bilimoria (1955) found that B. coagulans failed to grow in a medium containing inorganic nitrogen at 37° and 55°C. in the presence or absence of biotin. Studies with 2 strains at 55°C. showed that both required sulphur-containing amino acids and one strain, also, required thiamin and histidine. However, neither strain required these supplements at 37°C.

A summary of the nutritional requirements of B. coagulans found in the literature is given in Table 1. Although the vitamin and amino acid requirements of this organism are probably not numerous, much conflicting evidence indicates that there are many unexplained factors.

TABLE 1

The vitamin and/or amino acid requirements of *B. coagulans* as noted by various workers

| Reference | Requirements noted |
|---|---|
| Allen (1953) | Specific requirements not clearly defined. |
| Andersen and Werkman (1940) | Thiamin *or* riboflavin; arginine *or* threonine; glutamic acid; cystine. |
| Baker *et al.* (1955) | Folic acid. Strain No. 3084 recommended for assay of this vitamin with incubation at 55°C. |
| Bhat and Bilimoria (1955) | Thiamin, S-containing amino acids, and histidine required at 55° but not at 37°C. |
| Campbell and Williams (1953a) | Thiamin and biotin required at 36°, 45°, 55°C. Requirement for folic acid, niacin, valine, histidine, leucine, methionine, and tryptophan varied with incubation temperature. |
| Cleverdon *et al.* (1949) | Thiamin, biotin, niacin, cystine, and tryptophan required at 37° and 55°C. |
| Knight and Proom (1950)<br>Proom and Knight (1955) | Thiamin and biotin required at 45°C. Amino acids also required, but individual demands not shown. |

## EXPERIMENTAL METHODS AND MATERIALS

### Identification and Methods of Handling Cultures

Cultures of *Bacillus coagulans* were selected from a collection of spore-forming bacteria isolated from wash waters in Canadian tomato juice processing plants (Johns, 1953). This involved the screening of 177 cultures and the final identification of the *B. coagulans* strains. Cultures were purified by plating at least three times on thermoacidurans agar (Difco) (DTA), pH 5.0, in conjunction with microscopic observations. The organisms were then screened to determine their spoilage potentialities and to secure information on the selectivity of DTA.

The ability of the spores and of the vegetative cells of each organism to produce flat-sour spoilage in 5.0 ml. of tomato juice, pH 4.4, when incubated for 21 days at 37°C. was determined both aerobically and anaerobically. To fulfill the latter condition for the vegetative cells, tubes of tomato juice sterilized for 10 minutes under 15 lb. steam pressure were boiled to exhaust the dissolved oxygen, rapidly cooled in cold water (5°C), and inoculated with a 3.0 mm. loopful of washed cells from 24-hour DTA slant cultures grown at 37°C. These tubes were sealed immediately with sterile vaspar. For anaerobic inoculation with spores, saline suspensions were similarly prepared from DTA slopes after sporulation

was confirmed microscopically. Here, tubes of tomato juice inoculated with two 3.0 mm. loopfuls of suspension were heated for 10 minutes at 180°F. to destroy vegetative cells and exhaust the dissolved oxygen; subsequently, they were rapidly cooled and immediately sealed with vaspar. For aerobic studies, inoculated tubes remained unsealed. The following authentic strains of B. coagulans[1] were included for reference purposes: 43P, C2253, 711, and 7050.

Following incubation, tubes were generally examined macroscopically, microscopically, and pH determinations made with a Beckman model H2, glass electrode, pH meter. The type of reaction produced by each organism in tomato juice was compared with its reaction at 37°C. on DTA containing 0.004 percent bromcresol green indicator. Thirty-seven cultures representative of the varied reactions obtained in tomato juice were selected and identified according to the methods of Smith et al. (1952) with the following modifications:

(a) Yeast milk - tomato juice was excluded from this medium because of the variable results noted by Smith et al. in its presence.

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1. Supplied by Dr. C.S. Pederson, New York State Experimental Farm, Geneva, N.Y.

(b) Proteose peptone acid agar - DTA was substituted here since it is essentially the same in composition.

(c) Starch hydrolysis - Plates were flooded with Lugol's iodine solution instead of alcohol.

(d) pH determinations - the potentiometric method was substituted for the colorimetric method.

(e) Citrate utilization - Koser citrate medium (Difco) supplemented with 15 gm. agar and 0.08 gm. bromthymol blue indicator per liter was used in place of the modification of this medium employed by Smith *et al*.

The following authentic cultures of the genus *Bacillus* were included for reference purposes during the procedure of identification: *B. subtilis*, *B. polymyxa*, *B. macerans*, *B. cereus*, *B. cereus* var. *mycoides*, *B. pumilus*, *B. stearothermophilus*, and *B. coagulans*.

Fifteen cultures identified as *B. coagulans* were selected from the above group of cultures along with strains 43P, C2253, 711, and 7050 for nutritional studies. The following authentic strains were included in the study after their identity was confirmed by the methods of Smith *et al*.: strain 1604 supplied by Dr. A.H. Jones, Department of Agriculture, Ottawa, Canada, who isolated it from commercial tomato juice showing evidence of flat-sour spoilage; strain B-36

obtained from the National Research Council, Ottawa, Canada. Strain 12245 obtained from the American Type Culture Collection.

At first, cultures were carried on normal DTA. This medium proved unsatisfactory for certain cultures as evidenced by their sporadic response after storage for one month at 5°C. The difficulty was attributed to their frequent failure to sporulate on this medium and the subsequent dying off of the vegetative cells during storage. Consequently, cultures were carried routinely on nutrient agar (Difco) fortified with 0.25 percent yeast extract and 0.1 percent dextrose for nutritional studies (pH 6.8). This medium was a modification of one suggested by Becker and Pederson (1950) which resulted in earlier and more complete sporulation of B. coagulans than occurred on proteose-peptone acid agar.

Cells to be used for inoculum in nutritional studies were obtained from 20-24 hour broth cultures. In the preliminary work, cells were harvested by centrifugation, washed 3 times in 0.9 percent saline and resuspended in saline to give a final light transmittance of 75 percent at 545 mμ in a Luxtrol photoelectric colorimeter. In the determination of the vitamin requirements in semi-synthetic media, cells were washed in distilled water. Cells washed in M/20 phosphate buffer were employed in the determination of vitamin and amino acid requirements

in synthetic media and in the subsequent work on stimulation. A volume of 0.05 ml. was used to inoculate test media. An incubation temperature of 37°C. was used in all instances.

## Methods of Cleaning Glassware

Pipettes were soaked overnight in chromic acid cleaning solution, rinsed in running tap water in an automatic pipette washer for one day, rinsed thoroughly in distilled water, autoclaved in distilled water, and dried in a hot-air oven.

In the early work, pyrex test tubes and flasks were cleaned in hot $H_2SO_4$ containing $KNO_3$, rinsed in running tap water until neutral to bromthymol blue indicator, rinsed thoroughly in distilled water, autoclaved in distilled water, and dried in a hot-air oven. In later work, owing to the large number required, test tubes were detergent-cleaned with Alconox[1] in a manner similar to that described by Campbell and Williams (1953a).

## Methods of Estimating Growth

Growth was recorded as percent transmittance after 72 hours incubation using a Luxtrol photoelectric colorimeter at 545 mμ wavelength. In preliminary studies

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1. Manufactured by Alconox Inc., Jersey City, N.J.

on the influence of carbon source and initial pH, the incubation temperature was extended to 96 hours. In one case (Appendix VII), growth in semi-synthetic and synthetic media was also compared by titration with N/10 NaOH to the phenolphthalein endpoint.

## Media and Reagents

Vitamins, amino acids, trypsin 1-300, and vitamin-free enzymically-hydrolysed casein were obtained from Nutritional Biochemicals Inc., Cleveland, Ohio; vitamin-free casamino acids, micro inoculum broth, yeast extract, nutrient agar, and thermoacidurans agar were obtained from Difco Laboratories Inc., Detroit, Michigan; inorganic chemicals, dextrose, etc. were obtained from Fisher Scientific Company, Montreal, Quebec; vitamin and fat-free casein used in stimulation studies was a product of the British Drug House, Toronto, Ontario.

Metabolites and reagents were incorporated into media from stock solutions of appropriate strength prior to adjusting the final volume. Except in the preliminary studies on the influence of the initial pH level on development, all media were adjusted to pH 6.8-7.0 prior to sterilization. The pH was checked colorimetrically after sterilization using bromthymol blue.

# RESULTS

## Studies of the Spore-forming Bacteria Isolated from Tomato Wash Water

It may be noted in Fig. 1 (Appendix I-V) that only about one-third of the 177 spore-forming cultures isolated from tomato wash water produced sufficient acid in tomato juice to lower the pH significantly. This was true when grown under either aerobic or anaerobic conditions. Many of the cultures failed to affect the pH significantly and still others brought about a decided increase. However, those organisms which increased the pH of tomato juice failed to do so under anaerobic conditions. This evidence is of particular interest since all of these cultures were able to develop on thermoacidurans agar. This raises considerable question as to the value of this medium for detecting the presence of flat-sour producing organisms in tomato juice. The results of this experiment were similar when spores were used for inocula.

In the unsealed tubes, growth of the acid producers was manifested macroscopically by a sparse, delicately-flaky pellicle, with small white granules extending about ½ inch beneath the surface. Anaerobic growth of these organisms was evidenced microscopically by long, thin, filamentous cells. Acid production was usually accompanied by an off-flavor and odor.

Organisms failing to lower the pH of tomato juice did not grow in sealed tubes. They usually produced thick, cheesy pellicles and alkaline reactions. Extensive proteolysis of the juice by this latter group was apparent.

When the cultures were streaked on DTA containing bromcresol green, the acid producers were readily distinguished within 24 hours at 37°C. These, including the authentic flat-sour strains, rendered the medium yellow while the colonies per se were a distinctive deep yellow-green. Acid-tolerant, apparent non-acid producers either failed to affect the color indicator during growth or rendered the medium alkaline (blue). Increasing the dextrose concentration in DTA from 0.5 to 1.0 percent permitted the acid producers to reach their limiting hydrogen ion concentration; otherwise, they apparently first exhausted the dextrose and then either utilized the organic acids produced, or increased the pH by proteolytic action. The medium usually became more alkaline within 72 hours. Omission of phosphate buffer from the medium resulted in reduced acid production as shown by a slower change in and a reduced final intensity of the color.

Based on the methods of Smith *et al.* (1952), 19 of the 37 isolates selected for taxonomic study were identified as *B. coagulans*, 3 as *B. licheniformis* and 15 as *B. subtilis*.

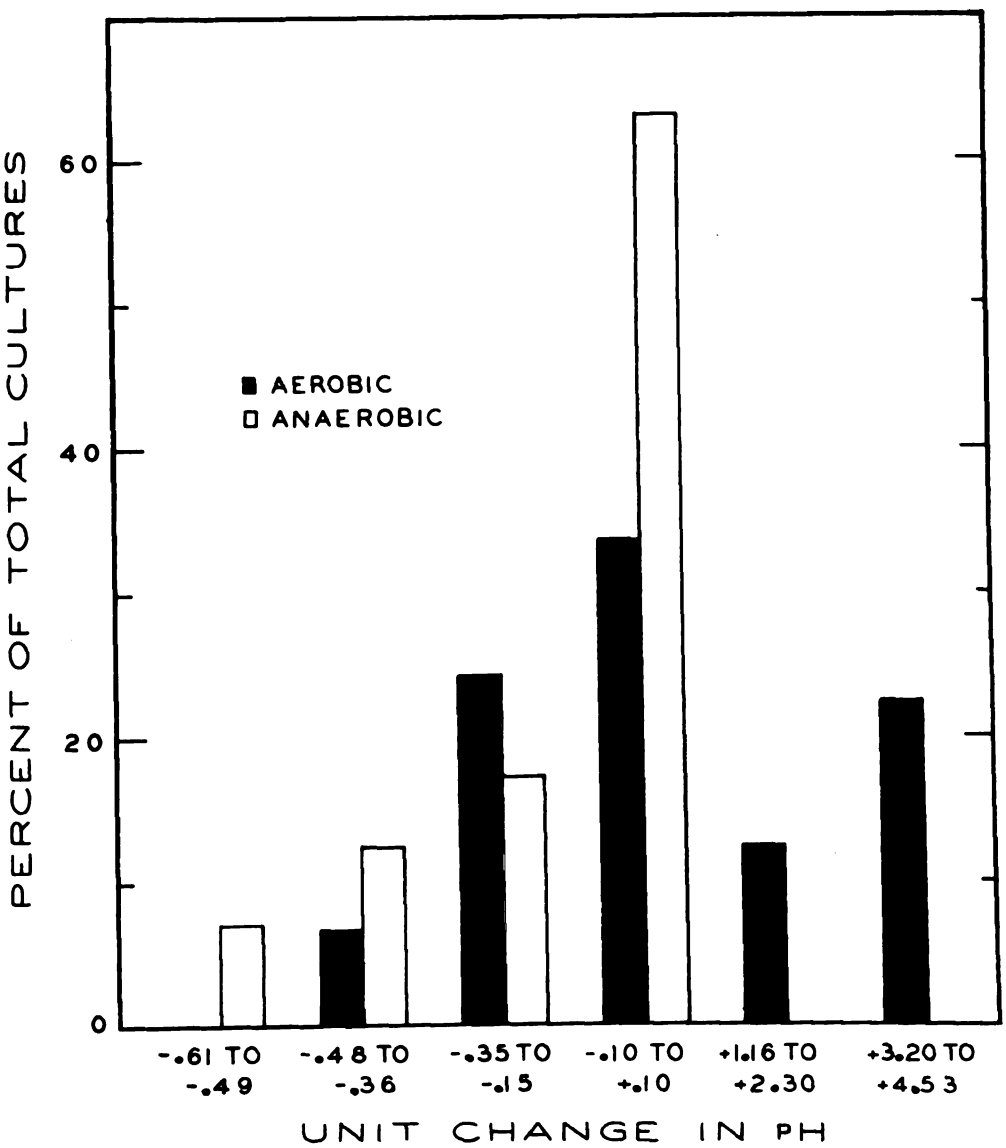


Figure 1. Distribution of spore-forming cultures from tomato wash water with respect to the unit change in pH produced in tomato juice.

## Preliminary Nutritional Studies

The existing controversy concerning the nutritional requirements of B. coagulans indicated that a preliminary study would be in order prior to the selection or development of a synthetic medium in which all strains would flourish consistently. Consequently, the following series of experiments was performed.

Effect of pH on development - Strains 1604, 43P, and C2253 were grown in 5.0 ml. of micro inoculum broth (Difco). Cells were then washed and resuspended in saline and inoculated into a medium of the following composition per 100 ml. (substrate CAY):

| | |
|---|---|
| Casamino acids (Difco) | 5.0 gm. |
| Yeast extract | 0.5 gm. |
| NaCl | 100 mg. |
| $K_2HPO_4$ | 100 mg. |
| $KH_2PO_4$ | 100 mg. |
| Salts solution X[1] | 0.5 ml. |

Aliquots of this medium were adjusted to pH levels of 7.5, 7.0, 6.0, and 5.5 and dispensed in 10.0 ml. amounts in cotton-plugged, optically-standardized test tubes. Following autoclaving, 0.25 ml. of sterile dextrose solution (0.4 mg/ml.)

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1. $MgSO_4.7H_2O$, 10.0 gm.; $FeSO_4$, 0.273 gm.; $MnSO_4.H_2O$, 0.379 gm.; distilled water 250 ml. (modified from Johnson. 1949.).

was added per tube. This gave a final concentration of about 1 percent.

It was evident from the results of this work (Figure 2, Appendix VI) that the strains of B. coagulans tested had a longer lag phase at pH 7.5 than at the lower pH's. However a greater number of cells was produced after extended incubation at pH 7.5 and 7.0 than at the two lower levels. Consequently, a pH of 7.0 was considered appropriate in subsequent studies.

Effect of different carbon sources on growth - The same experimental procedure described for pH studies was employed in these experiments except that individual aliquots of medium CAY adjusted to pH 7.0 were modified by adding 1.0 percent malate, succinate, fumarate, or citrate in place of glucose as a carbon source.

Table 2 shows that of the carbon sources tested, glucose was decidedly superior. Citrate was not utilized. Growth in the other sources was feeble and the cells were extremely difficult to disperse for accurate turbidity readings. Therefore, glucose was used routinely as a carbon source in all further studies.

Replacement of yeast extract - Good growth of each strain occurred in medium CAY but failed to occur when yeast extract was omitted. Therefore, a preliminary attempt was made to grow a few representative strains in medium CAY with a mixture of ten vitamins of the

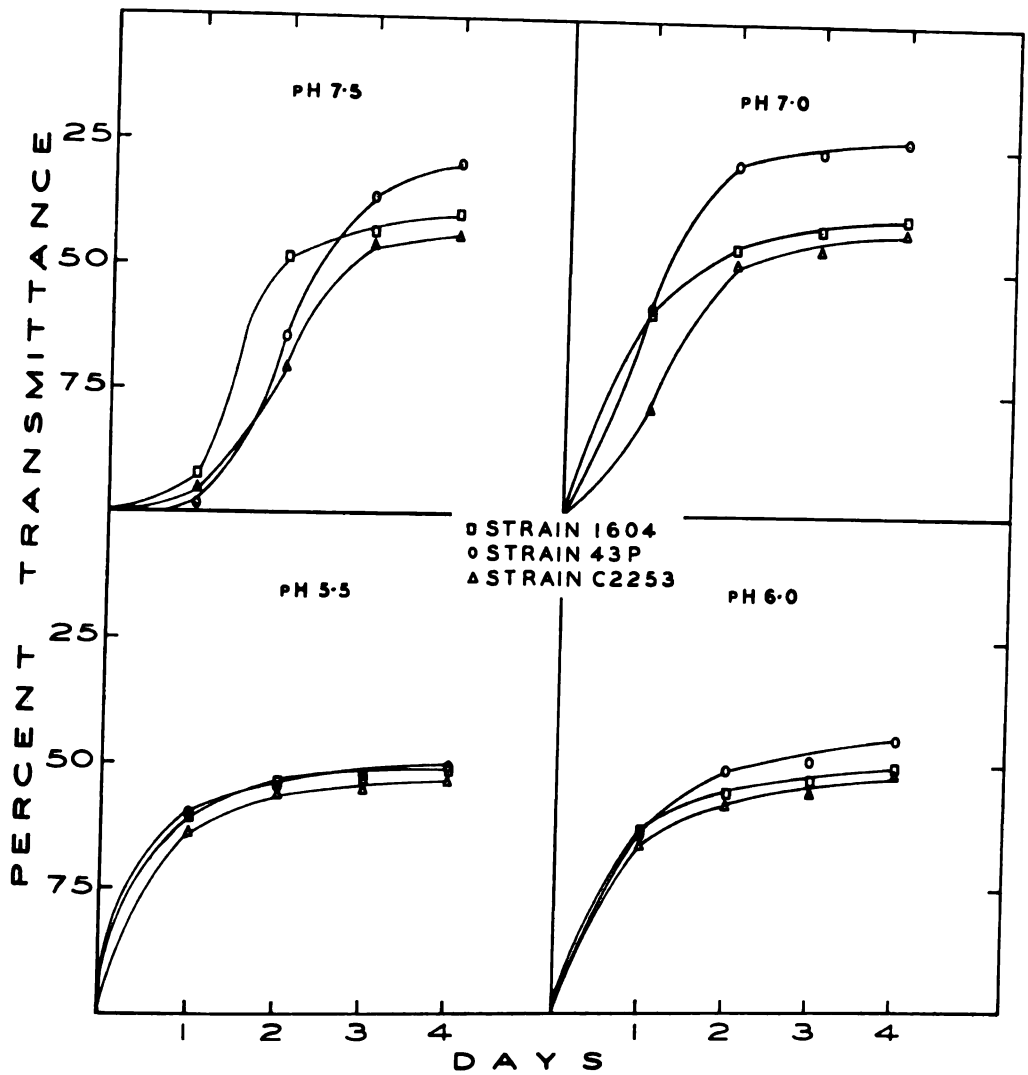



Figure 2. Effect of initial pH on the growth of B. coagulans.

TABLE 2

Growth of B. coagulans using different carbon sources

| Carbon source | Incubation time (days) | Culture | | |
|---|---|---|---|---|
| | | 1604 | 43P | C2253 |
| | | Percent transmittance | | |
| Glucose | 1 | 58.5 | 58.2 | 78.1 |
| " | 2 | 45.5 | 28.9 | 48.7 |
| " | 3 | 42.0 | 26.3 | 46.0 |
| " | 4 | 39.1 | 23.9 | 42.1 |
| Malate | 1 | 98.2 | 99.3 | 97.6 |
| " | 2 | --* | -- | 91.0 |
| " | 3 | -- | -- | 87.3 |
| " | 4 | 80.3 | 85.6 | 80.1 |
| Succinate | 1 | -- | 99.7 | 97.8 |
| " | 2 | -- | -- | 88.6 |
| " | 3 | -- | -- | 83.8 |
| " | 4 | 78.0 | 81.1 | 77.5 |
| Fumarate | 1 | 93.7 | 98.5 | 97.8 |
| " | 2 | -- | -- | 86.7 |
| " | 3 | -- | -- | 83.0 |
| " | 4 | 76.2 | 78.8 | 76.0 |
| Citrate | 1 | 99.5 | 100 | 100 |
| " | 4 | 99.5 | 100 | 100 |

* -- Growth difficult to disperse

B-complex plus a mixture of pyrimidines and purines replacing yeast extract. The inoculum was prepared by growing the organisms in medium CAY, and the cells were washed and resuspended in saline.

The results appeared promising in that partial replacement of yeast extract occurred with vitamins alone. Pyrimidines and purines usually provided slight stimulation but did not support growth in the absence of vitamins.

It now appeared feasible to attempt to determine the specific vitamin requirements of each strain. Medium CAY was modified by replacing yeast extract with the following vitamin complement to form the basal medium (medium CAV) for these tests.

| | Concentration/100 ml. |
|---|---|
| Thiamin.HCl | 50.0 µgm. |
| Pyridoxine.HCl | 50.0 µgm. |
| Pyridoxal.HCl | 50.0 µgm. |
| Ca pantothenate | 50.0 µgm. |
| Niacin | 50.0 µgm. |
| Riboflavin | 50.0 µgm. |
| Inositol | 50.0 µgm. |
| Choline | 50.0 µgm. |
| Paraaminobenzoic acid (PABA) | 50.0 µgm. |
| Pyridoxamine.HCl | 10.0 µgm. |
| Folic acid | 0.2 µgm. |
| Biotin | 0.1 µgm. |

Also, DL-tryptophan, 10.0 mg. and L-cysteine.HCl, 10.0 mg., were added per 100 ml. of substrate.

Tests for individual vitamin requirements were made by deleting individual vitamins from this medium and for pyridoximers by deleting them as a group. Medium CAY supplemented with tryptophan and cysteine with and without yeast extract were run as controls.

Although all strains grew as expected in the presence of yeast extract, growth in the CAV medium with the complete vitamin supplement was sporadic. This fact was difficult to account for in the light of the successful preliminary trials.

Attempts were made to improve growth in medium CAV by replacing cysteine with cystine and by adding the following substances per 100 ml. of medium individually: $CaCl_2$, 5.0 mg.; sodium oleate, 5.0 mg.; sodium acetate, 50.0 mg.; the ash from 0.5 gm. of yeast extract; biotin, 0.9 µgm. In addition, the influence of the concentration of casamino acids on growth was determined by preparing medium CAV with the following percentages of this component: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, and 2.0.

None of these modifications was effective in eliminating the sporadic results obtained in medium CAV. Cystine appeared more favorable than cysteine in the presence of the vitamin mixture but this effect was

not apparent in the presence of yeast extract. The addition of $CaCl_2$ or sodium acetate was inconsequential. More biotin favored one strain. Responses were markedly reduced by oleate and no growth occurred in the medium supplemented with yeast extract ash. Increasing the casamino acids concentration resulted in an increased response in medium CAY which fell off above the 1.0 percent level but growth remained sporadic in medium CAV.

Casamino acids vs. enzymically-hydrolyzed casein - Since the vitamin complement of medium CAV was fairly complete, it was believed that some other growth factor(s) must be limiting. Growth-promoting factors have been reported to be present in enzymically-hydrolyzed casein (Kitay and Snell, 1950; Kizer et al., 1955; Sprince and Woolley, 1945; Wright and Skeggs, 1944). Therefore, it seemed advisable to try substituting this amino acid source for the casamino acids. Media CAY and CAV were prepared along with the usual control medium without vitamins or yeast extract. Similar media were prepared in parallel with 0.5 percent enzymically-hydrolyzed casein (Nutritional Biochemicals Corp.) substituted for casamino acids. The inoculum was prepared as before in medium CAY. It was found that the strains tested grew markedly better in the media with enzymic hydrolysate than in those containing acid hydrolysate (Table 3). Further, the enzymic hydrolysate

TABLE 3

Growth of B. coagulans in enzymic vs. acid hydrolysates of casein

| Substrate | Culture | | | | |
|---|---|---|---|---|---|
| | 1604 | 711 | 61 | 13 | 88 |
| | Percent transmittance | | | | |
| Enzymic hydrolysate plus yeast extract | 44.5 | 14.1 | 17.4 | 19.2 | 24.7 |
| Acid hydrolysate plus yeast extract | 44.5 | 29.0 | 46.0 | 37.5 | 48.5 |
| Enzymic hydrolysate plus vitamins | 53.5 | 24.6 | 28.0 | 24.2 | 28.0 |
| Acid hydrolysate plus vitamins | 100 | 100 | 100 | 100 | 100 |

plus known vitamins generally supported better growth than the acid hydrolysate plus yeast extract. No growth was evident in medium CAV in this experiment although varying responses were observed in previous work. Further tests showed that all strains would produce consistently good growth in the media containing the enzymic hydrolysate either with yeast extract or vitamins. Trace growth, which often occurred in control tubes or deficient media, failed to increase on prolonged incubation and gradually disappeared in subculture. This precluded the possibility of adaptation or mutation. Repeated washing of inocula failed to eliminate trace growth.

Development of a synthetic medium - Attempts were made to cultivate each strain in a synthetic medium containing 24 amino acids in concentrations similar to their average in 0.5 gm. of casein (Katznelson, 1955). These were added to the semi-synthetic medium used to determine individual vitamin requirements in place of enzymically-hydrolyzed casein. Attempts were also made to grow the organisms in the medium of Campbell et al. (1953a). Neither of these media supported good growth of all strains and results were inconsistent. Consequently, studies were conducted in an effort to formulate a synthetic medium in which all strains would produce consistently good growth. The chemical

composition of enzymic casein hydrolysate was obtained from the manufacturer. From these data, a synthetic medium was devised which contained amino acids in approximately the same concentrations which would be obtained by the use of 0.5 percent enzymic hydrolysate. The complete medium was of the following composition per 100 ml.:

| | |
|---|---|
| L-lysine | 38.0 mg. |
| DL-isoleucine | 32.0 mg. |
| L-leucine | 52.5 mg. |
| DL-valine | 35.0 mg. |
| L-arginine | 16.5 mg. |
| DL-threonine | 20.0 mg. |
| DL-methionine | 12.5 mg. |
| L-cystine | 1.5 mg. |
| DL-phenylalanine | 22.5 mg. |
| L-histidine | 12.0 mg. |
| L-tryptophan | 4.5 mg. |
| L-glutamic acid | 102.0 mg. |
| Thiamin.HCl | 50.0 µgm. |
| Riboflavin | 50.0 µgm. |
| Niacin | 50.0 µgm. |
| Ca pantothenate | 50.0 µgm. |
| Pyridoxine.HCl | 50.0 µgm. |
| Pyridoxal.HCl | 50.0 µgm. |
| PABA | 50.0 µgm. |

| | |
|---|---|
| Pyridoxamine.HCl | 10.0 µgm. |
| Biotin | 1.0 µgm. |
| Folic acid | 2.0 µgm. |
| Glucose | 1.0 gm. |
| $NH_4Cl$ | 100.0 mg. |
| Na acetate | 50.0 mg. |
| NaCl | 100.0 mg. |
| $K_2HPO_4$ | 100.0 mg. |
| $KH_2PO_4$ | 100.0 mg. |
| Salts solution X | 0.25 ml. |
| Final pH 6.8-7.0 | |

A comparison was made of growth and acid production by 5 strains of B. coagulans in the synthetic medium, the enzymic hydrolysate plus yeast extract (medium CAEY), and the enzymic hydrolysate plus vitamins (medium CAEV). The latter medium contained the same vitamin complement added to the synthetic medium when growth in the two media was to be compared. It was found that medium CAEY supported slightly better growth than medium CAEV (Figure 3; Appendix VII). Also, growth in each of these media was better than in the synthetic medium. However, consistent and reproducible results were obtained in growth tests run with the synthetic. Therefore, this medium was used for vitamin and amino acid studies.

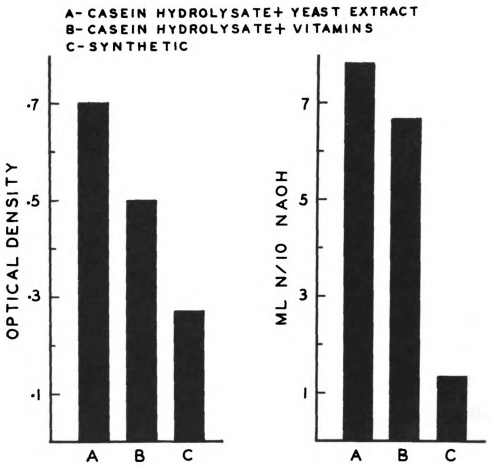



Figure 3. Growth of B. coagulans in synthetic vs. semi-synthetic media.

## Study of Vitamin Requirements

Studies in semi-synthetic media - On the basis of the foregoing work, a basal medium was devised for preparation of inocula and the testing of vitamin requirements. This medium had the following composition per 100 ml.:

| | |
|---|---|
| Enzymic casein hydrolysate | 0.5 gm. |
| NaCl | 100.0 mg. |
| $K_2HPO_4$ | 100.0 mg. |
| $KH_2PO_4$ | 100.0 mg. |
| Na acetate | 50.0 mg. |
| $NH_4Cl$ | 100.0 mg. |
| Salts solution X | 0.25 ml. |
| Final pH 6.9-7.0 | |

After autoclaving, 0.25 ml. of sterile dextrose solution (0.4gm/ml.) was added per tube. For use in preparing inocula, the basal medium was supplemented with 0.5 percent yeast extract. When used to determine vitamin requirements, the vitamin complement used in medium CAV was added. The biotin level was increased from 0.1 µgm to 1.0 µgm/100 ml. of medium since this appeared favorable in earlier studies with medium CAV.

The vitamin requirements were established by deletion of vitamins from the complete medium. The pyridoximers were deleted as a group. Folic acid and PABA were deleted both individually and together. The

remaining vitamins were deleted individually. In all cases, the complete semi-synthetic medium served as control.

All 22 strains of B. coagulans tested were found to require biotin and thiamin for significant growth (Table 4; Appendix VIII and IX). In addition, strain 1604 required niacin and strain 140 either folic acid or PABA. Pantothenate appeared stimulatory for many strains, especially strain 132 which showed much variation in response in its absence. Niacin and choline, also, appeared stimulatory for this strain. PABA and folic acid had little effect on growth when deleted individually; but when deleted together, strain 140 showed a requirement and growth of most strains was reduced. It was concluded that PABA and folic acid were interchangeable. Recently, Baker et al. (1955) reported this same relationship between folic acid and PABA for a single strain of B. coagulans.

Tests were then conducted to determine the ability of each strain to grow in the presence of minimal vitamin supplements. The design of this experiment may be noted in Table 5 (Appendix X). While limited growth of most strains was evident in the presence of the minimal vitamin mixtures, growth in general was reduced. No stimulation by pantothenate was noted even with strain 132. It is of interest that growth was obtained with

TABLE 4

Growth of B. coagulans upon deletion of vitamins from semi-synthetic medium

| Vitamin deleted | Group* | | |
|---|---|---|---|
| | I | II | III |
| | Percent transmittance | | |
| None | 37.1 | 34.9 | 49.3 |
| Thiamin | 96.5** | 98.6 | 98.0 |
| Pyridoximers | 38.9 | 41.3 | 51.8 |
| Pantothenate | 45.2 | 38.8 | 55.2 |
| Niacin | 41.9 | 99.1 | 52.2 |
| Riboflavin | 38.8 | 33.4 | 47.0 |
| Biotin | 96.2 | 96.7 | 97.8 |
| Inositol | 39.5 | 33.7 | 49.1 |
| Choline | 40.3 | 34.0 | 52.0 |
| PABA | 40.1 | 35.7 | 49.1 |
| Folic Acid | 39.3 | 31.0 | 46.9 |
| PABA, Folic Acid | 51.5 | 29.0 | 95.5 |
| All | 96.6 | 99.2 | 99.0 |

* Group I - Twenty Cultures (average)

Group II - Culture 1604

Group III - Culture 140

** All results underlined where a vitamin requirement is evident.

TABLE 5

Growth response of B. coagulans to minimal vitamin mixtures in semi-synthetic medium

| Vitamins present | Group* | | |
|---|---|---|---|
| | I | II | III |
| | Percent transmittance | | |
| All ten (control) | 42.3 | 45.7 | 46.8 |
| Thiamin, niacin, pantothenate, biotin | 57.1 | 42.5 | 69.9 |
| Thiamin, niacin, biotin | 62.0 | 47.4 | 74.8 |
| Thiamin, biotin, pantothenate | 60.9 | 99.0** | 76.1 |
| None | 96.2 | 99.3 | 97.5 |

* Group I - Twenty cultures (average)

Group II - Culture 1604

Group III - Culture 140

** All results underlined where definite vitamin deficiencies are evident.

culture 140 which previously failed to respond in the absence of folic acid and PABA. Sporadic responses with this culture also occurred in subsequent studies in synthetic media.

Vitamin requirements in synthetic medium - The procedures used in these experiments were essentially the same as described for the examination of the vitamin requirements in the semi-synthetic medium. The complete synthetic medium served as control in each case. Strain A.T.C.C. 12245, which had been reported to require folic acid (Baker et al., 1955), was included in this study as an additional reference organism.

The results of the studies of vitamin requirements in the synthetic medium were similar to those found with the semi-synthetic in that all strains of B. coagulans tested required biotin and thiamin and strain 1604 required niacin (Table 6; Appendix XI and XII). Strain 132, although stimulated by pantothenate, did not require it, and the stimulation afforded many other strains by pantothenate in the semi-synthetic medium was not evident in these results. These results differed greatly from the previous ones in that 19 of the 22 strains, including A.T.C.C. 12245, required folic acid or PABA. These vitamins had little or no effect on strain 1604 but afforded marked stimulation for strain 711 and especially for A.T.C.C. 7050. None of these strains, including

TABLE 6

Growth of B. coagulans upon deletion of vitamins from synthetic medium

| Vitamin deleted | Group* | | |
|---|---|---|---|
| | I | II | III |
| | Percent transmittance | | |
| None | 51.7 | 53.6 | 45.5 |
| Thiamin | 94.9** | 98.8 | 95.7 |
| Riboflavin | 51.9 | 52.8 | 45.8 |
| Niacin | 51.9 | 96.9 | 45.0 |
| Pantothenate | 52.3 | 55.1 | 44.6 |
| Pyridoximers | 52.3 | 52.0 | 45.6 |
| Biotin | 96.9 | 98.5 | 95.3 |
| Folic acid | 51.9 | 55.9 | 45.4 |
| PABA | 53.6 | 55.4 | 47.2 |
| PABA, Folic acid | 94.8 | 59.3 | 68.4 |
| All | 95.9 | 98.2 | 96.0 |

* Group I - Nineteen cultures (average)

Group II - Culture 1604

Group III - Two cultures (average)

** All results underlined where a vitamin requirement is evident.

A.T.C.C. 12245, required folic acid or PABA in the semi-synthetic medium. It was tentatively concluded that the enzymic casein hydrolysate either provided factors which partially replaced the folic acid or PABA requirement of B. coagulans or precursors from which this organism could synthesize these vitamins.

## Study of Amino Acid Requirements

The amino acid requirements were determined in the synthetic medium described previously except that riboflavin was omitted from it. Otherwise, the general procedure was essentially the same as employed in vitamin studies. However, instead of being deleted individually, the amino acids were deleted in groups based on their specific type as follows:

Group 1 - DL-valine, L-leucine, DL-isoleucine, and DL-threonine

Group 2 - DL-methionine, and L-cystine

Group 3 - L-glutamic acid

Group 4 - DL-phenylalanine, L-tryptophan, and L-histidine

Group 5 - L-arginine and L-lysine

All tests were performed in duplicate on at least two different days.

None of the strains developed in synthetic media when the nitrogen equivalent as $(NH_4)_2SO_4$ was substituted for amino acids.

The amino acid requirements appeared to be mostly non-specific. Of the 22 strains tested, only 4 required any specific group(s) of amino acids (Table 7; Appendix XIII and XIV). The deletion of Group 1 (DL-valine, L-leucine, DL-isoleucine and DL-threonine) from the medium had a pronounced effect on the growth of only one strain (No. 132). Four strains (C2253, 88, 43P, and 132) were found to require the presence of glutamic acid.

## Studies on Stimulation

Effect of DL-amino acids - A search was made for the factor(s) responsible for the relatively poor growth in the synthetic medium as compared to the semi-synthetic. The first idea investigated concerned the concentrations of some of the amino acids. In formulating the synthetic medium, amino acids were incorporated according to their percent occurrence in the enzymic hydrolysate without regard for the availability of D-isomers in racemic mixtures. Consequently, the growth of 4 strains in the synthetic medium containing twice the concentration of the complement of DL-amino acids used originally, was compared with growth in the normal medium, and with the semi-synthetic medium containing the enzymic hydrolysate. This procedure had no significant effect

TABLE 7

Growth of B. coagulans upon deletion of amino acids from synthetic medium

| Amino acids deleted | Group* | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| | Percent transmittance | | | |
| None | 46.3 | 54.3 | 57.0 | 44.0 |
| DL-valine, DL-threonine, L-leucine, DL-isoleucine | 55.0 | 57.7 | 86.7** | 55.2 |
| DL-methionine, L-cystine | 53.4 | 60.5 | 64.7 | 57.4 |
| L-glutamic acid | 59.3 | 96.2 | 88.4 | 98.0 |
| DL-phenylalanine, L-tryptophan, L-histidine | 47.4 | 57.6 | 72.6 | variable |
| L-arginine, L-lysine | 47.8 | 57.3 | 61.5 | variable |
| All | 95.1 | 95.0 | 96.7 | 98.6 |

* Group I - Eighteen cultures (average)

Group II - Two cultures (average)

Group III - Culture 132

Group IV - Culture 43P

** All results underlined where a definite amino acid requirement is evident.

on the growth response of the strains tested (Table 8).

Effect of non-dialyzable enzymic casein hydrolysate and of trypsin - Experiments were then conducted to determine if stimulation could be removed from the enzymic casein hydrolysate by dialysis and if any marked degree of stimulation was provided by trypsin 1-300. Under present conditions, the specific hydrolytic agent employed in preparing the enzymic hydrolysate was not known and unobtainable. However, Kizer et al. (1955) reported that factors concomitant with impure trypsin were stimulatory for certain lactic acid bacteria.

The normal synthetic medium and the semi-synthetic medium containing 0.5 percent of the enzymic hydrolysate were prepared as controls. Trypsin 1-300, inactivated by heating at 100°C. for 10 minutes, was added to a portion of the synthetic medium at the rate of 0.25 percent as a check on the possibility that stimulation might be associated with this enzyme preparation.

The effect of dialysis was determined as follows: 50 ml. of 5.0 percent enzymic hydrolysate were placed in a cellophane bag of 1-inch diameter and dialyzed in 16 liters of distilled water at 5°C. for 24 hours. The water was changed twice during this period and discarded. The semi-synthetic medium was then prepared with the non-dialyzable fraction of the casein hydrolysate substituted for the normal preparation. One-half of this

TABLE 8

Effect of increased concentration of DL-amino acids on the growth of B. coagulans in synthetic medium

| Substrate | Culture | | | |
|---|---|---|---|---|
| | 141 | 1604 | 10545 | 7050 |
| | Percent transmittance | | | |
| Casein hydrolysate plus vitamins | 22.4 | 25.1 | 26.7 | 64.5 |
| Normal synthetic substrate | 50.4 | 49.1 | 47.1 | 51.6 |
| Synthetic substrate with DL-amino acids doubled | 58.9 | 47.0 | 43.4 | 58.6 |

medium was supplemented with the amino acid complement used in the normal synthetic medium since it was assumed that the bulk of the amino acids were removed from the casein during dialysis.

The addition of trypsin to the normal synthetic medium had no significant effect on the growth of 3 strains of B. coagulans. Growth was markedly reduced in the medium prepared with the undialyzable fraction of the casein hydrolysate; however, this reduction in growth apparently did not result from a deficiency in amino acids (Table 9). Therefore, it appeared that stimulation was associated with a dialyzable factor(s) other than free amino acids.

Effect of various buffers - In a communication with the Nutritional Biochemicals Corporation, it was learned that the pH of their vitamin-free enzymic casein hydrolysate was lowered to about 3.0 (found to be about 4.0 as received) with acetic acid after hydrolysis and that the incorporation of this product in culture media generally provided sufficient acetate to satisfy the demands of most organisms employed for vitamin assay. The possibility came to mind that the acetate residue might contribute as an indirect stimulus by augmenting the inherent buffering capacity of the semi-synthetic medium with respect to acid produced from glucose by responsive strains. Investigations were carried out to check this

TABLE 9

Effect of trypsin and non-dialyzable casein hydrolysate on the growth of B. coagulans

| Substrate | Culture | | |
|---|---|---|---|
| | 141 | 10545 | 1604 |
| | Percent transmittance | | |
| Normal casein hydrolysate | 26.7 | 28.8 | 27.7 |
| Normal synthetic substrate | 51.0 | 49.4 | 47.8 |
| Synthetic substrate plus trypsin 1-300 | 45.6 | 49.2 | 48.7 |
| Dialyzed casein hydrolysate | 64.8 | 63.0 | 64.8 |
| Dialyzed casein hydrolysate plus amino acids | 53.4 | 45.5 | 50.6 |

possibility.

A trypsin hydrolysate of casein was prepared in general according to the methods of Kemmerer and Shapiro (1947) using vitamin and fat-free casein (British Drug House) and trypsin 1-300. After hydrolysis, aliquots of the hydrolysate were acidified to pH 4.0 with acetic acid, $H_3PO_4$, and HCl, respectively. Norit A was added for adsorption and the dialysates filtered under mild suction through Whatman No. 5 filter paper in a Buchner funnel. The respective filtrates were diluted to give a final concentration of 5.0 percent of original casein. The semi-synthetic medium was then prepared using each of these hydrolysates as well as the N.B.C. hydrolysate. In addition, the synthetic medium was prepared and aliquots were supplemented with acetic acid and $H_3PO_4$ at the rate they occurred in the media prepared with the corresponding hydrolysates. The normal synthetic medium served as control in parallel with that prepared with the HCl-acidified hydrolysate.

Media were dispensed in 5.0 ml. lots, autoclaved, and inoculated with strains 1604, 141, and 7050. In addition, the amount of N/10 HCl required to titrate duplicate 50 ml. portions of each medium from pH 7.0 to pH 3.0 was determined electrometrically. It is of interest that the quantity of acetic acid required to

acidify the laboratory-prepared hydrolysate was equivalent to the concentration of total volatile acids in the N.B.C. hydrolysate as determined by steam distillation and subsequent titration with N/10 NaOH to neutrality.

Figure 4 shows typical titration curves obtained by the gradual addition of N/10 HCl to 50.0 ml. quantities of the modified and unmodified synthetic and semi-synthetic media. Individual titration values appear in Appendix XVI. It should be kept in mind that growth in these media falls off at about pH 4.3 so that little significance is attached to buffer action below this level under present conditions. Apparently buffer action may play an important part in stimulation since those media showing greater buffering capacity generally support better growth (Figure 5; Appendix XV). This is particularly evident in the case of strains 141 and 1604 which produced considerably less growth in the HCl-acidified trypsin hydrolysate than in the same hydrolysate acidified with either $H_3PO_4$ or acetic acid, or in the N.B.C. hydrolysate. Growth of these strains in the latter three media approaches equivalence. The growth of strain A.T.C.C. 7050 was not enhanced in the medium containing the N.B.C. hydrolysate as compared with the normal synthetic medium, and less growth was obtained in the medium containing the acetic acid-acidified trypsin hydrolysate.

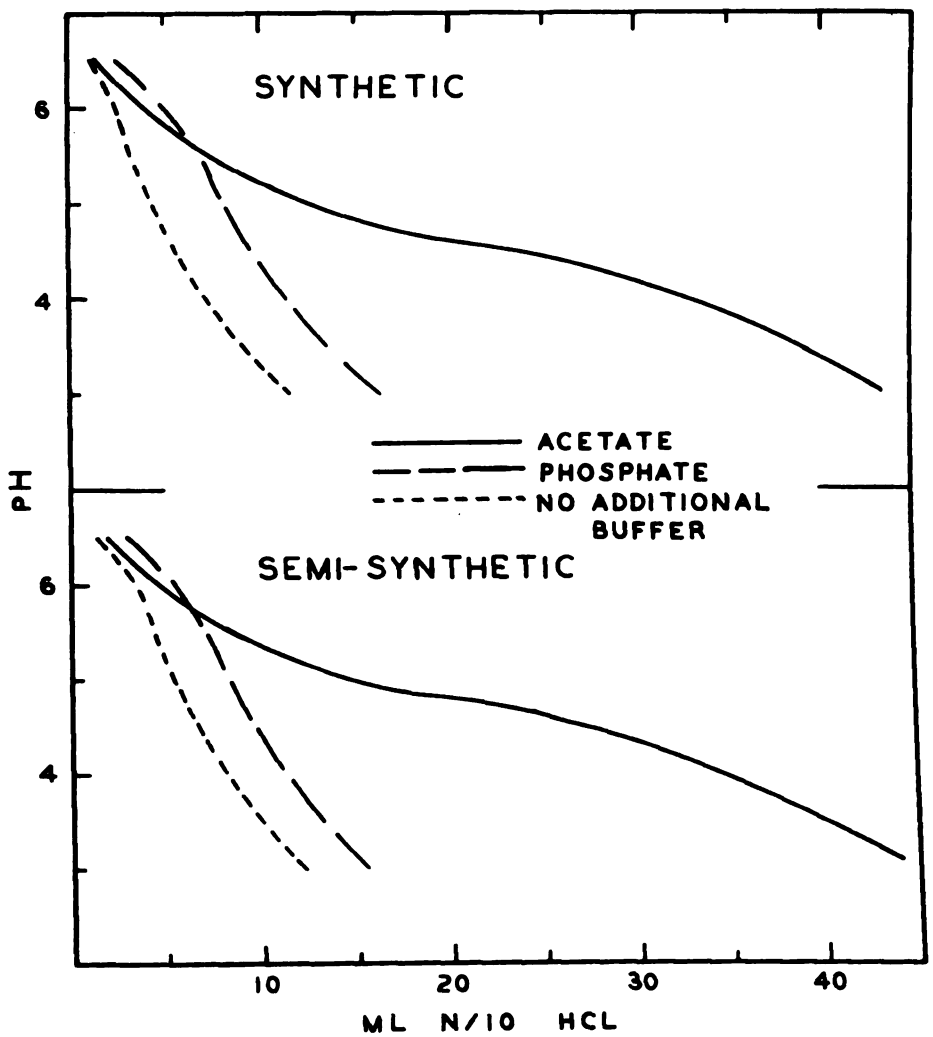
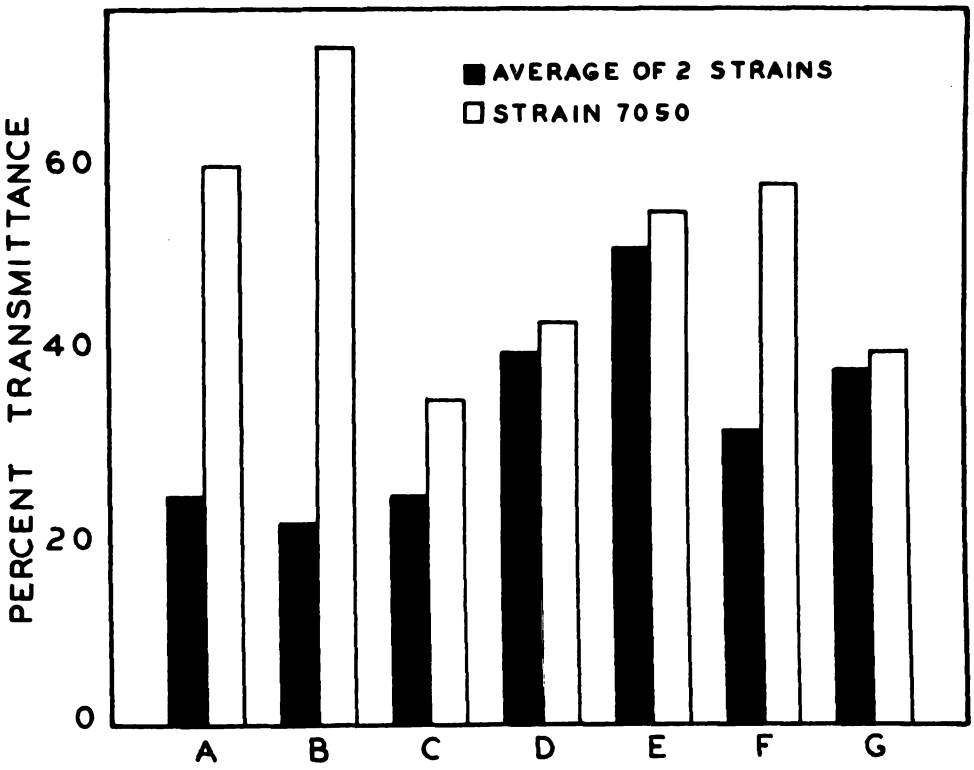



Figure 4. Titration curves for semi-synthetic and synthetic media with and without additional buffers.




A- N.B.C. HYDROLYSATE
B- TRYPSIN HYDROLYSATE + ACETATE
C- TRYPSIN HYDROLYSATE + PHOSPHATE
D- TRYPSIN HYDROLYSATE + HCL
E- SYNTHETIC MEDIUM
F- SYNTHETIC MEDIUM + ACETATE
G- SYNTHETIC MEDIUM + PHOSPHATE

Figure 5. Comparison of modified trypsin hydrolysates and synthetic media with N.B.C. casein hydrolysate.

In the synthetic medium, the response of all three strains was markedly enhanced by phosphate. In contrast, only strains 141 and 1604 reacted favorably to the supplement of acetate. In general, the addition of a suitable buffer to the synthetic medium results in growth either equivalent to or approaching that obtained in the corresponding semi-synthetic medium. Attempts were not made to establish optimum buffer concentrations because of inherent strain variation.

## DISCUSSION

In addition to providing strains of Bacillus coagulans for nutritional studies, this study brought to light an apparent weakness in thermoacidurans agar (Difco). Based on their ability to grow on this medium, each of the 177 isolates might be considered acid-tolerant and a potential spoilage hazard in commercial packs of tomato juice. However, only about 30 percent belonged to this select group. The remainder were of no apparent spoilage significance, especially in view of their inability to grow at a low oxygen tension. Therefore, the ability of bacteria to grow on proteose-peptone acid agar may not necessarily indicate their ability to spoil commercially-packed tomato juice as was suggested by Stern et al. (1942).

Results of this study suggest that the incorporation of a suitable indicator into thermoacidurans agar and increasing its dextrose concentration may enhance its value as a plating medium. Moreover, these modifications may find application in taxonomic studies on aerobic sporeformers since the ability to grow on this medium and lower its pH appears of greater value in segregating species than growth alone.

The study of the vitamin requirements of B. coagulans in semi-synthetic and synthetic media showed that three strains (1604, 711, and A.T.C.C. 7050)

required only biotin and thiamin. These observations agree with those of Knight and Proom (1950) and Proom and Knight (1955) who noted that 16 strains (including A.T.C.C. 7050) of this organism required these same growth factors in semi-synthetic and synthetic media.

Unfortunately, strains requested from Campbell *et al.* (1953a) for comparative purposes were unavailable. However, the results obtained in the synthetic medium and in one instance in the semi-synthetic medium (strain 140) support their observations that many strains may require folic acid in addition to biotin and thiamin. Although three strains (711, 7050, and 1604) did not require folic acid, two of the three were stimulated by this vitamin. This suggests that folic acid is of general importance in the nutrition of *B. coagulans*.

It is of interest that a difference in vitamin requirements in semi-synthetic vs. synthetic media was not observed by Knight and Proom (1950), Proom and Knight (1955). However, this study indicates that the test medium used is an important factor in nutritional studies with *B. coagulans*. This was evidenced by the general requirement for either folic acid or PABA in the synthetic but not in the semi-synthetic medium.

Only one strain tested was shown to require niacin in the present study. Except for single strains

reported to require niacin by Campbell and Williams (1953a) and by Cleverdon et al. (1949), no further reports in this respect were found in the literature. Therefore, the requirement for this growth factor appears rare within the species.

The amino acid requirements of B. coagulans were found to be generally non-specific. However, this is not surprising. Campbell and Williams (1953a) found that specificity in the amino acid requirements shown by B. coagulans at higher temperatures of incubation (45° and 55°C.) may disappear at a lower incubation temperature (36°C.). Also, Bhat and Bilimoria (1955) noted that certain metabolites necessary for growth at 55°C., were non-essential at 37°C. Nevertheless, the organisms still required organic nitrogen. Apparently, this species possesses synthesizing mechanisms which function at low temperatures but not at high.

This study indicated that glutamic acid may play an important role in the nutrition of B. coagulans, either as an essential metabolite or as a growth supplement. Allen (1953) and Andersen and Werkman (1940) noted that glutamic acid may be important in the nutrition of this organism.

In this study, most strains of B. coagulans grew poorly in synthetic media in the absence of glucose

indicating that amino acids serve primarily as a nitrogen source.

The lesser growth response obtained in the synthetic medium as compared with enzymatic digest (Nutritional Biochemicals Corp.) apparently did not result from a deficiency in L-amino acids; but, the results indicated that the concentration and proportion of DL-amino acids may be important with different strains. Also, stimulation did not appear to be associated with trypsin since its addition to the synthetic medium had a negligible effect on growth. Therefore, the factor(s) associated with certain proteolytic enzymes reported to be stimulatory for certain lactic acid bacteria by Kizer et al. (1955) did not appear to be in operation here.

When the synthetic medium or the laboratory prepared trypsin hydrolysates were supplemented with a similar concentration of acetate to that found in the N.B.C. hydrolysate, approximately the same degree of stimulation generally resulted for the strains tested. Since acetate could be replaced by phosphate, it is tentatively concluded that buffering action is primarily responsible for stimulation. This may explain why stimulation was removed by dialysis since acetate could readily pass through the membrane.

Noteworthy, is the failure of strain A.T.C.C.

7050 to show evidence of stimulation in media supplemented with acetate, although excellent growth resulted in its absence, or when media were supplemented with phosphate. It is hypothesized that the extent of growth of this strain was limited by the increasing amount of undissociated acetic acid accumulating during the gradual drop in pH resulting from glucose fermentation. Thus, it is assumed that variation in strain tolerance exists toward acetic acid, and the same may be true for other organic acids (Rice and Pederson, 1954). Phosphate appears to be the more suitable buffer in view of its relatively non-toxic character.

The sporadic results and frequent poor responses obtained using casamino acids as a nitrogen source, and the failure to obtain good growth of all strains in the medium of Campbell and Williams (1953), are not readily explainable. That the synthetic medium developed herein is considerably higher in total N than that of Campbell and Williams, and further differs in the type and proportion of amino acids present, might account for the different results. This idea is in line with the results of Proom and Knight (1955) who obtained growth of 16 strains of B. coagulans in a 14-amino acid medium, but not in a medium containing only 7 amino acids.

## SUMMARY

One-hundred and seventy-seven spore-forming bacteria isolated from the wash waters of Canadian tomato juice packing plants were screened to determine the ability of their spores and vegetative cells to grow aerobically and anaerobically in tomato juice. Although each organism grew well on thermoacidurans agar (Difco) (DTA), about 70 percent were not considered a commercial spoilage hazard owing to their failure to develop in tomato juice at a low oxygen tension. Aerobically, these cultures produced alkaline reactions in tomato juice and on DTA, whereas the remaining 30 percent generally produced acid reactions and could develop at a low oxygen tension. It was concluded that routine bacteria counts on DTA do not provide a true index of spoilage possibilities. However, results indicated that the addition of 0.004 percent bromcresol green and increasing the dextrose concentration to 1.0 percent rendered it more valuable as a plating medium for detecting bacteria capable of causing flat-sour spoilage of tomato juice.

Of 37 of the canning plant isolates selected for taxonomic study, 19 were identified as Bacillus coagulans, 3 as Bacillus licheniformis, and 15 as Bacillus subtilis.

The vitamin and amino acid requirements of 15 of the canning plant isolates identified as B. coagulans and 8 authentic strains of this organism were studied at 37°C. In a medium comprising vitamin-free acid hydrolyzed casein, yeast extract, glucose, and mineral salts, growth occurred earlier at pH 5.5 than at 7.5. Substitution of malate, succinate, fumarate or citrate for glucose in this medium indicated that citrate was not utilized whereas response to the remainder was feeble. The sporadic results obtained in this medium when a mixture of vitamins was substituted for yeast extract were not due to a deficiency of cysteine or tryptophan. This condition was corrected by substituting vitamin-free, enzymically-hydrolyzed casein for the acid hydrolysate.

Biotin and thiamin were required by all strains in the semi-synthetic medium. In addition, one strain also required niacin and another folic acid.

An additional requirement for folic acid (or PABA) was noted for most strains in a synthetic medium containing 12 amino acids. This probably reflected inherent differences in composition between the semi-synthetic and synthetic menstrua.

With few exceptions, the amino acid requirements were generally non-specific at 37°C. Glutamic acid

appeared essential for a few strains and provided stimulation for others.

The enhanced growth generally afforded by enzymic casein hydrolysate (Nutritional Biochemicals Corp.) was largely attributed to buffering action resulting from its high acetate content. Phosphate was considered a more suitable buffer for B. coagulans since one strain proved acetate sensitive.

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# APPENDIX

TABLE I

Final pH of tomato juice inoculated with spore-forming bacteria isolated from tomato wash waters. (Incubated 21 days at 37°C.)

| Culture No. | Vegetative cells | | Spores | |
|---|---|---|---|---|
| | Unsealed | Sealed | Unsealed | Sealed |
| Control | 4.35 | 4.50 | 4.35 | 4.45 |
| 2 | 4.36 | 4.48 | 4.52 | 4.48 |
| 3 | 8.21 | 4.69 | 8.35 | 4.50 |
| 4 | 8.55 | 4.45 | 8.48 | 4.49 |
| 5 | 4.41 | - | 4.45 | 4.47 |
| 6 | 4.33 | 4.40 | 4.40 | 4.46 |
| 7 | 4.35 | 4.40 | 4.45 | 4.40 |
| 8 | 4.44 | - | 4.49 | 4.48 |
| 9 | 4.35 | - | 4.40 | 4.50 |
| 10 | 4.42 | - | 4.40 | 4.39 |
| 11 | 8.75 | 4.75 | 8.32 | 4.50 |
| 11A | 8.42 | 4.45 | 8.45 | 4.50 |
| 12 | 5.76 | 4.49 | 5.38 | 4.49 |
| 13 | 3.95 | 4.08 | 3.99 | 4.00 |
| 14 | 4.29 | 4.65 | 4.33 | 4.37 |
| 15 | 5.68 | 4.48 | 5.15 | 4.50 |
| 16 | 4.35 | - | 4.48 | 4.48 |
| 17 | 8.45 | 4.65 | 7.78 | 4.52 |
| 18 | 5.75 | 4.47 | 5.24 | 4.49 |
| 19 | 4.01 | 3.98 | 4.37 | 4.48 |
| 20 | 4.38 | 4.42 | 4.45 | 4.50 |
| 21 | 4.35 | 4.50 | 4.40 | 4.49 |
| 22 | 4.36 | - | 4.45 | 4.45 |
| 23 | 4.25 | - | 4.30 | 4.40 |
| 24 | 8.62 | 4.51 | 8.64 | 4.52 |
| 25 | 5.72 | - | 5.45 | 4.48 |
| 26 | 4.36 | 4.58 | 4.45 | 4.49 |
| 27 | 4.00 | 4.10 | 4.36 | 4.48 |
| 28 | 5.51 | 4.68 | 5.35 | 4.52 |
| 29 | 5.82 | 4.45 | 5.46 | 4.49 |
| 30 | 4.01 | 4.18 | 4.08 | 4.18 |
| 31 | 8.60 | 4.65 | 7.92 | 4.50 |
| 31A | 8.17 | 4.45 | 7.65 | 4.48 |
| 32 | 4.05 | 4.23 | 4.12 | 4.14 |
| 33 | 4.10 | 4.21 | 4.25 | 4.28 |
| 34 | 4.29 | 4.32 | 4.26 | 4.43 |
| 35 | 4.36 | 4.40 | 4.41 | 4.46 |
| 36 | 4.38 | - | 4.40 | 4.48 |

TABLE II

Final pH of tomato juice inoculated with spore-forming bacteria isolated from tomato wash waters. (Incubated 21 days at 37°C.)

| Culture No. | Vegetative cells | | Spores | |
|---|---|---|---|---|
| | Unsealed | Sealed | Unsealed | Sealed |
| Control | 4.35 | 4.50 | 4.35 | 4.45 |
| 37 | 4.01 | 4.15 | 4.02 | 4.15 |
| 38 | 4.35 | 4.45 | 4.45 | 4.50 |
| 39 | 5.77 | 4.48 | 5.30 | 4.52 |
| 40 | 8.50 | 4.60 | 7.75 | 4.50 |
| 40A | 8.66 | 4.58 | 8.29 | 4.54 |
| 41 | 5.69 | 4.47 | 5.42 | 4.45 |
| 42 | 4.07 | 4.23 | 4.05 | 4.16 |
| 43 | 4.14 | 4.32 | 4.27 | 4.20 |
| 44 | 8.75 | 4.49 | 8.92 | 4.50 |
| 44A | 8.55 | 4.48 | 8.05 | 4.51 |
| 45 | 4.30 | 4.37 | 4.35 | 4.39 |
| 46 | 4.35 | 4.46 | 4.45 | 4.48 |
| 47 | 8.45 | 4.75 | 8.30 | 4.45 |
| 47A | 8.70 | - | 8.70 | 4.52 |
| 48 | 4.01 | 4.45 | 3.92 | 4.04 |
| 49 | 4.08 | 4.31 | 4.22 | 4.15 |
| 50 | 3.99 | 4.15 | 4.02 | 4.15 |
| 51 | 3.99 | 4.03 | 4.32 | 4.45 |
| 52 | 3.95 | 3.97 | 4.20 | 4.09 |
| 53 | 4.27 | - | 4.38 | 4.39 |
| 54 | 5.66 | 4.51 | 4.38 | 4.48 |
| 55 | 5.72 | - | 5.62 | 4.52 |
| 56 | 5.52 | 4.40 | 5.02 | 4.48 |
| 57 | 4.02 | 4.32 | 4.07 | 4.22 |
| 58 | 4.33 | 4.48 | 4.42 | 4.47 |
| 59 | 4.10 | 4.13 | 4.08 | 4.09 |
| 60 | 4.02 | 4.12 | 4.35 | 4.07 |
| 61 | 4.16 | 4.37 | 4.11 | 4.18 |
| 62 | 5.53 | 4.60 | - | - |
| 63 | 4.35 | 4.60 | 7.97 | 4.49 |
| 63A | 7.55 | 4.48 | 8.20 | 4.50 |
| 64 | 4.02 | 4.13 | 4.15 | 4.22 |
| 65 | 4.00 | 4.02 | 4.38 | 4.07 |
| 67 | 4.12 | 4.49 | - | - |
| 69 | 4.35 | 4.08 | 4.36 | 4.12 |
| 70 | 3.93 | 3.98 | 3.99 | 3.99 |
| 71 | 5.68 | - | 5.32 | 4.48 |
| 72 | 4.03 | 4.17 | 4.35 | 4.20 |

TABLE III

Final pH of tomato juice inoculated with spore-forming bacteria isolated from tomato wash waters. (Incubated 21 days at 37°C.)

| Culture No. | Vegetative cells | | Spores | |
|---|---|---|---|---|
| | Unsealed | Sealed | Unsealed | Sealed |
| Control | 4.35 | 4.50 | 4.35 | 4.45 |
| 73 | 4.35 | - | 4.48 | 4.46 |
| 74 | 4.08 | 4.22 | 4.15 | 3.98 |
| 75 | 4.11 | 4.22 | 4.27 | 4.15 |
| 76 | 4.37 | - | 4.45 | 4.53 |
| 77 | 4.34 | 4.45 | 4.50 | 4.48 |
| 78 | 4.07 | 4.45 | 4.35 | 4.22 |
| 79 | 4.40 | 4.50 | 4.38 | 4.48 |
| 79A | 4.35 | 4.49 | 4.45 | 4.51 |
| 80 | 4.38 | - | 4.50 | 4.50 |
| 81 | 4.03 | 4.22 | 4.07 | 4.25 |
| 82 | 4.03 | 4.30 | - | - |
| 83 | 5.74 | 4.48 | 5.23 | 4.48 |
| 84 | 4.02 | 4.19 | 4.05 | 4.10 |
| 85 | 4.04 | 4.18 | 4.48 | 4.52 |
| 86 | 4.40 | 4.40 | 4.45 | 4.42 |
| 87 | 4.40 | 4.48 | 4.38 | 4.45 |
| 88 | 4.07 | 3.98 | 4.10 | 3.95 |
| 89 | 4.37 | 4.37 | - | - |
| 90 | 4.37 | 4.65 | 4.42 | 4.50 |
| 91 | 5.75 | - | 5.88 | 3.96 |
| 92 | 4.28 | - | 4.32 | 4.35 |
| 93 | 4.35 | 4.32 | 4.33 | 4.40 |
| 94 | 6.00 | 4.50 | 5.82 | 4.45 |
| 95 | 6.65 | 4.53 | 4.35 | 4.46 |
| 95A | 4.38 | - | 4.45 | 4.48 |
| 96 | 4.26 | 4.35 | 4.32 | 4.45 |
| 97 | 4.32 | - | 4.42 | 4.45 |
| 98 | 4.05 | 4.22 | 4.04 | 3.99 |
| 99 | 4.30 | - | 4.33 | 4.40 |
| 100 | 4.10 | 4.08 | 4.07 | 4.04 |
| 101 | 5.81 | 4.42 | 5.52 | 4.47 |
| 102 | 8.78 | 4.75 | 8.02 | 4.52 |
| 103 | 3.96 | 4.02 | 4.00 | 3.95 |
| 105 | 4.06 | 4.22 | - | - |
| 106 | 5.76 | 4.51 | 4.06 | 4.18 |
| 107 | 8.31 | 4.53 | 5.28 | 4.48 |
| 108 | 4.37 | - | 7.70 | 4.50 |

TABLE IV

Final pH of tomato juice inoculated with spore-forming bacteria isolated from tomato wash waters. (Incubated 21 days at 37°C.)

| Culture No. | Vegetative cells | | Spores | |
|---|---|---|---|---|
| | Unsealed | Sealed | Unsealed | Sealed |
| Control | 4.35 | 4.50 | 4.35 | 4.45 |
| 109 | 8.75 | - | 7.95 | 4.53 |
| 110 | 4.10 | 4.18 | 4.13 | 4.03 |
| 111 | 4.05 | 4.18 | 4.35 | 4.12 |
| 112 | 8.42 | 4.53 | 8.66 | 4.50 |
| 112A | 8.32 | 4.75 | 8.72 | 4.48 |
| 113 | 4.42 | 4.39 | 4.44 | 4.52 |
| 114 | 4.02 | 3.90 | 4.00 | 3.92 |
| 115 | 4.35 | 4.45 | 4.49 | 4.48 |
| 116 | 4.37 | - | 7.85 | 4.50 |
| 116A | 8.07 | 4.48 | 8.35 | 4.43 |
| 117 | 5.70 | 4.45 | 5.05 | 4.50 |
| 118 | 4.35 | 4.39 | 4.40 | 4.47 |
| 119 | 4.02 | 4.15 | 4.08 | 4.05 |
| 120 | 8.50 | - | 8.21 | 4.52 |
| 121 | 4.35 | - | 4.41 | 4.49 |
| 121A | 4.36 | 4.51 | 4.49 | 4.49 |
| 122 | 5.60 | 4.47 | 5.52 | 4.49 |
| 123 | 8.15 | 4.75 | 8.22 | 4.49 |
| 124 | 8.56 | 4.48 | 8.23 | 4.50 |
| 124A | 8.48 | 4.52 | 8.18 | 4.50 |
| 125 | 4.27 | - | 4.29 | 4.49 |
| 126 | 4.35 | 4.38 | 4.38 | 4.45 |
| 126A | 4.37 | 4.45 | 4.45 | 4.47 |
| 127 | 3.91 | 3.91 | 3.98 | 3.93 |
| 128 | 8.58 | 4.46 | 8.50 | 4.51 |
| 128A | 8.39 | 4.62 | 8.30 | 4.50 |
| 129 | 8.40 | 4.48 | 8.42 | 4.51 |
| 130 | 4.26 | - | 4.36 | 4.38 |
| 131 | 3.96 | 4.04 | 3.99 | 3.99 |
| 132 | 4.02 | 4.12 | 4.02 | 3.98 |
| 133 | 3.97 | 4.02 | 4.38 | 4.14 |
| 134 | 4.07 | 4.00 | 3.95 | 4.02 |
| 135 | 3.87 | 4.08 | 3.92 | 3.95 |
| 136 | 8.85 | 4.43 | 8.45 | - |
| 136A | 8.75 | 4.64 | 8.55 | 4.52 |
| 137 | 8.56 | 4.49 | 8.68 | 4.49 |
| 137A | 8.82 | 4.48 | 8.47 | 4.39 |
| 138 | 4.20 | 4.35 | 4.37 | 4.48 |

TABLE V

Final pH of tomato juice inoculated with spore-forming bacteria isolated from tomato wash waters. (Incubated 21 days at 37°C.)

| Culture No. | Vegetative cells | | Spores | |
|---|---|---|---|---|
| | Unsealed | Sealed | Unsealed | Sealed |
| Control | 4.35 | 4.50 | 4.35 | 4.45 |
| 139 | 8.69 | 4.65 | 8.58 | 4.50 |
| 139A | 8.55 | 4.46 | 8.68 | 4.52 |
| 140 | 4.13 | 4.05 | 4.15 | 4.00 |
| 141 | 3.99 | 4.00 | 4.08 | 4.07 |
| 142 | 4.11 | 4.18 | 4.09 | 4.15 |
| 143 | 5.68 | 4.42 | 5.52 | 4.48 |
| 144 | 4.05 | 4.20 | 4.35 | 4.21 |
| 145 | 8.75 | 4.65 | 8.55 | 4.55 |
| 145A | 8.80 | 4.75 | 8.65 | 4.52 |
| 146 | 4.43 | - | 4.50 | 4.50 |
| 147 | 4.13 | 4.08 | 4.40 | 4.15 |
| 148 | 4.40 | 4.51 | 4.45 | 4.48 |
| 149 | 3.97 | 4.15 | 4.35 | 4.45 |
| 150 | 4.35 | 4.42 | 4.38 | 4.48 |
| 151 | 4.35 | 4.29 | - | - |
| 152 | 8.40 | 4.46 | 8.64 | 4.45 |
| 152A | 8.70 | 4.47 | 8.22 | 4.51 |
| 152B | 8.75 | 4.46 | 8.42 | 4.46 |
| 152C | 8.28 | 4.51 | 8.62 | 4.53 |
| 153 | 4.86 | - | 5.45 | 4.39 |
| 154 | 4.33 | 4.39 | 4.38 | 4.49 |
| 155 | 4.30 | - | 4.40 | 4.45 |
| 156 | 4.35 | 4.38 | 4.45 | 4.48 |
| 157 | 4.02 | 4.18 | 4.14 | 4.18 |
| 158 | 4.16 | 4.23 | 4.26 | 4.23 |
| 159 | 4.06 | 4.11 | 4.14 | 4.15 |
| 160 | 4.39 | - | 4.45 | 4.45 |
| 161 | 4.36 | 4.45 | 4.47 | 4.51 |
| C2253 | 4.02 | 4.15 | 4.07 | 4.15 |
| 711 | 4.08 | 4.13 | 4.12 | 4.10 |
| 7050 | 4.17 | 4.18 | 4.35 | 4.48 |
| 43P | 3.96 | 3.95 | 3.95 | 3.95 |

TABLE VI

Effect of initial pH on development of *B. coagulans* in glucose broth.

| Initial pH | Incubation (days) | Culture | | |
|---|---|---|---|---|
| | | 1604 | 43P | C2253 |
| | | Percent transmittance | | |
| 7.5 | 1 | 92.3 | 99.0 | 95.0 |
| " | 2 | 48.2 | 64.2 | 70.2 |
| " | 3 | 43.0 | 36.3 | 46.0 |
| " | 4 | 39.8 | 29.3 | 43.9 |
| 7.0 | 1 | 58.5 | 58.2 | 78.1 |
| " | 2 | 45.5 | 28.9 | 48.7 |
| " | 3 | 42.0 | 26.3 | 46.0 |
| " | 4 | 39.1 | 23.9 | 42.1 |
| 6.0 | 1 | 62.8 | 63.0 | 66.4 |
| " | 2 | 55.5 | 51.0 | 58.4 |
| " | 3 | 52.9 | 49.8 | 56.2 |
| " | 4 | 51.3 | 45.0 | 51.9 |
| 5.5 | 1 | 60.6 | 60.5 | 64.3 |
| " | 2 | 53.5 | 54.9 | 55.7 |
| " | 3 | 53.2 | 53.0 | 55.2 |
| " | 4 | 51.6 | 50.7 | 53.1 |

TABLE VII

Growth of B. coagulans in semi-synthetic vs.synthetic media.

| Medium | Culture | | | | |
|---|---|---|---|---|---|
| | 141 | 10545 | 103 | 1604 | 711 |
| | Percent transmittance | | | | |
| Casein hydrolysate plus yeast extract | 17.8 | 18.4 | 28.3 | 17.4 | 18.4 |
| Casein hydrolysate plus vitamins | 25.1 | 30.8 | 42.1 | 31.9 | 28.4 |
| Synthetic medium | 56.6 | 53.1 | 58.2 | 57.8 | 43.0 |
| | Ml. N/10 NaOH | | | | |
| Casein hydrolysate plus yeast extract | 8.94 | 8.62 | 6.74 | 7.00 | 7.80 |
| Casein hydrolysate plus vitamins | 6.80 | 7.86 | 5.58 | 5.44 | 6.62 |
| Synthetic medium | 1.06 | 1.22 | 0.72 | 1.64 | 1.35 |

TABLE VIII

Growth of B. coagulans upon deletion of vitamins from semi-synthetic medium.

| Vitamin deleted | Culture | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10545 | 70 | 103 | 127 | 1604 | 140 | 74 | B-36 | 7050 | 711 | 42 |
| | Percent transmittance | | | | | | | | | | |
| None | 33.5 | 27.2 | 38.5 | 26.5 | 34.9 | 49.3 | 45.7 | 40.8 | 64.9 | 26.3 | 44.2 |
| Thiamin | 97.1 | 97.1 | 95.0 | 97.2 | 98.6 | 98.0 | 95.8 | 90.0 | 98.1 | 98.9 | 97.8 |
| Pyridoximers | 35.5 | 30.2 | 41.0 | 27.3 | 41.3 | 51.8 | 42.5 | 37.9 | 62.2 | 32.2 | 50.7 |
| Pantothenate | 43.0 | 31.7 | 41.6 | 35.0 | 38.8 | 55.2 | 40.4 | 49.6 | 71.9 | 28.3 | 54.6 |
| Niacin | 35.1 | 31.7 | 42.4 | 36.5 | 99.1 | 52.2 | 40.2 | 42.4 | 70.2 | 27.3 | 53.5 |
| Riboflavin | 35.7 | 29.2 | 41.6 | 30.4 | 33.4 | 47.0 | 43.2 | 42.0 | 70.6 | 27.3 | 51.1 |
| Biotin | 92.4 | 93.0 | 96.5 | 96.2 | 96.7 | 97.8 | 98.5 | 95.9 | 97.6 | 91.7 | 99.2 |
| Inositol | 37.1 | 30.0 | 42.7 | 27.8 | 33.7 | 49.1 | 42.2 | 41.6 | 70.1 | 25.3 | 54.2 |
| Choline | 36.4 | 29.4 | 42.5 | 29.5 | 34.0 | 52.0 | 43.0 | 41.0 | 66.4 | 27.5 | 50.1 |
| PABA | 35.8 | 25.6 | 39.1 | 28.5 | 35.7 | 49.1 | 42.2 | 44.7 | 64.8 | 33.4 | 47.8 |
| Folic acid | 33.1 | 28.9 | 42.0 | 28.5 | 31.1 | 46.9 | 43.3 | 42.8 | 69.2 | 27.1 | 49.0 |
| PABA, Folic acid | 53.0 | 33.7 | 56.3 | 46.2 | 29.0 | 95.5 | 50.1 | 46.7 | 63.9 | 39.6 | 72.8 |
| All | 93.6 | 96.7 | 96.5 | 95.4 | 99.2 | 99.0 | 97.8 | 95.9 | 97.3 | 95.8 | 98.4 |

* All results underlined where a vitamin requirement was evident.

TABLE IX

Growth of B. coagulans upon deletion of vitamins from semi-synthetic medium.

| Vitamin deleted | Culture | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 141 | 48 | 88 | C2253 | 13 | 61 | 132 | 43P | 114 | 32 | 134 |
| | Percent transmittance | | | | | | | | | | |
| None | 29.0 | 27.2 | 41.1 | 37.2 | 26.2 | 24.5 | 54.7 | 36.4 | 36.0 | 41.2 | 40.6 |
| Thiamin | 93.2* | 99.0 | 93.8 | 99.6 | 99.1 | 99.0 | 94.1 | 97.6 | 93.3 | 94.7 | 99.5 |
| Pyridoximers | 30.3 | 30.1 | 40.2 | 41.3 | 27.5 | 23.8 | 65.7 | 35.6 | 34.4 | 43.8 | 45.9 |
| Pantothenate | 31.4 | 28.9 | 57.3 | 49.4 | 35.4 | 30.6 | 85.9 | 46.4 | 37.9 | 56.2 | 48.6 |
| Niacin | 33.5 | 27.6 | 43.8 | 46.1 | 30.4 | 25.1 | 71.5 | 39.6 | 38.8 | 46.5 | 56.6 |
| Riboflavin | 28.0 | 30.9 | 36.8 | 42.1 | 27.8 | 24.5 | 63.4 | 37.2 | 37.1 | 38.9 | 37.9 |
| Biotin | 94.1 | 98.5 | 98.5 | 93.1 | 93.1 | 96.9 | 98.9 | 97.2 | 97.4 | 96.1 | 98.5 |
| Inositol | 28.9 | 28.1 | 45.8 | 42.8 | 27.3 | 24.7 | 60.9 | 37.1 | 39.3 | 44.5 | 40.5 |
| Choline | 32.9 | 28.4 | 38.4 | 43.3 | 31.6 | 24.0 | 75.1 | 38.6 | 35.9 | 41.6 | 49.7 |
| PABA | 33.8 | 29.1 | 45.7 | 47.3 | 29.0 | 27.2 | 68.1 | 41.8 | 31.7 | 44.7 | 41.3 |
| Folic acid | 33.9 | 29.1 | 38.4 | 44.4 | 28.9 | 23.3 | 68.1 | 39.3 | 35.1 | 41.1 | 40.0 |
| PABA, Folic acid | 32.2 | 43.4 | 56.0 | 42.4 | 43.1 | 39.8 | 76.8 | 52.8 | 52.1 | 65.9 | 62.9 |
| All | 94.6 | 97.9 | 97.1 | 97.1 | 94.6 | 95.5 | 98.6 | 96.5 | 96.9 | 96.7 | 99.2 |

* All results underlined where a vitamin requirement was evident.

TABLE X

Growth response of B. coagulans to minimal vitamin mixtures in semi-synthetic medium.

| Culture | Vitamins present | | | | |
|---|---|---|---|---|---|
| | All ten | Thiamin Biotin Niacin Panto-thenate | Thiamin Biotin Niacin | Thiamin Biotin Panto-thenate | None |
| | Percent transmittance | | | | |
| 10545 | 34.3 | 52.8 | 59.3 | 57.9 | 93.3 |
| 70 | 30.1 | 46.2 | 47.2 | 48.9 | 92.1 |
| 103 | 54.9 | 62.5 | 63.7 | 68.4 | 97.9 |
| 127 | 28.4 | 47.1 | 55.7 | 51.9 | 95.4 |
| 1604 | 45.7 | 42.5 | 47.4 | 99.0 | 99.3 |
| 140 | 46.8 | 69.9 | 74.8 | 76.1 | 97.5 |
| 74 | 46.8 | 61.0 | 62.3 | 63.0 | 96.7 |
| B-36 | 47.4 | 59.3 | 62.0 | 62.4 | 92.9 |
| 7050 | 71.7 | 70.8 | 70.7 | 75.3 | 97.7 |
| 711 | 27.0 | 39.4 | 47.4 | 44.9 | 94.6 |
| 42 | 45.4 | 60.8 | 76.2 | 72.2 | 98.8 |
| 141 | 28.1 | 52.7 | 42.1 | 46.8 | 97.7 |
| 48 | 29.9 | 47.2 | 46.9 | 46.3 | 98.2 |
| 88 | 43.5 | 68.8 | 75.2 | 66.8 | 98.2 |
| C2253 | 48.3 | 47.7 | 55.9 | 52.6 | 95.0 |
| 13 | 33.5 | 42.3 | 57.9 | 52.5 | 93.1 |
| 61 | 24.3 | 50.3 | 56.4 | 56.8 | 96.1 |
| 132 | 72.9 | 85.2 | 92.2 | 88.8 | 97.8 |
| 43P | 45.3 | 52.5 | 69.8 | 61.1 | 97.4 |
| 114 | 34.8 | 48.3 | 51.9 | 44.7 | 96.4 |
| 32 | 51.1 | 67.7 | 72.0 | 72.4 | 96.2 |
| 134 | 49.1 | 79.8 | 75.9 | 84.0 | 98.9 |

TABLE XI

Growth of B. coagulans upon deletion of vitamins from synthetic medium.

| Vitamin deleted | Culture | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 141 | 48 | 88 | C2253 | 13 | 61 | 132 | 43P | 114 | 32 | 134 |
| | Percent transmittance | | | | | | | | | | |
| None | 58.4 | 48.5 | 59.5 | 51.9 | 43.2 | 36.9 | 63.7 | 43.6 | 52.1 | 58.0 | 53.3 |
| Thiamin | 93.5 | 97.4 | 94.7 | 93.9 | 95.0 | 96.6 | 96.6 | 99.3 | 92.4 | 96.2 | 96.6 |
| Riboflavin | 58.0 | 48.6 | 55.7 | 52.8 | 42.5 | 37.6 | 62.5 | 41.5 | 54.3 | 58.0 | 53.1 |
| Niacin | 59.3 | 49.3 | 60.0 | 50.9 | 41.0 | 39.0 | 64.7 | 46.1 | 52.2 | 57.5 | 54.7 |
| Pantothenate | 59.9 | 50.5 | 60.2 | 51.1 | 42.3 | 36.8 | 72.1 | 44.3 | 51.0 | 61.1 | 53.9 |
| Pyridoximers | 59.8 | 48.6 | 60.7 | 52.4 | 43.0 | 38.2 | 64.9 | 41.6 | 53.9 | 59.0 | 54.1 |
| Biotin | 99.1 | 97.2 | 96.2 | 92.4 | 99.7 | 96.7 | 99.2 | 99.1 | 98.5 | 98.3 | 97.7 |
| Folic acid | 59.9 | 49.9 | 58.7 | 52.1 | 41.7 | 35.8 | 63.8 | 45.7 | 50.7 | 56.9 | 51.9 |
| PABA | 58.7 | 50.1 | 57.6 | 51.8 | 42.2 | 35.2 | 64.6 | 52.4 | 52.0 | 56.4 | 55.1 |
| PABA, Folic acid | 93.2 | 92.6 | 92.5 | 96.1 | 94.3 | 92.9 | 97.0 | 95.8 | 95.9 | 98.5 | 96.4 |
| All | 94.1 | 98.9 | 94.3 | 96.2 | 94.8 | 98.7 | 95.8 | 96.5 | 96.8 | 98.3 | 93.5 |

* All results underlined where a vitamin requirement was evident.

TABLE XII

Growth of B. coagulans upon deletion of vitamins from synthetic medium

| Vitamin deleted | Culture | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10545 | 70 | 103 | 127 | 1604 | 12245 | 74 | B-36 | 7050 | 711 | 42 |
| | Percent transmittance | | | | | | | | | | |
| None | 53.4 | 39.2 | 62.0 | 46.2 | 53.6 | 50.5 | 52.8 | 54.6 | 59.1 | 31.9 | 54.5 |
| Thiamin | 93.2* | 93.4 | 94.9 | 94.3 | 98.8 | 92.8 | 92.5 | 96.8 | 93.6 | 97.8 | 93.4 |
| Riboflavin | 54.4 | 42.7 | 63.4 | 48.0 | 52.8 | 50.0 | 52.2 | 53.2 | 59.6 | 31.9 | 57.1 |
| Niacin | 56.0 | 38.1 | 63.2 | 44.9 | 96.9 | 48.7 | 52.9 | 52.3 | 58.9 | 31.1 | 54.6 |
| Pantothenate | 53.4 | 43.0 | 61.8 | 44.2 | 55.1 | 48.3 | 52.9 | 52.6 | 58.4 | 30.8 | 54.1 |
| Pyridoximers | 55.1 | 41.8 | 62.6 | 46.3 | 52.0 | 49.1 | 53.0 | 54.1 | 59.6 | 31.6 | 55.2 |
| Biotin | 98.7 | 94.3 | 97.9 | 95.6 | 98.5 | 94.4 | 96.5 | 93.2 | 96.9 | 93.6 | 95.9 |
| Folic acid | 54.4 | 42.8 | 59.6 | 46.9 | 55.9 | 49.6 | 54.0 | 53.8 | 59.9 | 30.8 | 57.2 |
| PABA | 48.8 | 41.5 | 62.4 | 47.5 | 55.4 | 70.4 | 54.5 | 58.7 | 61.1 | 33.3 | 58.7 |
| PABA, Folic acid | 92.7 | 93.2 | 92.6 | 96.3 | 59.3 | 93.1 | 95.6 | 92.8 | 80.8 | 55.9 | 98.9 |
| All | 94.1 | 94.5 | 95.4 | 96.4 | 98.2 | 95.3 | 97.4 | 93.0 | 94.8 | 97.1 | 98.7 |

* All results underlined where a vitamin requirement was evident.

TABLE XIII

Growth of B. coagulans upon deletion of amino acids from synthetic medium.

| Amino acids deleted | Culture | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 141 | 48 | 88 | C2253 | 13 | 61 | 132 | 43P | 114 | 32 | 134 |
| | Percent transmittance | | | | | | | | | | |
| None | 52.8 | 41.2 | 53.9 | 54.7 | 40.9 | 36.4 | 57.0 | 44.0 | 50.1 | 52.2 | 46.2 |
| All | 94.5* | 97.0 | 92.8 | 97.1 | 93.8 | 95.7 | 96.7 | 98.6 | 93.2 | 97.3 | 95.2 |
| DL-valine<br>DL-threonine<br>L-leucine<br>DL-isoleucine | 59.0 | 57.2 | 59.8 | 55.6 | 44.6 | 42.9 | 86.7 | 55.2 | 55.9 | 62.5 | 64.2 |
| DL-methionine<br>L-cystine | 54.5 | 52.5 | 66.2 | 54.8 | 49.5 | 49.9 | 64.7 | 57.4 | 54.0 | 56.5 | 50.7 |
| L-glutamic acid | 62.6 | 55.4 | 93.9 | 99.4 | 53.2 | 58.8 | 88.4 | 98.0 | 65.9 | 68.6 | 57.9 |
| DL-phenylalanine<br>L-tryptophan<br>L-histidine | 54.3 | 41.1 | 63.6 | 51.5 | 40.9 | 37.5 | 72.6 | Var.** | 49.3 | 53.1 | 48.2 |
| L-arginine<br>L-lysine | 63.2 | 45.8 | 64.1 | 50.4 | 44.4 | 38.2 | 61.5 | Var. | 50.6 | 53.7 | 49.8 |

* All results underlined where an amino acid requirement was evident.
** Growth variable.

TABLE XIV

Growth of B. coagulans upon deletion of amino acids from synthetic medium.

| Amino acids deleted | Culture | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10545 | 70 | 103 | 127 | 1604 | 12245 | 74 | B-36 | 7050 | 711 | 42 |
| | Percent transmittance | | | | | | | | | | |
| None | 45.7* | 41.1 | 56.9 | 42.0 | 47.6 | 47.3 | 46.3 | 46.2 | 53.8 | 32.3 | 54.9 |
| All | 93.3 | 93.1 | 92.8 | 96.8 | 97.7 | 93.4 | 95.2 | 93.0 | 96.4 | 97.2 | 96.5 |
| DL-valine<br>DL-threonine<br>L-leucine<br>DL-isoleucine | 50.6 | 47.7 | 62.9 | 47.8 | 51.9 | 50.1 | 61.2 | 54.5 | 72.7 | 44.7 | 58.9 |
| DL-methionine<br>L-cystine | 54.5 | 46.6 | 56.9 | 50.2 | 55.0 | 56.3 | 52.6 | 56.7 | 58.6 | 47.8 | 57.9 |
| L-glutamic acid | 62.8 | 54.6 | 69.8 | 59.3 | 55.6 | 54.1 | 55.8 | 64.4 | 64.4 | 35.8 | 67.8 |
| DL-phenylalanine<br>L-tryptophan<br>L-histidine | 49.1 | 39.9 | 59.3 | 43.2 | 49.3 | 45.5 | 49.0 | 49.9 | 57.6 | 29.5 | 57.0 |
| L-arginine<br>L-lysine | 48.4 | 42.9 | 57.0 | 45.2 | 50.0 | 40.5 | 50.5 | 52.7 | 55.7 | 20.1 | 51.5 |

* All results underlined where an amino acid requirement was evident.

TABLE XV

Comparison of modified trypsin hydrolysates and synthetic media with N.B.C. casein hydrolysate.

| Medium | Culture | | |
|---|---|---|---|
| | 141 | 1604 | 7050 |
| | Percent transmittance | | |
| N.B.C. casein hydrolysate | 24.6 | 23.5 | 59.5 |
| Acetic acid - acidified hydrolysate | 20.9 | 22.6 | 72.0 |
| $H_3PO_4$ - acidified hydrolysate | 23.6 | 24.8 | 34.5 |
| HCl - acidified hydrolysate | 34.3 | 45.1 | 42.6 |
| Normal synthetic medium | 51.5 | 49.5 | 54.1 |
| Synthetic medium plus acetic acid | 35.4 | 27.4 | 57.4 |
| Synthetic medium plus $H_3PO_4$ | 39.1 | 36.5 | 39.4 |

TABLE XVI

Titration of modified trypsin hydrolysates and synthetic media, and N.B.C. casein hydrolysate from pH 6.8 to pH 3.0.

| Medium | pH | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 6.5 | 6.0 | 5.5 | 5.0 | 4.5 | 4.0 | 3.5 | 3.0 |
| | Ml. N/10 HCl | | | | | | | |
| N.B.C. casein hydrolysate | 1.35 | 4.15 | 8.12 | 15.14 | 24.61 | 33.51 | 39.03 | 42.62 |
| Trypsin hydrolysate plus acetate | 1.80 | 4.74 | 8.46 | 15.20 | 24.91 | 34.14 | 40.11 | 44.01 |
| Trypsin hydrolysate plus phosphate | 2.67 | 5.53 | 7.15 | 8.40 | 9.88 | 11.50 | 13.26 | 15.66 |
| Trypsin hydrolysate plus HCl | 1.49 | 3.25 | 4.38 | 5.42 | 6.78 | 8.28 | 9.94 | 12.03 |
| Normal synthetic medium | 1.01 | 2.54 | 3.39 | 4.43 | 5.92 | 7.57 | 9.33 | 11.74 |
| Synthetic medium plus acetate | 1.08 | 3.57 | 7.08 | 13.87 | 24.21 | 33.83 | 39.87 | 44.10 |
| Synthetic medium plus phosphate | 2.33 | 5.21 | 6.74 | 8.01 | 9.67 | 11.51 | 13.52 | 16.60 |

## Date Due

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