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Growth of *Bacillus coagulans* in Chemically Defined Media[1]

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A nutritional study was made of five strains of *Bacillus coagulans* obtained from various culture collections. These five strains were descendants of two original isolates; three had been derived from one parent culture in years past and the other two were transfers from another parent culture. Therefore, the five cultures should have represented two distinct groups of genetically identical cultures. Three of the strains obtained from one culture collection had become methyl red-negative and sorbitol-negative and had gained abilities to hydrolyze gelatin and ferment arabinose. Nutritional requirements of the five cultures, determined at 37, 45, and 55 C, differed considerably among strains; however, thiamine and biotin were required by all cultures at all temperatures. Aspartic acid was stimulatory at 37 C and was required at 45 C; folic acid, basic amino acids, and certain other nutrilites were required at 55 C. Adenine supplementation was necessary for two strains at 55 C to prevent autolysis; this phenomenon is discussed. The response of these organisms to both serine and the basic amino acids at the three growth temperatures seems especially significant. The media devised for the growth of the five strains of *B. coagulans* used in this study permit excellent growth at three incubation temperatures.

Bacillus coagulans Hammer, as described by Smith, Gordon, and Clark (9), encompasses organisms that share characteristics of mesophilic as well as thermophilic sporeforming rods. The nutrition of *B. coagulans* has been studied by many investigators (1, 2, 4, 6, 7, 8), who have reported extremely divergent results. Nutritional requirements reported for *B. coagulans* have varied with the particular strains employed and techniques used.

The media previously reported in the literature would not support a satisfactory degree of growth of the five isolates used in this study. Therefore, the present investigation was initiated to determine the variability in physiological characteristics of *B. coagulans* caused by subculture of the organisms under the different conditions used in various laboratories, and to devise chemically defined media which would be suitable for production of cells for use in enzyme studies. The observed diversity of nutritional requirements at three different incubation temperatures was extensively examined, and from this research defined media were developed for five different strains of this organism at three temperatures of incubation.

MATERIALS AND METHODS

Organisms. Five strains of *B. coagulans* were used throughout this study. Strains 1A26, 1A37, and 1A71 were obtained from the Iowa State University culture collection and are designated according to the accession code used at this institution. *B. coagulans* strain 1A26 was originally obtained from N. R. Smith in 1949, 1A37 was obtained from the American Type Culture Collection (ATCC) in 1954, and 1A71 was obtained from the National Canners Association in 1962. These three strains correspond to ATCC strains 10545, 8038, and 8038, respectively. Two strains, 10545 and 8038, were obtained directly from the ATCC in 1965.

Physiological tests. The five strains of *B. coagulans* were subjected to physiological tests to characterize the extent of intrastrain (among different descendents from the same isolate) and interstrain (between different isolates) variation. In these studies, conventional diagnostic methods were used, and all cultures were incubated at 45 C. The results are given in Table 1.

Glassware. Glassware used for nutritional studies was soaked in acid-dichromate solution for 24 hr, and then rinsed with distilled water until a *p*H of 7 was obtained in the rinse water. The glassware was then heated for 24 hr in an American Sterilizer electric oven set at 95 C.

[1] The material in this paper was presented to the Graduate College of Iowa State University by Rosemarie Marshall in partial fulfillment of the requirements for the M.S. degree.
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TABLE 1. *Physiological properties of five strains of Bacillus coagulans at 45 C*[a]

Medium	10545	1A62	8038	1A37	1A71
Gelatin	−	+	−	+	+
Arabinose	−	+	−	+	+
Lysine decarboxylase	+	+	+	+	+
Rhamnose	+	−	−	−	+
Lactose	+	−	−	−	+
Litmus milk	Ac-R	Alk-R	Ac-R	Alk-R	Alk-R
Dulcitol	−	−	−	−	−
Sorbitol	+	−	+	−	−
Inositol	+	−	−	+	+
Maltose	−	−	−	−	−
Mannitol	−	−	−	−	−
Glucose	+	+	+	+	+
Methyl red	+	−	+	−	−
Voges-Proskauer	+	+	+	+	+
Nitrate	+	+	+	+	+

[a] See text for source of the strains. Symbols: − = negative in the character tested; + = positive in the character tested; Ac-R = acid and reduction; Alk-R = alkaline and reduction.

Nutritional studies. All cultures were grown for 24 hr on 2% Difco yeast extract-agar (YEA) slants. Cells were washed from the agar surface with physiological saline and were centrifuged on a Servall model SS-1 Supercentrifuge at 34,800 × *g* for 10 min. After three washings, the cells were suspended in sufficient physiological saline to give 65% transmission at 520 mμ on a Beckman/Spinco model 151 Spectrocolorimeter. A 0.1-ml amount of the washed-cell suspension was used for initial inoculation into 10 ml of medium. The initial light transmission of inoculated tubes was 98%.

The basal medium (8) was sterilized by autoclaving at 15 psi for 15 min. All amino acids were autoclaved at 10 psi for 10 min. The vitamins, adenine, and glucose were sterilized by passage through membrane filters (Millipore Corp., Bedford, Mass.). Stock solutions of organic constituents were sterilized individually and were added aseptically to 500 ml of the basal medium in 1-liter Erlenmeyer flasks. Finished media were dispensed aseptically into Pyrex tubes (16 by 150 mm) fitted with metal caps used on a standard aeration manifold apparatus. Six serial subcultures were made with each strain, at each of the three selected temperatures of incubation, in each of the media tested. This procedure precluded the possibility of recording growth responses due to the endogenous store of nutrients from the YEA slants. Growth was recorded as percentage of transmission after incubating the cultures in water baths for 24 hr at 37, 45, and 55 C. The growth response after six subcultures was recorded, and individual strains of this organism were then washed from the YEA slants used for growth of stock cultures. These cells were washed twice in saline, and were inoculated directly into the complete or optimal medium to detect the presence of variants which might have been selected during the subculturing process. All cultures were examined microscopically prior to subculturing to check for both cell morphology and sporulation.

Statistical methods. Data collected were subjected to a statistical analysis following the completely randomized design for each series of treatments. The analysis of variance was accomplished by conventional methods, and the treatment means were then subjected to a multiple-range test (5). Data consisted of the average responses of two replications of each treatment. All calculations were based on these averaged values of percentage of light transmission of cultures.

Chemicals. All amino acids, vitamins, and the adenine preparation used were obtained from Calbiochem, Los Angeles, Calif. Glucose was purchased from the Mallinkrodt Chemical Works, St. Louis, Mo.

The final concentration of each nutrilite used in these studies was as follows: biotin, 1.0 mμg/ml; folic acid, 0.1 μg/ml; niacin, 15.0 μg/ml; riboflavine, 15.0 μg/ml; thiamine, 0.5 μg/ml; adenine, 10.0 μg/ml; *p*-aminobenzoic acid (PABA), 50.0 mμg/ml; xanthine, 20.0 mμg/ml; DL-alanine, 0.16 mg/ml; DL-aspartic, 0.89 mg/ml; L-arginine-HCl, 0.03 mg/ml; L-cysteine, 0.02 mg/ml; L-glutamic, 0.14 mg/ml; glycine, 0.17 mg/ml; L-histidine-HCl, 0.02 mg/ml; DL-isoleucine, 0.40 mg/ml; L-leucine, 0.38 mg/ml; L-lysine-HCl, 0.24 mg/ml; DL-methionine, 0.06 mg/ml; L-proline, 0.11 mg/ml; DL-serine, 0.12 mg/ml; DL-threonine, 0.10 mg/ml; L-tyrosine, 0.03 mg/ml; DL-valine, 0.15 mg/ml; and glucose, 20 mg/ml.

RESULTS AND DISCUSSION

Three strains (1A26, 1A37, and 1A71) of *B. coagulans* used in this study were shown to vary from their original biochemical characterizations (see Table 1), e.g., in acquisition of the ability to hydrolyze gelatin and to ferment arabinose and in loss of the ability to give positive methyl red and sorbitol reactions. Strains 1A26, 1A37, and 1A71 grew suboptimally at 18 C as a lower incubation temperature limit, which is another indication of an extreme variation imposed by continuous cultivation. All five strains however, grew in the temperature range of facultative thermophiles, i.e., 30 to 55 C, and all strains complied with the usual morphological and cultural characteristics descriptive of *B. coagulans* (2), as well as with the biochemical reactions (except for those noted above).

Those media devised for the listed strains of this organism were found to support growth that is superior to that obtained on any previously reported defined medium. Analyses of variance and *F* tests performed on each series of treatments showed that different strains of this organism were responding in significantly different ways both to the different test media employed and to the different incubation temperatures. The multiple-range test on each series of treatments, with the use of ranked means, enabled the selection of an

TABLE 2. *Nutritional requirements of five strains of Bacillus coagulans at 37, 45, and 55 C*

B. coagulans strain designation[a]	Organic constituents[b]		
	37 C	45 C	55 C
1A26	Glutamic	Aspartic, glutamic, serine	Aspartic, glutamic, cysteine, methionine, arginine, histidine, riboflavine, folic acid, niacin, adenine
1A37	Glutamic	Aspartic, glutamic, cysteine, methionine, serine	Aspartic, glutamic, cysteine, methionine, arginine, histidine, tyrosine, folic acid, niacin, adenine
1A71	Aspartic, glutamic	Aspartic, glutamic, arginine, histidine, serine	Aspartic, glutamic, cysteine, methionine, proline, tyrosine, folic acid, niacin
10545	Proline, cysteine, methionine, threonine, alanine, arginine, valine, leucine, serine, folic acid	Proline, cysteine, methionine, threonine, arginine, serine	Proline, cysteine, methionine, threonine, alanine, arginine, valine, leucine, serine, folic acid
8038	Proline, arginine, cysteine, serine, methionine, folic acid, threonine	Arginine, histidine, methionine, cysteine, folic acid	Glutamic, histidine, arginine, cysteine, methionine, folic acid

[a] See text for sources of strains.
[b] Thiamine and biotin were required by all strains at all temperatures; glucose was added as carbon source in all media.

optimal medium for each given strain at each temperature used. The statistical treatment also suggested possible combinations of organic constituents which would successfully support growth of these strains. Over 200 media were tested for their ability to support growth; of these, 15 were found to be excellent for growth of the five strains under study at the temperatures specified.

Any further deletions from the organic constituents listed in Table 2 for each of the final media devised decreased growth significantly, indicating that the organic constituents used in the media are either required or are highly stimulatory. Table 3 shows turbidity readings and the mean reading over six subcultures in final media. Thiamine and biotin were found to be obligate growth requirements for all strains studied, which agrees with the general findings of other workers (4) for this organism. Glucose was satisfactory as the principal carbon source in all test media. Supplements which were found to be ineffective at any temperature included xanthine, PABA, glycine, and isoleucine.

It is of interest to note that the analogous organisms used in this study are very different in their physiological abilities (Table 1) and their nutritional capabilities (Table 2), and, therefore, presumably in their synthetic capacities.

An autolysis phenomenon was noted in cultures of 1A26 and 1A37 at 55 C. As cultures reached approximately 60% transmission after 8 hr in the test medium, they began autolyzing so that the tubes were substantially reduced in turbidity within 2 additional hr. It was found, however that addition of adenine would stabilize such cultures so that autolysis did not occur. Bacteriophages were not detected in these cultures nor was the autolysis associated with sporulation. Growth and sporulation occurred normally after addition of adenine. This phenomenon will be studied further.

An interesting phenomenon noted during this study was the differing nutritional status of the amino acid serine with the various strains tested. Serine inhibition was seen consistently, particularly with strains which have been present in the laboratory environment for considerable time. This amino acid was found to be nonessential, but not inhibitory, at 37 C for strains 1A26 and 1A37, whereas strain 1A71 was inhibited by it. At 45 C, serine was an obligatory requirement for growth, and could not be replaced by folic acid or glycine, singly or in combination; however, at 55 C serine was inhibitory for these three strains. The uses of serine in the oxidative metabolism of this organism have not as yet been studied.

TABLE 3. *Percentage of transmission readings (averaged values over replications) and mean percentage of transmission values of serial subcultures of Bacillus coagulans made at 24-hr intervals*

Temp of incubation	Strain designation[a]				
	1A26	1A37	1A71	10545	8038
C					
37	52	58	68	48	23
	51	71	62	50	66
	65	60	49	60	61
	60	73	46	63	59
	51	63	41	62	58
	43	55	50	51	56
	Mean, 54	63	53	56	54
45	52	66	64	63	75
	60	62	63	71	64
	65	71	54	70	52
	61	68	52	65	46
	54	67	56	64	47
	54	70	49	62	47
	Mean, 57	67	56	66	54
55	71	81	82	46	52
	71	72	78	53	46
	59	65	64	56	39
	50	51	52	49	52
	51	46	45	50	51
	46	42	42	48	50
	Mean, 58	59	61	50	48

[a] See text for source of the strains.

However, Campbell and Sniff (3) reported the inability of serine to replace the folic acid requirement for certain strains of this organism at 45 C. At 55 C, folic acid was used in place of serine by our strains; however, when both serine and folic acid were added at 55 C, growth could not be maintained for more than four subcultures. Apparently, serine exerts some effect such that continued growth and division are not possible in its presence. Campbell and Sniff (3) reported that serine inhibited growth of 13 of 20 strains of *B. coagulans* at 45 C. Grula (*personal communication*) has noted the same inhibitory effect of serine on his cultures, and has obtained evidence that the inhibition is due to an effect on the cytoplasmic membrane which inhibits cell division. Further work is needed in this area.

Reactions of 1A71 to arginine and histidine at the three temperatures of incubation are different (see Table 2). This strain exhibits a requirement for both of these amino acids at 45 C but not at 37 or 55 C. This strain would be well suited for nutritional studies with these amino acids.

It will be recalled that strains 1A26 and 1A37 are analogous to strain 8038, and that 1A71 is analogous to strain 10545, as designated by the ATCC and by the Iowa State University stock culture collection. However, it can be seen both from physiological studies and from nutritional requirements that strains 1A26, 1A37, and 1A71 tend to approach a similar pattern; those strains recently obtained from the ATCC also tend to be mutually similar. It is of interest to note changes in nutritional requirements of these organisms as the temperature of incubation is shifted away from the optimum (45 C). The three strains which have been maintained in the laboratory for a period of time have developed increased requirements as the temperature of incubation is increased from the optimum, and show decreased nutritional requirements as the temperature is lowered, whereas the requirements of 10545 and 8038 increase in complexity on either side of the optimum, i.e., intrastrain variation is minimized. These patterns indicate a great diversity between these "groups" of variants of the same isolate, and the extreme interstrain variation imposed by subculturing for a long period of time. Biochemical bases for these changes would, in all probability, be concerned with thermostability of the oxidative enzymes. The final optimal media devised support growth so profuse as to permit essentially 0% transmission after 48 hr of incubation. With these media, cultures approach complete sporulation in 72 hr.

Future work on these strains will include a study designed to gain knowledge of oxidative pathways of these strains and of the characteristics and thermostability of enzymes involved in these pathways.

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