THE NUTRITION AND PHYSIOLOGY OF THE GENUS PEDIOCOCCUS

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The genus *Pediococcus* has long been associated with a type of spoilage of beer called "sarcina sickness" which is characterized by turbidity, a low pH, and by a characteristic "sarcina odor". The odor has been shown to be due to the production of diacetyl by the organism (Shimwell and Kirkpatrick, 1939). Certain strains also have been shown to cause a ropiness in beer due to a heavy capsule formation. In recent years the importance of pediococci in fermenting vegetable products such as cucumbers and beans has been studied by Pederson (1949) who has published an extensive review of the genus.

The pediococci are gram positive, nonmotile, spherical cocci which tend to occur in packets of four cells, but which also are found singly and in pairs. They are facultative anaerobes and are homofermentative, producing large amounts of inactive lactic acid from carbohydrates. Nitrates are not reduced to nitrites and gelatin is not liquefied. In all earlier reports this genus has been characterized as catalase negative. Recently, it has been shown to produce catalase when grown in low concentrations of fermentable carbohydrates (Felton *et al.*, 1953).

In the 6th edition of *Bergey's Manual* the genus *Pediococcus* is included in the family *Micrococcaceae* as an appendix. It is now generally agreed that the genus should be included in the family *Lactobacteriaceae* (Tittsler *et al.*, 1952) although, admittedly, this is still open to question.

The work described in this paper was undertaken to obtain further information which might be of value in determining the true taxonomic position of the group. The minimum nutritional requirements have been determined, and the physiological reactions commonly attributed to the genus have been reviewed and others investigated.

During the course of this work it was determined by Felton and Niven (1953) and independently in this laboratory that the organism, formerly known as "*Leuconostoc citrovorum*, strain 8081", is actually a pediococcus. Because of the unique requirements of this organism for the so-called "citrovorum factor" the considerable amount of work done with this one strain now becomes more generally applicable to the genus *Pediococcus*. For this reason a brief review of the literature concerning this strain would seem appropriate at this time.

Dunn *et al.* (1947) first studied the nutrition of *L. citrovorum*, strain 8081, along with 22 other lactic acid bacteria and found sixteen amino acids to be either required or stimulatory for growth. In these experiments a concentrate of folic acid was used which probably contained sufficient "citrovorum factor" to support growth. Attempts to determine the vitamin requirements were without success, however, since in this case a synthetic folic acid was used and very slight growth was obtained (Shankman *et al.*, 1947).

Sauberlich and Baumann (1948) showed that this strain required an unknown growth factor found in liver extracts which they designated as the "citrovorum factor". A synthetic medium supplemented with small amounts of the liver extract supported growth, and it was noted also that high concentrations of folic acid could replace this unknown factor.

Snell *et al.* (1948) reported that thymidine was required by *L. citrovorum*. Although no strain number was mentioned, it is presumed that they were working with strain 8081. These workers also reported that the desoxyribosides of other pyrimidines and purines were ineffective in supporting growth (Kitay *et al.*, 1949). Oleic acid, pyridoxamine phosphate, LBF, and enzymatic digest of casein also were found to be ineffective.

A scheme has been proposed by Jukes *et al.* (1950) to explain the interrelationship of the "citrovorum factor", vitamin B_{12}, thymidine, and some of the other desoxyribosides in *Lactobacillus leichmannii* and *L. citrovorum*, strain 8081. Chang *et al.* (1951) have investigated the role of the "citrovorum factor" in the metabolism of *L. citrovorum*, strain 8081, and conclude that the factor in its free form apparently is not a functional coenzyme. It is concluded also that

the factor has some function in growth other than in the synthesis of nucleic acids.

Chemical studies have resulted in the identification and synthesis of the "citrovorum factor" which is known now as "folinic acid-SF" (Pohland *et al.*, 1951) and "leucovorin" (Roth *et al.*, 1952).

MATERIALS AND METHODS

Cultures. A total of 34 strains was used; 27 of these were obtained from Dr. C. S. Pederson. Three of these strains were obtained originally from the Techniche Hoogeschool, Delft, Holland, and were labeled as separate species and varieties: *Pediococcus damnosus*, *Pediococcus damnosus* var *perniciosus*, and *Pediococcus pentosaciens*. These were isolated initially from spoiled beer. Seven additional strains consisted of different cultures of *L. citrovorum*, strain 8081, obtained from various laboratories throughout the country.[1] These strains were obtained for the purpose of comparison with recognized strains of *Pediococcus*. They proved to be very similar except for one strain which was atypical on lactose, salicin, and ribose. Attempts to obtain cultures of beer pediococci from American brewery laboratories were unsuccessful. The four cultures which were obtained all proved to be gram positive cocci which were strongly catalase positive and aerobic. These appeared to be members of the family *Micrococcaceae*.

Methods. The standard techniques used in nutritional studies of microorganisms were employed. Stock cultures were maintained in tomato juice agar stabs and transferred every three months. The inoculum for studies in synthetic media was prepared by centrifuging an 18 to 24 hour tomato juice broth culture and washing three times with sterile distilled water. The cell suspension then was diluted to a faint turbidity, and one drop was used as the inoculum. All nutritional test media were tubed in 6 ml amounts in 16 by 150 mm pyrex test tubes with aluminum caps, and the results were obtained by turbidimetric measurements with a "kromatrol" colorimeter. Cultures were incubated at 30 C for 72 hours.

[1] Cultures of *Leuconostoc citrovorum*, strain 8081, were obtained from the following laboratories: American Type Culture Collection, Georgetown University, Washington, D. C.; National Institutes of Health, Bethesda, Maryland; American Meat Institute Foundation, Chicago, Illinois; Department of Chemistry, University of California, Los Angeles, California; Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario; Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York; and the Department of Animal Husbandry and Nutrition, Alabama Polytechnic Institute, Auburn, Alabama.

TABLE 1

Minimum synthetic medium

COMPONENT	CONC/ 10 ML	COMPONENT	CONC/ 10 ML
Glucose	100 mg	L-Cystine	2 mg
K_2HPO_4	40 mg	DL-Threonine	2 mg
Salts*	0.2 ml	DL-Methionine	2 mg
Na acetate	200 mg	DL-Valine	2 mg
NH_4Cl	20 mg	DL-Leucine	2 mg
Niacin	50 μg	DL-Isoleucine	2 mg
Ca pantothenate	10 μg	DL-Lysine	2 mg
Biotin	1 μg	L-Histidine	2 mg
Leucovorin	20 units	DL - Phenylalanine	2 mg
Pyridoxine	10 μg	L-Tyrosine	2 mg
Xanthine	50 μg	L-Proline	2 mg
Guanine	50 μg	Glycine	2 mg
Adenine	50 μg	DL-Alanine	2 mg
Thymine	50 μg	L-Arginine	2 mg
Uracil	50 μg	DL-Serine	2 mg
L-Tryptophan	2 mg	L - Glutamic acid	2 mg
		L - Aspartic acid	2 mg

* Salts: NaCl, 20.0 g; $MgSO_4 \cdot 7H_2O$, 0.8 g; $FeSO_4 \cdot 7H_2O$, 40.0 mg; $MnCl_2 \cdot 4H_2O$, 15.0 mg; dilute to 100 ml with H_2O.

RESULTS

Nutrition

Although *L. citrovorum*, strain 8081, has been grown on a synthetic medium (Sauberlich and Baumann, 1948; Kitay *et al.*, 1949), there have been no reports of a minimum synthetic medium for this strain or for any other strain of *Pediococcus*. Table 1 shows a medium which satisfies the minimum requirements of 32 of the 34 strains employed. The two strains which did not exhibit growth on this medium (2-170 and 4-89) were found to require riboflavin in addition to the other constituents.

The vitamin requirements were determined originally using "vitamin-free" acid hydrolyzed

TABLE 2

Amino acid requirements

AMINO ACID OMITTED FROM MEDIUM	OPTICAL DENSITY	
	Strain D-119	Strain 8081
None	87	87
L-Tryptophan	0	0
L-Cystine	2	3
DL-Threonine	0	0
DL-Methionine	9	10
DL-Valine	0	0
DL-Leucine	0	0
DL-Isoleucine	0	0
DL-Lysine	4	1
L-Histidine	0	0
DL-Phenylalanine	0	0
L-Tyrosine	0	0
L-Proline	0	0
Glycine	0	0
DL-Alanine	0	0
L-Arginine	0	0
DL-Serine	0	0
L-Glutamic acid	0	0
L-Aspartic acid	44	47
Asparagine	85	89
DL-Norleucine	89	85
L-Hydroxyproline	85	89

casein as an amino acid source and were found later to be the same when amino acids were used in place of the hydrolyzate. "Citrovorum factor", niacin, and pantothenic acids are absolute requirements whereas biotin and pyridoxine are merely stimulatory. Pyridoxine appears to be only slightly stimulatory since almost complete growth is obtained when this vitamin is omitted from the medium.

Other factors which were tested and found not to be required for maximum growth were: Riboflavin, thiamin, *p*-aminobenzoic acid, sodium thioglycolate, "tween 80", choline, inositol, vitamin B_{12}, strepogenin, and ascorbic acid.

Thymidine will replace the "citrovorum factor" in all strains, but maximum growth is never obtained. The desoxyribosides of xanthine and hypoxanthine, on the other hand, do not replace the factor. Sauberlich and Baumann (1948) originally reported that high concentrations of folic acid would substitute for the "citrovorum factor" resulting in maximum acid production although growth was slower. In our studies, 27 of the 34 strains studied were able to utilize folic acid; however, none of these attained maximum growth. This may be due to the fact that the criterion for growth in their study was acid production, whereas in ours, turbidity was used.

The requirements for vitamins and amino acids have been shown to be quite uniform for almost all strains studied. This does not appear to be the case with the purines and pyrimidines. Of the eleven strains investigated, seven showed moderate to good growth in the absence of all purines and pyrimidines. Those strains requiring these compounds exhibited greatest growth with either xanthine or guanine, whereas uracil and thymine were least effective. One strain (6-126) is unique in that only uracil will support growth. Because of this variation in the requirements of the various strains three purines and two pyrimidines have been included in the minimum synthetic medium although probably no one strain would require all of these.

The minimum amino acid requirements were determined for six strains using essentially the same method used with the vitamins. Table 2 gives the results obtained with two strains showing the optical density obtained when each amino acid was omitted from the medium individually. The results indicate that only norleucine and hydroxyproline are not required for growth, and that methionine and possibly lysine are stimulatory. Readings of three or less were considered to be insignificant. Aspartic acid appears to replace asparagine completely whereas asparagine only partially replaces aspartic acid. It is interesting to compare these results with those obtained by Dunn *et al.* (1947) in their work on *L. citrovorum*, strain 8081. This group reported that lysine, proline, norleucine, hydroxyproline, and norvaline were not required for maximum acid production and that methionine, tyrosine, and asparagine were stimulatory. The strain 8081 of *L. citrovorum* in our collection gave results essentially the same as those of the other pediococci.

Physiology

Pederson (1949) has determined the physiological reactions of 121 strains of pediococci isolated from fermenting cucumbers and beans and including some of the strains investigated in our work. All of our cultures were found to give reactions corresponding very closely with his results. The three cultures, originally isolated from beer, and all seven strains of *L. citrovorum*, strain 8081, proved to be almost identical with the

cultures isolated from fermenting vegetables. Although this group has been reported to be microaerophilic, agar shake cultures usually show an even growth throughout the tube, indicating that they are facultative anaerobes. Their oxygen relationship was determined further by growing the organisms in two flasks containing 100 ml each of tomato juice broth. One flask was placed on a shaker and the other was sealed with vaspar. After incubation at 30 C for 48 hours the cells were centrifuged off and measured both by turbidimetric methods and by dry weight, and the corresponding crops were found to be almost identical, indicating that good growth occurs under high oxygen tension.

In addition to the physiological reactions determined by Pederson, several others were determined. All 34 strains were found to ferment trehalose and ribose and to hydrolyze arginine and esculin. None fermented glycerol or sorbitol, and none hydrolyzed sodium hippurate or produced mucoid colonies on sucrose gelatin agar.

On horse blood agar the pediococci form small colonies which are surrounded by a very narrow zone of complete hemolysis. This zone frequently appears green to the unaided eye. However, on microscopic examination it can be seen that the colony is green whereas the hemolyzed area is colorless.

The direct oxidation of *p*-phenylenediamine in the presence of α-naphthol is considered to be a specific test for the presence of cytochrome c. This test has been applied to both intact cells on agar plates and to cell-free extracts of aerobically grown cells, and positive results were obtained in both cases. This would strongly suggest the presence of a cytochrome system in the pediococci.

Until very recently the pediococci have been considered to be catalase negative. Felton *et al.* (1953) have shown, however, that catalase is produced when the cells are grown in a medium containing small amounts of fermentable carbohydrates. It is interesting to note that Harrison and Hansen (1950) noticed a weakly positive catalase test for one of four strains of cultures tentatively considered by them to be pediococci and isolated from the cecal feces of turkeys. Although the catalase reaction is rather weak, it has been shown to be positive for most of our strains. Using the method described by Felton *et al.* (1953) only five cultures exhibited a positive reaction on APT agar (high glucose content), whereas on YTG (low glucose content) 28 strains gave a positive test. Inasmuch as all strains are very similar physiologically and nutritionally, it is probable that all strains would exhibit a positive catalase test if grown under the proper conditions.

Although other workers have reported final hydrogen ion concentrations of as high as pH 3.25 to 3.4, our cultures were unable to reach this level. Using a medium of 0.5 per cent tryptone, 0.5 per cent yeast extract, 2 per cent tomato juice, and 2 per cent glucose, the final pH obtained for 20 cultures tested was between 3.7 and 4.0.

It is interesting to note that all of the cultures used, including the three originally isolated from spoiled beer, showed no growth or extremely slight growth on beer. After decarbonation and neutralization the beer was still unable to support growth of the cultures. Evidently only certain types of beer are susceptible to spoilage, or only certain strains which are adapted for growth in beer will cause the spoilage.

DISCUSSION

Pederson (1949) has concluded, after extensive physiological studies, that all strains of pediococci studied by him should be considered as a single species. The results of this nutritional study would tend to support this view since very little variation is found in the nutritional requirements of the various strains. It is significant that the strains originally isolated from spoiled beer showed only very slight and inconsistent variation from the other strains studied. Pederson has suggested that the name *Pediococcus cerevisiae* as originally proposed by Balke should be retained.

Since these organisms are homofermentative and appear to be related more closely to the lactic acid bacteria than to any other group, their inclusion in the family *Lactobacteriaceae* would seem warranted. If this is done, however, the family characteristic will have to be changed to include organisms that divide in more than one plane. Shimwell and Kirkpatrick (1939) have proposed that this group be included in the genus *Streptococcus* inasmuch as the ability to divide in two planes and to form inactive lactic acid is not sufficient grounds for designating a new genus. Regardless of the merits of this ar-

gument, the positive catalase test now definitely excludes them from the genus. The strong oxidation of *p*-phenylenediamine by pediococci, indicative of the presence of cytochrome c, also separates them from the streptococci which are negative.

Recently, an interesting new genus, *Aerococcus*, has been described by Williams *et al.* (1953) that appears to be closely related to the genus *Pediococcus*. These genera appear to differ only in that the pediococci are smaller, produce a greater hydrogen ion concentration, hydrolyze arginine, and do not ferment mannitol. It is quite probable that a comparative study of these two groups would show them to be closely related and possibly members of the same genus.

SUMMARY

The minimum nutritional requirements of 34 strains of the genus *Pediococcus* have been determined, and additional physiological reactions of the group are reported.

The true identity of the so-called *Leuconostoc citrovorum*, strain 8081, as a pediococcus is confirmed, and the literature concerning this strain is reviewed.

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