# OPTIMUM TEMPERATURE FOR DIFFERENTIATION OF ESCHERICHIA COLI FROM OTHER COLIFORM BACTERIA

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During the past few years, several investigators have advocated the use of temperatures lower than 46°C. in the Eijkman test for *Escherichia coli*. Levine, Epstein and Vaughn (1934) suggested a temperature of 43° to 44°C. as optimum for selective growth of *Escherichia coli* in both the original Eijkman medium and in Standard Methods lactose broth. Their *Aerobacter* strains were markedly inhibited in both media at this temperature while some of the *Citrobacter* strains showed gas in the original Eijkman medium. None of the *Citrobacter* strains, however, produced gas at these temperatures in Standard Methods lactose broth of the A.P.H.A.

Skinner and Brown (1934) criticized the Eijkman test because strains of *Escherichia coli* failed to grow in Eijkman's or Bulir's medium if the tubes were placed directly in a water-bath at 46°C.

Wilson (1935), using the MacConkey medium, observed that practically all indol-positive strains of *Escherichia coli* grew well and produced gas from lactose at 44°C. None of his strains of *Aerobacter aerogenes* produced gas at this temperature and very few grew at all.

Minkevich, Alexandrov and Sobelova (1936) reported that a temperature of 46°C. did not hinder their *Escherichia coli* cultures from fermenting mannitol in Bulir's medium if inoculated heavily, but in many cases, they contended, the high temperature of 46°C. repressed growth of small numbers. Therefore, they proposed to make fermentation tests in Bulir's medium at 43° to 43.5°C.

Dodgson (1938) who has studied the application of the coliform index of pollution to shellfish for a number of years has concluded

"there can be no legitimate standard of purity for purified shellfish based on tests carried out at a temperature of 37°C." The practical limitations of using 37°C. for isolation of *Escherichia coli* from shellfish have been demonstrated time and again by extensive data on this subject by Perry and Hajna. Dodgson uses MacConkey's lactose bile medium at a temperature of 44°C.

The authors made a limited study in 1936 (unpublished) of incubation temperatures ranging from 40° to 49°C., for selective growth of *Escherichia coli* and other members of the coliform group. They used their own medium consisting of peptone, buffers, sodium chloride and glucose. Their observations led them to believe that a temperature greater than 45°C. (temperature of the medium, not of the incubator) was essential for the suppression of coliform types other than *Escherichia coli.*

One of the authors (A. A. Hajna, 1937) investigated the ability of bacteria of the genus *Escherichia* to decompose various carbohydrates other than glucose at 46°C. The substances tested were (monosaccharides) mannose, fructose, arabinose, xylose; (disaccharides) maltose, lactose, sucrose, trehalose; and (alcohols) mannitol, dulcitol and adonitol. It was found that with the exception of dulcitol and adonitol, all three substances were fermented at 46°C. as readily as at 37°C. They were substituted for glucose in the basic medium of Perry and Hajna mentioned above.

Hajna also observed that buffers enhanced gas production by the *Escherichia* organisms both at 37°C. and at 46°C. whereas meat extract restrained it. *Aerobacter* and *Citrobacter* cultures, however, were not tried.

## PURPOSE OF PRESENT STUDY

The primary purpose of the present investigation has been to determine if incubation at temperatures less than 46°C. could be used as a selective test for *Escherichia coli.* A second objective was the determination of the comparative value of MacConkey's broth (as suggested by Wilson and Dodgson) and the authors' modified Eijkman lactose broth at 44°C. Since glucose, lactose and mannitol have all been found valuable and have been recom-

mended at times, these three carbohydrates were used. Temperatures of 42°, 44° and 46°C. were considered to constitute an adequate range for the purpose of this study.

## CULTURES AND METHODS

Nearly all cultures[1] of the coliform group were freshly isolated from various materials under routine investigation. The *Aerobacter* and *Citrobacter* strains were alternately subjected to serial transfers in Koser's synthetic citrate medium and on Levine's eosin methylene-blue agar plates until their purity was beyond question. All of the cultures were grouped according to their biochemical reactions in various differential media (see table I).

Tests for indol were made with Ehrlich's reagent, on 18 to 24-hour cultures grown in Bacto-tryptone broth.

*Aerobacter aerogenes* was differentiated from *Aerobacter cloacae* by means of the hippurate test rather than by the time-consuming gelatin liquefaction test. Hippurate was considered to be hydrolyzed if cultures grew and caused definite turbidity in the synthetic medium devised for this test by Hajna and Damon (1934). If the medium remained clear at the end of three days incubation at 37°C., the reaction was considered negative.

The composition of the authors' modified Eijkman medium is:

| | |
|---|---|
| Peptone (Bacto) | 15 grams |
| Sodium chloride | 5 grams |
| Dipotassium hydrogen phosphate (anhydrous) | 4 grams |
| Potassium dihydrogen phosphate (anhydrous) | 1.5 grams |
| Lactose, glucose or mannitol | 3 grams |
| Distilled water | 1 liter |

The final hydrogen-ion concentration of the medium without titration is approximately 6.9.

The formula of the MacConkey broth as used by Wilson is:

| | |
|---|---|
| Peptone (Bacto) | 20 grams |
| Sodium chloride | 5 grams |
| Lactose | 10 grams |
| Sodium taurocholate | 5 grams |
| Distilled water | 1 liter |

[1] The authors wish to thank Dr. Ralph Tittsler of the University of Rochester, for some of the *Aerobacter* cultures and for 24 *Citrobacter* cultures.

Each liter of broth was titrated to pH 7.4 and filtered before 10 ml. of 1 per cent aqueous solution of neutral red was added.

Both media were tubed in approximately 5 ml. amounts, with inner fermentation tubes. The inoculated tubes of media were placed in wire test-tube racks throughout the experiments.

### GAS PRODUCTION FROM GLUCOSE, LACTOSE AND MANNITOL BY COLIFORM BACTERIA AT TEMPERATURES OF 42°, 44° AND 46°C.

All of the inoculations were made by a loop from 24-hour Bacto-tryptone broth cultures. Gas production was noted at 24 and 48 hours. The final readings as given in the tables are those made at 48 hours. Only one temperature (46°C.) was used for the *Escherichia* organisms since at that temperature, they fermented the carbohydrates and alcohols with ease and it was pointless to use lower temperatures. Incubation was in a Castle air incubator (the temperature being that of the medium, not of the incubator).

Most of the *Aerobacter aerogenes* strains produced gas from all three substances, glucose, lactose and mannitol at 42°C.; many at 44°C., but few at 46°C. The same temperature selection held for both the *Aerobacter cloacae* and the *Citrobacter* strains except that none of them produced gas from either glucose or lactose at 46°C.

On the other hand, all of the 1,374 *Escherichia* organisms produced gas from mannitol at 46°C.; only four failed in glucose and five in lactose, though growth was heavy. The strains failing at 46°C. were all isolated from raw sewage.

### COMPARISON OF MACCONKEY'S MEDIUM AND THE AUTHORS' MODIFIED EIJKMAN LACTOSE MEDIUM AT 44°C.

In the comparative study of MacConkey's broth with that of the authors, two tubes of each medium were inoculated from the same broth culture. One set of the tubes for each medium was incubated in the Castle air incubator while the other set was incubated in a water-bath. A United States Bureau of Standards thermometer was used to register correct temperatures. A

TABLE 1

*Gas production at 42°, 44° and 46°C. by coliform bacteria*

| COLIFORM GROUP | SOURCES OF STRAINS | BIOCHEMICAL REACTIONS IN DIFFERENTIAL MEDIA | | | | | | | | NUMBER OF STRAINS | TUBE TEMPERATURES OF MEDIUM | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MR | VP | I | Gly. | Gel. | Cit. | Uric | Hipp. | | 42°C. | | | 44°C. | | | 46°C. | | |
| | | | | | | | | | | | D | L | M | D | L | M | D | L | M |
| Aerogenes | Human feces 29, sewage 24, water 23, crabmeat 10, oysters 24, grain 5, soil 5. | − | + | − | + | − | + | + | + | 120 | 106 | 103 | 107 | 77 | 88 | 102 | 3 | 5 | 13 |
| Cloacae | Human feces 23, sewage 15, drinking water 12, crabmeat 2, oysters 8. | − | + | − | − | + | + | + | − | 60 | 14 | 26 | 39 | 5 | 4 | 10 | 0 | 0 | 1 |
| Citrobacter | Various | + | − | −42<br>+3 | +42<br>−3 | − | + | − | − | 45 | 39 | 16 | 37 | 22 | 5 | 29 | 0 | 0 | 1 |
| Escherichia | Human feces 395, animal feces 134, crabmeat 16, oysters 68, oyster waters 112, drinking waters 196, swimming pool waters 23, raw sewage 430. | + | − | +1,344<br>−30 | − | − | − | − | − | 1,374 | | | | | | | 1,370 | 1,369 | 1,374 |

Legend: D = dextrose, L = lactose, M = mannitol, MR = methyl red, VP = Voges Proskauer, I = indol, Gly. = glycerol, Gel. = gelatin, Cit. = citrate, Uric = uric acid test, Hipp. = hippurate.

fluctuation of 0.5°C. above and below 44°C. was noted in the water-bath, whereas in the air incubator the fluctuation in the tube temperature was 0.3°C.

Wilson contended that the temperature in a water-jacketed incubator is never quite uniform. He advocated the use of a water-bath in which the tubes are practically immersed, the water being set at 44°C. The authors' experience indicates that an air incubator is preferable to a water-bath. The lag of an hour or two before the tubes reach the high temperature, is actually desirable since it apparently permits the *Escherichia* organisms to acclimatize themselves to the higher temperature.

TABLE 2

*Gas production from lactose at 44°C. in two types of media by bacteria of the coliform group*

| COLIFORM GROUP | NUMBER OF STRAINS INOCULATED | TYPE OF INCUBATION | | | |
|---|---|---|---|---|---|
| | | Air (water-jacketed) | | Water-bath | |
| | | Eijkman | MacConkey | Eijkman | MacConkey |
| Aerogenes | 122 | 85 | 55 | 69 | 33 |
| Cloacae | 62 | 3 | 3 | 5 | 3 |
| Citrobacter | 45 | 1 | 1 | 0 | 0 |
| Escherichia | 66* | 66 | 64 | 66 | 50 |

* Of human origin.

All of the *Escherichia* strains (66 of human origin) produced gas from lactose in the authors' medium at 44°C. whether the tests were incubated in an air incubator or in a water bath, while in MacConkey's medium, 2 failed to produce gas from the incubator tubes and 16 from the water-bath tubes. Most of the *Aerobacter cloacae* (62) and *Citrobacter* (45) strains failed to produce gas at this temperature in both the water-bath and the air incubator. However, many of the *Aerobacter aerogenes* strains grew and produced gas at this temperature. Many more of the *Aerobacter aerogenes* organisms, however, were inhibited in MacConkey's than in the authors' medium.

Most of the tubes of MacConkey's medium had an average of about 10 to 20 per cent of gas in the inner tubes, in contrast to

the average of 30 to 60 per cent in the authors' medium. Very few of the cultures of the entire coliform group could be successfully re-isolated from MacConkey's medium at the end of 48 hours of incubation at 44°C. *Escherichia coli*, however, is readily recovered from the authors' medium after 96 hours and longer.

## DISCUSSION AND CONCLUSION

Shellfish, water, sewage and other materials usually harbor a mixture of various coliform bacteria. In order to isolate *Escherichia coli* from these substances, it is necessary to suppress the growth of other coliform types. The only method which has so far been found practical for the isolation of *Escherichia coli* from materials harboring other coliform organisms is incubation at a temperature high enough to suppress the growth of such interfering coliform types. At a temperature of 45° to 46°C., coliform types other than *Escherichia coli* usually fail to grow and produce gas from a suitable carbohydrate. *Escherichia coli* strains, on the other hand, seldom fail to grow and produce gas if a suitable medium is properly used. The isolation of *Escherichia coli* from such gas tubes by streaking on to plates of eosin methylene-blue agar medium is relatively simple. Almost pure cultures are usually obtained on such plates, whereas plates made from gas tubes obtained at temperatures of 44°C. or at 37°C. frequently contain a mixture of spreading mucoid colonies of *Aerobacter aerogenes* or *Aerobacter cloacae* which are apt to have overgrown any *Escherichia coli* present. Further difficulty may arise from inability to distinguish *Citrobacter* colonies from those of *Escherichia coli*. Temperatures between 45° and 46°C. inhibit the *Citrobacter* types and eliminate this difficulty.

The authors (1933) studied the unsatisfactory features of the Eijkman test. The original Eijkman medium contained a large amount of glucose which resulted in the rapid formation of a lethal amount of acid. They recommended the reduction of the carbohydrate to 0.3 per cent and the addition of phosphates to neutralize the acid formed from the carbohydrate. The modified Eijkman lactose broth has been found superior to MacConkey's. The superiority appears to be due to the lower

concentration of carbohydrate, the presence of suitable buffers, and to the use of a water-jacketed air incubator.

An air incubator, if properly controlled, has been found preferable to incubation in a water-bath since more strains of *Escherichia coli* grow and produce gas. A number of strains of *Escherichia coli* in our series, failed to produce gas from MacConkey's medium in a water-bath at 44°C., while many of the *Aerobacter aerogenes* types grew at this temperature. Most, but not all, of the *Escherichia coli* strains grew and produced gas at 44°C. in MacConkey's medium, in an air incubator, but more of the *Aerobacter aerogenes* strains also grew in the air incubator than in the water-bath.

Our findings are, therefore, not in complete agreement with Wilson's who claimed MacConkey's medium to be highly selective for *Escherichia coli* under these conditions. It should be borne in mind, however, that organisms of the *Aerobacter* group are not usually found, to a large extent, in shellfish except under certain conditions. For this reason, temperatures as low as 44°C. may be found practical even though not completely selective. Since in our experience, an incubator temperature between 45.5° and 46°C. (temperature of medium in tubes will be between 45.2 to 45.7°C.) is easily obtained with a good water-jacketed incubator, and since *Escherichia coli* is not suppressed to any appreciable extent in the authors' medium whereas practically all strains of *Citrobacter*, *Aerobacter cloacae* and *Aerobacter aerogenes* are suppressed, we believe this temperature is most satisfactory.

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