PROBLEMS IN THERMOPHILY

II. The Isolation and Characterisation of Therimophlic Bacilli

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A beginning has been made in this country in the isolation and identification of bacteria capable of growth at 55° C. The wide occurrence of these highly resistant bacilli is indicated by their presence in almost every sample of soil, water, laboratory dust and some canned fruits that were examined. Out of the ninety-five cultures isolated, thirty-six were identified as strains of *B. subtilis*, nine as strains of *B. coagulans*, five as strains of *B. megaterium*, four as strains of *B. firmus*, four as strains of *B. sphæricus*, three as strains of *B. brevis*, and one as a strain of *B. stearothermophilus*. The remaining isolates have been characterised and grouped according to their size, shape and other peculiarities but have not been identified upto the species. It has been pointed out that the number of isolates of each species isolated by us is no indication of their distribution in nature as the species obtained depend to a large extent on the method adopted for isolation.

INTRODUCTION

Although a considerable amount of work has been done on thermophilic bacilli in the United States and England, there has hardly been any attempt to study these micro-organisms from a tropical country like India where the prevailing temperatures are more suited to the growth and development of this group of organisms. As far as we are aware, Chopra[3-8] and Varma, *et al.*[13, 14] are the only workers who dealt with thermophilic bacilli in this country. Chopra, however, obtained the thermophilic cultures, *viz.*, *Bacillus thermophilus*, *Bacillus ærothermophilus* and *Bacillus thermoacidurans* from the Lister Institute, London. Varma and Laxminarayana[13] studied the microflora associated with the spoilage of boiled milk and isolated several sporeformers associated with its spoilage. Among their isolates was a strain of an obligate thermophile which they stated to have resembled *B. kaustophilus* Prickett. With these two exceptions there has been no attempt either to make a systematic study or to learn more about the nature or other unrevealed characteristics of this interesting group of bacteria. The results of the attempts made by us in the isolation and characterisation of thermophilic bacilli mainly from soils obtained from different parts of this country have been presented in this paper.

The sporeforming bacteria capable of growth at temperatures around 55° C. have been named differently by workers in the past but from the present-day view-point the most apt terms employed were those suggested by Cameron and Esty.[2] These workers used the terms " obligate thermophile " for bacteria capable of growth at 55° but not at 37° C. and " facultative thermophile " for those bacteria which could prolifer at 55° as well as at 37° C. or even at lower temperatures. This is the nomenclature we have adopted in presenting the result of our studies on the thermophilic bacilli which pose many problems among which one has been dealt by us in a previous communication.[1]

Materials, Methods and Results

The methods adopted for isolating thermophilic cultures from sources[1] such as soil consisted in inoculating a small amount of the source material into a tube containing either glucose broth or sterile milk and then heating it in boiling water for five minutes to destroy all vegetative forms. A loopful of the suspension in broth served as inoculum for isolation on glucose agar and in the case of the milk suspension, it had to be first incubated at 55° C. for 24 hours before a loopful could be streaked out on proteose-peptone acid agar for isolation purposes. The former method resulted in the isolation of strains of *B. subtilis*, *B. firmus*, *B. sphæricus*, *B. brevis* and other unidentified strains belonging to Group II, while the latter method gave strains identified as *B. coagulans*, *B. megaterium*, and only occasionally *B. subtilis*. These isolates were then studied by the procedures recommended by Smith, *et al.*[11] and the identity of a large number of them were established following the key proposed by these authors. The " Manual of Methods for Pure Culture Study of Bacteria " was another invaluable aid in the study of the cultural characteristics of thermophilic bacilli.[12]

Of the ninety-five isolates in our collection, sixty-two were identified as representing heat resistant members of the common mesophilic forms. The remainder were characterised but the identification was not possible due to the paucity of available information on this group of organisms. The identified isolates were the following:

B. subtilis species	36
B. coagulans	9
B. megaterium	5
B. firmus	4
B. sphæricus	4
B. brevis	3
B. stearothermophilus	1

TABLE I

Characteristics of Isolates falling into Group II

	Isolate No.										
	L 2	L 4	E 18	Centre	P 8	N 2	N 4	GS	Air 4	RHS	Mango
Gram's staining ..	−	−	−	−	−	−	−	+	−	−	−
Motility ..	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Production of Acid:*											
Glucose ..	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	+
Xylose ..	"	"	"	"	"	+	+	"	+	+	+
Arabinose ..	"	"	"	"	"	Alkaline	Alkaline	"	Alkaline	Alkaline	+
The V.P. Test ..	−	−	−	−	−	−	−	−	−	−	−
pH of Glucose broth ..	8·0	8·0–8·2	7·6	8·0	7·6	7·2	7·4	7·2	6·6–6·8	7·2	6·0–6·2
Production of Indol ..	−	−	−	−	−	−	−	−	−	−	−
Growth at 65° C. ..	−	−	+	−	−	+	+	+	+	+	+
Gelatin liquefaction ..	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis ..	+	+	+	+	+	−	−	−	−	−	−
Starch Hydrolysis ..	−	−	−	−	−	−	−	−	−	−	+
Nitrate Reduction ..	+	+	−	+	+	+	+	+	−	+	+
Identity ..	*B. brevis*	*B. brevis*	Unidenti-fied	*B. brevis*	Unidenti-fied	Unidenti-fied	Unidenti-fied	Unidenti-fied	Unidenti-fied	Unidenti-fied	*B. stearo thermo-philus*

* In organic basal medium.

The characteristics of *B. brevis*, *B. stearothermophilus* and several unidentified isolates belonging to Group II are presented in the accompanying table and those of representatives of the better known species are summarised below:—

Isolate Ag 1b:

Gram's staining: positive
Motility: motile
Growth on Glucose-nitrate agar: good, bluish black pigment produced
Pigment on Tyrosine agar: bluish black
Fermentation of sugars: acid only from glucose, xylose and arabinose in an organic basal medium
The V. P. Test: positive
pH of Glucose broth: 6·8–7·0
Gelatin Liquefaction: positive
Casein Hydrolysis: positive
Starch Hydrolysis: positive
Nitrate Reduction: nitrite produced
Action on Litmus Milk: alkalinity, reduction followed by proteolysis
Identity: *B. subtilis var. atterimus*

Isolate Nain:

Gram's staining: positive
Motility: motile
Monochrome staining with Aq. Fuchsin: even staining
Growth on Proteose-peptone acid agar: good
Growth in 5% NaCl broth: no growth
Growth on Glucose-nitrate agar: no growth
Pigment on Tyrosine agar: nil
Fermentation of sugars: acid only from glucose, xylose and arabinose in organic basal medium
The V. P. Test: positive
The M. R. Test: positive
Gelatin liquefaction: negative
Starch Hydrolysis: positive
Casein Hydrolysis: positive
Nitrate Reduction: negative

Action on Litmus Milk: reduction, acidity, coagulation and digestion
Identity: *B. coagulans*

Isolate L 1:

Gram's staining: positive
Motility: motile
Growth on glucose-nitrate agar: negative
Fermentation of sugars: acid only from glucose and xylose, alkalinity with arabinose in organic basal medium
The V. P. Test: positive
Gelatin Liquefaction: positive
Casein Hydrolysis: positive
Starch Hydrolysis: negative
Nitrate Reduction: nitrite produced
Action on Litmus Milk: acidity, coagulation, reduction and digestion
Identity: *B. firmus*

Isolate Bom 1c:

Gram's staining: positive
Motility: motile
Monochrome staining with Aq. Fuchsin: uneven staining
Growth on Proteose-peptone acid agar: good
Growth on Glucose-nitrate agar: nil
Pigment on Tyrosine agar: nil
Fermentation of sugars: acid only from glucose, xylose and arabinose in organic basal medium
The V. P. Test: positive
The M. R. Test: positive
Gelatin Liquefaction: negative
Starch Hydrolysis: positive
Nitrate reduction: negative
Action on Litmus Milk: alkalinity
Identity: *B. megaterium*

Isolate P 5:

Gram's staining: negative
Motility: motile

Fermentation of sugars: alkalinity with glucose, xylose and arabinose in organic basal medium

The V. P. Test: negative

pH of Glucose broth: 7·8

Gelatin Liquefaction: negative

Casein Hydrolysis: negative

Starch Hydrolysis: negative

Growth in 10% *NaCl broth:* negative

Nitrate Reduction: negative

Action on Litmus Milk: alkalinity

Identity: B. sphæricus

Discussion

It becomes obvious from the results presented that every source of material examined has in it thermophilic bacteria and this observation becomes significant when we view it from the standpoint of spoilage that occurs in the canned foods under certain conditions.

Of the ninety-five isolates studied fifty-eight fell into Group I and of these it was possible to identify fifty-four, the remaining four being unidentified owing to the fact that these died out during the course of work. In other respects, establishing the identity of isolates belonging to Group I did not present a problem.

Insofar as the organisms of Group II are concerned we are constrained to admit that their identification is a matter of considerable difficulty except for those isolates which could be referred to as *B. brevis* and *B. stearothermophilus*. The difficulty is largely due to the unsatisfactory state of affairs in the separation of sporeforming bacteria belonging to this group. The key presented by Smith *et al.*[11] utilises the presence or otherwise of amylase to separate the starch hydrolysing species *B. stearothermophilus*, *B. circulans*, and *B. alvei* from the starch non-hydrolysing species *B. laterosporus*, *B. brevis*, and *B. pulvifaciens*. Several of our isolates which can grow at 65° C. (temperature of water kept in the incubator was 65° C.) fail to utilise starch. Further, Smith *et al.*, have also reported that the only species of thermophilic bacteria that could grow at 65° C. were strains of *B. stearothermophilus*. Are these our strains then *B. stearothermophilus* or are they members of the starch non-hydrolysing species of Group II with optimum and maximum growth temperatures higher than what have been observed by previous workers? This remains to be seen. Of interest in this connection is the work of Jansen and Aschehoug[9] who reported the failure to utilise

starch by two of the nine strains of *B. circulans* they studied. A similar failure to utilise starch has also been reported by Marsh and Larsen[10] by two of the strains of *B. stearothermophilus* they examined.

The four isolates placed in Group III were, however, identified with ease as strains of *B. sphæricus*.

It is very significant to mention here that the number of isolates of each species in our collection is no indication of their distribution in nature. For example, the utilisation of an acid medium like proteose-peptone acid agar alone for isolation would result in isolating strains of *B. coagulans* with the consequential suppression of *B. subtilis*, even though the latter species is the most dominant of the commonly occurring sporeforming bacteria.

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