THE INFLUENCE OF GROWTH AND SPORULATION TEMPERATURE ON HEAT RESISTANCE OF A BACTERIAL NUCLEOSIDASE

P.K.AGRAWAL*, RAM NARAYAN AND K.G.GOLLAKOTA.

Department of Biochemistry, College of Basic Sciences & Humanities, G.B.Pant University of Agriculture & Technology**, PANTNAGAR(U.P.). INDIA.

Received January 31,1975

Employing Bacillus cereus T cultures capable of rapid growth and simultaneous sporulation, the influence of growth and sporulation temperature on the heat resistance of nucleosidase (EC.3.2.2.1) and of sporulating cells has been investigated. When the growth and sporulation temperature of the cultures is elevated from 30 ± 1 C to 34 ± 1 C, the heat resistance develops an hour earlier both in the nucleosidase (EC.3.2.2.1) and sporulating cells. A comparison has been made between the octyl alcohol resistant and the heat resistant nucleosidase (EC.3.2.2.1) activities. The data further confirm the previously reported close correlation between the development of heat resistance in the nucleosidase (EC.3.2.2.1) and in the sporulating cells themselves.

INTRODUCTION

A heat stable nucleosidase (EC.3.2.2.1) present in the cells and spores of Bacillus cereus T has been reported in our previous communication (1). This enzyme was found to be present in much greater amounts in the intact spores and in the germinated, octyl alcohol killed spores as compared to the vegetative cells, which contained the heat labile but octyl alcohol resistant form of nucleosidase (EC.3.2.2.1). We had also observed a close correlation between the development of heat resistance in the nucleosidase (EC.3.2.2.1) and that of heat resistance in the sporulating cells.

There are earlier reports to show that the thermo-resistance of the spores of Bacillus anthracis increased with the temperature of sporulation

---

* Present Address: Biochemistry Division, National Sugar Institute, KANPUR-208017(U.P.)INDIA.

**Formerly U.P.Agricultural University.

(2). Lechowich and Ordal (1960) could further demonstrate that elevated temperature of sporulation in Bacillus subtilis led to increased contents of dipicolinic acid, calcium, magnesium, manganese; all of them leading to the increased thermo-resistance of the spores (3). Further, alanine racemase and nucleosidase (EC.3.2.2.1) have been found to be intimately associated with a particulate fraction , which presumably is responsible for their thermal-resistant properties (4,5,6). The attachment of the nucleosidase (EC.3.2.2.1) to the spore cell wall may be the plausible explanation for its activity in the dormant spores which normally were considered impermeable (7).

In view of above reports, it was quite likely that elevating the growth and sporulation temperature may influence the heat resistance characteristics of the nucleosidase (EC.3.2.2.1) and its relationship, if any, with the heat resistance of the sporulating cells . The present communication describes such a study with Bacillus cereus T cultures cultivated at 30 ± 1 C and 34 ± 1 C,respectively.

MATERIALS AND METHODS

All investigations were done using materials and methods as described previously (1). For octyl alcohol treatment , the following procedure was adopted:-

To the 10 ml cell suspension, a drop of octyl alcohol was added and the cell suspension was shaken vigorously for 20 min . The cell suspension was then centrifuged at 8000 rpm for 10 min in a refrigerated superspeed centrifuge at a temperature of 0 to 4 C , cells washed twice with 10 ml aliquotes of ice cold, 0.2M phosphate buffer (pH 7.0); resuspended in 10 ml buffer, and finally used for assay of nucleosidase (EC.3.2.2.1 activity at 37 C.

The organism was grown at 30 ± 1 C and 34 ± 1 C, respectively;and

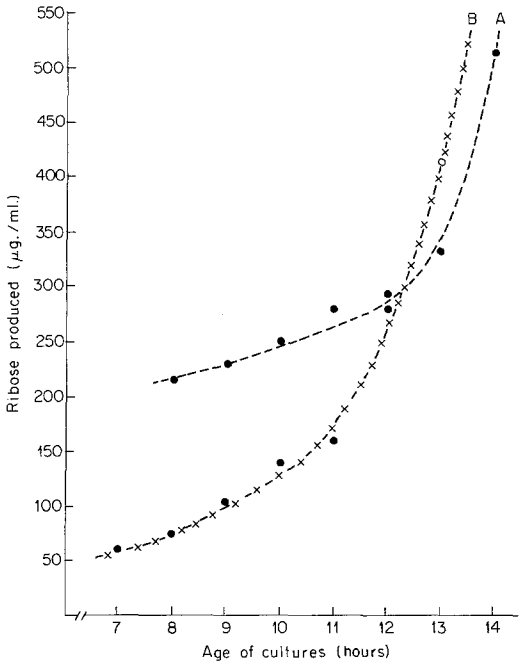

Fig.1.: Nucleosidase (EC.3.2.2.1) activity (in terms of ribose produced) in the sporulating cells (20 µg/ml) of different culture age, incubated with 600 µg adenosine at 75 C for 20 min. Symbols: A, enzyme activity in the cells grown at 30±1 C; B, enzyme activity in the cells grown at 34±1 C.

culture aliquotes drawn intermittently for determination of the nucleosidase (EC.3.2.2.1) activity and total viable/heat stable cell counts. A close check on the morphological state of the culture was kept by examining the stained smears microscopically.

RESULTS AND DISCUSSION

Influence of Elevated Growth And Sporulation Temperature on the Nucleosidase (EC.3.2.2.1) Activity at 75 C:

Fig.1 shows the increase in the nucleosidase (EC.3.2.2.1) activity (at 75 C) with the age of culture grown at 30±1 C and 34±1 C, respectively. It is apparent from the Fig.1 that enzyme activity in cells grown at 30±1 C increases linearly at a constant

Table 1: pH, morphology and appearance of sporulating cells grown at 30±1 C.

Age of Culture (Hours)	pH	Morphology And Appearance*
8	7.10	Heavy granulation, chains & clumps of cells formed, taking stain.
9	7.15	Cells reduced to the shape of spores, heavy granulation, clumps present, taking stain.
10	7.20	-do-
11	7.25	Granulation high, marked spore shape formed, stainability reduced.
12	7.325	Spore like bodies formed, becoming refractile, do not take stain.
13	7.40	Refractile spores in chains and clumps.
14	7.55	Completely refractile spores, in chains and clumps.

* Observed microscopically from crystal violet stained smears.

rate from 8 to 12 hours and exhibits a sudden spurt at 13 hours which persists. This abrupt increase in the enzyme activity, at 75 C, indicates very likely the development of heat resistance characteristics in the enzyme between 12 and 13 hours. It will be noted from Table 1 that the culture age of 12 and 13 hours corresponds to pH 7.325 and 7.4, respectively.

Fig. 1 also depicts that nucleosidase (EC.3.2.2.1) activity in the cells grown at 34±1 C increases linearly at a constant rate from 7 to 11 hours and, as in the previous case, increases abruptly soon after 11 hours , and continues increasing at a faster rate. In this case, the likely development of heat resistance characteristics in the enzyme appears to take place between 11 and 12 hours; that is an hour earlier. Table 2 indicates pH 7.3 and 7.4 at the culture age of 11 and 12 hours, respectively.

From above, it may be inferred that cells (grown at 34±1 C) of 11 to 12 hours culture age are in a physiological and biochemical

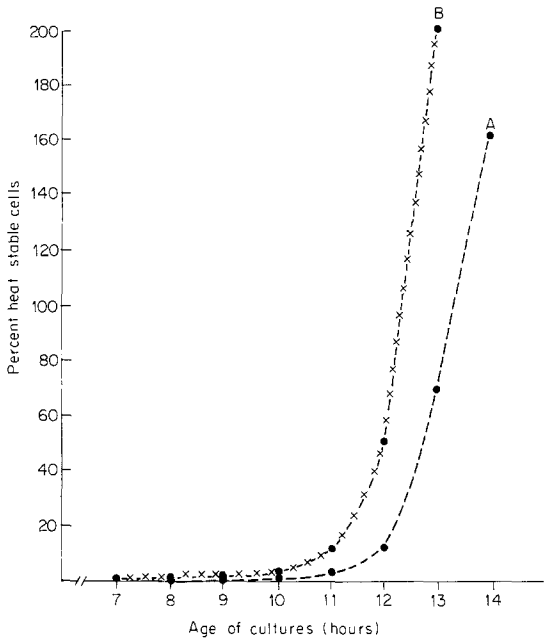

Fig.2.: Increase in heat stable cells (percent of total viable cells) of different culture age. Symbols: A, cells grown at 30±1 C; B, at 34±1 C.

---

state almost similar to that occurring at 12 to 13 hours culture age, had the cells were grown at 30±1 C. Further, it is also evident that nucleosidase (EC.3.2.2.1) in the sporulating cells grown at 34±1 C develops heat resistance one hour earlier than that in the cells grown at 30±1 C.

Influence of Elevated Growth And Sporulation Temperature on Heat Stable Cell Counts:

Fig.2 exhibits the increase in heat stable (heat treatment given at 80 C for 30 min , prior to plating) cells with age of culture. It is observed that, when cells are grown at 30±1 C, the heat stable cells are only 2-3 and 10 percent at 11 and 12 hours , respectively. There is a sudden increase in the percentage of heat stable cells from 12 hours onwards. From this it may be concluded that the development of heat resistance in the sporulating cells has taken place between 12 and 13 hours. From Table 1 ,

it will be noted that at 12 hours culture age sporulating cells have taken the shape of sporelike, refractile bodies which do not take stain. This morphological state at 12 hours is in close agreement with the development of heat resistance in the sporulating cells between 12 and 13 hours.

From Fig.2 it will also be noted that, when cells are grown at 34±1 C, the number of heat stable cells at 10 and 11 hours is very low (2-3 and 11 percent, respectively) and rises abruptly after 11 hours and continues to rise further. In this case again, it is evident that the sporulating cells have developed heat resistance between 11 and 12 hours , that is one hour prior to the cells grown at 30±1 C. From Table 2, it will be noted that at 11 hours culture age sporulating cells have taken the shape of sporelike, refractile bodies which do not take stain. This morphological state at 11 hours culture age is in close agreement with the development of heat resistance in the sporulating cells between 11 and 12 hours.

Table 2: pH, morphology and appearance of sporulating cells grown at 34±1 C.

Age of Culture (Hours)	pH	Morphology And Appearance*
7	7.00	Heavy granulation, cells in chains & clumps, Taking stain.
8	7.10	Sporelike bodies start forming , granulation heavy, cells in clumps, taking stain.
9	7.20	-do-
10	7.25	Sporelike bodies start becoming refractile, but take stain.
11	7.30	Clumps breaking, more refractile spores, do not take stain.
12	7.40	Spores in clumps and chains, completely refractile.
13	7.50	-do-
14	7.60	-do-

* Observed microscopically from crystal violet stained smears.

In Fig.2, percentage of heat stable cells higher than one hundred is due to the fact that on giving heat treatment to the cultures(heat treatment at 80 C for 30 min)chains and clumps of the sporulating cells are broken into individual cells,resulting into heat stable cell count higher than total viable cell count.

Comparison of Octyl Alcohol Resistant And Heat Resistant Nucleosidase (EC.3.2.2.1) Activities:

It has been demonstrated previously that octyl alcohol resistance in the sporulating cells of Bacillus cereus T developed earlier than heat resistance. It was also observed that heat resistant cells were also resistant to octyl alcohol but not vice versa(8). In our previous communication (1), we had reported that comparatively higher amounts of ribose was liberated by the nucleosidase (EC.3.2.2.1) present in the octyl alcohol killed cells incubated at 37 C than by the enzyme present in the normal cells, under similar conditions. It was due to the fact that the normal cells could further metabolize the ribose liberated by the nucleosidase (EC.3.2.2.1) whereas the octyl alcohol killed cells could not. It was also reported that no nucleosidase (EC.3.2.2.1) activity in the normal vegetative cells could be demonstrated when incubated at 75 C; thus providing evidence in favour of the fact that nucleosidase (EC.3.2.2.1) present in vegetative cells was resistant to octyl alcohol but not to heat.

Employing cultures grown at 30±1 C, a comparison has been made between the octyl alcohol resistant nucleosidase (EC.3.2.2.1) activity at 37 C and heat resistant nucleosidase (EC.3.2.2.1) activity at 75 C (Fig.3); in order to ascertain if the two activities were same or different. The graphic representation of the data shows that the two activities are parallel, in slope, to each other and are comparable. However, it is noted that at 37 C the activity is low than that at 75 C. It is quite likely that nucleosidase (EC.3.2.2.1) present in Bacillus cereus T possesses a high temperature optima thus liberating

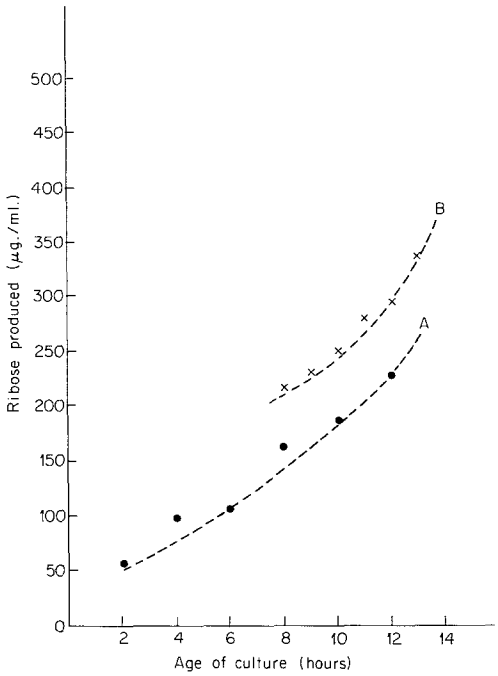

Fig.3.: Octyl alcohol resistant and heat stable nucleosidase (EC.3.2.2.1) activities (in terms of ribose produced),in cells grown at 30±1 C. Symbols: A, enzyme activity in the octyl alcohol killed vegetative cells (20/ug/ml), of different culture age, incubated with 600/ug adenosine at 37 C for 20 min.;B, enzyme activity in the normal sporulating cells (20/ug/ml), of different culture age, incubated with 600/ug adenosine at 75 C for 20 min.

---

greater amounts of ribose , on incubation with adenosine, at 75 C than at 37 C. Hence, the same enzyme species (θctyl alcohol resistant,heat labile),present in the earlier stages of growth & sporulation, probably acquires the resistance to high temperatures in the later stages. It is also evident from Figs.1 & 3 that octyl alcohol resistance in the nucleosidase (EC.3.2.2.1) develops much earlier than heat resistance.; whereas in case of sporulating cells it develops only one to two hours earlier (8).Although the possibility that nucleosidase (EC.3.2.2. present in Bacillus cereus T may be inherently stable to octyl alcohol can not be denied.

The results further confirm that there is a close correlation between

the development of heat resistance in the nucleosidase (EC.3.2.2.1) and that of heat resistance in the sporulating cells themselves, as reported earlier (1). When the growth and sporulation temperature is elevated by 4 C, this correlation is still valid but heat resistance, both in the enzyme and in the cells, occurs earlier by one hour.

ACKNOWLEDGEMENTS

We are grateful to the United States Agency for International Development for granting one of us (PKA) a Graduate Traineeship during the period of investigation.

REFERENCES

1.Agrawal,P.K.;Narayan,Ram and Gollakota,K.G.(1974).Biochem.Biophys. Res.Commun.60:111-117.
2.Weil,R.(1899).Arch.Hyg.35:355.
3.Lechowich,R.V.and Ordal,Z.J.(1960).Bacteriol.Proc.pp.44.
4a.Stewart,B.T.and Halvorson,H.O.(1953).J.Bacteriol.65:160-166.
4b.Stewart,B.T.and Halvorson,H.O.(1954).Arch.Biochem.and Biophys.49:168-178.
5a.Lawrence,N.L.(1955a).J.Bacteriol.70:577-582.
5b.Lawrence,N.L.(1955b).J.Bacteriol.70:583-587.
6.Nakata,H.M.(1956).M.Sc.Thesis,University of Illinois,Urbana,Ill.,U.S.A.
7.Halvorson,H.and Church,B.(1957).Bacteriol.Revs.21:112-131.
8.Gupta,R.K.;Narayan,Ram and Gollakota,K.G.(1970).Biochem.Biophys.Res. Commun.38:23-30.

***