

Growth of and trehalase activity in the thermophilic fungus *Thermomyces lanuginosus*

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Abstract. The thermophilic fungus, *Thermomyces lanuginosus*, was grown in a glucose-asparagine liquid medium. Optimal mycelial growth occurred at 50°C. The conditions for sporulation were different from those required for vegetative growth, the former being favoured by lower nitrogen level and temperature. Trehalase (α , α -glucoside-1-glucohydrolase, EC 3.2.1.28) was one of the most active glycosidases at 50°C. Non-sporulating mycelium had higher levels of this enzyme than the sporulating mycelium. Trehalase was synthesized constitutively and its activity appears to be controlled by catabolite repression.

Keywords. *Thermomyces lanuginosus*; thermophilic fungus; trehalase; sporulation; catabolite repression.

1. Introduction

Those fungi, which have a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C, have been arbitrarily defined as thermophilic fungi (Cooney and Emerson 1964). According to this definition, about 23 species of thermophilic fungi are known to date. Despite their widespread occurrence and potential economic importance, investigations on their physiology and biochemistry have been very few (Emerson 1968; Crisan 1973). There is no previous study of trehalase (α , α -glucoside-1-glucohydrolase, EC 3.2.1.28) from any thermophilic organism. A study was made of the optimal conditions for the production of trehalase in this fungus, with a view to purify this enzyme (Prasad and Maheshwari 1978) and compare its properties with trehalase from a mesophilic fungus. The present paper describes some characteristics of growth of the thermophilic fungus, *Thermomyces lanuginosus*, in a chemically defined medium.

2. Materials and methods

2.1. Chemicals

Trehalose, cellobiose, maltose, lactose, sorbose, galactose, *p*-nitrophenyl- α -glucoside, *p*-nitrophenyl- β -glucoside, *p*-nitrophenyl- β -galactoside were obtained from Sigma Chemical Company, St. Louis, MO, USA. Glucostat reagent was obtained from Worthington Biochemical Corporation, Freehold, NJ, USA. All other chemicals used were of the analytical reagent grade.

2.2. Isolation of *Thermomyces lanuginosus*

Samples of soil collected from various localities in India were used for the isolation of the thermophilic fungus, *Thermomyces lanuginosus*. The technique of collecting soil samples and the general procedures for the isolation of thermophilic fungi were as described by Maheshwari and Antony (1974) and Cooney and Emerson (1964). *T. lanuginosus* was isolated from all soil samples tested, numbering about 20. The soil samples screened were from exposed, shaded, cultivated and uncultivated areas. Colonies were purified by serial dilution technique and the thermophilic nature of the isolates was confirmed by their ability to grow at 50°C and inability to grow at room temperature (20°–28°C) on yeast extract-phosphate-starch-sulphate (YpSs) and yeast extract-glucose (YG) media (Cooney and Emerson 1964). The morphology of the isolates corresponded closely with the diagnosis given by Cooney and Emerson (1964). In the present study, an isolate *T. lanuginosus* RM-B, from horse-dung, was used. This fungus has also been referred to in literature as *Monotospora lanuginosa* (Crisan 1962) and *Humicola lanuginosa* (Cooney and Emerson 1964; Prasad and Maheshwari 1978). The presently accepted nomenclature for this fungus is *Thermomyces lanuginosus* (Samson and Tansey 1977).

2.3. Production of spores

Sporulating cultures were obtained by growing the organism on YpSs medium in prescription bottles at 40°C for 10 to 15 days in a humidified incubator followed by incubation at room temperature (20°–28°C) for 4 to 12 months. A spore suspension made in 0.05% (v/v) Triton X-100 in water from such cultures was used as inoculum for liquid cultures.

2.4. Culture conditions

A glucose-asparagine medium was used for the vegetative growth of the fungus. It contained (g/l) glucose 20, L-asparagine 4, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5 and 0.1 ml per litre of a trace element stock solution (Vogel 1964). The pH of the medium before sterilisation was 7.2. Static cultures were grown in 40 ml medium in 250 ml Erlenmeyer flasks and shake cultures (~ 200 rpm) were grown in 150 ml medium in 500 ml Erlenmeyer flasks, unless mentioned otherwise. Cultures were incubated at 50°C.

Sporulation was induced by reducing the concentration of L-asparagine to 0.5 g/l in the medium described above. These cultures were incubated at 40°C. Sporulation was quantitated by homogenising the sporulating mycelium from triplicate flasks in a stainless steel semi-microjar on a Waring blender for 3 min, following which the suspension was made up to volume and the number of spores in an aliquot of the suspension was counted in a haemocytometer.

Media were sterilised at 15 psi for 15 min. When various sugars were tested as carbon sources, these were filter-sterilised using Millipore filter (0.45 μ m) and added aseptically to the sterilised salt solution.

2.5. Enzyme assays

Since many glycosidases in fungi are bound to the cell-wall, activities were measured

in crude mycelial homogenates of the fungus. The mycelial homogenates were obtained by grinding washed mycelium with twice its weight of sand in 10 vol of 50 mM sodium acetate buffer (pH 5.5) in a chilled mortar.

Trehalase was assayed cuprimetrically by estimating glucose produced after the hydrolysis of trehalose (Nelson 1944). The reaction mixture contained 1.6 ml of 50 mM sodium acetate buffer (pH 5.5), 0.2 ml of 20 mM trehalose and 0.2 ml of crude mycelial homogenate in a total volume of 2 ml and incubated for 15 min at 50°C. The tubes were shaken regularly to disperse the homogenate.

Activities of cellobiase, lactase and maltase in the crude mycelial homogenate were measured by estimating the glucose formed using Worthington Glucostat reagent. The reaction mixture in a total volume of 2 ml was incubated at 50°C for 15 min, after which the enzyme was inactivated by placing the tubes in a boiling water bath for 10 min. The mixture was diluted to 4 ml with water and 2 ml of Glucostat reagent was added and incubated for 1 h at 37°C. The reaction was terminated by adding 0.1 ml of 4N HCl. Intensity of the colour was measured after 10 min using filter 42 in a Klett-Summerson colourimeter.

α - and β -Glucosidase and β -galactosidase were assayed for 15 min at 50°C, with *p*-nitrophenyl- α -glucoside, *p*-nitrophenyl- β -glucoside and *p*-nitrophenyl- β -galactoside as substrates, respectively. The assay system was similar to that described for trehalase. The reaction was stopped by the addition of 1 ml of 1 M Na₂CO₃. The solution was diluted to 6 ml with water and absorbance was measured at 405 nm.

One unit of trehalase activity is defined as the amount of enzyme which liberates one n mol of glucose per min at 50°C. For other glycosidases, one unit of activity is defined as the amount of enzyme which liberates one n mol of glucose or *p*-nitrophenol per min at 50°C. Specific activity is expressed as units/mg protein.

2.6. Protein estimation

Protein was estimated by the method of Lowry *et al* (1951) with bovine serum albumin as the standard.

2.7. Glucose in culture filtrate

Culture filtrate was cherry red in colour due to the secretion of pigment(s) during the growth of the organism. The filtrate was acidified with 1N HCl and the pigment was removed by extraction with ether. The solution was deionized by passing it consecutively through acidic and basic ion-exchange resins. Glucose was estimated in the eluate (neutral fraction) using Worthington Glucostat reagent.

2.8. Total carbon in culture filtrate

Total carbon in lyophilised culture filtrate was estimated by Pregl's method (Ingram 1962).

2.9. Total amino nitrogen in culture filtrate

Total amino nitrogen in the culture filtrate was estimated by Kjeldahl method (Ingram 1962).

3. Results

3.1. Growth and temperature characteristics

All isolates of *T. lanuginosus* tested failed to grow below 30°C even after prolonged incubation on YpSs and YG media. Yield of isolate RM-B in glucose-asparagine medium was comparable to that in a rich medium containing yeast extract (YpSs medium). This observation showed that the thermophilic growth of this organism is not associated with any unusual nutritional requirements. The minimum, optimum and maximum temperature for the growth of isolate RM-B were close to 30°, 50° and 60°C, respectively.

When the organism was grown in static cultures, increase in dry wt. ceased on day 6. An estimation of glucose in the culture filtrate revealed that all glucose had been completely metabolised. However, significant amounts of carbon- and nitrogen-containing compounds were present in the medium even after day 6 (figure 1). If the culture filtrate after day 6 was decanted, filter-sterilised and inoculated with spores, it supported further growth of the fungus. However, the yields in such culture filtrates (staled medium) were half of that produced in the fresh medium. When the fungus was grown in shake culture at 50°C, the growth was rapid and maximum growth was obtained on day 3 (330 mg/flask) as compared to day 6 in static culture (280 mg/flask).

3.2. Sporulation

The fungus did not sporulate in liquid glucose-asparagine medium containing 4 g/l L-asparagine or liquid YpSs medium at temperatures ranging from 37° to 55°C.

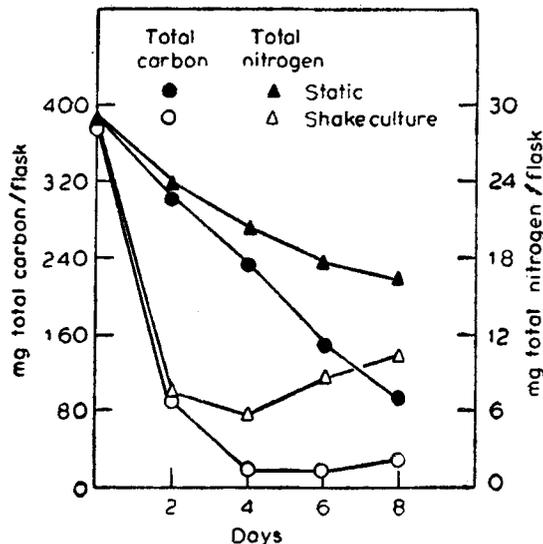


Figure 1. Nutrient utilisation by *Thermomyces lanuginosus* grown in static and shake cultures. Fungus was grown in glucose-asparagine medium (40 ml/250 ml Erlenmeyer flask) at 50°C. Maximum yield (dry wt) in static culture was 280 mg/flask as compared to 330 mg/flask in shake culture. Figures represent the amount of nutrients remaining in the medium.

Sporulation, however, occurred on agar medium of the same composition. The reason for the differential effect of liquid versus agar medium is not understood but may probably be due to differences in nutrient availability. Sporulation in liquid glucose-asparagine medium was, however, induced when the concentration of nitrogen source (L-asparagine) was reduced to 0.5 g/l. The degree of sporulation was influenced by the temperature of incubation. As shown in table 1, optimal sporulation was obtained at 40°C. Temperature for optimal sporulation (40°C) was different from that for vegetative growth (50°C). Sporulation also occurred in shake cultures (data not shown).

3.3. *Glycosidases*

Activities of all glycosidases in crude homogenates of non-sporulating mycelia increased during growth. Trehalase was one of the most active glycosidases on day 10 of culture (table 2) and this enzyme was, therefore, studied in detail. Five soil isolates and isolate RM-B of the fungus were compared for the specific activity of trehalase. RM-B produced trehalase of highest specific activity (data not given). In addition to high enzyme activity, it sporulated well on YpSs agar medium and produced visibly less pigment.

Table 1. Sporulation of *Thermomyces lanuginosus* in liquid glucose-asparagine medium in static cultures.

Temperature (°C)	Spores per flask ($\times 10^6$)
40	155
45	93
50	15

Number of spores per flask (average from three flasks) was estimated on day 15 of culture. One ml of a uniform suspension of spores was added into each flask containing 40 ml medium. The medium contained 0.5 g/l L-asparagine.

Table 2. Activities of glycosidases in *Thermomyces lanuginosus* RM-B.

Glycosidase	Specific activity	
	Day 5	Day 10
Trehalase (EC 3.2.1.28)	16	480
Maltase (EC 3.2.1.20)	180	433
Cellobiase (EC 3.2.1.21)	50	400
Lactase (EC 3.2.1.23)	8	114
Invertase (EC 3.2.1.26)	Not detected	
β -Galactosidase* (EC 3.2.1.23)	0.9	12
β -Glucosidase* (EC 3.2.1.21)	16	360
α -Glucosidase* (EC 3.2.1.20)	14	45

Fungus was grown in glucose-asparagine medium in static cultures at 50°C.

*One unit of enzyme activity was defined as the amount of the enzyme required to release one nmole of *p*-nitrophenol per min at 50°C.

3.4. Effect of carbon source on growth and trehalase activity

The fungus grew well on glucose, cellobiose, maltose, sucrose and trehalose. L-Asparagine was very poorly utilised as a carbon source. Growth was poor on sorbose, galactose and lactose. In carbon sources which produced good growth there was an initial delay in the development of trehalase activity (table 3).

It may be mentioned that invertase was not detected on day 5 or day 10 (table 2) although this fungus grew well on sucrose (table 3). It was later found that invertase in this fungus is an inducible enzyme and its activity decays rapidly following the utilisation of sucrose or raffinose (Palanivelu and Maheshwari, unpublished results).

3.5. Trehalase activity during growth in non-sporulating cultures

The relation between growth and levels of trehalase was studied by determining the sp. act. of the enzyme in crude mycelial homogenates. In static cultures, trehalase sp. act. was low till day 6, after which it increased rapidly. This burst in enzyme activity occurred after the disappearance of glucose in the medium and after growth

Table 3. Effect of carbon source on growth and trehalase activity in *Thermomyces lanuginosus* RM-B

Carbon source	Mycelial wet weight per flask		Specific activity	
	Day 5	Day 10	Day 5	Day 10
L-Asparagine	—	30	—	619
Cellobiose	460	960	14	406
Galactose	50	100	22	54
Glucose	610	900	29	644
Lactose	75	290	181	275
Maltose	590	720	66	286
Sorbose	12	32	266	247
Sucrose	560	910	3	208
Trehalose	500	1000	25	347

All compounds were used at concentration of 0.8% (w/v) carbon. 0.4% (w/v) L-Asparagine was used as nitrogen source. The fungus was grown in static cultures under non-sporulating conditions. — Not determined.

had ceased (figure 2). In five out of seven experiments, we observed two peaks of activity (day 8 or 9 and day 11 or 12), the former was less pronounced. The burst in enzyme activity was observed when autolysis had commenced. In contrast, in shake cultures the development of trehalase activity proceeded almost immediately, reaching a peak on day 5 or day 6, following which it declined (figure 3). Increase in trehalase activity in shake cultures also occurred after growth rate diminished and after most of glucose had been consumed.

Trehalase activity increased with age in both sporulating and non-sporulating mycelium, although to a very small extent in the sporulating mycelium (data not shown). The non-sporulating mycelium, however, had much higher levels of the

enzyme than sporulating mycelium, even at 40°C, the temperature which was found to be optimal for sporulation.

3.6. Effect of glucose on growth and trehalase activity

In static cultures, the burst in trehalase activity following the disappearance of glucose from the culture medium suggested that the enzyme might be under catabolite repression by glucose. This was corroborated by the observation that when the fungus was grown in a medium containing 0.5% instead of 2% glucose, there was no lag in trehalase development and trehalase had a higher specific activity (data not

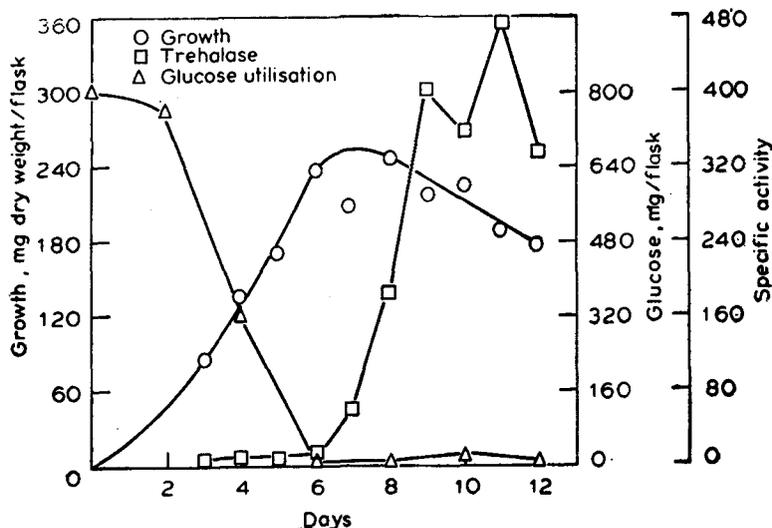


Figure 2. Growth and trehalase activity in relation to glucose utilisation in *Thermo- myces lanuginosus* grown in static cultures at 50°C

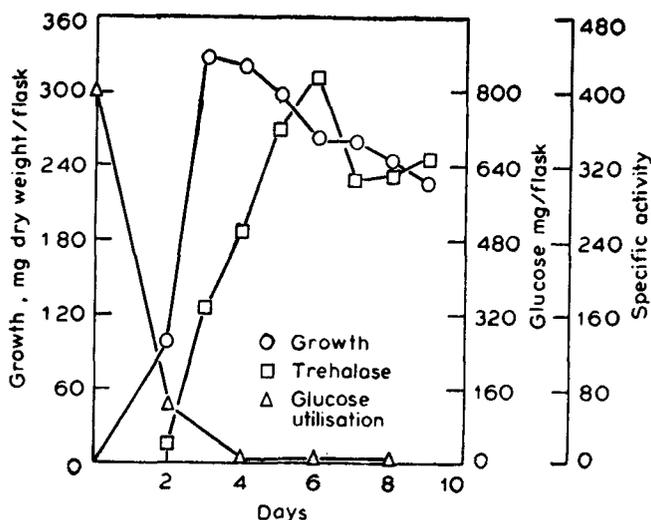


Figure 3. Growth and trehalase activity in relation to glucose utilisation in *Thermo- myces lanuginosus* grown in shake cultures at 50°C.

given). If increase in trehalase synthesis is triggered by the metabolism of glucose, addition of glucose should delay the burst in trehalase synthesis normally observed.

When fresh medium (40 ml) containing 2% glucose was aseptically added into culture flasks on day 4 or day 6 of growth (after decanting the staled medium), a new mycelial growth developed over the preformed mycelium. The new growth developed on the surface of the culture medium appressed to the preformed mycelial mat. Such mycelial growth was not readily apparent under the normal growth condition; however, the two mycelial mats separated when poured out. An overgrowth of mycelium also occurred if static cultures were merely disturbed (i.e., flasks were momentarily shaken) so as to cause the adhering edge of the mycelial mat to get dislodged from the side of the flask. Sufficient carbon and nitrogen was present in culture medium on day 6 for an overgrowth to form (figure 1).

Trehalase activity was measured separately in both preformed growth and the overgrowth produced in static cultures as a result of disturbing the cultures on day 6. The new overgrowth exhibited a pattern of trehalase activity similar to that in the undisturbed cultures (figure 4). In contrast, in the preformed mycelium the enzyme remained at a low level; the burst in trehalase activity which normally occurs in the undisturbed cultures was absent. The effect of addition of glucose was similar to that produced by disturbing the static cultures on day 6 (data not shown). The act of adding glucose solution into culture flasks itself caused a 'disturbance'.

Because of the difficulties in interpreting the data obtained with static cultures, the effect of glucose on trehalase activity was studied with mycelia grown in shake cultures. Mycelia pre-grown on mannitol were used to determine if addition of glucose inhibits trehalase activity under non-growing conditions (i.e., in the absence of a nitrogen source). As shown in table 4, addition of glucose reduced trehalase activity by more than 80% while the addition of substrate (trehalose) reduced enzyme activity by about 50%.

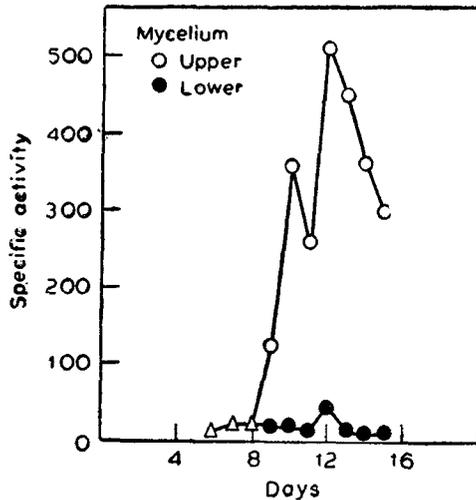


Figure 4. Trehalase activity in disturbed cultures. Fungus was grown in static culture. The culture flasks were momentarily shaken on day 6. This resulted in the development of overgrowth. Trehalase activity was measured separately in the two mycelial mats.

3.7. Growth and trehalase activity in staled medium

Staled medium from static cultures on day 6 of growth at 50°C was decanted off and filter-sterilised. *T. lanuginosus* was inoculated into 40 ml of this medium in 250 ml Erlenmeyer flasks and incubated at 50°C. Growth in staled medium was approximately half of that in fresh medium. In staled medium (lacking glucose) there was no inhibition of trehalase activity and this activity increased with growth.

4. Discussion

Although studies on thermophilic fungi are few, it does not appear that the nutritional requirements of the thermophilic fungi are more complex than that of mesophilic fungi. There are reports of microorganisms requiring supplementation for growth at higher temperatures (Ingraham 1962; Langridge 1963) and at lower temperatures (O'Donovan and Ingraham 1965). The simple nutritional requirements of *T. lanuginosus* should make this fungus suitable for biochemical investigations.

The cessation of growth of fungal colony in culture is usually ascribed to exhaustion of nutrients, production of staling factors, change in pH etc. These factors did not, however, appear responsible for the cessation of growth of *T. lanuginosus* observed in static cultures. Rather, cessation of growth in static cultures appeared due to depletion of dissolved oxygen. It was observed that by day 6 in static cultures, the mycelium had made an allround contact with the sides of the flask and this could have prevented diffusion of oxygen into the culture medium. Indeed, a disturbance of the culture on day 6 causing the adhering edge of the colony to dislodge resulted in the growth of a new colony. The profile of enzyme activity in the disturbed cultures changed. While this may not be a general phenomenon in fungi, nevertheless, this observation suggests caution in biochemical studies with static cultures.

There was a rapid and an increased utilization of nutrients and increased growth in shake cultures. Hanks and Sussman (1969a) observed that the mycelial growth in *Neurospora* ceased after 4 days even though sufficient carbon sources (0.5%) were available. In shake cultures, growth continued till a concentration of 0.05% sugar was reached. Reiming (quoted by Loginova and Tsaplina 1976) observed thermophiles to be economically less efficient. The amount of biomass produced was dependent on the amount of dissolved oxygen present in the medium. Deploey and Fergus (1975) have shown that thermophilic fungi cannot grow in atmospheres of pure nitrogen. Oxygen availability therefore is an important factor in thermophilic growth.

Table 4. Inhibition of trehalase activity in *Thermomyces lanuginosus* RM-B by glucose.

Treatment	Specific activity
50 mM Phosphate buffer, pH 7.0	421
+ 2% Glucose	83
+ 2% Trehalose	240

The fungus was grown in 2% mannitol in shake culture at 50°C for 12 h. Washed mycelium was incubated for 3 h with shaking at 50°C under conditions indicated. Enzyme activity was measured in the mycelial homogenates.

This study demonstrated that conditions for optimal sporulation are different from those for vegetative growth, being favoured by higher carbon: nitrogen ratio and lower temperature. Lower temperature may also affect sporulation in an indirect manner, by making more oxygen available. These factors may be important in growth and sporulation of *T. lanuginosus* in its natural habitat (soil).

Trehalase was one of the most active glycosidases in *T. lanuginosus*. Its substrate, trehalose, is present in the sporulating mycelium at a concentration of 1 to 3% of the dry weight (Prasad and Maheshwari, unpublished data). However, trehalose was not detected in hot ethanol-soluble extracts of non-sporulating mycelium from day 2 to day 13 of growth. Therefore, the significance of trehalase activity in non-sporulating mycelium is not understood. In fact, the role of trehalose in fungi is far from clear in spite of its being the most widely accumulated and major carbohydrate in fungal spores (Elbein 1974).

Irrespective of the carbon source used, levels of trehalase remained high. Indeed, even in carbon sources such as asparagine or sorbose which permitted only limited growth, trehalase activity was high (table 3). The possibility that the carbon sources are converted to trehalose and thereby induce trehalase synthesis is unlikely as trehalose was not detected in mycelium. In this thermophilic fungus, trehalose was detected only in spores. Furthermore, non-sporulating mycelium incubated in buffer alone had much higher trehalase activity than the mycelium incubated in the presence of the substrate (table 4). These observations show that trehalase in *T. lanuginosus* is a constitutive enzyme.

The activity of several glycosidases is controlled by catabolite repression (Horowitz and Metzenberg 1965). This situation may be applicable to trehalase in *T. lanuginosus* as well. Increase in trehalase activity occurred following the complete utilisation of glucose in the medium (figures 2 and 3) and the enzyme activity was greatly reduced by the addition of glucose (table 4). The addition of substrate reduced, rather than increased, trehalase activity, although the inhibition was less than that caused by glucose. Trehalose is not expected to form as extensive pools of glucose as glucose itself, thereby reducing the extent of inhibition.

Mandelstam (1962) found that when *E. coli* was grown on various carbon sources, the activity of constitutive (*i*⁻) β -galactosidase correlated with the doubling time of culture; the poorer the carbon source the higher the enzyme activity. A similar correlation was observed for trehalase in *N. crassa* (Hanks and Sussman 1969b) when cultures were harvested and assayed after 5 days of growth. In *T. lanuginosus*, the correlation mentioned above is apparent if day 5 data (table 3) are examined, however, the day 10 data show that such a correlation may not be real.

Acknowledgement

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