Appl Microbiol Biotechnol (1988) 29:485–493

Applied and Microbiology Biotechnology

Growth characteristics of a thermotolerant methylotrophic *Bacillus* sp. (NCIB 12522) in batch culture

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Summary. This contribution deals with problems associated with the culture of a thermotolerant methylotrophic *Bacillus* sp. The results reported clearly demonstrate why conventional enrichment/isolation procedures have, in the past, failed to allow such microbes to assert themselves. The catastrophic effect of carbon substrate (methanol) exhaustion on such cultures is clearly evidenced, but the effects of other nutrient exhaustion or limitations are demonstrated to be markedly less stringent. The failure of such cultures to complete the sporulation process when growing on methanol has important consequences with respect to their survival characteristics.

Introduction

During the late 1960's and the 1970's there was considerable interest in the production of single cell protein (SCP) using methanol as the process feedstock (Hamer 1979). Throughout that period repeated efforts were made to isolate an appropriate process microbe that could grow optimally in either the thermotolerant (45°–60° C) or the thermophilic (>60° C) temperature ranges for growth. With the exception of Snedcor and Cooney (1974), who reported some work with a mixed culture, and a claim in the patent literature by Hitzman (1976) concerning two *Bacillus* strains with temperature optima for growth of ca. 55° C, the objective of obtaining non-fastidious, thermotolerant process cultures for SCP production was elusive. It is frequently suggested that the reason that thermotolerant and thermophilic methylotrophic cultures cannot be enriched for and isolated is that methanol evaporates from aqueous growth media at such temperatures, clearly indicating a failure to understand the impact of vapour-liquid equilibria on substrate stripping (Hamer 1968).

Recently there has been a resurgence of interest in thermotolerant and thermophilic methylotrophs because of both their potential rôle in high temperature chemical industry wastewater treatment (Zlokarnik 1983) and, in the specific case of *Bacillus* spp., as recipient bacteria for the expression of genetic information transferred from other species for enhanced extracellular protein production. Therefore, the isolation of thermotolerant and thermophilic methylotrophs continues to be an important objective. Preliminary reports (Clement et al. 1986; Al-Awadhi et al. 1987; Brooke et al. 1987; Arfman et al. 1987) clearly indicate that enrichment for and isolation of thermotolerant methylotrophic bacilli is possible and Al-Awadhi et al. (1988) has described in detail the taxonomic characteristics of seven such *Bacillus* spp.

All researchers working with thermotolerant methylotrophic bacilli have noticed marked problems concerning the viability of inocula derived from batch cultures of these bacteria. The objective of this investigation is to study the growth characteristics of thermotolerant methylotrophic bacilli such that inocula viability problems can be understood and overcome, thereby allowing thermotolerant methylotrophic bacilli to be harnessed as process cultures. The specific aspects investigated include the pH and temperature spectra for rapid growth, methanol inhibition, iodonitrotetrazolium chloride (INT)-activity including the optimization of the INT method of Zimmermann et al. (1978) as modified by Lopez et al. (1986), for

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assessing viability in cultures of these bacteria under various growth conditions.

Materials and methods

Organism. Bacillus sp. NCIB 12522, a restricted facultative thermotolerant methylotroph isolated from a technical-scale aerobic thermophilic waste sewage sludge biotreater by Al-Awadhi et al. (1988) was used. The bacterium was maintained on 3M methanol agar slopes over 4–5 months at 4° C. Working stock cultures were maintained on 3M methanol agar plates incubated at 48° C and transferred every 2 days.

Batch culture medium. A synthetic buffered medium (pH 6.8), designated 3M, was used. This contained per litre: $Na_2HPO_4 \cdot 2H_2O$, 1.4 g; KH_2PO_4, 0.7 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; NaCl, 0.10 g; 1 ml trace element stock solution as described by Pfennig et al. (1981) with EDTA as chelating agent; 1 ml of vitamin stock solution as described by Egli et al. (1988). This medium was supplemented with methanol or other carbon substrates as specified subsequently. For solid medium, 3% Agar Noble (Difco, Detroit, USA) was added to the medium recipe because of the high experimental temperatures used.

Bioreactor and operating conditions. A fully instrumented 2.4 l (total volume) fermenter (Bioengineering AG, Wald, CH) was used for the experiments concerning temperature and growth kinetics. Instrumentation included pH control, dissolved oxygen monitoring, temperature control and impeller speed control. The pH of the culture was automatically maintained at 6.8 by addition of a mixture of 0.5 M NaOH and 0.5 M KOH. Temperature was maintained constant at 55° C for most batch experiments unless otherwise stated in the text.

pH spectrum measurements. These experiments were conducted in duplicate shake flasks at 55° C by using 3 M medium containing different ratios of 0.5 M $Na_2HPO_4 \cdot 2H_2O$ and 0.5 M KH_2PO_4 to give pH values of 5.9, 6.3, 6.6, 6.8, 7.1, 7.5 and 7.8. 0.5 g/l methanol was used as the carbon energy source.

Temperature spectrum measurements. These experiments were conducted in the 2.4 l bioreactor at different growth temperatures, 37°, 40°, 45°, 50°, 55° and 57.5° C. The pH was maintained at 6.8 and 1 g/l methanol was used as the carbon energy source. Duplicate runs were performed.

Methanol inhibition experiments. These experiments were conducted in duplicate shake flasks at 55° C with varying initial methanol concentrations of 0.1, 0.25, 0.5, 1.0, 5.0, 7.5, 10, 15, 20 and 25 g/l. The growth data for the exponential phase were used to calculate specific growth rate constants corresponding for each initial methanol concentration.

Analytical

Cell density/dry weight measurements. The optical densities of cultures were measured at 546 nm using a Uvikon 860 spectrophotometer (Kontron AG, Zürich, CH). Dry weights were measured on duplicate 10 ml samples of culture that were filtered through tared 0.22 μm Nuclepore filters and were dried overnight at 105° C to constant weight. Optical density measurements were correlated with dry weight measurements.

Protein. Intracellular protein was determined by using the Biuret method modified according to Munkres and Richards (1965) with bovine serum as the standard.

RNA. The RNA content of the cells was measured with the pentose specific orcinol method as described by Herbert et al. (1971).

Ammonium-Nitrogen. This was determined chemically by the addition of 5 ml of formaldehyde to 2 ml of cell free supernatant containing NH_4^+ and the protons generated in the reaction were titrated with 0.05 N NaOH solution.

Methanol. This was measured by using a gas chromatograph type R1A (Shimadzu Corp. Kyoto, Japan). The column used was 80/120 carbopack B/6.6% carbowax 20M, 2 m × 2 mm id. Column temperature was 80° C and the flow rate of the carrier gas (nitrogen) was 20 ml/min.

Oxygen consumption and carbon dioxide production. Oxygen and carbon dioxide concentrations in the effluent gas stream from the bioreactor were measured with a paramagnetic oxygen analyser (Oxymat 3, Siemens AG, FRG) and an infrared carbon dioxide analyser (Binos 1, Leybold-Heraeus GmbH, Hanau, FRG), respectively. Rates were then calculated on the basis of an inert gas balance.

INT-activity. This was measured after optimization of the iodonitro-tetrazolium chloride (INT) method for the bacterium used in this study. The optimized procedure was to add to a 2 ml sample of culture, 3.8 ml of buffered 3M medium (pH 6.8), 0.2 ml of methanol solution (0.55 M) and 1 ml of INT solution (0.2% w/v) sequentially. The treated sample was then incubated in the dark at the actual growth temperature of the culture for 15 minutes when the reaction was stopped by the addition of 1 ml of formaldehyde solution (37%). The sample was then centrifuged at 15000 *g* for 15 min. The resultant pellet was resuspended in 5 ml of dimethylsulfoxide and incubated for 30 minutes in the dark. The suspension was then centrifuged at 15000 *g* for 10 minutes and the extracted INT-formazan complex was measured at 460 nm in a Uvikon 860 spectrophotometer. In cases were the *Bacillus* sp. was grown on glucose, 0.2 ml of glucose solution (97 mM) was added instead of 0.2 ml of methanol solution, as described above. The basis for the optimization is reported under Results.

Results

pH spectrum

The results obtained from the series of duplicate shake flask experiments during exponential growth in a highly buffered medium at 55° C are shown in Fig. 1. During growth variations in pH were < 0.05 pH units. As can be seen in Fig. 1, the maximum specific growth constant increased rapidly between pH 5.9 and 6.6. The plateau representing the optimum pH was rather narrow, 6.6 to 6.8, whilst above 6.8 a rapid decline in the maximum specific growth rate constant occurred. Such behaviour with respect to medium pH is typical of the majority of neutrophiles.

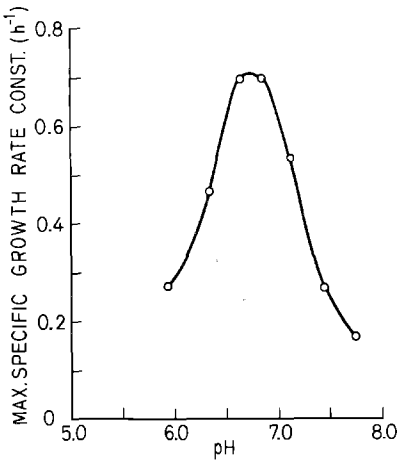

Fig. 1. The effect of culture pH on the maximum specific growth rate constant

Temperature spectrum

The results obtained from batch cultures in the 2.4 l bioreactor at a series of temperatures are shown in Fig. 2, where the maximum specific growth rate constant is shown with respect to temperature. A relatively broad temperature optimum between 45° and 55° C was observed. A marked decline in the maximum specific growth rate constant occurred above 55° C. Such behaviour with respect to temperature clearly indicates that the *Bacillus* sp. is a thermotolerant rather than a thermophilic strain. Essentially thermotolerant strains grow well both in the upper mesophilic range for growth (35°–45° C) as well as exhibiting a temperature optimum between 45° and 55° C, as indicated in Fig. 2. However, they fail to grow at temperatures above 60° C as discussed elsewhere (Al-Awadhi et al. 1988).

Methanol inhibition

The inhibitory effects of methanol on the maximum specific growth rate constant were determined during exponential growth in a series of duplicate shake flask experiments and the results are shown in Fig. 3. Growth on very low methanol concentrations, 0.05 and 0.10 g/l, occurred at only low values of the maximum specific growth rate constant 0.115 and 0.182 h^{-1}, respectively. At methanol concentrations between 0.5 and 5 g/l the maximum specific growth rate constant remained constant and no inhibition was evident, but concentrations of methanol >5 g/l partially inhibited the growth of the *Bacillus* sp., whilst concentrations >25 g/l totally inhibited growth, as can be seen in Fig. 3. The suboptimal specific growth rate constants observed at low methanol concentrations can be explained by the low affinity for methanol that is exhibited by thermotolerant methylotrophic bacilli (Arfman, private communication).

INT-activity

For the determination of the activity of cultures of the *Bacillus* sp. the INT method was used. This method is frequently used to differentiate between living and dead cells in cultures subjected to stress (Mason and Hamer 1987). However, with some bacteria apparently erroneous results can occur and obviously it is necessary to optimize the method for cases where this occurs (Dutton et al. 1986). In our work, initial results using the modified technique proposed by Lopez et al. (1986) to allow formazan crystal extraction and subsequent colourimetric determination of activity showed marked variability.

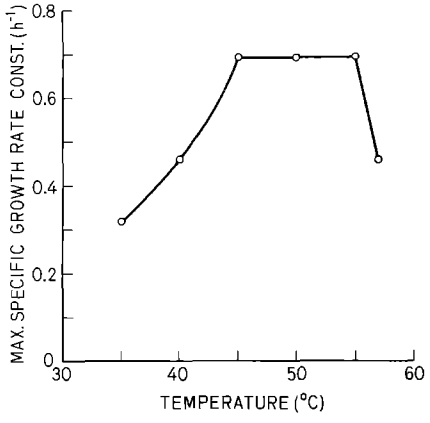

Fig. 2. The effect of temperature on the maximum specific growth rate constant

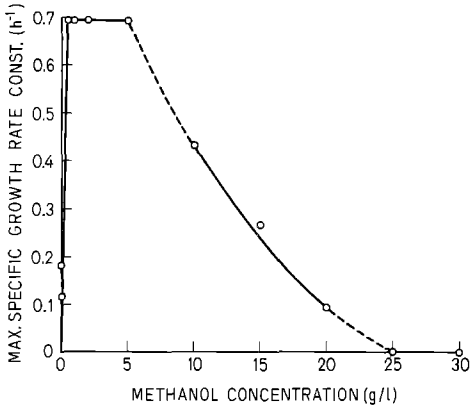

Fig. 3. The effect of methanol concentration on the maximum specific growth rate constant

The first question evaluated was the selection by Lopez et al. (1986) of tetrachloroethylene/acetone as the solvent for extraction and it was immediately clear that formazan extraction was incomplete. Dutton et al. (1986) proposed the use of dimethylsulfoxide as an alternative solvent for extraction primarily because of its greater ease of use and its miscibility with water. We found that additionally the formazan was completely extracted from the cells at short exposure times by using this solvent. Hence, we employed dimethylsulfoxide in our subsequent work. Some earlier papers describing work with the INT method suggested that the method could be sensitive to INT concentration, that there was in many cases a necessity to add additional carbon energy substrate in order to allow maximum activation of dehydrogenase activity, that the incubation time needed for the colour reaction to be complete was relatively extended and that the effects of culture concentrations, temperature and pH were all significant if method variability was to be eliminated. Therefore, it was necessary to investigate the effects of INT concentration, bacterial concentration, incubation time, methanol concentration, pH and incubation temperature on the test results. The results obtained are shown in Fig. 4. Clearly, from the results presented in Fig. 4, INT concentration, test temperature and test pH are critical factors as all show a marked maximum, whilst test incubation time, added carbon substrate (methanol) concentration and culture volume ultimately reach a plateau and, therefore, once a critical point is exceeded have little effect on the test results. The test procedure that was developed on the basis of these results was considered to be optimal for evaluation of the INT-activity of batch cultures of the thermophilic methylotrophic *Bacillus* sp. and is described under Methods.

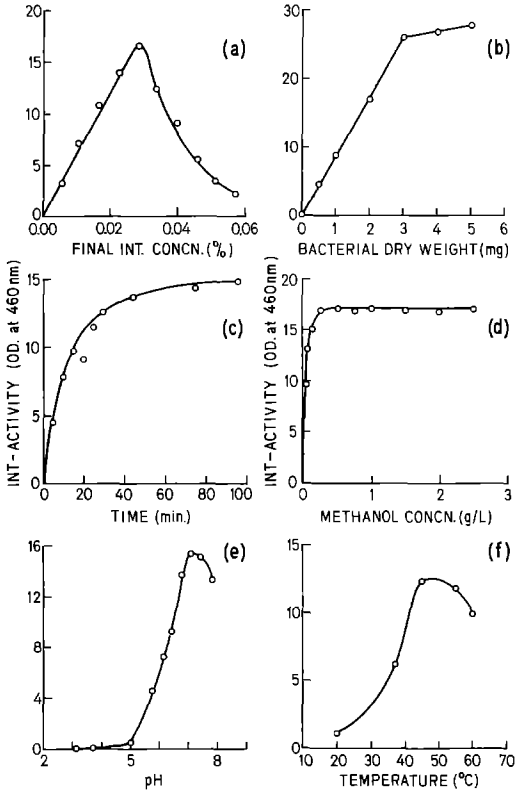

Fig. 4. The effect of test parameters on INT-activity: (**a**) final INT concentration; (**b**) bacterial dry weight; (**c**) incubation time; (**d**) methanol concentration; (**e**) pH; (**f**) temperature

Batch growth characteristics

The establishment of batch cultures of thermotolerant methylotrophic bacilli was fraught with difficulties. Frequently cultures failed to grow after inoculation from either plates or shake flasks and this seemed to be associated with inoculum age, suggesting that cells that had reached the stationary phase of growth became non-viable. Therefore, it was of obvious importance to understand the changes that occur in such cultures after either carbon energy substrate or nutrient exhaustion at the end of the exponential growth phase.

Typical results for a batch culture of the *Bacillus* sp. in the 2.4 l bioreactor at 55° C and pH 6.8 with 2 g/l methanol are shown in Fig. 5. Exponential growth occurred at a maximum specific growth rate constant of 0.69 h^{-1}. Upon exhaustion of methanol, growth ceased, but the typical stationary phase of the growth cycle where the dry weight of cells remains either constant or declines slightly due to maintenance requirements was absent. Instead, the culture entered the declining phase where the dry weight of cells decreased at a high rate, indicating simultaneous death and lysis as important features of carbon substrate starved cultures. INT-activity, somewhat surprizingly, did not coincide with either growth or respiration (Fig. 5a), but lagged by 1 h. The specific INT-activity, shown in Fig. 5b, remained constant during exponential growth, but immediately growth ceased it increased, peaked after one hour and then declined rapidly, within 4 h after the maximum value had been attained. Neither the protein nor RNA contents, shown in Fig. 5b, exhibited anomalous behaviour. Clearly, the absence of a stationary phase in the growth cycle and the rapid rate that occurs during the phase of

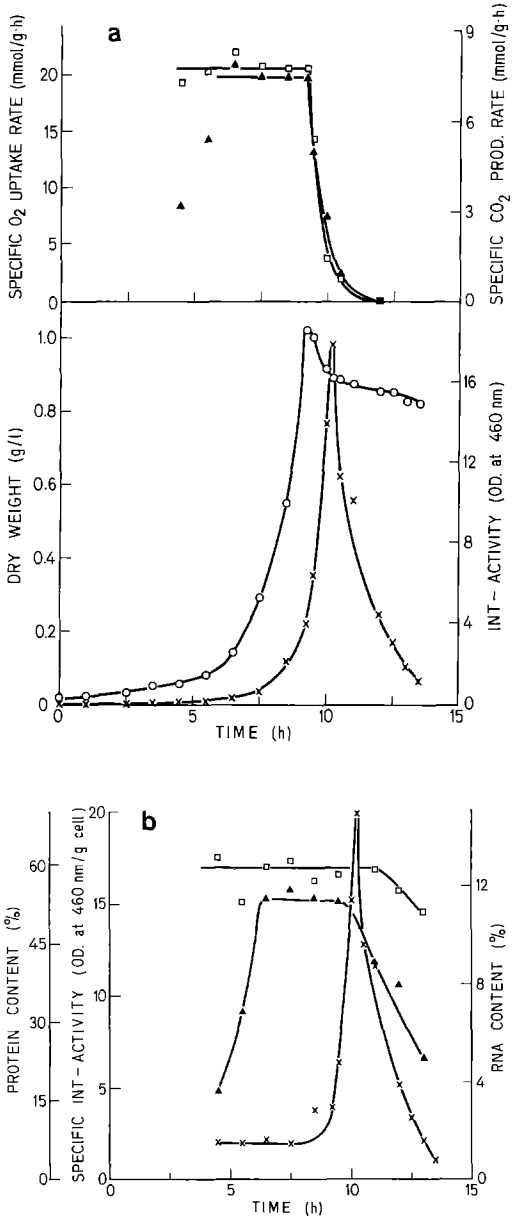

Fig. 5. Batch growth on methanol (2 g/l) at 55 °C and pH 6.8 (**a**) dry weight (○), INT-activity (×) specific oxygen uptake rate (□) and specific carbon dioxide production rate (▲) versus time; (**b**) protein content (□) specific INT-activity (×) and RNA content (▲) versus time

decline offer a partial explanation with respect to the question of culture aging, but the burst of INT-activity after the end of the exponential growth phases offers no evidence with respect to culture fastidiousness.

In order to further investigate this latter phenomenon, additional batch culture experiments were performed in the 2.4 l bioreactor in which a pulse of methanol was added to the bioreactor to give a methanol concentration of 0.5 g/l at a series of time intervals after termination of exponential growth. The elapsed times investigated were 1.5 h, 2 h and 3.75 h and the results obtained are shown in Fig. 6. At 1.5 h, the methanol pulse

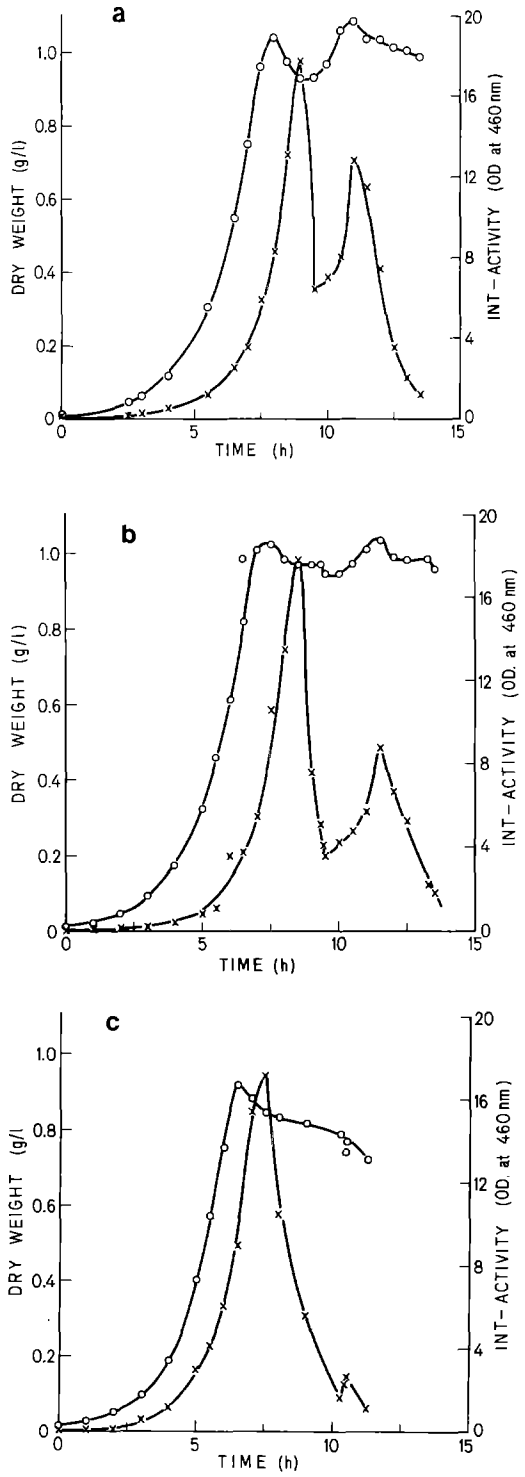

Fig. 6. Batch growth on methanol (2 g/l) at 55 °C and pH 6.8 showing the effect of a methanol pulse at various times after the end of the initial growth phase, dry weight (○) and INT-activity (×) versus time; (**a**) 1.5 h; (**b**) 2 h; (**c**) 3.75 h

resulted in both immediate secondary growth and a virtually coincident peak in INT-activity, followed by a further phase of decline with respect to culture density and a rapid decline in INT-activity after the second exhaustion of methanol. At 2 h, a similar response was observed, but the peak in INT-activity was much less pronounced. At 3.75 h, the methanol pulse give virtually no peak in INT-activity and in spite of the pulse, the phase of decline was uninterrupted.

In order to investigate other effects of changing environmental conditions such as pH, temperature, nitrogen source exhaustion in the presence of methanol and oxygen limitation, i.e., linear growth, on the growth of the culture, four additional batch culture experiments were performed.

In the experiment with uncontrolled pH and an initial methanol concentration, ca. 2 g/l, the pH fell from an initial value of 6.75 to a final value of 5.3 as shown in Fig. 7. The culture grew initially exponentially but growth deviated from the exponential pattern at pHs below 6.05. INT-activity was much reduced but essentially parallel with growth at pHs above 6.05, where it peaked and thereafter declined rapidly. Growth continued between pH 6.05 and pH 5.3 but exhibited on extended decelleration phase. Upon reaching a pH of 5.3, the culture entered a rapid declining phase in spite of methanol remaining present in the growth medium. Returning the pH to 6.75 did not result in any culture recovery. Such behaviour is clearly a significant factor when inocula are prepared under conditions of uncontrolled pH in only slightly buffered media.

No unexpected effects of reducing the growth temperature to 40° C were observed. Essentially, a similar pattern of growth to that at 55° C, but with reduced rates, was observed.

In the case where the nitrogen source (ammonia) was exhausted prior to complete utilization of the methanol, exponential growth ceased prior to nitrogen source exhaustion at an ammonia concentration of 15.8 mg/l nitrogen. At lower nitrogen concentrations, a less marked phase of decline was observed. INT-activity peaked some 0.75 h after the end of exponential growth and declined until it reached a plateau, suggesting that INT-activity is retained for an extended period of time.

The results obtained when the culture was subjected to oxygen limitation, i.e., when growth ceased to be exponential and became linear such that the growth rate was proportional to the oxygen transfer rate (Wilkinson and Hamer, 1972) are some of the most instructive. As can be seen from Fig. 8, the INT-activity increased only relatively slowly during oxygen limitation, but peaked 1 h after methanol exhaustion and then declined rapidly. These results suggest that the potential activity of cultures of the thermotolerant *Bacillus* sp. can be maintained by subjecting them to oxygen limitation in the presence of excess methanol as carbon energy substrate; thereby prolonging their growth phase and viability for use in the inoculation of subsequent batch cultures.

Finally, in order to differentiate between methanol specific and general aspects of the growth of the *Bacillus* sp., the bacterium was grown in the 2.4 l bioreactor under essentially the

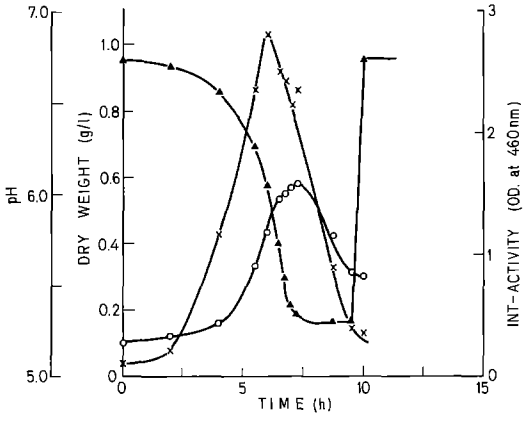

Fig. 7. Batch growth on methanol (2 g/l) at 55°C but without pH control, pH (▲) dry weight (O) and INT-activity (×) versus time

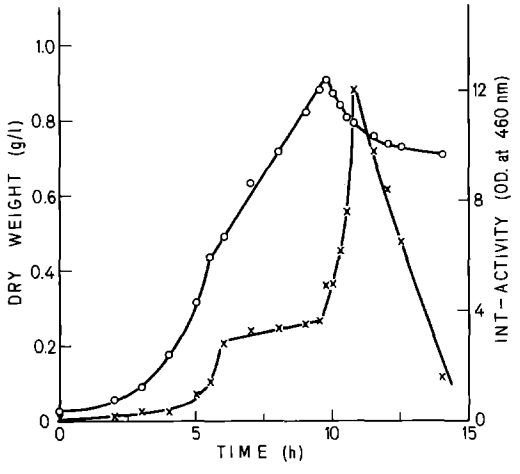

Fig. 8. Batch growth on methanol (2 g/l) at 55°C and pH 6.8 where oxygen becomes limiting after 5.5 h, dry weight (O) and INT-activity (×) versus time

same operating conditions that were used for growth on methanol but where methanol was replaced by glucose as the carbon energy substrate and where, in a glucose grown culture, the culture was, after exhaustion of glucose, subjected to a pulse of glucose 45 min after initial growth ceased. In the former case the behaviour of the culture was similar to that which occurred with methanol as carbon energy substrate, but the INT-activity, measured with added glucose instead of methanol, lagged only slightly behind growth. No stationary phase was evident and similar rates during the phase of decline were observed. However, with glucose as carbon energy substrate a few spores were observed some 24 h after the conclusion of growth in contrast with methanol grown batch cultures where no sporulation was observed. In the latter case, an immediate growth response to glucose addition occurred, indicating essentially similar behaviour to that observed in methanol grown cultures. When the glucose pulse was delayed to 1.5 h after the end of the inital growth no response occurred, suggesting that the degeneration of viability was more rapid with glucose as carbon energy substrate than with methanol.

Discussion and conclusions

The results that are reported for the growth characteristics of the thermotolerant methylotrophic *Bacillus* NCIB 12522 in batch culture indicate that the frequently encountered problem of inoculum viability must be associated with physiological changes that occur in cultures of the bacterium after growth ceases. Such changes are indicated by dramatic changes in INT-activity.

INT-activity measurements have been used for the evaluation of bacterial viability and activity (Mason et al. 1986; Mason and Hamer 1987). INT-activity measurements depend on the reduction of INT in microbial cells to form red formazan crystals. In aerobic bacteria, oxido-reduction dyes such as INT can compete along the electron transfer chain with oxygen for electrons from the oxidation of carbon energy substrates which are first transferred onto NAD^+ and subsequently fed into the respiratory chain. Therefore, INT-activity is thought to correlate with electron transfer activity.

In the case of *Bacillus cereus,* it has been reported that after termination of vegetative growth there is an increase in intra-cellular malic and succinic dehydrogenase activities (Hanson et al. 1963), which would seem to correspond with what is observed here after growth on methanol. In order to explain the increase in INT-activity, either one of two mechanisms would seem to be plausible. The first is that the capacity of the electron transfer system is increased by synthesis of one or more of the limiting enzymes, whilst the second is that the flux of electrons from methanol to NADH-dehydrogenase is a bottle-neck and increased INT-activity results from an increase in either the concentration or activity of methanol dehydrogenase. As the phenomenon is clearly methanol related the second alternative would seem more probable but elucidation requires detailed investigation of the biochemistry.

In many batch cultures of *Bacillus* NCIB 12522, culture viability is markedly reduced such that cells removed from batch cultures as inocula for subsequent cultures are frequently deficient with respect to their proposed use. From the results reported, the loss in viability applies particularly to batch cultures that are subject either to carbon energy substrate (methanol or glucose) exhaustion or to a sustained sub-optimal pH. In batch cultures where the nitrogen source is exhausted first, much higher levels of culture viability are maintained, as are they when cultures are grown oxygen-limited. In this latter case viability drops precipitously on carbon energy substrate (methanol) exhaustion. Therefore, it can be hypothesized that in order to retain culture viability, there is an obligate requirement for a carbon energy substrate.

Whilst it is generally accepted that different genera of bacteria exhibit different survival mechanisms (Dawes 1976; Harder et al. 1984), it is the mechanisms applicable to bacilli that must be considered here. When bacilli encounter nutritional deficiencies (starvation) or other stresses they typically produce resistant endospores.

It has been reported by Remsen et al. (1966) that β-phenethyl alcohol and by Freese and Heinze (1984) that ethanol inhibit sporulation. In the case of *Bacillus* NCIB 12522, Al-Awadhi et al. (1988) have reported an absense of sporulation when the bacterium is grown on methanol.

Sporulation results from a relatively extended sequence of some eight intra-cellular changes (Bernlohr and Leitzmann 1969; Freese and Heinze 1984). A concept that is used in the discussion of the sporulation sequence is commitment to sporulation (Sterlini and Mandelstam 1969; Cooney et al. 1975), essentially the point of no return during the differentiation process. Usually commitment to sporulation occurs some 1-2 hours after ex-

haustion of the carbon energy substrate (Cooney et al. 1975) and during the sporulation sequence rather than at its initiation. For *Bacillus* NCIB 12522, which sporulates with low frequency after growth on glucose, but fails to sporulate after growth on methanol, the results for batch growth on methanol suggest that commitment to sporulation occurs but the sporulation sequence does not proceed to completion. The continued increase in INT-activity for a further 1 h after methanol exhaustion followed by a dramatic decrease in INT-activity are consistent with such a hypothesis. In addition, the immediate reduction in culture dry weight after methanol exhaustion is typical for the growth of sporulating bacilli (Brown and Hodges 1974; Rowe et al. 1975) and corresponds to an initiation of sporulation. The results with subsequent pulses of methanol after initial exhaustion also support the hypothesis.

Therefore, the most probable reason that inocula taken from cultures of *Bacillus* NCIB 12522 after methanol exhaustion are non-viable is that they have proceeded beyond the point of commitment to sporulate, but in fact fail to produce spores, as a survival strategy, in spite of the fact that the vegetative cells die. This explanation also clearly indicates why such difficulty was encountered in isolating thermotolerant methylotrophic bacteria during the era of methanol based SCP process development.

Acknowledgements. Our thanks are due to Ms. H. Bolliger for drawing the figures and to Ms. R. Meisser for typing the manuscript. N. al A. was the recipient of a Kuwait Institute for Scientific Research (KISR) postgraduate scholarship.

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Received April 25, 1988 / Accepted July 7, 1988