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# Survival and activity of Klebsiella pneumoniae at super-optimal temperatures

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**Abstract.** The effect of temperature on a population of Klebsiella pneumoniae was examined together with the imposition of mild starvation conditions at temperatures of 35°, 41°, 49°, 55° and 60 °C. Results for changes in biomass, protein and metabolic activity are presented in terms of gross population changes and show that these parameters decline with increasing temperature. Increases in the amount of dissolved organic carbon, together with a decrease in the number of cells present with increasing temperature, suggest that death and lysis processes are occurring. Regrowth in the bioreactor occurred after returning the temperature to 35 °C and starting a flow of carbon and other nutrients. This was probably due to reinoculation from head space wall growth drainage rather than reversion of heat-stressed microbes. The consequences of this for thermophilic sludge treatment processes are discussed. The concept of endogenous metabolism is questioned with respect to it being a realistic description of the survival process.

## 1 Introduction

Using heat for rendering a population of microbes inert is commonly practised. Sterilization processes in the public health sector and pasteurization processes in the food and beverage industries have been used with considerable effect to achieve either reduction or total inhibition of microbes and their activity. However, the use of heat treatment has limited application in the waste-water and waste sludge treatment industries. Introduction of pasteurization processes in the treatment of sewage sludge have proved unsuccessful where inefficient or insufficient biodegradation of the organic material in the sludge allows the possibility of post-pasteurization reinfection either from organisms in aerosols or from other sources in the vicinity of the treated sludge.

At present, several novel treatment technologies are being assessed for their ability to achieve adequate removal of pathogenic organisms from waste sludge during treatment and at the same time achieve high levels of biodegradation and stabilization during exposure to high temperatures. Such processes operate in the thermophilic temperature range of 55 to 70 °C under either aerobic or anaerobic conditions. The target of such treatment processes with respect to hygienisation aspects of sludge are the bacteria, viruses, worm eggs and protozoa which can potentially result in serious diseases in humans and animals.

Bacteria of enteric origin such as Escherichia coli and Klebsiella pneumoniae have been used as indicators of more serious pathogens. Thus, E. coli and K. pneumoniae, which are both themselves potentially pathogenic, are monitored together with other coliforms to indicate the possible presence of more seriously pathogenic microbes such as Salmonella spp. and Vibrio spp. Bacteria are found that can grow or survive over a wide range of temperatures. Although most are found only at temperatures below 45 °C, others can survive and even grow at temperatures in excess of 100 °C [1]. The pathogenic microbes are of the former group and are killed or inactivated by temperatures above 50 °C, hence the potential for heat treatment technologies in the treatment of waste sludges.

How heat treatment affects microbes has been the subject of considerable attention. In E. coli physical changes in the cell surface have been described, whereby a weakening of the structure of the peptidoglycan results in extrusion of cell membrane-bound blebs following transfer to temperatures in excess of 50 °C [2]. Pellon et al. [3] and Pellon and Gomez [4] found extensive damage to the DNA of E. coli following a shift of temperature from 37 °C to 50 °C, with concomitant loss of viability. They showed that single and double strand breaks occurred in the DNA together with substantial physical association of protein to the DNA molecule, the DNA scissions resulting from the action of endo- and exo-nuclease activity [5]. Returning the microbes to 37 °C resulted in regrowth of the population, following a lag period of 40 min, during which time the DNA was repaired [6].

The ability to survive heat shocks has been shown to be dependent on the growth rate [7, 8], the nutritional status of the culture [9, 10] and the water activity in the bulk liquid [11]. Wu and Klein [9] found that a mixed bacterial

culture isolated from water showed a decreasing sensitivity to mild warming stress in relation to increasing time of nutrient starvation, whilst the reverse was true of E. coli.

The production of de novo enzymes and other proteins as a result of heat stress has also been described in considerable detail for a wide range of organisms [12–14], although the function of these proteins is yet to be elucidated.

In the aerobic thermophilic sludge biodegradation process hygienisation requires the destruction of the pathogenic microbes. It is known that microbial solids removal can occur at a high rate using this process [15, 16], but the fate of pathogenic microbes in such environments is largely unknown, especially when one examines only those effects caused by temperature, i.e., in the absence of thermophilic process microbes. The purpose of the investigation reported here was to examine the physiological changes of the enteric bacterium K. pneumoniae when introduced into a bioreactor maintained at temperatures above the maximum for growth of this bacterium, and the possibilities for such populations to regrow when returned to the optimum temperature for growth.

The accuracy of survival tests using agar-based cultivation methods is questionable, particularly when applied to stressed cells [17]. Therefore, an alternative technique for assessment of microbial activity was used here.

## 2 Materials and methods

### *2.1 Organism, cultivation medium and heat treatment*

Klebsiella pneumoniae, NCIB 418, was maintained by monthly subculture on plate count agar slopes incubated at a temperature of 35 °C and stored at a temperature of 4 °C.

K. pneumoniae was grown in a bioreactor of 2.5 $dm^3$ (MBR Bioreactor AG, Wetzikon, CH) in a mineral salts medium modified from the recipe of Evans et al. [18], such that it contained 50 $g/m^3$ of EDTA in place of citrate and used only 50% of the concentration of trace elements. It was also necessary to add 20 $g/m^3$ of polypropylene glycol as an antifoaming agent. 2.45 $kg/m^3$ of glucose were added, as the sole utilizable carbon energy source and was growth-limiting. The medium was added by means of a peristaltic pump and the flow rate monitored by weight displacement from the substrate reservoir bottle. The bioreactor was operated with a dilution rate of 0.10 $h^{-1}$. Spent broth was removed and the bioreactor volume was maintained by an overflow weir. The cultivation temperature was maintained constant at 35 °C and the pH-value was kept constant at 6.80 by controlled addition of a mixture of 1.5 M KOH and 1.5 M NaOH. Verification of culture purity was carried out by periodic microscopic observation and by cell cultivation on plate count agar followed by identification of single colonies using the API 20E test kit (API System S.A., Montalieu-Vercieu, F).

The effect of temperature on K. pneumoniae was investigated by continuously feeding cells from the cultivation bioreactor into a second, similar bioreactor maintained at various temperatures. The hydraulic residence time in the second bioreactor was maintained constant at 32 h ($D = 0.031\ h^{-1}$). The pH-value was maintained constant. The second bioreactor received no nutrient flow, except during regrowth experiments. Before sampling, at least five residence times were allowed so as to establish steady state conditions. The reactor was aerated by sparging with air.

### *2.2 Analyses*

Total biomass concentration was measured as dry weight by a filtration/gravimetric procedure using tared 0.4 µm Nucleopore filters. The filters were dried at 105 °C for 1 h before reweighing.

Dissolved organic carbon was determined in the filtrate obtained from dry weight determination after acidifying with concentrated HCl and removing the inorganic carbon by sparging with $N_2$ for 12 min in a TOCOR 2 total organic carbon analyser (Maihak AG, Hamburg, D).

Protein in the cells and in the extracellular medium was measured after centrifuging at 30,000 · $g$ using the Biuret method with bovine serum albumin as the standard as described by Herbert et al. [19].

Metabolic activity was measured using 2-(p-iodophenyl)-3-(-p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). 1 $cm^3$ of INT (2.0 $kg/m^3$ of aqueous solution) was added to a sample of 10 $cm^3$ immediately after removal from the bioreactor after suitable dilution in a 0.11 M NaCl, 0.02 M phosphate buffer at a pH-value of 6.80. 100 $mm^3$ of glucose in Evans mineral salts medium (glucose concentration 2.5 $kg/m^3$, were also added to provide an electron source. After 20 min of incubation 100 $mm^3$ of 37% formaldehyde were added to stop the reaction. The solution was centrifuged at 30,000 · $g$ for 25 min and the solids resuspended in 10 $cm^3$ tetrachloroethylene (40 vol%)/acetone (60 vol%) solution. After 30 min of incubation at room temperature in the dark, the solution was centrifuged at 30,000 · $g$ for 10 min and the absorption of the extracted INT-formazan measured at 490 nm.

Total cell number was assayed by means of the acridine orange direct count method. Suitable dilutions of the microbes were made using 0.11 M NaCl and 0.02 M phosphate buffer (pH 6.80). 5 $cm^3$ were filtered through pre-stained (1:15,000 sudan black in 50% absolute ethanol) 0.2 µm Nuclepore filters. 1 $cm^3$ of acridine orange solution (1:5000 in 6.6 mM phosphate buffer (pH 6.80) was added to the filter for 2 min, and then removed by filtra-

tion. Total cell counts were made by counting green and orange fluorescing cells with a Leitz SM-lux epifluorescence microscope (Leitz GmbH, Wetzlar, D).

All chemicals were of analytical grade and supplied by either Fluka (Buchs/SG, CH) or Merck (Darmstadt, D).

## 3 Results

The effects of heat on a population of K. pneumoniae were examined together with the effect of mild starvation conditions by feeding a steady state culture of the bacterium from a continuously fed bioreactor into a second bioreactor, which received no additional nutrient flow, but was maintained at a pH-value of 6.80 and aerated. The second bioreactor was maintained at each of a series of temperatures: at 35 °C (equivalent to the growth temperature in the first bioreactor), 41 °C, 49 °C, 55 °C and at 60 °C. Once steady state conditions had been established, samples were removed from the second bioreactor and examined with respect to dry weight, dissolved organic carbon concentration, metabolic activity, protein content, cell number and the accumulation of 260 nm absorbing compounds in the culture medium. The results of these analyses are shown in Figs. 1, 2 and 3.

As can be seen from Fig. 1, the dry weight decreases, as a result solely of starvation conditions, from 1.09 kg/m$^3$ in the first bioreactor to 0.99 kg/m$^3$ in the bioreactor maintained at the same temperature as the first (35 °C), but with no nutrient flow. Also noticeable is the further decrease in dry weight as a function of increasing temperature, such that at a temperature of 60 °C only 72.4% of the concentration of bacteria being fed into the bioreactor, on a dry weight basis, remained in the bioreactor at its outflow.

Simultaneous with the decrease in dry weight, there was a decrease in the number of cells present as determined by the acridine orange direct count. This reduction from the number present in the first bioreactor and the feed to the second bioreactor occurred under all conditions of starvation and heating. The extent of reduction was a function of temperature.

Attempts to enumerate stressed microbes have tended to use techniques requiring cell replication on an agar surface. Such techniques drastically underestimate the true number of microbes present, since failure to reproduce can occur purely through the physico-chemical characteristics of the agar medium, which differ drastically from conditions in submerged continuous cultures. Moreover, a time limit is usually enforced on such tests, thus further negating the application of such techniques for stress studies, where a period of adaptation is to be expected before growth of those microbes capable of repairing damage caused by heat, is likely to occur. As a consequence, a technique was used which measures metabolic activity of the culture. This technique is based on

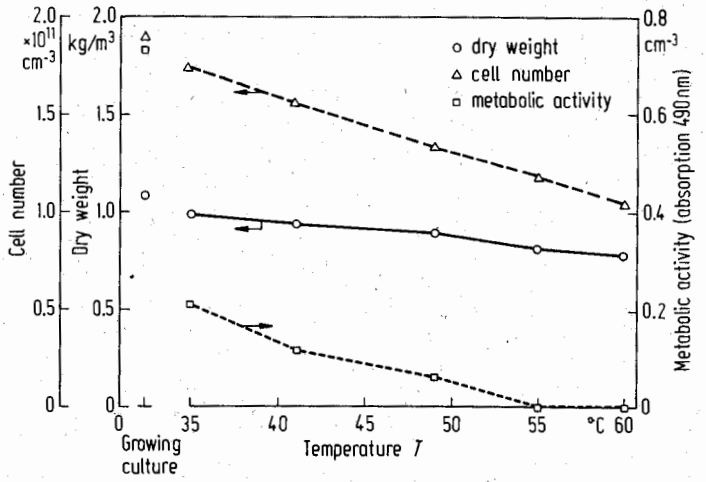


**Fig. 1.** Changes in dry weight, metabolic activity and cell number as a result of heat treating a culture of K. pneumoniae at various temperatures

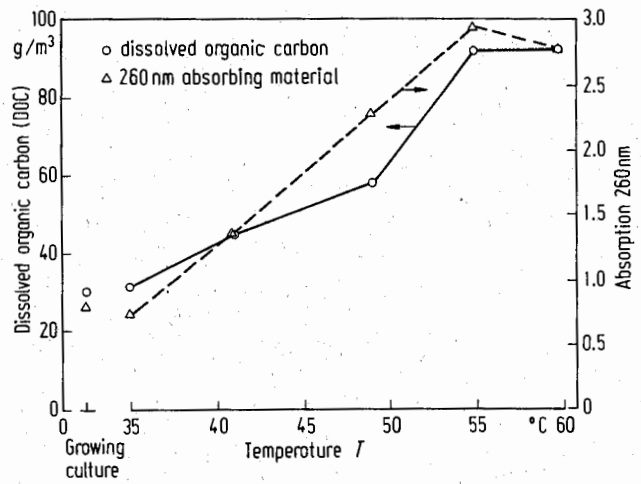


**Fig. 2.** Production of dissolved organic carbon and 260 nm absorbing material in steady state heat treated cultures of K. pneumoniae

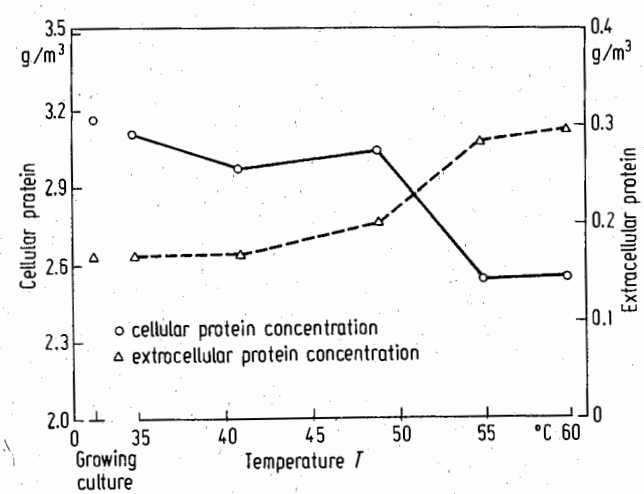


**Fig. 3.** Changes in cellular and extracellular protein concentrations in culures of K. pneumoniae exposed to super-optimal temperatures

the ability of the compound INT to be reduced by dehydrogenase enzymes to a water insoluble salt, INT-formazan, which is deposited internally in the cell. Several dehydrogenase enzymes are active in the electron transport system where INT competes with oxygen for electrons. Previous studies have shown that a large proportion of the total metabolic activity can be linked to electron transport system activity [20]. Application of this technique, which includes extraction of the INT-formazan salt from the cells and concentration measurement by absorption at 490 nm, produced the data shown in Fig. 1. Absorption at 490 nm has been shown to be proportional to the INT-formazan concentration [21]. A significant decrease in overall metabolic activity was found between microbes growing in the first bioreactor at 35 °C and those present in the second bioreactor at 35 °C, with a residence time of 32 h. Increasing the temperature resulted in further decrease in metabolic activity at 41 °C and 49 °C, although the levels found were significant in that they indicated the bacteria were still capable of metabolic reactions despite the super-optimal temperatures. At 55 °C and 60 °C the measured metabolic activity was extremely low, but not zero. Thus, despite temperatures in excess of the maximum where growth can occur, metabolic activity could still be detected.

Under starvation conditions at 35 °C, no increase in the amount of dissolved organic carbon (DOC) could be found, compared to that present in the first bioreactor (Fig. 2). However, with increasing temperature the amount of DOC present also increased to a peak value at 55 °C and 60 °C, equivalent to a threefold increase over that in the growing culture. This increase in DOC was mirrored by an increase in the amount of UV absorbing material (260 nm) (Fig. 2). For this parameter also there was similarity at 35 °C under starvation conditions to the amount found in the growth bioreactor and a steady increase with increasing temperature to 55 °C. The amount found at 60 °C was less than that present at 55 °C.

Cellular and extracellular protein concentrations are shown in Fig. 3. In both cases, there is little change in protein concentrations following starvation and heat treatment at 35 °C, 41 °C and 49 °C, but a significant reduction in cell-bound protein and an increase in extracellular protein occurred at 55 °C and 60 °C.

In the treatment of waste sludge containing pathogenic organisms and potential pathogens such as K. pneumoniae, the ability for heat-treated organisms to regrow when given nutrients at lower temperatures is of critical importance. This was examined by starting a sterile nutrient flow with a dilution rate of 0.08 $h^{-1}$ containing 2.5 $kg/m^3$ of glucose in an Evans mineral salts medium to the previously heat stressed cells in the second bioreactor after cooling the culture to 35 °C. In order to ensure that all bacteria in the bioreactor were subjected to the heat stress, the procedure followed was to stop the flow of bacteria from the growth bioreactor, and, only four hours later, to reduce the temperature and start the nutrient flow. The ensuing washout and regrowth was monitored by absorption at 546 nm (Fig. 4). This experiment was carried out after treatment at 49 °C, 55 °C and 60 °C and in each case regrowth occurred. After treatment at 49 °C, there was a long lag phase lasting approximately 7 h, whilst for the bacteria treated at 55 °C and 60 °C the lag phase was much shorter. The identity and purity of the regrown culture was checked using the API 20E identification system, and K. pneumoniae was identified as the only microbe present in all regrowth experiments.

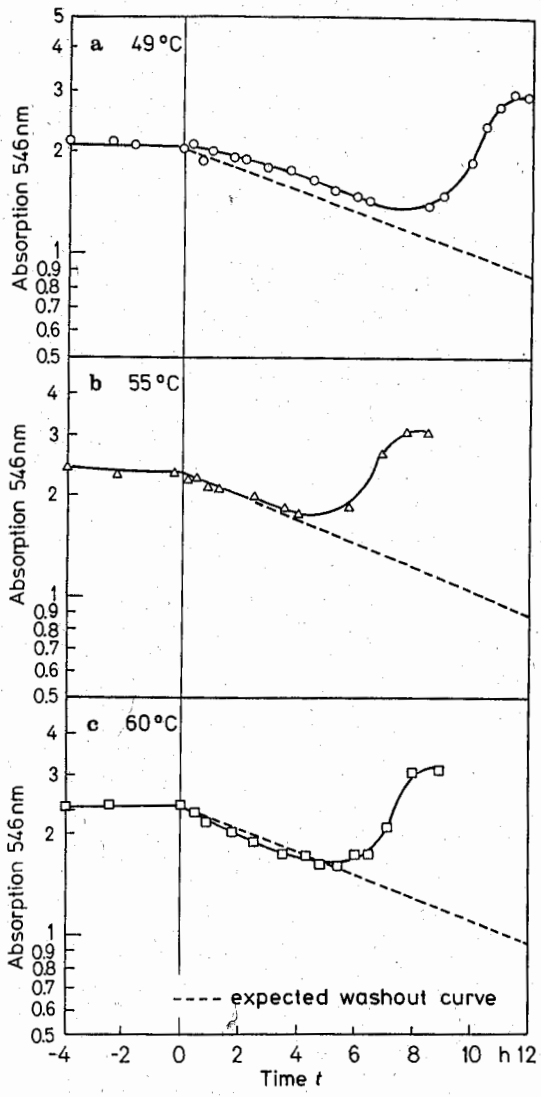


**Fig. 4.** Regrowth of heat treated cultures of K. pneumoniae. Continuous feed of microbes was halted four hours before returning the temperature to 35 °C and starting nutrient flow

## 4 Discussion

The results with respect to changes in various parameters as a result of heat shock, shown in Figs. 1, 2 and 3, are

presented in terms of mass per unit volume. In studies such as the present one, where the physiological matrix of the reference quantity is drastically different under different conditions, the use of this basis is questionable. Every indication suggests that death/lysis events are occurring in the culture, and therefore, release of cell contents into the extracellular medium is occurring. The acridine orange direct count also shows a clear decrease in the number of cells per $cm^3$ and thus expression of the amount of protein found on a mg per $cm^3$ basis does not take into account that successively fewer cells are present per $cm^3$ with increasing temperature. As shown in Fig. 5, the results for dry weight and cellular protein have been recalculated as examples and are expressed on a mg per cell basis. The trend here is the reverse of that described earlier. Whilst in terms of total cellular protein per $cm^3$ a reduction was measured, the amount per cell is higher under starvation conditions with a 32 h bioreactor residence time and under starvation/temperature treatment. Similarly, whilst the dry weight of 1 $cm^3$ of culture fluid decreases with increasing temperature, an increase in the dry weight of individual cells could be measured when calculated on a different basis.

Historically, the decrease in biomass found to be due to starvation has been ascribed to endogenous metabolism. Endogenous metabolism is defined as the summation of all metabolic reactions which occur when a cell is deprived of either compounds or elements which may serve specifically as exogenous substrates [22]. Comparison of the data for cells grown at 35 °C with nutrient flow and those maintained under starvation over a residence time of 32 h in the second bioreactor at 35 °C would seem to suggest that metabolism of endogenous substrates has brought about a reduction in the biomass. The occurrence of a reduction at 41 °C and 49 °C can be justified to some extent using this same hypothesis, which is based on the fact that a significant level of metabolic activity can still be measured. However, the extremely low level of metabolic activity found at 55 °C and at 60 °C, resulting from bacteria which had been in the bioreactor for an extremely short time, is insufficient to justify biomass reduction based on the endogenous metabolism hypothesis. The foregoing discussion taken together with the fact that protein in individual cells is not declining, as suggested by Fig. 3, but accumulating, leads to increasing reluctance to ascribe the observed effects to endogenous activity.

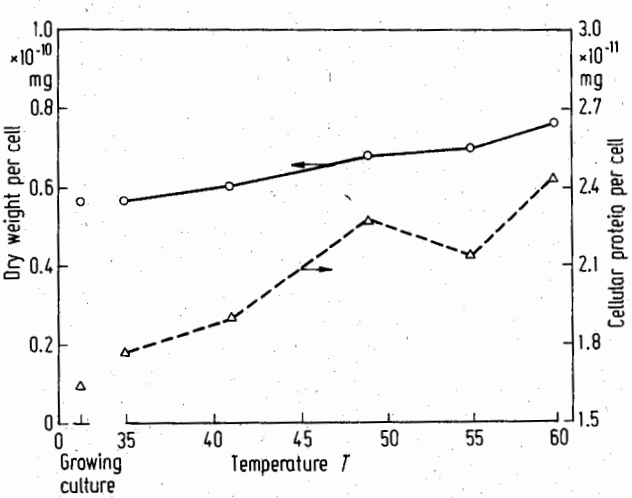


**Fig. 5.** Expression of protein and dry weight data in units per cell

Certainly the production of heat shock proteins has been well documented and may account for some of the increased values for protein per cell [23]. However, as in the example of endogenous degradation of protein and other cellular components, the low level of metabolic activity in the culture at 55 °C and 60 °C prohibits the extrapolation of this concept to the higher temperature conditions.

Evidence for lysis of the culture as a consequence of both starvation conditions and temperature effects are provided by the increase in UV absorbing material (protein, nucleic acids) and by the reduction in cell number. Both of these variables are unaffected by the difficulties described above, so that more reliance can be placed on these data. Thus the process of cryptic growth is expected to be occurring in those cultures in which the temperature does not inhibit the specific metabolic pathways involved. Cryptic growth has been described in detail by Mason and Hamer [24] for the microbe K. pneumoniae and may well be the reason for the apparent absence of accumulation of extracellular protein at 35 °C, 41 °C and 49 °C. At 55 °C and 60 °C it is also assumed that cryptic growth is not occurring.

A more likely explanation of the results reported here is that the permeability of the cell changes as a result of both the imposition of starvation conditions and the effects of temperature. As a result, the contents of the cell become concentrated due to water efflux, thus resulting in the higher concentrations of protein per cell. An effect of temperature on the microstructure of the cell contents was suggested many years ago by Hedén and Wyckoff [25], who showed that heating of E. coli to temperatures between 50 °C and 60 °C resulted in granulation of the cytoplasm. This effect began when cells were heated to 50 °C and was extensive in cells heated to 60 °C. But even in cells heated to 40 °C (from a growth temperature of 37 °C), some loss of protoplasmic homogeneity had already occurred. This change in the protoplasmic matrix was found to be irreversible above 50 °C, and partially reversible at 45 °C and 50 °C, when the cells were returned to their optimum growth temperature. A similar effect was observed more recently where under starvation conditions only, a marked decrease in the size of the protoplasm could be discerned with increasing time of starvation [26].

This change in permeability of the membrane may well be accompanied by a change in the ash content, thus accounting for an increase in the dry weight of K. pneumoniae on a per cell basis. At temperatures above 55 °C, either the cell membrane or the cell wall may become heat stabilized and prevent additional loss of internal contents, and this may be the reason why the amount of DOC released and the amount of 260 nm absorbing material decreases slightly between 55 °C and 60 °C.

Given these negative aspects with respect to the survival of K. pneumoniae at high temperatures, the data shown in Fig. 4 still need to be explained. The most likely reason for the regrowth of K. pneumoniae is that microbes were able to reinoculate the cooled culture from head space splash. Wall growth in the head space is commonly encountered in continuous culture vessels.

Hamer [27] suggested that in nonfoaming cultures some degree of microbial entrainment by bubble flotation followed by entrainment of microorganism containing droplets from bubble bursting at the liquid surface and collisions between the droplets and the vessel walls can lead to head space wall growth. Such microbes will be in an atypical environment compared to that in the bulk liquid and may therefore be exposed to much lower temperatures than prevalent in the bulk liquid. Hamer [27] calculated that in a bioreactor operating at 35 °C with a sparged air flow of 1 volume per volume of medium per minute, the volume of head-space drops formed as a result of bubbles bursting on the surface would be equal to 1.64 $dm^3$ per $dm^3$ of medium each hour.

A certain fraction of the droplets produced will collide with the fermenter walls and can result in a layer, often a thick layer, of cells in the head space wall area.

The consequence of this finding is that in any incompletely filled bioreactor, the head space will contain a source for reinoculation, by head space drainage, even under conditions of careful control. In this series of experiments, foaming was avoided by careful operation, including gradual changes in bioreactor temperature for enhanced temperature treatment. Moreover, the control of a small bioreactor of 2.5 $dm^3$ is better than that of an industrial bioreactor of 10 $m^3$. Thus, in the treatment of waste sludge, reinfection and regrowth may arise in treated sludge as a result of head space reinoculation in the bioreactor, particularly where only partial biodegradable organic matter stabilization has occurred. The low level of metabolic activity found at temperatures of 55 °C and 60 °C also suggests possibilities for regrowth in partially treated sludge when organisms short-circuit the thermophilic bioreactor in continuously fed processes. This is a feature that tends to be enhanced as system heterogeneity increases.

## 5 Conclusions

The use of temperature to kill pathogenic organisms in processes such as waste sewage sludge treatment requires effective stabilisation and sensible bioreactor design to minimise the danger of reinfection. In stress studies on microbes, the use of specific defined parameters to describe effects on individual microbes as opposed to overall parameters reveals different trends and casts doubts on the concept of endogenous metabolism as a realistic explanation of the survival process mechanism. Such processes can be better described in terms of death/lysis and cryptic growth phenomenon, together with changes in the physical properties of the cells.

## Acknowledgements

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