Doctoral Thesis

Kinetic and physiological aspects of bacterial growth at superoptimum temperatures

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KINETIC AND PHYSIOLOGICAL ASPECTS OF BACTERIAL GROWTH AT SUPEROPTIMUM TEMPERATURES

A dissertation submitted to the
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presented by
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Zürich 1990
TO MY PARENTS
AND LISA
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The following parts of this dissertation have been published or submitted for publication:

Chapter 4  
(The chapter represents 60% of this publication).

Chapter 5  

Chapter 7  

Parts of Chapter 2  
(sections 6 and 8)
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SUMMARY

Selected kinetic and physiological aspects of short and long term exposure of bacteria to superoptimum temperatures for growth have been investigated. The superoptimum temperature range is that between the highest optimum growth temperature and the maximum growth temperature. Such conditions are encountered as a result of overheating in both technical and natural environments and also during the application of temperature inducible expression systems for product formation by recombinant bacterial strains.

An overview concerning the effects of superoptimum temperatures on bacterial growth and physiology is provided. Whilst a considerable body of information is available on mechanistic physiological and molecular biological aspects of superoptimum temperatures, only very limited information exists on the impact of different cultivation conditions and growth media on the various processes taking place in bacteria that are exposed either continuously or transiently to such temperatures. Further, little systematic information exists on the kinetic response of growing bacterial populations during recovery from heat shocks after restoration to normal cultivation conditions.

A number of mathematical models exist for the description of the specific growth rate of bacteria in the superoptimum temperature range. Three unstructured models were critically investigated and compared with respect to their applicability for data fitting and model parameter estimation. The two mechanistic models investigated, which were based on extended Arrhenius functions where inactivation reactions were taken into account, resulted in limited correspondence with experimental data. However, parameter estimations gave gross variations. In contrast, the empirical "extended square root model" exhibited both a good correlation with experimental data and reproducible parameter values. The results indicate that mechanistic models require a degree of structure, in order to account for the complexity of processes taking place in the superoptimum temperature range.

The effects of local temperature fluctuations on overall culture performance were investigated in continuous cultures of Klebsiella pneumoniae, where fractions of the process culture were subjected to short term heat shocks, using a heat exchanger in a recycle system. Heat shocks in the superoptimum temperature range did not affect overall culture performance, whilst heat shocks involving supermaximum temperatures resulted in significant activity reduction of the heat shocked fraction. However, overall biomass
production remained essentially constant under the conditions investigated, a feature that was explained on the basis of culture segregation but which would not be expected in large-scale bioreactors.

The influence of defined heat shocks on the specific growth rate was quantitatively investigated and characterized in exponential phase cultures of *K. pneumoniae* growing in glucose mineral salts medium. Both heat shock temperature and exposure time-dependent transitory changes in the specific growth rate during recovery were evident. Cell viability determinations, based on colony counts, indicated complete survival from heat shocks in the superoptimum temperature range, whilst for heat shocks in the supermaximum temperature range recovery estimations were medium dependent. The kinetic response of the culture is explained on the basis of segregation in the bacterial population.

The expression of *htpG* heat shock gene expression was investigated in batch and continuous cultures of *Escherichia coli*, using a chromosomal *htpG-lacZ* gene fusion. Emphasis was given to the effect of the physiological culture status and growth medium on the *htpG* expression patterns. Stationary phase batch cultures exhibited a markedly reduced *htpG* gene expression when compared with exponentially growing cultures after an identical heat shock at a superoptimum temperature in glucose mineral medium. At a supermaximum temperature, no heat shock-induced *htpG* gene expression was found in the glucose minimal medium, but a significant response was observed under identical heat shock conditions in a complex medium.

Under continuous culture conditions at various temperatures, relative *htpG* gene expression patterns were essentially identical during steady state growth, irrespective of the dilution rate and growth medium used. In contrast, whilst the relative transient expression patterns after identical heat shocks in the superoptimum range were identical at different dilution rates in a given medium, significantly different responses were evident in various growth media at a fixed dilution rate. These results illustrate that, in addition to the effect of temperature *per se*, both the physiological status of the culture and the growth medium have a marked impact on *htpG* heat shock gene expression.
ZUSAMMENFASSUNG


Aus einer Literaturstudie geht klar hervor, dass in den meisten Untersuchungen über Auswirkungen von superoptimalen Temperaturen auf Bakterien das Hauptaugenmerk auf physiologische und mechanistisch, molekularbiologische Aspekte gerichtet war. Über die Einflüsse von verschiedenen Kultivationsbedingungen auf die Vielzahl von Prozessen, welche während kurzzeitiger oder konstanter Einwirkung von superoptimalen Temperaturen auf Bakterien stattfinden, sind wenig Informationen vorhanden. Auch ist über die kinetischen Auswirkungen von Hitzeschocks auf wachsende Bakterienpopulationen, speziell im Hinblick auf die Erholungsphase, wenig bekannt.


Die Auswirkungen von lokal en Temperaturschwankungen auf das Gesamtverhalten einer Bakterienkultur wurde unter kontinuierlichen Kultivationsbedingungen an Klebsiella...


1. GENERAL INTRODUCTION

The growth physiology of microbes is affected by a range of physical and physico-chemical factors. In aquatic environments, the primary physical factors are temperature, pressure and light, whilst the primary physico-chemical factors are hydrogen ion concentration (pH), oxidation-reduction (redox) potential and water activity. All these factors can adversely affect microbial growth but under given conditions a range exists for each factor within which optimum growth occurs. These ranges are not identical for the whole spectrum of microbes. In the case of temperature, pH, redox and water activity, the optimum ranges for an individual strain are narrow, whilst in the case of pressure per se, rather than of the secondary effects of pressure, the optimum range is rather broad. In the case of light, the wave-length range for the optimum growth of phototrophs is usually relatively narrow, but non-phototrophs have no obligate requirement for light, although high intensity light can impair their growth. Ultraviolet light damages all microbes. The present study concerns effects of temperature on the growth physiology of bacteria.

1.1 TEMPERATURE RANGES FOR GROWTH

Most bacteria can grow over a temperature range of some 30-40°C degrees, irrespective of whether they are psychrophiles, mesophiles or thermophiles (Ingraham et al. 1983). Although bacteria belonging to these three groups have distinct cardinal temperatures, i.e., minimum, optimum and maximum temperatures, the characteristic relationship between specific growth rate and temperature follows a similar pattern to that shown in Figure 1.1, where the resultant curve is subdivided into six distinct temperature ranges. The subminimum temperature range (I) is defined by its upper limit which is the lowest growth permissible temperature. Region (II) is the sub-Arrhenius range for growth, where the specific growth rate declines faster than expected from the Arrhenius relationship. Its lower limit is given by the minimum temperature for growth. In the Arrhenius range (III) a linear relationship occurs between the natural logarithm of the specific growth rate and the inverse of the absolute temperature, as predicted by the Arrhenius relationship for simple chemical reactions. Region IV, the optimum temperature range, is limited by the temperature where the curve deviates from the maximum of the Arrhenius range and by the highest optimum growth temperature, where the specific growth rate is a maximum. The superoptimum range (V), is characterized by the highest optimum growth temperature as its lower boundary and the maximum temperature for growth as its upper limit.
Temperatures exceeding the maximum temperature for growth belong to the supermaximum temperature range (VI).

Figure 1.1 Generalized Arrhenius plot for the specific growth rate of a bacterium and the corresponding characteristic temperature ranges: I, subminimal range; II, sub-Arrhenius range; III, Arrhenius range; IV, optimum range; V, superoptimum range and VI, supermaximum range. $T_{\text{min}}$, $T_{\text{opt}}$ and $T_{\text{max}}$ represent the minimum, optimum and maximum temperatures for growth.

Other proposals for the definition of characteristic temperature ranges with respect to specific growth rates have been suggested. Franks et al. (1980) proposed the existence of only four regions, excluding both the subminimum and optimum ranges. Ingraham et al. (1983) subdivided the Arrhenius plot into only three ranges, a low temperature range where the Arrhenius relationship is inapplicable, followed by a normal temperature range where the Arrhenius relationship is applicable with the highest optimum temperature as its upper boundary and a high temperature region above the highest optimum temperature for growth. The definition of an optimum range seems justified, since most bacterial strains exhibit optimum ranges of more than 5 C degrees within which the specific growth rate remains essentially constant (Mohr and Krawiec 1980, Al-Awadhi et al. 1988) or where the increase in the specific growth rate is slower than predicted by the Arrhenius
relationship (Farrell and Rose 1967, Mohr and Krawiec 1980). Van Uden and Madeira-Lopes (1975) have subdivided the superoptimum temperature range for the growth of mesophilic yeasts by taking cellular death into account and distinguishing between a first region in which no cellular death occurs, a second one where the specific growth rate is higher than the specific death rate such that net growth is apparent, a third region where transient growth for a limited time period is obtained after a temperature shift and, finally, a range where even an initial transient growth phase cannot be observed. An alternative subdivision of the superoptimum temperature range has been suggested by Pozmogova (1979) who introduced a supraoptimum temperature region where the temperature is somewhat higher than optimum and a submaximum range in which growth is very poor. However, no clear differentiation of either of these two ranges was presented.

1.2 OBJECTIVES OF THE PRESENT STUDY

In the investigations reported herein, attention has been focussed primarily on the superoptimum temperature range for bacterial growth. Chapter 2 comprises a mini-review of factors pertinent to bacterial growth at superoptimum temperatures. In Chapter 3, selected mathematical models that describe bacterial growth throughout the growth permissible temperature range are validated with respect to their utility for the superoptimum temperature range.

The physiological effects of short term heat shocks, in both the superoptimum and supermaximum temperature ranges for growth on portions of growing cultures of Klebsiella pneumoniae NCIB 418 are examined in Chapter 4, and used to evaluate the effect of temperature gradients on overall culture performance. In order to obtain quantitative data on the effects of heat shocks on growth kinetics, the recovery of exponentially growing cultures of K. pneumoniae NCIB 418 after heat shocks was investigated and reported in Chapter 5.

Because of the increasing potential for applications of temperature inducible expression systems for the controlled expression of genes in recombinant bacteria and the impact that such temperature treatments might have on the physiology of the process cultures, the effects of various heat shocks on the expression of a representative heat shock gene, htpG, in Escherichia coli JB23 was investigated under different growth conditions. The results from batch culture studies are discussed in Chapter 6, whilst those obtained under continuous culture conditions are presented in Chapter 7.
Chapter 8 places the overall findings of the investigations undertaken in perspective and emphasizes the conclusions that it has been possible to draw.

1.3 REFERENCES


2. GROWTH AT SUPEROPTIMUM TEMPERATURES

During growth in the superoptimum temperature range, the specific growth rates of all bacteria progressively decrease with increasing temperature until growth finally ceases. To account for this temperature-mediated reduction of the specific growth rate, various explanations have been proposed. Crozier (1925) developed a hypothesis that the temperature response of an overall process is determined by a so called "master reaction". Reactions, such as bacterial growth, which take place over a wide temperature range were considered to be controlled by more than one "master reaction", and breaks in the Arrhenius curve were interpreted as critical temperatures where a new "master reaction" becomes dominant for the overall process. About twenty years later, Hinshelwood (1946, cited in: Christophersen 1973) explained the growth optimum and subsequent decrease of growth rate on the basis of two coexistent competitive processes, one synthetic, the other degradative. Mitchell (1951) interpreted the decrease in specific growth rate above the optimum temperature as a combined result of cellular death and simultaneous growth of the remaining viable cells.

A close relationship between the structure and function of many biological macromolecules has been recognized for many years. Correlation between the maximum growth temperature and the minimum inactivation temperature for respiratory enzymes was reported by Edwards and Rettger (1937). Subsequently, various studies, seeking a molecular basis to explain the maximum growth temperature, were published. These were reviewed by Farrell and Rose (1967).

It has been concluded that temperature, as an environmental parameter, has either no (Schaechter et al. 1958, Bremer and Dennis 1987) or only very small (Ingraham 1962) effects on the macromolecular composition of bacterial cells. Since most of the experiments, upon which such statements were based, were carried out in batch cultures growing in the Arrhenius and optimum temperature ranges, such conclusions are questionable. Tempest and Hunter (1965) have clearly shown that the macromolecular composition of Aerobacter aerogenes (Klebsiella pneumoniae), grown either glycerol- or magnesium-limited in continuous culture at a fixed dilution rate, changed significantly in both the Arrhenius and optimum temperature ranges. In a similar study, using an obligately psychrophilic Pseudomonas sp., Harder and Veldkamp (1967) showed that changes occurred throughout the entire growth permissible temperature range. These results, which will be discussed in more detail later, show that the growth environment also has a significant effect on the response of a bacterium to temperature.
Since in bacterial cells, macromolecular components such as lipids, nucleic acids and proteins represent sum-parameters comprising various individual structural and functional elements, they will be considered in more detail with respect to their potential response to temperature in the superoptimum range for growth.

2.1 MEMBRANES

Optimum functioning of the cell membrane as the boundary between the exterior and the interior of a bacterium is strongly influenced by the physical state of the membrane's lipid bilayer. The physical state of phospholipid bilayers depends on their fatty acid composition and on environmental temperature. The two extreme states that can exist are a gel state at lower temperatures and a liquid crystalline state at higher temperatures. Transition between these two extreme states takes place over a relatively wide temperature range, characterized by an upper and a lower transition temperature. Transition temperatures usually vary with the growth temperature of the bacterium under consideration such that the membrane is maintained in the liquid crystalline state (Janoff et al. 1979 and 1980 and Reizer et al. 1985).

In most bacteria an increase in the growth temperature results in significant changes in membrane fatty acid composition. Generally, the relative proportion of unsaturated and short chain fatty acids decreases and the relative proportion of saturated and long chain species increases with increasing temperature (Russell 1984). Such changes have been reported for psychrophilic (McGibbon and Russell 1983), mesophilic (Ingraham 1987) and thermophilic (Reizer et al. 1985) bacteria. However, exceptions where only either small or no apparent changes in fatty acid composition occur, have been observed in some psychrophilic and psychrotrophic bacteria (Bakhoo and Herbert 1980) and in some mesophilic bacteria (Joyce et al. 1970). Whilst few studies have considered the superoptimum temperature range, a notable exception exists: the classical investigation of Escherichia coli by Marr and Ingraham (1962).

Gram negative bacteria have two membranes, a cytoplasmic membrane consisting of a symmetrical phospholipid bilayer interspersed with proteins, and an outer asymmetrical membrane consisting of an inner phospholipid layer and an outer lipopolysaccharide layer. In E. coli, the inner phospholipid layer is primarily composed of two unsaturated fatty acids, palmitoleic acid and cis-vaccinic acid, which both decrease in concentration with increasing growth temperature in the superoptimum temperature range and one saturated
fatty acid, palmitic acid, which increases in concentration with increasing growth temperature as shown in Figure 2.1 (Marr and Ingraham 1962). In contrast, the lipopolysaccharide layer is composed only of saturated fatty acids, primarily myristic acid and β-hydroxymyristic acid. During growth at optimum and superoptimum temperatures, the unsaturated fatty acids of the cytoplasmic membrane can, to a certain extent, be methylated (Ingraham 1987). Overath et al. (1970) have shown that the incorporation of exogenously supplied fatty acids, containing different hydrocarbon chain structures, into membranes, resulted in variations in the maximum growth temperature in an E. coli mutant which was unable to either synthesize or degrade unsaturated fatty acids.

Adjustment of the content of cis-vaccenic acid in the phospholipid layer with increasing growth temperatures is an interesting example of temperature mediated regulation resulting from the activity of β-ketoacyl-acyl carrier protein synthase II (de Mendoza and Cronan 1983). This enzyme catalyses the condensation of malonyl-ACP with the acyl-ACP ester of palmitoleic acid to produce cis-vaccenoyl-ACP, which competes with the saturated palmitoyl-ACP for position 1 of the phosphatidic acid. A lowered β-ketoacyl-ACP synthase II activity results in a reduced concentration of diunsaturated phospholipids.
thereby providing better membrane function at higher growth temperatures. This mode of
regulation allows immediate adaptation to changes in environmental temperature during
growth by ensuring the maintenance of optimum membrane function.

2.2 NUCLEIC ACIDS

The effect of temperature on the nucleic acid composition and content in bacteria has been
investigated in both batch (Schaechter et al. 1958) and continuous culture (Tempest and
Hunter 1965, Harder and Veldkamp 1967). For Salmonella typhimurium growing in
various media in batch culture it was shown that the total DNA and RNA contents in the
bacterial biomass remained constant in both the Arrhenius and optimum temperature
ranges for growth, but no experiments were performed at superoptimum temperatures. In
contrast, during the growth of A. aerogenes (K. pneumoniae) and a psychrophilic
Pseudomonas sp. within their respective Arrhenius and optimum temperature ranges at
fixed dilution rates in continuous culture, the RNA content decreased with increasing
temperature, whilst the DNA content remained essentially constant. In the case of the
Pseudomonas sp., growing in the superoptimum temperature range, the RNA content
increased markedly, whilst the DNA content increased only slightly. The increase in RNA,
which comprises predominantly rRNA, was explained by Harder and Veldkamp (1967) in
terms of compensation for impairment of protein synthesis at elevated temperatures.

2.2.1 DNA

Double stranded chromosomal DNA is considered to be thermostable within the growth
permissible temperature ranges exhibited by bacteria. Melting temperatures, where 50% of
the DNA examined is single stranded, are usually some 40 to 50 °C degrees above the
maximum growth temperature in the case of psychrotrophs and mesophiles. For E. coli the
DNA melting temperature is ca. 90°C (Knippers 1982). However, local unwinding of the
DNA double helix might already occur in the superoptimum temperature range, thereby
changing promoter activity and consequently gene expression patterns.
Goldstein and Drlica (1984) have shown that plasmid DNA supercoiling can be altered by
temperature changes even within the Arrhenius range for growth. Further, confirmation of
such effects at superoptimum temperatures has been provided by Ueshima et al. (1989),
who showed that, for E. coli, the in vitro rate of open complex formation in the P2
promoter region of the heat shock regulatory gene, rpoH, was enhanced when the temperature was increased from 32°C to 42°C, thus increasing transcription from this gene.

Temperatures only a few degrees above the maximum growth temperature for *E. coli* in complex medium have been reported to result in DNA single (Sedgwick and Bridges 1972) and double strand breaks (Woodcock and Grigg 1972). Such breaks have been shown to be induced by endonuclease II (Grecz and Bhatarakamol 1977). Structural changes, measured as altered sedimentation properties in sucrose gradients, resulting from either association or dissociation of proteins, have also been reported (Pellon *et al.* 1980, Pellon 1983).

Evidence exists that suggests that the DNA replication process is adversely affected during growth at superoptimum temperatures. For *E. coli* it was shown that mutations in dnaK and dnaJ (Itikawa and Ryu 1979, Wada *et al.* 1982), groES and groEL (Wada and Itikawa 1984) and grpE (Ang *et al.* 1986) heat shock genes interfere with growth particularly at superoptimum temperatures, due to DNA synthesis inhibition, indicating that certain heat shock proteins might either replace, support or provide essential functions in the DNA replication process at higher temperatures.

### 2.2.2 RNA

Total bacterial ribonucleic acid comprises three different species, i.e., stable rRNA and tRNA and unstable mRNA. Since the macromolecular structures of all these species exhibit distinct secondary and, at least in the case of rRNA and tRNA, tertiary structures which are intimately linked with their functional properties, exposure of bacteria to superoptimum temperatures can be expected to alter these structures. However, most of the experimental evidence for either structural alteration or denaturation of stable RNA has been obtained either from studies with washed cells in buffer at supermaximum temperatures or from *in vitro* studies with purified RNA species.

#### 2.2.2.1 rRNA and tRNA

Heating *E. coli* at 50°C results in a release of 260nm absorbing materials, which are constituents of RNA. Such losses result from partial rRNA denaturation, thereby making the rRNA accessible to ribonucleases which subsequently mediate its degradation, a matter that has been discussed in detail by Gomez (1977). Physical aspects of the thermal stability of the rRNA of *E. coli* and other bacteria have been investigated by Pace and Campbell...
(1967). As has been shown previously for DNA, the melting temperature of E. coli rRNA, which is 72°C, is significantly above the maximum growth temperature of the bacterium.

Early evidence for conformational changes in tRNA molecules at supermaximum temperatures was provided by Malcolm (1969), using tRNA from Micrococcus cryophilus. Malcolm (1969) also showed that the stability of tRNA was clearly temperature dependent within the growth permissible temperature range for this bacterium. Borisova et al. (1988) have reported a melting temperature of 74°C for E. coli tRNA^Phe.

The effects of temperature on the various synthesis parameters of stable RNA were investigated by Ryals et al. (1982), who showed that the amount of stable RNA per unit biomass in batch cultures of E. coli, grown in a glucose minimal medium in the Arrhenius and optimum temperature ranges between 20 and 40°C remained essentially constant. Similar results were reported earlier for S. typhimurium by Schaechter et al. (1958) for the Arrhenius range. Ryals et al. (1982) also showed that at 42°C a reduction of stable RNA in excess of 10% occurred, but whether this reflects a general trend for stable RNA in the superoptimum temperature range is unclear, since analyses were not conducted at other temperatures in the range. Further, their results indicated an over proportional increase in stable RNA chain elongation rates between 37°C and 42°C, but only insignificant changes in the specific growth rate in the same temperature range. These observations probably reflect a decrease in the stability of rRNA and tRNA with increasing temperature, thereby requiring compensation by a higher stable RNA synthesis rate, if high protein synthesis activity is to be maintained.

2.2.2.2 mRNA

The inactivation rates of various mRNA's such as lac mRNA (Kennell and Bicknell 1973), trpCBA mRNA and trpED mRNA (Wice and Kennell 1974) increase exponentially with increasing temperature in the Arrhenius and optimum ranges in E. coli. Further, it has been shown by Kaluza and Hennecke (1981) that the half life of nifHDK mRNA in K. pneumoniae was reduced about 3.6 fold at the superoptimum temperature of 39°C when compared with the stability at 30°C. This trend was also observed for other mRNA species in K. pneumoniae (Collins et al. 1986). However, the destabilizing effect of temperature on nifHDK mRNA was more pronounced than with other mRNA species and is probably mediated by the specific action of the nifA product. In contrast to the above findings, Erickson et al. (1987) reported a stabilizing effect of heat shocks at superoptimum temperatures on mRNA's of the heat shock regulatory gene rpoH in E. coli. When an
exponentially growing culture of *E. coli* was subjected to a step change from 30°C to 43.5°C, the synthesis of *rpoH* mRNA increased only two fold, whilst the level of *rpoH* mRNA increased 4 to 5 fold. These researchers suggested that this is either the result of a specific factor, which stabilizes *rpoH* mRNA or, alternatively, a stabilization effect due to increased translation.

An indication of a possible temperature-mediated alteration of mRNA secondary structure has been reported by Yanofsky (1985). In vivo studies concerning the regulation of the trp operon in *E. coli* revealed, that termination at the attenuator was significantly higher at 41°C than at 30°C. However, it is unclear, whether these results reflect better recognition of the termination signal by RNA polymerase or whether the antiterminator forms less readily at the higher temperature (Yanofsky 1985).

### 2.3 PROTEINS

The effect of elevated temperatures on enzyme activity has been the subject of numerous early investigations, but these will not be discussed here. For a review of this work Farrell and Rose (1967) should be consulted. In general, the loss of activity of important biosynthetic enzymes results in restricted growth in minimal media, but this can often be overcome by the addition of appropriate biochemical compounds. A well documented example of close correlation between a reduction in growth rate and enzyme inactivation at superoptimum temperatures is the reversible inactivation of homoserine transsuccinylase in *E. coli*, which results in inhibition of methionine synthesis (Ron and Davis 1971, Ron and Shani 1971).

The total amount of cellular protein remains essentially constant during growth of bacteria in batch cultures at temperatures in the Arrhenius, optimum (Schaechter *et al.* 1958) and superoptimum (Gounot *et al.* 1977 and Pozmogova 1979) temperature ranges. In contrast, a slightly different pattern is observed during the growth of bacteria in a chemostat. During glycerol-limited growth of *A. aerogenes* (*K. pneumoniae*) at a constant dilution rate, the total cellular protein content on a dry weight basis increases during growth at temperatures between 25° and 40°C, although during magnesium-limited growth the total amount of cellular protein remains essentially constant (Tempest and Hunter 1965). These temperatures include the Arrhenius, the optimum and the early superoptimum ranges. Unfortunately these researchers did not investigate the response in the entire superoptimum temperature range. However, Harder and Veldkamp (1967) showed that the protein content of a psychrophilic *Pseudomonas* sp. gradually increased during growth throughout its superoptimum temperature range.
2.3.1 Protein Synthesis

Although little change is observed in the total protein content, the concentration of individual proteins varies markedly with temperature in the superoptimum temperature range (Farrell and Rose 1967).

The introduction of two-dimensional high resolution gel electrophoresis (O'Farrell 1975 and 1977) for the detection of cellular proteins has provided an excellent tool for the quantitative measurement of individual cellular proteins. Herendeen et al. (1979) investigated the levels of 133 individual proteins, which represent some 70% of the total cellular protein of *E. coli* B/r during exponential growth in a rich medium at temperatures between 13.5° and 46°C. These researchers have grouped the proteins, on the basis of their temperature dependent levels, into various metabolic regulatory classes. Their most important conclusion was that the metabolic coordination within the Arrhenius range was primarily achieved by the modulation of enzyme activity rather than by modulation of the amount of enzyme present. In contrast, during growth in the optimum and superoptimum temperature ranges the levels of many proteins either decreased or increased, thereby indicating a need for major metabolic alterations to cope with the pertaining environmental temperatures. Temperature dependent regulation of the synthesis of individual proteins can occur at both the levels of transcription and translation, as illustrated below.

2.3.1.1 Transcriptional control

a) The heat shock response

A well known example of regulation at the level of transcription is the heat shock response in bacteria. The heat shock proteins are a group of proteins which increase in concentration with increasing temperature in the optimum and superoptimum temperature ranges for growth. In *E. coli* 17 such heat shock proteins have been identified (Neidhardt et al. 1984, Neidhardt and Van Bogelen 1987). Their levels, as a percentage of total cellular protein during batch growth in a nutrient rich medium are given in Table 2.1. These proteins are organized in a regulon, which is under the positive control of the *rpoH* gene that codes for a RNA polymerase sigma factor, $\sigma^{32}$ (Grossman et al. 1984). RNA polymerase associated with $\sigma^{32}$ selectively recognizes only heat shock promoters (Cowing et al. 1985). The regulation of the synthesis of $\sigma^{32}$ takes place predominantly at the level of transcription. The expression of the *rpoH* gene involves five promoters (Erickson et al. 1987 and Nagai et al. 1990). Four of these promoters are recognized by RNA polymerase containing $\sigma^{70}$.
Table 2.1 Levels of heat shock proteins as a percentage of total cellular protein in batch cultures of *E. coli* at different temperatures. The cultures were grown in a nutrient rich medium. (Data from Herendeen et al. 1979 and Neidhardt and Van Bogelen 1987).

<table>
<thead>
<tr>
<th>Protein α-numeric no.</th>
<th>Protein designation</th>
<th>Protein name</th>
<th>Mol wt</th>
<th>Gene</th>
<th>% of total cellular protein at 30°C</th>
<th>37°C</th>
<th>42°C</th>
<th>46°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B25.3</td>
<td>GrpE</td>
<td>25300</td>
<td>grpE</td>
<td>0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>B36.5</td>
<td>GroEL</td>
<td>62883</td>
<td>groEL</td>
<td>1.22 1.65 2.64 11.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B66.0</td>
<td>DnaK</td>
<td>69121</td>
<td>dnaK</td>
<td>1.09 1.41 1.99 4.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>B83.0</td>
<td>Sigma (α70)</td>
<td>70263</td>
<td>rpoD</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C14.7</td>
<td>htpE</td>
<td>14700</td>
<td>htpE</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>C15.4</td>
<td>GroES</td>
<td>10670</td>
<td>groES</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>C62.5</td>
<td>htpG</td>
<td>71000</td>
<td>htpG</td>
<td>0.13 0.26 0.5 0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>D33.4</td>
<td>htpH</td>
<td>33400</td>
<td>htpH</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>D48.5</td>
<td>htpI</td>
<td>48500</td>
<td>htpI</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>D60.5</td>
<td>Lysyl tRNA synthetase II</td>
<td>60500</td>
<td>lysU</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>F10.1</td>
<td>htpK</td>
<td>10100</td>
<td>htpK</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>F21.5</td>
<td>htpL</td>
<td>21500</td>
<td>htpL</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>F84.1</td>
<td>htpM</td>
<td>84100</td>
<td>htpM</td>
<td>0.04 0.07 0.07 0.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>G13.5</td>
<td>htpN</td>
<td>13500</td>
<td>htpN</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>G21.0</td>
<td>htpO</td>
<td>21000</td>
<td>htpO</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>H94.0</td>
<td>Lon, La</td>
<td>94000</td>
<td>lon</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>H26.5</td>
<td>DnaJ</td>
<td>40975</td>
<td>dnaJ</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

whilst one is recognized by RNA polymerase associated with σ^{24} (Wang and Kaguni 1989a). Differential expression of these promoters at various temperatures has been reported by Erickson *et al.* (1987). The transcription from the *rpoH* gene is further regulated by the DnaA protein, which has been shown to bind at two sites in the *rpoH* promoter region, thereby repressing transcription from two promoters (Wang and Kaguni 1989b). Another regulatory mechanism was recently shown by Nagai *et al.* (1990), who reported a catabolite sensitive promoter. *In vitro* transcription studies revealed that transcripts from this promoter were found only in the presence of cAMP and its receptor protein. Further, addition of glucose to cultures of *E. coli* growing on complex medium, resulted in partial inhibition of *rpoH* gene expression.

Transcription from heat shock genes depends on the concentration of the *rpoH* gene product, σ^{32}, in the cell (Straus *et al.* 1987). However, changes in σ^{32} concentration can be effected by both temperature dependent alterations in its stability (Tilly *et al.* 1989) and posttranscriptional stabilization of *rpoH* mRNA (Erickson *et al.* 1987). These regulatory mechanisms enable the bacterial cell to respond quickly to temperature changes in the
Table 2.2 Some major functions of heat shock proteins in *E. coli*.

<table>
<thead>
<tr>
<th>Protein name (α-num. des.)</th>
<th>Functions, activities, properties, phenotypes of mutations</th>
<th>References</th>
</tr>
</thead>
</table>
| GrpE (B25.3)              | - Involvement in nucleic acid synthesis. Neidhardt and Van Bogelen (1987)  
- Involvement in λ DNA and RNA synthesis.  
- Interaction with DnaK *in vivo* and *in vitro*; dissociation is ATP dependent.  
- Essential for growth at all temperatures. Ang and Georgopoulos (1989)  
- Mutations result in: ts growth at T>43.5°C; defective low copy number plasmid maintenance; more heat resistant compared to parent strain. Delaney (1990) | |
| GroEL (B56.5) and GroES (C15.4) | - Functional interaction between GroEL and GroES; association is ATP dependent; Weak ATPase activity (GroEL).  
- Association with ribosomes.  
- Essential for phage morphogenesis.  
- Necessary for growth at all temperatures.  
- Overproduction results in: Facilitation of LacZ hybrid protein export; Suppression of certain mutations; Facilitation of prokaryotic ribulose bisphosphate carboxylase assembly.  
- Mutations result in: Altered cell permeability; defective cell division; restricted DNA and RNA synthesis; defective proteolysis. Neidhardt and Van Bogelen (1987)  
- Essential for phagemorphogenesis. Fayet et al. (1990)  
| DnaK (B66.0)              | - Involvement in DNA initiation at least at high temperatures. Sakakibara (1988)  
- Interaction with GrpE *in vivo* and *in vitro*. Phillips and Silhavy (1990)  
- Mutations result in: Sensitivity to high and low temperatures; genetic instability; cell division defects; defective low copy number plasmid maintenance and chromosome segregation; defective proteolysis. Bukau and Walker (1989b)  
- Highly conserved during evolution. Bukau and Walker (1989b) | |
- Necessary for phage DNA replication. Tilly and Yarmolinsky (1989)  
- Mutations result in: Defective low copy number plasmid maintenance; defective proteolysis. Strauss et al. (1988) | |

optimum and superoptimum temperature ranges. The induction of the heat shock proteins after a rapid temperature step change from 37 to 42°C takes place within less than one minute (Yamamori and Yura 1980).

In addition to a temperature shift, several other agents such as e.g., ethanol, puromycin, viral infection, nalidixic acid and cadmium chloride can induce either a heat shock response or subsets of heat shock proteins (Neidhardt and Van Bogelen 1987).

The fact that heat shock proteins are present in all organisms suggests that they have important physiological functions. Evidence for their importance is also provided by the high degree of conservation of some of these proteins during evolution, manifested by high sequence homology between homologous proteins from prokaryotes and eukaryotes (Bardwell and Craig 1984 and 1987). A summary of major functions of the heat shock proteins of *E. coli* is presented in Table 2.2.
b) The htrA gene

Another example of heat inducible regulation of gene expression at the level of transcription is the expression of the htrA (degP) gene in E. coli which codes for a protein of 51 kD that is posttranscriptionally modified by cleavage to a 48 kD protein with an endopeptidase activity (Lipinska et al. 1990). The promoter of the htrA gene is recognized by RNA polymerase associated with σ^{24}, the sigma subunit which also recognizes one of the rpoH promoters. This establishes a link between these two temperature inducible systems, highlighting coordination of the response of the cell to higher temperatures. There is evidence that the htrA protein is localized either in the cell membrane or in the periplasmic space, where it has some proteolytic function. Its absence results in severe restriction of growth at superoptimum temperatures (Strauch et al. 1989).

2.3.1.2 Translational control

An example of temperature induced regulation of protein synthesis at the level of translation has been reported by Kuriki (1989). The expression of the amp gene, encoding for β-lactamase in E. coli, is repressed at the level of the initiation step of translation. It has been shown that the minimum DNA segment which is responsible for the repression includes both the Shine-Delgarno sequence and the initiation codon. Since expression of this gene is enhanced in vitro by a protein which is heat labile, it is assumed that inactivation of this protein is the cause for the repression of the amp gene at superoptimum temperatures. However, it is not clear whether this protein interacts with either the translation start signal region of the amp mRNA or is involved in the formation of the initiation complex between amp mRNA and ribosome for the binding of fMet-tRNA.

2.3.2 Protein Degradation

The degradation of intracellular proteins in bacteria is a fundamental process which contributes to many important physiological functions such as protein processing and secretion, regulation of the intracellular levels of proteins, elimination of abnormal peptides and provision of amino acids for reutilization for new protein synthesis (Potier et al. 1990). In E. coli the role of protein degradation as a regulatory function has been discussed by Gottesman (1987). During growth of bacteria at temperatures in the optimum and superoptimum range an increased proteolytic activity has generally been observed (St. John et al. 1978, Potier et al. 1987), suggesting that this is characteristic of growth at
superoptimum temperatures. Of particular interest is that an increase in proteolytic activity can be induced by the presence of abnormal proteins which is analogous with induction by elevated temperatures. The fact that proteolysis at higher temperatures is more pronounced might stem from an increase of partial protein unfolding and a subsequent loss of function (Goff et al. 1984). In *E. coli* the ATP dependent protease La, which is one of the heat shock proteins, has been shown to be able to selectively degrade abnormal, non-functional and certain normal proteins (Goff and Goldberg 1985) thereby intimately linking the heat shock system with protein degradation. Additionally, indirect involvement of the heat shock system in non-energy dependent protein degradation has been reported. It was shown that in *rpoH* and *lon* double mutants an additive stabilizing effect on certain recombinant proteins occurred (Miller 1987). Further, Straus et al. (1988) provided evidence that DnaK, DnaJ, GrpE and GroEL, which themselves have no proteolytic activity, were indirectly involved in protein degradation, since mutations in their genes resulted in decreased proteolysis both of polypeptide fragments generated by puromycin and of nonsense fragments of β-galactosidase. In contrast, over production of these proteins resulted in increased degradation of abnormal proteins.

Another example for a heat shock induced proteolytic enzyme is the *htrA* gene product which, as already mentioned, has a non-energy dependent proteolytic function in the periplasmic space (Lipinska et al. 1990).

The evidence presented clearly indicates that proteolytic activity at optimum and superoptimum temperatures for growth is a highly coordinated and important process that enables bacteria to withstand elevated temperatures.

### 2.4 CELL DIVISION AND SIZE

During growth at superoptimum temperatures approaching the maximum temperature for growth, many bacteria elongate and form filamentous cells as a result of their inability to septate and divide (Joakim and Inniss 1976, Donachie 1985). For the psychrotrophic bacterium, *Bacillus insolitus*, it was shown that DNA replication continued during filament formation, resulting in polynucleic filamentous cells (Ferroni and Inniss 1973). Return of such filamentous cells to temperatures in either the optimum or the Arrhenius temperature ranges resulted in cell division, requiring de novo enzyme synthesis (Ferroni and Inniss 1974, Joakim and Inniss 1976).

In *E. coli* mutants deficient in the heat shock response, cell division is inhibited at 42°C. Further, *dnaK*, *groEL* or *groES* mutants also form filamentous cells at superoptimum temperatures (Tsuchido et al. 1986). Mizusawa and Gottesman (1983) have shown that the
SulA protein, a cell division inhibitor formed during SOS response in *E. coli* (Walker 1987), is degraded by the lon protease. *Lon* gene mutants show a lethal SOS response, since the SulA protein can no longer be degraded (Gottesman 1987). Although these studies certainly confirm an indirect and possibly even a direct interaction between the heat shock response system and cell division it is not yet clear, how the inhibition of cell division is affected by superoptimum temperatures.

### 2.5 ENERGY METABOLISM

Almost fifty years ago Monod (1942) observed that the biomass yield coefficient for aerobic batch cultures of *E. coli* growing on glucose decreased at superoptimum temperatures (39 and 41°C), whilst it remained essentially constant in the Arrhenius and optimum temperature ranges for growth, i.e., between 20° and 37°C. In addition to a reduction in the biomass yield coefficient during growth at superoptimum temperatures, Senez (1962) reported that *A. aerogenes* (*K. pneumoniae*) grown in batch culture on glucose exhibited its maximum specific growth rate at 37°C, whilst the respiration rate of glucose reached its maximum at 42°C. Similar observations were subsequently made for various psychrophilic (Harder and Veldkamp 1967, Stokes and Larkin 1968, Inoue 1977), thermotolerant (Al-Awadhi *et al.* 1990) and thermophilic bacteria (Coultate and Sundaram 1975). The observed decreases in both the specific growth rate and biomass yield coefficient have been explained in terms of the various mechanisms discussed below (Franks *et al.* 1980).

#### 2.5.1 Maintenance Energy

The maintenance energy requirement of a bacterium is defined as the residual energy demand at zero growth rate, necessary for maintaining viability (Pirt 1975). It was shown for various bacteria that the maintenance energy requirement increases with increasing growth temperature, including temperatures within the superoptimum range for growth (Harder and Veldkamp 1967, Topiwala and Sinclair 1971, Esener *et al.* 1983, Pennock and Tempest 1988). However, few investigators provide any clear mechanistic explanation for such temperature induced changes in the basal energy demand. Recently, De Vrij *et al.* (1988) observed in a comparative study of the energy transducing properties of the cytoplasmic membranes of the mesophile, *Bacillus subtilis*, and the thermophile, *Bacillus stearothermophilus*, large differences between the membrane energy transduction
efficiency for these two species at their optimum growth temperatures. Further, in both species the capacity to generate a proton motive force by respiration declined with increasing temperature because of a positive temperature dependent increase in the H\textsuperscript{+} permeability of the membranes. These findings provide a possible explanation for the generally increased maintenance requirement for thermophilic bacteria in comparison with mesophilic bacteria. Further, it might also explain increased maintenance energy requirements in the superoptimum temperature range for the growth for bacteria from various temperature classifications.

2.5.2 Endogenous Metabolism

The increased protein turnover which is due to higher proteolytic activities in bacteria growing at superoptimum temperatures (see Section 2.3.2) can be expected to divert a considerable part of the energy produced from biomass production to the resynthesis of cellular proteins, thereby resulting in a reduction in the observed yield coefficient. The subject of endogenous metabolism has been discussed in a general context by Dawes and Ribbons (1964).

2.5.3 Temperature-Induced "Uncoupling" of Growth

It is frequently observed that in bacteria growing at superoptimum temperatures, catabolic processes become either partly or completely uncoupled from anabolic processes. This is manifested in decreases in both the maximum specific growth rate and the observed yield coefficient, whilst substrate consumption and respiration continue to increase with temperature, in accordance with an Arrhenius type relationship (Farmer and Jones 1976). Such behaviour can be accounted for by several mechanisms. For example, processes, similar to non-carbon energy nutrient depletion induced metabolic uncoupling, where either incomplete substrate oxidation or bypassing of energy generating reactions, which both result in excretion of metabolites, might occur (Tempest and Neijssel 1987). Alternatively, the decreased efficiency in energy transduction of bacterial membranes at higher temperatures (De Vrij et al. 1988), which results in both increased maintenance requirements and increased endogenous activity could also result in the same apparent response by a bacterium, thereby making any clear differentiation between these several possible mechanisms difficult.
A phenomenon that has been observed in exponentially growing anaerobic cultures of two Streptococcus cremoris strains in milk is the uncoupling of product formation from growth at both superoptimum and supermaximum temperatures (Breheny et al. 1975). After a temperature shift from the optimum temperature range for growth to either a superoptimum or a supermaximum temperature, either the specific growth rate decelerated or growth stopped after two hours, whilst lactic acid production continued at either the pre-shift rate or at a slightly reduced rate. Upon addition of yeast extract to cultures where growth ceased, growth was reestablished (Hillier et al. 1975), indicating that depletion of either one or more nutrients, which became essential for growth at elevated temperatures, caused the cessation of growth.

2.5.4 Cellular Death

The decrease in specific growth rate at superoptimum temperatures has also been attributed to cellular death (van Uden and Madeira-Lopes 1976). The concept of cellular death was extended by incorporation of exogenous processes such as cell lysis and subsequent cryptic growth which can be expected, under certain conditions, to significantly contribute to a reduction in biomass yield coefficient (Mason et al. 1986). However, significant cellular death might not occur in all bacterial cultures during their growth at superoptimum temperatures. Hakeda and Fukunaga (1983) observed that the energy charge of Vibrio sp. ABE-1 remained constant during growth in the superoptimum temperature range, whilst the ATP pool correlated positively with the specific growth rate. However, if a fraction of the population comprised dead cells, the total energy charge would be expected to be reduced, since the energy charge of dead cells is significantly reduced.

As already mentioned, clear differentiation between cellular death and effects attributed to endogenous metabolism is difficult. It will only be possible when appropriate methods for the estimation of the fraction of cells in each physiological state become available.

2.6 THE RESPONSE OF BACTERIA TO HEAT SHOCKS

2.6.1 The "Stress Cycle"

Whenever a bacterium is subjected to a temperature change within its growth permissible temperature range, the intracellular network of coordinated metabolic reactions will be
disturbed. The magnitude of such a disturbance depends primarily on the intensity of the heat shock. Critical factors are the magnitude of the temperature change, the range(s) within or over which the change occurs, the exposure time, the physico-chemical environment and the physiological state of the bacterium.

Figure 2.2 "Stress cycle": Generalized characteristic processes in a bacterial culture subjected to an environmental stress.

The effects of a heat shock on a bacterial culture involve a number of characteristic processes, which are of general validity when a culture is subjected to stress, irrespective of the nature of the stress involved. The processes taking place are described by the "stress cycle" shown in Figure 2.2. When any bacterium, in an active physiological state, is subjected to a sudden stress, three different results can occur. If the stress is minor, the bacterium becomes metabolically disturbed but retains the capacity either to return to its original physiological state or to adapt to the new physiological and environmental situations. In the case of a more pronounced stress, either reversible or irreversible damage to macromolecular cellular components occurs and the bacterium can be considered to be
injured. Such an injured bacterium either has the capacity to repair the cellular damage, thereby surviving the stress and entering the cycle as a metabolically disturbed cell or, alternatively, if irreversible damage is involved, the bacterium ultimately dies and lyces. In the case of severe damage, cellular death can be expected. However, when a bacterial population is considered, any particular stress usually results in the simultaneous appearance of all three types of stressed cells.

2.6.2 Heat Shock-Induced Metabolic Alterations and Damage

The effects of heat shocks on bacteria have been the subject of numerous investigations, particularly in the fields of food and dairy sciences, where preservation of products from spoilage and elimination of pathogenic bacteria are of primary concern. However, most investigations have been focussed on either pasteurisation/sterilization processes or sublethal heat injury, thereby considering, virtually exclusively, temperatures in the supermaximum range. A relatively recent discussion of this subject that details the pertinent literature has been provided by Andrew and Russell (1984).

An early study on the kinetic response of exponentially growing cultures of E. coli to step temperature increases (heat shocks) either within the Arrhenius and optimum temperature ranges or from the Arrhenius to the superoptimum range was published by Patterson and Gillespie (1972). After step increases in temperature in the Arrhenius and optimum ranges, the culture assumed a new specific growth rate which was characteristic for the new temperature immediately after the increase. In contrast, for step changes from 30°C to either 42 or 44°C, i.e., temperatures in the superoptimum range, transient adaptation periods for the specific growth rate from the initial to the final steady states were observed. The metabolic changes that occurred during such transient periods were complex, as indicated by marked alterations in the differential synthesis patterns of many proteins (Lemaux et al. 1978, Yamamori and Yura 1978). Amongst the proteins which exhibited the most pronounced alterations in their transient synthesis patterns were the heat shock proteins (see section 2.3). Most of the research effort concerning effects of heat shocks (step temperature changes) on growing bacteria was subsequently focussed on the molecular regulatory mechanisms and functions of these proteins. However, of special interest with respect to the "stress cycle" is the question of the kinetic and physiological response of either stressed growing or non-growing cultures after their return from either superoptimum or a supermaximum temperatures to the initial growth temperature. The processes that take place during such a restoration phase will clearly depend on the degree of metabolic disturbance and injury that results from the heat shock imposed. In Figure
2.3, temperature ranges for some important site specific damage and structural alterations of macromolecular cellular components, together with some metabolic changes that occur, are given for *E. coli*.

![Diagram](image)

**Figure 2.3** Ranges for temperature induced site-specific damage, alterations and metabolic changes for *E. coli*. Tmin, Topt and Tmax are minimum, optimum and maximum temperatures for growth in complex medium.

Heat shocks in the supermaximum temperature range can be expected to result in some degree of injury, such that repair and/or resynthesis of the lost function(s) will become necessary. The ability of any injured bacterium to recover a particular capacity depends on both the processes that are still functional within the cell and on the nutritional composition of the medium. Various aspects of these subjects have been discussed by Allwood and Russell (1970), Gomez (1977) and Andrew and Russell (1984).

### 2.7 EFFECTS OF THE GROWTH ENVIRONMENT

An important question with respect to bacterial growth at superoptimum and resistance to supermaximum temperatures concerns the impact of the physico-chemical properties of
the growth environment and of the resulting physiological state of a bacterium. It is well established that the maximum growth temperature for most bacteria is higher in nutrient rich media than in minimal media containing salts and a single carbon energy source. As a general rule, the richer the medium, the higher the maximum growth temperature. When bacteria are grown at elevated temperatures they can become auxotrophic for either one or several essential compounds. The reasons for such behaviour are commonly found in the inactivation of either one or several biosynthetic enzymes. An example, which illustrates this effect particularly well is the earlier mentioned requirement for methionine in *E. coli* and other closely related Enterobacteriaceae (Ron 1975) when growing at temperatures above 45°C. This effect was due to the reversible inhibition of homoserine trans-succinylase which prevented *de novo* synthesis of methionine at 45°C.

Combined effects of physico-chemical factors such as pH, salt tolerance/water activity and temperature have recently been systematically investigated with respect to the growth temperature range of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Beal et al. 1989) and *Listeria monocytogenes* (McClure et al. 1989). The more one or several of the factors deviated from their optima, the more pronounced was the adverse effect on the specific growth rate and biomass yield coefficient based on the carbon substrate.

The physiological state of a bacterium, as determined by its environment, is of critical importance with respect to its resistance to a heat shock, particularly in the supermaximum temperature range. Starved cultures of *E. coli* exhibit a greater resistance to heat treatment than exponentially growing cultures (Jenkins et al. 1988). Further, short heat shocking at superoptimum temperatures prior to a lethal heat treatment results in increased thermotolerance (Neidhardt et al. 1984). However, this phenomenon is not directly related to the induction of heat shock proteins (Van Bogelen et al. 1987), although absence of the heat shock response, as seen in *rpoH* mutant strains, results in cells that are unable to grow at temperatures above 37°C (Neidhardt and Van Bogelen 1987).

### 2.8 ASPECTS OF SUPEROPTIMUM TEMPERATURES IN PRACTICE

#### 2.8.1 Large Scale Bioprocesses

The scale of and mixing times in large bioreactors that are used for the manufacture of bulk microbial products frequently mean that operating conditions vary throughout the
bioreactor. When a bioreactor is scaled up, the surface area available for heat transfer reduces with increasing volume and can become insufficient to allow easy maintenance of the process temperature within the optimum range particularly under conditions where high levels of process intensity are sought. Such problems are amplified in stagnant zones in large scale bioreactors, when highly viscous non-Newtonian charges are involved (Hamer and Heitzer 1990). Further, temperature fluctuations, due to ineffective process cooling, have been reported to adversely affect ethanol production by *Zymomonas mobilis* (Benschoter and Ingram 1986).

In large technical-scale bioreactors of the type used for the aerobic treatment of industrial wastewater, large volumes of high temperature polluted wastewater are frequently dumped into the bioreactor, thereby subjecting a portion of the process culture to a heat shock. The period of time during which process microbes are subjected to temperature shocks in large-scale industrial production and wastewater treatment bioreactors is usually a matter of minutes rather than of either seconds or hours (Hamer and Heitzer 1990).

### 2.8.2 Temperature Inducible Expression Systems

The development of recombinant DNA technology has provided the possibility of cloning heterologous genes from various source-organisms into bacteria. This has opened a new dimension for the manufacture of a wide variety of proteins of commercial interest. However, the need for both high product concentrations and high productivities, dictated by the constraints of available separation technologies, means that the product of interest has to be produced in high amounts by the host bacterium. This frequently results in intracellular accumulation of the compound and subsequent growth inhibition, cellular death or vector instability (Murooka and Mitani 1985). Therefore, control over promoter activity is an essential feature of an expression vector. In principle, two basic systems are available for developing controllable expression vectors. These involve combined repressor/promoter systems which are either temperature sensitive or metabolite controlled as the *lac*, *trp* and *tac* promoter systems used in *E. coli* (Caulcott and Rhodes 1986). The relative ease with which temperature can be used as a control parameter makes temperature inducible expression systems especially interesting.

#### 2.8.2.1 The bacteriophage λ expression system

The most widely used temperature inducible expression system in bacteria, particularly *E. coli*, involves the powerful \( P_L \) and \( P_R \) promoters of the bacteriophage λ, which are both
controllable by the temperature sensitive repressor clg57. This repressor protein is

denatured at 42°C, thereby enabling transcription from these promoters, whilst at
temperatures below 37°C complete repression occurs.

In order to obtain high amplification of a heterologous gene under the control of either the
PL or PR promoter, high copy number vector plasmids must be used.

2.8.2.2 "Runaway plasmids"

A different approach for obtaining high gene amplification has been reported by Larsen et
al. (1984) who constructed a so called "runaway plasmid". The λ PR promoter in
combination with the thermosensitive clg57 repressor was inserted upstream of the
replication control genes in plasmid R1. At temperatures below 37°C this plasmid is
present in one copy per chromosome, whilst at 42°C, uncontrolled replication occurs,
resulting in excess of 1000 plasmid copies per cell.

With bacteria containing either of these two systems, a bioprocess can be conducted in two
distinct stages, i.e., a biomass production stage at ca. 30°C to attain a high cell density and
a product formation stage where the process culture is exposed to a temperature of 42°C,
for some hours.

2.8.2.3 Heat pulse induced promoter inversion

An alternative temperature inducible expression system involving a different induction
strategy has been constructed by Podhajska et al. (1985). In contrast to the previously
described systems, their system, based on irreversible promoter inversion, requires only a
short, transient induction period of 10 min at 42°C to trigger protein synthesis which then
can continue at 30°C. In the "OFF" phase, which is absolute, a strong promoter, which is
located on an att-nutL-p-att-N gene block, is facing away from the gene that should be
expressed, while in the "ON" phase the promoter is oriented towards the particular gene.
The two stage control of promoter activity is based on a physical rearrangement within the
genome. The first stage involves a brief derepression of an auxiliary int operon which is
controlled by a λ PL promoter, in combination with the temperature sensitive clg57
repressor, whilst in the second stage the int product inverts the promoter, which is cloned
between divergent attPOP and attΔPOΔP' sites. In order to increase expression efficiency
a strong antiterminator, nutL, in conjunction with the N gene product is placed downstream
of the major promoter. This expression system is claimed to have advantages over the
previously mentioned temperature inducible systems because of the short exposure time at
42°C needed for the process culture. This is less disadvantageous with respect to the physiological state of the host strain and where thermosensitive products are produced (Podhajska et al. 1985). A successful application of this system in combination with a lac promoter has recently been reported by Wong et al. (1989) for xylose isomerase production by E. coli.

2.8.2.4 Some general considerations

Despite the many advantages of temperature inducible expression systems, side effects of superoptimum temperatures, such as increased proteolysis and lowered growth efficiency, should not be overlooked (Stouthamer and Van Verseveld 1987, Anderson Da Silva and Bailey 1989). Particular consideration should be given to the heat shock response system not only because of its involvement in proteolytic activities, but also because of the energetic aspects of the formation of heat shock proteins at elevated temperatures (Caulcott and Rhodes 1986). It has been reported that during expression of human somatomedin-C in an E. coli rpoH, lon double mutant, accumulation of the hormone protein is improved because of reduced proteolytic activity (Buell et al. 1985).

Recently, applications of the λ promoter/repressor system on broad host range plasmid vectors in species other than E. coli have been reported (Leemans et al. 1987, Winstanley et al. 1989). However, since many of the host bacteria investigated have temperature maxima which are either close to or below 42°C, exposure to the temperature necessary for expression might be highly detrimental.

2.8.3 The Impact of Thermally Polluted Discharges on Natural Ecosystems

Despite considerable knowledge concerning the adverse effect of temperature fluctuations on many bacteria (Reichardt 1979, Tison 1980), protozoa (Martinez 1980), algae (Caims 1972) and higher organisms (Coutant and Goodyear 1972), the problem of thermal pollution of natural surface waters by cooling water and wastewater discharges is still of considerable importance in many parts of the world (Scott et al. 1985, Hamer et al. 1990). The resulting disturbances can either markedly alter species composition or reduce species diversity (Levin et al. 1972), thereby changing the composition of local ecosystems and, consequently, foodweb structures and interactions. In this respect any impact of heat on bacterial populations is of particular importance because of the major role of such populations in nutrient cycling, degradation and mineralisation.
In order to illustrate the effect of thermal pollution on bacterial populations and their activities in a freshwater system, the effect of temperature on lignocellulose mineralisation can serve as an example. In a recent study, Benner and McArthur (1988) have shown that an increase in water temperature from ca. 20°C to ca. 40°C, resulting from a cooling water discharge from a nuclear power plant, caused the optimum temperature for lignocellulose mineralisation to increase from 35 to 45°C within 9 to 27 days of the commencement of the discharge. Such a change clearly reflects an alteration in the composition of the bacterial population. However, when the discharge of cooling water stopped, the original temperature optimum for mineralisation was reestablished. These results have several implications. They provide clear evidence for the presence of thermotolerant bacteria in the environment, which only assert themselves under conditions where they have a selective advantage. One can conclude that this particular ecosystem has a potential for relatively rapid adaptation, as far as bacterially mediated lignocellulose mineralisation is concerned. However, possible secondary effects, such as increased mineralisation rates at higher temperatures, higher oxygen consumption rates, and changes in the physico-chemical properties in the environment, should not be overlooked. Physico-chemical properties that are particularly affected by temperature are solubility and diffusivity. This clearly shows the sensitivity of natural ecosystems to temperature perturbations and the complexity of their response.

Enforceable legislation for the control of thermally polluted discharges into natural surface waters is therefore necessary for minimizing negative environmental effects. A particularly good example of such legislation exists in Switzerland (Verordnung über Abwassereinleitungen 1975), where water quality criteria for surface waters define both a maximum temperature increase of 3°C degrees and an upper maximum temperature for the receiving water of 25°C. The reference temperature is that in the unimpacted water body. Additionally, no water discharge into surface waters must exceed 30°C and when cooling water is discharged into a lake, it must not change the natural mixing patterns and nutrient status of the lake. In adopting this formulation for definition of the reference state, an equal degree of protection is afforded to every surface water system in the country, irrespective of size.

2.9 REFERENCES


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3. MATHEMATICAL MODELS DESCRIBING THE SPECIFIC GROWTH RATE OF BACTERIA AT GROWTH PERMISSIBLE TEMPERATURES

3.1 INTRODUCTION

The first description of the relationship between rates of biological processes and temperature, based on the earlier published Arrhenius equation (Arrhenius 1889), which describes the influence of temperature on chemical reaction rates, was provided by Snyder (1907, cited in: Belehradek 1926). The effect of temperature on the specific growth rate of bacteria was then described by;

\[ \mu = A \cdot \exp(-\Delta E_a / R \cdot T) \]  

(3.1)

where \( \mu \) is the specific growth rate, \( A \), a frequency factor, \( \Delta E_a \), the activation enthalpy of the growth reaction, \( R \), the universal gas constant and \( T \), the absolute temperature. The major deficiency in such an approach is that equation (3.1) only applies to part of the growth permissible temperature range, i.e., some 10 - 20 C degrees in the suboptimum range were \( \mu \) increases with \( T \). Significant deviations from Arrhenius type behaviour first become evident at low temperatures and in the optimum temperature range, as a result of the highly complex nature of the biochemical processes responsible for growth at the lower and upper limits. Because bacterial growth is dependent on the coordinated interaction of a network of reactions mediated by enzymes and dependent on cellular structural elements which have different sensitivities to temperature, phenomena such as thermal inactivation and thermal modification need to be taken into account. A particular example where the maximum growth temperature is controlled by activity loss of a single biosynthetic enzyme is the reversible inactivation of homoserine trans-succinylase, which is essential for the synthesis of methionine in Escherichia coli (Ron and Shani 1971). However, the decrease of the specific growth rate at temperatures above the optimum for growth might not necessarily be due to activity changes of a single enzyme. In order to quantitatively describe the relationship between temperature and the specific growth rate of bacteria in both the optimum and superoptimum temperature ranges it is necessary to construct a mathematical model which takes temperature mediated inactivation processes into account. Since description of the growth of bacterial cultures is usually at the macroscopic level rather than at either the microscopic or molecular levels, unsegregated models are most frequently used. Further, most unsegregated models are also unstructured. The
establishment of realistic unsegregated structured models that mechanistically describe the effect of temperature on those aspects of bacterial physiology that determine growth requires experimental data at the microscopic and molecular levels that have yet to become available.

In this chapter, three existing unstructured unsegregated models that describe the effects of temperature on bacterial growth throughout the entire growth permissible temperature range are evaluated with respect to their applicability.

### 3.2 MATHEMATICAL MODELS

a) The Esener Model

In order to account for thermal inactivation, Esener et al. (1981) have modified the basic Arrhenius relationship. They assume that although bacterial growth is the result of a number of enzymic reactions, one specific enzyme, which can be present in either an active or an inactive form, determines the overall growth rate. By introducing such an activation/inactivation concept they were able to extend equation (3.1) with an expression that describes the active fraction of the growth limiting enzyme in terms of temperature dependence resulting in:

\[
\mu_{\text{max}} = \frac{A' \cdot \exp(-\Delta E_a/R*T)}{1 + K \cdot \exp(-\Delta E_i/R*T)} 
\]

(3.2)

where \( A' \) and \( K \) are constants, \( \Delta E_a \) is the activation enthalpy of the growth limiting enzymatic reaction and \( \Delta E_i \) the enthalpy change for the enzyme inactivation reaction. This equation results in a bell shaped curve for the relationship between the maximum specific growth rate and temperature, which asymptotically approaches the abscissa at both its limits.

b) The Fiolitakis Model

A different interpretation of the rapid decline of the specific growth rate at superoptimum temperatures has been proposed by Fiolitakis et al. (1987). In addition to thermal enzyme inactivation, repair processes that result in partial restoration of activity have been taken into account. Based on this, equation (3.1) was extended by an activity state variable, \( V \), resulting in:
\[ \mu = V \cdot \exp[K_0 \cdot \Delta E_a / (R \cdot (\theta + 273.16))] \]  \hspace{1cm} (3.3)

The activity state variable, \( V \), depends on the rate of damage, \( \sigma \), which is given in dimensionless form by equation (3.4);

\[ \sigma = \frac{1}{1 + \exp(\alpha_3 - \alpha_2 \cdot \theta)} \]  \hspace{1cm} (3.4)

where \( \alpha_2 \) and \( \alpha_3 \) are damage parameters and \( \theta \), the temperature. To account for repair, a repair rate, \( \rho \), expressed in terms of \( V \), can be expressed as;

\[ \rho = V \cdot \exp(-\beta_2 \cdot V) \]  \hspace{1cm} (3.5)

where \( \beta_2 \) is a repair parameter. It is assumed that microorganisms compensate for higher damage rates by lowering \( V \) such that at any steady state the condition \( \sigma = \rho \) is valid. The boundary conditions are given by:

i) If \( \sigma > \rho_{\text{max}} \) with \( \rho_{\text{max}} = 1/\left(e^{\beta_2}\right) \), then \( V = 0 \)

ii) If \( \sigma < \rho_{\text{min}} \) with \( \rho_{\text{min}} = \beta_3 \cdot \exp(-\beta_2 \cdot \beta_3) \), then \( V = \beta_3 = \text{constant} \)

The curve resulting from equation (3.3) approaches the abscissa asymptotically at suboptimum temperatures, whilst at superoptimum temperatures a point of no return in terms of repair can be found, such that the curve intersects the abscissa and the specific growth rate becomes zero.

c) The Ratkowsky Model

An alternative approach, proposed by Ratkowsky et al. (1982), involves a square root relationship between the maximum specific growth rate \( \mu \) and temperature \( T \).

\[ \sqrt{\mu} = b \cdot (T - T_{\text{min}}) \]  \hspace{1cm} (3.6)

In equation (3.6), \( b \) represents a regression coefficient, \( T \), the absolute temperature and \( T_{\text{min}} \), a theoretical minimum temperature for growth. In order to extend the application of equation (3.6) to the optimum and superoptimum temperature ranges for growth, Ratkowsky et al. (1983) modified equation (3.6) so that;
\[ \sqrt{\mu} = b(T - T_{\text{min}})(1 - \exp(c(T - T_{\text{max}}))) \]  

(3.7)

where \( c \) represents an additional regression coefficient and \( T_{\text{max}} \) is the theoretical maximum temperature for growth. The curve resulting from equation (3.7) is a parabola which intersects the abscissa at \( T_{\text{min}} \) and \( T_{\text{max}} \), respectively. More recently, McMeekin et al. (1987) have pointed out that equation (3.6) is a special case of a temperature relationship proposed by Belehradec (1935, cited in: McMeekin et al. 1987) for describing the relationship between the rate of development of a particular organism and temperature. A link was also shown to exist between equation (3.6) and equation (3.1) by McMeekin et al. (1987).

3.3 MATERIALS AND METHODS

Organisms: Klebsiella pneumoniae NCIB 418 and a thermotolerant Gram negative coccobacillus, NA17, isolated and characterized by Al-Awadhi (1989).

Growth conditions: K. pneumoniae was grown in batch culture in magnetically stirred flasks containing 200 ml of a mineral salts medium (Evans et al. 1970), modified by replacing citric acid with 55 mg l\(^{-1}\) Na\(_2\)EDTA. This medium was either supplemented with 1 g l\(^{-1}\) glucose (minimal medium) or with 0.5 g l\(^{-1}\) glucose, 2.5 g l\(^{-1}\) yeast extract and 5 g l\(^{-1}\) tryptic soy broth (complex medium). The medium was buffered at pH 6.8 with a Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) mixture (0.1 M for PO\(_4^{3-}\)). The growth temperatures in the minimal medium were 20, 25.1, 30, 35.5, 35.8, 38, 40.5, 41.8, 42.8, 44.7 and 46 °C, whilst those in the complex medium were 20, 25.1, 30, 35.5, 38, 38.7, 39.5, 40.8, 42.8, 46, 47.4 and 48 °C. NA17 was grown in batch culture in a bioreactor (Bioengineering AG, Wald, Switzerland) containing 2 l of a synthetic medium as described by Al-Awadhi et al. (1988), with 1 g l\(^{-1}\) ethanol as the carbon energy substrate. The impeller speed was controlled at 800 r.p.m., the aeration rate was 35 l h\(^{-1}\) and the pH was maintained constant at 6.8 by controlled addition of either an equimolar 1M NaOH/KOH mixture or a 10% (w/w) solution of H\(_3\)PO\(_4\). The growth temperatures used were 35, 40, 45, 50, 55, 57, 59 and 60°C.

Growth was determined from the increase in optical density measured at 546 nm. All the experiments were carried out in duplicate and data points represent mean values of the calculated specific growth rates.

Data for E. coli were taken from Herendeen et al. (1979) and for the thermotolerant methylotrophic Bacillus sp. NCIB 12522 from Al-Awadhi et al. (1988, 1990).
Best fit curves for the Esener and Ratkowsky models with respect to the experimental data were obtained by non-linear regression using the Marquardt-Levenberg algorithm. The values for V in the Fiolitakis model were obtained by solving the equation σ = ρ, using an algorithm that combines linear interpolation, inverse quadratic interpolation and bisection (Program: ZBREN/DZBREN by IMSL Inc. Math/library). Best fit curves for the experimental data were then obtained by applying the polytope algorithm (Program: UMPOL/DUMPOL by IMSL Inc. Math/library).

3.4 RESULTS

The description of the relationship between specific growth rate and temperature, with special reference to the optimum and superoptimum temperature ranges for growth, has been investigated for the three models by comparison with growth data for four different bacteria (Figure 3.1, 3.2, 3.3 and 3.4). The corresponding model parameters are given in Table 3.1, 3.2, 3.3 and 3.4.

In Figure 3.1A experimental data for K. pneumoniae grown in a glucose mineral salts medium and a complex medium are presented and compared with best fit curves according to the Esener model. From the experimental data it is evident that, in the nutrient rich complex medium, growth was faster at corresponding temperatures and both the

<table>
<thead>
<tr>
<th></th>
<th>A' [h⁻¹]</th>
<th>K [-]</th>
<th>ΔE_a [KJ mol⁻¹]</th>
<th>ΔE_i [KJ mol⁻¹]</th>
<th>T_opt [K]</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pneumoniae</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCIB 418 (Glucose)</td>
<td>2.83*10¹¹</td>
<td>3.04*10⁵⁵</td>
<td>66.40</td>
<td>331.13</td>
<td>308.4</td>
</tr>
<tr>
<td>NCIB 418 (Complex)</td>
<td>2.67*10¹¹</td>
<td>4.00*10⁵⁵</td>
<td>65.80</td>
<td>335.55</td>
<td>311.8</td>
</tr>
<tr>
<td>E. coli NC3</td>
<td>5.80*10¹³</td>
<td>1.51*10⁵⁰</td>
<td>79.80</td>
<td>302.54</td>
<td>312.1</td>
</tr>
<tr>
<td>NA17</td>
<td>1.90*10¹⁰</td>
<td>4.67*10⁵³</td>
<td>64.91</td>
<td>336.41</td>
<td>323.6</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>1.05*10¹⁰</td>
<td>1.66*10⁵⁴</td>
<td>62.28</td>
<td>340.60</td>
<td>324.2</td>
</tr>
</tbody>
</table>
optimum and maximum temperatures were higher than in minimal medium. Comparison of the experimental data with curves generated from the model indicate phenomenological correspondence for the Arrhenius and optimum temperature ranges. Under both growth conditions sufficient agreement is also observed in the lower part of the superoptimum temperature range. However, at superoptimum temperatures approaching the maximum temperature for growth, the model deviates from the experimental data, because it approaches the abscissa asymptotically. For *E. coli* (Figure 3.1B) and the thermotolerant coccobacillus NA17 (Figure 3.1C) essentially similar patterns between the model and the experimental data emerged. However, in the case of NA17 correspondence in the lower superoptimum temperature range was somewhat less good. For the thermotolerant *Bacillus* sp. NCIB 12522 (Figure 3.1D) the unusually broad temperature optimum could not be
Figure 3.2 Experimental data for *Klebsiella pneumoniae* NCIB 418 grown in glucose minimal medium and fitted curves according to the Fiolitakis model. The different curves were obtained by changing the initial parameter values for the fitting procedure (see Table 3.2).

Table 3.2 Model parameters, optimum and maximum temperatures obtained for the fitted curves (see Figure 3.2) according to the Fiolitakis model. The initial parameter values for each fit are given in brackets.

<table>
<thead>
<tr>
<th>K. pneumoniae NCIB 418 (Glucose)</th>
<th>$\alpha_2$ $[\text{[K}^{-1}\text{]}$</th>
<th>$\alpha_3$ [-]</th>
<th>$\beta_2$ [-]</th>
<th>$\beta_3$ [$\text{[ln h}^{-1}\text{]}$]</th>
<th>$K_0$ [$\text{[KJmol}^{-1}\text{]}$]</th>
<th>$\Delta E_a$</th>
<th>Sum of squares of dev.</th>
<th>$T_{\text{opt}}$ [$^{\circ}\text{C}$]</th>
<th>$T_{\text{max}}$ [$^{\circ}\text{C}$]</th>
</tr>
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<tbody>
<tr>
<td>(-----)</td>
<td>4.17 179.48 0.50</td>
<td>56.27</td>
<td>17.25</td>
<td>53.97</td>
<td>0.059</td>
<td>37.3</td>
<td>43.4</td>
<td></td>
<td></td>
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<tr>
<td>(1.8) (62.0) (0.5) (20.0) (20.0) (60.0) (6.53)</td>
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<td></td>
<td></td>
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<tr>
<td>(- - - - )</td>
<td>1.24 52.85 0.69</td>
<td>13.48</td>
<td>18.65</td>
<td>53.90</td>
<td>0.059</td>
<td>37.2</td>
<td>42.9</td>
<td></td>
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<tr>
<td>(1.5) (62.0) (0.5) (20.0) (20.0) (60.0) (2.12)</td>
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<td></td>
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<tr>
<td>(-------)</td>
<td>2.15 92.09 0.69</td>
<td>55.23</td>
<td>38.15</td>
<td>105.14</td>
<td>0.052</td>
<td>36.3</td>
<td>42.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.8) (62.0) (0.5) (20.0) (20.0) (60.0) (5.84)</td>
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</table>
Table 3.3 Model parameters, optimum and maximum temperatures obtained for the fitted curves (see Figure 3.3) according to the Fiolitakis model.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha_2$ [K$^{-1}$]</th>
<th>$\alpha_3$ [-]</th>
<th>$\beta_2$ [-]</th>
<th>$\beta_3$ [-]</th>
<th>$K_0$ [inh$^{-1}$]</th>
<th>$\Delta E_a$ [KJmol$^{-1}$]</th>
<th>$T_{opt}$ [°C]</th>
<th>$T_{max}$ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> NCIB 418</td>
<td>3.05</td>
<td>145.23</td>
<td>0.39</td>
<td>75.59</td>
<td>15.55</td>
<td>49.612</td>
<td>39.3</td>
<td>48.5</td>
</tr>
<tr>
<td>(Complex)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>E. coli</em> NC3</td>
<td>1.51</td>
<td>69.88</td>
<td>0.40</td>
<td>123.01</td>
<td>37.55</td>
<td>105.23</td>
<td>40.3</td>
<td>48.0</td>
</tr>
<tr>
<td>NA17</td>
<td>0.85</td>
<td>48.79</td>
<td>0.48</td>
<td>96.11</td>
<td>38.68</td>
<td>113.63</td>
<td>52.0</td>
<td>59.0</td>
</tr>
<tr>
<td><em>Bacillus sp.</em> NCIB 12522</td>
<td>1.96</td>
<td>115.06</td>
<td>0.26</td>
<td>61.73</td>
<td>13.89</td>
<td>49.11</td>
<td>52.7</td>
<td>(-)</td>
</tr>
</tbody>
</table>

accommodated by the model. The model parameters for the fitted curves are listed in Table 3.1. During fitting it was observed that the values of the final parameters were dependent on the initial parameter estimates, used to start the optimization routine. Convergence during the iteration procedure was frequently not obtained and variations of up to plus or minus one to two orders of magnitude were observed for the parameters $A'$ and $K$, whilst $\Delta E_a$ varied ± ca. 20% and $\Delta E_t$ within ± ca. 10%.

The best fit curves obtained, using the Fiolitakis model, are compared with the experimental data in Figure 3.2 and 3.3 and the corresponding model parameters are presented in Table 3.2 and 3.3. As has been already observed by applying the Esener model, the final parameter values obtained were markedly dependent on the initial parameter estimates. This behaviour is illustrated in Table 3.2 and it is evident, that by changing only one single parameter and keeping the remaining parameters constant, markedly different final parameter values result. However, the quality of the fit between the model curves and the experimental data, expressed as the sum of the squares of the deviations of the calculated curve from the experimental data points, were in the same range. Two essentially different types of curves result from the parameter estimates (Figure 3.2). Whereas one curve type gives a peak at the optimum temperature, the other curve exhibits a rounded shape around the temperature optimum. This behaviour can be explained by the fact that in the latter case the condition $V = \beta_3$ does not coincide with the temperature optimum. It is noticeable that the two peak forming curves are almost identical, although the damage and repair parameters are markedly different. The values for $K_0$ and $\Delta E_A$ remain essentially constant, but are entirely different from the estimates that apply to the rounded curve.
Figure 3.3 Experimental data and fitted curves according to the Fiolitakis model: (A) *Klebsiella pneumoniae* NCIB 418, grown in complex medium; (B) *Escherichia coli* NC3, grown in complex medium; (C) NA17, grown in a mineral salt medium with ethanol as carbon energy source; (D) *Bacillus* sp. NCIB 12522, grown in a mineral salt medium with methanol as carbon energy source.

In contrast to the Esener model, a maximum growth temperature can be described by the Fiolitakis model. The model curves decline precipitously to zero, when the damage rate, $\sigma$, equals the maximum possible repair rate, $p_{\text{max}}$ (Figure 3.3A to 3.3C). However, in Figure 3.3D the criterion $\sigma = p_{\text{max}}$ is not fulfilled, since $p_{\text{max}} > 1$, and $\sigma$ can only attain a maximum value of 1, so that damage will never equal $p_{\text{max}}$ and the curve will never intersect with the abscissa.

A comparison of experimental data with the best fit curves obtained from the Ratkowsky model is presented in Figure 3.4, where good correspondence between the model and the experimental data were obtained throughout the permissible growth range. In contrast to the Esener model, the Ratkowsky model results in curves that intersect the abscissa,
Figure 3.4 Experimental data and fitted curves according to the Ratkowsky model: (A) *Klebsiella pneumoniae* NCIB 418, grown in glucose minimal medium (●) and in complex medium (○); (B) *Escherichia coli* NC3, grown in complex medium; (C) NA17, grown in a mineral salts medium with ethanol as carbon energy source; (D) *Bacillus* sp. NCIB 12522, grown in a mineral salt medium with methanol as carbon energy source.

thereby defining both, a theoretical maximum and a theoretical minimum temperature for growth. The model parameters are given in Table 3.4. In all cases good convergence during fitting was obtained and parameter variations were negligible.

The optimum and, where possible, the maximum growth temperatures obtained from the different models are essentially coincident. In the case of the Esener model maximum temperatures could not be obtained.
Table 3.4 Model parameters obtained for the fitted curves (see Figure 3.4) according to the Ratkowsky model. The optimum temperatures were calculated by setting the first derivative of the model equation (3.7) to zero.

<table>
<thead>
<tr>
<th></th>
<th>( b ) [-]</th>
<th>( c ) [-]</th>
<th>( T_{\text{min}} ) [K]</th>
<th>( T_{\text{max}} ) [K]</th>
<th>( T_{\text{opt}} ) [K]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> NCIB 418 (Glucose)</td>
<td>0.063</td>
<td>0.119</td>
<td>284.1</td>
<td>319.2</td>
<td>309.9</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> NCIB 418 (Complex)</td>
<td>0.041</td>
<td>0.282</td>
<td>275.3</td>
<td>321.8</td>
<td>313.1</td>
</tr>
<tr>
<td><em>E. coli</em> NC3</td>
<td>0.041</td>
<td>0.271</td>
<td>277.2</td>
<td>322.4</td>
<td>313.5</td>
</tr>
<tr>
<td>NA17</td>
<td>0.025</td>
<td>0.291</td>
<td>292.4</td>
<td>332.9</td>
<td>324.9</td>
</tr>
<tr>
<td><em>Bacillus sp.</em> NCIB 12522</td>
<td>0.031</td>
<td>0.198</td>
<td>291.7</td>
<td>334.6</td>
<td>324.4</td>
</tr>
</tbody>
</table>

3.5 DISCUSSION AND CONCLUSIONS

The three models that have been considered differ in the philosophies adopted in their formulation. Whereas the Esener and the Fiolitakis models are mechanistic, the Ratkowsky model is essentially empirical. Further, the capacity of the last two models to describe microbial growth in the superoptimum temperature range is superior to that of the Esener model. The reason for this is that the Esener model implies an asymptotic approach to the abscissa. However, applications of this model in the literature (Esener et al. 1981, Saucedo-Castaneda et al. 1990) do not mention this deficiency and omit that portion of the curve where deviation is evident. The mechanistic basis for the Esener model is that one enzyme, present in equilibrium between its active and inactive forms, controls growth, and that enzyme deactivation, predominantly in the higher superoptimum temperature range, is quantified by thermodynamic considerations (Roels 1983). However, progressive deviation of model curves from experimental data at higher superoptimum temperatures clearly reflect the insufficiency of this oversimplified single enzyme approach.

An improvement from the mechanistic point of view was provided in the Fiolitakis model by the introduction of an activity state variable, which becomes zero, when repair processes cannot compensate for temperature induced damage. In contrast to the Esener
model, sufficient phenomenological correspondence between experimental data and model curves could be obtained in most cases throughout the superoptimum temperature range. However, a special case was observed where the model curve did not describe a maximum growth temperature. This was essentially due to an incomplete definition of the boundary conditions for $\beta_2$ which should exceed $e^{-1}$ in its value. Parameter estimations by both the Esener and the Fiolitakis models resulted in considerable variations which restrict the value of such models, particularly when they are used to estimate activation/inactivation energies. Further, the number of experimental data points and their distribution over the temperature range have a marked effect on such estimations.

Curves resulting from the Ratkowsky model were in agreement with experimental data over the entire temperature range for growth. The introduction of a theoretical maximum temperature, $T_{\text{max}}$, as a model parameter defines the upper limit for growth and coincides with the experimental observations. However, Ratkowsky et al. (1983) have emphasized the need for comprehensive data in the superoptimum temperature range when maximum growth temperature is to be accurately estimated. The calculated values obtained in the present study correspond with observed experimental values within 1 to 2 C degrees, thereby indicating the utility of the Ratkowsky model. Another interesting feature of the Ratkowsky model has been reported by McMeekin et al. (1987) who investigated the combined effect of reduced water activity, enhanced sodium chloride concentration and temperature on a halotolerant Staphylococcus sp. They found a linear relationship between $b^2$, where $b$ is defined in equation 3.6, and water activity, thereby allowing incorporation of water activity effects in the model.

Although a mechanistic basis for a mathematical model should be preferred to a non-mechanistic mathematical formulation, it is clear that the empirical Ratkowsky model has certain advantages over the mechanistic Esener and Fiolitakis models. For the Ratkowsky model, the values obtained for $T_{\text{min}}$ and $T_{\text{max}}$ can be considered as constants for any bacterium in a given medium, whilst the activation and inactivation energies in the Esener model and the activation energy in the Fiolitakis model are clearly functions of temperature. Even so, the estimation of optimum growth temperatures can be satisfactorily conducted with all three models. Such values correspond within 1 to 2 C degrees.

3.6 REFERENCES


4. SOME EFFECTS OF HEAT SHOCKS ON BACTERIAL GROWTH

4.1 INTRODUCTION

The widespread introduction of well instrumented laboratory scale bioreactors for the investigation of the growth of and product formation by bacteria has permitted the collection of process data under controlled operating conditions, i.e., constant temperature, pH and dissolved oxygen concentrations. Such studies allow the determination of optimum process conditions. However, in large technical scale bioreactors that are used either for the manufacture of bacterial products or wastewater treatment, the scale and mode of operation frequently mean that operating conditions are variable throughout the bioreactor. Hence, when considering process scale up, it is important to establish guide-lines that indicate the impact of changing operating conditions on the performance of process cultures.

It is generally agreed that bacteria exhibit relatively narrow temperature optima, usually between 3 and 6°C degrees. Aerobic growth of bacteria results in the production of large quantities of low grade heat which depends on the energy content of the carbon energy substrate utilized, on the degree of optimization of the yield coefficient achieved and on process intensity. When bioreactors are scaled up on the basis of geometrical similarity, the volume increases as the cube of a characteristic linear dimension whilst the surface area increases only as the square of the characteristic linear dimension. Therefore, with increasing scale, the surface area available for heat transfer reduces relative to volume and ultimately becomes insufficient to allow easy maintenance of the process temperature within the optimum range if similar levels of process intensity are to be maintained during large scale operation as can be maintained in the laboratory. Under such operating conditions, process performance can be adversely affected (Benschoter and Ingram 1986).

The degree will depend on mixing times and segregation phenomena in large scale bioreactors and, hence, the period of exposure of the process culture to superoptimum temperatures. Large scale loop type bioreactors will be particularly affected in this way, because of the relatively long mixing times applicable in such bioreactors (Schügerl and Sittig 1984).

In many large scale bioreactor designs, external cooling loops are now employed for temperature control. When the process culture passes through such loops it is subjected to a significant temperature gradient that could have kinetic and physiological consequences.
Because of the very extensive kinetic and physiological data available for *Klebsiella pneumoniae*, the effects of heat shocks on culture performance were studied using this bacterium growing on glucose in a defined mineral medium.

### 4.2 MATERIALS AND METHODS

Organism and culture maintenance: *Klebsiella pneumoniae* NICB 418 was maintained frozen in ampoules in liquid nitrogen for long term storage and prior to use maintained on plate count agar slopes (Difco Inc., Detroit, USA).

**Media:** *K. pneumoniae* was grown in a bioreactor in a defined mineral salts medium (Evans *et al.* 1970) modified by replacing citric acid with 55 mg l\(^{-1}\) EDTA-Na\(_2\). 2 g l\(^{-1}\) glucose were used as carbon energy substrate. As antifoam agent, 20 mg l\(^{-1}\) polypropylene glycol was added to the medium.

**Bioreactor system:** The bioreactor used was a 2.5 l total volume mini-bioreactor (MBR Bio Reactor AG, Wetzikon, CH) fitted with temperature, impeller speed and pH control, dissolved oxygen monitoring and both pumped medium inlet and culture overflow were so as to allow operation in the continuous flow (chemostat) mode. Both the oxygen and the carbon dioxide content of the effluent gas stream from the bioreactor could be measured on line with paramagnetic and infrared gas analysers, respectively. For all cultivation experiments, the culture pH was maintained at 6.8 by automatic addition of either an equimolar 2N NaOH/KOH mixture or a 10 percent (w/w) solution of H\(_3\)PO\(_4\). The operating temperature of the bioreactor was maintained constant at 35°C. The operating volume was 1.75 l, the impeller speed used was 1000 rpm and the inlet air flow rate was 54 l h\(^{-1}\).

![Figure 4.1 Heat exchanger: C, culture compartment; H, heating compartment; P, Pt 100 resistance thermometer. Dimensions are given in millimeters.](image)
In order to heat shock the growing culture by exposure to either superoptimum or supermaximum temperatures for short time intervals, a part of the culture was circulated, at a fixed flow rate of 1.21 h⁻¹, through an external heat exchanger (Figure 4.1), which had a process-side volume of 23 ml. The temperature of the heat exchanger was controlled by a thermostated external heating circuit. The temperatures of the culture entering and leaving the heat exchanger were measured with Pt100 resistance thermometers. Samples of culture could be taken both from the bioreactor and immediately after the heat exchanger before remixing. The system layout is shown in Figure 4.2.

**Figure 4.2** Scheme for the bioreactor system: A, acid reservoir; B, alkali reservoir; BR, bioreactor; CO₂, infrared carbon dioxide analyzer; F, air flow meter; F₁, air inlet filter; F₂, air outlet filter; H, heat exchanger; M, motor; MT, medium reservoir; O₂, paramagnetic oxygen analyzer; P₁ to 5, peristaltic pumps; pH, pH control; pO₂, oxygen partial pressure measurement; R, condenser; SP₁ to 3, sampling points; T₁, temperature controller; T₂, temperature measurement; WT, harvest tank.
Analytical: All analyses were performed on triplicate samples.

Bacterial biomass dry weights: These were determined by direct weighing using 0.2 μm Nuclepore filters (Nuclepore Inc., Pleasanton, USA).

Activity of cultures: This was determined using the iodonitrotetrazolium chloride (INT) method of Zimmermann et al. (1978) and modified by Lopez et al. (1986) to allow photometric measurement of formazan formation, after extraction with dimethyl sulfoxide, at 460 nm using a Uvikon 860 spectrophotometer (Kontron AG, Zürich, CH). The incubation temperature for the activity assays was 35°C. INT activity correlates with dehydrogenase activity and, hence, is considered to be an indirect measure of culture viability.

Glucose: This was measured enzymatically using the GOD-Period® method (Boehringer Mannheim GmbH, Mannheim, D).

Dissolved organic carbon: This was measured in filtered (0.2 μm) culture supernatant, after acidification, with a Tocor 2 analyser (Maihak GmbH, Hamburg, D).

Soluble absorbing matter: This was measured in filtered (0.2 μm) culture supernatant at 260 nm with a Uvikon 860 spectrophotometer.

Acetate: This was measured in filtered (0.2 μm) culture supernatant using a Shimadzu GC-RIA gas chromatograph fitted with a FID detector and a GP carbopack C/0.3% carbowax 20M/0.1% H₃PO₄ glass column, temperature programmed 145°C/2 min rate 5°C min⁻¹.

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, D) and an infrared carbon dioxide analyser (Binos 1, Leybold-Heraeus GmbH, Hanau, D), respectively. Rates were then calculated on the basis of an inert gas balance.

Table 4.1 Heat exchanger inlet and outlet temperatures.

<table>
<thead>
<tr>
<th>Heat shock condition</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet temperature [°C]</td>
<td>34.8</td>
<td>40.2</td>
<td>44.3</td>
<td>46.6</td>
<td>49.2</td>
</tr>
<tr>
<td>Outlet temperature [°C]</td>
<td>35.1</td>
<td>44.7</td>
<td>50.0</td>
<td>53.8</td>
<td>63.5</td>
</tr>
</tbody>
</table>

Bioreactor conditions: For the experiments a dilution rate of 0.68 h⁻¹, corresponding to ca. 56 percent of the critical value, was used. The inlet medium and outlet culture flow rates
were 1.21 h\(^{-1}\) and the flow rate through the heat exchanger was maintained constant at 1.21 h\(^{-1}\) such that the exposure time of the culture to heat was 1.15 min per passage. Heat shocks were imposed on the culture by subjecting it to elevated temperatures during passage through the heat exchanger. The inlet and outlet temperatures for the culture passing through the heat exchanger under the series of operating conditions employed are given in Table 4.1.

4.3 RESULTS

In Figure 4.3, the steady state dry bacterial biomass concentrations in the bioreactor during the different heat shock conditions, together with the corresponding inlet carbon energy substrate concentrations, are shown. Exposure of the culture to heat shock temperatures including and exceeding those for condition D, resulted in dense submerged wall growth in the bioreactor such that termination of the experiment became necessary.

![Figure 4.3](image)

**Figure 4.3** Steady state dry bacterial biomass concentrations (empty bars) and inlet medium substrate concentrations (hatched bars). Letters A to E represent the different heat shock conditions listed in Table 4.1.

In Figure 4.4, the specific INT activities of the suspended culture in the bioreactor and of the culture immediately after subjecting it to heat shock, before remixing, are given for the various conditions employed. The specific oxygen utilization and carbon dioxide production rates under each operating condition are given in Figure 4.5.
Figure 4.4 Specific INT dehydrogenase activity: Samples taken from the bioreactor (empty bars) and samples taken immediately after the heat exchanger (hatched bars). Letters A to E represent the different heat shock conditions listed in Table 4.1.

Figure 4.5 Specific respiration rates: Specific oxygen consumption rates (empty bars) and specific carbon dioxide production rates (hatched bars). Letters A to E represent the different heat shock conditions listed in Table 4.1.
Figure 4.6 Specific absorbance at 260 nm: Samples taken from the bioreactor (empty bars) and samples taken immediately after the heat exchanger (hatched bars). Letters A to E represent the different heat shock conditions listed in Table 4.1.

Figure 4.7 Specific dissolved organic carbon concentrations: Samples taken from the bioreactor (empty bars) and samples taken immediately after the heat exchanger (hatched bars). Letters A to E represent the different heat shock conditions listed in Table 4.1.
The specific absorbance results for soluble matter at 260 nm for each operating condition are given in Figure 4.6 for samples taken from both the bioreactor and the heat exchanger outlet prior to remixing. In Figure 4.7, the specific dissolved organic carbon concentrations present in the above mentioned samples are shown.

Acetate concentrations were consistently less than 10 mg l\(^{-1}\). Glucose utilization was complete under all conditions studied.

For growing cultures, heat shocks had no apparent effects either on the total biomass concentration (Figure 4.3) or on the specific respiration rates (Figure 4.5). Both the specific UV absorbing matter (Figure 4.6) and the specific dissolved organic carbon concentrations (Figure 4.7) in samples removed directly from the bioreactor were relatively constant except under the most severe heat shock condition E, when marked increases in both were observed. For the same samples, specific INT activity measurements gave corresponding results (Figure 4.4) with a significant decrease occurring only under heat shock condition E. In contrast, samples removed from the recirculation loop immediately after the heat exchanger exhibited markedly different results. In the case of both specific UV absorbing matter and specific dissolved organic carbon concentrations, a progressive increase occurred with increasing severity of heat shock (Figures 4.6 and 4.7), whilst the specific INT activity (Figure 4.4) showed a gradual decrease between condition A and D, but under condition E showed a precipitous decline to a value close to zero.

In Figures 4.8A and B, results are shown for the transient behaviour of culture density, residual glucose concentration and respiration rates during a temperature change in heat exchanger operation from condition A to condition E.

The recovery of a heat shocked culture, removed immediately after passage through the heat exchanger, under condition E, is compared with a non-heat shocked culture from condition A in Figures 4.9.

### 4.4 DISCUSSION

Most bacteria are unable to grow at temperatures > 10 C degrees above their maximum temperature for optimum growth. As far as growth at superoptimum temperatures is concerned, reduction in the observed specific growth rate constant with increasing temperature has been studied by Topiwala and Sinclair (1971). Although their results predict maximum temperatures for the growth of particular bacterial strains, the mechanisms responsible for the cessation of growth are not discussed. From the mechanistic viewpoint, Bergter (1983) has suggested that the maximum temperature at
Figure 4.8 Transient course of the culture density (Ο) and residual glucose concentration (Δ), (A); temperature profile of the heat exchanger inlet (——) and outlet (---) temperatures, (B); oxygen uptake rate (Ο) and carbon dioxide production (Δ) rate, (C).
which the growth of a particular bacterial strain is possible is the maximum temperature at which the cellular regulation system can compensate for temperature induced perturbations. Much earlier, Hedén and Wyckoff (1949) identified 50°C as the transition temperature between reversible and irreversible heat damage to the cell membrane of *Escherichia coli*, whilst Russell and Harries (1967) have shown that "short" exposure of washed suspensions of *E. coli* to temperatures >40°C results in increasing leakage of UV absorbing soluble matter from the cells, although they reported that it was only above 50°C that such leakage became significant.

![Graph](image_url)

**Figure 4.9** Recovery patterns of inocula of *K. pneumoniae* removed directly after temperature shocks from the heat exchanger loop. The heat exchanger outlet temperatures were 35.1°C (O) and 63.5 °C (Δ).

Short time heat shocking of growing cultures is expected to result in cell damage which will be a function of temperature, exposure time, strain, medium composition and the physiological status of the culture prior to heat shocking. As far as viability is concerned, cell damage manifests itself as either a reversible or an irreversible loss of viability when heat shocked cultures are returned to growth permissible temperatures. In the case of reversible damage, the concept of recovery time is frequently introduced, but does not explain the mechanisms involved in reacquisition of viability.
It is clear that heat shocks result in both the leakage of intracellular matter from the cells and a reduction in specific INT activity as shown from the analyses of samples removed from the recirculation loop. The results for samples removed from the bioreactor under heat shock conditions A through D suggest that recovery of the cells after returning to conditions suitable for optimum growth probably occurs and that the recovery time is relatively short. Further, these results also suggest that the cytoplasmic matter that leaks into the culture supernatant under such conditions is reutilized for cryptic growth, a phenomenon that has been shown for *K. pneumoniae* (Mason and Hamer 1987). However, the existence of a critical level of heat shock, above which recovery is severely restricted, is also evident. Under operating conditions where severe heat shock occurs, E, the marked reduction in the INT activity of the culture in the bioreactor suggests that a significant fraction of the cells in the bioreactor is, in fact, non-viable. This observation seems to be inconsistent with the essentially unchanged concentration of bacterial biomass present in the bioreactor, but can be explained by the effects of submerged wall growth (Topiwala and Hamer 1971; Wilkinson and Hamer 1974).

In experiments conducted under the severe heat shock condition, E, with *K. pneumoniae*, significant submerged wall growth was observed in the bioreactor. The results in Figure 4.9, clearly show that the recovery time of the suspended heat damaged cells exceeded the mean hydraulic residence time in the bioreactor. Under such operating conditions the rate of glucose consumption by suspended cells was markedly reduced, thereby leading to an increased availability of glucose for any cells that were attached to the walls of the bioreactor. Attached cells are subjected neither to heat shock nor to wash out. When significant submerged wall growth occurs, the attached cell biomass interaction with the suspended culture reaches a state of pseudo-equilibrium such that the quantity of attached active cell biomass remains essentially constant and any excess cells produced as a result of growth are discharged into the bulk liquid culture (Hamer 1973), thereby enhancing the fraction of viable cells in suspension. The continued existence of apparently non-viable cells in the bioreactor, in spite of wash out, results from the passage of viable cells discharged from the attached population through the heat exchanger, thereby subjecting them to serious damage. Wall growth effects, although particularly pronounced in laboratory scale bioreactors, will diminish in geometrically similar large scale bioreactors, where they are largely insignificant. This is in contrast to the effect of surface area:volume ratios on heat transfer capacity.

An apparent inconsistency in the results is the marginally enhanced specific respiration rate observed under severe heat shock condition E. However, this is artificial, because the specific respiration rates reported are based on the total (viable plus non-viable) suspended
cell biomass concentration and do not reflect the significant contribution of the attached biomass.

4.5 CONCLUSIONS

The tolerance of *K. pneumoniae* to heat shocks, as measured by recovery capacity, is considerable. Heat shocks at temperatures below the absolute maximum temperature for growth, i.e., that measured in complex media, are insignificant as far as cellular damage is concerned. This suggests that in large industrial scale bioreactors this bacterium is unlikely to suffer from major changes in its process performance as a result of moderate overheating.

However, generalizations about the possible impact and response of short term heat shocks on bacterial cultures should be undertaken with care. Data derived from a broad spectrum of potential process cultures are necessary.

4.6 REFERENCES


5. RECOVERY OF EXPONENTIALLY GROWING CULTURES OF Klebsiella pneumoniae NCIB 418 AFTER HEAT SHOKS

5.1 INTRODUCTION

The development of a better understanding of the response of bacteria to short term heat shocks at temperatures above their optimum for growth and product formation has recently gained in importance, because temperature inducible expression systems for the controlled expression of genes in recombinant bacteria are finding increasing application as alternatives to certain metabolite induced expression systems (Anderson Da Silva and Bailey, 1989). One such system involves the temperature-sensitive λ repressor cI857 with either a \( \lambda P_L \) or \( P_R \)-promoter, where, to obtain high degrees of expression, bacteria have to be subjected to a temperature shift to and maintenance at 42°C (Lastick et al., 1986). Another system consists of a lac promoter carried on an att-nulL-p-att-N gene block which inverts when the bacteria into which this has been introduced are subjected to 42°C for 10 min, thereby causing expression of the recombinant gene (Podhajska et al., 1985). The latter heat shocking could be accomplished by passage of the bacteria through a heat exchanger and subsequent return to a favourable growth temperature, as opposed to prolonged retention of the bacteria at a superoptimum temperature that, clearly, is potentially injurious to them. The purpose of the work described here was to study the effects of short term heat shocks on the growth kinetics of a bacterial population during subsequent recovery.

5.2 METHODS

Organism and cultivation conditions: Klebsiella pneumoniae NCIB 418 was grown in batch culture in a bioreactor (Bioengineering AG, Wald, Switzerland) with an operating volume of 1.4 l. The temperature and the impeller speed were controlled at 35°C and 800 r.p.m., respectively. The pH was maintained constant at 6.8 by automatic addition of either an equimolar 2M NaOH/KOH mixture or a 10% (w/w) solution of H\(_3\)PO\(_4\). The aeration rate was 35 l h\(^{-1}\). The growth medium was a defined mineral salts medium (Evans et al. 1970) modified by replacing citric acid with 55 mg l\(^{-1}\) Na\(_2\)EDTA, whilst 2.5 g l\(^{-1}\) glucose served as the sole carbon energy source. The medium used for the heat treatment and recovery experiments contained 1 g l\(^{-1}\) glucose and was buffered with a Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) mixture (0.1 M for PO\(_4^{3-}\)) at pH 6.8.
Heat treatment: Cells were removed from the bioreactor at a predetermined optical density of 1.8, measured at 546 nm, i.e., during the mid-exponential growth phase. Aliquots of 2 ml were immediately transferred into preheated stirred flasks of 100 ml total volume containing 20 ml of culture medium in which the glucose concentration was identical with that in the bioreactor at the time of inoculum removal. The temperatures used for heat shocking were 42, 45, 46.5, 48 and 51°C. Exposure times were either 1, 3 or 5 minutes. For each series of experiments a control was processed at 35°C. For recovery, the whole content of each flask was transferred into a second stirred 500 ml flask containing 180 ml of the same medium maintained at 35°C.

Growth measurement: Samples were removed at either 6 or 12 minute intervals and growth was measured as the increase in the absorbance at 546 nm (OD) using an Uvikon 860 spectrophotometer (Kontron AG, Zurich, Switzerland). The apparent specific growth rate constant (µ) at \( t = 0.5(t_2 + t_1) \) was calculated according to the equation:

\[
\mu(t_n) = \frac{[\text{OD}(t_2) - \text{OD}(t_1)]}{[0.5(\text{OD}(t_1) + \text{OD}(t_2))(t_2 - t_1)]}
\]  

(5.1)

where \( t \) is the time of sample removal in hours and the subscripts 1 and 2 represent initial and final conditions.

All the experiments were carried out either in duplicate or in triplicate and data points represent mean values.

Plate counts: PCA plates were prepared with 2.35 g l\(^{-1}\) plate count agar (Difco, Detroit, USA) and 13.5 g l\(^{-1}\) Bacto agar (Difco, Detroit, USA) supplemented with 1 g l\(^{-1}\) glucose and buffered at pH 6.8 with Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) (0.05 M for PO\(_4^{3-}\)). BG plates were prepared by addition of 15 g l\(^{-1}\) Bacto Agar to the mineral medium used for growth experiments, supplemented with 2 g l\(^{-1}\) glucose and buffered at pH 6.8. After sample dilution and plating, colonies were counted after incubation at 35°C for 48 hours. The counts were carried out in quintuplicate. The results presented are mean values.

5.3 RESULTS

Exponentially growing cultures of *K. pneumoniae* were subjected to heat shocks at either superoptimum (42 and 45°C) or supermaximum (46.5, 48 and 51°C) temperatures, for the growth of the bacterium on glucose as its sole carbon energy substrate. Response patterns were temperature and exposure time dependent as shown in Figures 5.1, 5.2 and 5.3. Superoptimum heat shocks were either insignificant (42°C) or resulted in a transitory, slightly exposure time dependent decrease of the specific growth rate constant (45°C). In
Figure 5.1 Time course of the specific growth rate during recovery of *K. pneumoniae* cultures after heat shocks at 42°C (A) and 45°C (B). Exposure times were 0 (○), 1 (□), 3 (+) and 5 (☆) minutes. The initial specific growth rate constants of the cultures before the heat treatments were, for (A) and (B), 1.23 h⁻¹.
Figure 5.2 Time course of the specific growth rate during recovery of *K. pneumoniae* cultures after heat shocks at 46.5°C (A) and 48°C (B). Exposure times were 0 (○), 1 (□), 3 (+) and 5 (★) minutes. The initial specific growth rate constants of the cultures before the heat treatments were 1.22 h⁻¹ (A) and 1.20 h⁻¹ (B).
Figure 5.3 Time course of the specific growth rate during recovery of *K. pneumoniae* cultures after heat shocks at 51°C. Exposure times were 0 (○), 1 (□), 3 (+) and 5 (★) minutes. The initial specific growth rate constant of the culture before the heat treatment was 1.21 h⁻¹.

Table 5.1 Recovery times for *K. pneumoniae* cultures subjected to several heat shocks for different exposure times; for definition of recovery time see section 5.3.

<table>
<thead>
<tr>
<th>Heat shock temperature [°C]</th>
<th>Recovery time [h]</th>
<th>Exposure time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>42</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>45</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>46.5</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>48</td>
<td>1.3</td>
<td>3.3</td>
</tr>
<tr>
<td>51</td>
<td>5.5</td>
<td>18.3</td>
</tr>
</tbody>
</table>
Figure 5.2 the responses of *K. pneumoniae* to heat shocks which are clearly in the supermaximum range (46.5 and 48°C) for growth in a glucose minimal medium are shown. After a heat shock at 46.5°C growth continued immediately after return to the initial growth temperature of 35°C. At 48°C an initial lag phase where no growth occurred, was followed by an acceleration phase with respect to the growth rate constant. The response to a heat shock at 51°C is shown in Figure 5.3. The recovery times exhibited by *K. pneumoniae* to attain the initial specific growth rate of the control, after the several heat treatments, are given in Table 5.1. Recovery time was defined as the time required for a heat shocked culture to attain the initial growth rate constant of the control.

![Graph](image.png)

**Figure 5.4 Survival of *K. pneumoniae* after heat shocks at 45°C (○), 48°C (□) and 51°C (△) on two different agars: (A) plate count agar, (B) glucose agar.**

In Figure 5.4 the survival patterns of *K. pneumoniae* plated both on plate count agar and on a minimal glucose agar after different heat shocks are shown. Heat treatments at 45°C for all exposure times investigated allowed complete recovery on both agars. In contrast, discrepancies in colony counts occurred at 48°C. Whilst heat treatment for 5 minutes allowed essentially complete recovery on plate count agar, an exposure time dependent reduction in survival was observed on minimal glucose agar. At 51°C a reduction in survival on both agars was observed, but was most pronounced on the minimal glucose agar.
In order to predict recovery after a finite heat shock, it is necessary to consider the change in total biomass, \( x_t \), during recovery after a heat shock for a segregated bacterial population comprizing a viable fraction \( x_v \) and a non-viable fraction \( x_n \). At time \( t \) this is given by

\[
x_t(t) = x_n + x_v(t) \tag{5.2}
\]

The viable cells contribution to growth at time \( t \) is given by

\[
x_v(t) = x_v(t=0)e^{\mu t} \tag{5.3}
\]

where \( \mu \) is the specific growth rate constant of the viable fraction.

Combination of equation (5.2) and (5.3) results in:

\[
x_t(t) = x_n + x_v(t=0)e^{\mu t} \tag{5.4}
\]

For a small time interval \( (t_{n+1} - t_n) \), where \( t_n \) and \( t_{n+1} \) are initial and final times respectively, the apparent growth rate \( \mu_a \) at time \( t_a = 0.5 (t_{n+1} + t_n) \), is given by:

\[
\mu_a(t_a) = \frac{[x_t(t_{n+1}) - x_t(t_n)]}{[0.5(x_t(t_{n+1}) + x_t(t_n)) (t_{n+1} - t_n)]} \tag{5.5}
\]

Figure 5.5 shows the results of a calculation for the prediction of recovery from a 5 minute heat shock at 48°C assuming a segregated bacterial population that initially comprizes a viable cell fraction of 0.3 and a non-viable fraction of 0.7. The relative ratios were estimated on the basis of colony counts observed on glucose agar. Calculations were performed for two different growth rate constants of the viable cell fraction: 1.22 h\(^{-1}\), the maximum value at 35°C and 0.9 h\(^{-1}\), which corresponded to the initial growth rate constant of the control. Neither of the simulations resulted in either reasonable quantitative or qualitative representations of the experimental response.

5.4 DISCUSSION AND CONCLUSIONS

The exposure of exponentially growing cultures of \( K. pneumoniae \) to various heat shocks results in both transitory reductions in the specific growth rate constant and significant lag phases, depending on shock intensity. Possible bases for such responses are that at superoptimum temperatures bacterial growth rate is generally reduced (Esener et al. 1981)
Figure 5.5 Recovery of *K. pneumoniae* after a 5 minute heat shock at 48°C. Comparison between experimental data (*) and responses based on calculations assuming a segregated population consisting of an initially constant non-viable fraction of 0.7 and a viable portion of 0.3 which was able to grow at a constant specific rate of either 0.9 h⁻¹ (dashed curve) or 1.22 h⁻¹ (continuous curve).

and that step changes in growth temperature can result in pronounced time delays with respect to adjustment of the specific growth rate constant (Ryu and Mateles, 1968; Ingraham, 1987).

Since the maximum growth temperature for *K. pneumoniae* in minimal medium with glucose as sole carbon energy substrate is between 45 and 46°C, response patterns to the heat shocks were divided into two groups (Franks *et al.* 1980), i.e., those in the superoptimum temperature range where balanced growth is still possible and those in the supermaximum temperature range where the maximum growth temperature is exceeded and growth does not occur. Short term heat shocks in the superoptimum temperature range result in recovery patterns which suggest that bacterial injury was insignificant, thereby confirming earlier results for *K. pneumoniae* when cells from a chemostat were heat shocked by passage through a heat exchanger (Heitzer *et al.* 1989). Although short term heat shocks in the superoptimum temperature region do not seem to cause major damage such temperatures can significantly alter the levels of many enzymes (Herendeen *et al.* 1979). These latter changes can occur very rapidly. Yamamori and Yura (1980) reported
that the induction of synthesis of heat shock proteins in *E. coli* after a step increase from 30 to 42°C was initiated within 60 seconds. Such energy consuming metabolic alterations probably take place at the expense of the maximum specific growth rate, an explanation consistent with the response observed here after subjecting *K. pneumoniae* to a heat shock at 45°C.

Heat shocks at supermaximum temperatures resulted in much more pronounced effects that were strongly temperature stress and exposure time dependent. Colony counts on minimal glucose agar for heat shocks at 48°C showed significant reduction in survival, suggesting that a part of the culture lost its capacity to recover, leading to the conclusion that recovery patterns must be interpreted by segregation of the bacterial population into a viable fraction and a non-viable fraction (Hamer and Heitzer, 1990). Calculations conducted for a 5 minute heat shock at 48°C on this basis resulted in the prediction of much faster recovery than was, in fact, observed (Figure 5.4), suggesting that the viable cell fraction must also have temporally lost its capacity to grow at its maximum rate, probably due to reversible damage. Earlier, Takano and Tsuchido (1982) concluded that growth delays in exponentially growing cultures of *E. coli*, after heat stress at supermaximum temperatures, were partially due to an increase in reversible injury to the viable fraction. For recovery after a supermaximum temperature shock, Daniels *et al.* (1984) reported that it took > 2 hours for complete restoration of normal protein synthesis in *Halobacterium volcanii*.

In the present experiments, the apparently non-viable fraction of the bacterial population present after heat shocks at 48°C should not be considered as a dead fraction, since recovery was found to be complete on protein rich plate count agar, on which the maximum growth temperature for *K. pneumoniae* is between 47 and 48°C. In contrast, heat shocks at 51°C resulted in reduced viable counts on both agar media, indicating that this heat treatment was lethal to a fraction of the population. The previous history of bacteria before subjecting them to a heat shock can significantly affect recovery. Jenkins *et al.* (1988) cited prior starvation as enhancing thermal resistance relative to exponentially growing bacteria. Also prior treatment at a superoptimum temperature when subjecting bacteria to supermaximum heat shocks enhances survival (Van Bogelen *et al.*, 1987; Ramsey, 1988). Another important question advanced by van Uden and Madeira-Lopes (1976), concerns the contribution of a non-viable fraction in a microbial population to substrate consumption.

From the present study it is clear that the choice of an appropriate host organism, when a recombinant strain with a temperature regulated expression system is constructed, depends on both its optimum and its maximum temperature for growth, particularly when growth after heat shocking is required.
5.5 REFERENCES


6. SOME ASPECTS OF HEAT SHOCK-INDUCED EXPRESSION OF THE \textit{htpG} GENE IN BATCH CULTURES OF \textit{Escherichia coli}

6.1 INTRODUCTION

The effects of short-term heat shocks in the superoptimum and lower supermaximum temperature ranges for growth on exponentially growing cultures of \textit{Klebsiella pneumoniae} have been shown to result in transiently reduced growth rates (Heitzer and Hamer 1990). These growth delays were clearly dependent on the magnitude of and the exposure time to a heat shock. It was shown earlier that heat shocks at superoptimum temperatures alter overall protein synthesis patterns in \textit{Escherichia coli} (Yamamori et al. 1978) and result in the induction of a set of specific heat shock proteins in less than one minute (Yamamori and Yura 1980). These proteins are positively regulated by a specific control gene, \textit{rpoH} (Neidhardt and Van Bogelen 1981), which codes for a RNA polymerase sigma factor ($\sigma^{32}$) that enables selective recognition of the heat shock gene promoters (Cowling \textit{et al}. 1985).

The sum of the 17 heat shock proteins known for \textit{E. coli} represent ca. 5% of the cellular protein at 37°C (Neidhardt \textit{et al}. 1984) and 8-10% at 42°C, estimated from data reported by Herendeen \textit{et al}. (1979). Such energy consuming transient synthesis of proteins is, in addition to other processes, expected to take place at the expense of maximum growth rate. With respect to industrial applications of temperature inducible expression systems for the synthesis of recombinant proteins, Caulcott and Rhodes (1986) have pointed out that such energetic aspects should also be considered during maximization of product yield coefficients.

In order to investigate the existence of a correlation and possible relation between growth delays and heat shock protein induction, the expression of a representative heat shock gene, \textit{htpG}, was studied in \textit{E. coli}. Although this gene codes for a particularly conserved protein amongst eucaryotes and procaryotes (Bardwell and Craig 1987), representing ca. 0.25% of the total cellular protein at 37°C, its function in \textit{E. coli} has yet to be elucidated (Bardwell and Craig 1988).

Since most of the experiments concerning heat shock gene expression that have been carried out so far were performed with exponentially growing batch cultures in nutrient rich media it was also of particular interest to determine how both the growth phase and the growth medium affect the expression pattern of the \textit{htpG} gene during heat shock.
Organism: *Escherichia coli* JB23. This strain was kindly provided by Prof. Dr. E.A. Craig. It contains a chromosomal substitution deletion mutation where the coding region of the *htpG* gene has been replaced by the coding sequence of the *lacZ* gene in a Lac⁻ mutant. The construction of this translational *htpG-lacZ* gene fusion has been described by Bardwell and Craig (1988).

Growth conditions: *E. coli* JB23 was grown in batch culture in a bioreactor (MBR Bioreactor AG, Wetzikon, Switzerland) with an operating volume of 2 l. The growth medium used was a defined mineral salts medium (Evans *et al.* 1970), modified by replacing citric acid with 55 mg l⁻¹ Na₂EDTA. The medium was supplemented with either 2 g l⁻¹ glucose (minimal medium) or with 1 g l⁻¹ glucose, 1 g l⁻¹ yeast extract and 1 g l⁻¹ brain heart infusion (complex medium). The growth temperature and the impeller speed were controlled at 37°C and 800 r.p.m., respectively. The pH was maintained constant at 7.0 by the controlled addition of either an equimolar 1M NaOH/KOH solution or a 10% (w/w) solution of H₃PO₄.

For all experiments an exponentially growing inoculum was used.

Heat treatment: To subject the culture in the bioreactor to defined heat shocks, programmed temperature profiles were used. These profiles involved temperature changes from 37 to 42 and 45°C for exposure times of 10 min. Heating and cooling times were 120 sec at 42°C and 210 sec at 45°C.

Sampling: Growth was measured as the increase in optical density of the culture measured at 546 nm. For dry weight measurements duplicate 5ml samples were filtered through pretared, 0.2μm pore diameter Nuclepore filters (Nuclepore Inc., Pleasanton, USA), dried to constancy and weighed. For the enzyme assay, 5ml samples were immediately frozen in liquid nitrogen and stored on ice.

Enzyme assay: Frozen cells were thawed on ice, washed once with 0.02 M Na₂HPO₄ buffer pH 7, centrifuged at 36000 g for 6 min at 4°C and resuspended in the same buffer. The cells were then disrupted by sonication at 0°C and kept on ice prior to assaying. β-galactosidase was assayed according to the procedure described by Miller (1972), modified so that the rate of increase in the absorbance was measured at 420 nm. Relative specific activities are expressed in arbitrary units and defined as enzyme activity per unit dry mass, divided by the specific activity before the heat shock. All relative specific values were normalized against corresponding values measured in a non-heat shocked control culture.
6.3 RESULTS

When a culture of *E. coli* JB23, growing exponentially in a glucose minimal medium, was subjected to a heat shock for 10 min at 42°C, an immediate increase of htpG gene expression was observed (Figure 6.1). Under such heat shock and growth conditions, the growth of the culture was unaffected. However, when the heat shock temperature was increased to 45°C, an immediate transient reduction of the specific growth rate, indicated as a delay in the increase of the optical density, occurred (Figure 6.2). In contrast to the gene expression pattern observed at 42°C, no heat shock induced increase of htpG gene expression was observed at 45°C. In order to test whether the stability of the fusion protein was responsible for this effect, a crude extract of *E. coli* JB23 was prepared and incubated at 45°C, and β-galactosidase activity was monitored at 15 min intervals. Only a 15 to 20% reduction in activity was observed after one hour, thus indicating that the fusion protein should be stable during the heat treatments used in the experiments.

![Figure 6.1](image)  
**Figure 6.1** Time course of the relative specific htpG gene expression during a heat shock in an exponentially growing culture of *E. coli* in glucose minimal medium. Gene expression was measured as specific β-galactosidase activity of the HtpG-LacZ fusion protein. The cultivation and heat shock temperatures were 37 and 42°C respectively, heat shock shock exposure time was 10 min and is marked by the dashed lines. (O), culture density; (Δ), relative specific β-galactosidase activity.

To investigate whether the type of medium employed had any effect on htpG heat shock gene expression, the same experiment was performed using a complex medium. The
Figure 6.2 Time course of the relative specific *htpG* gene expression during a heat shock in an exponentially growing culture of *E. coli* in glucose minimal medium. Gene expression was measured as specific β-galactosidase activity of the HtpG-LacZ fusion protein. The cultivation and heat shock temperatures were 37 and 45°C respectively, and heat shock exposure time was 10 min and is marked by the dashed lines. (○), culture density; (△), relative specific β-galactosidase activity.

results are shown in Figure 6.3, where a clear heat shock response can be seen. In addition, there was a significant period, ca. 0.5 h, during and immediately after the heat shock, in which the specific growth rate was retarded.

In Figure 6.4 the effect of the growth phase on the *htpG* heat shock gene expression pattern after 10 min exposure to 42°C is shown for an exponentially growing culture (A) and for a stationary phase culture (B), both in minimal medium. The stationary phase culture was heat shocked 45 min after it entered the stationary phase, i.e., after glucose exhaustion. The increase in the intracellular β-galactosidase level that was observed for the stationary phase culture was only ca. 20% of that observed for the exponentially growing culture.

6.4 DISCUSSION

The results presented clearly indicate that both the culture medium and the growth phase impact significantly on the *htpG* heat shock gene expression pattern in cultures of *E. coli* JB23 that are subjected to heat shocks. In a glucose mineral medium a marked response
In 1985, Grossman et al. observed that proteins shock heated at 45°C exist in some of the expression and response strings. At that temperature, the correlation between gene expression and protein synthesis is absent, and the process of translation initiation is essential since its effects are secondary. The absence of Met-tRNAf formation for this amino acid has been shown. Furthermore, methionine biosynthesis is inhibited at 45°C, and this is expected to have pronounced secondary effects, since this amino acid is required for the formation of fMet-tRNA which is essential for the initiation of the translation process. This absence might be responsible for the lack of heat shock gene expression at 45°C.

For E. coli, Ron and Shani (1971) have shown that methionine biosynthesis is severely inhibited at 45°C. Such a methionine depletion can be expected to have pronounced secondary effects, since this amino acid is required for the formation of fMet-tRNA which is essential for the initiation of the translation process, so that its absence might be responsible for the lack of heat shock gene expression at 45°C.

It has been suggested that a correlation between the stringent response and the expression of some of the heat shock proteins, including C62.5, exists (Grossman et al. 1985). In
Figure 6.4 Effect of the culture growth phase on the time course of the relative specific htpG gene expression in E. coli grown in glucose minimal medium. Gene expression was measured as specific β-galactosidase activity of the HtpG-LacZ fusion protein. The cultivation and heat shock temperatures were 37 and 42°C respectively, and heat shock exposure times were 10 min and are marked by the dashed lines. Response of an exponentially growing culture (A), and of a stationary phase culture (B).
contrast, Van Bogelen et al. (1987a) found that inhibition of isoleucine biosynthesis and concomitant cessation of growth did not result in increased C62.5 synthesis. Although no superimposed heat shock was involved in either of these sets of experiments, they serve to illustrate the variability of\textit{htpG} gene expression during different stringent response inducing conditions.

The difference in the responses of exponential phase and stationary phase cultures to an identical heat shock at 42°C raises questions concerning the regulation of\textit{htpG} gene expression and the possible functions of the native gene product. The primary regulator of heat shock gene transcription during a heat shock is the product of the\textit{rpoH} gene, $\sigma^{32}$, which associates with the RNA polymerase core enzyme (Grossman et al. 1984) and it is the RNA polymerase holoenzyme, $E_{o}$, that selectively recognizes heat shock gene promoters (Cowin et al. 1985). However, it was clearly shown that subsets of heat shock proteins can be selectively induced, depending on the environmental stimulus that is responsible (Van Bogelen et al. 1987b). Such results indicate that, in addition to $\sigma^{32}$, other regulatory systems, functioning at the level of transcription of individual heat shock proteins, play an important role.

In stationary phase cultures of\textit{E. coli}, carbon starvation results in the induction of some 30 starvation proteins. Two thirds of these proteins are regulated by cAMP (Schultz et al. 1988). At least two heat shock proteins, DnaK and GroEL, are also induced during carbon starvation, but their synthesis rate only increases three hours after glucose exhaustion (Groat et al. 1986). However, neither heat shock protein is regulated by cAMP. Since cAMP levels have an inverse relationship to energy availability, expression of cAMP regulated genes would be expected in the early stationary phase. Therefore, it seems unlikely that\textit{htpG} gene expression is under such a control, because it has been found in ancillary experiments, that the level of $\beta$-galactosidase remained constant for the first two hours of the stationary phase without heat shocking the culture. Hence, it seems probable that the\textit{htpG} gene is not expressed.

The reduced heat shock induced expression of the\textit{htpG} gene in stationary phase cultures compared to the response in exponentially growing cells, could reflect a functional role of the C62.5 protein in processes linked to growth at higher temperatures. The high degree of conservation of this protein in both eucaryotes and procaryotes is a strong indication for a similar function in both classes of organisms. For various eucaryotic organisms, homologous proteins, belonging to the HSP90 family, have been shown to associate with different types of proteins (Lindquist and Craig 1988). However, such a putative function in\textit{E. coli} seems unlikely to be essential for growth in the Arrhenius and optimum
temperature ranges, particularly since deletion strains are viable and able to grow with only slightly reduced specific growth rates, even at 42°C (Bardwell and Craig 1988).

The question of whether a causal relationship exists between specific growth rate retardation and heat shock protein formation cannot be conclusively answered, because at 42°C in glucose minimal medium, no significant retardation of specific growth rate was observed, whilst such an effect was evident at 45°C in complex medium. However, the amount of heat shock protein formed under both conditions was similar.

6.5 REFERENCES


7. SOME EFFECTS OF GROWTH CONDITIONS ON STEADY STATE AND HEAT SHOCK-INDUCED htpG GENE EXPRESSION IN CONTINUOUS CULTURES OF Escherichia coli

7.1 INTRODUCTION

Spatial and temporal heterogeneity of physical and chemical conditions are not only encountered in natural environments but also, depending on both scale and type of process, in technical bioreactor systems. One consequence of this is that complex transient physiological response patterns can result in the microbes involved. Of the various physical environmental parameters, temperature has for long been of major interest, due to its importance as a major control parameter for process optimization and also because of the fluctuations found in natural environments. Whilst our knowledge concerning growth of microbes at different temperatures is reasonably comprehensive (Ingraham 1987), our understanding of the effects of temperature changes (heat shocks), particularly within the optimum and superoptimum temperature ranges for growth remains incomplete.

Bacterial metabolism is affected in many ways by thermally mediated reversible or irreversible structural alterations to macromolecular cellular components. The overall macroscopic response of a growing bacterial culture to such structural alterations resulting from heat shocks is seen as transient changes in the specific growth rate, which has been shown to vary depending on the exposure time and magnitude of the heat shock (Heitzer and Hamer 1990). A highly coordinated adaptive response has also been shown to occur in all bacteria so far investigated as a result of temperature increases within the optimum and superoptimum temperature ranges for growth. This heat shock response involves an increase in the synthesis rates of a set of specific proteins. In Escherichia coli 17 such heat shock proteins have been described (Neidhardt and Van Bogelen 1987) but their functions are only partially known. The presence of these proteins at all growth temperatures (Herendeen et al., 1979 and Neidhardt et al., 1984) suggests a more fundamental role in cellular metabolism, than simply a function related to adverse environmental temperature conditions. Involvement in processes such as protein degradation (Goff et al. 1984), cell division (Tsuchido et al. 1986) and synthesis of macromolecules including phage development and protein assembly (Neidhardt and Van Bogelen 1987, Goloubinoff et al. 1989) have been reported. The heat shock response is under the positive control of the rpoH gene (Neidhardt and Van Bogelen 1981, Yamamori and Yura 1982), which codes for a RNA polymerase sigma factor, σ^{32}, (Grossman et al. 1984). The holoenzyme associated
with $\sigma^{32}$ selectively recognizes the promoters of heat shock genes (Grossman et al. 1984, Cowing et al. 1985). Here work is described concerning one of these heat shock genes, \textit{htpG}, which codes for the C62.5 protein. This protein represents ca. 0.26% of the total cellular protein of \textit{E. coli} during exponential growth at 37°C (Neidhardt et al. 1984). Although the amino acid sequence of this protein is highly conserved amongst eucaryotes and procaryotes (Bardwell and Craig 1987) its precise function in procaryotes is still unknown (Bardwell and Craig 1988), although some functions of the eucaryotic homologues have been reported (Lindquist and Craig 1988).

Most of the data concerning heat shock gene expression reported in the literature are derived from batch culture experiments under substrate and nutrient sufficient conditions during exponential growth so that the maximum specific growth rate possible for any given medium results. However, it is of interest to examine also the expression pattern of the \textit{htpG} heat shock gene at various imposed growth (dilution) rates in continuous culture with a defined glucose/mineral salts medium. In this manner the effects of heat shocks under either carbon or nitrogen limited growth can be investigated. Further, the effects of medium composition (nutrient status) on the heat shock response pattern of the continuous culture can be compared with growth in a complex medium.

7.2 MATERIALS AND METHODS

Organism: \textit{Escherichia coli} JB23. This strain was kindly provided by Prof. Dr. E. A. Craig. It contains a chromosomal substitution deletion mutation, called \textit{ΔhtpG1::lacZ}, where the coding sequence of the \textit{htpG} gene has been replaced by the coding sequence of the \textit{lacZ} gene in a Lac$^-$ mutant, resulting in an in frame fusion between the codons for amino acid 15 of C62.5 and amino acid 8 of $\beta$-galactosidase. A detailed description of the strain and its construction is given by Bardwell and Craig (1988).

Growth conditions: \textit{E. coli} JB23 was grown in continuous culture in a defined mineral salts medium (Evans et al. 1970) modified by replacing citric acid by 55 mg l$^{-1}$ Na$_2$EDTA. For carbon limited growth 1 g l$^{-1}$ glucose served as the sole carbon energy source. Nitrogen limitation was attained by using a molar carbon to nitrogen ratio of 16.67:1. Ammonium chloride was the nitrogen source. For experiments in a complex medium, the same Evans salts medium was supplemented with 0.5 g l$^{-1}$ glucose, 0.5 g l$^{-1}$ yeast extract and 0.5 g l$^{-1}$ brain heart infusion. An antifoam agent, 20 mg l$^{-1}$ polypropylene glycol, was added to all media.
Bioreactor: The bioreactor used was a computer controlled 2.5 l total volume mini-bioreactor (MBR BioReactor AG, Wetzikon, Switzerland) with an operating volume of 1.8 l. For cultivation the temperature was maintained at 32, 37 or 42°C. The stirrer speed was 800 r.p.m. and the air flow rate used was 50 l h⁻¹. The pH was maintained constant at 7.0 by the controlled addition of either an equimolar 1N NaOH/KOH or a 10% (w/w) H₃PO₄ solution. To operate the system as a chemostat, medium was continuously pumped into the reactor at a constant flow rate and spent medium was removed by an outlet pump controlled by the weight of the bioreactor.

Heat treatment: To subject the culture in the bioreactor to defined heat shocks, programmed temperature profiles were used. These profiles involved temperature changes from 37 to 39°C for 5 and 10 min exposure times and from 37 to 42°C for 5, 10 and 15 min exposure times. The times required for heating to and cooling from the higher temperature were 50 and 120 sec, respectively. Experiments were carried out at dilution rates of 0.23, 0.41 and 0.63 h⁻¹. Further, a culture growing at a dilution rate of 0.23 h⁻¹ was subjected to changes from 37 to 42°C where the final temperature was attained within either 2 min or 60 min and maintained.

Sampling: For dry weight measurements duplicate 5 ml samples were filtered through pretared, 0.2µm pore diameter Nuclepore filters (Nuclepore Inc., Pleasanton, USA), dried to constancy and weighed. For the enzyme assay 5 ml samples were immediately frozen in liquid nitrogen and stored on ice.

Enzyme assay: Frozen cells were thawed on ice, washed once with 0.02 M Na₂HPO₄ buffer pH 7 by centrifugation at 36000 g for 6 min at 4°C and resuspended in the same buffer. The cells were then disrupted by sonication at 0°C and kept on ice prior to assaying. β-galactosidase was assayed according to the procedure described by Miller (1972), modified such that the rate of increase in the absorbance at 420 nm was measured. Relative specific activities are expressed in arbitrary units and defined as enzyme activity per unit dry mass, divided by the specific activity during steady state growth.

Protein: The cellular protein content was determined according to a modified Biuret procedure described by Munkres and Richards (1965). The absorbance of the samples at 300 nm was compared to a bovine serum albumin standard.

All the experiments were carried out in triplicate in at least two independent series.

7.3 RESULTS

The relative htpG expression levels during steady state growth of Escherichia coli at 32, 37 and 42°C in carbon-limited, nitrogen-limited and complex medium are given in Table
Table 7.1 Relative htpG heat shock gene expression levels during steady state growth of *E. coli* at different temperatures under various growth conditions.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Growth rate [h⁻¹]</th>
<th>Relative htpG gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth temperature [°C]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>Carbon-limited</td>
<td>0.23</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>0.41</td>
<td>0.44</td>
</tr>
<tr>
<td>Nitrogen-limited</td>
<td>0.41</td>
<td>0.62</td>
</tr>
<tr>
<td>Complex medium</td>
<td>0.41</td>
<td>0.49</td>
</tr>
</tbody>
</table>

7.1. Under all growth conditions an essentially similar response was observed. In order to determine the effect of heat shock exposure time on htpG gene expression, cultures growing carbon-limited at 37°C at a dilution rate of 0.23 h⁻¹ were subjected to heat shocks at either 39°C for 5 and 10 min or at 42°C for 5, 10 and 15 min. The results, shown in Figures 7.1 and 7.2, indicate that increasing the exposure time resulted in higher final levels of the relative specific β-galactosidase activity for the exposure times investigated. The increase in intracellular β-galactosidase levels during heat shocks at 42°C (Figure 7.2), i.e., 8% after 5 min, 23% after 10 min and 45% after 15 min showed that proportionality between exposure time and htpG gene expression level did not exist. When the culture was returned to 37°C after each heat shock an immediate reduction of htpG gene expression was observed. By comparing the expected half-lives of β-galactosidase, assuming a basal expression level equivalent to the pre-heat shock steady state condition, with the observed values at different exposure times, it is evident that the reduction of the intracellular level of β-galactosidase after the heat shock at 42°C was not solely due to a growth rate dependent intracellular dilution effect (Table 7.2).

To investigate the effect of heating rate, cultures were subjected to either rapid (2 min) or slow (60 min) linear changes in temperature from 37 to 42°C. Both the temperature profiles used and the response patterns obtained are shown in Figure 7.3. As can be seen, the final relative specific β-galactosidase activity levels attained were similar and the transient response was closely coupled to the applied heating rate.

In Figure 7.4 changes in the relative specific β-galactosidase levels for cultures growing at 37°C at three different dilution rates, when subjected to a 10 min heat shock at 42°C, are shown. The relative response patterns during the heat shock were identical but the
Figure 7.1 Effect of heat shock exposure time on the relative specific htpG gene expression in E. coli growing carbon-limited in continuous culture at a dilution rate of 0.23 h\(^{-1}\). The growth and heat shock temperatures were 37 and 39\(^\circ\)C respectively, heat shock exposure times were 5 min (A), and 10 min (B) and are marked by the dashed lines.

subsequent reduction patterns differed markedly, showing a degree of dilution rate dependence. The results presented in Table 7.2 show significant discrepancies between observed and expected half life times. Table 7.3 shows that the specific \(\beta\)-galactosidase activities during steady state growth decreased with increasing dilution rate and that during a heat shock the increase in \(\beta\)-galactosidase level that occurred was proportional.

In order to investigate whether either the nature of the limitation in a defined mineral medium or use of a complex medium affected expression patterns, experiments under
Figure 7.2 Effect of heat shock exposure time on the relative specific hspG gene expression in E. coli growing carbon-limited in continuous culture at a dilution rate of 0.23 h⁻¹. The growth and heat shock temperatures were 37 and 42°C respectively, heat shock exposure times were 5 min (A), 10 min (B), 15 min (C), and are marked by the dashed lines.
Table 7.2 Half life times ($t_{1/2}$) for the HtpG-LacZ fusion protein in *E. coli* after various heat shocks at 42°C under different growth conditions; for definition of half life time see section 7.3.

<table>
<thead>
<tr>
<th>Growth/Heat shock conditions</th>
<th>Half life time for the HtpG-LacZ fusion protein</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Growth rate [h$^{-1}$]</td>
</tr>
<tr>
<td>Carbon-limited</td>
<td>0.23</td>
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<td>0.23</td>
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<td>0.23</td>
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<td></td>
<td>0.41</td>
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<td></td>
<td>0.63</td>
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<tr>
<td>Nitrogen-limited</td>
<td>0.41</td>
</tr>
<tr>
<td>Complex medium</td>
<td>0.41</td>
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</tbody>
</table>

carbon and nitrogen limitation and with a complex medium were carried out at 37°C and a dilution rate of 0.41 h$^{-1}$ that involved a 10 min heat shock at 42°C. The results are shown in Figure 7.5. The difference between the relative specific β-galactosidase activities during steady state and immediately after heat shock was ca. 40% under nitrogen limitation, ca. 20% during carbon limitation and ca. 30% in the complex medium. In addition, the specific activities observed depended on the medium used (Table 7.4). The steady state $htpG$ expression level during nitrogen limitation was less than 50% of that during carbon-limited growth, while the value obtained for the complex medium was similar to that for carbon limitation. To check whether the former was a function of the total cellular protein content, these were measured and found to be 62% under carbon limitation, 47% under nitrogen limitation and 59% in the complex medium. Hence, the pronounced reduction of the $htpG$ gene expression during nitrogen limitation did not correspond with the change in the overall protein content under such a growth condition. The amount of β-galactosidase formed during the ten minute heat shock did not show any pronounced difference between carbon and nitrogen limitation as might have been expected from the steady state values (Table 7.4). In contrast, in complex medium 40% more β-galactosidase was formed than in carbon-limited medium, although steady state $htpG$ expression levels shown in Table 7.4 were essentially identical. After returning the culture from the heat shock temperature to the normal growth temperature, large differences between both observed and expected values for the half life times of the β-galactosidase fusion protein were obtained as shown in Table 7.2.
7.4 DISCUSSION

In order to characterize the dynamic response of growing bacteria to moderate environmental changes it is essential that both growth conditions and medium composition are carefully defined and controlled. Here, results have been presented concerning steady state and heat shock induced htpG gene expression in continuous cultures of E. coli at different specific growth rates and in different media. The steady state htpG expression levels at different temperatures were consistent with the relative intracellular levels of the C62.5 heat shock protein reported by Herendeen et al. (1979) for exponentially growing
Figure 7.4 Effect of dilution rate on the relative specific *htpG* gene expression in *E. coli* growing carbon-limited in continuous culture at a dilution rate of 0.23 h\(^{-1}\) (O), 0.41 h\(^{-1}\) (Q) and 0.63 h\(^{-1}\) (+). The growth and heat shock temperatures were 37 and 42°C respectively, heat shock exposure was 10 min, and is marked by the dashed lines.

Table 7.3 Effect of dilution rate on steady and heat shock induced specific *htpG* gene expression in *E. coli* in a carbon-limited glucose mineral salts medium. The growth and heat shock temperatures were 37 and 42°C respectively, heat shock exposure was 10 minutes.

<table>
<thead>
<tr>
<th>Dilution rate [h(^{-1})]</th>
<th>0.23</th>
<th>0.41</th>
<th>0.63</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state level (A)</td>
<td>1656</td>
<td>1503</td>
<td>1350</td>
</tr>
<tr>
<td>Increase during 10 min heat shock (B)</td>
<td>340</td>
<td>313</td>
<td>283</td>
</tr>
<tr>
<td>Ratio (A/B)</td>
<td>4.87</td>
<td>4.80</td>
<td>4.77</td>
</tr>
</tbody>
</table>
Figure 7.5 Effect of growth medium composition on the relative specific htpG gene expression in *E. coli* growing either carbon-limited (○) or nitrogen-limited (□) in a glucose mineral salts medium and in a complex medium (+) at a dilution rate of 0.41 h⁻¹. The growth and heat shock temperatures were 37 and 42°C respectively, heat shock exposure was 10 min, and is marked by the dashed lines.

Table 7.4 Effect of growth medium composition on steady state and heat shock induced specific htpG gene expression in *E. coli* at a dilution rate of 0.41 h⁻¹. The growth and heat shock temperatures were 37 and 42°C respectively, heat shock exposure time was 10 minutes.

<table>
<thead>
<tr>
<th>Specific htpG gene expression</th>
<th>Specific β-galactosidase activity [ΔOD₄₂₀/8₀cells/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth medium</td>
<td>Carbon-limited</td>
</tr>
<tr>
<td>Steady state level (A)</td>
<td>1503</td>
</tr>
<tr>
<td>Increase during 10 min heat shock (B)</td>
<td>313</td>
</tr>
<tr>
<td>Ratio (A/B)</td>
<td>4.80</td>
</tr>
</tbody>
</table>
batch cultures of E. coli at corresponding temperatures. Different growth limitations resulted in marked changes in the specific htpG expression during steady state growth and this result is qualitatively consistent with the reduction in the total protein content. Schultz et al. (1988) have shown that during nitrogen starvation the intracellular levels of some heat shock proteins in E. coli change. For example, the DnaK level increases during nitrogen starvation whilst GroEL is not even induced. Although nitrogen starvation, where no growth takes place, is an entirely different physiological state from nitrogen limited growth, these results indicate that, when only one protein is considered, general conclusions concerning the expression patterns of the various genes of the heat shock regulon under different limiting growth conditions should be drawn with care.

Higher specific growth rates of E. coli in carbon limited medium resulted in a slight decrease of the specific β-galactosidase activity, which is in contrast to the results obtained by Pedersen et al. (1978) for the C62.5 protein levels at different growth rates in exponentially growing batch cultures. These authors report a positive but non-proportional relationship between specific growth rate and C62.5 level. However, since different specific growth rates were established by changing the medium composition, the growth conditions were sufficiently different from those in the present experiments to preclude direct comparison of results.

In the experiments described here, the effect of the growth medium on htpG expression during a heat shock could not be predicted from steady state data. The increase in the specific β-galactosidase level under nitrogen limited growth was only 15% less than under carbon limitation despite entirely different steady state levels. The fact that under both limitations the free amino acid pools are not markedly different in Gram-negative bacteria (Tempest et al. 1970) gives a plausible explanation for the similar heat shock protein synthesis rates during such short term heat shocks. Further, the response in complex medium, which was more pronounced than under carbon limitation, could result from an increased availability of metabolites. An increase in the extracellular amino acid concentration has been shown to increase the intracellular amino acid pool levels in E. coli (Britten and McClure 1962).

The response of the relative specific β-galactosidase level during a heat shock was equally rapid at the three dilution rates investigated. This implies that the regulation of htpG gene expression is growth rate dependent during both steady and transient states. When the culture was returned after a heat shock to the initial growth temperature of 37°C, an immediate reduction in the β-galactosidase synthesis rate was observed, irrespective of the
growth conditions, indicating a close and rapid temperature dependent regulation of \( htpG \) gene expression. The subsequent reduction of intracellular heat shock protein to the original level can be accounted for by at least two mechanisms. Proteolytic activity has been shown to increase in cells in which the heat shock response has been induced. One of the heat shock proteins, Lon, is known to be an important protease, specifically attacking abnormal and incomplete proteins (Goff et al. 1984, Straus et al. 1988). Alternatively, the assumption of rapid return to pre-heat shock synthesis rate might be invalid.

A final important question arising from these results concerns the mechanistic molecular basis for the regulation of \( htpG \) gene expression during both steady and transient states under different growth conditions. Since the level of active \( \sigma^{32} \), the product of the \( rpoH \) gene, limits transcription of heat shock genes (Tilly et al. 1989), the \( htpG \) gene expression will also depend on the regulation of the \( \sigma^{32} \) concentration. However, the issue is non-trivial since the expression of the \( rpoH \) gene in \( E. \ coli \) is a complex process involving a multiple promoter system (Erickson et al. 1987, Fujita and Ishihama 1987) and these promoters have been shown to be differently and differentially regulated (Wang and Kaguni 1989a, Erickson et al. 1987, Ueshima et al. 1989, Wang and Kaguni 1989b). Further, the stability of \( \sigma^{32} \) has been shown to be temperature dependent (Tilly et al. 1989). However, it is not known how different growth conditions affect these individual processes. The fact that under other stress conditions only subsets of these heat shock proteins are induced (Lindquist 1986) indicate that regulation other than only by \( \sigma^{32} \) concentration at the transcription level of the individual heat shock proteins might also play an important role.

7.5 CONCLUSIONS

The application of continuous culture techniques has been shown to be a valuable tool for investigating \( htpG \) heat shock gene expression under various controlled growth conditions. The results indicate that in addition to temperature as the major regulatory parameter of \( htpG \) expression, other growth environment dependent factors clearly affect both steady and transient state expression.

7.6 REFERENCES

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8. GENERAL DISCUSSION AND CONCLUSIONS

One of the most neglected aspects in process biotechnology is the question of the various heat transfer processes involved in controlling the temperature environment of the individual microbes in a bioreactor at a level coincident with the temperature optimum for their anabolic and catabolic processes. The optimum temperature range for growth and product formation by most microbes is narrow, usually only a few degrees. At temperatures only a few degrees in excess of the optimum for growth, a precipitous decline in the specific growth rate to zero occurs. The vast majority of microbes used in biotechnological processes are mesophiles. If process cooling is inadequate, the consequences are that either all or part of the process culture is subjected to superoptimum temperatures. Overheating problems in bioreactors are scale related and are frequently ignored during process research in small-scale laboratory bioreactors. Clearly, such process overheating results in temperature inhomogeneities in the bioreactor. The exposure time for process cultures to temperature gradients can be expected to be in the range of minutes rather than in the range of either seconds or hours.

The results in Chapters 5 and 6 provide evidence, that the effects of heat shocks within the superoptimum temperature range for growth in a given medium, have negligible consequences on the growth kinetics of both Klebsiella pneumoniae NCIB 418 and Escherichia coli JB23. Further, it was also evident, that continuous culture performance of K. pneumoniae NCIB 418, as judged by biomass formation, is not adversely affected, when portions of a process culture are repeatedly subjected to temperature gradients (Chapter 4). However, in these latter experiments cellular activity, measured by INT-reduction activity, was shown to be reduced in bacteria immediately after passage through a temperature gradient, indicating that metabolic changes did, in fact, occur. Results obtained for the htpG heat shock gene expression after a minor heat shock from 37 to 39°C for 5 minutes (Chapter 7) further illustrate the impact of minor temperature changes on cellular metabolism. Taking into account that the expression of the individual heat shock genes is under the control of a common regulatory gene, rpoH, and that at 37°C ca. 5% of the total cellular protein comprises heat shock proteins, one can conclude that the increased induction of heat shock proteins causes a significant change in the overall pattern of the bacterial metabolism. Therefore, even minor temperature changes can alter the physiological state of a bacterium.

Based on the concept of a "stress cycle", as introduced in Chapter 2, such cells can be considered to be metabolically disturbed rather than injured, since the short term changes
in their metabolism did not affect their specific growth rate. Thus, once returned to optimum conditions, restoration of the culture to its initial growth state is seen to occur immediately, if the specific growth rate is the only criterion considered. However, an increase of the stress intensity resulted in prolongation of the restoration period in both a temperature and exposure time dependent manner (Chapter 5). Although some of the experiments involved temperatures which were supermaximum for growth in glucose minimal medium, but still in the superoptimum temperature range when a complex medium was used, plate counts on a glucose minimal agar suggested significant exposure time dependent losses. In contrast, plating on complex agar indicated no reduction in the viable counts. With respect to physiological status, these data suggest that immediately after heat treatment no dead cells were present, since complete recovery was possible under appropriate conditions. The significantly different recovery pattern on glucose minimal agar can be explained in two ways. Assuming that the results are representative for the culture performance in a liquid medium, then additional processes such as cellular death and lysis have to be taken into account. Alternatively, the use of the plate count technique might not be an appropriate method for estimating viability in this temperature range in question, since the additional stress introduced by transfer from a liquid to a solid medium might prevent the growth of injured cells which could still reestablish growth if retained in an appropriate liquid medium.

A major question with respect to the adverse effects of temperature on cellular metabolism concerns the expression pattern and the role of heat shock proteins during heat shock and the restoration of equilibrium after such treatments. In order to address this question, the expression in E. coli of a representative heat shock protein, that is highly conserved during evolution, was investigated under various growth conditions (Chapters 6 and 7). The results emphasize the marked effects which the culture environment can have on the steady state htpG heat shock protein levels and also on the heat shock induced response. Further, the observations that in a stationary phase culture only minor htpG gene expression occurred after a heat shock when compared to an exponentially growing culture and that exposure to a supermaximum temperature, where growth immediately stopped, resulted in a complete absence of a response, raise two major questions:

i) Do these different expression patterns reflect a functional role of the native htpG heat shock protein?

ii) Is this a secondary effect involving an incapacity to produce the protein, due to environmental and physiological constraints, despite of its essential function?
With respect to the former question, the results obtained in Chapter 6 suggest a possible involvement in cellular processes linked with proteins or their synthesis rather than with DNA synthesis and growth *per se*. However, assuming linkage with protein synthesis through, for example, ribosomal functions, one might expect an increase in either the basal level or in the total amount formed during heat shock induction with increasing dilution rate, since the ribosome content increases with dilution rate in chemostat cultures. However, such behaviour was not observed and the level declined slightly with increasing dilution rate.

With respect to the latter question, the results obtained for cultures grown under chemostat conditions indicate that the steady state expression level under nitrogen-limited growth is much lower than under carbon limitation, thereby showing that the cell can afford to reduce its htpG protein level in a manner which corresponds with the generally reduced cellular protein level. However, the virtually identical amounts of heat shock protein formed during identical heat shocks under both operating conditions could reflect an immediate, fixed, characteristic response. Subsequent adjustment to the characteristic steady state level under nitrogen limitation occurs during prolonged exposure at 42°C. These results suggest the possible existence of long term fine control of the regulation during steady state growth under nitrogen-limited conditions, but during a heat shock, the proposed fine regulation does not operate, i.e., the cell functions on an "all or nothing principle". Of course, the ultimate question is whether both the steady state and the heat shock induced responses are exclusively regulated by the rpoH gene product, $\sigma^{32}$, with fine regulation mediated by the rpoH promoter system or whether additional transcriptional control, at the level of the individual heat shock genes, occurs. Indications that the latter is possible are provided by the patterns that result from induction by stress factors other than heat shock, where only subsets of the heat shock genes are expressed.

The implications of the heat shock response for the application of temperature inducible expression systems have been discussed in Chapter 2. Suggestions, for improving stability of heterologous proteins in host strains were to use *lon*, *rpoH* or double mutants. Unfortunately, this is not a complete solution to the instability problem. Recent findings that some of the heat shock proteins in *E. coli* can promote assembly of oligomeric proteins (Goloubinoff *et al.* 1989), can suppress mutations by interacting with the proteins affected (Van Dyk *et al.* 1989) and can facilitate export of *lacZ* hybrid proteins in *E. coli* (Phillips and Silhavy 1990) make the general advise to get rid of the heat shock response system (Caulcott and Rhodes 1986) questionable, when a temperature inducible expression
system for heterologous protein production is used. Under certain conditions, the presence of heat shock proteins is a probable advantage with respect to product stability.

Provided the response of the htpG gene is representative for other heat shock proteins, the reduced htpG gene expression observed during short term heat shocks, relative to long term exposure, with subsequent rapid reduction of the protein level to the pre-heat shock value (Chapter 7) make this an interesting observation with respect to process strategy, and enhances the possibilities for applications of the type of temperature inducible promoter-inversion expression systems that have been proposed by Podhajska et al. (1985). As discussed earlier, short heat shocks have negligible effects on growth rate and have markedly reduced adverse effects on product yield than does prolonged exposure to higher temperatures, as is needed for application of the lambda promoter system. A realistic process concept, using the former system, would be separation of biomass production from product formation by employing a two stage bioreactor system with an interstage heat exchanger for heat shocking.

The quantitative description of the relationship between bacterial growth and temperature in the superoptimum temperature range has been discussed on the basis of three different models (Chapter 3). The simultaneous application of these models for each data set clearly showed the descriptive superiority of the empirical Ratkowsky model over the mechanistic models of Esener and Fiolitakis. In order to obtain a more realistic mechanistic description of growth, a certain degree of structure is needed to account for the complex physiological processes that take place in the superoptimum temperature range. Structured models which took account of the physiological status of bacterial populations were first proposed by Ramkrishna et al. (1966) and Williams (1967). They divided the biomass into two compartments, one containing structural components and nucleic acids and the other containing the remaining biosynthetic enzymes.

Since temperature significantly affects structural elements and the activity of the macromolecular compounds in bacteria (Chapter 2), a similar approach is applicable for describing the relationship between temperature and specific growth rate, although it involves extensive simplifying assumptions. One possibility is to divide the biomass into three compartments as shown in Figure 8.1, where the B-compartment comprises all the biosynthetic enzymes for precursor synthesis, macromolecule synthesis occurs in the M-compartment, which contains precursor pools, reduction equivalents, adenylates, nucleic acids, ribosomes, lipids and cell walls, and the H-compartment comprises heat shock proteins. Separation of the heat shock proteins into their own compartment can be justified because:
Figure 8.1 Scheme for a three compartment model for the description of the relationship between bacterial growth and temperature throughout the growth permissible temperature range. S, substrate; B, H, M, compartments; $r_1$ to $r_4$, characteristic transfer rates. For a detailed description of the compartments and transfer rates see text.

i) They represent a significant fraction of the total cellular protein of E. coli during growth in the superoptimum temperature range (Herendeen et al. 1979, Neidhardt et al. 1984).

ii) At elevated temperatures, they are involved in major macromolecule synthesis processes (Neidhardt and Van Bogelen 1987).

Further, a correlation between the ability of a bacterial cell to produce heat shock proteins at temperatures $> 30^\circ$C and the maximum growth permissible temperature has been established by Yamamori and Yura (1982).

The interactions between the three compartments are also shown diagrammatically in Figure 8.1. At optimum and superoptimum growth temperatures, carbon energy substrate, S, is converted at a rate $r_1$, into the constituents of the M-compartment by catalytic action.
provided by the B-compartment and modulated by the heat shock proteins. The formation of biosynthetic enzymes in the B-compartment takes place at a rate $r_2$, which is under the influence of heat shock proteins at optimum and superoptimum temperatures. Turnover and degradation of the components of the B-compartment occurs at a rate $r_3$, which is again influenced by the heat shock proteins. Formation of the heat shock proteins occurs at a rate $r_4$.

The contribution of each compartment to the total biomass, is expressed as a weight fraction. Such a system can be described mathematically by applying the methodology of Harder and Roels (1982).

In order to validate the model, experimental data for optimum and superoptimum temperatures are required to establish the inter compartment and overall process stoichiometries. The major problem is the quantification of the transfer rates $r_j$ to $r_4$. This might be achieved by using $rpoH$ mutant strains with appropriate suppressors and comparing their responses with non-mutant parent strains.

Whilst the proposed model clearly involves over simplification, it should be of significant value for identifying critical reactions, thereby justifying either their inclusion or their exclusion from consideration. The ultimate value of the structured mechanistic model will be its ability to describe culture dynamics throughout the whole temperature range permissible for bacterial growth.

8.1 REFERENCES


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