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**Description and Mechanisms of Bacterial Growth Responses
to Water Activity and Compatible Solutes**

by

Karen Krist

BAgrSc (Hons.) University of Tasmania

**Submitted in fulfilment of the requirements for the degree
of Doctor of Philosophy**

University of Tasmania

November, 1997

*Accepted for publication
See*

DECLARATION

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ABSTRACT

Bacterial growth is inhibited by unfavourably low water activity conditions, however, the inhibition is partly alleviated by exogenous provision of compatible solutes. As compatible solutes exist in all food systems, describing and understanding this bacterial growth response is useful for food microbiologists concerned with limiting microbial growth on foods using water activity stress.

In the first application of predictive microbiology techniques to compatible solute growth responses, the four parameter square root model (Ratkowsky *et al.*, 1983) successfully described growth rate data collected for *Escherichia coli*, *Paracoccus halodenitrificans* and *Halomonas elongata*. The value of the parameter T_{min} was independent of the exogenous provision of compatible solutes, but $a_{w\ min}$ values and the observed minimum temperature for growth were lower where compatible solutes were provided.

Despite their accurate description of growth rate data, empirical square root models do not improve mechanistic understanding. A mechanistic explanation for the bacterial growth response to water activity and compatible solutes was examined using a substrate-limited batch culture technique, developed to measure cell yield. The cell yield of *E. coli* did not vary significantly with extracellular water activity or compatible solutes, except at water activity values close to the growth/no growth interface, indicating that water activity challenge is not an energetic burden for bacterial cells. Therefore, energetic limitation of growth was eliminated as a possible mechanistic explanation. Cell yield was also independent of incubation temperature, over most of the biokinetic range. The cell yield responses with water activity and temperature conditions were similar and consistent with a mechanistic, thermodynamic model (McMeekin *et al.*, 1993; Ross, 1993), thus the influence of water activity on microbial growth may be explained in terms of the thermodynamics of protein folding. This mechanism is consistent with contemporary models for the effect of compatible solutes on water structure close to the surface of macromolecules (Wiggins, 1990).

Examination of novel and published data using the thermodynamic model revealed a possible mechanism for the temperature and water activity limits for microbial growth. Large increases in the activation energy, close to the boundary between growth permissible and non-growth conditions, suggest a possible universal limiting activation energy for bacterial growth. This finding may provide a mechanistic basis for, currently empirical, growth/no growth models.

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CHAPTER 1: INTRODUCTION

1.1 Food Microbiology

Food is a valuable industry in Australia that caters to two markets: domestic and export. In 1996 Australian processed food and beverage exports were estimated to be worth \$10.8 billion per annum (Hooke, 1996). Therefore there is much interest in and effort expended to ensure that those markets are not jeopardised.

Food microbiology is a discipline that plays a role in maintaining food related export markets. For a food to be marketable, from a microbiological perspective, it must be fresh (have low numbers of spoilage bacteria) and safe (free from pathogens). Many importing countries enforce strict microbiological guidelines. In some cases (depending on the pathogen detected and importing country) if those guidelines are not met, not only will the current batch of product be rejected, but also future trade of that product may be prohibited.

For export markets with such countries to remain viable, Australian food products must therefore meet or exceed these microbiological criteria. This is difficult in itself, due to the small size and ubiquity of bacteria, but is made even more so by Australia's remote location, that means food must often be transported long distances to the export destination. This allows more time for bacterial growth and lapses in storage. These problems however are not new and there has already been much research within the food microbiology discipline towards meeting those needs. Research has included rapid method development, such as luminometry for the enumeration of bacterial cells and polymerase chain reaction (PCR) techniques for the detection of specific pathogens (Stannard and Wood, 1983; Theron *et al.*, 1986; McElroy *et al.*, 1996; Agersborg *et al.*, 1997; Meng *et al.*, 1997); improved media for isolation and identification of pathogens (Manafi, 1996) development of technology to record the temperature history of a product and therefore identify lapses in storage (time-temperature indicators) (McMeekin *et al.*, 1993) and; development of predictive models to forecast microbial growth (McMeekin *et al.*, 1993; Ross and McMeekin, 1994; Whiting and Buchanan, 1997). However, despite this large research effort the incidence of foodborne illness is rising and new pathogens continue to emerge (Maurice, 1994).

1.1.1 Foodborne pathogens

The term foodborne pathogen refers to any microbial agent that produces disease, where food is its mode of transmission. The disease produced by such agents varies from nausea to death depending on the pathogen, dose received and physiological state of the victim. Food poisoning by bacterial pathogens is due to either the consumption of the live pathogen or a toxin that has been liberated into the food.

The incidence of food-borne illness has been rising. In England and Wales food poisoning increased five-fold and in Europe as a whole it tripled, in the decade preceding 1994 (Maurice, 1994). Not only is the incidence of food poisoning rising, but also the number of agents recognised as responsible for food poisoning is increasing. Agents that have been recently recognised include *Campylobacter jejuni*, *Vibrio vulnificus*, *Listeria monocytogenes* and some strains of *E. coli* (Doyle, 1994). Verotoxigenic *E. coli* strains, in particular, have assumed enormous importance.

1.1.2 Pathogenic *E. coli*

E. coli can cause disease thus food poisoning due to *E. coli* can be very expensive; victims may require medical treatment, food outlets may face legal action, and in the case of international trade, export markets may be lost temporarily or permanently. Therefore understanding *E. coli* as a pathogen has become a priority for many microbiologists, both in the clinical and food disciplines.

Historically *E. coli* has been regarded by microbiologists and many other scientists as a useful research tool. It is popular because *E. coli* is easily accessible, most strains are not highly virulent, and it grows rapidly on defined media (Schaechter and Neidhardt, 1987). *E. coli* and its close relative *Salmonella typhimurium* are used in basic research in biochemistry, physiology and molecular biology. Hence, by 1987 approximately 1/3 of the gene products of *E. coli* had been studied in detail and their genes identified; 10% of the *E. coli* genome had been sequenced; map positions were known for 1/2 of its genes; and 80% of its metabolic pathways were identified (Schaechter and Neidhardt, 1987). For these reasons, and because it is a normal member of the mammalian gut flora, most people are familiar with the bacterium called *E. coli*.

In 1982 *E. coli* became known for more than being a useful research tool and member of the mammalian gut flora. In Michigan and Oregon (United States of America) there was an outbreak of *E. coli*-associated food poisoning. Forty-seven people fitted the case description, bloody diarrhoea and stomach cramps (haemorrhagic colitis), that could not be linked to any known gastrointestinal pathogen (Riley *et al.*, 1983). The outbreak was associated with eating hamburgers at fast-food restaurants belonging to the same restaurant chain. Studies at the time isolated *E. coli* of the serotype 0157:H7 from stool samples from many of the food poisoning victims, but not from controls. *E. coli* of the same serotype was also isolated from hamburgers made from a suspect lot of meat (Riley *et al.*, 1983). *E. coli* 0157:H7 had been implicated previously in enteric disease (Johnson *et al.*, 1983) but the outbreak in 1982 identified it as a pathogen conclusively.

Before the 1982 outbreak of *E. coli* 0157:H7, other pathogenic *E. coli* strains were known, grouped as:

- Enterotoxigenic *E. coli* (ETEC)
- Enteropathogenic *E. coli* (EPEC)
- Enteroinvasive *E. coli* (EIEC)

ETEC, EPEC and EIECs all cause diarrhoeal diseases, however those diseases differ in the clinical manifestation and pathogenic mechanism (Doyle and Padhye, 1989).

Enterotoxigenic *E. coli*, as their name suggests, produce enterotoxins that cause disease. There are two enterotoxins, and ETECs may produce one or both: heat-labile enterotoxin (LT) and non-antigenic, heat-stable enterotoxin (ST) (Levine *et al.*, 1983).

A loose definition applies to the pathogenic mechanism of EPECs; the EPECs are said to have a pathogenic mechanism that has '*not been proven to be related either to heat-labile enterotoxins or to Shigella-like invasiveness*' (Eldelman and Levine, 1983).

Enteroinvasive *E. coli* are characterised by their capacity to invade and multiply in epithelial cells, and cause the death of those cells (Doyle and Padhye, 1989).

When *E. coli* 0157:H7 was discovered it did not match the descriptions and clinical manifestations of these known groups of pathogenic *E. coli*. Therefore a new grouping was created: enterohaemorrhagic *E. coli* (EHEC) (Levine, 1987). Some

serotypes that had been categorised previously as EPEC, such as 0111 and 026, have since been reclassified as EHECs (Bettelheim, 1996), this can make some literature on the topic confusing.

Enterohaemorrhagic *E. coli*, as their name suggests, produce disease called haemorrhagic colitis that is characterised by abdominal cramps and bloody diarrhoea (Riley *et al.*, 1983, Griffin *et al.*, 1988). More serious complications may also develop: haemolytic uraemic syndrome (HUS), associated with kidney failure or; thrombotic thrombocytopenic purpura (TTP), associated with neurological dysfunction (Karmali *et al.*, 1985, Griffin *et al.*, 1988). The pathogenic mechanism of EHECs, like ETECs, is through toxins. In this case verotoxins 1 and 2 (VT1 and VT2), also known as Shiga-like toxin 1 (SLT1) and Shiga-like toxin 2 (SLT2). EHECs may produce one or both of these toxins (Johnson *et al.*, 1983; O’Brien *et al.*, 1983; Strockbine *et al.*, 1986).

Following the 1982 outbreak many more cases of *E. coli* 0157:H7-associated gastrointestinal disease have been reported, some are listed in Table 1.1. Increasing reports are probably due in part due to increased awareness that in turn results in better identification techniques and more fastidious reporting.

Table 1.1: Some reports of *E. coli* 0157:H7 associated gastrointestinal disease, since 1982.

Year	Location	No. Cases	Suspected Vehicle	Reference
1984	Nebraska, USA	34	Hamburger	Ryan <i>et al.</i> , 1986
1985	East Anglia, UK	24	Potatoes	Morgan <i>et al.</i> , 1988
1985	London, Canada	71	Sandwiches	Krishnan <i>et al.</i> , 1987
1988	Minnesota, USA	32	Hamburger	Belongia <i>et al.</i> , 1991
1989	Birmingham, UK	26	Sandwiches	Salmon <i>et al.</i> , 1989
1994	Wales, UK	8	Beefburger	Willshaw <i>et al.</i> , 1994
1995	Washington, USA	20	Salami	CDC*, 1995a
1996	Georgia + Tennessee, USA	10	Hamburger	CDC*, 1996
1996	Sakai City, Japan	6401	Radish sprouts	WHO†, 1996
1996	Scotland, UK	408	Meat	WHO†, 1997

*CDC = Centers for Disease Control and Prevention

†WHO = World Health Organisation

Because *E. coli* 0157:H7-associated disease has been reported often, and the outbreaks have been dramatic and received media attention, there has been a large amount of research interest in this serotype. Studies have included:

- Biochemical characteristics (Wells *et al.*, 1983; Doyle and Schoeni, 1984)
- Growth characteristics (Doyle and Schoeni, 1984; Abdul-Raouf *et al.*, 1993; Abdul-Raouf *et al.*, 1994; Rajkowski and Marmer, 1995)
- Growth models (Buchanan *et al.*, 1993; Buchanan and Bagi, 1994)
- Survival characteristics (Doyle and Schoeni, 1984; Rice *et al.*, 1992; Abdul-Raouf *et al.*, 1993; Zhao and Doyle, 1994; Clavero and Beuchat, 1996)
- Disease manifestations (Karmali *et al.*, 1983; Griffin *et al.*, 1988)
- Occurrence in retail foods (Doyle and Schoeni, 1987; Abdul-Raouf *et al.*, 1996; Bolton *et al.*, 1996)
- Reservoir identification (Martin *et al.*, 1986; Borczyk *et al.*, 1987)
- Detection, isolation and recovery method development (Szabo *et al.*, 1986; Padhye and Doyle, 1991; Bennett *et al.*, 1995; Czajaka and Batt, 1996; Czechowicz *et al.*, 1996; Restaino *et al.*, 1996; Wallace and Jones, 1996)
- Verotoxin investigation (Johnson *et al.*, 1983; O'Brien *et al.*, 1983; Scotland *et al.*, 1985; Weeratna and Doyle, 1991; Abdul-Raouf *et al.*, 1994)

This list of publications on *E. coli* 0157:H7 is by no means exhaustive. A very great research effort has been concentrated on this serotype.

The value of concentrating so much research effort on just one pathogenic serotype of *E. coli* should be questioned. The development of methods specific for detecting *E. coli* 0157:H7 has been criticised because it generally excludes other pathogenic *E. coli*. For example, the inability of *E. coli* 0157:H7 to ferment sorbitol has been exploited as a method for identifying this serotype (Wells *et al.*, 1983; March and Ratman, 1986). But other enterohaemorrhagic *E. coli* ferment sorbitol and therefore remain undetected by these methods (Gunzer *et al.*, 1992). This may be acceptable because 0157:H7 serotype is the main cause of haemorrhagic colitis and HUS (Desmarchelier and Grau, 1997). But, serious foodborne outbreaks due to other EHEC serotypes have occurred. In Italy *E. coli* of the serotype 0111:H- caused an outbreak of haemorrhagic colitis and HUS, during 1992 (Caprioli *et al.*, 1994). *E. coli* 0111:H- was also responsible for the first reported outbreak of *E. coli*-associated HUS, in Australia. The later outbreak was traced to the consumption of 0111:H- contaminated mettwurst (CDC, 1995b).

The criticisms of 0157:H7 directed research, therefore, appear valid. Other pathogenic serotypes have caused serious problems, and warrant equal investigation. However, besides performing research on specific serotypes, a useful adjunct would be to apply the principles of predictive microbiology (section 1.1.4). Application of predictive microbiology technology would allow the growth of *E. coli* (pathogenic or otherwise) to be forecast from knowledge of; the growth responses and limits of *E. coli* regarding conditions in its environment; the physiological reasons for those responses and limits and the physicochemical properties of the food. However, before predictive microbiology is considered, it is first necessary to describe typical growth responses of bacteria to conditions in their environment.

1.1.3 Environment and bacterial growth

Bacteria respond in different ways to different conditions in their environment. As for any other organism they may grow, survive or die, and their growth or death may be slow or rapid. Many conditions combine to determine these responses, including pressure, oxygen tension, carbon dioxide, nitrite and sulphite. However, three main factors that control bacterial growth responses in food environments, are temperature, pH (acidity/alkalinity) and water activity (saltiness/wetness).

Temperature

The influence of temperature on bacterial growth has been studied extensively. A typical bacterial growth rate curve, where temperature is the variable, is shown in Figure 1.1. Bacterial growth rate increases with increasing temperature until the optimum temperature for growth is reached, it then decreases, falling to zero rapidly. All bacterial growth rate curves, where temperature is the variable, are of this general form.

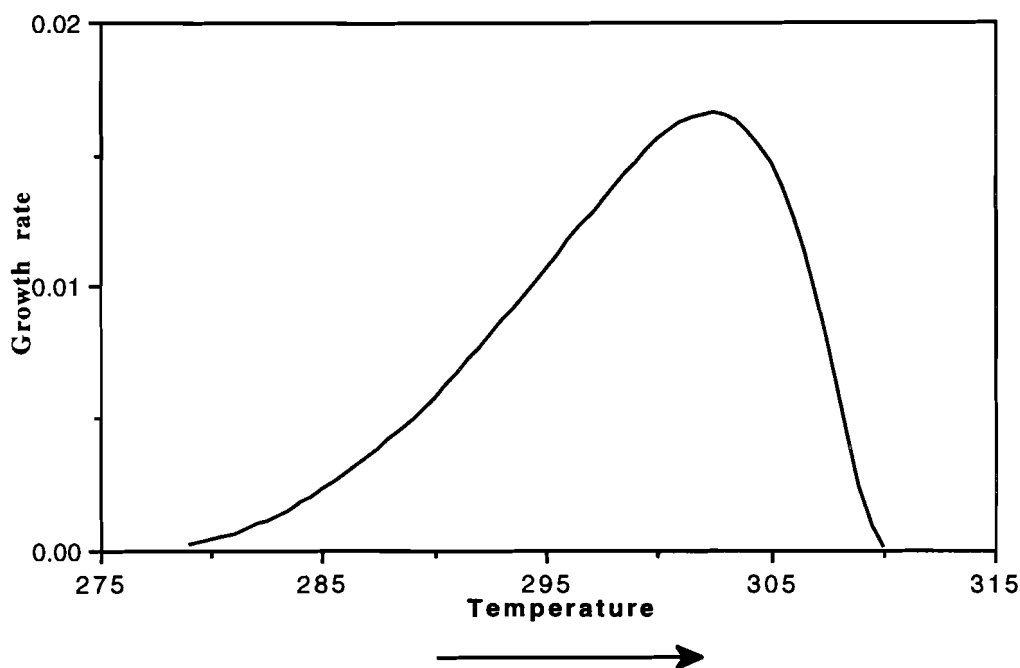


Figure 1.1: The growth rate response to temperature, typical of bacteria. The arrow indicates the direction of increasing temperature.

The growth rate response of bacteria to temperature conditions can be explained if bacteria are considered as chemical systems. In chemical systems, the usual effect of temperature is to accelerate reaction rates. The simplest explanation for this acceleration is ‘collision theory’, where increased temperature raises the kinetic energy of reagent molecules, increasing the likelihood that they will collide and react (Arrhenius, 1915). This explains the part of the temperature response curve where bacterial growth rate rises with increasing temperature. The reduction in growth rate above the optimum temperature can be explained by heat denaturation of proteins or other macromolecules (Sizer, 1943). Bacteria use proteins (enzymes) to catalyse their chemical reactions and the functioning of these enzymes is dependent on their being in an active conformation (native state). However, the conformation of enzymes is determined to some extent by extrinsic conditions including temperature, such that reversible heat and cold denaturation of enzymes occur, producing inactive conformations (denatured state). Therefore the reduction in growth rate close to the upper temperature limit for growth may be due to the heat denaturation of one or more enzymes. Heat denaturation times are much shorter than for cold denaturation, explaining the sharp reduction in growth rate at the high temperature growth limit (Sharpe and DeMichele, 1977).

pH

The effects of pH on bacterial growth are well described. A typical pH-response curve is shown in Figure 1.2. Bacterial growth rate remains fairly constant throughout most of the pH growth range (plateau) and falls dramatically to zero at both the acidic and alkaline pH limits for growth.

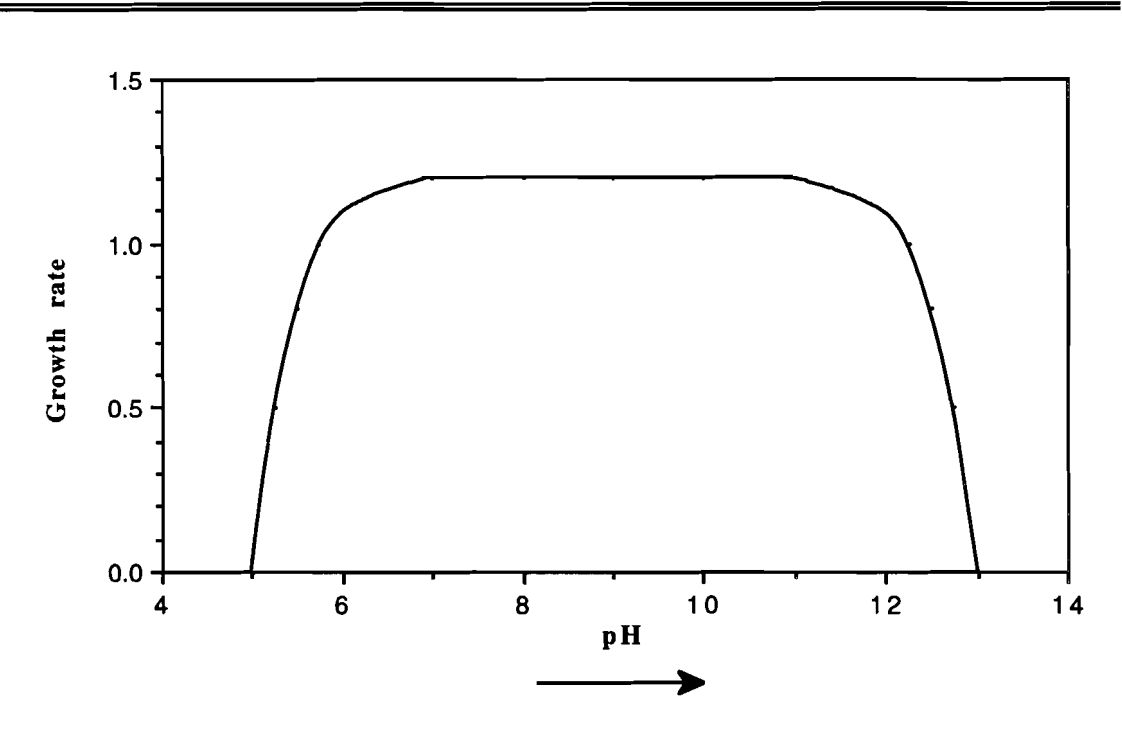


Figure 1.2: The growth rate response to pH, typical of many bacteria. The arrow indicates the direction of increasing extracellular pH values.

This pH-response curve can be explained by considering the mechanisms used by bacteria to adapt to pH stress. Bacteria cannot tolerate large changes in the intracellular pH, due to the destabilising effect that hydrogen ions have on biological macromolecules (Booth and Kroll, 1989). Therefore, when a pH stress is encountered the cell pumps hydrogen ions across its membrane to maintain a relatively constant intracellular pH. This means that the cellular machinery responsible for growth is maintained in a constant environment that is not affected by extracellular pH over most of the pH growth range, providing an explanation for the plateau portion of the pH-response curve (Figure 1.2). The points where growth rate drops rapidly, at the acidic and alkaline limits for growth, probably represent the points where stress due to extracellular pH becomes too great causing breakdown of the homeostatic

mechanisms, loss of control over intracellular pH and hence, markedly reduced growth rates (Presser *et al.*, 1997a).

Support for the above hypothesis on the physiological reason for pH limits for bacterial growth is gained from the bacterial growth response to organic acids. Organic acids are generally more effective than inorganic acids at inhibiting bacterial growth. This is because, unlike inorganic acids, the undissociated form of the organic acid can cross the cell membrane and dissociates once inside the cytoplasm, releasing hydrogen ions and lowering the intracellular pH (Corlett and Brown, 1980). That is, organic acids are more effective at disrupting the intracellular pH.

Water activity

Responses of bacteria to water activity (a_w) stress is the topic of this study, but before examining this, a definition of the water activity term is required. The terms water activity, osmotic pressure and solute concentration are often used interchangeably by microbiologists to refer to the availability of water to bacteria. Although each of these is related, they are different. These differences were explained, for aqueous solutions, by Scott (1957) as follows.

The term 'solute concentration' is self explanatory, although it may be expressed in different ways: percentage concentration (w/w), (v/v) or (w/v), molarity or molality (Brady and Holum, 1988).

- percentage concentration by weight, %(w/w); the number of grams of solute per 100 grams of *solution* (not solvent)
- percentage concentration by volume, %(v/v); used when both solute and solvent are fluids; the number of volumes of one component in 100 volumes of the entire solution
- weight/volume percentage concentration, %(w/v); the grams of solute in 100 mL of solution
- molarity or molar concentration, M; the number of mols of solute per litre of solution
- molality or molal concentration, m; the number of mols of solute per kilogram of solvent

High solute concentrations result in reduced water availability to bacteria.

The ‘water activity’ is a fundamental property of solutions, defined as:

$$a_w = \frac{p}{p_0} \tag{1.1}$$

where: p is the vapour pressure of the solution
 p_0 is the vapour pressure of the solvent

Because water activity is a ratio, it may have a value between zero and one. The water activity value for pure water is 1.000; when water activity equals zero there is no available water. All solutions and foods will have a water activity value that lies somewhere between those two extremes. The water activity values for a variety of aqueous solutions are given in Table 1.2.

Table 1.2: Water activity values for aqueous solutions of NaCl (15 - 50°C), LiCl₂ (5 - 45°C) and H₂SO₄ (0 - 55°C).

Solute Conc. (% w/w)	Water Activity		
	NaCl*	LiCl†	H ₂ SO ₄ †
2.0	0.989	0.983	0.992
4.0	0.977	0.964	0.984
6.0	0.964	0.943	0.976
8.0	0.950	0.950	0.966
10.0	0.935	0.889	0.955

* Chirife and Resnik (1984)

† Resnik and Chirife (1988)

The water activity of a solution changes with solute concentration. Table 1.2 illustrates that as solute concentration increases, the water activity of the solution decreases. This occurs because when solutes are dissolved in water, some of the water molecules become more ordered as they become oriented on the surfaces of the solute. This reduces the vapour pressure of the solution, since on average the water molecules then have less entropy, and hence the water activity is reduced. The effect of solute concentration on water activity can be mathematically expressed:

$$a_w = \frac{-vm\phi}{e^{55.51}} \tag{1.2}$$

where: m is the molal concentration of solute
 v is the number of ions generated by each molecule of solute
 (non-electrolytes, $v = 1$; NaCl, $v = 2$; H₂SO₄, $v=3$)
 ϕ is the molal osmotic coefficient
 (may be calculated or evaluated from measurements of
 boiling points, freezing points and osmotic pressure)

Note from Table 1.2, that the water activity at a given solute concentration varies depending on the solute used. Each solute has its own specific solute concentration/water activity relationship because water activity, according to Equation 1.2, is determined by a solute specific, molal osmotic coefficient and depends on the number of ions generated by each molecule of solute.

As water activity decreases, the availability of water to bacteria is reduced.

The 'osmotic pressure' of a solution is related to its water activity and includes the water activity term in its definition:

$$\text{Osmotic pressure} = \frac{-RT \ln a_w}{\bar{V}} \quad (1.3)$$

where: R is the universal gas constant, 8.314 JK⁻¹mol⁻¹
 T is the temperature (K)
 \bar{V} is the partial molar volume of water

Increased osmotic pressure literally means that the cell is subjected to an increased external pressure, or alternatively, a decreased internal pressure (Brown, 1976). The validity of using the term, due to disagreement on the physical implications of osmotic pressure, has been debated (Brown, 1976). The term, however, remains in use; increased extracellular osmotic pressure refers to a situation where the availability of water to bacteria is decreased.

Throughout this review the physiological responses of bacteria to water activity will be considered. The terms solute concentration, water activity and osmotic pressure will be used interchangeably to describe the availability of water to bacterial cells, however the differences between them in absolute terms is appreciated.

In a pattern resembling the case for temperature, bacteria grow fastest at their optimum water activity, and slow as conditions deviate from that optimum, until a growth rate of zero is reached. Figure 1.3 shows a typical water activity-response curve.

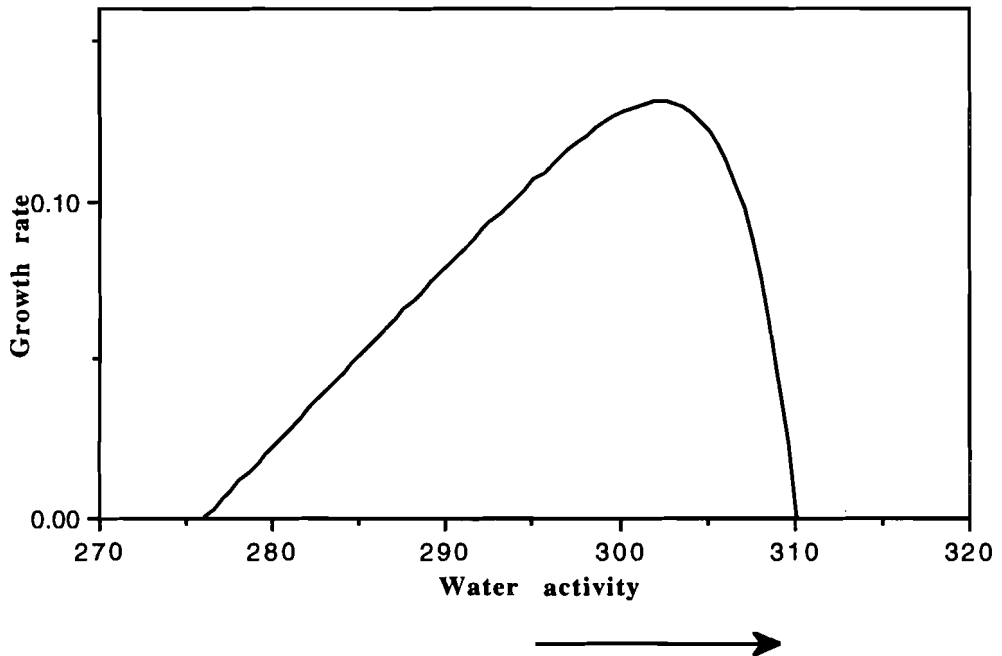


Figure 1.3: The growth rate response to extracellular water activity conditions, typical of many bacteria. The arrow indicates the direction of increasing water activity values. For some species the optimum water activity value approximates 1.000, hence, only the straight line portion of the curve is realised.

The physiological explanation for the water activity response curve is unknown. Cayley *et al.* (1991) correlated the growth rate of bacteria exposed to water activity stress with the amount of 'free water' inside the cell. Others have suggested that water activity alters the interaction of water with proteins (Arakawa and Timasheff, 1985; Wiggins, 1990; Cybulska and Doe, 1997). However, a complete interpretation of the observed water activity-growth response, as described for temperature and pH, has not been proposed to date.

Understanding the physiological response of bacteria to water activity constraints is important. Knowingly, or otherwise, everyone is aware of the inhibition

of bacterial growth by reduced water activity conditions. Perishable foods are stored in a dry place to increase their shelf life, and before the invention of refrigerators meat and fish were often preserved by salting. This technique of food preservation, by changing its water activity, is not new and has persisted through time. It remains in use in the food industry today; in abattoirs, for example, carcasses are allowed to dry during chilling. Also water activity, with pH, contributes to the microbial safety of fermented dried meat products like salami. Therefore the inhibition of bacterial growth by reduced water activity has important implications, especially regarding the growth of spoilage and pathogenic bacteria in foods.

1.1.4 Predictive microbiology

Predictive microbiology is based on the idea that bacteria respond reproducibly to known suites of environmental conditions; the preceding description of bacterial responses to conditions in their environment implied that this was true. Therefore, the growth of bacteria on foods can be predicted objectively from knowledge of the environmental history and intrinsic properties of the food. Each bacterial species has a specific, temperature-dependent growth rate that is the same each time it is grown provided all other conditions, such as water activity and pH, are constant. By knowing the specific, temperature-rate relationship for a bacterial species, predictions of the extent of growth through time are possible. This is called predictive microbiology.

Predictive modelling is not a recent concept. Scott (1937) reported on the growth of microorganisms on ox-muscle, as effected by temperature, and recognised the predictive value a knowledge of the bacterial growth rate with temperature would hold. But even before this time, simple predictive models had been developed and were in use in the food industry, the classic example being 'botulinum cook' (Esty and Meyer, 1922). This model describes the temperature-rate relationship for *Clostridium botulinum* death rather than growth. However the principle is the same: a given time-temperature combination results in a known change in bacterial numbers. The botulinum cook model was used to determine the time/temperature combination required for cooking canned foods to ensure their freedom from that pathogen. In a modified form the botulinum cook model remains in use (Stumbo *et al.*, 1983).

Despite its long history, the predictive modelling discipline did not attract wide attention until the 1980's. Ross and McMeekin (1994) attributed its revival at that time to three main factors; heightened public awareness due to a rise in the incidence of major food poisoning outbreaks at that time; the recognition by food microbiologists

that traditional microbiological culture techniques were too slow to be of predictive value; and increased access to and improvements in computers.

Kinetic and probability modelling have been the two main approaches to predictive microbiology (Ross and McMeekin, 1994). Kinetic models are mathematical descriptions for the extent and rate of growth of bacteria. In this approach, it is assumed that foods are an ideal environment for the growth of bacteria, where nutrient availability is not limiting. Hence, it is hypothesised that environmental factors including temperature, water activity and pH will dictate the rate and extent of microbial growth (Ross and McMeekin, 1994).

Kinetic models

Within the kinetic modelling category four main model types are recognised:

- Bělehrádek models
- Arrhenius type models
- Modified Arrhenius or Davey models
- Polynomial or Response Surface models

The development of these models has been reviewed (Heitzer *et al.*, 1991; Ratkowsky *et al.*, 1991; Ross and McMeekin, 1994), and therefore will not be reproduced here.

There have been many publications comparing different kinetic models. Most often Bělehrádek and Arrhenius type models have been compared. Examples include: Adair *et al.* (1989); Ratkowsky *et al.* (1991); Zweitering *et al.* (1991) and Alber and Shaffner (1992). Adair *et al.* (1989) used mean square error (MSE) to compare the Schoolfield, an Arrhenius type model (Schoolfield *et al.*, 1981) and square root (Ratkowsky *et al.*, 1982, 1983) models for eight different data sets. They found that the MSE for the Arrhenius type model was always smaller. But, using the f-test, Zweitering *et al.* (1991) could not statistically separate two Arrhenius and two Bělehrádek models.

Assessments of polynomial models have also been made. Little *et al.* (1992) compared a square root-type and a response surface model, with the latter giving closer predictions to the raw data, based on MSE. Polynomial models however, have generally been criticised because they are specific to the conditions under which they were produced (Baranyi *et al.*, 1996a).

Therefore, comparisons of kinetic models have been inconclusive. This has been attributed to the dependence of the results of such comparisons on the criteria chosen for comparison (Zweitering *et al.*, 1991) and the transformation of the data (Alber and Schaffner, 1992). The superiority of one kinetic model over any other has, therefore, not been unequivocally demonstrated. This has resulted in a situation where different predictive modelling research groups around the world have not reached a consensus and continue to use different types of kinetic models.

Probability models

Probability models predict the likelihood of a particular event. An example may be taken from Lindroth and Genigeorgis (1986). This model predicts the probability of a *C. botulinum* spore initiating growth and toxin production. Ross and McMeekin (1994) provide a review of probability models and their evolution, for which there has been less divergence compared to kinetic models.

Probability modelling differs from kinetic modelling because its main focus is not the extent of growth, but whether the bacteria will grow or not. This type of modelling is encouraged by the food industry, where although knowledge of microbial growth responses is useful in spoilage assessments and in the production of some foods, such as fermented meat products and yoghurt, they are less useful for pathogens. This is because, generally, far fewer numbers of pathogens are needed to cause disease than spoilage bacteria are needed to cause food spoilage. Therefore, in the case of pathogenic bacteria the initial concern for food producers will be whether the bacterium can grow, not the extent of growth. Recently it was suggested that probability and kinetic modelling are beginning to converge (Ross and McMeekin, 1994). Integration of the two modelling approaches has been used successfully to model the bacterial growth/no growth boundary (Ratkowsky and Ross, 1995).

Benefits

Regardless of the model type selected, the predictive modelling approach offers several benefits. Once a predictive model has been developed and validated the need for traditional microbiological analyses is reduced in many instances. This has benefits for the response time, because an immediate assessment of the effects of a processing or storage lapse is possible, as well as providing a cost saving. Also, predictive

microbiology has been promoted as a tool for the empowering of the HACCP (Hazard Analysis and Critical Control Point) approach (Ross and McMeekin, 1994). Further, through its incorporation into expert systems such as Food Micromodel (McClure *et al.*, 1994) and *Pseudomonas* Predictor (Neumeyer *et al.*, 1997), predictive microbiology allows non-experts to make objective judgements, can be used as an educational tool for food handlers or process operators, and can help in the design of new processes.

Criticisms

A major criticism of predictive models has been their generally empirical nature. Baird-Parker and Kilsby (1987) comment that, 'All models are pragmatic as we have no real understanding of the actual mechanisms'. That is, all models so far are summaries of observations that have no underlying mechanistic basis. Arrhenius models are often cited as having biological backgrounds. However, the Arrhenius equation (Equation 1.4) describes the relationship between temperature and first order chemical reactions. Microbial growth would be expected to be a more complex process than this, casting doubt on the mechanistic validity of Arrhenius modelling approaches (Stannard *et al.*, 1985). Additionally, in fitting Arrhenius models, the parameters are most often used as fitting parameters rather than estimates of biologically relevant parameters (Zweitering *et al.*, 1991).

$$\ln k = \ln A - \frac{E_a}{RT} \quad (1.4)$$

where: k is the specific reaction rate (growth rate in microbiology)
 A is the 'collision factor'
 E_a is the activation energy of the reaction
 R is the universal gas constant, $8.314 \text{ JK}^{-1} \text{ mol}^{-1}$
 T is the absolute temperature

Delegates at the 2nd International Conference on Predictive Microbiology, Hobart, Tasmania, February 1996, commented that mechanistic models would be more useful than empirical ones, and therefore that physiological studies were required to facilitate their development. This was also acknowledged by the conference organisers who included a symposium in the program devoted to microbial physiology and mechanistic modelling.

Mechanistic models

Researchers in predictive microbiology have for some time been exploring the mechanistic modelling approach; an example can be drawn from Chapter 10 of McMeekin *et al.* (1993), in which the authors build a new mechanistic model from the assumption that a single, unknown, rate-limiting enzyme determines microbial growth and by combining the Brandts (1967) and Murphy *et al.* (1990) functions (the Brandts function describes the activity of enzymes and the Murphy *et al.* function describes the thermal denaturation of proteins due to unfolding of hydrophobic groups within the protein core). The new mechanistic model was used successfully to produce realistic simulations of growth rate-temperature plots for psychrophilic, psychrotrophic, mesophilic and thermophilic bacteria. However, the model cannot be considered truly mechanistic because, as the authors acknowledge, not all of the parameters can be determined experimentally; a mechanistic modelling requisite of Heitzer *et al.* (1991).

To date no mechanistic model has been proposed to describe bacterial growth responses to extracellular water activity conditions. For the successful development of such a model a knowledge of the physiology of microorganisms in relation to water activity stress will be required.

1.2 Water Activity Stress Physiology

For survival and normal growth bacteria, like plant cells, need to maintain a positive turgor pressure. This is necessary because turgor pressure is thought to provide the stimulus for cell elongation and therefore growth (Koch, 1982). When a cell experiences a water activity stress due to osmotic upshock, the cell loses water due to osmosis. This occurs because the bacterial cell membrane is permeable to water and relatively impermeable to solutes. Therefore, water moves out to restore osmotic equilibrium and results in shrinkage of the cell; in extreme cases the cell membrane shrinks away from the cell wall (plasmolysis). For survival and growth, bacterial cells must counteract water activity stress and restore their turgid, pre-stress state.

To counteract and survive water activity stress, bacteria have evolved a number of physiological responses including; changes to the cell membrane, protein synthesis and adjustment of cytoplasmic water activity. The latter is the main physiological response to water activity stress and will therefore be the main focus of this review, but the other two responses will be considered briefly.

1.2.1 Cell membrane

The bacterial membrane, the main barrier to water and solute exchange between the cytoplasm and the external environment, plays an important role in the physiological response to water activity stress. This involves both components of the membrane - lipids and proteins. Phospholipids, the major lipid components of the bacterial cell membrane, must be maintained in the proper bilayer phase for normal functioning of the bacterial cell membrane. To achieve this the main change observed in response to high salinity involves a rise in the proportion of negatively charged phospholipids, often phosphatidylglycerol and/or glycolipids (Russell *et al.*, 1995) (the structure of phospholipids in membranes is explained briefly in Figure 1.4).

Changes in the membrane proteins, such as increased levels of solute transport proteins (porins), are likely during the osmoregulatory response. Changes in the protein components of the membrane have been noted (Kawaji *et al.*, 1979).

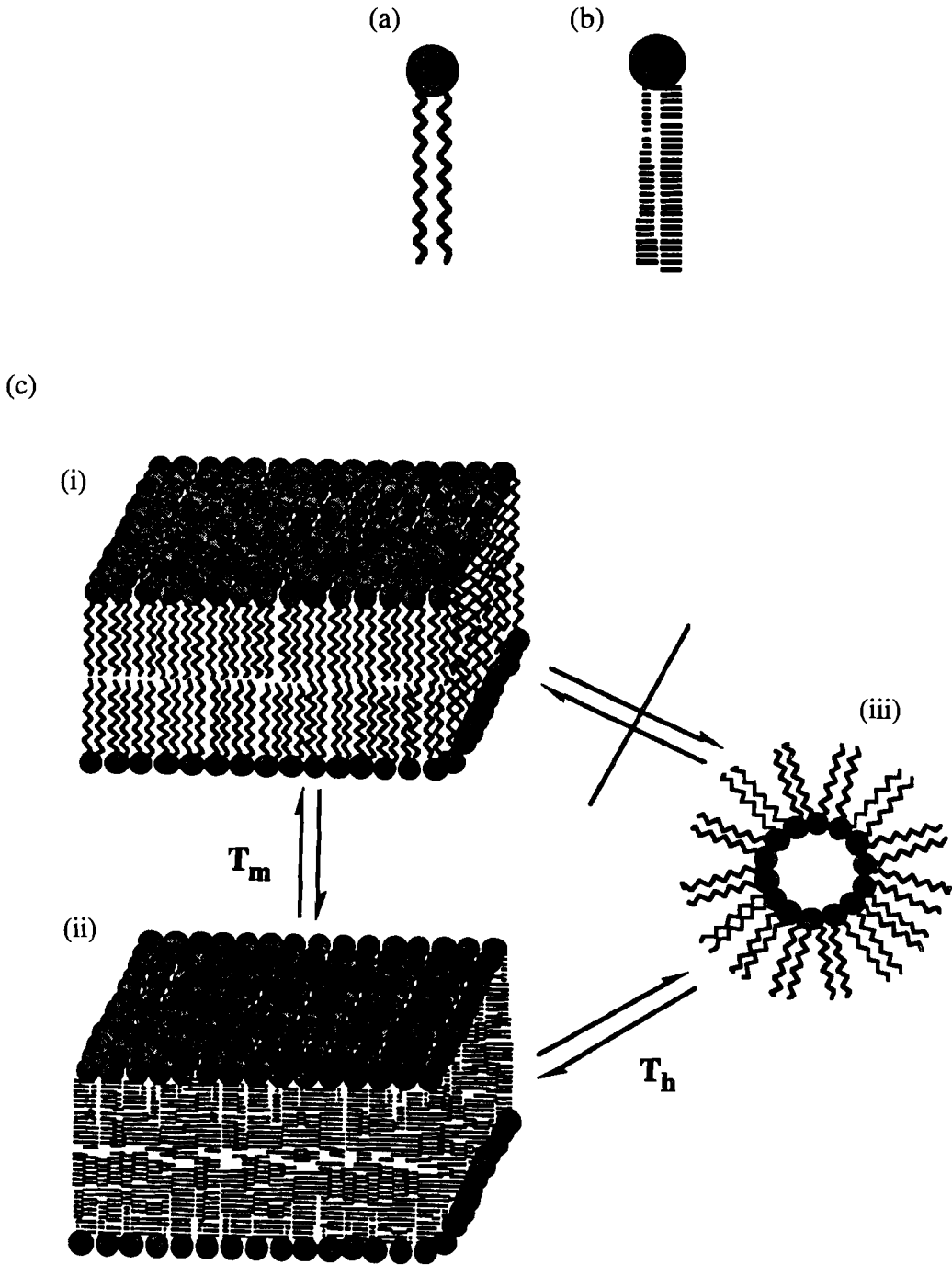


Figure 1.4: Schematic diagram of a phospholipid molecule; the head group is depicted as a ball and the hydrocarbon chains are undergoing (a) low levels of molecular motion and (b) high levels of molecular motion. Diagram (c) illustrates the lipid bilayer phase (i) that is required for normal membrane functioning. Changes in temperature and other environmental factors change the lipid phase; Figures (ii) and (iii). Therefore, for normal membrane functioning, changes in the chemical make-up of the lipids are necessary to overcome this effect. Rising solute concentrations result in changes in the head group composition. Adapted from Nichols (1996).

1.2.2 Protein synthesis

The synthesis of certain proteins is induced by osmotic stress (Kawaji *et al.*, 1979; Menzel and Roth, 1980; Laimins *et al.*, 1981; Csonka, 1982; Villarejo *et al.*, 1983; G    ver *et al.*, 1988; Hengge-Aronis *et al.*, 1991). Membrane porins have already been mentioned above and increased levels of those require protein synthesis. Other proteins are also required during the response to water activity stress, some of these are specifically synthesised during an osmotic challenge and others are produced constitutively; these will be dealt with in more detail in section 1.2.4. Like porins, many other osmotically induced proteins form the cellular machinery to facilitate a change in cytoplasmic water activity.

1.2.3 Cytoplasmic water activity

Bacteria adjust their cytoplasmic water activity using one of two strategies. These are known as the salt-in-cytoplasm type and the organic-osmolyte-in-cytoplasm type (Tr    per and Galinski, 1989).

Prokaryotes that use the salt-in-cytoplasm strategy accumulate potassium chloride to high intracellular concentrations. This is considered a primitive strategy because it does not provide a 'normal' cytoplasmic environment. Therefore adjustments are required in cellular physiology, especially regarding enzymes. The enzymes of prokaryotes that use the salt-in-cytoplasm strategy have additional negative charge that makes them stable at high solute concentrations but unstable at low solute concentrations (Mevarech *et al.*, 1977; Mevarech and Neumann, 1977). This makes it an inflexible system and, therefore, growth of prokaryotes that use this form of osmoadaptation is restricted to low water activity conditions. The *Halobacteriaceae*, members of the Archaea, are one group of prokaryotes that use the salt-in cytoplasm strategy for osmoadaptation and accordingly they display strict osmotic limits for growth: members of the genus *Halobacterium* require 3.0 to 5.2M sodium chloride for growth (Larsen and Grant, 1989). The salt-in-cytoplasm strategy therefore represents an evolution of specific cellular machinery that can operate in, and requires, a high ionic solute environment rather than a strategy to adapt truly in an environment where water activity fluctuates.

Most bacteria use the organic-osmolyte-in-cytoplasm strategy for osmoadaptation. In this strategy inorganic salts are excluded, while organic solutes are synthesised or accumulated in the cytoplasm from the environment. This is a more

flexible strategy for osmoadaptation because, unlike the salt-in-cytoplasm strategy, the organic solutes balance osmotic pressure but do not hinder the functioning of 'normal' salt-sensitive enzymes. Because they are compatible with enzymes, the organic osmolytes used in this osmoadaptation strategy are known as 'compatible solutes' (Brown, 1976).

Interestingly, the use of compatible solutes to adapt to a water activity stress is not limited to bacteria. Plants and animals also use the organic-osmolyte-in-cytoplasm strategy, and may provide an example of convergent evolution. Both the strategy and compatible solutes used are common to all groups (Yancey *et al.*, 1982). This suggests that compatible solutes have fundamental properties that make them suitable for use in osmoadaptation in all biological systems.

Compatible solute molecules have low molecular weights and polar functional groups. These properties make compatible solute molecules highly soluble, facilitating their accumulation to high intracellular concentrations. Also, compatible solutes are uncharged at normal cytoplasmic pH values (Trüper and Galinski, 1989). This property is required because high cytoplasmic ionic strength would be detrimental to enzymes. These restrictions on the molecular structure of compatible solutes limit the classes of compounds that are potential candidates.

Trüper and Galinski (1989), based on the above molecular criteria for compatible solutes, identified four classes of compounds as potential compatible solutes: sugars, polyols, amino acids and betaines. They then compiled a list of all compounds that had been implicated as compatible solutes, including those from plants and animals, and found that each of the four chemical classes was represented.

In bacteria the following classes of compounds are implicated in osmoregulation (Galinski, 1995):

- sugar polyol derivatives (eg. glucosyl glycerol)
- zwitterionic trimethyl ammonium and dimethylsulfonium compounds (betaines, thetines)
- natural amino acids (proline, glutamine)
- glutamine amide derivatives (*N*α-carbamoylglutamine amide)
- *N*-acetylated diamino acids (*N*δ-acetylornithine)
- ectoines (ectoine, β-hydroxyectoine)

All of these molecules obey the molecular criteria for compatible solutes and most belong to one of the four chemical classes identified by Trüper and Galinski (1989) or are derivatives thereof. Most of these compatible solutes are synthesised by halophilic and/or halotolerant bacteria only, but many, if exogenously present, are accumulated by non-halophiles in response to osmotic stress. Non-halophiles also use potassium glutamate and sugars such as trehalose as compatible solutes.

1.2.4 Case study - *E. coli*

E. coli maintains a positive turgor pressure in low water activity conditions using a rapid primary response, followed by a slower secondary response that is longer-term. During the primary response potassium ions are accumulated and glutamate is synthesised. In minimal media (devoid of compatible solutes) this is followed by trehalose synthesis, the secondary response. Trehalose is used as a compatible solute only if alternative compatible solutes, such as glycine betaine or proline, are not available extracellularly. However, if those are available and suitable uptake mechanisms exist, accumulation of extracellular compatible solutes substitutes for trehalose synthesis.

1.2.4.1 Primary response

Potassium

Potassium ions are very important in bacterial physiology. They are required for the activity of many enzymes (Steinbach, 1962) and, during osmotic stress, are accumulated by all bacteria as an immediate response (Epstein, 1986). The size of the potassium ion pool in *E. coli* is related directly to the external osmotic pressure. As the extracellular water activity falls the intracellular potassium ion pool increases (Epstein and Shultz, 1965). This relationship is linear, and is independent of the extracellular potassium ion concentration (Shultz and Solomon, 1961). Literature values for the absolute size of the potassium ion pool at any given extracellular water activity vary between authors (Epstein and Shultz, 1965; Dinnbier *et al.*, 1988; Cayley *et al.*, 1991). This variability is probably due to difficulties in the measurement of cellular volume. However, all authors agree that intracellular potassium ion levels increase with decreasing extracellular water activity and, therefore, that potassium ion

accumulation contributes to the maintenance of a positive turgor pressure in *E. coli* cells exposed to water activity stress.

The accumulation of potassium ions by *E. coli* during water activity stress has been attributed to increased influx of potassium ions via two major transport systems: TrkA and Kdp (refer to Appendix 1.1 for details on nomenclature of genes and gene products). The rate of efflux of potassium ions in these conditions remains unchanged, at the pre-stress rate (Meury and Kepes, 1981). Overall, therefore, net potassium ion uptake occurs.

The TrkA transport system is the main potassium ion transport system in *E. coli*, where there is no water activity stress. This system is produced constitutively in *E. coli* and has a high transport rate, but low affinity for the potassium ion (Rhoads *et al.*, 1976). The TrkA system is genetically complex, which is highlighted by confusion regarding the roles of various Trk gene products. For example, initial investigations of TrkD lead to the suggestion that this was a potassium transport system separate from TrkA, with a low transport rate (Epstein and Kim, 1971). Later all the *trk* genes, including *trkD* were said to affect a single transport system, TrkA (Walderhaug *et al.* 1987). Later still, TrkD was again divided off and is currently held to be a separate, but minor, potassium transport system (Bossemeyer *et al.*, 1989). Another example is that after many years of research into the single transport system called TrkA, there is now evidence that TrkA itself consists of two separate systems: TrkG and TrkH (Dosch *et al.*, 1991). Despite this confusion about the genetics of TrkA, its role as the main potassium ion transport system in *E. coli* remains undisputed.

Uptake of potassium ions by the TrkA transport system increases when there is a water activity stress (Rhoads and Epstein, 1978). Because TrkA expression is constitutive, this osmotic regulation of potassium ion uptake must act at the level of function rather than transcription. Exactly how this functional regulation is achieved is unknown, but it is thought to be mediated by the proton motive force (PMF). The PMF is required for the active accumulation of potassium by the TrkA system (Rhoads and Epstein, 1977); high PMF values are thought to be stimulatory and low PMF values inhibitory (Rhoads and Epstein, 1978). ATP is also necessary for the active accumulation of potassium ions, but does not play a regulatory role. ATP is used in the formation of a high-energy intermediate, without any net energy consumption (Rhoads and Epstein, 1978).

In normal circumstances the Kdp transport system of *E. coli* is not expressed (Laimins *et al.*, 1981). The primary function of this system is to scavenge potassium ions when the low affinity TrkA system is insufficient to meet the potassium ion needs of the cell. That is, when extracellular potassium ion concentration is low; the TrkA system is impaired due to mutation or; in conditions of low water activity. The Kdp system is a high affinity system, but has a slower transport rate than TrkA (Rhoads *et al.*, 1976). It is coded by five closely related genes - *kdpA*, *kdpB*, *kdpC*, *kdpD* and *kdpE* (Polarek *et al.*, 1992). KdpA, KdpB and KdpC are three inner membrane proteins, that make up the potassium ion transporter (Laimins *et al.*, 1978; Epstein *et al.*, 1990), whilst the remaining two gene products are involved in regulation of the Kdp system. KdpD is a membrane located sensor and KdpE a response regulator (Walderhaug *et al.*, 1992). The activity of the Kdp system is dependent upon ATP, but not on the proton motive force (Rhoads and Epstein, 1978).

Exposure to water activity stress causes the transcription of the *kdp* operon (Laimins *et al.*, 1981). This osmotic regulation of the Kdp system was thought to be effected through mechanical sensing of the turgor pressure, because there appeared to be no consistent correlation with either the external or internal potassium ion concentration (Laimins *et al.*, 1981). More recently it was suggested that KdpD, the environmental sensor, responds to the level of potassium and to the physio-chemical state of the cytoplasmic membrane (Sugiura *et al.*, 1994). However, the precise environmental stimulus for KdpD remains unknown. When a change in turgor pressure is detected, the KdpD protein phosphorylates KdpE to its active form that triggers *kdp* transcription (Walderhaug *et al.*, 1992).

Along with uptake systems, potassium ion efflux systems also operate in *E. coli*. Compared to transport systems, the potassium ion efflux systems KefA, KefB and KefC (Douglas *et al.*, 1991; Bakker *et al.*, 1987) have been less well studied. Because potassium ion efflux does not change during the primary response to osmotic stress (Meury and Kepes, 1981) detailed knowledge of the efflux systems is not required for the purposes of this review. Their role in adaptation to more favourable water activity conditions (Meury *et al.*, 1985), or in potassium ion efflux once the secondary response to osmotic stress occurs, has largely been ignored and requires further investigation.

Glutamate

Accumulation of large amounts of potassium ions during the primary response to osmotic stress requires, due to the physical need for electroneutrality, either simultaneous efflux of cations from cells or, influx or synthesis of anions. In *E. coli* electroneutrality during the primary response to osmotic stress is probably achieved by a combination of these.

The synthesis of glutamate in response to low water activity is a general phenomenon amongst non-halophiles (Tempest *et al.*, 1970). *E. coli* synthesises glutamate ($\text{COOCH}(\text{NH}_3)\text{CH}_2\text{CH}_2\text{COO}^-$) during the primary response to osmotic stress (Munro *et al.*, 1972; Measures, 1975; Richey *et al.*, 1987). The accumulation of glutamate in this circumstance has two benefits. Firstly it aids in maintaining intracellular electroneutrality and secondly it decreases the intracellular water activity. Hence it contributes to maintenance of positive turgor pressure. Despite repeated demonstrations that glutamate accumulation occurs during water activity challenge the regulation of glutamate accumulation at the molecular level is not understood. This is probably because glutamate, along with its role in the osmotic stress response, is involved in normal metabolism. Distinguishing between these roles is problematic. However, there must be some regulation to avoid futile cycling of glutamate during the primary response to osmotic stress; this issue requires further research.

The amount of glutamate synthesised, in general, does not match the amount of potassium ions accumulated (Richey *et al.*, 1987; Dinnbier *et al.*, 1988). Therefore, glutamate cannot solely account for charge balance during the primary response to osmotic stress and other factors are likely to contribute to maintenance of electroneutrality. Possibilities include putrescine efflux, and the synthesis or accumulation of other organic anions.

In *E. coli* the efflux of putrescine ($\text{CH}_2(\text{NH}_3)\text{CH}_2\text{CH}_2\text{CH}_2(\text{NH}_3)_3^{2+}$) is closely linked to increased water activity conditions and potassium ion accumulation (Munro *et al.*, 1972; Munro and Sauerbier, 1973). This loss of positively charged species is a possible means to achieve electroneutrality during osmotic stress. The excretion of an osmotically active solute during an osmotic stress response, however, at first glance appears to be a paradox. But, calculation of the 'trade-off' between putrescine efflux and potassium glutamate accumulation (accounting for the interplay between osmotic pressure, ionic strength and charge balance) is favourable (Munro *et al.*, 1972).

E. coli may also achieve charge balance by accumulation of anionic species that are synthesised intracellularly or accumulated from the extracellular environment. During water activity stress, organic ions other than glutamate are synthesised, although in relatively minor amounts. These include γ -glutamylglutamine and glutathione (McLaggan *et al.*, 1990). Also, organic anions (e.g. 3-morpholino-1-propanesulfonic acid - MOPS) from the extracellular medium are accumulated by *E. coli* during the primary response to osmotic stress (Cayley *et al.*, 1989). Both of these means contribute to charge balance, by increasing number of anions in the cytoplasm as well as increasing the intracellular osmotic pressure. In one calculation the accumulated glutamate and MOPS fully accounted for the required charge balance (Cayley *et al.*, 1991).

In conclusion it is pertinent to note that the accumulations of glutamate or other anions and the efflux of putrescine (Günther and Peter, 1979; Dinnbier *et al.*, 1988; Cayley *et al.*, 1989) are transient responses to low water activity conditions that occur before the long-term, secondary responses.

1.2.4.2 Secondary response

Trehalose

The secondary response to osmotic stress, by *E. coli*, involves the carbohydrate trehalose. Early investigations showed elevated levels of carbohydrates in *E. coli* subjected to low water activity (Roller and Anagnostopoulos, 1982). Also, mutants defective in trehalose synthesis exhibit reduced osmotolerance (Giæver *et al.*, 1988). Therefore, following the primary response to osmotic stress, trehalose is synthesised and accumulated to balance osmotic pressure (Strøm *et al.*, 1986; Larsen *et al.*, 1987).

Dinnbier *et al.* (1988) found that for *E. coli* trehalose synthesis begins approximately thirty minutes after osmotic upshock, by the addition of 0.5M sodium chloride, and after two hours trehalose replaces most of the potassium glutamate. The interplay between potassium ions, glutamate and trehalose may, however, be more complex than this. Using continuous culture Welsh *et al.* (1991) showed that following osmotic activation of trehalose synthesis, the potassium ion concentration remained high (at the level of the primary response to osmotic stress), and the concentration of glutamate depended on the experimental conditions. Only under

nitrogen limitation did trehalose replace glutamate. Regardless of whether trehalose replaces potassium, glutamate or both, trehalose synthesis and accumulation appear to be the secondary responses to osmotic stress by *E. coli* in circumstances where no alternative organic compatible solute is available extracellularly.

Trehalose is an osmotic stress protectant in *E. coli* when endogenously synthesised, but not when supplied externally (Strøm *et al.*, 1986; Larsen *et al.*, 1987). Two enzymes, trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase, catalyse the two-step pathway that is osmotically induced (Giæver *et al.*, 1988).

The synthesis of trehalose is regulated at two levels. At the transcriptional level the genes *otsA* and *otsB*, which code for trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase respectively, are stimulated during osmotic stress (Giæver *et al.*, 1988). Activation is dependent on the sigma factor encoded by *rpoS* (Kaasen *et al.*, 1992) (refer to section 1.2.5 for further details). Also, at the functional level, trehalose-6-phosphate synthetase is activated by potassium ions (Giæver *et al.*, 1988). Hence, transcriptional and functional regulation increases trehalose synthesis in response to low extracellular water activity. Trehalose synthesis during osmotic stress is not feedback inhibited, hence surplus trehalose is excreted into the periplasm. There a periplasmic trehalase (TreA) converts the excreted trehalose to glucose that is accumulated and reused (Styrvold and Strøm, 1991). TreA is also induced in osmotic stress conditions (Boos *et al.*, 1987). Thus a futile cycle of overproduction, excretion, degradation to glucose and reuse allows *E. coli* to regulate cytoplasmic trehalose levels during osmotic stress. Under normal conditions *E. coli* metabolises trehalose to glucose in the cytoplasm (Becerra de Lares *et al.*, 1977; Maréchal, 1984); this system is repressed in *E. coli* cells experiencing an osmotic stress, thereby avoiding a futile cycle of trehalose in the cytoplasm (Boos *et al.*, 1990).

The exclusive role of trehalose in osmotic adaptation has been questioned. Trehalose synthesis occurs under a variety of stress conditions (Van Laere, 1989; Wiemken, 1990; Hengge-Aronis *et al.*, 1991). Also trehalose synthesis requires *rpoS* for activation; *rpoS* is associated with gene expression upon the entry of cells into the stationary growth phase. Such evidence has lead several authors to question whether trehalose has a specific role in osmoadaptation, or is simply a general stress metabolite (Van Laere, 1989; Wiemken, 1990). However osmotolerance in *E. coli* is greatly reduced by mutations that result in the inability to synthesise trehalose (Giæver *et al.*, 1988). Therefore without exogenous compatible solutes, osmotically stressed *E. coli* cells do appear to use trehalose as a compatible solute.

To this point, this case study of the response of *E. coli* to low water activity conditions has been concerned with the physiological changes that occur in minimal media (devoid of exogenous compatible solutes). These physiological responses are summarised in Figure 1.5. The response to water activity stress differs when compatible solutes are exogenously provided.

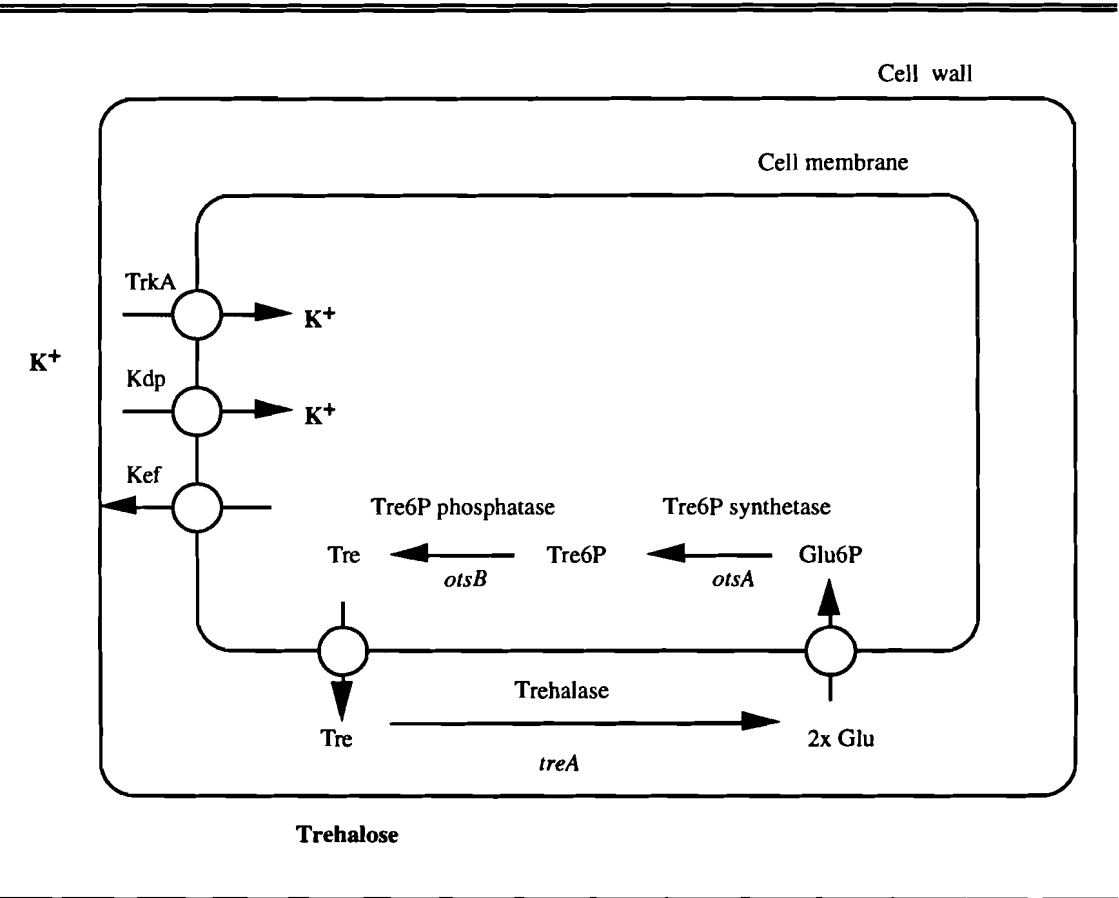


Figure 1.5: Summary of the main primary and secondary responses of *E. coli* to water activity stress, when compatible solutes are not available; adapted from Galinski (1995). TrkA, constitutive potassium ion uptake system; Kdp, inducible osmoregulated potassium ion uptake system; Kef, potassium efflux system; Glu, glucose; Tre, trehalose.

1.2.4.3 Exogenous compatible solutes

If compatible solutes are available extracellularly these will substitute for the responses described above. This review will concentrate mostly on the use of glycine betaine and proline in osmoregulation by *E. coli*; a brief description of the roles of ectoine and carnitine is also presented.

Betaine

Glycine betaine, generically known as 'betaine', is accumulated by *E. coli* in response to low water activity (Le Rudulier and Boulliard, 1983). Those authors showed that *E. coli*, and other enteric bacteria, accumulate betaine in response to osmotic stress. They also demonstrated that its accumulation removed the inhibition of growth due to osmotic stress. Unlike the situation with trehalose *E. coli* is unable to synthesise betaine. Therefore the use of betaine as an osmoprotectant depends on the extracellular availability and the presence of suitable uptake systems. Betaine is available widely in the environment, and *E. coli* is able to accumulate it using the ProP and ProU uptake systems (Cairney *et al.*, 1985a; Cairney *et al.*, 1985b). Despite the inability of *E. coli* to synthesise betaine *de novo*, it can accumulate precursors, such as choline and betaine aldehyde, and convert them to betaine.

It is likely that betaine is widely available to bacteria. From a laboratory perspective, many of the complex microbiological growth media used in laboratories contain yeast extract, which in turn contains betaine (Wohlfarth *et al.*, 1990). Considering foods as environments for bacterial growth, plants use betaine as a compatible solute themselves (Yancey *et al.*, 1982) - betaine was first discovered upon its isolation from sugar beet (Scheibler, 1869 cited in Galinski, 1993). Therefore foods of plant origin are likely to contain betaine. Also betaine is present in molluscs such as abalone (Olley and Thrower, 1977), fish muscle (Shewan, 1953), and in mammalian kidneys (Bagnasco *et al.*, 1986) where it is used in the osmoregulation of kidney cells, affording protection from the high solute concentration in urine. Interestingly this betaine also protects bacterial cells involved in urinary tract infections (Chambers and Kunin, 1985). Hence betaine is probably much more widely found in animals, and therefore foods derived from animals, than is currently realised. Finally, from an 'environmental' perspective, in marine environments betaine is accumulated by *E. coli* from sediments (Ghoul *et al.*, 1990); the source of this betaine is presumably detritus from decaying plant and animal matter or from bacteria capable of betaine synthesis. Therefore betaine appears to be widely available to *E. coli* for use as an osmoprotectant.

In *E. coli* betaine is accumulated using two transport systems: ProP (also known as PP II - proline porter II), derived from the *proP* gene, and ProU (also known as PP III - proline porter III), derived from the *proU* operon. The ProP and ProU systems were first found in *Salmonella typhimurium* (Menzel and Roth, 1980; Csonka, 1982; LeRudulier *et al.*, 1984), and as their name suggests were associated initially with proline transport. Later it was shown that both ProP and ProU were also

involved with the transport of betaine (Cairney *et al.*, 1985a; Cairney *et al.*, 1985b). A commonality between the osmotic response mechanisms of enteric bacteria was realised, and the *proP* and *proU* genes were also found in *E. coli* (Stalmach *et al.*, 1983; Gowrishankar, 1985). The roles of their gene products, ProP and ProU, as betaine transport systems in *E. coli* were also demonstrated (May *et al.*, 1986). ProP and ProU are low and high affinity transport systems for betaine respectively (Cairney *et al.*, 1985a; Cairney *et al.*, 1985b).

The ProP porter is a single protein that is embedded in the cytoplasmic membrane (Culham *et al.*, 1993). Energy for transport by ProP is derived from the proton motive force. Although ProP has a relatively low affinity for its substrates, it is thought to account for most of the solute uptake from the medium during the osmotic stress response by *E. coli* (Stirling *et al.*, 1989).

The ProU porter is structurally more complex than ProP; ProU is encoded by three genes: *proV*, *proW*, and *proX* for the proteins: ProV, ProW and ProX respectively (Gowrishankar, 1989; Stirling *et al.*, 1989). ProV is the energy coupling component of the ProU porter that hydrolyses ATP (May *et al.*, 1989). The ProV protein is associated with the inner side of the cytoplasmic membrane. The ProW protein is integrated into the membrane and, by analogy with other similar porter

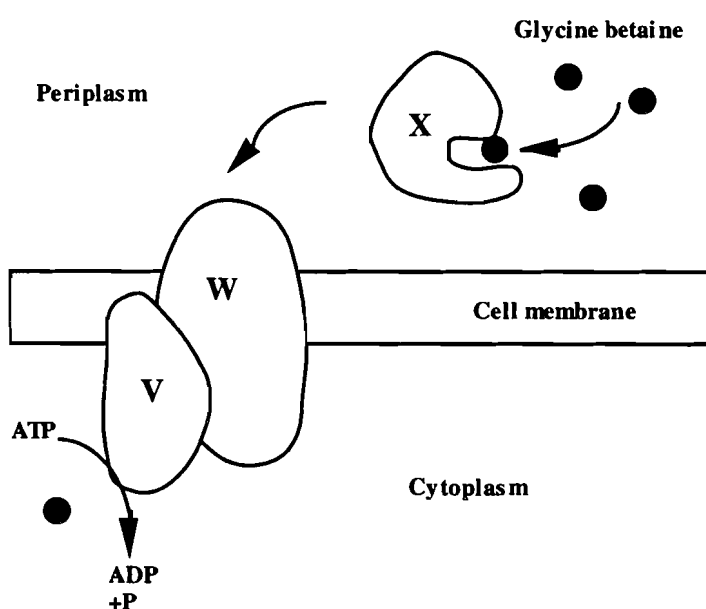


Figure 1.6: A schematic diagram of the operation of the multicomponent (ProV, ProW and ProX) ProU transport system; adapted from Lucht and Bremer (1994).

systems, is thought to couple with ProV to form the porter part of the ProU transport system (Higgins, 1992; Lucht and Bremer, 1994). Finally ProX is a periplasmic protein that captures and feeds betaine to ProW for transport inside the cell. The ProU porter is summarised in Figure 1.6. The primary function of ProU is thought to be the recapture and recycling of leaked osmolytes (Stirling *et al.*, 1989).

The concentration of betaine in the cytoplasm is correlated with extracellular water activity (LeRudulier and Bouillard, 1983); betaine levels increase when the water activity decreases. To achieve this control the betaine transport systems are osmotically regulated, both transcriptionally and functionally. ProP is produced constitutively in *E. coli*, but low water activity increases expression approximately three-fold (Cairney *et al.*, 1985a). Also high osmotic strength increases the activity of the ProP system (Milner *et al.*, 1988). Transcription of *proU* occurs at significant levels during osmotic stress only (Cairney *et al.*, 1985b). This osmotic control of ProP and ProU regulates betaine levels with osmotic stress. Other factors also contribute to the control of betaine levels including betaine efflux systems (Koo *et al.*, 1991) and the choline-betaine pathway (described below).

E. coli is not able to synthesise glycine betaine *de novo*. Although, previously, accumulation from complex media containing betaine or precursors has been confused with synthesis (Imhoff and Rodriguez-Valera, 1984). It is, however, capable of accumulating and converting the precursors - choline and betaine aldehyde (Strøm *et al.*, 1983). Osmoprotection by this means is called the choline-betaine pathway and is governed by a group of four (*bet*) genes.

The betaine precursors are not compatible solutes themselves, and therefore do not relieve osmotic stress unless converted to betaine (Styrvold *et al.*, 1986). The pathway for this conversion is summarised in Figure 1.7. The first step in the choline-betaine pathway requires oxygen, whilst the second does not. Therefore osmoprotection due to synthesis of betaine from choline is only possible in aerobic conditions, whilst both betaine and betaine synthesised from betaine aldehyde provide osmoprotection in anaerobic as well as aerobic conditions (Landfald and Strøm, 1986). Betaine and its precursors cannot be degraded by *E. coli* (Perroud and LeRudulier, 1985; Landfald and Strøm, 1986); therefore, the role of the choline-betaine pathway lies solely in osmoregulation.

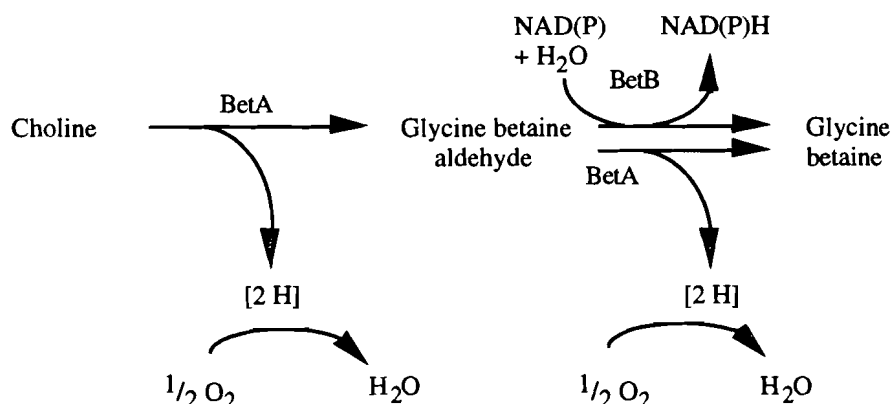


Figure 1.7: The choline-betaine pathway; adapted from Lucht and Bremer (1994). BetA, choline dehydrogenase; BetB, glycine betaine aldehyde dehydrogenase.

E. coli has two uptake systems for choline that, similarly to the transport systems for betaine, have low and high affinities for their substrate respectively (Styrvold *et al.*, 1986). Both of these systems are induced by osmotic stress, but do not require the presence of the substrate - choline. Osmotic induction is inhibited by chloramphenicol, therefore osmotic regulation of uptake is transcriptional, rather than activation of proforms (Styrvold *et al.*, 1986). The high affinity transport system (BetT) is encoded by the *betT* gene that has been sequenced (Andresen *et al.*, 1988; Lamark *et al.*, 1991). The BetT transport system is driven by the proton motive force (Lamark *et al.*, 1991) and along with osmotic regulation of *betT* expression, osmotic stress also increases BetT transport activity. Details of the low affinity system are lacking in the literature. Evidence for its existence comes from choline uptake kinetics data, and that low affinity uptake occurs in cells with mutations in the *betT* region (Styrvold *et al.*, 1986). It is also known that the low affinity system for choline transport is genetically distinct from the betaine transport systems (Strøm *et al.*, 1986) because mutants defective in both low and high affinity transport of choline have the full betaine uptake capacity (Styrvold *et al.*, 1986).

The two step oxidation of choline to betaine (Figure 1.7) in *E. coli* is catalysed by two enzymes (Landfald and Strøm, 1986). Both steps can be catalysed by a membrane-bound, oxygen dependent and electron transfer-linked choline dehydrogenase, that catalyses both steps at approximately the same rate. The second step can also be catalysed by a soluble, NAD-dependent glycine betaine aldehyde dehydrogenase. This betaine aldehyde dehydrogenase is highly specific for betaine aldehyde, and is a salt tolerant enzyme that retains 50% of its maximal activity at 1.2 M

potassium (Falkenberg and Strøm, 1990). The choline dehydrogenase and betaine aldehyde dehydrogenase are coded by genes denoted *betA* and *betB* respectively (Styrvold *et al.*, 1986; Andresen *et al.*, 1988). These two *bet* genes lie close to the *betT* gene in the *E. coli* genome.

The choline-betaine pathway is regulated osmotically; transcription of the *bet* genes is induced by osmotic stress (Landfald and Strøm, 1986; Eshoo, 1988). Whilst expression of the high affinity transport system does not require choline, full expression of the enzymes catalysing the conversion of choline to betaine requires the presence of choline in the growth medium (Landfald and Strøm, 1986).

Chloramphenicol evidence shows that regulation of *betA*, *betB* and *betT* is at the transcriptional level (Strøm *et al.*, 1986; Styrvold *et al.*, 1986), and depends on many factors: extracellular water activity and choline, as already noted, as well as betaine, temperature and oxygen (Eshoo, 1988). A fourth gene, *BetI* (close to *betA*, *betB* and *betT* in the genome), is implicated in the regulation of the pathway (Lamark *et al.*, 1991). *BetI* encodes a protein involved in the regulation of *betT* and possibly also plays a role in regulating *betA* and *betB* expression. These mechanisms contribute to regulation of intracellular betaine concentrations in *E. coli* in response to extracellular water activity conditions, when the choline-betaine pathway is active.

Proline

Proline can be used by *E. coli* as a sole nitrogen and carbon source or as a building block in protein synthesis. Additionally, proline plays a role in the osmotic stress response. Growth stimulation by proline of enteric bacteria in response to water activity stress was first demonstrated for *Salmonella oranienburg* (Christian, 1955a) and later, specifically for *E. coli* (Britten and McClure, 1962). Accumulated proline acts as a compatible solute under conditions of water activity stress by extending the water activity limit for growth, improving the growth rate and reducing the time after osmotic shift for resumption of growth (Dharies and Anagnatopolous, 1978; Csonka, 1981; Csonka, 1982). This role of proline is often overlooked because the improvements in growth produced by proline are qualitatively similar, but less dramatic, to those produced by betaine (LeRudulier and Bouillard, 1983; LeRudulier *et al.*, 1984; Cairney *et al.*, 1985a).

In *E. coli*, proline is transported by three porters. The main proline porter is PutP (Ratzkin *et al.*, 1978; Wood and Zadworney, 1979). This porter is specific for proline, is proline inducible and sodium dependent (Britten and McClure, 1962;

Rowland and Tristram, 1975; Ratzkin *et al.*, 1978; Cairney *et al.*, 1984). PutP is active under conditions of osmotic stress, but no more so than under normal conditions where proline is exogenously present (Dunlap and Csonka, 1985). Therefore PutP transport does not appear to have a specific role in osmotic accumulation of proline. The remaining two proline porters have been described for betaine transport - ProP and ProU. Both ProP and particularly ProU have higher affinities for betaine than proline (Grothe *et al.*, 1986). This has been speculated as the reason betaine is a more effective osmoprotectant than proline in *E. coli* (Milner *et al.*, 1987). This aside, proline is transported during osmotic stress by these two systems (Dunlap and Csonka, 1985). Of the two porters, ProP is the main contributor to proline accumulation, because it has a greater affinity for proline than does ProU (Milner *et al.*, 1987). Regulation by osmotic pressure of ProP and ProU, as described for betaine, contributes to the control of the intracellular concentration of proline during osmotic stress.

Unlike betaine, proline can be catabolised by *E. coli* (Frank and Ranhand, 1964). This has lead to speculation whether the catabolic pathway responsible (Figure 1.8) is repressed during osmotic stress to avoid futile cycles. Evidence suggests that osmotic stress does not reduce the rate of proline catabolism significantly, but does inhibit proline dehydrogenase activity in a non-specific way (Milner *et al.*, 1987; Csonka, 1988; Deutch *et al.*, 1989). Hence proline catabolism may antagonise proline uptake through ProP and ProU, the net result defining the intracellular concentration of proline during osmotic stress.

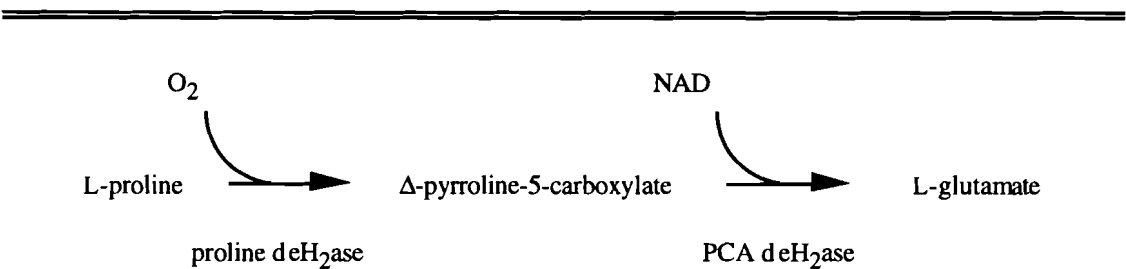


Figure 1.8: The catabolic pathway of proline degradation by *E. coli*. This two-step pathway converts proline to glutamate, the means by which proline is used as a sole carbon or nitrogen source. Enzymes proline deH₂ase (proline dehydrogenase) and PCA deH₂ase (pyrroline-5-carboxylic acid dehydrogenase) catalyse the pathway.

In *E. coli* most studies of exogenously supplied compatible solutes have been for proline and betaine. Also proline and betaine derivatives have been investigated Abdel-Ghaney *et al.*, 1993; Peddie *et al.*, 1994; Randall *et al.*, 1996; Reese *et al.*, 1996). Generally derivatives of proline and betaine are less effective as compatible solutes than their parent molecules, some are even antibiotic. Many of these investigations have been targeted at finding antibiotic types for use in treating urinary tract infections. Also studies on derivatives have been used to gain insights into the importance of certain structural components of compatible solutes to their efficacy as osmoprotectants and for transport by porters.

Proline, betaine and their derivatives are not the only exogenously supplied compatible solutes that *E. coli* can use. Other compatible solutes, if present in the external medium and suitable uptake mechanisms exist, can provide osmoprotection. Other compatible solutes that have been investigated for *E. coli* include, ectoine and carnitine.

Other compatible solutes

Osmoprotection of *E. coli* by exogenously supplied ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) has been demonstrated (Jebbar *et al.*, 1992). This compatible solute was first discovered as an osmoprotectant in *Ectothiorhodospira halochloris* (Galinski *et al.*, 1985), and later also in *Vibrio costicola* (Regev *et al.*, 1990). Ectoine protects *E. coli* from water activity stress as effectively as betaine. Osmotically stressed *E. coli* cells accumulate ectoine using both the ProP and ProU porters, although ectoine accumulation has been attributed mainly to ProP (Jebbar *et al.*, 1992). Like betaine, ectoine cannot be metabolised by *E. coli*, therefore its sole role is in osmoregulation. However, for other organisms protective properties other than for hyperosmolarity have been demonstrated, for example in *Streptomyces* species ectoine appears to protect cells from their own antibiotic (Inbar and Lapidot, 1988).

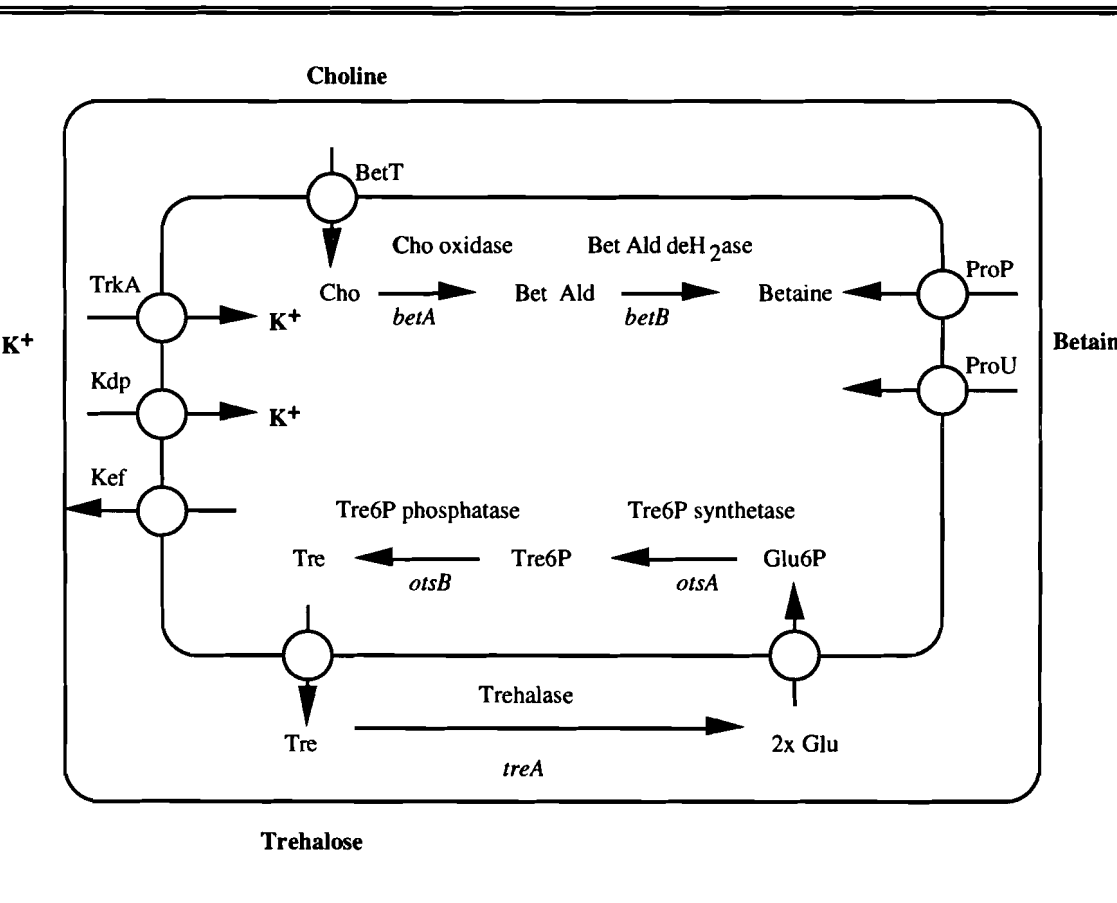
Carnitine (vitamin B_T) is accumulated by *E. coli* during osmotic stress, and relieves inhibition of growth due to that osmotic stress (Jung *et al.*, 1990). Carnitine is found widely in animals (Shewan, 1953; Fraenkel, 1954; Olley and Thrower, 1977) and therefore likely to be present in foods of animal origin. Its presence in foods, as in the case for betaine, makes carnitine especially relevant to this review. *E. coli* can metabolise carnitine to γ -butyrobetaine (Seim *et al.*, 1982 cited by Jung *et al.*, 1990). The pathway responsible for the conversion of carnitine to γ -butyrobetaine is,

Figure 1.9: (facing page) A schematic diagram summarising the main aspects of the physiological response of *E. coli* to water activity stress; adapted from Galinski (1995). TrkA, constitutive potassium ion uptake system; Kdp, inducible osmoregulated potassium ion uptake system; Kef, potassium efflux system; BetT, choline transport system; ProP and ProU, transport systems for compatible solutes; Cho; choline, Bet Ald, betaine aldehyde; Glu, glucose; Tre, trehalose.

however, inhibited during osmotic stress (Jung *et al.*, 1990). Therefore the accumulation of carnitine is responsible for the observed osmoprotection afforded by carnitine, not γ -butyrobetaine.

In conclusion, one point that should be highlighted is the high frequency with which reference to the ProP and ProU transport systems recur. In the general discussion of compatible solutes mention was made of the limited types of molecules that are used as compatible solutes in plants, animals and bacteria. Presumably due to structural similarities between these molecules, compatible solute porters are able to transport most or all of them. Therefore in *E. coli* it appears that the ProP and ProU porters satisfy the cells' solute accumulation needs, during the physiological response to low water activity, regardless of the type of compatible solute supplied.

A summary of the physiological responses of *E. coli* to water activity stress is given in Figure 1.9, that builds on Figure 1.5 by combining both the 'in house' response and the mechanisms involved when compatible solutes are available extracellularly.



1.2.5 General stress response

Most is known of general stress responses in *E. coli* for cells entering the stationary phase. Entry to the stationary phase coincides with the synthesis of a set of stress proteins. These stress proteins render *E. coli* more resistant to multiple stresses, hence the term 'general' stress response. Stress conditions for which protection is given include: temperature shock, oxidation, water activity stress, pH stress and starvation (Hengge-Aronis, 1993; Cheville *et al.*, 1996). Because this protection is also associated with the stationary phase, the resistance gained benefits cell survival, rather than growth. But the factor that regulates stationary-phase-induced resistance has also been implicated in stress responses during growth (Hengge-Aronis *et al.*, 1991).

One of the stationary phase induced genes in *E. coli*, *rpoS*, is important in regulating the general stress response during stationary phase. The *rpoS* regulon is fairly large and regulates many genes; early literature on the topic can be confusing because it was 'discovered' several times, each time being given a different name. Therefore *rpoS* has also been known as:

- katF* regulation of catalase HPII and exonuclease III (Loewen and Triggs, 1984; Sak *et al.*, 1989)
- appR* needed for expression of acid phosphatase (Touati *et al.*, 1986)
- nur* for near UV-resistance (Sammartano *et al.*, 1986)

These and many other stationary phase induced resistances are under the control of *rpoS*, and generally it is felt that the majority of *rpoS*-regulated genes are still unknown (Lange and Hengge-Aronis, 1991; McCann *et al.*, 1991). This regulation by *rpoS* is effected through its gene product, the putative sigma factor (σ^s).

In addition to stationary phase resistance, *rpoS* and σ^s have been implicated in the physiological response to water activity (Hengge-Aronis *et al.*, 1991). As previously stated the trehalose synthesis genes *otsA* and *otsB* require both osmotic stress and σ^s for expression. This in turn must lead to a consideration of the role of *rpoS* in stress responses in phases of growth other than the stationary phase. Research in this area is ongoing and currently little can be said definitively. For the purposes of this review it is sufficient to recognise that in addition to the specific physiological responses described, other more general stress responses may operate during osmotic stress. Some of the specific responses described may in fact be general stress responses. This is especially pertinent to the role of trehalose as a compatible solute during the secondary response to osmotic stress.

1.3 Compatible Solute Mechanisms

This review has described the role of compatible solutes in the physiological response to reduced water activity conditions solely as one of osmolyte, balancing intracellular and extracellular water activity so that the necessary turgor pressure is maintained. However, several lines of evidence suggest that their role may extend beyond this. Compatible solutes may also protect macromolecules during stress conditions.

Compatible solutes do not hinder the function of 'normal' salt sensitive enzymes (Pollard and Wyn Jones, 1979; Warr *et al.*, 1984). Also, compatible solutes protect enzymes from inhibitory salt concentrations (Luard, 1983; Warr *et al.*, 1984; Manetas *et al.*, 1986). Ionic salts denature proteins, probably due to electrostatic disruptions caused by ionic interactions between salts and amino acid residues (Arakawa and Timasheff, 1985). Compatible solutes protect against this denaturation. For example, Warr *et al.* (1984) showed that for glutamate synthetase from the marine cyanobacterium *Nodularia haveyana*, activity is inhibited by sodium chloride concentrations of 0.7 M and higher, but with 1.0 M betaine, glutamate synthetase activity is restored by up to 30%. This apparent protective ability of compatible solutes extends beyond water activity stress. Protection of macromolecules from freezing, heating and drying has also been observed (Back *et al.*, 1979; Gekko and Timasheff, 1981a; Paleg *et al.*, 1981; Coughlan and Heber, 1982; Lippert and Galinski, 1992; Carpenter, 1993). Enzymes from bacteria, plants and animals are all protected in this manner by compatible solutes. This provides further evidence for the convergent evolution of compatible solute molecules in these three very different types of organisms.

The mechanism by which the protective effect of compatible solutes is elicited is unknown. One observation that has been fundamental in the attempt to solve the puzzle is that compatible solutes are excluded from the immediate layer of water that surrounds the macromolecules that they stabilise (Arakawa and Timasheff, 1983). This observation has been termed preferential exclusion (Lee and Timasheff, 1981). Preferential exclusion has been observed for many molecules including: sugars, polyhydric alcohols, amino acids and betaines (Arakawa and Timasheff, 1983; Gekko and Timasheff, 1981a; Gekko and Timasheff, 1981b; Lee and Timasheff, 1981). Two models have been proposed to explain the preferential exclusion observation: the 'surface tension' model (Arakawa and Timasheff, 1985) and the 'high density water' model (Wiggins 1990). Both of these models have their foundations in simple

thermodynamic theories. They explain the preferential exclusion observation and provide insights into the stabilisation of proteins by compatible solutes.

1.3.1 Surface tension

The surface tension model incorporates Gibbs adsorption isotherm, Le Chatelier's principle and the observation that compatible solutes increase the surface tension of water. Increases in the surface tension of water caused by compatible solutes have been measured (Washburn *et al.*, 1926) and may be due to an increase in the surface free energy of water caused by compatible solutes. If true, Gibbs adsorption isotherm (which states that solutes that raise the surface energy are depleted at surfaces (Morowitz, 1970)) dictates that compatible solutes are excluded from the water-macromolecule interface. In this manner the surface tension model accounts for the preferential exclusion observation.

According to Le Chatelier's principle the preferential exclusion of compatible solutes is thermodynamically unfavourable. Le Chatelier's principle dictates that when a system in equilibrium is subjected to a disturbance that upsets equilibrium, the system responds in a direction that tends to counteract the disturbance and restore equilibrium in the system. Using Le Chatelier's principle the surface tension model can also be used to explain the stabilisation of proteins by compatible solutes. In the surface tension model the 'disturbance' is the exclusion of compatible solutes from the immediate surface of the protein. Therefore, to minimise the disturbance and restore equilibrium the volume from which compatible solutes are excluded must be minimised. That is, the area of the water-protein interface must be minimised. The surface area of globular proteins is smallest when they are in their native state (compared to the denatured state). Therefore, contact with the solvent systems under consideration should displace the native-denatured equilibrium towards the native. Hence proteins are stabilised by compatible solutes. This surface tension theory has been applied on several separate occasions for various types of molecules: sugars, polyhydric alcohols, salts and some amino acids (Gekko and Timasheff, 1981a; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983).

1.3.2 High density water

The high density water theory (Wiggins, 1990) provides an alternative and equally plausible explanation for the preferential exclusion observation and the stabilisation of proteins by compatible solutes. Simply, in this hypothesis a structurally modified type of water - high density water - exists around proteins and other macromolecules found in cells. This occurs because counterions belonging to macromolecules are unevenly distributed in the cellular water. The counterions tend to 'cling' closely to macromolecules, rather than floating free in the cytoplasm. This uneven distribution of counterions, from chemical potential laws, is expected to result in the formation of high density water around the macromolecules. The existence of high density water has been demonstrated in gel experiments using polyethylene glycol (Wiggins and Van Ryn, 1986).

Low density water, another type of structurally modified water is also a part of Wiggins (1990) theory. Due to the need for chemical equilibrium, the density of water that is not immediately adjacent to protein surfaces must change oppositely. That is, for every volume of high density water formed there must be an equal volume of low density water formed. However, in cells the volume occupied by high density water is small compared to the total volume of cellular water. Therefore, the density of the remaining water is mostly unchanged; most cellular water (excepting the high density fraction) is 'slightly low density' water or essentially 'normal' water.

The high density water around protein molecules, as its name suggests, is structurally very different to normal water, and has different properties. One difference is that it is less soluble for compatible solutes, therefore compatible solutes tend to be excluded from high density water. Preferential exclusion of compatible solutes from the immediate surface of proteins can therefore be explained using this model.

The high density water theory can also be used to explain the hydrophobic effect. High density water encourages the aggregation of hydrophobic molecules (Wiggins, 1990). It is therefore not a very great step to propose that high density water may also stabilise native structure of globular proteins, the folding of which is thought to be driven by the hydrophobic effect, by forcing hydrophobic moieties to the inside of the protein structure.

Two hypotheses, therefore, exist to explain protein stabilisation. These theories can be further extended to explain the promotion of growth by compatible solutes: if proteins are more stable, then the chemical reactions that result in growth

can occur more readily and growth rates will be improved. However, an alternate hypothesis for the mechanism of growth promotion by compatible solutes exists: the 'cytoplasmic volume' theory.

1.3.3 Cytoplasmic volume

This hypothesis, unlike the two presented above, does not explain the preferential exclusion of compatible solutes or stabilisation of proteins. But it does incorporate the preferential exclusion observation to explain the increase in growth rate observed with compatible solutes. Cayley *et al.* (1991) observed that growth rate and cytoplasmic volume are correlated in *E. coli* cells stressed by extracellular water activity, when the growth rate decreased with decreasing cytoplasmic volume. They also found that accumulation of compatible solutes increased the cytoplasmic volume, and that this was accompanied by an increase in growth rate. The growth rate and cytoplasmic volume correlations were equivalent when only water activity stress was applied and, when betaine was present. They propose therefore, that cytoplasmic volume determines growth rate. The mechanism proposed to explain this is that increased cytoplasmic volume reduces the molecular crowding produced by low levels of free water in the cytoplasm, allowing reactions to proceed at a faster rate, manifest as increased growth rates.

In the cytoplasmic volume hypothesis, not unlike the low and high density water model, there are said to be two types of water: bound water and free water. Compatible solutes are excluded from the bound water fraction that occurs around macromolecules (not unlike high density water). This exclusion is greater than is the case for the solutes that they replace (glutamate, potassium ions and MOPS in the case of *E. coli*) and so all the compatible solutes are forced into one section of the water (the free water). This means that compatible solutes make a greater contribution to the intracellular water activity than the solutes they replace (that are not, or less excluded from the bound water fraction), resulting in an influx of water. This line of reasoning has also been used to explain the greater efficacy of betaine as a compatible solute over proline. The preferential exclusion of betaine is greater than that for proline. Therefore, betaine makes a larger contribution to intracellular water activity than proline and is hence more effective at increasing the volume of water inside osmotically stressed cells.

Although, as stated at the outset of this section, the mechanism of protection by compatible solutes is not clear, it is encouraging that the hypotheses so far proposed

are compatible and do not exclude each other. This is highlighted by noting similarities between the cytoplasmic volume hypothesis and the high density water model. In the future it may be possible to draw those two hypotheses together, to describe the physiological effect of water activity on bacterial growth and the way in which compatible solutes modify it.

1.4 Objectives

Concerns over the rising incidence of foodborne disease have lead to increased research attention in related disciplines, such as predictive modelling. Predictive models are very useful for describing the responses of bacteria to many environmental conditions, but lack a mechanistic basis. A mechanisitic understanding of those responses would improve the reliability of predictive models. Therefore the objective of this study was to increase the mechanistic understanding of the bacterial growth response to extracellular water activity conditions. Initially, a mechanistic interpretation based on energy diversion from growth to maintenance in response to osmotic stress was developed and evaluated, using as a model organism *E. coli*, because of the importance of pathogenic strains in food poisoning world-wide.

CHAPTER 2: HYPOTHESIS DEVELOPMENT

2.1 Introduction

The growth rate of bacteria is inhibited by low water activity conditions, but the effect is partially reversed by compatible solutes. The mechanism for this is unknown, but accurate quantitative description of those effects may provide insights.

2.1.1 Square root models

The growth rate response of bacteria to extrinsic conditions can be quantitatively described using mathematical models, such as the square root model that was introduced briefly in section 1.1.4. It is a kinetic model, of the power function type. Square root models are also known as Bělehrádek models, after Jan Bělehrádek, who was the first to propose power function equations to describe the effect of temperature on the rate of biological processes (Bělehrádek, 1926, 1935):

$$y = \frac{a}{(t - \alpha)^d} \quad (2.1)$$

where: a, d are constants
 y is time
 t is temperature
 α is the temperature threshold, from which the temperature scale is reckoned (see Table 2.1)

Or, expressed in terms of rate, Equation 2.1 becomes:

$$r = \frac{1}{a}(t - \alpha)^d \quad (2.2)$$

where: a, d, t and α have been defined
 r is reaction rate (1/time), growth rate in the case of bacterial growth

The Bělehrádek equation has been used extensively by zoologists (Ross, 1987), but until recently had not been used in microbiology. Its use in microbiology began in 1982, when the square root model for bacterial growth was proposed (Ratkowsky *et al.*, 1982). Although unaware of doing so, the originators of the square root model had re-invented a special case of the Bělehrádek equation (Ross, 1987). The square root model, as it was originally proposed (Ratkowsky *et al.*, 1982), is shown below (Equation 2.3). By comparing Equations 2.2 and 2.3 it can be seen that the square root model is a special case of the Bělehrádek equation where the exponent equals 2 (parameter ' d '); b is $\sqrt{1/a}$ and T_o is α .

$$\sqrt{r} = b(T - T_o) \quad (2.3)$$

where: r is reaction rate at temperature T
 b is a constant, to be fitted
 T is temperature
 T_o is a conceptual temperature of no metabolic significance (theoretical temperature, where r is zero, see Table 2.1)

The square root model was based on the observation that a plot of the square root of the rate of nucleotide breakdown in carp muscle versus temperature was linear (Ohta and Hirahata, 1977). By chance the same relationship was observed to describe bacterial spoilage of fish (Olley, 1983). The square root model was then applied to other bacterial growth rate data and found to describe the effect of temperature on bacterial growth rate for many species well (Ratkowsky *et al.*, 1982). And, whilst empirical, the square root model gave superior descriptions of bacterial growth, compared to the Arrhenius equation (Equation 1.4) that had previously been used for this purpose (Ratkowsky *et al.*, 1982). No exception to the applicability of the square root model in the sub-optimal temperature region for any strain of bacteria or mould has yet been identified (McMeekin *et al.*, 1993).

The square root model, in its original form (Equation 2.3), was only relevant for growth of bacteria at temperatures below their optimum, often referred to as the sub-optimal temperature range. In order to describe the bacterial growth response over the entire temperature range for growth, Equation 2.3 was extended to describe the growth response for temperatures above the optimum (super-optimal temperatures) (Ratkowsky *et al.*, 1983):

$$\sqrt{r} = b' (T - T_{min}) \{1 - \exp[c(T - T_{max})]\} \tag{2.4}$$

where: r and T are as previously defined
 b' is a constant to be fitted
 T_{min} is a conceptual temperature, where r is zero, at the low temperature extreme of the biokinetic temperature range (see Table 2.1)
 c is a constant, to be fitted
 T_{max} is a conceptual temperature, where r is zero, at the high temperature extreme of the biokinetic temperature range

This extended version of the square root model has been termed the four parameter square root model (McMeekin *et al.*, 1993). The four parameter square root model describes growth of bacteria over the entire temperature range for growth, often referred to as the biokinetic range.

In graphical form Equation 2.4 describes the curve shown in Figure 2.1. When presented in this manner the parameters of the square root model can be visualised.

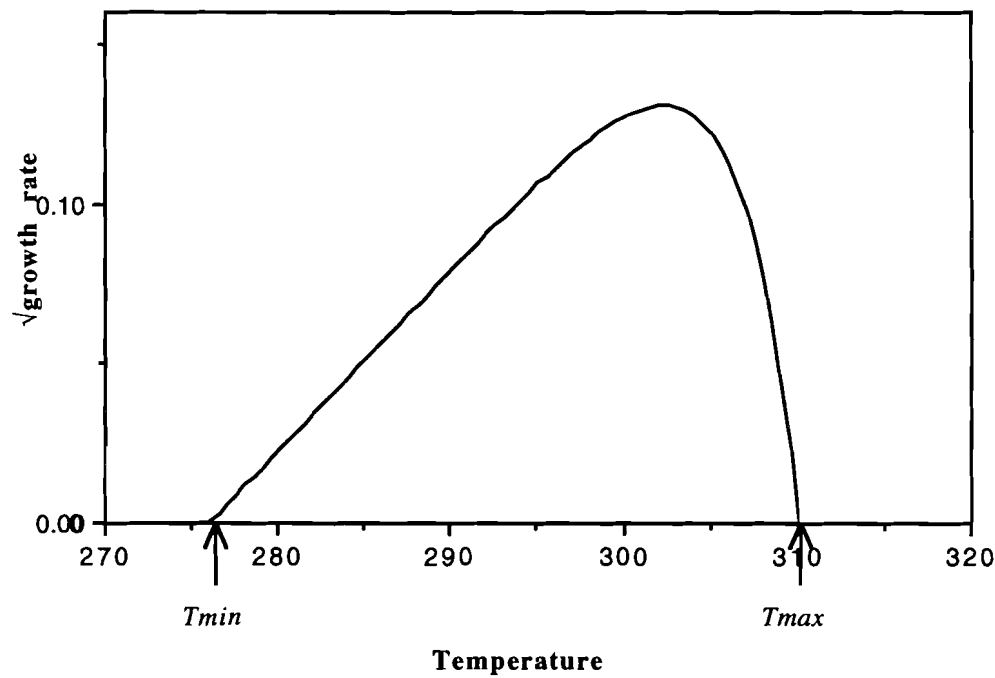


Figure 2.1: The line described by the square root model (Equation 2.4). When presented graphically, the parameters, T_{min} and T_{max} , of the four parameter square root model can be easily visualised.

Figure 2.1 shows that T_{min} is defined by the low temperature intersection of the square root of rate plot with the x-axis (also see Table 2.1). It should be stressed that T_{min} is a notional value; it is not the actual minimum temperature at which growth is observed. T_{min} is the theoretical temperature at which growth rate equals zero, notionally equivalent to Bělehrádek's temperature threshold. This definition of T_{min} has been stressed because some misuse of the parameter, probably due to misleading notation, has occurred (Ingraham *et al.*, 1983, Rosso *et al.*, 1993; Rosso *et al.*, 1995). Theoretically this notional T_{min} and the observed minimum temperature for growth should be close to identical. However, in practice this is not the case. Generally T_{min} is a few degrees lower than the observed minimum temperature for growth. For psychrophiles and psychrotrophs T_{min} is usually at or below 0°C. Therefore, freezing of water that in turn results in growth limiting water activities has been used to explain the non-convergence of T_{min} and observed minimum growth temperatures. However, for mesophiles and thermophiles this explanation is not valid, and data for these also show the anomaly between T_{min} and observed minimum growth temperatures. Therefore, some other explanation may be appropriate, possibly an inability of the protein synthesising apparatus to assemble correctly (Ross, 1987) or some other dysfunction at the metabolic level.

Although no biological significance for T_{min} has been claimed, it has been shown to be remarkably constant for any particular bacterial species. This has led to the proposal that T_{min} is an inherent property of microorganisms (McMeekin *et al.*, 1993). The constancy of T_{min} has been demonstrated for many species including, *Staphylococcus xylosus* (McMeekin *et al.*, 1987), *Halobacterium* spp. (Chandler and McMeekin, 1989a), *Aeromonas hydrophila* (Hayward, 1990), *L. monocytogenes* (Ross, 1993), *S. aureus* (Ross, 1993), *V. parahaemolyticus* (Miles 1994) and *Pseudomonas* sp. (Neumeyer, 1995). Also T_{min} is constant with changes in water activity and pH (McMeekin *et al.*, 1987; Chandler and McMeekin, 1989a; Ross, 1993; Neumeyer, 1995). This constancy of T_{min} has been exploited in square root modelling because it simplifies the modelling process. For any given species T_{min} need only be determined once for incorporation into a predictive model that is relevant for potentially all environmental conditions.

Similar to T_{min} , T_{max} is defined by the intersection of the square root of rate plot with the x-axis, but this time at the upper temperature limit. However, unlike T_{min} , T_{max} and the observed maximum temperature for growth are generally very similar.

Table 2.1: Clarification of the definitions of α , T_o and T_{min}

Parameter	Explanation
α	<ul style="list-style-type: none">• proposed by Belehradek (1935) as a temperature threshold from which temperature is reckoned.• has been equated with another term used by Belehradek: ‘biological zero’ (Ross, 1987). But, from original papers it is not clear whether α is biological zero. Therefore due to this confusion, and because the term biological zero implies that it is the temperature where biological processes cease, the term biological zero should be avoided.• defined as the theoretical temperature, where development time (y in Equation 2.1) is infinite. Determined by extrapolation.
T_o	<ul style="list-style-type: none">• proposed by Ratkowsky <i>et al.</i> (1982) as a temperature threshold from which temperature is reckoned.• defined as the theoretical temperature, where the growth rate (r in Equation 2.3) is zero. Determined by extrapolation; where regression line between \sqrt{r} and temperature, at sub-optimal growth temperatures, intersects the temperature axis.• notation considered confusing, the ‘0’ implies 0°C, which is incorrect.
T_{min}	<ul style="list-style-type: none">• proposed by Ratkowsky <i>et al.</i> (1983) as a temperature threshold from which temperature is reckoned.• originated as new notation for T_o, to overcome confusion with 0°C.• defined as the theoretical temperature, where growth rate (r in Equation 2.4) is zero. Determined by fitting Equation 2.4 (temperature intercept that gives the least mean square error of all parameters in Equation 2.4).• notionally equivalent to T_o, but in practice minor differences have been noted (Neumeayer <i>et al.</i>, 1997), due to differences in the method used for estimation.

Once square root modelling had been applied successfully to describe the effect of temperature on bacterial growth for the biokinetic range, the model was further extended to incorporate the effects of water activity (McMeekin *et al.*, 1987). This yielded the expression:

$$\sqrt{r} = d\sqrt{(a_w - a_{w\min})(T - T_{min})}$$

(2.5)

where:

r , T and T_{min} are as defined previously

d is a constant, to be fitted

a_w is water activity

$a_{w\min}$ is the theoretical minimum water activity for growth

Note that similar to T_{min} , $a_w min$ is also a notional value, defined at the point where a growth rate plot intersects the x-axis. However, in this case, water activity replaces temperature as the independent variable. This is illustrated graphically in Figure 2.2.

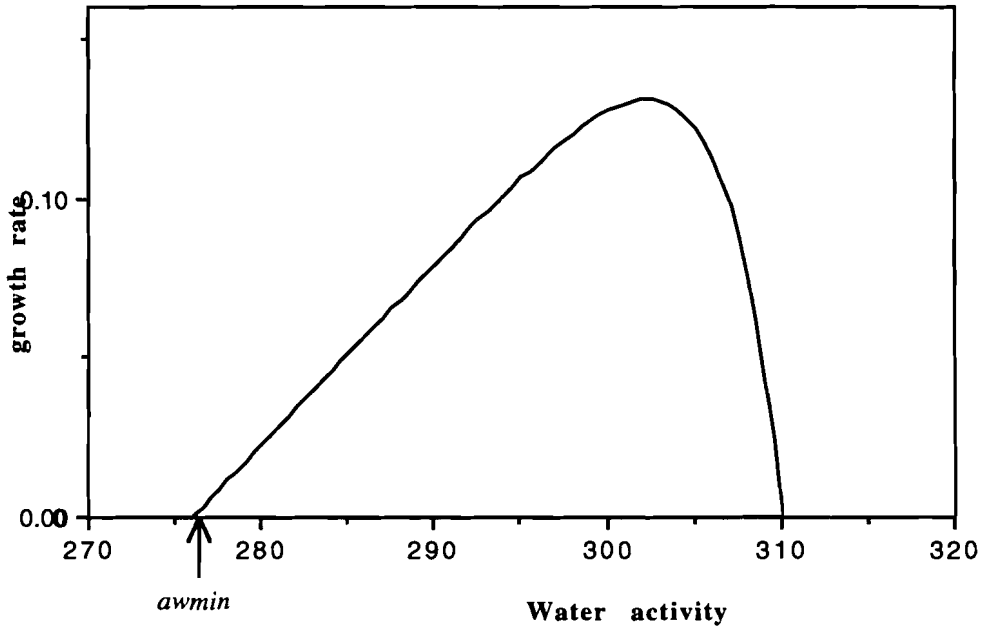


Figure 2.2: Graph showing the determination of the $a_w min$ parameter for use in Equation 2.4. For some species the optimum water activity value approximates 1.000, hence, only the straight line portion of the curve is realised.

Other square root models describing pH effects (Adams *et al.*, 1991) and a combined model for temperature, water activity and pH (McMeekin *et al.*, 1992) have also been developed. However, those models are not presented because they are not relevant to the analyses performed in this study.

2.2 Materials and Methods

For details of strains, media and equipment see Appendix A.

2.2.1 Influence of a_w and betaine on *E. coli* growth rate

A washed cell suspension of *E. coli* SB1 was prepared according to Appendix A.3 and inoculated into 150 mL minimal medium in 250 mL conical flasks, held at 37°C in shaking waterbaths (42 rpm \pm 10%). There were 30 flasks containing broths of varying water activity, due to NaCl; 18 of the flasks contained betaine (2 mM). This concentration of betaine was chosen upon advice from Dr. E.A. Galinski¹ and because similar studies on the osmoprotection of bacteria by compatible solutes have used millimolar quantities (LeRudulier and Bouillard, 1983; Beumer *et al.*, 1994). Water activity was measured using an Aqualab CX-2 immediately before inoculation. Each flask was randomly assigned to a position in one of four waterbaths (treatments are summarised in Appendix 2.1).

Growth was determined turbidimetrically (540 nm) at each sampling time by aseptically removing 4 mL aliquots from the flask and transferring to 10 mm diameter spectrophotometric tube. Turbidity was measured in a spectrophotometer (Spectronic 20) at intervals that corresponded to approximately a 5-10% decrease in percent transmittance (%T). Both the turbidity and time elapsed since inoculation were recorded at each sampling interval, permitting calculation of the change in %T since inoculation. Turbidity was monitored until the culture entered the stationary phase. Generation time was then calculated using fitted parameters obtained from a Gompertz equation (see Appendix A.3).

a_w min was determined by linear regression of growth rate versus extracellular water activity, performed using Cricket Graph 1.3.2.

¹ Dr. E.A. Galinski, Westfälische Wilhelms-Universität, Dept. of Biochemistry, Wilhelm-Klemm-Str.2, Münster, Germany, 48149.

2.2.2 Influence of a_w and betaine on growth for the biokinetic range

Inocula were prepared by growing *E. coli* SB1 overnight statically in minimal media at 37°C. This gave cells that were in the stationary phase of growth. To ensure no further change in numbers the flask was held on ice during inoculation, which took no longer than 1 hour. Inocula were transferred to glass, L-shaped tubes (L-tubes) containing 9 mL of minimal media, to give a final %T reading between 80 and 90%. There were four treatments: no added salt, no added salt with 2 mM betaine, 4% NaCl and 4% NaCl with 2 mM betaine. A water activity meter was not available, but the water activity of broths was calculated (Chirife and Resnik, 1984) to be 0.998, where no salt was added and 0.977, where 4% NaCl was added. Sixty tubes, 15 for each treatment, were held in a temperature gradient incubator (Advantec) set to generate a gradient between 0 - 50°C. Tubes containing no added salt were placed on one side of the temperature gradient incubator, alternating positions filled by betaine and non-betaine tubes. This arrangement was repeated for the tubes containing 4% NaCl on the other side of the temperature gradient incubator. Tubes were placed in the temperature gradient incubator 12 hours before inoculation so that the temperature of each tube had equilibrated. Growth was measured turbidimetrically at 540 nm. In this case aseptic removal of aliquots was unnecessary because L-tubes can be fitted into the spectrophotometer. The experiment was monitored for at least twice as long as the predicted lag time of cultures at the growth/no growth boundary (lag time predictions were based on data collected for cultures at temperatures above the growth/no growth boundary). The temperature of each tube was measured at the conclusion of the experiment using a digital Fluke thermometer.

Generation time was calculated from fitted parameters of a Gompertz equation (Appendix A.3). Also lag time was determined from the Gompertz parameters (Appendix A.3).

T_{min} and T_{max} were determined by fitting the four parameter square root model (Equation 2.4) using Ultrafit 3.0.

The above method was repeated to determine the effect of water activity and betaine on growth for the biokinetic range of *P. halodenitrificans* and *H. elongata*. Both of these bacteria are halotolerant, therefore the NaCl concentrations that represented optimal water activity and unfavourable water activity values were different. The four treatments for *P. halodenitrificans* were: 6% NaCl \pm 2 mM betaine (a_w : 0.964) and 15% NaCl \pm 2 mM betaine (a_w : 0.892), and for *H. elongata*: 8% NaCl \pm 2 mM betaine (a_w : 0.950), and 20% NaCl \pm 2 mM betaine (a_w : 0.752).

2.3 Results

2.3.1 Influence of a_w and betaine on *E. coli* growth rate at 37°C

The growth rate of *E. coli* declined as water activity conditions were made increasingly stringent (Figure 2.3). At the non-stress water activity of 0.998 the maximum specific growth rate was 0.019 min^{-1} (generation time: 54 min), whilst at water activity 0.972 the maximum specific growth rate was 0.0027 min^{-1} (generation time: 370 min). This relationship between extracellular water activity and growth rate was linear.

In the absence of water activity stress, the presence of betaine had no effect on *E. coli* growth rate. This was as expected because compatible solutes relieve stress due to water activity, but are not general growth enhancers. When water activity stress was applied, growth was enhanced by the exogenous presence of compatible solutes (Figure 2.3). At water activity 0.972 when no compatible solutes were provided, the growth rate was 0.0027 min^{-1} (generation time: 370 min) but when betaine was provided the growth rate was 0.0065 min^{-1} (generation time: 153 min). Growth rate, however, was not completely restored to the pre-stress level by betaine. Finally, it was noted that the minimum growth rate with and without betaine was the same, approximately 0.003 min^{-1} (generation time: 330 min).

2.3.2 Influence of betaine on $a_w \text{ min}$

Figure 2.3 was used to visualise the value of $a_w \text{ min}$. Where betaine was absent the $a_w \text{ min}$ value was 0.966, but when betaine was present it was 0.953. Therefore $a_w \text{ min}$ depends on the presence of compatible solutes; $a_w \text{ min}$ is lower when compatible solutes are present than when they are absent.

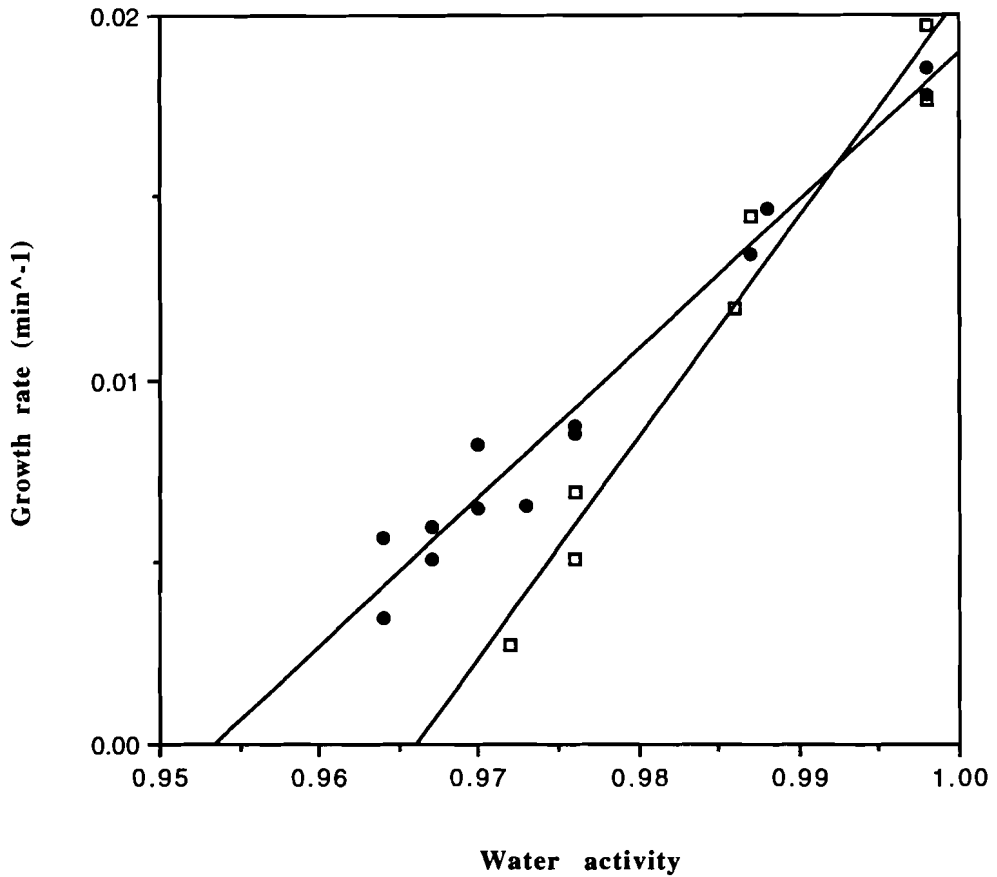


Figure 2.3: Growth rate of *E. coli* SB1 at 37°C as a function of water activity in the presence (●) and absence (□) of betaine. For experimental values refer to Appendix 2.1.1.

2.3.3 Influence of a_w and betaine on growth for the biokinetic range

The effect of reduced water activity and betaine on the growth rate of *E. coli* for the biokinetic range was determined (Figure 2.4). In this experiment only one level of water activity stress (0.977) was imposed. Figure 2.4 shows that this level of water activity stress markedly reduced the growth rate of *E. coli*. The reduction in growth rate due to unfavourable water activity conditions was partially, but not completely, reversed where betaine was added to the growth media. However, betaine had no effect on growth rate where no water activity stress was applied.

The growth rate response of *P. halodenitrificans* (Figure 2.5) and *H. elongata* (Figure 2.6) to unfavourable water activity conditions and betaine were essentially the same as those described for *E. coli*, although for *H. elongata* at the optimum water activity (0.950) a slight improvement in growth was observed when betaine was present.

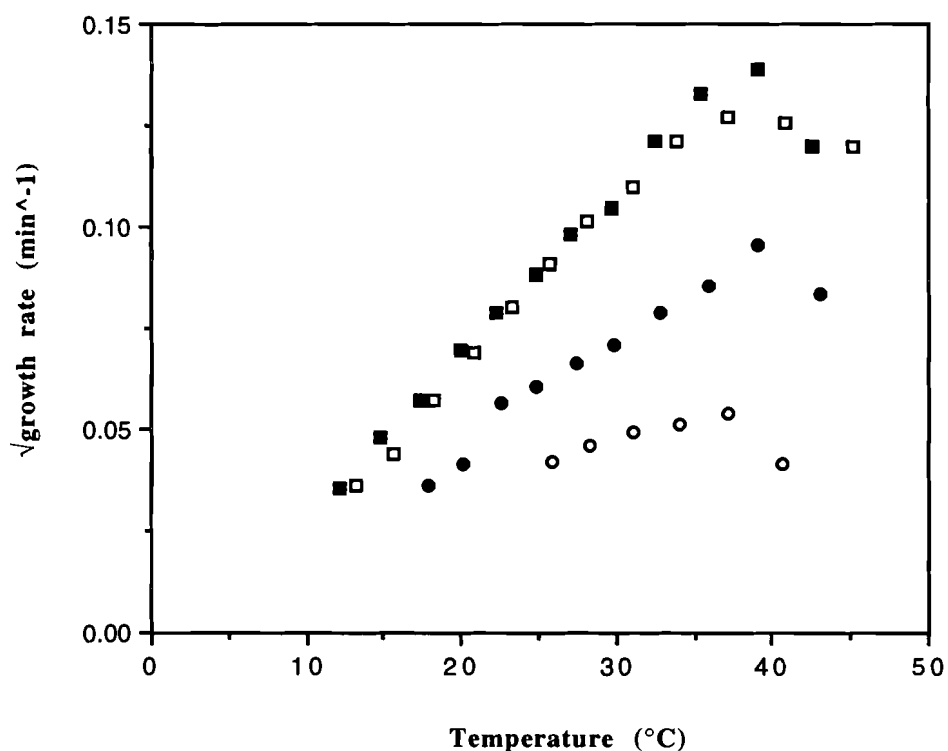


Figure 2.4: Square root of growth rate plot for *E. coli* SB1 in minimal medium, for the biokinetic range, for four water activity treatments:

(□), 0.998

(■), 0.998 + 2 mM betaine

(○), 0.977

(●) 0.977 + 2 mM betaine

For experimental values refer to Appendix 2.2.

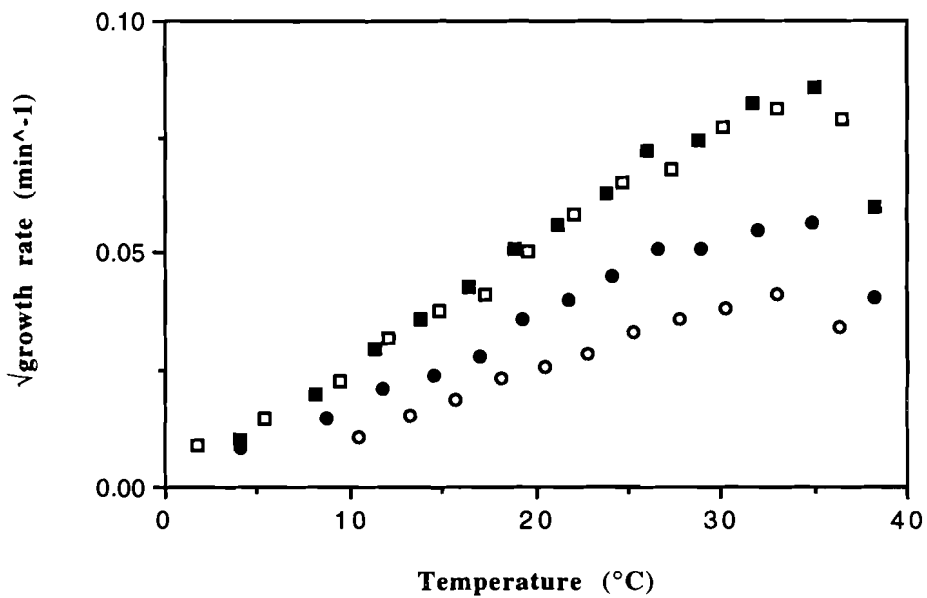


Figure 2.5: Square root of growth rate plot for *P. halodenitrificans* for the biokinetic range, for four treatments: (□), 0.964; (■), 0.964 + 2 mM betaine; (○); 0.892 and (●), 0.892 + 2 mM betaine. For experimental values refer to Appendix 2.3.

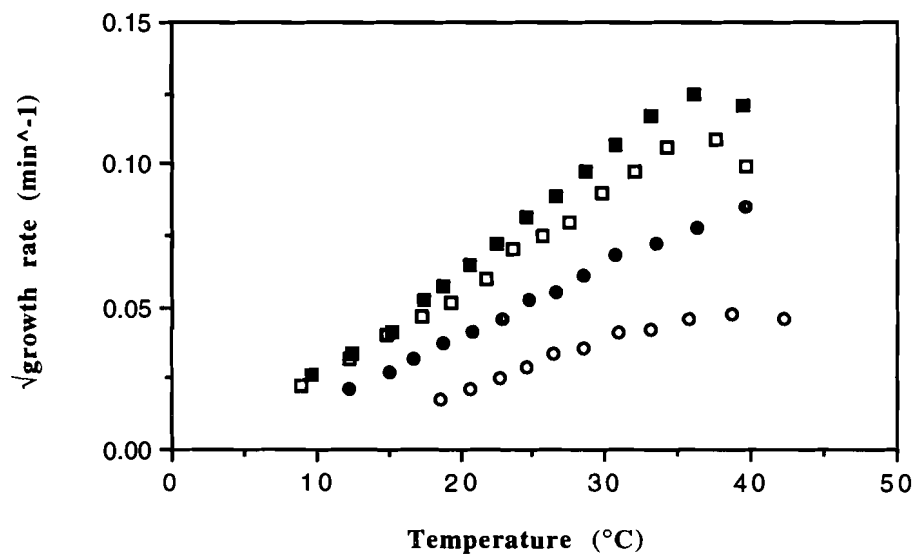


Figure 2.6: Square root of growth rate plot for *H. elongata* for the biokinetic range, for four treatments: (□), 0.950; (■), 0.950 + 2 mM betaine; (○), 0.752 and (●), 0.752 + 2 mM betaine. For experimental values refer to Appendix 2.4.

2.3.4 Influence of a_w and betaine on lag time

Lag time, the period from inoculation to when growth is first detected, depended on water activity. Figure 2.7 shows the lag times of *E. coli* SB1 cultures for the four treatments in the biokinetic range experiment. Cells that were exposed to salt stress resolved lag more slowly than those in favourable conditions. Interestingly however, at unfavourable water activities betaine did not effect the length of lag. The lag times for the 0.977 ± 2 mM betaine treatments appeared virtually identical.

The effects of unfavourable water activity on lag time were reproduced using *P. halodenitrificans* (Figure 2.8) and *H. elongata* (Figure 2.9). However, in both these cases, betaine reduced the lag time at unfavourable water activity values.

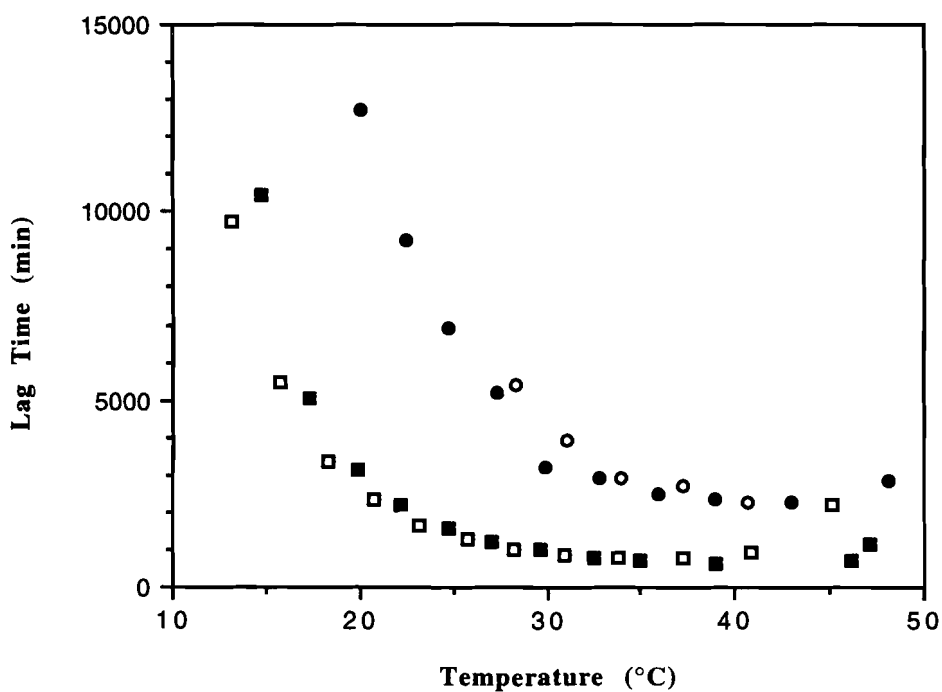


Figure 2.7: Lag time of *E. coli* SB1 across the biokinetic range, as a function of extracellular water activity and betaine:

(□), 0.998

(■), 0.998 + 2 mM betaine

(○), 0.977

(●) 0.977 + 2 mM betaine

For experimental values refer to Appendix 2.2.

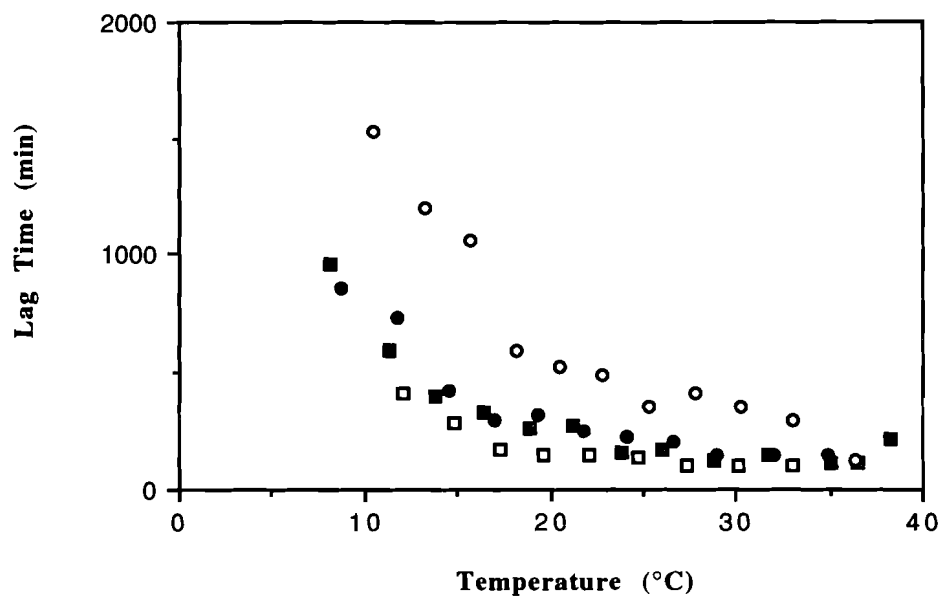


Figure 2.8: Lag time of *P. halodenitrificans* across the biokinetic range, for four treatments: (□), 0.964; (■), 0.964 + 2 mM betaine; (○); 0.892 and (●), 0.892 + 2 mM betaine. For experimental values refer to Appendix 2.3.

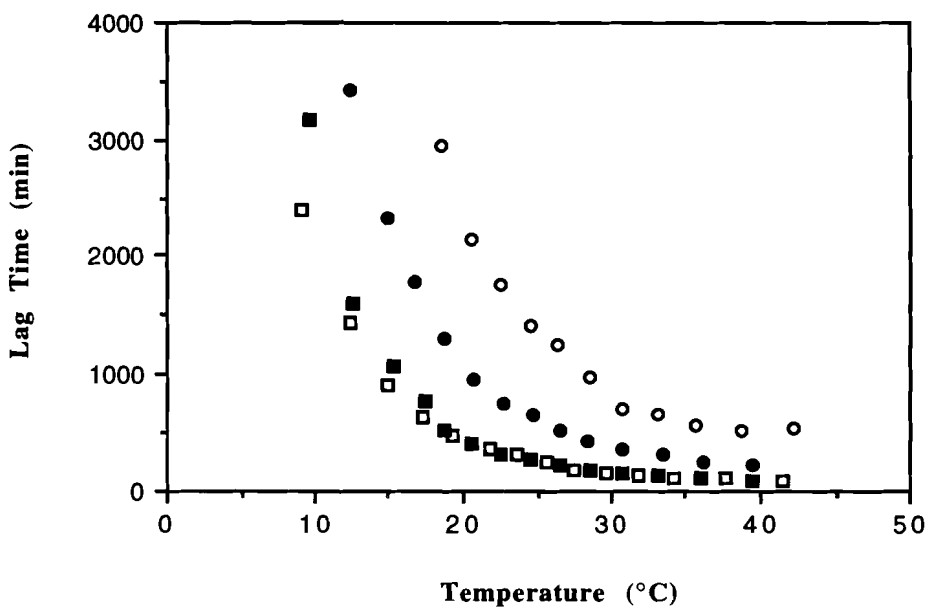


Figure 2.9: Lag time of *H. elongata* across the biokinetic range, for four treatments: (□), 0.950; (■), 0.950 + 2 mM betaine; (○); 0.752 and (●), 0.752 + 2 mM betaine. For experimental values refer to Appendix 2.4.

2.3.5 Influence of a_w and betaine on cardinal temperatures

Table 2.2 shows cardinal temperatures for growth of *E. coli* determined by applying the four parameter square root model to the data shown in Figure 2.4. For three treatments (0.998, 0.998 + 2 mM betaine and 0.977 + 2 mM betaine) the T_{min} values were very similar, averaging 4.3°C. For the high salt treatment however, T_{min} was -8.7°C. Confidence in this determination of T_{min} was low, because it had a very large confidence interval ($\pm 23.6^\circ\text{C}$, 95% confidence). This large confidence interval may be due to the limited amount of data available for fitting the four parameter square root model (six points), due to a large difference between T_{min} and the observed minimum temperature for growth.

The cardinal temperatures for *P. halodenitrificans* (Table 2.3) and *H. elongata* (Table 2.4) showed a similar trend to that for *E. coli*. T_{min} was constant with water activity treatments and betaine for *P. halodenitrificans*, without exception. For *H. elongata* T_{min} was constant with water activity and betaine except for the 0.752 water activity treatment. Similar to the case for *E. coli*, this may be due to a large difference between T_{min} (3.7°C) and the observed minimum temperature for growth (18.5°C).

2.3.6 Influence of a_w and betaine on the temperature range for growth

Figure 2.4 shows that the temperature range over which *E. coli* growth occurred was smaller where the water activity stress was applied. This effect, like the growth rate response, was partially reversed by betaine. Values of the minimum observed temperatures for growth for each treatment are detailed in Table 2.5.

It was not possible to determine the minimum temperatures for growth of *P. halodenitrificans* or *H. elongata* because, the design of those experiments did not permit certainty that the minimum temperature for growth was observed.

Table 2.2: Cardinal temperatures (and 95% confidence intervals) estimated using the four parameter square root model for *E. coli* SB1 data from Figure 2.4.

Treatment	T_{min} (°C)	T_{max} (°C)
0.997	6.2 ± 1.3	55.1 ± 3.3
0.997 + 2 mM betaine	3.2 ± 1.0	45.6 ± 1.2
0.977	-8.7 ± 23.6	42.9 ± 2.6
0.977 + 2 mM betaine	3.6 ± 4.4	45.6 ± 5.4

Table 2.3: Cardinal temperatures (and 95% confidence intervals) estimated using the four parameter square root model for *P. halodenitrificans* data from Figure 2.5.

Treatment	T_{min} (°C)	T_{max} (°C)
0.964	-0.8 ± 1.3	40.1 ± 5.9
0.964 + 2mM betaine	0.3 ± 0.9	40.1 ± 0.5
0.892	2.8 ± 1.1	39.3 ± 1.1
0.892 + 2 mM betaine	0.4 ± 1.8	40.7 ± 1.3

Table 2.4: Cardinal temperatures (and 95% confidence intervals) estimated using the four parameter square root model for *H. elongata* data from Figure 2.6.

Treatment	T_{min} (°C)	T_{max} (°C)
0.950	2.9 ± 1.0	41.9 ± 1.6
0.950 + 2 mM betaine	4.2 ± 0.8	42.2 ± 3.4
0.752	9.9 ± 2.4	51.2 ± 5.5
0.752 + 2 mM betaine	4.1 ± 1.2	53.8 ± 33.1

Table 2.5: Observed minimum temperatures for growth of *E. coli* SB1.

Treatment	Observed Minimum Temperature (°C)
0.998 (± 2 mM betaine)	12.1
0.977	25.8
0.977 + 2 mM betaine	17.8

2.4 Discussion

Unfavourable water activity conditions inhibit the growth of bacteria (reduce growth rate and increase lag time), but this inhibition is relieved when compatible solutes are available (section 1.2.3). These well-documented observations have been successfully reproduced here for *E. coli*, *P. halodenitrificans* and *H. elongata*, which gives confidence in the experimental method. One exception was noted, that could not be explained; for *E. coli* betaine did not appear to reduce the lag time at unfavourable water activity values. However, reductions were observed for *P. halodenitrificans* and *H. elongata*, therefore the observation for *E. coli* may be an anomaly.

2.4.1 Influence of betaine on $a_{w \min}$

The $a_{w \min}$ value was decreased by the presence of compatible solutes in the extracellular environment. This dependency of $a_{w \min}$ on compatible solutes has important implications for predictive modelling. In the square root modelling approach it is taken that each bacterial species has a specific $a_{w \min}$, dependant on the humectant, that is constant within the sub-optimal temperature range. This was established using data collected for *S. xylosus* (McMeekin *et al.*, 1987) and since has been applied successfully for other bacterial species including *S. aureus* (Ross, 1993) and *Pseudomonas* sp. (Neumeyer, 1995). The constancy of $a_{w \min}$ is useful and has been exploited in square root modelling because it simplifies the modelling process; by being a constant for any given bacterial species it need only be established once. However, this new data suggests that $a_{w \min}$ may not be constant.

Theoretically, this finding is cause for concern, however, in practice it may not be important. In practice square root models are generated first in complex laboratory growth media and then validated in real foods. Both the complex laboratory media and foods contain compatible solutes, therefore $a_{w \min}$ should be constant. However, a situation is conceivable where a square root model is developed in laboratory media containing compatible solutes that the bacterium is unable to accumulate. Then, if the foods used for validation contained a different array of compatible solutes that could be accumulated, the $a_{w \min}$ value may be different and the predictive model would fail.

Although $a_{w \min}$ is constant with varying temperature in the sub-optimal temperature range, shifts in $a_{w \min}$ with different humectants have been noted. In square root modelling this was first shown for *S. xylosus*, using two humectants:

sodium chloride and glycerol (McMeekin *et al.*, 1987; Chandler and McMeekin, 1989b). In those studies glycerol caused a greater reduction in growth rate than sodium chloride, at equivalent water activity values. Also, the $a_w \min$ value where glycerol was used as a humectant (0.908) was higher than that for sodium chloride (0.838).

Christian (1955b) also showed that some humectants were more inhibitory than others at equivalent water activity values, but this was before the evolution of square root modelling. That data showed that four different humectants, at equivalent water activity values, had different effects on the rate of growth of *S. oranienburg*. In decreasing order of inhibition those humectants were; glycerol, glucose, sucrose and a mixture of three inorganic salts. If that data is presented as plot of growth rate versus water activity (Figure 2.10), a similar trend to that demonstrated for *S. xylosus* emerges. That is, different humectants result in different $a_w \min$ values (Table 2.6). This is especially obvious when the $a_w \min$ value determined for the glycerol data is compared to those of the other three humectants.

Comparing the above described effects of humectants on growth rate with those for betaine, a pattern is revealed; osmotically active agents that cause a smaller reduction in growth rate at equivalent water activity values, have lower $a_w \min$ values. From a purely mathematical perspective this is a consequence of the method used to calculate $a_w \min$. $a_w \min$ is where the regression line between growth rate and water activity intersects the water activity axis. Therefore, osmotically active agents that cause proportionately smaller reductions in growth rate, have regression lines of shallower slope. Consequently such regression lines intersect the water activity axis at a lower value (than those for an osmotically active agent that causes a proportionately large reduction in growth rate).

A similar analysis can be applied to determine the influence of the species of compatible solute on the value of $a_w \min$. For example, generally betaine is more effective at relieving the inhibition of growth rate due to low water activity conditions than proline (Cairney *et al.*, 1985a). Therefore, the value of $a_w \min$ where proline is provided should be higher than the $a_w \min$ where betaine is present. This point should be noted regarding the earlier discussion of the influence of compatible solutes on $a_w \min$ and the potential consequences for square root predictive modelling.

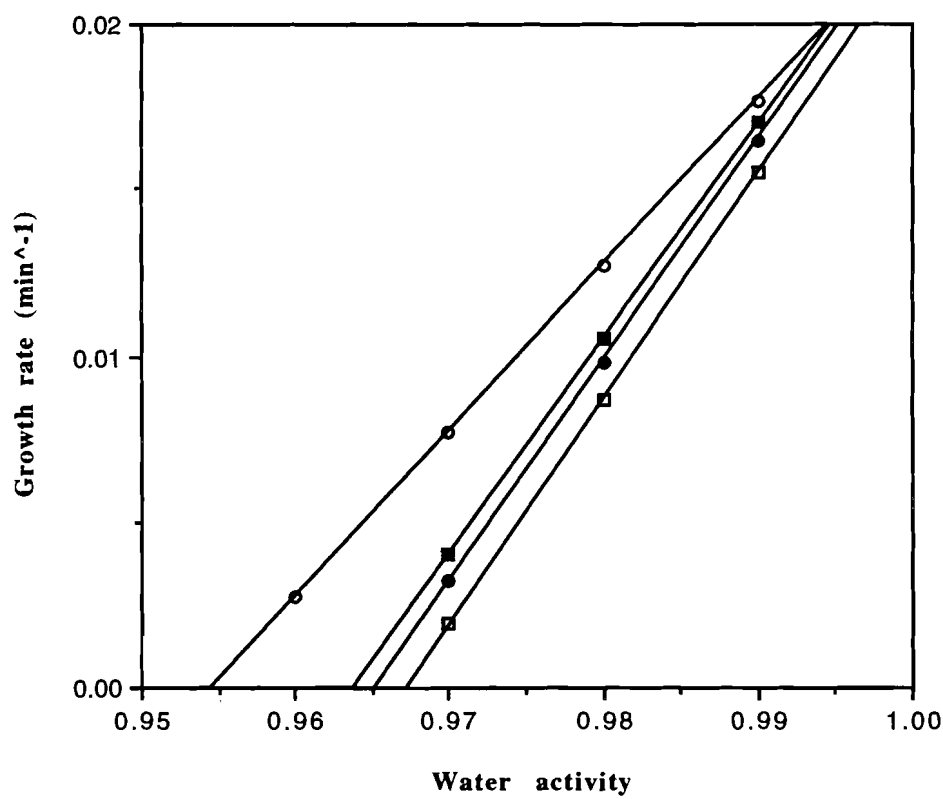


Figure 2.10: Growth rate of *S. oranienburg* as a function of extracellular water activity, adjusted using different humectants:
(□), NaCl-KCl-Na₂SO₄
(●), Sucrose
(■), Glucose
(○), Glycerol
Adapted from Christian (1955b) (Figure 1), to facilitate calculation of the $a_{w\ min}$ value; for complete details see Appendix 2.5.

Table 2.6: $a_{w\ min}$ values for *S. oranienburg*. The $a_{w\ min}$ value varies with humectant.

Humectant	$a_{w\ min}$
NaCl-KCl-Na ₂ SO ₄	0.967
Sucrose	0.965
Glucose	0.964
Glycerol	0.954

The similarity between effects of different humectants and compatible solutes can also be interpreted mechanistically. That different humectants cause different growth rates at equivalent water activity values, and therefore have different $a_{w\ min}$ values, indicates that their water activity lowering effect is not the only 'force' at work. If the effect of humectants on bacterial growth was purely due to lowered water activity, all humectants should cause the same reduction in growth rate at equivalent water activities. This additional 'force' is unknown but, could be a destabilising effect on biological macromolecules. A particular humectant may be more effective in this destabilisation because it is more permeable across the bacterial cell membrane or for some other unidentified reason. The observed effects of compatible solutes on growth rate and $a_{w\ min}$ complement this mechanistic interpretation. Compatible solutes can be thought of as 'depotentiating' humectants, making the humectants equivalent to some less inhibitory species - that is reflected in their effect on growth rate and hence $a_{w\ min}$.

2.4.2 Influence of a_w and betaine on cardinal temperatures

Excepting the result for the 0.977 treatment cardinal temperatures for *E. coli* growth and most importantly, for square root predictive modelling, T_{min} , were not changed by water activity or betaine. The average T_{min} calculated for the 0.998 ± 2 mM betaine and $0.977 + 2$ mM betaine treatments (4.3°C) is identical to that determined for *E. coli* M23 in nutrient broth (Ross² and Parker, *pers. comm*). The disparity in the T_{min} estimate for the 0.977 treatment may be due to the limited range of the data collected. Ross (1993) noted that there is a slight curvature in many square root plots. Therefore, T_{min} estimates depend on the range of data collected. This explanation may also apply for the inconsistency of the T_{min} estimate for the *H. elongata*, a_w : 0.752 treatment. For *P. halodenitrificans* at the stringent water activity treatment a relatively larger range of data was collected, hence the T_{min} estimate corresponded more closely with those for the other treatments. The constancy of T_{min} with water activity has been demonstrated frequently. This however, is the first report that T_{min} is constant with compatible solutes.

That T_{min} was constant with compatible solutes is important since the square root model depends on the constancy of T_{min} , as discussed in section 2.1.1.

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2.4.3 Influence of a_w and betaine on the temperature range for growth

An important observation from the *E. coli* data set is the value of the actual observed minimum temperatures for growth. Unlike T_{min} , this varied greatly between the four treatments. Due to care taken in the experimental design, this temperature range result is considered a real result, not an artefact of experimental design. The design involved two considerations: inoculum concentration and experiment duration.

Inoculum concentration, was an important consideration because at critical incubation conditions, reduced final population densities have been noted (Chandler and McMeekin, 1989b). Therefore it is conceivable that at these critical incubation conditions, if a sufficiently small inoculum is used, the turbidity at the final population density could be less than that detectable using turbidimetric methods. In this case, a false no growth response would be recorded. Traditional plate counting can be used to avoid such a no growth result. Alternatively, the problem can be overcome by using a large starting inoculum. In the current study sufficient starting inoculum was added to reduce turbidity to between 80 and 90 %T. This inoculum is already in the detectable range for turbidimetric methods, therefore if growth was possible it was detected.

The experiment duration was important because at critical low temperatures in particular, the growth rate is very slow and lag time long. Therefore, if experiments are not monitored for a sufficiently long period, growth at critical low temperatures may not be detected. In the current experiment this was avoided by using the square root model to predict the lag time (Figure 2.7) of treatments close to the growth/no growth boundary, and monitoring experiments well past that predicted lag time.

Food microbiology

The differences in the observed minimum temperatures for growth have important implications for the food microbiology discipline. A major factor in food preservation is temperature control. By storing food at low temperatures bacterial growth on that food is slower, hence extending the shelf life. However, refrigeration is expensive and keeping the so called 'cold chain' (the chain from food manufacturer to distributor to retailer to consumer) cold is difficult. Therefore, often it is necessary to use alternative food preservation procedures, such as reduced water activity, so that higher temperatures can be tolerated throughout the 'cold chain' with the same outcome (remaining shelf life). For example, according to the results in Figure 2.4

food may be stored at 12°C and 0.998 (a_w) or 18°C and 0.977 (a_w), with the same proliferation of *E. coli*. But, this is when *E. coli* accumulates compatible solutes. If the accumulation of compatible solutes were prevented then the same proliferation of *E. coli* would occur at 26°C and 0.977 (a_w). This is a hypothetical situation - 4% salt (a_w : 0.977) would render most foods inedible, but it is the principle that is of interest. In food preservation there is significant advantage to be gained from preventing osmoprotection of bacteria due to the accumulation of exogenous compatible solutes.

The potential advantages of a compatible solute free environment have been contemplated by other authors (Smith, 1996). Possibilities for achieving a compatible solute free environment include chelation of compatible solutes or addition of compatible solute porter inhibitors. Alternatively it may be possible to use the physiological response of bacteria to unfavourable water activity against themselves by addition of compatible solute analogues to foods. These analogues would be accumulated in response to osmotic stress but toxic to bacteria once absorbed. In the clinical microbiology discipline there has already been research interest in the development of toxic compatible solute analogues (Reese *et al.*, 1996). Therefore, there exists the potential to apply knowledge gained from those studies to problems in food preservation technology.

Mechanistic interpretation

Despite the fact that the inhibitory effect of water activity on bacterial growth has been known for a very long time, no mechanism has been shown. Not surprisingly however, a conventional wisdom has developed relating to these effects. Reference has often been made to the diversion of energy from growth to maintenance functions in relation to growth responses to unfavourable water activity conditions. Microbiologists consider that the energy budget of bacterial cells can be divided into two parts - energy used for growth and energy used for maintenance (section 3.1 considers the maintenance energy concept in detail). The maintenance component is used to maintain the status quo, by preserving or replacing unstable biological macromolecules (McGrew and Mallette, 1962).

The effect of unfavourable water activity conditions on the maintenance energy value has not been directly determined. Despite this however, a conventional wisdom that unfavourable water activity conditions increases cell maintenance energy has developed. The precise origin of this conventional wisdom could not be identified, but several examples of its application can be found.

One example can be drawn from McMeekin *et al.* (1993). Those authors interpreted observations on the growth of *S. xylosus* (McMeekin *et al.*, 1987) to conclude that unfavourable water activity conditions increased maintenance energy. Figure 2.11 shows the data used in this interpretation. From Figure 2.11 it was noted that as extracellular water activity increased, T_{min} was constant, but the minimum temperature at which growth was observed increased. This observation was analysed quantitatively by reconciling the Arrhenius (Equation 1.4) and square root equation (Equation 2.3) to give the expression:

$$E_a = \frac{2RT^2}{(T - T_{min})} \quad (2.6)$$

where: T_{min} is as previously defined
 E_a is the apparent activation energy
 R is the universal gas constant, $8.314 \text{ JK}^{-1}\text{mol}^{-1}$
 T is temperature (K)

(The full derivation of Equation 2.6 is reproduced in Appendix 2.6) Therefore, according to Equation 2.6, the apparent activation energy (the energy needed to overcome the temperature barrier and allow growth) is dependent on the distance between T_{min} and the observed minimum temperature for growth and can be calculated easily. The value of T_{min} for *S. xylosus* is 2.9°C (McMeekin *et al.*, 1987). And from Figure 2.11, the observed minimum temperature for growth was 7.4°C at the optimum water activity for growth (0.976) and 12.6°C at the unfavourable water activity of 0.848. Therefore, by applying Equation 2.6, in optimal conditions the maximum apparent activation energy hurdle for growth of *S. xylosus* that can be surmounted is 291 kJmol^{-1} compared to 140 kJmol^{-1} when water activity is adjusted to 0.848 using sodium chloride.

McMeekin *et al.* (1993) interpreted this reduction in the apparent activation energy for growth with increasingly unfavourable water activity using the maintenance energy diversion hypothesis. They proposed that cells exposed to unfavourable water activity conditions displayed a reduced ability to overcome the barrier to growth due to low temperature, in the order of 150 kJmol^{-1} for the above example. It should however be noted that the authors did warn that the experiment was not specifically designed to determine the observed minimum temperature for growth, and therefore could not be certain that the apparent activation energy determinations were valid.

The above interpretation probably originated from reports about the high energetic burden of synthesis and accumulation of compatible solutes (Csonka, 1989).

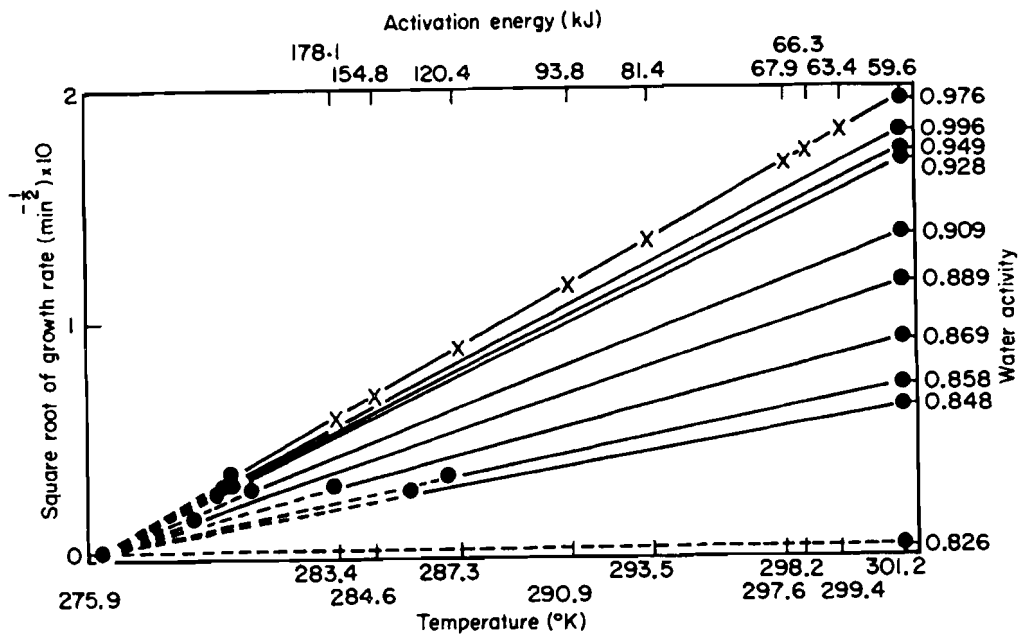


Figure 2.11: Square root of rate plot for *S. xylosus* CM21/3 at nine different water activities, reproduced from McMeekin *et al.* (1987). The minimum temperature at which growth was measured at each water activity is indicated by ●.

During the analysis summarised above, McMeekin *et al.* (1993) do make brief mention of diversion of energy from growth to compatible solute production. Support for this contention can be gained from the data generated in this thesis for *E. coli*. Table 2.7 (overleaf) shows the apparent activation energy values for *E. coli* growth, calculated using Equation 2.6. More confidence can be placed in these apparent activation energy values than those for the *S. xylosus* example above because of the care taken to identify the minimum temperature for growth.

If the same energy diversion hypothesis is applied, the same trend has been observed for *E. coli* as for the *S. xylosus* example. As water activity conditions become more stringent, the temperature barrier that can be overcome is smaller, indicating that energy has been diverted from growth to maintenance functions associated with the unfavourable water activity. Also, for the stringent water activity

Table 2.7: Calculated E_a values for *E. coli* growth, based on the observed minimum temperatures for growth, presented in Table 2.5.

Treatment	E_a (kJmol ⁻¹)
0.998	173
0.977	69
0.977 + 2 mM betaine	104

treatments the temperature barrier that could be overcome was smaller when compatible solutes were not provided. Therefore, the synthesis of compatible solutes appears to be a large drain on the energy budget of bacterial cells.

Before the development of Equation 2.6 the conventional wisdom relating to energy diversion from growth to maintenance during osmotic stress had appeared in the predictive modelling literature. This was with the advent of the Hurdle concept (Leistner, 1985). In this theory it is proposed that each unfavourable environmental condition imposed increases the energetic hurdle that must be overcome before growth is possible. It was in a climate where this idea was prominent that the above interpretations were generated. Therefore, the above application of the energy diversion hypothesis is not surprising.

Gould (1989) also used the energy diversion hypothesis when he wrote ‘food preservation procedures that involve the reduction of water activity.....exceed the osmoregulatory capacity of the cell, or in some way reduce the amount of energy available for osmoregulation’. This sentiment was reiterated in a later review (Knochel and Gould, 1995).

The rationale for the energy diversion hypothesis appears sound. Many changes in metabolism have been demonstrated to occur in response to unfavourable water activities. This was described in detail in Chapter 1. This combined with reports that these changes have a high energetic cost (Csonka, 1989), leads logically to the energy diversion hypothesis. Evidence for the validity of this energy diversion hypothesis however is lacking in the literature, since maintenance energy values for cells exposed to osmotic stress have not been determined. Indirect evidence, such as that presented above for *S. xylosus* and *E. coli* supports the energy diversion hypothesis, and led to the working hypothesis for this study.

2.5 Summary and Working Hypothesis

In summary the results presented in this Chapter indicate:

1. Literature observations on the variation of growth rate and lag time as a function of water activity and the exogenous presence of compatible solutes, can be reproduced successfully in the model system used throughout this thesis. This gives confidence in the experimental procedures used.
2. $a_w \min$ varies as a function of the exogenous presence of compatible solutes; $a_w \min$ is lower when compatible solutes are provided.
3. T_{\min} is constant as a function of the exogenous presence of compatible solutes.

The significance of observations (1), (2) and (3) regarding a mechanistic explanation of the growth response of bacteria to extracellular water activity, at this point is not clear. However, they are significant for food microbiology and predictive modelling.

4. The ability to overcome the low temperature barrier to growth is diminished as a function of increasing water activity stress.
5. Where compatible solutes are provided observation (4) is reduced in magnitude.

Observations (4) and (5) support the energy diversion hypothesis described in section 2.4.3. Combining this with the fact that the rationale for the energy diversion hypothesis appears sound, considering what is known of the changes in cell physiology when water activity stress is applied, the working hypothesis chosen here was:

Microorganisms exposed to water activity stress partition more energy into maintenance functions and therefore have less energy available for growth.

And:

De novo synthesis of compatible solutes, in response to unfavourable water activity conditions represents a large maintenance energy burden, that is reduced when they are provided extracellularly.

CHAPTER 3: ENERGY DIVERSION

3.1 Introduction

Bacteria allocate available energy to growth and non-growth functions, a concept that was introduced briefly in section 2.4.3. The energy used for growth is simple to measure as 'cell yield'. Measurement of the energy diverted to non-growth functions, termed 'maintenance energy', is more difficult. The term maintenance energy implies the energy used to maintain cellular integrity, but energy losses due to other functions that are not essential for cellular integrity, such as excretion of energy-rich molecules to the growth medium, are included.

3.1.1 Cell yield

'Cell yield' refers to the increase in biomass proportional to a known amount of energy substrate provided. In this study 'cell yield' is used more specifically to refer to the increase in optical density of a broth culture as a function of the initial amount of glucose added. The idea that these are proportional is not new and has been demonstrated on many separate occasions (Bauchop and Elsdén, 1960; Rosenberger and Elsdén, 1960). The most famous report that cell yield was directly proportional to the quantity of energy substrate provided was by Monod in 1942 (Russell and Cook, 1995). That study showed the dry weight of cells produced from batch culture of *E. coli*, *Bacillus subtilis* and *S. typhimurium* was proportional to the quantity of energy source added.

Cell yield is proportional to the amount of energy substrate provided because those substrates are metabolised and used with constant stoichiometry to provide the energy for growth. Bacterial energy metabolism produces a constant number of ATP molecules per molecule of substrate and, in turn, a constant number of ATP molecules are used up in growth related cellular reactions, such as macromolecule synthesis. Qualitatively the relationship between cell yield and amount of energy substrate was associated with ATP production from energy substrates in the 1950's. This began with the observation that the yield of cells from glucose fermentation by *Streptococcus faecalis* (now *Enterococcus faecalis*) was greater than that for *Leuconostoc mesenteroides* (DeMoss *et al.*, 1951). That difference was later attributed

to the use of different glucose fermentation pathways that gave different ATP/glucose ratios (Heath *et al.*, 1958; Hurwitz, 1958).

A quantitative relationship between cell yield and amount of ATP was established in the 1960's. For anaerobic culture of *Streptococcus faecalis*, *Saccharomyces cerevisiae* and *Pseudomonas linderi* (now *Zymomonas mobilis*) biomass was proportional to the quantity of ATP (Bauchop and Elsdén, 1960). This work led to the expression Y_{ATP} , the dry weight of cells produced per mol of ATP generated by catabolism. Y_{ATP} for the above three species equalled 10.5 g cells/mol ATP. Later a literature survey summarising results for 13 species and 47 Y_{ATP} determinations gave an average Y_{ATP} value of 10.6 ± 1.0 (Forrest and Walker, 1971). Therefore, the value for Y_{ATP} of ~10.5 g cells/mol ATP became accepted as a biological constant (Stainer *et al.*, 1976; Ingraham *et al.*, 1983; Gottschalk, 1986). The constant Y_{ATP} value was interpreted to indicate that the energetic cost of growth was similar for all microorganisms (Forrest and Walker, 1971; Brock and Madigan, 1991).

Subsequent scrutiny of Y_{ATP} as a biological constant revealed deficiencies in the concept. A review of literature shows that there was at least a five-fold range in Y_{ATP} values (Stouthamer, 1979). Russell and Cook (1995) noted that the value of 10.5 g cell/mol ATP, determined by Bauchop and Elsdén (1960), was an average derived from values ranging between 12.6 and 8.3. This variability in Y_{ATP} has been attributed to many factors including: technical difficulties in estimating ATP; energy source utilisation of carbon; changes in cell composition and a range of non-growth related energy expenditures (Stouthamer and Bettenhausen, 1973; Russell and Cook, 1995). In defence of the originators of the Y_{ATP} concept it should be noted that they intended Y_{ATP} to be calculated where: insignificant amounts of energy source carbon were assimilated into cell substance; the quantity of energy-yielding substrate consumed and the net ATP gain were known with certainty; and growth was limited by the supply of energy source (Tempest and Neijssel, 1984). Therefore some of the sources of variability listed above are nullified. However, it remains that non-growth related energy expenditures (maintenance energy) have the potential to affect Y_{ATP} values. Maintenance energy may differ between species or due to environment. Therefore Y_{ATP} , and consequently cell yield, may differ according to the extracellular conditions. Finally it should also be noted that theoretical Y_{ATP} values have not been observed in practice (Stouthamer, 1979). Diversion of energy to maintenance functions has also been invoked to explain this phenomenon (Stouthamer, 1979).

3.1.2 Maintenance energy

The concept of maintenance energy began with Duclaux in 1898 (Pirt, 1965), but despite this long history it appears to be poorly understood. Definitions of maintenance energy include the free energy provided to maintain the status quo (McGrew and Mallette, 1962) or the energy consumed for functions other than the production of new cell material (Pirt, 1965). Definitions of this type have, however, been criticised for their lack of mechanistic insight. Russell and Cook (1995) suggest that a more appropriate definition should include the nature of the maintenance functions. Currently this would be difficult and subjective; many maintenance functions have been suggested but they have not been completely characterised.

Turnover of macromolecules is one of the most commonly suggested maintenance functions (McGrew and Mallette, 1962; Marr *et al.*, 1963; Dawes and Ribbons, 1964; Stouthamer, 1979). Biological macromolecules are unstable and have a limited lifespan, therefore there is a continual turnover of protein, RNA and cell wall polymers. The cycle of breakdown (or denaturation) and rebuilding of macromolecules is needed to maintain the status quo and results in energy use without net increase in cell matter. Therefore, according to the above definitions, macromolecular turnover can be considered a maintenance function. However, despite the frequent references to macromolecular turnover as a maintenance function, evidence suggests that it can only account for a small to negligible part of the total energy expended on non-growth functions (Dawes and Ribbons, 1964; Tempest and Neijssel, 1984). This is especially true for growing cells that are thought to have negligible rates of turnover of macromolecules (Mandelstam, 1960). The rate of turnover of macromolecules is reportedly higher in starving cultures (5% per hour) but still is not considered a major part of the total maintenance energy expenditure (Dawes and Ribbons, 1964).

Osmoregulation is commonly noted as a maintenance function (Marr *et al.*, 1963; Ingraham *et al.*, 1983, Tempest and Neijssel, 1984) and the contribution that it makes to total maintenance energy is of particular interest to this thesis. From Chapter 1, for normal function, bacterial cells need to maintain the appropriate osmotic pressure, achieved by the transport and/or synthesis of solutes. These transport and synthetic processes will consume energy continually in both establishing and maintaining the solute gradients involved. Therefore osmoregulation is a potential sink for maintenance energy according to both definitions above.

Tempest and Neijssel (1984) observed increased respiration rates, for *Klebsiella aerogenes*, when osmotically stressed by elevated extracellular potassium concentration. That result was thought to indicate that maintenance of the potassium ion gradient was energetically expensive. Calculations suggested that 90% of the total maintenance energy was used to maintain the potassium ion gradient across the membrane. Also, in chemostat experiments, 1.0 M sodium chloride increased the maintenance energy of *Saccharomyces cerevisiae* compared to low salt controls (Watson, 1970). Both of those observations provide support for the working hypothesis (section 2.5).

Maintenance of near-neutral, intracellular pH may also be a maintenance function. Reduced cell yield and increased maintenance energy have been measured for *Listeria innocua* in the presence of acid stress (ter Steeg *et al.*, 1995).

Apart from the above most commonly recognised maintenance functions, other factors may contribute. These include motility, futile cycles, overflow metabolism, metabolic shifts and uncoupling (Tempest and Neijssel, 1984; Russell and Cook, 1995). Their consideration as actual maintenance functions, however, is to some extent confusing. Using the definition from Pirt (1965) all would result in spilling of energy without net gain in cell matter and therefore can be classified as maintenance functions. However, they are not required for the maintenance of the status quo and therefore are not strictly maintenance functions under alternative definitions (McGrew and Mallette, 1962). Regardless, these would result in energy expenditure on functions that are non-growth related and therefore would result in lower cell yields. This study proposes to use cell yield to indicate the diversion of energy from growth to maintenance, but any affect the above factors have on cell yield will also be included. Therefore cell yield may not be a true indicator of maintenance energy, but an indicator of how efficiently energy is channelled to growth versus other functions, including maintenance.

Endogenous metabolism

Endogenous metabolism requires brief mention because it is often confused with maintenance energy. Endogenous metabolism is the metabolic reactions that occur within starving, but living cells (Dawes and Ribbons, 1969). Some authors have failed to distinguish between endogenous metabolism and maintenance energy, resulting in criticism (Russell and Cook, 1995). The same energy consuming processes are thought to contribute to endogenous metabolism as for maintenance

energy, therefore the two are difficult to separate conceptually. Russell and Cook (1995) suggested that the term maintenance energy be used exclusively for populations in which most of the cells are able to grow, while endogenous metabolism should be used when growth is not possible. It would be equally valid to recognise that the magnitude of maintenance energy expenditure may differ with growth phase and to define energy expenditure during non-growth phases as 'starvation maintenance energy'. Cells are metabolically different when growing versus starving (an example concerning rate of turnover of macromolecules was given earlier) therefore maintenance during growth and non-growth phases is most likely quite different, and the distinction proposed by Russell and Cook (1995) is justified.

3.1.3 Estimation of maintenance energy

Both batch and continuous culture methods have been used to demonstrate and measure bacterial maintenance energy. Batch culture methods have historically met with limited success, whilst the relatively newer continuous culture technique has been more successful.

Batch culture

Batch culture is the closed system where bacterial growth occurs in a fixed volume, and with which food microbiologists are most familiar. In batch culture the growing bacteria continually change their extracellular environment until it is no longer able to support growth, due to exhaustion of an essential nutrient, or build up of inhibitory products.

By necessity (continuous culture had not been invented) the first attempts to measure maintenance energy were performed in batch culture. Again Monod features prominently in reviews of the topic (e.g. McGrew and Mallette, 1962; Pirt, 1965; Russell and Cook, 1995). Monod attempted to estimate maintenance energy from plots of cell yield (turbidity) versus the amount of energy substrate. A positive intercept of the plot with the energy substrate axis (where growth was zero) corresponded to the maintenance energy. However, the plots extrapolated to the origin, so he concluded that any maintenance energy was negligible (McGrew and Mallette, 1962). Similar studies, both before and following Monod, were also unsuccessful at estimating maintenance energy from batch culture experiments using both bacteria and fungal species (McGrew and Mallette, 1962). However,

maintenance energy was estimated for batch culture of *E. coli* using a different approach (McGrew and Mallette, 1962). Starting with turbid cultures, the minimum amount of glucose required to maintain turbidity at the starting level was determined and taken to indicate maintenance energy. The approach has, however, been criticised because the culture was not growing and therefore true maintenance energy (see '*Endogenous metabolism*') was not estimated (Russell and Cook, 1995). Also the frequency of feeding would have resulted in a feast and famine cycle, leading to overestimation of the maintenance energy by that method (Marr *et al.*, 1963).

Continuous culture

Continuous culture is an open system, where growth occurs in a constant volume that is continually turned over by addition of fresh media and removal of overflow. Once an equilibrium state is reached the cell number and nutrient status are constant, a situation referred to as the steady state. This type of culture is less common than batch culture, but has been applied for some research purposes and is common in industrial fermentation processes.

The most common type of continuous culture, the chemostat, allows control of population density and growth rate by manipulation of the flow rate and the concentration of a limiting, essential nutrient. The growth rate of bacteria is limited by very low substrate concentrations (Figure 3.1), in a manner that is similar to the limitation of reaction rate by substrate concentration in Michaelis-Menten kinetics.

Maintenance energy can be calculated by measuring the yield variation with growth rate (Pirt, 1965). Several mathematical derivations have been proposed. Table 3.1 provides a summary of the general approaches that have been applied.

Comparison of batch and continuous culture

Following its development, continuous culture has been the method of choice for investigations of the energy partitioning between growth and maintenance. But, in the current study batch culture was considered more representative of the conditions in food products, about which there has been some debate. Tempest (1970) criticised

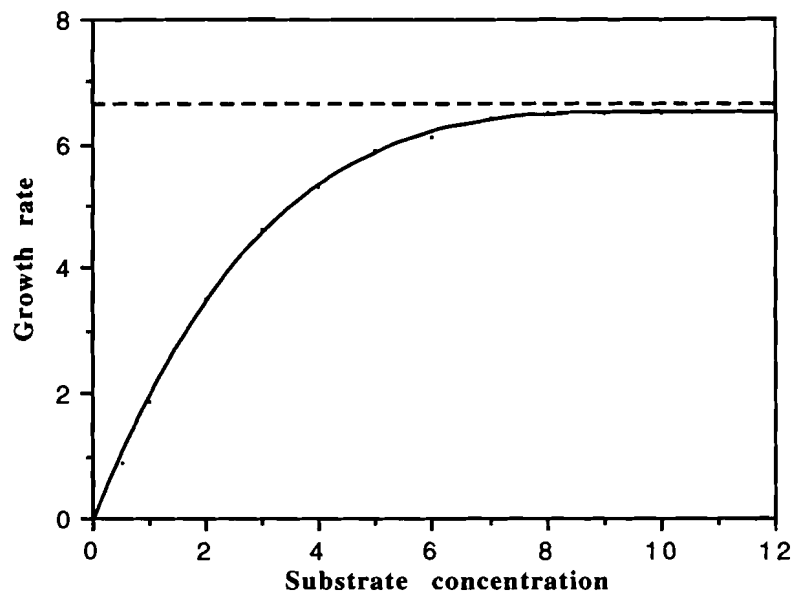


Figure 3.1: Low substrate concentrations limit the growth rate of bacteria. Chemostat cultures are designed so that growth rate is always substrate limited, allowing manipulation of the growth rate.

Table 3.1: Summary of the general approaches to estimation of maintenance energy from continuous culture. Note that this list is not exhaustive, but summarises the main approaches that have been described for estimating maintenance energy.

Approach	Comments
Herbert <i>et al.</i> (1956)	<ul style="list-style-type: none">• conceptualised maintenance energy as ‘negative growth’
Marr <i>et al.</i> (1963)	<ul style="list-style-type: none">• continued the negative growth theme• defined ‘specific maintenance rate’• determined by assuming that maintenance energy causes a decrease in the theoretical maximum growth rate (the growth rate that would occur if there was no maintenance)
Pirt (1965)	<ul style="list-style-type: none">• criticised the negative growth concept as artificial and indirect• defined the ‘maintenance coefficient’• determined by assuming maintenance energy does not influence growth rate, but causes a decrease in the theoretical maximum cell yield (the cell yield that would occur if there was no maintenance)
Neijssel and Tempest (1976)	<ul style="list-style-type: none">• conceptually similar to Pirt• mathematically differs from Pirt, resulting in different partitioning of the experimental error

batch culture as being unrepresentative of conditions in nature because:

- (1) organisms would rarely be confined in a closed and protected environment
- (2) rarely would nutrients be present in concentrations sufficient to support growth at its maximum possible rate
- (3) the natural environment generally would contain other species of organisms which would compete more or less effectively for the available nutrients.

Batch and continuous culture are equally unrepresentative of conditions in nature, considering criteria (1) and (3), therefore those criteria cannot be used to separate the two approaches. However, issue (2) is useful in distinguishing between batch and continuous culture approaches. Tempest (1970) argues that continuous culture is more representative because in nature it is most likely that some essential component will be present at a low, enzyme sub-saturating concentration resulting in sub-optimal bacterial growth rates. However, this study was approached from a food microbiology perspective, and evidence from this discipline suggests that this is not true. In the context of predictive microbiology, batch culture experiments are used to determine the maximum specific growth rates of microbes in complex, nutrient-rich media under defined environmental conditions. These maximum specific growth rates are then summarised into a predictive model that is used to forecast the growth of bacteria on foods, a 'natural' environment. Successful predictions of bacterial growth on foods have been achieved by these methods (Smith, 1985; Smith, 1987; Ross, 1996; Walls and Scott, 1996; Neumeyer *et al.*, 1997). Therefore it appears that in nature, or at least on foods, that bacterial growth occurs at the maximum specific growth rate until nutrient limitation or some other environmental condition becomes sufficiently unfavourable to prevent growth.

There have been several reports that the yield coefficients from glucose in batch, or glucose sufficient cultures, are less than those in continuous culture (Pirt, 1957; Marr *et al.*, 1963; Crabbendam *et al.*, 1985). This phenomenon has not been explained, but is of concern for this study because it implies that the metabolism of the cells is different using the two approaches. That there is a difference is no surprise; manipulation of growth rate by restricting the concentration of carbon source would be expected to result in metabolic adjustments. But it is undesirable to artificially alter metabolism in this way and then measure maintenance requirements. Therefore batch culture was considered most appropriate for this study, and a protocol to measure cell yield and maintenance energy in batch cultures was needed.

3.2 Protocol development

3.2.1 Materials and methods

Details of strain, media and equipment used are in Appendix A.

Cell yield (aerobic)

To determine the concentration of glucose that limited final optical density (cell yield) to within the observable range of the optical density scale, *E. coli* SB1 was grown in minimal media containing various glucose concentrations. Inoculum was prepared as a washed cell suspension (Appendix A.3), to avoid carry over of glucose from the inoculum to experimental broths of known glucose contents. Inoculum was transferred to L-tubes containing 9 mL of minimal media. Eleven glucose concentrations (Table 3.2) were tested, in duplicate. The 22 L-tubes were incubated in a 20°C constant temperature room, held in an Advantec shaking incubator that was operated isothermally.

Table 3.2: Glucose concentrations used in the development of an experimental protocol to measure cell yield for aerobic culture of *E. coli* SB1 in minimal media.

Treatment	x rec [*]	g glucose/ml	Symbol [†]
1	0.0001	1.0 x 10 ⁻⁷	
2	0.0003	3.0 x 10 ⁻⁷	
3	0.001	1.0 x 10 ⁻⁶	
4	0.003	3.0 x 10 ⁻⁶	
5	0.01	1.0 x 10 ⁻⁵	◆
6	0.03	3.0 x 10 ⁻⁵	■
7	0.1	1.0 x 10 ⁻⁴	●
8	0.3	3.0 x 10 ⁻⁴	■
9	1	1.0 x 10 ⁻³	×
10	3	3.0 x 10 ⁻³	□
11	10	1.0 x 10 ⁻²	+

^{*} Indicates the proportion of glucose added compared to the recommended amount. Manufacturers recommend 10 mL of 10% glucose solution per 1 L minimal medium.

[†] Indicates symbol (in Figure 3.2) that corresponds to each glucose treatment.

Growth was monitored turbidimetrically (section 2.2.1); %T and time elapsed since inoculation were recorded until at least three consecutive %T readings gave the same value $\pm 2\%$ ('optical density plateau'). This was assumed to indicate that available substrate had been exhausted. %T readings were converted to absorbance (OD) using Equation 3.1. Optical density was not measured directly because it is a log scale that was more difficult to read reliably on the analogue instruments available.

$$OD = 2 - \log(\%T) \quad (3.1)$$

Cell yield was calculated as the change in optical density from the starting value to the value at the optical density plateau.

Generation time was calculated using fitted parameters from a Gompertz equation (Appendix A.3) and by linear regression (Appendix 3.1).

Maintenance energy was estimated using two methods that are outlined in detail in Appendix 3.2.

Cell yield (anaerobic)

The protocol development described above was repeated for anaerobic culture of *E. coli* SB1. Some methodological adjustments were necessary due to the logistics of anaerobic culture. A washed cell suspension could not be prepared anaerobically, therefore a 48 hour culture of *E. coli* that was assumed (later demonstrated, Figure 3.4) to be glucose limited was used as inoculum. This was transferred to 20 mL of minimal media held in anaerobically prepared (Hungate, 1968) L-tubes. The L-tubes used were purpose designed³ to accept fitted butyl rubber stoppers, crimped in place by aluminium caps that provided a gas-tight barrier. Sodium thioglycollate (1.6 $\mu\text{g/L}$) with vitamin C (1.6 $\mu\text{g/L}$) was used as a reductant and resazurin (0.5 $\mu\text{g/L}$) as an oxygen indicator. Five glucose concentrations were tested (Table 3.3), in duplicate. The 10 L-tubes were incubated at 37°C, in an Advantec shaking incubator, operated isothermally.

³ Halu Glass Manufacturing Pty. Ltd., Whittons Road, Kettering, Tasmania, Australia, 7155.

Table 3.3: Glucose concentrations used in the development of an experimental protocol to measure cell yield for anaerobic culture of *E. coli* SB1.

Treatment	g glucose/ml	Symbol*
1	1.43×10^{-3}	■
2	2.86×10^{-3}	□
3	4.29×10^{-3}	●
4	5.72×10^{-3}	○
5	7.14×10^{-3}	+

* Indicates symbol (in Figure 3.4) that corresponds to each glucose treatment.

Validation

The protocol developed above, for aerobic culture, was validated using pH stress as a variable, with some methodological changes based on experience from the protocol development experiments. A washed cell suspension of *E. coli* SB1 (Appendix A.3) was inoculated into 150 mL minimal medium in 250 mL conical flasks, held at 37°C in Ratek shaking water baths. Twenty six flasks containing broths of varying pH were adjusted using hydrochloric acid to approximately the target pH, before autoclaving. After autoclaving, pH was measured using an Orion pH meter and recorded as the initial pH. Each flask was assigned randomly to a position in one of three waterbaths, as indicated in Appendix 3.3. Each waterbath was set to shake at $42 \pm 10\%$ oscillations per minute .

Growth, generation time and final optical density were determined in the same manner as described above.

At the end of the experiment culture purity and identity were assessed by streaking onto nutrient agar and eosin methylene blue agar.

3.2.2 Results

Cell yield

Figure 3.2 shows growth curves for *E. coli* SB1 in aerobic broths containing various glucose concentrations. Those turbidity plateaus, in which final optical density was less than 1.0, indicate substrate limitation of final population density. This substrate limitation was observed for those glucose concentrations tested below 1.0×10^{-3} g glucose/mL, the concentration recommended by the manufacturers of the minimal media. Each curve in Figure 3.2 that is substrate limited represents duplicate determinations; little variation in final cell yield was noted, indicating the high precision of cell yield estimates using this method. Where cell yield was substrate limited the relationship between initial glucose concentration and final cell population, measured as change in optical density, was linear (Figure 3.3).

Similar results (Figures 3.4 and 3.5) were obtained from anaerobic cultures. In the anaerobic experiment, substrate limitation of final population density was observed for all glucose concentrations tested. Cell yield estimates appeared more variable in the anaerobic experiments than the aerobic (especially for 7.14×10^{-3} g glucose/mL). This may be due to differences in methodology between the aerobic and anaerobic treatments that could not be overcome for logistical reasons. It was necessary to add sterile glucose solutions after media had been autoclaved. For broths prepared aerobically the glucose solutions were added using pipettes capable of precisely dispensing small volumes. However, for the anaerobic media, glucose solutions were administered as 0.2 mL aliquots using a needle and 1 mL graduated syringe. Therefore, initial glucose concentration was more precisely controlled for aerobically compared to anaerobically prepared media. Variability in initial glucose concentration would result in variability in final cell yield and could explain the increased variation that was noted in Figure 3.4.

Cell yield estimates by optical density were assessed by comparing the expected change in cell yield with that observed (Table 3.4). The expected change in cell yield was calculated from the known initial glucose contents of the media. Expected and actual changes were similar for cell yield determined by optical density.

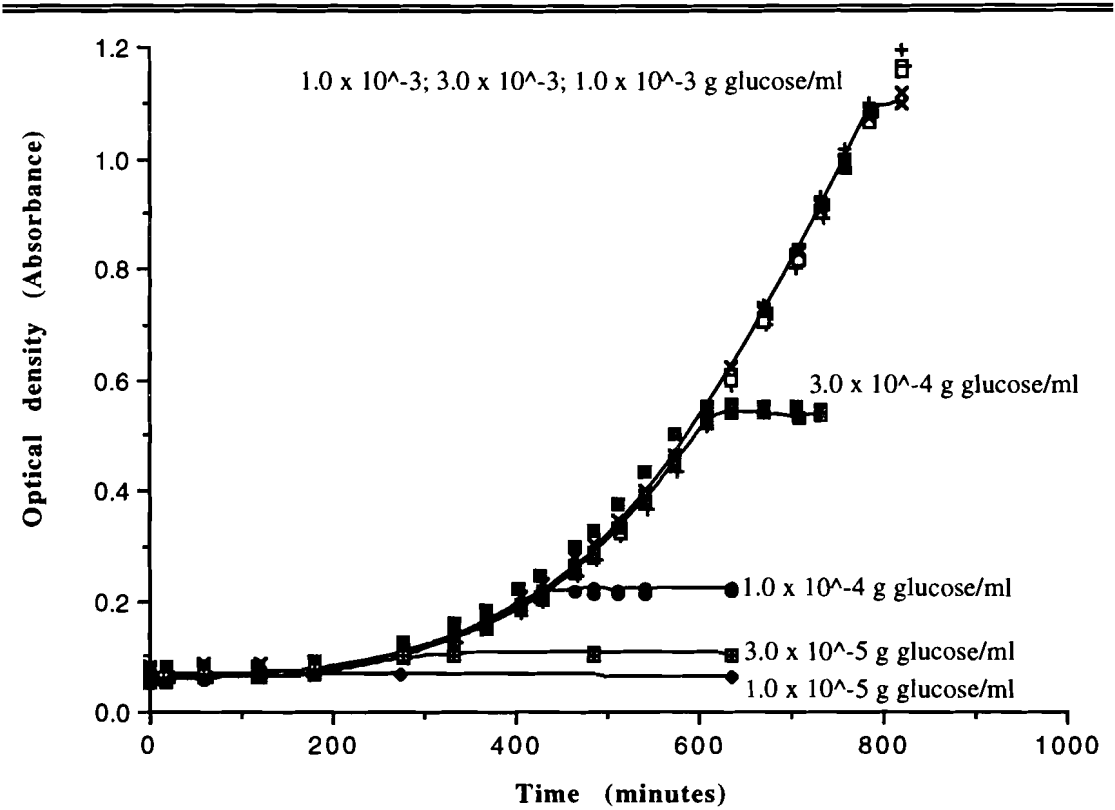


Figure 3.2: Growth curves for aerobic *E. coli* SB1 cultures in minimal media, at 20°C, containing different concentrations of glucose (as indicated). Curves were fitted using the interpolate function in Cricket Graph 1.3.2. For data refer to Appendix 3.4

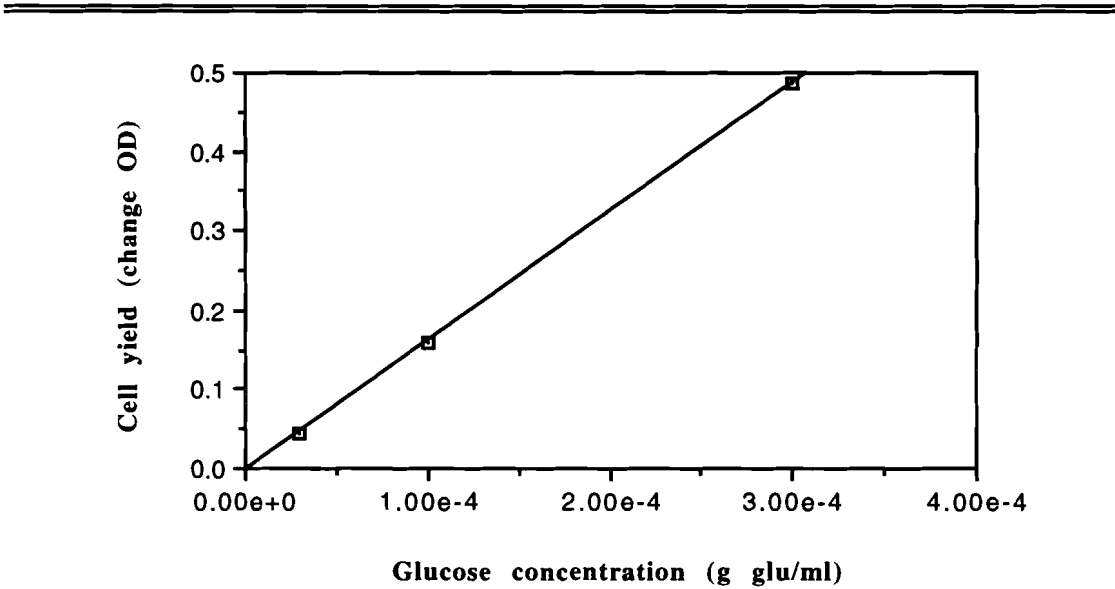


Figure 3.3: Cell yield, measured by optical density, as a function of initial glucose concentration, for aerobic culture of *E. coli* SB1, in minimal media at 20°C. The linear relationship is described by the regression line (Cricket Graph 1.3.2): $y = -4.4024 \times 10^{-3} + 1637.7x$, $r^2=1.000$.

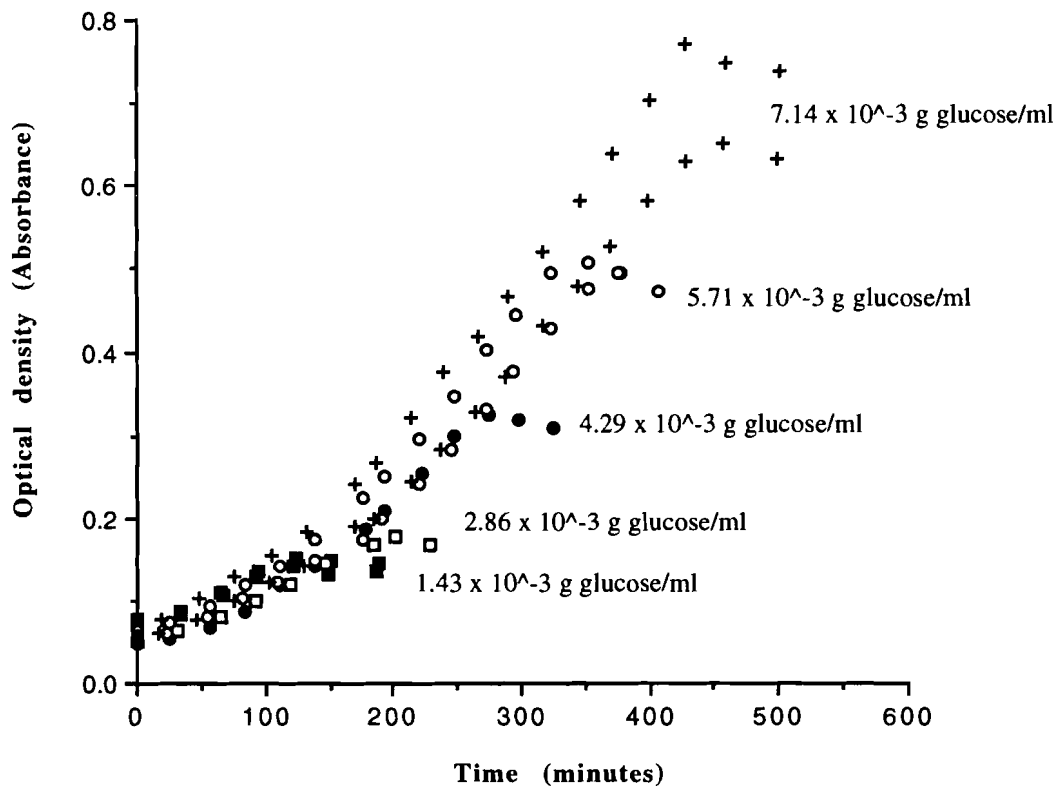


Figure 3.4: Growth curves for anaerobic *E. coli* SB1 cultures in minimal media, at 37°C, containing different concentrations of glucose (as indicated). For data refer to Appendix 3.5.

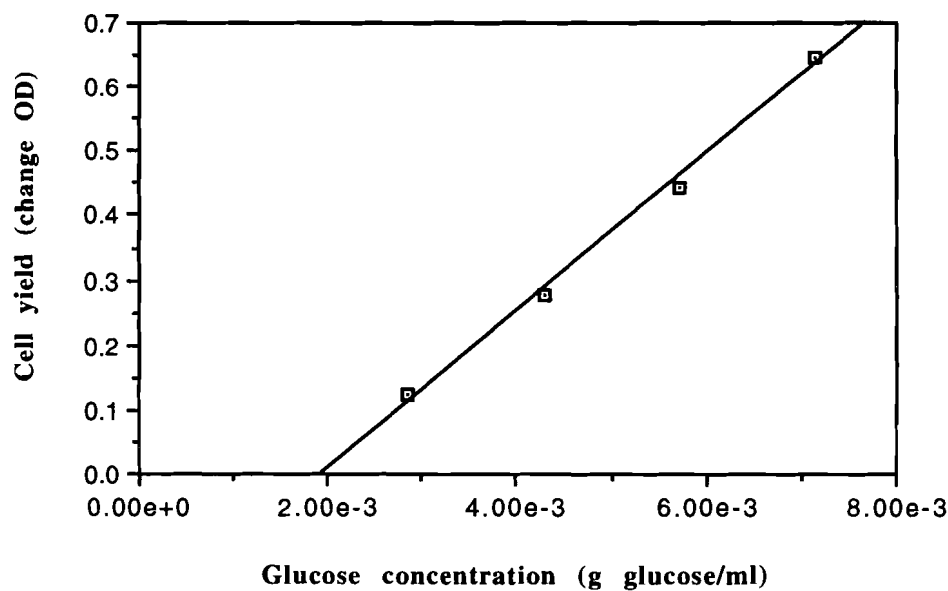


Figure 3.5: Cell yield, measured by optical density, as a function of initial glucose concentration, for anaerobic culture of *E. coli* SB1, in minimal media at 37°C. The linear relationship is described by the regression line (Cricket Graph 1.3.2): $y = -0.23464 + 121.28x$, $r^2 = 0.996$.

Table 3.4: Comparison of expected and observed cell yield estimates.

Comparison	Cell yield ratio	
	Expected	Observed
1*	3.33	3.53
2†	3.00	3.06

* comparison for treatments 6 and 7 (Table 3.2)

† comparison for treatments 7 and 8 (Table 3.2)

Growth rate

Figures 3.2 and 3.4 indicate that for the glucose concentrations tested, growth rate was not influenced; in Figure 3.2 all points fall along the same curve until substrate limitation causes growth to cease and, in Figure 3.4 all curves appear to have the same slope. Growth rates determined by two methods are shown in Table 3.5 and 3.6. These were analysed using residuals plot (Figure 3.6 and 3.7) to test for systematic errors. Residual plots are interpreted by observing the scatter of residuals (observed minus mean) about the mean. The absence of a pattern indicates random error, but patterns indicate systematic errors. For generation times estimated using the modified Gompertz function a pattern was observed: at low glucose concentrations and therefore low cell yield, residuals were above the mean whilst at high cell yields they tended to fall below the mean. This was true for data from both aerobic and anaerobic experiments. In contrast the linear regression data showed no obvious pattern.

Table 3.5: Generation time and growth rate of *E. coli* SB1 cultured aerobically in minimal media at 20°C, as a function of glucose concentration. The ‘a’ and ‘b’ refer arbitrarily to members of duplicate pairs. No observable growth occurred for treatments 1-6.

Treatment	Modified Gompertz		Linear Regression	
	Gen Time (min)	Growth Rate (min ⁻¹)	Gen Time (min)	Growth Rate (min ⁻¹)
7a	176	5.67 x 10 ⁻³	148	6.75 x 10 ⁻³
7b	168	5.95 x 10 ⁻³	145	6.90 x 10 ⁻³
8a	137	7.28 x 10 ⁻³	135	7.38 x 10 ⁻³
8b	140	7.13 x 10 ⁻³	147	6.80 x 10 ⁻³
9a	137	7.28 x 10 ⁻³	143	7.01 x 10 ⁻³
9b	145	6.89 x 10 ⁻³	155	6.46 x 10 ⁻³
10a	136	7.35 x 10 ⁻³	144	6.96 x 10 ⁻³
10b	136	7.36 x 10 ⁻³	140	7.12 x 10 ⁻³
11a	135	7.40 x 10 ⁻³	141	7.08 x 10 ⁻³
11b	135	7.42 x 10 ⁻³	143	7.01 x 10 ⁻³

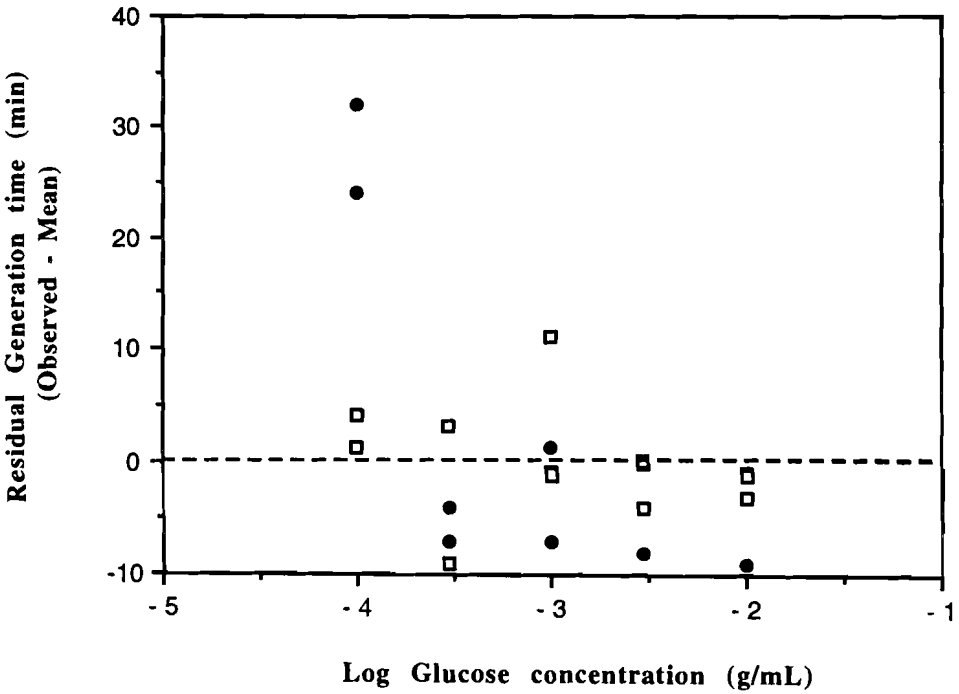


Figure 3.6: Residuals plot for generation times determined by modified Gompertz equation (●) and linear regression (◻), for aerobic culture of *E. coli* SB1.

Table 3.6: Generation time and growth rate of *E. coli* SB1, cultured anaerobically in minimal media at 37°C, as a function of glucose concentration. The ‘a’ and ‘b’ refer arbitrarily to members of duplicate pairs. Generation time could not be calculated for treatment 1 because there was insufficient growth (minimum change in %T of 30% is required). Duplicates for treatments 2 and 3 were unavailable due to broken culture vessels.

Treatment	Modified Gompertz		Linear Regression	
	Gen Time (min)	Growth Rate (min ⁻¹)	Gen Time (min)	Growth Rate (min ⁻¹)
2	164	6.10 x 10 ⁻³	100	1.00 x 10 ⁻²
3	115	8.27 x 10 ⁻³	101	1.13 x 10 ⁻²
4a	113	8.82 x 10 ⁻³	103	9.67 x 10 ⁻³
4b	125	7.99 x 10 ⁻³	109	9.17 x 10 ⁻³
5a	112	8.92 x 10 ⁻³	106	8.27 x 10 ⁻³
5b	121	8.24 x 10 ⁻³	104	8.83 x 10 ⁻³

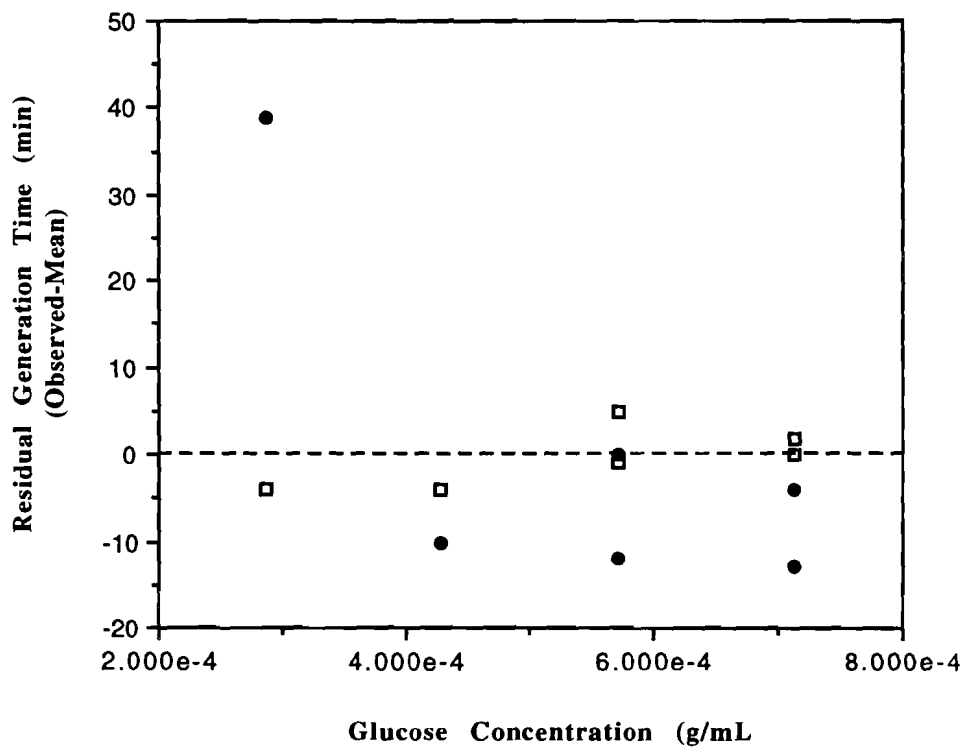


Figure 3.7: Residual plot for generation times determined by modified Gompertz equation (●) and linear regression (□) for anaerobic culture of *E. coli* SB1.

Maintenance energy

Table 3.7 shows maintenance energy estimates by two methods from the data collected in the protocol development section, for aerobic culture of *E. coli* SB1.

Table 3.7: Maintenance energy estimated by Method 1 and 2, as outlined in Appendix 3.2.

Parameter	Method	Estimate
Lag maintenance	1	3.3×10^{-16} g glucose.cell ⁻¹ .min ⁻¹
Lag maintenance	2	1.7×10^{-17} g glucose.cell ⁻¹ .min ⁻¹
Growth maintenance	2	2.8×10^{-15} g glucose.cell ⁻¹ .min ⁻¹
Energy cost/cell	2	2.2×10^{-13} g glucose.cell ⁻¹

Protocol validation

Figure 3.8a shows cell yield as a function of the starting pH of the media. Cell yield appeared to be unaffected by increasing acidity until pH 5, evidenced by the plateau portion of the curve. At initial pH < 5, cell yield declined rapidly. However, pH is a logarithmic scale, related to hydrogen ion concentration by the equation:

$$\text{pH} = -\log[\text{H}^+]$$

(3.2)

In this study the stress imposed by increasing acidity (concentration of hydrogen ions) is of interest, therefore, it is more appropriate to plot cell yield as a function of hydrogen ion concentration (Figure 3.8b). Figure 3.8b shows a linear decline in cell yield as a function of hydrogen ion concentraion due to increasing acid stress.

Growth rate (Figure 3.9) also declined with increasing hydrogen ion concentration, although, the magnitude of the effect was not great. The fastest recorded growth rate in this experiment was 0.0261 min⁻¹ (generation time: 38 min) whilst the longest was approximately half at 0.0138 min⁻¹ (generation time: 72 min). This lowest generation time was observed at pH 4.33. It should be noted that growth (change in optical density) was observed at pH values below pH 4.33 but less than one generation of growth occurred before substrate became limiting; growth rate values could not be reliably calculated from such data. No change in optical density was observed in broths that had an initial pH value below pH 4.09.

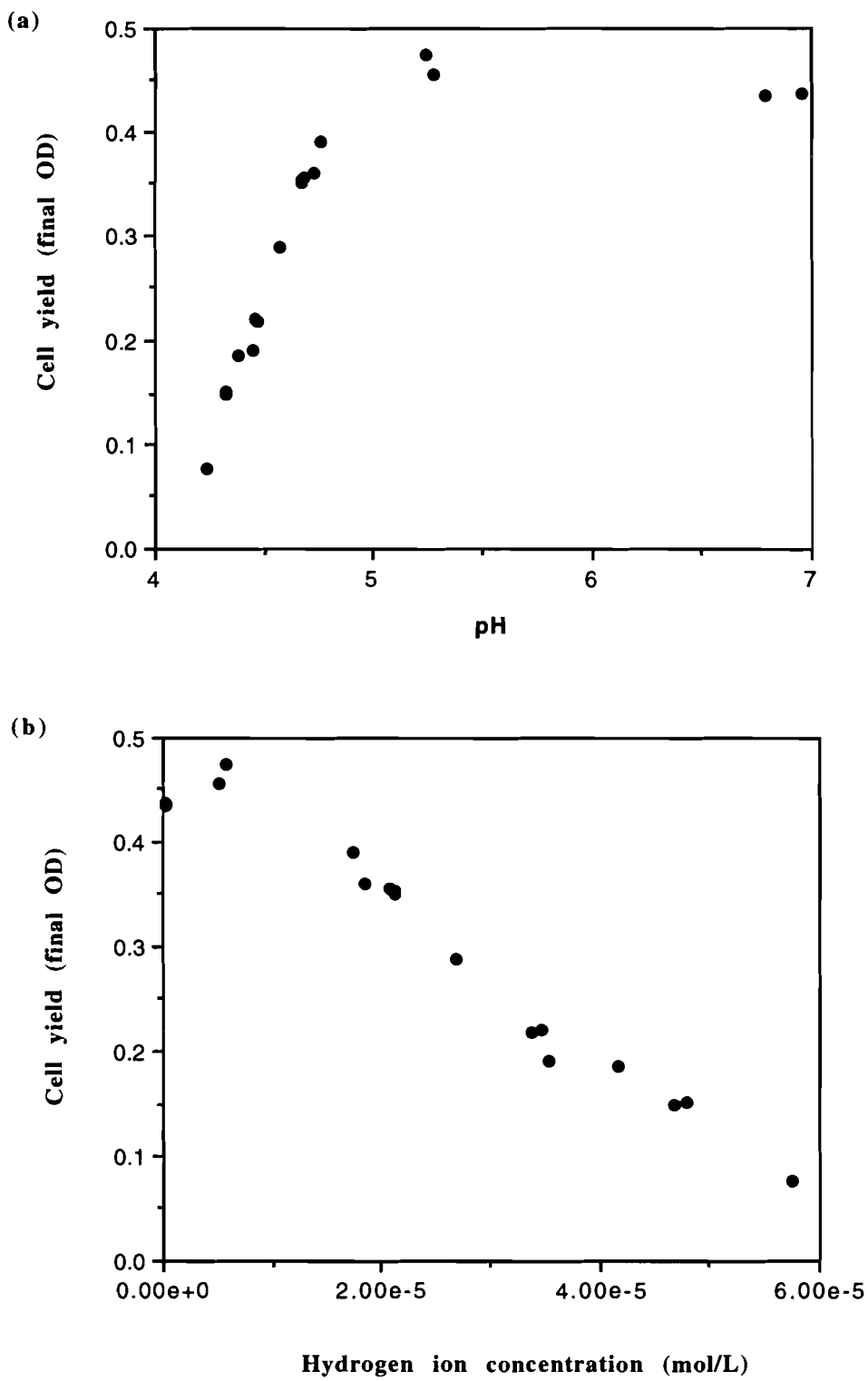


Figure 3.8: Cell yield of *E. coli* as a function of pH (a) and hydrogen ion concentration (b). Experimental data is available in Appendix 3.3.

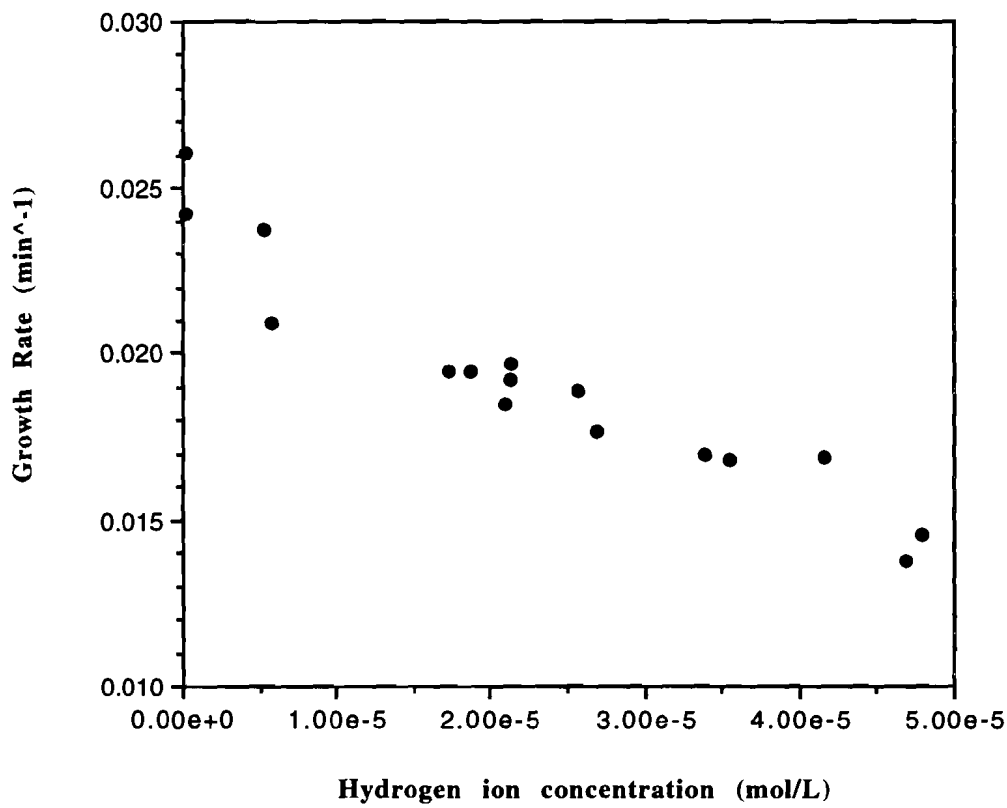


Figure 3.9: Growth rate of *E. coli* SB1 (determined by linear regression) as a function of hydrogen ion concentration. For data refer to Appendix 3.3.

3.2.3 Discussion

Measurement of cell yield

The linear relationship between change in optical density and initial glucose concentration (Figures 3.3 and 3.5) confirms that cell yield can be measured using optical density. The favourable comparison of observed and expected cell yield (Table 3.4) provides further support. This is in accord with several literature reports where optical density has been used to indicate cell yield (Bauchop and Elsdén, 1960; Rosenberger and Elsdén, 1960; Coultate and Sundaram, 1975), and is expected because optical density is proportional to biomass, within limits (Koch, 1981).

Other options for estimating cell yield are determining dry weight, determining total protein or viable plate counting. Optical density methods were preferred to each of these. Dry weight estimates of cell yield are directly related to optical density estimates, but are more labour intensive. Viable plate counting is also very labour intensive and only indicates the numbers of cells present, not actual cell yield (biomass). Cell size varies with growth rate and growth conditions (Pirt, 1957), therefore, the same biomass can be contained in many small cells or fewer large ones. Hence, different cell numbers are possible at equivalent cell yields. In contrast, optical density indicates biomass, and is relatively independent of cell size effects (Rosenberger and Elsdén, 1960). Exceptions occur only in extreme cases with large variations in cell size affecting optical density measurements (Koch, 1984).

The suitability of a protein assay as an indicator of cell yield is also questionable. Cell yield estimated in this way is susceptible to changes in cellular composition. The protocol under development was intended for measuring cell yield as a function of extracellular water activity, and cellular composition may change in response to such changes in the extracellular environment (Clark and Parker, 1984). Therefore, cell yield estimated by protein assay may be confounded.

One problem with measuring cell yield using optical density methods was noted; there were variations in the optical characteristics of individual L-tubes, due to scratches in the glass that accumulate with use. To overcome this problem the protocol was modified; subsequent experiments (section 3.3) were performed by culturing in conical flasks and, at each reading time, transferring small sub-samples to a spectrophotometric tube for measurement of optical density. Therefore, the same tube could be used for all optical density readings throughout the experiment, avoiding the

effects of variations in L-tubes. However, the modification was not logistically possible for the anaerobic experiments. Fortunately the anaerobic L-tubes were newer and had fewer scratches.

Substrate limitation of cell yield

From Figure 3.2 a glucose concentration of 5.0×10^{-4} g glucose/mL was chosen for all subsequent aerobic substrate-limited batch culture experiments. This was considered appropriate because final optical density fell in the approximately linear range of the optical density scale and was at the upper end of the range to leave the most scope for observing reductions in cell yield. It was assumed that cell yield would be maximal where no stress was applied, such as the conditions in the protocol development experiments. Therefore any deviations in cell yield, in response to applied stresses, were expected to be decreases. Similarly, from Figure 3.4, the limiting substrate concentration selected for anaerobic culture of *E. coli* SB1 was 5.71×10^{-3} g glucose/mL. Note that the quantity of glucose required for anaerobic culture of *E. coli* SB1 is approximately 10-fold greater than that for aerobic culture. This is in accord with the stoichiometry of ATP production from glucose (Brock and Madigan, 1991). Theoretically, respiration produces a net 38 ATP molecules per molecule of glucose, compared to 2 ATP molecules produced by fermentation; a factor of nineteen difference that approximately corresponds to the result noted above.

The growth rate of *E. coli* SB1 was unaffected by initial glucose concentration (discussed in more detail below). This implies that for growth at these concentrations (and therefore at the concentrations selected for incorporation into the experimental protocols) the membrane porters for glucose were saturated. Comparison of the K_m (Michaelis constant) for glucose uptake and the glucose concentration chosen confirms this. The K_m for glucose uptake by *E. coli* was estimated as 0.2 to 20 μ M (Postma, 1987), compared to the molar concentration of glucose chosen: 2.8 mM (or 28 mM for the anaerobic protocol), which is in large excess of the K_m for glucose uptake.

Other studies have been criticised for overlooking this problem (Marr *et al.*, 1963) and for this experimental protocol it was particularly important to ensure that the absolute amount of glucose present in the media limited cell yield, not the concentration and therefore the scavenging ability of the cell.

Estimation of growth rate

Several methods exist for estimating growth rate from turbidity data. The two used here were compared by residual plots (Figures 3.6 and 3.7). The pattern noted for growth rate calculated using fitted parameters from a Gompertz equation suggests that the Gompertz equation overestimated generation time (underestimated growth rate) at low cell yields. Therefore it is an inappropriate method for estimation of generation time for data generated by the substrate-limited batch culture method developed here.

The inadequacy of growth rate estimation using the Gompertz equation was confirmed (Figure 3.10) using data for *P. halodenitrificans* (Chapter 2). The growth curve was cropped systematically by increments of 10% (%T) until the minimum change in %T was 20%. Figure 3.10 shows growth rate estimated by both linear regression and the Gompertz approaches. Estimates using the Gompertz equation were unaffected by cell yield until final optical density was approximately 0.5 after which growth rate was increasingly underestimated with falling 'cell yield'. This effect was minimised when linear regression was used to estimate generation time. Figure 3.10 also suggests slight systematic overestimation of growth rate by the linear regression method at low cell yields, but this was much smaller in magnitude compared to the systematic errors associated with the Gompertz equation. Therefore growth rate estimation by linear regression was used for all subsequent growth rate determinations for data derived from substrate-limited batch cultures.

The systematic error detected for growth rate estimates using the Gompertz equation was not surprising because the Gompertz equation determines maximum specific growth rate by fitting a sigmoid curve to optical density data (Figure 3.11). This is appropriate for data derived from non-substrate limited cultures because they are sigmoid in shape. For substrate limited culture, however, the curve increases in a similar manner to their non-substrate-limited counterparts, but optical density readings plateau abruptly once the substrate has been exhausted (Figure 3.2).

Estimation of maintenance energy

Estimation of maintenance energy by method 1, mimics attempts by Monod (1942, cited by Russell and Cook, 1995) to estimate maintenance energy from batch culture, using a plot of glucose concentration versus cell yield and determining the positive intercept with the glucose axis. Despite finding a positive intercept in

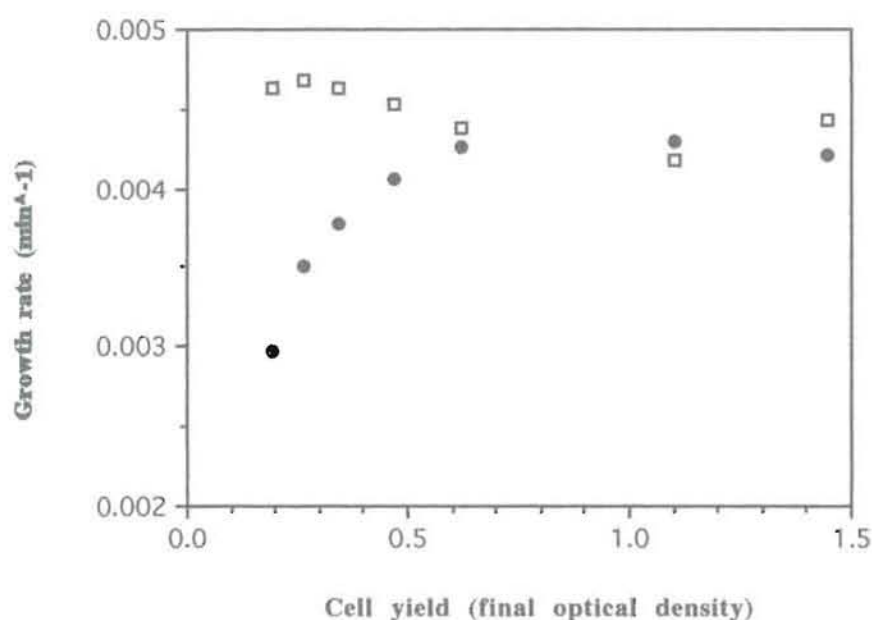


Figure 3.10: Systematic underprediction of growth rate by the Gompertz equation. Growth data for *P. halodenitrificans* at 6% NaCl and 24.8°C. Growth rate calculated using the modified Gompertz function (●), linear regression (□).

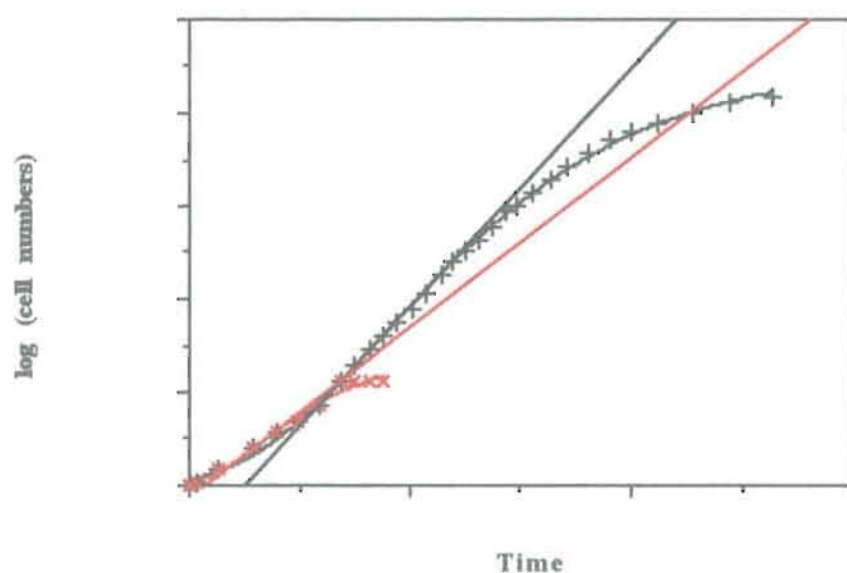


Figure 3.11: The Gompertz equation describes a sigmoid curve. This is appropriate for normal batch cultures that are not substrate limited (—), but is not appropriate for data derived from the substrate limited batch culture method developed here (—). The tangent, from which growth rate is estimated, is indicated for both data sets. The slope of the tangent is shallower for the truncated data set leading to underprediction of growth rate.

Figure 3.3, significant data rounding effects were noted. This is a consequence of the small magnitude of the displacement of the intercept from the origin; on a scale where 3.0×10^{-5} g glucose was the final data point, the intercept occurred at 2.7×10^{-6} g glucose (an order of magnitude difference). Therefore small changes (due to rounding) in the plotted values translated to significant changes in the intercept value. Hence, limited confidence was placed in the maintenance energy value determined by method 1. Further, this method estimates maintenance energy during the lag phase, which is likely to be different to the maintenance energy during growth (see section 3.1.2, *Endogenous metabolism*).

The estimate, by method 2, of maintenance energy during the exponential growth phase (2.8×10^{-15} g glucose/cell/minute) was large compared to published estimates. Tempest and Neijssel (1987) estimated the maintenance energy of *E. coli* from 3.5×10^{-17} g glucose/cell/minute to 1.5×10^{-16} g glucose/cell/minute. However, those estimates were determined from continuous culture experiments, where higher cell yields are obtained from equivalent amounts of glucose, than in batch fed cultures (Pirt 1957; Marr *et al.*, 1963). Although this phenomenon has not been explained it is well documented, and may explain the larger maintenance estimate obtained in this study.

From Table 3.7, according to estimates from method 2, maintenance represents 1.26% of the cost of cell synthesis. This is comparable to literature reports that 1.25% of the total energy expenditure by cells represents maintenance energy (Pirt, 1965).

However, it was noted that calculated values varied widely due to rounding of values obtained in the intermediate calculations. Small errors in large numbers (used in the calculation) translated to large errors in small numbers (the estimate). This highlighted the difficulties associated with estimating maintenance energy from batch culture experiments, therefore that line of investigation was discontinued.

Protocol validation

The linear decline in cell yield with increasing acid stress (Figure 3.8b), using a model in which energy is partitioned entirely between growth and maintenance, indicates that increased maintenance energy requirements can be measured qualitatively using the substrate-limited batch culture method. The homeostatic mechanisms that operate to maintain near-neutral pH are thought to be energetically

expensive (Booth, 1985). Therefore, if their activity increases, there will be an increased energy demand that requires diversion of energy from growth to maintenance functions, causing the reduction in cell yield. Hence Figure 3.8b shows successful validation of the substrate-limited batch culture technique.

However, the above analysis of the validation data has ignored effects of differences in lag and generation time between different acidity treatments. Changes in lag and generation time change the duration over which the cells must be maintained. This will affect the proportion of the total energy pool used for maintenance (but not the maintenance rate) in turn potentially affecting the cell yield. Fortunately, lag time was negligible for all acid treatments and therefore requires no further consideration for this example. From Figure 3.9 the generation time was only twice as great at the most stringent acidity condition than for optimal conditions. Combining this with the small magnitude of both estimated and reported maintenance energies of *E. coli* under optimal conditions means that generation time changes cannot be invoked to explain the observed changes in cell yield.

Increased maintenance requirements associated with acid stress, resulting in reduced cell yield have been noted previously. For *Listeria innocua* ter Steeg *et al.* (1995) found that maintenance energy expenditure increased exponentially with hydrogen ion concentration, from pH 6.0 to 4.25 and cell yield declined accordingly. This qualitatively corresponds with the cell yield response with acidity found here for *E. coli* SB1. However, ter Steeg *et al.* (1995) could not attribute the changes in cell yield solely to changing maintenance energy, because they also noted shifts in metabolic end products. That result is of concern for this study because it potentially undermines one of the assumptions made - that in substrate limited batch culture all of the energy, supplied as glucose, is used. Clearly, at least for *L. innocua* this is not true. Further, if the metabolic end-products change, either quantitatively or qualitatively, and consequently 'trap' different amounts of energy (as incompletely oxidised carbon compounds) then this would result in changes in cell yield (that are not associated with maintenance energy). This returns to an earlier theme (section 3.1.2); that cell yield may vary due to changes in maintenance energy, but also due to other non-growth, energy consuming processes including metabolic shifts. This highlights a problem that may confound cell yield results derived from substrate limited batch cultures.

3.3 Hypothesis Testing

3.3.1 Materials and methods

Details of strains, media and equipment are in Appendix A.

Cell yield

A series of experiments were conducted to examine the influence of extracellular water activity and compatible solutes on cell yield of *E. coli* SB1. Also, one experiment examined the influence of temperature on cell yield. In all experiments the substrate-limited batch culture protocol, developed in section 3.2, was used unless specifically stated otherwise.

The influence of water activity (adjusted using sodium chloride) and a compatible solute (2 mM betaine) on cell yield, during aerobic culture was determined for water activity values ranging between 0.998 and 0.956. Fifteen treatments were performed in duplicate, in four waterbaths set at 37°C. Treatments and random allocation to waterbaths are described in Appendix 2.1. A similar experiment was performed for anaerobic culture of *E. coli* SB1 when 18 duplicate treatments were incubated in a shaking Advantec incubator at 37°C. Treatments and their random allocation to positions in the incubator are detailed in Appendix 3.6.

In a fully replicated experiment the influence of moderate extracellular water activity on cell yield during aerobic culture of *E. coli* SB1 at 37°C was examined. Treatments, and their random allocation between two Ratek waterbaths are summarised in Appendix 3.7. Cell yield results were analysed using Analysis of Variance and least significant difference (LSD) was calculated from Equation 3.3 using Excel 5.0.

$$\text{LSD} = \text{tinv}(p, df) * \sqrt{\text{MSE}} * \sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)} \quad (3.3)$$

where:

tinv is the inverse of the Student's t-distribution

p is probability (level of significance)

df is degrees of freedom

MSE is mean square error

n_1 and n_2 are the number of replicates from which the two mean values were derived.

The influence of extended lag time on the cell yield of *E. coli* SB1 at 37°C was examined. Four treatments were performed in triplicate in two shaking waterbaths ($42 \pm 10\%$ oscillations per minute). Cell yield was measured for cultures that were held at non-growth permissible water activity conditions (0.966) for different durations and then shifted to permissible water activity conditions (~ 0.973) by the addition of glucose free minimal media (treatment B), glucose free minimal media containing betaine (treatment C) or by addition of betaine to a final concentration of 2 mM (treatment D). Control cultures (treatment A), where the initial water activity value was growth permissible (~ 0.973), were also included. Treatments, and their random allocation to waterbaths are summarised in Appendix 3.8.

Cell yield was determined as a function of temperature for *E. coli* SB1. Aerobic, substrate-limited batch cultures were incubated in L-tubes placed in an Advantec temperature gradient incubator. This experimental protocol was a compromise between the variation in optical characteristics between L-tubes and the large number of temperature treatments that could be generated using the Advantec shaking temperature gradient incubator. Temperatures ranged between 4.3°C and 46.8°C.

Adenylate energy charge

Parallel cultures of *E. coli* SB1 were grown to mid-exponential phase (monitored turbidimetrically) in 50 mL of minimal media made to manufacturer's specifications. The water activity of one of the cultures was adjusted using a concentrated NaCl solution (10 mL), so that the final salt concentration of the broth was 3.5%. Adenine nucleotide levels were monitored throughout and the energy charge (EC) calculated as:

$$EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]} \quad (3.4)$$

Adenine nucleotides were extracted and determined in duplicate. For the extraction of adenylates a 1 mL aliquot of culture was added to 1 mL of ice cold trichloroacetic acid/EDTA solution (10%/4 mM) (Kahru *et al.*, 1987) for ten minutes. In this state the mixture was stable and could be stored in the refrigerator until all of the samples were ready for analysis. Acid precipitable material was then removed by centrifugation at 6000 rpm for 10 minutes. The supernatant (containing extracted

adenine nucleotides) was adjusted with 2 M KOH to pH: 7.75 (the pH optimum for activity of firefly luciferase).

Adenine nucleotides were assayed with luciferin-luciferase using a Berthold Luminometer. 0.1 mL of extract was made up to 0.5 mL using assay buffer (20 mM Tris/2 mM EDTA, pH 7.75) in a disposable cuvette. ATP was then measured by automatic injection of 10 μ L luciferin-luciferase mixture, following light emission to the peak value, and comparison to a standard curve (200 fmol - 2 nmol, ATP). ADP and AMP were measured similarly by first converting them enzymatically to ATP, and calculating the difference. ADP and AMP were converted to ATP by incubating samples at 37°C for 15 minutes with 50 μ L of a solution containing 20 mM Tris, 0.5 mM phosphoenolpyruvate and 20 μ g pyruvate kinase (for ADP) and 50 μ L of a solution containing the above plus 25 μ g adenylate kinase (for AMP).

Death kinetics

E. coli SB1 was grown to mid-exponential phase (approximately 1.0×10^8 CFU/mL) in minimal media (made to manufacturer's specifications) containing 2% NaCl at 37°C and then challenged by a low water activity, by adding concentrated sodium chloride solutions until the desired water activity was reached. Following imposition of the water activity challenge the number of viable cells remaining was determined periodically by removing a 1 mL aliquot, preparing a dilution series in 0.1% peptone water and spreading 0.1 mL of appropriate dilutions, in duplicate, onto nutrient agar. After incubation for 18 hours at 37°C, colonies were counted manually. Two of these experiments were performed. In one the same water activity challenge (0.940) was applied to parallel cultures that differed only in the exogenous provision of 2 mM betaine. In the other parallel betaine present and absent treatments were also used, but the water activity shift applied was to 0.956 (betaine absent) and 0.948 (betaine present). This was approximately the same 'distance' (0.013 water activity units) beyond the water activity growth/no growth interface for *E. coli* SB1 in minimal media in the absence and presence of betaine respectively (Figure 3.12).

3.3.2 Results

Influence of extracellular water activity on cell yield

Figures 3.12 and 3.13, show the cell yield of *E. coli* SB1 as a function of extracellular water activity conditions, for aerobic and anaerobic cultures respectively. Generally, cell yield appeared independent of water activity conditions until a rapid decline occurred close to the water activity boundary between growth and no growth. Cell yield also appeared independent of the extracellular presence of betaine, except at growth limiting water activities. Where betaine was present the rapid decline in cell yield at the boundary between growth permissible and non-growth conditions occurred at a lower water activity value. This protective effect of betaine was observed for both aerobic and anaerobic cultures. Close to the growth/no growth interface, cell yield appeared to decline slightly before the sudden cell yield 'crash'. This was most obvious for the betaine present treatments (Figures 3.12b and 3.13b). The anomalous data point in Figure 3.13a (at a_w : 0.954) was noted and could not be explained, but represents less than one doubling.

Estimates of growth rate (Figure 2.3) and lag time (Figure 3.14a) were obtained for the aerobic substrate-limited batch culture experiment. From Chapter 2, the growth rate of *E. coli* SB1 falls linearly with reductions in the extracellular water activity. The lag time increased exponentially with increasingly stringent water activity conditions (Figure 3.14a). This relationship was linear when lag time data was transformed to the reciprocal of lag time (Figure 3.14b). This data transformation is equivalent to the calculation of growth rate from generation time. In Figure 3.14b, the water activity values where the regression lines intersect the water activity axis indicate where the lag time is infinite. When betaine was absent, lag time was infinite at water activity 0.970, compared to 0.961 where betaine was present. These water activity values correspond to the water activity values where cell yield dramatically declined in Figure 3.12.

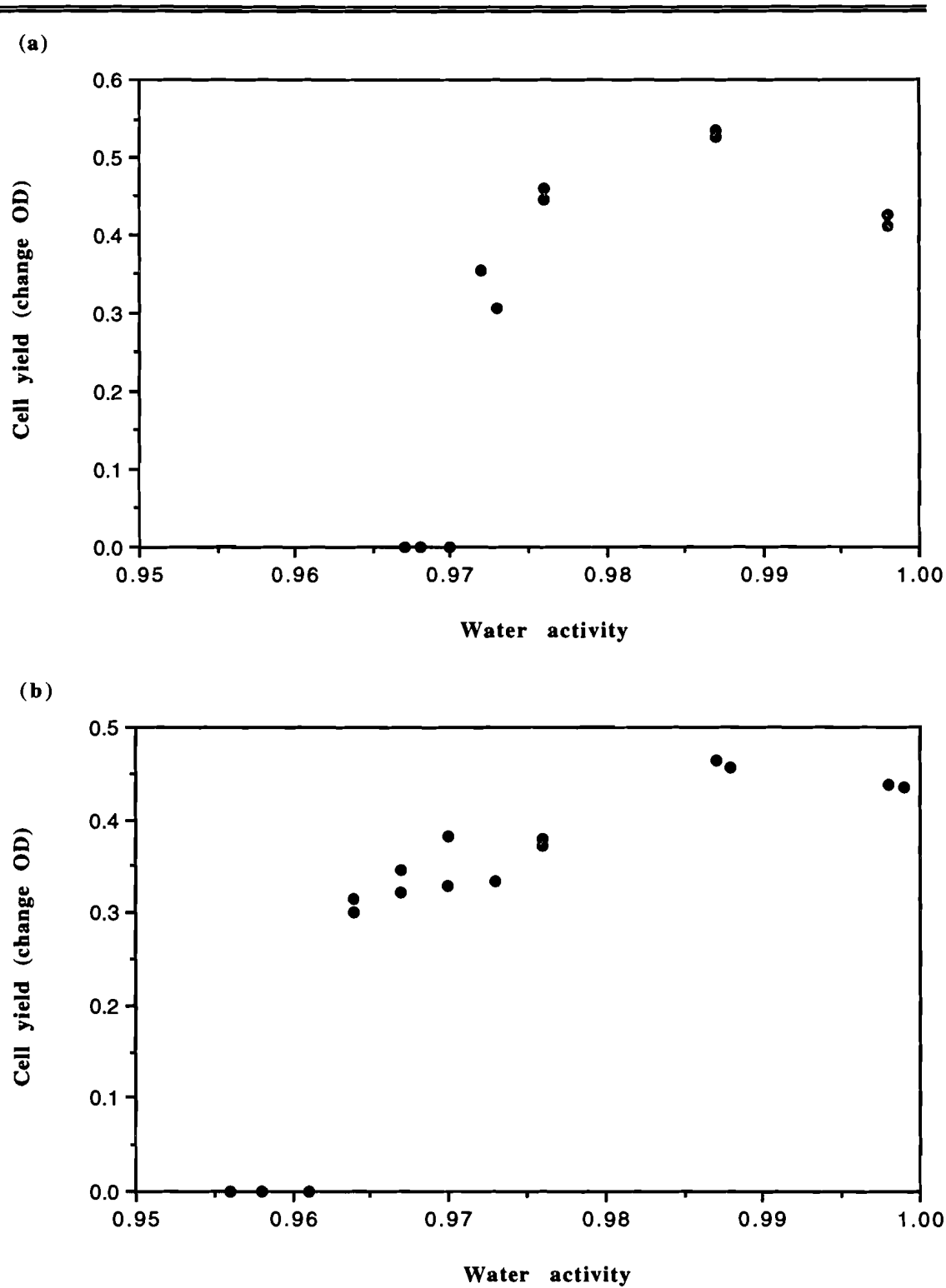


Figure 3.12: Cell yield during aerobic, substrate-limited batch culture of *E. coli* SB1 as a function of extracellular water activity and betaine; (a) betaine absent, (b) contains 2 mM betaine. Cell yield is approximately constant until a rapid decline at water activity values: 0.970 (betaine absent) and 0.960 (betaine present). For raw data see Appendix 3.9.

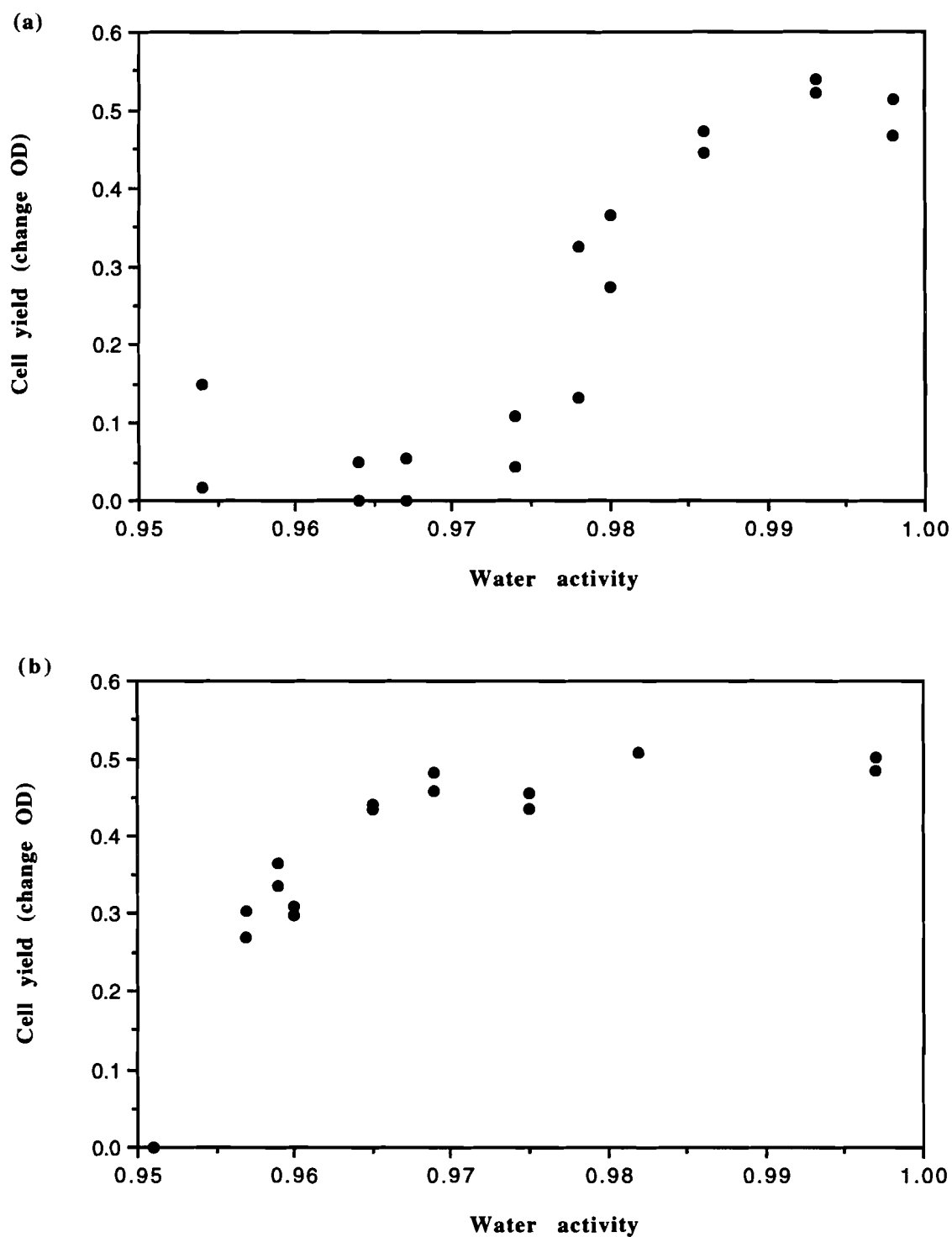


Figure 3.13: Cell yield during anaerobic, substrate-limited batch culture of *E. coli* SB1, as a function of extracellular water activity and betaine; (a) betaine absent, (b) contains 2 mM betaine. Cell yield is approximately constant until a rapid decline at water activity values: 0.980 (betaine absent) and 0.955 (betaine present). For raw data see Appendix 3.6.

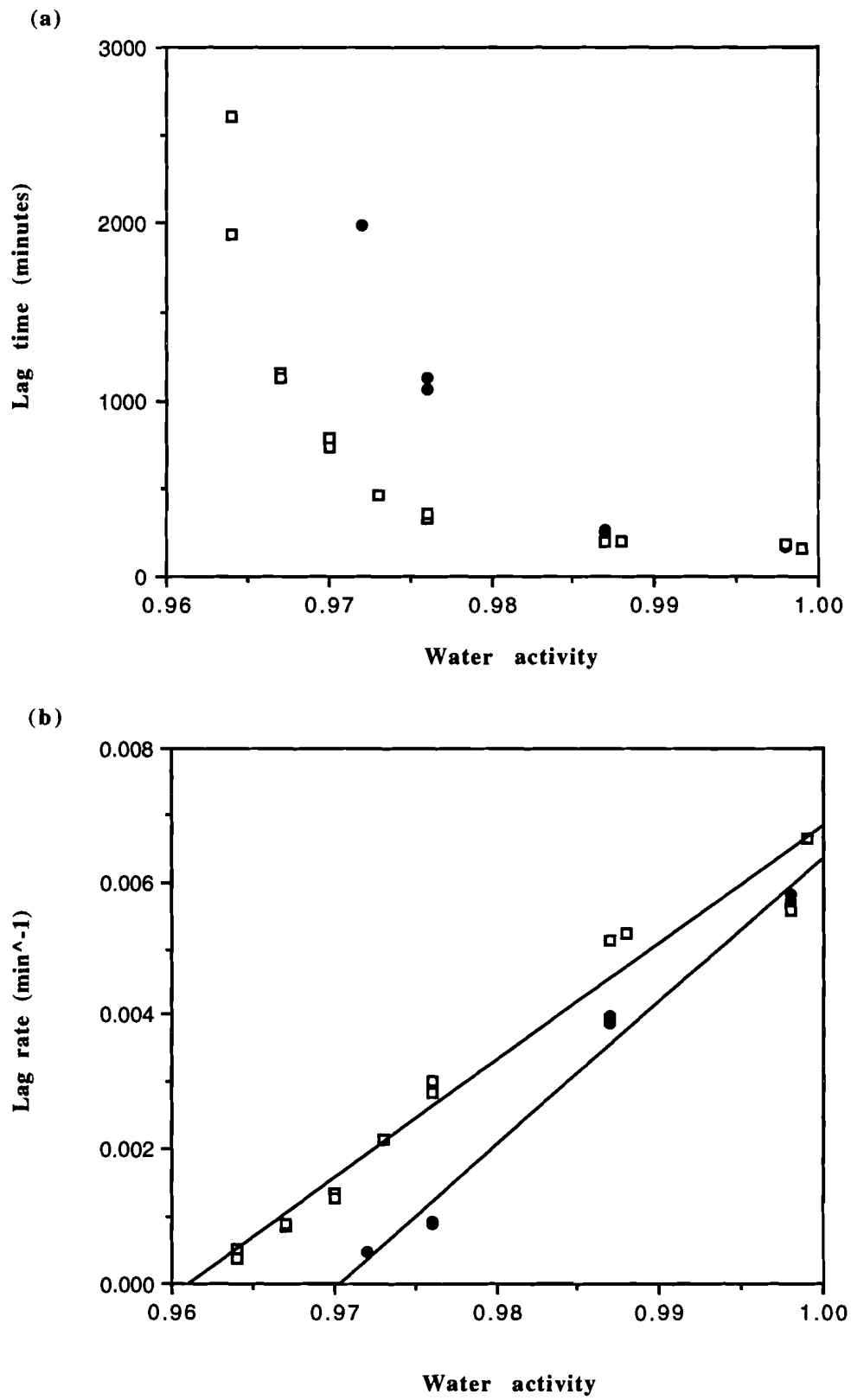


Figure 3.14: Effect of water activity and betaine on (a) lag time and (b) $1/\text{lag time}$ for *E. coli* SB1 cultured in minimal media at 37°C. (●) betaine absent, (□) contains 2 mM betaine.

Figure 3.15 shows the cell yield for *E. coli* SB1 in the substrate-limited hold and shift-up experiments. Cell yield was greatest where there was no holding treatment (A), however, the cell yields from all other treatments were indistinguishable. Therefore, cell yield appeared independent of the method used to adjust conditions from growth non-permissible to permissible. Also, the duration of the holding period did not appear to influence the final cell yield; holding period (ii) was twice as long as holding period (i).

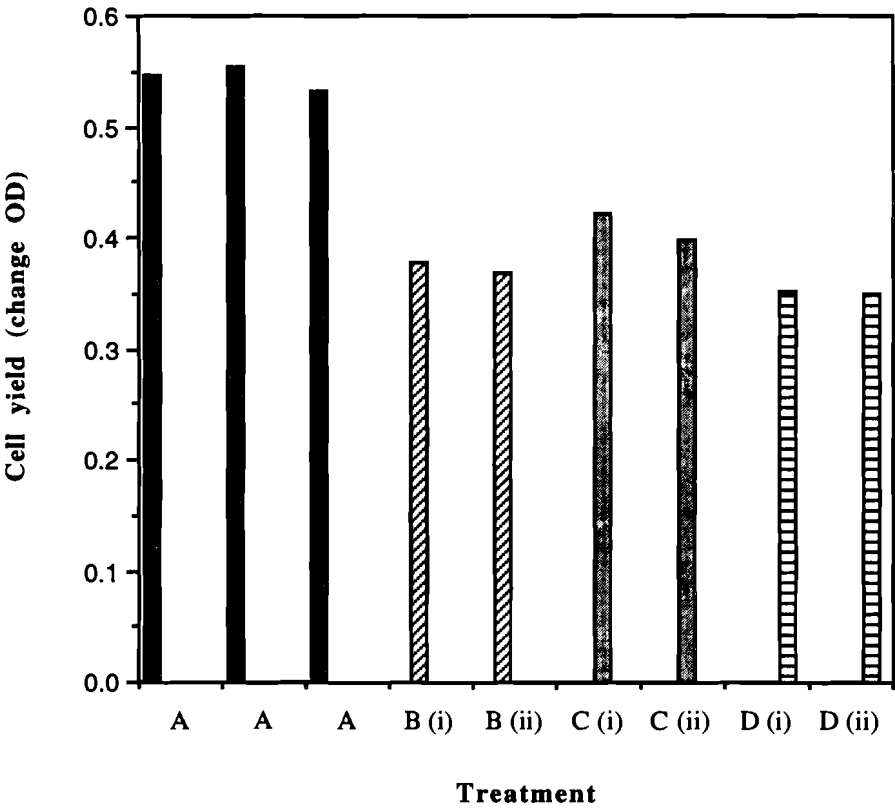


Figure 3.15: The influence of extended lag time on cell yield.

- (A) control, initial a_w : 0.973 (growth permissible, see Figure 3.12a)
- (B) initial a_w : 0.966 (growth non-permissible, see Figure 3.12a), shifted to 0.973 using glucose-free minimal media at (i) 4417 and (ii) 8630 minutes following inoculation.
- (C) initial a_w : 0.966, shifted to 0.973 using glucose-free minimal media containing betaine at (i) 4412 and (ii) 8892 minutes following inoculation.
- (D) a_w : 0.966, shifted to growth permissible conditions using betaine (Figure 3.12b) at (i) 4408 and (ii) 8629 minutes following inoculation.

For raw data see Appendix 3.8.

Influence of temperature on cell yield

Figure 3.16 shows the influence of incubation temperature on cell yield of *E. coli* SB1. Cell yield was largely independent of incubation temperature, except at high and low temperature extremes, where it declined sharply to zero.

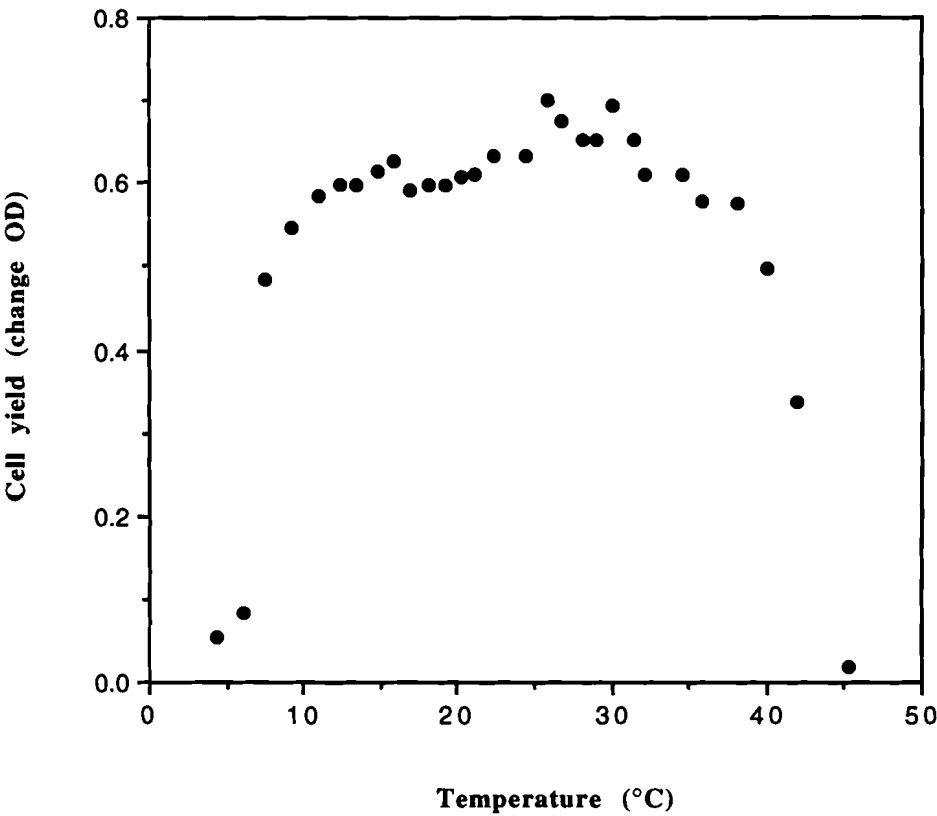


Figure 3.16: Influence of incubation temperature on cell yield of *E. coli* SB1 during aerobic, substrate-limited batch culture. For raw data see Appendix 3.10.

Correlation between growth rate and cell yield

Figures 3.17a, b and c show scatter plots between cell yield and growth rate for pH, water activity and temperature. For acid stress, both growth rate and cell yield decline linearly with hydrogen ion concentration, therefore the two are correlated (Figure 3.17a). The scatter plots for water activity (Figure 3.17b) and temperature (Figure 3.17c), show that growth rate and cell yield were not correlated.

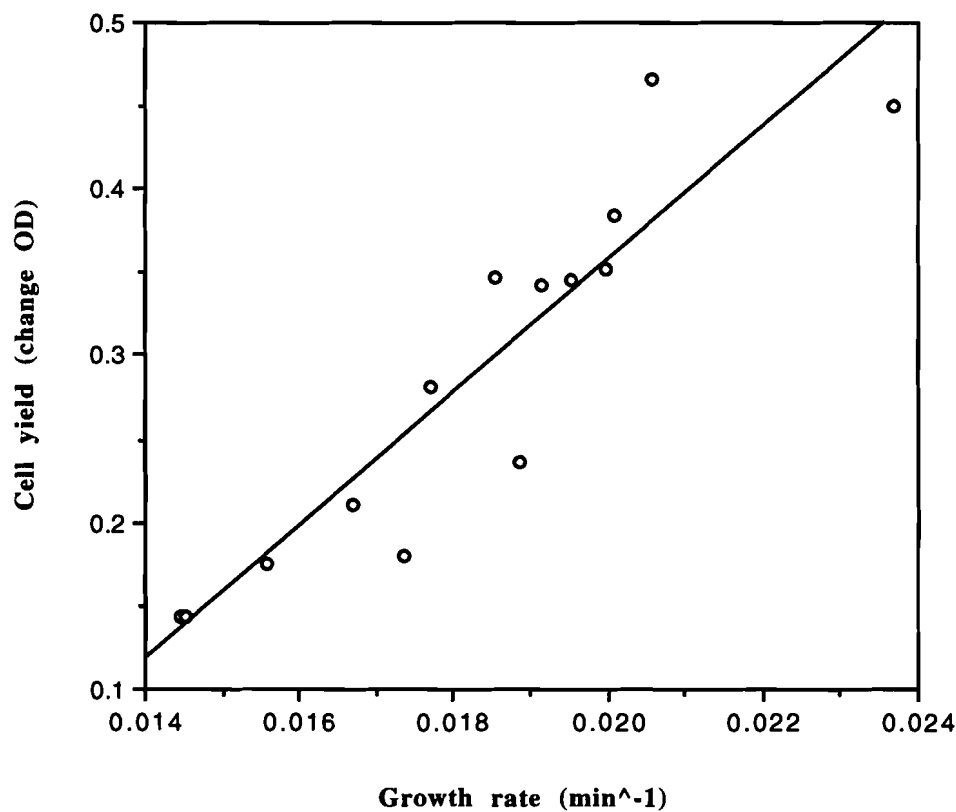


Figure 3.17a: Scatter plot indicating correlation between growth rate and cell yield for *E. coli* SB1, during pH stress ($r^2=0.840$).

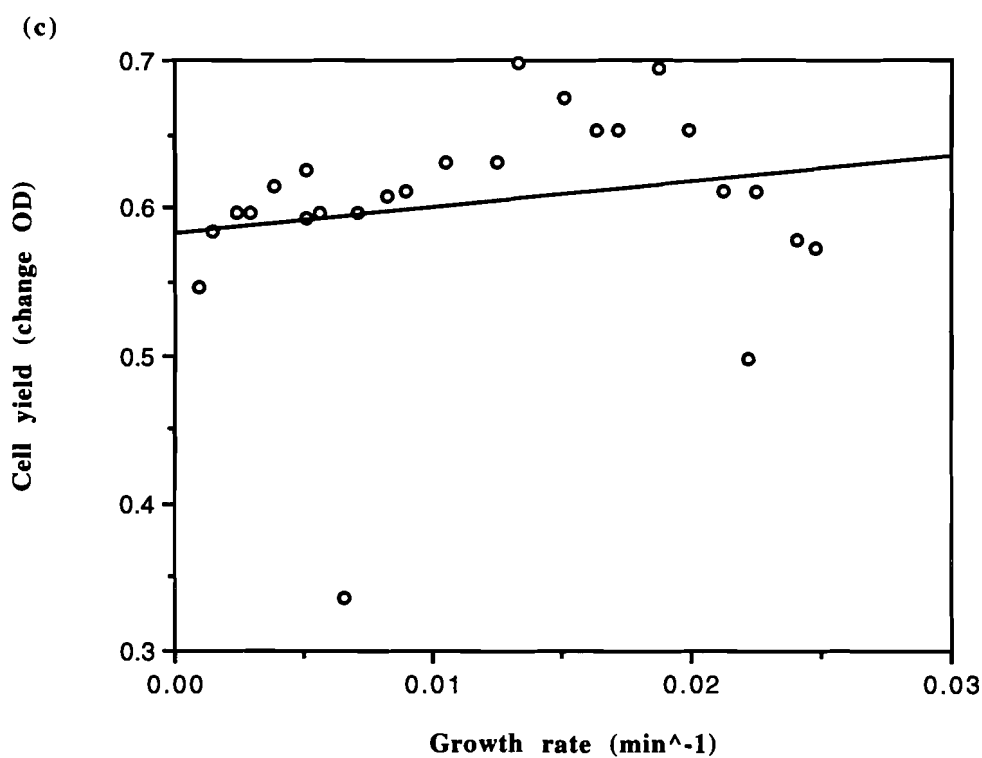
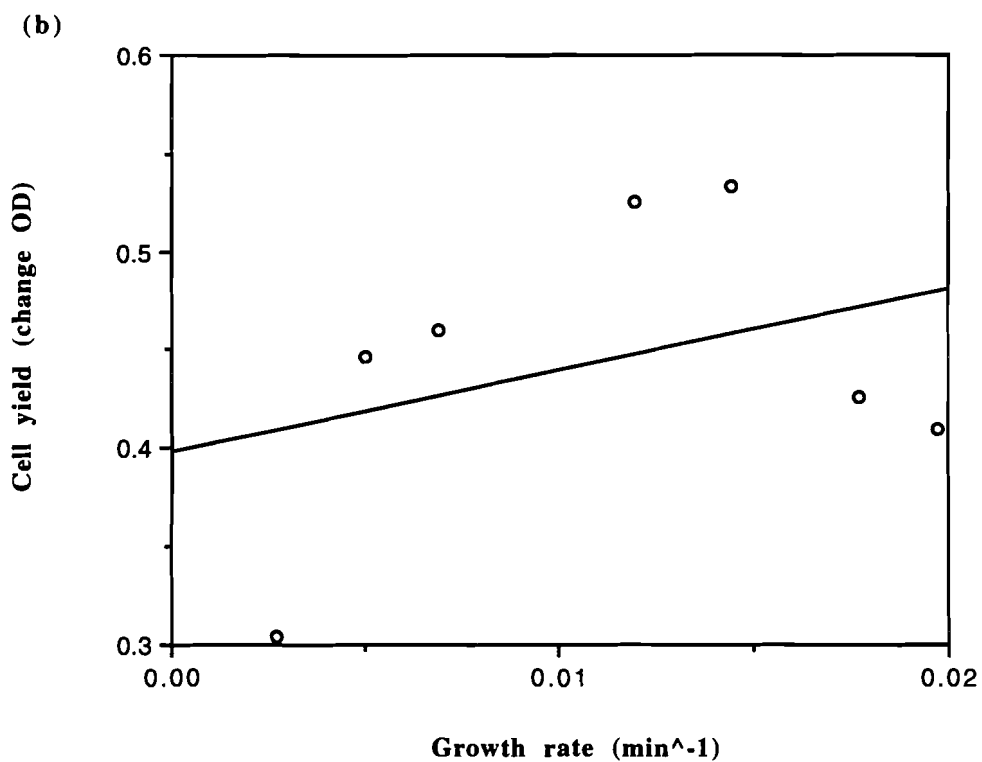


Figure 3.17a&b: Scatter plots between growth rate and cell yield for *E. coli* SB1 as a function of (b) extracellular water activity ($r^2=0.122$) and (c) incubation temperature ($r^2=0.035$).

Influence of moderate water activity conditions on cell yield

In Figure 3.12 slight increases in cell yield were noted at moderate water activity values. Table 3.8 shows the results of an experiment investigating that observation. There was no significant difference between cell yields for cultures without salt, with and without betaine. But significant differences ($P < 0.05$) between all other treatments. Moderate salt levels increased the cell yield of cultures compared to low-salt treatments. The effect was significant, but not as pronounced when betaine was provided. These differences were correlated with differences in generation time (Figure 3.18). Where generation time was longer, cell yield tended to increase (70% correlation).

Table 3.8: Comparison of cell yield and growth rate of *E. coli* SB1 for optimal and moderate water activity stress conditions, in the presence and absence of betaine. Means with the same superscript are not significantly different, all other treatment pairs are significantly different.

Treatment	n	Mean Cell Yield (final OD)	Mean Gen Time (min)
2% NaCl	6	0.5248 ^a	70.03 ^a
2% NaCl + 2 mM betaine	6	0.4475 ^b	58.53 ^b
0% NaCl	3	0.4187 ^c	49.17 ^c
0% NaCl + 2 mM betaine	3	0.4087 ^c	44.80 ^c

$$\text{LSD}(3v's3) = 0.0212 \quad \text{LSD}(3v's3) = 9.82$$

$$\text{LSD}(3v's6) = 0.0184 \quad \text{LSD}(3v's6) = 8.50$$

$$\text{LSD}(6v's6) = 0.0150 \quad \text{LSD}(6v's6) = 6.94$$

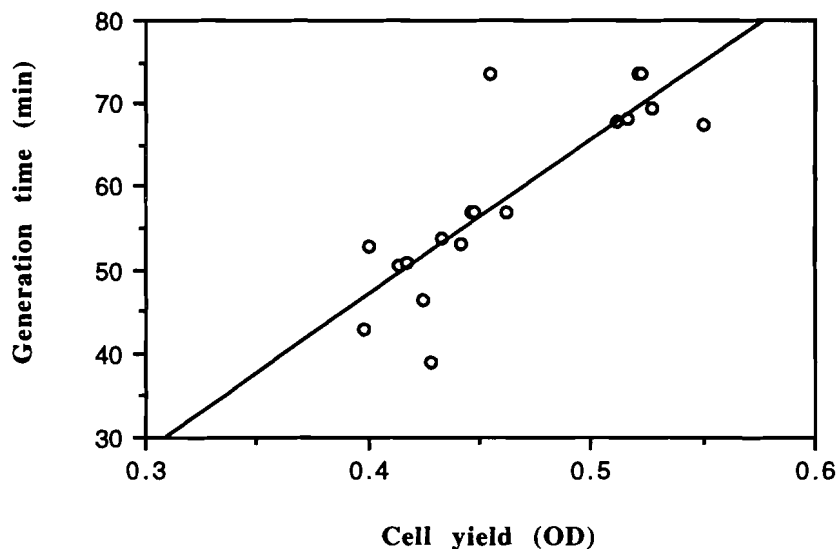


Figure 3.18: Scatter plot indicating correlation between generation time and cell yield for *E. coli* SB1 in moderate extracellular water activity conditions ($r^2=0.701$).

Adenlyate energy charge

Figure 3.19a shows a typical growth curve and energy charge values at various sampling times along the curve. From the growth curve, exponential and early stationary phases can be identified. Adenlyate energy charge was determined during the exponential and early stationary phases of growth. During the exponential growth phase the adenlyate energy charge was high and relatively stable (0.8-0.9), but it fell below 0.8 upon onset of the stationary growth phase.

Figure 3.19b shows a growth curve interrupted by water activity challenge. When salt was added there was an immediate and unexpected increase in turbidity (the peak immediately right of the dotted line) followed by a fall in turbidity, short recovery/lag phase and exponential growth at a slower rate (evidenced by the shallower slope of the curve). Throughout the salt shock and recovery period the energy charge was stable, at values comparable to those in Figure 3.19a. Therefore salt shock did not appear to effect adenlyate energy charge.

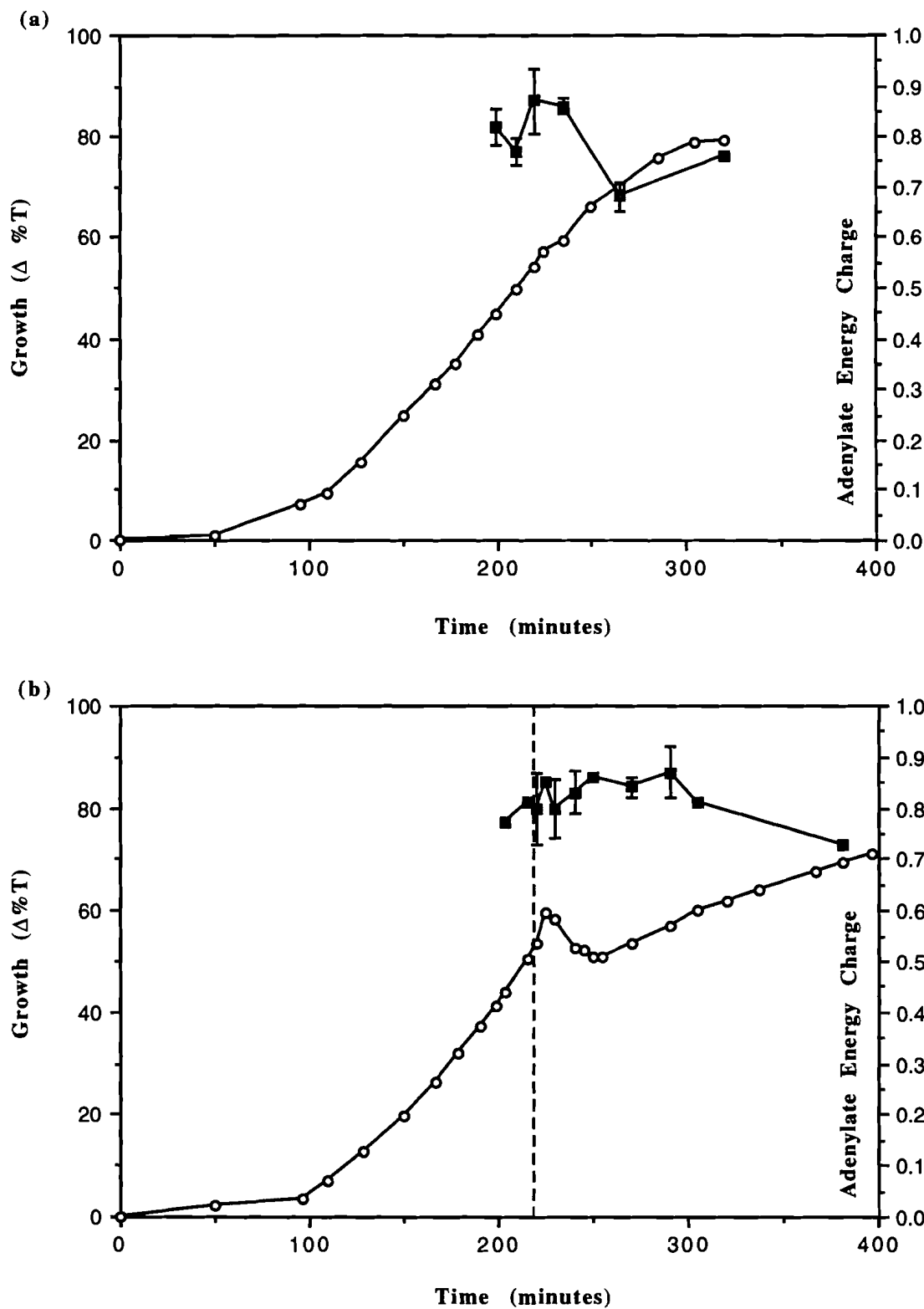


Figure 3.19: Growth curve (\circ) and adenylate energy charge (\blacksquare) for *E. coli* SB1 cultured in minimal media at 37°C, (a) optimal water activity conditions, (b) with osmotic challenge; the broken line indicates where osmotic challenge (3.5% NaCl) was applied. For raw data see Appendix 3.11.

Death kinetics

The survival curves (Figures 3.20) following water activity challenge, show an initial linear decline in cell number followed by a tailing effect, indicating that for the water activities and time frame involved reduction in numbers, but not elimination of viable cells, was possible. In both experiments the presence of betaine in the medium increased the death rate, as indicated by Figure 3.20 and the lower D-values (decimal reduction time) in Table 3.9. Also betaine resulted in greater total reductions in cell number. This is most obvious in Figure 3.20b.

The rate of death was faster at water activity values that were closer to the water activity limit for growth (according to Figure 3.12: 0.970, without betaine; 0.961, with betaine). This was clearest for the salt only treatments. The D-value is smaller where the water activity was 0.956 compared to 0.940, which are 0.014 and 0.030 water activity units respectively beyond the limiting water activity indicated by the cell yield ‘crash’ in Figure 3.12.

Table 3.9: Influence of water activity and betaine on the decimal reduction time of *E. coli* SB1 at 37°C, in minimal media, based on the initial steep slope in Figure 3.20.

Water activity	Betaine	D-value (min)
0.940	-	5881
0.940	+	2886
0.956	-	3561
0.948	+	2093

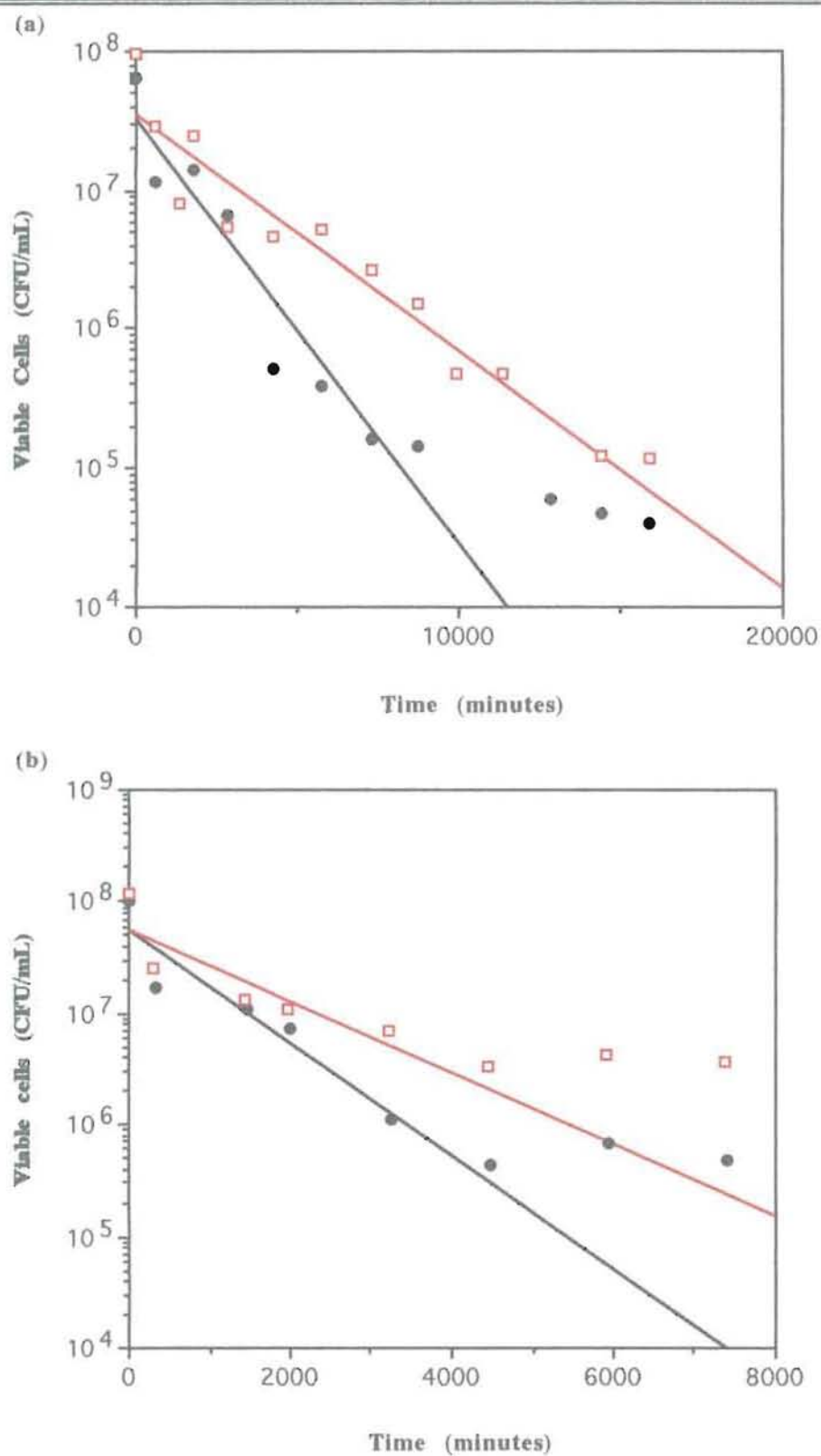


Figure 3.20: Survival curves for *E. coli* SB1 at 37°C in minimal media in the presence (●) and absence (□) of betaine, at 0.940 (water activity) (a), and equidistant from the growth/no growth interface (b). Regression lines based on the initial steep slope. For raw data see Appendix 3.12.

3.3.3 Discussion

Influence of water activity on cell yield

Cell yield was expected to decline linearly with increasingly stringent water activity conditions, in a manner similar to that observed in response to acid stress (section 3.2). This was anticipated because reduced water activity conditions should elicit progressive increases in the activity of the complex osmoregulatory processes described in section 1.2.4. In turn, this would result in energy diversion from growth to maintenance, to fuel those osmoregulatory processes. Such results would have been consistent with the working hypothesis developed in Chapter 2. However, the independence of cell yield on water activity (Figures 3.12 and 3.13), over most of the growth permissible water activity range, indicates that the amount of glucose converted to cell matter was largely independent of water activity. This implies that no energy is diverted from growth to maintenance or other non-growth related processes in response to non-optimal water activity conditions. Therefore, unless there are changes in metabolic end products that are exactly proportionate to those needed to meet increased maintenance energy demands (a highly unlikely scenario), the maintenance energy of *E. coli* SB1 was not significantly influenced by extracellular water activity conditions.

It had also been hypothesised that *de novo* compatible solute synthesis is a large energy burden for bacterial cells in low water activity conditions, therefore exogenous presence of betaine should improve cell yield. However, because cell yield was independent of water activity over most of the growth permissible range, no effect of the extracellular presence of betaine was observed on yield in that range. This is analogous to the result in section 2.3.3 where extracellular provision of betaine had no effect on growth rate when extracellular water activity was favourable (no improvement in response is possible where it is already maximal).

The independence of cell yield and growth permissible water activity also indicates that maintenance energy is a small component of the cell's total energy expenditure. In Figure 3.12, lag and generation times increase as water activity decreases, thereby increasing the duration over which cells must be maintained. Therefore, even if the maintenance rate (per cell per minute) is independent of water activity, at more stringent water activities the total amount of energy used for maintenance must have increased. The small magnitude of the maintenance energy is consistent with estimates in the literature (section 3.2.3) and in section 3.2.3.

The small reductions in cell yield noted for water activity conditions close to the boundary between growth and no-growth, may be due to increased lag and generation times. Alternatively it may indicate reduced cellular efficiency, due to increased wastage of substrate by increases or changes in metabolic end products, or metabolic uncoupling.

The independence of cell yield and water activity for *E. coli* SB1 was in contrast to the only literature report on the effects of water activity on cell yield that could be found (Watson, 1970). In that study, significant reductions in the cell yield were measured for *Saccharomyces cerevisiae* in response to water activity stress. However, the study used a respiratory mutant strain, and a single level of water activity stress (0.967). Information on the relative level of stress this represented compared to the water activity limit for growth was lacking. The water activity used was significantly higher than the reported minimum water activity (0.88) for growth of *S. cerevisiae* (VanDemark and Batzing, 1987). However, this water activity limit applies to the type-strain, as opposed to the mutant strain which may have different growth limits. If the water activity stress applied was close to the limit for growth of the mutant strain then the reduction in cell yield noted for *S. cerevisiae* may correspond to the yield 'crash' in this study (Figures 3.12 and 3.13). Unfortunately insufficient information was provided to resolve this question.

A report that 0.001 M methyl- β -D-thiogalactopyranoside (TMG) had no significant effect on the specific maintenance of *E. coli* (Marr *et al.*, 1963), is consistent with the cell yield result obtained here. TMG is a compound that causes the cell to 'leak', therefore cultures containing TMG increase solute accumulation activities. The insignificant effect of TMG indicated that solute accumulation is not energetically expensive.

Although cell yield data for most of the growth permissible range of water activities indicates that the mechanistic explanation for growth responses to water activity is not due to energetic limitations, the sudden decline in cell yield at critical water activity conditions may be due to a sudden energetic limitation that results in substrate exhaustion before the lag phase can be resolved. The lag phase represents a period of cellular regrouping, when all the enzymes and other factors essential for growth are assembled. If the energy substrate is exhausted before that regrouping is completed then growth would not occur. However, evidence from the hold-shift experiment (Figure 3.15) does not support this interpretation; cell yield appeared independent of the duration of the holding period (artificial lag). The lag time data is

consistent with the hold/shift results. The infinite lag time (Figure 3.14b) is indicated by the regression through data points derived where growth is possible, suggesting that even with continual substrate feeding, the lag phase would not be resolved. Therefore, the sudden decline in cell yield at critical water activity values is unlikely to be due to substrate exhaustion during the lag phase.

Influence of incubation temperature on cell yield

Figure 3.16 is consistent with reports that indicate cell yield is independent of growth temperature, except at limiting values (Senez, 1962). The sudden decline in cell yield at critical high and low temperatures is commonly observed. Even in 1942 Monod noted, for critical high temperatures, that this was a generally observed phenomenon (McGrew and Mallette, 1962).

The cell yield response with incubation temperature is consistent with master reaction models that describe the influence of temperature on microbial growth (Sharpe and DeMichele, 1977; Schoolfield *et al.*, 1981; Ross, 1993). Master reaction models assume that one enzyme in a metabolic sequence is a bottle-neck that is the rate limiting step and defines the growth rate. Reversible denaturation of the key enzyme at critical high and low temperatures is also assumed. The main features of the master reaction model of Ross (1993) are shown in Figure 3.21. According to Figure 3.21 the proportion of active key enzyme is constant across most of the growth permissible temperature range, but declines abruptly at critical high and low temperatures. This response coincides with the sudden decline in cell yield. Therefore the sudden decline in cell yield at critical temperatures may be due to denaturation of a key enzyme.

Not previously reported is the similarity of the cell yield response curves for temperature and water activity. This similarity may indicate that similar mechanistic explanations apply. The rapid decline in cell yield at critical water activity values due to denaturation of a key enzyme is plausible. Also, the lower critical water activity value where betaine is present is consistent with this interpretation, if compatible solutes stabilise protein molecules (Wiggins, 1990). These observations provide further impetus for rejecting the working hypothesis developed in Chapter 2.

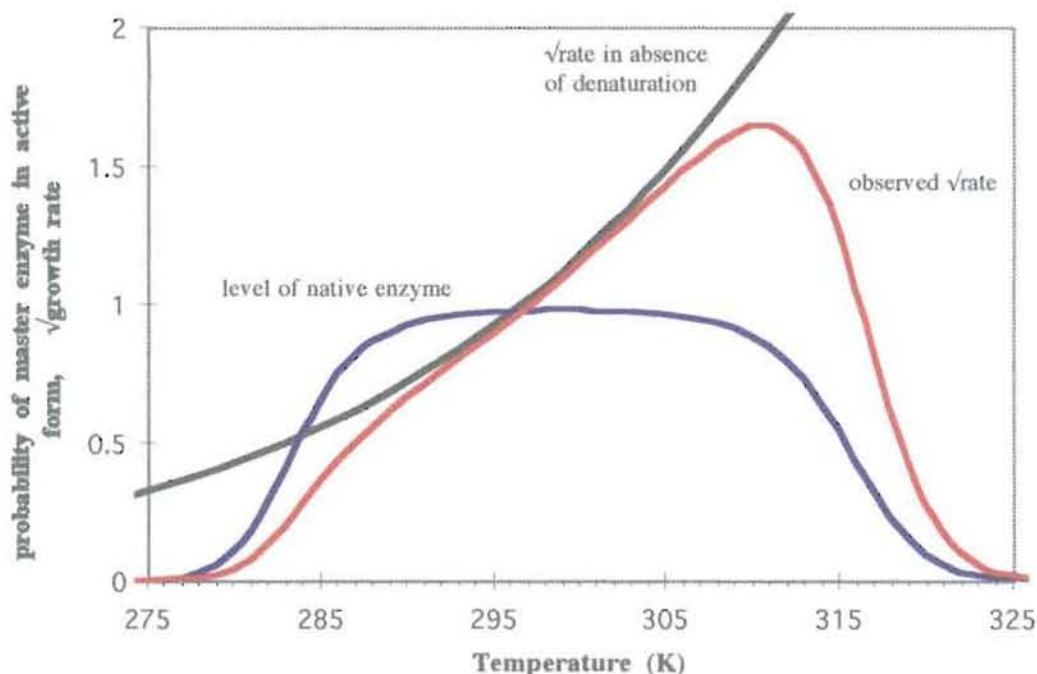


Figure 3.21: Diagrammatic representation of a master reaction model (McMeekin *et al.*, 1993; Ross, 1993). Bacterial growth (—) obeys linear Arrhenius kinetics (—) at temperatures where most of the putative key enzyme is in the native state. Deviations approaching the high and low temperature limits for growth occur due to denaturation of the key enzyme. The model was based on the hydrophobicity of denatured macromolecules.

Correlation between growth rate and cell yield

Precedent (Watson, 1970) suggests that reduced cell yield may be linked to an increased requirement for energy substrate for maintenance purposes that cause reduced growth rate. That is, cell yield and growth rate are coupled; changes in one would result in corresponding changes in the other. The correlation between growth rate and cell yield for acid stressed *E. coli* SB1 (Figure 3.17a) support this notion. However, comparison of cell yield and growth rate as a function of water activity (Figure 3.17b) and temperature (Figure 3.17c) indicates that growth rate and cell yield are not coupled. Even with dramatic reductions in growth rate, due to low temperature or low water activity, cell yield was not affected, until critical values close to the growth/no growth interface. Therefore, growth rate is not a reliable indicator of energetic limitation of bacterial growth.

Efficiency of Growth

Results shown in Figure 3.12 indicated the possibility that there may be slight enhancement of cell yield with moderate water activity stress. The results described in Table 3.8 confirmed this. The correlation in Figure 3.18 indicates that the cell yield response can be explained by increased generation times. When growth processes are slower they may be more efficient. This is in keeping with the most basic of physical principles, where processes that occur at the fastest possible rate are not necessarily the most efficient. Although having no bearing on the energy diversion hypothesis in this thesis, further work exploring growth rate and cell yield optima may be interesting because this finding indicates that the optimum conditions for maximising cell yield may be different from the conditions that are optimal for growth rate. Further evidence can be drawn from Figure 3.16, where cell yield was greatest between 25 and 30°C, but reduced at 40°C (the temperature where growth rate is maximum).

Adenylate energy charge

Adenylate energy charge indicates the available energy level of cells. Enzymes that regenerate ATP increase activity where the energy charge value is low (Atkinson, 1966) and ATP-using enzymes are more active when the energy charge value is high (Shen *et al.*, 1968). Therefore the energy charge of normal, growing cells should be maintained at a value where the activity of these two enzyme classes is half maximal, estimated to be around 0.8-0.9 (Ball and Atkinson, 1975). Many published energy charge estimates for bacterial cells are in this region (Chapman *et al.*, 1971; Bächli and Ettlinger, 1973; Kahru *et al.*, 1982; Hakeda and Fukunaga, 1983; Kahru and Vilu, 1983). The high and stable value for energy charge during exponential growth in Figure 3.19a is consistent with those reports.

The stability of the energy charge value during water activity challenge indicated that the salt-shock treatment did not pose a large energetic burden. Stresses that are energetically costly would be expected to reduce the ATP pool, at least temporarily until the cell's homeostatic mechanisms regain control. Reduced ATP levels would cause reduced energy charge values. The lack of adenylate energy charge response to water activity challenge is consistent with the interpretation of the cell yield data.

An additional observation on the results of the adenylate energy charge experiments is the immediate rise in turbidity when the salt shock was applied. This

was unexpected, because the salt was added in a 10 mL volume that, if anything, was expected to have a dilution effect, reducing the turbidity. However a literature search revealed that the temporary turbidity rise with salt-shock is a common phenomenon, probably due to changes in cell shape (Koch, 1984).

Death kinetics

Non-linear survival curves, resembling those in Figure 3.20, are observed often (Dean and Hinshelwood, 1966; Moats *et al.*, 1971; Whiting 1993). Most often microbiologists recall the logarithmic death rate, that they learned in first year microbiology class, which gives a linear relationship for plots of log survivors versus time. However, various other curves with 'shoulder' and 'tail' sections have been reported (Hansen and Riemann, 1963; Brown *et al.*, 1997). Therefore, the non-linear form of the survival curves in Figure 3.20 was not surprising.

Death due to exposure of vegetative cells to lethal temperatures (thermal death) is the most commonly studied class of microbial death kinetics. In Chapter 1 it was noted that 'predictive modelling' originated from a thermal death model (Esty and Meyer, 1922), and development of such models is also contemporary (Stephens *et al.*, 1994; Linton *et al.*, 1995; Baranyi *et al.*, 1996b; Fujikawa and Itoh, 1996). However, the death occurring in Figure 3.20 is not due to lethal heat treatment. The *E. coli* SB1 culture was held at 37°C and conditions were changed by adjusting the water activity to values that would not permit further growth. This appeared to induce (non-thermal) death. Recent interest in non-thermal death kinetics has been shown (Glass *et al.*, 1992; Beuchat *et al.*, 1994; Buchanan and Golden, 1995; Clavero and Beuchat, 1996; Buchanan *et al.*, 1997). However, many studies have been concerned with death due to pH and organic acids. Water activity only appears to be considered regarding multiple factor effects. For example, Cole *et al.* (1990) showed that moderate water activity conditions improved survival of *Listeria monocytogenes* during lethal pH challenge, but more stringent water activity conditions reduced survival.

Literature reports, showing the influence of non-growth permissible water activity at growth permissible temperature on survival, could not be found. However, that increasingly stringent water activity conditions improved survival was consistent with other literature reports that indicate low water activity conditions increase thermal resistance (Hansen and Riemann, 1963; Cotterill and Glauert, 1969, 1971; Gibson, 1973). Using models that assume thermal destruction of bacteria is due to the denaturation of nucleic acids, enzymes and other macromolecules (Hansen and

Riemann, 1963), it was proposed that the stability of proteins in conditions of low water activity could explain improved survival. The improved survival observed here with decreased water activity is also consistent with this death model.

That survival was improved at increasingly stringent water activity values was significant for the interpretation of survival data in the presence of betaine. Where betaine was present, the water activity limit for growth was 0.960 compared to 0.970 where betaine was absent (Figure 3.12). When water activity was adjusted to the same value for both betaine present and absent treatments (Figure 3.20a), the lethal water activity stress applied was relatively (to the respective water activity limits) less stringent for the betaine treatment. Therefore the faster death rate for the betaine treatment (Figure 3.20a), may be explained by the relative distance from growth permissive conditions. This possibility was examined in the second experiment (Figure 3.20b) where water activity was adjusted so that it was equidistant from the respective water activity limits. The death rate when betaine was present remained faster, although the magnitude of the difference was reduced, indicating that relative distance of lethal water activity from the growth limit could account for some, but not all the difference in death rate between treatments in Figure 3.20a.

That betaine appears to reduce survival is not consistent with the death model based on denaturation of macromolecules (above). According to general compatible solute theories, compatible solutes protect macromolecules, possibly by altering the structure of water immediately adjacent to those macromolecules (section 1.3.2). On this basis and according to the above death model, betaine should improve survival. An alternative model for death, and one that may be more applicable for non-thermal death is that non-growing cells, due to endogenous metabolism slowly use available substrates until there are none left to support them. Some cells die, releasing growth substrates for others, and so on, as the population slowly 'runs down'. Both the improved survival with increasingly stringent water activity and the increased rate of death with betaine are consistent with this model. Low water activity would slow down reaction rates, in a manner similar to the effect that lower temperature has on reactions, slowing the utilisation of substrates and therefore, the demise of the population. Betaine would increase these reactions, speeding the death rate.

The effect of compatible solutes on bacterial survival has implications for the food industry. Based on the alleviation of growth rate inhibition due to unfavourable water activity conditions by compatible solutes, the potential advantage of a compatible solute free environment was contemplated in Chapter 2. However, for bacterial cells during the death phase, the absence of compatible solutes improved

survival. Therefore, where conditions in foods prevent bacterial growth, the compatible solute free environment would be unfavourable from the food microbiologist's perspective.

Limiting bacterial growth and/or inducing bacterial death is a contemporary issue in food microbiology; evidenced by the proliferation of predictive modelling literature relating to these topics (Stephens *et al.*, 1994; Linton *et al.*, 1995; Ratkowsky and Ross, 1995; Baranyi *et al.*, 1996b; Fujikawa and Itoh, 1996; Presser *et al.*, 1997a). Within predictive modeling the observation that compatible solutes reduce survival suggests that compatible solutes must be included in media used for preparing growth boundary and survival models. The result should also sound a warning against generalisations based on empirical observations. From observing their effects on bacterial growth, compatible solutes can be 'stereotyped' as protective substances. Therefore, it is a logical progression to anticipate compatible solutes improve survival where environmental conditions prevent growth. However, the reverse is true. The actual effect of compatible solutes on survival during lethal water activity challenge could have been predicted by understanding the mechanism by which compatible solutes elicit their effects, but not from the empirical description of growth responses.

The increased death rate at non-growth permissive water activities by betaine is also of relevance to the energy diversion hypothesis. Growth rate declines with decreasing water activity which was thought to indicate that energy supply was limiting. Betaine improved growth rate and therefore was thought to free up energy supply. However, death, a process that presumably is not fuelled by energy, also occurs faster when betaine was added. Therefore it may be reasoned that energy does not necessarily limit growth rate either. This reasoning is consistent with experiments that have shown compatible solutes improve the reaction rate of pure enzyme systems where the rate is limited by water activity (Manetas *et al.*, 1986).

3.4 Summary

1. Using a simple substrate-limited batch culture technique it was possible to measure the yield of bacterial cells obtained during culture in different environmental conditions.
2. The cell yield of *E. coli* was independent of extracellular water activity and betaine, except at water activity values close to the growth/no growth interface, indicating that water activity challenge is not an energetic burden for bacterial cells. Therefore, energetic limitation of growth was eliminated as a possible mechanistic explanation for the effects of water activity on bacterial growth.
3. The cell yield of *E. coli* was independent of incubation temperature over most of the biokinetic range, in a pattern similar to that observed for water activity. The cell yield responses to water activity and temperature were consistent with a master reaction model (McMeekin *et al*, 1993; Ross, 1993). Therefore the influence of water activity on microbial growth may be explained in terms of protein folding.

Hence:

The working hypothesis (section 2.5) was rejected. The reason for the failure of that hypothesis and an alternative hypothesis are considered in Chapter 4.

CHAPTER 4: ACTIVATION ENERGY

4.1 Introduction

The energy diversion hypothesis (section 2.5) was based on the observation that the observed minimum temperature for bacterial growth was higher at more stringent water activity values. Therefore, calculated (Equation 2.6) apparent activation energies for growth at the boundary between growth and no growth were lower where unfavourable water activity conditions had been applied. This was interpreted to indicate that the cells exposed to unfavourable water activity conditions displayed a reduced ability to overcome the barrier to growth due to low temperature. However, that equation (McMeekin *et al.*, 1993) was based on the assumption that the apparent activation energy for bacterial growth is independent of extracellular water activity conditions. The validity of that assumption was questioned because the energy diversion hypothesis was rejected in Chapter 3.

The validity of the assumption that the apparent activation energy for growth is independent of the extracellular water activity is investigated in this Chapter using novel and published data sets for five bacterial species of varying halotolerance. This reveals that the assumption may be incorrect close to the low temperature growth/no growth boundary. Also a new hypothesis is developed for the mechanism that limits growth by temperature and water activity.

4.2 Materials and Methods

The apparent activation energy for bacterial growth, as a function of temperature and water activity, was calculated from Arrhenius plots for novel growth rate data for *E. coli* SB1, *P. halodenitrificans* and *H. elongata* (Chapter 2) and published growth rate data for *S. aureus* (Ross, 1993) and *S. xylosus* (Chandler, 1988). Calculations were made from raw data and by smoothing the data using second order polynomial fitting, the four parameter square root model (Equation 2.4) and a master reaction-type thermodynamic model (McMeekin *et al.*, 1993; Ross, 1993), based on the hydrophobicity of macromolecules:

$$rate = \frac{CT \exp(\Delta H^\ddagger / RT)}{1 + \exp(-n(\Delta H^* - T\Delta S^* + \Delta C_p[(T - T^*_H) - T \ln(T / T^*_S)]) / RT)} \quad (4.1)$$

where:

- C is a parameter whose value must be estimated
- ΔH^\ddagger is the activation enthalpy of the reaction catalysed by a key enzyme
- ΔC_p is the difference in heat capacity (per mol. amino acid residue) between the native and denatured state of the key enzyme
- T^*_H is the temperature (K) at which the ΔC_p contribution to enthalpy is 0.
- T^*_S is the temperature (K) at which the ΔC_p contribution to entropy is 0.
- ΔH^* is the value of enthalpy at T^*_H per mol-amino acid residue
- ΔS^* is the value of entropy at T^*_S per mol-amino acid residue
- T is temperature (K)
- R is the gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$)
- n is the number of amino acid residues in the key protein.

Traditional smoothing curves, such as Savitsky-Golay smoothing filters (Press *et al.*, 1992), were inappropriate because they require many more data points than were available and because they are non-parameterised. Parameterised functions were required to facilitate calculation of the apparent activation energy for growth.

For raw data, the apparent activation energy was calculated from the slope between adjacent data points on Arrhenius plots:

$$\ln k = \ln A + \frac{E_a}{RT}$$

$$\therefore \text{slope} = \frac{E_a}{R}$$

$$\therefore E_a = \text{slope} \times R \quad (4.2)$$

- where:
- k is reaction rate (growth rate in microbiology)
 - A is the collision factor
 - E_a is activation energy (J mol^{-1})
 - R is the gas constant ($8.314 \text{ J K mol}^{-1}$)
 - T is temperature (K)

Second order polynomials were fitted using Cricket Graph 1.3.2. Equations 2.4 and 4.1 were fitted using Ultrafit 3.0. Goodness of fits were compared by residual plots and residual mean square (RMS):

$$\text{RMS} = \sqrt{\frac{\sum(\text{residual})^2}{n}} \tag{4.3}$$

where: n is the number of residuals

Apparent activation energy was estimated from smoothed data, using equation 4.2, by determining the slope between smoothed values (on an Arrhenius plot), at 0.5°C intervals.

4.3 Results

Arrhenius plots of growth rate data for *E. coli* SB1, *P. halodenitrificans*, *H. elongata*, *S. aureus* and *S. xylosus* for various water activity conditions are presented in Figures 4.1 and 4.2. Straight line portions of those Arrhenius plots were noted, as indicated in Figure 4.2. For the *S. aureus* and *S. xylosus* data sets growth rates were similar at each water activity condition, so that plots were cluttered. Therefore for clarity, results for only the highest and lowest water activity were plotted in Figures 4.2d; results for an intermediate water activity (0.909) were also included in Figure 4.2e. Deviation of data points from the straight line appeared to occur at higher temperatures, with increasingly stringent water activity conditions (Figure 4.2).

Apparent activation energy values estimated from actual data are shown in Figure 4.3. Estimates were very variable, but some weak trends were noted. (apparent activation energy was stable for most of the biokinetic range, but increased and decreased sharply at the low and high temperature extremes of the biokinetic range respectively). This trend was most obvious for the a_w : 0.950 treatment for *H. elongata*, and the a_w : 0.963 treatment for *S. aureus*.

Example fits of each of the smoothing curves, for the *E. coli* SB1 data set, are shown in Figure 4.4. Table 4.1 lists the fitted parameter values (95% confidence intervals are available in Appendix 4.1) and RMS values for each of the smoothing curves, fitted to the five data sets. The RMS values were all low, indicating that all three models fitted the data closely. There were no consistent trends in RMS values, therefore the superiority of one model over another was not demonstrated by this method. Also, consistent fitting errors were not detected for any of the smoothing curves using residual plots (not shown).

Apparent activation energies estimated from smoothed curves are shown, as a function of temperature, in Figures 4.5, 4.6 and 4.7. Estimates of activation energy, from the second order polynomial model, decrease approximately linearly with increasing temperature (Figure 4.5). In general, decreasing water activity appears to increase the slope of the activation energy versus temperature plot.

The relationship between activation energy and temperature, when activation energy was estimated using Equation 2.4 (Figure 4.6) or Equation 4.1 (Figure 4.7) was sigmoid, with asymptotes at both high and low temperatures. Generally, estimates from Equation 2.4 were not affected by water activity conditions. But using Equation 4.1, the low temperature asymptote occurred at increasingly higher temperatures, with increasingly stringent water activity conditions.

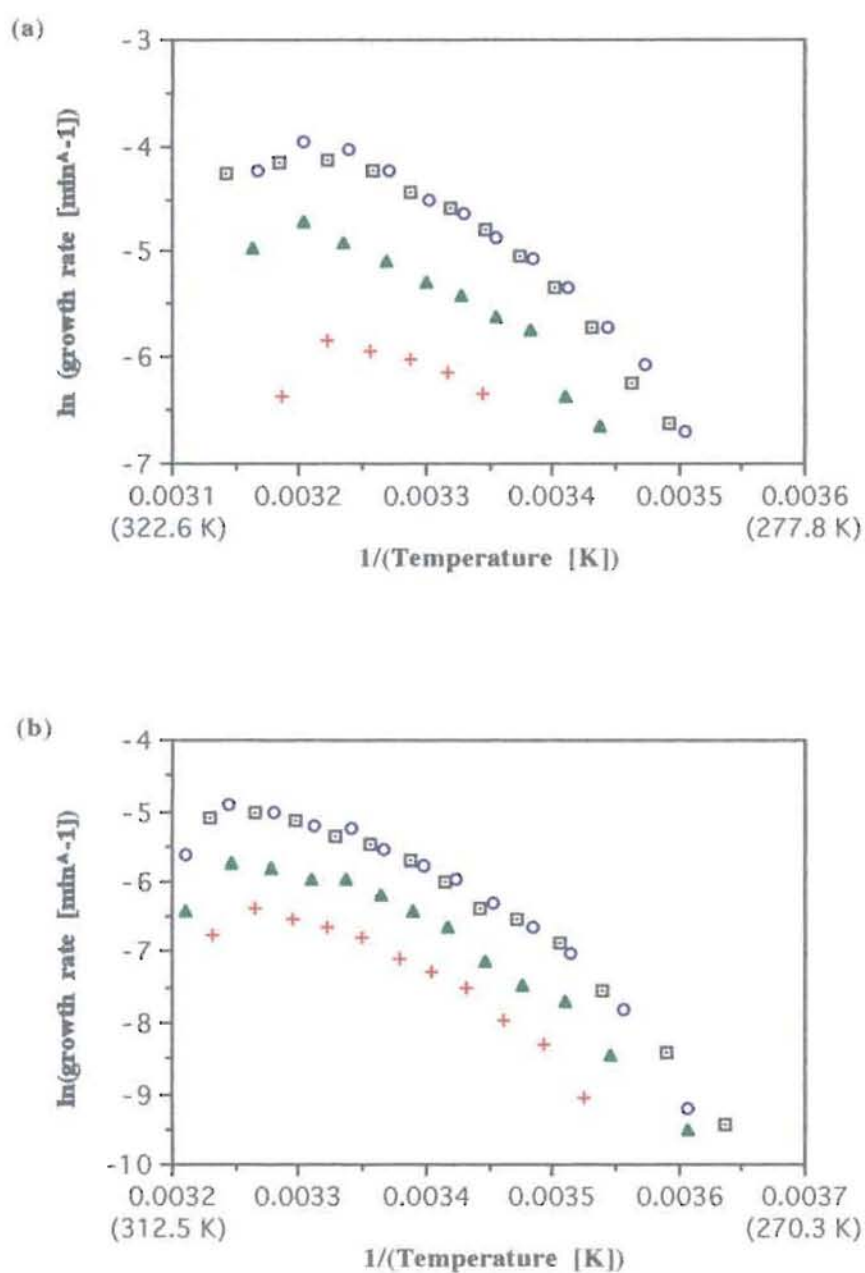
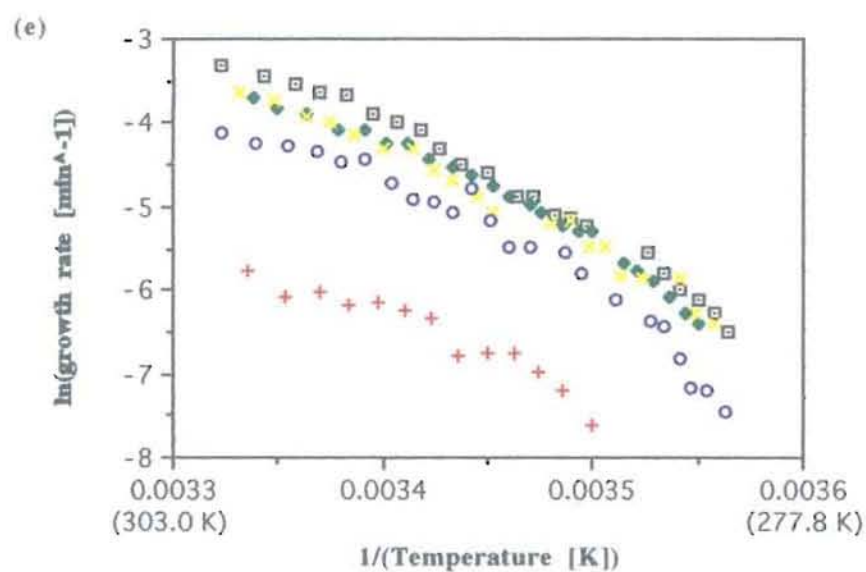
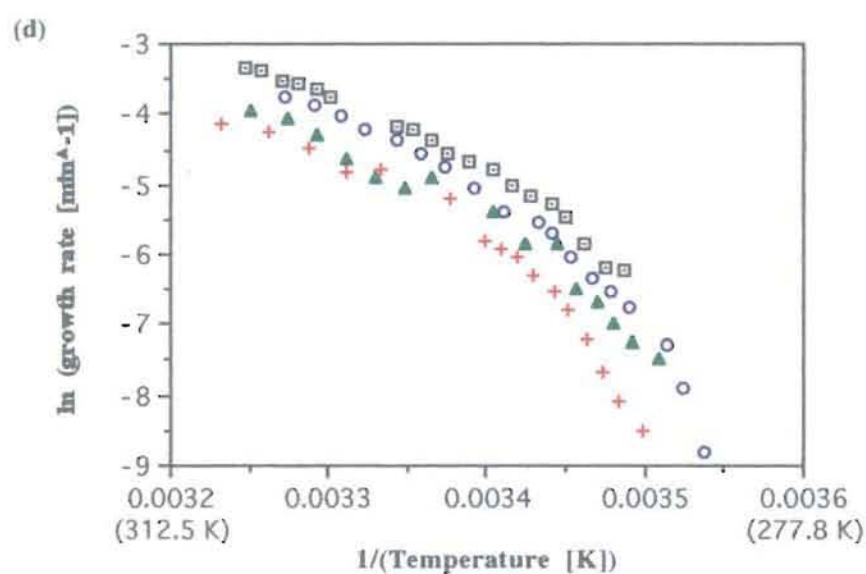
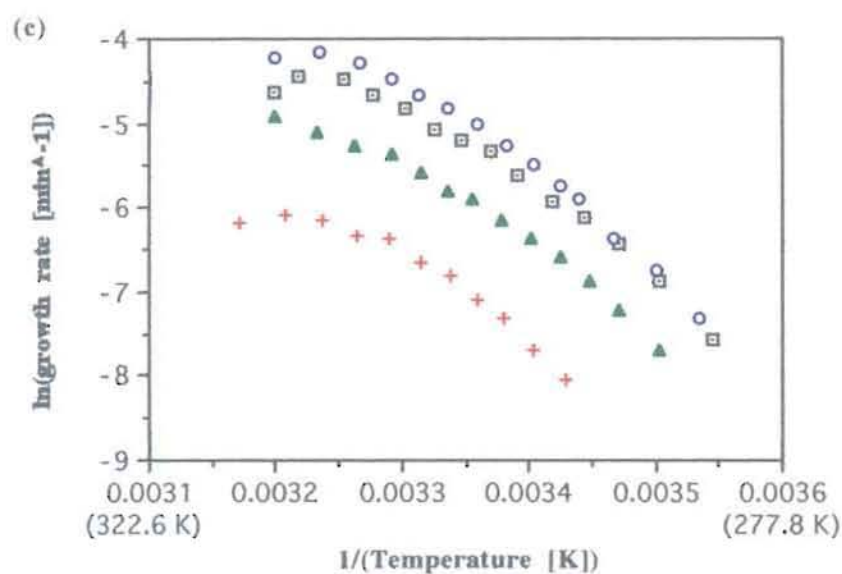


Figure 4.1: Arrhenius plots showing data used to estimate the apparent activation energies for bacterial growth (at various water activity values) in this chapter:

- (a) *E. coli*, (□) 0.998, (○) 0.998 + 2 mM betaine, (+) 0.977, (▲) 0.977 + 2 mM betaine
 (b) *P. halodenitrificans*, (□) 0.964, (○) 0.964 + 2 mM betaine, (+) 0.892, (▲) 0.892 + 2 mM betaine
 (c) *H. elongata*, (□) 0.950, (○) 0.950 + 2 mM betaine, (+) 0.752, (▲) 0.752 + 2 mM betaine
 (d) *S. aureus*, (□) 0.997, (○) 0.963, (▲) 0.935, (+) 0.914
 (e) *S. xylosus*, (□) 0.976, (◆) 0.949, (●) 0.928, (○) 0.909, (+) 0.848.



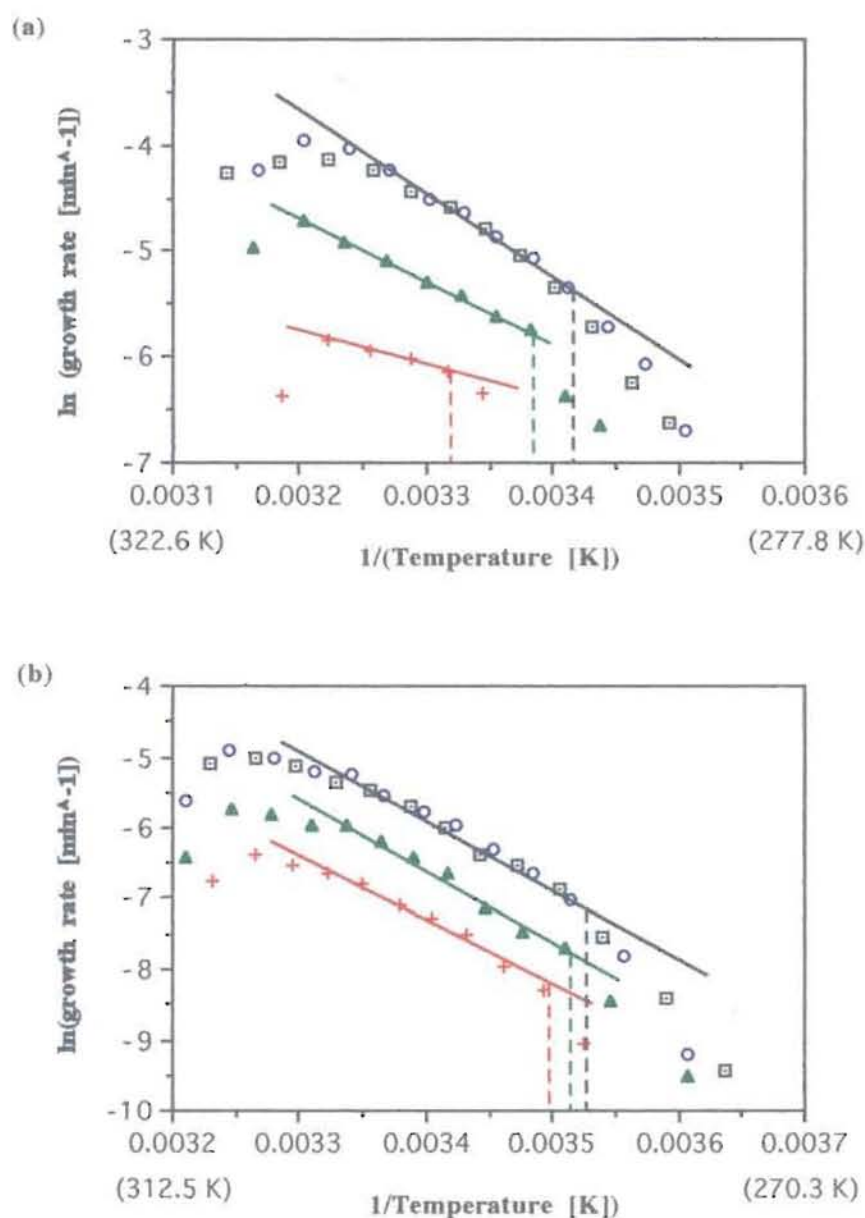
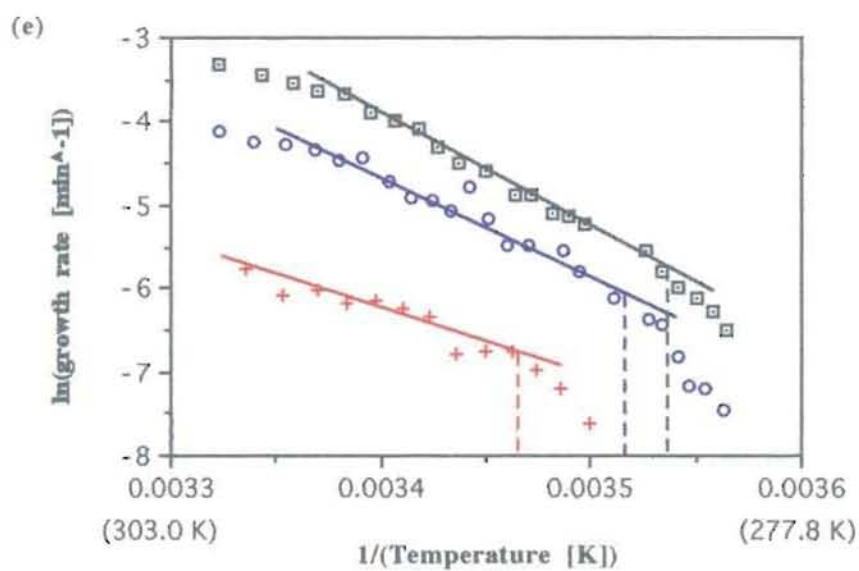
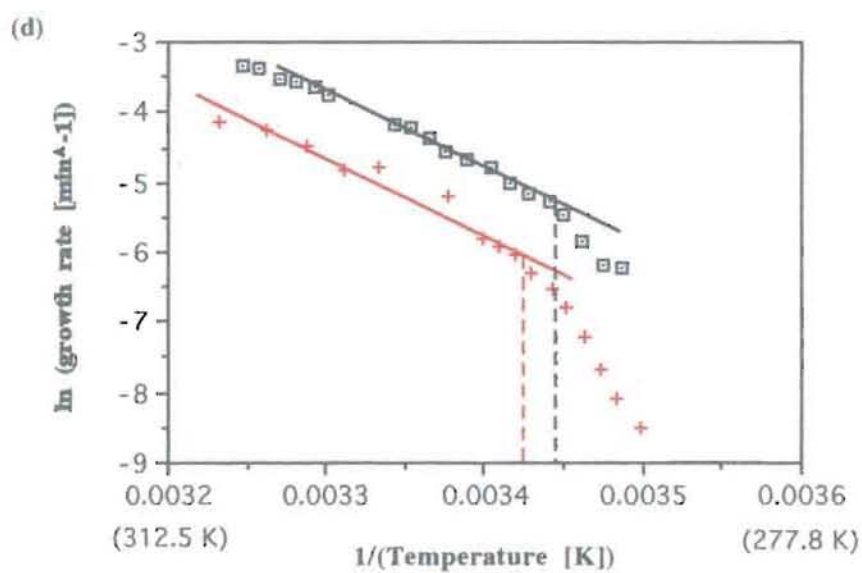
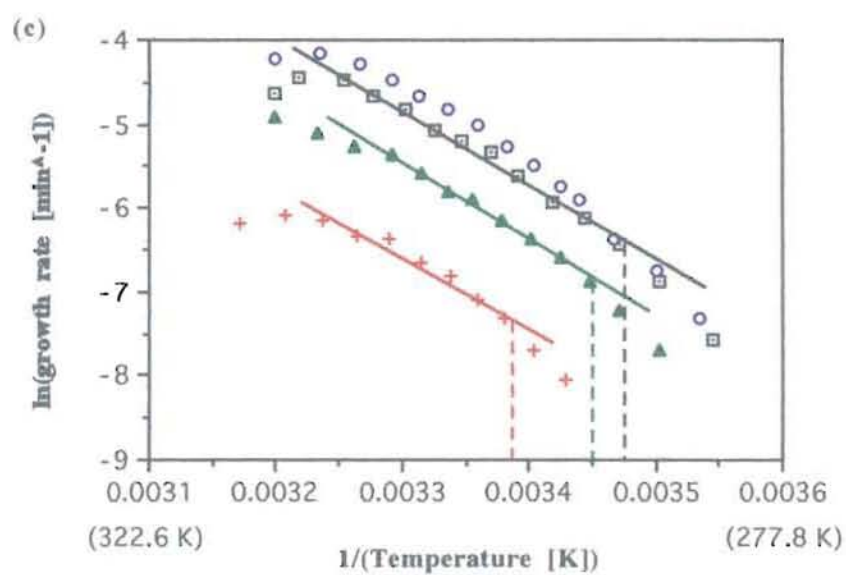


Figure 4.2: Arrhenius plots of bacterial growth data (at various water activity values) showing approximately linear portions (drawn 'by eye') of the curve for:

- (a) *E. coli*, (□) 0.998, (○) 0.998 + 2 mM betaine, (+) 0.977, (▲) 0.977 + 2 mM betaine
- (b) *P. halodenitrificans*, (□) 0.964, (○) 0.964 + 2 mM betaine, (+) 0.892, (▲) 0.892 + 2 mM betaine
- (c) *H. elongata*, (□) 0.950, (○) 0.950 + 2 mM betaine, (+) 0.752, (▲) 0.752 + 2 mM betaine
- (d) *S. aureus*, (□) 0.997, (+) 0.914
- (e) *S. xylosus*, (□) 0.976, (○) 0.909, (+) 0.848.

Vertical lines indicate the temperatures where data points appear to deviate from the linear response.



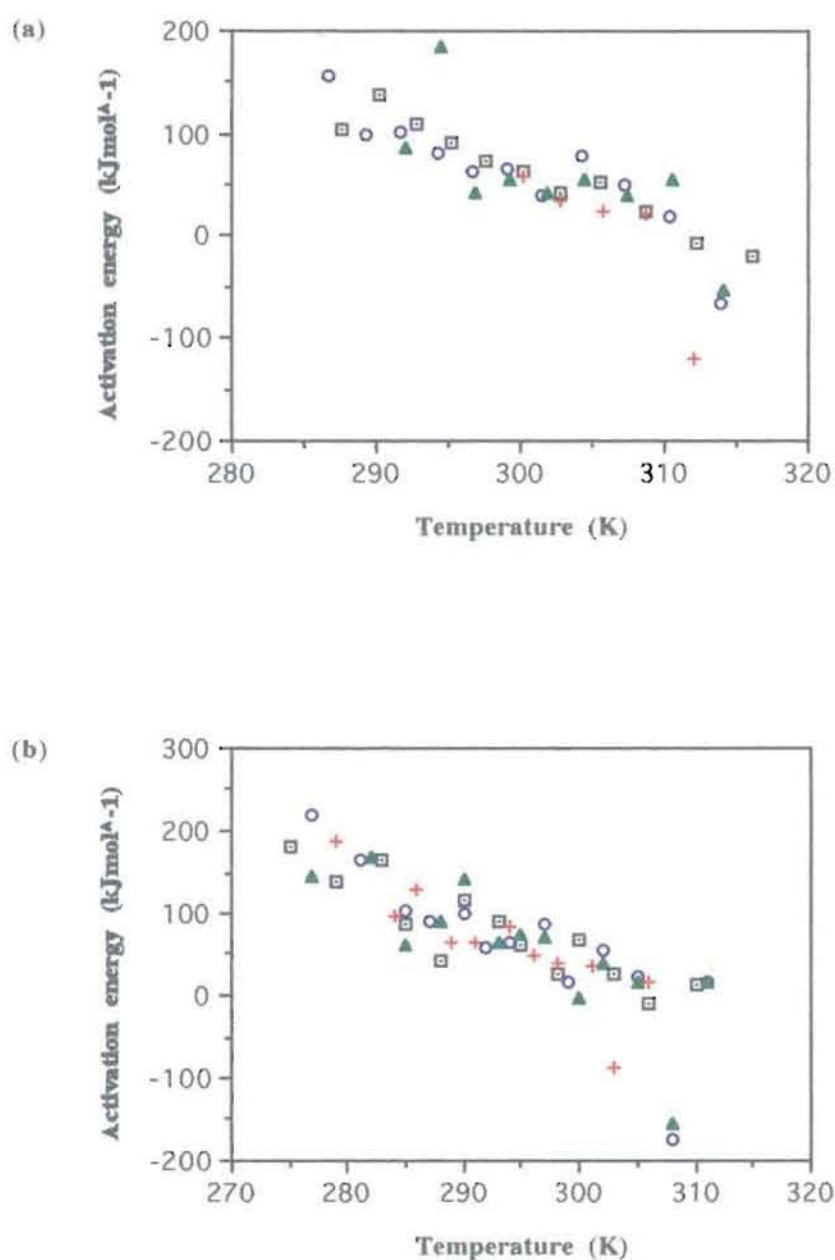


Figure 4.3: Apparent activation energy values estimated from growth rate data, at various water activity conditions:

- (a) *E. coli*, (□) 0.998, (○) 0.998 + 2 mM betaine, (+) 0.977, (▲) 0.977 + 2 mM betaine
 (b) *P. halodenitrificans*, (□) 0.964, (○) 0.964 + 2 mM betaine, (+) 0.892, (▲) 0.892 + 2 mM betaine
 (c) *H. elongata*, (□) 0.950, (○) 0.950 + 2 mM betaine, (+) 0.752, (▲) 0.752 + 2 mM betaine
 (d) *S. aureus*, (□) 0.997, (○) 0.963, (▲) 0.935, (+) 0.914
 (e) *S. xylosus*, (□) 0.976, (◆) 0.949, (●) 0.928, (○) 0.909, (+) 0.848.

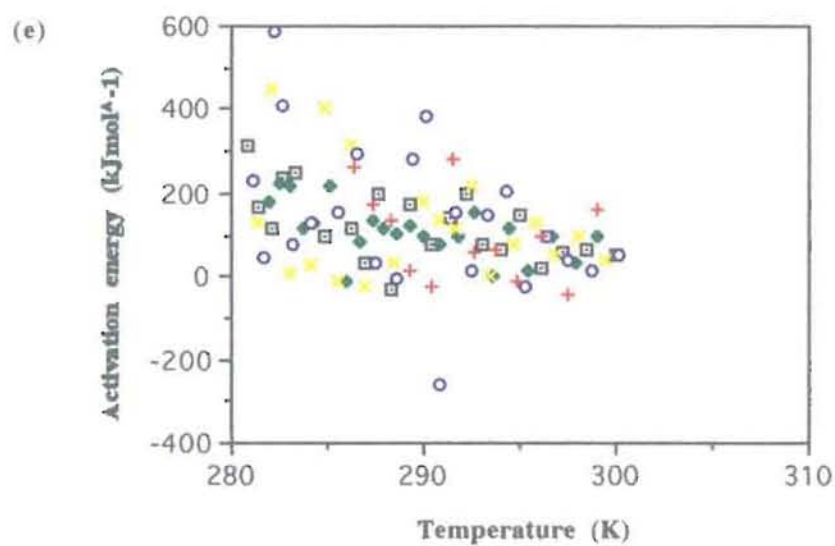
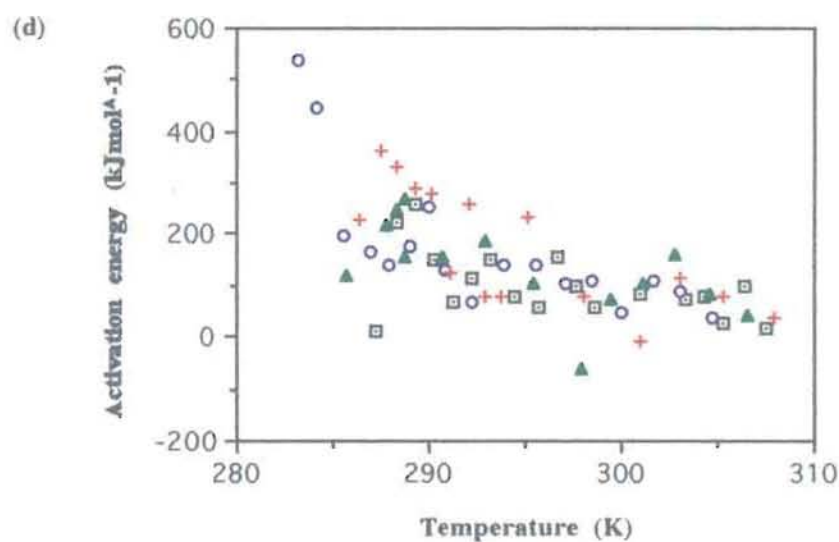
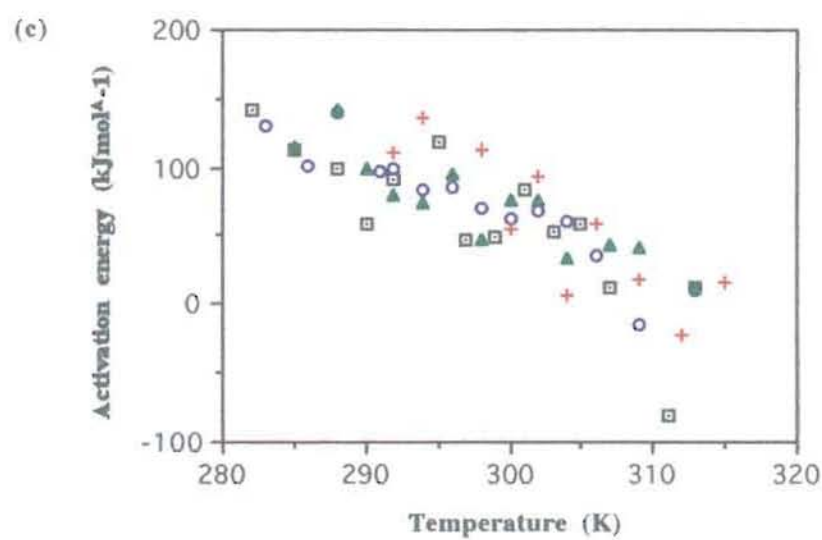


Figure 4.4: Example smoothing curves; (a) second order polynomial, (b) Equation 2.4 and (c) Equation 4.1, fitted to data for *E. coli* growth at various water activity conditions: (◻) 0.998, (◉) 0.998 + 2 mM betaine, (+) 0.977, (▲) 0.977 + 2 mM betaine.

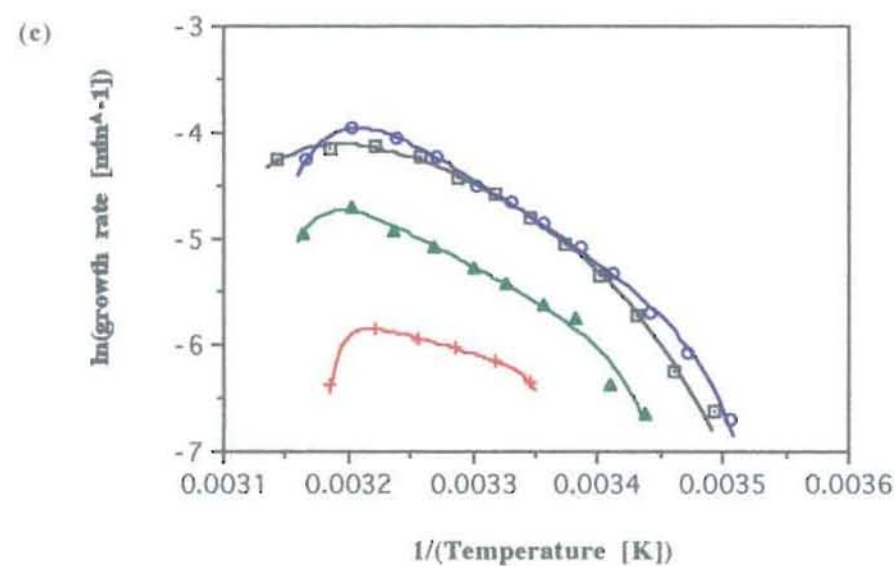
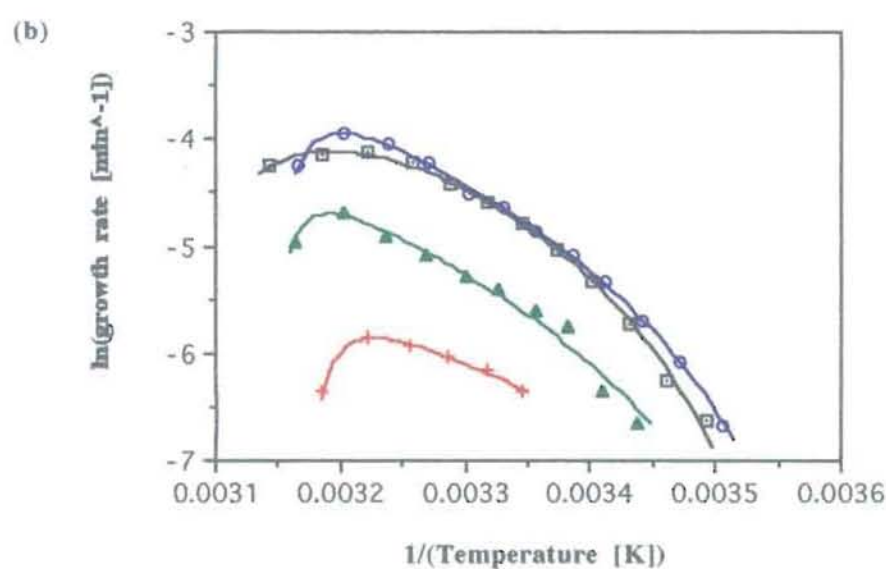
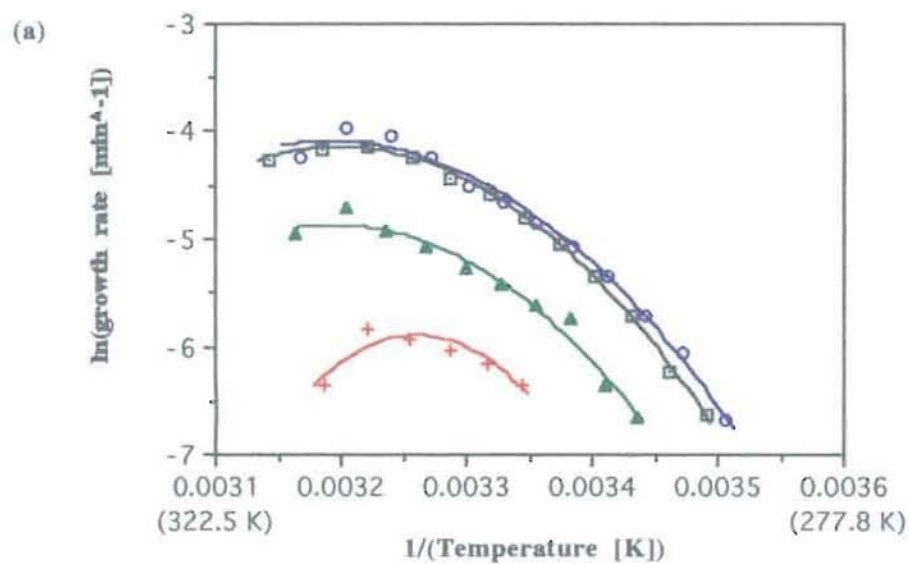


Table 4.1: Summary of parameters and goodness-of-fit of a second order polynomial equation, Equation 2.4 and Equation 4.1 for *E. coli*, *P. halodenitrificans*, *H. elongata*, *S. aureus* and *S. xylosus* data sets. For a summary and discussion of 95% confidence intervals see Appendix 4.1.

Treatment	2nd order polynomial*				Equation 2.4					Equation 4.1					
	a	b ($\times 10^{-5}$)	c ($\times 10^{-7}$)	RMS	b ($\times 10^{-3}$)	c	Tmin (°C)	Tmax (°C)	RMS	ΔC_p	ΔH^\ddagger ($\times 10^3$)	ΔH^*	C	n	RMS
<i>E. coli</i>															
0.998	-301.39	1.8593	-2.9074	0.0325	4.9737	0.0949	6.24	55.15	0.0507	76.0	45.4	5335.2	3.85×10^3	137	0.0645
0.998+B	-266.06	1.6422	-2.5735	0.0809	4.1086	0.4451	3.25	45.64	0.0317	72.5	61.9	5355.0	1.91×10^6	309	0.0359
0.977	-733.99	4.4568	-6.8478	0.0840	1.2278	0.5298	-8.68	42.88	0.0194	78.1	25.0	5326.1	1.6×10^{-1}	1495	0.0041
0.977+B	-317.61	1.9572	-3.0621	0.0928	2.7491	0.5638	3.76	45.65	0.0957	75.9	51.0	5343.5	1.09×10^4	449	0.0800
<i>P. halodenitrificans</i>															
0.964	-275.09	1.6726	-2.5899	0.0720	2.4611	0.5443	-0.78	40.12	0.2060	67.0	72.3	5349.1	9.26×10^7	148	0.0648
0.964+B	-412.12	2.4783	-3.7730	0.1437	2.6887	0.4874	0.34	40.12	0.0406	67.7	68.1	5355.7	1.31×10^7	357	0.1600
0.892	-418.53	2.5182	-3.8480	0.4930	1.4611	0.4010	2.76	39.28	0.6406	69.2	65.3	5349.4	9.66×10^5	492	0.0670
0.892+B	-356.52	2.1420	-3.2719	0.1483	1.8568	0.3528	0.4511	40.74	0.1680	66.7	76.3	5352.9	1.89×10^8	258	0.1383

<i>H. elongata</i>															
0.950	-222.34	1.3766	-2.1741	0.0729	3.3148	0.7098	1.06	41.88	0.0752	69.9	68.6	5359.0	1.76x10 ⁷	609	0.0870
0.950+B	-235.61	1.4650	-2.3177	0.0444	3.9969	0.6928	4.18	42.23	0.0933	70.8	74.3	5354.3	2.48x10 ⁸	279	0.1039
0.752	-388.42	2.3893	-3.7331	0.0385	2.0739	0.1272	9.94	51.18	0.0389	76.3	56.0	5334.9	2.95x10 ⁴	223	0.0487
0.752+B	-190.19	1.1892	-1.9075	0.0362	2.5094	0.2181	4.07	53.83	0.0338	72.3	65.3	5355.2	2.39x10 ⁶	441	0.0771
<i>S. aureus</i>															
0.997	-299.75	1.8741	-2.9606	0.0660	6.7980	0.8572	7.73	50.00	0.0642	71.3	85.5	5347.1	4.7x10 ¹⁰	470	0.0781
0.963	-602.28	3.6746	-5.6411	0.1592	6.1765	2.9474	7.99	45.00	0.1912	69.1	106.4	5345.8	1.7x10 ¹⁴	439	0.0832
0.935	-337.11	2.0969	-3.2987	0.1555	5.0477	2.3539	7.89	50.00	0.1541	71.5	89.2	5349.7	9.9x10 ¹⁰	490	0.1756
0.914	-715.92	4.3762	-6.7280	0.1404	5.3504	0.1813	10.33	50.00	0.1223	72.9	85.1	5342.7	1.7x10 ¹⁰	444	0.1573
<i>S. xylosus</i>															
0.976	-253.67	1.5774	-2.4794	0.0696	7.6982	1.1944	2.98	35.00	0.1359	64.0	108.6	5344.8	1.5x10 ¹⁵	380	0.0628
0.949	-404.64	2.4447	-3.7262	0.0513	6.5731	0.4737	2.35	35.00	0.0387	65.8	83.2	5344.0	3.5x10 ¹⁰	409	0.0397
0.928	-140.47	9.0810	-1.4928	0.0993	6.3819	12.662	2.26	35.00	0.1359	64.5	95.9	5345.3	5.1x10 ¹²	807	0.0940
0.909	-547.53	3.2832	-4.9597	0.1278	5.6795	0.2681	3.38	37.00	0.1115	65.9	85.2	5342.9	5.0x10 ¹⁰	437	0.1094
0.848	-521.49	3.1111	-4.6928	0.1123	2.6082	0.1980	3.25	37.00	0.1123	71.0	52.2	5350.2	1.24x10 ⁴	460	0.0986

* $\ln(\text{growth rate}) = a + b\left(\frac{1}{T}\right) + c\left(\frac{1}{T}\right)^2$

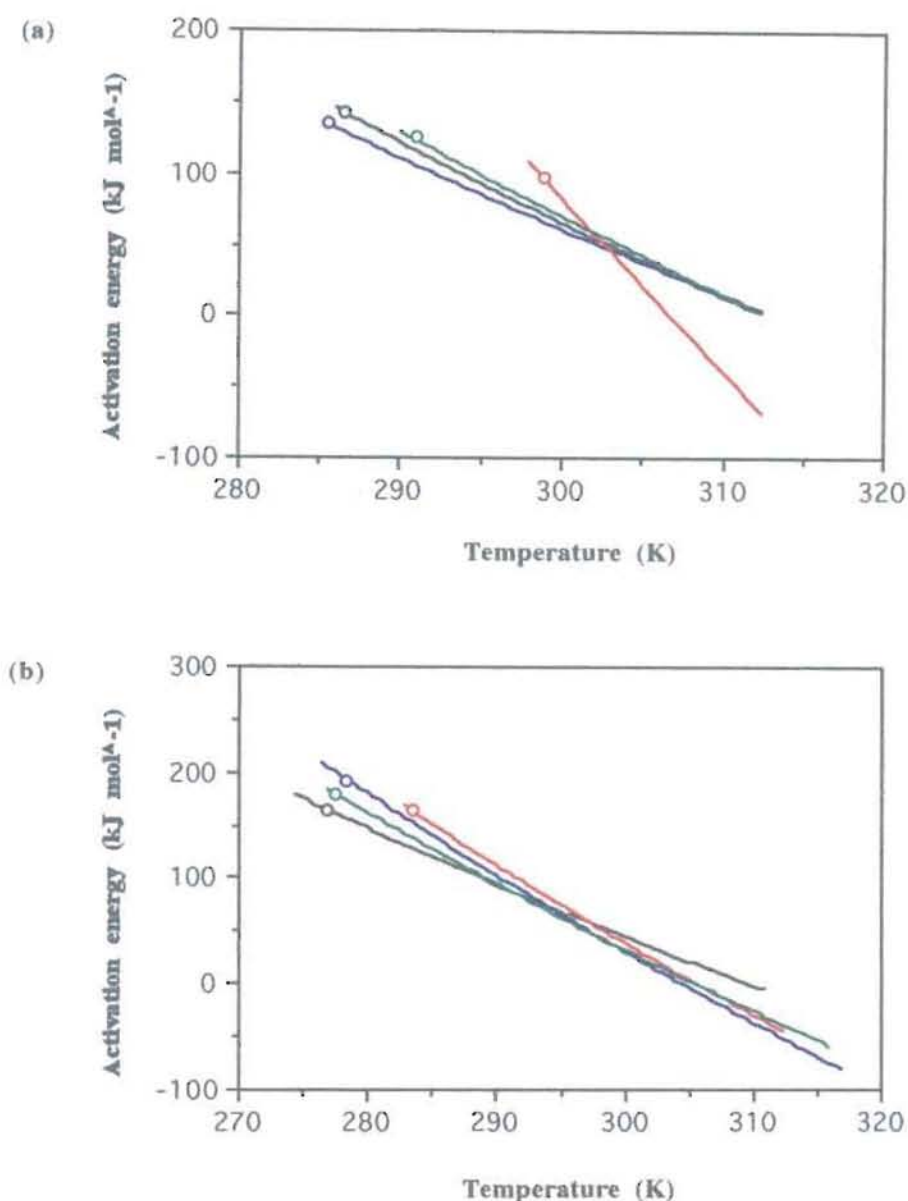
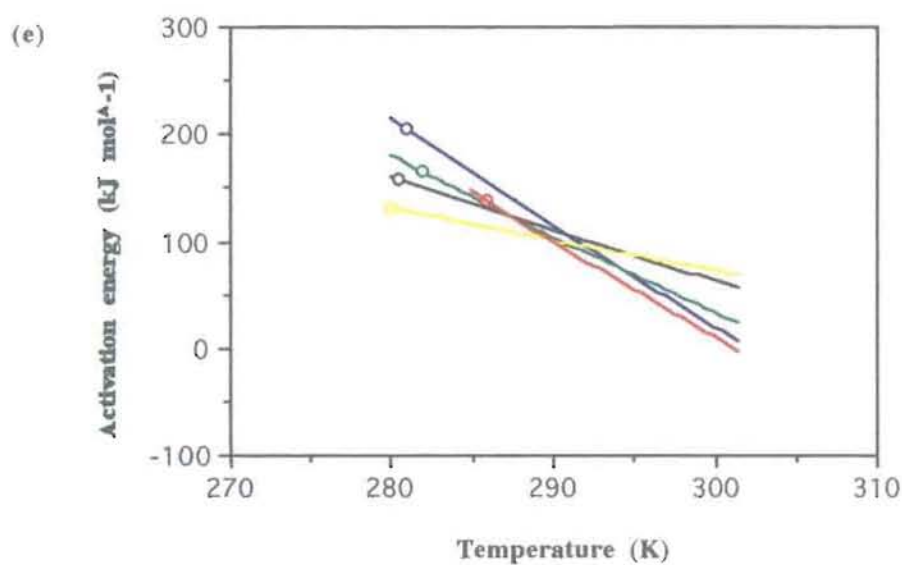
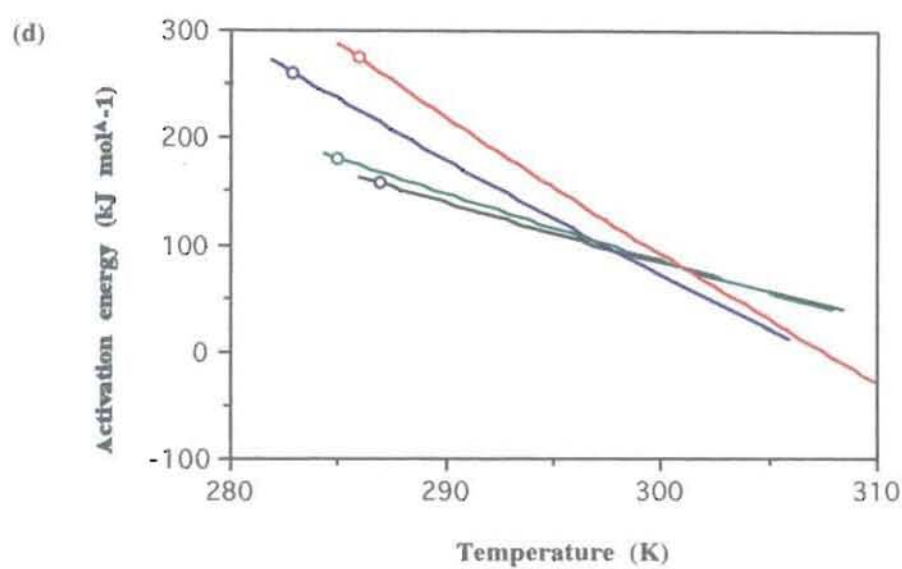
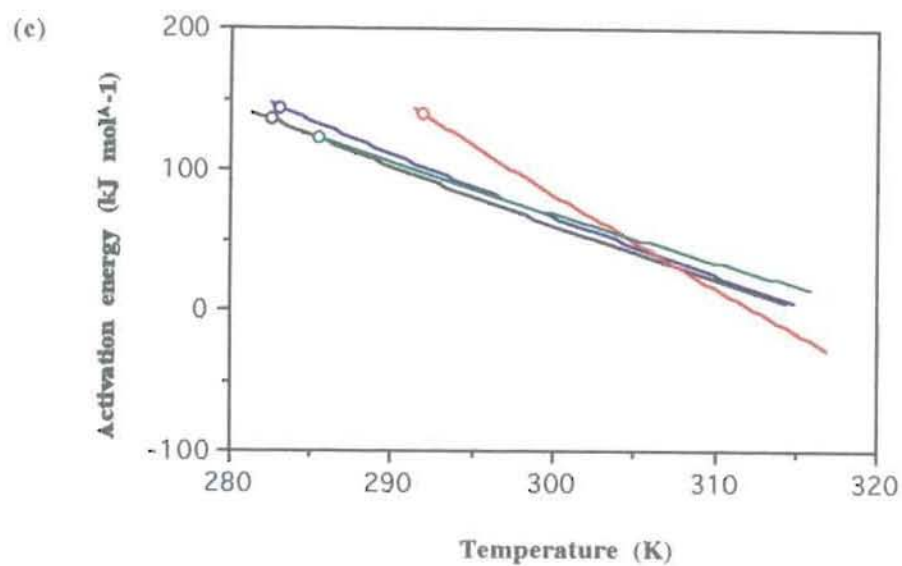


Figure 4.5: Apparent activation energies plotted as a function of temperature, estimated by smoothing raw data using second order polynomial equations, for bacterial growth rate at various water activity conditions:

- (a) *E. coli*, (—) 0.998, (—) 0.998 + 2 mM betaine, (—) 0.977, (—) 0.977 + 2 mM betaine
 (b) *P. halodenitrificans*, (—) 0.964, (—) 0.964 + 2 mM betaine, (—) 0.892, (—) 0.892 + 2 mM betaine
 (c) *H. elongata*, (—) 0.950, (—) 0.950 + 2 mM betaine, (—) 0.752, (—) 0.752 + 2 mM betaine
 (d) *S. aureus*, (—) 0.997, (—) 0.963, (—) 0.935, (—) 0.914
 (e) *S. xyloso*, (—) 0.976, (—) 0.949, (—) 0.928, (—) 0.909, (—) 0.848.

Open circles indicate the lowest temperature where growth rate data was available.



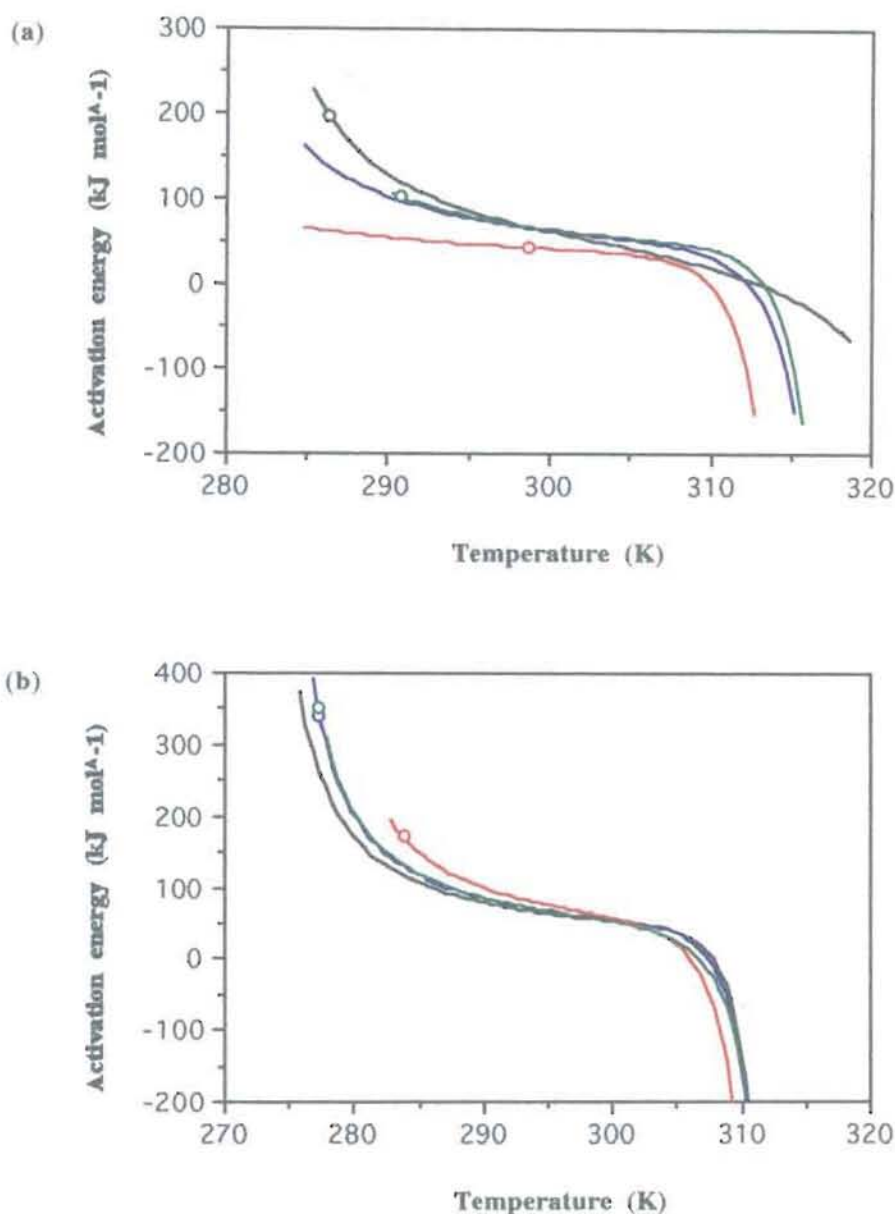
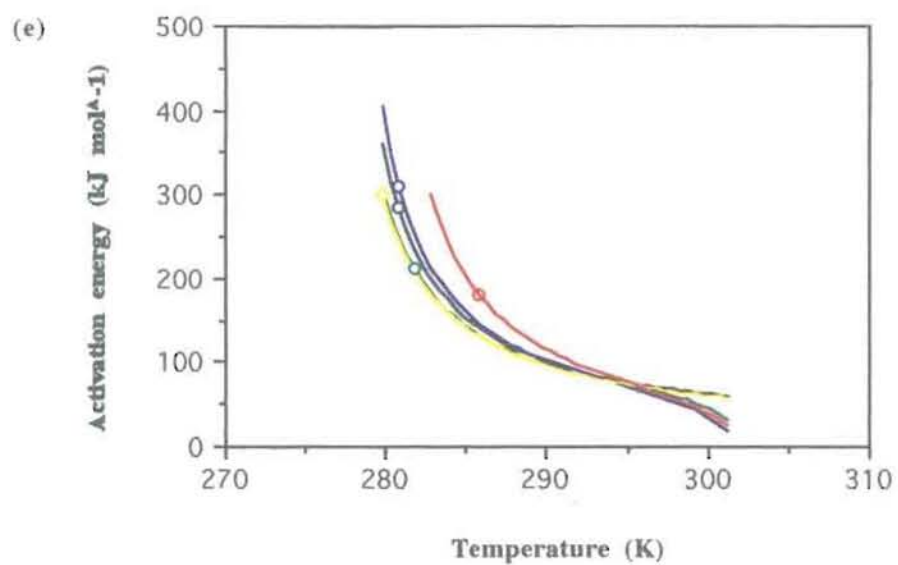
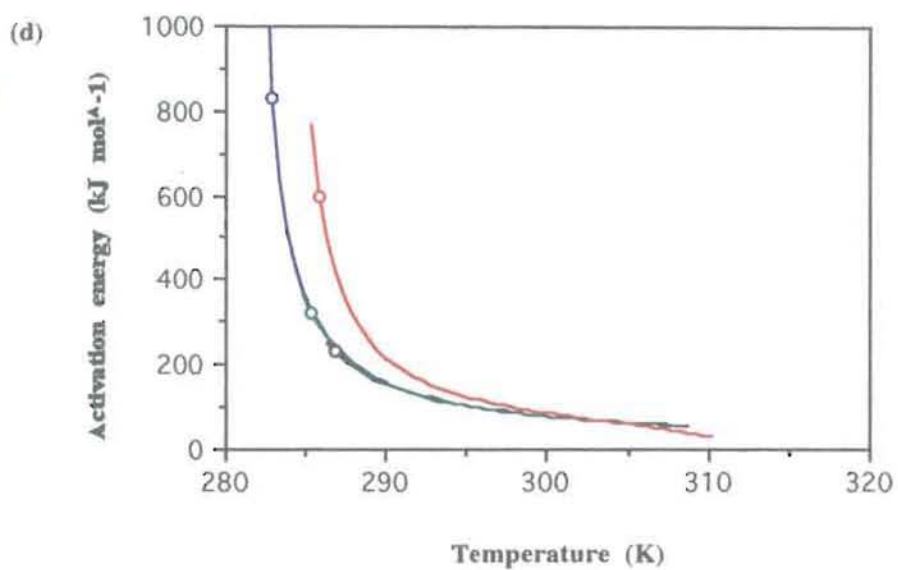
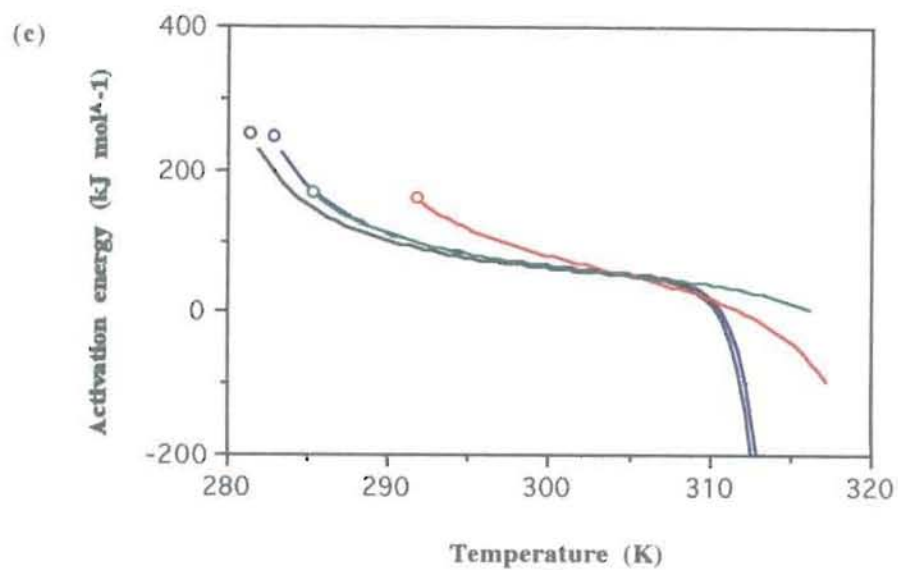


Figure 4.6: Apparent activation energy plotted as a function of temperature, estimated by smoothing raw data using Equation 2.4, for bacterial growth rate at various water activity conditions:

- (a) *E. coli*, (—) 0.998, (—) 0.998 + 2 mM betaine, (—) 0.977, (—) 0.977 + 2 mM betaine
 (b) *P. halodenitrificans*, (—) 0.964, (—) 0.964 + 2 mM betaine, (—) 0.892, (—) 0.892 + 2 mM betaine
 (c) *H. elongata*, (—) 0.950, (—) 0.950 + 2 mM betaine, (—) 0.752, (—) 0.752 + 2 mM betaine
 (d) *S. aureus*, (—) 0.997, (—) 0.963, (—) 0.935, (—) 0.914
 (e) *S. xylosus*, (—) 0.976, (—) 0.949, (—) 0.928, (—) 0.909, (—) 0.848.

Open circles indicate the lowest temperature where growth rate data was available.



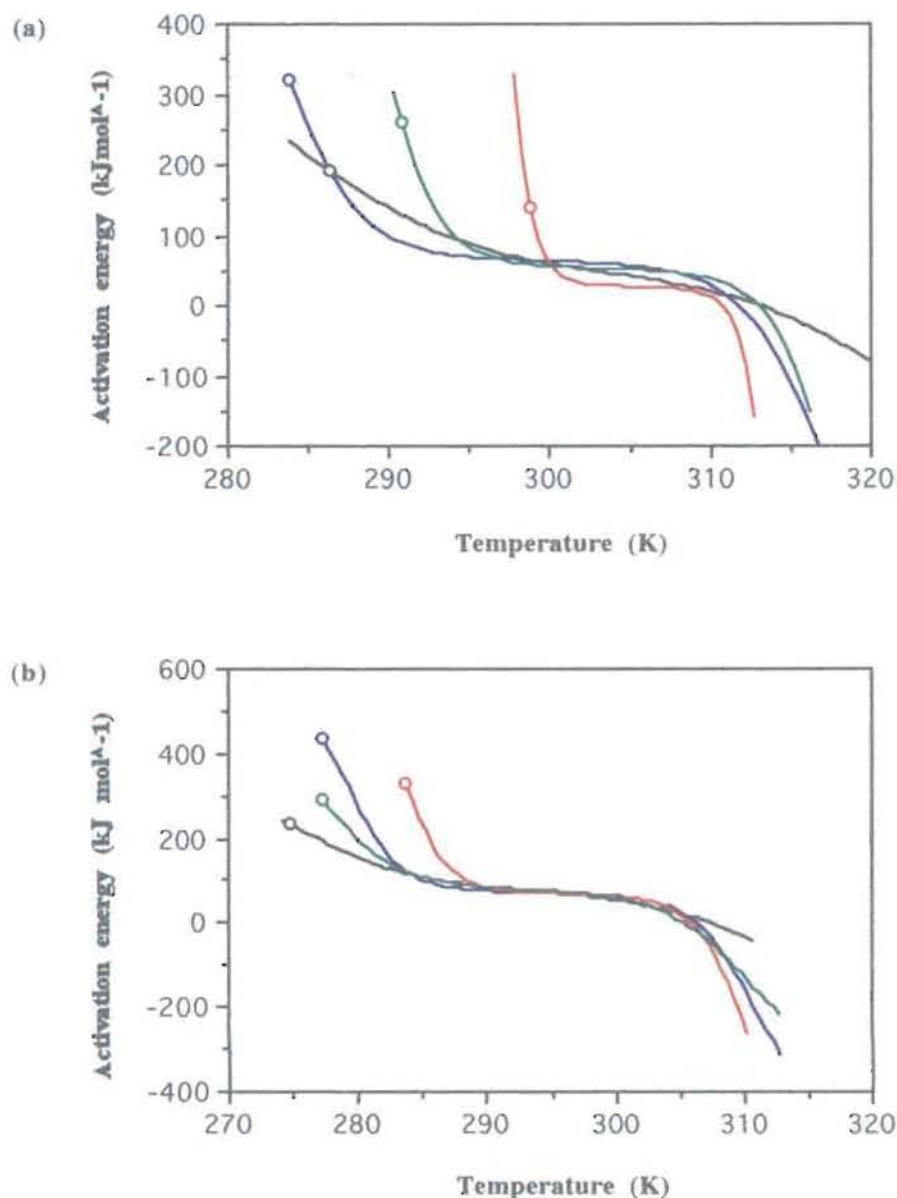
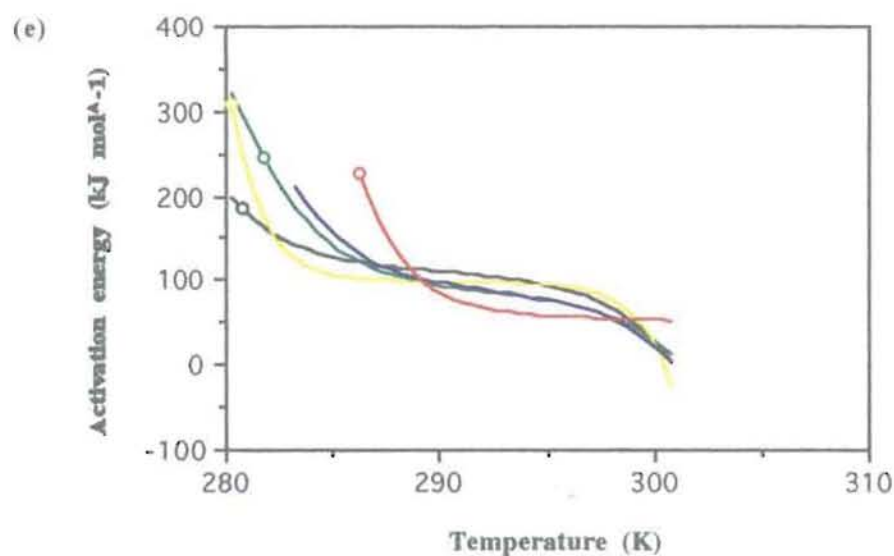
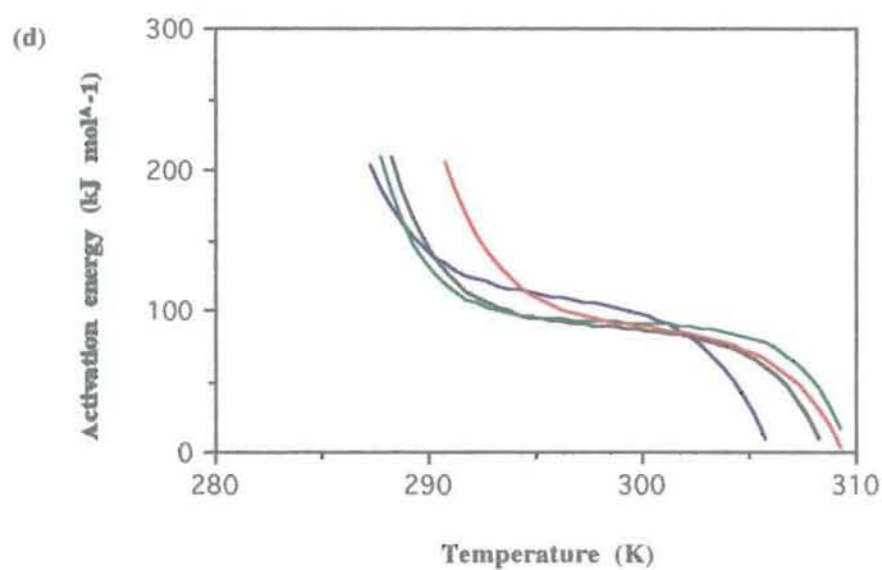
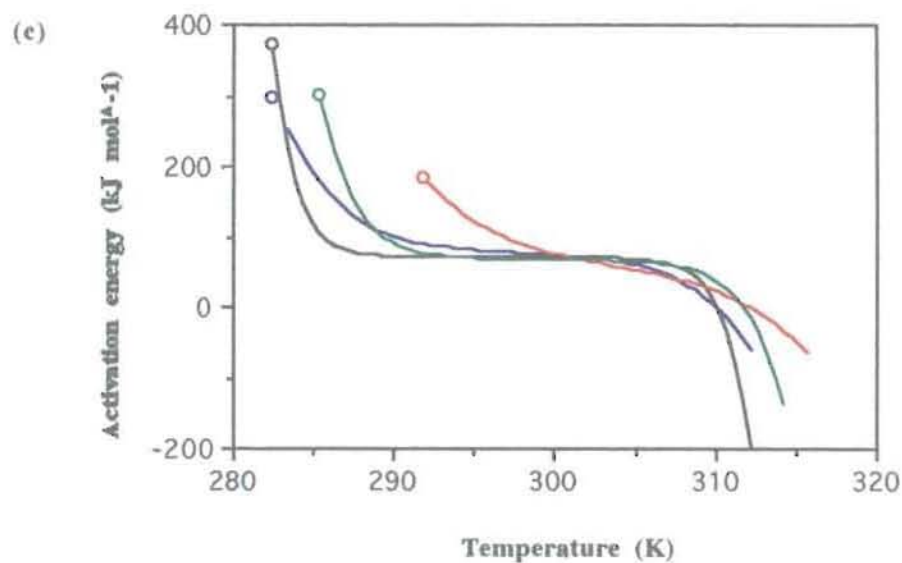


Figure 4.7: Apparent activation energies plotted as a function of temperature, estimated by smoothing using Equation 4.1, for bacterial growth at different water activity conditions:

- (a) *E. coli*, (—) 0.998, (—) 0.998 + 2 mM betaine, (—) 0.977, (—) 0.977 + 2 mM betaine
 (b) *P. halodenitrificans*, (—) 0.964, (—) 0.964 + 2 mM betaine, (—) 0.892, (—) 0.892 + 2 mM betaine
 (c) *H. elongata*, (—) 0.950, (—) 0.950 + 2 mM betaine, (—) 0.752, (—) 0.752 + 2 mM betaine
 (d) *S. aureus*, (—) 0.997, (—) 0.963, (—) 0.935, (—) 0.914
 (e) *S. xylosus*, (—) 0.976, (—) 0.949, (—) 0.928, (—) 0.909, (—) 0.848.

Open circles indicate the lowest temperature where growth rate data was available. These were omitted from curves in (d) and (e) because those curves were not extended to that point.



4.4 Discussion

4.4.1 Activation energy and water activity

The variability of the apparent activation energy estimates presented in Figure 4.3 highlights the difficulty associated with estimating the apparent activation energy for bacterial growth from raw data. Apparent activation energies were calculated by determining the slope between adjacent data points shown in Figures 4.1. However, the raw data did not form a perfect curve, due to natural variation and/or experimental error, hence the estimates varied widely. This made it difficult to observe trends in activation energy values as a function of temperature or water activity. Therefore, it was necessary to fit smoothing curves to the raw data.

Three curves (second order polynomial equation, Equation 2.4 and Equation 4.1) all fitted the data well. They could not be separated after assessment by RMS (Table 4.1) or residuals plot. Therefore, all were used to estimate activation energies for the various novel and published data sets. Activation energies estimated using the three different smoothing curves showed different trends as a function of temperature and water activity.

Second order polynomial equation

Apparent activation energy is approximately linearly related to temperature in Figure 4.5 because second order polynomial curves have steadily changing slopes and the magnitude of the biokinetic range is small. Predictive models that give this approximately linear relation between activation energy and temperature exist (Davey, 1989). However, those models mathematically require that the activation energy for growth of a given organism, at a given temperature, is independent of the extracellular water activity conditions. When such mathematical restrictions were avoided (by independently fitting second order polynomial equations to each data set; Figure 4.5), water activity appeared to influence the activation energy (generally the slope of the activation energy versus temperature curve was greater, the more stringent the water activity conditions).

Equation 2.4 (four parameter square root model)

The sigmoid relationship between activation energy and temperature in Figure 4.6 occurs because Equation 2.4 (as an Arrhenius plot) describes a smooth curve, the slope of which changes with temperature (Figure 4.8). At high, but suboptimal temperatures the change in slope is small, hence the curve approximates a straight line that is indistinguishable from linear Arrhenius kinetics (McMeekin *et al.*, 1993) and sometimes referred to as the normal physiological range (Ingraham, 1987). However, as the temperature decreases and begins to approach T_{min} the rate of change of the slope increases. Therefore, apparent activation energy estimates using Equation 2.4 are approximately constant for temperatures in the normal physiological range, but increase rapidly at lower temperatures. Also, at superoptimal temperatures there is a departure from linear Arrhenius kinetics, where growth rates decline rapidly and activation energy estimates become large and negative (this chapter is concerned

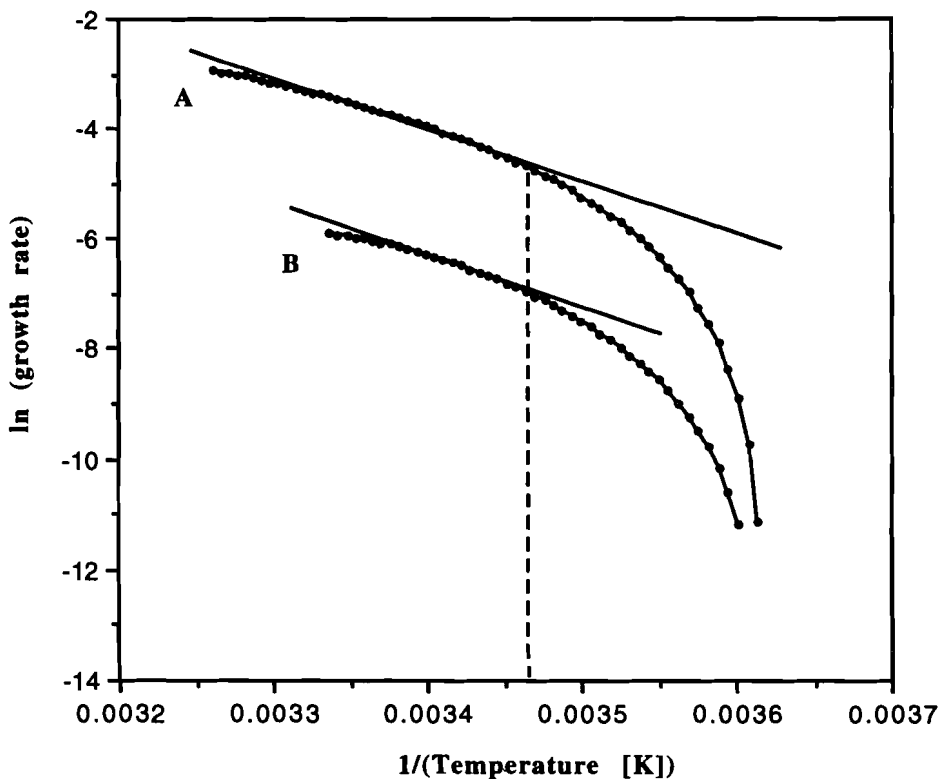


Figure 4.8: Arrhenius plots of Equation 2.4 simulating growth curves (A) at optimal water activity conditions and (B) at stringent water activity conditions. The broken line indicates the low temperature deviation of the square root model from linear Arrhenius kinetics. Curves A and B were simulated in Ultrafit 3.0 by fixing T_{min} and changing the 'b' and 'c' parameters.

only with the low temperature portion of the curve, hence no further mention of the superoptimal response will be made). This sigmoid relationship, between the apparent activation energy for bacterial growth and temperature, is more consistent with the trends noted in Figure 4.3 compared to the approximately linear relationship estimated using second order polynomial equations (Figure 4.5).

Similar to Davey models, square root models assume that the apparent activation energy for bacterial growth is independent of water activity conditions, as was generally observed in Figure 4.6. This is a mathematical consequence of assuming a fixed reference temperature (T_{min}). However, exceptions were noted: *E. coli* at 0.977, *P. halodenitrificans* at 0.892, *H. elongata* at 0.752 and *S. aureus* at 0.914. Each of those deviations can be accounted for by differences in T_{min} estimates (Table 4.1). Had T_{min} been specified when fitting Equation 2.4 (as required in predictive square root modelling) the deviations would have been eliminated. But, this assumption and result do not agree with the data presented in Figure 4.2. Deviation of the actual data points from linear (or approximately linear) Arrhenius kinetics appears to occur at higher temperatures when water activity conditions are more stringent. This is a mathematical impossibility in square root modelling (note that termination of the normal physiological range in Figure 4.8 occurs at the same temperature for both curve A and B). Therefore, the ability of the square root model to describe growth responses occurring outside the normal physiological range must be questioned. Inadequacies of the square root model in this temperature region have been noted previously. Ross (1993) noted a slight curvature in square root plots at suboptimal temperatures that may result in underestimation of growth rate for temperatures close to the low temperature growth/no growth boundary. Also, Kamperman (1994) showed that T_{min} estimates for pseudomonads were different when estimated using data for the entire suboptimal temperature range, compared to estimates from low temperature data (0 - 10°C).

Equation 4.1 (thermodynamic model)

The general shape of the Arrhenius curve described by Equation 4.1 (Figure 4.9) is similar to that for Equation 2.4, therefore the sigmoid relation between apparent activation energy and temperature (Figure 4.6) was expected. The thermodynamic model (as an Arrhenius plot) is the straight line Arrhenius equation in the normal physiological range with deviation from that straight line at low and high temperatures (corresponding to the high and low temperature asymptotes in Figure 4.7), due to denaturation of key enzymes or other macromolecules.

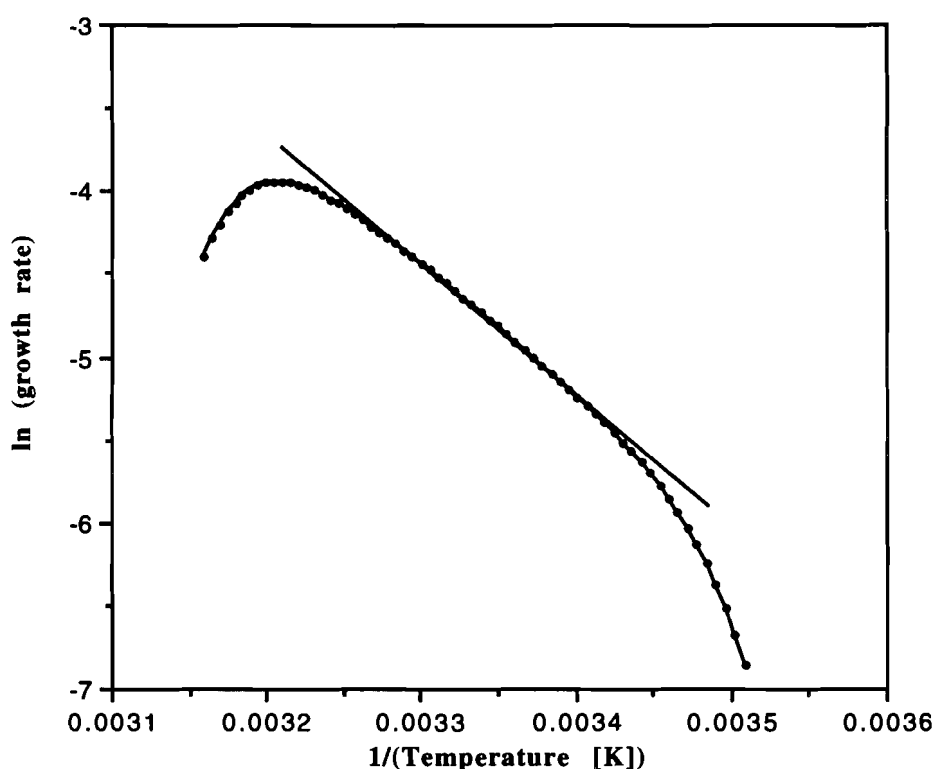


Figure 4.9: Arrhenius representation of the thermodynamic model (McMeekin *et al.*, 1993; Ross *et al.*, 1993).

The thermodynamic model has not yet been extended to include water activity effects on bacterial growth, therefore each curve summarised in Table 4.1 resulted from independent fits of the thermodynamic model to each data set. Consequently, the temperature at which deviation from linear Arrhenius kinetics occurs, and therefore where the low temperature activation energy asymptote occurs, was not fixed (unlike the case for square root models). The low temperature apparent activation energy asymptotes, when estimated using Equation 4.1, appear to occur at higher temperatures with increasingly stringent water activity conditions. That pattern is consistent with the raw data shown in Figure 4.2. Therefore, it appears that the activation energy for bacterial growth may be a function of both temperature and water activity, where extracellular water activity conditions define the low temperature boundary of the normal physiological range. Hence, if in the future Equation 4.1 is used to describe how growth responses of bacteria to temperature are modified by extracellular water activity conditions, understanding the interaction between temperature and water activity will be required.

4.4.2 Mechanistic basis

An influence of extracellular water activity conditions on the magnitude of the normal physiological range (resulting in altered activation energy values for bacterial growth at equivalent temperatures) is consistent with: master reaction models; current molecular theories on the influence of water activity and compatible solutes on water structure; and chemical literature concerning the influence of water activity on reaction rates.

Master reaction models

Equation 4.1 (McMeekin *et al.*, 1993; Ross, 1993), a master reaction model, predicts the deviation from linear Arrhenius kinetics for bacterial growth at low temperatures, due to cold denaturation of a key enzyme. Salt destabilises enzymes. Therefore, it is plausible that cold denaturation of enzymes occurs at higher temperatures when there is a water activity stress applied, resulting in the narrowing of the normal physiological range. Also, compatible solutes stabilise enzymes against the destabilising effects of salt (Luard, 1983; Warr *et al.*, 1984; Manetas, 1986). This would extend the normal physiological range for bacterial growth at unfavourable water activity values. Therefore, there is a possible mechanistic basis for the influence of water activity on the low temperature boundary of the normal physiological range.

Water structure

The high density water theory (Wiggins, 1990), introduced in section 1.3.2, implies that changes in extracellular water activity conditions can alter the kinetic state of the cytoplasm of bacterial cells. When exposed to altered water activity conditions an equilibrium state between the intracellular and extracellular water must be achieved; equilibrium of the chemical potentials, not strictly the water activities. Chemical potential is a function of water activity, temperature, pressure and molar volume (density). Within a cell it is impossible for temperature or pressure to change and Wiggins' (1990) assumes that insufficient amounts of water may exit the cytoplasm to balance the chemical potential, leaving just one degree of freedom: molar volume.

Where water activity values are low the chemical potential is also low. Therefore, for a cell stressed by low water activity conditions, the cytoplasm has a higher chemical potential than the extracellular environment. In this situation the

chemical potential of the cytoplasm may be reduced by expansion of the intracellular water. This expanded form of water, called low density water, tends to have many ice-like hydrogen bonds between water molecules. Therefore, low density water is more viscous than normal water, reducing the kinetic energy of the cytoplasm, without change in temperature. The activation energy is the minimum kinetic energy required for correctly oriented, colliding molecules to react (Arrhenius, 1915). Therefore, in unfavourably low water activity conditions that minimum kinetic energy would be realised at a higher temperature compared to when water activity conditions are favourable.

The high density water theory is also consistent with the observed influence of betaine on the apparent activation energy for bacterial growth (Figure 4.7). According to Wiggins' theory, compatible solutes increase the kinetic energy of the cytoplasm by increasing the amount of intracellular water and partitioning between low density and high density water in the cytoplasm, so that some of the low density water reverts to normal water (concomitantly reducing the viscosity).

Other supporting evidence

A precedent exists in the chemical literature that as water activity increases the activation energy tends to decrease. For example, the activation energy for thiamine loss in pasta decreased from 30.8 to 26.6 kcal/mole at water activities 0.44 and 0.65 respectively (Kamman *et al.*, 1981). Also the activation energy for rates of degradation of chlorophyll a and b decreased with increasing water activity conditions (LaJollo *et al.*, 1971).

4.4.3 Implications

Many predictive models (Ratkowsky *et al.*, 1983; Davey, 1989) assume the absence of interactions between temperature and water activity effects on bacterial growth rate. That assumption is undermined if both temperature and water activity influence the activation energy for bacterial growth. But, those models predict bacterial growth rate closely. Activation energy is however, the derivative of growth rate therefore, small errors in growth rate estimates translate to larger errors in activation energy estimates. Hence, square root models may approximate bacterial growth rate responses but not the activation energy response. Therefore, Equation 2.6 (the derivative of the square root model) proposed for estimating activation energy

(McMeekin *et al.*, 1993) is inappropriate, as were the interpretations based on it in Chapter 2 which led to the energy diversion hypothesis.

Observed shifts in the apparent activation energy for growth with water activity conditions (Figure 4.7) have led to a new hypothesis: that the minimum temperature for growth is defined by a critical activation energy value. Figure 4.10 re-examines Figure 4.7a. The solid black line, at 178 kJ mol^{-1} , indicates the predicted (by smoothing using Equation 4.1) activation energy at the temperature where growth was limited for the $a_w = 0.977$ without betaine treatment. From that critical activation energy value the minimum temperatures for growth were estimated for the remaining treatments (Table 4.2). The estimates match approximately with the observed minimum temperatures for growth, especially considering the limited amount of data

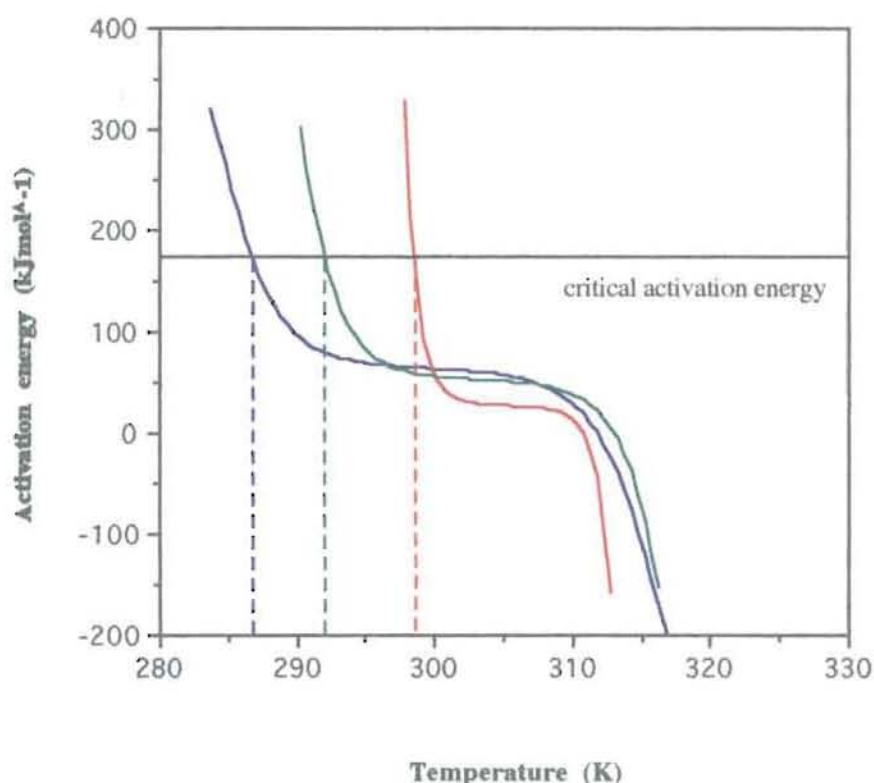


Figure 4.10: Apparent activation energy (estimated using Equation 4.1) for growth of *E. coli* at various water activity conditions: (—) 0.998 + 2 mM betaine, (—) 0.977 and (—) 0.977 + 2 mM betaine. The critical apparent activation energy (solid black line) was reckoned from the value at the minimum observed temperature for growth on the smoothed curve for the 0.977 treatment. This critical apparent activation energy value was then used to estimate the minimum temperatures for growth of the remaining two treatments (vertical, coloured lines).

available for fitting, and the steepness of the activation energy curves close to the growth/no growth interface. Therefore, the growth/no growth interface for temperature/water activity combinations may be due to an insurmountable, common activation energy. This analysis could not be performed for the other data sets because those experiments were not designed to detect the minimum observable temperatures for growth.

Table 4.2: Observed and predicted (using the common, critical activation energy concept) minimum temperatures for growth of *E. coli* SB1.

Treatment	Observed Minimum Temperature (°C)	Predicted Minimum Temperature (°C)
0.998 + 2 mM betaine	12.1	13.5
0.977 + 2 mM betaine	17.8	18.8
0.977*	25.8	25.8

* treatment used to define the critical activation energy for growth

Predictive modellers have noted that the growth/no growth boundary is clearly defined, however, the growth and death responses immediately adjacent to that boundary are extremely variable (McMeekin⁴, *pers. comm.*). The concept of a critical activation energy is consistent with the definite ‘cut-off’ between growth and no-growth conditions as well as the sudden decline in cell yield observed in Chapter 3 at critical water activity and temperature conditions.

The concept of a common, critical activation energy may also provide a mechanistic basis for growth/no growth interface models (Presser *et al.*, 1997b; Ratkowsky and Ross, 1995). Currently growth/no growth models are empirical, incorporating square root modelling parameters, including T_{min} . However, for good fits the T_{min} estimate cannot be fixed (Presser *et al.*, 1997b), providing further evidence that square root models are not appropriate for describing bacterial growth responses outside the normal physiological range. An interface defined by the critical activation energy would avoid such problems. The common, critical activation energy concept may also streamline the construction of interface models. If the critical activation energy for growth of a species was estimated from growth rate data collected at temperatures below the normal physiological range, it could be used to define other temperature and water activity combinations that limit bacterial growth.

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SUMMARY AND CONCLUSIONS

In a practical sense food microbiologists strive constantly to better satisfy consumer requirements. Consumers expect that their food is wholesome, fresh, safe and can be stored as such from purchase to consumption (often many days later). However, the longer perishable goods are stored the greater the opportunity for growth of spoilage organisms and pathogens, and the greater the food safety risk. The results of this thesis have highlighted an important consideration; practical solutions derive from understanding, not necessarily from empirical description. The influence of betaine on both growth and death rate, provides a good illustration. On the basis of empirical description of the increases in growth rate caused by compatible solutes for osmotically challenged cultures it was tempting to devise some means to prevent compatible solute uptake by bacteria on foods. However, betaine also accelerated the rate of death (when death was imposed by very high osmotic stress), therefore compatible solute uptake is antibacterial in that case. As discussed previously (section 3.3.3, *Death kinetics*) both responses would have been predicted from knowledge of the mechanisms, but not from empirical description.

The objective of this thesis (section 1.4) was to increase the mechanistic understanding of the bacterial growth response to extracellular water activity constraints. In Chapter 2 that response was successfully described using the square root model (Ratkowsky *et al.*, 1983). A mechanistic hypothesis was derived from that description, however, later that hypothesis failed. That failure highlighted that square root models are empirical, and while being useful for describing growth (especially within the normal physiological range) do not improve understanding.

This study did not elucidate the mechanism, but, from the cell yield experiments it is clear that water activity challenge is not an energetic burden for bacterial cells (Chapter 3), thereby eliminating the energy diversion hypothesis. The energy diversion hypothesis was based on the square root model, but has existed informally for some time (Csonka, 1989; Gould, 1989; Knochel and Gould, 1995).

The cell yield experiments also revealed the possibility that the mechanisms responsible for the bacterial responses to temperature and water activity conditions may be similar. Similar ranges of growth rates can be produced by these two factors, they show common trends in cell yield response and the molecular motion explanations of growth rate require their consideration in concert (section 4.4.2). In contrast the microbial response to extracellular pH constraints appears, on the basis of

growth rate and cell yield results, fundamentally different. However, further investigation is required.

Investigation of the reasons for the failure of the energy diversion hypothesis revealed a new hypothesis: critical apparent activation energy. Examination of five data sets provided some support for the new hypothesis, however more work is required. Few data sets were available where growth rate during water activity challenge had been measured across the biokinetic range. Most predictive models of microbial water activity responses have been constructed from data collected at normal physiological temperatures (Miles, 1994; Neumeyer, 1995). This is probably due most to practical considerations of experiment duration and temperature control. However, careful collection of growth rate data is now required for bacterial cultures, challenged by water activity, at temperatures close to the boundary of and below the normal physiological range. Such data would test the critical apparent activation energy concept. Also modelling of growth in that range may be important. If the boundary of the normal physiological range is determined by water activity (as indicated in Chapter 4) then the square root modelling assumption of the absence of interactions between water activity and temperature is inappropriate (at temperatures outside the normal physiological range); making square root models inappropriate for modelling growth rate at temperatures outside the normal physiological range, which may be important for models designed for refrigerated foods in particular.

Extracellular water activity conditions have profound influence over the growth of bacteria and are of great practical value in the food microbiologist's challenge to satisfy consumer expectations. However accurate manipulation of microbial growth (or survival) on foods using water activity will require a detailed understanding of the mechanisms that cause those responses, and with which we are only beginning to come to terms.

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APPENDIX 1.1: Nomenclature of genes and gene products.

Genes and their gene products often have the same terminology. They are identified in writing by italicising when referring to the gene (or operon), but not for the gene product. Genes referred to in the text of this thesis are:

E. coli osmoregulation:

<i>trkA</i>	encodes TrkA, a constitutive potassium transport system
<i>trkG</i>	together with <i>trkH</i> constitutes <i>trkA</i>
<i>trkH</i>	
<i>trkD</i>	encodes TrkD, a minor potassium transport system
<i>kdp</i>	encodes Kdp, an inducible, high affinity potassium transport system
<i>kdpA</i>	together with <i>kdpB</i> and <i>kdpC</i> encodes the potassium porter of Kdp
<i>kdpB</i>	
<i>kdpC</i>	
<i>kdpD</i>	together with <i>kdpE</i> regulates Kdp
<i>kdp E</i>	
<i>kefA</i>	KefA together with KefB and KefC, potassium efflux
<i>kefB</i>	
<i>kefC</i>	
<i>otsA</i>	encodes trehalose-6-phosphate synthetase (trehalose synthesis)
<i>otsB</i>	encodes trehalose-6-phosphate phosphatase (trehalose synthesis)
<i>treA</i>	encodes TreA, inducible, periplasmic trehalase (trehalose degradation)
<i>proP</i>	encodes ProP, a compatible solute (eg. betaine, proline) transporter
<i>proU</i>	operon encoding ProU, a compatible solute transporter
<i>proV</i>	together with <i>proW</i> and <i>proX</i> constitutes <i>proU</i>
<i>proW</i>	
<i>proX</i>	
<i>betA</i>	encodes choline dehydrogenase (choline-betaine pathway)
<i>betB</i>	encodes glycine betaine aldehyde dehydrogenase
<i>betT</i>	encodes BetT, a high affinity choline transport system
<i>betI</i>	regulates the choline-betaine pathway
<i>putP</i>	encodes PutP, the main proline porter

General stress response:

<i>rpoS</i>	encodes the putative sigma factor (σ^S) and regulates many genes associated with general stress responses. <i>rpoS</i> has also been known as: <i>katF</i> , <i>appR</i> and <i>nur</i>
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APPENDIX 2.1: Treatments and results for isothermal (37°C) experiment to determine the influence of extracellular water activity and betaine on the growth rate of *E. coli* SB1.

2A.1.1: Treatments used in isothermal (37°C) determination of *E. coli* SB1 growth rate as a function of extracellular water activity and betaine.

Flask	Waterbath	Water Activity	Betaine
1	2	0.998	-
2	3	0.998	-
3	4	0.998	+
4	1	0.999	+
5	3	0.987	-
6	2	0.986	-
7	3	0.987	+
8	1	0.988	+
9	3	0.976	-
10	2	0.976	-
11	4	0.976	+
12	3	0.976	+
13	1	0.973	-
14	3	0.972	-
15	2	NR	+
16	1	0.973	+
17	3	0.970	-
18	1	NR	-
19	2	0.970	+
20	1	0.970	+
21	4	0.967	-
22	2	0.968	-
23	2	0.967	+
24	1	0.967	+
25	1	0.964	+
26	2	0.964	+
27	2	NR	+
28	3	0.961	+
29	3	0.958	+
30	1	0.956	+

2A.1.2: Results obtained in isothermal (37°C) determination of *E. coli* SB1 growth rate as a function of extracellular water activity and betaine.

Water Activity	Gompertz Parameters				Gen Time (min)	Lag Time (min)
	A	B	D	M		
NaCl Only*						
0.998	3.45	2.20 x 10 ⁻²	59.69	220.1	50.9	174.6
0.998	4.53	2.05 x 10 ⁻²	57.30	223.9	56.6	175.2
0.987	5.98	1.49 x 10 ⁻²	64.87	324.1	69.1	256.7
0.986	4.83	1.27 x 10 ⁻²	62.95	329.7	83.6	250.7
0.976	3.30	7.39 x 10 ⁻³	61.81	1192.7	145.8	1057.3
0.976	3.55	5.37 x 10 ⁻³	62.29	1315.3	199.1	1129.1
0.972	3.31	3.25 x 10 ⁻³	55.36	2301.1	369.7	1993.8
With betaine†						
0.998	4.45	2.19 x 10 ⁻²	56.39	224.6	54.0	178.8
0.998	2.20	2.00 x 10 ⁻²	59.49	200.9	56.1	150.8
0.987	3.46	1.50 x 10 ⁻²	59.95	262.5	74.4	195.5
0.988	2.55	1.63 x 10 ⁻²	59.95	252.8	68.2	191.5
0.976	2.70	7.94 x 10 ⁻³	73.09	457.3	114.8	331.3
0.976	2.51	8.16 x 10 ⁻³	69.23	475.9	117.8	353.4
0.973	1.99	6.41 x 10 ⁻³	67.89	619.1	152.9	463.0
0.970	7.40	7.34 x 10 ⁻³	58.43	874.7	155.2	738.5
0.970	5.40	1.04 x 10 ⁻²	52.71	876.9	121.5	780.7
0.967	4.28	4.30 x 10 ⁻³	68.69	1333.3	197.8	1129.2
0.967	3.30	7.03 x 10 ⁻³	55.97	1290.5	169.2	1148.2
0.964	9.57	8.03 x 10 ⁻³	46.51	2058.8	178.3	1934.3
0.964	3.76	3.69 x 10 ⁻³	61.96	2874.1	291.1	2603.2

Equation for regression line between growth rate and water activity:

$$*y = -0.58403 + 0.60447x \quad r^2 = 0.967$$

$$†y = -0.38765 + 0.4065x \quad r^2 = 0.972$$

where: *y* is growth rate
x is water activity

APPENDIX 2.2: Results of experiment to determine the influence of extracellular water activity and betaine on the growth rate of *E. coli* SB1 for the biokinetic range.

Temp. (°C)	Gompertz Parameters				Gen Time (min)	Lag Time (min)
	A	B	D	M		
0.998						
13.2	1.91 x 10 ⁻²	9.60 x 10 ⁻⁴	91.8	10779.3	756	9738
15.7	2.35	1.37 x 10 ⁻³	94.2	6192.0	515	5463
18.3	1.85	2.20 x 10 ⁻³	98.7	3775.5	307	3320
20.8	3.12	3.33 x 10 ⁻³	95.5	2637.6	209	2338
23.2	3.51	4.75 x 10 ⁻³	90.7	1855.8	155	1645
25.7	4.15	6.22 x 10 ⁻³	88.8	1468.3	120	1308
28.2	2.61	7.35 x 10 ⁻³	93.0	1161.8	97	1026
31.0	2.13	8.74 x 10 ⁻³	91.4	976.8	83	862
33.8	2.93	1.04 x 10 ⁻²	93.0	844.9	69	749
37.2	3.89	1.12 x 10 ⁻²	96.2	859.4	62	770
40.8	4.47	1.10 x 10 ⁻²	95.2	1031.8	64	941
45.1	3.66	1.08 x 10 ⁻²	88.4	2286.3	70	2193
0.998 + 2 mM betaine						
12.1	8.69 x 10 ⁻²	7.40 x 10 ⁻⁴	112.1	11790.7	802	10439
14.8	2.39	1.66 x 10 ⁻³	93.0	5672.3	432	5069
17.3	1.86	2.20 x 10 ⁻³	100.1	3583.5	302	3130
19.9	3.42	3.44 x 10 ⁻³	93.3	2486.5	208	2195
22.2	4.20	4.61 x 10 ⁻³	90.3	1809.5	160	1592
24.8	2.31	5.56 x 10 ⁻³	93.4	1406.2	128	1226
27.1	2.74	6.62 x 10 ⁻³	96.5	1153.3	104	1002
29.7	2.91	7.61 x 10 ⁻³	96.2	918.4	91	787
32.5	3.69	1.09 x 10 ⁻²	89.0	796.1	68	704
35.5	4.76	1.41 x 10 ⁻²	83.0	727.7	57	657
39.0	5.17	1.54 x 10 ⁻²	83.0	764.1	52	699
42.6	3.19	9.11 x 10 ⁻³	105.1	1233.9	69	1124
0.977						
25.8	4.14 x 10 ⁻²	1.29 x 10 ⁻³	90.4	6031.3	572	
28.3	3.30	1.68 x 10 ⁻³	83.9	6020.9	471	5427
31.1	1.49	1.73 x 10 ⁻³	92.4	4460.0	416	3883
34.0	5.23 x 10 ⁻²	1.89 x 10 ⁻³	93.0	3458.0	380	2928
37.2	-1.25	2.13 x 10 ⁻³	90.0	3139.8	347	2671
40.7	-9.87	1.39 x 10 ⁻³	82.1	3005.9	582	2289

Temp. (°C)	Gompertz Parameters				Gen Time (min)	Lag Time (min)
	A	B	D	M		
0.977 + 2 mM betaine						
17.8	-4.65 E-2	9.40 x 10 ⁻⁴	92.0	13787.3	770	12723
20.1	-9.68 E-1	1.16 x 10 ⁻³	98.8	10084.0	581	9222
22.5	3.07	2.50 x 10 ⁻³	85.1	7279.4	313	6879
24.8	-2.47	2.54 x 10 ⁻³	95.6	5587.0	274	5193
27.4	2.71 E-1	3.19 x 10 ⁻³	92.4	3523.5	226	3210
29.9	-6.23 E-2	3.43 x 10 ⁻³	98.7	3205.4	197	2914
32.8	-5.04 E-1	4.14 x 10 ⁻³	100.3	2743.7	160	2502
35.9	-2.90 E-1	4.91 x 10 ⁻³	99.0	2533.4	137	2330
39.0	6.98 E-1	6.45 x 10 ⁻³	93.8	2417.0	110	2262
43.0	-2.34	4.52 x 10 ⁻³	103.0	3036.7	143	2815

APPENDIX 2.3: Results of experiment to determine the influence of extracellular water activity and betaine on the growth rate of *P. halodenitrificans* for the biokinetic range.

Temp. (°C)	Gompertz Parameters				Gen Time (min)	Lag Time (min)
	A	B	D	M		
0.964						
1.8	-2.52	2.31 x 10 ⁻⁴	23.86	2734.1	12081	200
5.4	-18.84	1.50 x 10 ⁻⁴	97.65	3594.6	4546	190
9.4	-7.80	3.52 x 10 ⁻⁴	102.13	2612.4	1852	400
12.1	-0.66	7.24 x 10 ⁻⁴	92.51	1651.4	994	408
14.8	-1.10	1.06 x 10 ⁻³	92.65	1143.0	675	291
17.3	-5.37	1.20 x 10 ⁻³	94.69	870.0	584	170
19.7	-2.96	1.76 x 10 ⁻³	96.08	647.3	393	147
22.1	-0.41	2.43 x 10 ⁻³	92.87	535.8	297	153
24.8	-0.58	3.07 x 10 ⁻³	91.31	448.6	237	140
27.3	-0.13	3.53 x 10 ⁻³	88.33	368.3	213	104
30.1	-0.13	4.33 x 10 ⁻³	90.80	327.5	169	107
33.0	1.56	5.03 x 10 ⁻³	86.58	295.3	153	103
36.5	0.51	4.86 x 10 ⁻³	85.58	310.7	160	111
0.964 + 2 mM betaine						
4	-17.62	1.19 x 10 ⁻⁴	54.98	1851.3	10177	
8.1	-6.30	2.26 x 10 ⁻⁴	119.59	4730.0	2464	959
11.4	-3.01	5.47 x 10 ⁻⁴	107.61	2258.9	1131	590
13.8	-1.44	8.74 x 10 ⁻⁴	100.96	1458.8	755	401
16.5	-0.95	1.33 x 10 ⁻³	94.18	1054.3	534	336
18.9	-0.72	1.83 x 10 ⁻³	94.79	786.2	383	262
21.2	-9.67	2.38 x 10 ⁻³	87.86	687.7	319	274
23.8	-0.45	2.71 x 10 ⁻³	96.08	509.8	256	159
26.1	1.20	3.74 x 10 ⁻³	92.33	435.0	193	172
28.8	0.88	3.81 x 10 ⁻³	95.16	380.5	184	128
31.7	1.17	4.98 x 10 ⁻³	89.72	347.7	149	149
35	1.28	5.31 x 10 ⁻³	91.65	299.8	137	115
38.3	3.07	2.48 x 10 ⁻³	98.08	602.1	273	212

Temp. (°C)	Gompertz Parameters				Gen Time (min)	Lag Time (min)
	A	B	D	M		
0.892 NaCl						
10.5	-9.71	7.00 x 10 ⁻⁵	110.21	11970.7	8631	1533
13.2	-5.23	1.71 x 10 ⁻⁴	94.87	6126.9	4105	1197
15.7	-4.21	2.71 x 10 ⁻⁴	84.95	4371.0	2892	1062
18.2	-8.15	3.94 x 10 ⁻⁴	92.41	2787.9	1829	593
20.5	-4.72	4.92 x 10 ⁻⁴	91.26	2312.8	1483	524
22.8	-4.77	6.16 x 10 ⁻⁴	89.72	1962.4	1205	494
23.5	-2.17	8.27 x 10 ⁻⁴	89.28	1439.4	902	353
27.8	-0.24	9.98 x 10 ⁻⁴	86.81	1350.6	769	407
30.3	-0.89	1.17 x 10 ⁻³	84.25	1169.2	677	359
33.0	-3.02	1.29 x 10 ⁻³	86.58	1020.5	595	297
36.3	-12.99	8.36 x 10 ⁻⁴	92.85	981.1	858	120
0.892 + 2 mM betaine						
4.0	-1.71	5.00 x 10 ⁻⁵	97.64	29210.4	13639	
8.8	-9.00	1.55 x 10 ⁻⁴	92.21	5806.3	4659	854
11.8	-5.18	3.10 x 10 ⁻⁴	97.25	3510.2	2209	735
14.5	-7.45	4.03 x 10 ⁻⁴	95.30	2438.8	1734	424
17.0	-8.95	5.62 x 10 ⁻⁴	94.33	1736.1	1256	297
19.4	-4.06	9.17 x 10 ⁻⁴	93.87	1296.3	774	317
21.8	-4.77	1.16 x 10 ⁻³	92.48	1026.9	622	251
24.1	-2.71	1.52 x 10 ⁻³	89.42	828.4	490	223
26.6	-2.41	1.99 x 10 ⁻³	86.40	675.0	387	202
29.0	-3.62	1.94 x 10 ⁻³	88.23	603.7	389	143
32.0	0.26	2.41 x 10 ⁻³	83.04	540.8	333	154
34.9	0.34	2.61 x 10 ⁻³	81.24	503.7	314	146
38.3	-23.72	1.06 x 10 ⁻³	102.73	325.7	610	

APPENDIX 2.4: Results of experiment to determine the influence of extracellular water activity and betaine on the growth rate of *H. elongata* for the biokinetic range.

Temp. (°C)	Gompertz Parameters				Gen Time (min)	Lag Time (min)
	A	B	D	M		
0.950						
9.0	0.84	4.20 x 10 ⁻⁴	81.37	4767.9	1948	2391
12.3	1.37	7.57 x 10 ⁻⁴	91.31	2767.1	963	1447
14.9	1.59	1.13 x 10 ⁻³	94.19	1802.8	627	917
17.2	1.94	1.61 x 10 ⁻³	91.84	1256.7	450	637
19.3	1.50	2.00 x 10 ⁻³	87.80	974.3	378	477
21.8	1.37	2.64 x 10 ⁻³	92.30	733.9	274	356
23.6	2.34	3.65 x 10 ⁻³	89.34	584.4	204	311
25.6	1.89	4.11 x 10 ⁻³	90.26	486.7	179	244
27.5	1.40	4.62 x 10 ⁻³	91.27	403.1	158	188
29.7	2.00	5.87 x 10 ⁻³	91.78	334.1	123	164
31.9	1.76	6.91 x 10 ⁻³	90.92	281.4	106	137
34.2	2.21	8.15 x 10 ⁻³	92.30	241.5	89	119
37.6	1.57	8.65 x 10 ⁻³	91.18	220.2	84	105
41.5	-0.61	6.15 x 10 ⁻³	106.75	245.0	101	87
0.950 + 2 mM betaine						
9.7	2.05	5.19 x 10 ⁻⁴	85.28	5103.7	1504	3177
12.5	0.86	8.07 x 10 ⁻⁴	95.08	2827.8	868	1589
15.2	0.62	1.22 x 10 ⁻³	93.63	1884.8	582	1066
17.5	1.58	2.16 x 10 ⁻³	83.96	1241.8	366	780
18.8	0.36	2.18 x 10 ⁻³	99.58	995.3	307	537
20.6	1.03	2.86 x 10 ⁻³	97.62	767.1	239	417
22.5	0.96	3.50 x 10 ⁻³	99.41	606.9	191	321
24.6	0.64	4.30 x 10 ⁻³	103.02	499.8	150	268
26.5	0.93	5.23 x 10 ⁻³	101.53	419.2	125	228
28.6	0.90	6.30 x 10 ⁻³	100.65	345.0	105	186
30.7	0.72	7.34 x 10 ⁻³	103.20	293.3	88	157
33.0	0.95	9.07 x 10 ⁻³	99.95	244.0	73	134
36.0	0.74	1.05 x 10 ⁻²	98.53	205.8	64	111
39.4	-0.32	9.60 x 10 ⁻³	101.95	198.6	68	95

Temp. (°C)	Gompertz Parameters				Gen Time (min)	Lag Time (min)
	A	B	D	M		
0.752						
18.5	-0.46	2.03 x 10 ⁻⁴	105.54	7765.2	3108	2945
20.6	0.92	3.40 x 10 ⁻⁴	87.33	5063.1	2243	2152
22.6	1.27	4.99 x 10 ⁻⁴	87.10	3752.8	1532.1	1757
24.5	1.47	6.26 x 10 ⁻⁴	88.40	3016.0	1203	1424
26.4	1.37	8.13 x 10 ⁻⁴	90.65	2495.3	903	1267
28.5	1.95	9.84 x 10 ⁻⁴	87.99	1998.1	769	984
30.8	0.35	1.25 x 10 ⁻³	91.57	1515.6	583	717
33.1	1.04	1.31 x 10 ⁻³	88.9	1427.8	572	668
35.7	1.26	1.57 x 10 ⁻³	90.34	1213.8	470	579
38.7	1.29	1.65 x 10 ⁻³	91.99	1140.3	439	537
42.2	0.85	1.61 x 10 ⁻³	85.66	1174.2	483	555
0.752 + 2 mM betaine						
12.3	0.32	3.38 x 10 ⁻⁴	90.19	6384.0	2184	3426
15.0	0.65	5.43 x 10 ⁻⁴	87.94	4171.5	1394	2330
16.8	0.41	7.81 x 10 ⁻⁴	88.68	3069.6	961	1789
18.8	0.52	1.01 x 10 ⁻³	90.4	2287.0	726	1302
20.8	0.45	1.26 x 10 ⁻³	91.00	1761.2	580	968
22.8	0.45	1.53 x 10 ⁻³	92.45	1411.4	471	757
24.8	0.25	2.00 x 10 ⁻³	91.75	1168.0	364	667
26.5	0.19	2.07 x 10 ⁻³	98.39	999.8	327	517
28.4	0.25	2.56 x 10 ⁻³	96.48	817.7	270	427
30.7	0.55	3.44 x 10 ⁻³	90.55	666.1	214	375
33.4	0.03	3.55 x 10 ⁻³	98.27	591.8	191	311
36.2	0.52	4.85 x 10 ⁻³	84.05	466.0	163	260
39.5	0.52	5.08 x 10 ⁻³	95.16	417.1	138	220

APPENDIX 2.5: Details on the construction of Figure 2.10, to enable calculation of the a_w min values for *S. oranienburg*.

2A.5.1: Figure 1 reproduced from Christian (1955b). Growth rates (2A.5.2) were read manually from an enlarged version of this figure.

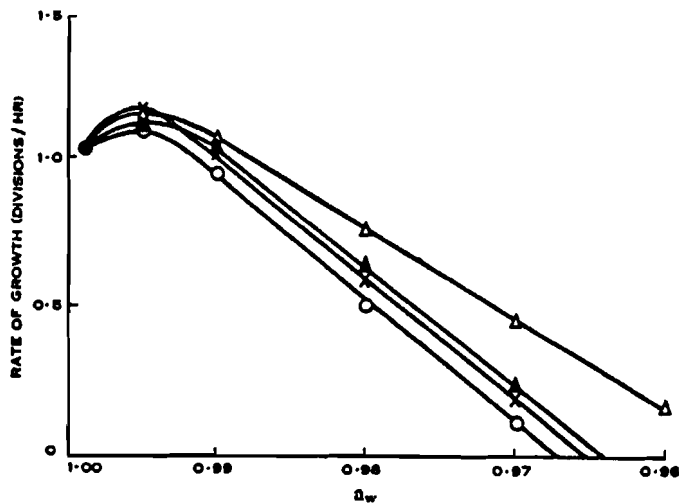


Fig. 1.—Relation between growth rate and a_w for *S. oranienburg* in glucose-salts medium using four methods for controlling a_w .
○ NaCl-KCl- Na_2SO_4 mixture in ratio of 5 : 3 : 2 moles.
× Sucrose. ▲ Glucose. ▴ Glycerol.

2.5.2: *S. oranienburg* growth rate data used for Figure 2.10.

Humectant	a_w	1	2	3	4
NaCl/KCl	0.990	0.930	1.55×10^{-2}	$y = -0.658 + 0.680x$	1.000
	0.980	0.519	8.64×10^{-3}		
	0.970	0.114	1.90×10^{-3}		
Sucrose	0.990	0.991	1.65×10^{-2}	$y = -0.641 + 0.664x$	1.000
	0.980	0.589	9.81×10^{-3}		
	0.970	0.193	3.21×10^{-3}		
Glucose	0.990	1.022	1.70×10^{-2}	$y = -0.626 + 0.650x$	1.000
	0.980	0.630	1.05×10^{-2}		
	0.970	0.240	4.01×10^{-3}		
Glycerol	0.990	1.063	1.77×10^{-2}	$y = -0.476 + 0.499x$	1.000
	0.980	0.761	1.27×10^{-2}		
	0.970	0.462	7.70×10^{-3}		
	0.960	0.164	2.74×10^{-3}		

- 1 divisions/hour, estimated from 2A.5.1
- 2 divisions/minute
- 3 equation describing the linear regression between water activity and column (2), (Figure 2.10)
- 4 regression coefficient for (3)

APPENDIX 2.6: Derivation of Equation 2.6, reproduced from McMeekin *et al.* (1993).

The differential form of the Arrhenius Law (Equation 1.4) is:

$$\frac{d \ln k}{d T} = \frac{E}{RT^2}$$

Taking the logarithm of both sides of the square root equation (Equation 2.3) and differentiating with respect to temperature:

$$\frac{d \ln k}{d T} = \frac{2}{(T - T_{\min})}$$

Hence it follows that the activation energy must be related to temperature by the expression:

$$\Delta E_{app} = \frac{2RT^2}{(T - T_{\min})}$$

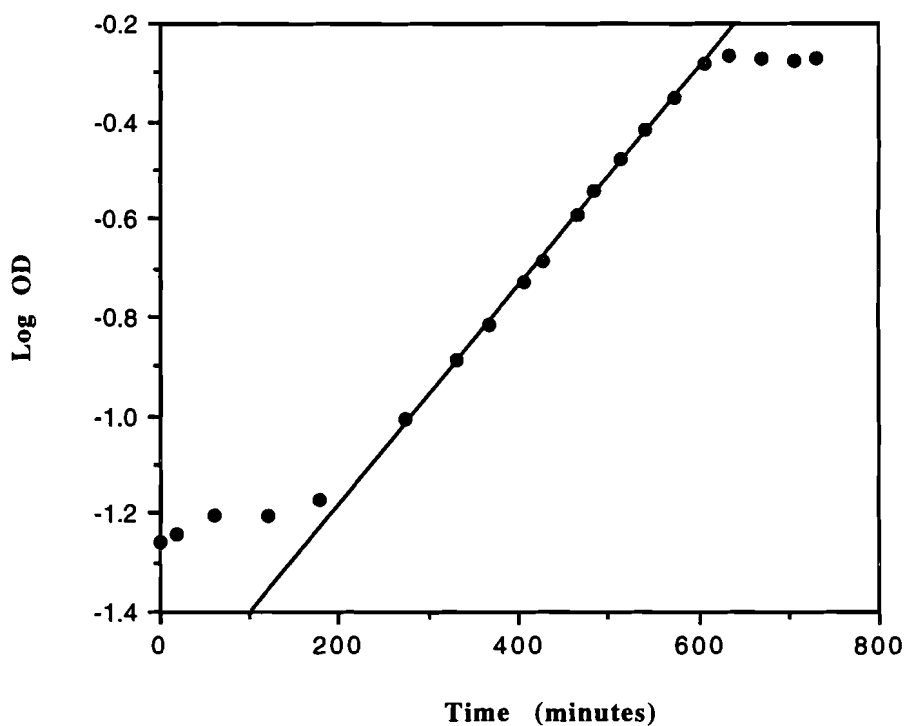
APPENDIX 3.1: Method for estimating generation time, lag time and T_f by linear regression.

The method used for estimating generation time by linear regression is described here, by example. The example uses data collected for aerobic growth of *E. coli* SB1 in minimal media containing 3.0×10^{-4} g glucose/mL (Table 3.4, treatment 8a).

3A.1.1: Growth data for *E. coli* SB1 cultured aerobically, in minimal media containing 3.0×10^{-4} g glucose/mL.

Time (min)	% T	OD	Log OD
0	88	0.05551733	-1.2555714
18	87.6	0.05749589	-1.2403632
59	86.6	0.06248211	-1.2042443
119	86.6	0.06248211	-1.2042443
178	85.6	0.06752624	-1.1705275
275	79.8	0.09799711	-1.0087867
332	74.2	0.12959609	-0.8874081
367	70.4	0.15242734	-0.8169371
405	65.2	0.1857524	-0.7310656
427	62	0.20760831	-0.6827553
465	55.6	0.25492521	-0.5935872
485	51.8	0.28567024	-0.544135
513	46.6	0.33161408	-0.479367
541	41.6	0.38090667	-0.4191814
573	35.8	0.44611697	-0.3505513
608	30	0.52287875	-0.281599
635	28.8	0.54060751	-0.2671179
671	29	0.537602	-0.2695391
708	29.4	0.53165267	-0.274372
732	29.2	0.53461715	-0.2719571

From 3A.1.1, time versus log OD was plotted (3A.1.2). On such a plot, data collected during the exponential growth phase forms a straight line. By fitting a straight line (linear regression, Cricket Graph 3.2.1) to that portion of the graph, generation time, lag time and time to reach final population density (T_f) can be calculated as follows:



3A.1.2: Growth curve for aerobic culture of *E. coli* SB1 in minimal media containing 3.0×10^{-4} g glucose/mL. The straight line was fitted to data in the exponential growth phase by linear regression in Cricket Graph 3.2.1.

The regression line in 3A.1.2 is described by the equation:

$$y = -1.6256 + 2.2213E-3x \quad (r^2=0.999) \quad (3A.1)$$

which is of the general form:

$$y = c + mx \quad (3A.2)$$

where

- y is logOD, equivalent to log(cell numbers)
- c is the y-axis intercept
- m is the slope
- x is time

Generation time

Generation time (GT), is the time for 1 cell to become 2 cells, or z cells to become $2z$ cells. Therefore, substituting $\log z$ (where z is cell numbers) for y in equation 3A.2, and rearranging equation 3A.2 gives:

$$\text{Time at } z \text{ cells: } x_z = \frac{\log z - c}{m} \quad \text{Time at } 2z \text{ cells: } x_{2z} = \frac{\log 2z - c}{m}$$

$$\text{Therefore: } GT = x_{2z} - x_z$$

$$\begin{aligned} &= \left[\frac{\log 2z - c}{m} \right] - \left[\frac{\log z - c}{m} \right] \\ &= \left[\frac{\log 2 + \log z - c}{m} \right] - \left[\frac{\log z - c}{m} \right] \\ &= \frac{\log 2}{m} \\ &= \frac{0.301}{m} \end{aligned} \quad (3A.3)$$

Substituting 'm' from equation 3A.1 into 3A.3, for the example:

$$GT = \frac{0.301}{2.221 \text{E}-3} = 135 \text{ minutes}$$

Lag time

Lag time is the time from inoculation to growth. Therefore, lag time is the value of x when the regression line in 3A.1.2 equals the starting y value. Rearranging equation 3A.2:

$$\text{Lag} = \frac{y_{(\text{initial})} - c}{m} \quad (3A.4)$$

Substituting values from 3A.1.1 and equation 3A.1, for the example:

$$\text{Lag} = \frac{-1.255 + 1.626}{2.221\text{E}-3} = 167 \text{ minutes}$$

Time to final population density (T_f)

T_f is the time from inoculation to where growth ceases. Therefore T_f is the x value when the regression line in 3A.1.2 equals the final y value. Rearranging equation 3A.2:

$$T_f = \frac{y_{(final)} - c}{m} \tag{3A.5}$$

Substituting values from 3A.1.1 and equation 3A.1, for the example:

$$T_f = \frac{-0.27 + 1.626}{2.221\text{E}-3} = 610 \text{ minutes}$$

APPENDIX 3.2: Methods 1 and 2 used to estimate maintenance energy in section 3.2. Example calculations are based on cell yield and growth data for the aerobic culture of *E. coli* SB1 at 20°C (section 3.2).

METHOD 1

Maintenance energy, or more strictly, endogenous metabolism, can be estimated by linear regression between cell yield and initial substrate concentration (Monod, 1942 cited in McGrew and Mallette, 1963); maintenance energy is indicated by the positive intercept of the regression line, with the substrate concentration axis. The rationale for this approach is that the substrate concentration, so determined, is just sufficient to maintain the inoculum until the time where the lag phase would be resolved, but not sufficient to support growth or maintain those cells beyond that time.

On this basis, for the example:

2.688×10^{-6} * g glucose is used to maintain,

5×10^7 † CFU/mL for

163 ± 12.6 § minutes, at which time the substrate has been exhausted.

* determined by linear regression between cell yield (change in optical density) and initial glucose concentration (Figure 3.3)

† estimate based on the assumption that an optical density value of 1.0 corresponds to 1.0×10^9 CFU/mL, and the relationship between optical density and cell number is linear. (*note: that this assumption was necessary indicates that determination of starting cell numbers should be incorporated into the experimental protocol for determining maintenance energy from substrate limited batch culture experiments*)

§ determined by linear regression between logOD and time (Appendix 3.1). The value shown is the mean value \pm standard deviation (n=10).

And therefore the maintenance energy of *E. coli* SB1, growing aerobically in minimal media is:

3.3×10^{-16} g glucose.cell⁻¹.minute⁻¹

METHOD 2

The second model for estimating maintenance energy from batch culture data was devised by the candidate's research supervisor, Dr. T. Ross. Based on the model:

energy used = lag maintenance (X) + growth maintenance (Y) + cell synthesis (Z)

where: energy used (g glucose)
 lag and growth maintenance (g glucose.cell⁻¹.minute⁻¹)
 cell synthesis (g glucose.cell⁻¹)

Unit analysis reveals that maintenance during lag and growth phases are a function of cell numbers and the duration for which those cells are supported, therefore a method to integrate those is needed. Giving the expression cell.minutes

Cell.minutes for the lag phase is:

$$\text{cell number (at inoculation) x lag time} \quad (3A.6)$$

The integral of cell numbers and time for the exponential phase is more complicated because cell number varies with time. The integral between two points on a curve is given by the difference between the values of the integral of the function that describes the curve. The exponential growth curve is described by the function:

$$N_t = N_o e^{\mu t}$$

where: N_t is the number of cells after time, t
 N_o is the initial number of cells
 μ is the specific growth rate (0.693/generation time)

Therefore cell.minutes for the exponential phase is:

$$\begin{aligned} & \int_{t_o}^{t_f} N_o \cdot e^{\mu t} \cdot dt \\ &= \left[\frac{N_o}{\mu} \cdot e^{\mu t_f} + C \right] - \left[\frac{N_o}{\mu} \cdot e^{\mu t_o} + C \right] \\ &= \left[\frac{N_o}{\mu} \cdot e^{\mu t_f} \right] - \left[\frac{N_o}{\mu} \cdot e^{\mu t_o} \right] \end{aligned} \quad (3A.7)$$

Values determined by applying equations 3A.6 and 3A.7 to data for growth at different glucose concentrations can be substituted into the model, allowing estimation of X, Y and Z, using simultaneous equations. For the example:

3A.2.1: Parameter values used in estimation of maintenance energy by method 2

a	Glucose conc.	3.0×10^{-5}	1.0×10^{-4}	3.0×10^{-4}
b	N_o	5.0×10^7	5.0×10^7	5.0×10^7
c	N_t	8.90×10^7	1.45×10^8	4.35×10^8
d	ΔN (c-b)	3.90×10^7	9.50×10^7	3.85×10^8
e	Lag time (min)	163	163	163
f	μ (0.693/GT; min^{-1})	4.81×10^{-3}	4.81×10^{-3}	4.81×10^{-3}
g	T_f (min)	256	434	603
h	Cell.minutes (lag)	8.15×10^9	8.15×10^9	8.15×10^9
i	Cell.minutes (exp)	7.51×10^9	2.79×10^{10}	7.59×10^{10}

a; g glucose/mL minimal media

b and **c**; estimates based on the assumption that an optical density value of 1.0 corresponds to 1.0×10^9 CFU/mL, and the relationship between optical density and cell number is linear.

e, **f** and **g**; determined by linear regression between logOD and time (Appendix 3.1). GT: generation time is 144 minutes.

h; calculated using 3A.6

i; calculated using 3A.7

Therefore:

$$3.0 \times 10^{-5} \text{ g glucose} = 8.15 \times 10^9 Z + 7.51 \times 10^9 X + 3.90 \times 10^7 Y \quad (3A.8)$$

$$1.0 \times 10^{-4} \text{ g glucose} = 8.15 \times 10^9 Z + 2.79 \times 10^{10} X + 9.50 \times 10^7 Y \quad (3A.9)$$

$$3.0 \times 10^{-4} \text{ g glucose} = 8.15 \times 10^9 Z + 7.59 \times 10^{10} X + 3.85 \times 10^8 Y \quad (3A.10)$$

3A.9 minus 3A.8:

$$7.0 \times 10^{-5} \text{ g glucose} = 2.04 \times 10^{10} X + 5.60 \times 10^7 Y \quad (3A.11)$$

3A.10 minus 3A.9:

$$2.0 \times 10^{-4} \text{ g glucose} = 4.80 \times 10^{10} X + 2.90 \times 10^8 Y \quad (3A.12)$$

(5.18 * 3A.11) minus 3A.12, to solve for X:

$$\begin{aligned} (3.63 \times 10^{-4}) - (2.0 \times 10^{-4}) &= [(10.6 - 4.80) \times 10^{10}] X \\ \therefore X &= 2.8 \times 10^{-15} \text{ g glucose.cell}^{-1}.\text{minute}^{-1} \end{aligned} \quad (3A.13)$$

The maintenance energy during the exponential growth phase is:

$$2.8 \times 10^{-15} \text{ g glucose.cell}^{-1}.\text{minute}^{-1}$$

substitute 3A.13 into 3A.12, to solve for Y:

$$\begin{aligned} 2.0 \times 10^{-4} \text{ g glucose} &= (4.80 \times 10^{10}) * (2.8 \times 10^{-15}) + 2.90 \times 10^8 Y \\ \therefore Y &= 2.2 \times 10^{-13} \text{ g glucose.cell}^{-1} \end{aligned} \quad (3A.14)$$

The energy cost per new cell synthesised is:

$$2.2 \times 10^{-13} \text{ g glucose.cell}^{-1}$$

substitute 3A.13 and 3A.14 into 3A.10, to solve for Z:

$$\begin{aligned} 3.0 \times 10^{-4} &= 8.15 \times 10^9 Z + (7.59 \times 10^{10} * 2.8 \times 10^{-15}) + (3.85 \times 10^8 * 2.2 \times 10^{-13}) \\ \therefore Z &= 1.7 \times 10^{-17} \text{ g glucose.cell}^{-1}.\text{minute}^{-1} \end{aligned}$$

The maintenance energy during the lag phase is:

$$1.7 \times 10^{-17} \text{ g glucose.cell}^{-1}.\text{minute}^{-1}$$

APPENDIX 3.3: pH validation of substrate-limited batch culture.

3A.3.1: Treatments for pH validation of substrate-limited batch culture.

Treatment	Waterbath	pH (target)	pH (actual)
1	3	7.00	6.94
2	3	7.00	6.79
3	3	5.50	5.28
4	2	5.50	5.24
5	2	5.00	4.76
6	1	5.00	4.73
7	2	4.90	4.67
8	2	4.90	4.68
9	3	4.80	4.67
10	1	4.80	4.57
11	1	4.70	4.59
12	1	4.70	4.47
13	3	4.60	4.45
14	3	4.60	4.38
15	2	4.50	4.32
16	3	4.50	4.33
17	1	4.40	4.20
18	2	4.40	4.24
19	2	4.30	4.11
20	1	4.30	4.09
21	1	4.20	4.00
22	3	4.20	4.00
23	1	4.10	3.91
24	2	4.10	3.92
25	2	4.00	3.81
26	1	4.00	3.77

3A.3.2: Data for pH validation of substrate-limited batch culture.

pH	[H ⁺]	ΔOD	Regression Line			GT (min)	Lag (min)
			c	m	r ²		
6.94	1.15x10 ⁻⁷	0.429	-2.12	7.29x10 ⁻³	0.999	41.29	2.33
6.79	1.62x10 ⁻⁷	0.429	-2.28	8.41x10 ⁻³	0.998	35.80	-0.36
5.28	5.25x10 ⁻⁶	0.451	-2.28	7.14x10 ⁻³	0.993	42.18	-10.81
5.24	5.75x10 ⁻⁶	0.466	-2.12	6.19x10 ⁻³	0.998	48.59	2.94
4.76	1.74x10 ⁻⁵	0.384	-2.14	6.05x10 ⁻³	0.998	49.78	-2.31
4.73	1.86x10 ⁻⁵	0.353	-2.13	6.01x10 ⁻³	0.998	50.05	-4.29
4.67	2.14x10 ⁻⁵	0.345	-2.13	5.88x10 ⁻³	0.997	51.22	5.12
4.68	2.09x10 ⁻⁵	0.347	-2.14	5.58x10 ⁻³	0.995	53.92	7.68
4.67	2.14x10 ⁻⁵	0.343	-2.14	5.76x10 ⁻³	0.992	52.26	5.94
4.57	2.69x10 ⁻⁵	0.281	-2.05	5.33x10 ⁻³	0.996	56.47	6.07
4.46	3.47x10 ⁻⁵	0.237	-2.15	5.68x10 ⁻³	1.000	52.98	-0.14
4.47	3.39x10 ⁻⁵	0.211	-2.03	5.02x10 ⁻³	0.989	59.92	3.45
4.45	3.55x10 ⁻⁵	0.180	-2.12	5.23x10 ⁻³	0.982	57.59	11.67
4.38	4.17x10 ⁻⁵	0.176	-2.03	4.68x10 ⁻³	0.992	64.25	3.51
4.32	4.79x10 ⁻⁵	0.144	-2.11	4.35x10 ⁻³	0.981	69.22	-9.40
4.33	4.68x10 ⁻⁵	0.144	-2.08	4.38x10 ⁻³	0.998	68.79	-64.64
4.20	6.31x10 ⁻⁵	0.054	-	-	-	-	-
4.24	5.75x10 ⁻⁵	0.072	-	-	-	-	-
4.11	7.76x10 ⁻⁵	0.025	-	-	-	-	-
4.09	8.13x10 ⁻⁵	0.031	-	-	-	-	-
4.00	1.00x10 ⁻⁴	0.023	-	-	-	-	-
4.00	1.00x10 ⁻⁴	0.025	-	-	-	-	-
3.91	1.23x10 ⁻⁴	0.026	-	-	-	-	-
3.92	1.20x10 ⁻⁴	0.014	-	-	-	-	-
3.81	1.55x10 ⁻⁴	zero	-	-	-	-	-
3.77	1.70x10 ⁻⁴	zero	-	-	-	-	-

APPENDIX 3.4: Data for aerobic, substrate-limited batch culture of *E. coli* SB1

Treatment	1a	1 b	2a	2 b	3a	3 b
Cell Yield						
Start OD	0.053	0.057	0.053	0.060	0.060	0.051
Final OD	0.059	0.062	0.062	0.063	0.064	0.061
ΔOD	0.006	0.005	0.009	0.003	0.004	0.010
Regression Line						
c	-	-	-	-	-	-
m	-	-	-	-	-	-
r ²	-	-	-	-	-	-
GT (min)	-	-	-	-	-	-
Lag (min)	-	-	-	-	-	-
Gompertz equation						
A	-	-	-	-	-	-
B	-	-	-	-	-	-
D	-	-	-	-	-	-
M	-	-	-	-	-	-
I	-	-	-	-	-	-
GT (min)	-	-	-	-	-	-
Treatment	4a	4 b	5a	5 b	6a	6 b
Cell Yield						
Start OD	0.055	0.075	0.052	0.052	0.054	0.055
Final OD	0.065	0.081	0.065	0.067	0.107	0.103
ΔOD	0.010	0.006	0.013	0.015	0.053	0.048
Regression Line						
c	-	-	-	-	-	-
m	-	-	-	-	-	-
r ²	-	-	-	-	-	-
GT (min)	-	-	-	-	-	-
Lag (min)	-	-	-	-	-	-
Gompertz equation						
A	-	-	-	-	-	-
B	-	-	-	-	-	-
D	-	-	-	-	-	-
M	-	-	-	-	-	-
I	-	-	-	-	-	-
GT (min)	-	-	-	-	-	-

Treatment	7a	7b	8a	8b	9a
Cell Yield					
Start OD	0.061	0.055	0.055	0.077	0.057
Final OD	0.223	0.215	0.541	0.553	1.097
Δ OD	0.162	0.160	0.486	0.476	1.040
Regression Line					
c	-1.542	-1.561	-1.626	-1.477	-1.560
m	2.031×10^{-3}	2.076×10^{-3}	2.221×10^{-3}	2.048×10^{-3}	2.109×10^{-3}
r ²	1.000	0.998	0.999	0.999	0.998
GT (min)	148.20	145.00	135.51	146.99	142.72
Lag (min)	162.84	147.33	166.58	177.0	147.93
Gompertz equation					
A	1.022	0.964	1.472	1.638	2.071
B	0.014	0.014	0.007	0.008	0.005
D	27.17	27.26	67.33	61.43	96.90
M	304.6	296.5	418.0	399.8	483.9
I	9	10	8	8	7
GT (min)	176.49	168.13	137.38	140.17	137.38
Treatment	9b	10a	10b	11a	11b
Cell Yield					
Start OD	0.080	0.057	0.052	0.057	0.060
Final OD	1.119	1.167	1.155	1.167	1.194
Δ OD	1.039	1.110	1.103	1.110	1.134
Regression Line					
c	-1.460	-1.569	-1.602	-1.608	-1.563
m	1.947×10^{-3}	2.094×10^{-3}	2.144×10^{-3}	2.132×10^{-3}	2.109×10^{-3}
r ²	0.999	0.997	0.998	0.997	0.998
GT (min)	154.64	143.73	140.38	141.14	142.72
Lag (min)	186.19	157.03	150.73	168.95	163.31
Gompertz equation					
A	1.057	0.984	1.517	0.865	0.768
B	0.005	0.005	0.005	0.005	0.005
D	93.57	99.94	100.6	101.4	98.15
M	490.8	491.7	494.3	502.3	485.0
I	6	6	7	7	6
GT (min)	145.22	136.11	135.90	135.11	134.77

APPENDIX 3.5: Data for anaerobic, substrate-limited batch culture of *E. coli* SB1

Treatment	1a	1 b	2	3
Cell Yield				
Start OD	0.076	0.072	0.052	0.048
Final OD	0.151	0.143	0.175	0.324
ΔOD	0.075	0.071	0.124	0.277
Regression Line				
c	-	-	-1.284	-1.259
m	-	-	3.021×10^{-3}	2.986×10^{-3}
r ²	-	-	0.999	1.000
GT (min)	-	-	99.64	100.82
Lag (min)	-	-	-1.24	-21.05
Gompertz equation				
A	-	-	0.2888	0.3196
B	-	-	0.017	0.012
D	-	-	23.85	47.38
M	-	-	88.12	136.29
I	-	-	7	7
GT (min)	-	-	163.94	114.69
Treatment	4a	4 b	5a	5 b
Cell Yield				
Start OD	0.066	0.057	0.076	0.057
Final OD	0.509	0.495	0.770	0.654
ΔOD	0.443	0.437	0.694	0.596
Regression Line				
c	-1.181	-1.234	-1.104	-1.234
m	2.910×10^{-3}	2.759×10^{-3}	2.840×10^{-3}	2.886×10^{-3}
r ²	0.994	0.995	1.000	0.996
GT (min)	103.44	109.09	105.97	104.30
Lag (min)	-0.70	-2.45	-5.73	-2.24
Gompertz equation				
A	-0.5822	-0.2303	-1.809	.778
B	0.009	0.008	0.008	0.007
D	66.30	70.09	75.46	76.05
M	160.39	190.00	164.48	191.13
I	6	7	5	6
GT (min)	113.36	125.13	112.08	121.40

APPENDIX 3.6: Treatments and data in determination of the influence of water activity and betaine on the cell yield of anaerobically cultured *E. coli* SB1.

Treatment	Position*	Water activity	Betaine	ΔOD
1	3, 37	0.997	-	0.468, 0.513
2	2, 24	0.993	-	0.540, 0.524
3	35, 47	0.986	-	0.445, 0.474
4	13, 46	0.980	-	0.273, 0.363
5	20, 29	0.978	-	0.324, 0.131
6	11, 44	0.974	-	0.042, 0.109
7	5, 38	0.954	-	0.017, 0.149
8	32, 48	0.967	-	zero, 0.053
9	10, 30	0.964	-	zero, 0.049
10	9, 14	0.997	+	0.483, 0.502
11	18, 23	0.982	+	0.508, 0.507
12	27, 43	0.975	+	0.455, 0.435
13	34, 36	0.969	+	0.458, 0.482
14	16, 22	0.957	+	0.303, 0.230
15	33, 45	0.965	+	0.440, 0.435
16	6, 17	0.959	+	0.366, 0.336
17	25, 41	0.960	+	0.311, 0.299
18	15, 28	0.952	+	zero, zero

* two positions in the incubator are given, referring to duplicate pairs

APPENDIX 3.7: Influence of moderate extracellular water activity on cell yield during aerobic culture of *E. coli* SB1.

3A.7.1: Treatments for determination of the influence of moderate extracellular water activity on cell yield during aerobic culture of *E. coli* SB1.

Treatment	Flask Number	Waterbath 1	Waterbath 2
0% NaCl + 2 mM betaine	1-3	2, 3	1
2% NaCl + 2 mM betaine	4-9	4, 5, 6	7, 8, 9
0% NaCl	10-12	12	10, 11
2% NaCl	13-18	15, 17, 18	13, 14, 16

3A.7.2: Data for determination of the influence of moderate extracellular water activity on cell yield during aerobic culture of *E. coli* SB1.

Flask	ΔOD	Regression Line			GT (min)
		c	m	r^2	
1	0.400	-2.098	5.72×10^{-3}	0.994	52.6
2	0.428	-2.441	7.72×10^{-3}	0.999	39.0
3	0.398	-2.275	7.04×10^{-3}	0.998	42.8
4	0.414	-2.177	5.30×10^{-3}	0.997	56.8
5	0.417	-2.151	5.28×10^{-3}	0.989	57.0
6	0.425	-2.221	4.09×10^{-3}	0.989	73.6
7	0.446	-2.136	5.59×10^{-3}	0.985	53.8
8	0.448	-2.177	5.67×10^{-3}	0.979	53.1
9	0.455	-2.104	5.30×10^{-3}	0.986	56.8
10	0.433	-2.169	5.97×10^{-3}	0.998	50.4
11	0.441	-2.129	5.92×10^{-3}	0.994	50.9
12	0.462	-2.186	6.51×10^{-3}	0.985	46.2
13	0.528	-2.128	4.33×10^{-3}	0.984	69.5
14	0.550	-2.172	4.46×10^{-3}	0.996	67.5
15	0.512	-2.111	4.44×10^{-3}	0.995	67.7
16	0.521	-2.108	4.09×10^{-3}	0.995	73.6
17	0.522	-2.110	4.09×10^{-3}	0.996	73.5
18	0.516	-2.129	4.41×10^{-3}	0.999	68.2

APPENDIX 3.8: Effect of extended lag time on cell yield of *E. coli* SB1.

Treatment	Waterbath	a_w (initial)	a_w (final)	ΔOD
A	1	0.973	-	0.546
A	2	0.973	-	0.554
B(i)	1	0.965	0.972	0.377
B(ii)	1	0.965	0.975	0.368
C(i)	2	0.966	0.974	0.422
C(ii)	2	0.965	0.975	0.397
D(i)	1	0.967	-	0.351
D(ii)	2	0.967	-	0.350

- A: initial water activity conditions were growth permissible (Figure 3.12).
- B: initial water activity conditions were growth non-permissible (Figure 3.12); shifted to growth permissible conditions using glucose-free minimal media.
- C: initial water activity conditions were growth non-permissible (Figure 3.12); shifted to growth permissible conditions using glucose-free minimal media containing betaine.
- D: initial water activity conditions were growth non-permissible (Figure 3.12); shifted to growth permissible conditions using betaine.

APPENDIX 3.9: Influence of extracellular water activity and betaine on cell yield of *E. coli* SB1.

Water activity	Betaine	ΔOD
0.998	-	0.410
0.998	-	0.426
0.987	-	0.534
0.987	-	0.525
0.976	-	0.460
0.976	-	0.446
0.973	-	0.305
0.972	-	0.354
0.970	-	zero
0.967	-	zero
0.968	-	zero
0.998	+	0.437
0.999	+	0.434
0.987	+	0.464
0.988	+	0.457
0.976	+	0.379
0.976	+	0.372
0.973	+	0.333
0.970	+	0.329
0.970	+	0.382
0.967	+	0.323
0.967	+	0.346
0.964	+	0.315
0.964	+	0.300
0.961	+	zero
0.958	+	zero
0.956	+	zero

APPENDIX 3.10: Data for the influence of temperature on the cell yield of *E. coli* SB1.

Temperature (°C)	ΔOD	Regression Line			GT (min)
		c	m	r ²	
4.3	0.054	-	-	-	-
6.1	0.084	-	-	-	-
7.6	0.484	-	-	-	-
9.2	0.546	-1.33	2.72x10 ⁻⁴	0.994	1107.55
11.0	0.584	-1.39	4.54x10 ⁻⁴	0.984	662.59
12.4	0.598	-1.46	7.27x10 ⁻⁴	0.958	414.16
13.5	0.598	-1.40	8.83x10 ⁻⁴	0.958	341.01
14.9	0.615	-1.44	1.15x10 ⁻³	0.972	261.97
15.9	0.625	-1.56	1.53x10 ⁻³	0.938	196.14
17.0	0.592	-1.39	1.53x10 ⁻³	0.990	197.04
18.1	0.597	-1.32	1.68x10 ⁻³	0.997	179.23
19.3	0.597	-1.30	2.12x10 ⁻³	0.997	142.05
20.2	0.607	-1.38	2.47x10 ⁻³	0.999	122.06
21.2	0.612	-1.38	2.71x10 ⁻³	0.999	111.07
22.4	0.632	-1.40	3.17x10 ⁻³	0.999	95.10
24.5	0.632	-1.37	3.75x10 ⁻³	0.998	80.30
25.8	0.699	-1.37	4.02x10 ⁻³	0.998	74.79
26.8	0.676	-1.40	4.54x10 ⁻³	0.999	66.28
28.1	0.653	-1.38	4.92x10 ⁻³	0.999	61.13
29.1	0.653	-1.37	5.17x10 ⁻³	0.999	58.25
30.0	0.694	-1.31	5.66x10 ⁻³	0.999	53.21
31.5	0.653	-1.35	5.99x10 ⁻³	0.999	50.23
32.1	0.612	-1.36	6.39x10 ⁻³	0.998	47.10
34.6	0.612	-1.36	6.78x10 ⁻³	0.998	44.42
35.8	0.579	-1.40	7.23x10 ⁻³	0.999	41.62
38.1	0.574	-1.41	7.46x10 ⁻³	0.998	40.34
40.1	0.497	-1.40	6.68x10 ⁻³	0.997	45.05
41.9	0.337	-1.24	1.98x10 ⁻³	0.983	151.99
45.2	0.020	-	-	-	-

APPENDIX 3.11: Data for determination of adenylate energy charge during water activity challenge.

Time (min)	Control		Salt Challenge	
	$\Delta\%T$	AEC \pm SD*	$\Delta\%T$	AEC \pm SD
0	0.1		0	
50	1.0		2.2	
96	7.0		3.7	
110	9.2		6.8	
128	15.6		12.6	
150	25.0		19.6	
167	31.0		26.2	
178	35.0		32.0	
190	40.8		37.2	
199	44.8	0.82 \pm 0.03	41.2	
204			44.0	0.77
210	49.4	0.77 \pm 0.03		
216			50.6	0.81
220	54.2	0.87 \pm 0.06	53.6	0.80 \pm 0.07
225	57.0		59.8	0.85 \pm 0.00
230			58.2	0.80 \pm 0.06
235	59.2	0.86 \pm 0.01		
240			52.8	0.83 \pm 0.04
245			52.0	
250	65.8		51.0	0.86 \pm 0.01
255			51.0	
265	69.8	0.68 \pm 0.03		
270			53.4	0.84 \pm 0.02
285	75.6			
290			57.0	0.87 \pm 0.05
305	78.6		60.0	0.81
320	79.0	0.76 \pm 0.00	62.0	
337			64.0	
349				
367			67.6	
381			69.4	0.73
397			71.0	

* adenylate energy charge \pm standard deviation (n=2)

APPENDIX 3.12: Raw data for death kinetics (Figure 3.20)

0.940		0.940 + betaine		0.956		0.948 + betaine	
Time (min)	Count	Time (min)	Count	Time (min)	Count	Time (min)	Count
0	9.50×10^7	0	6.45×10^7	0	1.17×10^8	0	1.01×10^8
570	2.95×10^7	570	1.15×10^7	300	2.60×10^7	330	1.67×10^7
1350	8.20×10^6	1770	1.42×10^7	1425	1.31×10^7	1455	1.08×10^7
1770	2.47×10^7	2850	6.65×10^6	1980	1.12×10^7	2010	7.20×10^6
2850	5.35×10^6	4290	5.25×10^5	3210	7.10×10^6	3240	1.12×10^6
4290	4.55×10^6	5760	3.90×10^5	4440	3.35×10^6	4470	4.45×10^5
5760	5.25×10^6	7290	1.61×10^5	5910	4.20×10^6	5940	6.65×10^5
7290	2.70×10^6	8760	1.43×10^5	7365	3.75×10^6	7395	4.70×10^5
8760	1.51×10^6	12810	5.90×10^4				
9930	4.80×10^5	14400	4.65×10^4				
11370	4.80×10^5	15870	4.05×10^4				
14400	1.25×10^5						
15870	1.17×10^5						

APPENDIX 4.1: Summary and discussion of 95% confidence intervals for the Equation 2.4 and 4.1 parameter estimates in Table 4.1.

The 95% confidence intervals for parameter estimates in Table 4.1 are summarised overleaf (4A.1.1).

For Equation 2.4 the confidence intervals for all parameters were small, except for some estimates of T_{min} and T_{max} . Those have already been discussed in section 2.4.2.

For Equation 4.1 generally, the confidence intervals for estimates of ΔC_p , ΔH^\ddagger and ΔH^* were small and those for C and n large. ΔC_p , ΔH^\ddagger and ΔH^* each define a single section of the bacterial growth curve (ΔC_p , the position of the temperature range, ΔH^\ddagger the steepness of the growth curve in the suboptimal temperature range and ΔH^* , the temperature range).

Parameter C (like ΔH^\ddagger) determines the steepness of the growth curve in the suboptimal temperature range. But, large changes in C are required before there is any noticeable effect on the shape of the curve, therefore the large confidence intervals for estimation of that parameter were not surprising. Interestingly for parameter n , when estimated to be ~ 400 , the confidence intervals were small, but when it deviated from that value (up or down) the confidence intervals increased.

The consequence of large confidence intervals for estimates of C and n was that no meaningful interpretations of the estimates of those parameters can be made (for the five data sets examined in Chapter 4).

4A.1.1: Summary of 95% confidence intervals for Equation 2.4 and 4.1 parameters in Table 4.1

Treatment	Equation 2.4				Equation 4.1				
	b	c	T _{min} (°C)	T _{max} (°C)	ΔC _p	ΔH [‡] (x10 ³)	ΔH [*]	C	n
<i>E. coli</i>									
0.998	7.05x10 ⁻⁴	4.45x10 ⁻²	1.30	3.26	2.87	16.8	22.7	2.54x10 ⁴	67
0.998+B	2.00x10 ⁻⁴	1.90x10 ⁻²	0.98	1.23	0.67	4.84	2.31	3.73x10 ⁶	77
0.977	7.77x10 ⁻⁴	7.49x10 ⁻¹	23.6	2.57	0.64	4.31	2.74	2.68x10 ⁻¹	814
0.977+B	5.42x10 ⁻⁴	1.23	4.40	5.44	1.42	13.0	5.05	5.63x10 ⁴	503
<i>P. halodenitrificans</i>									
0.964	1.73x10 ⁻⁴	9.14x10 ⁻¹	1.28	5.89	2.52	15.3	20.6	5.94x10 ⁸	93
0.964+B	1.25x10 ⁻⁴	1.51x10 ⁻¹	0.87	0.56	1.14	10.9	3.90	5.81x10 ⁷	121
0.892	9.82x10 ⁻⁵	1.65x10 ⁻¹	1.10	1.13	0.70	9.33	2.21	3.66x10 ⁶	192
0.892+B	1.90x10 ⁻⁴	1.86x10 ⁻¹	1.80	1.27	2.24	18.6	7.72	1.43x10 ⁹	106
<i>H. elongata</i>									
0.950	1.74x10 ⁻⁴	4.91x10 ⁻¹	1.06	1.64	0.75	7.70	2.86	5.43x10 ⁷	209
0.950+B	1.76x10 ⁻⁴	8.59x10 ⁻¹	0.80	3.41	1.07	7.92	4.18	7.93x10 ⁸	120
0.752	5.30x10 ⁻⁴	1.14x10 ⁻¹	2.43	5.51	2.42	18.6	12.8	2.17x10 ⁵	152
0.752+B	2.02x10 ⁻⁴	5.56x10 ⁻¹	1.17	33.1	1.18	8.00	3.72	7.65x10 ⁶	191

4A.1.1 cont....

Treatment	Equation 2.4				Equation 4.1				
	b	c	T _{min} (°C)	T _{max} (°C)	ΔC _p	ΔH‡ (x10 ³)	ΔH*	C	n
<i>S. aureus</i>									
0.997	2.22x10 ⁻⁴	9.94x10 ²	0.50	0.02	0.82	5.79	1.49	1.09x10 ¹¹	150
0.963	0.00	0.00	0.00	0.00	1.03	9.96	1.86	6.76x10 ¹⁴	169
0.935	4.53x10 ⁻⁴	1.32x10 ¹⁸	1.42	9.33x10 ⁷	3.37	17.6	6.27	7.05x10 ¹¹	393
0.914	5.61x10 ⁻⁴	8.79x10 ⁻²	0.87	0.18	1.56	16.0	3.02	1.09x10 ¹¹	170
<i>S. xylosus</i>									
0.976	4.32x10 ⁻⁴	5.22x10 ²	0.78	0.04	1.56	11.9	2.11	7.28x10 ¹⁵	189
0.949	2.44x10 ⁻⁴	2.26x10 ⁻¹	0.48	0.04	1.20	8.86	2.74	1.43x10 ¹¹	161
0.928	4.39x10 ⁻⁴	1.04x10 ¹⁷	1.07	1.09x10 ⁷	1.16	8.98	7.72	1.88x10 ¹³	1474
0.909	5.61x10 ⁻⁴	1.18x10 ⁻¹	1.04	0.17	1.88	21.2	3.89	4.35x10 ¹¹	391
0.848	1.16x10 ⁻³	1.94x10 ⁻¹	5.01	0.32	95.5	56.6	236	2.90x10 ⁵	4486

APPENDIX A: Common methods and materials

A.1 Materials

Organisms

E. coli SB1 was a commensal isolate used in undergraduate teaching and obtained from Ms. S. Bettiol (Division of Pathology, GPO Box 252-29, University of Tasmania, Hobart, Tasmania, Australia).

P. halodenitrificans and *H. elongata* were obtained from Ms. C. Mancuso Nichols (Australian Collection of Antarctic Microorganisms, Department of Agricultural Science, GPO Box 252-54, University of Tasmania, Hobart, Tasmania, Australia).

Media

Eosin methylene blue agar (Oxoid, CM69) was prepared and sterilised according to manufacturer's instructions from commercially prepared dehydrated media (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England).

Minimal medium for culture of *E. coli* and *H. elongata* was: *Minimal Broth Davis, w/o dextrose* (DIFCO, 0756-17-7), prepared and sterilised according to manufacturer's instructions (including addition of recommended concentration of dextrose, unless otherwise specified in the text) from commercially prepared dehydrated media (DIFCO Laboratories, Detroit MI 48232-7058, USA).

Minimal medium for culture of *P. halodenitrificans* was (after, Hochstein and Tomlinson (1984)):

NaCl	58.4 g
K ₂ HPO ₄	8.6 g
(NH ₄) ₂ SO ₄	1.0 g
MgSO ₄ .7H ₂ O	0.1 g
Sodium lactate	5.0 g
Thiamine hydrochloride*	0.05 mg
FeSO ₄ .7H ₂ O*	0.03 mg
L-methionine*	14.0 mg
KNO ₃	10 g
Water	1 L

The pH was adjusted to 7.4 using HCl and the broth sterilised by autoclaving (121°C for 15 minutes). After cooling * were added, filter sterile.

Nutrient agar was prepared from nutrient broth (Oxoid, CM1) and 15 gL⁻¹ Davis powdered agar (Davis Gelatine (Australia) Co., 28 Spring St., Botany, Sydney, Australia).

Chemicals

Reagents were obtained from a variety of chemical suppliers:

Adenylate kinase (M-3003)

ADP (A-6521)

AMP (A-1877)

ATP (A-5394)

Betaine (B-2629)

Bovine serum albumin (A-7906)

EDTA (E-5134)

L-methionine (M-1126)

(NH₄)₂SO₄ (A-2939)

Phosphoenol pyruvate (P-7002)

Pyruvate kinase (P-1506)

Resazurin (R-2127)

Sodium thioglycollate (T-0632)

Thiamine hydrochloride (T-4625)

Trichloroacetic acid (T-9159)

Tris (Trizma[®] sulfate, T-8379)

vitamin C (A-5960)

Firefly luciferase (FLE-50)

Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA.

D-glucose (783)

HCl (256)

K₂HPO₄ (398)

KNO₃ (791)

NaCl (7647-14-5)

Sodium lactate (738)

Ajax Chemicals, 9 Short St., Alburn, NSW, Australia, 7144.

FeSO₄·7H₂O (10112)

KOH (10210)

MgSO₄·7H₂O (10151)

AlanaR, BDH Chemicals (Australia) Pty. Ltd., Prices Highway, Port Fairy, Victoria, Australia, 3284.

Adenine energy charge reagents

Luciferase was prepared by dissolving 1 vial of FLE-50 in 20 mL of a solution containing (after, Lundin and Thore (1975)):

0.1% bovine serum albumin

10 mM MgSO₄·7H₂O

1 mM EDTA

(pH 7.4-7.5, using 2 M KOH)

Assay buffer was used for all sample preparations and dilutions, which contained (after, Lundin and Thore (1975)):

20 mM Tris

2 mM EDTA

(pH 7.75, using 2 M KOH)

Enzyme buffer was used for enzyme preparations (adenylate kinase and pyruvate kinase):

20 mM Tris

(pH 7.75 using 2 M KOH)

ATP, *ADP* and *AMP* were dissolved (2 mM) in assay buffer. 3 mL aliquots were administered into 5 mL plastic vials and stored frozen (-25°C). Vials were thawed and diluted, as required.

Phosphoenolpyruvate was dissolved (2.5 mM) in assay buffer. 1mL aliquots were administered into 5 mL plastic vials and stored frozen (-25°C). Vials were thawed and diluted, as required.

Software

Cricket Graph 1.3.2: Cricket software, Malvern, PA, USA

Excel 5.0: Microsoft Corporation, One Microsoft Way, Redmond, WA, USA

Ultrafit 3.0: ©Biosoft, 37 Cambridge Place, Cambridge, UK

A.2 Equipment

Centrifugation

Two centrifuges were used throughout this study:

'Easyspin' bench-top centrifuge, Sorvall® Instruments DuPont, DuPont Company, Biotechnology Systems Division, Wilmington, DE 19898, USA. (used in section 3.3: determination of adenylate energy charge)

Beckman J2-21 M/E Centrifuge, Beckman Instruments Inc., Spinco Division, 1050 Page Mill Road, Palo Alto, CA 94304, USA. (used in preparation of washed cell suspension, Appendix A.3)

Luminometer

Autolumat LB 953, EG & G Berthold, Wildbad 75323, USA.

pH meter

Orion pH meter 250A (Orion Research Inc. Boston, MA 02129, USA) with Activon AEP 433 flat tip probe (Activon Scientific Products Co. Pty. Ltd., 2A Pioneer Avenue, Thornleigh, PO Box 505, Pennant Hills, NSW, Australia, 2120).

Pipettors

A range of fixed and variable volume pipettors were used:

Fixopet: 100 µL (fixed), 1000 µL (fixed); Pluripet: 200-1000 µL, Kartell Spa Via, Delle Industrie, 1 20082 Noviglio, Milan, Italy.

Pipetman®: 20-200 µL, 200-1000 µL, Gilson Medical Electronics (France) S.A., B.P. 45-95400, Villiers-le-Bel, France.

Oxford Macro-set: 5-10 mL, Oxford Labware, Division of Sherwood Medical, St Louis, MO 63103 USA.

Spectrophotometry

Spectronic 20, Spectronic Instruments, Inc., 820 Linden Avenue, Rochester, NY 14625, USA.

Spectronic 20 1/2" test tubes, Milton Roy Company, USA.

Temperature gradient incubators

Two models of Advantec temperature gradient incubators were used:

Advantec TN.3, Advantec, Toyo Roshi International, California, USA. (required constant temperature room for isothermal operation)

Advantec TN-2148, Advantec MFS, Inc., 6691 Owens Drive, Pleasanton, CA 94588, USA. (capable of isothermal operation when ambient temperature is fluctuating)

Thermometer

Fluke® 51K/J (John Fluke Mfg. Co. Inc., 1150 W. Euclid Avenue, Palatine, IL 60067, USA) electronic thermometer with Iron-Constantan thermocouple bead probe. Calibration was checked periodically at 0°C and 100°C.

Waterbaths

Ratek SWB20D shaking waterbaths, Ratek Instruments Pty Ltd, Unit 1/3 Wadhurst Drive, Boronia, VIC, Australia, 3155.

Water activity meter

Aqualab CX-2, Decagon Devices, Inc., PO Box 835, Pullman, Washington 99163, USA.

A.3 Methods

Gompertz equation

The Gompertz equation describes a sigmoid curve that may be fitted to microbial growth data. The form of the Gompertz equation used throughout this thesis was:

$$\log N_{(t)} = A + D \exp\{-\exp[-B(t - M)]\}$$

where:

- t is time
- $N_{(t)}$ is population density at time t
- A is the initial level of bacteria
- D is the number of log cycles of growth
- M is the time when exponential growth rate is maximal and B is related to the slope of the curve at M such that:
 $\frac{BD}{e}$ is the slope of the tangent

Generation time and lag phase duration can be calculated from Gompertz parameters fitted to turbidity data, such that (after, McMeekin *et al.*(1993)):

$$\text{generation time} = \frac{66.59}{BD}$$

$$\text{lag time} = M - \frac{1}{B} + \frac{\log N_{(0)} - A}{\frac{BD}{e}}$$

Maintenance of cultures

E. coli SB1 was maintained on nutrient agar slopes, *P. halodenitrificans* on nutrient agar slopes containing 6% NaCl and *H. elongata* on nutrient agar slopes containing 8% NaCl. All were stored at 4°C and subcultured once every 2 months. Later *E. coli* SB1 was maintained at -70°C, on the surface of sterile plastic beads held in a 2 mL screw-cap vial.

Washed cell suspension

Washed cell suspensions were prepared by growing cultures to early stationary phase in nutrient broth. Cells were then harvested by centrifugation at 6000 rpm for 15 minutes. The supernatant was poured off and a loopful of culture was inoculated into fresh minimal medium (containing glucose) in side-arm flasks. This culture was monitored turbidimetrically (540nm) to mid exponential phase (~40%T), cells were then harvested by centrifugation as above. The supernatant was removed and the cells washed by resuspending in minimal medium (without glucose), followed by a third centrifugation step and resuspension in fresh minimal medium (without glucose).
