THE EFFECT OF TEMPERATURE UPON THE GROWTH AND METABOLISM OF AEROMONAS HYDROPHILA AND LACTOBACILLUS PLANTARUM IN PURE AND MIXED CULTURE.

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This is dedicated to my mother because I said I would, but principally because she taught me courage in the face of adversity.

THE EFFECT OF TEMPERATURE UPON THE GROWTH AND METABOLISM OF *AEROMONAS HYDROPHILA AND LACTOBACILLUS PLANTARUM IN PURE AND MIXED CULTURE.*

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Abstract.

The effects of temperature upon the growth and metabolism of pure and mixed populations of *Aeromonas hydrophila* and *Lactobacillus plantarum* were studied. Initially a medium was developed to provide unbiased support for both organisms. The effect of temperature upon lag phase, growth rate, and final population level between pure and mixed culture was investigated. Temperature effects were only found to be significant when comparing the final population levels of *Lb. plantarum* between pure and mixed culture. The lactobacilli exhibited a homofermentative to heterofermentative switch between pure and mixed culture. This was probably due to substrate competition from the aeromonad population in mixed culture

The metabolism of *Aer. hydrophila* has not been well described in the literature, compared to that of the lactobacilli. Due to the simplicity of the growth medium it was possible to determine the substrates relatively easily, although quantification required amino acid analysis. It was found that the organism utilized amino acids as primary substrates, switching to available carbohydrate as the population moved from growth to stationary phase. The principal product was found to be urea. During the stationary phase of population development it was interesting to note that the pH of the medium increased to well above the starting point of around 5.8. This was principally due to de-amination of the urea product. Growth temperature above recognized optimum (28°C) was found to affect the metabolic profile of this organism, leading to low final pH levels.

The pattern of temperature effect upon the metabolism of *Lb. plantarum* as expressed by growth yields showed a similar pattern to the final population levels. The ratio of lactate formed : dextrose utilized was reversed at the 10°C point. Growth of *Lb. plantarum* was not detected at the 5°C point.

A new third order polynomial model was developed to describe the lag phase of bacterial cultures across a temperature range. The new model was compared with two others from the literature. The new model was chosen based upon statistical results. The pattern exhibited by final population levels at the different temperatures showed a similar point of inflection to that expressed by the polynomial lag phase model. The growth rate was modeled with the Schoolfield model which was proven to be the closest estimate of the three models tested.

The theory of homeoviscous adaptation was used to explain the behavior patterns observed.

CONTENTS.

1.0 INTRODUCTION.

1.1 MICROBIAL GROWTH AND FOOD.

Food represents a nutrient rich environment capable of supporting the growth and development of a wide range of micro-organisms. The physical structure and chemical composition are crucial components of the natural selection process, combining to determine which species can develop.

The structure of food plays an important part in microbial development on foods. The tissues of plant and animal origin provide physical, and occasionally chemical, barriers to microbes [Boddy and Wimpenny, 1992]. A principal difference between plant and animal tissues is the ability of plants, whilst still healthy, to produce suberized periderm following damage. This provides continued protection from microbial development even after harvest. Animal tissues rapidly degrade following death, releasing more nutrient as a result of increased membrane permeability and offering less resistance to microbial colonization.

The physicochemical properties of a food are extremely important in determination of microbial colonization. Factors such as pH, temperature, partial pressures of various gases, and water potential can be modified and controlled by food processors to inhibit the development of a microbial community.

The pH level is of utmost importance to the development of any micro-organisms and can be used as a preservative. The pH of food products normally varies from neutral to acidic but can vary enormously on the microbial scale [Robinson *et al*, 1991]. The ability of bacteria to cause fluctuations of pH around small colonies of cells represents an area of concern to the food industry since colonies of otherwise inhibited pathogenic organisms may then develop in these zones [Robinson *et al*, 1991].

The concentration of hydrogen ions is of extreme importance since it affects the transmembrane potential of microbial cells and thus the performance of the organisms [Bulthuis *et al*, 1993]. Lactic acid bacteria are used to help prepare and preserve foods

such as salami, or sauerkraut giving distinctive organoleptic qualities to the foods which are not obtainable by any other technique. The initial pH of the substrate in these situations is of great importance to the end product. Ragout *et al* [1994] showed that the environmental pH could affect the fermentation balance of *Lactobacillus reuteri*. This has great significance since this organism is a component of the starter culture used to prepare sour dough. Disturbing the balance of the acetate : lactate ratio produced by this organism may result in poor product development and allow the growth of harmful organisms [Ragout *et al*, 1994]. Thus pH regulation of foods during and after preparation is extremely important to the development of microbial populations and their control.

Temperature is often used to extend the shelf life of foods by inhibiting microbial activity. Temperature has become increasingly important in food spoilage over recent years due to the public demand for less chemical preservation and fresher food. It is not completely understood how low temperature affects microbial cells although much work has looked at this effect [Gounot, 1991; Taillandier, *et al*, 1996]. It was proposed by Wiebe *et al* [1992] that growth at reduced temperature caused an increase in the energy demand of endogenous metabolism, suggesting an overall decrease in cellular efficiency at reduced temperatures. This may also be linked to the findings of Russell [1984, 1989] who showed that one facet of temperature adaptation was alteration of the fatty acid composition of the lipid membrane component. This process has been termed homeoviscous adaptation since it regulates the membrane fluidity and, consequently, the membrane function [Parola, *et al*, 1990; Ter-Kuile, *et al*, 1992]. Although homeoviscous adaptation has been used to describe the effects of fluctuating temperature upon the adaptation of microbial communities it has not yet been possible to incorporate this concept into the explanation of temperature effects upon growth rates or final population levels of microbial communities in foods.

Modified atmosphere packaging has also become increasingly important owing to the demand for lower levels of chemical preservatives in foods. Various modified atmospheres are used. The gases most prevalent are nitrogen (N_2), oxygen(O_2), and carbon dioxide (CO_2). Nitrogen is used as a filler to prevent the package collapsing as CO_2 dissolves into the product [Hotchkiss, 1988]. Oxygen maintains the fresh colour of the meat and inhibits the growth of anaerobic food poisoning organisms [Dixon and Kell, 1989] but does not usually increase the shelf life [Hotchkiss, 1988]. Carbon

dioxide inhibits the growth of a number of microbes and this has found increasing use in the preservation of foodstuffs from bacterial spoilage [Dixon and Kell, 1989]. Vacuum packaging has also been used to increase the shelf life of foods. The results of this packaging technique are thought to be caused by an increase in pCO_2 due to respiration of the meat and/or microbial activity [Enfors *et al*, 1979].

The inhibitory effect of CO_2 was first reported by Pasteur and Joubert [1877] and confirmed to be organism specific by Buchner in 1885. Buchner [1885] was able to demonstrate that CO_2 caused inhibition of Koch's vibrio whilst growth was obtained with *Salmonella typhi* [Dixon and Kell, 1989]. The site of action of CO_2 upon cells seems to be the membrane. Increased pCO_2 may cause changes in cell morphology, glucose uptake rates and amino acid absorption [Dixon and Kell, 1989]. One factor contributing to the growth-inhibitory effects of CO_2 has been alteration of membrane properties [Sears and Eisenberg, 1961]. The phase transition hypothesis [Lee, 1976] proposes that lipids form a gel phase anulus around functional membrane proteins and anaesthetics, carbon dioxide in this case, 'melt' the rigid lipid [Roth 1980]. There are also theories relating to the action of anaesthetics upon the membrane proteins [Dixon and Kell, 1989]. These theories describe a sequence of complex interactions between various electrostatic sites upon the membrane and the anaesthetic molecule. The conclusion of these theories relates to a change in or replacement of the annular lipid. Thus the membrane is still the site of action. If one were to apply Occam's razor[1] to these theories, those relating to alterations in membrane lipid composition would be selected due to the greater simplicity of the actions they describe.

Although carbon dioxide has the greatest effects upon a microbial population the inhibitory action is not so much due to control of the total microbial population as to the combination of two factors. 1) Increased pCO_2 extends the lag phase and reduces the growth rate of microbes which cause the most rapid deterioration of the product, and 2) elevated pCO_2 selects for lactic acid bacteria which are amongst the most CO_2 tolerant organisms [Dixon and Kell, 1989] and are known to have an antagonistic effect upon other bacteria [Schroder *et al*, 1980].

Thus, modified atmosphere packaging can be used as a control technique to limit microbial spoilage of food but is most effective when used in combination with

other preservative techniques, such as reduced temperatures [Eklund and Jarmund, 1983]. This is because those organisms which are not susceptible to an elevated pCO_2 may not be capable of growth at reduced temperature, such as the mesophilic *Lactobacillus* spp.

1.2 MODEL TYPES.

Use of the word 'model' in science indicates some form of representation of the real world [Laing, 1986]. Often this form is a mathematical equation or group of equations although there are more physical scientific models. Several different types of model have been used throughout this investigation. The discussion of model types will be limited here to those classes proposed by Laing [1986], table 1.1, which are pertinent to this study.

The first step in any model development is a breakdown of the subject to be studied into basic components [Laing, 1986]. This allows simplification of the system to the most relevant components. In the study of microbial food spoilage this initial step may be the development of a suitable medium and experimental apparatus designed to represent the original food environment, or the selection of an organism interacting with an environmental factor to be studied under a predefined set of conditions. These represent interpretive analogue models of the relevant features of the micro-organisms ecological niche. The usefulness of the model can be estimated from a comparison between population development in the laboratory and *in situ*. Such a comparison normally involves the use of statistics in order to provide impartial quantitative judgements however, the various population distributions used for the purpose of statistical analyses are themselves based upon assumptions which may also be tested. Each statistical analysis is dependent upon the relevance of the test to the system and it's model. Unfortunately each statistical analysis is rarely applicable to two different. This sequential testing soon reaches a point where further analysis can yield nothing of value and so comparison is usually limited to one or two different

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[1]Occam's razor, written several hundred years ago, may be translated from the Latin to read "entities should not be multiplied without necessity".

approaches. It is at this point that the step from analogue to computational model is made.

It is necessary to model the subjects environment in order to allow control of the relevant parameters, thus allowing investigation of the effects of varying each parameter individually or simultaneously, but always under control. It is the extreme complexity of an ecological niche which makes this step so important to the study of microbial ecology.

Isolation of the most relevant factors is not always obvious. Each bacterial species occupies a niche which can be termed an n-dimensional hypervolume [Wimpenny, 1981]. This volume is the physicochemical environment which exerts influences upon the development of the organism. It is multidimensional since each factor, such as pH, salinity, or population density, may be considered to exert some form of influence upon the overall population development and thus, represents a dimension. The complexity of the n-dimensional hypervolume represents the primary source of error in any ecosystem model. As previously stated, the first stage of model construction is simplification to the most relevant parameters. In an n-dimensional hypervolume all parameters must have some relevance, the degree of relevance depending upon the region within the volume under study. Thus the first stage of the model, resulting in simplification of the hypervolume (a reduction in n), must introduce error into the final result. The effect of each dimension upon each bacterial cell is currently un-measurable and so these effects are estimated from studies of microbial population development under controlled conditions. It is possible to estimate the error from comparisons between expected and observed parameter estimates however, only if the initial assumptions are correct will the error be low. The actual error estimate will vary from one experiment to the next and so only large differences between expected and observed parameter values indicate problems with the model set up.

The model types presented in table 1.1 are principally concerned with mathematics. The role of mathematics in microbiology has been increasing for many years. There is a great deal of interest in the development of mathematical models in the field of predictive microbiology [Hudson, 1992; Kell and Westerhoff, 1989; Buchanan and Phillips, 1990; Fernandez, *et al*, 1996]. Although the effects of physicochemical factors such as pH upon microbial populations has long been

recognised the availability of computers has, until recently, made objective studies upon these factors extremely complex. The increase of desktop computing over the last decade has led to an impressive collection of mathematical models [Fernandez, *et al*, 1996; Zwietering *et al*, 1992a; Palumbo, *et al*; 1991; Peters *et al*, 1991] describing the interactions between many variables and microbial species. As a direct result of an increased ability to rapidly manipulate large quantities of data the physical models have also become more complex.

Table 1.1 Principal model classifications from Laing [1986] with direct relevance to microbiology.

Type of Model	Comment	Example
IA Interpretive Analogue	Model is unlikely to physically resemble the original but is likely to give an analogous effect.	Experimental medium and/or apparatus.
CA Computational Analogue	These models are concerned with mathematical simulation and use simple mathematics.	The straight line relationship between time and the logarithm of an exponentially growing population.
CM Computational Mathematical	Mathematical estimation based upon observation and mathematics which are not especially complicated.	The determination of the coefficients of an equation for a population growth model by analysis of observations.
PM Pure Mathematical	Mathematical models using pure and applied mathematics.	Analysis of lag time determination by differentiation of the Gompertz growth equation [Zwietering *et al*, 1992a]

1.3 MICROBIAL GROWTH AND MATHEMATICAL MODELS.

The fact that temperature has an effect on microbial growth has never been in question. Much attention has been focused on microbial adaptation to varying temperature and how the adaptation affects microbial growth. Thus much of the

current research in the field of microbial food spoilage has been aimed at determination of the relationship between environmental factors, such as temperature, and the development of a microbial population [Hudson, 1992; Palumbo *et al*, 1991; Zwietering *et al*,1991 & 1992a]. This has resulted in a greater application of ecological concepts to microbiology than previously considered [Boddy and Wimpenny, 1992]. This has lead to consideration of microbial growth in terms of space and time in order to allow discussion of interactions between populations and, incorporation of the habitat, as a dynamic system, into the overview [Boddy and Wimpenny, 1992]. It can be seen from the example of Spanish green olive fermentation that all these factors are relevant to the study of microbial ecology. The substrate (olives) has a natural microflora which develops with time. This results in changing environmental conditions associated with the metabolism of differing microbial communities and time. The final result of olive fermentation is a complete suppression of undesirable Gram negative organisms by the environmental changes, principally a reduced pH value, caused by the development of a population of lactic acid bacteria (LAB) [Bobillo, 1991].

This raises the consideration of metabolic interactions between communities. There have been many studies which discuss how any one organism may develop on a single substrate in a controlled environment [Oxenburgh and Snoswell, 1965; van der Kooij and Hijnen, 1988; Stecchini, *et al*, 1993], which represents the classical microbiological approach. There are fewer studies describing the catabolites of these metabolic processes and even less discussion of successive community development, beyond acknowledging the effects of a change in pH. Inclusion of each additional factor can represent a considerable increase in the logistical problems of any investigation however, it should be remembered that they represent intrinsic characteristics of the microbial environment. Unfortunately, the very nature of the n-dimensional hypervolume that is a microbes environmental niche [Wimpenny 1981] means that we will probably never build a truly comprehensive model [Laing, 1986]. This proposes another problem since a complex model that is only half understood is a very dangerous tool predicting behaviour which can only be seen to be irrational after comparison with real data [Laing, 1986]. Thus a model which only approximates the real world may be better than one which fits accurately but fails only after confidence

has been established [Laing, 1986] provided that the limitations of the first model are known.

In opposition to these concepts are the ideas of resolution. A mathematical model should display the underlying trend without being affected by unimportant fluctuations in the prototype. This means that a sufficiently large number of data points are available for the model construction without taking readings after insignificant steps in the measured parameter. However, the decision on what size step is highly subjective and, when planning to construct a multivariate model the step size tends to increase since the number of experiments required rises exponentially with the number of parameters tested. This can lead to insufficient resolution of the model resulting in unreliable predictions of system behaviour.

Different approaches have been used in attempts to counter this logistical problem. Palumbo *et al* [1991] selected x factors and made growth measurements at y values of each factor. This meant that x^y experiments were conducted. The selection of the value for y was extremely important as it controlled the resolution of the data and, in this case, the proposed response surface model. This question of resolution is highly important. It is possible to connect two points, A and C, by a straight line but, only if the relationship is truly straight would a midpoint value, B, be found to lie upon this straight line. Thus the level of detail associated with each environmental variable is of vital importance to the final relationship, and the conclusions made therefrom.

Another approach was taken by Peters *et al* [1991] who utilised image analysis to measure the effects of four environmental factors varied simultaneously. With this technique it was possible to measure the growth of a microbial population, on a solid surface, at 37 values each for two of the factors and 6 values each for the remaining two factors, yielding 49,284 data points. Although impressive this approach was limited to the use of solid media and high population densities. The population density required for examination by this technique is irrelevant to the food environment and the approach itself does not allow for the study of mixed microbial communities.

De-resolution of the problem is an approach founded upon the fact that biology may be considered as a set of inherently complex systems which should be simplified whenever possible, if only because the scientific peer community expects it [Garfinkel, 1984]. This may have led to oversimplification of the subject areas being modelled. Occam's razor has been applied to argue that a simple model may be appropriate to

explain one set of experimental data, but may not be suitable to predict the reactions of a complex system [Garfinkel 1984]. The real problem is determination of sufficient or insufficient complexity. The metabolic control theory of Kacser and Burns [1973] and Heinrich & Rapoport [1974], helps go some way to resolving this debate by giving a logical balance between Occam's razor and complete consideration of all metabolic anastomoses within a living cell.

1.3.1 Modelling of Microbial Growth.

Brock [1984] defined growth as an orderly increase in all the cellular constituents and structures of an organism. In prokaryotic organisms growth results in cellular division and so an increase in population size. Studies of microbial population growth cycles have led to mathematical descriptions of the growth curve. The term mathematical model is applied to a set of equations which describe a prototype. The prototype is a representation of the system under study rather than the system itself [Salmon and Bazin, 1988]. The most basic microbial growth description (equation 1.1) gives a straight line equation for the increase in cell number as a function of time.

$$\frac{dX}{dt} = \mu \cdot X$$

Equation 1.1 A straight line description of the relationship between cell number (X) and elapsed time (t). μ is the specific growth rate constant [Brock *et al*, 1984].

Equation 1.1 is regarded as simplistic because it considers only the exponential growth phase of the population cycle, more complex models have been developed containing expressions to describe the lag and stationary phases (equation 1.2). The model most often referred to in the literature is that of Gompertz [1825].

$$y = a \cdot e^{[-e^{b-ct}]}$$

Equation 1.2 The Gompertz equation [Gompertz, 1825] which describes population growth (y) throughout the complete growth cycle. a, b, & c are mathematical parameters, t is time and e is the base of Napierian logarithms.

Until recently simplistic models held great attraction for researchers due to the lack of numerical analysis required to obtain growth parameter estimates however, the rapid development and power increase in small computers has led to a much wider use of the more complex models since the computer can perform the necessary regression in minutes, if not seconds. The increased computational power of desk top computers has led to more objective data gathering and an increase in the application of predictive modelling techniques to microbiology [Garfinkel, 1984]. For example, growth parameters are now usually determined by algorithmic regression of models, such as equation 1.2, onto experimental data, rather than the more traditional chartist estimates based on the opinion of the observer.

The Gompertz model (equation 1.2) was modified by Zwietering *et al* [1990] to replace the mathematical parameters with parameters conveying a biological meaning (equation 1.3).

$$y = A \cdot e^{[-e^{\frac{\mu e}{A}(\lambda - t + 1)}]}$$

Equation 1.3 The modified Gompertz equation [Zwietering *et al*, 1990]. L is the lag period (hours), μ is the specific growth rate, and A is the final population increase. t is the elapsed time (hours) and e is the base of Napierian logarithms (ln). y is the natural logarithm of the increase in population size following deduction of the initial population level.

Although this reparameterization of the original model appears to increase the complexity of the description it allows more rapid extraction of the data required and improves the statistical validity of that data [Zwietering *et al*, 1990]. Other sigmoid functions have been compared with this model and it was found that in almost all cases

the Gompertz model can be regarded as the best model to describe population growth data [Zwietering *et al*, 1990].

The fitting of growth models to population growth data allows rapid extraction of the growth parameters with mathematical objectivity however, such studies are only relevant to the particular conditions tested and as such they are of limited value for predictive modelling [Baird-Parker & Kilsby, 1987]. However, in spite of the large amount of microbial population growth data produced there is surprisingly little suitable for the purpose of predictive modelling. This is due to the wide range of prototype systems used for practical investigation of micro-organisms over the years [Baird-Parker & Kilsby, 1987]. This, and the variation between strains has led to a situation where new and old data may be compared but, short of making conclusions based upon general trends, there are too many inestimable variables, such as strain variation and medium composition, to allow direct assimilation of new and old data into one model.

The first step in modelling microbial food spoilage, as with all model types, is dissection of the system to generate a prototype containing only the factors known to be important [Laing, 1986]. This prototype may then be used to investigate the effects of varying one or more parameters upon the test organism. These effects may be seen as either a change in the population growth profile or metabolism.

1.4 THE ORGANISMS.

Selection of organisms for use in mixed culture studies may be considered a 'black art'. This stems from the enormous complexity of microbial ecosystems. As mentioned previously, microbial environments are dynamic n-dimensional hypervolumes. The structure, shape and dynamics of the hypervolume will be different for each species of organism. It is for these reasons that the early microbiologists found it necessary to isolate pure cultures in order to characterise these 'wee animalcules' as Leeuwenhoek described them in the 1680's [Brock *et al*, 1984]. These early requirements of microbiology have themselves been cultured such that mixed culture is something of an anathema to many modern microbiologists.

The principal reasoning behind these opinions may be considered thus:

> Microbial ecosystems have an extremely complex structure. The complexity is such that use of two organisms in mixed culture would provide less accurate results than pure culture studies, since final error values are the product of their components. More important than this, the use of polyxenic cultures can only provide information about the ecosystem function if one includes at least all major species present. Thus, monoxenic culture studies only 'fog' the understanding of these complex systems.

The selection of organisms for mixed culture studies is, therefore, a very difficult subject to quantify. In these times one must not only consider the relevance or application of the organisms to the system under study but also, one must consider the likelihood of funding for the proposed project. Fortunately, this last may, to a certain extent, be determined from a study of the literature which will contain references to all the most important areas of study, although the results of such an inquiry may not be to the taste of all.

The organisms chosen for this study reflect all these ideas. The candidates were chosen to reflect two distinct groups one might expect to find on any food type. The first, a lactic acid bacterium (LAB), was selected because of a ubiquitous nature and an involvement in many food fermentation processes [Bobillo, 1991]. Secondly, this organism was already under study at these laboratories and so there was already in existence an information base for the further study of this organism. The second, a Gram negative rod, was selected because this species may also be considered as ubiquitous and has been associated with food borne disease and opportunistic infection [Annapurna and Sanyal, 1977; Burke, *et al*, 1984b; Sorvillo, *et al*, 1989; Kirov, *et al*, 1990; Hinton and Bale, 1991; Schubert, 1991; Singh, *et al*, 1997]. During the development of the project this second organism, *Aeromonas hydrophila*, attracted attention because little of the physiology and metabolism of the organism had been elucidated and it appeared to be of increasing importance to the food industry.

1.4.1 *Lactobacillus plantarum.*

The genus *Lactobacillus* contains an heterogenous assemblage of organisms comprising Gram positive, nonmotile, chain forming rods. Many species have been classified as homofermentative, including *Lactobacillus plantarum*, but some are heterofermentative [Brock et al., 1984]. Lactobacilli have often been found in dairy produce, some strains being utilised to produce fermented foodstuffs such as kefir. The use of lactobacilli in food fermentations should not be underestimated, the majority of species being involved in at least one fermented product. The wide applications of this genus to the food industry is a result of both their acid and salt tolerance [Kandler & Weiss, 1986, Bobillo & Marshall, 1991]. These same qualities are also an important factor in food spoilage by lactobacilli, resulting in spoiled cheese [Pette & Beynum, 1943], and various spoilage factors in meats [Egan 1983].

These organisms have been utilised for hundreds of years, their metabolism within food sources being utilised as a means of increasing storage life and improving the organoleptic qualities of the product [Schillinger & Lucke, 1989]. They are not considered as pathogenic. Lactobacilli may be readily isolated from the gut of many animals, have been associated with mortality in salmonid fish [Shotts 1991] and one species, *Lb. jensenii*, has been isolated from human vaginal discharge and blood clot [Kandler & Weiss, 1986]. Indeed their presence in the intestine of humans and animals has led to the investigation of the use of preparations, containing various members of the genus, as an aid to recovery after gastrointestinal infections [Rehm 1983], although evidence of any beneficial effects has yet to be found and this area remains a controversial topic [Lauer *et al.*, 1980].

As a result of the enormous economic value represented by this group of organisms there is a large effort being made to understand LAB in general. Although once again there is little of this effort concentrated upon interactions with other organisms, a surprising fact since most of the fermentations carried out are performed by a mixed culture containing several types of LAB and, in the case of spoilage, perhaps more than 1 group of organisms [Kennes *et al.*, 1991] such as LAB spoilage of beer where the dominating population would be yeast.

Thus a great deal of attention has been focused on lactobacilli and their use in mixed culture fermentation of foodstuffs. These facts and the ubiquitous spread of *Lb. plantarum*, and other lactobacilli, throughout many environments makes this organism an ideal candidate for a mixed culture study.

Interactions of these organisms with the environment have been studied [Bobillo 1991; Suutari & Laakso, 1991] but, as indicated earlier, there is comparatively little information available on interactions with other micro-organisms [Kennes *et al.*, 1991; Weber, 1986] most of which has recently been concentrated on the effects of bacteriocins produced by a range of LAB [Foegeding *et al.*, 1992; Meisel et al., 1989; Schillinger & Lucke, 1989] upon other organisms associated with food spoilage and poisoning. This study has considered the effects of temperature upon interactions between *Lb. plantarum* and a Gram negative rod, *Aeromonas hydrophila*.

1.4.2 *Aeromonas hydrophila.*

Aeromonads are Gram negative rods divisible into two groups on the basis of motility. They exhibit extreme nutritional versatility. Little detail is known of the metabolism of these bacteria. Some strains are pathogenic and these seem to be limited to *Aeromonas hydrophila* and *A. sobria*, two of the motile species [Olivier *et al.*, 1981].

Aeromonads have long been associated with amphibians and fish [Gray 1984] but it is only in the last twenty years that *A. hydrophila* has been recognised as an opportunistic pathogen [Ketover *et al.*, 1973] and increasingly over the last ten years this organism has been associated with systemic infections in apparently healthy individuals [Trust & Chipman, 1979]. Almost all cases being after some form of water contact [Gray 1984]. The most frequent cases reported are acute diarrhoeal disease in several countries [Trust & Chipman, 1979] causing increasing concern as to the responsibility of this organism for food borne disease. Investigations into these infections have not been able to clearly identify the sources although several possibilities have been raised [Kirov *et al.*, 1990]. *A. hydrophila* has been isolated from many different sources around the world, including metropolitan drinking water

[Burke *et al.*, 1984b], and some slightly surprising sources such as a colonic irrigator seized by the Los Angeles Police Department [Sorvillo *et al.*, 1989].

Clearly, there is abundant evidence to indicate the potential public health significance of this organism and a large effort is being made to determine the implications of these findings. In spite of this however, there is a great paucity of knowledge concerning the metabolic behaviour of the organisms in this group. It has been suggested that this organism prefers fatty acids and amino acids as primary growth substrates over carbohydrates such as glucose [van der Kooij & Hijnen, 1988]. This is not especially surprising when one considers the natural environment of *A. hydrophila* where all nutrients normally exhibit low concentration levels and the competition for any nutrient must be high. Another physicochemical factor to the organisms advantage is the broad temperature range (4°C - 42°C) across which it can develop, with more than 50% of strains capable of initiating growth at 4-5°C [Payton 1987]. When considered with the toxigenic and widespread nature of this organism, its ability to grow on such a wide variety of substrates across such a wide temperature band should cause concern, especially when so little detailed information is available.

It should also be borne in mind that although this organism has been ubiquitous throughout the environment for a great many years it has only recently caused concern and, in spite of a great deal of attention, it is still not considered to be a high risk pathogen. In spite of this, the lack of knowledge and potential pathogenicity, when considered with the ubiquitous nature of the organism, make this bacterial species an ideal companion to *Lb. plantarum* for use in this study.

1.5 MIXED CULTURE.

Mixed culture is something of an anathema to many microbiologists. For many workers it represents an unnecessary increase in experimental complexity which should be avoided [Bergman, 1979]. In the food industry however, mixed cultures are frequently used [Weber 1986; Payton 1987] although the natural ecosystems that are the products represent a complex subject of study, especially at the microbial level. The main difficulties stem from the numerous interactions between the environment and the

microbes and the different species of microbe. The complexity of these interactions reduce any useful analysis of the system development. The simplest answer is to remove as many of the nonessential complexities as possible, for example reduction of the number of different micro-organisms to as low as possible, resulting in a system with only one complex variable, the growth and development of a population of one species of organism in a controlled environment. The simplest answer is not however, always the best answer. By the use of a mixed culture containing two organisms (termed monoxenic) it is possible to determine interactions between organisms [Kennes *et al.*, 1991], what outcome this will have on the environment, and how the environmental changes will feedback to affect the behaviour of the organisms. This is not possible with the classical pure culture (axenic) approach. By taking two separate organisms and monitoring their development individually it is assumed that a reasonable prediction of the behaviour of an identical system containing both organisms can be made, without the need to determine what the organisms have released into the environment to produce the monitored effects, such as a decrease in pH level. Time represents an intrinsic factor of all growth experiments and has more relevance in the monoxenic culture than the axenic. The rate of O_2 depletion caused by an initial large population of organism A may create conditions advantageous for development of organism B in a matter of hours in a monoxenic culture, as opposed to days for organism B in axenic culture. This time component could not even be estimated in an axenic sub-system. Axenic cultures show us how an organism has adjusted the population development due to environmental conditions but give little or no indication of how that organism will relate to the development of other species around it in mixed cultures. Monoxenic cultures simulate a larger part of the complex natural system and readily allow a reliable interpretation of the reactions that occur and a better understanding of the trophic relationships between micro-organisms without the need to recourse to much more complex polyxenic models.

1.6 AIMS OF THE INVESTIGATION.

The investigation was intended to research the impact of temperature on the population development of the two selected organisms with the aim of producing a useful model to describe the population interactions with the environment and each other.

The approach taken was to break the final target into component pieces and construct a foundation of knowledge from which the more complex mixed culture environment could be approached. This was done in several steps :

- Selection of a simple but suitable growth medium and temperature range.
- Pure culture investigation of the impact of temperature on the development of each population.
- Use of HPLC analysis to determine the effect of temperature on the metabolism of the organisms.
- Mixed culture investigation of the impact of temperature on the development of each population and a comparison with the pure culture observations.
- Interpretation of the mixed culture results based on the conclusions from the pure culture investigation.
- Development of a mathematical model to describe the relationship between observed interactions and the effects on population dynamics.

It was intended that a comprehensive model to describe expected metabolic interactions would be developed and incorporated with the model describing temperature effect upon population development.

2.0 METHODS AND MATERIALS.

2.1 MICRO-ORGANISMS.

2.1.1 Isolation of organisms.

Organisms used throughout this project were obtained from Oxford Brookes University Culture Collection. The first, *Aeromonas hydrophila* (OBUCC) B184, was originally isolated from chilled chicken at Oxford Brookes University. The second, *Lactobacillus plantarum* (OBUCC) L388, was isolated from fermenting green olive brine [Bobillo, 1991].

2.1.2 Maintenance of Cultures.

Pure cultures of both species were maintained as frozen stock on glass beads at -70°C according to the method of Jones *et al* [1984]. On the first day of each month a fresh glass bead from frozen stock was taken and resuscitated in either nutrient broth (LabM) medium (*Aer. hydrophila*) or MRS broth (LabM) medium (*Lb. plantarum*) at 30°C for 16 hours. The broth culture was then streaked onto agar and similarly incubated at 30°C for 16 hours. Resuscitated cultures were maintained at 4°C on nutrient agar (LabM) for *A. hydrophila* and MRS agar (LabM) for *Lb. plantarum* for 1 month.

2.1.3 Inoculum Preparation.

The resuscitated agar cultures were inoculated into the relevant broth medium and grown in a static incubator at 30°C. The population development was monitored

by turbidimetry in a spectrophotometer (Cecil CE272) at 650nm. When the turbidity had reached a level of between 0.4 and 0.5 units the population was judged to be in late exponential phase at a level of 10^8 cfu/ml. The culture was then diluted to provide a suspension containing of the order of 10^5 cfu/ml which was used for inoculation of the growth vessel(s) resulting in an initial population of approximately 2×10^3 cfu/ml.

2.2. CULTURE CONDITIONS.

2.2.1 Growth Media.

A. hydrophila inoculum and resuscitation cultures were grown in nutrient broth (LabM).

The modified MRS medium, termed GMRS, used in initial growth studies contained mixed peptones (10g/l), yeast extract (5g/l), beef extract (10g/l), glucose (2g/l), potassium phosphate (2g/l), sodium acetate (5g/l), triammonium citrate (2g/l), magnesium sulphate heptahydrate (0.02g/l), manganese sulphate tetrahydrate (0.05g/l). Pure cultures of both organisms were also grown in brain heart infusion (LabM) supplemented with potassium nitrate (10 - 0.05 g/l) and/or glucose (20 - 5 g/l) where indicated.

Pure and mixed culture experiments were carried out in a developed medium containing acid hydrolysed casamino acids 10 g/l (Sigma), yeast extract 3g/l (LabM), dextrose 5g/l (BDH) and supplemented with filter sterilised Rogosa salts after steam sterilisation. This medium was referred to as DECAYES.

2.2.2 Environmental Conditions.

2.2.2.1 Temperature Control.

The effect of temperature on the growth of both organisms in pure culture was investigated using screw capped 250ml. conical flasks each containing 20 ml. of

experimental medium. This allowed for maximum surface area: volume ratio, thus encouraging efficient exchange of gases between the medium and the head space. One sample was taken at each time point from each of two duplicate vessels. The flasks were grown either in a static cooled incubator (Astell Scientific JBF051), or in a static cooled water bath across a range of temperatures from 5°C to 30°C. Initial experiments were carried out at 30°C in either static (Astell Scientific JBF051), or shaken incubators.

2.2.2.2 pH Measurement and Control.

A sterilisable gel filled glass pH probe (Fermprobe) connected to an LH500 pH controller module (LH Engineering) was used to determine pH levels. The pH was adjusted, where required by the addition of 1.0 M NaOH (BDH).

2.2.3 Continuous Culture.

Lactate catabolism by *A. hydrophila* was investigated using continuous culture. The DECAYES medium (section 2.2.1) was supplemented with lactic acid across a range of concentrations from 0 to 25 mM and filter sterilised. Temperature was controlled at 25°C and pH maintained at 5.8. Control and monitoring of the chemostat was performed by a Nimbus computer (Research Machines) linked to an Anglicon Biosolo 2 controller (Control Techniques). The dilution rate was maintained at 0.15 hr^{-1} using a peristaltic pump (Watson-Marlow).

2.3 CELL CONCENTRATION DETERMINATION.

2.3.1 Viable Count.

Viable count estimations were performed on the cultures by dropping suitable dilutions onto the surface of an appropriate agar plate and spread across the surface with a glass spreader. The samples were diluted in Ringers solution (LabM). A volume of 0.1ml. of the diluted suspension was placed onto the agar plate by Gilson pipette. Estimation of viable count was expressed as colony forming units per ml.

Lactobacillus plantarum samples were taken using a U100 insulin syringe with a 28G needle. This was to ensure break up of chains formed by this organism. In mixed culture two 1ml samples were taken. The aeromonad sample was taken by pipette, and the *Lactobacillus* sample was taken by syringe. This was necessary to prevent forces in the syringe needle damaging the shear sensitive aeromonad.

The solid media used for the viable counts of the organisms were selected for the ability to inhibit the growth of either organism. *Lactobacillus plantarum* was cultured on MRS agar (LabM). *Aeromonas hydrophila* was cultured using a selective medium described by Palumbo *et al* [1985a]. This medium comprised phenol red agar base 31g/l (Difco), soluble starch 10 g/l (BDH). After sterilisation (appendix 2) the medium was cooled to below 50°C and ampicillin was added to achieve a concentration of 10µg/ml.

2.3.2 Preparation of Cell Free Extract.

The internal concentration of urea was determined by the inorganic analysis of a cell free extract. After harvesting the cells were centrifuged at 4000 rpm for 15 minutes in a cooled centrifuge set at 4°C. The supernatant from this sample was discarded into hypochlorite solution. The remaining cell pellet was resuspended in 9ml. of acid Ringers solution. This suspension was then transferred to a Mickle disintegrator vessel containing Ballotini beads. The vessel was then shaken in a Mickle disintegrator for 15 minutes. The vessels were then left to allow the beads to settle.

5ml of the supernatant was then removed and centrifuged as previously to remove any cell debris. The resulting supernatant was then filter sterilised and frozen at -20°C for future analysis.

Prior to analysis the supernatant was thawed and passed through a mixed ion exchange resin containing 50/50 of Amberlite IRA-93(OH) and IRA-120(H). This was to remove any contaminant salts which might affect the chemical reaction used to quantify urea, which has neutral charge. Dilution factors were calculated from analysis of identically treated controls containing known quantities of urea.

2.4 ANALYTICAL METHODS.

2.4.1 Glucose Concentration Determination.

2.4.1.1 Rapid Method.

This technique involved diluting a centrifuged (4000 rpm for 15 minutes) and 0.1μm filter sterilized sample 1:1 in deionised water. A drop of diluted sample was placed on a BM-Test Glycemie blood glucose strip (Boehringer Mannheim). This was left to react for 60 seconds and excess medium was removed with a medical wipe. The strip was then left in air for a further 60 sec to develop. Following development the strip was inserted into a Reflolux-S glucose meter (Boehringer Mannheim) and the glucose concentration determined in mmol/L.

2.4.1.2 High Performance Liquid Chromatography (HPLC).

Spent medium analysis was performed on each sample by HPLC using an aminex HPX-87H column (Biorad) at 40°C in a liquid chromatography oven (Anachem). Sulphuric acid diluted in HiPerSolv water (BDH) to 0.01N was used as the mobile phase at a flow rate of 0.6ml min^{-1}. over 20 min. The loop volume (50μl) was exceeded by a factor of 4 according to the overfilling technique described by

Rheodyne injection valves instruction manuals. Prior to injection each sample was diluted 50% v/v. Metabolites were detected by a refractive index detector (Bischoff model 8100) connected to a Nimbus computer (Research Machines) operating under Gilson 712 HPLC system control software.

2.4.2 Nitrite.

The nitrite assay used was the modified Griess-Ilosvay method from Vogel [1961]. Adaptations were made to allow for smaller sample volumes. Samples were prepared by pelleting cells at 4000 rpm for 15 minutes in a Denley refrigerated bench top centrifuge.

The prepared sample was chilled on ice for at least 10 minutes before the assay. At time zero, 0.2ml of reagent A (sulfanilamide 0.5g (Sigma) dissolved in 100ml of 20% HCl (Sigma)) was added and mixed. After five minutes incubation on ice 0.2ml reagent B (N-(1-napthyl)-ethylenediamine dihydrochloride 0.3g (Sigma) dissolved in 100ml of 1% HCl) was added and mixed. After a further 10 minutes the absorbance of the chromophore was read at 550nm against a blank prepared in the same way. Prechilling was necessary to stabilise the diazo-reaction between the sulfanilamide and the nitrite. Insufficient chilling results in incomplete dye-coupling and the chromophore decays in approximately 10 minutes. The timings involved in this reaction were extremely important to the accuracy of the test.

It should be noted that the sample used should not contain more than 4.0 mg $NaNO_2$ per litre

2.4.3 Ammonia Determination.

This assay was based upon Sigma Diagnostics procedure number 170-UV. The method involves the reductive amination of 2-oxoglutarate, using glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide (NADH). The

decrease in absorbance at 340nm due to oxidation of NADH is directly proportional to the ammonia concentration in the sample.

The purchased kit was found to give inaccurate results owing to the presence of lactate dehydrogenase in the reagent solution. The assay used therefore excluded this enzyme and was made up of 2-oxoglutarate 2mM (BDH), NADH 0.12mM (Sigma) dissolved in phosphate buffer at pH 7.4 [Cruickshank *et al.*, 1975]. A blank (deionised water) and control were included with each assay. The assay was carried out at 30°C using semi-micro silica cuvettes (Abinghurst) and a heated cuvette holder in the spectrophotometer (Cecil CE 272) at 340 nm. 1 ml. of reagent solution was added to each cuvette, then 0.066 ml. of diluted sample and mixed by gentle inversion. This was followed by temperature equilibration for 5 minutes and the first absorbance reading was taken (A_1). After equilibration 0.0066 ml. of enzyme solution (1200 U/ml of L-glutamate dehydrogenase (Sigma)) was added and mixed. The cuvettes were left to react for 10 minutes and the absorbance reading taken again (A_2). The test was considered accurate if an ammonia control solution (5µg/ml) (Sigma) was +/- 10% of the true value. Calculation of the ammonia present was as follows:

$$A_1 - A_2 = A_3$$

$$Factor = \frac{((vol\_in\_cuvette) \times 17)}{((vol\_in\_sample) \times 6.22)}$$

$$A_3 \times Factor = [NH_3](\mu g/ml)$$

where 17 = Weight (µg) of 1µmol. of ammonia, and 6.22 = millimolar absorbtion of NADH at 340nm.

2.4.4 Urea Concentration Determination.

2.4.4.1 Enzyme Assay.

This method was adapted from the assay outlined in section 2.4.3 and Sigma Diagnostics procedure number 67-UV.

The base reagent comprised 2-oxoglutarate 0.01M (Sigma) and NADH 0.35mM (Sigma) made up in phosphate buffer at pH 7.4 [Cruickshank *et al*, 1975]. A blank consisting of deionised water and control consisting either ammonia control solution 5µg/ml (Sigma), or glucose/urea nitrogen combined standard 10mg/dl urea concentration (Sigma) were used in each assay. A 1:1 mixture of both control solutions was also used. The reaction was carried out at 30°C.

A 1ml. aliquot of base reagent was placed in each semi micro silica cuvette (Abinghurst). Additions of 30µl. of blank, control, or sample were made and mixed by gentle inversion, followed by 5 minutes temperature equilibration. The absorbance reading (A_1) was then taken. Next was added 6µl. of the GLDH solution and mixed by gentle inversion. The absorbance was then monitored until a constant reading was obtained (30-50 minutes). The absorbance reading at this time was noted as A_2. A final addition of 40µl. jackbean urease 50 U/ml (Sigma) was made and mixed. After 30 seconds the absorbance was measured (A_3), and again after a total of 60 seconds (A_4).

This procedure allows calculation of ammonia levels in the sample according to section 2.4.3.1. Quantification of urea is based upon the following calculations:

$$A_2 - A_4 = \Delta A$$

where A_3 is a check on the linearity of the reaction.

$$\left(\frac{\Delta A/\min(sample)}{\Delta A/\min(control)}\right) \times [control] = [U]$$

where $[U]$ and $[control]$ are the urea and control concentrations in mg/dl.

2.4.4.2 Physical Assay.

A physical chemistry method for the quantification of urea was developed based upon the following chemical reaction.

$$CO(NH_2)_2 + 2HNO_2 \rightarrow 2N_2 + CO_2 + 3H_20$$

The reaction must be carried out in the presence of dilute HCl [Vogel, 1969]. Samples must be prepared as follows:

A mixed bed ion exchange resin, comprising 50/50 H^+ and OH^-, was used to remove all interfering ionic compounds. A 0.5ml sample was added to 0.5ml of the required nitrite solution (concentration dependent upon urea levels present). This was then incubated overnight with 9ml of dilute HCl to ensure that the reaction goes to completion. Following this the nitrite assay is performed as stated in section 2.4.2.1. The quantity of urea present was then calculated from a calibration curve of A_{550} against urea concentration and taking into consideration all dilution factors.

2.4.5 Dissolved Carbon Dioxide.

This assay was based upon the oxidation of NADH in the following reactions:

$$PhosphoenolPyruvate + HCO_3^- \xrightarrow{PEPC} Oxaloacetate + H_2PO_4$$
$$Oxaloacetae + NADH \xrightarrow{MDH} Malate + NAD$$

where PEPC = phosphoenol pyruvate carboxylase, and MDH is malate dehydrogenase.

Samples (1ml.) were removed from the growth vessel and the cells pelleted in a microfuge (13000g/30 seconds) as described in the Sigma diagnostics procedure number 131-UV.

Each assay consisted of a blank (deionised water), control, and a sample. Silica semi micro cuvettes (Abinghurst) were used in a cuvette holder heated to the assay temperature (30°C). To each cuvette was added 1ml. of the sample start reagent containing phosphoenol pyruvate 2.2mM, NADH 1.32mM, magnesium ions 10mM, PEPC (plant) 275U/l, MDH (porcine heart) 1540U/l in a buffer at pH 8.0. To this was added 10µl of blank, control, or sample and mixed gently by inversion. The cuvettes were then left to incubate for a period of 6 minutes. After this time the absorbance of all three cuvettes at 380nm was read. Carbon dioxide content was determined by the following calculation:

$$\frac{A_{blank} - A_{sample}}{A_{blank} - A_{stnd}} \times [Stnd.] = [CO_2]$$

where stnd = standard and [] is concentration (mM).

2.4.6 Organic Acids.

Quantification of organic acids was achieved by using the aminex HPX-87H column (Biorad) utilised for glucose analysis. This is a general column for carbohydrate and organic acid analysis. Combined with the refractive index detector used for glucose it was possible to quantify organic acids in the presence of glucose using the same separation and analysis methods as those in section 2.4.1.2.

2.4.7 Amino Acids.

Amino acid analysis was carried out by Mr. J. T. Griffiths of the University of Wales College, Cardiff using an Alpha amino acid analysis system (LKB Pharmacia).

2.5 NUMERICAL ANALYSIS.

2.5.1 Growth Parameter Estimation.

Growth parameters were determined using the software package Regress published by Blackwell Scientific. The growth data was entered as ln (N/N_0) with corresponding time points, where N is number of cells/ml at time t, and N_0 is number of cells/ml at time zero. The model chosen to estimate the growth curve was the modified Gompertz [Zwietering *et al*, 1990], equation 1 below.

$$y = A \cdot \exp^{\{-\exp^{[\frac{\mu_m \cdot e}{A}(\lambda - t)+1]}\}}$$

Equation 1. Where $y = \ln(N/N_0)$, μ = the specific growth rate (hr^{-1}), A = the final population level ($\ln(N/N_0)$), λ = the lag period (hours), and t = time (hours). The parameters were calculated by Regress using a Marquardt algorithm in an iterative process.

2.5.2 Growth Parameter Models.

The growth parameters chosen to compare effects of extrinsic factors upon the two organisms in pure and mixed cultures were μ, the specific growth rate, and λ, the lag period. Two models were selected to estimate each of these parameters. One from the literature and one developed during the course of this project in each case. Comparisons were made between the results for these two growth parameters and the effects of temperature upon the metabolism and final population density of *Lb. plantarum*.

2.5.2.1 Lag Phase.

The lag phase model from the literature is an hyperbolic function used widely in the literature [Davey, 1991; Zwietering *et al*, 1991]. It may be expressed as:

$$\ln(L) = C_0 + (C_1/T) + (C_2/T^2)$$

Equation 2. Where L = lag period (hours), T = temperature (°C), and C_0-C_2 are coefficients to be estimated.

The model developed in this project is a third order polynomial. The model may be expressed as:

$$\ln(L) = a + bT + cT^2 + dT^3$$

Equation 3. Where L = lag period (hours), T = temperature (°C), and a - d are coefficients to be estimated.

These models were fitted to the data in the Regress package where goodness of fit is estimated by the sum of squares and the standard deviation.

2.5.2.2 Growth Rate.

The model selected from the literature to estimate the effects of temperature upon the growth rate of the organisms was that of Ratkowsky, *et al*, 1982, with the general form:

$$\mu = [b(T - T_{min})]^2$$

Equation 4. Where μ is the specific growth rate, b is a Ratkowsky parameter ($°C^{-1}h^{-0.5}$) and T_{min} is the minimum temperature at which growth is observed (°C).

The model used to describe the relationship between growth rate and temperature for the purposes of this investigation was an asymmetric sigmoid curve (equation 5).

$$\mu = \mu_{min} + \frac{\mu_{max} - \mu_{min}}{(1 + (\frac{T}{T_i})^{-p})}$$

Equation 5. Where T_i is the inflection point of the asymmetric sigmoid curve (°C), μ_{min} and μ_{max} are the minimum and maximum growth rates ($hour^{-1}$), respectively, p is a regression constant.

These models were fitted to the data in the Regress package where goodness of fit was estimated by the sum of squares and the standard deviation.

2.6 STATISTICAL TESTS.

2.6.1 F Test.

The F-test was used to determine whether the two sample variances were drawn from populations with the same variance. This decides which of two methods are used for a t-test analysis of the data in the two samples. If the two samples are not homoscedastic then their ranges are significantly different. This may have repercussions when considering the effect of temperature upon growth rates in pure and mixed culture.

The F-test was performed by a comparison of $F_{(calc)}$ with $F_{(crit)}$. $F_{(calc)}$ was determined from equation 6.

$$F_{(calc)} = \frac{S_1^2}{S_2^2}$$

Equation 6. Where s_1 and s_2 are the two sample variances and $s_1^2 > s_2^2$

$F_{(crit)}$ is found from F distribution tables. The relevant table is selected by deciding upon the percentage significance level, e.g. 5%. The value of $F_{(crit)}$ is found by selecting the column headed by the degrees of freedom of s_1 and moving down the column to the row corresponding to the degrees of freedom of s_2. Generally, a one-tailed F-test is performed [Yeomans, 1982].

2.6.2 t Test.

The t test for sample means usually stipulates that the two populations are homoscedastic, although this is not an absolute requirement. Consequently these two situations require slightly different forms of the t test. The critical value for t is found from a table of the t distribution using $(n_1+n_2)-2$ degrees of freedom.

2.6.2.1 t Test For Non-homoscedastic Populations.

This is the simpler of the two forms of the t test and is shown by equation 7.

$$t_{(calc)} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2})}}$$

Equation 7. t test for non-homoscedastic populations.

2.6.2.2 t Test For Homoscedastic Populations.

This is the more complex of the two forms since it requires two calculations. The first, equation 8, is a preliminary to the actual test which has been shown in equation 9.

$$\sigma_{\bar{x}_1-\bar{x}_2} = \sqrt{(\frac{\sum(x_1-\bar{x}_1)^2 + \sum(x_2-\bar{x}_2)^2}{n_1+n_2-2}(\frac{1}{n_1} - \frac{1}{n_2}))}$$

Equation 8. Preliminary calculation for the t test on a homoscedastic population.

$$t_{(calc)} = \frac{\bar{x}_1 - \bar{x}_2}{\sigma_{\bar{x}_1 - \bar{x}_2}}$$

Equation 9. Final calculation for the t test on a homoscedastic population.

2.6.3 The χ^2 Test.

This test was used to examine the possibility of any significant difference between the frequency of occurrence of several categories in two or more samples. The comparison is achieved by using equation 10 to calculate the $\chi_{(calc)}^2$ statistic which is then compared with a theoretical value of χ^2 from a statistical table at the relevant level of significance.

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Equation 10. Where O is the observed frequency and E is the expected frequency.

2.6.4 Fisher's Z Transformation.

This test allows the comparison of an actual and a theoretical statistic value between -1 and +1. It is a logarithmic function which is approximately normal even though the sample may be small. It was originally developed by Professor Ronald Fisher, for whom it has been named, and was designed to compare the value of the regression coefficient r with the theoretical population coefficient of 1 or -1. In order to be used in this investigation the statistic compared was the standard deviation of the proposed model from test data obtained from the literature. This was compared to a theoretical population standard deviation of zero. The Z test was calculated from equations 11, 12 and 13 below. Equation 11 allows calculation of the standard error

of the normally distributed Z. Equation 12 was used to calculate Z_1 and Z_2 values. Z_1 represents the mean of the distribution based upon the standard deviation of the model from the data, and Z_2 represents the mean of the standard deviation (assumed to be zero) of the population from the data.

$$\sigma_z = \frac{1}{\sqrt{n-3}}$$

Equation 11. Where σ_z is the standard error of the Z distribution.

$$Z_a = \frac{1}{2} \cdot \ln\left(\frac{1+\sigma}{1-\sigma}\right)$$

Equation 12. Where σ may be either the determined value for the model fit or the proposed theoretical value. In each case the result may be termed Z_1 and Z_2 respectively for use in equation 13.

$$Z_{(calc)} = \frac{\sigma_z}{Z_1 - Z_2}$$

Equation 13. Determination of $Z_{(calc)}$ for comparison with tabulated values of Z.

The value of Z(calc) and Z(tab) were compared to determine acceptance or rejection of the null hypothesis which stated that the standard deviation of the model from the test sample data was not significantly different from the standard deviation of the population from the sample data, which was assumed to be zero.

3.0 *LACTOBACILLUS PLANTARUM.*

Lactobacillus plantarum is a ubiquitous Gram positive, chain forming rod. It has been very widely exploited in the food industry being associated with many food fermentations, for example Spanish green olives [Bobillo, 1991], or dry salami [Weber, 1986]. As a result of its extensive industrial use a great deal of effort has been put into elucidating the effects of environmental factors, such as salt tolerance [Bobillo, 1991; Quintanilla, *et al*, 1996], upon the metabolic activity of this organism[Rozes & Peres, 1996]. Growth rates of *Lactobacillus plantarum* have been investigated by Zwietering *et al.* [1990] who have used the organism to test the applicability of several bacterial population growth models with the conclusion that the modified Gompertz equation [Gompertz, 1825; Zwietering *et al.*, 1990] was statistically sufficient to describe the growth data (chapter 6).

Metabolic studies of *Lactobacillus plantarum* have revealed that during growth studies it is vital to maintain all factors constant, with the exception of the variable in question [Murphy & Condon 1984a & b]. The effect of varying levels of aeration on cultures of *Lactobacillus plantarum* have been shown to produce different growth patterns and metabolic activities[Murphy & Condon 1984a & b]. Archibald and Fridovich [1981] observed that optimal growth of this micro-organism on glucose occurred in the absence of O_2. Since this investigation was to use a facultative aerobe in mixed culture with *Lb. plantarum* the level of aeration was a significant consideration in the light of these results.

Optimum growth temperature for this organism has been reported as 30°C [Vaughn, 1975; Fleming, 1982; Vignolo *et al.*, 1988]. For these reasons growth of this organism was initially studied utilising shaken and static cultures with a variety of media at 30°C. The primary aim was initially to maximise the growth rate and final population level of both *Lactobacillus plantarum* and *Aeromonas hydrophila* for the later mixed culture studies.

3.1 INITIAL INVESTIGATIONS.

The first medium tested was brain heart infusion (LabM) supplemented with 2% w/v glucose. Growth was measured by optical density at 650nm. Growth of *Lactobacillus plantarum* was not encouraging compared to the aeromonad and this medium was not investigated further for this reason (see figure 3.1).

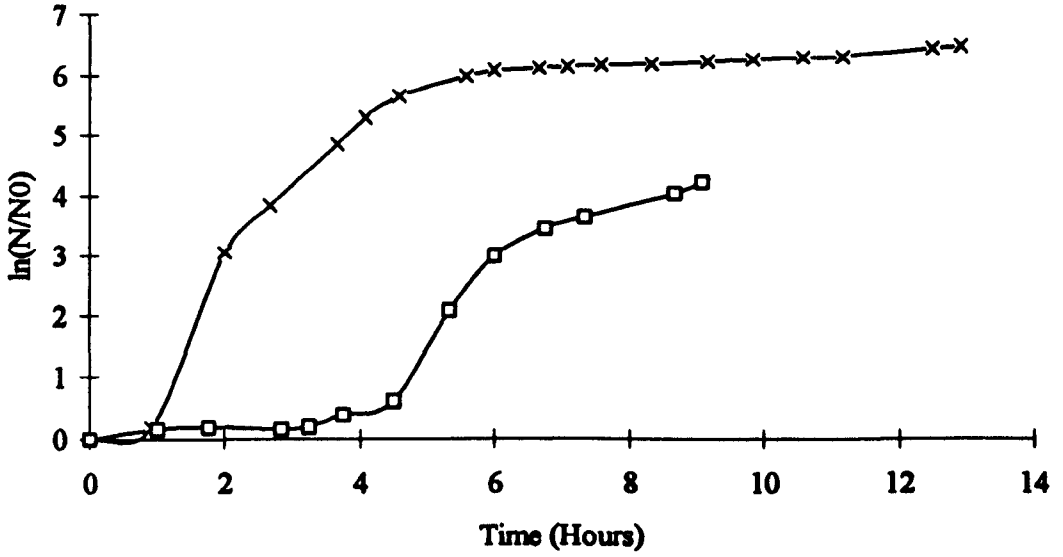

Figure 3.1 Growth of *Lactobacillus plantarum* □, and *Aeromonas hydrophila* X, in brain heart infusion with 2% w/v glucose in shake flasks at 30°C.

The next medium tested was MRS medium [de Man *et al.*, 1960], known to be highly suitable for all but the most fastidious lactobacilli [Bobillo, 1991]. This medium was found to be highly suitable for *Lb. plantarum* but only by omission of the Tween 80 component was it found to be suitable for the aeromonad. This modified medium was termed GMRS. Further pure culture investigations with the aeromonad resulted in the DECAYES medium development (see section 4.1) for determination of metabolic activity and growth parameters of both organisms in pure and mixed culture. This medium was found to be comparable to the GMRS medium (see figure 3.3) whilst containing fewer components, thus making it less complex and more economical than

the GMRS medium. Another advantage borne from the selection of DECAYES over GMRS medium was the reduction in Maillard reaction products formed during steam sterilisation. These reaction products are known to have a negative effect upon the development of an aeromonad population [Stecchini *et al*, 1991].

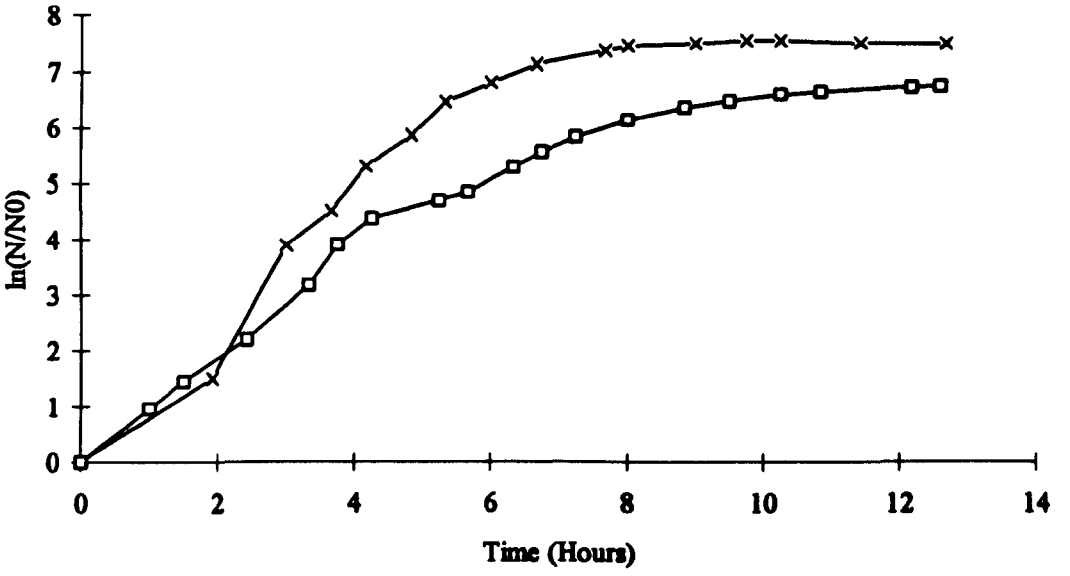

Figure 3.2 Growth of *Lactobacillus plantarum* □, and *Aeromonas hydrophila* X, in GMRS medium in shake flasks at 30°C.

3.2 SAMPLING TECHNIQUE VALIDATION.

Lactobacillus plantarum is a chain forming rod. For this reason it was deemed necessary to examine different sampling techniques in order to break up the chains for determination of viable counts by the spread plate technique described in section 2.3. The use of vortex mixers has been reported to be of use in the break up of chains of *Lactobacillus plantarum* [Zwietering *et al.*, 1991]. However to check that the shear forces generated by these vortex mixers would be sufficient to break a pair of cells into two separate, viable cells, it was decided to examine syringing the bacterial suspensions. The results of this test are shown in table 3.1.

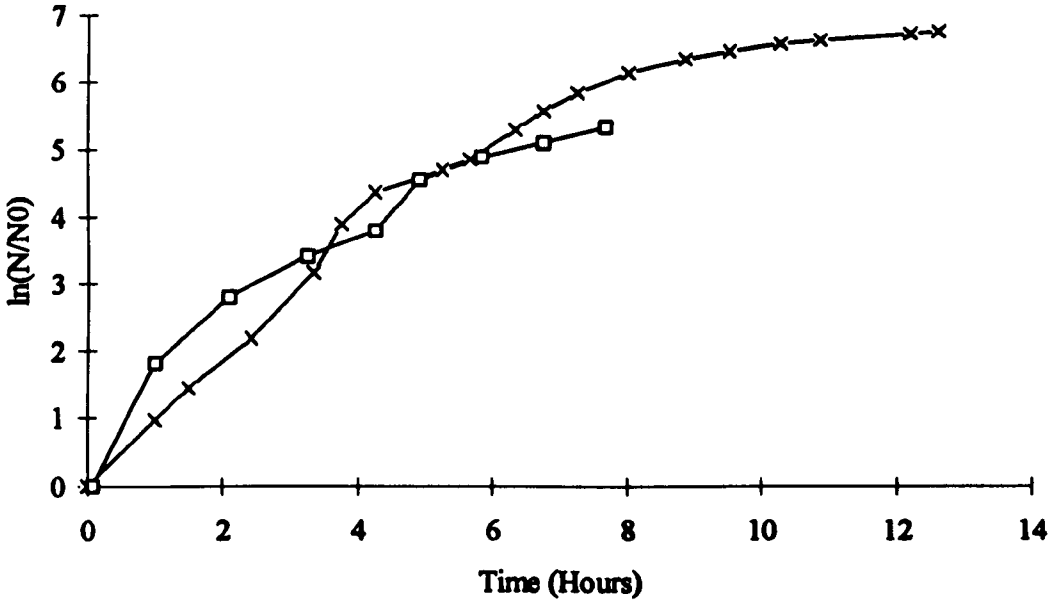

Figure 3.3 Growth of *Lactobacillus plantarum* at 30°C in GMRS medium X, and DECAYES □ medium.

Table 3.1 Frequency distribution of chain length with different treatments of a 4 hour *Lb. plantarum* culture in MRS broth grown at 30°C.

Chain Length (no. of cells)	Vortex (30 sec.)	Syringing (28G needle)
1	64	45
2	43	31
3	13	1
Viable Count (cfu/ml)	9.17×10^7	1.14×10^8

Each technique was performed upon the same culture. The resulting cell suspensions were examined microscopically using a haemocytometer (Neubauer). The frequency distribution of chain length for each technique is presented in table 3.1. This distribution was analysed using the χ^2 distribution at 5% significance (section 2.6). If $\chi^2 \geq \chi_{(calc.)}^2$ then the null hypothesis that no association exists between technique and chain length is proven. The analysis gave $\chi_{(calc.)}^2 = 6.54$ compared to the upper tail 5% critical point value $\chi^2 = 5.991$. Thus the null hypothesis was rejected.

This statistic shows, in combination with the viable counts presented in table 3.1, that the syringing technique is more likely to produce viable single cells from a chain of cells. Thus syringing was utilised for determination of *Lactobacillus plantarum* viable counts throughout this investigation.

3.3 THE EFFECT OF TEMPERATURE ON THE GROWTH OF *LACTOBACILLUS PLANTARUM.*

Initial investigations into the effects of temperature on the population development of a culture of *Lactobacillus plantarum* were effected using a cooled water bath and static flasks. Growth was monitored by optical density and converted to viable count with a calibration curve. The growth parameters are presented in table 3.2.

Table 3.2 Growth parameters (with standard errors) from initial studies of temperature effects upon cultures of *Lactobacillus plantarum.*

Temperature (°C)	Lag Time (hrs.)	Growth Rate ($hr.^{-1}$)
30	0.27 ± 0.58	0.43 ± 0.07
15	8.93 ± 0.90	0.28 ± 0.09
7	1.50 ± 25.19	-0.06 ± 0.21

In order to allow turbidometric population measurement a high initial cell concentration was used (10^7 cfu/ml). This resulted in a very short exponential phase which, combined with low sample numbers (≤ 7), lead to a poor fit of the Gompertz equation to the growth curve data. It was concluded that future studies should include more data points and start with a lower inoculum as this would improve the reliability of the fitted parameters.

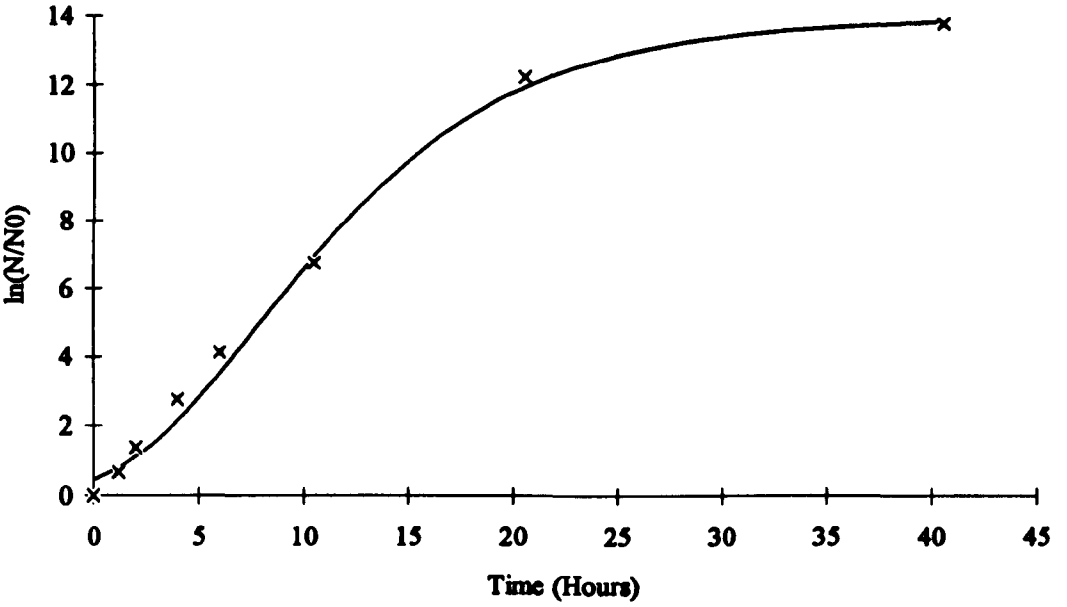

Figure 3.4 Growth of *Lactobacillus plantarum* at 30°C in DECAYES medium (x) and the fitted Gompertz equation (—).

Table 3.3 Growth parameters (with standard errors) for *Lactobacillus plantarum* at various temperatures. nd = not determined due to excessive lag period (>1100 hours).

Temperature (°C)	Lag Time (hrs.)	Growth Rate ($hr.^{-1}$)	Final Pop^{n}. Density (N/N_0)
30	1.15 ± 0.46	0.78 ± 0.06	13.98 ± 0.41
25	1.81 ± 0.95	0.48 ± 0.04	15.21 ± 2.55
20	4.85 ± 0.59	0.32 ± 0.01	15.32 ± 0.87
15	6.85 ± 2.52	0.17 ± 0.02	12.76 ± 0.54
10	0.47 ± 27.17	0.03 ± 0.01	8.77 ± 0.54
5	nd	nd	nd

Further studies of temperature effects on the growth of *Lactobacillus plantarum* were carried out in static flasks in a cooled incubator with an initial cell concentration of approximately 10^3 cfu/ml. Growth was monitored by spread plate evaluation of the viable population. The data points were analysed with the 'Regress' package (Blackwell Scientific) to provide the growth parameters in table 3.3. A

typical fit to the points is shown in figure 3.4. It was not possible to fit the Gompertz equation to the data from the 5°C growth experiment as this was below the minimum growth temperature for the organism (figure 3.5).

The estimated growth rates were compared with published growth rates [Zwietering *et al.*, 1991] (figure 3.6). The published growth rates are consistently greater than those estimated in this study. Since the two data sources for *Lactobacillus plantarum* were obtained from different media, it is the underlying trend revealing the similarity between the two data sets which is relevant.

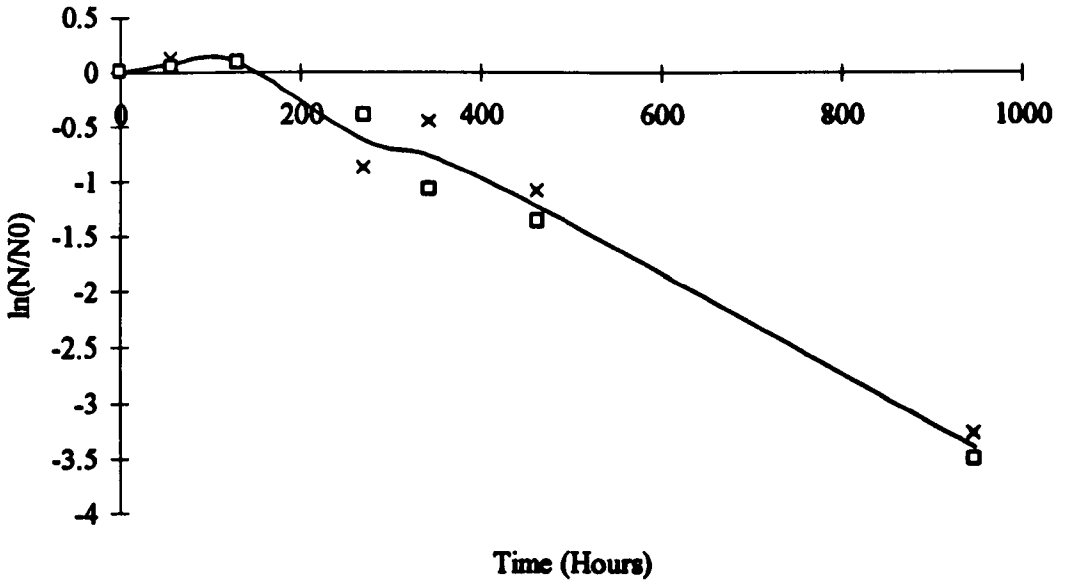

Figure 3.5 Duplicate *Lactobacillus plantarum* population development at 5°C in DECAYES medium. In both instances the population enters decline after approximately 150 hours. The line indicates the mean value of each datum pair.

The effect of temperature on the growth rate of *Lactobacillus plantarum* follows the expected hyperbolic increase as the incubation temperature is raised. The population did not develop at 5°C and went into decline between 150 and 250 hours after inoculation. The failure of the population to develop at 5°C was not unexpected given the results of the initial studies at 7°C and the published data of Zwietering *et al* [1991]. The lag time shows an inverse relationship between temperature and the rate of growth which will be discussed in greater detail in chapter 6. The lag time value

determined at 10°C has a very high standard deviation. This was due to extended time intervals between sample points at the reduced temperature causing less accurate estimations of growth parameters by the Regress software package.

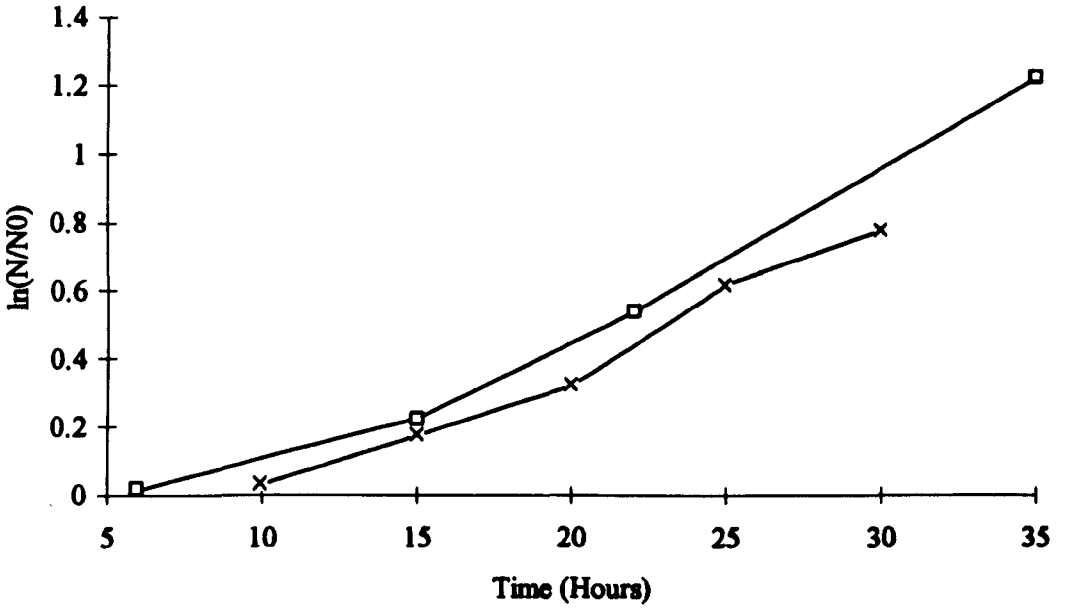

Figure 3.6 Comparison between published □, and estimated x growth rates of *Lactobacillus plantarum*. [Published data from Zwietering, *et al.* 1991]. Note the similarity of the underlying trend.

The final population densities followed a pattern with a maximum value between 20°C and 25°C, figure 3.7. This was important as it suggested that the most efficient temperature, i.e. the temperature conferring maximum level of growth on the available substrate, and the optimum growth temperature, i.e. the temperature at which the maximum growth rate was observed (30°C for *Lb. plantarum*), were different. This will be addressed further in chapter 6.

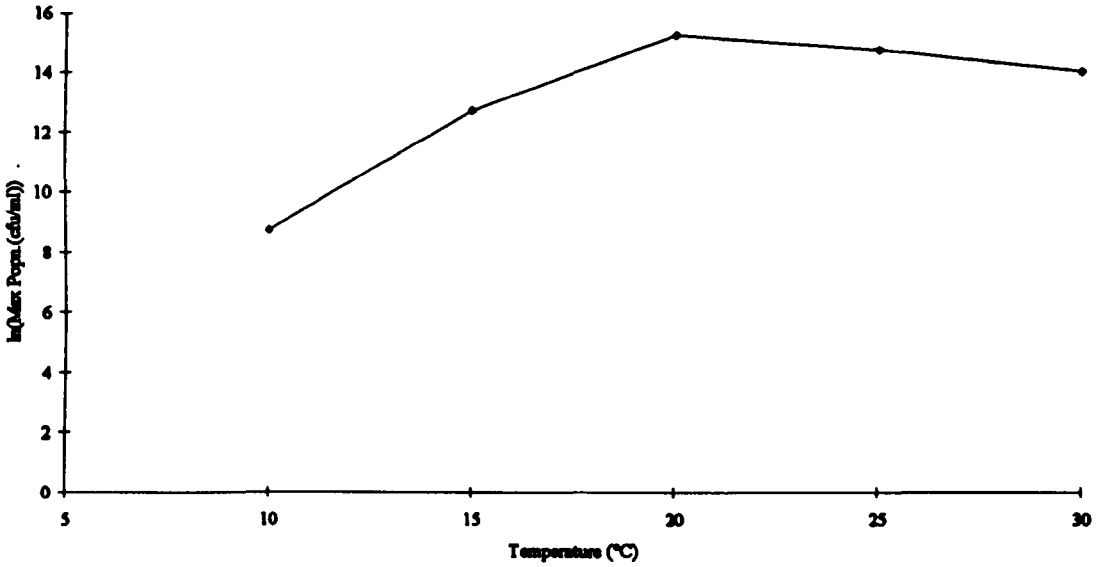

Figure 3.7 Fluctuations in final population density (ln(cfu/ml)) of *Lactobacillus plantarum* at the five growth temperatures investigated.

3.4 THE EFFECT OF TEMPERATURE ON THE METABOLISM OF *LACTOBACILLUS PLANTARUM.*

The catabolic product of *Lactobacillus plantarum* in pure culture was lactate at all temperatures above 5°C (for example 30°C, figure 3.8) and the final pH was between 3.41 (30°C) and 4.65 (10°C). At 5°C the population viability remained constant for approximately 125 hours and then declined. It was not possible to detect any metabolic activity by the HPLC analysis over the whole experimental period of 1017 hours.

It has been hypothesised by Wiebe *et al.* [1992] that at reduced growth temperatures bacteria exhibit an enhanced substrate requirement. This was important for the project since the aim was to produce a model relating bacterial growth to metabolic processes and environmental factors. The effects of extrinsic variables such as temperature upon the metabolism of the organism is therefore vital if the model were to be applied to environments such as food.

The proposal of Wiebe *et al.* [1992] implied that temperature would have to be included in the model if a non-linear relationship could be shown to exist between

metabolic activity and temperature. An analysis was performed relating bacterial dry weight to amount of dextrose utilised or lactate formed, figure 3.9, at the five temperatures which supported this organism. Comparison of final population size at the temperatures investigated, figure 3.7, yields a similar pattern to that shown in figure 3.9.

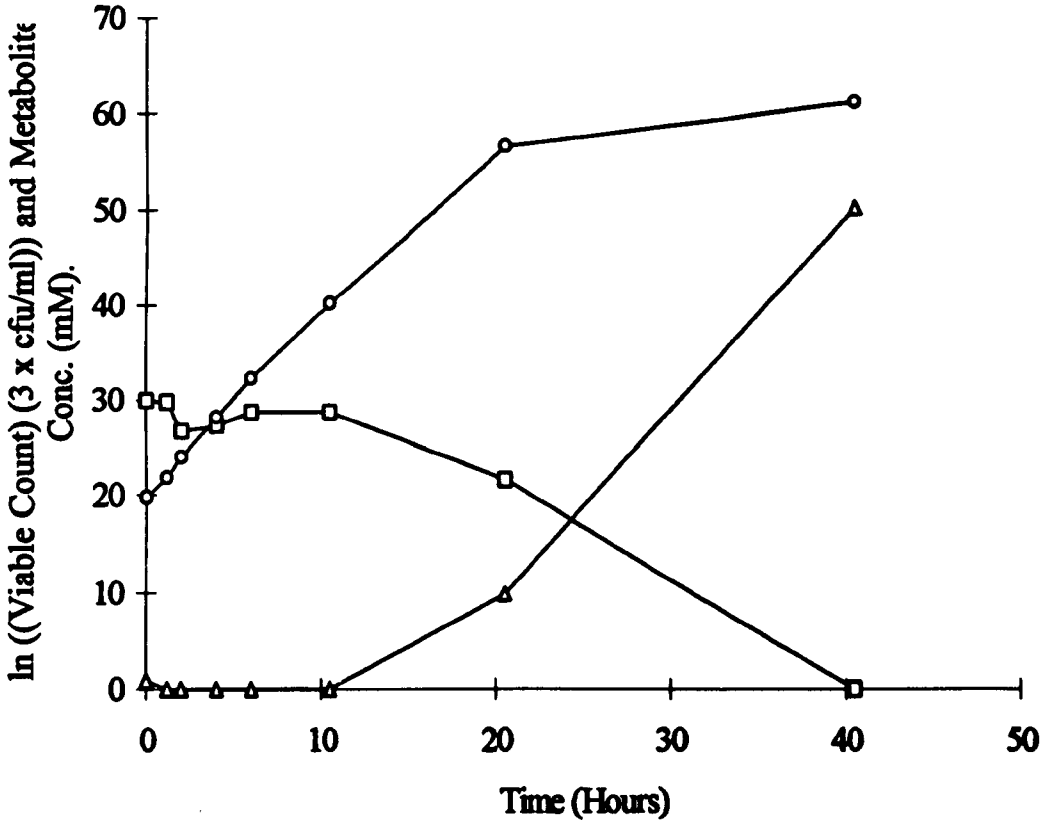

Figure 3.8 Dextrose utilisation □, and lactate production ○, with ln(viable count) Δ, of *Lactobacillus plantarum* at 30°C in DECAYES medium.

The result of this analysis supports the conclusion of section 3.3, that the most efficient temperature for growth lies within the 15°C to 25°C region. This indicates that the accepted optimum growth temperature for *Lactobacillus plantarum* (30°C as determined by growth rate studies) is not the most efficient temperature for growth. This finding will be discussed in greater detail in chapters 6, and 7.

The patterns of the population growth parameters produced in pure culture with this organism will be further analysed and discussed in chapters 5, and 6.

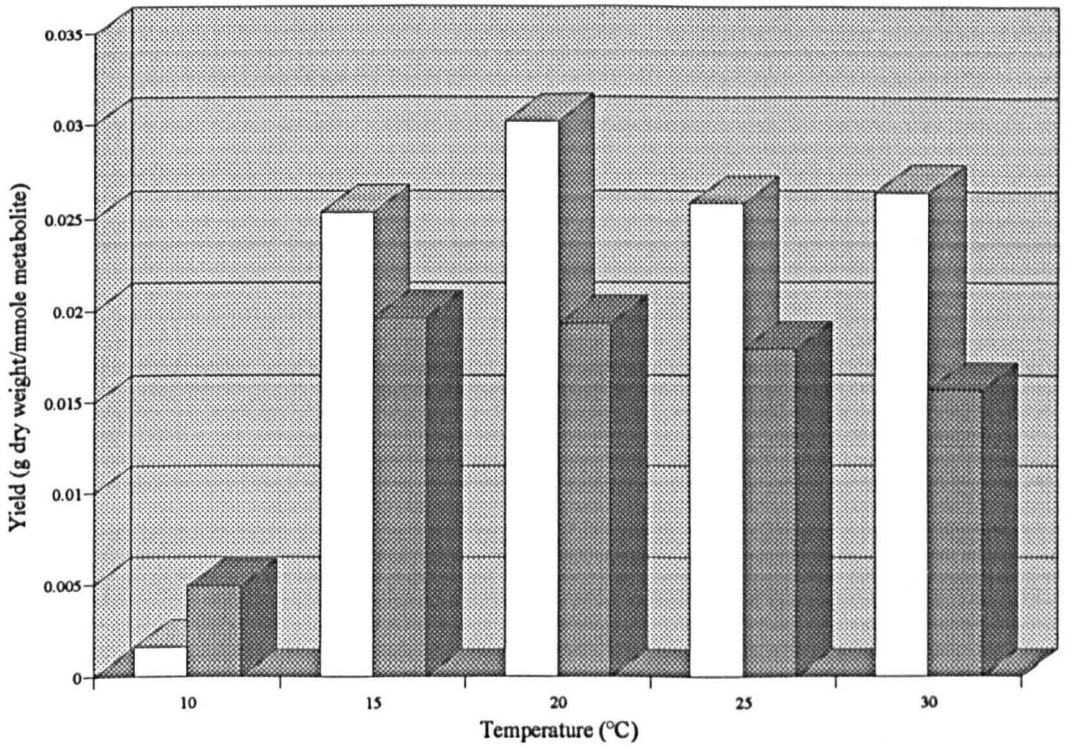

Figure 3.9 The yield of *Lactobacillus plantarum* at different temperatures. Yield presented as biomass produced per lactate formed (shaded) and dextrose utilised (gram dry weight/mmole metabolite).

3.5 CONCLUSIONS.

Growth of lactobacilli results in chain formation under the conditions used throughout this investigation. Studies of chain forming bacterial growth must therefore find the best way of disrupting the cell chain structure without reducing cell viability in order to define the viable count as precisely as possible. It was shown in section 3.2 that chain disruption by the use of vortex mixers has been improved upon. The use of a 28G needle attached to a syringe creates shear forces which have a significantly greater disruptive effect on the cell chains than those produced by a vortex mixer. The cell viability was not reduced by this technique. This more extensive chain breakage results in more accurate viable counts and therefore produces a closer fit of

growth parameters to the data obtained. It was noted that approximately 40% of the cells are left in chains of two, or more. This factor was not used to correct any data for the following reasons:

1. Such correction factors are not normally used in the available literature.
- Use of such a factor would, therefore, have made comparison between this study and published results less significant.
2. The data used was generated with a haemocytometer.
- This methodology is generally recognised as prone to operator error, thus any correction would be based upon potentially flawed data and so open to question.
- Haemocytometer counts are generally considered to be reliable when conducted by the same operator however, counts conducted by different operators usually differ.

The growth rate determined for this organism was shown to follow the same pattern as published data from Zwietering *et al*, [1992]. The differences between the published growth rates and those found in this investigation can be explained by the use of a different strain of the organism, different media and sampling techniques. Zwietering *et al.* [1990] used MRS medium which was developed to enhance the growth of lactic acid bacteria [de Man *et al.* 1960] such as *Lactobacillus plantarum*. The medium used in this investigation was developed to provide a growth environment for *Lactobacillus plantarum* which would also be suitable for the later mixed culture experiments. Zwietering *et al* [1991] used vortex mixers to disrupt the cell chains formed by *Lactobacillus plantarum* and so, by using a less efficient method for chain disruption than used in this study, found a wide range of growth parameter estimates for any given temperature. The other growth parameters found during the characterization of the behaviour of this organism in pure culture will be addressed more directly in chapter 6.

Figure 3.9 shows that when the growth temperature drops from 15°C to 10°C there is a substantial decrease in the yield per millimole of dextrose utilised. More significantly, figure 3.9 also shows that when more glucose is utilised at the lowest temperature (10°C) the relative amount of lactate produced decreases. This implies that a much greater percentage of the dextrose carbon skeleton is being otherwise utilised, since HPLC analysis would have revealed the presence of other likely acidic

end products such as acetate. The metabolism of this organism has been well described in the literature and it assumed that the missing carbon has been consumed in energy yielding metabolism (e.g. aerobic metabolism $\Rightarrow CO_2$). The hypothesis of Wiebe *et al.* [1992] states that reduced temperature causes an increase in the maintenance energy demand. The results here support this since there is a decreased growth yield in spite of greater substrate utilisation with a decrease in product formed. Further evidence in support of this hypothesis was found from the yield and final population size estimates which show that the lower temperature growth curves have a much lower final population level per unit of dextrose utilised. More interesting than this result was the apparent decrease in growth efficiency as the environment temperature was increased to the recognised optimum value for this organism (30°C).

The fatty acid composition of the bacterial cell membrane is known to fluctuate in response to environmental variation [Aaronson and Martin, 1983; Suutari and Laakso,1992]. Suutari and Laakso [1992] studied the effects of temperature on the fatty acid composition of *Lactobacillus fermentum*. They found that dihydrosterulic, oleic, and vaccenic acids showed the greatest variation with temperature. The published data [Suutari and Laakso,1992] showed a pattern similar to that observed here for the metabolic temperature adaptation of *Lactobacillus plantarum*. The growth rate of *Lactobacillus fermentum* continued to increase up to the recognised maximum after the pertinent fatty acid compositions had begun to plateau, approximately 10°C to 15°C earlier. Whilst it is not inconceivable that a relationship exists between the published observations [Suutari and Laakso,1992] and those presented here, a great deal more work should be done in this area before any significant conclusions are made.

4.0 *AEROMONAS HYDROPHILA.*

Organisms from the *Aeromonas* group have received recognition as agents of food-borne pathogenesis of concern to the food industry, public health officials, and consumers [Palumbo, *et al.*, 1991]. *Aeromonas hydrophila* is known to cause a variety of extra-intestinal diseases in man [Freij, 1987]. Although epidemiological and toxicological studies have suggested a link between this organism and gastroenteric disease, challenge studies have failed to confirm it [Morgan, *et al.*, 1985]. It has been reported that *Aeromonas hydrophila* has a ubiquitous nature and is capable of growth at refrigeration temperatures [Hudson, 1992; Pin, *et al*, 1996].

Aeromonas hydrophila was found on virtually all samples of fish, seafood, red meat and poultry tested by Palumbo, *et al* [1985a]. Callister and Agger [1987] reported that *Aeromonas hydrophila* was recovered from eleven out of twelve types of grocery store produce sampled. There is some evidence relating gastrointestinal disorders with the presence of psychrophilic strains of *Aeromonas hydrophila* in drinking water [Schubert, 1991]. The ability of this organism to grow at reduced temperatures has been widely reported in the literature [Eddy, 1960; Stecchini, 1993; Santos, *et al*, 1996]. The presence of this organism in food after spoilage at low temperature implies that this species is capable of competitive growth at refrigeration temperatures [Palumbo *et al.*, 1985]. As consumers demand food with less processing and fewer additives there is an increased emphasis on the use of temperature for food preservation; however, the ability of some micro-organisms to grow at 5°C (a temperature previously thought suitable to inhibit bacterial growth on foods [Palumbo *et al.*, 1991; Pin, *et al*, 1996]) has focused attention on the multifactorial approach [Palumbo *et al.*, 1991].

It has been reported that three strains of *Aeromonas hydrophila* isolated from three different sources (cooked mussels, milk, and a clinical isolate respectively) were predicted to behave differently when challenged with a multifactorial approach [Hudson 1992]. In contrast to this, Pin *et al* [1996] could discern no significant

difference between the behaviour of food and clinical isolates. The ecological history of the organism appears to influence both the thermal range and the salt sensitivity expressed in vitro by *Aeromonas hydrophila* [Stecchini, 1993]. This is an important result as it may help resolve the apparent incongruities found between the ubiquitous nature of the organism and the low number of clinical conditions to which it has been linked.

4.1 PRELIMINARY INVESTIGATIONS.

The aim of the project was to develop a model of mixed culture interactions, thus the ability to demonstrate that the growth profiles were reproducible was an inherent requirement. Reproducible growth of *Aeromonas hydrophila* was achieved during the initial investigations using brain heart infusion broth. Studies of the growth of *Lactobacillus plantarum* revealed that this organism would not grow in brain heart infusion and so another medium was investigated. This resulted in the use of GMRS (see section 3.1). Although this medium was found to be ideal for the *Lactobacillus* it was not possible to reproduce the growth curves of *Aeromonas hydrophila*, see figure 4.1. Although the conditions were considered identical there was obviously an uncontrolled parameter causing an unreliable population growth profile. An investigation of the literature revealed several possibilities which were resolved in turn.

It has been shown that *Aeromonas hydrophila* was more sensitive to acidic Maillard reaction products than some other food-poisoning micro-organisms [Stecchini *et al*, 1991]. The components of the GMRS medium were prone to caramelization during autoclaving and may have been responsible for fluctuations in the growth conditions.

The effect of this upon an *Aer. hydrophila* population was examined. Variations in GMRS glucose concentration and autoclaving regimes were compared. None of these combinations yielded a reproducible population growth profile for this organism and so a new medium (DECAYES) was developed with reduced Maillard reaction products after autoclaving. This was achieved by decreasing the levels of reducing sugars and amine groups in the medium compared to GMRS which has

several peptides and a relatively high dextrose level (see section 2.2.1). Thus DECAYES was developed with only casamino acids and a reduced dextrose level known to be sufficient to support the *Lactobacillus* without affecting the maximum potential growth rate. Further experiments showed that DECAYES supported the growth of both organisms and so would be suitable for use in mixed culture; however, the reproducibility of the aeromonad growth profile was not improved.

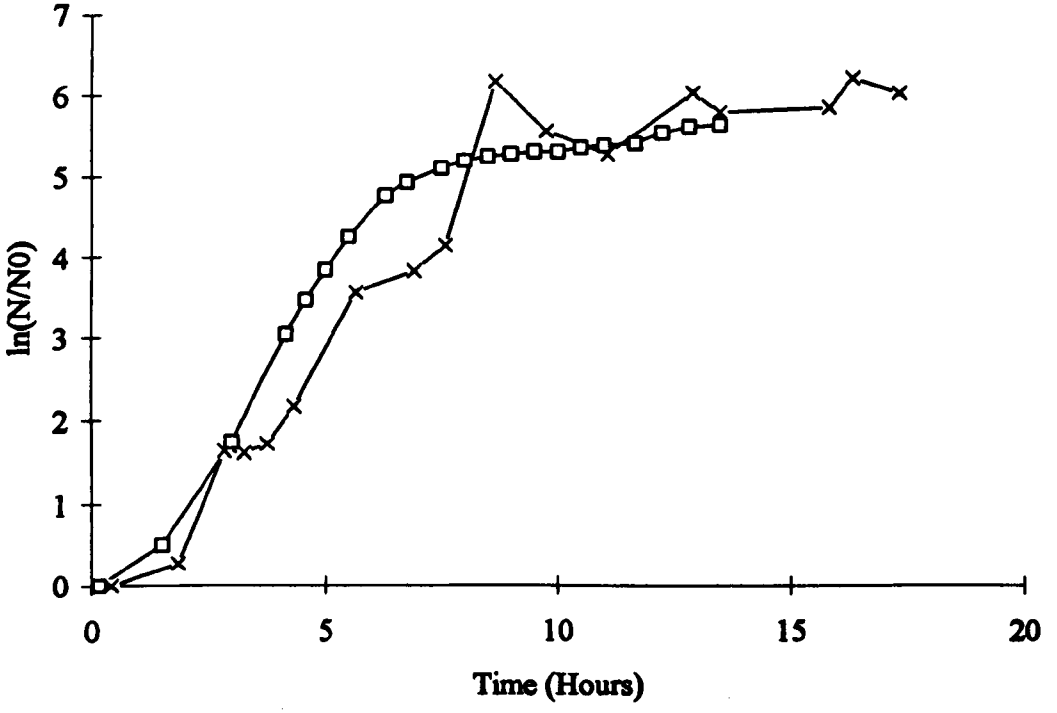

Figure 4.1 Two growth trials (A □, and B X) of *Aeromonas hydrophila* in GMRS broth under what were considered identical conditions (30°C in static flasks).

The use of the GMRS medium and development of the DECAYES medium had also seen the culture conditions optimised for *Lactobacillus plantarum*. This approach had been taken since it was known that the lactobacilli would grow more slowly than the aeromonad. *Lb. plantarum* has been classified as a facultative anaerobe [Gotz *et al*, 1980 a & b]. Thus in order to encourage the *Lactobacillus* population it had been decided to use a very low stir rate in the fermentation vessels,

providing sufficient homogeneity with a sufficient oxygen transfer rate (OTR) to allow aeromonad growth without saturating the broth with oxygen.

Reproducible aeromonad growth was finally achieved by utilising either high rates of agitation (shake flasks or stir rate) or creating a high surface area : volume ratio which permitted high oxygen transfer rates. The use of low stir rates and slight fluctuations in high inocula had lead to the situation where the *Aeromonas hydrophila* population reduced the dissolved oxygen concentration at varying rates causing unpredictable growth patterns. Use of cooled incubators with shake platforms was not possible as the heat generated by the shake platform caused the incubator temperature control to be unreliable. Thus, the static flask with high surface area : volume ratio was adopted.

This conclusion was the product of a concerted effort to systematically reduce the experimental components until the controlling factor was determined. Finally upon examination of the apparatus used it was possible to show that the rate of gaseous exchange was the uncontrolled factor throughout.

4.2 THE EFFECT OF TEMPERATURE ON THE GROWTH OF *AEROMONAS HYDROPHILA*.

Initial investigations into the influence of temperature on the growth of *Aeromonas hydrophila* were conducted in static flasks incubated in a cooled water bath. As in section 3.3 growth was monitored by optical density and converted to viable count by a calibration curve. The growth parameters were determined as described in section 2.5.1 (see table 4.1).

As occurred in section 3.3, low sample numbers and high initial population density resulted in poor statistical reliability of the growth parameter estimates obtained. Further experiments with low initial populations and increased sampling points were performed to obtain more dependable growth parameter estimates (see table 4.2). The lag phase and growth rate parameters for both organisms were compared (figures 4.2 and 4.3) and revealed the same patterns of temperature effect

upon both lag phase and growth rate although the aeromonad gave shorter lag times and faster growth rates than the *Lactobacillus*.

Table 4.1 Growth parameters from initial studies of temperature effects upon cultures of *Aeromonas hydrophila*.

Temperature (°C)	Lag Time (hrs.)	Growth Rate ($hr.^{-1}$)
30	0.79 ± 0.18	0.90 ± 0.072
15	8.19 ± 0.035	0.62 ± 0.0066
7	40.84 ±1.65	0.15 ± 0.02

There was no immediately obvious trend for the effect of temperature upon final population levels (figure 4.4). The value for 5°C has a large standard error and should not be considered reliable however, the value at 30°C is significantly lower than that for any of the lower temperatures. This was in marked contrast to that found for *Lb. plantarum* (figure 3.7) and may be as a consequence of the high nutritional versatility of this organism.

Table 4.2 Growth parameters for *Aeromonas hydrophila* at various temperatures.

Temperature (°C)	Lag Time (hrs.)	Growth Rate ($hr.^{-1}$)	Final Pop^{n}. Density $\ln(N/N_0)$
30	1.50 ± 0.51	1.34 ± 0.14	12.70 ± 0.41
25	1.79 ± 0.55	1.16 ± 0.10	15.15 ± 0.62
20	3.48 ± 0.36	0.619 ± 0.02	14.17 ± 0.16
15	5.49 ± 0.78	0.36 ± 0.015	14.75 ± 0.21
10	6.67 ± 1.67	0.16 ± 0.0074	14.21 ± 0.29
5	52.57 ± 17.75	0.099 ± 0.029	14.05 ± 2.16

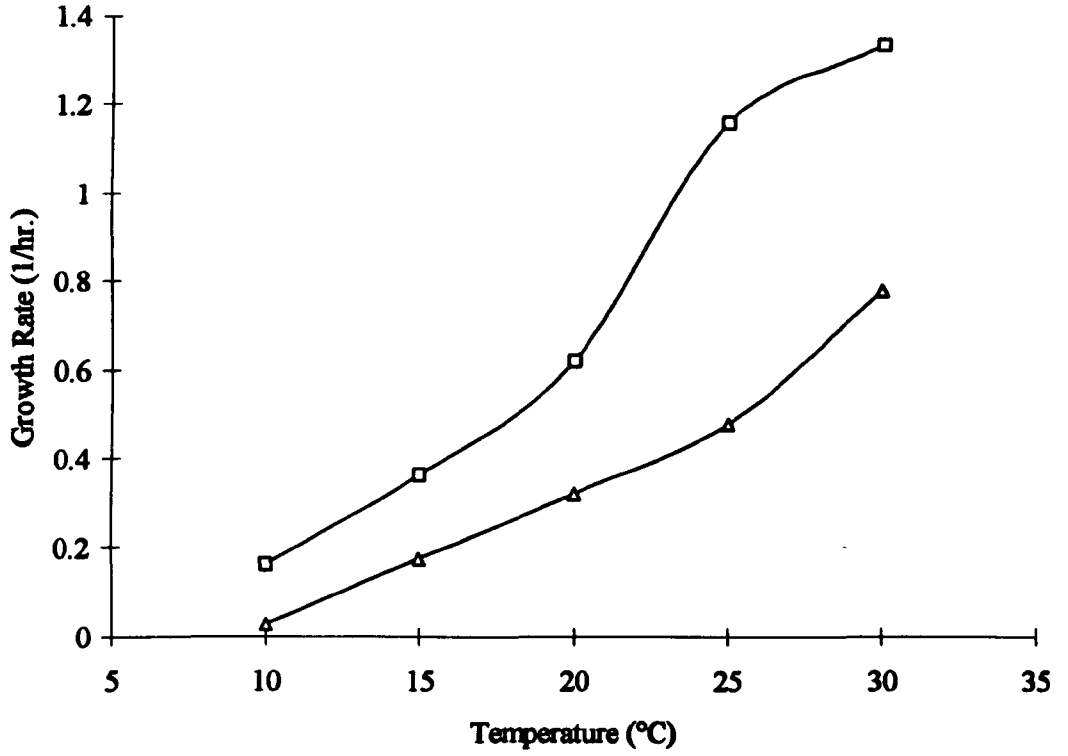

Figure 4.2 Comparison of pure culture growth rates at various temperatures for *Aeromonas hydrophila* □, and *Lactobacillus plantarum* Δ.

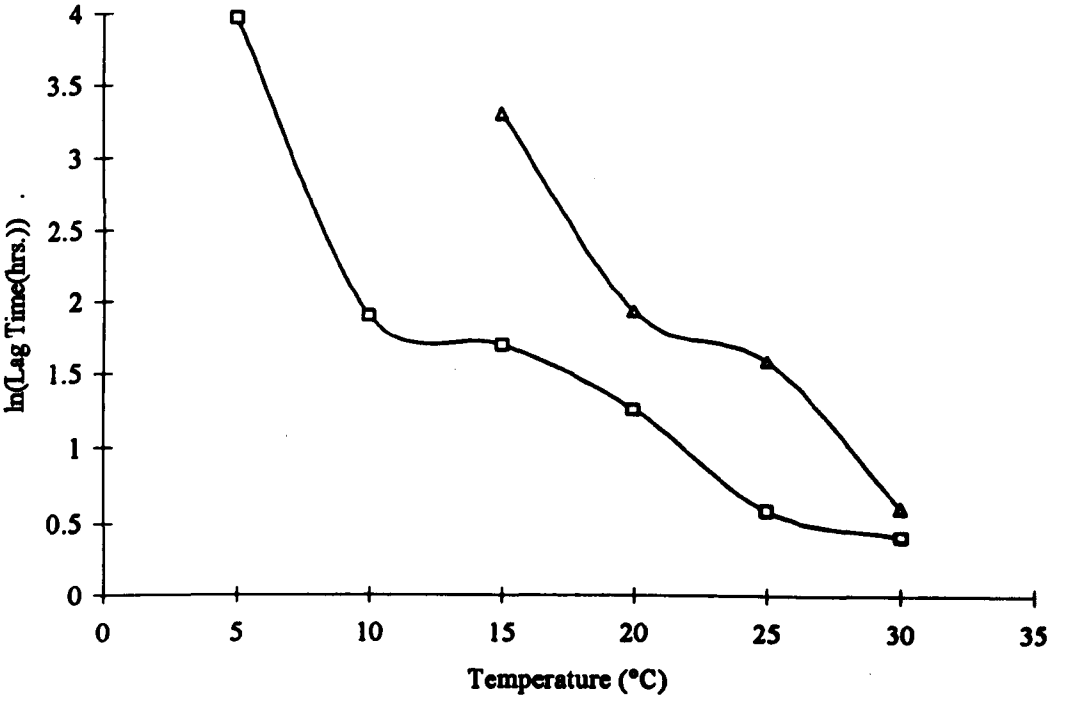

Figure 4.3 Comparison of pure culture lag times at various temperatures for *Aeromonas hydrophila* □ and *Lactobacillus plantarum* Δ.

It was concluded that since underlying trends were the same for each organism there was a likelihood that the temperature effects may be upon a common factor in both organisms, the cell membrane for example. However, it should also be remembered that any resemblance may be the broad similarity expected for a multi-reaction autocatalytic system with temperature-inactivable catalysts. The lag time data is plotted (figure 4.3) as the Napierian logarithm as suggested by Davey [1991]. This reveals a sigmoid curve to be discussed more completely in section 6.1. The same pattern was also observed with the *Lb. plantarum* data but the inflection point was much less obvious.

The observation that *Aeromonas hydrophila* can grow at 5°C is consistent with the published values for a food isolate which has shown a maximum specific growth rate of 0.04 $hr.^{-1}$ and lag period of 19.09 hours at 4°C and pH 5.5 [Hudson, 1992]. The published value was somewhat different from that obtained at 5°C for the strain used in this study (see table 4.2). Hudson [1992] compared three strains of *Aeromonas hydrophila* isolated from three different environments and concluded that each strain may be adapted to growth under different environmental conditions. Including the consideration that Hudson used a different medium from this study the apparent differences between the two data should not be considered significant.

4.3 THE METABOLISM OF *AEROMONAS HYDROPHILA*.

Bergey's Manual of Determinative Bacteriology [Schubert, 1986] refers to the organism as a chemo-organotroph with both respiratory and fermentative metabolism. During the development of the DECAYES medium it was observed that little, if any gas was being formed during population growth of this organism and no acidic odours were detected. This was surprising since the name *Aeromonas* means gas producing unit or monad. The pH of the various media tested was followed during growth of *Aeromonas hydrophila*. The pH value was seen to fall from an initial value of 5.8 to around 5.3 as the population developed through the exponential phase of growth.

Then as the population entered stationary phase the pH was seen to oscillate and in later studies in DECAYES medium, the final pH level rose to be well in excess of the

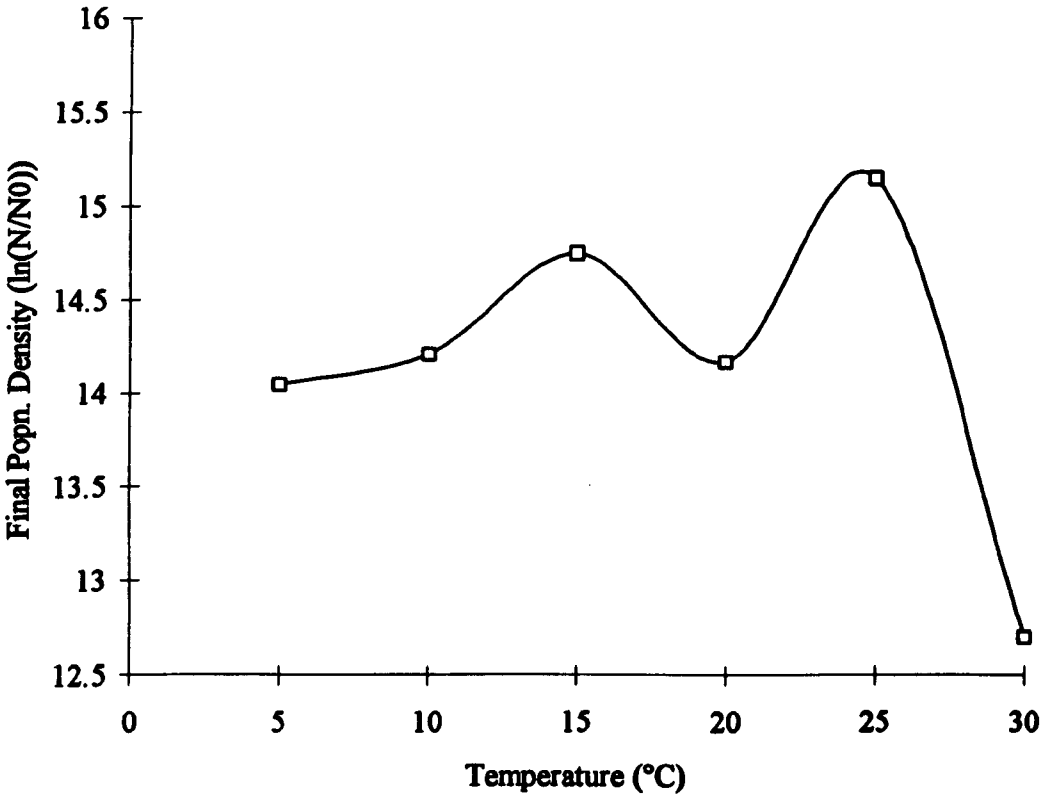

Figure 4.4 The effect of temperature (°C) upon the final population density (ln(N/N0)) of *Aeromonas hydrophila*. Growth at 30°C is above the published optimum (28°C [Hudson, 1992]) for this organism.

starting value (see figure 4.5). This finding was considered important since it indicated a probable shift in the metabolism of the aeromonad as the population developed from growth to stationary phase. The production or release of such a large quantity of basic product was an indicator that during stationary phase the aeromonad may have been utilising proteinaceous materials or amino acids and secreting ammonia as the end product. A Biorad protein assay revealed no significant fluctuations in the level of proteinaceous material and so it was concluded that the organism was most likely utilising single amino acids. It was decided, therefore, to investigate the metabolism of this organism since this would have a significant bearing on the mixed culture experiments.

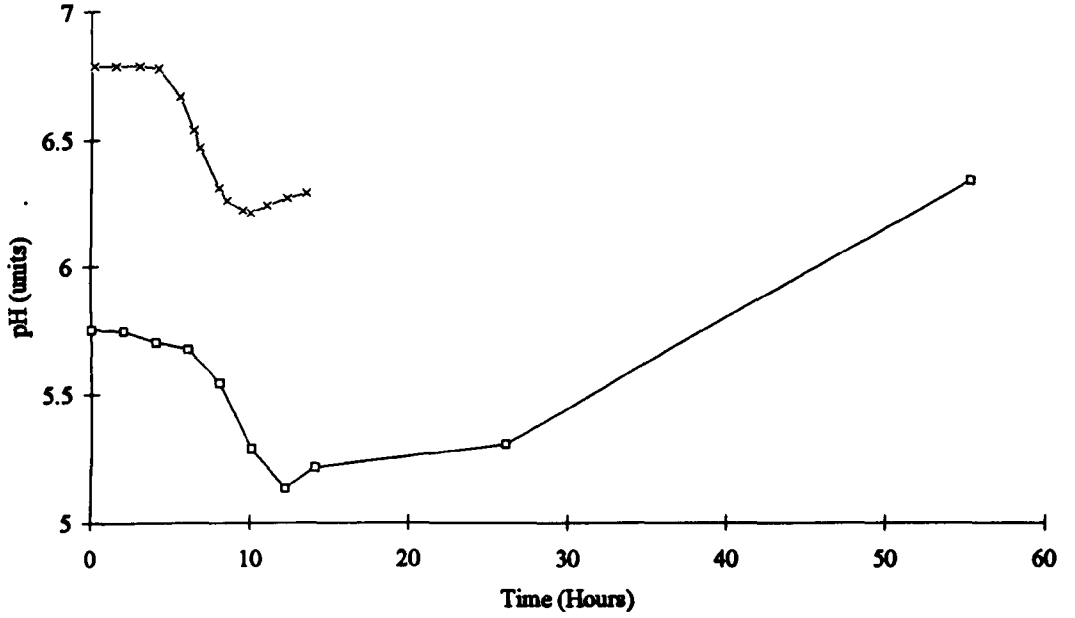

Figure 4.5 pH profiles for pure cultures of *Aeromonas hydrophila* in DECAYES (□) medium and GMRS (x) medium at 30°C.

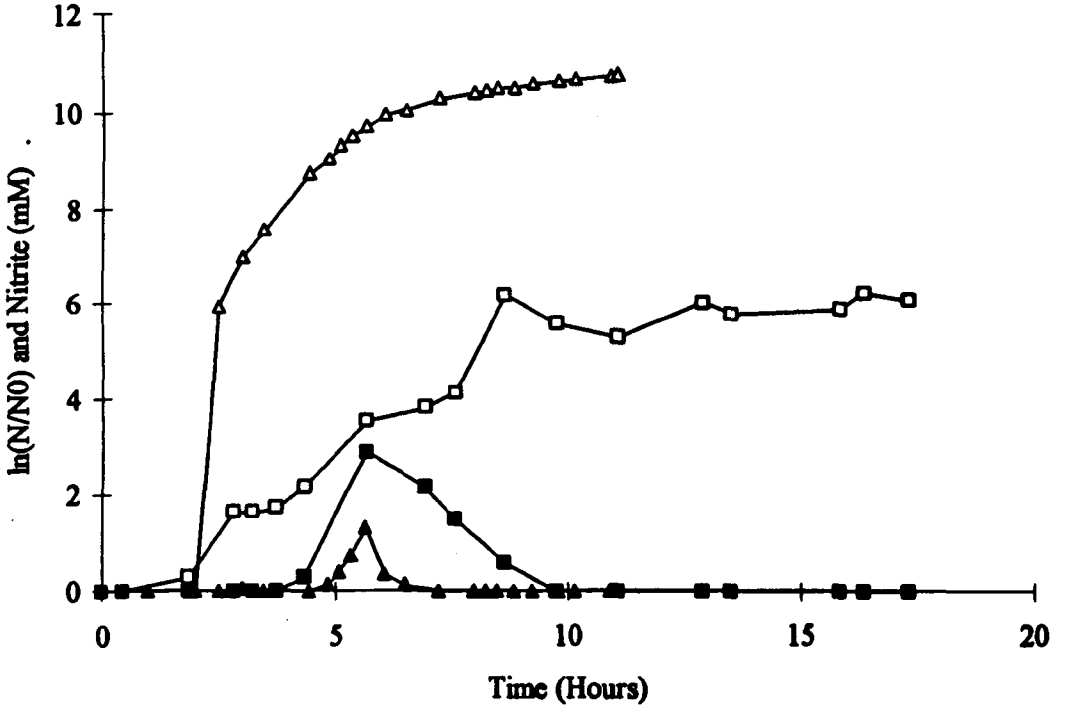

Figure 4.6 Investigation of nitrate reduction during two growth curves of *Aeromonas hydrophila* (A △, and B □) in GMRS at 30°C. The solid symbols reveal the nitrite peak expressed by the aeromonad during nitrate reduction at two different initial concentrations of nitrate. A : 1mM initial NO_3^-, B : 5mM initial NO_3^-. The disparity between the two growth curves (A and B) reflects the problems engendered by the unpredictable oxygen transfer rate.

With the aim of studying mixed cultures, efforts were made to follow the development of the aeromonad by monitoring reduction of nitrate to nitrite since the *Lactobacillus* was known to be incapable of this redox reaction [Bobillo, 1991]. As the population of *Aeromonas hydrophila* increased a rapid increase in nitrite levels was observed; however, around mid to late exponential phase, the concentration of the nitrite was seen to decrease sharply (see figure 4.6). To investigate the possibility of ammonia formation from nitrite, an assay was conducted on samples from a growth curve exhibiting the nitrite peak. The analysis showed that the ammonia levels fluctuated significantly in exponential phase and increased massively in stationary phase. The final ammonia levels detected were far in excess of the theoretical maximum for the reduction of the nitrite to ammonia. This was not surprising since the earlier experiments had shown that the organism was probably catabolising amino acids.

The excretion of ammonia explained the large rise in medium pH during stationary phase. The amount of background ammonia detected implied a large amino acid turnover since this was the only other significant nitrogen source, after the nitrate, in the DECAYES medium. This suggested that the organism was using the amino acids as a carbon source in the medium and not the carbohydrate provided. The pH reduction during exponential growth could be explained since the buffering capacity of the medium would be shifted by catabolism of various amino acids rather than secretion of acidic end products. HPLC analysis yielded data which showed that the organism was not using the dextrose during exponential growth. However, as the population entered stationary phase the dextrose level decreased rapidly. HPLC analysis provided no evidence of a metabolic end product at any point of the growth curve. Traces of organic acids were not at significant levels compared with the dextrose decrease exhibited during stationary phase. This is in agreement with the data of van der Kooij and Hijnen [1988] who found that *Aeromonas hydrophila* expressed a greater affinity for amino acids than carbohydrates when grown in drinking water supplemented with the relevant compounds. The use of amino acids as principle substrates by *Aeromonas hydrophila* B184 was confirmed in this laboratory with paper chromatography [Boardman, 1993]. There was no quantitative measurement possible with this method, however the result was definitive.

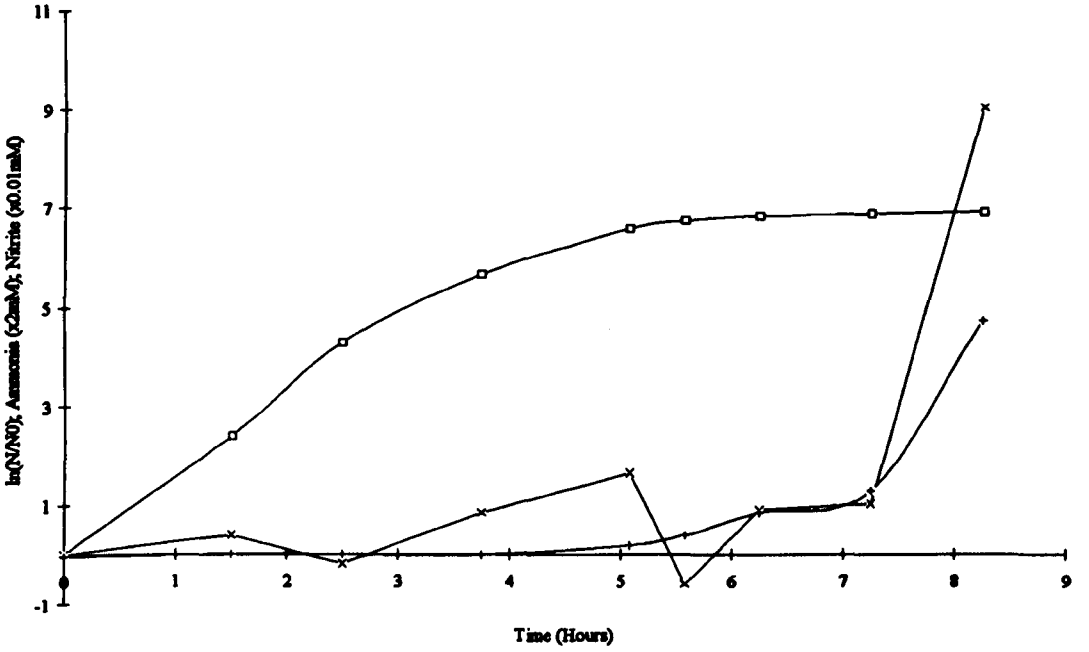

Figure 4.7 Fluctuation of ammonia, less initial background level, x (x 2mM) and nitrite + (x 0.01mM) levels with population development of *Aeromonas hydrophila* (□).

It was now established that *Aeromonas hydrophila* was utilising amino acids. It became necessary to try and detect an end product since this may have affected any mixed culture results. It has been shown that the organism releases large amounts of ammonia during stationary phase. No evidence of a final product from the amino acid carbon skeleton could be found. Results of growth studies at 25°C showed that the organism increased the level of dissolved CO_2 during the early stages of exponential growth, then reduced the level of dissolved CO_2 during late exponential phase. There was no gassing observed from cultures implying that carbon dioxide was not the principle catabolic product.

Although paper chromatography had yielded proof that the organism was utilising amino acids as the principle carbon source the separation achieved was insufficient to indicate which amino acids were being utilised. It was proposed that the organism would either utilise a range of amino acids potentially secreting a different end product for each or utilise one or two amino acids from the medium and

concentrate energy production along one or two pathways. Amino acid analysis was performed on several samples from a growth curve at 25°C to determine if *Aeromonas hydrophila* was utilising a specific amino acid or a range of amino acids (table 4.3). The results from this analysis of most interest are highlighted in table 4.3.

Table 4.3 Results of amino acid analysis of spent medium from a 25°C growth curve of *Aeromonas hydrophila*. Amino acid concentration (mM) at four time points. Standard deviation and other statistical values were not provided. Blank entries are unreliable results (on advice of analyser).

Time in hours (% Final Pop.)	0 0.001%	5 0.026%	7.5 0.173%	22 100%
Aspartate	**26**	**30**	**31**	**7.4**
Threonine	12.05	13.3	15.5	12.5
Serine	**20.55**	**22.75**	**24.7**	**0**
Glutamate	**59.65**	**67.5**	**68.2**	**65.7**
Proline	36.05	38.1	45.55	44.15
Glycine	24	2.45	10.75	12
Alanine	35.35	50.4	31	33
Valine	22.6	30.55	20.75	32.65
Methionine	3.55	4.1	4.65	5.25
Isoleucine	12.4	12.5	13.15	19.35
Leucine	29.65	30	31	39.9
Tyrosine	**4.4**		**4.95**	**8.5**
Phenylalanine	**14.35**	**13.65**	**7.8**	**3.8**
Histidine			1.1	
Lysine	15.05	17.75	17.15	30.05
Arginine			14.3	

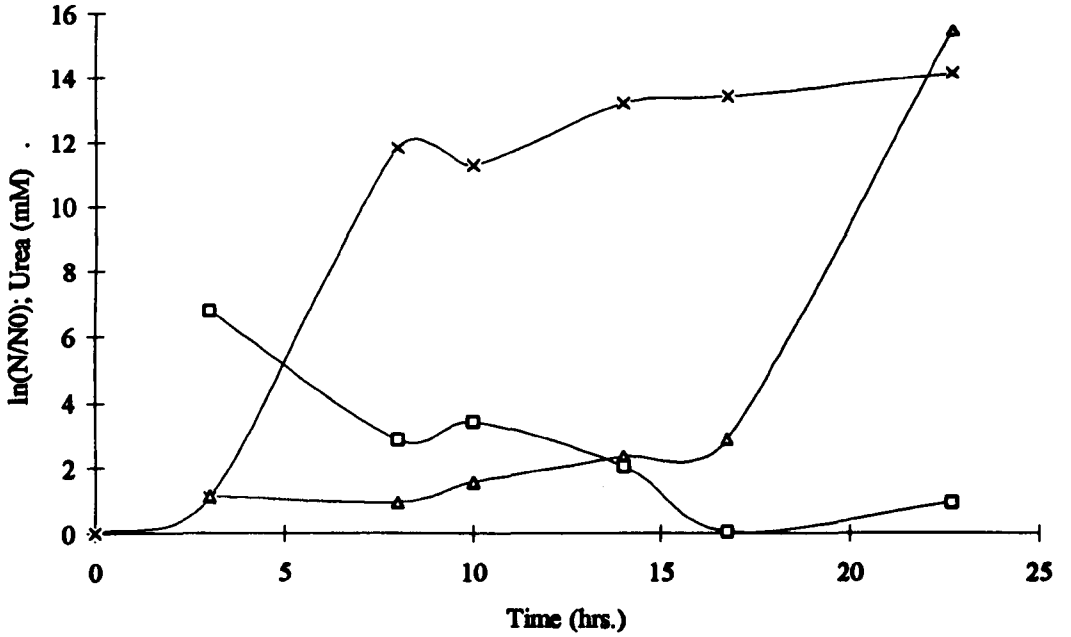

Figure 4.8 Internal (□ moles urea / g dry weight) and external (Δ mM) urea concentrations for *Aeromonas hydrophila* (x) growing at 25°C. The data suggests that this organism does not just secrete this end product but also utilises it during the lag and growth phases of population development.

Degradation of aspartate, serine and phenylalanine yields NH_4^+ and a carbon skeleton for use in the citric acid cycle, gluconeogenesis, and ketogenesis (phenylalanine only) (figure 4.9). The rises in the levels of glutamate and tyrosine supported these proposals. The relationship between these three amino acids and the fluctuating levels of glutamate and tyrosine has been shown in figure 4.9.

Although there was no trace of a primary catabolic product it was hypothesised that the organism may be producing urea and building the internal concentration of this product for use in ATP synthesis by a pathway similar to that described by Smith *et al* [1993]. These workers [Smith *et al*, 1993] found that *Ureaplasma urealyticum* could generate a transmembrane potential with resultant de novo ATP synthesis by internal urea hydrolysis creating an ammonia chemical potential. This hypothesis was tested experimentally by determination of the internal and external urea concentrations of an *Aeromonas hydrophila* population (figure 4.8) grown at 25°C. The apparent changes in the respective urea concentrations suggest that *Aer. hydrophila* may also be utilising urea in this way.

4.4 CONTINUOUS CULTURE.

Results of the mixed culture experiments and the HPLC analyses of the samples from those experiments to be reported in the next chapter (section 5.2) had shown that, for temperatures from 5°C to 25°C, the pH followed the profile seen for the aeromonad alone (figure 4.5); however, at 30°C, this was not true. At 30°C the final pH was below 4.0 which implied that the *Lactobacillus* had taken control of the fermentation in spite of the better growth kinetics exhibited by the aeromonad population. It was proposed that the *Lactobacillus* produced lactate at a faster rate at 30°C than the aeromonad could utilise. This would result in a continued decrease below the minimum pH tolerated by the aeromonad (approximately 5.3) [Margison, 1992; Boardman, 1993]. This could represent a significant interaction between the two micro-organisms in mixed culture, hence more information was required about the aeromonad's response to organic acid (lactate) levels. Continuous culture in DECAYES medium was used to simulate conditions where lactate was present at steadily increasing levels. This was achieved by maintaining the growth rate at 0.15hr.$^{-1}$ and increasing the concentration of lactate in the medium reservoir. As can be seen from figure 4.10 increases in lactate from 0 mM to 30 mM appeared to have little effect on the exponential *Aeromonas* population.

This cannot be related directly to the batch culture studies since HPLC analysis of continuous culture samples showed that the organism was utilising dextrose and producing a range of products (including lactate) detected by the refractive index detector connected to the aminex HPLC column (section 2.4.6). Whereas during the batch culture experiments the aeromonad did not utilise dextrose during exponential growth.

From the HPLC data presented in figure 4.10 it is possible to deduce that *Aeromonas hydrophila* was producing lactate since this compound was present at a level above 5mM when no lactate was added to the medium. Also, when the added lactate rose from 5mM to 10mM there was no significant fluctuation in the concentration of lactate within the culture vessel (approximately 7mM), implying that

the aeromonad was utilising the supplied lactate or had ceased to secrete lactate, either possibility giving the same effect. At levels above 10mM it can be seen that the concentration of lactate in the culture vessel begins to rise. This suggested that the organism had achieved a maximum level of lactate utilisation. The population level within the vessel had not begun to decline, thus the lactate had not reached a toxic level, although it should be remembered that pH control was used throughout.

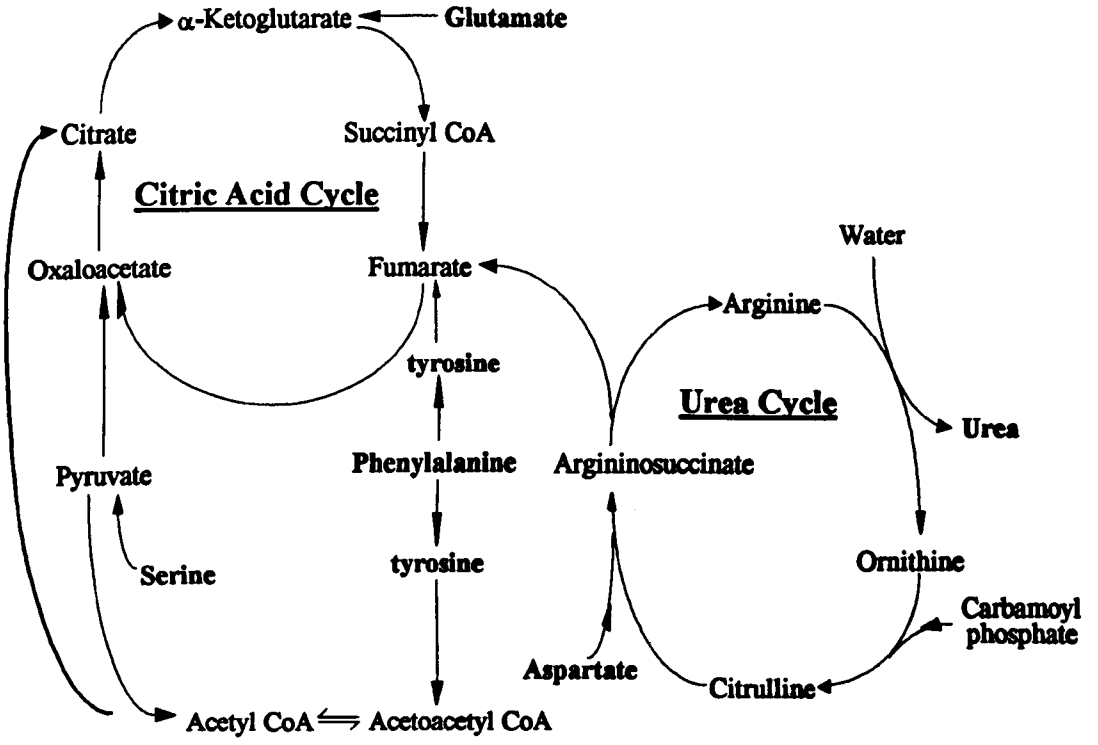

Figure 4.9 Possible metabolic pathways utilised by *Aer. hydrophila*. Principle substrates and catabolite in bold. Compare to Table 4.3.

During the initial growth studies and DECAYES medium development it was noted that the organism produced acidic end products as a result of oxygen limitation causing unpredictable growth behaviour. This is the most likely reason for the metabolic behaviour of the organism in continuous culture. Although a stir rate of 500rpm was maintained it was not possible to measure either the dissolved oxygen or the oxygen transfer rate. Since the culture had grown quickly during the batch phase and remained healthy throughout the continuous culture experiment the OTR was assumed to be sufficient.

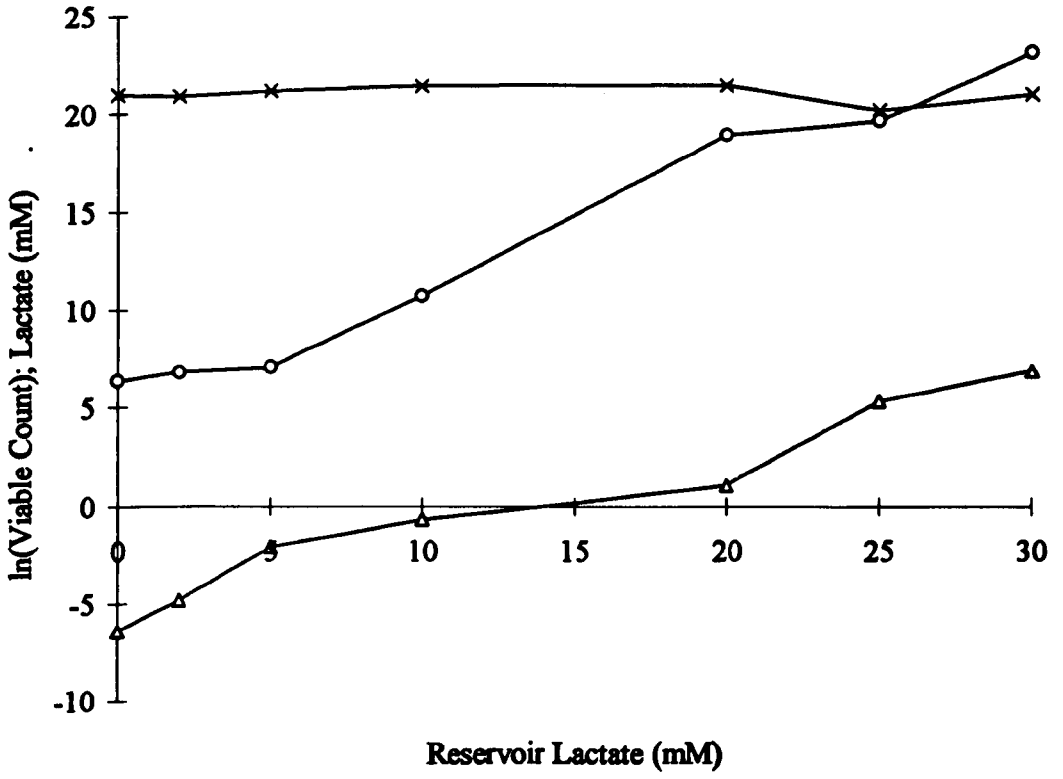

Figure 4.10 The effect of increasing lactate concentration (mM) upon an *Aeromonas hydrophila* population (X) in continuous culture. Also shown are the levels of lactate found in the spent medium from the culture vessel (o) and the difference between incoming and outgoing lactate levels (Δ).

Figure 4.10 shows that the steadily increasing concentration of lactate did not significantly affect the aeromonad population in continuous culture. It should be noted that the pH of the culture vessel was controlled at 5.8 with a mineral alkali. This may explain why relatively high concentrations of lactate (≤30mM) did not appear to have any inhibitory effect on an exponential *Aeromonas hydrophila* population.

It was not possible to even loosely elucidate the nature of all the catabolic compounds produced since this analysis was outside the scope of the investigation. The HPLC analyses indicated that the organism expressed a very low affinity for lactate being incapable of utilising concentrations below 5mM (figure 4.9). This is in agreement with the data published by van der Kooij and Hijnen [1988].

4.5 DISCUSSION.

Close inspection of the *Aeromonas hydrophila* data in figure 4.2 reveals that the $\Delta\mu$ from 20°C to 25°C is greater than that from 25°C to 30°C. This same observation is true for $\Delta\ln(L)$ in figure 4.3. The growth rate plot (figure 4.2) combined with the lag phase semi-logarithmic plot (figure 4.3) suggests that the optimum growth temperature for *Aeromonas hydrophila* B184 is between 25°C and 30°C. This conclusion is consistent with the published optimum growth temperature for a food isolate of around 28°C [Hudson, 1992]. The final population density parameters determined for the five temperatures investigated below 30°C showed little or no effect of temperature (figure 4.4). The drop in final population level at 30°C is consistent with the trend observed for *Lb. plantarum*. This result shows the organism to be well adapted to life across a wide temperature range and would perhaps be replaced by a pattern of behaviour similar to that expressed by *Lb. plantarum* (figure 3.7) only if a wider thermal range, including temperatures below 5°C, had been investigated.

The use of various chromatographic techniques has revealed that the organism utilised at least two different amino acids in preference to dextrose in batch culture with a sufficiently high oxygen concentration. The change in dissolved CO_2 (section 4.3) levels at different stages of population growth is presumed to be the result of the organism converting ammonia, from catabolised amino acids, into urea for possible energy production at a later stage. This hypothesis is supported by the difference between internal and external urea analyses (figure 4.8). The lag time for this experiment was estimated at 2.72 hours, which approximates the period of time before the internal urea concentration dropped sharply. The external urea concentration was observed to remain low until the population entered stationary phase. The initial high internal levels may have been built up by the starter culture population as it passed from exponential to stationary phase as a defence against a hostile environment. This supply of urea could then be used to generate a transmembrane potential with resultant *de novo* ATP synthesis [Smith *et al*, 1993]. This would provide the organism with a

competitive advantage over other microbes when the environmental conditions were once again favourable to population growth.

The amino acid analysis results show that the organism is not utilising any one amino acid and therefore may be producing a different product from each amino acid. This possibility has not been investigated.

The continuous culture data revealed that lactate will not significantly affect a growing population of *Aeromonas hydrophila*, under pH control, at concentrations below 30mM (figure 4.10). The data presented in figure 4.10 shows conclusively that this organism will utilise lactate since the amount of lactate found in the culture vessel was less than that supplied in the medium reservoir at the higher concentrations tested. At the lower concentrations (≤10mM) the effects of the lactate supplied, if any, were masked by the presence of lactate secreted following catabolism of dextrose present in the medium. Utilisation of dextrose during exponential growth in the chemostat was not expected after the batch culture investigations had revealed that *Aeromonas hydrophila* catabolised amino acids in preference to dextrose. This metabolic shift may have been caused by a low oxygen tension induced by an active large population over an extended period of time in spite of the stir rate (500 rpm).

5.0 MIXED CULTURE.

There have been many mixed culture studies of lactobacilli owing to the great involvement of this genus in both food production (e.g. kefir [Kennes *et al*, 1991]) and food spoilage (e.g. wine [da Cunha & Foster, 1992]). The essence of these studies has been the investigation of metabolic interactions between the various starter culture organisms [Hammes, 1986]. Few attempts have been made to quantify the metabolic competition to be found between competing organisms and the dynamics of a changing environment. Meisel *et al* [1989] investigated the effects of three starter organisms (*Lb. curvatus*, *Micrococcus varians*, and *Debaromyces hansenii*) upon the proliferation of *Staphylococcus aureus*. The staphylococci were seen to develop more slowly in the presence of the starter culture organisms than in their absence. This inhibition was cumulative with the diversity of organisms; however, no consideration was given to the mode of inhibition. This effect may be solely due to competition or, more likely, by a combination of different effects such as bacteriocins and competition. All of this work has revealed that a deeper understanding of how organisms interact metabolically with each other and the physico-chemical environment is required. Whilst there has been much discussion of the complexities involved in food fermentation [Nout & Rombout, 1992], there seems to be a distinct lack of objective data. In order to develop existing models and build new models, all aspects of growth must be measured [Zwietering, *et al*, 1992].

Microbial interactions may not be limited to purely chemical factors. The use of mixed microbial populations in the manufacture of solid and mixed phase food products (e.g. cheese and olives) demonstrates the possibility of variable interactions dependent upon physical factors such as time and the distance between two microcolonies. As an organism or microcolony develops it establishes gradients of both substrates and catabolites around itself. These gradients continuously change with time for as long as the colony population remains active [Boddy & Wimpenny, 1992] creating a highly dynamic system.

Interactions based on physical factors between two developing colonies of one or more bacterial species are extremely difficult to investigate as a result of the small quantities involved. Studies into this area are being conducted and should yield interesting results since the number of organisms currently being monitored can be as low as 50 bacterial cells per colony [MacKay and Peters, 1995].

5.1 POPULATION GROWTH PARAMETERS OF *AEROMONAS HYDROPHILA* AND *LACTOBACILLUS PLANTARUM* IN MIXED CULTURE.

In mixed culture the populations of both organisms were monitored using selective agars. The *Lactobacillus* population was grown on MRS agar [de Man *et al*, 1960] and the *Aeromonas* population was grown on starch-ampicillin agar [Palumbo *et al*, 1985]. The growth parameters of each population were determined with the 'Regress' software package using a Marquardt algorithm to fit the modified Gompertz

Table 5.1 Growth parameter estimates for *Aer. hydrophila* and *Lb. plantarum* in mixed culture at various temperatures.

	Temperature (°C)	Lag Time (hr.)	Growth Rate (μ/hr.)	Final Pop. Density ln(N/N0)
Aeromonas hydrophila	5	59.41 ± 14.23	0.12 ± 0.019	15.70 ± 0.82
	10	6.07 ± 2.46	0.19 ± 0.014	15.82 ± 0.91
	15	3.17 ± 3.21	0.32 ± 0.043	14.94 ± 0.40
	20	4.21 ± 0.68	0.84 ± 0.097	12.92 ± 1.10
	25	2.04 ± 0.54	1.16 ± 0.11	14.08 ± 0.45
	30	1.90 ± 0.38	1.51 ± 0.16	14.82 ± 1.94
Lactobacillus plantarum	10	7.98 ± 5.89	0.037 ± 0.007	3.06 ± 0.27
	15	5.21 ± 1.40	0.15 ± 0.012	6.34 ± 0.31
	20	2.74 ± 1.73	0.30 ± 0.063	5.80 ± 1.41
	25	3.62 ± 0.55	0.52 ± 0.024	13.44 ± 2.54
	30	1.30 ± 0.54	0.74 ± 0.094	8.48 ± 1.81

growth model [Zwietering *et al*, 1990] to the experimental data (section 2.5.1). The results of these analyses were extremely similar to the data obtained from the pure culture studies (figs. 5.2 - 5.5, tables 3.3, 4.2, & 5.1).

Since it was known from the pure culture studies that the two organisms utilise different chemical groups as a primary substrates (sections 3.4 and 4.3) it was hypothesised that the growth rates and lag times in pure and mixed culture would not be significantly different since substrate competition would not occur. The alternative hypothesis was that the differences are significant, thus demonstrating interaction between the two organisms. These possibilities represent the null hypothesis (H_0) and the alternative hypothesis (H_A) in figure 5.1.

To allow direct statistical comparison of the lag times or growth rates in pure and mixed culture it was necessary to weight the estimated parameter values. A factor of either temperature (°C) or inverse temperature, for lag time and growth rate values respectively, was used.

Table 5.2 Calculated and critical values for the statistical comparisons of the pure and mixed culture growth parameter estimates.

	F_{calc}	F_{crit}	t_{calc}	t_{crit}
Lag Phase (*Aer. hydrophila*)	1.176	10.97	0.061	± 1.11
Lag Phase (*Lb. plantarum*)	3.857	16	0.29	± 1.15
Growth Rate (*Aer. hydrophila*)	1.102	10.97	-0.39	±1.11
Growth Rate (*Lb. plantarum*)	1.068	16	0.064	± 1.15
Final Pop^n. Density (*Aer. hydrophila*)	1.30	10.97	0.98	± 1.11
Final Pop^n. Density (*Lb. plantarum*)	2.08	16	-2.74	± 1.15

This was done to balance the effects of the temperature upon the two growth parameters. It was not necessary to weight the final population density parameters. An F test demonstrated that the variances of all the parameter ranges in pure and mixed culture were homoscedastic for both organisms, table 5.2. Thus H_0 is proven when comparing the parameter variances of the pure and mixed culture methods. The t test utilised to compare the means of the weighted data ranges for the parameter estimates of both organisms in pure and mixed culture showed there to be no

significant differences between the lag time periods or growth rates in mixed and pure culture. Thus H_0 is accepted again.

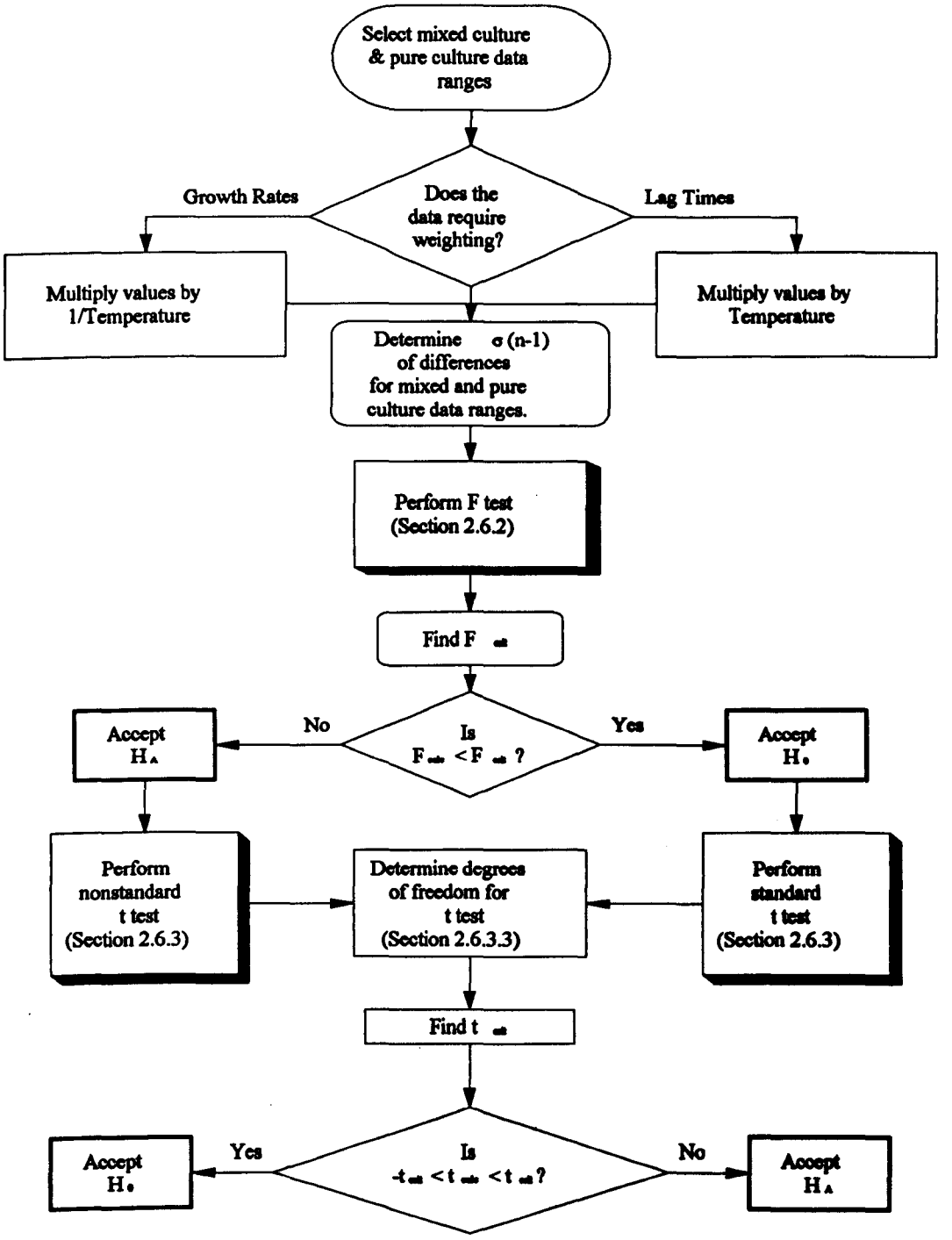

Figure 5.1 Flowchart describing the statistical analysis used to compare mixed and pure culture growth parameters.

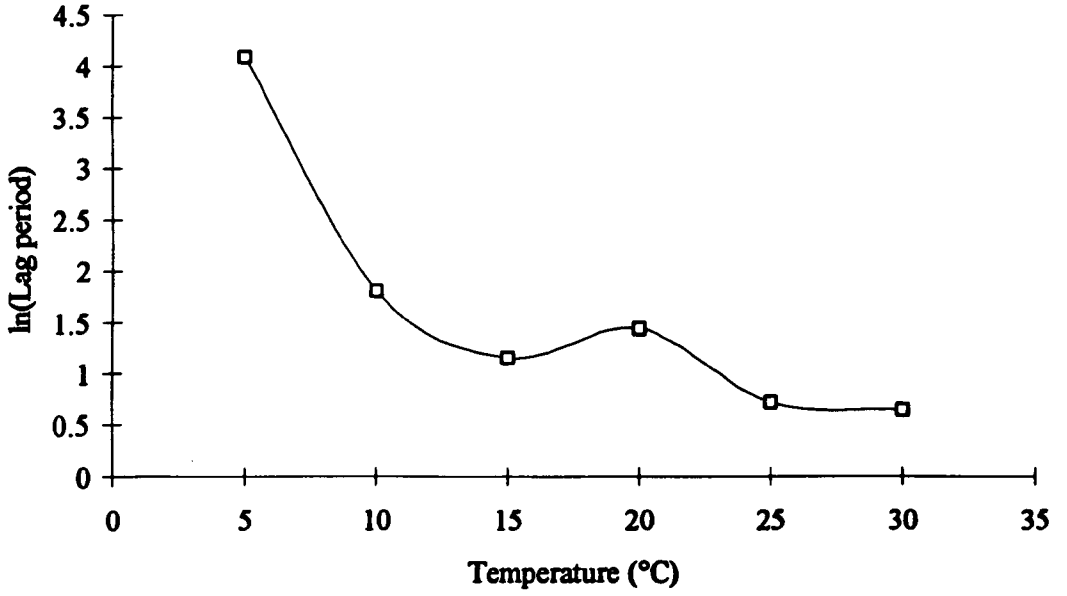

Figure 5.2 Lag phase of *Aer. hydrophila* in mixed culture with *Lb. plantarum* at various incubation temperatures.

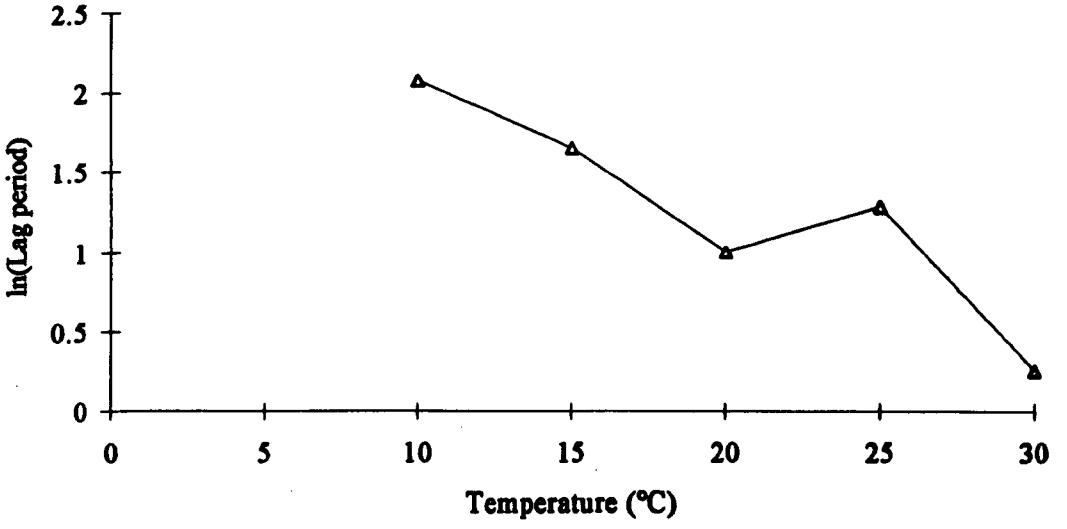

Figure 5.3 Lag phase of *Lb. plantarum* in mixed culture with *Aer. hydrophila* at various incubation temperatures.

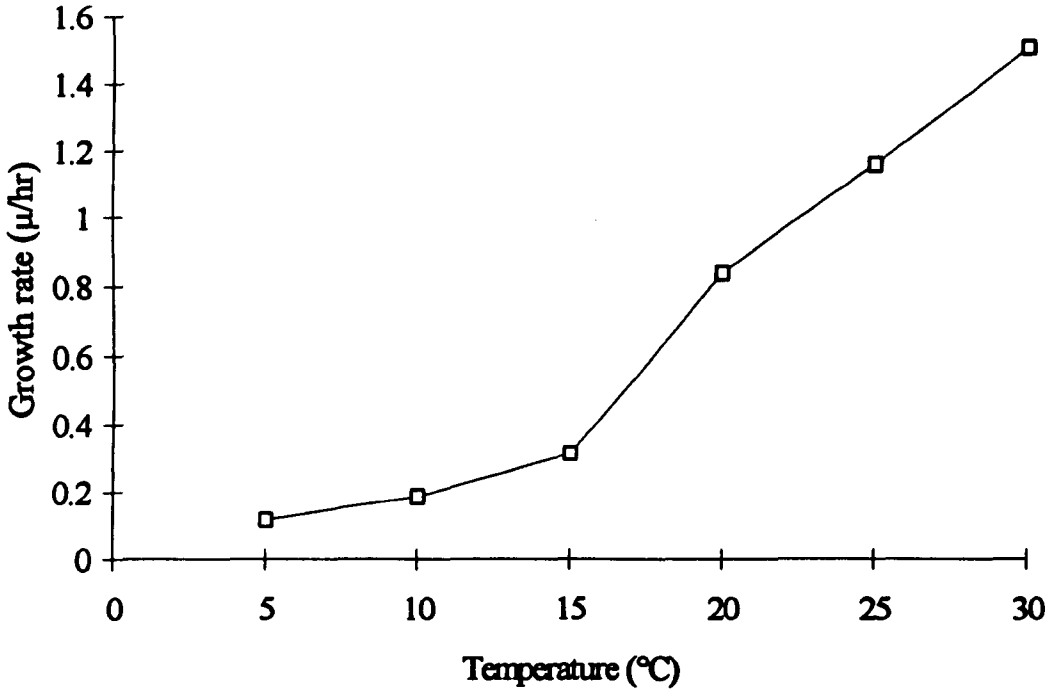

Figure 5.4 Growth rates of *Aer. hydrophila* in mixed culture with *Lb. plantarum* at various incubation temperatures.

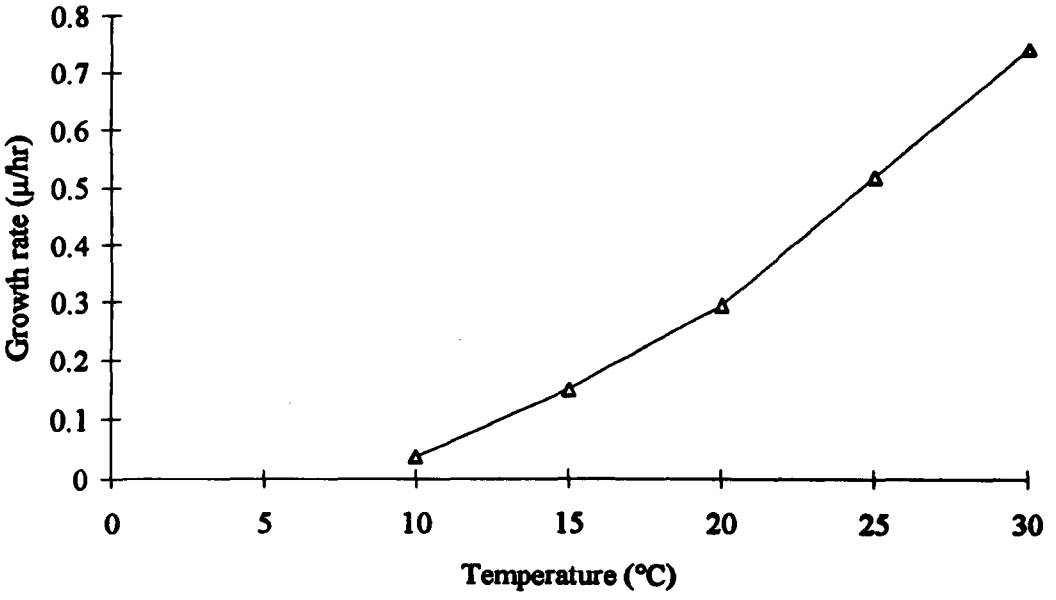

Figure 5.5 Growth rates of *Lb. plantarum* in mixed culture with *Aer. hydrophila* at various incubation temperatures.

Application of these statistical tests to the final population densities gave more interesting results. The t tests showed that there was a significant difference between the final population density of *Lb. plantarum* in mixed compared to pure culture. This was not true for *Aer. hydrophila* which showed similar levels in both pure and mixed culture.

5.2 METABOLIC INTERACTIONS OF *AEROMONAS HYDROPHILA* AND *LACTOBACILLUS PLANTARUM* IN MIXED CULTURE.

The primary substrates of each organism were known to be completely different (sections 3.4 and 4.3). *Lb. plantarum* is known to have a complex requirement for vitamins, amino acids and carbohydrates [Ruiz-Barba & Jiménez-Díaz, 1994] whilst the aeromonad has been shown to utilise a number of amino acids [section 4.3] for primary energy production with no apparent use of the available dextrose during population growth. These facts suggested that any interaction between the organisms should come from spatial relationships based on population density (such as propinquity) or an intercourse based upon catabolic products.

Although these ideas implied relatively few potential interactions, it should be remembered that microbial relationships multiply rather than add together [Boddy & Wimpenny, 1992]. The varying points of interaction are explained in further detail separately. An unfortunate consequence of widespread interaction will be that there is a certain amount of repetition from section to section. This will help relate the various attributes to each other and provide a basis for a general consideration of the interactions in section 5.3.

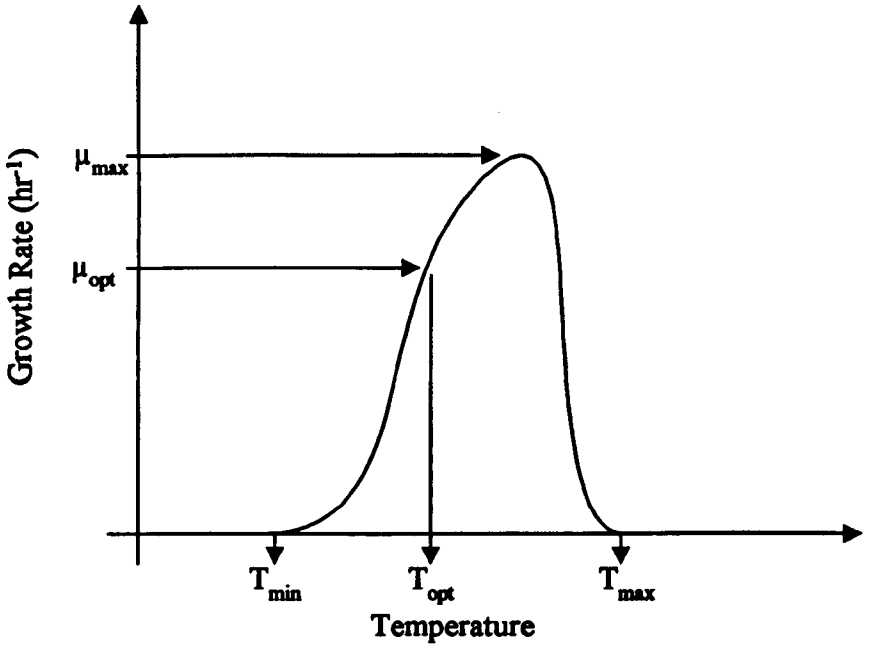

Figure 5.6 Schematic diagram showing the effect of temperature upon growth rate. Note that the optimum temperature (T_{opt}) does not produce the fastest growth rate (μ_{max}) [after Brock *et al*, 1984]. T_{min} & T_{max} denote the minimum and maximum viable temperatures. μ_{opt} denotes the growth rate corresponding to the most efficient rate of growth, as measured by the ratio of dry weight : substrate consumed.

5.2.1 Final pH.

After growth of an *Aer. hydrophila* population in pure culture the final pH was observed to rise above 6.0 having originally fallen from the initial value of 5.8 to a minimum of approximately 5.2. Growth of a *Lb. plantarum* population in pure culture caused the pH to fall from the initial value to below 4.0 at all temperatures investigated above 5°C.

In mixed culture at all temperatures from 5°C to 25°C, inclusive, the final pH ranged from 6.3 to 8.1, showing the aeromonad metabolism to have been dominant. This was not surprising as the growth parameter estimates had revealed the Gram negative aeromonad to express shorter lag times, higher growth rates, and greater final population density than the Gram positive lactic acid bacterium (table 5.1). At 30°C however, the final pH was observed to be 3.74, well below the minimum tolerated by the aeromonad. The growth parameter estimates for this temperature showed the same pattern as at the lower temperatures. This suggests that at the highest temperature investigated, either the aeromonad switched the metabolic behaviour from

that observed at the lower temperatures or the lactobacilli out competed the more advanced Gram negative population for the available glucose.

Examination of figure 5.6 shows that after an organism has achieved the optimum temperature for growth the growth rate continues to rise, at a slower pace, then slips into decline as the temperature continues to increase. Obviously the optimum temperatures for the two organisms are different. It was because the aeromonad, at 30°C, was being exposed to a temperature above the optimum temperature (28°C [Hudson, 1992]) that the lactobacilli were able to decrease the final pH to 3.74 in mixed culture. At 30°C *Lb. plantarum* was growing at the optimum temperature and so was utilising dextrose and excreting acids faster than at any other temperature investigated.

It was shown in section 3.4 and figures 3.7 and 3.9 that the optimum temperature for *Lactobacillus* growth was not the most efficient temperature. At 30°C the amount of acidic catabolite secreted per unit of carbohydrate metabolised will be greater than at the more efficient temperatures. This means that for the same population yield there was a greater pH decrease, hence the apparent contrast between the growth dynamics of the two populations and the final pH level observed at 25°C and 30°C.

5.2.2 *Lactobacillus plantarum* Yield.

The yield of *Lb. plantarum* in mixed culture was analysed, figure 5.7. The yield figures obtained were extremely low, ranging from 0.1% to 20% of the values in pure culture. The mixed culture values were highly inaccurate for two principal reasons.

1. The average ln increase in population size used for the mixed culture yield calculations was only 2.4 compared to 11.8 in the pure culture calculations (figure 3.9). This was because values for decreasing dextrose had to be taken before the aeromonad population had begun to enter stationary phase when it began to compete for the dextrose. The *Lb. plantarum* population had a longer lag phase and lower growth rate than the aeromonad so the population development was small when the

aeromonad had already passed μ_{max}. The same arguments for the unreliability of high lag period estimates proposed by Zwietering, *et al* [1991] apply here. The influence of the error upon the numerical values was greater than in the pure culture precisely because the numerical values used were small.

2. The dextrose concentration figures could be trusted since the HPLC gave an absolute value for the concentration. Although the aeromonad has been shown to utilise dextrose during stationary phase this effect was eliminated by the use of data solely from the experimental period prior to the Gram negative organism entering the stationary phase of population development. The continuous culture experiment (section 4.4) suggested that the affinity of *Aer. hydrophila* for lactate was approximately 6mM as this was the lowest level of lactate detected. The highest final lactate concentration found in mixed culture during growth of the *Aer. hydrophila* population was 6.7mM for a dextrose decrease of nearly 28mM! This meant that the aeromonad was utilising the lactate from the *Lactobacillus* giving a false low level of lactate formed.

As can be seen from figure 5.7, the yield per mmole lactate exceeds the yield per mmole dextrose utilised at all levels. This is the reverse of the situation in pure culture and would not be expected since one mole of dextrose will provide two moles of lactate.

Thus, the yield of lactobacillus cannot be quantitatively related to the metabolites in mixed culture because of interference from the *Aeromonas* population. During the HPLC analyses of samples from the mixed culture experiments it was also noted that the acetate : lactate ratio had inverted from that in *Lactobacillus* pure culture. The values of detected acetate in pure culture never rose above that of lactate but, in mixed culture the acetate concentration frequently exceeded the lactate concentration often achieving levels two or three times that of the lactate.

Analysis of the *Lb. plantarum* yield for acetate was not attempted since it was known that the aeromonad was utilising the lactate and this organism has a greater affinity for acetate than lactate [van der Kooij and Hijnen, 1988] potentially making the acetate concentration values from the mixed culture experiments less reliable than those for lactate.

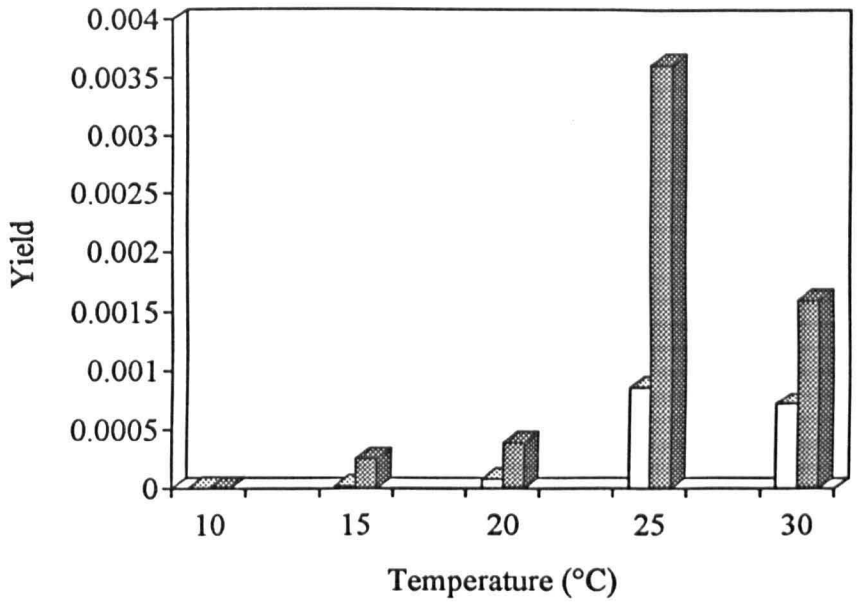

Figure 5.7 Yield of Lb. plantarum related to dextrose utilised and lactate formed (shaded) in mixed culture with *Aer. hydrophila* (g dry weight/mmole metabolite).

An explanation for the inversion of the lactate : acetate ratio would be a switch from homo- to hetero-fermentative metabolism by the lactic acid bacterium. This type of switch has been widely documented over recent years [Marshall, 1992; Kakouri and Nychas, 1994] and related to changes in environmental conditions [Sedewitz *et al*, 1984] such as glucose or oxygen limitation [Kakouri and Nychas, 1994]. Both glucose and oxygen may have become limited in these mixed culture experiments. As explained above, the evidence for the *Lb. plantarum* yield in mixed culture suggests that competition for the available dextrose may have occurred following the passage of the aeromonad population into stationary phase. Oxygen tensions in the growth media were not investigated. From the pure culture experiments the aeromonad was known to be sensitive to oxygen limitation (section 4.1) however, there was no suggestion from the mixed culture population growth curve profiles that oxygen became limited in mixed culture.

5.2.3 Organic Acid Uptake By *Aeromonas hydrophila.*

The final pH values discussed in section 5.2.1 indicated that there was a significant difference in acid secretion and utilisation rates between 25°C and 30°C in mixed culture. The rise in temperature from 25°C to 30°C achieves the optimum for *Lactobacillus plantarum* but crosses the optimum temperature for the aeromonad. It is suggested that crossing the optimum temperature value directly affected the ability of the aeromonad population to utilise organic acids (section 5.2.4). This in turn allowed the pH value to decrease below the minimum tolerated by the aeromonad thus inhibiting the continued characteristic pH profile expected during the development of the *Aer. hydrophila* population into the stationary phase. It was not possible to determine the uptake rate of any substrate by the *Aer. hydrophila* from the HPLC data obtained due to the complexity of the interactions which existed between the two organisms, although the difference between the rate of dextrose depletion in pure *Lb. plantarum* and mixed culture can be explained by the interference of the aeromonad.

5.2.4 Optimum Temperatures.

Figure 5.6 represents, schematically, the changes in growth rate with increasing temperature. The modifications do not prevent the growth rate rising with increased temperature but, as the growth rate approaches the maximum value there is a slowing in the rate of elevation. It is known that if an organism is challenged by temperature the inhibitory effects are offset by a change in the fatty acid composition of membrane lipids [Fukunaga and Russell, 1990]. This adaptation allows continued growth and development after a change in conditions. Fluctuations in the membrane fatty acid composition will affect the membrane fluidity and protein solubility.

It is proposed here that these changes directly affected the ability of the aeromonad to utilise the organic acids provided by the lactobacilli by altering the membrane function or performance or both. The adaptations to the *Lb. plantarum* membrane also promoted the growth and metabolism of this organism. The final result at 30°C was increased acid production by *Lactobacillus plantarum* and decreased acid

utilisation by *Aeromonas hydrophila* with overall control of the pH profile being taken by the lactobacilli.

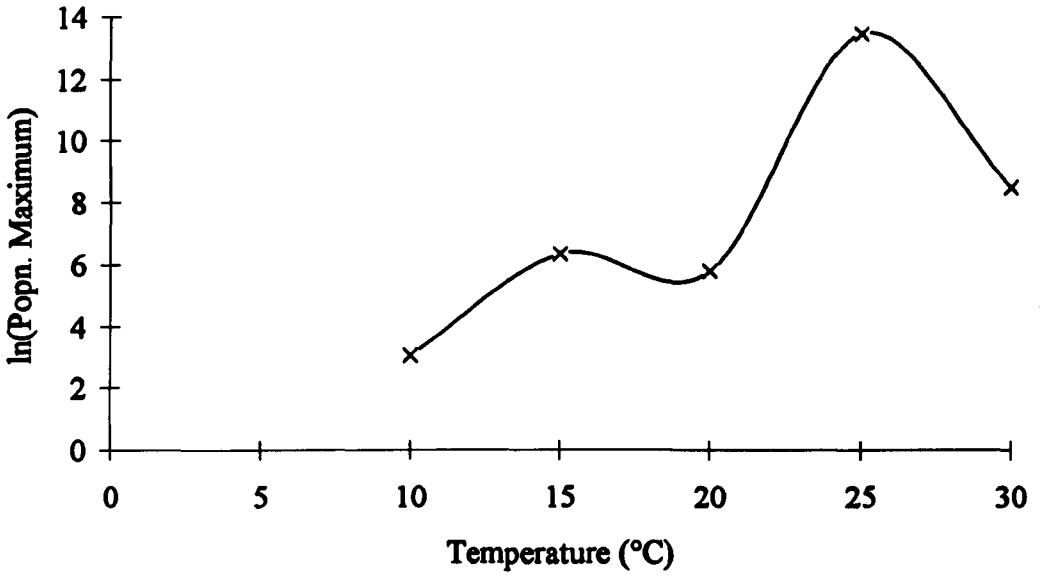

Figure 5.8 Final *Lactobacillus plantarum* population size X (ln (N/N_0)) at various temperatures in mixed culture. c/w Figure 3.7

5.2.5 Substrate Competition.

It has been shown that *Aer. hydrophila* utilised amino acids and *Lb. plantarum* utilised the available carbohydrate as primary growth substrates. Substrate competition occurred because of the changes in the aeromonad metabolism as the population progressed into stationary phase. At all temperatures from 5°C to 30°C the aeromonad entered stationary phase ahead of the lactobacilli. It was shown by HPLC analysis that the aeromonad switched from amino acid utilisation to catabolism of the dextrose as the organism progressed into stationary phase from exponential growth. In mixed culture this produced substrate competition between the two organisms since the *Lb. plantarum* population was still in the exponential phase of growth. The final effect of this interaction was a reduced final *Lb. plantarum* population in mixed

culture, compared to pure culture, as shown by figures 5.8 and 3.7. These figures do not take into account the changes in growth efficiency expressed by *Lb. plantarum* when growing heterofermentatively compared to homofermentatively. This suggests that the significance of the difference between final population levels of *Lb. plantarum* in pure and mixed culture was actually greater than that shown in figures 5.8 and 3.7 since the energy yield is greater from heterofermentative metabolism compared to homofermentative metabolism.

5.3 DISCUSSION.

The type of fermentative metabolism expressed by *Lactobacillus plantarum* is dependant upon several environmental factors [Sedewitz *et al*, 1984; Borch *et al*, 1991; Marshall, 1992]. Combined with the nutritional versatility of *Aer. hydrophila* this means that it has not been possible to quantitate any interaction between the two organisms tested in any detail.

The effect of pH has been implicated in the unpredictable nature of lactic acid bacterial metabolism and this factor must play an important role in this investigation since the known catabolites of the two organisms have antagonistic effects upon the final pH of the medium. The switch from homofermentative metabolism to heterofermentative metabolism in *Lactobacillus plantarum* has been linked to limitation of both glucose and oxygen [Murphy and Condon, 1984a; Borch *et al*, 1991; Kakouri and Nychas, 1994]. Oxygen limitation may have played a factor in the mixed culture studies since the aeromonad population grew faster than the lactobacilli but this is unlikely, due to experimental design, and was not investigated. There was no indication from the behaviour of the aeromonad that oxygen limitation became a factor in the mixed culture. The effects of differing oxygen affinities was not investigated and may have played a part. Glucose limitation may have played a part since the *Aer. hydrophila* population was large enough to successfully compete with the *Lactobacillus plantarum* for the available carbohydrate. This showed itself as a reduction in *Lactobacillus plantarum* final population size in mixed culture. A further point of interest shown by figures 5.8 and 3.7 was the effect of temperature upon the

final population level. It is shown clearly that the lactobacilli attain the highest population density at a temperature between 20°C and 25°C in both pure and mixed culture. In mixed culture (figure 5.8) the 20°C value is lower than expected. This was due to insufficient time for population growth, shown by a final dextrose value of 21.6mM after 21 hours. This excepted, the pattern was the same for pure and mixed culture.

The very low yield of *Lb. plantarum* per mmole lactate produced (figure 5.7) in mixed culture may be explained by a switch from homofermentative metabolism in pure culture to heterofermentative metabolism in mixed culture. The lactate and acetate concentrations in mixed culture did not exceed 4mM for a total decrease of approximately 25mM dextrose. The occurrence of acetate at levels usually above that of lactate was not expected, from the inspection of pure culture growth kinetics and HPLC analyses, and is indicative of heterofermentative metabolism. The heterofermentative metabolism expressed by *Lb. plantarum* would result in increased levels of acetate when compared to the pure culture studies. This was found to be the case throughout the six temperatures investigated. It has been shown that glucose limitation can cause lactic acid bacteria to switch from homo- to heterofermentative metabolism [Kakouri and Nychas, 1994]. Comparison of figures 5.8 and 3.7 shows clearly that the lactobacilli are glucose limited in mixed culture, possibly causing the metabolic switch.

Van der Kooij and Hijnen [1988] reported that, with the exception of acetate, *Aer. hydrophila* is poorly adapted to the utilisation of carboxylic acids at low concentrations. Increased levels of acetate in the medium may cause the aeromonad to utilise this compound in combination with the dextrose as the population entered the stationary phase. The increasing pH levels were only seen after the aeromonad had entered stationary phase. This may have been caused by a build up of internal urea during exponential growth for hydrolysis and resultant ATP synthesis during stationary phase as expressed by *Ureaplasma urealyticum* [Smith *et al*, 1993], or a release of external urease to prevent the environment becoming too acidic for the population. This does not however, explain the difference in final pH levels found between 25°C and 30°C in mixed culture.

The optimum growth temperature for *Aer. hydrophila* has been shown to be 28°C [Hudson, 1992], and 30°C for *Lb. plantarum* [Bobillo, 1991]. Growth of these

two organisms in mixed culture below 30°C followed the expected pattern with the aeromonad producing a high final pH as a result of ammonia release. At 30°C however, the final pH was observed to be lower than predicted by the earlier studies at lower temperatures. The aeromonad again expressed higher growth parameters than the lactobacillus at 30°C. This temperature may have induced changes in the metabolism of the aeromonad or the ability to adapt to a changing environment. These changes may have made it difficult for the organism to compete against the acid production of the lactobacillus. The rate of pH decrease at 30°C would be faster than at 25°C due to the decrease in metabolic efficiency associated with this change in temperature. The aeromonad population would not survive prolonged exposure to pH levels below 5.0 [Margison 1992]. Thus the lactobacilli continued to reduce the medium pH with little or no competition from the aeromonad. There are several suggestions explaining the final pH found at 30°C and it should be concluded that the final result was the product of several factors interacting rather than any one factor in particular.

The low *Lactobacillus plantarum* yield per mmole dextrose utilised (figure 5.6) was the result of the aeromonad population entering stationary phase and utilising the available carbohydrate causing a high dextrose utilised value for the apparent low growth of the *Lactobacillus* population at temperatures below 30°C. This effect was minimised in the calculations by utilising data from mixed culture experiments prior to the Gram negative population rising above 10^9 cfu ml^{-1}. However, this meant that low growth figures, with intrinsic high errors, were used in combination with false low values for lactate production. Together these factors show that a much more detailed study of *Aer. hydrophila* metabolism is required before any quantitative determinations of interaction between these two micro-organisms can be made. There has been no indication of the endpoint of the carbon skeleton from the metabolism of *Aer. hydrophila*, with the exception of urea. The development of both populations cannot account for the apparent discrepancy of final carbon catabolite levels. There was no apparent gassing from the medium during any experiment which implies that the missing carbon was present as an undetected solute. Further work upon the metabolism of *Aer. hydrophila* is required to provide a solution to this discrepancy. The metabolism of *Lactobacillus plantarum* has been examined in great detail by many workers and the production of lactate or acetate by either homo- or heterofermentative

metabolism explains the catabolites seen in this study. The utilisation of amino acids by *Aer. hydrophila* reveals high final pH levels due to the release of ammonia by this organism in pure and mixed culture.

Growth in mixed culture is predictable from pure culture studies at all temperatures investigated below 28°C, the optimum growth temperature for *Aer. hydrophila*. It is proposed that at 30°C, the optimum temperature for *Lb. plantarum*, the aeromonad metabolism is affected significantly since this is above optimum for this Gram negative organism. In combination with the decreased metabolic efficiency of *Lb. plantarum* at this temperature the adapted aeromonad metabolism was insufficient to counteract the effects of the acidic catabolites produced by *Lb. plantarum*. In this situation the medium pH continued to decrease below a level inhibitory to *Aer. hydrophila*.

It is a common principle of science that the simplest answer is often the true answer in spite of going against any or many commonly held principles [Desmond, 1975]. This argument is also known as Occam's razor. Although there are several factors which have been identified as producing the observations listed here, such as metabolic switching, changes in the growth efficiencies, and changes in control of the pH profile, we should be looking for the common factors between these processes and not specific solutions for each observation. In order to investigate the possibilities it was necessary to perform some mathematical analyses upon the available data.

6.0 MATHEMATICAL MODELLING.

A wide range of mathematical models have been developed and used to predict the probable outcome of microbial growth under conditions not previously tested [Baird-Parker & Kilsby, 1987; McClure, *et al*, 1994; Fernandez *et al*, 1996]. These models originate from a range of approaches discussed in chapter 1 (sections 1.2, 1.3, & 1.4). Each of the models has, to a certain extent, been shaped by the intended purpose [Garfinkel, 1984]. This has meant that the data requirements for the model development have also been shaped by the intended purpose. For example, the response surface model requires a wide spread of data across at least two variables. This means that either the investigator uses a reduced number of values for each variable or a great deal more time is spent gathering the required data. It is not surprising that the former route has usually been selected for reasons of both time and finance.

A great deal of discussion has been published upon determination of microbial population growth profile parameters [Enfors *et al*, 1979; Holmberg, 1982; Gibson *et al*, 1987; Buchanan *et al*, 1989; Duh & Schaffner, 1993; Stecchini *et al*, 1993] together with the development of models to describe population growth [Gompertz, 1825; Baird-Parker & Kilsby, 1987; Zwietering *et al*, 1990, 1991, 1992a]. These models have been used to describe the effects of different environmental factors upon a developing microbial population [Zwietering *et al*, 1991; Hudson, 1992; Duh & Schaffner, 1993; Stecchini *et al*, 1993]. Unfortunately, few of the models discussed in the literature have acquired any form of mechanistic explanation. After over 150 years there is still no explanation of why the Gompertz model [Gompertz, 1825], developed to describe human population development in 1825 [Sullivan & Salmon, 1972], gives such a good approximation to population growth. One true test of a model may be found in the ease of relating the model to established theories of the system behaviour. Alternatively, the model may not agree with preconceived concepts of the system. In this instance the model provides a vehicle for new hypotheses of system behaviour and suggests further research and the development of ideas. The fact that the Gompertz

equation may be applied to the growth of different populations, ranging from humans to prokaryotes [Gompertz, 1825; Zwietering *et al*, 1991], suggests that it may have some basis in a range of general population control factors, such as density, rather than any specific 'switch'.

The conception of ideas such as the metabolic control theory developed by Kacser and Burns [1973], and Heinrich and Rappoport [1974] enables the modelling of extremely complex systems by a developmental process. It is unlikely that a complete description of the anastomoses expressed by the metabolism of a single cell could yield a useful model. Due to the level of complexity such a simulation would have greater relevance to computer scientists researching processor multitasking capabilities than biologists.

By evolving an understanding of the controlling factors in a sequence of events and the probable feedback effects upon the system it is possible to determine the most important influencing factor, permitting a second generation model to incorporate the first by concentrating only upon the most relevant factors revealed by the first simulation. This approach has great application in biology since unnecessary variables may be eliminated from the model at an early stage. Another feature of this approach lays in the eliminated variables. If the simulation failed to predict a result it would be possible to return to earlier stages and identify the relevant feature quickly.

6.1 POPULATION GROWTH KINETICS.

A great many growth models have been published in recent years [Ratkowsky *et al*, 1982; Zwietering *et al*, 1990; Duh & Schaffner, 1993; etc.]. For this reason only three models have been compared for the lag phase and growth rate parameters, two from the literature and one new model. Selection of the models has been based upon the results of Zwietering *et al* [1991], and Duh & Schaffner [1993] who compared several different models in an effort to isolate the most appropriate for each growth parameter. A new model, developed during the course of this investigation, has been proposed for each population growth parameter. These new models have been based upon one unifying theory (homeoviscous adaptation [Zaritsky, *et al*, 1985; Herman, *et*

al, 1994]) explaining how temperature affects the initial development, growth and final population density of a prokaryotic population. This theory has also been related to the effects of temperature on the metabolism of the organisms used in this study. It is interesting to note that discussions homeoviscous adaptation were initially limited to eukaryotic organisms. More recently this theory has been discussed in relation to prokaryotic organisms [Nichols, *et al*, 1997].

6.1.1 LAG PHASE.

Lag time data has been shown to present large measuring errors at high numerical values [Zwietering *et al*, 1991]. Consequently, a logarithmic transformation of experimental data has been used throughout this study in order to limit the influence of this factor.

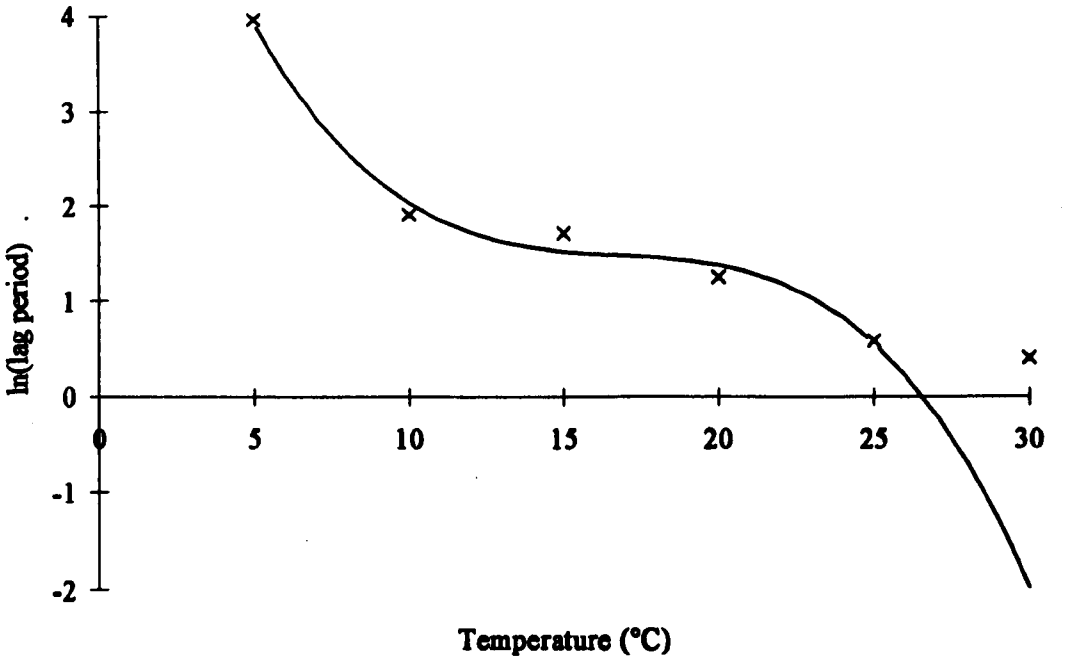

Figure 6.1 Lag time data for *Aer. hydrophila* in pure culture (plotted as ln(lag time(hr))) against temperature (°C). The new third order polynomial model has been fitted to the data from 5°C to 25°C (-).

Taking the chartist approach, it was noticed that the lag phase data of both *Aer. hydrophila* and *Lb. plantarum* (tables 3.3, 4.2, & 5.1) presented a sigmoid pattern when plotted as ln(lag phase) against temperature (°C) (figures 5.2, 5.3, 6.1, and equation 6.1)

A new third order polynomial was fitted to this shape (figure 6.1 and equation 6.1) and this model compared with two models from the literature; the modified Ratkowsky (equation 6.2) [Zwietering *et al*, 1991], and the hyperbolic (equation 6.3) [Gill *et al*, 1988].

$$\ln(L) = f + gT + hT^2 + kT^3 \qquad \text{equation 6.1}$$

Equation 6.1 New Polynomial Model.

$$\ln(L) = \ln[\{b(T - T_{min}) \cdot \{1 - e^{c(T - T_{max})}\}\}^{-2}] \qquad \text{equation 6.2}$$

Equation 6.2 Modified Ratkowsky Model [Zwietering *et al*, 1991]

$$\ln(L) = \frac{p}{(T - q)} \qquad \text{equation 6.3}$$

Equation 6.3 Hyperbolic Model [Smith, 1985; Gill *et al*, 1988]

L is lag time (hours), and T is temperature (°C). $T_{min/max}$ is minimum/maximum temperature at which growth is observed. b ($°C^{-1}\ h^{-0.5}$) and c ($°C^{-1}$) are Ratkowsky parameters. p indicates the decrease of L when T increases. q is the temperature at which $L = \infty$. All other symbols are regression parameters. Temperature is degrees Celsius for equations 6.1 and 6.3, and Kelvin for equation 6.2.

The data used for the model fitting was from the literature and represented three organisms: *Escherichia coli* [Smith, 1985], *Pseudomonas fluorescens* [Adair *et al*, 1989], and *Staphylococcus aureus* [Adair *et al*, 1989] shown graphically in figure 6.2. It was important for initial model validation that the data used was not connected to development of any of the tested models [Yeomans, 1982]. Following validation of

the lag period models, it was possible to use the data from this investigation to test the models used to describe other microbial growth parameters. This was due to the elucidation of a common feature of the models, an inflection point. Comparison of the lag period models was conducted by testing a null hypothesis that the standard deviation of the model from the data was not significantly different from zero. This has been based upon the assumption that the sample data represents the population sufficiently well such that the standard deviation of the sample from the population may be assumed to be zero. Thus the model may be shown to represent the population based upon the standard deviation of the model from the sample data.

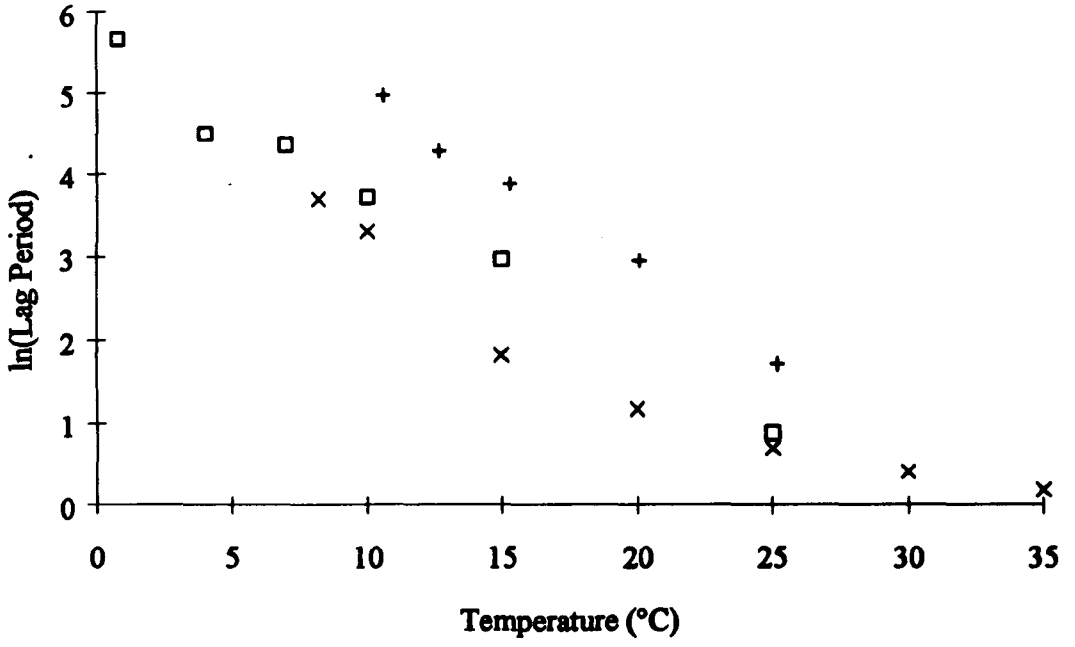

Figure 6.2 Graphical presentation of the lag period data used to test the models in this study. *Escherichia coli* (X), *Pseudomonas fluorescens* (□), and *Staphylococcus aureus* (+).

The test used was based upon Fisher's Z transformation. This is a logarithmic function of r, the coefficient of regression, yielding an approximately normal sampling distribution, even though the sample size may be small [Yeomans, 1982]. Figure 6.3 gives a schematic representation of the normal probability distribution for each independent variable value.

This test was applicable because all standard deviation values, of the models from the sample data, were below one. The standard deviation of the models from the data was determined according to equation 6.4.

When the polynomial model was regressed onto the lag time data from the *Aer. hydrophila* experiments, it was found that the regression coefficient rises from 0.96 to 0.99 if the data is restricted to temperatures below the optimum temperature. Accordingly, only population lag times determined below the optimum growth temperature for the organism concerned were used for testing of models. It was not possible to determine the coefficient of regression for each model since the derivation of these values was beyond the statistical scope of this project.

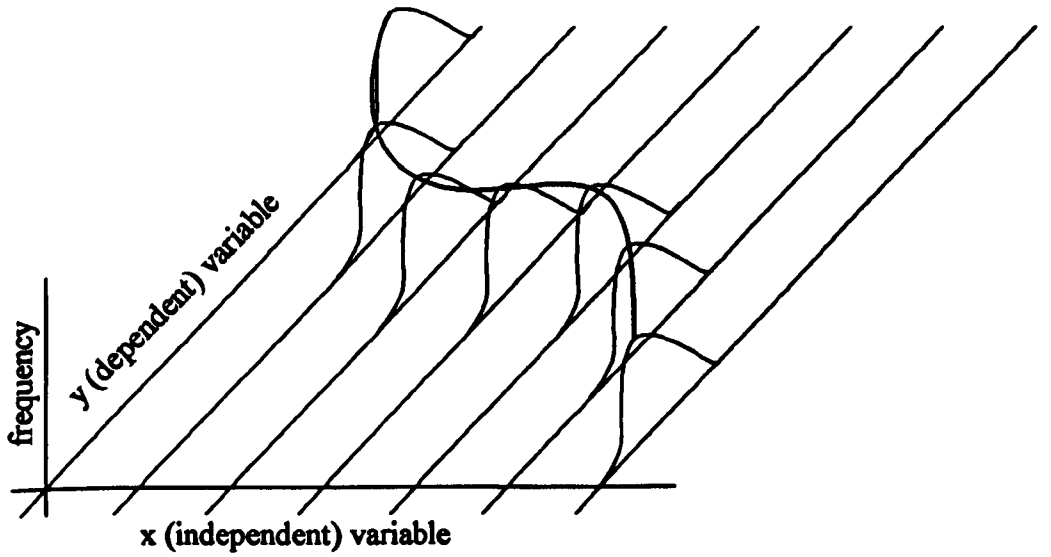

Figure 6.3 A schematic diagram showing the normal distribution of a bivariate population. A third order polynomial (thick line) has been fitted to the data points.

$$\sigma = \sqrt{\frac{ss}{df}}$$

Equation 6.4. Standard deviation of a model from the sample data. Where the degrees of freedom (*df*) = (number of points) - (number of parameters) and *ss* is the residual sum of squares.

The results of this z-analysis show quite clearly that the polynomial model has a better fit to the data than either of the other models for two out of three of the data sets (table 6.2). Control figures were calculated for all data sets, including those of *Lb. plantarum* and *Aer. hydrophila*, figure 6.4. It can be seen from these figures that the variance of the data sets is below the warning control figure with the exception of the data for *Ps. fluorescens*. By removing the first lag time value (288 hours at 0.8°C) from the pseudomonad data set the result can be adjusted to give a three out of three result in favour of the polynomial. The results of this are not presented since it is not a valid procedure within the constraints of the z test but demonstrates the effect of the high measuring error inherent to high lag time values even after logarithmic transformations [Zwietering *et al*, 1991].

Table 6.1. a) Calculated z values for the standard deviation of the fitted model to each of the three data sets and table 6.1b) the corresponding critical z values from a statistical table of the normal deviate [Yeomans, 1982].

a)

z_{calc}	Ratkowsky	**Polynomial**	Hyperbolic
Escherichia coli	0.417	**0.213**	0.709
Ps. fluorescens	2.065	**0.413**	1.327
Staph. aureus	0.918	**0.155**	0.527

b)

z_{tab}	Ratkowsky	**Polynomial**	Hyperbolic
Escherichia coli	0.337	**0.417**	0.239
Ps. fluorescens	0.019	**0.341**	0.092
Staph. aureus	0.179	**0.440**	0.298

These results have shown that the standard deviation of the polynomial model from the sample data was not significantly different from zero and the variation from zero of the two models from the literature has been shown to be significant. Thus, the polynomial model provides a better description of population lag time response to sub-optimum temperature than either of the published models considered here.

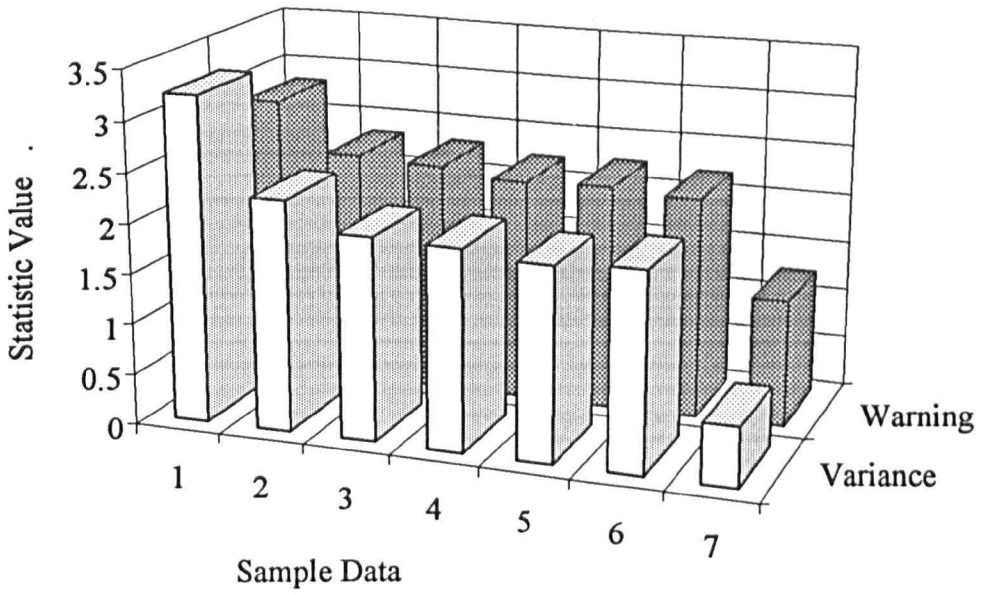

Figure 6.4 Sample data variance and warning statistic value (shaded) for 1) *Ps. fluorescens*, 2) *E. coli*, 3) *Staph. aureus*, 4) *Aer. hydrophila* (pure culture), 5) *Lb. plantarum* (pure culture), 6) *Aer. hydrophila* (mixed culture), 7) *Lb. plantarum* (mixed culture)

6.1.2 GROWTH RATES.

As with the validation of the lag period models (section 6.1.1) two previously published models were selected from the available literature and used to test the validity of the new growth rate model presented here. The models selected for comparison as descriptors of microbial growth rate behaviour at varying temperatures were:

1. Schoolfield (equation 6.5) [Schoolfield *et al*, 1981]
2. Modified Ratkowsky (equation 6.6) [Zwietering *et al*, 1991]
3. Asymmetric Sigmoid Curve. (equation 6.6)

The Schoolfield model is based upon absolute reaction rate theory and has been tested in many papers [Adair *et al*, 1989; Zwietering *et al*, 1991; Duh and Schaffner, 1993;]. It was constructed under the following assumptions: a) The total amount of all compounds in the cell is constant (i.e. balanced growth), and only one enzyme is

rate controlling. The rate controlling enzyme may be reversibly denatured at high and low temperatures. b) The total amount of rate controlling enzyme per cell is constant. c) The reaction rate of the cell growth controlling enzyme is zero order. d) The enzyme reaction and both the high- and low-temperature inactivation show an Arrhenius type of temperature dependency. These assumptions were put forward by Sharpe *et al* [1977], and Sharpe & DeMichele [1977], producing a model with highly correlated parameters. Schoolfield *et al* [1981] adapted this model and reduced the correlation between parameters resulting in equation 6.5.

$$\mu = \frac{\rho_{25} \frac{T}{298} e^{\left[\frac{H_A}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right]}}{1 + e^{\left[\frac{H_L}{R}\left(\frac{1}{T_{1/2L}} - \frac{1}{T}\right)\right]} + e^{\left[\frac{H_H}{R}\left(\frac{1}{T_{1/2H}} - \frac{1}{T}\right)\right]}}$$

Equation 6.5 The Schoolfield growth model [Schoolfield *et al*, 1981]. ρ_{25} is the growth rate μ at 25°C, T is the temperature, R is the universal gas constant, H is the enthalpy of activation, $T_{1/2L}$ and $T_{1/2H}$ are the low and high temperatures at which the enzyme is 50% inactivated respectively.

The Ratkowsky square root model was first developed for use in growth rate description [Ratkowsky *et al*, 1982] and later adapted to model the lag phase [Zwietering *et al*, 1991] (section 6.1.1). It has no theoretical or mechanistic foundation and was based on the observation that at lower temperatures the square root of the growth rate is linear with temperature [Ratkowsky *et al*, 1982; Zwietering *et al*, 1991].

$$\mu = [b(T - T_{min})]^2 \cdot [1 - e^{c(T - T_{max})}]$$

Equation 6.6 The modified Ratkowsky square root growth model [Zwietering *et al*, 1991]. T_{min} and T_{max} are the lower and upper temperatures where the growth rate μ is zero, *b* and *c* are regression coefficients.

The new model presented here is an asymmetric sigmoid curve. Selection of this model was based upon the chartist approach, as for the Ratkowsky and lag phase polynomial models. A symmetric model was also considered however, a consideration of the basic effects of temperature upon microbial growth soon yields the conclusion that the temperature dependant microbial growth rate profile must be asymmetric. Figure 6.4 shows that $\Delta\mu$ decreases above the optimum growth temperature with the new model fitted.

$$\mu = \mu_{min} + \frac{\mu_{max} - \mu_{min}}{(1+(\frac{T}{T_i})^{-p})}$$

Equation 6.7 The new growth model presented here. T_i is the inflection point of the asymmetric sigmoid curve (°C), μ_{min} and μ_{max} are the minimum and maximum growth rates ($hour^{-1}$), respectively, p is a regression constant.

Each of the models so far selected to describe growth rates has an inflection point. The polynomial model selected in section 6.1.1 also reveals an inflection point in the effect of temperature upon bacterial lag phases. The similarity between the inflection point of the growth rate and lag phase models has significance to the mechanistic explanation for the use of these models. As discussed in section 3.4, the most efficient temperature for growth is not the optimum temperature. A comparison of the inflection points in tables 6.2 and 6.3 shows that there is good agreement between the values for *Aer. hydrophila* in pure and mixed culture for both lag phase and growth rate analyses.

The discrepancy between the values for the lag phase and growth rates of *Lb. plantarum* is due to the use of data up to, but not beyond, the optimum growth temperature, i.e. 30°C, for the growth rate models. This has the effect of increasing the predicted maximum growth rate and thus shifting the model inflection point. In mixed culture this effect has been reduced by the competition for substrate existing between the two organisms with the obvious effect that this would have upon the potential growth rates of *Lb. plantarum* at the temperatures studied.

Use of the statistical comparison between models from section 6.1.1 revealed that all the models tested (equations 6.5 to 6.7) yielded the null hypothesis result. Thus the sample standard deviation was not significantly different from the population standard deviation (assumed to be zero). The results of this analysis showed that the Schoolfield model was the least applicable by comparison of differences between $z_{calc.}$ and tabulated values of the z statistic. This result was weighted by the effect of fitting 6 parameters for the Schoolfield model, compared to only 4 for the asymmetric and Ratkowsky models, to relatively few data points.

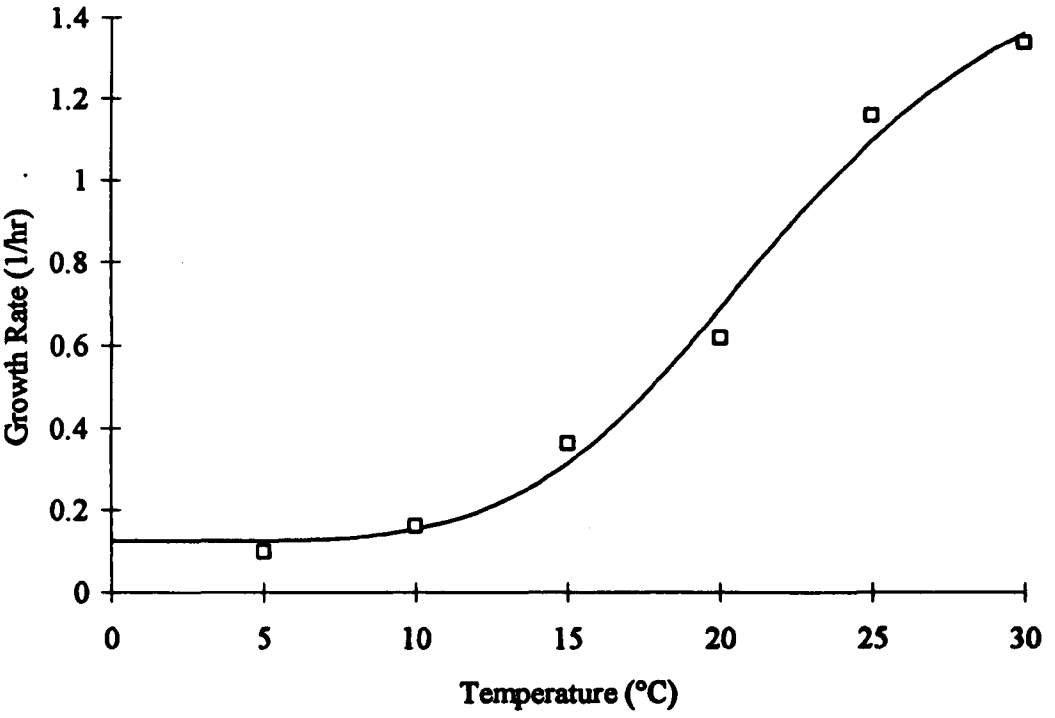

Figure 6.5 Growth rate data from *Aer. hydrophila* in pure culture at various temperatures. The asymmetric sigmoid curve (equation 6.7) has been fitted to the data (—).

Following this result an F-test was used to compare the variances of the models to the variance of the observations. The results of this test again showed that the model variation from the model mean value was not significantly different from the variance around the mean of the observed values. Taking this proof of homoscedasticity, a t-test was then performed to compare the model predicted means

with the experimental observations. This test confirmed that, with respect to growth rates, all three models provided reasonable fits to the experimental data.

Table 6.2 Inflection points (°C) for the polynomial model fitted to ln(lag time) data for *Aer. hydrophila* and *Lb. plantarum* in pure and mixed culture.

Inflection point (°C)	Pure Culture	Mixed Culture
Aer. hydrophila	21.11	20.72
Lb. plantarum	23.43	19.64

Table 6.3 Inflection points (°C) for the asymmetric sigmoid model fitted to growth rate data for *Aer. hydrophila* and *Lb. plantarum* in pure and mixed culture.

Inflection point (°C)	Pure Culture	Mixed Culture
Aer. hydrophila	22.25	22.00
Lb. plantarum	234.63	37.80

The conclusion that there is statistically no significant difference between the Schoolfield and the modified Ratkowsky model was also reached by Zwietering *et al* [1991] who selected the Ratkowsky model since it had fewer parameters. It is concluded that there is little or no difference between these models when applied to these data sets. It should be noted that the asymmetric sigmoid model does not allow expression of any high temperature decrease in growth rates and, as such, is not a useful model when applied to growth rate samples incorporating temperatures above optimum which clearly inhibit growth. The two published models do incorporate terms for this effect and so must be deemed equally useful.

6.1.3 Final Population Level.

Few researchers have considered the final population size. It has little application to the field of predictive microbiology since the attentions of workers in

this field are normally aimed at predicting the initiation and rate of microbial growth. Accurate predictions for time taken to achieve a given population level are of greater interest since this has more relevance to the food and medical industries than prediction of the potential final population size.

Examination of the ln(final population level) plotted against temperature revealed a non-linear pattern (figures 6.6a & b) for both *Aer. hydrophila* and *Lb. plantarum*. The curvature is clearest for the *Lb. plantarum* data which displays a distinct curvature. A parabolic curve was fitted to this data in order to provide an estimate of the population maxima. The maximum for *Lb. plantarum* pure and mixed culture data was 23.4°C and 23.9°C respectively.

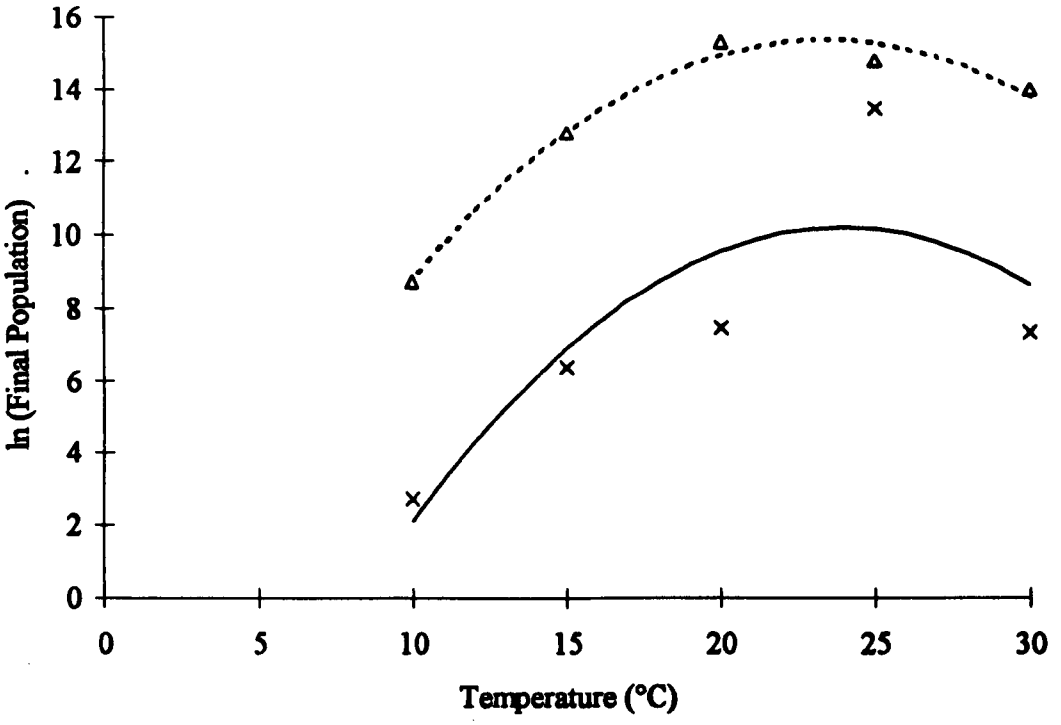

Figure 6.6a The effect of temperature upon final population levels of *Lb. plantarum* in pure (Δ) and mixed culture (X) with the quadratic curve fitted, pure (---) and mixed (—).

These values correspond with the inflection points for the lag phase polynomial model. The *Aer. hydrophila* final population data did not conform to this pattern as closely as the data from the *Lactobacillus*. The aeromonad pure and mixed culture

maxima were 17.5°C and 10.6°C respectively. The data for both organisms shows a decrease from 25°C to 30°C. The specific growth rate for the organisms over this same temperature change does not show a decrease. Rather there is a slight increase as each organism approaches or passes the optimum growth temperature. However the $\Delta\mu$ showed a decrease from 25°C to 30°C compared to the same size increment from 20°C to 25°C. The decrease in final population level signifies a decreased growth efficiency as the organism approaches the optimum growth temperature and represents further support for the idea that maximum growth efficiency does not yield maximum growth rate, as stated in sections 3.4 and 6.1.2.

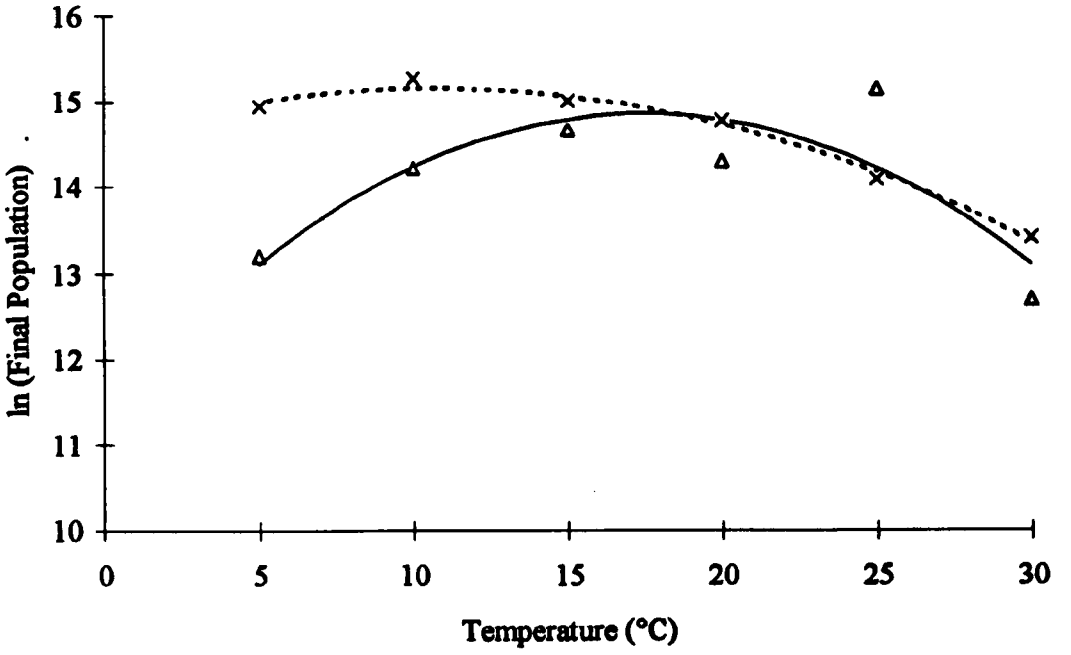

Figure 6.6b The effect of temperature upon final population levels of *Aer. hydrophila* in pure (Δ) and mixed culture (X) with the quadratic curve fitted, pure (—) and mixed (---).

At temperatures below 25°C there is no significant variation in the final population levels observed for *Aer. hydrophila*. The effect of the decreasing temperature on *Aer. hydrophila* may have been cushioned by several factors, such as the nutritional versatility of the psychrophilic aeromonad and the differences between the cell wall structures of Gram positive and Gram negative bacteria.

It was shown in section 3.4 that the yield of *Lb. plantarum*, based upon either dextrose use or lactate production, fluctuated with temperature (figure 3.9). These fluctuations reached a maximum between 15°C and 25°C. It has been repeatedly demonstrated that the optimum temperature is not the most efficient temperature for growth and this data concerning final population levels again supports this hypothesis. The hypothesis of Wiebe *et al.* [1992], that decreased temperatures caused a decrease in population energy efficiency concurs with the results presented here showing the peak efficiency to be below the recognised optimum temperature but well above the minimum temperatures investigated by these workers.

The data for the aeromonad final population level in pure culture shows very little variation from that of the mixed culture. The final population data from *Lb. plantarum* shows a marked difference between the pure and mixed cultures. This is the result of a stationary phase population of *Aer. hydrophila* switching to carbohydrate utilisation whilst the lactobacilli were still in growth phase. The resulting competition caused a reduction in the available carbohydrate and so effectively decreased the potential final population levels of *Lb. plantarum*.

6.2 METABOLISM.

Measurement of the effect of temperature upon the uptake of dextrose was performed by determining the rate of dextrose uptake, Δ[D]. The dextrose profile obtained from the HPLC analyses was inverted and the Gompertz growth model fitted to the resulting data [Membré & Tholozan, 1994]. As can be seen from figure 6.5 the Δ[D] shows a marked peak between 20°C and 30°C.

It was shown in chapter 4 that *Aer. hydrophila* utilised the available carbohydrate only after entering the stationary phase of population growth. In mixed culture, as previously shown, there was little or no difference between the growth parameters of either organism when compared to the pure culture data. This meant that the aeromonad achieved stationary phase long before the lactobacillus and resulted in competition for the available substrate. Obviously the effect of 10^9 cfu/ml switching from amino acid to carbohydrate utilisation had a marked effect upon the apparent

dextrose uptake rate. This is represented in figure 6.7 by the difference between the pure and mixed culture data points. The differences between these two profiles also has significance. The numerical difference between the pure and mixed culture data from figure 6.7, shown in figure 6.8, reveals that the ΔD of the stationary *Aer. hydrophila* population peaks in the same temperature band as the inflection points shown revealed by the growth parameter models.

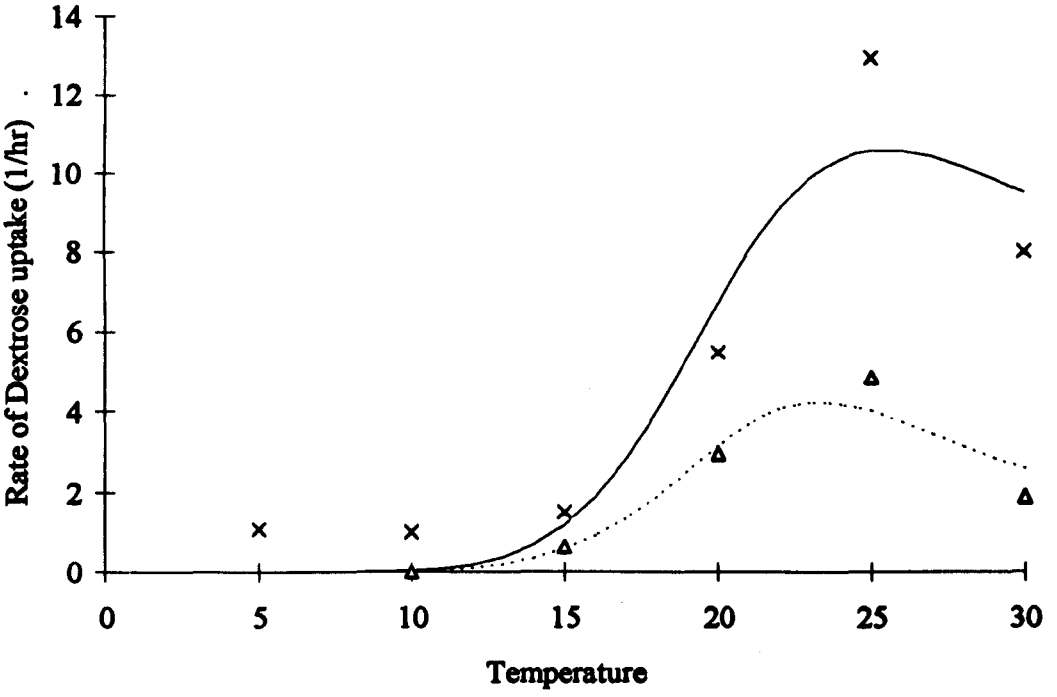

Figure 6.7 The effect of temperature upon the rate of dextrose uptake in pure culture (Δ) with *Lb. plantarum* and mixed culture (X) with *Lb. plantarum* and *Aer. hydrophila*. The Schoolfield growth model has been fitted to both data sets as this model contains expressions for substrate utilisation.

In figures 6.7 and 6.8 the Schoolfield model has been fitted to the data. This model was selected since it was based upon basic reaction rate and so more suited to the physical uptake rate than the modified Ratkowsky model which has no mechanistic or theoretical explanation [Ratkowsky *et al*, 1982]. These two figures demonstrate the non linear uptake rates by both the Gram positive and the Gram negative organisms

and further reinforce the conclusion that the most efficient temperature for growth and the optimum growth temperature have disparate values.

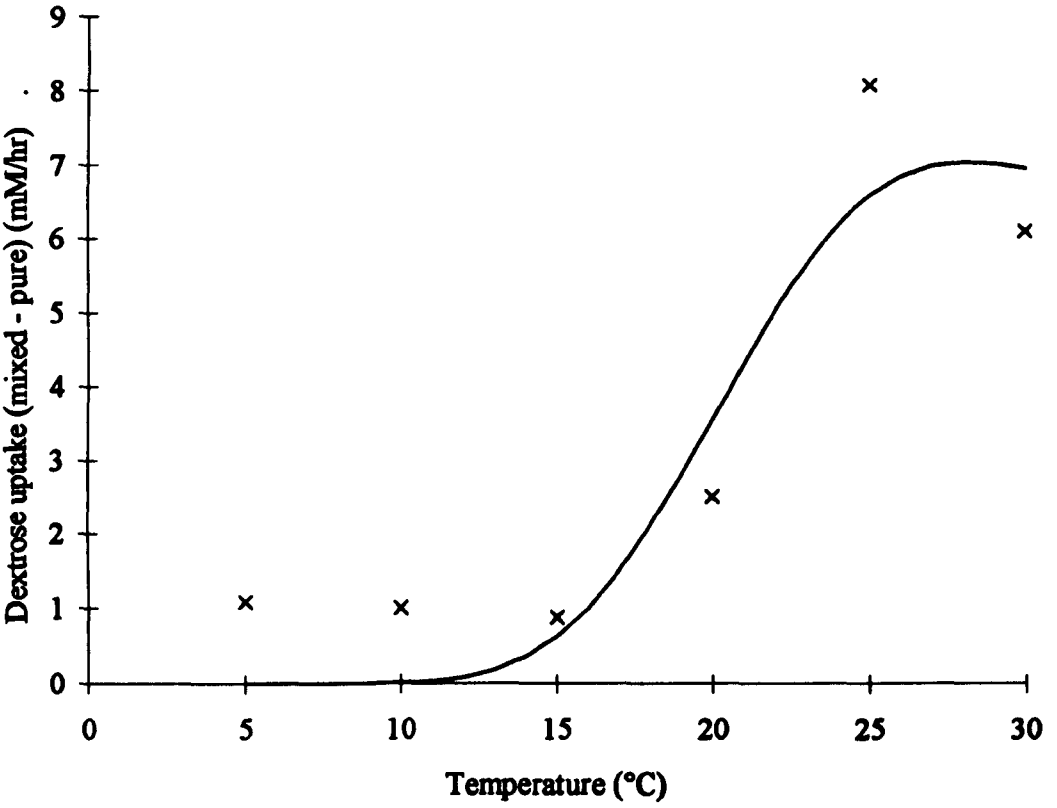

Figure 6.8 The difference between dextrose uptake rate in pure (*Lb. plantarum*) and mixed (*Lb. plantarum* with *Aer. hydrophila*) culture (Δ). The difference between the Schoolfield model estimates for the pure and mixed cultures has also been included (—). The non linearity of this curve has been attributed to variation in the uptake rate by each of the organisms at various temperatures. As can be seen from the graph there is a clear maximum between 20°C and 30°C.

6.3 DISCUSSION.

As stated in section 6.1 a hypothesis has been developed which provides an explanation for the use of models which exhibit a point of inflection. The hypothesis has been based upon the theory of homeoviscous adaptation and may be stated thus:

'The response of a prokaryotic population to fluctuations in environmental variables is based upon adaptations to the plasma membrane which results in changes to the fluid properties of the membrane. This causes changes in membrane transport efficiencies.'

The application of this theory to the profile of the lag phase against temperature has been based on the following assumptions:

1. The changes represent an optimisation of membrane function under the extant conditions, as proposed by the theory of homeoviscous adaptation.

2. Given that the changes in membrane composition affect the fluidity there should be an effect upon the function of proteins within the membrane. This would be the result of the function relating the solubility of a protein within a membrane to both the membrane composition and the protein conformation. Thus, changes in membrane composition must affect the physical performance of dynamic molecules such as transport proteins.

Applying these ideas to the lag phase polynomial explains the decrease in the rate of lag time change (ΔL) as the temperature approaches the inflection point. When the temperature increases the membrane adapts and becomes more fluid, and transport proteins would become more soluble. An increased number of transport proteins would convey a more rapid development of the cell into the growth phase since there would be no lack of substrates within the cell coupled to an increased metabolic activity due to the increasing temperature. The further increase in ΔL beyond the inflection point would be the result of further membrane adaptation to approach the maximum transport rate coupled to decreased metabolic efficiency. It should be borne in mind whilst considering the membrane fluidity changes that increased temperature must cause increased reaction rate kinetics. This would result in an apparent decrease in population lag phase duration up to the point of temperature inactivation.

The hyperbolic model proposed by Gill *et al* [1988] provides a suitable explanation for the lag phase based upon the idea of membrane fluidity adaptation. However, the physiological reasoning for this model does not explain the patterns observed for growth rates or final population levels nor does it allow for the gel-phase transitions inherent to the fluid mosaic model of membrane structure. The modified Ratkowsky model has little or no basis in biology and was developed using the chartist

approach. There is still no published theory to explain why this model fits either the lag time or growth rate data range. It should be understood that, by definition, there is an obvious inverse relationship between these two parameters since as lag time decreases the growth rate increases. If this were a true inverse relationship then an hyperbolic curve would describe a curve of μ against ln(Lag) very closely. This cannot be shown and despite the obvious similarities it should be assumed that the relationship has a more complex nature than just a unit inversion from hours to per hour. It must be assumed that whatever the reason for the fit of this model to either the growth rate, as originally proposed, or the lag time the relationship to the second parameter is based purely upon the simplistic inversion of units and does not represent a true description in spite of the statistical evidence.

The third order polynomial is a better description of the lag phase than the hyperbolic because it describes a rate of change with an inflection point which has been related to the rate of adaptation to changing temperature during the whole population growth profile and the rate of substrate uptake. It can also be stated that the modified Ratkowsky model has an inflection point however, the structure of this model does not allow for inflection over a data range as narrow as the growth temperature range of a bacterial population. Thus it must be concluded that the polynomial model provides the better description of population adaptation than either the hyperbolic or modified Ratkowsky models. This conclusion has a statistical basis and has been shown to concur with the physiological processes of lag time adaptation and population growth.

It was not possible to differentiate between the growth rate models statistically. The modified Ratkowsky model and the Schoolfield model were deemed to be the most suitable as they contain a function to describe high temperature cell inactivation. It may be that the Schoolfield description is closer purely because it has a greater number of parameters, six, than the modified Ratkowsky model, which has four. Although this argument must have some bearing on the discussion, it cannot be disputed that the rate of growth is generally considered to be related to one rate controlling process. The correlation between the inflection point found with the lag and growth models leads to the conclusion that this process may be linked to the membrane transport functions. The Schoolfield model has a physiological basis linked to the basic reaction rate kinetics of the Arrhenius equation [Arrhenius, 1889]. The effect of temperature upon metabolism was shown for *Lb. plantarum* in pure and mixed culture

(figure 6.7) but only be deduction in mixed culture for *Aer. hydrophila* (figure 6.8). Once again it was shown that an inflection point existed and that this point was below the recognised optimum growth temperature. The correlation between the inflection points found for the final population level, growth rate, lag phase and metabolism suggests that the adaptations resulting in these growth parameter profiles are inextricably linked to an efficiency factor in the growth and development of a prokaryotic population. The concept of growth kinetics relating to membrane fluidity explains the existence of an inflection point in the rate of population adaptation if one considers the gel phase transitions involved and the effect of this upon membrane protein solubility. This idea was implied by Wiebe *et al*, [1992] who proposed that organisms had a higher energy requirement growing at reduced temperatures. It is proposed here that the efficiency of the cell or a cell component is compromised at temperatures above and below the inflection point, resulting in an elevated energy demand by the cell.

It has been shown for several organisms that adaptation to adverse environmental temperatures is characterized by alteration of the fatty acid composition of the membrane lipids in order to maintain the membrane fluidity [Russell, 1984; McElhaney, 1993; Goverde *et al*, 1994]. The usual pattern is a shift towards a higher long chain, saturated fatty acid content with increased temperature [Goverde *et al*, 1994]. The overriding result of the use of models to investigate the physiology of these organisms has been the development of a unifying theory of growth, relating the processes of population development and final maturity to one rate defining process such as trans-membrane substrate transport.

7.0 CONCLUDING DISCUSSIONS.

7.1 METHODS AND MEDIA.

The final aim of this project was production of a model to describing the effect of temperature upon a mixed population of bacteria with relevance to the food industry. It was decided that this mixed population should comprise two organisms. A lactic acid bacterium due to the importance of this bacterial group in food production and spoilage. The second organism was selected due to the developing interest in *Aer. hydrophila* as both a pathogen and agent of food spoilage [Ketover *et al*, 1973; Gray, 1984]. It was perhaps unwise to select an organism about which so little was known, especially when metabolic interactions were to be an integral part of the investigation. The possible problems connected to the selection of the aeromonad came to light during the selection of a medium suitable for the growth of both organisms. The results from this study showed that *Aer. hydrophila* was susceptible to oxygen limitation (section 4.1) and revealed that the organism would not utilise dextrose as a primary substrate under the conditions tested (section 4.3). The final choice of medium was based upon the simplicity of the formulation which was intended to minimise Maillard reaction products whilst providing a nutrient source sufficiently complex to support the growth of the fastidious *Lb. plantarum* population. In order to reduce the level of Maillard reaction products formed during sterilisation the level of dextrose was reduced from 2% w/v to 0.5% w/v. An earlier study at these laboratories had shown that this level of carbohydrate would support the lactobacilli [Bobillo, 1991]. There was expected to be a slight decrease in cell growth efficiency associated with this decrease in available carbohydrate [Bobillo, 1991]. This was not expected to affect the project because there would be no variation of carbohydrate levels throughout the population growth trials.

The use of vortex mixers to disrupt chains of bacteria for viable count estimation has been widely used [Adams *et al*, 1991; Zwietering *et al*, 1991]. It is

generally accepted that not all chains will completely disrupt. It has been shown in this study that the use of a 28G syringe produces a greater degree of chain disruption with little or no reduction in population viability (table 3.2). This meant that the estimated viable count was closer to the actual level than could have been estimated from samples prepared with a vortex mixer. This was important for the project aims as it meant that the fitted growth parameters would be less susceptible to experimental error.

Another factor important to the reliability of growth parameter estimates was the initial population level. The time taken for a low initial population to develop to the final population level is obviously greater than that for a high initial count. The longer time base gives the population a higher likelihood of achieving the maximum specific growth rate before the culture conditions could induce any deceleration of growth rate into the stationary phase.

7.2 THE EFFECTS OF TEMPERATURE UPON POPULATION DEVELOPMENT OF *AEROMONAS HYDROPHILA* AND *LACTOBACILLUS PLANTARUM.*

A reduction of lag phase was the expected pattern with increasing temperature for both organisms. This was observed and as expected it was found that the relationship was non-linear (figs 4.3, 5.2 - 5.3). The available data from the literature revealed the same effect of temperature upon both organisms [Zwietering *et al*, 1991; Hudson, 1993]. It was observed that a third order polynomial curve fitted the data pattern for ln(lag period) against sub-optimum temperature. This curve revealed the existence of an inflection point which has not been previously reported. It was proposed that this inflection point related to the adaptation of the cell membrane since this represents the interface between the organism and the environment. It was beyond the scope of this project to determine this, however several workers [Russell, 1984; McElhaney, 1993; Goverde *et al*, 1994; Bowden, *et al*, 1996] have reported that extensive adaptations occur within the cell membrane during temperature adaptation.

Increasing temperature towards optimum increased the growth rate of the bacterial populations. This was also expected and a comparison of the data for *Lb. plantarum* revealed the same underlying pattern of growth rate behaviour as had been reported in the literature [Zwietering *et al*, 1991]. Although the actual values observed in this report were lower than the literature this was expected due to the different carbohydrate levels in the two media used (section 7.1). The overall effect of temperature upon the growth phase was found to be well described by either of two published models (section 6.1.2). The Schoolfield model was selected here because it was originally developed to relate the population growth rate to the cell function and chemical reaction rates. Zwietering *et al* [1991], when faced with a similar choice, selected the modified Ratkowsky model due to the lower number of parameters and greater simplicity of the model compared to that of Schoolfield *et al* [1981]. Although these are valid arguments, the increasing use of computers in modern research has meant that model complexity has become less relevant than several years ago. The number of parameters required by a model can be shown to relate to the degree of fit that the model describes. The effect of one more, or less, parameter is greatest when fitted to small data ranges. It was on this basis that Zwietering *et al* [1991] made their decision. The Ratkowsky model has no basis in theoretical biology [Ratkowsky *et al*, 1982] and so was discarded in favour of the Schoolfield model which was derived from absolute reaction rate theory.

The pattern of the temperature effect upon the final population level was not predicted. The hypothesis of Wiebe *et al* [1992] suggested that the final population level might have been expected to decrease at lower temperatures. Following this pattern one might have surmised that a similar decrease would occur at temperatures above the optimum growth temperature owing to high temperature inactivation. The appearance of such a decrease below the optimum growth temperature revealed a parabolic data pattern (section 6.1.3). This provided support for the use of the third order polynomial to describe the lag phase since the inflection point of both the third order polynomial lag model, and the parabolic final population model were similar. The decrease in final population level either side of the inflection point was highly apparent for the lactobacilli however, the aeromonad did not display such an obvious adaptation pattern. This may have been because the organism is psychrophilic although that might indicate that a sharp decrease would be observed at the higher

temperatures. This was not observed. The final population level of the aeromonad only showed suppression when the organism was grown at 30°C, slightly above the optimum temperature for this organism. The cell wall structure of *Aer. hydrophila* is markedly different from that of *Lb. plantarum*. This difference may have provided the Gram negative aeromonad with the means to withstand the variation of growth temperature to enable maximum growth potential at all temperatures observed.

7.3 THE EFFECT OF TEMPERATURE UPON THE METABOLISM OF *AEROMONAS HYDROPHILA* AND *LACTOBACILLUS PLANTARUM.*

The effect of temperature upon metabolism could only be quantified for *Lb. plantarum*. This was due to the nutritional versatility of *Aer. hydrophila*. Van der Kooij and Hijnen [1988] have shown that *Aer. hydrophila* does not use glucose in the presence of amino acids. A paper chromatographic method was used to prove that *Aer. hydrophila* B184 was utilising amino acids after it had been established that the organism was not utilising the available carbohydrate as primary substrate. Including the metabolism of this organism in the study would have meant use of commercial amino acid analyses and so was not possible due to financial constraints. Amino acid analysis was performed upon samples taken from a culture of *Aer. hydrophila* grown at 25°C. The results showed that *Aer. hydrophila* was utilising more than one amino acid during the growth phase. Although the catabolism used by *Aer. hydrophila* was not elucidated, it was possible to propose a pathway which would lead to the build up of urea (figure 4.9) from the known fluctuations in amino acid levels at 25°C.

It was shown that the principle end products of the aeromonad catabolism were urea and ammonia. The urea had been hard to detect due to the neutral nature of the compound. A chemical assay was developed which showed the build up of urea in the medium (fig 4.8). Unfortunately it was not possible to study the effects of temperature on the catabolites of *Aer. hydrophila* due to time constraints.

The metabolism of *Lb. plantarum* has been well studied [e.g. Murphy and Condon, 1984 a & b; Sedewitz *et al*, 1984; Montville *et al*, 1987a & b]. The organism

has been shown to express both homofermentative and heterofermentative metabolism under various conditions [section 5.2.2]. It was found that this lactic acid bacterium utilised an homofermentative metabolism during growth studies in single culture but switched to heterofermentative metabolism when grown in mixed culture with *Aer. hydrophila*. This switch was characterised by a switch in the [Lac]:[Ac] ratio of spent medium as detected by HPLC analysis. The cause for this change may have been any combination of several factors. It has been proposed that this change can be induced by limitation of glucose or oxygen, or both. In mixed culture the aeromonad showed no signs of oxygen limitation thus, the most probable cause for the change in basal metabolism of the *Lb. plantarum* was the decrease in dextrose concentration caused by the aeromonad utilising the available carbohydrate after entering stationary phase whilst *Lb. plantarum* was still in early exponential phase. Oxygen limitation was not determined but may have been responsible due to the spatial distribution of the two organisms in mixed culture. The aeromonad was motile and so evenly distributed throughout the medium however, the *Lb. plantarum* population was not. This probably resulted in development of an oxygen gradient from the surface of the medium downwards. Owing to the very high surface area : volume ratio (section 4.1) this effect was minimised but cannot be completely dismissed. The growth rate of *Lb. plantarum* was not decreased by the competition for dextrose as shown by the comparison of pure and mixed culture growth parameter estimates (section 5.1). The only observed effect was reduction of final population level (figs. 6.6 a & b). These results supported the finding of Bobillo [1991] who showed that although 0.5% w/v dextrose was limiting to total population yield it did not affect the growth rate. If the concentration of the substrate had been sufficiently low to affect the growth rate then statistical analysis would have revealed a significant difference between single and mixed culture estimates of μ. This would have meant that the nutrient supply was insufficient to satisfy all of the metabolic demands made by the developing population. That this was not the case implied that the nutrient carrier sites were fully occupied [Brock, *et al*, 1984]. Establishment of this fact was important for the proposed theory governing the underlying mechanisms behind the effect of temperature upon the growth profile of prokaryotic populations.

7.4 MIXED CULTURE INTERACTIONS.

The yield of *Lb. plantarum* could only be estimated for the single culture studies. This was because the aeromonad was utilising both primary substrate and catabolites of the lactobacilli in mixed culture. It was interesting to note that there was no detectable effect of this upon the aeromonad population development. The only apparent effect was upon the final population of *Lb. plantarum* as discussed above. Another factor in suppression of the final *Lb. plantarum* population level in mixed culture was the pH profile exhibited by *Aer. hydrophila* in single and mixed culture experiments where the pH level never dropped below pH 5.0 and was above pH 7.0 after population growth. The one exception to this rule was the mixed culture experiment at 30°C. During this growth experiment the pH fell to below pH 4.0 suggesting dominance of *Lb. plantarum*. As shown earlier there was no significant variation between the population parameters for mixed and single culture studies. The final population level of the aeromonad was lower at 30°C than at 25°C in both single and mixed culture. This suggested that for some reason either the aeromonad had not utilised the acidic catabolites of the lactobacilli or, that the lactobacilli had a secretion rate of acid end products much higher during growth at 30°C than 25°C. It can be seen from the lactate balance line on figure 4.1 that the aeromonad was capable of taking up lactate at the rate of approximately 6-7mmol/hour (assuming a population of $1x10^9$cfu/ml). If the lactate supply rate exceeds this capacity then the lactic acid level will increase and the pH decrease. Since 30°C is the optimum temperature for *Lb. plantarum* and above the optimum temperature for *Aer. hydrophila* it was concluded that the lactate secretion rate at the higher temperature exceeded the lactate uptake capacity of the aeromonad. It was concluded that this result was caused by a combination of decreased growth efficiency of *Lb. plantarum* , yielding a decreased [D]:[Lac] ratio, and the effect of exceeding the optimum growth temperature of *Aer. hydrophila*.

7.5 THE EFFECT OF TEMPERATURE ON POPULATION DEVELOPMENT : APPLICATION OF MATHEMATICAL MODELS.

As discussed in chapter 6 the effect of temperature upon a prokaryotic population can be related to changes in the membrane composition [Foot *et al*, 1983; Gliozzi *et al*, 1983; Russel, 1990; Killian *et al*, 1992; Puttmann *et al*, 1993]. Several mathematical models were shown to describe the effects of temperature upon the bacterial growth and metabolic profile [sections 6.1 and 6.2]. The common factor between the models describing the effect of temperature upon lag phase and growth rate was the description of an inflection point below the optimum growth temperature. This inflection point has been related to the growth efficiency of *Lb. plantarum*. Due to time and equipment constraints it was not possible to perform the necessary analyses relating the inflection points to the growth efficiency of *Aer. hydrophila*.

It has become generally accepted that there is one rate controlling step which governs the length of the lag phase and the growth rate, although the control for these two parameters may not be embodied by the same system. This idea has been based upon application of the Arrhenius temperature dependency model and Monod kinetics to explain cellular growth. The correlation between the various inflection points revealed by this study indicates that the rate controlling factor is related to the membrane composition and that it governs the whole microbial growth profile. The changes in membrane composition associated with the adaptation to varying temperature have been related to membrane fluidity [Leckband *et al*, 1994]. These changes in the membrane composition and fluidity affect the ability of the substrate carriers to operate. This in turn regulates the performance of the cell. The extent of these membrane changes, termed homeoviscous adaptation, regulates the length of time taken for a cell to develop from the lag phase into the growth phase and, by affecting the membrane transition temperature, the maximum specific growth rate and the final population level through the efficiency of the growth process.

The Schoolfield model used extensively throughout this investigation was based upon four assumptions. These assumptions provide a physiological explanation for the effect of temperature upon the bacterial growth profile. The assumptions may

be set out as follows. a) Each cell is in balanced growth, i.e. all compound concentrations within the cell are constant and, as previously stated, only one enzyme is rate controlling. This enzyme may be reversibly denatured by either high or low temperature. b) The concentration of the rate controlling enzyme is constant. c) The rate controlling enzyme has a zero order reaction rate. d) The enzyme reaction and both the high and low temperature inactivation show an Arrhenius type temperature dependency [Zwietering *et al*, 1991].

The first of these assumptions provides a base line for the model. The important statement of this assumption is that the enzyme may be reversibly denatured. The model presented here is based upon the known effect of temperature upon membrane fluidity [Farkas, *et al*, 1994; Leckband, *et al*, 1994]. This increase in fluidity, with increasing temperature, represents an adaptation towards an optimum level of performance and is termed homeoviscous. It is the result of a changing ratio of the long chain, saturated and cyclic fatty acid to short chain, unsaturated fatty acid components [Goverde, *et al*, 1994]. It is proposed that this changeover characterises the inflection point owing to homeoviscous adaptation and the distance between the external temperature and the membrane phase transition temperature. During the growth phase the growth rate of a microbial population is constantly changing. The maximum specific growth rate represents the maximum level obtained during the population development and is located at the inflection point of the growth curve. The rate of growth is generally considered to be dependent upon one rate controlling enzyme catalysed reaction as stated in the above assumptions. The modification of the membrane structure results in changes to the permeability [Russel, 1990; Goverde *et al*, 1994], dynamics, and transmembrane potential [Ter-Kuile *et al*, 1992; Bulthuis *et al*, 1993]. These changes cause a variation in the degree of protein solubility, which is based upon factors such as van der Waals forces, hydrophobic interactions and short range strong, specific, adhesive binding, and protein activity due to the conformational changes associated with substrate binding [Leckband *et al*, 1994]. Such changes cause variations in the substrate transmembrane transport rate since the effects apply to all proteins dissolved within the membrane. It was shown that the effect of temperature upon growth rate also revealed an inflection point [figure 6.4] and that this inflection point was close to that described by the lag phase polynomial model [tables 6.2 & 6.3]. The existence of the inflection point in both the lag phase and growth rate descriptions

implies a relationship and this may be connected to the changes in membrane composition. The effect of temperature upon the final population level also shows a dependence upon this inflection point which suggests a connection to cell growth efficiency. This relationship to cellular efficiency may be explained by the difference between the transition temperature (T_C) and the culture incubation temperature. If the incubation temperature is such that it is near to T_C then the performance of the membrane would be near optimum [Zakim *et al*, 1992; Leckband *et al*, 1994]. At the inflection point revealed in this study this would indicate a faster growth rate and better cell efficiency. This was supported by the dextrose uptake rate [figure 6.6] which also revealed an inflection point at approximately the same temperature as the previous models.

The second assumption of the Schoolfield model states that the concentration of the rate controlling factor should be constant. As discussed by many workers the membrane fluidity is determined by homeoviscous adaptation following adaptation to the environmental conditions [Gliozzi, *et al*, 1983; Foot, *et al*, 1983; MacDonald and Cossins, 1985; Zaritsky, *et al*, 1985; Parola, *et al*, 1990; Wiese, *et al*, 1994] . Once the membrane composition has optimised it remains in a dynamic equilibrium [Cribier *et al*, 1993]. In this situation the concentration of any compound is not relevant because the interaction is based upon physical processes within an equilibrated structure. Relating this to the effect of temperature upon the growth rate it is possible to see that, as temperature increases, when the optimum fluidity has been reached then the growth rate can no longer continue to change at the same rate. This results in a continued increase in rate of population growth but at an ever decreasing rate of change. Hence the sigmoid curve relating growth rate to temperature. At some point above the level of maximum growth rate the temperature begins to inactivate certain processes [Russel, 1990]. This causes the decrease which was described in chapter six by the Schoolfield and Ratkowsky models. Biological membranes are asymmetric [Lakos *et al*, 1990; Cribier *et al*, 1993] and any variation in the composition and behaviour of the membrane will affect the symmetry [Lakos *et al*, 1990; Kinnunen, 1991]. This disruption was shown to affect the dextrose uptake rate of *Lb. plantarum* [section 6.2]. The change in uptake rate may be the only factor directly relating the effect of temperature upon the cell membrane to the growth rate. This change in uptake rate governs the effect of temperature upon the final population level obtained.

For each substrate molecule utilised the cell must provide a certain degree of maintenance energy. As the rate of substrate uptake decreases, the percentage of each substrate molecule required for maintenance energy increases owing to the increased time base. This results in a decreasing cell efficiency and so, for the available substrate, reduces the final population level.

With regard to the third and fourth assumptions, this hypothesis represents interaction between several factors which could not be explained according to zero order reaction kinetics and does not follow an Arrhenius type temperature dependency [Zakim *et al*, 1992].

These proposals are a considerable simplification of the current theories regarding the controlling factors governing the three population growth phases. A consideration of the literature and the evidence presented here leads to the conclusion that the homeoviscous adaptation of bacteria to environmental conditions may be responsible for the shape of the population growth profile. This application of Occam's razor provides a greatly simplified basis for the continued modelling of the microbial response to environmental conditions.

This may have significance for the food preservation industry since it may be possible to affect the membrane fluidity of a mixed microflora without affecting the organoleptic qualities of a food and so, extend the shelf life of fresh food items. If this were possible it would yield huge financial gains for food processors and retailers. Such an application of the findings from this report would require extensive work which would only be justifiable if further verification of these results could be shown.

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