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GROWTH OF *STAPHYLOCOCCUS AUREUS* UNDER STRESS CONDITIONS

by

BRIAN ALLAN WINER

Submitted for the degree

M.Sc (Microbiology)

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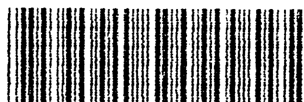
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I declare that the thesis herewith submitted by me for the degree of M.Sc (Microbiology) at the University of Pretoria has not been handed in for a degree to any other university.

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A B S T R A C T

GROWTH OF *STAPHYLOCOCCUS AUREUS* UNDER STRESS CONDITIONS

by

BRIAN ALLAN WINER

PROMOTER : Prof W H Holzapfel

DEPARTMENT : Microbiology and Plant Pathology

DEGREE FOR WHICH THESIS IS SUBMITTED : M.Sc (Microbiology)

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ABSTRACT

Staphylococcus aureus is considered as one of the most important food-borne, halotolerant pathogenic bacteria. An investigation was thus performed to determine parameters that could limit its growth in "Shelf Stable Meat Products".

Altogether 35 coagulase positive strains were isolated from fresh minced meat and "Shelf Stable Meat Products". Of the isolates 46% were found to be typable using the international basic human set of phages, indicating human origin.

Strains relatively sensitive and resistant to low a_w and pH were found. The minimal a_w for bacterial growth of the a_w sensitive and resistant strains were 0,866 and 0,846 respectively. However the type of humectant used to lower a_w greatly influenced bacterial growth. When investigating the effect of humectants, its strength for binding water, its bactericidal effects and the concentration of the humectant required to produce the desired a_w must be considered.

The inorganic acid HCl had the least effect on staphylococcal growth when compared to the organic acids. The minimal pH enabling bacterial growth of the pH sensitive and resistant strains was 5,1 and 5,0 respectively when an organic acid was used to adjust the pH, and 4,43 and 4,08 respectively when an inorganic acid was used. Acetic acid was the most effective organic acid for growth inhibition.

Lowering the a_w to 0,925 produced a much greater bacteriostatic effect than lowering the pH to 5,5. The greatest

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bacteriostatic effect was obtained at a_w 0,925 adjusted with sodium chloride and pH 5,5 adjusted with gluconic acid.

As the a_w was lowered, it was found that the amount of cardiolipin in the cell membrane increased, while the amount of phosphatidylglycerol decreased, irrespective of the humectant used to lower the a_w.

The average diameters of the cocci were found to increase from 0,87 μm to 1,25 μm when grown in BHI adjusted to a_w 0,925 with glycerol, propylene glycol, sucrose or sodium chloride and to 1,65 μm when the a_w was adjusted using sodium lactate.

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S A M E V A T T I N G

GROEI VAN *STAPHYLOCOCCUS AUREUS* ONDER GROEIBEPERKENDE TOESTANDE

deur

BRIAN ALLAN WINER

LEIER	:	Prof W H Holzapfel
DEPARTEMENT	:	Mikrobiologie en Plantpatologie
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SAMEVATTING

Staphylococcus aureus is bekend as een van die belangrikste voedselgedraagde, halotolerante patogene bakterieë. 'n Ondersoek is onderneem om die parameters te bepaal wat die groei daarvan in "Rakstabiele Voedselprodukte" (SSP) beperk.

Altesame 35 koagulase-positiewe stamme is in vars maalvleis en "Rakstabiele Vleisprodukte" geisoleër. Ses-en-veertig persent van die geïsoleerde stamme kon getipeer word deur gebruik van die internasionale basiese groep fage vir stamme van menslike oorsprong.

Stamme wat relatief sensitief en bestand teen a_w en pH is, is gevind. Die minimale a_w vir bakteriële groei van die a_w - "gevoelige" en "bestande" stamme was onderskeidelik 0,866 en 0,846. Die soort voghouer wat gebruik is om die a_w te verlaag, het bakteriese groei egter grootliks beïnvloed. By 'n ondersoek van die invloed van voghouers op bakteriegroei, moet die sterkte vir waterbinding, die bakterisidiese effek en die molariteit van die voghouer (nodig om die benodig a_w in te stel) in aanmerking geneem word.

Die anorganiese suur HCl het die groei van *S.aureus* die minste benadeel in vergelyking met organiese sure. Die minimale pH vir bakteriese groei van die pH - "gevoelige" en "bestande" stamme was onderskeidelik 5,1 en 5,0 wanneer 'n organiese suur gebruik is om die pH in te stel en onderskeidelik 4,43 en 4,08 vir 'n anorganiese suur. Asynsuur

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was die effektiefste organiese suur.

Verlaging van a_w na 0,925 het as sodanig 'n baie beter bakteriostatiese uitwerking gehad as pH-verlaging na 5,5. Die grootste bakteriostatiese uitwerking is gekry deur die a_w te verlaag met natriumchloried na a_w 0,925 en die pH na 5,5 met glukonsuur.

Namate die a_w verlaag is, is gevind dat die hoeveelheid kardiolipien in die selmembraan verhoog het, terwyl die hoeveelheid fosfatidielgliserol verminder het, onafhanklik van die soort voghouer wat gebruik is om die a_w te verlaag.

Die gemiddelde deursnee van die cocci het vergroot van 0,87 μm tot 1,25 μm wanneer dit gekweek is in BHI met a_w 0,925 (ingestel met gliserol, propileenglikol, sukrose of natriumchloried) en dit het vergroot na 1,65 μm wanneer natriumlaktaat gebruik is.

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ABBREVIATIONS

Ala	alanine
a_w	water activity
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CCM	Czechoslovakian Collection of Microorganisms
conc.	concentration
cm	centimeter
d	distance
DNA	Deoxyribonucleic Acid
DSM	Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms)
FDA	Food and Drug Administration (USA)
g	gram
G-C	Guanine-Cytosine
$Glc.NH_2$	glucosamine
Glu	glutamic acid
Gly	glycine
h	hour
HCl	hydrochloric acid
IMF	Intermediate Moisture Foods
Isoglu	isoglutamic acid
Lys	lysine
mmol	millimoles
mm	millimeters
mg	milligrams
min	minutes
ml	millilitres
mol	moles
Mur	muramic acid
mwt	molecular weight

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N	Normal
NaCl	sodium chloride
NB	Nutrient Broth
nm	nanometers
nt	not typable
$P_{\ddot{o}}$	vapour pressure of solution
P_s	vapour pressure of solvent
R.T.D.	Routine Test Dilution
sec	seconds
Ser	serine
SSP	Shelf Stable Products
ssp	species
Std 1	Standard 1
S	*Staphylococcus*
μl	microlitre
μm	micrometer
vol	volume
w/v	weight/volume
$\geq$	greater or equal to
$<$	less than
Cl.++	strong reaction
+,$\pm$	weak reaction

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CHAPTER 1

INTRODUCTION

Of the food-borne pathogens, *Staphylococcus aureus* represents the most halotolerant bacterium (Troller, 1973). This organism is associated with a wide variety of habitats, and due to its potential role in causing food poisoning a study was undertaken to determine parameters limiting its growth in "Shelf Stable Food Products".

The viability of *S.aureus* at reduced a_w is of concern to the food industry, particularly in view of increased development of Intermediate Moisture Foods (a_w 0,90 to 0,60) and Shelf Stable Products ($a_w \leq 0,95$). The inhibition of microorganisms in Shelf Stable Food Products does not depend on either a_w, pH, temperature, preservatives or competitive microbes, but on a combination of two or more of these parameters.

Water limitation techniques can be useful tools to preserve food materials not only from autodecomposition but also from microbial decomposition. The minimum a_w of about 0,86 for growth of *S.aureus* has been reported by several authors (Scott, 1957; Troller, 1973; Plitman *et al*., 1973).

In addition to the effect of water limitation on *S.aureus*, investigations have also been carried out using a combination of a_w and pH reduction to inhibit growth and toxin production of staphylococci. The type of acidulant used for pH

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adjustment has a significant effect on the growth of *S.aureus* (Tatini, 1973).

The purpose of this investigation was to isolate *S.aureus* strains from fresh and processed meats, to determine their relative sensitivity and resistance to a_w, pH and temperature and study some aspects possibly related to the mechanism of their resistance.

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CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL

Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) divides the family Micrococcaceae into three genera differing in the guanine plus cytosine (GC) content of their DNA (Schleifer and Kocur, 1973). The genus Micrococcus consists mainly of saprophytic, aerobic, coccal bacteria which produce acid by growth on glucose only under aerobic conditions. Their DNA guanine plus cytosine content is 66 to 77 mole %. Bacteria belonging to the genus Planococcus closely resemble micrococci, but differ in being motile and in their guanine plus cytosine content of 38 to 52 mol %. The genus Staphylococcus includes some pathogenic Gram-positive, catalase positive, non-motile, spherical bacteria which are facultative anaerobes, possessing the ability to ferment glucose anaerobically in a standard growth medium, with accompanying production of acid catabolites. Their DNA GC-ratio is 30 to 40 mole %.

The word Staphylococcus comes from the two Greek words staphylo (bunch of grapes) and coccus (a grain or berry). Thus the word Staphylococcus describes the morphological characteristics of the genus: spherical cells 0,5 to 1,5 µm in diameter occurring singly, in pairs and characteristically dividing in one plane to form irregular clusters. The cells are non-motile and no resting stages are known (Buchanan and Gibbons, 1974).

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The staphylococci can be divided into two main groups depending on their ability to produce coagulase: (i) potentially pathogenic staphylococci which are coagulase positive and parasitic, and (ii) saprophytic staphylococci which are coagulase negative (Holzapfel *et al.*, 1981). The coagulase positive staphylococci consist of *Staphylococcus aureus*, *Staphylococcus intermedius* and *Staphylococcus hyicus* ssp. *hyicus*. Of these *Staphylococcus aureus* is the most important potentially pathogenic *Staphylococcus* species.

S.aureus cells are spheres with a diameter of 0,8 to 1,0µm. When grown on solid medium their colonies are smooth, low convex, glistening and with an entire edge. Carotenoid pigments are produced by most strains giving cells ranging in colour from deep orange to pale yellow. When grown on Baird-Parker agar, black, glossy, vaulted colonies with a diameter of 1 to 1,5mm and with a narrow, white outer ring surrounded by a clear zone, 2 to 5mm in diameter broad, appear. The blackening is produced by the reduction of tellurite in the medium to tellurium. The clear zones that are produced around the colonies are due to lipolysis or proteolysis while the opaque areas produced within these clear zones are produced probably by lipase or lecithinase. When grown in broth it results in turbidity with a fine, easily suspended deposit. Frequently, a ring pellicle may be formed.

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2.2 CHARACTERISTICS OF *STAPHYLOCOCCUS AUREUS*

2.2.1 STRUCTURE AND COMPOSITION OF THE CELL WALL

The cell wall is the basis for several classical taxonomic characteristics. Not only does it contribute to the shape of the bacterium, but it is also responsible for the different stainability of a cell, for most of the serological behaviour and for phage adsorption (Schleifer and Kandler, 1972). The first known primary structure of the peptidoglycan of a Gram-positive bacterium was that of the peptidoglycan of *S.aureus* strain Copenhagen (Ghuysen *et al*., 1965).

The cell wall of *S.aureus* has two main components, the peptidoglycan and its associated teichoic acids (Schleifer and Kandler, 1972). The peptidoglycan consists of glycan residues and peptides (Schleifer and Kandler, 1972). The glycan moiety, made up of alternating β-1,4-linked N-acetylglucosamine and N-acetyl muramic acid residues is remarkably uniform (Ghuysen, 1968; Ghuysen and Strominger, 1963). The peptidoglycan moiety linked through its N-terminus to the carboxyl group of muramic acid, contains alternating L- and D- amino acids. A fragment of the primary structure of a peptidoglycan is shown in Figure 2.1. Usually L-alanine is bound to muramic acid followed by D- glutamic acid, which is linked by its α-carboxyl group to L-lysine to which the C-terminal D-alanine is attached. The sequence of the repeating unit of the peptide moiety of staphylococcal

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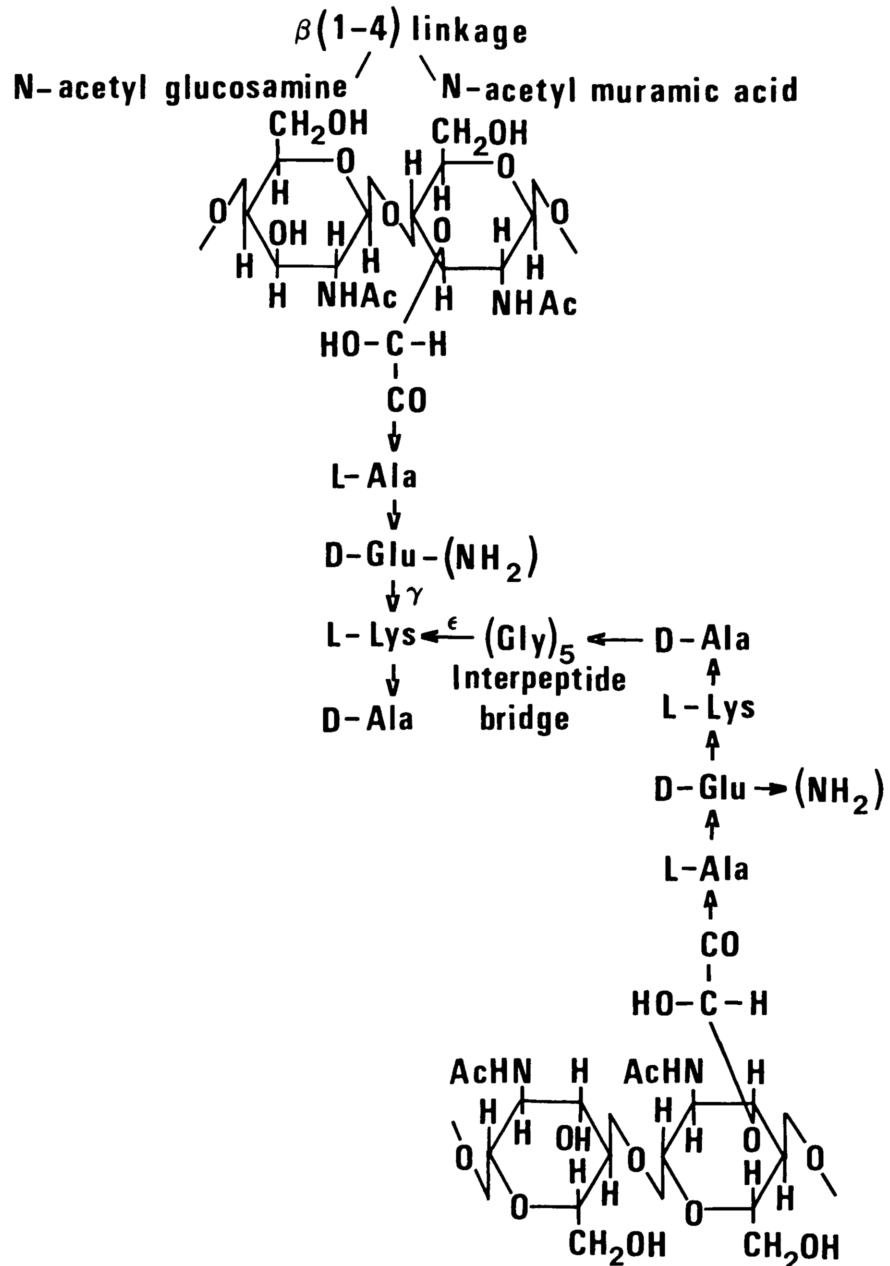

Figure 2.1 : Subunit of the primary structure of a *S.aureus* typical peptidoglycan (Schleifer and Kandler, 1972). Glu = glutamic acid; Lys = lysine; Gly = glycine; Ala = alanine; Ac = acetyl.

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murein is L-Ala-D-Isoglu-(NH_2)-N^{α}-L-Lys-(D-Ala)-N^{ϵ}-$(Gly)_5$-D-Ala-L-Lys (Giesbrecht *et al.*, 1976). The sequence L-Ala-D-Isoglu-L-Lys-D-Ala is designated a peptide subunit (Ghuysen *et al.*, 1965) and the pentaglycine that cross-links the peptide subunits from the α-amino group of the diamino acid (L-lysine) of one peptide subunit to the D-Ala carboxyl group of another peptide subunit is called the interpeptide bridge (Jarvis and Strominger, 1967).

Most variations of the peptide moiety of the peptidoglycan do not occur in the peptide subunit but in the interpeptide bridge and in the mode of cross-linking. The amino acid composition of the peptidoglycan is considered to be useful as a taxonomic tool (Cummins and Harris, 1956). Peptidoglycans with a high content of glycine are typical for staphylococci (Schleifer and Kandler, 1970). Most *S.aureus* strains contain a peptidoglycan cross-linked by penta - or hexaglycine bridges (Schleifer and Kandler, 1970). Schleifer (1969) found that when the medium contains an unusually high level of L-serine, then small amounts of glycine can be substituted by L-serine in the interpeptide bridges of *S.aureus*.

Teichoic acids are water-soluble polymers containing sugar, D-alanine residues and either glycerol or ribitol phosphates (Schleifer and Kandler, 1972). The point of attachment of teichoic acids in *S.aureus* (Figure 2.2) is through the 6 – hydroxyl group of muramic acid in the glycan chain (Schleifer and Kandler, 1972). In *S.aureus* cell walls,

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teichoic acid is joined to the peptidoglycan by a linking unit comprising three glycerol 1 - phosphate units attached to the 4 - position of N - acetylglucosamine. This engages through a phosphodiester group at position 1 with the 6 - hydroxyl group of muramic acid (Figure 2.2.). The acid-labile N-acetylglucosamine-1-phosphate linkage and the alkali-labile phosphodiester linkage at position 4 explain the ease with which teichoic acid can be split off from the peptidoglycan (Franklin and Snow, 1975).

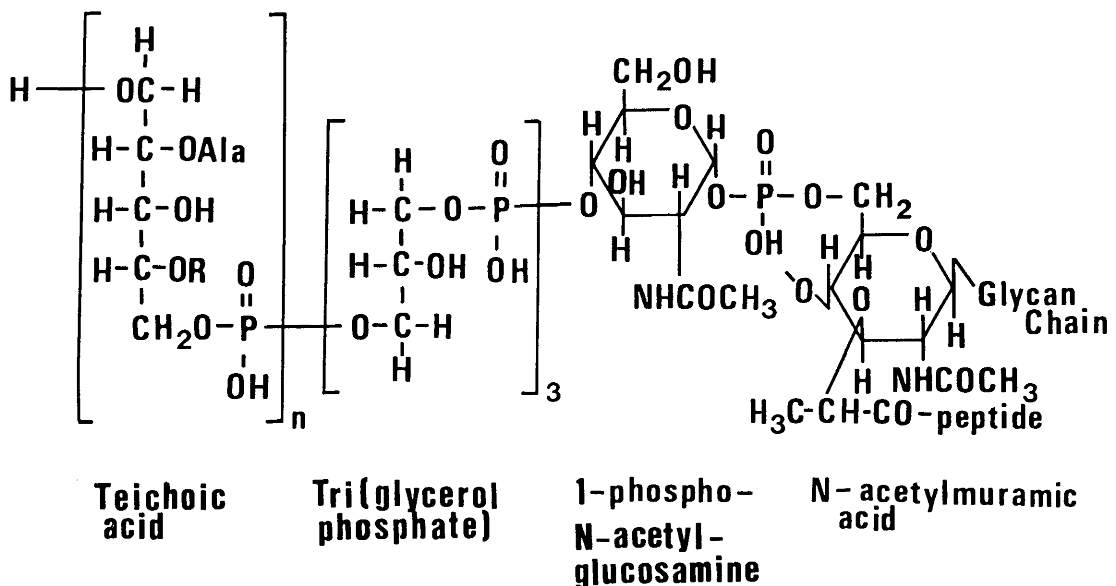

Figure 2.2 Teichoic acid and its linkage to peptidoglycan in the wall of S.aureus (Franklin and Snow, 1975). Ala=alanine.

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2.2.2 STRUCTURE AND COMPOSITION OF THE CELL MEMBRANE

Common to all cellular microorganisms is the permeability barrier provided by the cytoplasmic membrane. The most important feature of the cytoplasmic membrane is the lipid bilayer with its interspersed extrinsic and intrinsic proteins. The ratio of the different kinds of polar lipids in membranes is characteristic of the type of membrane system and the species (Lehninger, 1975).

Lipids are water insoluble organic biomolecules that can be extracted from cells and tissues by non-polar solvents such as chloroform, ether or benzene. They have several important biological functions, serving as structural components of membranes, storage and transport forms of metabolic fuel, protective coating on the surface of many organisms and as cell-surface components concerned in cell recognition (Lehninger, 1975).

The phosphoglycerides also known as the phospholipids or phosphatides, which posesses a polar head in addition to their nonpolar hydrocarbon tails, are the major components of cell membranes. Phosphatidylglycerol is often found in bacterial membranes as an amino acid derivative, particularly of L-lysine, which is esterified at the 3 position of the glycerol head group (Lehninger, 1975).

Diphosphatidylglycerol, also called cardiolipin, consists of a molecule of phosphatidylglycerol in which the 3' hydroxyl group of the second glycerol moiety is esterified

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to the phosphate group of a molecule of phosphatidic acid. The backbone of cardiolipin thus consists of three molecules of glycerol joined by two phosphodiester bridges; the two hydroxyl groups of both external glycerol molecules are esterfied with fatty acids (Lehninger, 1975).

Phosphatidylglycerol, O-aminoacylphosphatidylglycerol and cardiolipin are therefore all structurally related. They are all characteristically abundant in the cell membranes of bacteria.

The phospholipids of S.aureus U-71 have been identified as phosphatidylglycerol, lysylphosphatidylglycerol, phosphatidic acid, cardiolipin, phosphatidylethanolamine and phosphatidylglucose (Short and White, 1970; White and Frerman, 1967). These phospholipids together with the carotenoids, vitamin K_2 isoprenologues, and the glucolipids (monoglucosyldiglyceride and diglucosyldiglyceride), are the major components of the staphylococcal membrane (White and Frerman, 1967).

Short and White (1971) found that S.aureus accumulated cardiolipin and lost phosphatidylglycerol during the transition from the exponential growth phase to the stationary phase of growth. The minor lipids, phosphatidylethanolamine and phosphatidylglucose also accumulated, whereas the lysylphosphatidylglycerol content of the membrane remained constant as the stationary phase continued.

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During exponential growth, the proportions and total content of phospholipids per cell remained constant. Shifts in the phosphatidylglycerol, cardiolipin equilibrium suggest that these changes could be related to some important function which depends on lipid metabolism.

S.aureus is resistant to high concentrations of sodium chloride (NaCl) in the growth medium and this fact is used to isolate this organism selectively. The factors that permit S. aureus to grow in high Na^{+}-ion concentration may be achieved by adaptation of the cell barrier (cytoplasmic membrane or cell wall) or by changes in its internal enzymes (Kanemasa et al., 1972).

Kanemasa et al. (1972) found that the cardiolipin content of S.aureus increases, while phosphatidylglycerol and lysylphosphatidylglycerol diminish with increasing NaCl concentration of the culture medium. The major change in fatty acid composition lies in the proportion of branched acids, resulting in an increase of the branched fatty acids in cardiolipin when cultured in high NaCl medium. Kanemasa et al. (1972) also determined the total lipid content of the bacteria cultured in media containing 0,05%, 5% and 10% NaCl. The total lipid contents were found to be 6,8%, 6,85% and 7% of the dry cell weights respectively. Thus, hardly any influence of NaCl on the total lipid content was observed.

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Houtsmuller and Van Deenen (1965) observed that the pH of the medium is of primary importance for the ratio between phosphotidylglycerol and lysine-phosphatidylglycerol. Accumulation of lysine-phosphatidylglycerol was observed when the pH reached values below 5, thus inducing a stationary phase. However, when the cells were allowed to enter the stationary phase at pH7, phosphatidylglycerol was found to be the most prevailing phospholipid. This effect of a substitution of phosphatidylglycerol by lysine-phosphatidylglycerol was most pronounced and reproducible when the low pH was attained through the production of acid from glucose.

Houtsmuller and Van Deenen (1965) also studied the effect of the environmental pH on the membrane lipid composition and found that at pH 7,0 phosphatidyglycerol was the major phospholipid, whereas lysylphosphatidylglycerol prevailed at a pH below 5,0.

The phospholipid alterations observed are possibly a mechanism to protect the microorganisms against phospholipid degradation during stationary phase growth, for adaptation of the bacteria to new environmental conditions (Cronan, 1968), may act as a barrier against high level ionic strength or contribute to active transport (Kanemasa et al., 1972).

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Environmental factors, such as growth medium or age of the bacterial culture influence the qualitative and quantative composition of lipids, thus limiting their taxonomic value.

2.2.3 METABOLISM

Staphylococci are chemoorganotrophs and their metabolism is both respiratory and fermentative (Buchanan and Gibbons, 1974). While growing anaerobically they obtain energy from the glycolytic pathway and the enzymes of the Krebs cycle and the membrane-bound electron transport system are repressed (Collins and Lascelles, 1962; Strasters and Winkler, 1963). Addition of oxygen to the growing cultures causes induction of the Krebs cycle and the membrane-bound electron transport system (Frerman and White, 1967). Under anaerobic conditions the main product of glucose fermentation is lactic acid, while in the presence of oxygen, the main product is acetic acid with small amounts of CO_2 (Buchanan and Gibbons, 1974).

Coagulase positive staphylococci produce coagulase, hyaluronidase, DNase and acetoin (Devriese and Hájek, (1980). Acid is produced aerobically from fructose, galactose, mannose, ribose, maltose, sucrose, trehalose and mannitol (Kloos and Schleifer, 1975). *S.intermedius* and *S.hyicus* ssp. *hyicus*, both of which are coagulase positive, can be distinguished from *S.aureus* by their inability to produce acid from mannitol anaerobically

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(Devriese and Hajek, 1980).

2.3 STRESS FACTORS AFFECTING THE GROWTH OF *S. AUREUS*

2.3.1 THE EFFECT OF WATER ACTIVITY (a_w)

It is in wide agreement that a_w, by which the degree of availability of water in foods is expressed, is one of the most important factors determining microbial growth (Scott, 1957) and enzyme activity (Acker, 1962). Water activity may be expressed as the relationship of vapour pressure of the solution (P_s) to the vapour pressure of the solvent (P_o) at the same temperature (Troller and Christian, 1978).

To obtain comprehensive data on the water relations of a food, the a_w levels corresponding to a range of water contents must be determined. Water activity vs water content may be plotted to provide a water sorption isotherm (Troller and Christian, 1978) (Figure 2.3). The forces responsible for reducing water vapour pressure are not the same throughout the range of a_w values. A, B and C represent different types of water binding which may predominate. As water is added to a dry food, molecules are adsorbed onto appropriate sites until statistically at least all are occupied. This constitutes the water monolayer (Figure 2.3, region C). It is near the point of monolayer completion that given changes in water content have the most marked influence upon a_w.

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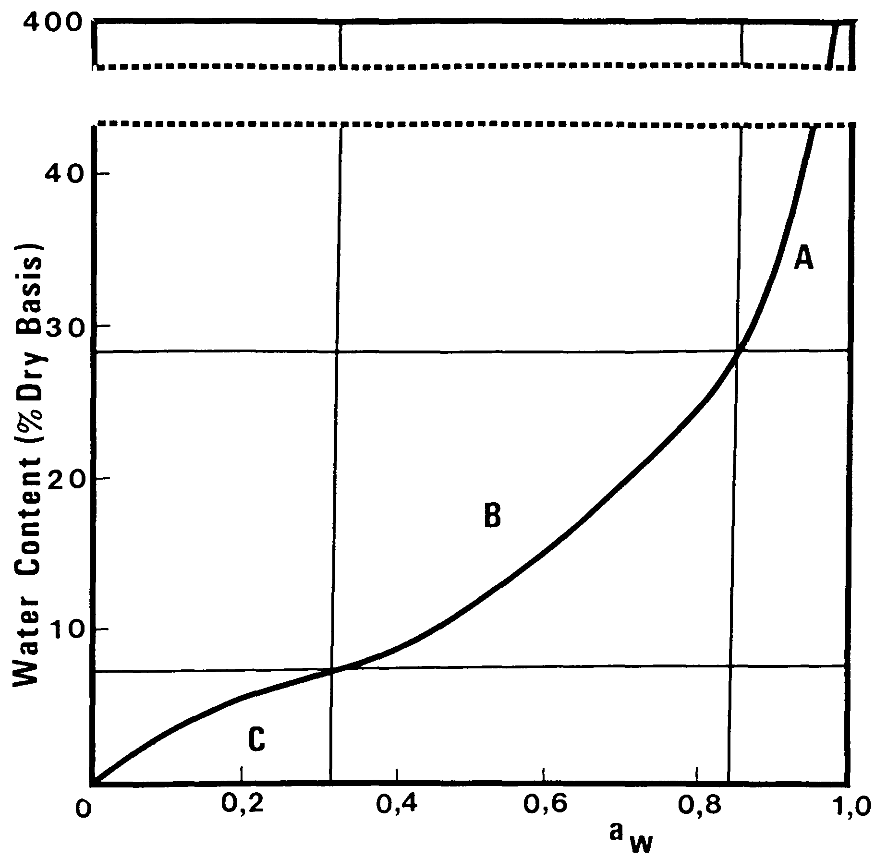

Figure 2.3 A generalized water sorption isotherm of a typical food material (Troller and Christian, 1978).

The water of the monolayer is thus very stable, behaving in many ways as part of the solid. The water in region B of the isotherm is less firmly bound than in the monolayer. Multilayer adsorption occurs and the solution of soluble components becomes important, modified by the nature of insoluble solids present. The water in regions B and C differs markedly from "free" water such as exists in region A. This latter, although mechanically trapped in the system, is subjected to only weak restrictive forces as indicated by the steepness of the isotherm (Troller and

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Christian, 1978).

The water content of a food is higher when a specific a_w is achieved by desorption of water from a moist material, than when the route is by adsorption from a dry food, This difference termed hysteresis (Labuza, 1968) is illustrated by the hysteresis loop in Figure 2.4.

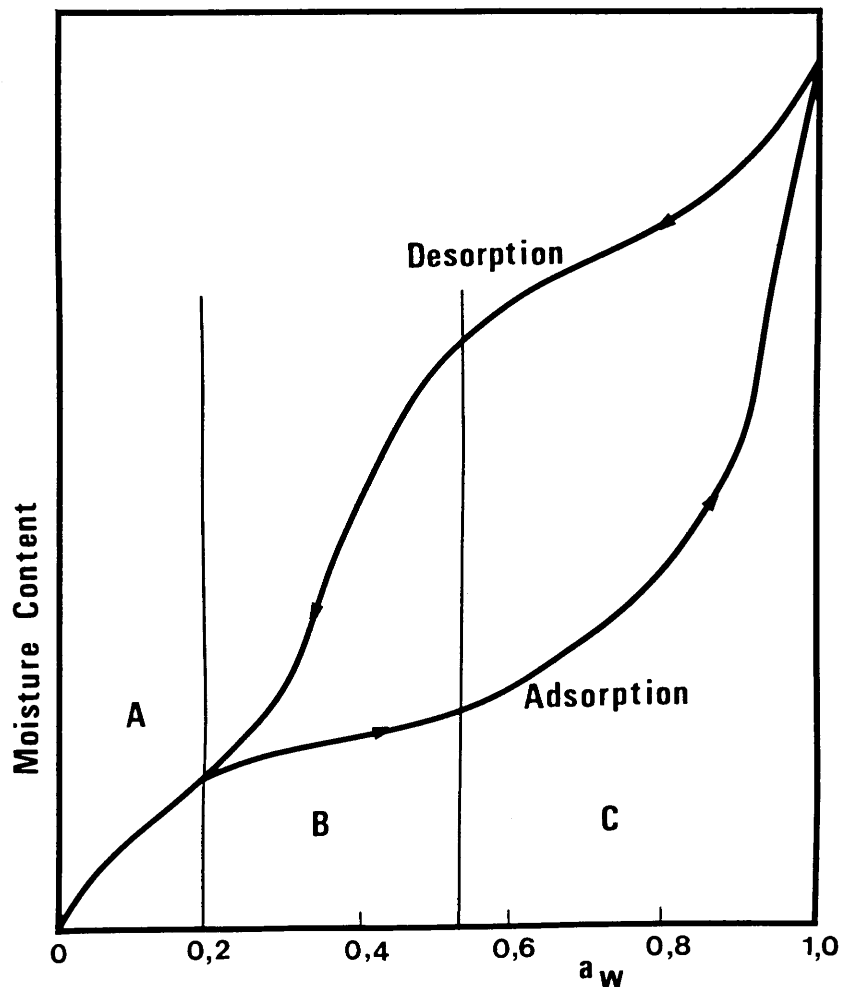

Figure 2.4 Generalized water sorption isotherm showing a"hysteresis loop"(Labuza, 1968).

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The hysteresis loop (Figure 2.4) terminates near the a_w of monolayer formation. Region C is due to condensation in pores (capillary effects), followed by dissolution of solute components. Thus, in theory, the course of water sorption by a dry material is first by the formation of a monolayer, followed by multilayer adsorption, then uptake into pores and capillary spaces, dissolution of solutes, and finally mechanical entrapment of water at the higher levels of a_w. These phases may overlap extensively and will differ in magnitude among foods depending upon chemical composition and structure (Labuza, 1968).

Water activity of food influences the multiplication, metabolic activity and also the resistance and survival of microorganisms (Scott, 1953). Based on a_w, foods can be divided into three categories (Leistner and Rödel, 1978).

(i) high moisture foods with a_w of 1,0 to 0,90 (below 0,95 - 'shelf stable products' (SSP))

(ii) intermediate moisture foods (IMF) with a_w between $<0,90$ and $\geq 0,60$ and

(iii) low moisture foods with $a_w < 0,60$.

Of the food-borne pathogens, S.aureus represents the most halotolerant bacterium (Scott, 1953). Selgelove and Dack (1951) showed that growth of S. aureus was inhibited when the moisture content of meat was 20% or lower. Scott

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(1953) found that maximal growth occurred at an a_w of 0,995 to 0,99 with a_w 0,86 and 0,91 as minimal a_w for growth under aerobic and anaerobic conditions respectively. Labuza et al., (1972) studied the viability of S.aureus in intermediate moisture pork prepared by adsorption and desorption methods. Pork slurry of a_w 0,84 adjusted with glycerol supported growth of S.aureus, whereas freeze-dried pork with a_w 0,90 did not support such growth. These authors thus concluded that the moisture content needed to yield the desired a_w was dependent on adsorption or desorption of water in the system. Table 2.1 shows literature data on the minimal a_w which allows S.aureus growth in laboratory media adjusted with different solutes (Vaamonde et al., 1982).

In Table 2.2 the minimal a_w required for the growth of a number of microorganisms is listed (Leistner and Rödel, 1978). Growth conditions were optimal using artificial substrates with the a_w adjusted with additives associated with food. However, results in the table will vary from author to author depending upon the solutes used to adjust the a_w or the moisture content of the substrate (Marshall et al., 1971; Kushner, 1971; Pawsey and Davies, 1976; Christian, 1981). From Table 2.2 it can be seen that of the food-poisoning bacteria, the lowest a_w is tolerated by S.aureus.

Some strains of S.aureus produce enterotoxins which can

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TABLE 2.1 LIMITING a_w VALUES FOR INHIBITION OF GROWTH OF *STAPHYLOCOCCUS AUREUS* IN LIQUID LABORATORY MEDIA ADJUSTED WITH DIFFERENT a_w CONTROLLING SOLUTES (VAAMONDE *ET AL*., 1982)

STRAIN	SOLUTE/S	TEMPERATURE (°C)	LIMITING a_w
-	Glycerol	30	0,89[a]
196 E	Glycerol	25	0,87[b]
S-6	Glycerol	25	0,865[b]
196 E	1,2-Propanediol	25	0,96[b]
S-6	1,2-Propanediol	25	0,95[b]
196 E	1,3-Butanediol	25	0,96[b]
S-6	1,3-Butanediol	25	0,97[b]
M 7/1	$NaCl:KCl:Na_2SO_4$ (5:3:2 mol)	25	0,887-0,870[c]
100	$NaCl:KCl:Na_2SO_4$ (5:3:2 mol)	30	0,864-0,860[d]
100	$NaCl:KCl:Na_2SO_4$ (5:3:2 mol)	25	0,887-0,870
M 7/1	$NaCl:KCl:Na_2SO_4$ (5:3:2 mol)	30	0,864-0,860[d]
-	NaCl	37	0,860[a]
ATCC 6538 P	Sucrose	35	0,867-0,864[e]
FM 1	Sucrose	35	0,876-0,843[f]
Various	Sucrose	30	0,88-0,86[g]
Various	3,44m Sucrose + salts mixture	30	0,86-0,84[h]
Various	4,07m Glucose + salts mixture	30	0,88-0,86[g]
Various	0,1m Glucose + salts mixture	30	0,88-0,86[g]
Various	$NaCl:KCl:Na_2SO_4$ (5:3:2 mol)	30	0,86-0,84[h]

[a] Limiting value for growth
[b] Limiting value inhibitory for growth
[c] Growth observed at 0,887 but not at 0,870 a_w
[d] Growth observed at 0,864 but not at 0,860 a_w
[e] Growth observed at 0,867 but not at 0,864 a_w
[f] Growth observed at 0,876 but not at 0,843 a_w
[g] Growth observed at 0,88 but not at 0,86 a_w
[h] Growth observed at 0,86 but not at 0,84 a_w
[i] $NaCl:KCl:Na_2SO_4$, 5:3:2 mol

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TABLE 2.2 MINIMAL a_w FOR MULTIPLICATION OF MICROORGANISMS ASSOCIATED WITH FOODS (LEISTNER AND RÖDEL, 1978)

a_w	BACTERIA	YEASTS	MOULDS
0.98	Clostridium (1), Pseudomonas+	-	-
0.97	Clostridium (2)	-	-
0.96	Flavobacterium, Klebsiella, Lactobacillus+, Proteus+, Pseudomonas+, Shigella	-	-
0.95	Alcaligenes, Bacillus, Citrobacter, Clostridium (3), Enterobacter, Escherichia, Proteus, Pseudomonas, Salmonella, Serratia, Vibrio	-	-
0.94	Lactobacillus, Microbacterium, Pediococcus, Streptococcus+, Vibrio	-	-
0.93	Lactobacillus+, Streptococcus	-	Rhizopus, Mucor
0.92	-	Rhodotorula, Pichia	-
0.91	Corynebacterium, Staphylococcus (4), Streptococcus+	-	-
0,90	Lactobacillus+, Micrococcus, Pedioccus, Vibrio+	Hansenula, Saccharomyces	-
0,88	-	Candida, Debaryomyces, Hanseniaspora, Torulopsis	Cladosporium
0.87	-	Debaryomyces+	-
0.86	Staphylococcus (5)	-	Paecilomyces
0.80	-	Saccharomyces+	Aspergillus, Penicillium, Emericella, Eremascus
0.75	Halophilic bacteria	-	Aspergillus+ Wallemia
0.70	-	-	Eurotium, Chrysosporium
0.62	-	Saccharomyces+	Eurotium+ Monascus

+Some strains; (1) Clostridium botulinum type C; (2) = Cl. botulinum type E and some strains of Cl. perfringens; (3) = Cl. botulinum type A and B and Cl. perfringens; (4) = anaerobic; (5) = aerobic.

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cause food-poisoning. The minimal a_w for *S.aureus* to produce enterotoxin A, B or C is 0,86 , 0,93 and 0,94 respectively (Leistner and Rödel, 1978). Thus, in intermediate moisture foods, staphylococcal enterotoxin production is possible and therefore the growth of *S.aureus* should definitely be avoided.

Plitman *et al*. (1973) studied the viability of *S. aureus* in intermediate moisture meats prepared by desorption and adsorption methods in order to determine differences between samples of the same a_w but different moisture contents. They found that glycerol inhibited growth of *S.aureus* at a_w 0,88 in adsorption samples and at a_w 0,865 in desorption samples. At a_w 0,92 in the samples prepared with 1,2-propanediol or 1,3-butanediol, the cells died rapidly. These results indicated that these two humectants have a bactericidal effect on *S.aureus* that cannot be solely explained in terms of their water-binding characteristics. High humectant concentration produces two effects: lowering the a_w and, superimposed on this, the specific action of the humectant molecules (Kushner, 1971).

Plitman *et al*. (1973) found that in brain heart infusion (BHI) broth, the minimal concentrations of humectants inhibitory for the growth of *S.aureus* were 40% for glycerol, 18% for 1,2-propanediol and 15% for 1,3-butanediol. These percentages give a_w values of

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0,865 , 0,95 and 0,97 respectively. These results again show that besides the water-binding properties of 1,2-propanediol and 1,3-butanediol, there is a bactericidal effect which depends on the chemical structure of the humectant molecule.

Vaalmonde *et al*. (1982) examined whether *S.aureus* ATCC 6538 P could grow below a_w 0,86 in laboratory media adjusted by the addition of l-proline, l-lysine, β -alanine, sorbitol, potassium chloride, sodium lactate and polyethylene glycol. They found that no growth occurred in any case at or slightly below a_w 0,86 indicating that the solutes all had inhibitory effects on *S.aureus* independent of their a_w lowering ability.

It is well known that *S.aureus* shows a strong resistance to a high concentration of NaCl and it can grow in a medium containing as much as 15% NaCl (Kanemasa *et al*., 1972). Kanemase *et al*.(1974) showed that *S.aureus*, despite proliferating in a high NaCl medium, has maintained constancy of Na^+ and K^+ concentrations within the cell. Consequently, when *S.aureus* is cultivated in the medium of a high NaCl concentration, it must adapt itself to acquire some special defence mechanism to high salt and high osmotic pressure in order to proliferate. Therefore, it seems only natural to consider that for the adaptation to environments, changes in the cell wall as well as in the structures and functional changes of the cell membrane should play an important role. Kanemasa

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et al. (1972) showed that cardiolipin increased with increasing a_w. However, the adaption to NaCl cannot be by phospholipid changes only. Thus Kanemasa et al. (1974)studied morphological changes and analysed thin sections of S.aureus.

From scanning electron microscopic observations Kanemasa et al. (1974) showed that there was no difference in shape, manner of cell division or surface structure, but there was a distinct difference in the size of S.aureus cell diameters; the diameter of S.aureus grown in 10% NaCl was 1,04μm as compared to 0,81μm when grown in 0,05% NaCl. This phenomenon seems to represent a defence mechanism to escape as much from the high NaCl condition as possible. In the intercellular space of S.aureus grown in 10% NaCl, there was a slimy substance thought to be the source of their viscosity.

Kanemasa et al. (1974)analysed thin sections of S.aureus, but found no marked difference in the thickness of the cell membrane. However, S.aureus grown in 10% NaCl had a cell wall thickness of about 38nm compared to 22nm when grown in 0,05% NaCl. This thickening of the cell seems to be related to adaptive changes as a non-specific barrier to preserve the cell structure against high osmotic pressure.

2.3.2 THE EFFECT OF pH

A factor profoundly affecting all microorganisms, including each individual cell of all plant and animal tissues, is

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the acidity or alkalinity of the fluid by which they are surrounded (Frobisher _et_ _al_., 1974). Different species of microorganisms require a pH or hydrogen ion concentration at which growth occurs at the fastest rate and an upper and lower pH at which no growth will occur. It is usually not possible to add acid to foods to lower the pH below that at which microorganisms will grow, since this would cause such products to be rejected because of taste. What is done, therefore, when acids are used, is to combine such treatment with other methods of preservation (Sykes, 1978).

In dealing with the antimicrobial activity of an acid, three aspects have to be considered (Ingram _et_ _al_., 1956):

(i) The effect solely of pH - this effect can be seen in an unbuffered system, where 0,1M HCl producing a pH of about 1, will kill virtually all microbes including yeasts and moulds, although most of these will be little affected by 0,1M acetic acid producing a pH near 3. However, this difference disappears in a strongly buffered system in which equivalent amounts of the two acids gives much the same pH (Ingram _et_ _al_., 1956).

(ii) The relations between the effects of dissociated and undissociated acid, which are related to the pH - often over a range within which pH itself has little effect on the growth of the relevant microorganisms. There is nevertheless a great alteration in the effect of an added preservative. When inorganic acids are used as

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preservatives, their action is due to their hydrogen ion concentration, while the organic acids owe this action mainly to the undissociated molecules (Sykes, 1978). The undissociated form of organic acids is more effective because uncharged molecular units generally penetrate cells more readily than electrically charged ions (Ingram _et al._, 1956).

(iii) Specific effects - these depend on differences between the molecules of the different preservatives in the ability with which they penetrate the cell membrane, the part of the cell which they attack and the chemical nature of that attack (Ingram _et al._, 1956).

Tatini _et al._(1971) found that _S.aureus_ growth could take place at pH values as low as 4,5 to 4,7 in certain food products. However, the type of acidulant used for pH adjustment has a significant effect on the lower pH limit of growth. They found that growth and enterotoxin A production by _S.aureus_ occurred at pH values of 5,0 or greater obtained with either lactic acid or hydrochloric acid in reconstituted nonfat dry milk, whereas neither growth nor enterotoxin production took place at pH 4,5 when adjusted with lactic acid.

2.3.3 THE EFFECT OF TEMPERATURE

Temperature is one of the most important factors influencing growth and survival of all living organisms (Frobisher _et al._, 1974). _S.aureus_ has a temperature

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growth range of 6,5° to 46°C, with an optimum range of 30° to 37°C and is thus classified as a mesophile (Buchanan and Gibbons, 1974).

In general, heat resistance of microorganisms is related to their optimum growth temperatures. The thermal destruction of microorganisms can be determined in terms of D-values for each organism. The D-value is the decimal reduction time, or the time required to destroy 90% of the organisms. This value is numerically equal to the number of minutes required for the survivor curve to traverse one log cycle. D-values may be used to reflect the relative resistance of microorganisms to heat (Jay, 1970). Staphylococci are usually sensitive to heat with a $D_{60°C}$ value of 3 minutes or less in buffer at pH7,0 (Buchanan and Gibbons, 1974).

Temperature can exert dual effects on microbial growth in the intermediate moisture range. It can affect the water requirement of microorganisms directly through its influence on physiological activities or through its effect on the a_w of foods (Lee et al., 1981). Moreover, reduction of a_w will result in increased D-values for thermal destruction of bacteria.

Lee et al. (1981) found that the minimal a_w supporting aerobic staphylococcal growth in bacon increased from 0,84 to 0,88 when the incubation temperature was decreased from 37°C to 20°C. From the isotherms obtained at

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these temperatures it could be seen that a bacon sample with an a_w of 0,84 at 37°C had an a_w of 0,87 at 20°C. Therefore it appears that the difference in isotherms was the major reason for the increased minimal a_w requirement for growth at 20°C. These results are in agreement with those of Lötter and Leistner (1978) who observed a lower minimal a_w required for growth at 30°C (0,867) than at 20°C (0,887).

Lee *et al*. (1981) studied the isotherms of raw chicken at three temperatures (5°,45° and 60°C), and reported that for samples with the same water content, the a_w value increased as the temperature increased throughout the range of a_w's studied (0,50 to 0,80).

2.3.4 COMBINED STRESS FACTORS - THE "HURDLE" EFFECT

The inhibition of microorganisms in IMF cannot solely depend on either a_w, pH, redox potential, temperature, preservatives or competitive microbes, but on a combination of two or more of these factors. How many of these "hurdles" and at what level they are needed for the stability of IMF depends not only on the type but also on the number of organisms present (Leistner and Rödel, 1978). IMF's have a disadvantage in relation to sensoric properties and a "chemical overloading" of the product. Thus "shelf stable" products which have an a_w between 0,95 and 0,90 could gain importance. A depression of a_w below 0,95 can be accomplished with

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legally permitted humectants which do not impair the organoleptic quality of the products (Leistner and Rödel, 1978).

If the growth of S.aureus is to be controlled, either an a_w of less than 0,86 for aerobic conditions, an a_w of less than 0,91 for anaerobic conditions (Scott, 1953), or a pH below 5,0 is required (Leistner and Rödel, 1978), since either one of these "hurdles" may protect the product.

Humectants such as glycerol or 1,2-propanediol can be used for the reduction of a_w, but when used in large quantities these impart unfavourable tastes to the food. Thus a_w, redox potential, temperature and pH should be used in combination with each other (Leistner and Rödel, 1978).

Nunheimer and Fabian (1940) investigated the effects of mixtures of acids with sucrose or NaCl solutions on six S.aureus strains. Only 5% NaCl plus 0,15 millequivalents of HCl was required to achieve a 50% reduction in growth; however, 11,5% NaCl was required to produce a similar growth reduction with 0,4 millequivalents of citric acid. These workers concluded that when a_w was used in combination with pH reduction to inhibit growth and toxin production of staphylococci, the organic acids were less effective when combined with NaCl than the inorganic acids.

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Calhoun and Frazier (1966) found that when S.aureus was heated to 60°C in phosphate buffer adjusted to a_w 0,950 with NaCl, there was increased heat resistance as compared to the organism in the control buffer at a a_w of 0,994. Yet, when S.aureus was heated in buffer adjusted to a_w of 0,950 with glucose, no increased protection was found.

Hughes and Hurst (1980) and Hurst et al. (1980) have shown that the addition of high levels of magnesium chloride, potassium chloride, glucose or sucrose to the growth medium of S.aureus permitted growth and enterotoxin formation at temperatures of at least 2°C higher than that of the unsupplemented growth medium. These authors suggested that the protective effect of the solutes were due to the osmotic effect of the non-penetrating Cl^- or sugar molecules. Studies on increased resistance to the bactericidal effect of heat in the presence of solutes thus suggests that the chemical nature of the solute is more important than the a_w.

The increased temperature required for the injury or death of microorganisms in the presence of certain solutes, is an important aspect for the safety of food products, particularly IMF's. If such foods were to receive marginal heat treatment (sufficient for foods of low solute content) organisms present might be injured rather than killed.

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Such injured staphylococci have been shown to recover, grow and produce toxins (Collins-Thompson *et al*., 1973 and Fung and Vandenbosch, 1975). Consequently, food with such characteristics should be processed sufficiently to ensure that all organisms have been killed rather than injured.

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C H A P T E R 3

MATERIALS AND METHODS

3.1 GROWTH MEDIA

Unless otherwise specified all media were sterilized at 121°C for fifteen minutes.

3.1.1 BAIRD-PARKER AGAR (BAIRD-PARKER, 1962)

Meat extract	5,0g	Glycine	12,0g
Peptone from casein	10,0g	Lithium chloride	5,0g
Yeast extract	1.0g	Agar	15,0g
Sodium pyruvate	10,0g		

- dissolve in 1 litre distilled water
- adjust to pH 6,8 ± 0,2 if necessary
- sterilize and then cool to 50°C
- add 50ml of egg yolk and 3ml of a filter-sterilized 3,5% aqueous solution of potassium tellurite.

3.1.2 NUTRIENT BROTH/AGAR (MERCK)

Meat extract	3,0g	Peptone from meat	5.0g

- dissolve in 1 litre distilled water
- adjust to pH 7,0 ± 0,2 if necessary
- to make nutrient agar add 12,0g agar-agar.

3.1.3 BRAIN HEART INFUSION BROTH (ROSENOW, 1919)

Infusion from brain	12,5g	D(+) glucose	2,0g
Infustion from heart	5,0g	NaCl	5,0g
Proteose peptone	10,0g	Na_2HPO_4	2,5g

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- dissolve in 1 litre distilled water
- adjust to pH 7,4 ± 0,2 if necessary.

3.1.4 STANDARD I NUTRIENT BROTH/ AGAR (MERCK)

Special peptone	15,6g	NaCl	5,6g
Yeast extract	2,8g	D(+) glucose	1,0g

- dissolve in 1 litre distilled water
- adjust to pH 7,5 ± 0,1 if necessary
- to make agar add 12,0g agar-agar.

3.1.5 PURPLE BROTH BASE (DIFCO)

Proteose-peptone No. 3	10,0g	NaCl	5,0g
Bacto-beef extract	1,0g	Bromo cresol purple	0,015g

- add to 1 litre distilled water
- adjust to pH 6,8 if necessary
- add 1% of the desired carbohydrate.

3.1.6 GELATIN AGAR PLATES (SMITH, GODAN AND CLARK, 1952)

Special peptone	15,6g	D(+) glucose	1,0g
Yeast extract	2,8g	Agar-Agar	12,0g
NaCl	5,6g	Gelatin	4,0g

- dissolve in 1 litre distilled water
- adjust to pH 7,4 if necessary.

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3.2 BACTERIAL CULTURES

3.2.1 AUTHENTIC CULTURES

Table 3.1 contains a list of authentic type cultures used in this study.

TABLE 3.1 AUTHENTIC BACTERIAL CULTURES USED IN THIS STUDY

CULTURE NUMBER	TYPE COLLECTION AND NUMBER	ORGANISM
S276	DSM NO. 20231	*S.aureus*
S292	CCM NO. 5739	*S.intermedius*
S297	FDA NO. 196E (ATCC NO. 3565)	*Staphylococcus* sp.+

+ known for the production of enterotoxin A; produces large amounts of beta-hemolysin (Casman *et al.*, 1963).

3.2.2 ISOLATES

3.2.2.1 ISOLATION PROCEDURES

From each sample, 20g was homogenised in a Colworth Model 400 stomacher together with 180ml of $\frac{1}{4}$-strength sterile Ringers solution. From each tube of a serial dilution 0,1ml was plated out onto Baird-Parker agar plates using the spread plate technique. The plates were then incubated for 24 hours at 37°C after which typical colonies were isolated from each sample. Subsequently these were taken and tested for coagulase reaction.

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3.2.2.2 STORAGE AND MAINTENANCE OF CULTURES

After isolation and purification, all strains were lyophilised. For this purpose cultures were cultivated on Standard I nutrient agar slants for 24 hours and the growth washed off into 1ml of sterile 10% skim milk containing 1% lactose. Into each of four sterile freeze dry ampoules (10cmx6,5mm), 0,25ml of bacterial suspension was inserted and lysophilised in an Edwards Model EF 03 centrifugal freeze drier. The ampoules were sealed under vacuum and then stored at room temperature.

Bacterial cultures maintained on either Baird-Parker agar or Standard I nutrient agar were sub-cultured monthly.

3.2.2.3 REVIVAL OF LYOPHILISED CULTURES

Lyophilised cultures were revived by aseptically opening an ampoule and transferring the contents with the aid of 0,5ml BHI broth into a test tube containing 5ml of this media. Cultures were incubated for 24 hours at 37°C and then plated out onto Baird-Parker agar plates to check for purity.

3.3 IDENTIFICATION TESTS

3.3.1 THE COAGULASE TEST

The production of coagulose was determined using the tube method of Gillespie (1943). To 0,5ml of coagulase plasma (Difco No. 0286-47), 0,1ml of an overnight bacterial culture was added. The tubes were incubated at 37°C and examined after two hours. Complete or partial coagulated

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plasma was interpreted as a positive result. Negative tubes were incubated for a further 24 hours before being classified as non-coagulase producers.

3.3.2 FERMENTATION OF CARBOHYDRATES

A few drops of an overnight culture of the test organism was added to a 1% solution of mannitol or maltose in purple broth base. The tubes were incubated for 24 hours at 37°C. Development of a yellow colouration was taken as a positive reaction (Harrigan and Mc Cance, 1976).

3.3.3 HYDROLYSIS OF GELATIN

Hydrolysis of gelatin was determined using the method of Smith, Godan and Clark (1952). The bacterial cultures were inoculated onto 0,4% gelatin agar plates. The plates were incubated at 37°C for 24 hours and then flooded with 5 to 8ml of mercuric chloride solution (15g of H_gCl_2 and 20ml of conc. HCl were dissolved in 100ml distilled water). Unhydrolysed gelatin forms a white opaque precipitate with the reagent while hydrolysed gelatin appears as a clear zone.

3.3.4 VOGES-PROSKAUER TEST

The Voges-Proskauer test was performed using the method of Barritt (1936). Tubes of glucose phosphate broth were inoculated with 0,1ml of an overnight bacterial suspension. The tubes were incubated at 37°C for 24 hours and 1ml of each culture were transferred aseptically into a sterile test tube. To each tube 0,5ml each of a 6% α- naphthol solution in 95% ethanol and a 16% potassium hydroxide solution was added and mixed. Development of

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a red colouration usually within ten minutes was taken as a positive reaction.

3.3.5 PHAGE TYPING

Bacterial cultures were phage typed by the South African Institute for Medical Research using the standard method of the Staphylococcus Reference Laboratory, London (1980). Single colonies were inoculated into 5ml volumes of nutrient broth and incubated for four to six hours at 37°C, or until there was visible growth. Nine-centimeter plates of nutrient broth agar were flooded with the culture and the excess fluid removed. The plates were dried for about thirty minutes at room temperature and the phages applied at RTD (routine test dilution) in a standard arrangement. The phage drops were allowed to dry and the plates incubated overnight at 30°C. Then the plates were examined for lysis and reactions recorded as follows irrespective of the size of the phage plaques:

1 to 19 plaques	±
20 to 50 plaques	+
50 plaques, confluent lysis	++

Strains that were non-typable (i.e. did not show one or more strong ++ reaction) were retested using phages 100 times stronger than RTD (RTDx100) and the results recorded as follows:

confluent lysis without secondary growth CL;
confluent lysis with secondary growth $\overline{CL}$;
inhibition reaction with or without superimposed plaques 0.

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3.4 BACTERIAL GROWTH UNDER STRESS CONDITIONS

3.4.1 INFLUENCE OF pH ON BACTERIAL GROWTH

Growth of *S.aureus* was tested in BHI broth adjusted to different pH values with HCl and the following organic acids: citric, ascorbic, lactic, tartaric, acetic and gluconic acid. For each acid tested, thirteen standard solutions of BHI (pH 7,2) were prepared. Twelve of these solutions were adjusted to the pH range 4,0 to 5,1 using 0,1 pH intervals (Figure 3.1), while the thirteenth solution was used as control. Eight milliliters of each solution was then dispensed into each test tube, sterilized at 121°C for twenty minutes and the pH values rechecked.

Into each tube, 0,1ml of an overnight bacterial culture was added. These tubes were then incubated at 37°C for seven days without shaking and bacterial growth measured in terms of absorbance at 560nm using a Gilford 250 spectrophotometer. The results were presented as a scale of pH vs absorbance.

3.4.2 INFLUENCE OF a_w ON BACTERIAL GROWTH

Using the data given in Table 3.2 to standardize a Nova Sina Model EEJA-6 water activity meter, standard graphs of percentage humectant (w/v) in BHI broth vrs a_w were drawn for the following humectants:

NaCl (10 to 24% : 1,71 to 4,11 mol),
glycerol (27,5 to 52,5% : 2,99 to 5,7 mol),
propylene glycol (12 to 32% : 1,58 to 4,20 mol),
sodium lactate (17,5 to 42,5% : 1,56 to 3,79 mol) and
sucrose (40 to 64% : 1,17 to 1,87 mol).

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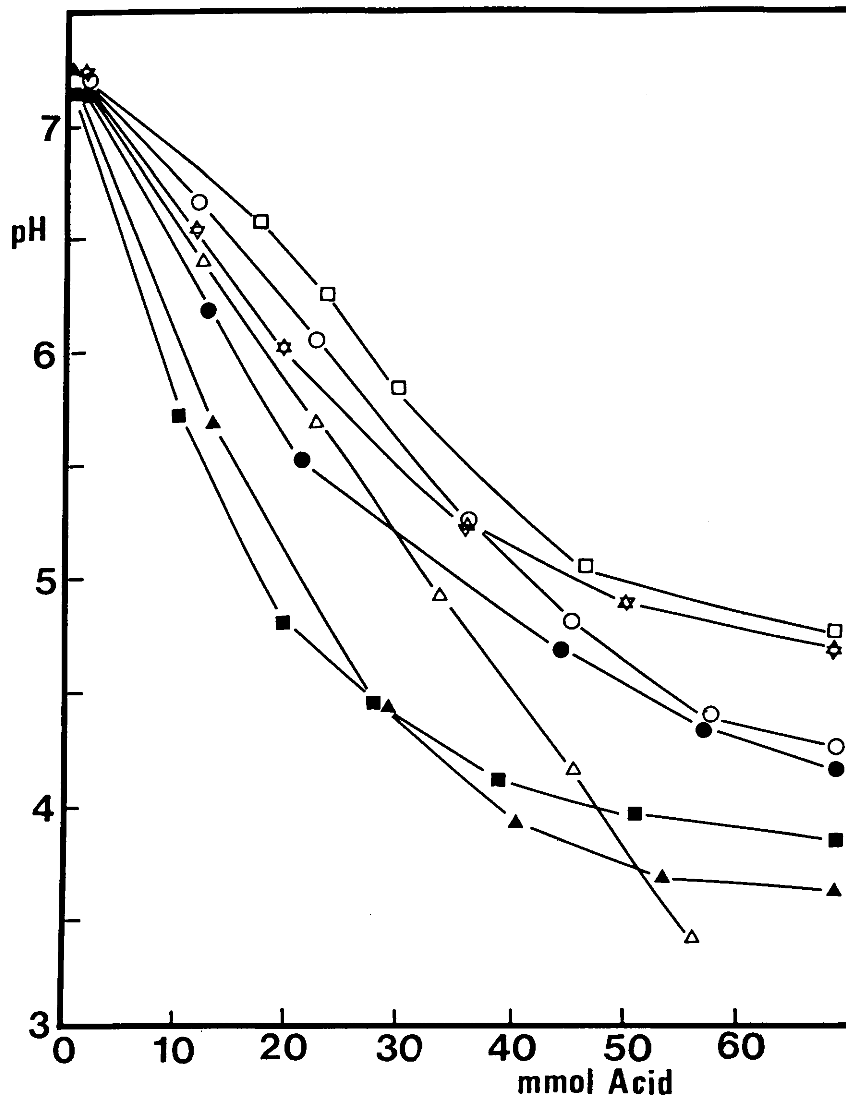

Figure 3.1 Standard curves of mmol acid vs pH in BHI.

■——■ citric acid; ○——○ ascorbic acid;
●——● lactic acid; ▲——▲ tartaric acid;
✡——✡ acetic acid; □——□ gluconic acid;
△——△ hydrochloric acid.

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TABLE 3.2 MOLALITY/% NaCl REQUIRED TO MAKE UP STANDARD a_w SOLUTIONS AT 25°C (PERSONAL COMMUNICATION: H. HECHELMANN, 1981, KULMBACH, GERMANY.)

MOLALITY	NaCl (%)	a_w-VALUE (+25°C)
0,2	1,15	0,993
0,4	2,28	0,986
0,6	3,39	0,980
0,8	4,47	0,973
1,0	5,52	0,966
1,2	6,55	0,960
1,4	7,56	0,953
1,6	8,55	0,946
1,8	9,52	0,938
2,0	10,46	0,931
2,2	11,39	0,924
2,4	12,30	0,916
2,6	13,19	0,908
2,8	14,06	0,901
3,0	14,92	0,893
3,2	15,75	0,885
3,4	16,58	0,876
3,6	17,38	0,858
3,8	18,17	0,860
4,0	18,95	0,851
4,2	19,71	0,843
4,4	20,45	0,834
4,6	21,19	0,825
4,8	21,91	0,816
5,0	22,61	0,807
5,2	23,31	0,798
5,4	23,99	0,788
5,6	24,66	0,779
5,8	25,31	0,769
6,0	25,96	0,760

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For each humectant range tested, the required concentration of humectant (w/v) was added to BHI broth. Eight milliliters of each solution was then dispersed into each test tube, sterilized at 121°C for twenty minutes and the a_w values checked. Into each tube, including control tubes containing BHI broth without humectant, 0,1ml of an overnight bacterial culture was added. These tubes were then incubated at 37°C for seven days without shaking, and bacterial growth measured in terms of absorbance at 560nm using a Gilford 250 spectrophotometer. The results were presented as a scale of a_w vs absorbance.

The specific effects of NaCl (10% : 1,71 mol), glycerol (26% : 2,82 mol), sucrose (56% : 1,64 mol), propylene glycol (18,8% : 2,47 mol) and sodium lactate (17,5% : 1,56 mol), all producing a_w 0,925 in BHI broth, on bacterial growth were determined by inoculating 200ml of the broth with a 1% overnight bacterial culture. The broths were incubated with shaking at 37°C. Samples were removed aseptically every three hours for fifteen hours and then after 24 and 27 hours. Bacterial growth was measured in terms of absorbance at 560nm using a Gilford 250 spectrophotometer and results presented as a scale of a_w vs absorbance.

3.4.3 INFLUENCE OF TEMPERATURE ON BACTERIAL GROWTH

These experiments were conducted with the aid of a temperature gradient incubator (Scientific Industries Inc.,

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New York). In all the experiments a temperature range of 2° to 60°C was used and a shaker speed of three was selected. Temperatures in the 30ml culture tubes were allowed to equilibrate for one hour before 0,15ml of an overnight bacterial culture was transferred into each of the 30 culture tubes, containing 15ml BHI broth. After 24 hours, bacterial growth was measured in terms of absorbance at 560nm using a Gilford 250 spectrophotometer. The results were presented as a scale of temperature vs absorbance.

3.4.4 D_{10} VALUES FOR HEAT DESTRUCTION OF CULTURES

The absorbance of 10^7 cells/ml was first determined: A flask containing 100ml BHI broth was inoculated with 1ml of an overnight bacterial culture. The flask was then incubated at 37°C on a bench shaker. At time zero and then after every half hour for seven hours, 2ml of each sample was removed aseptically. Half of the sample was used to make a serial dilution in $\frac{1}{4}$-strength Ringers while the absorbance of the remaining sample was measured using a Gilford 250 spectrophotometer. From each serial dilution, 0,1ml was plated out onto Standard I nutrient agar, incubated at 37°C for 24 hours after which colonies were counted.

D_{10} values at 60°C for heat destruction of the bacterial cultures were then determined as follows: One hundred milliliters of BHI broth was inoculated with 1ml of an overnight bacterial culture and incubated on a bench

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shaker until the absorbance (λ = 560nm) of the culture was approximately 1,4 (which corresponded to 10^7 cells/ml). One milliliter of this culture was then pipetted into each of 7 freeze drying ampoules (10cmx6,5mm) and these inserted into a water bath at 60°C. When the temperature of the culture reached 60°C, one ampoule was removed (time 0) and its entire contents diluted with 9mls of cold sterile $\frac{1}{4}$-strength Ringers (10^{-1} dilution) from which a serial dilution was made. After $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$, 3, and $3\frac{1}{2}$ minutes respectively, an ampoule was removed and processed as above. From each serial dilution 0,1ml was plated out onto Standard 1 nutrient agar, incubated at 37°C for 24 hours, after which colonies were counted. The results were presented as a scale of $\log_{10}$ (number of surviving cells/ml) vs time and the D_{10} value calculated.

3.5 BACTERIAL GROWTH UNDER COMBINED STRESS CONDITIONS

3.5.1 INFLUENCE OF pH AND POTASSIUM SORBATE ON BACTERIAL GROWTH

Growth of S.aureus was tested in BHI broth (initial pH 7,2) to which either 0,5, 1 or 2,5% potassium sorbate was added respectively and in BHI broth (initial pH 5,5 adjusted with conc. HCl) to which either 0,5, 1 or 2,5% potassium sorbate was added respectively. Flasks inoculated with a 1% overnight bacterial culture were incubated at 37°C on a bench shaker and bacterial growth measured in terms of absorbance at 560nm using a Gilford 250 spectrophotometer after twelve hours. The results

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were presented in tabular form.

3.5.2 INFLUENCE OF pH AND a_w ON BACTERIAL GROWTH

Growth of S.aureus was tested in BHI broth adjusted to a_w 0,925, using glycerol (26% : 2,82 mol), sodium lactate (17,5% : 1,56 mol), propylene glycol (18,8% : 2,47 mol), sucrose (56% : 1,64 mol) and NaCl (10% : 1,71 mol) and to pH 5,5 using citric, lactic, acetic, gluconic and hydrochloric acids. For controls humectant solutions (a_w 0,925) in BHI broth containing no added acid (pH 7,2) and acid solutions in BHI broth (pH 5,5) containing no humectant solution (a_w 0,985) were used. Flasks inoculated with a 1% overnight bacterial culture were incubated at 37°C on a bench shaker and bacterial growth measured in terms of absorbance at 560nm using a Gilford 250 spectrophotometer after 24 hours. The results were presented in tabular form.

3.6 CELL WALL ANALYSIS

3.6.1 ISOLATION AND PURIFICATION OF CELL WALLS

A modified method of Schleifer and Kandler (1967) was used. Flasks containing 400ml Std 1 nutrient broth were inoculated with 10ml of an overnight bacterial culture and incubated at 37°C on a bench shaker for 48 hours. The cells were harvested by centrifugation in a Sorval centrifuge at 8 000rpm for twenty minutes in a GSA rotor and the pellets frozen for one to two hours. The pellets were weighed and washed with distilled water into the tube of the cell mill

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so as to fill $\frac{1}{3}$ of the tube. The suspensions were heated for five minutes at 100°C to prevent autolysis and then the remaining $\frac{2}{3}$ of the tube was filled with glass beads 0,1 to 0,11 mm in diameter. The cells were broken in a Edmund Bühler Type Vi:2 cell mill for twenty minutes. The suspension was observed under the microscope to ensure that all the cells had been broken. The broken cells and beads were filtered through a sinterglass filter and the beads washed with the minimum of distilled water. The filtrate was harvested by centrifugation at 15 000rpm for twenty minutes in a SS-34 rotor in a Sorval centrifuge and the pellets washed in 200ml 0,1M phosphate buffer (pH 7,8). A knife point of trypsin (Boehringer and Mannheim No. 109827) as well as 10ml toluene were added, the flasks covered with parafilm and incubated overnight at 37°C. The suspension was harvested by centrifugation at 15 000rpm for twenty minutes in a SS-34 rotor and the pellet washed four times with distilled water. The pellet was frozen overnight in a plastic petri-dish and then lysophilised in an Edwards Model EF03 freeze drier for 48 hours.

3.6.2 TOTAL HYDROLYSIS OF CELL WALLS (HOLZAPFEL, 1969)

Ten milligrams of lysophilised cell walls were hydrolyzed with 1ml 4N HCl in a sealed glass ampoule for sixteen hours at 100°C. The hydrolyzates were dried in a hot water bath under nitrogen gas, washed twice with 1ml distilled water and

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then dissolved in 1ml citrate buffer, pH 2,2 for quantative analysis with the amino acid analyser. The composition of the citrate buffer is as follows:

39,2g of sodium citrate. $2H_2O$,
33,0ml of conc. HCl,
10,0ml of 2,2' thiodiethanol,
1,0ml of caprylic acid (octanoic acid),
43,83ml of L-2-amino-β-quanidino propionic acid
and 31,49 g of L-Nor-leucine were dissolved in a total volume of two litres distilled water and the pH adjusted to 2,2 if necessary.

For qualitative analysis the sample was dissolved in a few drops of distilled water.

3.6.3 PARTIAL HYDROLYSIS OF CELL WALLS (HOLZAPFEL, 1969)

Ten milligrams of lysophilised cell walls were hydrolyzed with 1ml 4N HCl in a sealed glass ampoule for three hours at 100°C. The hydrolyzates were dried in a hot water bath with nitrogen gas, washed once with 1ml distilled water and then dissolved in a few drops of distilled water.

3.6.4 QUANTATIVE DETERMINATION OF AMINO ACIDS (HOLZAPFEL, 1969)

One milliliter of totally hydrolyzed cell walls dissolved in citrate buffer (pH 2,2) were centrifuged in an Eppendorf 5412 bench centrifuge at 2 000rpm for ten minutes. Half a milliter of supernatant was then added to 4,5ml citrate buffer (pH2,2) and amino acid analysis was performed with a Beckman automatic amino acid analyzer using a double column system.

3.6.5 QUALITATIVE DETERMINATION OF AMINO ACIDS (HARPER AND DAVIS, 1979)

The amino acid composition of totally hydrolyzed cell walls dissolved in a few drops of distilled water were analyzed

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using a two-dimensional thin-layer chromatography system. For each analysis, 2 to 4mg of hydrolysate were spotted onto SS 2043 (Schleicher and Schül) paper keeping the sample spot size to a minimum. The first dimensional run was carried out in isopropanol : acetic acid : water (75 : 10 : 15 vol/vol) for 22 to 24 hours, and was repeated three times, the chromatograms being air dried after each run. The chromatograms were then inserted twice into the second system of 2 methylpyridine : water : NH_3 (70:28:2 vol/vol) for approximately 20 hours. After being air dried, the chromatograms were developed by being sprayed with ninhydrin reagent (5g of 2,2-dihydroxy-1,3-indanoian dissolved in 70ml conc. acetic acid and 930ml water saturated butanol) and dried in an oven at 100°C. The water saturated butanol was obtained by mixing 900ml butanol with 100ml water and removing the upper phase which is the water saturated butanol.

3.6.6 QUALITATIVE DETERMINATION OF SUGARS AND TEICHOIC ACID (TREVELYAN ET AL., 1950)

One-dimensional chromatography was performed on partially hydrolyzed cell walls on SS 2043 paper in butanol : pyridine : water : acetic acid (60:40:30:3) for 24 hours. The dried paper chromatogram was passed through a silver nitrate/acetone reagent solution (0,1ml of a saturated silver nitrate solution was dissolved in 20ml acetone and water was then added dropwise until the silver nitrate which separated on the addition of acetone had redissolved), dried and then sprayed with a 0,5N solution of sodium hydroxide

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in aqueous ethanol (8,3ml of a 30N solution of sodium hydroxide in water was added to 491,7ml ethanol). Excess silver oxide was dissolved by immersing the chromatogram in 6N ammonium hydroxide for a few minutes after which the chromatogram was washed for one hour in running water.

3.7 CELL MEMBRANE ANALYSIS

3.7.1 ISOLATION OF LIPIDS

The method of Bligh and Dyer (1959) as modified by Houtsmuller and van Deenen (1965) was used. The bacterial cells were harvested by centrifugation in a Sorval refrigerated centrifuge at 8 000rpm for twenty minutes and resuspended in 30ml phosphate buffer pH 7. A single phase system was obtained by the addition of 30ml chloroform and 65ml methanol and the solution stored at 2°C for 48 hours. The cells were removed by centrifugation (8 000rpm for twenty minutes in a Sorval centrifuge) and the supernatant solvent mixture mixed with a quarter of the volume each of water and chloroform. The aqueous phase not containing any appreciable quantity of phospholipid was removed and the chloroform layer was evaporated under reduced pressure. The phospholipids were then resuspended in 0,5ml of ethanol.

3.7.2 QUALITATIVE DETERMINATION OF LIPIDS

One-dimensional thin-layer chromatography on Silica Gel G glass plates was performed on lipid samples using chloroform : mehtanol : acetic acid : water (42:12:3,15:1) as solvent system (White and Frerman, 1967). The lipids were

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detected by spraying the plates with a 10% H_2SO_4 solution and then by charring them for five minutes at 100°C (Kanemasa et al., 1975).

3.8 METABOLIC STUDIES

3.8.1 DETERMINATION OF LACTIC ACID PRODUCTION

Lactic acid production and configuration was determined using the enzymatic-spectrophotometric method of Bergmeyer (1965). For each sample to be tested, 20 µl of sample was added to two plastic cuvetts to which 3,0ml glycine-hydrazine hydroxide buffer (45,6ml glycine, 100ml of 25% hydraziniumhydroxide and 1100ml distilled water; pH 9,0) and 200 µl NAD-solution (300mg NAD in 10ml distilled water) had been added. Two control cuvettes were prepared, each containing 3,0ml buffer, 200µl NAD-solution and 20 µl BHI broth. The solutions and sample were mixed by inversion and their absorbancies measured at 366 nm using a Gilford 250 spectrophotometer (0 hour value). To zero the instrument a solution of 3,0ml buffer 200 µl NAD-solution and 0,2ml distilled water was used. Into each sample and control cuvettes either 20 µl D-LDH or 20 µl L-LDH was added. The cuvetts were mixed by inversion, incubated at 37°C for one hour after which their absorbance was once again measured at 366nm (one hour value). The control value was subtracted from the 0 hour reading for each sample and the new zero value was subtracted from the one hour value. This value was then used to calculate the percentage lactate using the following

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formula:

$$\text{Lactic acid isomer (mg/ml sample)} = \frac{\Delta E \text{ corr} \times \text{Vcuv} \times \text{Verd} \times \text{mwt}}{\xi \times d \times V \text{ sample} \times 1\,000}$$

$$= \Delta E \text{ corr} \times \text{Verd} \times \text{Factor}$$

where Factor = 4,41818
ΔE corr = Absorbance difference between 0 and 1 hr
V cuvett in ml = 3,24
Verd = 1
mwt = 90
ξ = 3,30 at 366nm
V sample in ml = 0,02
d = 1cm

$$\% \text{ lactate} = \frac{\text{lactate isomer (mg/ml sample)}}{1\,000} \times \frac{100}{1}$$

3.8.2 DETERMINATION OF ACETIC ACID PRODUCTION

Acetic acid production was determined with the aid of a Fractovap Series 4100 Carlo Erba gas chromatograph. The separation was performed in a glass column (190 x 0,3 cm) containing Supeko GP 15% SP-1220/1% H_3PO_4 on Chromosorb 100/120 WAW. Operating conditions were : input 10; output 4; coarse 100; inject 160; oven temperature 125°C; detector temperature 270°C and carrier 0,2. A standard curve was obtained by analysing a series of 0,01 to 0,1% acetic acid dilutions and the results were presented as a scale of % acetic acid vs peak height. For each analysis 10 μl of sample were used.

3.9 MICROSCOPIC TECHNIQUES

3.9.1 SCANNING ELECTRON MICROSCOPY (KAY, 1967)

The standard method of fixation using 6% glutaraldehyde and 2% osmium tetroxide, followed by dehydration through a series of alcohols and finally by 1,1,2-trichlorotrifluoroethane,

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was used. Bacterial samples to be viewed were collected on cellulose millipore filters which were passed through the following solutions:

(i) 6% glutaraldehyde in 0,05M sodiumcacodylate buffer for 30 min (a 0,2M stock sodiumcacodylate buffer was made by adding 21,40g of $AsO_2Na.3H_2O$ to 500ml of distilled water and the pH adjusted to 7,2 to 7,4 with conc. HCl; to obtain 6% glutaraldehyde in 0,05M buffer, 25 ml of the 0,2M stock buffer was added to 24ml of 25% glutaraldehyde and 51ml distilled water and the pH adjusted to 6,8 to 7,4 using conc. HCl).

(ii) 0,05M sodiumcacodylate buffer pH 7,3 for 2x10 min.

(iii) 2% osmium in 0,05M sodiumcacodylate buffer pH 7,3 for 30 min (dissolve 0,5g OSO_4 in 25ml 0,05M Na-cacodylate buffer with stirring for one day).

(iv) 0,05M sodiumcacodylate buffer pH 7,3 for 2x10 min.

(v) Through 30%, 50%, 70% and 96% ethanol respectively for 10 min each.

(vi) 100% ethanol for 2x10 min.

(vii) 1,1,2-trichlorotrifluoroethane for 2x10 min.

The samples were then mounted onto stubs, coated with gold and viewed using a Hitachi S-450 Scanning Electron Microscope.

3.9.2 TRANSMISSION ELECTRON MICROSCOPY (KAY, 1967)

Bacterial cultures were negatively stained with phosphotungstic acid (PTA) pH 6 as follows: Carbon formvar films were put onto 200 mesh 3mm copper electron microscope grids. The grids were put on top of a drop of the bacterial culture for five minutes. The bacteria were stained for five

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seconds in PTA and the excess stain drained off using a piece of filter paper. Excess stain was removed by washing the grid twice in distilled water. The grids were dried and then viewed in a Hitachi H-600 Transmission Electron Microscope.

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C H A P T E R 4

RESULTS AND DISCUSSION

4.1 OCCURRENCE AND CLASSIFICATION OF COAGULASE POSITIVE STAPHYLOCOCCI IN MINCED AND PROCESSED MEATS

S.aureus has a potential role of causing food posioning in fresh and processed foods (Tatini, 1973). Thus a survey was undertaken in order to determine the occurrence of the organism in fresh minced meat and in shelf stable meat products. Tables 4.1 and 4.1a list the coagulase positive isolates that were obtained from fresh-minced meat and shelf stable meat products respectively.

The thirty-five coagulase positive isolates (Table 4.1 and 4.1a) and the three type strains (Table 3.1) were classified according to the scheme of Kloos and Schleifer (1975) as modified by Holzapfel *et al*. (1981). According to the modified scheme, coagulase, maltose and Voges-Proskauer positive reactions and a gelatinase negative reaction identifies the organism as *S.aureus* while coagulase, gelatinase and mannitol positive reactions with Voges-Proskauer and maltose negative reactions, identifies the organism as *S.intermedius*. From the results given in Tables 4.1 and 4.1a it can be seen that 88,5% of the coagulase positive isolates were *S.aureus*, while the remaining 11,5% were *S.intermedius*.

Table 4.1 shows that *S.aureus* can readily be isolated from fresh minced meat obtained from retail outlets in South Africa. Isolates of *S.aureus* are readily obtained from fresh minced meat due to the increased surface area of the

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TABLE 4.1 INFORMATION ON SOURCE, ISOLATION AND IDENTIFICATION OF COAGULASE POSITIVE BACTERIA RECOVERED FROM FRESH MINCED MEAT ON BAIRD-PARKER MEDIUM AND WHICH WERE USED IN THE STUDY

CULTURE NUMBER	RETAIL OUTLET	ORGANISM
S66	Inval Butchery	*S. aureus*
S72	Inval Butchery	*S. aureus*
S78	Northvaal Butchery	*S. aureus*
S144	Northvaal Butchery	*S. aureus*
S149	Arcadia Butchery	*S. aureus*
S150	Arcadia Butchery	*S. aureus*
S174	Renown Meat Market	*S. aureus*
S175	Renown Meat Market	*S. aureus*
S177	Park-Edge Butchery	*S. aureus*
S179	Park-Edge Butchery	*S. aureus*
S232	Northvaal Butchery	*S. aureus*
S240	Renown Meat Market	*S. aureus*
S241	Renown Meat Market	*S. aureus*
S242	Renown Meat Market	*S. aureus*
S243	Renown Meat Market	*S. aureus*
S244	Renown Meat Market	*S. aureus*
S255	Lynwood Manor Butchery	*S. aureus*
S256	Lynwood Manor Butchery	*S. aureus*
S257	Lynwood Manor Butchery	*S. aureus*
S258	Lynwood Manor Butchery	*S. aureus*

TABLE 4.1a INFORMATION ON SOURCE, ISOLATION AND IDENTIFICATION OF COAGULASE POSITIVE BACTERIA RECOVERED FROM SHELF STABLE SAUSAGES (WITH THE EXCEPTION OF S470 WHICH WAS ISOLATED FROM SAVORY MINCE) ON BAIRD-PARKER MEDIUM AND WHICH WERE USED IN THE STUDY

		INFORMATION ON SHELF STABLE SAUSAGES				
CULTURE NUMBER	DILUTION	REHEATED AT 80°C	STORAGE TIME (WEEKS) AT 37°C	INITIAL pH	INITIAL a_w	ORGANISM
S282				5,7	0,948	*S. aureus*
S283				5,7	0,948	*S. aureus*
S284				5,7	0,948	*S. aureus*
S285				5,7	0,948	*S. intermedius*
S286				5,7	0,948	*S. intermedius*
S422	-4	-	1	5,4	0,955	*S. aureus*
S424	-3	-	1	5,2	0,920	*S. aureus*
S425	-4	-	1	5,2	0,920	*S. intermedius*
S426	-4	-	1	5,2	0,920	*S. aureus*
S427	-1	+	2	5,2	0,920	*S. aureus*
S470	-3	+	1	5,1	0,890	*S. intermedius*
S482	-2	-	1	4,82	0,910	*S. aureus*
S483	-2	-	1	4,82	0,910	*S. aureus*
S486	-2	-	1	4,82	0,910	*S. aureus*
S487	-2	-	1	4,82	0,910	*S. aureus*

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product.

Table 4.1a shows that although staphylococci are usually sensitive to heat with a $D_{60°C}$ value of three minutes or less in buffer at pH 7,0 (Buchanan and Gibbons, 1974), they can be isolated from shelf stable products where the cooking process was at 70 to 75°C for one hour. Staphylococci can be isolated from processed meats due to the possible protective effect of fat on bacterial survival during heating (Smith *et al*., 1976) and due to protection afforded by solutes against heat-injury (Smith *et al*., 1982). The initial a_w and pH of the products were favourable for *S.aureus* multiplication, however no cultures were isolated after two weeks incubation. The highest number of organisms after one week's incubation was 10^4 bacteria/g while after two weeks, the highest number of organisms found was 10' bacteria/g. This thus indicates that after two weeks incubation, conditions due to competitive microbes or changes in environment were such so as to inhibit growth of *S.aureus* (Busta, 1976). During the period in which the staphylococci survive, they can metabolize and *S.aureus* can produce heat stable enterotoxins (Lötter and Leistner, 1978), thus the knowledge of the growth of *S.aureus* under various stress conditions is essential for the safe production of shelf stable products.

In order to determine the origin of the staphylococcal

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TABLE 4.2 PHAGE TYPING OF COAGULASE POSITIVE STAPHYLOCOCCI USING THE INTERNATIONAL BASIC HUMAN SET OF PHAGES

	R.T.D.								R.T.D. X 100																			
CULTURE	6	42E	47	53	54	75	77	83A	29	52	52A	79	80	3A	3C	55	71	95	6	42E	47	53	54	75	77	83A	84	94
S150	++		++		+	+			++										Cl	Cl	Cl	Cl	Cl		Cl			+
S175	+	±	++		+	+			++										Cl	Cl	Cl	±	Cl	Cl		+		
S282			++		+						+			+	+					+	Cl	+	++	++		++		
S283					nt				++	+	+			+	+					+	++	+	++	++		++		
S284					nt						+			±	±					+	Cl	+	++	++		++		
S285			++		±						+				+					++	Cl	++	++	++	±	++		
S286			++		+	+															Cl			++				
S422					nt										+									+	+		±	
S424					nt										+		±								±			
S426					nt										+		+							+	±			
S427					nt										++		±							+	±			
S470					nt										++		±							+				
S482					nt				++			+			++Cl		++							+				
S483					nt				++			+			++Cl													++
S486					nt				++			+			++Cl		++											++
S487					nt				++						++Cl		++											++

Cl. ++ = strong reaction nt = not typable

+, ± = weak reaction

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contamination all bacterial cultures were phage typed using the international basic human set of phages (Table 4.2). Of the bacteria recovered from fresh minced meat and shelf stable meat products 10% and 93,3% respectively were found to be typable indicating human origin leaving 90% and 6,66% non-typable strains respectively indicating that these were most probably of animal origin. The high percentage of typable strains isolated from shelf stable meat products is most likely due to the increased handling of the product during processing.

4.2 BACTERIAL GROWTH UNDER STRESS CONDITIONS

4.2.1 WATER ACTIVITY (a_w)

S.aureus is the bacterium having the greatest potential hazard to S.S.P. Knowledge of the lowest a_w permitting growth of S.aureus is thus of paramount importance when a food is to be preserved by lowering the a_w through the addition of solutes (Vaamonde, Chirife and Scorza, 1982). Thus the lowest a_w permitting growth of S.aureus was determined for all 35 isolated strains and the 3 type strains under static conditions.

Standard curves of the five humectants studied (glycerol, sodium lactate, sodium chloride, propylene glycol and sucrose),are given in Figure 4.1. All 38 strains were tested for their ability to grow in a range of a_w's adjusted with the five different humectants in order to isolate relatively a_w sensitive and resistant strains. The results of the growth of S258 (a_w sensitive) and S427 (a_w resistant)

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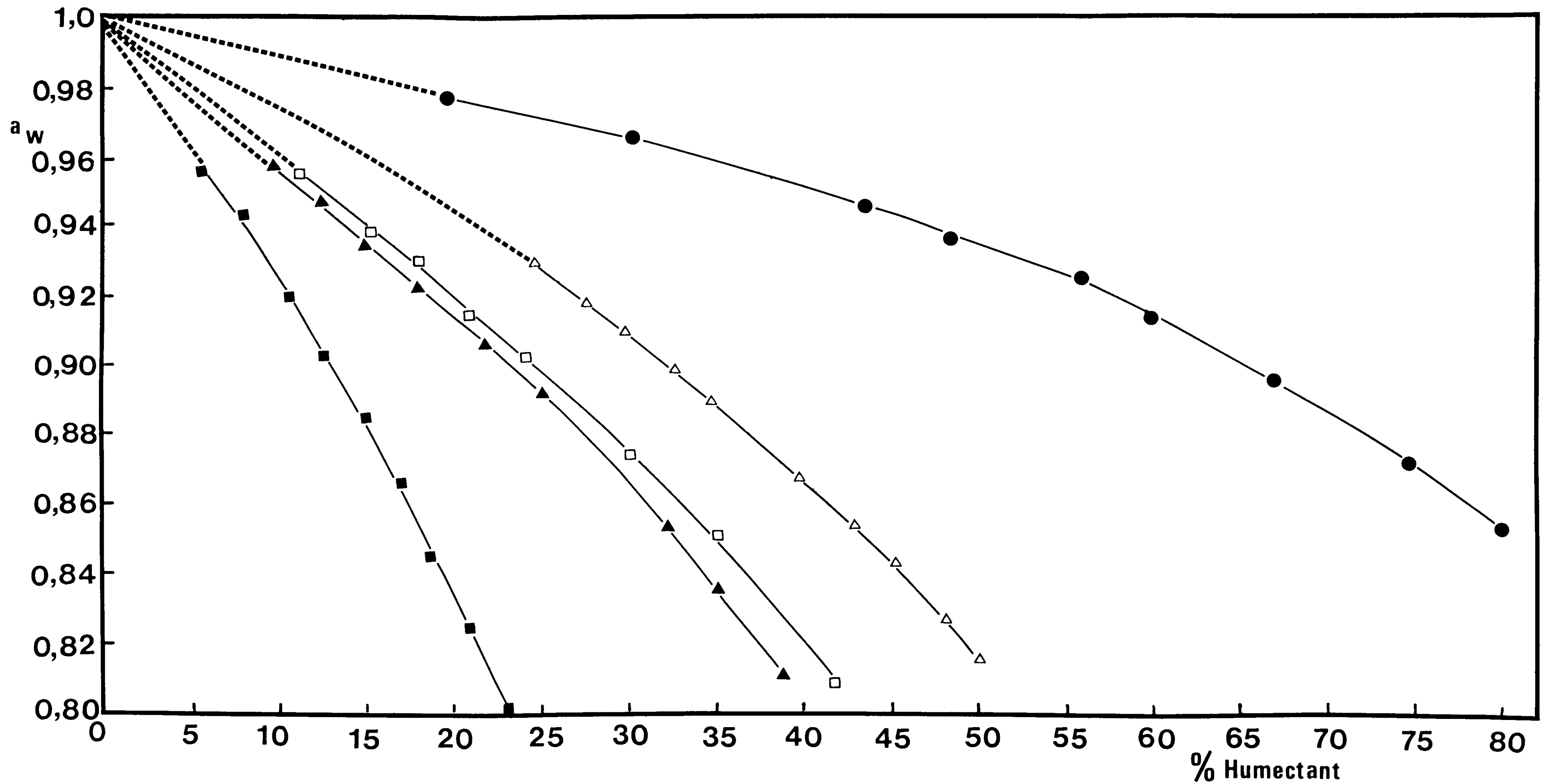

FIGURE 4.1 : Standard curves of % humectant vs a_w in BHI at 25°C.
▲——▲ sodium lactate; ■——■ sodium chloride;
△——△ glycerol; ●——● sucrose;
□——□ propylene glycol.

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are given in figures 4.2 and 4.3 respectively while the minimal a_w required for the growth of the a_w sensitive and resistant strains are given in Table 4.3

TABLE 4.3 MINIMUM a_w FOR THE GROWTH OF S258 (a_w SENSITIVE) AND S427 (a_w RESISTANT) USING DIFFERENT HUMECTANTS. GROWTH WAS MEASURED AFTER INCUBATION AT 37° C IN BHI UNDER STATIC CONDITIONS FOR SEVEN DAYS. (THE MINIMAL a_w WAS TAKEN AS THE a_w WHERE BACTERIAL GROWTH AS MEASURED BY ABSORBANCE AT 560NM WAS 0,1)

CULTURE NUMBER / HUMECTANT	S258	S427
sodium chloride	0,866	0,858
glycerol	0,871	0,845
propylene glycol	0,915	0,904
sodium lactate	0,886	0,900
sucrose	0,881	0,870

From Table 4.3 it can be seen that the minimum a_w for growth of S258 (a_w sensitive) was 0,866 as compared to 0,846 for S427 (a_w resistant). The minimal a_w for S258 was in agreement with the minimal a_w of 0,86 for growth of S.aureus as reported by Scott (1953) and by Lotter and Leistner (1978).

The minimal a_w for growth of S258 was achieved when sodium chloride was used as humectant while for S427 the minimal a_w for growth was found when glycerol was used as humectant. This thus seems to indicate that the type of humectant does influence bacterial growth.

From Figures 4.2 and 4.3 it can be seen that at a constant a_w adjusted using various humectants, bacterial growth varies to a great extent, suggesting that not only a_w but also the

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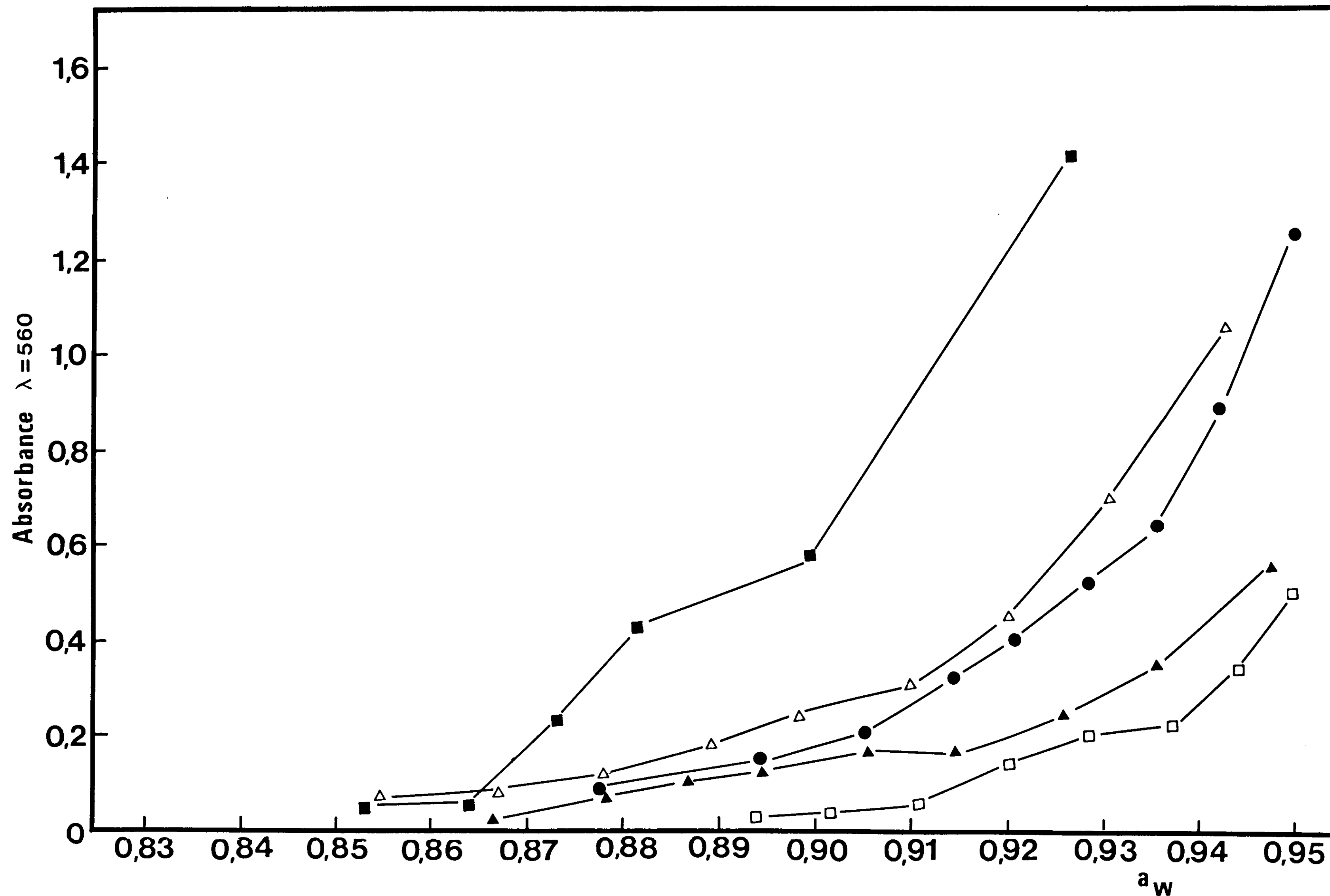

FIGURE 4.2 : Growth curves of S258 using different humectants to adjust the a_w. Growth was in BHI plus humectant at 37°C for 7 days.

■———■ NaCl; ●———● sucrose;
△———△ glycerol; □———□ propylene glycol;
▲———▲ sodium lactate.

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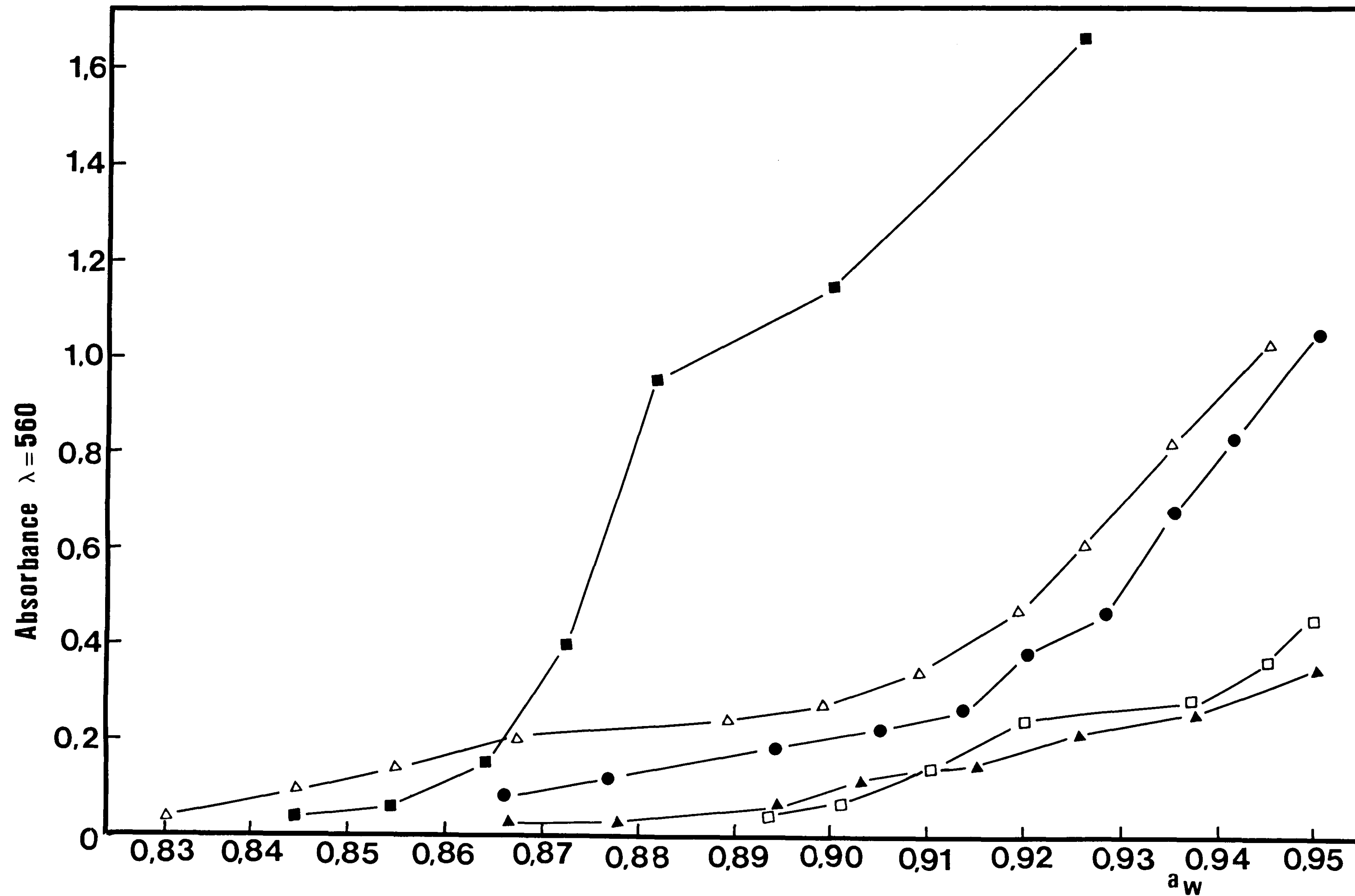

FIGURE 4.3 : Growth curves of S427 using different humectants to adjust the a_w. Growth was in BHI plus humectant at 37°C for 7 days.

■——■ NaCl; ●——● sucrose;
△——△ glycerol; □——□ propylene glycol;
▲——▲ sodium lactate.

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type of humectant plays an important role in controlling bacterial growth.

Scott (1953) initially noted that the minimal a_w which would allow staphylococcal growth was independent of the solutes employed to adjust the a_w of the medium. However Plitman *et al.* (1973) and Christian (1981) found that the a_w of the medium was not the only determining factor regulating the biological response of *S.aureus*, and the nature of the a_w controlling solute also had to be considered.

In order to determine the effect of humectants at a certain a_w level, *S.aureus* strains 258 and 427 were grown at a_w 0,925 with either 10,0% : 1,71 mol sodium chloride, 26% : 2,82 mol glycerol, 56% : 1,64 mol sucrose, 18,8% : 2,47 mol propylene glycol or 17,5% : 1,56 mol sodium lactate.

From Figures 4.4 and 4.5 it can be seen that although the a_w was constant at 0,925, bacterial growth varied greatly in the presence of different humectants. Thus these humectants must exert some kind of bacteriostatic effect on the growth of *S.aureus*. From Figures 4.4 and 4.5 it can be seen that sodium chloride and glycerol had the least bacteriostatic effect causing growth, as measured by an absorbancy of 2,2 to 2,4 after 27 hours, while sodium lactate, sucrose and propylene glycol had much stronger bacteriostatic effects enabling a growth of only 0,2 to 0,6 after 27 hours. The good growth observed with glycerol at a_w 0,925 could be due

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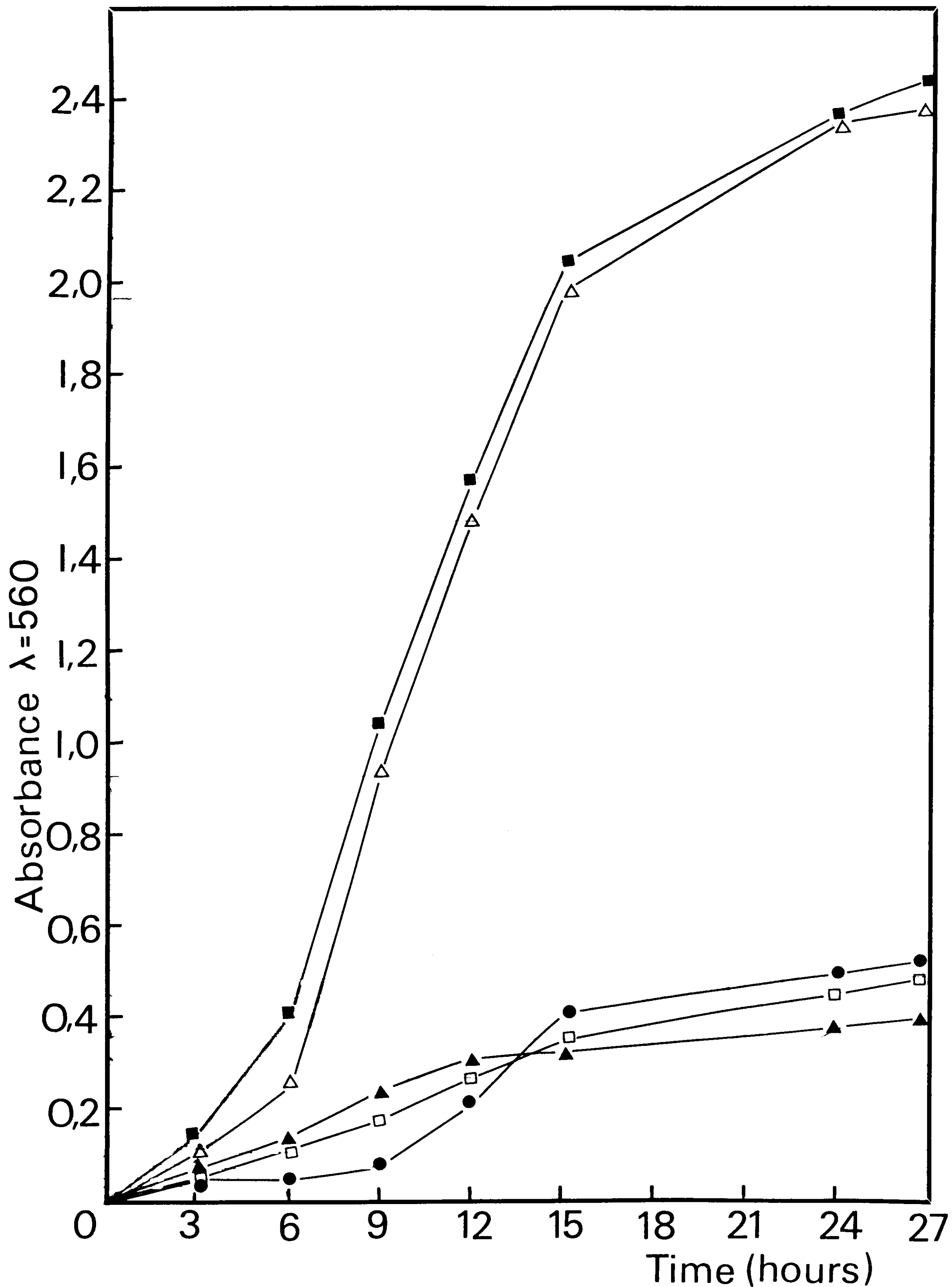

FIGURE 4.4 : Growth curves of S258 in BHI at a_w 0,925 adjusted using various humectants. Growth was at 37°C.

■———■ NaCl; △———△ glycerol;
●———● sucrose; □———□ propylene glycol;
▲———▲ sodium lactate.

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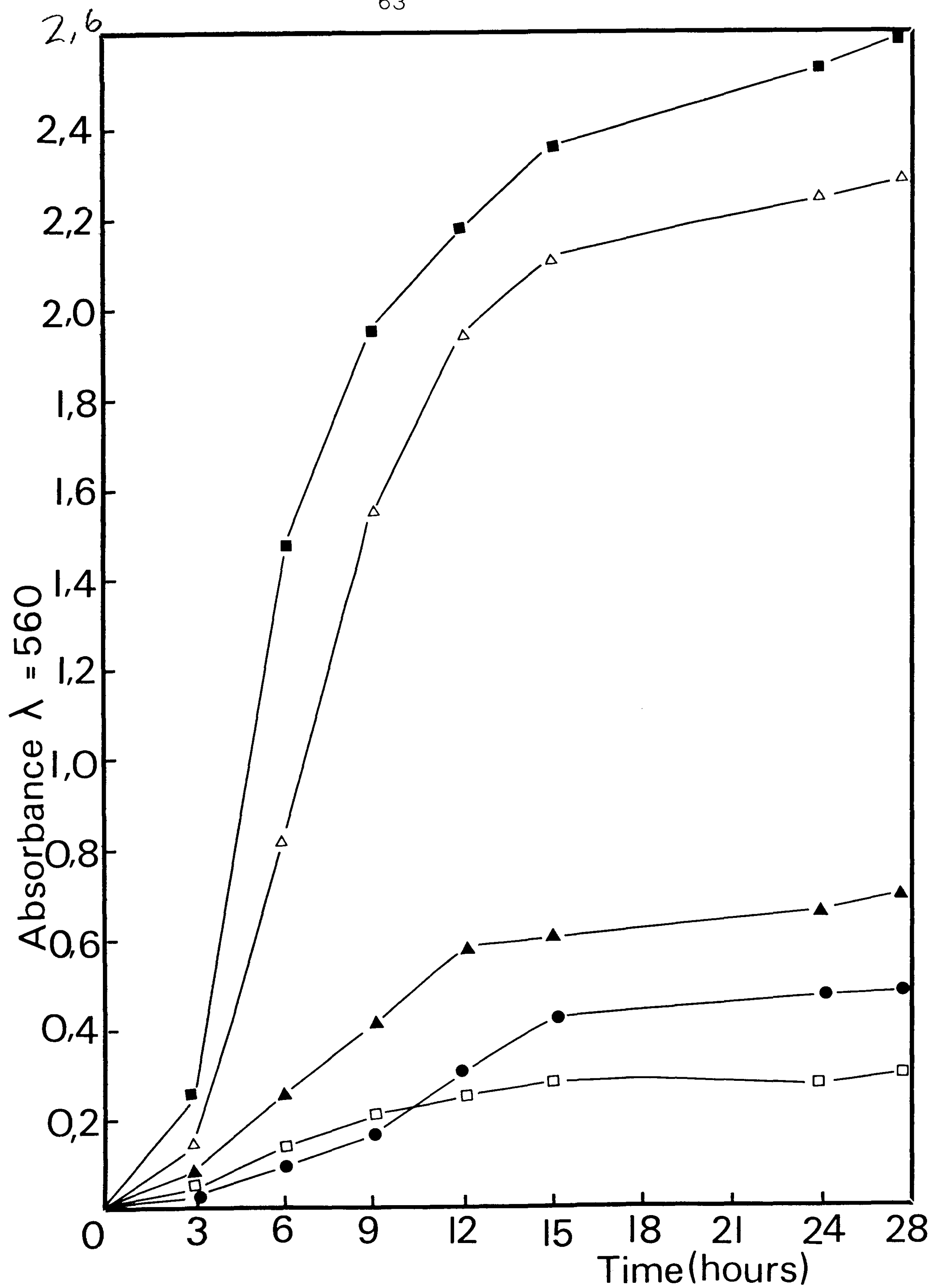

FIGURE 4.5 : Growth curves of S427 in BHI at a_w 0,925 adjusted using various humectants. Growth was at 37°C.

■———■ NaCl; △———△ glycerol;;
●———● sucrose; □———□ propylene glycol;
▲———▲ sodium lactate.

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to the fact that the organism could have utilized a portion of the glycerol as a carbon source and thus lowered the concentration of this compound with a concomitant increase in a_w (Troller, 1972).

High humectant concentration produces two effects: lowering of the a_w and in addition to this, a bacteriostatic effect as a result of the specific action of the humectant molecule (Kushner, 1971). However, a further factor to be considered is the percentage humectant needed to produce the required a_w and the state of the humectant. For sodium chloride and sucrose, both solids, 10% and 56% are required respectively to obtain a a_w of 0,925. For propylene glycol, sodium lactate and glycerol, all liquids, 18,8%, 17,5% and 26% are required respectively to obtain a_w 0,925. When comparing the amount of water added to adjust the a_w to 0,925 with sodium chloride or sucrose, approximately 90% water is required in the case of the sodium chloride as compared to only approximately 44% water for sucrose. Thus, although both solutions will have a_w 0,925, the sodium chloride solution will contain much more water than the sucrose solution.

For the liquid humectants the explanation is similar except for glycerol. A a_w 0,925 solution of glycerol requires 74% water while a_w 0,925 solutions of sodium lactate and propylene glycol require 82,5% and 81,2% water respectively. Thus growth in a_w 0,925 solutions of sodium lactate and propylene glycol should be better than in a_w 0,925 glycerol solutions. However, from Figures 4.4 and 4.5 it can be seen that this was not the case.

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There are two possible explanations for this phenomenon : (i) the glycerol is being used as a carbon source whereby its concentration may be lowered with a concomitant increase in a_w (Troller, 1972), (ii) sodium lactate and propylene glycol have stronger bacteriostatic effects on S.aureus than glycerol.

When considering the properties of humectants one must take the following three facts into consideration: (i) its strength for binding water, (ii) its bacteriostatic effects, (iii) the molarity humectant required to produce the desired a_w.

4.2.2 pH

In order to determine the minimum pH, the effect of inorganic and organic acids on S.aureus growth, all thirty-eight strains were grown under static conditions in a series of pH's adjusted using HCl as the inorganic acid and acetic, lactic, ascorbic, tartaric, gluconic and citric as the organic acids.

Figures 4.6 and 4.7 give the results for pH related growth of S144 and S470 relatively acid sensitive and resistant respectively. Table 4.4 summarizes these results by giving the minimal pH required for the growth of the sensitive and resistant strains when the pH was adjusted using various acids.

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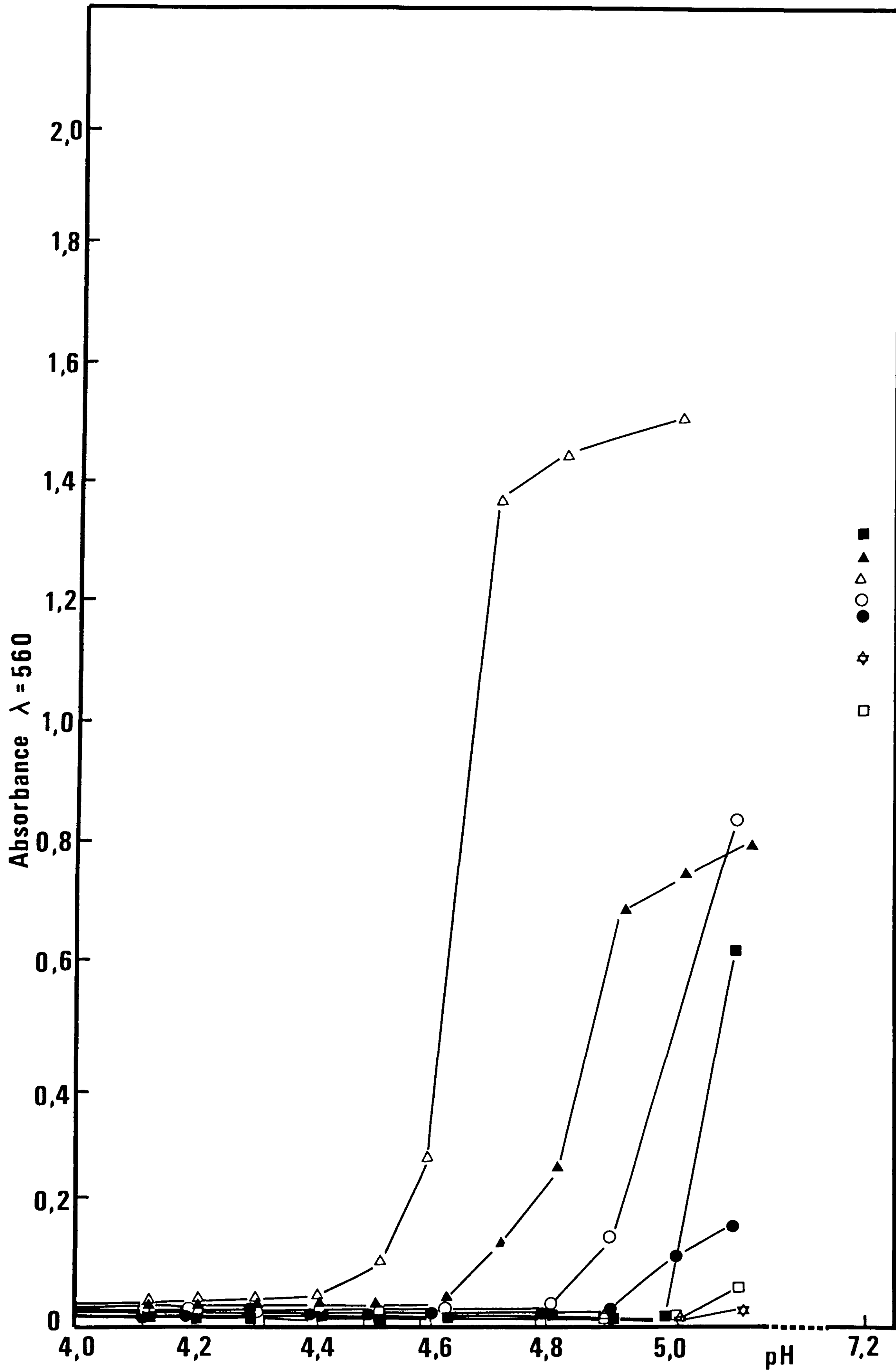

FIGURE 4.6 : Growth curves of S144 using different acids to adjust the pH. Growth was in BHI at 37°C for 7 days.
■——■ citric acid; ○——○ ascorbic acid;
●——● lactic acid; ▲——▲ tartaric acid;
✡——✡ acetic acid; □——□ gluconic acid;
△——△ hydrochloric acid.

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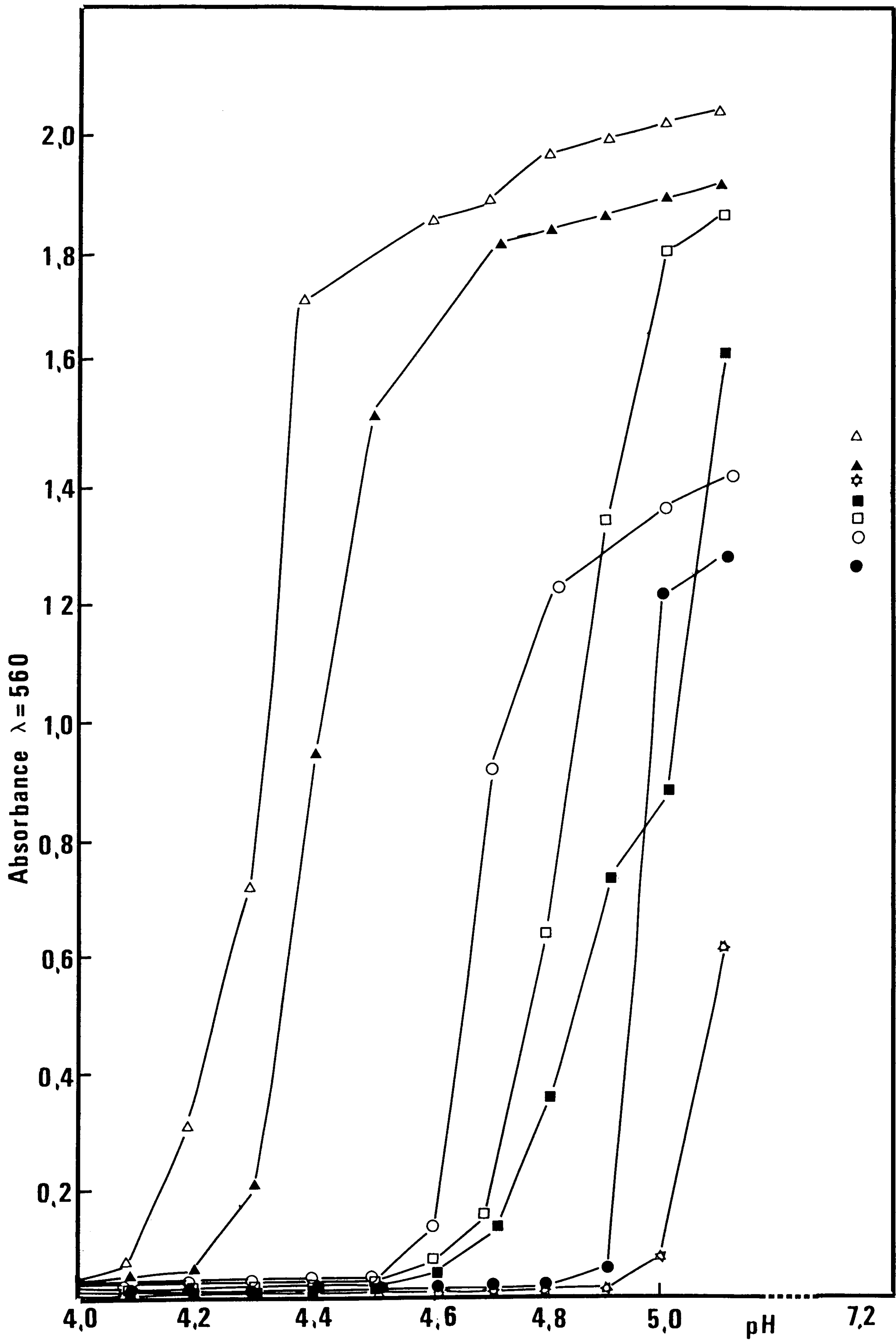

FIGURE 4.7 : Growth curves of S470 using different acids to adjust the pH. Growth was in BHI at 37°C for 7 days.
■———■ citric acid; ○———○ ascorbic acid;
●———● lactic acid; ▲———▲ tartaric acid;
✡———✡ acetic acid; □———□ gluconic acid;
△———△ hydrochloric acid.

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TABLE 4.4 MINIMAL pH FOR THE GROWTH OF S144 (pH SENSITIVE) AND S470 (pH RESISTANT) IN DIFFERENT ACIDS. GROWTH WAS AT 37°C IN BHI UNDER STATIC CONDITIONS FOR SEVEN DAYS. (THE MINIMAL pH IS TAKEN AS THE a_w WHERE BACTERIAL GROWTH AS MEASURED BY ABSORBANCE AT 560NM WAS 0,05)

ACID \ CULTURE NUMBER	S144	S470
Hydrochloric acid	4,43	4,08
acetic acid	5,10	5,0
lactic acid	4,95	4,91
ascorbic acid	4,86	4,52
tartaric acid	4,63	4,21
gluconic acid	5,10	4,57
citric acid	5,02	4,62

From Table 4.4 it can be seen that the inorganic acid, hydrochloric acid, had the least effect on staphylococcal growth (when used for pH adjustment), enabling growth of S144 and S470 down to a pH of 4,43 and 4,08 respectively. The most effective organic acid that effects the growth of *S.aureus* 144 is acetic acid followed by gluconic, citric, lactic, ascorbic and tartaric acids. For *S.aureus* 470, acetic acid is also the most effective, followed by lactic, citric, gluconic, ascorbic and tartaric acids.

Tatini *et al*. (1971) found that growth and enterotoxin A production by *S.aureus* occurred at pH 5,0 and above with either lactic or hydrochloric acid, whereas no growth or enterotoxin production took place at pH 4,5 when obtained with lactic acid. These results are in agreement with the results given in Figure 4.6 for *S.aureus* 144 where growth occurred at pH 4,95 and above when the pH was adjusted with lactic and hydrochloric acid respectively,

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while when the pH was adjusted to 4,5 growth occurred with hydrochloric acid but not with lactic acid.

In conclusion it can be noted that the type of acidulant used for pH adjustment has a significant effect on the lower limit of pH which will permit growth of *S.aureus*.

4.2.3 TEMPERATURE

The maximal, minimal and optimal growth temperatures were determined for all cultures. Figure 4.8 gives the growth curve of *S.aureus* 470. The maximal, minimal and optimal temperatures of the pH and a_w sensitive and resistant strains are summarized in Table 4.5

TABLE 4.5 OPTIMAL, MAXIMAL AND MINIMAL TEMPERATURE FOR THE GROWTH OF BACTERIAL CULTURES IN BHI AFTER INCUBATION FOR 24 HOURS

TEMPERATURE \ CULTURE	S144	S258	S427	S470
Optimal	34,5-36°	38°	38°	36,5°C
Maximal	50°C	51,2°C	50°C	52°C
Minimal	8°C	8°C	9°C	11,5°C

From the temperature growth curves, no exceptionally resistant or sensitive strains could be selected. All strains had a growth range of 11,5°C to 50°C with an optimal growth temperature of 34,5 to 38°C. These results are not in full agreement with those of Buchanan and Gibbons (1974) (who gave the growth range for staphylococci as 6,5 to 46°C with an optimum of 30 to 37°C), especially as far as the maximum temperature is concerned.

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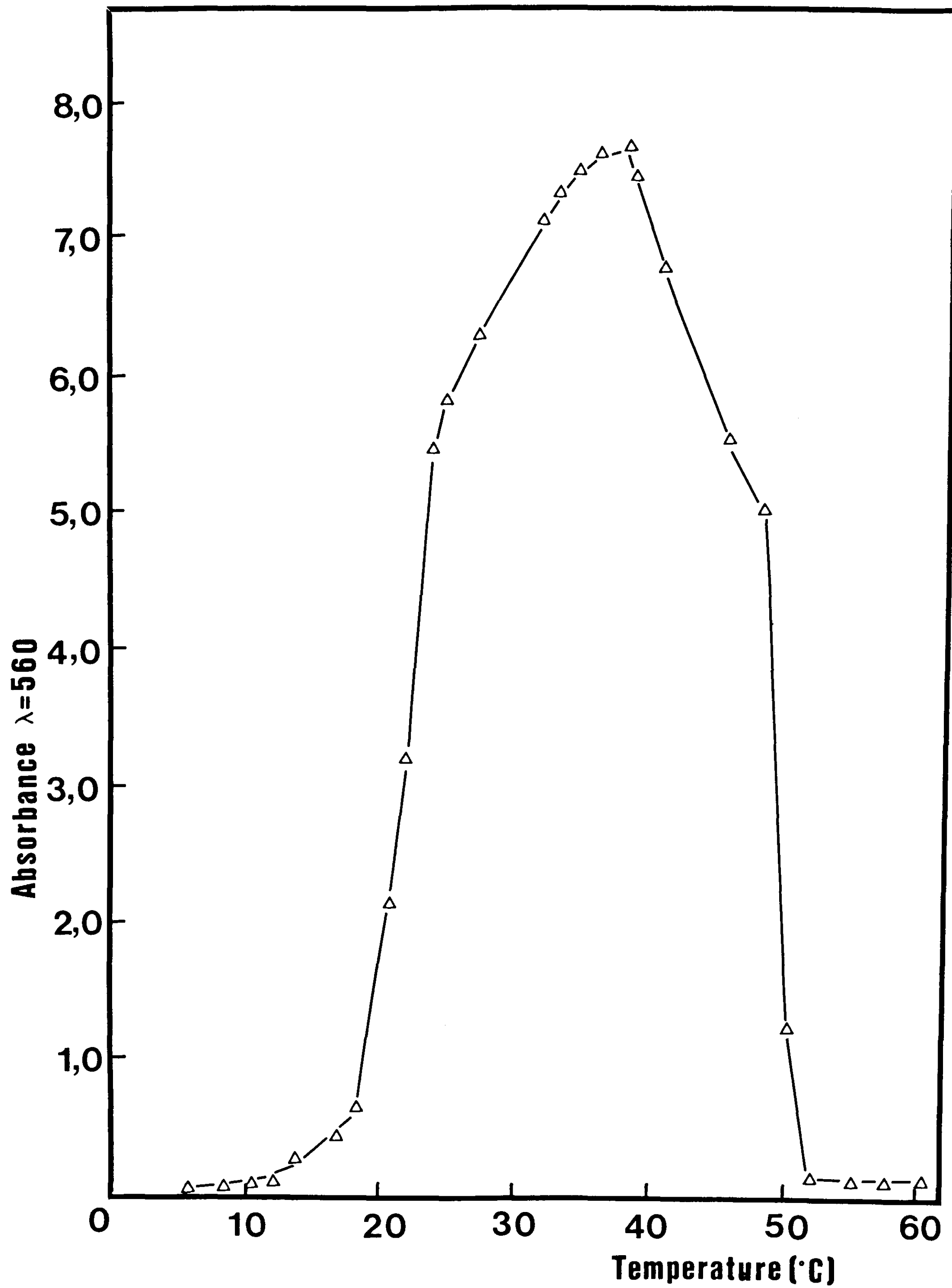

FIGURE 4.8 : Temperature growth curve of S470 in BHI after 24 h, as determined in a temperature gradient incubator.

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In order to examine the thermal destruction of the staphylococcal isolates, the D-values at 60°C were determined for all isolates. Figure 4.9 gives a graphical representation of the thermal destruction time of S.aureus strains 144, 258, 470 and 427. Table 4.6 gives the D_{10} at 60°C for four S.aureus strains.

TABLE 4.6 D_{10} VALUES AT 60°C FOR STAPHYLOCOCCI STRAINS. GROWTH WAS IN BHI AT 37°C FOR 3 H BEFORE THE $D_{60^\circ C}$ WAS DETERMINED

CULTURE	D_{10} AT 60°C
S144	5 min 30 s
S258	1 min 54 s
S427	2 min 27 s
S470	2 min 30 s

Staphylococci are usually sensitive to heat with a $D_{60^\circ C}$ value of three minutes or less (Buchanan and Gibbons, 1974). From Table 4.6 it can be seen that culture S144 is relatively resistant to heat with a $D_{60^\circ C}$ value of 5 min 30 s, although it had the lowest maximal and minimal growth temperatures (Table 4.5). However, S470 which had the highest maximal and minimal temperatures (Table 4.5) and was expected to have the highest D_{10} value, had a $D_{60^\circ C}$ value of only 2 min 30 s which is average for staphylococci. All the other cultures were more sensitive to heat with $D_{60^\circ C}$ values ranging from one to 2 min 27 s.

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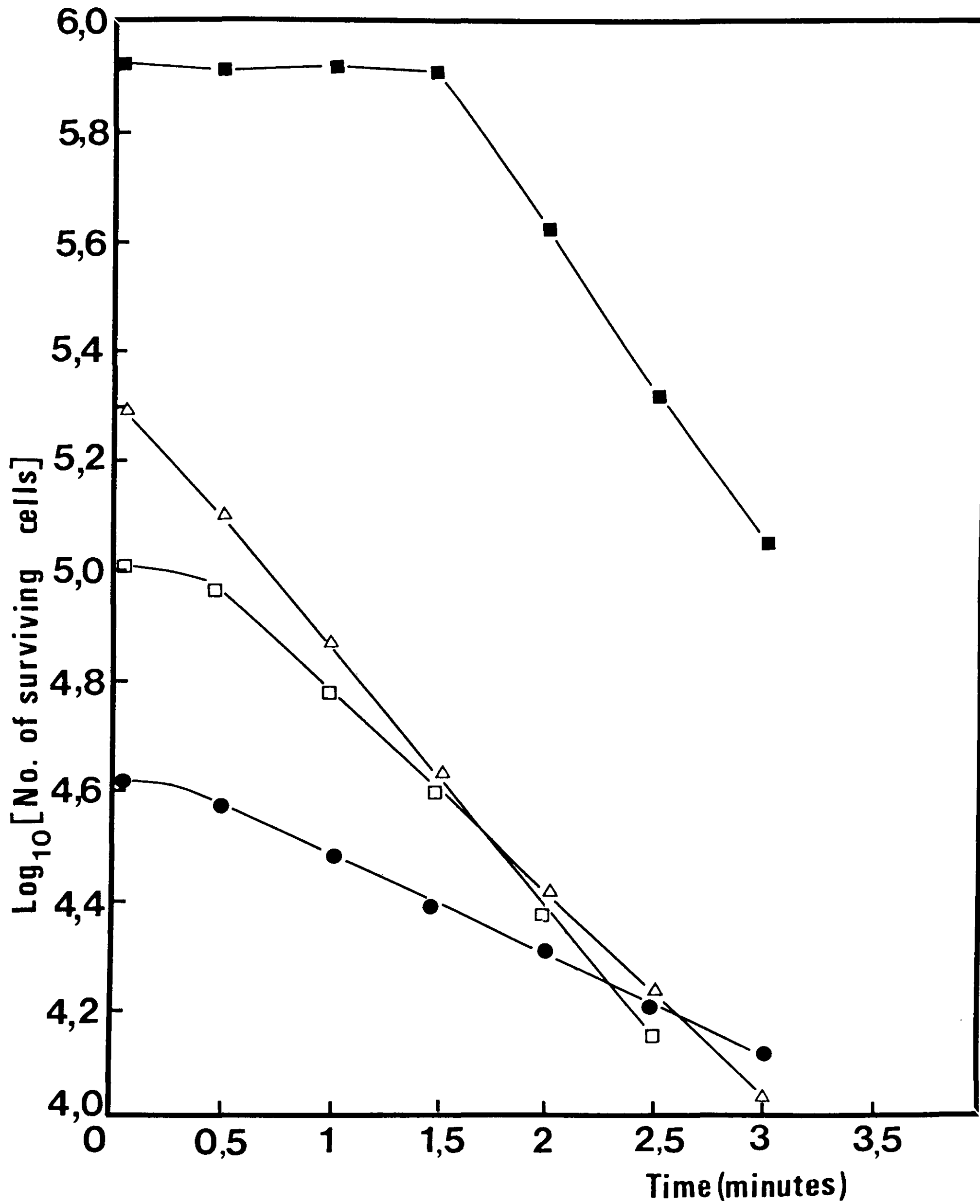

FIGURE 4.9 : Graphical determination of $D_{60°C}$ values for S144, S258, S427 and S470. Growth was in BHI at 37°C for 3 h (which gave approximately 10^7 cells/ml) before the $D_{60°C}$ value was determined.

■———■ culture 258; △———△ culture 470;
□———□ culture 427; ●———● culture 144.

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4.2.4 COMBINED EFFECT OF POTASSIUM SORBATE AND pH

Potassium sorbate is used in S.S.P. in order to control mould growth (Sofos and Busta, 1981). However potassium sorbate also produces bactericidal and bacteriostatic effects on bacteria (Laheller _et al._, 1981). Thus, because potassium sorbate was used in the products from which the staphylococci were isolated, its effect on staphylococcal growth was determined.

The a_w and pH sensitive and resistant strains were grown in 0,5%, 1% and 2,5% potassium sorbate in BHI with an initial pH of 7,3 (control) and 5,5 (initial pH of S.S.P.). The results of staphylococcal growth are given in Table 4.7

TABLE 4.7 COMBINED EFFECT OF POTASSIUM SORBATE AND pH ON 4 STAPHYLOCOCCAL STRAINS. GROWTH WAS FOR TWELVE HOURS IN BHI WITH AN INITIAL pH OF 7,3 AND 5,5 (ADJUSTED WITH HCl).

CULTURE / ABSORBANCE AFTER TWELVE HOURS	S144	S258	S427	S470
Initial pH 7,30				
0,5% potassium sorbate	2,18	2,20	2,19	2,18
1% potassium sorbate	2,08	2,12	2,12	2,16
2,5% potassium sorbate	1,36	1,65	1,48	1,68
Initial pH 5,5				
0,5% potassium sorbate	0,057	0,031	0,038	0,044
1% potassium sorbate	0,037	0,029	0,041	0,027
2,5% potassium sorbate	0,041	0,022	0,036	0,027

From Table 4.7 it can be seen that at pH 7,3 , the higher the concentration of potassium sorbate, the greater the inhibitory effect. However, this bacteriostatic effect

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was not very great. At an initial pH of 5,5 the bacteriocidal effect did not depend very greatly on the concentration of potassium sorbate because of the higher concentration of the undissociated molecule. Staphylocci were unable to grow in 0,5%, 1% or 2,5% potassium sorbate when the initial pH was 5,5 (adjusted with HCl). Thus, there is a definite pH influence on the effect of sorbate on bacterial growth. This is because potassium sorbate is effective only in the undissociated form, and so would be expected to be maximally effective at low pH (Troller and Christian, 1978). At pH 5,5 and 7,0 potassium sorbate is 15% and 0,6% undissociated respectively and this accounts for the inability of staphylococci to grow in 0,5%, 1% or 2,5% potassium sorbate where the initial pH was 5,5. Boylan _et al_. (1976) suggested that the increased effect of the preservative in the undissociated form is because it is highly lipid soluble and accumulates in the lipid structures of the cells inhibiting cell metabolism.

4.2.5 COMBINED EFFECT OF a_w AND pH

The inhibition of microorganisms in IMF depends to a large extent on a_w and pH (Leistner and Rödel, 1978). Thus, the combined effect of pH and a_w was studied. The a_w and pH resistant and sensitive strains were tested for their ability to grow at an a_w 0,925 and pH 5,5 adjusted with an inorganic acid and organic acids. The pH 5,5 and a_w 0,925 values were chosen as these are the initial values for the shelf stable meat products investigated. The

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degree of bacterial growth, as measured by absorbance at 560nm, in the various combinations of a_w and pH are given in Table 4.8

When the control values are compared in Table 4.8 it can be seen that the effect of lowered pH (pH 5,5) with a_w 0,985 had less effect on bacterial growth than when the a_w was lowered (a_w 0,925) but with pH 7,3. It can thus be concluded that lowering of a_w has a much greater effect than lowering of pH to 5,5. However there were two exceptions and these occurred when the a_w was lowered to 0,925 with glycerol and sodium chloride. The large amount of bacterial growth at a_w 0,925 adjusted with glycerol and sodium chloride can be attributed to the fact that the glycerol may be used as carbon source and thus lowered the concentration of this compound with a concomitant increase in a_w (Troller, 1972) and due to the protective effect of the sodium chloride (Smith, Benedict and Palumbo, 1982).

From the results it can be seen as expected that the pH and a_w "sensitive" strains S144 and S258 were more "sensitive" to combined a_w and pH than the pH and a_w "resistant" strains.

The organic acids were found to be more effective than the inorganic acid in the prevention of bacterial growth.
The most effective humectants when used at pH 5,5 were sodium lactate, propylene glycol and sucrose. The effect of these humectants is due to the bacteriostatic effect of the sodium lactate and propylene glycol and due to the high weight to volume ratio of sucrose.

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TABLE 4.8 BACTERIAL GROWTH (AS MEASURED BY ABSORBANCY AT 560NM AFTER 24 H) OF STAPHYLOCOCCAL CULTURES GROWN IN BHI AT a_w 0,925 AND pH 5,5. a_w WAS ADJUSTED USING FIVE DIFFERENT HUMECTANTS AND pH WAS ADJUSTED USING AN INORGANIC ACID AND ORGANIC ACIDS. ACID CONTROLS WERE AT pH 7,3 (NORMAL pH FOR BHI) WHILE HUMECTANT CONTROLS WERE AT a_w 0,985 (NORMAL a_w FOR BHI), BUT WITH A pH OF 5,5 ADJUSTED WITH HCl.

		pH 5,5					
a_w	ACID / HUMECTANT	CITRIC	LACTIC	ACETIC	GLUCONIC ACID	HCl	NO ACID pH 7,3
	S258						
0,925	glycerol	1,101	0,083	0,113	0,053	2,110	2,392
	sodium lactate	0,048	0,067	0,056	0,081	0,092	0,375
	propylene glycol	0,039	0,062	0,033	0,009	0,034	0,446
	sucrose	0,053	0,000	0,002	0,101	0,143	0,496
	sodium chloride	0,615	1,867	1,275	0,089	2,170	2,380
0,985	No humectant	2,396	2,474	2,447	1,932	2,506	2,801
	S427						
0,925	glycerol	1,962	1,585	0,263	0,245	2,117	2,232
	socium lactate	0,064	0,100	0,059	0,118	0,032	0,643
	propylene glycol	0,067	0,045	0,051	0,045	0,043	0,262
	sucrose	0,021	0,041	0,028	0,074	0,100	0,228
	sodium chloride	2,108	2,060	1,022	0,074	2,223	2,540
0,985	No humectant	2,290	2,384	2,312	1,350	2,462	2,732
	S144						
0,925	glycerol	1,821	1,304	0,103	0,091	2,009	2,336
	sodium lactate	0,064	0,029	0,075	0,432	0,031	0,870
	propylene glycol	0,036	0,000	0,057	0,072	0,063	0,331
	sucrose	0,071	0,064	0,058	0,091	0,103	0,287
	sodium chloride	1,031	1,587	1,103	0,084	2,147	2,496
0,985	No humectant	2,253	2,474	2,343	1,231	2,121	2,518
	S470						
0,925	glycerol	2,039	1,802	0,161	0,115	2,161	2,330
	sodium lactate	0,031	0,048	0,037	0,068	0,048	0,278
	propylene glycol	0,050	0,036	0,052	0,025	0,030	0,141
	sucrose	0,087	0,000	0,025	0,000	0,145	0,507
	sodium chloride	2,101	2,140	1,483	0,080	2,170	2,500
0,985	No humectant	2,270	2,443	2,440	1,455	2,484	2,725

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From Table 4.8 it can also be observed that when sodium chloride is used to lower the a_w to 0,925 together with a pH of 5,5 adjusted with gluconic acid bacterial growth was almost completely inhibited.

4.3 CELL WALL ANALYSIS

4.3.1 AMINO ACID COMPOSITION OF CELL WALLS

The amino acid composition of sixteen isolates were analyzed quantitatively and qualitatively. The strains analysed were: pH "sensitive" strains S78, S144, S276 and S297; pH "resistant" strains S422, S424, S427 and S470; a_w "sensitive" strains S177, S258, S426 and S482; a_w "resistant" strains S150, S175, S285, S427 and S487. Table 4.9 gives the molar ratios of the amino acid composition of the sixteen isolates studied. Figure 4.10 gives a diagramatic representation of a paper chromatographic separation of the amino acids obtained by total hydrolysis (4N HCl, 100°C, 16 hrs) of the cell wall of *S. aureus* 177.

The cell walls of all strains studied reveal a quite similar chemical composition (Table 4.9) (Schleifer *et al.*, 1976). All strains contained glycine, alanine, lysine, glutamic acid, glucosamine and muramic acid and small amounts of serine. Serine was not present in S422 and S487. The pepidoglycans of all strains are very high in glycine and thus belong to the L-Lys-Gly $_{5-6}$ type (Schleifer and Kandler, 1970).

From Table 4.9 it can be seen that (based on Glu = 1) S177 had 0,21 mol serine and 2,9 mol glycine as compared to an average value of 0,04 mol serine and 5,19 mol glycine for

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TABLE 4.9 : MOLAR RATIOS OF THE AMINO ACIDS IN THE PEPTIDOGLYCAN OF COAGULASE - POSITIVE STAPHYLOCOCCI ISOLATED FROM SSP AND FRESH MINCED MEAT (GLU = 1)

STRAINS	Ser	Gly	Ala	Lys	Glc.NH_2	Mur
S78	0,04	5,53	2,23	1,15	1,74	1,84
S144	0,05	5,76	2,24	1,16	1,88	1,83
S150	0,11	4,35	1,80	0,94	1,09	1,01
S175	0,13	4,23	1,89	0,90	1,43	0,99
S177	0,21	2,98	1,51	0,75	0,86	0,74
S258	0,07	3,97	2,01	0,89	1,36	1,12
S276	0,04	5,72	2,26	1,15	1,75	1,93
S285	0,03	5,85	3,17	1,39	2,13	0,97
S297	0,05	5,97	2,28	1,13	1,91	1,810
S422	-	5,78	2,02	1,10	1,90	1,87
S424	0,02	5,63	2,23	1,11	1,78	1,98
S426	0,01	4,65	2,01	0,96	1,47	1,11
S427	0,02	5,67	2,31	1,19	2,19	1,86
S470	0,03	5,61	2,17	1,11	1,77	1,93
S482	0,06	4,59	1,99	0,94	1,19	1,02
S487	-	4,86	2,11	1,00	1,32	0,96

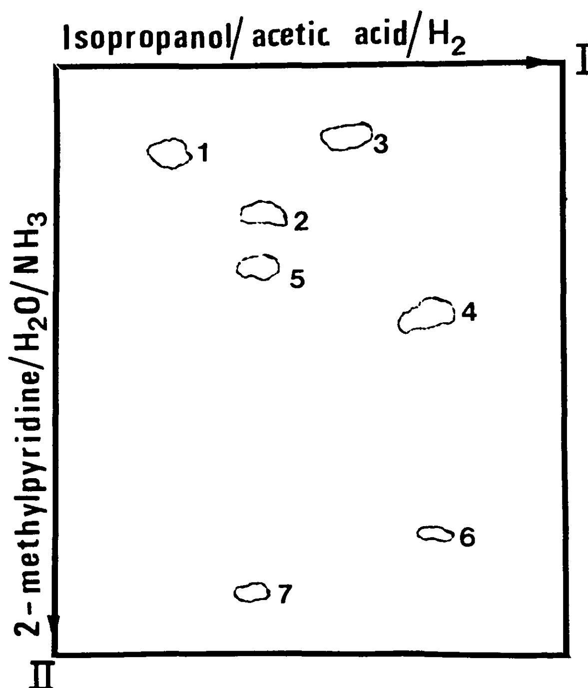

FIGURE 4.10 : Diagramatic representation of a two-dimensional paper chromatogram hydrolysate (4N HCl, 100°C for 16 h) of the cell wall of S.aureus 177. The first dimensional run was in isoproponal: acetic acid: water (75:10:15 vol/vol) and the second in 2-methylpyridine: water: NH_3 (70:28:2 vol/vol). 1. Lys; 2. Gly; 3. Glu; 4. Ala; 5. Ser; 6. Mur; 7. Glc NH_2

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the remaining fifteen strains. This abnormality can be explained by the observations of Schleifer *et al.* (1976) and Schleifer and Kandler (1970) who found that in some strains low amounts of glycine (0,1 to 0,2 mol/mol of glutamic acid) are replaced by L-serine. It can also be noted that S285 has 3,17 mol alanine as compared to an average value of 2,07 mol for the remaining fifteen strains. However, this abnormality is difficult to explain.

Two-dimensional paper chromatography of partial cell wall hydrolysates revealed the typical staphylococcal cell wall composition for all strains: L-Ala-D-Isoglu-(NH_2)-N^{α}-L-Lys-(D-Ala)-N^{ϵ} -$(Gly)_5$-D-Ala-L-Lys. Thus it can be concluded that the cell wall did not contribute to the relative a_w and pH resistance or sensitivity of the strains studied.

4.3.2 TEICHOIC ACIDS

The teichoic acids of the respective pH and a_w sensitive and resistant strains, S144, S470, S258 and S427, were examined qualitatively using a one-dimensional paper chromatogram system.

From Figure 4.11 it can be seen that all four strains examined contained a ribitol type of teichoic acid with mannose and glucose as sugars. This is in agreement with Baddiley *et al.* (1968) who found that typical coagulase-positive *S.aureus* strains contain a ribitol type of teichoic acid.

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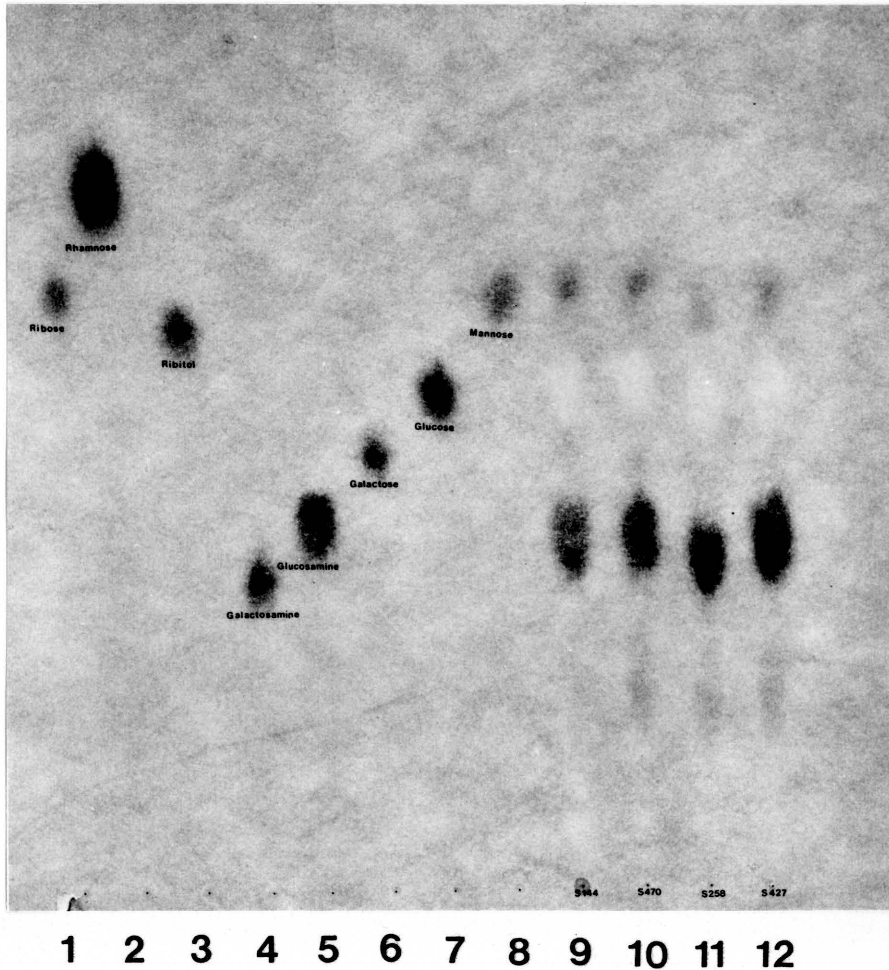

FIGURE 4.11 : One-dimensional paper chromatograph for the determination of sugars and polyols in the teichoic acids of S.aureus strains S144, S470, S258 and S427. Hydrolysis of the cell walls was with 1ml 4N HCl for 3 h at 100°C. Chromatography was in butanol: pyridine: water: acetic acid (60:40:30:3). 1. ribose; 2. rhamnose. 3. ribitol; 4. galactosamine; 5. glucosamine; 6. galactose; 7. glucose; 8. mannose; 9. S144; 10. S470; 11. S258. 12. S427.

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4.4 CELL MEMBRANE ANALYSIS

Kanemasa et al. (1972; 1975) found that when the a_w is adjusted with NaCl, the amount of cardiolipin in the cell membrane increased while the amount of phosphatidylglycerol decreased. Thus, in order to determine whether the lowered a_w or NaCl was responsible for the phospholipid change, S.aureus 427, grown in BHI at a_w 0,925 adjusted with NaCl, glycerol, sodium lactate, propylene glycol and sucrose, was examined for its phospholipid composition.

Bacterial growth was harvested after 24 hours, the phospholipids extracted and separated on Silica Gel G plates (Figure 4.12). From Figure 4.12 it can be observed that at an a_w 0,925 there was an increase in the amount of cardiolipin with a corresponding decrease in the amount of phosphatidylglycerol compared to S.aureus grown in BHI at a_w 0,98. This increase of cardiolipin and decrease of phosphatidylglycerol occurred independent of the humectant used to lower the a_w to 0,925.

It can thus be concluded that a_w alone and not the type of humectant used is responsible for the change in cardiolipin and phosphatidylglycerol concentration.

The phospholipid alterations observed are possibly a mechanism to protect the microorganism against phospholipid degradation during stationary phase growth or for adaptation of the bacteria to new environmental conditions (Cronan, 1968).

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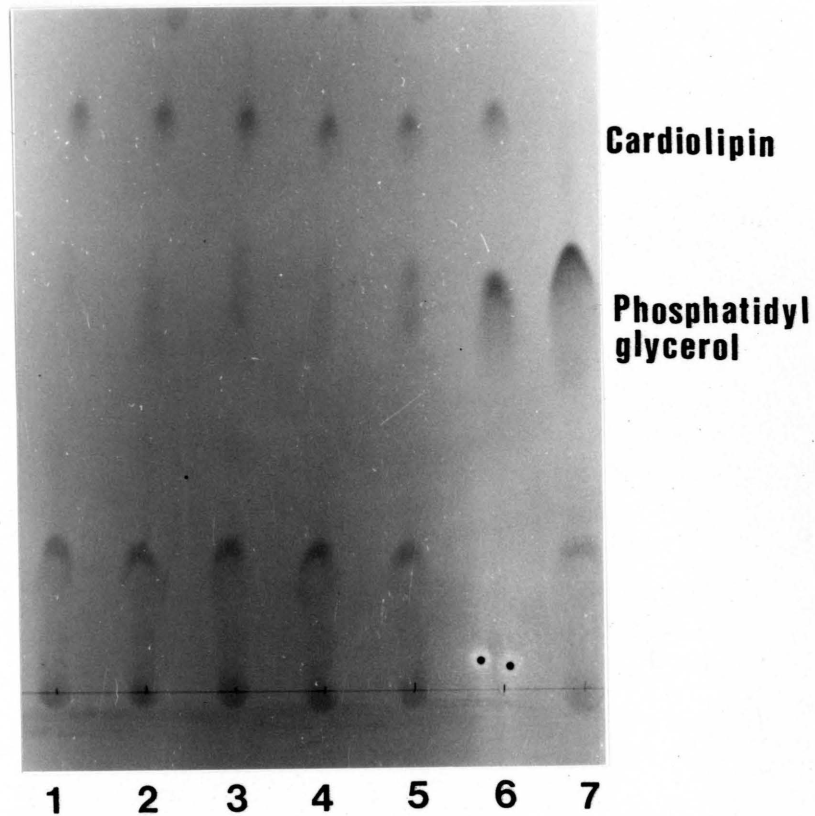

FIGURE 4.12 : One-dimensional chromatograph on Silica Gel G plates in chloroform: methanol: acetic acid: water (42:12:3,15:1) for the analysis of lipids of S427 grown in BHI only (7) and adjusted to a_w 0,925 with glycerol (1); sodium chloride (2); sucrose (3); propylene glycol (4); sodium lactate (5). Cardiolipin and α-phosphatidylglycerol were used as standards.

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The cardiolipin and branched fatty acids may also act as a barrier against high level ionic strength or contribute to active transport (Kanemasa _et al_., 1972).

4.5 METABOLIC STUDIES

In order to determine if there was any difference in metabolic products between the a_w sensitive and resistant strains S258 and S427 respectively, both strains were grown in BHI with shaking (aerobic conditions) and without shaking (anaerobic conditions) and the concentration of their main metabolic products analyzed (Figures 4.14a, 4.14b, 4.15a and 4.15b).

From all four figures it can be noted that, although bacterial growth was much greater aerobically than anaerobically (corresponding to an absorbancy of 2,2 and 1,6 respectively after nine hours), the resultant pH drop from 7,3 to either 6,3 or 6,4 did not reflect these differences. From the growth curves it can be observed that all cultures entered the stationary phase of growth after around eight hours. Thus, because the cultures grown aerobically and anaerobically had comparable growth curves resulting in similar pH drops, valid comparisons on metabolic products can be made between the a_w sensitive and resistant strains growing aerobically and anaerobically.

Under anaerobic conditions, the main product of glucose fermentation was lactic acid, while in the presence of air the main product was acetic acid with small amounts of CO_2

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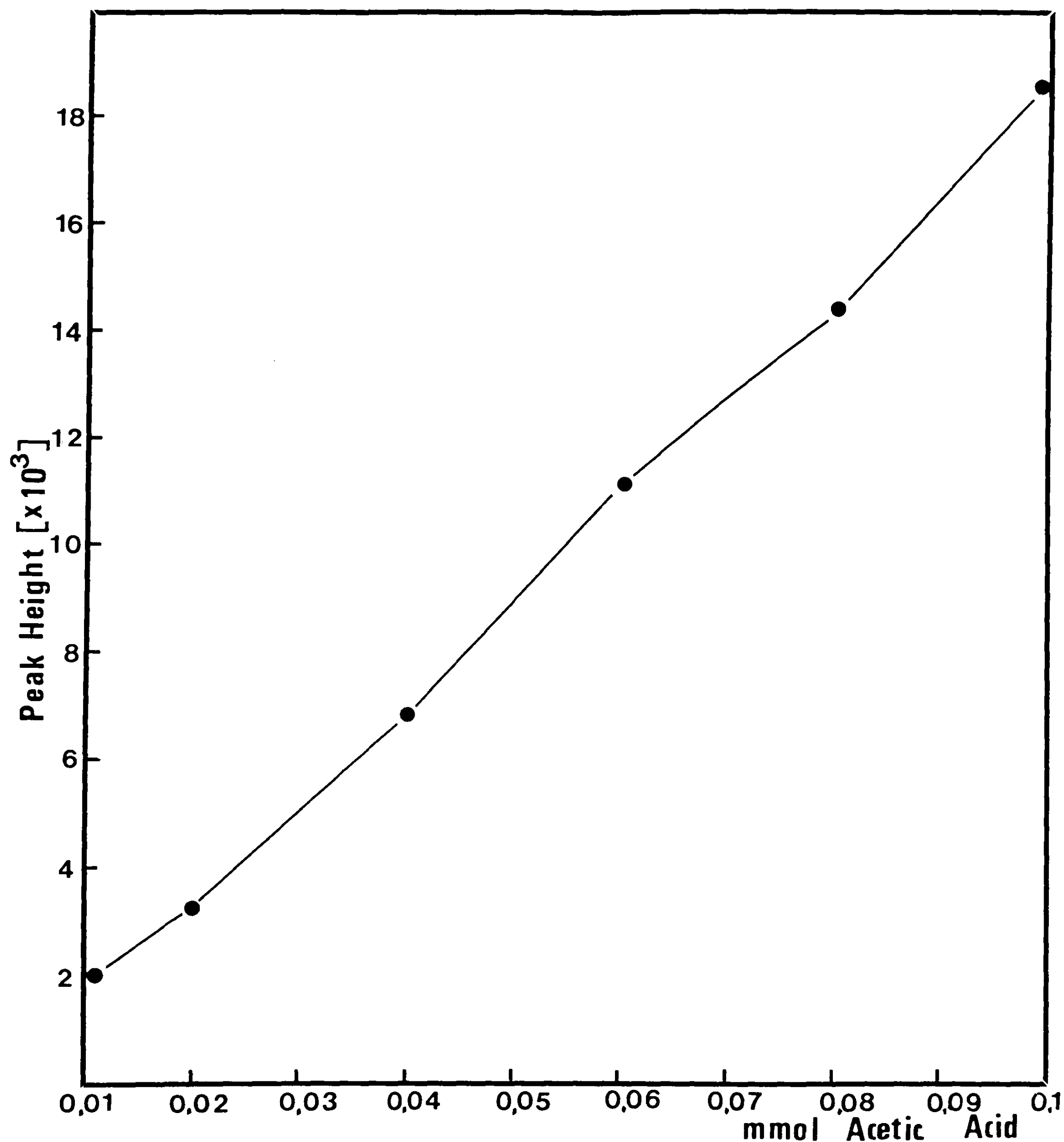

FIGURE 4.13 : Standard curve of mmol acetic acid vs peak height obtained using a Fractovap Series 4100 Carlo Erba Gas Chromotograph equipped with a glass column containing Supeko GP 15% SP-1220/1% H_3PO_4 on chromosorb 100/120 WAW. 10 µl of sample was injected.

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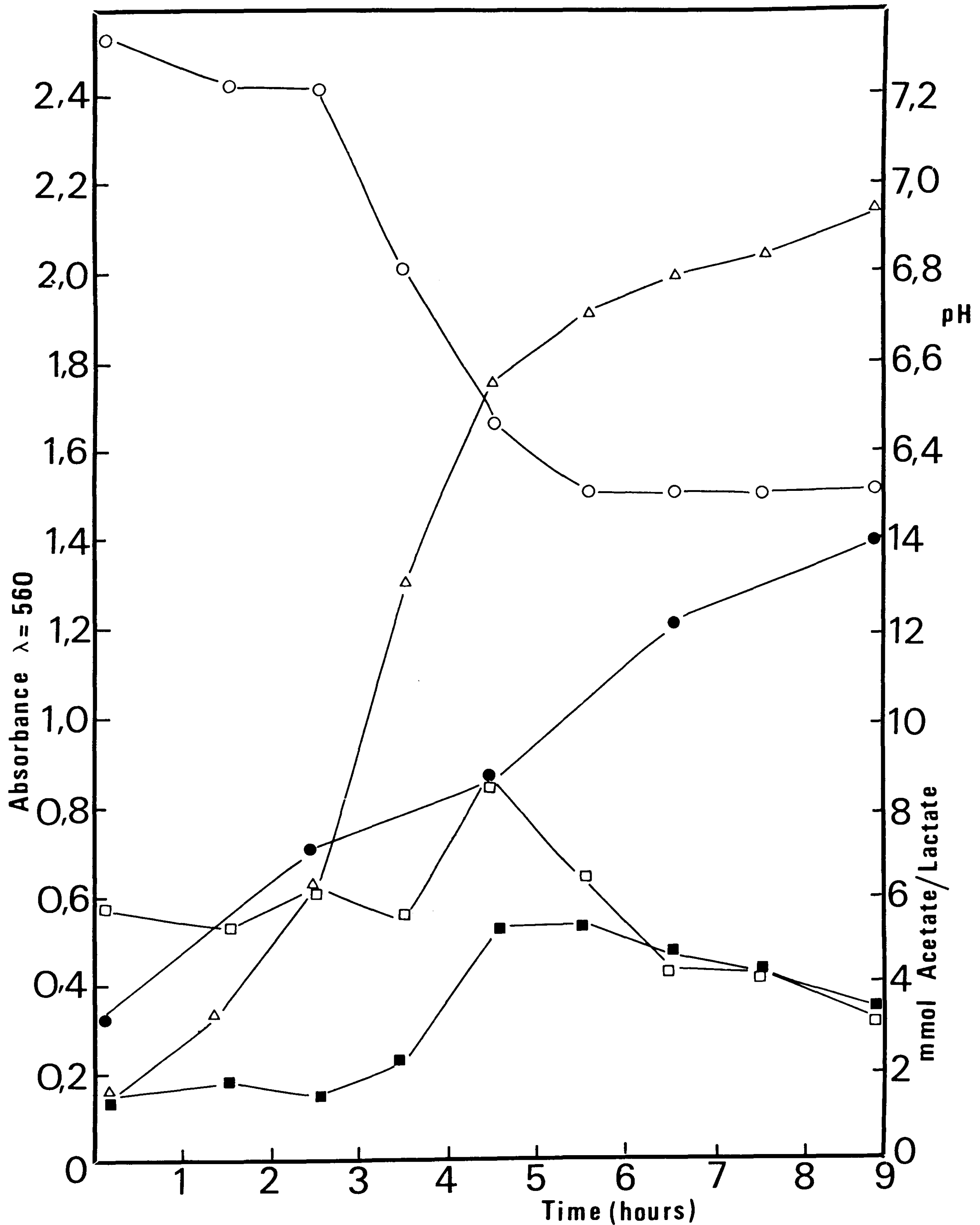

FIGURE 4.14 (a) : Growth curve of S258 grown under aerobic conditions showing change in lactic and acetic acid production and resultant pH drop.

△———△ Absorbance; ○———○ pH;
□———□ L(+)-lactic acid;
■———■ D(-)-lactic acid;
●———● acetic acid.

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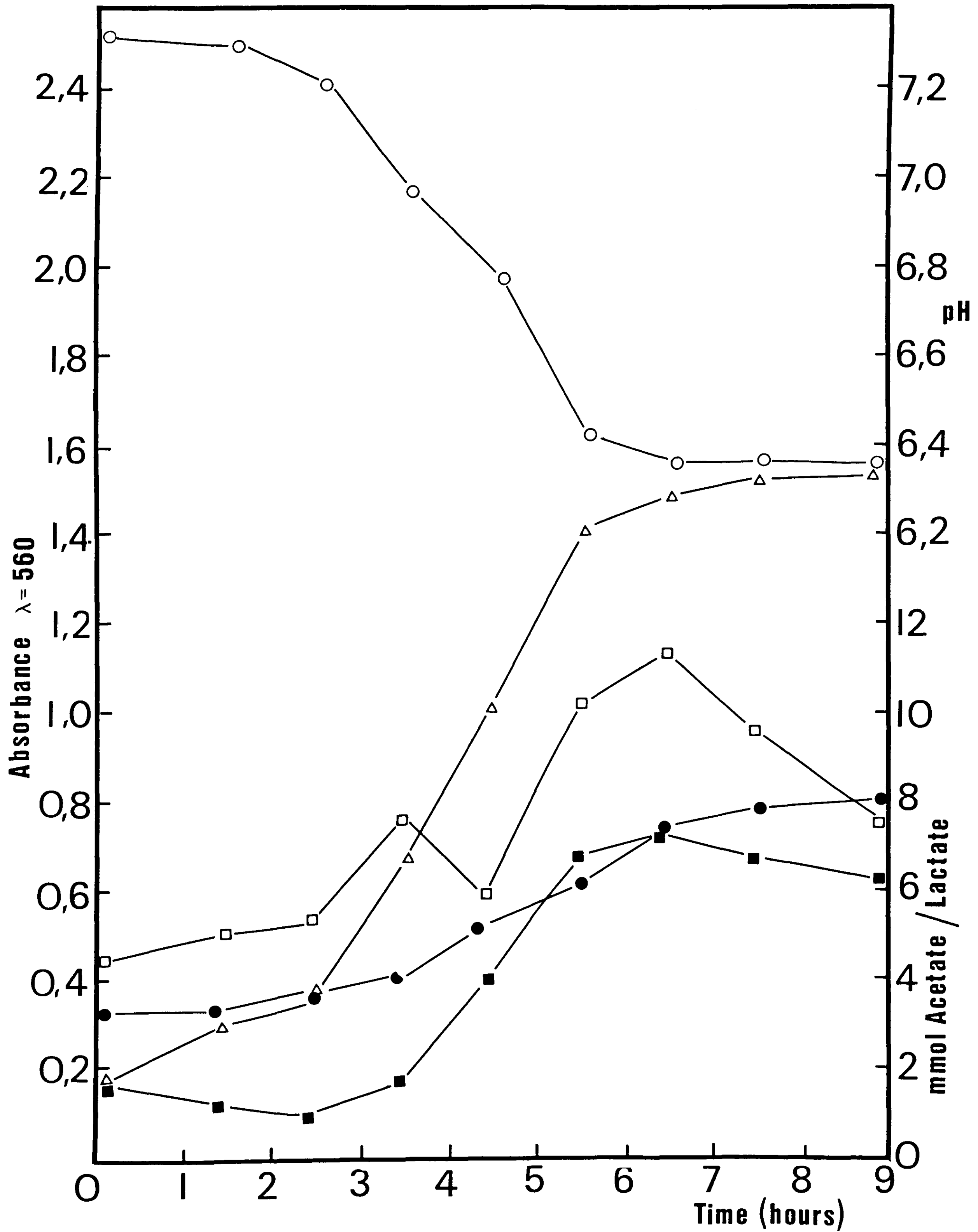

FIGURE 4.14 (b) : Growth curve of S258 grown under anaerobic conditions showing change in lactic and acetic acid production and resultant pH drop.
△———△ Absorbance; ○———○ pH;
□———□ L(+)-lactic acid;
■———■ D(-)-lactic acid;
●———● acetic acid.

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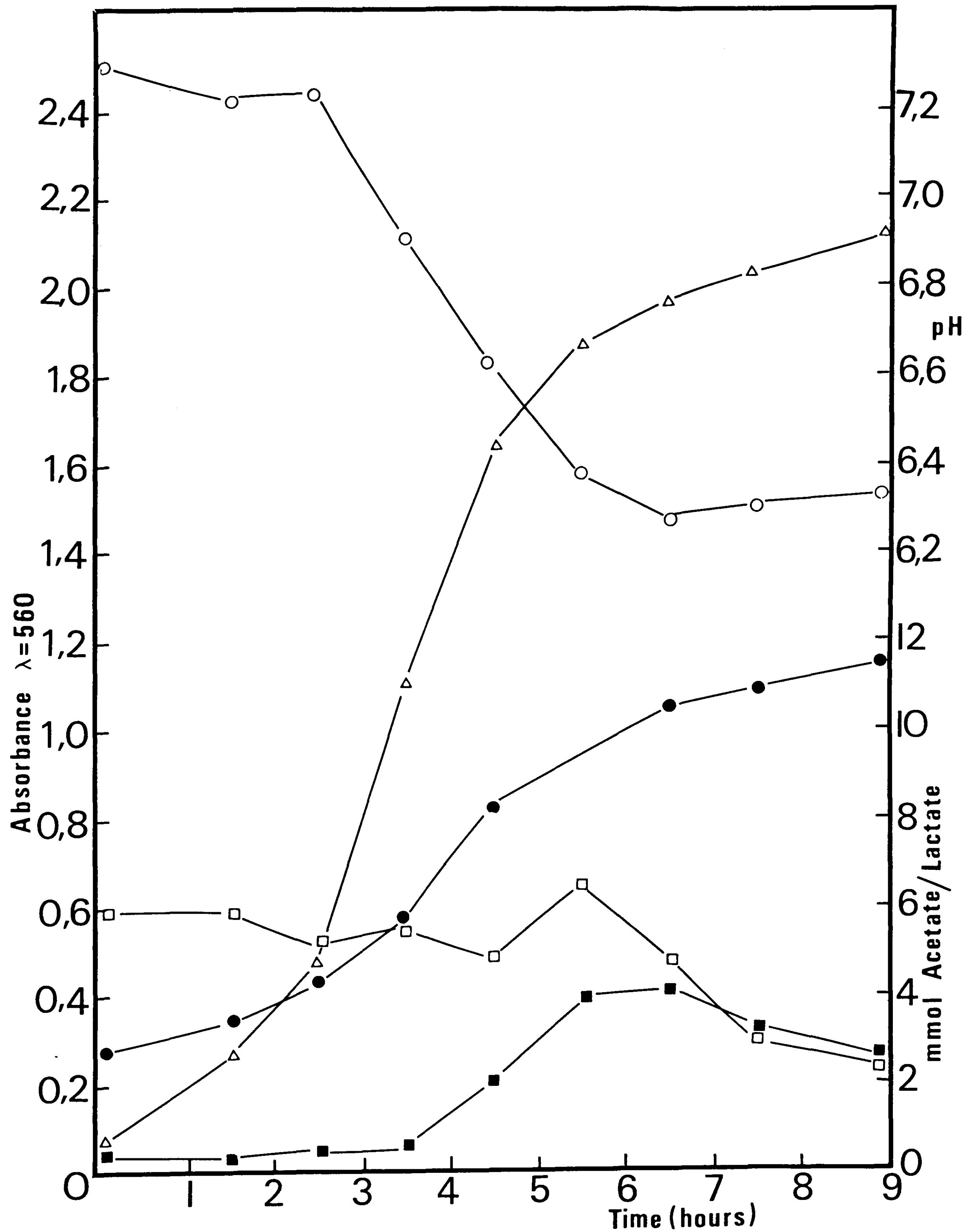

FIGURE 4.15 (a) : Growth curve of S427 grown under aerobic conditions showing change in lactic and acetic acid production and resultant pH drop.
△———△ Absorbance; ○———○ pH;
□———□ L(+)-lactic acid;
■———■ D(-)-lactic acid;
●———● acetic acid..

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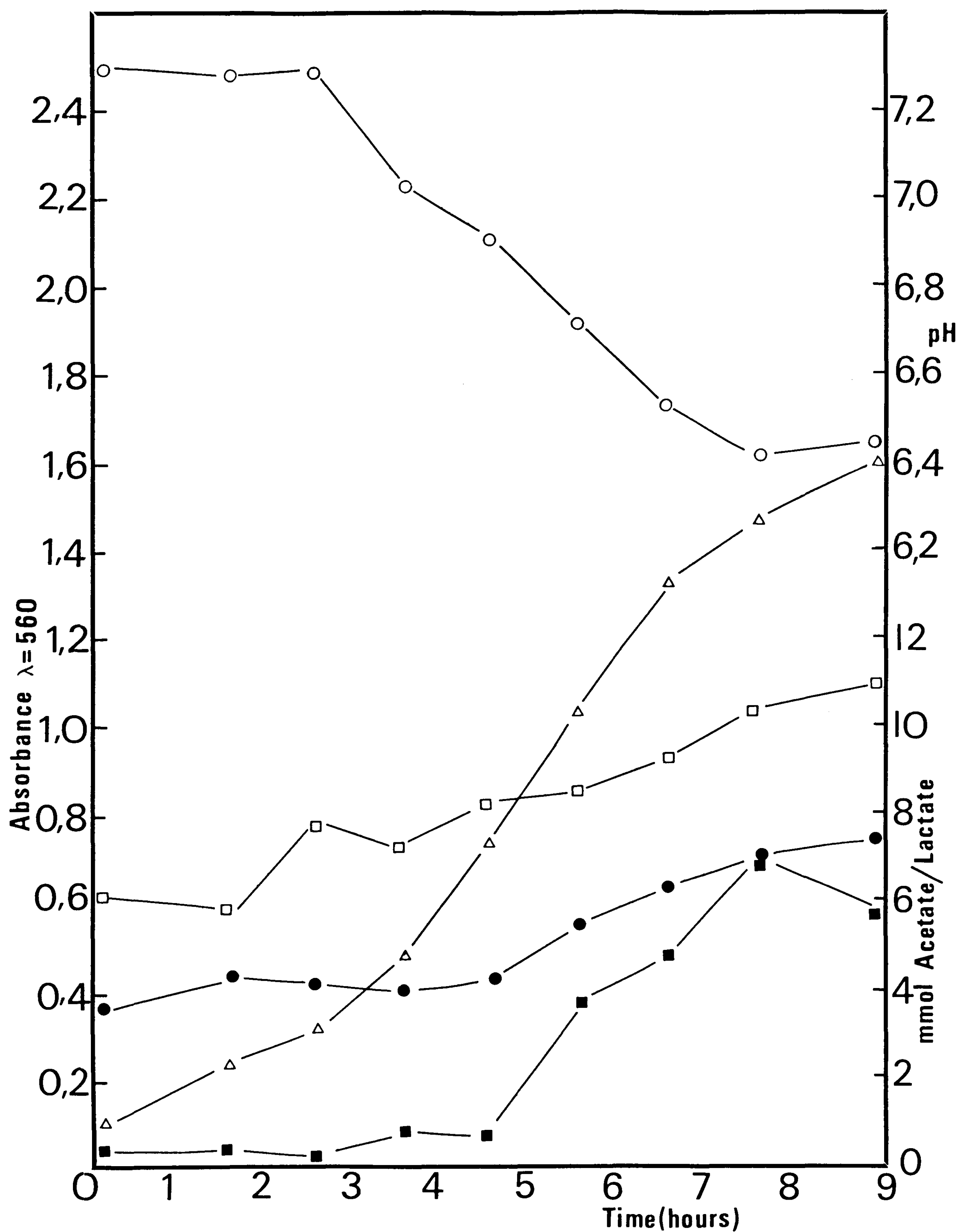

FIGURE 4.15 (b) : Growth curve of S427 grown under anaerobic conditions showing change in lactic and acetic acid production and resultant pH drop.
△———△ Absorbance; ○———○ pH;
□———□ L(+)-lactic acid;
■———■ D(-)-lactic acid;
●———● acetic acid.

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(Buchanan and Gibbons, 1974). Thus the concentrations of acetate and lactate were determined for S258 and S427 grown aerobically and anaerobically. Under aerobic conditions the concentration of acetate for S258 and S427 rose steadily from 3 mmol to 14 mmol and 11 mmol respectively after 9 h growth. The initial concentration of L(+)-lactate of 6 mmol was due to its presence in BHI. The concentration of L(+)-lactate remained relatively constant for S258 and S427 for the first 4½ to 5½ h after which period it was metabolized to result in a final concentration of 3 mmol after 9 h. D(-)-lactate was not produced for the first 3½ h for S258 and S427. During the next 3 h 4 mmol D(-)-lactate was produced and then presumably metabolized to give a final concentration of 3 mmol after 9 h. It can thus be concluded that under aerobic conditions energy is obtained mainly from the Krebs cycle and the membrane-bound electron transport system to produce acetate throughout the 9 h growth period. The glycolytic pathway was also active between 3½ and 6½ h growth to produce D(-)-lactate and L(+)-lactate which were subsequently partially metabolized to give final concentrations of around 3 mmol D(-)-lactate and L(+)-lactate.

Under anaerobic conditions the concentration of acetate for S258 and S427 remained constant at 3 mmol for the first 4½ h and then increased at a steady rate to give a final concentration of 8 mmol and 7 mmol respectively after 9 h. The concentration of D(-)-lactate remained constant for 3½ h for S258 and constant for 4½ h for S427. Its concentration increased sharply to 7 mmol over the next 3 h and then stabilized at

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6 mmol after the 9 h study period. For S427, the concentration of L(+)-lactate increased steadily from 6 mmol to 11 mmol during the 9 h growth period. However, for S258, the concentration of L(+)-lactate increased to 11 mmol after 6½ h growth and then decreased to give a final concentration of 7 mmol after 9 h. This decrease in L(+)-lactate acid concentration cannot be explained as there was not a concomitant increase in D(-)-lactate or acetate. It can thus be concluded that because conditions were not strictly anaerobic the enzymes of the glycolytic pathway and those of the Krebs cycle and the membrane-bound electron transport system were active resulting in production of acetate and D(+) and L(-)-lactate after 9 h.

It can thus be concluded that there is no difference in metabolism between the a_w "sensitive" and "resistant" staphylococci strains.

4.6 MICROSCOPIC OBSERVATIONS

S.aureus shows a strong resistance to high concentrations of humectants, and must adapt itself to acquire some special defence mechanism to high osmotic pressure in order to proliferate. Thus microscopic observations were done in order to determine if there were any changes in the surface structure of *S.aureus* a_w sensitive and resistant strains when grown in the presence of added humectants to BHI.

Scanning electron microscopic observations of *S.aureus* 258 and 427 (a_w sensitive and resistant respectively),

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were prepared after being grown in BHI only (control) and with added humectants glycerol, sodium lactate, propylene glycol, sucrose or sodium chloride to give a_w 0,925.

From the electron micrographs shown in Figures 4.16 and 4.17 no difference in the shape and surface structure could be observed. No difference was obtained in the average diameters of S258 and S427 when grown in the presence of varying humectants except for sodium lactate. The average diameter of S258 and S427 grown in BHI only was 0,87 µm. The average diameter of the cultures grown in the presence of 26% glycerol, 18,8% propylene glycol, 56% sucrose and 10% sodium chloride (all which gave an a_w 0,925) was 1 to 1,25 µm. However, when the cultures were grown in 17,5% sodium lactate (a_w 0,925) the average diameter was 1,62 µm. In Figures 4.16 and 4.17 a slime substance can be seen in the intercellular space, however, the slime should not be confused with the fibre strands of the filter paper on which the samples were prepared. These results compare favourably with those of Kanemasa et al. (1974) who observed from scanning electron microscopic observations that the diameter of S.aureus grown in 0,05% and 10% sodium chloride was in average 0,8 µm and 1,04 µm respectively.

This increase in diameter could represent a defence mechanism to escape from the high osmotic pressure and the large diameter obtained when the cocci are grown in the presence of 17,5% sodium lactate could cause the cells to burst and thus explain the bacteriocidal effect of sodium lactate (Kanemasa et al., 1974).

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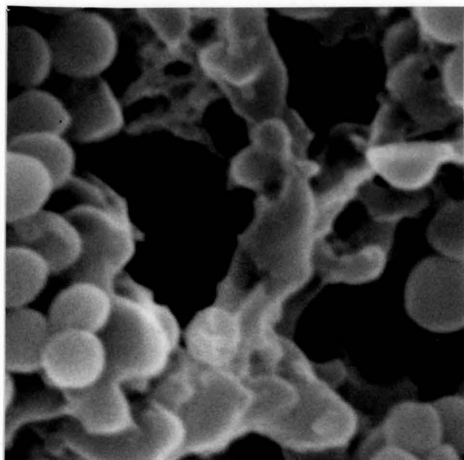
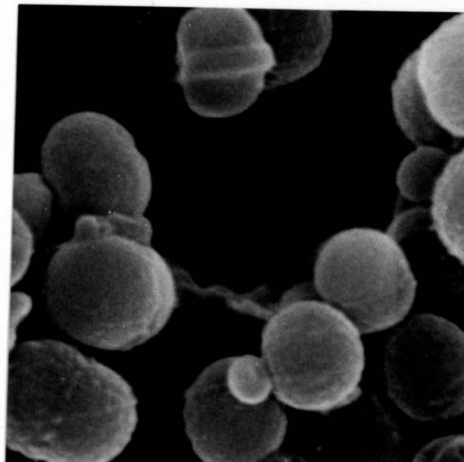
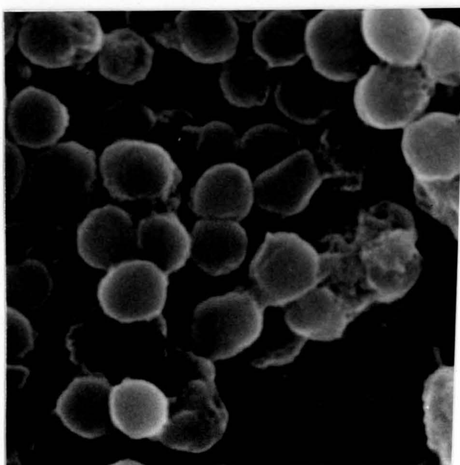


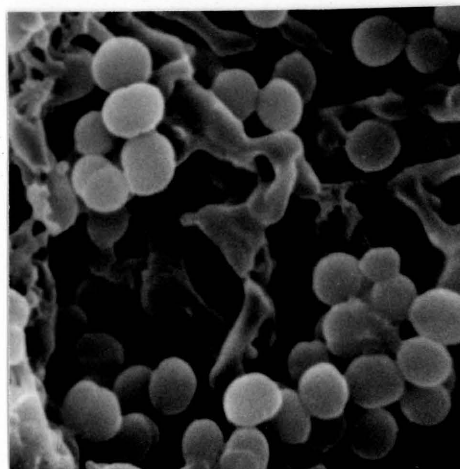
a: 26%/2,82 mol glycerol



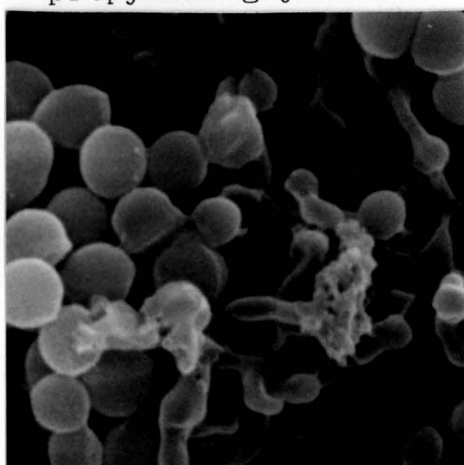
b: 17,5%/1,56 mol sodium lactate



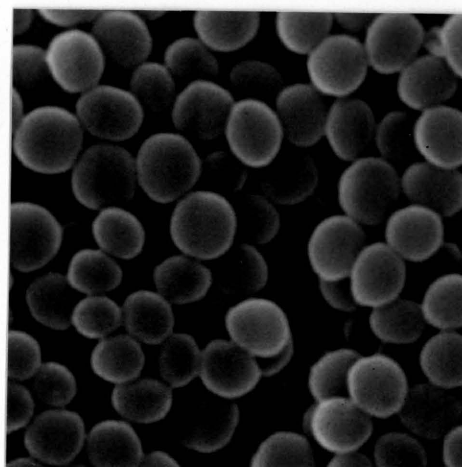
c: 18,8%/2,47 mol propylene glycol



d: 56%/1,64 mol sucrose



e: 10%/1,71 mol sodium chloride



f: control

μm

FIGURE 4.16 : Scanning electron micrographs of S.aureus 258 grown in BHI for 12 h (control) and at a_w 0,925 adjusted using various humectants.

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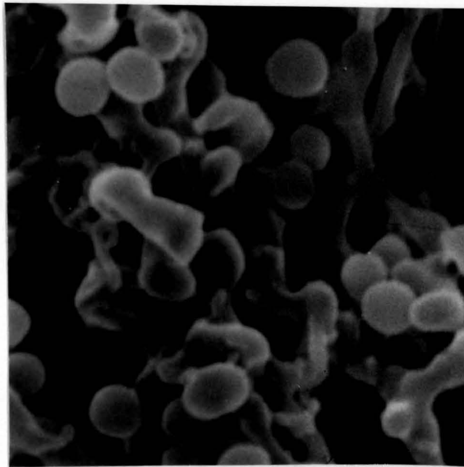
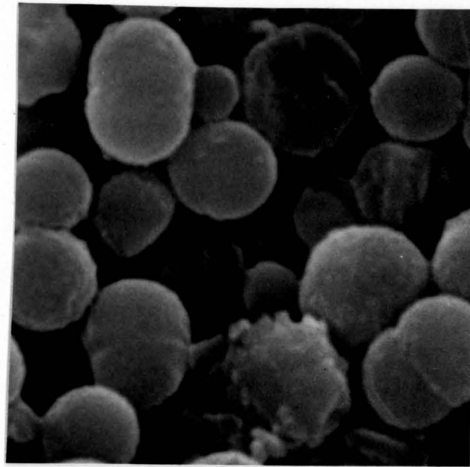
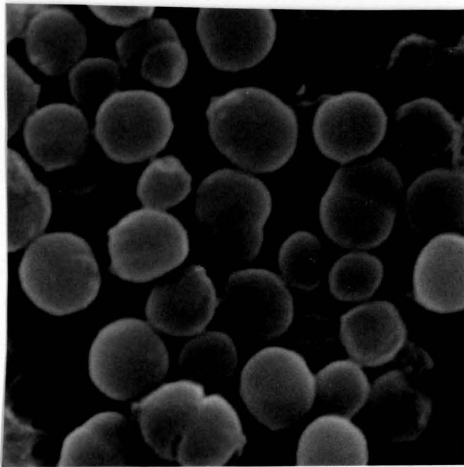


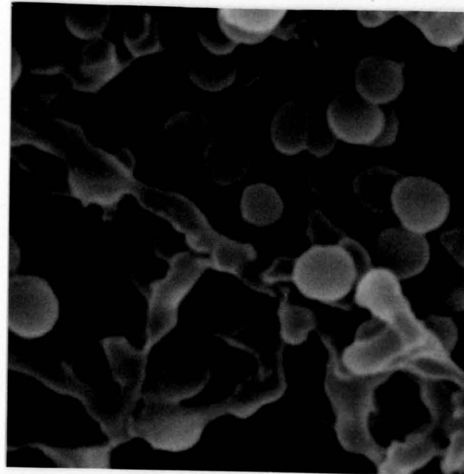
a: 26%/2,82 mol glycerol



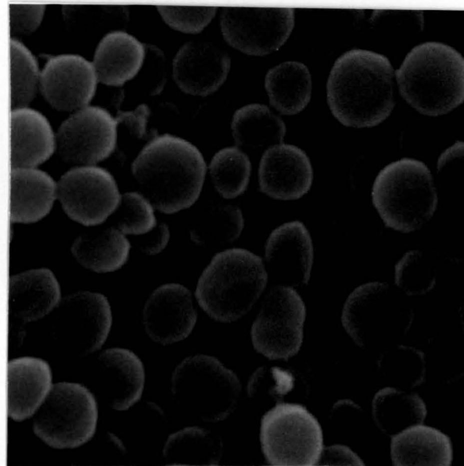
b: 17,5%/1,56 mol sodium lactate



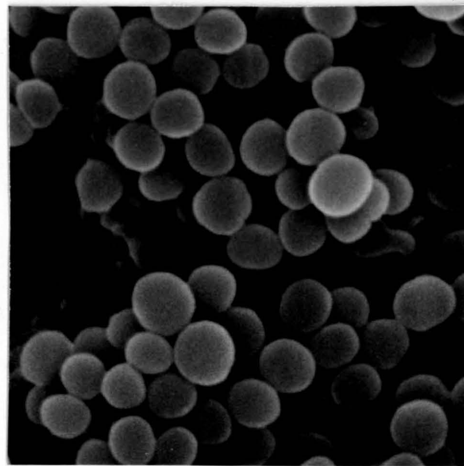
c: 18,8%/2,47 mol propylene glycol



d: 56%/1,64 mol sucrose



e: 10%/1,71 mol sodium chloride



f: control

μm

FIGURE 4.17 : Scanning electron micrographs of S.aureus 427 grown in BHI for 12 h (control) and at a_w 0,925 adjusted using various humectants.

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In Figures 4.16 and 4.17 cells can be seen that are in the process of separation. Amako and Vmeda (1977) studied the surface structure of the cell separation process of staphylococci and noted two distinct area : a rough and a smooth area, on the surface. However in the micrographs these surfaces are not very distinct but the border between these areas is clearly visible. The beginnings of the separation process of S.aureus 427 grown in BHI can also be observed in Figure 4.18, a negatively stained transmission electron micrograph.

It can be concluded that although humectants do increase the diameter of staphylococcal cells, they do not affect their process of cell division.

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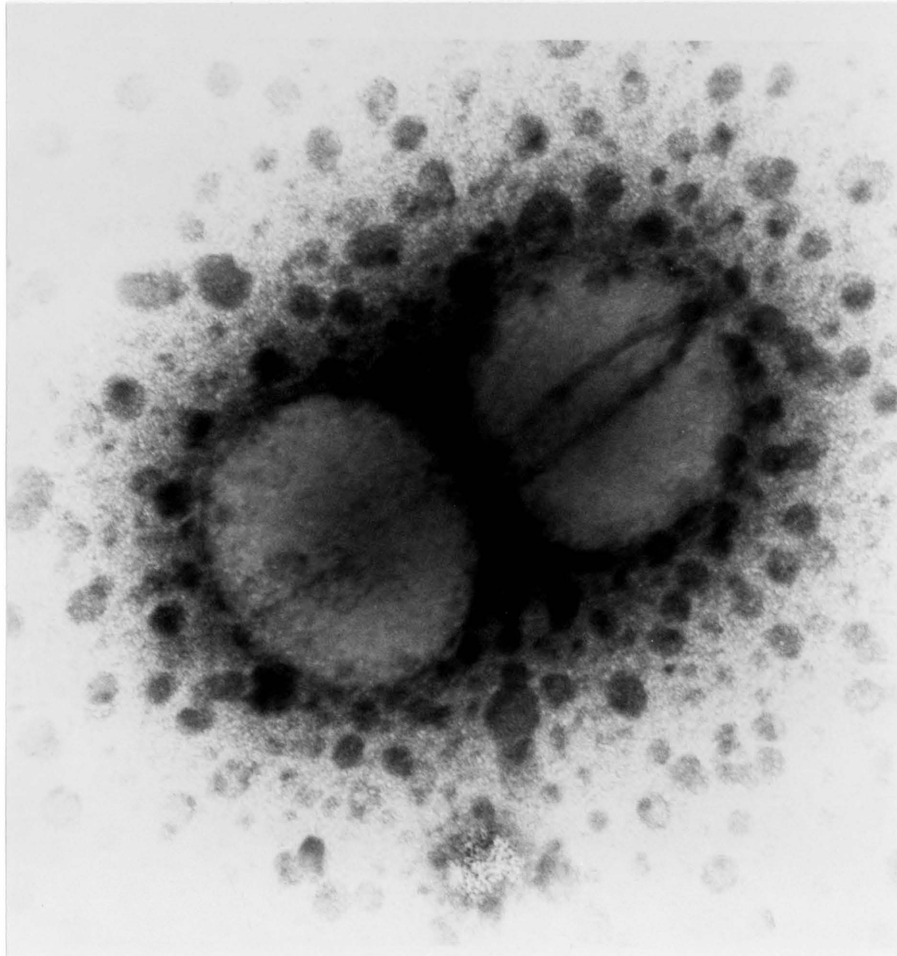

1 μm

FIGURE 4.18 : Transmission electron micrograph of a negatively stained (P.T.A, pH6) culture of S.aureus 427, grown in BHI for 8 h.

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C H A P T E R 5

SUMMARY AND CONCLUSION

Staphylococcus aureus, an important food-born pathogen was isolated from fresh and processed meats. During the experimental period thirty-five coagulase positive isolat were isolated. Of these isolates 88,5% were identified S.aureus, while the remaining 11,5% were found to be S. intermedius. S.aureus was isolated from S.S.P. up to tw weeks of storage. Phage typing using the international basic human set of phages of the isolates recovered from fresh minced meat and shelf stable meat products revealed that 10% and 93,3% respectively were typable indicating human origin, while the non-typable isolates were most probably of animal origin.

The thirty-eight strains were tested for their ability to grow in a range of a_w's adjusted with sodium lactate, glycerol, sodium chloride, propylene glycol and sucrose. The minimal a_w for growth of "sensitive" S258 and "resista S427 strains was 0,866 and 0,846 respectively. However, lowest a_w for growth of S258 was reached when sodium chlor was used as humectant as compared to glycerol for S427. This indicates that the type of humectant could influence bacterial growth. S.aureus 258 and S427 were grown at a_w 0,925 adjusted using glycerol, sodium lactate, propylen glycol, sucrose or sodium chloride. The results showed that although the a_w was constant, bacterial growth varied greatly in the presence of different humectants.

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The thirty-eight strains were tested for their ability to grow in a range of pH-values adjusted using the inorganic acid HCl, and the organic acids acetic, lactic, ascorbic, tartaric, gluconic and citric acid. The results showed that S144 and S470 were relatively acid "sensitive" and "resistant" respectively. The minimal pH for bacterial growth depended on the acid used. The inorganic acid, HCl, had the least effect on staphylococcal growth enabling growth of S144 and S470 down to a pH of 4,43 and 4,08 respectively, while the most favourable organic acid for inhibition of bacterial growth was acetic acid, enabling growth of S144 and S470 down to pH 5,10 and 5,0 respectively.

The optimal, maximal and minimal temperatures for growth of the 38 strains were determined using a temperature gradient incubator. No extremely temperature "resistant" or "sensitive" strains were isolated and all strains had a growth range of 8°C to 52°C with an optimal growth temperature of 34,5 to 38°C. D-values at 60°C were determined for all strains and showed strain S144 to be the most resistant with a $D_{60°C}$ value of 5½ min 30 s. Strain S258 was the most sensitive to heat with a $D_{60°C}$ value of 1 min 54 s.

The a_w and pH sensitive and resistant strains were grown in BHI in the presence of combinations of pH (5,5 and 7,3) and potassium sorbate (0,5%, 1% and 2,5%). It was found that, although increasing concentration of potassium sorbate did prevent bacterial growth at pH 7,3, the bacteriocidal effect of potassium sorbate was greatest when the initial pH was 5,5 irrespective of concentration.

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The a_w and pH sensitive and resistant strains were also grown in BHI in the presence of combinations of pH 5,5 adjusted with citric, lactic, acetic, gluconic or hydrochloric acid and a_w, adjusted using glycerol, sodium lactate, propylene glycol, sucrose or sodium chloride. The bacteriocidal effect of a lowered pH of 5,5 with a_w 0,985 is less than when a_w is lowered to 0,925 at pH 7,3. The best results at a_w 0,925 and pH 5,5 for inhibition of bacterial growth occurred when sodium chloride was used as humectant and when the pH was lowered using gluconic acid.

Quantitative and qualitive studies of the cell walls of selected isolates revealed the typical high glycine content in the peptidoglycans (5-6 mol Gly : 1 mol Glu). In *S.aureus* 177 small amounts of glycine were substituted by serine. All strains studied had typical *S.aureus* cell wall composition: L-Ala-D-Isoglu-(NH_2)-N^{α}-L-Lys-(D-Ala)-N^{ϵ}-$(Gly)_5$-D-Ala-L-Lys.

Studies of the phospholipids of *S.aureus* 427 cell membrane grown in BHI only and at a_w 0,925 adjusted with NaCl, glycerol, sodium lactate, propylene glycol or sucrose revealed that when the a_w was decreased to a_w 0,925 there was an increase in the amount of cardiolipin with a corresponding decrease in the amount of phosphatidylglycerol compared to *S.aureus* grown in BHI at a_w 0,98. The change in phospholipid composition occurred independent of the humectant used to lower the a_w.

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Metabolic products of S.aureus 258 and 427, (a_w "sensitive" and "resistant" respectively), were examined after aerobic and anaerobic growth. Aerobically the concentration of acetate of S258 and S427 after 9 h growth was 14 mmol and 11 mmol respectively, while the concentration of L(+)-lactate and D(-)-lactate was 3 mmol and 3,5 mmol respectively for S258 and 2,2 mmol and 2,4 mmol respectively for S427. Anaerobically the concentration of acetate (after 9 h) was 8 mmol and 7,5 mmol for S258 and S427 respectively while the concentration of L(+)-lactate and D(-)-lactate was 7,5 mmol and 6,1 mmol respectively for S258 and 11 mmol and 5,8 mmol respectively for S427.

Electron micrographs revealed that when S.aureus 258 and 427,(a_w"sensitive"and"resistant"respectively)were grown in a_w 0,925 lowered using sodium chloride, sodium lactate, propylene glycol, sucrose or glycerol, there was an increase in diameter of the cocci. However, no difference in the shape, surface structure or process of cell division was observed. The average diameter of the cocci grown in BHI was 0,87 μm. When either glycerol, propylene glycol, sucrose or sodium chloride were added to adjust the a_w to 0,925, the average diameter was 1 to 1,25 μm. However, when sodium lactate was used, the average diameter was 1.62 μm.

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In conclusion it can be noted that:

1. S.aureus can readily be isolated from fresh and processed meat.
2. Increased handling of meat may lead to higher S.aureus contamination of human origin.
3. Relatively a_w sensitive and resistant strains of S.aureus were isolated.
4. The type of humectant used to lower the a_w may influence the extent of growth by S.aureus.
5. When considering the effects of humectants the following facts must be considered

   (i) its strength for binding water
   (ii) its bacteriocidal effects
   (iii) the molarity of humectant required to produce the desired a_w

6. Relatively pH sensitive and resistant strains were isolated.
7. The inorganic acid HCl had the least effect on staphylococcal growth when compared to the organic acids.
8. Acetic acid was the most effective organic acid in preventing S.aureus growth.
9. No temperature sensitive or resistant strains were found from temperature growth curves.
10. S.aureus 144 was the most resistant to heat as determined by D-values at 60°C while S.aureus 258 was the most sensitive to heat (D_{60} values of 5½ min 30 s and 1 min 54 s respectively).
11. The pH affected the bacteriocidal properties of potassium sorbate on S.aureus to a greater extent than the concentration of sorbate as such. This emphasizes the importance of the dissociation constant of potassium sorbate in this respect.
12. Lowering the a_w to 0,925 had a more inhibitory effect on bacterial growth than a lowered pH of 5.5.

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13. The greatest bacteriocidal effect was obtained at a_w 0,925 adjusted with sodium chloride and pH 5,5 adjusted with gluconic acid.
14. Strains studied had typical cell wall composition of *S.aureus*, showing the pentaglycine interpeptide bridge in the peptidoglycan.
15. Cell wall studies also showed the presence of a ribitol teichoic acid as well as mannose and glucose as units of the cell wall polysaccharide.
16. As the a_w was lowered, the amount of cardiolipin in the cell membrane increased while the amount of phosphatidylglycerol decreased, irrespective of humectant used to lower the a_w.
17. No difference was found in lactic and acetic acid metabolism between the a_w "sensitive" and "resistant" staphylococcal strains under aerobic and anaerobic conditions.
18. In the presence of humectants, no difference in the shape, surface structure or process of cell division was observed but an increase in cell diameter.

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