THE DEVELOPMENT OF PEPTONE AND YEAST EXTRACT AS MICROBIOLOGICAL CULTURE MEDIA COMPONENTS

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THE DEVELOPMENT OF PEPTONE AND YEAST EXTRACT AS MICROBIOLOGICAL CULTURE MEDIA COMPONENTS

by

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This thesis is dedicated to my late father.

I certify that the thesis hereby submited, and the work presented therein, to the University of Pretoria for the degree of M.Sc. has not been previously submitted by myself in respect of a degree at any other University.

Signature: _____

Date: _____

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Degree	:	M.Sc. (Microbiology)

SUMMARY

Over 2000 formulated culture media were listed during the early 1930's. The current estimate lies between 6000 to 7000 different media. Unfortunately these media are all variations on a relatively few essential nutritional ingredients (e.g. peptones, tryptone and other extracts made from yeast, meat etc.) from unrevealed sources. Almost 3 tons of microbiologial culture media components are imported annually from Europe at a very high cost due to unfavourable exchange rates. Efforts were made to manufacture these expensive imported microbiological culture media components locally.

It is important to evaluate culture media components individually and in combination with other ingredients of culture media, because of unforeseen interactions which may occur with other defined and/or undefined culture media components. In this study the locally manufactured media were evaluated as follows:

- Physical and chemical evaluation (colour, clarity, solubility, setting properties and pH);

- Microbiological examination (sterility, heat-resistant microorganisms and primarily, the growth-supporting abilities).

Evaluation of the growth supporting abilities of culture media components and/or culture media was based on inoculating suitable pure cultures into solutions of the experimental raw materials and/or Nutrient broths. The growth of the test microorganisms was determined by measuring the optical density of the solutions over time. For the experimental agars, suitable pure cultures were spread plated onto the agars and colony sizes on the agar plates measured as an indication of growth of the test organisms. This was done as specified by the manufacturer of the standard agar media.

These results indicated that the locally manufactured peptones and yeast extract supported growth of all of the selected microorganisms similar to the standard. Hence, they can be used as culture media components. When the locally manufactured peptones and yeast extract were incorporated into culture media formulations, the experimental culture media results showed that the components were compatible and supported growth of the test organisms better than the standard culture media.

DIE ONTWIKKELING VAN PEPTONE EN GISEKSTRAK AS MIKROBIOLOGIESE KULTUUR MEDIA BESTANDELE

deur

SUPREME LETLAKANA SEBATA

Promotor : Prof T.E. Cloete

Departement : Mikrobiologie en Plantpatologie

Graad : M.Sc. (Mikrobiologie)

OPSOMMING

Ten minste 2000 geformuleerde kultuur media is beskryf gedurende die vroeë 1930's. Die huidige beraming dui op ongeveer 6000 to 7000 verskilende media. Die probleem is dat al hierdie media variasies is van 'n relatief klein hoeveelheid bestanddele (byvoorbeeld peptone, triptoon en ander ekstrakte gemaak van giste, vleis ens.) afkomstig van ongeïdentifiseerde bronne en vervaardigings prosesse. Hoë kostes is verbonde aan die ongeveer 3 ton mikrobiologiese kutuur-media komponente wat jaarliks vanaf Europa ingevoer word. Dit is vanweê die huidige swak wisselkoerse. Moeite is gedoen om die duur, ingevoerde mikrobiologiese kultuur-media komponente plaaslike te vervaardig.

Dit is belangrik om kultuur-media bestandele afsonderlik en in kombinasie met ander bestanddele van kultuur media te evalueer, aangesien onvoorsiene interaksies met ander gedefinieerde kultuur-media komponente mag plaasvind. In hierdie studie is die plaaslike vervaardigde kultuur-media komponente, as volg geëvalueer:

- Fisiese en chemiese evaluasie (kleur, helderheid, oplosbaarheid, stollings-eienskappe en pH);

- Mikrobiologiese evaluasie (steriliteit, hitte-bestande mikrobes, en primêr, die groei-onderhoudingsvermoë).

Geskikte reinkulture was geïnokuleer in oplossing van die roumateriale en/of voeding-soppe vir die evaluering van die groei onderhoudingsvermoë van kultuur-media komponente en/of kultuur media. Gevolglike groei van die toets-organismes was uitgedruk as optiese digtheid. Vir die eksperimentele agars, is geskikte reinkulture deur middel van die sprei-plaat metode op die agars uitgeplaat. Meting van die kolonie-grootes op die agar plate is as groei van die toets organismes uitgedruk, soos gespesifiseer deur die vervaardiger van die standaard agar media.

Die resultate dui daarop dat die plaaslik vervaardigde peptone en gisekstrak die groei van die meeste van die geselekteerde mikrobes onderhou op dieselfde wyse as die standaard. Plaaslik vervaardigde peptone en gisekstrak kan dus gebruik word as kultuur-media komponente. Met die insluiting van plaaslik vervaardigde peptone en gisekstrakte in kultuur-media formulasies was die eksperimentele kultuur-media se resultate verenigbaar en het dit die groei van die toets organismes beter onderhou as die standaard kultuur media.

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Before 1915 peptones were only manufactured in Germany for the commercial production of culture media. The nature and process of manufacture of these products have not been revealed. After stocks were exhausted, various American manufactures undertook to make peptones and culture media. American manufactures followed the same policy as the Germans did, in maintaining trade secrecy as to the process of manufacture and the nature of basic substrates (Leifson, 1943). The majority of culture media formulated (over 2000 were listed in 1930 and the current estimate is probably approximately 6000 to 7000 different media), are all variations on a relatively few essential nutritional ingredients, supplemented with a large number of selective, elective or indicative compounds (Bridson, 1978).

Few laboratories in the world produce their own culture media components and culture media. Laboratories in many countries import culture media components and/or culture media from reputable manufactures including Difco, Quest yeast, Oxoid, Solabia, Biolab, Orthana Kemisk, and Bio Springer. Almost 3 tons of microbiological culture media components are imported every year from Europe at a very high cost due to an unfavourable exchange rate. Hence, a great opportunity exist for import replacement of specifically: peptone, meat extract, meat pancreatic peptone, tryptone, malt extract and yeast extract. Replacement of these imported microbiological culture media components can be achieved by locally manufacturing these media components

and would be of great interest to the South African chemical/pharmaceutical companies, should the quality and cost be acceptable. The challenge of their manufacture lies not so much in the sophistication of the procedures required, but in the development of highly efficient methods using low cost raw or waste materials. The quality and the manufacturing efficiency of these products may also present export opportunities.

All reputable manufacturers of culture media carry out quality control inspections when selecting basic ingredients. Guaranteed reproducibility can, however, only be expected if the medium is prepared according to the directions of the manufacturer (Terplan and Zaadhof, 1982). Quality assurance and quality control of these media components and of the culture media which contains these components is of paramount importance, so that standards and specifications can be maintained. Standards and specifications should be maintained, because many laboratories use commercially prepared dehydrated media and rely on the manufacturer's quality assurance and quality control programmes. It cannot be emphasised too strongly that the user laboratory has considerable responsibility in following the manufacturer's instructions for preparing dehydrated media and should check the performance of autoclaves, waterbaths, etc. regularly (Blood, 1985).

The ingredients that were examined in this study (peptone and yeast extract) are classified as chemically undefined nutrients, extracts and hydrolysates. The fact that a substance is chemically defined does not automatically mean that it poses no problems as a microbiological ingredient (Costin, 1982). However, the problems are minor in comparison with the challenges

presented by the undefined microbiological components. This is because the exact chemical composition of the latter components are not known.

It is therefore important to test the components individually and in combination with other ingredients of culture media, because of unforeseen interactions which may occur with other defined and/or undefined components. If a microbiological component works well in a certain medium, it does not mean that it will perform equally well in another medium. For some ingredients, as many as six different complex media are necessary to ascertain whether they fulfil the majority of their utilisation purposes (Costin, 1982). Bridson and Brecker (1970) also stated that a full analysis of all the components in many natural undefined media components e.g. peptones, tryptone and extracts of meat yeast etc. is impossible. However, simple and cost-effective quality control programmes can be employed to study and evaluate undefined culture media components.

The aim of this project was to evaluate locally manufactured peptones and to devise a cost-effective method for the manufacturing of yeast extract and its evaluation for import replacement during the local manufacture of formulated culture media.

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CHAPTER 2

Literature review

1 Historical development of culture media

The importance of culture media was noticed just after the birth of microbiology in 1674, when Antony van Leeuwenhoek first saw "animolecules" (which we now recognise as protozoa and bacteria) in rainwater and pepper infusions (when trying to find out what made the pepper hot). It was also noticed by early investigators that when a perfectly clear infusion was allowed to stand for a time in a warm place, it became cloudy, and it was believed that the cloudiness was caused by the spontaneous development of microorganisms. Then, there were no satisfactory methods for culturing microbes artificially, or for separating one kind from another (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*, 1978; Ware and Hawker, 1979; Prescott *et al.,* 1993).

Between 1765 and 1776 Spallanzani described experiments which clearly showed the errors of Needhams conclusions in proving the spontaneous generation theory (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*, 1978; Ware and Hawker, 1979; Prescott *et al.,* 1993). During the years 1836 to 1854 Schulze, Schwann, Schroeder and von Dusch added on Spallazani's work on proving the spontaneous generation theory wrong, by conducting experiments that support sterility using Schwann flasks (with long banded necks), alkali, acids, flames and cotton plugs (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*,

Ware and Hawker, 1979; Prescott *et al.,* 1993). Culture media played an important role in proving the spontaneous generation theory wrong via experiments conducted by Louis Pasteur (1860-1861) and supplemented a few years later by those of John Tyndal (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*, 1978; Ware and Hawker, 1979).

During the end of the nineteenth century, improved techniques on handling and studying bacteria developed rapidly. In 1872 Schroeder observed pure culture colonies growing on potato slices. He also found that it was possible to select (suppress growth of other organisms) and differentiate (separate different components of the microbial mixture) mixed cultures inoculated onto potato slices (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*, 1978; Ware and Hawker, 1979; Prescott *et al.,* 1993). It would not have been possible for Robert Koch to convince the world in (1884) about the germ theory, if he was not able to isolate pure cultures, i.e. from Koch's second and fourth postulates respectively: "it must be isolated from a patient and grown in the laboratory apart from all other organisms" and "the same organism must be found again in the inoculated animals and recovered in laboratory cultures" (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*, 1978; Ware and Hawker, 1979).

Components with solidifying characteristics were also developed during Koch's time. Bredfeld added gelatin to infusions and allowed the infusion-gelatin to cool and solidify. Since gelatin liquefies readily when warmed, the temperature could not be allowed to rise above 28^0C. Moreover, human and animal pathogens grow slowly, if at all, at 28^0C.

Furthermore, some organisms digest and liquefies gelatin (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*, 1978; Ware and Hawker, 1979; Prescott *et al.,* 1993). It was Fanny Hesse who suggested agar as a solidifying agent (Atlas, 1993). Agar is a polysaccharide extracted from certain marine algae and it solubilizes only when heated to *ca.* 100^0C. It remains liquid until cooled to *ca.* 42^0C.

Prior to about 1915, commercial culture media production using raw materials from unrevealed sources was initiated in Germany. The nature of these products, and the process of manufacture has not been revealed. When stocks became exhausted, various American manufactures undertook to make peptones. Unfortunately, these manufacturers followed the same policy as the German manufacturers in maintaining trade secrecy as to the processes of manufacture and the nature of the basic substrates (Leifson 1943). Their products were designated by uninformative trade names. Culture media users appreciated the convenience and availability of products needed for scientific work, but the use of semi-secret proprietary products has never been in the interest of science. This secrecy has caused a major drawback and has ever since created a gap that would not be easily closed. The major reason why literature in this field is very limited, is because of the secrecy of the processes of manufacture and the nature of the basic substrates.

2 Microbiological culture media

Microbiological laboratory procedures of culture methods resemble an equation with at least three variables: the microorganisms, the substrate (medium) and the working methods (Costin, 1982). A prerequisite to the study of microorganisms is their cultivation under laboratory conditions. Extensive research has determined the nutritional requirements of bacteria and this information has resulted in the development of numerous media for their cultivation (Schlegel, 1992). Culture medium is a substrate or nutrient solution upon which microorganisms are cultivated in the laboratory (Carpenter, 1977). The nutrients can be the inorganic salt solution, on which the organism can scavenge sufficient carbon and nitrogen, or they may be very elaborate hydrolysates of protein plus the heat labile factors in blood serum or other tissue fluids. Such elaborate media may be mandatory to grow pathogenic organisms isolated from clinical specimens (Bridson, 1978). Because the nutritional requirements of bacteria vary widely, there are great differences in the composition of the culture media employed. However, the primary distinction is between defined media, prepared from known chemical constituents and undefined media, containing unknown natural mixtures of substances.

2.1 Types of culture media

The first solutions for cultivation of bacteria and other organisms consisted of natural materials such as plants and animal tissues. These solutions were prepared by soaking plant

material and animal tissues in water and obtaining liquids known as infusions in which microorganisms could grow. Pasteur was apparently the first to use culture media of known composition. A medium of this kind is known as chemically defined or synthetic medium (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.*, 1978; Ware and Hawker, 1979). This type of medium is designed for the cultivation of specific known types of microorganisms (Table 1.1) and can be reproduced at any time because the chemical formulae of all its constituents are exactly known.

Table 1.1 Synthetic medium for growth of *Escherichia coli* (Nester *et al.*, 1978)

Ingredient	**(g/l)**
Glucose	5.0
Dipotassium phosphate (K_2HPO_4)	7.0
Monopotassium phosphate (KH_2PO_4)	2.0
Magnesium Sulphate ($MgSO_4$)	0.08
Ammonium Sulphate [$(NH_4)_2SO_4$]	1.0
Water	1.000 ml

Certain nutritionally fastidious organisms require a dozen or more of accessory substances such as vitamins, growth factors, peptones, yeast extract etc. of unknown chemical composition (Table 1.2) (Kheshgi and Saunders, 1959 Nester *et al.*, 1978; Prescott *et al.*, 1993).

Table 1.2 Nutritious medium for the cultivation of a wide variety of bacteria (Biolab, 1996)

Composition	(g/l)
Beef extract	10.0
Peptone	10.0
Sodium chloride (NaCl)	5.0
Agar	15.0

These accessory substances in media are known as non-synthetic, complex or natural medium. Because the nutritional requirements of bacteria vary widely, there are great differences in the composition of the media employed for their cultivation. To meet these needs, the bacteriologist has available numerous media which on the basis of their application, or functions, may be classified as follows:

- **Enriched media**

Enriched media are mostly designed for the cultivation of human pathogens or other fastidious heterotrophic bacteria and contain mainly proteinaceous substrates such as peptones, meat- and yeast extract (Stolp and Starr, 1981). The addition of components such as blood, serum, or extract of plant or animal tissues to nutrient broth or agar provides additional nutrients so that the medium will support growth of fastidious heterotrophs (Nagel and Kunz, 1973; Pelczar *et al.*, 1977; Vassliadis *et al.*, 1982; Biolab, 1996).

- **Selective media**

Selective media are prepared by the addition of certain specific chemical substances to nutrient agar which will support the growth of one group of selected organisms and inhibit other unwanted organisms (Blood, 1985). Bile salts, or dyes like basic fuchsin and crystal violet favour growth of gram-negative bacteria, by inhibiting the growth of gram-positive bacteria, without affecting gram-negative organisms (Prescott *et al.,* 1993). MacConkey agar, Endo agar and Eosin methylene blue agar are widely used for the detection of *Escherichia coli* (Prescott *et al.,* 1993; Biolab, 1996). Other media may contain cellulose as the only source of carbon and energy, and are effective in the isolation of cellulose-digesting bacteria. The medium should exhibit negligible toxicity to bacteria belonging to the species sought, which may have been injured sublethally (Mossel, 1971; Nagel and Kunz, 1973; Harrewijn, 1982; Heeschen and Hahn, 1982; Kendall, 1982; Kusch, 1982; Biolab, 1996).

- **Differential media**

Differential media is produced by incorporating certain reagents or chemicals into culture media that might result in a kind of growth or change, after inoculation and incubation, which will permit differentiation between the types of bacteria. Haemolytic bacteria (e.g., many *Streptococci* and *Staphylococci* isolated from throats) produce clear zones around their colonies because of red blood cell destruction. This red blood cell destruction is known as haemolysis. The media used to differentiate between the haemolytic bacteria and the non-

haemolytic bacteria is Blood agar (Prescott *et al.,* 1993; Biolab, 1996). MacConkey agar is a selective and a differential media, because it contains lactose and neutral red dye. The lactose-fermenting colonies appear pink to red in colour and are easily distinguished from colonies of non-fermenters (Mossel, 1971; Nagel and Kunz, 1973; Nester *et al.*, 1978; Heeschen and Hahn, 1982; Prescott *et al.,* 1993; Biolab, 1996).

2.2 Bacterial growth in culture media

Due to the physiological diversity of microbial groups and the physico-chemical diversity of their micro-environment, no single medium has ever been able to provide growth requirements of more than 10% of the total number of bacteria in a natural habitat (Karl, 1986). When bacteria are inoculated into a suitable medium and incubated under appropriate conditions, growth will occur.

The term growth as commonly applied to bacteria and other microorganisms usually refers to changes in the culture of the cells rather than to changes in an individual organism (Poindexter, 1971; Pelczar *et al.*, 1977; Nester *et al.*, 1978). Growth is defined as the increase in dry mass of an organism (Poindexter, 1971), and an increase in cell number from the initial size of the inoculum (Pelczar *et al.*, 1977).Individual cells whose growth and division were studied with the aid of interference microscopy, which allows calculations of the mass of a growing cell at intervals, were observed to grow discontinuously (Poindexter, 1971).

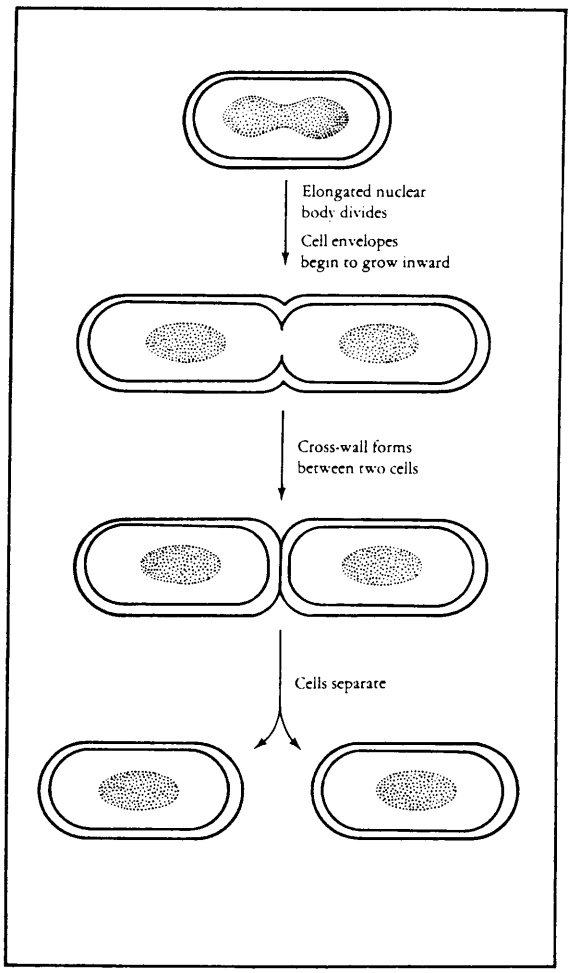


Fig 1.1 Diagrammatic representation of cell division. A single cell divides into two after it has doubled in size (Nester *et al.*, 1978).

The most common process, and no doubt the most important in the usual growth cycle of bacterial populations, is binary fission in which a single cell divides into two (Fig 1.1) (Atlas and Bartha, 1987a). The time interval required for the cell to divide, or for the population to

double is known as the generation time. Not all bacteria have the same generation time, at 37^0C. Some microorganisms such as *Escherichia coli* has a generation time of about 15-20 min; *Staphylococcus aureus* [27-30 min] and *Streptococcus lactis* [48 min]; *Lactobacillus acidophilus, Mycobacterium tubercolosis and Treponema pallidum* [48-80 min] (Van Denmark and Batzing, 1987).

2.2.1 Measurement of cell growth

Cell growth can be determined by numerous techniques based on the following types of measurement:

- **Direct methods**

Bacterial cell counts can be done directly by microscopy or an electronic particle counter and the cell mass directly weighed or the nitrogen content of the cell measured. These direct methods are not suitable for the purpose of this study, because of the elaborate procedures involved. It is easier to measure cell mass in liquid media, because centrifugation in order to separate the bacterial cells from the liquid media is simpler than washing off agar from the cells followed by drying, and weighing (Postgate, 1969).

- **Indirect methods**

These type of growth measurements are often more convenient than direct methods. The following methods are generally used :

* Light scattering or turbidity - the density of a population can be estimated by the amount of light scattered by the suspended cells

* Metabolic products - metabolic products are excreted in constant proportion to growth and some of these products are easily measured e.g., oxygen and carbon dioxide concentration

* Selected cell constituents - protein accounts for a fairly constant proportion of the dry weight of growing cells, and nitrogen accounts for a constant proportion of protein

* Linear measurements - the diameter and length of filamentous fungi which increases linearly with time and the rate is constant and

* Viable counts - estimation of growth rate by determination of multiplication rate is generally suitable for unicellular organisms that multiply by fission (Maillette, 1969).

Light scattering

Light scattering is the most widely used (Maillette, 1969) convenient and least complex method for estimating total microbiological material in a liquid medium. This method is widely used to determine the growth of selected bacterial cultures (Kheshgi and Saunders, 1959). More rapid techniques depend upon the fact that microbial cells scatter light that is striking them (Prescott *et al.,* 1993).

When light passes through matter, it is scattered apart from its original path by inhomogeneities present. If the inhomogeneities of interest are particles considerably larger than small molecules, scattering becomes relatively intense (Maillette, 1969). Light scattering is dependent upon the concentration, size and shape of the particles; the relative refractive indexes of particle and medium; and the wavelength of the incident light (Maillette, 1969). Bacteria scatter light primarily in the forward direction and hence most equipment can collect the scattered light by the detector system (Fig 1.2). The amount of scattering is proportional to the mass of cells present (Kheshgi and Saunders, 1959). Scattering is measured by passing a beam of light through the culture in an instrument containing a photocell that registers the amount of light that is scattered at a 90^0-angle (the turbidity meter) or the amount of light that passes through without being scattered (the photometer) (Fig 1.2).

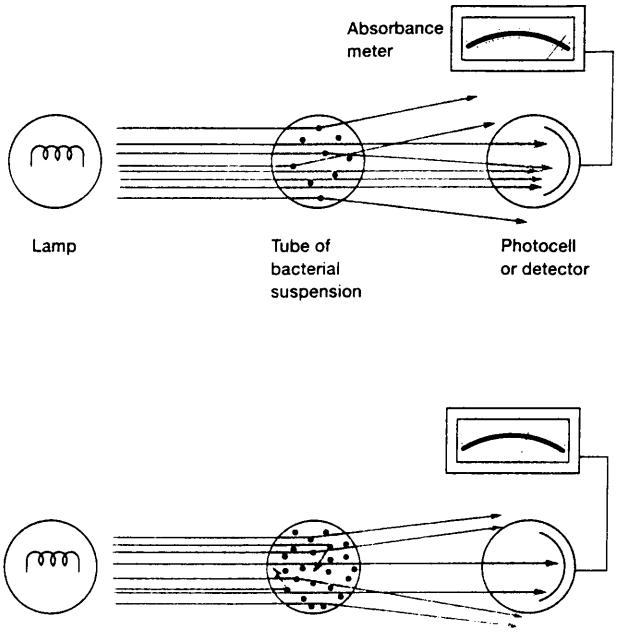


Fig 1.2 Measurement of bacterial mass by transmission of light. The amount of light which impinges on the detector is proportional to the number of bacteria in the suspension (Nester *et al.*, 1978).

The absorbency measurement routinely employed in microbiology is more related to total bacterial mass than to bacterial numbers (Maillette, 1969). Because of the wavelength dependency of light scattering, it can be helpful to change wavelengths either to a region of greater scattering for improvement of sensitivity or to a region in which light is not absorbed by coloured material (Maillette, 1969). Most spectrophotometers have wavelengths of between 350 to 800 nm and this flexibility is important, because different substances absorb light at different wavelengths (e.g. most bacteria absorb most of the light at 540-550 nm).

Spectrophotometers, even the simplest types, are convenient in this respect and have become the most widely used class instruments in microbiological turbidimetry. However, several problems may arise in turbidimetry, including light absorption, the need for calibration curves, collection of light in low scattering angles and the unwanted changes in the biological material (e.g. aggregating cells of *Pseudomonas aeruginosa* in high levels of phosphate) may introduce large uncertainties (Maillette, 1969).

Viable counts

Viable counts are used to determine the number of organisms in a population. The viable count of a microbial population is the absolute concentration of viable organisms present. The viability is the ratio of that number to the total concentration of microbes, dead or alive (Postgate, 1969). This method is based on the fact that a single living bacterium deposited on a solid nutrient medium is capable of multiplying to form two progeny and so on under conditions that are "optimal" for the cell concerned (Fig 1.1) (Postgate, 1969). Continued growth under these conditions on agar would result in visible colony formation (Hattori, 1988). The number of colonies that appear after a suitable period of incubation, represents the number of living or viable cells in the original suspension. Cells in the viable but nonculturable state do not form colonies on agar (Roszak and Colwell, 1987). This is supported by the large discrepancy between agar counts and viable counts determined microscopically (Kogure *et al.*, 1978).

2.2.2 Factors influencing microbial growth

A great variety of environmental factors that influence microbial growth can be enumerated. These can be divided into two broad categories: nutritional factors and the physical environment.

- **Nutritional factors**

For microorganisms to obtain energy and construct new cellular components, raw materials or nutrients must be available. Analysis of microbial cell composition shows that over 95% of cell dry weight is made up of a few major elements, of which the first six are components of carbohydrates, lipids, proteins and nucleic acids (Shlegel, 1988; Prescott *et al.*, 1993). Microorganisms also require trace elements including, manganese, zinc, cobalt, molybdenum, nickel and copper. Table 1.3 shows the principal roles of various elements in the nutrition of organisms.

To be useful in the synthesis of cell materials, a nutrient must possess a structure such that an organism can use the compound either as a whole or convert it to suitable structural units. The greater the synthetic abilities of an organism, the less complex are its nutritional needs. The food requirement of bacteria differ profoundly from species to species (Stephenson, 1949).

Table 1.3 Principal roles of various elements in the nutrition of organisms (Stanier *et al.*, 1966)

ELEMENT	PHYSIOLOGICAL FUNCTION
Hydrogen	Found in cellular water, organic cell materials and H_2 used as electron donor in metabolism.
Oxygen	Found in cellular water, organic cell materials and O_2 used as electron acceptor in respiration.
Carbon	Found in cell materials and used as electron donor or acceptor.
Nitrogen	Found in proteins and used as electron donor or acceptor.
Sulphur	Found in proteins and used as electron donor or acceptor.
Phosphorus	Found in nucleic acids, phospholipids etc. and is a principal agent in energetic coupling of reactions (as ATP).
Potassium	Principal inorganic cation and cofactor for some enzymes.
Magnesium	Found in chlorophylls. Used as inorganic cation and cofactor for enzymatic reactions, including those involving ATP.
Manganese	Inorganic cofactor for enzymes; sometimes replacing Mg.
Calcium	Major constituent in endospores and cofactor for enzymes.
Iron	Found in cytochromes; cofactor for enzymes and is electron donor (F^{++}).
Cobalt, Copper, Zinc, Molybdenum	Inorganic constituents of enzymes.

Nutritional types of microorganisms

All organisms require a source of energy, hydrogen and electrons for growth to take place. There are only two sources of energy available to organisms i.e. the light energy during photosynthesis and the energy derived from oxidising organic or inorganic molecules (Prescott *et al.,* 1993). Microorganisms can be grouped into nutritional classes based on how they satisfy this requirement (Table 1.4).

Carbon, hydrogen and oxygen

Carbon, hydrogen and oxygen are some of the vital nutrients that microorganisms require. Autotrophs can utilise CO_2 as their sole source of energy. For autotrophs the most widely used carbon source is the carbon dioxide which maybe supplied as bicarbonate within the medium (Atlas, 1993). Most heterotrophs use organic nutrients as a source of both carbon and energy. Carbohydrates, such as glucose, or other organic compounds, such as acetate, various lipids, proteins and hydrocarbons are included in media as sources of carbon for heterotrophs. These carbon sources can also serve as a supply for energy (Atlas, 1993). Some bacteria are exceedingly fastidious and catabolize few carbon compounds. These type of requirements can be provided by a simple enrichment media. Alcohols, pentoses, hexoses, disaccharides, trissacharides, various types of glycols, glycosides, acids, and hydroxyacids occur in predominantly proteinaceous medium components of biological origin such as many peptones, meat extract, yeast autolysate, serum and whole blood (Mossel, 1971).

Table 1.4 Major nutritional types of microorganisms (Prescott *et al.*, 1993)

Nutritional types	Sources of energy, hydrogen, electrons and carbon
Autotrophs	Carbon dioxide(CO_2)
Heterotrophs	Reduced, preformed, organic molecules
Phototrophs	Light
Chemotrophs	Oxidation of organic or inorganic compounds
Lithotrophs	Reduced inorganic compounds
Organotrophs	Organic molecules
Photolithotrophic autotrophs	Light, inorganic hydrogen and CO_2
Photoorganotrophic autotrophs	Light, organic hydrogen and organic carbon source or CO_2
Chemolithotrophic autotrophs	Chemical energy, inorganic hydrogen and CO_2
Chemoorganotrophic heterotrophs	Chemical energy(organic), organic hydrogen and organic carbon source

Nitrogen, phosphorus and sulphur

Nitrogen, phosphorus and sulphur, however, are essential compounds of the cell materials of all organisms, in which they occur principally in their reduced form as amino, phosphate and sulphide groups, respectively. Other compounds, such as ammonium ions, nitrite ions, elemental sulphur and reduced iron may be used as the sources of energy for the cultivation of autotrophs (Atlas, 1993). Nitrogen is needed for the synthesis of amino acids, purines,

pyrimidines, some carbohydrates and lipids. Phosphorus is present in nucleic acids, phospholipids, ATP and some proteins. Phosphates and metals, such as magnesium and iron are also necessary components of microbiological culture media. Phosphates may also serve as buffers to maintain the pH of the medium within the growth tolerance limits of the microorganisms being cultivated (Atlas, 1993). Photolithotrophic autotrophs often grow and reproduce when minerals and sources of energy, carbon, nitrogen, phosphorus, and sulphur are supplied (Prescott *et al.,* 1993). Sulphur is needed for amino acid cysteine, biotin and thiamine synthesis (Prescott *et al.*, 1993). The nitrogen sources used in culture media belong to classes: (1) well-defined inorganic salts, amino-acids and peptides; (2) reasonably standardised enzymatic digest of animal protein or soya flour, malt or yeast; (3) only crudely standardizable biologicals such as blood, serum, plasma, egg yolk, potato extract, corn steep liquor or rumen fluid which may have an additional value by supplying other growth factors (Kennedy *et al.,* 1955; Habeeb, 1959; Mossel, 1971).

Growth factors

Growth factors are organic substances required by a specific organism in addition to its principal sources of carbon and energy. These are substances that microorganisms cannot synthesise from its principal nutrients. Vitamins, amino acids, purines and pyrimidines for nucleic acid synthesis are some of the growth factors needed by bacteria. They are added to culture media either as individual compounds or mixtures of those, or in the form of liver or yeast extract (Stokes *et al.,* 1944).Knowledge of the functions of some common vitamins in

microorganisms makes possible quantitative growth-response assays for a variety of substances (Table 1.5) (Prescott *et al.*, 1993). Some microorganisms (e.g., *Enterococcus faecalis*) require as many as eight different vitamins for growth. Other growth factors such as heme (from haemoglobin or cytochromes) is required by *Haemophilus influenzae*, while some mycoplasmas need cholesterol (Prescott *et al.*, 1993).

Table 1.5 Functions of some vitamins in microorganisms (Prescott *et al.*, 1993)

Vitamin	**Function**
Biotin	Carboxilation One-carbon metabolism
Cyanocqoalamin (B_{12})	Molecular rearrangements one-carbon groups-carries methyl groups
Folic acid	One-carbon metabolism
Pantothenic acid	Precursor of coenzyme A-carries acyl groups(pyruvate oxidation, fatty acid metabolism)
Pyridoxine (B_5)	Amino acid metabolism(transamination)
Niacin (nicotinic acid)	Precursor of NAD and NADP-carry electrons & hydrogen atoms
Riboflavin (B_2)	Precursor for FAD and FMN-carry electrons or hydrogen atoms
Thiamine (B_1)	Aldehyde group transfer (pyruvate decarboxilation, α-keto acid oxidation)

3 Microbiological culture media components

Natural or undefined media contain a mixture of the breakdown products of hydrolysed protein. A wide variety of protein sources may be used to provide protein hydrolysates, including animal, plant and single cell proteins.

Table 1.6 A wide variety of protein sources available for culture media components

Source	Product
Meat and liver (fresh, frozen, dried)	Peptones, meat extracts, protein extracts, Myosate, liver digest, Thiotone etc.
Fish (fresh, dried)	Protein extracts, Peptamine, Amber MPH, OM-HAP
Casein	Tryptone, Casamino-acids, Trypticase, Casitone, N-Z amine, N-Z case, HY case
Gelatin	Solidifying agents, Gelysate
Keratin (horn, hair, feathers)	Protein extracts
Ground nuts	Peptones and protein extracts
Soya protein	Pryptone, Soytone, Soya peptone, Amber HSP
Cottonseed	Peptones, protein extracts, Amber O.M., Proflo, Pharmamedia
Sunflower seed	Peptones and protein extracts
Microorganisms (yeast, algae, bacteria)	Peptones, protein and yeast extracts
Guar protein	Peptones and protein extracts
Blood meal	Peptones, protein extracts, Amber O.M. - BHY
Corn gluten	Peptones and protein extracts
Egg albumin	Peptones and protein extracts

The quality of the hydrolysate will be no better than that of the original protein. Therefore quality specifications should be observed in the selection of the protein. Yeast extract is as important in culture media as the hydrolysates and it is a peptone like substance derived from

Saccharomyces. The commonly used *Saccharomyces* are obtained as bakers' or brewers' yeast.

3.1 Protein hydrolysates

The main microbiological applications of peptones are for the production of antibiotics, enzymes, toxin-toxoids, vaccines, starter cultures, cortisone or its precursors, and a great variety of packaged microbiological culture media (Leifson, 1943; Difco, 1953; Kheshgi and Saunders, 1959; Bridson and Brecker, 1970; Bridson, 1978). Because of such diversified applications, the evaluation of peptones on the basis of their end use was discarded, especially in view of the fact that in many instances methods of production and yields of such end products are not generally known (Kheshgi and Saunders, 1959).

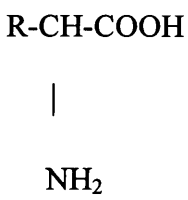
The nitrogen requirement of bacteria grown artificially was first met by the addition of natural occurring substances such as blood, urine and other body fluids to media. Naegeli was probably the first to use egg albumin, which he called "peptone" (Bridson and Brecker, 1970; Difco, 1953). However, it was later discovered that peptones obtained by partial digestion of proteins furnished organic nitrogen in a more readily available form. The term "peptone" as used in bacteriology signifies the product resulting from hydrolysis of protein substances (Leifson, 1943). As chemo-organotrophic bacteria must secrete proteolytic exoenzymes to hydrolyse protein before they can transfer the protein fraction back into the cell, it follows that all these organisms will utilise pre-hydrolysed protein more effectively (Bridson, 1978).

Since the substances used are not pure proteins, the resulting peptones may contain many other chemical substances, such as salts amines and complex organic substances, frequently of "accessory growth factors" (Leifson, 1943). Leifson (1943) also stated that in any given product, the amounts of these substances present will depend upon the nature of the substrate, the enzyme, the process of manufacture and whether or not substances have been added. He was later supported by Bridson (1978), by stating that the quality of the hydrolysates will be no better than that of the original protein. It follows therefore, that a quality specification should be observed in the selection of the protein to be used as original source of raw material.

Thus meat and offal (heart and liver) should be fresh from the abattoir or properly frozen and stored. Dried meat is commonly used in a form of meat meal. Temperatures below 50^0C and drying under negative pressure should be used to prevent overheating which leads to the Mailard reaction (caramelization of sugar) and inactivation of B-group vitamins (Bridson and Brecker, 1970).

3.1.1 Protein hydrolysis

Proteins are polymers of amino acids and are linked by peptide bonds, which have the following general structure:



Hydrolysis of proteins into peptides and amino acids can be accomplished by acid and enzymatic hydrolysis.

- **Acid hydrolysis**

Acid hydrolysis is carried out at high temperatures using mineral acids, e.g. hydrochloric and sulphuric acid. Acid hydrolysis yielded amino acids and hydrolysates must be neutralised before further processing. The neutralised hydrolysate is dicolourised, filtered and the filtrate evaporated in vacuum to a syrup containing approximately 85% total solids (Bridson and Brecker, 1970). The syrup may be stored and it is ultimately spray-dried to a powder. Acid hydrosylates require pH adjustment in odder to obtain a neutral product (Bridson, 1978).

- **Enzymatic hydrolysis**

This type of hydrolysis is carried out by a variety of proteolytic enzymes(proteases), such as pepsin, trypsin, and pancreatic or papain. The product of protein digestion by an enzyme is an ill-defined mixture of peptides of various sizes known as peptones (Bridson and Brecker, 1970). Although such peptones are undefined, controlling the process of digestion makes it possible to maximise the yields and to standardise the product so that growth characteristics are reproducible from batch to batch (Bridson and Brecker, 1970). It is also possible to determine the efficiency and speed of the enzymatic process during enzymatic hydrolysis (Habeeb and Shotton, 1956).

(a) Pancreatic (trypsin, chemotrypsin) enzymes are generally prepared from pig pancreas. Trypsin is active below pH 6.0 and chymotrypsin is stable below pH 7.0 (Bridson and Brecker, 1970). Both enzymes are destroyed automatically above pH values. The final products of pancreatic digestion are amino acids, peptides, and chemical peptones (Leifson, 1943).

(b) Pepsin is manufactured from mucosa of the stomach. The optimum pH pepsin activity is about pH 1.8. This enzyme does not hydrolyse esters or amides of α-amino acids (Bridson and Brecker, 1970). The final product of peptic digestion are mainly protease's and chemical peptones and only a relatively small amount of amino-acids are liberated (Leifson, 1943).

(c) Papain is the most widely used enzyme for proteolysis. It is obtained by tapping green, unripe fruit. After collecting and drying, the resulting latex exudate contains the enzyme. Papain is activated by reducing agents and inactivated by oxidising agents (Bridson, 1978). Papain is stable at pH 5.0, but becomes unstable below pH 3.0 and above pH 11.0 (Bridson and Brecker, 1970). It is also stable at temperatures around 70^0C and has a broad specificity.

(d) Ficin is obtained from the latex of tropical fig trees of the genus *Ficus* and extracted by solvent precipitation. This enzyme resembles Papain in many respects. Ficin has a different optimal pH value for hydrolysing protein depending on the substrate e.g. casein, either pH 6.7 or 9.5, gelatin, pH 5.0 (Whitaker, 1957). Ficin is relatively thermostable showing optimum activity at 62.5^0C, but becomes completely inactivated at 80^0C (Bridson, 1978).

(e) Bromelain is found in the pineapple plant *Ananas comosus.* This enzyme shows close similarity to Papain and Ficin, but it is not heat stable and that is why it loses activity at 55 ^{0}C. Its optimum pH is in the range pH 6-8.

(f) Microbial protease's are generally extracellular enzymes and are classified as acid, neutral and alkaline protease's. They are active in the pH range 2 to 5 and are stable in the pH range 2 to 6 (Bridson, 1978).

Literature on the techniques used for the preparation and properties of culture media components has never been published, because of the secrecy involved. Experienced workers in the field stated that the final choice of preparation technique should be based on simplicity, reproducibility, and nutritive qualities of the peptone (Leifson, 1943).

3.2 Yeast-extract

Debittered brewer's yeast and primary grown species of *Saccharomyces, Candida*, and *Kluyveromyces* are the raw materials for the manufacture of yeast autolysates, invertase, lactase, ribonucleic acids, isolated proteins and yeast glycan (Vosti and Joslyn, 1954b; Peppler, 1982). These products manufactured from yeast's can be used in food, beverage, pharmaceutical, and fermentation industries. There are basically two different methods of preparing yeast extracts. Autolysis is a process of self-digestion in which the cellular enzymes of viable yeast cells are induced by temperature to ferment some of the glycogen and solubulize a portion of yeast proteins on the cell walls, while hydrolysis is conducted by controlled cooking of yeast in a strong acid solution (Peppler, 1982).

Brewers' yeast are commonly obtained as a suspension of 25% w/v solids and may be stored at low temperatures, to retain viability, for up to two weeks (Bridson and Brecker, 1970). The yeast may be filtered through a fine screen and then washed to remove the beer. It is important that the yeast cells should be kept alive to prevent the loss of cell constituents during washing and filtration. Bakers yeast do not require de-battering and can be obtained

either in a slurry, delivered by bankers, or as compressed yeast in blocks. The use of fermentor, grown bakers yeast ensures a more reproducible source of yeast protein free from hop resins which are inhibitory to the growth of most bacteria (Bridson, 1978).

Yeast extract consist of a concentrated solution of hydrolysed yeast protein. It is produced by the autolytic reaction of the proteolytic enzymes of the cell on the yeast protein. The extract is basically a mixture of amino-acids and peptides, water-soluble vitamins, and carbohydrates. The carbohydrates of yeast are mainly glycogen and trehalose. These substances undergo enzymatic hydrolysis to glucose during the extraction process. Most of the previous investigations of yeast autolysis were carried out with poorly described strains of *Saccharomyces cerevisiae* whose conditions of growth, stage of growth, and previous storage were largely unknown (Vosti and Joslyn, 1954a). It is therefore advisable to know the type, source and the above mentioned factors described by Vosti and Joslyn (1954a) of the yeast. Yeast extract should have a meaty, souplike flavour, be free from bitter, burnt acid or other off-flavours, and dissolve in hot water to give a clear solution.

3.2.1 Yeast Autolysis

The methods of autolysis can be varied, to suit the manufacturers schedule and to maximise the yields of the autolysate. The principle of autolysis is based on inducing the enzymes within the viable yeast cells by slowly raising the temperature and subsequently solubilising the yeast cell walls (Peppler, 1982). The yeast is held in autolysing vessels and continuously

stirred. Autolysis of the cells is initiated by slowly raising the temperature to (30^0C-45^0C) and maintaining this temperature for a few hours, before raising to 75^0C.

Extraction is carried out by plasmolysis using 5% to 10% sodium chloride solution, that causes cell constituents to diffuse out through the cell wall or by centrifugation at low speed (Peppler, 1982). The extract is purified by filtration or filter pressing. Some extracts produce large quantities of free tyrosine which must be filtered off to obtain a bright filtrate (Bridson and Brecker, 1970). Powder yeast extract may be obtained from spray-dried liquor and self-dried in vacuum ovens (Fig 1.4). Other methods of autolysis are based on lysing the spheroplasts of the yeast cells by osmotic shock or detergents (Hutchison and Hartwell, 1967; Hinnen, *et al.,* 1978). These methods are expensive and are suitable for molecular biology studies and not yeast extract manufacture.

Cheaper and simpler methods are sometimes effective. Autoclaving of algae and desiccation of bacterial and yeast cells damage the cell walls to a varying extent and may increase nutritive value (Peppler, 1982). The damaging of yeast cell walls by autoclaving is effective, while purification and drying are performed using methods outlined in fig 1.4.

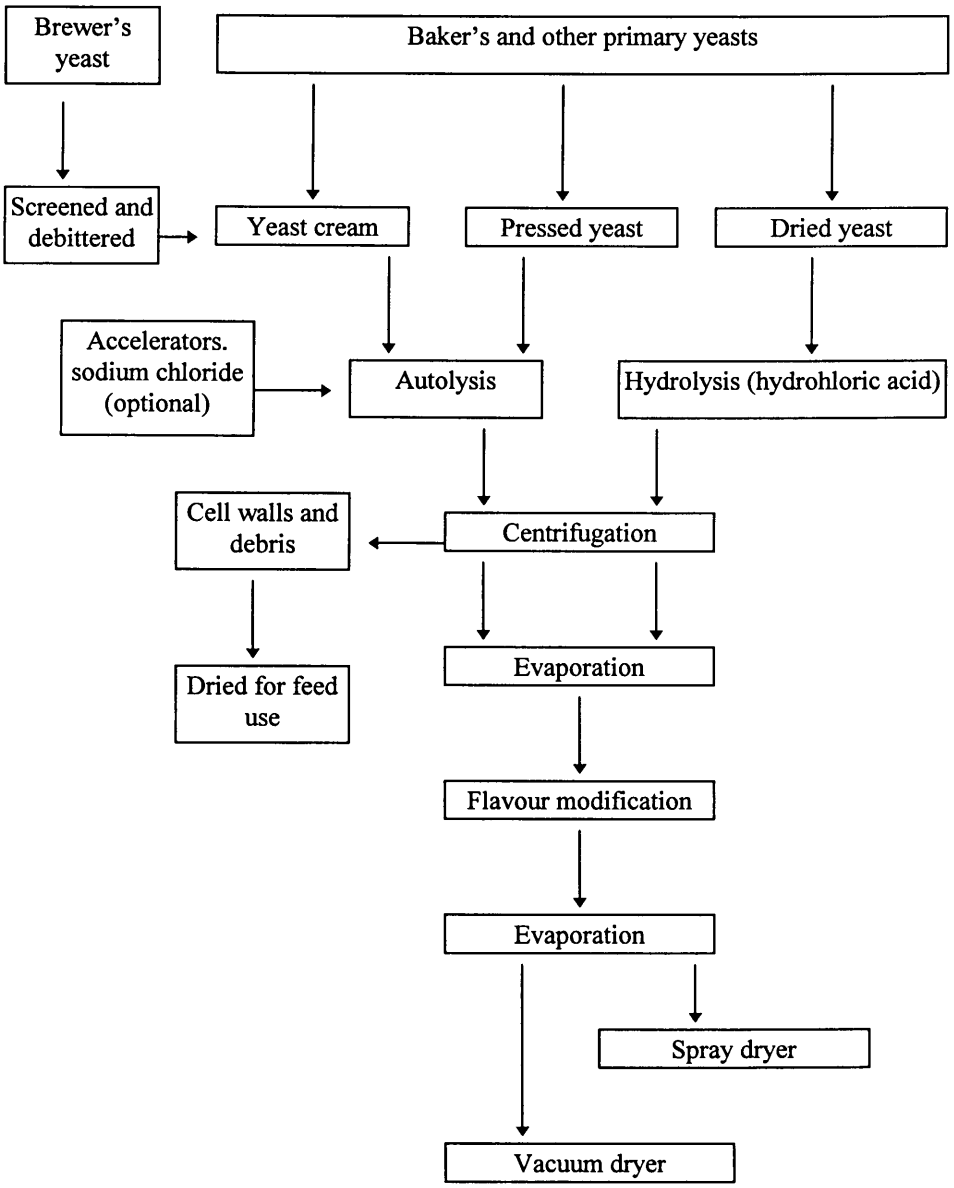


Fig 1.4 Process routes in the manufacture of yeast extracts (Peppler, 1982).

3.3 Factors affecting the quality of culture media components

- **Moisture content**

Moisture content is important because at 5% w/w microbial growth could take place during storage and will decrease the quality of a peptone (Costin, 1982). A sensitive indicator of moisture rising to critical level is the clumping together of powder components and eventual solidification of dehydrated culture media. Darkening and pH changes are other indicators of high moisture content. Darkening of colour and change in pH are indications of peptone degradation (Bridson and Brecker, 1970). It is therefore important to realise that proper storage is required. Storage conditions of components are similar to the conditions and precautions used to store finished medium.

- **Ash, salt (NaCl), phosphates and trace metals**

The ash content of peptones is composed of sodium chloride, phosphates, sulphates, silicates and metals oxides. The salt content is recognised either as sodium or potassium salts. Ash rises during pH changes in processing and is highest in acid-hydrolysed protein, unless special low salt processes are used. Raw materials hydrolysed by acids gain high sodium chloride contents during neutralisation of the pH (Bridson and Brecker, 1970). Phosphates do not only act as a buffer, but play an important part in bacterial metabolism (Bridson, 1978). Highly buffered peptones are not used in carbohydrate-indicator media, because small changes in pH

caused by fermentation might be masked. Trace metals including lead, silver, copper, and zinc show toxic effects in quiet small amounts (Bridson, 1978; Reviere, 1977).

- **Fermentable carbohydrates, lipids and vitamins**

Plant peptones and yeast extracts are usually high in carbohydrate content. The peptones used in carbohydrate fermentation should not contain fermentable carbohydrates (Reviere, 1977; Bridson, 1978). When lipids are present, the peptone becomes opalescent because they are insoluble in water. Vitamin content is usually stable. However, organisms may need additional vitamins which are then added in excess to the culture media (Bridson and Brecker, 1970).

4 Quality control of microbiological culture media and culture media components

In recent years it has become abundantly clear that quality is one of the most powerful techniques a company can use to ensure survival of its business in a competitive market place. Research in the United States and Europe has proved conclusively that the higher company's product quality (as perceived by its customers) the higher also its marked share (Cloete, 1985). In this study, the definitions for quality assurance and quality control, as defined by Costin (1982) at the IUMS in 1979 will apply. Quality assurance is defined as those procedures which lead to materials and techniques performing with desired and constant properties, while quality control is defined as the analytical steps necessary to demonstrate

that a certain material or procedure meets the desired requirements. Snell (1992) also clears the confusion about product evaluation and quality control: the former measures what a product is capable of and defines its operating characteristics, the latter simply shows that batches of the product are meeting pre-defined standards.

4.1 Quality control of culture media and culture media components

In a modern industrialised society the microbiologist has a very important role to play. Most manufacturing industries rely upon both qualitative and quantitative microbiological analysis to ensure that the raw materials used meet certain specifications, and also to check the quality of the whole manufacturing procedure and the final product. Since the quality control of culture media and culture media components are similar, it is therefore not necessary to discuss them separately. Media should be made to a recognised formula, which may be specified by regulatory or advisory bodies, or experts in the particular field, or may be the results of long experience in the user's laboratory. No amount of testing on the final product can make good the deficiencies in the original preparation and since the cost of preparing an unsatisfactory batch of medium is the same as that of preparing a good batch, care in the choice of formula and ingredients is essential. This is supported by Babu *et al.* (1978), who evaluated twenty-three blood culture media and found that the recovery rate of microorganisms depended not only upon the type of medium, but also upon the manufacture of the type of blood culture media components. Critical ingredients (e.g. bile salts) should be pre-tested in a pilot batch to ensure compatibility with other components.

It is often difficult to obtain a full specification for raw materials. Alterations to an apparently minor specification of a single raw ingredient can cause quite dramatic problems for the user and can render a medium completely useless (Kendall, 1982; Atlas, 1993). Since it has been noticed that a drastic change of microbial culture media components may lead to disastrous situations, it the therefore important to, (1) start looking for alternative sources of these microbiological culture media to gradually replace sources that might be exhausted with time, (2) bring these new sources into being, without affecting the culture media itself and the users results. Knowledge of constituent amino acids and peptides is desirable and is a necessary preliminary to obtaining uniformity in bacteriological culture media (Habeeb and Shortton, 1959).

4.1.1 Methods of evaluation of culture media and culture media components

The methods of evaluation includes the following tests : recording and sampling of the batch; physical testing (solubility, clarity, colour, compatibility with other ingredients, gel strength, diffusion rate etc.); chemical testing; pH; presence and level of organic and inorganic contaminants; and biological testing (Costin, 1982 and Snell, 1992). It is of paramount importance to follow three principles outlined by Costin (1982) at the International Union of Microbiological Societies (IUMS) in 1979, in odder to make a minimal quality control program cost-effective, rapid and practical. The three principles were described as follows:

The sample must be representative

An adequate sample is one which is representative of a well identified and delineated batch. The sample should be prepared under sterile conditions.

The examination should start with the simplest eliminatory test

These are generally physical criteria e.g., if a given peptone sample is not clearly soluble, there is no reason to proceed to further complicated chemical and biological tests, because the batch will be rejected for this technical reason.

Biological tests should have priority over chemical tests

If a biological test gives aberrant results although physical and chemical tests are satisfactory, the lot must be rejected. If the biological test is passed but certain chemical tests fail, acceptance of the lot may still be possible.

4.1.1.1 Physical and chemical tests

Colour and odour of solutions of prepared media/components are checked before and after sterilisation, because excessive heating produces an unwanted increase in acidity. Gel strength of agar is affected by sterilisation conditions, especially pH. In general physical and

chemical tests should rapidly detect errors, preferably before distribution and sterilisation. Faulty batches may then be discarded without the need to await results of culture tests. Instruments such as the Gelometer and the LFRA Texture Analyser (C.S. Stevens and son Ltd. U.K) provide a good measure of gel strength of the medium. Other factors besides gel strength, which affect results, include storage temperature of agar before testing, depth of agar, shape of container and conformation of the probe. There is at present no standard condition for this test and it is doubtful if a need exists outside media manufactures for such elaborate quality control methods. A simple piece of equipment which measures the depth of penetration of a weight when placed on an agar surface is the van der Bijl penetrometer.

4.1.1.2 Biological tests

Since culture media are designed to grow microorganisms, the ultimate test of their quality must be an assessment of their suitability for this purpose. For the functioning of the ecosystem, consisting of the medium and its inoculum, clearly the most important parameters are those connected with the medium itself, in other words its compositional characteristics (Mossel, 1971). Besides the quality of its raw material, the performance of a culture media depends first on the so-called "intrinsic" attributes (available nutrients, redox potential, acidity, water activity, initial pH and its change depending on the buffer capacity, and antimicrobial substances fortuitously formed e.g. during excessive heating). In addition "extrinsic" factors during incubation (e.g. temperature, gaseous atmosphere) and "implicit" factors (vitality of the microorganisms, synergism, and antagonism) can influence the

performance of a given medium (Mossel *et al.*, 1980). As many as possible of these variables should be kept under control.

A very important consideration is the selection of appropriate microorganisms for checking the performance of culture media. In many cases the media need only be inoculated with pure cultures of one species or genus. It should be mentioned, however, that microbial populations may mutually influence each other's further development in a synergistic or antagonistic way (Atlas and Bartha, 1987b). Therefore it seems to be more realistic to use mixed cultures prepared from stock cultures.

In the ecological sense, inoculation is one of the most essential facets of the use of culture medium, because part of its use introduces, in principle, both the organisms whose isolation or enumeration is the aim of the procedure and contaminants (Mossel, 1971). The evaluation of media to determine the size of inoculum required for growth was not reported, until Brinkley and Huber (1978) recognised that the inoculum size was a significant factor in susceptibility testing. Regardless of the type of microorganism used, the amount of inoculum should also be standardised (Terplan and Zaadhof, 1982).

The performance of the media can be determined by adequate statistical evaluation of the results, e.g. logarithmic transformation and calculation of the differences resulting in different batches. In this way, more reliable information on the performance of the media can be obtained than using only stock cultures. Performance tests, which should be done with each

new batch or lot of medium, can confirm that the medium tested is satisfactory and specifically does what it is designed to do. Moreover undue variations between successive batches can be detected.

Methods have been devised to asses the performance of culture media/ components. The classic methods rely on inoculating suitable pure cultures onto, or in the media under examination. In the case of solid media colony counts are mostly made; for liquid media, dilution to extinction leading to "titre's" is practised (Mossel, 1971; Mossel *et al.*, 1980). The other method used to measure growth of test organisms is optical density (OD), (Kheshgi and Saunders, 1959). When monitoring non-selective media, test cultures should include very robust and rather fastidious organisms. In examining selective media, a spectrum of organisms of varying robustness which are expected to grow on the media should be included (Mossel, 1971; Mossel *et al.*, 1980).

4.2 Preparation of culture media and culture media components

Because most ingredients which enter into composition of dehydrated culture media are dry materials, powders and granules, the colour, odour, particle size and water content of the powders or granules before preparation should be within an acceptable range (Costin, 1982; Snell, 1992). The preparation of culture media entails the processes involved from when the media is in powder or granule form to when the media is finished.

4.2.1 Water

Although culture media provide considerable protection from toxic effects of water, only distilled or deionized water should be used. Additionally the water must be free from substances that might inhibit or favour microbial growth (traces of dissolved metals, residual chlorine or chloramine in water prepared from chlorinated water supplies, bacterial metabolites in deionized water). The dehydrated media from commercial suppliers should be soaked in the water for about 15 min to reduce the amount of heating necessary to obtain complete solution and hence to prevent unnecessary decomposition of ingredients. Whereas agar media must be heated to boiling point for complete solution, liquid media are readily soluble in water at room temperature (Terplan and Zaadhof, 1982).

4.2.2 pH

The pH of a prepared medium/component should be measured and if necessary, adjusted, though preparation of media with dehydrated material will usually require no further adjustment. Generally, thermal sterilisation will change the pH of the medium/component by lowering it (Mossel, 1971). The pH of the medium/component is extremely important for the growth of microorganisms, the majority preferring media/components which are approximately neutral, although some require distinctly acid medium. The choice of the acids or bases used for adjustment of the pH of culture media/components is important, since some possess antimicrobial properties which must be considered. Calcium carbonate is not

preferred as a base because it is opaque under alkaline conditions when no chelating agent is present. When acid or chelating agent are introduced, $CaCO_3$ dissolves and the medium/ component becomes clear (Keston and Rosenberg, 1967). Lactic acid and acetic acid posses intrinsic antimicrobial properties, whereas citric acid and tartaric acid show no other inhibitory effects than those resulting from their ability to reduce the pH of the medium/component (Mossel, 1971).

4.2.3 Sterilisation

As the medium on which the desired microorganisms grow must be free of all other organisms, it must be sterilised by autoclaving. Routine checks on the efficiency of the autoclave by use of the filter-paper strips impregnated with spores of vegetative microorganisms or by other acceptable sterility tests are recommended. Various times and temperatures are used, but the generally recommended treatment is at 121^0C for 15 min. It should be borne in mind that culture media/components are always affected to a greater or lesser extend by any heat treatment, e.g., resulting in a breakdown of nutrient constituent. No medium should be subjected to more heating than is necessary. The actual duration of such a heating will depend on the size of the load to be sterilised and the size and nature of the individual containers, as well as the relative amount of steam available.

Some ingredients (e.g. heat-labile carbohydrates) must be prepared apart from the base medium to avoid undesirable degradation or reactions which would otherwise take place

during normal autoclaving. These ingredients should be sterilised separately e.g. by filtration or by autoclaving separately and then added with aseptic precautions (e.g., laminar-flow bench) to the cooled sterilised basal medium. Some media must on no account be autoclaved because their performance would be decreased by doing so. Examples include violet red bile agar and bismuth sulphite agar. The pH of the medium, depending on the formulation, may change after autoclaving and therefore must be checked, preferably with a temperature compensating electrode and adjusted, if necessary.

4.3 Precautions to be taken when using culture media

Several points have to be considered when using culture media. Firstly, dehydrated media should be dated when received in the laboratory and when opened or first used. The media should be stored in tightly capped containers (to avoid any absorption of water, oxidation or contamination of the powder, which may lower the efficiency of the medium) (Martin, 1991). Storage should be at temperatures below 25^0C or, if appropriate, refrigerated. The expiry date provided by the manufacturer must be respected. If the media becomes caked or discoloured before expiry date, it should be discarded. After accurately weighing the desired quantity of the dehydrated media, it must be rehydrated with the correct volumes of water.

A record sheet should be kept for each batch of medium prepared on which should be noted the source, batch number or weight or volume of ingredient used, together with details of

sterilisation and the person preparing the medium. All the information requested on this form should be at hand during the preparation of the medium.

4.4 Storage of finished medium

If the finished media are not used immediately, it can be kept for a limited period (some media even longer than one month if they are stored in sealed plastic bags). Generally media are stored in the dark at ca. 4^0C. Some media e.g. thioglycollate-medium is better kept at room temperature. Storage will inevitably lead to changes and these have to be kept under check. Media showing signs of water loss, discoloration, microbial growth or any colour change, should be discarded at once. If solid media poured into Petri dishes for use in surface culturing are not used within few days, there is a risk that it will dry out at the surface. Freshly prepared media have a water activity value of approximately 0.99 (Mossel, 1971). In refrigerated rooms, the relative humidity will not frequently exceed 0.85, whereas in stores at room temperature this value is mostly 0.60 or lower (Mossel, 1971). Miles-Misra methods and ecographic techniques have been devised to evaluate the shelf life of stored media, and can be used to assure the quality of broth medium (Mossel, 1971; Brinkley and Huber, 1978; Mossel *et al.,* 1980).

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CHAPTER 3

PEPTONE AND YEAST EXTRACT MANUFACTURE FOR USE IN MICROBIOLOGICAL CULTURE MEDIA

ABSTRACT

A peptone and/or yeast extract which sufficiently supports growth of bacteria will be suitable for use in microbiological culture media. The quality of culture media components is directly dependent on the quality of its original raw material and the method of its manufacture. Autoclaving was used as a cost-effective method for the lysing of yeast cells during the manufacture of yeast extract. The performance and quality of locally manufactured peptone and yeast extract was evaluated, by inoculating suitable pure cultures into solutions of the raw materials. The growth of the test organisms was photometrically measured. The growth-supporting ability of the peptones and yeast extract was comparable to the commercially available products used as the standard.

1 INTRODUCTION

Culture media played an important role in the development of microbiology, viz. in proving the spontaneous generation wrong via experiments conducted by Louis Pasteur during the year (1860-1861) and supplemented a few years later by those of John Tyndal and Schroeder (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.,* 1978; Ware and Hawker, 1979). Components with solidifying characteristics were developed during Koch's time, when Bredfeld added gelatin to infusions and allowed the infusion-gelatin to cool and solidify. It was Fanny Hesse who suggested agar as a solidifying agent, thus replacing gelatin, because gelatin liquefies readily when warmed above 28^0C, while agar liquefies only when heated to *ca.* 100^0C and remains liquid until cooled to *ca.* 42^0C (Burdon and Williams, 1969; Carpenter, 1977; Nester *et al.,* 1978; Ware and Hawker, 1979; Atlas, 1993; Prescott *et al.,* 1993).

The production of commercial culture media is dated to as early as *ca.* 1915. During those years, commercial culture media was produced using raw materials from unrevealed sources. The policy of manufacturers to maintain trade secrecy as to the processes of manufacture and the nature of basic substrates was maintained (Leifson, 1943). Even today, the policy of culture media manufacturers to maintain trade secrecy is still maintained. Hence, no recent publications on the subject exist. For the sake of quality and consistency of culture media components, culture media manufacturers should at least identify a culture media component by its source, the type of preparation and purification, the identification reactions and the principal physical, chemical and biological characteristics (Costin, 1982).

The raw materials that micro-organisms demand for growth vary widely from the few simple carbon, nitrogen and inorganic compounds required by oligotrophic organisms, to the very specific growth factors (glutamine, cocarboxylase, etc.) demanded by some exacting strains (Bridson, 1978). Yeast extracts and peptones contain most common nutrients that microorganisms demand, including amino-acids, peptides, vitamins and carbohydrates (Habeeb and Shotton, 1956; Habeeb, 1959; Bridson and Brecker, 1970; Bridson, 1978).

Brewer's and baker's yeast are used as substrates for the manufacture of yeast extract (Vosti and Joslyn, 1954a; Vosti and Joslyn, 1954b; Bridson and Brecker, 1970; Peppler, 1982). Yeast extract consists of a concentrated solution of hydrolysed yeast protein. The carbohydrates of yeast are mainly glycogen and trehalose. These substances undergo enzymatic hydrolysis to glucose during the extraction process (Vosti and Joslyn, 1954a; Vosti and Joslyn, 1954b; Bridson and Brecker, 1970; Peppler, 1982). Yeast extract is produced by:

* physically lysing the yeast cells,
* enzymatic digestion of the yeast cell wall,
* plasmolysis using a strong sodium chloride solution and
* autolysis using the autolytic reaction of the proteolytic enzymes of the cell on the yeast protein.

A peptone is the product resulting from the hydrolysis of a protein substrate (Leifson, 1943). Fresh meat, either as carcass muscle or offal (liver and heart) is widely used as a substrate for peptones (Bridson and Brecker, 1970). Hydrolysis of proteins into peptides, amino-acids, salts, amines and vitamins can be accomplished by:

* acid hydrolysis and/or
* enzymatic hydrolysis.

Since the substrates used for peptone and yeast extract manufacturing are not pure proteins, the end-products may contain many chemical substances that support or inhibit microbial growth (Bridson, 1978). It is therefore necessary to evaluate culture media components primarily for their bacterial growth supporting ability. Culture media components can be evaluated by physical testing (solubility, clarity, colour); chemical testing (pH) and biological testing (presence of microbiological contaminants and growth-promoting properties). Keshgi and Saunders (1959) developed a classical method to compare microbiological peptones based on inoculating suitable pure cultures into solutions of the raw materials and measuring the growth of the test organisms as optical density. This method is still useful in determining the growth-promoting abilities of culture media components.

The aim of this study was to devise a method for the manufacturing of yeast extract and to evaluate both the locally produced peptone and yeast extract for import replacement. A commercial peptone and yeast extract was used as a standard. The practical approach to measuring the criteria of peptone quality is to determine its ability to support the growth of certain strains of bacteria, that are known to be demanding in their nutritional requirements (Bridson, 1978). The other aspect is to use the simplest eliminatory method present to examine a peptone, before proceeding to the time consuming microbiological examination (Bridson and Brecker, 1970; Costin, 1982). This approach was followed during this study.

2 MATERIALS AND METHODS

2.1 Raw materials used for peptone and yeast extract manufacture

Three peptone paste samples were obtained from a local manufacturer (Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa) and labelled T1, T2 and T5. A commercially available peptone powder, labelled PAB0082, was obtained from Biolab (11 Fedlife Park, Tonetti street, Midrand, South Africa) and used as a standard. A commercially available yeast extract powder, labelled PAB0133, was obtained from Biolab and used as a standard for comparison with the locally manufactured yeast extract powder (MPYE).

2.2 Method of yeast extract manufacture

A 20% (m/v) of spent yeast, obtained from the South African Breweries(SAB), solution was prepared by suspending 20g of the spent yeast into 100 ml distilled water. Yeast extract was manufactured following this procedure (Fig 3.1):

* Autolysis of the yeast cells was achieved by autoclaving 20% (m/v) of spent yeast (SAB) at 121°C for 30 min.
* The product was refrigerated at temperatures below 7°C for 24h.
* The product was centrifuged at 4000 rpm for 20 min at 20°C, the supernatant was decanted, then bowl centrifuged at 1200 rpm for 20 min at 20°C to remove the remaining solids.
* The supernatant was spray-dried with an inlet temperature of 180°C and an outlet temperature of *ca.* 102°C, to yield MPYE.

The above mentioned method was preferred to other methods, because no sophisticated and expensive equipment was needed during the lysing process. This method yielded *ca.* 24% (m/v) yeast extract (MPYE) based on the dry weight of the spent yeast cells.

2.3 Physical and chemical evaluation of yeast extract and peptone

2.3.1 Yeast extract

The colour and odour of MPYE were observed. Yeast extract solutions were aseptically prepared by dissolving 1.0 g of the yeast extracts into 100 ml of 0.5% (m/v) sodium chloride (NaCl) solution. The pH of the solutions was measured and adjusted to 7.5 ± 0.1 at 20 ± 1.0 °C according to the standard. The pH was adjusted with 1.0 M sulphuric acid and 1.0 M sodium hydroxide and measured, using a Beckman 34 pH meter. The solutions were then autoclaved at 121 ± 1.0 °C for 15 ± 2.0 min. Solubility and colour of MPYE in solution were examined after dissolving 0.5 g, 1.0 g and 2.0 g into 100 ml of distilled water, and autoclaving at 121 ± 1.0 °C for 15 ± 2.0 min.

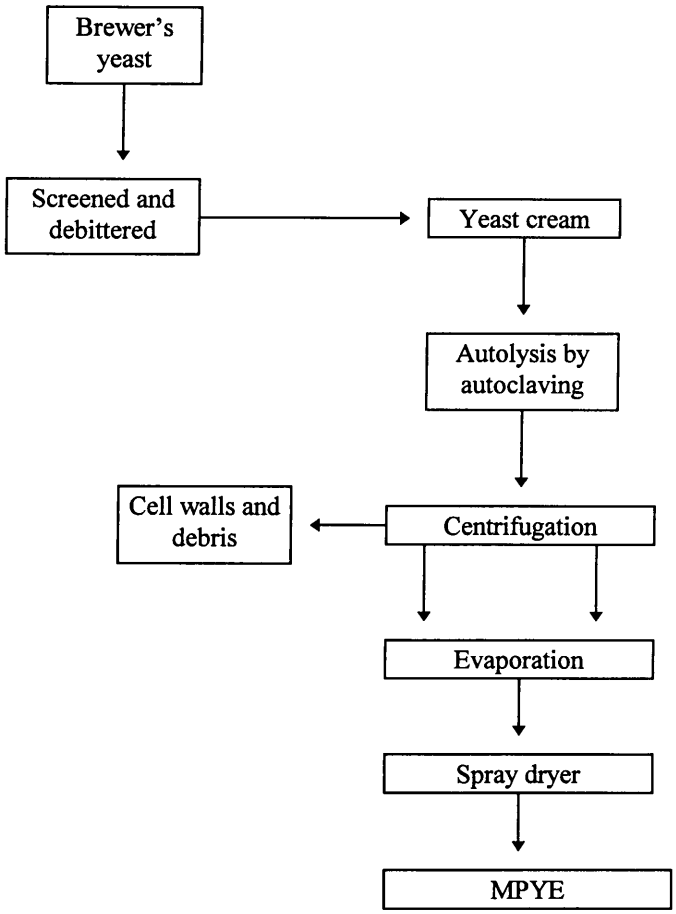

Fig 3.1 Process routes in the manufacture of MPYE.

2.3.2 Peptones

A comparison of the colour and odour of T1, T2 and T5 was not possible because these products were supplied as pastes compared to the standard which was supplied as a powder. The International Union of Microbiological Sciences (IUMS) specified that 1.0g peptone powder or granules (96% solids) should be suspended in 100 ml of 0.5% (m/v) sodium chloride solution for their analysis as microbiological culture media components (Costin 1982). Since the experimental peptones were supplied as pastes (80% solids), the dry mass had to be determined before suspending into the 0.5% (m/v) sodium chloride solution. The dry mass of

the pastes was determined by drying the pastes at 55^0C for 72h and weighing. The mass of the pastes in *g* was then related to the initial paste in *ml*. The calculated ratios were as follows: 2.0 ml of T2 and T5 was equivalent to 1.0 g, while 3.0 ml of T1 was equivalent to 1.0 g. The peptone solutions were aseptically prepared by dissolving 1.0 g of the peptones and yeast extracts into 100 ml of 0.5% (m/v) sodium chloride (NaCl) solution. The pH of the solutions was measured and adjusted to 7.5 ± 0.1 at 20 ± 1.0 °C to be the same as that of the standard. The pH was adjusted with 1.0 M sulphuric acid and 1.0 M sodium hydroxide and measured, using a Beckman 34 pH meter. The solutions were then autoclaved at 121 ± 1.0 °C for 15 ± 2.0 min. Solubility and colour of both the peptone solutions were examined by dissolving 0.5 g, 1.0 g and 2.0 g into 100 ml of distilled water, then autoclaved at 121 ± 1.0 °C for 15 ± 2.0 min.

2.4 Microbiological examination

2.4.1 Microbial content (Bioburden)

The solutions of the peptone and yeast extract were aseptically prepared without being autoclaved, and incubated at 37°C. After 24h the solutions were serially diluted and then spread plated onto Nutrient agar plates. Autoclaved peptone and yeast extract solutions were inoculated with sterile Ringer's solution and *Escherichia coli* pure cultures. These suspensions were used as negative and positive controls respectively. Total plate counts were made after 24h of incubation at 37°C.

2.4.2 Growth-promoting properties of yeast extract and peptone

Fresh bacterial cultures were prepared by growing *Escherichia coli, Pseudomonas aeruginosa, Shigella sonnei, Streptococcus faecalis* and *Staphylococcus aureus,* obtained from the Department of Microbiology and Plant Pathology culture collection, at the University of Pretoria and *Streptococcus lactis* obtained from the South African Bureau of Standards on Nutrient agar for 24h at 37°C. Suspensions were prepared by inoculating 3 to 4 colonies of each bacterial culture into 10 ml quarter strength Ringer's solution. Suspensions were serially diluted from 10^{-1} to 10^{-9} dilution. 0.01 ml suspension of the dilution similar to opacity of the 0.5 McFarland standard (Biolab) were inoculated into 100 ml of a 1.0% (m/v) peptone and yeast extract solution. The solutions were inoculated in triplicate, incubated in the side armed flasks at 37°C and shake incubated at 60 rpm (Fig 3.2). The side arm of the flasks fit into the cuvette slot of a Spectronic 20 spectrophotometer (Milton Roy Company) and was used to measure absorbance at 546 nm at 30 min intervals for ± 6h. Peptone and yeast extract inoculated with sterile Ringer's solution were used as negative controls.

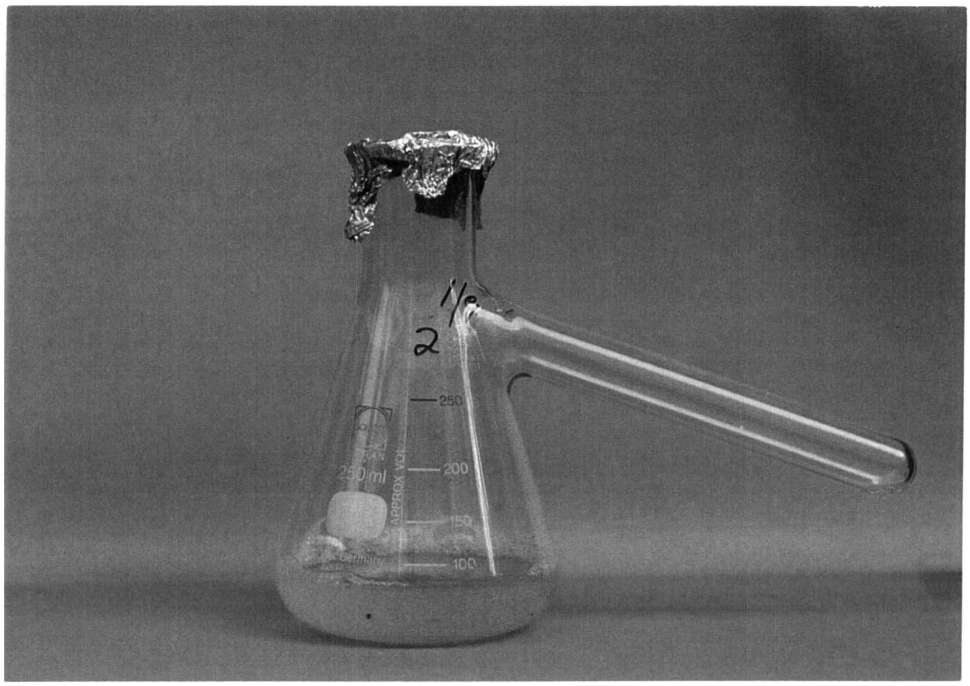

Fig 3.2 Flasks used to determine growth promoting properties of the peptones and yeast extract using spectrophotometry.

3 RESULTS

3.1 Physical and chemical analysis of the experimental yeast extract and peptone

3.1.1 Yeast extract

The colour of MPYE in powder form was light-brown and similar to the colour of the standard (Table 3.1). The odour was typical for that of yeast extract. 1.0% (m/v) MPYE was completely soluble in 0.5% (m/v) sodium chloride solution and had the same colour as the standard (Table 3.1).

Table 3.1 Physical observations of yeast extract after preparation

Characteristic	Specific component	
	Standard	MPYE*
Solubility	Completely soluble	Completely soluble
Colour	Clear, straw	Clear, straw

MPYE* - the experimental yeast extract.

3.1.2 Peptones

1.0% (m/v) of all the peptones (T1, T2, T5 and the standard) was completely soluble in 0.5% (m/v) sodium chloride solution, except T5 which became clear after filtration through a 125 mm Whatman filter paper (Table 3.2). All the peptones had a colour similar to that of the standard, excepting for T1 which had a pale straw colour (Table 3.2).

Table 3.2 Physical observation of peptones after preparation

Characteristic	Peptones			
	Standard	**T1 peptone**	**T2 peptone**	**T5 peptone**
Solubility	Completely soluble	Completely soluble	Completely soluble	Filtered*
Colour	Clear, straw	Clear, pale straw	Clear, straw	Clear, straw

Filtered*-Filtered through a 125 mm Whatman filter paper in odder to remove insoluble material.

3.2 Microbiological examination of the experimental yeast extract and peptones

3.2.1 Microbial content (Bioburden)

Total plate counts for T1, T2 and T5 were 4.4 x 10^3, 3.9 x 10^3 and 4.0 x 10^3 cfu/g respectively. This was less than the 4.7 x 10^3 cfu/g for the standard (PAB0082). The total plate counts for MPYE was 4 x 10^1 cfu/g which was lower than the 4 x 10^2 cfu/g for the standard (PAB0133).

3.2.2 Growth-promoting properties of yeast extract

Escherichia coli reached a maximum absorbance value of 1.8 in 240 min when cultured in MPYE and a maximum absorbance reading of 1.5 after 210 min when grown in the standard (PAB0133) (Fig 3.3). When cultured in the standard, *Staphylococcus aureus* reached a maximum absorbance reading of 1.2 in 270 min, while in MPYE growth was supported to give a maximum absorbance reading of *ca.* 1.0 in 180 min (Fig 3.4). *Streptococcus lactis* had its growth sustained in PAB0133 for 300+ min at an absorbance reading of *ca.* 0.27, which was also the absorbance reading at 0 min. The absorbance reading of *Streptococcus lactis* when grown in MPYE fluctuated between absorbance values of 0.25 and 0.15 for 300+ min (Fig 3.5).

3.2.3 Growth-promoting properties peptones

The growth of all bacteria cultures grown in the experimental peptones were comparable to those of their respective standards. Some bacterial growth readings approached the maximum absorbance value after 240 min, whereas others reached their maximum absorbance readings

after 360 min. On an average basis, T2 had the highest final absorbance reading compared to the other peptones, including the standard (Fig 3.6-3.9). T1 and T5 supported growth of all the test organisms, alternating, depending on the specific bacteria, either below or above the standard.

In T2 *Streptococcus faecalis* reached a maximum absorbance reading of 0.85 in 240 min, whereas in T5 it reached its maximum absorbance reading of 0.55 in 330 min. In T1 and PAB0082 it had a maximum absorbance reading of less than 0.3 (Fig 3.6).

Staphylococcus aureus reached a maximum absorbance reading of 2.0 in 300 min when grown in T2, while in T5 and PAB0082 it had the same absorbance reading of *ca.* 1.5 in 360 min. T1 supported the growth of *Staphylococcus aureus* to a maximum absorbance reading of 1.4 in 420 min, which was just below 1.6 the maximum absorbance reading of T5 and PAB0082 after the same period (Fig 3.7).

Pseudomonas aeruginosa showed the same growth pattern in all the peptones (Fig 3.8). In T2 *Pseudomonas aeruginosa* reached a maximum absorbance reading of 1.5 in 240 min. In T5 and PAB0082 it reached a maximum absorbance reading of *ca.* 1.4 in 270 min, whereas in T1 it reached its maximum absorbance reading of 1.2 in 270 min (Fig 3.8).

Shigella sonnei grew very well in T2, and managed to reach a maximum absorbance reading of *ca.* 1.5 in 270 min, whereas in the other peptones, including the standard, it grew up to a maximum absorbance reading of just below 1.0 over the experimental period of 420 min (Fig 3.9).

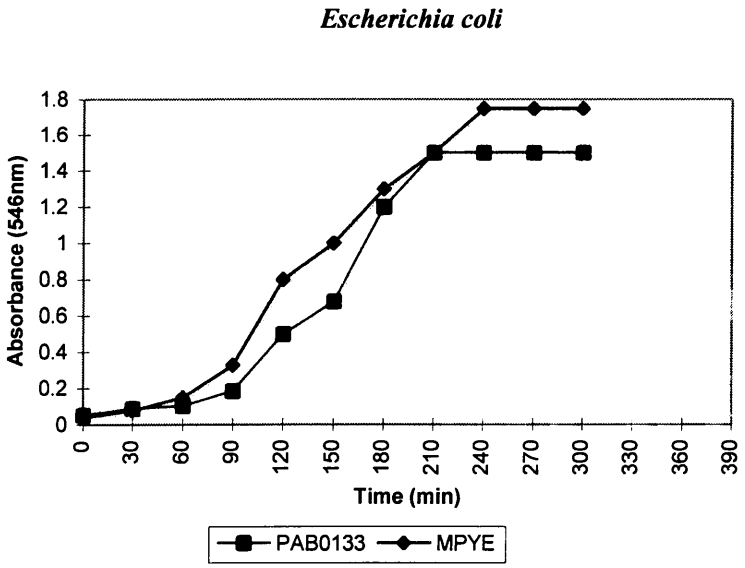

Fig 3.3 The growth of *Escherichia coli* in MPYE and PAB0133 (standard).

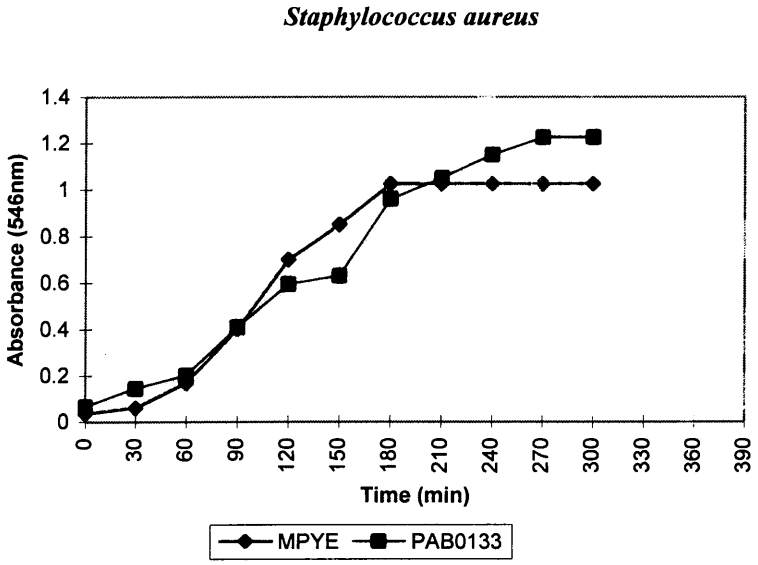

Fig 3.4 The growth of *Staphylococcus aureus* in MPYE and PAB0133 (standard).

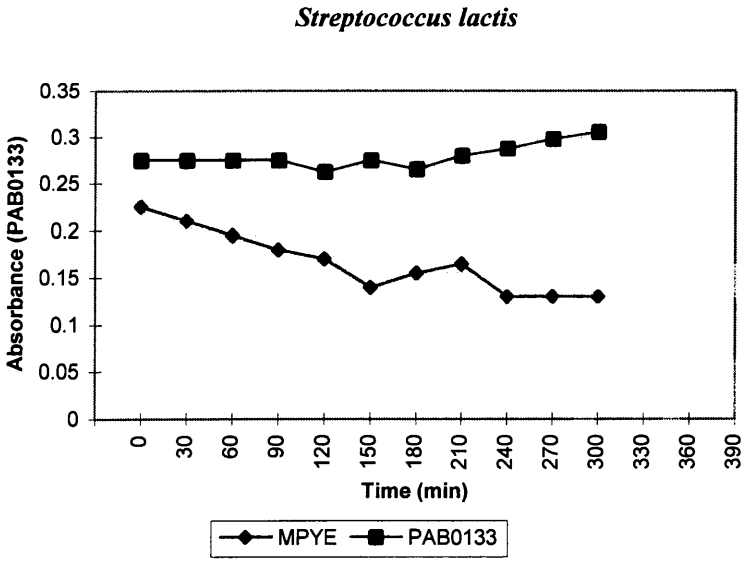

Fig 3.5 The growth of *Streptococcus faecalis* in MPYE and PAB0133 (standard).

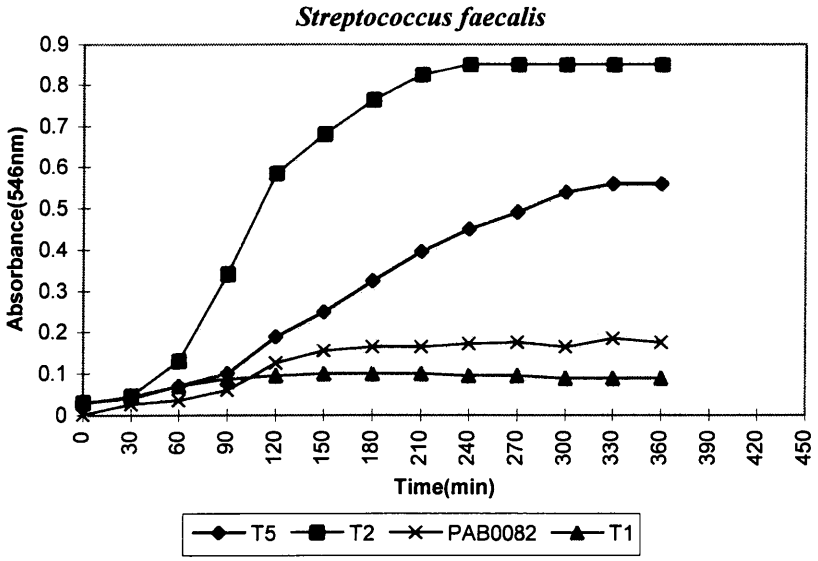

Fig 3.6 The growth of *Streptococcus faecalis* in T1, T2, T5 and PAB0082 (standard).

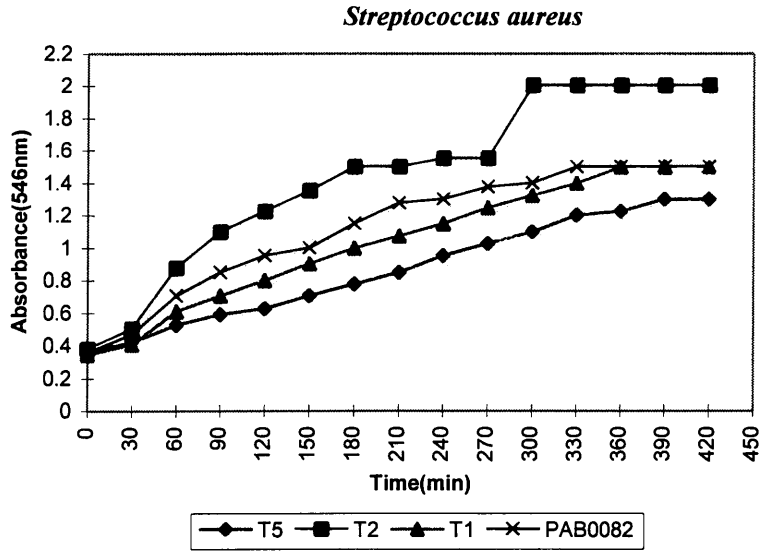

Fig 3.7 The growth of *Staphylococcus aureus* in T1, T2, T5 and PAB0082 (standard).

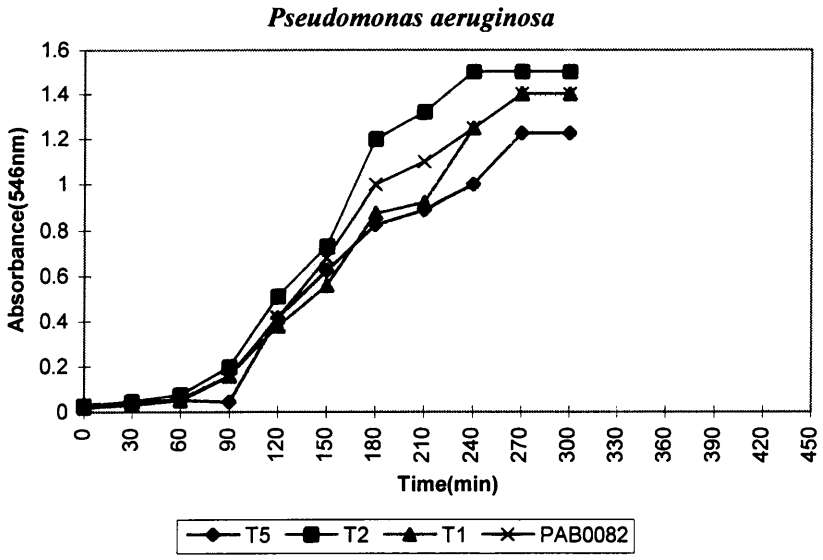

Fig 3.8 The growth of *Pseudomonas aeruginosa* in T1, T2, T5 and PAB0082 (standard).

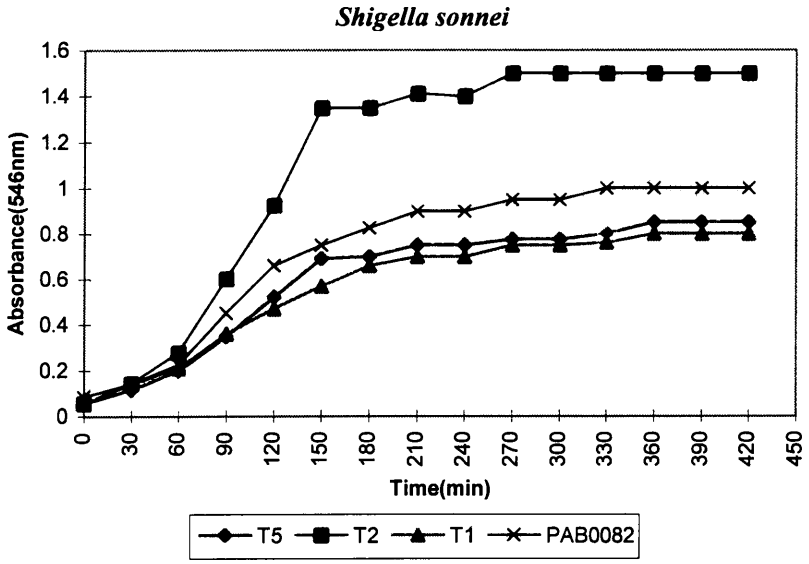

Fig 3.9 The growth of *Shigella sonnei* in T1, T2, T5 and PAB0082 (standard).

4 DISCUSSION

4.1 Physical and chemical properties of the experimental yeast extract and peptones

- **Peptones**

Table 3.1 shows that all locally manufactured peptones had similar colour and solubility properties to the standard. 1.0% (m/v) of T1, T2 and PAB0082 (standard) were completely soluble in 0.5% (m/v) sodium chloride solution. 1.0% (m/v) T5 was not 100% soluble and the insolubles had to be removed using a 125 mm Whatman filter paper. Solubility was attributed to the origin of the raw material and the method of manufacture.

According to IUMS specifications, dark colours are not acceptable for microbiological culture media components. Dark components will yield dark culture media and therefore during microbiological examinations the absorbance of broths and the observation of colony morphology on the agars would be difficult (Costin, 1982). The colour of T2, T5 and PAB0082 was similar. T1 had a slightly different colour than the other peptones. It was concluded that the physical properties of the experimental peptones were similar to the physical properties of PAB0082 (standard).

- **Yeast extract**

The colour of powders play a very important role in the physical examination of microbiological culture media components, because dark powders of the components are unacceptable to be used in culture media production (Bridson and Brecker, 1970; Bridson, 1978; Costin, 1982). Dark components will yield dark culture media and therefore during microbiological examinations the absorbance of broths and the observation of colony morphology on the agars would be impossible (Costin, 1982). MPYE (powder) had an acceptable colour, because it was comparable to the standard.

1.0% (m/v) MPYE and PAB0133 (standard) were completely soluble in 0.5% (m/v) sodium chloride solution and had the same colour after solubilization. This indicated that no caramelisation, due to excessive heating, took place.

4.2 Microbiological properties of the experimental yeast extract and peptones

4.2.1 Microbial content (Bioburden)

The required microbial content for culture media as specified by the IUMS should be less than 5000 cfu/g (Costin, 1982). The microbial content also determines the quality of a component, because once the microbial content is above 5000 cfu/g, the raw material can not be used as a culture media component. This is because of the fact that a large number of bacteria and fungi in the culture media components could for instance make the media component unsuitable for analytical purposes (e.g. vitamin assay), because the substances contained in the microbial cells

can interfere in the test. Spores within the culture media component will also make the formulated medium unstable. The total plate counts for T1, T2, T5, and the standard were 4.4 X 10^3, 3.9 X 10^3, 4.0 X 10^3 and 4.7 X 10^3 cfu/g respectively. MPYE had a total plate count of 4.0 X 10^1 cfu/g and PAB0133 (standard) 4.0 X 10^2 cfu/g. This means that the experimental components had a microbial content within an accepted range for culture media components.

4.2.2 Growth-promoting properties of the experimental yeast extract and peptones

- **Yeast extract**

Escherichia coli reached the highest maximum absorbance of *ca.* 1.75 when grown in MPYE for 210 min, compared to the absorbance reading of 1.5 in *ca.* 210 min when grown in PAB0133 (Fig 3.3). This might be due to the nature of *Escherichia coli* and the growth supporting ability of MPYE. *Escherichia coli* is a motile bacteria that grows well on the usual laboratory media in both the presence and absence of oxygen and its metabolism can either be respiratory or fermentative (Orskov, 1981; Holt *et al.,* 1994). From this basis, it is therefore easy to assume that *Escherichia coli* does not have sophisticated nutritional demands.

There was no difference between the absorbance readings of *Staphylococcus aureus* when grown in MPYE and PAB0133 (Fig 3.4). It was concluded that the growth supporting abilities of the two yeast extracts was the same for *Staphylococcus aureus*.

Streptococcus lactis did not grow in either MPYE and PAB0133 (Fig 3.5). However, the growth of *Streptococcus lactis* was sustained better in PAB0133 (standard) than in MPYE.

Streptococcus lactis is a nutritionally fastidious bacteria, it requires a complex medium containing amino acids such as isoleucin, valine, leucine, histidine, methionine, arginine, and proline and vitamins (niacin, Ca-panto-thernate and biotin) (Mundt, 1986). The latter was probably the reason why both the standard and the experimental yeast extract did not yield good growth of *Streptococcus lactis.*

From the results in Fig 3.3-3.5, one can deduce that nutritionally PAB0133 and MPYE are almost the same. MPYE could be used in culture media production, because it supported growth the same as the standard and showed no physical difference.

- **Peptones**

Streptococcus faecalis grew best in T2 (Fig 3.6). The facultative anaerobic streptococci require amino-acids, peptides and proteins, carbohydrates, fatty acids, vitamins, and purines and pyrimidines, in addition to inorganic ions (Devirese, 1981). The growth of *Streptococcus faecalis* might be due to T2 providing all the nutritional requirements of *Streptococcus faecalis*.

Staphylococcus aureus grew very well and gave the highest absorbance reading when grown in T2, compared to the other experimental peptones including the standard (Fig 3.7). This might be because *Staphylococcus aureus* is a facultative anaerobe, and it grows more rapidly and abundantly under aerobic conditions (Bailey and Scott, 1974; Kloos and Schleifer, 1981; Kloos and Schleifer, 1986). These aerobic conditions under which *Staphylococcus aureus* grow in abundance were provided during experimentation and these might be the reason why

Staphylococcus aureus gave a high absorbance reading. The other reason might be because *Staphylococcus aureus* occur in pairs, short chains or clusters.

Pseudomonas aeruginosa showed the same growth pattern in all the peptones, but grew best in T2 (Fig 3.8). There was no difference between T1, T5 and PAB0082 (Fig 3.8). The reason for the good growth of *Pseudomonas aeruginosa* in all the peptones was attributed to its growth requirements. *Pseudomonas aeruginosa* is not a fastidious microorganism and thus it may even grow in a low nutritional environment (e.g. water). Pseudomonads can utilise organic acids and can be characterised by a marked degree of tolerance for agents that are deleterious to most other organisms (Holt *et al.,* 1994). Hence, it was not surprising that the growth yield for *Pseudomonas aeruginosa* was similar in all the peptones.

Shigella sonnei reached its maximum absorbance reading of 1.5 in 270 min when grown in T2. This was higher than the maximum absorbance readings in the other experimental peptones, including the standard (Fig 3.9). *Shigella* does not grow in a synthetic medium containing only salts and a simple carbon source, unless glucose, amino acids, purines, pyrimidines, nicotinic acid and other vitamins are added (Rowe and Gross, 1981). It was therefore concluded that T2 provided the nutrients required by *Shigella sonnei* for growth.

T2 was nutritionally superior compared to the other peptones tested in this study, because it supported growth better than all the experimental peptones including PAB0082 (standard). T1 and T5 alternated in their growth supporting ability of the different microorganisms i.e. *Streptococcus faecalis* grew better in T5 compared to T1, while *Pseudomonas aeruginosa* grew

better in T1 compared to T5. T1 and T5 were nevertheless comparable to the standard in terms of growth-promoting properties.

Our results also support Bridson and Brecker (1970) and Costin (1982), who indicated that biological criteria have priority over physical and chemical tests, because T1, T5 and MPYE complied to the physical tests, but performed inconsistently above and/or below the standard in microbiological tests.

The methods used in this study proved to be reproducible and practical. These methods can also be used as a valuable tool for (1) detecting variations between batches of culture media components, (2) for checking out intended modifications in the production processes, and (3) for evaluating the effect on the nutritive value of a potential component from the manufacturing process.

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CHAPTER 4

LOCALLY MANUFACTURED PEPTONE AND YEAST EXTRACT AS CULTURE MEDIA COMPONENTS

ABSTRACT

The performance of culture media depends on many variables including the quality of its components. This study was aimed at evaluating selected culture media containing locally manufactured peptone and yeast extract. The evaluation of culture media was performed by incorporating peptone and yeast extract into formulations of Nutrient agar, Nutrient broth and MacConkey agar and assessing their performance. The performance was evaluated by inoculating suitable pure cultures into broths or onto agars. Growth of test cultures in broths was measured photometrically, while colony sizes of the test cultures was measured for agars. The culture media containing locally manufactured peptone and yeast extract had similar growth supporting abilities as the standard.

1 INTRODUCTION

The determination of bacterial numbers is a basic prerequisite in microbiology and yet the study of microorganisms in nature remains one of the least understood areas of microbial research (Karl, 1986). To grow microorganisms in the laboratory, whether on a small or large scale, a nutrient environment or culture medium is required, which will satisfactorily supply all the essential elements required for growth of microorganisms (Bridson and Brecker, 1970; Atlas, 1993) in proportions similar to those occurring in the microbial cells (Reviére, 1977). Due to diversity of microorganisms and their diverse metabolic pathways, there are numerous media employed for their cultivation. The composition of a nutrient agar for enumeration of viable bacterial cells in a specific environment should, therefore, be tailored to the specific nutritional requirement of the relevant bacteria or of the majority thereof (Brözel, 1990).

When complex constituents of the culture media are assembled for the purpose of isolation, enrichment or selection, the nitrogen-carbohydrate basis are considered (Bridson and Brecker, 1970). Amino acids, carbohydrates and vitamins provided by peptones and yeast extracts can be used in culture media as energy sources (Bridson and Brecker, 1970; Mossell, 1971). The mineral and inorganic ion contents of culture media are often ignored in complex media containing peptones and extracts (Bridson and Brecker, 1971). Bridson and Brecker (1970) also stated that the deliberate addition of selective compounds in culture media may profoundly affect even those organisms for whom the medium is specifically designed.

For the functioning of the ecosystem, consisting of the medium and its inoculum, clearly the most important parameters are those connected with the medium itself, in other words its compositional characteristics (Mossel, 1971; Mossel *et al.*, 1980). Besides the quality of its raw material, the performance of a culture media depends first on the so-called "intrinsic" attributes (available nutrients, redox potential, acidity, water activity, initial pH and its change depending on the buffer capacity, and antimicrobial substances fortuitously formed e.g. during excessive heating). In addition "extrinsic" factors during incubation (e.g. temperature, gaseous atmosphere) and "implicit" factors (vitality of the microorganisms, synergism, and antagonism) can influence the performance of a given medium (Mossel, 1971; Mossel *et al.*, 1980). As many as possible of these variables should be kept under control when using culture media.

An alteration to a minor specification of a single raw material can render a medium completely useless (Kendall, 1982). Even a slight difference in the composition of a medium can result in dramatically different growth characteristics of microorganisms (Atlas, 1993). Careful considerations should therefore be given when formulating new culture media. Hence, it is important to evaluate culture media especially when new ingredients are introduced.

Since culture media are designed to grow microorganisms, the ultimate test of their quality must be an assessment of their suitability for this purpose. The performance of new formulations of culture media is evaluated by inoculating suitable pure cultures into broths or onto agars. The growth of test cultures in broths was measured photometrically (Keshgi and Saunders, 1959) and colony sizes of the test cultures was measured from the agars. An alternative would be to use

lengthy agar or tube-dilution techniques i.e. Miles-Misra method described by Mossel (1971); Mossel *et al.* (1980); Fossum (1982). The Miles-Misra technique of viable counting is a surface-plating method, which is based on diluting bacteria in broth to about six to eight decimal dilutions. One drop from each dilution is spotted onto six nutrient agar plates with numbered segments and six to eight drops are then spotted onto each plate using a calibrated Pasteur pipette to deliver the drops. The pipettes are prepared by cutting the tips to a standard external diameter of 0.91 mm. After incubation the number of colonies at a suitable dilution are counted and the numbers in the original suspension calculated from the mean of the counts on six plates (Fossum, 1982).

Large quantities of microbiological culture media components are imported from Europe at a very high cost and therefore increasing the price of culture media. The only solution to this problem is to locally develop culture media components and subsequently culture media for import replacement. The local manufacturing of these culture media components and culture media may also present export opportunities. This study was therefor aimed at evaluating selected microbiological culture media, containing locally manufactured peptone and/or yeast extract. This was done by incorporating peptone and yeast extract into known formulations of Nutrient agar, Nutrient broth and MacConkey agar and assessing their physical and microbiological performance.

2 MATERIALS AND METHODS

2.1 Sources of the selected culture media and culture media components

Nutrient broth, Nutrient agar and MacConkey agar were obtained from Biolab (Fedsure Park, Tonnetti street, Midrand, P.O Box 1998, Halfway House, 1685, South Africa) and used as standards. All the components in the formulations of the media used were obtained from Biolab, excluding peptone (T2) and yeast extract (MPYE) which were obtained from the University of Pretoria. Tables 4.1 to 4.3 indicate formulations, required pH and preparation procedures of the standard media used and that of experimental media with substituted components.

2.2 Physical and chemical examination of the culture media formulations

Experimental culture media were compared with the standard media. All examinations were performed in triplicate.

2.2.1 Colour and odour of powders

The colour and odour of powder preparations were observed. The colour and odour of Nutrient broth C and Nutrient agar C could be compared with the standard after incorporating MPYE powder. The comparison of the colour and odour of Nutrient broths A and B, Nutrient agar A and B, and MacConkey agar A was not possible because T2 was supplied in a paste form, making it

impossible to compare the finished product, however once these products were prepared (solubillized and autoclaved) a comparison was possible.

2.2.2 Preparation of the experimental broths and agars

- **Nutrient broths**

The Nutrient broths were prepared by suspending 16g of all the components in 1.0*l* distilled water (Table 4.1) (Biolab, 1996). The suspensions were then frequently stirred until completely soluble and then poured into final containers. After media preparation the pH of all the Nutrient broths were measured and adjusted to 7.1 ± 0.1 at 20 ± 1.0 °C using the Beckman 34 pH meter. The Nutrient broths were sterilised by autoclaving for 15 min at 121°C.

- **Nutrient agar**

The Nutrient agars were prepared by suspending 31 g of all the components in 1.0*l* dH_2O (Table 4.3) (Biolab, 1996). The Nutrient agar suspensions were brought to boil with frequent stirring until completely dissolved. After media preparation, the pH of all the Nutrient agars were measured and adjusted to 7.1 ± 0.1 at 20 ± 1.0 °C using the Beckman 34 pH meter. The Nutrient agars were autoclaved at 121°C for 15 min.

- **MacConkey agar**

MacConkey agars were prepared by suspending 50 g of all the components in 1.0*l* dH_2O (Table 4.3) (Biolab, 1996). The MacConkey agar suspensions were brought to boil with frequent stirring until completely dissolved. After media preparation the pH of all the MacConkey agars were measured and adjusted to 7.1 ± 0.1 at 20 ± 1.0 °C using the Beckman 34 pH meter. The MacConkey agars were autoclaved at 121°C for 15 min.

2.2.3 Solubility, colour and setting properties of the prepared culture media

The solubility and colour of the Nutrient broths, Nutrient agars and MacConkey agars after preparation was observed. The setting ability of the experimental Nutrient agars and MacConkey agars were tested by pouring prepared agar into Petri-dishes, and then incubating them at 37^0C. After 24h the Petri-dishes were observed, to confirm the setting abilities.

Table 4.1 Nutrient broth product composition (Biolab, 1996)

Components of Nutrient broth (standard)	**Amount (g)**
Peptone (PAB 0082 & PAB 0126)	5.0
NaCl	8.0
Yeast Extract (PAB 0133)	2.0
Beef extract	1.0
Components of Nutrient broth A	**Amount (g)**
T2	5.0
NaCl	8.0
PAB 0133	2.0
Beef extract	1.0
Components of Nutrient broth B	**Amount (g)**
T2	5.0
NaCl	8.0
MPYE	2.0
Beef extract	1.0
Components of Nutrient broth C	**Amount (g)**
PAB 0082 & PAB 0126	5.0
NaCl	8.0
MPYE	2.0
Beef extract	1.0

Table 4.2 Nutrient agar product composition (Biolab, 1996)

Components of Nutrient agar (standard)	**Amount (g)**
Agar	15.0
Peptone (PAB 0082 & PAB 0126)	5.0
NaCl	8.0
Yeast extract (PAB 0133)	2.0
Beef extract	1.0
Components of Nutrient agar A	**Amount (g)**
Agar	15.0
T2	5.0
NaCl	8.0
PAB 0133	2.0
Beef extract	1.0
Components of Nutrient agar B	**Amount (g)**
Agar	15.0
T2	5.0
NaCl	8.0
MPYE	2.0
Beef extract	1.0
Components of Nutrient agar C	**Amount (g)**
Agar	15.0
Peptone (PAB 0082 & PAB 0126)	5.0
NaCl	8.0
MPYE	2.0
Beef extract	1.0

Table 4.3 MacConkey agar product composition (Biolab, 1996)

Components of MacConkey agar (standard)	**Amount (g)**
Peptone (PAB 0082 & PAB 0126)	20.0
Agar	13.5
Lactose	10.0
NaCl	5.0
Bile Salt no 3	1.5
Neutral Red	0.03
Crystal Violet	0.001
Components of MacConkey agar A	**Amount (g)**
T2	20.0
Agar	13.5
Lactose	10.0
NaCl	5.0
Bile Salt no 3	1.5
Neutral Red	0.03
Crystal Violet	0.001

2.3 Microbiological examination of the culture media formulations

2.3.1 Sterility test

Samples of all the sterilised media and broths were poured into Petri dishes and flasks respectively and incubated at 25^0C and 37^0C for 24, 36 and 48h. Sterile media inoculated with pure cultures of *Escherichia coli* and Ringer's solution were used as positive and negative controls respectively. After the incubation period total plate counts were done for solid media and absorbance for the broths was measured at 546 nm using a Spectronic 20 spectrophotometer (Milton Roy Company). All the tests were performed in triplicate.

2.3.2 Growth-promoting properties of the experimental culture media

Fresh cultures were prepared by growing bacterial cultures on Nutrient agar obtained from Biolab for 24h at 37^0C. *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloaceae, Staphylococcus aureus, Bacillus subtilis, Proteus mirabilis, Pseudomonas aeruginosa* and *Shigella sonnei* were obtained from the Department of Microbiology and Plant Pathology culture collection, at the University of Pretoria. Suspensions were prepared by inoculating 3-4 colonies of each bacterial culture into 10 ml quarter strength Ringer's solution. The suspensions were serially diluted from 10^{-1} to 10^{-9} dilution, in preparation for the inoculum used in the absorbency measurements of broths and colony size measurements of agars.

- **Absorbance measurements**

Absorbance measurements were done for all the Nutrient broths. 0.01 ml suspensions of the respective bacterial dilutions, similar to the opacity of the 0.5 McFarland standard, were inoculated, into the Nutrient broth in the side-arm flasks (Fig 4.1). The flasks were incubated at 37^0C and at the same time mixing was achieved by shaking at 60 rpm. After 24h the absorbencies were measured at 546 nm using a Spectronic 20 spectrophotometer (Milton Roy Company). Nutrient broths inoculated with sterile Ringer's solution were used as controls.

- **Colony size measurements**

Colony sizes were measured on the Nutrient agars and MacConkey agars. 0.01 ml suspensions of the respective bacterial dilutions, similar to the opacity of the 0.5 McFarland standard, were spread plated onto, agar Petri-dishes containing the respective medium. The plates were incubated at 37^0C. After 24h colony sizes were measured using a steel ruler (lineal) obtained from Biolab. Nutrient agars and MacConkey agars, which were inoculated with sterile Ringer's solution, were used as controls.

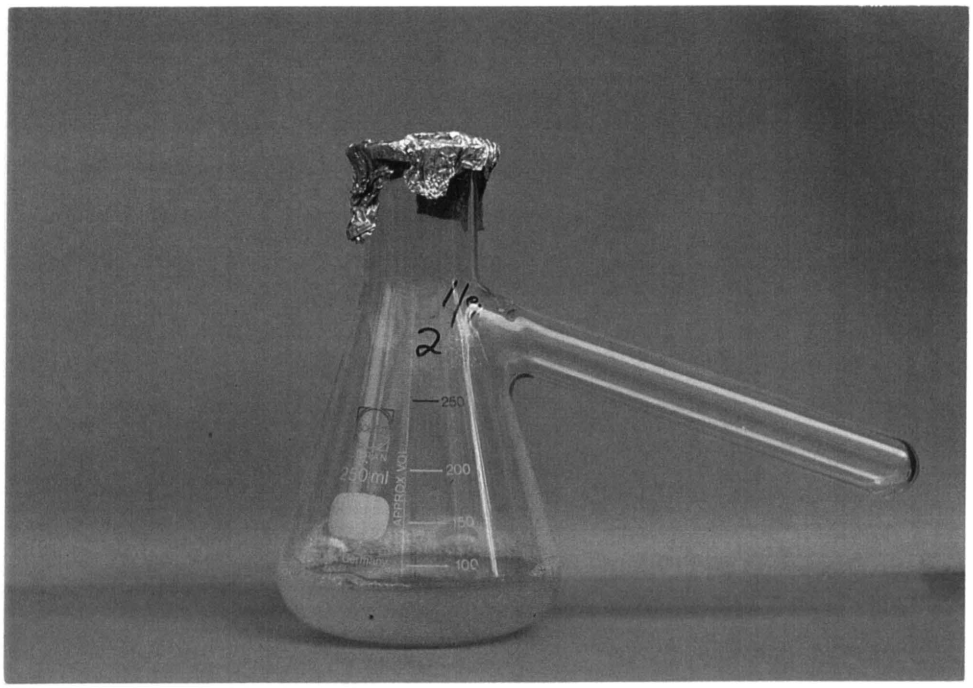

Fig 4.1 Flask used to determine the growth supporting ability of the experimental broths.

3 RESULTS

3.1 Physical and chemical examinations of the experimental culture media

3.1.1 Colour and odour of powders

The colour of the experimental Nutrient broth C and Nutrient agar C in powder form were light-brown and beige to dim-brown. This was similar to those of their respective standards. The odour

of Nutrient broth C and Nutrient agar C were typical of the Nutrient broth (standard) and Nutrient agar (standard).

3.1.2 Solubility, colour and setting properties of the prepared culture media

Physical properties of the experimental culture media, showed that there was no difference when compared to their standards (Tables 4.4 to 4.6). Solubility and colour of all the experimental Nutrient broth formulations including the standard Nutrient broth were the same (Tables 4.4). The experimental Nutrient broths were completely soluble when suspended in distilled water.

The experimental Nutrient agars and the standard Nutrient agar were completely soluble after boiling and frequent stirring. The colour of Nutrient agar A and the standard were slightly opalescent, beige, while Nutrient agar B and Nutrient agar C had a beige colour. All the experimental Nutrient agars solidified at 37 ± 1.0°C after 24h (Table 4.5).

The experimental MacConkey agar and the standard MacConkey agar were completely soluble after boiling and frequent stirring (Table 4.6). The experimental MacConkey agars and the standard MacConkey agar were clear, reddish-purple in colour. The MacConkey agar and the standard MacConkey agar solidified at 37 ± 1.0°C after 24h (Table 4.6).

Table 4.4 Physical observations of the experimental Nutrient broths after preparation

Characteristic	Specific medium			
	Standard	Broth A	Broth B	Broth C
Solubility	Completely soluble	Completely soluble	Completely soluble	Completely soluble
Colour	Clear, pale-straw	Clear, pale-straw	Clear, straw	Clear, pale-straw

Table 4.5 Physical observations of the experimental Nutrient agars after preparation

Characteristic	Specific medium			
	Standard	Agar A	Agar B	Agar C
Solubility	Completely soluble	Completely soluble	Completely soluble	Completely soluble
Colour	Slightly opalescent, beige	Slightly opalescent, beige	Beige	Beige
Setting properties	Solidified at (37 ± 1.0)°C	Solidified at (37 ± 1.0)°C	Solidified at (37 ± 1.0)°C	Solidified at (37 ± 1.0)°C

Table 4.6 Physical observations of the experimental MacConkey agars after preparation

Characteristic	Specific medium	
	Standard	Agar A
Solubility	Completely soluble	Completely soluble
Colour	Clear, reddish-purple	Clear, reddish-purple
Setting properties	Solidified at (37 ± 1.0)°C	Solidified at (37 ± 1.0)°C

3.2 Microbiological examination

3.2.1 Sterility test

There was no growth of microorganisms after the sterile media was incubated at 25^0C and 37^0C for 24, 36 and 48h. Growth was only visible on plates and tubes inoculated with *Escherichia coli* as a control for all the experiments. This indicated that the sterilised media was indeed sterile and that there were no heat resistant microorganisms in any of the experimental culture media components.

3.2.2 Growth-promoting properties of the experimental culture media

- **Nutrient broths**

The growth of *Escherichia coli* was within the standard deviation of the standard Nutrient broth when grown in Nutrient broth A and Nutrient broth B. Nutrient broth C did not support the growth of *Escherichia coli,* indicated by an absorbance reading below the standard deviation of the standard Nutrient broth (Table 4.7).

The growth of *Klebsiella pneumoniae,* was within the standard deviation of the standard Nutrient broth when grown in Nutrient broth C. Nutrient broth B and Nutrient broth A had an absorbance reading below the standard deviation of the standard Nutrient broth and therefore did not support the growth of *Klebsiella pneumoniae* better than the Nutrient broth C and the standard Nutrient broth (Table 4.7).

The growth of *Pseudomonas aeruginosa* in Nutrient broth A was above the standard deviation of the standard Nutrient broth and this showed that Nutrient broth A was nutritionally superior compared to the other experimental Nutrient broths including the standard Nutrient broth. Nutrient broth B and Nutrient broth C had an absorbance reading which did not deviate from the standard nutrient broth.

In Nutrient broth C, *Staphylococcus aureus* grew above the standard deviation indicating that Nutrient broth C was nutritionally superior compared to the other experimental Nutrient broths (Table 4.7). The growth of *Staphylococcus aureus* was within the standard deviation of the standard Nutrient broth when grown in Nutrient broth B. Nutrient broth A had an absorbance reading below the standard deviation of the standard Nutrient broth and therefore did not support the growth of *Staphylococcus aureus* satisfactorily compared to the other Nutrient broths including the standard Nutrient broth (Table 4.7).

There was no absorbance reading and subsequently no growth in the Nutrient broths which were inoculated with sterile Ringer's solution (control). This indicated that the results obtained reflect the true growth of the inoculated microorganism in the Nutrient broths.

In general, there was no significant difference between the growth supporting ability of Nutrient broth B and the standard. This is because Nutrient Broth B supported the growth of all the test microorganisms consistently better than the standard. Nutrient broth A and Nutrient broth C had poor growth supporting abilities, because two of the test microorganisms inoculated on Nutrient broth A and Nutrient broth C grew below the standard deviation (Table 4.7).

- **Nutrient agars**

Bacillus subtilis grew exceptionally well on all the experimental Nutrient agars including the standard Nutrient agar. When *Bacillus subtilis* was grown in all of the above mentioned Nutrient agars, it grew up to a point where the colonies became "spreaders" and were therefor too big to be measured.

Nutrient agar B supported growth of *Enterobacter cloaceae* better than all the Nutrient agars including the standard, because its colony size was larger than the standard deviation of the Nutrient agars (Table 4.8). The growth of *Enterobacter cloaceae* in Nutrient agar A and Nutrient agar C was comparable to the standard Nutrient agar. Nutrient agar A and Nutrient agar C had the same colony size of 2.0 mm and was similar to the colonies measured on the standard Nutrient agar.

Nutrient agar A and Nutrient agar B supported the growth of *Escherichia coli* better than Nutrient agar C and the standard Nutrient agar, because the colony sizes on Nutrient agar A and Nutrient agar B were bigger than those of the other experimental Nutrient agars including the standard Nutrient agar (Table 4.8). *Escherichia coli* grew better in the standard Nutrient agar and Nutrient agar C, because there was no significant difference in the colony sizes of *Escherichia coli* on the standard Nutrient agar and Nutrient agar C (Table 4.8).

Nutrient agar A and Nutrient agar B supported the growth of *Klebsiella pneumoniae* better than Nutrient agar C and the standard Nutrient agar, because the colony sizes on Nutrient agar A and

Nutrient agar B were bigger than those of the other experimental Nutrient agars including the standard Nutrient agar (Table 4.8). *Klebsiella pneumoniae* grew better in the standard Nutrient agar and Nutrient agar C (Table 4.8). In general, *Klebsiella pneumoniae* grew better in all the experimental Nutrient agars. All the Nutrient agars supported its growth without deviating from the standard Nutrient agar (Table 4.8).

Nutrient agar B supported the growth of *Pseudomonas aeruginosa* better than the other Nutrient agars, because the colony sizes on Nutrient agar B were bigger than those of the other Nutrient agars including the standard Nutrient agar (Table 4.8). The growth of *Pseudomonas aeruginosa* was the same on the standard Nutrient agar, Nutrient agar A and Nutrient agar C, because there was no difference in the colony sizes on all the compared Nutrient agars (table 4.8).

Staphylococcus aureus had its growth supported in all the experimental Nutrient agars. The colony sizes of *Staphylococcus aureus* in all the experimental Nutrient agars were similar to the colony sizes of the standard Nutrient agar, because the colony sizes were within the standard deviation of the standard Nutrient agar (Table 4.8).

There were no colonies formed and subsequently no growth on all the Nutrient agars which were inoculated with sterile Ringer's solution (control). This showed that the results obtained reflect the true growth of the inoculated microorganisms on the Nutrient agars.

In general, there was no difference between all the Nutrient agars, except for the fact that Nutrient agar B was nutritionally superior, because it supported the growth of most microorganisms above the standard deviation (Table 4.8).

- **MacConkey agars**

Table 4.9 shows that there was a negligible difference in the growth of the test organisms in all the experimental MacConkey agars and the standard MacConkey agars, by both appearance and size of their colonies.

Proteus mirabilis had the same colony sizes on both the standard MacConkey agar and MacConkey agar A. *Proteus mirabilis* also had colourless colonies on both the standard MacConkey agar and MacConkey agar A. The colourless colonies are attributed to the fact that *Proteus mirabilis* did not ferment lactose within the selective media.

There was no significant difference in the colony sizes of *Escherichia coli* on MacConkey agar A and the standard MacConkey agar. The *Escherichia coli* colonies were pink-red and had turbid zones indicating that *Escherichia coli* fermented the lactose within the media.

Pseudomonas aeruginosa had the same colony sizes on both the standard MacConkey agar and the MacConkey agar A. *Pseudomonas aeruginosa* had colourless colonies on both the standard

MacConkey agar and the MacConkey agar A. The colourless colonies are attributed to the fact that *Pseudomonas aeruginosa* did not ferment lactose within the selective media.

Shigella sonnei had the same colony sizes on both the standard MacConkey agar and MacConkey agar A. *Shigella sonnei* did not ferment lactose and thus had colourless colonies.

Staphylococcus aureus did not grow in either the standard MacConkey agar or the MacConkey A.

Escherichia coli and Proteus mirabilis (mixed culture) both grew on the standard MacConkey agar and MacConkey A. *Proteus mirabilis* had colourless colonies, while *Escherichia coli* had pink-red colonies with turbid zones. This indicated that *Proteus mirabilis* did not ferment lactose, while *Escherichia coli* fermented the lactose within the media.

There was no growth and subsequently no colonies formed on any of the MacConkey agars which were inoculated with sterile Ringer's solution (control).

These results reflect the true growth of the inoculated microorganisms on the MacConkey agars. The experimental MacConkey agar proved to be both a selective and a differential medium, because the MacConkey agar inhibited the growth of gram-positive bacteria and differentiated between lactose fermenters and non-lactose fermenters in the same way as the standard MacConkey agar.

Table 4.7 Absorbance readings of bacteria inoculated into Nutrient broths measured after incubation at 37°C for 24h

Organism	Absorbance at 546 nm				Standard deviation
	Standard	Broth A	Broth B	Broth C	All broths*
Escherichia coli	0.475	0.630	0.590	0.390	0.109
Klebsiella pneumoniae	0.050	0.015	0.025	0.050	0.018
Pseudomonas aeruginosa	0.230	0.310	0.260	0.200	0.047
Staphylococcus aureus	0.050	0.020	0.120	0.145	0.059
Control	No growth	No growth	No growth	No growth	0.000

All broths* - Standard, broth A, broth B and broth C.

Table 4.8 Colony size of bacteria plated onto Nutrient agar after incubation at 37°C for 24h

Organism	Approximate colony size (mm)				Standard deviation
	Standard	Agar A	Agar B	Agar C	All agars*
Bacillus subtilis	Spreader	Spreader	Spreader	Spreader	0.00
Enterobacter cloaceae	2.3	2.0	3.0	2.0	0.472
Escherichia coli	3.0	4.0	3.5	3.2	0.252
Klebsiella pneumoniae	3.3	4.0	3.5	2.0	0.853
Pseudomonas aeruginosa	3.2	3.1	4.0	3.0	0.457
Staphylococcus aureus	2.0	1.5	1.0	1.0	0.500
Control	No growth	No growth	No growth	No growth	0.000

All broths* - Standard, broth A, broth B and broth C.

Table 4.9 Appearance and colony size of bacteria plated onto MacConkey agars incubated at 37°C for 24h

Organisms	Approximate colony size (mm) and colour		Standard deviation
	Standard	Agar A	All agars*
Proteus mirabilis	2.0 (colourless)	2.0 (colourless)	0.000
Escherichia coli	3.6 (pink-red, turbid zones)	2.5 (pink-red, turbid zones)	0.778
Pseudomonas aeruginosa	3.0 (colourless)	3.5 (colourless)	0.354
Shigella sonnei	3.0 (colourless)	3.0 (colourless)	0.000
Staphylococcus aureus	No growth	No growth	0.000
E. coli and P. mirabilis	All grew (pink-red, turbid zones and colourless)	All grew (pink-red, turbid zones and colourless)	0.000
Control	No growth	No growth	0.000

All broths* - Standard, broth A, broth B and broth C.

4 DISCUSSION

The performance of culture media depends on many variables including the quality of its components. Compatibility of microbiological components is one of the most important factors, because incompatibility of components in formulations of culture media announces its presence by a visual change in appearance of the media i.e. precipitation, opalescence, colour changes and loss of gel strength (Bridson and Brecker, 1970; Costin, 1982).

4.1 Physical and chemical properties of the culture media formulations

4.1.1 Colour and odour of powders

The colour and odour of Nutrient broth C were typical of the standard Nutrient broth. The colour and odour of Nutrient broth C was similar to their corresponding standards. This was because the components were compatible and yielded an acceptable colour and odour.

4.1.2 Solubility, colour and setting properties of the prepared culture media

All the Nutrient broths, Nutrient agars and MacConkey agars were completely soluble after preparation. These indicated that the components added to the experimental culture media formulations were compatible with the other components in solution.

All the Nutrient broths, Nutrient agars and MacConkey agars had almost the same colour as their respective standard.

Setting of the agars at 37 ± 1.0 ^{0}C after 24h showed that the components were compatible. Incompatibility of the components would have interfered with the setting ability of the agar.

4.2 Microbiological properties

4.2.1 Sterility of the experimental culture media

Sterility of the media proved that there were no contaminants and specifically no heat resistant organisms in any of the raw materials.

4.2.2 Growth-promoting properties of the experimental culture media

It is important to know that slight changes in the formulation of a culture media may result in products, possessing entirely different nutritive properties from the standard medium (Leifson, 1943; Kendall, 1982; Atlas, 1993). The literature states that, a medium is evaluated by its ability to support growth of different microorganisms (Leifson, 1943; Keshgi and Saunders, 1959; Bridson and Brecker, 1970; Bridson, 1978; Costin, 1982). Therefore, if a medium supports growth of bacteria better than another similar medium, the preferred media would be the one that supports the best growth.

- **Nutrient broths**

Escherichia coli is motile and grows well in a usual laboratory media in both the presence and absence of oxygen and its metabolism can either be fermentative or respiratory (Ørskov, 1981). It is therefore necessary to assume that the medium that supports the growth of *Escherichia coli* similar to the standard are nutritionally of a high quality. Nutrient broth C gave an absorbance reading which was below the standard deviation of the standard Nutrient broth and therefore it was not a good culturing medium for *Escherichia coli.*

Klebsiella pneumoniae grew best in the standard Nutrient broth, Nutrient broth B and Nutrient broth C. This indicated that Nutrient broth C provided necessary nutrients required for the growth of *Klebsiella pneumoniae.*

Pseudomonas aeruginosa grew well in all the Nutrient broths and had superior growth in Nutrient broth B and Nutrient broth A. These might be due to the growth requirements of *Pseudomonas aeruginosa.* Pseudomonads can utilise organic acids and are characterised by a marked degree of tolerance for agents that are deleterious to most other microorganisms (Devirese *et al.,* 1981). *Pseudomonas aeruginosa* is not a nutritionally fastidious microorganism and can therefore grow in low nutrient environments (i.e. water). All the Nutrient broths provided more than enough nutrients to the test *Pseudomonas aeruginosa* strain used.

There was no significant difference for the growth of *Staphylococcus aureus* in Nutrient broth C, Nutrient broth A, Nutrient broth B and the standard Nutrient broth. This might be due to the fact that Nutrient broths provided all the nutrients required by *Staphylococcus aureus*. *Staphylococcus aureus* is a facultative anaerobe which grows more rapidly and abundantly under aerobic conditions (Kloos and Schleifer, 1981; Kloos and Schleifer, 1986). The other reason might be that the components are compatible in formulation and therefore do not form substances that inhibit the growth of *Staphylococcus aureus*.

From the results, it was concluded that Nutrient broth B would be preferred over the other experimental Nutrient broths. This is because Nutrient broth B supported growth of all the microorganisms consistently, mostly within and above the standard deviation. The other Nutrient broths were inconsistent, because they supported growth sometimes above and mostly below the standard deviations.

- **Nutrient agars**

Bacillus subtilis was a fast grower and formed a "spreader" in all the Nutrient agars. This might be due to the compatibility of the components, and that Nutrient agars provided the nutrients that *Bacillus subtilis* demand for optimum growth.

Bacillus subtilis, Enterobacter cloaceae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and *Staphylococcus aureus* grew in all the Nutrient agars. The Nutrient agars supported

growth of all the above mentioned bacteria. This was not surprising since the above mentioned bacteria are not nutritionally fastidious and thus the Nutrient agars would have provided more than enough nutrients for their growth. The other reason is that the components were compatible in formulation and did not form substances that inhibits the growth of the above mentioned bacteria.

Nutrient agar B would be the preferred medium, because it supported the growth of all the microorganisms better than all the other experimental Nutrient agars including the standard Nutrient agar.

- **MacConkey**

MacConkey agar is a selective agar medium used for the isolation of *Salmonella, Shigella* and coliform bacteria from foods, urine, faeces, sewage etc. (Atlas, 1993; Biolab, 1996).

Proteus mirabilis, Escherichia coli, Shigella sonnei and *Pseudomonas aeruginosa* grew on the MacConkey agar formulations indicating that the medium provided necessary nutrients for these bacteria.

Staphylococcus aureus did not grow on the standard MacConkey agar and MacConkey agar A, because it is a gram-positive bacteria and it was inhibited by the crystal violet within the media. This result emphasises the similarities of the standard MacConkey agar and MacConkey agar A.

Escherichia coli growth was indicated by red colonies with turbid zones. This showed that *Escherichia coli* fermented lactose to acid. This was indicated by the phenol red indicator, which becomes red in acid conditions. The turbid zones formed indicated that the bacteria was an *Enterobacteriaceae.*

In general, it was concluded that the experimental MacConkey agar A was similar to the standard MacConkey agar in terms of its selecting and differentiating abilities.

The results obtained from this study indicated that, although the nutritive properties of the experimental media were different from the standard media, the growth supporting ability of the experimental media were similar to the standard media.

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CHAPTER 5

GENERAL CONCLUSION

It would be in the interest of the South African economy and that of neighbouring countries if imported microbiological culture media components from Europe, are manufactured in South Africa. To accomplish this, however, newly developed culture media components have to be evaluated individually and/or in formulations of culture media. Evaluation of culture media components is important, because slight changes in the formulation of a culture media can result in products possessing entirely different nutritive properties from the original (Leifson, 1943; Kendal, 1982; Atlas, 1993). Because this study was demand-driven, it was concerned with the ability of the culture media components to be used in culture media production and the growth-supporting abilities of the culture media thereof.

The evaluation of growth supporting abilities of culture media components and/or culture media was done by inoculating suitable pure cultures into solutions of the experimental raw materials and/or Nutrient broths, and determining the growth of the test microorganisms by measuring optical density of the solutions over time. For the experimental agars, suitable pure cultures were spread plated onto the agars and colony sizes on the agar plates measured as growth of the test cultures, as specified by the manufacturer of the standard agar media. The methods used in this study to evaluate both the locally manufactured peptones and yeast extract proved to be reproducible and practical. These methods can also be used as a valuable tool for (1) detecting variations between batches of culture media components, (2) for checking intended

modifications in the production processes, and (3) for evaluating the effect on the nutritive value of a potential component from the manufacturing process.

The results obtained indicated that the locally manufactured peptones and yeast extract supported growth of all of the selected microorganisms similar to the standard. Hence, they can be used as culture media components. When the locally manufactured peptones and yeast extract were incorporated into culture media formulations, the experimental culture media results showed that these were compatible and supported the growth of the test organisms better than the standard culture media. These results indicated that, although the nutritive properties of the experimental culture media were different from the standard media, the growth supporting abilities of the experimental culture medium were similar and that was the most important determining factor. It was indicated in the results that T2 was nutritionally superior compared to the other tested peptones and could be used in full scale culture media production.

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"I am a man who in the eager pursuit of knowledge forgets his food and in the joy of its attainment forgets his sorrows, and who does not perceive that old age is coming on."

Anonymous.