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# DEVELOPMENT OF NEW CEREAL-BASED PROBIOTIC FOODS


**By**

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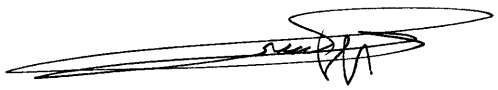
*I would like to dedicate this dissertation to my parents who have devoted their lives to their children's education.*

*I have seen that no one can write any text in a day without saying the following: it could have been better if this was changed, or it could have been preferred if this was added, or it would be more precise if this was moved from one place to the other, or it could sound better if this was removed, and this suggests that nothing is perfect.*

**E**mad **A**l-**D**en **A**l-**A**sfhane (1098-1181)

# DECLARATION

No part of the work included in this dissertation has been submitted in support of an application for another degree or qualification of this or any other institute of learning.



**M**ohammed **E**brahim **A**l-**A**seeri

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# Development of New Cereal-Based Probiotic Foods

## ABSTRACT

In the last two decade, the relation between the human health and his food has been given special attention. This interest together with other factors (such as consumers' education level) leads to an obvious increasing in the demand for balanced foods with extra-health benefits. In the past five years 10% of all new products in the EU make reference to health. It has been recently proved that probiotic products (defined as those that contain a single or mixed culture of certain microorganisms) could affect beneficially the consumer health. The European probiotic market is estimated to be of approximately 370 M Euro and will probably increase in the coming years.

The main purpose of this work is to produce new cereal based probiotic foods with suitable aroma, flavour and pH. This requires selecting suitable types of both cereal grains and probiotic microorganisms and finding the most appropriate methods (physical and chemical) to monitor fermentation. Malt grains and malt extracts were selected as the fermentation media while *Lactobacillus casei* shirota and the *Saccharomyces cerevisiae* were chosen as the probiotic microorganisms. Four different media were prepared, analysed and used for fermentation with two different types of inocula settings (pure and mixed cultures). The effects of the different fermentation media and the inocula on the final product characteristics such as FAN concentration, pH level, phosphorus and lactic acid content were investigated. The aroma and flavour of the fermented drink was also tasted. It was found that the microorganisms inoculated could grow in all media with different growth rates as was indicated by the measured parameters (e.g. phosphorus and pH). It was also found that the pH values for the final products were within the acceptable consumption range. According to the results of the tasting sessions, two of the fermented products were found to have acceptable aroma and flavour but the rest were in need of further improvement (e.g. by addition of sugar or saccharine).

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACDP | Advisory committee on dangerous pathogens |
| ADP | Adenosine-5'-diphosphate |
| ATP | Adenosine-5'-triphosphate |
| Catal. | Catalase produced, Tables 2.2 and 2.9 |
| CFU | Colony forming unit(s) |
| D | Label for (-) isomer, Table 2.9 |
| DNA | Deoxyribonucleic acid |
| EBC | European brewery convention |
| EU | European union |
| FAN | Free amino nitrogen |
| FOSHU | Foods for special dietary use |
| GRAS | Generally recognised as safe |
| HG | Hazard group |
| HPLC | High performance liquid chromatography |
| L | Label for (+) isomer, Table 2.9 |
| LAB | Lactic acid bacteria |
| M | Finished malt without rootlets, Table A.1-1 |
| MRS | *Lactobacillus* agar according to DeMan, Rogosa and Sharpe |
| MYGP | Yeast agar made out of malt extract, yeast extract, glucose and peptone |
| $NAD^+$ | Nicotinamide adenine dinucleotide (oxidised form) |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NCYC | National collection of yeast cultures |
| $P_i$ | Inorganic orthophosphate |
| PTFE | Polytetrafluoroethylene |

| | |
|---|---|
| R | Coefficient of determination |
| Rpmi | Type of agar medium |
| rpm | Revolutions per minute |
| SCFA | Short chain fatty acids |
| SEM | Scanning electron micrograph |
| sp. | Specie |
| spp. | Species |
| ssp. | Sub-specie |
| STD | Standard |
| subsp. | Sub-specie |
| subssp. | Sub-species |
| TCW | Thousand corn dry weight, Table A.1-1 |
| TKN | Total Kjeldahi nitrogen |
| TVC | Total viable cells, Table 4.2 |
| UHT | Ultra high temperature |
| VTT | Valtion Teknillinen Tutkimuskeskus<br>(Biotechnology and Food Research Institute, Finland) |

# LIST OF SYMBOLS

| Symbol | | SI Units |
|---|---|---|
| $A_{430}$ | Absorbance of sample at a wave length of 430 | nm |
| $A_{460}$ | Absorbance of sample at a wave length of 460 | nm |
| $A_{570}$ | Absorbance of sample at a wave length of 570 | nm |
| $A_{S570}$ | Absorbance of standard solution at a wave length of 570 | nm |
| $A_{730}$ | Absorbance of sample at a wave length of 730 | nm |
| C | Lactic acid concentration in fermentation, Equation B.6-1 | $\mu g\ mL^{-1}$ |
| d | Dilution factor of sample, Equation B.2-1 | - |
| F | FAN concentration in sample, Equation B.2-1 | $mg\ L^{-1}$ |
| LPhos | Total amount of phosphorus in liquid sample, Equation B.4-1 | $mg\ L^{-1}$ (liquid) |
| N | Total amount of nitrogen in sample, Equation B.3-1, Tables A.2-1 and A.2-2 | $mg\ g^{-1}$ (solid) or $mg\ mL^{-1}$ (liquid) |
| SPhose | Total amount of phosphorus in solid sample, Equation B.5-1 | $mg\ g^{-1}$ (solid) |
| w | Weight or volume of the sample, Equations B.3-1 and B.5-1 | g (solids) or mL (liquid) |

# 1

# INTRODUCTION

# 1. INTRODUCTION

Since the beginning of human civilisation there was an intimate companionship between the human being, his fare and the fermentative activities of microorganisms that goes back to the beginning of recorded history and almost certainly existed long before then. These fermentative activities have been utilised in the production of fermented foods and beverages, which are defined as those products that have been subordinated to the effect of microorganisms (bacteria, fungi or yeast) or enzymes to cause desirable biochemical changes. Fermented foods are produced all around the world with various manufacturing techniques, raw materials and different types of microorganisms.

Probiotic foods are defined as those that contain a single or mixed culture of microorganisms that affect beneficially the consumer's health. Recently, fermented foods and beverages have been given special attention and in particular that categorised as probiotic. This attention resulted from the fact that many different health benefits are attributed to the consumption of such a food products, which contain living microorganisms, commonly lactic acid bacteria.

The concept of consuming foods that contain healthy components is gaining ground in some countries such as the United States, Finland and Japan. Because of that, a lot of scientific researchers and legislators are actively working to expand the potential market for such foods in the European Union, the USA and Japan. The driving force behind this trend is that the prospective markets for such products in the United States and Japan combine to a total of $ 22.4 billion (Senders, 1998; The Japanese market,

1998). Same research has been carried out to investigate potential raw materials other than milk that could be used as media to grow probiotic microorganisms. Cereal grains are considered to be one of the most important fermentation media because they are a major source of dietary nutrients for people all over the world, despite of their deficiency in some basic nutrients.

Several methods can be used to ameliorate the nutritional quality of cereals, but probably one of the best ones is fermentation. Fermentation plays an important role in the manufacturing of cereal-based foods (e.g. bread and beer) because it is the simplest and most economical means to increase the nutritional value and utilisation of cereals. However, few attempts have been made to investigate the biochemical and nutritional changes during the fermentation of different types of cereals.

The malt grains and their derivatives (malt extracts) have been chosen due to the fact that during malt processing certain chemical changes take place which produce a suitable medium for microbial growth. In addition, malt has a very desirable flavour and reasonable price.

This work aims to investigate the possibility of fermenting malt grains and their derivatives by using two types of microorganisms (*Lactobacillus casei* shirota and *Saccharomyces cerevisiae*), and to determine whether such microorganisms will remain viable after fermentation in order to obtain a probiotic food. The taste and aroma of the fermentation products will also be taken into consideration to evaluate their potential commercial application.

# 2

# LIERATURE SURVEY

# 2. LITERATURE SURVEY

Cereal grains are major source of dietary nutrients, although the nutritional quality of cereals and sensory properties of their products are sometimes inferior or poor in comparison with milk and milk products, but other wise they are excellent foods. The reasons behind this are the lower protein content, the deficiency of certain essential amino acids, the lower starch availability, the presence of certain antinutrients and the coarse nature of the grains (Chavan and Kadam, 1989).

Several methods have been used to ameliorate the nutritional qualities of cereals. These include genetic improvement, amino acid fortification, and supplementation of complementation with protein concentrates and isolates or other protein-rich sources, such as grain legumes and defatted oil seed meals. The processing technologies used to improve the nutritional properties of cereals include cooking, sprouting, milling, and fermentation. Fermentation and sprouting are widely and routinely used in the Orient and in the Far East Asia to process grain legumes. However, such processing in the case of cereals is not very common except for brewing and baking. Lately, research shown that fermentation can be effectively used for improving the sensory and nutritional qualities of cereals and their products (Chavan and Kadam, 1989).

Fermentation is one of the oldest and most economical methods of producing and preserving foods (Chavan and Kadam, 1989; Billings, 1998). Microbial fermentations have played an important role in food manufacturing for thousands of years. Since the beginning of human civilisation there was an intimate companionship between human being, his fare and the fermentative activities of microorganisms. This companionship

goes back to the beginning of recorded history, and almost certainly existed long before (Imire, 1976; Sanders, 1992; F.A.O., 1998), with evidences dating back to 2500 BC. This has been shown in the form of paintings of the ancients Iraqis, the Sumerians, adding an inoculum to milk in order to complete the fermentation successfully (Power and Moore, 1994). Another evidence arose from the Roman civilisation; it has been marked down by the Roman historian Plinio who supported the use of fermented milks in the treatment of various pathological forms of gastrointestinal infections (O'Sullivan *et al.*, 1992).

Fermentation provides a natural way to preserve food products, to reduce volume of material to be transported, to destroy undesirable factors, enhance the nutritive value, improve appearance, colour and test of some foods, to salvage material otherwise not usable for human consumption, reduce energy required for cooking, and to make a safer product. The kind of raw material used for fermentation, methods or fermentation conditions, and sensory qualities of end products differ greatly from culture to culture around the world. The technologies for the industrial production of fermented products from milk, meat, fruits, and vegetables are well developed. The technology of fermentation of cereals for the commercial production of alcoholic beverages, such as beers, is also well known, although innovations to make the process more simple, economical, and efficient through biotechnology are currently under way. Such approaches have occasionally been applied to produce fermented foods from cereals, legumes or oil seeds on a commercial amount.

In the last twenty years, the number of books and articles dealing with indigenous fermented beverages and foods found around the whole world have rapidly increased (Steinkraus, 1983). Ever since, fermented products and the utilisation of

microorganisms have gained attention all over the world, and scientific researches are actively being carried out in the European Union (EU), Japan and the USA (Hirahara, 1998; Pagni, 1998).

Nowadays, the utilisation of microorganisms by the modern biotechnology industry represents a multi-billion-dollar business world-wide with implementations ranged from production of alcohol to waste recycling (Power and Moore, 1994). For example; the size of the market in the United State has been estimated at $ 13.4 billion (Sanders, 1998) and in Japan the market is worth $ 9.0 billion (The Japanese market, 1998). In the European, some countries (such as Finland) have placed big budgets to improve nutritional and eating quality of foods by the utilisation of microorganisms (Mattila-Sandholm, 1998b).

## 2.1 Fermented foods

Bacteria, yeasts, and molds have been utilised in the production of fermented foods for centuries around the world (Harlander, 1992). Yoghurt was first produced long time ago in the Middle East as a mechanism for preserving milk in hot climates. Yoghurt is produced using a food grade bacterial culture known as *Lactobacilli.* Its action thickens milk and helps preserve it. Beer and wine are other long-standing examples of fermented products (Quorn, 1997a).

Fermented products (foods or beverages) are defined as those products that have been subordinated to the effect of microorganisms (bacteria, fungi, or yeasts) or enzymes to cause desirable biochemical changes (Harlander, 1992; Garbutt, 1997).

The microorganisms used may be the microflora indigenously present on animal or vegetable products that serve as the substrates for fermentation, or these may be added as starter cultures (Harlander, 1992).

Several literature reviews on fermented foods are available in the literature since the late seventeenths of this century. Some reviews tried to cover the topic in general; e.g. the famous review of Steinkraus in 1983, while other authors base their reviews on the regions; e.g. Soni and Sandhu review in 1990 and Tanasupawat and Komagata in 1995.

## 2.2 Cereal-based fermented foods

Cereal grains are a very important source of nutrients for people all around the world, and they played a main role in human nutrition.

As previously mentioned, different methods have been used to produce foods with high nutritional quality from cereals. This work focuses on the use of fermentation, which is considered one of the oldest and most important methods of food processing.

This section will briefly cover the most common cereal-based fermented foods (both traditional and modern); their names, substrates, microorganisms involved, nature of use and their regions will be reviewed. The applications of different types of cereals will also be discussed in order to provide clear examples on cereal-based fermented foods and their ways of preparations.

The preparation of many indigenous or traditional fermented foods and beverages from food grains remains even today a household art except for a few products such

as nan and soy sauce that is prepared on a commercial scale (Chavan and Kadam, 1989). Some of the more common indigenous fermented foods prepared either from cereals or cereals-legume/oil seed mixtures traditionally consumed in different regions of the world are listed in Table 2.1.

In most of these products the fermentation is natural, and involves mixed cultures of, yeasts, bacteria and fungi. The common fermenting bacteria are species of *Leuconostoc, Lactobacillus, Streptococcus, Pediococcus, Micrococcus and Bacillus.* The mold specie involved is *Aspergillus*, although species of *Paecilomyces, Cladosporium, Fusarium, Penicillium* and *Trichothecium* are also found in certain products. The common fermenting yeasts are species of *Saccharomyces*, which results in alcoholic fermentation.

Lactic acid bacteria is a name of a group of bacteria belonging to a variety of genera (see Table 2.2) used in food fermentations. Its comprises mainly bacteria whose primary metabolic end product from carbohydrate metabolism is lactic acid, although poor lactate producers are sometimes included due to their association with food fermentation (Sanders, 1992). The amount of lactic acid or alcoholic formed and the changes in pH and acidity are scarcely measured or standardised during the preparation of such products at the domestic level. The fermentation products domestically prepared are often used as breakfast foods, snacks, flavouring and seasoning agents, colorants, desserts, beverages and porridges. Very few products such as tape, kisra idli, panky, jamin-bang, ogi, pozol, injera, dalaki, and bogobe are consumed as staples.

**Table 2.1** The most common indigenous cereal and cereal-legume–based fermented foods and beverages (Chavan and Kadam, 1989; Soni and Sandhu, 1990; Harlander, 1992; Adams, 1997; Sankaran, 1997; Knorr, 1998).

| Product | Substrates | Microorganisms involved | Nature of use | Regions |
|---|---|---|---|---|
| Dhokla | Rice or wheat and Bengal gram | *Leuconostoc mesenteroids, Lactobacillus fermenti, Streptococcus faecalis* | Breakfast or snack food | Northern India |
| Idli | Rice and black gram | *Leuconostoc mesenteroids, Lactobacillus fermenti, Lactobacillus delbrueckii, L. lactis, Streptococcus lactis, S. faecalis, Pediococcus cerevisiae*, yeast | Breakfast food | India, Sri Lanka |
| Miso | Wheat/rice/ barley and soybeans | *Aspergillus oryzae, Streptococcu faecalis, Pediococcus acidilactici, P. Halopilus, Micrococcus* sp., *Bacillus* sp., *Saccharomyces rouxii,* Other yeasts | Seasoning | Japan, China, Taiwan, U.S. |
| Shoyu (soy sauce) | Wheat and soybeans | *Aspergillus oryzae, Saccharomyces rouxii, Pediococcus halophilus, Lactobacillus delbrueckii* | Seasoning | Japan, China, Taiwan, U.S. |
| Dosa | Rice and Bengal gram | *Leuconostoc mesenteroids, Lactobacillus delbrueckii, L. fermenti, Streptococcus faecalis, Pediococcus*, yeasts | Breakfast or snack food | India |
| Hopper | Cereal/legume | *Pediococcus, Streptococcus, Leuconostoc* | Breakfast or snack food | India |
| Vada | Cereal/legume | *Pediococcus, Streptococcus, Leuconostoc* | Breakfast or snack food | India |
| Papadam | Cereal/legume | *Pediococcus, Streptococcus, Leuconostoc* | Breakfast or snack food | India |
| Adai | Cereal/legume | *Pediococcus, Streptococcus, Leuconostoc* | Breakfast or snack food | India |
| Kecap | Wheat and soybean | *Aspergillus oryzae, Lactobacillus* sp., *Hansenula* sp., *Saccharomyces* sp. | Flavouring agent | Indonesia |
| Tao-si | Wheat and soybeans | *Aspergillus oryzae* | Seasoning | Philippines |
| Taotjo | Roasted wheat meal or glutinous rice and soybeans | *Aspergillus oryzae* | Condiment | East India |
| Tauco | Cereals and soybeans | *Rhizopus oligosporus* or *Aspergillus flavus* | Seasoning | Indonesia, West Java |
| Sierra rice | Rough rice | *Aspergillus flavus, A. candidus, Bacillus subtilis* | Brownish-yellow dry rice | Ecuador |
| Ang-kak | Rice | *Monascus purpureus* | Dry red powder as colorant | Chain, Southeast Asia |
| Busa | Rice or millet | *Lactobacillus* sp., *Saccharomyces* sp. | Liquid drink | Syria, Egypt, Turkestan |

| Product | Substrates | Microorganisms involved | Nature of use | Regions |
|---|---|---|---|---|
| Lao-chao | Rice | *Rhizopus oryzae, R. Chinensis, Chlamydomucor oryzae, Saccharomycopsis* sp. | Paste, soft, juicy, glutinous consumed as such, as dessert or combined with eggs, seafood | Chain, Indonesia |
| Puto | Rice | LAB | Solid paste as seasoning agent, snack | Cambodia, Philippines |
| Tape | Rice or cassava | *Saccharomyces cerevisiae, Hansenula anomala, Rhizopus oryzae, Chlamydomucor oryzae, Mucor* sp., *Endomycopsis fibuliger* | Soft, solid staple | Indonesia |
| Anarshe | Rice | LAB | Breakfast, sweetened snack food | India |
| Torani | Rice | *Hansenula anomala, Candida quilliermondii, C. tropicalis, Geotrichum candidum* | Liquid as seasoning for vegetables | India |
| Kanji | Rice and carrots | *Hansenula anomala* | Liquid added to vegetables | India |
| Banku | Maize cassava | LAB, yeasts | Dough as staple | Ghana |
| Chicha | Maize | *Aspergillus* sp., *Penicillium* sp., yeasts, bacteria | Spongy, eaten with vegetable | Peru |
| Jaminbang | Maize | Yeasts, bacteria | Bread, Cake-like | Brazil |
| Kaanga-Kopuwai | Maize | Yeasts, bacteria | Soft, slimy eaten as vegetables | New Zealand |
| Kenkey | Maize | *Lactobacillus* sp., *Clostridium* sp., *Acetobacter* sp. | Mush, steamed eaten with vegetables | Ghana |
| Mahewu | Maize | LAB | Liquid, sour, non-alcoholic drink | South Africa |
| Injera | Sorghum, maize, wheat, | *Candida guilliermondii* | Bread-like staple | Ethiopia |
| Bouza | Wheat | LAB, yeasts | Thick, acidic liquid | Egypt |
| Merissa | Sorghum | *Saccharomyces* sp. | Liquid drink | Sudan |

| Product | Substrates | Microorganisms involved | Nature of use | Regions |
|---|---|---|---|---|
| Ogi | Maize, sorghum | LAB, *Cephalosporium, Fusarium, Aspergillus* and *Penicillium* spp., *Saccharomyces cerevisiae, Candida mycoderma* | Paste as staple or breakfast, weaning babies | Nigeria, West Africa |
| Pozol | Maize | Molds, Yeasts, bacteria | Spongy dough, water diluted and drunk as basic food | South-eastern Mexico |
| Rabdi | Maize | *P. acidilactici, Bacillus* sp., *Micrococcus* sp. | Semisolid mash eaten with vegetables | India |
| Pito | Maize, Guineacorn | *Lactobacillus* sp., *Leuconostoc* sp., *Saccharomyces* sp., *Candida* sp., *G. candidum* | Liquid drink | Nigeria |
| Jalebies | Wheat flour | *Saccharomyces bayanus*, LAB | Pretzel-like syrup-filled confection | India, Nepal, Pakistan |
| Minchin | Wheat gluten | *Paecilomyces, Aspergillus, Cladosporium, Fusarium, Syncephalastum, Penicillium* and *Trichothecium* ssp. | Solid as condiment | China |
| Nan | Unbleached wheat flour | *Saccharomyces cerevisiae, Lactobacillus, Streptococcus* ssp. | Solid as snack | India, Pakistan, Afghanistan, Iran |
| Kurdi | Wheat | Not known | Solid, fried crisp, salty noodles | India |
| Tarhana | Parboiled wheat meal and yoghurt (2:1) | LAB | Solid powder, dried seasoning for soups | Turkey |
| Kishk | Wheat, milk | LAB, *Bacillus* ssp. | Solid, dried balls, dispersed rapidly in water | Egypt, Syria, Arab world |
| Homanatto | Wheat flour | *Aspergillus oryzae, Streptococcus* spp. | Raisin-like soft, flavouring agent, for meat, fish as snack | Japan |
| Burukutu | Sorghum and cassava | LAB, *Candida* ssp., *Saccharomyces cerevisiae* | Creamy drink with suspended solids | Nigeria |
| Munkoya | Kaffir corn, millet or maize plus roots of munkoyo | LAB, yeasts | Liquid drink | Africa |

| Product | Substrates | Microorganisms involved | Nature of use | Regions |
|---|---|---|---|---|
| Sorghum beer | Sorghum | LAB, yeasts | Liquid drink, acidic, weakly alcoholic | South Africa |
| Bogobe | Sorghum | Not known | Soft porridge staple | Botswana |
| Kisra | Sorghum | LAB | Staple as bread | Nigeria |
| Dalaki | Sorghum | Not known | Thick porridge | Nigeria |
| Feni | Sorghum | Not known | Breakfast, snack food | India |
| Braga | Millet | Not known | Liquid drink | Romania |
| Darassum | Millet | Not known | Liquid drink | Mongolia |
| Thumba | Millet | *Endomycopsin fibuliger* | Liquid drink | Eastern India |
| Natto | Legume | *Bacillus subtilis var. natto* | Seasoning | Japan, China |
| Tempe | Legume | *Rhizopus oligosporus* | Seasoning | Japan, China |
| Koko | Maize, sorghum | *P. acidilactici* | Breakfast or snack food | Japan, Philippines |
| Crackers | Wheat | *Saccharomyces cerevisiae* | Solid as snack | India, Pakistan, Afghanistan, Iran |
| Boza | Wheat, millet, maize and other cereals, sugar or saccharine | *Lactobacillus* ssp., *Saccharomyces cerevisiae*, *Leuconostoc* ssp. | Thick, sweet, slightly sour beverage | Albania, Turkey Bulgaria, Romania |
| Busaa | Maize | *Lactobacillus helveticus, L. salivarus, L. casei, L. brevis, L. plantarum, L. buchneri, Saccharomyces cerevisiae, Penicillium damnosus* | Alcoholic beverage | Kenya |
| Chee-fan | Soybean wheat curd | *Mucor* sp., *Aspergyllus glaucus* | Cheese-like product, eaten fresh | China |
| Chinese yeast | Soybeans | *Mucoraceous* molds, yeasts | Eaten fresh with rice | China |
| Kaffir beer | Kaffir corn | *Lactobacillus delbrukii, L. plantarum, L. casei, Sacch. cerevisiae, Kloeckera apiculata, Leuconostoc* spp. | | South Africa |
| Sekete | Mize | *Sacch. cerevisiae, S. chevalieri, S. elegans, Lactobacillus plantarum, Lactococcus lactis, Bacillus subtilis, Aspergillus niger, A. flavus, Mucor rouxii* | Alcoholic beverage | Nigeria |
| Soybean milk | Soybeans | LAB | Drink | China, Japan |

**Table 2.2** Genera of lactic acid bacteria (McKay and Baldwin, 1990; Oberman and Libudzisz, 1996; Suskovic *et al.*, 1997; Batt, 1998).

| Genera of LAB | Cell form | Catal. | Gram (+/-) |
|---|---|---|---|
| *Lactobacillus* | Rods (Bacilli; coccobacilli) | - | + |
| *Streptococcus* | Spheres in chains (Cocci) | - | + |
| *Pediococcus* | Spheres in tetrads (Cocci) | - | + |
| *Lactococcus* | Cocci | - | + |
| *Leuconostoc* | Spheres in chains (Cocci) | - | + |
| *Bifidobacterium* | Branched rods | - | + |
| *Carnobacterium* | Cocci | - | + |
| *Enterococcus* | Cocci | - | + |
| *Sporolactobacillus* | Rod | - | + |
| *Lactosphaera* | Cocci | - | + |
| *Oenococcus* | Cocci | - | + |
| *Vagococcus* | Cocci | - | + |
| *Aerococcus* | Cocci | - | + |
| *Weissella* | Cocci | - | + |

The type of bacterial flora developed in each fermented food depends on the water activity, pH, salt concentration, temperature and the composition of the medium. Lactic acid bacteria are perhaps the most widespread and desirable microorganisms in food fermentations. They convert most of the available carbohydrates into lactic acid and small amounts of acetic acid, which result in a lower pH value of the product (Tanasupawat and Komagata, 1995). They are acid forming, which stimulates the digestion process (Billings, 1998).

During cereal fermentations, ranges of volatile compounds are formed (see Table 2.3), which contribute to a complex blend of flavours in the products (Chavan and Kadam, 1989).

**Table 2.3** Compounds formed during cereal fermentation (Chavan and Kadam, 1989).

| **Organic acids** | | **Alcohols** | **Aldehydes and ketones** | **Carbonyl compounds** |
|---|---|---|---|---|
| Butyric | Heptanoic | Ethanol | Acetaldehyde | Furfural |
| Succinic | Isovaleric | *n*-Propanol | Formaldehyde | Methional |
| Formic | Propionic | Isobutanol | Isovaleraldehyde | Glyoxal |
| Valeric | *n*-Butyric | Amy alcohol | *n*-Valderaldehyde | 3-Methyl butanal |
| Caproic | Isobutyric | Isoamyl alcohol | 2-Methyl butanol | 2-Methyl Butanal |
| Lactic | Caprylic | 2,3-Butanediol | *n*-Hexaldehyde | Hydroxymethyl furfural |
| Acetic | Isocaproic | β-Phenylethyl alcohol | Acetone | |
| Capric | Pleargonic | | Propionaldehyde | |
| Pyruvic | Levulinic | | Isobutyraldehyde | |
| Plamitic | Myristic | | Methyl ethyl ketone | |
| Crotonic | Hydrocinnamic | | 2-Butanone | |
| Itaconic | Benzylic | | Diacetyl | |
| Lauric | | | Acetoin | |

### 2.2.1 Applications of cereal-based fermentation in food and drink production

The microbial fermentation, either natural or with pure cultures, of cereals, legumes, oil seeds meals, and their mixtures is known to ameliorate their nutritional values, sensory properties, and functional qualities. However, many fermented foods are traditionally prepared and consumed all over the world without much knowledge about their nutritional quality (Chavan and Kadam, 1989).

Fermentation may give the most simple and economical means to increase the quality and utilisation of cereals. Until now, few attempts have been done to study and investigate the biochemical and nutritional changes during cereal fermentation. In the following sections, a very brief discussion on some traditional cereal-based fermented foods and beverages is presented as examples of possible applications of fermented cereals. The raw materials used and the methods of preparations are also explained.

### 2.2.1.1 Indigenous foods

Traditional fermented foods prepared from most common types of cereals (such as rice, wheat, corn and sorghum) are well known in many parts of the world. Some are consumed as colorants, spices, beverages and breakfast or light meal foods, while few are used as main foods in the diet.

### A. Rice-based fermented foods

**Idli:** It is an old fermented food used as breakfast in southern India (Sankaran, 1997). It is prepared by steaming a fermented better or dough from different proportions of rice and black gram (Chavan and Kadam, 1989; Soni and Sandhu, 1990; Sankaran, 1997). The preparation, physiochemical and microbiological changes, organoleptic characteristics, nutritional quality and used of other idli preparation have been reviewed by Soni and Sandhu in 1990, Beuchat in 1983, Reddy and his team in 1980 (Steinkraus, 1983; Chavan and Kadam, 1989), Ramkrishnan in 1977 and by Purushothaman *et al.* in 1977 (Purushothaman, 1977; Chavan and Kadam, 1989). Mukherjee and his assistants have identified the bacteria present in fermented idli batter which include *Lactobacillus delbrueckii, L. fermenti, L. lactis, Streptococcus faecalis, Pediococcus cerevisiae*, in addition to *Leuconostoc mesenteroides* (Ramkrishnan, 1977; Purushothaman, 1977; Chavan and Kadam, 1989; Soni, and Sandhu, 1990). The yeasts, such as *Oidium lactis*, *Torutopsis holmii*, *T. Candida* and *Tricholsporon pullulans* have also been implicated (Chavan and Kadam, 1989; Soni, and Sandhu, 1990). In 1961 Rao reported an increase in the contents of all essential amino acids of idli during his observations on some fermented foods (Chavan and Kadam, 1989; Steinkraus, 1995). Rajalakshmi and Vanaja have found in 1967 that fermentation of idli batter appears to have a significant effect on elimination of

reduction of antinutrients, such as phytic acid, enzyme inhibitors and flatus sugars (Chavan and Kadam, 1989; Steinkraus, 1995).

**Dosa:** This is another popular traditional fermented product consumed as breakfast or snack food in India. It is very similar to idli batter. Following fermentation, dosa is quickly fried as a thin, fairly crisp pancake and eaten directly. Sankaran (1997) and Soni and Sandhu (1990) published excellent reviews on the Indian fermented food dosa. Chavan and Kadam (1989) approached dosa in their study 'Nutritional Improvement of Cereals by Fermentation'. On the other hand, Ramkrishnan (1977) and Purushothaman *et al.* (1977) provides a good study about dosa batter fermentation. The Sankaran review (1997) covered the microbiological, the physical and the biochemical changes for dosa during fermentation as well as the nutritive value.

**Dhokla:** It is a fermented product prepared from legume dhals or mixture of rice and chickpea flour. The fermented batter is poured into a graced pie tin and steamed in an open steamer (Ramkrishnan, 1977; Purushothaman, 1977; Chavan and Kadam, 1989). Rajalakshmi and vanaja (1967) and Sankaran (1997) reported the improvement in dhokla contents when fermented. Similarly, Aliya and Geervani (1981) observed a significant improvement in the biological value and net protein utilisation of dhokla due to fermentation (Chavan and Kadam, 1989). Dhokla ingredients, details of manufacturing, fermentation procedure, physical and biochemical changes during fermentation are well explained and reviewed by Ramkrishnan in 1977 and Purushothaman and his colleagues in the same year. In 1997 another review has been published by Sankaran.

## B. Wheat-based fermented foods

**Soy Sauce or Shoyu:** This fermented product is mainly used as an all-purpose seasoning in Japan, China and the Far East countries (Yokotsuka and Sasaki, 1991). Two distinct basic processes are used to prepare soy sauce. The Japanese soy sauces are prepared by fermentation with microorganisms and the Chinese sauces involve chemical modifications of the ingredients. Only Japanese soy sauces are true traditional fermented foods. More than 84% of the Japanese soy sauce contain equal proportions of wheat and soybean (Yokotsuka, 1977, 1986; Chavan and Kadam, 1989; Yokotsuka, 1991). The steps involved in the preparation of the ingredients in shoyu have been well described by Beuchat (1978, 1983), Ogawa and Fujita (1980), and Yokotsuka (1977, 1986, 1991). *Aspergillus oryzae* and *A. soyae* strains are widely used in shoyu fermentation (Yokotsuka, 1977, 1986; Chavan and Kadam, 1989; Yokotsuka, 1991). The effects of feeding shoyu for long-term have been studied by Yokotsuka and Sasaki (1981) and by MacDonald and Duck (1976) (Chavan and Kadam, 1989). Steinkraus *et al.* (1985), Franta *et al.* (1986), Ayres *et al.* (1987) and Rowan *et al.* (1997) have covered the microbiological aspect of shoyu. Furthermore, Fukushima (1988) and Yokotsuka (1991) have written a detailed, comprehensive review on soy sauce (Yokotsuka, 1977, 1986, 1991).

**Kishk (Fugush):** It is a fermented food prepared from parboiled wheat and milk and consumed in the Arabian World and Iran (Chavan and Kadam, 1989). The milk undergoes a lactic fermentation, and the lactic acid bacteria responsible for this fermentation include *Lactobacillus plantarum, L. casei and L. brevis* as reported by Beuchat in his study in indigenous fermented foods published in 1983 (Abd-el-Malek and Demerdash, 1977; Mahmoud, 1977; Morcos, 1977a; Chavan and Kadam, 1989).

The product is stored in the form of dried balls, brownish in colour with a rough surface and hard texture. Kishk is a balanced food with excellent preservation quality, richer in B vitamins than either wheat or milk, and well adapted to hot climates by its content of lactic acid (Abd-el-Malek and Demerdash, 1977; Mahmoud, 1977; Morcos, 1977a). The practical nutritional importance of kishk has been determined by Morcos and his teamwork (1973a), who found that kishk improves the basic cereal protein diet by adding animal protein in a highly acceptable form. In addition, kishk prolongs the availability of the animal protein by converting it along with wheat to a stable food. A full description about kishk, its microbiological activities and its biochemical changes during fermentation have been reviewed and studied by Abd-el-Malek and Demerdash (1977), Mahmoud (1977) and Morcos (1977a).

**Tarhana (Trahanas):** Tarhanans is a fermented food made from crushed wheat and fermented sheep milk (Chavan and Kadam, 1989) boiled together, dried and stored in the form of biscuits (Economidou and Steinkraus, 1977). The fermentation process and the type of product is very similar to kishk. The sheep milk yoghurt contains *S. thermophilus* and *L. bulgaricus* as the major fermenting organisms. Since tarhana consist mainly of wheat and milk protein, it is of high nutritional value (Economidou and Steinkraus, 1977). An excellent scientific survey done by Economidou and Steinkraus (1977) covers several important points about trahana, such as, the preparation steps, the microbiological and biochemical changes during fermentation, the nutritional value and fermentation controls.

## C. Corn-based fermented foods

**Ogi:** It is porridge prepared and consumed from fermented corn in West African countries (Chavan and Kadam, 1989). It has a very smooth texture similar to a hot blancmange and acid taste reminiscent of yoghurt (Banigo, 1977; Onyekwere and Akinrele, 1977; Moss *et al.*, 1984; Onyekwere *et al.*, 1989a). Ogi is consumed as porridge (pap) which is considered as the most important traditional food for weaning infants and the major breakfast cereal for adults (Banigo, 1977; Onyekwere and Akinrele, 1977; Moss *et al.*, 1984; Onyekwere *et al.*, 1989b). The sour porridge fermentation takes place without the intentional addition of any inoculum and the microorganisms in ogi have been isolated and identified by Akinrele in 1970 (Banigo, 1977; Onyekwere and Akinrele, 1977; Moss *et al.*, 1984; Chavan and Kadam, 1989; Onyekwere *et al.*, 1989a). Banigo and Muller (1972) obtained the microbiology of fermentation of ogi and the biochemical changes occurring in the substrates during its fermentation. They also covered in their study the nutritional value of ogi (Banigo, 1977; Onyekwere and Akinrele, 1977; Moss *et al.*, 1984; Chavan and Kadam, 1989; Onyekwere *et al.*, 1989a). Problems in industrialisation of ogi fermentation are covered in a closely related paper by Onyekwere *et al.* (1989a, 1989b).

**Chicha:** This is a fermented corn product widely consumed in South America (Chavan and Kadam, 1989; Steinkraus, 1995). Chicha is a unique fermentation in which, traditionally, saliva serves as the source of amylase for conversion of starch to fermentation sugars (Escobar, 1977; Escobar *et al.*, 1977). Soriano (1938) and Gomez (1949) reported that yeasts, particularly *S. cerevisiae*, and bacteria of the genus *Lactobacillus, Leuconostoc, Acetobactor* with various molds such as *Aspergillus* were the primary fermenting organisms in chicha (Escobar, 1977; Escobar *et al.*, 1977). In

1983 Beuchat followed the steps for the production of chicha and found that a natural lactic acid fermentation occurs, giving a slightly acidic taste to the end product (Chavan and Kadam, 1989). Escobar *et al.* (1977) reported that the nutritive value of chicha is relatively high due to the high content of vitamin B and protein.

## D. Sorghum-based fermented foods

**Injera (Enjera):** This is a leavened, flat, round traditional bread made from different cereals consumed in Ethiopia (Chavan and Kadam, 1989; Odunfa and Oyewole, 1998). Tef is the major cereal ingredient in Ethiopian enjera (Vogel *et al.*, 1977; Gashe *et al.*, 1982; Dirar, 1993a; Ashenafi, 1994). The sorghum grains used to prepare injera are dehulled manually or mechanically and milled to flour. The microorganisms involved in fermentation of injera are mainly yeasts. Some fungi including *Pullaria, Aspergillus, Penicillium, Rhodotorula, Hormodendrum, Candida* and number of unidentified bacteria have been isolated and identified by Stewart and Getachew in 1962 (Vogel *et al.*, 1977; Gashe *et al.*, 1982; Dirar, 1993a; Ashenafi, 1994). Enjera is rich in calcium and iron, so it is expected to have a very high nutritional value. A complete review about the manufacturing details, microbiological activities, biochemical and nutritive changes occurring during enjera fermentation has been reviewed by Vogel *et al.* (1977), Gashe *et al.* (1982), Dirar (1993a) and Ashenafi (1994).

**Kisra (Aseeda or Aceda):** This is a thin pancake like leavened bread prepared from sorghum flour (Odunfa and Oyewole, 1998). It is a staple food in the diet of most people in the Arabian Gulf, Sudan (Abdel Gadir and Mohamed, 1977; Chavan and Kadam, 1989; Dirar, 1993b) and Iraq. The main microorganisms isolated in kisra

are *Lactobacillus* sp., *Acetobacter* sp. and *S. cerevisiae*. Other microorganisms may be present but they have not been identified (Abdel Gadir and Mohamed, 1977; Chavan and Kadam, 1989; Dirar, 1993b). El-Tinay and his teamwork (1979) have studied the biochemical changes occurring during the fermentation of sorghum flour for kisra preparation (Chavan and Kadam, 1989). They found there is a slight increase in the protein and fibre content during fermentation, as well as an appreciable drop in starch and sugars concentration.

### 2.2.1.2 Fermented beverages

In addition to the cereal-based fermented foods there are several types of cereal-based fermented drinks produced around the world, which will be covered in brief in this section. They can be both alcoholic and non-alcoholic beverages, and they can be classified based on the raw materials used or the type of fermentation involved in the manufacturing process. Alcoholic beverages can be classified into wines and beers, while the great majority of non-alcoholic fermentations are souring, mainly lactic acid fermentations (Chavan and Kadam, 1989; Fleet, 1998).

**Beers:** They are commonly made from cereals. The technology for production of European barley beer and the biochemical and microbiological changes that take place during malting, fermentation and subsequent processing and storage are well documented (Hules *et al.*, 1980). In 1982 Novellie has compiled information about a few types of South African beer (Chavan and Kadam, 1989), and Haggblade and Holzapfel (1989a, 1989b) have provided a comprehensive review on both traditional processing and industrialisation of Africa's indigenous beer. The main microorganism involved in beer fermentation is *Saccharomyces* spp. (Chavan and Kadam, 1989;

Fleet, 1998). The nutritional considerations of beer have been reviewed by Nout (1981), Sanni (1993), Steinkraus (1996), Potter and Hotchkiss (1997), Fleet (1998) and Godfrey (1998).

**Sake (Rice Wines):** This is a traditional fermented alcoholic beverage consumed particularly in Japan and china (Lotong, 1998) prepared from rice. The brown rice is polished and steamed. A part of steamed rice is used to grow *A. Oryzae* which produces different types of enzymes required for sake brewing. The seed mash is traditionally obtained by natural lactic acid fermentation involving various aerobic bacteria, wild yeasts, lactic acid bacteria, and sake yeasts (Chavan and Kadam, 1989; Yokotsuka and Sasaki, 1991; Lotong, 1998). Before used it is diluted with water in order to adjust the taste and the colour. The best reference about sake can be found in the reviews done by Muraki (1972), Kodama (1977), Yoshizawa and Ishikawa (1988), and Media-Akita (1997).

**Egyptian Bouza:** This is a fermented alcoholic wheat beverage known since the times of the pharaohs (Morcos *et al.*, 1973b; Morcos, 1977b). It is a light yellow, thick, sour alcoholic drink consumed mainly in Egypt, Turkey and in some Eastern Europe countries (Odunfa and Oyewole, 1998). Since the beginning of the century the Egyptian ministry of Public Health no longer issues licenses for bouza shops, since Islamic religious beliefs are opposed to the consumption of alcohol (Morcos, 1977b). The preparation steps of bouza, have been reviewed by Morcos (1977b). The biochemical changes of wheat occur during bouza fermentation have been studied by Morcos *et al.* (1973b) who found that the low pH (3.9-4.0) and the high acidity of bouza indicate a fermentation by lactic acid bacteria and the alcohol is due to yeast

fermentation (Odunfa and Oyewole, 1998). The protein content of bouza ranges from 1.5-2.0% and due to the alcoholic fermentation involved in its formation, a significant contribution of vitamin B can be expected.

**Non-Alcoholic fermented beverages:** Although the yeast fermented alcoholic beverages are widely used, traditionally prepared non-alcoholic products are also common in some regions of the world. They are usually sour, being lactic acid the main souring agent. The high acid concentration of such beverages acts as a preserver. Maize, sorghum, and millets in particular are often excellent sources of *Lactobacilli* (Chavan and Kadam, 1989). Mahewa, amahewu and magou are some of the traditional non-alcoholic sour beverages made from corn meal and consumed in Africa (Chavan and Kadam, 1989) and some Arabian Gulf countries.

The major control microorganism in the spontaneous fermentation is *S. lactis* (Schweigart, 1970). The nutritional value of such fermented products is sometimes poor, but it can be easily increased. For example, by the addition of fish flour to mahewu, the protein contents increase which leads to a great improvement in its nutritional properties. Hesseltine (1979) reviewed the traditional and modern processes of making mahewu (Chavan and Kadam, 1989). The microbiology and the biochemical changes of mahewu during its fermentation have been studied by van der Merwe and his colleagues (1965) and by Schweigart in his work published in 1970.

## 2.3 Functional foods

The concept of providing functional foods containing healthful components is gaining ground in some countries such as the United States, Finland and Japan. Functional

foods are defined as those modified foods that provides a health benefit beyond satisfying traditional nutrient requirements (see Table 2.4). Recently, many new terms

**Table 2.4** Definitions of functional food (Wood, 1997; EarthNet SuperHealth Products, 1998; Hirahara, 1998; Mattila-Sandholm and Salminen, 1998; Roberfroid, 1998; Sanders, 1998; Arnold, 1999; Short and Merryfield, 1999; Roberfroid, 1999).

| Term | Definition |
|---|---|
| Functional food | A modified food or food ingredient that provides a health benefit beyond satisfying traditional nutrient requirements. |
| Designer foods or (FOSHU) | Foods that are expected to have certain health benefits and have been claiming that a person using them for a specified health use may expect to obtain the health use through the consumption thereof or they can be defined as foods designed to meet a particular objective:<br>❑ Being suitable for a special diet<br>❑ Stimulating a particular function<br>❑ Treating an illness, such as:<br>• Pharmafoods<br>• Nutraceutical |
| Pharmafoods | A special type of food dictated in the United States that fulfil the following conditions:<br>❑ Must be used under medical supervision<br>❑ Must be for a disease with well defined, specific nutrient characteristics<br>❑ Based on recognised scientific principles<br>❑ Must provide medical evaluation |
| Nutraceutical | A nutraceutical is a food or part of a food that offers medical and/or health benefits including prevention or treatment of disease. |
| Prebiotic | A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health (Molin, 1999). |
| Probiotic | A mono or mixed culture of microorganisms which when applied to animal or man affect the host beneficially. |
| Synbiotic | A mixture of prebiotic and probiotic. |
| Novel food | A new or novel product which is either produced in a different way or has a different composition from that of previously marketed products. |

have been developed in this field such as designer foods, prebiotic foods, probiotic foods and synbiotic foods. All types of foods defined by any of these terms can be considered as functional food, but with some differences in the main definition of each term and that due to their beneficial effects on the host (man or animal) (see Table 2.4) (Schaafsma and Huis in't Veld, 1996).

### 2.3.1 Probiotic foods

The use of live microbial products (foods or beverages) for animal feed applications has increased over the last fifteen years and today it represents a section of the fermentation industry that is very quickly expanding. The predominate fields of employment of live microbial in animal feed and forage are as probiotics and silage inoculants which form the bulk of the market in Europe (Power and Moore, 1994).

The term probiotic is derived from the Greek language, and means "for life" (being the antonym of antibiotic). In the last thirty years the term probiotic has had different definitions. It was first used in 1965 by Lilly and Stillwell to characterise substances produced by one protozoan that provoked the growth of another (O'Sullivan *et al.*, 1992; Power and Moore, 1994; Introducing Probiotics, 1998). Sperti in 1971 has used it to describe tissue extracts that stimulated microbial growth (O'Sullivan *et al.*, 1992). In 1974, without taking into consideration the earlier definitions, Parker used the word "probiotic" to describe animal feed supplements - organisms and substances which have a beneficial influence on the host animal by contributing to its intestinal microbial balance. Fifteen years later, in 1989, Roy Fuller redefined probiotics as a live microbial supplement that beneficially affects the host animal by improving its intestinal microbial balance. This revised definition highlighted the significance of

live microorganisms as the necessary component of an effective probiotic (O'Sullivan *et al.*, 1992; Introducing Probiotics, 1998). However, the definition of probiotic has since then bordered to cover examples of treatments with live bacteria that are not controlled by mouth (medical injections) and that do not exert their influence through the intestinal microflora. Huis In't Veld and Robert Havenaar at the Netherlands Organisation for Applied Scientific Research have expanded the definition of probiotic to a mono- or mixed culture of live microorganisms that applied to man or animal (e.g. as dried cells or as a fermented product), affect beneficially the host by improving the properties of the indigenous microflora (O'Sullivan *et al.*, 1992; Shortt, 1998).

In other words, probiotic are live bacteria that can resist the rigorous human digestive system, which includes of course surviving the stomach acidity and bile (Shortt, 1998). This definition indicates that probiotic products contain live microorganisms and ameliorate the health conditions of the host by exerting beneficial effects in the mouth or gastrointestinal tract (taken as food or capsules) in the upper respiratory tract or in the urgential tract (O'Sullivan *et al.*, 1992).

Sanders (1998) has provided the latest definition of probiotic as a mono or mixed culture of microorganisms which when applied to animal or man affect the host beneficially. The definition is obviously derived from Huis in't Veld and Robert Havenaar's definition.

In recent years an increased emphasis has been placed on studies of the probiotic activity of lactic acid bacteria, which may play an important role in improving the

intestinal microflora balance of the host (man or animals) (Suskovic *et al.*, 1997). The attention has generally focused on the exploitation of strains belonging to the genera *Bifidobacterium* and *Lactobacillus*, although bacteria from other genera have also been included (O'Sullivan *et al.*, 1992).

### 2.3.2 Effects of probiotic products on the human and animal health

Probiotics in the form of fermented milk products have been consumed all over the world probably for thousands of years. In this century many different health benefits have been claimed result from the consumption of foods containing live microorganisms, specifically lactic acid bacteria (Scheinbach, 1998). In 1906 Elie Metchnikoff first provided a scientific explanation of the beneficial influences of lactic acid bacteria present in yoghurts. He propounded that the consumption of large amounts of yoghurt containing *Lactobacillus* ssp. would result in the replacement of toxin producing bacteria existing in the intestine, and would thus result in better health and an increased lifetime. In 1906, Henri Tissier advised the administration of *Bifidobacteria* to infants suffering from diarrhoea, He believed that the *Bifidobacteria* displace putrefactive bacteria responsible for gastric upset, while re-establishing themselves as the preponderant intestinal microorganism (Ganguli, 1988; O'Sullivan *et al.*, 1992; Introducing Probiotics, 1998; Scheinbach, 1998).

Recently, the concept of providing functional foods containing healthful components rather than getting rid of potentially detrimental ones (e.g. saturated fat) is gaining ground in some countries such as the United States and Japan. Functional foods, designer foods (or foods for specified health use such as pharmafoods or medical foods and nutraceuticals) are synonyms for foods with elements that can inhibit and

remedy diseases (see Table 2.4). A probiotic may also be functional food, but more specifically it is a live microbial feed supplement that beneficially affects the host, man or animal, beyond rectifying for traditional nutrient insufficiency by improving its intestinal balance (Schaafsma and Huis in't Veld, 1996). Consequently, it may be considered as a functional food with the particular property of containing live, beneficial microorganisms. Regulation of the intestinal microbial balance arises from the rivalry among the various bacterial species that survive the passage through the upper gastrointestinal tract and colonise the human colon (see Figure 2.1 and Figure 2.2). A health advantage can also result from the ability of an ingested microorganism to donate an enzyme to the small intestine, e.g. β-galactosidase (lactase), that the host needs (see Figure 2.2) (Scheinbach, 1998). The healthful fermentation activities of the microorganisms in the large intestine (human colon) have been described by Hoover in 1993 (see Figure 2.3).

Over the last 90 years several studies have been done supporting the therapeutic benefits of probiotics in humans and animals' health (Huis in't Veld and Havenaar, 1991). These healthy and nutritional effects are listed in Table 2.5 (O'Sullivan *et al.*, 1992; Introducing Probiotics, 1998).

The mechanisms by which these benefits are derived are not yet fully understood (Ganguli, 1988; Introducing Probiotics, 1998), but according to several studies by various scientists, probiotics could improve health through one or more of the following mechanisms (Conway, 1996; Richardson, 1996; Holzapfel *et al.*, 1998; Introducing Probiotics, 1998; Scheinbach, 1998):

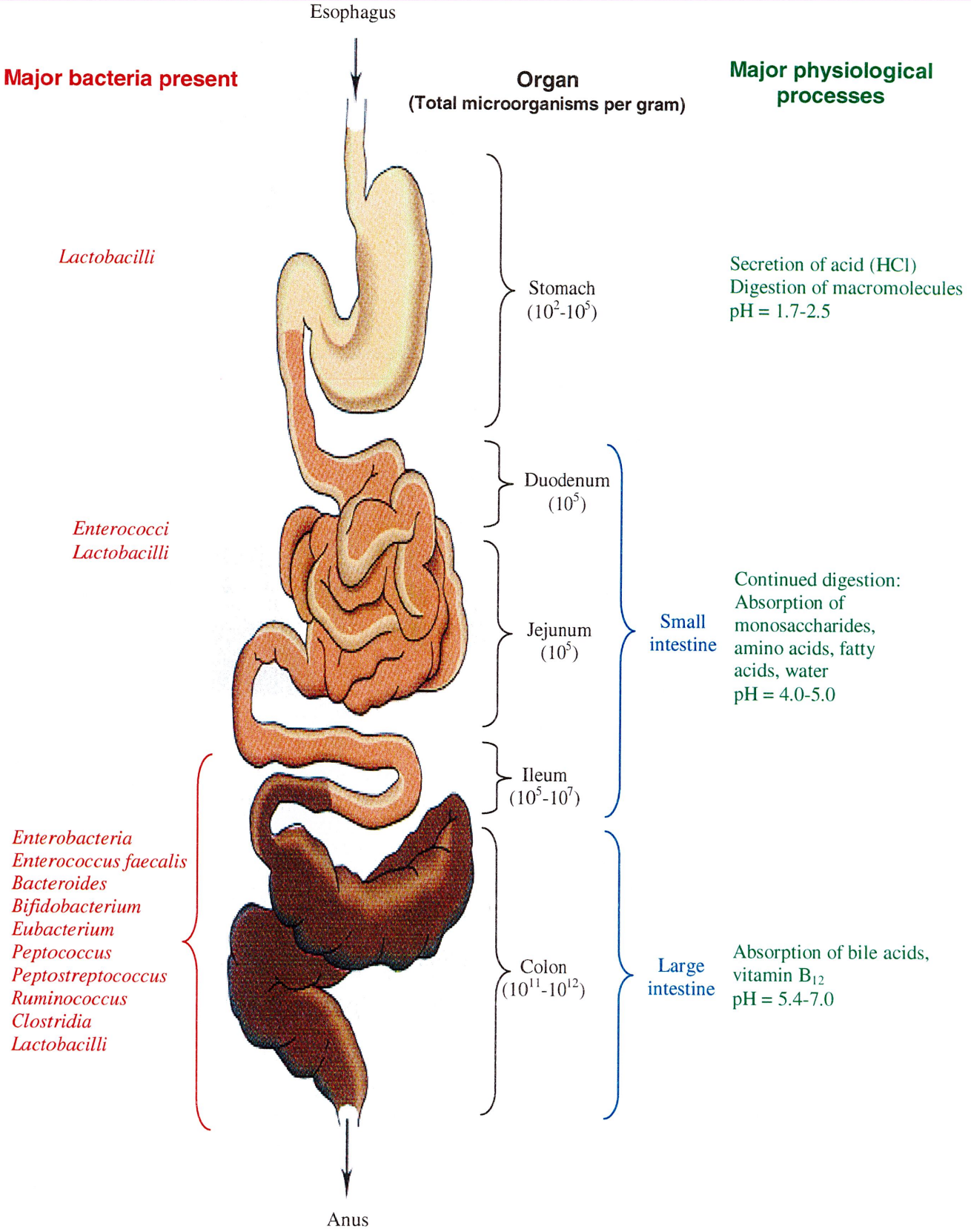



**Figure 2.1** The human gastrointestinal tract showing the functions of the different organ and the distribution of microorganisms (Marshall, 1991; Madigan *et al.*, 1997; Lee *et al.*, 1999).

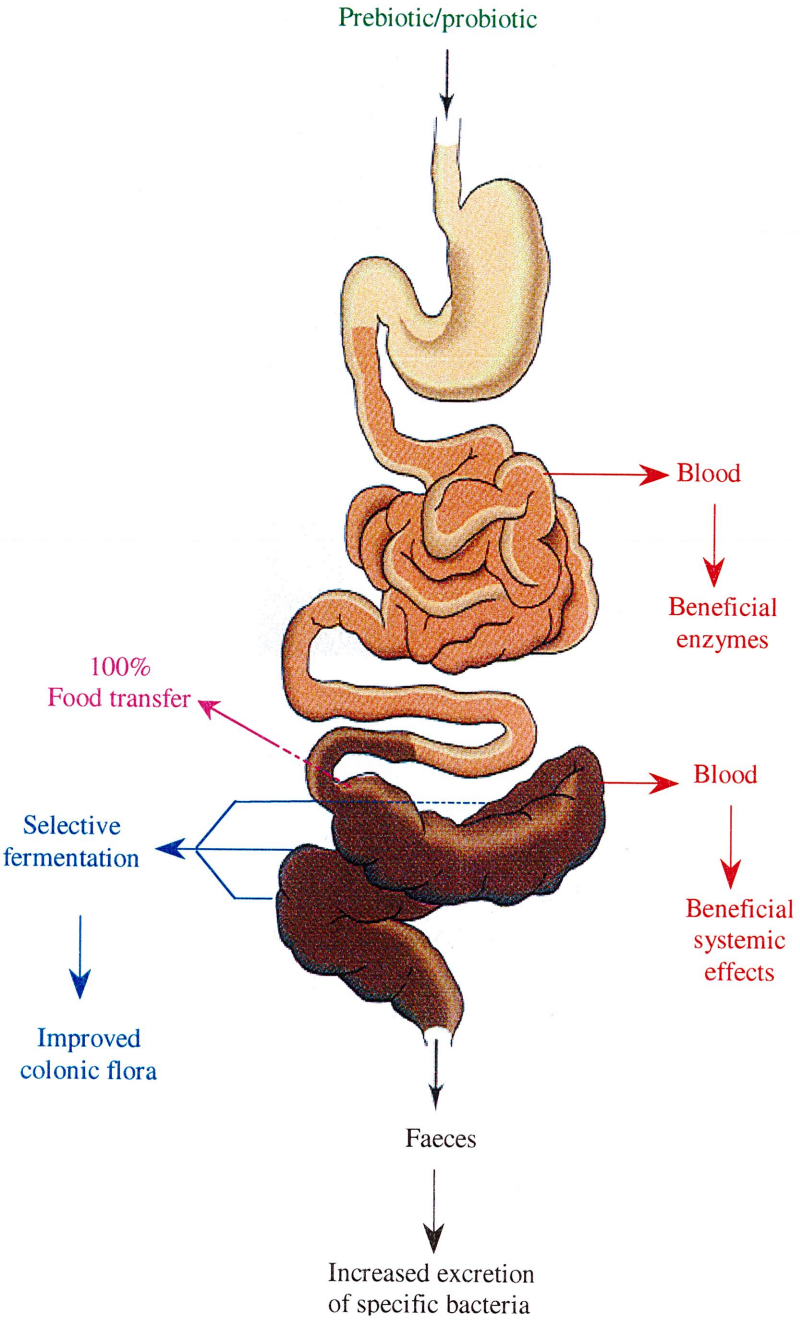



**Figure2.2** Diagrammatic representation of the probiotic and prebiotic concepts (Gibson and Beaumont, 1996).

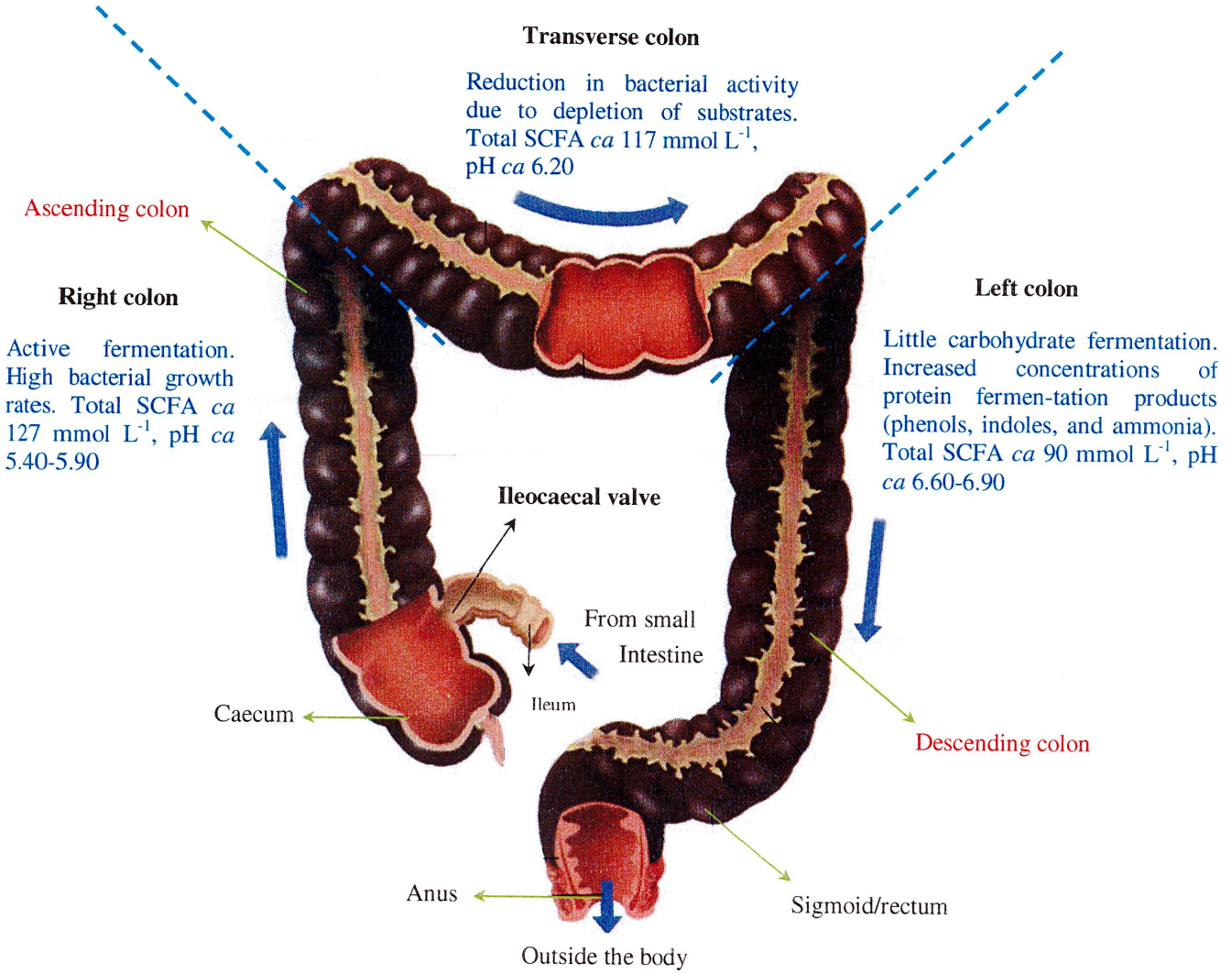



**Figure 2.3** Fermentation activities of bacteria in the large intestine and food pathway in the human colon (Hoover, 1993).
**SCFA** Short chain fatty acids

**Table 2.5** Claimed beneficial effects and therapeutic applications of probiotic bacteria in humans and animals (Marteau *et al.*, 1990; Daly, 1991; Hose and Sozzi, 1991; O'Sullivan *et al.*, 1992; Hoover, 1993; Marks, 1996; Richardson, 1996; Kikuchi-Hayakawa *et al.*, 1997; Wood, 1997; Mattila-Sandholm, 1998a; Scheinbach, 1998; Introducing Probiotics, 1998; Shortt, 1998; Arnold, 1999).

| **Beneficial effects** |
|---|
| Maintenance of normal intestinal and urogenital microflora |
| Alleviation of lactose intolerance |
| Reduction of serum cholesterol levels |
| Anticarcinogenic activity |
| Stimulation of the immune system |
| Inhibition of microbial pathogens |
| Antimutagenic activity |
| Inhibition of Candida albicans |
| Improved nutritional value of food. Lactobacilli and Bifidobacteria have been reported to produce folic acid, niacin, nicotinic acid, thiamin, riboflavin, pyridoxine, biotin, vitamins K, B1, B2, B6, B12, promote the calcium absorption and increase the bioavailability of other nutrients. |
| **Therapeutic applications** |
| Protection against traveller's diarrhoea |
| Prevention of infantile diarrhoea |
| Reduction of antibiotic-induced diarrhoea |
| Protection from colon/bladder cancer |
| Reduction of side effects of hepatic encephalopathy |
| Aid in cases of hypo- and hyperchlorohydria |
| Prevention of osteoporosis |
| Reflorastation post antibiotic therapy |
| Treatment of infantile diarrhea |
| Reduction in AIDS related diarrhea |
| Reducing incidence of intestinal and vaginal candidiasis |
| Reduction of lactose intolerance |
| Reducing fecal mutagenicity |
| Reducing fecal bacterial enzyme activity |
| Reduction of constipation symptoms |
| Prevention and Reduction of hypercholesterolaemia |
| Prevention and Reduction of occurrence of urogenital infections in women |

- Competition for substrates.
- Actuation of the immune system.
- Formation of acids, hydrogen peroxides, carbon dioxide, ethanol, or bacteriocins bactericidal to groups that negatively impact health.

- ❍ Competition with pathogens for mucosal binding sites; for adhesion receptors (Salminen *et al.*, 1996).
- ❍ Release of substances antagonistic to enteropathogenic microorganisms such as *Lactocidin, Lactobicillin, Acidolin* which considered as antagonistic substances.
- ❍ Production of short chain fatty acids (SCFAs) which are important due to their different effects such as:
  - Preferential fuel for mucosalcells.
  - Stimulation of colonic epithelial cell growth.
  - Acetic acid (the most plentiful SCFA produced by *Bifidobacteria*) has antimicrobial activity against yeasts, molds and certain types of bacteria.
- ❍ Inhibition of certain carcinogen-producing faecal bacterial enzymes.
- ❍ Reduction of colonic pH inhibiting the growth of acid-sensitive, undesirable microorganisms.
- ❍ Production of lactase.
- ❍ Association with some antimicrobial factors such as low redox potential, nutrient depletion, crowding and diacetyl.
- ❍ Synthesis of vitamins and growth factors for host intestinal cells.

Justification for health claims comes mainly from animal studies. While there is some evidence to demonstrate these processes occur *in vitro* and in animal models, the effectiveness of probiotics in humans remains thoughtful due to the lack of well-designed nutritional pathways.

Probiotic products consumed by humans' fall into three different classes:

- ❍ Infant foods.
- ❍ Cultured milks.
- ❍ Pharmaceutical preparations.

About 75% of the wet weight of human fecal output is composed of bacteria and each gram contains at least 1.0 x $10^{11}$ organisms of at least 50 genera, belonging to over 400 different species, which form the most complex microbial ecosystem that ever known (Scheinbach, 1998). Among this enormous number of species of intestinal origin, the most repeatedly utilised are *Bifidobacterium bifidum, Bifidobacterium infantis, Lactobacillus casei, Lactobacillus acidophilus, Enterococcus faeium* and *Propionibacterium freudenreichii* subsp. *Shermanii.* A more extensive list of bacteria used either in dairy or pharmaceutical probiotic preparations is presented in Table 2.6.

### 2.3.3 Characteristics of a good probiotic

The use of lactic acid bacteria (LAB) in foods has a long history and *Lactobacilli* have generally recognised as safe (GRAS) (Lee and Salminen, 1995; Holzapfel *et al.*, 1998). Many scientists have recently reviewed the safety LAB. They concluded that the frequency of occurrence of lactic acid bacteria as opportunistic bacteria is extremely rare (Lee and Salminen 1995; Conway, 1996). On the other hand, the occurrence of any virulence factors in LAB remains to be shown. The International Union of Microbiological Societies reviewed the literature and concluded that the clinical data do not support the fact that *Lactobacilli* and related organisms represent such risks. However, as new probiotic preparations also include *Bifidobacteria,*

**Table 2.6** Most common strains currently used in probiotic products (Ganguli, 1988; O'Sullivan *et al.*, 1992; Allen *et al.* 1996; ASM News, 1996; Conway, 1996; Suskovic *et al.*, 1997; Candida and Probiotics 1998; Holzapfel *et al.*, 1998; Introducing Probiotics, 1998; Knorr, 1998; Salminen and Von Wright, 1998; Scheinbach, 1998; Shortt, 1998; Lee *et al.*, 1999).

| ***Bifidobacterium* spp.** | |
|---|---|
| *B. adolescentis* | *B. bifidum* |
| *B. brevis* | *B. longum* |
| *B. animalis* | *B. infantis* |
| *B.thermophilum* | *B. breve* |
| *B. lactis* | |
| ***Lactobacillus* spp.** | |
| *L. fermentum* | *L. bulgaricus* |
| *L. johnsonii* | *L. crispatus* |
| *L. salivarius* | *L. bifidus* |
| *L. rhamnosus* or GG | *L. acidophilus* |
| *L. reuteri* | *L. plantarum* |
| *L. helveticus* | *L. casei* subsp. *rhamnosus* |
| *L.gallinarum* | *L. brevis* |
| *L. delbrueckii* subsp. *bulgaricus* | *L. gasseri* |
| *L. cellobiosus* | *L. vitulinus* |
| *L. collinoides* | *L. cremoris* |
| *L. ruminis* | *L. dextranicum* |
| *L. lactis* | *L. thamnosus* |
| *L. lactis* biover *oliacetylactis* | *L. casei* |
| *L. casei* shirota | |
| **Other** | |
| *Propionibacterium freudenreichii (Non-LAB)* | *Lactoccus lactis* subssp. *lactis* and *cremoris* |
| *Enterococcus faecium* | *Enterococcus faecalis* |
| *Saccharomyces cerevisiae (Yeast)* | *Saccharomyces boulardii (Yeast)* |
| *Sporolactobacillus inulinus* | *Escherichia coli (Non-LAB)* |
| *Leuconostoc mesenteroides* subsp. *dextranium* | *Leuconostoc paramesenteroides or lactis* |
| *Bacillus cereus (Non-LAB)* | *Pediococcus pentosaceus* |
| *Pediococcus acidilactici* | *Pediococcus halophilus* |
| *Streptococcus diacetilactis* | *Streptococcus salivarius* subsp. *thermophilus* |
| *Streptococcus cremoris* | *Streptococcus faecium* |
| *Streptococcus lactis* | *Streptococcus equinus* |

*Entercocci, Propionibacteria* and even *Saccharomyces* strains, it is important to verify the safety of new probiotic strains. Although this is an important aspect, no firm guidelines exist for a safe testing (Dale, 1991). Salminen and his teamwork

(1993) provide an example of a suitable safety assessment procedure. The safety assessment should include:

- ❍ The risk of microbial invasion (infectivity).
- ❍ Basic toxicology: Development of harmful metabolites in the intestine.
- ❍ Intestinal mucosa degradation.
- ❍ Provide epidemiological data and evidence on safety.

The European 'novel food' guidelines provide a further guidance about safety. Johansson *et al.* (1993) found that a further evidence of safety can be assured by selecting strains of human origin and strains that are relatively common in the human intestinal tract (Lee and Salminen, 1995). Such strains have been isolated and readministered to human subjects, and include *Lactobacillus plantarum*, *L. reuteri* and *L. casei* subsp. *rhamnosus*. On the other hand, Goldin and his group work documented that bacteria commonly found in yoghurt are rarely isolated from the lower parts of the intestinal tract (Lee and Salminen, 1995).

In 1998, Seppo Salminen and Atte von Wright suggested safety assessment for new probiotic strains consisting of nine main points:

- ❍ Selection of strains that are non-pathogenic members of the normal healthy human gastrointestinal microflora.
- ❍ Characterisation of the intrinsic properties of microorganisms and strains selected for probiotic use, e.g. adhesion factors, degradation of intestinal mucus, plasmid content, antibiotic resistance and its possible transfer, enzyme profile.
- ❍ Analysis of the metabolic end products of the microorganisms.

- Assessment of the acute and subacute effects of ingestion of extremely large amount of the microorganisms.
- Estimation of the *in vitro* infective properties of probiotic microorganisms using cell lines and human intestinal mucus degradation. Assessment of infectivity in animal models, e.g. immunocompromised animals or lethally irradiated animals.
- Assessment of probiotic effects on the composition of human intestinal microflora.
- Careful recording of side effects (if any) during human volunteer studies and clinical studies in disease-specific states.
- Epidemiological and post-marketing surveillance of populations ingesting large amount of newly introduced probiotic microorganisms for infections, comparison to the use of traditional strains.
- In addition to the above mentioned safety measures eventual genetically modified strains and strains derived from animals should be subjected to the EU novel food regulations and required safety assessment.

Very similar safety assessments have been published in 1997 by the PROBDEMO group (Mattila-Sandholm, 1997).

Kim (1988), Fuller (1989), Renner and Munzner (1991) and Singh *et al.* (1997) have outlined the required characteristics that nominee organism should have if they are to be used as "dietary adjuncts" (O'Sullivan *et al.*, 1992). Ganguli (1988), Hose and Sozzi (1991), O'Sullivan with his companions (1992), Richardson (1996),

Kalantzopoulos (1997), Kelly (1998) and Mattila-Sandholm and Salminen (1998) have also reported these features and they found that the selected organisms should:

- ❍ Be of human origin.
- ❍ Non-pathogenic and non-toxic to animals and humans (Marteau and Rambaud, 1996).
- ❍ Have high tolerance to bile and low acidity.
- ❍ Produce lactic acid during fermentation.
- ❍ Facile augmentation *in vivo*.
- ❍ Facile augmentation *in vitro*.
- ❍ High stability at room temperature, individually or when mixed with the feed.
- ❍ Lack of potential for mating with pathogenic microorganisms.
- ❍ Normal inhabitants of site of application; produce metabolites inhibitory or antagonistic to the indigenous microflora (which try to prevent colonisation by exogenous microorganisms) (Candida and Probiotics, 1998).
- ❍ Susceptible of surviving and growing in the site of application.
- ❍ Exert beneficial effect(s) on host.
- ❍ Maintain their viability and activity throughout product manufacture and storage.
- ❍ Maintain a good flavour and aroma profile after fermentation.
- ❍ Generally recognised as safe; have GRAS status.
- ❍ Technologically usable.
- ❍ Must not be contaminated with other microorganisms that may be pathogenic.

- ❍ Capable to stick to the mucosal surface of the gastrointestinal tract (to prevent being washed out by peristalsis) (Candida and Probiotics, 1998).
- ❍ Anticholesterolaemic effects.
- ❍ Antitumour activity (Rowland, 1996).

Relying on such characteristics, most of the probiotics are selected from the group of lactic acid bacteria (Fuller, 1987) of which the *Lactobacillus* species are the most important as shown previously in Table 2.6.

Huist In't Veld and Shortt (1996) have developed a schematic representation of a rational for strain selection criteria, which summarises the above mentioned considerations (see Figure 2.4). Another selection criteria has been developed by Fazel *et al.* (1999a) for probiotic strain selected from microbial ecosystems (see Figure 2.5).

Jeroen Hugenholtz (1993) has found that lactic acid bacteria involved in food fermentations have only one main property in common: a limited ability to metabolise food-borne substrates. He also reported that the main activity of lactic acid bacteria is to metabolise the sugars present as fast as possible to the end products (lactic acid).

## 2.4 Overview of the fermentation processes using probiotic microorganisms

It is obvious from the market and the articles published in the field that the most common probiotic products are dairy products (see Table 2.7), which is probably due to the long history between the human kind and the milk.

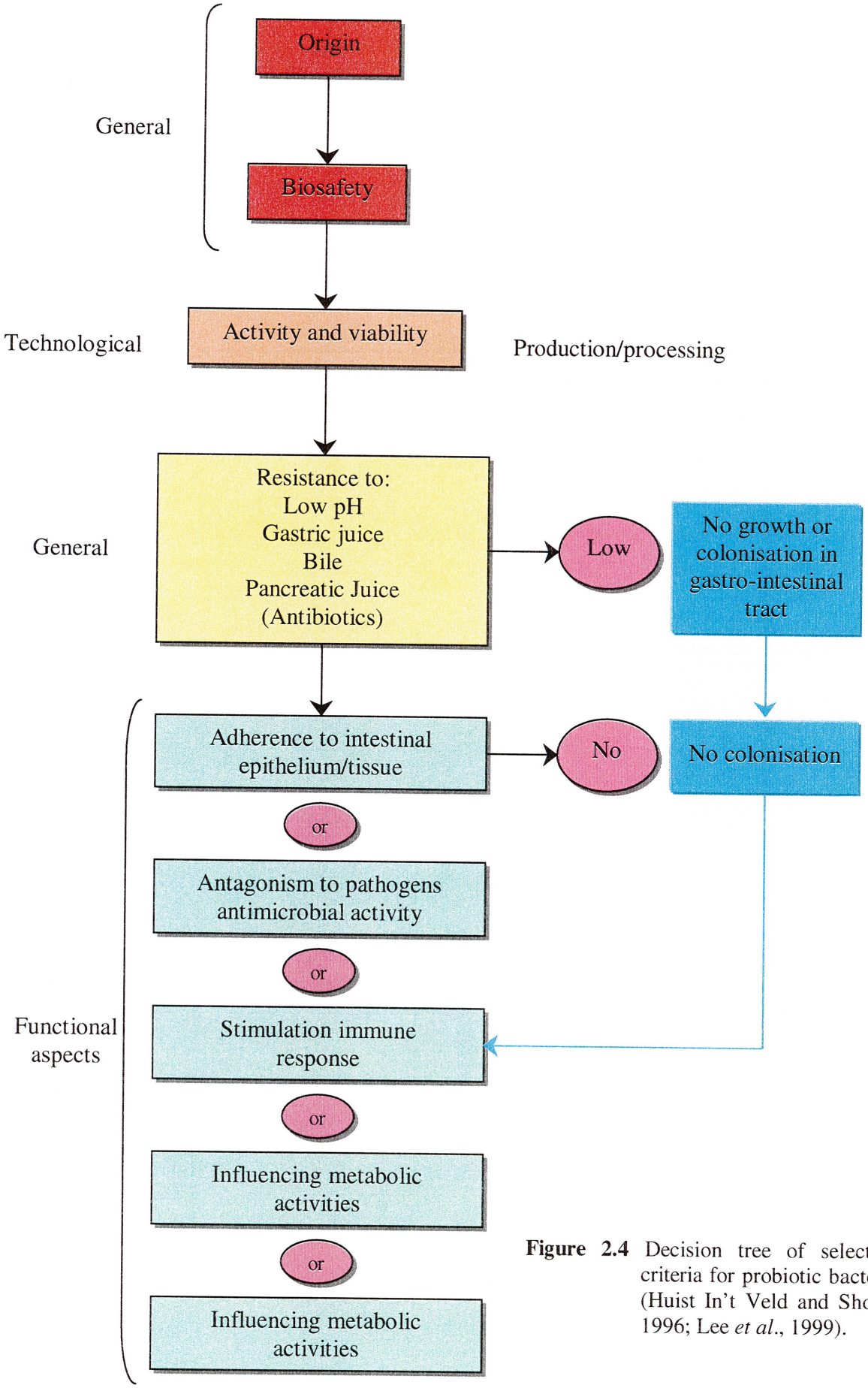



**Figure 2.4** Decision tree of selection criteria for probiotic bacteria (Huist In't Veld and Shortt, 1996; Lee *et al.*, 1999).

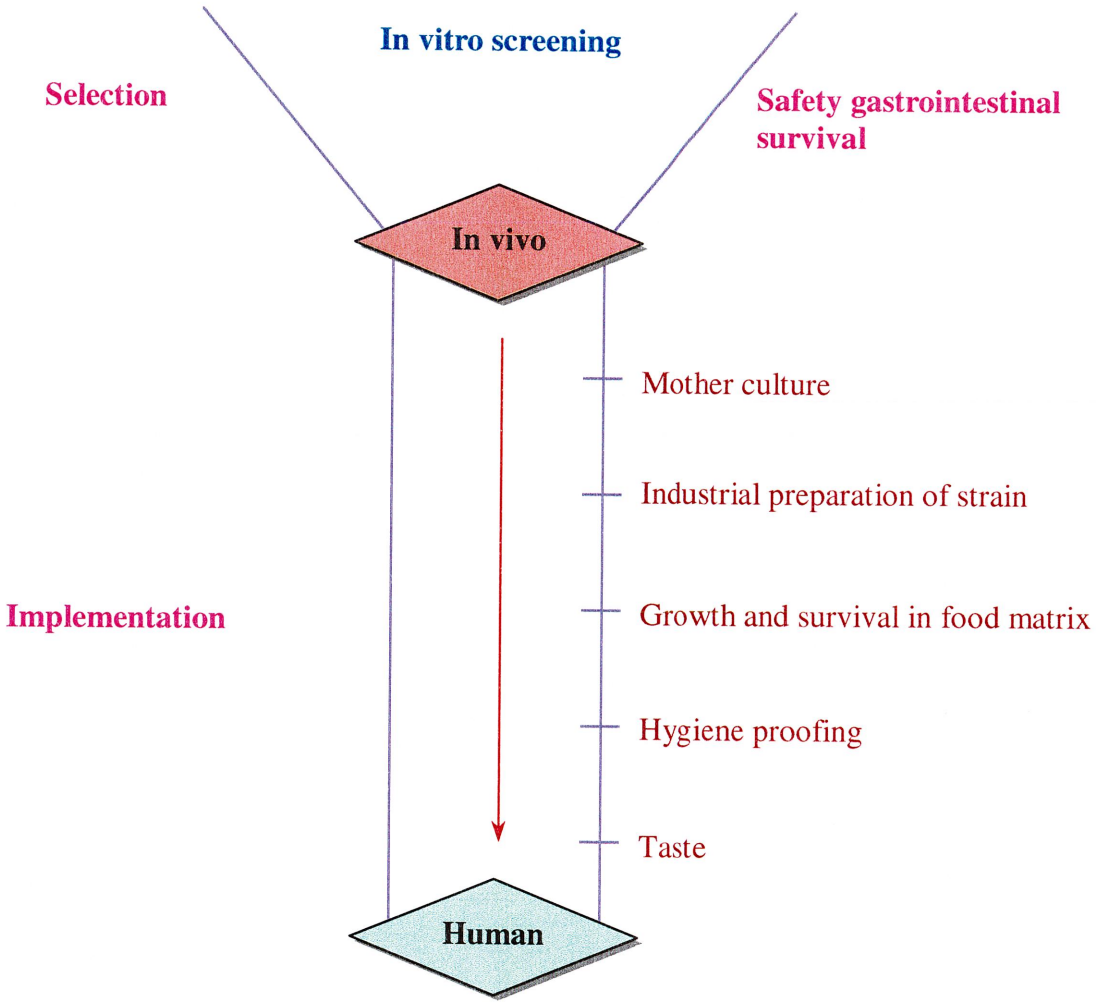



**Figure 2.5** Probiotic strain selection criteria (Fazel *et al.*, 1999a).

Milk is the fluid secreted by the mammary glands of mammals for the primary purpose of nourishing their young. Since humans also nurse their young, the idea of using milk from animals as human food probably developed as soon as man was able to domesticate animals. At the present time, the milk of dairy cows, sheep, goats, camels, water buffalo, reindeer and mares is used in various parts of the world.

**Table 2.7** Some common commercial products containing probiotic bacteria (O'Sullivan *et al.*, 1992; Klein *et al.*, 1998; Lee *et al.*, 1999; Vaillant, 1999).

| ***Dairy products*** | | |
|---|---|---|
| **Product name** | **Country of origin** | **Probiotic bacteria** |
| Gefilac | Finland | *L. casei* subsp. *rhamnosus* |
| Gefilus | Finland | *S. thermophilus, L. bulgaricus, L. casei GG* |
| Biola | Norway | *L. acidophilus, B. bifidum B. lactis, L. GG* |
| Bra-Mjoelk | Sweden | *L. acidophilus L. reuteri* |
| Cultura AB-Yoghurt | Denmark | *L. acidophilus, B. bifidum* |
| ACO-yoghurt | Switzerland | *S. thermophilus, L. bulgaricus, L. acidophilus* |
| Sym Balance | Switzerland | *L. reuteri, L acidophilus, L. casei, Bifidobacteria* |
| Aktifit | Switzerland | *S. thermophilus, L. casi, L. acidophilus, B. bifidum* |
| Biokys | Czechoslovakia | *B. bifidum, L. acidophilus, P. acidilactici* |
| Bifidus milk | Various countries | *B. bifidum* or *B.longum* |
| Acidophilus milk | Various countries | *L. acidophilus* |
| Fyos | Various countries | *L. casi rhamnosus* |
| LC1 | Various countries | *S. thermophilus, L. bulgaricus, L. johnsonii* |
| Actimel | Various countries | *S. thermophilus, L. bulgaricus, L. casi* Imuntass |
| Little Swallow | United Kingdom | *L. acidophilus, B. bifidum* |
| Leisure Live | United Kingdom | *L. acidophilus, B. bifidum* |
| Erivan Acidophilus Yoghurt | USA | *L. acidophilus* |
| Crunch N-Yoghurt | USA | *S. thermophilus, L. bulgaricus, L. acidophilus* |
| Classic Flavor | USA | *L. acidophilus* |
| Biogarde | Germany | *S. thermophilus, B. bifidum, L. acidophilus* |
| Bifighurt | Germany | *B.longum, S. thermophilus* |
| Vifit | Germany | *S. thermophilus, L. bulgaricus, L. acidophilus, B. bifidum* |
| LA7 | Germany | *L. acidophilus* |
| Bio Pot | Germany | *L. acidophilus, B. bifidum* |
| Bulla AB Live | Singapore | *L. acidophilus, B. bifidum* |
| Yoplait | Singapore | *L. acidophilus* |

| Product name | Country of origin | Probiotic bacteria |
|---|---|---|
| Ski | Singapore | *L. acidophilus, Bifidus* |
| Paigen Twinu | Thailand | *L. acidophilus,* |
| Pauls | Indonesia | *L. acidophilus* |
| Vita Charm | Indonesia | *L. bulgaricus, L. acidophilus* |
| Ofilus | France | *S. thermophilus, L. bulgaricus, L. acidophilus, B. bifidum* and/or *B.longum* |
| Fresh and hard cheese | France | *L. acidophilus, L .casei , B. bifidum* |
| Raw sausage | France | *L. acidophilus, L .case, Bifidobacterium* |
| Mil-Mil/E | Japan | *B. breve* strain Yakult, *L. acidophilus, S. thermophilus* |
| Bifiel | Japan | *B. breve* strain Yakult, *L. lactis, S. thermophilus* |
| Yakult | Japan | *L. casei* strain shirota |
| Sofuhl | Japan | *L. casei* strain shirota , *S. thermophilus* |
| Joie | Japan | *L. casei* strain shirota |
| Onaka He GG | Japan | *S. thermophilus,L. bulgaricus, L. GG* |
| Morinaga Caldus/Bifidus | Japan | *B. longum, L. acidophilus* |
| Chamyto | Brazil | *L. johnsonii, L. helveticus* |
| Vitagen | Malaysia | *L. acidophilus* |
| Bio-Garde | Australia | *L. acidophilus, B. bifidum* |
| Yoplus | Australia | *Bifidus* |
| Vaalia | Australia | *L. acidophilus, B.lactis ,L. GG* |
| ***Pharmaceutical preparations*** | | |
| Ventrux Acido | Sweden | *S. feacium* |
| Infloran Berna | Switzerland | *L. acidophilus, B. infantis* |
| Bioforin | Switzerland | *S. feacium* |
| LGG | USA | *L. GG* |
| Lactinex | USA | *L. bulgaricus, L. acidophilus* |
| Lactopriv | Germany | Bifidobacterium ssp. |
| Lactana-B | Germany | Bifidobacterium ssp. |
| Omniflora | Germany | *L. acidophilus, B. longum, E. coli* |
| Euga-Lein | Germany | Bifidobacterium ssp. |
| Syerlac | France | *L. acidophilus, B. bifidum, L. bulgaricus* |
| Levenin | Japan | *L. acidophilus, B. infantis, E. faecalis* |

Because milk is designed to satisfy the nutrient requirements of the young, rapidly growing animal, it is generally an excellent source of protein, calories in the form of fat and carbohydrates, vitamins and minerals. Milk is not only very nutritious for human, it is also an excellent substance for various microorganisms. Fortunately, milk is generally seeded unintentionally with *Lactobacilli* and *Streptococci*, resulting in a rather rapid souring of untreated milk at usual ambient temperatures. This natural acid fermentation curdles the milk and preserves it from putrefaction and spoilage.

Soured milk is consumed in products such as buttermilk following churning to produce butter, yoghurt, kefir and koumiss (Tietze, 1999). It is also the intermediate product in the production of many types of cheeses. As Steinkraus (1996) mentioned in his book, these products are important not only in parts of the developing world but in developed countries as well where, in addition, cream and ice cream can be described as staples in the diet.

Fermented milks by themselves or combined with cereal offer a mean of preserving highly nutritious foods at much lower costs than canning, freezing and artificial dehydration.

Fresh milk sours naturally. The final product, whether sour milk, yoghurt or cheese, is determined by the work conditions during fermentation. The most basic reaction occurring is the utilisation of the milk sugar lactose to produce lactic acid, lowers the pH and literally preserves the milk against spoilage.

By controlling the conditions of fermentations, primarily temperature, the natural microbial flora can be altered to produce a wide variant of products. The addition of a small amount of previously fermented product introduces large numbers of the desired microorganisms, greatly enhancing the chances of obtaining the proper fermentation. Like this, stable mixed populations of microorganisms are established and propagated for many years. With careful handling these empirically selected milk cultures yield remarkably consistent, healthful products.

An excellent review of cheese and cultured milk products have been written by Kosikowski (1977) who classifies fermented milks into four types (see Table 2.8) depending on the kind of microorganisms involved, growing conditions and amount of titratable acid formed.

**Table 2.8** Types of fermented milks (Steinkraus, 1996; Potter and Hotchkiss, 1997).

| Fermented Milk | Examples |
|---|---|
| Acid/Alcohol | Kefir and koumiss |
| High acid | Bulgarian sour milk |
| Medium acid | Acidophilus milk and yoghurt |
| Low acid | Cultured butter milk and cultured cream |

In the following sections, a very brief discussion of some common probiotic products (food or beverage) currently available in the market are presented as examples.

## 2.4.1 Dairy probiotic products

**Yakult:** It is a dairy product containing probiotic bacteria consumed and produced mainly in Japan since 1941 (O'Sullivan *et al.*, 1992; Klein *et al.*, 1998; Shortt, 1998; Yakult Europe, 1999). It has a delicious refreshing taste and it considered as a type of

yoghurt (Arnold, 1999). The microorganism involved in Yakult fermentation is the lactic acid bacteria called *Lactobacillus casei* shirota (Shirotam) (O'Sullivan *et al.*, 1992; Klein *et al.*, 1998). Yakult is consumed by more than 23 million people world wide mostly for breakfast (Yakult Europe, 1999).

There is a lack of scientific reviews on Yakult, its microbiological and biochemical changes and its nutritional value. According to some articles, and because of its probiotic strains, it has been found that Yakult provides several beneficial effects (see Table 2.5) and the most common one is its ability to maintain and improve the environment in the intestinal balance of the consumer (Oberman and Libudzisz, 1996; Japanscan, 1999). The effects on the human immune system exerted by *Lactobacillus casei* shirota, which presented in some fermented milk as well as Yakult, have been reviewed by Tanaka (1996), Havenaar *et al.* (1998) and by Mattila-Sandholm and Salminen (1998).

Jin and his teamwork (1999) have found that Yakult contains most of the essential amino acids. Yakult is marketed in small plastic bottles each of 65ml capacity. Consumption of one bottle of Yakult supplies the human body by 51 kcal, which the same energy provided by an apple and each small bottle contains approximately 6.5 billion live bacteria.

The production process of Yakult follows the following steps (Oberman and Libudzisz, 1996; Yakult Europe, 1999):

Factory laboratory:

- Mother culture preparation: A small a mount of skimmed milk, water and dextrose is mixed, sterilised and cooled to 37°C, then *Lactobacillus casei* Shirotam is added.
- Storage: It is stored in incubators for two days while it multiplies and then it is transferred to the 'seed room' where the large-scale culture production takes place.

Fermentation section:

- Skimmed milk powder, sugar, dextrose and water are UHT sterilised for three seconds at 120°C and transferred into 26,000 litre tanks.
- The mixture is cooled to 98°C and held at that temperature for 90 minutes while caramelisation takes place.
- The mixture is cooled to 37°C and then about 30 litres of the culture is added.
- The mixture is held for about five days and it is stirred occasionally.
- Bacteria levels and acidity of the mixture will rise (pH decrease). The object of fermentation is to achieve a pH at which the probiotic microorganisms can survive the acidity of the human stomach.
- When the pH and the microorganism concentration are optimum, the mixture is put through a homogeniser to create a smooth fluid product.
- Then the mixture moved to 24,000 litre mixing tanks and cooled down to 5 °C to stop fermentation.

Final preparations:

- When the fermentation process is stopped, the product, which has one-month shelf life from bottling, has to remain at 5 °C throughout the chill chain.
- Sugar is added at a 'taste maker', along with nature-identical citrus and vanilla flavours.
- A final check with a hygrometer indicates how much water must be added to achieve the right consistency.
- The water is filtered, UV treated and mixed with the product in-line.

**Yoghurt (Yogurt, Yahourth, Robah or Zabadii):** It is a general expression referring to sour milks of different types. The term is also used to describe a specific kind of fermented milk in which *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are the primary fermenting organisms (Kosikowski, 1982; Potter and Hotchkiss, 1997). It is a well-known acidophilus cultured food and it has been eaten for centuries all over the world. Because of its acidic nature, it is very beneficial in the absorption of iron and also supports the growth of valuable intestinal bacteria. Fresh yoghurt contains $1.0 \times 10^9$ organisms per gram, but this number declines upon storage (Batt, 1998). The titratable acidity range from 0.85 – 0.90%, which it is considered as a medium acidity (see Table 2.8) (Kosikowski, 1982; Batt, 1998).

Yoghurt bacteria show some influence on immunological parameters. The consumption of yoghurt has been associated with a decrease in allergic symptoms in elderly. Recent studies on yoghurt have documented its beneficial influences on

humans and animals. Good reviews on yoghurt, its microbiological and biochemical changes, healthful features, nutritional value and its types are provided by Kosikowski (1982), Oberman and Libudzisz (1996), Kalantzopoulos (1997) and Batt (1998).

### 2.4.2 Cereal-based probiotic products

There are also some cereal-based fermented products that considered as probiotic products, which have high nutritional value and beneficial effects on human health (Gotcheva, 1998; Holzapfel *et al.*, 1998; Bioferme Oy, 1999).

**Yosa:** It is a new snack food made from oat bran pudding cooked in water and fermented with LAB and *Bifidobacteria* and then flavoured with sucrose or fructose and fruit jam (Salovaara, 1996). It is mainly consumed in Finland and other Scandinavian countries. It has a texture and a flavour similar to yoghurt (Tuorila *et al.*, 1998) but it is totally free from milk or other animal products and it comes in different flavours depending on the type of fruit jam used. It is lactose-free, low in fat, contains β-glucan and it is suitable for vegetarians (Tuorila *et al.*, 1998; Bioferme Oy, 1999).

Yosa is a healthy addition to any diet because it contains oat fibre (Tuorila *et al.*, 1998) and probiotic LAB which can maintain and improve the environment in the intestinal balance of the consumer. Oat fibre is also a good source of β-glucan, which can lower the cholesterol levels in the consumer blood, which in turn can reduce the risk of heart disease (Bioferme Oy, 1999).

**Boza:** It is a colloid suspension, from light to dark beige, sweet, slightly sharp to slightly sour, non-alcoholic beverage consumed daily in Bulgaria, Albania, Turkey, Romania. It is made from wheat, rye, millet, maize and other cereals mixed with sugar or saccharine and its origin dates many ages back (Gotcheva, 1998). Due to its pleasant taste, flavour, and high nutritional values, boza has become a very popular beverage consumed as everyday food by people of all ages. Gotcheva (1998) has found that the main microorganisms involve in boza formation are *Lactobacillus fermentum, L. brevis*, *Leuconostoc paramesenteroides* or *lactis,* and *Saccharomyces cerevisiae*.

The industrial production process of boza follows the following steps (Gotcheva, 1998):

- The grains are separated carefully from all types of adulterants.
- Then they are washed and cooked in an autoclave for about two hours at 4 – 5 atmospheres (or 30 – 40 minutes at 170 °C). The cooking time depends on the grain quality, autoclave volume, pressure and temperature. The proportion of water to grain is about 3 to 1 volume parts, and during cooking process grain turns to a mash. Cooking is finished when the mixture turns from light to dark beige and has a pleasant specific bread taste and flavour with a touch of caramel.
- Afterwards the mash is gradually mixed with cold water at a ration of 1 to 1 volume parts. The better and quicker is the cooling, the higher is the quality. The cooling process lasts 2 – 3 hours.

- Extra amount of water is added when the mixture temperature lies between 12 and 15°C. The amount of water depends on the dry matter, which is supposed to be over 7%.
- The mash is percolated and then stored at 4°C.
- Compressed bakers' yeast (usually contaminated with LAB) is added as inoculum.
- Sugar or saccharine is added before bottling.

Boza is good for consumption at every stage of the fermentation until pH drops to about 3.5.

## 2.5 Microorganisms with probiotic properties

Different product types or supplements containing viable microorganisms with probiotic properties are commercially available either in lyophilised from or as fermented food commodities. *L. acidophilus* and *L. casei* strain shirota probably have the longest history among known bacteria strains. Nowadays commercial probiotic products with *Lactobacillus* ssp. are the most common ones, followed by *Bifidobacterium* sp., some other LAB genera and even a few non-lactic bacteria (see Table 2.9). These non-lactics, however, are rarely used in dairy or other food commodities, but find their application rather as lyophilised or encapsulated pharmaceutical preparations (Holzapfel *et al.*, 1998).

With the emphasis mainly on 'novel-type' fermented dairy products, a steadily increasing range of yoghurt-like products is available on the European and Japanese markets (Holzapfel *et al.*, 1998). *Sterptococcus thermophilus*, although generally

**Table 2.9** Microorganisms used in probiotic products (LAB , non-LAB and yeasts) (Fischer, 1895; Winslow *et al.*, 1917; Cowan, 1973; Rogosa *et al.*, 1975; Sneath, 1986; Prescott *et al.*,1993; Schaechter *et al.*, 1993; Noda *et al.*, 1994; Sneath *et al.*, 1994; Ingraham and Ingraham, 1995; Oberman and Libudzisz, 1996; Madigan *et al.*, 1997; Stanley, 1997; Gotcheva, 1998; Hammes and Ganzle, 1998; Lucke, 1998; Mainzer *et al.*,1998; Black, 1999; Lee *et al.*, 1999; Fisher and Arnold, 1999; Stanier *et al.*, 1999).
**Catal.** Catalase produced
**D** Label for (-) isomer
**L** Label for (+) isomer

| ***Bacteria*** | | | | | |
|---|---|---|---|---|---|
| **Name** | **Cell form** | **Gram +/-** | **Catal.** | **Metabolism** | **Lactic acid configuration (D/L)** |
| *B. adolescentis* | Branched rods | + | - | Heterofermentative | DL |
| *B. brevis* | " | + | - | No information | " |
| *B. animalis* | " | + | - | Heterofermentative | " |
| *B. lactis* | " | + | - | " | " |
| *B. bifidum* | " | + | - | " | " |
| *B. longum* | " | + | - | " | L |
| *B. infantis* | " | + | - | " | DL |
| *B. breve* | " | + | - | " | L |
| *B. thermophilum* | " | + | - | " | DL |
| *L. fermentum* | Rods (Bacilli, coccobacilli) | + | - | " | " |
| *L. johnsonii* | " | + | - | Homofermentative | D |
| *L. salivarius* | " | + | - | Heterofermentative | L |
| *L. rhamnosus* or GG | " | + | - | Homofermentative | Not determined |
| *L. reuteri* | " | + | - | Heterofermentative | Not determined |
| *L. helveticus* | " | + | - | Homofermentative | DL |
| *L.gallinarum* | " | + | - | No information | Not determined |

| *L. delbrueckii* subsp. *bulgaricus* | " | + | - | Homofermentative | D |
|---|---|---|---|---|---|
| *L. cellobiosus* | " | + | - | Heterofermentative | Not determined |
| *L. collinoides* | " | + | - | " | Not determined |
| *L. ruminis* | " | + | - | Homofermentative | L |
| *L. lactis* | " | + | - | " | D |
| *L. lactis* biover *oliacetylactis* | " | + | - | No information | Not determined |
| *L. casei* shirota | " | + | - | Homofermentative | DL |
| *L. bulgaricus* | " | + | - | " | DL |
| *L. crispatus* | " | + | - | " | " |
| *L. bifidus* | " | + | - | No information | Not determined |
| *L. acidophilus* | " | + | - | Homofermentative | DL |
| *L. plantarum* | " | + | - | Heterofermentative | DL |
| *L. casei* subsp. *rhamnosus* | " | + | - | " | L |
| *L. brevis* | " | + | - | " | DL |
| *L. gasseri* | " | + | - | Homofermentative | DL |
| *L. vitulinus* | " | + | - | " | D |
| *L. cremoris* | " | + | - | No information | Not determined |
| *L. dextranicum* | " | + | - | " | " |
| *L. thamnosus* | " | + | - | " | " |
| *L. casei* | " | + | - | Homofermentative | " |
| *Bacillus cereus (Non-LAB)* | Oval (Rod, encapsulated) | + | - | Heterofermentative | " |
| *Lactoccus lactis* subssp. *lactis* and *cremoris* | Cocci | + | - | " | DL |
| *Enterococcus faecium* | " | + | - | Homofermentative | L |
| *Enterococcus faecalis* | " | + | - | " | L |

| | | | | | |
|---|---|---|---|---|---|
| *Leuconostoc mesenteroides* subsp. *dextranium* | Spheres in chains (Cocci) | + | - | Heterofermentative | D |
| *Leuconostoc paramesenteroides or lactis* | " | + | - | " | D |
| *Sporolactobacillus inulinus* | Rod | + | - | Homofermentative | D |
| *Escherichia coli (Non-LAB)* | Rod | - | - | Heterofermentative | " |
| *Pediococcus acidilactici* | Spheres in tetrads (Cocci) | + | - | Homofermentative | DL |
| *Pediococcus halophilus* | " | + | - | " | " |
| *Pediococcus pentosaceus* | " | + | - | " | " |
| *Streptococcus salivarius* subsp. *thermophilus* | Spheres in chains (Cocci) | + | - | " | L |
| *Streptococcus cremoris* | " | + | - | " | " |
| *Streptococcus diacetilactis* | " | + | - | " | " |
| *Streptococcus faecium* | " | + | - | " | " |
| *Streptococcus equinus* | " | + | - | " | " |
| *Streptococcus lactis* | " | + | - | " | " |
| *Propionibacterium freudenreichii (Non-LAB)* | Pleomorphic rods | + | - | Not homo-or hetero lactic fermentation | DL |

***Yeasts***

| **Name** | **Budding** | **Shape** | **Spores** | **Metabolism** | **Lactic acid configuration (D/L)** |
|---|---|---|---|---|---|
| *Saccharomyces cerevisiae* | Multipolar | Spherical oval | 4 | Heterofermentative | Not determined |
| *Saccharomyces boulardii* | " | " | " | Homofermentative | L |

associated with these 'mild' yoghurt types, is applied for technical reasons and sustains the low acid fermentation typical of these novel yogurt types. Probiotic strains for most of the probiotic products (foods and beverages) are generally derived from the gastro-intestinal tract of the adult human host. Strains of autochthonous *Lactobacillus* ssp., associated with the human gut, are applied in these products, being *Lactobacillus salivarius* the only exception (see Table 2.9). Particularly *L. casei* and *L. acidophilus* group appear to have special value as probiotic agents. Formerly considered as one species, *L. acidophilus* was shown to be heterogeneous, first by Lerche and Rruter in 1962, and later, on the basis of DNA homology groups, by Laure and his team in 1980 and by Johnsons group in the same year (Holzapfel *et al.*, 1998).

An important difference between subgroups of the LAB lies in the nature of the products formed during the fermentation of carbon source. One group, which is known as homofermentative (homolactic), produce practically a single fermentation product (lactic acid), while the other group, which called heterofermentative (heterolactic), produces other products, mainly ethanol and $CO_2$ as well as lactate (Garbutt, 1997; Madigan *et al.*, 1997; Lindquist, 1999). The metabolic pathways of the two groups have been studied by several authors and it can be found in most of the biological and microbiological books or even in some food science books. The metabolic pathways for both homofermentative and heterofermentative LAB are shown in Figure 2.6 and Figure 2.7, respectively.

Pronounced differences have been observed by Marteau *et al.* (1990) in the survival rate of probiotic stains passing through the stomach and upper intestinal tract (Holzapfel *et al.*, 1998). *In vitro* model studies, taking account the low pH in the

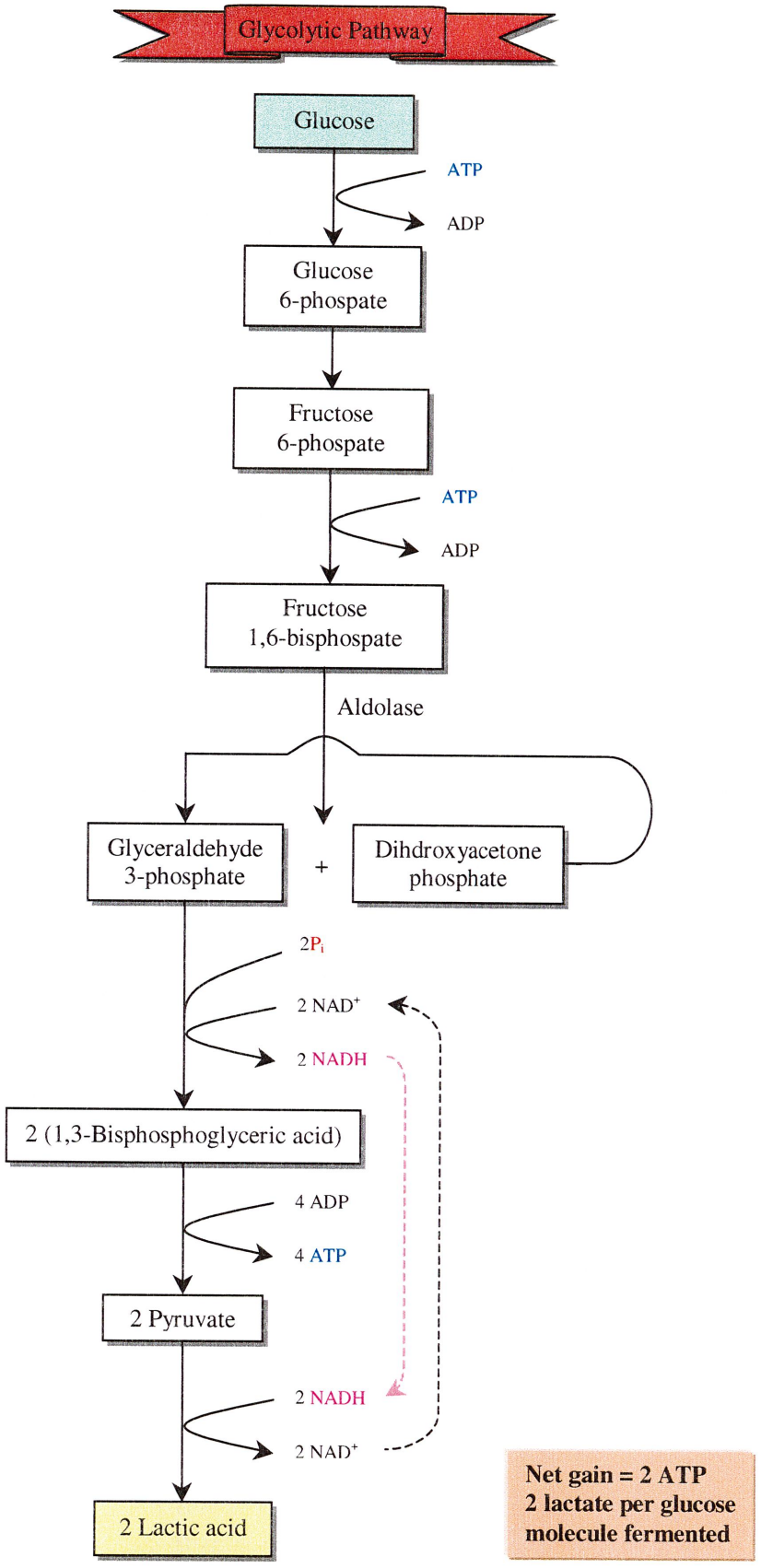



**Figure 2.6** The fermentation of glucose in homofermentative LAB (Homolactic acid) (Bol and de Vos, 1997; Madigan, 1997).

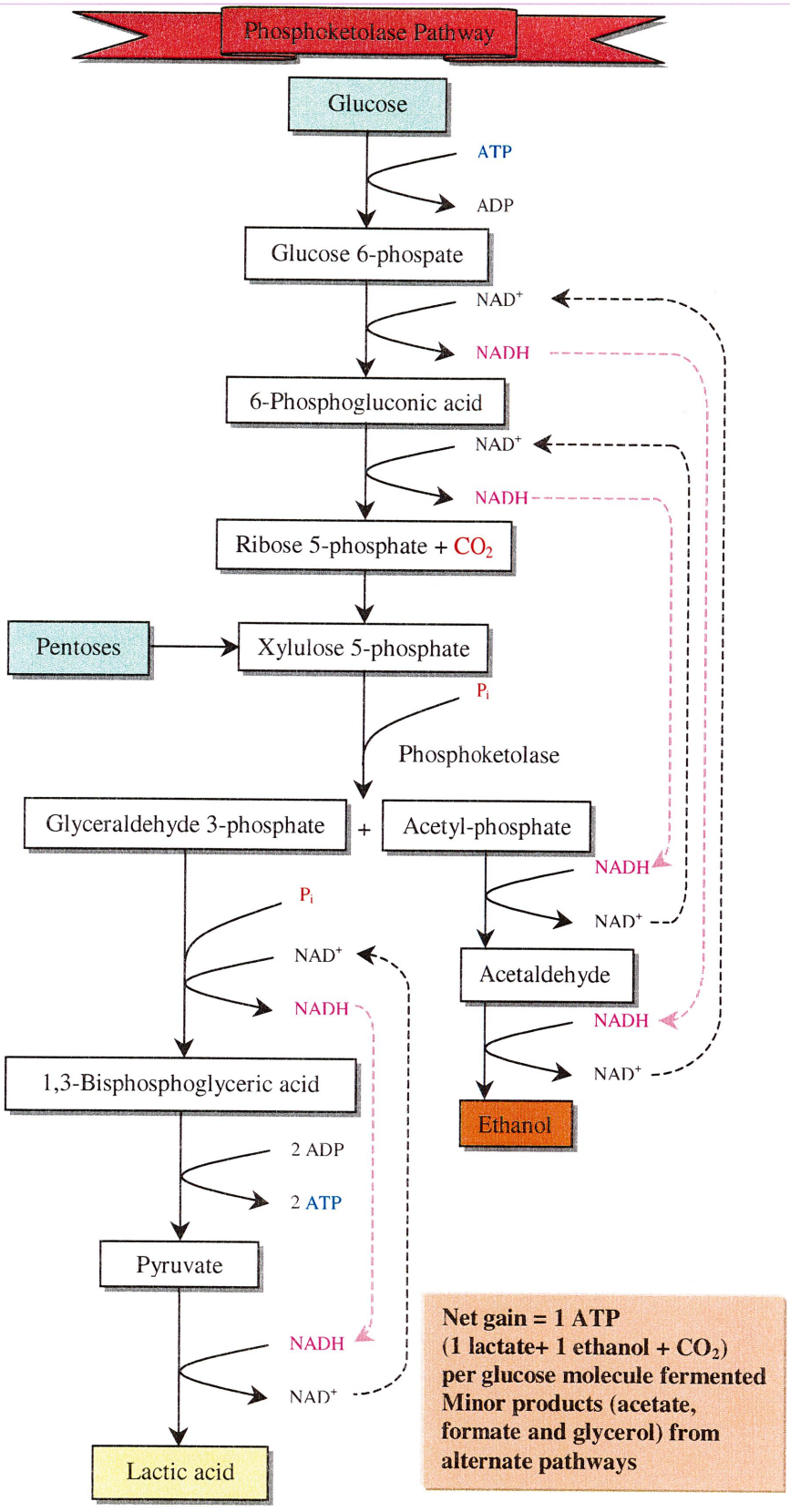



**Figure 2.7** The fermentation of glucose in heterofermentative LAB (Heterolactic acid) (Bol and de Vos, 1997; Madigan, 1997).

human stomach (1.70-2.50) (Dressman *et al.*, 1990; Hyde and Smith, 1999) and the toxic effects of the bile salts show marked differences in the sensitivity of commercial strains (Huis In't Veld and Shortt, 1996; Holzapfel *et al.*, 1998).

*In vitro* methods are suitable for screening some of the characteristics required in the good probiotic microorganisms. However, it is important to note that results from such studies may not be representative of the actual *in vivo* situation (see Table 2.10). For example, the survival of microorganisms at low pH in a test tube is not a reflection of what happens in the stomach, where much more complex physiological conditions exist. Therefore, it is a major advantage to have a dynamic model that mimics in vivo gastrointestinal conditions as closely as possible (Mattila-Sandholm *et al.*, 1997; Dressman, 1999). Researchers at the Nutrition and Food Research in the Netherlands have developed such a model. Details of the *in vitro* gastrointestinal model have been studied and reviewed by Huis In't Veld and Shortt (1996).

The possibility of growing the microorganism at an industrial scale has most important practical consequences for the manufacturer. Strains should be adapted to a suitable carrier or fermentable substrate, e.g. milk or cereal-based medium, and the final product should have an acceptable shelf life and sensory attributes such as colour, taste, aroma and texture. Probiotic strains claimed to be present in a product should remain viable in sufficiently high number and retain their metabolic activity even beyond the 'use-by' date.

**Table 2.10** Minimum pH value that allows the initial growth of various microorganisms (Piard and Desmazeaud, 1991; Prescott *et al.*, 1993; Garbutt, 1997; Nemcova, 1997).

| **Gram negative bacteria** | **pH Limit** |
|---|---|
| *Escherichia coli* | 4.4 |
| *Klebsiella pneumoniae* | 4.4 |
| *Proteus vulagris* | 4.4 |
| *Pseudomonas aeruginosa* | 5.6 |
| *Salmonella paratyphi* | 4.5 |
| *Salmonella typhi* | 4.0-4.5 |
| *Vibrio parahaemolyticus* | 4.8 |
| *Campylobacter jejuni* | 2.3-5.8 |
| *Shigella* sp. | 4.5 |
| | |
| **Gram positive bacteria** | **pH Limit** |
| *Basillus cereus* | 4.9 |
| *Clostridium botilinum* | 4.7 |
| *Enterococcus* sp. | 4.8 |
| *Lactobacillus* sp. | 3.8-4.4 |
| *Micrococcus* sp. | 5.6 |
| *Staphylococcus aureus* | 4.0-4.2 |
| *Lactococcus lactis* | 4.3-4.8 |
| *Brevibacterium linens* | 5.6 |
| *Listeria monocytogenes* | 5.5 |

## 2.6 Future prospects

Probiotics clearly have certain effects on human health, and many medical conditions are currently being carried out to investigate the use of probiotic. Several large-scale projects examining the role of probiotics in health and disease are underway and the European Union is currently supporting various probiotic projects. Nine European research partners, including Nestle', Arla, Valio, Hansen and researchers in Finland, the Netherlands, Italy and Ireland, are collaborating in issues such as the safety and effects of probiotics in healthy individuals and patients with inflammatory bowel disease. Furthermore, in Canada, Australia, New Zealand, Japan, Finland and the United State many probiotic research initiatives are underway. For example, Todd

Klaenhammer's group in Raleigh, North Carolina, is carrying out studies on the functional activities of probiotics (Klaenhammer, 1998), Dan O'Sullivan's group at the University of Minnesota is working on methods of tracking probiotic bacteria through the intestine and Mattila-Sandholm's team in VTT centre, Finland, has a program with a big budget to improve nutritional and eating quality of foods by utilisation of enzymes and LAB (Mattila-Sandholm, 1998b; Pagni, 1998).

Recent advances in methodologies provide more powerful tools to support the effects of the probiotic products, for example the molecular methods to distinguish strains and to track probiotic strains during *in vivo* trials. With the advent of new technologies, scientific researches efforts world wide are now focusing on the development of probiotic products containing bacteria selected for their specific functional characteristics. Exploiting probiotic bacteria provides an opportunity for food scientists to collaborate with biomedical researchers to develop healthy food products used for particular nutritional uses, and medical foods for therapeutic use (Short, 1998).

# 3

# OBJECTIVES

# 3. OBJECTIVES

A market survey on most common bioproducts containing probiotic microorganisms available in the UK and in the EU (see Table 3.1) shows that most of the bioproducts currently available in the markets are not cereal-based foods, with the exception of Boza and Yosa. In addition to that it has been found that there are no cereal-based bioproducts currently in the UK market (see Table 2.7).

**Table 3.1** Most common bioproducts contain in probiotic microorganisms in the UK and the EU (Hartles *et al.*, 1977; Mogilevsky, 1977;Rose, 1982; Mensah, 1993; Garbutt, 1997; Potter and Hotchkiss, 1997; Bioproducts, 1999; Douglas, 1999; Goff, 1999; Miloslav, 1999; Scheinbach, 1999; Watts, 1999; Vaillant, 1999).

| **Bioproduct (Foods, beverages and pharmaceutical preparations)** | **Bioproduct (Foods, beverages and pharmaceutical preparations)** |
|---|---|
| BioFlavour | Swiss cheese |
| Boza | Blue vein cheese |
| Yakult | Spirulina |
| Cheddar cheese | LC1 (vanilla flavour) |
| Typical yoghurt | Yoghurt clabbered milk (Soy Bean Milk) |
| Yosa | Cream butter |
| Soy Sauce | Fermented coffee |
| Cream cheese | Fermented cocoa |
| Unpinned cheeses | High-fat cream |

The main objective of this work is to develop new cereal-based fermented foods that can be categorised as probiotic with suitable aroma, flavour and pH. This requires selecting suitable types of both cereal grains and probiotic microorganisms and finding the appropriate chemical and physical methods to investigate whether this target can be achieved. Physical and chemical tests will be carried out during fermentation (pH measurements, FAN concentration, phosphorus concentration, lactic acid concentration, sugar content, viable cells count, total cell count and dry weight), and the more appropriate ones will be selected.

In order to investigate whether the aroma and the flavour of the products will be acceptable, tasting sessions will also be carried out. A suitable sensorial test procedure and form will therefore need to be developed to cover all the related aspects of the tasting.

# 4

# MATERIALS AND METHODS

# 4. MATERIALS AND METHODS

## 4.1 Inocula

The inocula used for fermentation in this work were a single type of LAB which is the *Lactobacillus casei* shirota, and a mixture of the LAB and yeast (*Saccharomyces cerevisiae*). The mixture LAB-Yeast has sometimes a synergetic effect and is commonly used in the food industry (e.g. in the production of sour bread). The following paragraphs contain brief explanation about the two microorganisms used in this study and why they have been selected.

### 4.1.1 Bacteria

The *Lactobacillus casei* shirota was chosen for its ability to produce lactic acid, and due to its classification as probiotic bacteria. More and above, this strain is categorised as HG1 – ACDP (ACDP, 1995), which means that it belongs to the hazard group number 1 (see Table 4.1), so it is safe and can be used for food fermentations with no restrictions.

The cells of *Lactobacillus casei* shirota vary from long and slender; sometimes bent rods, to short chain; often coryneform coccobacilli (Sneath *et al.*, 1994). *Lactobacillus casei* shirota rods generally have dimensions of 0.7-1.5 x 2.0-4.0 $\mu$m, often with square ends and tending to form chains (Winslow *et al.*, 1917; Sneath *et al.*, 1994). It has a suitable growth temperature range between 2-53°C, but the optimum temperature range is 30-40°C. The optimum pH usually lies within 5.5-6.2, but growth generally occurs at 5 or less (Sneath *et al.*, 1994; Madigan *et al.*, 1997; Stanier *et al.*, 1999).

**Table 4.1** Hazard groups description for both bacteria and yeasts (ACDP, 1995).

| Hazard Groups | Description |
|---|---|
| *Hazard Group 1* | A Biological agent unlikely to cause human disease |
| *Hazard Group 2* | A Biological agent that can cause human disease and may be a hazard to employees; it is unlikely to spread to the community and there is usually effective prophylaxis or effective treatment available |
| *Hazard Group 3* | A Biological agent that can cause severe human disease and presents a serious hazard to employees; it may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available |
| *Hazard Group 4* | A Biological agent that cause severe human disease and is a serious hazard to employees; it is likely to spread to the community and there is usually no effective prophylaxis or treatment available |

*Lactobacillus casei* can been found in dairy products, grains, meat and fish products (Winslow *et al.*, 1917; Sneath *et al.*, 1994), and it can be isolated from milk, cheese, sour dough, human intestinal tract (Winslow *et al.*, 1917; Sneath *et al.*, 1994; Stanier *et al.*, 1999). The strain *Lactobacillus casei* shirota that used for this study has been obtained from a Japanese beverage known as Yakult.

### 4.1.2 Yeast

*Saccharomyces cerevisiae* (NCYC 239) was used due to its classification as probiotic microorganism and due to its ability to grow on sugar-containing media, such as malt extract, molasses, wood sugars or spent sulphite liquor by utilising sugars in the approximate sequence: sucrose, glucose, fructose, maltose and maltotriose (Atkinson and Mavituna, 1991). *Saccharomyces cerevisiae* can not utilise lactose and therefore it can not produce lactic acid (Kriger van Rij, 1984; Alvarez, 1999). Besides, this strain is categorised as a non-hazardous microorganism or as HG1 – ACDP (ACDP, 1995), which means that it belong to the hazard group number 1 (see Table 4.1) and it

has been used for the production of commercial fermented foods and beverages long time ago, so it is safe and can be used for food fermentations with no restrictions.

The cells of *Saccharomyces cerevisiae* have spherical oval shape with a cream colour and generally they have 50-75 μm diameter (Kriger van Rij, 1984; Alvarez, 1999). The suitable growth temperature range between 0-42°C, but the optimum temperature range between 28-36°C. The optimum pH usually falls between 4.3-7.5, but growth generally occurs at 4.5-5.0 (Atkinson and Mavituna, 1991; Madigan *et al.*, 1997; Stanier *et al.*, 1999).

*Saccharomyces cerevisiae* can produce acetic acid in commercially scales and due to that it is used in the production of vinegar, preservative in food industry; chemical foodstocks and in the polymer industry. In addition to that, *Saccharomyces cerevisiae* has the ability to produce ethanol in large quantities and it is used as the main microorganism in the production of the most alcoholic beverages and fuel extender (Atkinson and Mavituna, 1991).

## 4.2 Fermentation media

Malt grains and malt extracts have been used as the main raw materials for fermentation. Due to its chemical composition and the chemical changes that occur during the malting of barley (see Table A.1-1, A.2-1 and A.2-2), malt is a very suitable medium for microbial growth. In addition to that, malt has a very desirable flavour.

The malt extracts used have been supplied by the company PureMalt products, Ltd. (Haddington, Scotland), which is specialist in manufacturing of malt ingredients for the food, brewing and beverage industries. The company also produces malt, malt flour, malt beer concentrates and beverage concepts.

The Malt grains have been provided by Scottish Courage Brewery (Moss side, Manchester). The malt was produced by PaulsMalt and it is categorised as Regina (STD) No. 040943-045. For more information about malt and the malting process see Appendix A.

Two different types of malt extract have been used for fermentation: the media called KL3 and B-Syrup. Only the KL3 product specifications have been provided by the company and it is presented in Table 4.2. B-Syrup is a development product.

## 4.3 Analytical methods

During fermentation, a sample of broth of approximately 15 mL was taken every 12 hours approximately, of which about 0.1-0.2 mL was used for cell plate counting. The remaining quantity was used to measure the pH of the broth. Then the sample volume was adjusted to 10 mL in a centrifugal tube in order to separate the *Lactobacillus casei* shirota from the medium. The filtrate was then used to measure the following parameters:

- Free amino nitrogen content (FAN).
- Total nitrogen content (for the pre-fermented media only).
- Phosphorus in liquid samples.
- Total phosphorus content (for the pre-fermented media only).

- Lactic acid content.
- Sugar types and content.

**Table 4.2** Product specifications for the KL3 malt extract (PureMalt Products Ltd.,1999).
**EBC** European brewery convention
**TVC** Total viable cells

| | | |
|---|---|---|
| **Description** | | |
| A hot water extract of kiln dried barley malt, concentrated by vacuum evaporation, free from additives. | | |
| **Appearance:** Light brown, viscous syrup. Free from extraneous matter. | | |
| **Odour:** Characteristically malty. Free from burnt and foreign odours. | | |
| **Flavour:** Consistent characteristics, malty. Free from fermented, strong or off flavours. | | |
| **Analytical Parameters** | | |
| **Solids** | Brix % | 80-82 |
| **Colour** | EBC comparator | <160 |
| **pH** | 10% w/v | 4.8-5.8 |
| **Microbiological parameters** | | |
| TVC | 30 °C | <10000/g |
| Yeasts | | <500/g |
| Moulds | | <500/g |
| *Free from pathogenic bacteria* | | |
| **Shelf life/Storage** | | |
| Nine months unopened. Once opened use within 6 weeks at ambient storage. | | |
| 48 hours if product is exposed to environment. | | |

## 4.3.1 pH measurements

The pH value of each sample was measured in the filtrate using a Corning No. 155 pH/ion meter in a temperature range of 25-30°C. The pH meter has been calibrated by using two different standard solutions at two different pH values (4 and 7). For more details about standardisation of the pH cell and measurement procedure see Appendix B (B.1)

### 4.3.2 Free amino nitrogen determination

FAN (free amino nitrogen) content was measured from the filtered medium using the ninhydrin colourimetric method recommended by the European Brewery Convention (EBC) in 1987 (Wang, 1999a). This method is based on the colour reaction between ninhydrin and amino acid at pH ranged between 6.6–6.8. The reaction yield can be estimated by measuring the absorbency of the sample at 570 nm with a spectrophotometer (PYE UNICAM PU 8600 UV/VIS; Philips). For more details about the sample preparation, analysis and calculation procedures see Appendix B (B.2).

### 4.3.3 Total Kjeldahi nitrogen (TKN)

The Nessler's spectrophotometery method was used for the determination of the total nitrogen content in the sterilised medium. This method is based on the colour-reaction between nitrogen and mercuric iodide, and the colorimetric method involves the application of a digestion apparatus (Hatch, USA) specially designed for the analysis. Details about sample preparation, experiment procedure and calculation procedure are well explained in Appendix B (B.3).

### 4.3.4 Determination of phosphorus in the liquid sample

This method relies on the destruction of an organic compound by acid digestion and the colorimetric determination of the formed phosphate. The destruction yield can be estimated by measuring the absorbency of the sample at 730 nm with a spectrophotometer (PYE UNICAM PU 8600 UV/VIS; Philips). For more details

about the sample preparation, measurements and calculation procedures see Appendix B (B.4).

### 4.3.5 Determination of total phosphorus

This method is based on a principle similar to that used for the determination of phosphorus in the liquid phase, but uses different experimental and calculation procedure. The Nessler's spectrophotometery method was used for the determination of the total phosphorus content in the sterilised fermentation medium. This method is based on the colour-reaction between phosphorus and mercuric iodide, and the colorimetric method involves the application of a digestion apparatus (Hatch, USA) specially designed for the analysis. For more details about sample preparation, experiment procedure and calculation see Appendix B (B.5).

### 4.3.6 Lactic acid determination

The determination of lactic acid produced during fermentation has been developed by Barker and Summerson in 1941. The method is based on the chemical fact that while heated with concentrated sulphuric acid, lactic acid is converted quantitatively into acetaldehyde, which produces a colour when reacts with p-hydroxydiphenyl in concentrated sulphuric acid. The colour intensity can be quantified by measuring the absorbency of the sample at 570 nm with a spectrophotometer (PYE UNICAM PU 8600 UV/VIS; Philips). For details about sample preparation and experiment procedure see Appendix B (B.6).

### 4.3.7 Sugar analysis

The HPLC technique was used to analyse the sugar content. Quantitative analysis by HPLC clearly requires that a relationship is established between the magnitude of the detector signal and the concentration of a particular solute in the sample, the former being measured by either the corresponding peak area or the peak height (Jeffery, 1991). A liquid chromatography column (5000 LC-12 MM.I.D) (Varian, USA) was used to analyse the samples. The column was operated at a flow rate of 1.0 mL $min^{-1}$, 3.0 atmospheric pressure and 25°C. For more details about sample preparation and experimental procedure see Appendix B (B.7).

### 4.3.8 Viable cells count

The spread plate technique (surface count method) is often used instead of pour-plate method in the food analysis because the growth of some organisms may be impaired if agar is used at too high temperature. It is also easier to subculture colonies from surface plates than from pour plates.

0.1 mL or a suitable amount of the sample is placed using an autopipette in the centre of a well-dried plate of suitable medium and spread with a loop all over the agar surface. The plate is incubated at 37°C during 48 hours, and the colonies are then counted.

When counting colonies, difficulties arise with large smears of small colonies caused when a large viable unit is broken up but not dispersed during manipulation. Such units should be treated as single units, but other colonies could be seen and counted through smears.

This technique was used to count the number of viable cells present in each sample. For more details about sample preparation, experimental and counting procedure see Appendix B (B.8).

### 4.3.9 Total cells count (slide count)

The slide count was performed using a special type of microscopic slide known as Hemocytometer or Helber counting chamber. This slide has an area in the centre called platform surrounded by a ditch. The platform is lower than the remainder of the slide by a defined depth and it has been divided into 400 small squares of known dimensions. The top of the slide is ground so that when an optically plane cover glass is placed over the centre depression, the depth is uniform and the volume over each small square is known. The concentration of cells per volume can be found by counting the number of cells per certain number of squares. For more details about sample preparation, experimental and counting procedure see Appendix B (B.9).

### 4.3.10 Dry weight

The dry weight method was applied to investigate cells growth and component hydrolysis during fermentation. Calibrated 10 mL centrifuge tubes (Fisons, England) were used to measure sample volumes due to the high solid content in the fermentation broth. After being centrifuged in a centrifuge (IEC Centra-MPYR) at 5000 rpm and 2°C for 30 minutes, supernatant from 10 mL sample was collected for component analyses while solids were resuspended in glass vials of known weight by using distilled water and then stored in a 80-110°C oven for at least 5 days. After

being dried the samples cooled for 30-40 minutes in a desiccator before being weighed to 0.0001g. For more details about this method see Appendix B (B.10).

## 4.4 Sensorial analysis and food tasting

Food tasting is not an easy and simple job. Sensorial analysis required a number of characteristics and conditions on the people who taste and in the environment around them. For reasons proposes, sensory analysis uses an expert panel (6 to 12 assessors trained to mark the intensity of each specific attributes) to determine descriptive profiles. In the quality control field, sensory analysis uses trained panels (12 to 20 assessors trained to discriminate products) to test product with triangular, A non-A, Duo-Trio tests (Demyttenaere and Masson, 1999).

Besides the human eye, nose and mouth as a detector, there are some important conditions and factors that greatly influence the tasting process and the food acceptance. Those factors can be divided into three categories (Cardello *et al.*, 1985; Kahkonen *et al.*, 1997): sensory properties of the food, individual characteristics and context of food consumption.

The most general tasting guidelines are illustrated in the following (Lusina, Inc.1999):

- Place: comfortable with access to rinsing facilities.
- Time: before meal, mid-morning is preferred, early evening is also good and must have lots of time to enjoy it.
- Glasses (Container): tulip shape is preferred and must be perfectly clean (without smells or residues).

- Product: no more than 2-3 different products should be tried per tasting and arrangement should be made for blind tasting (pour samples without knowing the name of the product).
- Results: should be record on a 'Tasting Form' (see Table 4.3).

**Table 4.3** Tasting form (Renaissance Vineyard & Winery, 1998; Lusina, Inc., 1999).

| ***Sense-organ*** | ***Examined Part*** | ***Comments*** | ***Rating*** |
|---|---|---|---|
| Eyes | Appearance (clarity and colour) | | 0 to 5 |
| Nose | Aromas/Bouquet (purity and complexity of fragrance) | | 0 to 10 |
| Mouth | Body (texture and weight in the mouth) | | 0 to 10 |
| Mouth | Flavour Intensity (power) | | 0 to 5 |
| Mouth | Complexity (variety of flavour nuances) | | 0 to 10 |
| Nose and Mouth | Balance (harmony appreciation) | | 0 to 10 |
| Mouth | Finish (length and firmness of aftertaste) | | 0 to 10 |
| | General Quality (did you like it?) | | 0 to 20 |
| | If you know that it contains live microorganisms, do you still like it? | | 0 to 10 |
| | Do you want to try it again? | | 0 to 10 |

*Note:* Rating number indicates your opinion, so the higher the rating number is, the more satisfied you are.

Date: __ / __ / _____ Product code: _____ Total points scored = /100

**Procedure**

Samples from each product were tasted and rated according to their appearance, aromas, body, flavour intensity, complexity, balance, finish, general quality, acceptance as probiotic and aptitude to be tried again on seven evaluation points (see Table 4.4), as has been suggested by Kahkonen *et al.* (1997) and Demyttenaere and Masson (1999).

Assessors who tasted the product need to fill in a tasting form. The average scored by each product was then calculated in order to classify them according to the values shown in the evaluation table (see Table 4.4).

**Table 4.4** Evaluation of total points scored in the sensorial test (Cardello *et al.*, 1985; Kahkonen *et al.*, 1997; Demyttenaere and Masson, 1999).

| Range of Total Points Scored | Divisions |
|---|---|
| Less than 40 | Completely disagree |
| 40-50 | Somewhat disagree |
| 50-60 | Disagree a little |
| 60-70 | Neither agree or disagree |
| 70-80 | Agree a little |
| 80-90 | Somewhat agree |
| 90-100 | Completely agree |

The tasting sessions of all products were carried out in the mid-morning in a comfortable place, the Satake centre food grade standard laboratory, which has the required rinsing facilities. The products were served in clean glasses and no more than two products were tasted per day. The assessors have filled the tasting forms of each product and the final results are shown in the following chapter, (Chapter 5).

# 5

# RESULTS AND DISCUSSION

# 5. RESULTS AND DISCUSSION

Series of analyses were carried out in order to monitor some basic parameters during eleven shaking flasks and nine bioreactor fermentations. These analyses include pH measurement, cell counting, chemical analysis (measurement of phosphorus, FAN, lactic acid and glucose concentrations) and tasting sessions for the fermentation product. The aim of the experiments was to find the optimum fermentation time while the taste of the fermentation broth is still good, and simultaneously to give evidence that the fermentation medium still contains viable probiotic microorganism.

The fermentations carried out in this work can be divided into two types. The first one can be called shaking flask fermentations or the inoculum preparation (in 500 mL flasks), and the second type can be called main fermentations or bioreactor fermentations (carried out in 2 L glass fermenters).

## 5.1 Fermentation media preparation

The two types of the malt extract; KL3 and B-Syrup, have been used as two different fermentation medium. Beside that, two other fermentation media have been prepared from the malt grains. The first one, which has been called whole malt grains medium, was prepared by steeping the grains in water for 1 hour. Then the grains were cooked/boiled with tap water for 8 hours in an autoclave (Rodwell-Ensign, UK) at 121◦C, and the mash then cooled to room temperature. The mashes were then passed through a sieve of 710 μm (Endecotts Ltd., England) and the filtrate was used as the fermentation medium.

The second type, which has been called whole milled malt grains, was prepared from the flour of the malt grains. The malt grains flour was produced by using the Satake hammer mill fitted with a sieve of 500 μm. Then the malt flour were cooked/boiled with tap water for 2 hours in the autoclave at 121◦C. Then the mash was cooled to room temperature and used directly as fermentation medium.

Details of the fermentation medium preparation (such as ratios of the materials from which the medium made, sterilisation periods and total volumes for both shaking flask and bioreactor fermentation) will be indicated in the following sections.

## 5.2 Inocula preparation (shaking flask fermentations)

The microorganism (*Lactobacillus casei* shirota) was taken from the Japanese beverage known as Yakult, while the yeast (*Saccharomyces cerevisiae*) was taken from an original mother culture. Yakult was used indirectly as the main inoculum for the medium to be fermented. It has been used directly as the inoculum substance in the preparation of the main inoculum medium (shaking flask fermentation).

Liquid MYGP medium was used in order to invigorate the *Saccharomyces cerevisiae* taken from the mother culture before the inoculation step (Wang, 1999b). The medium was composed of:

- Malt extract (Oxoid, England) 3.0 g $L^{-1}$
- Yeast extract (Oxoid, England) 3.0 g $L^{-1}$
- Glucose (Fluka, England) 10.0 g $L^{-1}$

- Peptone (Oxoid, England) 5.0 g $L^{-1}$
- Distilled water 200 mL

The unadjusted pH of the MYGP medium was between the range 5.4-5.6.

Four shaking flasks of 200 mL capacity of the MYGP medium were prepared and sterilised for about 15 minutes at 121◦C (Wang, 1999b; Cooper, 1999). Then they were inoculated with the *Saccharomyces cerevisiae* and placed over a suitable rotary glass shaker (HT-INFORS-AG-CH 4103 Botimingen, England) operating at 200 r.p.m. The shaking flasks were incubated at 30◦C which falls within the optimum growth temperature range for *Saccharomyces cerevisiae*. The required invigoration period for the *Saccharomyces cerevisiae* in this medium ranged between 24 to 48 hours (Wang, 1999b).

Fermentations 1 to 8 and fermentation 10 (see Table 5.1) all have been inoculated with Yakult. All fermentations were carried out in a 500 mL shaking flask. The fermentation media were made from different mixtures of malt/water, and sterilised for a period of time at 121◦C (see Table 5.1). Each fermentation was then inoculated with Yakult (10-15% of the total volume) as suggested by Stanbury *et al.* (1998), Wang (1999b) and Cooper (1999). Then the medium was placed on a suitable rotary glass shaker (HT-INFORS-AG-CH 4103 Botimingen, England) operating at 280 r.p.m. The shaking flasks were incubated at 33◦C, which lies within the optimum growth temperature range for *Lactobacillus casei* shirota.

Fermentations 9 and 11 were made of mixtures with different malt/water ratios, and were also sterilised at 121◦C (see Table 5.1). These two fermentations were inoculated

**Table 5.1** Preparation data of the shaking flask fermentation media.

| Fermentation No. | Fermentation Medium | Malt to Water Ratio | Sterilisation Period (h) | Total Volume (mL) |
|---|---|---|---|---|
| 1 | B-Syrup | 1 mL : 1 mL | 0.25 | 235.3 |
| 2 | B-Syrup | 1 mL : 1 mL | 0.25 | 235.3 |
| 3 | KL3 | 1 mL : 1 mL | 0.25 | 235.3 |
| 4 | KL3 | 1 mL : 1 mL | 0.25 | 235.3 |
| 5 | B-Syrup+KL3 | 0.5 : 0.5 : 1 mL | 0.25 | 235.3 |
| 6 | Whole malt grains | 8.2 mL : 1 g | 8.00 | 235.3 |
| 7 | Whole milled malt grains | 5.6 mL : 1 g | 2.00 | 235.3 |
| 8 | Whole malt grains | 8.2 mL : 1 g | 8.00 | 235.3 |
| 9 | Whole malt grains | 8.2 mL : 1 g | 8.00 | 235.3 |
| 10 | Whole milled malt grains | 5.6 mL : 1 g | 2.00 | 235.3 |
| 11 | Whole milled malt grains | 5.6 mL : 1 g | 2.00 | 235.3 |

with the MYGP medium containing the yeast *Saccharomyces cerevisiae* (approximately 10% of the total volume) as has been suggested by Stanbury *et al.* (1998) and Wang (1999b). Then they were also placed on a suitable rotary glass shaker (HT-INFORS-AG-CH 4103 Botimingen, England) operating at 280 r.p.m. The shaking flasks were incubated at 33◦C, which also lies within the optimum growth temperature range for *Saccharomyces cerevisiae*·

## 5.3 Bioreactors preparation (main fermentations)

Each of the main fermentation media was made from a mixture of malt/water sterilised for a period of time at 121◦C (see Table 5.2). The medium was then

inoculated with the main inoculum (see Table 5.2) by 10-15% of its total volume (Stanbury *et al.*, 1998; Wang, 1999b). The medium was put into a two-litre glass bioreactor with a flee (magnetic or stirrer bar). The bioreactor was then placed in a water bath with a heater set at 34.5°C in order to keep the temperature of the fermentation medium at 33°C. The water bath containing the bioreactors was also had a magnetic stirrer to mix the medium thoroughly at 400-550 r.p.m. (see Figure 5.1 and 5.2).

**Table 5.2** Preparation data of the bioreactor fermentations.

| Fermentation No. | Fermentation Medium | Main Inoculum | Malt to Water Ratio | Sterilisation Period (h) | Total Volume (mL) |
|---|---|---|---|---|---|
| 12 | B-Syrup | 1 | 1 mL : 1 mL | 0.25 | 1000 |
| 13 | B-Syrup | 2 | 1 mL : 1 mL | 0.25 | 1000 |
| 14 | KL3 | 4 | 1 mL : 1 mL | 0.25 | 1000 |
| 15 | Whole malt grains | 6 | 8.2 mL : 1 g | 8.00 | 1000 |
| 16 | Whole milled malt grains | 7 | 5.6 mL : 1 g | 2.00 | 1000 |
| 17 | Whole malt grains | 8 and 9 1 : 1 | 8.2 mL : 1 g | 8.00 | 1000 |
| 18 | Whole milled malt grains | 10 and 11 1 : 1 | 5.6 mL : 1 g | 2.00 | 1000 |

## 5.4 Characteristics of fermentation media

Some analyses were made to the four different materials used as fermentation media before the inoculation. These analyses involve the measurement of the pH, solid contents, density, total nitrogen, FAN, total phosphorus, phosphorus in liquid, lactic acid and sugar content. The results obtained by these analyses are shown in Table 5.3.

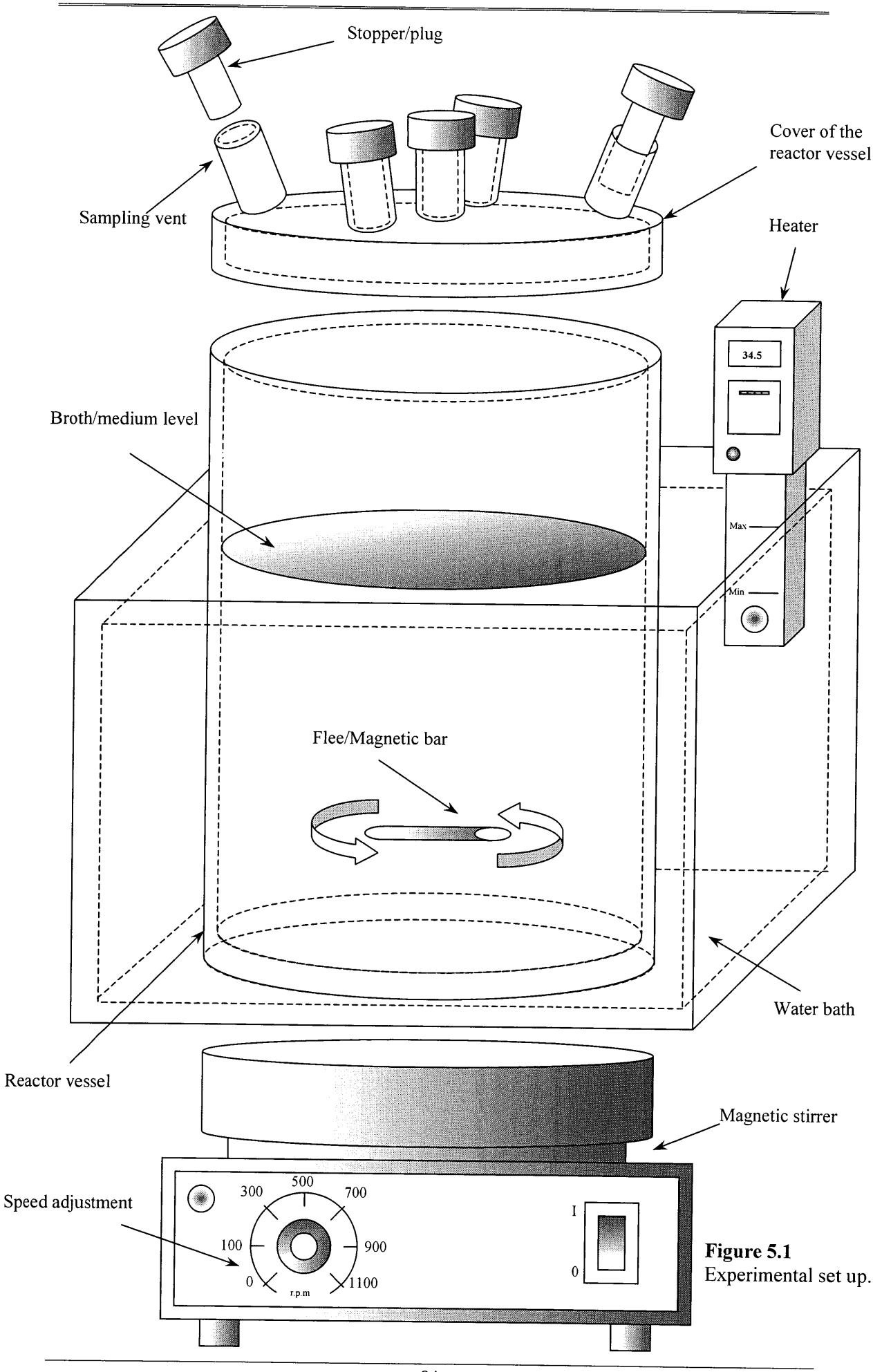



**Figure 5.1**
Experimental set up.

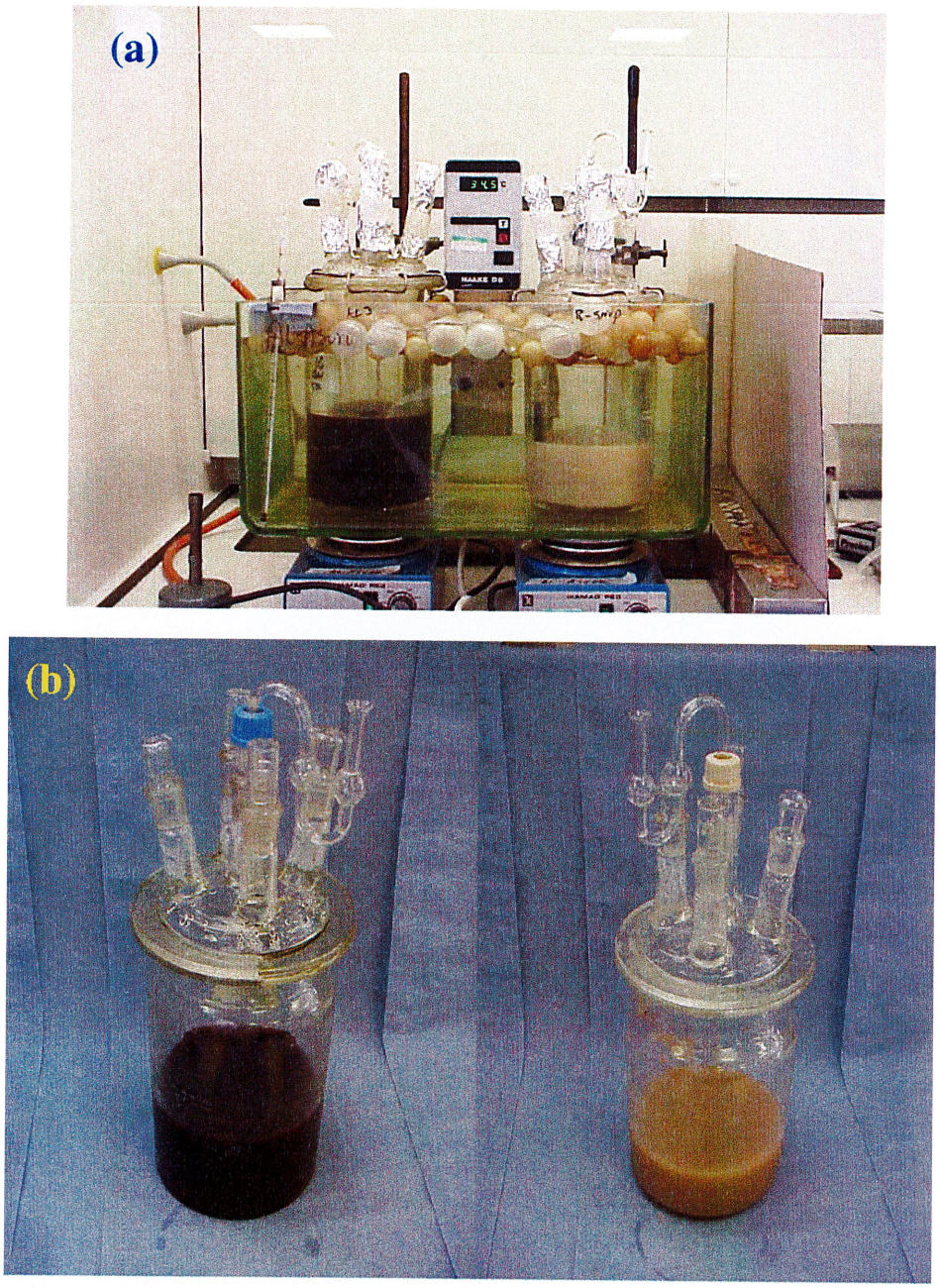



**Figure 5.2** Actual experimental set up. (a) Complete set up laminar flow cabinet. (b) Glass bioreactors of two litres capacity with two of the fermentation media used (KL3 and B-Syrup).

**Table 5.3** Analysis for the four different fermentation media.

| Measured Parameters | Sterilised Fermentation Medium | | | |
|---|---|---|---|---|
| | **B-Syrup** | **KL3** | **Whole Malt Grains** | **Whole Milled Malt Grains** |
| pH | 4.85 | 5.24 | 4.71 | 5.41 |
| Solid Contents (g $L^{-1}$) | 804.1 | 539.1 | 58.400 | 193.50 |
| Density (g $L^{-1}$) | 1044.0 | 1180.0 | 990.90 | 1208.2 |
| Total Nitrogen (mg $g^{-1}$) | 0.5353 | 7.6528 | 10.479 | 11.514 |
| Protein '6.25*N' (mg $g^{-1}$) | 3.3460 | 47.830 | 65.496 | 71.963 |
| FAN (mg $L^{-1}$) | 149.48 | 1421.4 | 117.65 | 89.465 |
| SPhos (mg $g^{-1}$) | 0.2245 | 2.0230 | 1.5469 | 2.0198 |
| LPhos (mg $L^{-1}$) | 229.43 | 357.95 | 136.39 | 214.27 |
| Lactic Acid (mg $L^{-1}$) | 142.67 | 201.15 | 186.41 | 174.46 |
| Sugar Concentration | | | | |
| **Before Sterilisation** | | | | |
| Fructose (g $L^{-1}$) | - | - | - | - |
| Glucose (g $L^{-1}$) | 2.0125 | 3.1085 | - | 0.3465 |
| Sucrose (g $L^{-1}$) | 0.9980 | 1.3940 | - | 1.6801 |
| Maltose (g $L^{-1}$) | 17.515 | 21.841 | - | 0.9023 |
| **After Sterilisation** | | | | |
| Fructose (g $L^{-1}$) | 0.3734 | 0.5420 | - | - |
| Glucose (g $L^{-1}$) | 2.0492 | 3.1305 | 0.2530 | 0.3310 |
| Sucrose (g $L^{-1}$) | 0.8454 | 1.3810 | 1.3345 | 1.8730 |
| Maltose (g $L^{-1}$) | 16.270 | 16.646 | 0.701 | 0.8680 |

From the results obtained, it is clear that the total amount of nitrogen available in each of the four media was much more than that required for both the *Lactobacillus casei* shirota and *Saccharomyces cerevisiae* to grow, since the minimum amount of nitrogen required by *Saccharomyces cerevisiae* is about 0.05 mg $g^{-1}$ (Wang, 1999b), and much less than that is required by *Lactobacillus casei* shirota (Wang, 1999b). In order to prove that, four sets of agar plates have been prepared from the four media and have been inoculated with *Saccharomyces cerevisiae*. After 12 hours the *Saccharomyces cerevisiae* colonies appeared on the surface of all the plates which gives strong evidence about the suitability of the nutrition media. These experiments also prove

that the amounts present of other nutrients such as the total phosphorus and sugar are more than sufficient for the growth of the microorganisms used.

## 5.5 Analysis of the main inocula (Yakult)

Some experimental analyses have also been made on the Yakult drink, which was used indirectly as the main inoculum for the shaking flask fermentations. The measured values are shown in Table 5.4.

**Table 5.4** Analyses of Yakult.

| Measured Parameters | Yakult |
|---|---|
| pH | 3.68-3.73 |
| Solid Contents (g $L^{-1}$) | 34.100 |
| Density (g $L^{-1}$) | 1074.0 |
| Total Nitrogen (mg $g^{-1}$) | 1.7580 |
| Protein '6.25*N' (mg $g^{-1}$) | 11.173 |

## 5.6 Monitoring of the fermentation processes

This section intends to discuss the experimental in terms of the microorganisms growth and the different media used. In addition, results from different runs will be compared and contrasted when appropriate.

### 5.6.1 Plate counting (viable cell count)

As it has been reported by Winslow *et al.* (1917) that *Lactobacillus casei* shirota colonies has a white to very light yellow colour, and their growth in the fermentation broth causes heavy turbidity. It was almost impossible to benefit from the viable count (plate counting) technique because it was not possible to distinguish between the agar plate medium (solid malt extract) and the colonies, even when other types of agar

medium, such as MRS (Sigma) or Raka-Ray (Sigma), were used. This technique was therefore not used.

### 5.6.2 Slide counting (total cells count)

In this work it was assumed that the *Lactobacillus casei* shirota would display Monod growth kinetics in free cell culture. Based on that, the results obtained from the slide counting technique are not acceptable as can be seen from Figure 5.3, where the number of cells curve does not follow the typical Monod curve.

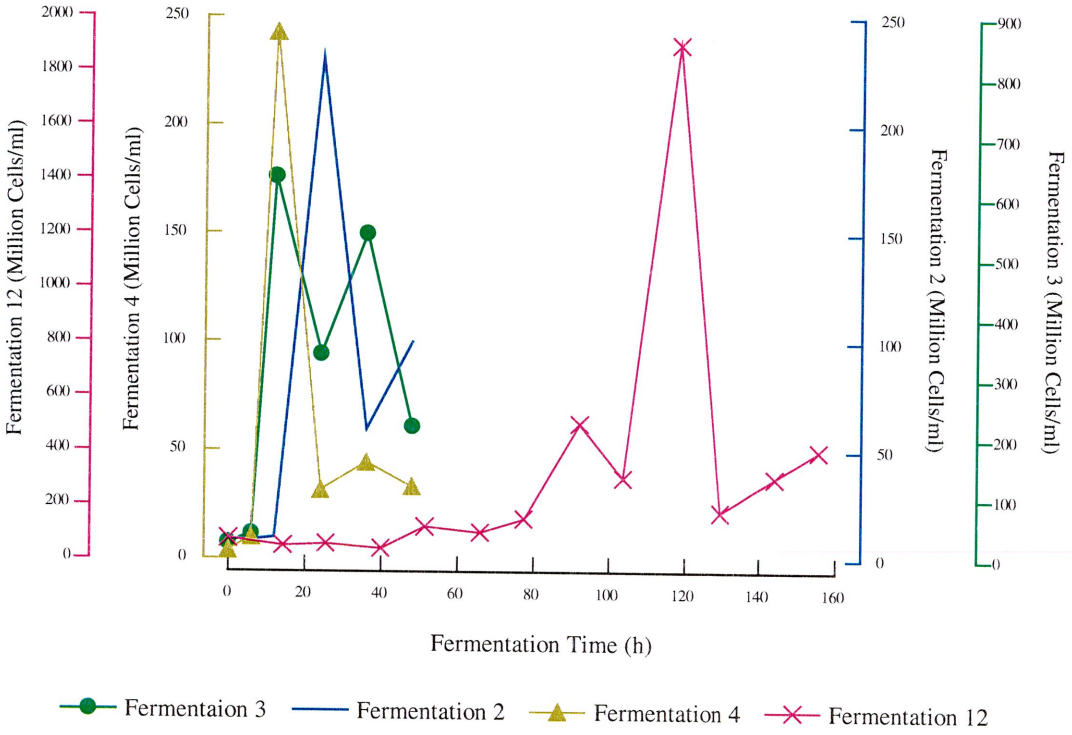



**Figure 5.3** Analysis of total cell count during some B-Syrup and KL3 fermentations (Slide counting for the *Lactobacillus casei* shirota).

Such results might have been caused by inefficient mixing, inaccurate preparation of the dilution reagent and the difficulty of counting bacteria under the microscope due to their very small size. Therefore, the slide counting techniques was not used for the rest of the fermentations samples analyses.

### 5.6.3 Experimental analyses

The chemical analyses (which include pH measurements, free amino nitrogen, phosphorus in liquid sample, lactic acid, sugar analysis and dry weight) have been perform in almost all the fermentation samples. Tables of the results obtained are given in Appendix C. These results have also been plotted against the fermentation time. The graphs provide a good understanding of the fermentation process and the optimum fermentation time.

Fermentations 1 and 12 (a shaking flask and a bioreactor fermentation respectively) were intended to be trial runs to obtain preliminary about the suitable parameters to be used to follow fermentation.

The analyses for free amino nitrogen, phosphorus and sugars were chosen because the microorganisms need these basic substrates to grow. As a results of that, any change in the measured amount of any of these compounds during the fermentation can indicate whether the cells are growing or not and can be helpful in estimating the rate of the fermentation process.

As a consequence of the problems faced in using the plate counting and the slide counting techniques, the dry weight technique was chosen as an alternative method to obtain the growth curve based on the total number of cells.

The lactic acid analysis was used to measure the production of lactic acid, a major product of the homofermentative LAB used. On the other hand, the pH measurement was also used to indicate the acidity level of the fermentation broth. The lactic acid concentration and the level of acidity play a big role in both the concentration of viable cells and the taste of the final product.

### 5.6.3.1 Evolution of fermentation parameters

Figure 5.4 shows the changes of the monitored parameters during the second shaking flask fermentation of B-Syrup; samples were taken every 6 or 12 hours. The dry weight increases by 0.0853 g $mL^{-1}$ which is an indication of the growth of the microorganisms. In addition to that, glucose and phosphorus concentrations decrease from 3.843 g $L^{-1}$ and 239.8 mg $L^{-1}$ to 1.994 g $L^{-1}$ and 226.3 mg $L^{-1}$ respectively, due to the consumption of these substances by the growing cells mainly during the exponential phase. Over a fermentation time of 48 hours the pH value falls from 4.08 to 4.03 as a result of the lactic acid produced by the *Lactobacillus casei* shirota, which increases from 170.11 to 521.39 mg $L^{-1}$. On the other hand, the measured amount of FAN decreases slightly during the first 12 hours and then it drops in a linear manner to reach its minimum value after 36 hours. After that, the FAN concentration starts to increase.

The slight change in the FAN at the beginning of the process is due to the lag phase, which is considered as an adaptation phase for the microorganism. During this phase thousands of enzymatic reactions occur inside the cells in order to adapt themselves to the new environment where growth takes place but no new cells are produced.

Moreover, through this growth phase, cells do not need to absorb FAN; which is required for the protein production, while they need to absorb the phosphorus and the glucose in very small amounts. Phosphorus is needed during the lag phase because it contributes to all the enzymatic reactions occurring while the glucose is required as an energy source for the cells. In other words, phosphorus and glucose are consumed as maintenance substrates during the lag phase, but during the exponential phase they are used as substrates for growth, producing mainly new biomass and lactic acid.

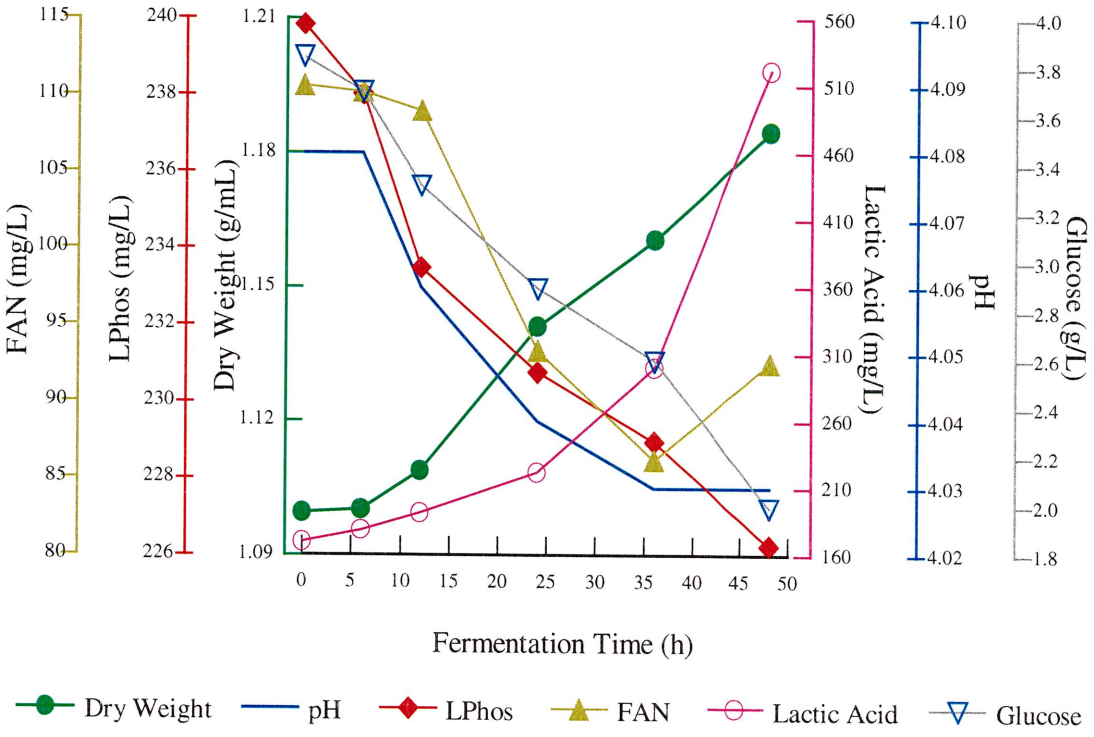



**Figure 5.4** Analyses during B-Syrup shaking flask fermentation (Fermentation 2; inoculated with *Lactobacillus casei* shirota).

The changes in FAN concentration observe could then be due to two different mechanisms. The linear decreases in the FAN concentration was due to its consumption as the main nitrogen source for the cells and this decrease was considered as a direct indicator of cell growth. The increase in the FAN after 36 hours could be due to one or more of the following reasons. The first is that the LAB could be used to produce protease during fermentation. Protease is an enzyme produced by specific types of microorganisms under certain conditions to hydrolyse proteins into FAN. The idea of the protease production (and therefore FAN) could also be explained in two different ways. The first one is if the protease is produced at the beginning of the fermentation process as a primary metabolism with low activity and it accumulates until the level of protease activity is sufficient to produce. A FAN increase means that the protease production rate of FAN at certain point is higher than the rate of FAN consumption by the growing cells. The second possibility is based on the assumption that protease was produced as a secondary metabolism when the stationary phase was reached. This phase represents the maximum possible cell concentration where the cells grow to their maximum size and then they start to produce protease. The above two possible explanations have been proposed because there were no information in the literature confirming whether the *Lactobacillus casei* shirota is a protease producer or not.

The second possible explanation for the increase in FAN is cell autolysis. This is based on the assumption that at certain point the old cells start to autolysis by losing their cell walls and that leads to the release of the internal cell contents, which are mainly proteins and amino acids, to the fermentation broth.

In the rest of the fermentations, the parameters followed similar trends to those observed in Figure 5.4 (see Figure 5.5 to Figure 5.15), with the exception of the glucose concentration. In fermentations 4, 5 and 13 (see Figures 5.5, 5.6 and 5.11) the glucose concentration follows a pattern similar to that seen in Figure 5.4. However, in fermentations 6 and 15 (see Figures 5.7 and 5.13) the glucose concentration continually increases. On the other hand, in fermentations 7, 14, and 16 (see Figures 5.8, 5.12, and 5.14) the glucose concentration first increases, but at some point it decays to the end of fermentation.

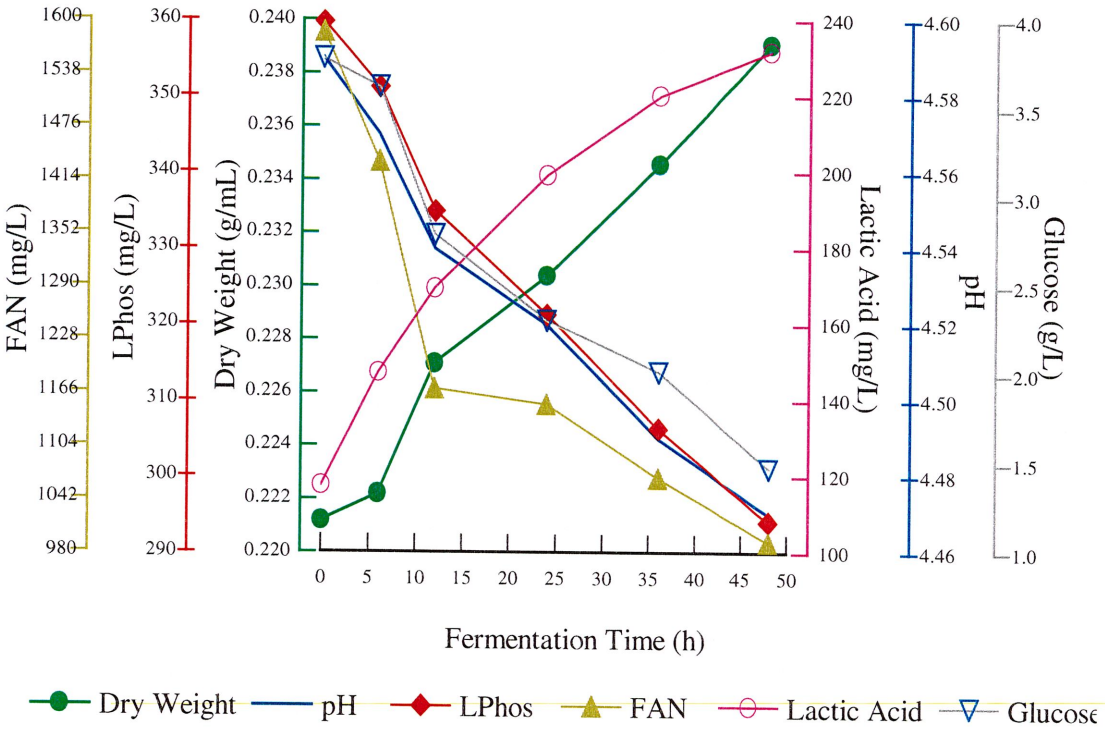



**Figure 5.5** Analyses during KL3 shaking flask fermentation (Fermentation 4; inoculated with *Lactobacillus casei* shirota).

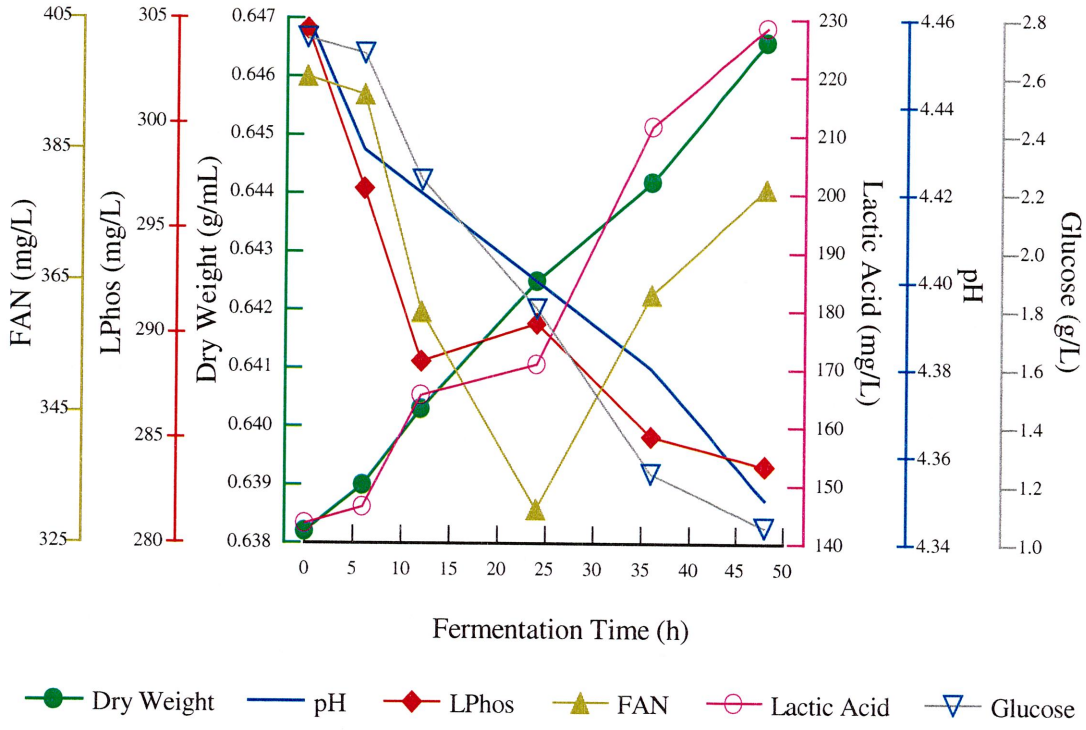



**Figure 5.6** Analyses during a mixture of KL3 and B-Syrup shaking flask fermentation (Fermentation 5; inoculated with *Lactobacillus casei* shirota).

The decrease in glucose concentration is an indication that the rate of the production of glucose by hydrolysis of starch is less than the rate of glucose consumption by the microorganisms or that the amount of starch available in the media is too little to sustain a sufficient rate of glucose production by hydrolysis.

The previous experiments helped in recognising the most suitable parameters to use in order to monitor the cell growth, which were mainly the phosphorus concentration and the pH level. Therefore, in fermentations 7 to 11 and 16 to 18, the parameters measured were only these.

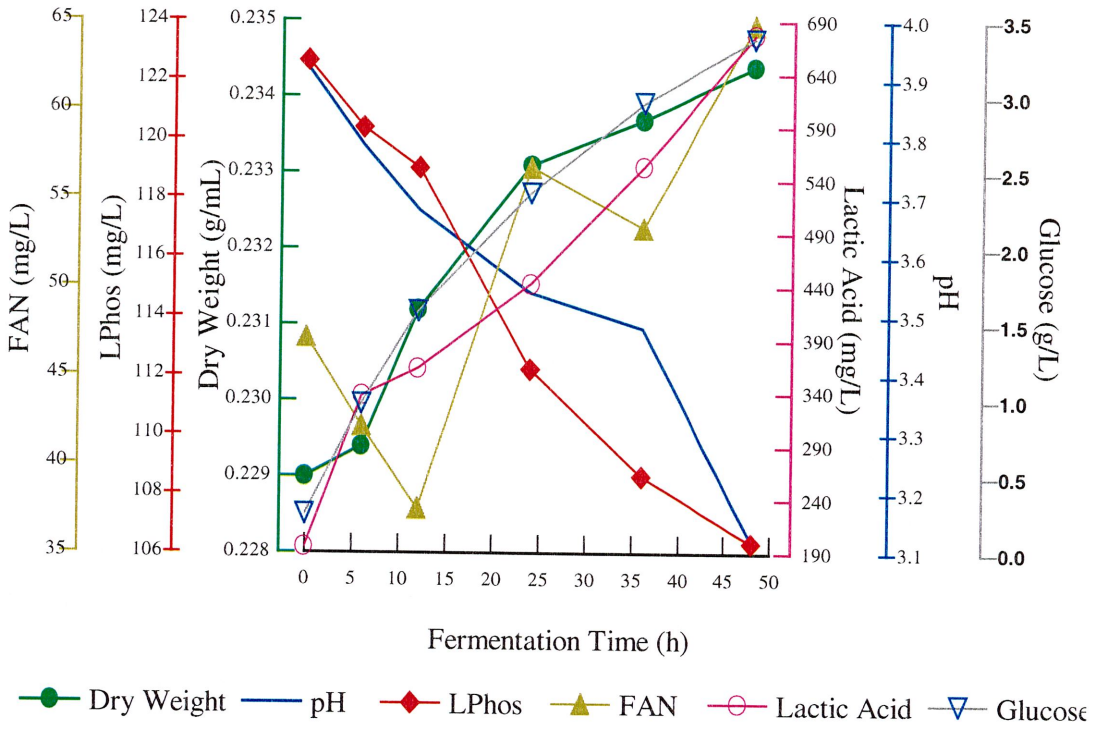



**Figure 5.7** Analyses during whole malt grains shaking flask fermentation (Fermentation 6; inoculated with *Lactobacillus casei* shirota).

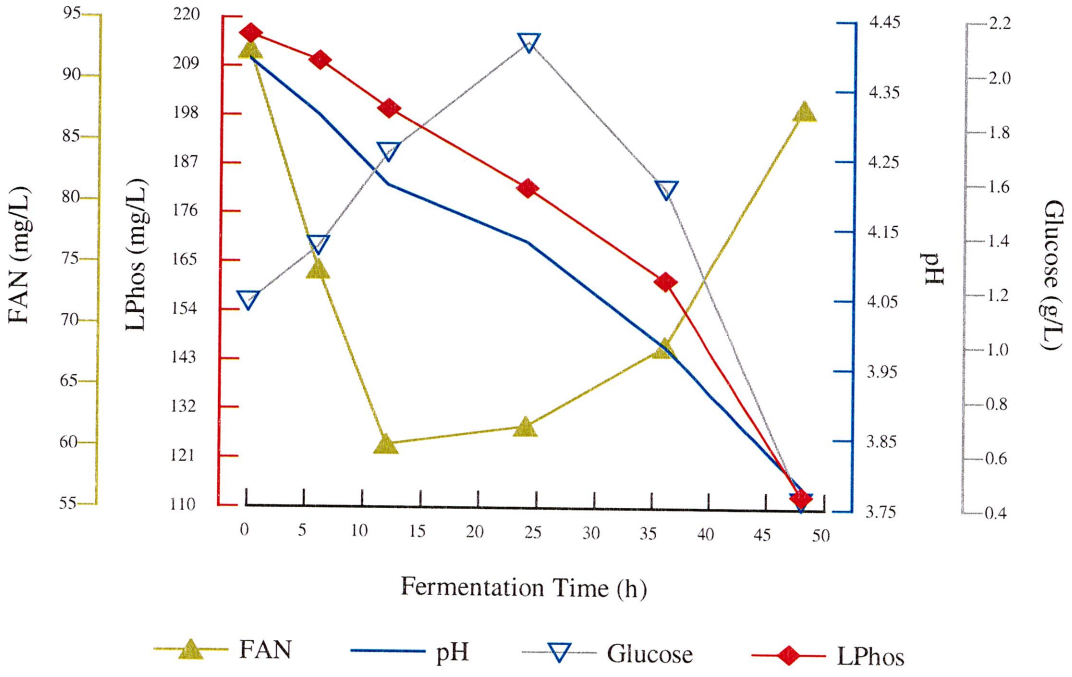



**Figure 5.8** Analyses during whole milled malt grains shaking flask fermentation (Fermentation 7; inoculated with *Lactobacillus casei* shirota).

Figure 5.9 shows the changes in the monitored parameters for two different shaking flask fermentations, fermentations 8 and 9. Both fermentations have the same medium, whole malt grains (see Table 5.1), but they were inoculated with two different microorganisms. Fermentation 8 was inoculated directly with Yakult as the main source for the *Lactobacillus casei* shirota while fermentation 9 was inoculated with the MYGP medium that contains *Saccharomyces cerevisiae*.

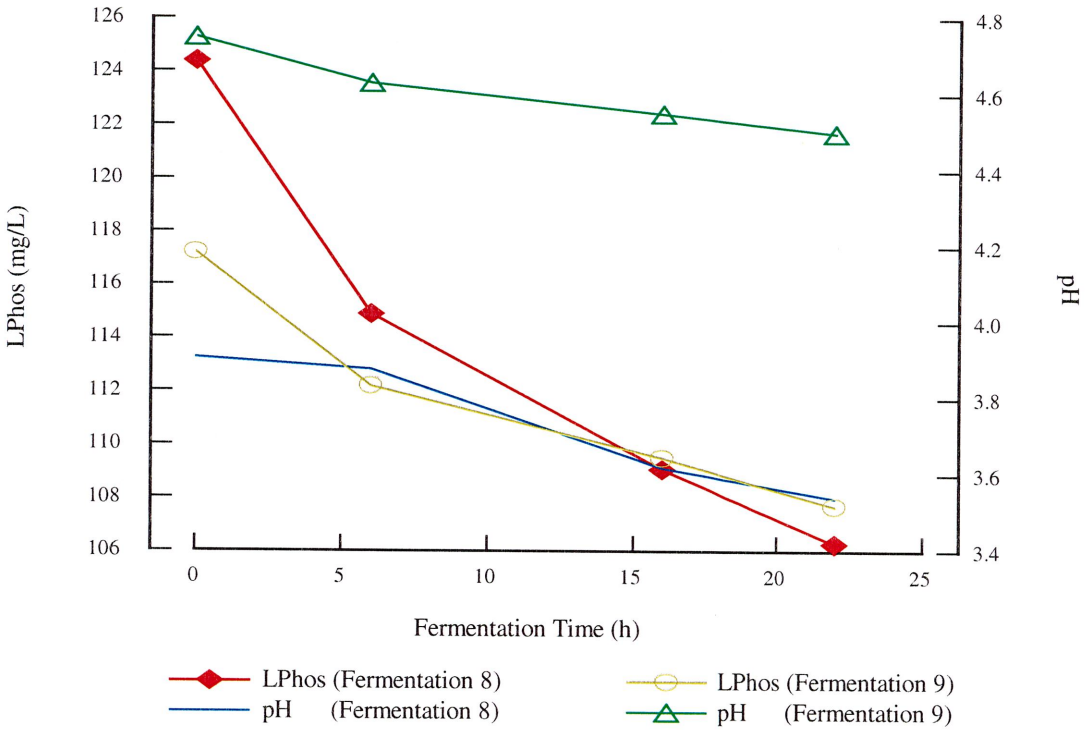



**Figure 5.9** Phosphorous concentration and pH during whole malt grains shaking flask fermentations (Fermentation 8 and 9; inoculated with *Lactobacillus casei* shirota and *Saccharomyces cerevisiae*, respectively).

As can be seen in Figure 5.9, the pH of the MYGP medium is higher than that of the medium inoculated with Yakult, but in both cases the pH values decrease during the fermentation process. The phosphorus concentration in both fermentation media decreases from 124.4 and 117.2 mg $L^{-1}$ to 106.3 and 107.7 mg $L^{-1}$ respectively.

The same tendency was observed in similar fermentations, fermentation 10 and 11, where the whole milled malt grains was used as fermentation medium instead of whole malt grains (see Figure 5.10).

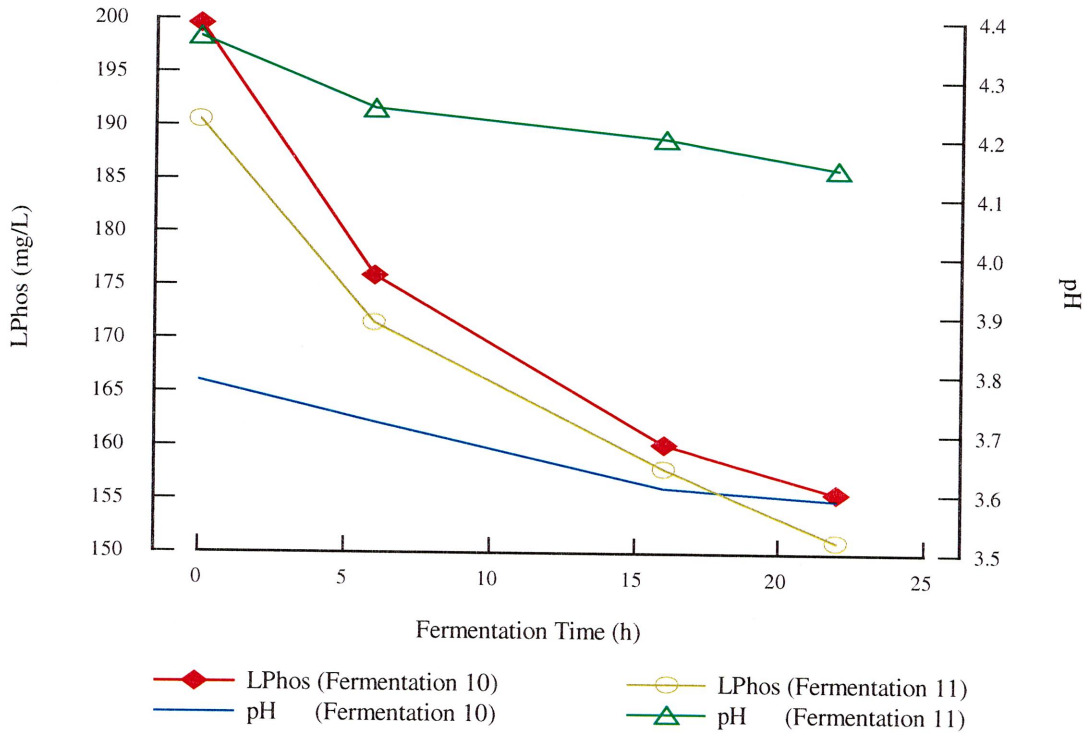



**Figure 5.10** Phosphorous concentration and pH during whole milled malt grains shaking flask fermentations (Fermentation 10 and 11; inoculated with *Lactobacillus casei* shirota and *Saccharomyces cerevisiae*, respectively).

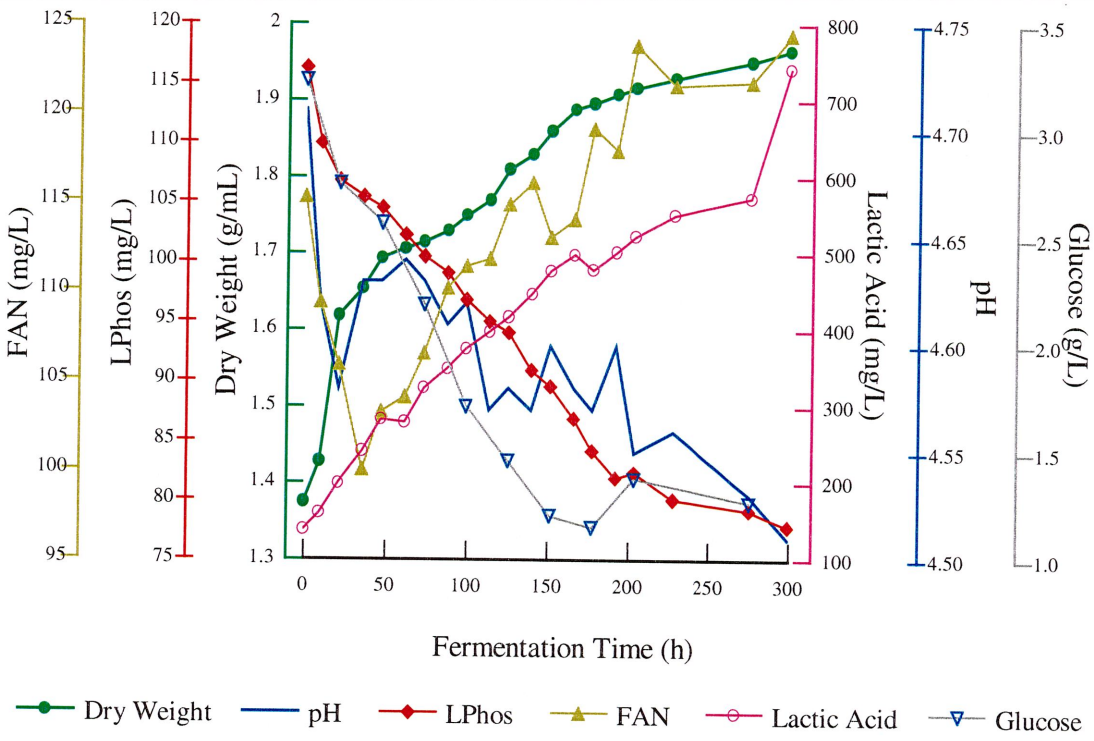



**Figure 5.11** Analyses during B-Syrup bioreactor fermentation (Fermentation 13; inoculated with *Lactobacillus casei* shirota).

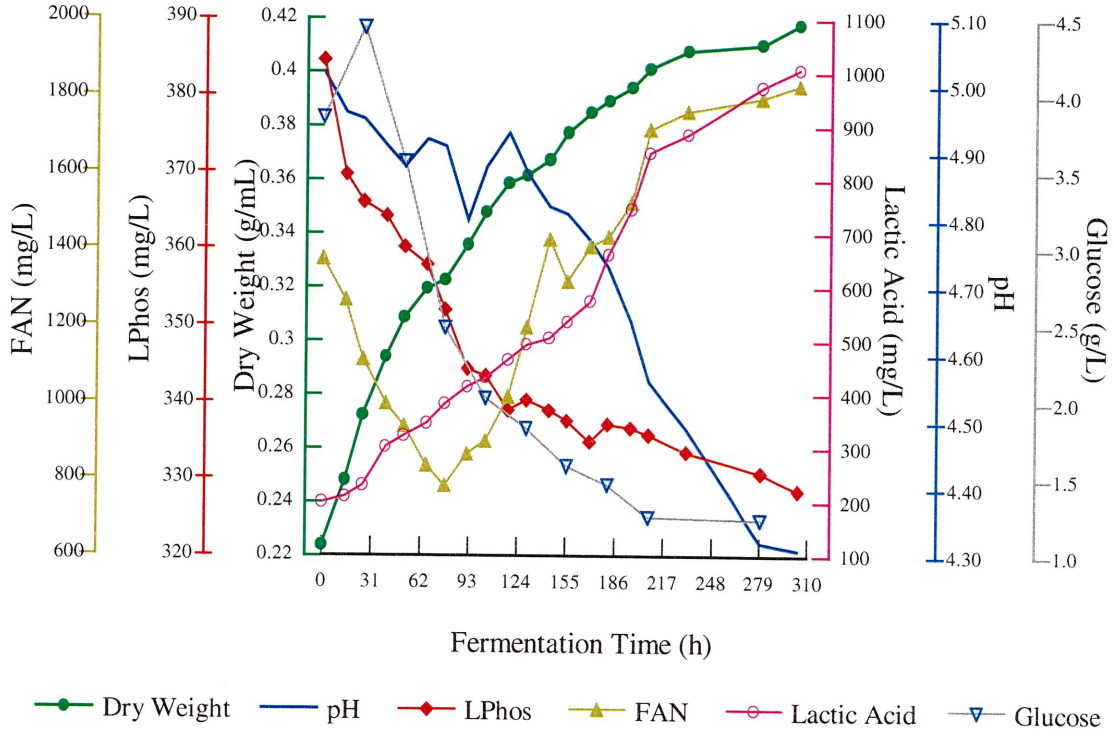



**Figure 5.12** Analyses during KL3 bioreactor fermentation (Fermentation 14; inoculated with *Lactobacillus casei* shirota).

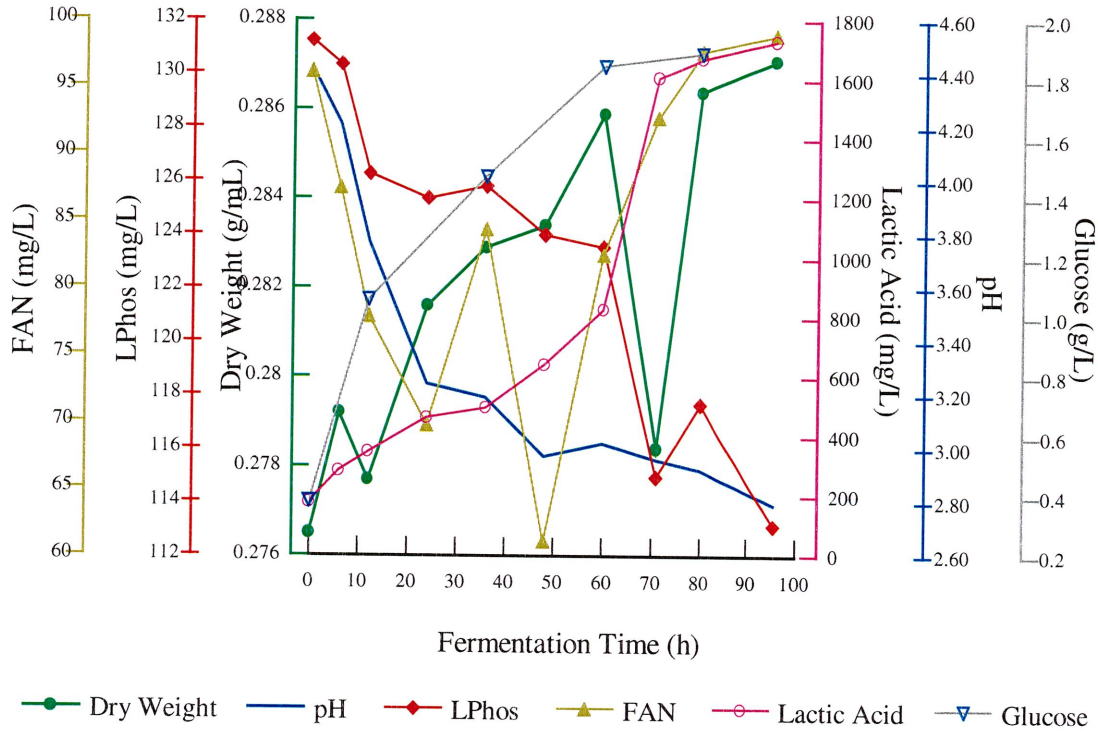



**Figure 5.13** Analyses during whole malt grains bioreactor fermentation (Fermentation 15; inoculated with *Lactobacillus casei* shirota).

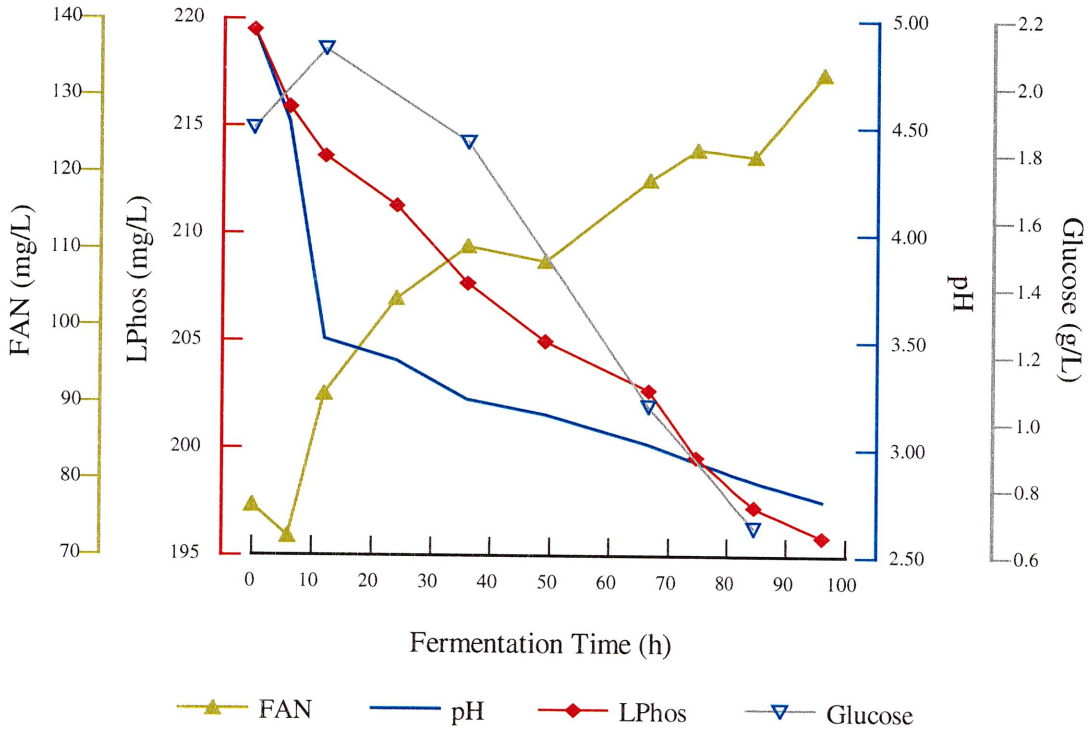



**Figure 5.14** Analyses during whole milled malt grains bioreactor fermentation (Fermentation 16; inoculated with *Lactobacillus casei* shirota).

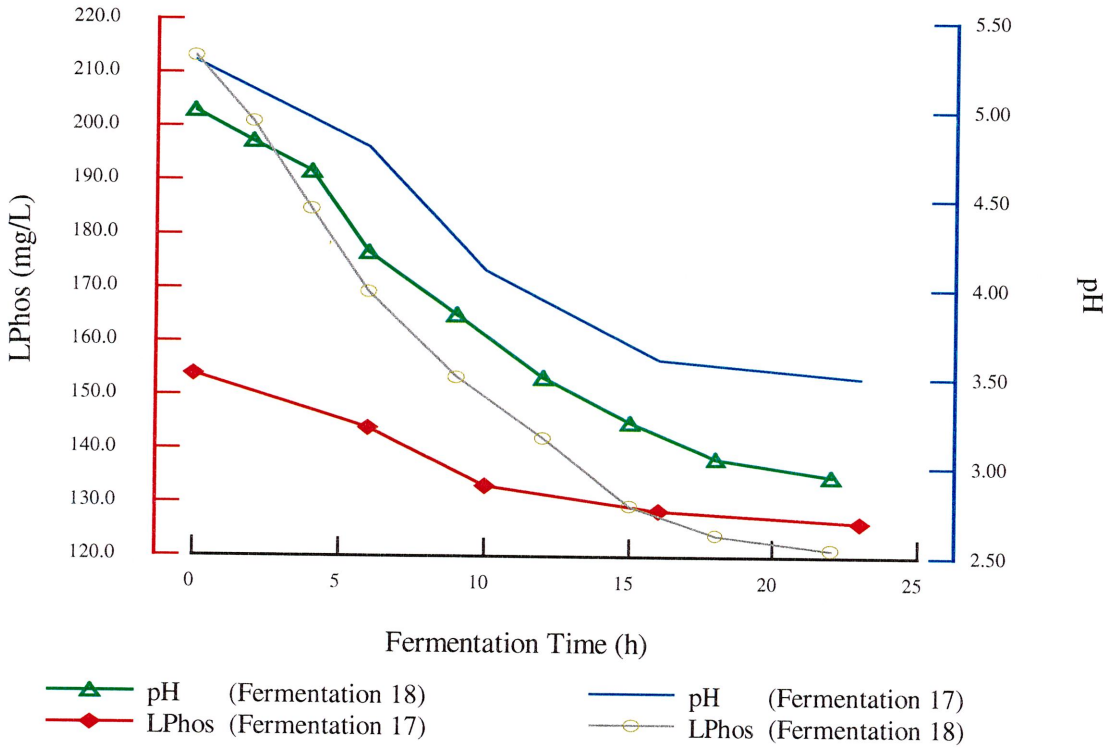



**Figure 5.15** Phosphorus concentration and pH during whole malt and whole milled malt grains bioreactor fermentations (Fermentation 17 and 18; inoculated with a mixture of *Lactobacillus casei* shirota and *Saccharomyces cerevisiae*).

### 5.6.3.2 Comparison between fermentation media

In the following figures four of the parameters monitored in all fermentations (both using shaking flasks and bioreactors, 18 fermentations in total) will be compared (see Figures 5.16 to 5.23). New data obtained from fermentations 1, 3 and 12, not discussed before, will also be included.

In Figures 5.16 and 5.17 all the pH profiles are shown. It can be observed (see Figure 5.17) that the pH fall is significant at the beginning of fermentation (up to around 30 h), but then it decreases at a smaller rate. This behaviour agrees with what has been found in the literature particularly with the studies published by Sanders (1992), Tanasupawat and Komagata (1995), and Billings (1998).

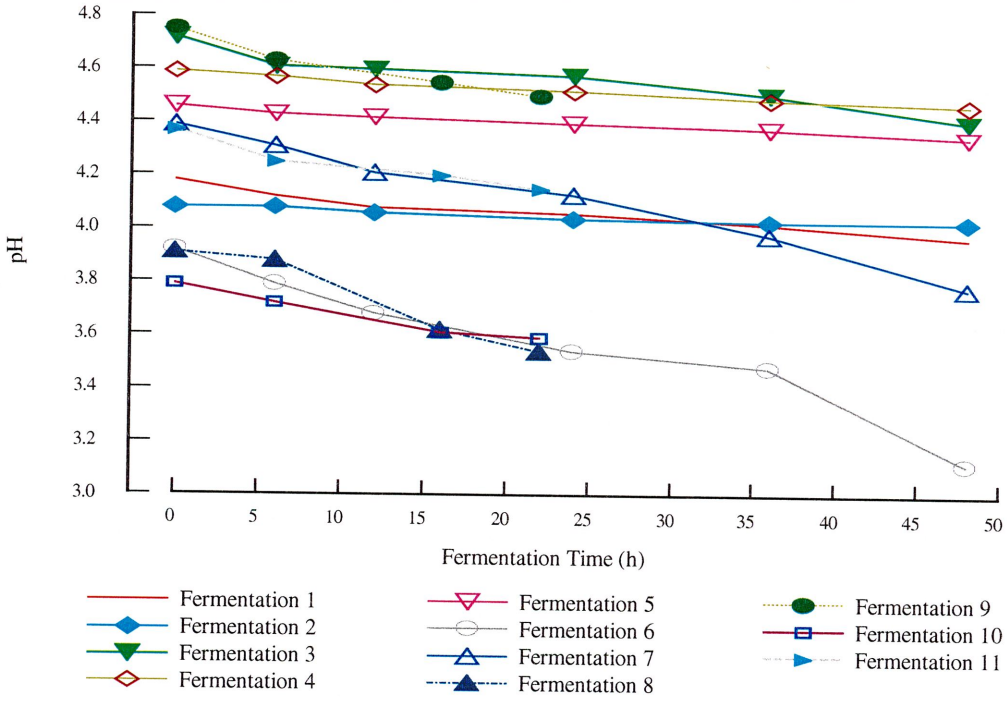



**Figure 5.16** pH profiles for all shaking flask fermentations using different fermentation media and microorganism.

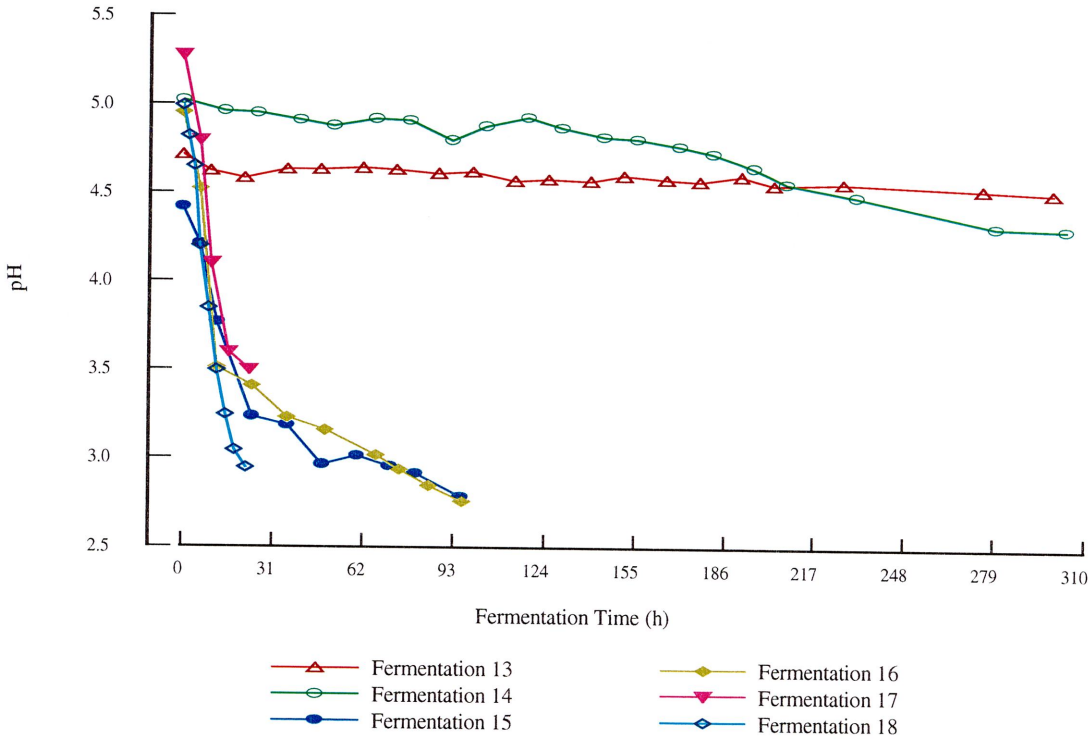



**Figure 5.17** pH profiles for all bioreactor fermentations using different fermentation media and microorganism.

From Figures 5.16 and 5.17, it can be easily seen that each fermentation medium had a specific pH value at the beginning of the fermentation and at the end. The highest initial pH value among the shaking flask fermentations was for the KL3 medium (about 5.02) while the lowest value was for the whole milled malt grains (about 3.59). The lowest and highest initial pH values measured in the bioreactors were for the whole malt grains medium in fermentations 15 and 17 which were inoculated with *Lactobacillus casei* shirota. The lowest pH value reached during all the shaking flask and bioreactor fermentation was about 2.76, which is considered as an acceptable value for both the human consumption and for LAB growth.

It is important to mention that fermentations 1 and 2 use the same fermentation medium (B-Syrup) and are inoculated with the same microorganism (*Lactobacillus*

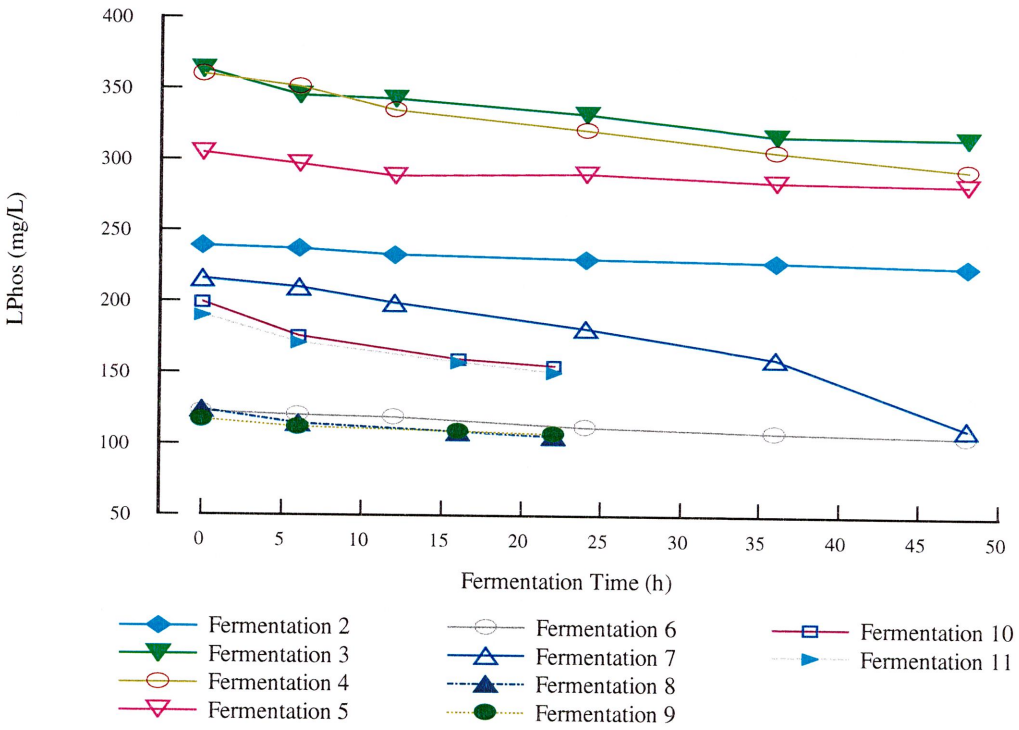



**Figure 5.18** Phosphorus consumption profiles for shaking flask fermentations using different fermentation media and microorganism.

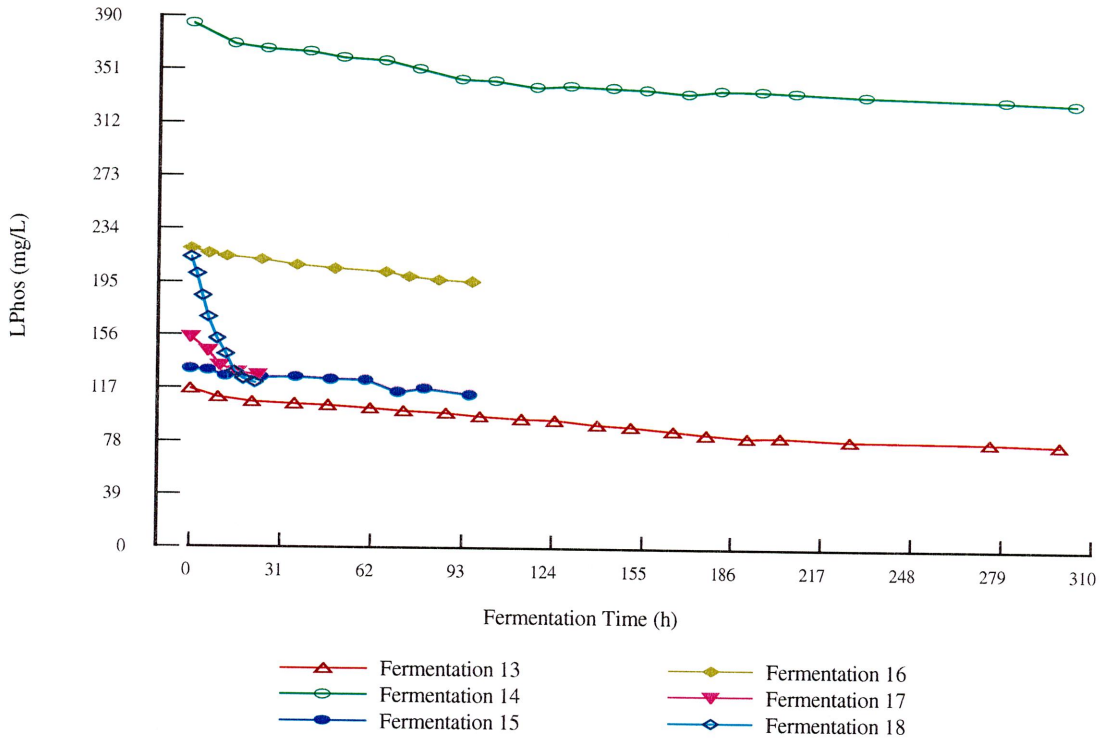



**Figure 5.19** Phosphorus consumption profiles for bioreactor fermentations using different fermentation media and microorganism.

*casei* shirota), and they have been monitored to ensure the repeatability of the results. Fermentations 3 and 4, and fermentations 12 and 13 were also carried out under the same conditions for the same reason.

Figures 5.18 and 5.19 show all the phosphorus concentration profiles. It can be noted they follow a tendency similar to that observed in the case of the pH. The explanation for this behaviour has already been discussed in section 5.6.3.1. This results agree with those reported by Sanders (1992) and Billings (1998). The KL3 medium appears to have the highest phosphorus concentration, both at the beginning and the end of fermentation.

The highest phosphorous consumption rate (see Figure 5.19) was obtained in fermentation 18, where it decreased from 213.1 to 121.3 mg $L^{-1}$. This effect could be due to the use of a mixed culture (*Lactobacillus casei* shirota and *Saccharomyces cerevisiae*) for the fermentation of whole milled malt grains.

The nitrogen concentration was measured as free amino nitrogen (see Figures 5.20 and 5.21). In all cases the FAN first decreases for approximately 60 hours (which is an indication of cell growth), but then the concentration starts to increase (see Figure 5.21). The possible reasons for this behaviour were discussed in the previous section. Again, the KL3 medium shows the highest FAN concentration if compared with the other media.

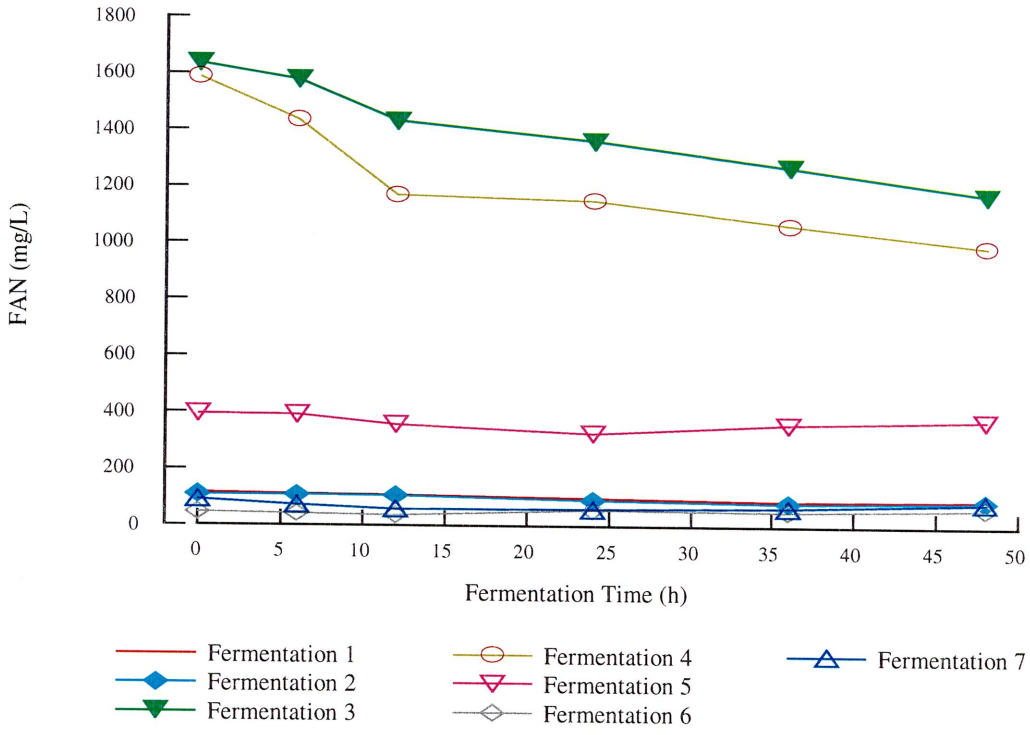



**Figure 5.20** FAN consumption profiles for shaking flask fermentations using different fermentation media.

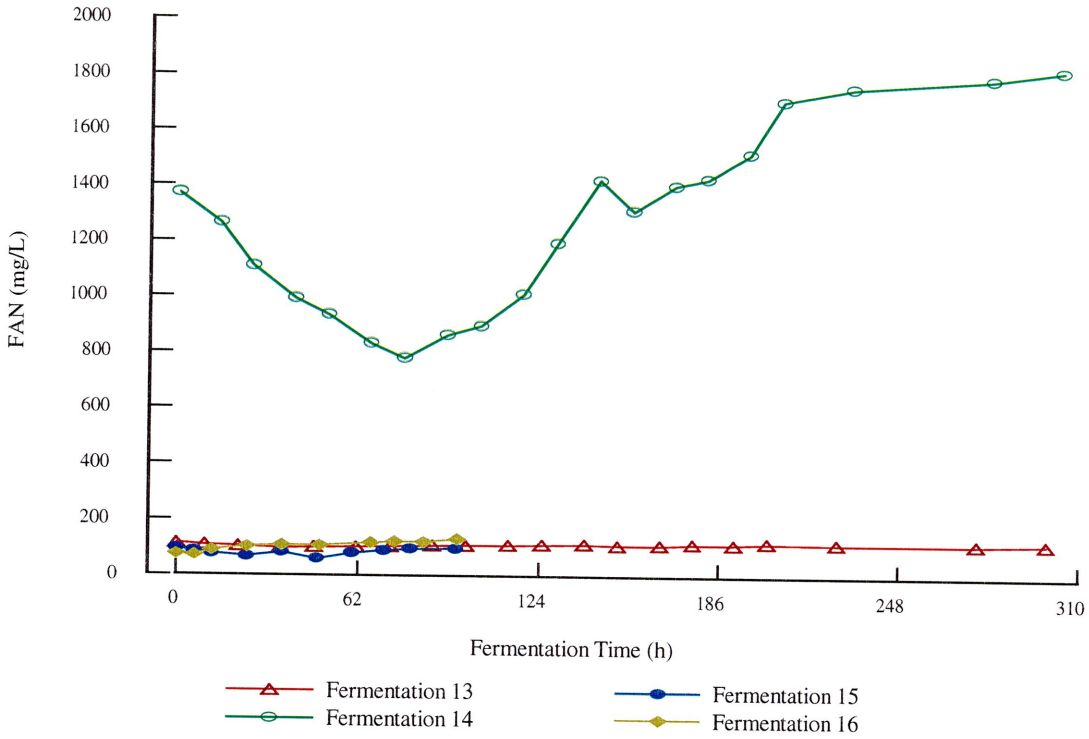



**Figure 5.21** FAN consumption profiles for bioreactor fermentations using different fermentation media.

Figures 5.22 and 5.23 indicate that lactic acid is produced even during the lag phase. This could be due to the fact that in the case of LAB, lactic acid has also been classified as one of the products directly associated with the generation of energy (energy metabolism), as shown in the results obtained by Sanders (1992), Hugenholtz (1993), Doran (1995), Tanasupawat and Komagata (1995), Garbutt (1997), Madigan *et al.* (1997) and Billings (1998).

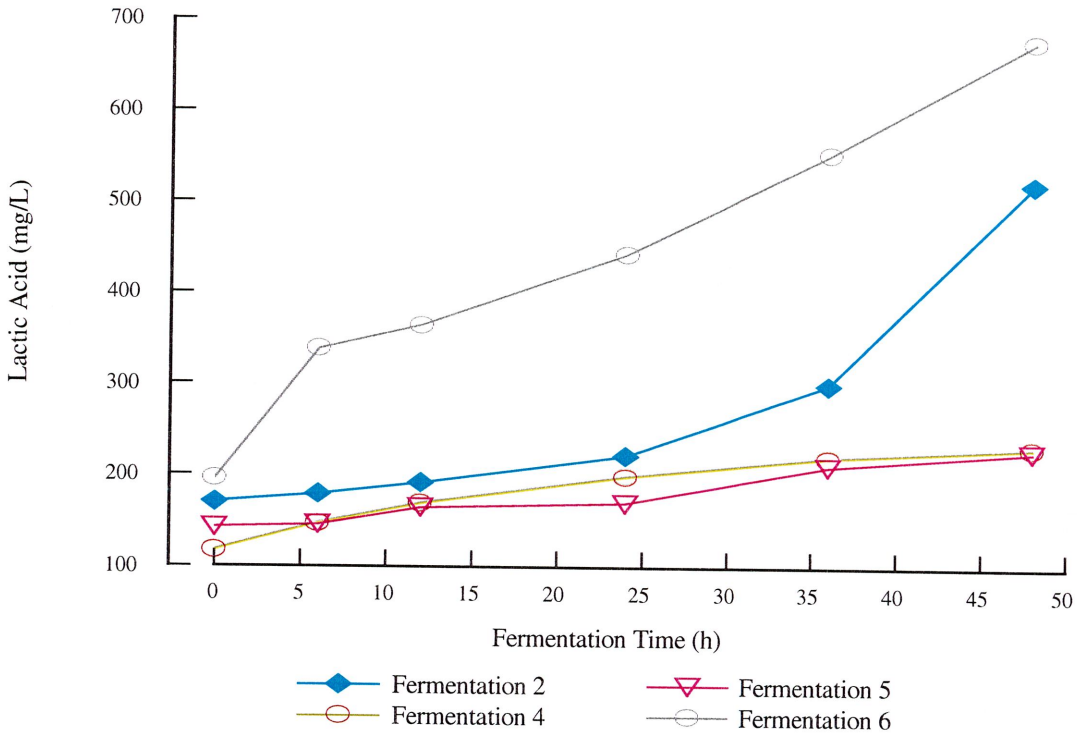



**Figure 5.22** Lactic acid production profiles for shaking flask fermentations using different fermentation media.

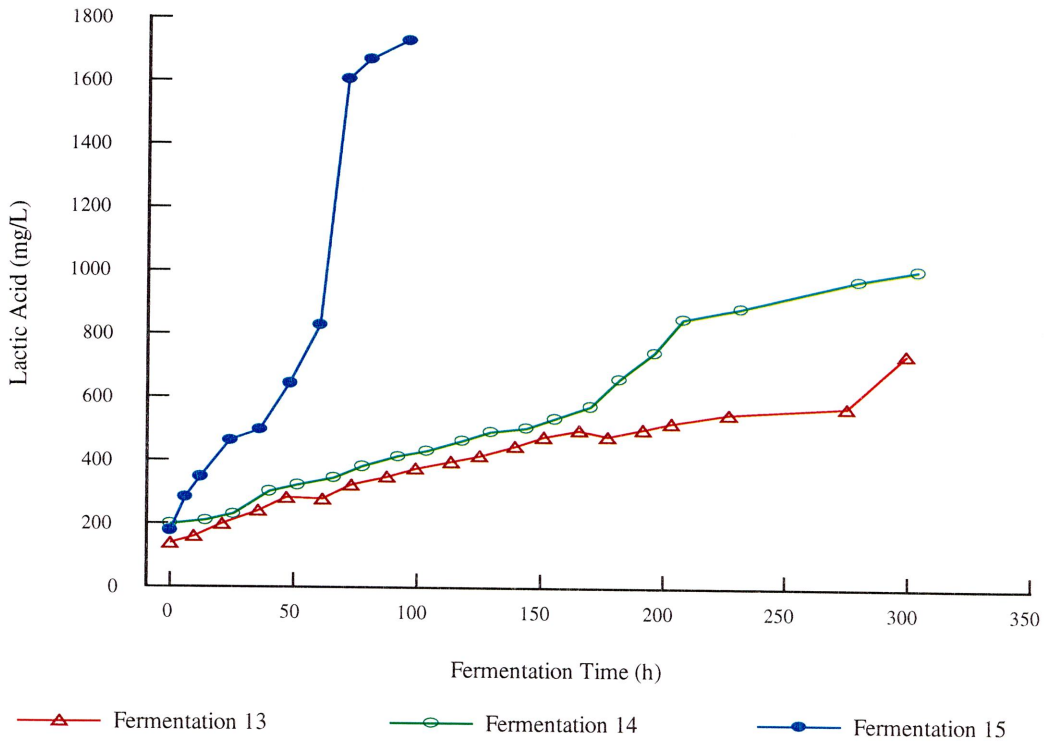



**Figure 5.23** Lactic acid production profiles for bioreactor fermentations using different fermentation media.

The measured values of the lactic acid in the shaking flasks show that the highest production rate occurs in fermentation 4, which contains KL3 as a fermentation medium and *Lactobacillus casei* shirota as an inoculum. In the case of the bioreactor fermentations the whole malt grains medium used in fermentation 15 had the highest concentration and the fastest production rate, which indicates the suitability of this medium to be fermented by the *Lactobacillus casei* shirota.

In addition to the lactic acid effect on flavour, lactic acid bacteria (LAB) can produce some organic molecules in very small quantities that may also influence the flavour of the fermented food (Garbutt, 1997).

The following figure (Figure 5.24) shows the actual shape of *Lactobacillus casei* shirota under the microscope. These two pictures were taken for a sample prepared from one of the fermented products using the SEM system.

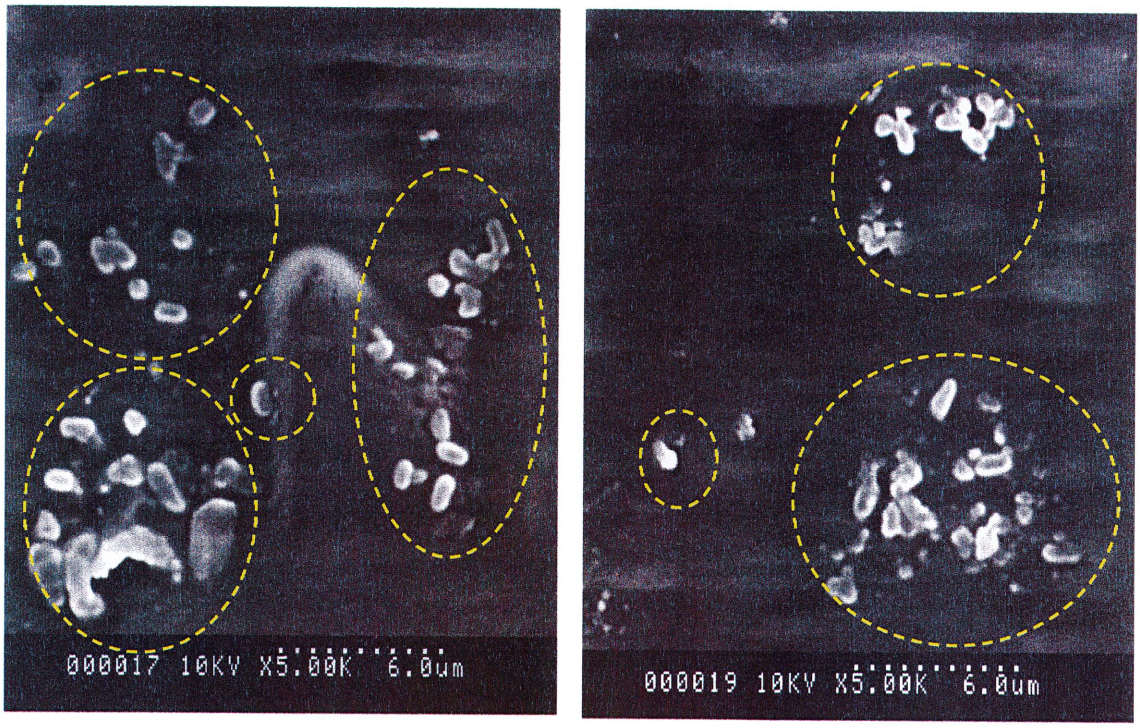



**Figure 5.24** Images of *Lactobacillus casei* shirota in one of the fermentation broth taken by SEM system (Scanning electron micrograph).

## 5.6.4 Sensorial tests

The results of the sensorial tests performed on each of the fermented products are illustrated in Table 5.5.

The final evaluation shows that the most preferable fermented drinks were the B-syrup/KL3 mixture and the KL3 in that order, but their final evaluation was not completely satisfactory due to their bitter taste.

**Table 5.5** Results of sensorial tests.

| | No. of Assessors | General Comments | Average Points Scored | Final Evaluation |
|---|---|---|---|---|
| Products inoculated with *Lactobacillus casei* shirota | | | | |
| B-Syrup | 6 | Fine, Slightly bitter | 59.2 | Disagree a little |
| KL3 | 6 | Good, Pleasant, Nice, Not bad | 60.2 | Neither agree or disagree |
| B-Syrup and KL3 | 4 | Fine, Nice, Not bad | 61.6 | Neither agree or disagree |
| Whole malt grains | 10 | Unacceptable, Bad | 21.4 | Completely disagree |
| Whole milled malt grains | 6 | Very dense, Bitter, Horrible | 21.5 | Completely disagree |
| Products inoculated with a mixture of *Lactobacillus casei* shirota and *Saccharomyces cerevisiae* | | | | |
| Whole malt grains | 2 | Horrible, Very bad | 11.5 | Completely disagree |
| Whole milled malt grains | 1 | Horrible | 11 | Completely disagree |

The following figure (Figure 5.25) shows four different pictures with samples of all fermented products. In each picture (a, b, c or d) the left sample represents the unfermented medium while the one in the right-hand side represents the final fermented drink. The colour of most media lightly changes after fermentation. KL3 samples became darker than the original one (picture a) while the colour of the B-Syrup (picture b) became lighter but with higher turbidity. For the case of whole malt

grains (picture c) it was difficult to observe the difference in the colour. The medium presented in picture (d) (whole milled malt grains) showed a certain degree of change in the colour and turbidity probably due to the precipitation of malt particles during fermentation. That was the reason why it was not possible to perform dry weight analysis for this medium.

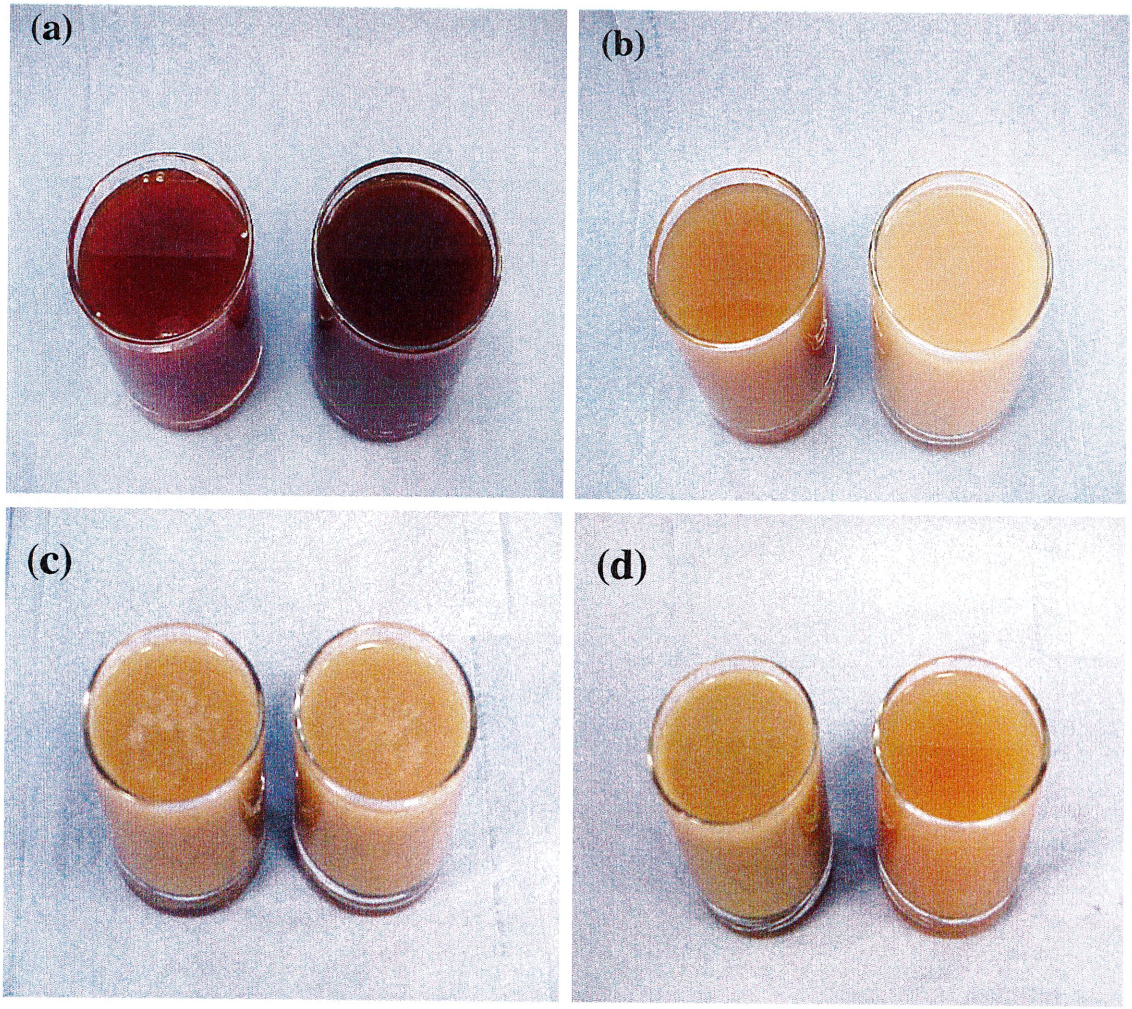



**Figure 5.25** The un-fermented (left) and fermented media (right). (a) KL3, (b) B-Syrup, (c) Whole malt grains and (d) Whole milled malt grains. All were inoculated with *Lactobacillus casei* shirota.

# 6

# CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

# 6. CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

## 6.1 Conclusions

According to the results discussed in Chapter 5, the following main conclusions can be drawn:

1. The chemical and physical analyses show that the four different malt grain media are suitable for the growth of the selected probiotic microorganisms. Any of these media could therefore be used to produce probiotic foods both using single or mixed culture (*Lactobacillus casei* shirota and/or *Saccharomyces cerevisiae*).

2. Different parameters were monitored during fermentation (pH measurements, FAN concentration, phosphorus concentration, lactic acid concentration, sugar content, viable cells count, total cell count and dry weight). The measurement of the phosphorus concentration and the pH seem to be the most appropriate parameters to monitor fermentation.

3. The fermentation time required to produce the new probiotic products could be obtained from the phosphorus concentration and the pH profiles, also taking into account the results obtained from tasting sessions.

4. The pH measurement indicates that the minimum pH values, which are indications of the lactic acid concentrations, for all media lied within the acceptable consumption range.

5. The sensorial analysis indicates that two of the chosen media (KL3 and B-Syrup/KL3) produce acceptable probiotic products, though these could probably improved by using some external additives such as sugar. For the other fermented foods (such as B-Syrup, whole malt and whole milled malt grains) the taste was not acceptable.

## 6.2 Suggestions for further work

Although the present study has generated promising information on the production of new cereal based probiotic foods, further work needs to be carried out to investigate the effect of some other factors on the behaviour of the fermentation parameters and on the aroma and flavour of the final products. The suggested further work could include:

1. To study the changing of the raw materials by using other types of cereals or cereals mixture.

2. To use some other probiotic microorganisms that may improve the sensorial properties of the fermented products.

3. To study the possibility of using additives at the end of fermentation (e.g. sugar, flavouring, etc.).

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# APPENDICES

# APPENDIX A: FERMENTATION MEDIA

This section contains a brief description about malt grains, malt processing and the common chemical and physical characteristics of these grains.

## A.1 Malt

Malt is a grain that has been softened and prepared by germination to the point were roots and steam just begin to appear (Potter and Hotchkiss, 1997). Smith (1995) has defined the malt as the final product after kilning that is cleaned to remove the dried rootlets and given a degree of polishing. Barley is the chief cereal, which is malted. It is soaked in water for about two days and then gently dried to stop growth yet leave the enzyme activity intact and that done by exposed it to air either in a layer on a malting floor or, in most recent installations, in a cylindrical or a large air-conditioned rectangular containers (Clarke *et al.*, 1978; Potter and Hotchkiss, 1997). Germination results in activation of enzymes that convert starches in the malted barley and in other cereal grains into sugars (see Table A.1-1), which can be easily fermented by yeast during the fermentation step. This is necessary because yeasts can not utilise the starch in the cereal grains for conversion to ethanol and $CO_2$ (Potter and Hotchkiss, 1997; Tom and Vince, 1999, Paddock Wood, 1999).

## A.2 Malting process

In the first step of malting process the barley has to be steeped in tanks of cool water until the grains absorbed the maximum amount of moisture. Next the barley spread over the malting floor where germination takes place. Regularly

**Table A.1-1** Changes in the carbohydrate composition of barley during traditional malting (Briggs *et al.*, 1981).
**M** Finished malt without rootlets
**TCW** Thousand corn dry weight

| Carbohydrate | Raw Barley | Steeping (days) | | | Germination (days) | | | | Kilning (days) | | | | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *1* | *2* | *6* | *9* | *1* | *2* | *3* | *4* | |
| *Soluble carbohydrate* | | | | | | | | | | | | | |
| Fructose | 0.027 | 0.012 | 0.027 | 0.042 | 0.100 | 0.097 | 0.132 | 0.145 | 0.189 | 0.267 | 0.297 | 0.262 | 0.194 |
| Glucose | 0.016 | 0.012 | 0.015 | 0.031 | 0.085 | 0.143 | 0.173 | 0.178 | 0.283 | 0.312 | 0.300 | 0.285 | 0.462 |
| Sucrose | 0.301 | 0.208 | 0.298 | 0.265 | 0.276 | 0.370 | 0.540 | 0.624 | 0.519 | 0.713 | 0.965 | 0.755 | 1.315 |
| Maltose | - | - | 0.008 | 0.012 | 0.026 | 0.147 | 0.179 | 0.123 | 0.166 | 0.109 | 0.103 | 0.109 | 0.257 |
| Maltotriose | - | - | - | - | - | - | 0.052 | 0.063 | 0.069 | 0.055 | 0.055 | 0.059 | 0.148 |
| Glucodifructose | 0.033 | 0.040 | 0.043 | 0.047 | 0.029 | - | - | - | - | - | - | - | - |
| Raffinose | 0.058 | 0.054 | 0.077 | 0.050 | 0.014 | - | - | - | - | - | - | - | - |
| Fructosan | 0.226 | 0.584 | 0.459 | 0.370 | 0.401 | 0.410 | 0.621 | 0.842 | 0.752 | 0.494 | 0.477 | 0.374 | 0.352 |
| Insoluble carbohydrates | | | | | | | | | | | | | |
| Glucosan | 0.725 | 0.577 | 0.711 | 0.824 | 0.869 | 0.876 | 1.611 | 2.161 | 1.396 | 1.216 | 1.036 | 1.129 | 0.613 |
| Araban | 0.047 | 0.016 | 0.030 | 0.039 | 0.025 | 0.039 | 0.120 | 0.148 | 0.130 | 0.141 | 0.112 | 0.115 | 0.110 |
| Xylan | 0.050 | 0.020 | 0.030 | 0.039 | 0.031 | 0.058 | 0.139 | 0.182 | 0.156 | 0.175 | 0.168 | 0.153 | 0.142 |
| Starch | 25.74 | 24.72 | 24.48 | 25.06 | 24.63 | 24.43 | 22.06 | 20.70 | 20.95 | 20.65 | 20.90 | 21.50 | 20.79 |
| Blue value | 0.326 | 0.331 | 0.317 | 0.308 | - | 0.310 | - | 0.381 | 0.371 | 0.377 | 0.364 | 0.374 | - |
| TCW (g) | 39.1 | 39.2 | 38.8 | 38.7 | 38.6 | 38.9 | 37.6 | 37.6 | 37.2 | - | - | 36.8 | 35.3 |

the barley grains turned to enable the excess moisture to evaporate. The growing shoot, the acrospire, has to be prevented to protrude from the end of the barley kernel. When that achieved, the malt can be considered as a fully "modified malt" (see Table A.1-1, A.2-1 and A.2-2) (Harrel and Dirks, 1955; Tom and Vince, 1999).

**Table A.2-1** The chemical composition of European malt (Briggs *et al.*, 1981).

| Fraction | Proportions (% dry weight) | Fraction | Proportions (% dry weight) |
|---|---|---|---|
| | Malt | | Malt |
| Starch | 63-65 | Albumin (salt-soluble protein) | 0.5 |
| Sucrose | 1-2 | Globulin (salt-soluble protein) | 3 |
| Reducing sugars | 0.1-0.2 | Hordein (protien) | 3-4 |
| Other sugars | 1 | Glutelin (protien) | 3-4 |
| Soluble gums | 1-1.5 | Amino acids and peptides | 0.5 |
| Hemicelluloses | 8-10 | Nucleic acids | 0.2-0.3 |
| Cellulose | 4-5 | Minerals | 2 |
| Lipids | 2-3 | Other substances | 5-6 |
| Crude 'protein' (N x 6.25) | 8-11 | Starches analysis (See Table A.2-2) | |

**Table A.2-2** Analysis of starches from malt (Briggs *et al.*, 1981).

| Starches Analysis | |
|---|---|
| Moisture | 14.7% |
| 'Protein content' (N x 6.25%) | 2.05 |
| Ratio of glucose residues, Non-terminal : Terminal | 22:1 |
| 'Blue value' with iodine | 0.409 |
| Amylose content | 29.2 |
| Ratio of amylopectin residue, Non-terminal : Terminal | 15-16 : 1 |
| Amylose (g/1000 corns) | 6.37 |

After that, the malted barley transferred to a kiln where it dried and roasted. The differing moisture contents, kiln temperatures and kiln times provide the maltster with the means to produce many different types of malt (Harrel and Dirks, 1955; Tom and Vince, 1999). The malting process is shown in Figure A.1-1.

The chemical changes occurs during malting have been reported by Briggs *et al.* (1981) who found that the gross chemical changes observed during malting are the net result of the degradation of reverse substances, the interconversion of materials in the living tissues, the flux of materials to the embryo and the losses occasioned by:

- Leaching during steeping.
- Oxidation to carbon dioxide and water.
- The separation of rootlets.

Also they found that the total malting usually losses amount between 6-12% of the original dry weight of the barley.

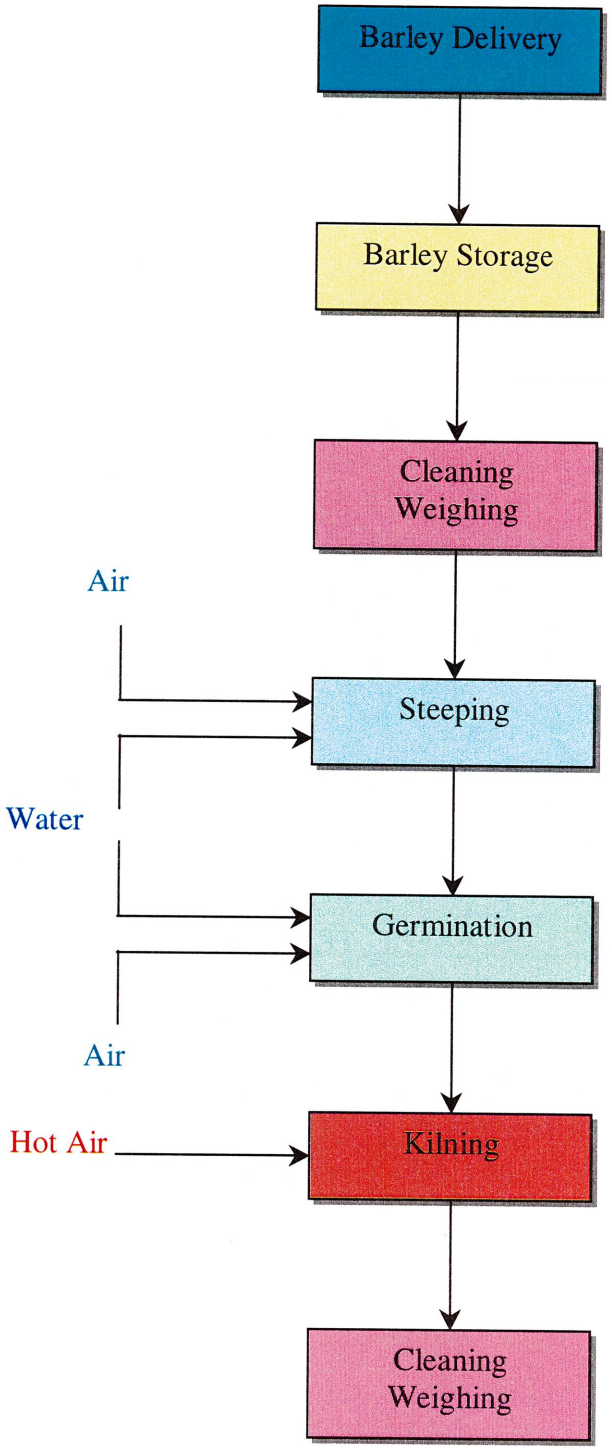



**Figure A.1-1** Malting process flow diagram (Warpala, 1998).

# APPENDIX B: ANALYTICAL METHODS

This section contains a full description about the sample preparation and the experiment procedures used in the analytical methods.

## B.1 pH measurement

The standardisation of the pH cell and the pH measurements procedures are well explained in the following (Bates, 1966; Jeffery *et al.*, 1991):

### Standardisation of the pH Cell (pH meter calibrations)

The pH meter should be standardised routinely with two solutions (BDH, England) the pH values of which bracket the pH of the unknowns to be determined. The standards with which the instrument is adjusted should have pH values between 2.5 and 11.5 at room temperature. They should be warmed or cooled as necessary to match within 2 °C the temperature of the unknowns.

Wash the electrodes and sample cup carefully with distilled water and dry gently with clean absorbent tissue. Immerse the electrodes in the first standard solution and set the temperature compensator of the instrument to the temperature of the solutions whose pH is to be measured. Bring the instrument to balance and adjust the standardisation control until the meter is again balanced at the known pH of the standard at the appropriate temperature. Repeat the procedure with successive portions of the first standard until

replacement of the solution causes no change in the position of balance. Wash the electrodes by distilled water and dry as before.

Repeat this entire procedure with the second standard solution, reading the pH of the solution without changing the position of the standardisation control. Replace the solution and repeat the measurement until successive readings agree within ± 0.02 pH unit. The glass electrode and measuring assembly are judged to be operating satisfactorily if the reading of the second buffer agrees within 0.02 unit with the appropriate value of the standardised solution being used.

**Test solution**

The procedure for measuring the pH of unknowns is the same in every respect as that outlined for the second standard solution. Successive measurements on buffered solutions should agree to 0.02 unit or better; with water or poorly buffered solutions values.

## B.2 Free amino nitrogen determination

**Reagents preparation**

**Glycine stock solution:** Weight exactly 0.1072 g glycine (Fisons, England) and dissolved it in distilled water in a 100 mL volumetric flask to give an accurate concentration of 1.072 g $L^{-1}$ (preparation of 1% glycine solution). The solution was stored at 4°C in a refrigerator.

**Glycine standard solution:** 1.0 mL glycine stock solution was diluted to exactly 100 mL in a volumetric bottle to result in a FAN

concentration of 2 mg $L^{-1}$. The solution was stored at 4°C in a refrigerator.

**Colour reagent:** Dissolve approximately 49.71 g $Na_2HPO_4.2H_2O$ (Fisons, England), 5.0g ninhydrin (Aldrich Chemical Co. Ltd.) and 3.0 g fructose (Philip Harris Biological Ltd.) in 700 mL distilled water to form a solution, which was made up to one litre in a volumetric flask after pH was carefully adjusted to 6.70 by introducing into solution of 50.0 g $KH_2PO_4$ (Fisons, England) in 200 mL of distilled water. The solution was stored in an amber glass bottle at 4°C in a refrigerator and it was effective in two weeks (Wang, 1999a; Wang, 1999b).

**Dilution reagent:** Dissolve 2.0 g $KIO_3$ (AnlaR., England) in 200 mL distilled water in one litre volumetric flask, add 384.0 mL absolute ethanol (Fisons, England) and fill the with distilled water to the mark in order to form a one litre solution. The solution was stored at 4°C in a refrigerator.

## Procedure

**Sample treatment:** During tentative analysis, the spectrophotometer (PYE UN ICAM PU 8600 UV/VIS; Philips) was found to give the greatest accuracy in a reading range below 1.5. Samples with a reading above 1.5 were, therefore, further diluted with distilled water and reanalysed. Throughout the project, the samples for FAN analysis were the supernatant from various sources, such as fresh medium, sterilised medium and fermentation broth. The dilution factor for these samples was found suitable in a range from 150 to 400. A volume of 2 mL of

diluted sample was introduced into three screw neck test tubes (160x16; Fisons, England) with rigid plastic caps having inert PTFE lined rubber discs (Wang, 1999a; Wang, 1999b).

**Blank sample:** A volume of 2 mL distilled water was transferred to a test tube.

**Standard:** A volume of 2 mL glycine standard solution was placed into three test tubes.

**Determination:** One millilitre of the colour reagent was added to all test tubes of samples, blank and standard. After the caps were screwed on to prevent evaporation, the test tubes were placed in a water bath of boiling water for exactly 16 minutes. Then the test tubes moved to another water bath to cool at 20°C for 20 minutes. A volume of 5 mL dilution reagent was equally introduced into every tube and well mixed before the measurement of the absorbency at 570 nm comparison with that of the blank (Wang, 1999a; Wang, 1999b).

## Calculation

The FAN concentrations in the samples were calculated by:

$$F = \frac{A_{570\,nm} \times d \times 2}{A_{S570\,nm}} \qquad \text{(B.2-1)}$$

where,

$A_{570\,nm}$: Absorbance of sample at a wave length of 570 nm

d: Dilution factor of sample

2: FAN concentration in glycine standard solution (mg $L^{-1}$)

$A_{S570\ nm}$: Absorbance of standard solution at 570 nm (=0.430) (Wang, 1999a)

F: Free amino nitrogen concentration in sample (mg $L^{-1}$)

## B.3 Total Kjeldahi nitrogen (TKN) (total nitrogen)

### Procedure

To determine total nitrogen content in solids samples of less than 0.1 g were required, whereas for liquid materials sample sizes up to a maximum of 2 mL were sufficient depending on nitrogen content expected. In order to maintain the best accuracy, a four-digit balance was used to weigh not only solid but also liquid samples directly into the specially designed 100 mL digestion flask. Operated at 440°C with the application of firstly 4 mL concentrated sulphuric acid ($H_2SO_4$) then about 10 mL 50% hydrogen peroxide ($H_2O_2$) (Fisons, England) as digestion reagents in the Digesdahl (Hach, USA) the digestion procedure time was about 10 minutes. After being cooled for about 5 minutes the digest was diluted to 100 mL mark with distilled water. By recording the weigh on the four-digit balance, 1.0 mL of the well-mixed diluted digest was transferred into a 25 mL volumetric flask, in which about 20 mL distilled water was then added. After three drops of Mineral Stabiliser (Camlab, Cambridge) and three drops of Dispersing Agent (Camlab, Cambridge) were added the content was further diluted to the mark. A bright green colour was produced by introducing 1.0 mL of Nessler Reagent (Camlab, Cambridge). Finally, the absorbency was measure at 460 nm (PYE UN ICAM PU 8600

UV/VIS; Philips) against a blank produced following the same procedure from distilled water insted of the digest. Using glycine as material of known nitrogen contents a standard curve (see Figure B.3-1) was produced (Jeffery *et al.*, 1991; Wang, 1999a; Wang, 1999b).

**Standard curve**

- **Reagents preparation**

  **Glycine stock solution:** dissolve about 0.10 g (weight to 0.0001 g) glycine in distilled water in a 100 mL volumetric flask.

  **Glycine standard solutions:** dilute glycine stock solution to 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%.

Digest standard solution and measure absorbance following the procedure above. Then plot absorbance ($A_{460nm}$) vs. Nitrogen contents (see Figure B.3-1) in the standard solutions and calculate slope and intercept at the standard curve by least square method.

**Calculation**

Determine the total amount of nitrogen in the sample by:

$$N = \frac{A_{460\,nm} - 0.0009}{0.0515} \times \frac{1}{w} \qquad \text{(B.3-1)}$$

where,

$A_{460\,nm}$: Absorbance of sample at a wave length of 460 nm

0.0515: Slope of the standard curve (Wang, 1999b)

0.0009: Intercept of the standard curve (Wang, 1999b)

w: Weight or volume of the sample (g of solids or mL of liquid)

N: Total amount of nitrogen (mg $g^{-1}$ in solids or mg $mL^{-1}$ in liquid)

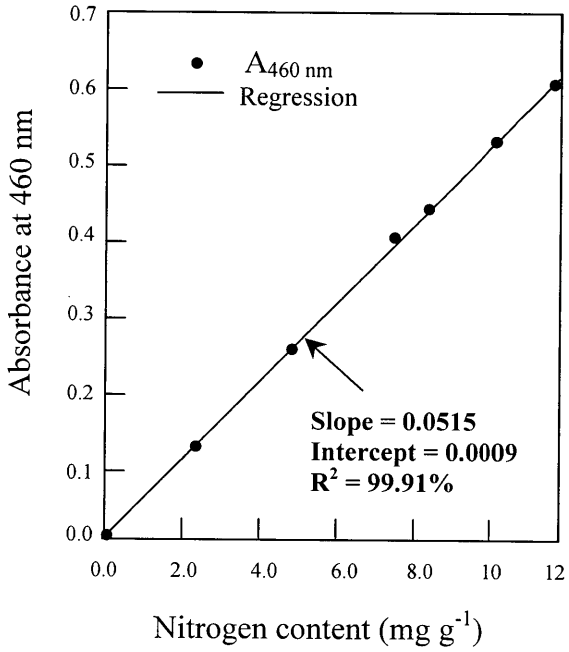



**Figure B.3-1** Standard curve of Nessler's method (Wang, 1999a).

## B.4 Determination of phosphorus in the liquid phase

Mainly to simplify the analytical procedure, phosphorus concentration in liquid samples was measured by the method described by Herbert and his colleges (1971). The principle of this method involves release and oxidation of organically combined phosphorus to produce phosphate, formation of phosphomolybdate by reaction with ammonium molybdate and reduction of

the phosphomolybdate under controlled conditions to form a blue compound, the concentration of which is measured spectrophotometrically (Jeffery *et al.*, 1991; Wang, 1999a).

**Procedure**

Samples were diluted 50 times by adding 0.1 mL sample and 4.9 ml distilled water into a test tube (100x12 mm). Then 0.4 mL 70% (v/v) perchloric acid (Fluka, UK) was introduced into the tube. The tube was shaken for 20 seconds on a vortex mixer before the addition of 0.3 mL 1% ascorbic acid, which was freshly prepared from 10% (w/v) stock solution. Following another 20 seconds shaking, the tube was charged with 0.4 ml 5% (w/v) ammonium molybdate solution and then mixed for a further 30 seconds. Finally, after the colour developed for 30 minutes, the absorbency was measured with the spectrophotometer at a wavelength of 730 nm using distilled water as blank. Phosphorus concentration in the samples was calculated from equation (B.4-1) which based on the linear relationship between phosphorus concentration and absorbency obtained using *di*-potassium hydrogen phosphate as a known phosphorus compound (see Figure B.4-1) (Wang, 1999a).

**Calculation**

Determine the amount of phosphorus in the liquid sample by:

$$\text{LPhos} = \frac{A_{730\,nm} - 0.011}{0.1348} \times 61 \qquad \text{(B.4-1)}$$

where,

$A_{730\ nm}$: Absorbance of sample at a wave length of 730 nm

0.1348: Slope of the calibration curve

0.011: Intercept of the calibration curve

61: Dilution factor of sample

LPhos: Total amount of phosphorus in liquid sample (mg $L^{-1}$)

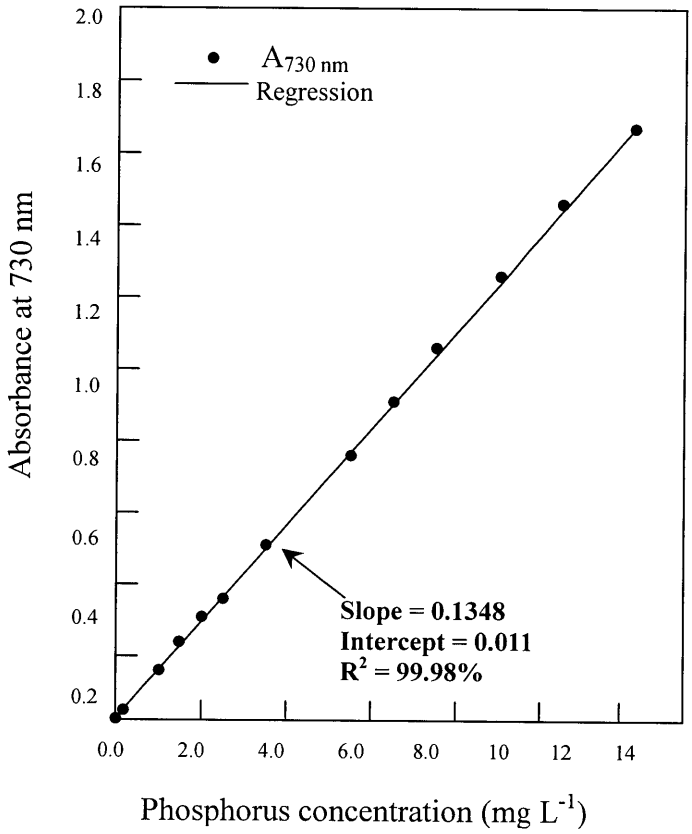



**Figure B.4-1** Standard curve of phosphorus assay in liquid samples at 730nm (Wang, 1999a).

## B.5 Determination of total phosphorus

To determine the total phosphorus concentration in an organic source, such as the malt, the molybdovanadophoric acid colorimetric method, originated by Kitson and Mellon (1944), was used. The principle of this method is the reaction of ammonium molybdate in an acidic environment with vanadate and orthophosphate to form molybdovanadophosphoric acid. The produced

heteropoly acid is yellow in colour and obeys the Beer-Lambert law over a wide range of phosphorous concentrations. To avoid interference from other components, samples from various sources were digested with a mixture of concentrated sulphuric acid and concentrated perchloric acid (70%) (Fluka, UK) before the complex formation was attempted.

Samples were directly weighed into the digesdahl flask with the best accuracy. In the analysis procedure, 3.0 mL of concentrated sulphuric acid and 0.50 mL of 70% perchloric acid (Fisons, England) were added into the digesdahl flask. The mixture was then boiled in the digesdahl flask at 440-450 °C until a colourless liquid was produced. After being cooled for 10 minutes, about 20 mL distilled water was added then re-cooled for another 5 minutes before 5 mL of 1% (w/v) ammoniumvanadate (Fisons, England) in 2 N nitric acid (Fisons, England) and 10 mL of 10% (w/v) ammonium molybdate (Fisons, England) were added. The solution was finally diluted to the mark with distilled water. After exactly 20 minutes for the colour to fully develop, the absorbency was measured with a spectrophotometer (PYE UNICAMP PU 8600 UV/VIS) at 430 nm against distilled water. Phosphorus concentration was calculated by using equation (B.5-1) that based on the standard curve obtained using *di*-potassium hydrogen phosphate (Fluka, UK) as a known phosphorus material (see Figure B.5-1).

**Calculation**

Determine the total amount of phosphorus in the solid sample by:

$$\text{SPhos} = \frac{A_{430\,nm} - 0.0437}{0.3909} \times \frac{1}{w} \qquad \text{(B.5-1)}$$

where,

$A_{430\ nm}$: Absorbance of sample at a wave length of 430 nm

0.3909: Slope of the calibration curve

0.0437: Intercept of the calibration curve

w: Weight of sample (g)

SPhos: Total amount of phosphorus in solid sample (mg $g^{-1}$)

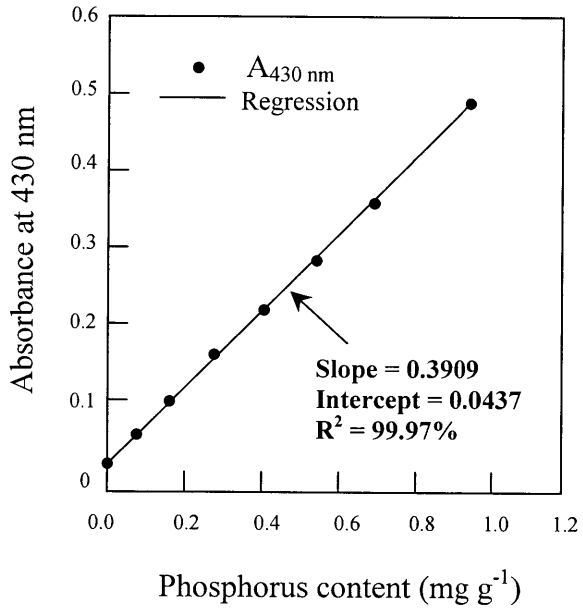



**Figure B.5-1** Standard curve of phosphorus assay in solid samples at 430nm (Wang, 1999a).

## B.6 Lactic acid determination

The reagents preparation and the procedure for this experiment have been taken from an article wrought by Aurand (1966), while the procedure for the standard curve preparation was found in another article published in 1941 by Barker and Summerson.

**Reagents preparation**

**(25% $CuSO_4$ solution):** dissolve 25.0 g $CuSO_4.5H_2O$ (Fluka, UK) in distilled water and dilute to 100.

**(5% $CuSO_4$ solution):** dissolve 25.0 g $CuSO_4.5H_2O$ (Fluka, UK) in distilled water and dilute to 100.

**($Ca(OH)_2$ solution):** grind 300.0 g CaO (Fluka, UK) with distilled water to a cream in a mortar then transfer it to a container with water and dilute with 1400 mL of distilled water.

***p*-Hydroxydiphenyl reagent:** dissolve 1.50 g *p*-hydroxyliphenyl in 10 mL of 5% (w/v) NaOH (Fluka, UK) then heat up and stir the mixture until dissolved. Dilute the mixture to 100 mL with mater and store it in a dark glass bottle.

**Primary lactate standard:** transfer 0.1067 g lithium lactate ($C_3H_5O_3Li$) (Fluka, UK), accurately weighed, into a one litre volumetric flask then dissolve and dilute to the mark with distilled water.

**Dilute lactate standard:** transfer 3.0, 6.0, 9.0 and 12.0 mL of the Primary lactate standard solution to four 100.0 mL volumetric flasks and diluting with distilled water to the mark. These solutions contain the equivalent of 3, 6, 9 and 12 μg lactic acid per mL.

**Procedure**

1.0 mL broth sample was diluted with distilled water to approximately 35 mL in a 50 mL volumetric flask and then 0.50 mL of 5% $CuSO_4$ solution was added to the diluted broth sample, while swirling. Next the flask contents were heated up to 45– 47°C by rapid swirling in a water bath at approximately 90°C and 5.0 mL of the 25% $CuSO_4$ solution was added to the flask and maintained for 10 minutes at 45–

47°C. After that a 5.0 mL $Ca(OH)_2$ suspension was added to the broth sample and the whole solution was diluted to the mark with distilled water. The flask was shook vigorously and held for an additional 10 minutes at 45–47°C. Then, the flask was cooled to 20°C, and the volume was adjusted in a centrifugal tube and filtered.

After filtration, 1.0 mL of filtrate was transferred into a Pyrex tube (25 mm x 150 mm) with 1 drop of 5% $CuSO_4$ solution and exactly 6.0 mL of concentrated $H_2SO_4$. Then, the tube was placed in boiling water for 5.0 minutes and then it cooled to 20°C in iced water.

Two drops of *p*-hydroxyliphenyl reagent were added and mix thoroughly to ensure a fine suspension of the reagent that is insoluble in acid. The mixture was held at 30°C and after 15 minutes another drop of *p*-hydroxyliphenyl reagent was added and mixed well again and the mixture was held for an additional 15 minutes at 30°C. Next to that, the tube was transferred to a boiling water bath for 1.5 minutes to dissolve excess suspended reagent then it was cooled to the room temperature. Finally, measure $A_{570}$ against distilled water (blank sample).

**Standard curve**

Transfer 1.0 mL of each of the four dilute standard solutions containing 3, 6, 9 and 12 μm lactic acid in a Pyrex tube, respectively

and develop the colour as described above for the filtrate then measure $A_{570}$ against distilled water as a blank sample.

**Determination of lactic acid concentration by:**

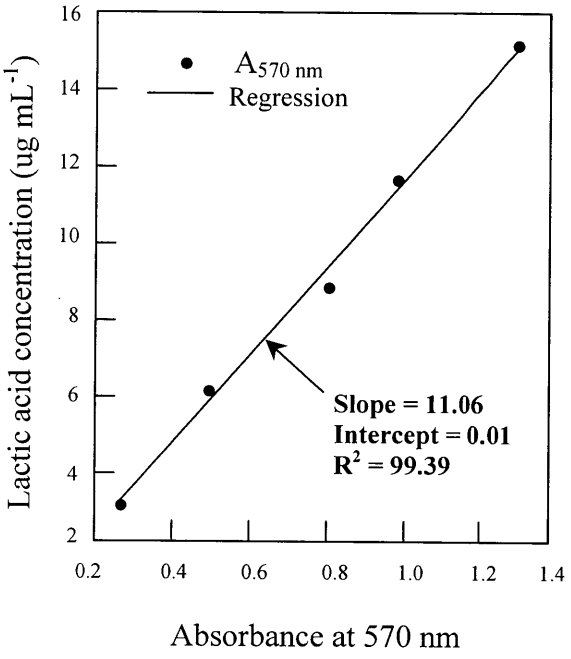



**Figure B.6-1** Standard curve for the determination of lactic acid concentration (Barker and Summerson, 1941; Wang, 1999a).

**Calculation**

Determine the lactic acid concentration in fermented broth by:

$$C = ((A_{570\,nm} \times d \times 11.06) + 0.01) \times 1.06 \qquad \text{(B.6-1)}$$

where,

C: Lactic acid concentration in fermentation ($\mu g\ mL^{-1}$)

$A_{570\,nm}$: Absorbance of sample at a wave length of 570 nm

d: Dilution factor of sample (fermentation broth)

11.06: Slope of the standard curve

0.01: Intercept of standard curve

1.06: Overall multiplication factor (overall correction factor)

*Note:*

There are two correction factors to be applied to all results read from the standard curve due to the allowance has been made for sample dilution:

- For the volume occupied by the precipitate in the volumetric flask.
- For the partial retention of lactic acid by the precipitate.

## B.7 Sugar analysis

The mobile phase preparation and the procedure for this experiment have been given and described by the laboratory technician.

**Mobile phase (HPLC solution) preparation**

**(80:20% v/v $CH_3CN$ solution):** dilute 800 mL of $CH_3CN$ (Acetonitrile) (Fluka, UK) with de-ionised water/HPLC water to approximately 1000 mL in a one litre glass bottle and mix the solution thoroughly.

**Standard samples preparation**

**(50g of sample / 1 litre of HPLC water):** dilute 1.0 g of each type of samples in a 20 mL of de-ionised water and keep each of them in a

sterilised universal bottle to any avoid contamination, then mix them thoroughly until all the malt extract or the original sample dissolve or disappears completely (Jeffery *et al.*, 1991). Then add a specific amount of the material that its signal need to be defined by weighed this amount to 0.0001 g for solid, or by using an accurate pipette in the case of liquid.

**Samples preparation**

**(50g of sample / 1 litre of HPLC water):** dilute 1.0 g of each type of samples in a 20 mL of de-ionised water and keep each of them in a sterilised universal bottle to any avoid contamination, then mix them thoroughly until all the malt extract or the original sample dissolve or disappears completely (Jeffery *et al.*, 1991).

**Procedure**

The mobile phase solution has been filtered by using nylon membrane filter (Phenix, UK) and a vacuum vessel as shown in Figure B.7-1. First of all, the vacuum vessel has been cleaned by 25–30 ml of the HPLC solution, then about 40–70 mL of the HPLC solution were filtered in order to prepare the nylon filter for the major filtration. The filtered amounts have been transferred to the main HPLC solution vessel and refilter the whole HPLC solution (major filtration).

About 2.0-3.0 mL of each of the standard samples was filtered before injecting them into the liquid chromatography column (5000LC-12

MM.I.D.) (Varian, USA), by using a special type of nylon membrane and a suitable syring (Phenix, UK) (see Figure B.7-2).

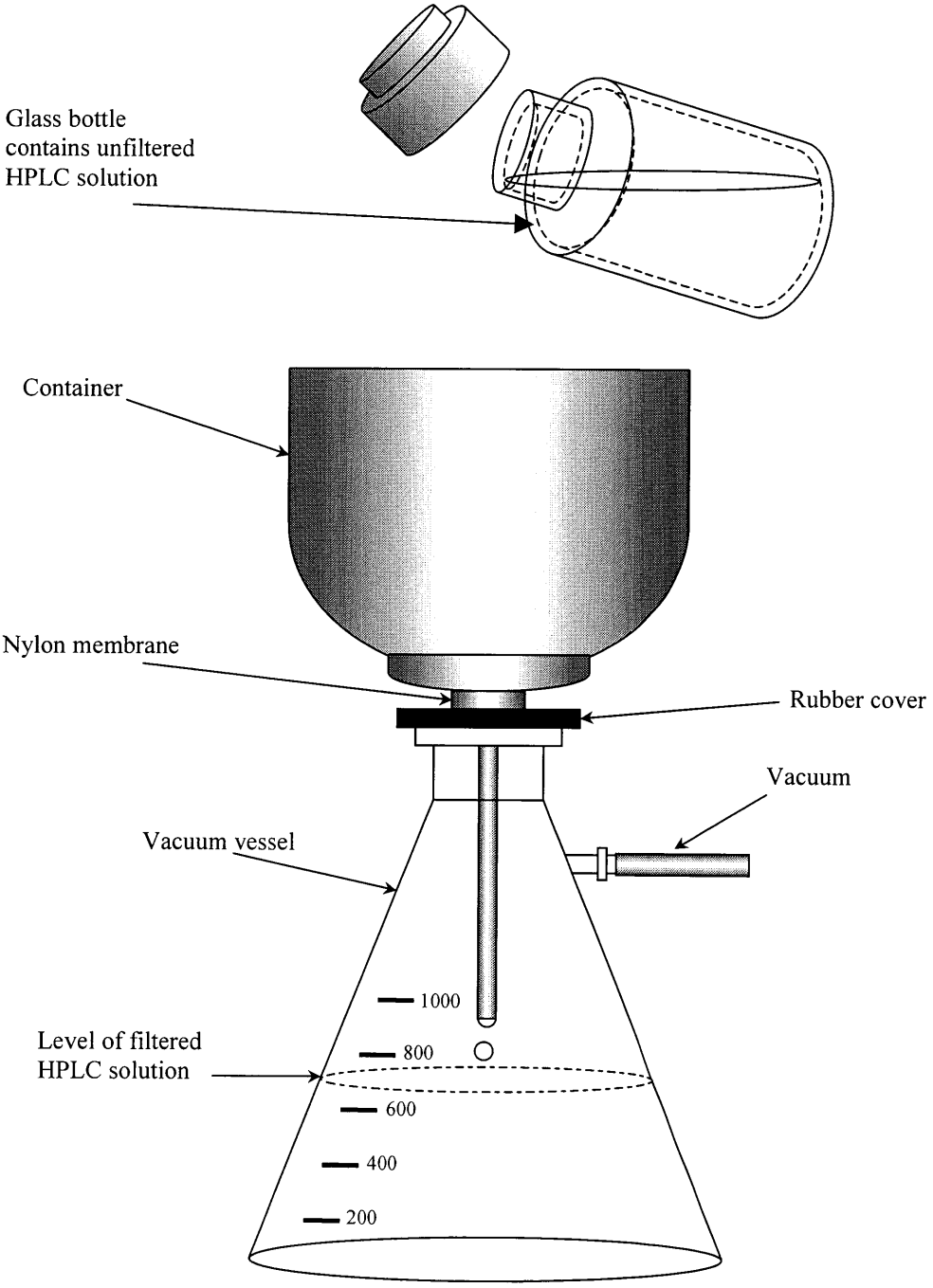



**Figure B.7-1** Filteration process of the HPLC solution.

Approximately 100 μL of the filtrated standard sample has been injected into the injection point of the liquid chromatography column twice. The first 100 μL was injected when the discharge bottom was on, in order to clean the path of the injection point, while the second injection; the main injection, was done when the charge bottom was on (see Figure B.7-3).

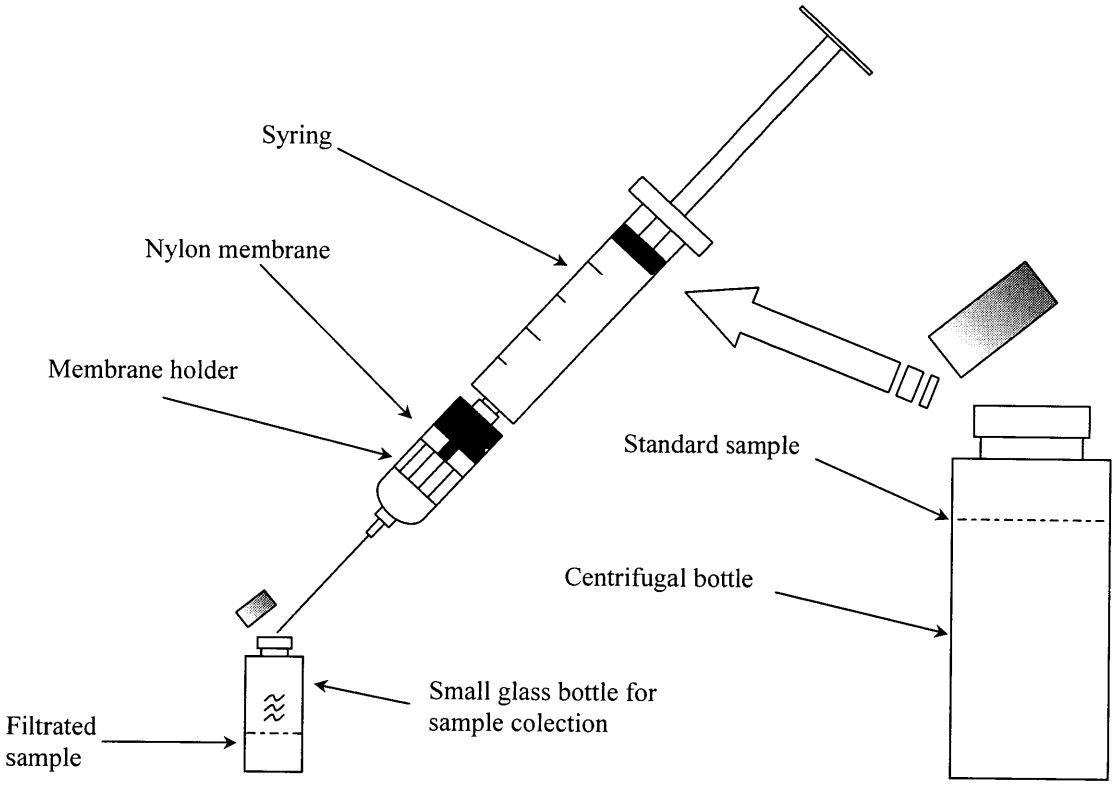



**Figure B.7-2** Standard sample filteration.

The HPLC system was operated at at a flow rate of 1.0 mL $min^{-1}$, 3.0 atmospheric pressure and 25°C. The HPLC analysis for each of the injected samples was detected by a differential refractrometer (Waters 410) (Waters, USA) and then recorded directly and printed out (see Figure B.7-3) in the form of signals (see Figure B.7-4) each of which

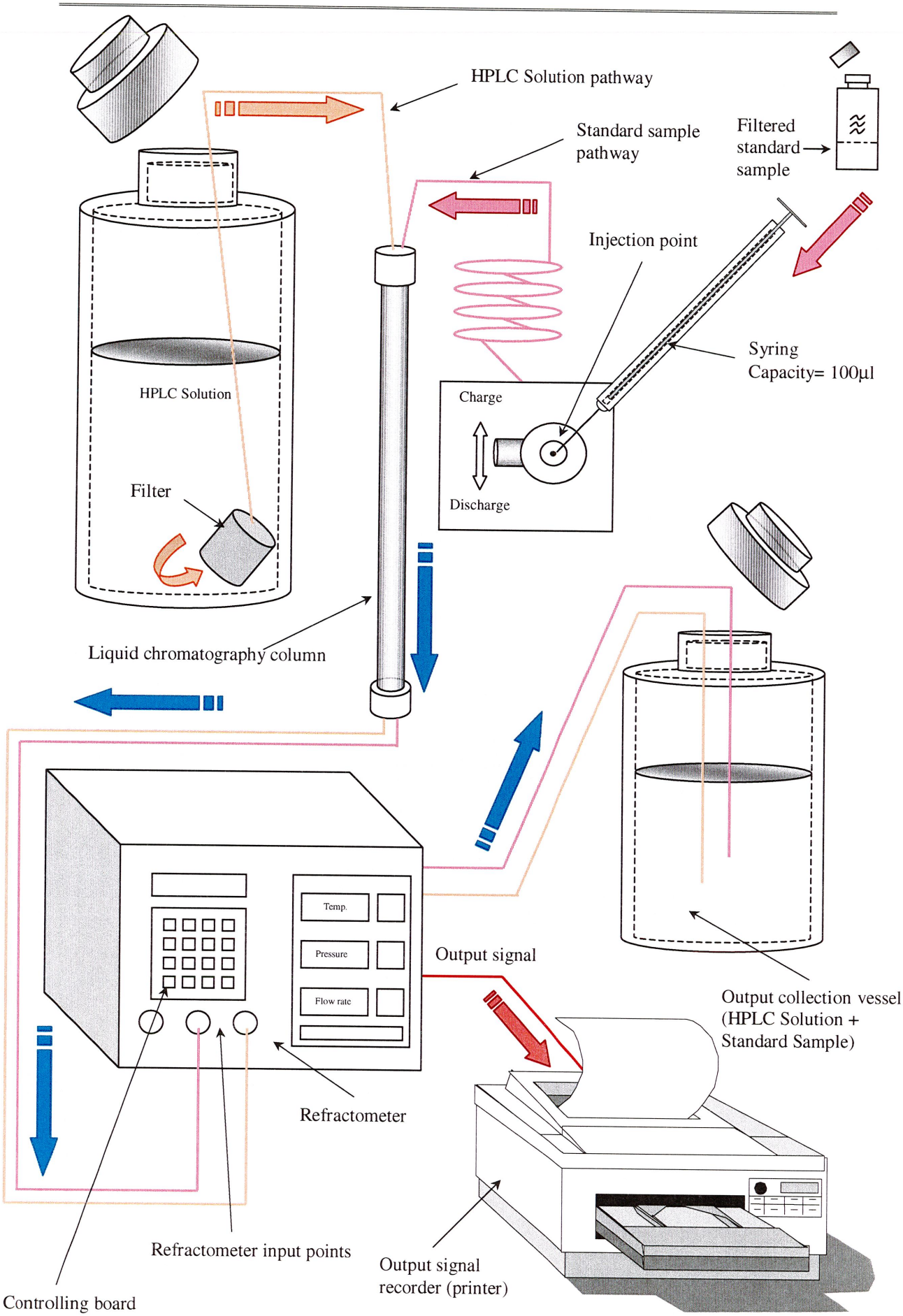
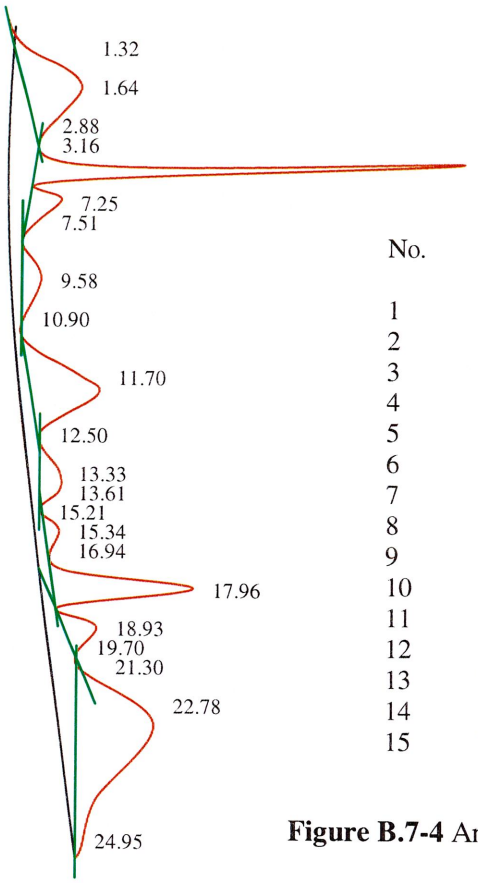



**Figure B.7-3** Sugar analysis sitting by using liquid chromatography column.




| No. | Rate | Area | Conc. |
|---|---|---|---|
| 1 | 1.32 | 485280 | 1.605 |
| 2 | 1.64 | 418129 | 1.382 |
| 3 | 3.62 | 21379903 | 70.690 |
| 4 | 7.25 | 618006 | 2.043 |
| 5 | 7.51 | 882938 | 2.919 |
| 6 | 9.58 | 1145116 | 3.786 |
| 7 | 11.70 | 533133 | 1.763 |
| 8 | 12.50 | 61768 | 0.204 |
| 9 | 13.33 | 495617 | 1.639 |
| 10 | 13.61 | 642003 | 2.123 |
| 11 | 16.94 | 54452 | 0.180 |
| 12 | 17.96 | 524401 | 1.734 |
| 13 | 20.30 | 76443 | 0.253 |
| 14 | 22.78 | 2233117 | 7.384 |
| 15 | 24.95 | 8387 | 0.276 |

**Figure B.7-4** An example of HPLC analysis printed record.

stand for a special kind of material that present the sample being analysed. In most cases small signals appear between the major signals as noises. In order to distinguish between the major signals and the noise signals and to define each of the signals correctly; the materials being representative, the standard samples were used. The standard samples which contain a defined concentration of the material being tested or analysed can be helpful in two ways. The first one; as it has been mentioned before, is to identify the signals. The second way is to make sure from the concentration recorded by the HPLC system.

Finally, the HPLC system produces a complete record for each sample with the following information:

- The time at which each of the signals appear (the rate).
- The total number of signals.
- The area of each signals by integrating the area under each signal and the equivalent concentration in g $L^{-1}$.

The final results can be found from the recorded data (see Figure B.7-4) or can be calculated by comparing the area under each curve or signal and its equivalent concentration with the same values found for the standard samples, which has a defined concentration.

## B.8 Viable cells count

### Culture transfer method

An inoculating loop was used for simple transfer and a sterile pipette was used to transfer a known volume.

Aseptic precautions in either case (see Figure B.8-1):

- Sterilise inoculating loop in flame, allow to cool, or fit a sterile tip to an autopipette (handle at the top only).
- Remove the tap from the culture tube, holding it in the crook of your little finger, do not put it down. Flame the neck of the tube briefly, dip sterile loop in liquid or scrape up cells off solid or pipette up measured volume then flame neck briefly and replace the cap.

- Remove cap from the recipient tube, holding it in the crook of your little finger, do not put it down. Flame the neck of the tube briefly; dip loop in liquid or stroke across agar or deliver sample from pipette, without splashing then flame neck briefly and replace the cap.

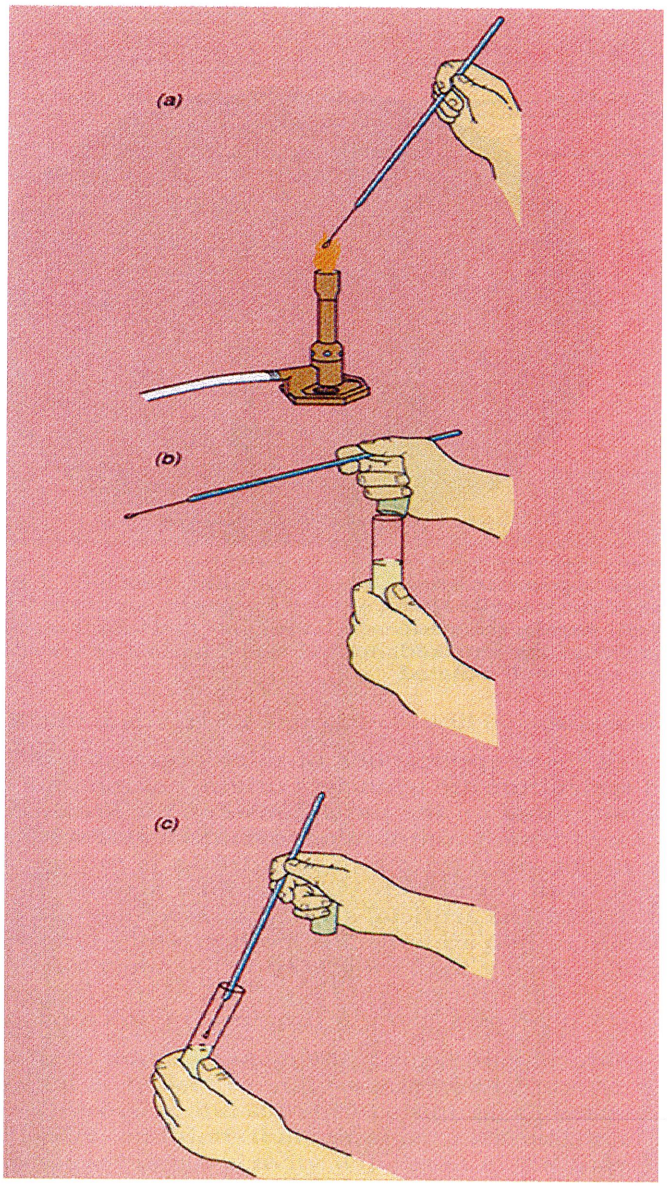



**Figure B.8-1** Aseptic transfer method. (a) Loop is heated until red-hot and cooled in air briefly. (b) Tube is uncapped. (c) Sample is removed and tube is recapped. Sample is transferred to a sterile tube. Loop is reheated before being taken out of service (Madigan *et al.*, 1997).

**Spread plate technique**

Dilute the cell suspension (sample) to about 2000 cells per ml by using transfer methods described above. If there is no clue to cell number make a series of tenfold dilutions covering a range from 0 to $10^4$ fold and make spread plates from all of them in the hope that one contained about 1000 cell. Dip the glass spreader in 70% ethanol, flame and set aside to cool supported by the handle. Do not let the spreader bar touch anything. Use a sterile autopipette tip to transfer 0.1 mL cell suspension to the middle of a dry agar plate. Lift the lid as little as possible (see Figure B.8-2 b). Then, touch spreader bar to agar surface away from the drop of culture to make sure it is cool, then use it to spread the 0.1 mL over the entire surface of the agar, right up to the edges, with a sweeping motion. Do not leave the 0.1 mL drop more than 30 seconds before spreading it or it may dry into the agar as a concentrated spot. After that dip spreader in 70% ethanol and flame to disinfect. Stand plate for few minutes to let sample soak into the agar, then incubate inverted to avoid condensation dripping onto the agar (see Figure B.8-2).

**Viable cells count and the dilution procedure**

For counting an 0.1 mL sample on a petri dish a suspension of around $10^3$ cells per ml will give 100 cells per dish. This can economise on equipment by only counting dilutions likely to give a suitable count.

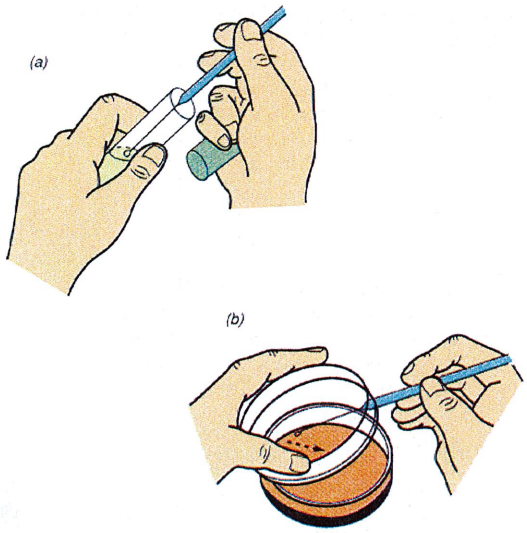



**Figure B.8-2** Spread plate technique. (a) Loop is sterilized and then a loopful of inoculum is removed from tube. (b) streak is made over a sterile agar plate, spreading out the organisms (Madigan *et al.*, 1997).

It has been found from the experience that if a sample is not visibly turbid it may contain anything from 0 to $10^6$ cells per mL and a wider range of dilutions should be counted. The dilution series is as follows (see Figure B.8-3):

Add 0.1 mL original culture to a tube containing 9.9 mL sterile buffer and mix thoroughly ($10^2$ dilution). Then take 0.1 mL of $10^2$ dilution, add to 9.9 mL sterile buffer and mix thoroughly ($10^4$ dilution). After that take 1.0 mL of $10^4$ dilution, add to 9.0 mL sterile buffer and mix thoroughly ($10^5$ dilution). Next take 1.0 mL of $10^5$ dilution, add to 9.0 mL sterile buffer and mix thoroughly ($10^6$ dilution). Next to that, take 1.0 mL of $10^6$ dilution, add to 9.0 mL sterile buffer and mix thoroughly

($10^7$ dilution). Finally, leave all plates 5-10 minutes to dry, then incubates inverted at 37 °C.

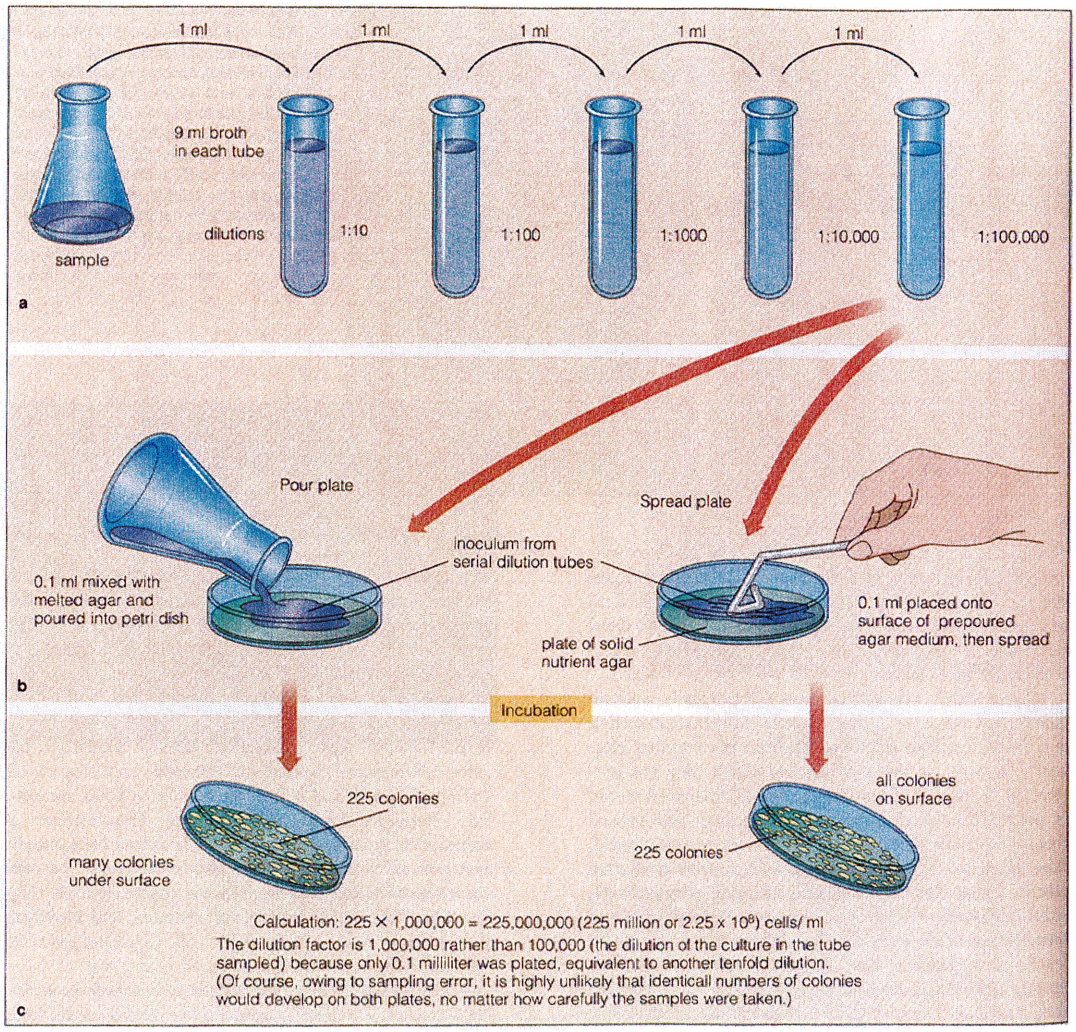



**Figure B.8-3** A plate count is a direct measurement and gives a viable count. First the sample is serially diluted by transferring 1 mL of the sample to 9 mL of sterile medium and mixing thoroughly. This process is repeated until an appropriate dilution is obtained (in this example 1:100 000 ($10^5$)). Then 0.1 mL is added to a plate of nutrient agar either by spreading it on the surface (spread plate method) or by pouring it with the medium (pour plate method). The plates are incubated. The colonies that develop are counted. For statistical reasons, plates should contain between 30 and 200 or 300 colonies. In the example, the plate has 225 colonies (Ingraham and Ingraham, 1995).

### Counting colonies

To count on a simple colony counter the following points must be observed:

Plates with between 30 and 200 colonies must be selected and the open dish, glass side up, should be placed over the illuminated screen then the colonies can be counted using a 75 mm magnifier and a hand-held counter. The glass above each colony should be marked with a felt tip pen.

The colony or viable count per ml can be calculated by multiplying the average number of colonies per countable plate by the reciprocal of the dilution.

Preferably the colonies from at least two dilutions should be counted and from that the CFU in the original sample can be calculated. 'Colony forming units per mL' (CFU) or 'viable count per mL' must be reported.

If all plates contain more than 200 colonies rule sectors, e.g. of one-quarter or one-eighth of the plate, the colonies number in these should be counted and included the sector value in the calculations.

## B.9 Total cell count (slide count)

### Hemocytometer or counting chamber technique

The Helber counting chamber is a slide 2-3 mm thick with an area in the centre called the platform and surrounded by a ditch. The platform

is 0.02 mm lower than the remainder of the slide (see Figure B.9-1). The top of the slide is ground so that when an optically plane cover glass is placed over the centre depression, the depth is uniform. On the platform an area of 1.00 $mm^2$ is ruled so that there are 400 small squares each 0.025 $mm^2$ in area (see Figure B.9-2). The volume over each small square is 0.02 mm x 0.0025 $mm^2$, *i.e.* 0.000 05 $mm^3$ (Jones *et al.*, 1998; Collins *et al.*, 1999).

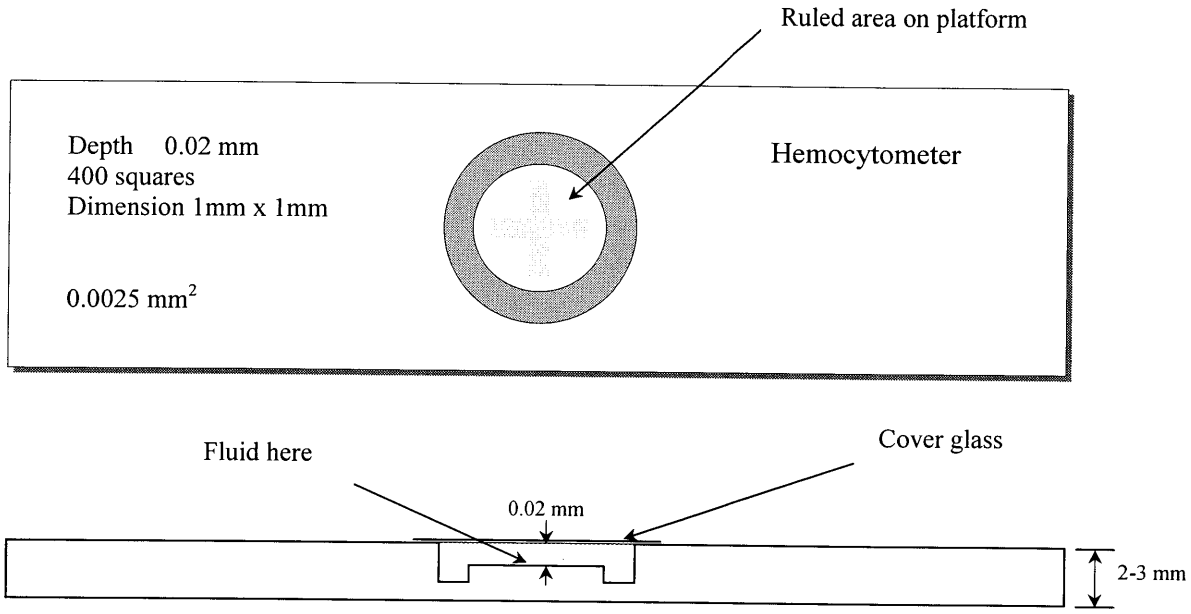



**Figure B.9-1** Bacterial counting chamber (depth is exaggerated) (Collins *et al.*, 1999).

Add a few drops of the well-mixed suspension to be counted on the ruled area. The suspension must be diluted; using ¼ strength Ringer's solution, so that when the counting chamber is filled there will be a possibility of finding an acceptable number of cells per small squares. This requires initial trial and error.

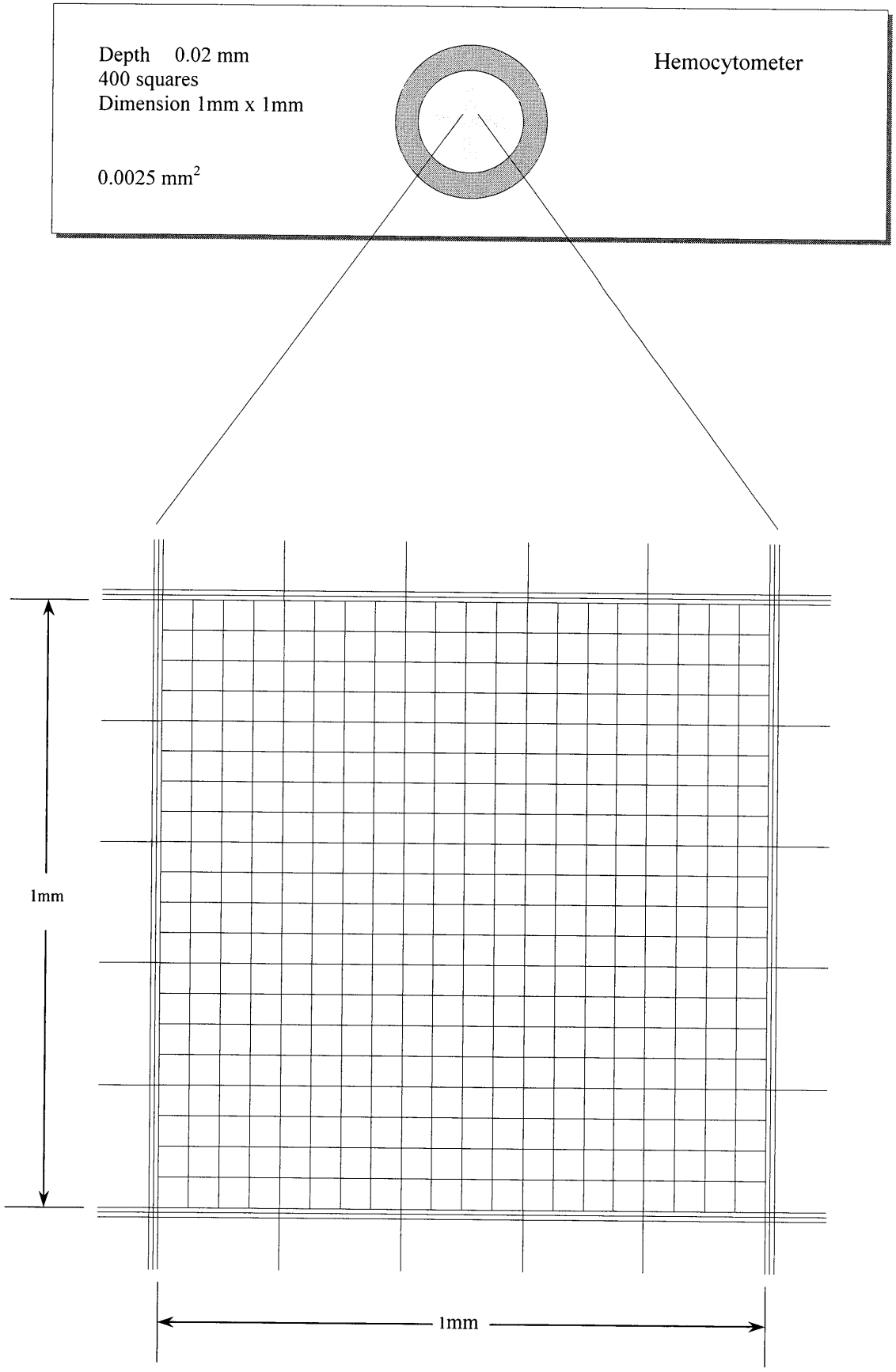



**Figure B.9-2** Hemocytometer and the counting square.

**Dilution buffer preparation**

**(¼ strength Ringer's solution):** Dissolve one of the Ringer tablets in a 500 mL of double distilled water and sterilise the solution in the autoclave at 121°C for 15 minutes. This solution is used to avoid cells shocks during dilution.

Place a loopful of suspension on the ruled area and apply the cover glass, which must be clean and polished. The amount of suspension must be such that the space between the platform and the cover glass is just filled and no fluid runs into the ditch; this requires practice. If the cover glass is applied properly, Newton's rings will be seen. Allow five minutes for the bacteria to settle ml (Collins *et al.*, 1999).

Examine with a 4-mm lens with reduced light, dark field or phase contrast if available. Count the bacteria in 50-100 squares selected at random. Divide the count by number of squares counted. Multiply by ($2 \times 10^7$) and by the original dilution factor to obtain the total number of bacteria/ml. Repeat twice more and take the average of the three counts ml (Collins *et al.*, 1999).

With experience, reasonably accurate counts can be obtained but the chamber and the cover glass must be scrupulously cleaned and examined microscopically to make sure bacteria are not left adhering to either.

## B.10 Dry weight

### Procedure

Calibrated 10 mL centrifuge tubes (Fisons, England) rather than pipettes were used to measure sample volumes due to the high solid content in the fermentation broth. After being centrifuged in a (IEC Centra-MPYR) centrifuge at 5000 rpm and 2°C for 30 minutes, supernatant from 10 mL sample was collected for component analyses. In the samples from the early stage of fermentation, i. e. until 300-308 hours after inoculation, solids were resuspended in glass vials of known weight by using distilled water and then stored in a 80-110°C oven for at least 5 days in order to dry out the solids completely. After being dried the samples cooled for 30-40 minutes in a desiccator before being weighed to 0.0001g. The results were calculated from the difference of weights.

# APPENDIX C: TABLES OF RESULTS

This section contains the analytical results obtained from each fermentation process. The results of the shaking flasks and the bioreactor fermentations are illustrated in the following.

# APPENDIX C: TABLES OF RESULTS

**Table C.1** Analyses for B-Syrup shaking flask fermentation (Fermentation 1; inoculated with *Lactobacillus casei* shirota in the pilot plant).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | 8.80E+06 | 4.18 | - | - | - | 0.193 | 0.191 | 0.187 | 0.190 | 116.1 |
| 2 | 6.0 | 8.20E+06 | 4.12 | - | - | - | 0.190 | 0.182 | 0.184 | 0.185 | 113.1 |
| 3 | 12.0 | 9.50E+06 | 4.08 | - | - | - | 0.184 | 0.177 | 0.179 | 0.180 | 109.8 |
| 4 | 24.0 | 2.70E+08 | 4.06 | - | - | - | 0.163 | 0.165 | 0.168 | 0.165 | 100.9 |
| 5 | 36.0 | 2.10E+08 | 4.02 | - | - | - | 0.155 | 0.151 | 0.150 | 0.152 | 92.72 |
| 6 | 48.0 | 2.30E+08 | 3.97 | - | - | - | 0.157 | 0.164 | 0.158 | 0.160 | 97.39 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | 3.756 | 0.650 | 11.639 | - | - | - | - |
| 2 | 6.0 | - | 3.458 | 0.641 | 11.052 | - | - | - | - |
| 3 | 12.0 | - | 2.986 | 0.547 | 10.251 | - | - | - | - |
| 4 | 24.0 | - | 2.605 | 0.425 | 8.432 | - | - | - | - |
| 5 | 36.0 | - | 2.055 | 0.346 | 7.384 | - | - | - | - |
| 6 | 48.0 | - | 1.763 | 0.287 | 6.179 | - | - | - | - |

**Table C.2** Analyses for B-Syrup shaking flask fermentation (Fermentation 2; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | 9.10E+06 | 4.08 | 9.7419 | 10.841 | 1.0995 | 0.187 | 0.185 | 0.172 | 0.181 | 110.6 |
| 2 | 6.0 | 8.50E+06 | 4.08 | 8.2900 | 9.4312 | 1.1002 | 0.186 | 0.175 | 0.181 | 0.181 | 110.2 |
| 3 | 12.0 | 9.67E+06 | 4.06 | 8.1334 | 9.3182 | 1.1089 | 0.179 | 0.183 | 0.174 | 0.179 | 108.9 |
| 4 | 24.0 | 2.31E+08 | 4.04 | 6.5132 | 10.841 | 1.1412 | 0.153 | 0.149 | 0.157 | 0.153 | 93.33 |
| 5 | 36.0 | 5.96E+07 | 4.03 | 6.6596 | 9.4312 | 1.1607 | 0.151 | 0.135 | 0.138 | 0.141 | 86.21 |
| 6 | 48.0 | 1.00E+08 | 4.03 | 6.7123 | 9.3182 | 1.1848 | 0.147 | 0.156 | 0.152 | 0.152 | 92.51 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | 3.843 | 0.691 | 12.030 | 0.540 | 239.8 | 0.292 | 170.11 |
| 2 | 6.0 | - | 3.701 | 0.665 | 11.894 | 0.536 | 238.0 | 0.307 | 178.85 |
| 3 | 12.0 | - | 3.315 | 0.582 | 10.513 | 0.526 | 233.5 | 0.329 | 191.67 |
| 4 | 24.0 | - | 2.893 | 0.474 | 8.644 | 0.540 | 230.8 | 0.381 | 221.96 |
| 5 | 36.0 | - | 2.596 | 0.410 | 7.213 | 0.536 | 228.9 | 0.515 | 300.02 |
| 6 | 48.0 | - | 1.994 | 0.337 | 6.580 | 0.526 | 226.3 | 0.895 | 521.39 |

**Table C.3** Analyses for KL3 shaking flask fermentation (Fermentation 3; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}1$ | $A_{570}2$ | $A_{570}3$ | $A_{ave}$ | F |
| 1 | 0.0 | 2.55E+07 | 4.72 | - | - | - | 0.883 | 0.875 | 0.881 | 0.880 | 1636 |
| 2 | 6.0 | 4.10E+07 | 4.61 | - | - | - | 0.845 | 0.859 | 0.843 | 0.849 | 1579 |
| 3 | 12.0 | 6.34E+08 | 4.60 | - | - | - | 0.781 | 0.762 | 0.769 | 0.771 | 1433 |
| 4 | 24.0 | 3.40E+08 | 4.58 | - | - | - | 0.736 | 0.717 | 0.749 | 0.734 | 1365 |
| 5 | 36.0 | 5.40E+08 | 4.51 | - | - | - | 0.707 | 0.687 | 0.663 | 0.686 | 1275 |
| 6 | 48.0 | 2.20E+08 | 4.41 | - | - | - | 0.631 | 0.626 | 0.643 | 0.633 | 1178 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | 0.250 | 4.574 | 1.975 | 8.719 | 0.814 | 363.4 | - | - |
| 2 | 6.0 | 0.331 | 4.056 | 1.854 | 8.699 | 0.774 | 345.3 | - | - |
| 3 | 12.0 | - | 3.870 | 1.641 | 7.273 | 0.769 | 343.0 | - | - |
| 4 | 24.0 | 0.533 | 3.481 | 1.575 | 6.169 | 0.746 | 332.6 | - | - |
| 5 | 36.0 | - | 2.617 | 1.224 | 5.703 | 0.713 | 317.7 | - | - |
| 6 | 48.0 | - | 1.909 | 1.027 | 5.274 | 0.711 | 316.8 | - | - |

**Table C.4** Analyses for KL3 shaking flask fermentation (Fermentation 4; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}1$ | $A_{570}2$ | $A_{570}3$ | $A_{ave.}$ | F |
| 1 | 0.0 | 1.35E+07 | 4.59 | 8.2580 | 8.4792 | 0.2212 | 0.821 | 0.875 | 0.862 | 0.853 | 1586 |
| 2 | 6.0 | 3.51E+07 | 4.57 | 6.7253 | 6.9475 | 0.2222 | 0.791 | 0.770 | 0.753 | 0.771 | 1435 |
| 3 | 12.0 | 8.74E+08 | 4.54 | 6.5663 | 6.8054 | 0.2271 | 0.594 | 0.661 | 0.632 | 0.629 | 1170 |
| 4 | 24.0 | 1.14E+08 | 4.52 | 6.6791 | 6.9095 | 0.2304 | 0.617 | 0.626 | 0.615 | 0.619 | 1152 |
| 5 | 36.0 | 1.60E+08 | 4.49 | 6.5127 | 6.7473 | 0.2346 | 0.567 | 0.582 | 0.571 | 0.573 | 1066 |
| 6 | 48.0 | 1.20E+08 | 4.47 | 6.5663 | 6.8054 | 0.2391 | 0.541 | 0.526 | 0.533 | 0.533 | 992.2 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | 0.432 | 3.784 | 2.075 | 9.297 | 0.806 | 359.8 | 0.202 | 117.68 |
| 2 | 6.0 | - | 3.623 | 1.960 | 9.836 | 0.787 | 351.2 | 0.253 | 147.39 |
| 3 | 12.0 | - | 2.791 | 1.722 | 8.405 | 0.751 | 334.9 | 0.291 | 169.53 |
| 4 | 24.0 | - | 2.308 | 1.614 | 7.080 | 0.721 | 321.3 | 0.342 | 199.24 |
| 5 | 36.0 | - | 2.017 | 1.221 | 6.554 | 0.688 | 306.4 | 0.378 | 220.21 |
| 6 | 48.0 | - | 1.474 | 0.994 | 5.942 | 0.661 | 294.1 | 0.398 | 231.86 |

**Table C.5** Analyses for KL3 and B-Syrup shaking flask fermentation (Fermentation 5; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight (g $mL^{-1}$) | | | FAN (mg $L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | - | 4.46 | 6.4879 | 7.1261 | 0.6382 | 0.626 | 0.663 | 0.659 | 0.649 | 396.1 |
| 2 | 6.0 | - | 4.43 | 6.5492 | 7.1882 | 0.6390 | 0.636 | 0.645 | 0.654 | 0.645 | 393.4 |
| 3 | 12.0 | - | 4.42 | 6.7621 | 7.4024 | 0.6403 | 0.594 | 0.587 | 0.591 | 0.591 | 360.3 |
| 4 | 24.0 | - | 4.40 | 8.1379 | 8.7804 | 0.6425 | 0.541 | 0.553 | 0.530 | 0.541 | 330.2 |
| 5 | 36.0 | - | 4.38 | 6.7028 | 7.3470 | 0.6442 | 0.595 | 0.589 | 0.601 | 0.595 | 362.9 |
| 6 | 48.0 | - | 4.35 | 6.6549 | 7.3015 | 0.6466 | 0.612 | 0.645 | 0.607 | 0.621 | 379.0 |

| Sample No. | Time (h) | Sugar Concentration (g $L^{-1}$) | | | | Phosphorus (mg $L^{-1}$) | | Lactic Acid (mg $L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | 2.732 | 0.878 | 9.389 | 0.684 | 304.5 | 0.246 | 143.32 |
| 2 | 6.0 | - | 2.680 | 0.863 | 9.220 | 0.667 | 296.9 | 0.251 | 146.23 |
| 3 | 12.0 | - | 2.246 | 0.779 | 8.886 | 0.649 | 288.7 | 0.284 | 165.45 |
| 4 | 24.0 | - | 1.804 | 0.621 | 7.511 | 0.653 | 290.5 | 0.293 | 170.70 |
| 5 | 36.0 | - | 1.240 | 0.514 | 6.681 | 0.641 | 285.1 | 0.363 | 211.47 |
| 6 | 48.0 | - | 1.055 | 0.406 | 5.870 | 0.638 | 283.7 | 0.392 | 228.37 |

**Table C.6** Analyses for whole malt grains shaking flask fermentation (Fermentation 6; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}1$ | $A_{570}2$ | $A_{570}3$ | $A_{ave.}$ | F |
| 1 | 0.0 | - | 3.92 | 6.4806 | 6.7096 | 0.2290 | 0.052 | 0.049 | 0.051 | 0.051 | 47.13 |
| 2 | 6.0 | - | 3.79 | 6.7663 | 6.9957 | 0.2294 | 0.047 | 0.045 | 0.044 | 0.045 | 42.17 |
| 3 | 12.0 | - | 3.68 | 6.6930 | 6.9242 | 0.2312 | 0.040 | 0.042 | 0.039 | 0.040 | 37.51 |
| 4 | 24.0 | - | 3.54 | 6.5132 | 6.7463 | 0.2331 | 0.059 | 0.053 | 0.071 | 0.061 | 56.74 |
| 5 | 36.0 | - | 3.48 | 6.5972 | 6.8309 | 0.2337 | 0.059 | 0.055 | 0.058 | 0.057 | 53.33 |
| 6 | 48.0 | - | 3.12 | 6.5693 | 6.8037 | 0.2344 | 0.069 | 0.065 | 0.075 | 0.070 | 64.80 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | 0.252 | 1.320 | 0.513 | 0.282 | 122.6 | 0.335 | 195.16 |
| 2 | 6.0 | - | 0.978 | 1.250 | 0.508 | 0.277 | 120.4 | 0.580 | 337.89 |
| 3 | 12.0 | 0.266 | 1.592 | 1.174 | 0.486 | 0.274 | 119.0 | 0.624 | 363.52 |
| 4 | 24.0 | - | 2.368 | 1.143 | 0.455 | 0.259 | 112.2 | 0.760 | 442.75 |
| 5 | 36.0 | - | 2.963 | 0.974 | 0.416 | 0.251 | 108.6 | 0.949 | 552.85 |
| 6 | 48.0 | 1.592 | 3.386 | 0.456 | 0.365 | 0.246 | 106.3 | 1.162 | 676.93 |

**Table C.7** Analyses for whole milled malt grains shaking flask fermentation (Fermentation 7; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight (g $mL^{-1}$) | | | FAN (mg $L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | Ave. | F |
| 1 | 0.0 | - | 4.39 | - | - | - | 0.109 | 0.091 | 0.098 | 0.099 | 92.40 |
| 2 | 6.0 | - | 4.31 | - | - | - | 0.084 | 0.081 | 0.075 | 0.080 | 74.42 |
| 3 | 12.0 | - | 4.21 | - | - | - | 0.067 | 0.063 | 0.064 | 0.065 | 60.16 |
| 4 | 24.0 | - | 4.13 | - | - | - | 0.063 | 0.069 | 0.067 | 0.066 | 61.71 |
| 5 | 36.0 | - | 3.98 | - | - | - | 0.075 | 0.077 | 0.068 | 0.073 | 68.22 |
| 6 | 48.0 | - | 3.78 | - | - | - | 0.085 | 0.107 | 0.091 | 0.094 | 87.75 |

| Sample No. | Time (h) | Sugar Concentration (g $L^{-1}$) | | | | Phosphorus (mg $L^{-1}$) | | Lactic Acid (mg $L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | 3.535 | 1.151 | 1.920 | 1.230 | 0.489 | 216.3 | - | - |
| 2 | 6.0 | 3.249 | 1.358 | 1.970 | 1.460 | 0.476 | 210.4 | - | - |
| 3 | 12.0 | 2.833 | 1.703 | 2.112 | 1.896 | 0.452 | 199.6 | - | - |
| 4 | 24.0 | 1.714 | 2.110 | 2.413 | 2.338 | 0.413 | 181.9 | - | - |
| 5 | 36.0 | 1.164 | 1.572 | 3.742 | 2.637 | 0.367 | 161.1 | - | - |
| 6 | 48.0 | 0.763 | 0.428 | 5.232 | 3.550 | 0.260 | 112.7 | - | - |

**Table C.8** Analyses for whole malt grains shaking flask fermentation (Fermentation 8; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | - | 3.91 | - | - | - | - | - | - | - | - |
| 2 | 6.0 | - | 3.88 | - | - | - | - | - | - | - | - |
| 3 | 16.0 | - | 3.62 | - | - | - | - | - | - | - | - |
| 4 | 22.0 | - | 3.54 | - | - | - | - | - | - | - | - |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | - | - | - | 0.286 | 124.4 | - | - |
| 2 | 6.0 | - | - | - | - | 0.265 | 114.9 | - | - |
| 3 | 16.0 | - | - | - | - | 0.252 | 109.1 | - | - |
| 4 | 22.0 | - | - | - | - | 0.246 | 106.3 | - | - |

**Table C.9** Analyses for whole malt grains shaking flask fermentation (Fermentation 9; inoculated with *Saccharomyces cerevisiae*).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | - | 4.75 | - | - | - | - | - | - | - | - |
| 2 | 6.0 | - | 4.63 | - | - | - | - | - | - | - | - |
| 3 | 16.0 | - | 4.55 | - | - | - | - | - | - | - | - |
| 4 | 22.0 | - | 4.50 | - | - | - | - | - | - | - | - |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | - | - | - | 0.270 | 117.2 | - | - |
| 2 | 6.0 | - | - | - | - | 0.259 | 112.2 | - | - |
| 3 | 16.0 | - | - | - | - | 0.253 | 109.5 | - | - |
| 4 | 22.0 | - | - | - | - | 0.249 | 107.7 | - | - |

**Table C.11** Analyses for whole milled malt grains shaking flask fermentation (Fermentation 11; inoculated with *Saccharomyces cerevisiae*).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | FAN |
| 1 | 0.0 | - | 4.37 | - | - | - | - | - | - | - | - |
| 2 | 6.0 | - | 4.25 | - | - | - | - | - | - | - | - |
| 3 | 16.0 | - | 4.20 | - | - | - | - | - | - | - | - |
| 4 | 22.0 | - | 4.15 | - | - | - | - | - | - | - | - |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | - | - | - | 0.432 | 190.5 | - | - |
| 2 | 6.0 | - | - | - | - | 0.390 | 171.5 | - | - |
| 3 | 16.0 | - | - | - | - | 0.360 | 157.9 | - | - |
| 4 | 22.0 | - | - | - | - | 0.345 | 151.1 | - | - |

**Table C.12** Analyses for B-Syrup bioreactor fermentation (Fermentation 12; inoculated with *Lactobacillus casei* shirota in the pilot plant).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | 1.80E+07 | 5.05 | - | - | - | 0.189 | 0.194 | 0.191 | 0.191 | 116.7 |
| 2 | 10.0 | 1.95E+08 | 4.89 | - | - | - | 0.184 | 0.179 | 0.181 | 0.181 | 110.6 |
| 3 | 16.5 | 2.60E+08 | 4.72 | - | - | - | 0.181 | 0.178 | 0.179 | 0.179 | 109.4 |
| 4 | 31.0 | 3.50E+08 | 4.61 | - | - | - | 0.173 | 0.177 | 0.173 | 0.174 | 106.3 |
| 5 | 42.5 | 3.20E+08 | 4.73 | - | - | - | 0.166 | 0.162 | 0.167 | 0.165 | 100.7 |
| 6 | 57.0 | 4.20E+08 | 4.62 | - | - | - | 0.160 | 0.159 | 0.162 | 0.160 | 97.80 |
| 7 | 68.5 | 5.90E+08 | 4.60 | - | - | - | 0.164 | 0.163 | 0.171 | 0.166 | 101.3 |
| 8 | 83.0 | 3.60E+08 | 4.55 | - | - | - | 0.174 | 0.170 | 0.169 | 0.171 | 104.3 |
| 9 | 94.5 | 2.00E+09 | 4.53 | - | - | - | 0.177 | 0.178 | 0.172 | 0.176 | 107.2 |
| 10 | 109.0 | 5.50E+09 | 4.54 | - | - | - | 0.181 | 0.180 | 0.175 | 0.179 | 108.9 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | 0.188 | 3.079 | 0.193 | 14.167 | - | - | - | - |
| 2 | 10.0 | - | - | - | - | - | - | - | - |
| 3 | 16.5 | 0.312 | 2.448 | 0.334 | 9.694 | - | - | - | - |
| 4 | 31.0 | - | - | - | - | - | - | - | - |
| 5 | 42.5 | 0.286 | 1.384 | 0.476 | 8.851 | - | - | - | - |
| 6 | 57.0 | - | - | - | - | - | - | - | - |
| 7 | 68.5 | 0.254 | 1.365 | 0.612 | 7.633 | - | - | - | - |
| 8 | 83.0 | - | - | - | - | - | - | - | - |
| 9 | 94.5 | 0.209 | 1.965 | 0.840 | 15.561 | - | - | - | - |
| 10 | 109.0 | - | - | - | - | - | - | - | - |

**Table C.12** *(Continue)*

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight (g $mL^{-1}$) | | | FAN (mg $L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 11 | 120.5 | 4.60E+09 | 4.51 | - | - | - | 0.179 | 0.185 | 0.182 | 0.182 | 111.0 |
| 12 | 135.0 | 5.20E+09 | 4.47 | - | - | - | 0.183 | 0.187 | 0.186 | 0.185 | 113.1 |
| 13 | 146.5 | 5.10E+09 | 4.44 | - | - | - | 0.187 | 0.191 | 0.189 | 0.189 | 115.3 |
| 14 | 161.0 | 5.00E+09 | 4.36 | - | - | - | 0.182 | 0.189 | 0.194 | 0.188 | 114.9 |
| 15 | 172.5 | 4.60E+09 | 4.38 | - | - | - | 0.191 | 0.194 | 0.179 | 0.188 | 114.7 |
| 16 | 187.0 | 6.00E+09 | 4.37 | - | - | - | 0.189 | 0.197 | 0.198 | 0.195 | 118.7 |
| 17 | 198.5 | 6.10E+09 | 4.41 | - | - | - | 0.204 | 0.201 | 0.192 | 0.199 | 121.4 |
| 18 | 222.5 | 5.10E+09 | 4.38 | - | - | - | 0.211 | 0.214 | 0.203 | 0.209 | 127.5 |
| 19 | 270.5 | 4.80E+09 | 4.35 | - | - | - | 0.213 | 0.210 | 0.206 | 0.210 | 127.9 |
| 20 | 294.5 | 4.40E+09 | 4.32 | - | - | - | 0.209 | 0.215 | 0.197 | 0.207 | 126.3 |

| Sample No. | Time (h) | Sugar Concentration (g $L^{-1}$) | | | | Phosphorus (mg $L^{-1}$) | | Lactic Acid (mg $L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 11 | 120.5 | 0.547 | 1.263 | 0.428 | 7.052 | - | - | - | - |
| 12 | 135.0 | - | - | - | - | - | - | - | - |
| 13 | 146.5 | 0.725 | 1.370 | 0.349 | 10.045 | - | - | - | - |
| 14 | 161.0 | - | - | - | - | - | - | - | - |
| 15 | 172.5 | 0.384 | 1.734 | 0.333 | 9.228 | - | - | - | - |
| 16 | 187.0 | - | - | - | - | - | - | - | - |
| 17 | 198.5 | - | 1.837 | 0.206 | 8.764 | - | - | - | - |
| 18 | 222.5 | - | - | - | - | - | - | - | - |
| 19 | 270.5 | - | 1.533 | 0.198 | 6.647 | - | - | - | - |
| 20 | 294.5 | - | - | - | - | - | - | - | - |

**Table C.13** Analyses for B-Syrup bioreactor fermentation (Fermentation 13; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | 1.67E+07 | 4.71 | 8.4141 | 9.7892 | 1.3751 | 0.187 | 0.188 | 0.192 | 0.189 | 115.3 |
| 2 | 9.5 | 2.00E+08 | 4.62 | 10.232 | 11.66 | 1.4282 | 0.178 | 0.183 | 0.177 | 0.179 | 109.4 |
| 3 | 21.0 | 1.30E+09 | 4.58 | 6.5008 | 8.1195 | 1.6187 | 0.162 | 0.184 | 0.175 | 0.174 | 105.9 |
| 4 | 35.5 | 4.00E+08 | 4.63 | 6.6130 | 8.2672 | 1.6542 | 0.161 | 0.167 | 0.164 | 0.164 | 100.0 |
| 5 | 47.0 | 5.00E+08 | 4.63 | 6.5163 | 8.2105 | 1.6942 | 0.169 | 0.173 | 0.166 | 0.169 | 103.3 |
| 6 | 61.5 | 1.50E+08 | 4.64 | 6.5353 | 8.2421 | 1.7068 | 0.172 | 0.174 | 0.166 | 0.171 | 104.1 |
| 7 | 73.0 | 1.00E+09 | 4.63 | 6.5337 | 8.2493 | 1.7156 | 0.177 | 0.176 | 0.171 | 0.175 | 106.5 |
| 8 | 87.5 | 4.50E+08 | 4.61 | 6.7015 | 8.4317 | 1.7302 | 0.179 | 0.181 | 0.182 | 0.181 | 110.2 |
| 9 | 99.0 | 1.10E+09 | 4.62 | 6.6156 | 8.3658 | 1.7502 | 0.183 | 0.184 | 0.181 | 0.183 | 111.4 |
| 10 | 113.5 | 4.50E+09 | 4.57 | 6.4928 | 8.2629 | 1.7701 | 0.188 | 0.186 | 0.176 | 0.183 | 111.8 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | 3.237 | 0.215 | 16.290 | 0.268 | 116.3 | 0.238 | 138.66 |
| 2 | 9.5 | - | - | - | - | 0.254 | 109.9 | 0.275 | 160.21 |
| 3 | 21.0 | 0.112 | 2.756 | 0.270 | 14.852 | 0.247 | 106.8 | 0.342 | 199.24 |
| 4 | 35.5 | - | - | - | - | 0.244 | 105.4 | 0.415 | 241.77 |
| 5 | 47.0 | 0.184 | 2.571 | 0.352 | 11.360 | 0.242 | 104.5 | 0.486 | 283.13 |
| 6 | 61.5 | - | - | - | - | 0.237 | 102.3 | 0.480 | 279.63 |
| 7 | 73.0 | - | 2.190 | 0.568 | 8.700 | 0.233 | 100.5 | 0.557 | 324.49 |
| 8 | 87.5 | - | - | - | - | 0.230 | 99.10 | 0.601 | 350.12 |
| 9 | 99.0 | 0.171 | 1.716 | 0.617 | 8.128 | 0.225 | 96.84 | 0.646 | 376.34 |
| 10 | 113.5 | - | - | - | - | 0.221 | 95.03 | 0.684 | 398.47 |

**Table C.13** *(Continue)*

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 11 | 125.0 | 3.90E+09 | 4.58 | 6.6404 | 8.4511 | 1.8107 | 0.192 | 0.189 | 0.184 | 0.188 | 114.9 |
| 12 | 139.5 | 2.25E+09 | 4.57 | 6.7089 | 8.5394 | 1.8305 | 0.191 | 0.193 | 0.187 | 0.190 | 116.1 |
| 13 | 151.0 | 2.50E+09 | 4.6 | 6.6121 | 8.4733 | 1.8612 | 0.172 | 0.189 | 0.195 | 0.185 | 113.1 |
| 14 | 165.5 | 4.80E+09 | 4.58 | 6.5977 | 8.4874 | 1.8897 | 0.192 | 0.185 | 0.184 | 0.187 | 114.1 |
| 15 | 177.0 | 3.90E+09 | 4.57 | 6.6581 | 8.5556 | 1.8975 | 0.190 | 0.196 | 0.200 | 0.195 | 119.2 |
| 16 | 191.5 | - | 4.60 | 6.7418 | 8.6512 | 1.9094 | 0.199 | 0.184 | 0.197 | 0.193 | 117.9 |
| 17 | 203.0 | - | 4.55 | 6.5441 | 8.4619 | 1.9178 | 0.202 | 0.209 | 0.198 | 0.203 | 123.8 |
| 18 | 227.0 | - | 4.56 | 6.6097 | 8.5401 | 1.9304 | 0.201 | 0.205 | 0.192 | 0.199 | 121.6 |
| 19 | 275.0 | - | 4.53 | 6.5403 | 8.4926 | 1.9523 | 0.195 | 0.207 | 0.197 | 0.200 | 121.8 |
| 20 | 299.0 | - | 4.51 | 6.5981 | 8.5652 | 1.9671 | 0.203 | 0.198 | 0.211 | 0.204 | 124.4 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 11 | 125.0 | 0.547 | 1.462 | 0.446 | 7.318 | 0.219 | 94.12 | 0.717 | 417.70 |
| 12 | 139.5 | - | - | - | - | 0.212 | 90.96 | 0.769 | 447.99 |
| 13 | 151.0 | 0.725 | 1.206 | 0.362 | 7.205 | 0.209 | 89.60 | 0.821 | 478.28 |
| 14 | 165.5 | - | - | - | - | 0.203 | 86.88 | 0.857 | 499.25 |
| 15 | 177.0 | 0.384 | 1.152 | 0.310 | 8.884 | 0.197 | 84.17 | 0.823 | 479.45 |
| 16 | 191.5 | - | - | - | - | 0.192 | 81.91 | 0.863 | 502.75 |
| 17 | 203.0 | - | 1.379 | 0.256 | 9.071 | 0.193 | 82.36 | 0.898 | 523.14 |
| 18 | 227.0 | - | - | - | - | 0.188 | 80.10 | 0.946 | 551.10 |
| 19 | 275.0 | - | 1.267 | 0.217 | 8.684 | 0.186 | 79.19 | 0.984 | 573.24 |
| 20 | 299.0 | - | - | - | - | 0.183 | 77.83 | 1.274 | 742.17 |

**Table C.14** Analyses for KL3 bioreactor fermentation (Fermentation 14; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight (g $mL^{-1}$) | | | FAN (mg $L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 1 | 0.0 | 7.50E+07 | 5.02 | 6.5650 | 6.7891 | 0.2241 | 0.719 | 0.743 | 0.753 | 0.738 | 1374 |
| 2 | 14.3 | 5.00E+07 | 4.96 | 6.5274 | 6.7756 | 0.2482 | 0.686 | 0.689 | 0.667 | 0.681 | 1266 |
| 3 | 25.5 | 5.71E+07 | 4.95 | 6.7231 | 6.9956 | 0.2725 | 0.598 | 0.609 | 0.587 | 0.598 | 1113 |
| 4 | 40.0 | 4.00E+07 | 4.91 | 6.5690 | 6.8631 | 0.2941 | 0.553 | 0.522 | 0.532 | 0.536 | 997 |
| 5 | 51.5 | 1.20E+08 | 4.88 | 6.6406 | 6.9493 | 0.3087 | 0.511 | 0.508 | 0.494 | 0.504 | 938 |
| 6 | 66.0 | 1.00E+08 | 4.92 | 6.7118 | 7.0313 | 0.3195 | 0.463 | 0.461 | 0.424 | 0.449 | 836 |
| 7 | 77.5 | 1.50E+08 | 4.91 | 6.6924 | 7.0151 | 0.3227 | 0.432 | 0.404 | 0.426 | 0.421 | 783 |
| 8 | 92.0 | 5.00E+08 | 4.8 | 6.5969 | 6.9327 | 0.3358 | 0.463 | 0.477 | 0.457 | 0.466 | 866 |
| 9 | 103.5 | 3.00E+08 | 4.88 | 6.8074 | 7.1553 | 0.3479 | 0.488 | 0.491 | 0.472 | 0.484 | 900 |
| 10 | 118.0 | 1.90E+09 | 4.93 | 6.5761 | 6.9349 | 0.3588 | 0.534 | 0.570 | 0.532 | 0.545 | 1015 |

| Sample No. | Time (h) | Sugar Concentration (g $L^{-1}$) | | | | Phosphorus (mg $L^{-1}$) | | Lactic Acid (mg $L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | 0.587 | 3.854 | 2.857 | 9.337 | 0.861 | 384.6 | 0.362 | 198.96 |
| 2 | 14.3 | - | - | - | - | 0.828 | 369.7 | 0.381 | 209.40 |
| 3 | 25.5 | 0.411 | 4.442 | 2.830 | 8.199 | 0.820 | 366.0 | 0.419 | 230.28 |
| 4 | 40.0 | - | - | - | - | 0.816 | 364.2 | 0.550 | 302.27 |
| 5 | 51.5 | - | 3.569 | 1.067 | 8.415 | 0.807 | 360.2 | 0.587 | 322.61 |
| 6 | 66.0 | - | - | - | - | 0.802 | 357.9 | 0.629 | 345.69 |
| 7 | 77.5 | 0.330 | 2.487 | 2.074 | 9.389 | 0.789 | 352.0 | 0.695 | 381.96 |
| 8 | 92.0 | - | - | - | - | 0.772 | 344.3 | 0.753 | 413.84 |
| 9 | 103.5 | - | 2.028 | 1.977 | 8.364 | 0.770 | 343.4 | 0.785 | 431.42 |
| 10 | 118.0 | - | - | - | - | 0.760 | 338.9 | 0.845 | 464.40 |

**Table C.14** *(Continue)*

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}$ 1 | $A_{570}$ 2 | $A_{570}$ 3 | $A_{ave.}$ | F |
| 11 | 129.5 | 1.75E+08 | 4.87 | 6.6357 | 6.9975 | 0.3618 | 0.643 | 0.620 | 0.667 | 0.643 | 1197 |
| 12 | 144.0 | 3.00E+08 | 4.82 | 6.6826 | 7.0502 | 0.3676 | 0.777 | 0.762 | 0.760 | 0.766 | 1426 |
| 13 | 155.5 | 4.00E+08 | 4.81 | 6.7000 | 7.0777 | 0.3777 | 0.695 | 0.747 | 0.68 | 0.707 | 1316 |
| 14 | 170.0 | 0.00E+00 | 4.77 | 6.7279 | 7.1131 | 0.3852 | 0.784 | 0.722 | 0.763 | 0.756 | 1407 |
| 15 | 181.5 | 0.00E+00 | 4.73 | 6.7194 | 7.1091 | 0.3897 | 0.748 | 0.765 | 0.795 | 0.769 | 1431 |
| 16 | 196.0 | - | 4.65 | 6.6897 | 7.0843 | 0.3946 | 0.822 | 0.815 | 0.817 | 0.818 | 1522 |
| 17 | 207.5 | - | 4.56 | 6.7081 | 7.1098 | 0.4017 | 0.875 | 0.932 | 0.955 | 0.921 | 1713 |
| 18 | 231.5 | - | 4.49 | 6.4804 | 6.8887 | 0.4083 | 0.902 | 0.972 | 0.964 | 0.946 | 1760 |
| 19 | 279.5 | - | 4.32 | 6.7836 | 7.1943 | 0.4107 | 0.976 | 0.954 | 0.963 | 0.964 | 1794 |
| 20 | 303.5 | - | 4.31 | 6.6921 | 7.1104 | 0.4183 | 0.989 | 0.967 | 0.992 | 0.983 | 1828 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 11 | 129.5 | - | 1.831 | 1.704 | 7.682 | 0.763 | 340.3 | 0.897 | 492.98 |
| 12 | 144.0 | - | - | - | - | 0.760 | 338.9 | 0.919 | 505.07 |
| 13 | 155.5 | - | 1.586 | 1.190 | 6.063 | 0.757 | 337.5 | 0.974 | 535.29 |
| 14 | 170.0 | - | - | - | - | 0.751 | 334.8 | 1.044 | 573.76 |
| 15 | 181.5 | - | 1.465 | 1.022 | 5.493 | 0.756 | 337.1 | 1.201 | 660.05 |
| 16 | 196.0 | - | - | - | - | 0.755 | 336.6 | 1.356 | 745.23 |
| 17 | 207.5 | 0.273 | 1.254 | 0.938 | 4.678 | 0.753 | 335.7 | 1.548 | 850.75 |
| 18 | 231.5 | - | - | - | - | 0.748 | 333.5 | 1.611 | 885.37 |
| 19 | 279.5 | - | 1.238 | 0.879 | 3.900 | 0.742 | 330.7 | 1.772 | 973.85 |
| 20 | 303.5 | - | - | - | - | 0.737 | 328.5 | 1.833 | 1007.4 |

**Table C.15** Analyses for whole malt grains bioreactor fermentation (Fermentation 15; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}1$ | $A_{570}2$ | $A_{570}3$ | $A_{ave.}$ | F |
| 1 | 0.0 | - | 4.42 | 6.7149 | 6.9914 | 0.2765 | 0.088 | 0.108 | 0.114 | 0.103 | 96.12 |
| 2 | 6.0 | - | 4.21 | 6.7096 | 6.9888 | 0.2792 | 0.109 | 0.086 | 0.087 | 0.094 | 87.44 |
| 3 | 12.0 | - | 3.77 | 6.5875 | 6.8652 | 0.2777 | 0.100 | 0.074 | 0.077 | 0.084 | 77.83 |
| 4 | 24.0 | - | 3.24 | 6.7235 | 7.0051 | 0.2816 | 0.079 | 0.068 | 0.078 | 0.075 | 69.77 |
| 5 | 36.0 | - | 3.19 | 6.5968 | 6.8767 | 0.2829 | 0.084 | 0.08 | 0.108 | 0.091 | 84.34 |
| 6 | 48.0 | - | 2.97 | 6.7835 | 7.0518 | 0.2834 | 0.069 | 0.074 | 0.054 | 0.066 | 61.09 |
| 7 | 60.0 | - | 3.02 | 6.5127 | 6.7716 | 0.2859 | 0.088 | 0.091 | 0.087 | 0.089 | 82.48 |
| 8 | 71.0 | - | 2.96 | 6.5765 | 6.8476 | 0.2784 | 0.101 | 0.095 | 0.103 | 0.100 | 92.71 |
| 9 | 80.0 | - | 2.92 | 6.7683 | 7.0426 | 0.2864 | 0.109 | 0.112 | 0.094 | 0.105 | 97.67 |
| 10 | 95.5 | - | 2.79 | 6.7284 | 7.0067 | 0.2871 | 0.111 | 0.098 | 0.110 | 0.106 | 98.91 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | 0.381 | 1.877 | 0.710 | 0.301 | 131.2 | 0.305 | 177.69 |
| 2 | 6.0 | - | - | - | - | 0.299 | 130.3 | 0.487 | 283.71 |
| 3 | 12.0 | 0.310 | 1.060 | 1.060 | - | 0.290 | 126.2 | 0.596 | 347.21 |
| 4 | 24.0 | - | - | - | - | 0.288 | 125.3 | 0.795 | 463.13 |
| 5 | 36.0 | 0.822 | 1.473 | 0.783 | 0.253 | 0.289 | 125.8 | 0.853 | 496.92 |
| 6 | 48.0 | - | - | - | - | 0.285 | 123.9 | 1.104 | 643.14 |
| 7 | 60.0 | 0.832 | 1.846 | 0.922 | 0.213 | 0.284 | 123.5 | 1.422 | 828.39 |
| 8 | 71.0 | - | - | - | - | 0.265 | 114.9 | 2.760 | 1607.8 |
| 9 | 80.0 | - | 1.890 | 0.624 | 0.192 | 0.271 | 117.7 | 2.869 | 1671.3 |
| 10 | 95.5 | - | - | - | - | 0.261 | 113.1 | 2.972 | 1731.3 |

**Table C.16** Analyses for whole milled malt grains bioreactor fermentation (Fermentation 16; inoculated with *Lactobacillus casei* shirota).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}1$ | $A_{570}2$ | $A_{570}3$ | $A_{ave.}$ | F |
| 1 | 0.0 | - | 4.95 | - | - | - | 0.080 | 0.083 | 0.084 | 0.082 | 76.59 |
| 2 | 6.0 | - | 4.52 | - | - | - | 0.074 | 0.079 | 0.081 | 0.078 | 72.56 |
| 3 | 12.0 | - | 3.51 | - | - | - | 0.089 | 0.111 | 0.094 | 0.098 | 91.16 |
| 4 | 24.0 | - | 3.41 | - | - | - | 0.116 | 0.104 | 0.114 | 0.111 | 103.6 |
| 5 | 36.0 | - | 3.23 | - | - | - | 0.119 | 0.121 | 0.116 | 0.119 | 110.4 |
| 6 | 49.0 | - | 3.16 | - | - | - | 0.115 | 0.118 | 0.117 | 0.117 | 108.5 |
| 7 | 66.5 | - | 3.02 | - | - | - | 0.127 | 0.133 | 0.124 | 0.128 | 119.1 |
| 8 | 74.5 | - | 2.94 | - | - | - | 0.132 | 0.134 | 0.131 | 0.132 | 123.1 |
| 9 | 84.5 | - | 2.85 | - | - | - | 0.129 | 0.132 | 0.133 | 0.131 | 122.2 |
| 10 | 96.0 | - | 2.76 | - | - | - | 0.144 | 0.142 | 0.143 | 0.143 | 133.0 |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | 1.873 | 2.413 | 1.269 | 0.496 | 219.5 | - | - |
| 2 | 6.0 | - | - | - | - | 0.488 | 215.9 | - | - |
| 3 | 12.0 | 1.714 | 2.110 | 2.431 | 2.338 | 0.483 | 213.6 | - | - |
| 4 | 24.0 | - | - | - | - | 0.478 | 211.3 | - | - |
| 5 | 36.0 | 1.204 | 1.833 | 3.347 | 3.550 | 0.470 | 207.7 | - | - |
| 6 | 49.0 | - | - | - | - | 0.464 | 205.0 | - | - |
| 7 | 66.5 | - | 1.046 | 3.679 | 3.753 | 0.459 | 202.7 | - | - |
| 8 | 74.5 | - | - | - | - | 0.452 | 199.6 | - | - |
| 9 | 84.5 | - | 0.685 | 5.522 | - | 0.447 | 197.3 | - | - |
| 10 | 96.0 | - | - | - | - | 0.444 | 195.9 | - | - |

**Table C.17** Analyses for whole malt grains bioreactor fermentation (Fermentation 17; inoculated with both *Lactobacillus casei* shirota and *Saccharomyces cerevisiae*).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}1$ | $A_{570}2$ | $A_{570}3$ | $A_{ave.}$ | F |
| 1 | 0.0 | - | 5.27 | - | - | - | - | - | - | - | - |
| 2 | 6.0 | - | 4.79 | - | - | - | - | - | - | - | - |
| 3 | 10.0 | - | 4.10 | - | - | - | - | - | - | - | - |
| 4 | 16.0 | - | 3.60 | - | - | - | - | - | - | - | - |
| 5 | 23.0 | - | 3.50 | - | - | - | - | - | - | - | - |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | - | - | - | 0.351 | 153.9 | - | - |
| 2 | 6.0 | - | - | - | - | 0.329 | 143.9 | - | - |
| 3 | 10.0 | - | - | - | - | 0.305 | 133.1 | - | - |
| 4 | 16.0 | - | - | - | - | 0.295 | 128.5 | - | - |
| 5 | 23.0 | - | - | - | - | 0.290 | 126.3 | - | - |

**Table C.18** Analyses for whole milled malt grains bioreactor fermentation (Fermentation 18; inoculated with both *Lactobacillus casei* shirota and *Saccharomyces cerevisiae*).

| Sample No. | Time (h) | Slide counting (Cells $mL^{-1}$) | pH | Dry weight ($g\ mL^{-1}$) | | | FAN ($mg\ L^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Empty | Dry | Net | $A_{570}1$ | $A_{570}2$ | $A_{570}3$ | $A_{ave.}$ | F |
| 1 | 0.0 | - | 4.99 | - | - | - | - | - | - | - | - |
| 2 | 2.0 | - | 4.82 | - | - | - | - | - | - | - | - |
| 3 | 4.0 | - | 4.65 | - | - | - | - | - | - | - | - |
| 4 | 6.0 | - | 4.20 | - | - | - | - | - | - | - | - |
| 5 | 9.0 | - | 3.85 | - | - | - | - | - | - | - | - |
| 6 | 12.0 | - | 3.50 | - | - | - | - | - | - | - | - |
| 7 | 15.0 | - | 3.25 | - | - | - | - | - | - | - | - |
| 8 | 18.0 | - | 3.05 | - | - | - | - | - | - | - | - |
| 9 | 22.0 | - | 2.95 | - | - | - | - | - | - | - | - |

| Sample No. | Time (h) | Sugar Concentration ($g\ L^{-1}$) | | | | Phosphorus ($mg\ L^{-1}$) | | Lactic Acid ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Glucose | Sucrose | Maltose | $A_{730}$ | LPhos | $A_{570}$ | C |
| 1 | 0.0 | - | - | - | - | 0.482 | 213.1 | - | - |
| 2 | 2.0 | - | - | - | - | 0.455 | 200.9 | - | - |
| 3 | 4.0 | - | - | - | - | 0.419 | 184.6 | - | - |
| 4 | 6.0 | - | - | - | - | 0.385 | 169.2 | - | - |
| 5 | 9.0 | - | - | - | - | 0.350 | 153.4 | - | - |
| 6 | 12.0 | - | - | - | - | 0.325 | 142.1 | - | - |
| 7 | 15.0 | - | - | - | - | 0.297 | 129.4 | - | - |
| 8 | 18.0 | - | - | - | - | 0.285 | 124.0 | - | - |
| 9 | 22.0 | - | - | - | - | 0.279 | 121.3 | - | - |