CHARACTERISATION AND UTILISATION OF MICROBES IN THE PRODUCTION OF FISH SAUCE AND PASTE

BEATRIX LUBBE

Thesis presented in partial fulfillment for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE

In the Department of Food Science, Faculty of Agricultural and Forestry Sciences
University of Stellenbosch

Study Leader: Prof. T.J. Britz
Co-study Leader: Mr. D.S. Basson

December 2000

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

_____

Beatrix Lubbe

ABSTRACT

Fermented fish products are popular food products mainly consumed and produced in Southeast Asia. These products are not produced in South Africa, and those available to the public are imported. The main action during the production and fermentation of this sort of product, is that of proteolysis, either by the bacteria or enzymes naturally present in the fish.

The prevalent microbes present in six fermented fish samples from Bangkok (Thailand) and seven from Khon Kaen (Thailand), were determined, and using numerical methods, clustered into similarity groups using the calculated dendrogram distance (D_D) technique to determine their relation to reference strains. Forty-seven different bacterial strains were isolated, but no yeasts, moulds or lactic acid bacteria were found. Five Gram-negative, oxidase-positive species, five different *Staphylococcus* species and nine different endospore-forming species of the genus *Bacillus,* were isolated and identified using the API systems. The data indicated that members of the genus *Bacillus* were the prevalent organisms in all the products examined.

The isolates were also scanned for general enzyme activity using the API Zym technology, and the production of proteases was investigated using the Standard Methods Caseinate and the Universal Protease Substrate methods. It was found that most of the isolated organisms produced protease, which is of major importance in the production of fermented fish products.

Proteolytic cultures from the fermented fish products, as well as lactic acid starters, were used in the production of a fermented fresh water fish product. Production parameters including: glucose, inoculum, moisture content and incubation time, were evaluated in order to select optimum fermentation conditions. Fermentation efficiency was determined by measuring the final pH, titratable acid and the free amino nitrogen content. Optimum efficiency was obtained with 5% (w/w) added glucose at a moisture level of 150 ml water per 100 g fish.

A factorial design (3 x 3 x 3) was used to indicate viable trends to facilitate optimisation of the fermentation process. The main effects, two-factor and three-factor interactions were calculated. Optimum trends obtained were a

glucose concentration of 5% (w/w), inoculum concentration of $1x10^8$ cfu.ml^{-1}, an incubation period of 15 days and temperature of 30°C. Three lactic acid starters (226 - *Lactobacillus plantarum,* 140 - *Lactococcus diacetylactis* and 407 - *Pediococcus cerevisiae)* were selected as they produced some of the best fermentation results and are safe to use in food. It was found that a combination of all three strains (226, 140 and 407) yielded the best results.

By using the above parameters, an acceptable product was produced in terms of consistency, colour and aroma. Further studies need to be conducted to optimise the safety of the product as well as the flavour, both chemically and sensorically optimisation of the product.

UITTREKSEL

Gefermenteerde visprodukte is populêre voedselprodukte in die lande van Suidoos-Asië. Die produkte word nie in Suid-Afrika geproduseer nie, maar slegs ingevoer. Die hoof aksie tydens die fermentasie proses is proteolise deur die bakterieë en ensieme wat natuurlik teenwoordig is in vis.

Die oorheersende mikrobes teenwoordig in ses gefermenteerde vis produkte van Bangkok (Thailand) en sewe van Khon Kaen (Thailand), is bepaal. Numeriese metodes is gevolg om die isolate in groepe te sorteer en te groepeer deur gebruik te maak van die berekende dendrogram afstand (D_D) tegniek om hul verwantskap ten opsigte van die verwysingsorganisme te bepaal. Sewe-en-veertig verskillende bakterieë is geïsoleer, maar geen fungi of melksuurbakterieë is geïdentifiseer nie. Vyf Gram-negatiewe, oksidase-positiewe spesies, vyf verskillende *Staphylococcus* spesies en nege verskillende endospoorvormende spesies van die genus *Bacillus,* is geisoleer en geïdentifiseer deur gebruik te maak van die API CHB sisteme. Die data het getoon dat lede van die genus *Bacillus* die oorheersende organismes was.

Die isolate is daarna ondersoek vir algehele ensiemaktiwiteit deur van die API Zym tegnologie gebruik te maak. Daar is veral klem gelê op die protease aktiwiteit en dit is gemeet deur van die "Standard Methods Caseinate Agar" metode asook die "Universal Protease Substrate" metodes gebruik te maak. Daar is gevind dat die oorgrote meerderheid organismes proteolitiese ensieme produseer wat belangrik is in die produksie van gefermenteerde visprodukte.

Kulture wat geïsoleer is uit gefermenteerde visprodukte asook melkssuurkulture is gebruik vir die produksie van 'n gefermenteerde varswater visproduk. Produksieparameters insluitende: glukose-, inokulum- en voginhoud asook inkubasie tyd is ondersoek om die optimum fermentasie kondisies te bepaal. Optimum effektiwiteit is gevind by 'n 5% glukose konsentrasie en vogvlakke van 150 ml water per 100 g vis.

'n Faktoriale ontwerp (3 x 3 x 3) is gebruik om die optimum kondisies te bepaal. Die hoof effekte asook die twee faktor en drie faktor interaksies is bereken. Optimum neigings is gevind by 'n glukose konsentrasie van 5%,

inokulum konsentrasie van $1x10^8$ kve.ml^{-1}, 'n inkubasie tydperk van 15 dae en temperatuur van 30°C. Drie melksuurbakterieë (226 - *Lactobacillus plantarum*, 140 - *Lactococcus diacetylactis* en 407 - *Pediococcus cerevisiae)* is gekies aangesien hulle die beste resultate gelewer het en veilig vir gebruik in voedselprodukte is. Daar is gevind dat die drie melksuurkulture saam in kombinasie die beste fermentasie resultate opgelewer het.

Deur gebruik te maak van die bogenoemde fermentasie kondisies, kon 'n aanvaarbare produk, in terme van kleur en geur, gelewer word. Verdere studies moet gedoen word om die veiligheid asook die geur, chemies asook sensories, te optimiseer.

dedicated to my parents

CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

ACKNOWLEDGEMENTS

My sincere gratitude to the following for their invaluable contributions:

Prof. T.J. Britz of the Department of Food Science, University of Stellenbosch, for his invaluable guidance and enthusiasm as my Study Leader;

Mr. D.S. Basson of the Department of Food Science, University of Stellenbosch, for his advice and input as my Co-study Leader;

Mr. D. Brink, Mr. R. de Wit and their co-workers from the Aquaculture Research Unit, University of Stellenbosch, for the supply of literature as well as trout needed for this study;

Professor J.J. Joubert of the Department of Medical Microbiology, University of Stellenbosch, for the supply of fermented fish samples from Thailand;

Mr. G.O. Sigge, Ms. L. Maas, Ms. M. Reeves, Ms. C. van Schalkwyk and Mr. E. Brooks of the Department of Food Science, University of Stellenbosch, for technical support;

The National Research Foundation (NRF) and the University of Stellenbosch for financial support;

My fellow post-graduate students as well as the staff of the Department of Food Science for their support and friendship;

My parents, family and close friends, especially Annemarie, Jaco, Marisa, Riet and Rikus, for their endless encouragement and motivation throughout my studies; and

My Heavenly Father for giving me courage and strength.

CHAPTER 1

INTRODUCTION

Fermented food is defined as "palatable products which are prepared from raw or heated materials and which acquire their characteristic properties by a process in which microorganisms are involved" (Buckenhüskes, 1993; Hammes, 1990). Fermentation is not only a preservation method, but also improves the taste, aroma, visual appearance, texture, consistency, shelf-life and safety of the raw materials (Buckenhüskes, 1993). Some of the health benefits associated with fermented products include: anticancer activity; improvement of intestinal motility; lowering of serum cholesterol; and benefits to lactose intolerant individuals (Alm, 1982; Friend & Shahani, 1983; Mann, 1977; Naidu, 1999). Fermented food is certainly of great value to the human in providing healthy, long-life products.

Various traditional fermented foods are produced and consumed in the South Eastern countries of Asia. In Thailand alone, more than 20 different kinds of fermented foods including fish, meat, vegetables and fruit are produced (Tanasupawat, 1993a and b). Since most of the food consumed in these regions are bland and starchy products such as rice, fermented fish and meat products they do provide a rich source of protein and amino acids to the diet of the population (Tanasupawat & Komagata, 1995).

Fermented fish sauces and pastes, in particular, are rich in flavour and aroma and strongly improve the taste of rice and cereal meals. Adding to the above advantages, these products are cheap and are, therefore, popular among the whole of the population (Toole, 1998). Considering the above, it can be concluded that fish sauces and pastes have the potential to become popular products in South Africa as well. These products can be used to provide a cheap, healthy and tasty food source to the mass market stopping problems such as malnutrition and famine. No literature on the manufacture of fermented fish products in South Africa has been found and the market for these products should be exploited.

Trout is one of the most popular fresh water fish species in South Africa. The market for this fish is currently growing exponentially and trout is sold as a

fresh or smoked product. It has been estimated that approximately 8% of trout is of substandard quality and the main cause for this is faulty colour and texture caused by gaping and not by spoilage (Brink, 1999). Such trout, however, are still suitable for human consumption and could possibly be used for the production of a processed fermented product rather than being thrown away. This simple method may provide a solution in South Africa to avoid the loss of a useful protein source. Fermented products include fish sauces and pastes and are produced by the action of microbial and natural fish proteases.

The aim of this study was to firstly isolate and characterise the proteolytic active microbes present in fish sauces and pastes from Thailand. This was done by examining the morphological as well as the metabolic characteristics of each isolate. Secondly, these or other suitable isolates were used in the production of a trout based fish sauce and paste. The optimisation of this method was also examined.

References

Alm, L. (1982). Effect of fermentation of lactose, glucose, and galactose content in milk and suitability of fermented milk products for lactose intolerant individuals. *Journal of Dairy Science,* **65**, 346-352.

Brink, D. (1999). The Department of Genetics, University of Stellenbosch, Personal communication.

Buckenhüskes, H.J. (1993). Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. *FEMS Microbiology Reviews*, **12**, 253-272.

Friend, B.A. & Shahani, K.M. (1983). Antitumor properties of lactobacilli and dairy products fermented by lactobacilli. *Journal of Food Protection,* **47**, 717-723.

Hammes, W.P. (1990). Bacterial starter cultures in food production. *Food Biotechnology,* **4**, 383-397.

Mann, G.V. (1977). A factor in yoghurt which lowers cholesterolemia in man. *Atherosclerosis,* **26**, 335-340.

Naidu, A.S., Bidlack, W.R. & Clemens, R.A. (1999). Probiotic spectra of lactic acid bacteria (LAB). *Critical Reviews in Food Science and Nutrition,* **38** (1), 13-126.

Tanasupawat, S., Okada, S., Kozaki, M & Komagata, K. (1993a). Characterization of *Pediococcus pentosaceus* and *Pediococcus acidilactici* strains and replacement of the type strain of *P. acidilactici* with the proposed neotype DSM 20284, request for an opinion. *International Journal of Systematic Bacteriology,* **43**, 860-863.

Tanasupawat, S., Okada, S., Suzuki, K., Kozaki, M & Komagata, K. (1993b). Lactic acid bacteria , particularly heterofermentative lactobacilli, found in fermented foods in Thailand. *Bulletin of the Japan Federation of Culture Collection,* **9**, 65-78.

Tanasupawat, S. & Komagata, K. (1995). Lactic acid bacteria in fermented foods in Thailand. *World Journal of Microbiology and Biotechnology,* **11**, 253-256.

Toole, A. (1998). Traditional fermented foods. *Food Info*, **6**, 2-3.

CHAPTER 2

LITERATURE REVIEW

A. South African fresh water fish industry

The total world production of fish and shellfish amounted to 23.5 million tons in 1997 (Brink, 1997). Africa and South Africa's contribution to this total were respectively 0.34% (w/w) and 0.019% (w/w). Despite this small contribution, the South African aquaculture industry is rapidly growing and in 1997 generated revenue in excess of R71 million and employed 2 200 people. The aquaculture production values for the period 1993 to 1997, are shown in Table 1 (Brink, 1997).

Trout was imported to South Africa for the first time during 1896 mainly for angling purposes, but the value of trout as a food source was only recognised in 1970. Today, the annual trout production is about 1 200 tons and is mostly produced at local trout farms. Approximately 600 tons of this total production is sold as plate-size trout and consumed freshly. Smoked filleted salmon contributes 300 tons of the total and the rest is sold as live fish for angling purposes (Brink, 1999).

No trout has been exported from South Africa up to now. The main reason for this is the low production level as well as the long transport distances from the market areas. The production of trout in South Africa is also very low in comparison to other trout producing countries. Trout is, however, gaining popularity in the tourism sector, catering business and among the health conscious as it is considered a high quality "table fish" and a large variety of products can be made from this fish (Brink, 1999).

Trout production has been relatively constant except for a low production figure in 1995. This was ascribed to the high summer temperatures and poor rainfall during that specific year. There are still several possibilities for the expansion of the trout farming industry in southern Africa by developing new trout cage cultures. South African trout-based sport fishing is also growing exponentially and therefore, trout fingerling production is necessary for restocking purposes (Brink, 1997). South Africa is a producer of eyed ova and in 1997 exported approximately 50 million ova to the UK and Denmark (Brink, 1997).

Table 1. South African aquaculture production values for the period 1993 to 1997 (Brink, 1997).

Species	Production in metric tons					Production value (x R 000)
	1993	1994	1995	1996	1997	1997
Rainbow trout	1 020	1 020	770	790	1 050	14 175
Catfish	50	50	10	20	35	280
Ornamental	1.0 m*	1.5 m*	2.0 m*	3.5 m*	5.3 m*	20 000
Tilapia	60	60	25	15	20	200
Carp	22	22	30	12	35	280
Other fish	5	4	10	12	15	150
Crocodiles	800**	1 500**	2 500**	5 000**	8 700**	8 700
Freshwater crayfish	1	2	1	2	1	70
Waterhawthorn	300	300	310	320	85	850
Mussels	2 345	2 700	2 256	2 088	2 500	11 250
Oysters	480	520	339	339	450	9 765
Abalone	1	2	1	7	10	2 000
Prawn	36	51	86	86	70	3 080
Seaweed	-	-	4	8	10	200
Total tonnage and ex-farming value: 1997					4 281	71 000

* number of fish in millions, ** number of skins, R1 = 17 US cents

It has been estimated that 8% of the South African produced trout is of sub-standard quality. The major causes of this problem are, faulty colour and texture, mainly caused by gaping and not by spoilage (Brink, 1999). To prevent such losses, methods should be developed to stop gaping or to preserve and utilise these fish. Fermentation is one method that can be used to produce a valuable product that has a long shelf-life as well as certain health qualities. Such products could, therefore, be produced from otherwise unusable fish to generate an income and prevent wastage.

B. Fermented foods

Fermentation is one of the oldest and certainly one of the most economical methods of producing and preserving foods (Battcock & Azam-Ali, 1998; Hull *et al.*, 1992). Various fermented foods of both plant and animal origin, make an essential part of the human diet in many parts of the world (Toole, 1998). Bread, cheese and wine are some of the indigenous fermented foods that have been produced and consumed for thousands of years and are strongly linked to the culture and tradition of especially village communities in rural households (Battcock & Azam-Ali, 1998). Fermented food probably originated in Asia where it is still extremely popular due to the outstanding organoleptic qualities. Taste, aroma, visual appearance, texture, shelf-life and product safety are the characteristics of fermented foods that distinguishes them from raw foods (Buckenhüskes, 1993).

Fermentation can be defined as "a process in which chemical changes are brought about in an organic substrate through the action of enzymes produced by microorganisms" (Jay, 1996). Fermentation is also a relatively efficient, low energy preservation technique that increases the food's shelf-life and decreases the need for refrigeration or other forms of preservation. This technique is, therefore, appropriate for use in developing countries and remote regions where there is a limited access to sophisticated equipment (Battcock & Azam-Ali, 1998).

The main reason for fermentation in the early days was to preserve food for later use when the raw product would not be available. Although efficient heat sterilising and refrigerating methods take the place of this preservation method today, fermentation is still used for the production and preservation of food even in

countries where there is an abundance of mass-produced food (Buckenhüskes, 1993; Hull *et al.*, 1992). Today, the large-scaled and controlled production of fermented products is possible due to the development of modern food processing technologies (Naidu *et al.*, 1999).

During fermentation bacteria, yeasts and fungi or combinations of these are used to metabolise carbohydrates and to produce products such as alcohol, antimicrobial agents and organic acids which contribute to the preservative action of fermented food. Fermented foods are mostly prepared from raw or heated materials using microorganisms to provide the necessary characteristics required in the final product (Buckenhüskes, 1993). Fermentation does not only involve the production of preservative and antibiotic ingredients, organic acids, carbon dioxide and alcohol, but also results in desirable chemical and physical changes to the substrate. The metabolites that form during fermentation are important for the improvement of the flavour of the product as characteristic tastes and aromas can only be obtained by the fermentation process (Buckenhüskes, 1993; Pederson, 1971). Fermentation can also be used to produce: natural pigments that can act as food colourants; amino acids from carbon rich substrates; wine, beer and distilled alcoholic beverages; CO_2 by yeasts; essential vitamins such as vitamin B_{12}; and single cell proteins that are normally used as animal protein sources. Fermentation can, therefore, be seen as a natural method of food preservation rather than that of a chemical preservation (Pederson, 1971). The character of each type of fermented food is different and is determined by the quality and nature of the food, the changes that occur due to the action of different inherent enzymes, the changes that occur as a result of the microbial fermentation and the interactions between these products and the constituents of the food (Pederson, 1971).

Fermentation of raw food with microorganisms, especially bacteria, can also be associated with food contamination and spoilage. However, the fermentation process has more advantages than disadvantages for the food industry, with cheese and yoghurt as examples of the most popular and best-selling products produced during the fermentation of milk. Waste products from the paper, pulp and even petroleum industries, if they contain a suitable carbon source, can even be used as the growth medium for yeasts during the production of vital products (Pretorius, 1994). The fermentation process can improve food safety by eliminating harmful

toxic compounds and the prevention of growth of food spoilage and poisoning organisms (Battcock & Azam-Ali, 1998). Some of the disadvantages associated with fermentation are discolouration, hydrolysis of pectin bridges in fruit causing it to become soft, the production of aflatoxins and toxic amines leading to food poisoning (Gilliland, 1985; Jay, 1996; Nagodawithana, 1993).

Adding to all the various advantages of fermented foods are the positive health aspects associated with these products. Some health-related aspects of food fermented with specifically lactic acid bacteria include microbial interference, physiological effects, supplementary effects, immunomodulatory effects, anti-tumor effects as well as the clinical management of various disorders. Many intestinal disorders caused by disturbed intestinal conditions and increased gut permeability can be treated with probiotic lactic acid bacteria that can colonise the intestine and survive gastric conditions. Some physiological effects in the human body improved by lactic acid bacteria include the absorption of nutrients, improvement of lactose utilisation (if milk is a substrate), improvement of intestinal motility, increased digestibility, reduction of serum cholesterol levels in the blood, anti-diabetic effects and management of osteoporosis (Gilliland, 1989; Naidu, 1999; Oberman & Libudzisz, 1998). The nutritional value of fermented foods is elevated by the supplementation of the B-complex vitamins in lactic fermented foods (Naidu, 1999; Oberman & Libudzisz, 1998). Some disorders that may be relieved in the presence of lactic acid bacteria are diarrhoea, diaper dermatitis and even bowel disorders (Naidu, 1999). Lactic acid bacteria can also be used in the production of high quality fermented fish products in order to increase their nutritional and sales value.

C. Fermented fish products

Origin and characteristics

Fermented fish and crustacean products are popular staple foods and condiments in many oriental countries where they contribute to a significant portion of the dietary protein consumed (Avery, 1952; Crisan & Sands, 1975; Sanceda *et al.*, 1990; Toole, 1998). In Northern Europe, these products are mainly consumed as a condiment (Jay, 1996) in contrast with the people of Southeast-Asia where it is

mainly used to add variety and nutrition to the otherwise bland starchy diets (Beddows, 1998).

Fermented foods that are traditionally consumed in the Asian diet include fermented fish sauces and pastes, and fermented soy products such as soy sauce and miso. More than 20 different fermented products are available in Thailand and include fermented fish, meat, vegetables and fruit (Tanasupawat *et al.*, 1993a and b). The production of these products originated in China probably about 3000 years ago and spreaded to Japan and the rest of the Asian countries (Naidu *et al.*, 1999). Lactic acid bacteria are mainly responsible for the souring and ripening of these products (Tanasupawat *et al.*, 1993a and b).

In the Philippines, the monsoon season causes the fish to be seasonal in the different areas of the islands. In order to distribute the fish throughout the whole island, fish must be transported to all the remote areas. Due to inadequate transportation and refrigeration facilities, it is difficult to always supply a good product. Ice and refrigeration are very expensive and, therefore, not used frequently. Furthermore, very little canning is done because of the high cost and scarcity of cans. As a result of all these limiting factors, more inexpensive methods must be used to preserve fish. In the Asian countries, fishermen usually catch any fish, regardless of size, maturity or type. The larger fish are usually used fresh and the smaller fish are processed by either drying, salting, smoking, steaming, pickling or fermenting the fish (Avery, 1952). The fish used to prepare fermented fish products are usually less than 160 mm long and are caught inshore by small fishing vessels using purse seines. In these cases, spoilage will begin before the fish reach the shore or processing plant, mainly due to poor hygienic conditions as a result of the absence of refrigeration facilities (Saisithi *et al.*, 1966).

The traditional production method for the production of fish sauces and pastes are unhygienic and no care is normally taken to obtain good hygiene or control parameters. The fish is usually bottled and kept at room or warmer temperatures, for example in the vicinity of a fireplace and the temperature can, therefore, range from 30 to 50°C. Many consumers are, however, reluctant to buy a product produced under unhygienic conditions in fear of the health implications (Ijong & Ohta, 1996). However, the occurrence of food poisoning directly from fermented fish products is rare, but a lowering in salt concentration, in response to

consumer demand, may enhance the growth of pathogens or spoilage organisms and increase the risk of food infections and poisoning (Toole, 1998). The process, therefore, lacks quality control and would fail normal safety and quality standards such as those set for microbiological safety (Ijong & Ohta, 1996).

Fermented fish products are a valuable supplement to cereal food such as rice. It can supply up to 7.5% of an individual's total nitrogen intake and may serve as a rich source of various nutrients, vitamins and minerals. Examples of fermented fish products include Nuoc-nam, Nam-pla, Bu-du, Ka-pi, Tai-pla and Pla-ra. It has been reported that Nuoc-nam and Nam-pla can contain 27.5 - 28.9 g.100 ml^{-1} salt, 0.6 - 2.0 g N.100 ml^{-1} organic nitrogen and 0.2 - 0.7 g N.100 ml^{-1} ammonia nitrogen (Saisithi *et al.*, 1966). These products have pH values that range from 4.7 to 6.6 and sometimes even reach a pH of 8.0 and the total microbial counts can range from 1.8×10^2 to 8.9×10^7 cfu.g^{-1} (Tanasupawat & Komagata, 1995).

During fermentation, insoluble fish protein is mainly converted into a soluble form due to proteolysis. The main products of the fermentation action are amino acids and small polypeptides as well as ammonia and other low molecular mass nitrogenous compounds. As the fermentation process continues, the free amino acid and polypeptides content increases steadily up to 45 days whereafter it stabilises (Beddows, 1998).

No evidence of the manufacture of fermented fish products can be found in South Africa. Some fermented fish sauces are, however, imported on a small scale and sold in mainly the health shops. A study on the use of fermentation of fresh water fish, such as trout, could be of value in providing a low-cost, healthy product to aid in value adding and new product development.

Production and products

A flow sheet summarizing the basic method used for fish sauce and paste production is given in Fig. 1. The main preservative action in fermented fish is the large quantity of salt (10 - 20 % w/w) added during the processing phase. During the salting period, fish and salt are packed in layers or mixed thoroughly and left for a few months until the fermentation process is completed. The addition of salt initiates the dehydrating action, which reduces the moisture level and water activity to such a point where spoilage cannot take place. It is also known that salt, on its

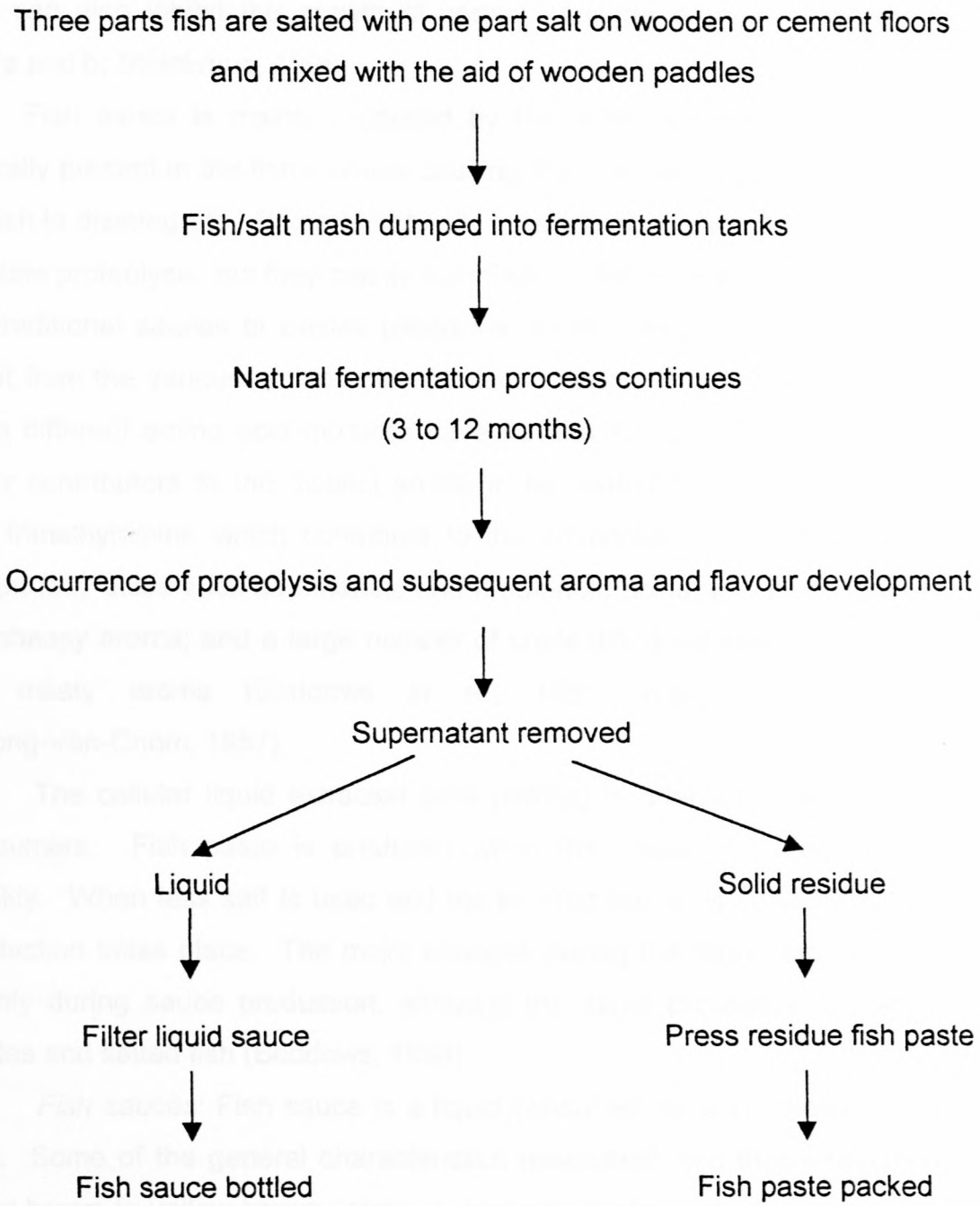


Figure 1. Flow sheet of the general method used to produce fermented fish sauce and paste (Baens-Arcega, 1977a and b; Steinkraus, 1996).

own, can also inhibit the growth of certain spoilage organisms (Baens-Arcega, 1977a and b; Steinkraus, 1996).

Fish sauce is mainly produced by the action of the proteolytic enzymes naturally present in the fish material causing the internal membranes and muscle of the fish to disintegrate. Bacteria naturally present on or in the fish material can also facilitate proteolysis, but they mainly contribute to the unique aromas and flavours of the traditional sauces or pastes (Beddows, 1998). The unique flavours can also result from the various amino acids produced during fermentation and proteolysis, since different amino acid mixtures have various flavours (Beddows, 1998). The major contributors to the distinct aroma in fermented fish products are: ammonia and trimethylamine which contribute to the ammoniacal character; low-molecular weight fatty acids such as ethanoic and n-butanoic acids, which are responsible for the cheesy aroma; and a large number of unidentified volatiles which contribute to the meaty aroma (Beddows *et al.*; 1980; Dougan & Howard, 1975; Truong-Van-Chom, 1957).

The cellular liquid extracted after pickling is often used as a sauce by the consumers. Fish paste is produced when the sauce is extracted regularly or quickly. When less salt is used and the pickling period is very short, "salted" fish production takes place. The major changes during the fermentation of fish occurs mainly during sauce production, although the same processes are applicable to pastes and salted fish (Beddows, 1998).

Fish sauces: Fish sauce is a liquid consumed as a condiment together with rice. Some of the general characteristics associated with these sauces include a clear brown to yellow-brown colour, a very salty taste, a sharp, meaty aroma and a protein content of 9.6 to 15.2%. The production of fermented fish sauces date back to ancient times when the Romans consumed products by the name of "Garum and Alec" (Badham, 1854). Today, an old type of sauce known as Garos (Kelaiditis, 1949) is still made in Greece from fish liver. A list of various traditional fish sauces is given in Table 2.

Nouc-mam is one of the most popular sauces known and is generally consumed by a large proportion of the Vietnamese population and added to rice and other cereals to add taste to their usual bland diets. This sauce has a distinctive

Table 2. Traditional fermented fish sauces (Beddows, 1998).

Country of origin and product name	Fermentation process	Fermentation period
Japan		
Shottsuru	5:1 fish:salt + malted rice and koji (3:1)	6 months
Uwo-shoyu	5:1 fish:salt + malted rice and koji (3:1)	6 months
Ika-shoyu	5:1 fish:salt + malted rice and koji (3:1)	6 months
Korea		
Jeot-kal	4:1 fish:salt	6 months
Vietnam		
Nuoc-mam	3:1 to 3:2 fish:salt	4 - 12 months
Nuoc-mam-gau-ca	10:1 fish liver:salt	Boiled after 8 days
Thailand		
Nam-pla	5:1 fish:salt	5 - 12 months
Malaysia		
Budu	5:1 to 3:1 fish:salt + palm sugar + tamarind	3 - 12 months
Bakasang	5:2 fish:salt	3 - 12 months
Burma		
Ngapi	5:1 fish:salt	3 - 6 weeks
Philippines		
Patis	3:1 to 4:1 fish:salt	3 - 12 months
Indonesia		
Katjap-ikan	5:1 fish:salt	6 months
India/Pakistan		
Colombo-cure	6:1 gutted fish:salt + tamarind	12 months
Hong Kong		
-	4:1 fish:salt	3 - 12 months
Greece		
Garos	9:1 fish liver:salt	8 days
France		
Pissala	4:1 fish:salt	2 - 8 weeks
Anchovy	2:1 beheaded/gutted fish:salt	6 - 7 months

meaty and cheesy aroma, salty taste and has a clear brown colour (Beddows, 1998). Similar to Nouc-mam, Nam-pla is produced more commercially in Thailand. Some products, such as Budu (Malaysia) have additives such as tamarind and palm sugar which sweetens the sauce and darkens the colour (Beddows, 1998).

Fish pastes: Fish pastes are more commonly and widely produced than fish sauces and are consumed raw or cooked and eaten with rice and vegetables. Many of the pastes are the result of a fermentation process, but the processing period is shorter than that of fish sauces. Pastes are usually manufactured from larger fish as well as fish unsuitable for sauce production and generally has a pH of about 5.1 (Beddows, 1998). A large variety of fish pastes can be found and include: Bagoong, Tinabal and Balbakwa (Philippines); Par-hoc (Cambodia); Padec (Laos); Blachan or Bleachon and Trassi-udang (Malaysia); Sidal (Pakistan); and Shiokara (Japan) (Jay, 1996). The fish:salt ratio in pastes ranges from 3:1 to 4:1. Different additives including spices, flavourants and colourants can also be added to pastes to improve the taste and appearance. Microorganisms probably assist more in tissue breakdown than with sauce production and are mainly involved in flavour and aroma development (Beddows, 1998). Some of the flavour compounds found in fish pastes are volatile fatty acids (formic, ethanoic, propanoic, isobutanoic and *n*-pentanoic acids), ammonia, trimethylamine and mono-and dimethylamines (Ooshira & Koreeda, 1960; Teshima *et al.*, 1967).

Fermented rice and shrimp/fish and salted fish: "Balao-balao" is a traditional food of the Philippines and is a cooked rice and shrimp product which is fermented for 7 to 10 days in a 20% (w/v) salt brine. "Burong isda" is a similar fermented product, but the shrimp is substituted with fish. Other fish products associated with rice are better known as Naresushi or Funasushi.

Biochemical changes and the hydrolysis of proteins

During sauce and paste production, many changes take place and these changes have to be studied if the process is to be optimised. The same changes do not occur with the manufacturing of all the products as fish species vary and the microorganisms involved in the fermentation process may differ according to their environment. The most important phase during production is the conversion of

insoluble fish protein into a soluble form, which is then called the fish sauce. The agents responsible for this conversion are enzymes present either on or in the fish, or produced by naturally occurring microorganisms. The main products that form during proteolysis are amino acids and polypeptides which all contribute significantly to the flavour of the product. Low molecular nitrogen products as well as ammonia can also be produced as by-products (Beddows, 1998). During Nuoc-mam and Patis fermentation over 120 days, the total nitrogen increases steadily up to 2.38% of which the organic nitrogen (including soluble protein, polypeptides, amino acids and ammonia) is 2%. Approximately 84% of the total nitrogen was organic. Of the total nitrogen, 49% was present as free amino acids, 14% as polypeptides and 17% as ammonia (Uyenco *et al.*, 1953). Similar results with Budu were obtained by Beddows *et al.* (1979) who reported that 78% of the total nitrogen was organic.

During fermentation, extensive tissue degradation takes place mainly due to the action of the proteolytic and lipolytic enzymes from the muscle and viceral tissue. The bacterial action involved in the fermentation process of fish is minimal and involves the conversion of any carbohydrates to lactic acid. This was proved by Beddows *et al.* (1979) who used antibiotics to inhibit the growth of microorganisms during the fermentation process. In their study, no microbial growth occurred and the level of nitrogenous products was not altered.

Aroma and flavour

Microorganisms indigenous to the fish and those tolerant to the high salt concentration in the curing brine, can contribute to the aroma and flavour of fermented fish products. As the degradation of the fish tissue continues, low molecular weight compounds are released which also contribute to the flavour and aroma of the sauces (Beddows, 1998).

Compounds involved in flavour formation are not as well characterised as those involved in aroma and many aroma compounds can contribute to form a specific flavour. The aroma of Nam-pla is classified into three major groups or notes (Dougan & Howard, 1975): i) ammoniacal notes formed by an excess of ammonia and trimethylamine (Beddows *et al.*, 1979; Uyenco *et al.*, 1953); ii) cheesy notes due to the low molecular weight fatty acids present in all sauces (Abe &

Tsuyuki, 1969; Beddows *et al.,* 1980); and iii) a meaty aroma which is formed by a large number of volatiles and has not yet been attributed to a specific compound (Dougan & Howard, 1975).

Some of the volatiles found in Nuoc-nam include ethanol, acetone, 2-propanol, butanone, butanol, propanoic acid, butanoic acid and acetic acid (Tanaka & Shoji, 1994). These aroma compounds are the result of the combined action of various bacteria and cannot as yet be ascribed to a specific species. In contrast, the only volatiles found in sauces produced under sterile conditions are ammonia and trimethylamine (Beddows, 1998).

The taste of fermented fish products is mainly salty but volatile compounds as well as different amino acids, nucleotides and biogenic amines can also contribute to form specific flavours. The concentration of amino acids and peptides, which is determined by the action of proteolytic enzymes, mainly influences the flavour of the product (Beddows, 1998).

D. Bacterial populations

Microbes naturally present in fermented fish products: Several bacterial species have been isolated from fermented fish products. They include bacteria from the genera *Bacillus, Enterobacter*, *Moraxella*, *Pseudomonas*, *Lactobacillus*, *Staphylococcus*, *Micrococcus*, *Streptococcus* and *Pediococcus*. Species of *Bacillus,* which has been reported to be the predominant genus, includes *Bac. cereus*, *Bac. circulans*, *Bac. licheniformis*, *Bac. megaterium*, *Bac. pumilus* and *Bac. subtilus*. The second most numerous isolates were species from the genera *Micrococcus* and *Pediococcus*. A few yeasts have also been isolated and include members of the species *Debaromyces hansenii* and *Hansenula anomala* var. *anomala.* These isolates are believed to be present either due to unhygienic production methods or to their natural presence on the raw fish product (Crisan & Sands, 1975; Ijong & Ohta, 1996).

Most of the bacteria found in fermented fish products, such as those species from the genus *Bacillus,* are aerobic endospore-forming halophiles, and are more abundant at salt concentrations of 10% than at lower concentrations. Their optimum salt concentration is 20% and the optimum pH where they can survive is 6.5 - 7.5.

They also appear to grow better in a more humid atmosphere (75% humidity) and survive by obtaining their nutrients from the fish proteins and/or fish extractives (Saisithi *et al.*, 1966). Bacteria from the genus *Bacillus,* especially *Bac. anthracis* and *Bac. cereus,* are generally known as pathogens to the human and should not be present in food related products. Infections caused by members of the genus *Bacillus* include eye infections by *Bac. thuringiensis* (Samples & Buettner, 1983) and fatal meningitis by *Bac. sphaericus* (Allen & Wilkenson, 1969). Since high ambient temperatures and the pH of the media may favour undesirable microorganisms, high salt concentrations (30 - 33%) are essential during the initial stages of the fermentation process to ensure the inhibition of these organisms (Toole, 1998).

Microbes generally used for fermentations: Lactic acid bacteria have traditionally been used for the production of fermented foods. This began as a natural process in products such as milk, where the nutrients available, and environmental conditions selected for the growth of these particular microorganisms (Campbell-Platt, 1994). Lactic acid bacteria have advantages that make them very useful and functional in fermented food production. One of these is the production of probiotics by *Lactobacillus bulgaricus*, *Lactococcus lactis*, *Lc. salivarius*, *Streptococcus thermophilus*, *Enterococcus faecium*, *E. feacalis*, and *Bifidobacterium* species. (Naidu *et al.*, 1999). These bacteria as well as various other lactic acid bacteria could possibly be used in the production of fermented fish products to improve their value and taste. This implies the use of fermentation for the development of a product that is unknown in a country such as South Africa.

E. Discussion

The development of a new product holds a risk for many companies. Studies have shown that the population of the USA are reluctant to change their eating habits while favourite foods of ten years ago are still favourite foods today. People still prefer soft drinks, hamburgers, pizza and fries. These habits are nevertheless changing and people now demand a larger variety of foods that are healthy and cost-effective to prepare (Hollingsworth, 1996). People are also looking for food that have characteristics such as innovative ingredients, nutritional value, sensory qualities, convenience and value for money (Anonymous, 1995).

Catering for the mass market in South Africa has been underexploited during the past few years. This mass market is very poor and cannot afford expensive food (Hughson, 1993). They buy food with low prices and low standards of quality and then usually do not get all the essential nutrients from their diet (McGill, 1988). A cheap, healthy product is the answer for this market (Hughson, 1993). The development and production of a South African product such as fermented fish sauce or paste offers exciting opportunities in for new product development. This is certainly a product that would be very popular throughout the population due to its cost-effectiveness, nutritional value and sensory qualities.

Fermented fish pastes and sauces could possibly lead to the development of new products that have a very characteristic flavour and aroma as well as helping to prevent fish product losses due to spoilage and wastage.

F. References

Abe, T. & Tsuyuki, H. (1969). Studies on the organic acids in 'shottsuru' (fish sauce). *Journal of Food Science and Technology, Japan.* **16**(12), 560-565.

Allen, B.T. & Wilkenson, H.A. (1969). A case of meningitis and generalized Schwartzman reaction caused by *Bacillus sphaericus. Johns Hopkins Medical Journal,* **1235**, 8-13.

Anonymous. (1995). With flying colours. *Food Review*, **22**(1), 17-19.

Avery, A.C. (1952). Preservation of very small fish in the Philippine Islands. *Food Technology*, **6**, 4-5.

Badham, C.D. (1854). In: *Prose Halieutics; or Ancient and Modern Fish Tattle.* P. 552. London: Parker and Sons.

Baens-Arcega, L. (1977a). Process of making bagoong in the Philippines. *Symposium on Indigenous Fermented Foods*, Bangkok, Thailand.

Baens-Arcega, L. (1977b). Patis, a traditional fermented fish sauce and condiment of the Philippines. *Symposium on Indigenous Fermented Foods*, Bangkok, Thailand.

Battcock, M. & Azam-Ali, S. (1998). Opportunities for fermented food products in developing countries. *Food Chain,* **23**, 3-4.

Beddows, C.G. (1998). Fermented fish and fish products. In: *Microbiology of Fermented Foods, Volume 1*, 2nd ed. (Edited by B.J.B. Wood) Pp. 417-429. London: Elsevier Applied Science Publishers.

Beddows, C.G., Ardeshir, A.G. & Wan Johari bin Daud, B. (1979). Biochemical changes occurring during the manufacture of Budu. *Journal of the Science of Food and Agriculture,* **30**, 1097-1103.

Beddows, C.G., Ardeshir, A.G. & Wan Johari bin Daud, B. (1980). Development and origin of the volatile fatty acids in Budu. *Journal of the Science of Food and Agriculture,* **31**, 86-92.

Brink, D. (1997). *Fourth Congress of the South African Aquaculture Association*, Stellenbosch, September 1997.

Brink, D. (1999). Department of Genetics, University of Stellenbosch, Personal communication.

Buckenhüskes, H.J. (1993). Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. *FEMS Microbiology Reviews*, **12**, 253-272.

Campbell-Platt, G. (1994). Fermented foods - a world perspective. *Food Research International*, **27,** 253-257.

Crisan, E.V. & Sands, A. (1975). Microflora of four fermented fish sauces. *Applied Microbiology*, **29**(1), 106-108.

Dougan, J. & Howard, G. (1975). Some flavouring constituents of fermented fish sauces. *Journal of the Science of Food and Agriculture,* **26**, 887-894.

Gilliland, S.E. (1985). *Bacterial Starter Cultures for Foods*. Boca Raton, Florida: CRC Press.

Gilliland, S.E. (1989). Acidophilus milk products: a review of potential benefits to consumers. *Journal of Dairy Science,* **72**, 2483-2494.

Hollingsworth, P. (1996). Food trends: diversity and choice dominate. *Food Technology,* **50**(5), 40.

Hughson, L. (1993). A worthwhile risk. *Food Review*, **20**(3), 32-33.

Hull, R.R., Conway, P.L. & Evans, A.J. (1992). Probiotic - a new opportunity. *Food Australia*, **44**, 112-113.

Ijong, F.G. & Ohta, Y. (1996). Physiochemical and microbiological changes associated with Bakasang processing - a traditional Indonesian fermented fish sauce. *Journal of the Science of Food and Agriculture*, **71**, 69-74.

Jay, J.M. (1996). Fermentation and fermented dairy products. In: *Modern Food Microbiology*, 5th ed., Pp. 131–145. New York: Chapman & Hall.

Kelaiditis, G.C. (1949). Technological research on salted sardine and anchovy produced in Greece. *Praktika of Hellenic Hydrobiological Institute,* **3**, 5.

McGill, A.E.J. (1988). Food processing: a consuming passion. *Productivity SA*, **14**(4), 9-11.

Nagodawithana, T. (1993). Yeast-derived flavours and flavour enhancers. *Food Industries of South Africa*, **46**(11), 9-15.

Naidu, A.S., Bidlack, W.R. & Clemens, R.A. (1999). Probiotic spectra of lactic acid bacteria (LAB). *Critical Reviews in Food Science and Nutrition,* **38** (1), 13-126.

Oberman, J.S.H. & Libudzisz, Z. (1998). Fermented Milks. In: *Microbiology of Fermented Foods, Volume 1,* 2nd ed. (Edited by B.J.B. Woods) Pp. 339-345. London: Elsevier Applied Science Publishers.

Ooshira, K. & Koreeda, H. (1960). Studies on 'katsuo-shiokara'. *Memoires of the Faculty of Fisheries, Kagoshima University,* **8**, 56.

Pederson, C.S. (1971). *Microbiology of Food Fermentations.* Westport, CT: AVI.

Pretorius, P.J. (1994). Mikrobiologie - die vakgebied op die voorpunt van navorsing en ontwikkeling. *Spectrum*, **32**(4), 61-64.

Saisithi, P., Kasemsarn, B-O., Liston, J & Dollar, A.M. (1966). Microbiology and chemistry of fermented fish. *Journal of Food Science*, **31**, 105-110.

Samples, J.R. & Buettner, H. (1983). Ocular infection caused by a biological insecticide (*Bacillus thuringiensis*). *Journal of Infective Diseases,* **148**, 614.

Sanceda, N.G., Kurata, T. & Arakawa, N. (1990). Overall quality and sensory acceptance of a lysine-fortified fish sauce. *Journal of Food Science*, **55**(4), 983-988.

Steinkraus, K.H. (1996). Fermented fish-shrimp sauces and pastes. In: *Handbook of Indigenous Fermented Foods.* (Edited by K. H. Steinkraus) Pp. 565-654. New York: Marcel Dekker Inc.

Tanaka, T. & Shoji, Z. (1994). Low-molecular-weight volatiles emitted from Vietnamese fish sauce (nuocmam). *Kyoritsu Joshi Daigaku Seikatsu Kagakuka Kiyo,* **37**, 1-4.

Tanasupawat, S., Okada, S., Kozaki, M. & Komagata, K. (1993a). Characterization of *Pediococcus pentosaceus* and *Pediococcus acidilactici* strains and replacement of the type strain of *P. acidilactici* with the proposed neotype DSM 20284, request for an opinion. *International Journal of Systematic Bacteriology,* **43**, 860-863.

Tanasupawat, S., Okada, S., Suzuki, K., Kozaki, M & Komagata, K. (1993b). Lactic acid bacteria, particularly heterofermentative lactobacilli, found in fermented foods in Thailand. *Bulletin of the Japan Federation for Culture Collection,* **9**, 65-78.

Tanasupawat, S. & Komagata, K. (1995). Lactic acid bacteria in fermented foods in Thailand. *World Journal of Microbiology and Biotechnology,* **11**, 253-256.

Teshima, S., Kanazawa, A & Kashiwada, K. (1967). Studies on volatile fatty acids and volatile bases in 'shiokara' II. Some changes on the process of ripening of Ika-shiokara. *Bulletin of the Japanese Society of Science of Fisheries,* **34**, 163.

Toole, A. (1998). Traditional fermented foods. *Food Info*, **6**, 2-3.

Truong-van-Chom. (1957). The determination of amino and ammonia nitrogen in Nuoc-mam. *Proceedings of the 9th Pacific Scientific Congress, Bangkok, Thailand,* **5**, 136-138.

Uyenco, V.I., Lawas, P.R., Briones, P.R. & Taruc, R.S. (1953). Mechanics of 'bagoong' (fish paste) and patis (fish sauce) processing. *Proceedings of the Indo-Pacific Fish Council,* **4**(II), 210-222.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF THE MICROBIAL POPULATIONS IN FERMENTED FISH PRODUCTS

Summary

The microbial populations present in six fermented fish samples from Bangkok (Thailand) and seven from Khon Kaen (Thailand) were enumerated, characterised, identified and using numerical methods, clustered into similarity groups. The fermented fish samples were serially diluted and enumerated using seven different media: Yeast Lactate Agar; MRS; Tomato Juice Broth Agar; Potato Dextrose Agar; Plate Count Agar; Violet Red Bile Agar; and Nutrient Agar, to determine the prevalent organisms present in the fermented products. The average bacterial count was found to vary from 51 to 492 000 cfu.g^{-1} of fermented fish sample. No yeasts, moulds or lactic acid bacteria were found and 47 different bacterial strains were isolated from the highest dilutions. These were taken as representatives of the prevalent populations. Each isolate was purified and identified using the API system. Five different *Staphylococcus* species and nine different endospore-forming species of the genus *Bacillus,* were isolated and identified using the API Staph and API CHB systems. Five Gram-negative, oxidase-positive species were also isolated and identified with the API 20 NE method. The data indicated that members of the genus *Bacillus* were the prevalent organisms. All the isolates were compared with known reference strains and then clustered into groups using the calculated dendrogram distance (D_D) technique to determine their relation to the reference strains.

Introduction

Fermented fish and crustacean products are popular condiments in many oriental countries where they contribute to a significant portion of the dietary protein consumed (Avery, 1952; Crisan & Sands, 1975; Sanceda *et al.*, 1990; Toole, 1998). These products are mainly used to add nutritional variety to the otherwise bland

starchy diets of the people in Southeast-Asia (Beddows, 1998). Small fish are usually preserved by fermentation while the larger size fish are eaten. The general method of fermentation includes combinations of drying, salting, smoking, steaming, pickling and fermentation (Avery, 1952).

Fish sauces are known by various names (Saisithi *et al.,* 1966). The general method used during preparation is to mix three parts fish or crustacean with one part salt and allowing the natural fermentation process to proceed for periods of a month to a year. The supernatant is then filtered and cured for about three months and then bottled (Crisan & Sands, 1975; Sanceda *et al.*, 1990). The final product is a clear dark-brown solution and has the required traditional characteristic flavour and aroma (Saisithi *et al.*, 1966). Fish pastes are also found in the Southeast Asian countries but the role of the fermenting microorganisms during the production of fish pastes, is minimal (Beddows, 1998). The microorganisms present during fermentation are mainly halophylic aerobic endospore-formers such as strains of the genus *Bacillus*. A few streptococci, micrococci and staphylococci may also be present (Jay, 1996; Toole, 1998) and *Pseudomonas* has also been reported in Korean fermented seafoods (Sands & Crisan, 1974).

The main fermentation action during the production of fish pastes and sauces is by enzyme action. During the degradation of the fish tissue, amino acids, polypeptides, ammonia and low molecular weight nitrogenous compounds are released which contribute to the flavour and aroma of the sauces. Bacteria contribute to the breakdown of proteins and lipids, which then strongly influence the unique flavour of each of the products. Since high ambient temperatures and the neutral pH of the fish substrates may favour undesirable microorganisms, high salt concentrations (30 - 33% w/v) are essential during the initial stages of the fermentation process to ensure the inhibition of spoilage and pathogenic organisms. The occurrence of food poisoning from fermented fish products is rare. However, a lowering in the salt concentration, in response to consumer demand, may enhance the growth of pathogens and spoilage organisms and also lead to an increase in food poisoning (Toole, 1998).

The aim of this study was to enumerate and then isolate, characterise and identify the prevalent microbial communities in different fermented fish products obtained from local markets in Bangkok and Khon Kaen, Thailand.

Materials and methods

Fermented fish sample preparation

Six samples (10 g each) of fermented fish from Bangkok (Thailand) and seven samples (10 g each) from Khon Kaen (Thailand), were examined (Table 1). The samples were obtained from each city's local market by Professor J.J. Joubert (Department of Medical Microbiology, University of Stellenbosch, personal communication, 1998).

The samples were stored at -18°C and were characterised according to colour and product type. A series of microbial dilutions of the fish samples were made in sterile saline and the dilutions plated out on the respective media. One ml of each of the diluted samples was inoculated in the various media by means of the pour plate technique (Harley & Prescott, 1993a). The plates were all incubated at 30°C for 24 - 48 h.

Isolation media

Seven media including: Yeast Lactate (YEL); MRS; Tomato Juice Broth Agar (TJBA); Potato Dextrose Agar (PDA); Plate Count Agar (PCA); Violet Red Bile Agar (VRBA); and Nutrient Agar (NA), were used for the enumeration and isolation of bacteria and yeast from the 13 fermented fish samples. YEL-medium was used for the isolation of lactate utilising bacteria and consisted of ($g.l^{-1}$): yeast extract, 5.0; sodium lactate (50%), 20.0; peptone, 2.0; KH_2PO_4, 10.0; Tween 80, 1 ml; and agar, 12.0 (Riedel & Britz, 1993). MRS-medium (Biolab) was used for the isolation of lactic acid bacteria and consisted of ($g.l^{-1}$): MRS powder 50.0; KH_2PO_4, 10.0; and agar, 12.0. PDA-medium (Biolab) was used for the isolation of yeast and moulds and consisted of ($g.l^{-1}$): potato extract, 4.0; dextrose, 20.0; and agar, 15.0. The PCA-medium (Biolab) was used for the aerobic plate counts and consisted of ($g.l^{-1}$): tryptone, 5.0; yeast extract, 2.5; dextrose, 1.0; and agar, 14.5. Nutrient agar (Biolab) was used for the selection of *Bacillus* species and consisted of ($g.\ l^{-1}$): meat extract, 1.0; yeast extract, 2.0; peptone, 5.0; NaCl, 8.0; and agar, 12.0. The TJBA (Difco) was used for the isolation of fastidious lactic acid bacteria, and consisted of ($g.l^{-1}$): tomato juice broth, 20.0; casein peptone, 10.0; cysteine hydrochloride, 0.5; ammonium citrate, 3.5; glucose, 5.0; Tween 80, 1 ml; and agar, 12.0 (Tracey &

Table 1. Characterisation of fermented fish samples obtained from Bangkok and Khon Kaen.

Source	Number	Colour	Product type
Bangkok	B1	Red-brown	Paste
Bangkok	B2	Pink-brown	Whole small fish
Bangkok	B3	Red-brown	Whole small fish
Bangkok	B4	Grey-brown	Thick sauce
Bangkok	B5	Light brown	Paste
Bangkok	B6	Light brown	Sauce
Khon Kaen	K1	Light brown	Sauce
Khon Kaen	K2	Light brown	Sauce
Khon Kaen	K3	Pink-brown	Paste
Khon Kaen	K4	Brown	Sauce
Khon Kaen	K5	Brown	Sauce
Khon Kaen	K6	Dark brown	Sauce
Khon Kaen	K7	Pink	Paste

Britz, 1987). The pH was set at 7.5 before sterilisation. VRBA (Biolab) was used for the isolation of coliforms and consisted of ($g.l^{-1}$): peptone, 7.0; yeast extract, 3.0; lactose, 10.0; bile salts no. 3, 1.5; NaCl, 5.0; neutral red, 0.03; crystal violet, 0.002; and agar, 12.0. All the media were prepared according to the suppliers' instructions and all, with the exception of the VRBA, were sterilised at 121°C and 100 kPa for 15 min.

Enumeration and selection of isolates

The number of colony forming units (cfu) were counted on the plates in the dilutions containing more than 30 and less than 300 colonies (Harley & Prescott, 1993b). Each different colony, at the highest dilution, was selected and streaked out on the specific media and incubated at 30°C for 24 h. After incubation, plates of each sample were visually compared to select plates containing different colonies. Each of the selected colonies was microscopically examined for purity before lypholisation using the method described by Joubert & Britz (1987).

Identification of the isolates

Gram and endospore stains were done on each purified isolate and the morphology was determined (Harley & Prescott, 1993c). The Gram-positive cocci were further identified using the API Staph system (API system S.A., La Balme le Grottes, 38390 Montalieu Vercieu, France). Oxidase-positive Gram-negative rods were identified using the API 20 NE system (API system S.A., La Balme le Grottes, 38390 Montalieu Vercieu, France). The following additional tests were done on the aerobic endospore forming strains (Gordon *et al.*, 1973): catalase; casein and starch hydrolysation; Voges-Proskauer; pH in Voges-Proskauer broth; growth in anaerobic agar; growth at 50° and 65°C; growth in 7% NaCl and growth at pH 5.7; utilisation of citrate; and acid and gas production from glucose.

The identity of the Gram-positive and Gram-negative rods was confirmed using Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986), identification Keys "1 and 2" described by Gordon *et al.* (1973) and the API 50 CHB and the API 20 E identification systems (API system S.A., La Balme le Grottes, 38390 Montalieu Vercieu, France).

Microlog software (Biolog Inc., USA) was used to facilitate clustering and

interpret the groupings of the bacteria identified with the API Staph, API 20 NE and API 50 CHB identification systems. The data sets consisted of the characteristics of the isolated bacteria as well as the characteristics of specific reference organisms (API system S.A., La Balme le Grottes, 38390 Montalieu Vercieu, France). One-dimensional plots were created on the basis of the calculated dendrogram distances (D_D) and the different isolates were clustered in relation to their closest relatives. Isolated organisms were compared to reference organisms obtained from the Food Science Culture Collection, in order to determine if they clustered in the same group.

Results and discussion

Characterisation of the fermented fish samples

The fish samples were characterised according to colour, aroma and product type, and the results summarised in Table 1. It was found that the predominant odour of all of the fish was that of a characteristic cheesy and meaty type which is a general characteristic of fermented fish products (Abe & Tsuyuki, 1969; Beddows *et al.*, 1980; Dougan & Howard, 1975). The colour of the samples varied from pink to reddish brown. Both fish sauces and pastes were included in the samples. It was observed that some of the sauces contained very small fish or pieces of fish.

Enumeration

The average viable microbial counts ($cfu.g^{-1}$) obtained from the triplicate plates with the highest dilution value of each medium type are shown in Table 2. Only plates containing 30 to 300 colonies were selected and counted. No colonies were formed on the VRBA, which was taken as an indication of the absence of coliforms or related enterobacterial organisms.

Problems encountered with the count and selection of the isolates included the growth of spreader organisms (Table 2), which covered the agar surface totally within a few hours and prevented the subsequent counting of colonies. In most cases these spreaders were found to be the only type of organism present on the specific plate. Some of the isolates clearly only grew as pinpoint colonies beneath the surface of the agar, which made them extremely difficult to isolate.

Table 2. Average viable bacterial counts ($cfu.g^{-1}$) of triplicate plates of the highest dilutions of the 13 different fermented fish samples cultivated in the various media.

Source	Media					
	NA	**PCA**	**YEL**	**TJBA**	**MRS**	**PDA**
B1	81 250	75 000	200 000	76 000	10 200	9 360
B2	*Spreader	35 500	520	256	18 000	3 080
B3	53	233	57	51	61	256
B4	322	400	292	324	1 920	970
B5	*Spreader	1 852	140	108	60	-
B6	550	402	414	360	452	*Spreader
K1	*Spreader	-	63	327	-	*Spreader
K2	1 020	1 440	1 700	757	-	430
K3	*Spreader	1 170	*Spreader	790	**Mould	1 160
K4	492 000	100 000	119 000	76 000	8 200	19 800
K5	9 000	16 800	6 860	120 000	2 660	3 400
K6	*Spreader	235	108	57	400	160
K7	*Spreader	80	328	285	405	394

*The evaluation of colonies on these plates was prevented by the growth of spreader organisms or

** moulds which completely covered the plates within a few hours.

NA = Nutrient Agar
PCA = Plate Count Agar
YEL = Yeast Extract Lactate Agar
TJBA = Tomato Juice Broth Agar
MRS = MRS Agar
PDA = Potato Dextrose Agar

The average cfu.g^{-1} counts, as shown in Table 2, ranged from 51 (TJBA-medium) to 492 000 (NA-medium). In most cases, the agar plates were overgrown with bacteria after only 24 hours. This phenomenon could be ascribed to the fast growing endospore forming bacilli from the genus *Bacillus*. These organisms grow extremely fast, resulting in the domination on the media and preventing slower growing organisms especially on the TJBA and MRS media.

According to the data summarised in Table 2, the highest enumeration value was obtained on the NA-medium and this was later correlated to the high number of aerobic endospore forming bacilli isolated from this medium type. Members of the genus *Bacillus* are also known to be spreaders explaining the high numbers of spreaders found on the NA-medium. It was found that the numerical numbers per medium varied from sample to sample. Some had low numbers for all the media (B3, B4, B5, B6, K1, K2, K6 and K7) while for others the numbers were high (B1, B2, K3, K4 and K5) but no specific correlation was found for sauces or pastes.

Identification of isolates

No yeasts or moulds were isolated from the PDA-medium that was used specifically for this purpose. One mould was, however, isolated from the MRS-medium. This was considered a contaminant, since it was only present on one of the triplicate plates.

Forty-seven Gram-positive and Gram-negative bacteria were isolated from the 13 fermented fish samples and the isolated strains were identified and grouped as follows:

Gram-positive cocci: Ten of the strains isolated from four of the products, were found to be Gram-positive and catalase-positive cocci. The comparison data and strain characteristics obtained using the API-Staph method are shown in Tables 3 and 4. The identification profile for this group was found to vary from an acceptable 75.7% for the *Staphylococcus simulans* strains to a very good identification of 97.8% for the *Sta. aureus* strain since the identification percentage in this case was high. The presence of *Staphylococcus* in the fish product was surprising as the presence of these organisms has, so far as can be accertained, only once been reported in the literature by Saisithi *et al.* (1966).

Some of the bacteria from the genus *Staphylococcus* are generally known to

Table 3. Identification results of the Gram-positive, catalase-positive staphylococci using the API Staph system.

Isolate number	Identification	Isolation media	Identification percentage and acceptability	
B2 1N	*Staphylococcus aureus*	NA	97.8 %	Very good
B2 1Y	*Sta. hominis*	YEL	83.5 %	Acceptable
B2 2Y	*Sta. simulans*	YEL	75.7 %	Acceptable
K2 3N	*Sta. simulans*	NA	75.7 %	Acceptable
K2 4N	*Sta. caprae*	NA	97.5 %	Very good
K2 1P	*Sta. schleiferi*	PCA	92.8 %	Good
K4 1P	*Sta. schleiferi*	PCA	96.0 %	Good
K5 2P	*Sta. schleiferi*	PCA	92.8 %	Good
K5 2T	*Sta. caprae*	TJBA	97.5 %	Very good
K5 2Y	*Sta. schleiferi*	YEL	96.0 %	Good
Sam 1	*Kocuria varians*	SMCA	97.4%	Very good
Sam 2	*Koc. varians*	SMCA	97.4%	Very good

Numbering: First two digits - sample number; third digit - isolate colony number; and fourth digit - isolation media (N-NA, P-PCA, Y-YEL, T-TJBA, and M-MRS)

Isolation media: SMCA - Standard methods caseinate agar (see Chapter 4)

Table 4. Characterisation of the staphylococci strains using the API Staph identification system.

Test	K2 4N	K5 2T	B2 1Y	B2 1N	K2 1P	K5 2P	K5 2Y	K4 1P	B2 2Y	K2 3N	Sam1	Sam2
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Endospores present	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
No substrate	-	-	-	-	-	-	-	-	-	-	-	-
Acidification of:												
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	-	-	-	+	+	+	-	-	-	-	-	-
Maltose	-	-	+	+	-	-	-	-	-	-	-	-
Lactose	-	-	-	+	?	?	-	-	+	+	-	-
D-trehalose	+	+	+	+	+	+	+	+	+	+	-	-
D-mannitol	-	-	-	+	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-
D-melibiose	-	-	-	-	-	-	-	-	-	-	-	-
Potasium nitrate	-240+	+	+	+	+	+	+	+	+	+	-	-
β-naphthyl-acid phosphate	+	+	+	+	+	+	+	+	+	+	+	+
Sodium pyruvate	+	+	+	+	+	+	+	+	+	+	-	-
Acidification of:											-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	+	+	-	-	-	-	+	+	-	-
α-methyl-D glucoside	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl-glucosamine	-	-	+	+	-	-	+	+	+	+	-	-
Arginine	+	+	+	+	+	+	+	+	+	+	-	-
Urea	-	-	+	+	-	-	-	-	+	+	-	-

be pathogens and under the correct conditions can cause human infections (http://vm.cfsan.fda.gov/~mow/chap3.html). *Staphylococcus aureus, Sta. hominis* and *Sta. simulans* are inhabitants of the human skin and may cause skin infections. *Staphylococcus aureus* is known to cause food poisoning and less than 1.0 µg of the *Staphylococcus* exotoxin produced can cause symptoms of nausea, vomiting, retching, abdominal cramping and extreme physical weakness (http://vm.cfsan.fda.gov/~mow/chap3.html). *Staphylococcus caprae* has previously been isolated from goats milk (Sneath *et al.*, 1986) where members of these species were able to survive in the presence of 10% (w/w) NaCl and found to mainly require amino acids as nitrogen and nutritional source. Fermented fish products would, therefore, be an ideal growth habitat for these organisms to flourish in and under the correct conditions, produce harmful enterotoxins (Miller & Fung, 1973).

Members of the genus *Staphylococcus* should not be present in food due to their pathogenic potential. Not more than 10 cfu.g^{-1} of *Sta. aureus* are allowed in any fish product according to the South African food legislation (Standards Act, 1993). The FDA & EPA guidance level for *Sta. aureus* in food products state that no enterotoxin or otherwise not more than 1 x 10^4 members of the genus *Staphylococcus* are allowed (FDA & EPA guidance levels, 1995). Contamination and poor hygienic conditions under which the fermented fish (pastes and sauces) products examined in this study were processed probably were the cause of the presence of this group in the fermented fish products.

Two additional strains (Table 4) that were Gram-positive cocci (Sam 1 and Sam 2) and originally isolated from fermented fresh trout (Mapstone, 1998), were also included in this group and compared with the staphylococci from the fermented products. However, they differed from the staphylococci strains and were identified as *Kocuria varians* at an identification percentage of 97.4%.

Gram-negative, oxidase-positive rods: The API 20 NE identification results and the characteristics of the eight Gram-negative, catalase-positive, oxidase-positive rod shaped isolates are shown in Tables 5 and 6. These organisms occurred in seven of the 13 products examined (Table 1). The identification of the *Stenotrophomonas maltophilia* isolates was found to be doubtful as only a low identification of 63.1% was obtained. The other identification results varied from acceptable (81.3%) to an excellent identification (99.9%) for the

Table 5. Identification of the Gram-negative, catalase-positive, oxidase-positive rods using the API 20 NE method.

Isolate number	Identification	Isolation media	Identification percentage and acceptability	
B3 3T	*Sphingomonas paucimobilis*	TJBA	81.3 %	Acceptable
B5 1M	*Stenotrophomonas maltophilia*	MRS	63.1 %	Bad
K1 3N	*Agrobacterium radiobacter*	NA	99.9%	Excellent
K1 4T	*Sphingomonas paucimobilis*	TJBA	81.3 %	Acceptable
K2 2N	*Sphingomonas paucimobilis*	NA	81.3 %	Acceptable
K3 1N	*Vibrio alginolyticus*	NA	87.1 %	Acceptable
K6 4P	*Chryseomonas luteola*	PCA	90.3 %	Good
K7 1Y	*Stenotrophomonas maltophilia*	YEL	63.1 %	Bad

Numbering: First two digits - sample number; third digit - isolate colony number; and fourth digit - isolation media (N-NA, P-PCA, Y-YEL, T-TJBA, and M-MRS)

Table 6. Characterisation of the non-enteric, Gram-negative rods using the API 20 NE identification system.

Test	K1 3N	B5 1M	K7 1Y	K3 1N	K2 2N	B3 3T	K1 4T	K6 4P
Gram stain	-	-	-	-	-	-	-	-
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Endospores present	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Potassium nitrate	+	+	+	+	-	-	-	+
Tryptophan	-	-	-	-	-	-	-	-
Glucose acidification	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-	-
Urea	+	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+
Gelatine	-	+	+	+	+	+	+	+
β-galactosidase	+	-	-	-	+	+	+	+
Assimilation of:								
Glucose	+	+	+	+	+	+	+	+
Arabinose	+	+	+	-	+	+	+	+
Mannose	+	+	+	+	-	-	-	+
Mannitol	+	+	+	+	+	+	+	+
N-acetyl-glucosamine	+	+	+	+	+	+	+	?
Maltose	+	+	+	+	+	+	+	+
Gluconate	+	-	-	-	+	+	+	+
Caprate	-	-	-	-	-	-	-	-
Adipate	-	-	-	-	-	-	-	-
Malate	+	+	+	+	+	+	+	+
Citrate	-	+	+	-	?	?	?	+
Phenyl-acetate	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+

Agrobacterium radiobacter strain.

Sphingomonas paucimobilis, Agr. radiobacter and *Steno. maltophilia* are known to be able to cause human infections and *Chryseomonas luteola* has been reported to cause food spoilage (Membre & Kubaczka, 1998). In contrast, *Vibrio alginolyticus* is a normal inhabitant of seawater but, when conditions are suitable, can cause wound and ear infections in humans (Twedt *et al.,* 1969).

No guidance levels for bacteria of this group are available in the South African Food legislation (Law on Standards, 1993). The FDA & EPA guidance level for pathogenic *Vibrio* species is, however, not more than 1 x 10^4 cfu.g^{-1} per food product (FDA & EPA guidance levels, 1995). The occurrence of these bacteria in fish may possibly either be natural or caused by bad hygienic conditions during or after processing. Since they may, however, be harmful to the human, food containing members of these species should not be consumed.

Gram-positive and negative, endospore-forming rods: A total of 61.7% of the isolates obtained during this study, were aerobic, endospore-forming rods from the genus *Bacillus.* The 29 strains were isolated from 11 of the 13 fish samples and identified using the API 50 CHB and API 20 E systems. The identification results and characteristics are shown in Tables 7, 8 and 9.

Two additional Gram-positive endospore forming strains (Sam 3 and Sam 4) that had been isolated from fermented trout (Mapstone, 1998) were included in the study in order to compare salt water fermented fish isolates with fresh water fish isolates. These two strains were identified as *Bacillus firmus* and *Brevibacillus brevis.*

The identified species of the isolates from the salt water fermented fish included members of the *Bac. cereus, Bac. mycoides, Bac. licheniformis, Bac. megaterium, Bac. pumilus, Bac. anthracis, Bac. amyloliquefaciens, Bac. coagulans, Bac. lentus, Bac. subtilus* and *Bac. circulans.* Identification of this group ranged from a very doubtful 43.2% for *Bac. coagulans* to an excellent identification (99.9%) for the *Bac. megaterium* and *Bac. cereus* isolates. The occurrence of bacteria from the genus *Bacillus* in fermented fish samples has also been reported by Crisan & Sands (1975), Sands & Crisan (1974) and Saisithi *et al.* (1966). In all these different studies, it was found that *Bacillus* was the prevalent organism in fermented fish samples.

Table 7. Identification results of the Gram-positive and negative endospore-forming rods using the API 50 CHB and API 20 E systems.

Isolate number	Identification	Isolation media	Identification percentage and acceptability	
B1 1Y	*Bacillus subtilus*	YEL	98.3 %	Very good
B1 2Y	*Bac. amyloliquefaciens*	YEL	49.8 %	Doubtful
B2 1P	*Bac. anthracis*	PCA	87.5 %	Acceptable
B2 4Y	*Bac. mycoides*	YEL	79.2 %	Acceptable
B3 1T	*Bac. cereus*	TJBA	99.9 %	Excellent
B3 2N	*Bac. mycoides*	NA	79.2 %	Acceptable
B3 2Y	*Bac. licheniformis*	YEL	99.5 %	Excellent
B4 1P	*Bac. coagulans*	PCA	43.2 %	Doubtful
B4 2P	*Bac. amyloliquefaciens*	PCA	49.8 %	Doubtful
B4 2T	*Bac. mycoides*	TJBA	79.2 %	Acceptable
B4 3T	*Bac. cereus*	TJBA	73.8 %	Acceptable
B4 4T	*Bac. megaterium*	TJBA	98.8 %	Very good
B4 3Y	*Bac. megaterium*	YEL	99.9 %	Excellent
B5 3T	*Bac. megaterium*	TJBA	98.8 %	Very good
B6 1Y	*Bac. cereus*	YEL	99.9 %	Excellent
B6 4Y	*Bac. cereus*	YEL	73.8 %	Acceptable
B6 1M	*Bac. cereus*	MRS	73.8 %	Acceptable
B6 2T	*Bac. cereus*	TJBA	73.8 %	Acceptable
K1 1P	*Bac. cereus*	PCA	54.4 %	Bad
K1 1M	*Bac. cereus*	MRS	62.4 %	Bad
K1 1T	*Bac. mycoides*	TJBA	79.2 %	Acceptable
K1 2T	*Bac. amyloliquefaciens*	TJBA	65.4 %	Bad
K3 2T	*Bac. mycoides*	TJBA	64.8 %	Bad
K5 1M	*Bac. cereus*	MRS	62.4 %	Bad
K6 1T	*Bac. mycoides*	TJBA	79.2 %	Acceptable
K6 2P	*Bac. amyloliquefaciens*	PCA	65.4 %	Bad
K6 3P	*Bac. mycoides*	PCA	79.2 %	Acceptable
K6 5P	*Bac. lentus*	PCA	82.9 %	Acceptable
K7 1T	*Bac. amyloliquefaciens*	TJBA	49.8 %	Doubtful
Sam 3	*Bac. firmus*	SMCA	99.6%	Excellent
Sam 4	*Brevibacillus brevis*	SMCA	99.8%	Excellent

Numbering: First two digits - sample number; third digit - isolate colony number; and fourth digit - isolation media (N-NA, P-PCA, Y-YEL, T-TJBA, and M-MRS)

Isolation media: SMCA - Standard methods caseinate agar (see Chapter 4)

Table 8. Results of tests performed on the Gram-positive and negative, endospore-forming rods (Gordon *et al.*, 1973).

Isolate	Catalase	VP	pH in VP >7	Anaerobic agar	50°C	65°C	Starch	7% NaCl	pH 5.7	Citrate	Casein	Acid in glucose
B1 1Y	+	+	-	-	+	-	+	+	+	+	+	+
B1 2Y	+	+	-	-	+	-	+	+	+	-	+	+
B2 1P	+	+	+	-	+	-	+	+	+	-	+	+
B2 4Y	+	+	-	-	+	-	+	+	+	+	+	+
B3 1T	+	-	-	+	+	-	+	+	+	+	+	+
B3 2N	+	+	-	-	+	-	+	+	+	+	+	+
B3 2Y	+	+	-	-	+	+	+	+	+	+	+	+
B4 1P	+	+	-	-	+	-	+	+	+	-	+	+
B4 2P	+	+	-	-	+	-	+	+	+	-	+	+
B4 2T	+	+	-	-	+	-	+	+	+	+	+	+
B4 3T	+	+	-	-	+	-	-	+	+	+	+	+
B4 4T	+	+	-	-	-	-	+	+	+	-	+	+
B4 3Y	+	+	-	-	-	-	+	+	+	-	+	+
B5 3T	+	+	-	-	-	-	+	+	+	-	+	+
B6 2T	+	+	-	-	+	-	-	+	+	+	+	+
B6 1Y	+	-	-	+	+	-	+	+	+	+	+	+
B6 4Y	+	+	-	-	+	-	-	+	+	+	+	+
B6 1M	+	+	-	-	+	-	-	+	+	+	+	+
K1 1P	+	+	-	-	+	-	-	+	+	-	+	+
K1 1M	+	+	-	-	+	-	+	+	+	-	+	+
K1 1T	+	+	-	-	+	-	+	+	+	+	+	+
K1 2T	+	+	-	-	+	-	+	+	+	-	+	+
K3 2T	+	+	-	-	+	-	+	+	+	-	+	+
K5 1M	+	+	-	-	+	-	+	+	+	-	+	+
K6 1T	+	+	-	-	+	-	+	+	+	+	+	+
K6 2P	+	+	-	-	+	-	+	+	+	-	+	+
K6 3P	+	+	-	-	+	-	+	+	+	+	+	+
K6 5P	+	+	-	-	+	-	+	+	+	-	-	+
K7 1T	+	+	-	-	+	-	+	+	+	-	+	+

Table 9. Characterisation of the various strains of the genus *Bacillus* using the API 50 CHB and API 20 E identification systems.

Test	B5 3T	B4 4T	B4 3Y	B1 1Y	B3 1T	K1 1P	B6 4Y	B6 1M	B4 3T	B6 2T	K1 1M	K5 1M	B3 2N	B2 4Y	K6 3P
Gram stain	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Endospores Present	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of:															
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	?	?	?	?	?	?	+	+	+	+	+
D-Xylose	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	?	?	-	+	+	-	-	-	-	-	-	-	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	?	?	+	+	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
α-Methyl – D- mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
N-acetyl glucosamine	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Arbutine	+	+	+	+	?	+	+	+	+	+	+	+	-	-	-
Esculine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicine	+	+	+	+	?	+	+	+	+	+	-	-	-	-	-

Table 9. (continued)

Test	B5 3T	B4 4T	B4 3Y	B1 1Y	B3 1T	K1 1P	B6 4Y	B6 1M	B4 3T	B6 2T	K1 1M	K5 1M	B3 2N	B2 4Y	K6 3P
Cellobiose	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Saccharose	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inuline	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Melezitose	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Amidon	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
Glycogene	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Turanose	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
2-ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ortho-nitro-β-D-galactopyranoside	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Arginine	-	-	-	+/-	+	-	-	-	-	-	+	+	+	+	+
Lysine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium citrate	-	-	-	+	+	-	+	+	+	+	-	-	+	+	+
Sodium thiosulphate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Tryptophane (Deaminase)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophane (Indole)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Kohn's gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+

Table 9. (continued)

Test	B4 2T	K6 1T	K1 1T	K3 2T	B2 1P	K6 2P	K1 2T	B1 2Y	K7 1T	B4 2P	K6 5P	B3 2Y	B4 1P	Sam3	Sam4
Gram stain	+	+	+	+	+	+	-	+	+	-	-	+	-		
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Endospores present	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of:															
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-
D-Xylose	-	-	-	-	-	+	+	-	-	-	-	?	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
D-Mannose	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-
Mannitol	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
Sorbitol	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-
α-Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-
N-acetyl glucosamine	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Amygdaline	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-
Arbutine	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-
Esculine	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Salicine	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-

Table 9. (continued)

Test	B4 2T	K6 1T	K1 1T	K3 2T	B2 1P	K6 2P	K1 2T	B1 2Y	K7 1T	B4 2P	K6 5P	B3 2Y	B4 1P	Sam3	Sam4
Cellobiose	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Lactose	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-
Melibiose	-	-	-	-	-	+	+	+	+	+	+	-	+	-	?
Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?
Inuline	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	+	+	+	+	+	-	-	+	-	-
Amidon	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
Glycogene	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	-	-	-	-	-	+	+	+	+	+	-	+	+	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2-ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ortho-nitro-β-D galactopyranoside	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Arginine	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
Lysine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium citrate	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-
Sodium thiosulphate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Tryptophane (Deaminase)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophane (Indole)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Kohn's gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Nitrate reduction	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-

One of the distinguishing characteristics of the genus *Bacillus,* is the production of endospores under aerobic conditions. These endospores enable this bacterial group to survive harsh environmental conditions such as a low pH and very high salt concentrations (Doi & McGloughlin, 1992). Bacteria from the genus *Bacillus* are indigenous to soil and some, *Bac. anthracis* and *Bac. cereus,* may occur in food where they cause spoilage or are extremely pathogenic to humans causing diseases such as anthrax and gastroenteritis (Sneath *et al.*, 1986).

The standard Food Legislation for fish products in South Africa contains no specific reference or specifications for the presence or level of members of the genus *Bacillus* (Law on Standards, 1993). The FDA & EPA guidance levels only state that the presence of any pathogen, like *Bac. cereus,* is considered as a potential health hazard (FDA & EPA guidance levels, 1995). Members of this genus are not expected to be found in food, but they may be present due to contamination or bad hygiene or even poor quality of the raw product. Their survival in fermented fish products can be validated by their tolerance to high concentrations of NaCl and a low pH (Sneath *et al.*, 1986).

Inter- and intra-relationships

The isolate characteristics used to facilitate the clustering of related bacteria and the subsequent plotting of one-dimensional dendrograms are summarised in Tables 4, 6, 8 and 9. The data of the additional tests recommended by Gordon *et al.* (1973) for the identification of members of the genus *Bacillus* are shown in Table 8 and were also taken into consideration in the clustering process of the *Bacillus* strains. The relationship positions of the major groups included known reference strains (Table 10) as well as the isolated bacteria, and are illustrated as dendrograms in Fig. 1, 2 and 3. The clustering groupings obtained are as follows:

Gram-positive cocci (Fig. 1): Four major groups were found for the Gram-positive cocci during the dendrogram analysis. The first group consisted of the fresh water fish *Kocuria* strains and their reference strain (S6). These isolates were clustered in a distance of two D_D from their reference strain showing the high similarity between them. This high similarity to a reference strain was also observed with *Sta. aureus,* which was clustered right next to its reference strain (S3).

Staphylococcus caprae and *Sta. schleiferi* were clustered together as a group

Table 10. Reference strains used during the dendrogram clustering process.

Dendrogram number	API Number	Strain
Gram-positive cocci	API Staph	
S1		*Staphylococcus caprae*
S2		*Sta. hominis*
S3		*Sta. aureus*
S4		*Sta. schleiferi*
S5		*Sta. hominis*
S6		*Kocuria varians*
Gram-negative non-enteric rods	API 20 NE	
NE1		*Agrobacterium radiobacter*
NE2		*Vibrio alginolyticus*
NE3		*Stenotrophomonal maltophilia*
NE4		*Sphingomonas paucimobilis*
NE5		*Chryseomonas luteola*
Gram-positive endospore-forming rods	API 50 CHB and API 20 E	
B1		*Bacillus megaterium (Strain A)*
B2		*Bac. megaterium (Strain B)*
B3		*Bac. subtilus*
B4		*Bac. cereus (Strain A)*
B5		*Bac. cereus (Strain B)*
B6		*Bac. mycoides*
B7		*Bac. anthracis*
B8		*Bac. amyloliquefaciens*
B9		*Bac. lentus*
B10		*Bac. licheniformis*
B11		*Bac. coagulans*
B12		*Bac. firmus*
B13		*Brevibacillus brevis*

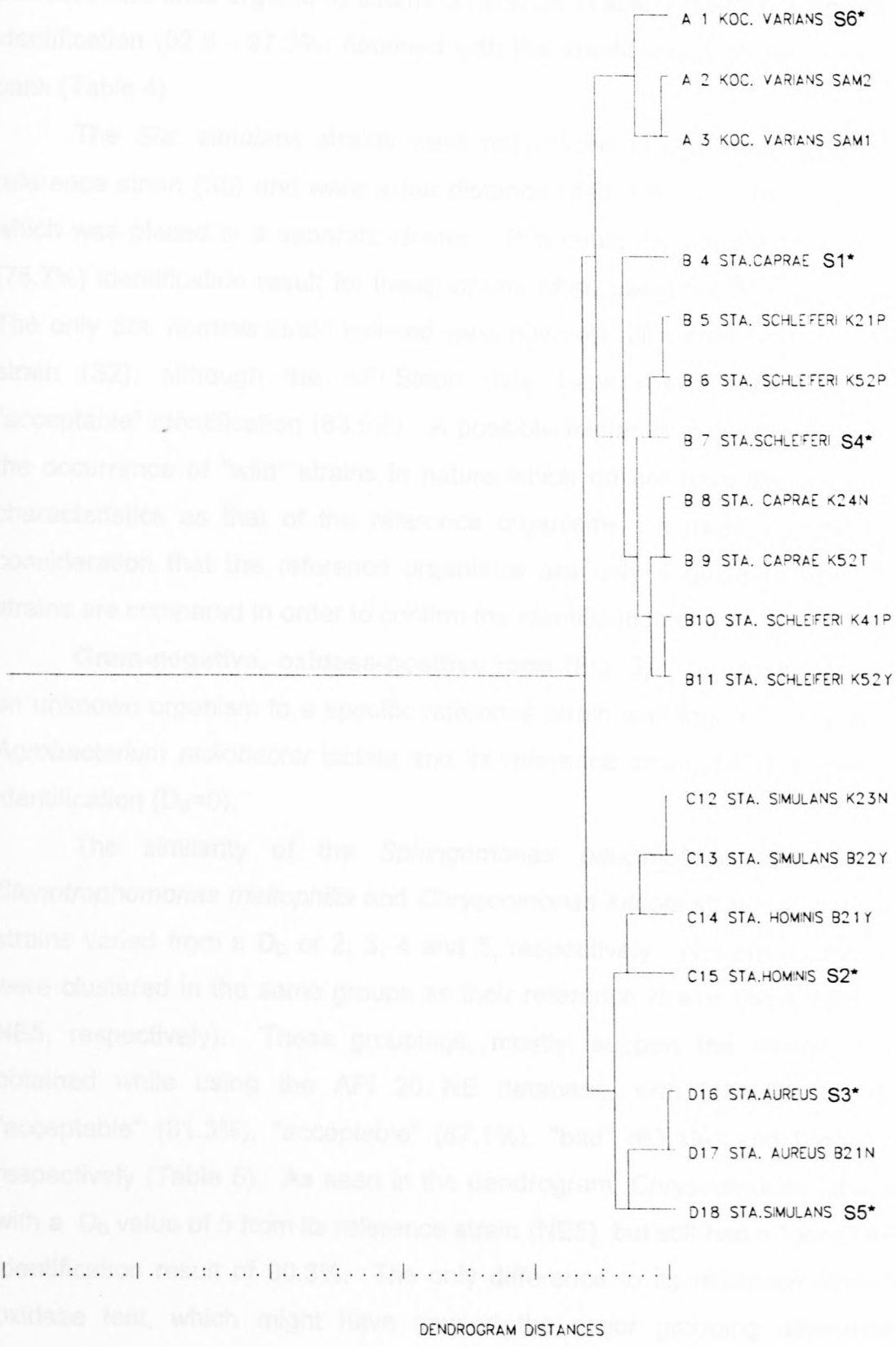


Figure 1. Dendrogram indicating the clustering of the Gram-positive cocci strains in relation to their *reference strains (Table 10).

Koc. = Kocuria, Sta. = Staphylococcus.

indicating a fairly close relationship. These isolates were also grouped near to the standard reference organisms strains S1 and S4 (Table 10) which supports the high identification (92.8 - 97.5%) obtained with the identification of the API-Staph data bank (Table 4).

The *Sta. simulans* strains were not placed in the same cluster as their reference strain (S5) and were a fair distance of four D_D from the reference strain which was placed in a separate cluster. This could explain the only "acceptable" (75.7%) identification result for these strains when using the API-Staph data bank. The only *Sta. hominis* strain isolated was, however, clustered next to its reference strain (S2), although the API-Staph data bank results also only gave an "acceptable" identification (83.5%). A possible explanation for this phenomenon is the occurrence of "wild" strains in nature which do not have the same metabolic characteristics as that of the reference organisms. It must also be taken into consideration that the reference organisms are only a guide to which unknown strains are compared in order to confirm the identification status.

Gram-negative, oxidase-positive rods (Fig. 2): The closest association of an unknown organism to a specific reference strain was found for this group. The *Agrobacterium radiobacter* isolate and its reference strain (NE1), showed a 100% identification (D_D=0).

The similarity of the *Sphingomonas paucimobilis, Vibrio alginoliticus, Stenotrophomonas maltophilia* and *Chryseomonas luteola* strains to their reference strains varied from a D_D of 2, 3, 4 and 5, respectively. Not one of these isolates were clustered in the same groups as their reference strains (NE4, NE2, NE3 and NE5, respectively). These groupings, mostly, support the identification status obtained while using the API 20 NE database, with identification results of "acceptable" (81.3%), "acceptable" (87.1%), "bad" (63.1%) and "good" (90.3%), respectively (Table 5). As seen in the dendrogram, *Chryseomonas luteola,* ended with a D_D value of 5 from its reference strain (NE5), but still had a "good" API 20 NE identification result of 90.3%. The only difference to its reference strain was the oxidase test, which might have caused the major grouping difference in the dendrogram (API 20 NE).

Gram-positive and negative, endospore-forming rods (Fig. 3): The isolates in this section and their reference strains were clustered into four major

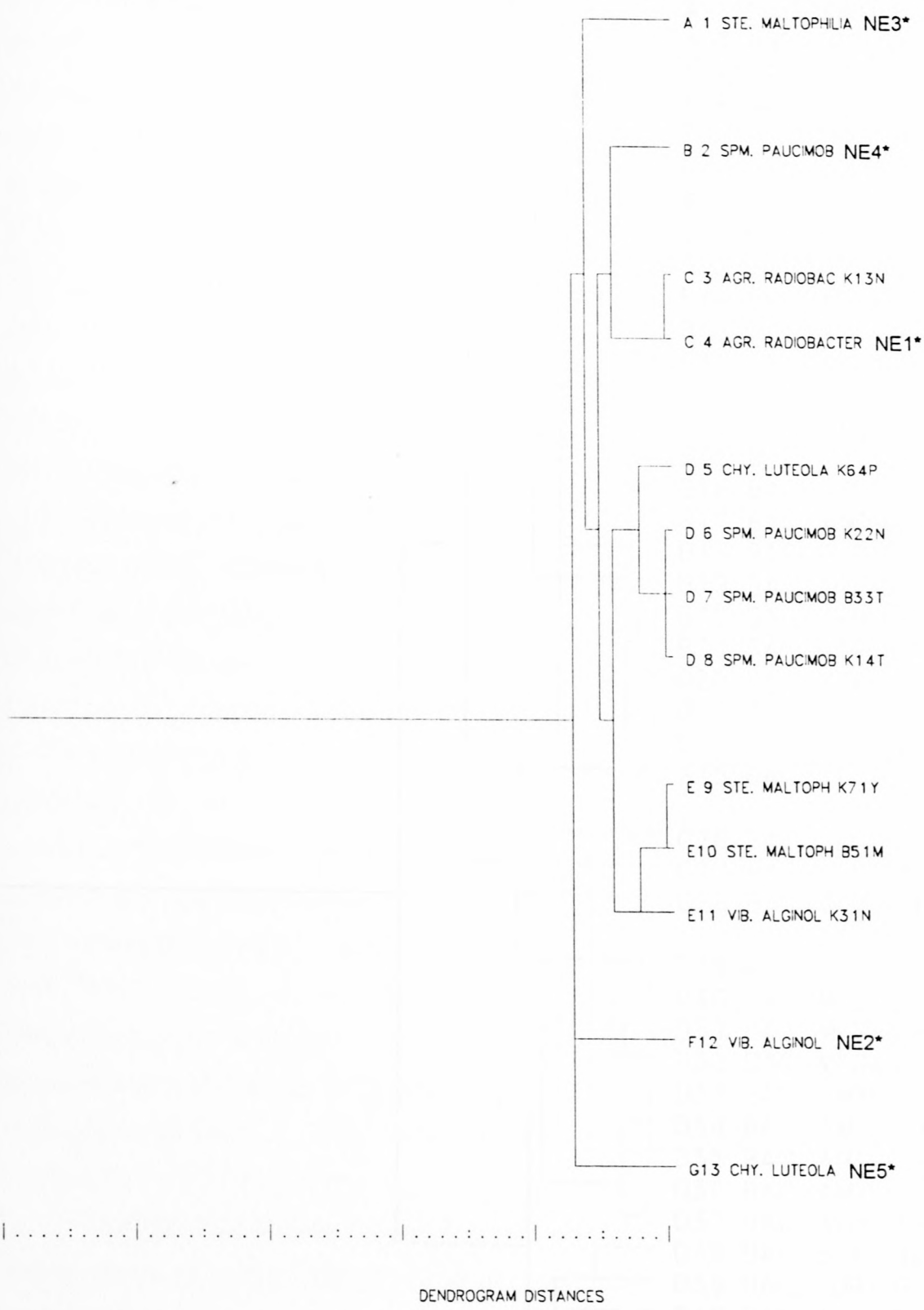


Figure 2. Dendrogram indicating the clustering of the Gram-negative non-enteric rods in relation to their *reference strains (Table 10).

Ste. = Stenotrophomonas, Spm. = Sphingomonas, Agr. = Agrobacterium, Chy. = Chryseomonas, Vib. = Vibrio alginoliticus

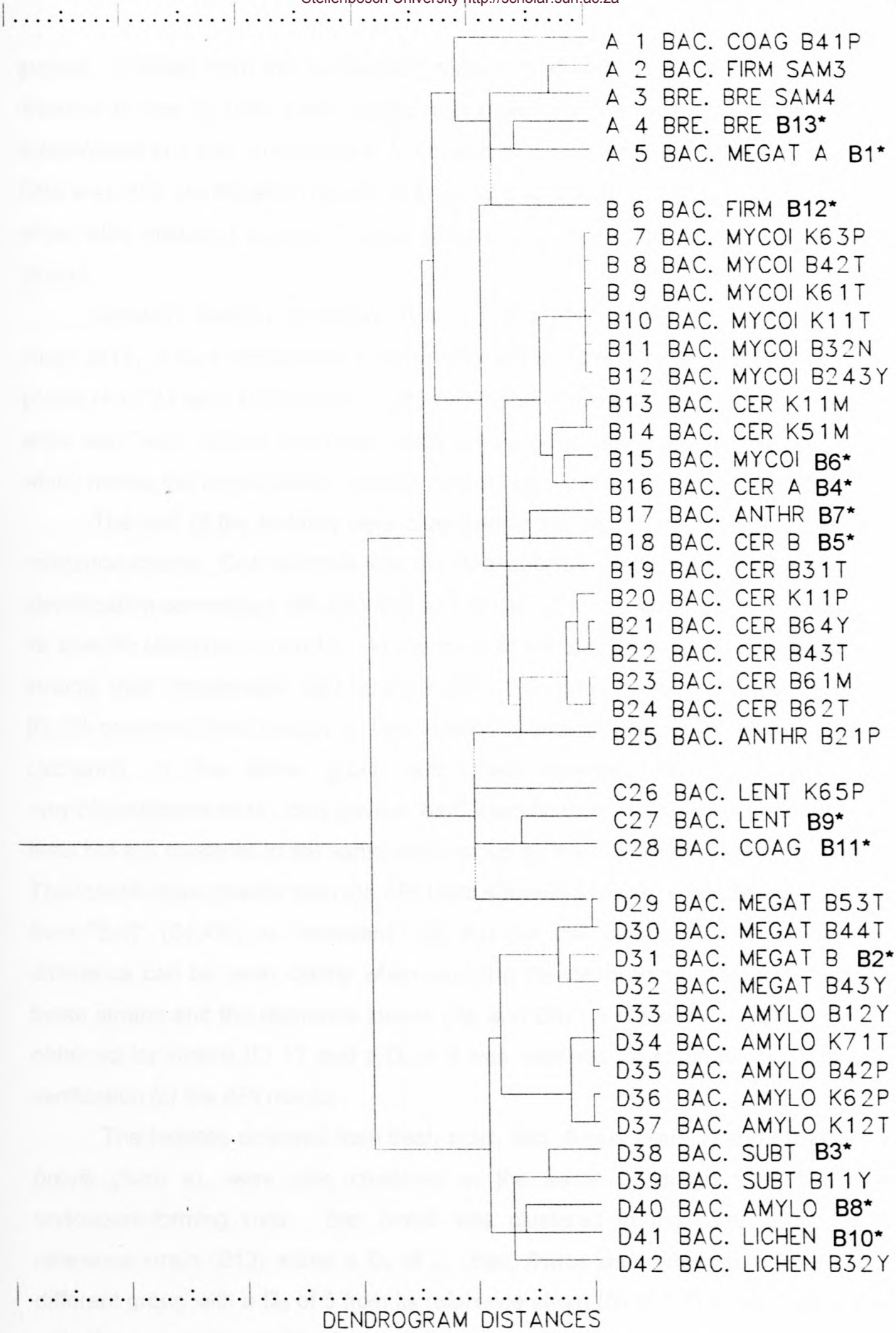


Figure 3. Dendrogram indicating the clustering of the Gram-positive cocci strains in relation to their *reference strains (Table 10).

Bac. - Bacillus, Bre. = Brevibacillus

groups. Isolates from the fermented products that were found at a dendrogram distance of one D_D from their specific reference strains were *Bac. anthracis, Bac. licheniformis* and *Bac. megaterium.* In support of the dendrogram distances, the API CHB and 20 E identification results of 87.5, 99.5 and 98.8%, respectively, were high which also indicated a close relation between the reference strains and isolated strains.

Although *Bacillus coagulans* (B4 1P) was only one D_D from its reference strain (B11), it was clustered in a different group to its reference strain. A doubtful profile (43.2%) was obtained during the API tests for this strain. This problem might arise with "wild" strains when they were compared to reference laboratory strains, which makes the identification process very difficult.

The rest of the isolates were clustered in the same groups as their specific reference strains. One example was the *Bac. subtilus* strain that had a "very good" identification percentage (98.3%) and was clustered in the same general group with its specific reference strain B3. In the case of the *Bac. mycoides* and *Bac. lentus* strains, their "acceptable" API 50 CHB and 20 E identification results of 79.2 and 82.9% confirmed their relation to their reference strains (B6 and B9) as well as their clustering in the same group with their reference strains. The *Bac. amyloliquefaciens* strain, only gave a "bad" identification profile (65.4%) with the API tests but still clustered in the same major group as their reference strain (B8).
The identification results from the API tests showed a range of percentages varying from "bad" (54.4%) to "excellent" (99.9%) for the *Bac. cereus* strains. This difference can be seen clearly when studying the dendrogram distances between these strains and the reference strains (B5 and B6). A close distance of 2 D_D was obtained for strains B3 1T and a D_D of 5 was obtained for strain K1 1P. This is a verification for the API results.

The isolates obtained from fresh trout, *Bac. firmus* (Sam 3) and *Brevibacillus brevis* (Sam 4), were also clustered in the same dendrogram as the other endospore-forming rods. *Bre. brevis* was clustered in the same group as its reference strain (B13) within a D_D of 2. *Bac. firmus* was, however, clustered in a different group with a D_D of 5 from its reference strain (B12). This does not comply with the excellent results obtained from the API tests (99.6%) and once again illustrates the differences between "wild" environmental isolates and reference

strains mostly from clinical sources as is used in the API data bank.

Conclusion

In this study, the microbial populations present in a range of fermented fish products obtained from Thailand were enumerated and the product colonies isolated and characterised. The data showed that the population consisted of a variety of members of the genera *Bacillus, Staphylococcus* as well as some non-enteric, oxidase positive rods (*Stenotrophomonas maltophilia, Chryseomonas luteola, Vibrio alginoliticus, Sphingomonas paucimobilis* and *Agrobacterium radiobacter*). The majority of the bacteria isolated from the thirteen different fermented fish samples were found to be members of the genus *Bacillus*. The occurrence of this wide range of organisms in the fermented fish products was not expected especially since some of them are of serious pathogenic nature. These, however, probably survived in these products due to the high tolerance to salt concentrations. Bacteria from the genera *Bacillus* as well as *Staphylococcus,* have previously been isolated from fermented fish products by Crisan & Sands (1975), Sands & Crisan (1974) and Saisithi *et al.* (1966). The pathogens isolated in these studies were *Sta. aureus, Bac. anthracis* and *Bac. cereus.*

The average viable bacterial count ($cfu.g^{-1}$) was mostly within the limits set for South African standards with a maximum of not more than 1×10^5 $cfu.g^{-1}$ (Standards Act, 1993). No coliforms were present, which eliminates the possibility of fecal contamination. It was, however, unexpected to find potential pathogens such as *Sta. hominis, Sta. aureus, Bac. cereus,* and *Ste. maltophilia* in the fermented fish. The occurrence of *Bac. anthracis* in one of the samples is of great concern since these bacteria produce a fatal toxin. The presence of these pathogens are most probably caused by post contamination or poor hygiene during processing and handling. A too low salt concentration in the product may also have had an influence on the survival of these isolates. It is obvious that the processing of fermented fish products in the Eastern countries is not always a controlled or very hygienic process.

Since the aim of this study was to isolate and characterise the microbial populations in fermented fish products with the possibility of using selected strains

in fish fermentations, it was expected that some of the isolates would be lactic acid bacteria. The isolates in this study have been isolated during the final stages of fermentation and lactic acid bacteria might have been present during the initial stages of fermentation and died of as a result of the low pH of fermented fish products.

The use of GRAS (generally regarded as safe) bacteria, such as lactic acid bacteria, should be considered to be used in the fermentation process to inhibit the growth of pathogens. To avoid spoilage or the occurrence of potential pathogens, the pH, salt concentration and storage temperature during processing need to be controlled. All these parameters need to be investigated and optimised to ensure the production of a safe and cost-effective product.

References

Abe, T. & Tsuyuki, H. (1969). Studies on the organic acids in 'shottsuru' (fish sauce). *Journal of Food Science and Technology, Japan.* **16**(12), 560-565.

Avery, A.C. (1952). Preservation of very small fish in the Philippine Islands. *Food Technology*, **6**, 4-5.

Beddows, C.G., Ardeshir, A.G. & Wan Johari bin Daud, B. (1980). Development and origin of the volatile fatty acids in Budu. *Journal of the Science of Food and Agriculture,* **31**, 86-92.

Beddows, C.G. (1998). Fermented fish and fish products. In: *Microbiology of Fermented Foods, Volume 1*, 2nd ed. (Edited by B.J.B. Wood) Pp. 417-429. London: Elsevier Applied Science Publishers.

Crisan, E.V. & Sands, A. (1975). Microflora of four fermented fish sauces. *Applied Microbiology*, **29**(1), 106-108.

Doi, R.H. & McGloughlin, M. (1992). What is a *Bacillus*? In: *Biology of Bacilli: Applications to Industry.* (Edited by R.H. Doi and M. McGloughlin) Pp. 1-21. Stoneham, MA: Butterworth-Heinemann.

Dougan, J. & Howard, G. (1975). Some flavouring constituents of fermented fish sauces. *Journal of the Science of Food and Agriculture,* **26**, 887-894.

FDA and EPA guidance levels (1995). In: *Fish and Fishery Products Hazard and Controls Guide*, 2nd ed. Regulation 21 CFR 123. Compliance Program 7303.842.

Gordon, R.E., Haynes, W.C. & Hor-Nay Pang, C. (1973). The Genus *Bacillus*. In: *Agricultural Handbook No. 427*. Pp. 97-98. Washington: Government Printing Office.

Harley, J.P. & Prescott, L.M. (1993a). Pour plate technique. In: *Laboratory Exercises in Microbiology*, 2nd ed. Pp. 30-31. Dubuque: Wm. C. Brown Publishers.

Harley, J.P. & Prescott, L.M. (1993b). Determination of Bacterial Numbers. In: *Laboratory Exercises in Microbiology*, 2nd ed. Pp. 40-43. Dubuque: Wm. C. Brown Publishers.

Harley, J.P. & Prescott, L.M. (1993c). Bacterial Morphology and Staining. In: *Laboratory Exercises in Microbiology*, 2nd ed. Pp. 47-69. Dubuque: Wm. C. Brown Publishers.

Jay, J. M. (1996). Fermentation and fermented dairy products. In: *Modern Food Microbiology*, 5th ed. (Edited by D.R. Heldman) Pp. 131–145. New York: Chapman & Hall.

Joubert, W.A. & Britz, T.J. (1987). A simple and inexpensive method for the long-term preservation of microbial cultures. *Journal of Microbiological Methods*, **7**, 73-76.

Law on Standards (1993). Voorgestelde verpligte spesifikasie vir bevrore vis, bevrore skulpdiere en bevrore produkte wat daaruit verkry word, No. 622. *Government Gazette,* **395**(18858), 283-369.

Membre, J-M. & Kubaczka, M. (1998). Degradation of pectic compounds during pasteurised vegetable juice spoilage by *Chryseomonas luteola*: a predictive microbiology approach. *International Journal of Food Microbiology*, **42**(3), 159-166.

Mapstone, S.J. (1998). Department of Food Science, University of Stellenbosch, Personal communication.

Miller, R.D. & Fung, D.Y.C. (1973). Amino acid requirements for the production of enterotoxin B by *Staphylococcus aureus* S-6 in a chemically defined medium. *Applied Microbiology,* **25**, 800-806.

Riedel, K.H.J. & Britz, T.J. (1993). *Propionibacterium* species diversity in aerobic digesters. *Biodiversity and Conservation*, **2**, 400-411.

Saisithi, P., Kasemsarn, B-O., Liston, J & Dollar, A.M. (1966). Microbiology and chemistry of fermented fish. *Journal of Food Science*, **31**, 105-110.

Sanceda, N.G., Kurata, T. & Arakawa, N. (1990). Overall quality and sensory acceptance of a lysine-fortified fish sauce. *Journal of Food Science*, **55**(4), 983-988.

Sands, A. & Crisan, E.V. (1974). Microflora of fermented Korean seafoods. *Journal of Food Science*, **39**, 1002-1005.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. (1986). Endospore-forming Gram-positive rods and cocci. In: *Bergey's Manual of Systematic Bacteriology Volume 2*, 1st ed. (Edited by P.H.A. Sneath) Pp. 1104-1208. Baltimore: Williams & Wilkins.

Toole, A. (1998). Traditional fermented foods. *Food Info*, **6**, 2-3.

Tracey, R.P. & Britz, T.J. (1987). A numerical taxonomic study of *Leuconostoc oenos* strains from wine. *Journal of Applied Bacteriology*, **63**, 523-532.

Twedt, R.M., Spaulding, P.L. & Hall, H.E. (1969). Morphological, cultural, biochemical, and serological comparison of Japanese strains of *Vibrio paraemolyticus* with related cultures isolated in the United States. *Journal of Bacteriology,* **98**, 511-518.

CHAPTER 4

DETERMINATION OF THE ENZYMATIC ACTIVITY OF BACTERIA ISOLATED FROM FERMENTED FISH PRODUCTS

Summary

The cultures isolated from the fermented fish products were scanned for general enzyme activity using the API Zym technology. The production of proteases as well as protease activity of the isolates were investigated using the Standard Methods Caseinate Agar method and the Universal Protease Substrate method. It was found that most of the isolated organisms produced the enzyme protease, which is important in the production of fermented fish products. Results of the two methods used to detect protease activity could be compared. The Universal Protease Substrate method seems to be more accurate in indicating the presence of proteases, since it gives a direct measurement of the amount of protease present by measuring the activity.

Introduction

Fermented fish sauces and pastes are products indigenous to the countries of Southeast-Asia and contribute to a large portion of the dietary protein consumption (Sanceda *et al.*, 1990; Toole, 1998). The basis for the production of fermented fish products is the indirect use of enzymes, specifically proteases, which can break down proteins into amino acids and other nitrogen containing products (Steinkraus, 1996). The excessive protease activity occurring naturally in fish causes the rapid softening of the fish muscle (Ashie *et al.*, 1996), which results in the liquid phase containing free amino acids. Some of these proteases include cathepsins, alkaline proteases, trypsin-like and digestive enzymes and other fish muscle proteases (Jiang *et al.*, 1992; Linder *et al.*, 1988; Makinodan *et al.*, 1985; Yamashita & Konagaya, 1992).

Proteases indigenous to fish are mainly active during the fermentation process. There is also the possibility that enzymes derived from microorganisms

present in or on the fish play a role in the hydrolysis of the substrate leading to the production of low molecular weight compounds, which could contribute to the characteristic flavour and aroma of the product (Steinkraus, 1996; Toole, 1998). The source of these microorganisms is probably the fish itself, but can also be from the added salt or even from the environment. Since many bacteria produce proteases and amino acid decarboxilases, they could possibly be used in the production process to assist in the proteolysis process, enhancing the degradation of the proteins and subsequent production of free amino acids. If the natural fermentation process continues for very long periods, from six months to a year, these amino acids could produce amines, which are usually abundant in fermented fish products (Boeker & Snell, 1972; Fardiaz & Markakis, 1979). The addition of proteolytic bacteria can, therefore, be used to speed-up the fermentation process and produce amines in a shorter time period leading to a faster production rate and possibly a superior product.

The aim of this study was to examine the protease activity of bacteria isolated from fermented salt fish samples and from fresh water trout.

Materials and methods

Test isolates

The 33 bacteria strains used in this study (Table 1) were isolated from fermented fish products (Chapter 3 of this thesis) and were cultivated on Plate Count Agar (PCA) (Merck) at 35°C for 24 h and then examined for enzymatic activity. Plate Count Agar (PCA) was used as growth media as all the strains grew well on it. Four proteolytic cultures, isolated from fresh trout, were also included in this study (Mapstone, 1998). These isolates were identified as: *Kocuria varians* strain Sam 1; *Kocuria varians* strain Sam 2; *Bacillus firmus* strain Sam 3; and *Brevibacillus brevis* strain Sam 4.

Proteolytic activity - Standard Methods Caseinate Agar method

Standard Methods Caseinate Agar (SMC-agar) was used to examine all the test isolates (Table 1) for proteolytic activity according to the method described by Martley *et al.* (1970). The SMC-agar was prepared by making a sodium citrate

solution (4.41 $g.l^{-1}$) and dividing it into two 500 ml volumes. The reagents added to the first 500 ml solution were (g): pancreatic digest of casein, 5; yeast extract, 2.5; glucose, 1; and agar, 15. Sodium caseinate (10 g) was added to the second 500 ml sodium citrate solution and the two 500 ml volumes were then combined and sterilised at 121°C and 100 kPa for 15 min. A 20 ml volume of sterile $CaCl_2.6H_2O$ (219.1 $g.l^{-1}$) (w/v) was added to the SMC-agar and stirred gently to prevent bubble formation. Active cultures of the test isolates were spotted onto SMC-agar plates and the plates incubated at 30°C for 24 - 47 h. Bacterial colonies surrounded by a clear zone were taken as an indication of casein breakdown due to proteolysis and were identified as proteolytic bacteria.

Proteolytic activity - quantitative method

The proteolytic activity of the bacterial isolates was also determined using the Universal Protease Substrate method (Boehringer Mannheim). This method is based on the detection of free resorufin-labelled peptides released due to proteolytic activity on resorufin-labelled casein. These peptides can be detected spectrophotometrically as they cannot be precipitated by trichloroacetic acid. The concentration of resorufin-labelled peptides is thus equivalent to the proteolytic activity (Boehringer Mannheim). This method was used to determine the quantity of proteases produced by the various isolates and to compare these results with those obtained with the SMC-agar method.

The following reagents were prepared: i) Substrate Solution - casein, resorufin-labelled (4 $g.l^{-1}$) in redistilled water; ii) Incubation Buffer - 0.2 M Tris-HCl, pH 7.8, 0.02 M $CaCl_2$; iii) Sample Solution - Standard Methods Caseinate Broth (SMC-broth) inoculated with specific bacterium; iv) Stop Reagent - trichloroacetic acid (50 $g.l^{-1}$) in redistilled water; v) Assay Buffer - 0.5 M Tris-HCl, pH 8.8; and vi) Blank Solution - sterile SMC-broth.

The assay procedure was carried out by preparing a sample blank with 50 µl Substrate Solution, 50 µl Incubation Buffer and 100 µl Blank Solution. Each test sample consisted of 50 µl Substrate Solution, 50 µl Incubation Buffer and 100 µl Sample Solution. The blank as well as the test samples were incubated at 30°C for 24 h.

After incubation, 480 µl of the Stop Reagent was added to each solution causing the precipitation of all the resorufin-labelled casein and the released peptides to stay in solution. The solution was then incubated at 37°C for 10 min, centrifuged for 5 min at 10 000 rpm and 400 µl of the supernatant, containing the released peptides, pipetted into 1 ml cuvettes. A volume of 600 µl of the Assay Buffer was added to each cuvette. The absorbance of each sample was read immediately against the blank at room temperature and a wavelength of 574 nm on a Spectronic 20 Genesys spectrophotometer.

Detection of the general enzymatic activity - API Zym method

In order to identify the proteolytic and lipolytic enzymes produced by the various isolates, the enzymatic activities of each was examined using the API Zym method (API system S.A., La Balme le Grottes, 38390 Montalieu Vercieu, France). This semi-quantitive micromethod allows the systematic and rapid study of 19 enzymatic reactions using very small sample quantities. The different enzymes produced by each strain inoculated in the microtubes, were read by observing the colour changes after 4 h. The intensity of each colour was taken as an indication of the activity of each of the enzymes present and was measured by comparing it to a colour chart included in the kit (Fig. 1). The different enzyme activities determined with this method are also listed in Fig. 1.

Results and discussion

Proteolytic activity of test isolates

The results obtained with the SMC-agar and the Universal Protease Substrate (UPS) methods are summarised in Table 1. Both methods were repeated and the table contains only the average values of duplicate repeats. These results give an indication of the proteolytic activity of the 33 bacteria isolated from fermented salt water fish and four from fresh water trout. It is important to compare the results of the two methods since the SMC-agar method only indicates the presence of proteolytic enzymes whereas the UPS-method allocates a quantitative value to the grade of proteolytic activity. A comparison of

Table 1. Comparison of the SMC-agar and Universal Protease Substrate (UPS) methods for the detection of proteolytic activity of 37 isolates from fish products.

Isolate number	Isolate	Source	SMC-agar method	UPS method Absorption (574nm)	
K2 4N	*Staphylococcus caprae*	Fish sauce	-	0.002	Low
K5 2T	*Sta. caprae*	Fish sauce	-	0.000	Absent
B2 1Y	*Sta. hominis*	Whole small fish	-	0.003	Low
B2 1N	*Sta. aureus*	Whole small fish	-	0.019	Low
K2 1P	*Sta. schleiferi*	Fish sauce	-	0.005	Low
K5 2P	*Sta. schleiferi*	Fish sauce	-	0.005	Low
K5 2Y	*Sta. schleiferi*	Fish sauce	-	0.003	Low
K4 1P	*Sta. schleiferi*	Fish sauce	-	0.002	Low
B2 2Y	*Sta. simulans*	Whole small fish	-	0.002	Low
K2 3N	*Sta. simulans*	Fish sauce	-	0.003	Low
K1 3N	*Agrobacterium radiobacter*	Fish sauce	+	0.116	High
B5 1M	*Stenotrophomonas maltophilia*	Fish paste	+	0.778	High
K7 1Y	*Ste. maltophilia*	Fish paste	+	0.645	High
K3 1N	*Vibrio alginoliticus*	Fish paste	+	1.738	Very high
K2 2N	*Sphingomonas paucimobilis*	Fish sauce	+	0.145	High
B3 3T	*Spm. paucimobilis*	Whole small fish	+	0.145	High
K1 4T	*Spm. paucimobilis*	Fish sauce	+	0.008	Low
K6 4P	*Cryseomonas luteola*	Fish sauce	+	1.207	Very high
B5 3T	*Bacillus megaterium*	Fish paste	+	0.008	Low
B4 3Y	*Bac. megaterium*	Fish sauce	+	0.000	Absent
B1 1Y	*Bac. subtilus*	Fish paste	+	1.388	Very high
B3 1T	*Bac. cereus*	Whole small fish	+	0.755	High
K1 1P	*Bac. cereus*	Fish sauce	+	0.089	Low
B6 4Y	*Bac. cereus*	Fish sauce	+	1.445	Very high
K5 1M	*Bac. cereus*	Fish sauce	+	1.129	Very high
B3 2N	*Bac. mycoides*	Whole small fish	+	0.069	Low
K3 2T	*Bac. mycoides*	Fish paste	+	0.688	High
B2 1P	*Bac. anthracis*	Whole small fish	+	1.063	Very high
K6 2P	*Bac. amyloliquefaciens*	Fish sauce	+	1.459	Very high
B1 2Y	*Bac. amyloliquefaciens*	Fish paste	+	1.938	Highest
K6 5P	*Bac. lentus*	Fish sauce	+	0.057	Low
B3 2Y	*Bac. licheniformis*	Whole small fish	+	0.164	High
B4 1P	*Bac. coagulans*	Fish sauce	+	0.026	Low
Sam 1	*Kocuria varians*	Fresh trout	+	0.979	High
Sam 2	*Koc. varians*	Fresh trout	+	0.037	Low
Sam 3	*Bac. firmus*	Fresh trout	+	0.296	High
Sam 4	*Brevibacillus brevis*	Fresh trout	+	0.081	Low

+ proteolytic activity - no proteolytic activity

the data from the two methods can also be used to determine the accuracy and repeatability of the methods.

By the repetition of both methods, the similarity and significance of the results of the two different methods could be determined. Results with a 95% similarity rate are considered as significant. The same results using the SMC-agar method, were obtained after two duplicate trials. The student T-test was performed to determine the significance of the UPS-method results. Data sets consisted of the absorption values for each isolate. This test assesses whether the means of the two groups of results are statistically different by judging the difference between the two means relative to the variability of their scores. A low value of 0.026 was obtained for this test indicating a similarity rate of more than 95%. The mean values for the data sets as well as the standard deviation values were almost equal with values of 0.445, 0.443 and 0.607, 0.592, respectively. There were, therefore, no significant difference between the duplicate sets of the UPS results which indicates that the UPS method is reliable and is repeatable (Anonymous, 2000a).

The SMC-agar method data indicated that all the staphylococci evaluated in this study tested negative for protease activity (Table 1). These findings were confirmed with the UPS-method, as the absorption values for the staphylococci were very low to absent, indicating no or almost no protease activity. The *Staphylococcus aureus* strain (B2 1N) however, tested more positive for protease activity with the UPS-method, with an absorbance value of 0.019, the highest of the staphylococci strains.

The remaining isolates tested positive for proteolytic activity and formed large zones of proteolysis on the SMC-agar. However, a few of the isolates's (Table 1) absorpsion values on the UPS-method were not comparable to the results obtained with the SMC-agar method. *Sphingomonas paucimobilis* (K1 4T) and the *Bacillus megaterium* strains (B5 3T and B4 3Y) gave very low or no absorption values of 0.008, 0.008 and 0.000, respectively. One *Sphingomonas* strain (K1 4T) however, did test positive with the SMC-agar test and may have lost its ability to produce the protease enzyme which explains the low absorption values for the UPS-method.

Figure 1. API Zym colour chart indicating the numerical values to be given for each colour intensity.

The fresh water trout strains (Sam 1, Sam 2, Sam 3 and Sam 4) all tested positive for proteolytic activity with the SMC-agar method. These four strains were isolated as the only proteolytic bacteria present on fresh water trout (Mapstone, 1998). The proteolytic results were confirmed with the UPS method which indicated proteolytic activity although it was low for *Kocuria varians* (strain Sam 2) and *Brevibacillus brevis* (strain Sam 4).

It is clear from the data in Table 1, that the other strains isolated from the fermented salt water fish products are proteolytically active and the majority of them were *Bacillus* strains. Certain strains had absorption values of >1.000 with the *Bac. amyloliquefaciens* strain B1 2Y being the highest.

The role of the non-enterics and *Bacillus* strains are highlighted by these results. They were all isolated at the end stage of the fish fermentation which indicated their possible role in protein degradation. The presence of proteolytic organisms, in these products is extremely important since the essential action during the production of fermented fish products is that of proteolytic activity and subsequent tissue degradation. The strong proteolytic attributes (>1.000) of some of the isolates examined in this study, with the exception of the staphylococci, suggests that they might have been active in the protein degradation of the fish, producing amino acids and low molecular nitrogenous products.

Detection of general enzymatic activity

The results of the API Zym tests are summarised in Table 2. The API Zym system was used to detect the specific enzyme profiles of a selection of the isolates. Only one representative of each major clustering group (Chapter 3 of this thesis) was examined with this method. A value ranging from zero to five was allocated to each enzyme tested to indicate the enzyme activity, where five was very active and zero indicated no activity. Values were allocated according to the reference colours on the colour chart given in Fig. 1. A wide variety of enzymes, ranging from lipases and esterase to proteases were tested with this technique to possibly predict which substrate in fish could probably be degraded and what products would be formed.

Table 2. Enzyme activity as shown by the API Zym tests of the selected 22 bacterial isolates (A value from 0 through to 5 is allocated to the grade of activity, where 5 represents strong activity)

Enzymes	*Sta. caprae* K2 4N	*Sta. hominis* B2 1Y	*Sta. aureus* B2 1N	*Sta. schleiferi* K2 1P	*Sta. simulans* B2 2Y	*Koc. varians* Sam 1	*Bac. firmus* Sam 3	*Bac. brevis* Sam 4
Phosphatase alcaline	2	0	2	2	1	2	2	1
Esterase (C4)	2	2	1	2	1	2	2	2
Esterase Lipase (C8)	2	1	2	2	1	2	2	4
Lipase (C14)	0	0	0	0	0	0	0	0
Leucine arylamidase	0	3	1	0	0	4	4	5
Valine arylamidase	0	1	0	0	0	0	0	1/2
Cystine arylamidase	0	1/2	1/2	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	1	0	0
Phosphatase acid	5	2	5	5	5	3	1	1/2
Naphtol-AS-B1-phosphohydrolase	1	1	1	1	1	1	1	1
α galactosidase	0	0	0	0	0	0	0	0
β galactosidase	0	0	3	1	1/2	0	0	0
β glucuronidase	0	0	1	0	0	0	0	0
α glucosidase	2	2	3	1	2	3	0	4
β glucosidase	4	5	0	5	5	0	0	0
N-acetyl-β glucosaminidase	0	0	0	0	0	0	0	0
α mannosidase	0	0	0	0	0	0	0	0
α fucosidase	0	0	0	0	0	0	0	0

Table 2. (continued)

Enzymes	*Agr. radiobacter* K1 3N	*Ste. maltophilia* K7 1Y	*Vib. alginolyticus* K3 1N	*Sph. paucimobilis* K2 2N	*Chy. luteola* K6 4P	*Bac. subtilus* B1 1Y	*Bac. cereus* B3 1T
Phosphatase alcaline	5	1	2	2	4	1	1
Esterase (C4)	2	2	2	2	1	3	2
Esterase Lipase (C8)	1	2	2	2	1	2	1
Lipase (C14)	0	0	0	0	0	0	1/2
Leucine arylamidase	2	0	0	0	1	1	5
Valine arylamidase	1/2	0	0	0	1	1	1
Cystine arylamidase	0	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	3	0	0	1/2
Phosphatase acid	1	1	1/2	2	1	1	3
Naphtol-AS-B1-phosphohydrolase	1	1	1	1	1	1	1
α galactosidase	0	0	0	1	0	0	0
β galactosidase	3	0	0	1	0	1	0
β glucuronidase	0	0	0	0	0	0	0
α glucosidase	0	0	0	1/2	1	2	1
β glucosidase	0	1	0	0	0	0	0
N-acetyl-β glucosaminidase	0	0	0	0	0	0	0
α mannosidase	0	0	0	0	0	0	0
α fucosidase	0	0	0	0	0	0	0

Table 2. (continued)

Enzymes	*Bac. megaterium* B4 3Y	*Bac. mycoides* B3 2N	*Bac. anthracis* B2 1P	*Bac. amyloliquefaciens* K6 2P	*Bac. lentus* K6 5P	*Bac. licheniformis* B3 2Y	*Bac. coagulans* B4 1P
Phosphatase alcaline	1	1	1	5	5	5	1
Esterase (C4)	2	2	2	1	1	2	2
Esterase Lipase (C8)	3	2	2	3	1	2	2
Lipase (C14)	0	0	1/2	0	0	0	0
Leucine arylamidase	1/2	5	5	0	0	5	0
Valine arylamidase	0	1/2	1/2	0	0	0	0
Cystine arylamidase	0	0	0	0	0	0	0
Trypsin	0	1/2	0	0	0	0	0
Chymotrypsin	3	1/2	1/2	0	0	1	1
Phosphatase acid	2	1	5	0	1	2	0
Naphtol-AS-B1-phosphohydrolase	1	1	1	1/2	1	1	1
α galactosidase	1	0	0	0	0	0	0
β galactosidase	1	0	0	0	1	4	3
β glucuronidase	0	0	0	0	0	0	0
α glucosidase	2	1	1	3	0	1	0
β glucosidase	0	0	1	1/2	0	1/2	0
N-acetyl-β glucosaminidase	0	0	0	0	0	0	0
α mannosidase	0	0	0	0	0	0	0
α fucosidase	0	0	0	0	0	0	0

The enzymes that are generally considered to be important in the fish fermentation process and on which will be the focused, are the lipolytic and proteolytic enzymes, specifically esterase lipase, lipase, trypsin, chymotrypsin and the arylamidases. The enzymes esterase lipase and lipase, are functional in the breakdown of lipids to produce fatty acids which contribute to the distinct flavours and aromas characteristic of fermented fish products (Anonymous, 2000b). The low-molecular weight fatty acids present in all pastes and sauces are responsible for the "cheesy notes" in fermented fish products (Abe & Tsuyuki, 1969; Beddows *et al.*, 1980). Trypsin, chymotrypsin and the arylamidases are all from the peptidase family and cause the breakdown of proteins and peptides which results in the release of amino acids and amines in fish sauces and pastes (Anonymous, 2000c, d and e). A picture of three different API Zym test results are presented in Figure 2.

All the representative isolates were found to produce esterase lipase but only *Bacillus cereus* (B3 1T), *Bac. mycoides* (B3 2N) and *Bac. anthracis* (B2 1P) had the ability to produce lipase. None of the staphylococci were found to produce trypsin or chymotrypsin, which is in agreement with the results of the SMC-agar and UPS-methods where no proteolytic activity (Table 1) was detected for these strains. The non-enteric rods did not produce high peptidase values except for the *Sphingomonas paucimobilis* (K2 2N) strain that produced chymotrypsin. Isolates of the genus *Bacillus* produced a variety of arylamidases and also chymotrypsin, but only *Bac. mycoides* (B3 2N) produced trypsin.

Considering the bacteria isolated from fresh water trout, only *Kocuria varians* (Sam 1) produced the enzyme chymotrysin. None of the strains, however produced trypsin, the main proteolytic enzyme. Only leucine arylamidase of all the arylamidases was produced by all three strains from the fresh water trout (Sam 1, Sam 3 and Sam 4).

The API Zym method gives a clear indication of the specific enzymes that could be involved in the proteolytic and lipolytic process during fish fermentation. The quantitative value given to each enzyme aids in showing the grade of activity of the enzyme tested for. This method does not focus entirely on proteolytic enzymes as in the case of the SMC-agar and UPS-methods, but examines a wider range of enzymes that could be active.

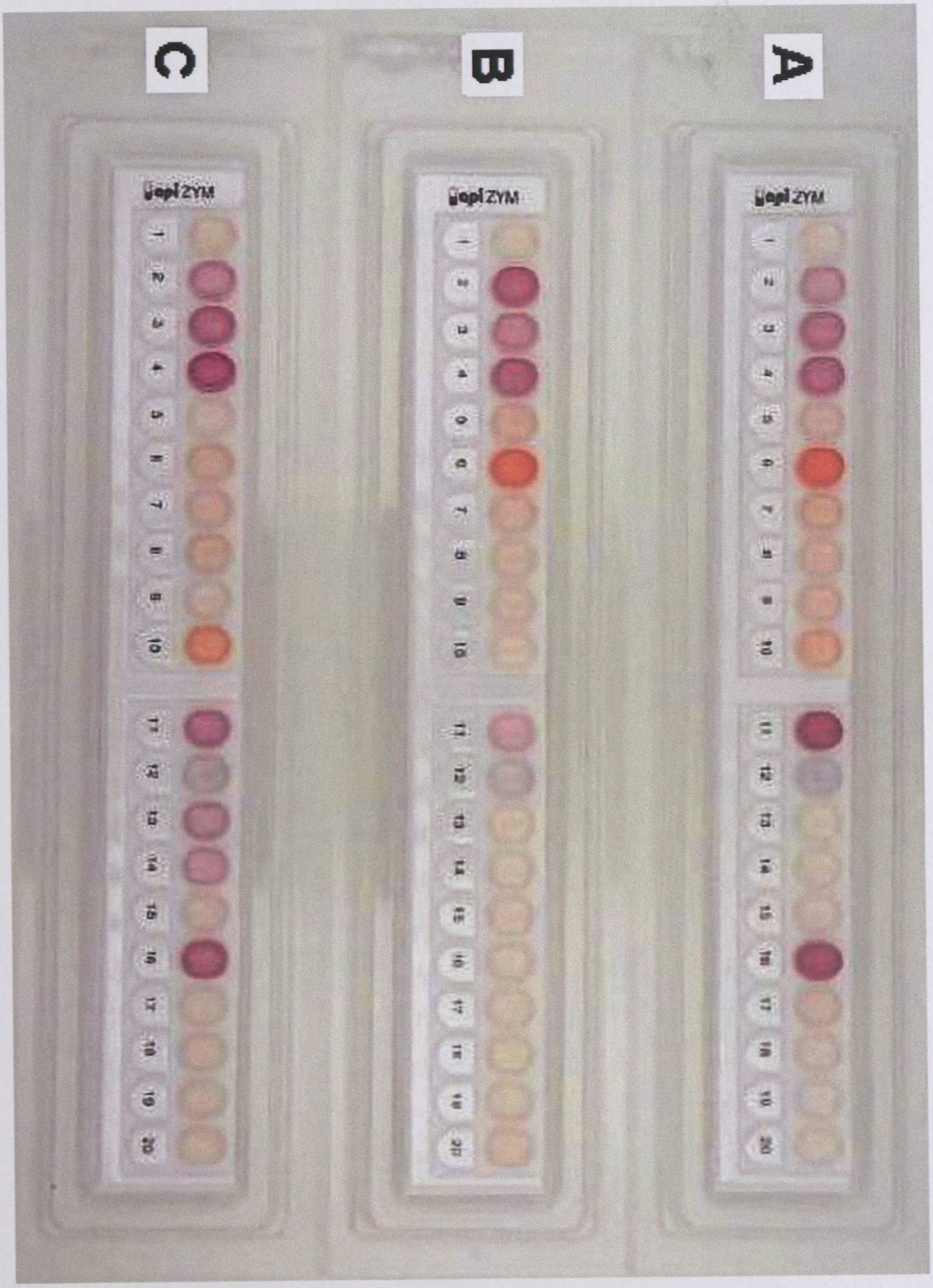


Figure 2. An example of the API Zym results for A - *Kocuria varians* (Sam 1), B - *Bacillus firmus* (Sam 3) and C - *Bac. megaterium* (B4 3Y). The form of this figure is to compliment the data given in Figure 1.

Conclusions

In this study various bacteria that were isolated from fresh water trout as well as from fermented salt water fish products, were examined for proteolytic as well as general enzyme activity. The three methods used were the Standard Methods Caseinate Agar-method (SMC-method), Universal Protease-method (UPS method) and the API Zym-system. These methods were also compared and evaluated for significance.

The main role of bacteria in fermented fish products is the utilisation of their proteolytic characteristics. This enables them to liquefy the fish tissue by the degradation of proteins and the subsequent formation of fish sauce and paste. The SMC and UPS-methods were best suited for use in these conditions, since they test solely for protease activity and not for general enzyme activity as in the case of the API Zym-system.

The majority of bacteria isolated from the fermented fish were from the genus *Bacillus*. These organisms also had the highest proteolytic activity, as seen from the data presented in Table 1. The *Staphylococcus* did not produce any proteolytic enzymes as can be seen in the results of the SMC-agar and UPS-methods. Although the SMC-agar method is merely qualitative and the UPS-method quantitative, their results compared well with a variation of only 1.11% and these results were mostly confirmed by the API Zym system data.

It can be concluded that the majority of bacteria isolated from fermented fish products are proteolytically active and assist in the degradation of fish tissue and the subsequent release of free nitrogenous compounds altering the taste and texture of the product. The data from these tests may be used in future studies to select proteolytic isolates which could assist in the production of fermented fish products. It must be noted that the strains used in this study were isolated at the end of the fermentation process and that other microbes which are essential for the process might only be present during the initial stages of fermentation.

References

Abe, T. & Tsuyuki, H. (1969). Studies on the organic acids in 'shottsuru' (fish sauce). *Journal of Food Science and Technology, Japan.* **16**(12), 560-565.

Anonymous. (2000a). The Student - T Test for Two Samples. 11-08-2000. http://fonsg3.let.uva.nl/Service/Statistics/2Sample_Student_t_Test.html

Anonymous. (2000b). NiceZyme View of Enzyme: EC 3.1.1.3. 07-08-2000. http://www.expasy.ch/cgi-bin/nicezyme.pl?3.1.1.3

Anonymous. (2000c). NiceZyme View of Enzyme: EC 3.4.11.6. 07-08-2000. http://www.expasy.ch/cgi-bin/nicezyme.pl?3.4.11.6

Anonymous. (2000d). NiceZyme View of Enzyme: EC 3.4.21.4. 07-08-2000. http://www.expasy.ch/cgi-bin/nicezyme.pl?3.4.21.4

Anonymous. (2000e). NiceZyme View of Enzyme: EC 3.4.21.1. 07-08-2000. http://www.expasy.ch/cgi-bin/nicezyme.pl?3.4.21.1

Ashie, I.N.A., Simpson, B.K. & Ramaswamy, H.S. (1996). Control of endogenous enzyme activity in fish muscle by inhibitors and hydrostatic pressure using RSM. *Journal of Food Science,* **61**, 350-361.

Beddows, C.G., Ardeshir, A.G. & Wan Johari bin Daud, B. (1980). Development and origin of the volatile fatty acids in Budu. *Journal of the Science of Food and Agriculture,* **31**, 86-92.

Boeker, E.A. & Snell, E.E. (1972). Amino acid decarboxilase. In: *The Enzymes. Vol. 6.* (Edited by P.D. Boyer). New York: Academic Press

Jiang, S-T., Wang, Y-T. & Chen, C-S. (1992). Lysosomal enzyme effects on the post-mortem changes in tilapia (*Tilapia nolotica X T. aurea)* muscle myofibrils. *Journal of Food Science,* **57**, 227-279.

Fardiaz, D. & Markakis, P. (1979). Amines in fermented fish paste. *Journal of Food Science*, **44**, 1562.

Linder, P., Angel, S., Weiburg, Z.G. & Granit, R. (1988). Factors inducing mushiness in stored prawns. *Food Chemistry,* **29**, 119-132.

Makinodan, Y., Toyohara, H. & Niwa, E. (1985). Implication of muscle alkaline proteinase in the textural degradation of fish meat gel. *Journal of Food Science,* **50**, 1351-1355.

Mapstone, S.J. (1998). Department of Food Science, University of Stellenbosch, Personal communication.

Martley, F.G., Jayashankar, S.R. & Lawrence, R.C. (1970). An improved agar medium for the detection of proteolytic organisms in total bacterial counts. *Journal of Applied Bacteriology,* **33,** 200-204.

Sanceda, N.G., Kurata, T. & Arakawa, N. (1990). Overall quality and sensory acceptance of a lysine-fortified fish sauce. *Journal of Food Science*, **55**(4), 983-988.

Steinkraus, K.H. (1996). Fermented fish-shrimp sauces and pastes. In: *Handbook of Indigenous Fermented Foods.* (Edited by K. H. Steinkraus) Pp. 565-654. New York: Marcel Dekker Inc.

Toole, A. (1998). Traditional fermented foods. *Food Info*, **6**, 2-3.

Yamashita, M. & Konagaya, S. (1992). Differentiation and localisation of catheptic proteinases responsible for extensive proteolysis of mature chum salmon muscle (*Oncorhynchus keta). Comparitive Biochemistry and Physiology.* **103B**, 999-1003.

CHAPTER 5

IMPACT OF THE ADDITION OF PROTEOLYTIC STARTER CULTURES ON THE PRODUCTION PARAMETERS DURING LABORATORY SCALE FERMENTATION OF FRESH WATER TROUT

Summary

Cultures isolated from fermented fish products from Thailand, as well as lactic acid starters, were used in the production of a fermented fresh water fish product. Different production parameters, including glucose (0.5, 1.0, 2.5 and 5.0% w/w) and inoculum concentration ($1x10^6$, $1x10^7$, $1x10^8$ and $1x10^9$), moisture (50 ml, 100 ml and 150 ml per 100 g fish) and inoculum content (1% and 10% v/v), two temperatures (30° and 35°C) as well as incubation time, were examined and compared to select the optimum fermentation conditions. Fermentation efficiency was determined by measuring the final pH, titratable acid and the free amino nitrogen content. It was found that the optimum efficiency was obtained with 5% (w/w) added glucose at a moisture level of 150 ml water per 100 g fish. A factorial design was then used to determine combinations of the fermentation conditions in order to indicate viable trends to facilitate optimisation of the fermentation process. The main effects, two-factor and three-factor interactions were calculated. Optimum trends obtained were a glucose concentration of 5% (w/w), inoculum concentration of $1x10^8$ cfu.ml^{-1} and an incubation period of 15 days at 30°C. The three lactic acid starters (226 - *Lactobacillus plantarum,* 140 - *Lactococcus diacetylactis* and 407 - *Pediococcus cerevisiae)* were selected as they produced some of the best fermentation results and are safe to use in food fermentations. Two further studies were conducted to compare the fermentation results of combinations of the starters added to the fish. It was found that a combination of all three strains (226, 140 and 407) gave the best data and that the data improved when the fermented products were reinoculated at days 8, 15 and 23 with the starters.

Introduction

Fermented fish products are traditionally produced in Southeast Asia and are mainly used as condiments, adding taste to rice and other bland, starchy foods. It is also a

rich source of protein. Although a variety of fermented fish products are available and known by different names, they can be categorised into two main product groups, namely fish sauce and paste (Sanceda *et al.*, 1986).

Fish sauce is a clear brown liquid hydrolysate of salted fish obtained after a fermentation period of 6 to 12 months (Sanceda *et al.,* 1996) and usually contains about 20 $g.l^{-1}$ nitrogen of which 80% is in the form of amino acids (Lafont, 1955). In the traditional manufacturing process, fish is mixed with salt (3 - 10 parts fish to one part salt), placed in earthenware pots and allowed to stand for 1 to 15 months at temperatures of 30° to 35°C (Avery, 1952; Dougan & Howard, 1975). In some cases glucose is added to accelerate the fermentation process. At the end of the fermentation period, the proteinaceous liquid is separated and sold as fish sauce and the solid mass is used as paste (Avery, 1952).

The demand for fermented fish sauce in the Philippines, for local use as well as export, is increasing rapidly (NEDA, 1992). Due to the long fermentation process (6 - 12 months), the demand for the product far exceeds the supply (Sanceda *et al.,* 1996) and thus a shorter, controlled fermentation process would be very advantageous provided that the original quality is retained.

The liquefaction of the fish tissue is primarily caused by the action of exogenous enzymes produced by bacteria as well as endogenous enzymes from fish viscera (Saisithi *et al.*, 1966). The specific group of enzymes responsible for this action is the proteolytic enzymes. The addition of these enzymes to fish followed by a drop in pH, will cause a subsequent acceleration of the liquefication process (Uyenko *et al.*, 1952). A low pH and high salt concentration will, furthermore, help preserve the sauce by preventing the growth of spoilage and pathogenic organisms (Sanceda *et al.*, 1996).

The aim of the present study was to examine the impact of the addition of different lactic and proteolytic bacteria on the production parameters for the laboratory production of fermented fish using fresh water rainbow trout as substrate.

Materials and methods

Selection of test starter cultures

A variety of test strains were selected to use in the production of the fermented fish sauce and paste. The selection (Table 1) consisted of lactic acid bacteria as well

Table 1. Bacterial strains used in the production of the fermented fish products.

Strain	Species	Source
140	*Lactococcus diacetylactis*	-
897	*Lactobacillus acidophilus*	-
226	*Lb. plantarum*	-
407	*Pediococcus cerevisiae*	-
Sam 1	*Kocuria varians*	Fresh trout - Stellenbosch
B5 1M	*Stenotrophomonas maltophilia*	Fermented fish paste - Bangkok
K6 4P	*Chryseomonas luteola*	Fermented fish sauce - Khon Kaen
B5 3T	*Bacillus megaterium*	Fermented fish paste - Bangkok
B1 1Y	*Bac. subtilus*	Fermented fish paste - Bangkok
B3 1T	*Bac. cereus*	Fermented fish - Bangkok
B3 2N	*Bac. mycoides*	Fermented fish - Bangkok
K6 2P	*Bac. amyloliquefaciens*	Fermented fish sauce - Khon Kaen
K6 5P	*Bac. lentus*	Fermented fish sauce - Khon Kaen
B3 2Y	*Bac. licheniformis*	Fermented fish - Bangkok

as certain proteolytic bacteria isolated from fish sauces and pastes (Chapter 3 of this thesis).

Standard growth curves

Test cultures 140 (*Lactococcus diacetylactis)*, 897 (*Lactobacillus acidophilus)*, 226 (*Lactobacillus plantarum)*, 407 (*Pediococcus cerevisiae)* and Sam 1 (*Kocuria varians)* were grown in sterile MRS broth consisting of ($g.l^{-1}$): MRS (Biolab), 50 and KH_2PO_4, 10. The pH was set at 7.1. The remaining cultures (Table 1) were cultivated in sterile Nutrient Broth (Biolab) which consisted of ($g.l^{-1}$): meat extract, 1; yeast extract, 2; peptone, 5; and NaCl, 8. A series of microbial dilutions, from 10^{-1} through to 10^{-8}, were made in sterile physiological salt solution and the microbial members enumerated using the pour plate technique. The absorbance of each dilution was measured at 540 nm in a spectrophotometer (Spectronic® 20 Genesys™) against the blank. A standard absorbance versus count curve was determined for each strain.

Preparation of trout

Rainbow trout (*Oncorhynchus mykiss*) from the University of Stellenbosch trout farm in the Jonkershoek Valley, were used in the production of the fermented fish products. The fresh fish were gutted and filleted after which each fillet was washed in clean water. The fish was then macerated using a sterile meat grinder into pieces of approximately 10 mm and 10% (w/w) coarse NaCl was added.

Experimental Study 1: Influence of two glucose concentrations

In this study, the ground fish was divided into two batches of equal weight, and 0.5 and 5% (w/w) glucose was added to the two batches, respectively. 100 g of the ground fish mixture was placed in sterile Erlenmeyer flasks and covered with a tin foil cap. No additional moisture was added. A 1% (v/v) inoculum (1×10^6 $cfu.ml^{-1}$) of either *Lactococcus diacetylactis* (140), *Lactobacillus acidophilus* (897), *Lactobacillus plantarum* (226), *Pediococcus cerevisiae* (407) or *Kocuria varians* (Sam 1), was added to the respective duplicate flasks. Only these five cultures were selected for the first study in order to optimise the fermentation process. Duplicate control flasks, without any added cultures, were also included. The flasks were incubated at 35°C for 30 days. The pH and titratable acidity (%TA) were measured on days 0, 10 and 30.

Experimental Study 2: Influence of varying moisture content

The trout, with salt, was prepared as described in Study 1, but only a 5% (w/w) glucose concentration was added. A 100 g fish mixture was placed in each sterile Erlenmeyer flask and inoculated with each starter culture as in Study 1. The duplicate fish flasks were divided into two groups and 50 ml sterile distilled water was added to the first group and 100 ml sterile distilled water to the second group. The flasks were incubated at 35°C for 30 days. The pH and %TA were measured on days 0, 10 and 30.

Experimental Study 3: Comparison of the impact of different bacterial strains on the production parameters

After the preparation of the trout, with salt, as described in Study 1, glucose at a 5% (w/w) concentration was added and 100 g of the fish mixture placed in sterile 250 ml containers. The volume of moisture added was increased to 150 ml per 100 g fish as the sauce in Study 2 was still too dry. The inoculum concentration and volume was also increased to 10% (v/v) inoculum (1×10^7 cfu.ml^{-1}) to ensure the growth of the starter cultures. The strains, as listed in Table 1, were separately added to the fish in the different containers. The caps of the containers were screwed on firmly and incubated at 35°C for 30 days. The pH and %TA was measured every 5 days. Total free amino nitrogen (FAN) was also measured on every 10th day, according to the Sorensen method (AOAC 24.043, 12th ed.). Free amino nitrogen can be used as an indication of the proteolytic activity during fermentation since this is the most important reaction that leads directly to the production of fermented fish products.

Experimental Study 4: Influence of different glucose concentrations and the addition of a buffer on the production parameters

In this study, the trout was prepared in duplicate as described in Study 1 but different glucose concentrations (1.0%, 2.5% and 5.0% w/w) were added. An additional test was conducted by adding 5.0% (w/w) glucose as well as 1.117 g Na_2HPO_4 per kg fish (value according to government legislation). All the production parameters, as done in Study 3, were repeated and the FAN also measured on every fifth day in order to determine correlations between the drop in pH and increase in FAN and to monitor the proteolytic activity more accurately.

Experimental Study 5: Optimisation of production parameters using a 3 x 3 x 3 factorial design

Fermented fish was manufactured as previously described using the five most suitable strains identified in Studies 1 to 4. They were selected on their ability to produce a high concentration of acid, low final pH as well as free amino nitrogen. These strains were identified as *Bacillus mycoides* (B3 2N), *Lactobacillus plantarum* (226), *Kocuria varians* (Sam 1), *Lactococcus diacetylactis* (140) and *Pediococcus cerevisiae* (407). The product produced with the use of these cultures was subjected to combinations of different production factors. These included: varying glucose concentrations (1.0, 2.5, and 5.0% (w/w)); varying inoculum concentrations ($1x10^7$, $1x10^8$, $1x10^9$ cfu.ml^{-1}); and different incubation times (0, 15 and 30 d). These variables were selected to determine their impact on the final pH, titratable acid (%TA), and free amino nitrogen (FAN) values of the final fermented product. A control was included for each experimental set.

A 3 x 3 x 3 factorial design at two temperatures (30° and 35°C) was used to evaluate the quantitative effects of the variables, individually and in combination, for each of the five bacterial strains. The Yate's algorithm (Box *et al.,* 1978) was used to calculate the response of the effects and interactions of glucose concentration (C), inoculum concentration (I) and incubation time (T). The main effect (either C or I or T) was defined as the influence of a specific variable on a response averaged over the span of the other variables. Positive two or three factor interaction values would imply an enhancement of the final response when the two or three variables are increased simultaneously.

Experimental Study 6: Investigation of the use of different starter combinations for fermented fish production

Fermented fish was produced using the three optimum fermentation conditions obtained from the factorial design results in Study 5. The procedures were repeated using 5% (w/w) glucose, 10% salt (w/w) and a 10% (v/w) starter inoculum at a concentration of $1x10^8$ cfu.ml^{-1}. The moisture level was kept at 150 ml water per 100 g fish. The following single or culture combinations were used: 226 (*Lc. plantarum);* 140 (*Lc. diacetylactis);* 407 (*Pc. cerevisiae*); 226 and 140; 226 and 407; 140 and 407; 226

and 140 and 407; and a control sample containing no starter culture. The fermentations were performed at 30°C and the pH, %TA and FAN were measured on days 0, 15 and 30.

Fermented fish was also produced in larger volumes of 500 and 1000 ml using a combination of cultures 226, 140 and 407, as the starter. This Experimental Study was done in duplicate.

Experimental Study 7: Confirmation of production parameters and influence of reinoculation

Fermented fish was produced, as in Study 6, with 10% NaCl (w/w), 5% glucose (w/w), 150 ml moisture per 100 g fish and 10% (v/w) inoculum) using only the combination of the three cultures (*Lc. plantarum* 226, *Lc. diacetylactis* 140 and *Pc. cerevisiae* 407). The fish was incubated at 30°C. These three cultures were selected as a combination starter because they were all three lactic acid bacteria and safe to use in fermented foods. The fish was again reinoculated on days 8, 15 and 23 with the selected combination starter, to ensure the growth of the culture. The aim of this study was to confirm the production parameters as determined in Study 6.

Results and discussion

Experimental Study 1: Influence of two glucose concentrations

In the first study only five strains representing both lactic acid producers and proteolytic bacteria, were chosen to determine changes in the production parameters. Different glucose concentrations (0.5 and 5.0% w/w) were used in this study to determine the optimum fermentation conditions for the bacterial starters as glucose is an essential carbon for especially the lactic acid bacteria as they utilise this source to produce acid.

The changes in pH and %TA over the incubation period for each of the five strains are illustrated in Fig. 1 and 2. An addition of 0.5% (w/w) glucose (Fig. 1) to the fish resulted in an increase of the pH over the 30 day period for all the strains, whereas the %TA hardly increased. Normally lactic acid bacteria can utilise glucose as carbon source and produce lactic acid as one of their end-products, which in turn results in a drop in pH. In this study, the increase in pH and almost no change in titratable acid, was ascribed to the too low concentration of glucose (0.5% w/w) present in the fish.

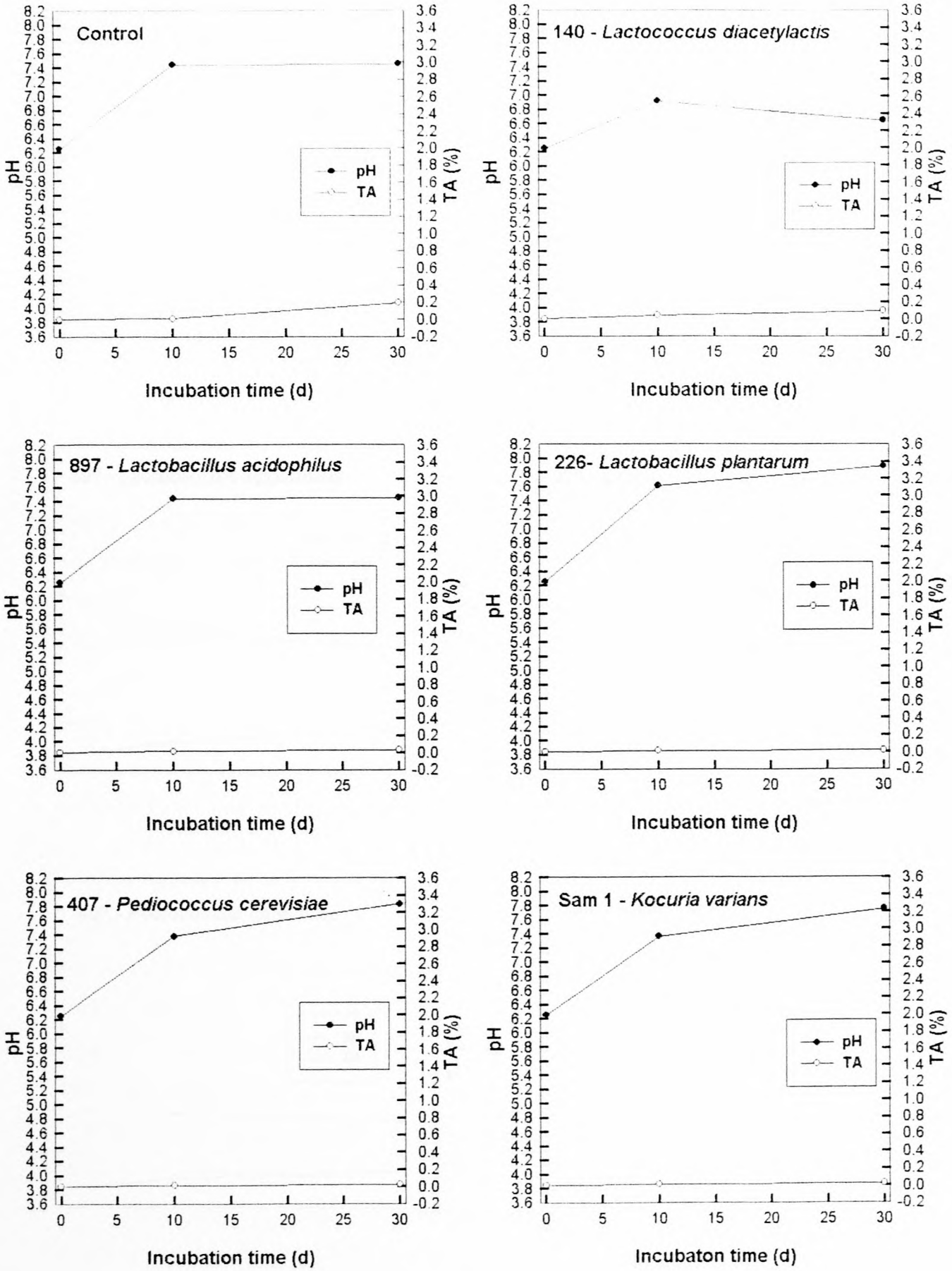


Figure 1. Influence of five different bacterial strains on pH and %TA over the 30 day incubation period with 0.5% (w/w) glucose.

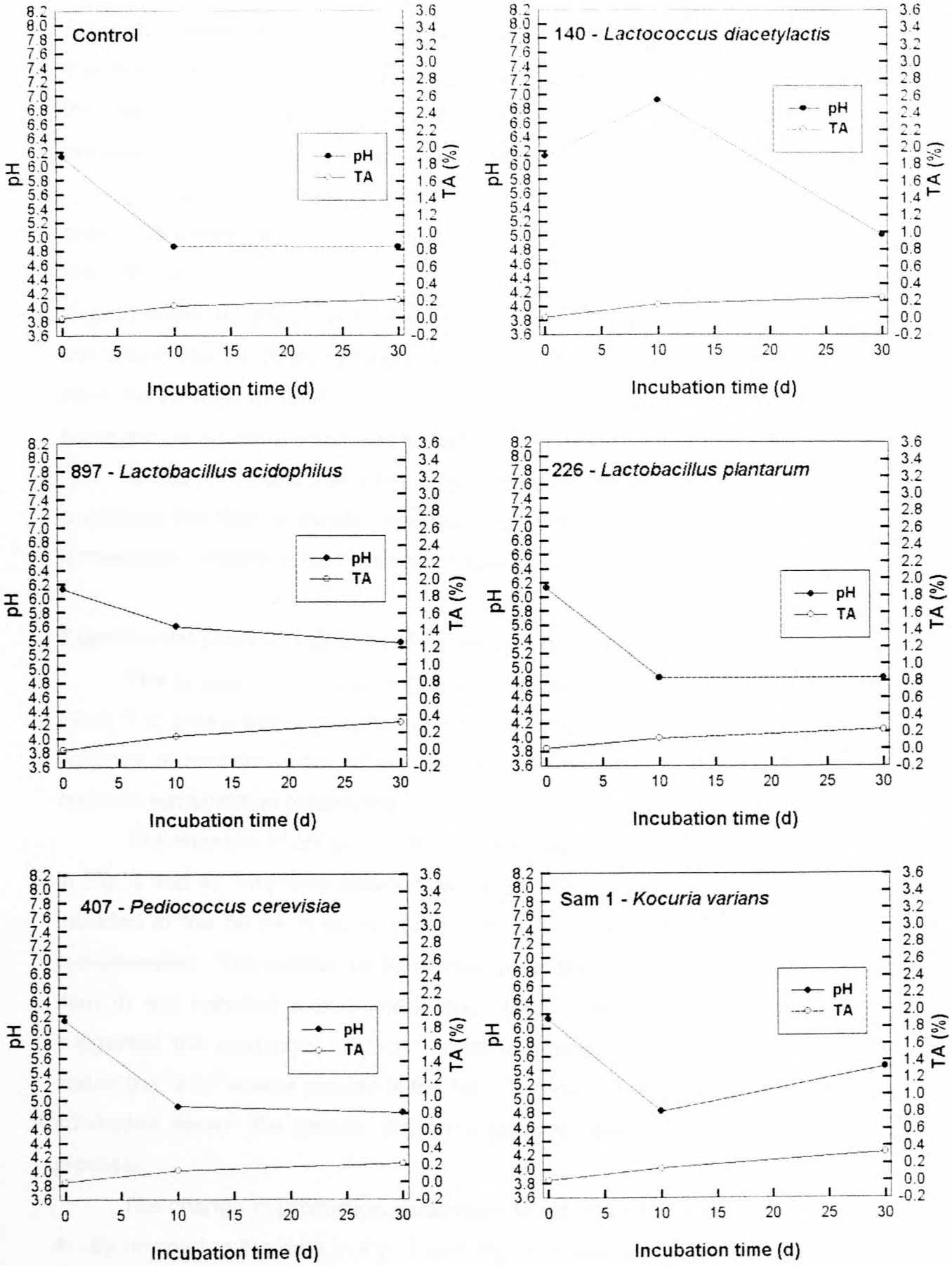


Figure 2. Influence of five different bacterial strains on pH and %TA over the 30 day incubation period with 5.0% glucose added.

Since the experimental graphs did not differ from the control graph, it was concluded that there probably was not enough glucose for the bacteria to grow on and produce the required lactic acid. The increase in pH was ascribed to the lack of lactic acid production and probable production of nitrogen compounds.

In contrast, the higher glucose content (5.0% w/w) (Fig. 2) resulted in most cases in a drop in pH and a more noticeable increase of titratable acid probably due to the more favourable conditions for the production of lactic acid. The resulting profiles (Fig. 2) however, still did not differ noticeably from the control profile. This led to the conclusion that the bacteria naturally present in fish were still the dominant population. After the 30 days incubation time, the fish appeared dried-out and did not look like a paste and no sauce or liquid was formed.

It was concluded that a higher glucose concentration improved the fermentation conditions but that additional moisture had to be added to the fish to enhance the fermentation conditions for the starter bacteria.

Experimental Study 2: Influence of varying moisture content

The glucose concentration in this study was set at 5% (w/w) since it was found in Study 1 to give a better response in the production parameters. In this study, varying volumes of moisture were added (50 and 100 ml per 100 g fish) to determine the optimum fermentation parameters.

The changes in pH and %TA over the incubation time of 30 days are illustrated in Fig. 3 and 4. The data obtained for strain 140 (*Lactococcus diacetylactis)* was not included in the 50 ml moisture study (Fig. 3) as this batch was spoiled by a mould contamination. The addition of 50 ml moisture to the fish resulted in a larger drop in pH than in the previous experimental study (Fig. 1 and 2). This larger pH change suggested the production of lactic or other organic acids by the inoculated starter and/or the "wild" strains present in the fish. The data clearly indicates that more humid conditions favour the growth of microorganisms and accelerates the fermentation process.

The change in production parameters when 100 ml was added, is shown in Fig. 4. By comparing the data in Fig. 3 and Fig. 4, it was concluded that the formation of acid increased as the moisture increased. Although the pH drop for both the 50 and 100 ml moisture additions were large and very similar, the %TA's obtained for the 100

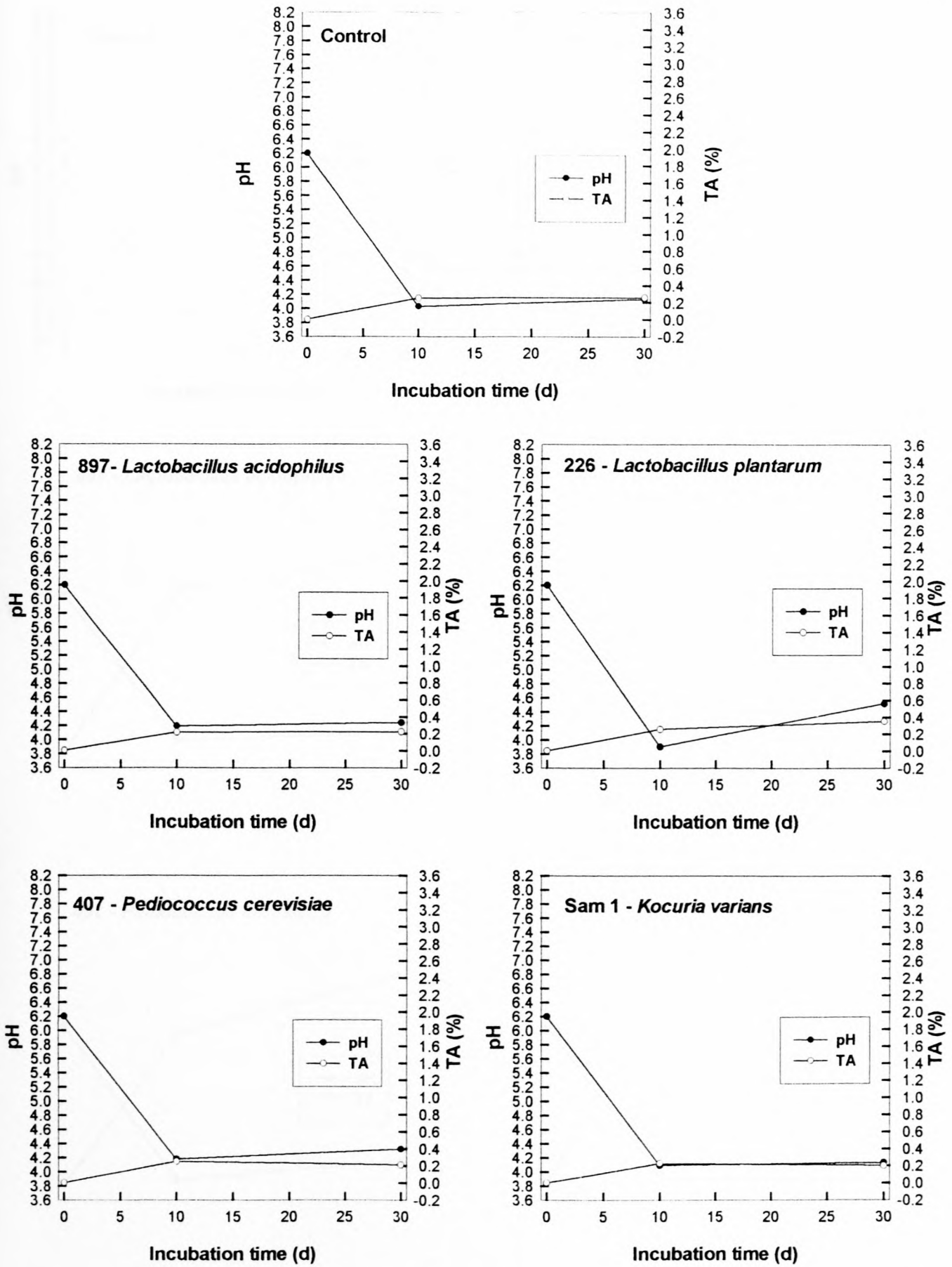


Figure 3. Influence of four different bacterial strains on pH and %TA over the 30 day incubation period with 50 ml water added per 100 g fish (the data for strain 140 - *Lactococcus diacetylactis* were left out due to spoilage of the fish by a mould).

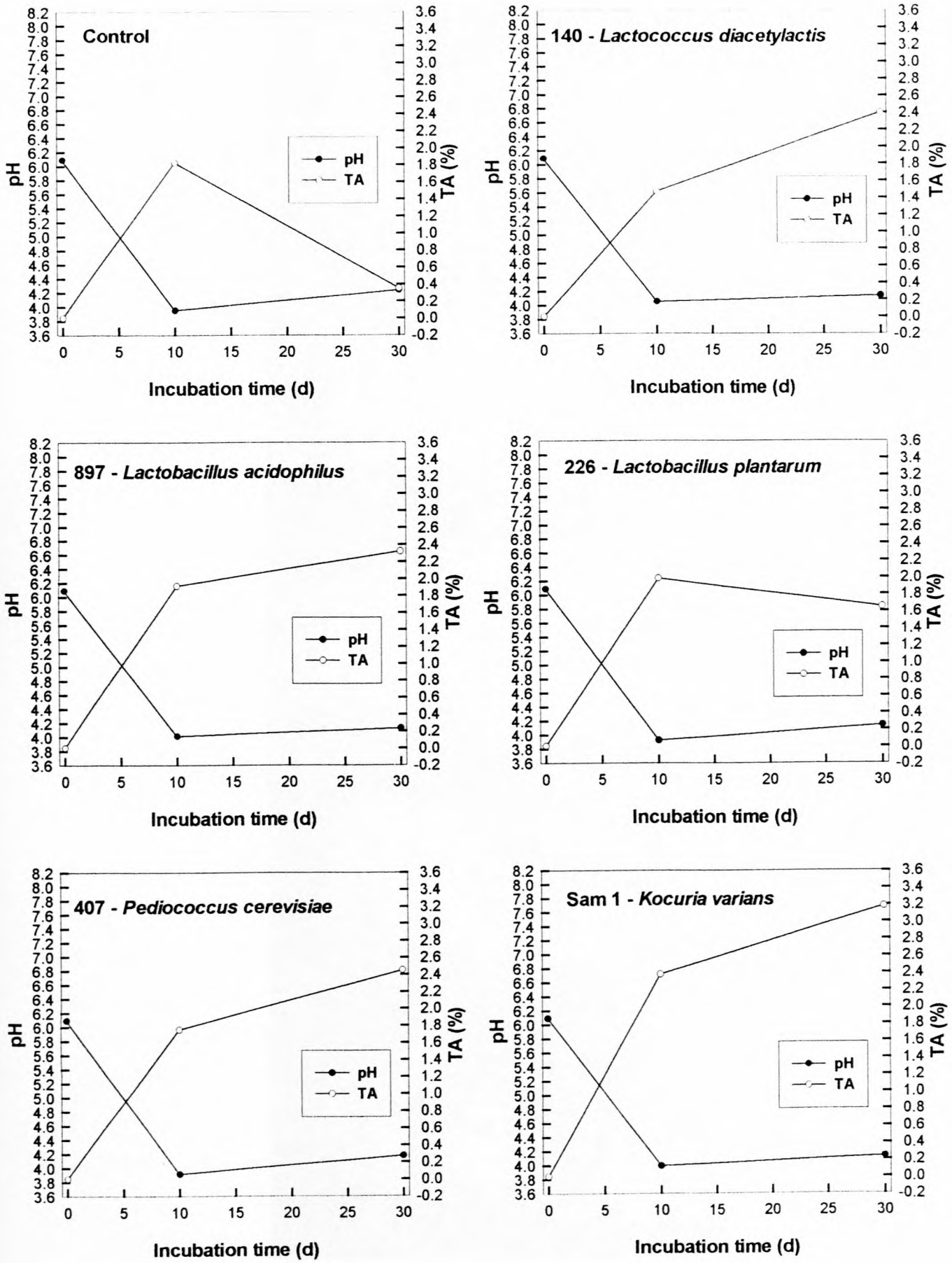


Figure 4. Influence of five bacterial strains on pH and %TA over the 30 day incubation period with 100 ml water added per 100 g fish.

Figure 5. Picture of the fermented fish as produced in Experimental Studies 1 and 2. A = 0.5% (w/w) glucose added, B = 5.0% (w/w) glucose added, C = 50 ml moisture per 100 g fish, D = 100 ml moisture per 100 g fish, and E = strain 140 spoiled by mould growth.

ml moisture addition (Fig. 4) were much greater than those found for the 50 ml moisture addition (Fig. 3).

It was interesting to observe that there was only a small difference between the drop in pH of the control in comparison with the samples with added starter cultures. It appears from the data that the lactic acid bacteria and other starters did not have a strong influence on the fermentation process. However, in the case of the %TA, it was clear that the presence of the starters did influence the acid production (%TA), especially after day 10, in comparison to the decrease of the %TA after day 10 for the control.

In the case of the addition of the *Kocuria varians* strain Sam 1, it was found that the %TA differed from the other starters and was much higher with a final acidity at day 30 of 3.2. This organism was previously isolated from fresh trout (Table 1) (Mapstone, 1998) and the natural occurrence of it in trout plus the addition of a fairly high inoculum, might have resulted in a competitive advantage leading to the increased production of acids causing an increase in the %TA.

Photographs of the fermented fish product obtained from Study 1 and Study 2 can be seen in Fig. 5 (A = 0.5% (w/w) glucose, B = 5.0% (w/w) glucose, C = 50 ml moisture per 100 g fish, D = 100 ml moisture per 100 g fish and E = sample 140 spoiled by a mould). It is clearly discernible that the two samples (A and B) from Study 1 were very dried out and that the samples containing moisture were more humid and saucy.

From this experimental study, it was concluded that a higher moisture content improved the fermentation process in terms of acid production and also caused the fish product to appear more liquid and saucy. An even higher moisture content might improve growth conditions for the cultures and this should be investigated. It can also be speculated that an increase in inoculum concentration and inoculum volume might enhance the growth of the starters, consequently resulting in an increased production of lactic or other acceptable acids.

Experimental Study 3: Comparison of the impact of different bacterial strains on the production parameters

In this study, an increased moisture level (150 ml per 100 g fish), higher inoculum concentrations ($1x10^7$ cfu.ml^{-1}) and inoculum volumes (10 ml) were used to produce the fermented fish product. These parameter values were chosen to insure more favourable fermentation conditions than those used in Experimental Studies 1

and 2. The impact of 14 different proteolytic strains (Table 1) (Chapters 3 and 4 of this thesis) on the production parameters, was determined in this study.

The results of the changes in pH, %TA and FAN over the incubation period of 30 days, are summarised in Table 2 and illustrated in Fig. 6. A picture containing two samples from this study can be seen in Fig. 7. Both the samples have a smooth, saucy appearance which is an indication of the liquefaction of the fish tissue.

In most cases (Fig. 6), the addition of the different starters gave the same pH profiles as was found for the control. The biggest drop in pH was by culture 226 (*Lb. plantarum)* with a final value of 3.74 on day 10. This bacterium also produced a large amount of acid, presumably lactic acid, which resulted in the highest titratable acid value of 3.55 on day 30. Other starter cultures with similar pH drops but slightly lower %TA's were strains 407 (*Pc. cerevisiae)*, B3 2N (*Bac. mycoides)* and K6 2P (*Bac. amyloliquefaciens).*

Although a higher concentration and volume of inoculum, was used, the resulting fermentation profiles of the control sample again did not differ drastically, with a few exceptions, from most of the experimental profiles (Fig. 6). This led to the conclusion that the natural microorganisms present in fish, even under the more competitive conditions, still dominated the fermentation process. This is also the possibility that the sudden drop in pH and subsequent increase in acids may have inhibited the growth of all the bacteria. It was thus speculated that the addition of a buffer may help to solve this problem.

The most important reaction that takes place during the production of fermented fish is proteolysis and the subsequent production of amino components which strongly contribute to the aroma and taste of the final product. The aim of this study was also to compare the acid and free amino nitrogen formation by the different proteolytic bacteria as well as the lactic acid bacteria. Again starter 226 (*Lb. plantarum)* was found to be the best FAN producer with a production of 132 mg FAN. From the data obtained in this study, it was concluded that: cultures 226, 407, B3 2N and K6 2P; a higher moisture level of 150 ml; and a glucose level of 5% (w/w) gave the best results in terms of best pH changes, %TA and good FAN production.

Further methods, such as glucose concentration and incubation temperatures, should still be investigated to find the optimum conditions for the fermentation process.

Table 2. Results indicating the changes in pH, %TA and FAN over the incubation period of 30 days for the different strains.

Isolate	Time (d)	pH	%TA	FAN (mg)
Control	0	6.2	0.0	25
	5	4.0	1.9	
	10	4.2	2.1	96
	15	4.3	2.6	
	20	4.3	2.7	103
	25	4.3	3.1	
	30	4.3	3.3	109
Lc. diacetylactis 140	0	6.2	0.0	25
	5	4.0	1.7	
	10	4.1	1.9	89
	15	4.2	2.3	
	20	4.3	3.0	106
	25	4.2	2.6	
	30	4.3	3.2	112
Lb. acidophilus 897	0	6.2	0.0	25
	5	4.1	2.5	
	10	4.2	2.5	94
	15	4.3	2.3	
	20	4.3	2.8	100
	25	4.2	2.6	
	30	4.2	3.4	115
Lb. plantarum 226	0	6.2	0.0	25
	5	3.9	2.4	
	10	3.7	2.8	91
	15	3.9	3.2	
	20	3.9	3.2	108
	25	3.8	3.2	
	30	3.8	3.6	132
Pc. cerevisiae 407	0	6.2	0.0	25
	5	3.8	1.5	
	10	4.0	1.9	99
	15	4.3	2.6	
	20	4.3	2.7	103
	25	4.2	2.8	
	30	4.2	2.7	117

Isolate	Time (d)	pH	%TA	FAN (mg)
Koc. varians S1	0	6.20	0.0	25
	5	4.07	2.2	
	10	4.15	1.9	94
	15	4.26	1.9	
	20	4.32	2.5	106
	25	4.27	2.7	
	30	4.28	2.7	117
Ste. maltophilia B5 1M	0	6.20	0.0	25
	5	4.17	2.1	
	10	3.96	2.0	90
	15	4.28	2.5	
	20	4.34	2.3	104
	25	4.24	2.4	
	30	4.31	2.8	118
Chy. luteola K6 4P	0	6.20	0.0	25
	5	3.99	1.6	
	10	3.94	1.6	98
	15	4.29	2.3	
	20	4.30	2.5	111
	25	4.23	2.5	
	30	4.26	2.9	118
Bac. megaterium B5 3T	0	6.20	0.0	25
	5	4.08	2.1	
	10	4.13	2.1	94
	15	4.24	2.6	
	20	4.28	2.7	103
	25	4.25	3.0	
	30	4.27	3.2	116
Bac. subtilus B1 1Y	0	6.20	0.0	25
	5	4.18	2.7	
	10	4.00	1.7	98
	15	4.18	2.3	
	20	4.29	2.7	108
	25	4.22	2.4	
	30	4.32	3.2	120

Isolate	Time (d)	pH	%TA	FAN (mg)
Bac. cereus B3 1T	0	6.2	0.0	25
	5	4.2	2.2	
	10	4.1	2.3	85
	15	4.2	2.9	
	20	4.30	2.7	93
	25	4.2	2.4	
	30	4.3	2.4	103
Bac. mycoides B3 2N	0	6.2	0.0	25
	5	4.0	1.6	
	10	3.8	1.7	80
	15	4.2	1.5	
	20	4.2	2.0	92
	25	4.2	2.5	
	30	4.3	2.6	99
Bac. amyloli-quefaciens K6 2P	0	6.2	0.0	25
	5	4.0	2.2	
	10	3.8	2.0	99
	15	4.3	2.4	
	20	4.3	2.6	104
	25	4.3	2.4	
	30	4.3	2.6	120
Bac. lentus K6 5P	0	6.2	0.0	25
	5	3.9	1.8	
	10	4.1	1.8	90
	15	4.2	2.2	
	20	4.3	2.7	96
	25	4.2	2.5	
	30	4.2	2.7	109
Bac. licheniformis B3 2Y	0	6.2	0.0	25
	5	4.3	2.0	
	10	4.2	2.3	91
	15	4.3	2.6	
	20	4.3	2.6	95
	25	4.3	2.2	
	30	4.3	3.1	108

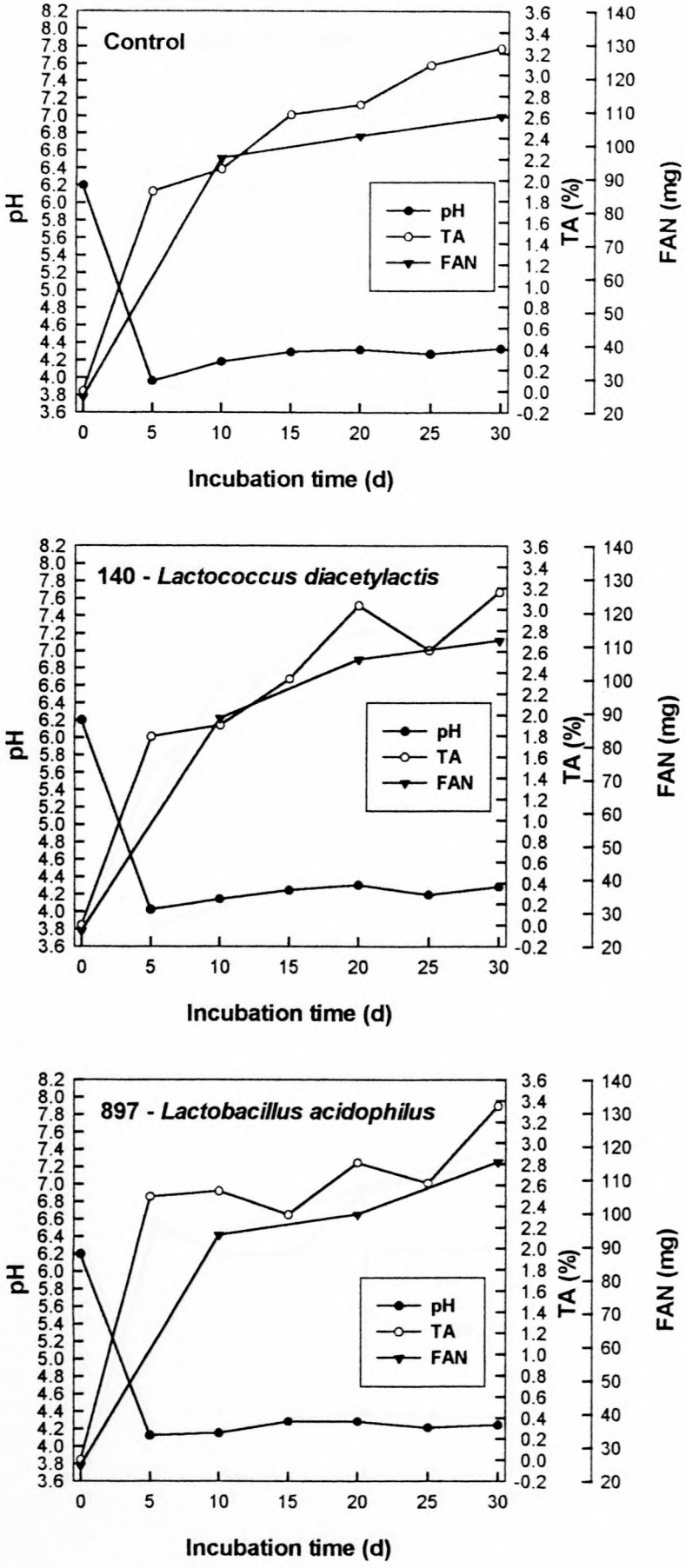
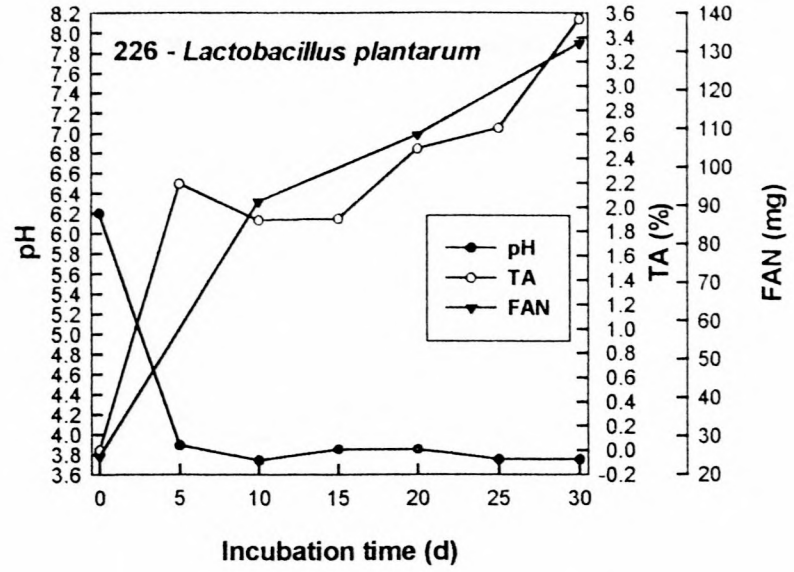
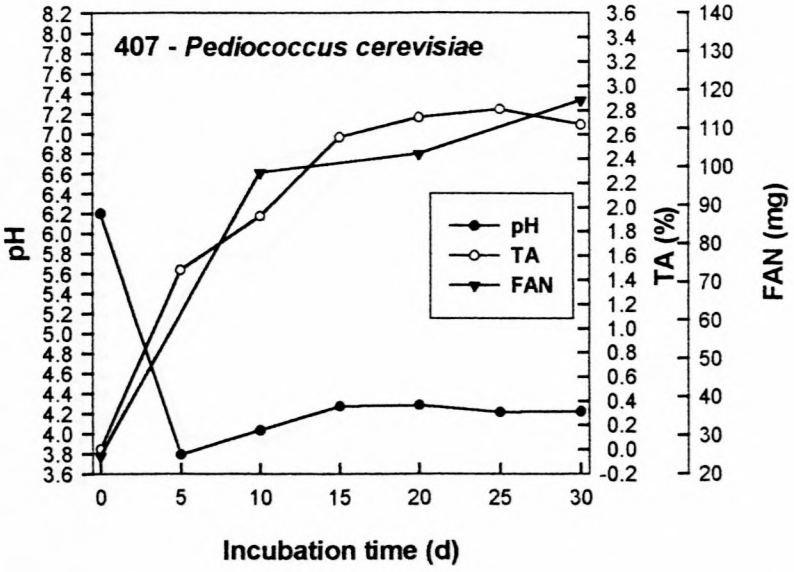


Figure 6. Influence of different bacterial strains on pH, %TA and FAN over the 30 day incubation period with 150 ml water added per 100 g fish.





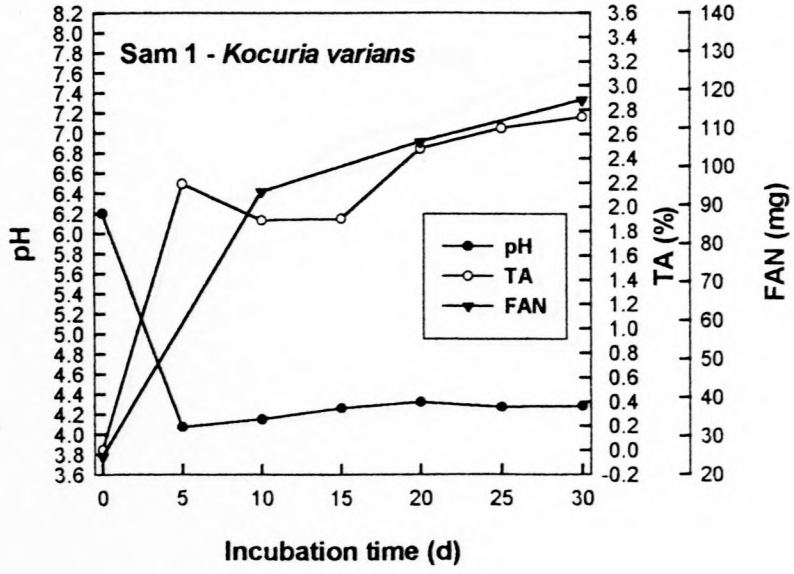
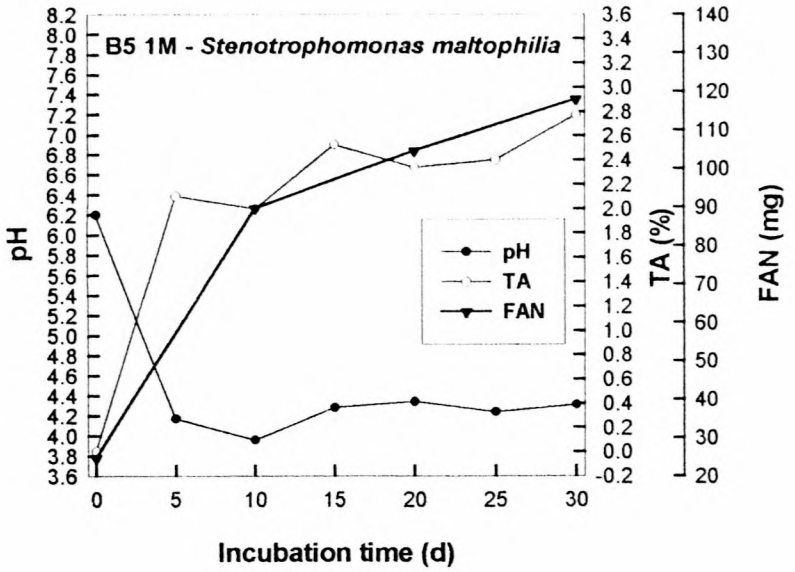
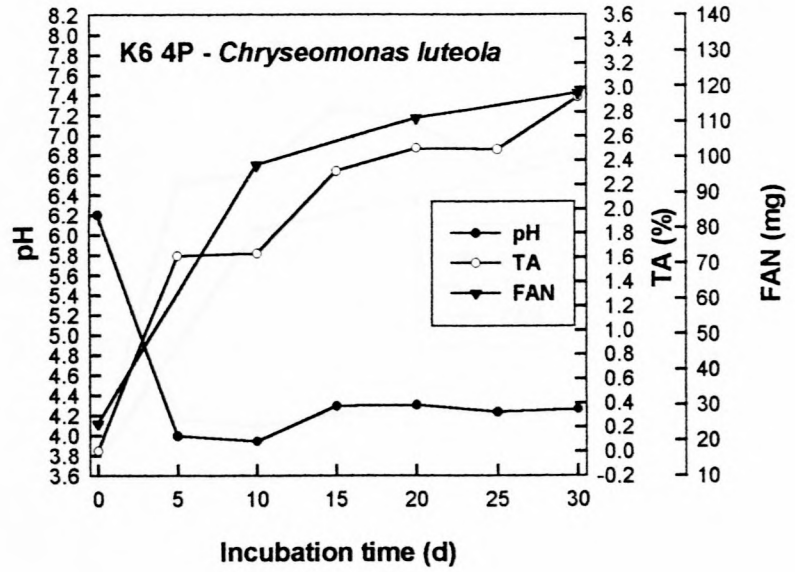


Figure 6. (continued).





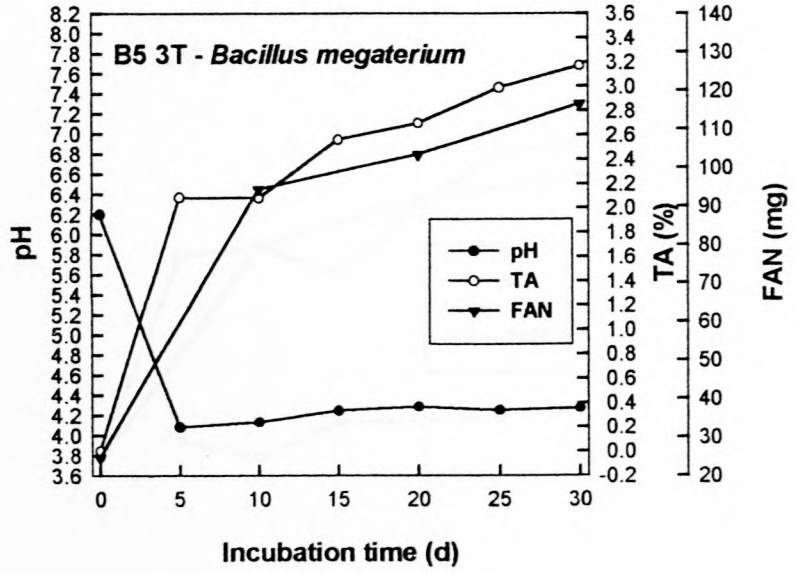
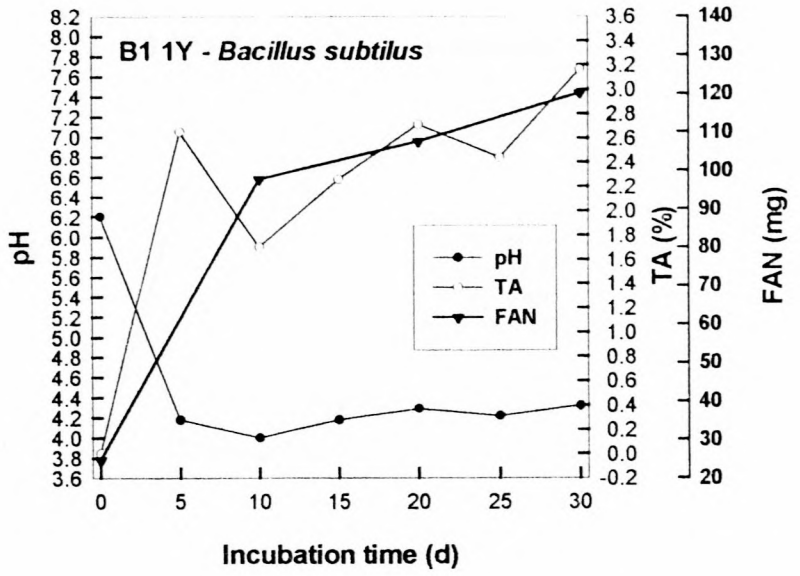
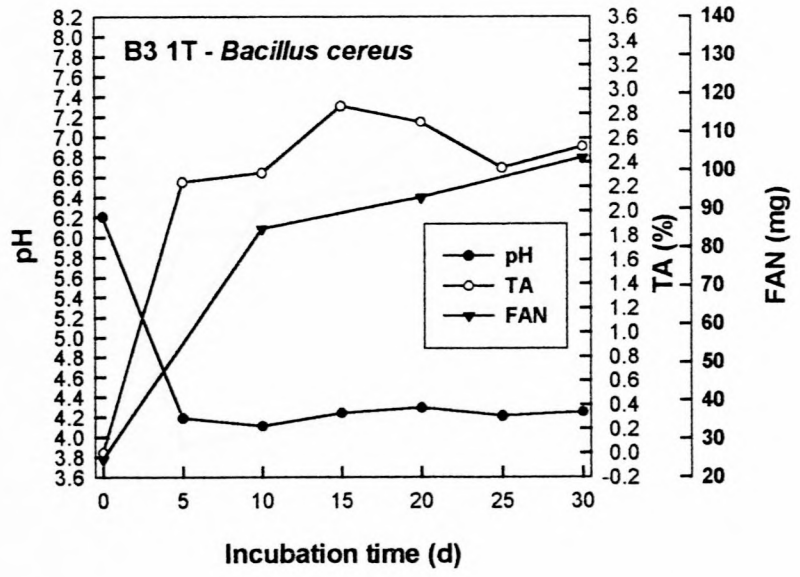


Figure 6. (continued).





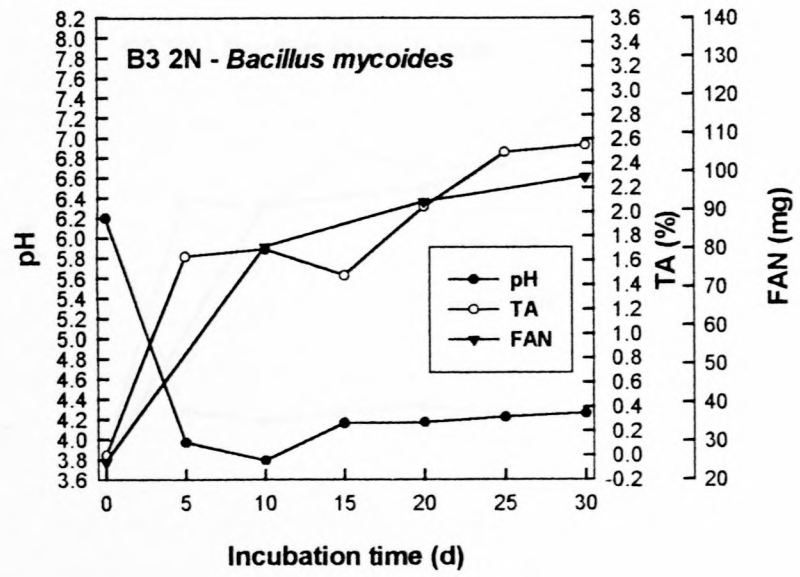
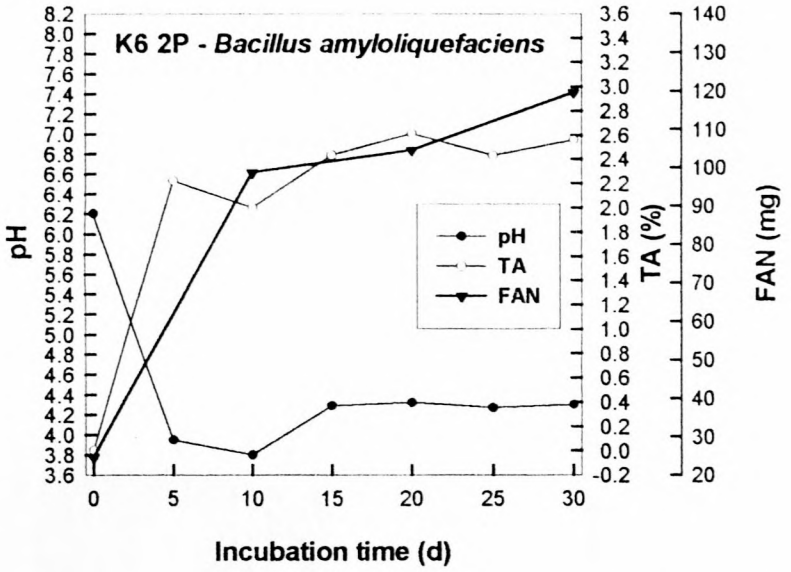
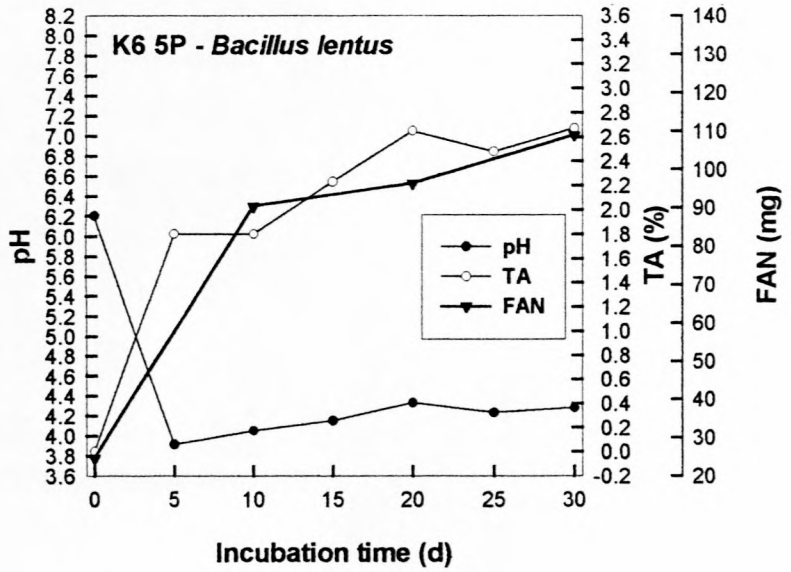


Figure 6. (continued).





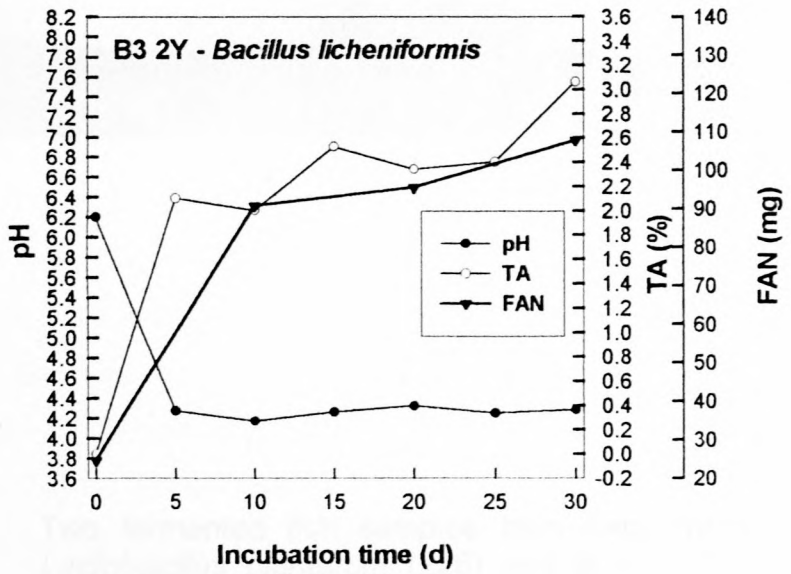


Figure 6. (continued).

Figure 7. Two fermented fish samples from Experimental Study 3. A = *Lactobacillus plantarum* (226) and B = *Lactococcus diacetylactis* (140).

Experimental Study 4: Influence of different glucose concentrations and the addition of a buffer on the production parameters

In this study, the reactions of the 14 selected strains (Table 1) to different glucose concentrations (1.0, 2.5 and 5.0% w/w) and 150 ml added water, were evaluated. A buffer was also added to one of the fish batches to determine if the high level of acid and low pH really plays a role on the growth and metabolism of the bacteria.

The results of the changes in pH, %TA and FAN during the fermentation period of 30 days with added glucose as well as an added buffer, are summarised in Tables 3.1 to 3.4 and the subsequent results are illustrated in Fig. 8 to 11, respectively. By comparing the profiles of the different glucose concentrations, it was found that, the higher the glucose concentration, the larger the drop in pH was. The increased glucose concentration also resulted in an increase in acid production (%TA) and higher free amino nitrogen (FAN) production.

Isolates that produced high levels of FAN combined with a low pH with the 1% added glucose were 226 (*Lb. plantarum),* 140 (*Lc. diacetylactis)* and 407 (*Pd. cerevisiae)* (Fig. 8). At the 2.5% glucose concentration, 140, 226, 407, B3 1T (*Bac. cereus)* and B3 2N (*Bac. mycoides)* were the most suitable isolates resulting in the best FAN/pH combination (Fig. 9). *Lactobacillus plantarum* (226) was the best lactic acid bacterial isolate in the trial where 5% glucose was used (Fig. 10). The pH was lowered to 3.8, the %TA was 3.2, and the FAN production was 128 mg. Other isolates that produced high levels of acid, a low pH as well as good FAN production in this trial were B3 2N, B3 1T, K6 4P, B5 1M, 140, 897 and Sam 1 (*Koc. varians).*

In Study 3 it was speculated that an initial sharp drop in pH might cause the inhibition of the growth and metabolism of the inoculated bacteria and prevent them degrading the proteins with subsequent production of free amino nitrogenous products. Although a buffer was added to the last batch of fish (Table 3.4 and Fig. 11), the data clearly showed that it did not prevent the fast lowering of the pH.

It can be concluded from the above data that a higher level of glucose is essential for a higher production of free amino nitrogen as well as the high acid production and fast lowering of the pH that is needed to act as a preservative for the product. The isolate that performed well under these conditions was the *Lb. plantarum* strain (226) followed by some of the other lactic acid bacteria, namely *Pc. cerevisiae* (407) and *Lc. diacetylactis* (140).

Table 3.1. Results indicating the changes in pH, %TA and FAN over the incubation period of 30 days for 1.0% (w/w) glucose added.

Isolate	Time (d)	pH	%TA	FAN (mg)
Control	0	6.3	0.0	24
	5	6.0	0.6	52
	10	6.1	1.2	66
	15	5.9	1.7	
	20	5.9	1.8	62
	25	6.0	1.8	
	30	6.0	1.8	81
Lc. diacetylactis 140	0	6.3	0.0	24
	5	4.9	0.7	43
	10	4.9	1.1	72
	15	5.4	1.6	
	20	5.5	2.0	67
	25	5.5	1.8	
	30	5.4	2.0	105
Lb. acidophilus 897	0	6.3	0.0	24
	5	6.0	0.6	60
	10	6.2	1.1	66
	15	6.2	1.7	
	20	6.2	1.8	94
	25	6.4	2.1	
	30	6.5	2.2	100
Lb. plantarum 226	0	6.3	0.0	24
	5	5.0	0.6	45
	10	5.7	1.2	76
	15	5.6	2.2	
	20	5.6	2.1	86
	25	5.7	2.5	
	30	5.7	2.6	92
Pc. cerevisiae 407	0	6.3	0.0	24
	5	4.8	0.7	44
	10	5.1	1.1	65
	15	5.5	1.9	
	20	5.4	2.1	84
	25	5.5	2.2	
	30	5.4	2.2	103

Isolate	Time (d)	pH	%TA	FAN (mg)
Koc. varians S1	0	6.3	0.0	24
	5	5.6	0.7	49
	10	5.9	1.4	86
	15	6.0	2.0	
	20	5.9	2.0	90
	25	6.0	2.3	
	30	6.2	2.4	98
Ste. maltophilia B5 1M	0	6.3	0.0	24
	5	5.9	0.5	32
	10	5.7	1.1	63
	15	5.8	1.5	
	20	5.9	1.7	62
	25	5.9	1.8	
	30	6.0	1.8	93
Chy. luteola K6 4P	0	6.3	0.0	24
	5	5.6	0.6	40
	10	5.7	1.2	71
	15	5.8	2.1	
	20	5.9	2.2	99
	25	6.0	2.3	
	30	6.1	2.4	100
Bac. megaterium B5 3T	0	6.3	0.0	24
	5	5.5	0.7	49
	10	5.6	1.0	63
	15	5.4	1.7	
	20	5.7	1.8	68
	25	5.9	2.0	
	30	5.9	2.0	93
Bac. subtilus B1 1Y	0	6.3	0.0	24
	5	5.5	0.6	43
	10	5.5	1.0	62
	15	5.8	1.4	
	20	5.7	2.5	66
	25	5.7	2.5	
	30	5.6	2.5	76

Isolate	Time (d)	pH	%TA	FAN (mg)
Bac. cereus B3 1T	0	6.3	0.0	24
	5	5.2	0.6	38
	10	5.4	1.1	64
	15	5.6	1.8	
	20	5.5	2.3	75
	25	5.6	2.3	
	30	5.5	2.3	101
Bac. mycoides B3 2N	0	6.3	0.0	24
	5	5.5	0.7	35
	10	5.7	1.0	60
	15	5.8	1.4	
	20	5.8	1.6	61
	25	5.9	1.9	
	30	6.0	2.0	81
Bac. amyloli-quefaciens K6 2P	0	6.3	0.0	24
	5	5.4	0.6	48
	10	5.4	1.1	82
	15	5.9	1.4	
	20	5.9	1.7	84
	25	5.8	2.4	
	30	5.8	2.4	108
Bac. lentus K6 5P	0	6.3	0.0	24
	5	5.7	0.5	67
	10	5.7	1.2	71
	15	5.6	1.9	
	20	5.8	2.6	93
	25	5.8	2.4	
	30	5.8	2.5	104
Bac. licheniformis B3 2Y	0	6.3	0.0	24
	5	5.6	0.5	36
	10	5.7	0.9	51
	15	5.6	1.5	
	20	5.6	2.1	59
	25	5.7	2.1	
	30	5.6	2.1	104

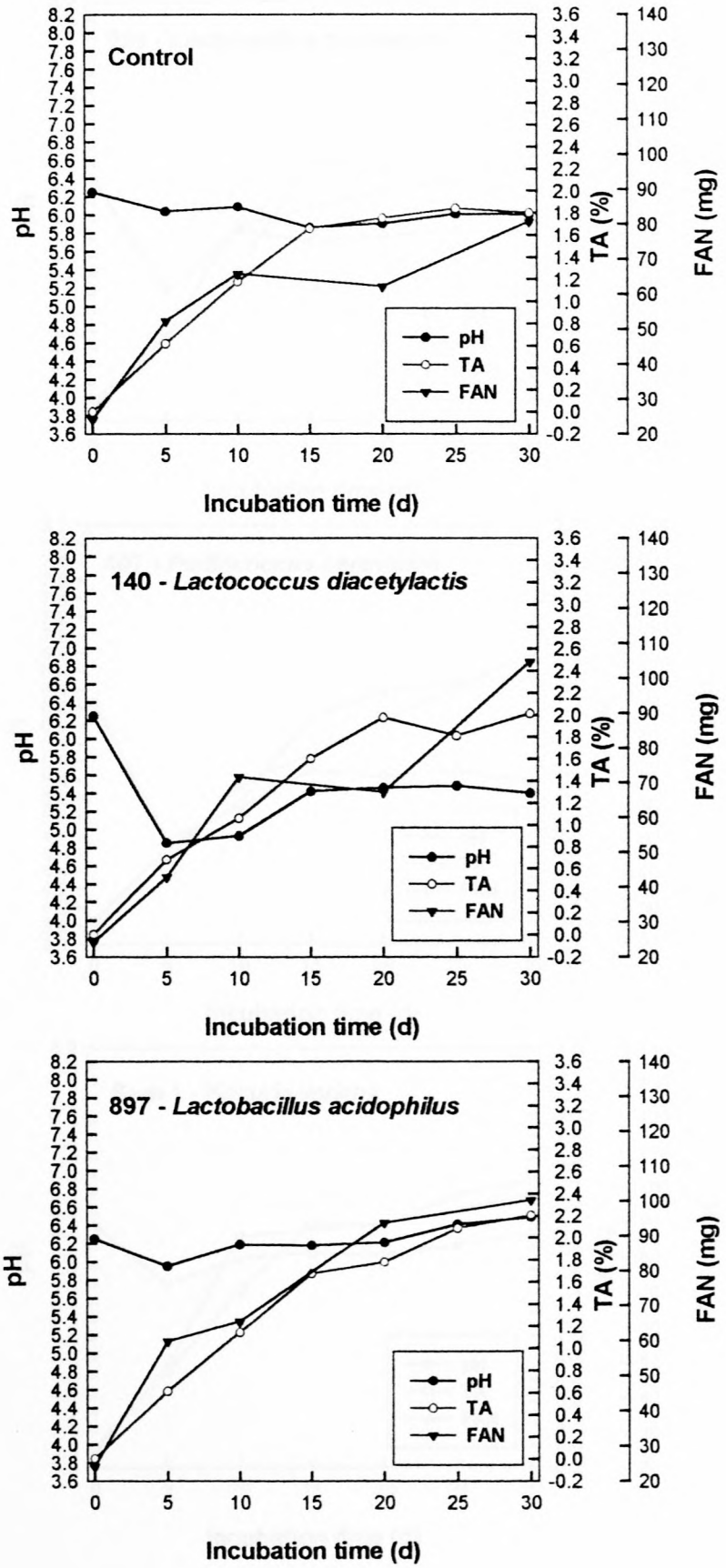


Figure 8. Influence of different bacterial strains on pH, %TA and FAN over the 30 day incubation period with 1.0% (w/w) glucose.

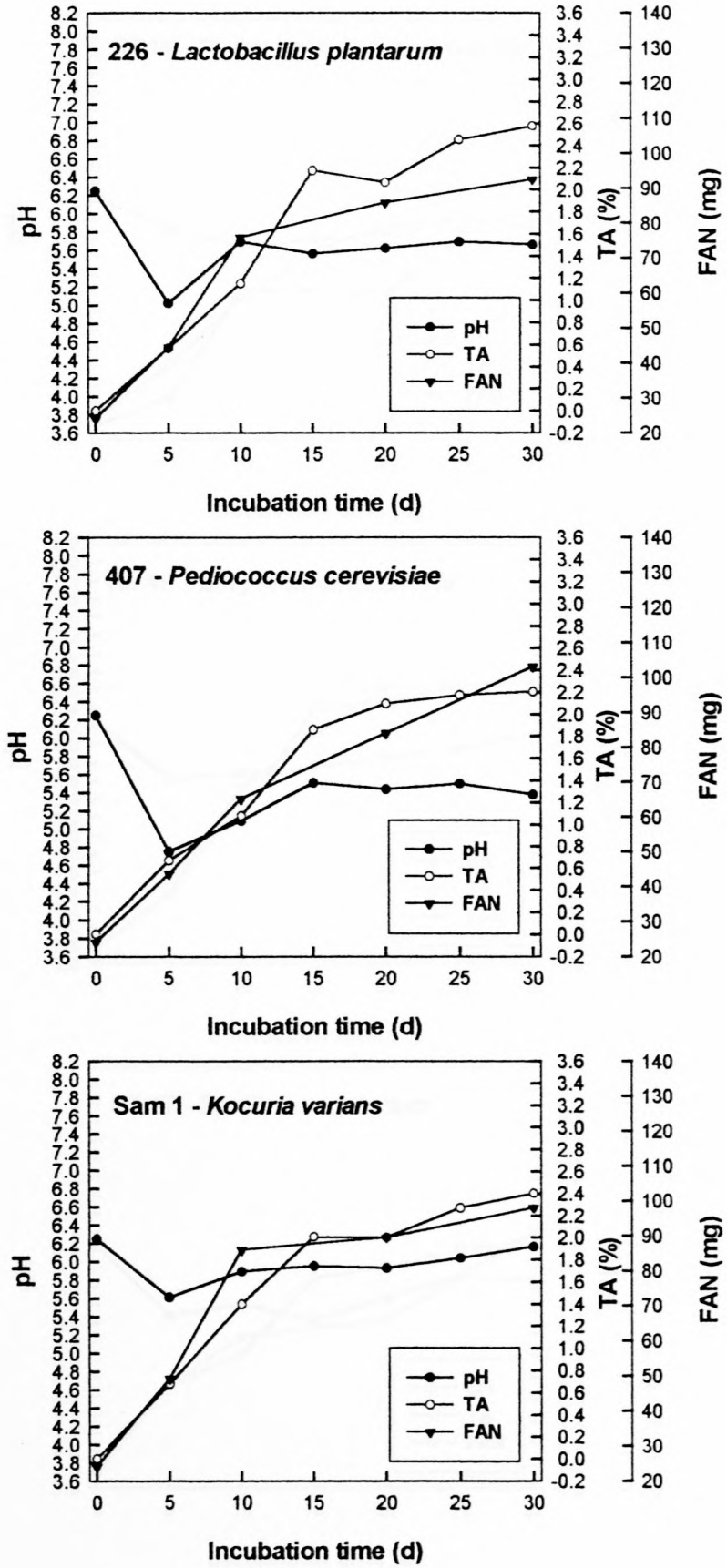


Figure 8. (continued).

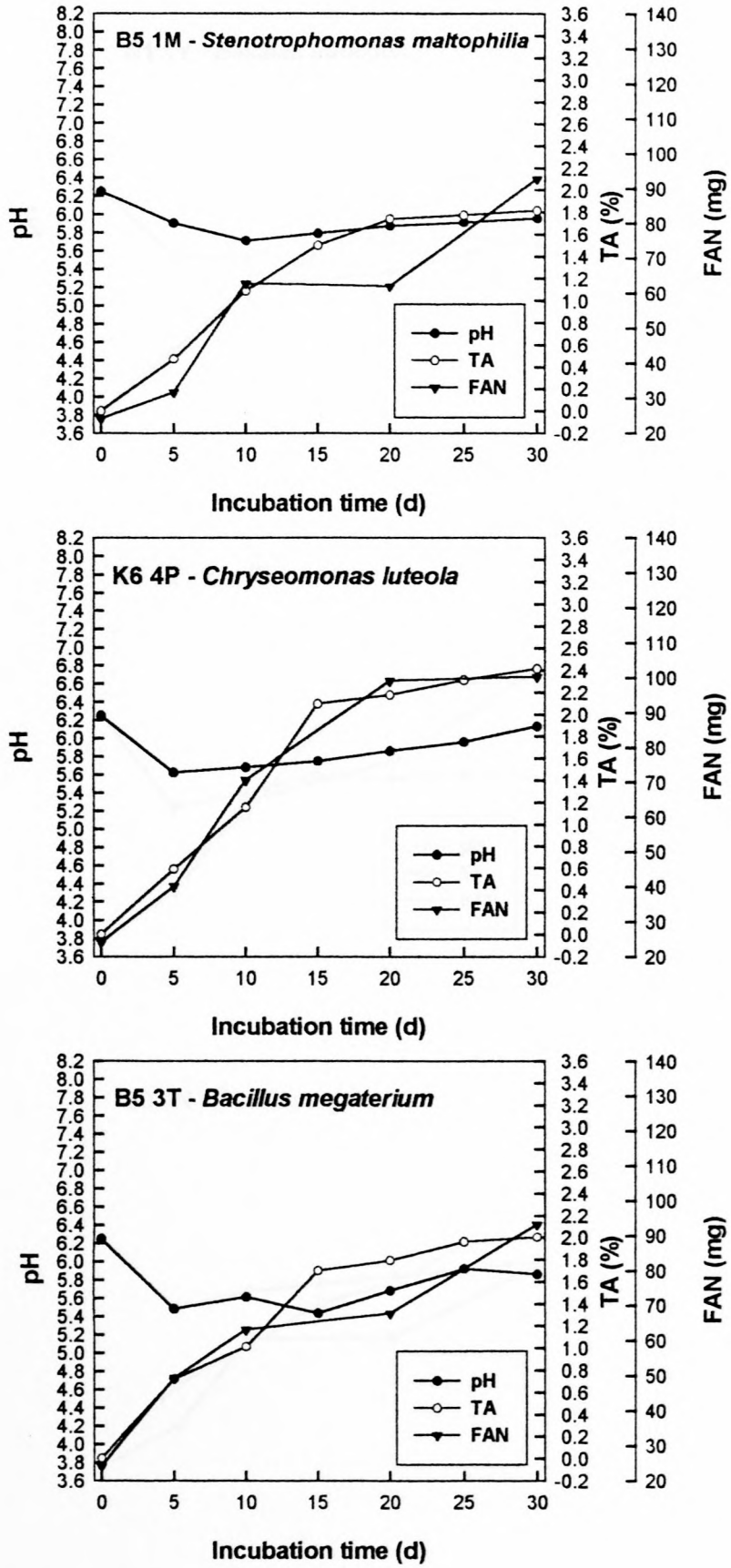


Figure 8. (continued).

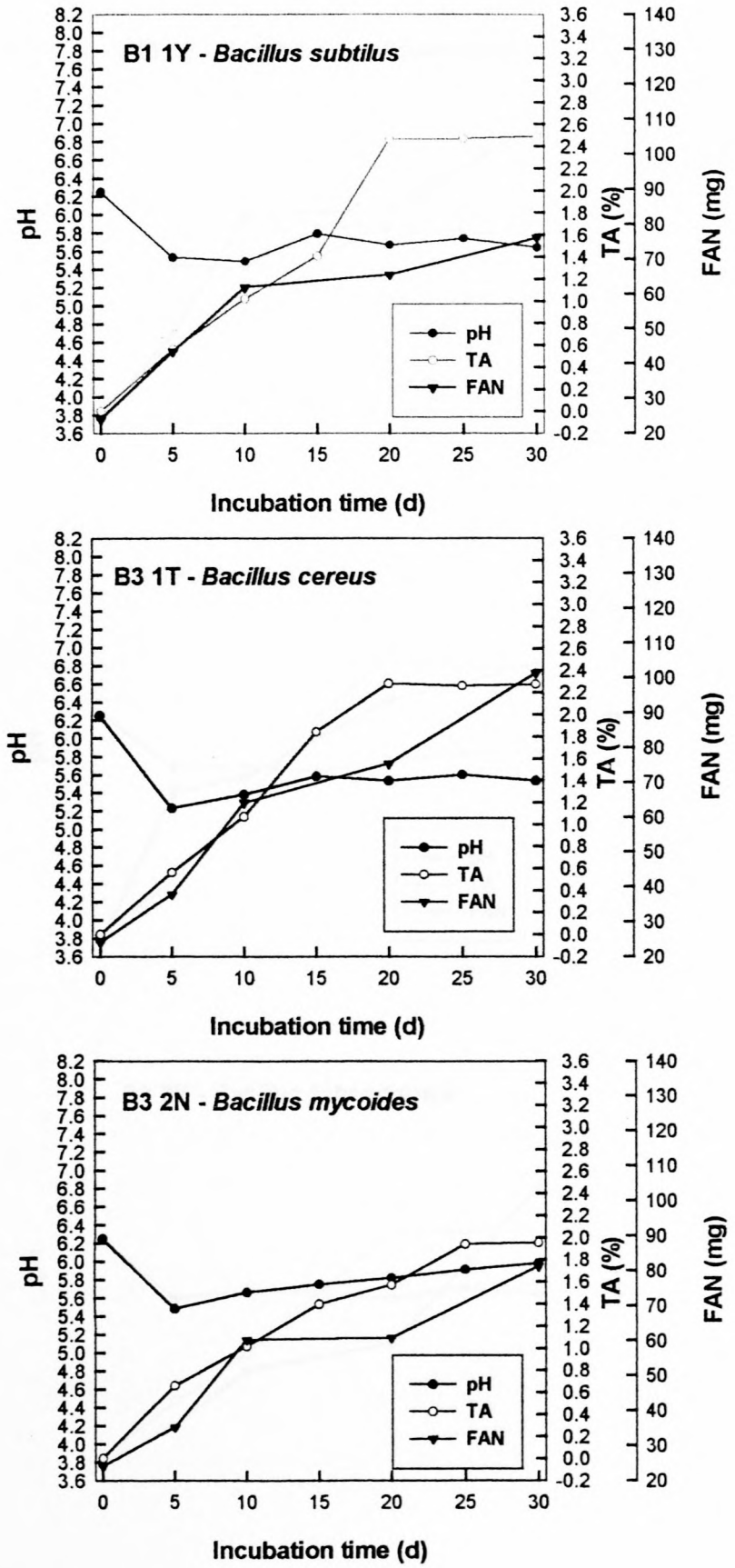


Figure 8. (continued).

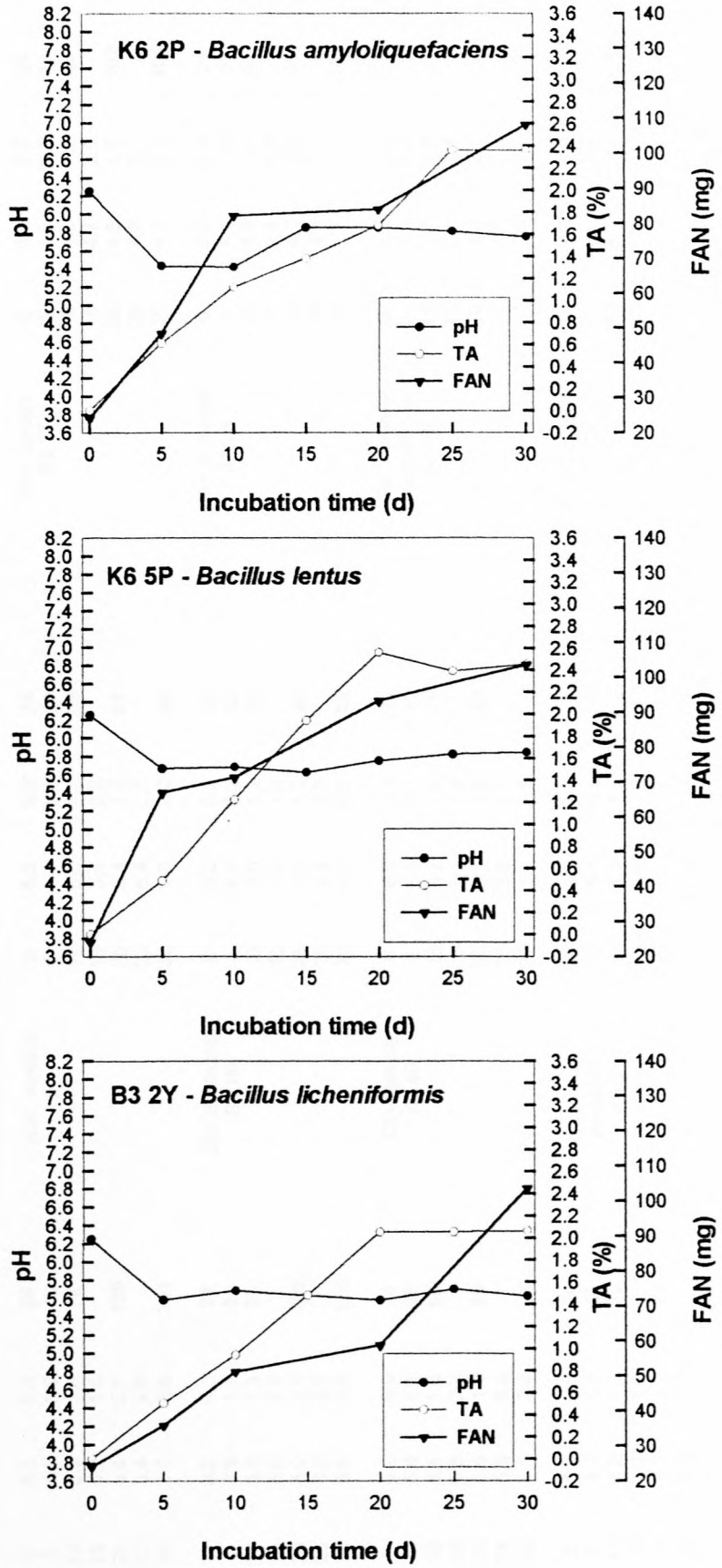


Figure 8. (continued).

Table 3.2. Results indicating the changes in pH, %TA and FAN over the incubation period of 30 days for 2.5% (w/w) glucose added.

Isolate	Time (d)	pH	%TA	FAN (mg)
Control	0	6.2	0.0	24
	5	4.9	0.9	46
	10	5.0	1.6	72
	15	5.2	1.5	
	20	5.4	2.1	100
	25	5.4	2.6	
	30	5.3	2.6	101
Lc. diacetylactis 140	0	6.2	0.0	24
	5	4.5	1.1	54
	10	4.8	1.3	85
	15	5.0	2.0	
	20	5.0	2.0	106
	25	5.5	2.5	
	30	5.8	2.5	116
Lb. acidophilus 897	0	6.2	0.0	24
	5	5.0	1.1	66
	10	5.4	1.0	66
	15	5.6	2.3	
	20	5.5	1.9	99
	25	5.6	2.0	
	30	5.5	2.2	100
Lb. plantarum 226	0	6.2	0.0	24
	5	4.1	1.2	55
	10	4.2	1.3	70
	15	4.3	1.9	
	20	4.2	2.0	84
	25	4.4	2.3	
	30	4.2	2.3	93
Pc. cerevisiae 407	0	6.2	0.0	24
	5	4.4	1.3	61
	10	4.3	1.5	81
	15	4.4	2.2	
	20	4.2	2.0	103
	25	4.4	2.2	
	30	4.2	2.2	105

Isolate	Time (d)	pH	%TA	FAN (mg)
Koc. varians S1	0	6.2	0.0	24
	5	5.0	0.9	59
	10	5.0	1.1	69
	15	5.4	2.2	
	20	5.4	1.8	87
	25	5.4	2.1	
	30	5.3	2.2	96
Ste. maltophilia B5 1M	0	6.2	0.0	24
	5	5.0	0.9	52
	10	5.0	1.2	66
	15	5.2	1.8	
	20	5.3	1.7	89
	25	5.4	1.9	
	30	5.2	2.0	90
Chy. luteola K6 4P	0	6.2	0.0	24
	5	5.2	0.7	42
	10	5.7	1.4	76
	15	5.5	1.8	
	20	5.6	1.8	99
	25	5.8	2.2	
	30	5.7	2.2	114
Bac. megaterium B5 3T	0	6.2	0.0	24
	5	5.3	0.8	52
	10	5.7	1.1	67
	15	5.6	2.2	
	20	5.7	1.7	116
	25	5.9	2.4	
	30	5.8	2.5	118
Bac. subtilus B1 1Y	0	6.2	0.0	24
	5	5.2	1.2	64
	10	5.8	1.1	65
	15	5.6	1.2	
	20	5.7	2.1	84
	25	5.8	1.9	
	30	5.7	1.9	95

Isolate	Time (d)	pH	%TA	FAN (mg)
Bac. cereus B3 1T	0	6.2	0.0	24
	5	4.5	0.7	43
	10	4.5	1.2	87
	15	4.7	2.0	
	20	4.6	1.7	102
	25	4.7	2.1	
	30	4.6	2.2	104
Bac. mycoides B3 2N	0	6.2	0.0	24
	5	4.4	1.9	51
	10	4.6	1.3	87
	15	4.8	1.8	
	20	4.7	2.0	95
	25	4.9	2.1	
	30	4.7	2.2	101
Bac. amyloli-quefaciens K6 2P	0	6.2	0.0	24
	5	5.4	0.8	50
	10	5.7	1.0	74
	15	6.0	1.6	
	20	6.2	2.0	111
	25	6.5	2.1	
	30	6.6	2.1	112
Bac. lentus K6 5P	0	6.2	0.0	24
	5	5.0	0.8	50
	10	4.8	1.3	80
	15	4.9	1.7	
	20	4.9	1.7	94
	25	5.0	1.7	
	30	5.0	1.8	95
Bac. licheniformis B3 2Y	0	6.2	0.0	24
	5	5.1	0.7	42
	10	5.5	1.2	57
	15	5.5	1.7	
	20	5.5	1.8	85
	25	5.6	1.8	
	30	5.5	1.9	88

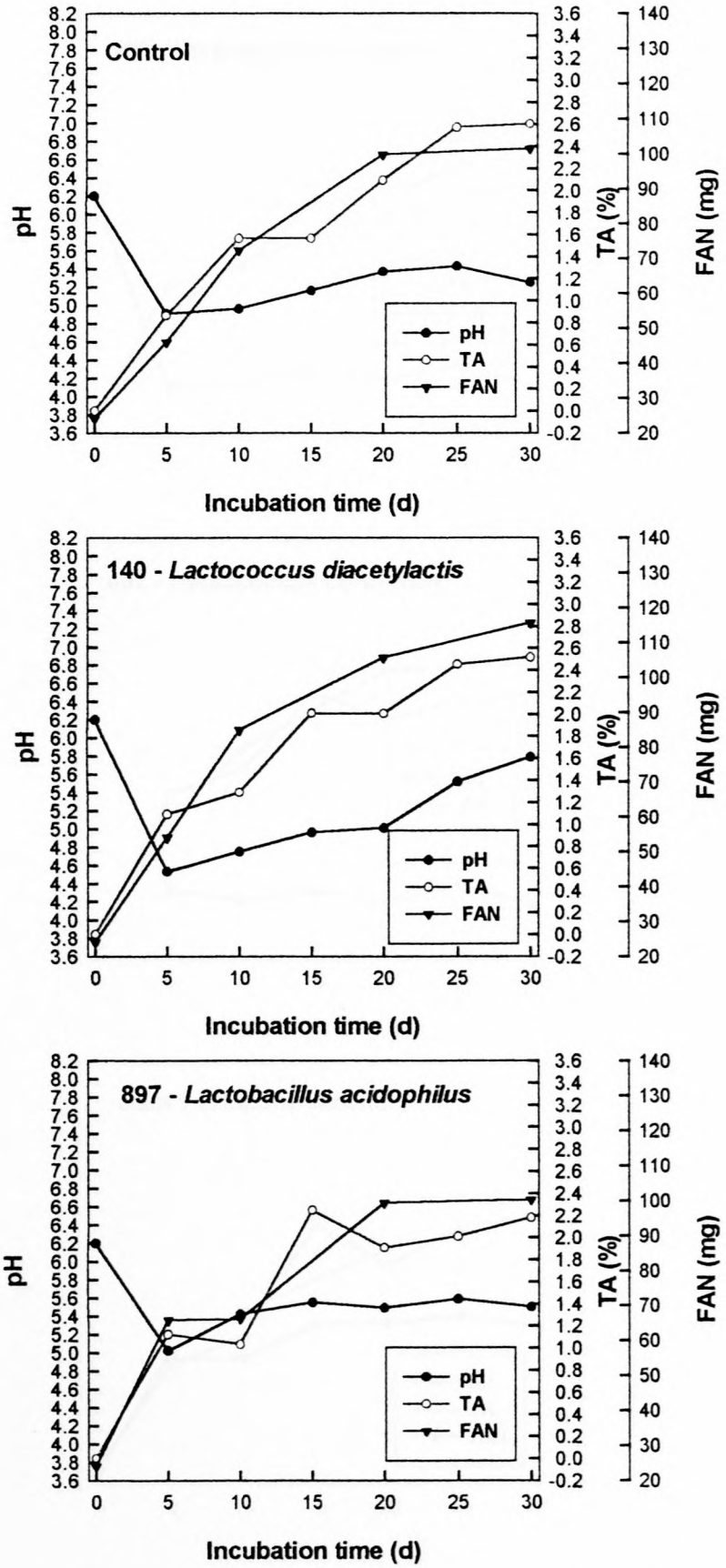


Figure 9. Influence of different bacterial strains on pH, %TA and FAN over the 30 day incubation period with 2.5% (w/w) glucose.

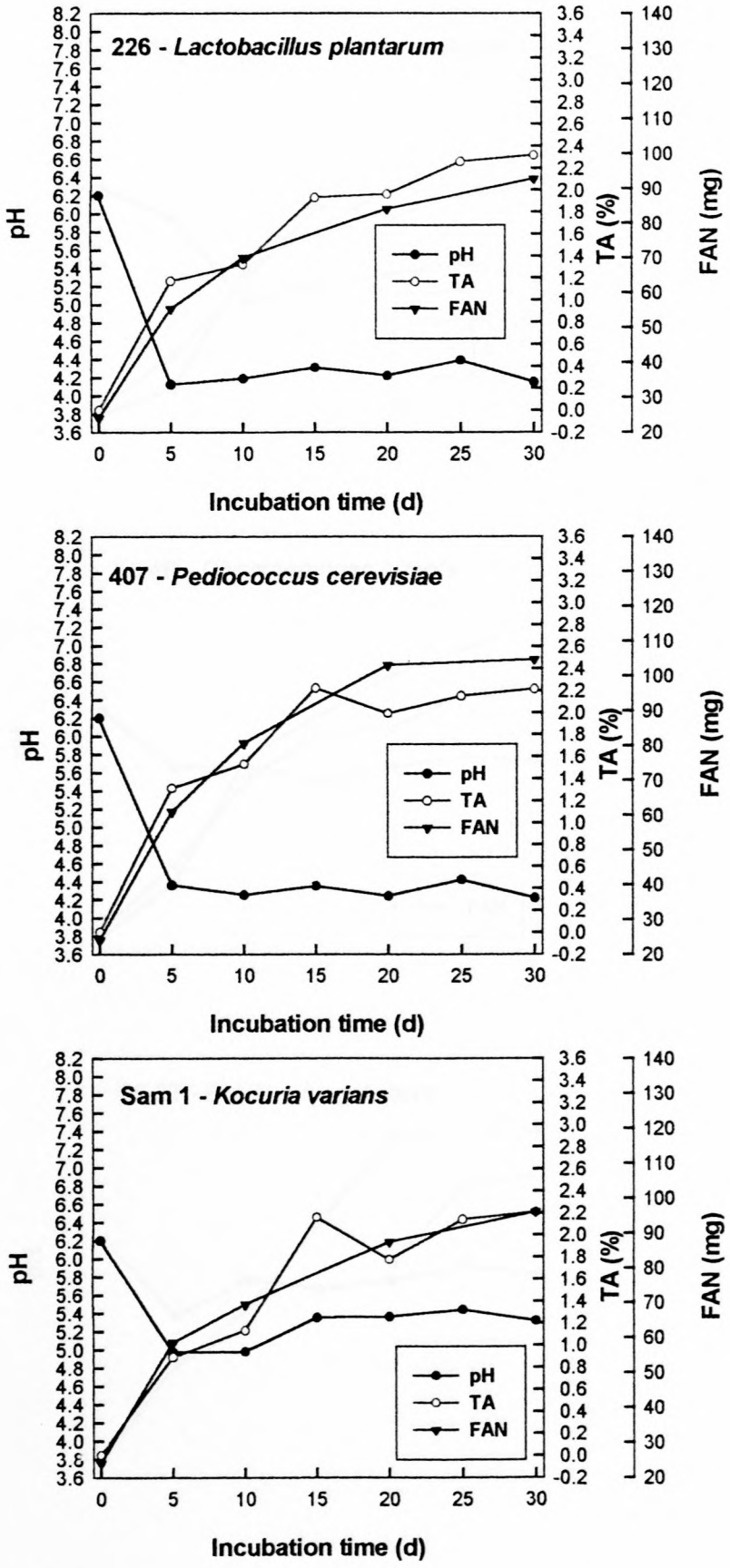


Figure 9. (continued).

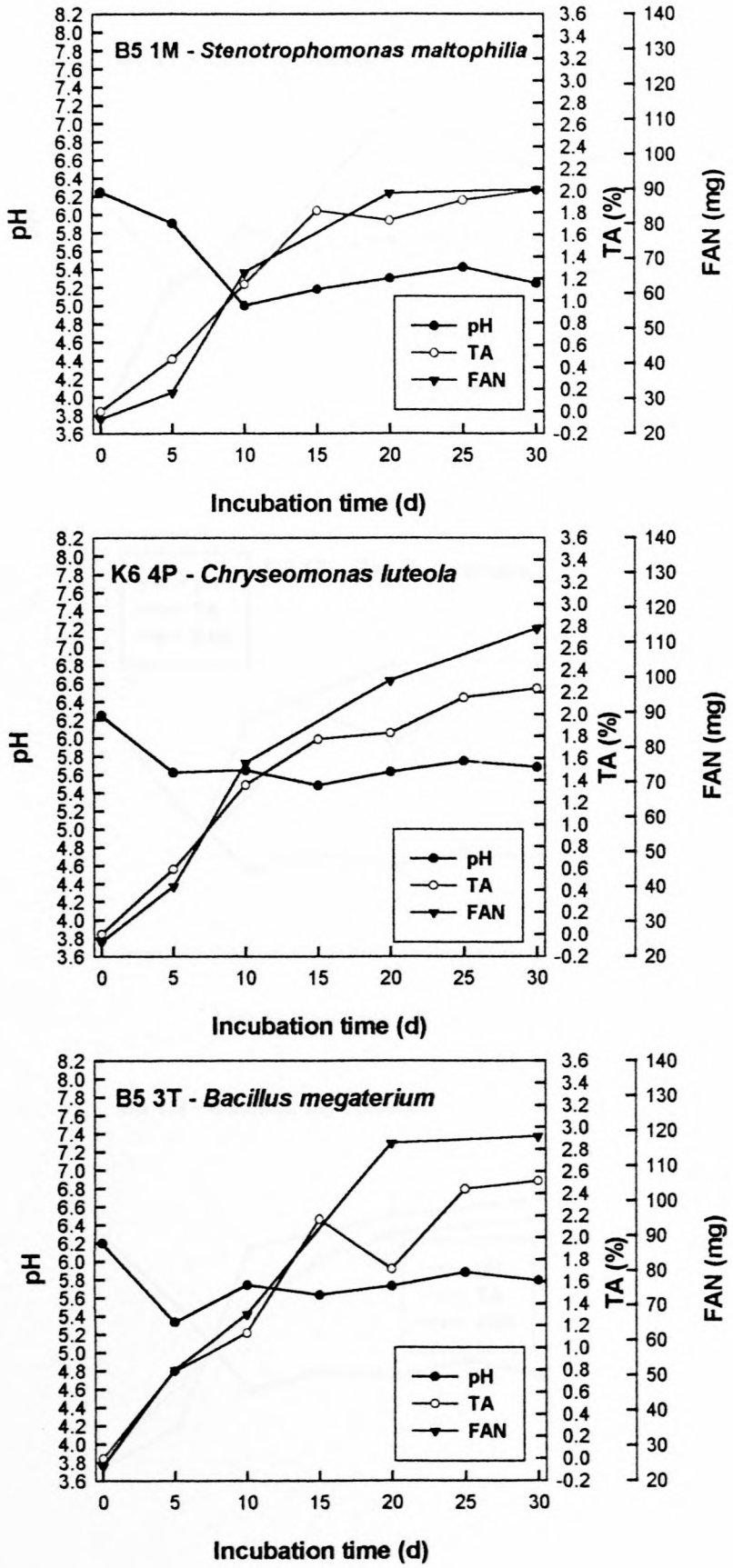


Figure 9. (continued).

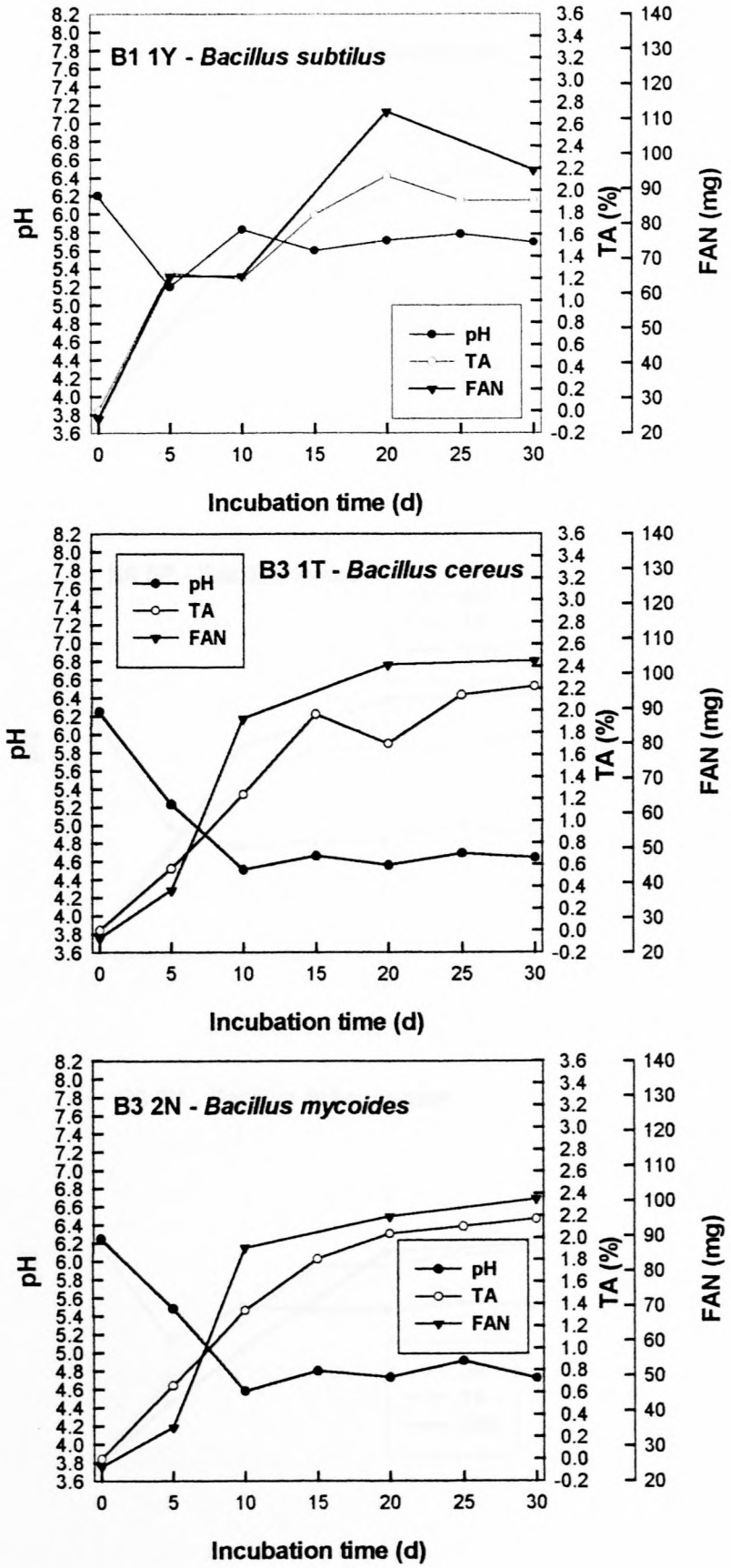


Figure 9. (continued).

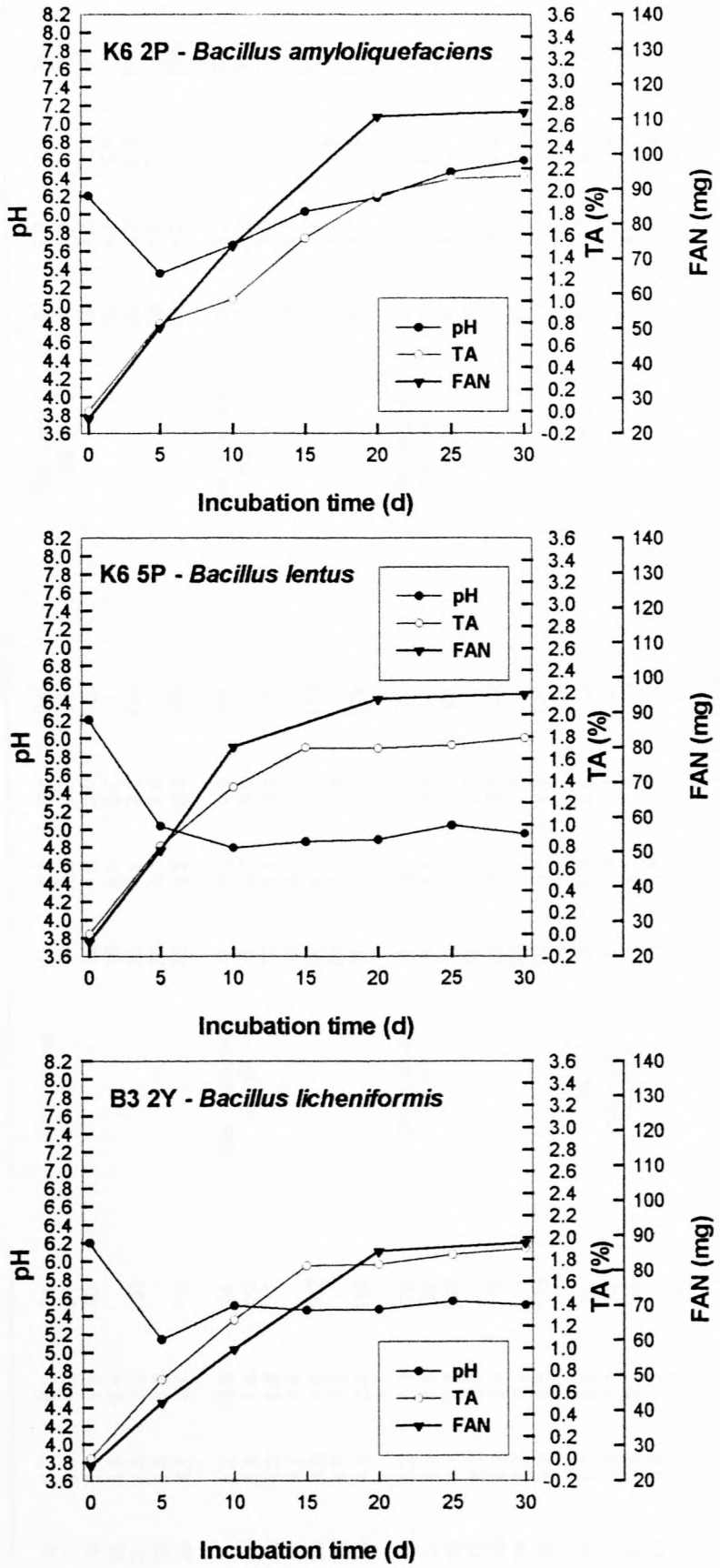


Figure 9. (continued).

Table 3.3. Results indicating the changes in pH, %TA and FAN over the incubation period of 30 days for 5.0% (w/w) glucose added.

Isolate	Time (d)	pH	%TA	FAN (mg)
Control	0	6.2	0.0	24
	5	4.4	1.0	61
	10	3.9	1.9	90
	15	3.9	2.4	
	20	4.0	2.5	103
	25	4.0	2.6	
	30	4.1	2.7	113
Lc. diacetylactis 140	0	6.2	0.0	24
	5	4.1	1.0	50
	10	4.2	1.8	77
	15	4.1	1.8	
	20	4.3	2.0	107
	25	4.2	2.1	
	30	4.4	2.2	115
Lb. acidophilus 897	0	6.2	0.0	24
	5	4.4	0.8	59
	10	4.1	2.0	101
	15	4.1	2.3	
	20	4.2	2.4	115
	25	4.2	2.4	
	30	4.2	2.5	116
Lb. plantarum 226	0	6.2	0.0	24
	5	3.9	1.5	72
	10	3.8	2.5	98
	15	3.8	3.3	
	20	3.9	3.2	114
	25	3.9	3.1	
	30	3.9	3.3	128
Pc. cerevisiae 407	0	6.2	0.0	24
	5	4.6	0.8	58
	10	4.4	1.4	98
	15	4.4	2.3	
	20	4.4	2.0	106
	25	4.4	2.5	
	30	4.5	2.6	112

Isolate	Time (d)	pH	%TA	FAN (mg)
Koc. varians S1	0	6.2	0.0	24
	5	4.0	1.2	71
	10	4.0	2.0	90
	15	4.0	2.7	
	20	4.1	2.8	123
	25	4.0	2.5	
	30	4.2	2.8	126
Ste. maltophilia B5 1M	0	6.2	0.0	24
	5	4.7	0.6	47
	10	4.1	2.2	90
	15	4.2	2.5	
	20	4.3	2.4	115
	25	4.2	2.3	
	30	4.2	2.3	129
Chy. luteola K6 4P	0	6.2	0.0	24
	5	4.4	0.9	59
	10	4.1	1.9	90
	15	4.0	2.7	
	20	4.1	2.5	132
	25	4.1	2.5	
	30	4.1	2.5	134
Bac. megaterium B5 3T	0	6.2	0.0	24
	5	5.0	1.1	67
	10	5.1	1.2	77
	15	5.5	1.8	
	20	5.6	1.9	84
	25	5.5	2.5	
	30	5.6	2.5	125
Bac. subtilus B1 1Y	0	6.2	0.0	24
	5	4.5	1.2	59
	10	4.0	1.7	86
	15	4.0	2.5	
	20	4.1	2.4	76
	25	4.1	2.3	
	30	4.2	2.3	102

Isolate	Time (d)	pH	%TA	FAN (mg)
Bac. cereus B3 1T	0	6.2	0.0	24
	5	4.3	1.3	89
	10	4.1	2.0	100
	15	4.1	2.8	
	20	4.2	2.9	105
	25	4.2	2.4	
	30	4.2	2.5	125
Bac. mycoides B3 2N	0	6.2	0.0	24
	5	4.2	1.0	57
	10	3.8	2.2	92
	15	3.9	3.0	
	20	3.9	2.6	98
	25	3.9	2.5	
	30	4.0	2.6	115
Bac. amyloli-quefaciens K6 2P	0	6.2	0.0	24
	5	5.0	0.7	48
	10	4.4	1.4	81
	15	4.4	2.1	
	20	4.3	2.2	79
	25	4.2	2.5	
	30	4.3	2.5	103
Bac. lentus K6 5P	0	6.2	0.0	24
	5	4.2	1.0	54
	10	4.0	1.9	94
	15	4.1	2.2	
	20	4.1	2.3	87
	25	4.1	2.3	
	30	4.2	2.4	128
Bac. licheniformis B3 2Y	0	6.2	0.0	24
	5	5.4	0.7	59
	10	6.2	1.6	75
	15	6.0	3.0	
	20	6.2	2.1	81
	25	6.2	2.1	
	30	6.2	2.2	118

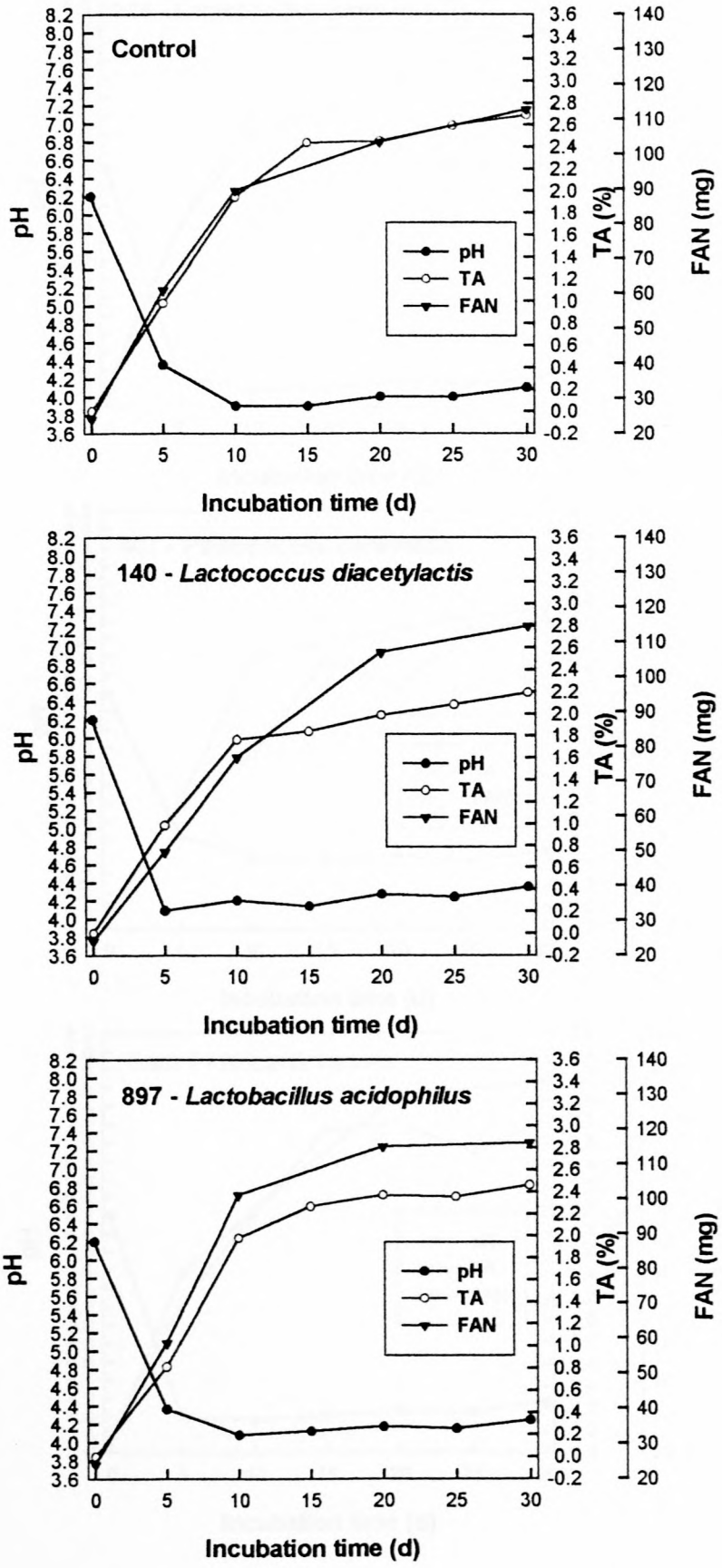


Figure 10. Influence of different bacterial strains on pH, %TA and FAN over the 30 day incubation period with 5.0% glucose.

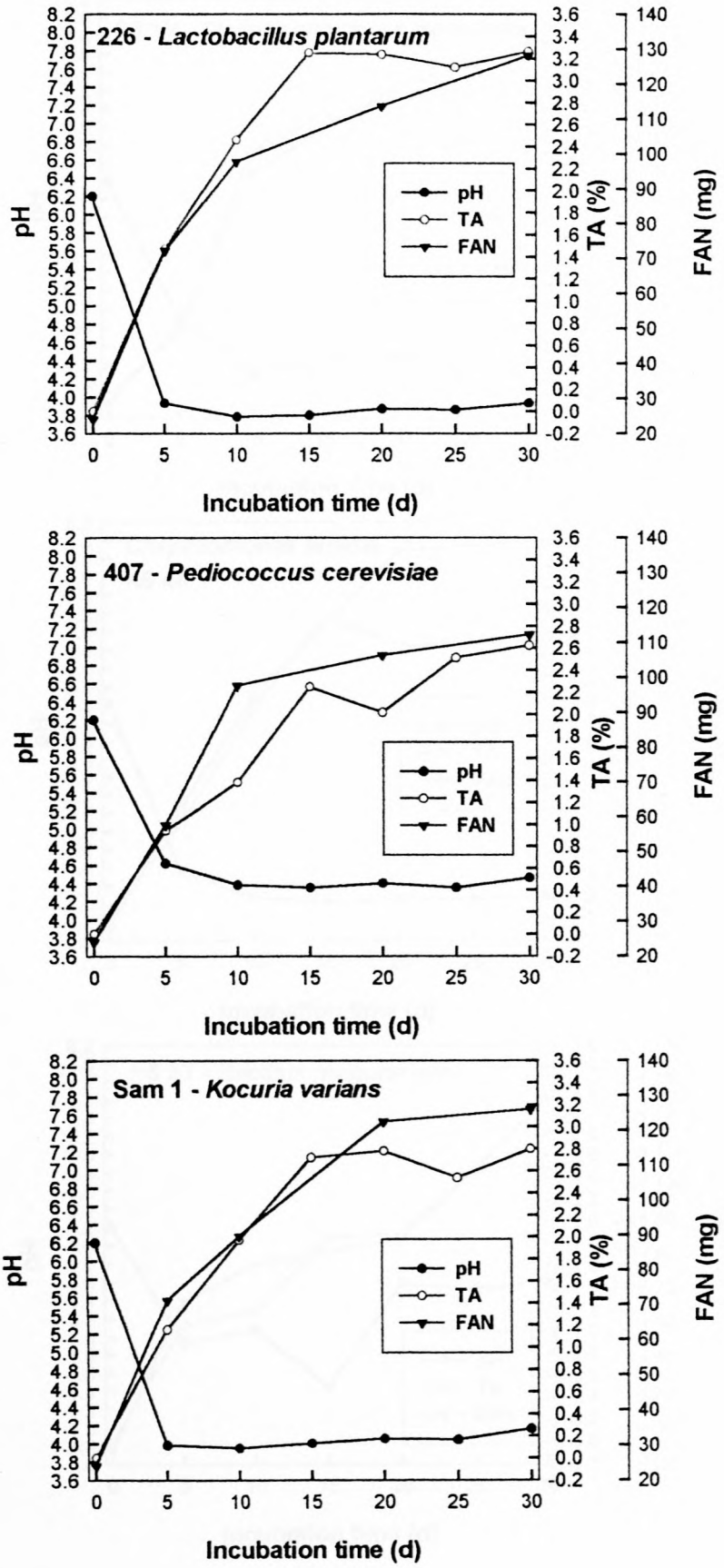


Figure 10. (continued).

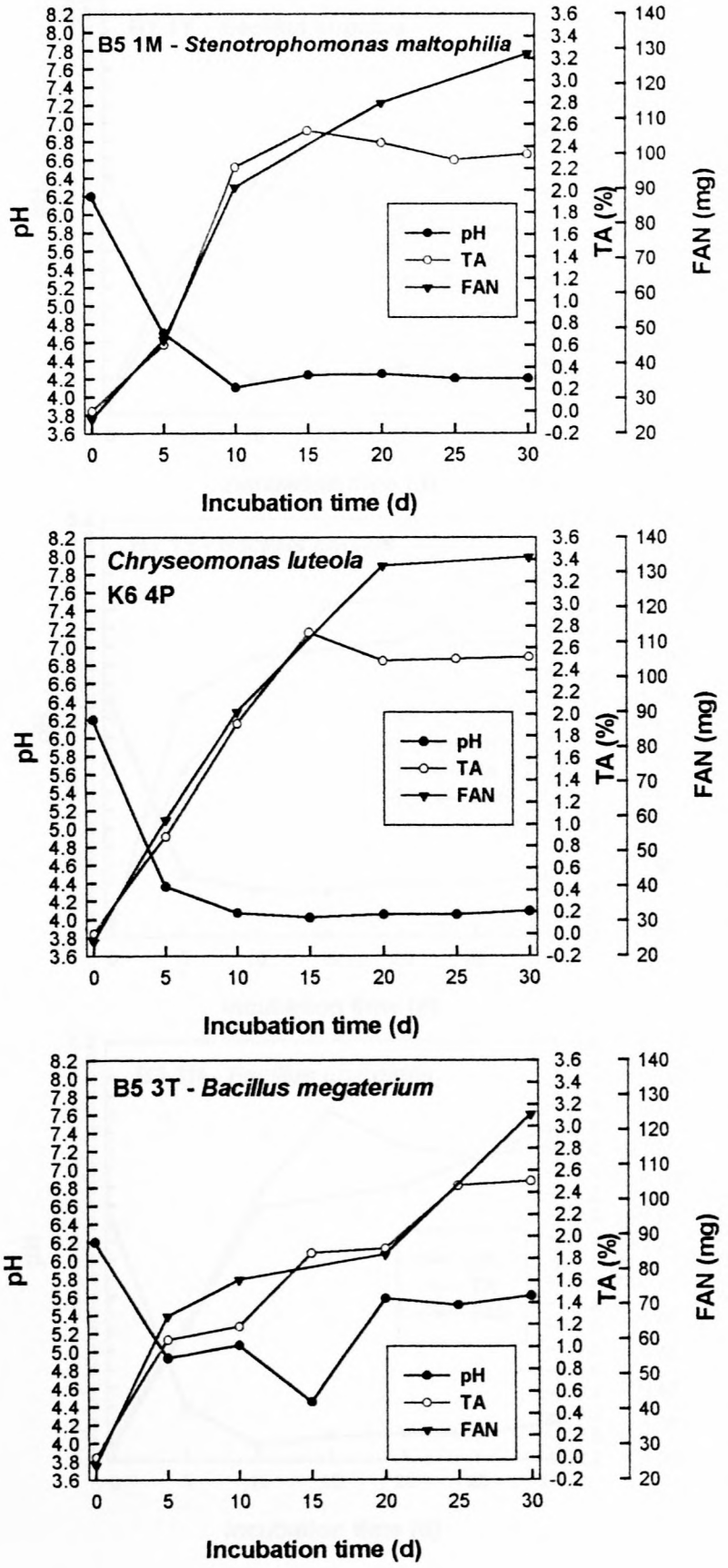


Figure 10. (continued).

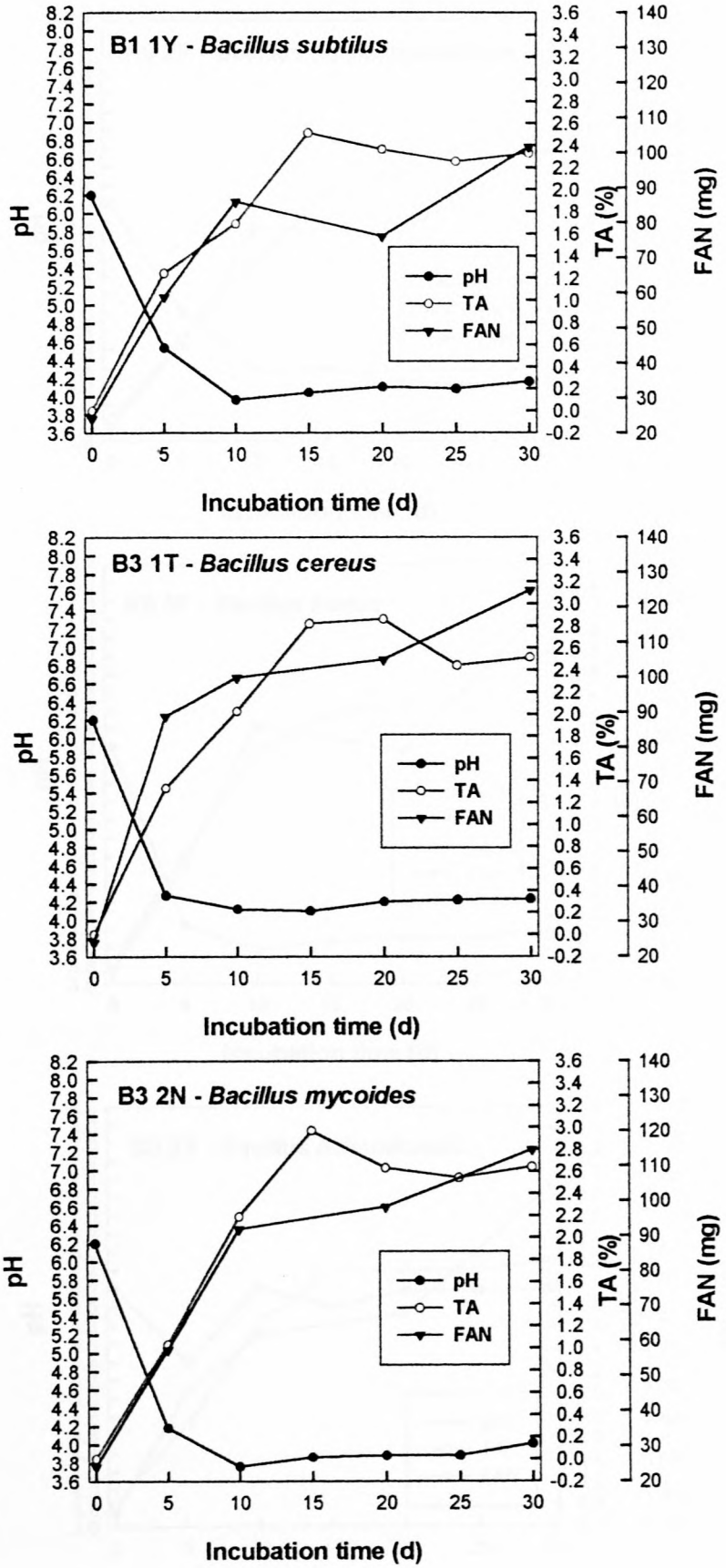


Figure 10. (continued).

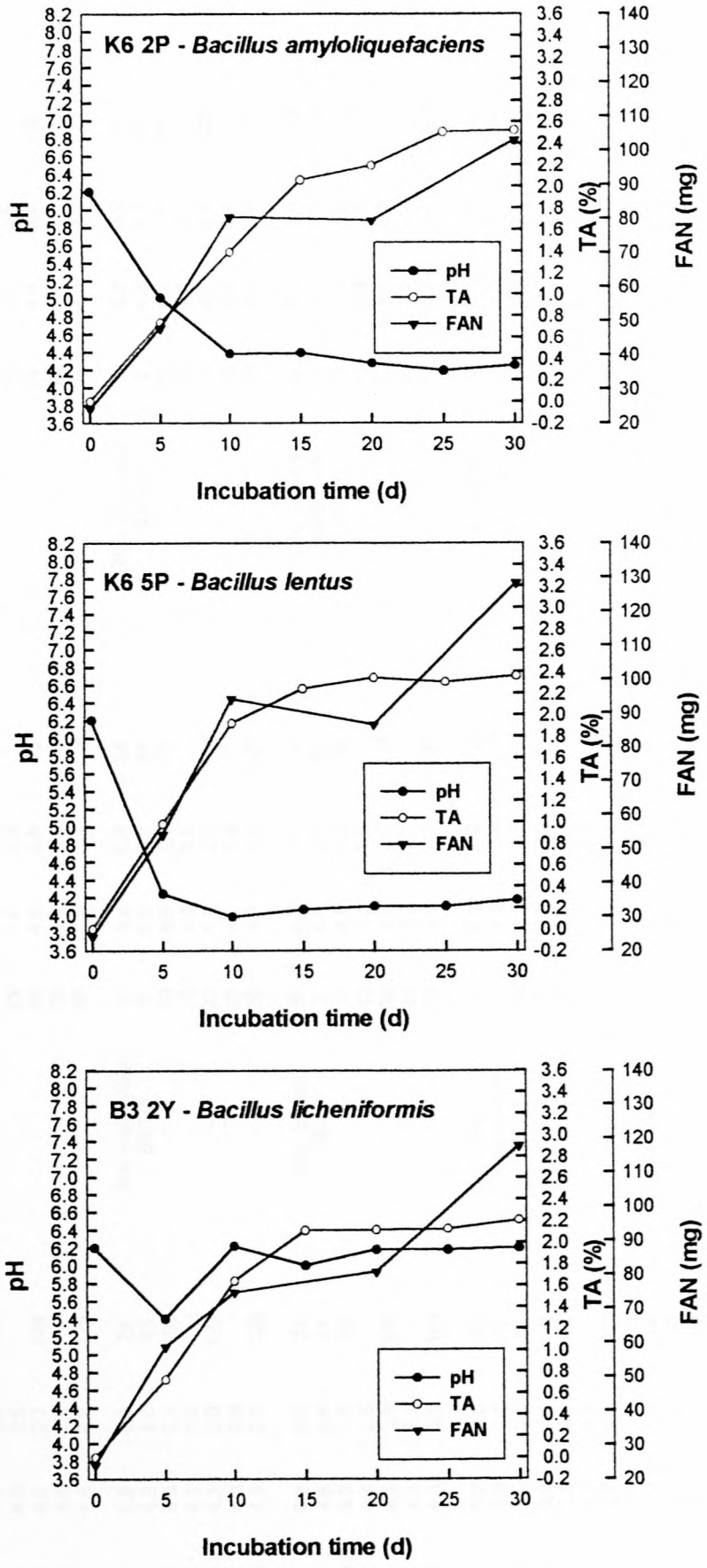


Figure 10. (continued).

Table 3.4. Results indicating the changes in pH, %TA and FAN over the incubation period of 30 days for 5.0% (w/w) glucose added plus a buffer.

Isolate	Time (d)	pH	%TA	FAN (mg)
Control	0	6.2	0.0	24
	5	4.6	1.0	69
	10	4.4	1.6	89
	15	4.5	2.5	
	20	4.6	2.3	129
	25	4.4	2.2	
	30	4.4	2.2	129
Lc. diacetylactis 140	0	6.2	0.0	24
	5	4.3	1.0	53
	10	4.0	2.1	108
	15	4.1	1.1	
	20	4.1	2.5	123
	25	4.0	2.6	
	30	4.0	2.7	125
Lb. acidophilus 897	0	6.2	0.0	24
	5	4.6	0.9	61
	10	4.3	1.7	96
	15	4.4	2.5	
	20	4.4	2.2	108
	25	4.3	2.1	
	30	4.3	2.1	109
Lb. plantarum 226	0	6.2	0.0	24
	5	3.9	1.1	49
	10	4.0	2.1	91
	15	3.9	3.1	
	20	3.8	3.1	108
	25	3.7	2.9	
	30	3.7	2.9	125
Pc. cerevisiae 407	0	6.2	0.0	24
	5	4.1	0.9	40
	10	4.0	1.7	83
	15	3.9	2.9	
	20	3.8	2.5	116
	25	3.8	2.5	
	30	3.7	2.5	123

Isolate	Time (d)	pH	%TA	FAN (mg)
Koc. varians S1	0	6.2	0.0	24
	5	4.0	0.9	63
	10	4.0	1.9	92
	15	4.0	2.8	
	20	4.0	2.5	13
	25	3.9	2.4	
	30	4.0	2.4	116
Ste. maltophilia B5 1M	0	6.2	0.0	24
	5	5.0	0.6	44
	10	4.8	1.3	75
	15	4.6	2.5	
	20	4.5	2.5	106
	25	4.3	2.4	
	30	4.3	2.4	121
Chy. luteola K6 4P	0	6.2	0.0	24
	5	4.9	0.8	41
	10	4.5	1.2	76
	15	4.5	2.5	
	20	4.3	2.5	126
	25	4.2	2.3	
	30	4.2	2.3	127
Bac. megaterium B5 3T	0	6.2	0.0	24
	5	4.9	0.8	46
	10	4.2	1.8	99
	15	4.3	2.6	
	20	4.4	2.4	107
	25	4.2	2.2	
	30	4.2	2.2	128
Bac. subtilus B1 1Y	0	6.2	0.0	24
	5	4.7	1.0	54
	10	4.5	1.3	81
	15	4.4	2.2	
	20	4.3	2.3	82
	25	4.1	2.2	
	30	4.1	2.3	112

Isolate	Time (d)	pH	%TA	FAN (mg)
Bac. cereus B3 1T	0	6.2	0.0	24
	5	4.2	1.3	68
	10	4.0	1.9	105
	15	4.0	2.5	
	20	4.0	2.5	132
	25	3.8	2.9	
	30	3.9	3.0	133
Bac. mycoides B3 2N	0	6.2	0.0	24
	5	4.2	1.7	52
	10	4.2	1.6	82
	15	4.0	2.3	
	20	4.1	2.4	120
	25	4.0	2.4	
	30	4.0	2.4	122
Bac. amyloli-quefaciens K6 2P	0	6.2	0.0	24
	5	4.8	0.8	62
	10	4.3	1.9	89
	15	4.4	2.5	
	20	4.4	2.5	119
	25	4.3	2.4	
	30	4.3	2.4	119
Bac. lentus K6 5P	0	6.2	0.0	24
	5	4.8	0.9	52
	10	4.3	1.5	99
	15	4.4	2.5	
	20	4.4	2.4	116
	25	4.2	2.3	
	30	4.2	2.3	119
Bac. licheniformis B3 2Y	0	6.2	0.0	24
	5	4.2	0.8	42
	10	4.2	1.3	74
	15	4.1	2.3	
	20	4.1	2.6	114
	25	4.0	2.4	
	30	4.0	2.4	117

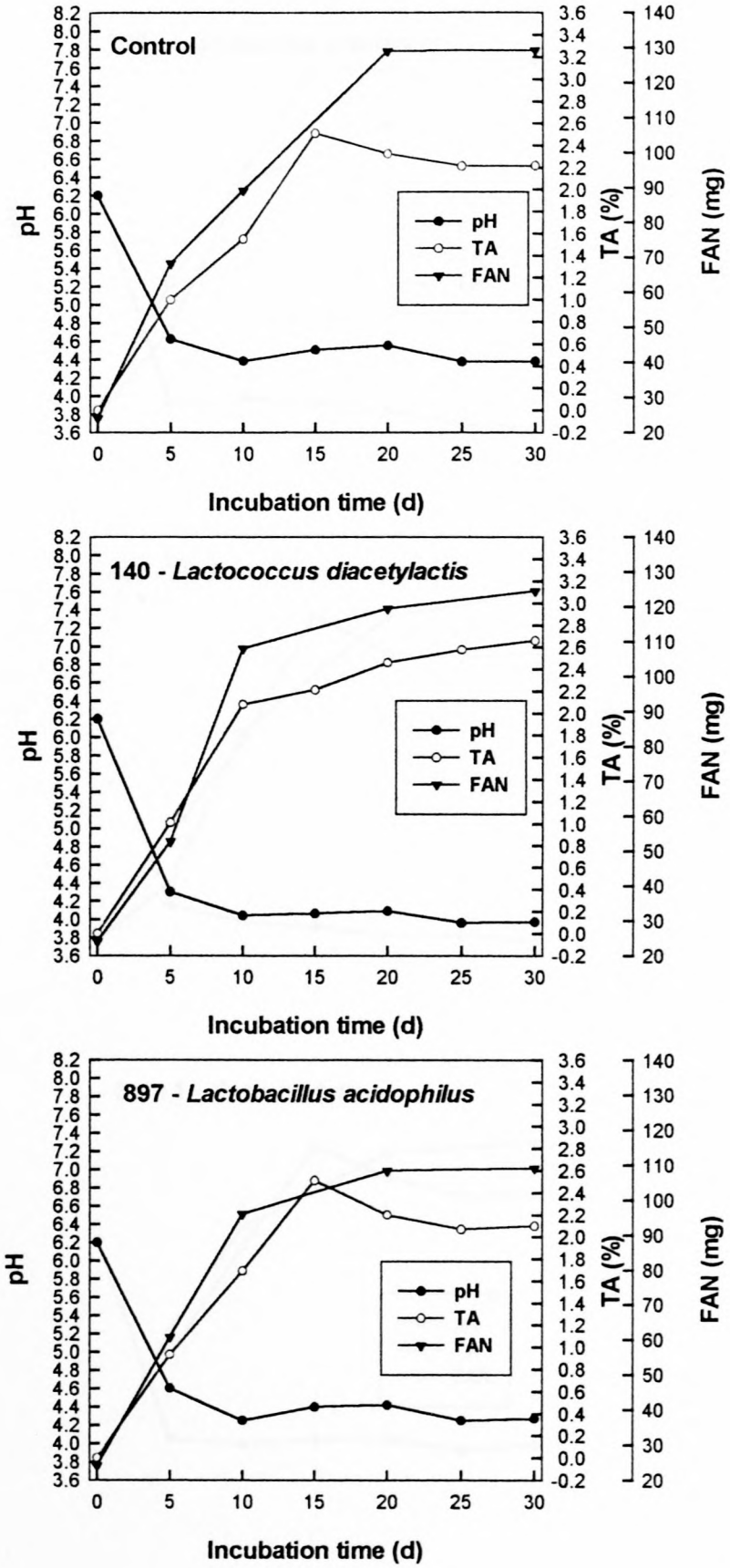


Figure 11. Influence of different bacterial strains on pH, %TA and FAN over the 30 day incubation period with 5.0% glucose and a buffer.

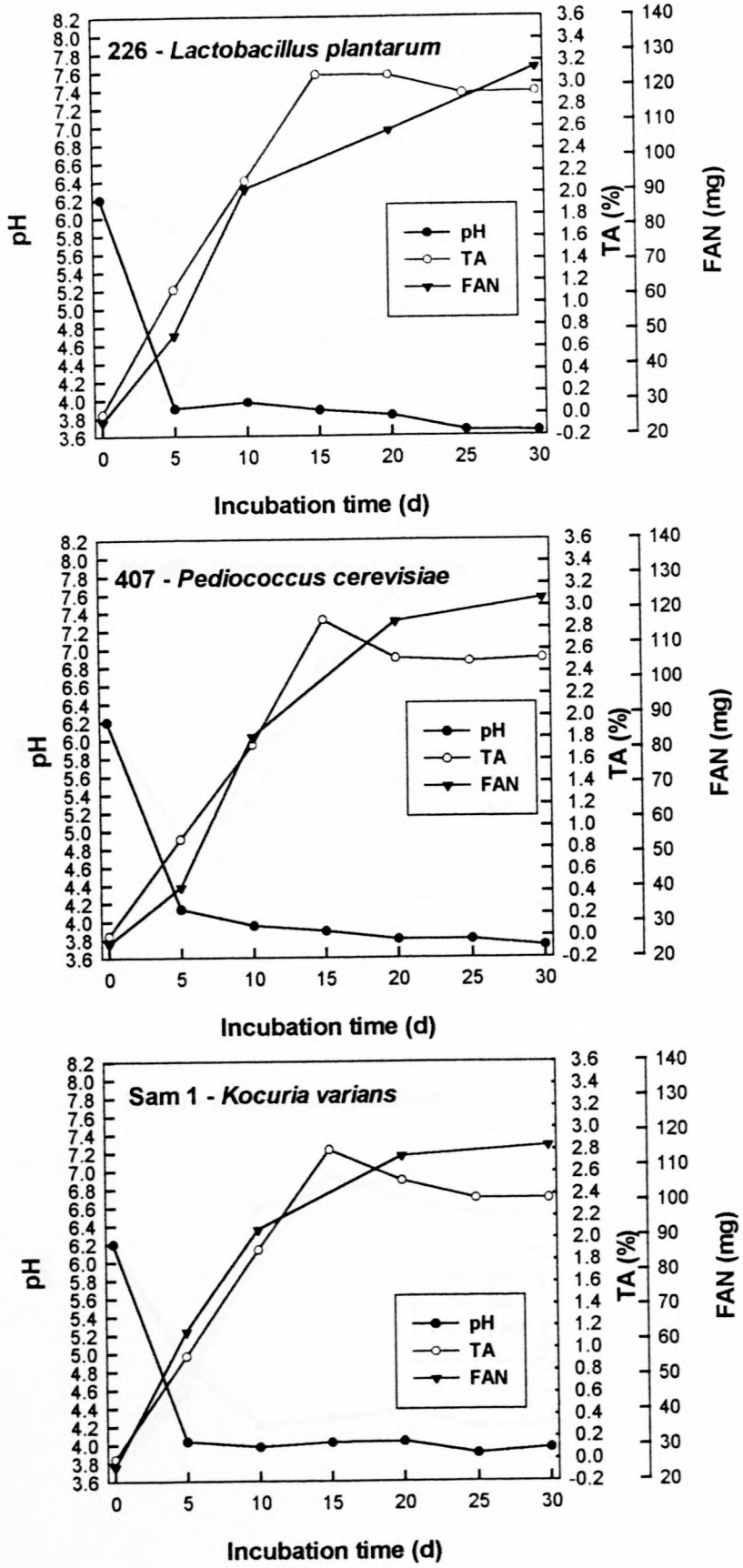


Figure 11. (continued).

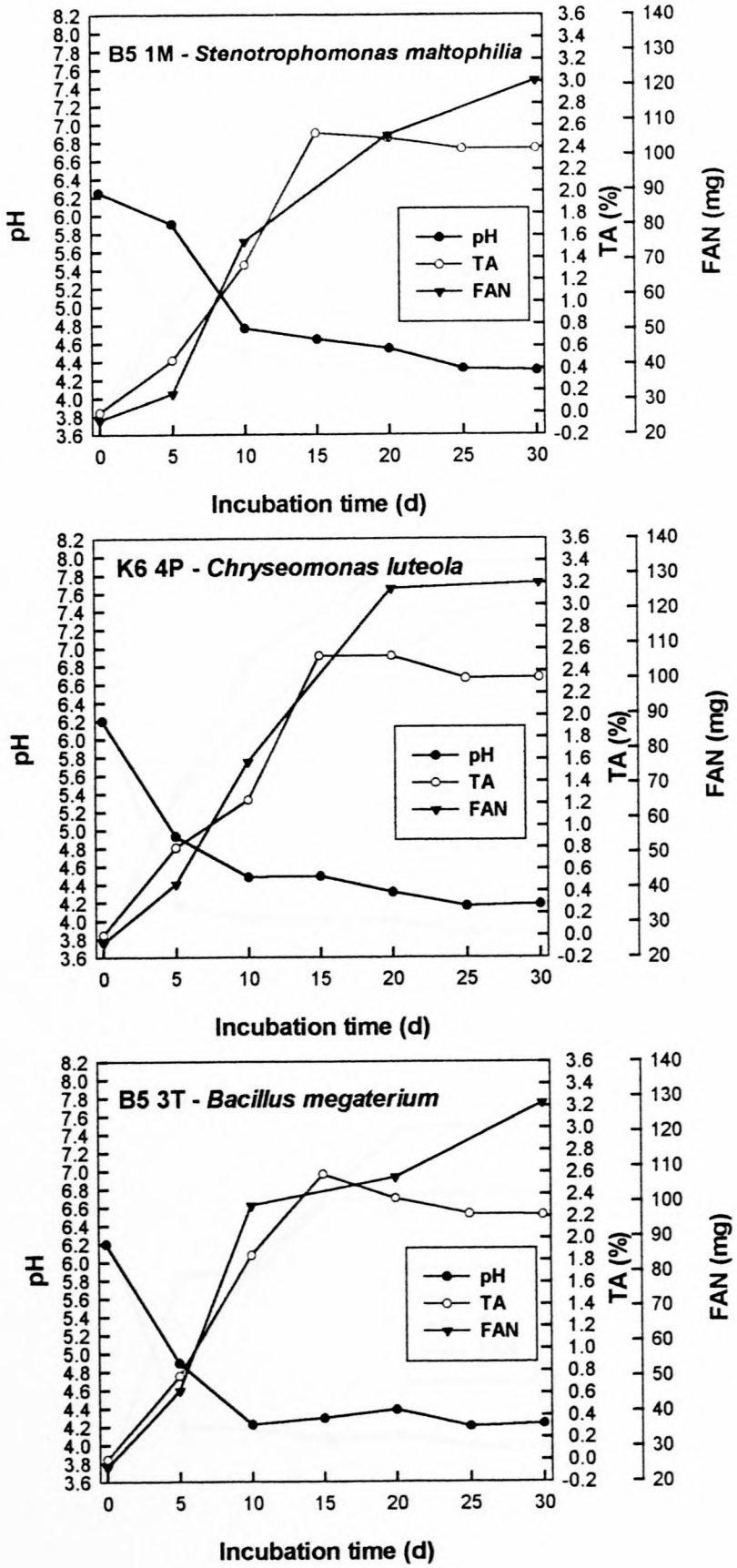


Figure 11. (continued).

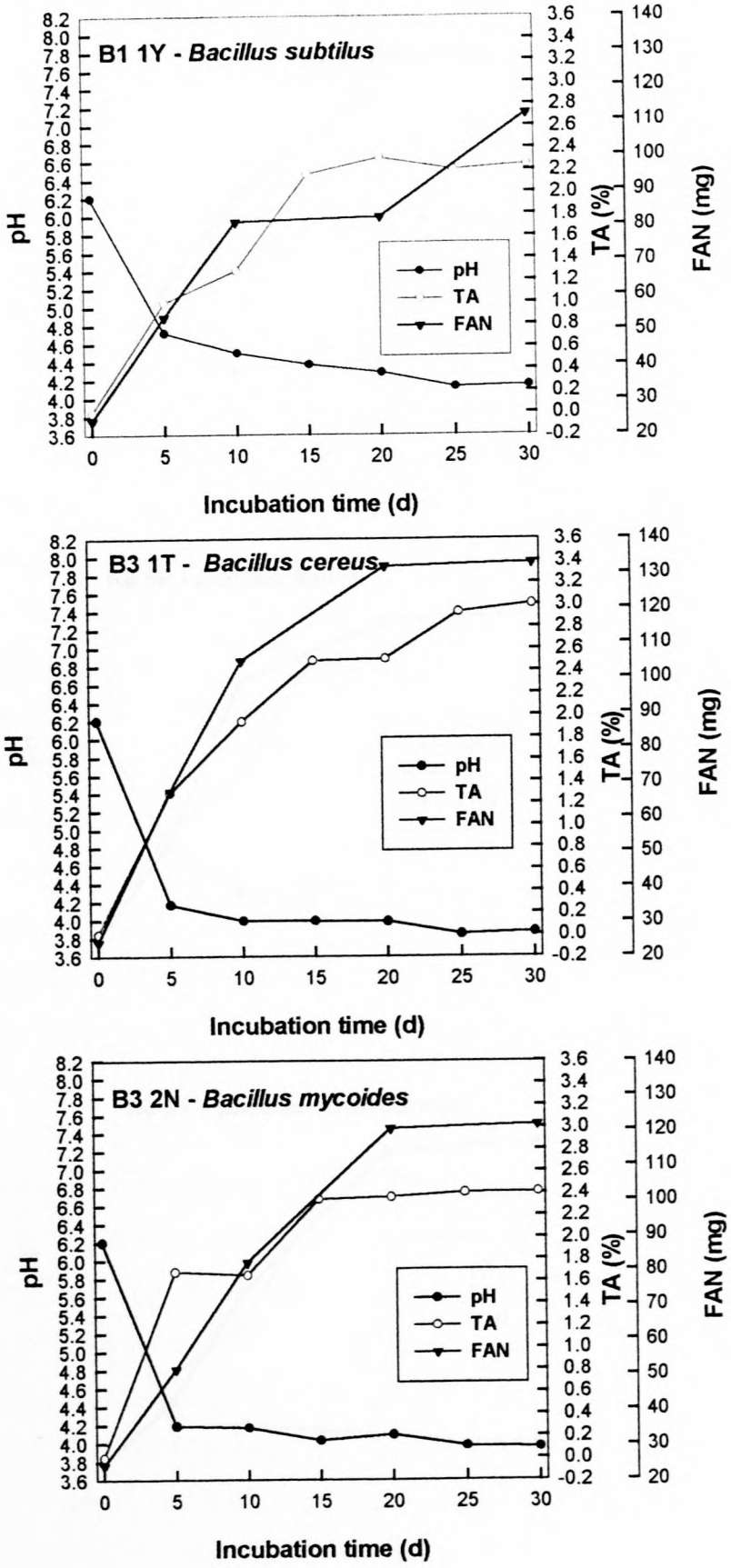


Figure 11. (continued).

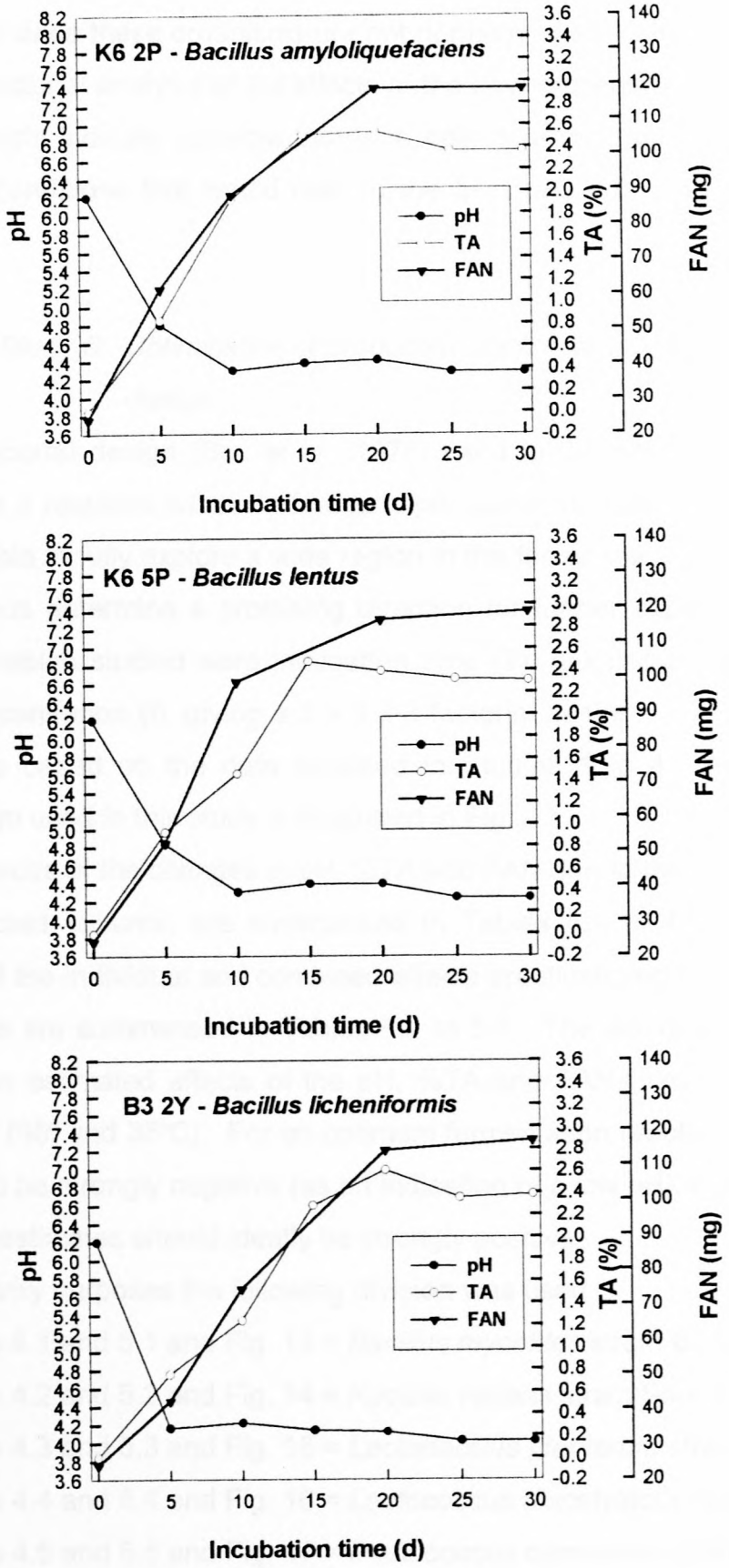


Figure 11. (continued).

Further trials including bacteria of the genus *Bacillus* are not usually recommended since these organisms are not normally used in the fermentation of food products. A factorial analysis of the effects of the environmental changes in production parameters might indicate valuable trends to help determine the optimum growth and fermentation conditions that would lead to the best pH, %TA and FAN fermentation results.

Experimental Study 5: Optimisation of production parameters using a 3 x 3 x 3 factorial design

The factorial design (Box *et al.,* 1978) used in this study can be of practical importance as it requires relatively few runs per factor studied. Although the specific design is unable to fully explore a wide region in the factor space, it can indicate major trends and thus determine a promising direction for further experimentation. In this study the variables studied were incubation time (T), glucose concentration (C) and inoculum concentration (I), giving a 3 x 3 x 3 factorial design. The selection of these variables was based on the data obtained in Studies 1 to 4. An example of the factorial design used in this study is illustrated in Fig. 12.

The results of the changes in pH, %TA and FAN during the incubation period for the five selected cultures, are summarised in Tables 4.1 to 4.5. The schematical illustrations of the individual and combined effects are illustrated in Fig. 13 to 17, while the responses are summarised in Tables 5.1 to 5.5. The isolates were evaluated by comparing the estimated effects of the pH, %TA and FAN (mg) variance at the two temperatures (30° and 35°C). For an optimum fermentation reaction, the estimates for the pH should be strongly negative (as an indication of a low pH) and for both the %TA and FAN the estimates should ideally be strongly positive.

For clarity purposes the following division was used:

- Tables 4.1 and 5.1 and Fig. 13 = *Bacillus mycoides* strain B3 2N;
- Tables 4.2 and 5.2 and Fig. 14 = *Kocuria varians* strain Sam 1;
- Tables 4.3 and 5.3 and Fig. 15 = *Lactobacillus plantarum* strain 226;
- Tables 4.4 and 5.4 and Fig. 16 = *Lactococcus diacetylactis* strain 140; and
- Tables 4.5 and 5.5 and Fig. 17 = *Pediococcus cerevisiae* strain 407.

Summaries of the optimal fermentation conditions indicated by the statistical and quantitative evaluation data are summarised in Tables 6.1 to 6.3.

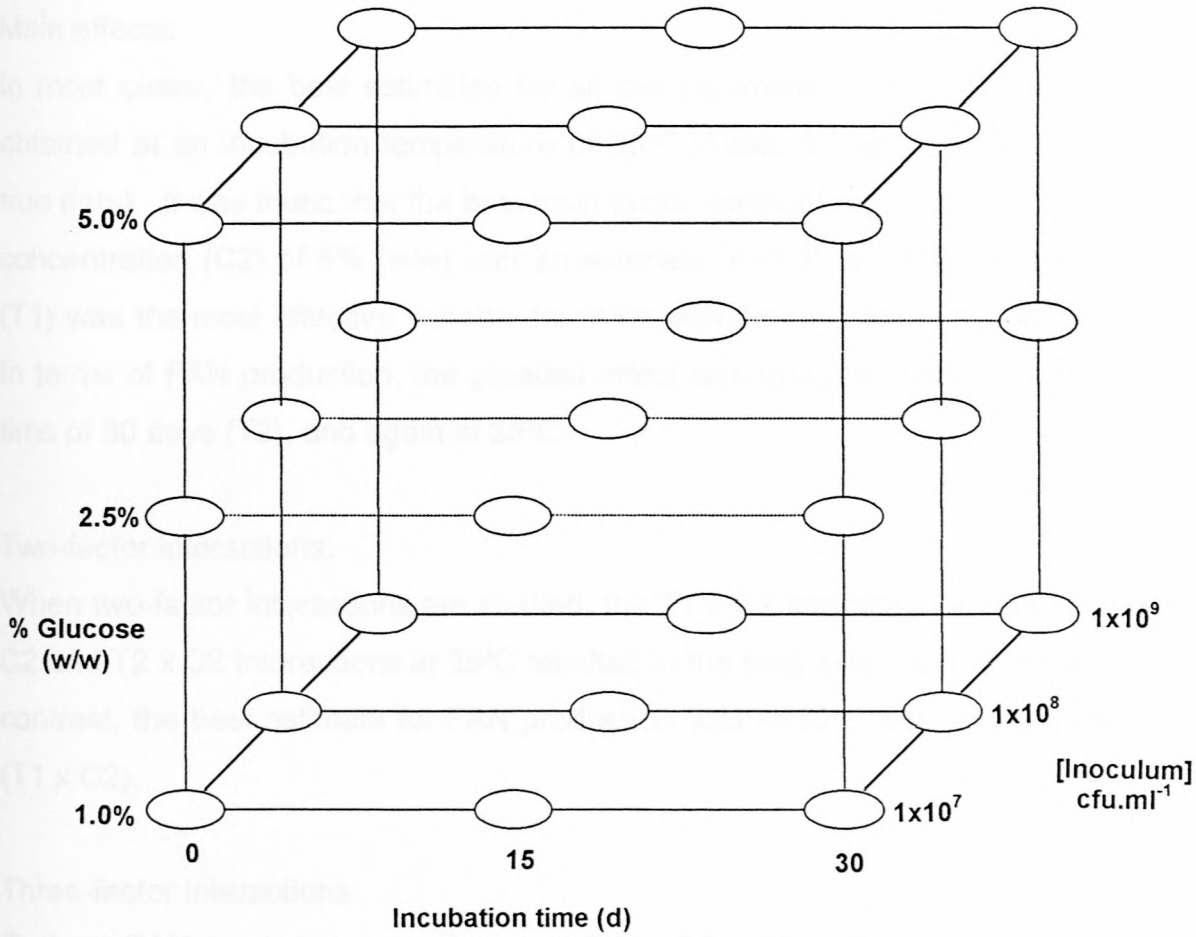


Figure 12. The factorial design used in this study.

Data evaluation

Bacillus mycoides strain B3 2N

Main effects:

In most cases, the best estimates for all the parameters (pH, %TA and FAN) were obtained at an incubation temperature of 35°C (Table 5.1 vs Table 4.1)(estimates vs true data). It was found that the best main factor estimate regarding pH, was a glucose concentration (C2) of 5% (w/w) with an estimate of -1.23 at 35°C. The effect of time (T1) was the most effective variable for %TA with an estimate of 10.27, also at 35°C. In terms of FAN production, the greatest effect was brought about after an incubation time of 30 days (T2), and again at 35°C.

Two-factor interactions:

When two-factor interactions are studied, the T1 x C2 interaction at 30°C and the T1 x C2 and T2 x C2 interactions at 35°C resulted in the best estimates for pH and %TA. In contrast, the best estimate for FAN production was at 30°C with an estimate of 10.08 (T1 x C2).

Three-factor interactions:

Optimal FAN production was found at T1 x C2 x I2 at 30°C (11.68) and the best three-factor interaction for %TA was T2 x C2 x I2 (0.12) also at 30°C. However, the best interaction for pH was T2 x C2 x I2 at 35°C (-0.23).

When considering the data of the effects and interactions, the three-factor interactions indicate that optimal estimates for pH are at an incubation period (T2) of 30 days, glucose concentration (C2) of 5% (w/w), an inoculum concentration (I2) of $1x10^9$ cfu.ml^{-1} and at an incubation temperature of 35°C. However, in terms of pH, the data indicates that incubation time of 30 days will not effect any of the final results considering the fact that most of the main products, such as lactic and other organic acids, are formed during the initial stages of the fermentation. Optimal estimates for %TA are T2 x C2 x I2 at both the temperatures with an estimate of 0.12 and 0.10 at 30° and 35°C, respectively, thus it was concluded that temperature did not strongly impact on the %TA. For FAN, the optimum three-level interaction was T1 x C2 x I2 at 30°C.

Table 4.1. Data obtained during the fermentation of rainbow trout using *Bacillus mycoides* (B3 2N) at the different experimental conditions.

Time (d)	Inoculum concentration ($cfu.ml^{-1}$)	[Glucose] (%) (w/w)	Incubation temperature					
			30°C			35°C		
			pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
0	$1x10^7$	1.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^7$	2.5	6.0	0.3	8	6.0	0.3	8
0	$1x10^7$	5.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^8$	1.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^8$	2.5	6.0	0.3	8	6.0	0.3	8
0	$1x10^8$	5.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^9$	1.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^9$	2.5	6.0	0.3	8	6.0	0.3	8
0	$1x10^9$	5.0	6.0	0.3	8	6.0	0.3	8
15	$1x10^7$	1.0	6.1	0.9	80	5.1	1.0	108
15	$1x10^7$	2.5	5.6	0.8	75	4.8	1.1	96
15	$1x10^7$	5.0	4.4	1.3	83	4.1	1.8	109
15	$1x10^8$	1.0	6.1	1.0	85	5.9	1.0	84
15	$1x10^8$	2.5	5.9	0.8	89	6.1	0.8	96
15	$1x10^8$	5.0	4.2	1.4	92	4.3	1.4	101
15	$1x10^9$	1.0	6.6	0.5	53	6.2	0.9	110
15	$1x10^9$	2.5	6.2	0.7	89	6.0	0.7	93
15	$1x10^9$	5.0	4.8	1.3	104	3.8	1.8	111
30	$1x10^7$	1.0	6.1	1.1	95	5.8	1.0	104
30	$1x10^7$	2.5	6.1	1.1	96	5.5	1.4	109
30	$1x10^7$	5.0	4.6	1.4	93	4.2	1.7	120
30	$1x10^8$	1.0	6.1	1.0	96	5.8	0.8	111
30	$1x10^8$	2.5	5.9	1.1	103	5.9	1.0	109
30	$1x10^8$	5.0	4.3	1.5	95	4.4	1.5	107
30	$1x10^9$	1.0	6.5	0.9	105	6.3	0.8	111
30	$1x10^9$	2.5	6.0	1.0	109	6.1	0.8	116
30	$1x10^9$	5.0	5.3	1.7	108	3.8	1.9	116

Table 5.1. Statistical evaluation of the effects and interactions of time, inoculum concentration and glucose concentration at different temperatures on changes in pH, %TA and FAN with *Bacillus mycoides* (B3 2N) as starter culture.

Effects	Interactions	Estimates at 30°C			Estimates at 35°C		
		pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
Main effects							
Time (T)	0 - 15 d (T1)	-0.46	7.84	41.46	-0.80	10.27	51.73
	0 - 30 d (T2)	-0.35	0.91	91.96	-0.71	0.93	103.32
Inoculum (I)	$1x10^7$ - $1x10^8$ cfu.ml^{-1} (I1)	-0.03	0.02	4.19	0.25	-0.17	-4.26
	$1x10^7$ - $1x10^9$ cfu.ml^{-1} (I2)	0.28	-0.07	5.05	0.24	-0.11	1.04
[Glucose] (C)	1.0 - 2.5% (w/w) (C1)	-0.18	0.02	5.08	-0.13	0.05	-1.02
	1.0 - 5.0% (w/w) (C2)	-1.09	0.35	6.63	-1.23	0.52	3.96
Two-factor interactions							
Time and [glucose]	T1xC1	-0.18	0.00	5.82	-0.13	-0.04	-2.97
(TxC)	T1xC2	-0.89	0.27	10.08	-0.93	0.34	3.04
	T2xC1	-0.10	0.03	1.80	-0.07	0.11	1.45
	T2xC2	-0.74	0.26	-0.14	-0.92	0.44	2.89
Time and inoculum	T1xI1	0.01	0.03	4.53	0.28	-0.11	-5.28
(TxI)	T1xI2	0.24	-0.10	1.23	0.22	-0.06	0.12
	T2xI1	-0.06	0.00	1.75	0.10	-0.14	-1.11
	T2xI2	0.19	0.00	6.34	0.14	-0.10	1.44
[Glucose] and inoculum	C1xI1	0.02	0.01	2.36	0.26	-0.09	2.64
(CxI)	C1xI2	-0.09	0.07	7.08	0.12	-0.12	-0.81
	C2xI1	-0.09	0.04	0.84	0.03	-0.09	-0.78
	C2xI2	0.01	0.16	8.58	-0.30	0.08	-1.75
Three-factor interactions							
Time, [glucose] and inoculum	T1xC1xI1	0.06	-0.01	2.14	0.28	-0.06	5.92
(TxCxI)	T1xC1xI2	0.00	0.08	9.95	0.15	-0.07	-1.14
	T1xC2xI1	-0.08	0.02	0.88	0.00	-0.09	3.98
	T1xC2xI2	-0.04	0.11	11.68	-0.21	0.02	0.18
	T2xC1xI1	-0.02	0.02	1.40	0.11	-0.09	-1.96
	T2xC1xI2	-0.13	0.02	0.67	0.03	-0.10	-0.07
	T2xC2xI1	-0.06	0.04	0.38	0.04	-0.04	-5.16
	T2xC2xI2	0.05	0.12	1.20	-0.23	0.10	-2.81

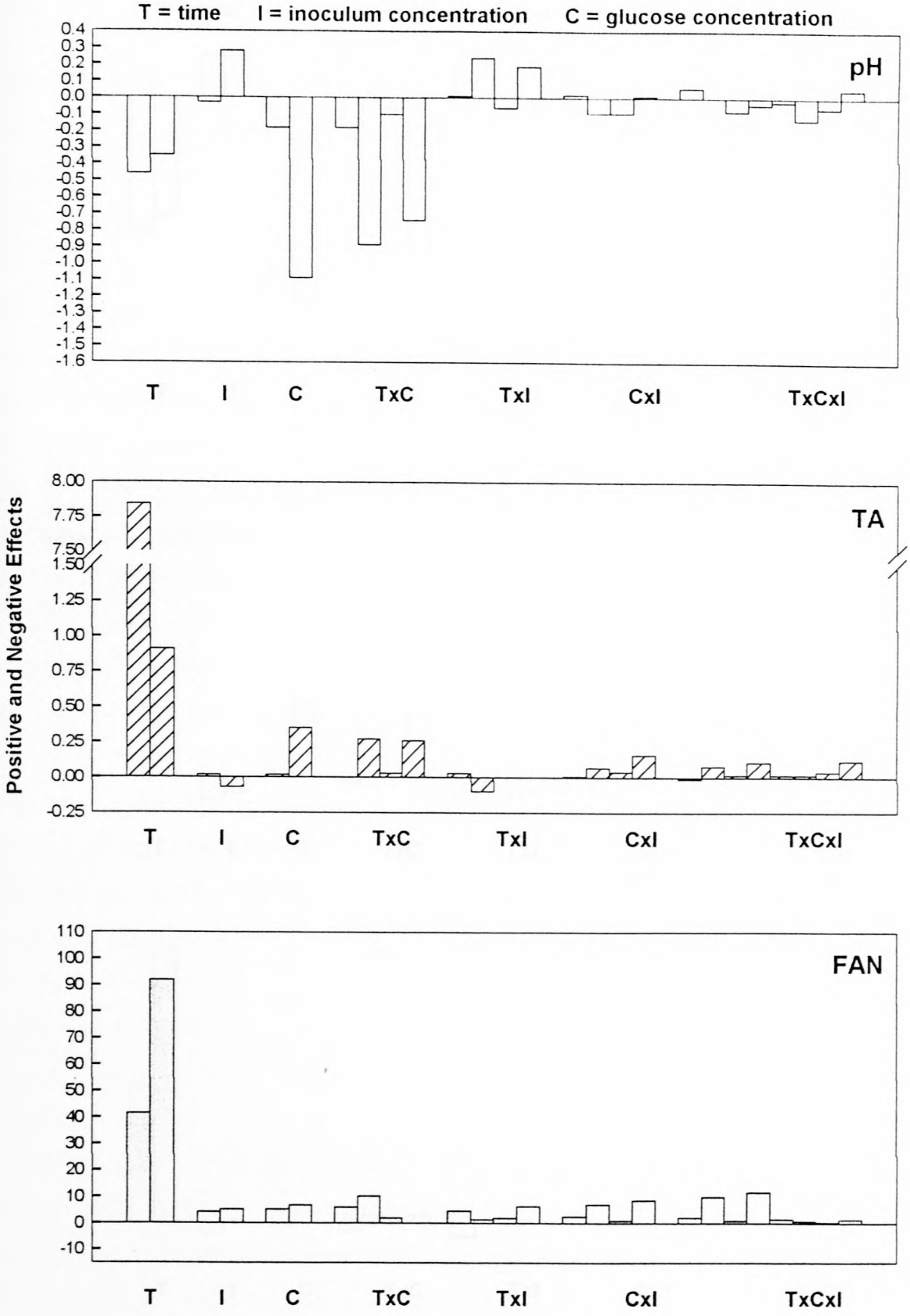


Figure 13 a. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 30°C by *Bacillus mycoides* (B3 2N).

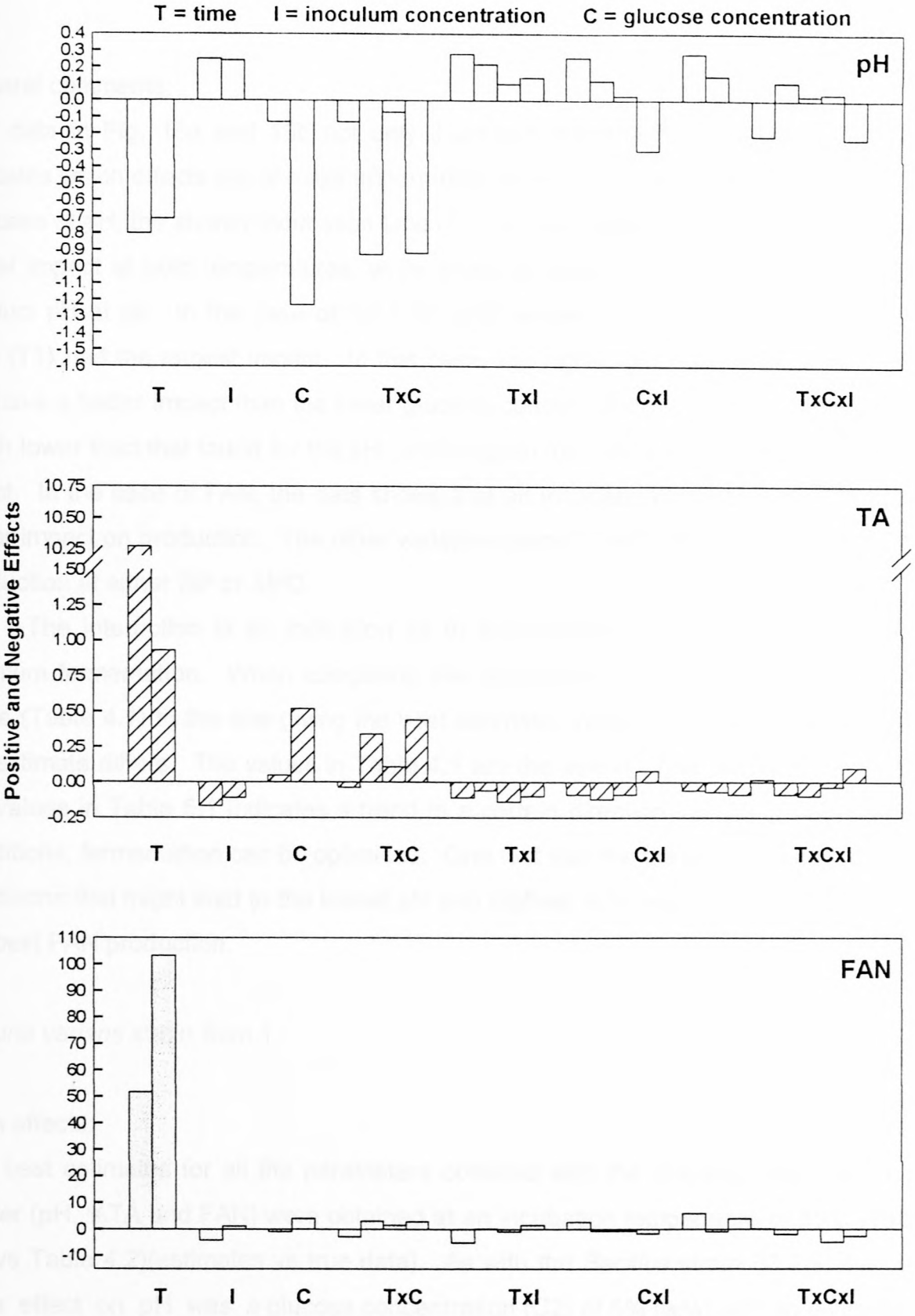


Figure 13 b. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 35°C by *Bacillus mycoides* (B3 2N).

General comments:

The data in Fig. 13a and 13b not only illustrates the effects of variables but also indicates which effects are of major importance and specific trends can be deduced. In the case of pH, the shorter incubation time (T1) and largest glucose content (C2) had a larger impact at both temperatures, while inoculum size did not positively impact the product pH at all. In the case of %TA for both temperatures, the shorter incubation time (T1) had the largest impact. In this case, the higher glucose concentration (C2) did have a better impact than the lower glucose concentration (C1) but the impact was much lower than that found for the pH. Once again the inoculum size was not a major effect. In the case of FAN, the data shows that an incubation time of 30 d (T2) had a major impact on production. The other variables were not of major importance for FAN production at either 30° or 35°C.

The interaction is an indication as to the conditions which would lead to an optimum fermentation. When comparing the interaction resulting in the highest FAN value (Table 4.1) to the one giving the best estimate (Table 5.1), it was observed that the estimate differs. The values in Table 4.1 are the actual experimental results while the values in Table 5.1 indicates a trend in a certain direction indicating under which conditions, fermentation can be optimised. One fact that the trends indicated, was that conditions that might lead to the lowest pH and highest %TA were different to those for the best FAN production.

Kocuria varians strain Sam 1

Main effects:

The best estimates for all the parameters obtained with the *Kocuria* strain Sam 1 as starter (pH, %TA and FAN) were obtained at an incubation temperature of 35°C (Table 5.2 vs Table 4.2)(estimates vs true data). As with the *Bacillus* strain B3 2N, the best main effect on pH was a glucose concentration (C2) of 5% (w/w) with an estimate of -1.33 at 35°C. For %TA, the incubation time of 15 d (T1) resulted in the highest effect of 9.74 at 35°C. In terms of FAN production, an incubation time period of 30 d (T2) resulted in the best estimate of 94.28 at 35°C.

Table 4.2. Data obtained during the fermentation of rainbow trout using *Kocuria varians* (Sam 1) at the different experimental conditions.

Time (d)	Inoculum concentration (cfu.ml^{-1})	[Glucose] (%) (w/w)	Incubation temperature					
			30°C			35°C		
			pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
0	1x10^{7}	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{7}	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^{7}	5.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{8}	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{6}	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^{8}	5.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{9}	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{9}	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^{9}	5.0	6.0	0.3	8	6.0	0.3	8
15	1x10^{7}	1.0	5.6	0.8	123	5.8	0.7	70
15	1x10^{7}	2.5	5.3	1.0	79	5.1	1.0	91
15	1x10^{7}	5.0	5.9	1.1	84	3.9	1.9	117
15	1x10^{8}	1.0	6.0	0.9	94	5.8	0.9	92
15	1x10^{8}	2.5	5.2	1.0	81	4.8	1.0	94
15	1x10^{8}	5.0	3.7	1.6	78	3.7	1.9	109
15	1x10^{9}	1.0	5.6	0.7	70	5.9	0.8	96
15	1x10^{9}	2.5	5.2	1.0	88	5.4	0.9	88
15	1x10^{9}	5.0	3.8	1.9	85	3.8	2.0	104
30	1x10^{7}	1.0	5.8	1.2	98	5.8	0.8	83
30	1x10^{7}	2.5	5.3	1.3	99	5.3	1.2	102
30	1x10^{7}	5.0	5.8	1.2	104	3.8	2.0	110
30	1x10^{8}	1.0	6.0	0.8	102	5.7	1.0	95
30	1x10^{8}	2.5	5.4	0.9	101	5.3	1.2	105
30	1x10^{8}	5.0	3.6	1.9	104	3.8	2.1	110
30	1x10^{9}	1.0	5.9	0.9	100	5.9	0.9	103
30	1x10^{9}	2.5	5.3	1.0	104	5.6	1.0	106
30	1x10^{9}	5.0	3.7	2.0	95	3.9	2.0	106

Table 5.2. Statistical evaluation of the effects and interactions of time, inoculum concentration and glucose concentration at different temperatures on changes in pH, %TA and FAN with *Kocuria varians* (Sam 1) as starter culture.

Effects	Interactions	Estimates at 30°C			Estimates at 35°C		
		pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
Main effects							
Time (T)	0 - 15 d (T1)	-0.86	8.39	42.25	-1.11	9.74	50.81
	0 - 30 d (T2)	-0.82	0.97	92.75	-1.00	1.06	94.28
Inoculum (I)	$1x10^7$ - $1x10^8$ cfu.ml^{-1} (I1)	-0.42	0.06	-3.15	-0.06	0.04	3.56
	$1x10^7$ - $1x10^9$ cfu.ml^{-1} (I2)	-0.45	0.10	-5.09	0.08	-0.01	3.31
[Glucose] (C)	1.0 - 2.5% (w/w) (C1)	-0.35	0.09	-3.78	-0.37	0.12	5.17
	1.0 - 5.0% (w/w) (C2)	-0.92	0.48	-4.17	-1.33	0.76	13.04
Two-factor interactions							
Time and [glucose]	T1xC1	-0.26	0.09	-6.35	-0.35	0.07	2.46
(TxC)	T1xC2	-0.63	0.36	-6.61	-1.01	0.57	12.02
	T2xC1	-0.27	0.05	0.68	-0.20	0.11	5.30
	T2xC2	-0.76	0.36	0.36	-0.99	0.57	7.54
Time and inoculum	T1xI1	-0.31	0.11	-5.60	-0.08	0.01	2.77
(TxI)	T1xI2	-0.36	0.12	-7.18	0.05	0.00	1.62
	T2xI1	-0.33	-0.02	0.88	-0.02	0.06	2.57
	T2xI2	-0.32	0.04	-0.46	0.07	-0.02	3.34
[Glucose] and inoculum	C1xI1	-0.12	-0.03	4.86	-0.06	-0.07	-4.67
(CxI)	C1xI2	-0.05	0.00	10.72	0.05	-0.09	-7.45
	C2xI1	-0.85	0.27	3.25	-0.02	-0.05	-7.22
	C2xI2	-0.71	0.33	7.08	-0.04	-0.01	-10.49
Three-factor interactions							
Time, [glucose] and inoculum	T1xC1xI1	-0.13	-0.05	7.85	-0.10	-0.04	-4.69
(TxCxI)	T1xC1xI2	-0.03	0.01	15.39	0.04	-0.06	-6.94
	T1xC2xI1	-0.66	0.10	5.91	-0.04	-0.05	-7.75
	T1xC2xI2	-0.53	0.21	13.50	-0.05	0.01	-9.54
	T2xC1xI1	-0.05	-0.01	-0.55	0.00	-0.07	-2.31
	T2xC1xI2	-0.04	-0.01	0.69	0.03	-0.08	-4.24
	T2xC2xI1	-0.61	0.30	-1.04	0.00	-0.03	-3.08
	T2xC2xI2	-0.54	0.29	-2.88	0.00	-0.03	-6.20

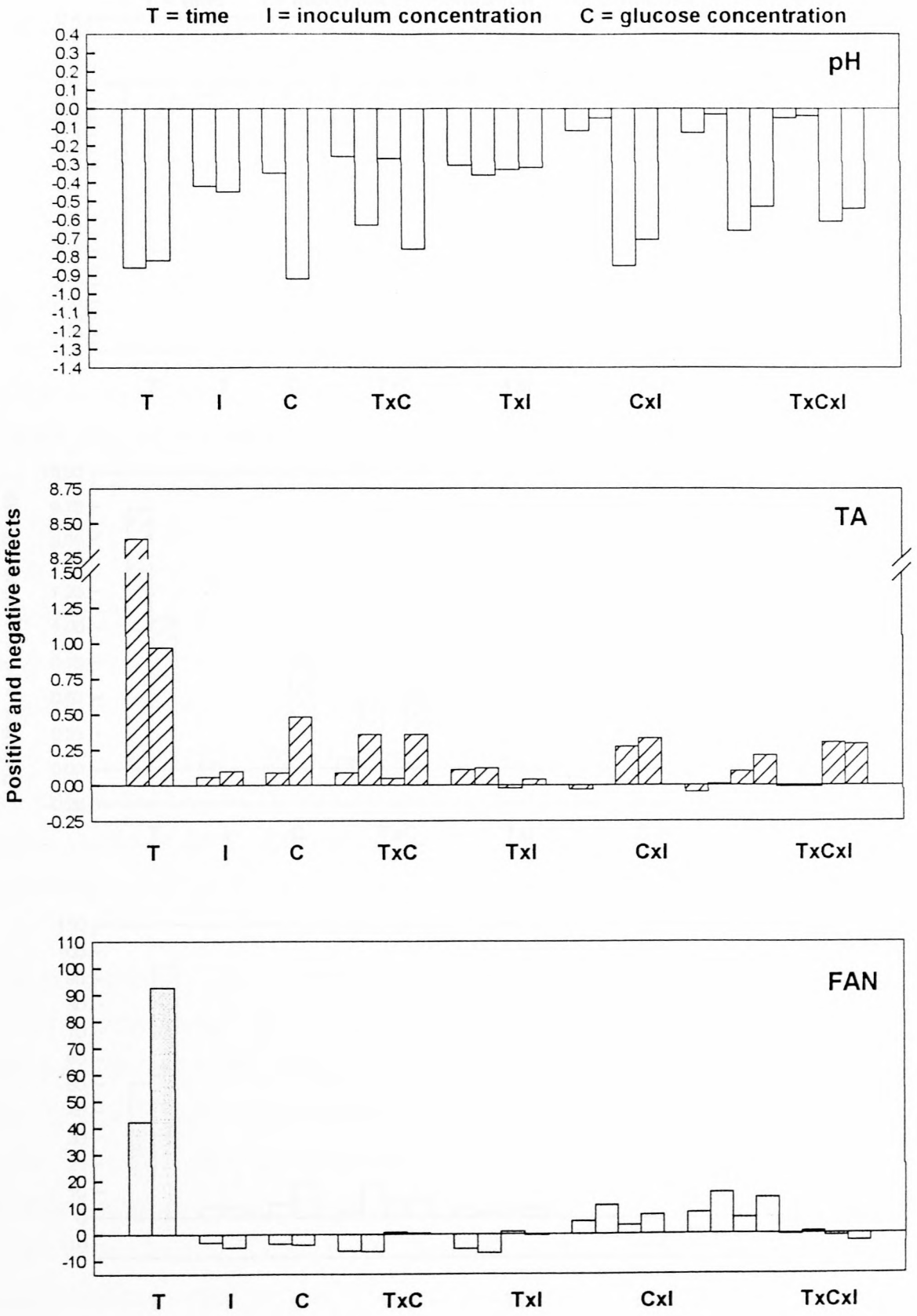


Figure 14 a. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 30°C by *Kocuria varians* (Sam 1).

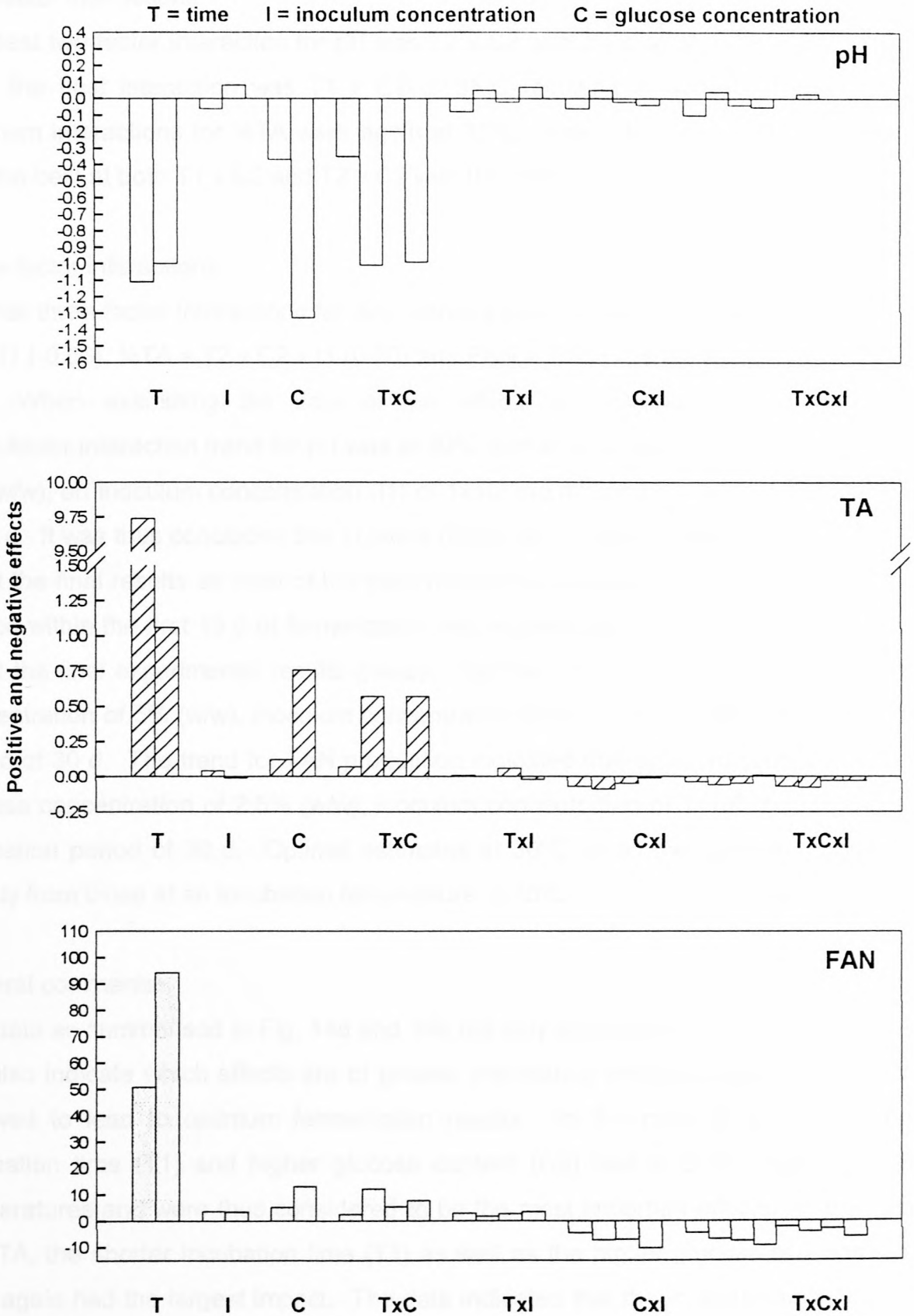


Figure 14 b. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 35°C by *Kocuria varians* (Sam 1).

Two-factor interactions:

The best two-factor interaction for pH was T2 x C2 with a value of -0.99 at 35°C. For FAN, the best interaction was T1 x C2 at 35°C resulting in an estimate of 12.02. Optimum interactions for %TA were again at 35°C. The estimates for this parameter was the best at both T1 x C2 and T2 x C2 with the same value of 0.57 in both cases.

Three-factor interactions:

Optimal three-factor interactions for *Koc. varians* were all at 30°C. For pH it was T1 x C2 x I1 (-0.66), %TA = T2 x C2 x I1 (0.30) and FAN = T1 x C1 x I2 (15.39).

When examining the data of the effects and interactions, the optimum three-factor interaction trend for pH was at 30°C and at a glucose concentration (C2) of 5% (w/w), an inoculum concentration (I1) of $1x10^8$ cfu.ml^{-1} and an incubation time (T1) of 15 d. It was thus concluded that in terms of pH, an incubation time of 30 d would not effect the final results as most of the main metabolic products, such as lactic acid, are formed within the first 15 d of fermentation and a prolonged incubation time would not effect the final experimental results greatly. Optimal %TA was obtained at a glucose concentration of 5% (w/w), inoculum concentration of $1x10^8$ cfu.ml^{-1} after an incubation period of 30 d. The trend for FAN production indicated that optimum conditions are a glucose concentration of 2.5% (w/w), inoculum concentration of $1x10^8$ cfu.ml^{-1} and an incubation period of 30 d. Optimal estimates at 30°C for all the parameters differed greatly from those at an incubation temperature of 35°C.

General comments:

The data as summarised in Fig. 14a and 14b not only illustrate the effects of variables, but also indicate which effects are of greater importance and which trends should be followed to lead to optimum fermentation results. In the case of pH, the shorter incubation time (T1) and higher glucose content (C2) had a larger impact at both temperatures and were thus considered to be the most important effects. In the case of %TA, the shorter incubation time (T1) as well as the higher glucose concentration (C2) again had the largest impact. The data indicated that the inoculum size (I) on its own had no major impact. In the case of FAN production, the data showed that an incubation time of 30 d (T2) had the only major impact on production. The other variables were not of major importance for FAN production at either 30° or 35°C.

It must be remembered that an interaction is only an indication as to the conditions leading to an optimum fermentation. The actual experimental data in Table 4.2 differs from the estimates in Table 5.2 but the values in Table 5.2 clearly indicate a trend in a certain direction and that for FAN production incubation time was the most important.

Lactobacillus plantarum strain 226

Main effects:

The best estimates for all the parameters (pH, %TA and FAN) were all obtained at an incubation temperature of 35°C (Table 5.3 vs Table 4.3)(estimates vs true data). The optimum estimate for pH, was a glucose concentration (C2) of 5% (w/w) with a value of -1.49 at 35°C. In terms of %TA, the greatest effect was brought about after an incubation time of 15 d (T1) also at 35°C. The effect of time (T2) was the most effective variable for FAN with an estimate of 92.28 again at 35°C. However, at 30°C the incubation time (T2) resulted in a very similar estimate of 91.47.

Two-factor interactions:

The best estimates for both pH and %TA was T2 x C2 at 35°C with estimated values of -1.16 and 0.69, respectively. The optimum FAN production effect was, however, at 30°C and an interaction of T1 x C1 (6.23).

Three-factor interactions:

The estimated values for the three-factor interactions were very low indicating that this starter might not be very effective as a fish fermenter. Optimum estimates for all the parameters were obtained at 30°C. The best value for pH was T2 x C2 x I1 (-0.21). Optimum conditions for %TA were obtained at T1 x C2 x I1 (0.10) and for FAN, T1 x C1 x I1 (2.81).

When considering the data of the effects and interactions, the three-factor interactions indicate that the optimal estimate for pH were -0.21 (T2 x C2 x I1). This value is very close to the value for the second best interaction (T1 x C2 x I1) of -0.20. Incubation time (T1 and T2) is the only parameter that differs in these interactions and this fact was thus taken as an indication that a lengthening of the incubation time would not result in much improvement of the pH data.

Table 4.3. Data obtained during the fermentation of rainbow trout using *Lactobacillus plantarum* (226) at the different experimental conditions.

Time (d)	Inoculum concentration (cfu.ml^{-1})	[Glucose] (%) (w/w)	Incubation temperature					
			30°C			35°C		
			pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
0	1x10^7	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^7	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^7	5.0	6.0	0.3	8	6.0	0.3	8
0	1x10^8	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^8	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^8	5.0	6.0	0.3	8	6.0	0.3	8
0	1x10^9	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^9	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^9	5.0	6.0	0.3	8	6.0	0.3	8
15	1x10^7	1.0	5.7	1.0	72	5.8	0.8	83
15	1x10^7	2.5	4.2	1.3	78	4.5	1.2	95
15	1x10^7	5.0	3.7	2.0	84	3.6	1.9	96
15	1x10^8	1.0	6.5	0.6	69	5.6	0.9	87
15	1x10^8	2.5	4.4	1.2	86	4.3	1.5	91
15	1x10^8	5.0	3.7	2.1	89	3.7	2.0	95
15	1x10^9	1.0	5.3	0.8	76	6.0	1.0	104
15	1x10^9	2.5	4.3	1.4	90	4.0	1.5	91
15	1x10^9	5.0	3.8	1.9	80	3.7	2.0	101
30	1x10^7	1.0	5.7	1.2	96	5.9	0.8	85
30	1x10^7	2.5	4.4	1.3	97	4.8	1.2	111
30	1x10^7	5.0	3.6	2.1	99	3.6	2.2	107
30	1x10^8	1.0	6.6	1.0	99	5.7	0.7	95
30	1x10^8	2.5	4.7	1.2	101	4.6	1.4	100
30	1x10^8	5.0	3.7	2.2	98	3.6	2.1	102
30	1x10^9	1.0	5.5	1.0	101	6.2	0.8	104
30	1x10^9	2.5	4.7	1.3	108	5.4	1.0	96
30	1x10^9	5.0	5.7	1.2	96	5.9	0.8	85

Table 5.3. Statistical evaluation of the effects and interactions of time, inoculum concentration and glucose concentration at different temperatures on changes in pH, %TA and FAN with *Lactobacillus plantarum* (226) as starter culture.

Effects	Interactions	Estimates at 30°C			Estimates at 35°C		
		pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
Main effects							
Time (T)	0 - 15 d (T1)	-1.39	8.37	39.82	-1.43	10.33	48.57
	0 - 30 d (T2)	-1.26	1.20	91.47	-1.19	1.10	92.28
Inoculum (I)	$1x10^7$ - $1x10^8$ cfu.ml^{-1} (I1)	0.25	-0.04	1.73	-0.10	0.05	-0.71
	$1x10^7$ - $1x10^9$ cfu.ml^{-1} (I2)	0.01	-0.04	2.74	0.07	0.04	2.36
[Glucose] (C)	1.0 - 2.5% (w/w) (C1)	-0.96	0.23	5.21	-0.86	0.32	2.94
	1.0 - 5.0% (w/w) (C2)	-1.43	0.75	3.74	-1.49	0.83	5.26
Two-factor interactions							
Time and [glucose]	T1xC1	-0.79	0.25	6.23	-0.77	0.27	0.54
(TxC)	T1xC2	-1.04	0.61	5.94	-1.07	0.55	2.99
	T2xC1	-0.66	0.10	1.59	-0.52	0.21	3.87
	T2xC2	-1.10	0.52	-0.33	-1.16	0.69	4.90
Time and inoculum	T1xI1	0.18	-0.06	1.57	-0.08	0.08	-0.17
(TxI)	T1xI2	-0.04	-0.02	1.92	-0.06	0.10	3.55
	T2xI1	0.20	-0.01	1.03	-0.06	0.00	-0.89
	T2xI2	0.05	-0.03	2.19	0.16	-0.04	0.00
[Glucose] and inoculum	C1xI1	-0.20	0.05	1.92	0.03	0.07	-4.81
(CxI)	C1xI2	0.17	0.07	2.15	-0.06	0.00	-9.82
	C2xI1	-0.27	0.11	0.49	0.10	-0.02	-3.10
	C2xI2	0.14	0.02	-2.81	-0.07	-0.04	-6.43
Three-factor interactions							
Time, [glucose] and inoculum	T1xC1xI1	-0.15	0.07	2.81	0.01	0.04	-2.03
(TxCxI)	T1xC1xI2	0.13	0.06	1.97	-0.17	0.04	-6.20
	T1xC2xI1	-0.20	0.10	2.00	0.07	-0.02	-0.94
	T1xC2xI2	0.13	0.02	-2.26	-0.03	-0.03	-3.90
	T2xC1xI1	-0.16	0.00	0.07	0.02	0.06	-5.18
	T2xC1xI2	0.12	0.04	1.26	0.08	-0.04	-8.54
	T2xC2xI1	-0.21	0.07	-1.26	0.08	-0.02	-3.71
	T2xC2xI2	0.08	0.01	-1.96	-0.06	-0.02	-5.74

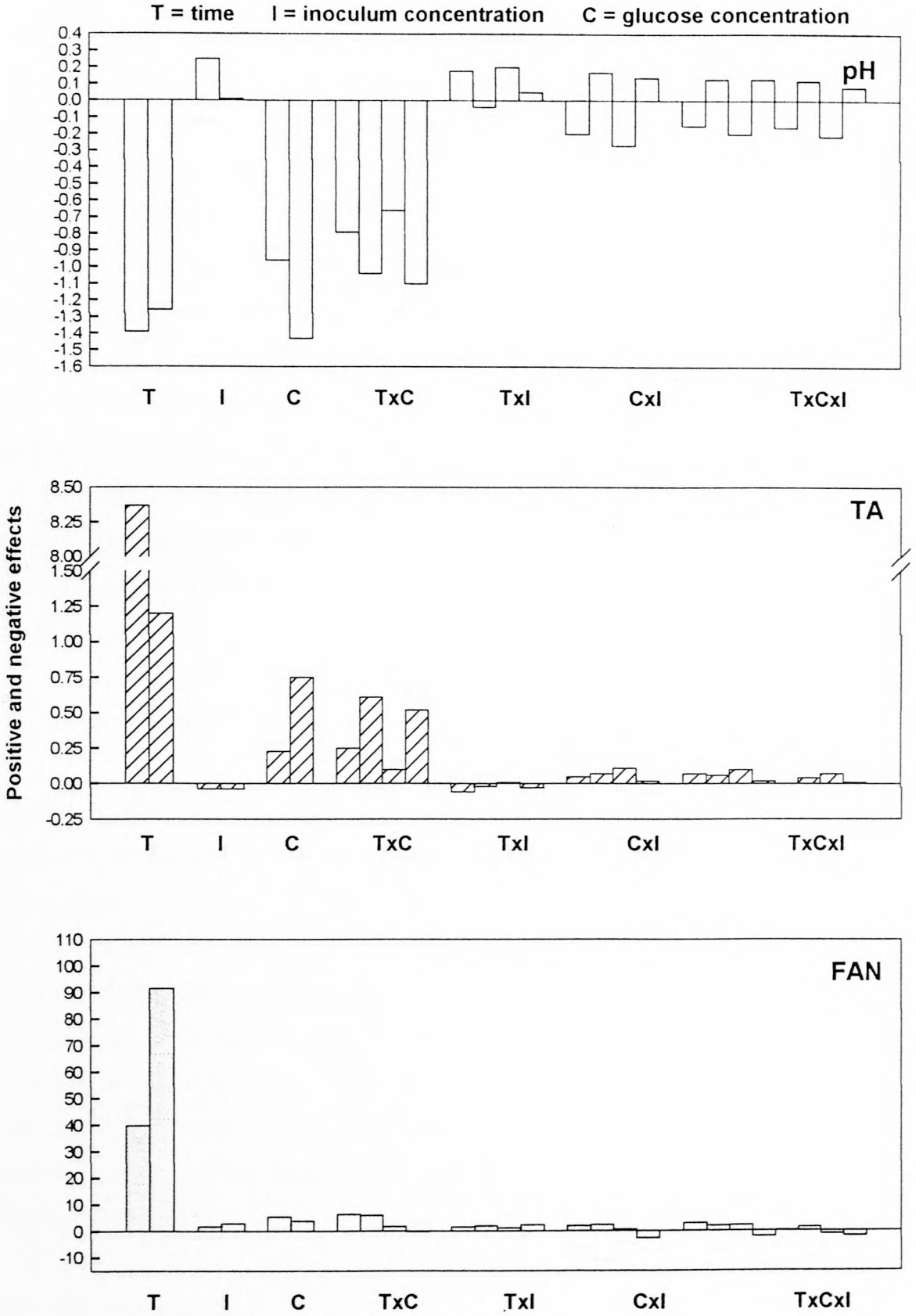


Figure 15 a. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 30°C by *Lactobacillus plantarum* (226).

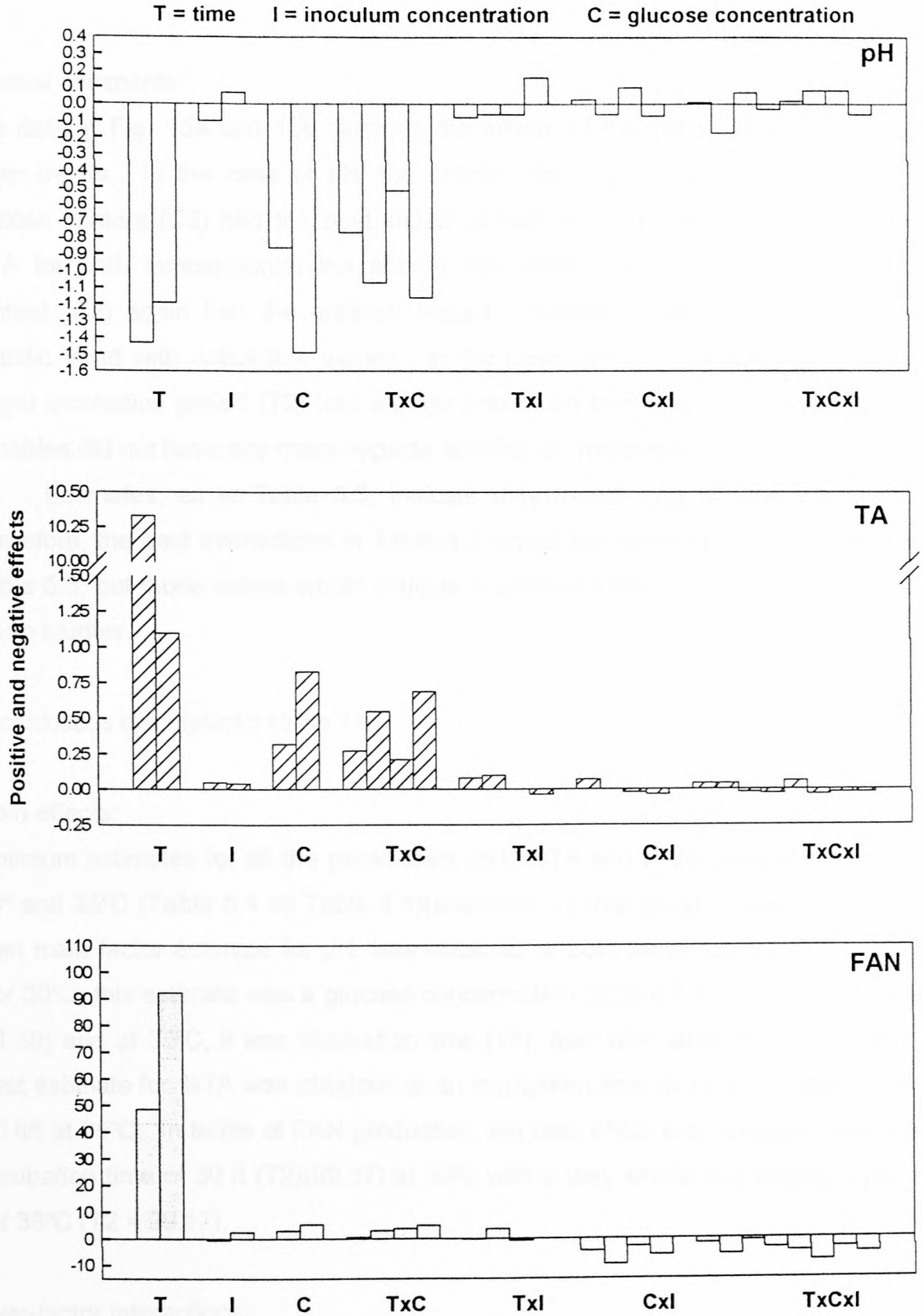


Figure 15 b. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 35°C by *Lactobacillus plantarum* (226).

General comments:

The data in Fig. 15a and 15b illustrate the effects of the variables and indicate the major trends. In the case of pH, the shorter incubation time (T1) and the largest glucose content (C2) had the best impact at both the temperatures. In the case of %TA for both temperatures, the shorter incubation time (T1) and higher glucose content (C2) again had the greatest impact. Inoculum size (I) did not indicate a specific trend with noticeable values. In the case of FAN, the data shows that the longer incubation period (T2) had a major impact on fermentation, whereas the other variables did not have any major impacts at either of the temperatures.

Estimates, as in Table 5.3, indicate only trends and not the actual values. Therefore, the best interactions in Table 4.3 would not necessarily be the same as in Table 5.3, but those values would indicate a positive trend that can be followed up in future studies.

Lactococcus diacetylactis strain 140

Main effects:

Optimum estimates for all the parameters (pH, %TA and FAN) were obtained at both 30° and 35°C (Table 5.4 vs Table 4.4)(estimates vs true data). It was found that the best main factor estimate for pH, was obtained at both temperatures (30° and 35°C). For 30°C, this estimate was a glucose concentration (C2) of 5% (w/w) with a value of (-1.38) and at 35°C, it was incubation time (T1), also with an estimate of -1.38. The best estimate for %TA was obtained at an incubation time of 15 d (T1) with a value of 10.86 at 35°C. In terms of FAN production, the best effect was brought about after an incubation time of 30 d (T2)(99.37) at 30°C with a very similar but slightly lower value for 35°C (T2 = 99.17).

Two-factor interactions:

The best two-factor interaction regarding pH was T2 x C2 with a value of -1.12 at 30°C. Optimum interactions for both %TA and FAN, were T1 x C2 at 35°C resulting in estimates of 0.58 and 7.99, respectively.

Table 4.4. Data obtained during the fermentation of rainbow trout using *Lactococcus diacetylactis* (140) at the different experimental conditions.

Time (d)	Inoculum concentration ($cfu.ml^{-1}$)	[Glucose] (%) (w/w)	Incubation temperature					
			30°C			35°C		
			pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
0	$1x10^7$	1.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^7$	2.5	6.0	0.3	8	6.0	0.3	8
0	$1x10^7$	5.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^8$	1.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^8$	2.5	6.0	0.3	8	6.0	0.3	8
0	$1x10^8$	5.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^9$	1.0	6.0	0.3	8	6.0	0.3	8
0	$1x10^9$	2.5	6.0	0.3	8	6.0	0.3	8
0	$1x10^9$	5.0	6.0	0.3	8	6.0	0.3	8
15	$1x10^7$	1.0	5.5	0.8	80	5.7	0.8	90
15	$1x10^7$	2.5	4.6	1.2	89	5.3	1.0	100
15	$1x10^7$	5.0	3.9	1.8	89	3.7	2.1	103
15	$1x10^8$	1.0	6.3	0.7	99	5.5	1.0	89
15	$1x10^8$	2.5	4.1	1.4	89	4.2	1.3	85
15	$1x10^8$	5.0	3.7	1.8	99	3.8	1.9	105
15	$1x10^9$	1.0	5.5	0.7	67	5.7	0.8	87
15	$1x10^9$	2.5	4.9	1.0	80	4.2	1.3	81
15	$1x10^9$	5.0	3.9	1.7	95	3.7	2.0	106
30	$1x10^7$	1.0	5.9	1.1	102	5.9	0.9	104
30	$1x10^7$	2.5	4.8	1.1	104	5.4	1.2	121
30	$1x10^7$	5.0	3.6	2.3	117	3.8	2.0	114
30	$1x10^8$	1.0	6.1	1.2	101	5.5	1.2	106
30	$1x10^8$	2.5	4.3	1.3	105	4.2	1.4	103
30	$1x10^8$	5.0	3.6	2.3	113	3.8	1.9	109
30	$1x10^9$	1.0	5.8	1.2	106	5.9	0.8	108
30	$1x10^9$	2.5	5.1	1.5	111	4.3	1.2	95
30	$1x10^9$	5.0	3.8	2.1	109	3.7	2.0	105

Table 5.4. Statistical evaluation of the effects and interactions of time, inoculum concentration and glucose concentration at different temperatures on changes in pH, %TA and FAN with *Lactococcus diacetylactis* (140) as starter culture.

Effects	Interactions	Estimates at 30°C pH	TA (%)	FAN (mg)	Estimates at 35°C pH	TA (%)	FAN (mg)
Main effects							
Time (T)	0 - 15 d (T1)	-1.29	9.46	43.37	-1.38	10.86	47.46
	0 - 30 d (T2)	-1.24	1.29	99.37	-1.28	1.13	99.17
Inoculum (I)	$1x10^7$ - $1x10^8$ cfu.ml^{-1} (I1)	-0.02	0.04	2.96	-0.30	0.08	-3.89
	$1x10^7$ - $1x10^9$ cfu.ml^{-1} (I2)	0.07	0.00	-1.24	-0.25	0.02	-5.47
[Glucose] (C)	1.0 - 2.5% (w/w) (C1)	-0.81	0.20	2.31	-0.75	0.22	0.24
	1.0 - 5.0% (w/w) (C2)	-1.38	0.70	7.32	-1.31	0.73	6.49
Two-factor interactions							
Time and [glucose]	T1xC1	-0.62	0.24	1.79	-0.55	0.18	0.02
(TxC)	T1xC2	-0.96	0.51	5.99	-0.96	0.58	7.99
	T2xC1	-0.60	0.07	1.68	-0.57	0.15	0.35
	T2xC2	-1.12	0.54	4.99	-1.01	0.51	1.75
Time and inoculum	T1xI1	0.02	0.02	5.00	-0.20	0.06	-2.36
(TxI)	T1xI2	0.04	-0.05	-2.51	-0.18	0.04	-3.14
	T2xI1	-0.05	0.04	-0.56	-0.25	0.07	-3.48
	T2xI2	0.07	0.04	0.65	-0.19	-0.02	-5.06
[Glucose] and inoculum	C1xI1	-0.32	0.09	-2.60	-0.29	0.00	-5.60
(CxI)	C1xI2	0.13	0.02	1.19	-0.36	0.05	-7.62
	C2xI1	-0.19	0.01	-1.63	0.12	-0.13	-0.72
	C2xI2	0.06	-0.05	1.32	-0.01	0.00	-1.10
Three-factor interactions							
Time, [glucose] and inoculum	T1xC1xI1	-0.30	0.10	-4.68	-0.24	0.04	-3.40
(TxCxI)	T1xC1xI2	0.09	-0.03	1.08	-0.29	0.06	-3.82
	T1xC2xI1	-0.23	0.03	-2.10	0.05	-0.09	0.70
	T1xC2xI2	0.03	0.00	4.84	-0.02	-0.02	1.61
	T2xC1xI1	-0.19	0.03	0.77	-0.20	-0.04	-5.01
	T2xC1xI2	0.10	0.06	0.70	-0.26	0.02	-7.60
	T2xC2xI1	-0.05	-0.02	-0.35	0.12	-0.11	-1.79
	T2xC2xI2	0.06	-0.08	-2.87	0.00	0.02	-3.26

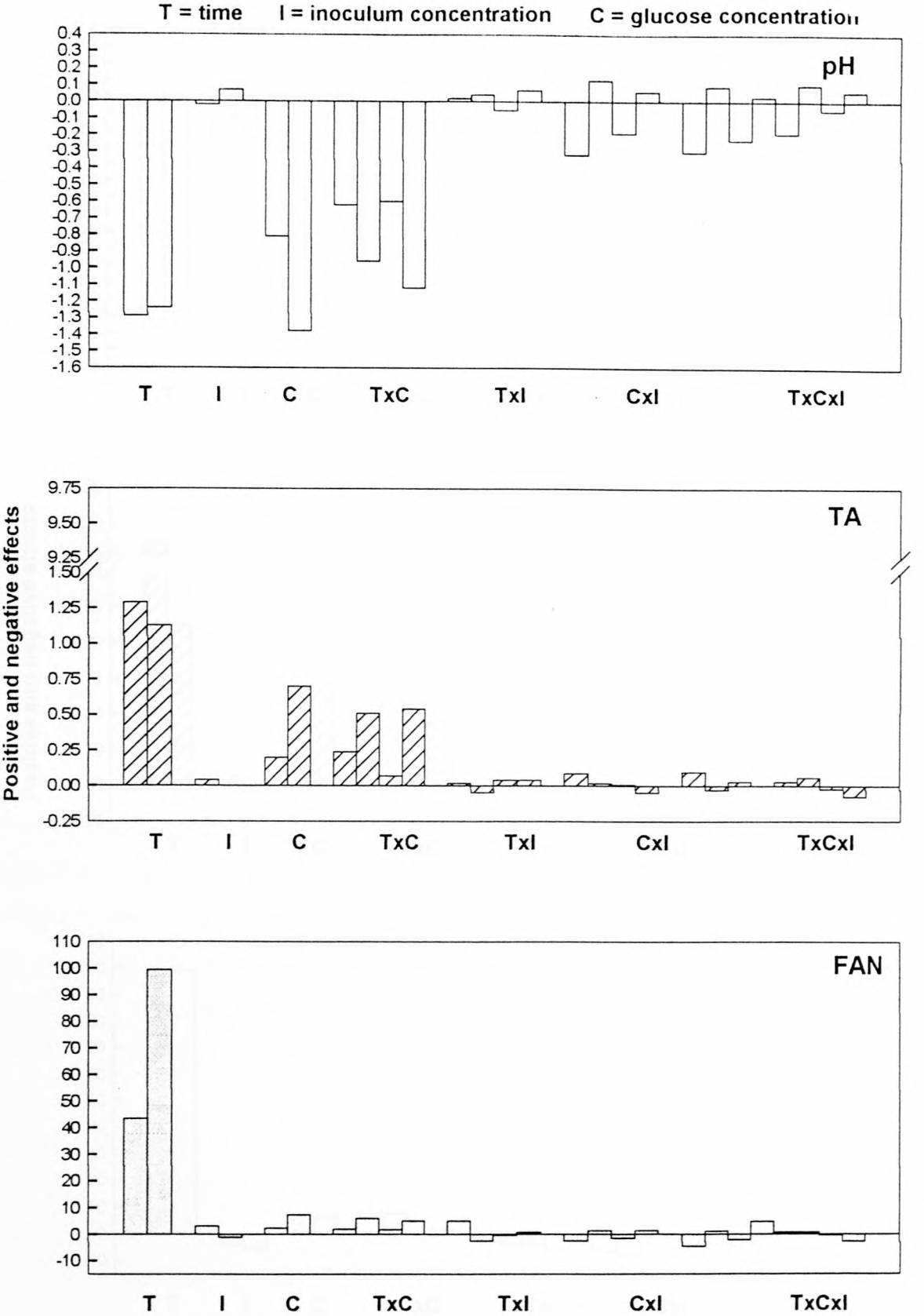


Figure 16 a. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 30°C by *Lactococcus diacetylactis* (140).

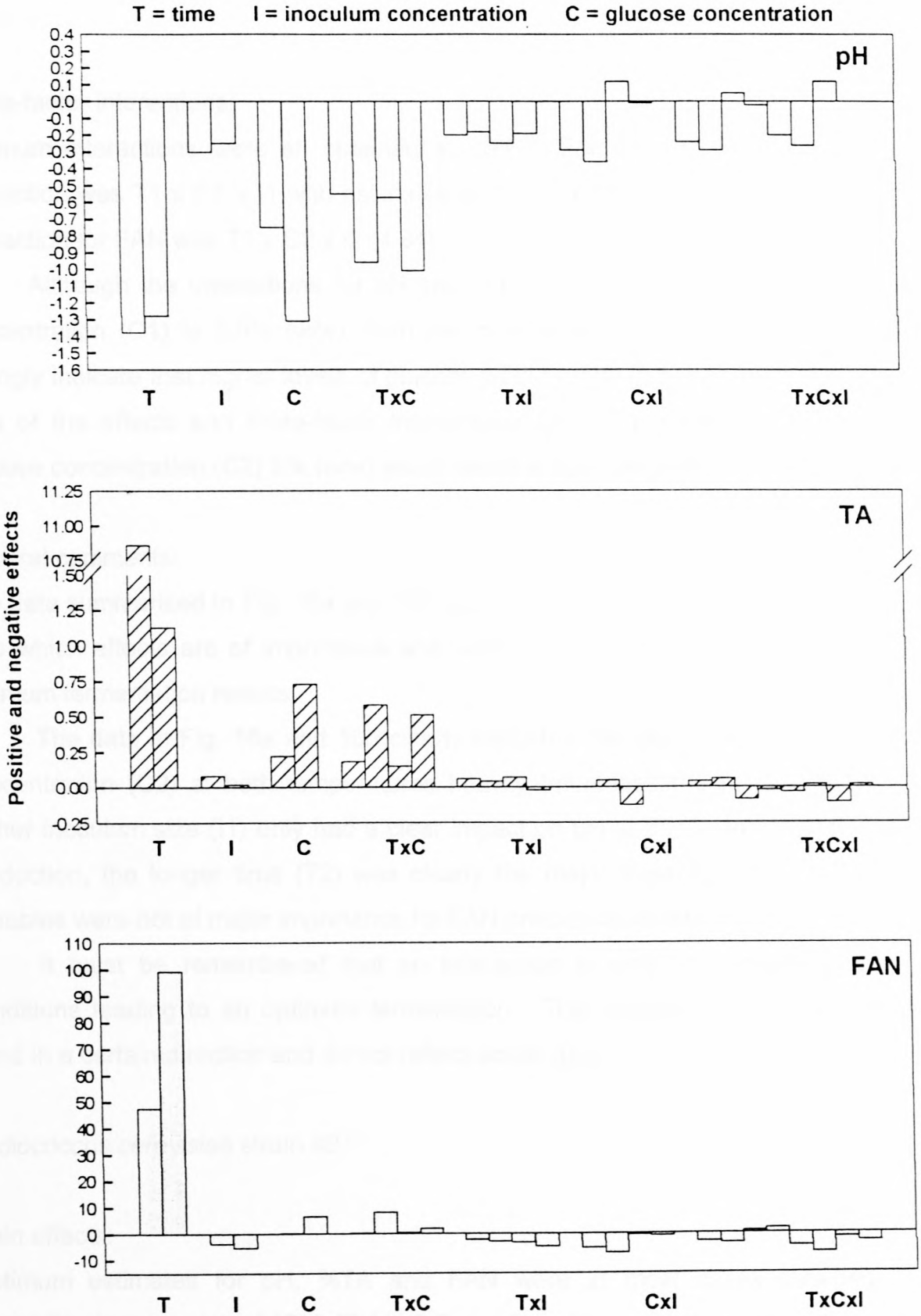


Figure 16 b. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 35°C by *Lactococcus diacetylactis* (140).

Three-factor interactions:

Optimum interactions were all obtained at 30°C. For pH and %TA, the optimum interaction was T1 x C1 x I1 with estimates of -0.30 and 0.10, respectively. The best interaction for FAN was T1 x C2 x I2 (4.84).

Although the interactions for pH and %TA indicate that the optimum glucose concentration (C1) is 2.5% (w/w), both the main effects and two-factor interactions strongly indicate that higher levels of glucose would result in better fermentation. The data of the effects and three-factor interactions for pH also indicate that a higher glucose concentration (C2) 5% (w/w) would result in optimum FAN production.

General comments:

The data summarised in Fig. 16a and 16b not only illustrate the effects of variables but also which effects are of importance and which trends should be followed to obtain optimum fermentation results.

The data in Fig. 16a and 16b clearly indicates that both time (T1) and glucose concentration (C2) at both temperatures heavily influenced the pH and %TA. The higher inoculum size (I1) only had a clear impact on pH at 35°C. In contrast, for FAN production, the longer time (T2) was clearly the major impacting factor. The other variables were not of major importance for FAN production at either 30° or 35°C.

It must be remembered that an interaction is only an indication as to the conditions leading to an optimum fermentation. The values in Table 5.4 indicate a trend in a certain direction and do not reflect actual data.

Pediococcus cerevisiae strain 407

Main effects:

Optimum estimates for pH, %TA and FAN were in most cases obtained at an incubation temperature of 35°C (Table 5.5 vs 4.5)(estimates vs true data). It was found that the best main factor estimate for pH, was an incubation time of 15 d (T1) with an estimate of -1.44 at 30°C. For %TA, the best estimate (10.01) was obtained at 35°C and also an incubation time of 15 d (T1). FAN production reached an optimum estimate after 30 d (T2) with a value of 99.12 at 35°C.

Table 4.5. Data obtained during the fermentation of rainbow trout using *Pediococcus cerevisiae* (407) at the different experimental conditions.

Time (d)	Inoculum concentration (cfu.ml^{-1})	[Glucose] (%) (w/w)	Incubation temperature					
			30°C			35°C		
			pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
0	1x10^{7}	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{7}	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^{7}	5.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{8}	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{8}	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^{8}	5.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{9}	1.0	6.0	0.3	8	6.0	0.3	8
0	1x10^{9}	2.5	6.0	0.3	8	6.0	0.3	8
0	1x10^{9}	5.0	6.0	0.3	8	6.0	0.3	8
15	1x10^{7}	1.0	5.1	1.1	87	5.9	0.7	88
15	1x10^{7}	2.5	4.4	1.3	85	4.3	1.5	94
15	1x10^{7}	5.0	4.1	1.4	81	4.0	1.4	90
15	1x10^{8}	1.0	5.6	0.9	83	5.7	0.7	88
15	1x10^{8}	2.5	4.2	1.3	83	4.2	1.1	89
15	1x10^{8}	5.0	4.1	1.4	75	4.0	1.2	74
15	1x10^{9}	1.0	5.3	0.8	76	5.3	0.8	80
15	1x10^{9}	2.5	4.2	1.2	92	4.1	1.2	81
15	1x10^{9}	5.0	4.1	1.4	91	4.0	1.2	65
30	1x10^{7}	1.0	5.7	1.0	100	6.0	0.6	88
30	1x10^{7}	2.5	4.8	1.2	103	4.5	1.3	109
30	1x10^{7}	5.0	4.3	1.4	101	4.0	1.7	124
30	1x10^{8}	1.0	5.7	1.1	98	6.3	0.6	89
30	1x10^{8}	2.5	4.8	1.5	103	4.5	1.2	110
30	1x10^{8}	5.0	4.0	1.7	109	3.6	2.4	123
30	1x10^{9}	1.0	5.6	0.9	98	5.4	1.0	93
30	1x10^{9}	2.5	4.3	0.9	89	4.2	1.4	110
30	1x10^{9}	5.0	4.0	1.8	116	3.8	1.8	119

Table 5.5. Statistical evaluation of the effects and interactions of time, inoculum concentration and glucose concentration at different temperatures on changes in pH, %TA and FAN with *Pediococcus cerevisiae* (407) as starter culture.

Effects	Interactions	Estimates at 30°C			Estimates at 35°C		
		pH	TA (%)	FAN (mg)	pH	TA (%)	FAN (mg)
Main effects							
Time (T)	0 - 15 d (T1)	-1.44	9.04	42.56	-1.39	10.01	40.31
	0 - 30 d (T2)	-1.25	0.99	93.83	-1.32	1.06	99.12
Inoculum (I)	$1x10^7$ - $1x10^8$ cfu.ml^{-1} (I1)	-0.07	0.07	-0.69	-0.06	0.01	-2.27
	$1x10^7$ - $1x10^9$ cfu.ml^{-1} (I2)	-0.11	-0.03	0.49	-0.21	0.04	-4.86
[Glucose] (C)	1.0 - 2.5% (w/w) (C1)	-0.77	0.18	1.40	-0.99	0.37	7.49
	1.0 - 5.0% (w/w) (C2)	-0.93	0.38	3.35	-1.25	0.58	7.57
Two-factor interactions							
Time and [glucose]	T1xC1	-0.53	0.16	2.38	-0.72	0.28	1.34
(TxC)	T1xC2	-0.61	0.24	0.22	-0.83	0.27	-4.65
	T2xC1	-0.62	0.10	-0.28	-0.76	0.27	9.89
	T2xC2	-0.79	0.33	4.81	-1.04	0.60	16.01
Time and inoculum	T1xI1	0.03	-0.02	-2.02	-0.07	-0.09	-3.50
(TxI)	T1xI2	-0.01	-0.07	0.98	-0.15	-0.06	-7.62
	T2xI1	-0.13	0.13	0.98	-0.02	0.11	0.09
	T2xI2	-0.16	0.01	-0.23	-0.17	0.12	0.33
[Glucose] and inoculum	C1xI1	-0.21	0.07	0.62	-0.02	-0.05	-0.95
(CxI)	C1xI2	-0.12	0.04	1.08	0.11	-0.11	-1.75
	C2xI1	-0.13	0.07	1.02	-0.09	0.10	-2.94
	C2xI2	-0.05	0.14	6.26	0.15	-0.10	-4.63
Three-factor interactions							
Time, [glucose] and inoculum	T1xC1xI1	-0.19	0.05	0.29	0.04	-0.08	-1.21
(TxCxI)	T1xC1xI2	-0.10	0.08	4.56	0.10	-0.09	-1.51
	T1xC2xI1	-0.14	0.05	-0.78	0.05	-0.03	-3.72
	T1xC2xI2	-0.04	0.07	5.33	0.13	-0.07	-4.35
	T2xC1xI1	-0.12	0.06	0.63	-0.07	0.00	-0.21
	T2xC1xI2	-0.08	-0.02	-2.94	0.07	-0.07	-1.12
	T2xC2xI1	-0.06	0.06	2.31	-0.18	0.18	-0.70
	T2xC2xI2	-0.04	0.14	4.06	0.10	-0.07	-2.59

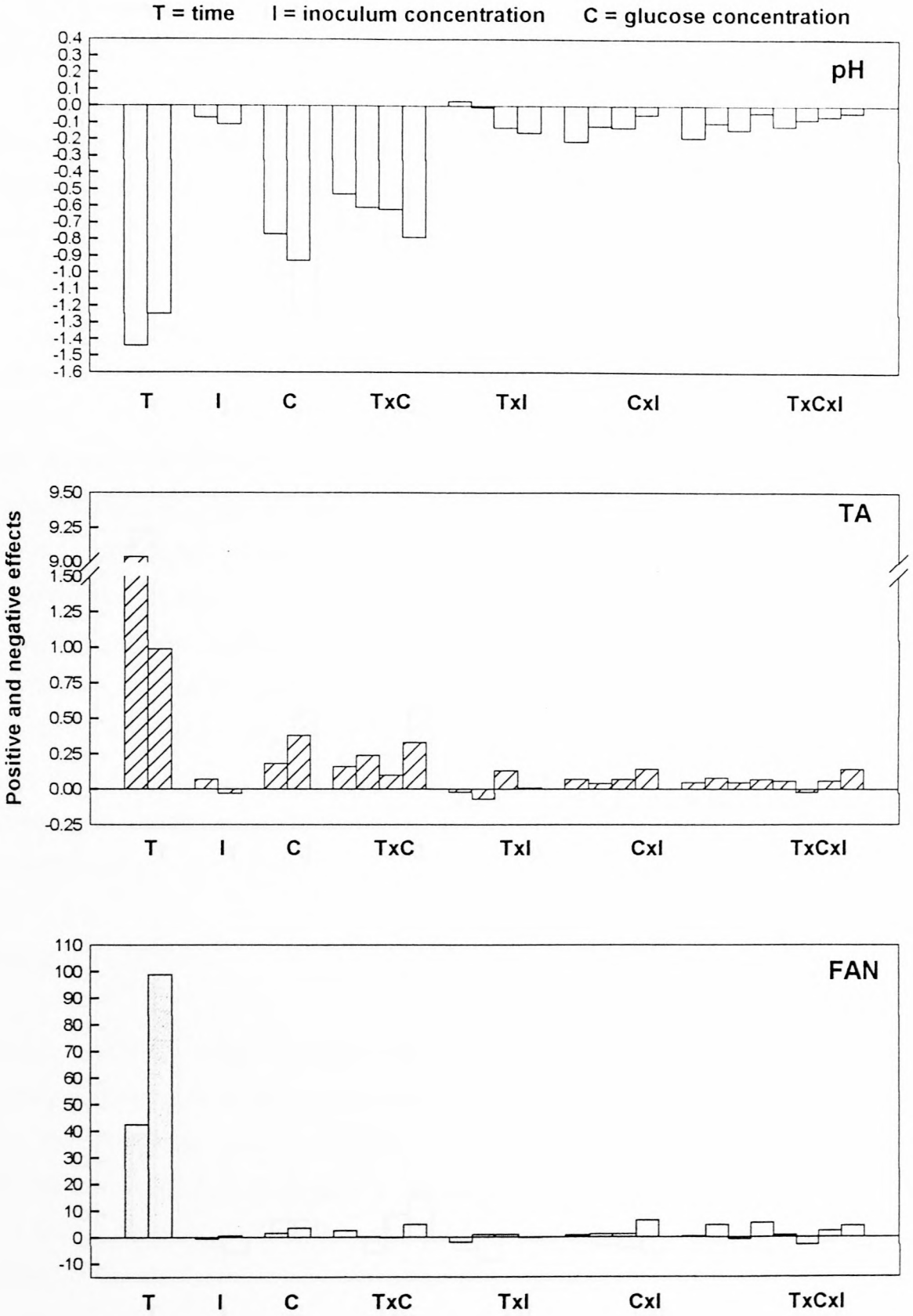


Figure 17 a. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 30°C by *Pediococcus cerevisiae* (407).

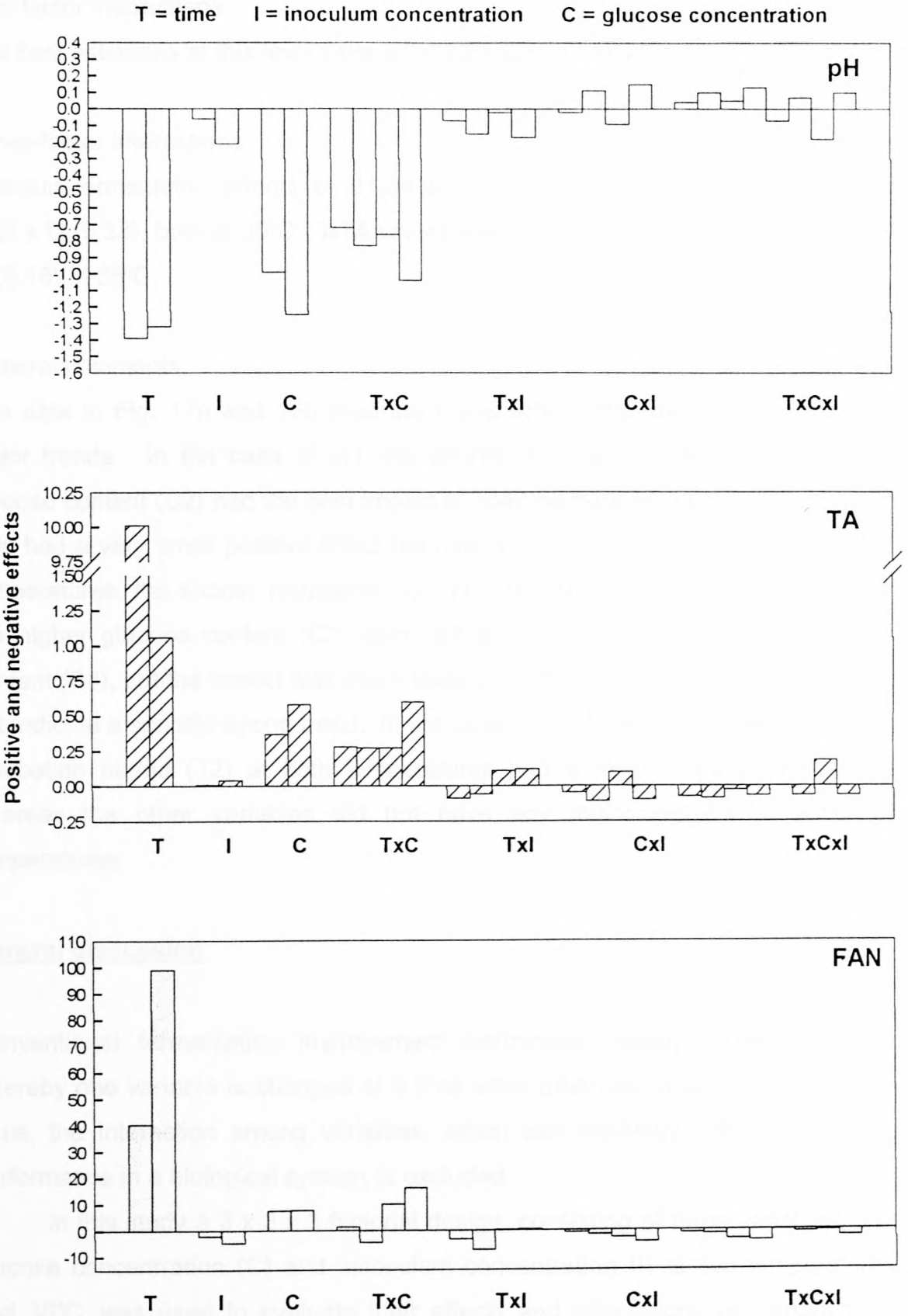


Figure 17 b. Histograms illustrating the Effects of variables, individual and in combination, on the pH, %TA and FAN changes at 35°C by *Pediococcus cerevisiae* (407).

Two-factor interactions:

The best estimates at this level were all at T2 x C2 and at 35°C.

Three-factor interactions:

Optimum fermentation effects for pH were found at T1 x C1 x I1 (-0.19) and for FAN T1 x C2 x I2 (5.33), both at 30°C. %TA values were the best at an interaction of T2 x C2 x I1 (0.18) at 35°C.

General comments:

The data in Fig. 17a and 17b illustrate the effects of the variables and indicate the major trends. In the case of pH, the shorter incubation time (T1) and the higher glucose content (C2) had the best impact at both the temperatures. Inoculum size (I2) only had a very small positive effect but mostly at 35°C. In the case of %TA for both temperatures, the shorter incubation time (T1) had the greatest impact. In this case, the higher glucose content (C2) also had a bigger impact than the lower glucose content (C1), but the impact was much lower than that for the pH. Inoculum size (I) did not indicate a specific strong trend. In the case of FAN, the data shows that the longer incubation period (T2) at both temperatures had a major impact on fermentation, whereas the other variables did not have any major impacts at either of the temperatures.

General discussion

Conventional fermentation improvement techniques usually employ a procedure whereby one variable is changed at a time while other parameters are kept constant. Thus, the interaction among variables, which can markedly influence fermentation performance in a biological system, is excluded.

In this study a 3 x 3 x 3 factorial design, consisting of three variables, time (T), glucose concentration (C) and incoculum concentration (I) at two temperatures, 30° and 35°C, was used to evaluate their effects and interactions on certain important process responses - final pH, %TA and FAN production. The two most important responses are a low final pH and high FAN content. This evaluation technique has found widespread application in industry and gives an indication of major trends which can lead to further process improvements.

Table 6.1. Summary of conditions that indicate major trends for changes in pH.

	Bac. mycoides B3 2N		*Koc. varians* S1		*Lb. plantarum* 226		*Lc. diacetylactis* 140		*Pc. cerevisiae* 407	
	30°C	35°C	30°C	35°C	30°C	35°C	30°C	35°C	30°C	35°C
Main effects										
Time	T1 (-0.46)	T1 (-0.80)	T1 (-0.86)	T1 (-1.11)	T1 (-1.39)	T1 (-1.43)	T1 (-1.29)	T1 (-1.38)	T1 (-1.44)	T1 (-1.39)
Concentration	C2 (-1.09)	C2 (-1.23)	C2 (-0.92)	C2 (-1.33)	C2 (-1.43)	C2 (-1.49)	C2 (-1.38)	C2 (-1.31)	C2 (-0.93)	C2 (-1.27)
Inoculum	I1 (-0.03)	I2 (0.24)	I2 (-0.45)	I1 (-0.06)	I2 (0.01)	I1 (-0.10)	I1 (-0.02)	I1 (-0.30)	I2 (-0.11)	I2 (-0.21)
Two-factor interactions										
Time and glucose	T1xC2 (-0.89)	T1xC2 (-0.89)	T2xC2 (-0.76)	T1xC2 (-1.01)	T2xC2 (-1.10)	T2xC2 (-1.16)	T2xC2 (-1.12)	T2xC2 (-1.01)	T2xC2 (-0.79)	T2xC2 (-1.04)
Time and inoculum	T2xI1 (-0.06)	T2xI1 (-0.06)	T1xI2 (-0.36)	T1xI1 (-0.08)	T1xI2 (-0.04)	T1xI1 (-0.08)	T2xI1 (-0.05)	T2xI1 (-0.25)	T2xI1 (-0.16)	T2xI2 (-0.17)
Glucose and inoculum	C1/2xI1/2 (-0.09)	C2xI1 (-0.30)	C2xI1 (-0.85)	C1xI1 (-0.06)	C2xI1 (-0.27)	C2xI2 (-0.07)	C1xI1 (-0.32)	C1xI2 (-0.36)	C1xI1 (-0.21)	C2xI1 (-0.09)
Three-factor interactions	T2xC1xI2	T2xC2xI2	T1xC2xI1	T1xC1xI1	T2xC2xI1	T1xC1xI2	T1xC1xI1	T1xC1xI2	T1xC1xI1	T2xC2xI1
Estimates at three-factor level	-0.13	-0.23	-0.66	-0.10	-0.21	-0.17	-0.30	-0.29	-0.19	-0.18
pH values	6.00-6.04	6.00-3.80	6.00-3.70	6.00-3.73	6.00-3.69	6.00-4.00	6.00-4.13	6.00-4.15	6.00-4.15	6.00-3.56
pH decrease	-0.04	2.20	2.30	2.27	2.31	2.00	1.87	1.85	1.85	2.44

Table 6.2. Summary of conditions that would result in the greatest increase in %TA.

	Bac. mycoides B3 2N		*Koc. varians* S1		*Lb. plantarum* 226		*Lc. diacetylactis* 140		*Pc. cerevisiae* 407	
	30°C	35°C	30°C	35°C	30°C	35°C	30°C	35°C	30°C	35°C
Main effects										
Time	T1 (7.84)	T1 (10.27)	T1 (8.39)	T1 (9.74)	T1 (8.37)	T1 (10.33)	T1 (9.46)	T1 (10.86)	T1 (9.04)	T1 (10.01)
Concentration	C2 (0.35)	C2 (0.52)	C2 (0.48)	C2 (0.04)	C2 (-0.75)	C2 (5.26)	C2 (0.70)	C2 (0.73)	C2 (0.38)	C2 (0.58)
Inoculum	I1 (0.02)	I2 (-0.11)	I2 (0.10)	I1 (0.76)	I1/I2 (-0.04)	I1 (2.36)	I1 (0.04)	I1 (0.08)	I1 (0.07)	I1 (0.04)
Two-factor interactions										
Time and glucose	T1xC2 (0.27)	T2xC2 (0.44)	T1/T2xC2 (0.36)	T1/T2xC2 (0.57)	T1xC2 (0.61)	T2xC2 (0.69)	T2xC2 (0.54)	T1xC2 (0.58)	T2xC2 (0.33)	T2xC2 (0.60)
Time and inoculum	T1xI1 (0.03)	T1xI2 (-0.06)	T1xI1 (0.12)	T2xI1 (0.06)	T2xI1 (-0.01)	T1xI2 (0.10)	T2xI1/2 (0.04)	T2xI1 (0.07)	T2xI1 (0.13)	T2xI3 (0.12)
Glucose and inoculum	C2xI2 (0.16)	C2xI2 (0.08)	C2xI2 (0.33)	C2xI2 (-0.01)	C2xI1 (0.11)	C1xI1 (0.07)	C1xI1)0.09)	C1xI2 (0.05)	C2xI2 (0.14)	C2xI1 (0.10)
Three-factor interactions	T2xC2xI2	T2xC2xI2	T2xC2xI1	T1xC2xI2	T1xC2xI1	T2xC1xI1	T1xC1xI1	T1xC1xI2	T1xC1xI1	T1xC1xI2
Estimates at three-factor level	0.12	0.10	0.30	0.01	0.10	0.06	0.10	0.06	0.10	0.06
%TA values	0.280-1.700	0.280-1.948	0.280-1.926	0.280-2.120	0.280-2.064	0.280-1.360	0.280-1.440	0.280-1.275	0.280-1.440	0.280-1.275
%TA yield	6.070	6.957	6.879	7.571	7.371	4.857	5.143	4.554	5.143	4.554

Table 6.3. Summary of conditions that would result in the greatest increase in FAN.

	Bac. mycoides B3 2N		*Koc. varians* S1		*Lb. plantarum* 226		*Lc. diacetylactis* 140		*Pc. cerevisiae* 407	
	30°C	35°C	30°C	35°C	30°C	35°C	30°C	35°C	30°C	35°C
Main effects										
Time	T2 (91.96)	T2 (103.32)	T2 (92.75)	T2 (94.28)	T2 (91.47)	T2 (92.28)	T2 (99.37)	T2 (99.17)	T2 (93.83)	T2 (99.12)
Concentration	C2 (6.63)	C2 (3.96)	C1 (-3.78)	C2 (13.04)	C1 (5.21)	C2 (5.26)	C2 (7.32)	C2 (6.49)	C2 (3.35)	C2 (7.57)
Inoculum	I2 (5.05)	I2 (1.04)	I1 (-3.15)	I1 (3.56)	I2 (2.74)	I2 (2.36)	I1 (2.96)	I1 (-3.89)	I2 (0.49)	I1 (-2.27)
Two-factor interactions										
Time and glucose	T1xC2 (10.08)	T1xC2 (3.04)	T2xC2 (0.68)	T1xC2 (12.02)	T1xC1 (6.23)	T2xC2 (4.90)	T1xC2 (5.99)	T1xC2 (7.99)	T2xC2 (4.81)	T2xC2 (16.01)
Time and inoculum	T2xI2 (6.34)	T1xI1 (0.12)	T2xI1 (0.88)	T2xI2 (3.34)	T2xI2 (2.19)	T1xI2 (3.55)	T1xI1 (5.00)	T1xI1 (-2.36)	T1/2xI1/2 (0.98)	T2xI2 (0.33)
Glucose and inoculum	C2xI2 (8.58)	C1xI1 (2.64)	C1xI2 (10.72)	C1xI1 (-4.67)	C2xI1 (0.49)	C2xI1 (-3.10)	C2xI2 (1.32)	C2xI1 (-0.72)	C2xI2 (6.26)	C1xI1 (-0.95)
Three-factor interactions	T1xC2xI2	T1xC1xI1	T1xC1xI2	T2xC1xI1	T1xC1xI1	T1xC2xI1	T1xC2xI2	T1xC2xI2	T1xC2xI2	T2xC1xI1
Estimates at three-factor level	11.68	5.92	15.39	-2.31	2.81	-0.94	4.84	1.61	5.33	-0.21
FAN	8.120-103.544	8.120-95.704	8.120-88.060	8.120-105.280	8.120-86.100	8.120-95.480	8.120-95.172	8.120-106.456	8.120-91.280	8.120-109.760
FAN yield	12.750	11.786	10.845	12.966	10.603	11.759	11.720	13.110	11.241	13.517

A summary of the conditions that indicate major trends for changes in pH, %TA and FAN production are summarised in Tables 6.1 to 6.3. The data obtained with the 3 x 3 x 3 factorial design showed that the manipulation of the most important environmental factors (Effects) had a prominent impact on the metabolic activity of the starter cultures, especially in terms of the final pH, %TA and FAN. The two- and three-factor interactions were found to have less dramatic effects when compared to the main factor interactions. However, interpretation of the three-factor results presented valuable data in terms of practical implementation. The main factor interactions were found to be valuable in the sense that they provided insight into the main expression of the variables contributing to the three-factor interaction results.

The most positive main estimates for the studies were the glucose concentration and the starter incubation time, and specifically at 35°C. Inoculum concentration was found not to be a strong estimate. In contrast, at the three-factor level these two variables had the strongest impact at 30°C, suggesting an important interacting effect.

Inoculum concentration did not seem to play an important part in the fermentation process, as no definite results were obtained regarding this parameter. Incubation time proved to be important for FAN. A period of 30 d was needed for optimum FAN production, although the optimum results for pH and %TA were after only 15 days. An incubation temperature of 30°C seemed to be the most effective. It is of importance to note that the products incubated at this temperature were also found to be more liquid with a better colour and more buttery aroma.

From the data obtained and trends indicated in the study, the following production parameters were selected for the next study and included a 5% glucose concentration, $1x10^8$ cfu.ml^{-1} inoculum concentration, incubation temperature of 30°C, a higher moisture content of 150 ml per 100 g fish and an incubation period of 30 d.

Experimental Study 6: Investigation of the use of different starter combinations for fermented fish production

In this study the macerated fish, with salt, was fermented using the "trend indicators" as obtained in Study 5. These optimum trends included the use of 5% glucose, 10% inoculum with a $1x10^8$ cfu.ml^{-1} concentration and incubation for 30 d at 30°C. The results of changes in pH, %TA and FAN during incubation period are summarised in Table 7 and the data illustrated in Fig. 18. The three lactic acid

Table 7. Data obtained during the fermentation of rainbow trout using single as well as combinations of starter cultures.

Starter	Days	Run 1			Run 2		
		pH	%TA	FAN	pH	%TA	FAN
Control	0	6.2	0.0	13	6.2	0.0	13
	15	4.4	1.6	88	4.4	1.3	78
	30	4.5	2.0	91	4.5	1.7	88
226 - *Lb. plantarum*	0	6.2	0.0	13	6.2	0.0	13
	15	4.3	1.4	83	4.0	1.4	62
	30	4.4	1.9	88	4.0	2.2	83
140 - *Lc. diacetylactis*	0	6.2	0.0	13	6.2	0.0	13
	15	4.4	1.2	91	4.5	1.1	76
	30	4.4	1.7	101	4.7	1.5	86
407 - *Pd. cerevisiae*	0	6.2	0.0	13	6.2	0.0	13
	15	4.2	1.1	70	4.4	1.2	69
	30	4.3	1.7	115	4.4	1.5	88
226 and 140	0	6.2	0.0	13	6.2	0.0	13
	15	4.0	1.5	64	3.9	1.7	99
	30	4.0	2.2	97	4.0	2.5	103
226 and 407	0	6.2	0.0	13	6.2	0.0	13
	15	4.0	1.4	73	3.9	1.8	80
	30	4.2	2.0	85	4.0	1.9	91
140 and 407	0	6.2	0.0	13	6.2	0.0	13
	15	4.6	1.2	85	4.4	1.3	93
	30	4.8	1.5	116	4.4	1.8	109
226 and 140 and 407 (250 ml)	0	6.2	0.0	13	6.2	0.0	13
	15	4.0	1.4	82	4.0	1.4	66
	30	4.0	2.3	116	4.0	2.2	97
226 and 140 and 407 (500 ml)	0	6.2	0.0	13	6.2	0.0	13
	15	4.0	1.4	69	4.0	1.2	61
	30	4.1	2.4	113	4.0	2.2	92
226 and 140 and 407 (1000 ml)	0	6.2	0.0	13	6.2	0.0	13
	15	4.2	1.6	107	4.1	1.6	89
	30	4.2	2.3	112	4.1	2.2	94

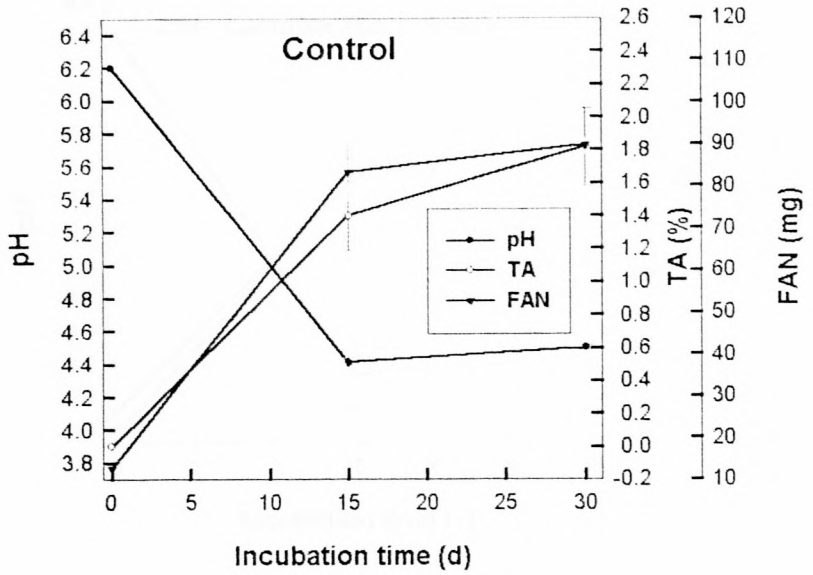
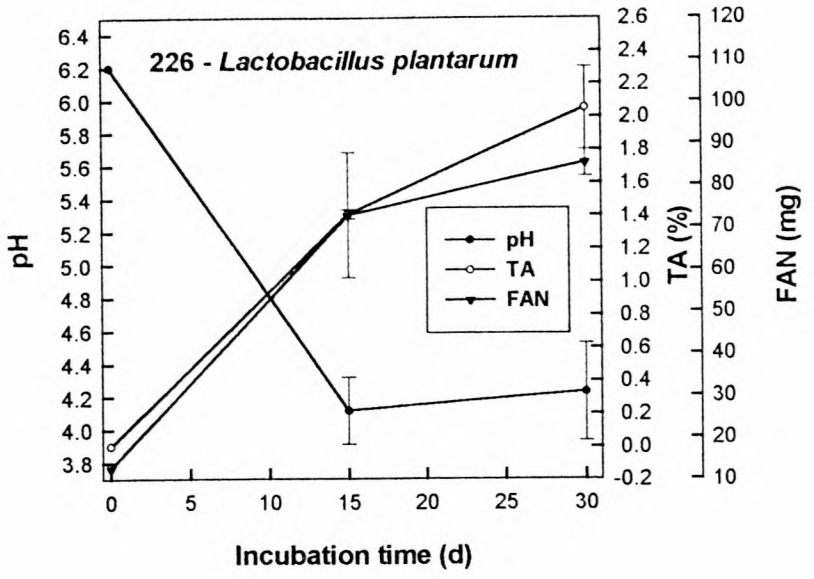
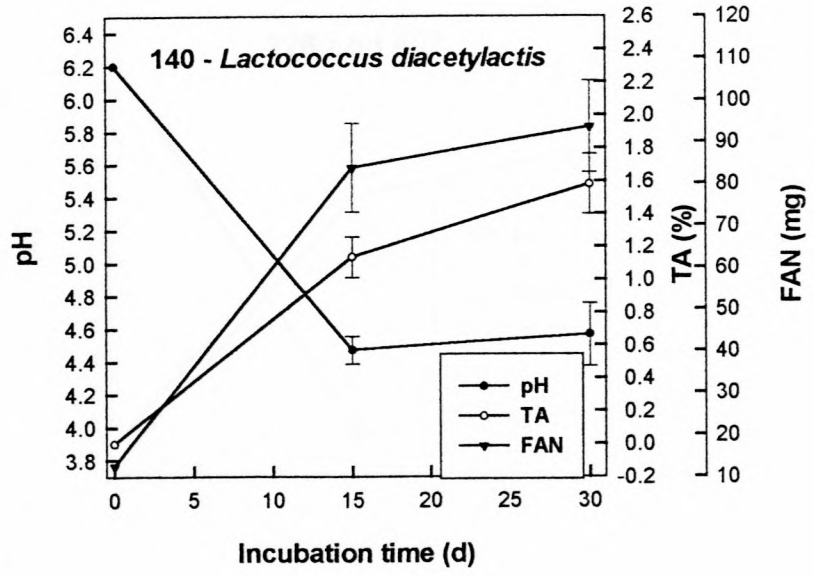


Figure 18. Changes in pH, %TA and FAN during the incubation period for the samples. As the Study was done in duplicate, the error bars indicate the variance of the results. 226 = *Lactobacillus plantarum*, 140 = *Lactococcus diacetylactis* and 407 = *Pediococcus cerevisiae.*





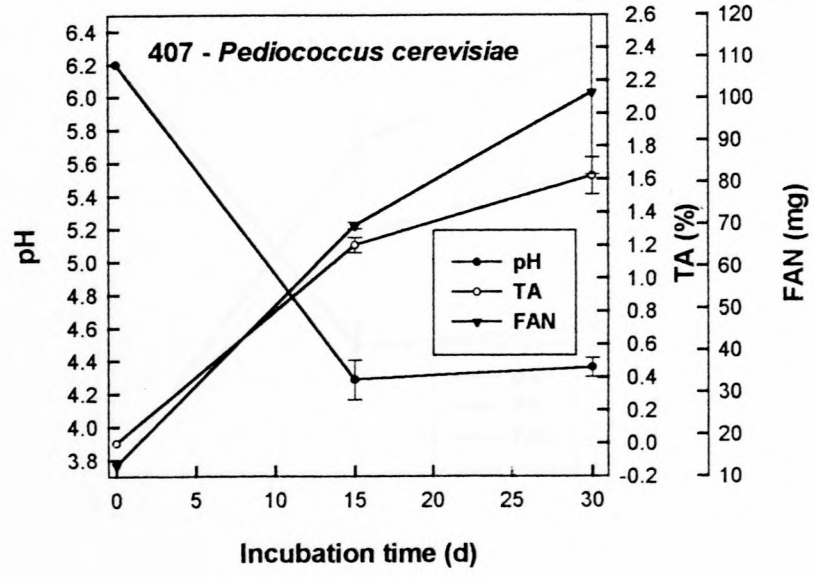
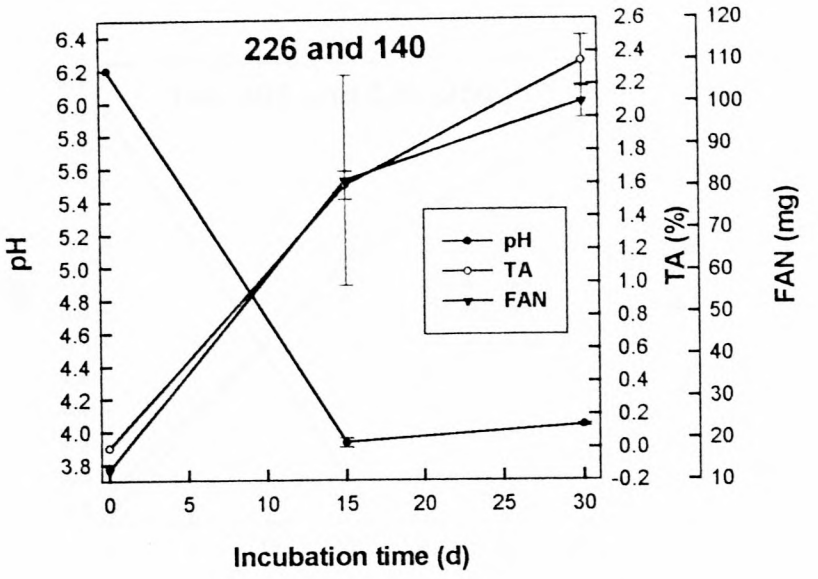
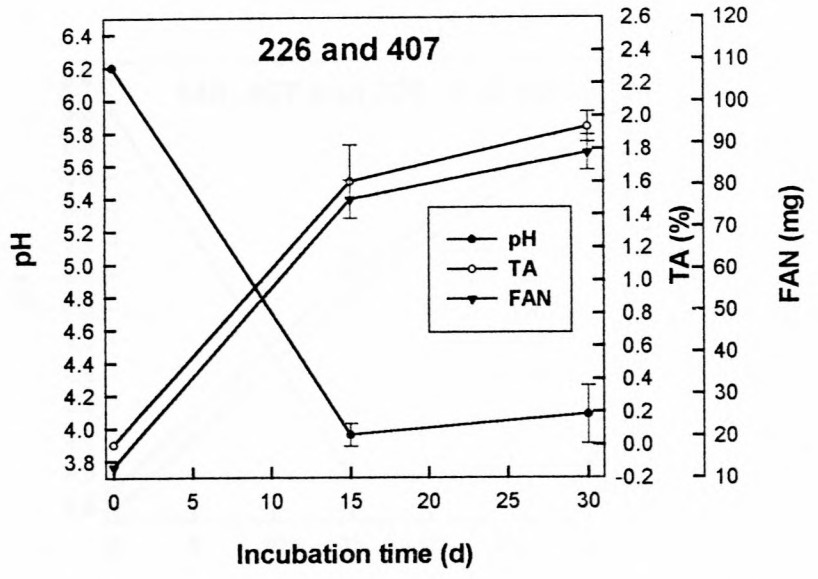


Figure 18. (continued).





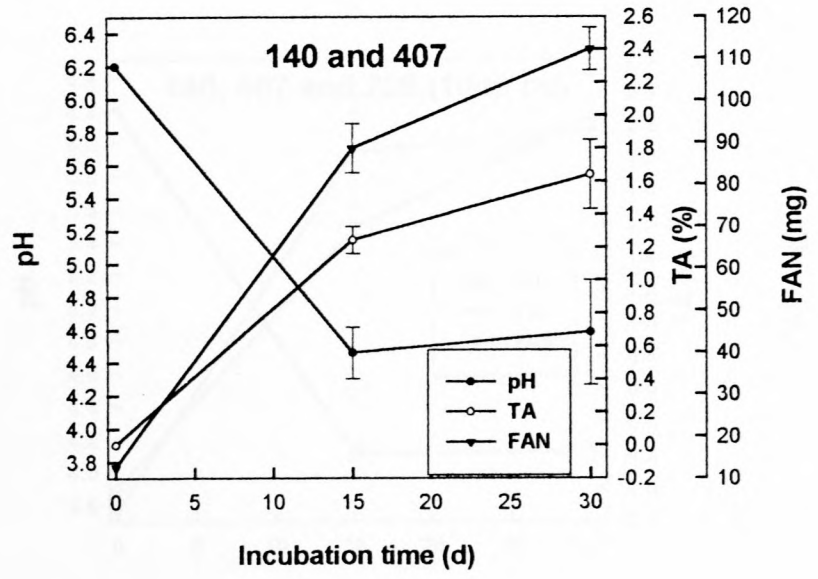
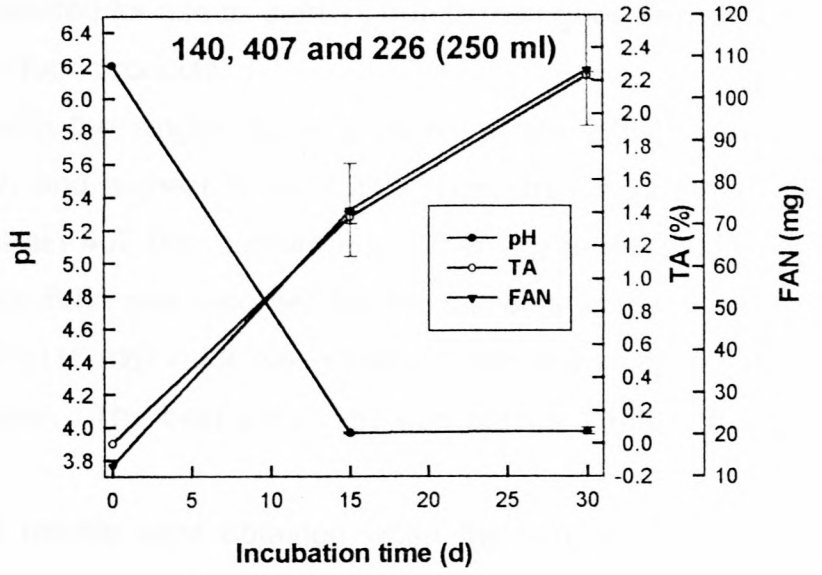
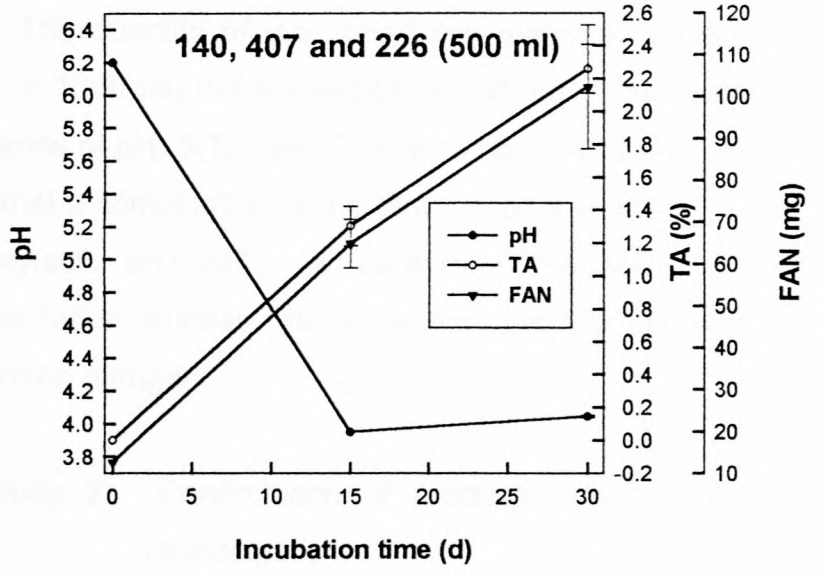


Figure 18. (continued).





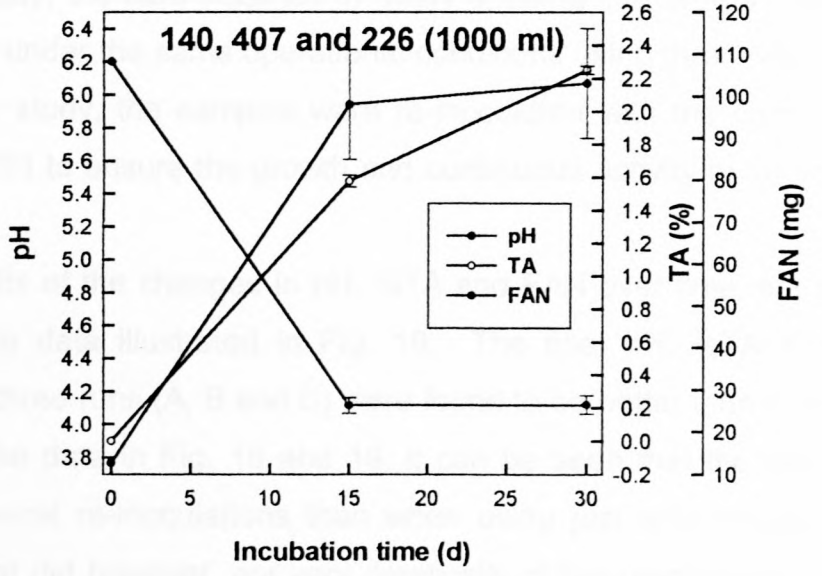


Figure 18. (continued).

bacteria were selected for use as starters due to their good fermentation results as well as their safety in food products.

When using the single starter cultures, strain 226 (*Lb. plantarum)* gave the lowest pH (4.02) and highest %TA (2.23). This strain however, did not produce as much FAN as strain 407 (*Pc. cerevisiae).* When a starter containing two strains was used, the highest %TA was recorded for the samples which included starter 226, but the highest FAN (116 mg) value was obtained from the sample containing strains 140 and 407 as starter. The best pH (3.96) was obtained with strains 226 and 407 as starter.

The best results were obtained when the fish was inoculated with a starter containing all three strains. In one duplicate run, an excellent amount of 116 mg FAN was produced. The quantity of macerated fish material that was used (volumes of either 250, 500 or 1000 ml) did not appear to influence the fermentation results and similar data in terms of pH, %TA and FAN were obtained for all three volumes. It was thus concluded that a combination of the three lactic acid strains (226 - *Lb. plantarum,* 140 - *Lc. diacetylactis* and 407 - *Pc. cerevisiae*) resulted in a more optimum and acceptable fermentation process with a low final product pH, high levels of titratable acids and free amino nitrogen.

Experimental Study 7: Confirmation of production parameters and influence of re-inoculation

In this study, the data obtained in Study 6, using the three starter combination, were reaffirmed under the same operational conditions using three separate runs (A, B and C). In this study, the samples were re-inoculated with the combined starter on days 8, 15 and 23 to ensure the growth and continuous activity of the members of the starter.

The results of the changes in pH, %TA and FAN over time, are summarised in Table 8 and the data illustrated in Fig. 19. The final pH, %TA and FAN results obtained for all three runs (A, B and C) were found to be better than that of the control. By comparing the data in Fig. 18 and 19, it can be seen that the acid production is higher after several re-inoculations than when using just one inoculation (Study 6). The FAN content did however, not vary drastically of the re-inoculations. It was thus concluded that continuous inoculation only aids in enhancing the acid production by

Table 8. Data obtained during the fermentation of rainbow trout using combinations of starter cultures.

Run	Days	pH	%TA	FAN
Control	0	6.2	0.0	12
	15	4.4	1.3	84
	30	4.5	1.7	91
A	0	6.2	0.0	12
	15	4.1	2.1	100
	30	4.1	2.6	107
B	0	6.2	0.0	12
	15	3.9	2.0	106
	30	3.9	2.4	112
C	0	6.2	0.0	12
	15	4.3	1.9	103
	30	4.3	2.5	104

* Key: A, B and C represent the three runs in this Experimental Study

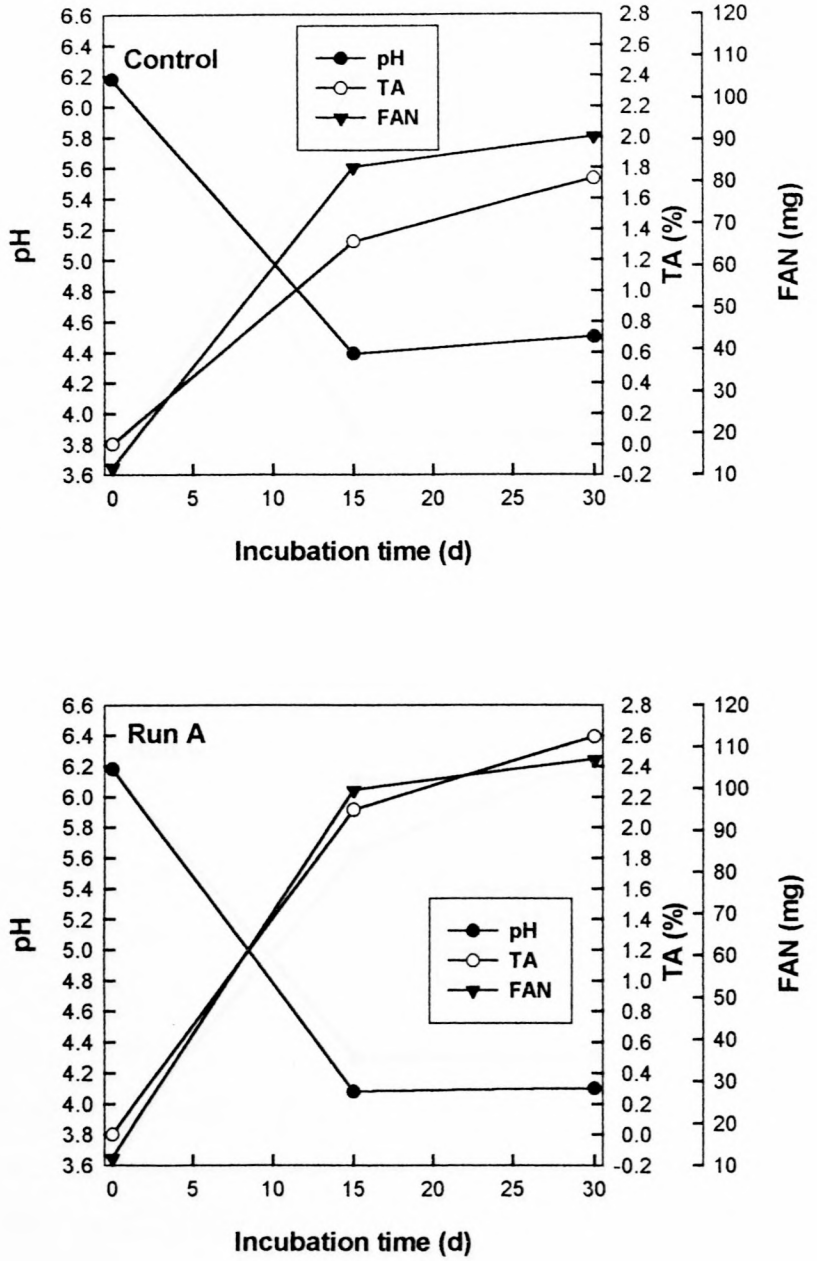
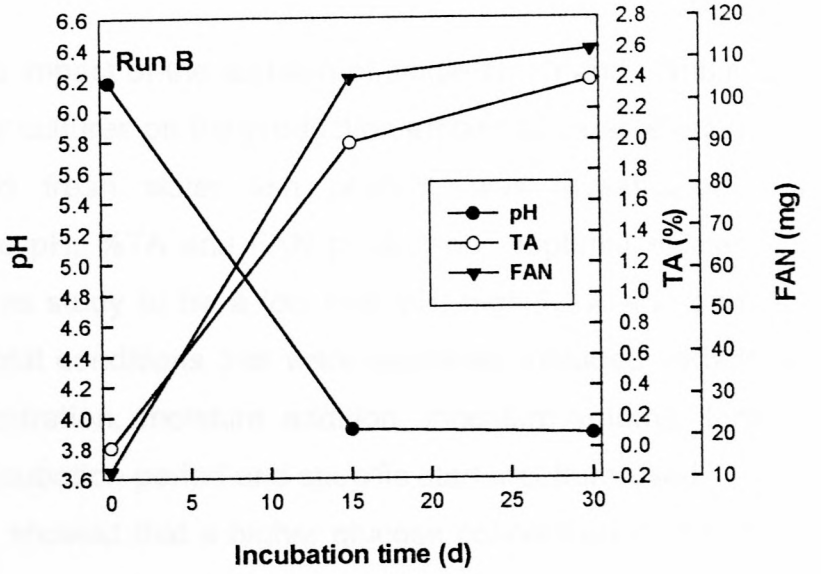


Figure 19. Changes in pH, %TA and FAN during the 30 day incubation period for the control sample and the samples of the three runs (A, B and C).



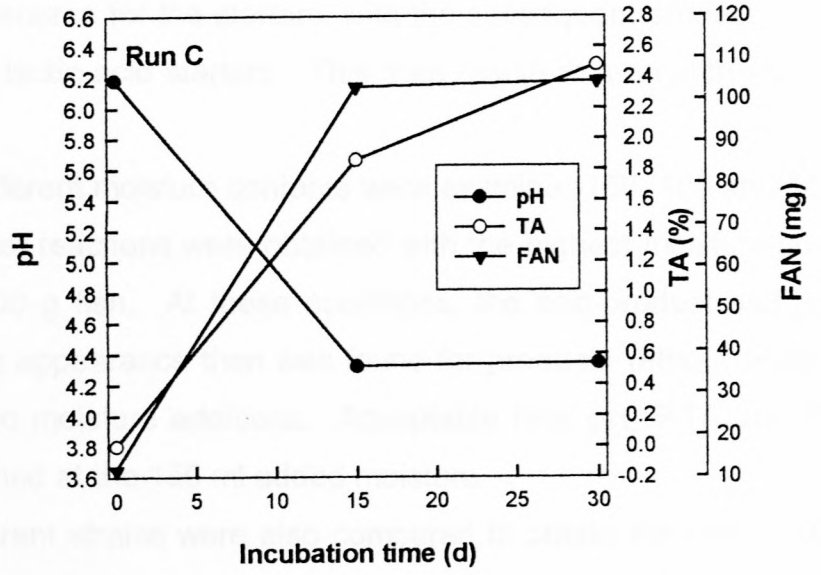


Figure 19. (continued).

the lactic acid bacteria but it does not influence proteolysis and the subsequent amino nitrogen production.

Conclusions

In this study, the impact of the addition of single strain and combinations of proteolytic and lactic starter cultures on the production efficiency parameters used for a laboratory scale fermented fresh water fish product, was investigated. The production parameters were pH, %TA and FAN production. Optimal efficiency conditions were considered in this study to be a low final pH, high %TA and enhanced FAN values. The environmental conditions that were examined included variations in glucose and inoculum concentration, moisture addition, inoculum volume, temperature variation, extending the incubation period and specific starter culture used.

The data showed that a higher glucose concentration of 5.0% (w/w) resulted in the largest pH drop and best acid production for all the starters used. The main reason for this was that the added glucose probably served as an easily utilisable primary carbon source for the starters, with the subsequent production of lactic acid in the case of the lactic acid starters. This then resulted in an increase in the titratable acids.

Three different moisture contents were examined (50, 100 and 150 ml per 100 g fish) and the best reactions were obtained with the highest moisture content of 150 ml moisture per 100 g fish. At these conditions, the end-product had a smoother and more sauce-like appearance than was found for products without added moisture and for the other two moisture additions. Acceptable final pH, %TA and FAN production were also obtained at the 150 ml added moisture.

The different strains were also compared to obtain the most suitable starter for fermentation parameter enhancement. A selection of suitable strains included strains of *Lactococcus diacetylactis, Lactobacillus plantarum, Pediococcus cerevisiae, Kocuria varians* and *Bacillus mycoides*. These starters were examined statistically to obtain the optimum trends for the fermentation conditions and starters. A 3 x 3 x 3 factorial design was used to determine the trends to follow to obtain optimum fermentation results.

The production parameters that were selected for the following study were a 5% glucose concentration, $1x10^8$ cfu.ml^{-1} inoculum concentration, incubation temperature

of 30°C and an incubation period of 30 d. For food safety reasons, the *Bacillus* and *Kocuria* strains were not included in the final selection. The three lactic starters (*Lactobacillus plantarum* 226, *Lactococcus diacetylactis* 140 and *Pediococcus cerevisiae* 407) were selected and the optimum fermentation results were obtained when they were combined as a starter.

References

AOAC. (1975). *Official Methods of Analysis* (12 th Ed.). Association of Official Chemists, Washington DC, USA.

Avery, A.C. (1952). Preservation of very small fish in the Philippine Islands. *Food Technology*, **6**, 4-5.

Box, G.E.P, Hunter, W.G. & Hunter, J.S. (1978). Factorial designs at two levels. In: *Statistics for Experimenters*. New York: John Wiley & Sons. Pp. 306-351.

Dougan, J. & Howard, G. (1975). Some flavouring constituents of fermented fish sauces. *Journal of the Science of Food and Agriculture,* **26**, 887-894.

Lafont, R. (1955). Valeur alimentaire des sauces de poisson. In*: Proceedings of Indo-Pacific Fish Council*, 15th Meeting, Bangkok, Thailand, Sections II and III, p. 163.

Mapstone, S.J. (1998). Department of Food Science, University of Stellenbosch, Personal communication.

NEDA. (1992). National Economic Development Authority. National Statistics Office. Manila, Philippines.

Saisithi, P., Kasemsarn, B-O., Liston, J & Dollar, A.M. (1966). Microbiology and chemistry of fermented fish. *Journal of Food Science*, **31**, 105-110.

Sanceda, N.G., Kurata, T. & Arakawa, N. (1986). Study of the volatile compounds of fish sauces - Shottsura, Nampla and Noucman. *Agricultural and Biological Chemistry,* **50**, 1201-1208.

Sanceda, N.G., Kurata, T. & Arakawa, N. (1996). Accelerated fermentation process for the manufacture of fish sauce using histidine. *Journal of Food Science,* **61**(1), 220-222.

Uyenco, V.I., Lawas, P.R., Briones, P.R. & Taruc, R.S. (1953). Mechanics of 'bagoong' (fish paste) and patis (fish sauce) processing. *Proceedings of the Indo-Pacific Fish Council,* **4**(II), 210-222.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Fermentation is one of the oldest and certainly one of the most economical methods of producing and preserving foods (Battcock & Azam-Ali, 1998; Hull *et al.,* 1992). Various fermented foods, including fermented fish products, make an essential part of the human diet in many parts of the world (Toole, 1998). Fermented fish products can be used as a supplement to cereal food supplying an individual with up to 7.5% of the total recommended nitrogen intake as well as various vitamins and minerals (Saisithi *et al.,* 1966).

South Africa has a large fishing industry, but as far as is known, no fermented fish products are manufactured in South Africa, and fermented fish products that are offered for sale to the public are generally imported from Southeast Asia. Thus, there does appear to be a market for fermented fish sauces and pastes in South Africa but very little information is available on the starters and production conditions needed to produce an acceptable product.

With this in mind, fermented fish products were obtained from Thailand (with the kind co-operation of Prof. J.J. Joubert, 1998, personal communication) and the microbial population examined. In the study, the microorganisms present in 13 different fermented fish sauces and pastes obtained from Bangkok and Khon Kaen (Thailand), were determined. The population was found to consist of members of the genera *Baciillus, Staphylococcus,* as well as some non-enteric, oxidase positive rods, *Stenotrophomonas maltophilia, Chryseomonas luteola, Vibrio alginoliticus, Sphingomonas paucimobilis* and *Agrobacterium radiobacter.* The prevalent population was found to be members of the genus *Bacillus* and these represent strains of the species *Bac. anthracis, Bac. mycoides, Bac. amyloliquefaciens, Bac. licheniformis, Bac. cereus, Bac. lentus, Bac. subtilus* and *Bac. coagulans.*

Some of the isolates such as *Bac. anthracis, Bac. cereus, Sta. hominis* and *Sta. aureus* were not expected to be found due to their potential pathogenic nature and potential role in food poisoning. This was a matter of concern, and it was concluded that the fermented products examined were manufactured under either unhygienic or uncontrolled processing conditions, which could have been the

cause of these high numbers of spoilage/pathogenic types. Surprisingly, no lactic acid bacteria were found as was expected for a fermented product. They probably did not survive due to the low pH (3.8 - 4.2) conditions of the final product.

During fermentation, insoluble fish protein is converted into a more soluble form as a result of proteolysis. The main end-products are usually amino acids, small polypeptides, ammonia and other low molecular mass nitrogenous compounds (Beddows, 1998). Microbes and proteolytic enzymes that are naturally present in fish, are the main cause of tissue breakdown and the subsequent liquefaction of the proteins (Beddows, 1998). Since enzymatic activity is an important aspect of fish fermentation, a range of enzyme activities of the isolates from the Thailand fermented fish products, were investigated. The methods used in the determination of the enzymatic activity of the bacteria isolated from the fermented fish products included, the API Zym technology, the "Standard Methods Caseinate Agar" and the "Universal Protease Substrate" methods. It was found that all the isolates, with the exception of the staphylococci, were proteolytic and the activity was found to vary from 0.008 to 1.938 units. The absence of proteolytic activity by the staphylococci strains, is an indication that these organisms probably did not play a role in the fermentation process, but were present due to contamination or as a result of unhygienic processing conditions. In the case of the proteolytic activity, these are bacteria that can thus assist in the degradation of the fish tissue and might be used in the production of fermented fish products.

Fermented fish products are traditionally produced by adding one part salt to 3 - 10 parts fish and leaving it to ferment for 1 - 15 months at temperatures ranging from 30° to 35°C (Avery, 1952; Dougan & Howard, 1975). In some cases, glucose is added to accelerate the fermentation process (Avery, 1952). In this study, experimental studies were conducted to determine the optimum fermentation conditions for the production of fermented fish products by varying the glucose, inoculum and moisture contents. A selection of proteolytic bacteria that had been isolated from the Thailand fermented fish products, as well as lactic acid bacteria (*Lactobacillus plantarum, Lactococcus diacetylactis, Lactobacillus acidophylis and Pediococcus cerevisiae),* were used as starters in the production of a fresh water fish product. Fresh water trout was selected due to its availability in this region, its good quality and popularity under consumers. It was found that the optimum

fermentation conditions were, a glucose concentration of 5% (w/w) and a moisture content of 150 ml water per 100 g fish. A 3 x 3 x 3 factorial design was used to determine the optimum trends in terms of inoculum concentration, incubation time and temperature, and glucose concentration. This factorial technique was mainly used to indicate the major trends that would give an indication of optimum fermentation conditions.

The best main effects were obtained at an incubation temperature of 35°C, but as soon as more parameters were considered simultaneously (two- and three-factor interactions), the optimum effects were at 30°C. FAN was the most important fermentation parameter analysed as it indicates the rate of proteolysis. The results proved that the best fermentation under conditions used in this study would take place at a glucose level of 5% (w/w) and an inoculum concentration of $1x10^8$ cfu.ml^{-1} for 30 days at a temperature of 30°C. The strains that were the most effective in the fermentation process and the more likely to be used in a fermentation process, were *Lactococcus plantarum* 226, *Lactococcus diacetylactis* 140 and *Pediococcus cerevisiae* 407. Their optimum fermentation action was obtained when they were combined as a starter.

Recommendations and future research

The optimisation of the fermentation process can still be improved by following the trends indicated in the factorial design study more closely. This future study could be combined with evaluations regarding the combinations and combination ratio of cultures that will be used as starters. This should provide further valuable information regarding proteolysis and parameter optimisation.

There is also no strong proof that the starter cultures used in this study survived the fermentation process. High acid conditions probably inhibited the growth of the lactic acid bacteria and may have allowed other microorganisms to out grow and out-compete the added starters. Re-inoculation might be a way of keeping the starter population from dying out. Molecular methods could be used to determine the microbial populations and their activity levels present, during the various stages of fermentation.

Some of the microorganisms naturally present in fish may be spoilage organisms or even pathogens such as *Staphylococcus aureus* and *Bacillus cereus,* as was found in this study. Should the starters not survive during the fermentation process, these unacceptable organisms might increase in number and cause serious food poisoning scenarios. Before such a fermented product is introduced to the general market, it is essential to make sure that no toxins or harmful bacteria are present.

The aroma and flavour compounds should be evaluated chemically and this followed up by sensory evaluation and by gas chromatography. This would eliminate off-flavours and unacceptable aromas. Furthermore, a market and economical study can also be useful to determine the target customers and their taste preferences, and the most acceptable packaging options.

Considering all the above, the data from this study and provided that other safety aspects have been satisfied, it should be possible to produce a tasty, cost effective, acceptable and safe fermented fresh water fish product.

References

Avery, A.C. (1952). Preservation of very small fish in the Philippine Islands. *Food Technology*, **6**, 4-5.

Battcock, M. & Azam-Ali, S. (1998). Opportunities for fermented food products in developing countries. *Food Chain,* **23**, 3-4.

Beddows, C.G. (1998). Fermented fish and fish products. In: *Microbiology of Fermented Foods, Volume 1*, 2nd ed. (Edited by B.J.B. Wood) Pp. 417-429. London: Elsevier Applied Science Publishers.

Dougan, J. & Howard, G. (1975). Some flavouring constituents of fermented fish sauces. *Journal of the Science of Food and Agriculture,* **26**, 887-894.

Hull, R.R., Conway, P.L. & Evans, A.J. (1992). Probiotic - a new opportunity. *Food Australia*, **44**, 112-113.

Saisithi, P., Kasemsarn, B-O., Liston, J & Dollar, A.M. (1966). Microbiology and chemistry of fermented fish. *Journal of Food Science*, **31**, 105-110.

Toole, A. (1998). Traditional fermented foods. *Food Info*, **6**, 2-3.