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MICROBIOLOGICAL SOLID STATE FERMENTATION OF APPLE POMACE BY YEAST AND FUNGUS

A thesis presented in partial fulfilment of the requirements for the degree of Master of Technology in Biotechnology at Massey University, Palmerston North, New Zealand.

by HISHAMUDIN RAHMAT
1991

Abstract

The enrichment of apple pomace prepared by the mechanical juice extraction process using *Candida utilis* Y15 was demonstrated. The organic protein content increased from 4.80% to 6.51% per dry weight of apple pomace with near total exhaustion of reducing sugar available. Using regression equation based on crude fibre content of the fermented apple pomace, the pomace may be more suitable as a feed for ruminants than for monogastrics animals.

The citric acid production by *Aspergillus niger* NRRL 328 on apple pomace prepared by the enzymatic juice extraction process was also demonstrated. *Aspergillus niger* NRRL 328 was found to produce limited amounts of citric acid. Additions of three percent methanol (v/w) stimulated citric acid production significantly. Highest level of citric acid production was observed with addition of 4% (v/w) oil and 3% methanol, which was approximately 44% of the sugar consumed. The production of citric acid seemed to be nitrogen limiting. No citric acid was produced with any exogenous nitrogen addition. The optimum inoculum size was found to be 1×10^5 to 1×10^6 spores per 20 gram of apple pomace.

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HISHAM RAHMAT 31st. March 1991.

- 'The beginning of a new chapter in life' -

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1 Introduction

The New Zealand Apple and Pear Marketing Board currently processes approximately 150,000 tonnes of apple pomace per year. Of this, a waste stream of 35,000 tonnes a year is generated from the juice extraction operation. This residue, apple pomace, consists of flesh, skins, stalks and pips has a high sugar level, about eighty percent moisture content and poses a considerable waste disposal problem. At present, it is trucked from factories to landfill and some is used as pigfood.

A process flow diagram (Clear apple juice concentrate production) is shown in Figure 1. In 1992 apple season, the Board will change its juice extraction operation from a mechanical to an enzymic process whereby the pomace will undergo enzymic degradation and water extraction to liberate sugars. The modified process will also include screening of the pomace so that separate fractions are produced i.e. dejuiced flesh and a mixed of pips, stalks and peel fraction. Enzymes used will be predominantly pectolytic but some side-activities including hemicellulases, arabinases and cellulases are present. The resulting pomace has significantly less sugar and potentially a modified polysaccharides composition (Marks, J., personal communication).

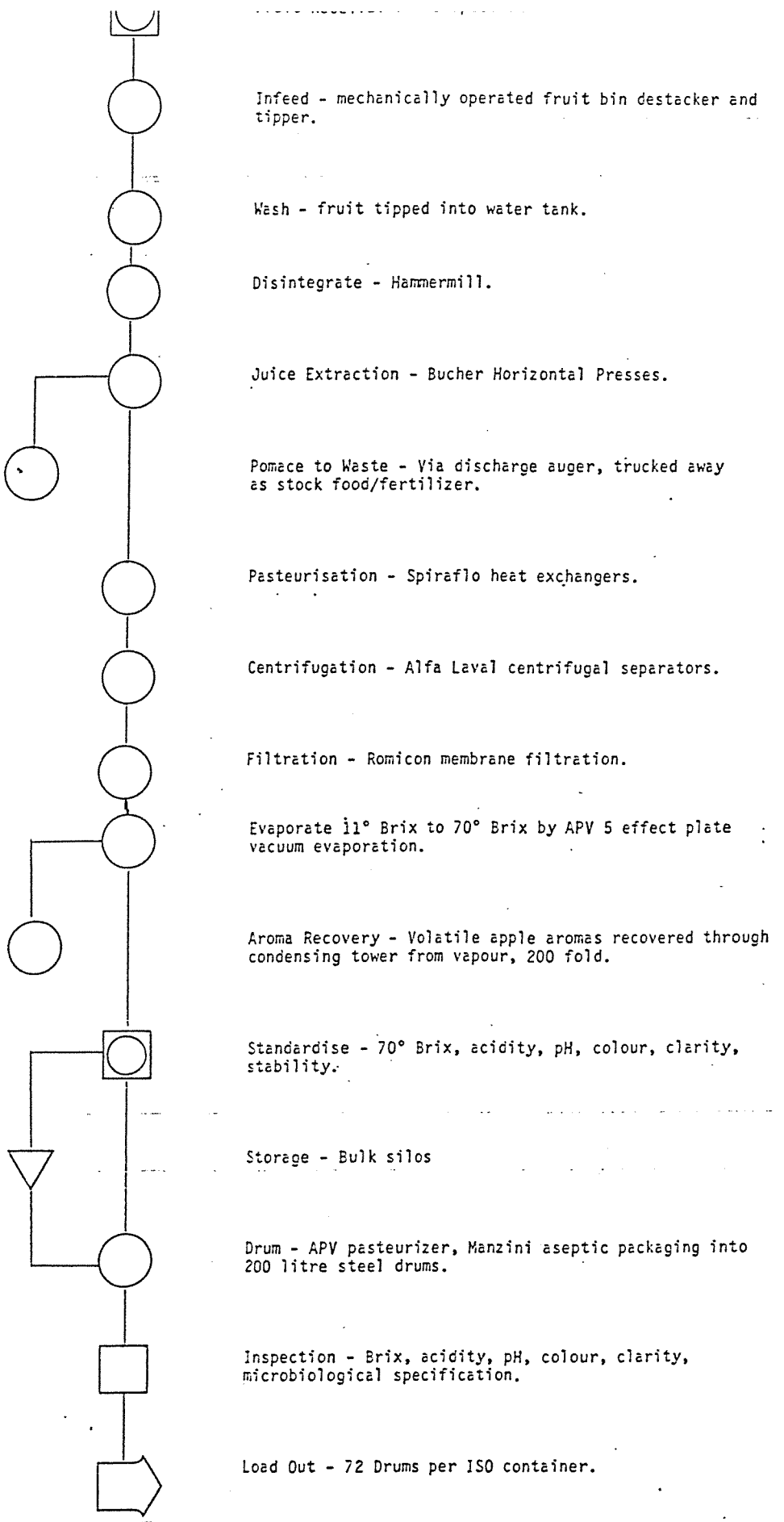


Figure 1 A process flow diagram (Clear apple juice concentrate) - (New Zealand Apple and Pear Marketing Board).

(N.B. From this point onwards, apple pomace from mechanical and enzymic juice extraction operation will be termed old and new apple pomace respectively).

Disposing apple pomace economically has always been a problem. Some of the possibilities of disposing apple pomace are as follows:-

i) Landfill
ii) Stockfeed
iii) Microbiological modification for stockfeed
iv) Pectin production
v) Organic acids and fuel production

1.1 Landfill

This method of disposal is currently being employed by the New Zealand Apple and Pear Marketing Board. Alternative options are currently being investigated by the board since this method poses ecological and environmental problem.

1.2 Stockfeed

Apple pomace itself is not a very nutritious stockfeed due to lack of certain important nutrients notably protein, assimilable carbohydrate and vitamins and also seasonal availability. It also faces competition from existing stockfeed in terms of pricing. Due to its high moisture content of about eighty percent, the cost of drying could be very high and this limits its potential as a cheap stockfeed.

1.3 Microbiological modification for stockfeed

Numerous investigators have investigated the potential for microbiologically modifying or enriching pomace as stockfeed. Rossi *et al.*(1988) has shown that a selected strain of the fungus *Fusarium culmorum* can be grown on a mixture of orange peel powder and wheat straw. The fungal protein was up to 16.32 gram/kg pomace after sixty hour of propagation with an average productivity of 0.16 gram/kg pomace/hour. Solomon *et al.*(1988) has shown that the growth of *Saccharomyces cerevisiae* could be supported on cashew apple pomace with the production of about forty percent protein. Examples of other investigations include the use of sugar beet pulp (Grajek, 1988; Bajon *et al.*, 1985), wheat straw (Laukevics *et al.*, 1984), apple pomace (Hang *et al.*, 1988b), palm oil solid fraction (Martinet *et al.*, 1982), rice straw waste (Han *et al.*, 1974; Han *et al.*, 1976; Han *et al.*, 1978), olive black water (Ercoli *et al.*, 1983), pineapple cannery effluent (Prior, 1984), shellfish waste chitin (Revah-Moiseev *et al.*, 1981) and cheese whey (Sandhu *et al.*, 1983).

This option of utilizing apple pomace is a promising one since it may produce a higher quality but cheap protein source suitable as a substitute for existing animal feeds.

1.4 Pectin production

Pectin is a mixture of methyl esterified galacturonan, galactan and araban. The galacturonan molecules are linked chemically to some of the galactan and araban molecules.

Pectin is mainly used as a gelling agent in the preparation of jellies and similar food products (Merck, 1968).

Bomben *et al.*(1971) and Bomben *et al.*(1973) have described pectin production from apple pomace. The high pectin content of apple peel made itself potentially suitable for use as thickening and flavouring agent in apple pies to replace starch that is presently used as a thickener. A flow diagram of process for making apple peel powder is shown in Figure 2. Since apple peel is only about fifteen percent of total apple pomace, this process is incomplete and could still pose an environmental problem.

1.5 Organic acids and fuel production

Hang (1982) and Jewell *et al.*(1984) reviewed the potential uses of apple pomace for production of fuels and food-grade chemicals. These include ethanol, biogas (primarily methane gas) and citric acid. Production of ethanol and biogas would not be commercially viable since it will face competition from a cheaper source of energy from petro-chemical industry. The production of citric acid could be a viable option since it is a widely used acid and currently New Zealand is importing all the citric acid need for the food industries.

1.6 Objective of this thesis

Based on the above informations, microbial protein for stockfeed and citric acid production on apple pomace will be investigated further in this study.

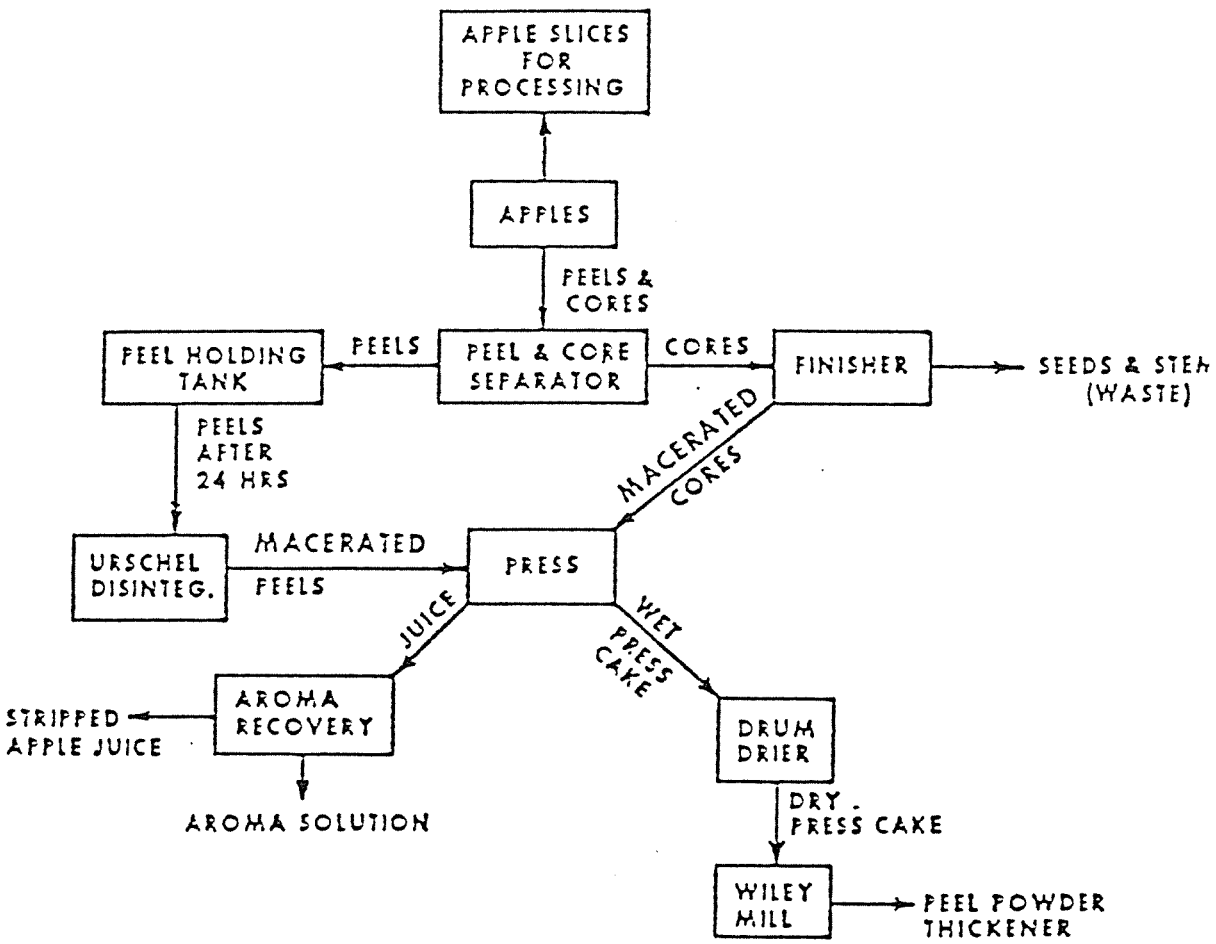


Figure 2 A flow diagram of apple peel powder production (Bomben *et al.*, 1973).

2 Literature Review

2.1 Protein enrichment of apple pomace

2.1.1 World outlook for stockfeed

By the turn of the century, the world population is estimated to be near two billion people (Ledward *et al.*, 1983). With the ever increasing population, the food shortages that are being experienced in certain parts of Africa and Asia will be more acute. This factor, coupled with a poor nutritional value of the available food causes gross malnutrition. The problem is, how to sustain not only an adequate supply of food in the face of increasing populations but also in the face of diminishing resources that are needed to produce this food. Additional and confounding issues include the loss of land to erosion and population pressure for highways and housing (Altshul, 1968; Brown, 1968; Munro, 1968; Pimentel *et al.*, 1983)

Table 1 shows that the world can meet the protein requirement of all the populations but factors such as unequal distribution, high cost of transportation, lack

Table 1 : Available protein (g) per capita per day.

(Ranjhan, 1983)

Region	Supplies percent of requirement	available supplies Total	vegetable	animal
World	108	69.3	44.8	24.4
Advanced Countries				
North America	138	105.7	33.7	72.0
West Europe	128	94.2	41.0	53.2
Oceania	128	107.3	33.7	73.6
Others	108	85.2	45.6	39.7
Developing Countries				
Africa	94	54.9	44.3	10.6
Latin America	107	65.2	8.5	26.7
Near East	100	74.0	59.5	14.4
Far East	102	49.6	42.0	7.6
Asia	92	58.3	46.2	12.1
Others	105	DNA	DNA	DNA
Centrally Planned Economies				
USSR and Eastern Europe	131	102.8	51.6	51.2
Asia	94	63.0	49.7	13.4

DNA : Data not available

of foreign exchange, political or environmental reasons aggravate protein shortages in some parts of the world.

Recently, an increasing demand for high quality protein such as meat and meat products has been observed in the developed and developing countries fuelled by rising standard of living and per capita income (Goldblith, 1970; Waterlow, 1970; Goldberg, 1985). Table 2 shows the

increasing consumption of meat from 1961 to 1980.

With this increasing consumption of meat, it also increases the feed needed to produce the meat and in some cases, human grade proteins are being used as feed; such in the case of feeding barley to stocks (Ganzin, 1974; Clough *et al.*, 1983).

Currently, thousands of tonnes of waste are being generated each year. Some of the waste can be upgraded for feeding animals by chemical or biological methods (Ledward *et al.*, 1983; Tolan, 1983). But the upgrading has to be done in such a way that the resulting feed is of high nutritional value to the animal and at a low cost to farmers (Blair, 1968; McKenzie, 1968; Gaden, 1974; Hammonds, 1975; Levinson *et al.*, 1975; Bull *et al.*, 1979; Slater *et al.*, 1979; Rockwell, 1976; Bellamy, 1983; Wilson *et al.*, 1983; Wiseman *et al.*, 1983)

Table 2 : World meat consumption ('000 tonnes)

(Snowdon, 1984)

	Beef & veal	Lamb & Mutton	Pig meat	Poultry	Total
1961-1965 (average)	31,878	6,547	29,420	11,693	79,539
1966	35,334	6,777	33,609	12,156	87,869
1967	36,826	6,999	34,907	12,842	91,567
1968	38,621	7,180	35,494	13,266	94,552
1969	39,706	7,136	35,764	14,233	96,831
1970	40,190	7,251	38,329	15,572	101,334
1974-1976 (average)	45,486	7,188	45,574	19,729	117,968
1977	48,052	7,190	48,013	22,137	125,383
1978	48,014	7,521	49,853	23,817	129,196
1979	46,780	7,624	53,020	25,847	133,263
1980	46,385	7,929	55,081	27,174	136,561
% change (1965/65 to 1980)	+45	+21	+87	+132	+72

2.1.2 Apple pomace as a stockfeed

Various advantages and disadvantages of using apple pomace as a sole feed or as a supplement to a ration for animals had been reported. The advantage of using apple pomace as a feed is the low cost and in some cases, negligible cost associated with it since apple pomace is itself an agricultural waste.

Dreyer *et al.*(1979) found that apple pomace due to its high fibre content, could be used as a bulking agent in beef feed. The resulting animal was said to have a substantial low level of body fat content, suggesting that apple pomace could be useful in feed to give leaner meats.

This was supported by Bowden *et al.*(1959) when pigs were fed with apple pomace. They found that the pigs produced low fat ham cuts with no apparent effect to the carcass quality, with apple pomace supplemented up to twenty percent of the total feed. However, excessive feeding of apple pomace led to reduced feeding efficiency and slower weight gain.

Physiological problems had been encountered when feeding apple pomace to various stages of growth of animals. The problems ranging from poor calving performance and even death. Feeding large quantity of apple pomace to the animal produced deformed or dead offsprings of cows and lambs. Pesticide residues on apple pomace may have been the cause although some reports indicated otherwise (Snowdon, 1984; Fuller *et al.*, 1989; Wang *et al.*, 1989).

2.1.3 Single cell protein

The most common way of improving protein content of a potential stockfeed is by growing microorganisms on the material to provide source of protein. This protein is termed "single cell protein". The word "single cell protein" was invented at Massachusetts Institute of Technology in May 1966 by Professor Carroll Wilson (Scrimshaw, 1968). It refers to the cells of microorganisms such as bacteria, yeasts, moulds, algae, actinomycetes and higher fungi grown in large scale culture systems for use as protein sources in human food or animal feeds (Enebo, 1970; Humphrey, 1975; Mateles, 1975; Mateles, 1979;

Goldberg, 1985).

During World War I, in Germany, *Saccharomyces cerevisiae* was grown on molasses solely for consumption as a protein supplement. *Candida utilis* (*Torula* yeast) was also grown in hardwood liquors from pulp and paper industries, about 15,000 tonnes of the yeast were used each year as a meat substitute and meat extender, first for the army and prisoners-of-war, and later for civilian population. Later, after the war, in 1920, straw supplemented with inorganic nitrogen fertiliser was used to grow *Aspergillus fumigatus* for animal feed, and other fungi were grown by submerged culture method for human consumption.

Information on SCP production has significantly increased due to several factors. The first and most important reason is that an expectation of high economic returns from the selling of a relatively cheap but high quality protein source has driven commercial interest and research. Concomitantly, the increasing government and public concern for the quality of the environment reinforced the need to provide proper and quick solutions for the disposal of organic wastes of high biochemical and chemical oxygen demands (Goldberg, 1985). Other factors propelling the technology forward were based on political reasons, shortage of foreign exchange and scientific curiosity in developing a new technology. A final factor was due to the successful cultivation of microorganism on petrol fractions; a process which efficiently utilised various inexpensive carbon sources to produce good quality

microbial protein (Vogt, 1970).

2.1.4 Microorganisms

The most important characteristics which are desirable in microorganisms to be used in SCP production are (Bressani, 1968; Marr, 1968; Rockwell, 1976) :-

- high growth rate
- growth on simple media with no requirement for expensive growth factors
- high yield coefficient on a carbon substrate
- high affinity for the carbon substrate
- ability to utilize complex substrate and/or mixture of carbon substrates when required
- ability to grow at high cell densities
- resistance to substrate and/or product toxicity
- stable growth in continuous culture
- high optimum growth temperature
- stable fermentation with resistance to contamination
- capability of genetic modification
- use ammonia as nitrogen source
- pH tolerant
- protein, fat and carbohydrate content of high quality
- low nucleic acid content
- high nutrient content
- high digestibility
- absence of toxicity
- good taste

- ease of recovery
- amenable to further processing, eg; drying, without changes of colour, texture, smell, etc.

Potential microorganisms can be divided into four main categories : bacteria, algae, moulds/fungi and yeasts.

2.1.4.1 Bacteria

The advantages of using bacteria in producing microbial protein are; firstly, higher growth rate and biomass yield from carbon sources than any other organisms.Higher productivity lowers the oxygen requirement and amount of heat evolved per unit of cell produced and thus will lower the cost of aeration and cooling of the fermenters which are parts of operating costs. Secondly, higher protein contents than any other organisms and lastly, better quality of the protein in terms of higher concentration of sulphur amino acids and lysine (Bunker, 1968; Goldberg, 1985).

Some of the disadvantages are the very small size of bacteria which can cause difficulties during the separation stage, the relatively high content of nucleic acids which are unsuitable for human consumption, the unpleasant odour produced by some bacteria and lack of assurance that the type of bacteria chosen would not include pathogenic species.

2.1.4.2 Yeasts

Studies on yeasts in manufacture of and use in food have been carried out quite extensively (Bunker, 1968; Mateles, 1975; Goldberg, 1985). At present, three types of yeast products are commonly available; brewer's yeast which is the by-product of the beer industry and the dried baker's yeast which are of the same genus; *Saccharomyces*. The dried *Saccharomyces cerevisiae* yeast is superior to the brewer's yeast as it does not require debittering and has a higher content and more uniform level of vitamins. While the *Torula* yeast or *Candida utilis* is of a different strain and is capable of assimilating almost all carbohydrate supplied for energy including pentoses, whereas *Saccharomyces cerevisiae* is not capable of utilizing pentose carbohydrate (Oura, 1974).

It is well known that yeasts have a low concentrations of sulphur amino acids but they contain a high level of other essential amino acids namely lysine, tryptophan and threonine. Yeasts are a good source of B group vitamins and have also a small amounts of vitamin E and provitamin D. The quantities of these vitamins vary with strain, specific conditions of cultivation, carbon source and methods of production. They are relatively low in nucleic acid content, low toxicity and greater acceptance by consumers.

2.1.4.3 Fungi

The study of fungi has been mainly concentrated for the production of organic acids and antibiotics. Only recently

the possibility of cultivating fungi as a protein source has been investigated (Litchfield, 1968; Laukevics *et al.*, 1984; Bajon *et al.*, 1985; Grajek, 1987; Durand *et al.*, 1988; Lu *et al.*, 1988; Wainwright, 1990; Friendich *et al.*, 1990).

These researchs have shown that :-

1) the growth rates of fungi are usually slower than those of yeast and bacteria
2) their protein content is usually lower than that of bacteria and yeasts
3) fungal protein is often deficient in sulphur amino acids
4) the problem of digestibility of fungal cell wall
5) fungi are more of an unknown entity with respect to nutrition and toxicology.

However two important advantages of using fungi are

1) most fungi produce a range of carbohydrate hydrolysing enzymes and thus are able to grow on complex raw materials such as cellulose, hemicellulose and starch
2) fungi can usually be recovered by simple filtration, which offers a significant reduction in capital and processing costs.

2.1.4.4 Algae

Modern sewage treatment facilities have been employing the use of algae and other microorganisms to degrade wastes. Owing to their photosynthetic abilities, algae can

be an attractive source of SCP. Since carbon dioxide is readily available, there is no need of any addition of carbon as an energy source and there are even some species that are able to fix atmospheric nitrogen.

Algal growth rates are much lower than those of bacteria and yeasts but the final collection procedure is easier. The production of algal biomass is conducted in a large open pool to provide a huge area for good supply of sunlight, carbon dioxide and nitrogen through the surface of the pool but contamination can be a problem. Algal protein is low in sulphur amino acids, however, the lysine content is high. (Goldberg, 1985; Batt *et al.*, 1984; Laws *et al.*, 1983; Nigam *et al.*, 1981; Vonshak *et al.*, 1983).

2.1.5 Carbon sources for SCP production

Carbohydrate containing byproducts or wastes of agriculture or industry have always been a traditional source of carbon for SCP production. Most of these waste contain a large proportion of readily assimilable sugars, starch or cellulosic material (Bunker, 1968; Gaden, 1974; Rockwell, 1976).

A real major advance of SCP technology has been in petrochemical industry. The technology originated in an attempt to desulphurize petroleum fractions. This had a limited success, but through the work, it was observed that yeasts, particularly *Candida lipolytica*, would grow on petroleum fractions, utilizing the n-alkanes as sole carbon sources. Now, some petrochemical companies in Russia and

Czechoskovakia have made the production of microbial protein from hydrocarbons the main goal.

Many raw materials have been considered as carbon and energy sources for SCP production. They can be divided into three main groups; CO_2, fossil mass (petroleum, natural gas, coal and oil shale) and renewable biomass (lignocellulose, starch and sugar).

As the raw material cost accounts for a major portion in SCP production which can be up to 60%, the raw material basis becomes very important. The raw material offering the greatest commercial advantages depends upon the factory location, substrate availability and competitive cost. Competitive cost means both cheaper raw materials and a better process in terms of favourable scale of operation, highest product yield, lower energy costs and lower capital investment (Gaden, 1974; Resmini, 1974).

The SCP production utilizing carbon dioxide and fossil mass as carbon sources will not be reviewed in detail here. (Blanch *et al.*, 1973; Goldberg *et al.*, 1976; Gutierrez *et al.*, 1978; Humphrey, 1967; Kapelli *et al.*, 1976; Leao *et al.*, 1982; Leao *et al.*, 1984; Moo-Young *et al.*, 1971; Mor *et al.*, 1968; Munk *et al.*, 1969; Repaske, 1966; Resmini, 1974; Samuelov *et al.*, 1982a; Samuelov *et al.*, 1982b; Siegel *et al.*, 1984; Tani *et al.*, 1980; Wilkinson *et al.*, 1974; Yoshida *et al.*, 1971).

2.1.5.1 Renewable resources

Various types of renewable raw materials have been used as important industrial feedstocks in the organic chemical and fermentation industries. They are not only renewable, but also adaptable, cheap, widely available and in most cases, a waste product (Rockwell, 1976; Lipinsky, 1974). The major component of the renewable biomass of relevance to SCP production is the carbohydrate fraction.

Carbohydrate substrates are divided into two major classes; saccharides and polysaccharides. Example of raw materials containing saccharide substrates include molasses, whey, sulphite waste liquor and waste of fruit and vegetables. Polysaccharide rich substrates are of two types; starchy and cellulosic. Starchy materials, such as grains or cassava, are usually chemically or enzymatically treated in order to convert the starch to assimilable sugars. Cellulosic materials, although abundant, normally require hydrolysis of the cellulose to saccharides. Typical examples are wood wastes, bagasse, corn cobs and oat hulls.

For microbial protein production, the carbohydrate substrates used mostly are agricultural wastes or byproducts. Advantages are their abundance, renewable, low cost and also as a mean of relieving waste disposal problem. But the substrates suffer from fluctuations in quality, availability and cost due to seasonal harvests and climatic conditions.

2.1.5.1.1 Saccharide substrate

Saccharide substrate are polyhydroxy aldehydes and polyhydroxy ketones with empirical formula $(CH_2O)_n$, or their derivatives. The most abundant monosaccharide is the six carbon (hexose) sugar, D-glucose, which is also the building block of the most abundant polysaccharides. Other important hexoses are fructose, galactose and mannose.

Pentose (five-carbon) sugars occur in plants both as free sugars and in the form of polysaccharides (pentosan such as hemicellulose). Most important in nature from a quantitative point of view, are D-xylose and L-arabinose found in plant polysaccharides (Bunker, 1968).

The most common disaccharides are sucrose, found in sugar cane and sugar beet, maltose, which is formed as an intermediate product of the action of amylase on starch, lactose, found in milk but otherwise not occurring in nature, and cellobiose, formed as an intermediate product of cellulose hydrolysis.

2.1.5.1.2 Polysaccharide substrates

2.1.5.1.2.1 Starch

Starch is the major storage carbohydrate of plants and is produced commercially from corn, wheat, barley, rice, potatoes, tapioca and sago.

Starches are mixtures of two different types of polysaccharides. The minor components (about 25%) is the - amylose molecule, which consists of long linear chains,

in which the D-glucose units are bound in β(1 -> 4) linkages. The chains vary in molecular weight from a few thousand to five hundred thousand. Amylose is not truly soluble in water but form hydrated micelles in which the polysaccharide chain is twisted into a helical coil with six monomer units per turn. The major component (about 75%) of starch, amylopectin, is a highly branched polymer which molecular weight may be as high as one million, consisting of many short β(1 -> 4) linked branches of approximately twenty D-glucose units each, attached through β(1 -> 6) branch points. Amylopectin, too, yields colloidal or micellar solutions (Lawson *et al.*, 1978).

Starch is difficult to handle owing to its insolubility in water. Its inaccessibility, especially for yeast is due to differing types of granule structure. Thus, starchy materials must be converted to mono and disaccharides to be suitable as substrates for SCP production. This hydrolysis can either be catalyzed by enzymes or by dilute acids (Jarl, 1969).

In most commercial processes, the starchy material is hydrolysed chemically or enzymatically, and then fermented with a yeast strain to form the SCP product. One of these processes was based on a dual yeast fermentation system, in which the yeast *Saccharomyces* (*Endomycopsis*) *fibuliger* produces α- and β- amylases for hydrolysis of starch to glucose and maltose, which enables co-growth of *Candida utilis.* Because of the faster growth of the later yeast, the percentage of *S. fibuliger* cell solids is only 4% of

the harvested biomass, which is intended for animal feed use. However, the process practically was limited by the intermittent availability of the waste stream from potato processing operations, and it was finally discontinued (Senez, 1984; Goldberg, 1985).

2.1.5.1.2.2 Lignocellulosic raw material

The major components of agriculture residue are primarily cellulose and hemicellulose, which are the structural wall polysaccharides of most green plants. These are the most plentiful renewable organic compounds on earth (Ander *et al.*, 1984; Durand *et al.*, 1984; Leisola, 1984a; Leisola, 1984b; Johnson *et al.*, 1989).

Cellulose from natural sources is frequently associated with other polymers such as hemicellulose, lignin and pectin (a polymer containing D-galacturonic acid), which protect the cellulose fibres from the environment (Buchert *et al.*, 1989; Burns *et al.*, 1989).

Hemicellulose is not related structurally to cellulose, but is a polymer of D-xylose in β(1 -> 4) linkage, with side- chains of arabinose and other sugars i.e. glucose, galactose and mannose.

Lignin is found in all fibrous plant and comprises from 14 to 30% of the dried plant residue. This material is the structural glue that binds filaments of cellulose into fibre for cell integrity and rigidity. Lignin is a three dimensional polymer and its structural backbone is composed of phenyl propanoic monomeric units, linked together in a

variety of complex chemical bonds. In plants, lignin is formed by peroxidase-mediated free radical polymerization of hydroxylated and/or methoxylated cinnamyl alcohols including coniferyl sinapyl and p-coumaryl alcohols. The relative contribution of each varies with the plant species, but in all cases lignin contains numerous and complex intermonomeric linkages (Bergeron *et al.*, 1989; Lawson *et al.*, 1978).

Most processes for the utilization of cellulosic raw material in microbial SCP production have three stages in common. They employ some means of pretreatment in order to effect at least some initial molecular size reduction and more often to cause a disassociation of cellulose and lignin. Furthermore, they involve acid and/or enzymatic hydrolysis (depolymerization) of the cellulose and hemicellulose to produce monodisaccharides. Finally, they employ fermentation to produce the SCP product. A host of technical problems are encountered in the fermentation of cellulosic materials is :-

- polymers of glucose or xylose are not readily available to microorganisms
- lignin and hemicellulose degradation are difficult to bacteria
- enzyme inhibitors and/or microbial inhibitors
- insoluble in both a crystalline and an amorphous state
- difficult to,agitate in suspensions above 100 gram/ litre cellulosic materials

- virtually impossible to measure microbial growth
- use of solid substrate is difficult relative to liquid substrates in continuous cultures, difficult to provide homogenous conditions, increased sterility problems, higher power, and hence higher capital inputs in pretreatment etc.
- sampling and line plugging problems.

Other nutritional requirements for biomass production.

In addition to the carbon source, all microorganisms require sources of energy, nitrogen, minor elements (phosphorus, potassium, sulphur, magnesium), and trace elements, and may require supplemental nutrients such as vitamins. The nutritional needs of the microorganisms are diverse as they differ in their inherent ability to synthesize essential growth factors from simple nutrients.

2.1.6 Energy sources

Energy is made available to microorganisms in several different ways (Rockwell, 1976; Jones *et al.*, 1984; Goldberg, 1985). Photosynthetic algae and bacteria possess photosynthetic pigments which allow utilization of solar energy. Others, called chemolitotrophs, obtain energy by oxidation of specific inorganic substrate e.g. nitrites, nitrates, sulphates and ferrous salts. These include bacteria belonging to the genera *Thiobacillus, Nitrosomonas, Nitrobacter, Hydrogenomonas, Alcaligenes* and *Desulfovibrio*. Lastly, the largest group of microorganisms, the heterotrophs or chemoorganotrophs, gain their energy

by catabolism of organic substrates.

2.1.7 Sources of nitrogen

Microorganisms show great diversity in their ability to utilize nitrogen sources (Rockwell, 1976; Goldberg, 1985). Many microorganisms are autotrophic for nitrogen, being able to grow on nitrate, ammonia, urea and sometimes on gaseous nitrogen as the sole source of nitrogen, but others need this element to be supplied as amino acids (protein hydrolysates) or as purine and pyrimidine bases. Microorganisms requiring amino acids may be nutritionally deficient, either being unable to synthesize amino groups or failing to synthesize certain amino acids.

The nitrogen is metabolized to provide mainly protein, nucleic acids and cell wall polymers, and it constitutes up to 12% of dry weight of bacteria and 10% of fungal dry weight.

2.1.8 Sources of minor elements

Microorganisms require a number of inorganic ions in micro and millimolar concentrations for optimal growth. The role played by these ions is twofold :- enzymatic - as the catalytic centre of an enzyme, as an activator or stabiliser of enzyme function, or to maintain physiological control by antagonism between activators and deactivators, and structural - the ions act to neutralize electrostatic forces present in the various cellular anionic units. These two roles of any ion are important in defining the optimum

concentrations for growth (Rockwell, 1976).

The phosphorus source for SCP production is usually supplied either as phosphoric acid or as soluble phosphate salts. The phosphate is mostly incorporated into nucleic acids, phospholipids and cell wall polymers, though it occasionally may accumulate in the cells as polymetaphosphate. The phosphorus content of bacterial cells is about 1.5% of dry weight, but this increases at high growth rates and at low temperatures, which reflect changes in RNA and phospholipid
content.

Potassium, magnesium and sulphur are required for various specific purposes in the cells, such as coenzymes (potassium), active metal in chlorophyll (magnesium), components of amino acids (sulphur) and components of coenzymes (sulphur), membranes (magnesium) and RNA (potassium).

Trace elements such as manganese, copper, zinc, molybdenum, cobalt and boron i.e. those elements that are required in minute concentrations, play important roles as constituents of enzymes or coenzymes.

2.1.9 Vitamins

Many organisms are exacting in their nutritional requirements because they are unable to synthesize complex organic compounds or "vitamins" that are in most cases, constituents or precursors of enzymes and coenzymes. The water soluble vitamins which are important to

microorganisms are thymine, riboflavin, pantothenic acid, pyridoxine, nicotinic acid, biotin, p-aminobenzoic acid, folic acid and cobalamin. It is important that vitamins be supplied to the medium in minute quantities (1 - 50 µg/litre)whenever necessary. In undefined media, yeast extract is commonly used as a source of vitamins (Goldberg, 1985).

2.1.10 Fermented apple pomace as stockfeed

Having feed available for livestock throughout the year ordinarily involves the storage of large amounts of feed. Since apple pomace is seasonal, it is essential that the material be properly stored to avoid damage to the feed with loss of nutrients and possible harm to the consuming livestocks.

One of the major factors influencing the effectiveness of a feed storage is that of moisture content. Fermented apple pomace has to be dried to attain a moisture level of about 10%. An excess amount of moisture will cause such feed to heat and if not given prompt attention, they will become mouldy and unsuitable for feeding purposes. The amount of moisture that a feed can safely tolerate will vary depending on atmospheric temperature, the humidity and the amount of air circulation through the feed (Cullison, 1979).

2.1.11 Effect of heating on feed

The main components of a feed are protein and carbohydrate. The essential building blocks for proteins are amino acids. There are large number of interactions between the different amino acid residue which give rise to secondary, tertiary and quaternary protein structures. The most common types of interactions are hydrogen bonds, non-peptide covalent bonds, hydrophobic interactions and salt linkages. Among these interactions, hydrogen bonds play a major role in maintaining the protein structure, so it is not surprising that a radical change in structure usually occurs on heating. This is known as denaturation. If the heat supplied to the protein is excessive, then covalent bonds may rupture leading to thermal degradation. As the result, most of the bonds maintaining the conformation of the protein will rupture and the protein will take up a predominantly random-coil structure and all enzymes with specific conformation will lose their activity (Ledward, 1979).

When heat is applied to mono- and oligosaccharides, browning occurs. The extent of such browning depends on a variety of factors such as the temperature, concentration and the presence of other materials. In general terms, the browning reaction is one of dehydration in the first instance, leading to a fuller complex series of reactions and end-products but these are not yet fully understood.

The effect of heat on simple sugars has often been considered as falling into three categories :

1) caramelization

2) pyrolysis

3) the Maillard reaction

Caramelization was said to result from loss of water from the sugar molecule to give 'anhydro sugars', perhaps followed by further unknown reactions to give brown-coloured degradation and polymerisation products. Pyrolysis implies severe heating of sugar molecules which again results brown colorations, carbon-carbon linkages probably being broken. While the Maillard reaction occurs when carbohydrates are heated in the presence of amines; again browning occurs, and this is usually to a greater extent than when carbohydrates are heated alone, or in the presence of acid or alkali (Greenwood *et al.*, 1979).

The reaction and development of browning is depended on a number of factors such as temperature, pH, concentration, water activity, oxygen supply and the nature of the raw materials. The higher the temperature, the greater the extent of browning also the higher the concentration of reactants, the greater the extent of the reaction. Water activity can greatly affect the browning rate, often there is an activity range over which browning proceeds optimally.

Especially, when protein is present, dehydration or browning reactions can damage the nutritional value of the product. Lysine and arginine, and to lesser extent other

amino acids such as tryptophan, cystine and histidine, are often lost in such browning reactions. There is also the possibility that browning reactions can give rise to toxic materials although knowledge of this browning product is still imprecise (Holdsworth, 1979; Priestley, 1979).

2.1.12 Feeding of heat damaged material to animals

It was generally agreed that reduced digestibility of heat damaged materials in animals was an adequate explanation for the reduction found in the nutritional value of the material (Boctor *et al.*,1968; Nesheim *et al.*,1967; Tanaka *et al.*,1975; Nitsan *et al.*,1976; Varnish *et al.*,1975; Percival *et al.*,1979; Oste *et al.*,1984; Shirley *et al.*, 1975). Although various works had been done with animal trials, it was still unclear the factors or mechanism that were responsible to this reduced in digestibility.

Oste *et al.*(1984) suggested that the change in the physical state of proteins upon heating might make them less soluble and perhaps less susceptible to the digestive enzymes. Using rat trials, fructose-L-tryptophan, an early Maillard reaction product formed from tryptophan and glucose, in the rats' urine, which suggested that this compound was absorbed by the large intestine but excreted without being metabolized.

A procedure of estimating the available lysine in animal-protein foods had been developed (Carpenter, 1960). Lysine is often the factor limiting the protein quality of the

mixed diets for animals. The procedure involved estimating lysine units in foods whose ϵ-amino groups would undergo a reaction with fluorodinitrobenzene. The reason was that those lysine units in proteins of heat-processed foods whose ϵ-amino groups are bound to other groups, and so were unable to react, were likely also to be nutritionally unavailable. Modification to the Carpenter's available lysine method was made to take into account of the losses of compounds especially of plant materials in reaction by adding a correction factor (Booth, 1971).

2.1.13 Data on feed

For efficient animal production, a high intake of food is needed. Feeds which are not palatable, difficult to eat, bulky, or of low digestibility can lower the gross production efficiency (Miller, 1968; Oser, 1975; Rha, 1975; Shacklady, 1975; Young *et al.*, 1975; Cullison, 1979; Maletto, 1984; Calloway, 1974; Davis, 1974; de Groot, 1974; Hibino *et al.*, 1974; Hoogerheide, 1974; Milner, 1974; Taylor *et al.*, 1974; Van der Wal, 1983). Knowledge of such properties is especially important for feeds which are used as the whole diet or as a considerable part of it. Such negative properties are not so serious if used in small quantities or mixed with other ingredients which dilute or even mask these effects. In ruminants, speed and degree of degradation of feed in stomach to a large extent determine voluntary intake. Other additional factors can also lower voluntary intake e.g. higher heat load of the feed, higher

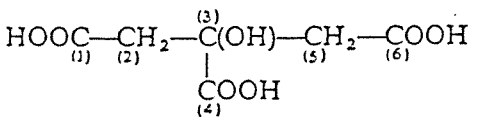
cell wall content with higher degree of lignification, lower contents of nitrogen and easily fermentable carbohydrates and higher infestation with intestinal parasites (Van Es, 1983).

Next to intake, digestibility is another very important factor determining a feed's nutritive value. This is not only because digested nutrients can be used by the animals for maintenance and production, but also because there often exists a positive relationship between digestibility and the efficiency of utilization of the digested nutrients, especially with regard to energy metabolism. A feed's nutritive value is also determined by its nitrogen content. For monogastrics, it concerns the content of digestible limiting amino acids and for ruminants, information on apparent digestible crude protein (Bender, 1970; Oser, 1970; King, 1970).

Data on mineral and vitamin contents are not very important in determining a feed nutritive value as these show much variation due to soil and climate. Moreover, a ration's mineral or vitamin deficiency can be corrected easily.

2.2 The production of citric acid by Aspergillus niger

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) [77-92-9] has the following structure :-



It crystallises from water in two forms, namely anhydrous and monohydrate. When crystallization occurs at a temperature exceeding 36.6°C the anhydrous form is produced. The monohydrate is formed at the lower temperature. The monohydrate was at one time the only form commercially available but increasing amounts of the anhydrous acid are now used. It is essential in dry-mix formulations and has a lower tendency to cake on storage (Milsom, 1985).

Citric acid is one of the most commonly used acids in the food and pharmaceutical industry because of its ease of assimilation and low toxicity. Most of the world's present supply is produced by fermentation with selected strains of *Aspergillus niger*, which yield up to ninety percent (w/w) citric acid from sucrose.

At the beginning of this century, citric acid production of about 10,000 tonnes per year was obtained by pressing citrus fruits, which contain about seven to nine percent

citric acid, and precipitation as the calcium salt. This process comprised the processing of about 30 to 40 tonnes of lemons to obtain one tonne of citric acid. Italy as a citrus fruit producing country almost had a monopoly until the advent of citric acid fermentation (Rohr *et al.*, 1981). It was the work of Currie (1917) that opened the way for the industrial production of citric acid using aspergilli fungi. The most important finding was that *Aspergillus niger* could grow well at pH values around 2.5 to 3.5 and that citric acid was abundantly produced at pH values even lower than 2.0. Currie also found that yields of over sixty percent could be achieved within fermentation times as short as one to two weeks.

In 1986, the world produced 350,000 tonnes of citric acid of which eighty percent was from submerged fermentation and twenty percent was from surface fermentation (Sevella *et al.*, 1988). The bulk of citric acid produced today is used mainly as an acidifying and flavour enhancing agent, i.e. in soft drinks, fruit juices and in beer manufacture, candies, jam and desserts. It serves also as buffer and pH stabiliser as well as a fat antioxidant in a variety of foods, where citrate salts are often employed.

For the manufacture of pharmaceuticals, some sixteen percent of the total citric acid production is used for products such as : Seltzer-type effervescent tablets and powders, syrups, elixirs and astringents.(Meyrath *et al.*, 1967).

The New Zealand Apple and Pear Marketing Board process

approximately 150,000 tonnes of apples per year, generating a waste stream of 35,000 tonnes of apple pomace. At present, it is trucked from factories to landfill. Because of its physical nature, apple pomace is not amenable to submerged fermentation. For submerged fermentation to occur, it is necessary to make a five parts dilution of the pomace. The yield of citric acid from the dilute apple pomace mash will too low to recover economically.

Furthermore in 1992 apple season, the New Zealand Apple and Pear Marketing Board will change its juicing operation from mechanical to enzymic process. The resulting pomace will have significantly less sugar. Less sugar in the pomace will render the submerged fermentation even more uneconomical.

In the correct use of the term, solid state fermentation refers to the growth of microorganisms on solids material without the presence of free liquid. While the presence of moisture is necessary in solid state fermentation, it exists in an absorbed or complexed form within the solid matrix (Cannel *et al.*,1980).

The major advantages of using solid state fermentation rather than submerged fermentation include (1) the yields are much higher than those in liquid media, (2) the space taken up by the fermentation vessel required is small relative to yield of products because less water is used and the substrate is concentrated and (3) the operating costs are much lower than those of liquid phase fermentation. (Hesseltine, 1972).

2.2.1 Mechanism of citric acid accumulation in microorganisms

The metabolic pathways by which simple mono- and di-saccharides are converted to citric acid are shown in Figure 3. Both the Embden-Meyerhof glycolysis and the citric acid cycle are involved.

The citric acid cycle is the mechanism by which acetate is oxidised to two molecules of carbon dioxide in most, if not all, living cells. In the normal way, one molecule of acetate is condensed with a molecule of oxaloacetate to give a molecule of citrate. The citrate is metabolised via iso-citrate to oxalosuccinate which loses a molecule of carbon dioxide to yield α-ketoglutarate which in turn loses a further molecule of carbon dioxide to give succinate. The succinate is further metabolised via fumarate and malate to regenerate oxaloacetate. If the citric acid cycle is interrupted at citric acid, no oxaloacetate is regenerated to condense with acetyl coenzyme A and so form citrate, and it is necessary to consider another so-called anapleurotic reaction to produce oxaloacetate. The anapleurotic reaction, at least in *Aspergillus niger* is the carboxylation of pyruvate by the enzyme pyruvate carboxylase (Milsom *et al.*, 1985; Rohr *et al.*, 1981).

Since *Aspergillus niger* produces an invertase and since hexokinase will act on both glucose and fructose, sucrose can also act as a source of citric acid. It remains, however, to be explained why citric acid accumulates and why the whole citric acid cycle is not active during the

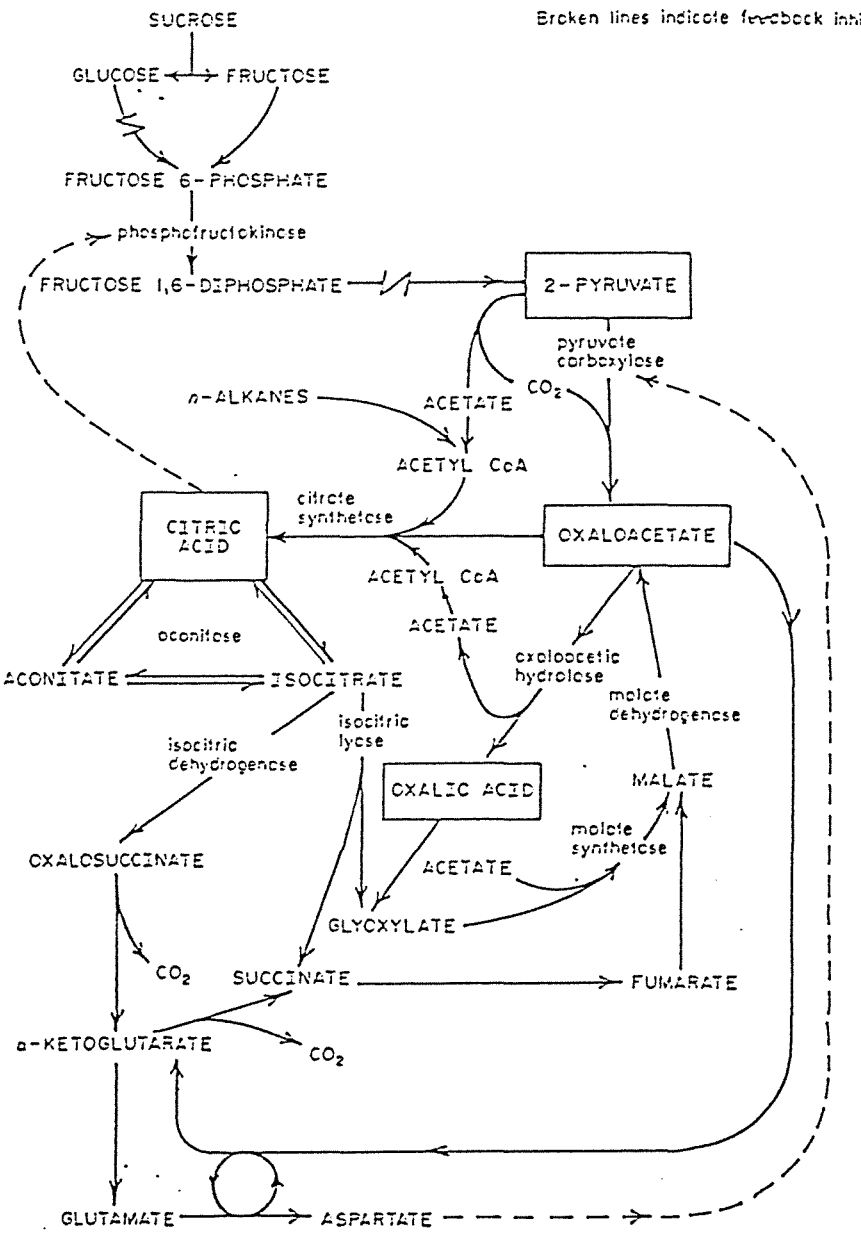


Figure 3 Metabolic pathways of citric acid cycle (Milsom *et al.*, 1985)

citric acid fermentation.

Most strains of *Aspergillus niger* under all conditions of growth do not accumulate citric acid in the quantities required for an economic commercial process. Two factors are of prime importance, namely strain selection and the maintenance of the correct conditions of fermentation, especially in terms of nutrition.

2.2.2 Environmental factors

The environmental factors affecting the production of citric acid are as follows:-

i) Organisms
ii) pH
iii) Temperature
iv) Nutrient
v) Trace metals
vi) Methanol

2.2.2.1 Organisms

Strains of *Aspergillus niger* have usually given most successful results, both in the laboratory and on an industrial scale (Hang *et al.*, 1985; Hang *et al.*, 1988). However, not all wild type strains of *Aspergillus niger* are suitable for citric acid production under optimum conditions of fermentation. Strains may be isolated from appropriate geographic locations and screened for citric acid production using shake-flask cultures. Many of these isolates produce high yields, possess uniform biochemical

characteristics, are easily cultivated and produce a negligible quantity of undesirable end-products such as oxalic acid. Promising cultures are improved by mutation. Suspension of spores are treated with mutagens e.g. UV light, X-rays, N-methy-N'-nitro-N-nitrosoguanidine or other chemicals. The treated suspensions are plated on a suitable solid medium and single colonies isolated. These colonies after further propagation are screened in shake-flask cultures. The best isolates are exposed to further treatment using the same or another mutagen.

Hannan *et al.*(1973) found that *Aspergillus niger* isolated following ultraviolet and gamma radiation mutants gave surprisingly high yields of citric acid, viz. 110% to 118% of yields from sucrose when compared on dry basis. This was also confirmed by Gardner *et al.*(1956) and Trumpy *et al.*(1963). Islam *et al.*(1986) used several gamma ray induced mutants of *Aspergillus niger* propagated on trays at a semi pilot scale and achieved inconsistent citric acid production results. A simple method of mutants selection had been devised by James *et al.*(1956), using absorbent paper soaked in an indicator medium. The paper culture greatly facilitated the isolation and characterization of mould colonies with economy of space, media and equipment.

Doelger et al.(1934) has shown that better results were obtained by seeding only one-fourth to one half of the surface area of a medium with spores. Uniform germination of the spores was prevented if the entire surface was seeded, with the result that ungerminated spores became

embedded in the mycelial mat. It was believed that the ungerminated spores exerted a toxic effect toward citric acid production.

A successive transfer of spores from one medium lot to another of the same uniform composition may stimulate the mould to produce a large yield of citric acid (Doelger *et al.*, 1934).

2.2.2.2 pH

For a successful submerged fermentation process, the final pH value must be between 1.5-2.8. Values over 2.8 will increase the formation of oxalic acid and thus decrease citric acid production while pH values below 1.5 will cause low growth rate of mycelium (Sodeck *et al.*, 1981; Millis *et al.*, 1963; Rossi *et al.*, 1988; Doelger *et al.*, 1934). It also reduces the risk of contamination by other organisms. A higher pH is favoured during the initial stages of the fermentation since this facilitates spore germination. However, the pH decreases during fermentation as the citric acid accumulates in the weakly buffered medium (Berry *et al.*, 1977). Hang *et al.*(1984) and Hang *et al.*(1986) did not make any pH adjustment to the substrate of apple pomace and grape pomace before or during fermentation. An adjustment of pH is quite difficult to accomplish on solid state fermentation since a good contact between the pH electrode and the substrate while maintaining mixing must be made in order to measure the concentration of hydrogen ions.

Dilution can cause inaccuracies of pH measurement since a dilution factor of ten will cause a shift of one pH unit. The initial pH of apple pomace is about 4.5 and it decreases with storage. It is low enough to inhibit any contamination from bacteria and if the initial inoculum is large, contamination from yeasts and other molds can be reduced also.

2.2.2.3 Temperature

Temperature is an important factor in the control of the physiology of *Aspergillus niger*. Temperature of 28-30°C have been proposed to obtain high yields and rapid rates of accumulation of citric acid (Berry *et al.*, 1977). At this temperature, there can be a significant moisture loss from the substrate. Too rapid evaporation retards the formation of citric acid by the organism; therefore it is best to employ covered containers for the fermentation, allowing only a minimum of air circulation, or in open containers at a relative humidity of at least 80% (Doelger *et al.*, 1934). The latter technique however can carry the danger of contamination from air. When the fermentations were run in Erlenmeyer flasks with nonabsorbent cotton stopper, evaporation was most rapid during the early stages of the fermentation while the mat of *Aspergillus niger* had not completely covered the surface of the medium used. Most of the reduction in volume took place after the mat had formed as a direct consequence of the process of respiration.

Respiration releases much carbon dioxide through sugar content oxidation, resulting in lesser citric acid production (Doelger *et al.*, 1934). Hang *et al.*(1987) observed that the rates of sugar consumption and citric acid production based on a dry weight were much greater at higher moisture (50-60%) than at lower moisture (40%) values. However, in the absence of the additive methanol and using the same strain of organism, citric acid production decreased sharply as the substrate moisture level increased (60-75%). This represents a problem for the fermentation of apple pomace by solid state since its initial moisture content is about 80%.

2.2.2.4 Nutrient

If unlimited growth of *Aspergillus niger* is permitted by use of a rich culture medium, the amount of citric acid accumulated in the medium is minimal. If, however, growth is restricted, citric acid is produced in amounts equivalent to 70 to 90% (w/w) as based on the sucrose supplied. In the hypothetical ideal situation in which no fungal growth occurs and no carbon dioxide or other by-product is produced, the yield would be 123%. Thus, under favourable conditions, up to about 75% of sucrose supplied appears as citric acid. It was found that sucrose was the most favourable sugar source followed by glucose, fructose and then lactose for the production of citric acid by *Aspergillus niger*. No citric acid was produced from galactose. In fact, the presence of galactose or a product

of galactose metabolism caused inhibition of citric acid production, and also reduced the rate of glucose utilization (Hossain *et al.*, 1984; Hossain *et al.*, 1985; Klasson *et al.*, 1989).

When sucrose is employed for fermentation, theoretically 1 molecule of the sugar produces 2 molecules of citric acid or 1 gram of sucrose produces 1.12 grams of citric acid. However, not all of the sucrose in the medium is available for direct conversion to citric acid by the organism; as much of it is utilised in the formation of the mat or mycelium and some is lost in the form of carbon dioxide as a result of the organism's respiration activity (Doelger *et al.*, 1934).

Considerable hydrolysis of the sucrose can occur when any sucrose-containing substrate is autoclaved in the presence of acid or at low pH. Any acid produced in such a media will be less than that produced by fermenting a medium containing unhydrolyzed sucrose. The reason for this can possibly be explained by assuming that the best enzyme production leading to the production of citric acid is stimulated by a splitting of the sucrose, while the monosaccharide present interferes with the normal sequence of changes from sucrose to citric acid (Doelger *et al.*, 1934).

Millis *et al.*(1963) observed that there is an effect on yield of citric acid on adding various lipids to the fermentation medium. Fatty acids with less than 15 carbon atoms inhibited growth and no citric acid was produced.

However, natural oils with a high content of unsaturated fatty acids and oleic acid itself, when added at 2% (v/v) to the media, increased the yield of citric acid by about 20%. It was suggested that unsaturated lipids act as alternative hydrogen acceptors to the oxygen during the fermentation and thus improve the yield of citric acid.

Aspergillus niger seems to produce citric acid only under nitrogen-limiting conditions (Heinrich *et al.*, 1982; Berry *et al.*, 1977; Eikmeier *et al.*, 1984; Russell, 1988). Ammonium nitrate, ammonium sulphate, ammonia, sodium nitrate and potassium nitrate have all been used as nitrogen sources for citric acid fermentation. The use of ammonium carbonate has been shown to minimise the formation of pigment and slime and also facilitates the extraction procedure. It was further reported that ammonium carbonate suppressed the oxalic acid formation (Berry *et al.*, 1977).

Tomlinson *et al.*(1950) reported on the effect of trace elements from the degree of cleanliness of the glasswares used in the fermentation. The data showed a large variation in citric acid yield encountered when the only known variable was a difference in the degree of cleanliness of the glassware i.e. washed with soap and chemically cleaned. It was found that if the glassware was washed with soap and water and reused, the high yield first obtained could not be duplicated; in fact, the yields fell significantly. This strongly suggested the loss of some essential nutrients from the glassware during repeated use.

2.2.2.5 Trace metals

Many variations in the requirements of the citric acid fermentation for metals have been reported in the literature. If high yields are to be obtained, these need to be investigated each time a new strain or substrate is used. Four metals have been investigated quite extensively: manganese, copper, zinc and iron (Berry *et al.*, 1977; Heinrich *et al.*, 1982; Kubicek *et al.*, 1982; Rohr *et al.*, 1981; Trumpy *et al.*, 1963; Kristiansen *et al.*, 1982; Prescott and Dunn, 1959; Tomlinson *et al.*, 1950; Tomlinson *et al.*, 1951; Sanchez-Marroquin *et al.*, 1970; Islam *et al.*, 1986; Currie, 1917).

2.2.2.6 Methanol

The use of methanol to enhance the production of citric acid by solid state fermentation has been reported by Hang *et al.*(1987a) and Hang *et al.*(1987b). Increasing the concentration of methanol resulted in marked increase in the production of citric acid. *Aspergillus niger* produced the greatest amount of citric acid at a concentration of 3% of methanol. Methanol at concentrations above 3%; however, exerted an inhibitory effect on citric acid production. Furthermore, its stimulating action on citric acid production decreased markedly as the substrate moisture decreased.

Methanol is not assimilated by *A.niger*, and its exact nature in stimulating the production of citric acid is still not known. It is likely that methanol affects the

permeability properties and enables greater excretion of citric acid (Hang *et al.*, 1987a).

2.2.3 Commercial production of citric acid

The details concerning the commercial production of citric acid are kept secret. Generally, three processes can be distinguished (Milsom *et al.*, 1985; Rohr *et al.*, 1981). These are:-

i) Submerged process

ii) Surface process

iii) Koji process

2.2.3.1 Submerged process

Submerged citric acid fermentation can be performed using both conventional stirred reactors and tower fermenters (Dawson, 1986; Rohr, 1983). Increasingly, the latter is the preferred type of bioreactor offering a number of advantages as compared to the deep tank stirred fermenter. These are, its lower price, the possibility of building larger reactors, operation without large rotating units with less risk of contamination and better conditions for working with suspended solids.

Owing to the corrosive action of citric acid and the sensitivity of *Aspergillus niger* to traces of metals that could be dissolved from the construction materials, special coatings for the wall materials of the reactors or employing especially acid resistant steel are used. Various

mechanised systems provide aeration and mixing.

Inoculation is performed in several ways. If a spore suspension is used, a surface active substance is incorporated as an aid in evenly suspending the hydrophobic spores throughout the fermentation medium. It has been proved useful to incubate the spore suspension for six to eight hours in a saline solution with added surface active substances prior to inoculation thus shortening the fermentation cycle. Alternatively, it is possible to propagate the inoculum in a small seed fermenter in a dilute fermentation medium and transferring the pellets formed aseptically to the production fermenter. This too may shorten the fermentation cycle by about twelve hours.

2.2.3.2 Surface process

In the classic process for the manufacture of citric acid, the culture solution is kept in shallow pans and the fungus develops as a mycelial mat on the surface of the medium. This type of process is thus termed surface or shallow pan fermentation (Layokun *et al.*, 1986; Rohr, 1983; Roukas *et al.*, 1986).

The system consists of fermentation rooms in which a large number of trays are mounted one over the other in stable racks. The trays are generally made of high purity aluminium or of special qualities of stainless steel. The tray sizes vary from 2 m x 2.5 m x 0.15 m to 2.5 m x 4 m x 0.15 m with usable liquid depths of 0.08 to 0.12 m, in other cases with a depth up to 0.25 m. This means that

liquid volumes with weights of 0.4 to 1.2 tonnes per tray have to be supported by the rack construction. Provision is made for continuous filling and draining by appropriate overflow devices.

The fermentation rooms are provided with an effective aeration (air circulation) system which mainly serves the purpose of temperature regulation and only to a lesser extent that of supplying oxygen and controlling humidity. Air is introduced into the fermentation room in an almost laminar flow manner through outlets evenly placed at all levels of the room. Apart from this, the rooms are almost closed. Wall and floor covering materials are washable and resistant to disinfectants as well as acids. This is of importance to enable operation under sterile conditions. Contamination is mainly caused by yeasts and lactic acid bacteria.

Inoculation is performed in different ways. The necessary number of conidia is introduced as a suspension added to the cooled medium, or dry conidia may be mixed with the air blown over the trays. The quantity of conidia for inoculation is in the range of 100 to 150 mg per m^2 of medium. Alternatively, cell material of a small surface culture may be disintegrated in a homogenizer and then added aseptically to the fermentation medium. Germination of conidia requires one to two days during which a decrease in temperature has to be compensated by the introduction of heated moist air.

2.2.3.4 Koji process

The simplest process for citric acid fermentation has been developed in Japan. It largely resembles a solid state fermentation (Rohr, 1983). The raw materials are starch containing residual pulps from starch manufacture. The starchy material is placed in suitable trays and soaked with water until a water content of 65 to 70% is reached. After removal of excess water, the mass undergoes a steaming process yielding a fairly sterile starch paste which is placed in trays or simply on the floor of the fermentation compartment and is then inoculated by spreading conidia of *Aspergillus niger* over the material. Since the trace elements present in the raw material cannot be removed by standard techniques, organisms have been selected which accumulate citric acid in the presence of various trace metals. The pH of the mass is about 5.5 and the incubation temperature is 30°C. Initially, the starch is saccharified by the fungal amylase and subsequently converted to citric acid resulting in a changes of the pH to values below 2.0. Alternatively, the saccharification of starch may be performed by separate or simultaneous treatment with appropriate enzyme preparations such as commercial α-amylases.

Under optimum conditions, the process is completed after about ninety hours. The resulting acid containing mass is crushed and extracted with warm water in a simple counter-current apparatus yielding a 4% (v/v) citric acid solution which is further processed to calcium citrate by standard

procedures (Lakshminarayana *et al.*, 1975).

3 Materials and methods

3.1 Materials

3.1.1 Microbiological media

Malt extract (ME) agar (CM59) and broth (CM57), MacConkey agar (CM7) and Nutrient agar (CM135) were obtained from Oxoid Limited, Basingstoke, Hampshire, England. Peptone water (M38100) and Bile Aesculin Azide agar (M05500) were obtained from Gibco Diagnostics, Madison, Wisconsin, U.S.A. Reinforced Clostridial agar (11564) was obtained from BBL Microbiology System, Becton Dickinson and Co., Cockeysville, U.S.A. Potato dextrose agar (PDA) (0013-01-4) was obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

3.1.2 Chemicals

All the chemicals used were of analytical grade supplied by BDH Chemicals New Zealand Ltd., Palmerston North, New Zealand.

3.1.3 Organism

Candida utilis Y15 was obtained from Biotechnology Department Culture Collection, Massey University, Palmerston North, New Zealand.

Candida utilis is a food grade yeast. According to

Barnett *et al* (1983), *Candida utilis* is the asexual state of *Hansenula jadinii*. Other names for *Candida utilis* are *Candida guilliermondii, Candida nitratophila, Cryptococcus utilis, Saccharomyces jadinii, Torula utilis and Torulopsis utilis*. It can be isolated from human, animals, flowers and distillery. It formed white or cream coloured colonies on nutrient agar plate; multilateral budding (Barnett *et al.*,1983)

The culture was maintained on malt extract agar slopes. It was streaked monthly onto a fresh malt extract agar plate and incubated for 48 hours at 30°C. Single colonies were also picked and inoculated onto agar slopes, incubated at 30°C for 48 hours and stored at 4°C.

Aspergillus niger NRRL 328 was kindly supplied by Dr. S.W. Peterson, United States Department of Agriculture, Agricultural Research Service, Midwest Area, Preoria, Illinois, U.S.A. The culture was grown on a potato dextrose agar slant at 34°C for seven days. A spore inoculum was prepared by adding about five ml of sterile distilled water to the slant and shaking vigorously for one minute. The number of spores was determined using haemocytometer counting method. The spores were stored in a nutrient broth with glycerol (30% v/v) at -20°C.

3.1.4 Apple pomace

The old and the new apple pomace were obtained from New Zealand Apple and Pear Marketing Board, Hastings, New

Zealand and is a solid waste arising from juicing operations. Old apple pomace was the pomace prepared by the older juice extraction technology i.e. mechanical operation while new apple pomace was prepared by the new technology i.e. enzymatic extraction which consisted only flesh part of apple. They were packed into plastic bags of about one kilograms each and stored at -20°C until needed.

3.1.5 Fermentation containers

3.1.5.1 Fermentation by *Candida utilis*

Fermentation was carried out in 1 litre Agee Preserving jars. A doubled layered mutton cloth was used as a top for the jar. This allowed air flow during the fermentation.

3.1.5.2 Fermentation by *Aspergillus niger*

Fermentation by *Aspergillus niger* was carried out in 250 ml Erlenmeyer flasks.

3.2 Methods

3.2.1 Media preparation

All media used for growing *Candida utilis* and cultivation of *Aspergillus niger* spores were made up according to the manufacturers' instructions.

3.2.2 Media, chemicals and apparatus sterilisation

Media, apple pomace and fermentation bottles were autoclaved at 121°C for 15 minutes. Apple pomace was not placed into the fermentation bottles prior to sterilisation

due to the nature of the top of the jars that were used. Since the mutton cloth top is pervious to steam during autoclaving, the condensed steam will significantly alter the moisture content of the apple pomace.

Miscellaneous glassware was sterilised in a hot-air oven at 160°C for two hours.

3.2.3 Cleaning of glassware

All glassware was washed in hot Pyroneg(R) solution (Diversey Wallace Company, Auckland, New Zealand.), rinsed in hot tap water followed by distilled water, and then dried using hot air in drying cupboard.

3.2.4 Cell cultivation

Malt extract broth was used as a medium for growing *Candida utilis*. A loopful of the culture was transferred aseptically from a maintenance slope to a 250 ml Erlenmeyer flask containing 100 ml of the broth. The flask was then incubated at 30°C overnight on a Lab-line junior orbit shaker (Lab-line Instruments Inc., Illinois, U.S.A.) at 150 rpm.

3.2.5 Cell harvesting

Candida utilis that has been grown overnight was harvested aseptically by centrifugation using Clandon T52.1 centrifuge at 6000 U/min for 10 min. The supernatant was discarded and the cells were washed once by resuspending

in deionised water and centrifuging once again. This would wash any trace of the broth from the culture. The cells were then resuspended in approximately an equal volume of deionised water.

3.2.6 Fermentation

3.2.6.1 Fermentation by *Candida utilis*

150 g of the autoclaved apple pomace was loaded into a fermentation bottle and 1% (v/w) of the above *Candida utilis* was used as the inoculum. For a nitrogen source, 1% (w/w) of ammonium sulphate was used. The ammonium sulphate was added to the apple pomace after autoclaving since there could be a possibility of the ammonium sulphate disintegrating by heat during the autoclaving. The apple pomace was then mixed well to give an even distribution of material in the bottle.

Twelve jars were employed for each fermentation to provide enough material for subsequent analyses. The fermentation was conducted at ambient temperature in the laboratory. (Figure 4 and 5).

Samples were taken every twelve hours for a period of 72 hours. Before fifty grams of sample was taken, the apple pomace in the bottles was mixed well for ten seconds to give a relatively presentative sampling. The following analyses were conducted for each sample:-

i) pH measurement

ii) Cell count

iii) Total reducing sugar determination

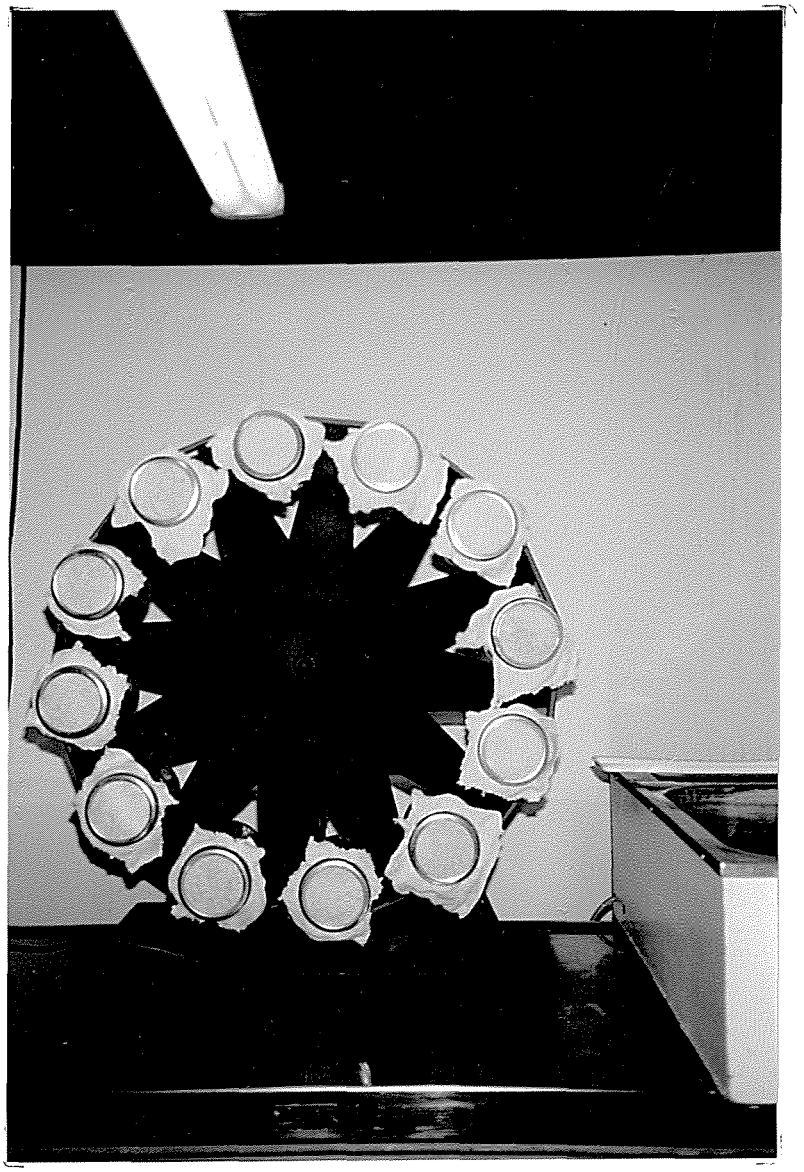


Figure 4 Fermenter (frontal view)

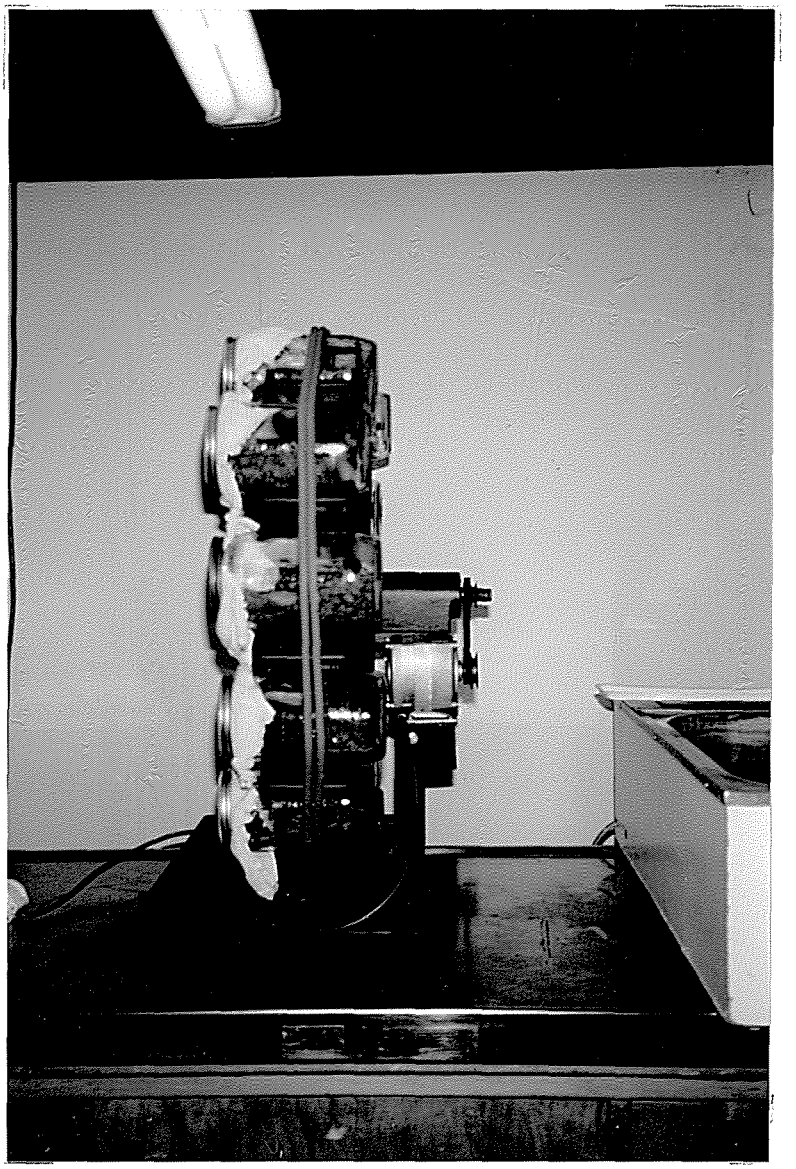


Figure 5 Fermenter (side view)

iv) Total nitrogen determination

v) Total inorganic nitrogen determination

vi) Total organic nitrogen and crude protein determination.

3.2.6.1.1 pH measurement

pH measurement of samples were performed using an Orion Research model 701A/ digital Ionalyzer, supplied by Watson Victor Ltd, New Zealand. It was not possible to measure the pH of a solid substance such as apple pomace accurately since a good contact between the material and the pH electrode must be made. In order to overcome this problem, a relative pH measurement was employed. This does not give an accurate or 'true' measurement of pH but gives an indication of the pH changes during the fermentation.

One gram of apple pomace sample was diluted using CO_2 free water. The water was prepared by boiling deionised water to remove any dissolved carbon dioxide gas. It was then quickly cooled to room temperature before being used as dilutant for the apple pomace sample. One gram of apple pomace sample was diluted with nine ml of the prepared water to give 1:10 dilution and the pH was then measured.

3.2.6.1.2 Cell count

Yeast cells were suspended in water by diluting one gram of apple pomace sample and nine ml of peptone water and mixed briefly using a vortex shaker Lab-line Super Max No. 1288, Lab-line Instruments Inc., Illinois. Serial dilutions

were made in peptone water and the yeast cells were plated out on malt extract agar medium. The plates were incubated at 30°C for 48 hours. After incubation, the total number of colonies was counted. The result was then expressed as $\log_{10}$ no. of yeast cells per gram of wet apple pomace; assuming that each colony was formed from a single yeast cell.

3.2.6.1.3 Total nitrogen determination

The total nitrogen determination was based on Kjeldahl method. (Wilson, M.,1990 - personal communication)
The method used in general involves 2 steps:-

i) The sample was digested by heating with concentrated sulphuric acid containing potassium sulphate to raise the boiling point and a catalyst selenium. Under this condition, carbonaceous material was oxidised and nitrogen was converted to ammonium sulphate.

ii) Colorimetric method was used to determine the nitrogen content of the digested sample.

3.2.6.1.3.1 Kjeldahl digestion procedure (Rock *et al.*,1986)

4.0 g of K_2SO_4, 1.0 g of $CuSO_4$ and 0.03 g of Na_2SeO_4 were weighed into a labelled 200 ml Kjeldahl flask. Each sample was analyzed in duplicate. About 6 - 7 grams of sample was used per flask. To each flask, ten ml of concentrated sulphuric acid was added from a burette with a couple of glassbeads to prevent excessive bumping. The flasks were

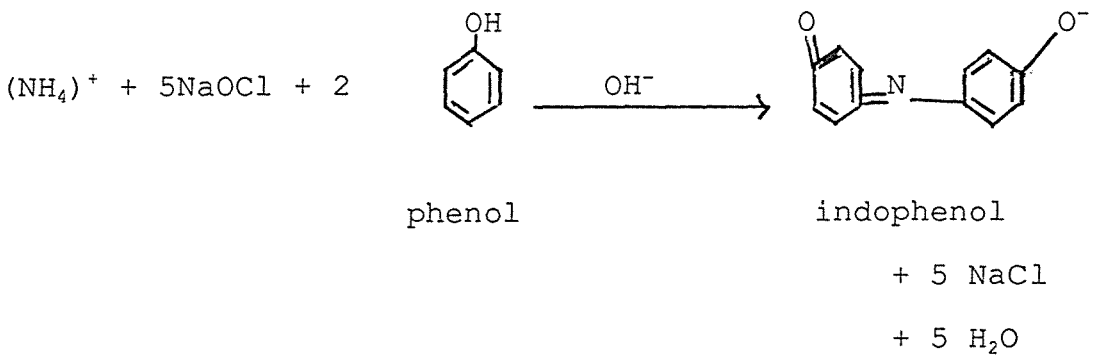
placed in a digestion rack in a fume cupboard. The mixture was boiled gently until the boiling was constant then the heater was turned up gradually. The mixture was boiled vigorously until a clear digest was observed, i.e. until all carbon had disappeared. The contents were shaken frequently during this time to speed up the digestion.

The digests were left to cool slightly to a manageable hotness before proceeding further. If the digests were left to cool for too long at room temperature, salt crystals formed. When this happened, 5 - 10 ml of distilled water was added and the digest was heated gently, with shaking, over a small flame to redissolve the salts. Care was taken since water was added to a solution of concentrated acid.

The contents of the flasks were transferred quantitatively to two labelled fifty ml volumetric flasks, and made up to the volume with distilled water and mixed thoroughly.

3.2.6.1.3.2 Determination of the nitrogen content of the digest (Rock et al.,1986)

Berthelot procedure for estimating ammonia was used. The principle of the Berthelot procedure was that ammonia $(NH_4)^+$ and hypochlorite in alkaline conditions formed indophenol which dissociated in alkali solution to form a blue colour that absorbed at 560 nm. Nitroprusside is added as a catalyst.



The very acidic digest was first neutralised but great care was taken not to make the mixture alkaline since if this occurred, free NH_3 would be released to the atmosphere. Two ml of the acidic digest was accurately pipetted into two 200 ml beakers, as duplicate. About 80 ml of distilled water was added and mixed. The pH was then adjusted to about pH 2.5 by adding 5 M KOH dropwise.

The contents of the beakers were quantitatively transferred into two labelled 100 ml volumetric flasks. The beakers were rinsed with several lots of water. These were added to the flasks, then made up to volume and mixed thoroughly by inversion. Duplicate aliquots of 0.25, 0.5 and 1.0 ml of the neutralised digests were pipetted into labelled test tubes and made up to 1.0 ml with distilled water. A series of standards containing 0 - 0.005 mgN, using a standard $(NH_4)_2SO_4$ solution (0.005 mgN/ml) was also prepared and made up to 1.0 ml with distilled water.

The reagents used were made up as follows:-

i) Phenol-nitroprusside reagent

10 grams phenol

0.05 grams sodium nitroprusside dihydrate

Dissolved in 1 litre of deionised water and stored in an amber bottle at 4°C. The reagent is stable for up to two months.

ii) Alkaline hypochlorite

5 grams sodium hydroxide

0.42 grams sodium hypochlorite . Janola solution of about 3% hypochlorite was used; 14 ml of Janola solution was added to 1 litre of solution.

Dissolved in 1 litre of deionised water and stored in an amber bottle at 4°C. The reagent is stable for up to two months.

iii) Standard $(NH_4)_2SO_4$ 5 μgN/ml

A stock standard $(NH_4)_2SO_4$ of 50 μgN/ml was made by dissolving 118 mg of $(NH_4)_2SO_4$ in 500 ml deionised water. This solution was diluted 1:10 for each assay to get a working standard of 5 μgN/ml.

2.5 ml of phenol-nitroprusside solution was added to all tubes and mixed by inversion. Then 2.5 ml of alkaline-hypochlorite solution was added and mixed again by inversion. The test tubes were placed in a waterbath at 37°C for 20 minutes. The absorbance of standards and unknowns were read at 560 nm and a standard curve was prepared. Philips PU8625 UV/VIS spectrophotometer with 1 cm light-path perspex cuvette was used to determine the absorbance.

The amount of total nitrogen was then expressed as gram nitrogen per 100 gram of dry apple pomace. The value of

this should not significantly different from one sample to the other in a fermentation with identical conditions since no nitrogen was added or removed from the system during the fermentation.

3.2.6.1.4 Extraction of soluble sugar and inorganic nitrogen

Twenty gram of apple pomace sample was diluted with 80 ml of deionised water in a conical flask to give a dilution of 1:10. The mixture was shaken occasionally for one hour and filtered through Whatman no.54 filter paper. A pestle was used to compress any remaining liquid off the filter paper. The filtrate was then centrifuged at 6000 rpm for 10 minutes in a Clandon T52.1 centrifuge and the supernatant was collected. About 10 ml of the supernatant was then filtered through a Millipore AP prefilter and Millipore HA filter 0.45μm. The filtered sample was stored at 4°C if they could not be analyzed immediately.

3.2.6.1.5 Total reducing sugar analysis

The modified Dinitrosalicylic acid method used for estimating the amount of reducing sugar in the dried apple pomace was based on colorimetric method.(Sumner 1946 with Miller modification 1959)

The reagents used were :-

i) 2% w/v sodium hydroxide solution.

ii) Modified Miller reagent

2 gram of 3,5-dinitrosalicylic acid and 0.4 gram phenol were weighed into a beaker. Then 100 ml of the 2% w/v sodium hydroxide solution was added into the beaker and stirred to dissolve.

iii) 0.1% w/v sodium sulphite solution.
100 ml of this solution was freshly prepared for every sugar analysis.

iv) 40% w/v Rochelle salt solution. (potassium-sodium tartrate)

100 ml of sodium sulphite solution was added to 100 ml of modified Miller reagent and was named as solution A. A series of standards was prepared using the standard glucose solution of 10 μmoles/ml with distilled water used as dilutant. Three ml of the standards and sample was pipetted into labelled test tubes. Then three ml of solution A was added to each tube and mixed. The tubes were heated in a boiling water bath for 15 min. One ml of Rochelle salt solution was added to the test tubes and the test tubes were quickly cooled under running tap water to ambient temperature. The absorbance was read at 580 nm using Philips PU8625 UV/VIS Spectrophotometer with 1 cm light path perspex cuvette. A standard curve was prepared from the standards and the unknowns were read against the curve. The sugar concentration of the unknowns was then calculated.

3.2.6.1.6 Inorganic nitrogen analysis (Rock et al.,1986)

Berthelot procedure for estimating ammonia was used (as for total nitrogen analysis). No pH adjustment was made since the filtrate was mildly acidic. The amount of inorganic nitrogen was expressed as gram nitrogen per 100 gram of dried pomace. Inorganic nitrogen was measured to give the amount of unutilized ammonium sulphate for the growth of *Candida utilis* during fermentation.

3.2.6.1.7 Organic nitrogen analysis

Total organic nitrogen was calculated from total nitrogen minus total inorganic nitrogen. It was expressed as gram nitrogen per 100 gram dry pomace and then converted to gram crude protein per 100 gram of dry pomace by multiplying with the factor 6.25, which assumed that proteins in the apple pomace sample contained 16% nitrogen and all the organic nitrogen is derived from protein. The term crude protein was used since these assumptions might not be true for apple pomace. The crude protein content gave a fair indication of the changes in protein content of the apple pomace during fermentation.

3.2.6.2 Fermentation by *Aspergillus niger*

New apple pomace (20 g) was loaded into 250 ml Erlenmeyer flasks and autoclaved at 121°C for 15 minutes. Each flask was inoculated with 1 x 10^6 spores.

Duplicate runs were carried out to investigate the effects of factors listed below on the production of citric

acid :-

i) Methanol additions

ii) Ammonium sulphate as nitrogen source additions

iii) Inoculum size

iv) Moisture content of apple pomace

v) Temperature of fermentation

vi) Oil additions

3.2.6.2.1 Methanol additions

Apple pomace was prepared as above. Methanol was added to the apple pomace at a concentration of 0 - 5% (v/w). The apple pomace was mixed well with a stirring rod and lightly pressed to form a single uniform layer at the bottom of the flasks. The flasks were incubated at 30°C for five days.

3.2.6.2.2 Ammonium sulphate additions

Apple pomace was prepared as above. 0 - 5% (w/w) of ammonium sulphate were added to the apple pomace. Also 0 and 3% (v/w) of methanol were added in separate runs as comparison between fermentation with and without methanol addition. The flasks were incubated at 30°C for five days.

3.2.6.2.3 Natural oil additions

Apple pomace was prepared as above. 0 - 5% (v/w) of soyabean oil were added to the apple pomace. Also 0 and 3% (v/w) of methanol were added to the separate runs as comparison between fermentation with and without methanol addition. The flasks were incubated at 30°C for five days.

3.2.6.2.4 Inoculum size

Instead of inoculum size of 1 x 10^6 spores, 1 x 10^5, 1 x 10^7 and 5 x 10^7 spores per 20 gram of apple pomace were used as inoculum. Also 0 and 3% (v/w) of methanol were added to the separate runs as comparison between fermentation with and without methanol addition. The flasks were incubated at 30°C for five days.

3.2.6.2.5 Temperature of fermentation

The apple pomace was prepared as above. Also 0 - 3% (v/w) of methanol were added in separate runs as comparison between fermentation with and without methanol addition. The flasks were incubated at 25°C, 30°C and 37°C.

3.2.6.2.6 Moisture content of apple pomace

Apple pomace was dried to about 10% moisture content using an oven dryer model Sirocco supplied by Davidson Ltd., Belfast, Northern Ireland. The temperature of drying was 65°C with air speed of 0.8 m/s. The dried apple pomace was stored in an air-tight container away from sunlight.

The dried apple pomace was rehydrated to 40%, 50%, 60%, 70% and 80% moisture content with sterile distilled water and inoculated as above. Also 0 and 3% (v/w) of methanol was added as comparison between fermentation with and without methanol addition. The flasks were incubated at 30°C for 5 days.

3.2.6.2.7 Extraction of citric acid and sugar

At the completion of the fermentation, the fermented materials were extracted with 40 ml of boiling water. This helped to soften the fungal mycelia in the apple pomace for extraction. The mixture was shaken and left on the bench for 45 minutes with occasional shaking. The extract was filtered through Whatman no.54 filter paper under vacuum. A pestle was used to squeeze any remaining liquid from the apple pomace. The extract was analyzed for total reducing sugar and citric acid.

3.2.6.2.8 Citric acid determination (Hossain *et al.*,1986)

The concentration of citric acid was determined by an HPLC method, employing High Performance Liquid Chromatograph Model 590 supplied by Waters Associates Inc., Maple St., Milford, MA., U.S.A. equipped with Millipore Waters 740 Data Module Integrator.

The solvent system was a 20 g/l potassium dihydrogen phosphate solution prepared using deionised water and adjusted to pH 2.46 with orthophosphoric acid. Apple pomace extracts were filtered through a prefilter and Whatman 0.45 μm filter paper before 50 μl was analyzed for citric acid concentration. C_{18} Radial Pak column of 8 mm inside diameter was used in the system.

3.2.7 Drying of apple pomace

Fermented and unfermented apple pomace were dried in an oven dryer model Sirocco, Davidson Ltd., Belfast, Northern

Ireland. Since heat from drying could have a negative effect on the nutritional value of the apple pomace, a series of test of drying conditions was conducted. Air speed of 0.8 m/s and drying temperature ranging from 40°C to 135°C which corresponded to the minimum and maximum heating capability of the oven dryer respectively. The arbitrary air speed of 0.8 m/s was chosen as not to blow away the apple pomace due to excessive air speed although this reduced the efficiency of drying.

Two kilograms of apple pomace was spread out into a layer of about four cm deep on a stainless steel tray. The initial moisture content was determined by drying a sample in a hot air oven at 105°C overnight. Final moisture content that must be achieved was between 10 - 12% as at this low level of water content it prevented microbial spoilage and also for ease of handling.

3.2.8 Grinding of dried apple pomace

The size reduction of dried apple pomace was done using Grinder Thomas Scientific, U.S.A., with 0.5 mm sieve attached. The ground apple pomace was then stored in an air-tight container, kept away from direct sunlight.

3.2.9 Freeze-drying of wet apple pomace

Wet apple pomace was freeze dried using Cuddons Freeze Dryer FD57, Cuddon Ltd, New Zealand. The working conditions of the freeze drier were 0 - 10°C shelf temperature, vacuum pressure of 0.12 mBar for 4 days. The dried sample was

stored in an air-tight container, kept away from direct sunlight.

3.3.0 Determination of FDNB-available lysine by modified Carpenter procedure (Booth, 1971; Wilson, M., personal communication)

The reagents used were:-

i) Mono ε-N-dinitrophenyl-lysine hydrochloride monohydrate (DNP-L). It has a molecular weight of 366.77 and contained the equivalent of 39.85% of lysine (Lys). 314 mg were dissolved in 250 ml of 8.1 M HCl, ten ml of which was diluted with water to 100 ml. This diluted solution was used as a standard for the tests described below.

ii) 1-Fluoro-2,4-dinitrobenzene (FDNB). About 0.4 ml of FDNB in 15 ml of ethanol was used for each test. Enough ethanol was added to the FDNB in a conical flask and dissolved with shaking. A fresh solution was made for every determination.

iii) Methoxycarbonyl chloride (methyl chloroformate) (MCC) was not stable indefinitely. The bulk was kept in a refrigerator at 4°C, and a small amount in a bottle.

iv) NaHCO3 solution was made from 80 gram in 1 litre of distilled water.

v) Peroxide free diethyl ether (Vogel's).

The peroxide was removed by shaking 1 litre of

ether with 10 - 20 ml of a concentrated solution of an iron (II) salt prepared by dissolving 100 gram of iron (II) chloride in a mixture of 42 ml of concentrated hydrochloric acid and 85 ml of distilled water.

vi) Phenolphthalein solution was made from 400 mg per litre of 60% ethanol.

vii) NaOH solution (120 g/l) was used to raise the pH to 8.5 for the MCC reaction. The solution was kept in a plastic bottle.

viii) Buffer (pH 8.5) was made by dissolving 19.5 gram of $NaHCO_3$ and 1 gram of Na_2CO_3 in 250 ml of distilled water and the pH adjusted. This buffer becomes more alkaline by losing CO_2 to the air so a well-filled bottle was used and kept stoppered.

About 1 - 2 gram of dried sample was weighed into a 100 ml round-bottomed flask. Four anti-bump glassballs were placed in the flask. Ten ml of the $NaHCO_3$ solution was added. The flask was gently shaken by hand and carefully left to stand until the sample was fully wetted.

Fifteen ml of the FDNB solution was added to the flask. The flask was stoppered and shaken, gently at first, for at least two hours. Halfway through the shaking period, the flask was twirled to disperse the sample and to ensure that all particle were wetted by the FDNB solution. Then the ethanol, but not all the water, was evaporated on a heating mantle until the flask lost about 12.5 gram in weight.

When the mixture cooled, 30 ml of 8.1 M HCl was added which neutralised the $NaHCO_3$ and made to a total volume of 40 ml and a final concentration of 6 M. The mixture was refluxed gently for 16 hours.

The heat was turned off. The condenser was washed with a little water and the flask disconnected. The content was filtered, while still hot, through a Whatman no.541 filter paper, into a 250 ml volumetric flask. The digestion flask and the residue were washed thoroughly with hot water until the total filtrate was almost 250 ml. When the filtrate had cooled, it was made to volume and mixed.

Two ml of the filtrate was pipetted into each of two stoppered test tubes, A and B. The content of tube B was extracted with about 5 ml of peroxide-free diethyl ether. As much of the ether was discarded safely by suction. The tube was placed in a hot waterbath (about 80°C) until effervescence from the residual ether had ceased, and cooled. A drop of phenolphthalein solution was added and then NaOH solution from a dropping pipette until the first pink appeared. Two ml of carbonate buffer (pH 8.5) was added. Under the fumehood, five drops (about 0.01 ml. each) of MCC were added. The tube was firmly stoppered and shaken vigorously. The pressure was released cautiously. After about 8 minutes, 0.75 ml of concentrated HCl was added dropwise with caution at first, and then with agitation to prevent frothing. The tube was shaken occasionally. The solution was extracted with ether as described above for four times, and the tube was kept in hot waterbath. The

tube was then cooled and the content made up to ten ml volume with distilled water.

During the pauses between manipulations of tube B, tube A was extracted with diethyl ether. Residual ether was removed as described above and the content made up to ten ml with 1 M HCl.

The absorbances of both A and B were read at 435 nm against water, using Philips PU8625 UV/VIS spectrophotometer. Reading A minus reading B (the blank) is the net absorbance attributable to DNP-L.

From the diluted standard solution of DNP-L, two ml was pipetted into each of duplicate pairs of tubes A and B and the procedure was as described above. This was done routinely along with each set of analyses. The nett absorbance was used as reference for calculating unknowns.

The result was calculated as follows:

$$c = \frac{ws \times Au \times v \times 100 \times 100}{wu \times As \times a \times [cp]}$$

c = content as gram lys / 16 gram Nitrogen

ws = weight of standard, expressed as mg lysine in two ml, which is 0.1

wu = weight of sample in mg.

As = nett absorbance of standard.

Au = nett absorbance of unknown

v = volume of filtered hydrolysate, which is 250 ml.

a = aliquot of filtrate, two ml.

cp = crude protein, 6.25 x gram Nitrogen / 100 gram material

3.3.1 Stockfeed analysis (Smith, W.,1990 - personal communication)

The stockfeed analysis done on the dried apple pomace were:-

i) Ash and organic matter

ii) Percentage nitrogen (using Kjeldahl method of section 3.2.6.3)

iii) Gross energy using bombcalorimetry

iv) Percentage fat using Soxhlet extraction

v) Percentage neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin

vi) Amino acids analysis.

3.3.1.1 Ash and organic matter

Dried apple pomace was weighed accurately into a dry beaker. The beaker was then placed in a muffle furnace Wild Barfield, Furnesco, New Zealand at 500°C overnight. The beaker was removed from the furnace and cooled in a dessicator. The beaker containing apple pomace ash was weighed.

$$\text{Percentage ash} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100\%$$

Percentage organic matter was calculated from 100% minus percentage ash. All percentage were expressed on dry matter basis.

3.3.1.2 Gross energy

The value of gross energy of the apple pomace sample was done using a bombcalorimeter, Gallenkamp Autobomb, Watson Victor Ltd., New Zealand. About 0.5 gram of sample was weighed accurately for each analysis and done in duplicate. The value was expressed as Joule per gram of dry pomace.

3.3.1.3 Percentage of ADF, NDF and lignin determination (Rock et al.,1986)

The procedure employed was a rapid gravimetric method which was an alternative to full carbohydrate fractionation. It was much faster but tended to be less specific.

The reagents used were:-

i) Neutral detergent (ND) solution

60 gram sodium lauryl sulphate

37.22 gram Na_2EDTA

13.62 gram Na tetra borate decahydrate

9.12 gram Na_2 hydrogen phosphate anhydrous (Na_2HPO_4)

Each was weighed into a two litre conical flask and dissolved in 1.5 litre of distilled water. Then twenty ml of ethylene glycol was added, made up to two litre with distilled water and stirred.

The pH was checked to be in the range of 6.9 - 7.1 and adjusted with NaOH or HCl if necessary.

ii) α-amylase solution (Sigma, Bacterial/crude, type X1A) . One gram of α-amylase was dissolved in 30 ml distilled water in fifty ml volumetric flask. Ten ml of ethoxyethanol was added and made up to fifty ml with distilled water. The solution was stored in the refrigerator and replaced about every week.

iii) Acid detergent (AD) solution

One litre of distilled water was put into a two litre conical flask. 56 ml of concentrated H_2SO_4 was added carefully and made up to two litre with distilled water to give 5% (w/w) H_2SO_4. Fourty gram of CTAB was weighed into a large beaker, dissolved with 5% H_2SO_4 and made up to two litres with the remaining acid. The solution was left overnight to completely dissolved.

iv) 72% H_2SO_4 (w/w) solution.

326 ml of concentrated H_2SO_4 was added slowly to 170 ml of distilled water in a 600 ml beaker. When the solution had cooled, it was made up to 500 ml with distilled water.

v) Filtered drum acetone.

Day 1 :- A set of ten or twelve labelled crucibles was placed in a furnace at 500°C for two hours and cooled in a dessicator. The crucible weight was recorded as cc. One

gram of dried and ground (to 1 mm) sample was accurately weighed in duplicate into labelled 400 ml beakers. The weight of sample was recorded as W. Fifty ml of ND solution was added into the beaker, then covered with a watchglass and brought to boil on a hotplate which had been set to 400°C initially and latter to 300°C prior to boiling. The beakers were left to simmer for 30 minutes.

Another fifty ml of ND solution and two ml of -amylase solution were added. The beakers were brought to boil and then simmered again for 30 minutes.

The hotplate was turned off and the samples were then filtered through correspondingly numbered crucibles while the mixture was still hot. All samples were rinsed off from the watchglass and beaker sides with hot water.

The residue was washed several times until no detergent was present and then dried overnight in 105°C oven.

Day 2 :- The crucibles were cooled in a dessicator and weighed as ccNDR (=crucible + ND residue).

The crucible was put sideways into a 600 ml beaker and covered with AD solution and watchglass. As previously, it was boiled gently on hotplate for one hour. The hotplate was turned off. With an aid of a tweezers, the crucible sides and bottom were washed using a washbottle filled with hot distilled water to remove all samples from the crucible into the beaker. The crucible was placed on a vacuum system under low suction and the sample was filtered into the crucible, washed several times with hot distilled water to remove all detergent then dried in 105°C overnight.

Day 3 :- The samples were removed from oven, cooled in a dessicator and weighed as ccADR (=crucible + AD residue). The crucible was put upright in a 150 ml beaker and 5 - 10 ml of 72% H_2SO_4 was added. The samples were stirred to a smooth paste with the rounded-end of a glassrod. More acid was added to half fill the crucible. The sample in the crucible was kept covered with acid for three hours, with occasional stirring and refilling from their respective beakers. Sample stuck to the rod was rinsed into the crucible with hot distilled water. The sample was rinsed off from the sides and was washed five times with hot distilled water. The crucibles were then placed in 105°C oven overnight.

Day 4 :- The dried crucible was cooled in dessicator and the weight was recorded as ccSAR (=crucible + sulphuric acid residue)

The crucibles were then placed in a furnace at 500°C overnight.

Day 5 :- The crucible was cooled in a dessicator and weighed as ccASH (=crucible + ash).

The calculations were carried as below :-

Acceptable error between duplicates

$$\% \text{ NDR} = \frac{\text{ccNDR} - \text{cc}}{\text{W}} \times 100\% \qquad <= 3\%$$

$$\% \text{ ADR} = \frac{\text{ccADR} - \text{cc}}{\text{W}} \times 100\% \qquad <= 5\%$$

$$\% \text{ lignin} = \frac{\text{ccSAR} - \text{ccASH}}{\text{W}} \times 100\% \qquad <= 8\%$$

% cellulose = % ADR - % lignin

% hemicellulose = % NDR - % ADR.

3.3.1.4 Estimation of percentage fat by Soxhlet method (Rock et al.,1986)

The term fat is usually applied to the triglyceride component of lipids, whereas the term "crude fat" may include other lipids, or lipid soluble substances e.g. fat soluble vitamins which may be present.

The procedure used involved the extraction of fat from a dried sample by petroleum ether in the Soxhlet extraction apparatus which recycled the solvent automatically and intermittently through the sample.

Two labelled round-bottomed flasks were dried in an oven at 70°C for 30 minutes, cooled in a dessicator and weighed. Two Soxhlet thimbles were weighed and about 5.0 gram of dried sample was accurately weighed into each one. A small amount of cotton wool was placed in the top of the thimbles to stop the sample from floating out, and the thimbles were put in two extractors.

About 100 ml of petroleum ether was poured into a round-

bottomed flask which was connected to the extractor, and this in turn connected to the condensor on an electric-mantle unit. The heater switch was turned on and when boiling started, it was adjusted to maintain an even but not excessive boiling rate. The evaporated ether condensed into the extraction apparatus and then siphoned back to the round-bottomed flasks, taking the extracted fat with it. This process was continued for at least six hours. The thimbles were then removed and the ether was collected into the extractors, leaving the flasks containing the extracted fat. The flasks were dried in an oven at 70°C, cooled in a dessicator and weighed. The difference in the weight of the flask before and after the extraction process gave the weight of fat extracted from the sample and this was then converted to percentage of crude fat in the sample on a dry weight basis.

3.3.1.5 Amino acids analysis

Dried samples were sent to Feed Evaluation Unit, Monogastric Research Centre, Massey University, Palmerston North for analysis. The hydrolysis time was 24 hours at 110'C.

3.3.1.6 Microbiological analysis

The micrbiological analysis on the dried fermented apple pomace was conducted according to the recommendations given by de Groot (1974). The information about specific selective media for the tests were given by Oxoid and Gibco

Media Manual.

The recommended tests were :-

i) Total aerobic count

ii) Fungal spores

iii) Enterobacteriaceae

iv) Clostridia group

v) Enterococci

3.3.1.6.1 Total aerobic count

Serial dilutions of the dried apple pomace were prepared using peptone water, pour-plated on Oxoid Nutrient agar (CM135) and incubated at 30°C for 48 hours.

3.3.1.6.2 Fungal spores

Serial dilutions of the dried apple pomace were prepared using peptone water, pour-plated on Oxoid Malt extract agar (CM59) and incubated at 30°C for 48 hours.

3.3.1.6.3 Enterobacteriaceae

Serial dilutions of the dried apple pomace were prepared using peptone water, pour-plated on Oxoid MacConkey agar (CM7), incubated at 37°C for 48 hours.

3.3.1.6.4 Clostridia group

Serial dilutions of the dried apple pomace were prepared using peptone water, pour-plated on BBL Reinforced Clostridial agar (11564), incubated at 37°C for 48 hours.

3.3.1.6.5 Enterococci

Serial dilutions of the dried apple pomace were prepared using peptone water, pour-plated on Gibco Bile Aesculin Azide agar (M05500) and incubated at 34°C for 48 hours.

4 Fermentation Of Apple Pomace With *Candida utilis*

4.1 Biochemical analyses of wet fermented apple pomace

The fermentation of *Candida utilis* on apple pomace was carried out in one litre Agee Preserving jars for 72 hours. Samples were taken every 12 hours for the analyses of sugar concentration, yeast number, relative pH, ammonium sulphate concentration and total nitrogen concentration. The ambient temperature around the fermentor where the fermentation took place was also noted.

The apple pomace was found to form clumps or "balls" after a few hours of rotation of the fermenter. Since the production of biomass was an aerobic process, the pomace was mixed for ten seconds, just before sampling was carried out. This did not only break up the clumps of apple pomace but also facilitated a good sampling. A small portion of apple pomace was taken from each individual jar and then mixed together to give a total representative sampling of a particular time point.

Although temperature could have a significant effect on the fermentation, the temperature of the laboratory was found to be fairly constant, ranging from the lowest of 19°C to the highest of 23°C with 20°C being the most common temperature. The central heating system of the laboratory maintained the temperature close to this value. This

enabled the fermentation of *Candida utilis* to be carried out at ambient temperature in the laboratory without the need of using an incubator or a hot-room.

It was found that samples taken had to be analyzed immediately. Storage at -20°C for too long a period caused the cells of apple pomace to rupture and liquid was released, resulting in mushy and sticky samples which were difficult to be analyzed giving inaccurate results. Due to this predicament, the samples were kept at 4°C for a maximum of four days in airtight, water proof labelled containers.

The plot depicting the growth of *Candida utilis* and changes in relative pH versus time is shown in Figure 6. Since the inoculum used was from an actively growing yeast culture, the plot does not show a typical sigmoid curve of the growth of yeast which starts with a lag phase, a phase which a yeast adjusted itself to a new environment. The use of this actively growing inoculum shortens the duration of the fermentation time considerably. The log phase of the growth reached its maturity at about 60 hours with the exhaustion of available reducing sugar in the apple pomace (Figure 7).

A plot of protein and nitrogen concentration versus time is shown in Figure 8. It was noted that the inorganic nitrogen i.e. ammonium sulphate was not being utilised appreciably during the first 24 hours of the fermentation although the sugar concentration decreased from 46% to 38%. Comparing the graph of Figure 8 and the amino acids profile

Table 3 : Fermentation characteristics of apple pomace

Time(h)	relative pH	$\log_{10}$ yeast/ g wet pomace	reducing sugar g/100g dry pomace	Organic Nitrogen g/100g dry pomace	Inorganic Nitrogen g/100g dry pomace	True Protein g/100g dry pomace
0	4.52	4.90	46.97	0.774	1.198	4.838
12	4.21	6.48	42.44	0.796	1.176	4.975
24	4.21	6.90	38.68	0.796	1.176	4.975
36	3.80	7.30	34.47	0.915	1.057	5.719
48	2.89	7.70	23.81	0.966	1.006	6.038
60	2.71	8.30	5.77	1.019	0.953	6.369
72	2.67	8.30	3.19	1.041	0.931	6.506

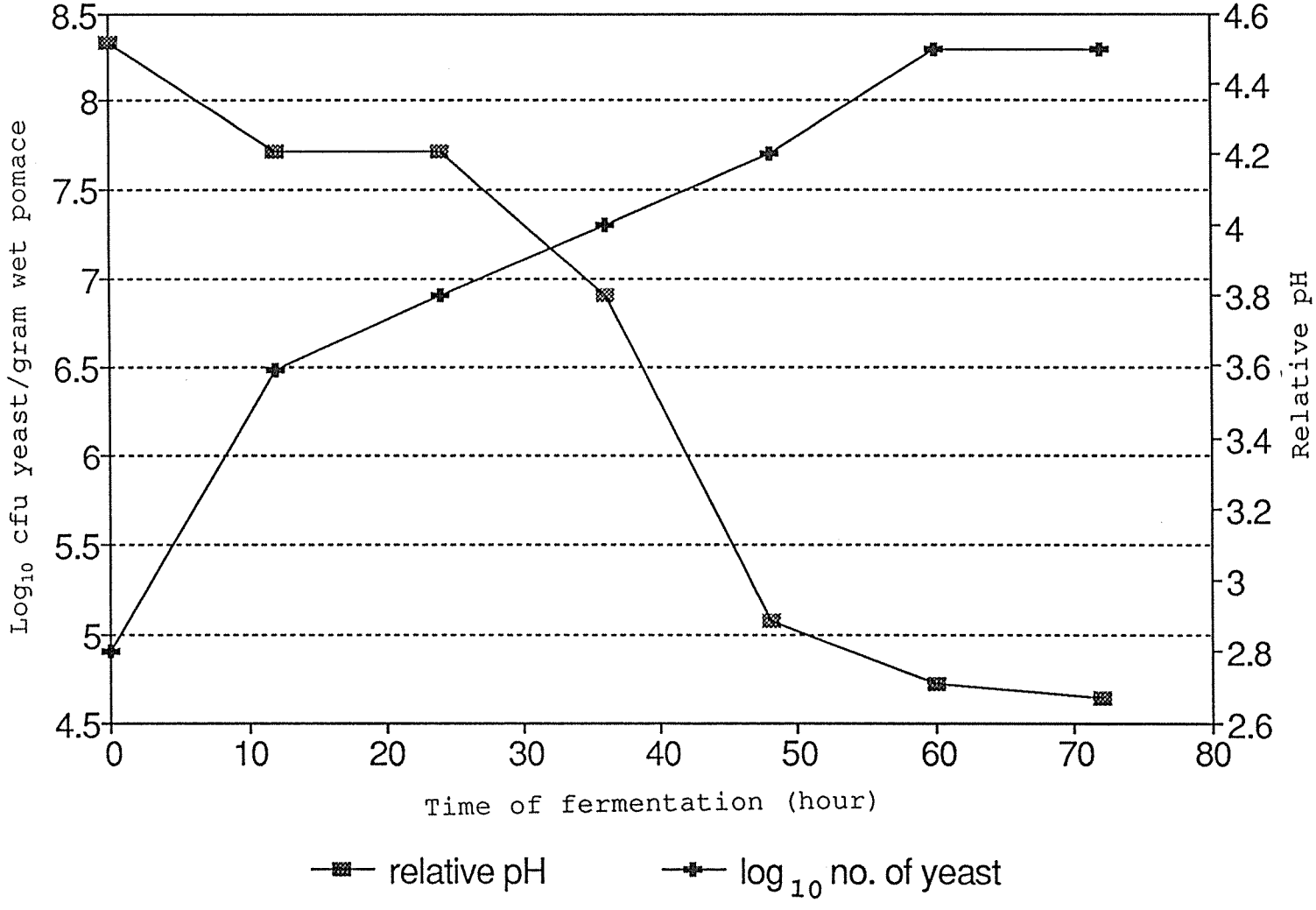


Figure 6 Plot of relative pH and $\log_{10}$ cfu yeast/gram wet pomace vs time

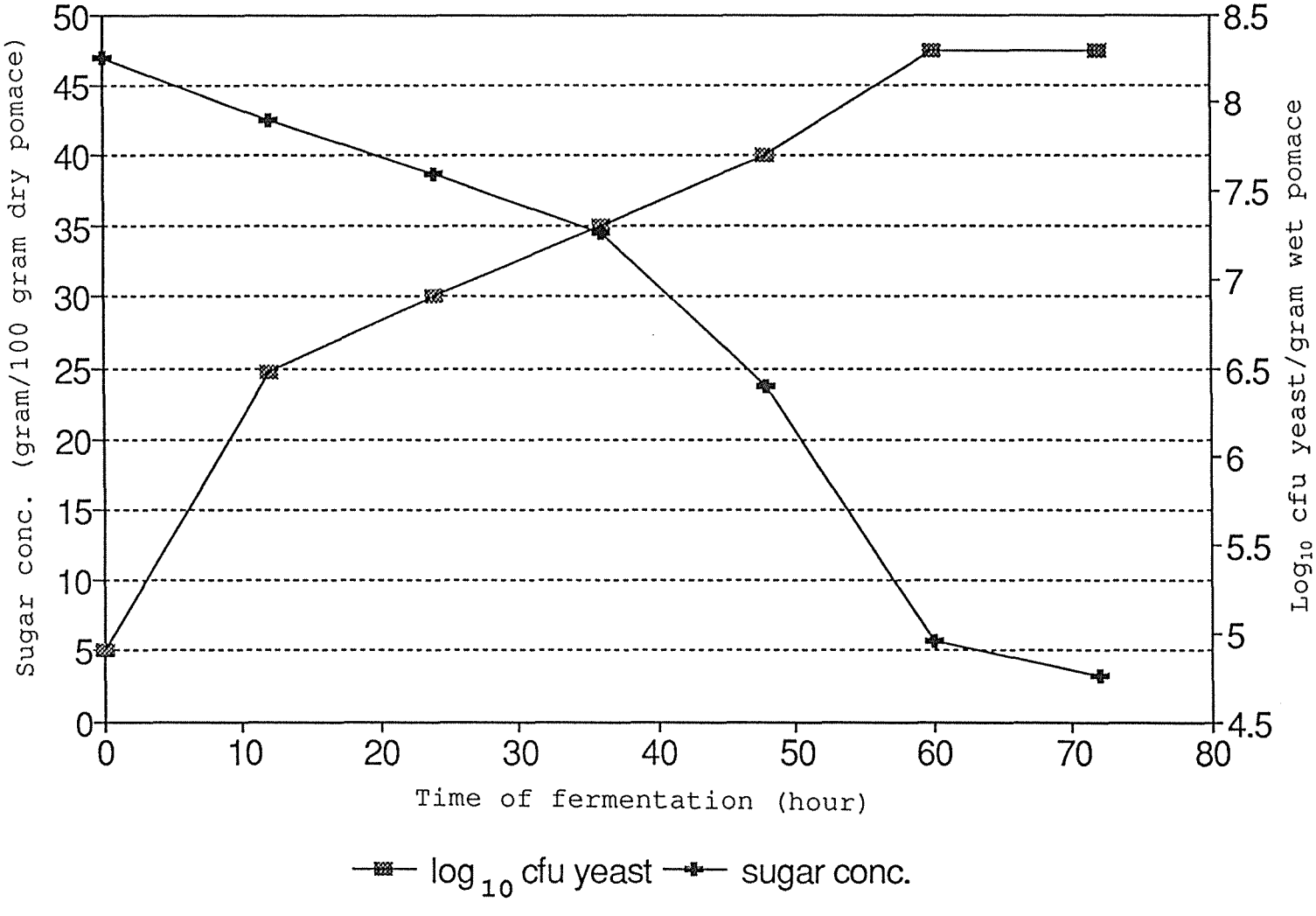


Figure 7 Plot of sugar conc. and $\log_{10}$ cfu yeast vs time

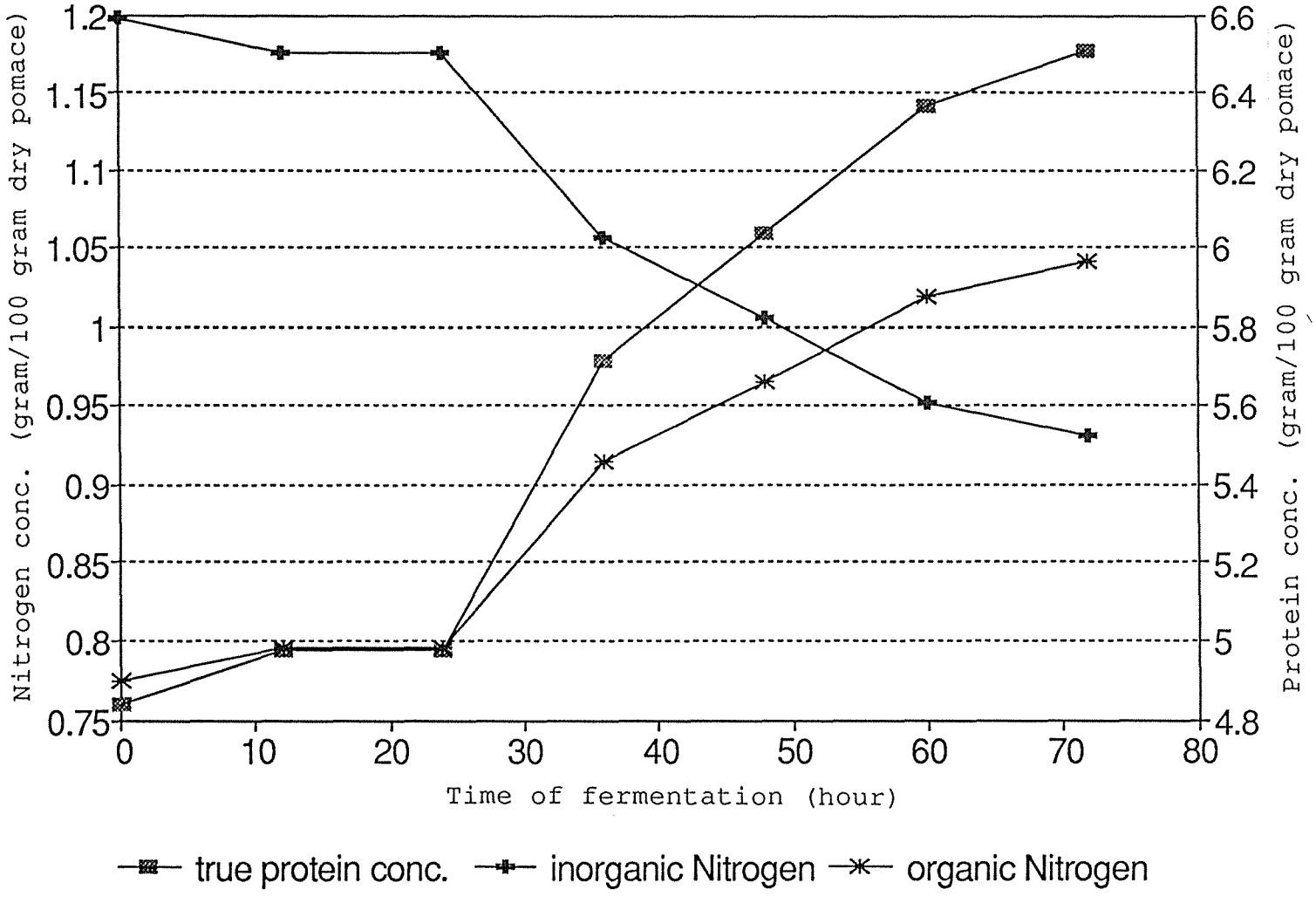


Figure 8 Plot of nitrogen conc. (gram N/100 gram dry pomace and protein conc. (gram/100 gram dry pomace) vs time

(Table 4) of apple pomace, it can be deduced that for the first 24 hours of the fermentation, *Candida utilis* did not use the ammonium sulphate that had been provided but instead the amino acids that were present in the apple pomace. This suggests that it was easier for the *Candida utilis* to assimilate and incorporate the already synthesised amino acids into cellular protein than to assimilate and incorporate the ammonium sulphate.

Also it was noted that the drop in relative pH was not very significant during the first 24 hours although the drop was expected to be proportional to the growth of *Candida utilis.* This could be due to the buffering effect of ammonium sulphate in the apple pomace. Another reason could be that during this time *Candida utilis* used the available energy and nutrients to produce more biomass, not by- or end- products that could lower the pH of the apple pomace.After 24 hours, when *Candida utilis* started to use the supplied ammonium sulphate as the building blocks for protein synthesis actively, the relative pH dropped from 4 to 2.5 at 60 hours and then levelled off till the end of the fermentation i.e. at the 72nd. hour. During about the same period of time, the inorganic nitrogen content decreased from 1.176 to 0.953 gram N/ 100 gram dried pomace.

At the end of the fermentation (72 hours), *Candida utilis* utilised 93% of the original 46% reducing sugar/gram dried pomace and 22.3% of 1.198 gram N/100 gram dried pomace to increase the true protein content from 4.80 to 6.51 gram

protein/100 gram dried pomace.

4.2 Effect of drying on fermented apple pomace

For the fermented apple pomace to be suitable as animal feed, its moisture content had to be reduced to 10% (w/w). Thus, drying operation using an oven dryer was employed. This prevents microbial degradation and also eases handling. The negative side of drying was the decrease of nutritional value due to the destruction of nutrients e.g. amino acids. Also the Maillard reaction which occurred during drying operation could cause off flavours, poor colour and the loss of certain essential nutrients (Oste *et al.*,1984; Percival, 1979). The use of the Carpenter method (Booth modification) in estimating the available lysine in fermented apple pomace was employed because lysine has been often the factor limiting protein quality.

Figure 9 shows the plot of available lysine versus drying temperature for the fermented apple pomace. The data shown was based on true protein i.e. only organic protein. There was a slight decrease in available lysine with increase in temperature of drying, and it dropped from 8.65 gram N/16 gram N in freeze-dried fermented apple pomace to 7.53 gram/16gram N of fermented apple pomace dried at 135°C. This corresponded to a decrease of about 13% of the total available lysine. This was surprisingly low although the temperature employed was 135°C and could be explained by Figure 7. At the end of the 72 hour fermentation period only about 7% of the total reducing sugar was left in the

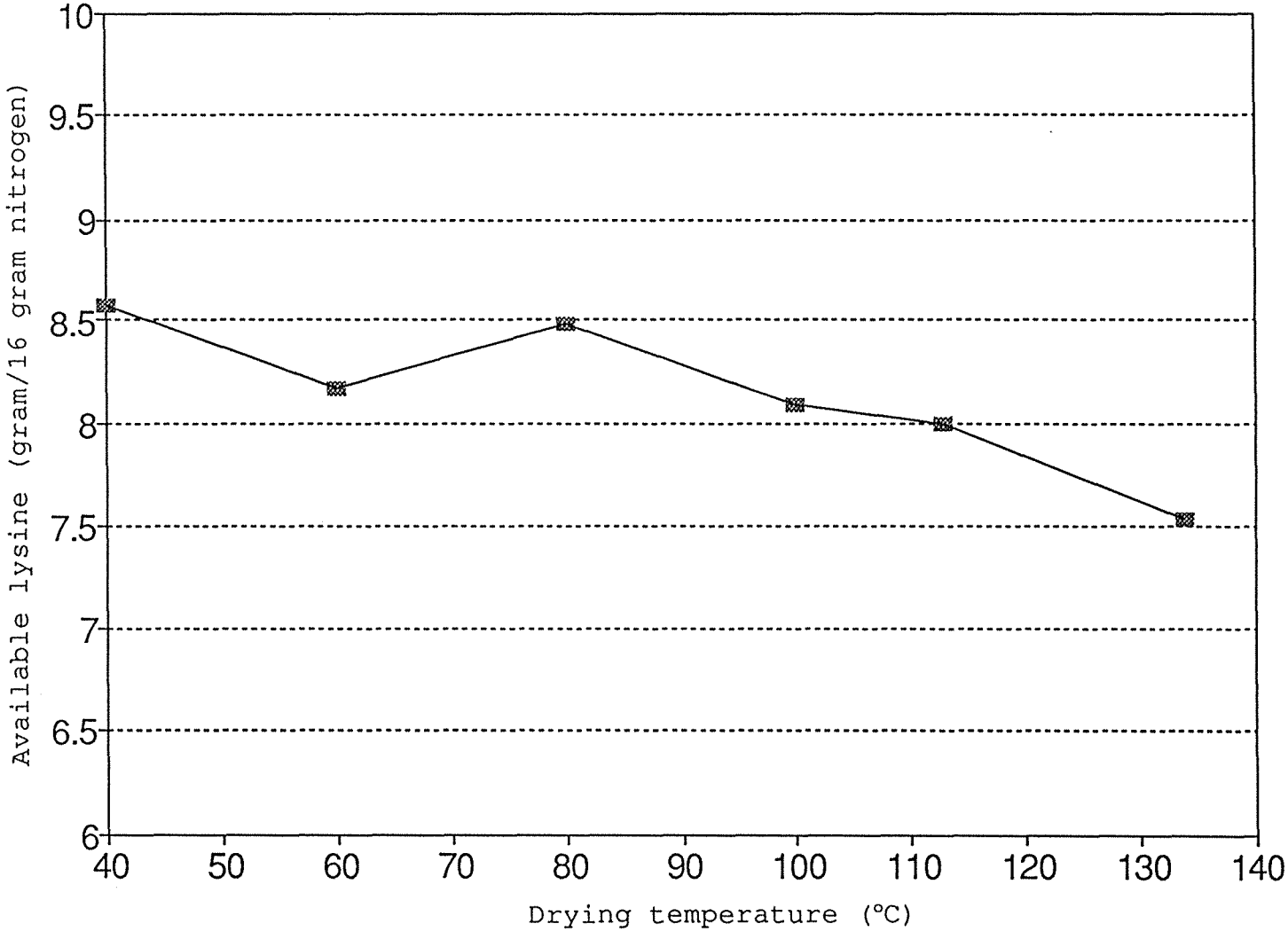


Figure 9 Plot of available lysine vs drying temperature

fermented apple pomace. This residue of reducing sugar could bind to a small percentage of amino acids to make them nutritionally unavailable (Ledward, 1979).

Based on these observations, the highest temperature of the oven dryer of 135°C was employed for subsequent drying operations since this temperature gave the fastest drying operation without any significant decrease in available lysine content in the apple pomace.

4.3 Stockfeed analyses of dried fermented apple pomace

A variety of tests was conducted on fermented and unfermented apple pomace (Table 4). A list of nutritive values of barley is also shown as comparison. Amino acids profiles for fermented and unfermented apple pomace are shown in Table 5.

Although the crude protein content of the fermented apple pomace was slightly higher than those of barley, it is consisted of two significant parts; one being organic or true protein content and the other the inorganic or non-protein nitrogen content, at 1.041 gram N/100 gram dry pomace and 0.931 gram N/100 gram dry pomace respectively. Although protein from non-protein nitrogen can be synthesized by ruminants through microbial action in the rumen, it must be fed along with a readily fermentable carbohydrates to supply the energy for the formation of the protein molecules (Cullison, 1979).

Table 4 Nutritive evaluation of dried fermented and unfermented apple pomace and barley as comparison.

	Unfermented apple pomace	Fermented apple pomace	Barley
Dry matter %	89.94	89.34	88.32
Organic matter %	98.36	97.53	86.6
Nitrogen %	0.762	2.096	
Crude protein*	4.80	13.100	12.16
True protein %	4.80	6.51	
Gross energy	19.036 kJ/g	19.270 kJ/g	
Crude fat %	2.463	3.208	1.9
Fibre %- lignin	7.910	12.084	3.2
-hemicellulose	15.233	19.434	3.2
-cellulose	8.523	11.418	9.7

* calculated by percentage Nitrogen x 6.25

(Nutritive value of barley was kindly supplied by Dr.W.Smith of Animal Science Department. Massey University)

The amino acids profile for true protein is given in Table 5. From the table, most essential amino acids requirement are met by the fermented apple pomace, except for sulphur containing amino acid, methionine. These results were consistent with other findings (Goldberg,

Table 5 : Amino acids profile of apple pomace.

	% air dry basis		% true protein (from Table 3)			
Amino acid	apple pomace	fermented pomace	apple pomace	fermented pomace	recommended FAO	*Candida utilis* (Hodge 1991)
essential						
Threonine	0.152	0.367	2.34	5.64	2.8	2.40
Valine	0.184	0.389	2.83	5.98	4.2	2.99
Methionine	0.051	0.096	0.78	1.47	2.2	0.65
Isoleucine	0.155	0.319	2.38	4.90	4.2	2.30
Leucine	0.267	0.571	4.10	8.77	4.8	3.65
Phenylalanine	0.160	0.355	2.46	5.45	2.8	2.10
Histidine	0.188	0.335	2.89	5.15		0.96
Tryptophan	DNA	DNA	DNA	DNA	1.0	
Lysine	0.164	0.488	2.52	7.50	4.2	3.79
Arginine	0.199	0.553	3.06	8.49		2.04
non-essential						
Aspartic acid	0.484	0.673	7.43	10.34		4.16
Serine	0.193	0.435	2.96	6.68		2.40
Glutamic acid	0.534	1.093	8.20	16.79		6.89
Proline	DNA	DNA	DNA	DNA		1.46
Glycine	0.191	0.384	2.93	5.90		4.73
Alanine	0.201	0.453	3.09	6.96		1.42
Cystine	DNA	DNA	DNA	DNA	2.0	0.10
Tyrosine	0.121	0.311	1.86	4.78		1.80
Ammonia	0.121	0.652				
TOTAL	3.361	7.474				

1985; Batt, 1984) which noted that yeast cell proteins lacked sulphur containing amino acids. The requirement for methionine could be met by cystine but this was not known for the fermented apple pomace due the unavailability of data on the complete profile of amino acids of the material. Comparing the amino acids profile before and after the fermentation, it was found that there was more than two fold increase in the total amino acid in the fermented apple pomace with nearly twice an increase in the percentage of lysine content. This profiles gave an initial indication of the relatively high nutritional value of the fermented apple pomace.

The fermented apple pomace has a gross energy value of 19.27 kJ/gram of dried apple pomace. Although the gross energy value has no direct relationship to the feed's digestible, metabolizable or net energy values, it did indicate the maximum energy stored in the material.

The fibre analysis (Table 5) was found to be slightly higher in the fermented apple pomace than unfermented apple pomace. This increase in fibre could be due to the yeast cell wall. Although an accurate structure of yeast cell wall has not been satisfactorily modelled, approximate composition of yeast cell wall has been investigated. Yeast cell wall has been found to consist mainly of glucans, mannan and chitin. A large proportion of the fibre came from the apple pomace and this should not significantly change during the fermentation due to the incapability of *Candida utilis* to assimilate these materials.

Hemicellulose is a group of substances which chemically lie between sugars and starch on one side and cellulose on the other. Most of such substances are more digestible than cellulose but less digestible than sugars and starch. Cellulose is a principal constituent of the cell wall of apple pomace. It can be digested fairly effectively by ruminants. While lignin is not a true carbohydrate but usually considered along with polysaccharides. Lignin is of no known nutritive value except as bulking agent. From the percentage of fibre shown above, about 12.1% of the dried fermented apple pomace would be nutritionally unavailable and this figure could increase if they blocked the nutritional components of this product. Crude fibre tended to decrease the digestibility of nutrients chiefly by protecting constituents of the material from attack by digestive or microbial enzymes, to a greater extent in monogastric animals than in ruminants.

Regression equations for estimating the extent of crude fibre depression of the digestibility of a crude containing feed in animal are as follows (Bondi,1987) :-

for ruminants : $Y = 90 - 0.85 X$

for horses and pigs : $Y = 90 - 1.60 X$

for poultry : $Y = 90 - 2.30 X$

where X = percentage crude fibre in dry matter,

Y = coefficient of digestibility of the organic matter.

So, from Table 1, total crude fibre for dried fermented

apple pomace = 42.936%).

for ruminant : Y = 53.50

for horses and pigs : Y = 21.30

for poultry : Y = -8.75

Based on these regression equations, the dried fermented apple pomace was not suitable for poultry feeding at all and to certain extent to horses and pigs as well, while ruminants can digest this material to a "fair" degree. On the basis of crude fibre, the dried fermented apple pomace was at par with wheat straw but with slightly higher level of crude protein (Table 6). This could be an advantage as a bulking agent so as to produce stock with leaner meats (Snowdon, 1984).

Microbiological analysis was also done on the dried apple pomace that had been stored in airtight containers for four months at ambient temperatures and kept away from direct sunlight.

Ten fold (v/v) dilutions with peptone water for pour-plating were done. The mixture was difficult to be pipetted due to its high suspended solids. It had to be left to settle until enough supernatant could be removed. Dilution of 100 fold (v/v) amounts were then used for pour-plating and from this level of dilution, no colony was formed on any of the media after incubation.

Table 6 : Composition of various selected feeds (Cullison 1979)

	Composition Of Feeds				
	% fed basis	% Dry basis			
	Dry matter	Ash	Crude Fibre	Crude fat	Crude Protein
Dehydrated alfalfa leaf kennel	92.3	12.2	20.1	3.4	22.6
Blood meal	89.3	4.9	0.7	1.5	89.8
Barley grain	89.0	3.4	6.0	1.9	13.0
Dried beet pulp	90.6	5.3	20.1	0.6	9.6
Wet beet pulp	11.3	4.7	30.1	2.1	11.7
Carrots	12.9	9.7	9.1	1.4	10.3
Fresh citrus pulp	18.3	7.7	12.6	3.3	6.6
Alsike clover hay	67.7	8.7	30.1	2.7	14.2
Corn bran	88.7	2.2	10.9	5.1	9.1
Cottonseed hulls	90.2	2.9	45.0	1.6	4.4
Ground cottonseed	92.7	3.8	18.2	24.7	24.9
Oats grain	88.9	3.8	11.9	5.1	13.2
Milo grain	88.9	2.0	2.5	3.2	12.2
Sunflower meal	93.0	8.3	11.6	3.1	50.3
Wheat straw	87.8	7.2	43.6	1.5	3.7
Wheat grain	88.9	2.1	2.8	2.1	13.4

Table 7 : Microbiological analysis of the dried fermented apple pomace

	Dried fermented apple pomace	Recommended level (Groote 1974)
Total aerobic count	absent	$< 10^4$ / g
Mould spores	absent	< 10 / g
Enterobacteriaceae	absent	absent in 0.1g
Clostridial group	absent	$< 10^2$ / g
Enterococci	absent	$<10^2$ / g

Based on these results (Table 7), it was concluded that dried fermented apple pomace was free from contamination by pathogenic microorganisms.

Although, chemical analyses cannot be used as a sole indication of stockfeed suitability, the fermented apple pomace did show a good prospects as a stockfeed but does require further testing in animal feeding trials.

5 Citric Acid Fermentation From Apple Pomace By *Aspergillus niger*

Six experiments were carried out to investigate factors affecting the solid state production of citric acid by *Aspergillus niger* on the new apple pomace. They were :-

i) Methanol additions
ii) Ammonium sulphate addition
iii) Spore inoculum
iv) Natural oil additions
v) Temperature of fermentation
vi) Moisture content of apple pomace

5.1 Effect of methanol addition

Methanol at varying concentration of 0 - 5% (v/w) was added to the apple pomace prior to fermentation. The result is shown in Table 8. The most noticeable effect of methanol on the fermentation was the slow growth and sporulation of *Aspergillus niger* on apple pomace. To citric acid accumulation to an extent suitable for a commercial process, neither full growth nor sporulation must take place. Observations could be drawn that when full growth or sporulation was allowed to take place, most of the available sugar would be converted to producing biomass and some would be lost via respiration. *Aspergillus niger* did not start to sporulate until the fifth day at five percent

methanol (v/w). Increasing the concentration of methanol produced a marked increase in the citric acid production. *Aspergillus niger* produced the greatest amount of citric acid at three percent methanol (v/w) at 249 gram/kg of utilized sugar which corresponded to a yield of 24.9%. However, addition of methanol of more than three percent (v/w) had an inhibitory effect on citric acid production resulting in no citric acid accumulation. It was suggested (Rohr *et al.*, 1981) that the addition of methanol reverses the negative effect of trace metals on citric acid formation. The exact nature of the effect is unknown, but since trace metals are involved in membrane composition, it was reasonable to assume some action of the methanol on the cytoplasmic membrane.

5.2 Effect of ammonium sulphate addition

Ammonium sulphate was added to the apple pomace to provide a nitrogen source to the *Aspergillus niger*. 0 - 5% (w/w) of ammonium sulphate was added to the apple pomace. The result is shown in Table 9. No citric acid was produced at each concentration of ammonium sulphate and it has been suggested that *Aspergillus niger* produces citric acid only under nitrogen limiting conditions (Berry *et al.*, 1977; Heinrich *et al.*, 1982; Eikmeier *et al.*, 1984). The protein content of the apple pomace was about three percent on dry weight basis (N.Z Apple and Pear Marketing Board). It could be concluded that at this level of existing protein in apple pomace, it was sufficient as a nitrogen source for

the fermentation without the need for any exogenous nitrogen source supplementation. The stimulating effect of methanol on the citric acid production also seemed to diminish with any external nitrogen addition.

5.3 Level of spore inoculum

An experiment on the spore density in the inoculum was carried out to determine the optimum level of spore inoculum for citric acid production. The largest inoculum that could be used was 5×10^7 spores per 20 gram of apple pomace since inocula larger than this would alter the moisture content of the apple pomace quite significantly. The results are shown in Table 10. It was found that the optimum number of spores needed was between 1×10^5 to 1×10^6 spores per 20 gram of apple pomace. Most work on the solid state production of citric acid have used 2×10^6 spores per forty gram of apple pomace (Hang *et al.*, 1984; Hang *et al.*, 1987). Higher number of spores seem to produce more biomass and a faster rate of sporulation. Again, methanol had a dramatic effect in stimulating *Aspergillus niger* to produce citric acid.

5.4 Effect of natural oil addition

Soya bean oil (natural oil) was added at 0 - 5% (v/w) to the apple pomace before fermentation. The results of these experiments are shown in Table 11. No citric acid was produced at oil concentration of 1 to 3% (v/w). At 5% (v/w) the surface of the apple pomace looked and felt oily. The

growth and sporulation were not affected but no citric acid was produced. Limited amounts of citric acid were produced at three percent (v/w) of oil with three percent (v/w) methanol. An increase in citric acid was observed at four percent (v/w) oil with addition of three percent (v/w) of methanol. This was increase of about twenty percent from no addition of oil but three percent (v/w) methanol. Thus oil alone was not affective but oil with methanol did stimulate acid production. Millis *et al.*(1963) suggested that unsaturated lipids act as alternative hydrogen acceptor although her observations were conducted on submerged cultures. Furthermore she also used a different strain of *Aspergillus niger*. Thus direct comparisons of her results and these ones are difficult.

5.5 Effect of temperature of fermentation

From Table 12, no citric acid was produced at fermentation temperature of 25°C and 37°C. At 25°C, the growth of *Aspergillus niger* was seen to be slightly retarded and with addition of three percent (v/w) methanol, the growth was seriously limited until the fourth day of fermentation when any obvious growth could be observed. In contrast, at temperature of fermentation of 37°C, the growth was more pronounced with subsequent of heavy sporulation after two days of fermentation. Growth or production of biomass was favoured at higher temperature of 37°C.

5.6 Effect of moisture content of apple pomace

No growth and citric acid production was observed at an apple pomace moisture content of 40%, 50% and 60% (Table 13). The sugar utilization was reduced to a minimum in all cases. Although growth was observed at moisture contents of 70% and 80%, no citric acid was produced. The sporulation at 70% and 80% moisture content was not very pronounced compared to that seen at 90% moisture content. This observation on citric acid production was markedly different from the work reported by Hang *et al.*(1987) when it was found that from 45% to 75% (w/w) moisture content the citric acid yield increased from about 55% to 70% based on the amount of fermentable sugar consumed when 3% (v/w) of methanol was added. But the yield of citric acid decreased from 45% to 20% when no addition of methanol was made.

Table 8 Amount of citric acid produced at various methanol additions

	Residual sugar gram / 100 gram dry pomace	Yield of citric acid gram / kg sugar consumed
methanol % (v/w)		
0	0.96	0
1	0.4	0
2	0.54	124
3	0.42	249
4	0.62	0
5	0.97	0

Table 9 Amount of citric acid produced at various ammonium sulphate and methanol additions

		Residual sugar gram / 100 gram dry pomace	Yield of citric acid gram / kg sugar consumed
% ammonium sulphate	% methanol		
0	0	0.78	0
	3	1.00	267
1	0	0.72	0
	3	0.67	0
2	0	1.03	0
	3	0.58	0
3	0	0.92	0
	3	1.06	0
4	0	0.60	0
	3	0.67	0
5	0	0.97	0
	3	0.49	0

Table 10 Amount of citric acid produced at various spore inocula and methanol additions

		Residual sugar gram / 100 gram dry pomace	Yield of citric acid gram / kg sugar consumed
spore level	% methanol		
1 x 105	0	0.85	0
	3	0.95	404
1 x 107	0	0.78	0
	3	0.82	1
5 x 107	0	0.71	0
	3	0.98	16

Table 11 Amount of citric acid produced at various natural oil and methanol additions

		Residual sugar gram / 100 gram dry pomace	Yield of citric acid gram / kg sugar consumed
% oil	%methanol		
0	0	0.57	0
	3	0.43	344
1	0	0.85	0
	3	0.92	0
2	0	0.68	0
	3	0.71	0
3	0	1.06	0
	3	0.78	149
4	0	0.96	0
	3	0.99	443
5	0	0.94	0
	3	0.96	0

Table 12 Amount of citric acid produced at various temperatures of fermentation °C and methanol additions

		Residual sugar gram / 100 gram dry pomace	Yield of citric acid gram / kg sugar consumed
Temperature	% methanol		
25°C	0	1.76	0
	3	2.80	0
30°C	0	1.82	0
	3	1.40	371
37°C	0	0.66	0
	3	0.56	0

Table 13 Amount of citric acid produced at various moisture contents of apple pomace and methanol additions

		Residual sugar gram / 100 gram dry pomace	Yield of citric acid gram / kg sugar consumed
% moisture content	% methanol		
40	0	4.97	0
	3	5.12	0
50	0	4.99	0
	3	4.95	0
60	0	3.40	0
	3	5.09	0
70	0	1.38	0
	3	1.01	0
80	0	1.36	0
	3	1.38	0

6 Conclusion

It was found that the enrichment of apple pomace prepared by the older juice extraction technology could be carried out successfully using *Candida utilis* Y15. The organic protein content increased from 4.80% per dry weight of apple pomace to 6.51% with near total exhaustion of reducing sugar available. The overall amino acid content of the fermented apple pomace was above the recommended FAO value except for sulphur amino acids i.e. methionine and cystine.

Although the fermented apple pomace still contained a high proportion of crude fibre, this could be a bonus in producing lean meats (Bowden *et al.*,1959; Dreyer *et al.*, 1979). Estimation based on crude fibre content of the fermented apple pomace suggests that it may be more suitable as a sole feed for ruminants than for monogastric animals such as pigs although it could be used as a supplement in pigs diet.

More work will have to be done which should incorporate animal trials to investigated further the potential of fermented apple pomace as stockfeed.

Aspergillus niger NRRL 328 was found to produce limited amounts of citric acid via solid state fermentation of apple pomace prepared by a newer juice extraction technology. The factors that seemed to have profound effects on citric acid production were concentration of

methanol, oil, ammonium sulphate and spore inoculum size.

The addition of 3% (v/w) methanol to the apple pomace fermentation increased citric acid production. High methanol concentration (4 - 5% v/w) retarded and to certain extent inhibited the growth of *Aspergillus niger*. The cellular action of methanol was not determined but it was suggested that it could affect the cytoplasmic membrane of *Aspergillus niger*.

The highest citric acid production was with 4% (v/w) oil and 3% (v/w) methanol which gave about 44% yield based on sugar consumed. No citric acid was produced at all with any ammonium addition. The production of citric acid by *Aspergillus niger* seemed to be nitrogen limiting.

The level of inoculum was another factor that influenced the production of citric acid by *Aspergillus niger*. The optimum level of inoculum was 1×10^5 to 1×10^6 spores per 20 gram of apple pomace. Higher spore numbers caused rapid vegetative growth and subsequent sporulation with no citric acid produced.

Vegetative growth and sporulation were observed to take place profusely in almost all of the experiments carried out. To encouraged citric acid production, *Aspergillus niger* must be made constrained so as not to produce biomass to any large extent but to accumulate citric acid. It is recommended the use of mutation techniques using *Aspergillus niger* NRRL 328 should be investigated further in future work on the production of citric acid from apple pomace.

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Appendix

Table A1 : Analysis of available lysine content of fermented apple pomace at various temperature of drying

Temperature of Drying (°C)	Available lysine (gram / 16 gram Nitrogen *)
freeze-dried	8.65
40	8.57
60	8.17
80	8.49
100	8.09
113	8.00
134	7.5

* based on percentage true protein (Table 4)