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Biotechnology of Whey using Yeast Fungi Isolated from Local Environment

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BIOTECHNOLOGY OF WHEY USING YEAST FUNGI ISOLATED FROM LOCAL ENVIRONMENT

A Thesis

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The potential threat of whey pollution to the soil environment and ultimately groundwater resources in UAE promoted the elements of concern to the utilization of the whey waste. This work was conducted in Al-Ain area to isolate new fermenting yeast strains, from local soils, capable of fermenting whey lactose.

Soil samples were collected from two locations in Al-Ain area (near Al-Ain dairy farm & Al-Markhania area) and from a neighboring area (Al-Buraimi) based on the soil nature and ecological parameters. Inorganic elements and organic residues of the soil samples were characterized. Results indicated that Al-Buraimi soil is rich in nitrate nitrogen, reactive iron (878 mg kg^{-1}), sulphur and organic carbon. Al-Ain Dairy Farm soil is rich in ammonia nitrogen (26mg Kg^{-1}) and magnesium oxide. Al-Markhania soil is rich in phosphorus and potassium.

Organic analysis indicated that Al-Markhania soil is the richest in organic residuals of plant debris. This results in providing the most favourable organic environment for the growth and proliferation of microorganisms. The highest soil conductivity was recorded in Al-Buraimi area (1.0740dS m^{-1}). This is attributed to the high content of soil minerals. The pH value indicated that all the soil samples were within the neutral to slightly alkaline range [7.10-7.80]. The type of whey used in the study was classified according to pH (6.62) and acidity (0.10%) as sweet whey and the salt content was found to be 0.20%.

Standard and enrichment isolation methods in which the whey lactose is used as a carbon source were used to isolate the potential whey-fermenting yeasts from the soil. The highest number of isolated yeasts was obtained from Al-Markhania soil (rich in organic matter), followed by Al-Buraimi soil (rich in minerals). The lowest number of yeast has been observed in AI-Ain Dairy Farm soil which is poor in organic nutrients (non cultivated desert soil). Yeast isolates number (2, 14 and 20), all isolated from Al-Markhania soil, have shown strong fermentative ability on whey broth. Out of these three isolates, the highest ethanol concentration was achieved by the yeast isolate (number 20). It was tentatively identified to species level in accordance to morphological, cultural, sexual and physiological criteria as described by Barmet, (1984). The identification of isolate number (20) was confirmed by the National

Collection of Yeast Cultures (NCYC, UK) and as a result of this study, it has been referred to as *Kluyveromyces marxianus* NCYC 2886.

Yeast fermentation conditions [temperature and whey lactose concentrations] were studied so as to reach optimal ethanol yields. *K. marxianus* NCYC 2886 was subjected to various temperatures ranging between 30°C to 50°C. The results revealed that ethanol production increases in parallel with the elevation of temperature from (30°C, 35°C, 40°C and 45°C). The optimal ethanol production was achieved at 40°C. Therefore it could consequently be referred to as a thermotolerant whey fermenting yeast. Reduction of ethanol production was observed at high temperatures exceeding 50°C.

Eight lactose concentrations, in whey, in the range of 0.31-11.05%, were employed to investigate the effect of lactose concentrations on the fermenting ability and efficiency of the yeast strain *Kluyveromyces marxianus* NCYC 2886 in producing ethanol. The optimal lactose concentration found to enhance ethanol production is 2.5% contained in 50% diluted whey. The increase in lactose concentration from 2.50% to 4.95% slightly decreased the ethanol yield and ultimately withstood lactose utilization.

With the aim of optimizing ethanol yield, different chemical amendments were subjected to study so as to test their ability in enhancing whey fermentation at 40°C using 50% diluted whey. Addition of 0.20 g of yeast extract in 100 ml of 50% whey concentration resulted in yielding 6.55% ethanol compared to the non-amended whey (5.33%). This was the highest yield of ethanol obtained. Ethanol concentrations were found to be similar (6.10%) when 1.20 g/100 ml whey of both beef extract and peptone were used. Addition of 0.03 g of potassium di-hydrogen phosphate (KH_2PO_4) resulted in producing 6.35% ethanol. The above supplements are significantly increased the ethanol production and considered as enhancers.

In contrast, the addition of higher concentration of yeast extract (1.20 g) significantly decreased ethanol yield to 3.80% compared to the non-amended whey (5.33%). A clear inhibition of ethanol yield was observed when magnesium sulphate ($MgSO_4$) alone was used as a supplement in whey fermentation. Urea has shown inhibitory effect on whey fermentation by the yeast strain *K. marxianus* NCYC2886. Di-potassium hydrogen phosphate (K_2HPO_4) ions appeared to exert a significant inhibitory effect on fermentation process compared to the non-amended whey (5.33%). on addition of mesoinositol (0.10% w/v) and pantothenate (0.0001% w/v)

the ethanol concentrations yield were 3.57% and 3.55%, respectively. Calcium carbonate ($CaCO_3$) (0.20% w/v) led to the production of 3.99% ethanol which is significantly low compared to the non-amended whey (5.33%). Manganese chloride ($MnCl_2$) added to 50% cheese whey (final concentration was 1.0 g/L) resulted in low production of ethanol at 40°C (3.53%). This result may be attributed to the addition of higher concentration of $MnCl_2$. The addition of high linoleic acid concentration (1% w/v) to 100 ml of 50% whey resulted in obvious reduction of ethanol production where 3.55% ethanol was yielded.

The results obtained by using riboflavin (0.0001% w/v), pyridoxin-HCl (0.0001% w/v), aminobenzoic acid (0.0002% w/v), malt extract (0.2% w/v), ammonium sulphate [$(NH_4)_2SO_4$] (0.05% w/v), Tween 80 (30 µL), nicotinic acid (0.0005 % w/v), thiamin (0.0001% w/v), peptone (0.20% w/v) and beef extract (0.20% w/v) indicated no significant effect on the whey fermentation process at 40°C compared to the non-amended whey (5.33%).

The reduction efficiency of Chemical Oxygen Demand (COD) obtained for the fermented 50% whey, with and without yeast extract addition at 40°C, were 59% and 46% respectively.

SCIENTIFIC CONTRIBUTIONS BASED ON THE THESIS FINDINGS

- **Al-Muhairi M. S., M. H. Soliman, K. El-Tarabily,** and **M.O.El-Obeid (2001)**

  "Potential utilization of waste whey in U.A.E."- A poster work accepted to be displayed at *NIZO Dairy Conference on Food Microbes: from knowledge to application* (Ede, The Netherlands : 13-15 June 2001), Sponsored by Elsevier Science Co. (UK).

- **Al-Muhairi M. S., M. H. Soliman, K. El-Tarabily** and **M.O.El-Obeid (2000)**

  "Whey treatment by using yeast fungi isolated from local soil environment". A technical paper presented at the *Workshop on Waste Treatment Strategies in U.E.A.* (Al-Ain : April. 2000), Sponsored by Al-Ain Municipality.

- **Granted a UNSCO-MIRCEN Scholarship for a Short-Term Training** Program On *Scale Up of Fermentation Processes* (Cairo:6-14/november.2000).

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NOTE

Besides the work carried out in this thesis, the candidate Mouza Suhail Al-Muhairi pursued postgraduate studies for the partial fulfillment of the M.Sc. degree in Environmental Science in the following topics:

A. Core courses:

Environmental Science I.

Environmental Science II.

Environmental Law.

Social Impact Assessment.

Seminar.

Applied Statistics.

B. Special courses:

Environmental Microbiology.

Applied Systems Ecology.

Food and Water Pollution.

Selected Topics in Biological and Agricultural Science.

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Mouza Suhail Al-Muhairi

CHAPTER I
Introduction

Chapter One

INTRODUCTION

The agro-alimentary industry produces a considerable amount of organic by-products that are often discarded as wastes to find their way to the surrounding environments, although they are rich with different organic components that could be used for the extraction of valuable products or for obtaining edible biomass (Maullu *et al.*, 1999). Among the most important of these wastes, is the cheese industry by-product such as whey which contains primarily lactose, different proteins and various soluble minerals.

Whey which has a high biological oxygen demand (BOD) of 4-5 X 10^4 ppm, remains a major disposal problem to be considered in most milk-producing countries in Europe, Australia, New Zealand, America and Canada (Maullu *et al.*, 1999). The continuous land disposal of cheese whey is going to endanger the physio-chemical and biological properties of soil and also will lead to serious water pollution. Therefore, countries will suffer from whey pollution sooner or later unless more attention is given for finding practical solutions to combat this potential problem at its infancy (Ben Hassan & Ghaly, 1994).

Whey has long been considered as an enormous by-product of casein and cheese manufacture. Its disposal is either by feeding to the farm animals or running to waste in streams or onto the land. Such disposal can cause hazardous pollution to the soil and the water. In the past few decades, the environmental awareness and public pressure, coupled with a recognition of the inherent value of whey components have resulted in the development of processes for the conversion of liquid whey into a range of value-added products (Zadow, 1993). In addition, the economical feasibility of whey utilization is entirely attributed to its useful ingredients (Szczodrak *et al.*,

1997). Whey ingredients enter into a wide spectrum of industrial and commercial applications which are supported by the continuing technological advancements in the field of organic waste recycling (Maullu *et al.*, 1999). In industry, whey ingredients can effectively be used as infant and animal food supplements, as a source of lactose, as fermentation substrates, as well as to produce a wide variety of value-added products such as alcohol, biogas, enzymes, pharmaceuticals, nutraceuticals and single cell protein (Marwaha & Kennedy, 1988). Whey is also used in xanthan gum, a thickening and stabilizing agent of calcium magnesium acetate which is used as deicer. Furthermore, whey is used in the production of ethanol and other non-alcoholic beverages, aromas, plant hormones such as gibberelic acid and some enzymes such as polygalactonase and α-amylase (González-Siso, 1996).

The most recent application of whey is the production of biodegradable plastic which in turn delivers a double benefit to the environment. It keeps the waste from polluting water and soil and the plastic returns safely to the environment (Coleman, 1990). Deproteinized whey permeate has also been studied as a substrate for the yeast *Phaffia rhodozyma* which produces large amounts of β-carotene astaxantin which is used to colour eggs in the farms or salamonids in aquaculture (González-Siso, 1996).

Whey is also used in the production of some therapeutic agents such as immunopotentiators, antitumors and antiparasitics. Some experimental studies revealed that whey has a potential antitumor effects. An *in vitro* assay showed that the specially formulated whey protein concentrate which is called "Immunocal" appears to exert an inhibitory effect not only on the initiation of cancer growth but also on its progression, particularly on breast cancer (Kennedy *et al.*, 1995). The manufacture of lactose and associated whey-based industries has often been seen as an effective means of overcoming and averting some of the environmental problems caused by whey disposal (Zadow, 1984).

During the last two decades, the image of cheese whey has been rapidly changed from a bothersome by-product to a highly prized organic resource of a wide spectrum of industrial and nutritional applications (Jelen, 1994). This is because of the increase in the public environmental safety awareness and the commitment of the industrial sector to manufacture environmentally friendly products (Jelen, 1994). The bioconversion of whey waste to value-added products has been achieved by means of microbial biotechnology, genetic engineering and fermentation processes.

Over the last decade, U.A.E have introduced and established modern dairy-based industry and related livestock activities in which whey is regarded as a polluting waste. Yet, it has not been utilized in any commercial applications.

1.1 Aims of the thesis

No previous economical, microbiological or environmental studies have been conducted in the U.A.E. on the utilization of the cheese whey disposals. The objectives of the present study endeavor to investigate the following:

i- To isolate and identify yeast fungi from UAE soils capable of utilizing and fermenting whey to produce alcohol.

ii- To conduct a comparative study for the fermentation abilities and alcohol production of the isolated yeasts under different whey concentrations and at different temperatures. This is to optimize the fermentation conditions to reach the maximum alcohol production.

iii- To improve the microbial fermentation ability and alcohol production by adding a variety of nutrients (simple and complex) to the fermentation media.

iv- To investigate the effectiveness of yeast fermentation process in reduce the pollution potential of cheese whey.

CHAPTER II
Literature Review

Chapter Two

LITERATURE REVIEW

2.1 Dairy-based industry in U.A.E.

United Arab Emirates has witnessed the establishment of dairy-related industries in the early 1968 when Dubai Emirate initiated this activity. Later on, during the period between 1970-1996, a rapid expansion emerged throughout the country when Dagdaga Dairy Farm in Ras-Alkhaimah in 1978, Al-Ain Dairy Farm in 1980 and Al-Rawabai Dairy Farm in Dubai in 1990 established their own industries. These pioneer factories have paved-the-way to other milk-based industries to launch from a solid foundation.

The new industries are namely Gulf & Safa (Dubai-based), Milco (Abu Dhabi-based), Lacnore (Sharjah-based) and recently Marmum (Dubai-based) in 1996 (A. El- Sedeeq, Al-Ain Dairy Farm, personal communication). Recently, the UAE milk-based industry achieved a considerable turnover and consequently contributed significantly in the food industry and national income (*courtesy of Al-Ain Dairy Farm, 1998 report).*

Today, there are six major players in fresh milk market in UAE. The two major ones are Al-Ain Dairy (Al Ain-based) and Al-Rawabi (Dubai-based). The amount of milk produced from the local dairy industry in 1998 was 110 million liters (Fresh milk 58, Recombined (laban & yoghurt) 40 and UHT milk 12). The market size in UAE fresh dairy market of milk and milk products in each emirate in 1998 was 41% in Abu Dhabi, 36% in Dubai and 23% in Sharjah & other emirate (*courtesy of Al-Ain Dairy Farm, 1998 report).*

More than 25 dairy farms exist in Al-Ain. This constitutes the majority of the farms in the UAE. The number of cows in these dairy farms are more than 12,000 whereas the domestic cows are only 300-500. Annually, these farms produces 75 million liters of milk whereas the annual total milk

products are produced from the equivalence of 60 million liters. Cheese and milk powder are produced from an amount of 7.3 million liters milk yearly (*courtesy of Al-Ain Dairy Farm, 1998 report).*

Al-Ain area yields annually the major amount of whey waste in UAE. It is estimated to be around 3 to 6 million liters. Since most of the local whey waste finds its way to sewerage and soil as shown in (Figure 1-A & 1-B), both components of the natural environment will ultimately be suffering from gradual accumulation of whey disposal. Serious environmental problems would shortly occur as a result of whey pollution. This might arise in the near future unless this problem is urgently and seriously acknowledged and effective solutions are considered and put into action.

2.2 Historical background of whey

The use of whey as a beverage has been recorded since the time of Hippocrates of ancient Greece (Jelen, 1994). It has been used as a component of hair tonics and burn salves during the middle ages (Yang & Silva, 1995). Since the 1960s, great efforts have been made to fractionate and use the main constituents of whey independently such as protein. Despite such efforts, only about 50% of the world whey products being turned to good accounts (Moulin & Galzy, 1984). In 1974, 32.5 billion pounds of whey were produced in the USA and over half of that amount was disposed of as waste. This represents a pool of 1.6 billion pounds of lactose, which if converted into usable food products could be of sizable monetary value to the dairy industry (O'Leary *et al.*, 1977).

The increase in size of cheese industry, the necessity for the reduction of the Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of the effluent, along with the need to maximize returns on raw material have encouraged producers to seek new ways of using the whey (Moulin & Galzy, 1984). In the past, whey was concentrated or dried for

Figure 1 (A): Improper management of whey disposed in a local factory.

Figure 1 (B): Eutrophic lake caused by the nutrients present in whey which stimulate the growth of algae.

animal feed. Recently, new technologies have been innovated to utilize whey waste into a variety of biotechnology-based applications such as the production of biomass and single-cell protein (Mansour *et al.*, 1993; Maullu *et al.*, 1999), ethanol and other alcohols (Ferrari *et al.*, 1994; Ghaly & El-Taweel, 1997; Banat *et al.*, 1998), glycerol (Rapin *et al.*, 1994), biofuels (Quereshi & Maddox, 1991), bioingredients (Belem & lee, 1998a) and antitumor drugs (Kennedy *et al.*, 1995).

2.3 Definition of whey

Whey is defined as an opaque greenish-yellow liquid remaining in the cheese vat following the precipitation and removal of milk casein in cheese making process (Xu *et al.*, 1987; Valentas *et al.*, 1991; González-Siso, 1996). In dairy industry, two main types of whey are produced depending on the lactic acid content of the whey and on the procedures that are used in the casein precipitation (Mawson, 1994). These types are designated as acid and sweet whey (Jelen, 1994).

2.4 Types of whey

González-Siso (1996) classified whey on the basis of acidity and pH values. These classes are sweet (acidity: 0.10-0.20 & pH: 5.8-6.6), medium acid (acidity: 0.20-0.40 & pH: 5.0-5.8) and acidic whey (acidity: 0.40-0.60 & pH: 4.0-5.0). Each class generally reflects the lactic acid content of the whey. Spreer (1998), further classified whey into three types based on the way of casein coagulation either by acid or enzymatic means. These three types are:

i- ***Acid or quark whey***: This is a milk serum obtained during the acidic process. It is rich in calcium and phosphorus content. It is produced by the solubilization of the calcium phosphate complex of the casein micelle at the acidic pH range which is used for the acid coagulation of the casein (Jelen, 1994).

ii- ***Sweet or rennet whey***: This is a calcium lactate-free whey. This type is obtained by the enzymatic coagulation of whey casein. Sweet whey can

not be easily converted into acidic type even with post-acidification. It is obtained from the manufacture of most hard and semi hard cheeses including Cheddar, Swiss, Gouda, and Mozarella (Jelen, 1994).

iii- ***Industrial grade whey***: This is obtained when protein coagulation is carried out with acids other than lactic acid (e.g. acetic acid or mineral acids such as sulphuric and hydrochloric acids).

2.5 Chemical composition of whey

The interest in industrial utilization of whey has grown in the late 1930s, the matter which triggered off a series of analytical investigations to study its composition as well as its physio-chemical ingredients which are of industrial interest and economical value. Many researchers have focused on the nature and composition of whey (Bajpai *et al.,* 1991; Ben-Hassan *et al.*, 1992; Ferrari *et al.*, 1994; Mawson, 1994; González-Siso, 1996; Spreer, 1998).

Whey represents about 85-95% of the milk volume and retains nearly 55% of the milk nutrients (Xu *et al.,* 1987; Rapin *et al.*, 1994). González-Siso (1996) investigated the whey major abundant nutrients and identified their percentages. Among the most important nutritious components are lactose (4.5-5% w/v) which is fairly constant but depends on the amount converted to lactic acid soluble proteins, mainly β-lactoglobulin (0.6-0.8% w/v), which largely depends on the milk coagulum and its treatment (Scott, 1985). Whey also contains a small amount of fat (0.4-0.5% w/v). High fat content in the cheese milk leads to high fat content in the whey. Whey must be decreamed at fat content of $> 0.1\%$. Cream is either processed into whey butter or used in the fat standardization of cheese milk (Spreer, 1998). Whey also contains minerals and salts such as NaCl, KCl and Ca salts. There concentrations are fairly constant unless chemicals such as nitrates (Zadow, 1993; Mawson, 1994; Rapin *et al.*, 1994) or calcium chloride or hydroxide (Scott, 1985) were added to the milk.

Whey also contains appreciable amounts of other components such as

lactic and acetic acids, non-protein compounds and vitamins B and C. Both vitamin B and lactoflavin, cause the greenish yellow colour of whey. The amount of lactose, lactic acid and minerals content varies depending upon the source of milk, the type of cheese and variations in processing methods used (Zall, 1979). Whey composition also varies according to its origin (ewe, goat or cow) as well as to the cheese-making technique employed as illustrated in (Table 1) (Moulin & Galzy, 1984). It is noteworthy to mention that whey obtained from ewe's milk contains more than twice the amount of protein found in that obtained from cow's milk (Moulin & Galzy, 1984).

2.6 Economical potentials of whey

About 9 kg of whey is generated from the production of 1 kg of cheese (Belem & Lee, 1998a). The different components of whey have been utilized in manufacturing many value-added products of commercial significance. The most valuable whey components from nutritional standpoint are whey proteins, water-soluble vitamins especially riboflavin and some nutritionally important minerals. Proteins from either sweet or acid whey are the best proteins for human food use (Jelen, 1994). The nutritive value of whey protein is quite high compared with other sources of proteins such as milk, beef and potato as measured in terms of biological value. The high biological value of whey protein depends on the high contents and availability of essential amino acids compared to other types of proteins (Guirguis *et al.*, 1993). The nutritional importance of such amino acids that is obtained from external sources is due to the fact that both human and animals can not synthesize these amino acids (Guirguis *et al.*, 1993).

Large amounts of wasted cheese whey yearly find their way to sewerage and land to raise serious environmental pollution. The conversion of useful products and nutritive ingredients from wasted cheese whey (Table 2) requires many applications and processes. Bajpai *et al.* (1991) shed lights on the need to develop new uses of cheese whey and its nutritionally valuable derivatives

Table 1: Comparison of different types of liquid whey (g/L) according to Février & Burdin (1977) cited in (Moulin & Galzy, 1984).

Crude type	*Cow Rennet*	Cow *Mixed*	Cow *Lactic*	Ewe *Rennet*	Goat *Lactic*
Density	1.239	1.0247	1.0245	1.0234	1.0269
Dry matter	70.84	70.49	65.76	83.84	62.91
Dry matter without lipids	65.78	67.11	64.91	77.38	62.51
Lactose	51.81	50.84	45.25	50.98	39.18
Total nitrogen	1.448	1.454	1.223	2.933	1.466
Non-protein N_2	0.368	0.414	0.536	0.796	0.669
Ammonia N_2	0.041	0.090	0.140	0.129	0.176
Urea nitrogen	0.141	0.095	0.070	0.139	0.122
Lactic acid	0.322	2.226	7.555	1.763	8.676
Citric acid	1.298	1.095	0.260	1.032	0.157
Ash	5.525	5.888	7.333	5.654	8.361
Phosphorus	0.412	0.470	0.649	0.545	0.703
Calcium	0.466	0.630	1.251	0.494	1.345
Potassium	1.455	1.491	1.485	1.281	1.812
Sodium	0.505	0.537	0.528	0.616	0.433
Chlorides, NaCl	2.195	2.208	2.092	2.368	3.287

in order to demonstrate the benefit of cheese whey as a promising biomass resource of economical potentials. Schoutens & Groot (1985) estimated the production costs of fermentatively produced iso-propanol-butanol-ethanol mixture (IBE) from whey permeate in comparison with the same products produced from oil. They concluded that the production cost from the former is more economically feasible at a large scale when new technologies such as cell immobilization and membrane process are used. Kemp & Quickenden (1988) acknowledged whey as a worthy alternative for producing biogas methane to be used directly in the main system providing in-house return on capital investment. Whereas, Quereshi & Maddox (1991) re-assessed the economical viability of acetone-butanol-ethanol (ABE) mixture produced from whey fermentation. They found that the membrane process allowed an economically viable alternative and the substrate cost has a major effect on the product's price. Anaerobic semicontinuous digestion of whey was applied to produce high quality biogas with no traces of H_2S (Kosaric & Asher, 1985; Lebrato *et al.*, 1990).

Whey also plays a significant role as an additive in fermenting solid food wastes such as starchy waste products like bread, biscuits, potato chips and flour (Kumar *et al.*, 1998). Whey-derived products have a wide spectrum of uses and applications in human foodstuff and animal feeding (Zall, 1979). Whey is recently utilized in the production of bio-fuel by means of microbial fermentation techniques (Spreer, 1998). It can also contribute indirectly in the elimination of some contaminants. It was found that mercury-resistant bacteria can grow in lactose solution while lactose can be used as carbon and energy source for cell synthesis of these microorganisms and this consequently can be used in the biological detoxification of mercury process with efficiency greater than 95% (Xu *et al.*, 1987).

Zadow (1993) revised the different processing options for treating cheese whey. These options fall into four main areas which are:

Table 2: Examples of some cheese whey by-products obtained by the action of different microorganisms.

MICROORGANISM	BY-PRODUCTS	REFERENCES
Kluyveromyces fragilis	i- Biomass, SCP ii- Lactic acid iii- Lipids iv- Wine v- Oligosaccharides vi- RNA derivatives	i- Yang & Silva, (1995); Kiers *et al.*, (1998). ii- Stockar & Marison, (1989). iii- Maullu *et al.*, (1999). iv- Yang & Berggren, (1976). v- Belem & Lee, (1997a). vi- Belem & Lee, (1997b).
Kluyveromyces lactis	i- Aromas Gibberelic acid ii- Lactic acid iii- Lysozyme enriched biomass	i- Gonzalez-Siso, (1996). ii- Kosaric & Asher, (1985). iii Maullu *et al.*, (1999).
Phaffia rhodozym	β-Carotene astaxanthin	Gonzalez-Siso, (1996).
Saccharomyces zygosaccharomyces	Ethanol and Biomass	Polman *et al.*, (1996).
Genetically engineered *Saccharomyces cerevisiae*	i- Fructose-di-Phosphate ii- Polygalacturonose iii- Ethanol	i- Gonzalez-Siso, (1996). ii- Gonzalez-Siso, (1996). iii- Terrell *et al.*, (1984).
Rhodotorula glutinis	i- Carotenoids ii- Fermented whey concentrates iii- Food ingredients	i- Frengova *et al.*, (1994). ii- Gandhi & Patel, (1994). iii- Huffman, (1996).
Candida pseudotropicalis	Biomass and Ethanol	Marlene *et al.*, (1982); Ghaly & El-Taweel, (1997).
Trichosporon beigelli	Lipids and Biomass	Tahoun *et al.*, (1987).
Propioibacterium acidopropionia	i- β-Galactosidase ii- Alcohol iii- Deicer iv- Ethanol v- Methane vi- Non-alcoholic beverages vii- Propionic acid	i- Bojorge *et al.*, (1999). ii- Chen, (1981). iii- Huang & Yang, (1998). iv- Ghaly & El-Taweel, (1997). v- Desai &Madamwar, (1994). vi- Kosaric & Aher, (1985). vii- Yang *et al.*, (1994).
Lactobacillus acidophilus	Fermented whey beverage (acidowhey)	Gandhi & Patel, (1994).
Bacillus spp.	i- α-amylase ii- 2,3 Butanol iii- Acetate or Acetic acid iv- Glycerol v- Oraganic acids vi- Xanthan gum	i- Bajpai *et al.*, (1991) ii- Gonzalez-Siso, (1996). iii- Huang & Yang, (1998). iv- Jenq *et al.*, (1989). v- Stockar & Marison, (1989). vi- Kosaric & Asher, (1985); Gonzalez-Siso, (1996).
Aspergillus niger	Oxalic acid	Bohlmann *et al.*, (1998).

i- The processes concerned with simple removal of water by spraying to produce whey powder.

ii- The processes concerned with increasing the ratio of protein by ultra-filtration and fractionation for the manufacture of protein isolates and heat treatment for lactoalbumin.

iii- The processes concerned with utilization of lactose in cheese whey by the treatment with lactase heat/acid lactose hydrolysis, or by means of fermentation to produce lactic and acetic acids and alcohol, in addition to single-cell protein (SCP).

iv- The processes designed to alter the mineral composition of the product by means of electrodialysis and ion exchange techniques for the manufacture of demineralized products.

While these methods produce useful products, many of them are economically marginal because of their sizeable expenditures necessary for equipment, labour, and energy (Xu *et al.*, 1987). In some countries, milk production is seasonal. Therefore, the production does not satisfy the need of whey-converting factories to run in an economical term.

Spreading whey on land is purely a disposal route. However, whey is non-toxic and is valuable as a fertilizer for improving soil texture. It also contains the vital plant nutrients such as nitrogen, phosphorus, and potassium in proper proportions (Strudsholm & Andersen, 1985; Yang & Silva, 1995). Therefore, spraying whey onto the fields is being practiced by many cheese makers, especially in rural areas (Jelen, 1994).

Disposal of cheese whey on the agricultural lands as a fertilizer or animal feed has changed during the past 20 years due to the changes in farming techniques (Scott, 1985). However, whey must not be applied in excess, as the annual limit is around 5 cm/yr. Moreover, spreading of whey on the agricultural lands is no longer an option for many dairies because of recent environmental regulations (Yang & Silva, 1995).

2.7 The biological oxygen demand (BOD)

BOD is defined as the total amount of oxygen that would be consumed if all organic matter in one liter of water is oxidized by microorganisms. It represents an indirect measure of the biodegradable organic matter in an aqueous sample. However, the test of BOD is not achieved directly because the dissolved oxygen which is consumed by the microorganisms while degrading the organic matters is the parameter which is measured rather than the depletion of the matters themselves (Boardman, 1994). If the organic wastes were not treated properly they would create a serious oxygen deficiency in the water environment by means of increasing the BOD level. The presence of some dissolved ions or compounds in the sample may inhibit microorganisms degrading the organic matter. This results in an artificially low or absence of BOD (Boardman, 1994).

Samples may be seeded by microorganisms to compensate for those problems. The absence of a required nutrient will make the BOD test invalid (Boardman, 1994). Raw sewage may give BOD readings in hundreds while a food process waste may give BOD readings in thousands. Several experimental methods have been conducted to reduce the BOD created by effluents of dairy industries. Among the most effective methods is lactose fermentation which causes severe BOD if not well treated (Zadow, 1984).

2.7.1 BOD of whey

Although several possibilities for cheese whey utilization have been assayed over the last 50 years, approximately half of the world whey production is not well treated. However, it is discarded as an effluent into municipal sewerage system and soil (González-Siso, 1996). Whey is characterized by high organic matter content, exhibiting a high BOD of 4-5 X 10^4 ppm compared to sewage (350-400 ppm), household waste (400 ppm), and post-waste-water treatment (20 ppm) (González-Siso, 1996).

Whey is fairly difficult to be treated biologically or digested by some

organisms because lactose is the major carbon source (Valentas *et al.*, 1991). About 90% of whey BOD is due to the presence of lactose. The early the whey is processed, the higher the quality of the final product (Spreer, 1998). One thousand kg of raw whey which is discharged into sewage treatment plant per day can impose a load approximately equal to that obtained from 470 persons (Zall, 1979) the matter which represents a very high value in comparison to other human-produced wastes. For every kg of raw whey discharged into a stream, about 4500 kg of unpolluted aerated water is required for its biological oxidation (Zall, 1979).

2.8 Other aspects of whey pollution

Some types of whey are acidic and therefore, potentially corrosive. Such types have disposal liability and exert a costly burden on sewerage or industrial effluent treatment systems. Untreated whey is a detriment to the environment and may create other expenses (Zall, 1979). An approach is urgently needed to meet the problem of water pollution due to the increasing volumes of whey production (González-Siso, 1996). Aerobic yeast fermentation of cheese whey is eventually used to reduce its potential pollution as well as to produce value-added products such as bioethanol and SCP. The lactic acid concentrations in different whey streams affects the oxygen demand reduction achieved in the process (Mawson, 1994).

Long-term land disposal of whey can cause many environmental pollution problems as reported by many authors. Nitrogen is soluble in water and may be subject to leaching into groundwater. Thus, it becomes a threat both to human and animal health (Ben-Hassan & Ghaly, 1994). It has been reported that the continuous disposal of cheese whey can endanger the physical and chemical structure of the soil, decreases the crop yield, kills vegetation, produces a stench and creates serious water pollution problems (Ben-Hassan & Ghaly, 1994; Belem & Lee, 1998a).

Reports on total chloride content of liquid dairy waste such as whey,

whey permeate and contaminated milk revealed that the high concentration levels of chloride in drinking water may be injurious to human health and cause kidney and heart problems. Such concentration levels of chloride in soil and groundwater can cause serious problems to the crops which result in reduced ability of water absorption, poor yield, burn of leaf margin and early leaf drop (Strudsholm & Andersen, 1985).

2.9 Limitation of whey utilization

The large-scale of whey utilization has been firstly investigated in the industrial countries. However, the technologies used were not effective.
Little amounts of whey production were fully utilized. This is attributed to many factors. These factors includes the mineral content and low pH of casein whey (Zadow, 1993); lack of environmental regulations (Spreer, 1998); the cost of energy used for processing or that used in the biological waste treatment (Guirguis *et al.*, 1993; *Spreer*, 1998); the perishable nature of cheese whey (Guirguis *et al.*, 1993) and the seasonal production of whey with peak and trough periods throughout the year (Guirguis *et al.*, 1993). In addition, the high level of lactose (75% of the total solids) which is difficult to be treated by biological means is also a limiting factor (Valentas *et al.*, 1991; Zadow, 1993).

2.10 Fermentation of whey

Fermentation is a term derived from the Latin verb *Fevere* (to boil) which describes a complex transformation of material via the metabolic activity of microorganisms or via an enzymatic reaction using enzymes obtained from microorganisms (Sengha, 1993). It is also known as the first industrial process to produce microbial metabolites such as the production of CO_2, ethanol and single-cell protein (SCP) as biomass. This biomass can be separated and dried as a feed concentrate (Spreer, 1998).

Whey, an interesting source of lactose which is necessary for the microbial growth and assimilation has long been found to be a useful substrate in fermentation industries. Several distilleries producing ethanol from whey

are in commercial operation in Ireland, the USA and particularly New Zealand where 50% of the whey production is used to produce ethanol (González-Siso, 1996).

In whey fermentation industry, the production of ethanol from whey containing the original lactose concentration is not economically attractive because the level of ethanol obtained is about 2% the matter which makes the recovery process of ethanol too expensive (González-Siso, 1996). In addition, potable ethanol has a good economic return because it can be used as the substrate for vinegar production (Yang & Silva, 1995).

To overcome some of these disadvantages, many researchers have given a great deal of attention to the economical utilization of cheese whey using different approaches such as: extractive fermentation; the use of continuous bio-reactors with cell recycling or cell immobilization; the introduction of genetically engineered microorganisms (GEMs) and the addition of many different complex nutrients into the fermentation media to improve the quality and quantity of the products obtained from cheese whey fermentation (Ferrari *et al.*, 1994; McMillan, 1997).

2.10.1 Microorganisms involved in the whey fermentation

The treatment of cheese whey by fermenting microorganisms has received a wide scientific and industrial attention (González-Siso, 1996). The targeted whey ingredient is lactose which furnishes favourable conditions as a carbon source for fermenting microorganisms to work effectively (Spreer, 1998). Microbiologists have identified some genera as whey fermenting microorganisms. These includes *Kluyveromyces, Saccharomyces, Candida* and *Lactobacillus* spp. (Mawson, 1994). Previous studies have identified *Kluyveromyces marxianus* (formerly known as *K. fragilis*) as the most promising genus for the conversion of lactose to ethanol and it is the microorganism of choice for most commercial plants (González-Siso, 1996). The rate of lactose fermentation by *K. marxianus* was reduced by sugar

concentrations above 2% and by accumulation of ethanol (González-Siso, 1996).

González-Siso, (1996) reported that *Saccharomyces cerevisiae* was not a promising candidate for whey fermentation because it lacks the lactose permease system which is the membrane-lactose barrier that controls the entry of sugars into the cells, as well as the intracellular enzymes for lactose hydrolysis and β-galactosidase. This reduces its ability to ferment lactose directly into ethanol.

2.10.2 Enhancement of fermentation ability

Many researchers conducted experimental and applied studies for the production of alcohol from cheese whey fermentation using different strains of bacteria and yeasts. Cooperation has been bridged between the microbiologists and genetic engineers to solve problems encountering the economical utilization of whey by enhancing the fermentation ability of bacteria and yeast strains by means of genetic engineering (González-Siso, 1996) or by the addition of chemicals to achieve an effective approaches to increase the yield of fermentation. Cell immobilization techniques have also been used (Stockar & Marison, 1989).

2.10.2.1 Genetic engineering

Genetic engineering of microorganisms has emerged as a preferable solution and have been widely used in improving the ability of candidate microorganisms, particularly the yeast, in performing whey conversion by means of fermentation (Szczodrak *et al.*, 1997). Yeast is now beginning to offer the researchers a considerable advantages over bacteria (mainly *Escherichia coli*). Yeasts are inexpensive to grow on an industrial scale and recent studies have yielded new information on its genetics at a molecular level. Because of the larger cell size of yeasts relative to bacteria, the separation and down-stream processing have also become more efficient and cost effective (Nagodawithana, 1994). Thus, the genetic transformations of

industrial microbes extended the ability to produce products of high commercial quality which extended far beyond the traditional approaches such as hybridization and mutations.

Unfortunately, *Saccharomyces cerevisiae* can not metabolize lactose. Due to this inability, several approaches have been tried to construct genetically engineered *S. cerevisiae* strains which are able to metabolize lactose and produce ethanol (Sreekrishna & Dickson, 1985; Domingues *et al.*, 1999). One approach, is the protoplast fusion which results in the hybridization of the strains *S. cerevisiae* and *Kluyveromyces lactis* (Domingues *et al.*, 1999). Another approach, is the use of recombinant DNA techniques which results in the expression of the genes that code for the β-galactosidase and lactose permease system of *K. lactis* (González-Siso, 1996; Tahoun *et al.*, 1999), *E. coli* (Alberghina *et al.*, 1991; Porro *et al.*,1992) and *Aspergillus niger* (Ramakrishnan & Hartley, 1993) in *S. cerevisiae.* In addition to ethanol, the genes that code for the β-galactosidase of *A. niger* produce salts of fructose-diphosphate which are used in pharmacology (González-Siso, 1996).

The disadvantages of this approach is that the recombinant yeast are very slow growing and have reduced genetic stability and therefore, the yields are low (González-Siso, 1996). Another trial was conducted by Guimaraes *et al.* (1992) in which lactose was successfully converted to ethanol by using recombinant *E. coli* containing *Pdc* and *adhB* genes obtained from *Zymomonas mobilis.* Recently, the cloning and the expression of the gene from *Candida pseudotropicalis* in *S. cerevisiae* cell have been reported (Domingues *et al.*, 1999).

2.10.2.2 Chemical amendments

Whey permeate (the residue after ultrafiltration of whey) is low in organic nitrogen source which is needed for the growth of many industrial microorganisms. In order to improve microbial fermentation of whey, many

chemicals were added to the fermentation media to enhance the rate of fermentation as shown in (Table 3).

2.10.2.3 cell immobilization

Immobilization of the yeast or bacterial cells or the enzyme lactase is used for the enhancment of fermentation ability. The enzyme might be immobilized directly onto various types of membranes or inorganic-support matrices (Stockar & Marison, 1989). Many commercial lactase preparations are available in both soluble and immobilized form, but their prices are usually quite expensive for the purpose of effluent treatment (Berruga *et al.*, 1997). The immobilized cells may have high productivity even in plain whey permeate because of the high cell density and the reduced growth requirements (Yang & Silva, 1995). González-Siso (1996) described an interesting alternative method which involved the hydrolysis of lactose by β-galactosidase produced from another microorganism and subsequent fermentation by *Saccharomyces cerevisia*. This process can be developed in two steps or in only one step with mixed cultures or with the enzyme and yeast co-immobilized. Gonzáles-Siso (1996) illustrated that β-galactosidase co-immobilization seems to be a promising alternative for industrial batch fermentation of cheese whey lactose into ethanol. *S. cerevisiae* has been co-immobilized with the enzyme β-galactosidase in order to convert deproteinized cheese whey during batch fermentation to ethanol. The disadvantages of this method are the high price of β-galactosidase and the failure of this enzyme to hydrolyze all the lactose. Ward *et al.* (1995) described an alternative approach to the use of mixed cultures by using ethanol-producing yeast *K. marxianus* to produce ethanol at 45°C on starch-containing media supplemented with amylase obtained from the thermophilic fungus *Talaromyces emersonii*. The mixed cultures were capable of ethanol and enzyme production in the same fermentation vessel at 45°C.

Table 3: Examples of different chemical amendments used to enhance fermentation processes.

Chemical amendments	STRAINS	SUBSTRATES	POTENTIAL EFFECTS	REFERENCES
Element: Cu, Fe, Mn, Zn; Vitamins: nicotinamide, folic acid, riboflavin, thiamine	*Candida utilis*	Whey	Improving biomass production with accumulated contents of fats and lipids	Abu-Hamid, (1994)
Yeast extract; Ergosterol	i- *Saccharomyces frgilis* ii- *Kluyveromyces frgilis*	i-Whey reconstituted from acid whey powder ii-Lactose in sweet whey	i- Pronounced effects on ethanol and mass production ii- Improving ethanol production by whey-supplemented media, and maintaining pH	Chen & Zall, (1982) Zertuche & Zall, (1985)
Vitamins: biotin, thiamine HCl Ca-pantothenate, nicotinic acid, myoisositol, para-aminobenzoic acid, pyridoxin HCl	*Kluyveromyces lactis*	Glucose	Regulation of alcoholic ferment-ation under aerobic conditions in batch-chemostat culture	Kiers *et al.*, (1998)
Yeast extract; $(NH_4)_2SO_4$, K_2HPO_4	*Candida pseudotropicalis*	Cheese whey	i-Yeast extract achieved high ethanol production, ii-$(NH_4)_2SO_4$ & K_2HPO_4 at high concentrations initiated cell growth and reduce lactose consumption	Ghaly & El-Taweel, (1995 b)
Yeast extract; $(NH_4)_2SO_4$; KH_2PO_4, Fatty acids	*Kluyveromyces marxianus IMB3*	Molasses	Increased ethanol yield by means of optimizing media known as (Simplex Optimization Method)	Gough *et al.*, (1996)
Solid & soluble coal; Humic acid; Yeast extract	*Saccharomyces cerevisiae; Zygosaccharomyces rouxii*	Dextrose	i-Chelation effects. ii-Doubled ethanol/biomass production	Polman *et al.*, (1996)
Mn^{2+}	*Kluyveromyces marxianus IMB3*	Lactose	Increased efficiency of the conversion of lactose to ethanol at 45°C	Brady *et al.*, (1995)
Urea; Yeast extract	*Kluyveromyces fragilis; Kluyveromyces lactis*	Whey permeate	Improved ethanol production at optimal fermentation conditions	Zayed & Foley, (1987)
$MnCl_2$; Electrical field	*Kluyveromyces marxianus IMB3*	Lactose	Doubled ethanol production at 45oC supported by electrical field	Simpson *et al.*, (1995)
Surfactants: Tween 20, 60, 80; Na lauryl sulphate; triton X100; caprylic acid	Methanogenic bacteria	Mixture of: whey, poultry waste and cattle dung	Improved aerobic digestion of the mixture and methane content	Desai & Madamwar, (1994)
Soya flour; Yeast cell walls; Glycine; Yeast extract	*Saccharomyces cerevisiae*	Glucose	The ethanol productivity was enhanced by 50%	Bafrncova *et al.*, (1999)
Thermostable fungal β-galacto-Sidase	*Kluyveromyces marxianus IMB3*	Malt extract agar	Increased substrate access to the biocatalytic unit at 45°C	Barron *et al.*, (1994)
Soya flour	*Saccharomyces cerevisiae*	Beet molasses	Increased the final concentration and reduced fermentation times	Li X., (1995)
Tween 80; Linoleic acid	*Saccharomyces uvarum; Saccharomyces cervisiae*	Wort; Glucose	Enhanced the excretion of ethanol from yeast cell	Panchal & Stewart (1981)
Nisin	*Kluyveromyces marxianus Y113*	Whey permeate; Sulphuric acid casein	i-Have no adverse effects on the fermentation performance of the commercial yeast strains. ii-Effective agent for controlling natural bacterial population present in whey during distillery	Mawson & Costar, (1993)
$CaCO_3$; $CaCl_2$; NaCl	Free cell of *Zymomonas mobilis* (ZM_4; mutant ZMI_2)	Glucose; Sucrose	Improved ethanol production in high sugar concentrations at 45°C	Sreekkumar & Basappa, (1992)

2.11 Major by-products of whey fermentation

2.11.1 Ethanol

Ethanol production from agricultural products has been in practice for the past 80 years. In recent years, ethanol production from renewable sources has emerged as an alternative clean fuel in comparison to fossil fuel that emits a large amount of greenhouse gases to the atmosphere (Kumar *et al.*, 1998). Bio-ethanol may be obtained from different bio-mass sources including agricultural by-products and food processing waste which contains starch, sugar or cellulose (Kumar *et al.*, 1998). Bio-ethanol can be used as a flexible transportation fuel due to its positive characters such as its high solubility in water and biodegradability. It is also relatively low in toxicity (McMillan, 1997).

Ethanol production from whey lactose can be an effective waste treatment process by means of reducing its BOD value and generating credit by-product sales (Ferrari *et al.*, 1994). Ethanol and CO_2 which are used in various applications such as beverage production, or as fuel, or as an industrial solvent are manufactured from whey on a large industrial scale in some countries (Mawson, 1994; Spreer, 1998).

The economic viability of obtaining ethanolic fuel by yeast fermentation, particularly in warm climates, is hindered by the high energy input requirement in order to maintain operating temperature between 25°C and 35°C. Temperatures above this range will result in thermal inactivation of the fermenting yeast strains employed in the system. To overcome such a problem, thermotolerant strains of ethanol-producing yeast are recommended (Fleming *et al.*, 1993). It is generally accepted that the inhibitory effect of ethanol does increase with the increasing temperature (Anderson *et al.*, 1986). Reductions in ethanol production at high temperature can be attributed to enhanced inhibition by the ethanol itself. The temperature dependence of

ethanol inhibition of the yeast has extensively been discussed by Hughes *et al.* (1984).

The addition of ethanol to the fermentation culture is found to be less toxic than the produced ethanol (Thatipamala *et al.*, 1992). Reasons proposed for this phenomenon are that the build up of toxic by-products, the depletion of nutrients and the intracellular accumulation of ethanol in the suspensions of fermenting yeast cells are less than or equal to those in the extra-cellular environment. There is an intracellular accumulation of ethanol during the early stages of yeast fermentation. As fermentation proceeds, both intracellular and extra-cellular ethanol concentrations become similar. In addition, increases in the osmotic pressure are associated with increased intracellular accumulation of ethanol (D'Amore *et al.*, 1988).

Ethanol concentration is clearly of major influence on the fermentation process. High concentrations usually decrease lactose utilization, the relative growth rate and the ethanol yield (Walker & O'Neill, 1990).

2.11.2 Single-cell protein (SCP) or biomass production

The term single-cell protein (SCP) refers to the dried cells of the microorganisms such as actinomycetes, algae, bacteria, yeast and molds grown in a large-scale culture systems (Mansour *et al.*, 1993). SCP is primarily used as an animal-feed supplement for cows and poultry. It is also used in human food and human dietetic nutrition such as sport diets and baby meal formulae with certain limitations (Harden, 1996). SCP has been commercially produced since the 1940s for the improvement of food and feeding industries by means of using fermenting-yeasts such as *Kluyveromyces* spp. e.g. (*K. marxianus, K. fragilis, K. lactis*), *Candida, Saccharomyces* and *Torulopsora* spp. (González-Siso, 1996).

Consumers attitude towards the use of single cell protein in food will have to be considered. Very little information exists on how consumers will react to SCP as a human food (Kosaric & Asher, 1985). Unfortunately, the

market demand for yeast SCP appears limited, the matter which discourages many cheese manufacturers to attempt such a process (Zall, 1979).

Despite the very attractive features of SCP as a nutrient for human, there are some problems that limit its uses in a global scale. For instance, the individual taste and customs make microorganisms less attractive as a food substance to many people.

The conversion of whey lactose to biomass can be achieved by a single-stage fermentation using a pure yeast strain or a mixture of cultures for the purpose of optimizing the utilization of all carbon source available during the fermentation process (Mawson, 1994).

Fermentation may take place directly in the farm using ultrafiltration (UF) process to concentrate the milk. Then fungi may be used to avoid excess nucleic acid content which would render the end product (SCP) toxic when it is used as animal feed (Yang & Silva, 1995). It is also possible to convert the carbohydrate of cheese whey into alcohol and SCP by means of culturing lactose with splitting yeast.

Depending on the quality of the whey used, it is sometimes necessary to add nitrogen and phosphorus sources. The processes of biomass production are operated at high temperature (>30° C) and low acidity (pH 3.0-3.5) so as to minimize contamination. In this way, pasteurization of the incoming whey is avoided (Mawson, 1994). Thermotolerant yeast strains such as *K. marxianus* are well adapted to work under these conditions. The biomass may be recovered by centrifugation, or it may be plasmolyzed by heating at 85°C and finally it is dried in cylinders or atomization towers (Mawson, 1994).

The biomass is rich in lysine. It contains 48-52% proteins, essential amino acids and B-group vitamins (González-Siso, 1996). The nutritional qualities of yeast are well recognized and are related to the amino acids, sterols, fatty acids and vitamins composition.

González- Siso (1996) reported that the whole whey was not

completely used in the process of producing SCP. The fermenting-yeast utilized and assimilated the carbon source provided by whey lactose leaving a considerable amount of unutilized whey proteins. These proteins can promote yeast flocculation which inhibits fermentation.

2.12 Methods used in whey fermentation

Whey can be fermented by four common different methods each of which has its own advantages and disadvantages. These methods are namely: batch, fed batch, continuous culture and extractive fermentation.

2.12.1 Batch culture

In this method, the reactor is filled with a sterile nutrient substrate and inoculated with the microorganism. The culture is allowed to grow until no more product is being made. The culture goes through a lag phase, exponential growth, stationary phase and death phase. Depending on what the product is, the useful part of the growth cycle can be anyone of these four stages. Usually it is the growth or stationary phase (Bains, 1993).

2.12.2 Fed-batch culture

The batch culture is fed with a batch of nutrients before it gets into stationary phase so as to secure a continuous nutrients supply (Bains, 1993). The reactor is filled until the volume of medium achieves the maximum capacity. While the substrate is feeding the reactor, the medium is constantly diluted. This is to avoid the increase in lactose concentration in the reactor and to allow a high biomass yields (Belem & Lee, 1998a). Fed-batch culture is a better alternative approach to ferment media which contain high lactose. It is used so as to prevent the catabolite repression of the respiratory enzyme synthesis due to the high sugar content (Ferrari *et al.*, 1994; Belem & Lee, 1998a).

Increase in lactose concentration during fermentation can eventually lead to the accumulation of pyruvate and promote greater glycolytic fluxes resulting in the reduction of the final biomass yield. Fed-batch fermentation

could reduce the pollution potential of highly concentrated whey. Belem & Lee (1998a) showd that more than 95% of lactose in the medium was consumed by *K. marxianus* during fed-batch fermentation.

2.12.3 Continuous culture

A continuous closed loop system for yeast aerobic fermentation was first described in 1977 to produce a single cell protein. This can be converted under anaerobic conditions to produce alcohol (Zall, 1979). Continuous fermentation has proven to be quite successful compared to batch fermentation. In continuous culture, the fermenter is fed continuously with nutrients and the culture medium is removed continuously. This has some advantages over fed-batch systems in that the culture conditions are always the same (Belem & Lee, 1998a).

Several researchers reported that the decreased number of yeast cells and viability during continuous fermentation is caused by the substrate and ethanol inhibition. Therefore, it is essential that yeast viability should remain high during the continuous fermentation of whey (Ghaly & El-Taweel, 1997).

2.12.4 Extractive fermentation

The accumulation of dissolved toxic products which are produced by the fermentation slows down and ultimately cease up the growth and the production of fermenting microorganisms. A logical approach to increase the fermenter productivity is to remove the inhibitory compounds as soon as they are formed rather than after the fermentation process ends (Minier & Goma, 1982; Kang *et al.*, 1990).

In alcoholic fermentation, the major limitation of the conventional process does come from ethanol inhibition (Minier & Goma, 1982; Kang *et al.*, 1990). Extractive fermentation provides a suitable solution to overcome such a problem. It is a technique based on circulating biocompatible and immiscible organic solvent through the fermentation vessel to remove any

inhibitory products. These products are then separated independently from the aqueous phase.

This technique has successfully been employed with *Saccharomyces cerevisiae, Zymomonas mobilis,* and *Clostridium acetobutylicum*. However, the extractive fermentation is applicable to cultures where the fermentation products can cause inhibition and/or repression on its own synthesis or affect the microbial growth (Minier & Goma, 1982). Jones *et al.* (1993) described the characteristics of the solvent used in the extractive fermentation as follows: low aqueous miscibility; low emulsion-forming tendencies; chemical and thermal stability under fermenter conditions and favorable qualities of product recovery.

2.13 Fermenting yeast

2.13.1 Yeast Taxonomy

The classification of yeast began in 1837 following the assignment of the genus named *Saccharomyces* to yeast. Barnett *et al.* (1984) reviewed the chief characteristics used to classify yeasts that based on the work of Van Rij done in 1964 where yeasts are classified in three classes of higher fungi. These classes are namely: Ascomycetes, Basidiomycetes and Deuteromycetes.

Most yeasts in nature belong to the classes Ascomycetes (sporogenous or ascosporogenus) and Fungi imperfecti (asporogenous). Only a few belongs to the class Basidiomycetes (Nagodawithana, 1994; Priest & Campbell, 1996).

2.14 Genus *Kluyveromyces*

Kluyveromyces belongs to Ascomycetous yeasts (ascosporogenus yeasts) that can reproduce sexually by means of ascospore formation, or asexually by means of multilateral budding and fusion. It is composed of both homo- and heterothallic species with spherical or ellipsoidal ascospores and homothallic species producing kidney-shaped ascospores. Ascus formation is generally preceded by isogamic or heterogamic conjugation. Large numbers of the ascospores occur in the asci of the strains of *Kluyveromyces* (Belem &

Lee, 1998b). Asci may be multi-spored, each with 1-60 spherical, oval or reniform ascospores (Barnett *et al.,* 1984) as in *K. polysporus.* Sporulation process of *Kluyveromyces* species, in particular the early liberation of mature ascospores, was judged to be sufficiently distinctive to justify a separate genus in the previous classification of yeast (Priest & Campbell, 1996). *Kluyveromyces* species are considered among the most beneficial yeast of economical and industrial value (Table 4). The most important application of the different *Kluyveromyces* strains is in the fermentation industry, particularly whey. Thus, *Kluyveromyces* are well known as whey-fermenting yeast.

The history of *Kluyveromyces* taxonomy is characterized by a series of interesting developments which are rooted from the creation of the genus. They are then prompted by the discovery of *K. polysporus* by Van der Walt in 1956. In 1965, several species were included and were considered as members of *Saccharomyces sensu* in 1970 (Lachance, 1998). *Kluyveromyces* is conveniently divided into three major groups (Lachance, 1998) designated as group A, B and C.

Group A comprises *Kluyveromyces africanus, K. bacillisporus, K. blattae, K. delphensis, K. lodderae, K. phaffii, K. polysporus* and *K. yarrowii.* Whilst, Group B comprises *K. aestuarii, K.dobzhanskii, K. lactis, K. marxianus* and *K. wickerhamii* and Group C comprises *K. thermotolerans* and *K. waltii.*

2.14.1 Kluyveromyces marxianus

K. marxianus was traditionally found in kefir grain. Kefir is an alcoholic acid milk drink made from the milk of cow, goat, and sheep which is mainly consumed in Russia. Thus, *K. marxianus* is currently considered as an efficient milk fermenting yeast which is formerly named *Kluyveromyces fragilis.* However, according to the classification of Van Rij, *K. marxianus* and *K. frgilis* have been merged as a single species *K. marxianus var. marxianus* (Belem & Lee, 1998b).

The definition of Van der Walt (1970) to *K. marxianus* was altered to reflect the ability of certain species to hybridize. Following that view, *K. marxianus* encompassed several varieties of taxa among which recognized herein as distinct species were *K. dobzhanskii* and *K. lactis*. However, the current name of *K. marxianus* has been given by Hansen & Van der Walt in 1971 (Lachance, 1998). The species definition used takes into account hybridization ability in combination with genetic structure of the genus population. This is because species delineation in *K. marxianus* has been controversial (Lachance, 1998).

Hack & Marchant (1998b) also defined *K. marxianus* as a respiratory yeast. It grows at 37°C-40°C as subglobulose, elipsoidal to cylindrical shaped cells (2.0-5.5 x 3.5-10) μm and pseudomycelium is normally formed. Only little amounts of glucose can be converted into ethanol under aerobic conditions. The available informations on physiological properties, isoenzyme distribution, ecological affinities and nuclear genome have clearly indicated that *K.marxianus* is evolutionarily a distinct taxa (Lachance, 1998). Important known technological characteristics of *K. marxianuse* are the production of B1, B2 and B12 vitamins, carbon dioxide and esters. Sugars which are subject to fermentation by *K. marxianus* are glucose, galactose, sucrose, lactose, and raffinose.

K. marxianus and *K. lactis* are closely related strains. They differ from each other only in that *K. marxianus* can grow in a wide range of temperature (Belem & Lee, 1998b).

2.14.2 Ethanol and heat tolerance of Kluyveromyces

Members of the genus *Kluyveromyces* are generally more thermotolerant than the species of *Saccharomyces* and *Candida* (Fleming *et al.*, 1993).

Banat *et al.* (1996) described the capability of *K. marxianus* to grow at 49°C. The strain *K. marxianus* IMB3 has shown thermotolerance ability more

Table 4: By-products produced by *Kluyveromyces marxianus* from raw materials.

RAW MATERIALS	BY-PRODUCTS	REFERENCES
Cellulosic biomass	i- β-Galactosidases ii- Ethanol	i- Barron *et al.*, (1995). ii- Bothast & Saha, (1997); Barron *et al.*, (1994).
Jerusalem artichoke	Ethanol	Margaritis & Bajpai, (1982).
MyFM medium	Ethanol	Hack & Marchant, (1998a).
Molasses	Ethanol	Li, (1995); Gough *et al.*, (1996).
Straw	Ethanol	Boyle *et al.*, (1997).
Sugarcane (syrup or molasses)	Ethanol	Anderson *et al.*, (1986).
Whey	i- β-Galactosidaes ii- Ethanol iii- Glycerol iv- Oligopeptides v- Oligosaccharides vi- Ribonucleotides vii- Yoghurt	i- Bojorge *et al.*, (1999). ii- Marwaha & Kennedy, (1984); Tin & Mawson, (1993). iii- Jenq *et al.*, (1989); Rapin *et al.*, (1994). iv- Belem *et al.*, (1999). v- Belem & Lee, (1997a). vi- Belem *et al.*, (1997). vii- Penna *et al.*, (1997).

than other strains (Hack & Marchant, 1998a). This property allows *K. marxianus* to be used successfully in fermentation industry. Particularly in ethanol production, the matter which might compensate for its lower tolerance to ethanol compared to *S. cervisiae* (Lachance, 1998).

Margaritis & Bajpai (1982) investigated ethanol inhibition kinetics in *K. Marxianus*. They found that high initial ethanol concentration inhibited *K. marxianus* specific growth-rate but had no effect on the final ethanol concentration, cell yield, or sugar utilization. They also reported that ethanol tolerance level in *K. marxianus* was similar to that reported for *S. cerevisiae*. On the other hand, Rosa & Sá-Correia (1992) found lower ethanol tolerance in *K. marxianus* compared to that in *S. cerevisiae*. They correlated these findings to the activities of the plasma membrane enzyme ATPase. Lower ethanol tolerance in other closed strains such as *K. fragilis* has also been reported to be due to the inhibition of the carbon source transport system (Banat *et al.*, 1998).

CHAPTER III
Materials & Methods

Chapter Three

MATERIALS & METHODS

3.1 Materials

3.1.1 Soil samples

Soil samples used in this study were collected from three different sites in November 1998. The sites were near Al-Ain dairy farm factory, Al-Markhania and Al-Buraimi areas. Soil samples were collected from four different locations within each site from a depth of up to 10 cm. The samples were bulked and mixed to ensure uniformity and then passed through a 1 cm mesh sieve to remove stones and were stored in seallable plastic bags at 5°C. Three sub-samples were used as replicates for each soil.

3.1.2 Whey samples

Samples of cheese whey were obtained from Al-Ain dairy farm factory, Al-Ain, UAE. Each batch was collected in a 500 ml sterile bottle. The samples were stored at –20°C in order to reduce microbial and enzymatic degradation.

3.1.3 Media & Chemicals

***Yeast malt extract agar (YM agar)* (Hägler *et al.*, 1993)**

Yeast extract (Oxoid)	3.00 g
Peptone (Oxoid)	5.00 g
Malt extract (Difco)	3.00 g
Glucose (ADH)	10.00 g
Distilled water	1000 ml
Chloramphenicol (Sigma)	250 mg dissolved in 10 ml of 95% ethyl alcohol
pH	5.50
Agar (ADH)	20.00 g

Yeast malt extract broth (YM Broth) **(Hägler *et al.*, 1993)**

Yeast extract (Oxoid)	3.00 g
Peptone (Oxoid)	5.00 g
Malt extract (Difco)	3.00 g
Glucose (ADH)	10.00 g
Distilled water	1000 ml
Chloramphenicol (Sigma)	250 mg dissolved in 10 ml of 95% ethyl alcohol
pH	5.50

Corn-meal Tween 80 agar **(Koneman *et al.*, 1979)**

Cornmeal (Difco)	17.00 g
Tween 80 (Sigma)	3.00 ml
Distilled water	1000 ml
Agar (ADH)	3.00 g

2% Glucose-yeast extract peptone broth **(Wickerham, 1951)**

Glucose (ADH)	20.00 g
Peptone (Oxoid)	10.00 g
Yeast extract (Oxoid)	5.00 g
Distilled water	1000 ml
pH	6.00

Endospore stain **(Johnson & Case, 1989)**

Malachite green	5%
Safranin	0.5%

***Rose-bengal chloramphenicol agar* (Oxoid)**

Mycological peptone	5.00 g
Glucose	10.00 g
Dipotassium phosphate	1.00 g
Magnesium sulphate	0.50 g
Rose bengal	0.05 g
Chloramphenicol (Sigma)	250 mg dissolved in 10 ml of 95% ethyl alcohol
Distilled water	1000 ml
pH	7.20
Agar (ADH)	20.00 g

***Yeast nitrogen base liquid assimilation medium* (Wickerham, 1951)**

Yeast nitrogen base (Difco)	6.70 g
Distilled water	100 ml

***Fermentation basal medium* (Wickerham, 1951)**

Yeast extract (Oxoid)	4.50 g
Peptone (Oxoid)	7.50 g
Distilled water	1000 ml

Whey broth (WB)

Whey	1000 ml
pH	6.62

Whey agar (WA)

Whey	1000 ml
pH	6.62
Agar (ADH)	20.00 g

***Vitamin-free yeast base medium* (Wickerham, 1951)**

Ammonium sulphate	5.00 g
Dextrose (ADH)	10.00 g
Potassium phosphate dibasic	0.15 g
Magnesium sulphate	0.50 g
Sodium chloride	0.10 g
Calcium chloride	0.10 g
L-Histidine monohydrochloride	10.00 mg
DL-Methionine	20.00 mg
DL-Tryptophane	20.00 mg
Boric acid	500 µg
Copper sulphate	40.00 µg
Potassium iodide	100 µg
Ferric chloride	200 µg
Manganese sulphate	400 µg
Sodium molybdate	200 µg
Zinc sulphate	400 µg
Potassium phosphate monobasic	0.85 g
Distilled water	1000 ml
Agar (ADH)	16.70 g

***2% Glucose-yeast extract peptone agar* (Wickerham, 1951)**

Glucose (ADH)	20.00 g
Peptone (Oxoid)	10.00 g
Yeast extract (Oxoid)	5.00 g
Distilled water	1000 ml
pH	6.00
Agar (ADH)	20.00 g

Yeast infusion **(Lodder & Kreger-Van Rij, 1952)**

Compressed yeast	200 g
Tap water	1000 ml

60% glucose –yeast extract agar **(Wickerham, 1951)**

Glucose (ADH)	60.00 g
Yeast infusion	40 ml
Distilled water	100 ml
Agar (ADH)	3.00 g

Basal medium with 1% NaCl **(Lodder & Kreger-Van Rij, 1952)**

Powdered yeast extract (Oxoid)	5.00 g
Glucose (ADH)	20.00 g
Peptone (Oxoid)	10.00 g
Sodium chloride	10.00 g
Distilled water	1000 ml

Glucose-chalk agar **(Van der Walt & Yarrow, 1984)**

Glucose (ADH)	50.00 g
Chalk ($CaCO_3$, light precipitate)	5.00 g
Yeast infusion	1000 ml
Agar (ADH)	20.00 g

Arbutin agar **(Lodder & Kreger-Van Rij, 1952)**

Arbutin	0.50 g
Yeast infusion	100 ml
Sterile 1% ferric ammonium citrate solution.	2-3 drops
Agar (ADH)	2.00 g

***Urea hydrolysis agar* (Van der Walt & Yarrow, 1984)**

Peptone (Oxoid)	1.00 g
Glucose (ADH)	1.00 g
Sodium chloride	5.00 g
Potassium dihydrogen phosphate (ADH)	2.00 g
Phenol red	0.012 g
Distilled water	1000 ml
Filter sterilized urea solution (20%)	0.5 ml
pH	6.80
Agar (ADH)	20.00 g

***Gorodkowa agar* (Phaff *et al.*, 1978)**

Glucose (ADH)	2.50 g
Meat extract	10.00 g
Sodium chloride	5.00 g
Distilled water	1000 ml
Agar (ADH)	20.00 g

***50% glucose –yeast extract agar* (Wickerham, 1951)**

Glucose (ADH)	50.00 g
Yeast infusion	50 ml
Distilled water	100 ml
Agar (ADH)	3.00 g

***Malt extract gelatin* (Wickerham, 1951)**

Powdered malt extract (Difco)	100 g
Gelatin (ADH)	120 g
Distilled water	1000 ml

***Yeast carbon base liquid assimilation medium* (Wickerham, 1951)**

Yeast carbon base (Difco)	11.70 g
N-source:	
Potassium nitrate or	0.78 g
Sodium nitrite or	0.26 g
Ethylamine hydrochloride	0.64 g
Distilled water	1000 ml

***Ascospore agar* (Koneman *et al.*, 1979)**

Potassium acetate (ADH)	10.00 g
Yeast extract (Oxoid)	2.50 g
Dextrose (Oxoid)	1.00 g
Distilled water	1000 ml
Agar (ADH)	20.00 g

3.2 Methods

3.2.1 Soil characteristics

Soil pH was measured in a mixture of one part soil to five parts 0.01 M $CaCl_2$, and saturation paste extracts were used to measure electrical conductivity and soluble cations (Rayment & Paulitz, 1992).

Soluble cations in the saturation paste extracts were extracted as described by Carter (1986) and measured by inductively coupled plasma atomic emission spectrometry (ICPAES) as described by Zarcinas *et al.* (1987).

Organic carbon; available phosphorus and potassium; extractable sulphur; ammonium and nitrate nitrogen and reactive iron were measured as described by Walkely & Black (1934), Colwell (1965), Blair *et al.* (1991), Searle (1984), and Tamm (1922), respectively.

3.2.2 Whey analysis

Prior analysis, samples of whey were kept at room temperature for 24 hrs so as to allow complete thawing.

3.2.2.1 Whey components

Measurement of fat, protein, lactose, total solids and the non-fatty solids contents in crude whey were performed by using FTIR (Fourier Transfer Infrared) spectrometer Milko-scan (FT. 120; Foss electric; Denmark). The samples were analyzed directly without sample preparation.

3.2.2.2 Acidity

The acidity of whey (as lactic acid) was measured by titrating 5 ml sample with 0.1 M sodium hydroxide where 1 ml 0.1M NaOH is equal to (0.009008 g) Lactic acid. Phenolphthalein was used as indicator. The volume of 0.1 M NaOH used in titration was 0.60 ml. The acidity was calculated using the following equation

$$\frac{\text{Volume of NaOH (ml)} \times 0.009008 \times 1}{\text{Volume of sample (ml)}}$$

3.2.2.3 Salt content

The salt content as sodium chloride, was measured volumetrically by titrating 2 ml sample with 0.1 N silver nitrate (NaCl equivalent weight is equal to 58.44). Potassium chromate (5%) was used as indicator. The volume of $AgNO_3$ used was 0.80 ml.

Salt content was calculated using the following equation:

$$\frac{\text{Volume of } AgNO_3 \text{ (ml)} \times 0.1 \times 58.44 \times 100}{\text{Volume of sample (ml)} \times 1000}$$

3.2.2.4 pH value

The pH value was measured by a CD 7400 pH-meter; Brand WPA; England.

3.2.3 Isolation of yeast from soil

Two methods were carried out in order to isolate yeast fungi from freshly sampled soils.

3.2.3.1 Standard isolation method

The yeast population of the three freshly sampled soils were estimated using the soil dilution plate method (Johnson & Curl, 1972). The soils were air dried for 4 days at 28°C to reduce the number of viable vegetative bacterial cells (Williams *et al.*, 1972). Three 10 g replicates of each soil were dispensed into 100 ml of sterile 0.1 % (w/v) agar (Gibco BRL, Paisley, Scotland) solution in de-ionized water containing 20 g glass beads (3 mm diameter). The soil suspension was shaken using gyratory shaker (Model G76, New Brunswick Scientific-Edison, N.J., U.S.A.) at 200 rpm at 28°C for 1 hr. After shaking, the soil suspension was placed in an ultrasonic cleaner (model: B-221, 185 warr, Branson Cleaning Equipment Company, U.S.A.) at a frequency of 55,000 cycles s^{-1} for 20 sec. Ten-fold dilutions were made in sterile de-ionized water and 0.2 ml were spread with a sterile glass rod over the surface of the yeast malt agar medium (YM) supplemented with 250 mg L^{-1}

chloramphenicol (Sigma). Ten plates were used per dilution. The plates were dried in a laminar flow cabinet for 30 min and then incubated at 28°C ± 2. Yeast colonies were counted after 4 days and were expressed as colony forming units (cfu) g dry^{-1} soil.

All colonies were then transferred onto YM agar plates and stored in 10% glycerol (cryoprotectant) at –20°C (Schaefer *et al.*, 1985).

3.2.3.2 Enrichment isolation method

Autoclaved whey broth was used as enrichment isolation medium. Five grams of soil samples were transferred into 250 ml Erlenmeyer flasks containing 100 ml whey broth and incubated at 28°C ± 2 on a rotary shaker at 280 rpm for 48 hrs.

Ten-fold dilutions were made in sterile de-ionized water and 0.2 ml were spread with a sterile bent-glass rod over the surface of whey agar plates. Ten plates were used per dilution. The plates were dried in a laminar flow cabinet for 30 min and then incubated at 28°C ± 2 and colony count was carried out after 4 days. Yeast colonies were expressed as colony forming units (cfu) g dry^{-1} soil as described above.

3.2.4 Preliminary fermentation test

All yeast isolates obtained from section 3.2.3 were preliminary screened for their ability to ferment whey and to produce ethanol using whey broth as a medium. Aliquots (10 ml) of the whey broth were pipetted into sterile cotton-plugged (150 × 12 mm) tubes carrying Durham tubes of (50 × 6 mm) and the tubes were autoclaved for 15 min at 15 lbs overpressure. Yeast cultures (48 hrs) were suspended in 4.5 ml sterile water and 0.1 ml of this suspension was inoculated into the autoclaved whey. The tubes were incubated at 28°C ± 2 for 72 hrs. The fermentation was observed by the presence of gas in the Durham tubes. Only three powerful yeast isolates were choosen for further study and were classified as a strongly fermentative isolates. These isolates were

stored in 10% glycerol at –20°C and in YM slant at 4°C until required.

3.2.5 Selection of the best ethanol producing isolates

Quantitative whey fermentation was conducted for each of the three isolates selected in section 3.2.4 in 22 ml screw top hole capped clear vials with PTFE/Silicone septa (USA), containing 20 ml of 100 % whey of 4.95% lactose concentration and pH of 6.62. Yeast cultures (48 hrs) were suspended in 4.5 ml sterile water and 0.1 ml of this suspension was inoculated into the autoclaved whey. The tubes were incubated at 28°C ± 2 for 72 hrs and they were regularly shaken. The ethanol content yielded by each isolate was measured according to the procedure described below in section 3.2.6.

3.2.6 Determination of ethanol production

Ethanol concentrations in the fermentation experiment were determined after 3 days of incubation (section 3.2.5) by using Chrom-pack model CP-9001 gas chromatograph GC; Holland equipped with a 1.5 m long OD ¼ in, ID 2 mm glass column, packed with chromosorb W.AW. The injector temperature was 175°C. Oven temperature was 85°C and nitrogen was employed as a carrier gas. The flow rate of the carrier gas was 180 kpa. The combustion gases were hydrogen and air. The gas chromatograph output signal was connected to a computer. Samples of 1 µl size were injected using a 1 µl syringe. Ethanol concentrations in the samples were determined from a calibration curve of known standard solutions.

3.2.7 Identification of yeast isolates

From data obtained in section 3.2.5 and 3.2.6, only one isolate (number 20) was choosen for further study. This isolate produced the maximum ethanol and it was identified by using two methods:

1- The kit identification system for yeast identification (3.2.7.1).

2- The morphological (3.2.7.2), cultural (3.2.7.3), sexual (3.2.7.4) and

physiological (3.2.7.5) characteristics recommended by Barnett *et al.* (1984).

The identification was then confirmed by the National Collections of Yeast Cultures (NCYC, UK). The yeast isolate, which has been sent to the NCYC for confirming the identification, was stored in a slant of YM agar.

3.2.7.1 Yeast kit identification

Yeast isolate number (20) was identified using ID 32C identification kit system (BioMerieux, Marcy-l'Etoile, France) using a standardized and miniaturized assimilation tests with a specially adapted database.

3.2.7.1.1 Preparation of the inoculum

Several identical colonies were removed from the culture plate and a suspension with turbidity equivalent to 2 McFarland was obtained in 2 ml of sterile distilled water. Aliquots (250 µl) of the preceding suspension was inoculated into C Medium.

3.2.7.1.2 Inoculation of the strip

The strip was inoculated by distributing 135 µl of the C Medium. The electronic pipette was used to inoculate the yeast suspension from C Medium in each cupule.

3.2.7.1.3 Strip Reading

Reading of the strip was carried out after 48 hrs using the automatic ATB reader. The reader detected growth in each cupule and the results were sent to a computer for identification.

3.2.7.1.4 Strip identification

Results were transmitted to the computer and interpreted by the corresponding identification software.

3.2.7.1.5 Composition of C Medium

C Medium is composed of ammonium sulfate (5 g), mono-potassium phosphate (0.31 g), Dipotassium phosphate (0.45 g), disodium phosphate (0.92 g), sodium chloride (0.1 g), calcium

chloride (0.05 g), magnesium sulfate (0.2 g), histidine (0.005 g), trytophane (0.02 g), methionine (0.02 g), agar (0.5 g), trace elements (10 ml) in 1 liter of water and the final pH was adjusted to 6.5-6.7.

3.2.7.2 Morphological characteristics

3.2.7.2.1 Formation of pseudodomycelium & true mycelium

Slide culture technique was used for the detection of pseudodomycelium and true mycelium formation. A petri plate containing a U-shaped glass-rod support on which two glass slides were placed, was sterilized by dry heat at 180°C for 3 hrs. Corn-meal tween 80 agar was melted and poured into a second petri plate. The glass slides were quickly removed from the glass rod with a flame-sterilized pair of tweezers and dipped into the agar. They were replaced on the glass-rod support. After solidification of the agar on the slides, the yeast isolate was very lightly inoculated in three lines along each slide and a sterile coverslip was placed over a part of the lines. Some sterile water was poured into the petri plate to prevent the agar from drying out. The culture was then incubated at 25°C for 4 to 5 days. The slides were taken out of the petri plate and the agar was wiped off the back of the slide. The areas of the inoculation lines under and around the coverslip were studied and microphotographs were made using an olympus BH-2 microscope (Olympus optical co., Ltd, Tokyo, Japan).

3.2.7.2.2 Formation of ballistospores

Liquified corn-meal agar (10 ml) was poured into a petri plate. After setting, the plate was inoculated with the yeast isolate along two diameters at right angles. The plate was inverted over another petri plate bottom containing malt agar on which a sterile slide was placed. One of the lines was positioned over the slide and the two plates were taped together along the entire circumference. A piece of moistened sterile cotton was placed inside to raise the humidity. Incubation was performed

at 18-20°C. The discharged spores, if present, germinate to form colonies on the bottom of the plate and then collected on sterile glass slide for direct microscopy examination.

3.2.7.3 Cultural characteristics

3.2.7.3.1 Growth in liquid medium

The appearance of the growth in 30 ml quantities of 2% glucose-yeast extract-peptone broth in Erlenmeyer flasks after 2-3 days growth at 28°C was recorded. The flasks were kept at room temperature and the growth was again examined 2 and 4 weeks after inoculation.

3.2.7.3.2 Growth on solid medium

The appearance of the streak culture on 2% glucose-yeast extract-peptone agar was recorded after 2-3 days cultivation at 28°C and again after 2 and 4 weeks at 28°C. The isolate was also described after 2-3 days cultivation on Rose- bengal chloramphenicol agar at 28°C.

3.2.7.4 Sexual characteristics

3.2.7.4.1 Characteristics of asci and ascospores

The isolate was first brought to the active growth state by subculturing on yeast malt extract agar (YM) for 2 days at 28°C. The sporulation media (ascospore agar) were lightly inoculated with the isolate and then incubated at 25°C for 3 days before being examined microscopically for the first time. Materials that have not sporulated were then maintained at room temperature and examined at weekly intervals for at least 4-6 weeks. Ascospore formation was verified by staining the heat fixed preparations.

3.2.7.5 Physiological characteristics

3.2.7.5.1 Fermentitive utilization of carbon compounds

Aliquots (2 ml) of the fermentation basal medium were pipetted into sterile cotton-plugged (150 × 12 mm) tubes containing Durham tubes of (50×6 mm) and the tubes were autoclaved for 15 min at 15 lbs overpressure. After sterilization 1 ml of various aqueous membrane filter-sterilized sugar solutions

were added aseptically to the tubes each at a time. Aqueous solutions (6%) of the sugars were prepared except for raffinose where 12% was employed. These sugars included (D- glucose, D-galactose, sucrose, maltose, cellobiose, α-trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch and methyl α- D glucopyranoside). The yeast culture (48 hrs) was suspended in 4.5 ml of sterile water and 0.1 ml of this suspension was inoculated into the fermentation media. The tubes were incubated at 28°C and the fermentation was observed by the presence of gas in the Durham tubes and by changing in the colour of the bromothymol blue indicators over a period of 24 days (Van der Walt, 1970).

3.2.7.5.2 Oxidative utilization of carbon compounds

For the carbon assimilation test, only high grade standardized products were used before proceeding with the test. The yeast isolate was first brought to an active growth state by transferring it on YM agar at 28°C for 3 days. Cell suspensions were made up with 3 ml sterile distilled water in a 16 mm tube. Yeast nitrogen base (6.7 g) was dissolved with 1 g of one of the following carbohydrates separately (D-glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, α-trehalose, lactose, melibiose, raffinose, melezitose, L-arabinose, D-arabinose, D-ribose and L-rhamnose) in 100 ml distilled water. This solution was sterilized by filteration (47 cm diameter, 0.45 μm pore size, Millipore, Australia. Pty limited, N.S.W., Australia). One ml aliquots of this medium was pipetted in 9 ml amounts of sterile distilled water in 16 mm cotton plugged tubes. Tubes were incubated at 28°C after inoculating with 0.4 ml of the cell suspensions and observations were made at weekly intervals over 4 weeks. The degree to which the various carbon compounds were utilized was determined by placing the tubes which had been vigorously shaken to disperse all yeast growth, against a white card bearing lines drawn with black Indian ink. When the growth in the tubes completely obliterated the lines, it was recorded as (+ + +). When the lines

appeared as diffused bands, the growth was rated as (+ +). When the bands were distinguishable as such but have indistinct edges it was recorded as (+). The absence of growth was indicated as (-). A (+ + +) or (++) reaction within 4 weeks was considered to be positive and a (+) reaction as very weak or negative.

3.2.7.5.3 Assimilation of nitrogen compounds

The medium was prepared by dissolving 11.7 g yeast carbon base (Difco), together with requisite amount of the nitrogen source in 100 ml distilled water. Where the nitrogen source was a nitrate, 0.78 g potassium nitrate was used. While when it was a nitrite, 0.26 g sodium nitrite was used and where it was primary amino-nitrogen, 0.64 g ethylamine hydrochloride was used. The medium was first filter-sterilized and then aseptically distributed in 0.5 ml amounts into 4.5 ml amounts of sterile distilled water in 16 mm cotton-plugged tubes which have been autoclaved for 15 min at 15 lbs overpressure. Blank tubes were prepared by adding 0.5 ml amounts of filter-sterilized basal solution. The medium has been shaken thoroughly before use. The nitrogen assimilation test tubes were incubated at 28°C for 4 weeks after inoculating with 0.4 ml of cell suspensions. A second tube was inoculated with one loopful from the original one. Growth was detected by eye observation. When the second tube showed a (+ +) to (+ + +) reaction, this has been taken as that the yeast isolate was capable of utilizing the nitrogen source. When a (+) reaction was scored, the yeast was considered to be incapable of utilizing the nitrogen source.

3.2.7.5.4 Splitting of arbutin

A slant of arbutin agar was inoculated with an actively growing culture of the yeast isolate and was incubated at 28°C. An uninoculated tube served as control. When arbutin was splitted, a dark brown color developed in the agar within 2-7 days.

3.2.7.5.5 Growth in vitamin-free medium

The test solution was prepared by pipetting aseptically 0.5 ml of the vitamin free yeast base solution into 4.5 ml sterile water in 16 mm cotton plugged tubes. The media were mixed by shaking and were inoculated with cell suspensions in sterile distilled water and the tubes were incubated at 28°C for 7 days. A second vitamin free tube was inoculated from the first tube and incubated at 28°C for 7 days. Yeast isolate which gave a (+ + +) reaction in the second tube was considered as capable of synthesizing all the vitamins while tubes which scored (+) or less were considered as incapable of synthesizing all vitamins for growth.

3.2.7.5.6 Growth on media of high osmotic pressure

The growth of the tested isolate was examined in 50% and 60% (w/w) glucose-yeast extract agar. Actively growing cultures were inoculated as a streak on autoclaved 50% and 60% glucose agar, dispensed in 5-6 ml aliquots in 16 mm cotton plugged tubes. The tubes were incubated at 28°C for 4 weeks and were examined for growth.

3.2.7.5.7 Sodium chloride tolerance test

The test was performed in sets of cotton plugged tubes containing 5 ml of basal medium with 1% (w/v) sodium chloride increments to give a range of concentrations varying from 0% to 10%. The tubes were inoculated and incubated at 25°C on a rotary shaker. The lowest concentration of sodium chloride at which no visible growth after 3 days has occurred, was recorded as the limit of sodium chloride tolerance.

3.2.7.5.8 Growth at elevated temperature

3.2.7.5.8.1 Growth at 37°C

The growth at 37°C was tested by the method described by Kreger-Van Rij, (1964). Yeast isolate was inoculated on YM agar and the plates were incubated at 37°C for 2-4 days.

3.2.7.5.8.2 Maximum growth temperature

Tubes containing glucose yeast extract peptone broth were inoculated with the yeast isolate. The inoculated tubes were incubated in water baths operated at temperature range between 30 to 50 with temperature differences of 1°C. The maximum temperature for growth was located between the lowest temperature at which no growth after 7 days had occurred and the next lowest temperature (Van der Walt & Yarrow, 1984).

3.2.7.5.9 Acid production

The yeast isolate was inoculated onto slants of glucose-chalk agar and then incubated at 28°C. Acid production was reported as positive when sufficient amount of acid has been formed to clarify the opaque medium. If not, the reaction was considered as weakly positive (Van der Walt, 1970).

3.2.7.5.10 Production of extracellular amyloid compounds

After 3-4 weeks, carbon assimilation test cultures which initially contained sugars, were examined for the presence of amyloid compounds or starch. One drop of diluted Lugol's iodine solution was added per tube and the tube-contents were shaken. Amyloid materials showed color reaction varying in intensity from blue to purple or green (Van der Walt, 1970).

3.2.7.5.11 Hydrolysis of urea

The yeast isolate was inoculated onto urea agar (Van der Walt & Yarrow, 1984) and incubated at 25°C. Cultures were observed daily up to 5 days. The reactions were recorded as positive after the appearance of deep pink colour (Van der Walt, 1970). The medium was prepared by dissolving 1.5 g of urea agar in 90 ml distilled water which was sterilized by autoclaving at 115°C for 15-20 min at 15 lbs overpressure and cooled to 50-55°C. After autoclaving, 0.5 ml of a 40% filter-sterilized urea

solution was added to the cooled urea agar medium under aseptic conditions. The content was mixed thoroughly and 10 ml were distributed aseptically in sterile cotton-plugged tubes as described by Van der Walt, (1970).

3.2.7.5.12 Fat splitting or lipolytic activity

Fat from fresh beef suet was melted out and then filtered and sterilized by autoclaving. Aliquot (0.5 ml) of sterile fat was pipetted into sterile warm Petri plate which was tilted to ensure a uniform layer on the bottom. The plate was then placed in the fridge for 2 hrs. A sterile Gorodkowa agar (18-20 ml) (Phaff *et al.*, 1978), containing 0.1% chalk was melted and cooled to 40°C and then carefully layered over the fat. The medium was then left to set. The plate was inoculated and incubated at 25°C. The result was taken after few days by the appearance of opaque deposit of the calcium salt formed by the liberated fatty acids under the inoculation streak (Van der Walt, 1970).

3.2.7.5.13 Ester production

The liquid medium was prepared by dissolving 5% (w/v) glucose in yeast infusion. After dispensing in cotton-plugged flask, the medium was autoclaved for 15 minutes at 15 lbs overpressure. A plate culture of the yeast isolate was inoculated into 50 ml Erlenmeyer flask containing 20 ml of 5% glucose solution in a yeast infusion as described by Van der Walt & Yarrow, (1984). The flask was incubated for 3-5 days at 25°C. Ester production was detected by odour (Van der Walt, 1970).

3.2.7.5.14 Cycloheximide (actidione) resistance

Ten milligrams cycloheximide (Sigma) were dissolved in 90 ml distilled water and then sterilized by filtration. The filtered sterilized solution (4.5 ml) was dispensed into sterile 16 mm cotton plugged tubes in each of which 0.5 ml of tenfold concentrated yeast-nitrogen based was added. After inoculation, the tubes were incubated at 28°C and were

regularly shaken and observed weekly for 3 weeks. Yeast cultures which gave growth reaction of (+ +) or (+ + +) within 7 days, were taken to be highly resistant to cycloheximide (Van der Walt, 1970).

3.2.7.5.15 Gelatin liquefaction

A mixture of 100 g powdered malt extract and 120 g gelatin was dissolved in 1 liter distilled water over moderate heating, dispensed in tubes and sterilized by autoclaving for 15 min at 10 lbs overpressure. The melting and solidification temperature of the medium ranged between 25-27°C. The liquefaction medium was tested in the tubes and the medium was then incubated at 18°C. The test was recorded positive when the gelatin was transformed from solid or gel state to the liquid state.

3.2.8 Optimization of fermentation conditions for ethanol production

With the aim of investigating the effect of different lactose concentrations on the fermenting ability of yeast isolate (number 20), samples of full whey (100%) which is equivalent to lactose concentration of 4.95 %, water diluted whey (50%, 25%, 12.50%, and 6.25%) which are equivalent to lactose concentrations of 2.50%, 1.24%, 0.62% and 0.31% respectively, have been studied. This is in addition to three samples of full whey (4.95% lactose concentration) supplemented with 1.53 g/l, 3 g/l and 6.1 g/l powdered lactose. These three samples were analyzed for total lactose concentration according to the method in (section 3.2.2.1) and the results obtained indicated concentrations of 6.48%, 7.95% and 11.05% lactose respectively.

All the above mentioned samples, were inoculated with 1 ml of yeast inoculum and the tubes were incubated at 30, 35, 40, 45 and 50°C for 3 days. Four replicates were used for each sample.

The lactose content in all these samples was measured as described in section 3.2.2.1. Ethanol production was also carried out for all samples as described in section 3.2.6.

3.2.9 Addition of different chemical amendments

To increase the yield of fermentation and alcohol production using the yeast isolate number (20), 50% whey was used as a substrate and different amendments were employed. These amendments used were Tween 80 (300 µl/1L), magnesium sulphate $MgSO_4$ (0.6 g/1L), dipotassium hydrogen phosphate K_2HPO_4 (3 g/1L), urea (0.5 g/1L), peptone (2 & 12 g/1L), potassium dihydrogen phosphate KH_2PO_4 (0.3 g/1L), yeast extract (2 & 12 g/1L), beef extract (2 & 12 g/1L), linoleic acid (10 ml/1L), malt extract (2 g/1L), manganese chloride $MnCl_2$ (1 g/1L), calcium carbonate $CaCO_3$ (2 g/1L), ammonium sulphate $(NH_4)_2SO_4$ (0.5 g/1L), mesoinositol (1 g/1L), thiamin (0.001 g/1L), aminobenzoic acid (0.002 g/1L), pantothenate (0.001 g/1L), pyridoxin-HCl (0.001 g/1L), nicotinic acid (0.005 g/1L) and riboflavin (0.001 g/1L). All media were autoclaved at 121°C for 15 min except mesoinositol, thiamin, aminobenzoic acid, pantothenate, pyridoxin-HCl, nicotinic acid, and riboflavin. Those were sterilized by membrane filtration (0.2 µm). Four amended 20 ml (50% whey) replicates were used for each amendment. The tubes were then incubated at 40°C for 3 days. Ethanol concentrations were determined according to the procedure described in section 3.2.6.

3.2.10 COD analysis

This analysis was carried out to measure the amount of oxygen that is equivalent to the organic material present in a whey sample. An open reflux method (Clesceri *et al.*, 1998) was used in this determination where a known amount of potassium dichromate ($K_2Cr_2O_7$) was digested and the unreacted $K_2Cr_2O_7$ was then titrated with ferrous ammonium sulphate, FAS, $[Fe(NH_4)_2(SO_4)_2.6H_2O]$. The oxidizable matter was then calculated in terms of oxygen equivalent. Therefore, this test is based on the fact that all organic compounds can be oxidized with a strong oxidizing agent under acid conditions. The glassware and equipment required in this determination were

reflux apparatus, pipettes (class A with wide-bore), burette (25 ml) and a blender.

For measuring COD of whey sample, 25 ml of 0.04167 M $K_2Cr_2O_7$ was added to a known volume of sample (5 ml) and mixed, followed by the addition of 5 ml $HgSO_4$ solution. Sulphuric acid (70 ml) reagent was then carefully added and mixed well. The refluxing flask was attached to the condenser. The open-end of the condenser was covered with a small beaker to prevent any potential contamination from entering the refluxing mixture. The mixture was refluxed for 2 hrs with continuous cooling by water. The reflux condenser was disconnected to dilute the mixture to about twice its volume with distilled water. The mixture was then cooled down to room temperature and the excess $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate (FAS) using Ferroin (2 to 3 drops) as indicator. The colour change from blue-green to reddish-brown was considered as the endpoint.

The COD in mg O_2/L was calculated using the following formula:

$$\text{COD as mg } O_2/L = \frac{(A-B) \times M \times 8000}{\text{mL sample before dilution.}}$$

where:

A= mL of FAS used for blank

B= mL of FAS used for sample

M= molarity of FAS

8000= milliequivalent weight of oxygen x 1000 mL/L

3.2.11 Statistical analysis

Analysis of variance was carried out using superanova (Abacus Concepts, Inc., Berkeley, California, U.S.A.) to evaluate the effect of different temperatures, whey and lactose concentrations and the addition of different chemical amendments on the rate of ethanol production. Significant differences between means were determined by Duncan's New Multiple Range Test at $p = 0.05$.

CHAPTER IV
Results & Discussion

Chapter Four

RESULTS & DISCUSSION

The main goal of this investigation was the isolation of strongly active fermenting yeast isolate from the local soils. The isolated strains could be used to produce valuable products, such as ethanol, from sweet whey locally produced in Al-Ain dairy farm. Optimization of the fermentation conditions (whey concentration, temperature and the addition of lactose and chemical amendments) in order to achieve the maximum ethanol production was also one of the major concerns of this study.

Al-Ain area was the investigated area of the present study. It is located in the eastern part of Abu-Dhabi Emirate, 140 km east of Abu-Dhabi, the capital of the United Arab Emirates. Al-Ain area is considered as the largest and the most ancient oasis of the Arabian Peninsula which is endowed by plentiful supply of fresh groundwater derived from northern Oman mountains (Al-Shamsei, 1993). Although it is located in the arid belt of the world, Al-Ain provides the most favorable area in the UAE for agricultural and related activities. This is enhanced by the rich fresh water and fertile soil.

The soil samples were collected from two different locations of Al-Ain area (Al-Ain Dairy Farm & Al-Markhania) and a neighbouring area across the borders of the city of Al-Ain (Al-Buraimi). The choice of the three locations was based on the apparent differences in the soil nature and ecology. This was an attempt of having a wider chance in obtaining the most suitable yeast isolate which satisfies the aims of this study.

4.1 Soil Analysis

The soil samples were analyzed for the purpose of characterization of the inorganic elements and organic residues (section 3.2.1). The chemical and physical characteristics of the three soils are summarized in (Table 5).

Table 5: General characteristics of the three soil samples collected from Al-Ain dairy farm, Al-Markhania and from Al-Buraimi area in November 1998.

Chemical characteristics	Al-Ain Dairy Farm	Al-Markhania	Buraimi
Texture	Sandy	Sandy	Loamy
Colour	Brown	Brown	Greenish brown
pH level ($CaCl_2$)	7.800	7.400	7.100
Conductivity (dS m^{-1})	0.1500	0.4960	1.0740
Organic carbon (%)	0.310	0.620	1.130
Reactive Iron (mg kg^{-1})	129.000	150.000	878.000
Sulphur (mg kg^{-1})	21.500	52.400	243.000
Potassium (mg kg^{-1})	230.000	670.000	152.000
Phosphorus (mg kg^{-1})	96.000	243.000	184.000
Ammonium nitrogen (mg kg^{-1})	26.000	2.000	5.000
Nitrate nitrogen (mg kg^{-1})	8.000	73.000	97.000
Chloride (mg kg^{-1})	0.025	0.008	0.000
Sodium oxide (meq $100g^{-1}$)	0.565	0.856	0.823
Magnesium oxide (meq $100g^{-1}$)	17.735	5.844	4.570
Calcium oxide (meq $100g^{-1}$)	21.810	22.116	20.800

The results indicated that Al-Ain Dairy Farm soil was rich in magnesium and ammonium nitrogen. It is known that ammonium nitrogen is subject to volatilization or fixation in the soils. Its accumulation also depends on the microorganisms requirement of nitrogen for growth. Ammonium nitrogen is released during decomposition of soil organic matter (Paul & Clark, 1989).

Al-Markhania soil was rich in potassium and phosphorus. Continuous addition of phosphorous fertilizer or animal manure cause enrichment in the soil phosphorous, carbon and nitrogen while Al-Buraimi soil was found to be rich in nitrate nitrogen, organic carbon, reactive iron and sulphur.

The soil matrix adsorption of the microbial enzymes is known to raise the pH to higher values. Therefore the pH measurement is considered to be an important criteria for predicting the capability of soils to support microbial reactions (Paul & Clark, 1989). The pH values for the three soil samples subject of this study, were found to be in the neutral to slightly alkaline range. Yeast can readily grow at values of 7.8 and often even higher, but optimum growth is normally found somewhere in the range between pH 4.5 and 6.5 (Phaff *et al.*, 1978).

The soil texture, of the three samples, has been analyzed and the data presented in (Table 5). The result indicated that no great differences in the soil texture of the three studied samples. The texture is considered as an important factor affecting the occupancy of the organisms and their capability to move and graze in the soil microflora (Atlas & Bartha, 1993).

The highest soil conductivity was recorded in Al-Buraimi soil (1.0740 dS m^{-1}) the matter which is attributed to the high content of soil minerals. Soil sample collected from Al-Ain dairy farm contained 21-fold less reactive iron than that obtained from Al-Markhania. The slight increase of reactive iron in Al-Markhania is possibly due to the fact that Al-Markhania soil is irrigated with treated waste water. Such waters are normally characterized by

containing high metal levels. It has also been observed that the value of reactive iron in Al-Buraimi soil (878 mg kg^{-1}) is far more than that obtained in Al-Ain soils.

Low levels of metals are normally a characteristic of sandy soils. In such soils the mobile inorganic nutrients can easily be leached out of the surface soil environment or are lost with water movement through the soil (Prescott *et al.*, 1990). From nutrient content standpoint, Al-Markhania soil was found to be rich in organic residuals of fermented dates debris in addition to the phosphorus, which serve as a primary energy source for microbial oxidation, resulting in providing the most favourable organic environment for the growth and proliferation of microorganisms, whereas Al-Ain Dairy Farm soil can be classified as a poor nutrient contained soil (non-cultivated desert soil).

4.2 Analyses and Characteristics of Whey

The acidity and pH tests were performed so as to categorize the type of the whey sample used in this study. The data obtained classified the sample as sweet whey (Theoretical range of acidity: 0.10-0.20 & pH: 5.8-6.6). This goes in line with the fact that sweet whey is the main by-product of soft cheese. The chemical characteristics of the whey samples are summarized in (Table 6).

Acidity of whey sample as lactic acid (0.1%), has been done as described in section 3.2.2 and the pH (6.62) was measured as described in section 3.2.2.4. Some studies reported that the high pH value is a favourable factor for fermentation of whey lactose into ethanol (Tu *et al.*, 1985). The pH usually affects whey fermentation patterns mainly through the transport of lactose across the cell membrane of the fermenting yeast and through the permease enzyme which is then hydrolyzed by β-galactosidase into glucose and galactose. β-galactosidase does work well at pH 6.5. Many studies considered it as an optimum pH value and considered the faster fermentation processes as those carried out at high pH values (Tu *et al.*, 1985). The salt content of the

whey was measured (0.2%) and calculated as described in section 3.2.2.3. The value obtained for salt content was found to give the greatest effect on pH stabilization during whey fermentation to enhance ethanol production by using free cells of yeast strain *Zymomonas mobilis* (Sreekumar & Basappa, 1992). No similar studies were conducted using the yeast strain *Kluyveromyces marxianus*.

The amount of lactose in the whey sample was found to be at the higher range (Theoretical range: 4.5-5.0%), while the fat content was low (Theoretical range: 0.4-0.5%) (Table 6). This could be attributed to the low fat content in the cheese milk. Thus, decreaming is not required to utilize such quality whey in industry.

4.3 Yeast Isolation

Using the standard isolation techqniue, Al-Markhania soil had a significantly ($p \leq 0.05$) higher number of colony forming units (cfu) than Al-Buraimi and Al-Ain Dairy farm soils (Table 7). This may be explained on the basis of organic matter content of the soil.

Al-Markhania soil was collected from underneath the date trees. The location was full of dates and was high in sugar content. Anderson & Watson (1986) isolated wild yeast strains from sugar cane mill which is rich in glucose and cane syrup. The isolated yeast was identified as *K. marxianus*. In general, sugar is the best energy source for yeasts (Frazier & Westhoff, 1995).

By applying the whey enrichment method, also Al-Markhania soil showed a significantly ($p \leq 0.05$) higher number of colony forming units (cfu) than the other two soils, but the number was much less than those obtained by the standard isolation method (Figure 2-A & 2-B). This can be explained on the basis of selective growth on whey medium. In the standard isolation method, the carbon source was glucose which was shown to be easily assimilated by yeasts compared to lactose and this may explain the high number of yeasts on yeast malt glucose agar compared to whey agar.

Table 6: Chemical characteristics of whey collected from Al-Ain dairy farm factory.

Component	Mean value
Fat	**0.210%**
TS	**7.736%**
SNF	**7.520%**
Protein	**1.013%**
Lactose	**4.950%**
Salt (NaCl)	**0.200%**
Acidity	**0.100%**
pH	**6.620**

TS= Total Solids
SNF= Solids Non Fat

Table 7: The number of yeast colony forming unit isolated using standared and enrichment methods. Values are means of four replicates and values in parenthesis are the standard error of the means. Values with the same letter within a raw are not significantly different ($p > 0.05$) according to Duncan's New Multiple Range Test.

Soil sampling locations	**Colony counting /CFU***	
	Standared isolation method	**Enrichment isolation method**
Al-Markhania	29615000 ± (2.785 E6) *c*	31830.000 ± (705.668) *c*
Al-Buraimi	22500000 ± (1.19 E6) *b*	22755.000 ± (283.622) *b*
Al-Ain dairy farm	65750.000 ± (48094.308) *a*	01475.000 ± (131.498) *a*

*CFU= Colony Forming Unit

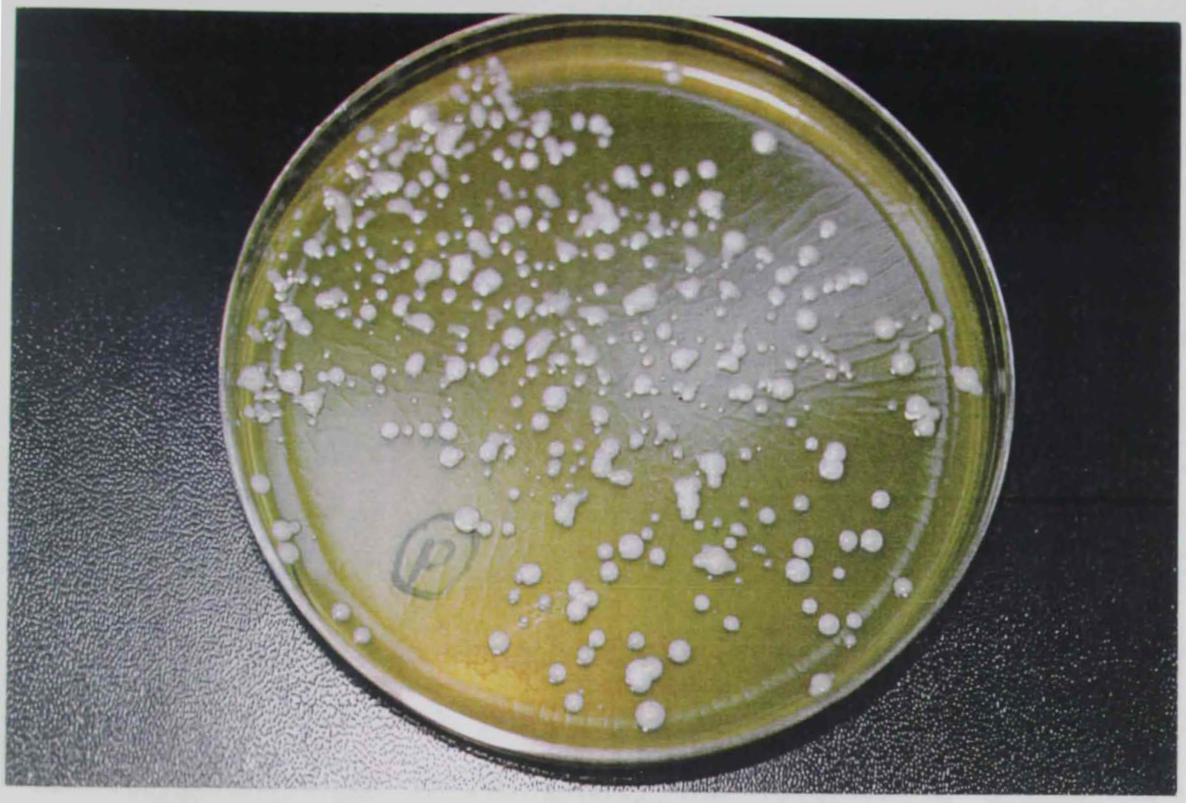

Figure 2 (A): Culture on yeast malt agar (YM) after 4 days incubation at 28°C showing total count of yeast isolated from Al-Markhania area by using the normal method of isolation.

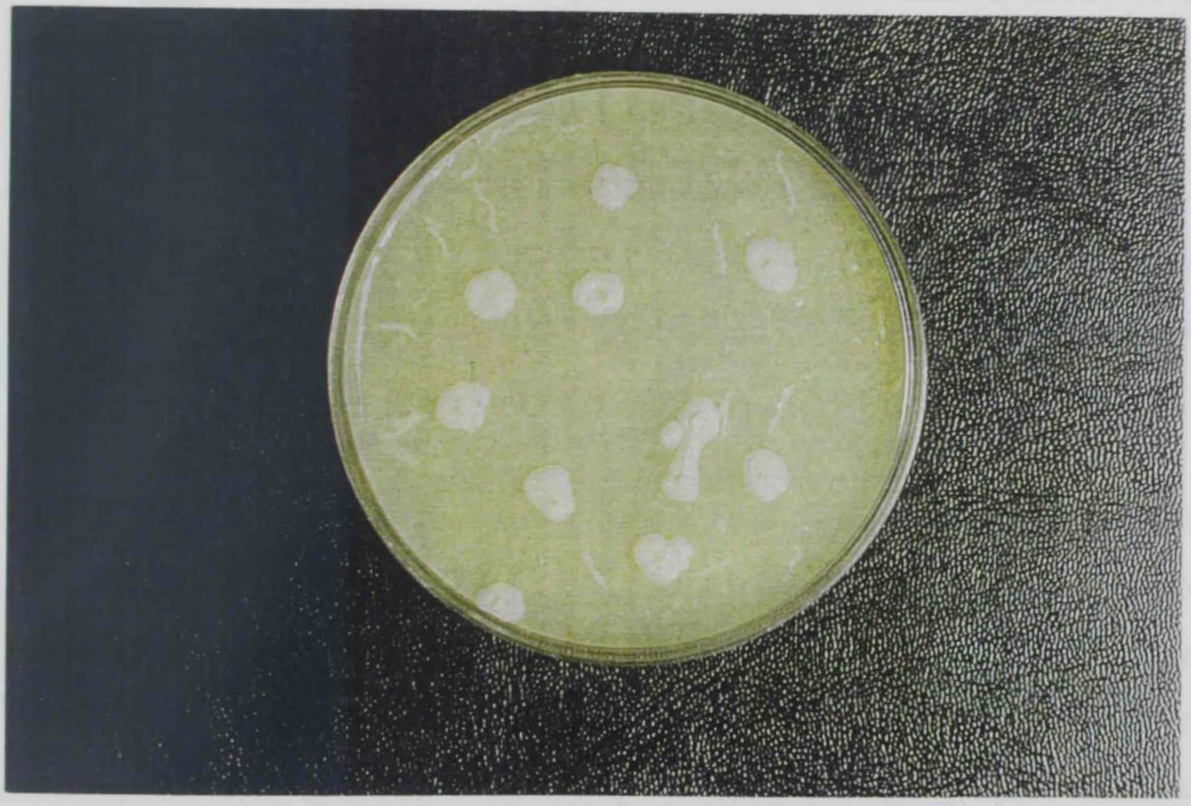

Figure 2 (B): Culture on whey agar (YA) after 4 days incubation at 28°C showing total count of yeast isolated from Al-Markhania area by using the normal method of isolation.

The enrichment and the standard isolation methods used in the present study resulted in isolating thirty nine yeast isolates, from Al-Markhania, Al-Buraimi and Al-Ain dairy farm soils. All isolates were preliminary screened, as described in section (3.2.4) with the aim of testing their fermentation ability on whey broth. The results indicated that yeast isolates number (2, 14, and 20), which were isolated from Al-Markhania soil, have shown a strong fermentation ability on whey broth (Table 8). They yielded the maximum gas in the Durhum tubes.

A further study was conducted for the isolates number (2, 14, and 20) to select the highest ethanol producing strain from whey fermentation. The method described in section (3.2.5) was designed as a step for the chromatographic method which was employed in the measurements of the exact amounts of ethanol produced by each isolate (section 3.2.6).

The results gave a clear comparison for the fermentative ability of the three whey-fermenting yeast isolates (Table 9) and (Figure 3). The highest ethanol concentration was achieved by the yeast isolate number 20 followed by isolates (14 and 2). Therefore, isolate number (20) was considered as the most suitable isolate for the purposes of this study. This was due to its high capability of growth in whey broth and its high fermentation ability on whey lactose.

4.4 Yeast Identification

Yeast isolate number 20 was primarily identified using the identification kit as described in section (3.2.7.1). The strip readings generated by automated system indicated that the strain was *Candida kefyr*. This is the imperfect state of *Kluyveromyces marxianus*, where the yeast can not form sexual ascospores and reproduce only by asexual blastospores. *Kluyveromyces marxianus* is the perfect state of *Candida kefyr* where it can reproduce by sexual ascospores as well as asexual blastospores. The automated kit system was not capable of distinguishing the appreciable differences between perfect and imperfect states

Table 8: The fermentation abilities of the isolated yeast strains from the three soil samples (Al-Markhania, Al-Buraimi, and near Al-Ain dairy farm).

Yeast #	Source	Whey broth The amount of gas in Durham tubes
1	Al-Markhania	+3
2	≈	+5
3	≈	+2
4	≈	+3
5	≈	+4
6	≈	+3
7	≈	—
8	≈	—
9	≈	+4
10	≈	—
11	≈	+3
12	≈	+2
13	≈	+3
14	≈	+5
15	≈	—
16	≈	—
17	≈	+3
18	≈	+2
19	≈	+2
20	≈	+5
21	≈	—
22	≈	—
23	Al-Buraimi	+3
24	≈	—
25	≈	+1
26	≈	+1
27	≈	+4
28	≈	+2
29	≈	+2
30	≈	+2
31	≈	+2
32	≈	+2
33	≈	+2
34	Al-Ain dairy farm	+1
35	≈	+3
36	≈	—
37	≈	+2
38	≈	+1
39	≈	+2

- = no fermentation; +1 = very weak fermentation, +2 = weak fermentation; +3 = mild fermentation; +4 = moderate fermentation; +5 = strong fermentation.

Table 9: Ethanol production by isolates 2, 14 and 20 isolated from Al-Markhania soil. Values are means of four replicates and values in parenthesis are the standard error of the means. Values with the same letter within a raw are not significantly different ($p > 0.05$) according to Duncan`s New Multiple Range Test.

Yeast isolates	Ethanol production %
2	3.432 ± (0.118) *a*
14	4.800 ± (0.324) *b*
20	5.093 ± (0.309) *b*

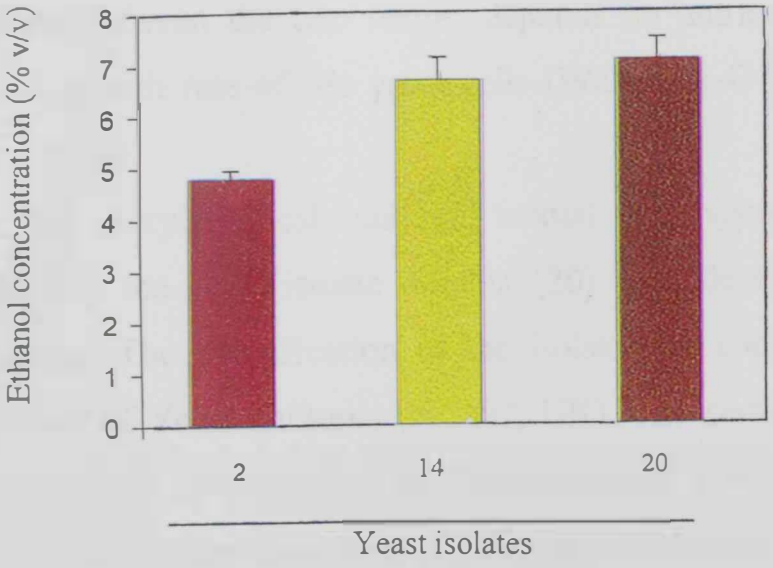

Figure 3: Ethanol production by the three fermentative yeast isolates (2, 14 and 20) after 3 days on whey broth medium incubated at 28°C. Ethanol content was measured by Gas chromatography. The bars represents the standard errors of the mean.

and therefore, the system is highly not recommended to identify yeast strains to species level (Chris, NCYC, personal communication) and should be confirmed by morphological, cultural, sexual and physiological criteria as described in sections (3.2.7.2, 3.2.7.3, 3.2.7.4, and 3.2.7.5). The morphological features of the yeast isolate (number 20) are characterized by an extensive, well developed regularly branched pseudomycelium with few oval blastospores under aerobic conditions. This morphology is considered as filamentous i.e. F-form as shown in (Figure 4-A & 4-B).

The growth of the yeast isolate number (20) in glucose-yeast extract-peptone broth as a liquid media was cream, non flocculent deposit with partial white ring after one week while the growth of the individual colonies on solid media such as glucose-yeast extract-peptone agar and Rose-bengal exhibited smooth cream pale beige slightly shiny colonies and smooth cream pink colonies, respectively (Figure 5-A & 5-B). Clear transformations between yeast-like budding mode (Y-form) (Figure 6) and filamentous mycelial mode (F-form) (Figure 4), were evidently obvious in isolate number (20). The transition between the two forms, depends on nutrients, oxygen availability and the growth rate of the yeast cells (Walker & O'Neill, 1990; O'Shea & Walsh, 1996).

From the morphological, cultural, sexual and physiological characteristics (Table 10), the yeast isolate number (20) was identified as *Kluyveromyces marxianus*. The identification of the isolate was confirmed by the National Collection of Yeast Cultures (NCYC, UK). The certificate issued by NCYC confirming the identification of *Kluyveromyces marxianus* is shown in page (70). In this confirmation step, NCYC gave a reference number to this specific isolate and it was registered as *Kluyveromyces marxianus* NCYC 2886. *Kluyveromyces marxianus* proved its ability to ferment a wide range of carbohydrates. Among the most important carbohydrates is whey lactose which is ultimately utilized to produce ethanol (Marwaha & Kennedy, 1984;

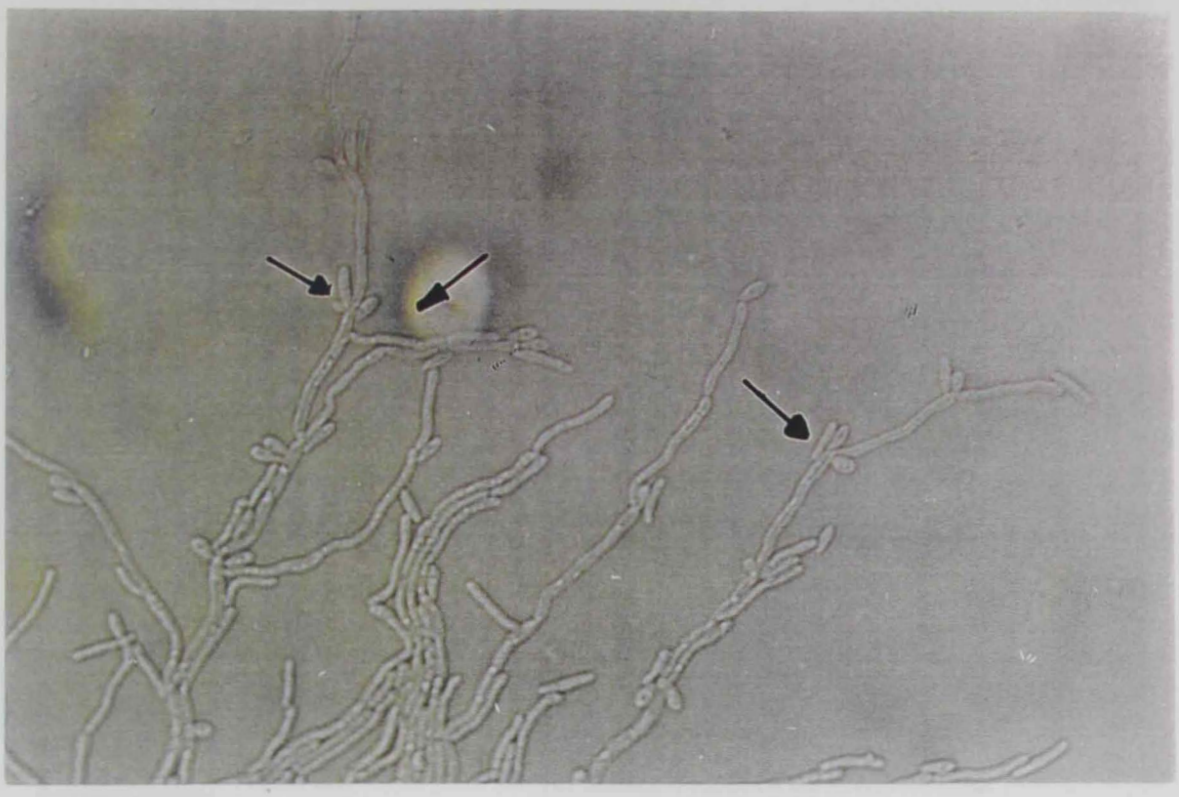

Figure 4 (A): Formation of branched pseudomycelia (arrows) with elongated cells when cultured on corn-meal agar at 25°C for 4 days.

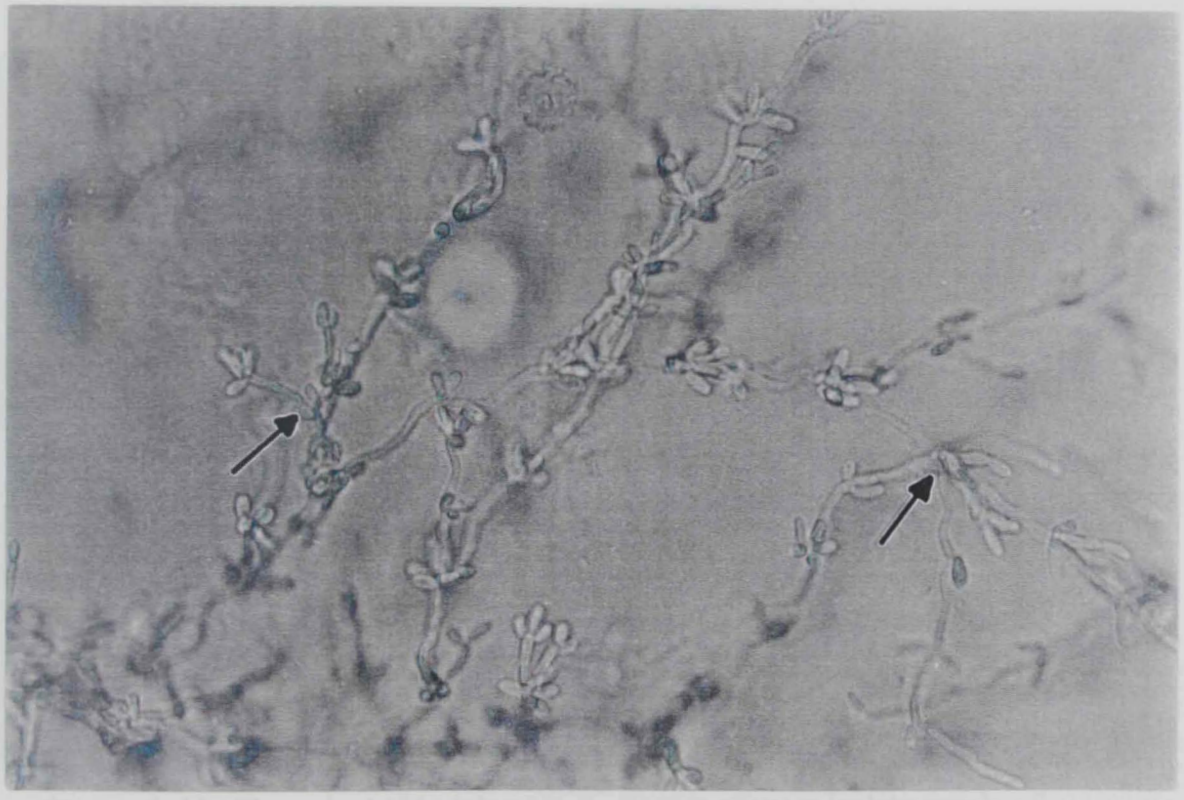

Figure 4 (B): Extensive, well developed regularly branched pseudomycelia (F-form) (arrows) with oval blastospores cultured at aerobic conditions on corn-meal at 25°C for 4 days.

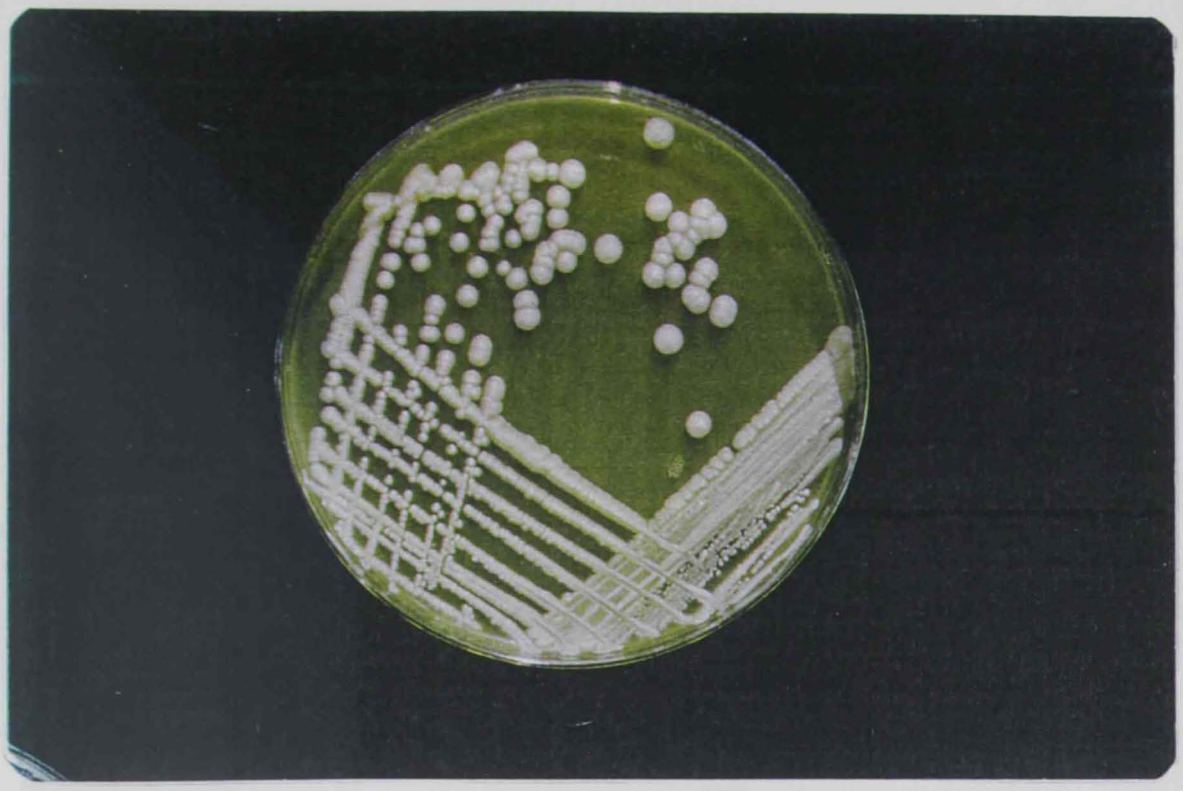

Figure 5 (A): Three days old pale beige, cream colonies of yeast isolate number 20 (*Kluyveromyces marxianus*) cultured on glucose-yeast extract-peptone agar at 25°C.

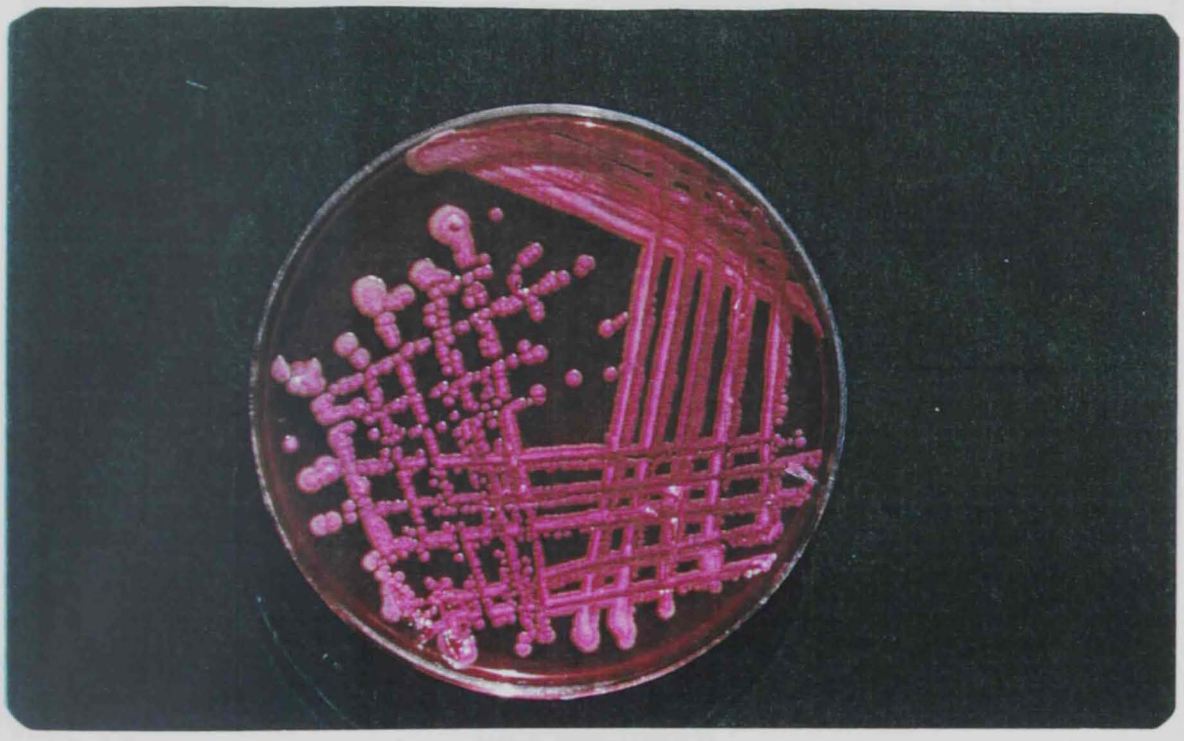

Figure 5 (B): Three days old pink, cream colonies of yeast isolate number 20 (*Kluyveromyces marxianus*) cultured on rose-bengal chloramphenicol agar at 25°C.

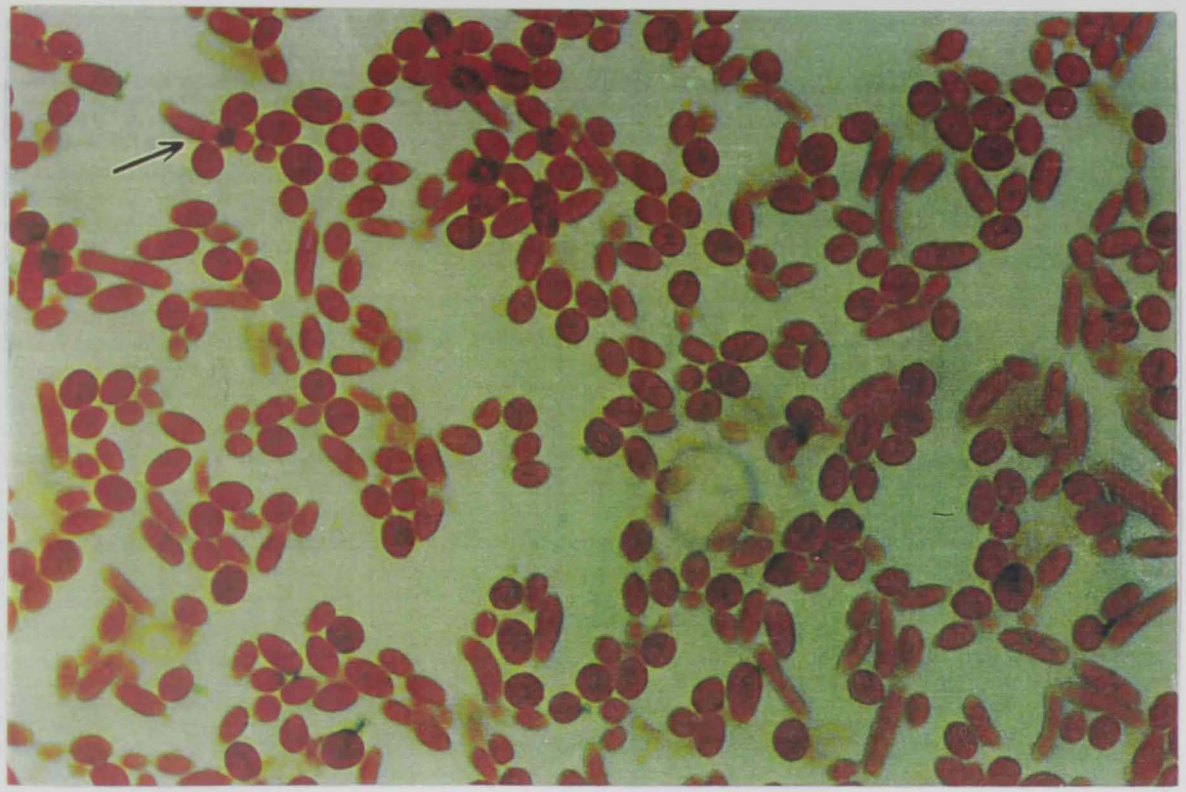

Figure 6: Oval to elongated cells (Y-form) of isolate number 20 (*Kluyveromyces marxianus*) occurring singly and in chains (arrows show multipolar budding).

Table 10: Characteristics of the genus *Kluyveromyces marxianus* (isolate number 20).

Characteristics	*Kluyveromyces marxianus*
Morphological & cultural Characteristics	
Budding cells	+
Splitting cells	-
Apical budding	-
Cell spherical, oval, cylindrical	+
Cells of other shapes	-
Filamentous	+
Pseudohyphae	
Sexual Characters	
Septate hyphae	-
Ascosporogenous	+
Ascospores oval, reniform/crescent	+
Ascospores hat or saturn	-
Ascospores of other shapes	-
Arthrospores	-
Ballistospores	-
Chlamydospores	-
Endospores	-
Fermentation of Carbohydrates	
D-Glucose	+
D-Galactose	+
Maltose	-
Methyl α-D-Glucopyranoside	-
Sucrose	+
α,α-Trehalose	-
Melibiose	-
Lactose	+
Cellobiose	-
Melezitose	-
Raffinose	+
Soluble starch	-
Inulin	-
Assimilation of Carbon Sources (growth in)	
D-Glucose	+
D-Galactose	+
L-Sorbose	-
D-Ribose	-
Xylose	+
L-Arabinose	+
D-Arabinose	-
L-Rhamnose	-
Sucrose	+
Maltose	-
α,α-Trehalose	-
Methyl α-D-Glucopyranoside	-
Cellobiose	+
Salicin	+
Melibiose	-
Lactose	+
Raffinose	+
Melezitose	-
Inulin	+
Soluble starch	-
Glycerol	+
Erythritol	-
Ribitol	+
Galactitol	-
D-Mannitol	+
D-Glucitol	+
mesoinositol	-

continued

Characteristics	*Kluyveromyces marxianus*
D-Glucono-1,5-lactone	+
Lactic acid	+
Succinic acid	+
Citric acid	+
Methanol	-
Ethanol	+
Ethylamine	+
D-Glucosamine	-
Xylitol	+
Cadaverine	+
L-Lysine	+
Assimilation of Nitrogen Compounds (growth in)	
Potassium nitrate	-
Ammonium sulphate	+
Growth in Vitamin-free Supplemented Media	
Growth sans vitamins	-
Growth sans insositol	+
Growth sans pantothenate	v
Growth sans biotin	-
Growth sans thiamin	+
Growth sans pyridoxine	v
Growth sans niacin	-
Growth sans folic acid	+
Growth sans PABA	+
Osmotic Ability to Grow in Glucose	
Growth in 50%	+
Growth in 60%	-
Growth at elevated temperature	
Growth at 37^0C	+
Growth at 40^0C	+
Cycloheximide Tolerance	
0.01% growth	+
0.1% growth	+
Production of Capsular Materials	
Pink colonies	-
Miscellaneous	
Urease activity	-
Salt tolerance (NaCl)	+
Starch formation	-
Acid production	-
Arbutin hydrolysis	+
1% Acetic acid growth	-
Lipolytic activity	-

Codes in table: + = positive; - = negative; v = variable

Continued

The certificate of *Kluyveromyces marxianus* identification issued by the National Collection of Yeast Cultures (NCYC).

NCYC no. 2886 Species KLUYVEROMYCES MARXIANUS Strain 20 In-House No. D3033

Deposited by MOZA AL-MUHAIRI, AL AIN, UNITED ARAB EMIRATES. Date AUGUST 1999

Isolated by MOZA AL-MUHAIRI

Source SOIL, UAE

Information IDENTIFICATION CONFIRMED BY 26S rDNA SEQUENCE ANALYSIS.

References

Morphology on YM medium

Broth:	Cells @ 48 h	CELLS OVAL TO ELONGATED, OCCURRING SINGLY & IN BRANCHED PSEUDOMYCELIAL TYPE CHAINS. MULTIPOLAR BUDDING. (3-6)×(5-18)μ.
	Culture @ 21 d	CREAM. NON-FLOCCULENT DEPOSIT. PARTIAL WHITE RING @ 1 WEEK.
Agar	Cells @ 48 h	CELLS OVAL TO ELONGATED, OCCURRING SINGLY & IN CHAINS. MULTIPOLAR BUDDING. (3-6)×(4-18)μ.
	Culture @ 21 d	CREAM/PALE BEIGE. SMOOTH. SLIGHTLY SHINY.

Pseudo/true Mycelium CMA: AEROBIC: EXTENSIVE FRINGES OF FAIRLY-WELL DEVELOPED, REGULARLY BRANCHED MYCOCANDIDA TYPE PSEUDOMYCELIUM. A FEW OVAL BLASTOSPORES.
ANAEROBIC: EXTENSIVE. WELL-DEVELOPED, REGULARLY BRANCHED MYCOCANDIDA TYPE PSEUDOMYCELIUM. NO BLASTOSPORES.
PDA: AEROBIC: AS CMA: ANAEROBIC.
ANAEROBIC: AS CMA: ANAEROBIC (IN PATCHES ONLY).

Ballistospores -VE

Arthrospores -VE

Endospores -VE

Sexual Spores @ 3 WEEKS

KAc: -VE

GORODKOWA: } 1-4 PER ASCUS. REINIFORM/CRESCENT SHAPED

	Negative	Positive
Fermentation		
D-Glucose	☐	☑
D-Galactose	☐	☑
Sucrose	☐	☑
Maltose	☑	☐
Cellobiose	☑	☐
α,α-Trehalose	☑	☐
Lactose	☐	☑
Melibiose	☑	☐
Raffinose	☐	☑
Melezitose	☑	☐
Inulin	☑ V. WEAK	☐
Soluble starch	☑	☐
Methyl α-D glucopyranoside	☑	☐
Assimilation		
D-Glucose	☐	☑
D-Galactose	☐	☑
L-Sorbose	☑	☐
Sucrose	☐	☑
Maltose	☑	☐
Cellobiose	☐	☑
α,α-Trehalose	☑	☐
Lactose	☐	☑
Melibiose	☑	☐
Raffinose	☐	☑
Melezitose	☑	☐
Inulin	☐	☑
Soluble starch	☑	☐
Xylose	☐	☑
L-Arabinose	☐	☑
D-Arabinose	☑	☐
D-Ribose	☑	☐
L-Rhamnose	☑	☐
Ethanol	☐	☑
Glycerol	☐	☑
Erythritol	☑	☐

	Negative	Positive
Ribitol	☐	☑
Galactitol	☑	☐
D-Mannitol	☐	☑
D-Glucitol	☐	☑
Methyl α-D-glucopyranoside	☑	☐
Salicin	☐	☑
Lactic acid	☐	☑
Succinic acid	☐	☑
Citric acid	☐	☑
myo-inositol	☑	☐
D-Glucono-1,5-lactone	☐	☑
D-Glucosamine	☑	☐
Methanol	☑	☐
Xylitol	☐	☑
Ammonium sulphate	☐	☑
Potassium nitrate	☑	☐
Ethylamine	☐	☑
Cadaverine	☐	☑
L-Lysine	☐	☑
Miscellaneous		
Vitamin free growth	☐	☐
0.01% cycloheximide growth	☐	☑
0.1% cycloheximide growth	☐	☑
50% glucose growth	☐	☑
60% glucose growth	☑	☐
1% Acetic acid growth	☑	☐
Lipolytic activity	☑	☐
Acid production	☑	☐
Growth at 37°C	☐	☑
Growth at 40°C	☐	☑
Arbutin hydrolysis	☐	☑
Urease activity	☑	☐
Starch production	☑	☐
Salt tolerance	☐ 10%	☑
Max. growth temperature		
Min. growth temperature		
Optimum growth temperature		

Tin & Mawson, 1993).

4.5 Optimization of Fermentation Conditions

With the aim of maximizing the optimum ethanol production from whey fermentation by the yeast isolate *K. marxianus* NCYC 2886 (isolate number 20), the method described in section 3.2.8.2 was applied.

4.5.1 Temperature

In this study, the ethanol production increased parallel with the elevation of temperature from 30°C to 40°C. This indicates that temperature control is a critical factor for maximum ethanol production. The optimum ethanol production from whey fermentation by *K. marxianus* was achieved at 40°C (Table 11) and (Figure 7). Therefore it could consequently be referred to as a thermotolerant whey fermenting yeast (Sree *et al.*, 2000). This temperature, coincides with that obtained by Banat *et al.* (1998), working on *K. marxianus*. Many workers reported that the optimum temperature for ethanol production from whey fermentation by yeasts such as *Candida pseudotropicalis*, is ranging between 30°C and 35°C (Zayed & Foley, 1987; Roukas & Lazarides, 1991).

Optimum ethanol production at high temperature is possibly due to the effect of temperature on shortening the log phase, the matter which allows to reach the maximum growth stage at a high rate (Banat *et al.*, 1998). In addition, the elevated temperature is thought to increase the fluidity in yeast membrane (D'Amore *et al.*, 1989). This helps in making the yeast to respond to high temperatures by means of changing their fatty acid composition.This in turn, leads to higher saturated esterified fatty acids in yeast cell membrane. This phenomenon is usually associated with a decrease in the amount of membrane phospholipids to maintain optimal membrane fluidity for cellular activities. This is considered as a part of an adaptive response (D'Amore *et al.*, 1989; Banat *et al.*, 1998).

Table 11: Ethanol production by *Kluyveromyces marxianus* NCYC 2886 (isolate number 20) at different lactose (whey) concentrations and temperatures. Values are means of four replicates and values in parenthesis are the standard error of the means.

Temp.	Treatment 1**	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7	Treatment 8
30°C	1.010 ± (0.014) *a* B#*	1.938 ± (0.031) *ab C*	3.705 ± (0.099) *c B*	5.280 ± (0.057) *d BC*	4.210 ± (0.217) *cd B*	3.268 ± (0.545) *bc AB*	4.350 ± (0.058) *cd BC*	3.138 ± (0.311) *bc B*
35°C	1.087 ± (0.049) *a C*	1.905 ± (0.018) *b C*	3.487 ± (0.070) *c B*	5.500 ± (0.309) *e BC*	4.400 ± (0.238) *d B*	4.410 ± (0.451) *d B*	2.928 ± (0.492) *c AB*	5.025 ± (0.400) *d BC*
40°C	1.055 ± (0.021) *a BC*	1.935 ± (0.010) *a C*	3.580 ± (0.053) *ab B*	7.650 ± (0.071) *c D*	5.827 ± (0.365) *b C*	9.835 ± (2.652) *d C*	9.29 ± (0.573) *d D*	6.595 ± (2.154) *bc C*
45°C	1.037 ± (0.005) *a BC*	1.763 ± (0.035) *ab B*	3.480 ± (0.106) *bc B*	5.700 ± (0.678) *cd B*	4.510 ± (0.330) *de C*	6.685 ± (1.132) *e BC*	6.780 ± (1.248) *e CD*	5.188 ± (0.109) *cde BC*
50°C	0.290 ± (0.004) *a A*	0.343 ± (0.014) *bcd A*	0.303 ± (0.006) *ab A*	0.333 ± (0.006) *bc A*	0.370 ± (0.028) *cd A*	0.330 ± (0.018) *abc A*	0.340 ± (0.004) *bcd A*	0.382 ± (0.009) *d A*

**Treatment (1): 6.25% whey = 0.31% lactose; Treatment (2): 12.50% whey = 0.62% lactose; Treatment (3): 25% whey = 1.24% lactose; Treatment (4) 50% whey = 2.50% lactose; Treatment (5): 100% full whey = 4.95% lactose; Treatment (6): 100% full whey + 1.53% lactose powder = 6.48% lactose; Treatment (7): 100% full whey + 3.00% lactose powder = 7.95% lactose; Treatment (8): 100% full whey + 6.1% lactose powder = 11.05% lactose.

*Values with the same lower case within a row are not significant (p> 0.05) different according to Duncan's New Multiple Range Test.

#Values with the same upper case within a column are not significant (p> 0.05) different according to Duncan's New Multiple Range Test.

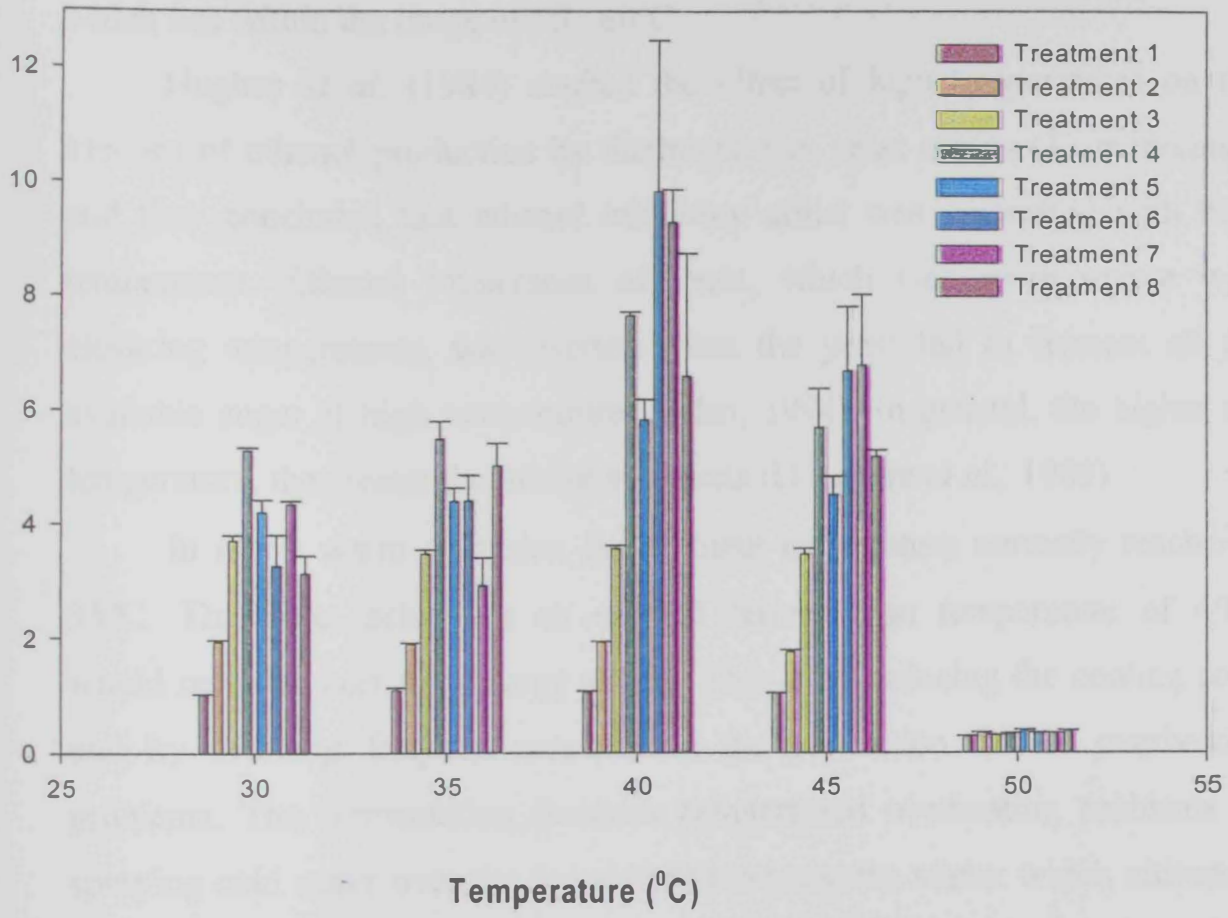

Figure (7): The effect of different concentrations of whey lactose as a carbon source and temperature range on the rate of ethanol production, by *Kluyveromyces marxianus* NCYC 2886 (isolate number 20). The bars represents the standard errors of the mean.

Treatment 1: 0.31% lactose concentration; Treatment 2: 0.62% lactose concentration; Treatment 3: 1.24% lactose concentration; Treatment 4: 2.50% lactose concentration; Treatment 5: 4.95% lactose concentration; Treatment 6: 6.48% lactose concentration; Treatment 7: 7.95% lactose concentration; Treatment 8: 11.05% lactose concentration.

The amount of ethanol yielded at temperatures higher than 40°C (45-50°C) was significantly reduced as shown in (Table 11) and (Figure 7). These results indicated the reduction in the fermentation rate at such high temperature. Such reduction can be explained by the inhibitory effect of the produced ethanol and/or to enzymatic activities, the optimal temperature of which lies within the range of 50 - 60°C.

Hughes *et al.* (1984) studied the effect of high temperatures on the kinetics of ethanol production by thermotolerant yeast strains (*K. marxianus*) and they concluded that ethanol inhibitory effect was associated with high temperature. Ethanol intolerance of yeast, which was more severe with elevating temperatures, was exerted when the yeast fail to ferment all the available sugar at high temperatures (Jelen, 1994). In general, the higher the temperature, the greater the inhibitory effects (D'Amore *et al.*, 1989).

In many warm countries, the ambient temperature normally reaches ≥ 35°C. Therefore, achieving an optimal fermentation temperature of 40°C would result in cost and energy saving. This is by reducing the cooling costs and by avoiding frequent cessation in the production due to overheating problems. The fermentation factories counteracted overheating problems by spraying cold water over the fermentation vessels, the matter which ultimately increase production cost (D'Amore *et al.*, 1989; Sree *et al.*, 2000). Therefore, it is rather more advantageous from economical and technical standpoints to operate the fermentors with the thermotolerant yeast, capable of fermenting whey at a reasonably high temperature such as 40°C for the process of ethanol production.

4.5.2 Whey Lactose Concentration

The optimal lactose concentration which is believed to enhance ethanol production at the optimum temperature (40°C) was found to be present in the 50% diluted whey (2.5% lactose) (Table 11) and (Figure 7). This means that the available raw material is doubled and this may add an economic value. It

has also been observed that the increase in lactose concentration from 2.5% to 4.95% non significantly decreased the ethanol yield and ultimately withstood lactose utilization. The result obtained in this study, can be classified as quite satisfactory in comparison to the results obtained by many researchers such as Schaefer *et al.* (1985); Grubb & Mawson (1993); and Domingues *et al.* (1999) who found the optimal concentration of lactose medium to range between 3.7 and 5%.

The inhibitory effect of high concentrations of lactose and the produced ethanol on fermenting yeast strains have been a major concern of fermentation researchers (Szczodrak *et al.*, 1997). Ghaly & El-Taweel (1995a) studied such effects on *Candida pseudotropicalis*. They found that high concentration of lactose had an inhibitory effect on the specific growth rate, lactose utilization and ethanol production rate.

Schaefer *et al.* (1985) reported the same effect on *Kluyveromyces fragilis*. They observed that a marked reduction in lactose utilization took place when the concentration of lactose medium increased from 50 to 150 g/L. Similar works of other researchers such as Marlene *et al.* (1982); Marwaha & Kennedy (1988); Tin & Mawson (1993); Berruga *et al.* (1997) and Domingues *et al.* (1999) revealed that the inhibitory effect was attributed to enzymatic activities of lactase at high concentrations of lactose medium along with the produced ethanol.

Berruga *et al.* (1997) presumed that lactose would act as an inhibitor on its own carrier (the yeast cells) due to low lactase activity. They observed that when a very high initial external lactose concentrations were applied, a very low galactose level was obtained.

4.6 Addition of different chemical amendments

Fermentation industries have long been paying special attention to nutrient supplements. Such supplements proved that they could play key roles in enhancing fermentation processes it work as a catalysts for the purpose of

scaling up fermentation processes and improving the productivity and the target product yields (Brady *et al.*, 1995 & Gough *et al.*, 1996). In this study, different chemicals were added to whey (50%) at 40°C in order to test their ability to enhance whey fermentation by *K. marxianus* with the aim of optimizing ethanol yield. 50% whey and 40°C were choosen because they gave the best alcohol production rate (Table 11) and (Figure 7). The effects of different chemical amendments on cheese whey fermentation and alcohol production by *K. marxianus* are summarized in (Table 12) and (Figure 8).

4.6.1 Yeast Extract

In this study, the addition of 0.2 g of yeast extract to 100 ml of 50% whey, incubated at 40°C resulted in obtaining 6.55% ethanol. This was the highest ethanol yield obtained. It is considered as significant ($p \leq 0.05$) increase in ethanol production by *K. marxianus* compared to non-amended whey (5.33%). This result coincides with the results obtained by Anderson *et al.* (1986) who worked on *Kluyvermyces sp.* The concentrations of yeast extract they used, lied within the range of 0.2-0.7% (w/v). It has been observed that the yeast growth as well as ethanol production increased simultaneously with the yeast extract concentration.

Such parallel relationship could be explained by the fact that yeast extract provides large amounts of vitamins and amino acids which are involved in maintaining the yeast cell integrity for long-term even if the cell growth ceased and consequently, maintain the optimal ethanol production (Chen & Zall, 1982; Zayed & Foley, 1987; Roukas & Lazarides, 1991). It has been stated by Anderson *et al.* (1986), that the addition of yeast extract to whey at 40°C successfully eliminated the initial death phase.

Yeast extract is the product of yeast autolysis. It has been extensively used as an enhancer for maintaining optimal ethanol production from fermentation of a wide variety of biomass resources such as cheese whey. It is considered by fermentation industry as a very potent enhancer.

Table 12: Chemical amendments used in the modification of 50% whey (2.50% lactose) fermentation at 40°C by *Kluyveromyces marxianus* NCYC 2886. Values are means of four replicates and values in parenthesis are the standard error of the means.

	Chemical amendments used	Concentration q / 100 ml whey	Ethanol production
1	Control	-	5.325 ± (0.155) *d e f g**
2	Yeast extract	0.20 (g)	6.550 ± (0.225) *j*
3	KH_2PO_4	0.03 (g)	6.350 ± (0.311) *i j*
4	Peptone	1.20 (g)	6.115 ± (0.217) *h i j*
5	Beef extract	1.20 (g)	6.125 ± (0.236) *h i j*
6	Nicotinic acid	0.0005 (g)	6.038 ± (0.124) *g h i j*
7	Thiamin	0 0001 (g)	5.988 ± (0.053) *f g h i j*
8	Peptone	0.20 (g)	5.918 ± (0 215) *f g h i j*
9	Beef extract	0.20 (g)	5.700 ± (0.647) *f g h i*
10	Pyridoxin–HCl	0.0001(g)	5.547 ± (0.394) *e f g h*
11	Malt extract	0.20 (g)	5.380 ± (0.306) *e f g h*
12	Riboflavin	0.0001 (g)	5.330 ± (0.252) *d e f g*
13	Tween 80	30 (µL)	5.275 ± (0.025) *d e f*
14	Aminobenzoic acid	0.0002 (g)	4 913 ± (0.276) *d e*
15	$(NH_4)_2SO_4$	0.05 (g)	4.868 ± (0.425) *d e*
16	$CaCO_3$	0.20 (g)	3.990 ± (0.201) *b c*
17	Yeast extract	1.20 (g)	3.825 ± (0.214) *b*
18	Pantothenate	0.0001 (g)	3.548 ± (0.364) *b*
19	Mesoinositol	0.10 (g)	3.568 ± (0.274) *b*
20	Linoleic acid	1.00 (ml)	3 550 ± (0.676) *b*
21	$MnCl_2$	0.10 (g)	3.525 ± (0.338) *b*
22	$MgSO_4$	0.06 (g)	3.350 ± (0.343) *b*
23	K_2HPO_4	0.30 (g)	2.350 ± (0.104) *a*
24	Urea	0.05 (g)	2.350 ± (0.104) *a*

* Values with the same letter within a column are not significant (p> 0 05) different according to Duncan's New Multiple Range Test.

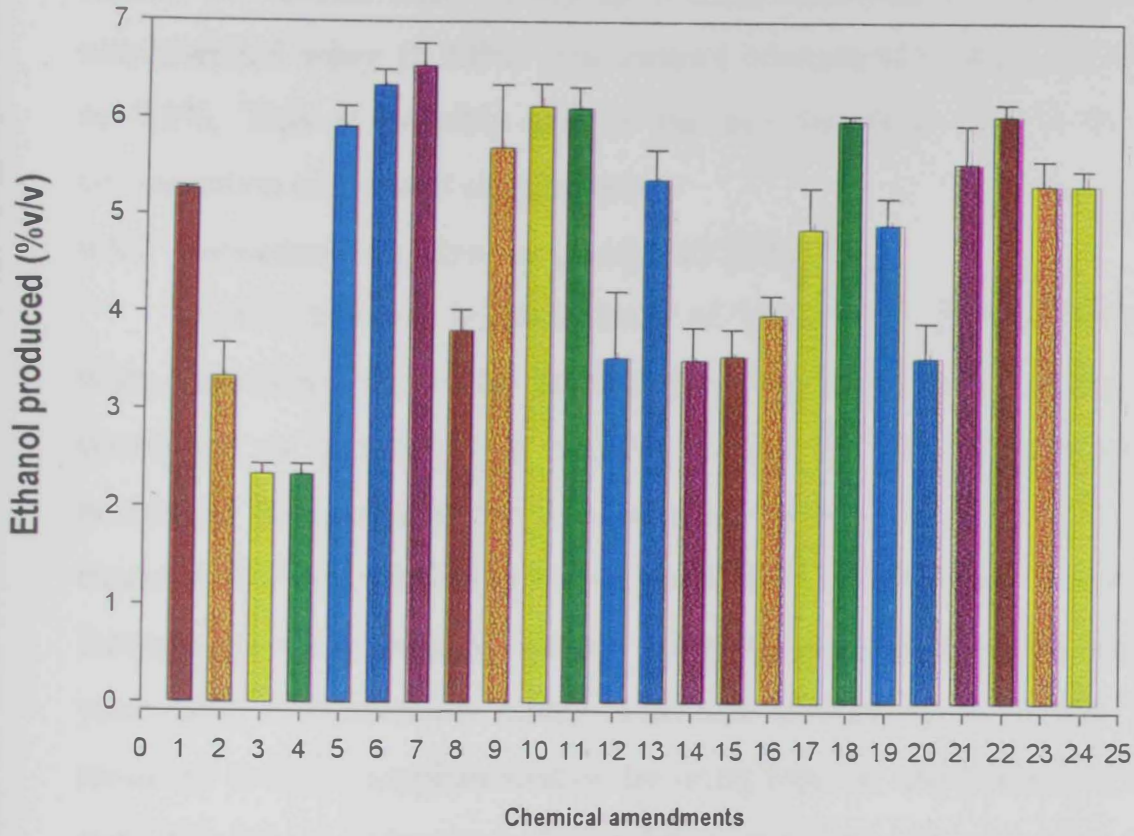

Figure (8): The effect of different chemical amendments on the rate of ethanol production, by *Kluyveromyces marxianus* NCYC 2886 (isolate number 20). The bars represents the standard errors of the mean.

1: Tween 80; 2: $MgSO_4$; 3: K_2HPO_4; 4: Urea; 5: Peptone (0.20 g); 6: KH_2PO_4;
7: Yeast extract (0.20 g); 8: Yeast extract (1.20 g); 9: Beef extract (0.20 g);
10: Beef extract (1.20 g); 11: Peptone (1.20 g); 12: Linoleic acid; 13: Malt extract;
14: $MnCl_2$; 15: Mesoinositol; 16: $CaCO_3$; 17: $(NH_4)_2SO_4$; 18: Thiamin;
19: Aminobenzoic acid; 20: Pantothenate; 21: Pyridoxin-HCl; 22: Nicotinic acid;
23: Riboflavin; 24: Control.

In the present study, the addition of 1.2% yeast extract to 50% whey, resulted in a noticeable reduction in ethanol production by *K. marxianus*. The ethanol production significantly ($p \leq 0.05$) decreased in comparison to the non-amended whey (5.33%). The ethanol concentration obtained was as low as 3.8%. This is possibly due to the cell inhibition caused by the high concentration of nutrient supplements.

4.6.2 Potassium dihydrogen phosphate (KH_2PO_4)

In the present study, the addition of 0.03 g of KH_2PO_4 to 100 ml of 50% whey, incubated at 40°C, resulted in obtaining 6.35% ethanol. This is considered as significant ($p \leq 0.05$) increase in ethanol production by *K. marxianus* compared to the non-amended whey (5.33%). KH_2PO_4 plays an essential role in establishing the required ionic environment for the yeast cell through providing bulk K^+ cations which in turn, increase the resistance of yeast cells to ethanol i.e. ethanol-tolerance (Gough *et al.*, 1996). They also modified nutrient supplementation by using $MgSO_4$ and linseed oil in parallel with KH_2PO_4 to enhance molasses fermentation by *Kluyvermyces marxianus* IMB3 at 45°C.

4.6.3 Magnesium Sulphate ($MgSO_4$)

A significant reduction ($p \leq 0.05$) in the ethanol yield by *K. marxianus*, compared to the non-amended whey (5.33%), was observed when an amount of 0.06 g $MgSO_4$ alone was added to 100 ml of 50% whey (incubated at 40°C) (Table 12) and (Figure 8). $MgSO_4$ proved the ability of increasing ethanol tolerance of many fermenting yeast strains through stabilising some glycolytic enzymes involved in the fermentation process. This is when complemented with other nutrient supplements (D'Amore *et al.*, 1989). It has been observed that when $MgSO_4$ is complemented with other nutrient supplements such as KH_2PO_4 or linseed oil, its potential enhancement to fermentation increased obviously (Gough *et al.*, 1996). This is due to the fact that the magnesium transportation by yeast has been reported to be dependent on the presence of

potassium (D'Amore *et al.*, 1989; Gough *et al.*, 1996).

4.6.4 Urea

Urea, as a nitrogen source, has shown a reduction in whey fermentation by the yeast isolate *K. marxianus*. This was indicated by a significant ($p \leq 0.05$) decrease in ethanol production compared to the non-amended whey (5.33%). This may be attributed to the fact that *K. marxianus* lacks enzymatic machinery to produce urease. However, the effect of 0.5 g/L urea concentration on fermentation using *Kluyveromyces fragilis* was weak enough to increase ethanol production (Zayed & Foley, 1987).

On the other hand, it has been reported that the mixture of urea with yeast extract exerted a potent effect on whey fermentation by *Saccharomyces cerevisiae* to maximise ethanol production (Roukas & Lazarides, 1991).

4.6.5 Dipotassium Hydrogen Phosphate (K_2HPO_4)

Di-potassium hydrogen phosphate exerted a significantly ($p \leq 0.05$) inhibitory effect on fermentation of 50% whey, at 40°C, by the yeast strain *K. marxianus* compared to the non-amended whey (5.33%). This result coincides with the findings of Ghaly & El-Taweel (1995b) where they used *Candida pseudotropicalis* to ferment cheese whey supplemented with 0.005% or 0.01% w/v of K_2HPO_4. They found that the increased concentration of potassium in the cheese whey inhibited the yeast cell growth and fermentation rate. Increasing K^+ may have caused inhibition of hexokinase,enolase, membrane ATPase and nucleic acid uptake.

4.6.6 Peptone and Beef extract

The effects of both Peptone and Beef extract on whey fermentation were found to be similar in this study.

The application of peptone or beef extract (1.2 g/100 ml of 50% whey), incubated at 40°C, led to significant ($p \leq 0.05$) increase in alcohol production by *K. marxianus* compared to the non-amended whey (5.33%). In contrast, the application of lower doses of these two supplements (0.2 g/100 ml), did not

lead to significant increase in alcohol production.

Beef extract is prepared entirely from fresh beef meat, whereas peptone is derived from peptic juice of the bile. Therefore, peptone is known to be rich with digestive enzymes. Both extracts have amino nitrogen concentration which reaches 2.5% (w/w). This is probably the reason for their enhancement property.

4.6.7 Calcium Carbonate ($CaCO_3$)

In the present study, the fermentation of 50% cheese whey by *K. marxianus* with the addition of 0.20% w/v $CaCO_3$ led to the production of 3.99% ethanol. This significant ($p \leq 0.05$) decrease in alcohol production, compared to the non-amended whey (5.33%) indicated an inhibitory effect of $CaCO_3$ at the concentration used. Sreekumar & Basappa (1992) investigated the effect of different concentrations of $CaCO_3$ in the fermentation of glucose medium by the strain *Zymomonas mobilis*. They concluded that 0.20% w/v of $CaCO_3$ was the optimum for utilization of glucose and the production of ethanol.

4.6.8 Manganese Chloride ($MnCl_2$)

$MnCl_2$ was added to 50% cheese whey (1.0 g/L) at pH 6.62 prior to the fermentation by the yeast isolate *K. marxianus*. The result obtained for ethanol production was extremely low (3.53%) compared to the non-amended whey (5.33%). This significant ($p \leq 0.05$) decrease in alcohol production, may be attributed to the addition of high concentration of $MnCl_2$, the matter which resulted in inhibition rather than stimulation (Banat, Ulster University, Ireland, personal communication).

It has been stated by Brady *et al.* (1995) that the addition of Mn^{2+} ions to enzyme preparations contributed significantly to enzyme stability at 45°C. This in turn resulted in increased efficiency of yeast strain *K. marxianus* IMB3 to convert lactose to ethanol by whey fermentation. Simpson *et al.* (1995) treated the same yeast strain with single electric field pulses of 2.4 k V/cm.

They obtained an increase of ethanol from 25% to 35% on 4% (w/v) lactose-containing media. On using electrical pulses followed by addition of 1 mM $MnCl_2$ (0.19 g/L) to lactose-containing media, ethanol production was almost doubled within thirty hours period.

4.6.9 Ammonium Sulphate [$(NH_4)_2SO_4$]

In this study, the ethanol production (4.87%) after the addition of 0.05% $(NH_4)_2SO_4$, as a supplement to cheese whey fermentation, indicated no significant effect on ethanol production by *K. marxianus* compared to the non-amended whey (5.33%). This result goes in line with that obtained by Gough *et al.* (1996), working on molasses fermentation by *K. marxianus* IMB3. They reported that the addition of nitrogen in the form of $(NH_4)_2SO_4$ did not affect the ethanol production rate.

Several workers reported a significant inhibitory action to be exerted by $(NH_4)_2SO_4$ in the production of ethanol from whey fermentation. Guimaraes *et al.* (1992) reported that $(NH_4)_2SO_4$ limit the *E. coli*-based fermentation of sweet whey. Ghaly & El-Taweel (1995b) also observed an inhibitory action exerted by adding $(NH_4)_2SO_4$ on the growth and survival of the yeast strain *Candida pseudotropicalis* during whey fermentation process.

4.6.10 Unsaturated Fatty Acids

In this study, the addition of high linoleic acid concentration such as 1% (w/v), to 50% whey, incubated at 40°C, resulted in significant ($p \leq 0.05$) reduction in ethanol production by *K. marxianus* compared to the non-amended whey (5.33%) and only 3.55% ethanol was yielded. Panchal & Stewart (1981) reported that the addition of low linoleic acid concentration such as 0.1% (w/v) to the fermentation medium caused a significant increase in the ethanol production using the strain *Saccharomyces cerevisiae*. Such a contradictory finding to what has been obtained in this study, can be explained by the adverse effect of high linoleic acid concentration on yeast membrane to increase its permeability or by causing re-entry of ethanol into yeast cell

(Panchal & Stewart, 1981). Unsaturated fatty acids such as linoleic acid are well known to increase the fluidity of yeast cell membrane by the enhancement of both the efflux of ethanol from yeast cell and influx of nutrients into the yeast cell (Panchal & Stewart, 1981).

4.6.11 Tween 80 (*Polyoxyethylene sorbitan monooleate*)

The addition of 30 µL of Tween 80 to 50% whey incubated at 40°C, did not exert any significant effect on the ethanol production by *K. marxianus* compared to the non-amended whey (5.33%). A significant increase in ethanol yield has been observed by Panchal & Stewart (1981) using 0.2% (w/v) Tween 80 in a fermentation medium containing 40% (w/v) glucose to produce ethanol by employing *Saccharomyces cervisiae* as a fermenting yeast strain. Tween 80 is categorised as a non-ionic oleic acid-based surfactant. It is believed to enhance the permeability of yeast cell membrane and also improve enzyme productivity.

The neutral effect of Tween 80 observed in this study can be explained by the enhancement of the permeability of yeast cell membrane which is counteracted by the induction of rigidity to cell membrane by the produced ethanol (Ingram, 1976).

4.6.12 Malt Extract

In this study, the addition of 0.2% (w/v) malt extract to 50% whey incubated at 40°C showed no significant effect on ethanol production by *K. marxianus* compared to the non-amended whey (5.33%). However, no previous studies in the literature surveyed, have been conducted to verify the actual effects of malt extract as nutrient supplement on the fermentation of whey or other biomass wastes.

Malt extract which is prepared by extracting the soluble products from sprouted grain followed by treatment with low temperature evaporation to reach dryness, conserves both the nitrogenous and carbohydrate constituents. It is recommended for the use in media for the growth of yeast and moulds

(Bridson, 1990).

4.6.13 Vitamins

Vitamins are essential compounds for maintaining yeast growth and functions. The appropriate amount of each vitamin was added to 100 ml of 50% whey so as to obtain the concentrations stated below. Each sample was inoculated with 0.1 ml of *K. marxianus* and incubated at 40°C. On addition of mesoinositol (0.10% w/v) and pantothenate (0.0001% w/v), the ethanol concentrations yield were 3.57% and 3.55%, respectively. Therefore, they were considered as significantly ($p \leq 0.05$) inhibitors to cheese whey fermentation by the yeast strain *K. marxianus* compared to the non-amended whey (5.33%). The results obtained using riboflavin (0.0001% w/v), pyridoxin-HCl (0.0001% w/v), aminobenzoic acid (0.0002% w/v), nicotinic acid (0.0005 % w/v) and thiamin (0.0001% w/v) indicated no significant effect on the whey fermentation process. The concentrations of the ethanol produced are shown in (Table 12) and (Figure 8).

The addition of the individual vitamins mesoinositol and pantothenate as supplements resulted in significantly ($p \leq 0.05$) inhibiting alcohol production. In a previous study carried out by Kiers *et al.* (1998), a mixture of vitamins contained the above mentioned vitamins as components acted as enhancer rather than inhibitor. This is probably due to a synergistic action which has been exerted by the enhancers. In addition to that the fermenting medium and the microorganisms (*K. lactis*) which have been used by the researchers are different from that used in the present study.

Results obtained in the present study, can be interpreted on the bases that the effect of the individual vitamin enhancers on whey fermentation process by the yeast isolate *K. marxianus* is not comparable to that exerted by 0.20% of yeast extract which is known as a rich source of vitamin B complex. It is believed that the combination of vitamins when used in whey

fermentation processes would enhance better ethanol yield (Guimaraes *et al.*, 1992).

4.7 Chemical Oxygen Demand (COD) analysis

The reduction of COD was examined by the open reflux titrimetric method as described in section (3.2.10). The chemical oxygen demand analyses were performed on the 50% whey samples before and after fermentation by *K. marxianus* at 40°C. The reduction efficiency of chemical oxygen demand which is defined as: The difference between the initial and final COD values divided by its initial COD value (Moresi *et al.*, 1980), was calculated and found to be 46 %. Blending and mixing so as to homogenize the samples in all stages was found to be quite essential. The effect of the addition of 0.20 g yeast extract has also been studied. The efficiency of COD removal due to the addition of this enhancer has been increased to 59 %. Ben-Hassan & Ghaly, (1994) reported that aerobic fermentation of cheese whey using the yeast *K. fragilis* was successful in reducing the total chemical oxygen demand to 42%, while Urbina *et al.* (2000), using a mixed culture made up of *Torulopsis cremoris* and *Candida utilis,* achieved 95.8% COD removal. The addition of surfactants like Tween 80 has been reported to lower the COD value to 26%, while silica gel and sodium lauryl sulphate reduced the COD to 31.97% (Desai & madamwar, 1994).

One of the aims of this study, was to investigate the effectiveness of yeast fermentation to reduce the pollution potential of cheese whey as measured by the reduction in Biological Oxygen Demand (BOD) value. The BOD determination is a crucial environmental index for monitoring organic pollutants in wastewater. The chemical oxygen demand (COD) has been used as a substitute for the measurement of the BOD because of the many disadvantages involved in measuring the later. The main disadvantages of BOD determination are the long time required to perform the analysis (5 days), compared to (2 hrs) for COD, the poor

precision and indeterminable accuracy of the method (Jirka & Carter, 1975). Furthermore, the experimental procedure of BOD is very tedious (Lee *et al.*, 1999). Chemical oxygen demand is defined as: The amount of oxygen equivalents consumed in the oxidation of organic compounds by strong oxidizing agents such as dichromate or permanganate (Lee *et al.*, 1999).

CHAPTER V
Conclusion & Recommendation

Chapter Five

CONCLUSION & RECOMMENDATION

5.1 Conclusion

The issue of whey waste seems not to draw the attention of dairy-based industry hitherto. This is possibly the reason for the absence of local studies of such nature. Therefore, this study is based entirely upon the scientific findings of researchers working outside UAE.

The candidate yeast involved in this study was isolated from natural local soil environment. The choice of locations for soil sampling was based on the apparent variations in the physico-chemical properties and biological nature of the soils.

Logical relationships were found to be established between the activity of yeast population growth and availability of specific elements and compounds. The soil characterized by high organic residuals of fermented date debris in addition to phosphorus was found to be the most favorable environment for the growth and proliferation of yeast fungi.

The laboratory test for fermentation ability and identification of yeast isolates nominated *K. marxianus* (isolate number 20) as the most powerful fermenting yeast strain among the other isolates. Identification of *K. marxianus* was then confirmed by NCYC (UK) and as a result of this study it has been referred to as *K. marxianus* NCYC 2886.

Optimization of fermentation conditions for maximum ethanol production by *K. marxianus* using locally produced sweet whey as a substrate has been reached. The maximum ethanol production by *K. marxianus* has been achieved at 40°C, using 50% diluted whey (2.5% lactose concentration).

The operation of fermenters with a thermotolerant yeast capable of fermenting whey at a high temperature such as 40°C for the process of ethanol production is rather more advantageous and could add value to the economical

importance of this study. Another economical value is the optimal lactose concentration (2.5%) which is contained in the 50% diluted whey. This means that the available raw material is doubled in the amount.

The effect of twenty three chemical amendments on cheese whey fermentation and ethanol production by *K. marxianus* has been studied at the optimum conditions at 40°C. Different effects have been shown, four amendments such as yeast extract (0.20 g /100 ml whey) behaved as enhancers. Ten amendments such as riboflavin (0.0001 g/ 100 ml whey) showed no significant effect, whereas the rest nine amendments such as urea (0.05 g/100 ml whey) exerted an inhibitory effect.

Reduction efficiency of COD to 46% has been achieved by applying the optimum fermentation conditions outlined in this study. This achievement has even been improved when 0.20 g of yeast extract has been mixed with 100 ml of 50% whey. The COD reduction efficiency obtained due to this amendment was as high as 59%.

5.2 Recommendation

The environmental awareness coupled with the recent recognition of inherent value of whey components resulted in the development of alternative methods for the effective reduction of pollution potential of waste whey and the production of valuable products. Ethanol and other valuable whey-based products are obtained by the application of reliable and effective waste treatment processes to the disposed whey. From these processes, the fermentation which has been used in this study to produce ethanol, resulted in reducing the residual COD of whey by a significant percentage (59 %).

The findings of this study paved the way for further related Studies. The followings can be suggested for future works:

- The other yeast strains isolated in this study from the local soils, can be screened to find the most suitable strains to form a mixed synergistic culture with *K. marxianus* NCYC 2886 in an attempt to increase the fermentation process potential for the production of more ethanol yield.
- The full spectrum of the yeast strains isolated from the local soils, in this study, can be screened in an attempt for employing the promissing strains in the fermentation processes of other food waste such as molasses, vegetables and dates.
- To reveal the relationship between the feeding stuff of the dairy cows and the composition of whey.
- Biotechnological investigations in the field of genetic engineering is essential. This is to produce yeast strains, capable of producing higher ethanol yield.
- The application of the fermentation process achieved in this study in a bench scale fermenter is recommended with the best chemical amendment obtained in the present study. This can be considered as a step to apply the process in a large industrial scale.

- The morphological and metabolic changes in the yeast *K. marxianus* NCYC 2886 during fermentation of whey can be studied. These studies are targeted to find the suitable solutions for any inhibitory factors affecting ethanol production.
- The toxicity levels of most of the organic and inorganic soil components in relation to the yeast population which have not been touched in the literature is an important area of research to be dealt with.

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ARABIC SUMMARY

لقد تم التمكن في هذه الدراسة من زيادة كفاءة تخفيض Chemical Oxygen Demand في تخمر محلول الشرش (٥٠%) عند ٤٠ درجة منوية إلى ٤٦% بدون مضافات وإلى ٥٩ % عند إضافة مستخلص الخميرة بتركيز ٠،٢٠غرام في ١٠٠مل من محلول الشرش (٥٠%).

تم استخدام ثمانية تركيزات من اللاكتوز في الشرش تتراوح بين ٣١،. % - ٥،. ١١وذلك لمعرفة تأثير تركيزات اللاكتوز في مقدرة *Kluyveromyces marxianus* NCYC 2886 على التخمر وانتاج الكحول حيث وجد أن تركيز اللاكتوز بنسبة ٢،٥ % في محلول الشرش (٥٠%) هو التركيز الأمثل للحصول على أعلى نسبة من الكحول.

كما تمت دراسة مواد متعددة كمواد مضافة في محاولة لرفع نسبة الكحول المنتج لقدر اكبر من الحد الأقصى الذي تم التوصل إلية بدون استخدام هذه الإضافات. لقد وجد أن إضافة ٠،٢٠ جرام من مستخلص الخميرة (Yeast extract) إلى ١٠٠مل من محلول الشرش أدي إلى الحصول على ٦،٥٥ % من الكحول بالمقارنة إلى (٥،٣٣) في البيئات الغير مضاف إليها مواد مغذية. وهذه النسبة تعتبر أعلى نسبة تم الحصول عليها في هذه الدراسة. وعند إضافة كلا من مستخلص اللحم والببتون (Beef extract & Peptone) بنسبة ١،٢٠ جرام /١٠٠مل من الشرش ، تم الحصول على نتائج متشابهة من الكحول (٦،١٠%). بينما أدت إضافة ٠،٠٣% جرام من البوتاسيوم داي هيدروجين فوسفات (KH_2PO_4) إلى إنتاج ٦،٣٥ % من الكحول.

وعلية تعتبر هذه المضافات محفزات للتخمر وانتاج الكحول في الظروف التي تم اختيارها لإجراء التجارب. في الجانب الآخر فان إضافة تركيز أعلى من مستخلص الخميرة (١،٢٠%) أدى إلى انخفاض نسبة الكحول إلى (٣،٨٠%). بإضافة المغنيسيوم سلفيت ($MgSO_4$) دون خلطها بمضافات أخرى ، لوحظ تثبيط واضح في إنتاج الكحول وكذلك الحال عند إضافة اليوريا (Urea). لقد أدت إضافة الداى بوتاسيوم هيدروجين فوسفيت (K_2HPO_4) أيضا إلى تثبيط في عملية التخمر وبالتالي اثر في إنتاج الكحول. كما وأدت إضافة Mesoinositol (٠،١٠% وزن/ حجم) و Pantothenate (٠،٠٠٠١% وزن/ حجم) إلى إنتاج ٣،٥٧% و ٣،٥٥% من الكحول على التوالي. إضافة ٠،٢٠% وزن/ حجم كالسيوم كاربونيت ($CaCO_3$) أدى إلى إنتاج ٣،٩٩% من الكحول. بإضافة منغنيز الكلور يد ($Mncl_2$) لمحلول الشرش (٥٠%) بتركيز نهائي (١،٠ جرام / لتر)، تم الحصول على نسبة متدنية من الكحول (٣،٥٣%) عند درجة الحرارة المثلى (٤٠ درجة منوية). يمكن أن تعزي مثل هذه النتيجة الغير متوقعة للتركيز العالي من منغنيز الكلورايد. كذلك إضافة تركيز عالي (١% وزن/ حجم) من حمض لينوليك (Linoleic acid) أدى إلى انخفاض واضح في الكحول المنتج (٣،٥٥ %).

إن إضافة (٠،٠٠٠١% وزن/ حجم) من الرايبوفلافين (Riboflavin) ، (٠،٠٠٠١% وزن/ حجم) من بايريدوكسين هيدوكلورايد (Pyridoxin-HCl) ،(٠،٠٠٠٢% وزن/ حجم) من أمينو بنزويك أسيد (Aminobenzoic acid) ، (٠،٠٢% وزن/ حجم) من مستخلص الشعير (Malt extrect) ، (٠،٠٥% وزن/ حجم) من الامونيوم سلفيت [(NH4)2So4]، (٣٠ ميكروليتر) توين ٨٠ (Tween 80) ، (٠،٠٠٠٥ % وزن/ حجم) من النيكوتينك أسيد (Nicotinic Acid) ،(٠،٠٠٠١ % وزن/ حجم) من الثيامين (Thiamin) ، (٠،٢٠% وزن/ حجم) من الببتون (Peptone) و (٠،٢٠ % وزن/ حجم) من مستخلص اللحم Beef extract بالنسب التي تمت الإشارة إليها قرين كل مضاف عند درجة حرارة (٤٠ درجة منوية)، لم تؤدي إلى تأثير ذا أهمية تذكر في عملية التخمر وبالتالي في إنتاج الكحول.

الملخص العربي

إن المخاطر التي تنطوي على تلوث التربة و المياه الناتج من تصنيع الأجبان بدولة الإمارات العربية المتحدة أدى إلى التفكير في إيجاد طريقة للتخلص الآمن لمخلفات صناعة الاجبان (الشرش) في الدولة . لقد تم إجراء الدراسة بمنطقة العين بهدف عزل بعض سلالات الخميرة الجديدة من التربة المحلية والقادرة على تخمير سكر اللاكتوز الموجود في الشرش بغرض الحصول على الكحول.

تم تجميع عينات التربة من موقعين بمنطقة العين (بجوار مزرعة أبقار العين ومنطقة المرخانية) هذا بالإضافة لمنطقة مجاورة وهي (البريمي) ولقد تم اختيار المواقع بناءً على الاختلافات في طبيعة التربة والخصائص البيئية لها، و تم إجراء التحاليل الخاصة بالمكونات العضوية واللاعضوية للتربة. هذا وقد أشارت النتائج بغنى تربة البريمي بنيتروجين النترات والحديد النشط والكبريت والكربون العضوي. ومن نتائج الدراسة أيضا ارتفاع نيتروجين النشادر وأكسيد الماغنسيوم في تربة مزرعة أبقار العين. أما تربة المرخانية فقد تميزت بارتفاع نسبة المواد العضوية الناتجة من مخلفات النبات، ومثل هذه المكونات تمثل البيئة العضوية المثالية لنمو الخمائر وغيرها من الميكروبات. أشارت النتائج الخاصة بالأس الهيدروجيني بأن عينات التربة التي أخضعت للدراسة تقع بين المدى المتعادل والقلوي (٧،١٠ – ٧،٨٠). يندرج الشرش الذي خضع لهذه الدراسة بناءً على خصائص الأس الهيدروجيني (٦،٦٢) ودرجة الحموضة (١، ٠%) تحت نوعية الشرش الحلو. هذا وقد كانت نسبة الملح في عينة الشرش (٢، ٠%).

طرق العزل التي تم استخدامها لعزل الخميرة القادرة على تخمير الشرش من التربة كانت عبارة عن الطريقة القياسية وطريقة الإثراء حيث تم استخدام لاكتوز الشرش كمصدر للكربون. تم عزل العدد الأكبر من الخمائر من تربة المرخانية ثم تربة البريمي ومن ثم تربة مزرعة أبقار العين التي احتوت على الحد الأدنى من الخمائر والتي تعتبر تربة صحراوية غير مزروعة تفتقر إلى المكونات العضوية. لقد أثبتت العزلات رقم (٢،١٤ و ٢٠) التي تم عزلها من تربة المرخانية قدرة كبيرة على تخمير الشرش وباستخدام هذه العزلات تم الحصول على أعلى معدل للكحول باستخدام العزلة رقم (٢٠). تم توصيف هذه العزلة باستخدام التحاليل المورفولوجية والزراعية والجنسية والفسيولوجية. قد تم تأكيد ومطابقة التعريف بواسطة ال National collection of yeast cultures (NCYC, U.K.) وقد تم الإشارة إليها في التقرير باسم :

Kluyveromyces marxianus NCYC 2886.

لقد تمت دراسة ظروف التخمير من حرارة وتركيزات لاكتوز الشرش للوصول للحد الأقصى من إنتاج الكحول. تم إخضاع *Kluyveromyces marxianus* NCYC 2886 لدرجات حرارة متفاوتة (٣٠،٣٥،٤٠،٤٥،٥٠ درجة منوية) وقد أشارت النتائج بارتفاع معدل إنتاج الكحول ارتفاعا مطردا مع ارتفاع درجات الحرارة (ما بين ٣٠ إلى ٤٠ درجة منوية) وقد تم الحصول على الحد الأقصى من الكحول عند درجة حرارة ٤٠ درجة منوية. أما في درجات حرارة تفوق ال٥٠ درجة منوية لوحظ انخفاض إنتاج الكحول.

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عمادة الدراسات العليا

برنامج ماجستير علوم البيئة

عنوان الرسالة: **التقنية الحيوية لشرش الجبن باستخدام بعض سلالات الخميرة المعزولة من التربة المحلية**

اسم الطالبة : موزة سهيل محمد عبدالله المهيري

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بسم الله الرحمن الرحيم

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التقنية الحيوية لفرش الجبن باستخدام بعض سلالات الخميرة المعزولة من التربة المحلية

رسالة مقدمة من الطالبة

موزة سهيل محمد عبدالله المهيري
بكالوريوس في العلوم تخصص علوم الحياة
كلية العلوم -جامعة الإمارات العربية المتحدة

استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم
(علوم البيئة)

جامعة الإمارات العربية المتحدة

يونيو (٢٠٠١)