

**ISOLATION AND MOLECULAR
CHARACTERIZATION OF YEAST STRAINS FROM
KENYAN CHEESE INDUSTRIES AND THEIR
POTENTIAL UTILIZATION IN BIOETHANOL
PRODUCTION FROM WHEY**

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**Isolation and Molecular Characterization of Yeast Strains from
Kenyan Cheese Industries and their Potential Utilization in
Bioethanol Production from Whey**

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**A thesis submitted in partial fulfilment for the Degree of Master
of Science in Biotechnology in the Jomo Kenyatta University of
Agriculture and Technology**

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DECLARATION

This research thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my family especially my parents Mr. and Mrs. George
Kimani Muraga

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LIST OF ABBREVIATIONS

BLAST – Basic Local Alignment Search Tool

DNA – deoxyribonucleic acid

dNTPs – deoxynucleotide triphosphates

D1/D2 – domain of the 26S nuclear ribosomal RNA (nrRNA)

HPLC – High-Performance Liquid Chromatography

KCC – Kenya Cooperatives Creameries

IBR – Institute of Biotechnology Research

ILRI – International Livestock Research Institute

NCBI – National Center for Biotechnology Information

PCR – Polymerase chain reaction

RFLP – Restriction Fragment Length Polymorphism

RNA – Ribosomal nucleic acid

UV – Ultraviolet

YEPD – Yeast Extract Peptone Dextrose

YEPL – Yeast Extract Peptone Lactose

ABSTRACT

The use of Bio-wastes like whey as raw materials for production of ethanol is gaining importance due to the environmental impact and exhaustion of fossil fuel sources making renewable fuel alternatives highly attractive. The objective of the study involved the isolation, characterization and identification of yeast strains from the Kenyan Cheese Industries exhibiting robust whey-lactose fermentation to ethanol and their potential application in bioethanol production from whey, Yeast strains were isolated using PDA and YEPL Medias from samples collected from KCC, Sameer and Browns cheese dairy industries. Genetically, the variable D1/D2 domain of the large subunit (26S) ribosomal DNA of the isolates were amplified by the polymerase chain reaction (PCR), sequenced and compared with known 26S rDNA sequences in the GenBank database, the rDNA fragment containing the internal transcribed spacers (ITS1 and ITS2) and 5.8S-ITS rDNA were PCR-amplified, sequenced and the PCR product digested with the enzymes *Hinfl*, and *HaeIII*. Twenty eight out of forty two pure isolates were found to have fermentative ability. The sequence analysis of the variable D1/D2 domain of the 26S rDNA, showed that the isolates belonged to *Kluyveromyces*, *Yarrowia*, *Pichia* and *Candida*. In addition, the unique variability in the size and profiles of the amplified product and in the restriction patterns enabled differentiation between the isolates. The assessment of fermentative performance of 4 yeast strains showed that *K. marxianus* strain BM4, *K. marxianus* strain BM9 and *K. lactis* strain P41 had higher ethanol productivity of 5.52, 4.92 and 5.05% w/v,

respectively, from whey at pH 4.5 and 30°C while *P. cactophila* strain YB2 produced 1.40% w/v ethanol at 10% whey sugar and pH 4.5 at 30°C. It was concluded that molecular methods based on the sequences of the 26S rDNA D1D2 domain and the ITS region were rapid and precise compared with the physiological method for the identification and typing of these species, The yeast strains isolated also showed potential in whey to Bio-ethanol production.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Recently, there are increased public concerns and ecological awareness on climate change; air quality issues and security of supply have led to renewed interest on new less carbon intensive/non-fossil based fuels that are generated in a sustainable way with minimum greenhouse gas production. In an attempt to meet this goal, the renewable energy sources that include wind, solar, geothermal and biofuels are being explored (Pimentel, 2006). Presently, ethanol (ethyl alcohol, bioethanol) is the most employed liquid biofuel in terms of volume and market value, either as a fuel or as a gasoline enhancer (Patzek & Pimentel, 2005; Hill *et al.*, 2006; Renewable Fuels Association, 2011). Furthermore, there are ongoing efforts aiming at increasing yield and energy efficiency of ethanol production from biomass as a potential fossil fuel substitute that is both renewable and environmentally friendly (Andrietta *et al.*, 2007). The world bioethanol production is estimated to reach about 85.2 billion liters (Renewable Fuels Association, 2012), with USA and Brazil being the highest producers. On average, 73% of produced ethanol worldwide corresponds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol (Sanchez & Cardona, 2008).

The massive utilization of fuel ethanol in the world requires that its production technology be cost-effective and environmentally sustainable. However,

the current first generation process of bioethanol production is based on fermentation of starch and sugar from maize and sugar cane, which only constitutes about 1-2% of the total plant biomass. Although there has been a steady rise in bioethanol production, the merits of resource-intensive crops-to-bioethanol process in terms of net emissions, cost, and energy balance remains under debate (Patzek & Pimentel, 2005; Hill *et al.*, 2006; Fargione *et al.*, 2008). In addition, the “fuel vs. food” debate that suggests that growing food crops for biofuel negatively impacts the world’s poorest populations has also risen (Collins, 2008; Mitchell, 2008; Tenenbaum, 2008). This has led to renewed momentum in the search for new and cost-effective alternative non-food sources or second generation processes feedstock for bioethanol production.

In most developing countries, agricultural, industrial and urban wastes have widespread abundance and relatively cheap procurement cost, and is thus potential feedstocks for production of cheap bioethanol (Prasad *et al.*, 2006). For example, the production of ethanol from lignocellulosic biomass (corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corn cob, oat hull, corn fiber, woodchips and cotton stalk; energy crops such as switch grass and Alfa Alfa, and various weeds such as *Saccharum spontaneum*, *Lantana camara*, *Eichhornia crassipes* (water hyacinth), etc.) has become one of the attractive alternatives (Joshi *et al.*, 2011). However, a major obstacle to industrial-scale utilization of lignocelluloses lies in the inefficient deconstruction of plant biomass owing to the recalcitrant nature of the lignin and current pretreatment methods being generally expensive, energy intensive, and

relatively inefficient (Lynd *et al.*, 2008). In addition to lignocelluloses, municipal and agro-industrial wastes such as cotton linters, spent sulfite liquor, wastes from vegetable and fruit industries, coffee waste and cheese whey have been proposed as important abundant and cheap feedstocks (Prasad *et al.*, 2006).

Whey as the main by-product of cheese industry is increasingly becoming an attractive source of many bioactive valuable compounds (Guimaraes *et al.*, 2010). It is characterized by abundant amounts of lactose (ca. 5% w/v) and other milk nutrients, which represents a significant environmental problem as a result of its high biological demand. Consequently, due to the large lactose surplus generated, its conversion to bio-ethanol has long been considered as a possible solution for whey bioremediation. The fermentation of whey lactose using yeasts has been frequently reported (Porro *et al.*, 1992; Compagno *et al.*, 1993; Grba *et al.*, 1998; Barba *et al.*, 2001) and the ability to metabolize lactose to ethanol has been demonstrated in *Kluyveromyces lactis*, *K. marxianus*, and *Candida pseudotropicalis* (Breunig *et al.*, 2000; Fukuhara, 2006). Presently, there are a few established industrial processes to produce ethanol from whey utilizing these yeast strains, which has been done in some countries, namely Ireland, New Zealand, United States and Denmark (Siso, 1996; Pesta *et al.*, 2007). But several challenges and limitations inhibit the process of utilization of whey lactose to ethanol. Yeast strains exhibiting lactose fermenting ability are still rare in nature (Fukuhara, 2006), and therefore, there is an urgent research need to isolate, identify and characterize novel microbial strains that can ferment lactose to ethanol with very high efficiency to increase the industrial

attractiveness of whey-to-ethanol fermentation.

1.2 Problem Statement

Due to high demand for energy in Kenya, there is urgent need to identify and utilize new renewable energy sources. In Kenya, whey from dairy industry, represent one of the major environmental pollution agents due to its high lactose and nutrient content. Whey-to-bioethanol bioprocess represents a novel and attractive way for industrial cheese waste utilization and bioremediation. However, efficient production of ethanol from whey is dependent on suitable yeast strains capable of efficient hydrolysis of whey lactose to its constituent monosaccharides and the subsequent metabolism to bio-ethanol. Consequently, there is need to research and develop novel indigenous yeast strains capable of efficient whey-to-bioethanol fermentation and amenable to local conditions.

1.3 Justification of the Study

The increasing depletion of petroleum fossils and the corresponding spiraling global price in addition to increased public awareness to conserve environment, warrants the search for new alternative sources of energy. Ethanol represents one alternative renewable source of the energies that Kenya can use to mitigate its high dependence on fossil fuels and help in enhancing the attainment of vision 2030 goals of energy sustainability. However, for the ethanol to be attractive, it must be cost effective. Utilization of industrial wastes for bio-ethanol production such as whey, a byproduct from the dairy industry that contributes to environmental pollution, would

help solve disposal problems at the same time provide a source of energy. In Kenya there are several cheese producing industries, and so far there has been no documented report on industrial utilization of produced cheese whey. Furthermore, there is no research report on isolation of novel lactose fermenting yeast strains of industrial significance. Hence the need to carry out this study on the isolation, molecular identification and characterization of indigenous yeast strains capable of converting whey to ethanol.

1.4 General Objective

To produce bioethanol from whey using yeast strains isolated from Kenya's cheese industries

1.4.1 Specific Objectives

1. To isolate and characterize yeasts strains exhibiting efficient whey lactose conversion to bioethanol from Kenya's cheese industries
2. To identify the ethanol-producing isolates by using sequences of D1/D2 domains of 26S rRNA gene and phylogenetics analysis
3. To differentiate between the isolates by amplifying rDNA fragment containing the 5.8S ITS1-ITS2 and restriction analysis

1.5 Hypotheses

1. There exist yeast strains that can ferment whey lactose as carbon source to yield bio-ethanol.
2. The yeast strains that ferment whey lactose are not different from already known yeast strains.
3. The yeast strain that ferment the whey lactose can produce considerable quantity of ethanol

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Energy Needs

Fossil fuels, in particular petroleum, have for a long time been an abundant and cheap raw material for the production of fine chemicals and, more important, transport fuels (van Maris *et al.*, 2006). However, petroleum is a non renewable resource and with the depletion of this crucial energy reserve it is clear that current supply can no longer meet the ever-increasing global energy demands (Sanchez & Cardona, 2008). Recurring crises in major crude-oil producing areas such as the Middle East and the Niger delta, and spectacular growth experienced within the major Asian economies, especially China in recent years, among other factors have helped push crude-oil prices constantly above \$60 per barrel (Skeer & Wang, 2007; Sanchez & Cardona, 2008). This has raised concerns about the security of oil supplies, requiring national governments to reconsider their dependence on foreign oil reserves (van Maris *et al.*, 2006; Fofana *et al.*, 2009). Similarly, Kenya faces a crippling dependency on petroleum fuel imports, and thus incurs a huge import bill and these imports account for about 33% of the total import bill (Mohammud, 2011) It is also forecasted that development projects under Vision 2030 will increase demand on Kenya's energy supply (Kenya Vision 2030 document). Thus, the country's economy is exposed to global oil supply risk that is unsustainable in the long term.

The combustion of fossil fuels has led to a steady increase in the levels of greenhouse gas emissions which are a major cause of climate change, particularly global warming (Ragauskas *et al.*, 2006). Currently, motor vehicles account for 70% of global carbon monoxide emissions and 19% of carbon dioxide emissions worldwide, and it is projected that the number of motor vehicles are to increase to 1.3 billion by 2030 and to more than 2 billion vehicles by 2050. Such growth will in no doubt affect the availability of global oil reserves as well as the stability of ecosystems and global climate (Balat & Balat, 2009). For the world and Kenya to achieve energy sustainability, these concerns require intensified efforts to diversify our energy sources and focus more on alternative clean and carbon-neutral fuels that can be sustainable in the long term.

2.2 Bioethanol as renewable energy source

Among the renewable energy alternatives, one solution is to harness solar energy in the form of plant biomass to produce biofuels (Sánchez & Cardona, 2008). Biofuels refers to any solid, liquid or gaseous fuel that is predominantly produced from biomass through biochemical or thermochemical processes (Balat, 2007). Biomass sources include plant matter and lignocellulosic residues, such as forestry and agricultural by-products as well as municipal wastes (Balat & Balat, 2009).

Ethanol produced through the fermentation of sugars is currently the most predominant liquid biofuel and is already a well-established biofuel in the transport

and industry sectors of some countries, notably Brazil, the USA and the European Union (Galbe & Zacchi, 2007). The world's ethyl alcohol production has reached about 70 billion litres per annum, with the USA and Brazil accounting for close to 90% of the global output (Renewable Fuels Association, 2011). The European Union, China, India, Canada and Thailand are also major bioethanol producing countries.

An in-depth look at the Kenyan bioethanol scenario shows that the concept of using ethanol as a fuel is not entirely new. Such efforts date back to production of power alcohol in 1982 up to 1991, where it used to be blended (10%) in premium and super gasoline. The raw materials, cane molasses, are sourced from the Kisumu Western Region (Chemilil, Sony, Kibos, Mumias and Nzoia areas). Currently, there are two large scale producers of bioethanol in Kenya namely: Agro Chemicals and Food Co. Ltd (AFC) in Muhoroni and Spectre International in Kisumu/Lake Victoria with a total production capacity of 55,000 and 60,000 litres per day, respectively. Furthermore, a major expansion that will increase the production capacities to >230,000 liters per day is underway (GTZ, 2008).

Bioethanol is presently produced from sugar sources such as sugar cane juice (Brazil), molasses (India, Egypt and Kenya) and sugar beet (France), and also starch sources such as maize (USA, Canada), wheat (Germany, Spain, and Sweden) and cassava (Thailand) (Antoni *et al.*, 2007; Purwadi *et al.*, 2007). However, these raw materials, which require prime agricultural land for cultivation and which are also used for human food and animal feed, will not be sufficient to meet the rising demand

for fuel ethanol (Hahn-Hägerdal *et al.*, 2006; Chang, 2007). Moreover, their utilization as ethanol feedstock has also led to an increase in global food prices (Frow *et al.*, 2009).

To its merit, bioethanol production can be considered as a good strategy for management of agricultural, industrial and urban wastes. Industrial wastes such as spent sulfite liquor, vegetable and fruit industries wastes, and cheese whey have also been identified as potential abundant feedstock sources of low cost bioethanol production (Prasad *et al.*, 2006). To harness the advantages, however, the technological and economical challenges related increasing yields and energy efficiency of ethanol production facing the waste-to-ethanol processes must be addressed.

2.3 Cheese Whey

The dairy industry represents an important part of the food processing industry and contributes significant liquid process residues that can be used for the production of ethanol. Cheese whey, a by-product of dairy industries, generally refers to the watery part that is formed during the coagulation of milk during cheese making process. It consists of about 85–95% of the milk volume and retains 55% of milk nutrients that includes lactose (4.5–5% w/v), soluble proteins (0.6–0.8% w/v), lipids (0.4–0.5% w/v), mineral salts (8–10% of dried extract) and appreciable quantities of other components, such as lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins (Siso, 1996; de Glutz, 2009)

(Table 1). Due to high volumes produced and its high organic matter content, cheese whey represents an important environmental problem. The high biological oxygen demand (BOD) due to its lactose fraction makes biological treatment of cheese whey very expensive, particularly for the small cheese factories (Kisaalita *et al.*, 1990; Saddoud *et al.*, 2007).

Table 1. Whey Characteristics^a

Compound	g/kg
Albumin	4.98
Peptides	0.83
Lipids	0.41
Lactose	38.1
Lactic acids	0.08
Citric acids	1.66
Monovalent cationic salts	1.66
Polyvalent cationic salts	1.01
Monovalent ammonium salts	0.92
Polyvalent ammonium salts	0.99

^aAdapted from de Glutz (2009)

Whey can be considered to have vast potential as a source of value added products since it retains most of the milk nutrients, which includes soluble proteins, peptides, lipids, lactose, minerals and vitamins. There are numerous reports where cheese whey has been utilized for production of valuable chemicals such as organic acids (lactic, acetic), bioactive whey proteins and peptides, single cell protein,

methane, and cheese whey powder (Yang & Silva, 1995; Siso, 1996; Audic *et al.*, 2003; Pesta *et al.*, 2007). However, the lactose-rich effluent that remains after separation of proteins and other interesting bioactive components is still a major environmental concern and solutions for its bioremediation are needed. Since there is a large surplus of lactose, its bioconversion to bulk commodities such as bio-ethanol has long been considered as a possible alternative solution.

2.3 Whey-to-Ethanol Process

The whey-to-ethanol process generally utilizes deproteinated whey from casein manufacture or total milk protein production as feedstock (Hamilton, 1998). Prior to fermentation, the feedstock is concentrated, from 4 to 8% lactose, by reverse osmosis (Gibson, 2009). Fermentation of the concentrate feedstock takes about 24 h and is achieved using *Kluyveromyces* spp., to attain an ethanol content of about 4%.w/v Distillation and water removal is then employed to obtain the different ethanol grades (Gibson, 2009). Fermentation of whey into ethanol involves initial hydrolysis of the lactose sugar into glucose and galactose, whereby the glucose is converted into fructose-1,6-bisphosphate implying the utilization of two molecules of ATP. Fructose-1,6-bisphosphate is then cleaved into two triose phosphate. Glyceraldehyde-3-phosphate is oxidized by the reduction of NAD⁺, and then esterified with inorganic phosphate resulting in 1, 3-bisphosphoglyceric acid which is then converted to pyruvic acid with the production of two molecules of ATP. NADH, in the process regenerates NAD required for continued functioning of the glycolytic

pathway. Most fermentations result in the production of several major end-products, the formation of which is influenced by environmental and physiological factors and is a property of each individual organism. Apart from ethanol other products, such as acetate, butyrate, butanol, isopropanol, propionate, lactate, glycerol or butanediol, can be formed by metabolism of pyruvate from multiple microorganisms via different pathways. (Fernandez *et al.*, 1995).

In Ireland, New Zealand, United States and Denmark, there are established industrial processes for whey-to-ethanol production (Siso, 1996; Pesta *et al.*, 2007). For example, Carbery Milk Products Ltd of Ireland has developed a whey-to-ethanol process for the production of both potable ethanol and fuel ethanol for E85 and E5 petrol blends, with estimated production of about 11 000 mT of ethanol per year (Doyle, 2005; Ling, 2008). According to Ling (2008), Carbery process was later adopted by plants in New Zealand and the United States. In New Zealand, Anchor Ethanol Ltd, operates three whey-to-ethanol plants, which produce around 17 million liters of ethanol per year; producing eight different ethanol grades that includes potable ethanol for beverages to anhydrous alcohol for E10 blend petrol fuels (Thiele, 2005). The major markets for produced ethanol ranges from pharmaceutical, cosmetics, industrial solvents as well as food and beverages applications, with a substantial proportion of the produce being exported (Hamilton, 1998; Thiele, 2005). These few examples illustrate the potential utilization of cheese whey as a feedstock for the industrial production of bioethanol.

Despite these examples of industrial implementation, the fermentation technology must be further improved in order to enhance the attractiveness of whey-to-ethanol bioprocesses. Although there has been a long research and development effort in this field, there is still the need to identify and develop microbial strains that ferment lactose to ethanol with very high efficiency.

2.4 Role of Yeast in Fermentative Processes

Lactose, a major sugar carbohydrate in whey, is a disaccharide consisting of glucose and galactose. In nature, yeast strains that can assimilate lactose aerobically are ubiquitous. However, those that ferment lactose are rather rare (Fukuhara, 2006). A few species that have been reported to ferment lactose includes *Kluyveromyces lactis*, *K. marxianus*, and *Candida pseudotropicalis* and the fermentation of whey lactose to ethanol, particularly using these yeasts, has been frequently referred in the literature (Barba *et al.*, 2001; Ozmihci & Kargi 2007a; Rech & Ayub 2007; Guimares *et al.*, 2008; Guimares *et al.*, 2010). These yeast strains ability to ferment lactose is the due to expression of β -galactosidase (β -GAL) activity that facilitate initial lactose hydrolysis to glucose and galactose that are eventually metabolized downstream to ethanol. Generally, lactose hydrolysis can be achieved by acid hydrolysis, enzymatically or utilization of yeast strains capable of expressing β -GAL activity. Unfortunately, acid hydrolysis can form some byproducts that may inhibit the fermentation, and enzymatic hydrolysis will add expense to the process. Therefore, there is a great need to isolate yeast strains possessing the β -GAL activity and

evaluate their ability to ferment lactose.

Yeasts contribute largely in the production of fermented food products such as wine, beer and bread. In dairy industry they play an important role in proteolysis, lipolysis, and fermentation of lactose, ripening of cheese and production of other milk products such as yoghurt, contributing to their organoleptic properties (Jakobsen & Narvhus, 1996; Addis *et al.*, 2001). In addition, several yeasts have been proposed as novel probiotic microorganisms, biocontrol agents and producers of functional ingredients (Fleet, 2007; Gatesoupe 2007; Chi *et al.*, 2009; Banker *et al.*, 2009).

2.4.1 Yeast Ecology

The probable role of yeasts as probable agents of pollution, bioremediation or biological pest control, in an environmental ecosystem, has been well studied. For example, the use of microbial antagonists as agents of protective agents has been under investigation leading to the use of some naturally occurring yeast such as *Candida oleiphila*, *C. sake*, *C. saltoana* and *Cryptococcus albidus* in commercial products (Brakhage & Turner, 1995; Poggeler, 2001). It is well documented that yeasts are not arbitrarily distributed throughout the biosphere. However, they form communities of species as defined by their habitat, which is the actual place where an assembly of yeasts lives, and by the niches of its component species. The niche includes all physical chemical or biotic factors required for successful existence. The nature of yeasts generally limits them in the range of habitats they can occupy. The mineral, nutrient as well as organic source of carbon and energy differs between

habitats and influence the type of yeasts present in that habitat (Phaff & Starmer, 1980; Phaff, 1986; Lachance & Starmer, 1998).

Several reports show that yeasts grow typically in moist environments where there is an abundant supply of simple, soluble nutrients such as sugars and amino acids. (Lachance & Starmer 1998) They are found in widely different aquatic and terrestrial sources, the atmosphere as well as certain restricted habitats. They may also be found associated with the body of certain animals since they act as intestinal commensals.

2.4.2 Lactose Fermenting Yeasts

Some of the known lactose fermenting yeasts are *C. pseudotropicalis* (*C. kefir*) and *Kluyveromyces* species. *K. lactis* is one of the mostly studied yeast species and making it a model system for comparative studies with *S. cerevisiae* (Breunig *et al.*, 2000). Although not commonly used for ethanol production, *K. lactis* has been exploited for other biotechnological applications such as the production of heterologous proteins using cheese whey as culture media (Maullu *et al.*, 1999). The ability of this yeast to metabolise lactose results from the presence of a lactose permease (encoded by the *LAC12* gene) and a β -galactosidase *LAC4* gene) (Rubio-Teixeira, 2006) (Figure 1).

In the same genus, *K. marxianus* has also received attention due to its biotechnological potential and advantages over *K. lactis*. *K. marxianus* isolates originate from an enormous variety of habitats, accounting for the species broad

metabolic diversity and consequent wide range of biotechnological applications (Fonseca *et al.*, 2008). A significant advantage of some *K. marxianus* strains is their ability to grow and ferment at elevated temperatures (> 40°C). Some thermotolerant *K. marxianus* strains have been reported to be capable of growing aerobically at 52°C, on lactose and whey permeate (Banat & Marchant, 1995). This property affords cost savings, due to reduced cooling cost, in ethanol production bioprocesses (Fonseca *et al.*, 2008).

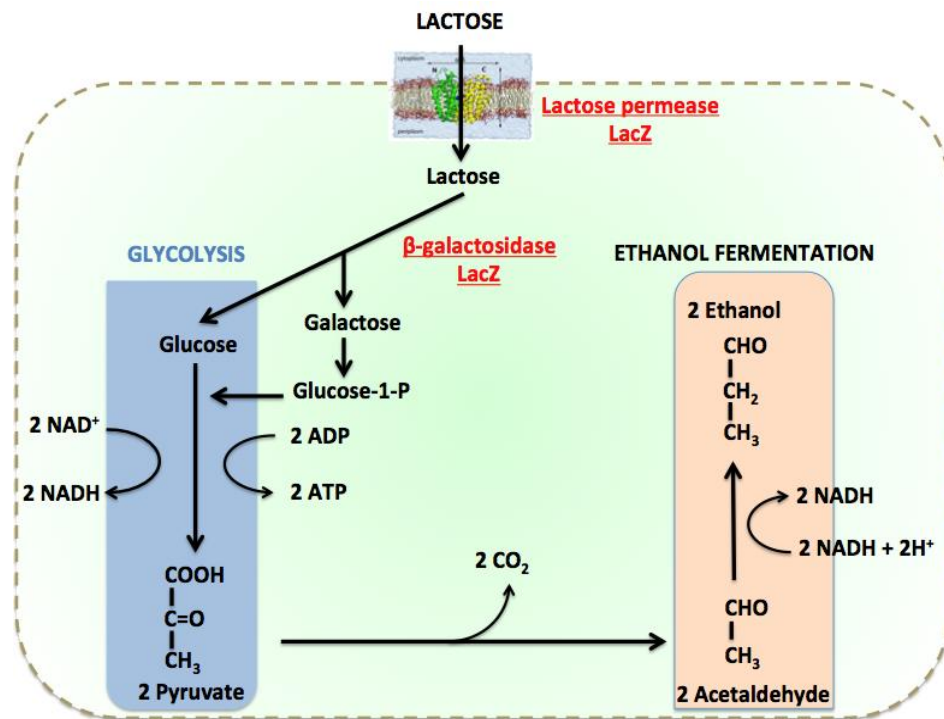


Figure 1. Ethanol fermentation of the yeast strain *Kluyveromyces marxianus*. The importance of lacY (lactose permease) and lacZ (β -galactosidase) genes in whey fermentation to bioethanol is highlighted.

There is an abundance of information regarding lactose/whey alcoholic fermentation using *Kluyveromyces* yeasts (Lachance, 1998; Fonseca *et al.*, 2008; Silveira *et al.*, 2005). A typical process is illustrated in Figure 1. Inhibitory effects and associated problems in the fermentation of concentrated lactose/whey media have been reported by various authors. Predominantly, slow fermentation and high residual sugar have been noted, when the initial lactose concentration is increased, above 100 to 200 g l⁻¹ (Dale *et al.*, 1994; Silveira *et al.*, 2005; Zafar *et al.*, 2005; Ozmihci & Kargi, 2007a). These problems have been attributed to osmotic sensitivity and low ethanol tolerance, as well as inhibition by high salts concentration (Grubb & Mawson, 1993; Zafar *et al.*, 2005). The level of such effects seems to be strain-dependent, although the fermentation conditions, in particular oxygen and other nutrients availability, may also play a key role in this regard.

In terms of fermentation efficiency, *C. pseudotropicalis* strains have been selected by some studies as the most efficient, in conversion of whey lactose in whey to ethanol, among lactose fermenting yeasts (Ghaly & El-Taweel, 1995; Szczodrak *et al.*, 1997). As in *Kluyveromyces* spp., *C. pseudotropicalis* strains are repressed by high lactose concentrations and ethanol (Ghaly and El-Taweel, 1995; Szczodrak *et al.*, 1997). Ghaly and El-Taweel, (1995), undertook studies on the effects of nutrients (ammonium sulphate, dipotassium hydrogen phosphate) on yeast extract supplementation and the effect of lactose concentrations in *C. pseudotropicalis* this yeast strain when used in fermentation of whey with reagent grade lactose of 10-20%

in a 5 l batch bioreactor yielded an ethanol productivity of 0.7-1.0 g l h⁻¹ and an ethanol titre of 40-45g l⁻¹ while when whey supplemented with yeast extract and 15% lactose in a continuous bioreactor yielded an ethanol productivity of 1.4 g l h⁻¹ with an ethanol titre of 58 g l⁻¹. Direct lactose fermenting yeasts generally suffer from low conversion to ethanol and have poor tolerance to ethanol causing product inhibition even at concentrations as low as 2%, consequently causing high energy costs due to distillation required to concentrate the ethanol (Rosa & Sa-Correia 1992; Dale *et al.*, 1994; Fonseca *et al.*, 2007). Therefore, there is an urgent need to isolate and identify novel yeast strains or engineer the promising and/or existing strains with robust fermentative abilities to ensure the achievement of an efficient and optimized whey fermentation processes.

2.5 Yeast Isolation and Identification

2.5 .1 Yeast Isolation

Yeasts usually occur in the presence of molds and/or bacteria (Yarrow, 1998), necessitating use of selective techniques for their recovery. Similar to other heterotrophic living organisms, yeasts require carbon, nitrogen, phosphorous, trace elements and growth factors as sources of nutrition. In yeast isolation, the media used should permit the yeast to grow while suppressing molds and bacteria. Most isolation methods require the use of media, which is acidified using either hydrochloric acid or phosphoric acid like yeast malt agar acidified to a pH of 3.7. According to Lachance & Starmer (1998), acidified media is preferred over media incorporated with

antibiotics and fungistatic agents; fungistatic agents should be used with caution since it may also inhibit certain yeasts (Yarrow, 1998).

2.5.2 Yeast Identification

Classical culture-based diagnostic methods use morphological characteristics of yeasts (size, colour and shape of the colony), as well as biochemical (fermentation of selected carbohydrates, assimilation of carbon or nitrogen from selected organic compounds, acid production, etc.). These methods, however, require long time waiting for the final score, some lasting up to 1–2 weeks. Therefore, laboratories are increasingly choosing rapid diagnostic tests, such as API[®] Candida, API[®] 20C AUX, and ID32C[®] that shorten identification time to 24–48 h. Commercial tests are based on evaluation of selected biochemical properties with assigned values, which in turn are given a numerical code designating the species (Katarzyna, 2011). The limitation of rapid diagnostic methods is that they are designed mainly for the identification of medical yeast isolates (Fricker-Hidalgo *et al.*, 1996). However, yeast identification using a combination of the laborious and time consuming identification techniques to molecular taxonomy with improved speed and accuracy in identification due to their established and comprehensive databases for comparisons of strains have been reported (Kock *et al.*, 1985; Viljoen *et al.*, 1986; Cottrell & Kock, 1989; Miller *et al.*, 1989; Botha *et al.*, 1992; Botha & Kock, 1993).

These techniques have also found application in production environments such as in monitoring the succession of active yeast species during wine production

(Guillamón *et al.*, 1998; Esteve-Zarzoso *et al.*, 1999), in analysis of restriction fragment length polymorphism of the ITS region, allowing for detection and quantification, of different yeast species (Querrol & Ramon, 1996; Vasdinyei & Deak, 2003).

Studies by Cai *et al.* (1996), James *et al.* (1996), Kurtzman (1992) and Kurtzman (1993), have demonstrated that the complex ITS (internal transcribed spacer) regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved), are useful in measuring close fungus genealogical relationships (Figure 2). This is due to their ability to exhibit far greater interspecific differences than the 18S and 25S rRNA genes. Ribosomal regions evolve in a concerted fashion and hence show a low intraspecific polymorphism and a high interspecific variability (Li, 1997). This has proved very useful in the classification of *Saccharomyces* species (Huffman *et al.*, 1992; Molina *et al.*, 1992; Valente *et al.*, 1996; Wyder & Puhán, 1997), *Kluyveromyces* species (Belloch *et al.*, 1998) and, recently, for the identification of a small collection of wine yeast species (Guillamón *et al.*, 1998).

The use of two universal and two species-specific primers derived from the D1/D2 region of the 26S rDNA and subsequent sequencing of this domain allows for rapid and accurate species identification (Hong *et al.*, 2001; Herzberg *et al.*, 2002; Scorzetti *et al.*, 2002; Daniel & Meyer, 2003). According to Frutos *et al.* (2004), the use of D1/D2 domain is generally accepted as the main tool for yeast taxonomy

allowing for identification of new ascomycetous yeasts previously not recognized as novel through use of conventional identification techniques (Kurtzman, 2000).

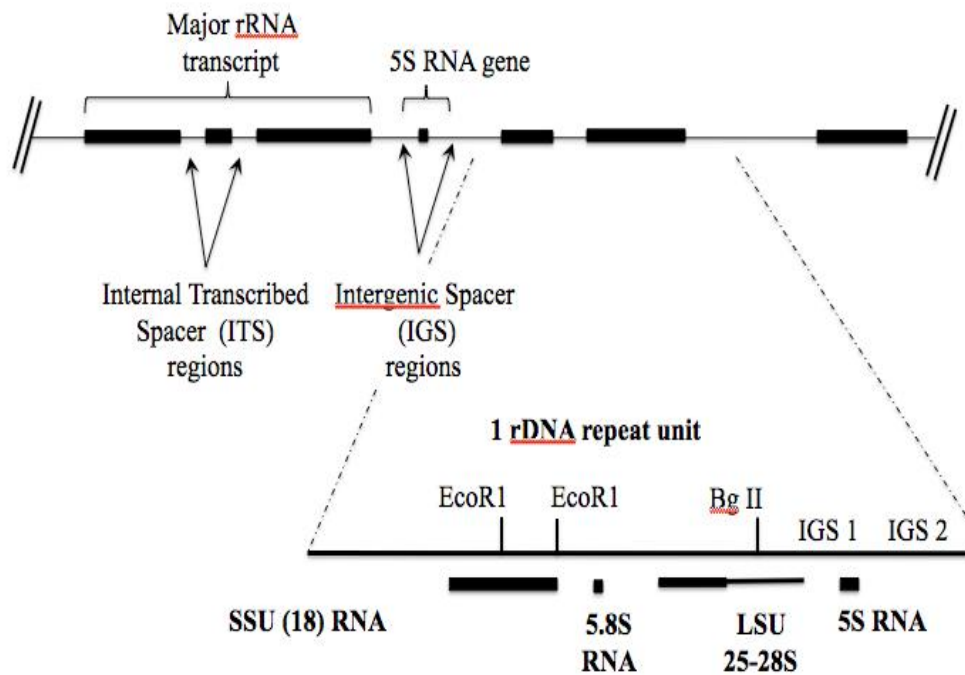


Figure 2. Schematic representation of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) (Adapted from Gargas & De Priest, 1996).

Databases of the D1/D2 sequences are available for all currently recognized ascomycetous and basidiomycetous yeasts. This extensive database makes species identification much easier and could serve as reliable and practical criteria for identification of most known yeasts (Kurtzman & Robnett, 1998; Kurtzman, 2001; Wesselink *et al.*, 2002; Abliz *et al.*, 2004; Guffogg *et al.*, 2004; Hesham *et al.*, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

The Dairy waste samples (whey, wastewater and swabs) were collected from KCC, Sameer, and Browns cheese industry in Nairobi and Kiambu counties. Eleven samples were collected from KCC, four samples from Sameer Dairy Factory Ltd and six samples from Browns Cheese Industry Ltd (Table 2). Laboratory work was carried out at Jomo Kenyatta University and Technology (JKUAT) in Kiambu County, and Kenya Industrial Research and Development institute (KIRDI) in Nairobi, Kenya.

3.2 Study Design

A cross sectional study which involved purposive sampling was used. The study area was divided into strata based on the different dairy industries KCC, Sammer and Brown cheese industries. Samples were collected from various sites that were chosen purposively based on points of collection and disposal of waste products.

3.3 Sampling and Collection of Samples

Whey, wastewater and swabs samples were collected from New KCC (Kenya Cooperatives Creameries), Sameer Agriculture in Industrial Area and Browns Cheese Industry Ltd in Limuru. Samples were collected randomly at four different points and

they were then thoroughly mixed to constitute a composite sample. Collection dates of the samples were recorded and then transported under cold storage at 4°C in autoclaved sterile Borosil bottles to the laboratory for analysis.

Table 2. Summary of Sampling done at various sites

Samples	KCC	Sameer	Browns	Total
Whey samples	4	3	4	11
Waste water	2		2	4
Swabs	5			5
Total	11	3	6	20

3.4 Growth, Isolation and Evaluation of Fermenting Ability of Yeasts

Collected samples were used for isolating yeast on YEPD (yeast extract peptone dextrose) agar medium containing yeast extract 1.0%, peptone 2.0%, dextrose 2.0%, pH 5.5 supplemented with 50 mg chloramphenicol antibiotic and PDA (potato dextrose agar) media also supplemented with 50 mg chloramphenicol antibiotic were used in order to inhibit growth of bacteria. Plates were incubated at 30°C for 48 h. After incubation different colonies were picked up (each colony represented one isolate) on the basis of colony shape and color (Barnett *et al.*, 2000). The colonies picked were further purified by streaking 3 times on YEPD media.

Yeast isolates were evaluated for their ability to ferment lactose by incubating the cultures at 30°C in test tubes containing YEPL (yeast extract peptone lactose) medium for selective isolation of lactose utilizing yeasts with inserted Durham tubes. MacConkey broth supplemented with bromophenol purple dye was used to test for acid production and fermentation of the yeast isolates. The accumulation of gas in the Durham tubes and colour change from purple to yellow was used to score for positive lactose fermenting ability of yeast isolates.

3.5 Phenotypic Characterization of Yeast Isolates

3.5.1 Morphology Characteristics

According to Barnett *et al.* (2000) and Yarrow (1998), yeast isolates were identified phenotypically on the basis of colony morphology, colour and elevation on YEPD (yeast extract peptone dextrose agar) media and photographed under a contrast microscope.

3.5.2 Cellular Morphology

Preliminary characterization by simple staining (using lactophenol cotton blue dye and gram iodine solution) of each of the isolates was done (Kango 2010). Briefly, isolates were picked and suspended on a drop of water on microscope slide on both sides of the slide followed by flooding with few drops of gram iodine solution on one side and phenol cotton blue solution on the other side. The slides were then covered

with cover slips and incubated at 30°C for 10 min then observed under a light microscope at ×100 magnification under a microscope.

3.6 Physiological Characterization of Yeast Isolates

3.6.1 Growth at Different Temperature

To determine the ability of the isolates to grow at varied temperature ranges, each isolate was inoculated in duplicate in 50 ml YEPL (yeast extract peptone lactose) broth medium containing yeast extract 1.0%, peptone 2.0%, lactose 2.0%, and incubated at the five different temperatures (25, 30, 35, 42, and 47°C). Two uninoculated bottles for each temperature were used as controls. Optical densities at 600nm were determined using a spectrophotometer at intervals of 24, 36, and 72 h as a measure of growth.

3.6.2 Growth at Different pH Ranges

To determine the ability of the isolates to grow at varied pH ranges, YEPL (yeast extract peptone lactose) broth medium containing yeast extract 1.0%, peptone 2.0%, lactose 2.0%, was prepared separately in conical flasks and the pH adjusted to 3.0, 4.0, 4.5 5.5 and 6.0 using Sulphuric acid. Isolates were inoculated in duplicates in 50 ml universal bottles containing YEPL broth at different pH and incubated at 30°C. The growth was determined after 24, 36, and 72 h by measuring the OD_{600nm}.using a spectrophotometer. Two uninoculated bottles for each pH value were used as negative.

3.7 Biochemical Characterization

Biochemical characterization based on the ability of yeast isolates to utilize various carbon sugars were carried out using API[®] 20C AUX Kit (bioMérieux, Marcy l'Etoile, France) according to manufacturer's instructions. Briefly, using a wire loop a portion of a 24 h old yeast colony was picked by successive touches and inoculated in sterile 0.85% NaCl, and 100 µl aliquot of the solution was transferred into API C medium where it was gently homogenized with a pipette avoiding formation of bubbles. Each capsule containing the test sugar (D-Glucose, Glycerol, calcium 2-Keto-Gluconate, L-Arabinose, D-Xylose, Adonitol, Xylitol, D-Galactose, Inositol, D-Sorbitol, Methyl- α D-Glucopyranoside, N-Acetyl-Glucosamine, D-Celiobiose, D-Lactose, D-Maltose, D-Sucrose, D-Trehalose, D-Melezitose and D-Raffinose) was filled with API C medium inoculated with each yeast isolate and incubated at 30°C for 72 h, utilization of carbon source was seen as change in turbidity in the ampoules.

3.8: Molecular Genetic Characterization

3.8.1 DNA Extraction for PCR

DNA extraction was carried out using Gentra Puregene[®] Yeast/Bacteria kit (Qiagen, USA) according manufacturer's instructions. 1 ml overnight cell suspension of the 28 yeast isolates was transferred to a 1.5 ml Eppendorf tube on ice and centrifuged for 5 s at 13,000 x g to pellet cells and the supernatant carefully discarded by pouring. 300 µl cell suspension solution was added and pipetted prior to the

addition of 1.5 μ l lytic enzyme solution. The mixture was again mixed by inverting the tube several times followed by incubation at 37°C for 30 min. After incubation, it was centrifuged for 60 s at 13,000 x g to pellet cells and the supernatant discarded by pouring. 300 μ l cell lysis solution was then added and mixed by pipetting up and down then 100 μ l protein precipitation solution was added, vortexed vigorously for 20 s at high speed followed by centrifugation at 13,000 x g for 3 min.

One ml Isopropanol (Scharlab S.L., Spain) was pipetted into a clean 1.5 ml microcentrifuge tube and the supernatant from previous step added by careful pouring, mixed by inverting several times, and then centrifuged for 1 min at 13,000 x g. The supernatant was carefully discarded by draining the tube on a clean piece of absorbent paper. 300 μ l of 70% ethanol (Scharlab S.L., Spain) was added and inverted several times to wash DNA pellet, centrifuged for 1 min at 13,000 x g and the supernatant discarded and the tube drained on a clean piece of absorbent paper, and then air dried for 5 min.

100 μ l DNA hydration solution was added and mixed by vortexing for 5 s at medium speed, 1.5 μ l RNase solution was added and again mixed by vortexing followed by pulse spinning to collect liquid. It was then incubated at 37°C for 30 min followed by further incubation at 65°C for 1 h to dissolve the DNA. The samples were incubated at room temperature overnight with gentle shaking upon which the presence of DNA was checked on 1% agarose and visualized under ultraviolet by

staining with ethidium bromide. The remaining volume was stored at -20°C. The genomic DNA was used as template for subsequent PCR amplifications.

3.8.2 Partial Sequencing of the Gene Coding for 26S rRNA

To identify and determine the correct phylogenetic position of the isolates, a sequence analysis of the variable D1/D2 domain of the large subunit (26S) ribosomal DNA was performed. Fragments containing about 600 - 650 bp of the 26S rRNA were amplified by PCR using forward and reverse primers: NL-1 (5'-GCA TAT CAA TAA GCG GAG GAAAAG- 3') and NL-4 (5'-GGTCCG TGT TTC AAG ACG G- 3') (Hesham *et al.*, 2006). In 50 µl reaction volume containing 2.5 µl of each forward and reverse primers, 2.0 µl template, 25 µl Taq mix (Roche, USA) and 18 µl PCR water The PCR was run for 35 cycles with an initial denaturation at 95°C for 4 min, followed by denaturation at 95°C for 60 s, annealing at 52°C for 60 s, an extension at 72°C for 2 min and a final extension at 72°C for 7 min and holding at 4°C (Ramos *et al.*, 2005). PCR products were separated on a 2% agarose gel containing 5 µl of ethidium bromide and visualized under UV light. The sizes of the PCR products were estimated by comparing them to a 100 bp Gel pilot DNA molecular weight marker (Qiagen, USA).

3.8.2.1 Phylogenetic Analysis

The 26S sequences of isolates were used for a BLAST search in the EMBL/GenBank databases (www.ncbi.nlm.nih.gov/BLAST/). The 26S sequence of the isolates were further aligned and compared to published 26S rRNA sequences

using the taxonomy browser of the National Center for Biotechnology Information (NCBI; Bethesda, MD) and GenBank. A phylogenetic tree was constructed with molecular evolutionary genetic analysis (MEGA), version 4.0 (Tamura *et al.*, 2007) using a neighbor-joining algorithm and the Jukes–Cantor distance estimation method with bootstrap analyses where 1000 replicates were performed (Saitou & Nei, 1987).

3.8.3 Amplification of 5.8S-ITS rDNA

Internal transcribed spacer regions (ITS1 and ITS2) and 5.8 S of nuclear ribosomal DNA was amplified according to method described by White *et al.* (1990). The reaction mixture for PCR amplification for all the isolates was prepared using 50 µl volume containing 2.5 µl ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3'), 2.5 µl ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), 2.0 µl template, 25 µl Taq mix (Roche, USA) and 18 µl PCR water. The PCR conditions was: 40 cycles including an initial denaturation at 95°C for 4 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 2 min followed by final extension at 72°C for 7 min and holding at 4°C. PCR products were separated on a 2% agarose gel containing 5 µl of ethidium bromide and visualized under UV light. The sizes of the PCR products were estimated by comparing them to a 100 bp Gel pilot DNA Molecular weight marker (Qiagen, USA).

3.8.4 Purification of Samples for Sequencing

Amplicons amplified using NLI and ITS primers were purified using Roche Sequencing Purification kit according to manufacturer's instructions (Roche, USA) and taken to ILRI for sequencing.

3.8.5 Restriction Analysis of the PCR Products

Amplified internal transcribed spacer regions (ITS1 and ITS2) and 5.8S of nuclear ribosomal DNA was used for restriction analysis. An aliquot of PCR product 10 µl were digested with 2 µl buffer, 1 µl enzyme and 9 µl PCR water in 22 µl reaction volume, according to manufacturer instructions the restriction enzymes used were *HaeIII* and *HinfI*(Roche USA). PCR aliquots digested with *HaeIII* were incubated 37°C for 1 h then deactivated at 65°C for 10 min, *HinfI* was incubated for fourteen hours then deactivated at 65°C for 10 min. Digested DNA fragments were separated by gel electrophoresis in 3% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel for 2 h at 80 V and compared to a 100 bp marker from (Promega, USA).

3.9 Chemical Analysis

3.9.1 Proximate Composition

3.9.1.1 Crude Protein

Crude protein content of whey (N x 6.25) was determined according to the improved Kjeldahl method (Approved Method 46-12A; AACC, 2000) with slight modifications. 1 g of dried sample was accurately weighed in a nitrogen free-filter

paper and placed in a Kjeldhal flask upon which 1 tablet of Kjeldhal catalyst and 5 ml of concentrated H₂SO₄ were added to the flask. The mixture was digested in a fume cupboard for about 2 h until a clear solution was obtained. A blank sample of only a filter paper, Kjeldhal catalyst and H₂SO₄ was also digested. After cooling, distilled water was added to increase the volume of the mixture to three-quarters of the flask. The flask was connected to the distillation unit after adding 1 ml phenolphthalein and 10 ml 40% NaOH solution. Distillation was carried out until a drop of distillate did not react with Nessler's reagent placed in a test tube. The distillate was collected in a 400 ml conical flask containing 50 ml 0.1 N HCl solution and 2-3 drops methyl orange indicator. The excess HCl solution in the distillate was back titrated with 0.1 N NaOH. The percent nitrogen was calculated as follows:

$$\% \text{ Nitrogen} = \frac{(\text{Titre} - \text{Blank}) \times \text{N HCl} \times \text{Factor} \times \text{Ammonia mass}}{\text{Mass / Volume used}}$$

Protein content was calculated by multiplying the percent nitrogen by 6.25.

3.9.1.2 Total Carbohydrates

Reagents phenol 80% w/v in water was prepared by adding 20 g water to 80 g phenol (Ferak, Berlin) and concentrated H₂SO₄. 1 ml of sample was initially diluted in a 100 ml volumetric flask. In a test tube, 1 ml of diluted sample, 1 ml of water and 0.05 ml of 80% phenol was added and mixed on a vortex test tube mixer. To each tube five 5 ml of concentrated H₂SO₄ was added and mixed on a vortex mixer. The

tubes were then allowed to cool and mixed again using the vortex mixer. The absorbance of the treated samples was read at 490 nm against standards containing 100, 200, 300, 400 and 500-ppm lactose.

3.9.1.3 Total Ash and Mineral Profiling

The ash content of whey was measured according to AOAC Approved Method 942.05 (AOAC, 1984). Approximately 200 ml of sample was taken and dried at 105°C overnight then 5 g of each sample was weighed into a porcelain crucible and placed in a temperature controlled furnace preheated to 550°C. The sample was held at this temperature for 2 h. The crucible was then transferred directly to a desiccator, cooled and weighed. Ash content was reported as a percentage of the whole sample.

For mineral profiling of the samples, 10 ml of sample was taken and 20 ml of 50% HCl was added and the sample was heated until a dark color was observed. The heated sample was filtered and made up to 100 ml using distilled water and the concentrations of Ca, Fe, Zn, Bo and Co determined using an AA-6300 atomic absorbance spectrophotometer (Shimadzu Scientific Instruments, Columbia, USA).

3.9.2 Chemical Oxygen Demand (COD)

0.4 g mercuric sulfate (HgSO_4) crystals with few granules of anti-bumping, 20 ml of x1000 diluted sample and 10 ml standard potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) solution were placed in a refluxing flask connected to the condenser. 30 ml concentrated H_2SO_4 containing silver sulfate (Ag_2SO_4) was added slowly through the open end of the condenser and mixed thoroughly by swirling while adding the acid. The mixture was refluxed for 2 h, cooled and then 90 ml of distilled water added

through the condenser. A blank consisting of 20 ml distilled water instead of sample was treated in the same manner. The excess dichromate was titrated with standard ferrous ammonia sulfate/Mohr`s salt $[(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$ in presence of 2-3 drops ferroin indicator. The end point was indicated by the sharp colour change from blue-green to reddish brown.

$$\text{COD} = \frac{(\text{Titre} - \text{Blank}) \times \text{Oxygen atomic mass} \times 0.25 \times 1000 \times \text{dilution factor}}{\text{Aliquot used}}$$

3.9.3 pH and Total Titratable Acidity (TTA)

The pH meter was calibrated using standardized buffer solutions of acidic and basic values of 4.01 and 9.08 at 25°C before use (TOA pH Meter HM-7B, Tokyo, Japan). The TTA was determined according to Lonner *et al.* (1986). Briefly, approximately 10 ml sample was pipetted into a conical flask and 2 drops of phenolphthalein indicator added. Titration was done using 0.1M NaOH to a faint pink color for 1 min and compared against a white background. The titre volume was noted and used for calculations of TTA, which was expressed as percentage lactic acid as follows;

$$\% \text{ Lactic Acid} = A \times 0.009 \times 100/V$$

where: **A** = ml of 0.1 NaOH required for the titration; and **V** = ml of sample taken for the test. 0.009 is a Constant

3.9.4 Total Soluble Solids (BRIX)

Total soluble solids of the samples were read using a refractometer, which was thoroughly cleaned before use. A small amount of distilled water was placed on the prism of the refractometer and the value read for zeroing machine followed by drying the lens using tissue paper. For Brix measurement, small amount of sample placed on the prism and the value read as a percentage, and the refractometer thoroughly before subsequent reading of samples.

4.0 Optimization of Whey Fermentation using Yeast Isolates

4.1 Effect of substrate concentration, Temperature and inoculums size

Whey collected from Browns Cheese Industry was adjusted to 10, 12 and 15% Brix using commercial lactose (Oxoid) and fermented using various yeast isolates at 30, 35 and 40°C and at different yeast concentration of 10 and 20% for 120 h. Samples were taken periodically after every 24 h for sugar, Total soluble solids and pH were determined as described in 3.9.3 and 3.9.4. The remaining samples were centrifuged at 4000 rpm for 10 min and the supernatant kept in the refrigerator prior to ethanol determination using HPLC.

4.2 Ethanol Determination

The amount of ethanol in the fermented whey samples was determined in 10 AT High Performance Liquid Chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with 10 A refractive index detector (Shimadzu Corp., Kyoto, Japan). Using discovery[®] HSC18 (Supelco-USA) reverse phase column at a temperature of 30°C,

and 0.005M H₂SO₄ as a mobile phase at a flow rate of 0.7 ml/min with a refractive index detector and control temperature of 40°C. Samples were filtered with a 0.45mm membrane filter prior to injection into the machine with 1, 2, 4, 6 and 10% ethanol (Scharlab S.L., Spain) as standard.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Growth, Isolation and Evaluation of Fermenting Ability of Yeasts

In this study, 42 different yeast strains were isolated from twenty samples that included whey, wastewater and swabs. On the basis of morphology characteristics, the isolates were grouped into 5 groups on the basis of colony morphology (Table 3, Figure 3 and 4). All the isolates were white to cream in color, with most being round in form and only seven isolates being undulate. A greater percentage of the isolates had raised elevation with only two isolates having convex elevation. Also, most of the isolates had entire margin with seven isolates being undulate (Table 3).

Twenty eight yeast isolates were found to have lactose-fermenting ability due to their ability to reduce pH of MacConkey media from 7.4 to 5 during fermentation (figure 6). In addition, these isolates were able to ferment lactose when grown on YEPL broth producing gas (Figure 5) These strains were further characterized on the basis of morphological (Table 3) and physiological characteristics (Figure 7 and 8) and also identified using several biochemical tests, including ability to ferment different carbon sugars and molecular characteristics.

Table 3. Phenotypic characterization of yeast isolates

Group*	Colony characteristics				
	Form	Colour	Margin	Elevation	Identity
I	Round	White	Entire	Raised	BM4, BM1, P45, M4, P2, P43, M11, BS4
II	Round	Cream-White	Entire	Convex	P42, YC5
III	Round	Cream	Entire	Raised	P3A, PB9, PB10, PC2, P21, YC1, YC4, YB2, BM9, YC2, P3
IV	Irregular	Cream-White	Undulate	Raised	P41, M3, P44, P22, M15, YC6
V	Round	White	Undulate	Raised	M5

NB:* Shows grouping on the basis of colony Morphology



Figure 3: Colony Morphology of strain P2 (*Kluyveromyces* species)



Figure 4: Cellular Morphology of strain P2 (*Kluyveromyces* species)

Table 4. Mean growth of Yeasts under different pH ranges showing significant differences of the isolates at each pH range

Isolate	pH3.0	pH 4.0	pH 4.5	pH 5.5	pH 6
BM1	1.05 ^a	0.84 ^a	1.26 ^{abc}	0.93 ^a	0.52 ^{bc}
BM4	0.91 ^a	0.50 ^a	1.01 ^{abcdef}	0.74 ^a	0.81 ^{abc}
BM9	0.47 ^a	0.61 ^a	0.91 ^{abcdef}	1.18 ^a	0.84 ^{abc}
BS4	0.73 ^a	0.56 ^a	0.82 ^{abcdef}	0.98 ^a	1.28 ^{ab}
M11	1.00 ^a	0.52 ^a	1.39 ^{ab}	0.91 ^a	1.02 ^{abc}
M15	0.86 ^a	0.81 ^a	1.22 ^{abc}	0.97 ^a	1.12 ^{abc}
M3	0.72 ^a	0.64 ^a	1.14 ^{abcde}	1.16 ^a	1.24 ^{ab}
M4	0.53 ^a	0.57 ^a	0.64 ^{bcdef}	1.38 ^a	1.11 ^{abc}
M5	1.23 ^a	0.95 ^a	1.14 ^{abcde}	1.22 ^a	1.64 ^a
P 42	0.87 ^a	0.52 ^a	1.14 ^{abcd}	1.15 ^a	1.05 ^{abc}
P2	0.76 ^a	0.38 ^a	1.00 ^{abcdef}	0.79 ^a	0.96 ^{abc}
P21	0.68 ^a	0.82 ^a	1.19 ^{abc}	1.23 ^a	1.02 ^{abc}
P22	0.99 ^a	0.62 ^a	1.20 ^{abc}	1.23 ^a	1.17 ^{abc}
P3A	0.79 ^a	0.49 ^a	1.01 ^{abcdef}	0.77 ^a	0.86 ^{abc}
P41	0.70 ^a	0.49 ^a	1.32 ^{ab}	1.13 ^a	0.97 ^{abc}
P43	0.91 ^a	0.67 ^a	1.55 ^a	1.19 ^a	1.25 ^{ab}
P44	1.06 ^a	0.68 ^a	0.87 ^{abcdef}	0.96 ^a	0.71 ^{bc}
P45	0.87 ^a	0.61 ^a	1.21 ^{abc}	1.08 ^a	0.60 ^{bc}
PB10	0.34 ^a	0.93 ^a	0.48 ^{cdef}	0.90 ^a	1.01 ^{abc}
PB9	0.52 ^a	0.58 ^a	1.15 ^{abcd}	0.92 ^a	0.86 ^{abc}
PC2	0.57 ^a	0.93 ^a	1.09 ^{abcdef}	0.82 ^a	0.98 ^{abc}
PC3	0.84 ^a	0.60 ^a	0.97 ^{abcdef}	1.24 ^a	0.79 ^{abc}
YB2	0.25 ^a	0.61 ^a	1.40 ^{ab}	0.96 ^a	1.03 ^{abc}
YC2	0.72 ^a	0.54 ^a	0.94 ^{abcdef}	0.92 ^a	0.36 ^c
YC4	0.89 ^a	0.54 ^a	0.88 ^{abcdef}	0.75 ^a	0.76 ^{bc}
YC5	0.61 ^a	0.60 ^a	0.95 ^{abcdef}	0.91 ^a	0.87 ^{abc}
YC6	0.79 ^a	0.80 ^a	1.60 ^a	0.75 ^a	0.97 ^{abc}
YCI	0.50 ^a	0.54 ^a	0.92 ^{abcdef}	1.02 ^a	0.83 ^{abc}

Table 5. Mean growth of Yeasts under different temperature ranges showing significant differences of the isolates at each Temperature

Isolate	25°C	30°C	37°C	42°C	47°C
BM1	0.79 ^{bcd} e	1.18 ^a	1.47 ^{ab}	0.96 ^{ab}	0.95 ^{ab}
BM4	0.71 ^{cde}	1.41 ^a	1.61 ^a	1.24 ^a	0.78 ^{abcd}
BM9	1.45 ^a	1.22 ^a	1.31 ^{abcdefg}	0.57 ^{ab}	0.52 ^{bcd} e
BS4	0.78 ^{bcd} e	1.26 ^a	0.75 ^{fghi}	0.48 ^{ab}	0.44 ^{cde}
M11	0.74 ^{bcd} e	1.33 ^a	1.38 ^{abcdef}	0.48 ^{ab}	0.23 ^e
M15	1.45 ^a	1.71 ^a	0.93 ^{bcd} efgi	0.51 ^{ab}	0.54 ^e
M3	1.44 ^a	1.33 ^a	1.10 ^{abcdefg} hi	0.75 ^{ab}	0.91 ^{abc}
M4	0.63 ^{de}	1.12 ^a	1.32 ^{abcdefg}	0.65 ^{ab}	0.35 ^{de}
M5	0.68 ^{cde}	1.63 ^a	1.27 ^{abcdefg}	1.19 ^{ab}	0.84 ^{abcd}
P 42	1.28 ^{abcd}	1.05 ^a	1.44 ^{abcd}	0.64 ^{ab}	0.43 ^{cde}
P2	0.89 ^{abcde}	0.92 ^a	1.20 ^{abcdefg} h	1.20 ^{ab}	1.02 ^a
P21	0.87 ^{abcde}	1.37 ^a	0.56 ^{hi}	0.55 ^{ab}	0.48 ^e
P22	1.40 ^a	1.45 ^a	1.42 ^{abcde}	0.81 ^{ab}	0.25 ^e
P3A	0.54 ^e	1.14 ^a	1.28 ^{abcdefg}	0.66 ^{ab}	0.25 ^e
P41	1.32 ^{abc}	1.37 ^a	1.17 ^{abcdefg} hi	0.95 ^{ab}	0.51 ^{bcd} e
P43	1.37 ^{ab}	1.44 ^a	1.60 ^a	0.85 ^{ab}	0.19 ^e
P44	0.51 ^e	1.56 ^a	1.44 ^{abc}	0.53 ^{ab}	0.22 ^e
P45	0.75 ^{cde}	1.29 ^a	0.78 ^{fghi}	0.49 ^{ab}	0.50 ^{bcd} e
PB10	0.74 ^{bcd} e	1.13 ^a	1.04 ^{abcdefg} hi	0.51 ^{ab}	0.21 ^e
PB9	1.08 ^{abcde}	1.05 ^a	0.71 ^{ghi}	0.62 ^{ab}	0.40 ^{de}
PC2	1.13 ^{abcde}	1.50 ^a	1.44 ^{abc}	0.45 ^{ab}	0.28 ^e
PC3	0.68 ^{cde}	0.60 ^a	0.80 ^{cefghi}	0.68 ^{ab}	0.47 ^{cde}
YB2	0.68 ^{cde}	1.27 ^a	0.77 ^{fghi}	0.35 ^b	0.17 ^e
YC2	0.73 ^{bcd} e	1.28 ^a	0.62 ^{hi}	0.72 ^{ab}	0.34 ^{de}
YC4	0.75 ^{bcd} e	1.26 ^a	1.17 ^{abcdefg} hi	0.44 ^{ab}	0.59 ^{bcd} e
YC5	0.88 ^{abcde}	1.04 ^a	1.44 ^{abcd}	0.59 ^{ab}	0.51 ^{bcd} e
YC6	0.79 ^{bcd} e	1.45 ^a	1.03 ^{abcdefg} hi	0.69 ^{ab}	0.24 ^e
YCI	0.61 ^{de}	1.30 ^a	0.93 ^{bcd} efghi	0.58 ^{ab}	0.43 ^{cde}

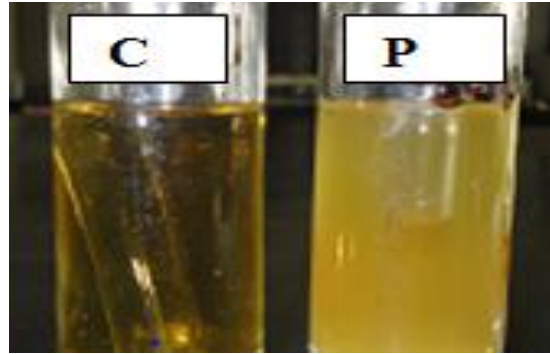


Figure 5. Gas production by lactose fermenting yeast grown on YEPL media. C shows a control where there is no gas formation and P showing positive isolates with gas formation

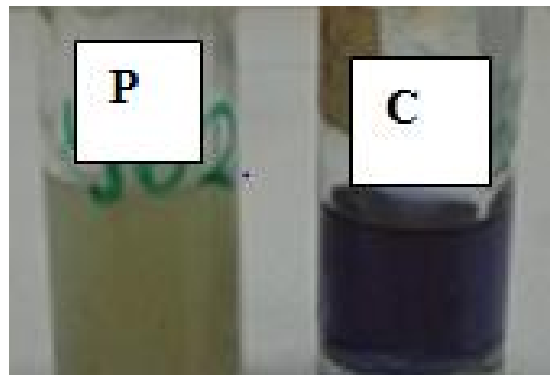


Figure 6. Acid production by yeast isolates cultured on MacConkey media. C denotes control while P is positive isolate that changed the colour of the media after fermentation

4.2 Physiological Characterization

The ability of yeast isolates exhibiting lactose-fermenting ability to grow under different temperature levels and pH ranges was evaluated. The mean growth of the 28 yeasts isolates showed an optimal growth at 30°C and generally good growths at 25 and 37°C that was observed after 24 and 36 h after incubation (Figure 7 and Table 5). However, there was minimal growth observed at 42 and 47°C for 24 h and subsequent decreased growth at 36 and 72 h. Figure 8 and Table 4 depicts the ability of isolated yeast strains to grow under different pH ranges. It was also observed that the mean growth of most yeasts isolates was highest at pH range of 4.5-6.0. Furthermore, yeast isolates were also able to grow at pH 3 and 4.

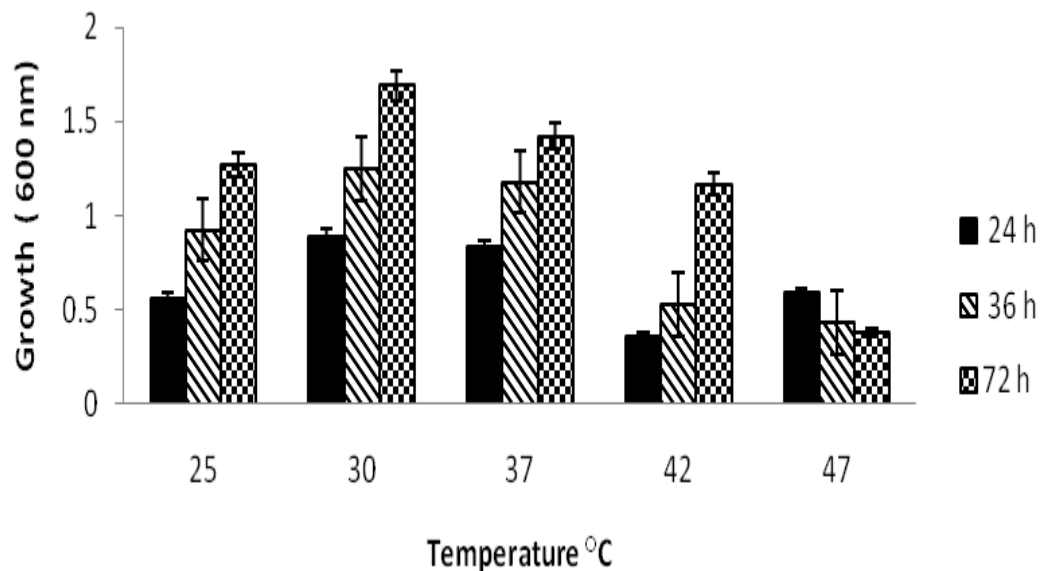


Figure 7. Effect of Temperature on growth of dairy industry yeasts

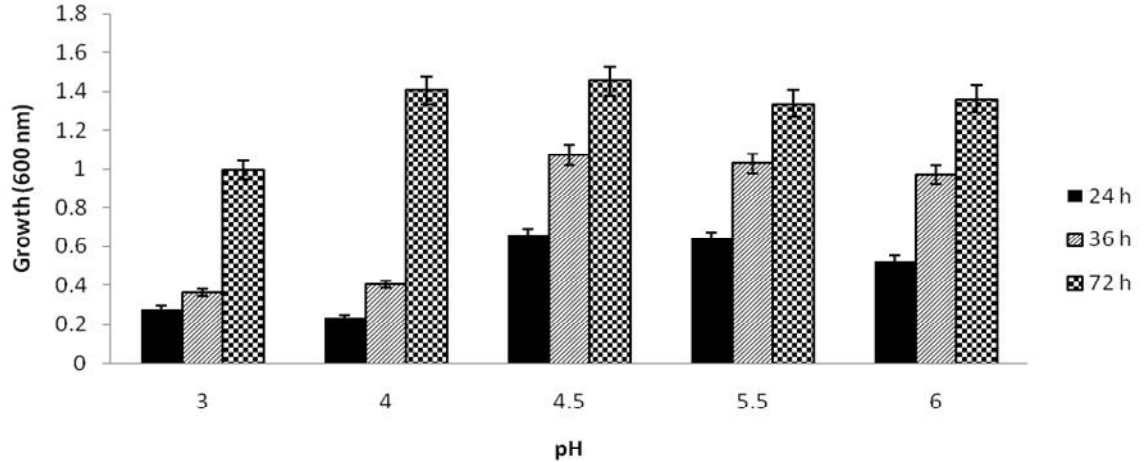


Figure 8. Effect of pH on growth of dairy industry yeasts

4.3 Biochemical Characterization of Dairy Industry Yeasts (API® 20C AUX)

All the 28 yeast isolates that had showed ability for lactose fermentation were subjected to various biochemical tests using an API® 20C AUX kit. The API 20C system (bioMérieux, Marcy l’Etoile, France)

All isolates were able to utilize glucose, glycerol, calcium 2-ceto gluconate, maltose, saccharose, trehalose and raffinose while only two isolates BM9 and PB9 were not able to assimilate xylitol. Only 6 isolates (PC2, BM4, M11, P2, BM9 and P21) were able to utilize adonitol (Table 6). Twelve isolates were negative for inositol and this were YC1, BM1, P41, P42, BM9, YC5, M5, YC6, PC3, P3A, M4 and YC2 whereas isolate BM9 and P3A were negative for sorbitol (Table 6). Three isolates were negative for MDG namely P41 YC5 and M4. PC2, BM4, BM1, P41, P42, BM9, P22, YC5, M4, P45, P21, YC2 and PB9 were positive for melezitose (Table 4). Based on results achieved through the use of AP1 kit, the closest relatives

of the yeast isolates were mainly of clinical nature as shown in Table 6, which differed from the results of molecular characterization.

Table 6. Biochemical characterization of yeast isolates showing their closest relative on the basis of API kit

	PC2	BM4	YC1	BM1	M11	M15	YC4	P2	P41	BS4	P42	BM9	P22	YC5	M5	YC6	PC3	P3A	M4	P45	P21	YC2	PB9
Assimilation of Carbohydrates																							
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ceto-G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+	+	.	+	+	+	+	.	+
Adonitol	+	+	.	.	+	.	.	.	+	.	.	+	+	.
Xylitol	+	+	.	.	+	.	.	.	+	.	.	+	.	.	.	+	+	.
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	.	.	+	+	+	+	+	+	.	.	+	.	.	.	+	+	.	.	+	.	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	.	+	+	+	+
MDG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+
NAG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Celiobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	+	+	+	+	.
D-Melezitose	+	+	.	+	+	.	+	+	+	+	+	.	.	.	+	+	+	+	+
D-Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Identity by API kit (Closest relative)	<i>C. famata</i>	<i>C. famata</i>	<i>C. albidus</i>	<i>C. albidus</i>	<i>C. famata</i>	<i>C. humicola</i>	<i>C. humicola</i>	<i>C. famata</i>	<i>C. quilliermondii</i>	<i>T. asahii</i>	<i>C. albidus</i>	<i>T. asahii</i>	<i>C. humicola</i>	<i>C. laurentii</i>	<i>C. laurentii</i>	<i>T. mucooides</i>	<i>T. mucooides</i>	<i>T. mucooides</i>	<i>C. laurentii</i>	<i>C. humicola</i>	<i>T. Mucooides</i>	<i>C. laurentii</i>	<i>T. mucooides</i>

4.4 Molecular Genetic Characterization

In order to identify and determine the correct phylogenetic position of the yeast isolates, molecular genetics identifications were performed. In this way, the genomic DNA was extracted from the all isolated yeast strains, and primers NL1 and NL4 were used for the amplification and sequencing of the D1/D2 region of the 26S rRNA gene. As shown in (Figure 9), the size of the amplified 26S rRNA gene were about 600 to 650 bp for all the yeast isolates, which is the expected size of 26S rRNA regions.

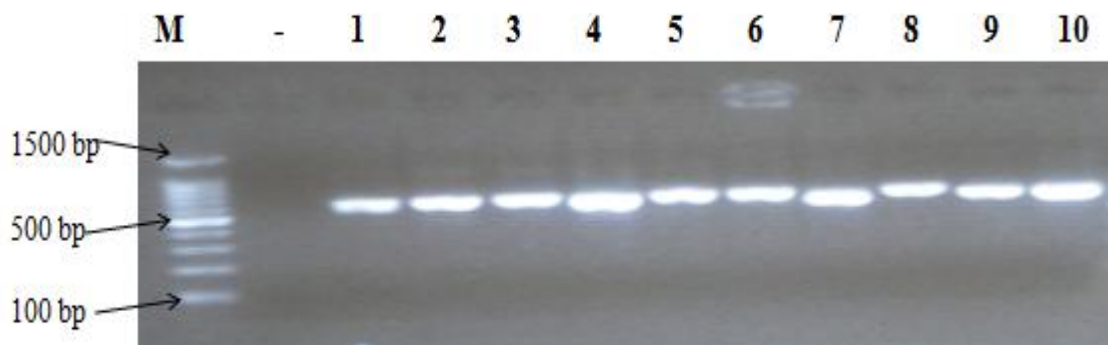


Figure 9. Amplified DNA of D1/D2 domain of isolates run in 2% (W/V) agarose gel. Lanes: M, 100bp marker; -, negative control; 1, BM2; 2, PC2; 3, BM1; 4, YC6; 5, PB9; 6, P41; 7, BS4; 8, M11; 9, YB2; and 10, P21.

The obtained sequence data for the yeast isolates were compared with the sequences of 26S rRNA regions available in Genbank for each by means of BLAST search of the National Center for Biotechnology Information (NCBI) databases. Alignment of the 26S rRNA gene sequences of these isolates with sequences obtained

by doing a BLAST search showed different similarity to different yeast species (Table 7).

4.4.1 Phylogenetic Analysis of the of D1/D2 domain 26S rDNA and 5.8S-ITS rDNA region Sequences

To confirm the position of each strain in phylogeny, a number of sequences were selected from Genbank database for the construction of a phylogenetic tree using Chromas Pro (Technelysium Ltd) and MEGA4 software (Tamura *et al.*, 2007). Phylogenetic analysis demonstrated that the isolates belonged to various genera.

As shown in (Figures 10, 11 and Appendices 1-4), the phylogenetic trees indicated that isolates BM9, BM4, BM2, M11, P2, YC4 and *Kluyveromyces marxianus* shared one clade cluster with similarities 99%. Therefore, these isolates were identified as *K. marxianus*. In contrast, isolate P41 and *K. lactis* shared one clade cluster with the similarity 98% and was thus identified as *K. lactis* (Figure 12).

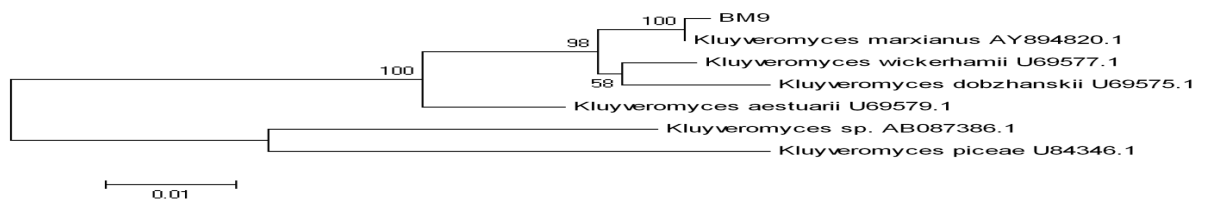


Figure 10. Phylogenetic tree for *Kluyveromyces marxianus* strain BM9 and related species constructed by the neighbor-joining method based on the D1/D2 domain of LSU rRNA gene sequences. Segments corresponding to an evolutionary distance of 0.01 are shown with bars. Accession numbers for sequences are also shown in the phylogenetic tree.

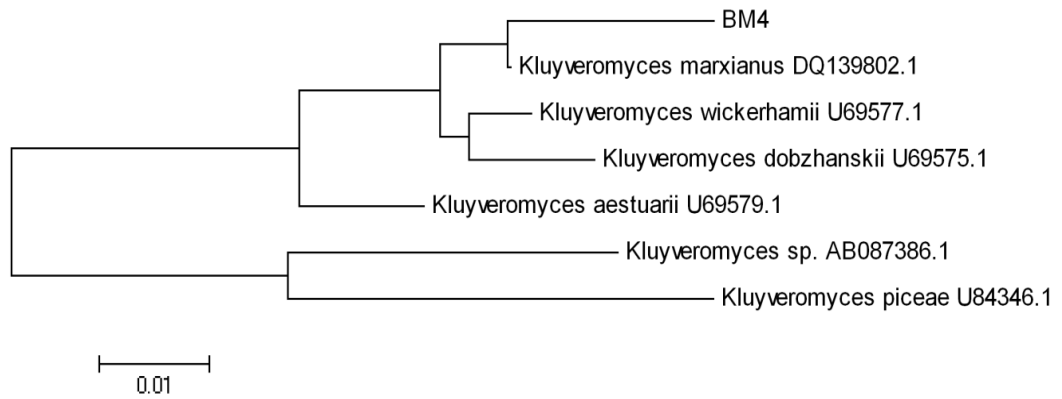


Figure 11. Phylogenetic tree for *Kluyveromyces marxianus* strain BM4 and related species

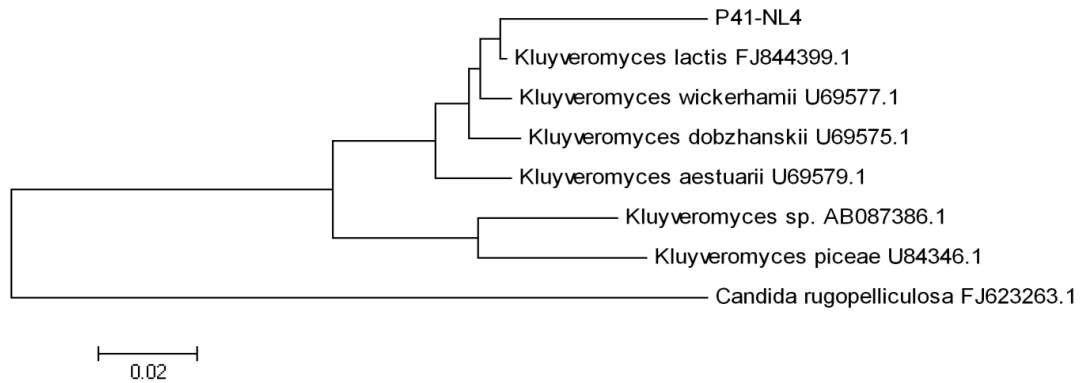


Figure 12. Phylogenetic tree for *Kluyveromyces lactis* strain P41 and related species

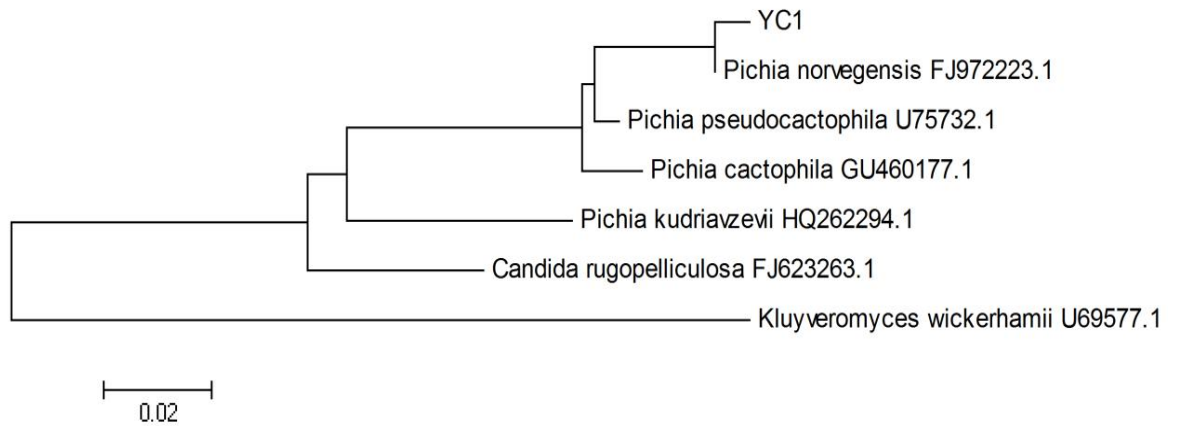


Figure 13. Phylogenetic tree for *Pichia norvegensis* strain YC1 and related species

For isolates YC1, YB2, YC6, PC2, PB9, and PB10, the phylogenetic trees (Figures 13 and 14, and Appendices 5-8) were constructed by selected sequences representing *Pichia* sp. from Genbank database. While isolates YC1, YC6 & PC2 and *Pichia norvegensis* shared one clade cluster with 99% similarity, isolates YB2, PB9 and PB10 were identified as *Pichia cactophila* due to sharing of a clade cluster with 99% similarity. On the basis of phylogenetic analysis, isolates YC5, BM1, & P22 were identified to belong to genera *Candida*, where isolate YC5 and BM1 shared 94 and 99% similarity, respectively, with *Candida inconspicua* while P22 and P21 had 99% similarity with *Candida tropicalis* (Figures 15, 16, and 17, and Appendices 9-13). On the other hand, phylogenetic analysis of isolates M3, P42, P43 and P45 revealed that these isolates were closely related to *Yarrowia lipolytica* with similarity 99% similarities.

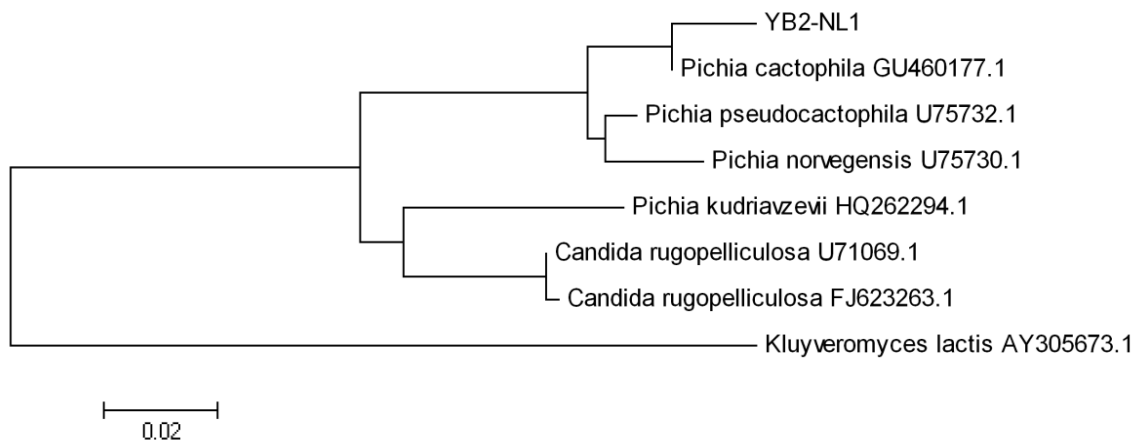


Figure 14. Phylogenetic tree for *Pichia cactophila* strain YB2 and related species.

During the study, some isolates which included isolates BS4, M4, M5, M15, P45 and PC3 could not be correctly identified using the sequences of the D1/D2 domain of the 26S rDNA gene. It has previously been demonstrated that the 5.8S-ITS rDNA analysis is a reliable routine technique for the differentiation of yeasts at species level. Consequently, the sequences of the internal transcribed spacers (ITS1 and ITS2) of 5.8S-ITS rDNA were also performed and the phylogenetic analysis applied for the identification of the above isolates.

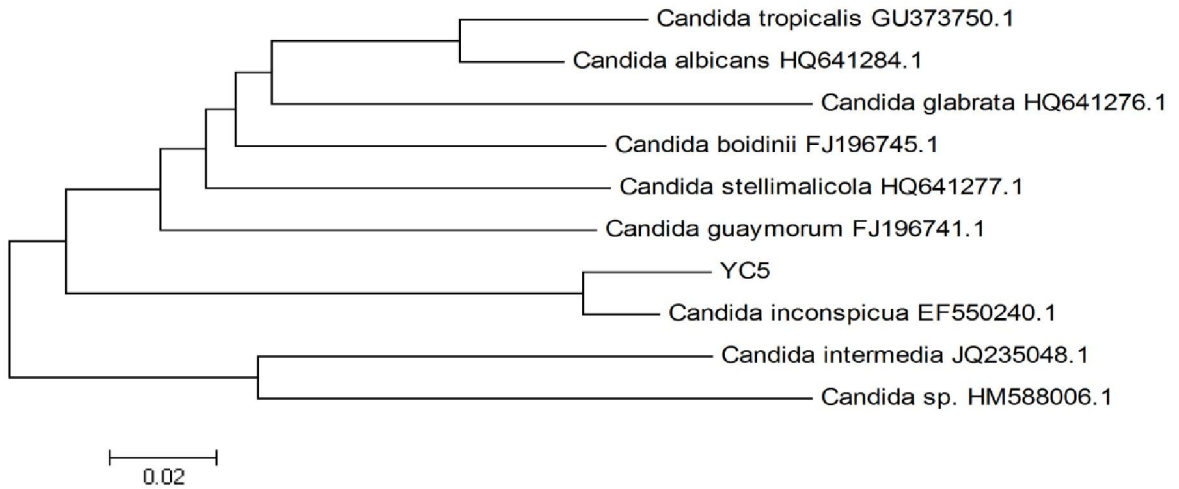


Figure 15. Phylogenetic tree for *Candida inconspicua* strain YC5 and related species

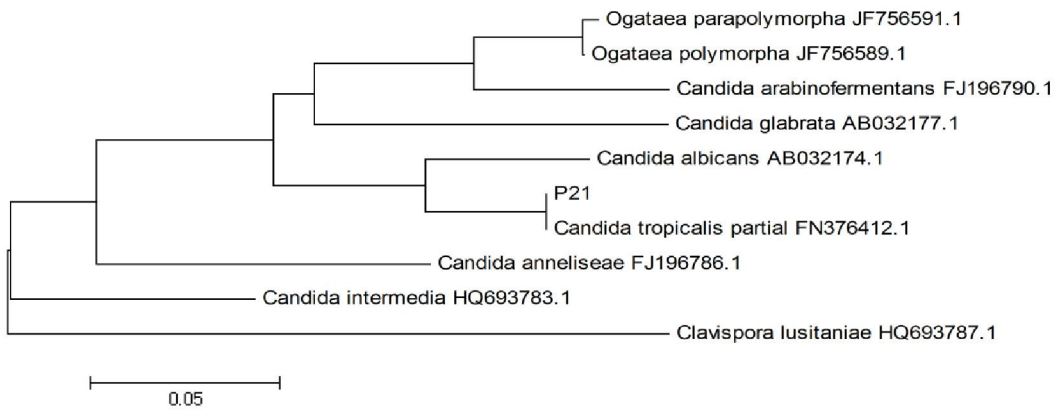


Figure 16. Phylogenetic tree for *Candida tropicalis* strain P21 and related species

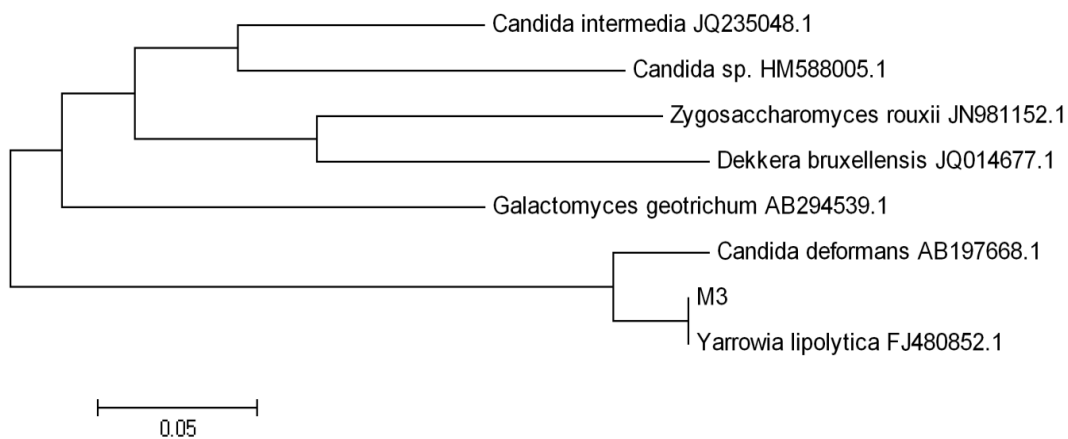


Figure 17. Phylogenetic tree for *Yarrowia lipolytica* strain M3 and related species

BLAST results revealed that the six isolates BS4, M4, P21, M5, M15, P45 and PC3 were identified as *K. marxianus*, *C. catenulate*, *C. tropicalis*, *Y. lipolytica*, *Y. lipolytica*, *Y. lipolytica*, and *P. cecembensis*, respectively, due to homologies >95% for all the isolates (Table 7 and Appendices 14-19). The results obtained from the ITS region show that this gene region is a useful marker for identification of yeast species.

The identity of the isolates based on the homologies of the sequences of the 26S rDNA, D1/D2 domain, and ITS region with those deposited in the Genbank databases is summarized in Table 7. Therefore, the results of the study is consistent with other studies that have reported that molecular methods based on the combination of sequences of the 26S rDNA, D1/D2 domain, and ITS region are rapid and precise tools when compared with physiological methods for the identification and typing of yeast species (White *et al.*, 1990).

Table 7. Identification of yeast isolates by comparative sequence of the D1/D2 domains of 26S rDNA and the 5.8S-ITS rDNA^a region.

Isolate	Identity (%)	Closest Relative (Accession Number)
BM1	99%	<i>Candida inconspicua</i> (U71062.1)
BM2	99%	<i>Kluyveromyces marxianus</i> (FJ896140.1)
BM4	99%	<i>Kluyveromyces marxianus</i> (DQ139802.1)
BM9	99%	<i>Kluyveromyces marxianus</i> (AY894820.1)
BS4 ^a	99%	<i>Kluyveromyces marxianus</i> (JX174415.1)
M3	99%	<i>Yarrowia lipolytica</i> (FJ480852.1)
M4 ^a	99%	<i>Candida catenulata</i> (JN8370 95.1)
M5 ^a	95%	<i>Yarrowia lipolytica</i> (HE660067.1)
M11 ^a	99%	<i>Kluyveromyces marxianus</i> (HQ436414.1)
M15	99%	<i>Yarrowia lipolytica</i> (FJ515197.1)
P2	99%	<i>Kluyveromyces marxianus</i> (HQ436414.1)
P21	99%	<i>Candida tropicalis</i> (FN376412.1)
P22	99%	<i>Candida tropicalis</i> (GU373750.1)
P41	98%	<i>Kluyveromyces lactis</i> (FJ844399.1)
P42	99%	<i>Yarrowia lipolytica</i> (FJ480852.1)
P43	99%	<i>Yarrowia lipolytica</i> (GU373759.1)
P45 ^a	99%	<i>Yarrowia lipolytica</i> (DQ659346.1)
PB9	99%	<i>Pichia cactophila</i> (GU460184.1)
PB10	99%	<i>Pichia cactophila</i> (GU460181.1)
PC2	99%	<i>Pichia norvengensis</i> (FJ972223.1)
PC3 ^a	95%	<i>Pichia cecembensis</i> (EU315768.1)
YB2	99%	<i>Pichia cactophila</i> (GU460184.1)
YC1	99%	<i>Pichia norvengensis</i> (FJ972223.1)
YC4	99%	<i>Kluyveromces marxianus</i> (GQ121676.1)
YC5	94%	<i>Candida inconspicua</i> (EF550240.1)
YC6	99%	<i>Pichia norvengensis</i> (AB278168.1)
YC2	99%	<i>Pichia norvengensis</i> (FJ972223.1)

4.4.2 Discrimination of Yeast Species by PCR-RFLPs of the 5.8S-ITS rDNA Region

To determine the species, a PCR-RFLP analysis of the 5.8S-ITS rDNA region was also used to identify 15 yeast isolates (Figure 18, 19 and 20). The 5.8S-ITS rDNA region was initially PCR-amplified using primers ITS1 and ITS4. As shown in Figure 14, PCR products showed a unique variation for the different species analysed, for example, 750 bp for *K. marxianus*, 500 bp for *P. norvergensis* and 400 bp for *Y. lipolytica*. Fifteen different restriction patterns were generated by using two restriction enzymes *Hinf*I and *Hae*III, whereby the sizes of the PCR product and restriction fragments were visually estimated by comparison with a 100-bp DNA ladder (Figure 19 and 20). When using *Hinf*I, the amplicons of strains P42, P43, M3, YC6, YB2, YC2 and PB9 gave restriction fragments of 190 and 290 bp, while strain BM1 gave resultant fragments at 200 and 280 bp from the original 500 bp amplicon. Similarly, *Hae*III digestion of 510 bp amplicon in strain PC3 resulted in two fragments of 210 and 300 bp. In contrast, the digestion of BM2, M11, BS4, and BM4 PCR product gave fragments of 300, 220, 130 and 100 bp (Table 8). When *Hae*III was used, no restriction digestion was observed for isolate BM1, P42, P43 and M3, with two fragments of 400 and 110 bp being observed for strain PC3. On the other hand, YC6 digestion gave two bands at 380 and 100 bp while BM2 gave three bands at 100, 200 and 350 bp and M11 giving fragments at 100, 210 and 480 bp. Finally, BS4 and BM4 had fragments of 650 and 100 bp with strains YB2 and YC2 giving 380 and 100 bp, while isolate P3A had fragments 400 and 100 bp (Table 8). These

results demonstrated that heterogeneity in the sequence of the 5.8S-ITS rDNA region can be used for the discrimination between closely related yeast isolates.



Figure 18. Amplified DNA of 5.8 S ITS DNA of 14 isolates run in 2% (W/V)

agarose gel. Lanes: M, 100 bp marker; -, negative control; A, PB9; B, M15; C, BS4; D, BM2; E, BM4; F, P2; G, PC2; H, YC2; I, YC1; J,P42; K, P21; L, YB2; M, P45; and N, P43.

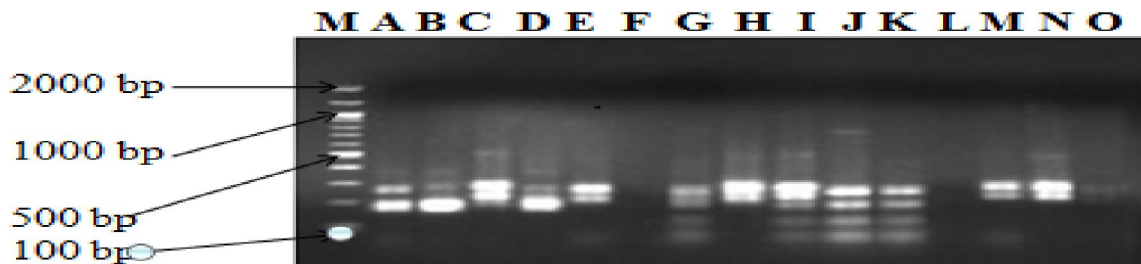


Figure 19. Amplicons digested with *HinfI* restriction enzyme. Lanes: A, P42; B, P43; C, PC3; D, M3;E, BM1; F,P2; G, BM2; H, YC6; I, M4; J, BS4; K, BM4; L, P21; M, YB2; N, YC2; and O, PB9.

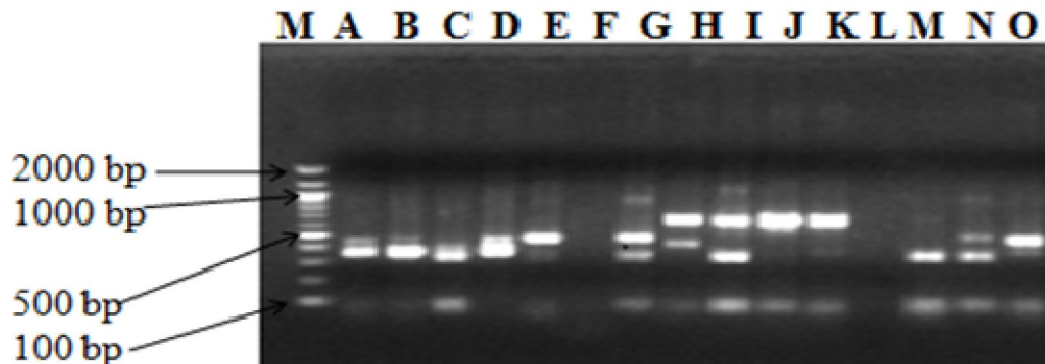


Figure 20. Amplicons digested with *Hae*III restriction enzyme. **Lanes: A, P42; B, P43; C, PC3; D, M3; E, BM1; F, P2; G, YC6; H, BM2; I, M4; J, BS4; K, BM4; L, P21; M, YB2; N, PB9; and O, P3A.**

Table 8. Length in bp of the PCR-amplified products of 5.8S-ITS rDNA region and ITS-RFLP obtained with two restriction endonucleases

Lane	Isolate	PCR product length (bp)	Restriction fragment length (bp)	
			<i>Hinf</i> I	<i>Hae</i> III
A	P42	480	190+290	480
B	P43	480	190+290	480
C	PC3	510	210+300	400+110
D	M3	480	190+290	480
E	BM1	500	200+280	500
H	YC6	480	190+290	380+100
G	BM2	750	300+220+130+100	100+300+350
I	M11	750	300+220+130+100	60+210+480
J	BS4	750	300+220+130+100	650+100
K	BM4	750	300+220+130+100	650+100
M	YB2	480	190+290	380+100
N	YC2	480	190+290	380+100
0	PB9	480	190+290	ND ^a

ND^a not detected

4.5 Ethanol Fermentation by Isolated Yeast Strains

4.5.1 Characterization of whey Samples

The whey used in the study had a COD of 68 000 mg/l, the protein content (dry weight basis) of 1.02%, ash content of 0.55%, and lactic acid content of 0.69% (Table 9). The mineral content included: boron, 40 mg/l; iron, 4.5 mg/l; calcium,

28.37 mg/ml and zinc content of 1.52 mg/l. However, no cobalt was detected while the initial total soluble solid was 5.3% with a total carbohydrate content of 5.3 mg/l and pH of 5.2.

Table 9. Characteristics of whey used for ethanol fermentation

Properties	Amount
Crude protein (dry weight) (%)	1.02
Chemical Oxygen Demand (COD) (mg/l)	68,000
Ash content (%)	0.55
Lactic acid (%)	0.69
Boron (mg/l)	40
Iron (mg/l)	4.5
Calcium (mg/l)	28.37
Zinc (mg/l)	1.52
Cobalt (mg/l)	ND ^a
Total Soluble solids (TSS) (%)	5.3
pH	5.2
Total carbohydrate (mg/ml)	5.9

ND^a not detected

4.5.2 Ethanol Fermentation

Four yeast strains chosen initially due to their robust lactose fermentation ability (*K. marxianus* strain BM9, *K. marxianus* strain BM4, *K. lactis* strain P41, and *P. cactophila* strain YB2) were used for further bioethanol production experiments from industrial whey.

As shown in Table 10, fermentation using *K. marxianus* strain BM9 yielded an ethanol concentration of 4.91 and 1.28% at pH 4.5 and 6, respectively. At different sugar concentrations, the strain also yielded ethanol concentrations of 4.08, 2.10, and 1.74% at 10, 12, and 15% Brix sugar concentration, respectively. Culturing at 35°C resulted in the highest ethanol production compared to a yield of 0.82 and 0.50% when fermentation was carried at 30°C and 40°C, respectively. Ethanol production of 1.02 and 0.62% was achieved at inoculation rates of 10 and 20% yeast concentrations, indicating that the rate of yeast inoculation did not result in significant differences in ethanol production. In contrast, *K. marxianus* strain BM4 yielded an ethanol concentration of 5.25 and 2.63% at pH 4.5 and 6, respectively. In addition, ethanol concentration at different sugar concentrations were 4.02, 1.58 and 3.06% when cultured in whey with adjusted Brix at 10, 12 and 15%, respectively. However, no significant differences were observed at different temperatures (0.84, 0.68, and 0.58 ethanol concentration at temperatures 30, 35 and 40°C, respectively) and yeast inoculation rates (1.83 and 1.46 % ethanol yield at 10 and 20%, respectively (Table 10).

Similar to *K. marxianus* strain BM4 and *K. marxianus* strain BM9, both *K. lactis* strain P41 and *P. cactophila* strain YB2 also showed higher ethanol productivity at pH 4.5 compared to pH 6.0 (Table 10). At pH 4.5, the ethanol productivity for *K. lactis* strain P41 was 5.05% while 3.33% ethanol was realized at pH 6.0. Likewise, ethanol productivity at pH 4.5 by *P. cactophila* strain YB2 was almost double than that at pH 6.0. However, *P. cactophila* strain YB2 did not show any significant differences in ethanol productivity at different fermentation temperatures. In contrast, *K. lactis* strain P41 gave higher ethanol production (1.91%) at 30°C compared to lower ethanol values of 0.48 and 0.56% at 35 and 40°C, respectively. The sugar concentration (% Brix) of whey had similar effect on ethanol production for the two strains, where higher ethanol productivity was achieved at when whey was adjusted to 12% Brix.

Table 10. Effect of different cultural conditions on ethanol production from whey by *K. marxianus* strain BM4, *K. marxianus* strain BM9, *K. lactis* strain P41 and *P. cactophila* strain YB2 after 120 h*

Culture Condition	Ethanol Production (% w/v)			
	<i>K. marxianus</i> strain BM4	<i>K. marxianus</i> strain BM9	<i>K. lactis</i> strain P41	<i>P. cactophila</i> strain YB2
Initial pH				
4.5	5.25 ± 0.83	4.91 ± 0.86	5.05 ± 0.14	1.40 ± 0.00
6.0	2.63 ± 0.04	1.28 ± 0.52	3.33 ± 0.58	0.77 ± 0.21
Lactose (% Brix)				
10	4.02 ± 0.01	4.08 ± 0.12	1.40 ± 0.31	0.91 ± 0.00
12	1.58 ± 0.02	2.10 ± 0.53	2.21 ± 0.20	2.72 ± 0.01
15	3.06 ± 0.01	1.74 ± 0.03	1.10 ± 0.12	0.60 ± 0.01
Temperature (°C)				
30	0.84 ± 0.07	0.82 ± 0.02	1.91 ± 0.07	0.44 ± 0.01
35	0.68 ± 0.08	2.79 ± 0.01	0.48 ± 0.01	0.47 ± 0.00
40	0.58 ± 0.08	0.50 ± 0.04	0.56 ± 0.15	0.50 ± 0.05
Yeast (w/v)				
10	1.83 ± 0.33	1.02 ± 0.72	0.71 ± 0.01	0.30 ± 0.06
20	1.46 ± 0.01	0.62 ± 0.02	0.52 ± 0.06	0.56 ± 0.15

*The results are the means of three separate experiments consisting of three replicates each.

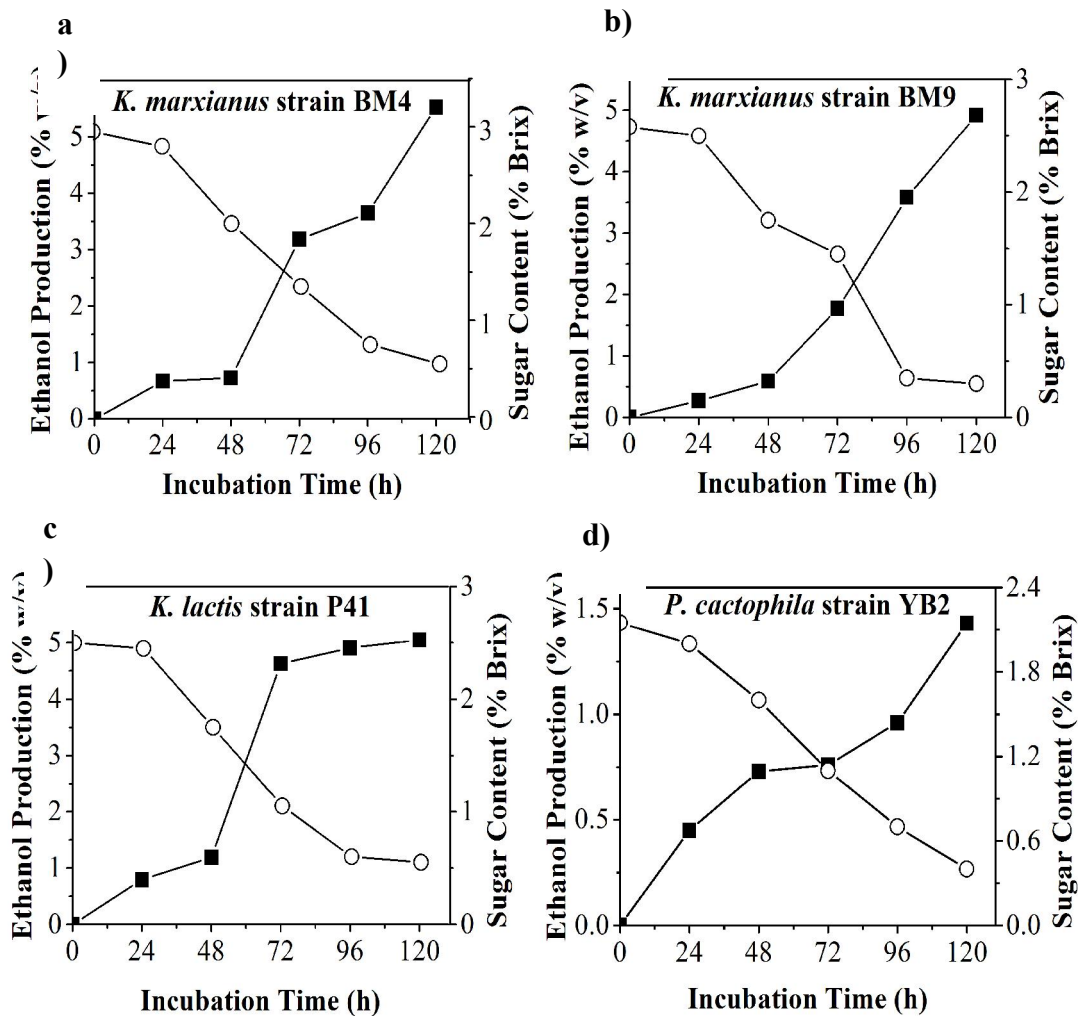


Figure 21. Ethanol productivity (■) versus sugar depletion (○) in whey by selected yeast isolates over varied incubator times. The cultural condition included 10% lactose supplementation, 10% yeast inoculation with whey pH at 4.5

Comparatively, the best condition for ethanol fermentation for *K. marxianus* strain BM9 was achieved at pH 4.5 with a yield of 4.92% ethanol with concurrent reduction of the Brix of 5.3 to 0.3% after 120 h of culturing (Figure 21b). In contrast, the optimum conditions for ethanol production by *K. marxianus* strain BM4 was achieved by culturing in whey supplemented with 10% sugar concentration, 10% yeast concentration at pH of 4.5 and incubated at 30°C temperature. The highest ethanol production observed (5.05%) for *K. lactis* strain P41 was achieved when the strain was cultured at pH 4.5 (Figure 21c). Similarly, the best fermentation condition for *P. cactophila* strain YB2 was observed at pH 4.5 leading ethanol productivity of 1.40% at 120 h (Figure 21 d).

In summary, all the strains showed high ethanol fermentation and productivity at pH 4.5 compared pH 6.0 (Table 10). In the terms of ethanol productivity capacity, *K. marxianus* strain BM4 had a more robust ethanol production ability compared to other strains under study, producing the highest ethanol concentration of 5.525% after 120 h fermentation (Figure 21a), followed by 5.05% for *K. lactis* strain P41, *K. marxianus* strain BM9 and *P. cactophila* strain YB2 with 4.92 and 1.40% ethanol achieved after 120 h fermentation at pH 4.5 (Table 10 and Figure 21).

4.6. DISCUSSION

4.6.1 Biochemical and Physiological Characterization of Yeasts

The aim of this study was to isolate yeast strains from Kenyan dairy industries for subsequent characterization using cultural, biochemical and molecular genetics approaches, and to assess their biotechnological potential in production of ethanol. According to Barnett *et al.* (2000) *Yarrowia* species are usually round, walnut hat or Saturn shaped ascospores usually formed after mating pairs of compatible strains, while *Kluyveromyces* species are smooth, round or reniform ascospores while *Pichia* species usually have variation in ascospores from round hat or Saturn shaped. The morphology of *Kluyveromyces* species have also been described by Kurtzman & Fell (1998) as ovoidal, ellipsoidal and cylindrical to elongate. They may form pseudomycelium but true hyphae are not produced.

The yeast strains isolated in this study were evaluated for the ability to grow under different pH and temperature conditions. The isolates were able to grow optimally at 25, 30 and 37°C with reduced growth at 42 and 47°C. The best pH for growth of the strains was at pH 4.5, 5.5 and 6.0. Reports by Soichi *et al.* (2001) on early death at medium acidification and survival after low pH adaptation in *Cryptococcus neoformans* indicated early growth phase death in susceptible strains at pH 3 was attributable to release of cytoplasm through weakened parts of the cell wall and shrinkage due to dysfunction of plasma membrane at low pH. It is well known that the metabolic activities of yeasts are greatly affected by the temperature at which they grow, whereby temperatures above the optimum causes lower growth rate,

oxygen solubility and change in the cellular composition of yeasts, while an increase in temperature does not inhibit substrate uptake nor does it significantly alter enzymes levels (Slapack *et al.*, 1987). Most yeast species are mesophilic, hence cultures are usually incubated at temperatures of between 20-25°C, although for taxonomic studies 25°C is preferred. Optimum temperatures for growth are higher for some yeast and lower for others while higher temperatures in the range of 30-37°C, are often required for yeasts that are strictly associated with warm-blooded animals (Yarrow, 1998). According to Priest & Campbell (2002), yeasts grow in simple media that contains fermentable carbohydrates to supply energy and carbon skeleton for biosynthesis, adequate nitrogen for protein synthesis, minerals and one or more growth factors. However, sources of carbon may include monosaccharides, disaccharides and trisaccharides. Therefore, carbon assimilation is an important criterion in the taxonomy and identification of yeasts, which depend on organic carbon sources for the energy supply and growth. For example, galactose as a nutrient can be utilized by yeasts in the absence of glucose in the medium; the utilization of galactose indicating the expression of Gal genes, while utilization of carbon sources; sucrose, lactose and cellobiose show expression of genes that activate the synthesis of enzymes invertase, beta-galactosidase and beta glucosidase (Ogawa *et al.*, 2000; Yun *et al.*, 2001; Jimoh *et al.*, 2012).

4.6.2 Molecular Characterization of Dairy Yeasts

Traditionally, yeasts have been identified based on morphological, physiological and biochemical characteristics. According to Barnett *et al.* (2000) and

Guillamón *et al.* (1998), morphological traits and physiological abilities used for identification and characterization of yeast species and strains is strongly influenced by culture conditions and may consequently give false results. In addition, these methods are laborious and time consuming. Thus, molecular biology techniques as alternative and additional methods are increasingly becoming important tools in solving industrial problems.

It has been reported that molecular methods based on the sequences of the 26S rDNA, D1/D2 domain, and ITS region are rapid and precise when compared with physiological methods for the identification and typing of yeast species (Hesham *et al.*, 2006). Sequencing of the D1/D2 of the large-subunit 26S ribosomal DNA is now widely accepted as a standard procedure for yeast identification. Moreover, a 600 bp length of the D1/D2 domain of the 26S rDNA contains sufficient variation to define individuals at the species level (Kurtzman *et al.*, 1998; Frutos *et al.*, 2004; Hesham *et al.*, 2006; Hesham *et al.*, 2009; Hesham *et al.*, 2011). In this study a combination of restriction-fragment length polymorphism (RFLPs) of 5.8S-ITS rDNA region and sequencing of the domains D1/D2 of the 26S rRNA gene was utilized for the discrimination and identification of yeast isolates from the Kenyan Cheese Industries.

Consistent with the results of this study, many studies using both cultural and molecular genetic identification of yeast isolates originating from different dairy products show that *Kluyveromyces* species is most predominant (Vasdinyei & Deak, 2003; Lopandic *et al.*, 2006; El-Sharoud *et al.*, 2009). A study by Vallian *et al.*

(2004) isolated 6 isolates of *K. lactis* and 4 strains of *K. marxianus* that were characterized with appreciable β -galactosidase enzyme activity and tested their potential in single cell proteins (SCP) production. In Brazilian artisanal cheese, *K. lactis* has been shown as the dominant yeast species (Borelli *et al.*, 2006), while *K. marxianus* has been reported as one of the dominant yeast strains in traditional Egyptian dairy products (El-Sharoud *et al.*, 2009). Some strains of thermotolerant *K. marxianus* species have also been isolated from soil samples and their potential in industrial ethanol production demonstrated (Hack & Marchart, 1998). Other habitats where this strain has been isolated include Bantu beer, milk of mastitic cow, asthmatic expectoration and maize meal. Using sequences of D1/D2 domain of 26S rRNA gene, Lopandic *et al.* (2006) showed that *K. marxianus* was one of the most frequently isolated species from various farmhouse milk products available on the Austrian market, although *K. lactis* was also a common isolate.

Other yeast strains isolated in this study such as *Y. lipolytica* and *C. catenulata* have been isolated in cheese in Brazil (Fadda *et al.*, 2004; Borelli *et al.*, 2006). Qing *et al.* (2010) also showed that the dominant species in Qula were *P. fermentas* but *C. zeylanoides* and *P. cactophila* were the major population in milk cake from Yunnan in China. Another study by Vasdinyei & Deak (2003), carried out to check the biodiversity of yeast strains isolated from Hungarian dairy products, classified 62 yeast strains into 26 species and showed that the major strains were *D. hansenii*, *G. candidum*, *Y. lipolytica*, *K. lactis*, and *C. catenulata*. This study also showed that *P. norvergensis*, *P. cactophila*, *P. cecembensis*, *C. inconspicua*, *C.*

tropicalis and *Y. lipolytica* were also present in Kenyan Cheese Industries and occurs together with *K. marxianus* and *K. lactis*.

The polymorphism of the rDNA internal transcribed spacer (ITS), covering the conserved 5.8S rRNA gene region and the two variable flanking regions ITS1 and ITS2, has been used in many studies to identify yeast species through a PCR-RFLP based method (Guillamón *et al.*, 1998; Esteve-Zarzoso *et al.*, 1999). For example, amplification of the ITS region of 5.8S ribosomal DNA give unique amplicon sizes that has been used for rapid identification of yeast strains in dairy products, wine, honey and various foods (Fernandez-Espinar *et al.*, 2000; Carvalho *et al.*, 2005; Martin *et al.*, 2007). Using ITS primers to amplify 5.8S rDNA of 92 isolates from Turkish white cheese, Tansel & Fusun (2009) were able to distinguish between 15 strains of *Y. lipolytica*, 15 strains of *K. marxianus* and 25 strains of *D. hansenii*, through the resultant single fragments of 360, 740 and 640 bp, respectively. Consistent with these results, amplification of 5.8S rDNA with ITS primers in this study gave unique amplified bands of approximately 750, 400 and 500 bp that were important in discriminating *Kluyveromyces*, *Y. lipolytica* and *P. norvengensis*, respectively.

A study carried out by Esteve-Zarzoso *et al.* (1999) showed that *Kluyveromyces* species and *Y. lipolytica* were approximately 740 and 380 bp, respectively, which compared favorably to approximately 750 and 400 bp, respectively, observed in this study. Bockelmann *et al.* (2008), also have reported

amplicon sizes of 726, 375 and 500 bp for *K. marxianus*, *Y. lipolytica* and *P. norvengensis*, respectively, which is consistent with values reported in our study. The restriction fragments comprising the internal transcribed spacer (ITS) and the 5.8S rRNA has been used in identification of yeast species where species belonging to the same genus, show very similar restriction patterns (Carvalho *et al.*, 2005). In this study, RFLP pattern of the different yeast strains isolated made it possible to differentiate among the studied species using a pattern of bands which were characteristic of each genus (Table 6). In a study by Carvalho *et al.* (2005), RFLP of ITS region was used in differentiate seven yeast species belonging to six different yeast genera isolated from honey. In addition, the restriction pattern of the 5.8S-ITS region has also been used to differentiate 132 yeast species belonging to 25 different genera isolated from food and related genera (Esteve-Zarzoso *et al.*, 1999). Consistent with the results of this study, it has been reported that restriction of *Y. lipolytica* 5.8S-ITS fragment by enzyme *Hae*III results to no fragment being observed (Esteve-Zarzoso *et al.*, 1999). The analysis of generated RFLP fragments therefore confirmed that this method could be used to differentiate most of the yeast species from Kenyan cheese industries.

4.6.3 Conversion of whey to ethanol by isolated Yeast Strains

Waste biomass though widely utilized as a raw material for bioethanol production (Gong *et al.*, 1999; Hari *et al.*, 2001; Nigam, 2001), presents challenges as it is deemed expensive. The process is cost intensive as it requires separation of lignin from cellulose, hydrolysis of cellulose to sugars, fermentation of sugar solution to

ethanol and separation of ethanol from water. Therefore, there is an impetus to search for inexpensive and widely available raw materials for bioethanol production.

Fermentation of cheese whey has been extensively investigated and credited to several yeasts (Lark *et al.*, 1997; Lukondeh *et al.* 2005; Ozmihci & Kargi, 2007b; Zhang & Lynd, 2007). For example, *K. marxianus* strains are already being employed in commercial production (Ling, 2008). In this study, cheese whey was fermented at different pH and temperature conditions to yield bioethanol by 4 different yeast strains exhibiting robust lactose-fermenting ability. Our results on fermentations are comparable to those reported by Kadar *et al.* (2011) where 0.51 g ethanol/g lactose yield was achievable by fermentation of whey type 1 at pH 4.5 and 30°C. Thus, the pH has significant effect on the ethanol yield during whey that may be attributable to the yeast using energy to pump out H⁺ ions instead of channeling it for biomass production in response to low whey pH values. Consequently, a higher ethanol yield is obtained because more lactose is used for production of energy instead of formation of biomass.

In terms of culturing temperature, *K. marxianus* strain BM4 and *K. lactis* strain P41 exhibited optimal ethanol production at 30°C, while *K. marxianus* strain BM9 and *P. cactophila* strain YB2 were at 35 and 40°C, respectively. These results slightly deviates from the studies undertaken by Grba *et al.* (2002), where optimal temperature was 34 °C for ethanol production with *K. marxianus*. Ling (2008) and Grba *et al.* (2002) investigated the suitability of five different strains of *K. marxianus*

for alcoholic fermentation of deproteinized whey. The selection of yeast strains was performed at different cultivation conditions: temperature ranging between 30-37°C, lactose concentration of between 5 and 15%, and pH between 4.5-5.0. High temperature alcoholic fermentation of whey has also been demonstrated by Kourkoutas *et al.* (2002) using *K. marxianus* strains MB3. However, Zoppellari & Bardi (2012) found that the best performances for ethanol production by *K. marxianus* were reached at low temperatures (28°C), although high temperatures are also compatible with good ethanol yields in whey fermentations.

Strains of *Kluyveromyces* have been considered the most appropriate for bio-conversion of lactose in whey (Barnett *et al.*, 2000; Caralcho & Spencer 1990). However, incomplete or slow fermentations have been observed for many *Kluyveromyces* strains when concentrated whey or lactose-enriched substrates have been employed (Grubb & Mawson, 1993). This is consistent with results of this study where increases in lactose concentration (% Brix) were concomitantly associated with lower ethanol productivity in all the strains tested. These effects have been attributed to the toxicity of the ethanol produced and/or to inhibition by high salt concentrations, resulting in elevated osmotic pressure (Grubb & Mawson, 1993). Furthermore, it has been reported that increases in lactose concentrations can lead to the accumulation of pyruvate resulting from the greater glycolytic flux in these yeasts, thereby causing a reduction in final biomass yields.

The development of biotechnological applications with *K. marxianus* has been motivated by a number of advantages which includes its ability to grow on a wider range of substrates, at higher temperatures, its specific growth rates (Bellaver *et al.*, 2004). In this study, it was found that ethanol productivity by two strains of *K. marxianus* and one strain of *K. lactis* from whey was higher than that of *P. cactophila*. Several yeasts belonging to the genus *Pichia* such as *P. farinosa*, *P. fermentans* and *P. stipitis* are known to produce ethanol from glucose (Ando *et al.*, 1998). The most promising yeasts that have the ability to use both pentose and hexose sugars are *P. stipites*. There are no reports in literature on utilization of *P. cactophila* for ethanol production from cheese whey. Therefore, this study represents a first report whereby *P. cactophila* strain has been applied in the ethanol production from cheese whey.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The isolation of yeasts strains from dairy industry implied that Kenyan cheese industries harbor novel lactose-fermenting yeasts that are genetically diverse, albeit, not very different from yeasts isolated by other studies from dairy products.
2. Using PCR amplification and phylogenetic analysis, the isolates were clustered into four different genera namely; *Kluyveromyces*, *Pichia*, *Yarrowia*, and *Candida*.
3. Restriction pattern analysis of ITS region using *HaeIII* and *HinfI* permitted a fast and effective identification of yeast species under study.
4. The study also demonstrated the potential of whey fermentation to bio-ethanol by yeast strains *K. marxianus* strain BM4 yielded an ethanol content of 5.52%, *K. marxianus* strain BM9 4.91%, *K. Lactis* strain P41 .5.05% and *P. cactophila* strain YB2 yielded an ethanol content of 1.40% at pH 4.5
5. The study highlighted the feasible application of the yeast isolates in valorization of whey a common pollutant in the local cheese industries.

5.2 Recommendations

The findings of this study recommend that:

- i. More research needs to be carried out on other parameters that can enhance ethanol production efficiency of the isolated strains to maximize their productivity.
- ii. Further characterization need to be carried out to determine genetic differences of the isolates, including characterization of the other byproducts produced during whey fermentation by the isolated yeasts to determine the purity of bio-ethanol produced.

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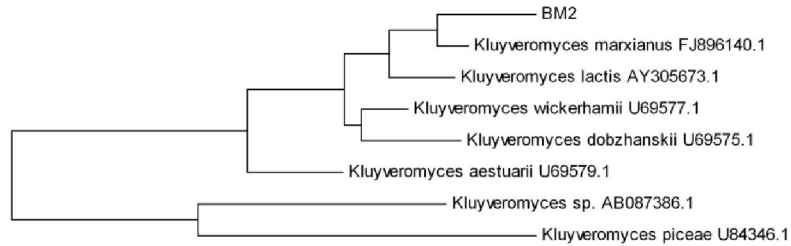
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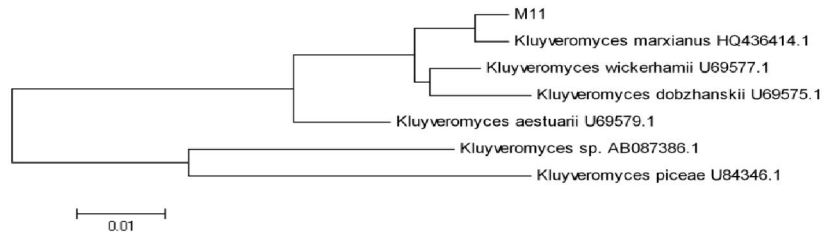
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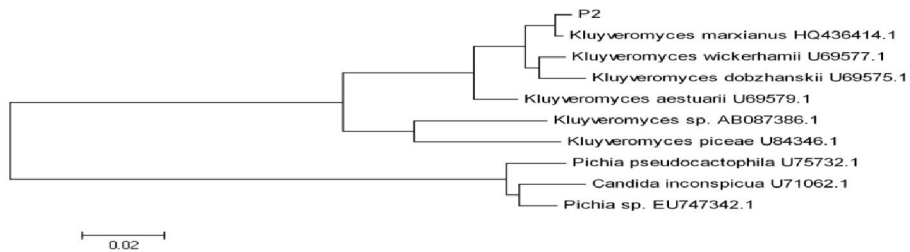
APPENDICES



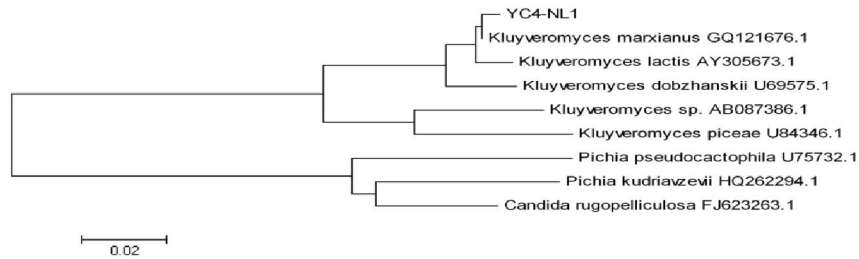
Appendix 1. Phylogenetic tree for *K. marxianus* strain BM2 and related species



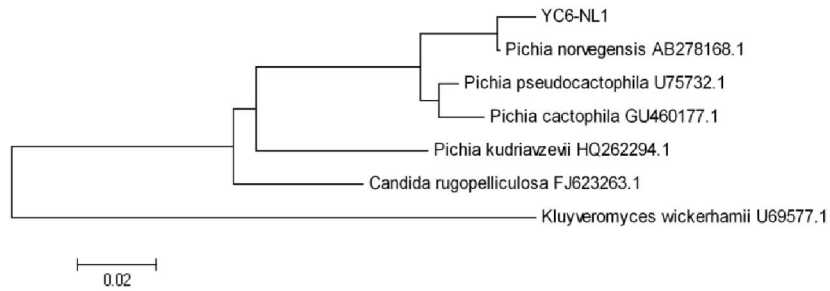
Appendix 2. Phylogenetic tree for *K. marxianus* strain M11 and related species



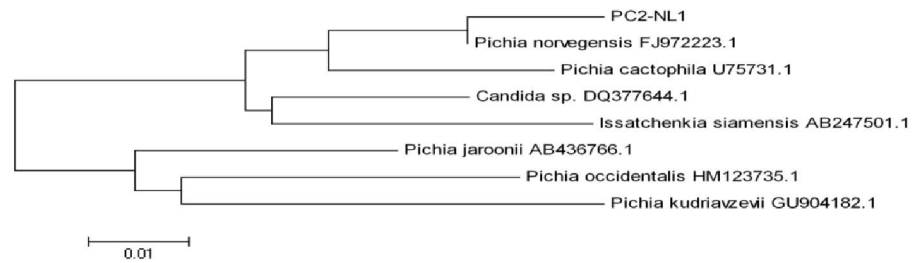
Appendix 3. Phylogenetic tree for *K. marxianus* strain P2 and related species



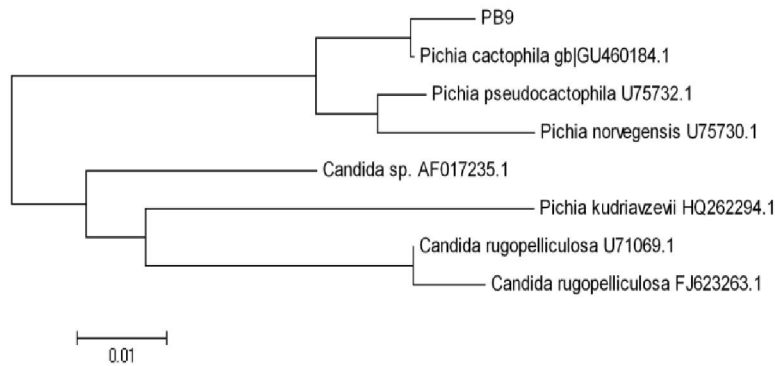
Appendix 4. Phylogenetic tree for *K. marxianus* strain YC4 and related species



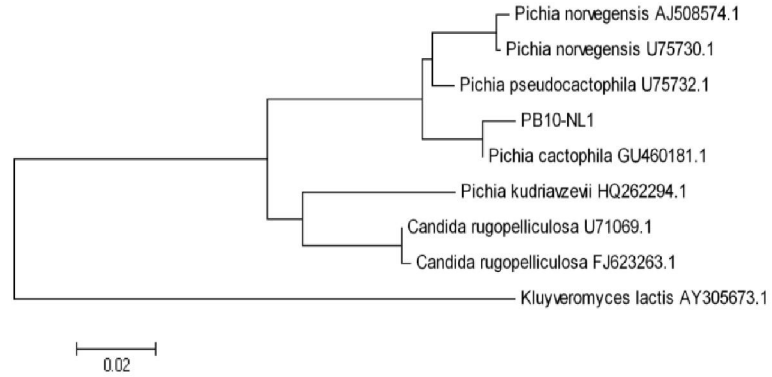
Appendix 5. Phylogenetic for *Pichia norvegensis* YC6 and related species



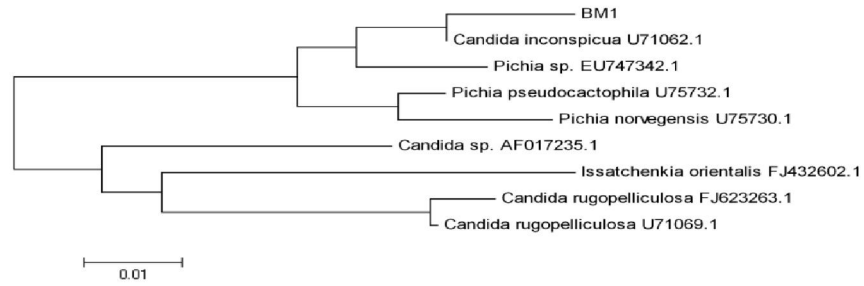
Appendix 6. Phylogenetic tree for *P. norvegensis* strain PC2 and related species



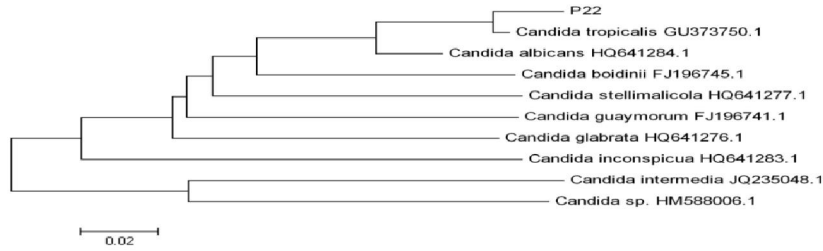
Appendix 7. Phylogenetic tree for *Pichia cactophila* strain PB9 and related species



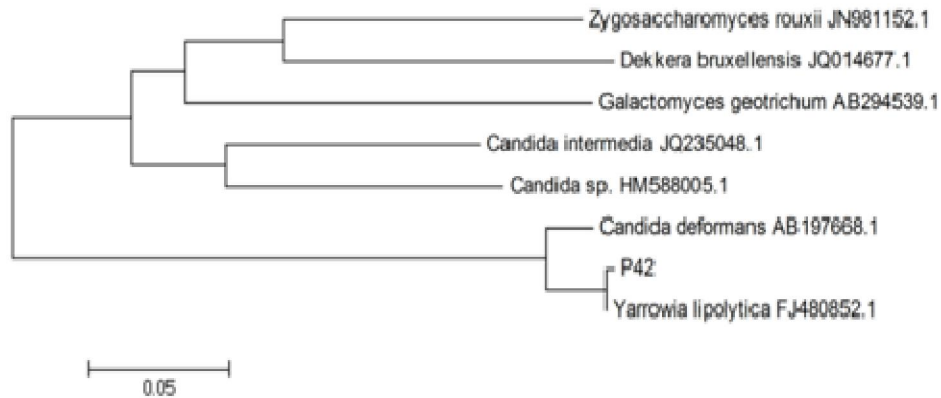
Appendix 8. Phylogenetic tree for *Pichia cactophila* strain PB10 and related species



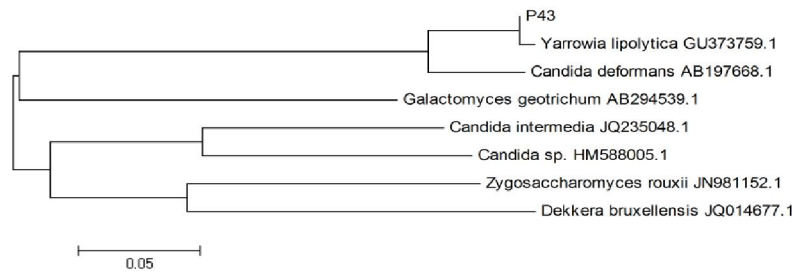
Appendix 9. Phylogenetic tree for *Candida inconspicua* strain BM1 and related species



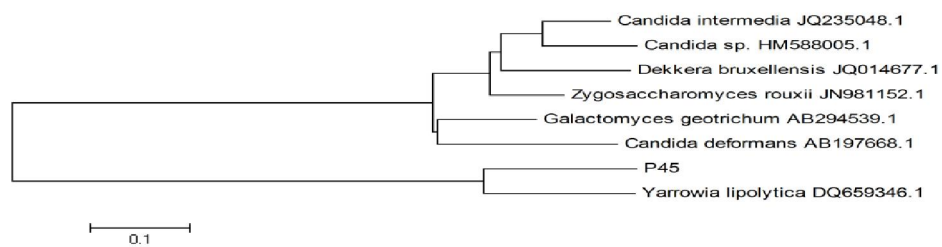
Appendix 10. Phylogenetic tree for *Candida tropicalis* strain P22 and related species



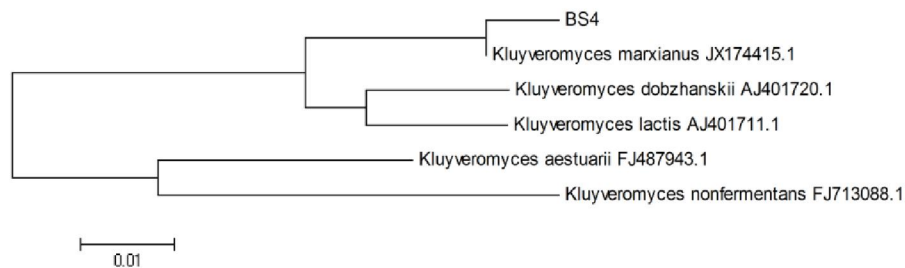
Appendix 11. Phylogenetic tree for *Yarrowia lipolytica* strain P42 and related species



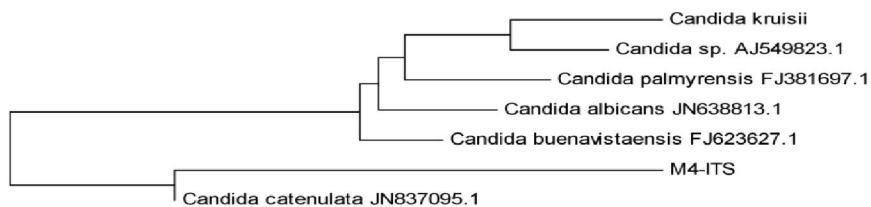
Appendix 12. Phylogenetic tree for *Yarrowia lipolytica* strain P43 and related species



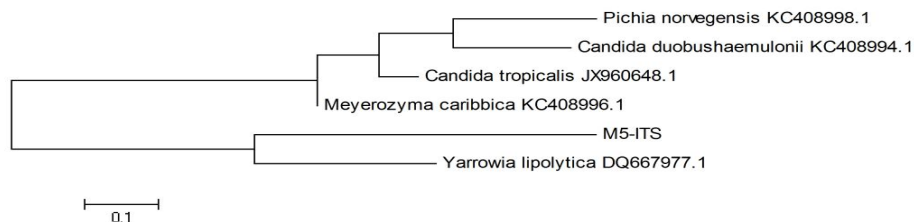
Appendix 13. Phylogenetic tree for *Yarrowia lipolytica* strain P45 and related species



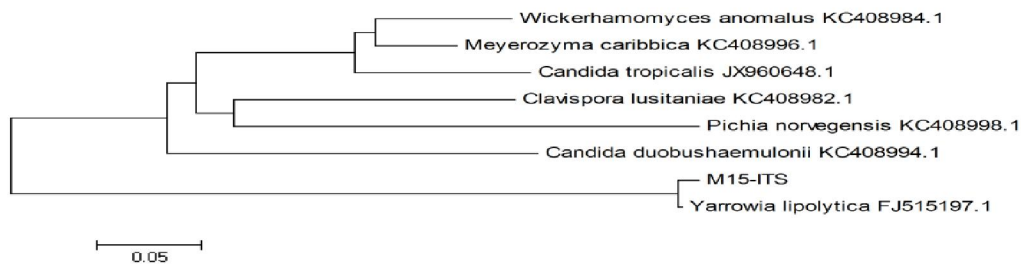
Appendix 14. Phylogenetic tree for *K. marxianus* strain BS4 and related species



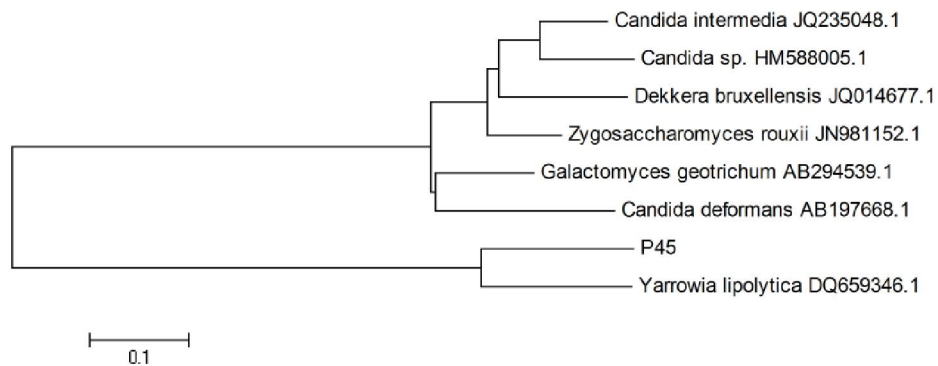
Appendix 15. Phylogenetic tree for *Candida catenulate* strain M4 and related species



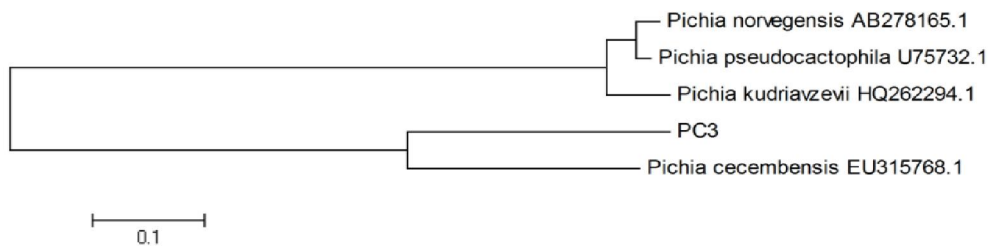
Appendix 16. Phylogenetic tree for *Yarrowia lipolytica* strain M5 and related species



Appendix 17. Phylogenetic tree for *Y. lipolytica* strain M15 and related species



Appendix 18. Phylogenetic tree for *Yarrowia lipolytica* strain P45 and related species



Appendix 19. Phylogenetic tree for *Pichia cecembensis* strain PC3 and related species

Score	Expect	Identities	Gaps	Strand	Frame
1007 bits(545) 0.0() 555/559(99%) 4/559(0%) Plus/Plus					
Query	4	ACCGGGCATTGCCTTAGTACGCGAGT	AGCGGCAAAAGCTCAAATTTGAAATCTGGC	61	
Sbjct	31	ACCGGGATTGCCTTAGTACGCGAGT	AGCGGCAAAAGCTCAAATTTGAAATCTGGC	89	
Query	62	GTCTTCGACGTCGAGTTGTAATTTGAAGAAGGCGACTTTGTAGCTGGTCCTTGCTATG	121		
Sbjct	90	GTCTTCGACGTCGAGTTGTAATTTGAAGAAGGCGACTTTGTAGCTGGTCCTTGCTATG	149		
Query	122	TTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGATCCCAGTTATT	181		
Sbjct	150	TTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGATCCCAGTTATT	209		
Query	182	TGTAAGTGCCTTCGACGAGTCGAGTTGTTGGGAATGCAGCTCTAAGTGGGTGGTAAAT	241		
Sbjct	210	TGTAAGTGCCTTCGACGAGTCGAGTTGTTGGGAATGCAGCTCTAAGTGGGTGGTAAAT	269		
Query	242	TCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGA	301		
Sbjct	270	TCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGA	329		
Query	302	TGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTTGTTGAAAGGGAAGGGCAT	361		
Sbjct	330	TGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTTGTTGAAAGGGAAGGGCAT	389		
Query	362	TTGATCAGACATGGCGTTTCTTCGCTTCGCTGGGCCAGCATCAGTTTAGCGGTTGG	421		
Sbjct	390	TTGATCAGACATGGCGTTTCTTCGCTTCGCTGGGCCAGCATCAGTTTAGCGGTTGG	449		
Query	422	ATAAATCCTCGGGAATGTGGCTCTGCTTCGGTAGAGTGTTATAGCCCGTGGGAATACAGC	481		
Sbjct	450	ATAAATCCTCGGGAATGTGGCTCTGCTTCGGTAGAGTGTTATAGCCCGTGGGAATACAGC	509		
Query	482	CAGCTGGGACTGAGGATTGCGACTTTGTCAAGGATGCTGGCGTAATGGTTAAATGCCGC	541		
Sbjct	510	CAGCTGGGACTGAGGATTGCGACTTTGTCAAGGATGCTGGCGTAATGGTTAAATGCCGC	569		
Query	542	CCGTCTTGACCAACGGAC	560		
Sbjct	570	CCGTCTTGACCAACGGAC	587		

Appendix 20. Sequence alignment of the isolate BM9 against D1/D2 of 26S rDNA sequence data of *Kluyveromyces marxianus* showing 4 base pair substitutions

Score	Expect	Identities	Gaps	Strand	Frame
977 bits(529) 0.0() 534/536(99%) 2/536(0%) Plus/Minus					
Query	15	CAGCATCCTTG--AAAAGTCGCAATCCTCAGTCCCAGCTGGCTGTATTCCCACGGGCTAT			72
Sbjct	549	CAGCATCCTTGACAAAAGTCGCAATCCTCAGTCCCAGCTGGCTGTATTCCCACGGGCTAT			490
Query	73	AACACTCTACCGAAGCAGAGCCACATTCCCGAGGATTTATCCAACCGCTAAAACCTGATGC			132
Sbjct	489	AACACTCTACCGAAGCAGAGCCACATTCCCGAGGATTTATCCAACCGCTAAAACCTGATGC			430
Query	133	TGGCCAGCGAAAGCCGAAGCAAACGCCATGTCTGATCAAATGCCCTTCCCTTTCAACAA			192
Sbjct	429	TGGCCAGCGAAAGCCGAAGCAAACGCCATGTCTGATCAAATGCCCTTCCCTTTCAACAA			370
Query	193	TTTCACGTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCTTCCATCACTGTA			252
Sbjct	369	TTTCACGTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCTTCCATCACTGTA			310
Query	253	TTCGCTATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGC			312
Sbjct	309	TTCGCTATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGC			250
Query	313	TGCATTCCCAACAACCTCGACTCGTCGAAAGCACTTTACAATAACTGGGATCCTCGCCA			372
Sbjct	249	TGCATTCCCAACAACCTCGACTCGTCGAAAGCACTTTACAATAACTGGGATCCTCGCCA			190
Query	373	CACGGGATTCTCACCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGGACCAGCTAC			432
Sbjct	189	CACGGGATTCTCACCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGGACCAGCTAC			130
Query	433	AAAGTCGCCTTCTTCAAATTACAACCTCGGACGTCGAAGACGCCAGATTTCAAATTTGAGC			492
Sbjct	129	AAAGTCGCCTTCTTCAAATTACAACCTCGGACGTCGAAGACGCCAGATTTCAAATTTGAGC			70
Query	493	TTTTGCCGCTTCACTCGCCGTTACTAAGGCAATCCCAGGTTGGTTTCTTTTCCTCCG			548
Sbjct	69	TTTTGCCGCTTCACTCGCCGTTACTAAGGCAATCCCAGGTTGGTTTCTTTTCCTCCG			14

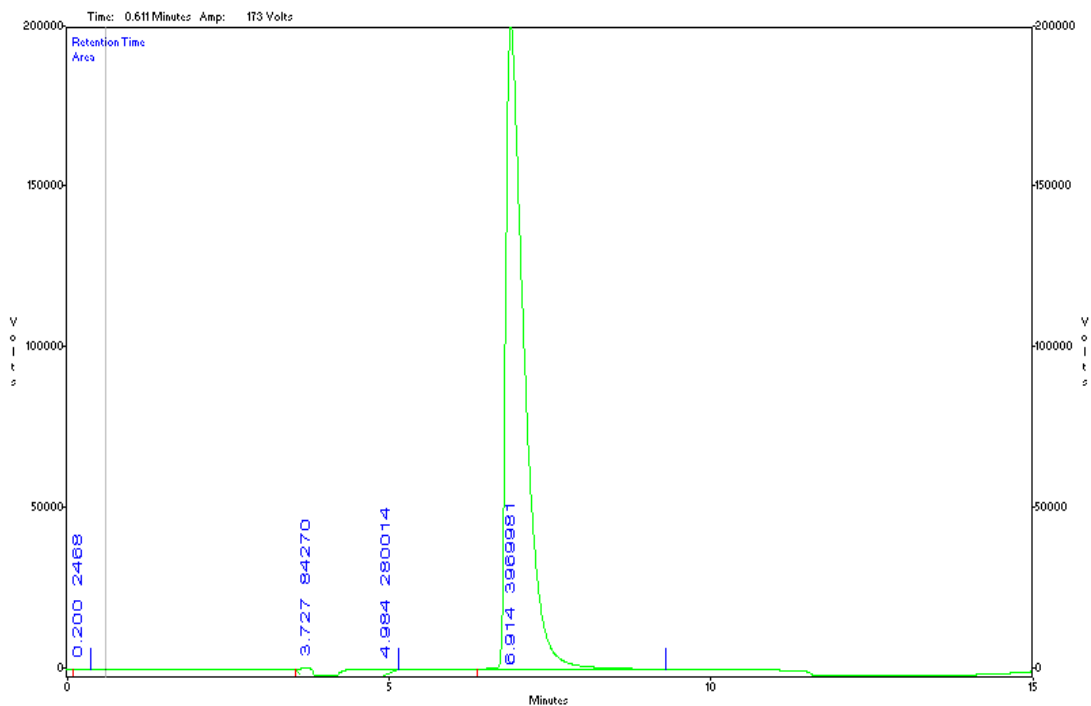
Appendix 21. Sequence alignment of the isolate BM4 against D1/D2 of 26S rDNA sequence data of *Kluyveromyces marxianus* showing 2 base pair substitutions

Score	Expect	Identities	Gaps	Strand	Frame	
861 bits(466)	0.0()	492/504(98%)	3/504(0%)	Plus/Minus		
Features:						
Query	41	CCAGCTGGCTGTATTCCC	CGGGCTATAAC	CTCTACCGAA	CAGAGCCACATTCCCGAG	98
Sbjct	504	CCAGCTGGCTGTATTCCC	CGGGCTATAAC	CTCTACCGAA	CAGAGCCACATTCCCGAG	445
Query	99	GATTTATCC	ACCGCTA	TAAGT	GATGCTGGCCAGCGAAAGCCGAAACGCCATGTC	157
Sbjct	444	GATTTATCC	ACCGCTA	TAAGT	GATGCTGGCCAGCGAAAGCCGAAACGCCATGTC	385
Query	158	TGATCAAATGCCCTTCCCTTTCAACAATTT	ACGTA	CTTTTCACTCTCTTTTCAAAGTT		217
Sbjct	384	TGATCAAATGCCCTTCCCTTTCAACAATTT	ACGTA	CTTTTCACTCTCTTTTCAAAGTT		325
Query	218	CTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTT				277
Sbjct	324	CTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTT				265
Query	278	AGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAACAACCTCGACTCGTCGAAAGCA				337
Sbjct	264	AGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAACAACCTCGACTCGTCGAAAGCA				205
Query	338	CTTTACAAATAACTGGGATCCTCGCCACACGGGATTCTCACCCCTCTATGACGTCCTGTTC				397
Sbjct	204	CTTTACAAATAACTGGGATCCTCGCCACACGGGATTCTCACCCCTCTATGACGTCCTGTTC				145
Query	398	CAAGGAA	TATAAAC	GAGGACCAGCTACAAAGT	CACCTTCTCAAATTACAACCTCGGACGT	457
Sbjct	144	CAAGGAA	CATAGAC	GAGGACCAGCTACAAAGT	TACCTTCTCAAATTACAACCTCGGACGT	85
Query	458	CGAAGACGCCAGATTTCAAATTTGAGCTTTTGCCGCTTCACTCGCCGTT	CTAAGGCA	CT		517
Sbjct	84	CGAAGACGCCAGATTTCAAATTTGAGCTTTTGCCGCTTCACTCGCCGTT	CTAAGGCA	AT		25
Query	518	CCCGGTTGGTTTCTTTTCCTCCGC				541
Sbjct	24	CCCGGTTGGTTTCTTTTCCTCCGC				

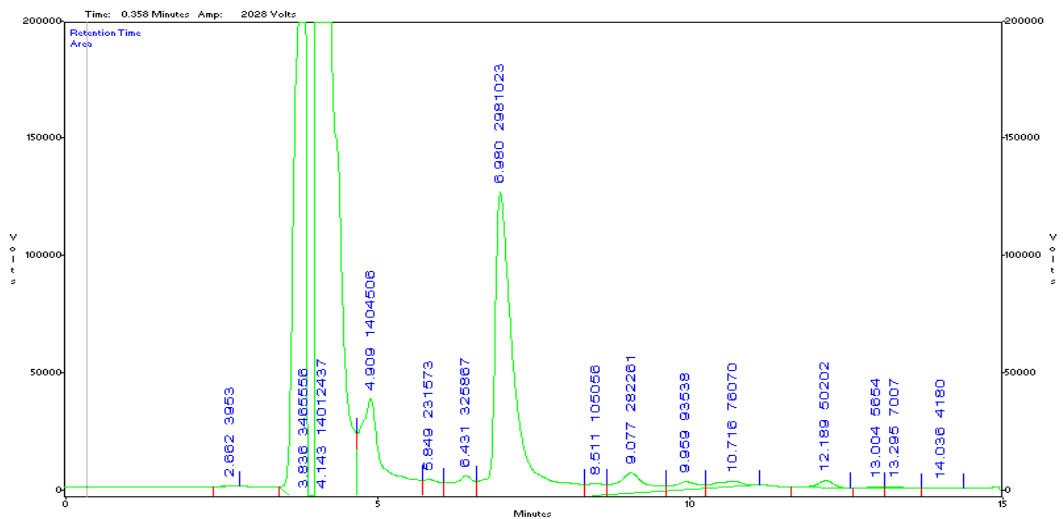
Appendix 22. Sequence alignment of the isolate P41 against D1/D2 of 26S rDNA sequence data of *Kluyveromyces lactis* showing 11 base pair substitutions

Score	Expect	Identities	Gaps	Strand	Frame
1042 bits(564) 0.0() 571/574(99%) 2/574(0%) Plus/Plus					
Features:					
Query	13	CTCAGTAGCGGCGAGTG	-AGCGGCAAGAGCTCAGATTTGAAATCGCAGC	AC	CATGCTGCG 71
Sbjct	9	CTCAGTAGCGGCGAGTGA	AGCGGCAAGAGCTCAGATTTGAAATCGCAGC	-G	CATGCTGCG 67
Query	72	AGTTGTAGATTGCAGGTGGGAGAGTCTGCGTAGGCCGGTGTGGAAGTCCCTTGGAAACAGG			131
Sbjct	68	AGTTGTAGATTGCAGGTGGGAGAGTCTGCGTAGGCCGGTGTGGAAGTCCCTTGGAAACAGG			127
Query	132	GCGCCACTGAGGGTGAGAGCCCCGTGCGATGCCACGCTCTACGTTTTGTACTCCCCTCTG			191
Sbjct	128	GCGCCACTGAGGGTGAGAGCCCCGTGCGATGCCACGCTCTACGTTTTGTACTCCCCTCTG			187
Query	192	ACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCATCTAAGGCTAA			251
Sbjct	188	ACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCATCTAAGGCTAA			247
Query	252	ATACTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGA			311
Sbjct	248	ATACTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGA			307
Query	312	AAAGAGAGTGAAACAGCACGTGAAATGTTGAAAGGGAAGGGTATTGGGCCCGACATGGG			371
Sbjct	308	AAAGAGAGTGAAACAGCACGTGAAATGTTGAAAGGGAAGGGTATTGGGCCCGACATGGG			367
Query	372	AATCGCGCACCGCTGCTCCTTGTGGGCGGCGCTCTGGGCTTTTCCTGGGCCAGCATCGGT			431
Sbjct	368	AATCGCGCACCGCTGCTCCTTGTGGGCGGCGCTCTGGGCTTTTCCTGGGCCAGCATCGGT			427
Query	432	TCTTGCTGCAGGAGAAGGGGCTGTGGAATGTGGCTGCCGCGCTTTGCCGGGGCAGTGTTA			491
Sbjct	428	TCTTGCTGCAGGAGAAGGGGCTGTGGAATGTGGCTGCCGCGCTTTGCCGGGGCAGTGTTA			487
Query	492	TAGCCACTGGGCCAGATGCTGCGTGTGGGGACCGAGGACTGCGGCCGCAAGGTCTCGGAT			551
Sbjct	488	TAGCCACTGGGCCAGATGCTGCGTGTGGGGACCGAGGACTGCGGCCGCAAGGTCTCGGAT			547
Query	552	GCTGGCACAAACGGCGCAATACCGCCCGTCTTGAA			585
Sbjct	548	GCTGGCACAAACGGCGCAATACCGCCCGTCTTGAA			581

Appendix 23. Sequence alignment of the isolate YB2 against D1/D2 of 26S rDNA sequence data of *Pichia cactophila* showing 3 base pair substitutions



Appendix 24. HPLC chromatogram of 6% for ethanol



Appendix 25. HPLC chromatogram of ethanol yield for BM4