“Diversity among the yeasts isolated from naturally fermented cashew apple juice”

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DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

by

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Work carried out under the guidance of

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(Guide)

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Dedicated to

My loving Parents

Mr. Nagesh Prabhu Khorjuvenkar

Mrs. Geeta N. Prabhu Khorjuvenkar

My sister

Diksha N. Prabhu Khorjuvenkar

Certificate

Certified that the research work embodied in this thesis entitled **"Diversity among the yeasts isolated from naturally fermented cashew apple juice"** submitted by **Ms. Supriya Nagesh Prabhu Khorjuvenkar** for the award of **Doctor of Philosophy** degree in **Microbiology** at Goa University, Goa, is the original work carried out by the candidate herself under my supervision and guidance.

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Statement

I hereby state that this thesis for Ph.D. degree on "**Diversity among the yeasts isolated from naturally fermented cashew apple juice**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

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Abbreviations

4-MUG	4-methylumbelliferyl-b-d-glucoside
BHI	Brain Heart Infusion
BIS	Bureau of Indian Standards
BLAST	Basic Local Alignment Search Engine
$CaCl_2$	Calcium chloride
CAJ	Cashew apple juice
CellA	Cellulase Activity
CMC	Carboxy Methyl Cellulose
CO_2	Carbon dioxide
Conc. H_2SO_4	Concentrated Sulphuric acid
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
EEA	Extracellular Enzyme Activity
FDA	Food and Drug Administration
$FeSO_4.7H_2O$	Ferrous sulphate
FSSAI	Food Safety and Standards Authority of India
G.I	Geographical Indication
GlucA	Glucosidase Activity
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
ICAR	Indian Council of Agricultural Research
IS	Indian Standards

ITS	Internal Transcribed Spacer
$KMnO_4$	Potassium permanganate
LipA	Lipase Activity
$MgSO_4.7H_2O$	Magnesium sulphate
MTCC	Microbial Type Culture Collection
mtDNA	Mitochondrial DNA
$Na_2S_2O_3$	Sodium thiosulphate
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PDA/PDB	Potato Dextrose Agar/Potato Dextrose Broth
PectA	Pectinase Activity
PrA	Protease activity
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid
SDA	Starch Degrading Activity
TA	Titratable Acidity
UrA	Urease Activity
VA	Volatile Acidity
YEPG	Yeast Extract Peptone Glucose
YPD	Yeast extract Peptone Dextrose

Units of Measurement

cfu	colony forming unit
g	Gram
g/L	gram per Litre
gm/L	gram per Litre
IU	International Units
kg	Kilogram
M.T	Metric Tonnes
mg	Milligram
mg/L	milligram per litre
nm	Nanometer
rpm	rotation per minute
µg/kg	microgram per kilogram
µg/L	microgram per litre

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CHAPTER 1

General Introduction

Microbial diversity which provides a large pool of resources is the key to human survival and economic well being. Fermentation and maturation processes are governed by varied active diverse populations of prokaryotic and lower eukaryotic microorganisms and in turn impact the quality of the end product (Barbuddhe et al., 2013). Isolation of the yeasts has been attempted from varied sources for their use in industries. Fermentation using yeasts is an ancient process known to make fermented beverages by humans (Pretorius et al., 1999; Pretorius, 2000). Virtually any fruit (e.g., mulberry, watermelon, banana, mango, pineapple, grapes, passion fruit, papaya) can be processed into an alcoholic beverage through spontaneous fermentation caused by wild yeasts that are present on the fruits/fruit juice (Parameswari et al., 2015; Darias-Martin et al., 2003; Duyen et al., 2013; Verzera et al., 2008; Reddy et al., 2010; Selli et al., 2003; Dhar et al., 2013; Hossain et al., 2010; Nzabuheraheza and Nyiramugwera, 2014; Li et al., 2011; Kumar and Mishra, 2010; Santiago-Urbina et al., 2011). The process involves an alcoholic fermentation of sugars that yields alcohol and carbon dioxide (Dudley, 2004). However, the current indiscriminate use of these resources threatens their accessibility as important food and feed products (Deenanath et al., 2012). Therefore, there is search for alternative substrates for fermentation, which are inexpensive, economical and easily available as an energy source (Rocha et al., 2006). Cashew apples make an attractive agro-industrial and alternative unusual waste product as substrate for fermentation products including bioethanol production due to its carbohydrate content (Rocha et al., 2006; Deenanath, 2014).

Cashew (*Anacardium occidentale* L.) is a native crop of Brazil (Honorato et al., 2007; Luz et al., 2008) and widespread in other tropical countries (Nam et al., 2014).

Cashew, an important commercial cash crop of India, is also considered as a 'Gold Mine' in the waste lands. It was introduced in India by Portuguese during 16th century mainly to check erosion covering around an area of 5,12,000 hectars (Chandrasekharan and Jeyakumar, 2014). From thereon, it was extended to other parts of the country. The primary producers of cashew are the states of Maharashtra, Kerala, Andhra Pradesh, Odisha, Tamil Nadu, West Bengal and Karnataka. Cashew is also grown in other areas like, Gujarat, Andaman and Nicobar Islands, Chhattisgarh and Goa. Cashew apple is a pseudofruit. The total cashew apple production in India is ~ 56 Lakh M.T. (Bhakta, 2010) with about 2,32,000 MT of cashew apple production in Goa. As per rough estimates, one acre of cashew plantation produces 2240 Kg of cashew apples yielding about 117 Litres of feni (Bhakta, 2010). Total national waste is estimated at 14.5 crore Barrels of feni which can be converted to either potable alcohol or for petroleum mixing. Thus, 56 lakh tonnes cashew apples can be used to get 29 crore Barrels cashew Feni, or 14.5 crore liters of alcohol of commercial value worth Rs. 362 crores (Bhakta, 2010). The cashew apples produced in the country go unutilised/waste. Therefore, it is a big national waste. But in Goa they are completely utilised for making beverage “feni”. In 2009, feni has been given recognition as Geographical Indication (GI) (Rangnekar, 2009).

Cashew apple is a soft fibrous fruit that yields highly nutritious juice (Cavalcante et al., 2005) which is also good for health (de Lima et al., 2014). Cashew apple juice contains high amount of fermentable sugars, minerals and vitamins (Desai et al., 2012). Cashew apple has been reported to contain more vitamin C (three to six folds) as compared to orange (Akinwale, 2000; Costa et al., 2009; Lowor and Agyente-Badu, 2009). It can be utilized for the preparation of several value added products such as juice,

feni, wine, dried cashew apple, syrup and jam (Suganya and Dharshini, 2011). As it contains high vitamin C and sugar, it is totally edible, with enjoyable flavor aroma and high nutritive value. Natural and processed cashew apple juices (CAJ/cajuina) are amongst the most popular juices in North-east Brazil (Cavalcante et al., 2005). It is also cheap and easily available (Honorato et al., 2007; Silveira et al., 2012), which makes it a suitable substrate for production of alcoholic beverages (Desai et al., 2012; 2013).

In India, Goa is the only state where it is used to produce one of the common distilled alcoholic beverage "Feni" (Mandal et al., 1985). Besides Feni making, it is used as a substrate for production of mannitol (Fontes et al., 2009), lactic acid (Silveira et al., 2012), dextransucrase (Honorato and Rodrigues, 2010), biosurfactant (Rocha et al., 2007) and ethanol (Pinheiro et al., 2008).

In spite of these characteristics, cashew apple is a typical instance of food wastage in many cashew producing countries with heavy losses (Filgueiras et al., 1999; Rocha et al., 2006). Cashew cultivation is intended for production of cashew nuts, while, a huge amount of cashew apples are allowed to waste in the plantations after removing the nut (Honorato et al., 2007). These facts together with its richness in fermentable sugar turn cashew apple juice also forms an exciting and cheap culture medium for alcohol production (Pinheiro et al., 2008; Desai et al., 2012). For centuries together, the fermentation of cashew apple juice has remained traditional and non scientific leading to inconsistency and poor olfactory and chemical quality parameters of the distillate.

Yeasts play very important role in alcoholic fermentation (Van Dijken et al., 1986; Pretorius, 2000). Yeasts are involved in different processes including industrial production of ethanol, production of single cell protein, leavening of dough for breadmaking and for wine production (Layokun, 1984; Amachukwu et al., 1986;

Okagbue, 1988; Osho and Odunfa, 1999).Yeasts have also been isolated from fermenting cassava tubers (Okafor, 1977; Oyewole and Odunfa, 1988). Owing to such different biological actions, yeasts are widely used in varied range of applications extending beyond the food sector (Hatoum et al., 2012). Carbohydrates present in agricultural products are widely used for alcohol production employing ideal yeast strains (Brooks, 2008). As such, a small number of yeast strains have been found to possess significant characteristics for alcohol production (Hacking et al., 1984). Hence, there is a requirement to search the prospect of native strains of yeasts to fulfill the national demands of use of yeast with desirable qualities in wine making industry.

The diversity and identification of yeast species in fermented cashew apple juice can be useful as a first approach to evaluate potential characteristics valuable in industry, biotechnology, or biotransformation. Yeasts provide enzymes during bioconversion which act upon and convert the organic compounds into other compounds (Goulas et al., 2012; Ennouali et al., 2006; Long and Ward, 1989). This concept is also applicable in alcohol production from cashew apple wherein microbes biotransform the cashew apple content into alcohol and other volatile compounds. Many studies have observed the favorable and unfavorable influences of yeasts and their potential application in fermentations (Reddy et al., 2010). Many reports have indicated the use of different starter cultures and native yeasts in fermented beverages with considerable variations in chemical composition and concentration of the flavor compounds depending upon the metabolic activity varying with yeast strain/species, could be produced (Lambrechts and Pretorius, 2000; Romano et al., 2003). Studies have also highlighted the influence of non-*Saccharomyces* yeasts on the sensory characters of fermented beverages (Romano et al., 2003; Egli et al., 1998; Soden et al., 2000).

However, it has been shown that although some volatile aroma substances arise from constituents of the fruits, during fermentation by yeasts, many of these compounds are altered and a significant fraction of flavor essences are formed (Sampaio et al., 2011). In an investigation to identify the aroma volatiles, 71 volatiles were identified in cashew apple juice which was evaporated off and recovered in the water phase (Sampaio et al., 2011). Forty seven volatile factors were odour active. Alcohols such as heptanol, trans-3-hexen-1-ol and 3-methyl-1-butanol were recovered in the cashew water phase and represented about 42% of the total area of chromatogram. It imparted green grass and fruity aroma to the water phase. Esters constituted mainly ethyl 2-hydroxyhexanoate, ethyl trans-2-butenoate and ethyl 2-methylbutanoate and covered 21% of the total chromatogram area. Esters imparted the fruity/cashew-like smell of the water phase. Gas chromatography-mass spectrometry analyses of a Brazilian cashew apple variety revealed numerous volatiles such as esters, terpenes, hydrocarbons, carboxylic acids, aldehydes, alcohols, ketones, lactones and norisoprenoids (Bicalho and Rezende, 2001; Bicalho et al., 2000). Therefore, in this study, an attempt was made to explore the volatile metabolites producing abilities of yeasts isolated from naturally fermented cashew apple juice.

Unlike *Saccharomyces* species, the non-*Saccharomyces* yeasts have been reported to produce and exude many enzymes such as esterases, lipases, β-glucosidases, proteases, cellulases to the periplasmic space and in the culture medium (Cordero Otero et al., 2003).

Enzymes produced from yeasts are extensively available in nature and are commonly used in detergents, foods, pharmaceuticals, leather and textile manufacture, and waste treatment (Zhao, 2012; Carrasco et al., 2012) for various enzyme-based

biotransformational activities converting complex substrates into simple useful products. Production of enzymes from yeasts has been studied mainly for their biotechnological and biotranformational properties and their implications in the alcohol industries (Buzzini and Martini, 2002; Strauss et al., 2001).

Yeast cells have been utilized in biotransformation of ricinoleic acid to γ-decalactone (peach-like flavour compound), conversion of carbohydrates to alcohol and carbon dioxide, biotransformation of lignin (e.g: *Sporobolomyces roseus*), biotransformation of castor oil into decalactone, conversion of xylose into xylitol or production of zymocin (Thanh, 2009). Microbial transformation of terpenes in the synthesis of (+)-citronellol from (+)-citronellal is mediated by yeast cells (Khor and Uzir, 2011). Conversion of pectin (*Pichia methanolica*) and conversion of xylan to oligosaccharides (xylobiose and xylotriose) have been attempted (Nakagawa et al., 2005). Besides these enzymes, there are many enzymes which are important in the alcoholic industries i.e. amylase in saccharification (de Souza and Magalhaes, 2010; Pandey et al., 2000; van der Maarel et al., 2002), bread and bakery (Hirose et al., 2009; Pandey et al., 2000; de Souza and Magalhaes et al., 2010), protease, pectinase, cellulase (preventing haziness in juice, solves clarification and filtration problems of fruit juice, increases juice volume, paper and pulp industry, textile industry) (Lagace and Bisson, 1990), urease (prevent formation of ethyl carbamate during alcohol fermentation), lipase (role in fatty acid esters formation) (Esteve-Zarcoso et al., 1998) and glucosidase (releases fruit-bound terpenol giving aroma compound to the finished alcohol) (Williams et al., 1982; Gunata et al., 1990, Vasserott et al., 1995; Winterhalter and Skouroumounis, 1997). Therefore, assessing the yeasts for such enzymes is of much importance. In this

context, particular attention needs to be given to the wild flora, which can be of great interest for enzyme production. Explorations of biodiversity in the search for new biocatalysts by selecting yeasts from fermented cashew apple juice represents a method for discovering new enzymes which may permit the development of bio-catalysis on an industrial scale. Currently, there is great interest in finding yeast species that are not yet known as interesting producers of inputs to industry in general, and also in biotechnological processes can replace many chemical processes.

Yeasts have always remained potential candidate for an extensive array of applications beyond the food sector due to their varied biological activities. Apart from their most important contribution to enzyme production, few yeast species reveal strong activity against microbes and are being exploited as novel agents in minimizing the foods spoilgae medicine and pharmaceuticals. These hostile activities of yeasts towards unwanted bacteria, and fungi are now extensively known (Hatoum et al., 2012).

Conventional methods employed in the identification of yeasts are painstaking, extensive and often not able to differentiate at species level and thus offer uncertain identifications which limit their use in categorization (Huppert et al., 1975; Casey et al., 1990; Suh et al., 2006; Shokohi et al., 2010; Rajkumari et al., 2014; Sumitra Devi and Maheshwari, 2014). To overcome the drawbacks of the conventional methods, molecular markers are widely employed for taxonomy and genetic description of yeasts (Querol et al., 1992; Buzzini et al., 2007). Identification of yeasts is done by the 26S rDNA sequencing and comparing with biochemical tests. In contrast to conventional methods, the application of molecular methods is a consistent approach for the identification of yeasts (Lopandic et al., 2006; Taqarort et al., 2008). Several investigations have

demonstrated enough differences in the D1/D2 domain of the yeasts to predict intra- and inter-species associations (Kurtzman and Robnett, 1997, 1998; Fell et al., 2000). Therefore, the D1/D2 domain of the 26S rDNA gene has been widely targeted for identification of the yeasts isolated from diverse sources (Chavan et al., 2009). Besides this, use of the 5.8S rRNA gene and the two internal transcribed spacers as sequence targets has been intended for identification of yeasts isolated from diverse sources (Las Heras-Vazquez et al., 2003).

In this study, owing to the industrial importance of yeast biodiversity, an attempt was made to isolate and characterize the flora of yeasts from naturally fermented cashew apple juice. Further, the capability of these yeasts to secrete enzymes such as amylase, urease, pectinase, protease, β-glucosidases, cellulases and lipase was attempted. The alcohol producing ability along with alcohol tolerance and their contribution in volatile profile in the finished beverage were also studied. Non-*Saccharomyces* yeasts were also screened for their alcohol tolerance and effect on beverage.The study was proposed with the following objectives.

Objectives*:*

1. To isolate yeasts from naturally fermented cashew apple juice.
2. To characterize the yeast for their biochemical and bio-transformational properties.
3. To determine the diversity among yeast using genotypic methods.

CHAPTER 2

Review of Literature

Fermented foods made by action of several microbes are a part of cultural heritage and essential part of our diet. Several types of fermented foods are consumed around the world. Indeed, it is likely that the methods of preparation of traditional foods and beverages varies from region to region (Kabak and Dobson, 2011; Benkerroum, 2013). The scope of food fermentation ranged from alcoholic beverages, fermented milk and vegetable products producing overall nutritious and safe products with appealing qualities.

The fermentation techniques considered to have been developed over many years for preservation of food use during times of shortage, to impart pleasing flavour in foods, and to make the foods less toxic (Motarejmi, 2002; Valyasevi and Rolle, 2002; Chaves-Lopez et al., 2014).

2.1 History of fermentation

The history of fermentation as well as the role of microorganisms in the fermentation can be traced back to the era of Louis Pasteur. Since then the fermentation process is evolving with time. Agricultural food crops such as corn, wheat, sugar beets, barley, sorghum, rice, corn, and sugar cane, as well as barley straw, barley husks, corn stover, sugar cane bagasse, and switch grass have been utilized as raw sources for fermentation to prepare alcoholic beverages (Kim and Dale, 2004; Linde et al., 2008, 2007; Najafi et al., 2009; Balat and Balat, 2009).

Due to presence of easily utilizable sugars, fruits are important substrates for several beverages including alcoholic beverages (Hossain and Fazliny, 2010; Nzabuheraheza and Nyiramugwera, 2014; Li et al., 2011; Kumar and Mishra, 2010; Santiago-Urbina et al., 2011). Traditionally, several alcoholic beverages are made from fruits such as - apple, grapes, pineapple, cashew apple, jackfruit, banana etc.

An attractive and unconventional agro-industrial waste crop, with undetected but significant possibility for use as a bioethanol substrate, is cashew apple (Deenanath et al., 2013; Honorato et al., 2007; Honorato and Rodrigues, 2010; Luz et al., 2008; Rocha et al., 2006). In Goa, one of the common local alcoholic beverages is prepared from cashew apple juice known as Feni (Battcock and Azam, 2001; Mandal et al., 1985).

2.2 Cashew apples

The cashew (*Anacardium occidentale* L.) tree is a perennial plant native to Northeastern Brazil (Honorato et al., 2007; Luz et al., 2008; Assuncao and Mercandate, 2003). Cashew is a major cash crop in India especially Goa, introduced by Portuguese during 16th century. Cashew plantations are now spread over a number of states of India.

Cashew tree with cashew apples and nuts.

Though cashew nuts are famous worldwide, the consumption of cashew apples is not that common due to its unpleasant phenolic taste. The mature fruits contain ample pulp, pliable peel, high sugar content and stronger flavor and are without seeds (Cavalcante et al., 2005). The detailed composition of the cashew apple is as mentioned in Table 2.1. Certain phenolic compounds present in fruit pulp contribute to the

astringency decreasing its pleasantness and acceptability for taste purpose (Adou et al., 2012b; Rufino et al., 2010). However, the fruit has been shown to possess several nutritionally important components. The presence of vitamin C, tannin, and polyphenolic compounds gives cashew apple rich antioxidant properties (Adou et al., 2012b; Morton, 1987; Kubo et al., 2006). Cashew apples have very interesting nutritional facts:

Table 2.1 Chemical and nutritional composition of cashew apple (per 100 gram) (Oduwole et al., 2001; Assuncao and Mercadante, 2003; Rocha et al., 2007).

Constituents	Amount
Moisture	87.80%
Protein	0.20%
Fat	0.10%
Carbohydrate	11.60%
Crude fibre	0.90%
Calcium	10 mg/100g
Phosphorous	10 mg/100g
Iron	0.2 mg/100g
Vitamin	261 mg/100g
Minerals	200 mg/100g
Thiaine	0.02 mg/100g
Nicotinic acid	0.4 mg/100g
Vitamin A	39 IU
Riboflavin	0.5mg/100g

It has high amount of reducing sugars such as fructose and sucrose (Pereira and Rodrigues, 2012; Luz et al., 2008). Such sugars can be used in the production process of ethanol (Pinheiro et al., 2008). Several studies have shown the richness of the apple juice that contains minerals, vitamin especially three to six times more vitamin C than orange juice (Cavalcante et al., 2005; Santos et al., 2007; Assuncao and Mercandante, 2003, Sivagurunathan et al., 2010; Adou et al., 2012a; Lavinas et al., 2008) and ten times more than pineapple juice (Akinwale, 2000). It contains 0.099% vitamin B2, 0.24% vitamin C,

0.041% calcium, 0.011% phosphorous and 0.003% iron, minerals, ascorbic acid, antioxidant, and some amino acids (Cavalcante et al., 2005; Wharta et al., 2004; Rufino et al., 2010; Akinwale, 2000; Campos et al., 2002; Price et al., 1975; Assuncao and Mercandente, 2003; Adou et al., 2011, 2012a; Sivagurunathan et al., 2010). The high tannin content of cashew apple makes it an appropriate therapy for upper respiratory track infection such as sore throat as well as in some cases of gastrointestinal illnesses in Brazil and Cuba (Morton, 1987).

Cashew apples are also known for several medicinally important properties such as anti-tumor activity (Kubo et al., 1993; Melo-Cavalcante et al., 2003, 2008) and inhibition of gastric pathogens such as *Helicobacter pylori* (Kubo et al., 1999). Due to cost effectiveness and huge availability, cashew apples can be very cheap source of substrate in the manufacture of industrially important products such as surfactants (Giro et al., 2009; Rocha et al., 2007), mannitol (Fontes et al., 2009), alcohol (Mohanty et al., 2006; Prommajak et al., 2014; Rabelo et al., 2009) and syrups made up of cashew apple juice (Guilherme et al., 2007; Lavinas et al., 2008).

Fresh juice of cashew apples and the processed juice products (*cajuína* in Portuguese) have been shown to possess antimicrobial, anti cancer and anti-tumor activities (Cavalcante et al., 2005; Kubo et al., 1999; Melo-Cavalante et al., 2003; Kozubek et al., 2001).

Cashew apple juice can be consumed fresh and also as juices, pulp, wine and preserves (Nam et al., 2014). Cajuina in Brazil is well known product made up of fresh cashew apple juice as well as from processed juice (Melo-Cavalcante et al., 2003). The powder made up of fruits of cashew apple and guava (*Psidium guajava* L.) residues after removing the juice can be utilized as source of lipids, and vitamin C (Costa et al., 2009).

The cashew apples have a high amount of volatile compounds. Forty eight volatile composites are well known in cashew apple juice, of which the predominant compounds include esters (42%) and aldehydes (14%) (Garruti et al., 2003; Franco et al., 2005). These would be readily available in the fermentation medium and help in fermentation processes (Chagas et al., 2007; Honorato et al., 2007). Though ashew apple is an agricultural waste, it is a cost effective substrate for microbial growth (Honorato and Rodrigues, 2010). The fermented cashew apple juice produced by the growth of *Lactobacillus acidophilus* was showed to be a good probiotic for human health (Giang et al., 2013). Rufino et al. (2010) assessed cashew apple and acerola as sources of antioxidants, dietary fibre and phenolic compounds. Pinho et al. (2011) produced hamburgers of high nutritional quality that is rich or have high dietary fiber content and is low in fat with partial substitution of the meat with cashew apple residue powder. Wine has also been produced from cashew apples (Li and Li, 1999; Li et al., 1999).

Despite these attributes, pulp of the cashew apple is usually discarded during harvest, which is wasteful (Pereira, 2011; Sivagurunathan et al., 2010) and cashew tree cultivation activity is directed at producing cashew nuts (Chagas et al., 2007), thus rendering the cashew apples as strong instance of food wastage in the countries growing cashew (Oduwole et al., 2001), losses being as high as 90% since their general decomposition process does not produce any value of relevant by-products in soil (Honorato et al., 2007; Filgueiras et al., 1999; Luz et al., 2008; Pinheiro et al., 2008).

Due to highly perishable nature of the fruit, they are very susceptible to injury and rapid microbial deterioration once harvested (Nanjundaswamy et al., 1985). These factors together with the difficulties in the collection and transport of ripe fruits have deterred the commercial utilization of cashew apple (Nanjundaswamy et al., 1985).

2.3 Uses of Cashew apple

Although cashew apple do not have many economic values as a fruit, it has been explored as a raw material for several industrial purposes. The industrial uses has been presented in Table 2.2: Industrial uses of cashew apple.

Table 2.2 Industrial uses of the cashew apple.

Cashew apple product	**Industrial use/Active properties**	**Reference**
Cashew apple juice	General: Health drinks (especially as Vitamin C supplement), ice-cream, sport drink, juice, jams, candies, vinegar	Sobhana and Mathew, 2014; Suganya and Dharshini, 2011
	Alcoholic: Cajuina, feni, wine	Suganya and Dharshini, 2011
	Medicinal: Prebiotics, medium for production of hyaluronic acid, proteins with IgE reactive epitopes, including cashew nut legumin and vicilin	Vergara et al., 2010; Agrarius, 2014; Oliveira et al., 2014; Comstock et al., 2008
	Substrate for production of mannitol andsurfactant production, production of ester, terpenes, hydrocarbons, carboxylic acid, aldehyde, alcohol, ketones, lactones and nonisoprenoids, substrate for dextransucrase production	Fontes et al., 2009; Rocha et al., 2006; Apine and Jadhav, 2015; Araujo et al., 2011; Chagas et al., 2007
Cashew apple powder	Supplement for biscuit-type cookies	Ogunjobi and Ogunwolu, 2010

Table 2.2 Industrial uses of the cashew apple (*continued*)

Cashew apple product	**Industrial use/Active properties**	**Reference**
Cashew apple juice	Antibacterial, antifungal, antimutagenic, anticlastogenic, antioxidant, cytotoxic	Vivek et al., 2014; Melo-Cavalcante et al., 2008; Melo-Cavalcante et al., 2011; Queiroz et al., 2011;
Cashew apple bagasse	Source of sugars for ethanol production, tannase production	Rodrigues et al., 2011; Rodrigues et al., 2007
Cashew apple waste	Livestock feed, substrate for vermin composting	Rale, 1985

2.4 Significance of cashew apples in Goa

In Goa, the cashew plantations are grown in large area (Table 2.3)

Table 2.3 Area and production of cashew apples in Goa.

State	**Area**	**Productive area**	**Production**	**Productivity**
Goa	55,000 ha	55,000 ha	26,000 MT	473 kg/ha

The production and harvesting of cashew apples are anticipated for cashew nuts due to its economic significance. Therefore, the cashew nuts produced become the primary products. Due to abundance and juicy nature, conventionally, cashew apples have been utilized to prepare liquors by locals. Till date, this production is based on very traditional way and described here (http://cazulofeni.com/about-feni/category/feni-production-process/)

2.5 Steps in Feni production

Step 1: Collection of cashew apples

The remnants of cashew apples are generally collected or purchased in bulk. If nuts are attached, then are manually separated from cashew apples. The fruits are washed and then either stored or immediately used for juice extraction process.

Cashew apples with nuts

A woman separating cashew apples and at Shiridao cashew field

Step 2: Extraction of juice

For small scale, the cashew apples are generally crushed manually in a shallow tank to obtain the juice. While, to obtain juice from larger scale cashew apples manual bench-press machines are used.

Manual crushing of cashew apples at Shiridao

The juice extracted by manual crushing at Shiridao

Step 3: Natural fermentation

The extracted juice is filtered through muslin cloth and transferred to tanks or barrels. The juice produced is then allowed to ferment naturally.

Step 4: Distillation

The fermented juice is then distilled traditionally by putting fire under the earthen pots. The distillate is checked for its alcohol content by observing the over-flame produced in

the presence of match-stick fire. Based on the time of flame getting produced, the 'degree' of feni is decided.

Earthen pot containing extracted cashew apple juice for distillation at Shiridao

Step 5: Packaging and selling

Based on the degree of alcoholic content, Feni is packed in glass or plastic bottles and sold locally. The four major stakeholders in the feni industry are distillers, bottlers, whole sellers and retailers. Around 3/4rth of distillers sell their produce to bars directly, and bottlers direct their sales through whole sellers.

Table 2.4 Distribution of the supply-side of the cashew feni industry (Sahu, 2012)

Category	Numbers
Number of cashew zones	1532
Licensed stills	2656
Retailers	6589
Whole sellers	94
Bottlers	19

The current feni production is highly traditional as well as unhygienic. The primary fermentation is carried out by several different uncontrolled and innate microflora. Goa produces approximately 6 Lakh barrels or 40 thousand Kolso of *Feni*. Therefore, *Feni* is rated as the most popular and highly consumed alcoholic beverage produced by fermenting cashew apple juice (Bhakta, 2010). Recently Cashew Feni got its GI registration as a specialty alcoholic beverage from Goa (Rangnekar, 2009).

Yet, several issues such as variety, region of cultivation, weather, farming practices, ripeness at harvest (Drake and Eisele, 1997), and storing conditions (Drake et al., 2002; Drake and Eisele, 1999) are mostly highlighted as responsible factors for changes in chemical formulations of apple juice, thus, ultimately there may be presence of diverse microbial flora responsible for preparing value added product especially fermented beverage.

2.6 Naturally fermenting microorganisms in juice of cashew apple

Microorganisms present on the surface of cashew apples get added during crushing of cashew apples. Fruit surface has microflora depending on the ripeness, sugar content, climatic condition. As the fruit ripens, the sugar and nutrient content from the fruit tissues leach to the surface and thus The fruit surface contains diverse microbial flora including bacteria, yeast and fungi (Pereira and Rodrigues, 2012). If the fruit is overripe might get damage dur to fungal growth or by fruitfly *Drosophila* resulting in the more diverse microbial flora. Of this list of microbes, yeasts are known as responsible factors for alcohol fermentation (Esteve-Zarcoso et al., 1998; Donalies et al., 2008) because of sugar content, acidity of fruit. Therefore, the yeast flora that gets developed subsequently determines the taste and flavour (Mafart, 1986; Le Quere and Drilleau, 1993; Suarez et al., 2005). Sharma et al. (2012) have summarised the role of yeasts in

volatile metabolites production in various fermentation processes such as alcoholic beverages, single cell proteins, fermented cheese and yoghurt.

Few studies have been reported on microbial flora in cashew apple juice (CAJ). Barros et al. (2014) reported the presence of *Hanseniaspora* yeast in CAJ which produced the ethanol. Another study by Osho (2005) isolated two *Saccharomyces* spp. including three different strains of *S. cerevisiae* and one *S. uvarum* from fermentation medium i.e. CAJ. Desai et al. (2013) identified seven morphologically different strains of *Candida* spp. acting as good starter cultures for commercial production of alcoholic beverages from the cashew apple. Apart from CAJ, Aderiye and Mbadiwe (1993) isolated strains of genera *Leuconostoc, Lactobacillus, Pediococcus, Aspergillus, Rhizopus*, and few other yeast strains from freshly extracted cashew apple pomace. *S. cerevisiae* sp. was found in CAJ (Adesioye, 1991). In fact, the natural flora of cashew apple juice might have great difference due to the geographical area where it is grown and also because of the processing methods used. Analyses of yeasts in raw Brazilian cashew kernel revealed the presence of *Pichia guillermondii* (3.2×10^2 cfu/ sample) (Freire and Offord., 2002), *Candida utilis, Saccharomyces cerevisiae* on variety of cashew (*Anacardium occidentale)* (Rale et al., 1985), *Candida* spp. from cashew apple from west coast and Konkan region of India (Desai et al., 2013). Nutritional quality of cashew apples have been reported earlier but microbial aspect is not studied (Krishnaswamy et al., 1973), those studied are not studied further (Friere and Kozakiewicz, 2005). Microbial aspect of cashew apple fermentation have not received much attention as it has remained a household small scale unorganized business in Goa. Feni being geographical restriction to Goa, not much is known about the microbes that are involved. Barbuddhe et al. (2011) made an attempt previously determining the

natural flora of cashew apple in order to understand the microbes that take part in the production of Feni. The study showed yeasts as a dominant (>78%) microflora followed by bacteria and fungi. The study could identify the majority of the yeasts involved as *Saccharomyces cerevisiae, Pichia species* and *Issatchenkia orientalis (Pichia kudriavzevii)*. Other yeasts species isolated were *Candida ethanolica, Lachancea fermentatii* and *Pyrenochaeta nobilis* (Barbuddhe et al., 2011). Several yeast, however, remained unidentified.

2.7 Yeast: a microorganism in natural fermentation

Yeasts are single celled eukaryotic organisms that are ubiquitous in nature (Arroyo-Lopez et al., 2012) and different geographical conditions nurture different yeast flora (Longo et al., 1991). Various geographical conditions like, precipitation, temperature, type of soil , growth and nutritional availability such as natural soil nutrient content, fruit maturity, physical damage to the fruit surface because of birds, pests and fungi, mechanical injury, use of fungal and insect control agents contribute overall to the diversity of yeasts (Chavan et al., 2009; Chatterjee et al., 2011).

The isolation of different yeasts has been reported from several different sources. Grape juice is highly studied due to its commercial significance in wine production. If raw grape juice is considered, yeasts such as *Candida intermedia, C. parapsilosis, Hanseniaspora occidentalis, H. vineae, Pichia fermentans, Saccharomycopsis crataegensis* and *Hanseniaspora uvarum* have been reported to be observed commonly (Arias et al., 2002; Parish and Haggins, 1989; Hatcher et al., 2000). Several other yeasts such as *Rhodotorula mucilaginosa, Saccharomyces cerevisiae, Candida stellata, Zygosaccharomyces rouxii, Torulaspora delbrueckii, Issatchenkia orientalis, H. occidentalis, Lodderomyces elongisporus, Kluyveromyces theromotolerance,*

Hanseniaspora guillermondii, Candida glabrata, Pichia anomala, Candida, tropicalis, Candida magnoliae, Candida maltosa, Candida parapsilosis, Candida tropicalis, Clavispora lusitaniae, Cryptococcus humicolus, C. laurentii, Pichia membranaefaciens, and *Sporidiobolus sabnonicolor Clavispora lusitaniae, Pichia anomala, Hanseniaspora uvarum, Pichia fermentans, S.cerevisiae, S.unisporus*, and *Trichosporon asahii* have reported from fruit juices (Las Heras-Vazquez et al., 2003). *Candida haemulonii, Cryptococcus aerius, Aureobasidium pullulans var. melanogenum, Aureobasidium* sp., *C. flavescens, Cryptococcus* sp. and *Rhodosporidium paludigenum* were isolated from Mango (Gana et al., 2014). *S. cerevisiae, S. kluyveri, S. exigus, S. dairnensis, S. ludwigii, S. octosporus* and *S. unisporus* have reported from citrus juice (Qureshi et al., 2007). Characteristic yeast spp. observed in citrus juices were *Candida parapsilopsis, C. stellata, C. intermedia, Saccharomyces crataegensis, S. cerevisiae, Torulaspora delbrukii* and *Zygosaccharomyces ruoxii* (Hatcher, et al., 2000; Arias et al., 2002).

Pichia membranifaciens, Saccharomycodes ludwigii, Kloeckera apiculata, Metschnikowia pulcherrima, Candida species (Candida stellata and Candida raghi), Issatchenkia orientalis and *Rhodotorula* spp. were isolated from Malbec grape berries (Combina et al., 2005a). *Rhodotolula mucilaginosa, Issatchenkia orientalis, Pichia guilliermondii, Aureobasidium pullulans, Saccharomycodes ludwigii, Candida tropicalis, P. fermentans, Zygosaccharomyces bailii, Hanseniaspora uvarum, Hanseniaspora opuntiae, Candida stellata* and *Erythrobasidium hasegawianum* have been islated from fermenting pineapple juice (Chanprasartsuk et al., 2013). *Hanseniaspora osmophilia, Pichia membranaefaciens, Candida* spp., *Rhodotorula* spp., and *Saccharomyces cerevisiae* were isolated from grape berries (Parish and Caroll, 1985)

and *C. membranifaciens, P. guillerimondii* and *R. mucilaginosa* from fresh apple (Gholamnejad et al., 2010; Fard et al., 2012).

Eleven diverse species of yeasts comprising of seven genera were isolated from grape vines in India as: *Candida azyma, Candida quercitrusa, Debaryomyces hansenii, Hanseniaspora guilliermondii, H. viniae, H. uvarum, Issatchenkia orientalis, I. terricola, Pichia membranifaciens, Saccharomyces cerevisiae* and *Zygoascus steatolyticus*. *H. guilliermondii* was the most prevalent spp. while *S. cerevisiae* was reported infrequently in the six vine varieties (Chavan et al., 2009).

Yeast strains were recovered from healthy and rotten fruits (Bhadra et al., 2007; Peter et al., 2005; Rao et al., 2007a; Tournas and Katsoudas, 2005). *S. cerevisiae* were found rarely in intact berries and vineyard soils and occurred in small quantiy on healthy, good quality grapes (Martini, 1993; Pretorius, 2000). However, it had been hypothesized that injured berries were the natural reservoirs of yeasts, comprising *S. cerevisiae* (Mortimer and Polsinelli, 1999). *S. cerevisiae, Candida castellii, C. fructus, C. intermedia, C. krusei, C. tropicalis, G. candidum, H. anomala, K. apiculata, P. membranifaciens, P. ohmeri, S. chevalieri, S. uvarum , K. africanus, T. delbrueckii* and *R. grainis* were isolated from alcoholic beverages (Sanni and Lonner, 1993). *S. cerevisiae, R. mucilaginosa, Candida colliculosa, C. utilis, C. magnolia, Trichosporon asahii, R. glutinis, C. pelliculosa* and *Cryptococcus albidus* had been isolated from fermented beverages (Jimoh et al., 2012). *Pichia manshurica, P. fermentans* and *P. anomala* were isolated from decayed mangosteen fruit (Bhadra et al., 2008). Yeasts of the genera *Sporobolomyces, Saccharomyces, Rhodotorula, Cryptococcus, Candida* and *Pichia*, among others, have been recoverd from fresh and spoiled fruits (Péter et al., 2005; Tournas and Katsoudas, 2005; Rao et al., 2007a).

2.8 The use of media for yeasts isolation procedures

The yeasts isolation has been evolved from raw simple nutrients based agars to specific media. Since yeast isolation has been studied from ancient times, there have been many isolation methods and culture media for isolation and identification. Menna (1957) used modified Sabouraud agar. Oxytetracycline Glucose Agar base medium has used for selective isolation of yeasts (Singh et al., 2013), YPD medium having propionic acid and Rose Bengal were used as an isolation media for yeast from fermented citrus molasses (Oliveira et al., 2006). Isolation of yeasts on yeast extract malt extract agar has been reported (Gana et al., 2014; Mamum-Or-Rashid et al., 2013; Chanchaichaovivat et al., 2007). Peptone Dextrose agar was also used (Patil and Patil, 2006). Potato dextrose agar (PDA) has been used widely for isolation of yeasts (Beech and Davenport, 1971; Mokhtari et al., 2011; Verma et al., 2010).

2.9 Characterisation of Yeasts

As yeasts are important from commerical and public health point of view, the yeast isolates are generally characterised based on area of interest.

2.9.1 Identification of yeasts

Traditionally, yeasts characterization based on their microscopic observations, growth morphologies, phenotypes and biochemical characteristics provide ease for exact identification and differentiation (Chatterjee et al., 2011; Tikka et al., 2013). These analyses are cumbersome, approximately 50–100 tests are necessary to identify most yeasts to species level reliably (Lin and Fung, 1987). Preliminary step in identification is microscopic analysis which can be done by staining (Mamum-Or-Rashid et al., 2013). Identification was based on an established scheme of biochemicals (fermentation and

assimilation patterns in durhams tube) (Kreger-van Rij, 1984). The recovered yeasts were confirmed as described (Yarrow, 1998; Kurtzman and Fell, 1998). These analysis included colony morphology, pseudomycelium, pellicle formation, growth at different temperatures of 30, 35, 37 and 42^{o}C, growth in high sugar content such as 50% glucose, growth at high salt concentrations (upto 10%). Ready made test strips AP120C AUX determining the carbon assimilation and differentiating different yeasts species are also available commercially.

The identification of yeasts was generally performed by physiological and morphological standard protocols as per Kreger-van Rij (1984) and Kurtzman and Fell (1998). In this identification process, over 75 tests need to be performed (Table 2.5). Consequently, the traditional conventional methodology for yeast identification is labor intensive and time-consuming (Giudici and Pulvirenti, 2002). With the advacement in technology, several novel methods have been introduced for the identification of yeasts. Biochemical test kits are widely spread for yeast identification. However, it has been reported that these systems are not appropriate for identification of environmental isolates (Ramani et al., 1998). Morphological and physiological characteristics were the traditional system of yeast identification (Barnett et al., 1990) altered by molecular applications (Esteve-Zarcoso et al., 1998).

Hence, with the introduction of PCR-based methodologies, microbial identification has got a new milestone (Ness et al., 1993; Paffetti et al., 1995; de Barros Lopes et al., 1996), which are now popular for identification of yeasts because they could be used to classify yeasts rapidly up to genus and species level and reliable identification. These are time efficient and less tedious which provide reliable data in very short time (Kurtzman and Fell, 1998; Barnett et al., 2000; Fernandez-Espinar et al., 2006).

Table 2.5 Conventional physiological tests of yeast (Kreger-van Rij, 1984; Kurtzman and Fell, 1998)

Assimilation	Fermentation	Cultural characteristics	Microscopy
Glucose, Galactose, L-Sorbose, Sucrose, Maltose, Cellobiose, Trehalose, Lactose, Melibiose, Raffinose, Melezitose, Inulin, Soluble Amides, D-Xylose, L- And D-Arabinose, D-Ribose, L-Rhamnose, Dglucosamine, N-Acetyl-D-Glucosamine, Methanol, Ethanol, Glycerol, Erythritol, Ribitol, Galactitol (Dulcitol), D-Mannitol, Dsorbitol, ∝-Methyl-D-Glucoside,Salicin, D-Gluconic Acid, DL-Lactic Acid, Sodium Succinate, Sodium Citrate, Inositol, Hexadecane, ∝-Ketoglutaric acid, Xylitol, L-Arabinitol, Propane 1.2 Diol, Butane 2.3 Diol, Lysine, Ethylamine, Potassium Nitrate, Cadaverine, Creatine Glucosamine	Glucose Sucrose Maltose, Galactose, Raffinose, Lactose, Trehalose, Melezitose Cellobiose Inulin,	Starch formation, Growth On Presence of Cycloheximide (1%, 0.1% And 0.01%), Growth On Media Containing 50% Of Glucose, Growth At Different Temperatures (40°c, 37°c, 34°c, 25°c, 19°c, 17°c), Growth On vitamin-free medium, Growth On 10% Nacl With 5% Glucose,	Production of a true mycelium, Production of pseudo-mycelium, Vegetative Reproduction by budding or splitting, Production of Ascospores, Production Of Basidiospores , Growth On 5% Malt Extract (Scoring Cell And Colony Shape).

Of the various molecular approaches available, RFLP analysis of the ITS region of rDNA, sequencing of the D1/D2 domain of the 26S rDNA and sequencing of the ITS region of rDNA are finding most application (Kurtzman and Robnett, 1998). ITS-RFLP analysis was reported to be the most convenient method for yeast identification because of its speed (10 hr), simplicity, and cheaper cost and at the same time identifying large group of yeast (Esteve-Zarcoso et al., 1999; Granchi et al., 1999; Chen et al., 2000; Fernandez-Espinar et al., 2000; Guillamon et al., 1998). The mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) as well as restricted fragment length polymorphims (RFLP) analysis were used to recognize the yeasts recovered from two different fermentative processes in the same winery (Guillamon et al., 1998; Sabate et al., 1998). Automated DNA sequencing analysis has been studied (Lee et al., 1992; Parker et al., 1995; Demeter et al., 1998).

2.9.2 Biotransformation by Yeasts

Since last two decades, the transformation of compounds by microorganisms has attracted considerable attention. Microorganisms are a good sources of biomolecules such as enzymes, fatty acids, pigments, metabolites and antibiotics of great commercial and industrial importance (Bull et al., 2004).

Yeasts exhibit a variety of enzyme activities that convert a broad range of compounds (Buzzini and Vaughan-Martini, 2006; Strauss et al., 2001; Arroyo-Lopez et al., 2012) which have tremendous applications in the field of biotechnology. Therefore, yeasts can be efficiently utilized as reservoir of many types of potential enzymes which originated special research interest to explore the extracellular enzymes and their activities in food grade yeasts (de Vuyst et al., 2009; Araujo et al., 2011).

Amylase **(**EC 3.2.1.1, α-1,4-glucan-glucanohydrolase):

Amylases are well known for hydrolysis of starch polymer at α-1,4 glycosidic bond to get varied products such as dextrins and smaller polymers of glucose, maltose or maltotriose (Gupta et al., 2003; Swargiari and Baruah, 2012). Amylases are usually derived from several microbes such as fungi, yeasts and bacteria, out of which yeast amylase are more stable (Pandey et al., 2000). However, very few species of yeasts exhibits the production of amylase. The amylases from yeasts have been utilized to obtain maltodextrin, modified starches, or glucose and fructose syrups, fuel ethanol, alcoholic beverages and sweetener ranging from food industry, starch industry, bread and bakery industry to the production of biofuels (Pandey et al., 2000; Hirose et al., 2009; de Souza and Magalhaes et al., 2010). Production of ethanol from starchy biomass using *Saccharomyces cerevisiae* have been reported previously (Satyanarayan et al., 2004; Pervez et al., 2014; Prakash and Jaiswal, 2009). These yeast based amylases are also

important for brewing, preparation of cakes, fruit juices, starch syrups as well as industrial application such as laundry, porcelain detergents, paper industry (van der Maarel et al., 2002; Pandey et al., 2000; Aiyer, 2005; de Souza and Magalhaes, 2010; Rosell et al., 2001).

Since each and every diverse application requires unique properties of amylase, it is crucial to hunt the biodiversity for novel reservoir of these enzymes. Although yeasts are not among the industrial producers of amylases, the enzyme is widely distributed in many naturally occurring yeast species (Gupta et al., 2003; de Mot et al., 1984). Numerous yeast species recovered from low temperature and tropical atmospheres have found to possess the amylase activity (Brizzio et al., 2007; Buzzini and Martini, 2002; Skorupa et al., 2002). Commercially important amylase have been obtained from *Debaryomyces nepalensis*, *Brandoniozyma complexa*, *Hypopichia burtonii*, *Candida wangnamkhiaoensis*, *Kodamaea ohmerii*, *Pichia anomala*, *Pseudozymahubeiensis*, *P. prolifica*, and *P. pruni* (Gana et al., 2014). An alpha-amylase secreted by *Pichia burtonii* strain 15-1 isolated from a traditional starter murcha of Nepal was studied and nucleotide sequence was determined (Kato et al., 2007; Takeuchi et al., 2006). A study by Arttirilmansi (2013) observed the maximum amylase production at 30°C and pH 5.5 by *S. fibuligera* useful in starch saccharification (Hostinova, 2002), bakery industry, starch liquefaction, paper industry, detergent industry, and medical analysis.

Table 2.6 Amylase production capacity of yeast

Sr. No	***Yeast species***	***Amylase production***	***References***
1	*C. parapsilosis*	14.68 Units/ml	Oliveira et al., 2015
2	*R. mucilaginosa*	25 Units/ml	
3	*C. glabrata*	25.39 Units/ml	
4	*C. flavus*	0.25 Units/ml	Wanderley et al., 2004

Protease

Proteases are hydrolytic enzymes which contribute to the breakdown of peptide bonds in proteins into peptides and amino acids (Najafi et al., 2005; Vermelho et al., 2008). Depending on the target of action, proteases are divided into exo- and endo-peptidases. They are also grouped as serine, aspartic, cysteine and metallo proteases on the basis of the functional group of the enzyme and the active site of the enzymes (Rao et al., 1998). Proteases constitute approximately 60% of the total industrial enzyme market and applicable in various industries such as food processing, brewing industry, pharmaceuticals, leather processing, silver retrieval in the X-ray film industry, industrial waste treatment and as detergent additives for removal of the variety of organic materials such as, food, blood, and other body secretions as well as hard stains (Rao et al., 1998; Poza et al., 2001; Dias et al., 2010; Haki et al., 2003; Sumantha et al., 2006). Due to the action of proteases, raw foods get converted into nutritionally significant food products and it also makes the flavour changes with the direct proteolytic cleavages and/or by their conversion into volatile compounds. They also might be important as nutritious entities that have an influence on microbial communications throughout the fermentation procedure (Bolumar et al., 2005). Protease producing yeasts could be used in alcohol and beverage industry for juice clarification and easy filtration. In brewing, beer contains unassimilated protein particles which cause haze formation on cooling (Bishop, 1975; Charalambous, 1981) thereby affecting the quality of beer. In such case, protease hydrolyses undesired protein components.

The rapid protease activity have been observed from *Candida caseinolytica* (Poza et al., 2001), *Kluyveromyces* and *Candida* species (Koelsch et al., 2000; Poza et al., 2001). *Saccharomyces* spp., *Pichia farinosa*, *Candida humicola*, *C. pulcherrima*,

Kloeckera apiculata, *Pichia anomala*, *Rhodotorula bacarum*, *Leucosporidium antarcticum*, *Aureobasidium pullulans*, *Metschnikowia pulcherima*, *Hanseniaspora guillerimondi*, *Torulaspora delbruckii*, *Candida stelleta*, *Torulopsis magnolia*, *Yarrowia lypolitica*, *Cryptococcus* sp., *Kloeckera* spp.and *Hanseniaspora* spp. have been found to exhibit protease activity (Kim, 2010; Dizy and Bisson, 2000; Rao et al., 2011; Li et al., 2009). The protease production by *K. apiculate* have been also utilised to degrade protein residues in the wine Rensburg and Pretorius (2000).

Table 2.7 Protease production capacity of yeast

Sr. No	***Yeast species***	***Protease production***	***Reference***
1	*C. matritensis*	2.40 Units/ml	Rodarte et al., 2011
2	*M. reukaufii*	72.5 Units/ml	Jing et al., 2010

The cold active proteases obtained from *C. humicola* and *L. antarcticum* have been applied in detergents and soap powder of ‘‘cold washing’’ processes, food preparations and pharmaceutical fields (Margesin et al., 2008). The yeasts, *Kloeckera apiculata*, *Metschnikowia pulcherrima*, and *Torulaspora magnoliae* secrete proteases that work also in acidic conditions, were observed to be significant at the degradation of the proteins present in the wine in both wine and model solutions (Lagace and Bisson, 1990). The proteases also has been obtained from extremophilic yeast such as *Rhodotorula mucilaginosa*, *Cryptococcus adeliensis*, *Guehomyces pullulans* and *Metschnikowia australis* (Duarte et al., 2013).

Urease

Fermented foods and alcoholic beverages such as sake, beer and wine and distilled spirits are assessed to contain many toxic compounds such as ethyl carbamate (Varga et al., 2011) in the μg/L or μg/kg range (Canas et al., 1989; Ough, 1976; Wittkowski, 1997), formed by reaction of urea and alcohol during alcoholic fermentation, that could be harmful to humans as it is carcinogenic (Jiao et al., 2014; Canas et al., 1989; Alcarde et al., 2012).

Initially, urea carboxylases carboxylase urea into allophanate (Whitney and Cooper, 1973). In the fermentation reaction, the major enzyme precursors are generally produced with arginine metabolism by lactic acid bacteria or by *Saccharomyces cerevisiae* (Jiao et al., 2014). Urease is an enzyme, which hydrolyzes urea into ammonia and carbon dioxide (Varga et al., 2011). Urease was also highly found in yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) (Persike et al., 2002). *I. orientalis* isolated from Korean traditional nuruk showed urease production on Christensen urease agar (Lee and Park, 2012).

Table 2.8 Urease production capacity of yeast

Sr. No	***Yeast species***	***Urease production***	***Reference***
1	*C. tropicalis*	1.82 Units/ml	Bharathi and Meyyappan, 2015
2	*S. bayanus*	1.66 Units/mg	Balcerek and Szopa, 2006

Pectinase

Pectins are a structural hetero-polysaccharides and found largely in the parts of higher plants such as the middle lamellae and primary cell walls. The raw material that contains pectin adds turbidity, stickiness and viscosity to the solution. Therefore, raw

material containing pectin needs to be treated. Pectinase is a hydrolytic enzyme that cleaves down pectin. In the worldwide trades, the pectinase enzymes share 25 percent of total food enzymes. Pectinolytic enzymes are important particularly considering extraction and clarification of fruit juice, scouring of cotton, plant fibre degumming, vegetable oil extraction, wastewater treatment, fermentations of tea and coffee, paper bleaching and in the alcoholic beverages and food industries (Jayani et al., 2005; Rombouts and Pilnik, 1980; Gummadi and Panda, 2003; Kashyap et al., 2001; Hoondal et al., 2002; Baracat-Pereira et al., 1994; Henriksson et al., 1999). The pectinase degrades the pectin, which otherwise acts as an impeded in the filtration processes (Fernandez-Gonzalez et al., 2004). The pectinase is deliberately used in some processes of beverages to increase the colour intensity (Busse-Valverde et al., 2011; Pinelo et al., 2006).

Pectinases are produced mainly by bacteria, fungi and plants (Jayani et al., 2005; Alana et al., 1990; Pascualli et al., 1991; Fogarty and Kelly, 1983; Sakai et al., 1993), molds from rotten citrus products and by yeasts (Blanco et al., 1999; Fernandez-González et al., 2004; Oliveira et al., 2006; Alimardani-Theuil et al., 2011).

Enzyme production varies in yeasts species as well as between strains within same species (Table 2.6) (Masoud and Jespersen, 2006). Till date the pectic enzymes for industrial purposes have been extracted from moulds and bacteria (Sakai et al., 1993), the pectolytic action of yeasts has, however, been considered with conflicting outcomes (Charoenchai et al., 1997). Reports are largely lacking which investigated the yeast species associated with citrus juice producing pectinases (Parish and Haggins, 1989; Deak and Beuchat, 1993). Depending on strain, yeasts such as *Kluyveromyces marxianus*, *Trichosporon penicillatum*, *Saccharomycopsis* and *Saccharomyces*

cerevisiae have demonstrated pectinolytic activity (Blanco et al., 1999; Alimardani-Theuil et al., 2011) whereas, *K. thermotolerance* and *C. rugopelliculosa* showed a little activity. *S. cerevisiae* was found utilizing potato waste by producing pectinase and cellulase (Afifi, 2011). In addition, the tray fermentation of West African cocoa beans rich in pectin was accomplished by *S. cerevisiae* and *P. membranifaciens* (Jespersen et al., 2005). Notable pectolytic activity was reported in *Saccharomyces fragilis* and *Candida tropicalis*, whereas, *Saccharomyces thermantitonum*, *Torulopsis kefyr* and *Torulopsis lactosa* have shown less activity (Sakai et al., 1984).

Table 2.9 Pectinase production capacity of yeast

Sr. No	***Yeast species***	***Pectinase production***	***Reference***
1	*S. cerevisiae*	52..68 Units/ml	Arevalo-Villena et al., 2011
2	*K. marxianus*	0.98 Units/ml	Almeida et al., 2003
3	*A. pullulan*	1.16 Units/ml	Merin et al., 2012

There are various substrates utilized for pectinase screening including citrus pectin (Oliveira et al., 2006; Thangaratham and Manimegalai, 2014; Reddy and Sreeramulu, 2012; Venkata Naga raju and Divakar, 2013; Priya and Sashi, 2014), polygalacturonic acid (McKay, 1988; Oliveira et al., 2006) using Ruthenium red (Oliveira et al., 2006; Reddy and Sreeramulu, 2012), CTAB (Azad et al., 2013; Mukesh Kumar et al., 2012), Potassium iodide iodine solution (Thangaratham and Manimegalai, 2014; Venkata Naga raju and Divakar, 2013; Priya and Sashi, 2014; Anisa et al., 2013), cetrimide (Sandhya and Kurup, 2013).

Cellulase

Cellulose is an organic compound that consist of linear chain of hundreds to few thousands of β linked D-glucose. As the D-glucose is the simplest sugar, conversion of cellulose into D-glucose is necessary to utilize the bounded glucose. Cellulases enzymes break down cellulose (polymer of β-1,4 linked glucose units) into small glucose units which has several potential applications, such as the production of bioenergy and biofuels as well as application in the textile, laundry, and pulp and paper industry, food and feed industry, brewing, and agriculture (Castro and Pereira, 2010; Kuhad et al., 2011; Rai et al., 2012; Brindha et al., 2011). In industry, cellulases have found important applications of conversion of cellulose to fermentable sugars and ethanol (Rai et al., 2012). Cellulase performs enzymatic hydrolysis of the lignocellulose materials (Kitagawa et al., 2011) resulting to alcohol/biofuel (Nakatani et al., 2013). The cellulase enzymes have been reported from yeasts such as *Trichosporon cutaneum,T. pullulans, C. tropicalis* (Sulman and Rehman, 2013). Substrates used for this test could be CMC (Chen et al., 2004), cellulose (Goldbeck et al., 2012, 2013; Chen and Jin, 2006) using Congo red.

Table 2.10 Cellulase production capacity of yeast

Sr. No	***Yeast species***	***Cellulase production***	***Reference***
1	*C. laurentii*	0.11 Units/ml	Otero et al., 2015
2	*Candida sp*	44.84 Units/ml	Rai et al., 2012
3	*S. cerevisiae*	17.24 Units/ml	Sahoo and Shirnalli, 2015

Lipase

Lipases are carboxyl ester hydrolases which are responsible for catalysis of the

hydrolysis of triacylglycerol and fatty acid (Thevenieau et al., 2007; Rashid et al., 2009). Yeast lipases study deals with *Candida cylindracea, C. rugosa, Candida antarctica* and *Candida lypolytica* (now *Yarrowia lipolytica*) having its use in lipid stain digesters (Lock et al., 2007; Vakhlu and Kour, 2006; Fadiloglu and Erkmen, 2002; Fickers et al., 2005; D'Annibale et al., 2006; Rajendran et al., 2008), *Pseudozyma antarctica* (Shivaji and Prasad, 2009), and in species of *Cryptococcus* and *Rhodotorula, Candida*, *Yarrowia*, *Saccharomyces*, *Pichia*, *Debaryomyces* (Koilery and Keerthi, 2012; Vakhlu and Kour, 2006), *Aureobasidium* sp., *Candida haemulonii*, *Aureobasidium pullulans* var. melanogenum, *Cryptococcus aerius*, *C. flavescens*, *Cryptococcus* sp. and *Rhodosporidium paludigenum* isolated from mango (Gana et al., 2014). Industrial applications of lipases include roles in the food industry, resolution of pharmaceuticals, biodiesel, acylated flavonoids, biocide making, biosensor modulation, biosurfactants, speciality organic syntheses, resolution of racemic mixtures, and chemical analyses, hydrolysis of fats and oils, alteration of fats, waxes, synthesis of lipid-derived flavours and fragrances, and treatment of olive mill wastewater (Pandey et al.,1999; Larios et al., 2004; Xu et al., 2005; Rajeshkumar et al., 2013; Watanabe et al., 2002; Hsu et al., 2002; Salis et al., 2003; Passicos et al., 2004; Sharma et al., 2001).

Table 2.11 Lipase production capacity of yeast

Sr. No	***Yeast species***	***Lipase production***	***Reference***
1	*C. lipolytica*	7.9 µ/ml	Paskevicius, 2001
2	*Candida sp*	279 Units	Hou, 1997

Beta- glucosidase

Flavour is the impact of aroma and taste imparting distinguishing characteristic to fruit derived beverages and is dependant on complexity of different volatile components

within it (Lambrechts and Pretorius, 2000). This includes flavors contributed by various factors or stages such as varietal, prefermentative, fermentative and post fermentative (Schreier, 1979; Rapp, 1998). β-glucosidases form a key group among the glycoside hydrolases (Henrissat and Davies, 1997) which play a role in expression of flavor in the food by liberating aroma-rich terpenes or several secondary metabolites in a free, volatile and flavourless non-volatile forms (Gunata et al., 1990, Vasserott et al., 1995; Winterhalter and Skouroumounis, 1997), providing the varietal character to the beverage (Rapp and Versini, 1991).

To date, isolation of β-glucosidases has been attempted from plants, grapes or from micro-organisms like fungi (Cabaroglu et al., 2003; Takki et al., 2005; Vaithanomsat et al., 2011; Zahoor et al., 2011; Ahmed et al., 2013; Mfombep et al., 2013) and yeasts (Barnett et al., 1956).

Apart from *Saccharomyces*, non-*Saccharomyces* yeasts such as *P. anomala* have been studied for glucosidase activity (Spagna et al., 2002; Rosi et al., 1994) and found to possess interesting properties like altering the fermentation dynamics, composition and flavour of wine (Soden et al., 1998; Riou et al., 1998; Riccio et al., 1999). Most research on intra and extracellular production of β-glucosidase in yeasts such as *S. cerevisiae* and especially non-*Saccharomyces* yeasts from the genera *Kloeckera*, *Hanseniaspora*, *Brettanomyces*, *Debaryomyces*, *Candida*, *Metschnikowia*, *Pichia*, *Torulaspora* and species *Hanseniaspora valbyensis*, *Brettanomyces anomalus*, *Zygosaccharomyces*, *Hanseniaspora uvarum*, *Trichosporon asahii*, *Pichia fermentans* have found the activity

during intial stages of grape juice fermentation (Manzanares et al., 2000; McMahon et al., 1999; Mendes-Ferreira et al., 2001; Wang et al., 2011; Fernandez et al., 2000; Arroyo et al., 2011).

Table 2.12 β-glucosidase production capacity of yeast

Sr. No	***Yeast species***	**β-glucosidase *production***	***Reference***
1	*S. cerevisiae*	43,996.10 U mg-1 protein	Wanapu et al., 2012
2	*S. cerevisiae*	79 nmol	Fia et al., 2005
3	*C. albicans*	21 Units/ml	Polacheck et al., 1987

Several substrates have been employed for screening for this enzyme including various carbon sources such as arbutin esculin and 4-MUG on Yeast Nitrogen Base (Rosi et al., 1994; Charoenchai et al., 1997; Fia et al., 2005; Manzanares et al., 2000).

2.10 Antimicrobial activity of yeast against pathogenic bacteria

Microbial spoilage in foods causes risky health problems and product losses due to contamination caused by wild type yeasts, bacteria, fungi or molds during fermentations done by *Saccharomyces cerevisiae* (Adebayo et al., 2014; Agrawal and Prakash, 2013; Adebayo-Oyetoro et al., 2013). Antagonistic microorganisms prevent/inactivate food borne pathogens and spoilage microorganisms by producing antimicrobial metabolites (Buyuksirit and Kuleasan, 2014). Yeasts known as killer yeast are known to produce toxic proteins or glycoproteins that can inhibit bacteria and yeast species with the help of binding of double-stranded DNA viruses that is located in the cytoplasm of yeast cells, plasmids and chromosomal genes responsible for the production of some toxins on the cell surface and kill microorganisms (Magliani et al., 2004). Antimicrobial substances secreting strains of yeasts from different genera have

been isolated from varied sources and the toxins produced by different yeast strains show antimicrobial activity, inhibiting the growth of mold and bacteria as well as growth of other yeast strains (Roostita et al., 2011; Li et al., 2012). Different yeast species namely, *Candida saitoana, C. oleophila, Debaryomyces hansenii, Metschnikowia fruticola, M. pulcherrima, Pichia anomala, Rhodotorula glutinis* have been tested for their antimicrobial properties (Sharma et al., 2009). The species *Pichia anomala* has been found to secrete a killer toxin (Pikt) which has antifungal activity against *Brettanomyces/Dekkera* sp. (Ingeniis et al., 2009). The yeast species *Candida apis, C. bombicola, C. fructus, C. krusei, C. sorbosa, Hanseniaspora uvarum, Issatchenkia occidentalis, Kloeckera apis, Kluyveromyces marxianus, Pichia membranifaciens, Pichia ohmeri-like, and Sporobolomyces roseus* were found to be killer yeasts (Abranches et al., 1997). The yeasts *Gluconobacter asaii*, *Candida sp., Discosphaerina fagi* and *Metschnikowia pulcherrima* were found to inhibit the growth of *L. monocytogenes,* whereas, all four except *Candida* spp, inhibited the growth of *S. enterica* on fresh-cut apple tissue (Leverentz et al., 2006). *C. intermedia* and *Kluyveromyces marxianus* repressed growth of *L. monocytogenes* (Georges et al., 2006) and *S. cerevisiae* was found to reduce *L. monocytogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Rajkowska et al., 2012). Antagonisitc activity of *Candida* species was also seen by Kim et al. (1989) against *Pseudomonas syringae*. Antimicrobial activity against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* has been demonstrated by *Candida parapsilosis* (Roostita et al., 2011).

2.11 Alcohol fermentation

Fermentation for alcohol production is carried out naturally by yeasts in sequential manner with the growth of non-*Saccharomyces* yeast at intial stages wherein

they abundantly commence spontaneous fermentation and are metabolically active (Comitini et al., 2011; Lema et al., 1996; Schutz and Gafner, 1993). The nature and variety of species are unpredictable (Fleet, 2003) which initiate the fermentation and produce plethora of aromatic compounds and die off due to low alcohol tolerance (Combina et al., 2005b; Di Maro et al., 2007; Fleet, 1998) and fermentation medium is dominated by *Saccharomyces* yeast (Heard and Fleet, 1985). The yeasts from the genera *Kloeckera/Hanseniaspora* and also from other genera such as *Candida, Pichia* and *Metschnikowia* initiate the fermentation of grape juice (Povhe Jemec et al., 2001; Lema et al., 1996). After 3-4 days of fermentation, these yeasts are inhibited, and are substituted by the extremely fermentative yeast (*Saccharomyces cerevisiae*) which continue and conclude the fermentation process (Martini, 1993; Pretorius, 2000).

Survey of literature showed that *Saccharomyces* species were predominant members of the community which persisted and completed the fermentation (Fleet, 1990; Martini, 1993; Osho, 2005). Despite this fact, Desai et al. (2012) reported the presence of *Candida* species to be predominant in case of cashew apple juice. Various yeasts species further to *S. cerevisiae* have been reported in the fermentation of different cereal products such as sourdough breads impacting activities responsible for flavors. Prominent contributors have been reported to be *S. exiguus*, *C. humicola/C. milleri*, *T. delbrueckii*, *Pichia* species, *Debaryomyces hansenii, S. cerevisiae, S. uvarum* and other species of *Candida* (Meroth et al., 2003; Hammes et al., 2005). Species typically belonging to the genera such as *Hanseniaspora, Candida, Torulaspora, Metschnikowia* and *Kluyveromyces* have been reported to originate from the surface of the berry skin and the winery environment (Fleet et al., 1984; Fleet and Heard, 1993). Alongside these

yeast genera, *Issatchenkia* (Van Zyl and du Plessis, 1961) and *Pichia* (Kurtzman and Fell, 1998) may also be found at the early stage of fermentation.

2.12 Alcohol tolerance by yeasts

Alcoholic fermentation is studied extensively from grapes involving combined activities of different yeasts species. These yeasts grow in sequence all the way through the fermentation course which is commenced by different species of *Candida*, *Debaryomyces*, *Hanseniaspora*, *Pichia*, *Kloeckera*, *Metschnikowia*, *Schizosaccharomyces*, *Torulospora*, and *Zygosaccharomyces* that subsist in nature on the surface of grapes (Pretorius et al., 1999). The growth of yeasts is usually restricted to the initial 2 or 3 days of fermentation owing to the osmotic pressure generated by the added glucose. The later phases of alcohol fermentation are governed by the alcohol-tolerant strains of the *Saccharomyces* sensu strict group of yeasts mainly *Saccharomyces bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorians* (Henderson and Block, 2014).

This chain of actions happen as during fermentation, yeasts especially non-*Saccharomyces* yeasts at relatively low concentrations undergo alcohol stress which affects its cell structure and function, decrease their growth rate, cell viability, amino acid and glucose transport systems, increase membrane fluidity and consequential decrease in membrane integrity and cell membrane structure and function (Gibson et al., 2007; Ding et al., 2009; Stanley et al., 2010), slowing down cell division, diminishing cell volume and specific growth rate, while high ethanol concentrations lessen cell vitality and boost cell death (Birch and Walker, 2000).

For a yeast strain to be useful for industrial purpose, requires specific physiological properties such as sugar tolerance and invertase activities (Ekunsanmi and

Odunfa, 1990). Ethanol tolerance is an important character for use in industrial ethanol production (Jimenez and Benetez, 1986; Thammasittirong et al., 2013).

Alcohol tolerance plays very important role in successful and complete fermentation. Yeast which cannot tolerate alcohol lead to a stuck fermentation (Malherbe et al., 2007).

Even though the most robustly fermenting and more ethanol tolerant species of *Saccharomyces* lead the fermentation (Fleet and Heard, 1993; You et al., 2003), comparatively high ethanol concentrations slow down cell growth and viability, diminishing the fermentation productivity and ethanol yield (Aguilera et al., 2006). *S. cerevisiae strain* studied by Ghareib et al. (1988) was found to tolerate 13% ethanol but growth was prevented at 14% ethanol. Seven strains of *S. cerevisiae* obtained from different fruit sources showed alcohol tolerance levels between 7%-12% (Tikka et al., 2013). *Saccharomyces cerevisiae* TA and C2 strains isolated by Ali and Khan (2014) were high ethanol tolerant (tolerated 14% ethanol) whereas, *S. cerevisiae* isolated from banana peels tolerated 6%-12% (Brooks, 2008). Various strains yeasts *W. anomalus*, *Candida* spp., and *P. anomala* tolerated upto 15% alcohol (Lee et al., 2011). *S. cerevisiae* strains isolated from nuruk, cashew apple, and soil (Osho, 2005; Kang et al., 2000; Jung et al., 2008) could grow in a medium containing 15% alcohol. Yeast strain (*Saccharomyces cerevisiae*) from the sources such as grapes, molasses, musambi, cashew apple, distillary effluent, sorghum, sugarcane and it is used for the study of ethanol tolerance at various concentrations from 6-14.5%. Among these, the yeast strains isolated from molasses and cashew apple showed the highest tolerance upto 12.5% and strains from sorghum had least with 10% (Priya et al., 2011).

Exploitation of proficient yeast strains exhibiting superior ethanol tolerance in the alcoholic fermentation would reduce the costs of distillation and in turn the profitability taken as a whole process (Chandrasena et al., 2006).

There are few studies available on beverage production from cashew apple juice. Fully ripe cashew apples were used for production of wine (Mohanty et al., 2006). The titratable acidity (TA) changed from 0.24 g/ml from must to 1.2 g/ml in finished wine, increase in TA was associated with decrease in pH from 4.6 to 2.9 (Mohanty et al., 2006). Sparkling wine from cashew apple with alcohol content of 7.72%, pH 3.6, total acidity 0.59 g/ml was produced by (Abreu, 1997). Similarly, commercially available *S. cerevisiae* and three other wine yeasts were used for fermenting cashew apple juice. It was observed that wine produced from *S. cerevisiae* had good prospect for beverage industry (Osho and Odunfa, 1999). In a later study, clarified cashew apple juice was fermented with *S. bayanus* at 18°C and 30°C (Garruti et al., 2006).

As per Bureau of Indian Standards specifications for Cashew Feni and Giriappa (1996), cashew feni contains major aroma-based compounds and should be tested for these compounds (total and volatile acidity, esters, aldehydes, higher alcohols, alcohol and presence/absence of methanol).

Seventy one aroma volatiles were identified in cashew apple juice evaporated off and recovered in the water phase during its concentration (Sampaio et al., 2011). Different volatile compounds were seen in cashew apple and it varied as per the methodology used or to different varieties of cultivars or geographical regions (Franco and Janzantti, 2005).

Table 2.13 Composition of Feni

Constituents	Composition according to Giriaapa, 1996	Composition required according to BIS Specification of Feni
Alcohol (%)	43	42
Volatile acidity	19 gm/100 L absolute alcohol	60 gm/100 L absolute alcohol
Total solids	0.14%	-
Esters	244.2 gm/100 L absolute alcohol	50 gm/100 L absolute alcohol (Minimum reqirement)
Aldehydes	28 gm/100 L absolute alcohol	25 gm/100 L absolute alcohol
Higher alcohol	196.2 gm/100 L absolute alcohol	present
Furfural	5 gm/100 L absolute alcohol	-

Source: IS: 14326 (2005): https://archive.org/details/gov.in.is.14326.2005

However, it has been shown that though few volatile aroma essences arise from constituents of the fruits (e.g., grape) (Schreier et al., 1976), majority of the compounds found in wine are produced during fermentation by yeasts (Margalith and Schwartz, 1970). Many of these compounds are transformed and a considerable portion of beverage flavor substances are formed during yeast fermentation by conversion of sugars into alcohols, CO_2 and numerous secondary end products that together contribute to the delicacy and uniqueness of the character of the beverage (Nykanen, 1986; Lambrechts and Pretorius, 2000).

Natural fermentation is a complex process involving variations in fermentation processs affected by various factors, including the native microbial flora, the fruit variety, climatic conditions, and the process (Chira et al., 2011; Green et al., 2011;

Castilhos et al., 2012; Sharma et al., 2009; Cuadros-Inostroza et al., 2010; Hernandez-Orte et al., 2009; Romano et al., 2003). The major fractions of compounds that outline the "fermentation bouquet" are the balance of volatile compounds such as esters, organic acids and higher alcohols to some extent, aldehydes (Rapp and Versini, 1991). The preferable flavors of high quality end product are based on a balance of volatile constituents such as acids, alcohols, aldehydes, ketones, and esters (Ribereau-Gayon, 1978). These volatile metabolites are often formed only in trace amounts, yet these are of paramount importance for the flavor profile and organoleptic perception of a beverage (Verstrepen et al., 2003a, b).

The volatile compounds produced during the process of alcoholic fermentation depend on the type of yeast species as well as on the particular strain of the yeast within the species (Cabrera et al., 1988; Romano, 1998; Lambrechts and Pretorius, 2000). Although *S. cerevisiae* is involved in the alcoholic fermentation producing acetaldehydes, isoamyl acetate, ethyl acetate, isobutanol, n-propanol, amylic alcohol (Longo et al., 1992; Garde-Cerdan et al., 2006), the occurrence of non-*Saccharomyces* species might be significant because secondary metabolites are produced by these species, which can contribute to the final quality of the beverage (Esteve-Zarcoso et al., 1998). Reports are available demonstrating the non-*Saccharomyces* yeast strains modifying wine flavor and improving product quality through the release of secondary products such as aroma volatiles responsible as sensory attributes (Hansen et al., 2001; Heard, 1999; Soden et al., 2000; Dorneles et al., 2005). The quality of the end product of the fermentation process can, therefore, be difficult to predict and can vary depending on the significant role played by the natural yeast strains present during spontaneous fermentation (Matapathi et al., 2004; Sharma et al., 2012).

Various reports are available demonstrating the contribution of different non-*Saccharomyces* yeasts such as *Hanseniaspora, Candida, Pichia, Metschnikowia, Kluyveromyces, Schizosaccharomyces* and *Issatchenkia* in addition to *S. cerevisiae* and *S. bayanus*, to the aroma complexity of the final product (Ciani et al., 2010; Ciani and Ferraro, 1998; Jolly et al., 2006; Mendoza and Farias, 2010; Fleet, 2003; Pretorius, 2000).

It has been suggested that fermentation using mixed cultures of the non-*Saccharomyces* species with *S. cerevisiae* contributed positively to the final quality attributes such as taste and flavour of wine (Gil et al., 1996; Lema et al., 1996; Ciani and Ferraro, 1998; Soden et al., 2000). Many reports imply that the yeast interaction using mixed culture fermentation generate unexpected compounds and/or different levels of fermentation products affecting the final quality attributes (Howell et al., 2006; Anfang et al., 2009). Ciani et al. (2010) confirmed this by employing the controlled inoculation of *Saccharomyces cerevisiae* starter cultures and non-*Saccharomyces* yeasts, it had been proved to be a possible way towards improving the intricacy and improving the desirable characteristics of the fermented beverages.

2.12.1 Total acidity and Volatile fatty acid

Tartaric acids has been described as the most important organic acids in wine playing a vital role in the organoleptic quality and the physical, biochemical and microbial stability of wine (Volschenk et al., 2006). This acid enters in the beverage mostly from the fruit itself but the concentrations of tartrates in the fruits varied widely among fruit varieties (Kliewer et al., 1967). In grapes, the average concentrations of L-Tartaric acid are about 5 to 10 g/L (Ruffner, 1982).

According to Indian Standard Cashew Feni — Specifications, the volatile acid content of feni should be 60 g/100 L of absolute alcohol. If feni contains more than 60 g/100 L of absolute alcohol of acetic acid then it may mask the aroma of an alcoholic beverage leading to vinegar-like taste. In wine, near its flavour threshold of 0.7-1.1 g/L acetic acid becomes objectionable, depending on the style of wine, but may not be higher than 1.0-1.5 g/L, as specified in different countries (Eglinton and Henschke, 1999).

Volatile acid production

Acetic acid is formed early in fermentation and its production stops as soon as the sugar is fermented (Erasmus et al., 2004). Increasing amounts of acetic acid is produced below pH 3.2 and at values more neutral than pH 4 (Hanneman, 1985; Delfini and Cervetti, 1987).

2.12.2 Higher alcohol

The higher alcohol form the major group of fermentative group of aroma compounds in alcoholic beverages and are minor products of fermentation during alcohol production (Amerine et al., 1980). The higher alcohols are negatively perceived when their concentration in wine exceeds 400 mg/L (Rapp and Mandery, 1986). The concentration of the higher alcohols may vary from slightly less than 100 mg/L to a concentration higher than 500 mg/L (Nykanen, 1986).

Higher alcohols are made up of aliphatic (propanol, isobutyl alcohol, active amyl alcohol and isoamyl alcohol) and aromatic alcohols (major one is phenethyl alcohol) (Nykanen et al., 1977). Quantity of higher alcohols in alcoholic beverage depends on yeast strain (Giudici et al., 1990). Higher alcohols production preceds ester formation

during fermentation (Soles et al., 1982) with the association of the esters of higher alcohols with pleasant aromas.

Higher alcohol production

Various yeast strains contribute to the variation in the higher alcohol content (Giudici et al., 1990; Rankine, 1967). Antonelli et al. (1999) found two *S. cerevisiae* yeasts producing high quantities of 3- ethoxy-propanol. Mateo et al. (1991) reported that higher alcohol produced from musts of Monastrell grapes varied depending on different yeasts involved in fermentation such as *C. valida, Brettanomyces bruxellensis, Rhodotorula aurantica, H. uvarum, K. apiculata, Dekkera intermedia, S. cerevisiae var. capensis, S. cerevisiae var. chevalieri, S. cerevisiae var. bayanus* and *S. cerevisiae var. cerevisiae.*

2.12.3 Esters

Esters are volatile compound that impart pleasant fruity smell to fermented beverages (Verstrepen et al., 2003a). Esters are one of the largest and most important groups of compounds affecting flavor. Many of the esters found in alcoholic beverages are generated by yeasts during later part of fermentation (Herraiz and Ough, 1993) as secondary products of sugar metabolism (Peddie, 1990; Fujii et al., 1994). Ester formation is yeast strain dependent (Nykanen and Nykanen, 1977; Peddie, 1990; Daudt and Ough, 1973; Gammacurta et al., 2014). Besides acetic acid, ethyl acetate contributes significantly to the volatile character to beverge if present in higher level, as in case of wine (150 to 200 mg/L) imparting spoilage character to wine (Amerine and Cruess, 1960). Ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl caproate and 2-phenylethyl

acetate are the main esters that are associated with fruity, pear-drops, banana, apple and flowery aromas, respectively (Peddie, 1990).

Ester production

Non-*Saccharomyces* yeast strains are efficient in the production of esters which varies with the strain (Gammacurta et al., 2014; Rojas et al., 2001). Rojas et al. (2003) compared the ester production by using pure cultures of *S. cerevisiae* and mixed culture of *Hanseniaspora guilliermondii* and *Pichia anomala* co-cultures with *S. cerevisiae* and revealed an increase in acetate ester concentrations when compared to the pure cultured *S. cerevisiae*. These results were later confirmed by Viana et al. (2008). According to Nykanen (1986) *H. anomala* and *C. krusei* yeasts produces more ethyl acetate than *S. cerevisiae*, *S. pombe* and *P. membranifaciens.*

2.12.4 Aldehydes

Acetaldehyde is the major volatile compound of alcoholic fermentation found in alcoholic beverages (Rusu Coldea et al., 2011; Geroyiannaki et al., 2007), making upto more than 90% of the total aldehyde content in wines and spirits. It contributes both pleasing and negative character to the alcoholic product (Garza-Ulloa et al., 1976).

Aldehydes production

S. cerevisiae strains producing comparatively high levels of acetaldehyde (from 50 to 120 mg/L) have been reported (Fleet and Heard, 1993), whereas, *K. apiculata*, *C. krusei*, *C. stellata*, *H. anomala* and *M. pulcherima* produced low levels (from non detectable amounts to 40 mg/L) of acetaldehyde. The low activity of alcohol dehydrogenase in *S. bombicola* leads to excess of acetaldehyde production (Ciani et al., 2000). *S. cerevisiae* rapidly utilizes this excess aldehyde (Ciani and Ferraro, 1998).

Movement of acetaldehyde between *S. cerevisiae* and *S. bayanus* has been reported (Cheraiti et al., 2005). In mixed fermentations for acetaldehyde production, the interactions between *S. cerevisiae, K. thermotolerans* and *T. delbrueckii* have been observed (Ciani et al., 2006; Bely et al., 2008).

2.12.5 Alcohol production and methanol content

Besides ethanol which is the important criteria of any alcoholic beverage, methanol is also a part of an alcoholic beverage (Bindler et al., 1988) which occurs naturally in low concentration without causing any harm (Paine and Davan, 2001; Badolato and Duran, 2000). Presence of methanol due to the pectinolytic enzymes in ciders and wine have been reported (Mangas et al., 1993; Revilla and Gonzalez-San Jose, 1998). There are no reports of the presence of pectin in cashew apple juice but cashew apple residues left after juice extraction are rich in pectin (10%) (Nair, 2010; Giriappa, 1996) and can be used for making icecream, jam, jellies (Giriappa, 1996). Still another study has shown that cashew apple contains pectic substance which has the methoxyl content of 3.5% of anhydrouronic acid of 45.105 and jelly grade of 75 (Majumder, 2013).

CHAPTER 3

Isolation and identification of yeast using phenotypic and sequence analysis

3.1. Introduction

Diverse yeasts have been isolated from a range of fruit surfaces and fermented juices and products (Nyanga et al., 2013; Boboye et al., 2009; Chavan et al., 2009; Matapathi et al., 2004; Maragatham and Paneerselvam, 2011a,b; Obasi et al., 2014). *Saccharomyces cerevisiae* has been considered as the most prominent microorganism on many fruits responsible for fermentation (Albertin et al., 2011; Martinez et al., 2014). Osho (2005) has reported the presence of *S. cerevisiae* and *S. uvarum* from fermenting cashew apple juice, while, Barros et al. (2014) isolated yeast species belonging to genus *Hanseniaspora.* Desai et al. (2012) isolated *Candida* species from fresh cashew apple juice and were regarded as a prominent organism for the first and second grade alcohol production and alcoholic beverage production from cashew apple. The diversity of the yeasts varies depending on climatic conditions, fruit conditions, variety of fruits source, geographical locations (Longo et al., 1991; Arias et al., 2002, Chavan et al., 2009).

Traditionally, morphological characteristics and physiological properties of yeasts species have been employed for their identification and characterization (Kreger-van Rij, 1984; Barnett et al., 2000) besides biochemical characterization (Esteve-Zarcoso et al., 1999). However, these conventional procedures needs some 60 to 90 analyses to be performed making them complex, laborious, and lengthy process (Deak, 1995; Deak and Beuchat, 1996). In some instances even around 100 tests need to be performed to achieve a consistent identification of yeasts at the species level spanning over a period of 1-3 weeks to get the final results (Krejer-van Rij, 1984; Lin and Fung, 1987). For discrimination of the strains the morphological characters and physiological features were compared (Barnett et al., 1990 ; Krejer-van Rij, 1984), however, in some instances

this can infer to a wrong classification of species or misidentification of the strains (Spencer et al., 2011; Taverna et al., 2013).

During the past decade, with the introduction of molecular biology techniques, there has been a radical change in the identification of yeasts (Esteve-Zarcoso et al., 1999). These techniques included direct PCR amplification of conserved oligonucleotide primers, followed by restriction digestion of the amplified products employing endonucleases (Las Heras-Vazquez et al., 2003). In the present study, 26S rDNA sequencing was used to identify diverse yeasts isolated from fermented cashew apple juice. It has been suggested that the divergence in D1/D2 domain of yeast genome is usually enough to determine individual species (Kurtzman and Robnett, 1998).

Cashew apples are readily available as agricultural waste in Goa, however, regardless of their high sugar contents and other basic nutrients needed for growth of yeasts, these are not used to their fullest extent as culture medium for yeast strains and substrates for alcohol production. In this study, we attempted to isolate and identify yeast strains from naturally fermented cashew apple juice and evaluate their characteristics. In order to study the diversity of yeasts from naturally fermented cashew apple juice, yeast isolation followed by characterization of yeast isolates was performed by biochemical and genotypic analysis.

3.2. Materials and methods

The research work was carried out at the Microbiology Laboratory, Department of Animal Science, ICAR Research Complex for Goa, Old Goa, India.

3.2.1. Study area and origin samples

Naturally fermented cashew apple juice samples were collected from different

locations in Goa namely, Old Goa, Shiridao, Pomburpha, Galvar, Ucassaim, Karmali, Keri, Ponda, Thivim, Mandur, Agarwada, Vodla and Pajir and processed for isolation of yeasts. The places of collection were situated distantly and the units were owned by different persons (Table 3.1) engaged in extracting juice and distilling cashew feni by traditional method .

Table 3.1 Distance in km of sampling locations from Old Goa (Headquarter)

Sampling location	***Unit Name***	***No. of samples processes***	***No. of yeast isolates picked up***	***Distance between the sampling sites from Old Goa (Headquarter) (km)***
Old Goa	***Field***	***10***	***35***	***2***
Shiridao	***Field***	***3***	***10***	***11***
Galvar	***Field***	***2***	***8***	***27***
Ucassaim	***Field***	***2***	***10***	***24***
Thivim	***Cazcar Heritage Distillery***	***2***	***16***	***29***
Karmali	***Field***	***2***	***7***	***2***
Ponda	***Field***	***4***	***12***	***20***
Keri	***Field***	***6***	***25***	***49***
Mandur	***Field***	***3***	***14***	***5***
Agarwada	***Field***	***1***	***6***	***37***
Pomburpha	***Field***	***2***	***8***	***30***
Vodla	***Field***	***3***	***16***	***25***
Pajir	***Field***	***2***	***9***	***26***

All the units were practicing natural way of fermentation of extracted cashew apple juice and traditional way of distillation of the fermented juice. Except one at Thivim (Cazcar Heritage Distillery), no unit was using controlled way of fermentation.

3.2.2. Collection of samples

A total of 42 naturally fermented cashew apple juice samples were collected from different locations in Goa in sterile screw capped tubes. Approximately 15 ml of naturally fermented juice samples were collected in clean, dry sterile screw cap tubes and transferred to the laboratory under chilled conditions (2-8° C) and processed for yeast isolation within 24 h of collection.

3.2.3. Isolation of yeast strains

Samples were inoculated by spread plate method on Potato Dextrose Agar (PDA; Himedia) as described earlier (Warnasuriya et al., 1985; Verma et al., 2010). The plates were incubated at 28°C for 2 days (Mokhtari et al., 2011). The colonies appeared on agar plates were subcultured for further purification. Pure colonies were isolated and were further characterized.

3.2.4. Morphological characterization

Each of the purified colonies of yeasts were assessed for their colony characteristics mostly shape, colour, margin, texture (Chavan et al., 2009; Goralska, 2011; Spencer et al., 2011), and microscopic characteristics (yeast cell shape, presence/absence of budding and pseudohyphae) by lactophenol-cotton blue staining (Heritage et al., 1996; Haw et al., 2013; Elias et al., 2009) under 100x light microscope.

3.2.5. Biochemical characterization of yeasts

Biochemical characteristics of selected yeast strains were analysed according to procedures described earlier (Barnett et al., 1983; Kurtzman and Fell, 1998; Lodder, 1970; Barnett and Pankhurst, 1974; Kurtzman et al., 2011). The biochemical tests included: growth at 37^{O}C, glucose utilization at 50% (w/v), fermentation using 10 sugars (glucose, galactose, maltose, raffinose, lactose, sucrose, xylose, trehalose, mannitol and mannose), assimilation using 18 sugars (glucose, galctose, maltose, sucrose, rhamnose, cellobiose, lactose, salicin, ribose, mellibiose, melezitose, xylose, inulin, L-arabinose, D-arabinose, L-sorbose, erythritol and adonitol), and assimilation of nitrate. Utilization of glucose, sucrose, maltose, galactose and lactose as carbon sources by yeast strains was performed in Durham tubes in fermentation basal medium containing the particular sugar having the pH indicator. For determining the ability of growth at 37°C, the yeast strains were cultured on the PDA medium.

3.2.6. Identification by DNA sequencing

The genomic DNA from 50 selected yeast isolates was extracted by using Yeast DNA isolation kit (Genei, Bangalore). Following DNA quantification, the 26S rDNA gene fragment was subjected to amplification using PCR. A PCR-based method developed by de Barros Lopes et al. (1998) that permitted both intraspecies delineation and species recognition of yeast isolates was employed. In detail, 25 µl PCR reaction contained 12.5 µl 2x ReadyMix^{TM} PCR master mix (Sigma), 50 ng of DNA and 50 pmol of each primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Taverna et al., 2013; de Barros Lopes et al, 1998), 50 ng DNA template making the final volume to the 25 µl by molecular grade water. The cycling conditions included the 25 cycles each of denaturation at 94 °C for 1

min, annealing at 50 °C for 2 min, and extension at 74 °C for 1 min. For initial denturation of DNA template 3 min at 94 °C and a final 5 min extension at 74 °C were done. The isolates were subjected to amplification of the 5.8S rRNA gene and the two internal transcribed spacers (ITS) (Las Heras-Vazquez et al., 2003). Amplified products were purified and sequenced commercially (Merck-GeNei, Bangalore). The sequences obtained were BLAST searched in NCBI database. Sequences of the isolates those showed more than ≥0.98 identity values were confirmed as yeast species (Barghouthi, 2011).

3.3. Results

3.3.1. Isolation of yeast species

Forty-two samples of naturally fermented cashew apple juice were screened for yeasts on PDA agar. Based on the sizes and characteristics of the colonies, 176 yeast isolates were obtained (Table 3.2). The highest number of yeast isolates were isolated from samples collected from Old Goa, followed by Keri.

Predominantly isolated colonies exhibited smooth surfaces with irregular shape. The morphological data of the investigated strains is described and represented in Table 3.3. Wide variation in the colour of colonies was observed ranging from creamy white, white, off white, creamy, creamish yellow, orange and buff pink, and yellowish brown (Figure 3.1). Wide variation among the colonies of yeasts was observed in terms of surface, margin and colour. Smooth, irrregular and creamy white colonies were predominant.

Table 3.2: Number of isolates isolated from naturally fermented cashew apple juice.

Source of sample collection	No. of samples processed	No. of isolates picked up
Old Goa	10	**35**
Shiridao	3	**10**
Galvar	2	**8**
Ucassaim	2	**10**
Thivim	2	**16**
Karmali	2	**7**
Ponda	4	**12**
Keri	6	**25**
Mandur	3	**14**
Agarwada	1	**6**
Pomburpha	2	**8**
Vodla	3	**16**
Pajir	2	**9**
TOTAL	**42**	**176**

Variation was also observed in the shapes of the yeast cells such as spherical, elongate, cylindrical, globose, oval and spherical (Table 3.3) (Figure 3.1).

Based on distinguishing colony and microscopic characteristics, 50 yeast strains were selected from a total of 176 yeast isolates and tested for their biochemical characteristics.

3.3.2. Biochemical characterization yeasts

Each of the yeast strains were subjected to physiological studies using fermentation of carbon sources using 10 sugars, assimilation using 18 sugars, assimilation of nitrate and growth in presence of 50% glucose and at 37°C (Tables 3.4 and 3.5). Variable reactions were seen in fermentation and assimilation patterns for the sugars tested.

Table 3.3 Colony and microscopic characteristics of representative yeast isolates isolated from naturally fermented cashew apple juice.

Culture code	Colony characteristics					Microscopic characteristics	
	Shape	**Colour**	**Margin**	**Elevation**	**Texture**	**Cell shape**	**Budding/Pseudohyphae**
ICAR G1	Irregular	Brown	Undulate	Raised	Smooth, shiny	Oval	Budding
ICAR G10	Irregular	Yellowish	Undulate	Raised	Smooth, dry	Oval, Cylindrical	Budding
ICAR G30	Irregular	Cream	Undulate	Flat	Smooth, shiny	Oval	Budding
ICAR G50	Circular	White	Entire	Umbonate	Smooth, dry	Oval, Cylindrical, Globose, Elongate	Budding
ICAR G16	Circular	Yellowish brown	Entire	Convex	Smooth, shiny	Oval	Budding and pseudohyphae
ICAR G23	Irregular	Orange	Undulate	Flat	Smooth, shiny	Oval, Spherical	Budding

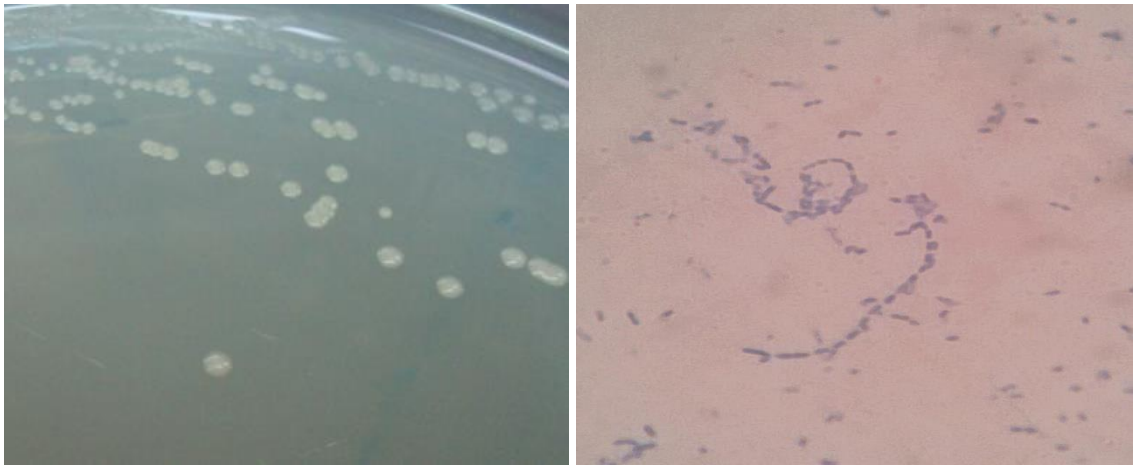
Table. 3.4. Fermentation patterns and growth characteristics of yeast isolates

Culture code	Identity	Fermentation using sugars																				Growth in presence of 50% glucose	Growth at 37°C
		Glucose		Glucose		Galactose		Galactose		Maltose		Maltose		Raffinose		Raffinose		Lactose		Lactose		Sucrose		Sucrose		Xylose		Xylose		Trehalose		Trehalose		Mannitol		Mannitol		Mannose		Mannose			
		A		G		A		G		A		G		A		G		A		G		A		G		A		G		A		G		A		G			
ICAR G1	*P. kudriavzevii*	+		+		+		±		-		-		-		-		-		-		-		-		-		-		-		-		+		±		+	+
ICAR G2	*P. occidentalis*	+		+		+		±		-		-		+		±		-		-		±		±		-		-		-		-		+		±		+	±
ICAR G3	*C. ethanolica*	±		±		±		±		-		-		-		-		-		-		-		-		-		-		-		-		-		±		-	+
ICAR G4	*P. galeiformis*	+		+		±		±		-		-		-		-		-		-		-		-		-		-		-		-		-		+		±		-	±
ICAR G5	*P. manshurica*	+		+		±		±		-		-		-		-		-		-		±		±		-		-		-		-		-		+		+		-	-
ICAR G6	*P. kudriavzevii*	+		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+		+		+	+
ICAR G7	*P. kudriavzevii*	+		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+	+
ICAR G8	*P. kudriavzevii*	+		+		-		-		+		-		+		-		-		-		-		-		-		-		-		-		-		+		+		+	+
ICAR G9	*P. kudriavzevii*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+		±		±	+
ICAR G10	*P. manshurica*	±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	+
ICAR G11	*Pichia spp.*	+		+		±		±		-		-		±		±		-		-		+		±		-		-		-		-		-		+		±		±	+
ICAR G12	*P. manshurica*	+		+		+		+		-		-		±		±		-		-		+		±		-		-		-		-		-		±		±		-	+
ICAR G13	*P. galeiformis*	+		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	+
ICAR G14	*P. galeiformis*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		±		±		-	+
ICAR G15	*I. orientalis*	+		+		+		±		-		-		-		-		-		-		-		-		-		-		-		-		-		+		+		+	+
ICAR G16	*P. membranifaciens*	±		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+	+
ICAR G17	*I. orientalis*	+		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+		-		±	+
ICAR G18	*I. orientalis*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+		+		±	+
ICAR G19	*P. manshurica*	+		+		+		-		±		-		+		±		-		-		+		+		-		-		-		-		-		±		±		-	-
ICAR G20	*P. manshurica*	+		+		+		+		+		+		+		+		-		-		+		+		-		-		-		-		-		+		+		±	±
ICAR G21	*S. cerevisiae*	+		+		-		-		+		-		+		+		-		-		+		+		-		-		-		-		-		+		+		+	+
ICAR G22	*P. kudriavzevii*	+		+		±		±		-		-		-		-		-		-		-		-		-		-		-		-		-		±		+		+	+
ICAR G23	*R. mucilaginosa*	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	+
ICAR G24	*Pichia spp.*	+		+		±		+		-		-		+		+		-		-		±		±		±		±		-		-		-		±		+		±	-
ICAR G25	*P. kudriavzevii*	+		+		±		±		-		±		+		+		-		-		-		-		-		-		-		-		-		±		±		±	-
ICAR G26	*P. kudriavzevii*	±		-		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		±	+
ICAR G27	*P. kudriavzevii*	±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+	+
ICAR G28	*I. terricola*	+		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		±		-		-	±
ICAR G29	*L. fermentatii*	+		+		+		+		-		-		+		±		-		-		+		+		-		-		±		±		-		-		+		+	+
ICAR G30	*P. kudriavzevii*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	+
ICAR G31	*P. kudriavzevii*	+		+		±		±		-		-		-		-		-		-		-		-		-		-		-		-		-		±		±		+	+
ICAR G32	*P. kudriavzevii*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+		+		+	+
ICAR G33	*I. orientalis*	+		+		+		±		-		-		±		±		-		-		±		±		-		-		-		-		-		+		+		±	+
ICAR G34	*P. manshurica*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	+
ICAR G35	*P. kudriavzevii*	±		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	+
ICAR G36	*P. kudriavzevii*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+		+		+	+
ICAR G37	*P. kudriavzevii*	±		±		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	+
ICAR G38	*P. kudriavzevii*	+		+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+		+		+	+
ICAR G39	*P. kudriavzevii*	+		+		±		±		-		-		±		±		-		-		-		-		-		-		-		-		-		-		+		-		+	+
ICAR G40	*P. kudriavzevii*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		±		±		+	+
ICAR G41	*I. terricola*	+		+		-		-		-		-		-		-		-		-		±		±		-		-		-		-		-		-		-		-	-
ICAR G42	*P. kudriavzevii*	+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		±		±		+	+
ICAR G43	*I. orientalis*	+		+		+		+		-		-		-		-		-		-		-		-		-		-		-		-		-		±		±		+	+
ICAR G44	*S. cerevisiae*	+		+		+		+		±		-		+		+		-		-		+		+		-		-		-		-		-		+		+		+	-
ICAR G45	*S. cerevisiae*	+		+		+		+		-		-		+		+		-		-		+		+		±		±		±		±		±		-		+		+		+	±
ICAR G46	*I. orientalis*	+		+		+		+		-		-		-		-		-		-		+		±		-		-		-		-		-		-		+		+		+	+
ICAR G47	*S. cerevisiae*	+		+		+		+		±		±		±		+		-		-		+		+		-		-		+		±		±		±		+		+		+	+
ICAR G48	*S. cerevisiae*	+		+		+		+		+		+		+		+		-		-		+		+		-		-		+		±		-		-		+		+		+	-
ICAR G49	*R. mucilaginosa*	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-		+	+
ICAR G50	*S. cerevisiae*	+		+		+		+		+		+		+		+		-		-		+		+		-		-		-		-		-		+		+		-	-

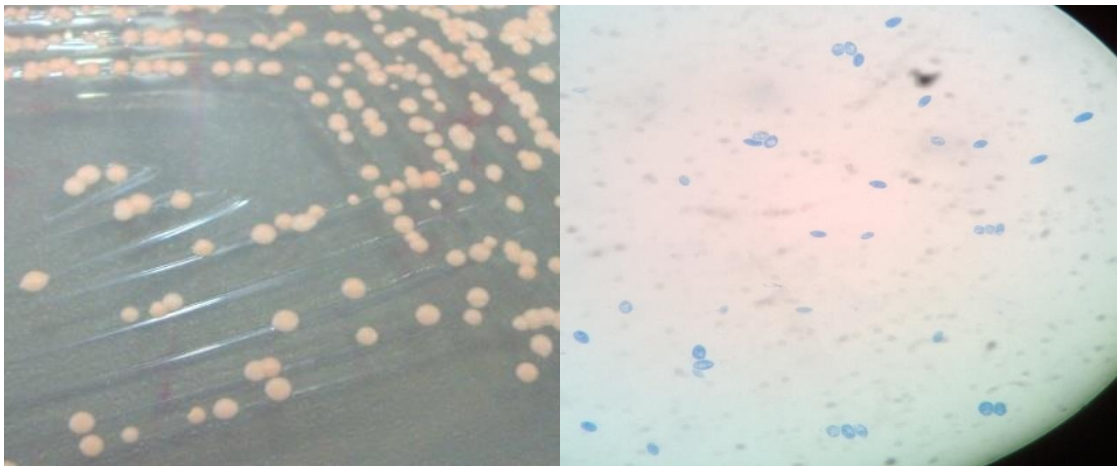
Table 3.5 Assimilation patterns of the yeast isolates.

Culture code	Identity	Assimilation of sugars																			Nitrate utilization
		Glucose	Galactose	Maltose	Sucrose	Rhamnose	Cellobiose	Lactose	Salicin	Ribose	Mellibiose	Melezitose	Xylose	Inulin	L-arabinose	D-arabinose	Ethanol	L-sorbose	Erythritol	Adonitol	
ICAR G1	*P. kudriavzevii*	+	+	+	+	-	-	-	-	-	±	-	±	±	±	±	+	-	-	-	-
ICAR G2	*P. occidentalis*	+	±	±	±	-	-	-	-	-	-	-	-	-	-	-	+	±	-	-	-
ICAR G3	*C. ethanolica*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G4	*P. galeiformis*	+	+	±	+	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-
ICAR G5	*P. manshurica*	+	+	+	+	±	-	-	-	-	±	±	-	±	±	-	+	-	-	-	-
ICAR G6	*P. kudriavzevii*	+	±	±	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G7	*P. kudriavzevii*	+	-	±	+	±	-	-	-	-	±	-	-	-	-	-	+	-	-	-	-
ICAR G8	*P. kudriavzevii*	+	±	±	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G9	*P. kudriavzevii*	+	+	-	±	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G10	*P. manshurica*	+	+	±	+	-	-	-	-	-	-	-	-	-	±	±	±	-	-	-	-
ICAR G11	*Pichia spp.*	+	+	±	±	±	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-
ICAR G12	*P. manshurica*	+	+	+	+	-	-	-	-	-	±	-	±	-	±	±	+	-	-	-	-
ICAR G13	*P. galeiformis*	+	+	-	±	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G14	*P. galeiformis*	+	+	-	-	-	-	-	-	-	-	-	±	-	-	-	+	-	-	-	-
ICAR G15	*I. orientalis*	+	+	±	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G16	*P. membranifaciens*	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ICAR G17	*I. orientalis*	+	+	-	±	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G18	*I. orientalis*	+	-	-	-	±	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G19	*P. manshurica*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ICAR G20	*P. manshurica*	+	±	±	+	±	-	-	-	-	-	±	-	-	-	-	+	-	-	-	-
ICAR G21	*S. cerevisiae*	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G22	*P. kudriavzevii*	+	+	+	+	±	-	-	-	-	±	-	-	-	±	±	+	-	-	-	-
ICAR G23	*R. mucilaginosa*	+	+	±	+	±	±	-	+	-	-	-	+	-	+	+	+	-	-	-	-
ICAR G24	*Pichia spp.*	+	±	±	±	-	-	-	-	-	±	-	-	±	-	-	±	-	-	-	-
ICAR G25	*P. kudriavzevii*	+	-	±	-	-	-	-	-	-	±	±	+	-	±	±	±	-	-	-	-
ICAR G26	*P. kudriavzevii*	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-
ICAR G27	*P. kudriavzevii*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-
ICAR G28	I. terriola	+	±	-	±	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G29	L. fermentatii	+	+	±	±	±	±	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G30	*P. kudriavzevii*	+	+	±	-	±	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G31	*P. kudriavzevii*	+	±	±	±	±	-	-	-	-	-	-	±	-	-	-	+	-	-	-	-
ICAR G32	*P. kudriavzevii*	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G33	*I. orientalis*	+	+	+	+	±	-	-	-	-	-	±	-	-	-	-	+	-	-	-	-
ICAR G34	*P. manshurica*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	±	+	-	-	-	-
ICAR G35	*P. kudriavzevii*	+	+	-	±	-	-	-	-	-	-	-	-	±	-	±	+	-	-	-	-
ICAR G36	*P. kudriavzevii*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G37	*P. kudriavzevii*	+	-	-	±	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G38	*P. kudriavzevii*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G39	*P. kudriavzevii*	+	±	-	±	-	-	-	-	-	-	-	-	-	-	-	+	±	-	-	-
ICAR G40	*P. kudriavzevii*	+	-	-	-	-	-	-	-	-	±	-	-	-	-	-	+	-	-	-	-
ICAR G41	*I. terriola*	+	+	-	+	±	±	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G42	*P. kudriavzevii*	+	+	-	-	-	-	-	-	-	-	-	-	-	±	±	+	-	-	-	-
ICAR G43	*I. orientalis*	+	±	+	+	-	-	-	±	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G44	*S. cerevisiae*	+	±	±	+	-	-	-	-	±	-	-	-	-	-	-	+	-	-	-	-
ICAR G45	*S. cerevisiae*	+	±	+	+	-	-	-	-	-	-	-	±	-	-	-	+	-	-	-	-
ICAR G46	*I. orientalis*	+	+	±	±	-	-	-	±	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G47	*S. cerevisiae*	+	+	-	±	±	-	-	-	±	-	±	-	-	±	-	+	-	-	-	-
ICAR G48	*S. cerevisiae*	+	+	+	+	±	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G49	*R. mucilaginosa*	+	±	-	±	±	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-
ICAR G50	*S. cerevisiae*	+	±	+	+	±	-	-	-	±	-	-	±	-	±	-	+	-	-	-	-

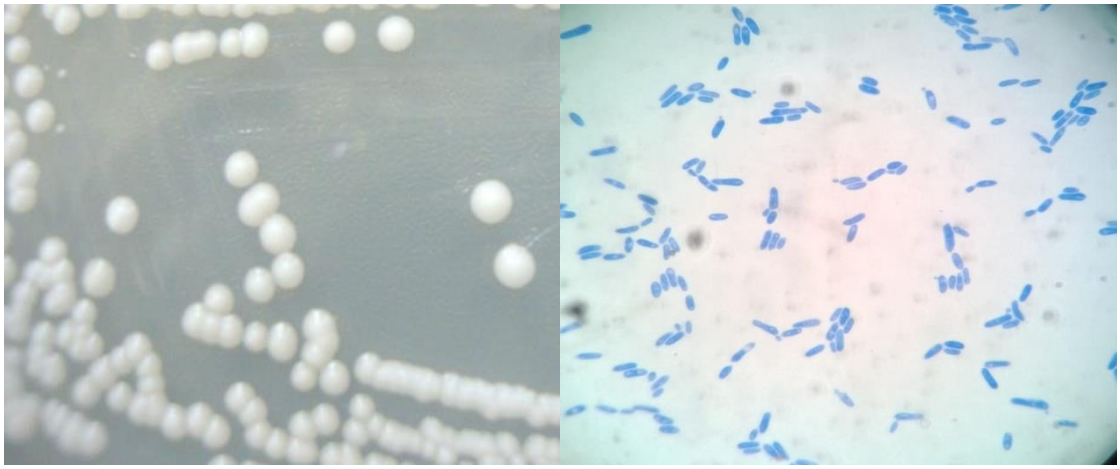
Figure 3.1 Colony characteristics of representative yeast isolates.



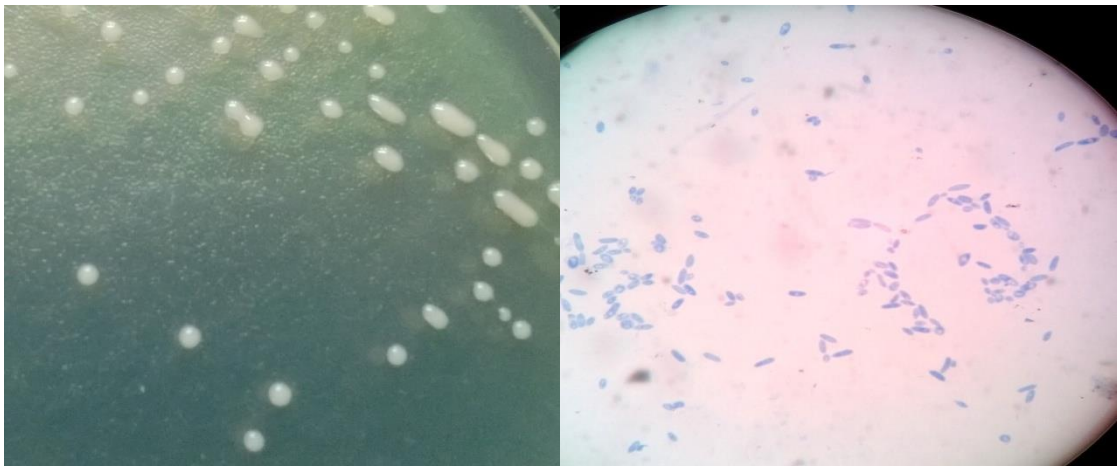
Colony and microscopic characters of *P. membranifaciens*.



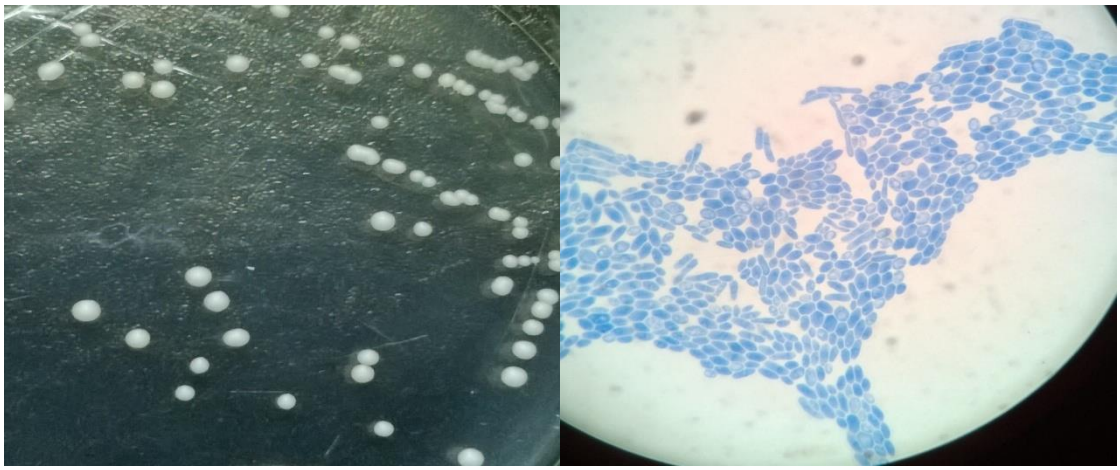
Colony and microscopic characters of *R. mucilaginosa*.



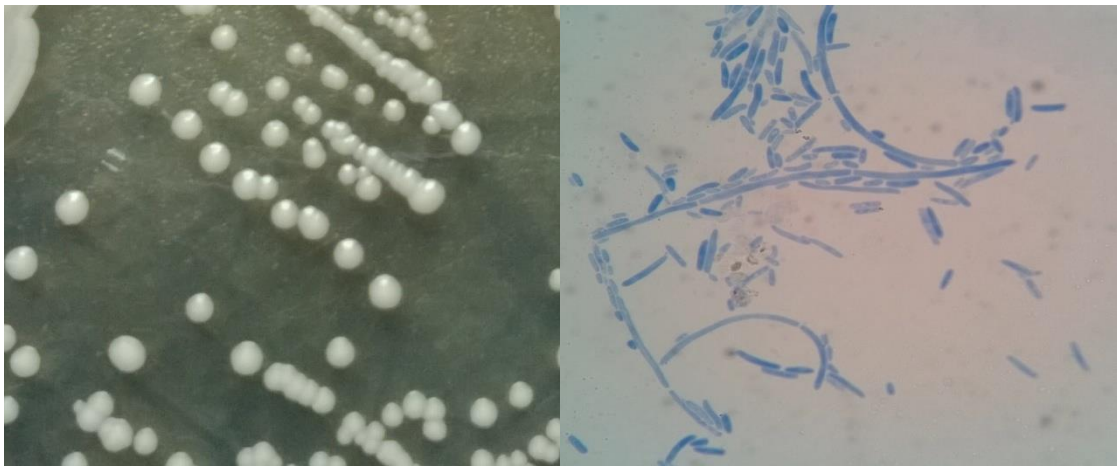
Colony and microscopic characters of *P. kudriavzevii*.



Colony and microscopic characters of *P. galeiformis*.



Colony and microscopic characters of *S. cerevisiae*



Colony and microscopic characters of *I. orientalis*

The biochemical analysis of the isolates showed that twenty five strains could grow in the presence of 50% glucose, nine showed weak growth and rest sixteen could not grow. Eight isolates however did not grow at 37°C. However, wide variation in assimilation profiles was observed among these strains.

3.3.3 Identification of yeasts by sequencing

The fifty yeasts strains selected on the basis of morphology and biochemical characteristics were subjected to sequence and phylogenetic analysis. BLAST analysis of the nucleotide sequences of the fifty yeast strains were identified showing >98% similarities**.** The identification of the 50 isolates studied has been given in Table 3.6. Overall, the sequences revealed the yeast flora belonging to genera *Saccharomyces*, *Rhodotorula, Candida, Pichia, Issatchenkia* and *Lachancea,* with *Pichia* being the dominant genus. The partial sequences were submitted to the GeneBank with the accession numbers given in Table 3.6. Specific biochemical tests together with sequencing identified isolates as *Saccharomyces cerevisiae, Pichia kudriavzevii, P. membranifaciens, P. galeiformis, P. manshurica, P. occidentalis, Issatchenkia terricola, I. orientalis (P. kudriavzevii), Candida ethanolica, Lachancea fermentati,* and *Rhodotorula mucilaginosa*. Out of 50, the majority of the yeasts identified were *Pichia kudriavzevii* (n=19), followed by *Pichia manshurica* (n=6) *Issatchenkia orientalis* (n=6) and *Saccharomyces cerevisiae* (n=6) (Table 3.7). Two yeast isolates belonging to genus *Pichia* could not be identified to species level owing to their low similarity (<94%). These isolates might represent novel species.

Table 3.6: Identification of yeasts based on their 26S rDNA sequence analysis. The 26S rDNA sequences obtained were BLAST searched and isolates showing >0.98 Identity value were considered as confirmed species. (Barghouthi 2011; de Barros Lopes et al., 1998)

Sr. no	Culture code	Identified species	Accession No.
1	ICAR G1	*Pichia kudriavzevii*	KM234478.1
2	ICAR G2	*Pichia occidentalis*	KM234444.1
3	ICAR G3	*Candida ethanolica*	KM234475.1
4	ICAR G4	*Pichia galeiformis*	KM234452.1
5	ICAR G5	*Pichia manshurica*	KM234474.1
6	ICAR G6	*Pichia kudriavzevii*	KM234473.1
7	ICAR G7	*Pichia kudriavzevii*	KM234470.1
8	ICAR G8	*Pichia kudriavzevii*	KM234468.1
9	ICAR G9	*Pichia kudriavzevii*	KM234467.1
10	ICAR G10	*Pichia manshurica*	KM234471.1
11	ICAR G11	*Pichia spp.*	KP223717.1
12	ICAR G12	*Pichia manshurica*	KM234469.1
13	ICAR G13	*Pichia galeiformis*	KM234450.1
14	ICAR G14	*Pichia galeiformis*	KM234447.1
15	ICAR G15	*Issatchenkia orientalis*	KR259304
16	ICAR G16	*Pichia membranifaciens*	KM234477.1
17	ICAR G17	*Issatchenkia orientalis*	KR 259307
18	ICAR G18	*Issatchenkia orientalis*	KR 259308
19	ICAR G19	*Pichia manshurica*	KM234439.1
20	ICAR G20	*Pichia manshurica*	KM234451.1
21	ICAR G21	*Saccharomyces cerevisiae*	KM234476.1
22	ICAR G22	*Pichia kudriavzevii*	KM234466.1
23	ICAR G23	*Rhodotorula mucilaginosa*	KP223715.1

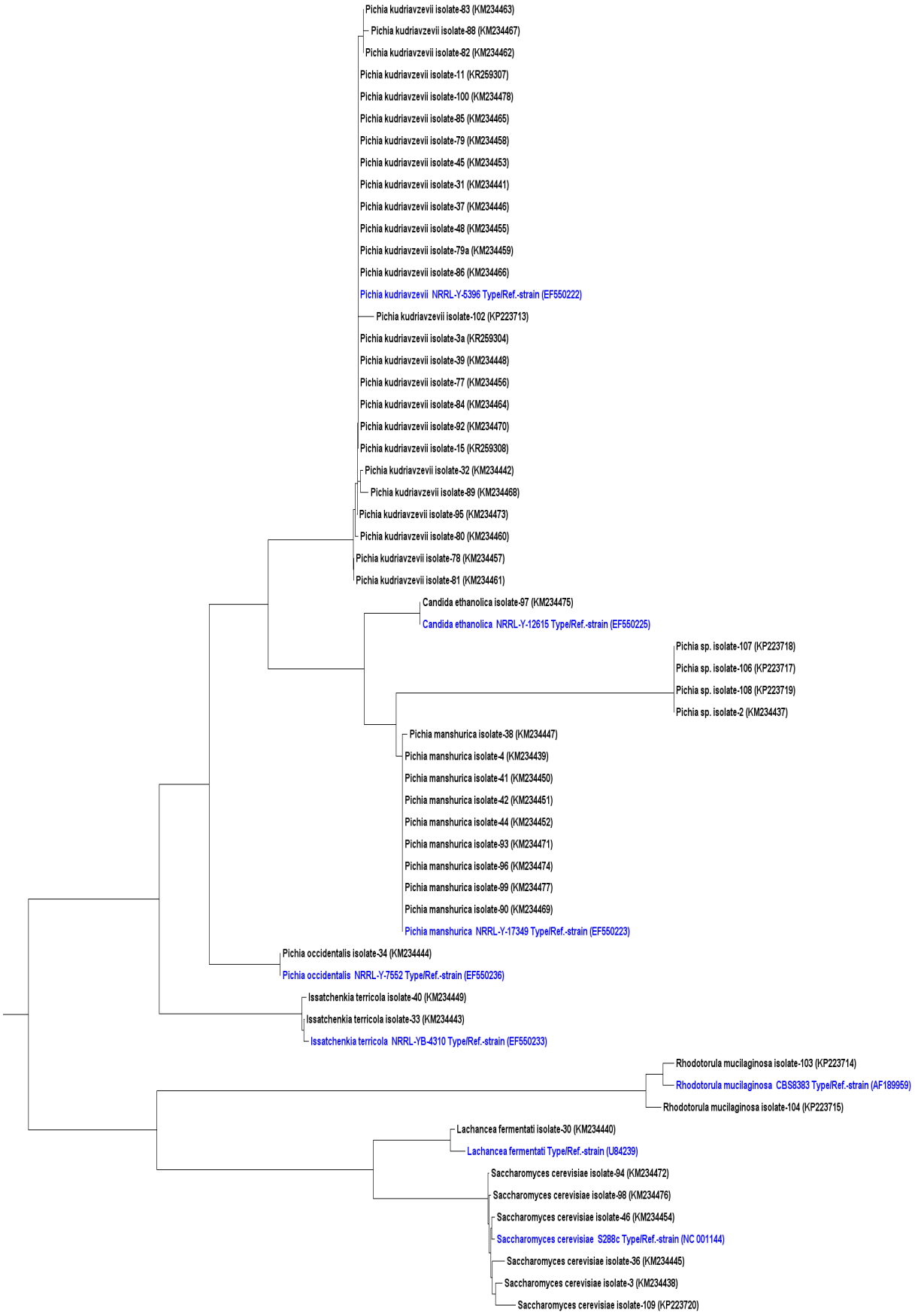
24	ICAR G24	*Pichia spp.*	KP223718.1
25	ICAR G25	*Pichia kudriavzevii*	KM234453.1
26	ICAR G26	*Pichia kudriavzevii*	KM234465.1
27	ICAR G27	*Pichia kudriavzevii*	KM234464.1
28	ICAR G28	*Issatchenkia terricola*	KM234449.1
29	ICAR G29	*Lachancea fermentatii*	KM234440.1
30	ICAR G30	*Pichia kudriavzevii*	KM234448.1
31	ICAR G31	*Pichia kudriavzevii*	KM234463.1
32	ICAR G32	*Pichia kudriavzevii*	KM234462.1
33	ICAR G33	*Issatchenkia orientalis*	KP223713.1
34	ICAR G34	*Pichia manshurica*	KM234437.1
35	ICAR G35	*Pichia kudriavzevii*	KM234446.1
36	ICAR G36	*Pichia kudriavzevii*	KM234461.1
37	ICAR G37	*Pichia kudriavzevii*	KM234442.1
38	ICAR G38	*Pichia kudriavzevii*	KM234460.1
39	ICAR G39	*Pichia kudriavzevii*	KM234459.1
40	ICARG40	*Pichia kudriavzevii*	KM234458.1
41	ICAR G41	*Issatchenkia terricola*	KM234443.1
42	ICAR G42	*Pichia kudriavzevii*	KM234457.1
43	ICAR G43	*Issatchenkia orientalis*	KM234456.1
44	ICAR G44	*Saccharomyces cerevisiae*	KM234472.1
45	ICAR G45	*Saccharomyces cerevisiae*	KM234454.1
46	ICAR G46	*Issatchenkia orientalis*	KM234441.1
47	ICAR G47	*Saccharomyces cerevisiae*	KP223720.1
48	ICAR G48	*Saccharomyces cerevisiae*	KM234445.1
49	ICAR G49	*Rhodotorula mucilaginosa*	KP223714.1
50	ICAR G50	*Saccharomyces cerevisiae*	KM234438.1

Table 3.7. Yeasts identified by the 26S rDNA and ITS sequence analysis.

Species	**Number**
Candida ethanolica	1
Issatchenkia terricola	2
Lachancea fermentati	1
Pichia galeiformis	3
Pichia kudriavzevii	19
Pichia manshurica	6
Pichia membranifaciens	1
Pichia occidentalis	1
Pichia sp.	2
Rhodotorula mucilaginosa	2
Saccharomyces cerevisiae	6
Issatchenkia orientalis (P. kudriavzevii)	6
Total	50

The phylogenetic tree representing isolated yeast strains and some reference strains is shown in Figure 3.2.

Fig. 3.2. The phylogenetic tree representing isolated yeast strains and some reference strains.



The phylogenetic analysis of the identified yeast strains (Fig. 3.2) revealed grouping of the strains according to species and genera. The type strains of the each of the yeast strains were also included in the analysis. The phylogenetic analysis of the sequences revealed different clades for different species of yeasts isolated. The *Pichia* species formed a separate clade along with *Issatchenkia*. Other genera were showed separate branching. *I. orientalis* and *P. kudriavzevii* clustered together. The analysis showed that the genus *Issatchenkia* was phylogenetically similar to *Pichia*.

3.4 Discussion

Microbial species are found in nature in abundance. It is difficult to culture and isolate many of the organisms and considered as viable but non-culturable. In this study naturally fermented cashew apple juice samples were examined for types of yeast flora. Identification of yeast species using phenotypic assays can give unreliable results (Arroyo-Lopez et al., 2006; Bezerra-Bussoli et al., 2013). Phenotypic characteristics may be influenced by conditions of the culture and may provide indecisive results (Yamamoto et al., 1991). The intra-species strain variability might be the primary reason for the misidentification of these strains (Chavan et al., 2009).

Yeast identification employing assimilation tests alone may not be reliable as a universal means for identification. Numerous novel species, intraspecies variability of strains and conflicting assimilation profiles might be responsible for misidentifications (Rohm and Lechner, 1990). However, assimilation tests still have a role in primary screening and aiding the other methods in identification such as sequencing (Pathania et al., 2010).

Limitations exhibited by conventional methods could be prevailed over by using molecular markers for identification, taxanomy and genetic analysis of yeasts (Pathania et al., 2010) which also offer identificaion of highly variable regions in DNA fragments simultaneouly. Yeast identification using DNA sequencing method is the most preferred (Valente et al., 1999). Many researchers have followed D1/D2 sequencing method for yeast identification (Bhadra et al., 2008). The sequence analysis of the 5.8S rRNA gene and internal transcribed spacers (ITS) have been used for yeast identification (Las Heras-Vazquez et al., 2003; Chavan et al., 2009).

Identification of the 50 yeasts strains was done using biochemical and blast searches of the similarity in nucleotide sequences. *Rhodotorula mucilaginosa*, *Issatchenkia orientalis* and *P. membranifaciens* were tentatively identified based on colony and microscopic and biochemical characteristics and further identification was carried out based on nucleotide sequences. It was difficult to differentiate other strains among *Issatchenkia*, *Pichia* and *Candida* using biochemical tests. They have ascribed it to conflicting biochemical characteristics and variations among the strains (Chavan et al., 2009). Molecular identification of *R. mucilaginosa* and *P. membranifacines* corresponded to their biochemical characteristics.

Several researchers reported yeasts from fruits and fruit juices (Bhadra et al., 2007; Chavan et al., 2009). Natural foods such as honey, fruit juices, milk were found to harbour the genera of yeasts such as *Saccharomyces, Issatchenkia, Candida, Rhodotorula, Kluyveromyces, Pichia, Trichosporon, Kloeckera* and *Zygosaccharomyces* (Ivo, 1982; Bai et al., 2010; Carvalho et al., 2010; Hakim et al., 2013; Moreira et al., 2001; Stratford et al., 2002; Mushtaq et al., 2004; Gadaga et al., 2000; Ebabhi et al.,

2013; Mirbagheri et al., 2012; Saleh, 2013; de Melo et al., 2007). While isolating yeasts from grape berries, *P. membranifaciens, Saccharomycodes ludwigii, Candida* species, *I. orientalis* and *Rhodotorula* spp. were confirmed (Combina et al., 2005a). Isolation of *Pichia* spp., *Candida pulcherrima, C. lambica, C. sake, Rhodotorula* spp. and *Debaryomyces polymorphus* have been reported from samples of fruit salads and pasteurized fruit juice (Tournas et al., 2006). *Saccharomyces cerevisiae* has been isolated from orange fruit juice (Las Heras-Vazquez et al., 2003), ogol, a traditional Ethiopian honey wine, fermentation (Teramoto et al., 2005).

Isolation of *Pichia kudriavzevii* has been previously reported from various sources such as sour doughs, fermented butter-like products, the starter culture of Tanzanian fermented togwa, the African fermented products like cassava lafun and a Ghanaian cocoa bean heap, fermented juices like pineapple and orange, and grapes (Chan et al., 2012). Significantly, *P. kudriavzevii,* was earlier reported as a potential bioethanol producer (Chan et al., 2012), and these yeasts isolates obtained in this study too were from fermented cashew apple juice inferring the potential for alcohol production.

P. manshurica is generally recognized as wine spoilage yeast, was also found to be dominant from cashew apple juice (Saez et al., 2011) and decaying mangosteen fruit (Bhadra et al., 2008). *P. manshurica* produced volatile phenols causing intense odour and bitter test (Saez et al., 2011).

Issatchenkia orientalis also synonymously known as *Pichia kudriavzevii* and an anamorph of *Candida krusei*, is a budding yeast involved in chocolate production (Bezerra-Bussoli et al., 2013; Satyanarayana and Kunze, 2009).

Lachancea fermentati has been previously isolated from coconut water and reconstituted fruit juice (Maciel et al., 2013). *P. membranifaciens* has been isolated from decaying mangosteen fruit (Bhadra et al., 2008) and grapes (Masih et al., 2001).

Though, *Rhodotorula* species were previously considered as non-pathogenic, these have emerged as an opportunistic pathogens (Capoor et al., 2014) and recovered from many environmental sources (Wirth and Goldani, 2012). *R. mucilaginosa* has been frequently detected from foods and beverages such as peanuts, apple cider (Fard et al., 2012; Tournas et al., 2006; Mokhtari et al., 2011), cherries (Venturini et al., 2002), fresh fruits and juices (Gholamnejad et al., 2010), fermented beverages (Jimoh et al., 2012), sugarcane (de Azeredo et al., 1998), cheese (Senses-Ergul et al., 2006), sausages (Gardini et al., 2001), edible molluscs (Kajikazawa et al., 2007), and crustaceans (Eklund et al., 1965). The species have been isolated from air, fresh and seawater and milk of goats (Nagahama et al., 2006; Libkind et al., 2003; Callon et al., 2007). As *R. mucilaginosa* has been reported as a cause of onychomycosis, a dermatological problem in immunocompetent patients, its occurrence in the fermented beverages is hazardous (Marcel et al., 2009). Regardless of the deleterious consequences of the occurrence of the genus *Rhodotorula*, these carotenoid biosynthetic yeasts were easily identifiable by distinctive colonies ranging from cream coloured to orange, red, pink or yellow (Krinsky, 2001).

Earlier, genetic relationships among species of the genus *Pichia* and *Issatchenkia* had been examined using phylogenetic analysis of gene sequences (Kurtzman et al., 2008). For this analysis, nucleotide sequence divergence in the genes coding for large and small subunit rRNAs and for translation elongation factor-1a was considered. It was reported that the species of *Issatchenkia* were members of the *Pichia membranifaciens* clade and their transfer to *Pichia* was proposed (Kurtzman et al., 2008). The name *Pichia*

kudriavzevii was proposed to replace *orientalis* (Kurtzman, et al., 2008). It was suggested that due to conflicting phenotypes of the newly proposed genera, their recognition required gene sequence analyses. The *Pichia* spp. (accession nos. KP223717, KP223718) formed a separate cluster.

In conclusion, diverse yeasts were isolated from naturally fermented cashew apple juice. Characterization using biochemical and molecular methods, yeast strains isolated from naturally fermented cashew apple juice yielded 11 different species belonging to six genera from 13 sampling locations from Goa.

CHAPTER 4

Biotransformational characterization–based enzyme activity, fermentation and antimicrobial activity

4.1. Introduction

Microbes are considered as the huge reservoir of the industrially important enzymes which stimulated the need to explore the extracelluar enzyme activity (EEA) in different microbes including food grade yeasts (de Mot, 1990; Bilinski and Stewart, 1990; Burden and Eveleight, 1990). Yeasts have been studied for enzyme capabilities, amongst them oenologically important species were more extensively analyzed for the extracelluar enzyme production, purification, characterization and other capabilities (Rosi et al., 1994; Charoenchai et al., 1997; Strauss et al., 2001). However, the environmentally originated yeasts were neglected for their enzyme production capabilities (Burden and Eveleight, 1990). Yeasts with potential of producing enzymes such as amylase, cellulase, protease, lipase, pectinase, beta glucosidase and urease have applications in the biotechnological sectors, food, biofuel and detergent industries as well as in wine and alcohol industry (Buzzini and Martini, 2002; Strauss et al., 2001; Margesin and Feller, 2010; Zhao, 2012). Many studies have been undertaken on yeast characterizations in alcohol industry with respect to enzyme production and volatile metabolites (Lee et al., 2011; de Souza and Magalhaes, 2010; Shigechi et al., 2004; Kim et al., 2013; Teugjas and Valjamae, 2013; Krisch et al., 2010; Villena et al., 2007; Daenen et al., 2004; Daenen et al., 2008a,b; Wanapu et al., 2012). In this study, we screened the yeasts recovered from naturally fermented cashew apple juice for their

ability to produce industrially important extracellular enzymes such as, amylases, lipases, proteases, pectinases, urease, glucosidase and cellulases.

Amylase is an extracelluar enzyme which can be useful in many industries including in textile for starch saccharification, food, brewing as well as distilling industries, apart from analytical chemistry, clinical and medicinal uses (Gupta et al.,

2003; de Souza and Magalhaes et al., 2010; Kandra, 2003; Pandey et al., 2000). Protease enzyme helps in preventing haze formation during beer making (Lopez and Edens, 2005). Ureases significantly help in beverage making by preventing the creation of carcinogenic compound, ethyl carbamate formed during fermentation and also found in distilled spirits (Canas et al., 1989). Pectinolytic yeasts can be used to clarify and extract juice before fermentation thus, further reducing the use of costly strainers machines to extract the juice of pectinolytic fruits and other items (Roldan et al., 2006). Pectinases increase the release of colour and aroma compounds in musts both before and during fermentation and at the same time, improves maceration, clarification and reduces the filtration time and ensures proper filteration which was seen earlier wherein strains of *S. cerevisiae* reduced the filtration time by half during the winemaking process (Servili et al., 1992; Blanco et al., 1997). Cellulases are highly specific biocatalysts that act in synergy to release sugars, especially glucose which is of great industrial interest due to the potential for its conversion to, for example, bioethanol (Chen and Jin, 2006). It has been assumed that yeasts which hydrolyse certain monoterpenes, can alter the physical properties of wines and also increase the specific organoleptic characteristics (Fia et al., 2005), therefore, the role of β-glucosidase activity of yeast cultures which can be used for wine production has been highlighted significantly; however, there are no studies available which illustrate the glucoside hydrolase activities of the yeasts involved in

cashew apple juice fermentations. Mostly because of their probable significant use in biotechnological processes and fermentation, yeasts have become progressively deliberate in current years in terms of role of enzyme activities during alcohol fermentation for imparting aroma volatile flavor compounds as well as biotransformation of raw material substrate into finished product. Antagonistic property of yeasts against

bacteria is yet unresolved. Studies are lacking on yeasts from cashew apple for their enzyme activities, antimicrobial activity and biotransformation of cashew apple juice into alcohol.

Explorations of biodiversity in the search for new biocatalysts by selecting yeast from fermented cashew apple juice represents a method for discovering new enzymes which may permit the development of bio-catalysis on an industrial scale. Such naturally occurring yeasts have been explored worldwide for different biotranformational capacities (Hong et al., 2002; Tikka et al., 2013; Hagler et al., 2013). Also, as per different geographical locations, the types of yeasts and their biotranformational capabilities found to be different (Vilanova et al., 2011).

In addition to their major contribution to enzyme production, some yeasts exhibiting antagonistic behavior against the unwanted bacteria as well as fungi are highlighted significantly (Hatoum et al., 2012; Buyuksirit and Kuleasan, 2014) which help food processing industries to introduce it as biocides in order to control the food spoilage and also in medicine and pharmaceuticals. Yeasts can be used as antagonist against undesirable microbes, major foodborne pathogens such as endospore forming *Clostridium* species such as *C. butyricum* and *C. tyrobutyricum* (Fatichenti et al., 1983). Several studies have been carried considering this aspect employing, for *S. cerevisiae*

var. *boulardii,* however, studies are lacking involving other genera or species for their antagonistic abilities. Yeasts generally show antagonistic action against other microbes primarily by competing for available nutrients, changes in medium composition by extracellular by-products such as ethanol, exudation of antimicrobial compounds such as killer toxins or "mycocins" and pH of the growth medium due to growth of the yeast (Suzuki et al., 2001; Golubev, 2006; Young and Yagiu, 1978). Viljoen et al. (2006) reported the action of organic acids, volatile acidity, hydrogen peroxide excreted by yeast species. Volatile thermolabile toxic extract "amine" has been reported as inhibitory to *E. coli* and *S. aureus* (Viljoen et al., 2006). Ripke et al. (2004) reported unsaturated fatty acid of yeast to be inhibitory to *S. aureus*. Studying naturally occurring yeasts may explore more capabilities with industrial importance.

Throughout alcoholic fermentation process, the yeast cells come across numerous stress environments like increase in temperature, variations in osmotic conditions, high concentration of ethanol and the presence of competing organisms (Attfield, 1997). It has been speculated that yeasts might be having certain specific physiological properties which enable to analyse the stress and quickly respond against the particular stress without losing the vaiablilty of the strains in the fermentation medium (Bauer and Pretorius, 2000).

In alcohol fermentation, high amount of alcohol above a critical level acts as a toxicant to yeast cells, thus, limiting the alcohol production therefore, alcohol tolerance plays an important role in high alcohol production (Stanley et al., 2010). Although *Saccharomyces* is prominent yeast due to its high alcohol tolerating ability, ethanol produced might cause stress to yeast species (Pratt et al., 2003; Carlsen et al., 1991). Yeast species isolated from naturally fermented cashew apple juice were used for fermentation in controlled conditions and production of a distilled product. All the

essential and desirable qualities required for production of a fermented product like faster growth and quick fermentation ability, high flocculation potential, considerable osmotic balance, increased ethanol tolerance ability and significant thermo-tolerance are not present in single yeast strain, however, some yeast strains may have significant ability for ethanol production (Panchal et al., 1982; Hacking et al., 1984). In this study,

yeasts with high alcohol tolerance (10% to 15%) and wide range of carbohydrate fermentation and utilization ability were selected for fermentation (Kumsa, 2010; Spencer and Spencer, 1997).

In addition to the production of alcohol by fermentation, yeasts impart different flavours to these products throughout the fermentation as a result of production of wide variety of compounds by different types of means and activities such as using fruit juice ingredients, extraction of flavoring constituents from juice soilds while producing ethanol and other types of solvents, transformation of neutral fruit juice compounds into flavour rich compounds through production of enzymes, production of numerous flavour active, minor metabolites (e.g. volatiles and flavor compounds and volatile sulphur compounds), and autolytic dilapidation of dead yeast cells by autolysis (Cole and Noble, 1997; Lambrechts and Pretorius, 2000). An attempt was made to explore the volatile metabolites producing ability of yeasts, isolated previously from fermented cashew apple juice when inoculated as monocultures and its comparison with the traditionally distilled feni sample (field sample). However, information is largely lacking on the enhancement of eminence of traditionally produced product i.e., feni in Goa by optimal choice of yeasts. Information is also largely lacking on chemical properties of feni with respect to its quality.

One of the objective of the study was to observe the effect of the yeast species on the quality of the distilled end product in terms of the formation of metabolites (total acids, volatile acid, esters, higher alcohols, aldehydes, ethanol and methanol). Yeast strains that emanate exceptional flavors are of importance in alcohol industry (Pretorius, 2000). Therefore, it is significant to know the possible differences in volatile production

by different strains of yeasts which may help to select the optimum strain to make an authentic and interesting product.

4.2. Materials and Methods

4.2.1. Testing of yeasts for production of enzymes

4.2.1.1. Yeast cultures

Fifty yeast strains (representing of 11 species of 6 genera) (Table 4.1) identified using 26S rDNA sequencing method were tested for their enzyme activities. Yeast strains were plated on Potato Dextrose Agar plate and incubated for 2 days at room temperature and used for the tests.

Table 4.1: Yeast species tested for enzyme production.

Yeast species	No. of strains
C. ethanolica	1
I. orientalis	6
P. manshurica, P. galeiformis	9
P. kudriavzevii	19
P. membranifaciens	1
P. occidentalis	1
R. mucilaginosa	2
S. cerevisiae	6
L. fermentatii	1
I. terricola	2
Pichia spp.	2

4.2.1.2. Media and screening procedure

The yeasts isolates to be tested were grown for 24 h and the colonies were spot inoculated on the prepoured solidified agar plates. The agar plates were observed for the occurrence of enzyme producing activity after incubation at room temperature for 2–5 days. The experiments were repeated thrice.

Amylase activity

Yeast strains were monitored for their capability to hydrolyse starch on a amylase activity medium (g/L) [Peptone, 5; Soluble starch, 5; Yeast extract, 5; MgSO4.7H2O, 0.5; FeSO4.7H2O, 0.01; NaCl, 0.01 and agar, 15] plates, pH 6.0 (Arttirilmasi, 2013). After appearance of growth, the petri dishes were submerged with Lugol's iodine solution [Gram's iodine solution: 0.1% iodine and 1% potassium iodide] (Yarrow, 1998). A colony surrounded by a pale yellow zone in an otherwise blue medium specify starch degrading activity (Fossi et al., 2009; Li et al., 2007a, b; Pushpalatha and Bandlamori, 2014).

Protease activity

Extracellular production of protease was assayed on potato dextrose agar containing 10 g/L skim milk powder and 15 g/L agar (Charoenchai et al., 1997; Duarte et al., 2013; Geok et al., 2003). A clear zone around the yeast colony demonstrated the protease activity.

Urease activity

Urease activity was determined on Christensen urea medium as described by Seeliger (1956) and Roberts et al. (1978). Color change of the media from orange to pink was observed indicating a positive result.

Pectinase activity

The production of extracellular pectinase enzymes was checked on the medium containing yeast extract (1%), peptone (2%), citrus pectin (1%) and (2%) agar, pH 5.5 (Strauss et al., 2001; Oliveira et al., 2006). After appearance of growth, plates were submerged with hexadecyl trimethyl ammonium bromide (1%) (Biely and Slavikova,

1994; Hankin and Anagnostakis, 1975). A yeast colony surrounded by a clear zone in an otherwise opaque medium indicated degradation of the pectin.

Cellulase activity

Cellulase production was detected on YEPG medium containing 5 g/L carboxymethyl cellulose (Teather and Wood, 1982; Goldbeck et al., 2012; Sethi and Gupta, 2014). After incubation period, the occurrence of extracellular cellulases was observed by flooding with 0.1% Congo red solution and counter staining with 1 M HCl for 15 min.

Lipase activity

Lipase activity was assayed as per the method described (Chi et al., 2009; Slifkin, 2000). Tween-80 Agar plates containing Tween-80 were prepared as per the method described by Gopinath et al. (2005). Yeast strains were tested on medium containing peptone (10%), NaCl (5%), $CaCl_2$ (0.1%) and agar (1.5%) and 5 ml of separately sterilized Tween 80 (1%) with final pH 6.0 (Atlas and Parks, 1993). Phenol red agar plates prepared by adding phenol red (0.01% w/v) to the above described medium were also used to assess the test organisms. . A change in color of phenol red was used as an indication of the enzyme activity.

β-glucosidase activity

β-glucosidase activity was screened on aesculin agar containing 1 gm aesculin, 0.5 gm ferric ammonium citrate, 5 gm peptone and 1 gm yeast extract (pH 5.0). Before pouring the plates, 2 ml of filter sterilized 1% ammonium ferric citrate solution was added. Colonies showing activity were identified by the discoloration of the media to a

brown colour indicated the enzyme activity (Mende-Ferreira et al., 2001; Lock et al., 2007; Strauss et al., 2001; Kim et al., 2010).

4.2.2. Antimicrobial activity of yeasts against pathogenic bacteria

A study was conducted to gauge the antimicrobial activity of yeasts against pathogenic bacteria. Fifty isolates selected as described earlier were screened for their antimicrobial activity against pathogens namely, *Listeria monocytogenes* MTCC 1143, *Salmonella typhi*, *Staphylococcus aureus* MTCC 1144 and *Escherichia coli* 8143. The bacterial strains were obtained from Microbial Type Culture Collection, Chandigarh, India. Each pathogen grown in Brain-Heart Infusion (BHI) broth was spread onto BHI agar plates. To determine the antimicrobial activity, yeast isolates were grown for 48 h at 28^{o}C in 2 ml of Potato-Dextrose Broth. Cell-free extract was obtained by pelleting the yeast cells at 8000g for 10 min. The supernatant was aspirated by pipette and transferred to screw cap vials. Sterile filter paper discs were taken, cell-free yeast extract (20 µl/disc) was placed, dried and placed in each BHI agar plate inoculated with the pathogen. The inoculated plates were incubated at 37°C for 1-2 days and observed for zone of inhibition.

4.2.3. Screening of yeasts for alcohol tolerance

Alcohol production is one of the best biotransformational capability exhibited by yeasts. All 50 yeasts were tested for their alcohol tolerating abilities as described by Lee et al. (2011). In brief, increasing amount of alcohol (from 5-25%) in 5 ml of potato dextrose broth (PDB) was prepared in test tubes. Freshly grown cultures of yeasts were added (0.1 ml). After thorough mixing the tubes were incubated for 48 h at 28^{O}C. Tubes were observed for the growth by measuring the turbidity of the culture at 600 nm by

spectrophotometer. A negative control was maintained without adding any culture to tubes of each conc. of 5-25%. A positive test control was maintained by inoculating cultures to just PDB (without alcohol). Increase in the optical density with the increasing incubation time of the culture was considered as the evidence of active growth. The alcohol concentration at which the growth of the yeast was just stalled was regarded as the highest concentration of ethanol that the strain could tolerate. Strains showing the growth in medium containing 10% ethanol (v/v) or more were selected for production of volatile metabolite study. All the experiments were performed in triplicates.

4.2.4. Biotransformation of cashew apple juice into volatile metabolites

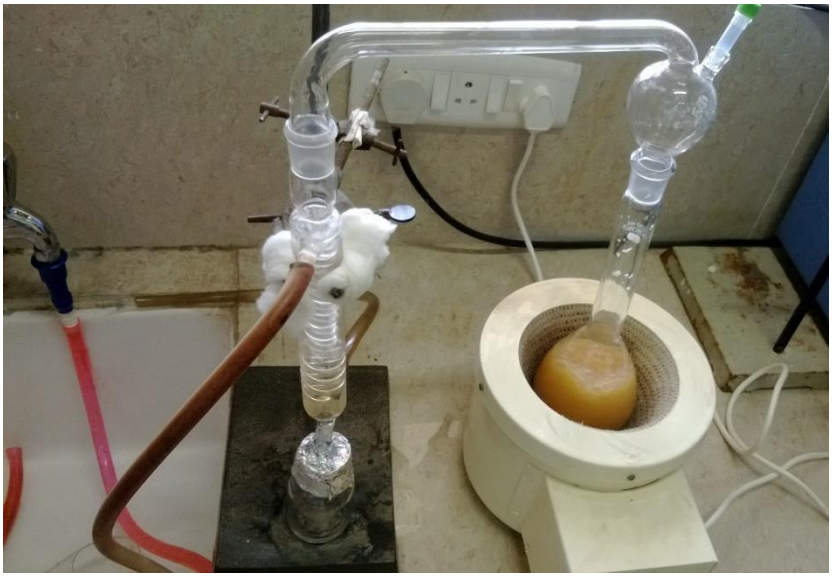
The fully ripe, fresh and unspoiled cashew apples were collected from cashew plantation of ICAR Research Complex for Goa, Old Goa and Pomburpa area in Goa. The collected apples were washed 2-3 times with sterilized water to remove soil debris and other dust particles. The juice was extracted by squeezing the washed cashew apples through muslin cloth under aseptic conditions under laminar flow to avoid possible airborne contaminations within 24 h after collection. Sterile hand gloves were used during extraction of the juice. The fresh cashew apple juice was distributed in 250 ml quantity in sterile conical flasks and used for fermentation (Attri, 2009; Neelaknandan and Usharani, 2009). Twenty characterized yeast isolates exhibiting high ethanol

tolerance were cultured and used individually for fermentation of cashew apple juice. Yeast cells were cultured in PDB at room temperature with continuous shaking at 150 rpm for 24 h in order to obtain high cell density (Alvarenga et al., 2011; Mohanty et al., 2006).

Fermentation of cashew apple juice was carried out in sterile conical flasks each separately inoculated with different yeast species with 5% inoculum for 2-3 days at

ambient temperature (28-30°C) (Attri, 2009; Joshi et al., 2009; Gibbons and Weastby, 1986; Many et al., 2014; Joseph et al., 2010). The fermentation was allowed for 2-3 days till the bubbling in the fermenting juice was minimal. Once the fermentation was complete (no bubbling), the cashew apple juice fermented with each yeast isolate was distilled at 80°C in glass distillation unit (Sathees Kumar et al., 2011) to obtain distillate. The distilled product was chemically analysed for different parameters at Food and Drug Administration (FDA) Laboratory, Government of Goa.

Figure 4.1: Distillation unit used for distillation of fermented cashew apple juice.



The distilled products were analysed according to Indian Standard Cashew Feni-Specification (IS 14326:2005), for the following characteristics as specified by FDA.

i) Total acids (IS:14326, 2005)

The distilled sample (50 ml) was mixed with 200 ml of neutralized distilled water and titrated against standard 0.05N sodium hydroxide solution using phenolphthalein as an indicator.

Total acids were calculated using the following formula:

Total acidity, expressed as tartaric acid, gm/100 litres of absolute alcohol =

$$\frac{0.00375 \times V \times 100 \times 1000 \times 2}{V_1}$$

where V = volume in ml of standard sodium hydroxide used for titration, and V_1 = alcohol, % by volume

ii) Volatile acidity (IS:14326, 2005)

Fifty ml distillate sample was titrated against standard alkali using phenolphthalein as an indicator.

Calculation:

1 ml of standard NaOH equivalent to 0.003 g acetic acid

Volatile acidity expressed as acetic acid, gm/100 litres of absolute alcohol =

$$\frac{V \times 100 \times 1000 \times 0.003 \times 2}{V_1}$$

Where, V = volume of alkali in ml and V_1 = alcohol, % by volume

iii) Esters (IS:14326, 2005)

To the neutalised distillate obtained from volatile acidity determination, 10 ml standard alkali was added and was refluxed on steam bath for 1 h. It was cooled and the excess of alkali was back titrated with standard sulphuric acid. Simultaneously, a blank was run taking 50 ml distilled water in place of distillate in same way.

The difference in titration value in ml of standard acid solution gives equivalent ester.

Calculation:

1 ml of standard alkali equivalent to 0.0088 g ethyl acetate.

Esters expressed as ethyl acetate, gm/100 litres of absolute alcohol = $\frac{V \times 100 \times 1000 \times 0.0088 \times 2}{V_1}$

Where, V = difference in ml of standard acid used for blank and expt, and V_1 = alcohol, % by volume

iv) Higher alcohol (IS:14326, 2005)

Small graduated cylinder previously rinsed with spirit to be tested was filled with 10 ml of spirit, and 1 ml of 1% salicyclic aldehyde and 20 ml conc. H_2SO_4 were added to it. It was allowed to position for 24 h at room temperature. Change in the colour was noted. The colours developed after reaction indicated the amount of higher alcohol as follows:

Colour	Amount
Light yellow	Only traces
Yellow-brown	About 0.01% (v/v)
Brown	0.02-0.03%
Red	About 0.05-0.1%
Dark red-black	Above 0.1%

v) Aldehydes (IS:14326, 2005)

Fifty ml distillate and 10 ml bisulphite solution was added in iodine flask and was kept in dark place for 30 min with occasional shaking. Later, 25 ml iodine solution was

added to it and excess iodine was back titrated against standard sodium thiosulphate solution using starch indicator. A blank test was run using 50 ml distilled water in place of liquor in same way.

The difference in titration value in ml of sodium thiosulphate solution gave the equivalent aldehydes.

Calculation:

1 ml of standard $Na_2S_2O_3$ is equivalent to 0.0011 g acetaldehyde

Aldehydes expressed as acetaldehyde, gm/100ltrs of absolute alcohol

$$= \frac{0.0011 \times 2 \times V \times 100 \times 1000}{V_1}$$

Where, V = difference in ml of standard sodium thiosulphate used for blank and expt , and V_1 = alcohol, % by volume

vi) Alcohol and methanol detection

Alcohol content was determined by measuring the specific gravity of the distillate (Pyknometer method) (IS: 3752, 1967, 2005; FSSAI: 2012).

Cashew distillate obtained was used for alcohol content detection. Clean and dry pyknometer was weighed along with the stopper at 20°C (W). It was then filled with a distillate upto brim and closed with stopper. Distillate which was spilled out was wiped with filter paper and weighed at 20°C (W_1). Then, the distillate was removed and pyknometer was washed with distilled water and was filled with distilled water in the same manner as described above and measured weight (W_2).

$$\text{Specific gravity} = \frac{W1-W}{W2-W}$$

The value obtained here was used to find out the corresponding alcohol percent by volume from the table showing specific gravity v/s alcohol content.

Methanol was detected as per IS: 323, 1959.

Two ml of distillate was diluted with 3 ml distilled water. To this diluted distilled sample, 2 ml of 3% $KMnO_4$ (dissolved in H_3PO_4 acid) was added and allowed to stand for 10 min. Later, 2 ml oxalic acid (dissolved in 50% H_2SO_4) was added and tube was shaken until solution became colourless. In the end, 5 ml of Schiff's reagent was added to it and allowed to stand for 30 min. Formation of violet olour indicated the presence of methanol, whereas, appearance of no colour indicated absence of methanol.

4.3. Results

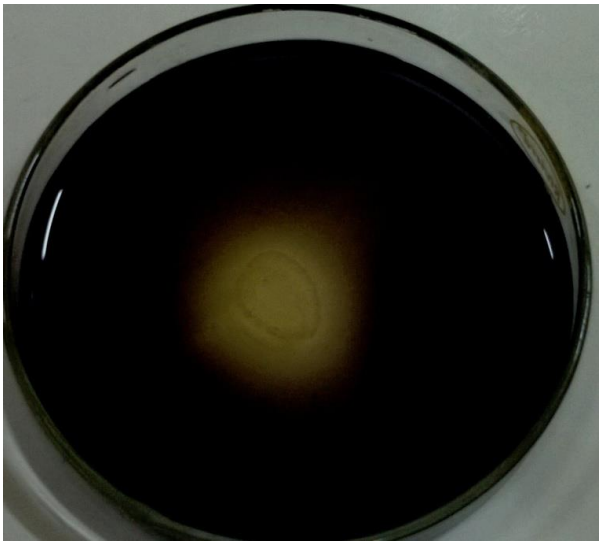
4.3.1. Screening of yeasts for enzymatic activity

The extracellular enzymatic activities (EEA) exhibited by different yeasts are given in Table 4.2.

Amylase activity

Fifty yeast isolates were assayed for amylase activity on Starch Agar medium with soluble starch as the only carbon source. Following the incubation and staining of the plates with lugol's solution, clear zones appeared around growing yeast colonies indicating starch hydrolysis (Figure 4.2). One strain each of *I. orientalis* and *P. kudriavzevii* demonstrated the ability to hydrolyse starch, while, two of nine strains of *P. manshurica* showed amylase activity. Strains of *C. ethanolica, I terricola, P. membranifaciens, P. occidentalis, S. cerevisiae, L. fermentatii, R. mucilaginosa* and *P. galeiformis* did not show amylase activity.

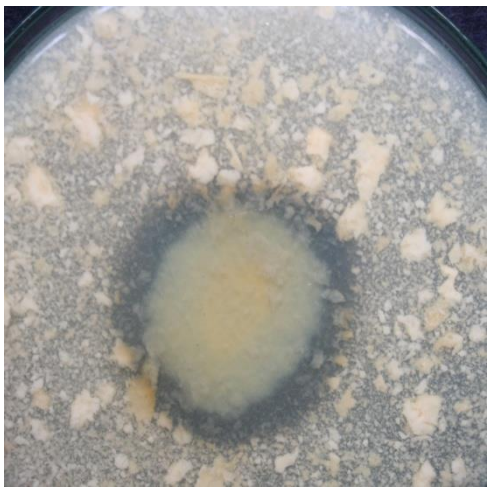
Figure 4.2. The yeast strain showing amylase activity on Starch agar medium with soluble starch as the only carbon source.



Protease activity

Fifty yeast strains were evaluated for their potential to secrete proteases using Skim milk agar plates. Following the incubation, the plates were observed for clear zones around growing yeast colonies indicating protease production (Figure 4.3). Of these isolates, both *R. mucilaginosa* showed this activity, 4 of 6 *I. orientalis* strains and 9 of 19 *P. kudriavzevii* strains were positive for protease activity. Two of 9 *P. manshurica* and 1 out of 6 *S. cerevisiae* and one of 2 *I. terricola* were also positive. *C. ethanolica, P. galeiformis, P. membranifaciens, P. occidentalis, Pichia spp.* and *L. fermentatii*, did not protease activity at all when grown on skim milk agar.

Figure 4.3. The yeast strains showing protease activity.



Urease producing yeasts

Urease activity shown by yeast strains is depicted in Figure 4.4. *P. occidentalis* showed urease activity. Also three of six *I. orientalis*, eight of 19 *P. kudriavzevii* strains, one of nine *P. manshurica* and both *Pichia* spp. showed positive activity. *C. ethanolica*, *P. galeiformis, P. membranifaciens, R. mucilaginosa, S. cerevisiae, I. terricola* and *L. fermentatii*, did not show urease activity at all.

Figure 4.4. The yeast strain showing urease activity**.**

Pectinase activity

Figure 4.5 illustrates pectin-degrading activity by a yeast strain indicating transparent haloes of pectinase activity against an opaque background. In the present work, manifestation of pectinase activity by the isolates was indicated by formation of a colourless halo. Strains of *C. ethanolica* and *R. mucilaginosa* showed pectinase activity. While, four of 6 *I. orientalis* strains, four of 9 *P. galeiformis/P, manshurica*, four of 19

P. kudriavzevii, two of 6 *S. cerevisiae*, one of 2 *I. terricola* and one of 2 *Pichia* spp. also showed pectinase activity.

Figure 4.5. The yeast strain showing pectinase activity.

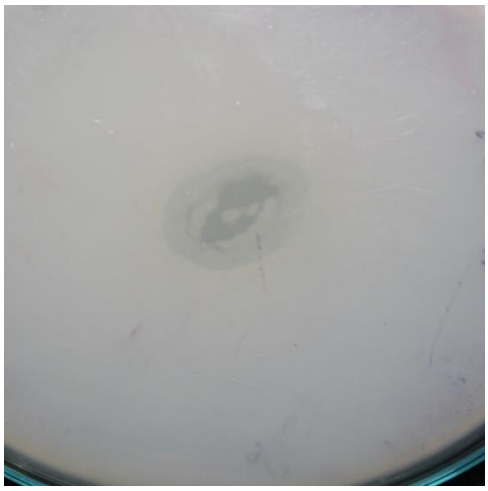


Table 4.2 Profile of extracellular enzymatic activities shown by yeasts isolated from naturally fermented cashew apple juice.

Species	No. of strains	SDA	PrA	UrA	PecA	CellA	LipA	GlucA
C. ethanolica	1	0	0	0	1	1	0	0
I. orientalis	6	1	4	3	4	2	5	2
P. manshurica and P. galeiformis	9	2	2	1	4	5	5	3
P. kudriavzevii	19	1	9	8	4	3	17	3
P. membranifaciens	1	0	0	0	0	0	1	0
P. occidentalis	1	0	0	1	0	0	1	0
R. mucilaginosa	2	0	2	0	1	0	2	1
S. cerevisiae	6	0	1	0	2	1	4	1
I. terricola	2	0	1	0	1	0	2	1
L. fermentatii	1	0	0	0	0	0	0	0
Pichia spp	2	0	0	2	1	0	2	1
Total	50	4	19	15	18	12	39	12

SDA, Starch-degrading activity; PrA, Protease activity; UrA, Urease activity; PectA, pectinase activity; CellA, Cellulase activity; LipA, lipase activity; GlucA, Glucosidase activity.

Cellulase activity

For selection in solid medium, 50 yeasts were grown in petri dishes in order to identify those able to degrade carboxymethyl cellulose (CMC). After incubation, the plates were revealed with a Congo red solution and halo formation was analyzed. The formation of a clear halo surrounding the colony indicates hydrolytic activity. Figure 4.6 illustrates degradation of CMC by a yeast strain indicated by orange-yellow haloes of cellulase activity against the red background. *C. ethanolica* showed cellulase activity whereas, two of 6 *I. orientalis* strains and five of 9 *P. galeiformis/P. manshurica*, three of 19 *P. kudriavzevii* and one of 6 *S. cerevisiae* strains were positive. *P.*

membranifaciens, P. occidentalis, Pichia spp., R. mucilaginosa, I.terricola and *L. fermentatii*, did not show cellulase activity.

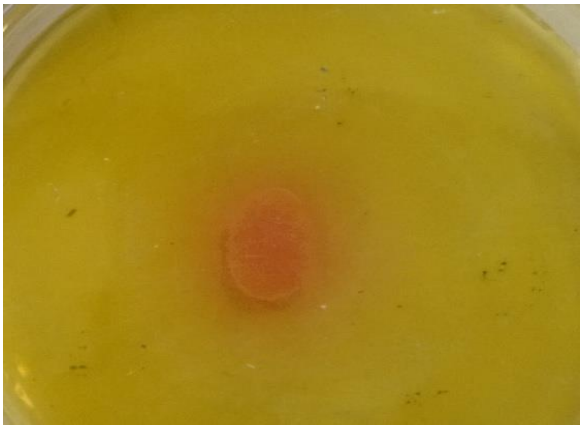
Figure 4.6. The yeast strains showing cellulase activity.

Lipase activity

Lipase activity was detected in 50 strains on medium containing Tween 80 as a carbon source. Clear zone around the colony was indicted as depicted in Figure 4.7. In the present study, five of 6 *I. orientalis* strains showed lipase activity, five of nine *P. galeiformis/ P. manshurica*; 17 of 19 *P. kudriavzevii,* all of the *Pichia* spp., *P.*

membranifaciens, I. terricola, P. occidentalis and *R. mucilaginosa* showed positive lipase activity. Four of 5 *S. cerevisiae* strains were positive. *C. ethanolica* and *L. fermentatii* did not show any activity.

Figure 4.7. The yeast strain showing lipase activity.



β-glucosidase activity

In the present study, esculin was used as a substrate. Fifty yeast isolates were screened for the presence of β-glucosidase activity on agar plates containing esculin as carbon source. Following incubation for 2-3 days at 25°C, the presence of β-glucosidase was evidenced by the appearance of a dark brown colour of the colonies (Figure 4.8); the intensity of the colour was higher in three days. *R. mucilaginosa* strain showed the positive activity. Two of 6 *I. orientalis* and three of 9 *P. galeiformis. P. manshurica* strains showed glucosidase activity. Three of 19 *P. kudriavzevii,* one of 6 *S. cerevisiae,* one of 2 *I. terricola,* and one of 2 *Pichia* spp. were also positive. *C. ethanolica, P. membranifaciens, P. occidentalis* and *L. fermentatii,* did not show glucosidase activity. Results for screening, presented in Table 4.2, showed that enzyme production was strain and species dependent. No other yeast isolate showed the enzyme activity.

Figure 4.8. The yeast strain showing β-glucosidase activity.

4.3.2. Antimicrobial activity of yeasts against pathogenic bacteria

The isolates were screened for their antimicrobial activity against bacterial pathogens, namely *Listeria monocytogenes*, *Salmonella typhi*, *Staphylococcus aureus* and *Escherichia coli*. Out of 50 yeasts tested, only one isolate (ICAR G16), previously identified as *Pichia membranifaciens* could show the significant (18 mm) zone of clearance against *S. aureus* (Figure 4.9). However, the cell free extract of *P. membranifaciens* ICAR G16 could not inhibit other pathogens tested. All other yeasts did not show any antibacterial activity.

Figure 4.9. Antimicrobial activity of *Pichia membranifaciens* against *Staphylococcus aureus*

4.3.3. Alcohol tolerance test

Ethanol tolerance of the yeast strains (n=50) isolated from fermented CAJ was tested. Data presented in Table 4.3 indicates that *S. cerevisiae* and *P. galeiformis* strains showed the highest ethanol tolerance (15%) as compared to other strains. The one strain of *C. ethanolica*, twelve strains of *P. kudriavzevii*, three strains of *P. manshurica*, two strains of *P. galeiformis*, four strains of *I. orientalis* and two *Pichia spp*, showed tolerance at 5% (v/v) ethanol whereas, seven strains of *P. kudriavzevii*, one *P.*

occidentalis, one *P. membranifaciens*, two *R. mucilaginosa*, one *L. fermentatii*, two strains of *I. orientalis*, three strains of *P. manshurica*, five strains of *S. cerevisiae* and two strains of *I. terricola* tolerating 10% alcohol. The yeasts could tolerate at least 5% of ethanol.

Table 4.3: Alcohol tolerance of yeasts

Culture code	Yeast species	Alcohol tolerance (%)	Culture code	Yeast species	Alcohol tolerance (%)
ICAR G1	*P. kudriavzevii*	10%	ICAR G26	*P. kuriavzevii*	5%
ICAR G2	*P. occidentalis*	10%	ICAR G27	*P. kuriavzevii*	5%
ICAR G3	*C. ethanolica*	5%	ICAR G28	*I. terricola*	10%
ICAR G4	*P. galeiformis*	5%	ICAR G29	*L. fermentatii*	10%
ICAR G5	*P. manshurica*	5%	ICAR G30	*P. kudriavzevii*	5%
ICAR G6	*P. kudriavzevii*	10%	ICAR G31	*P. kudriavzevii*	5%
ICAR G7	*P. kudriavzevii*	10%	ICAR G32	*P. kudriavzevii*	5%
ICAR G8	*P. kudriavzevii*	5%	ICAR G33	*I. orientalis*	5%
ICAR G9	*P. kudriavzevii*	10%	ICAR G34	*P. manshurica*	10%
ICAR G10	*P. manshurica*	5%	ICAR G35	*P. kudriavzevii*	5%
ICAR G11	*Pichia sp*	5%	ICAR G36	*P. kudriavzevii*	10%
ICAR G12	*P. manshurica*	5%	ICAR G37	*P. kudriavzevii*	5%
ICAR G13	*P. galeiformis*	5%	ICAR G38	*P. kuriavzevii*	10%
ICAR G14	*P. galeiformis*	15%	ICAR G39	*P. kuriavzevii*	10%
ICAR G15	*I. orientalis*	5%	ICAR G40	*P. kuriavzevii*	5%
ICAR G16	*P. membranifaciens*	10%	ICAR G41	*I. terricola*	10%
ICAR G17	*I. orientalis*	5%	ICAR G42	*P. kuriavzevii*	5%
ICAR G18	*I. orientalis*	10%	ICAR G43	*I. orientalis*	10%
ICAR G19	*P. manshurica*	10%	ICAR G44	*S. cerevisiae*	10%
ICAR G20	*P. manshurica*	10%	ICAR G45	*S. cerevisiae*	15%
ICAR G21	*S. cerevisiae*	10%	ICAR G46	*I. orientalis*	5%
ICAR G 22	*P. kudriavzevii*	5%	ICAR G47	*S. cerevisiae*	10%
ICAR G23	*R. mucilaginosa*	10%	ICAR G48	*S. cerevisiae*	10%
ICAR G24	*Pichia sp*	5%	ICAR G49	*R. mucilaginosa*	10%
ICAR G25	*P. kudriavzevii*	5%	ICAR G50	*S. cerevisiae*	10%

4.3.4. Biotransformation of cashew apple juice into volatile metabolites

Twenty yeast isolates exhibiting high alcohol tolerance and good fermentative characteristics were selected for fermentation of cashew apple juice in controlled conditions in order to study the profile of volatiles produced. Once the bubble formation on surface layer of the juice was minimal, the fermented juice was distilled. Under traditional system of fermentation method used by local feni producers, the same

approach is being followed. The distillate was tested for quality parameters such as total acidity, volatile acidity, esters, higher alcohols, aldehydes, alcohol and methanol content as per Indian Standard Cashew Feni Specification. Data is depicted in the Table 4.4.

Initially, the acidity of the distilled product was checked. Result of the analysis showed that all 20 yeast isolates produced total acidity within the threshold limit i.e., upto 200 gm/100 ltr of absolute alcohol. The sample from traditionally distilled product showed higher total acidity than that shown by the samples fermented under controlled condtions. Total and volatile acids in the distilled product produced by various yeast species are tabulated in the Table 4.4. *P. membranifaciens, P. galeiformis, P. manshurica, P. kudriavzevii, S. cerevisiae, I. terricola, P. occidentalis, I. orientalis, C. ethanolica* and *L. fermentatii* used in cashew apple juice fermentation showed difference in total and volatile acids in the distilled product. The range of total acidity and volatile acidity of *S. cerevisiae* (27-36 g/100 L absolute alcohol) and (19.2-26.4 g/100 L absolute alcohol) was observed in this study.

Variations were noticed in the total and volatile acids produced by different strains as *P. occidentalis* 45 g/100 L absolute alcohol and 33.6 g/100 L absolute alcohol, *I. orientalis* (27-39 g/100 L absolute alcohol) and (21.6-33.6 g/100 L absolute alcohol), *I. terricola* (51 g/100 L absolute alcohol) and 40 g/100 ltr absolute alcohol, *L. fermentatii*

78 g/100 L absolute alcohol and 33.6 g/100 L absolute alcohol, *P. galeiformis* (57 g/100 L absolute alcohol) and (14.4 g/100 L absolute alcohol), *P. kudriavzevii* (27-48 gm/100 L absolute alcohol) and (21.6-40 g/100 L absolute alcohol), *P. manshurica* (33 g/100 L absolute alcohol) and (21.6 g/100 L absolute alcohol) and *R. mucilaginosa* 36 g/100 L absolute alcohol and 48 g/100 L absolute alcohol.

Higher alcohol, aldehydes and esters

Certain sensory characteristics (fruity flavor) are influenced by higher alcohols although these form a comparatively smaller quantity of the total essences. The end products obtained after fermentation with all the 20 yeasts showed the presence of higher alcohol.

Ester and aldehyde contents observed were : *P. occidentalis* (35.2 and 24.64 g/100 L absolute alcohol), *I. orientalis* (21.12 and 13.3-15.84 g/100 L absolute alcohol), *I. terricola* (28.16 and 13.2 g/100 L absolute alcohol), *L. fermentatii* (21.12 and 23.76 g/100 L absolute alcohol), *P. galeiformis* (14.08 and 22 g/100 L absolute alcohol), *P. kudriavzevii* (14.08-35.2 g/100 L absolute alcohol) and (4.4-24.64 g/100 L absolute alcohol), *P. manshurica* (28.16 and 24.64 g/100 L absolute alcohol), *R. mucilaginosa* (49.28 and 19.36 g/100 L absolute alcohol) and *S. cerevisiae* (21.12-35.2 and 18.48-22 g/100 L absolute alcohol). The results (Table 4.4) showed that there were significant differences among the various distilled products in respect of ester and aldehyde concentrations whereas, higher alcohols were not significantly different. *P. occidentalis*, *P. kudriavzevii* and *S. cerevisiae* had the highest ester concentrations, while *P. kudriavzevii* and *P. galeiformis* the least. But the ester content was within the limit range as per IS : 14326: 2005.

Alcohol content

The specific gravity values obtained are noted in Table 4.5. These specific gravity values were matched with the chart of specific gravity v/s alcohol (%) from IS: 3752, 1967, 2005. The value of W = 12.863.

The ranges of ethanol content reported in this study were: *P. galeiformis* (21.24%), *L. fermentatii* (30.29%), *I. orientalis* (21.42 - 32.01%), *P. manshurica* (11.85%), *I. terricola* (10.92%), *S. cerevisiae* (35.92 – 41.19%), *P. kudriavzevii* (22.34-

34.52%), *P. occidentalis* (24.86%) and *R. mucilaginosa* (10.09%). The result showed that, all 20 yeast isolates produced significantly different alcohol content. In this study, none of the yeasts produced methanol (Table 4.4).

Table 4.4: Quality of distilled end product produced by various yeast isolates.

Sr.No	Culture code	Yeast species	Characteristics						
			Total acid (g/100 ltr absolute alcohol)	Volatile acid (g/100 ltr absolute alcohol)	Esters (g/100 ltr absolute alcohol)	Higher alcohol	Aldehydes (g/100 ltr absolute alcohol)	Ethanol yield (%)	Methanol
			Requirement: Max 200	Requirement: Max 60	Requirement: Min 50		Requirement: Max 25		
1	ICAR G1	*P. kudriavzevii*	27	40	35.2	red-black	22	22.34	absent
2	ICAR G2	*P. occidentalis*	45	33.6	35.2	red-black	24.64	24.86	absent
3	ICAR G7	*P. kudriavzevii*	27	31.2	35.2	red-black	19.36	26	absent
4	ICAR G9	*P. kudriavzevii*	48	26.4	21.12	Red	4.4	30.29	absent
5	ICAR G14	*P. galeiformis*	57	14.4	14.08	red-black	22	21.24	absent
6	ICAR G20	*P. manshurica*	33	21.6	28.16	red-black	24.64	11.8	absent
7	ICAR G18	*I. orientalis*	27	33.6	21.12	red-black	15.84	21.42	absent
8	ICAR G21	*S. cerevisiae*	33	26.4	35.2	red-black	22	35.92	absent
9	ICAR G23	*R. mucilaginosa*	36	48	49.28	red-black	19.36	10.09	absent
10	ICAR G29	*L. fermentatii*	78	33.6	21.12	red-black	23.76	30.29	absent
11	ICAR G36	*P. kudriavzevii*	30	21.6	14.08	red-black	19.36	25.83	absent
12	ICAR G38	*P. kudriavzevii*	39	33.6	35.2	red-black	7.04	33.59	absent
13	ICAR G39	*P. kudriavzevii*	45	40	21.12	red-black	18.48	23.25	absent
14	ICAR G41	*I. terricola*	51	40	28.16	red-black	13.2	10.92	absent
15	ICAR G44	*S. cerevisiae*	33	26.4	28.16	red-black	21.12	39.9	absent
16	ICAR G45	*S. cerevisiae*	27	21.6	21.12	red-black	18.48	41.19	absent
17	ICAR G43	*I. orientalis*	39	21.6	21.12	red-black	13.2	32.01	absent
18	ICAR G48	*S. cerevisiae*	36	26.4	28.16	red-black	21.12	38.63	absent
19	ICAR G6	*P. kudriavzevii*	33	26.4	35.2	red-black	24.64	34.52	absent
20	ICAR G50	*S. cerevisiae*	30	19.2	28.16	red-black	20.24	40.58	absent
21	Sample collected from feni unit		60	45.6	63.36	red-black	25.52	41.73	absent

*Requirement as per IS 14326 : 2005

Table 4.5: Alcohol content (%) in distilled samples

Culture code	Yeast species	Weight of pyknometer with distillate (W1)	Weight of pyknometer with distilled water (W2)	Specific gravity (W) W1-W / W2-W	Alcohol (%)
ICAR G1	*P. kudriavzevii*	22.17	22.43	0.9728	22.34
ICAR G2	*P. occidentalis*	22.15	22.43	0.97	24.86
ICAR G7	*P. kudriavzevii*	22.15	22.45	0.968	26
ICAR G9	*P. kudriavzevii*	22.15	22.5	0.9636	30.29
ICAR G14	*P. galeiformis*	22.23	22.48	0.974	21.24
ICAR G20	*P. manshurica*	22.28	22.43	0.9843	11.85
ICAR G18	*I. orientalis*	22.17	22.42	0.9738	21.42
ICAR G21	*S. cerevisiae*	22	22.42	0.956	35.92
ICAR G23	*R. mucilaginosa*	22.3	22.43	0.9864	10.09
ICAR G29	*L. fermentatii*	22.15	22.5	0.9636	30.29
ICAR G36	*P. kudriavzevii*	22.104	22.4	0.9689	25.83
ICAR G38	*P. kudriavzevii*	22.07	22.46	0.9593	33.59
ICAR G39	*P. kudriavzevii*	22.19	22.46	0.9718	23.25
ICAR G41	*I. terricola*	22.32	22.46	0.9854	10.92
ICAR G44	*S. cerevisiae*	21.97	22.45	0.9499	39.9
ICAR G45	*S. cerevisiae*	21.95	22.45	0.9478	41.19
ICAR G43	*I. orientalis*	22.1	22.47	0.9614	32.01
ICAR G48	*S. cerevisiae*	21.97	22.43	0.9519	38.63
ICAR G6	*P. kudriavzevii*	21.99	22.39	0.958	34.52
ICAR G50	*S. cerevisiae*	21.96	22.45	0.9488	40.58
Sample collected from feni unit		21.96	22.47	0.9469	41.73

4.4. Discussion

Methods used for screening for finding suitable micro-organisms that can produce bioactive molecules has been an important fact highlighted in the reviews (Steele and Stowers, 1991; Bull et al., 1992). Progress in genetics and microbial physiology has significant impact on enzyme production technology. Therefore, assaying for search of enzymes could reveal well-defined yeasts for industrial purposes. Amongst the various physical parameters, the pH of the growth medium in which the organisms are grown has an important function to play which induce morphological alterations in the organism and in enzyme secretion (Prasanna Kumar et al., 2004; Blanco et al., 1999). The change

in pH may also affect the stability of the product during the growth of the organism in the medium (Gupta et al., 2003).

Enzymes derived from organisms are exceedingly efficient and precise biocatalysts and are known to be more capable than chemical catalysts which are used in organic reactions because of their stability at broad range of pH and temperature (Rai et al., 2012; Gurung et al., 2013). Majority of the substrates which have been employed in industrial processes are synthesized artificially. This requires continuous search for new enzymes able to perform the specified reactions. Worldwide, there has been an increasing interest and necessity in searching new prospective microbial enzyme producers to develop more novel, sustainable and economically competitive production processes (Adrio and Demain, 2014). Isolation and identification of microorganisms from natural habitats and searching of a strain by suitable plate-screening methods has been the useful and successful technique for the finding of new enzymes (Ghani et al., 2013; Meera et al., 2013). Production of enzymes for use by industries by indigenous microorganisms has contributed to the industrial bioprocesses (Adrio and Demain, 2014). Thus, testing of microorganisms for the selection of suitable strains is an important preliminary step in the making of preferred metabolites (Ghani et al., 2013; Arttirilmasi, 2013). Since yeasts were isolated from fermented cashew apple juice, the enzyme activities screened were those significant in alcohol and beverage industry besides other biotechnological and industrial sectors.

In the present study, potential of yeasts isolated from naturally fermented cashew apple juice have been identified to produce a broad range of EEA. The yeasts strains were found to exhibit the enzyme activities for β-glucosidase, lipase and protease production. In the present investigation, the starch degrading activity was shown by less

number of the strains. Earlier studies have demonstrated contradictory results on starch degrading activity prolies by yeasts (Charoenchai et al., 1997; Strauss et al., 2001). In an earlier study, the yeasts isolated from the cassava fermentation did not degrade starch (Lacerda et al., 2005). The yeasts isolated were also capable of secreting amylase. In this study, except *P. kudriavzevii, I. orientalis and P. manshurica*, none of the other yeast strains secreted amylase. The yeast strains isolated from Pozol fermentation, a traditional Mexican maize food were not able to produce extracellular amylase (Ampe et al., 1999). The amylase has been used widely in starch based industries (Sivaramakrishnan et al., 2006; de Souza and Magalhaes, 2010). Efficient amylase production has been reported from a number of microbial sources (Rao et al., 2007; Hostinova, 2002; Fossi et al., 2005). The enzyme, amylase has wide industrial application in beverage, food processing, pharmaceutical and detergent industries. Therefore, there has been increasing interest among the scientists and industrial researchers to exploit newer sources such as novel microorganisms which can be explored for amylase production.

The proteases produced by yeasts have important role in beverage industry in minimizing the chill haze formation in bottled beer (Dizy and Bisson, 2000; Strauss et al., 2001). Microorganisms producing proteases are studied widely (Cohen et al., 1975; Singh et al., 2012). However, some proteases derived from yeasts have been tried for different probable applications (Poza et al., 2001; Rosi et al., 1987). In genetics and biochemistry studies for intracellular proteolysis, *Saccharomyces cerevisiae* has been used (Jones, 1984). Studies are largely lacking exploring the possibility of screening the extracellular proteolytic activity among certain strains. Proteolytic activity in culture supernatants of a *Saccharomyces uvarum* (*carlsbergensis*) strain has been reported (Maddox and Hough, 1970). The occurrence of proteolytic yeasts (Table 4.2) was

usually lesser than that reported in this study (Strauss et al., 2001). In this study, *R. mucilaginosa* showed proteolytic activity which was similar to the findings of Duarte et al. (2013). Proteolytic activity was observed in *Pichia membranifaciens* (Fernandez et al., 2000). In earlier studies, none of the *S. cerevisiae* strains examined, have been found to produce proteases (Ahearn et al., 1968; Alessandro and Federico, 1980; Foda and Din, 1979; Kamada et al., 1972). In this study, only one out of six *S. cerevisiae* strains gave positive protease activity. Earlier studies reported the absence of extracellular acid protease activity among different species of *Saccharomyces* (Bilinski et al., 1987). However, the existence of exocellular proteases in *Saccharomyces* have been reported (Conterno and Delfini, 1994; Moreno-Arribas et al., 1996; Iranzo et al., 1998). The findings of the present study demonstrated the potential of yeasts isolated from naturally fermented cashew apple juice to produce enzymes having industrial applications. The enzymes also represent a huge resource to be exploited for biotechnological purposes.

Out of 50 yeasts screened, fifteen yeast isolates showed urease production. Urease producing yeasts could be used in alcohol and beverage industry for inhibiting ethyl carbamate formation which otherwise is carcinogenic (Francis, 2006). When urea is present in the juice or formed from arginine in high amount, it may react with ethanol formed during fermentation leading to formation of ethylcarbamate which is a carcinogen (Coulon et al., 2006). Therefore, urease production would be effective in preventing this phenomenon.

Pectinase activity was rare in *S. cerevisiae*. Poondla et al. (2015) isolated *S. cerevisiae* from fruit waste producing pectinase enzyme which have prospects of application in fruit peel pectin degradation and in many industrial sectors. Sahay et al. (2013) isolated psychrotrophic *Rhodotorula mucilaginosa* PT1 strain exhibiting

pectinase production as also observed in this study, which could be applied to wine production and juice clarification at low temperature. The ability to secrete pectinase by *S. cerevisiae* has been reported previously (Gainvors et al., 1994a; Blanco et al., 1999). In the present study, two out of six *S. cerevisiae* strains showed pectinase production. Isolation of *S. cerevisiae* strain secreting polygalacturonase, pectin lyase and pectin methylesterase has been described (Gainvors et al., 1994a).

Many cellulolytic microorganisms and their enzyme systems have been studied extensively for the enzymatic conversion of cellulosic substances (Gilkes et al., 1991; Bey et al., 2011; Boonmak et al., 2011). In the present investigation, all yeast species except *P. membranifaciens, P. occidentalis, Pichia spp., R. mucilaginosa, I. terricola* and *L. fermentatii* contributed in degrading CMC.

Lipases produced by yeasts are of industrial importance mainly with application in detergent and dairy industries (Sharma et al., 2001). Maximum isolates of *I. orientalis* showed lipase activity which was also reported earlier by Costas et al. (2004). Lipase-producing species were identified as *Rhodotorula mucilaginosa* by Duarte et al. (2011). Similar yeast species isolated by Charoenchai et al. (1997) from wines also showed lipase activity. Kurtzman and Fell (1998) reported lack of lipase activity by *Lanchancea fermentatii*. One isolate of *P. galeiformis* did not show lipase activity. Earlier, Rodrigues-Gomes et al. (2010) reported the absence of lipase activity by *P. galeiformis* isolated from rice olives. *S. cerevisiae* also showed lipase activity as reported earlier (Jandrositz et al., 2005).

Enzyme producing yeasts add sensory characteristics to fermentation product (Charoenchai et al., 1997). One of the enzymes expressing fermentative flavor is glucosidase. Non-*Saccharomyces* wine yeasts containing glucosidase activity have been

reported earlier (Arroyo et al., 2011). However, no information was available on ability of *I. orientalis, C. ethanolica* and *P. galeiformis* to exhibit glucosidase activity. It has been assumed that yeasts having the ability to hydrolyse bound monoterpenes may change the sensory characters of wines and even improve their organoleptic profile (Lomolino et al., 2006; Wang et al., 2013; Li et al., 2013). We studied the β-glucosidase activity of yeast strains isolated from fermented cashew apple juice.

The utilization of esculin in the medium as sole carbon source for yeast growth with supplementation of ferric ammonium citrate was regarded as dependable method for detecting β-glucosidases (Mendes-Ferreira et al., 2001). Apart from *S. cerevisiae* (Mateo and Di Stefano, 1997), non-*Saccharomyces* species of yeasts have been found to produce glucosidases in ample quantity (Rosi et al., 1994; Miklosy and Polos, 1995). These findings were confirmed in the present study. In this study, two *I. orientalis* isolates showed glucosidase activity, whereas, one *I. terricola* isolate showed enzyme activity as was also found from isolate from grapes by Gonzalez-Pombo et al. (2011). On the other hand, *C. ethanolica, L. fermentatii, P. membranifaciens* and *P. occidentalis* did not show any glycosidase activity. In this study, only one *S. cerevisiae* strain produced this enzyme which is in accordance with previous reports showing less *S. cerevisiae* activity on arbutin and thus exhibiting β-glucosidase activity (Spagna et al., 2002; Rodriguez et al., 2004). Yeast with β-glucosidase becomes an interesting property for bioflavouring especially by non-Saccharomyces yeast (Gonzalez-Pombo et al., 2011; Mateo et al., 2011). *S. cerevisiae* isolated from grape pomace and other sources produced glucosidase activity which imparted flavours to grape-derived alcoholic beverages (Lomolino et al., 2006; Delcroix et al., 1994; Rosi et al., 1994). *Candida* and *Pichia* also contribute in producing glucosidase. In a study carried out by McMahon et al. (1999), β-glucosidase

production by strains of the genera *Candida, Pichia, Saccharomyces* was determined, however, one strain of *Saccharomyces* showed the activity.

A pronounced β-glucosidase activity was demonstrated by non-*Saccharomyces* yeasts (Daenens et al., 2008a, b). The glucosidases have wide significance in the wine industry with their ability to liberate flavour compounds from glycosidically bound non-volatile precursors in wine (Ubeda and Briones, 2000; Maicas and Mateo, 2005; Arevalo et al., 2007). Our results open the possibility for the use of these strains to improve the aromatic characteristics of the fermented beverages with regard to the liberation of terpenes.

In the present study, *P. membranifaciens* isolated from cashew apple juice showed antimicrobial activity against *S. aureus*. It is a virulent pathogen causing severe infections involving multiple organs and systems (Archer, 1998). Biological control/inhibition of pathogen using antimicrobial yeast isolated from fermented cashew apple juice has been studied. Since *P. membranifaciens* could inhibit this pathogen, it can be used as a probiotic yeast. The inhibitory activity of the yeast might be due to the presence of bioactive compounds present or due to secretion of killer toxin which is detrimental to the survival of the cells of different bacteria (Prabhakar et al., 2012; Heard and Fleet, 1987). Recently, a killer toxin from *P. kudriavzevii* RY55 has been shown to exhibit antibacterial activity against several human pathogens such as *Escherichia coli, Enterococcus faecalis, Klebsiella spp., S. aureus, Pseudomonas aeruginosa* and *P. alcaligenes* (Bajaj et al., 2013).

P. membranifaciens had been found to exhibit antagonistic activity against parasitic fungus *Botrytis cinerea* (Masih et al., 2001). In an another study, killer toxin of *P. membranifaciens* was used as a biocontrol agent against *Botrytis cinerea* (Santos and

Marquina, 2004). *P. membranifaciens, C. famata* and *R. mucilaginosa* isolated from fruits showed antagonistic action against *Penicillium digitatum* (causative agent of fruit rot of orange) (Ghosh et al., 2013). *P. membranifaciens* isolated from olive brines was found to secrete a killer toxin, which inhibited spoilage yeast, *Brettanomyces bruxellensis* (Santos et al., 2009). There are no studies available showing antimicrobial activities of *P. membranifaciens* isolated from fermented cashew apple juice. This study provided important insights towards the utilization of the concealed oenological potential of *P. membranifaciens* isolated from naturally fermented cashew apple juice. The results obtained demonstrated the value of yeast as a antibacterial inhibiting pathogenic strain of *S. aureus*. The results of the study may lead to the development of novel antimicrobial chemotherapeutic agents and new bio-based safer candidates for food preservation.

It has been well established that at the commencement of fermentation, yeasts are subjected to high sugar concentration and as the ethanol is produced, both the sugar and ethanol causes stress to the yeast strain. As ethanol concentration in media increases, there is usually reduction in growth of the organism. Ethanol is known to act as an inhibitor to yeast cells by decreasing membrane fluidity and denaturation of membrane proteins inducing loss of cell survival and inhibition of both yeast growth and different transport systems (Jimenez and Benitez, 1988; Ding et al., 2009). In other way, fermentation remains incomplete or poor, with low ethanol yield, the separation process by distillation and absorption might have some problems. High amount of ethanol has three detrimental effects on yeasts, it decreases the rate of growth, induces damage to the mitochondrial DNA in yeast cells and inactivate some important enzymes required for alcohol production i.e. hexokinase and dehydrogenase (Ibeas and Jimenez, 1997) ultimately slowing the glycolytic reaction (Millar et al., 1982), and of fermentation and

its cell viability, cell member structure and function. Therefore, some efforts are needed to be taken to increase the ethanol yield. *S. cerevisiae* can adapt to high alcohol concentration (Olson and Hhn-Hagerdal, 1996). Thus, ethanol tolerant yeast strains are beneficial in order to achieve high fermentation efficiency and finally a high yield of ethanol (Bauer and Pretorius, 2000).

Thus, search for yeast strains having a elevated resistance to ethanol stress is of immense importance for their economic value for traditional brewing. *S. cerevisiae* was found to be a superior yeast tolerating alcohol (Alexandre et al., 1994) but there are very few reports on high alcohol tolerance of non-*Saccharomyces* yeasts. We examined the ethanol tolerance of the yeast strains in PDB medium with 5%-25% ethanol concentration. The range of ethanol tolerance obtained in the present study was 5-15% which correlated with the previous report by Benitez et al. (1983). The physiological basis for ethanol tolerance in yeasts remains obscure (Nowak et al., 1981).

The present study observed variation in ethanol tolerance among isolated yeasts. *S. cerevisiae* and *P. galeiformis* demonstrated the highest ethanol tolerance (15%) as compared to other yeasts. It could be because yeasts could synthesize chaperone proteins in response to high alcohol (Ding et al., 2009). Two strains of *Saccharomyces* species Strain TBY1 and strain TGY2 showed tolerance of 15.5% (v/v) and 16% (v/v) of ethanol (Kumsa, 2010) which was more as compared to *S. cerevisiae* tested in this study. In an attempt to check the viability of yeast cells under different concentrations of ethanol, Tikka et al. (2013) reported seven strains of *S. cerevisiae* obtained from different fruit sources showing a range of tolerance levels between 7-12%, with maximum of 12% ethanol tolerance by one of the strain. Two of the yeast strains *S. cerevisiae* and *P. galeformis* tolerated 15% ethanol which is in accordance with earlier

report (Sathees Kumar et al., 2011) wherein, *S. cerevisiae* isolated from toddy tolerated higher ethanol concentration. Similar results were reported by Khaing et al. (2008) and Moneke et al. (2008) and observed that the *S. cerevisiae* (KY1 and KY3) and Orc 2 strains tolerated up to 15% of ethanol in the medium and *S. cerevisiae* (KY2) and Orc 6 tolerate up to 20% of ethanol. *S. cerevisiae* isolated from oil palm wine tolerated ethanol at 10% (Nwachukwu et al., 2006), by strain isolated from raffia palm wine at 20% (Bechem et al., 2007) and by strain isolated from fermenting cassava tuber yeasts (Ekunsanmi and Odunfa, 1990) have been reported. Interestingly, none of the strains except *S. cerevisiae* and *P. galeformis* showed growth at 15% ethanol concentration. Bulawayo et al. (1996) and Bechem et al. (2007) observed similar inhibition of growth at 15% alcohol concentration for other yeasts. Another study reported growth of all isolates at 6% (v/v) ethanol, with *S. cerevisiae* tolerating the highest concentration of 12% (v/v) (Maragatham and Panneerselvam, 2011a,b).

Desai et al. (2012) reported *Candida* spp. strains IS1, IS3 and IS7 as ethanol tolerant. The present work demonstrated that although ethanol production was traditionally associated with yeasts, not all yeasts were able to tolerate higher percentage of ethanol. Yeast strains isolated from grapes by Unaldi et al. (2002) showed the maximum alcohol tolerance upto 9% (v/v) whereas, *Saccharomyces* sp. isolated from date palm showed 12% alcohol tolerance (Gupta et al., 2009).

This high ethanol tolerance may be due to lipid content of yeast plasma membrane (Ingram and Bhuttke, 1984; Vanegas et al., 2012). Evaluation of tolerance by various yeasts indicated that isolates were having varying potential of alcohol tolerance. Ethanol tolerance is an important attribute of yeasts in the field of alcohol production helping in improving the fermentation rate and alcohol production.

Yeasts play an important role in biotransformation of carbohydrates (fruit sugar) into alcohol which is a way of preservation and also this act prevents the wastage of agriculturally waste fruits (Fernando and Loreto, 1997). Use of some tropical fruits for wine production using yeast strains has been reported (Obisanya et al., 1987; Ndip et al., 2001; Ezeronye, 2004). Yeasts are the major group of organisms responsible for alcoholic fermentation, which is a complex microbial reaction involving the sequential development of various species of microorganisms (Combina et al., 2005). Besides *Saccharomyces*, non-*Saccharomyces* yeasts which survive for 1-2 days in the initial stages of fermentation produce secondary metabolites (Esteve-Zarcoso et al., 1998). Non-*Saccharomyces* die off due to ethanol production (Fleet, 1984; Heard and Fleet, 1985), therefore, ethanol tolerant yeasts need to be selected for fermentation. Use of potential yeast strains with superior ethanol tolerance to get better ethanol yields in the fermented wash would decrease the distillation costs and hence the profitability of the entire process (Chandrasena et al., 2006).

During fermentation, yeasts convert sugars to ethanol and carbon dioxide as well as produce numerous flavor compounds during fermentation that determine the alcohol quality (Raineri and Pretorius, 2000). Investigation concerning fermented alcoholic beverages indicated that the main compounds (alcohol, higher alcohols, esters and aldehydes) which contribute to flavor were formed during fermentation by yeasts (Dato et al., 2005; Patel and Shibamoto, 2003). Various different yeast species and strains are involved in imparting volatile flavors (Estevez et al., 2004; Molina et al., 2009; Nurgel et al., 2002; Oliveira et al., 2005; Perez-Coello et al., 1999; Patel and Shibamoto, 2003; Swiegers and Pretorius, 2005).

Desirable sensory characteristics have been contributed by the growth of non-*Saccharomyces* yeasts and diverse indigenous *Saccharomyces* strains, while, products produced by single strain are supposed to be simpler and less interesting (Fleet, 1990; Lema et al., 1996). *Saccharomyces* and non-*Saccharomyces* species have been shown to impact specific flavor and aroma compounds (Zoecklein et al., 1997; Otero et al., 2003; Romano et al., 2003). The concentrations of majority of the volatile compounds were significantly influenced by the type of the inoculated yeast strain, due to the metabolic disparities between strains. Various *S. cerevisiae* strains which are found to produce various volatile compounds have been generally used in alcoholic fermentation of grapes (Alvarez and Garcia, 1984; Aragon et al., 1998).

A 5% inoculum was inoculated for cashew apple juice fermentation which according to Gibbons and Westby (1986) results in rapid ethanol production and the lower inocula might result in lowered final yeast populations and increased fermentation times. Twenty yeast isolates with high alcohol tolerance and good fermentative characteristics selected for alcohol production were checked for biotransformational characteristics.

Fine aroma compounds are usually formed during alcoholic fermentation (Wondra and Berovic, 2001). The yeast strain suitable for production of fermented beverage should have high fermentability, tolerance to ethanol, sedimentation property and no effect on titratable acidity (Joshi et al., 2009). The flavor profile of the end product depends on yeast strains (Chen and Xu, 2010). In the present study, the fermentation efficiency of the distillate was influenced by yeast strains as had been reported earlier (Joshi et al., 2009). Distillates produced from different yeast strains demonstrated significant variation in quality parameters. As reported earlier (Vilanova

and Massneuf-Pomarede, 2005) significant differences in quality parameters of wines produced from different yeast strains were observed.Volatile acidity (VA) is present naturally in the fruit juice and most is formed by yeast during fermentation (Vilela-Moura et al., 2011). It should be within a threshhold limit. According to Bureau of Indian Standards, feni should contain VA within the range upto 60 gm/100 L of absolute alcohol. If it is more than the limit, finished beverage leads to a sharp vinegary flavour and if its very poor, beverage leads to poor and dull taste. The results of present study showed that, all 20 yeast isolates produced VA within the threshold limit. The sample traditionally produced product (field sample) showed higher VA. It could be because of the metabolic effects and interactions among mixed culture (yeast-yeast or yeast-bacteria interaction) present during fermentation (Petravic-Tominac et al., 2013). Even acetic acid bacteria present in mixed culture fermentation could be another possibility for high level of acetic acid (Vilela-Moura et al., 2011) in the sample collected from field. Volatile acidity of the wines may vary depending on the yeast strains used (Lambretchs and Pretorius, 2000; Fleet, 1990; Petravic-Tominac et al., 2013). Volatile acidity was found to be lower than that seen in pure cultures of non-*Saccharomyces* yeast as observed in mixed or sequential cultures of *H. uvarum/S. cerevisiae* (Ciani et al., 2006). On the other hand, *R. mucilaginosa* produced more level of VA than the field sample and might be attributed to its nature as spoilage organism.

In this study, the distilled products obtained by different yeasts were colourless. *P. membranifaciens, P. galeiformis, P. manshurica, P. kudriavzevii, S. cerevisiae, I. terricola, I. occidentalis, I. orientalis, C. ethanolica* and *L. fermentatii* used in cashew apple juice fermentation showed difference in total and volatile acids in the distilled product. The range of total acidity and volatile acidity of *S. cerevisiae* (0.27-0.36%) and

(0.19-0.26%) observed in this study has been reported earlier by Okunowo et al. (2005) in case of orange juice. The highest total acidity as tartaric acid was 0.75% with *S. cerevisiae* from sugarcane molasses and least was 0.59% with *S. cerevisiae var. ellipsoideus.* The volatile acidity as acetic acid was 0.25% with *S. cerevisiae* from sugarcane molasses, 0.17% with *S. cerevisiae var. ellipsoideus* and least as 0.16% with *S. cerevisiae* from yam. Study by Attri (2009) found increased titratable acidity, volatile acidity, esters and decreased aldehydes with alcohol yield of 8.25% in cashew apple wine produced by fermentation using *S. cerevisiae*. On cashew apple juice fermentation with *S. cerevisiae*, cashew wine was slightly yellowish, acidic in taste (titratable acidity [1.21 g tartaric acid/100 mL]), high in tannin and low in alcohol (7%) concentration (Shuklajasha et al., 2005). The physical and chemical characters of mango wines fermented by *S. cerevisiae* CFTRI 101 showed total acidity (0.6-0.7%), volatile acidity (0.1-0.2), higher alcohols (131-343mg/l) and esters (15-35mg/l) (Reddy and Reddy, 2009). Sorghum beer produced by fermentation of sorghum using *S. cerevisiae* and *I. orientalis* showed characteristic physicochemical properties of beer. Titratable acidities by *S. cerevisiae* and *I. orientalis* were 0.86% and 0.81%, co-fermentation produced 0.89% acidity (Lyumugabel et al., 2014). Okunowo et al. (2005) identified that the percent TTA was 0.85 with *S. cerevesiae* from sugarcane molasses and 0.84 with *S. cerevesiae* from yam and 0.79 with *S. cerevisiae var. ellipsoideus.*

Maturity of fruits governs the synthesis of higher alcohols during fermentation. Certain sensory characteristics (fruity flavor) might be influenced by higher alcohols in spite of their comparatively lesser amount of the total substances. The concentrations of amino acids, yeast strain used, fermentation temperature and pH influences the level of higher alcohols in final product (Swiegers and Pretorius, 2005). Fermentation process

may change the aroma of fruit juice as there is production of yeast volatiles and the induced metabolism of original fruit volatiles (Reddy and Reddy, 2009). This study identified yeast species like *I. terricola*, *I. orientalis*, *I. occidentalis* which were initially found to be unrelated with the wine making process (Di Maro et al., 2007). Giudici et al. (1990) examined different strains of *S. cerevisiae* showing different ability of higher alcohol. Some of these have been infrequently isolated previously from wine fermentations (Pallmann et al., 2001; Sabate et al., 2002; Di Maro et al., 2007). However, Clemente-Jimenez et al. (2004) reported *I. orientalis* strain showing high amount of higher alcohols after *S. cerevisiae* during wine fermentation. In contrast, *I. terricola* demonstrated lesser fermentative power (Clemente-Jimenez et al., 2004). Wondra and Berovic (2001) reported higher alcohols in the range of 263.5 mg/L-738.4 mg/L and oxidation of alcohol to aldehyde resulted in excess amount of aldehydes in wine leading to oxidized and acidic taste.

Aldehydes are important compounds for aroma and taste of alcoholic beverages present naturally in raw materials such as cashew apple juice (Sampaio et al., 2013; Osorio and Cardeal, 2013) but much of it is produced by yeasts during fermentation (Pigeau and Inglis, 2007; Frivik and Ebeler, 2003) or through chemical oxidation reactions (Liu and Pilone, 2000; Osorio and Cardeal, 2013). Its production varies from yeast species and strains. In the present study, aldehyde production using all the 20 yeast isolates was found to be within permissible limits making it desirable character of distilled beverage. The field sample showed higher aldehyde contents. According to BIS, feni should contain aldehyde within the range upto 25 gm/100 L of absolute alcohol. Aldehyde production indicate incomplete alcoholic fermentation or oxidation of alcohol

(Amerine et al., 1980), but high concentrations may be toxic to humans, since these are considered to be responsible for the disagreeable effects.

The percentage of esters depends on components of distillate and the distillation process (Amerine, 1974). Esters are a group of volatile aromatic compounds responsible for fruity flavor and thus increasing the quality of fermented beverages and have low thresholds (Saerens et al., 2007). Most esters are the secondary metabolites produced by the yeasts during fermentation (Saerens et al., 2010; Sumby et al., 2010). *Pichia* sp. strains increased the ester production level in beer fermentation and it was affected by many factors like variety of fruit, clarification and fermentation conditions (Reddy and Reddy, 2009). Ester formation also depends on sugar fermentation in relation to alcohol acetyl transferase activity which is governed by composition of fatty acid of yeast cell membrane (Yoshioka and Hashimoto, 1984, 1983). In the present study, all 20 yeast isolates produced esters within the threshold limit. The field sample showed higher ester concentrations. Ester levels are largely determined by the amounts of corresponding alcohol produced by the yeast since alcohol or higher alcohol and acetic acid are substrates for ester formation (Calderbank and Hammond, 1994; Lilly et al., 2000; Pires et al., 2014) which might have resulted in high level. Spoilage organisms may also contribute to higher ester formations (du Toit and Pretorius, 2000). The spoilage organisms may be responsible to produce off flavor or vinegary flavor which include species and strains of the yeast of the genera *Brettanomyces, Candida, Hanseniaspora, Pichia, Zygosaccharomyces,* the lactic acid bacterial genera, *Lactobacillus, Leuconostoc, Pediococcus,* and the acetic acid producing bacterial genera *Acetobacter* and *Gluconobacter* (Mas et al., 2014).

In case of 20 distillates which were produced by using single cultures showed less ester production, which could be because of lower alcohol/higher alcohol production which are substrates required for ester formation. In some cases, the ester levels were low which could be attributed to low expression of enzymes responsible for ester formation (Calderbank and Hammond, 1994; Lilly et al., 2000). But overall, ester productions were less than the minimum limit range mentioned as per IS but the range varied from yeast to yeast.

Various strains of native yeasts competent of producing ethanol have been isolated from various local sources including molasses sugar mill effluents, locally fermented foods and fermented pineapple juice (Rose, 1976; Anderson et al., 1986; Ameh et al., 1989; Eghafona et al., 1999). The use of ideal microbial strain, appropriate fermentation substrate and suitable process technology influence the yield of ethanol employing microbial fermentation.

Variability in alcohol yield was reported in many studies (Gorzynska et al., 1973). Different *S. cerevisiae* strains tolerating 12% ethanol found to yield 12.5% and 8.9% ethanol after fermentation whereas, strain tolerating 3% ethanol was found to yield 12.06% ethanol (Gupta et al., 2009). Alcohol yield by *S. cerevisiae* and *Trichoderma* sp. on sweet potato was 47g/100g sugar consumed (Swain et al., 2013). Aerobic yeasts of the genera *Pichia* and *Williopis* are found to produce less alcohol (Erten and Campbell, 2001). Cabrera et al. (1988) found ethanol production by the four *Saccharomyces* strains on fermentation of sterile musts from Pedro Ximénez grapes to be similar. In the present study, the distillates produced after fermentation with monocultures of yeasts showed variation in the amount of alcohol production. Some yeasts produced very little amount of alcohol. It could be due to oxidation of alcohol to acetaldehyde or might be due to

inherent ability of yeast to produce less alcohol even though had high alcohol tolerating ability. Culture of *P. manshurica* even though tolerated 10% alcohol, it produced only 11.8% alcohol. In case of culture with *R. mucilaginosa*, ester synthesis was very high compared to other yeast species, however, it was less than the limit required. It could be because ester production is mainly the characteristic of acetic acid bacteria (Mas et al., 2014). Therefore high ester concentration in field sample, compared to other monoculture yeast distilled samples may be dur to the presence of acetic acid bacteria in natural distilled sample (field sample) whereas distilled sampled produced yeast monoculture had less effect in ester production. However, each yeast (except *R. mucilaginosa*) even though produced little alcohol, these could produce volatile compounds as necessary criteria for yeast selection for fermentation on industrial scale. The early stages of fermentation is dominated by *R. mucilaginosa* , and its occurrence along with the *Pichia* species later in fermentation could add to difficulty but also decrease the wine quality (Deak and Beuchat, 1993; Loureiro and Malfeito-Ferreira, 2003).

In majority of the studies reported, the favored candidate for industrial production of ethanol has been *S. cerevisiae.* Ethanol yield by *S. cerevisiae* and *I. orientalis* in sorghum beer production was 4.34% and 1.49% and co-fermentation yielded 4.56% (Lyumugabel et al., 2014). It has been reported that, the total alcohol produced was the least with *S. cerevisiae var. ellipsoideus* and the highest with *S. carlsbergensis* (Okunowo et al., 2005) with the optimum pH ranging from 3.81 for *S. cerevisiae var. ellipsoideus* and 3.71 for *S. cerevisiae.* Yeasts isolated from traditional dough products produced alcohol content of 11.3-11.5% (Kumsa, 2010). Alcohol content produced by *S.*

cerevisiae was in the range of 6-11% which was similar to *S. cerevisiae* isolated from ripe banana peels exhibiting the best attributes for ethanol production by tolerating 6 - 12% (v/V) ethanol (Brooks, 2008). *S. cerevisiae* producing 8-12% alcohol in Papaya pulp alcoholic fermentation has been reported (Maragatham and Paneerselvam, 2011a,b). Tomato wine produced by *S. cerevisiae* from tomato juice fermentation resulted in alcohol yield of 10-12% (Many et al., 2014). Another study by Kim et al. (2008) found ethanol (7.8%) from grapemust fermentation using *I. orientalis* and *S. cerevisiae*. Pineapple juice fermentation by *S. cerevisiae* produced (8.4%) ethanol in the final product (Panjai et al., 2009). Production of mango wine from mango fermentation using *S. cerevisiae* produced 6.3%-8.5% ethanol (Reddy and Reddy, 2009). It was reported that wine yeasts could grow well at 10% (V/V) and fairly well at 15% (V/V) ethanol (Benitez et al., 1983). In this study the ethanol tolerance was found to be less than previous reports (Benitez et al. 1983). Alcohol production from cashew apple juice using *S. cerevisiae* with 15.64 g/l of ethanol concentration has been reported (Karuppaiya et al., 2010). Neelakandan and Usharani (2009) showed bioethanol production from cashew apple juice using *S. cerevisiae* with 7.62% ethanol. *S. cerevisiae* isolated from banana peels on fermentation reduced 5.8% alcohol at 37°C (Brooks, 2008).

Methanol in high concentration has been reported in alcoholic beverages obtained from fermented fruits distillates especially the beverages which are traditionally home-made and not tested chemically (Croitoru et al., 2013). It occurred naturally at a low level in most alcoholic beverages without causing harm (Paine and Davan, 2001). It is naturally present in fruit in form of pectin wherein during fruit fermentation or due to enzyme addition, pectin methylesterase breaks down pectin releasing methyl ester group producing methanol in beverage (Micheli, 2001), which is poisonous leading to health

risk if present in high amount in any food product (Croitoru et al., 2013). In the process of distillation of fermented fruits, methanol is distilled together with ethanol due to their similar physicochemical properties. That is the reason why all alcoholic beverages obtained from fermented fruits may contain methanol which is undesirable (Zocca et al., 2007), due to its high toxicity for humans. In the present study, the methanol concentration was measured in the distillates. None of the yeasts produced methanol. It could be because either distillation was conducted carefully and completely avoiding the methanol or cashew apples used for juice extraction were not too ripe or cashew apples were low in pectin.

No detectable methanol levels were found in the all the distillate samples. The strains of *S. cerevisiae* generally do not produce methanol but the hydrolysis of pectin present in fruits i.e. maceration of the fruit juice along with skin peels could contribute to production of methanol (Amerine et al., 1980).

In conclusion, the yeast strains subjected to fermentation using fresh cashew apple juice as substrate have their own impact on quality of the end product and produced a beverage with balanced volatile compounds. None of the yeast strains showed negative quality parameters. Among all the used strains, *Saccharomyces* as well as non-*Saccharomyces* strains were found to be efficient in fermentation. It was observed that fermentation behaviour and quality was influenced by the yeast strains used. Among the identified yeast strains, *S. cerevisiase* as well as *P. galeiformis, P.kudriavzevii* and *I. orientalis* performed well and can be potentially used for obtaining quality products. Several studies have demonstrated the impact of non-*Saccharomyces* yeasts on composition of the beverages, with promising aromatic development as reviewed by Ciani et al. (2010). Results of the present study have demonstrated the potential of non-

Saccharomyces yeast strains to be used in mixed starter cultures together with *S. cerevisiae*, and may be considered as a strategy to improve complexity of the beverages. The naturally fermented cashew apple juice can be explored as source of novel yeasts capable of producing enzymes for industrial applications. The yeasts have shown high alcohol tolerance. Cashew apples, considered as agricultural waste, have potential to be used for substrate for enzyme production. The traditional distilled product of Goa "Feni", has been produced in highly traditional way. There have been no attempts to improve the quality of the product through manipulation of the microflora for controlled fermentation and also done in the most unhygienic way. The main limitations perceived by the enterpreneurs involved in Feni industry include lack of uniformity in quality, highly traditional, non-standardized processes for juice extraction, fermentation, distillation and disparity in these processes. Also there is lack of consistency in product quality varying from enterpreneur to enterpreneur. Fermentation of cashew apple juice under possible controlled condtions with naturally occurring yeast strains may help to improve the quality of the product. This will also help maintain the uniform quality of the product as well to create a brand. This in turn will help to earn more per unit area of cashew plantations.

Chapter 5

Summary and Conclusion, and Future needs for research

Diversity of yeasts isolated from the naturally fermented cashew apple juice samples from various locations in Goa was studied. The isolates were characterized using various biochemical tests and the the 26S rDNA/ITS sequencing. The yeasts were characterized for various functional attributes such as enzyme activities, antimicrobial activity as well as effects of fermentation practices using monocultures which produced a variety of volatile and aroma in the end products. The diversity of non-*Saccharomyces* species present during the natural cashew apple juice fermentation had been shown to be broad, and their diversity ranged from genera; *Pichia, Saccharomyces, Issatchenkia, Rhodotorula, Candida* and *Lanchancea*. Diverse yeast flora explored in fermenting cashew apple juice in various locations in Goa were identified as *P. membranifaciens, P. kudriavzevii, P. galeiformis, P. manshurica, P. occidentalis, I. orientalis, I. terricola, C. ethanolica, L. fermentati, S. cerevisiae* and *R. mucilaginosa*. Non-*Saccharomyces* yeasts showed the potential to exhibit various extracellular enzyme activities. Yeast species showed various enzyme-based biotransformational abilities (amylase, protease, urease, pectinase, lipase, cellulase and β-glucosidase) having industrial applications. Enzymes produced by the yeast species associated with naturally fermented cashew apple juice need to be utilized to initiate expected biotransformations for industrial purposes. *Pichia membranifaciens* showed antimicrobial activity against *Staphylococcus aureus* and could be used against staphylococcal infections. Isolation of high ethanol tolerant yeasts from fermented cashew apple juice is an attractive alternative other than the traditional *Saccharomyces* spp. Most of the isolated yeast strains showed 10-15% alcohol tolerance. *S. cerevisiae* and *P. galeiformis* strains showed the higher ethanol tolerance and offered the potential to improve yields of ethanol in the distillaries which in turn can reduce the costs of distillation.

Fermentation employing 20 different yeast strains isolated from naturally fermented cashew apple juice showed a varying influence on the compositions and volatile aroma compounds in distilled end products. The flavor and aroma components estimated from the distilled products from fermentation with monocultures of yeasts were within the acceptable limits as per standard of Bureau of Indian Standards for cashew feni (IS 14326 (2005)) distilled in the state of Goa. Cashew apple juice distillate using *S. cerevisiae* and *P. kudriavzevii* used for fermentation produced higher yields of alcohol. This provides another good alternative for the alcoholic beverage industry. Besides *Saccharomyces*, this study also helped to yield novel strains of yeasts and tap their potential for use as cultures in alcohol industry.

It was evident that by employing non-*Saccharomyces* yeasts together with *S. cerevisiae* might prove an useful strategy to enhance the organoleptic properties of the beverages. The yeasts used for fermentation as monocultures did not produce negatively perceived volatile compounds (e.g. acetic acid) in extreme amount and produced desirable esters flavor bound precursors and alcohol quality. To gain further insight into the many potential benefits that may come from the co-inoculation of non-*Saccharomyces* and *S. cerevisiae*, more investigations need to be conducted to establish the importance of use of selected cultures in mixed culture fermentation. Moreover, the interactions that occur between the species should be investigated using several approaches, ranging from the biochemistry behind the relevant pathways which the yeast employs to the physical environment in which fermentation occurs. In this manner, a more holistic interpretation of what occurs in mixed fermentations can be made.

This study revealed the need for exploitation of agriculturally and industrially neglected by-products which are under utilized as substrates for alcohol production as

well as for enzymatic processes. In this study, diverse yeasts isolated from naturally fermented cashew apple juice with appreciable fermentation attributes were characterized. The yeasts also had the ability to enhance ethanol yield minimizing the cost of production. Cashew apples are available in abundance in Goa and adjoining areas and thus serve as readily available raw material for the isolation of yeasts having industrial importance.

The needs for future research

Despite the use of fermentation of foods as a method for food preparation and preservation of foods since ancient times, the mechanisms for transformations using the microbial and enzymatic progressions were basically unknown. In the recent years, there has been increased interest to study these processes at industrial level and their application in industries and subsequent commercialization. Yeasts through fermentation processes provide a great scope and potential for their use in meeting the increasing demand worldwide for food through efficient utilization of under utilized food and feed resources. The indigenous microflora particularly yeasts play an important role in the production of fermented food products, their preservation. It is essential that this age old wisdom is preserved and utilized to its fullest extent to meet the growing demand of foods worldwide.

A large number of microbes including yeasts are unculturable on laboratory media and regarded as viable but non-culturable. This phenomenon offers limitations to our understanding of the physiology, genetics, and ecology of microbes. Molecular biological techniques, namely, metagenomics may provide valuable insights about these microbes. This can help in identification of novel microorganisms paving way for

suggesting novel biocatalysts and biosynthetic pathways. Antimicrobial activity detected in this study could be further explored by studying the proteomics of antimicrobial compound and to elucidate the complex cellular responses of *Pichia membranifaciens* to antimicrobial compound which could give insights in the mode of action of this antimicrobial compound.

Having observed the inherent attributes in feni such as astringent smell, lack of original fruity flavor, inconsistent yield due to disparity in the methods of fermentation, there is need to research these issues. The fermentation process used for feni production needs uniformity in tune with commercial attributes and consistent output of quality and yield. Fermentations using mixed cultures such as *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts may provide a possible way towards improving the intricacy and improving the specific characteristics of the end product produced from fermented cashew apple juice. Except in the state of Goa, cashew apples are not wholly utilized anywhere else. This huge resource goes waste in spite of its value as a source of nutrients and a substrate for alcohol production and warrants due attention.

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APPENDIX

Appendix

1. Potato Dextrose Agar

Components	Quantity
Potato infusion	200 g
Dextrose	20 g
Agar	15 g
Distilled water??	1000 ml
pH	5.6

2. **Fermentation Basal medium**

Components	Quantity
Yeast Extract	4.5 g
Peptone	7.5 g
Distilled water	1000 ml
Bromothymol blue	4 ml stock solution per 100 ml of 100 ml of fermentation basal medium

Stock solution

Bromothymol blue	50 mg/75 ml distilled water

3. **Amylase Activity medium**

Components	Quantity
Peptone	5 g
Soluble starch	5 g
Yeast extract	5 g

MgSO4.7H2O	0.5 g
FeSO4.7H2O	0.01 g
NaCl	0.01 g
Agar	15 g
Distilled water	1000 ml

4. Lugols iodine solution

Iodine	0.1%
Potassium iodide	1%

5. Christensen urea agar

Composition	Quantity
Peptic digest of animal tissue	1 g
Dextrose	1 g
Sodium chloride	5 g
Disodium phosphate	1.2 g
Monopotassium phosphate	0.8 g
Phenol red	0.012 g
Agar	15 g
Distilled water	1000 ml
pH	6.8

Suspend 24.01 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 10 lbs pressure (115°C) for 20 minutes. Cool to

50°C and aseptically add 50 ml of sterile 40% Urea Solution (FD048) and mix well. Dispense into sterile tubes and allow to set in the slanting position. Do not overheat or reheat the medium as urea decomposes very easily.

6. 0.1% Congo red solution

Composition	Quantity
Congo red	0.1 g
Distilled water	100 ml

7. Aesculin agar

Composition	Quantity
Aesculin	1 g
Ferric ammonium citrate	0.5 g
Peptone	5 g
Yeast extract	1 g
Distilled water	1000 ml
pH	5.0

Before pouring the plates, 2 ml of filter sterilized 1% ammonium ferric citrate solution was added to the above 100 ml medium.

8. Brain Heart Infusion (BHI) broth/agar

Components	Quantity
Brain Heart, Infusion from solids	8 g

Peptic digest of animal tissue	5 g
Pancreatic digest of casein	16 g
Sodium chloride	5 g
Glucose	2 g
Disodium hydrogen phosphate	2.5 g
Agar*	15 g
Distilled water	1000 ml
pH	7.4±0.2

*For preparation of BHI agar

PUBLICATIONS

Poster Presentation

Prabhu Khorjuvenkar, S.N., Doijad, S.P., Poharkar, K., Raorane, A. and Barbuddhe, S.B. (2013) Identification and characterization of yeast isolated from naturally fermented cashew apple juice in Goa. Poster presented at 54th Annual Conference of Association of Microbiologists of India (AMI), Rohtak.