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.