BIOCHEMICAL AND MOLECULAR GENETIC ASSESSMENT OF YEAST STRAINS USED BY CERTAIN TRIBAL COMMUNITIES OF ASSAM IN ALCOHOL PRODUCTION

A THESIS
SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
Doctor of Philosophy

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November 2009

I DEDICATE THIS THESIS

TO

MY WIFE AND SON

Dr. (MRS) MINATI DAS

AND

RANIT

Abstract

Mankind has been practicing fermentation as a means of food processing since antiquity. It is recorded that since 9,000 years ago yeasts have been used for making bread, wine, beer, sake etc (Akadaa, 2002). Fermentation is the oldest of all biotechnological process. The study of yeast could be traced back to 1838 AD only (Vaughan-Martini, 1995). The importance of yeast in food and beverage industry was realized only in 1860.

Almost every tribal community especially the Sub-Himalayan Mongoloid tribes has their distinct types of rice beer. These are produced at household level using traditional techniques with limited or no knowledge of microbiology. Therefore, traditional beer production system encountered limitations associated with different stages and aspects of fermentation process such as inadequate grading and cleaning of raw materials, lack of code of hygiene, lack of proper knowledge of the culture of the process and their characteristics, absence of any control over the process variables such as pH, temperature, humidity and water quality etc, insufficient product protection due to lack of packaging, possibility of contamination with food borne pathogen, etc. Besides lack of knowledge of proper nutritional and toxicological implications of fermentation and its products also add to the limitations of traditional fermentation.

The tribal communities of NE-India produce traditional rice beer since time immemorial. They produce and preserve the starter culture for future use in the form of dried ball or cake locally called as *pithaguti, bakhar, emao, thapp, obpob* etc by the respective tribes. The product of the different tribes are known by different names such as *hanj* of Ahom, *zu* of Bodo, *hor* of Karbi, *apong* of Mising and *suje* of Tiwa.

Till date no serious attempt has been made to gather systematic information on the spectrum of microorganisms like yeast and fungi associated with the traditional fermented rice beer of Assam. But for the production of an assured quality product such knowledge is very essential. To improve the

trustworthiness of the traditional rice beer fermentation process, biotyping of each appropriate yeast strains responsible for fermentation should be a priority. A properly documented database on suitable strains of yeast and fungi responsible for the fermentation process to produce traditional beer would be an asset for the food and beverage industry.

In this study starter cultures used by five different tribal communities of Assam were collected, analyzed for moisture content, pH and microbial load. Moisture content of Ahom culture was found to be the highest (13.15%) and that of Bodo the lowest (12.32%). pH of all the cultures was acidic. Ethanol content of the sample was found within the range of 4.6 (Bodo)-5.5% (v/v) (Ahom). Again laboratory products produced by using the same cultures were also found to contain 4.5 (Kaarbi)-6.0 % (v/v) (Tiwa). The total yeast load (cfu) of Mising culture was the highest (1.93×10^8) and lowest in Karbi (1.14×10^7). The yeast isolates AF1 belongs to *Mucor indicus,* AF2 and BF2 *Amylomyces rouxii*, BF1, KF2, MF1 and MF2 *Rhizopus oryzae* and KF1, TF1 and TF2 *Mucor circinilloides.*

Aerobic growth test with carbon sources, growth test with nitrogen sources, semi-anaerobic fermentation of sugars, test of vitamin requirement, growth in high osmotic pressure, growth in the presence of cycloheximide, diazonium Blue B test etc were done to identify the yeast isolates. Using the conventional dichotomous keys (Barnett *et al.*, 2000), isolates AY0, AY1, AY2 and BY2 were found to be either *S. cerevisiae* or *S. paradoxus*. It was confirmed as *S. cerevisiae* as it can grow in vitamin free medium whereas *S. paradoxus* cannot grow on vitamin free medium (Jespersen *et al.,* 2000). Again by following the same keys isolates KY0, KY1 and TY1 were identified as *S.bayanus, S. cerevisiae, S. pastorianus or S. microellipsoides.* All these species, except *S. microellipsoides,* belonged to *Saccharomyces sensu stricto group*. Based on key no. 2 provided by Barnett *et al.* (2000) these species could not be distinguished from each other. Isolates BY0(A), BY0(B), BY0(C) and TY0 identified as *Issatchenkia orientalis.* Isolates BY1 and MY0 were identified as *D. hansinii, D. maramus* or *D. nepalensis.* These species could not

be distinguished from each other based on the morphological and biological tests (Barnett 2000). Isolates KY2, TY2 and TY3 were identified as *P. anomala* but isolates MY1 and MY2 are identified as either *P. anomala* or *P. sydowiorum* as these two species are indistinguishable from each other based on the keys.

In contrast to the ambiguity of the biochemical characterization of yeast isolates, molecular taxonomy using the PCR based techniques was found to be useful. Genomic DNA of the yeast isolates was isolated by employing the modified Harju *et al.* (2004) protocol. The Dendogram obtained by using SPSS package showed grouping of the isolates as that of the morphological grouping except BY0(B) isolate, which is not within the group but closely related to the group. Clustering of the data obtained from PCR-RFLP of the yeast isolates and that of the reference strain revealed that the isolates AY0. AY1, AY2, BY2, KY0, KY1 and TY1 belonged to *S. cerevisiae,* BY0(A), BY0(B), BY0(C) and TY0 to *I. orientalis,* BY1 and MY0 to *D. hansinii* and KY2, MY1, MY2, TY2 and TY3 to *P. anomala.* It was of imperative that RFLP analysis of the ITS spacer and 5.8S rRNA was regarded to be a fast and reliable method for the identification of yeasts species.

DECLARATION

I hereby declare that the thesis entitled "BIOCHEMICAL AND MOLECULAR GENETIC ASSESSMENT OF YEAST STRAINS USED BY CERTAIN TRIBAL COMMUNITIES OF ASSAM IN ALCOHOL PRODUCTION" being submitted to the Department of Molecular Biology and Biotechnology, Tezpur University, is a record of original research work carried out by me. Any text, figure, method or result that are not of own devising are appropriately referenced in order to give credit to the original author(s). All sources of assistance have been assigned due acknowledgement. I also declare that neither this work as a whole nor a part of it has been submitted to any other university or Institute for any other degree, diploma or award.

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CERTIFICATE BY THE PRINCIPAL SUPERVISOR

This is to certify that the thesis entitled "BIOCHEMICAL AND MOLECULAR GENETIC ASSESSMENT OF YEAST STRAINS USED BY CERTAIN TRIBAL COMMUNITIES OF ASSAM IN ALCOHOL PRODUCTION" submitted to Tezpur University in the Department of Molecular Biology and Biotechnology, under the School of Science and Technology in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of research work carried out by Mr. Khanindra Ratna Barman under my personal supervision and guidance.

All help received by him from various sources have been duly acknowledged.

No part of this thesis has been reproduced elsewhere for award of any other degree.

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CERTIFICATE OF THE EXTERNAL EXAMINER AND ODEC

This is to certify that the thesis entitled "BIOCHEMICAL AND MOLECULAR GENETIC ASSESSMENT OF YEAST STRAINS USED BY CERTAIN TRIBAL COMMUNITIES OF ASSAM IN ALCOHOL PRODUCTION" submitted by Mr. Khanindra Ratna Barman to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us on 21.05.10..... and found to be satisfactory.

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Acknowledgement

It is a matter of immense pleasure and joy to express my heartfelt gratitude to my supervisor, Prof. Bolin Kumar Konwar, Department of Molecular Biology and Biotechnology and Dean School of Science and Technology, Tezpur University, Napaam, who has guided me throughout the entire work. Without his constant inspiration, encouragement and constructive criticism, this arduous task would not have been possible for me.

I would like to express my sincere thanks to Prof. A. K. Mukherjee, HoD, Department of Molecular Biology and Biotechnology and Prof. T.K. Majhi, Department of Chemical Sciences, Tezpur University, Napaam for their kind advice and constant encouragement during the tenure of my research period.

I express my deep sense of gratitude to Dr. Manabendra Mandal and Dr. S. K. Ray, Department of Molecular Biology and Biotechnology, Tezpur University, for their kind help in carrying out few important analyses.

It is a great pleasure to offer my sincere thanks to all faculties and staff members of the Department of MBBT, specifically Mr. Pranab Mudoi, Sarat Konwar, Samar Kakati, Dipak Deka and D. Bania for their support during the entire course of investigation.

Special thanks are due to my friends Dr. Dhiren Choudhury, Dr. Jitu Buragohain, Dr. Ranjan Kandali, Dr. Koushik Das, Dr. Rupam Kataki, Dr. Kishore Das, Dr. Robin Doley and Dr. Naba kr Bordoloi for their friendly advices and constant support. I am also grateful to my junior Sudhir, Jyoti Prasad, Pinki, Krishna and Pranjal for their cooperation.

I would like to express my deep sense of gratitude to every member of my family specially to my wife Dr. Minati Das and son Ranit for their constant help and inspiration.

I also like to acknowledge the help received from my friends Hridoy Choudhury, Khanin Das and Chandra Jyoti Pegu for their help in collecting the samples.

The financial assistance received from the Agriculture Department, Government of Assam by providing me study leave to carry out this work is duly acknowledged.

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Date: 03-11-2009 (Khanindra Ratna Barman)

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Abbreviation and symbols

A_{260}	Absorbance at 240 nm
A_{280}	Absorbance at 280 nm
@	At the rate of
AFLP	Amplified fragment length polymorphism
BC	Before Christ
BLAST	Basic Local Alignment Search Tool
bp	Base pair
^0C	Degree celsius
ca	Circa meaning about
Ca	Calcium
CaCl	Calcium chloride
cfu	Colony forming unit
CHEF	Contour-clamped homogeneous electric field
Cm	Centimeter
CMA	Corn meal agar
CO_2	Carbon dioxide
cv.	Cultivar
DBB	Diazonium blue B
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
rDNA	Ribosomal DNA
mtDNA	Mitochondrial DNA
DNTP	Deoxynucleotide triphosphate
δ	Delta
EDTA	Ethylenediaminetetraacetic acid
et al.	et alia (and others)
etc.	et cetera (and so on)
$FeCl_3$	Ferric chloride
Fig.	Figure

GC	Gas chromatography
g	Gram
x g	Gravity (multiplies of, as in centrifugal field)
% (G+C)	Percentage of guanine plus cytocine
h	Hour
HCL	Hydrochloric acid
H_3BO_3	Boric acid
H_2O	Water
ITS	Integenic transcribed spacer
kb	Kilobase
Kg	Kilogram
KH_2PO_4	Potassium didydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
KI	Potassium iodide
l	Liter
LAB	Lactic acid bacteria
λ	Lamda (Greek)
M	Molar
Mbp	Mega base pair
Mfg.	Manufacturer
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Milliliter
mM	Milimolar
$MnSO_4$	Manganese sulphate
MSP	Microsattelite primer
MYGP	Malt extract, yeast extract, glucose, peptone
µg	Microgram
µl	Microliter

µm	Micrometer
mm	Millimeter
mM	Millimolar
MS	Mass spectra
NA	Nutrient-Agar
ng	Nanogram
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$(NH_4)_2SO_4$	Diammonium sulphate
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PCA	Principal compound analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
%	Percent
PFGE	Pulse field gel electrophoresis
pg	Picogram
PI	Potassium iodide
ppm	Parts per million
RAPD	Rapidly amplified Polymorphic DNA
:	Ratio
RFLP	Random fragment length polymorphysm
RFE	Rotating field electrophoresis
RNA	Ribonucleic acid
rRNA	Ribosomal
rpm	Revolution per minute
SA	Starch agar
SDS-PAGE	Sodium dodecyl sulfate PAGE
SE	Standard error
Sec	Seconds

SIM	Simplified identification system
SN	Serial number
sp.	Species
Syn.	Synonym
TAE	Tris acetic acid ethylenediaminetetraacetic acid
TBE	Tris boric acid ethylenediaminetetraacetic acid
TE	Tris EDTA
tris	Tris(hydroxymethyl)-aminomethane
U	Unit
UBA	Universal beer agar
UPGMA	Unweight Pair Group Method using Arithmetic Average
UV	Ultra violet
UV/VIS	Ultra violet visible
V	Volt
VJA	V8 juice agar
v/v	Volume/volume
WLN	Wallerstein Laboratory nutrient media
w/v	Weight/volume
YM	Malt, yeast, glucose, peptone agar medium
YMA	Yeast morphology agar
YCB	Yeast carbon base
YNB	Yeast nitrogen base
$ZnSO_4$	Zinc sulfate

Chapter 1

Introduction

Fermentation is an inexpensive technology of food preservation that also improves its nutritional value and organoleptic properties. Moreover, it also detoxifies and destroys undesirable factors present in raw food such as phytates, polyphenols, tannins etc.

Mankind has been practicing fermentation as a method of food processing since antiquity. Beer was produced in Sumeria before 7000 B.C. and in Assyria before 3500 B.C. Babylonian clay tablets show detailed recipes of beer making in 4300 B. C. In Asia, using yeasts milk was converted to *kefir* and *koumiss* for many centuries (Kurtzman and Fell, 1990). It is the oldest of all biotechnological processes. The origin of biotechnology can be traced back to the production of beer by fermentation of cereals. It has its early records in South-East Asia, where China is regarded as the cradle of mold-fermented food; and in Africa where the Egyptians developed the concept of combined brewery-bakery (Hesseltine, 1981). The early Egyptian beers were probably quite similar to some of the traditional opaque sorghum, maize or millet beer found in various African countries. Although, the use of yeasts in food and beverage production dates back to ancient days, the importance of yeasts in food and beverage industry was realized only in 1860.

Almost every ethnic tribal community has their distinct type of beer. Tribal communities of North-East India are no exceptions. They have the practice of producing beer in their households. The production and consumption of beer become a part of their socio-cultural life. Besides using as inebriating drinks, products are also a part of their socio-cultural occasions such as naming ceremony, marriage and burial of dead body etc.

The traditional rice beer of Ahom community is known in local language by various names such as *hanj, lau or hanjpani.* The microbial culture used for the preparation of *hanj* is known as *hanjpitha* or *pithaguti.* The plants

Clerodendron viscosum (dhopat tita), *Glochidion lanceolanium* (pani modhori), *Naravelea zeylanica* (gopsoi), *Centella asiatica L* (manimuni), *Dentela repens* (bonjaluk), *Scoparia dulcis* (chenibon), *Cyclosorus extensa Bh Ching* (biholongoni), roots of *Xanthoxylum hamiltonianum Wall* (tezmori), leaves of *Artocarpus heterophyllus* (jackfruits), *Desmodium laxiflorum* (beoni sabota), *Rubus alceifolius* (*jetulipoka*), *Acanthus leucostachys* (naga dorob), *Saccharum officinarum L* (kuhiar), *Ananus comosus* (matikathal) etc are mostly used for the preparation of *hanjpitha*. The weathered leaves and other parts of medicinal plants are mixed with required amount of cleaned, washed and dried rice (approximately 1:3 proportion) and then ground in to a fine powder. Rice may either be fine grained (*lahi*) or glutinous (*bora*). One or two (depending on size) old *hanj pitha* is grounded in to powder and mixed thoroughly with the rice-leaves powder. A little amount of clean water is sprinkled on the mixture and then made it in to small balls (*laru or pithaguti*) or flattened cakes (*hanj-pitha*). The balls or cakes are dried under the sun for a short time and then dried by keeping over a machan or *dhuashang* over the fire place by wrapping with *beholongini* (*Cyclosorus extensa Bh Ching*) leaves. *Beholongini* leaves act as repellent of insect pest as well as microorganisms.

Rice beer (*hanj or hanjpani*) is made from any fine quality rice. However, the *hanj* made from *bora* (sticky or glutinous) rice produces the best beer having an attractive flavour and light yellowish colour. The preparation of *hanj* is described bellow:

1. The rice is cooked as per the normal procedure. However, the *bora* (glutinous) rice is cooked in steam (*bhapatdiya* or *chenwadiya*) and then allowed to cool.
2. The beer cake or ball (*hanj-pitha*) @ 40-50g/kg of rice is ground into a fine powder and mixed uniformly with the cooked or steamed rice.
3. Filter or clean tap water is sprinkled over the mixture for soaking.
4. The mixture is put in a bamboo basket placing clean banana leaves at the bottom of the basket and mounted on a wide mouthed earthen urn (kalash).

5. The microbial inoculum in the beer cake acts on the water soaked rice and converts it into alcohol after two to three days.

6. The beer juice percolates into the collecting urn.

7. The collected juice, called as *rahi,* is poured over the fermented rice mixture to enhance fermentation and get quick production.

8. The whole mix on 3rd day is transferred in to the earthen urn and allowed to ferment for another 2-3 days.

9. The beer is collected for consumption by pouring from the urn.

10.The night before the collection, the required volume of water is poured into the mixture by a clean and dry muslin cloth as sieve.

11. The *hanj* can be collected several times by adding water prior to collection of the day. However, towards the end the taste becomes flat.

At the end of the whole process of beer making, the left over substrate is fed to pig or cattle as nutritious feed. The earthen urn (*kalah*) to be used in beer making has to be cleaned thoroughly and then dried by putting over a fire place. The urn has to be dried completely and a layer of smoke with dark colour has to be formed.

Bodos (Kasaris), like many of the sub-Himalayan hill tribes, undoubtedly have a certain weakness for what may be looked upon as their national beverage, *zu*, a form of rice-beer. They consume the beverage in large quantities on special occasions like wedding, funeral, *bihu* and more particularly on the occasion of feasting rice of the first harvest at the end of the season "*mikham gadan zanai*" (Endle, 1975).

Bodos prepare "*zu*" for the immediate consumption. The inoculum required in the preparation of *zu* is known as '*emao* or *bakhar*'. It is prepared by using a definite proportion of husked rice, leaves of jackfruit tree, leaves of a wild shrub *bhetai* and leaves of poisonous ferns. All these ingredients are ground in to a fine powder and passed through a fine sieve. The powder so prepared is mixed with water so as to make a more or less tenacious paste, which is divided in to small pieces sufficient to make solid discs, about 3 inches in diameter, having an inflated central region. The discs are sprinkled freely with

the powder prepared by grinding old-preserved discs of some weeks standing and are covered with rice straw for a brief time period. The discs are placed on a bamboo platform inside the house for 3-4 days and then put under bright sunshine for another 4-5 days to make them well dried. Finally, the discs find their way into a wide mouthed earthen urn (kalah). The urn is kept on a bamboo machan erected over the fire place at a height of 5-7 feet until required for use.

The process of *zu* preparation can be described as follows: 3-4 kg rice is cooked using an iron or brass utensil. The cooked rice spread out on a bamboo mat and allowed to cool. Two *emao* discs are ground in to a fine powder and mixed carefully with the cooked rice. The inoculated rice is transferred into a clean-dry earthen urn (*kalah*). The vessel with its content is then placed on a bamboo platform erected at a height of about 5 feet over the household fire place and kept for 3-4 days keeping the mouth open for the first 1-2 days and then covered for the remaining days. Rice beer secreted inside the urn become ready to serve after adding water '*ad libitum*'. The beer is poured through a crude sieve made of rice straw after shaking the vessel vigorously.

Traditional beer usually lacks the ensured price status contrary to that of the refined quality of the high-tech industrial product. Some people even regard it as backward or poor peoples' drink. Moreover, inadequate grading and cleaning of the raw materials, short shelf life, unhygienic preparation procedure, crude handling and processing, insufficient product protection due to lack of packaging, lack of quality standard, fear for the food borne pathogen are the major causes for the poor appeal of the traditional beer among the elite classes of the society.

Household traditional fermented beverages play an important role in the socio-economic sphere of the ethnic tribal groups and they remain to be popular only among the low income group people.

Beer has been produced following traditional practices with limited knowledge on microorganisms and/or enzymes for thousands of years. Almost every tribal community preserve the fermentation culture for future use as wine cake or '*pithaguti*' or '*emao,* or '*bakhar*', prepared by mixing rice flour with

grounded leaves of several plant species and inoculating old culture in to it. So far, no serious attempts have been made to study the microorganisms associated with the traditional home-made rice beer. Since 8,000 years ago, yeasts have been used for making bread, wine, beer, sake etc (Akada, 2002), the study of yeast could be traced back to 1838 AD only (Vaughan-Martini *et al.*, 1995). As a matter of fact, biotechnology has evolved with the traditional food fermentation process in the developing countries like India. The age-old tradition of beer or wine making as the household rural technique is still in use. But, in order to meet the current and future challenges by the traditional beer or wine, the developing countries must strive to acquire capabilities through modern biotechnological tools and technologies.

Traditional beverage production system encountered limitations associated with different stages and aspects of the fermentation process such as lack of good manufacturing practice or code of hygiene, lack of proper knowledge of the culture of the process and their characteristics, absence of any control over the process variables such as pH, temperature, humidity, water quality etc. Moreover, lack of knowledge of proper nutritional and toxicological implications of fermentation and its products also add to the list of limitations of traditional fermentation (Iwuoha and Eke, 1996).

In the traditional beer production, scientific and technological skill applied is poor standard. In many cases, although specific microorganisms predominate, natural fermentation plays an important role in the process. For improving the knowledge base, a thorough investigation on the microbiological aspect of even the least known product is essential. To determine the biotechnological worth of each organism, they have to be isolated, characterized, studied and preserved. Moreover, as the quality of the end product is largely dependent on the active microorganisms, their role needs to be clearly understood. These factors may transform the traditional beer production art in to a technology to incorporate the objective-based methods of process control. With the use of improved starter cultures, acceptability of the new product and the improvement of traditional beer/wine can be achieved.

Use of alternative plant materials such as jackfruit, banana, glutinous rice honey etc as substrate may be tried to satisfy people who like novel products and tastes. Moreover, this may lead to traditional beer production with maximum substrate utilization, minimum production cost, product with improved nutritional value and improved quality.

Microorganisms such as bacteria, yeast and at least some filamentous fungal species play important role in fermented food and beverage production. Although traditional beer production is practiced using starter culture, it is prone to natural microflora under the non-sterile condition. The environment resulting from chemical composition of the raw material, fermentation temperature, absence or presence of oxygen etc causes a gradual selection of microorganisms responsible for the desired product characteristics. Thus, the natural fermentation process is suited to the local situation. The variety of microorganisms playing important role in the natural fermentation process can impart attractive flavours that are hard to achieve under complete aseptic condition and using pure culture.

But the list of disadvantages of the natural fermentation is also long. It is a difficult task to control natural fermentation for commercial purpose. The presence of a significantly large number of microflora may accelerate the spoilage after completion of the fermentation process. To reduce the risk of natural fermentation "back-slopping", a portion of the previous batch of fermentation dough is used to inoculate the next batch as in the case of sourdough and curd preparation process. It is also important to investigate the effect of inoculum enrichment on product characteristics and consumer acceptance. Therefore, the development of a gradually evolved and suitable fermentation starter will be an attractive proposition for use in the home and the small-scale fermentation under non-sterile or semi-sterile condition. But, the best proposition to stabilize fermentation under the non-sterile condition is the multistrain dehydrated starter, which can be stored at ambient temperature enabling more flexibility. These types of homemade starter are in use for most of the fermentations. Although, these are homogeneous and their doses being

convenient, they may possess some spoilage causing bacteria and yeasts. This requires quality monitoring of the inoculum used and of the fermentation process in which it is used.

Industrialization of appropriate indigenous process of traditional beer production at village level with appropriate and affordable technology may help the poor who originated and preserved the process.

Traditionally yeasts were classified into groups based on comparison of morphological traits and physiological features (Barnett *et al.* 2000). But, in some cases this method leads to incorrect identification of strains. Recent advancements in molecular biology lead to the application of new methods for the identification and characterization of yeasts and other microorganisms.

Meyen, using binomial nomenclature system, gave the name *Saccharomyces cerevisiae* to the yeast species. *Saccharomyces* means sugar mold and *cerevisiae* means beer from *kerevigia* (Gaelic) or *cervoise* (French). Rees first described the genus *Saccharomyces* in his book that described all fungi capable of alcoholic fermentation. He differentiated *S. ellipsoideus* as the yeast fermenting fruit juices from *S. pastorianus* as the brewing yeast. Later, Hansen from Carlsberg brewery applied the pure culture technique to yeast for the isolation and maintenance. Guilliermond described 20 *Saccharomyces* species applying the system for yeast classification based on cell morphology and on a few physiological tests mainly based on fermentation and assimilation of different compound as the sole carbon or nitrogen source (Rainieri *et al.*, 2003).

The approaches in yeast identification have significantly changed in the recent few decades due to the rapid increase in basic biological knowledge, enormous technological advances and increased interest in the practical applications and biodiversity of this important microbial group. Although, conventional methods are still employed largely, several rapid yeast identification methods based on the specificity of nucleic acid sequences or molecular techniques have been developed that allow for strain classification on all taxonomic levels. These methods are considered more authentic as well as

reliable. But, the oldest tool of microbiology, the microscope, is still a fundamental accessory for studies involving yeast biology, biodiversity and taxonomy (Vaughan-martini, 2003).

Molecular taxonomy is the term used to designate the modern method of classification of yeast and other microbes based on the study and analysis of the informational macromolecules DNA and RNA. The vast array of methods examining the various classes of DNAs or RNAs allow for the classification of yeast as well as other microbes at different taxonomic levels, permitting us to understand microbial evolution and to arrange them according to their ancestral relationship. The main advantage of the molecular taxonomy over the conventional methods of classification is its consistency. In fact, genomic data are constant and independent of the cultural status or growth conditions. This characteristic of molecular taxonomy offers vast scope for more effective classification schemes for fermenting yeasts.

Besides biochemical characterization, molecular biology techniques provide alternative and additional methods and are becoming an important tool in the identification of yeast species. Now-a-days, molecular genetic techniques for the classification, identification and ecological study of yeast and other microbes are becoming an important tool. Pulse field gel electrophoresis (PFGE), a molecular method of DNA analysis provides direct evidence of chromosomes and allows fingerprinting of yeast DNA. The number and size of chromosomal DNA obtained as an electrophoretic pattern using PFGE is considered to be a species-specific method of characterization. Now-a-days, polymerase chain reaction (PCR) protocols are widely employed in fungi identification. Polymorphism related to intra-specific variations in yeast is commonly studied using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). Both methods provide different banding patterns that are useful for distinguishing strains. The PCR of some repeated sequences typical of yeast genomes, such as the δ, τ and σ regions, allow an amplified sequence polymorphism to be obtained, which is useful for the identification of individual strains of *Saccharomyces cerevisiae* (Arlorio *et*

al., 1999). In fungi including the yeast, the structure of the ribosomal region comprises repeated clusters of coding regions. The coding sequences for 17S, 25S and 5.8S rRNAs are separated by the internal transcribed spacer (ITS) regions. The ITS region of the rDNA contains variable non-coding regions that are useful for distinguishing genera and species.

Characterization of yeast strains used in fermentation is necessary for the quality control during production to determine whether the used strain is identical to the original one and to ensure that the fermentation process is actually maintained by the inoculated yeast strain. To improve the trustworthiness of the traditional rice beer fermentation process, biotyping of appropriate yeast strains responsible for the fermentation should be a priority. A properly documented regional or sub-regional databank on suitable strains of yeast responsible for the fermentation of traditional beer would be an asset for the scientist working of the fermented food and beverages.

Till date, no serious study has been made to gather information on the spectrum of microorganisms associated with the traditionally fermented rice beer of Assam and adjoining states of North Eastern region. But, such knowledge is essential for the production of a stable product with better quality. In distant as well as recent past, there were no authentic data on the technical, nutritional, quality control and economic aspects of traditional rice beer of Assam. The aim of the present study is to study the microflora associated with the traditionally produced and used starter culture (inoculum) for making the rice beer.

Objectives

On the basis of the above mentioned information, the present investigation was taken up with the following objectives:

1. To isolate yeast strains from the starter culture '*pithaguti*' or '*emao*' *or* '*bakhar*' of rice beer.
2. To characterize them on the basis of colony behaviour, morphology, and biochemical test and at molecular level using probe-based RAPD.
3. To produce beer using the selected yeasts from various raw materials.

Chapter 2

Review of Literature

Yeast was one of the first microbes to study scientifically. Antonie Lavoisier, a chemist, first described the phenomenon of alcoholic fermentation. He also studied how sugar is converted to carbonic acid gas and spirit of wine. He estimated ethanol content by distillation and the production of CO_2 by dissolving in alkali. Several types of traditional beverages have been produced throughout the world from time immemorial, especially in the developing countries, at household level using age-old techniques. There are many inter continental as well as regional variations in the preparation of beverages. Research in to the processing techniques of the traditional fermented beverages is still in its infancy. The biological agents of beer and wine fermentation are yeasts belonging mainly to the genus *Saccharomyces* having the ability to accumulate ethanol. Like in most of the oriental countries, in Assam too, microbial inocula in the form of hardball or flattened cake made from flour of starchy rice grains are used as starter culture. Unfortunately, starter cultures of all the tribes have not been studied scientifically till date. In this context, attempt is made to present a review of the available literature on various aspects of traditional beverages, focusing mainly on characterization of the yeast species involved, from morphological to molecular aspects.

2.1 Work done

2.1.1 Works on morphological, physiological and biochemical traits

Ahearn *et al.* (1960) using standing and shaken cultures for assimilation of carbon sources found that the use of agitation was to reduce the incubation time from 3 to 1 week or less. Non-fermentative yeast particularly those of the

genus *Rhodotorula,* which commonly assimilate carbohydrate by adaptive or slow growth were found to increase their assimilation under agitation.

Deak (1986) proposed a simplified scheme for the identification of yeast. This identification scheme was based on the results of a few selected tests such as assimilation of or aerobic growth on maltose, raffinose, galactose, cellobiose, trehalose, xylose, erythritol, mannitol, and nitrate, fermentation of glucose, growth in the presence of 0.01% cycloheximide and production of urease. The tests were supplemented by the microscopic observation of cell morphology. In the first stage of the scheme, using eight standard tests, strains were grouped into 16. In the second stage 2-8 additional tests were performed to each group to identify one strain from each group. This two-step identification scheme was simple and economic and even time saving as only two Petri dishes and four test tubes were needed to initiate identification of one strain.

Gao and Fleet (1988) studied the ethanol tolerance of yeast strains isolated from *pito,* a cereal-based alcoholic beverage of Ghana.

Rohm and Lechner (1990) used and evaluated a simplified identification and computer based method to identify 12 reference and 382 yeast strains isolated from cultured milk products. The simplified key failed to account for species variability with regard to physiological, morphological, and sexual reproduction characteristics. They opined that a reevaluation of the basic theoretical assumption of the simplified key could only confirm the practical results and indicate the identification method to be unsatisfactory.

Vaughan-Martini and Martini (1993) tried to distinguish the strains of ten recognized species of the genus *Saccharomyces*. After complete physiological analysis, they constructed a taxonomic key to the genus Saccharomyces. They concluded that except *S. dairensis* and *S. exiguous* containing heterogeneous taxa, strains of all other species could be distinguished with the help of physiological tests.

Iwuoha and Eke (1996) reviewed the Nigerian indigenous fermented food and discussed the traditional status, and the technical and scientific steps introduced to develop them to a modern processed food. The major problems usually faced by the ethnic art of producing traditional alcoholic beverages were related mainly to the production environment, microbiology in processing, process control and nutritional and toxicological status.

Morais *et al.* (1997) studied the characterization and succession of yeast population associated with the spontaneous fermentation of sugarcane 'aguardente', a traditional Brazilian alcoholic beverage. Preparation of aguardente by spontaneous fermentation of sugarcane juice is a traditional art in Minas Gerais state of Brazil. A starter ferment *'pe'de cuba'* was produced by various methods including the development of fermentative micro-biota in the sugarcane juice alone, or mixing sugarcane juice with rice, rotting fruits, crushed corn and other substrates. They observed rise in the yeast counts in the starter ferment in 24 h and reached the maximum in 5 days. *Saccharomyces cerevisiae* together with *Candida sake* presented high population counts at the end of the starter culture production. During fermentation, the rise in the count of *Saccharomyces cerevisiae* corresponded to the disappearance of the most yeast species inoculated with the juice including the *Kloeckera* sp. at the end of the fermentation cycle.

Ramano *et al.* (1997) using secondary product formation as a tool, studied the strain diversity in non-*Saccharomyces* yeast. The diversity of yeast species and strain was monitored by determining the formation of secondary products of fermentation such as acetaldehydes, ethyl acetate and higher alcohol. They isolated 30 strains *Kloeckera apiculata,* 20 *Candida stellata,* 8 *Candida valida* and 20 *Zygosaccharomyces fermentii.* Within each species, the strains were distinguishable in phenotype through the production of different amounts of by-products. At different stages of spontaneous fermentation, different phenotypes of non-*Saccharomyces* yeast were presented, characterized by consistent differences in some by-products involved in the wine bouquet.

Van-der-Aa Kuhle and Jesperson (1998) tried to detect wild yeasts in brewery yeast samples using six different selective principles like MYGP (malt extract, yeast extract, glucose, peptone) agar supplemented with 195 ppm $CuSo_4$, lysine medium, crystal violet medium, actidione (1.0 ppm) medium, WLN medium and UBA (universal beer agar) medium. API ID 32C kit was also used to determine the remaining carbon compound assimilations. *S. cereviisiae* wild yeasts were isolated by all five principles. Non-*Saccharomyces cerevisiae*

wild yeasts were more successfully detected on the $CuSo_4$ medium and lysine medium. From these selective media, 126 wild yeast isolates belonging to *S. cerevisiae* (57%) and 101 non-*S. cerevisiae* brewing yeasts drawn from 45 breweries were isolated.

Gadaga *et al.* (1999) reviewed the traditional fermented food and beverages of Zimbabwe. This review included fermented products ranging from fermented maize porridges, fermented milk products, non-alcoholic cereal based beverages, alcoholic beverages from sorghum or millet malts, fermented wild fruit beverages etc.

Lee and Fujio (1999) analyzed the sample and investigated the micro flora therein of *benh-men*, a traditionally produced starter from Vietnam. They found that *benh men* samples were acidic in nature with a mean pH of 5.76. Samples contain moisture of 13.6% (w/w). The observed total mold count of *benh men* sample was 13 X 10^6 cfu/g fresh sample, whereas total yeast count was 4.3 X 10^6 cfu/g. They isolated mold species such as *Rhizopus oryzae, Mucor indicus, Mucor circinilloides and Amylomyces rouxii.* The isolated yeast species included *Saccharomyces cerevisiae, Hypopichia burtonii, Saccharomycopsis fibuligera, Pichia anomala and Candida* sp.

Sefa-Dedeh *et al.* (1999) isolated and characterized yeast from *pito,* a traditional cereal-based alcoholic beverage of Ghana using biochemical and physiological methods. They identified 21 strains belonging to *Saccharomyces cerevisiae*, *Candida tropicalis*, *Torulaspora delbrueckii, Schizosaccharomyces pombe* and *Kluvyeromyces africanus.* Malt extract 0.1% and peptone gave less than 20% decrease in cell viability of all isolates. At relatively low temperature, growth increased with increasing pH, but a decrease was observed with increasing pH at high temperature for *Saccharomyces cerevisiae* and *Candida tropicalis*. All isolates demonstrated good growth at 10% ethanol concentration over a period of 8 days incubation except *K. africanus*, in which growth was completely inhibited after 4 days of incubation. The quality indices of the beverage produced using *Saccharomyces cerevisiae* as the single-starter organism compared favourably with that of the traditional brew.

Comi *et al.* (2000) isolated 70 *Saccharomyces cerevisiae* strains from grapes of different zones of Collio, Italy. Upon chemical analysis of the different parameters such as the production of ethanol, glycerol, acetic acid, higher alcohols and ethyl acetate when inoculated sterile grape-must showed different levels of production of n-propanol and 3-methyl-1-butanol by the yeast strains. The production of ethyl acetate, isobutanol and 2-methyl-1-butane was also considered as parameter in differentiation.

Ezeronye and Okerentugba (2000) studied the physiological variants of yeast isolates from palm wine and further selected suitable strains for genetic improvements. They also studied ethanol concentration on laboratory scale and ethanol tolerance of the isolated yeast strains.

Gomes *et al.* (2000) carried out a comparative study to obtain a better and reliable identification of industrial yeasts. Five strains from Brazilian fuel alcohol industry and another five yeast strains isolated from five major beer industries were evaluated by the methods. It was observed that differential media, containing different dye at different concentrations, was unable to detect any difference among the fuel alcohol industrial yeasts. However, three of five beer isolates were identified by the method. Keeping in view the factors like efficiency, reproducibility, simplicity and cost effectiveness, differential media is simple and affordable but time-consuming and less efficient.

Jespersen *et al.* (2000) while studied the phenotypic and genotypic diversity of *Saccharomyces* contaminants isolated from *lager* breweries and their phylogenetic relationship with brewing yeasts found that *Saccharomyces* sensu stricto complex yeasts were not easily identified phenotypically as they were found to be very close to each other. Ability to grow at 37^0 C was used to differentiate *S. cerevisiae* from *S. pastorianus* although in some cases it was questionable. Growth in the vitamin free medium was used to identify *S. bayanus.* The isolate identified as *S. cerevisiae* by ITS-PCR RFLP was also observed to grow in the vitamin free medium. The study established that the current taxonomy within the *Saccharomyces* sensu stricto complex was not fully explored. The phenotypic differentiation between the species appeared to be

based on the well-characterized laboratory strains, but not fit for the characterization of isolates selected from other sources such as food and processing plants.

Naumov *et al.* (2000) studied yeasts associated with some French wine and conclude that the distribution of *Saccharomyces bayanus* var. *uvarum* was connected with low temperature climatic condition and /or wine technologies in which most of the fermentations were at least partially carried out at low temperature (10-15^0 C). He also concluded that based on the distribution of *Saccharomyces bayanus* var. uvarum in different wine making processes; it was probable that cryo-tolerance was an important trait in determining the ecology of the yeast.

Freydiere and Odds (2001) reported about the concern for the standardization of commercial kits for the yeast identification. The readily available yeast identification kits were abundantly used in brewing microbiology and particularly in clinical mycology. This was due to easy availability, time saving and accuracy. The accuracy of API ID 32C identification system (bioMerieux, France) was reported to be more than 94% and. But, studies on the system conducted in the USA gave notably less impressive results. Again, just the opposite situations occurred to the RapID Yeast Plus system (Innovative Diagnostic System, USA.) that it was evaluated negatively in Europe. Whereas the system was recommended for routine use on the basis of its rapid and accurate performance in three US studies. This type of disagreements for the performance of the same system on the two sides of Atlantic might be due to geographical differences (Ramani *et al,* 1998) that might not be the complete explanation of the fact.

Sipiezki *et al.* (2001) studied the diversity of yeast flora in a spontaneously fermenting sweet white wine of a Tokaj winery. They observed that the non-*Saccharomyces* yeasts, later replaced by a heterogeneous *Saccharomyces* population, dominated the first phase of fermentation. The dominating species was *Sacchromyces bayanus*. The study did not recommend any strain to be inoculated for the fermentation of Tokaj wines. They concluded

that the combination of the positive factors of the yeast strains might result in a strain with a favorable profile of metabolites and high genetic stability.

Teramoto *et al.* (2001) analyzed a sample of *boza*, an indigenous beer of Egypt and determined its characteristics. They observed that *boza* contains 3.8 % (v/v) ethanol, along with volatile esters and higher alcohols. Although they could not isolate any viable yeast from *boza,* but could isolate a yeast strain, capable to carry fermentation, from wheat bread and identified it to be *Candida krusei.* The study reported that the rice sake made with the isolated yeast strain contained 11.7 % ethanol.

Teramoto *et al.* (2002) collected samples of *zutho*, an indigenous alcoholic beverage of Nagaland, made with sprouted rice grain and subjected it to analytical and microbiological characterization. They reported that *zutho* is a whitish porridge like slurry containing 5.0 % (v/v) ethanol along with slight amount of volatile esters and higher alcohol. *Zutho* was found to have the unique aroma having characteristics similar to those of Japanese sake and sprouted rice sake. The pH and acidity of *zutho* was assayed to be 3.6 and 5.1, respectively. They isolated a yeast strain designated as *Saccharomyces* Naga 97 and is found to be capable of ethanol fermentation.

Fleet (2003) reviewed the yeast interaction and wine flavor and concluded that wine was the product of many diverse interactions between yeasts, fungi and bacteria.

Mugula *et al.* (2003) studied the characteristics of *togwa*, a Tanzanian fermented food. *Togwa* is produced by natural fermentation of a mixture of lactic acid bacteria (LAB) fermented sorghum, millet or maize gruels with sorghum or millet malt flour. The microbial population of *togwa* was dominated by *Lactobacillus brevis, L. fermentum, L cellobiosus, Weissela confusa, Pediococcus pentosaceus, and L. plantarum* (LAB). Although the species were isolated from all types of *togwa* and at all culture stages, *L. plantarum* dominated the final stages of fermentation. Yeast species isolated from *togwa* were identified as *Issatchenkia orientalis* (50%), *S. cerevisiae* (23%), *Candida tropicalis* and *C. pelliculosa.* A co-metabolism between yeast and LAB, where

the bacteria provides the acidic environment for the growth of yeast and the yeast provides vitamins and other factors to the bacteria was proposed. Acidity increased during the fermentation.

Nissen and Arneborg (2003) studied the cause of early death of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyce scerevisiae*. The study revealed that *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii* yeast strains died of earlier than the *S. cerevisiae* in mix culture fermentation of *K. thermotolerans* with *S. cerevisiae* and *T. delbruečkii*. It was also concluded that the early death, which was seemed to be no-apoptotic in nature, was not due to the nutrient depletion or accumulation of toxic substances but seemed to be mediated by a cell-to-cell contact mechanism at high cell densities of *S. cerevisiae* and due to a lesser ability of *K. thermotolerans* and *T. delbrueckii* to compete for space.

Rainieri *et al.* (2003) reviewed the systematic-genetic diversity and evolutionary aspects of *S. cerevisiae* sensu stricto group. Classification of *Saccharomyces* group was a problematic task especially at species level. Different workers proposed many classification methods over the years and accordingly names of individual strains and species were also a subject of revision. This fact creates confusion among the scientists and industry.

Romano *et al.* (2003) studied the function of yeast species and strains in wine flavor. They assumed that the wine quality would be determined by the interaction among grapes, yeasts and technology used. In the study, the metabolic profiles of some strains belonging to some of the most frequently encountered wine yeast species such as *S. cerevisiae, H. uvarum, C. stellata, S. ludwigii and Z. fermentatii* were observed. It was observed that the wine flavor is determined by a wide variety of compounds with different aromatic properties. Although major volatile products of yeast fermentation play minor role in determining wine flavor and other compounds such as organic acids, higher alcohols, esters and to a lesser extent acetaldehydes contribute to the product flavor. Although, use of pure culture fermentation ensures reproducibility and reduces the risk of spoilage along with predictable control of

fermentation and quality, it might also deprive the product of some desirable metabolites contributing product flavor. It would therefore be advantageous to formulate and use mixed starter culture.

Tamang (2003) studied the biochemical and microbial characteristics of the *'marcha'* and *'jaanr'* and isolated sixty strains of filamentous moulds such as *Mucor circinelloides, Mcor sp. Rhizopus chinensis and Rstolonifer*; 203 strains of amylolytic and alcohol producing yeasts viz: *Saccharomycopsis fibuligera, Hansenula anomala and S. cerevisiae,* and 163 strains of lactic acid bacteria.

Thapa and Tamang (2004) characterized the fermented finger millet beverage *kodo ko jaanr* of the Eastern Himalayan region. A total of 161 strains of microorganisms were isolated from 40 samples of *kodo ko jaanr*, of which 81 isolates were yeast and 80 lactic acid bacteria (LAB). Following the taxonomic keys described by Kreger-van-Rij (1984) and Kurtzman and Fell (1984), yeast strains belonging to four species viz. *Sccharomyces cerevisiae* Meyen ex Hansen, *Pichia anomala, Candida glabrata and Saccharomycopsis fibuligera* (Lindner) were identified. Moreover, bacterial strains of *Pediococcus pentosaceous* Mees and *Lactobacillus fermentans* Kandler were also detected in the samples.

Casalone *et al.* (2005) studied *Saccharomyces cerevisiae* natural population for studying colony morphology and pseudohyphal growth using several media and techniques. The strains showed widespread ability to form filamentous-pseudohyphal structure in response to different stressing stimuli. Ethanol and isoamyl alcohol were found to be good inducers of filamentous growth and invasiveness for most of the strains from natural populations. But, the degree of responsiveness to stimuli was found to vary greatly within strains. Some strains needed no stimuli and formed filaments and showed invasiveness on all agarified media. But, some other strains only occasionally invaded agar media and never showed filamentous growth. Tetrad analysis of some of the strains showed that at least part of the polymorphism was due to heterozygosity. The genetic control of the rough, filamentous and invasive traits are complex

and performed by different, probably interacting, dominant and recessive genes. The frequency of well-structured fluffy colonies was very rare in the natural colonies. This was in contrast to the general statement that the smooth phenotype was the result of yeast domestication under the laboratory condition. Moreover, cells of a strain covered by the mycelia of a contaminating unidentified mold showed string invasiveness indicating that the fungal colony exerts stimuli to produce invasive hyphae.

Tsuyoshi *et al.* (2005) studied the microflora of *marcha* collected from Sikkim, India. *Marcha* is an amylolytic starter to produce traditional alcoholic beverages. In this study, 20 yeast strains isolated from six samples of *marcha* were identified by phenotypic methods. Preliminary phenotypic characterization and grouping was done by using API test kit. Representative strains from each group were subjected to molecular phylogenetic analysis to obtain their phylogenetic position. In this phylogenetic and phenotypic studies of the yeast isolated from *marcha* demonstrated more diversified microflora than previously recognized. The yeast species isolated and identified were *S. bayanus, C. glabrata, S. fibuligera, S. captularis, P.burtonii* and *P. anomala.* All samples tested were either starch degraders or ethanol producers.

Gonzalez *et al.* (2007) analyzed the influence of hybrid yeast on 2 different grape-must fermentations at 4 different temperatures (14, 18, 22 and 32^0 C) by studying the volatile compounds, sugar assimilation and other characteristics influencing the enological properties of wine caused by the impact of yeast. Hybrid yeasts behaved particularly well at 14, 18, 22^0 C and the commercial strain of *Saccharomyces cerevisiae* was found to be better adapted at higher temperatures. Hybrid yeasts showed moderate behaviour in the production of glycerol, acetic acid and malic acid but they were greater producer of higher alcohol.

Hrnandez *et al.* (2007) applying a biochemical method identified and characterized yeasts isolated from the fermentation of seasoned green table olive. It was revealed that in the fresh olive, *Cryptococcus sp.* was dominant while in higher brine concentrations *Pichia anomala, Kluyveromyces marxianus*

and Saccharomycese cerevisiae were dominant. Unlike yeasts isolated from fresh olive, the strains obtained from the olive-brine mostly showed low pectolytic activities. Some of the strains also exhibited lypolytic activities and their assimilation or production of organic acids in the brine.

2.1.2 Works on molecular genetic assessment of yeast

Baleiras Couto (1995) tested the suitability of the random amplified polymorphic DNA (RAPD) assay and the restriction enzyme analysis of PCR amplified rDNA for the identification of common spoilage causing yeasts *Zygosaccharomyces bailli, Z. rouxii, Saccharomyces cerevisiae*, *Candida valida* and *C. lypolitica.* Both techniques proved to be adequate for the identification of yeasts. RAPD could provide less stable patterns than restriction pattern analysis of PCR amplified rDNA. Principal compound analysis (PCA) was applied successfully for clustering the RAPD patterns. The primer of choice was shown to be important with respect to the discriminatory power of the RAPD method. Some primers used enabled discrimination on the subspecies level. It was concluded that from this study, both typing methods could be applied for yeast taxonomic purpose.

Paffetti *et al.* (1995) applied RAPD and RFLP in molecular assessment of genomic DNA isolated from yeast strains belonging to the genera *Saccharomyces* and *Zygosaccharomyces.* Upon analysis of the data they concluded that the RAPD fingerprinting combined with the analysis of RFLP can provide unambiguous type assignment. It was observed that 13 strains, except one, showed RAPD or RFLP pattern very similar to that of the *S. cerevisiae* strain DBVPG-6173 and in UPGMA consensus tree these strain clustered together at a low level of divergence which suggested that these were of the same species. Again high intragenus and intraspecies variability was observed in case of two *Z. fermentii* and *Z. bailli* type strains as well as *Z. bailli* F39 strain. It was concluded that RAPD analysis with multiple primers is a powerful tool in yeast typing and in defining genetic relatedness among strains.

Versavaud *et al.* (1995) studied the variability among the strains, their phylogenetic affinity and biogeographic distribution by using the electrophoretic karyotyping, mitochondrial DNA restriction length polymorphism analyses and PCR amplification of the interspread repeats of the wild *S. cerevisiae* enological strains. By the above molecular method of differentiation, they identified a limited number of *S. cerevisiae* strains responsible for the fermentation process. They also concluded that one strain is omnipresent over the entire area surveyed and there was little correlation between the geographical location and the genetic affinity.

Nadal *et al.* (1996) studied the molecular polymorphism distribution of phenotypically distinct populations of wine yeast strains. Electrophoretic karyotyping and mtDNA restriction analysis were used to study the polymorphisms of the 124 yeast strains isolated from spontaneously fermenting grape must. *Hin* fI restriction endonuclease was used for the restriction analysis and to group 116 of 120 yeast strains according to the restriction patterns. The analysis of *Saccharomyces* clones isolated revealed a large degree of mtDNA polymorphism. At least 24 different mtDNA restriction patterns were observed. Three mt DNA restriction patterns accounted for more than 70% of the isolates and the remaining 30% strains showed mtDNA restriction patterns that were unique or rare. It was interpreted that the appearance of strains with the same mtDNA restriction patterns and similar phenotypic characteristics but with somewhat different karyotype profile might be an indication that the isolated strains were not originated from a single cell but from a pre-existent population of different, related yeast clones. The presence of such a mosaic of phenotypically distinct natural yeast populations, perhaps, reflect an adaptation to specific microenvironment- having very important implications for the ecology and the biotechnological use of wild yeast strains. The correlation between different relevant phenotypes and specific mtDNA polymorphism could facilitate the isolation and characterization of wild yeast population with the desired metabolic and genetic traits.

Mozina *et al.* (1997) developed a quick method for differentiating *Saccharomyces* sensu stricto and *Torulaspora* yeasts isolates on the basis of their PCR ribotyping. In the study 18S rDNA with ITS1and 25S rDNA PCR product of 28 *Saccharomyces* sensu stricto and *Torulaspora* yeasts and their anamorph forms were digested with *Hae* III, *Msp* I, *Hin* fI and *Cfo* I. Using combination of 2 restriction enzymes, specific ribotyping pattern of 6 species were found. PCR ribotyping offers a convenient tool for quick identification of yeast isolates. However, on the basis of rDNA fragments and/or restriction enzymes chosen for the analysis, it does not seem possible to predict the degree of variability in the rDNA repeats in different taxonomic groups of yeasts.

Guilliamon *et al.* (1998) applied the molecular genetic tools for analyzing the diversity of wine yeast species during spontaneous fermentation. They identified 33 wine yeast species and strains based on the restriction patterns generated from the region spanning the internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA gene. It was observed that the PCR products of this rDNA region showed a high length variation for the different species. The size of the PCR products and the restriction analysis with three restriction endonucleases (*Hin* fI, *Cfo* I and *Hae* III) yielded a specific restriction pattern for each species with very few exceptions. When the rRNA gene region was digested with *Hin* fI, each of the species exhibited a specific pattern, with the exception of the species that have both perfect and imperfect stages including *Saccharomyces* sibling species, which showed the same pattern. Although, same results were observed with the *Cfo* I enzyme, it differed from the earlier by showing the same restriction pattern in the case of *Rhodotorula glutinis* and *R mucilaginosa*. Restriction digestion with *Hae* III showed almost similar results but an important difference to the other two enzymes was that the type strains of *S cerevisiae* exhibited a pattern different from that showed by S. *bayanus* and *S. pastorianus*, which also showed the same pattern. They observed another important characteristics of the enzyme that it did not recognize any restriction site in the ribosomal region of most of the *Candida* species and of *Hanseniaspora uvarum* and its imperfect form *Kloeckera apiculata*.

Sabate *et al.* (1998) conducted an ecological study of *S. cerevisiae* strains in spontaneous alcoholic fermentation in the same winery over 2 consecutive years (1994 and 1995). Upon analysis of 200 *Saccharomyces* colonies in both years, 60 and 66, different mtDNA restriction patterns were found, respectively. It was also observed that 21 patterns were common for both fermentations. *S. cerevisiae* strains were differentiated by restriction with *Hinf*I restriction enzyme. It was also observed that *Rsa* I restriction enzyme produced species specific patterns which allowed the identification of all isolates as *S. cerevisiae.* A sequential substitution of *S. cerevisiae strains* was observed throughout the fermentation. Population dynamics of *S. cerevisiae* strain was very similar and the same strains were predominant in both the years with different climatic conditions implied that these dynamics were not influenced by the climatic conditions.

Torriani *et al.* (1999) investigated the genotypic and phenotypic diversity of *Saccharomyces* sensu stricto strains isolated from 'amerone' wine at the end of the first fermentation and before ageing. Adding 3 different primers singly, RAPD amplifications were carried out. RAPD products were analyzed by electrophoresis on 1.4 % (w/v) agarose. TAE gel stained with ethidium bromide was used for the purpose. Restriction analysis of mtDNA was used to detect genetic variability among isolates. *Rsa*I and *Hin*f1 endonucleases differentiated *S cerevisiae* and *S. bayanus* species and showed to be discriminated at inter-specific level. The mtDNA restriction patterns revealed the presence of different profiles of *S. cerevisiae* and *S. bayanus.* Both RAPD-PCR and mtDNA restriction analysis of *Saccharomyces* strains from amerone wine revealed different genotypic patterns even in the same winery. Moreover the species were differently distributed in the wineries.

Erlorio *et al.* (1999) investigated the sequence variability of the ITS regions between two *Triticum* species by the PCR with ITS1/ITS4 primers and found a single fragment of 650 bp. They observed a high degree of conservation in this region, as there was no DNA length polymorphism between these 2 species. In the case of *S. cerevisiae* the same PCR showed amplification band of

800bp. Amplification with ITS1/ITS2 universal primers gave a characteristic band of about 300 bp for *T. aestivum* and 450 bp for *S. cerevisiae*. This study revealed that the amplification carried out with primers (ITS1/ITS2) and (ITS1/ITS4) obtained from ITS region of the fungal genome enables to distinguish between genomic DNA from *Triticum* sp and *Saccharomyces species.* Ribosomal region of ITS1 of *S. cerevisiae* was chosen as the target sequence and the primer positions were empirically designed by comparison with sequence data from the ITS1 ribosomal sequence of *S. cerevisiae* and *Triticum* sp. in order to obtain a 300 bp fragment. This primer, when used in conventional PCR experiment, specifically amplified the *S. cerevisiae* template giving a single fragment of 300 bp as expected. But, the samples of the *T. aestivum* amplified with these primers did not show any amplification. This approach using SC1/SC2 specific primers provided a rapid and sensitive method for confirming the presence of the yeast *S. cerevisiae* in bakery product.

Fernandez *et al.* (1999) studied characteristic of non-*Saccharomyces* yeast isolated from spontaneous fermentation by physiological and molecular methods employing PCR-RFLP. The region between 18S and 28S rRNA genes was amplified using specific internal transcribed spacers ITS1 and ITS4 primers. The 47 non-*Saccharomyces* isolates produced 13 different genotypic profiles showing that PCR/RFLP can be more discriminating. The information supplied by the two methodologies was very similar. PCR/RFLP could be used to correct the erroneous identification by phenotypic and in some cases to achieve intra-species differentiation.

Granchi *et al.* (1999) after performing ITS-PCR with ITS1 and ITS4 primers and restriction digestion of the PCR product with five endonucleases viz. *cfo* I, *Dra* I, *Eco* RI, *Hae* III and *Hin* fI showed at least one species-specific restriction pattern for each species with the exception of four *Saccharomyces* sensu stricto strains and the anamorphic and teleomorphic forms. This method was said to be rapid, easy and highly reproducible. This method was also found to be time saving as it suppress the amplicon electrophoresis step and by

performing a parallel amplicon digestion *Dra* I, and *Hae* III directly in the PCR buffer.

Gutierrez *et al.* (1999) studied the ecology of spontaneous fermentation in one winery in La Rioja, Spain during five consecutive years using mtDNA restriction analysis. The number of different strains detected for each vintage and the appearance frequency varied from one year to another. Although, a small number of strains were observed in consecutive years, only 1 strain was present in all 5 years studied.

Arguelles (2000) reviewed the physiological role of trehalose in bacteria and yeasts. It was opined that in yeast and filamentous fungi large amount of trehalose were stored both as reserved carbohydrates and protector against stress challenges to cells. But, in contrast to the bacterial cells yeast cells are largely unable to grow on trehalose as carbon source.

Attfield and Kletsas (2000) analyzed 4 strains of bakers' yeasts for their hyper-osmotic responses. It was observed that 2 strains produced strong fermentative activity in medium with low osmotic stress while the other 2 strains more similar fermentative activities even with higher osmotic stress. It was also observed that the strains that were inhibited by higher sucrose concentration were unable to produce significant amount of glycerol under hyper-osmotic conditions while the yeasts strains that were not inhibited significantly by higher sucrose concentration produced a considerable amount of glycerol. The strains that produced significant amount of glycerol also exhibited efficient expression of the glycerol-3-phosphate dehydrogenase gene *GPD1*. These novel data on the molecular responses of industrially relevant strains of bakers' yeasts are prerequisites for designing strategies for improving the performance of industrial yeasts in high sugar concentration media.

Comi *et al.* (2000) tested a numbers of strains for their genetic variability in mtDNA by using restriction enzyme analysis with *Hin* fI and *Rsa* I. Eight profiles on the 70 *Saccharomyces cerevisiae* strains were obtained using *Hin* fI restriction enzyme, while 9 restriction patterns were obtained with *Rsa* I endonuclease. It was observed from this study that strains isolated from the

same grape had different profiles and were grouped in to clusters, whereas strains isolated from different grapes showed the same mtDNA restriction fragments and formed specific groups. Moreover, a very weak relatedness is observed between the degree of genetic relatedness and the chemical results.

Echeverrigaray *et al.* (2000) employed RAPD for characterizing and examining the relationship among the commercial winery yeast strains. Random DNA amplification was performed using eight selected primers. This study found 7 to 15 bands of amplified DNA with sizes ranging from 300 to 2500 bp. Strains that appear identical in the amplification with one primer could be separated in reaction with a different primer. The combined information from several reactions of the species leads to distinguish the strains from each other.

Ezeronye and Okerentugba (2000) studied the genetic and physiological variants of yeast isolates from palm wine and further select suitable strains for genetic improvements. They also studied the ethanol concentration on the laboratory scale and ethanol tolerance of the isolated yeast strains.

Fernandez-Espinar *et al.* (2000) applied PCR-RFLP analysis of the region spanning ITS1-ITS2 and the adjoining 5.8S rRNA genes for the identification of newly accepted species of the *Saccharomyces* sensu lato complex, viz, *S. kunashirensis, S. martiniae, S. rosinii, S. spencerorum* and *S. transvaalensis* as well as *Saccharomyces* flor yeasts responsible for aging sherry wine. Amplification of DNA and restriction digestion of the amplified DNA with *Alu* I, *Cfo* I, *Dde* I, *Hae* III, *Hin* dIII, *Hin* fI, *Hpa* II, *Nde* II and *Scr* FI restriction endonucleases showed a diversity in the length of the amplified region or in the restriction pattern of the *Saccharomyces* sensu lato complex, which allowed unequivocal identification of these species. But, 2 species, *S. bayanus* and *S. pastorianus* of the *Saccharomyces* sensu lato complex could not be differentiated based on their restriction pattern probably due to the hybrid nature of the *S. pastorianus*. The flor yeast strain exhibited different restriction patterns from those of the typical *Saccharomyces cerevisiae.* This might be due to the presence of a 24 bp deletion fixed in the ITS region of the flor yeasts. It

was concluded that 5.8S-ITS region analysis offers a convenient tool for the fast identification of yeast species.

Gomes *et al.* (2000) after a comparative study of four methods, differential culture media, RAPD, SDS-PAGE and CHEF remarked that karyotype analysis is difficult, expensive and time consuming, but of limited efficiency and of the lowest reproducibility for the differentiation of strains within a species. SDS-PAGE although was efficient, rapid and inexpensive method; large number of bands and small distances between them made its analysis a difficult task requiring technical expertise. Considering all these factors, the RAPD analysis was the most appropriate method of yeast identification. The comparative cost effectiveness, high number of analyzable and polymorphic variables and the absence of environmental effects made it the most efficient for yeast identification.

Jespersen *et al.* (2000) studied the phenotypic and genotypic diversity of *Saccharomyces* contaminants isolated from *lager* breweries and their phylogenetic relationship with brewing yeasts. In the study, *Saccharomyces* sensu stricto complex yeasts were not easily identified phenotypically as they were found to be very close to each other. Ability to grow at 37^0 C was used to differentiate *S. cerevisiae* from *S. pastorianus* although in some cases it was questionable. Growth in vitamin free medium was used for the identification of *S. bayanus.* But, the isolate identified as *S. cerevisiae* by ITS-PCR RFLP was also able to grow in the vitamin free medium. Amplification of ITS region followed by digestion with *Hae* III was found to be useful for the differentiation between *S. cerevisiae* and *S. bayanus* along with identification of some inter-species hybrids. But, the restriction profiles of the inter-species hybrids were not identical to *S. pastorianus* (*S. cerevisiae* X *S. bayanus*) type strains. Chromosome length polymorphism was useful to group majority of the isolates in to either *S. cerevisiae* or *S. pastorianus/bayanus.* Majority of the *Saccharomyces* brewing contaminants possessed more than two *MAL* loci which lead to the conclusion that these were closely related to brewing and bakery yeast but differed from wine and fermented indigenous African food, which

possessed only two *MAL* loci. The study showed that the current taxonomy within the *Saccharomyces* sensu stricto complex was not fully explored.

Kurzai *et al.* (2000) evaluated the discriminatory potential of four phenotype-based methods, biochemical assimilation patterns and a novel PCR test against sequencing rRNA coding genes. This study achieved an unequivocal classification of 133 clinical yeast isolates by sequencing the V3 variable region of the large ribosomal subunit-coding gene. Differentiation of the isolates were done by using a discriminating PCR based on the analysis of two structural genes *C. albicans PHRI* and its structural homologue *cdPHRI.* After comparison with the previous two methods, this corresponded 100% to the rDNA sequence information.

Pretorius (2000) observed that the genetic diversity among the strains of *Saccharomyces* sensu stricto was a matter of interest. Most strains of *Saccharomyces* sensu stricto complex or other *Saccharomyces* species showed high levels of polymorphism. Strains of a particular species might show a variety of characteristics that are strain specific.

Tornai-Lehoezki and Dlauchy (2000) differentiated brewing yeast strains using different molecular techniques. In this study RFLP patterns analysis of 18S rRNA coding DNA was done in order to group ale and lager strains. Traditionally ale and lager (top and bottom fermenting) yeast strains were delimitated based on differences in morphology, physiology and fermentation technology which, however, were not sufficiently constant and vary to a greater or lesser extent. Rotating field gel electrophoresis (RFE) of larger than1300 kb size bands proved to be sufficient for separating ale and lager brewing yeast strains. Moreover from this study, it was observed that RAPD analysis using properly designed primers is a suitable method to distinguish not only the type and synonym type strains of *S. cerevisiae* and *S. pastorianus* but also ale and lager fermenting strains from each other. But, 18S rRNA coding DNA restriction fragment length polymorphisms (RFLP) did not fulfil the earlier promise since all studied brewing yeast strains showed the same RFLP pattern

as the type strain and synonym type strain of *S. cerevisiae*, which they were clearly different from the type strain and synonym of *S. pastorianus.*

Abdelgadir *et al.* (2001), while characterized dominant micro flora of Sudanese traditionally fermented milk *rob,* used ITS-PCR profile along with morphological, physiological techniques. In this study after analysis of ITS-PCR profile 9 groups of lactic acid bacteria and 2 groups of yeasts were found.

Molecular biology techniques used for the identification of yeasts are the modern approaches and include DNA-DNA hybridization (Vaughan-Martini, 1989), identification of DNA sequence polymorphisms (Seehaus *et al.*, 1985), mitochondrial DNA (mtDNA) restriction analysis (Lee *et al.*, 1985; Querol *et al.*, 1992; Guillamon *et al.*, 1994; Versavaud *et al.*, 1995; Comi *et al.*, 2000), comparison of chromosomal DNA profiles (Shutz and Garner, 1994; Naumov *et al.*, 2001), ribosomal DNA sequencing (Huffman *et al.*, 1992; Molina *et al.*, 1992), analysis of random amplified polymorphic DNA by RAPD-PCR (Echeverrigaray *etal.*, 2000) as well as polymorphic chain reaction restriction fragment length polymorphism (PCR-RFLP) (Naumova *et al.*, 2003).

Fernandez-Espinar *et al.* (2001) studied 45 different commercial *Saccharomyces* wine yeast strains by combining restriction analysis of *Hin* fl endonuclease, electrophoretic karyotyping and PCR amplification of δ sequences. It was observed that maximum discriminatory power was achieved when results of these three techniques were combined and analyzed. Mitochondrial DNA restriction analysis using *Hin* fl and *Dde* I endonucleases showed 17 strains as unique out of 45 commercial *S. cerevisiae* strains. The remaining 28 strains could only be identified in to 8 groups. These strains were then additionally analyzed by electrophoretic karyotyping and PCR amplification of δ sequences. This resulted in identification of 13 individual strains from among 28 strains. Thus strain might have the same restriction pattern, chromosomal profile and PCR products. This study recommended that PCR is a convenient method for rapid identification of yeast strains but when results from different companies need to be compared, mtDNA restriction analysis is recommended.

Guera *et al.* (2001) studied the genetic diversity of *S. cerevisiae* strains during the 24 h fermentation cycle for the production of the artisanal Brazilian *cachaca* (*aguardente*) by employing PFGE and RAPD-PCR with primers E11 and M13. The study found a high degree of genetic polymorphism among the strains within a 24 h fermentation cycle. All the strains of a distillery showed distinct electrophoretic profiles that might be due to recombination, either reciprocal or non-reciprocal between homologous chromosomes of different sizes. (Nadal *et al.,* 1991). It was also opined that the molecular diversity found in *S. cerevisiae* strain might be due to the unique characteristics of *cachaca* fermentation process such as short fermentation cycle (24-36 h), higher environmental temperature (25-40^{0}C), higher alcohol percentage (7%) etc which exerts a strong selective pressure over the strains. Moreover, differences among *S. cerevisiae* strains observed in RAPD-PCR might be due to the existence of a large number of individual genotypes within the species probably resulting from point mutation or small deletions /insertions.

Hansen and Jakobsen (2001) studied the technological and taxonomical characteristics of *Saccharomyces cerevisiae* associated with blue veined cheese. The ITS-PCR profiles with ITS1 (5′-TCC GTA GGT GAA CCT GAG G 3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC 3′) primers showed a high degree of similarity between the examined isolates. These strains were tested for the assimilation of acids related to cheese for growth in the presence of different NaCl concentrations and then enzymatic activity. Phenotypically they were identified as *Saccharomyces* sensu stricto complex. Genotypically all strains were similar although chromosome polymorphisms were observed. They observed that all the strains were similar in assimilation and fermentation of residual sugars and organic acids naturally formed in cheese. These had similar lypolytic activity although they differ in the tolerance of different NaCl concentration.

Torija *et al.* (2001) analyzed the grape related indigenous yeast population dynamics in two Spanish wine producing regions for three consecutive years. This study was able to identify the non-*Saccharomyces* yeast

species by RFLPs of the rDNA and *Saccharomyces* yeast species by RFLPs of mtDNA. They also, like some other workers, observed that the non-*Saccharomyces* yeast species were involved during the early stages of fermentation and whilst *Saccharomyces* dominated towards the end of the alcoholic fermentation.

Cadez *et al.* (2002) employed three molecular methods, RAPD-PCR analysis, electrophoretic PFGE karyotyping and RFLP of the PCR amplified ITS regions-ITS1, ITS2 and the intervening 5.8S rDNA for determining inter and intra-specific relationship of 74 yeast strains isolated from sources and/or geographically distinct regions. RAPAD –PCR was done using OPA-13 (5/-*CAG CAC CCA C*-3/), *(ATG)* $_5$, *(GTG)* $_5$ and *M13* (5/-*GAG GGT GGC GGT* TCT-3/) primers and the profiles of each strain were combined in a composite fingerprint using Gel Compar 3.1. Similarities between combined fingerprints were clustered using the Pearson product-moment correlation coefficient (r). Cluster analysis of the pair wise values was generated using UPGMA algorithms. PCR-RFLP analysis of ITS region with two restriction enzymes allowed discrimination of all species studied. It was observed that the strains that were specific on the basis of high DNA homology were variable with regard to growth at 34^0 C and 37^0 C. As De Morais *et al.* (1992) suggested that variability in the ability to grow at higher temperature may be a consequence of adaptation to environment. The cluster analysis of the combined RAPD-PCR fingerprint revealed groups that agreed with those obtained by DNA–DNA homology studies. Each cluster represented a currently accepted species in the genus *Hanseniaspora* and one separated cluster of five strains represented a group of strains physiologically indistinguishable from *H. uvarum.* The RAPD-PCR analysis did not reflect phylogenetic relationship between the species, not even the relationship between the closest related species *H. vinae* and *H. osmophila* sharing 40% DNA–DNA homology. It was concluded that RAPD-PCR method was only useful for revealing the relationship among strains within the species of *Hanseniaspora* due to its high-resolution capacity. Moreover,

restriction analysis of rDNA is a reliable and rapid method for the identification of *Hanseniaspora-Kloeckera* isolates.

Mitterdorfer *et al.* (2002) used chromosomal karyotyping by PFGE, RAPD-PCR and ITS-RFLP and showed that *S boulardii* yeast share molecular characteristics clustering them within the species *Saccharomyces cerevisiae* and concluded that *S boulardii* could not be regarded as a distinct species.

Cavalieri *et al.* (2003) collected residue from inside one of the earliest wine jars of ancient Egypt, dated to 3000 B. C. recovered from tomb U-j of king Scorpion I. This residue was subjected to ribosomal DNA extraction, amplification and sequencing under condition designed to minimize the risk of contamination with DNA from modern organisms, in a laboratory and using equipment previously unexposed to modern *S. cerevisiae*. The extracted DNA was used for PCR amplification using primers ITS1 and ITS4, which amplify a region of *ca.* 840 bp across the genomic region between 18S and 28S ribosomal RNA genes that include ITS1, 5.8S ribosomal DNA and ITS2. The PCR products, with primer ITS1 and ITS4, of 540, 580 and 840 were purified and sequenced. A BLAST search of GenBank using 540 bp sequence revealed a close match with that of an ITS1-5.8S rRNA-ITS2 sequence of a fungal clone T2709 recovered from the cloning of the Iceman "Otzi" discovered near the Hauslabjoch formation in the Otztal Alps in 1991 and dated to 3350-3300 B. C. The 840 bp fragment corresponds in size to the region encompassing *S. cerevisiae* chromosome 12 coordinates. Again BLAT search of GenBank revealed that 850 bp sequences most closely matches the sequence from modern *S. cerevisiae.* The results indicate that the organism in the residue is nothing but *S. cerevisiae* and was probably responsible for the wine fermentation by at least 3150 B.C.

Libkind *et al.* (2003) characterized 64 carotenogenic yeast strains using both physiological and molecular methods. Molecular characterization of the isolates was based on the mini/micro satellite primed PCR techniques (MSP-PCR) employing the $(GTC)_5$ and M13 primers. The sequence analysis of the D1/D2 domains of the 26 rDNA was employed to confirm identification and in

the characterization of the unidentified MSP-PCR groups. Based on the findings of this study, it was recommended that the MSP-PCR techniques proved to be rapid, low cost and reliable that allowed the accurate identification and characterization of *ca.* 90% of yeast isolates studied.

Naumov *et al.* (2003) genetically analyzed nine Far-East Asian *Saccharomyces* isolates based on the fact that six sibling species of *Saccharomyces* sensu stricto complex differ in the sequence of the ITS1 and ITS2 spacers and differentiated on the basis of analysis of the restriction fragment length of the region. The amplification of 5.8S ITS fragment of seven isolates and six test cultures showed that the PCR product had the same size of 80 bp. The study placeed those species having four different *Hae* III restriction fragments (320, 230, 170 and 130 kb) and two different *Hpa* II restriction fragment (730 and 120 kb) as *Saccharomyces cerevisiae.* The isolates with same *Hae*III restriction fragments but not having any *Hpa* II restriction site were identified as *Saccharomyces paradoxus.* The third category of isolates with three different sized (490, 230 and 130 kb) restriction fragments with *Hae* III but same *Hpa* II restriction fragment with *S. cerevisiae* were identified as *S. bayanus.* The study concluded that species *S. bayanus* was likely to be associated with the exudates of a particular broad-leaved tree. Moreover, seasonal and climatic temperature factors could play an important role in the ecogeographical distribution of cryophilic *S. bayanus* yeast.

Naumova *et al.* (2003) explained the taxonomic position of 24 phenotypically different yeast strains used in the production of African sorghum beer with type culture of six *Saccharomyces* sensu stricto complex species and the hybrid taxon *S. pastorianus.* In this study the taxonomic position of the yeast species of African sorghum beer was elucidated and were differentiated by using the discriminating power of RFLP analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal gene. Out of 24 phenotypically different strains only 4 strains sporulated and were subjected to tetrad analysis. Again out of these 4 sporulating strains, 3 showed very low ascospore viability and one produced non-viable ascospores.

Rainieri *et al.* (2003) reviewed the molecular taxonomic approaches employing molecular methods for the classification of yeast. This included the determination of the mole percentage of guanine and cytosine (% G+C) and DNA reassociation studies that were the molecular methods used previously. Now-a-days, electrophoretic karyotyping (Johnston and Mortimer, 1986; Fernandez-Espinar *et al.*, 2001), RFLP (Guillianom *et al.*, 1994; Tornai-Lehoezki and Dlauchy, 2000; Torija *et al.*, 2001), PCR (de Barros Lopes *et al.*, 1998; Ryu *et al.*, 1998), AFLP (de Barros Lopes *et al.*, 1999; Azumi *et al.*, 2001) and sequencing (Valente *et al.*, 1999) are used to distinguish yeast at species and even at strain level.

Ribosomes are important phylogenetic indicators as their nucleotide sequence is highly conserved and also they are present in almost all cellular organisms displaying some function. Analysis of highly conserved region of rRNA such as rRNA small subunits may help in distinguishing distantly related yeast taxa (Kurtzman and Robnett, 1991) and analysis of the areas showing high rate of divergence such as ITS region may help in genotypically closely related species (Oda *et al.*, 1997 and Guilliamon *et al.* 1998) or even strains of the same species.

Almost all *Saccharomyces* sensu stricto species were successfully differentiated with the analysis of the ITS regions with the exception of *S. bayanus* and *S. pastorianus*. The identity of the *S. bayanus* and *S. pastorianus* is always a matter of concern. It is considered that *S. bayanus* includes wine and cider strains (Naumov *et al.*, 2001; Sipiezki *et al.*, 2001) along with grape must contaminant (Giudici *et al.*, 1999) whereas *S. pastorianus* includes lager brewing strains. Both the species are extremely heterogeneous which includes strains of hybrid and non-hybrid nature. But, they share some common properties such as ability to grow at low temperature and inability to grow at temperature higher than 37^0 C, active transport system etc. On the basis of physiological and molecular biological tests, *S. bayanus* was divided into two groups: i) the *S. bayanus* group which includes *S. bayanus* type strains CBS 380 and some others, and ii) the *S. uvarum* group which includes several species.

Both conventional as well molecular taxonomy distinguished *S. uvarum* from *S. bayanus*. But, the fact is that the *S. bayanus* type strain itself contains genetic materials from different yeasts. More recent studies showed that *S. bayanus* type strains CBS 380 contains genomic DNA from *S. uvarum* and from the same non- *Saccharomyces* parental strains as that of lager brewing strains.

Most *Saccharomyces* sensu stricto yeast species showed a high degree of polymorphism in their electrophoretic karyotyping as well as variability in the DNA sequence of some gene. The high level of variability among the strains of *Saccharomyces* sensu stricto complex being homothallic, were attributed to the fact that there might occur spontaneous mutation, genomic renewal and mitotic crossing over. Natural inter-specific hybrids were reported in several instances (Casaregola *et al.,* 2001; Pedersen, 1986; Hansen and Kielland-Brandt, 1995). Vaughan-Martini and Kurtzman (1985) on the basis of studies on the chromosome and genetic composition of lager brewing strains belonging to *S. carlsbergensis* (currently *S. pastorianus*) established it to be the hybridized product of *S. cerevisiae and S. bayanus.* Later it was confirmed that the non-*Saccharomyces* parental strain was *S. monacensis* currently a synonym of *S. pastorianus* (Hansen and Kielland-Brandt, 1995). Joubert *et al.* (2000) demonstrated that *S. monacensis* to be a hybrid and the non- *Saccharomyces* contributor could be a specific strain held at NRRl (NRRLY1551). Caseregola *et al.* (2001) confirmed these findings.

Succi *et al.* (2003) studied the presence of yeasts in Southern Italian sourdough from *Triticun aestivum* flour. This is a traditional bread making process using "sourdough" as the starter. Sourdough is produced- without the use of baker's yeast. Although this method requires longer time for fermentation, but people prefers it for its sensorial characteristics and longer shelf life. They studied the phenotypic characteristics of the yeast isolates and identified them by readymade identification kit. Isolated DNA was amplified in a mastercycler gradient using M13 (5′-GAGGGTGGCGGTTCT-3′) and RF2 (5′-CGGCCCCTG-3′). They separated the amplification products by electrophoresis

on 1.8 % (w/v) agarose gel in 0.5X TBE buffer. This study concluded RAPD to be a useful for the identification of yeasts.

van Kuelen *et al.* (2003) observed the yeast population associated with the spontaneous fermentation of three different wines of Lake-Erie region of the USA. They tested the samples for nitrogen and carbon assimilation and sugar fermentation. Later they analyzed the part of the large subunit rDNA segments and RFLP of amplified DNA and then compared the sequence of the D1/D2 region of the 26 rDNA from selected fermentation isolates and also of the three winery isolates with that of the known sequences of the same region of various yeast species present in the Gene bank. They concluded that genera were easily identified based on the sequence identity and suggested that the species was not identical to the one with the highest degree of identity score from the Gene-bank.

Vasddinyei and Deak (2003) identified yeast isolates collected from various Hungarian dairy products using the simplified identification system (SIM) and restriction fragment analysis of PCR amplified 18S rDNA with the neighboring ITS1 region. Considering the disadvantages of identifying yeast by conventional methods, such as, by determining morphological, physiological and biochemical characteristics that requires considerable experience and skill along with performance and evaluation of some 60 to 90 specified tests. In this study, the suitability of SIM procedure, ribotyping and RAPD analysis for the rapid characterization of the yeast isolates were described. SIM is mostly used for the identification of yeasts that occur frequently in food. They also tried intra-specific typing in order to differentiate strains according to their origin. This study confirmed the efficiency of restriction enzyme analysis of PCR amplified 18 rDNA with the neighboring ITS region (ITS-PCR, rybotyping) for rapid identification of yeast at species level. This study also successfully differentiated the strains of *Debariomyces hansinii* species through RAPD analysis. RAPD analysis with M13 primer produced bands differentiating between strains within the same species.

Brzezowski and Robak (2004) differentiated contaminating yeasts in brewery by PCR based techniques – RAPD and RFLP analysis. Keeping in view the selected disadvantages like long waiting period and inaccuracy of results of selective solid media, molecular biology techniques provide several alternative time saving and accurate methods. RFLP patterns of PCR amplified rRNA gene fragments and RAPD were successfully used for the identification of yeasts. The results obtained in RAPD analysis with primer 21 allowed the separation of the tested strains into 16 groups. The comparison of results from PCR-RFLP of rDNA and RAPD analysis indicated that both PCR based techniques should be used together. The number and type of restriction enzymes used in PCR-RFLP and sequence of primer used in RAPD analysis determine the level of discrimination of yeast strains.

Clemente-Jimenez (2004) isolated naturally occurring micro flora of "Valle-del Andarax" area of Spain during spontaneous fermentation of six grapes varieties and identified them using PCR-RFLP of the ITS region. The volatile profile of the wine from each variety of grape was also determined. Yeast isolates were identified according to ITS polymorphism. The ITS1 and ITS4 primers amplified the region between 18S rRNA and 28S rRNA. The isolates showed different product sizes ranging between 375-880 bp. The PCR products were digested with *Cfo* I, *Hae* III, and *Hin* fI restriction enzymes. From 7 different profiles, 5 were identified after comparing the molecular mass of the restriction products. The identified yeast species were *C. stellata, Issatchenkia terricola, Metschnikowia pulcherrima, Pichia fermentus* and *S. cerevisiae.* The sixth profile was identified as *H. uvarum* after an additional restriction analysis with *Dde* I endonuclease. The identity of the seventh profile was confirmed when the PCR products from seven groups were sequenced and compared with the available DNA sequence database. The seventh profile showed very high sequence homology to *I. orientalis.* From this study, it was concluded that to emulate natural fermentation, it was advisable to start with low fermentation yeast that appeared in the initial stage of fermentation such as *H. uvarum,* followed by a typical yeast of tumultuous or late fermentation such as *I.*

orientalis, M. pulcherrima or *P. fermentans* and finishing with a yeast that consumes all remaining sugar, such as *S. cerevisiae*.

Esteve-Zarzoso *et al.* (2004) studied the characteristics of yeast strains isolated from the velum formed on the surface of cherry wine during ageing. Employing mtDNA restriction analysis and karyotyping, chromosomal profiles were determined by CHEF technique using a DRIII apparatus with the standard chromosome of *S. cerivisiae* as marker. From this study, it was observed that the restriction analysis of 5.8S ITS region with *Cfo* I showed that these strains from cherry wine velum identified as *S. rouxii (Zygosaccharomyces rouxii)* contained the ITS1 deletion, typical of *S. cerevisiae* "flor" yeast. This ITS deletion was present in *S. cerevisiae var. beticus, cheresiensis and montuliensis.* Together with this, the restriction patterns obtained with *Hae* III and *Hin* fl suggested that these strains belong to a single group. But the strains *S. cerevisiae* var. *beticus, cheresiensis, montuliensis* were indistinguishable by sequence data and they were proposed to belong to a unique group rather than four different races of *S. cerevisiae.* But, this study could not establish the criteria based on the fermentation of seven carbon compounds to differentiate races within *S. cerevisiae*. Hence, the physiological analysis might not be a reliable method either for the identification and characterization of yeast or for the differentiation among the races of *S. cerevisiae* "flor" yeast.

Pulvirenti *et al.* (2004) identified the dominant yeast strains in homemade sourdough of Italy using PCR-RFLP of internal transcribed spacer region. These data were also confirmed by phenotypic tests. The strains belonging to *S. cerevisiae* were identified to strain level by analysis of the δ region. The study defined dominant species as the one whose number of vital cells is greater than the sum of the cells of any other species present. It was observed that the dominant species in homemade sourdough could differ from each other. This might be due to the selective pressure by the sourdough rebuilding method on the yeast species present that determine the dominance of one species over the other. The study reported that besides *S. cerevisiae* other

species such as *Candida humilis, C. milleri, I. orientalis, S. exigus* etc are also present in significant quantities in sourdough.

Sujaya *et al.* (2004) isolated, characterized and identified 51 yeast strains from fermented mash of Balinese rice wine "*brem*" fermented with five different type of starters, *ragi tape* on the basis of their internal transcribed spacers ITS region and 18S rDNA sequences. These yeast strains were preliminarily identified by sequencing their ITS regions using primers ITS5 and ITS4 which gave amplicon sizes of *ca.* 850, 600 and 500 bp. In some cases where ITS region could not be sequenced directly, 18S rDNA sequencing supported the identification. It was noted that due to the presence of a heterocopy of ITS in the rDNA, the ITS region could not be directly sequenced. This might probably be due to the heterothallic nature of the strains containing two locus systems in one locus gene. The phylogenetic analysis of the yeast isolates revealed that these are closely related to *S. cerevisiae, S. glabrata, Pichia anomala* and *Issatchenkia orientalis.* Out of these *S. cerevisiae* was the species capable of growing and producing the highest amount of ethanol in the fermentation and obviously responsible for the ethanol content of *brem.*

Torriani *et al.* (2004) developed a multiplex PCR assay for the specific identification and differentiation of *S. cerevisiae*, *S. bayanus*, and their hybrid *S. pastorianus*. This study demonstrated the use of multiplex PCR as a valuable tool for the rapid and accurate identification of members of the *Saccharomyces* sensu stricto complex. In this process, after an intensive analysis of sequence data that revealed nucleotide variability useful for the design of specific primer, 2 sets of specific primers YC1f-YC2r and YB1f-YB2r were designed by comparison of the YBRO33w region sequences available in current databases for *S. cerevisiae* and *S. bayanus*. The multiplex PCR protocol was optimized in relation to the annealing temperature and the time of primer addition. Addition of the primer YB1f-YB2r to the reaction mixture at the 10^{th} PCR cycle was found to be fundamental for the success of the assay. The most stringent condition of amplification used was the annealing temperature of 59^0 C instead of 57^0 C, which resulted in the absence of the fragment (515 bp) specific for *S.*

paradoxus. The study confirmed the use of multiplex PCR assay to attain rapid identification of the most common *Saccharomyces* sensu stricto yeasts involved in the industrial fermentation process.

Baleiras-Couto (2005) tried to use restriction enzyme digest of PCR amplified fragments of 26S rRNA as a routine methodology to monitor non-*Saccharomyces* yeast species diversity during red wine fermentation. These results were confirmed by sequencing the region D1/D2 of 26 rDNA. Amplification of the DNA fragment coding 26S rDNA was done using NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and LR6 (5′-CGC CAG TTC TGC TTA CC-3′). PCR amplified products were digested with restriction enzymes *Mse* I, *Hin* fII, *Apa* I, *Bge* II, *Bam* HI and *Eco R*I according to manufacturer's direction. Amplification results showed a fragment of either 1000 bp or 1160 bp for the 121 non-*Saccharomyces* strains. The level of discrimination in restriction enzyme digest differed on the restriction enzyme used. *Mse* I, *Apa* I and *Hin* fII were found to be most discriminant. Combining the results of the restriction patterns obtained with these three restriction enzymes, 19 different profiles were generated. The numbers of strains presenting each combine profile varied from one strain up to 49. Upon sequencing of the D1/D2 region 16 strains corresponding to 10 different restriction profiles showed high D1/D2 sequence similarity higher than 99.3% with the type strain of respectively 10 different species. It was concluded that application of restriction enzyme digest of amplified 26S rDNA is a reliable tool to differentiate wine yeast species as confirmed by the sequence data. No correlation between the experimental condition and the specific patterns could be obtained, as most of the yeast groups were present in various experimental conditions.

Capece *et al.* (2005) characterized wild apiculate yeast strains isolated from "Aglianico del Vulture" grape must using both molecular and physiological methods. They identified most of the species as *Hansiniaspora uvarum* while using restriction analyses of 18S rDNA as a tool to identify strains at species level. Evaluation of polymorphism of these species was done by using

RAPD and evaluation of technological traits. Thus, RAPD analyses clustered the wild *H. uvarum* strains in four main genetic groups and a very high phenotypic variability confirmed the genetic polymorphism. It was observed that the technological traits were strain dependent as they differed significantly among the strains. This study also revealed that the cluster analysis of the RAPD-PCR fingerprints showed five distinct groups characterized by a very low degree of similarity among the strains. The influence of the environment on the genetic patrimony of the population was observed, as there was a certain correlation between the groups and the strain isolation region.

Gil *et al.* (2005) constructed a *Saccharomycese* strain and studied the phenomenon of overproduction of exoglucanase encoded by the *EXGI* gene and also investigated the possible role of this enzyme in increasing wine aroma through the release of a glycoside precursor. It was observed that overproduction of the major exoglucanase of *Saccharomycese cerevisiae* lead to an increase in the aroma of wine.

Nisiotou and Gibson (2005) surveyed the typical Greek wine for the presence of culturable yeasts and identified the isolated yeast strains using the 5.8S–ITS rDNA sequence analysis. Culturable yeasts were isolated from 2 out of 5 filtered high alcohol content wines and from 5 oak barrel stored wines. After amplification of the 5.8S–ITS rDNA sequences all isolates were grouped in to 4 based on the size of the amplicons. Based on a stringent criterion of ≥99% sequence identity to other yeast strains available in the Genbank database, 16 isolates were identified as *Brettanomyces bruxellensis*, *S. cerevisiae* and *Rhodotorula pinicola.* It was evident from this study that the combination of ITS1 and ITS2 regions could identify most of the wine isolates at the species level. Intra-species variability was also detected among some isolates which might be due to nucleotide insertions or deletions in either ITS1 or ITS2 region. It was concluded that both ITS regions were more efficient than 5.8S in distinguishing between wine isolates at species level. As direct sequencing become more available, utility of 5.8S–ITS sequence analysis for quality control assay of market wine is suggested.

Pina *et al.* (2005) applied two PCR based approaches, RAPD and PCR fingerprinting to identify the source of contamination in a carbonated orange juice chain. The discriminatory RAPD with primer P24 and PCR fingerprinting with primer $(GTG)_5$ and $(GAC)_5$ was respectively 0.68, 0.73 and 0.66. By combining these 3 primers, 29 composite profiles were generated. Thus, the combination of PCR fingerprinting and RAPD assays showed to be very useful for the discrimination among the yeast species and to track the route of contamination.

Arroya-Lopez *et al.* (2006) used molecular identification methodology of restriction pattern analysis of both PCR amplified 5.8S rRNA genes and internal transcribed spacers ITS1 and ITS2 and restriction analysis of these PCR products with *Cfo* I, *Hae* III, *Hin* fI and *Scr* FI restriction enzymes. Restriction profiles generated with these restriction enzymes and also, in some case analyses of 26S rRNA gene allowed identification of yeasts isolated from table olive. These techniques allowed identification of three yeast species *Issatchenkia occidentalis, Geotrichum candidum and Hansiniaspora guilliermondii* that were not previously reported from fermented olive. Thus, the PCR amplification of 5.8S rRNA gene with ITS region along with 26S rRNA gene led to the conclusion that *Saccharomyces cerevisiae* and *Candida boidini* were the most frequent species in green seasoned olive and processed black olive, respectively.

Piskur *et al.* (2006) reported the origin of the genes encoding alcohol dehydrogenase and the process of ethanol accumulation in *Saccharomyces cerevisiae.* It was found that *Saccharomyces* yeasts owe their competitiveness to a combination of several properties including fast growth, efficient glucose repression, good ability to produce and consume ethanol and a tolerance for several environmental stresses such as high ethanol concentration and low oxygen level. These properties are unevenly distributed among the different modern yeast but are uniquely combined, specialized to perfection, regulated and coordinated through an efficient network in *Saccharomyces cerevisiae.*

Senses-Ergul *et al.* (2006) characterized some yeast strains isolated from food such as fermented and non-fermented home made carrot, homogenized

black carrot by traditional and molecular tests. With the help of traditional identification tests, 22 isolated yeast strains were grouped into 11 genera. Moreover, the pattern obtained by the restriction of the ITS-18S rRNA gene with *Msp* I and *Hae* III restriction endonuclease revealed that all the species differed in patterns whereas strains belonging to the same species gave similar digestion patterns by using *Msp* I. With the help of RAPD analysis performed within the same species, discrimination of *Metschnikowia pulcherrima* strain could be achieved. For the assimilation of 18S rDNA genes with the neighbouring ITS region NS 1 (5′-GTA GTC ATA TGC TG CTC-3′) and ITS 2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) primer pair was used. *Msp* I and *Hae* III restriction endonucleases were used separately to digest the amplification products of ITS-PCR. As it was observed that several important species could give similar restriction patterns, more restriction enzymes should be used to differentiate these species besides conventional differentiation techniques.

Walczak *et el.* (2007) isolated and identified fifteen wild yeast strains from two factories of a lager brewing company in Poland. To differentiate the isolates, randomly amplified polymorphic DNA (RAPD) with (GTG)5, (GAC)5, (GACA)4 microsatellite primers and M13 core sequence (50-GAG GGT GGC GGT TCT-30) were chosen. On the overall patterns, all isolates identified as *C. sake*, except one, were regrouped in one cluster. Collection strain *C. sake* CBS 617 was similar in 46% to the cluster with six isolates. The second reference strain *C. sake* CBS 159 and the Strain No. 1 were regrouped with other *Candida* species (collection strains) showing, respectively, only 20% and 42% of similarity to other *C. sake* strains.

Valles *et el.* (2008) isolated 350 yeast colonies from a cider cellar in Asturias (Spain) and were identified by rDNA ITS-RFLP restriction analysis. *Saccharomyces* spp. strains were characterized by mitochondrial DNA (mtDNA) restriction analysis. They identified and tested. fifty-four different *Saccharomyces* spp. to ascertain their capacity to carry out secondary fermentation of sparkling ciders.

Kopsahelis *et el.* (2009) tested the fermentation performance by both a cryotolerant and a thermophilic yeast (strain AXAZ-1) isolated from grapes in Greece in an extremely wide temperature range (3-40^{0C}). Sequence analysis of the 5.8S internal transcribed spacer and the D1/D2 ribosomal DNA (rDNA) regions assigned isolate to *Saccharomyces cerevisiae*. Yeast cells growing planktonically were capable of fermentation in a wide temperature spectrum (3^0C to 38^0C). Strain AXAZ-1 is very promising for the production of ethanol from low cost raw materials, as it was capable to perform fermentations of high ethanol concentration and productivities in both low and high temperatures.

Stringini *et al.* (2009) investigated the occurrence and diversity of yeast species during tapping and fermentation of palm wine from Cameroon. They used primers specific for δ sequences and minisatellites of genes encoding the cell wall. The molecular characterization of the *S. cerevisiae* isolates at the strain level showed a wide intraspecies biodiversity during the different steps of the tapping process. Indeed, 15 different biotypes were detected using a combination of three primer pairs, which were well distributed in all of the samples collected during the tapping process, indicating that a multistarter fermentation takes place in this particular natural, semi-continuous fermentation process.

Tofalo *et el.* (2009) performed the molecular identification of 78 isolates by a combination of PCR-RFLP of the 5.8S ITS rRNA region and sequencing of the D1/D2 domain of the 26S rRNA gene. In addition, the isolates were also differentiated by RAPD-PCR. Only a restricted number of osmotolerant yeast species, i.e. *Candida apicola*, *Candida zemplinina* and *Zygosaccharomyces bailii*, were found throughout all the fermentation process, while *Saccharomyces cerevisiae* prevailed after 15 days of fermentation.

Chapter 3

Materials and Methods

Rice beer is an age old drink of the tribal communities. Since time immemorial the ethnic communities produce the beer using the traditional know-how. It occupies an indispensable role in their socio-cultural life. Systematic study on the nutritional composition and microbiological aspects of the traditional rice beer has not been done in North East India except some sporadic studies in Sikkim and Nagaland. So, a pragmatic approach has been planned to study and gather knowledge and information about it that may help in future improvement of this inebriating social drink.

3.1 Collection of Starter Culture

Starter cultures used by different ethnic communities for making rice beer were collected from different places of Assam. Starter culture of Ahom community was collected from Namrup and Tinsukia; Bodo from Bagariguri and Udalguri; Karbi from Rajapathar and Bakulia, Karbi Anglong district; Mising from Dhakuakhana of Lakhimpur district and that of Tiwa (Lalung) from Barmanipur village of Morigaon district.

Starter cultures in the form of cakes (*pitha*) or balls (*guti*) were collected using sterile polythene bags. Each packet was labeled properly mentioning culture details and date of collection.

3.2 Analysis of Starter Culture

3.2.1 Determination of moisture Content

The moisture content of the cultures (*pitha* or *guti*) was determined by taking the initial weight just after the collection. Sample from each tribe was ground in to a fine powder and dried in an oven at 42° C till attaining a constant

weight. The difference in fresh and dry weight of culture was converted to per cent moisture content.

3.2.2 Determination of pH

One starter culture cake/ball (*pitha* or *guti*) from each of the samples was ground in to a fine powder. An amount of 5 g of the powder was added to 25 ml of neutralized deionized water and then vortexed for 5 minutes. The pH of the suspension was measured and recorded using a Cyberscan 510 digital pH meter. Triplicate readings were taken for accuracy.

3.2.3 Determination of ethanol content in traditional rice beer

Ethanol content of the rice beer collected from different tribal communities as well as the one produced in the laboratory using the isolated yeast cultures was determined by using a GC-Mass spectrometer (Varian).

3.3 Isolation of microflora

3.3.1 Isolation of yeast and fungi

A starter culture cake/ball from each of the samples was ground in to a fine powder in a sterile environment. An amount of 5 g powder from each sample was homogenized in 25 ml of sterile distilled water. The suspension was serially diluted to 10^{-5} with sterile water. An aliquot of 100 µl from each of the dilutions was cultured on *malt-yeast-glucose-peptone*-agar (YM agar; Himedia, M424) plate. The plates were incubated at 25°C for 48 h. Yeast colonies appeared in the culture plates were divided in to 18 groups on the basis of their phenotypic characters. Pure cultures were established from the distinct single colonies by the conventional streaking method. Density of yeast cells was determined with the help of a colony counter (Lapiz, Medica Instrument Mfg. Co., Mumbai) and expresses in terms of colony forming unit (cfu) per g of fresh sample. The isolated strains were maintained on YM slants at -4^{0}C and also in 20% glycerol at -20^{0}C.

In the case of isolation of fungi after the dilution-plating on potato dextrose agar (PDA; Himedia, M096), the plates were incubated at 30°C for 72 h. The population of fungal colonies were calculated with the help of a colony

counter (Lapiz, Medica Instrument Mfg. Co., Mumbai) and expressed in terms of cfu (colony forming unit) per g of fresh sample. Pure culture of fungi were maintained at PDA slants and kept at – 4^0C.

3.4 Morphological characterization

3.4.1 Calibration of the microscope

Calibration factor for one ocular division of Leica ATC 2000 microscope for 40X magnification was determined following the formula described below: One division on ocular micrometer (mm) is equal to known distance between 2 line on stage micrometer divided by the no of division coinciding on ocular micrometer. Calibration factor was found to be 2.5 μm.

3.4.2 Morphological characterization of yeast

Colony and cell (microscopic) characters of the yeast strains were studied by growing them on YM agar plates and YM broth, respectively, for 48-72 h. To induce sporulation of the yeast strains, Corn Meal Agar (CMA) (Himedia, M146), Yeast Morphology Agar (YMA) (Himedia, M138) and V8 Juice Agar (VJA) (Himedia; M638) media were used following the procedure of Barnett *et al.* (2000). Microscopical observations in respect of cell shape, dimension, budding, presence or absence of hyphae or pseudohyphae, reproductive structures were recorded.

3.4.3 Characterization of Fungi

The colony morphology of the fungal strains was examined after growing cultures on (PDA) for 72 h. Cellular observations were carried out under 10 x 40X magnification of a compound microscope following staining the specimen with lactophenol cotton blue. The fungal isolates were studied using the taxonomic keys described by Hesseltine (1991) and Lee and Fujio (1999). These were photographed with a manually operated camera (Cosina C1s) mounted over trinocular microscope (Leica ATC 2000).

The starch hydrolyzing activity of fungal strains was assessed for preliminary screening by growing them in 2% Starch Agar (SA) (Himedia,

M107S) plates for 48 h at 25°C. Plates were then flooded with 1:5 Lugol's iodine solution to check for the zone of hydrolysis.

3.5 Microscopic observation of vegetative cells

3.5.1 Non-filamentous vegetative cells

An aliquot of a freshly growing culture (1day-old) was inoculated in a 100 ml Erlenmeyer flask containing 30 ml of YM broth and then incubated at 25^0C with continuous shaking (250 rpm). After 24 h cell mass was concentrated by centrifuging 1.5 ml culture for 1 min at 8000 x *g*. Yeast cells were stained with methylene blue and observed under the microscope (Leica ATC 2000) at 10X x 40X and 10X x 100X magnification.

Observed cells were photographed with a (Cosina C1s) manually operated camera. Photonegatives were developed and printed. Photopositives were scanned with a HP Scanjet 3670 scanner.

3.5.2 Filamentous vegetative cells

A piece of sterile filter paper was placed in to a sterile Petri dish and a sterile glass rod was put over it. Two sterile microscopic slides were dipped separately in to warm potato dextrose agar (PDA) media kept in a wide and deep tube. The slides were drained and replaced over the glass rod support in the Petri dish. These slides were lightly inoculated with actively growing culture along the length and a sterile coverslip was put over a portion of the inoculated agar. The same were incubated at 25^0C. After wiping the agar from the back of each slide they were observed under microscope every alternate day to assess the presence of filamentous growth.

3.5.3 Microscopic observation for ballistoconidia

Yeast from a growing young culture (1day-old) was inoculated in a Petri dish containing Corn Meal Agar (CMA) medium in straight lines laid out at right angles. Each inoculated Petri dish was inverted over another Petri dish containing the same medium and a sterile microscopic slide placed over the media. The two Petri dishes were tied together all round the circumference and incubated at 20^0C for 3 weeks. The lower Petri dish was observed periodically to

see the presence of colony formed from the discharged ballistoconidia of the upper Petri dish. The slide was observed under the microscope to locate the presence of ballistoconidia.

3.5.4 Microscopic observation for ascospores

An actively growing young yeast culture (1day-old) was inoculated in to a Petri dish containing ascosporulation-medium V8 agar (Himedia) and (CMA) (Himedia, M146), and incubated at 25^0C for 3 days. Aliquots from this culture were observed up to 6 weeks under the microscope at 10X x 40X and 10X x 100X magnification for the presence of ascospores.

3.6 Biochemical characterization

3.6.1 Aerobic growth response in different carbon sources

Aerobic growth test or assimilation test was done to examine the ability of the yeast species to use organic compounds as the sole source of carbon. The test was done in test tubes of 180 mm x 16 mm size containing yeast nitrogen base and 50 mM of the test substrates. Nitrogen base with D-glucose was the positive control and without carbon source as negative control. The yeast strain, grown overnight on YM agar, was suspended in nitrogen base to get *ca.* $25x10^6$ cells ml^{-1}. An aliquot of 100 µl of this suspension was inoculated in a test tube containing 10 ml of YNB and test substrate and incubated at 25^0C in continuous shaking (250 rpm) condition. The growth of culture tube was measured spectrometrically at every alternate day up to 1 week and at weekly interval there after up to 4 weeks.

3.6.2 Aerobic growth response in different nitrogen sources

To examine the ability of the yeast strains to use nitrogen compounds for aerobic growth 5mM of the test substrate was taken in 10 ml of yeast carbon base. This was inoculated with an aliquot of 100 µl from a suspension of $25x10^6$ cells ml^{-1}. The pH of the media was adjusted to 6.5 as toxic nitrous acid may form at pH values below 6. The inoculated tubes were incubated at 25^0C in continuous shaking condition. The growth of the culture tube was measured

spectrometrically at every alternate day up to 1 week and at weekly interval thereafter.

3.6.3 Determination of the ability to use sugar anaerobically

In to a 50 ml-test tube 15 ml yeast extract medium at a concentration of 0.5% (w/v) was taken along with 50 mM of the test sugar. The test sugars were sterilized by autoclaving. One small test tube with the same medium was put in to the large one in inverted position and sterilized. No sugar was added to the negative control. Tubes were inoculated with 100 µl of actively growing yeast suspension of *ca.* 10^7 cells ml^{-1} and incubated at 25^0C for 7 days with regular shaking to sediment the yeast growing on the upper part of each tube and examined for the appearance of bubbles of gas. Emission of CO_2 replaces the media inside the inner tube and was regarded as the positive.

3.6.4 Vitamin requirement by yeasts

To determine the requirement of vitamins, yeast strains were cultivated initially in medium having no vitamins. Culture medium was taken in a series of test tubes, each with one vitamin and the yeast strains were tested for its growth. Vitamins added to each of the media are biotin, folic acid, *myo-inositolI, p*-aminobenzoic acid, nicotinic acid, calcium pantothenate, pyridoxine hydrochloride, riboflavin and thiamin hydrochloride.

3.6.5 Production of extracellular starch like compounds

One drop of Lugol's iodine solution was added to each yeast culture tube having the liquid culture medium, which showed positive results in the presence of a sugar. The positive result was highlighted with appearance of blue/purple/green colour.

Preparation of Lugol's iodine

Iodine 5 gm and 10 gm of potassium iodide were dissolved in 10 ml of distilled water and the volume was made up to 100 ml. The solution was diluted with distilled water in the ratio of 1:5 for subsequent use.

3.6.6 Growth at high osmotic pressure

To assess the osmotic tolerance, yeast strains were grown in yeast extract agar medium containing 50 and 60 % (w/v) D-glucose and 10 and 16 % (w/v)

Sodium chloride. Slants of yeast extract agar were prepared and were inoculated with actively growing (1day-old) yeast strains and incubated at 25^0C for growth. Observations were recorded up to 4 weeks of culture.

3.6.7 Growth in cycloheximide containing medium

Test tubes (180 mm x 16 mm size) containing yeast nitrogen and 50 mM of D-glucose were supplemented with filter sterilized cycloheximide solution to result the concentration of 0.1% and 0.01% (w/v). Yeast strains, grown overnight on YM agar medium was suspended in nitrogen base to get *ca.* $25x10^6$ cells ml^{-1}. An aliquot of 100 µl of this suspension was inoculated in a test tube and incubated at 25^0C in continuous shaking (250 rpm) condition. The growth of the yeast strains in cultures tube was measured spectrophotometrically at every alternate day up to 7 days and thereafter at weekly interval up to 4 weeks.

3.6.8 Test for urea hydrolysis by urease enzyme

The test was carried out to determine the activity of urease enzyme secreted by the yeast strains. Urea broth was dispensed in aliquot of 0.5 ml in to test tubes and was kept in deep freeze for 6 weeks. A loopful of cells from an actively growing 1day-old culture was suspended in to it and incubated at 37^0C. Tubes were observed at every half an hour interval up to 4 h for change to red colour. Appearance of red colour indicate the presence of urease activity.

3.6.9 Diazonium blue B (DBB) test

A positive response to DBB is indicative of the presence of basidiomycetous yeast. A 10 days-old culture of yeast strains on YM agar was held at 55^0C for 5 h and then flooded with DBB reagent. As and when the culture turned dark red within 2 min at room temperature, it was regarded as positive.

Preparation of Diazonium blue B (DBB) reagent

Diazonium blue B salt @ 1 mg/ml was added to cold 0.1 M tris-HCl buffer (pH 7) and mixed thoroughly. The reagent was kept ice cold and used within few min of the preparation before it discolours.

3.6.10 Composition of various media

i) YM (*malt-yeast-glucose-peptone*-agar)

Dried yeast extracts	3.0 g
Dried malt extracts	3.0 g
Mycological Peptone	5.0 g
D-glucose	10.0 g
Water	1000 ml
Unadjusted pH	~5.5

Agar 20% was added for solid medium

ii) CMA (corn meal agar)

Yellow corn meal 12.5 g was stirred with 300 ml of water at 60^0C for 1 h. Filtrate was diluted to 300 ml and 3.8 g agar was added to it. The medium was sterilized by autoclaving at 120^0C for 15 min.

iii) V-8 juice agar

This medium contains a mixture of juices from several vegetables and baker's yeast. In a vessel 14 g agar was dissolved in 340 ml of water. In another vessel 350 ml of V-8 (Campbell Camden, N. J. USA) juice was well mixed with 5 g of compressed yeast previously dispersed in 10 ml of distilled water. The content was heated for 10 min in steam and the pH was adjusted to 6.8 at 20^0C. The content of both vessel were mixed and autoclaved.

iv) PDA (potato dextrose agar)

Potatoes were washed thoroughly, peeled and finely grated. 100 g of these potato was soaked overnight in 300 ml of water in a refrigerator, filtered through muslin cloth. The filtrate was autoclaved for 1 h at 120^0C. Then 230 ml of the autoclaved extract was added to 730 ml of distilled water, 20 g of D-glucose and 20 g of agar. The medium was sterilized at 120^0C for 15 min.

v) Chemically defined media

a) (YMA) Yeast Morphology Agar

The medium contains all ingredients as listed below and agar 2% (w/v).

b) YNB (Yeast Nitrogen Base)

It contains all ingredients listed below: except 5 g $(NH_4)_2SO_4$ and no L-Asparagine or no D-glucose.

c) YCB (Yeast Carbon Base)

It contains ingredients as listed bellow; without major source of nitrogen, but with 1 mg L-histidine, 2 mg DL-methionine and 2 mg DL-tryptophan.

d) Vitamin free medium

The medium contains all ingredients of chemically defined media listed bellow, except 5 g $(NH_4)_2SO_4$, no L-Asparagine and no growth factors :

Table 3.1 List of ingredients of Chemically defined media

Source	**Compound**	**Quantity**
Nitrogen sources	$(NH_4)_2SO_4$	3.5 g
	L-Asparagine	1.5 g
Carbon source	D-glucose	10.0 g
Amino acids	L-Histidine	10.0 mg
	DL-Methionine	20.0 mg
	DL-Tryptophan	20.0 mg
Growth factors	Biotin	20.0 µg
	Folic acid	2.0 µg
	Myo-inositol	10.0 mg
	p-aminobenzoic acid	200.0 µg
	Ca pantothenate	2.0 mg
	Pyridoxine HCl	400.0 µg
	Riboflavin	200 µg
	Thiamin HCl	400.0 µg
	Nicotinic acid	400.0 µg
Trace element	H_3BO_3	500.0 µg
	$CuSO_4.5H_2O$	40.0 µg
	KI	100.0 µg
	$FeCl_3.6H_2O$	200.0 µg
	$MnSO_4.4H_2O$	400.0 µg
	$Na_2MoO_4.2H_2O$	200.0 µg
	$ZnSO_4.7H_2O$	400.0 µg

Salts	KH_2PO_4	850.0 mg
	K_2HPO4	150.0 mg
	$MgSO_4.7H_2O$	500.0 mg
	NaCl	100.0 mg
	$CaCl_2.6H2O$	100.0 mg

3.6.11 Identification of the yeast strains based on biochemical and morphological tests

Yeast strains were identified based on the identification keys as described by Barnett *et al.* (2000). The physiological and biochemical characterization of the yeast strains were also carried out following the procedures and keys described by Deak (1986), Teramoto *et al.* (2001) and Tamang and Sarkar (1995).

3.7 Molecular genetic characterization

3.7.1 Yeast genomic DNA isolation

Burst and Grab protocol described by Harju *et al.* (2004) with certain modification, was used for the isolation of genomic DNA of the yeast strains.

1. Actively growing (20-24 h) 5ml culture (YM broth) was taken in a microcentrifuge tube) appendorf and pelleted by centrifuged at 13,500 rpm at room temperature.
2. Added lysis buffer 300 µl (25 TritonX-100, 1% SDS, 100 mM NaCl, 10 mM tris-HCl (pH 8) and 1mM EDTA (pH 8) in to the pellet.
3. Submerged the tube in to a liquid nitrogen bath for 2 min and transfer to a hot water bath of 95 ^{0}C for 1 min. Repeat the cycle and vortexed for 30 sec.
4. Added 300 µl chloroform and vortexed for 2 min.
5. Centrifuged at room temperature for 10 min at 13500 rpm.
6. Transfer upper aqueous layer to another microcentrifuge tube containing 500 µl ice cold 100% ethanol.
7. Incubated at room temperature for 5 min.

8. Centrifuged at room temperature for 15 min at 13500 rpm. Removed supernatant.
9. Washed the pellet with 0.5 ml of 70% ethanol. Spin down by centrifuging at room temperature for 15 min at 13500 rpm. Removed supernatant.
10. Air-dry the pellet at room temperature.
11. Resuspended the pellet in 50 µl TE buffer.
12. Added 3 µl of RNAse A (10mg/ml) and incubated at 37^0C for 30 min and stored at 4^0 C.

3.7.2 DNA quantification and purity test by UV spectrophotometry

1. An aliquot of 5 µl of DNA sample was put in to a quartz cuvette and made up the volume to 3 ml.
2. The cuvette was placed in to the UV- spectrophotometer (Beckman DU® 530 Life Science UV/VIS spectrophotometer) and absorption was measured at 260 nm and at 280 nm along with the ratio of OD_{260} and OD_{280}.
3. DNA concentration was calculated by using the relationship of soluble standard DNA being 1 O.D. at 260 nm = 50 µg/ml.
4. The ratio between the absorption data at 260 nm and 280 nm was calculated to check the purity of the isolated DNA. A good DNA preparation exhibits a ratio value (ratio) in between 1.75 and 2.0.

3.7.3 Electrophoresis of yeast genomic DNA

1. Agarose 0.8% solution was prepared in a 250 ml conical flask by adding 49 ml of distilled water, 1ml of TAE buffer (1x) and 0.4g of agarose powder.
2. The solution was put in a microwave oven for 1-3 min until the agarose was fully dissolved. Prior to the solidification of the gel, 2 µl of ethidium bromide (final concentration of 0.5 µg ml^{-1}) was added to the solution.
3. The pre-cleaned gel tray was cello-taped in both ends and the comb was placed in the tray approximately 2.5cm from the end.

4. The gel solution was poured in to the tray to a depth of approximately 5 mm and then allowed to solidify for about 30 min at room temperature.
5. After the solidification of the gel, the comb and cello-tapes were removed. The gel was then put in the electrophoresis tank and then covered with about 250 ml of 1x TAE buffer or until the wells of the gels were submerged.
6. From each digestion mixture, DNA sample (5 µl) was taken and mixed with 5 µl of bromophenol blue. All samples were then loaded into wells of the gel.
7. Marker DNA (3 µl λ *Hind* III digested DNA) was loaded on the extreme left side of the gel or on both sides of the gel. The gel was electrophoresed at 150V for 1 h until the dye marker migrated to two third distance of the gel.
8. After electrophoresis, the DNA was visualized in a trans-illuminator and documented by taking photographs in a Gel Doc system (BIO RAD Gel Doc 1000).

3.7.4 Reagents, chemical and solution

10 M TrisHCl (pH 8.0)

Tris base 121.1 g was dissolved in 80 ml of distilled water (dH_2O). The pH was adjusted to 8.8 by adding concentrated HCl. The solution was allowed to cool to room temperature. The volume was adjusted to 1 *l*. The solution was dispensed in aliquots, sterilized by autoclaving and stored at room temperature.

5 M NaCl

NaCl 292.2 g was dissolved in 800 ml of water and adjusted the volume to 1 *l*. Dispensed into aliquots and sterilized by autoclaving.

10 % SDS

Electrophoresis grade SDS 10 g was dissolved in 900 ml of distilled water, heated to 68 ^{0}C for complete dissolving. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl. The volume was adjusted to 1 *l* and dispensed into aliquots.

10 % (v/v) TritonX-100

TritonX-100 10 ml was dissolved in 90 ml of distilled water. The solution was mixed and sterilized by Millipore filter paper, stored in dark condition at room temperature.

0.5 M EDTA

$Na_2EDTA.2H_2O$ 186.1 g was dissolved in 800 ml of distilled water and stirred vigorously. The pH was adjusted to 8.0 with 10 M NaOH. The volume was adjusted to 1 *l*, sterilized by autoclaving and stored at room temperature.

5X TBE buffer

Tris 5.4 g, boric acid 2.75 g and 0.5 M EDTA 2 ml (pH 8.8) were mixed in distilled water and make up he volume to 100 ml. The solution was stored at room temperature.

Bromophenol blue (loading dye)

(6 X loading dye, 20 ml)

Glycerol (autoclaved) 10 ml was added to 5 ml of 1 X TBE. To the solution, 50 mg of bromophenol blue was added, the volume adjusted to 20 ml and stored at 4^0C

Ethidium bromide (10 mg/ml)

Ethidium bromide 100 mg was dissolved in 10 ml sterile distilled water, mixed properly and then stored at 4^0C in darkness.

3.7.5 Random Amplified Polymorphic DNA (RAPD) analysis

RAPD-PCR was done according to the method described by Succi *et al.* (2003) and Torriani *et al.* (1999) with some modification. The PCR reaction mixture (25 µl) was prepared as follows:

1. 10 mM Tris-HCl (pH 8.3)
2. 200 µM DNTP mixture
3. 1.5 mM $MgCl_2$
4. 20 ng primer
5. 80 ng DNA sample for Primer M13, 20 ng for Primer 21 and Primer RF2
6. 2 U *Taq* DNA polymerage
7. 2.5 µl 10 X PCR buffer without $MgCl_2$.

Amplifications were performed in a mastercycler gradient, GeneAmp PCR System 9700 (Applied Biosystem) using the following primers and amplifications:

Primer M13 (5′-GAG GGT GGC GGT TCT- 3′)

Primer 21 (5′ GCT CGT CGC T-3′)

Initial denaturation at 94^0C for 2 min

40 cycles at 94^0C for 1 min

Annealing at 45^0C for 20 sec

Extension at 72^0C for 2 min

Final extension at 72^0C for 5 min

Amplification products were separated by gel electrophoresis on 1.4 % (w/v) agarose gel in 0.5 X TBE buffer.

RAPD-PCR a profile for both the primer were combined together for each of the strain and dendrogram was constructed that allowed classification of the yeast strains based on their similarity level.

3.7.6 Restriction Fragment Length Polymorphism (RFLP)

PCR-RFLP analysis of the internally transcribed ribosomal spacer region and the 5.8S rDNA was done according to the method described by Naumova *et al.* (2003) with some modifications. The PCR reaction mixture (50 µl) was prepared as follows:

1. 100 ηg genomic DNA
2. 0.2 mM of each DNTP
3. 50 pM of each primer ITS1 and ITS4
4. 5 µl 10 X PCR buffer with 15mM $MgCl_2$
5. 2.5 U *Taq* DNA polymerage

Amplifications were performed in a mastercycler gradient GeneAmp PCR System 9700 (Applied Biosystem) using the following primers and amplifications:

Primer ITS1 (5′-TCC GTA GGT GAA CCT GCG G- 3′)

Primer ITS4 (5′-CCT CCG CTT ATT GAT ATG C- 3′)

Initial denaturation at 94^0C for 3 min

30 cycles at 94^0C for 2 min

Annealing at 60^0C for 1 min

Extension at 72^0C for 1 min

Final extension at 72^0C for 5 min

Amplification products were separated by gel electrophoresis in 1.2% (w/v) agarose gel at 65 V in 0.5 X TBE buffer.

RFLP assay was carried out with *Hin*fI, *Hpa*II and *Hae*III restriction endonucleases purchased from Bangalore Gennie, India. Amplified DNA (10 µl) was digested with 2 units (U) restriction endonuclease at 37^0C overnight. The restriction digests were visualized by electrophoresis in 2.5 % (w/v) agarose gel at 65 V in 0.5 X TBE buffer for 2 h. Similarity among various profiles resulting from the digestion with eadonucleases and separation by gel electrophoresis was determined by Jaccard coefficient and also by clustering of strains which was accomplished by using Unweight Pair Group Method with arithmetic average (UPGMA).

Chapter 4

Results

The present study highlighted the morphological, physiological, biochemical as well as molecular characterization of the microflora (yeasts) present in the starter cultures used by the selected tribal communities of Assam for producing traditional rice beer. All these aspects were interesting and essential for the study of the traditional beer for their future improvement.

4.1 Analysis of Starter Culture

4.1.1 Collection

The starter cultures used by the selected tribal communities of the state were collected and the same are described in Table 4.1.

Table 4.1. Starter culture of traditional beer made by the selected tribes of Assam

Sl. No.	Tribe	Local name of culture	Place of collection
1	Ahom	*Pitha guti or Hajguti*	Namrup and Tinsukia
2	Bodo	*Emao*	Bagariguri and Udalguri
3	Karbi	*Thaap*	Rajapathar and Bakulia, Karbi Anglong
4	Mising	*Obpob*	Dhakuakhana, Lakhimpur
5	Tiwa (Lalung)	*Chooh*	Bormanipur, Morigaon

4.1.2 Determination of moisture content and pH of starter culture

Moisture content and pH of the starter cultures were determined and data are presented in Table 4.2

Table 4.2 Moisture content and pH of the different starter cultures

Sl. No.	Tribe	Moisture content % (w/w) ± SE	pH ± SE
1	Ahom	13.15±0.027	5.47±0.145
2	Bodo	12.32±0.943	5.25±0.095
3	Karbi	12.74±0.218	5.72±0.036
4	Mising	12.41±0.506	5.63±0.053
5	Tiwa (Lalung)	12.95±0.211	5.26±0.036

4.1.3 Determination of ethanol content

Ethanol content of the rice beer produced from the common rice (cv Aijong) by the respective community based villages and that of the laboratory scale production was estimated and the same is presented in Table 4. 3.

Table 4.3. Ethanol content of the rice beer produced by the tribal communities and that of the laboratory produce

Sl No	Tribe	Ethanol content (%v/v)	
		Sample collected from production site	Laboratory scale produce
1	Ahom	5.5	5.8
2	Bodo	4.6	4.9
3	Karbi	4.8	4.5
4	Mising	5.3	5.5
5	Tiwa (Lalung)	4.7	6.0

4.2 Isolation of microflora

4.2.1 Isolation of yeast and fungi and their population count

Yeast and fungal strains were isolated from the starter cultures, and their population density determined. Data obtained are presented in Table 4.4.

Table 4.4 Yeast and fungal load in the starter cultures of respective communities

Sl. No.	Tribe	Total yeast count (cfu) gm^{-1}	Total fungi count (cfu) gm^{-1}
1	Ahom	1.50×10^8	5.50×10^6
2	Bodo	1.12×10^8	5.25×10^6
3	Karbi	1.14×10^7	4.80×10^6
4	Mising	1.93×10^8	4.95×10^6
5	Tiwa (Lalung)	1.07×10^8	5.34×10^6

The pure cultures isolated from the starter cultures are described in Table 4.5. Altogether 18 isolates of yeasts and 10 isolates of fungi were obtained from 5 starter cultures.

Table 4.5 Yeast and fungi isolated from starter cultures from the selected tribal communities of Assam

Sl. No.	Tribe	Yeast strains	Fungi strains
1	Ahom	AY0, AY1 and AY2	AF1 and AF2
2	Bodo	BY0(A), BY0(B), BY0(C), BY1 and BY2	BF1 and BF2
3	Karbi	KY0, KY1 and KY2	KF1 and KF2
4	Mising	MY0, MY1 and MY2	MF1 and MF2
5	Tiwa (Lalung)	TY0, TY1, TY2 and TY3	TF1 and TF2

4.3 Morphological characterization

4.3.1 Morphological characters of yeast

Colony morphology of the yeast isolates was studied and data obtained are presented in Table 4.6.

4.3.2 Morphological characters of fungi

Fungal isolates were cultured in PDA medium at 30^0C for 72 h. Colony morphology of the isolates was studied and the same are presented in Table 4.7.

4.4 Biochemical test

4.4.1 Aerobic growth test with carbon sources

Yeast species could be distinguished by their ability to utilize certain organic compounds as major source of carbon like sugars, and organic acids. The tests, called as assimilation test, were carried out to assess the ability of the isolated yeast strains to utilize certain compounds for aerobic growth. Results of the tests are tabulated in Table 4.8.

4.4.2 Aerobic growth test with nitrogen sources

About 25% of all species of yeast utilize nitrate, which is usually a uniform feature of all strains within the species. Therefore, the feature was used as a valuable tool for identifying yeasts. Besides, some other nitrogen compounds like nitrite, ethylamine and L-lysine, were also tested for the identification of yeasts. Results are presented in Table 4.9.

4.4.3 Semi anaerobic fermentation

Yeast isolates were tested for their ability to ferment sugars including D-glucose semi-anaerobically. Results of such tests are presented in Table 4.10.

Table 4.6 Colony morphology of yeast isolates

Characteristics	Yeast strains					
	AY0	AY1	AY2	BY0(A)	BY0(B)	BY0(C)
Colonial morphology	Whitish mucoidal	Whitish to creamy mucoidal	Whitish mucoidal, slightly chalky	Whitish mucoidal	Creamy mucoidal	Whitish mucoidal, wrinkled
Cell shape	Spherical to oval	Spherical to oval	Spherical to oval	Oval	Oval to elliptical	Oval to cylindrical
Dimension	5.8 x 6.3 μm	6.0 x 6.7 μm	5.7 x 6.6 μm	5.3 x 7.5 μm	6.1 x 7.9 μm	5.8 x 7.6 μm
Budding	Terminal	Terminal	Terminal	Terminal	Terminal	Terminal
Pseudohyphae	Not observed	Not observed	Not observed	Simple	Simple	Simple
True hyphae	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Reproductive structure	Asci	Asci	Asci	Asci	Asci	Asci

Table 4.6 contd.

Characteristics	Yeast strains					
	BY1	BY2	KY0	KY1	KY2	MY0
Colonial morphology	Whitish sticky	Whitish to creamy butyrous	Whitish butyrous,	Whitish to cream	Whitish to creamy butyrous	Whitish mucoidal
Cell shape	Spherical to oval	Oval	Spherical to oval	Spherical to oval	Spherical to oval	Spherical to oval
Dimension	5.3 x 6.8 µm	5.0 x 6.7 µm	6.3 x 7.8 µm	6.5 x 7.4 µm	5.9 x 7.1 µm	6.2x 7.6 µm
Budding	Terminal	Terminal	Terminal	Terminal	Terminal	Terminal
Pseudohyphae	Yes	No	No	No	No	No
True hyphae	No	No	No	No	No	No
Reproductive structure	Asci	Asci	Asci	Asci	Asci	Not observed

Table 4.6 contd.

Characteristics	Yeast strains					
	MY1	MY2	TY0	TY1	TY2	TY3
Colonial morphology	Creamy butyrous	Whitish	Whitish to creamy	Whitish to cream	Ivory white	Whitish cream,
Cell shape	Spherical to oval	Spherical to oval	Oval, cylindrical	Spherical to oval	Spherical to oval	Spherical to oval
Dimension	5.8 x 7.4 µm	6.0 x 7.7 µm	6.7 x 7.6 µm	5.9 x 7.8 µm	6.0 x 7.7 µm	6.7 x 8.0 µm
Budding	Terminal	Terminal	Terminal	Terminal	Terminal	Terminal
Pseudohyphae	No	No	Simple	No	No	No
True hyphae	No	No	No	No	No	No
Reproductive structure	Asci	Asci	Asci	Asci	Asci	Asci

Table 4.7 Colony characters of fungal isolates

Characteristics	Fungal isolates				
	AF1	AF2	BF1	BF2	KF1
Colonial morphology	Tuft like whitish abundant mycelia	Whitish cottony appearance	Abundent aerial mycelia with blackish spore	Whitish cottony	Brownish with scare aerial mycelia
Reverse view of the plate	Ivory white to brownish	Whitish to greyish	Greyish	Whitish	Ivory white
Sporangiophores	Hyaline brownish	Brownish to yellowish	Brownish with local swealing	Hyaline brownish	Hyaline brownish
Sporangia	Blackish dehiscent 45 µm in dia	Blackish abortive	Blackish dehiscent 136 µm dia	Blackish	Blackish dehiscent
Columellae	Subglobose to ellipsoidal	Applanate	Ellipsoidal	Applanate	Ellipsoidal to globose
Sporangiospore	Ellipsoidal 4.7 x 5.3 µm	Irregular shaped	Ellipsoidal to angular 8.3 x 6.1 µm	Ellipsoidal	Ellipsoidal 4.6 x 5.1 µm
Chlamydospores	Not observed	Scare	Mostly intercalary	Abundant both terminal & intercalary	Scare, intercalary
Zygospores	Blackish with stellate	Not observed	Brownish	Not observed	Brownish
Growth at 37° C	+	+	+	+	+

Table 4.7 cont.

Characteristics	Fungal isolates				
	KF2	MF1	MF2	TF1	TF2
Colonial morphology	Abundent aerial mycelia with blackish spore	Abundent aerial mycelia with blackish spore	Profuse aerial mycelia with blackish spore	Brownish to yellowish with scare aerial mycelia	Whitish aerial mycelia
Reverse view of plate	Whitish to greyish	Greyish	Greyish	Yellowish	Ivory white
Sporangiophores	Hyaline to brownish	Brownish	Hyaline brownish	Hyaline brownish	Hyaline brownish
Sporangia	Blackish abortive132 µm in dia	Blackish dehiscent 145 µm dia	Blackish 142 µm in dia	Blackish dehiscent	Blackish dehiscent
Columellae	Ellipsoidal	Ellipsoidal	Applanate	Subglobose to ellipsoidal	Subglobose
Sporangiospore	Ellipsoidal 8.0 x 5.6 µm	Ellipsoidal 8.3 x 5.8 µm	Ellipsoidal 7.8 x 5.9 µm	Ellipsoidal 6.7 x 4.3 µm	Ellipsoidal 4.6 x 5.2 µm
Chlamydospores	Scare	Scare	Iintercalary	Very scare, intercalary	Not observed
Zygospores	Brownish with projection	Brownish	Brownish	Blackish with stellate projection	Blackish
Growth at 37° C	+	+	+	+	+

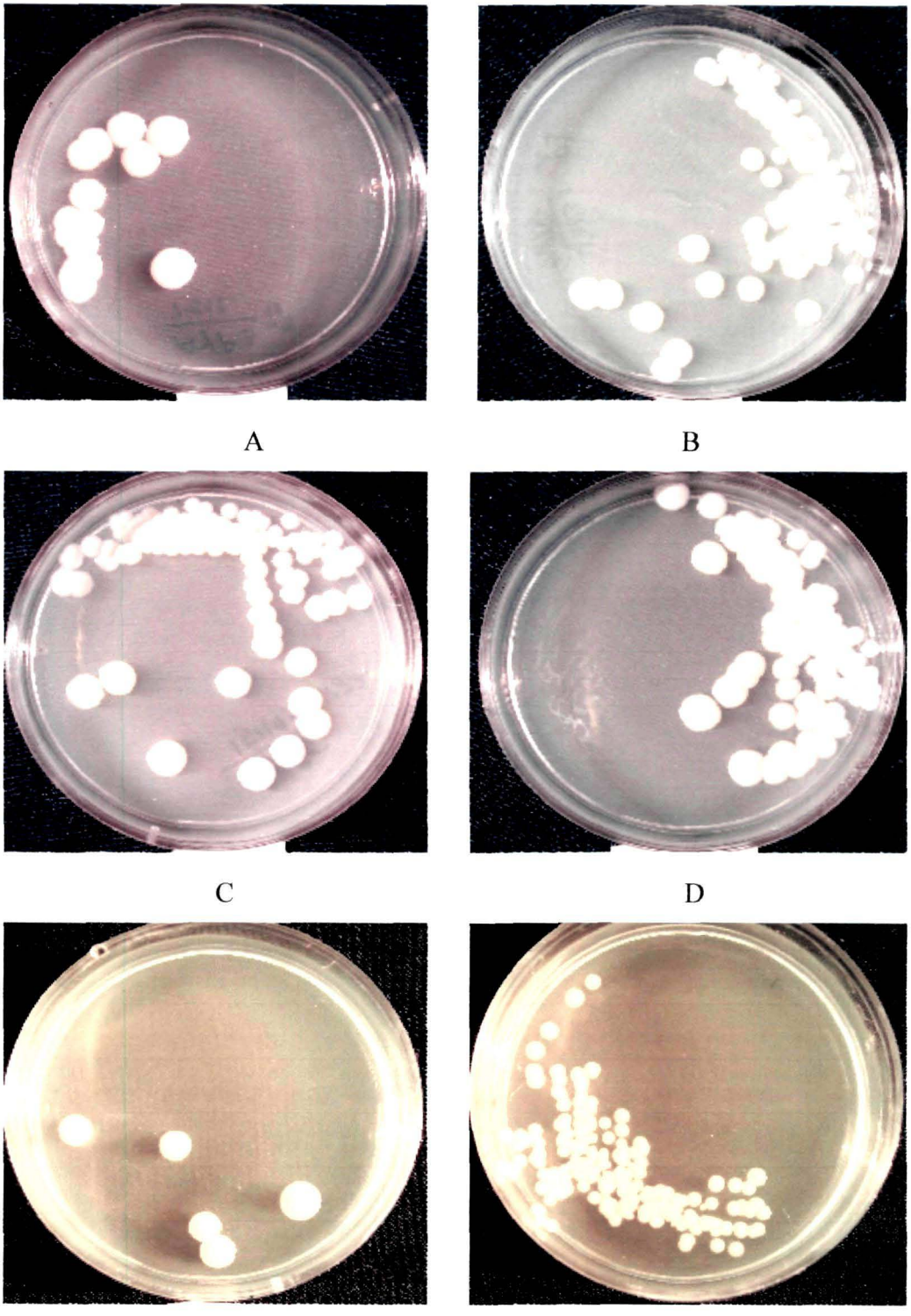


Plate 4.1 Yeast colony
A- AY0; B- AY1; C- AY2; D- BY2; E- KY0; F- KY1

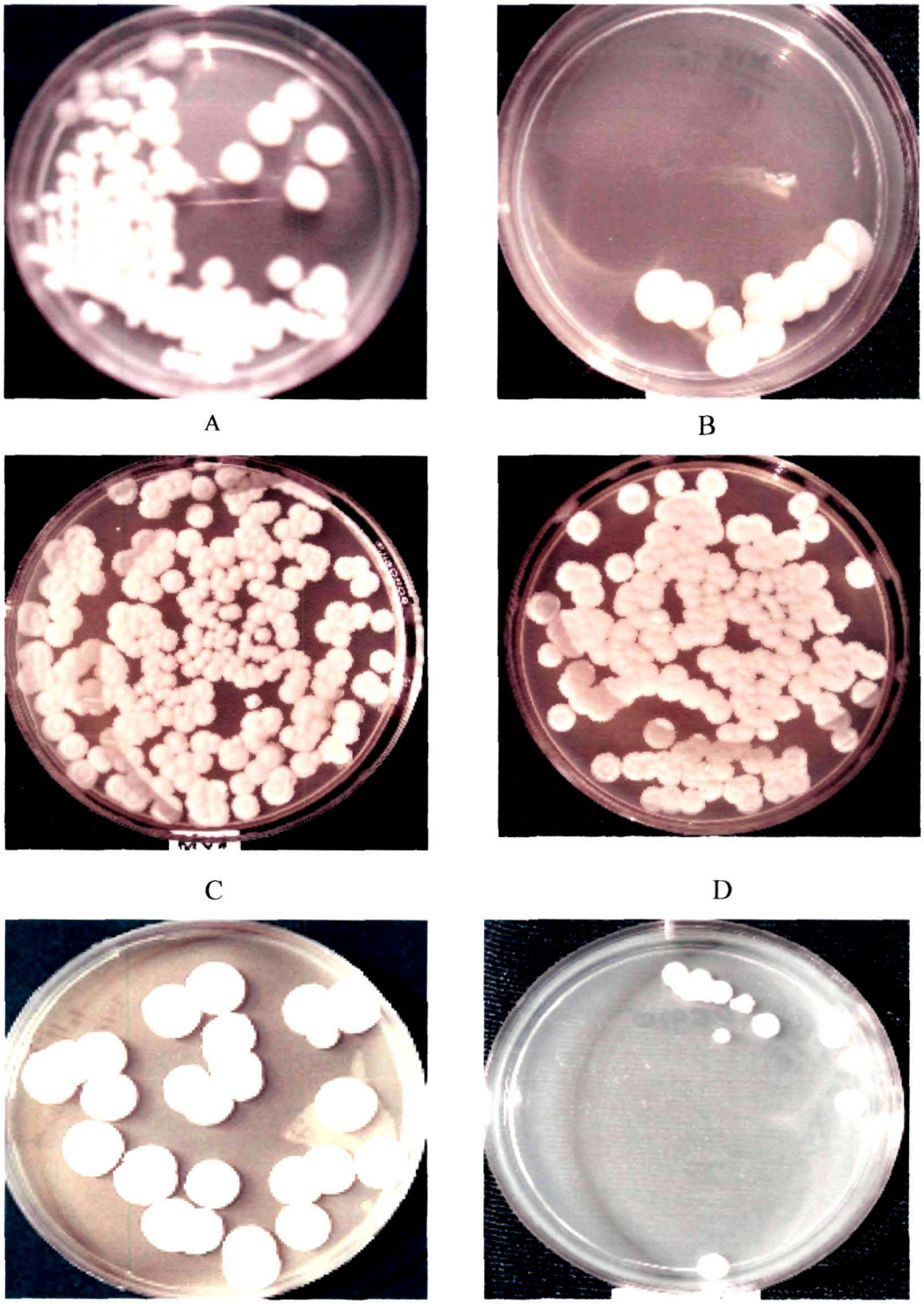
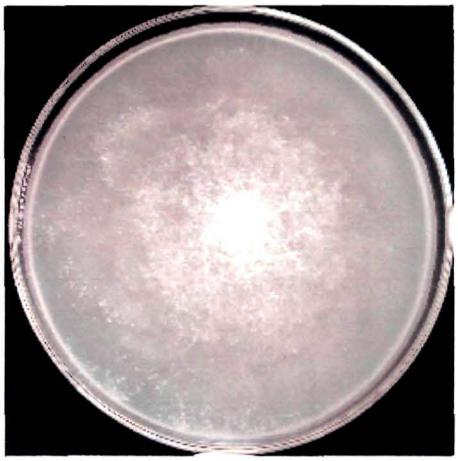


Plate 4.2 Yeast colony
A-KY2; B- MY0; C- MY1; D- MY2; E- TY0; F- TY1

A



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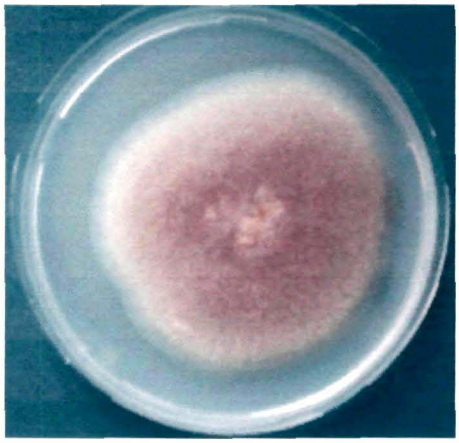

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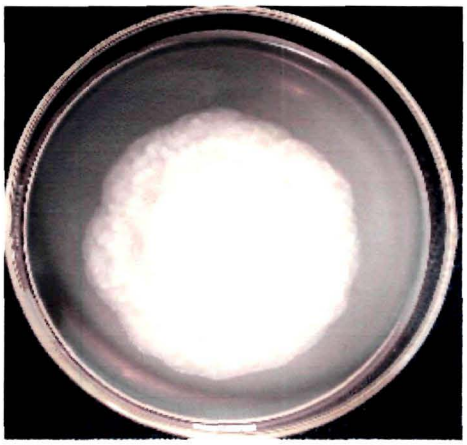

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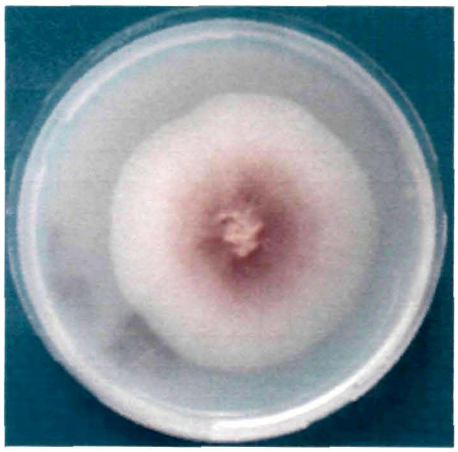
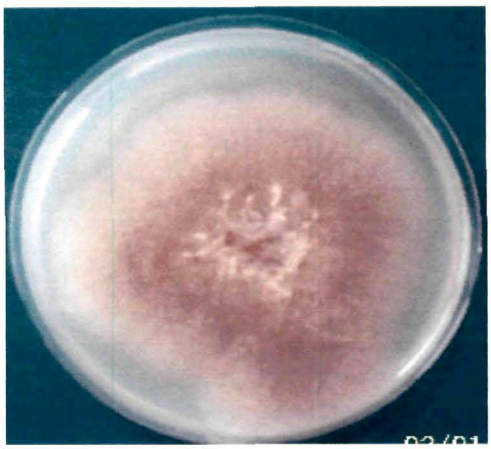


E

Plate 4.3 Fungal colony

A-AF1; B- AF2; C- BF1; D- BF2; E- KF1



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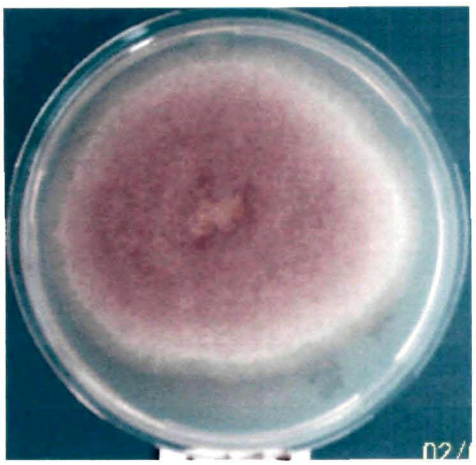

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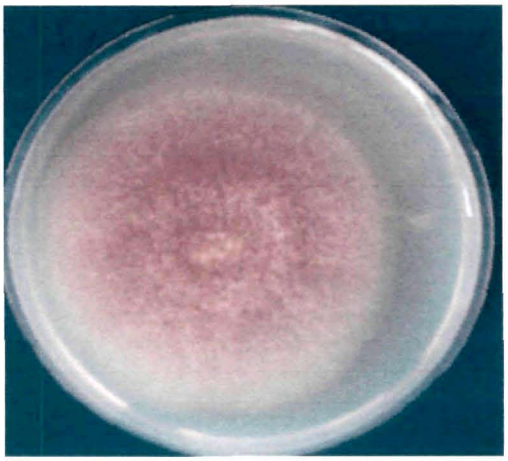

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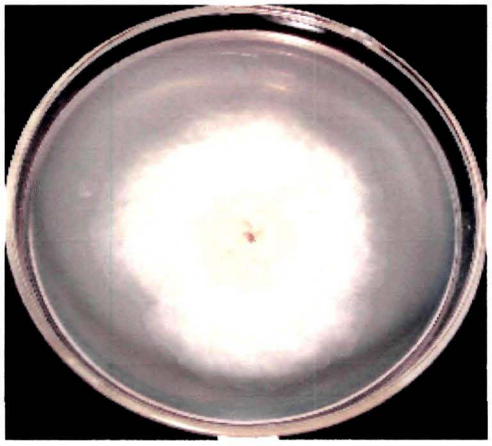

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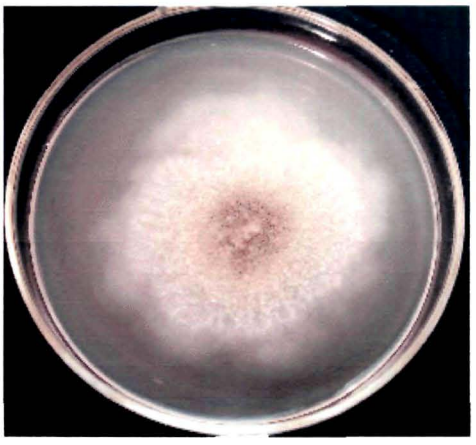
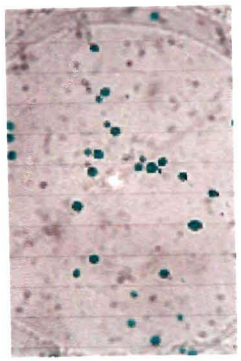


E

Plate 4.4 Fungal colony

A -KF2; B- MF1; C- MF2; D- TF1; E- TF2



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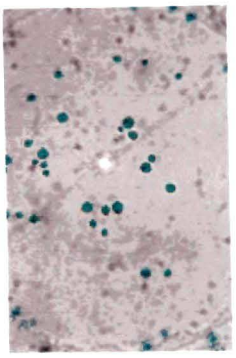

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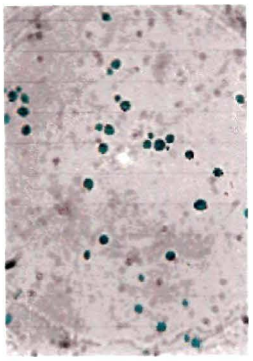

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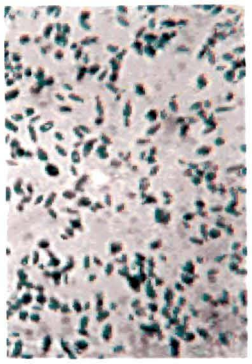

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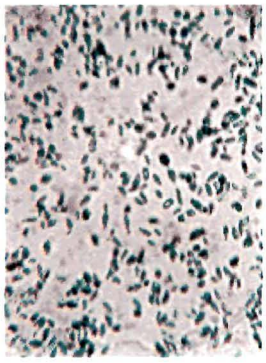

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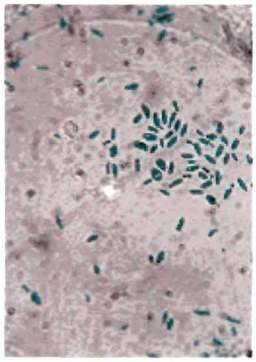
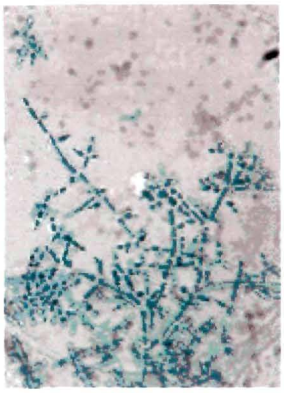
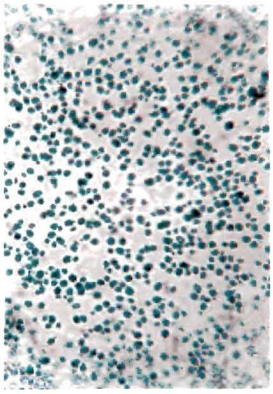


F

Plate 4.5 Micrograph of yeast isolates
A- AY0; B-AY1; C- AY2; D- BY0(A); E- BY0(B); F- BY0(C)
Magnification: 400X



A



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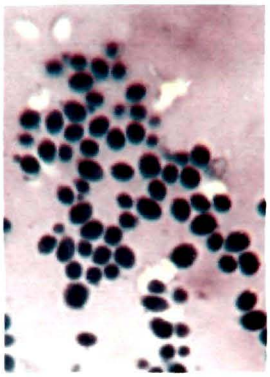

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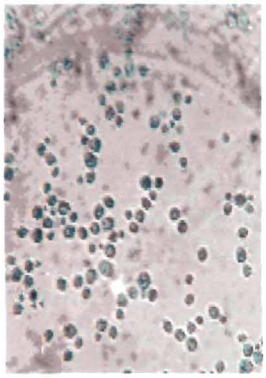

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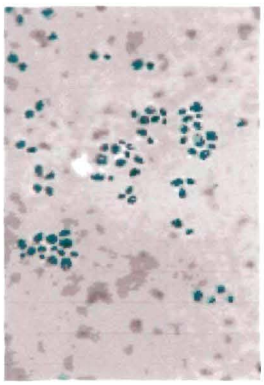

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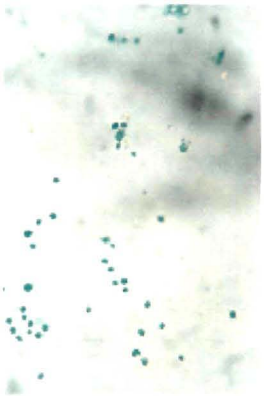


F

Plate 4.6 Micrograph of yeast isolates
A- BY1; B- BY2; C- KY0; D- KY1; E- KY2; F- MY0
Magnification : KY0 - 1000X, Others 400X

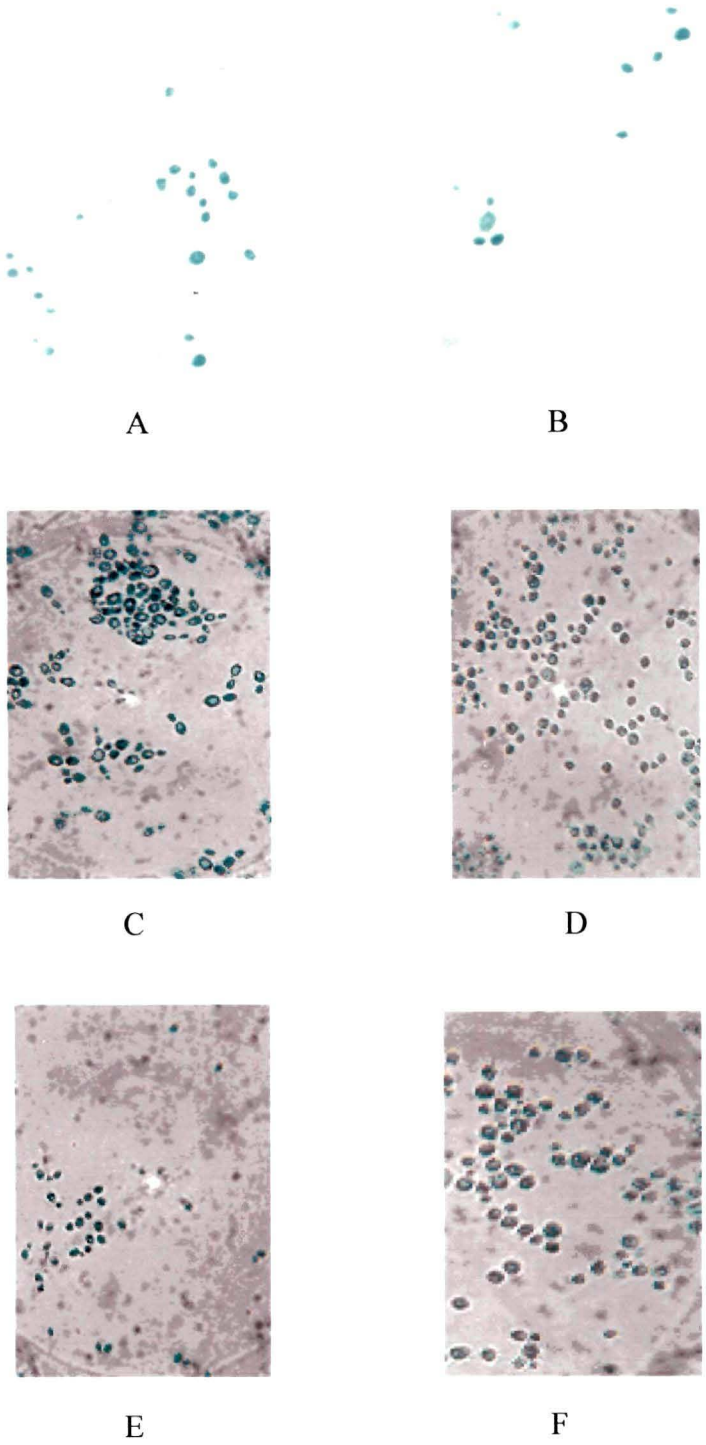
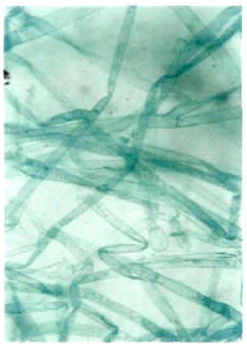
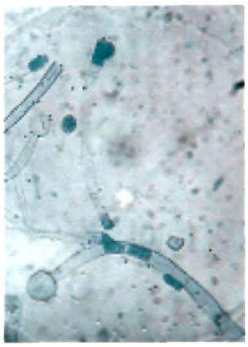


Plate 4.7 Micrograph of yeast isolates
A- MY1; B- MY2; C- TY0; D- TY1; E- TY2; F- TY3
Magnification : 400X



A



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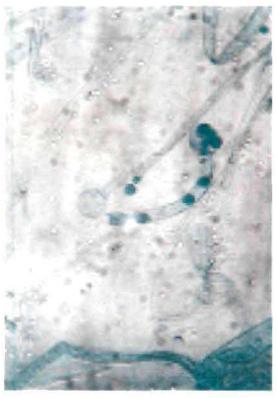
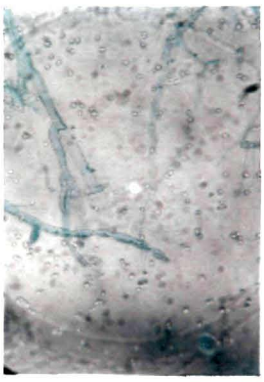
C

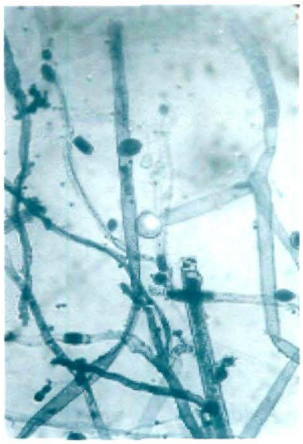


D

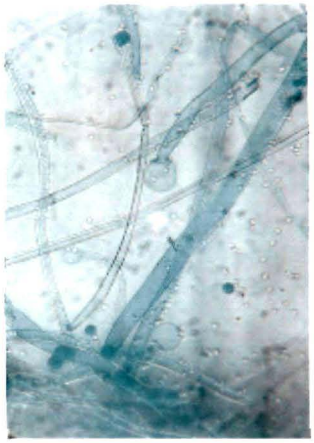


E

Plate 4.8 Micrograph of Sporangiophore of Fungal isolates
A- AF1; B- AF2; C- BF1; D- BF2; E- KF1
Magnification : 400X



A



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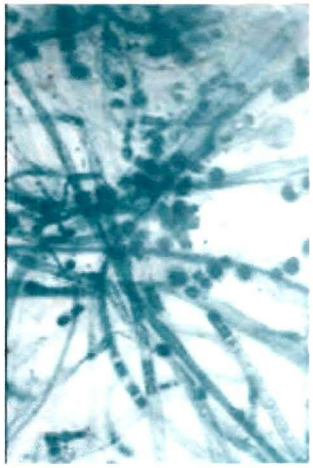
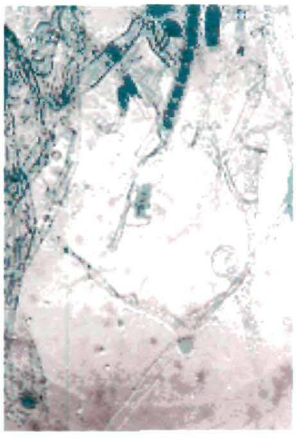
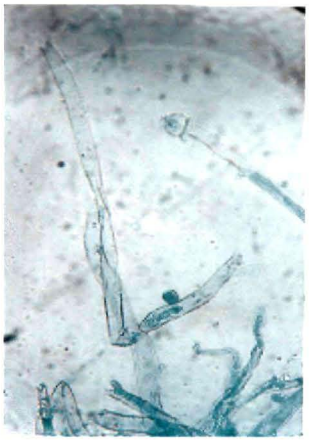


C

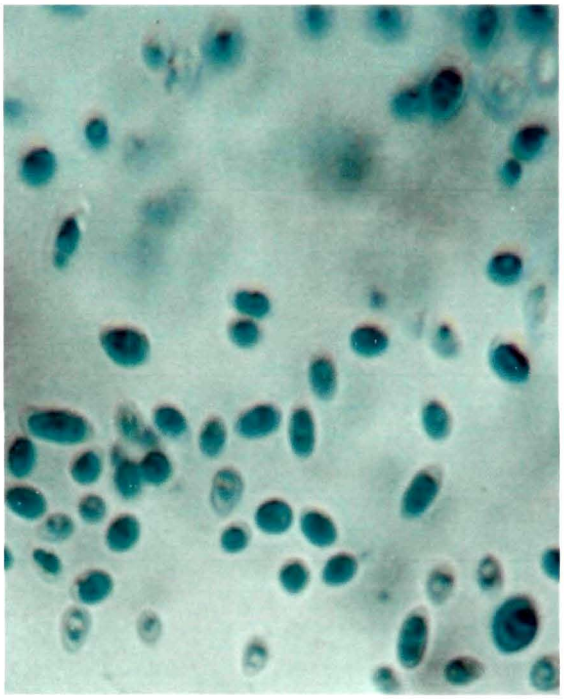


D

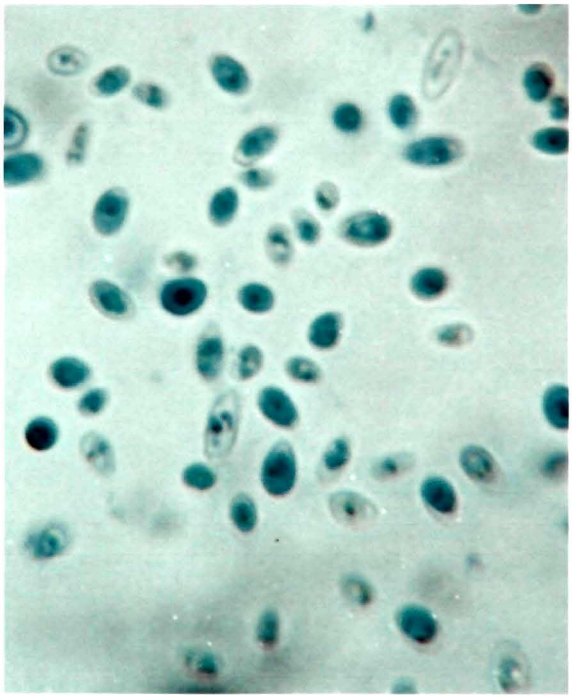


E

Plate 4.9 Micrograph of sporangiophore of Fungal isolates
A- KF2; B- MF1; C- MF2; D- TF1; E- TF2
Magnification : 400X



Ascospores of AY0



Ascospores of KY0

Plate 4.9 Micrograph of ascospores
Magnification : 400X

Table 4.8 Aerobic growth tests with carbon sources

Strain	Assimilation reaction															
	D-glucose	D-galactose	L-sorbose	D-glucosamin	D-ribose	D-xylose	L-arabinose	D-arabinose	L-Rhamnose	Sucrose	Maltose	α-α-Trehalose	Me-α-D-glucose	Cellobiose	Salicin	Arbutin
AY0	+	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
AY1	+	+	–	–	–	–	–	–	–	+	+	+	–	–	–	–
AY2	+	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
BY0(A)	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
BY0(B)	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
BY0(C)	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
BY1	+	+	+	+	–	+	+	–	–	+	+	+	+	+	+	+
BY2	+	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
KY0	+	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
KY1	+	–	–	–	–	–	–	–	–	+	D	D	+	–	–	–
KY2	+	+	–	–	–	+	–	–	–	+	+	+	+	+	+	+
MY0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
MY1	+	+	–	–	–	+	–	–	–	+	+	D	+	+	+	+
MY2	+	+	–	–	–	+	–	–	–	+	+	+	+	+	+	D
TY0	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TY1	+	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
TY2	+	+	–	–	–	+	–	–	–	+	+	+	+	+	+	+
TY3	+	+	–	–	–	+	–	–	–	+	+	–	+	+	+	+

+ positive, – negative, D delayed positive growth after 1 week

Table 4.8 contd.

Strain	Assimilation reaction															
	Melibiose	Lactose	Raffinose	Melezitose	Inulin	Starch	Glycerol	Erythritol	Ribitl	Xylitol	L-Arabinose	D-glucitol	D-mannitol	Galactitol	*Myo*-inositol	D-glucono-1-5-lactone
AY0	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
AY1	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
AY2	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
BY0(A)	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
BY0(B)	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
BY0(C)	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	+
BY1	–	–	+	+	–	–	+	+	+	+	+	+	+	+	–	+
BY2	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–
KY0	+	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–
KY1	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–
KY2	–	–	+	+	–	–	+	–	–	+	–	D	+	–	–	+
MY0	+	–	+	+	–	–	+	+	+	+	+	+	+	+	–	+
MY1	–	–	+	+	–	–	+	+	–	+	+	+	+	–	–	D
MY2	–	–	+	+	–	–	+	+	–	+	D	+	+	–	–	D
TY0	–	–	–	–	–	–	D	–	–	D	–	–	–	–	–	+
TY1	+	–	+	D	–	–	–	–	–	–	–	–	D	–	–	D
TY2	–	–	+	+	–	–	+	+	+	+	–	+	+	–	–	+
TY3	–	–	+	+	–	–	+	–	+	+	–	+	+	–	–	+

+ positive, – negative, D delayed positive growth after 1 week

Table 4.8 contd.

Strain	Assimilation reaction														
	2-keto-D-gluconate	5-keto-D-gluconate	D-gluconate	D-glucuronate	D-galacturonic	D-L-Lactate	Succinate	Citrate	Methanol	Ethanol	Propane-1-2-diol	Butane-2-3-diol	Quinic acid	D-glucarate	D-galactonate
AY0	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
AY1	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–
AY2	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
BY0(A)	–	–	–	–	–	+	+	+	–	+	–	–	–	–	–
BY0(B)	–	–	–	–	–	+	+	+	–	+	–	–	–	–	–
BY0(C)	–	–	–	–	–	+	+	+	–	+	–	–	–	–	–
BY1	+	–	–	+	–	–	+	–	–	+	–	–	–	–	–
BY2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KY0	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–
KY1	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
KY2	+	–	–	–	–	+	D	–	–	+	D	–	–	–	–
MY0	+	–	+	+	–	+	+	+	–	+	D	–	–	–	–
MY1	+	–	+	–	–	+	+	+	–	+	+	+	+	–	–
MY2	–	–	–	–	–	+	+	+	–	+	+	+	+	–	–
TY0	–	–	–	–	–	+	+	+	–	+	–	–	–	–	–
TY1	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–
TY2	+	–	–	–	–	+	+	+	–	+	+	+	+	–	–
TY3	+	–	–	–	–	+	+	+	–	+	+	–	+	–	–

+ positive, – ,negative, D delayed positive growth after 1 week

Table 4.9 Ability to use nitrogen compounds for aerobic growth

	Nitrogen compounds									
Strain	Nitrate	Nitrite	Ethylamine	L-lysine	Cadaverine	Creatine	Creatinine	Glucosamine	Imidazole	Tryptophane
AY0	–	–	–	–	–	–	–	–	–	–
AY1	–	–	–	–	–	–	–	–	–	–
AY2	–	–	–	–	–	–	–	–	–	–
BY0(A)	–	–	+	+	+	–	–	–	–	–
BY0(B)	–	–	+	+	+	+	+	–	–	+
BY0(C)	–	–	+	+	+	–	–	–	–	–
BY1	–	–	–	–	+	+	–	–	–	–
BY2	–	–	–	–	–	–	–	–	–	–
KY0	–	–	–	–	–	–	–	–	–	–
KY1	–	–	–	–	–	–	–	–	–	–
KY2	+	–	–	+	–	–	–	–	–	–
MY0	–	–	–	–	+	+	–	–	–	–
MY1	+	–	–	–	–	–	–	–	–	–
MY2	+	–	+	–	+	–	–	–	–	–
TY0	–	–	+	+	+	–	–	–	–	+
TY1	–	–	–	–	–	–	–	–	–	–
TY2	–	–	–	–	–	–	–	–	–	–
TY3	–	–	+	–	–	–	–	–	–	–

+ positive, – negative, D delayed positive growth after 1 week

Table 4.10 Test for semi anaerobic fermentation

	semi anaerobic fermentation													
Strain	D- Glucose	D- Galactose	Maltose	Me-α-D-Glucoside	Sucrose	α-α-Trehalose	Melibioe	Lactose	Cellobiose	Melezitose	Raffinose	Inulin	Starch	D-Xylose
AY0	+	+	+	+	+	–	–	–	–	+	+	–	–	–
AY1	+	+	–	+	+	–	–	–	–	+	+	–	–	–
AY2	+	+	+	+	+	–	–	–	–	+	+	–	–	–
BY0(A)	+	–	–	–	–	–	–	–	–	–	–	–	–	–
BY0(B)	+	–	–	–	–	–	–	–	–	–	–	–	–	–
BY0(C)	+	–	–	–	–	–	–	–	–	–	–	–	–	–
BY1	+	–	–	–	–	–	–	–	–	–	–	–	–	–
BY2	+	+	–	+	+	–	–	–	–	+	–	–	–	–
KY0		+	+	–	+	–	–	–	–	–	–	–	–	–
KY1	+	+	–	+	+	–	–	–	–	–	–	–	–	–
KY2	+	+	+	–	+	–	–	–	–	–	D	–	D	–
MY0	+	–	–	–	+	–	–	–	–	–	–	–	–	–
MY1	+	D	+	+	+	–	–	–	–	–	D	–	–	–
MY2	+	D	+	+	+	–	–	–	–	–	D	–	–	–
TY0	+	–	–	–	–	–	–	–	–	–	–	–	–	–
TY1	+	+	+	+	+	–	–	–	–	–	–	–	–	–
TY2	+	D	D	+	+	–	–	–	–	–	D	–	–	–
TY3	+	D	D	–	+	–	–	–	–	–	D	–	D	–

+ positive, – negative, D delayed positive growth after 1 week

4.4.4 Vitamin requirements

The test was performed on the basis of differences in the requirement for the exogenous supply of certain vitamins. Yeasts differ from each other in their requirements for vitamins. Results of such tests for the ability of yeasts to grow in the absence of each single vitamin or without any of them were presented in Table 4.11.

4.4.5 Growth at different temperatures

Yeast strains could be differentiated on the basis of their ability to grow at different temperatures. The results of the tests are presented in Table 4.12.

4.4.6 Growth in the presence of cycloheximide

Cycloheximide or actidione stops the growth of many eukaryotes by inhibiting the synthesis of protein in 80S ribosomes (Vazquez, 1979). Yeast isolates were grown on liquid media containing the chemical and the results obtained are presented in Table 4.13.

4.4.7 Growth at high osmotic pressure

Some yeast can grow at high concentration of sugar and salts. The results of such tests carried out are presented in Table 4.13.

4.4.8 Urea hydrolysis

Urease activity of yeast is used for identifying yeast strains. Some yeast strains have the ability to hydrolyse urea. The results of tests carried out are presented in Table 4.13.

4.4.9 Diazonium Blue B test

Basidiomycetous yeasts show positive response to diazonium blue B reaction. Yeasts isolated were tested for the reaction and the results obtained are presented in Table 4.13

Table 4.11Growth of yeasts with or without vitamins

	Growth of yeasts with or without vitamins									
Strain	Without vitamin	Without *myo*-inositol	Without Pantothenate	Without Biotin	Without Thiamin	Without Biotin and Thiamin	Without Pyridoxine	Without Pyridoxine and Thiamin	Without Niacin	Without ρ-aminobenzoate
AY0	+	+	+	_	+	_	+	+	+	+
AY1	+	+	_	+	+	_	+	+	+	D
AY2	+	+	+	+	+	_	+	_	+	D
BY0(A)	+	V	V	_	V	V	+	V	V	V
BY0(B)	+	V	V	_	V	V	+	V	V	V
BY0(C)	_	V	V	_	V	V	+	V	V	V
BY1	+	+	+	_	+	_	+	+	+	+
BY2	+	+	+	_	+	_	+	+	+	+
KY0	+	+	+	+	+	_	+	D	+	D
KY1	+	+	+	+	+	_	+	D	+	D
KY2	+	+	+	+	+	+	+	+	+	+
MY0	_	+	+	_	+	_	+	+	+	+
MY1	+	+	+	+	+	+	+	+	+	+
MY2	+	+	+	+	+	+	+	+	+	+
TY0	+	V	V	_	V	V	+	V	V	V
TY1	_	+	+	+	+	_	+	D	+	D
TY2	+	+	+	+	+	+	+	+	+	+
TY3	+	+	+	+	+	+	+	+	+	+

+ positive, - negative, V variable and D delayed positive growth after 1 week

Table 4.12 Growth of yeast isolates at different temperature

Growth of yeasts isolates at various temperatures (^{0}C)							
Strains	25^{0}C	30^{0}C	35^{0}C	37^{0}C	40^{0}C	42^{0}C	45^{0}C
AY0	+	+	+	+	–	–	–
AY1	+	+	+	+	–	–	–
AY2	+	+	+	+	–	–	–
BY0(A)	+	+	+	+	+	–	–
BY0(B)	+	+	+	+	+	–	–
BY0(C)	+	+	+	+	+	–	–
BY1	+	+	+	+	–	–	–
BY2	+	+	+	+	–	–	–
KY0	+	+	+	+	–	–	–
KY1	+	+	+	+	–	–	–
KY2	+	+	+	+	–	–	–
MY0	+	+	+	+	–	–	–
MY1	+	+	+	+	–	–	–
MY2	+	+	+	+	–	–	–
TY0	+	+	+	+	+	–	–
TY1	+	+	+	+	–	–	–
TY2	+	+	+	–	–	–	–
TY3	+	+	–	–	–	–	–

+ positive, - negative

Table 4.13 Growth of yeasts isolates in the presence of cycloheximide, at high osmotic pressure, urea hydrolysis and Diazonium blue B test

Strains	Growth at high osmotic pressure and some additional tests							Additional tests	
	Growth in cycloheximide		Growth in 1% acetic acid	Growth in glucose		Growth in NaCl		Urea hydrolysis	Diazonium blue B test
	0.01% (w/v)	0.1% (w/v)		50% (w/v)	60% (w/v)	10% (w/v)	16% (w/V0		
AY0	–	–	–	+	+	–	–	–	–
AY1	–	–	–	+	+	–	–	–	–
AY2	–	–	–	+	+	–	–	–	–
BY0(A)	–	–	–	+	–	+	–	–	–
BY0(B)	–	–	–	+	–	+	–	–	–
BY0(C)	–	–	–	+	–	D	–	–	–
BY1	–	–	–	+	+	+	–	–	–
BY2	–	–	–	+	+	–	–	–	–
KY0	–	–	–	+	+	–	–	–	–
KY1	–	–	–	+	+	–	–	–	–
KY2	–	–	–	+	+	+	–	–	–
MY0	–	–	–	+	+	+	–	–	–
MY1	–	–	–	+	+	+	–	–	–
MY2	–	–	–	+	+	+	–	–	–
TY0	–	–	–	+	–	+	–	–	–
TY1	–	–	–	+	+	–	–	–	–
TY2	–	–	–	+	+	+	–	–	–
TY3	–	–	–	–	–	+	–	–	–

+ positive, - negative and D delayed positive growth after 1 week

4.5 Identification of yeast isolates

Based on the biochemical characterization and microbiological observations of the yeast strains studied and also by following the conventional dichotomous identification keys described by Barnett *et al.* (2000) the isolates were tentatively identified as described bellow:

1. AY0- *Saccharomyces cerevisiae/S. paradoxus* (1)
2. AY1- *Saccharomyces cerevisiae/S. paradoxus* (1)
3. AY2- *Saccharomyces cerevisiae/S. paradoxus* (1)
4. BY0(A)-*Issatchenkia orientalis* (2)
5. BY0(B)- *Issatchenkia orientalis* (2)
6. BY0(C)- *Issatchenkia orientalis* (2)
7. BY1-*Debaryomyces hansenii/D. maramus/D. nepalensis* (3)
8. BY2- *Saccharomyces cerevisiae/S. paradoxus* (1)
9. KY0- *Saccharomyces cerevisiae/S, bayanus/S. pastorianus/ S. microellipsoides.* (1)
10. KY1- *Saccharomyces cerevisiae/S, bayanus/S. pastorianus/ S. microellipsoides* (1)
11. KY2- *Pichia anomala* (4)
12. MY0- *Debaryomyces hansenii/D. maramus/D. nepalensis* (3)
13. MY1- *Pichia anomala/P. sydowiorum* (4)
14. MY2- *Pichia anomala/P. sydowiorum* (4)
15. TY0- *Issatchenkia orientalis* (2)
16. TY1- *Saccharomyces cerevisiae/S, bayanus/S. pastorianus/ S. microellipsoides* (1)
17. TY2- *Pichia anomala* (4)
18. TY3- *Pichia anomala* (4)

4.6 Molecular genetic assessment

4.6.1 Genomic DNA isolation and purification

Studies on the genomic DNA reflect the genome size and also allows for reasonable speculation about the number of genes present. Genomic DNA obtained through the modified protocol was of better quality than that obtained through the original protocol. The yield and quality of the isolated genomic DNA are presented in Table 4.14.

Table 4.14 Yield and quality of yeast genomic DNA

Isolates	OD_{260}	OD_{280}	OD_{260}/ OD_{280}	Yield µg/ µl DNA sample
AY0	0.284	0.160	1.775	2.84
AY1	0.238	0.131	1.817	2.38
AY2	0.326	0.176	1.852	3.26
BY0(A)	0.205	0.113	1.814	2.05
BY0(B)	0.369	0.119	1.785	3.69
BY0(C)	0.201	0.114	1.763	2.01
BY1	0.250	0.139	1.794	2.50
BY2	0.309	0.170	1.817	3.09
KY0	0.256	0.145	1.766	2.56
KY1	0.257	0.142	1.810	2.57
KY2	0.363	0.193	1.881	3.63
MY0	0.213	0.119	1.785	2.13
MY1	0.258	0.141	1.830	2.58
MY2	0.226	0.125	1.808	2.26
TY0	0.354	0.186	1.904	3.54
TY1	0.258	0.138	1.870	2.58
TY2	0.268	0.151	1.775	2.68
TY3	0.223	0.126	1.849	2.23

The isolate BY0(B), followed by KY2, TY0, AY2 and BY2 showed the highest yield of DNA content with 3.69, 3.63, 3.54, 3.26 and 3.09 $\mu g.\mu l^{-1}$ respectively. The isolates BY0(C) yielded the least amount of DNA with 2.01 $\mu g.\mu l^{-1}$.

4.6.2 Electrophoresis of yeast genomic DNA

The isolated and purified genomic DNAs were electrophoresed in 0.7% agarose gel. The banding patterns thus obtained are presented in Fig. 4.1- 4.3. The size of the genomic DNA of the yeast isolates did not vary significantly. The average size of the genomic DNA was found to be more than 23.13 kb.

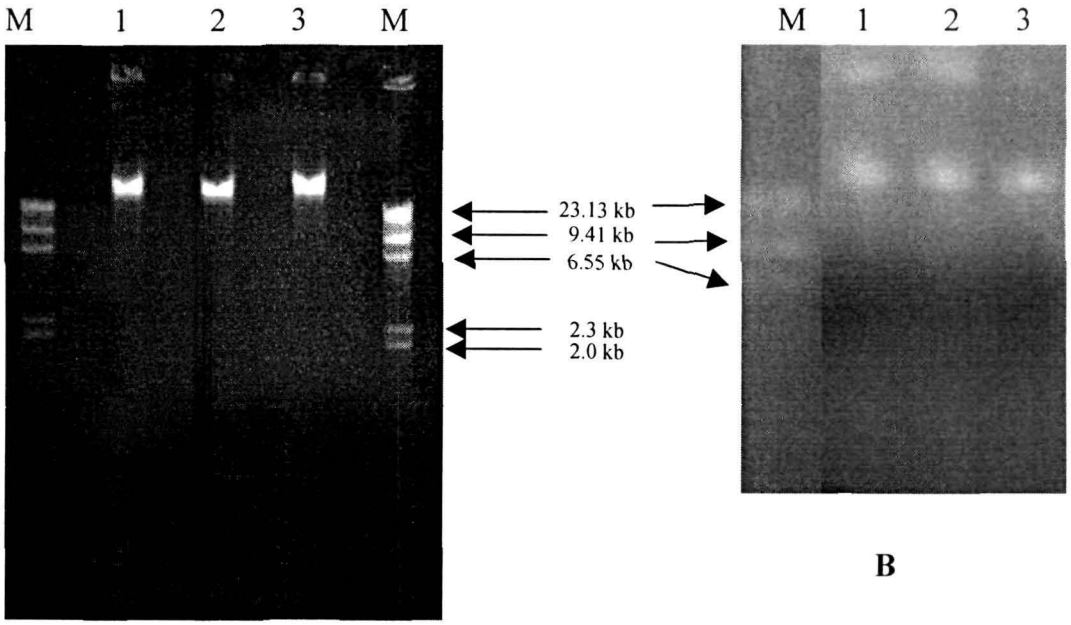


Fig. 4.1 Genomic DNA of yeast isolates **(A)** Lane M: Marker DNA (*Hin*dIII digested λ-DNA), Lane 1: AY0, Lane 2: AY1, Lane 3: AY2, **(B)** Lane M: Marker DNA, Lane 1: BY0(A), Lane 2: BY0(B) and Lane 3: BY0(C)

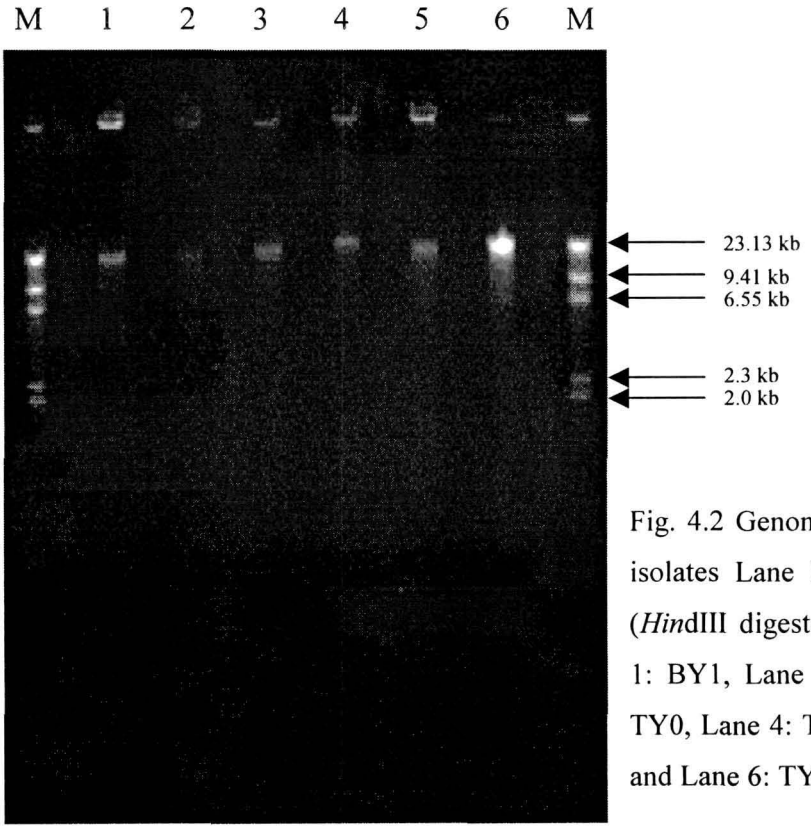


Fig. 4.2 Genomic DNA of yeast isolates Lane M: Marker DNA (*Hin*dIII digested λ-DNA), Lane 1: BY1, Lane 2: BY2, Lane 3: TY0, Lane 4: TY1, Lane 5: TY2 and Lane 6: TY3

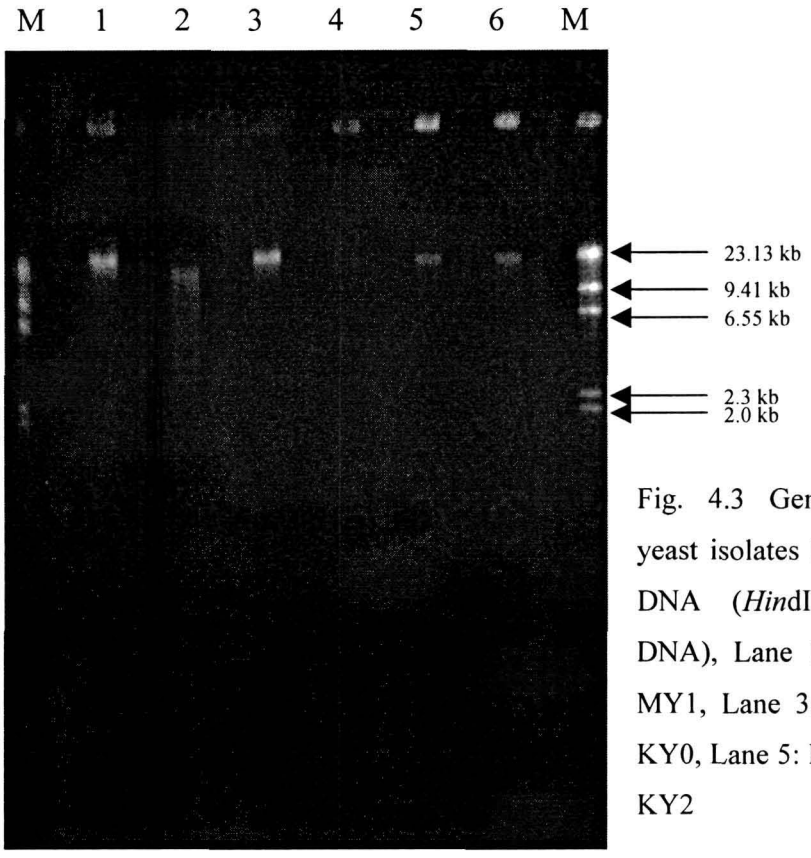


Fig. 4.3 Genomic DNA of yeast isolates Lane M: Marker DNA (*Hin*dIII digested λ-DNA), Lane 1: MY0, Lane2: MY1, Lane 3: MY2, Lane 4: KY0, Lane 5: KY1 and Lane 6: KY2

4.6.3 PCR-based RAPD profiling of yeast genomic DNA

The isolated genomic DNA of yeast strains were subjected to PCR-amplification with the universal primers like M13 and 21. The amplified DNA samples were run in 1.4% agarose gel electrophoresis. The amplified DNA and their banding patterns are shown in Fig. 4.4 and 4.5.

Yeast isolates AY0, AY1, AY2, BY0(A), BY0(B), BY0(C),BY2, KY0, KY1, TY0 and TY1 showed common band of size 1.3 kb and that of AY0, AY1, AY2, BY2, KY0 and KY1 isolates shared a common band of 0.8 kb in the case of M13 primer. The isolates AY0, AY1, AY2, BY2, KY0 also shared a common band of 0.85 kb on use of the primer 21. Again BY0(A), BY0(B), BY0(C) and TY0 shared a common band size of 1.1 kb and 0.75 bp respectively when the primer M13 and 21 were used.

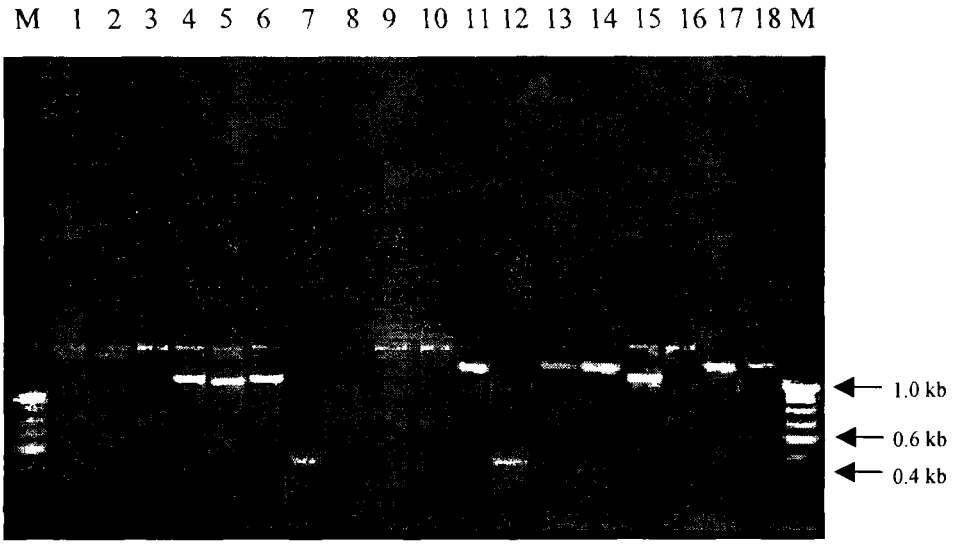


Fig. 4.4 RAPD-PCR profile of the yeast isolates using primer M13 Lane M: Marker DNA (100 bp DNA ladder, Bangalore Gennie), Lane 1: AY0, Lane 2:AY1, Lane 3: AY2, Lane 4: BY0(A), Lane 5: BY0(B), Lane 6: BY0(C), Lane 7: BY1, Lane 8: BY2, Lane 9: KY0, Lane 10: KY1, Lane 11: KY2, Lane 12: MY0, Lane 13: MY1, Lane 14: MY2, Lane 15: TY0, Lane 16: TY1, Lane 17: TY2 and Lane 18: TY3

The sole band size of 0.6 kb was shown by isolates BY1 and MY0 for both primers. Other isolates KY2, MY1, MY2, TY2 and TY3 shared a common band size of 1.2 kb in the case of the primer M13 and 0.65 kb in the case of primer M13. All other band sizes were not shared by a group of isolates.

4.6.4 Phylogenetic relationship based on RAPD profiles among isolates

The similarity among various profiles obtained from the gel separation of the PCR amplified products was determined by Jaccard coefficient and presented in Table 4.15.

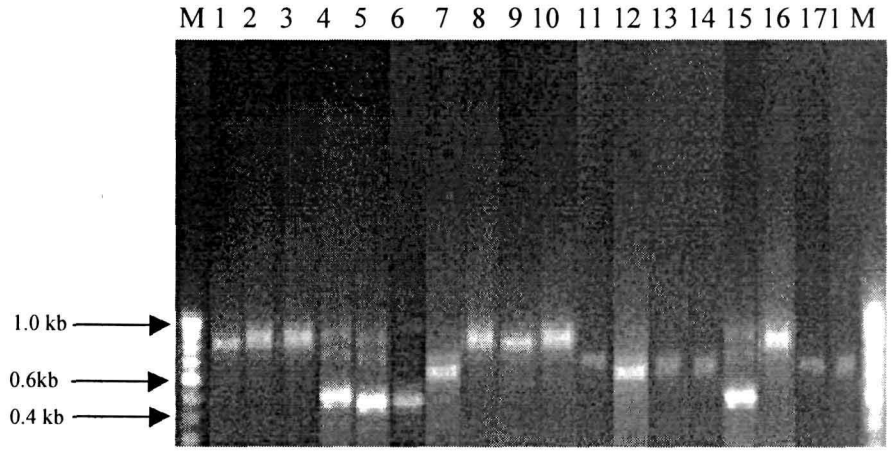


Fig. 4.5 RAPD-PCR profile of the yeast strains Primer 21 Lane M: Marker DNA (100 bp DNA ladder, Bangalore Gennie), Lane 1: AY0, Lane 2: AY1, Lane 3: AY2, Lane 4: BY0(A), Lane 5: BY0(B), Lane 6: BY0(C), Lane 7: BY1, Lane 8: BY2, Lane 9: KY0, Lane 10: KY1, Lane 11: KY2, Lane 12: MY0, Lane 13: MY1, Lane 14: MY2; Lane 15: TY0, Lane 16:TY1, Lane 17: TY2 and Lane 18: TY3

Table 4.15 Jaccard's similarity co-efficient matrix for RAPD

Case	1:AY0	2:AY1	3:AY2	4:BY0(A)	5:BY0(B)	6:BY0(C)	7:BY1	8:BY2	9:KY0	10:KY1	11:KY2	12:MY0	13:MY1	14:MY2	15:TY0	16:TY1	17:TY2	18:TY3
1:AY0		1.000	0.750	0.125	0.125	0.143	0.000	1.000	0.750	0.750	0.000	0.000	0.000	0.000	0.125	0.750	0.000	0.000
2:AY1			0.750	0.125	0.125	0.143	0.000	1.000	0.750	0.750	0.000	0.000	0.000	0.000	0.125	0.750	0.000	0.000
3:AY2				0.143	0.143	0.167	0.000	0.750	1.000	1.000	0.000	0.000	0.000	0.000	0.143	1.000	0.000	0.000
4:BY0(A)					1.000	0.800	0.000	0.125	0.143	0.143	0.000	0.000	0.000	0.000	1.000	0.143	0.000	0.000
5:BY0(B)						0.800	0.000	0.125	0.143	0.143	0.000	0.000	0.000	0.000	1.000	0.143	0.000	0.000
6:BY0(C)							0.000	0.143	0.167	0.167	0.000	0.000	0.000	0.000	0.800	0.167	0.000	0.000
7:BY1								0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
8:BY2									0.750	0.750	0.000	0.000	0.000	0.000	0.125	0.750	0.000	0.000
9:KY0										1.000	0.000	0.000	0.000	0.000	0.143	1.000	0.000	0.000
10:KY1											0.000	0.000	0.000	0.000	0.143	1.000	0.000	0.000
11:KY2												0.000	1.000	1.000	0.000	0.000	1.000	1.000
12:MY0													0.000	0.000	0.000	0.000	0.000	0.000
13:MY1														1.000	0.000	0.000	1.000	1.000
14:MY2															0.000	0.000	1.000	1.000
15:TY0																0.143	0.000	0.000
16:TY1																	0.000	0.000
17:TY2																		1.000
18:TY3																		

Dendogram constructed using the RAPD-PCR data with the help of SPSS software package is presented in Fig 4.10. It reveals that the yeast isolates could be broadly grouped in to two. The first group includes Isolates AY0, AY1, AY2, BY1, BY2, KY0, KY1, MY0 and TY1. The other group includes BY0(A), BY0(B), KY2, MY1, MY2, TY0 TY2 and TY3. The isolate BY0(C) clubbed with BY0(A) and TY0 of the second group and formed a small group of their own. Within the first group the isolates AY1, AY2, BY2 and KY0 formed another small group.

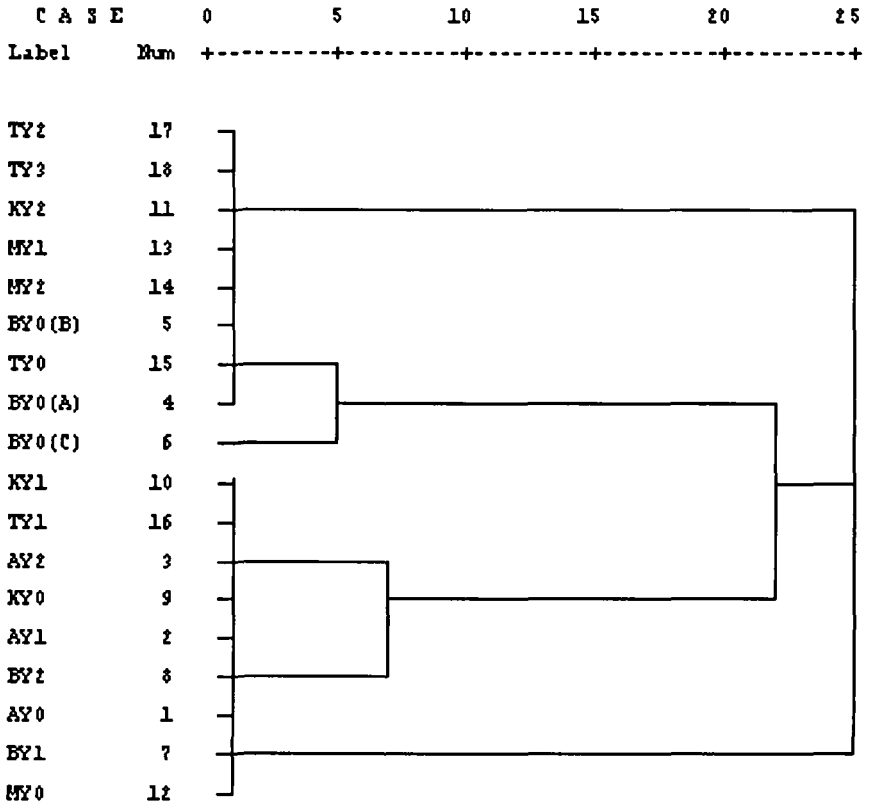


Fig. 4.6 Dendrogram using RAPD data

4.6.5 ITS-PCR of the genomic DNA

ITS spacer region and 5.8S rDNA was amplified using the primer ITS1 and ITS4. The amplified products were separated by electrophoresis in 1.2% (w/v) agarose gel. The resultant fragments were found to be 825-850 bp for AY0. AY1, AY2, BY2, KY0 and TY1; 600-640 bp for KY2, MY1, MY2, TY2 and TY3; 500-525 bp for BY0(A), BY0(B), BY0(C) and TY0; and 475 bp for BY1 and MY0. The profile is presented in Fig 4.6

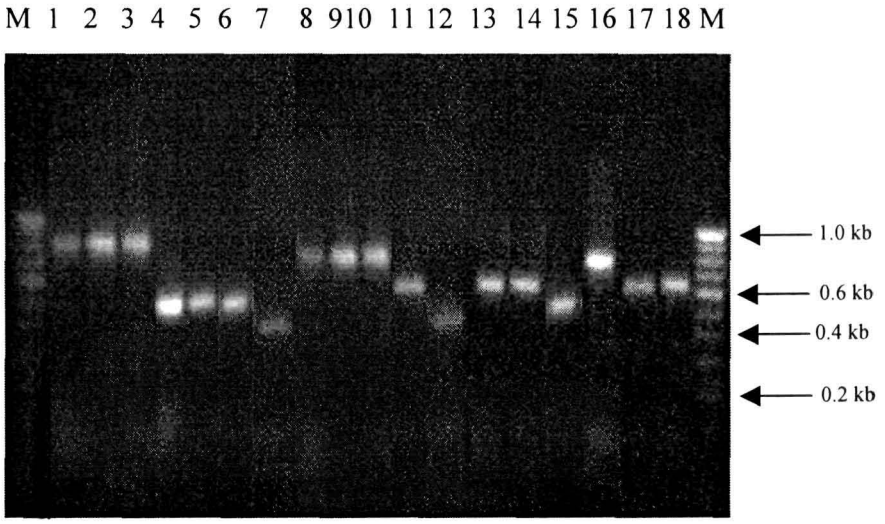


Fig. 4.7 Gel separation of ITS-PCR product (primer pair ITS1 and ITS4) Lane M: Marker DNA (100 bp DNA ladder, Bangalore Gennie), Lane 1: AY0, Lane 2: AY1, Lane 3: AY2, Lane 4: BY0(A), Lane 5: BY0(B), Lane 6: BY0(C), Lane 7: BY1, Lane 8: BY2, Lane 9: KY0, Lane 10: KY1, Lane 11: KY2, Lane 12: MY0, Lane 13: MY1, Lane 14: MY2, Lane 15: TY0, Lane 16; TY1, Lane 17: TY2 and Lane 18: TY3

4.6.6 RFLP analysis of ITS spacer and 5.8S rRNA gene region

The amplification product of the ITS spacer region and the 5.8S rRNA region with ITS1 and ITS4 primer were further restriction digested using the restriction endonucleases *Hae*III, *Hin*fI and *Hpa*II. The restriction digested products were separated by gel electrophoresis in 2.5% ((w/v) agarose gel and visualized under UV light.

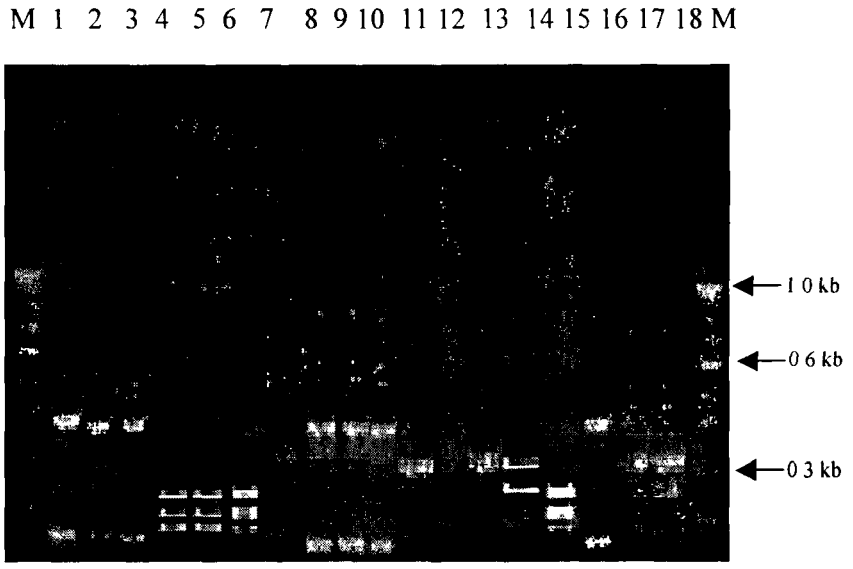


Fig. 4.8 Restriction fragments of PCR amplified 5.8S rRNA of yeast strains with Lane M: Marker DNA (100 bp DNA ladder, Bangalore Gennie), Lane 1: AY0, Lane 2: AY1, Lane 3: AY2, Lane 4: BY0(A), Lane 5: BY0(B), Lane 6: BY0(C), Lane 7: BY1, Lane 8: BY2, Lane 9: KY0, Lane 10: KY1, Lane 11: KY2, Lane 12: MY0, Lane 13: MY1, Lane 14: MY2, Lane 15; TY0,Lane 16: TY1, Lane 17: TY2 and Lane 18: TY3

4.6.7 Phylogenetic relationship based on RFLP profiles among isolates

The phylogenetic relationship or similarity among various profiles resulting from the restriction digestion of PCR amplified products with the restriction enzymes and separation by gel electrophoresis was determined by Jaccard coefficient and is presented in Table 4.17.

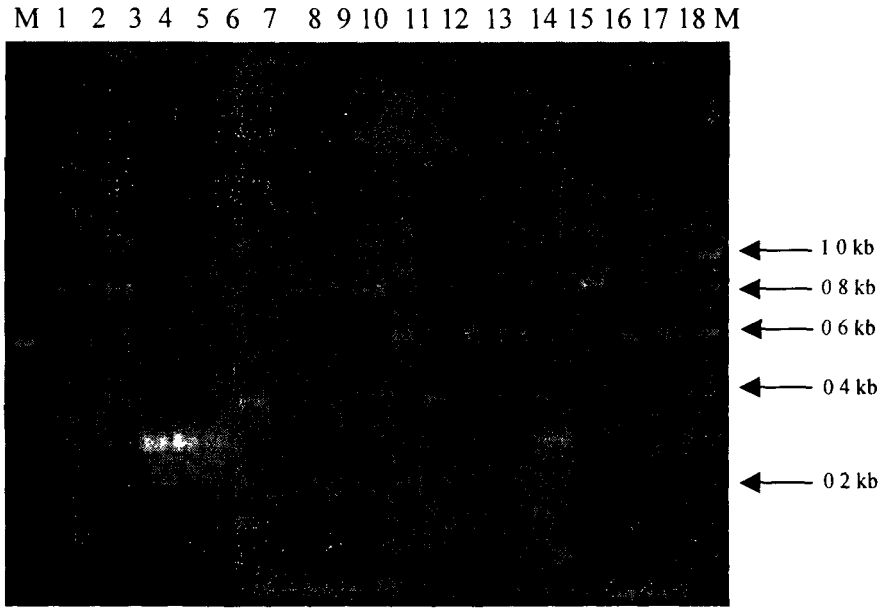


Fig. 4.9 Restriction fragments of PCR amplified 5.8S rDNA of yeast strains with *Hpa*IILane M: Marker DNA (100 bp DNA ladder, Bangalore Gennie), Lane 1: AY0, Lane 2: AY1, Lane 3: AY2, Lane 4: BY0(A), Lane 5: BY0(B), Lane 6: BY0(C), Lane 7: BY1, Lane 8: BY2, Lane 9: KY0, Lane 10: KY1, Lane 11: KY2, Lane 12: MY0, Lane 13: MY1, Lane 14: MY2, Lane 15: TY0, Lane 16: TY1, Lane 17: TY2 and Lane 18: TY3

The dendogram constructed using the PCR-RFLP data with the help of SPSS software was found to be useful in grouping and identification of the yeast isolates in comparison to that of the reference strain. Dendogram for clustering of the isolates were constructed from the data obtained from restriction digestion of the PCR products by using the "Unweighted Pair Group Method using arithmetic average (UPGMA)" and is presented in Fig. 4.11.

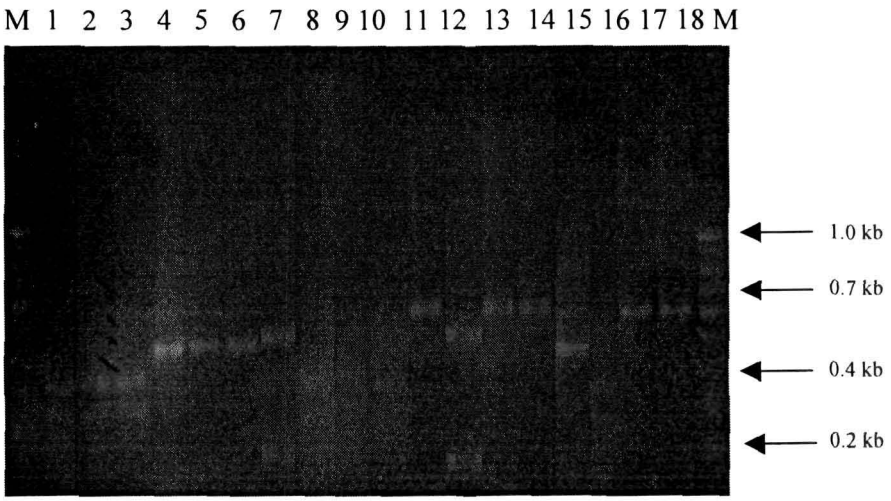


Fig. 4.10 Restriction fragments of PCR amplified 5.8S rDNA of yeast strains with *Hae*III Lane M: Marker DNA (100 bp DNA ladder, Bangalore Gennie), Lane 1: AY0, Lane 2: AY1, Lane 3: AY2, Lane 4: BY0(A), Lane 5: BY0(B), Lane 6: BY0(C), Lane 7: BY1, Lane 8: BY2, Lane 9: KY0, Lane 10: KY1, Lane 11: KY2, Lane 12: MY0, Lane 13: MY1, Lane 14: MY2, Lane 15: TY0, Lane 16: TY1, Lane 17: TY2 and Lane 18: TY3

Table 4.16 Length in bp of 5.8S-ITS region of the yeast strains amplified by PCR and digestion with the restriction enzyme

Strains	bp of 5.8S-ITS	Restriction enzymes		
		HaeIII	*Hin*fI	*Hpa*II
AY0	825	325+230+170+125	375+365+110	850
AY1	825	325+230+170+125	375+365+270+110	850
AY2	825	325+230+170+125	375+365+270+110	850
BY0(A)	525	370+90	225+160+145	300
BY0(B)	525	370+90	225+160+145+300	300
BY0(C)	525	370+90	225+160+145	300
BY1	475	400+120+75	315	400
BY2	825	325+230+170+125	375+365+110	850
KY0	825	325+230+170+125	375+365+110	850
KY1	825	325+230+170+125	375+365+110	850
KY2	600	615	310+270	600
MY0	475	400+120+75	315	400
MY1	600	615	310+270	600
MY2	600	615	310+270	600
TY0	525	370+90	225+160+145	300
TY1	825	325+230+170+125	375+365+270+110	850
TY2	600	615	310+270	600
TY3	600	615	310+270	600

Table 4.17 Jaccard's similarity co-efficient matrix for RFLP

Case	1:AY0	2:AY1	3:AY2	4:BY0(A)	5:BY0(B)	6:BY0(C)	7:BY1	8:BY2	9:KY0	10:KY1	11:KY2	12:MY0	13:MY1	14:MY2	15:TY0	16:TY1	17:TY2	18:TY3	19:S.c	20:I.o	21:D.h	22:P.a
1:AY0		0.889	0.889	0.000	0.000	0.000	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.889	0.000	0.000	1.000	0.000	0.000	0.000
2:AY1			1.000	0.000	0.000	0.000	0.000	0.889	0.889	0.889	0.083	0.000	0.083	0.083	0.000	1.000	0.083	0.083	0.889	0.000	0.000	0.000
3:AY2				0.000	0.000	0.000	0.000	0.889	0.889	0.889	0.083	0.000	0.083	0.083	0.000	1.000	0.083	0.083	0.889	0.000	0.000	0.000
4:BY0(A)					1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.833	0.000	0.000
5:BY0(B)						1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.833	0.000	0.000
6:BY0(C)							0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.833	0.000	0.000
7:BY1								0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
8:BY2									1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.889	0.000	0.000	1.000	0.000	0.000	0.000
9:KY0										1.000	0.000	0.000	0.000	0.000	0.000	0.889	0.000	0.000	1.000	0.000	0.000	0.000
10:KY1											0.000	0.000	0.000	0.000	0.000	0.889	0.000	0.000	1.000	0.000	0.000	0.000
11:KY2												0.000	1.000	1.000	0.000	0.083	1.000	1.000	0.000	0.000	0.000	0.400
12:MY0													0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
13:MY1														1.000	0.000	0.083	1.000	1.000	0.000	0.000	0.000	0.400
14:MY2															0.000	0.083	1.000	1.000	0.000	0.000	0.000	0.400
15:TY0																0.000	0.000	0.000	0.000	0.833	0.000	0.000
16:TY1																	0.083	0.083	0.889	0.000	0.000	0.000
17:TY2																		1.000	0.000	0.000	0.000	0.400
18:TY3																			0.000	0.000	0.000	0.400
19:S.c																				0.000	0.000	0.000
20:I.o																					0.000	0.000
21:D.h																						0.000
22:P.a																						

Clustering of data revealed that isolates AY0, AY2, BY2, KY0 and TY1 belonged to the same group. Again AY0, BY2, KY0 and KY1 formed a group with *S. cerevisiae* reference strain. Isolates BY0(A) and BY0(B) formed a group with *I. orientalis* reference strain. BY0(A), BY0(B), BY0(C) and TYO isolates closely related to the *I. orientalis*. Isolates KY2, MY1 and MY2 formed a group with *P. anomala* reference strain. These isolates along with TY2 and TY3 were found to be very closely related to *P. anomala* reference strain. Isolates BY1 and MY0 formed a group with *D. hansinii* reference strain.

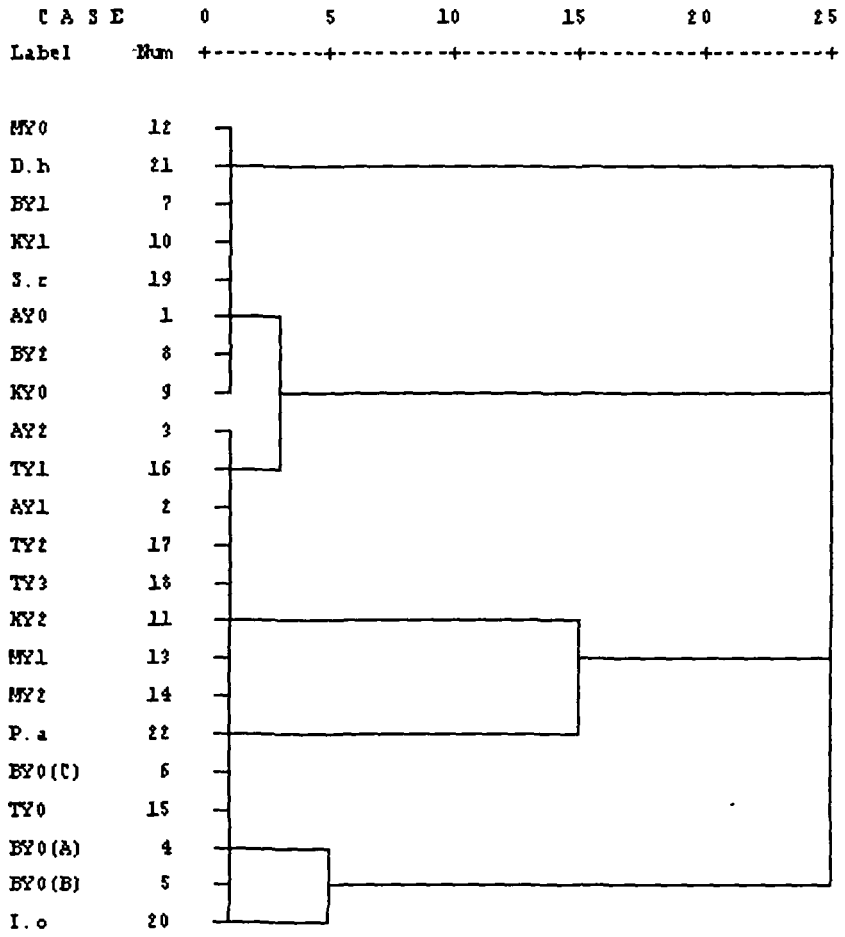


Fig. 4.11 Dendrogram using RFLP data

4.7 Experiments of yeast strains with locally available substrate

Performance of the isolated yeast strains were tested by using some locally availably substrates like common rice, *bora* (glutinous) rice, ripped banana, honey and ripped jackfruits. The isolate TY1 produced the highest amount of ethanol (6.0, 8.2, 5.8 and 5.1 %w/v) when common rice, *bora* rice, honey and jackfruit, respectively were used as the substrate. In the case of jackfruit besides TY1 and AY1, the isolate KY1 also produced the same quantity of ethanol. In the case of banana, the isolate AY0 produced the highest amount of ethanol (4.9% w/v). Results of these experiments are presented in Table 4.14 and Fig 4.12.

Table 4.18 Production of ethanol (%) by yeast strains in different locally available substrate

Strains	Ethanol production (%) by yeast isolates in substrates				
	Common rice	*Bora* rice	Honey 20% (w/v)	Jackfruit	Banana
AY0	4.5 ± 0.26	6.2 ± 0.20	5.5 ± 0.20	4.9 ± 0.30	4.9 ± 0.45
AY1	5.0 ± 0.43	5.2 ± 0.32	5.0 ± 0.02	5.1 ± 0.35	4.7 ± 0.20
AY2	4.9 ± 0.26	4.8 ± 0.40	4.9 ± 0.26	4.6 ± 0.25	4.2 ± 0.26
BY0(A)	3.6 ± 0.43	3.5 ± 0.25	3.2 ± 0.20	3. 1± 0.15	3.2 ± 0.11
BY0(B)	3.1 ± 0.20	3.4 ± 0.17	3.5 ± 0.30	2.9 ± 0.23	3.1 ± 0.20
BY0(C)	4.1 ± 0.20	3.6 ± 0.50	3.2 ± 0.25	3.3 ± 0.23	2.6 ± 0.20
BY1	3.1 ± 0.36	3.5 ± 0.20	3.1 ± 0.20	2.5 ± 0.26	2.8 ± 0.30
BY2	4.6 ± 0.43	4.7 ± 0.37	5.4 ± 0.30	4.9 ± 0.26	4.2 ± 0.30
KY0	4.9 ± 0.26	5.2 ± 0.30	5.0 ± 0.17	4.8 ± 0.26	4.0 ± 0.20
KY1	5.0 ± 0.40	5.7 ± 0.36	5.0 ± 0.17	5.1 ± 0.40	4.0 ± 0.26
KY2	4.1 ± 0.25	4.3 ± 0.26	4.5 ± 0.40	4.6 ± 0.36	3.6 ± 0.43
MY0	3.2 ± 0.30	3.2 ± 0.15	2.9 ± 0.26	3.0 ± 0.25	2.6 ± 0.20
MY1	4.2 ± 0.23	4.4 ± 0.30	3.2 ± 0.45	3.3 ± 0.41	3.8 ± 0.26
MY2	4.1± 0.11	3.2 ± 0.32	3.4 ± 0.15	3.7 ± 0.20	3.2 ± 0.41
TY0	3.5 ± 0.26	3.8 ± 0.30	3.5 ± 0.20	3.9 ± 0.40	3.0 ± 0.20
TY1	6.0 ± 0.26	8.2 ± 0.26	5.8 ± 0.30	5.1 ± 0.35	4.9 ± 0.26
TY2	4.6 ± 0.40	4.9 ± 0.36	3.3 ± 0.26	4.6 ± 0.30	3.6 ± 0.30
TY3	4.1 ± 0.30	4.3 ± 0.20	3.9 ± 0.36	4.5 ± 0.36	3.8 ± 0.37

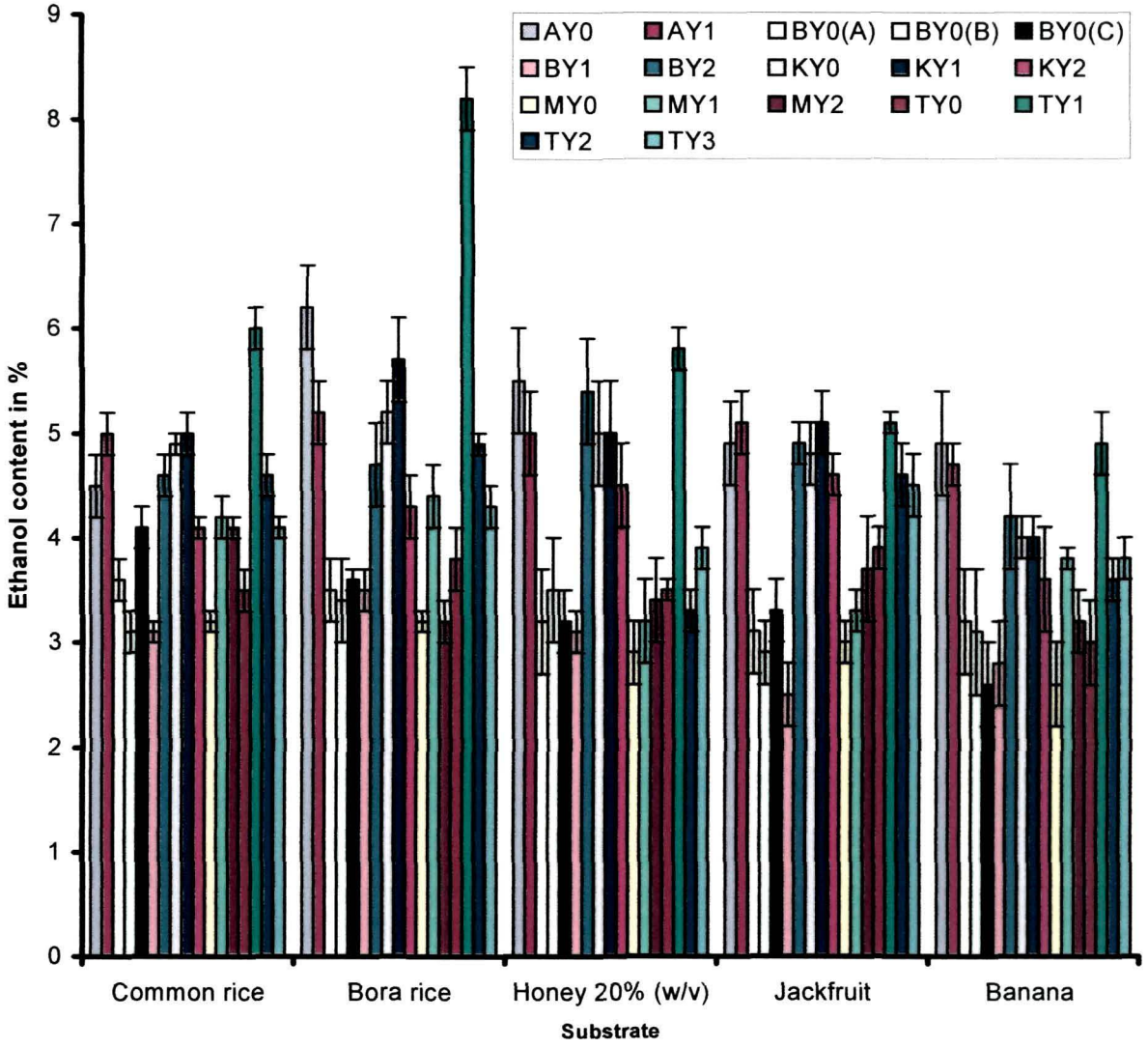


Fig. 4.12 Percentage of ethanol production by the yeast strains on different substrates

Chapter 5

Discussion

5.1 Analysis of Starter Culture

5.1.1 Collection of Starter Culture

Assam, the main state of North East India, is the habitat of several tribal communities. The Ahoms are mainly distributed in the Upper Assam district of Dibrugarh, Jorhat, Sibsagar, Tinsukia, Lakhimpur, Dhemaji, Golaghat etc; Bodos in the Lower Assam districts of Kokrajhar, Sirang, Bongaigaon, Barpeta, Baksa, Nalbari, Udalguri, Darrang and part of Sonitpur and Karbi Anglong etc. and Karbis in Karbi Anglong district. Mising tribe is distributed in the Upper Assam district of Lakhimpur, Dhemaji, Sibsagar, Sonitpur etc; Tiwa or Lalung tribe in the central Assam districts of Morigaon, Nagaon and a part of Karbi Anglong. Starter cultures were collected from the places of the traditional habitats, keeping in view the representative districts of the community concerned.

5.1.2 Determination of moisture content

Moisture content of different starter cultures studied was found to be within the range of 12.32 - 13.15% (w/w) which favours the growth and preservation of the mould and the yeast. These findings were found to be similar to that of other oriental (Asian) fermentation starter cultures (Tamang and Sarkar, 1995; Lee and Fujio, 1999).

5.1.3 Determination of pH of the starter culture

All the starter cultures were found to be acidic in nature. The pH of Karbi culture was the highest (5.72%) whereas Bodo culture was the lowest (5.25%). Such low acidic pH is generally preferred by acidophilic microorganisms like fungi and yeast. Similar findings were reported in *banhmen* and *murcha* by Lee and Fujio (1999) and Tamang and Sarkar (1995), respectively.

5.1.4 Determination of ethanol content

Samples of the end product ready for consumption were collected and analysed for the ethanol content and the same was found to be within the range of 4.6 - 5.5% (v/v). Again, the laboratory products made by using the same cultures were found to contain almost same amount of alcohol 4.5 - 6.0 % (v/v). It is probable that ethanol concentration beyond this level is toxic to the yeast itself. Several workers reported similar range of ethanol content of the traditional beer such as in *"doro"* by Madovi (1981), in *"pito"* by Bansah (1990) and in *"otika"* by Iwuoha and Eke (1996).

5.2 Isolation of microflora

5.2.1 Isolation of yeast and fungi and their population count

Serial dilution and plating in suitable media lead to the isolation of 18 yeast and 10 fungal types from the starter culture samples. The population of yeast was the highest in the case of cultures collected from Mising tribe (1.93 x 10^8 cfu.ml^{-1}) and least in Karbi culture (1.14 x 10^7 cfu.ml^{-1}). The population density of fungal isolates was the highest in the case of Ahom culture (5.50 x 10^6 cfu) and the least in the case of Karbi culture (4.80 x 10^6 cfu). These findings were in agreement with the observations made by several workers like Hadisepoetro *et al.* (1979), Del Rosario (1980), Tamang and Sarkar (1995), Hesseltine *et al.* (1988) and Le and Fujio (1999). No bacterial growth was observed during the incubation of the dilution plates. It might be due to the faster growth of yeast which prevented the growth of bacteria. However, other workers found the presence of lactic acid bacteria in amylolytic starter cultures (Hesseltine *et al.*, 1988; Tamang and Sarkar, 1995). Hesseltine (1985) and Lee and Fujio (1999) did not observe any bacterial growth during the study of fermentation starter cultures of Asian countries and microflora of *banh man*, a Vietnamese starter culture.

5.3 Morphological characterization

5.3.1 Morphological characters of yeast

Based on the morphological characters, the yeast strains were grouped in to four, group I: AY0, AY1, AY2, BY2, KY0, KY1 and TY1; group II: BY0(A), BY0(B), BY0(C) and TY0; group III : BY1 and MY0 and group IV : KY2, MY1, MY2, TY2 and TY3. Yeast isolates of group I exhibited whitish to creamy mucoidal colony morphology, spherical to oval cell with terminal budding. Smooth oval to round 1- 4 ascospores were observed in the group. Group II isolates were whitish mucoidal, oval to cylindrical with terminal budding. This group of yeasts showed simple pseudohyphae on CMA medium. Asci contained 1-2 round shaped ascospores. Group III isolates were whitish and slightly sticky and spherical to oval cells with terminal budding. Although BY1 showed the presence of simple pseudohyphae on CMA medium, no isolates showed true hyphae. Reproductive structure was persistent asci with 1 or 2 round to oval ascospores. The colonies of group IV isolates were of white to creamy coloured, spherical to oval cells with terminal budding. No isolate showed true hyphae. In CMA medium KY2, MY1 and MY2 isolates showed pseudohyphae. Asci with 4 hat-shaped ascospores were observed. Almost similar observations were reported by Lee and Fujio (1999), Tsuyoshi *et al.* (2005) and Barnett *et al.* (2000).

5.3.2 Morphological characters of fungi

Most of the fungi imperfecti including Zygomycetes were identified by observing their soma or asexual reproductive structures. The fungal strain AF1 showed tuft of whitish mycelia and ivory white to brownish colour when viewed from the reverse side of the plate containing PDA medium. Hyaline brownish sporangiophore with blackish powdery dehiscent sporangia and ellipsoidal sporangiospores were observed. Collumelae were observed to be sub-globose. No chlamydospore was observed but blackish zygospores with stellate projection observed. Good Growth was observed at 37^0 C

The fungal isolates AF2 and BF2 were whitish cottony and whitish to grayish when viewed from the reverse of the plate containing PDA medium.

These isolates exhibited brownish to yellowish sporangiophore; blackish abortive sporangia with irregular shaped sporangiospores. Collumelae were applanate and very scare chlamydospores were observed. The ideal growth temperature was found to be 37°C.

Fungal isolates BF1, KF2, MF1 and MF2 showed abundant aerial mycelia with blackish spores and greyish colour in the reverse of the plate while grown on PDA medium.

Observations on isolates KF1, TF1 and TF2 revealed brownish to yellowish scare aerial mycelial surface and yellowish or ivory white reverse surface. Sporangiophores were hyaline to brownish with dehiscent and persistent, brownish or blackish sporangia. Collumelae were ellipsoidal to globose in the case of KF1 and globose and subglobose in the case of TF1 and TF2, respectively. Sporangiospores were ellipsoidal for all 3 isolates while intercalary chlamydospores were observed in the case of KF1and TF1 isolates. Brownish to blackish zygospores were observed in all 3 isolates. Based on these morpho-physiological characteristics, all ten fungal isolates were identified to be belonging to 4 species. Isolates AF1 belonged to *Mucor indicus;* AF2 and BF2 to *Amylomyces rouxii*; BF1, KF2, MF1 and MF2 to *Rhizopus oryzae*; and KF1, TF1 and TF2 to *Mucor circinilloides*. These results were in agreement with the findings of other workers like Ellis *et al.* (1976), Schipper (1984) and Hesseltine (1991).

5.4 Biochemical test

5.4.1 Growth test with different chemicals

Most of the yeast species could be distinguished by their ability to utilize or assimilate certain organic compounds as major sources of carbon like sugars and organic acids etc. These tests were carried out to assess the ability of the isolated yeast strains to utilize certain compounds for the aerobic growth. Yeast identification and taxonomy are, in many instances, still open problems, especially when it concerns natural isolates, owing to the poor reliability of the conventional classification methods based mainly on morphology of asci or

ascospores, fermentation of sugar and assimilation of carbon or nitrogen compounds. This led in the past to the repeated appearance or disappearance of species or genera as in the case of species *S. bayanus* and the genus *Zygosaccharomyces*. But, yeast identification still remains to be conventional as supporting means of modern molecular biological technique.

In this investigation yeast isolates were mainly characterized on the basis of result of 99 biochemical or physiological tests along with summaries of microscopical appearance and information on the sexual reproduction. This method of identification of yeast was followed by Barnett *et al.* (2000). The tests used for the identification of yeast isolates included the assimilation of carbon, aerobic growth test with nitrogen sources, semi-anaerobic fermentation of sugars, requirement for the exogenous supply of certain vitamins, growth at high osmotic pressure and at different temperatures, in the presence of cycloheximide, urea hydrolysis and diazonium Blue B test. The conventional dichotomous identification keys were followed. These keys were based on results of the tests and observations on each species listed by Barnett *et al.* (2000).

No yeast ferments sugar unless it also ferments D-glucose and all yeast that ferments glucose also ferments D-fructose and D-mannose. This generalized conclusion was made by Kluyver (1931) much before it was biochemically explained.

5.5 Identification of yeast isolates

5.5.1 Identification of Group I isolates

By following the key no. 2 employed for the identification of yeast that fermented D-glucose, isolates AY0, AY1, AY2 and BY2 were found to be either *S. cerevisiae* or *S. paradoxus*. It was confirmed as *S. cerevisiae* as it could grow in vitamin-free medium whereas *S. paradoxus* could not grow on the same medium. Similar observation was reported by Jespersen *et al.*, (2000). Again by following the same key, isolates KY0, KY1 and TY1 were identified as *S.bayanus, S. cerevisiae, S. pastorianus or S. microellipsoides.* All these species,

except *S. microellipsoides,* belonged to *Saccharomyces sensu stricto group.* Based on the same key provided by Barnett *et al.* (2000) these species could not be distinguished from each other.

5.5.2 Identification of Group II isolates

The yeast isolates BY0(A), BY0(B), BY0(C) and TY0 were identified as *Issatchenkia orientalis*. Based on Barnett *et al.* (2000) key no. 2 these were initially identified either as *I. occidentalis or I. orientalis*. But, these two species could be differentiated on the basis of growth on media without biotin. *I. occidentalis* showed positive growth whereas *I. orientalis* negative growth on the medium. In the present study, all isolates showed negative result in the test, as such the isolates were identified as *I. orientalis*.

5.5.3 Identification of Group III isolates

The yeast isolates BY1 and MY0 were initially identified either as *Candida saitoana, Debaryomyces. hansinii, D. maramus* or *D. nepalensis.* These isolates did not belong to *Candida saitoana* as the species did not produce ascospores. In the present study, these isolates showed ascospores. Nevertheless, these isolates were identified as either *D. hansinii, D. maramus* or *D. nepalensis* as they could not be distinguished from each other based on the morphological and biological tests Barnett *et al.* (2000).

5.5.4 Identification of Group IV isolates

The yeast isolates KY2, TY2 and TY3 of this group were identified as *Pichia anomala*. Isolates MY1 and MY2 were identified as either *P. anomala* or *P. sydowiorum* based on the keys of Barnett *et al.* (2000).These two species were indistinguishable from each other.

5.6 Molecular genetic assessment

5.6.1 Genomic DNA isolation and purification

Genomic DNA obtained through the modified protocol was of better quality than that obtained through the original protocol of Harju *et al.* (2004). In the study, instead of ice cold ethanol, the liquid nitrogen was used in the lysis of the yeast cells for the genomic DNA isolation. Spectrophotometric measurement

of the isolated DNA samples at 260 nm and 280 nm resulted a desirable absorbance ratio (OD_{260}/ OD_{280}) of 1.76 - 1.90 indicating an insignificant level of contamination by protein, RNA or other organics. Therefore, the modified protocol might be considered as effective for obtaining high yield of quality DNA.

5.6.2 Electrophoresis of yeast genomic DNA

The isolated and purified genomic DNAs of the yeast isolates were electrophoresed in 0.7% agarose gel. The size of the genomic DNA of the yeast isolates did not vary significantly. The average size of the genomic DNA was found to be higher than that of 23.13 kb.

5.6.3 PCR-based RAPD profiling of yeast genomic DNA

Analysis of the RAPD-PCR data with the help of SPSS software package helped in grouping the yeast isolates into two broad groups. The first group included isolates AY0, AY1, AY2, BY1, BY2, KY0, KY1, MY0 and TY1. The other group included BY0(A), BY0(B), KY2, MY1, MY2, TY0, TY2 and TY3. Isolates BY0(C) clubbed with BY0 (A) and TY0 of the second group formed a small group. Within the first group AY1, AY2, BY2 and KY0 formed another small group. In this classification, isolates BY1 and MY0 of group III based on the morphological criteria were close to group I. Except BY0(C) all other isolates of the morphological group II and IV formed the second group based on the RAPD-PCR.

5.6.4 ITS-PCR of genomic DNA

ITS spacer region and 5.8S region was amplified using the primer ITS1 and ITS4. The amplified products were separated by electrophoresis in 1.2% (w/v) agarose gel. The resultant fragments were found to be 825-850 bp for AY0. AY1, AY2, BY2, KY0, KY1 and TY1; 600-640 bp for KY2, MY1, MY2, TY2 and TY3; 500-525 bp for BY0(A), BY0(B), BY0(C) and TY0; and 475 bp for BY1 and MY0. Based on the ITS-PCR band sizes of the genomic DNA, yeast isolates belonging to group I were identified as *S. cerevisiae*, group II as *I. orientalis*, group III as *D. hansinii* and group IV as *P. anomala*. Sujaya *et al.*

(2004) also found similar results while studying yeast isolates isolated from *brem.*

5.6.5 PCR- RFLP profiles of yeast isolates

Studies on the banding patterns and clustering of the data obtained from PCR-RFLP revealed that the isolates AY0, AY2, BY2, KY0 and TY1 belonged to the same group. Again AY0, BY2, KY0 and KY1 formed a group with *S. cerevisiae* reference strain. Isolates BY0(A) and BY0(B) formed a group with *I. orientalis* reference strain. BY0(A), BY0(B), BY0(C) and TYO isolates closely related to *I. orientalis*. Isolates KY2, MY1 and MY2 formed a group with *P. anomala* reference strain. These isolates along with TY2 and TY3 were very closely related to *P. anomala* reference strain. Isolates BY1 and MY0 formed a group with *D. hansinii* reference strain. These studies were carried out by Granchi *et al.* (1999) and Naumova *et al.* (2003) and confirmed the identification of yeast strains. It was imperative to the RFLP analysis of the ITS-spacer and 5.8S rRNA gene region that was shown to be fast and reliable method for the identification of yeasts. These findings were also in conformity with Esteve-Zarzso *et al.* (1999).

It was observed that either for the identification and characterization of the yeast, or for the differentiation among strains of *S cerevisiae* flor yeast, the analysis of physiological abilities was not a reliable method (Esteve-Zarzoso, 2004).

5.6.6 Experiments of yeast strains with locally available substrates

The yeast isolates were cultured on some locally available substrates like common and glutinous rice, honey, jackfruit and banana to determine their efficiency to produce ethanol. The isolate TY1 produced the highest amount of ethanol (6.0, 8.2, 5.8 and 5.1% w/v, respectively) when common rice, *bora* rice, honey and jackfruit, respectively were used as the substrate. Thus, the isolate could be the most efficient among all isolates. Thus, production of ethanol with the use of the isolate TY1 as a single starter compared favourably with that of the traditional brew. These findings were supported by the findings of Sefa-Dedeh *et al.*(1999). In the case of jackfruit, besides the isolate AY1; isolates

KY1 and TY1 also produced the same quantity of ethanol. In the case of banana, the isolate AY0 produced the highest amount of ethanol (4.9% w/v). Kuboye *et al.* (1978) found similar result using the over ripen banana as the substrate for *agadagidi* production. This might be due to the preference by AY0, AY1, KY1 and TY1 of sugars like fructose present in these fruits.

Chapter 6

Conclusion and future works

6.1 Conclusion

From the present investigation the following conclusions have been drawn:

1. The starter cultures collected from tribal communities were acidic in nature and its moisture content favours preservation of the yeast and mould.
2. Ethanol content in the finished products collected from the sites ranged from 4.6-5.5% (v/v) and that of laboratory products 4.5 - 6.0% (v/v).
3. The yeast and fungal load (cfu) of the starter culture was found to be 1.14×10^7 - 1.93×10^8 and 4.80×10^6 - 5.50×10^6, respectively. Altogether 18 isolates of yeasts and 10 isolates of fungi were obtained from 5 starter cultures collected.
4. Based on morphological characters, yeast isolates were grouped in to 4 and fungal isolates identified belonging to 4 species.
5. Yeast isolates were subjected to assimilation tests, growth in nitrogen sources, semi-anaerobic fermentation tests, growth at different temperatures, high osmotic pressure, in the presence of cycloheximide or actidione and urea hydrolysis etc for the identification.
6. Based on the morphological and biochemical tests, yeasts isolates were identified as follows: AY0, AY1, AY2 and BY2 as *Saccharomyces cerevisiae,* KY0, KY1 and TY1 as *S. bayanus, S. cerevisiae* or *S. pastorianus*, BY0(A), BY0(B), BY0(C) and TY0 as *Issatchenkia orientalis*, BY1 and MY0 as either *Debaryomyces hansenii, D. maramus* or *D. nepalensis*, and MY1 and MY2 as either *Pichia anomala* or *P. sydowiorum.*

7. A modified protocol was standardized to obtain high yield of quality genomic DNA.
8. RAPD-PCR based grouping of the yeast isolates at the molecular level could not coroborate the morphological grouping, but ITS-PCR profiles could.
9. PCR-RFLP profile study and comparison of it with that of the reference strains allowed to identify the yeast strains belonging to four different species.
10. The yeast strains AY0, AY1, KY1 and TY1 (*S. cerevisiae*) were found to be potential ethanol producer while used on various locally available raw materials.

6.2 Future works

Starter cultures used by all other ethnic tribes of the region might be investigated to enhance the knowledge base. Each and every yeast, fungus and bacterium should be characterized and preserved to establish their biotechnological worth.

More primers and oligonucleotides might be used to study the yeast strains of the region. Extensive molecular characterization might be taken up with a large number of primers and oligonucleotides to cover the yeast strains of the entire region.

Attempts should be made to transform the traditional beer production art into a technology-based small scale industry incorporating objective-based method of process control.

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* Original not seen