NTNU

Norwegian University of
Science and Technology

Isolation and genotypic characterization of different yeast strains from Nepalese mixed culture (Murcha)

Kshitiz Bhusal

Biotechnology
Submission date: December 2017
Supervisor: Per Bruheim, IBT

Norwegian University of Science and Technology
Department of Biotechnology and Food Science

Foreword

This master thesis was delivered at the Department of Biotechnology and Food Science at the faculty of Natural Science and Technology at Norwegian University of Science and Technology (NTNU) on 31st of December 2017.

I would like to thank my main supervisor Per Bruheim who has been very supportive and helpful in every problematic situation. His immense support and motivation at the time I had been dragging behind due to health condition mean a lot to me. Likewise, I am also grateful to the support from student advisor Jo Esten Hafsmo, who had been helpful and supportive during all the administrative work during sick leave and until the submission.

I would also like to thank my co-supervisor Olav Vadstein and Ingrid Bakke for their knowledge and help during lab works. An exceptional thanks to Truls Rasmussen for guiding me during lab work and helping me with sequence analysis and Clone Manager and, Zdenka Bartosova for her guidance in headspace GC-MS and HPLC. I would also like to remember the help provided by Silje Malene Olsen during synthetic must preparation.

I would also like to thank my family and friends, who motivated and pushed me to finish my master's degree.

Declaration of Compliance

I declare that this is an independent work according to the exam regulations of the Norwegian University of Science and Technology (NTNU).

Trondheim, December 31, 2017
Kshitiz Bhusal

Abstract

14 different yeast strains were isolated from the Nepalese yeast cakes (murcha) of six different places varying in geographical condition. The isolation was entirely random based on the morphologies of the colonies, were genotypically characterized using the ITS and LSU region. The fermentation was carried out with modified synthetic must and concentrations of sugars, acid and ethanol was analyzed using HPLC and composition of volatiles were determined using headspace GC-MS. A genetic analysis was made using the internal transcribed spacer (ITS) sequencing to identify the species and establish genetic relationships between the strains. The isolated strains were classified as Vanderwaltozyma, Wickerhamomyces and Saccharomyces after LSU sequencing.

Candida inconspicua from Baglung showed lesser gravity fall during fermentation. Maximum utilization of sugars was shown by Saccharomyces while strains of Saccharomycopsis utilized less sugars. Thus, establishing themselves with high and low ethanol producers respectively. The production of ester compounds is found to be very less except for ethyl acetate. Ethyl acetate production was observed very high in most of the strains.

Table of Contents

Contents

Foreword ii
Declaration of Compliance ii
Abstract iv
Table of Contents v
List of Tables vii
List of Figures viii
Abbreviations ix
1. INTRODUCTION 1
2. Theory 1
2.1 History of Alcohol in Nepal 1
2.2 Home-made alcoholic beverages in Nepal 2
2.3 Murcha/Marcha (Yeast cakes) 3
2.4 Yeast phylogeny 4
2.5 Polymerase Chain Reaction 5
2.6 Fermentation 5
2.7 High-Performance Liquid Chromatography (HPLC) 7
2.8 Headspace Gas Chromatography-Mass Spectrometry 7
3.1 Sample Collection 9
3.2 Reviving of the dried yeast 9
3.3 Freeze Stock for the Original Sample 9
3.4 Growth Rate 10
3.5 Genetic Analysis 10
3.5.1 DNA Isolation 10
3.5.2 Polymerase chain reaction 11
3.5.3 Post-PCR 13

3.5.4 PCR cleanup 13
3.5.5 Analyzing the sequence data 14
3.6 Fermentability Test 14
3.7 Analysis of Volatile and Non-volatile Compounds 16
3.7.1 Headspace Gas Chromatography-Mass Spectrometry 16
3.7.1.1 Preparation of Internal Standard 17
3.7.1.2 Sample 18
3.7.1.3 Quantification of compounds 19
3.7.2 High-Performance Liquid Chromatography 19
3.7.2.1 Preparation of Standard mix 19
3.7.2.2 Sample 20
4.1 Isolation 21
4.2 Genotypic Characterization 23
4.3 Fermentation 25
4.4 Sugar, Alcohol and Organic Acid Quantification with HPLC 27
4.5 Headspace GC-MS 30
5.1 Species identification based on ITS and LSU sequencing 34
5.2 Fermentation 35
5.3 Alcohol, ester and acid production 35
References: 38

List of Tables

Table 3.1 PCR primers used for ITS and LSU sequencing along with the melting temperature. 10
Table 3.2: PCR reaction volume [µL] used for different reagents for single sample in ITS amplification 11
Table 3.3: PCR reaction volume [µL] used for different reagents for single sample in LSU amplification 12
Table 4.1 Species identification through RDP database for consensus ITS region 24
Table 4.2 Species identification through RDP database for consensus LSU region 25
Table 4.3 Sugar, alcohol and organic acid quantification with HPLC for all yeast samples 27
Table 4.4: Quantification of volatile compounds by headspace GC-MS for the samples (part a). 30
Table 4.5: Quantification of volatile compounds by headspace GC-MS for the samples (part b). 31

List of Figures

Figure 2.1 Map of rRNA gene and the ITS region along with SSU and LSU region. 4
Figure 2.2 Overview of metabolic activities of Saccharomyces during fermentation 6
Figure 3.3: PCR program ran for ITS region 12
Figure 3.4:PCR program ran for LSU region 13
Figure 4.1: Map of Nepal showing the places of the sample collection.. 21
Figure 4.2 Microscopic photos taken of some yeast strains from different places of Nepal. 22
Figure 4.3 A phylogenetic tree constructed from consensus sequence of ITS region 23
Figure 4.4 Fall in specific gravity over time (hours) of eight yeast samples 26
Figure 4.5 Fall in specific gravity over time (hours) of seven yeast samples 26
Figure 4.6 Consumption of glucose by S.cerevisiae and C.fabianii 28
Figure 4.7 Consumption of fructose by S.cerevisiae and C.fabianii 29
Figure 4.8 Production of ethanol by S.cerevisiae and C.fabianii 29
Figure 4.9 Concentration (mg/L) of major compounds detected in headspace GC-MS for C. fabianii from various places 32
Figure 4.10 Concentration (mg/L) of major compounds detected in headspace GC-MS grouped together 33

Abbreviations

BKT Bhaktapur

BLAST Basic Local Alignment Search Tool

BLG Baglung

DRN Dharan

ESTD External standard

EtOH Ethanol

GC Gas chromatography

HPLC High-performance liquid chromatography

HS GC-MS Headspace gas chromatography-mass spectrometry

ISTD Internal standard

ITS Internal transcribed spacer

KTR Kritipur

LAB Lactic acid bacteria

LC Liquid chromatography

LOQ Limit of quantification

LSU Large subunit

m/z Mass-to-charge ratio

ME Malt extract

MQ Milli-Q

MRS DeMan, Rogosa and Sharpe

MS Mass spectrometry

MSA Multiple sequence alignment

MTG Mustang

NPY Nepalese Yeast

OD Optical density

PCA Principal component analysis

PCR Polymerase chain reaction

PKR Pokhara

ppm Parts per million

QC Quality control

RDP Ribosomal Database Project

RT Retention time

SSU Small subunit

WA Wort Agar

1. INTRODUCTION

Sandwiched between India and China, a small land-locked country, Nepal is however very much diverse in geography and socio-cultural traditions. Within the average distance of 193 kilometers of north-south distance, altitude varies from 60 meters to 8848 meters. There are 126 caste/ethnic groups speaking 123 different languages at present [(Statistics 2012)].

Each ethnic group follows their unique tradition and festivals, celebrated with their unique style of food and beverages. Some of the groups also produce their specific fermentative alcohol used in their festivals. They ferment different substrate like maize, rice, wheat, millet, etc. to create many varieties of un-distilled and distilled alcoholic drinks. These alcohols are mostly popular among the people who cannot afford expensive bottled drinks.

Many incidents including the death of people drinking local alcohols had also been reported in the past years. But production from such local areas has not ended, and large mass is still drinking these local products.

There has been very less research regarding these yeasts cakes from Nepal. There has always been a quest in search of new yeast varieties. Through this study, we could assume to explore at least some unique characteristics in yeast. Thus, samples from the diverse ethnic groups and geographical location were taken as much possible. It is of interest to isolate, characterize and allow them to ferment and analyze the fermented products through HPLC and head-space GC-MS.

2. Theory

2.1 History of Alcohol in Nepal

The very first thing we must understand is *Caste* system when it comes to consumption and usage of alcohol in Nepal. During the rule of King Jayasthiti Malla (1382-95 A.D), he tried to classify people based on the type of occupation. The working castes ‘sudhras’ could drink, but the Brahmins (priest), Chhettri (warrior) and Baishya (traders) were prohibited to drink according to the caste system. (Subba et al., 1995) [(Dhital, Subedi et al. 2001)]

The different ethnic groups like Tharus, Gurungs, Tamangs, Newars, Rais, Limbus, etc come under working castes. Although there is no inscription of usage of beverages from Licchhavi period, in the middle period Newars started serving alcohol (Madira) among food items and rituals. (Vaidya, 1993)

The use of foreign alcohol was known to be popular after the visit of Janga Bahadur Rana (prime minister) to England in 1850. However, it was only limited to the noble class and people at the powerful political position.

Nepali soldiers recruited in the British and Singapore Army and remarkable involvement in First and Second World war was the prominent reason for popularizing the drinking culture. This had highly inspired both the rural and urban population of some ethnic group to establish *Bhatti* (traditional pub) as a source of income. Later, it became very common to youths to join Indian, Myanmar, Singapore armed forced. The traveling of general people to the outside world in search of jobs or education started transforming society gradually, and they began accepting drinking. ((Shah 2000)cited in (Dhital, Subedi, et al. 2001)

Later, the establishment of Mahendra Sugar Mills and Jawalakhel Distillery in 1963 and 1972 respectively, industrial production of alcoholic beverages was commenced which was boosted by democratic movement of 1989.

2.2 Home-made alcoholic beverages in Nepal

The homemade alcoholic beverages can be categorized into two types: Non-distilled mild alcoholic beverages and Distilled Alcoholic beverages. *Jand, Chhyang, Tumba* are the different type of undistilled beverages whereas *Local Raksi* is a common name for distilled alcohol across the country [Thapa N, 2015].

The essential stages in this solid-state fermentation while preparing undistilled beverages are liquefaction and saccharification of cereal starch provided by filamentous molds and yeasts that are present in murcha [J.P, 2010c]. The former being carried out mainly by *Rhizopus spp*. and *Sachharomycopsis fibuligera* and the later by *Sachharomycopsis fibuligera* and *S. cerevisiae* (Thapa and Tamang, 2006). These undistilled beverages are taken as high-calorie food in the Himalayan regions of Nepal (Thapa and Tamang, 2004).

Raksi is an ethnic alcoholic beverage that is prepared by distilling fermented cereal beverages using murcha. It is known to be the drink of the Himalayas with the aromatic characteristic with an alcohol content of 22–27% (v/v) [M. Kozaki,

2000]. Aeylaa is a strong distilled alcohol mainly prepared by Newar community and served during their rituals and street festivals

The alcohol content of beverages in Nepal varies with the ingredients, place of production and ethnicity of people. It has been observed that ethanol concentration of both distilled and non-distilled alcohol vary a lot in samples. From some study, it has been found to be less than 40% and 18.9% for distilled and non-distilled respectively [Thapa N, 2015].

2.3 Murcha/Marcha (Yeast cakes)

Murcha or Marcha are the indigenous starters that are used to ferment the locally produced beverages in Nepal. These are known by different names according to ethnic groups and mostly prepared by Rai, Limbu, Tamang, Gurung, Newar, and Tharu communities in Nepal. With the long history of the existence of these starters, there is no evidence of the time it gets started. The ingredients and process of making starters is kept secret that not daughters, only daughters-in-law are taught.

This starter culture is a mixed type which comprises various saccharifying molds, fermentative yeast, and acidifying lactic acid bacteria where the loads were found to be 10^6 CFU/g, 10^8 CFU/g, and 10^7 CFU/g respectively (Karki 1986), (J. P. Tamang 1995).

The general method of preparation involves soaking of rice (*Oryza sativa*) in water for 8-10 hours and crushing into fine powder. Mixing of different ingredients, like roots, leaves, and flowers from various *murcha* plants, ginger, dry red chili and previously prepared *murcha* is performed. Water is added to the mixture and kneaded into cakes of different shape and size. Then, they are kept for fermentation for 1-3 days. The distinctive aroma and swollen structure of cakes specify the end of fermentation which is sun-dried for next few days.

Among the 42 plants known to be used in the preparation of these starters, *Plumbago zeylanica, Buddleja asiatica, Vernonia cinerea, Polygala arillata,* etc. are major murcha plants. The use of plants in *marcha* is based on the specific ethnic groups and distribution of plants (J.B. KC 2001).

Although various groups of microorganisms are present, selective growth of desirable microflora is crucial that includes S.cerevisiae, S. fibuligera, C. versatilis, Rhizopus sps and P. pentosaceus (Shrestha et al.2002). The two main types of *marcha* are *Mana* and *Manapu.*

2.4 Yeast phylogeny

Fungi are known to be the most diverse kingdom on earth including more than 1.5 million species of the ecosystem. They are an integral part of the environment, acts as decomposers, governs carbon cycling, plant nutrition and behave like a parasite to various organisms. (Toju, 2012)

From the gene sequence analysis of yeast's 18s ribosomal DNA, internal transcribed spacer (ITS), s26 rDNA and cytochrome oxidase II reveals a vast difference in phenotypic character and genotype. Thus, creating conflict upon the current classification of yeast species into genera and families which is majorly based on the morphology of vegetative cells and sexual states. (Kurtzman, 2012)

At present, ITS regions have been the dominant tool for sequencing and basis for identification of fungal sequences in community ecology (Peay et al., 2008) & (Schoch et al., 2012). It has also been beneficial in the identification of fungi at the species level, although proper consideration is required to analyze data due to its hyper-variable nature (Nilsson et al., 2012). So, small subunit (SSU) or large subunit (LSU) sequences of rRNA gene complex are taken as alternatives to ITS regions.

The figure below shows the map of ITS region in rRNA genes that lie between SSU and LSU. 5.8S gene divides ITS region into two segments ITS1 and ITS2. Although it can be amplified as a single sequence with the primers ITS-F KYO2 and ITS4 (Kurtzman, 2012), the same primers we have used during the PCR work.

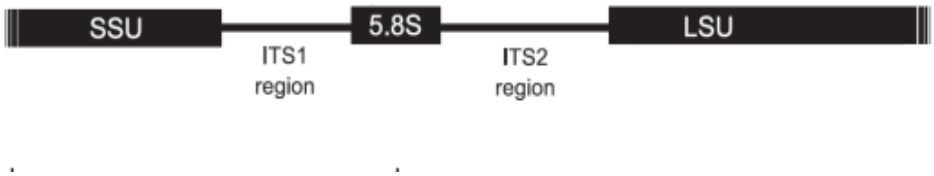


Figure 2.1 Map of rRNA gene and the ITS region along with SSU and LSU region.

2.5 Polymerase Chain Reaction

The process of PCR cycle is explained in major three steps: denaturation, annealing, and extension. At first step, the template DNA is allowed to denature by heating. Secondly, the specific primers bind to the template strands that are complementary at the annealing temperature. Thirdly, the primers are then elongated to the long chain by DNA polymerase. The chain elongation is always performed from 5' to 3', where new nucleotides are added to free 3'-OH ends. The most commonly used DNA polymerase enzyme is extracted from thermophilic bacteria *Thermus aquaticus* which is stable at high temperature. The important parameter in PCR cycle is a temperature which is controlled at every step. (Allison, 2007).

2.6 Fermentation

Living organisms undergo cellular respiration to produce chemical energy, which is obtained in the form of adenosine triphosphate (ATP). This energy-producing process may or may not require oxygen, some organisms like yeast (facultative anaerobes) could produce energy in both condition. In the presence of oxygen, it performs cellular respiration whereas undergoes alcohol fermentation in its absence. (Campbell et al., 2008)

Fermentation is a balanced redox reaction in which single substrate of different proportion oxidize and reduce to produce energy, without the presence of external electron acceptor. (Todd, 1999). Energy yield through oxidative phosphorylation is always more, thus is a preferred pathway. In the absence of oxygen, yeast switch to fermentative metabolism where sugars are converted to intermediate compound pyruvate, which ultimately yields CO_2 and ethanol. (Madigan et al., 2015)

Yeast also excretes various compounds during fermentation along with ethanol and carbon dioxide. The figure will show a schematic summary of main metabolic pathways during beer fermentation, which can also be correlated to any fermentation cycle. (Bokulich. & Bamforth, 2013)

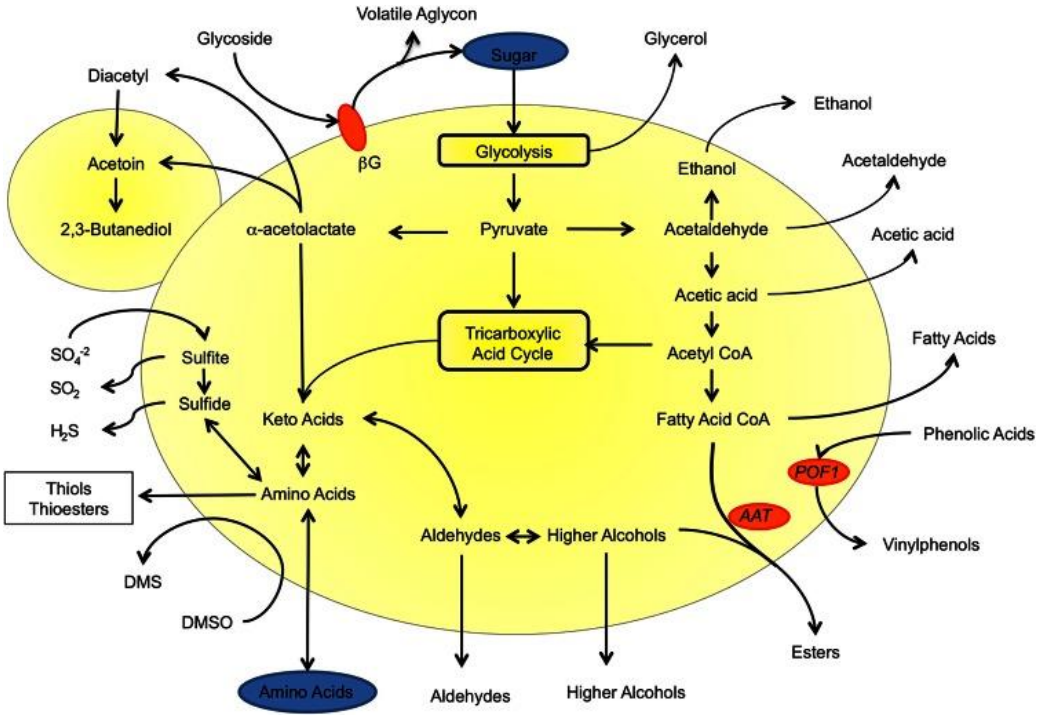


Figure 2.2 Overview of metabolic activities of Saccharomyces during fermentation

Despite the difference in the concentration, almost all species of yeast produces the substances like glycerol, vicinal diketones alcohols, esters, organic acids, fatty acids and various sulfur-containing compounds. These parameters vary depending upon the fermentation conditions like pitching rate, temperature, duration of fermentation, etc. (Bokulich. & Bamforth, 2013).

A range of esters is also produced by the action of alcohol acetyltransferase enzyme on higher alcohols and acetyl-coenzyme A (acetyl-CoA) (Procopio et al. 2011 & Verstrepen et al., 2003). Amount of oxygen and unsaturated fatty acids in fermenting substrate plays an important role in this. (Moonjai et al., 2002)

2.7 High-Performance Liquid Chromatography (HPLC)

Chromatography is a technique to separate the different analyte from a mixture. This method allows to separate different analyte creating a difference in retention time of in the solution, which is fed as a mobile phase to pass through the stationary phase. Thus, various molecules are eluted at different retention time, which is recognized by the detectors (Moldoveanu & David, 2013).

In HPLC, the stationary phase is a column packed with small porous particle where the liquid mobile phase is introduced in a high pressure by a pump. The detectors used in the process are of two types: refractive index (RI) and ultraviolet (UV) detectors (Moldoveanu & David, 2013). Carbohydrates being non-fluorescent and low absorption with UV makes it suitable to detect with RI detectors (Lethonen & Hurme, 1994). Poor selectivity, lack of sensitivity and dependency in various factors like temperature and flow rate are drawbacks of RI detectors despite its popularity (Ferreira, 2009). Organic acids are analyzed using UV detectors at 200-210nm. Moreover, there could be significant interference from other organic substance and difficulty in quantifying accurately, unless samples are very pure. (Shimadzu protocol)

2.8 Headspace Gas Chromatography-Mass Spectrometry

The headspace GC-MS method is used to determine the concentration and analysis of the volatile organic compounds. It also follows the universal principle of chromatography where the gaseous analyte is transported through the column by a carrier gas (mobile phase) (Harris, 2010).

The space within the sealed vials with the analyte is headspace which will be transformed into a vapor phase by heating. The preset temperature of the system will convert the analyte into the vapor phase, reaches the equilibrium, and then the sample is analyzed (Sithersingh & Snow, 2012). A certain amount of vapor is extracted from the headspace in equilibrium and transferred to a column by an inert carrier gas (generally Helium) in a split ratio to increase resolution. Thus, volatiles in the headspace is separated by their boiling point. The Mass Spectrometry (MS) that are the detectors provide the mass-to-charge ratio (m/z) of the ions continuously that are separated (Grayson, 2016).

After samples are separated from the chromatography column, they pass through a mass spectrometer, where they are ionized and fragmented by the ion source. The bombardment by energetic electrons, ionize the molecule and allows ions to fragment when further bombarded. Then, ions pass through mass analyzer where they are sorted by m/z ratio (Uggerud et al., 2003).

By knowing the concentration of samples used in the internal samples, the concentration of the compound in the sample can be measured. The internal standard method also corrects for losses during separation and measuring concentration. Thus, it is referred as the most accurate method for quantification in GC-MS (Sparkman, Penton, & Kitson, 2011).

3.**Methods**

3.1 Sample Collection

The yeast cakes (*murcha*) were collected from different places varying in geographical location ranging from low altitude to higher mountain ranges. The famous local alcohol producers (i.e., based on a recommendation from people nearby) were visited, dried yeast cakes were collected, and transferred into the sterile vials (VWR 89039-656)**.**

3.2 Reviving of the dried yeast

The yeast cakes were crushed into the fine powder within the collection tubes with the sterile rods, 1 and 2 grams (approximately) transferred into two different tubes with 10mL of sterile water. The observation of time zero was made through the microscope. Later, tubes with samples were incubated at 110 rpm and 22^0C. After 4 hours, one mL of each sample 1 and 2 were transferred into fresh liquid 5% Malt Extract medium. The growth of the cells was measured by Optical Density (OD_{600}).

3.3 Freeze Stock for the Original Sample

The cultures were grown for 48 hours in 5 % ME medium in incubator flasks (Bellco 250 mL at 22˚C at 180 rpm (Infors AG CTA-41+ Bottminsen Minitron incubator). All content was transferred to sterile 50 mL centrifuge tubes (VWR 89039-656). The tubes were centrifuged (Eppendorf Centrifuge 5804R) for 5 minutes at 3000 rpm. The supernatant was discarded, and seven mL fresh sterile 5 % ME medium was added to the centrifuge tube with 3 mL sterile Glycerol (Sigma-Aldrich), performed in sterile bench. The yeast was resuspended in the ME/glycerol mixture, and aliquoted in Cryotubes (Thermo Scientific CryoTubeTM Nunc 375353) and stored at -80^0C.

3.4 Growth Rate

250 µL of freeze stock original sample were inoculated in 30 mL 5 % sterile ME medium. The optical density was measured in a spectrometer (VWR V-1200) at 600 nm with disposable cuvettes (Plastibrand 1.5 mL Semi-Micro Disposable cuvettes 7590 15) every 60 minutes. If the spectrometric reading obtained was beyond the linear range, the samples were diluted with 5 % non-sterile ME medium.

3.5 Genetic Analysis

3.5.1 DNA Isolation

After growth of yeast cells (48-72 h), they were subjected to DNA isolation using the MO BIO DNA isolation kit (PowerSoil 12888-100). The concentration of isolated DNA was measured with NanoDrop before amplification with PCR. The amplification of entire ITS region was carried out using forward primer ITS-F KYO2 (used in ITS1 region) and reverse primer ITS4 (used in ITS2 region). The amplification of LSU region was carried out using forward primer NL-F and reverse primer LSU-R.

Table 3.1 PCR primers used for ITS and LSU sequencing along with the melting temperature.

Name[a]	Primer Sequence	Tm [^{0}C]
ITS-F_KYO2	5'-TAGAGGAAGTAAAAGTCGTAA-3'	52.6
ITS4-R	5'-TCCTCCGCTTATTGATATGC-3'	61.5
NL-F	5'-TTGCCTTAGTAACGGCGA-3'	62.0
LSU-R	5'-TTGTGCACCTCTTGCGAG-3'	64.1

a F = forward; R = reverse.
b The melting temperature, Tm, was calculated by the supplier Sigma-Aldrich using the nearest neighbor method (Rychlik, Spencer, & Rhoads, 1990).

3.5.2 Polymerase chain reaction

Thermo Scientific Arktik Thermal Cycler PCR apparatus was used, and the PCR protocol programmed into the apparatus for ITS region is given in Table 3.2 and Figure 3.1 and LSU region.

Table 3.2: PCR reaction volume [µL] used for different reagents for single sample in ITS amplification

Name	Volume(µL)	End concentration
10X Reaction buffer with $MgCl_2$	2.5	1X
10mM dNTP	0.5	200 µM
BSA	0.75	
100 µM ITS-F_KYO2 Primer	0.125	0.5 µM
100 µM ITS4-R Primer	0.125	0.5 µM
Taq DNA Polymerase	0.125	
DNA template	2.5	1 ng/ µL
Filtered MQ-water	18.375	
Total	25.0	

A master mix was created by mixing all above items besides DNA template and divided equally into each PCR tubes within chilled trays. The tubes were marked, and finally, DNA template was added. The tubes were centrifuged (Galaxy Mini, VWR) for few seconds to settle down the samples attached to the walls and to remove air bubbles. For negative control, tubes without DNA-template were also placed with the samples in each run.

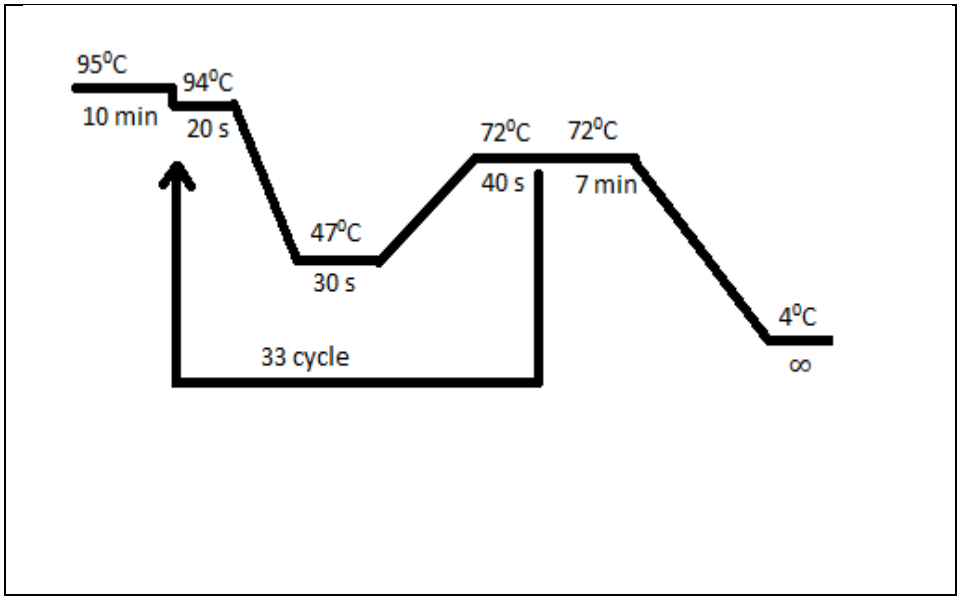


Figure 3.3: PCR program ran for ITS region

Table 3.3: PCR reaction volume [µL] used for different reagents for single sample in LSU amplification

Name	Volume	End concentration
10X Reaction buffer with $MgCl_2$	2.5	1X
10mM dNTP	0.5	200 µM
BSA	0.75	
100 µM NL-F Primer	0.125	0.5 µM
100 µM LSU-R Primer	0.125	0.5 µM
Taq DNA Polymerase	0.125	
DNA template	5.0	2 ng/ µL
Filtered MQ-water	15.875	
Total	25.0	

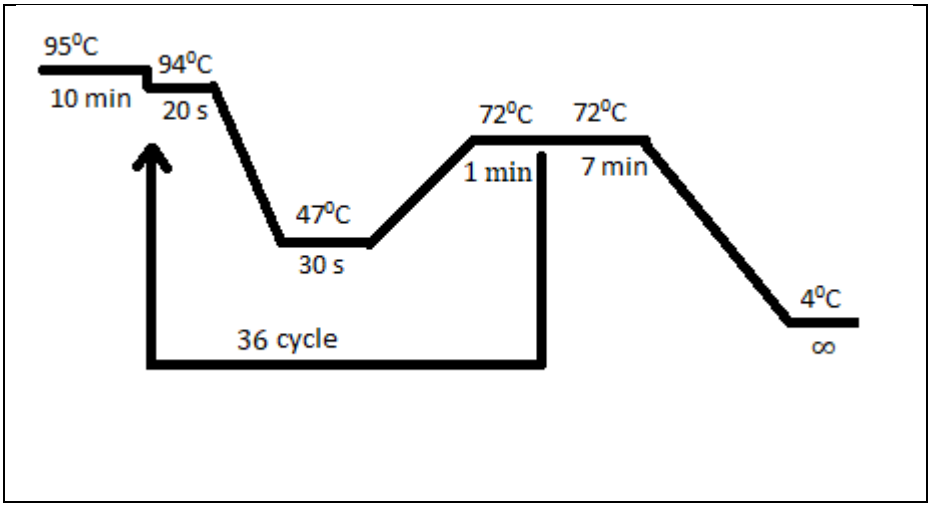


Figure 3.4:PCR program ran for LSU region

3.5.3 Post-PCR

After completion of PCR cycle, the samples are run through agarose gel electrophoresis for the confirmation. 1% (w/v) Agarose (Lonza, SeaKem LE) was dissolved in 1x TAE buffer with the addition of 5µL of GelRed (Biotium 41003) per 100 mL of gel. 5 µL PCR product was loaded with 1 µL 6x DNA loading buffer dye (Thermo scientific) into different wells along with 1Kb Plus DNA ladder marker (Thermo scientific GeneRuler) to either side to know the size of amplified DNA. Electrophoresis was programmed at 140 V for 45 minutes. After completion, the gel was visualized under UV light. GeneSnap software was used to take an image of gel inside G: box (Syngene).

3.5.4 PCR cleanup

The purification of PCR product was carried out using QIAquick PCR Purification Kit. The protocol provided was followed by the process attached in Appendix. The concentration of DNA after purification was checked using NanoDrop.

The samples were then prepared for sequencing in 1.5 mL Eppendorf tubes. Two separate tubes were prepared for each DNA samples using forward and reverse primer of the target regions, and labeled with barcodes. The samples were then sent to GATC Biotech Biotech (https://www.gatc-biotech.com/en/index.html) for LIGHTrun Sanger sequencing.

3.5.5 Analyzing the sequence data

The sequences obtained from GATC Biotech were inspected through Chromas software, where the quality of the chromatograms was analyzed visually. The FASTA files of forward and reverse primer sequence were extracted from the Chromas and processed through Clone Manager Professional 9. The forward sequence was aligned with the inverted reverse sequence to achieve the consensus sequence. Some mismatches observed were manually edited observing the quality of chromatograms from forward and reverse sequences. Chromatograms data from Chromas has been attached in the Appendix.

The reviewed sequences (consensus sequence) obtained after editing with Clone Manager Professional 9 for ITS and LSU regions were uploaded to the RDP (http://rdp.cme.msu.edu/classifier/classifier.jsp) for identification and classification. For ITS region, the 'Warcup Fungal ITS trainset 2' was used, and 'Fungal LSU training set 11' was used for LSU region.

3.6 Fermentability Test

Fermentability of the different yeast samples was observed in the modified synthetic must. The composition and method of preparation are categorized in the Table 3.1. The weight of the empty falcon tubes was measured at the beginning. Then, 35 mL of synthetic-must was pipetted into the vials (in sterile bench). The weight of the vials was taken, then the yeast cells from the culture flask were transferred into the tubes. The calculation for cells added is listed in the Appendix, the initial concentration in the growing vials was set $2.0* 10^6$ cells per mL. The initial weight at time zero was noted. The vials were then incubated at 22°C at 180 rpm. The weight of the vials was noted in every 8 hours for 48 hours and every 24 hours afterward. The end of fermentation was observed through the graph plot (weight vs hours of incubation). After the change in weight of tubes came near to constant, the tubes were centrifuged to remove the yeast cells. Then, the medium was transferred into separate vials and stored at -20 °C until HPLC and headspace GC-MS analysis. Some of the samples were taken out of the incubator after certain hours of incubation and stored to perform the analysis at desirable hours of incubation.

Table 3.4: Composition and preparation of Modified Synthetic Must for fermentability test

Compounds	Amount [g or ml]	Consideration
Step A		
Glucose	100 g	Sugars were dissolved at 371.5 g of H_2O (autoclaved 20 min at 120°C)
Fructose	30 g	
Sucrose	30 g	
Lactose	40 g	
Water	371.5 g	
Step B		
Citric acid (. H_2O)	5.47 g	pH was adjusted to 3.3 with NaOH Water adjusted to 484.5 g Autoclaved for 20 min at 120°C
Malic acid	0.5 g	
Tartaric acid	1.0 g	
KH_2PO_4	0.75 g	
$MgSO_4.7H_2O$	0.5 g	
$CaCl_2$	0.117 g	
NaCl	0.2 g	
NH_4Cl	0.153 g	
Water adjusted to	484.5 g	
Step C		
Amino acid stock sol. (100 mg N/l)	3.5 ml	Sterilization of these factors were performed by filtration and added at last.
Oligo-elements (Stock sol.)	1.0 ml	
Vitamins (stock sol.)	10.0 ml	
Anaerobiosis factors (stock sol.)	1.0 ml	
Finally, the volume was adjusted to 1 L and allowed to dissolve for homogeneity		

3.7 Analysis of Volatile and Non-volatile Compounds

3.7.1 Headspace Gas Chromatography-Mass Spectrometry

The volatile compounds at the end-point of fermentation in modified synthetic-must were performed using a 7890 A GC System coupled with the 7000 Series Triple Quadrupole GC-MS system (Agilent Technologies), with a Teledyne Tekmar HT3TM Static and Dynamic Headspace System autosampler. The experimental conditions for the static headspace system and the chromatographic conditions for the GC-MS are presented in the table. The instrumental control, data acquisition, and processing was performed through MassHunter software, while HT3 TekLink was used for autosampler.

Table 3.5: Experimental conditions for the static headspace system.

Parameter	Condition
Constant heat time	On
GC cycle time[a]	53.00 min
Valve oven temp.	105 °C
Transfer line temp.	110 °C
Standby flow rate	25 mL/min
Platen/Sample temp	60 °C
Platen temp. equil. time	0.50 min
Sample equil. time	20.00 min
Pressurize	9 psig
Pressurize time	2.00 min
[a]The GC cycle time can vary; = Time of GC separation (31.95 min) + time needed to cool down the column (15-25 min).	

Table 3.6: Chromatographic parameters for GC-MS

Parameter	Condition		
Column	Agilent J&W DB-624 UI, 30 m × 0.25 mm, 1.4 µm		
Carrier	Helium, constant flow, 1.8 mL/min		
Oven	Temperature	Hold time	Rate
	35 °C	5.66 min	8.8 °C/min

	100 °C	1.7 min	13.3 °C/min
	220 °C	3.39 min	22.1 °C/min
	250 °C	3.43 min	End
Inlet	220 °C, Split ratio 5:1		
MSD	Scan 30-400 amu, scan time 70 (ms/cycle)		
	Source temp. 230 °C		
	Quad temp. 150 °C		
	Solvent delay 4.5 min		

3.7.1.1 Preparation of Internal Standard

The internal standard (ISTD) used in all samples was 4-methyl-2-pentanol. At first, the stock solution of ISTD was prepared by dissolving 20-30 mg into 3-5 mL of ethanol (EtOH) in a 20-mL volumetric flask, thus ranging from 1.0-1.5 mg/mL. The volumetric flask was filled with MQ water to fulfill the desired concentration and mixed thoroughly. Then, the stock solution was diluted to create a working solution of ISTD with a concentration of 100 µg/mL, which was divided into aliquots and stored at -80 °C.

A calibration series of different alcohols and esters were prepared for the headspace GC-MS analysis. The various compounds were categorized into three types: Mixture A, B, and C, the details are presented in the table below. The compounds were accurately weighed in 20 mL volumetric flask to meet their preset ranges as in the table and filled with specified ratios of EtOH and distilled water. Moreover, Appendix includes the exact weights used.

5 mL of Mix A, 2 mL of Mix B, 2 mL of Mix C, was added in a separate flask and filled with water until 100 mL to make stock solution of 40 ppm. Then, it was used for serial dilution ranging from 0.2-40 ppm, as shown in Table. 5 mL of each prepared calibration solution was added into HS vials, along with 100 µL of ISTD. Then, the vials were quickly capped (Agilent Technologies Hdspc Al crimp cap, PTFE/si sep 20 mm 5183-4477) with a crimper (20 mm Crimper - Agilent tech 5040-4669).

Table 3.7: Composition of compounds in Mixture A, B, and C to create a stock solution for the calibration series.

Compounds	Weight range [mg]	Volume [mL]
Mixture A		
1-propanol	45-50	20 (EtOH/H2O, 50:50 v/v)
Ethyl acetate	45-55	
Isobutyl alcohol	35-45	
Isoamyl alcohol	65-75	
Active amyl alcohol	40-50	
Mixture B		
1-butanol	35-45	20 (EtOH/H2O, 80:20 v/v)
Ethyl propionate	30-40	
Propyl acetate	20-30	
Acetal	20-30	
Isobutyl acetate	20-30	
Mixture C		
Ethyl butyrate	20-30	20 (EtOH/H2O, 80:20 v/v)
Isoamyl acetate	18-25	
Ethyl hexanoate	18-25	
Ethyl octanoate	20-30	
Ethyl decanoate	40-50	

3.7.1.2 Sample

At first, the samples were shaken vigorously so that the CO2 could escape. 5 mL of the sample was carefully and slowly pipetted to a 22 mL HS vials to avoid bubbles and ensure accurate measurement. Then, 100 µL of working solution of ISTD was added. The vials were closed promptly and analyzed immediately.

3.7.1.3 Quantification of compounds

The identification and quantification of various compounds in the samples was carried out with MassHunter Quantitative Analysis (QQQ) software. Based on the relative response of compounds and ISTD, the concentration of various compounds was determined, giving a calibration curve, which was set into quadratic. The maximum concentration that could be calculated from calibration curve was 131,440 µg/mL, above which it would show greater. The different compounds, their RT, the equation and coefficient of determination (R^2) of the calibration curves are presented in the Table below.

3.7.2 High-Performance Liquid Chromatography

HPLC analysis was performed to measure the concentration of sugars, ethanol, and acids. Shimadzu HPLC system which was coupled to both RI and UV (210 nm) detectors, with a column (Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) operated at 45 °C, and 5mM H_2SO_4 (flow rate: 0.6 mL/min) as a mobile phase was used.

The sugars and ethanol were analyzed with RI detector, while UV detector was used for analyzing carboxylic acids, acetic and succinic acid. External single-point standard (ESTD) was used in the calibration assuming analyte concentration does not vary widely in the samples. The standard solution was run twice at the start and calibrated at every 10^{th} sample run. The example of how the concentration was calculated has been mentioned in the Appendix.

3.7.2.1 Preparation of Standard mix

A set of nine stock solutions (glucose, fructose, sucrose, maltose, lactose, acetic acid, glycerol, Ethanol and succinic acid) was prepared to make a standard mix for the experiment. The volume of each compound taken from the stock to prepare standard mix are listed in the Table below. 10 mL of standard mix was prepared, which was total volume taken from stock for each compound and the remaining volume fulfilled by MQ-water. Finally, it was filtered through 0.2 µm syringe filter before use.

Table 3.8: The compounds included in the standard solution with their stock concentration, volume used and final concentration in 10mL volumetric flask used for the HPLC analysis.

Compounds	Conc. Of Stock [g/L]	Volume [mL]	Dilution factor	Final conc. [g/L]
Glucose	50	1	10	5
Fructose	50	1	10	5
Sucrose	50	1	10	5
Maltose	50	1	10	5
Lactose	50	1	10	5
Glycerol	25	1	10	2.5
Ethanol	50	2	5	10
Acetic acid	16	0.375	26.7	0.6
Succinic acid	50	0.3	33.3	1.5

3.7.2.2 Sample

The two types of samples after fermentation in modified synthetic-must were prepared for HPLC run, undiluted and 10X diluted. The filtered sample with 0.2 µm syringe filter was diluted with filtered MQ-water. The prepared samples in tubes were wrapped with parafilm and stored at -20 °C until the HPLC run.

4 Results

The different samples of yeasts cakes collected during a summer visit to Nepal include samples from six places including eastern lowland of Dharan to the western high-altitude region of Mustang. Figure 4.1 marked with yellow points shows the places of our sample collection. Although some research regarding morphological characteristics and microbial activities have been performed, the yeast cakes (murcha) has not been studied at genotypic level.

It is difficult to study all the microflora present in that mixed culture genotypically, this study will mainly be based on the random selection (based on morphology) of different yeast strains and extract the genotypic sequence of ITS and LSU region, observe the sugar fermentability and analysis of volatile compounds produce by those strains through HPLC and headspace GC-MS.

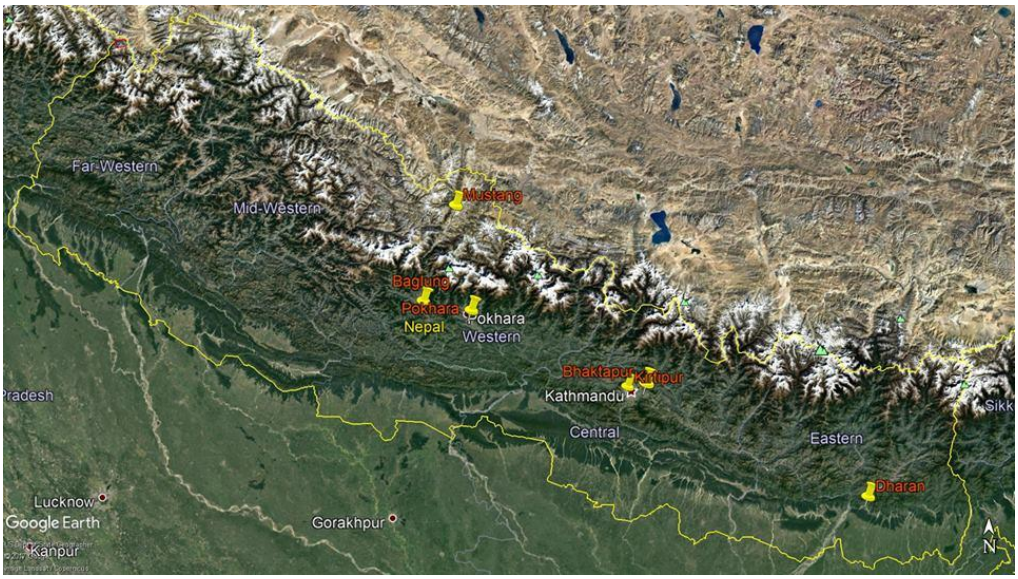


Figure 4.5: Map of Nepal showing the places of the sample collection

4.1 Isolation

After growing the different *murcha* samples of Nepal in 5% Malt Extract medium and series of plating in MRS and WA medium, fourteen different yeast strains were isolated. The isolation was mainly based on the phenotypic difference in the

colony. At least two different types of colonies were picked from a sample of each place. Some of the pictures of culture plates during isolation are presented in Appendix.

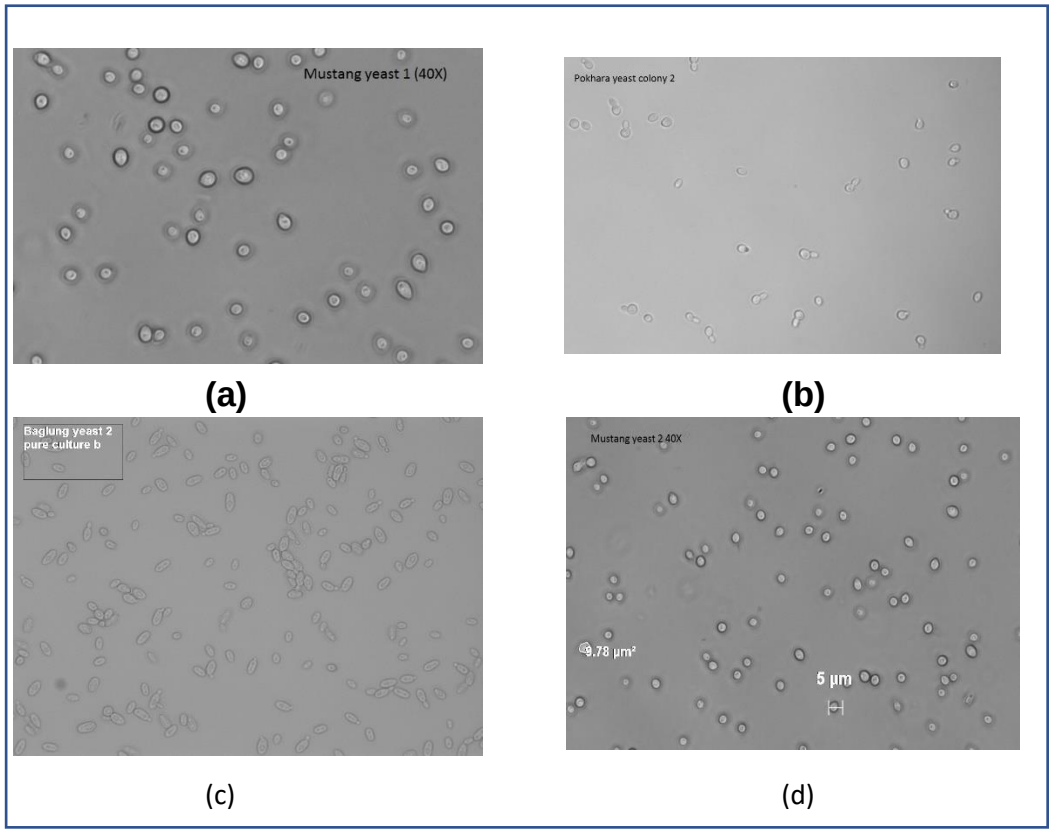


Figure 4.6 Microscopic photos taken of some yeast strains from different places of Nepal. Here a. 40X view of NPY4_MustangY1 b. 10X view of NPY3_PokharaY2 c. 40X view of NPY5_Baglung Y2 d. 40x view of NPY4_MustangY2

Through Figure 4.2 some of the pure cultures of the yeast strains has been presented. As some of the variation can be observed in the size of yeasts, here we assume the variation in size is due to their presence in a different stage of the growth cycle.

4.2 Genotypic Characterization

The sequencing data from GATC Biotech were observed through Chromas software to analyze the quality of the chromatograms. These sequences were further processed with Clone Manager to build a consensus sequence.

These FASTA sequences were uploaded to MUSCLE web link from EMBL-EBI software data base for multiple alignment to construct a phylogenetic tree. The Figure 4.3 shows the cladogram representation for ancestral relationships between the yeast's ITS region.

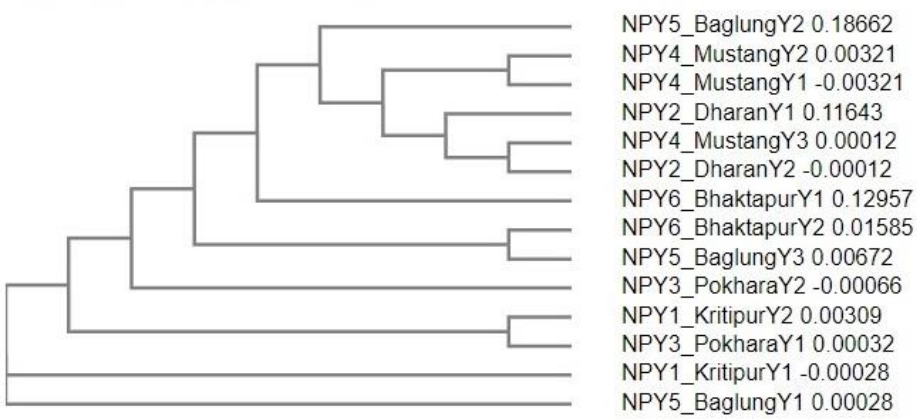


Figure 4.7 A phylogenetic tree constructed from consensus sequence of ITS region

The closely related yeast strains are presented more near to each other. As observed in the figure, we can say Kritipur Y2, and Pokhara Y1 share a common ancestor. Likewise, Bhaktapur Y2 and Baglung Y3; Mustang Y3, Dharan Y2, and Dharan Y1; Mustang Y2 and Y1 are close to each other.

The consensus ITS sequence for each strain were then uploaded to RDP Classifier. The database for 'Warcup Fungal ITS trainset 2' was used for sequence similarity of different yeast species. The results are presented in Table 4.1 with the score for each species. Out of 14 different strains, five were classified as *Cyberlindnera fabianii*, two as *Saccharomycopsis fibuligera*, two as *Kodamaea ohmeri*, two as *Wickerhamomyces anomalus*. Three of the strains were classified as *Pichia burtonii*, *Candida inconspicua*, and *Saccharomyces cerevisiae*.

The LSU sequence for all yeast strains was not possible due to the poor quality of chromatograms and inability of extracted DNA from NPY2_DharanY1 for PCR amplification with LSU primers. The consensus LSU sequence for available strains was then uploaded to RDP Classifier for 'Fungal LSU training set 11' database search.

Table 4.4 Species identification through RDP database for consensus ITS region

S.N.	Sample	ITS sequence	Score
1	NPY1_Kritipur Y1	Cyberlindnera fabianii	100
2	NPY1_Kritipur Y2	Cyberlindnera fabianii	100
3	NPY2_Dharan Y1	Pichia burtonii	100
4	NPY2_Dharan Y2	Kodamaea ohmeri	100
5	NPY3_Pokhara Y1	Cyberlindnera fabianii	100
6	NPY3_Pokhara Y2	Cyberlindnera fabianii	100
7	NPY4_Mustang Y1	Saccharomycopsis fibuligera	100
8	NPY4_Mustang Y2	Saccharomycopsis fibuligera	100
9	NPY4_Mustang Y3	Kodamaea ohmeri	100
10	NPY5_Baglung Y1	Cyberlindnera fabianii	100
11	NPY5_Baglung Y2	Candida inconspicua	100
12	NPY5_Baglung Y3	Wickerhamomyces anomalus	100
13	NPY6_Bhaktapur Y1	Saccharomyces cerevisiae	100
14	NPY6_Bhaktapur Y2	Wickerhamomyces anomalus	100

Out of 11 results obtained from LSU region as listed in Table 4.2, 8 strains were classified as Vanderwaltozyma, two as Wickerhamomyces and one as Saccharomyces.

Table 4.5 Species identification through RDP database for consensus LSU region

S.N.	Sample	LSU sequence	Score
1	NPY1_Kritipur Y1	Vanderwaltozyma	100
2	NPY1_Kritipur Y2	Vanderwaltozyma	100
3	NPY2_Dharan Y1		
4	NPY2_Dharan Y2	Vanderwaltozyma	100
5	NPY3_Pokhara Y1	Vanderwaltozyma	100
6	NPY3_Pokhara Y2		
7	NPY4_Mustang Y1	Vanderwaltozyma	100
8	NPY4_Mustang Y2		
9	NPY4_Mustang Y3	Vanderwaltozyma	100
10	NPY5_Baglung Y1	Vanderwaltozyma	100
11	NPY5_Baglung Y2	Vanderwaltozyma	100
12	NPY5_Baglung Y3	Wickerhamomyces	100
13	NPY6_Bhaktapur Y1	Saccharomyces	100
14	NPY6_Bhaktapur Y2	Wickerhamomyces	100
Some of the samples were not been able to analyze the LSU sequence due to poor resolution of chromatograms and lack of PCR amplification			

4.3 Fermentation

The fermentation was carried out in modified synthetic must with the syringe inserted at the lid in the tube, incubated at 22^0C at 180 rpm. The decrease in weight was calculated to know the pattern of gravity fall over hours. The Figure 4.4 and 4.5 shows the fall of gravity for all 14 strains, eight included in Figure 4.4 and remaining seven presented in Figure 4.5. The number in the graph is the short representation for the yeast strains as described in Table 4.1 and Table 4.2.

The initial weight of empty tubes was deducted from the weight at time zero, divided by the volume (35mL) of synthetic must gave the specific gravity for each tube. The graph shows the fall of gravity was rapid for first 120 hours and gradually slow afterward. After the fall of gravity in tubes was near to constant (280 hours), the fermentation was stopped.

The graphs from Figure 4.4 shows that gravity fall is gradual for most of the yeast strains excepts for number 8 (NPY4_Mustang Y2).

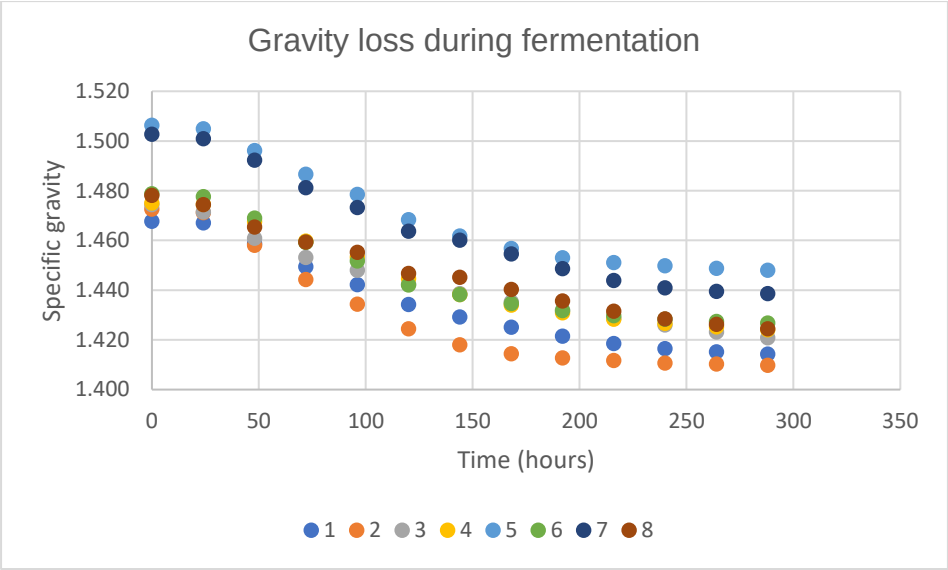


Figure 8.4 Fall in specific gravity over time (hours) of eight yeast samples

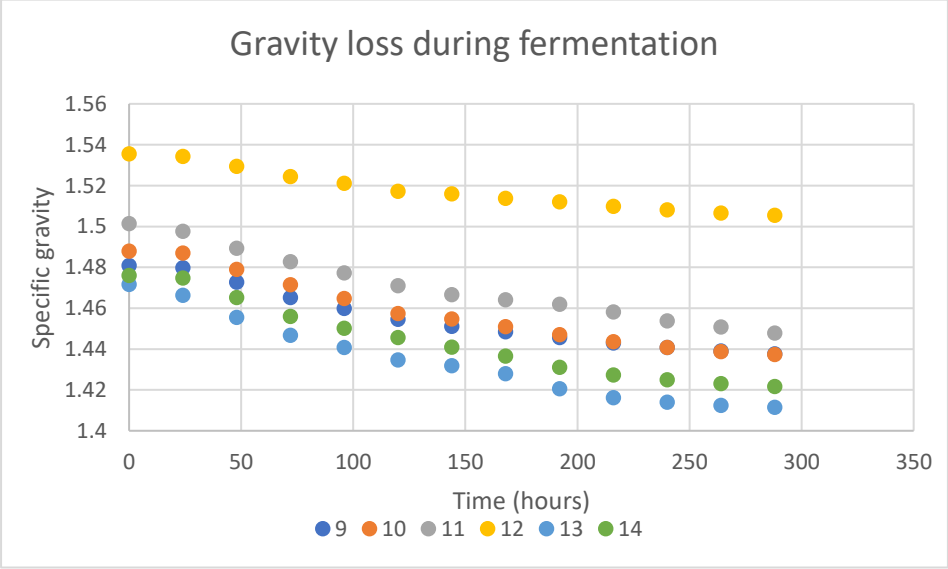


Figure 4.9 Fall in specific gravity over time (hours) of seven yeast samples

Figure 4.5 shows that the fall is not quite distinctive for number 12 (NPY5_Baglung Y3). The fall in gravity is very less (below 0.02) for this strain. Although the gravity fall of other yeast strains seems evident, it is not entirely smooth for number 11 (NPY5_Baglung Y2).

4.4 Sugar, Alcohol and Organic Acid Quantification with HPLC

The concentration of sugars, alcohols and organic acid calculated through HPLC analysis are presented in Table 4.3. The raw data and calculations for the concentration of different analytes are mentioned in Appendix. The consumption of glucose and fructose was less than expected. Only the NPY6_Bhaktapur Y1 consumes all glucose and most of the fructose. For other strains, consumption is carried out utmost still some residual glucose and fructose were present. The yeast strains from Mustang (NPY4_Mustang Y1, Y2, and Y3) consumed very less of both glucose and fructose. Lactose was not consumed by any of the yeast strains giving the endpoint concentration very similar to the start concentration.

The concentration of ethanol produced by most of the strains is promising (greater than 45 g/L) except for those less sugar consuming strains (NPY4_Mustang Y1, Y2, and Y3). The concentration of ethanol produced by these low sugar consuming strains is in the range of 25-28 g/L, which is nearly half the concentration of other yeast strains. The concentration of glycerol, acetic acid, and succinic acid are very less as expected.

Table 4.6 Sugar, alcohol and organic acid quantification with HPLC for all yeast samples. The concentrations of compounds are presented in following the order: 1. Glucose 2. Fructose 3. Lactose 4. Glycerol 5. Ethanol 6. acetic acid and 7. Succinic acid is given in g/L.

Sample	Concentration [g/L]						
	1	2	3	4	5	6	7
NPY1_KTR Y1	8.3331	13.398	40.554	1.857	53.368	1.85	1.269
NPY1_KTR Y2	1.7777	9.8294	41.351	1.757	56.893	2.65	1.678
NPY2_DRN Y1	9.5202	14.034	40.244	2.920	53.151	0.72	1.184
NPY2_ DRN Y2	12.560	14.898	39.579	1.205	45.052	1.27	1.614
NPY3_PKR Y1	7.0094	13.519	40.261	1.732	54.093	1.82	1.297
NPY3_PKR Y2	11.152	14.788	40.082	1.736	45.320	1.97	1.409
NPY4_MTG Y1	58.874	21.352	38.608	1.063	25.040	0.66	1.799

NPY4_MTG Y2	63.411	23.143	39.704	1.396	26.446	0.81	1.301
NPY4_MTG Y3	14.833	21.273	40.787	1.453	43.206	0.93	1.765
NPY5_BLG Y1	15.332	14.997	40.039	1.830	45.356	1.27	1.078
NPY5_BLG Y2	6.1406	8.8627	40.541	4.261	46.341	0.35	0.608
NPY5_BLG Y3	6.1897	13.823	39.181	2.566	46.734	2.16	1.886
NPY6_BKT Y1	0	7.6137	37.520	3.411	57.733	1.20	2.533
NPY6_BKT Y2	3.8786	13.668	39.299	1.445	47.234	3.25	1.219

The two yeast strains were selected at the beginning of fermentation to analyze the sugar degradation over time: Kritipur Y1 (NPY1_KTR Y1) and Bhaktapur Y1 (NPY6_BKT Y1). The selection was made on the basis that *Cyberlindnera fabianii* was a common yeast strain and *Saccharomyces cerevisiae* being the only best strain isolated.

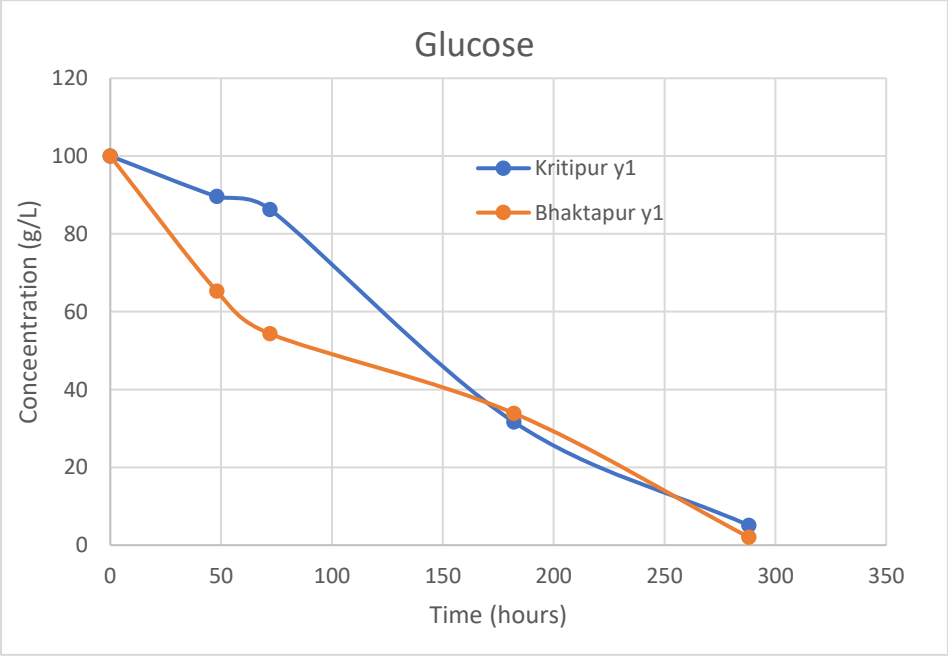


Figure 4.10 Consumption of glucose by *S.cerevisiae* and *C.fabianii*

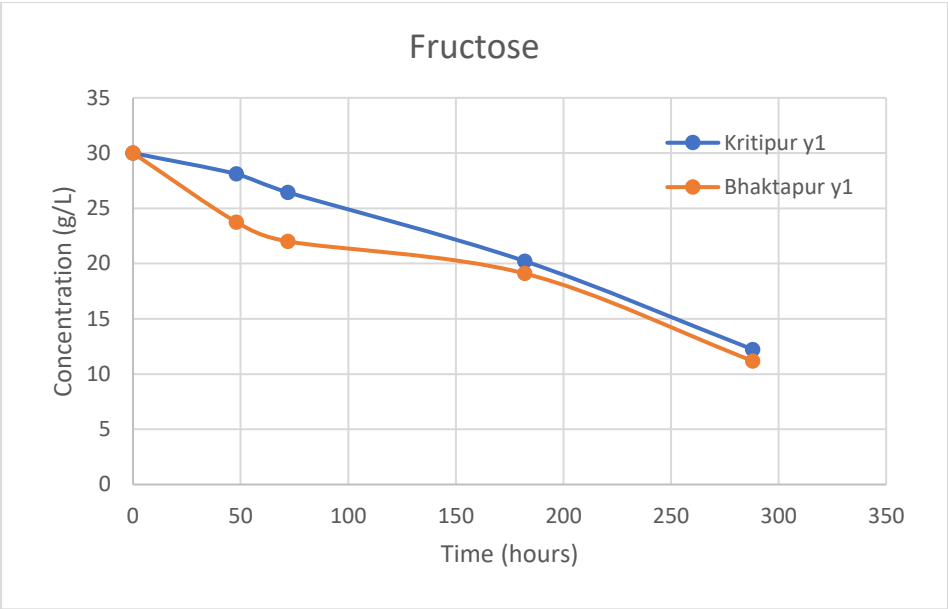


Figure 4.11 Consumption of fructose by *S.cerevisiae* and *C.fabianii*

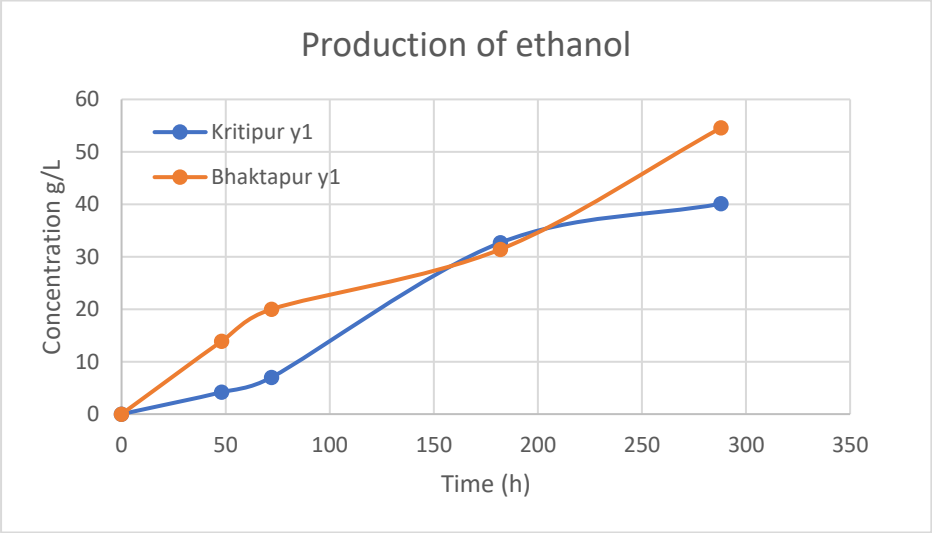


Figure 4.12 Production of ethanol by S.cerevisiae and C.fabianii

4.5 Headspace GC-MS

Table 4.7: Quantification of volatile compounds by headspace GC-MS for the samples (part a). The compounds are numbered as follows: 1. 1-Propanol 2. Ethyl acetate 3. Isobutyl alcohol 4. 1-Butanol 5. Ethyl propionate 6. Propyl acetate

Sample	Concentration [mg/L]					
	1	2	3	4	5	6
NPY1_KTR Y1	44.35	131.45	85.68	1.00	4.90	2.9
NPY1_KTR Y2	38.07	131.45	111.79	0.97	3.17	1.6
NPY3_PKR Y1	47.40	131.45	96.85	0.76	2.91	1.3
NPY3_PKR Y2	42.03	131.45	90.65	0.63	4.07	2.8
NPY5_BLG Y1	51.03	131.45	58.56	1.12	3.94	2.1
NPY2_DRN Y1	21.39	131.45	88.90	0.33	0.40	0.1
NPY2_ DRNY2	24.43	13.13	82.39	0.52	0.00	0.0
NPY4_MTG Y3	24.64	12.33	50.08	0.36	0.00	0.0
NPY4_MTG Y1	28.33	10.53	43.34	0.34	0.00	0.0
NPY4_MTG Y2	22.41	11.00	41.97	0.29	0.00	0.0
NPY5_BLG Y2	9.43	131.45	124.99	0.27	1.37	0.1
NPY5_BLG Y3	32.73	131.45	57.03	1.28	1.09	2.0
NPY6_BKT Y2	29.97	131.45	60.18	0.62	1.16	1.8
NPY6_BKT Y1	32.03	35.93	68.63	0.61	0.23	0.0

Through headspace GC-MS, 15 compounds were analyzed; 1-propanol, ethyl acetate, isobutyl alcohol, 1-butanol, ethyl propionate, propyl acetate, acetal, isoamyl alcohol, active amyl alcohol, isobutyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate. The concentration of all these compounds produced by the sample yeast strains is tabulated in Table 4.4 and Table 4.5.

The concentration for the highest limit of detection through the calibration range was 131,440 µg/mL. Most of the samples contained a high level of ethyl acetate, far beyond the highest range in calibration. The concentration was set to be the maximum limit of detection for this compound. The dilution of sample and re-injection to headspace setup for detection would have solved this problem. But we didn't re-run because this was observed for a single compound. The samples that were below the LOQ were set to zero. In most samples, the compounds ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate were below the LOQ and were set zero. Only Bhaktapur Y1 sample (NPY6_BKT Y1) contained 0.03 mg/L of ethyl butyrate and ethyl hexanoate. Kritipur Y1

(NPY1_KTR Y1) and Baglung Y1 (NPY5_BLG Y1) showed 0.03 mg/L and 0.02 mg/L of ethyl butyrate, while the concentration of other samples remained below LOQ.

Table 4.8: Quantification of volatile compounds by headspace GC-MS for the samples (part b). The compounds are numbered as follows: 7. Acetal 8. Isoamyl alcohol 9. Active amyl alcohol 10. Isobutyl acetate 11. Ethyl butyrate 12. Isoamyl acetate 13. Ethyl hexanoate

Sample	Concentration [mg/L]						
	7	8	9	10	11	12	13
NPY1_KTR Y1	2.03	67.54	70.28	0.84	0.03	6.03	0.00
NPY1_KTR Y2	1.27	86.36	72.02	0.61	0.00	4.35	0.00
NPY3_PKR Y1	0.85	73.06	68.73	0.38	0.00	2.36	0.00
NPY3_PKR Y2	1.68	60.40	58.20	0.82	0.00	4.60	0.00
NPY5_BLG Y1	0.60	48.68	58.45	0.36	0.02	3.57	0.00
NPY2_DRN Y1	2.37	46.20	16.79	0.07	0.00	0.77	0.00
NPY2_ DRNY2	3.43	52.01	14.55	0.00	0.00	0.15	0.00
NPY4_MTG Y3	0.19	34.46	14.43	0.00	0.00	0.36	0.00
NPY4_MTG Y1	0.08	39.86	13.73	0.00	0.00	0.40	0.00
NPY4_MTG Y2	0.14	30.07	11.79	0.00	0.00	0.28	0.00
NPY5_BLG Y2	0.74	72.27	33.31	0.16	0.00	0.62	0.00
NPY5_BLG Y3	0.74	103.03	45.80	0.23	0.00	1.57	0.00
NPY6_BKT Y2	1.03	81.12	31.70	0.22	0.00	1.22	0.00
NPY6_BKT Y1	5.76	171.20	63.00	0.00	0.03	0.26	0.03

The comparison of the compounds with larger concentration detected through headspace GC-MS by same species belonging to different samples are presented in Figure 4.9 and Figure 4.10.

Figure 4.9 shows the comparison between the concentration of compounds produced by *C. fabianii* (identified in five yeast strains). The graph shows the concentration of 1-propanol, ethyl acetate, and active amyl alcohol is almost similar. The concentration of ethyl acetate is presented same as their concentration was beyond the highest level of detection and kept same. Isobutyl alcohol concentration is higher for KTR Y2 sample followed by PKR Y1, PKR Y2, and KTR Y1. BLG Y1 has concentration nearly half of the concentration of KTR Y2.

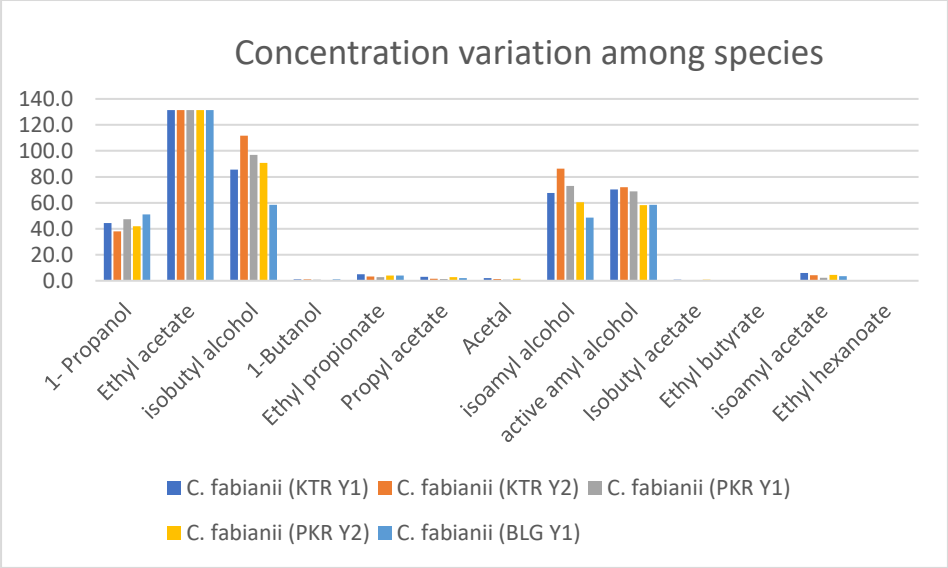


Figure 4.13 Concentration (mg/L) of major compounds detected in headspace GC-MS for C. fabianii from various places

From Figure 4.10 the concentration for 1-propanol is higher for *Sm. fibuligera* from Mustang Y1 compared to that from Mustang Y2. Likewise, it is slightly greater to *W. anomalus* from BLG Y3 compared to that from BKT Y2. There is not much variance in ethyl acetate concentration within the species for all three yeast strains. 2-methyl-1-propanol concentration is distinctively greater for *K. ohmeri* from DRN Y2 sample compared to MTG Y3. *W.anomalus* from BLG Y3 showed greater 2-methyl-1-butanol concentration compared to BKT Y2.

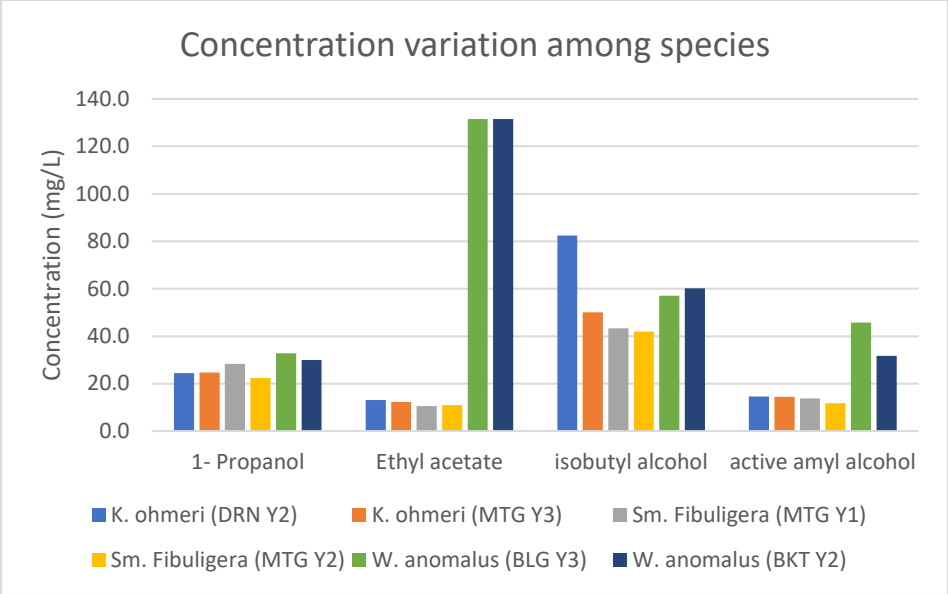


Figure 4.14 Concentration (mg/L) of major compounds detected in headspace GC-MS grouped together

5. Discussion

5.1 Species identification based on ITS and LSU sequencing

The species identified through sequencing of ITS and LSU region support the dominance of non-Saccharomyces yeast in the population of isolated strains (see Table 4.1 and 4.2). The isolated strains include; non-Saccharomyces species: *Cyberlindnera fabianii, Pichia burtonii, Kodamaea ohmeri, Saccharomycopsis fibuligera, Candida inconspicua, Wickerhamomyces anomalus* and a Saccharomyces species: *Saccharomyces cerevisiae*. The isolation was entirely random based on the visual morphological difference in the colonies. However, some of the strains were not able to map their LSU region and thus, making it difficult to confirm the species only with ITS sequence.

The LSU primers could not amplify the DNA extracted from *Pichia burtonii* and thus unable to extract sequence information. The primers were designed based on sequences from Saccharomyces and closely related species, which may have been the reason for the lack of amplification. While checking through the RDP classifier database, no evidence of LSU sequence has been found for this species. Thus made us claim the species only by the ITS sequence.

The LSU sequence gave a result for three main genera: Vanderwaltozyma, Wickerhamomyces, and Saccharomyces. The species *C. fabianii, K. ohmeri, Sm. fibuligera, C.inconspicua* were also categorized to genus: Vanderwaltozyma. This again unveils the hypervariability of ITS region and questions the sole reliability in it (Nilsson et al., 2012). The results from LSU will be more dependable in a sense they are long base pair sequence compared to ITS.

The poor resolution of data during ITS and LSU sequencing was another obstacle during identification. Some limitation regarding ITS includes, the taxonomic resolution being low for some species and problems in designing fungus-specific PCR primers (Porras-Alfaro et al., 2014).

The concentration of DNA needed for sequencing a LSU region is significantly high due to use of several primers. The use of several primers ease us to map the region more accurately. In this case, primarily the desired concentration was difficult to obtain even after concentration for each reagents in PCR reaction was tripled. The different approaches were made: increasing elongation time in PCR program, increasing a number of cycles to 36 and increasing template DNA in PCR reaction. Although we receive desired concentration of DNA, the approach might have affected some way in the resolution of obtained LSU data. Thus, it was not possible to use data from Dharan Y1, Pokhara Y2 and Mustang Y2 for LSU sequencing, we rely on their ITS data only.

5.2 Fermentation

The process was carried out with the same synthetic-must medium, same pitching rate of initial yeast cells, incubated at the same temperature, confirming similar fermentation condition for all samples. The pattern of gravity loss is similar for the same species as identified. The gravity fall shown by *C. fabianii* is almost identical regardless the yeast cakes they are isolated from. The rapid fall of gravity in early stages of fermentation is due to consumption of simple sugars.

The residual glucose concentration in the samples is high in case of *Sm. fibuligera* yield less ethanol. Also described in section 2.2 this strain plays an important role in liquefaction and saccharification of starch. Despite being able to degrade starch with glucoamylases, many strains are found to have low tolerance for ethanol (Ma et al, 2000). Also, this strain has been used widely with the co-culture of high ethanol producing strains (Abouzied et al, 1987), reflects the strain is not efficient ethanol producer. Thus, it justifies the ethanol concentration, being low compared to other strains of yeast isolated.

5.3 Alcohol, ester and acid production

The concentration of ethyl acetate has been maximum for those species utilizing more sugars except for *S. cerevisae*, whose concentration is low compared to other sugar utilizing strains. The increased production of ethyl acetate could be due to high gravity of the synthetic must (Piddocke et al, 2009) or higher availability of fatty acid precursor in medium, or should be active biosynthetic enzyme in those strains (Saerens, et al., 2008).

While analyzing the level of ester production in these strains, ethyl acetate level is much higher for *C. fabianii* species. And, isoamyl acetate produced are in range 3.5-6.3 mg/L for these species which are high compared to 1.2 mg/L threshold desired in case of lager beers (Meilgaard, 1975; Dufour and Malcorps, 1994). Concentration for isobutyl acetate and ethyl butyrate are less. Analysis of ester compounds from all the strains suggests that they can produce esters to smaller extent excepts for ethyl acetate.

The concentration of higher alcohols (1-propanol, isobutyl alcohol, isoamyl alcohol) are also observed low for less sugar utilizing species and higher for those having less residual sugars. Different species showed varied supremacy for higher alcohols. These all yeast strains could be the microflora of single fermentation process, as they are the isolates of mixed culture. Thus, they may result in

significantly greater concentration of higher alcohols, which could possess harmful effects on health.

Temperature is an important parameter to be considered in fermentation. The analysis of the yeast strains should have been performed on varying temperature, which would have given better understanding on the production of higher alcohols, and esters. These parameters have been reported to be high at fermentation running at elevated temperature (Saerens et al., 2008). An increase in 10^0C has reported up to 24% more amyl alcohol, 39% more isobutanol and 17% less n-propanol (Rankine, 1967).

The concentration of acetic acids and succinic acids are low with almost all species. The ethanol production is favored by low acetic acid concentration (Greetham, 2014). The acids concentration from HPLC analysis proves relatively low presence, thus higher ethanol concentration was observed.

The individual sugar degradation curve from two species: *S. cerevisiae* and *C. fabianii* does not explain more information about how the sugars were used during fermentation. As glucose being simple sugar will always be preferred than fructose, where Saccharomyces exhibits better utilization. The presence of more residual fructose at the end suggest the inability of the species of its maximum utilization.

6. **Conclusion**

14 different yeast strains were isolated from the Nepalese yeast cakes (murcha) of six different places varying in geographical condition. The isolation was entirely random based on the morphologies of the colonies grown repeatedly on WA and MRS medium to establish pure culture. Those isolated strains were genotypically characterized using the ITS and LSU region. Out of 14 different strains, five were classified as *Cyberlindnera fabianii*, two as *Saccharomycopsis fibuligera*, two as *Kodamaea ohmeri*, two as *Wickerhamomyces anomalus*. Three of the strains were classified as *Pichia burtonii*, *Candida inconspicua* and *Saccharomyces cerevisiae*. LSU characterization narrow down the genera into three: Vanderwaltozyma, Wickerhamomyces and Saccharomyces.

Phylogenetic tree based on ITS sequence was performed with neighbor joining method, showed linkage of same species and nearby ancestors making two types of lineage finally linked at the Saccharomyces at the center.

Fermentation was carried out for each yeast strain using modified synthetic must. Overall fermentation was observed with smooth fall of gravity except for Baglung Y2, also Baglung Y3 resulting in very less gravity fall.

HPLC analysis showed that maximum utilization of sugars was achieved with Saccharomyces while strains of Saccharomycopsis were the least utilizing ones. Thus, establishing themselves with high and low ethanol producers respectively. A comparative study for degradation of sugars by two selected strains: *S. cerevisiae* and *C. fibuligera* was performed in which the former strains showed rapid consumption of glucose, thus exhibiting exponential production of ethanol compared to latter strain.

The production of ester compounds is found to be very less except for ethyl acetate. Some of the strains are found to produce significant amount of isoamyl acetate as well. Meanwhile, concentration of other esters compounds is minimum, high production for ethyl acetate could be assumed to be precursor driven.

The variation in concentrations of different higher alcohols by each strain does not implies any significant value. Although, these strains are result of isolation from a mixed culture, thus, these strains could possess the possibility to produce higher alcohols of high concentrations, when allowed to ferment collectively.

References:

Dhital, R., G. Subedi, Y. B. Gurung and P. Hamal (2001). ALCOHOL AND DRUG USE IN NEPAL. Kathmandu, Nepal, Child Workers in Nepal Concerned Centre (CWIN)**:** 102.

J. P. Tamang, S. P. (1995). "Microflora of murcha: An amylolytic fermentation starter." Microbios 81: 327.

J.B. KC, D. K. S., B.K. Rai (2001). "Plants used in murcha preparation in Eastern Nepal." J. Hill Res. Sikkim Sci. Soc. 14(2).
Karki, T. B. (1986). Murcha In: "A Concise Handbook of Indigenous Fermented Foods in the ASCA Countries". Canberra, The Govt. of Australia.

Shah, S. (2000). Alcohol, State and Women. Himal. Kathmandu, Nepal, Himal. 10

Statistics, C. B. (2012). "National Population and Housing Census 2011." NPHC 2011 **01**(November, 2012): 263.

Vaidya, T. R. e. a. (1993). Social History of Nepal. New Delhi, Anmol Publication.

Nilsson,R.H., Tedersoo, L., Abarenkov, K., Ryberg,M., Kristiansson, E., Hartmann, M., Schoch, C.L., Mylander, J.A.A., Bergsten, J., Porter, T.M., Jumpponen, A., Vaishampayan, P., Ovaskainen, O., Hallenberg, N., Bengtsson-Palme, J., Eriksson, K.M., Larsson, K.H., Larsson, E., Koljalg, U., (2012). Five simple guidelines for establishing basic authenticity and reliability of newly generated fungal ITS sequences. MycoKeys 4, 37e63.

Peay, K.G., Kennedy, P.G., Bruns, T.D., (2008). Fungal community ecology: a hybrid beast with a molecular master. BioScience 58, 799e810.

Schloss, P.D., Gevers, D., Westcott, S.L., 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNAbased studies. PLOS One 6, e27310.

Kurtzman, C., P, Mateo, R., Q. Kolecka, A., Theelen, B., Robert, V., Boekhout, T. (2015). Advances in yeast systematics and phylogeny and their use as predictors of biotechnologically important metabolic pathways. FEMS Yeast Res. 2015 Sep;15(6). pii: fov050. doi:10.1093/femsyr/fov050. Epub 2015 Jun30.

Allison, L. A. (2007). Recombinant DNA Technology and Molecular Cloning. In Fundamental molecular biology (Chap. 8, pp. 180–231). Blackwell Publishing.

Todd H. Wiedemeier, Hanadi S. Rifai, Charles J. Newell, John T. Wilson, (1999). Natural Attenuation of Fuels and Chlorinated Solvents in the Subsurface by, ISBN: 978-0-471-19749-2, Mar 1999 617 pages

Campbell, N., Reece, J., Urry, L., Cain, M., Wasserman, S., Minorsky, P., Jackson, R., (2008). Biology. 8th ed. Pearson Education, San Francisco

Bokulich, N. A. & Bamforth, C. W. (2013). The Microbiology of Malting and Brewing. Microbiology and Molecular Biology Reviews, *77*(2), 157–172. doi:10.1128/MMBR.00060-12

Procopio S, Qian F, Becker T. (2011). Function and regulation of yeast genes involved in higher alcohol and ester metabolism during beverage fermentation. Eur. Food Res. Technol. 233:721–729.

Verstrepen KJ, Derdelinckx G, Dufour JP, Winderickx J, Pretorius IS, Thevelein JM, Delvaux FR. (2003). The *Saccharomyces cerevisiae* alcohol acetyl transferase gene ATF1 is a target of the cAMP/PKA and FGM nutrient-signalling pathways. FEMS Yeast Res. 4:285–296.

Moonjai N, Verstrepen KJ, Delvaux FR, Derdelinckx G, Verachtert H. (2002). The effects of linoleic acid supplementation of cropped yeast on its subsequent fermentation performance and acetate ester synthesis. J. Inst. Brew. 108:227–235

Moldoveanu, S. C. & David, V. (2013). Basic Information about HPLC. In Essentials in modern hplc separations (Chap. 1, pp. 1–51). Elsevier. doi:10.1016/B978-0-12-385013-3.00001-X

Lethonen, P. & Hurme, R. (1994). Liquid Chromatographic Determination of Sugars in Beer By Evaporative Light Scattering Detection. Journal of the Institute of Brewing, 100, 343–346.

Analytical Methods for Organic Acids
https://www.shimadzu.com/an/hplc/support/lib/lctalk/54/54intro.html accessed: December 10, 2017

Harris, D. C., (2010). Quantitative Chemical Analysis. Eighth Edition. NewYork: W. H. Freeman.

Ferreira, I. M. (2009). Quantification of Beer Carbohydrates by HPLC. In V. R. Preedy(Ed.), Beer in health and disease prevention (Chap. 103, pp. 1067–1073). San Diego: Academic Press. doi:10.1016/B978-0-12-373891-2.00103-6

Sithersingh, M. J. & Snow, N. H. (2012). Headspace-Gas Chromatography. In C. Poole (Ed.), Gas chromatography (Chap. 9, pp. 221–233). Elsevier Inc. doi:10.1016 /B978-0-12-385540-4.00030-4

Grayson, M. A. (2016). A History of Gas Chromatography Mass Spectrometry (GC/MS). In The encyclopedia of mass spectrometry (pp. 152–158). Boston: Elsevier. doi:10.1016/B978-0-08-043848-1.00020-1

E. Uggerud, S. Petrie, D. K. Bohme, F. Turecek, D. Schröder, H. Schwarz, D. Plattner, T. Wyttenbach, M. T. Bowers, P. B. Armentrout, S. A. Truger, T. Junker, G. Suizdak, Mark Brönstrup. (2003).Topics in Current Chemistry: Modern Mass Spectroscopy, Springer-Verlag pp. 1-302, 225., Berlin.

Sparkman, O. D., Penton, Z. E., & Kitson, F. G. (2011). Quantitation with GC/MS. In Gas chromatography and mass spectrometry (2nd ed., Chap. 6, pp. 207–218). Amsterdam: Academic Press. doi:10.1016/B978-0-12-373628-4.00006-X

Porras-Alfaro, A., Liu, K. L., Kuske, C. R., & Xiec, G. (2014). From genus to phylum:Large-Subunit and Internal Transcribed Spacer rRNA Operon Regions Show Similar Classification Accuracies Influenced by Database Composition. Applied and Environmental Microbiology, 80(3), 829–840. doi:10.1128/AEM.02894-13

Choo, J. H., Hong, C. P., Lim, J. Y., Seo, J.-A., Kim, Y.-S., Lee, D. W., … Kang, H. A. (2016). Whole-genome de novo sequencing, combined with RNA-Seq analysis, reveals unique genome and physiological features of the amylolytic yeast *Saccharomycopsis fibuligera* and its interspecies hybrid. Biotechnology for Biofuels, *9*, 246. http://doi.org/10.1186/s13068-016-0653-4

Ma, Y.-J., Lin, L.-L., Chien, H. R. and Hsu, W.-H. (2000), Efficient utilization of starch by a recombinant strain of *Saccharomyces cerevisiae* producing glucoamylase and isoamylase. Biotechnology and Applied Biochemistry, 31: 55–59. doi:10.1042/BA19990080

Abouzied MM, Reddy CA. (1987).Fermentation of starch to ethanol by a complementary mixture of an amylolytic yeast and *Saccharomyces cerevisiae*. Biotechnol Lett.; 9:59–62. doi: 10.1007/BF01043395

Meilgaard M.C. (1991). The flavor of beer. MBAA Techn Quart. 28:132–141.

Dufour J.-P., Malcorps P. Ester synthesis during fermentation: enzymes characterization and modulation mechanism. In: Campbell I., Priest F. G., editors. *The Institute of Brewing*; 1994. pp. 137–151., and. In Proceedings of the 4th Aviemore Conference on Malting, Brewing and Distilling, and (eds). London, UK:

Rankine, B.C. (1967). Formation of higher alcohols by wine yeasts, and relationship to taste thresholds. J.Sci. Food Agric., 18: 583-589. Doi: 10.1002/jsfa.2740181208

Greetham D. (2014). Presence of Low Concentrations of Acetic Acid Improves Fermentations using *Saccharomyces cerevisiae*. *J Bioprocess Biotech* 5:192 doi: 10.4172/2155-9821.1000192

Saerens, S. M. G., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M., & Delvaux, F. R. (2008). Parameters affecting ethyl ester production by Saccharomyces cerevisiae during fermentation. Applied and Environmental Microbiology, *74*(2),454–461. doi:10.1128/AEM.01616-07

Appendices

Appendix A

1. Isolation of yeast strains

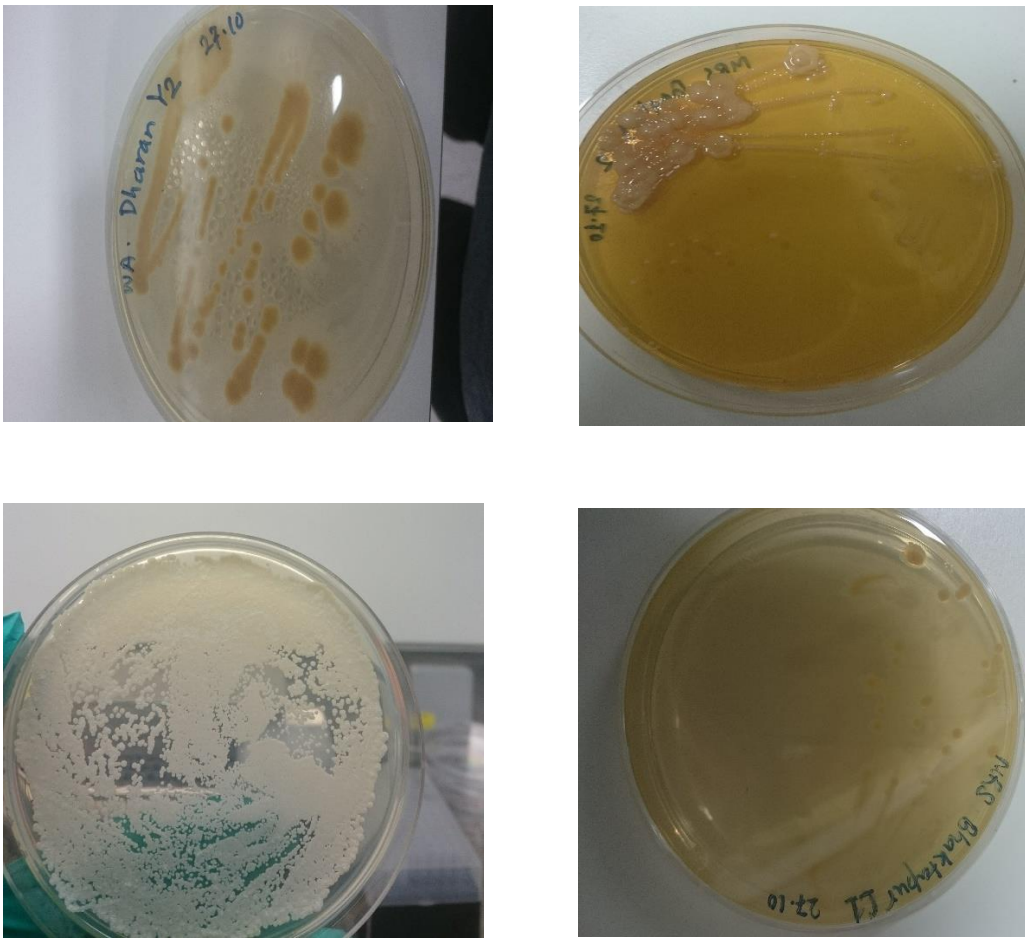
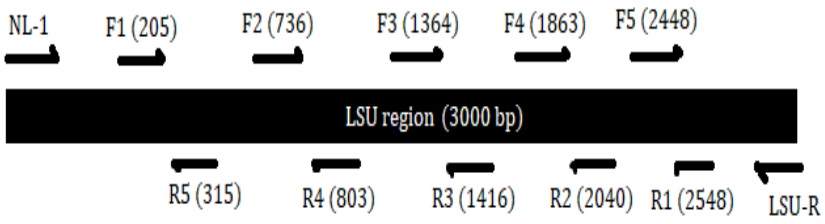


Figure: Plating in agar plates during isolation of yeasts strains.

2. **Figurative representation of the all primers used for LSU region.**



3. **Running agarose gel electrophoresis**

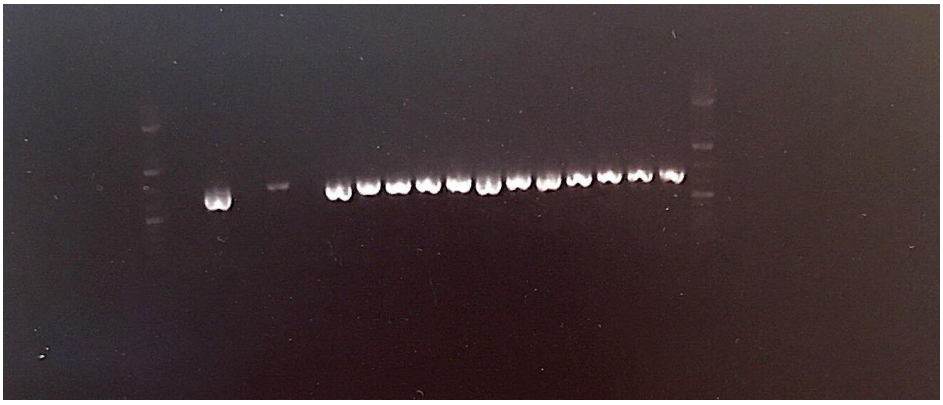
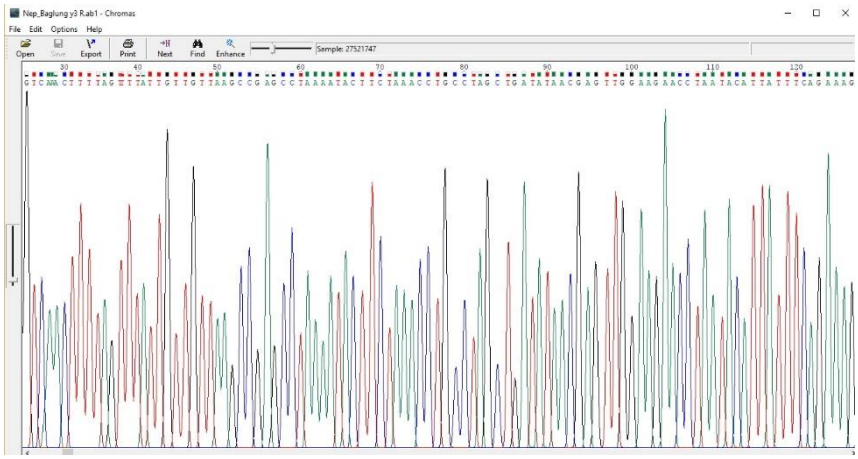


Figure: Confirming the PCR amplification through agarose gel electrophoresis for yeast strains**.**

4. Scanning sequencing results through Chromas



Appendix B

1. **Synthetic Must composition in detail**

Glucose	100 g
Fructose	30 g
Sucrose	30 g
Lactose	40 g
Citric acid	5.0 g
Malic acid	0.5 g
Tartaric acid*	3.0 g

*could be reduced if problems with precipitation

KH_2PO_4	0.750 g
K_2SO_4	0.500 g
$MgSO_2$ 7 H_2O	0.250 g
$CaCl_2$ 2 H_2O	0.155 g
NaCl	0.200 g

Nitrogen (140 mg N assimilable / l)

NH_4Cl (40 mgN/l)	0.153 g
Amino acids stock solution (100 mgN/l)	3.5 ml
Oligo-elements (stock 1000X)	1 ml
Vitamins (stock 100X)	10 ml
Anaerobiosis factors (stock 1000X, warm 70ºC)	1 ml
H_2O distillate	Csp 1 L

Adjust the pH at 3.3 with NaOH

Autoclave 20 min. a 120ºC before adding the stocks (amino acids, vitamins, oligoelements, and anaerobiosis factors). Could also be sterilized by filtration.

Oligo-elements stock solution (1000X, 1 L)

$MnSO_4$, H_2O	4 g
$ZnSO_4$, 7 H_2O	4 g
$CuSO_4$, 5 H_2O	1 g
KI	1 g
$CoCl_2$, 6 H_2O	0.4 g
H_3BO_3	1 g
$(NH_4)_6Mo_7O_{24}$	1 g
H_2O destil·lada	csp 1L

Sterilise by filtration and store at 4ºC.

Anaerobiosis factors stock solution (100X, 100 ml)

Ergosterol	1.5 g
Oleic Acid	0.5 ml
Tween 80	50 ml
Ethanol	cps 100 ml

Warm at 70 ºC to dissolve.

Store at 4ºC.

Amino acids (in 1 litre buffer solution Na_2CO_3 2%). (Stock solution at 28.8 mg N / l)

Add in the compounds in following order:

		Observations:
Tyrosine (Tyr)	1.95 g	heat at 100ºC
Tryptophan (Trp)	17.50 g	70ºC
Isoleucine (Ile)	3.25 g	70ºC
Aspartic acid (Asp)	4.42 g	

Glutamic acid (Glu)	11.95 g	
Arginine (Arg)	36.80 g	
Leucine (Leu)	4.80 g	increase Tº
Threonine (Thr)	7.54 g	
Glycine (Gly)	1.82 g	
Glutamine (Gln)	49.92 g	
Alanine (Ala)	14.56 g	
Valine (Val)	4.42 g	
Methionine (Met)	3.12 g	
Phenylalanine (Phe)	3.77 g	
Serine (Ser)	7.80 g	
Histidine (His)	3.38 g	
Lysine (Lys)	1.69 g	
Cysteine (Cys)	2.08 g	
Proline (Pro)	59.93 g	(not assimilable by yeast)
H_2O	cps 1 L	

Sterilise by filtration and store at -20ºC in aliquots.

Vitamins stock solution (100X, 1L)

Myo-inositol	2 g
Pantothenate calcium*	0.15 g
Thiamine, hydrocloride	0.025 g
Nicotinic acid	0.2 g
Pyridoxine*	0.025 g
Biotine*	3 ml**
H_2O destil·lada	csp 1L

* Vitamins stored at cold temperature.

* *Stock solution of biotine at 100 mg/l, stored at -20ºC.

Sterilise by filtration and store at -20ºC in aliquots.

2. Calculation for volume of yeast added during fermentation

Sample	Cell count	Dilution factor	No. of cells in culture (per ml)	Volume required (ml)
NPY1_KTR Y1	196	920	1803200000	0.038819876
NPY1_KTR Y2	193	920	1775600000	0.039423294
NPY2_DRN Y1	133	920	1223600000	0.057208238
NPY2_ DRN Y2	239	920	2198800000	0.031835547
NPY3_PKR Y1	371	920	3413200000	0.020508614
NPY3_PKR Y2	255	25	63750000	1.098039216
NPY4_MTG Y1	179	25	44750000	1.56424581
NPY4_MTG Y2	265	920	2438000000	0.028712059
NPY4_MTG Y3	101	920	929200000	0.07533362
NPY5_BLG Y1	157	920	1444400000	0.04846303
NPY5_BLG Y2	65	920	598000000	0.117056856
NPY5_BLG Y3	173	920	1591600000	0.0439809
NPY6_BKT Y1	278	920	2557600000	0.027369409
NPY6_BKT Y2	85	920	782000000	0.089514066

Appendix C

1. Calculation of concentration of standard lactose and glucose through HPLC data

			ID	tR	Area	Height	ID	tR	Area	Height
RI02.CHR	std	11:40:38	**laktose**	7.69	604.84	39.62	**glukose**	9.01	608.96	39.77
RI15.CHR	STD	17:12:42	**laktose**	7.69	603.77	40.04	**glukose**	9.01	612.76	40.21
RI26.CHR	STD	21:53:40	**laktose**	7.69	608.22	40.07	**glukose**	9.01	613.28	40.25
RI37.CHR	STD	02:34:41	**laktose**	7.69	619.12	40.47	**glukose**	9.01	615.89	40.48
RI48.CHR	STD	07:15:46	**laktose**	7.69	586.94	39.3	**glukose**	9.01	603.08	39.67
RI59.CHR	STD	11:57:46	**laktose**	7.69	604.82	39.84	**glukose**	9.01	609.83	40.03
RI66.CHR	std	14:56:43	**laktose**	7.68	610.93	40.47	**glukose**	9.01	618.1	40.76
RI77.CHR	STD MIX	19:37:38	**laktose**	7.68	603.63	39.77	**glukose**	9	606.08	39.97
RI88.CHR	STD MIX	00:18:35	**laktose**	7.68	585.85	39.26	**glukose**	9	602.45	39.73
RI99.CHR	STD MIX	04:59:37	**laktose**	7.68	624.35	40.65	**glukose**	9	622.84	40.8
RI109.CHR	STD MIX	09:15:09	**laktose**	7.68	612.72	40.23	**glukose**	9	616.36	40.41
SD				0.004979	11.14297	0.444877		0.00481	6.107585	0.377298
Average				7.685455	605.9264	39.97455		9.006364	611.7845	40.18909
RSD				0.064789	1.838997	1.112901		0.053412	0.998323	0.938807

The area of the samples is known from the HPLC run. The highlighted area is the average area of standard which will be used for the calculation as the RSD which must be less than 5 % (to ensure 95% confidence level). The area of known concentration of standard solution is compared with the area of chromatograms for the given samples. Thus, concentration of given sample can be known from it.

Appendix D

1. Data for Figure 4.4 and Figure 4.5

Samples	0	24	48	72	96	120	144	168	192	216	240	264	288
1	1.468	1.467	1.459	1.449	1.442	1.434	1.429	1.425	1.421	1.418	1.416	1.415	1.414
2	1.473	1.471	1.458	1.444	1.434	1.424	1.418	1.414	1.413	1.412	1.411	1.410	1.410
3	1.474	1.471	1.461	1.453	1.448	1.442	1.439	1.435	1.432	1.429	1.426	1.423	1.421
4	1.475	1.474	1.468	1.460	1.453	1.445	1.438	1.434	1.431	1.428	1.427	1.425	1.424
5	1.506	1.505	1.496	1.487	1.478	1.468	1.462	1.457	1.453	1.451	1.450	1.449	1.448
6	1.479	1.478	1.469	1.459	1.452	1.442	1.438	1.435	1.432	1.430	1.428	1.427	1.427
7	1.503	1.501	1.492	1.481	1.473	1.464	1.460	1.455	1.449	1.444	1.441	1.439	1.439
8	1.478	1.474	1.465	1.459	1.455	1.447	1.445	1.440	1.436	1.432	1.428	1.426	1.424
9	1.481	1.480	1.473	1.465	1.460	1.455	1.451	1.448	1.446	1.443	1.441	1.439	1.438
10	1.488	1.487	1.479	1.471	1.465	1.457	1.455	1.451	1.447	1.444	1.441	1.439	1.437
11	1.501	1.498	1.489	1.483	1.477	1.471	1.466	1.464	1.462	1.458	1.454	1.451	1.448
12	1.536	1.534	1.529	1.524	1.521	1.517	1.516	1.514	1.512	1.510	1.508	1.507	1.505
13	1.472	1.466	1.456	1.447	1.441	1.435	1.432	1.428	1.421	1.416	1.414	1.412	1.411
14	1.476	1.475	1.465	1.456	1.450	1.446	1.441	1.436	1.431	1.427	1.425	1.423	1.422

Fall in specific gravity over time (hours) of yeast samples

2. Data for Figure 4.6, 4.7 and 4.8

Glucose		Fructose		Ethanol	
Kritipur y1	Bhaktapur y1	Kritipur y1	Bhaktapur y1	Kritipur y1	Bhaktapur y1
100	100	30	30	0	0
89.60715	65.28426268	28.107161	23.7613265	4.17616776	13.87273089
86.311031	54.34873963	26.443735	22.0073798	5.84701246	19.98972165
31.672685	33.90936596	20.221875	19.1015752	32.6824775	31.41565624
5.0975719	2.054245408	12.223646	11.1839013	40.1059366	54.5903666

3. Data for Figure 4.9 and 4.10

	1- Propanol	Ethyl acetate	2-methyl-1-p	1-Butanol	Ethyl prop	Propyl acetat	Acetal	3-methyl-	2-methyl-	Isobutyl a	Ethyl buty	3-methyl-	Ethyl hexanoate
Cyberlindnera fabianii	44.35	131.45	85.68	1.00	4.90	2.95	2.03	67.54	70.28	0.84	0.03	6.03	0.00
Cyberlindnera fabianii	38.07	131.45	111.79	0.97	3.17	1.65	1.27	86.36	72.02	0.61	0.00	4.35	0.00
Cyberlindnera fabianii	47.40	131.45	96.85	0.76	2.91	1.30	0.85	73.06	68.73	0.38	0.00	2.36	0.00
Cyberlindnera fabianii	42.03	131.45	90.65	0.63	4.07	2.80	1.68	60.40	58.20	0.82	0.00	4.60	0.00
Cyberlindnera fabianii	51.03	131.45	58.56	1.12	3.94	2.18	0.60	48.68	58.45	0.36	0.02	3.57	0.00
Pichia burtonii	21.39	131.45	88.90	0.33	0.40	0.14	2.37	46.20	16.79	0.07	0.00	0.77	0.00
Kodamaea ohmeri	24.43	13.13	82.39	0.52	0.00	0.00	3.43	52.01	14.55	0.00	0.00	0.15	0.00
Kodamaea ohmeri	24.64	12.33	50.08	0.36	0.00	0.00	0.19	34.46	14.43	0.00	0.00	0.36	0.00
Saccharomycopsis fibuligera	28.33	10.53	43.34	0.34	0.00	0.00	0.08	39.86	13.73	0.00	0.00	0.40	0.00
Saccharomycopsis fibuligera	22.41	11.00	41.97	0.29	0.00	0.00	0.14	30.07	11.79	0.00	0.00	0.28	0.00
Candida inconspicua	9.43	131.45	124.99	0.27	1.37	0.17	0.74	72.27	33.31	0.16	0.00	0.62	0.00
Wickerhamomyces anomalus	32.73	131.45	57.03	1.28	1.09	2.05	0.74	103.03	45.80	0.23	0.00	1.57	0.00
Wickerhamomyces anomalus	29.97	131.45	60.18	0.62	1.16	1.86	1.03	81.12	31.70	0.22	0.00	1.22	0.00
Saccharomyces cerevisiae	32.03	35.93	68.63	0.61	0.23	0.00	5.76	171.20	63.00	0.00	0.03	0.26	0.03