ORIGINAL RESEARCH ARTICLE

Isolation of Yeast from Soil and Different Food Samples and Its Characterization Based on Fermentation

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Abstract

Yeasts are eukaryotic microorganisms which can also be used for bioethanol production. In this modern era of increasing demand for energy and fuel, the microbial biosynthesis of ethanol has gained importance. In this study, the potential yeasts for ethanol production from pentose and hexose sugars were identified. Yeasts were isolated from soil and different food samples. They were identified and characterized based on cell morphology (e.g., mode of cell division and spore shape) and physiology (e.g., sugar fermentation tests). Furthermore, quantification of ethanol and cell concentration was performed throughout the fermentation. Spot plate count method was followed to determine the viable yeast count whereas Potassium dichromate oxidation method was used for determining the ethanol concentration. Six different species of yeasts were cultured in three sets of broth for 24, 48, 72, and 96 hours for bioethanol production. The yeasts isolated from black and green grapes relatively synthesized higher concentration of ethanol.

Key words: Yeast, Fermentation, Ethanol, Potassium Dichromate Oxidation Method, YEPDA.

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Introduction

Yeasts are single-celled microorganisms that are classified, along with molds and mushrooms, as members of the Kingdom Fungi [1, 2]. Although yeasts are unicellular organisms, they possess a cellular organization similar to that of higher organisms, including humans. Specifically, their genetic content is contained within a nucleus. This classifies them as eukaryotic organisms, unlike their single-celled counterparts, bacteria, which do not have a nucleus and are considered prokaryotes [3, 4].

Yeast is widely dispersed in nature with a wide variety of habitats. They are commonly found on plant leaves, flowers, and fruits, as well as in soil. Yeasts are also found on the surface of the skin and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites. Yeasts are responsible for several types of infections including oral thrush, vaginitis, urinary tract infection, endocarditis, respiratory syndromes, meningitis, etc. The common "yeast infection" is typically caused by *Candida albicans*.

Beside infections, yeast is very useful in commercial application. Yeast has long been considered to be the organism of choice for the production of alcoholic beverages, bread, and a large variety of industrial products [1, 5, 6]. This is based on the ease with which the metabolism of yeast can be manipulated using genetic techniques, the speed with which it can be grown to high cell yields (biomass), the ease with which this biomass can be separated from products and the knowledge that it is generally recognized as safe (GRAS).

The budding yeast, *Saccharomyces* cerevisiae and other yeast species have long been used to ferment the sugars of rice, wheat, barley, and corn for the production of alcoholic beverages such as beer and wine [7,8]. There are two major types of brewing yeast, top-fermenting ale yeast and bottom-fermenting lager yeast. Top-fermenting yeast such as *S. cerevisiae* rise to the surface during fermentation and are used to brew ales, porters, stouts and wheat beers. In contrast, *S. pastorianus*, (formerly known as *S. carlsbergensis*) is a bottom-fermenting yeast, used to make lager beer. Lager yeasts grow best at lower temperatures. As a result they grow more slowly, produce less surface foam, and therefore typically settle to the bottom of the fermenter [9, 10].

S. cerevisiae or baker's yeast has long been used as a leavening agent in baking. Baker's yeast ferment sugars present in dough, producing carbon dioxide and ethanol. Murcha (locally available yeast) is the complex mixture of various types of microorganisms along with yeast. Murcha has been traditionally used in fermentation and

commonly found in Nepal, Bhutan and even some parts of India.

Yeasts are being used in the petrochemical industry where it has been engineered to produce biofuels such as ethanol, diesel and jet fuel precursor. They are also used in the production of enzymes, lubricants and detergents. Food additives including colorants, antioxidants, and flavor enhancers can also be produced using the yeast cells. Likewise, it is used in the production of pharmaceuticals including antiparasitics, anticancer compounds and bio-pharmaceuticals such as insulin, vaccines, and nutraceuticals.

Another important characteristic of yeasts being used as model organisms is that they replicate quickly and are easy to manipulate genetically. The doubling time for yeast is about 90 minutes, Furthermore, the study of genome and its organization have been completed [11,12].

Methodology

Sample Collection

Four samples were collected from local market and two from Department of Microbiology, Pinnacle Academy, Lalitpur, Nepal and Central Department of Biotechnology, Tribhuvan University (TU), Nepal. Collected samples and subcultures were given different codes for the convenience. A: Mango peel; B: Black Grapes sample; C: Banana Peel; D: Soil Sample; E: Green grapessubculture from Pinnacle Academy); F: Subculture of TU.

Sample Processing

The samples were washed with distilled water and were crushed using mortar and pestle. One gram of finely crushed sample was weighed and serially diluted up to 10^{-5}.

Isolation and Identification of Yeast

One ml of sample from each dilution was spread on YEPDA media to isolate yeast. Morphological characteristics were studied on the basis of colour, texture, margin, elevation. Simple staining was performed to elucidate the morphology and arrangement of yeast cells and budding.

Subculture of isolated yeast

Subculture of isolated yeast was done in the YEPDA plates for further study and preservation.

Spot Plate Technique

Counting of viable yeast was done by spot plate method. 5 µl of serially diluted samples was inoculated with the help of micropipette on the marked area on YEPDA media. Plates were labelled according to the dilution factor and incubated at 37°C for 24 hrs. Individual colonies in the most dilute samples were counted and the number of viable cells in the original culture was calculated.

Preparation of Broth

Three sets of broth (1-xylose, 2- glucose, 3- xylose + glucose) were prepared for each sample and its fermentative characteristics were studied.

Inoculation in Broth

All isolated yeasts from each sample were inoculated in three series of broth and kept at shaker for 4-5 days with regular monitoring. Absorbance was taken at 560nm at the interval of 24 hours. Smell of the alcohol was also monitored at the same interval.

Measurement of ethanol concentration using solvent extraction and Potassium dichromate oxidation method

Positive samples with characteristic alcoholic smell were further monitored for estimation of methanol concentration. 1ml of each positive sample was mixed with 1ml Tributyl phosphate (TBP) and was centrifuged at 10,000 rpm for 5 minutes and 950µl of upper TBP was transferred to new centrifuge tube. 950 µl of potassium dichromate was added, vortexed for 2-3 minutes and centrifuged at 10,000 rpm for 5 minutes. Then upper layer was discarded, lower layer was pipetted out and its absorbance was taken at 595 nm using YEPD broth as blank [13, 14, 15].

Preparation of standard ethanol Solution

Standard ethanol solution was prepared by mixing different aliquots of 75% ethanol from stock to make up to 5ml by mixing required amount of ethanol and distilled H_2O.

Table 1: Morphological characteristics of isolated Yeasts

Sample	Morphological characteristics					
	Colour	Shape	Margin	Elevation	Texture	Consistency
A	Cream	Oval	Lobate	Non	Smooth	Mucoid
B	White	Round	Entire	Elevated	Smooth	Mucoid
C	White	Oval	Entire	Non	Smooth	Mucoid
D	White	Round	Entire	Non	Smooth	Mucoid
E	Light –orange	Oval	Entire	Elevated	Smooth	Mucoid
F	cream	Oval	Lobate	Non	Smooth	Mucoid

Results and discussion

Morphological characteristics of isolated Yeasts

The morphological characteristics were studied from the colonies isolated on YEPDA media.**(Table 1)**

Simple staining of yeast

The yeast appeared as round or oval cells that were dark purple in color and budding were also visible under microscope. **(Table 2)**

Table 2. showing the Color and shape of the isolated Yeasts

Sample	Staining Characteristics	
	Shape	Color
A	Oval shaped	Purple in color
B	Oval and round	Purple colored yeast with buds
C	Oval shaped	Purple colored yeast with buds
D	Round shaped	Purple in color
E	Oval shaped	Light purple colored yeast with buds
F	Oval shaped	Purple in color

Counting of viable yeast by Spot plate method

Each row represents a dilution series from a different yeast culture. The same volume of diluted culture is used for each spot. The dilution series was designed so that the most dilute spots contain a small number of individual colonies that can be distinguished from one another, typically less than 15. The colonies were counted respectively. **(Table 3)**

Determination of growth curve of Yeasts

Absorbance of broth with inoculums added was taken from day first to day fourth at the interval of 24 hours at 565nm. All the readings of three sets of broth of all series of samples with comparable chart are as follows:

Table 3: Table showing the number of yeast by spot plate technique

Sample	Dilution factor	Colony count	No of cells /100µl
A	10^3	14	14×10^3
B	10^4	12	12×10^4
C	10^5	8	8×10^5
D	10^4	10	10×10^4
E	10^4	9	9×10^4
F	10^5	6	6×10^5

Yeast Extract, Peptone and Xylose

Yeast Extract, Peptone and Glucose

Yeast Extract, Peptone, Xylose and Glucose

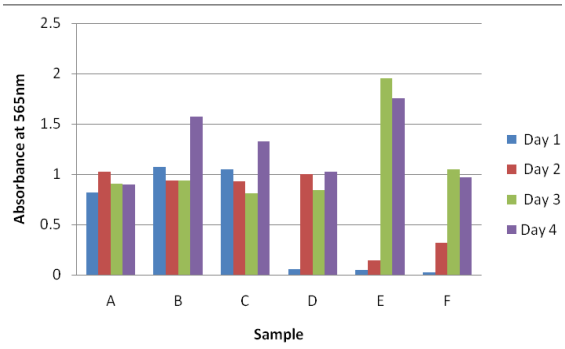

Figure 1: Graph showing the Absorbance of different Samples in the broth containing Peptone and Xylose from first day up to fourth day.

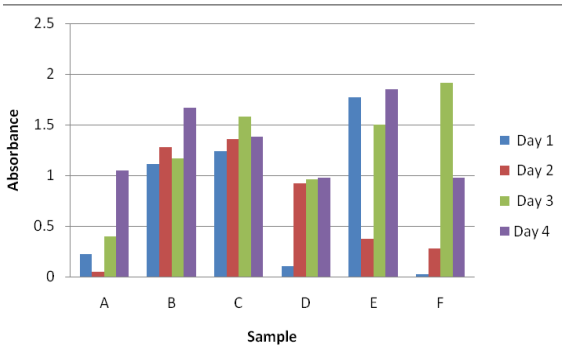

Figure 2: Graph showing the Absorbance of different Samples in the broth containing Peptone, and Glucose from first day up to fourth day.

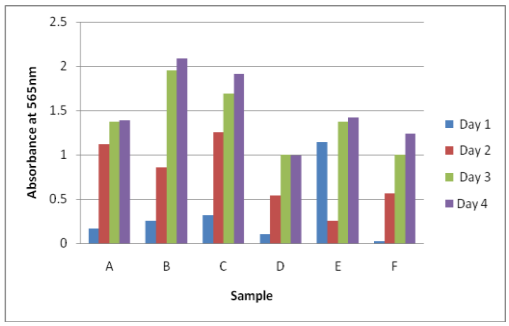

Figure 3: Graph showing the Absorbance of different Samples in the broth containing Peptone, and xylose +Glucose from first day up to fourth day.

Monitoring of Alcoholic smell

The flasks were monitored for alcohol production by smelling. There was presence of characteristic alcoholic smell in all the sets of conical flask expect 1 which contains xylose along with yeast extract and peptone **(Table 4)**.

Table 4: Absorbance of each broth with respective sample and detection of their characteristic alcoholic smell.

Sample	Broth	Absorbance at 595nm
A	1	-
	2	-
	3	-
B	1	-
	2	0.841
	3	0.629
C	1	-
	2	0.714
	3	0.542
D	1	-
	2	0.621
	3	0.428
E	1	-
	2	0.913
	3	0.624
F	1	-
	2	0.357
	3	0.601

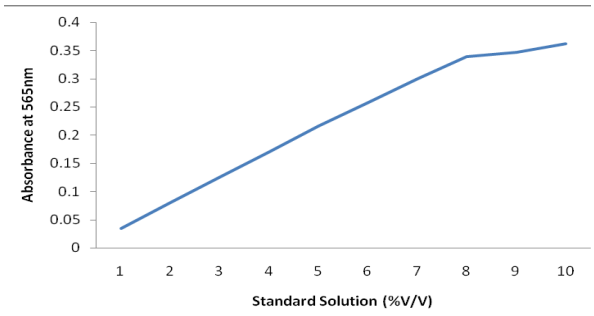

Figure 4. Absorbance of Standard ethanol solution

Potassium dichromate testing

Absorbance of all the positive samples and standard ethanol solution were taken at 595nm. Absorbance were taken for those samples only which gave characteristic alcoholic smell. Since, there was absence of alcoholic smell in sample A; its absorbance was not taken.

Comparison with the standard ethanol

By comparing the absorbance of sample with standard ethanol solution, it was found that all the isolated yeast were highly fermentative.

Discussion

The different fermentative yeasts were isolated from different samples. The maximum of Yeast colonies were obtained from both grape samples, which is mostly used in fermentative purposes.

It is also found that when yeast is grown in liquid medium, the culture follows a well established pattern for microbial growth. Cultures are usually started by inoculating media with a small number of cells. A lag phase follows the inoculation, during which cells become acclimated to the new environment and begin to condition the media with their own metabolites. Lag phase is followed by an exponential, or log phase, when the number of cells increases exponentially. During this phase, the cells are in actively growing stage with high metabolic activities, making this phase a desirable stage for the generation of primary metabolites including ethanol.

During the study, six carbon compound glucose, five carbon compound xylose and their mixed culture has been used and were screened for their suitability for fermentation. The broth containing only five carbon compound or pentose sugar xylose didn't show the fermentative characteristics, which was confirmed by the absence of characteristic alcoholic smell. The broth containing glucose and the other broth containing glucose and xylose has shown the fermentative characteristics.

Sample E show growth in all the media but however show high growth in the broth containing peptone and glucose and hence it's highly fermentative. Thus, yeasts utilize glucose a major carbon source for fermentation.

From the above graph, Sample B and C show relatively similar growth in all the media but it shows high growth in medium containing peptone and glucose. Thus, Sample B and C prefer peptone and glucose as a major carbon source.

Sample A and B utilize all carbon sources for its metabolism. Sample F uses glucose as a major

source. Thus, we can conclude, yeasts utilize six carbon compounds as a fermentative source.

The ethanol was extracted from the aqueous phase using the solvent. The ethanol in the solvent phases moved to an acidic aqueous phases and subsequently reacted with dichromate. The ethanol concentration was measured from the increase in green color from orange at 595nm against blank. The solvent extraction in this ethanol measurement is a crucial step because glucose, yeast extract, peptone, and glycerol can cause a change in color through reaction with dichromate. In other words, without the ethanol extraction step, the measured ethanol concentration in the culture medium may be tainted by a direct reaction between the dichromate reagent and other component in the culture medium.

Many solvents have previously been used for the selective extraction of ethanol, particularly for the ethanol extraction fermentation. Primary aliphatic alcohol (eg. n-decanol, n- dodecanol) is a representative solvent for extracting ethanol from a culture broth and benzyl alcohol has been used as an alternative to distillation. In this study, however, non alcoholic solvents TBP was investigated in order to find better solvent for the extraction of ethanol from a culture broth. Moreover, phase separation after solvent extraction was inhibited when the culture medium was used to prepare the ethanol standard solution. However, TBP made a distinct interface between TBP and water and showed a linear standard ethanol curve in various media [14, 15].

In the measurement of ethanol concentration of the yeast culture, the data from the solvent extraction-dichromate oxidation method were similar to those from gas chromatography [16, 17]. Therefore, this ethanol assay format is practically useful for the selection of a strain having high productivity, the development of a bioethanol production process, and monitoring and control in alcoholic beverage production.

Finally, sample E can be used as a yeasts source for fermentation. The further characterization of Sample E is essential for the future use

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References

1. Alfenore S, Molina-Jouve C, Guillouet SE, Uribelarrea JL, Goma G, Benbadis L: **Improving ethanol production and viability of Saccharomyces cerevisiae by a vitamin feeding strategy during fed-batch process**. Appl Microbiol Biotechnol, 2002. **60**:67–72.
2. Alfenore S, Cameleyre X, L Goma G, Molina-Jouve C, Guillouet SE: **Aeration strategy: A need for very high ethanol performance in *Saccharomyces cerevisiae* fed-batch process**. Appl Microbiol Biotechnol, 2004 **63**:537–542.
3. Barnett JA, Payne RW, and Yarrow D: **Yeasts: Characteristics and Identification.** 3rd edn. Cambridge: Cambridge University Press. 2000
4. Boekhout T, Robert V, and Smith MT: **Yeasts of the world. Morphology, physiology, sequences and identification.** World Biodiversity Database CD-ROM Series. ETI: University of Amsterdam. 2002
5. Abouzied M, Reddy CA:. **Direct fermentation *of* potato starch *to* ethanol *by* coculture *of Aspergillus niger and*** Saccharomyces ***cerevisiae***. Appl. Environ. Microbiol, 1986 **52**(5):1055-1057
6. Adesanya OA, Oluyemi KA, Josiah Adesanya RA, Ofusori DA, Bankole MA. **Ethanol production by Saccharomyces cerevisiae from cassava peels hydrolysate**. Internet J. Microbiol. **5**:1.
7. Barnett JA: **The entry of D-ribose into some yeasts of the genus Pichia**. J. Gen. Microbiol. 1975 **90**(1):1-12.
8. Boulton C and Quain D: **Brewing Yeast and Fermentation.** Oxford: Blackwell Science Ltd. 2006
9. Kirsop B: **Maintenance of yeast cultures" in Yeast Biotechnology.** (Allen & Umwin, Boston, Massachusetts. 1987
10. Chanchaichaovivat A, Ruenwongsa P, Panijpan B: **Screening and identification of yeast strains from fruits and vegetables: Potential for biological control of postharvest chilli anthracnose (*Colletotrichum capsici*).** Biol. Contr. 2007 **42**:326–335.
11. M. Raines, **Advanced Yeast Culturing Kit Instruction**. Booklet (Brewers Resource, Camarillo, California,) 1992
12. Martini A: **Biodiversity and conversation of yeasts.** Biodiver. Conserv. 1992 **1**(4):324-333.
13. Senthilraja P, Kathiresan K, Saravanakumar K: **Comparative analysis of bioethanol production by different strains of immobilized marine yeast**. J. Yeast Fungal Res. 2011 **2**(8):113–116.

14. Hyun-Beom Seo, Hyun-Joo Kim, Oh-Kyu Lee, Ji-Hye Ha, Hyeon-Yong Lee, Kyung-Hwan Jung: **Measurement of ethanol concentration using solvent extraction and dichromate oxidation and its application to bioethanol production process.** J indust. microb. Biotech. 2009 **36**(2):285-292
15. Pilone GJ: **Determination of ethanol in wine by titrimetric and spectrophotometric dichromate methods: collaborative study.** Association of official Analytical chemists. 1985 68(2):188-90.
16. Seo HB, Kim HJ, Lee OK, Ha JH, Lee HY, Jung KH: **Measurement of ethanol concentration using solvent extraction and dichromate oxidation and its application to bioethanol production process.** J Ind Microbiol Biotechnol. 2009 **36**(2):285-292. Epub 2008 Nov 7.
17. Martínez-Nieto P, Vanegas-Hoyos M, Zapata-Pineda M, Robles-Camargo J: **Hydrolysis of *Eicchornia crassipes* and *Egeria densa* for Ethanol Production by Yeasts Isolated from Colombian Lake Fúquene**. International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering 2013 **7**(1)